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OPERATION OF AN INSECTARY

PRACTICAL MANUAL

AEDES AEGYPTI REARING PROCEDURES AND BASIC PRINCIPLES OF BIOSAFETY

Operation of an Insectary
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Aedes aegypti Rearing Procedures and Basic Principles of Biosafety

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CONTENT

ACRONYMS

ACL	Arthopod Containment Level
ASTMH	American Society of Tropical Medicine and Hygiene -
BMLB	Biosecurity Guide in Microbiology and Biomedicine Laboratories
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
CENAPRECE	Centro Nacional de Programas Preventivos y Control de Enfermedades, Gobierno de México.
PECET	Programa de estudio y control de enfermedades tropicales, Universidad de Antioquia, Colombia.
PPE	Personal Protective Equipment
SOP	Standard Operating Procedure
WHO	World Health Organization

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I. Insectary Operation

Chapter I

1. DEFINITION OF INSECTARY

A well-defined and strictly controlled space where colonies of live insects are reared and housed in environmentally stable conditions. It is common for an insectary to use entire rooms that have floor-to-ceiling walls and full-sized and tight-fitting doors. The host institution mandates which species are kept in the insectary, and this determines the microclimate parameters for the insect rearing rooms, along with the biosafety measures that should be followed. An insectary is a strictly controlled space, with restricted access, where the microclimate conditions are adapted to the insect species kept there and the function given to the insects originating from there.

For an insectary to be considered operational, several microclimate variables should be strictly controlled, such as temperature, relative humidity, and photoperiod (total hours of light vs. hours of darkness). These parameters are described in greater detail in the following sections of this document.

An insectary is essential for the rearing and maintaining insects used in basic, applied, or operational research. For example, the rearing of urban mosquitoes that are widely distributed in tropical and subtropical areas, such as *Aedes aegypti,* and the maintenance of live colonies of this species, has been a common procedure at institutions linked with public health programs. Existence of the mosquito colonies ensures enough biological material to be used in efficacy tests for products such as insecticides, repellents, and other prevention or control tools. Such tests should follow standard protocols, which require controlled spaces (laboratories and insectaries), quality procedures (standard protocols), and a large number of biological organisms generally of the same age.

Even when local conditions of countries in tropical and subtropical climates approach what is required to maintain live mosquito (Culicidae) colonies in a given space, and live mosquitoes can be kept in handmade cages, any room designated for testing of the live colonies must be properly adapted for entomological work. The establishment of an insectary must follow clear parameters that guarantee that the maintenance of live mosquito colonies does not represent any risk for the staff or for the community where the insectary is placed. This means that various protection measures and environmental control factors must be consistently established, evaluated, and maintained in such a way that any result or information originating from the insectary has been produced with scientific rigor and high quality standards.

For an insectary to be fully functional, it should have separate rooms for the immature and adult phases of the species being maintained. The separate rooms should have functional doors to isolate the live individuals maintained there, enforce restricted access to said spaces, and prevent the insects that are the subject of study from escaping. The above implies that the ideal microclimate for the different developmental phases of an insect may vary greatly. For example, in the case of adult colonies of mosquitoes, maintaining a high relative humidity (70% - 80%) is key to the survival of female mosquitoes; because the larval forms of the mosquitoes are aquatic, they do not need to be in a room with high relative humidity.

The spaces dedicated to an insectary are of great importance, as are spaces dedicated to public health laboratories used for biological, microbiological, and other research. These controlled spaces should have not only a certain physical infrastructure (walls and functional doors), but also other elements needed for public health studies, staff training, and the creation of reference collections. Insectaries and laboratories are fundamental to countries' public health programs as these spaces support the operations research required to make decisions on mosquito vector control. The following sections present different technical materials and recommendations that are necessary for an insectary to be functional and adequate for public health studies.

Chapter I

2. MATERIALS AND EQUIPMENT

For an insectary to meet the expected goals and be an acceptable space in which to conduct studies on medically important insects (such as Culicidae mosquitoes), it needs to meet minimum requirements for equipment and materials. The most important tools for insectary use are described below:

1.2.1 FURNITURE AND SURFACES

In an environment where hygiene and safety are essential, the selection of furniture and surfaces is of great importance. This calls for rust-proof material that can be frequently cleaned and resist the high temperature and humidity inside the rearing rooms [1]. Thus, the preferred materials for insectary furniture are stainless steel, fiberglass, and plastic.

Shelving structures should be easily adjustable and be made of independent units. If not fixed to walls, shelves with wheels are preferred. There should be enough space between each unit to facilitate cleaning. Metallic or plastic shelves are preferable to wooden shelves since metal and plastic can be cleaned regularly and can withstand the high temperatures and humidity that are common inside insectary rooms.

All spaces designated for laboratory work should use modern materials that are thermo-stable, resistant, easily cleanable, non-porous, and anti-corrosive surfaces. Such modern materials include phenolic and epoxy resins which are synthetic polymers that provide long-lasting countertops for laboratory use [2]

1.2.2 MATERIALS AND EQUIPMENT FOR ENTOMOLOGICAL WORK

Basic supplies needed for the proper functioning of the insectary are described below (Figures 1-14).

Figure 1. Stereoscope

Also known as a dissecting microscope or a stereoscopic microscope. The stereoscope is designed to produce a three-dimensional image of the item being observed.
The stereoscope possesses a limited magnification capacity, normally from 10x to 80x.
The magnification obtained depends on the capacity of the eyepieces and the objective lenses that each brand and model includes in its component parts.

Figure 2. Optical microscope

Also called a compound optical system. It is used to observe particles and objects not visible to the naked eye.
The compound microscope enables the replication and magnification of the image by way of a lens assembly. It is composed of ocular and objective lenses. The objective lens projects an image of the sample, which is then magnified by the ocular lenses.
Magnification ranges from 4x to 2000x.

Figure 3. Humidifier

Maintains a healthy climate for the mosquitoes kept in live colonies.
A device that releases water vapor to increase the levels of relative humidity in the air/room.
Humidifiers are used in the rooms where live adult mosquito colonies are maintained

Figure 4. Thermo-hygrometer

A measuring instrument used to determine the relative humidity and environmental temperature.
This tool should be kept inside the rooms where temperature and relative humidity must be monitored. The daily record of these parameters should be made on paper forms. This item is indispensable to monitor the microclimate inside the insectary.

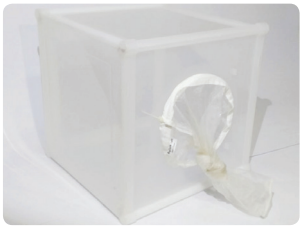
Figure 5. Analytical balance

A high-precision laboratory instrument used to measure small masses, generally from ten thousandths of a gram (0.0001 g) up to 200 grams.

Figure 6. Thermometer

An instrument used to measure temperatures with a high level of accuracy. Used to measure the temperature of liquids or equipment such as a cooler or refrigerator (which should be kept at a temperature of 4°C).

Figure 7. Metal and/or plastic cages



A cubed box covered with fine mesh with a front access opening with long sleeves that allow the handling of specimens while preventing their escape. It is used to conserve live adult mosquito populations under strict supervision.
The mesh covering the cage may be metallic or plastic, with fine pores (gaps in the net) to prevent the adult mosquitoes from escaping. The internal structure of the cage can be made of plastic, galvanized iron, or even wood.
The most common size for adult cages is 30 cm^3 (30 cm long x 30 cm wide x 30 cm deep), to maintain an approximate population of 250–500 living specimens.

Figure 8. Plastic tray

Rectangular-shaped white trays made of enameled metal or plastic.
This document refers to this item as "larval rearing trays." They are used to maintain *Ae. aegypti* larvae.

Figure 9. Plastic containers (ovitrap)

A 250 ml (approx.) wide-mouth container, which is placed inside the cage to collect eggs from the gravid females.
These containers can be used in the field for the same purpose, with the only difference being that field ovitraps tend to be larger (up to 500 ml).

Figure 10. Plastic containers (cups)

A 250 ml (approx.) wide-mouth plastic container used to place the pupae inside the adult cages.

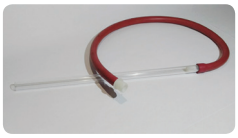
Figure 11. Pasteur pipettes (disposable plastic)

Used to transfer liquids or handle immature mosquitoes. If the lower opening is cut, it can be used to easily transfer larva or pupae from one container to another. Generally, they are available in sterile forms and in various volumes that range from 1 to 10 ml.

Figure 12. Absorbent filter paper, paper towel, or oviposition surface

Used as an oviposition substrate, which provides a rough and humid surface for the adherence of eggs from an *Ae. aegypti* female.

Figure 13. Buccal suction tube or buccal aspirator



Used to transfer adult mosquitoes from one container to another.
The most basic version consists of a transparent acrylic tube and a latex hose with an acrylic nozzle.
To prevent any insects from being swallowed accidentally by the person handling the insects, the acrylic tube is protected with a fine net (mesh or cloth) placed over the opening.

Other important items include:

- Timer (used to regulate periods of light and darkness in the insectary rooms)
- Magnifying glass (to facilitate the observation of the different stages of mosquito development; large adjustable magnifying glasses affixed to a table or desk, or a handheld magnifying glass, can be used)
- Cotton (preferably cotton balls)
- Mosquito net (fine-pored, to prevent the escape of adult insects)
- Manual counter (to count and record the number of eggs)
- Measuring spoons (to provide food to the rearing larval trays)
- Permanent markers (to mark materials depending on the type of strain and geographical origin)
- 70% alcohol (to clean the work area surfaces). To store and conserve biological specimens (such as larvae and pupae), ethanol at higher concentrations (80% - 95%) should be used

Figure 14. Test tube

A volumetric measuring instrument that consists of a cylinder graduated in cubic centimeters or millimeters.
Used to hold liquids and to measure volumes with greater accuracy. There are test tubes of varying capacities, which range from 5 ml to 1 l. They are generally made of glass, although there are also plastic test tubes for the same purpose.

- A pH neutral soap or detergent that is free of phosphates (recommended for cleaning laboratory material)
- Cleaning cloths, exclusive for use in each insectary room
- General cleaning supplies

Chapter I

3. CHARACTERISTICS OF INSECTARY FACILITIES

The main goal of an insectary is to create a safe space for the insects being reared, and for the personnel working in it. The insectary facilities should have certain characteristics to make it an appropriate space for insect rearing and management. Some technical recommendations are outlined below:

1.3.1 INSECTARY LOCATION

The insectary should be segregated from free-access areas, and primarily, far from areas designated for the storage of chemical products such as insecticides. The external walls of each room inside the insectary should be labeled so that all staff can identify and be informed about the restricted areas where live insects are maintained or when activities are being carried out that require a minimal disruption. Whenever possible, the insectary should be located in a separate building in areas with a minimum flow of people; in other words, isolated from office areas or areas that are open to the general public [3].

DISTRIBUTION OF WORK AREAS

Work areas inside the insectary will be distributed according to the type of insects intended for colonization and maintenance, and the laboratory activities necessary to meet the objectives of the intended studies, as well as those established by the institution that manages the insectary [4]. In addition, there are national and international regulations issued by the Pan-American Health Organization (PAHO) and the World Health Organization (WHO) that are considered to be standard guidelines for entomology labs and for equipment used in public health operations. These guidelines are the central theme of Chapter III of this document.

INSECTARY DOORS

An insectary should have one access point for entry and exit, ideally a double-door vestibule (Figure 15A, B) where the doors cannot open simultaneously, to keep the insects in the rearing rooms from escaping. If the insectary has two rearing spaces or rooms containing immature-phase and adult-phase mosquitoes, both rooms should have a labeled and independent door.

Figure 15. Examples of safety vestibules with double doors

A) Main access door to the insectary; B) Space between the main door and the secondary door.

Image source: ZAP Honduras Entomology Laboratory, El Paraíso Health Region, Honduras.

Additional safety barriers may be installed to complement this door system. Barriers include curtains or a safety closure. For example: a plastic or fabric curtain could cover the entry door in a rearing room.

PORTABLE BOOTHS

When the physical space of an insectary is limited and the staff work with different strains in a single room, the installation of portable booths is recommended (Figure 16A - C). These portable booths serve to keep the different strains of the same species isolated from each other, and thus keep each strain free from contamination. Usually, different populations of the same species come from different geographical locations, and to maintain their characteristics the personnel should keep such populations or strains isolated until the time of testing.

The portable booths are viable containment tools as they can be produced locally and made-to-measure. The booths typically are a large-sized portable cage (suggested measurements: 214 cm high x 144 cm long x 108 cm wide) with a wooden base structure and front door access.

Figure 16. Portable booths

A) Portable booths placed within one insectary room. B) and C) are suggested measurements for height, length, and width of the booths.

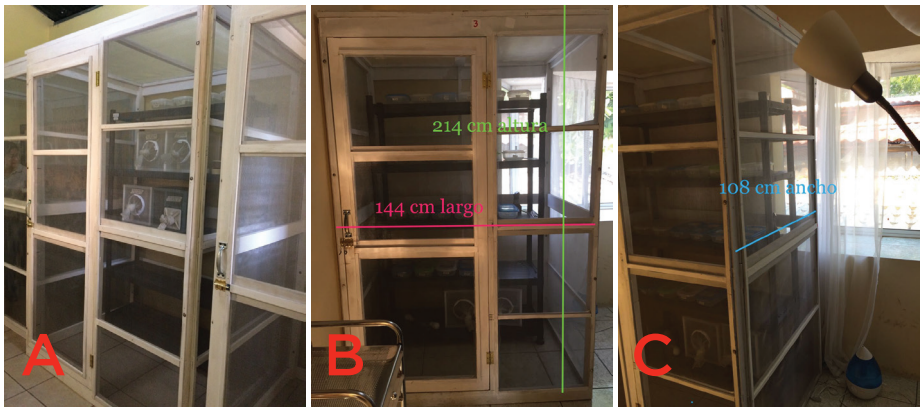


Image source: ZAP Guatemala

INSECTARY WINDOWS

The general recommendation is to either eliminate or seal any windows in the rearing rooms, unless the natural light the windows let enter is needed to regulate the photoperiod of the insects under study. All insectary windows should be sealed in such a way that they cannot be opened and protected with a screen or fine mesh, to prevent arthropods from entering or leaving [5].

INTERIOR SURFACES

The interior wall surfaces should be smooth and painted in a light color so that a loose arthropod can be easily seen, recaptured, or eliminated. Bright finishes, ideally resistant to chemical disinfectants, are recommended. The floors also should be of a light color, smooth, and bare, and the ceilings should be as low as possible to easily detect and capture flying insects [3].

The insectary's interior should be designed or arranged in a way that facilitates the constant cleaning of work area surfaces. Walls, shelves, tabletops, countertops, and floors should be cleaned regularly – at minimum, once a week – with warm, soapy water to remove fungi and microorganisms [1]. Eating or storing of food products by the insectary's staff must be strictly controlled; food and drink are prohibited inside the insectary area proper..

HYDRAULIC FACILITIES

Hydraulic facilities provide the insectary with permanent running water, which is needed to perform routine procedures, and to clean materials, equipment, and work areas. Ideally, the insectary should have a sink should be installed in at least one of the rearing rooms (preferably in the immature mosquito rearing room).

DRAINAGE SYSTEM

When there is a drainage system in the area designated for the insectary, the drains should be covered with a fine, preferably metallic mesh to prevent insect pests, such as cockroaches that live in the pipes, from entering the insectary. Furthermore, when disposing of biological material, mainly dead larvae and immature mosquitoes, insectary staff must follow the procedures outlined in Chapter III of this document; for example, biological material must never be disposed of down pipes or drains.

ILLUMINATION

The photoperiod and light intensity affect the development of the different life cycle stages of Aedes mosquitoes; in the larval phases, light fosters their metabolic development, and it is of particular importance in the adult phases to stimulate development of physiological processes such as mating and oviposition [6]. For this reason, the photoperiod (hours of light followed by periods of darkness) used for live mosquito colonies should be constant and stable. The stability of the photoperiod is one of the most significant variables for ensuring the healthy development of the mosquito colonies (feeding behavior, oviposition, development of immature mosquitoes, etc.).

In the insectary, a cycle of 14 hours of daylight and 10 hours of darkness is most suitable for rearing the majority of mosquito species [7], although, for practical purposes, cycles of 12 hours of daylight and 12 hours of darkness have also been documented as successful [6]. In regions where the climate is predominantly warm, with temperatures ranging from 28 to 30°C, the facilities can be conditioned in such a way that the colonies are exposed to a natural photoperiod (from 11 to 13 hours of light, depending on the time of year). This can be achieved by installing (sealed) windows with translucent glass that allows sunlight to enter, without directly exposing the live organisms of the colonies to bright sunlight [4].

TEMPERATURE

Temperature is the most important extrinsic factor that affects larval growth rates and the metabolic rates of adults. It also influences the feeding rate and oviposition of the female specimens. The optimum temperature is that in which development occurs with minimum mortality and loss of fertility in the resulting adults [7].

The recommended optimum temperature for *Ae. aegypti* colonies is 27 ± 3°C [7]. The temperature greatly influences blood digestion and the development of ovaries in *Ae. aegypti* females; low temperatures tend to increase the duration of these processes [8]. Continual temperature fluctuations are harmful to larval development, as temperatures below 20°C delay the growth of the larvae [9], and high temperatures can be lethal to the immatures. For this reason, temperature control is important for attaining a productive mosquito colony in all developmental phases.

RELATIVE HUMIDITY

Relative humidity of the insectary is another factor that has an essential role in the longevity of adult mosquitoes. It is very important in the rearing and maintenance of Aedes mosquito colonies. In addition, low relative humidity increases the duration of the gonotrophic cycle. Relative humidity is a critical variable for the survival of adult *Ae. aegypti,* and a range of 70% to 80% is suggested [7]. The mosquito colonies that are established in spaces without controlled humidity are subject to a high mortality of adults specimens.

Several methods are suggested to generate humidity inside an insectary. The optimal choice will depend on the needs and the locale of each case.

The available options are:

- Vapor injection into the central ventilation system
- A greenhouse-type mist humidifier
- Room or household humidifiers
- Small steam generators

VENTILATION SYSTEM

The air intake system is important to maintain arthropods [3] and to provide adequate working conditions for the insectary staff. All ventilation ducts must be covered with a mesh or screen that prevents the entrance or exit of insects through the ducts. The installation of air conditioning systems that blows toward the interior of the insectary's rearing rooms is not recommended. Although air conditioning is used to keep the human staff in an ideal temperature, the microclimate it creates is not ideal for maintaining *Ae. aegypti* mosquito colonies.

1.3.2 INTERNAL RULES AND REGULATIONS

Every insectary should have a written set of rules and technical procedures and considerations, compiled in a physical document and readily available for every staff member to read and sign, confirming that they understand all the specified procedures required for the maintenance of the live insect colonies. Some work instructions are outlined below to provide a set of rules and facilitate insectary operation:

ACCESS TO THE INSECTARY

Access to the insectary rooms must be restricted; in other words, only the staff members in charge of the insect colonies and room cleaning, as well as the group supervisors and managers may have access to this space. It is very important that access by non-authorized personnel be strictly controlled, because they have not received the proper training and could facilitate the escape of insects, or carry in an external contaminating agent.

Ideally, as part of its established rules regarding the use of an insectary, the institution should keep a registration book in which all the individuals who enter the facilities to work must register their name, date, and time of entry and exit. This registry is important for controlling the flow of people inside the colony rooms; these records will allow supervisors to corroborate that the working hours for maintenance of the insect colonies have been strictly followed, including when such work falls on weekends or holidays.

All the staff should be thoroughly familiar with the operating and administrative procedures, as well as with the adequate use of the work equipment and established biosafety and biosecurity measures [3]. (See Chapter III to review the biosafety and biosecurity measures).

Chapter I

4. MAINTENANCE OF THE *AEDES AEGYPTI* STRAINS

When strains of live colonies or populations of the same species from different geographical origins are maintained in an insectary, contamination between the species must be avoided. For example, when populations of *Ae. aegypti* originating from different cities or municipalities are maintained simultaneously, contamination between strains is possible. This can happen when mosquito cages containing both strains are located in the same room. Adult individuals of one mosquito strain can escape their cage and invade the cage of another strain; they can also interact with individuals of a different strain. It is impossible to visually detect when two strains of *Ae. aegypti* originating from different geographical regions mix, given that individuals of the same species have the same morphological characteristics. Thus, the staff members tasked with maintaining live colonies of *Ae. aegypti*, should not keep populations of different geographical origins (strains from different localities) in the same room, because the risk of cross-contamination between populations is high. Even when a staff member has vast experience in handling colonies, it is impossible to prevent adult mosquitoes from occasionally escaping. The identity of a strain of organisms, or a population of organisms, originating from a specific location is determined in the final instance by its genetic composition; contaminated populations have little value, especially if their only distinctive feature is their geographic origin [1].

In ideal conditions, to prevent contamination between species or between geographically different populations of the same species, their physical isolation (physical barriers) is recommended in different rooms (see section above regarding portable booths). Maintaining the purity of the mosquito colonies depends on the careful attention to adequate technical procedures, such as the use of labels on trays or cages indicating the geographic origin of the population, date of collection or egg hatching dates, and log of the filial generation of each strain.

Moreover, these procedures must be coupled with the cleaning of transfer materials during different phases, such as pipettes used to transfer larvae or pupae and suction tubes to transfer adults. Covering breeding trays with netting also reduces the risk of contamination.

1.4.1 RECOMMENDATIONS TO PREVENT THE CONTAMINATION OF SPECIES

- The larvae, pupae, and adult mosquitoes can become attached to transfer instruments (such as pipettes and suction tubes), and using these same instruments while performing maintenance on another strain may result in contamination between different populations or strains. To avoid this, use materials that have been carefully washed with a low concentration solution of soap and water and visually examine the transfer instruments to be used. Ideally, the different geographic strains of mosquitoes should have exclusive materials and tools designated to their handling, duly marked with the name of that strain.

- Eggs can become attached to plastic ovipositor containers, and if these same containers are reused in other cages without first being decontaminated, this may result in the transfer of eggs and contamination between strains. To avoid this, thoroughly wash each container with soap solution and water to ensure they are totally decontaminated before reusing them. Otherwise, separate the materials used for each strain and clearly mark all the items so that it is clear to which mosquito strain they belong.
- Avoid placing pupae in the wrong cages. Use colored tape or different colored markers to identify each strain and mosquito cage; this will facilitate the visualization of the different strains of mosquitoes and reduce "human error" by the staff members managing the colonies.
- Free-flying adult mosquitoes represent a contamination risk because the gravid females might deposit their eggs on uncovered trays. To avoid such a situation, do everything possible to prevent mosquitoes from escaping, and keep breeding trays covered with netting. If they do escape, capture them with a mechanical suction device, or eliminate them with an electric insect-killing racket or an alcohol spray (commercial concentration of alcohol 70%). A captured mosquito cannot be returned to one of the colony cages because a staff member has no way to determine – without a doubt – where the mosquito originated and thus to which cage it should be returned.
  The staff members in charge of maintaining the live insect colonies (in this case of Culicidae mosquitoes) should be organized, methodical, and detail-oriented. It is essential that the staff members in charge of the live mosquito colonies mark the cages, trays, and other biological material (with labels stating the origin of the mosquito strain, species, hatching or breeding date, feeding dates, etc.). Additionally, the staff members must keep all the containers and transfer instruments clean from any eggs, larvae, pupae, or adults in such a way that no risk of contamination exists between strains of the same species.

Remember: If mosquitoes escape capture them with a mechanical suction device, or eliminate them with an electric insect-killing racket or an alcohol spray (commercial concentration of alcohol 70%). A captured mosquito cannot be returned to one of the colony cages because a staff member has no way to determine – without a doubt – where the mosquito originated and thus to which cage it should be returned.

1.4.2 SPECIES HARMFUL TO MOSQUITO COLONIES

One important aspect in maintaining an insectary is the control of factors related to pests or species that may be harmful to the mosquito colonies.

Insect infestations of an insectary are a major concern because they can easily deplete an adult mosquito colony over the course of one night.

Tapinoma melanocephalum: This is an ant species that damages agricultural crops and insectaries; it generally eats *Ae. aegypti* adults and eggs if it has easy access to the spaces where breeding trays or storage of oviposition papers (or substrates) are located [10]. These ants can be found in all seasons and can eat *Ae. aegypti* eggs whether they are stored on paper strips or in the containers after the oviposition process.

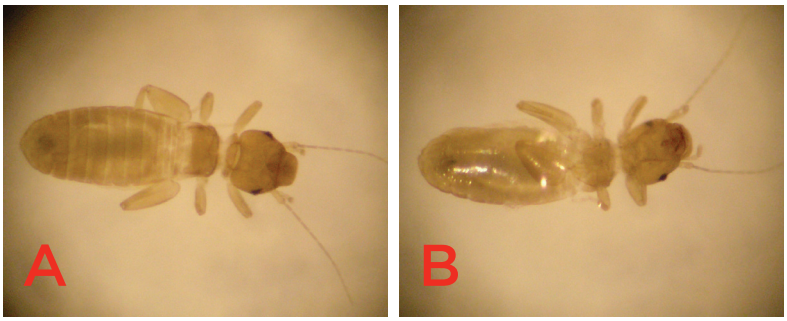
Nesticodes rufipes: This is a type of arachnid that is widely distributed in tropical and subtropical regions. It feeds on various arthropod species, and, as such, is considered a significant natural predator of *Ae. aegypti* [11, 12]. Its webs contain a paralyzing substance that paralyze mosquitoes on contact, which increases its capture efficiency [13]. The species has been found inside *Culex quinquefasciatus* and *Ae. aegypti* mosquito cages [14].

Genus *Liposcelis:* Better known as "booklice" or "barklice" because their morphology is similar to that of insects commonly known as lice (Figure 17 A, B). Although the common name for this insect associates them with books, these insects are widely distributed and inhabit a variety of diverse environments in their search for organic matter of any type as a source of food. They are frequently found in food stored in cupboards or pantries, containers or cardboard boxes, moldy paper, books, and in damp environments inside homes.

Figura 17. *Liposcelis* sp

A) Dorsal view on the left, and B) ventral view on the right.
Approximate size 1–4 mm.

Image source: Courtesy of C. Torres Gutierrez, Entomologist ZAP Project.



These insects belong to the taxonomical order named *Psocoptera*. Psocoptera eat plant and animal-based materials such as fungus mycelium, mold, cereal grains, pollen, dead insects, and insect eggs, and therefore are considered a hazard to the insectary. Generally, they infest in large numbers the papers with *Ae. aegypti* eggs when this type of material has not been stored correctly. One particular characteristic of the females of this genus is they are parthenogenetic organisms (individuals develop from an unfertilized ovum) [15].

Common cockroaches: *Periplaneta americana*, are considered as mechanical vectors of pathogens and may be found in insect rearing rooms [7]. Cockroaches are common urban pests and usually search for spaces with favorable humidity and temperature, for which reason, the food used to feed mosquito larvae must always be kept in plastic or glass containers that can be sealed shut.

Other pests include vertebrate animals, such as rodents, which are also considered as urban pests. The presence of rodents in insect colony rooms can introduce contaminating agents (microorganisms) to the rearing rooms, as well as eat the food generally used to feed the immature Culicidae.

The easiest way to minimize pests in the insectary is to decrease or eliminate the conditions attracting them, such as food and shelter. Even the standard cleaning procedures of surfaces and shelves that are required for all rearing rooms as a preventive measure against urban pests might be insufficient on their own. The staff assigned to the insectary must give prompt notice when there is evidence of the presence of pests so that the supervisors can evaluate the best measures to take in each case (cover holes in walls, fix doors that do not fit properly, seal windows, cover drainage areas, etc.). Thus, it is recommended to use water traps on lower shelf and table surfaces to trap circulating ants, lice, or other insects in the insectary rooms, and to keep paper and paper towels with mosquito eggs stored in sealed bags or plastic boxes with tight lids.

1.4.3 SANITARY HYGIENIC MEASURES

In line with the technical experience of the authors of this document, as well as with the scientific literature published on the matter, we list below a set of sanitary-hygienic measures aimed at promoting the best practices for mosquito rearing and for the maintaining live insects, safeguarding the health of the researchers, and protecting the biological material reproduced in the facilities:

- Eating, drinking, smoking, or applying makeup is prohibited inside the insectary. Food should be stored outside the insectary in places or refrigerators designated for that purpose.
- The staff working in the area must use the required protective and hygiene products, such as: laboratory coats, gloves, and face masks. These items should be used exclusively in the insectary rooms.
- Work in an orderly manner. The work tables and countertops must remain clean at all times, free of books, boxes, or unnecessary accessories while work is being performed.
- Wash hands thoroughly (using soap and water) before and after handling biological material.
- Any instruments used in the insectary must be manually cleaned with abundant running water with or without a soap solution (low concentration of soap). When a soap solution is needed, follow with a thorough rinse with running water. The frequent use of soap is recommended only for tabletops, shelves, and other work areas, not for materials that will come in contact with larvae or adult mosquitoes on a daily basis.
- If the mosquito cages are made of plastic or acrylic materials, they must be washed and frequently disinfected, using detergent solutions (avoid the use of pure detergents) followed by rinsing with running water, and should be dried afterward by exposing them to sunlight to take advantage of the bactericidal effects of the sun's ultraviolet rays. Alternatively, a hairdryer can be used (exclusively for the use of the colonies), in such a way that the cages are fully dried to prevent the growth of microorganisms (fungus).
- Any supplies with insecticides or materials used to handle insecticides must be stored in a separate space, far from the insect colonies. Do not expose any materials of the insectary (cages, trays, pipettes, etc.) to materials sprayed with insecticides under any circumstances.
- Label or mark all containers containing biological materials; this information must include the insect genus or species, collection date, date admitted to the insectary, geographic origin, and methodology of collection in the field, as well as the name of the personnel in charge of the specimens.
- The temperature and relative humidity must be logged daily on paper forms prepared for that purpose. This ensures the availability of the information in a physical document, by using lab forms or notebooks, thereby allowing the display and analysis of these records in a consistent and frequent manner.

Chapter I

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II. Rearing Procedures for *Aedes aegypti*

Chapter II

1. LIFE CYCLE AND HANDLING PROCEDURES OF LIVE COLONIES OF *AEDES* MOSQUITOES IN AN INSECTARY

2.1.1 BIOLOGY, ECOLOGY, AND MORPHOLOGY OF *AEDES AEGYPTI*

This section discusses the biology, ecology, and morphology of *Ae. aegypti,* as well as the steps to maintain live colonies and to rear specimens in an insectary.:

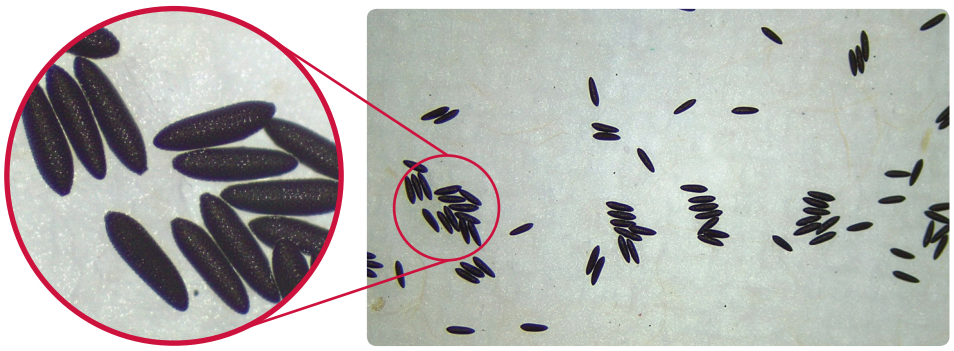
EGGS

Females of the genus *Aedes* lay their eggs individually on damp substrates located near bodies of water or in containers of water. In the case of *Ae. aegypti,* oviposition often occurs in manmade containers found in or near human dwelling places, such as plastic canteens with standing water, domestic tanks, or other containers in which water collects. The published literature indicates that the primary factors that determine the place of *Ae. aegypti* oviposition are linked to chemical, visual, and tactile stimuli. The female *Ae. aegypti* obtains information about the potential oviposition sites through their chemical and mechanical receptors once they approach or come into contact with an aquatic environment. It is known that the metabolites produced by different microorganisms, mainly bacteria living in water bodies but also the volatile substances released by different plant species and decomposing organic matter, are attractive to female *Ae. aegypti* and other Culicidae species (olfactory stimuli) [1, 2].

Ae. aegypti eggs are oval-shaped to the naked eye. Right after the eggs are laid, their color is white but after a few hours of exposure to the environment and oxygen, the white eggs darken to black, as a result of the melanization process that occurs approximately two hours after females oviposit [3] (Figure 18). The eggs are fertilized during oviposition and embryonic development is generally completed within 48 hours, if the environment is suitable, ideally humid, and warm [4]. The length of each egg is approximately 1 mm [5].

Figure 18. *Aedes aegypti* eggs

Magnification of *Aedes aegypti* eggs (using a stereoscope).



Source: ZAP El Salvador, El Paisnal Entomology laboratory.

As long as the embryo has fully developed inside the egg, the egg can enter into a dormant state. It will remain viable for several months under dry conditions (the literature has recorded viability ranging from one to six months, although this varies depending on the microclimate where eggs were laid). Dormant *Ae. aegypti* eggs will hatch once the environmental conditions become ideal again. It is worth noting that the development of the embryo depends entirely on the location where it is deposited [5, 6, and 7]. The egg's protective membrane or chorion is responsible for its dormant property; once it comes in contact with water where the bacterial action on the organic matter decreases the oxygen tension, it is stimulated to hatch [4, 8].

In wild environments, *Ae. aegypti* females lay their eggs separately, one by one, and generally use various substrates and containers to spread out the batch of eggs, even when eggs come from the same lot or oviposition period. The females generally seek to oviposit across multiple sites [5]. Complete embryonic development inside an egg takes two to three days, at which time the first larval stage is ready to hatch [3].

2.1.2 GATHERING AND HANDLING OF EGGS

Ae. aegypti eggs can be collected in various ways. The most common are the following:

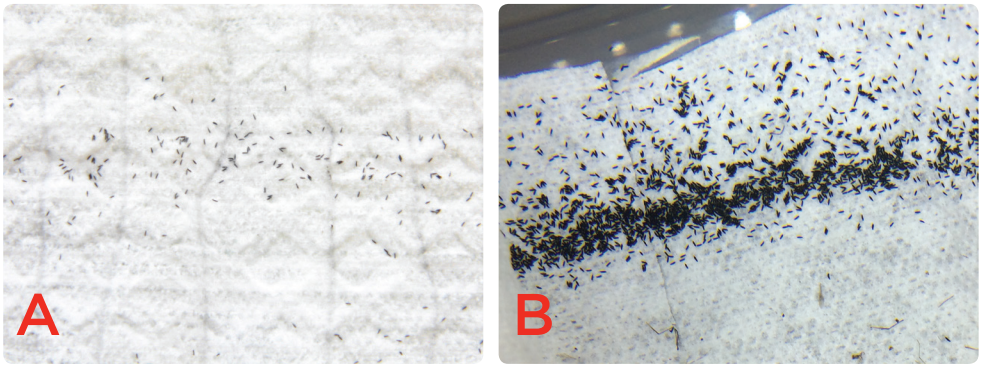
1. Use of ovitraps in the field and collection of the eggs through the use of paper towels (see Figure 19), or other oviposition substrate such as a piece of wood or fabric (pellon). The oviposition substrate may vary depending on the field protocol chosen.
2. Eggs can be collected from *Ae. aegypti* colonies once these insects have been established in an insectary (insect colonies serve different purposes and should be identified). The recommendations to gather eggs from an *Ae. aegypti* colony are described in the following sections of this chapter.

The next section describes the procedures for keeping *Ae. aegypti* eggs – regardless of their origin – viable under lab conditions and to activate oviposition and continue the life cycle of the mosquitoes under controlled conditions, until a new generation is obtained

Figure 19. ***Aedes aegypti*** **Eggs**

A) *Aedes aegypti* eggs laid in a paper towel used in a field ovitrap. B) Oviposition substrate obtained from live colonies under insectary

Image sources: ZAP El Salvador, El Paisnal Entomology laboratory (A), ZAP Guatemala Insectary (B).



2.1.3 ACTIVATING *A. AEGYPTI* EGGS

When the eggs come from field collections through the use of ovitraps, it is expected that the oviposition substrates (paper towels, fabric, or other) may contain eggs of various ages (from hours to days old) that might have been laid by different females. Because the eggs may be of various ages, hatching may occur at different times. Hence, some eggs may hatch within a few minutes after coming into contact with water, whereas others may hatch within one or two days [4].

Factors such as dry conditions, egg predation by other animals, or inadequate handling of this material may affect egg viability (full development and hatching), resulting in the loss of the biological material sampled in the field. When the eggs are collected via oviposition substrates (paper towels or others) and subsequently stored and transported from the field to the lab, the handling of the biological material is of great importance, and in these cases standard protocols to activate or store them must be implemented to ensure their physical integrity [9].

Ae. aegypti eggs may be activated using distilled water, boiled water, or dechlorinated tap water. Ideally, a small amount of food for the larvae, or yeast, should be added to the water used, which will act as a stimulus for microbial activity and thereby create favorable conditions for hatching.

The number of eggs that should be activated will depend on the quantity of mosquitoes needed for subsequent colony maintenance or bioassays. It should be noted that the viability of the *Ae. aegypti* oviposition is on average 70 - 80%; that is, out of the total number of eggs activated or deposited in a water container, and fortified with some type of larval food, only a percentage of them will effectively hatch and develop into first-stage larvae.

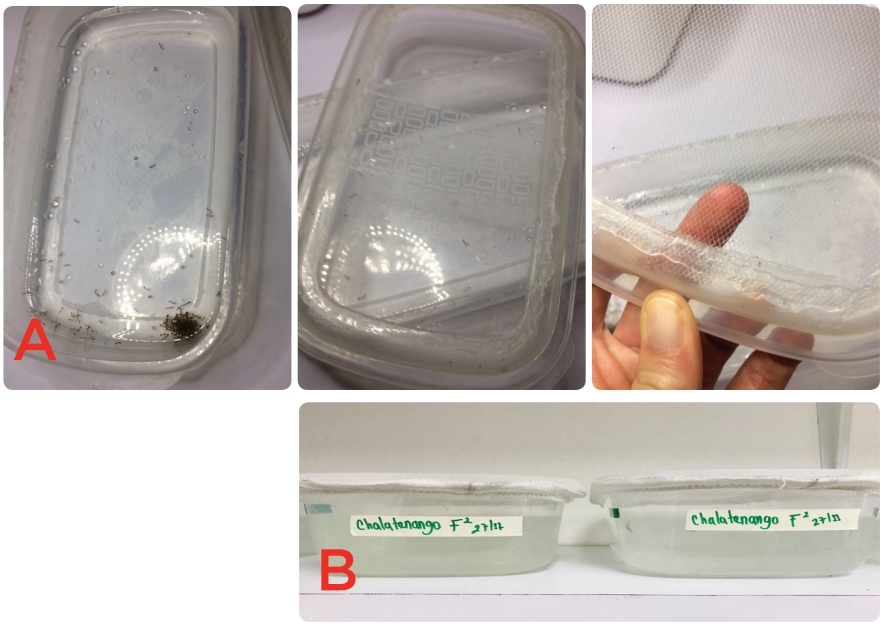
When the oviposition is obtained from female mosquitoes maintained in cages of established *Ae. aegypti* colonies, the eggs' viability will depend primarily on the total amount of food ingested (blood ingested) and the size of each female; as such, this can increase or decrease depending on the particular conditions of each colony, including the microclimate variables.

Once a cluster of eggs has been obtained from ovitraps or lab colonies, the following steps should be taken:

- Prepare a container in which to activate the *Ae. aegypti* eggs. Fill the incubation tray with boiled and cooled water or dechlorinated water, ensuring that the papers containing the eggs are completely submerged. If regular tap water is used, the chlorine content may kill the *Ae. aegypti* first-stage larvae. A fast and practical way to reduce the chlorine content in any volume of tap water is to leave the water in an uncovered plastic container for at least 12 hours, which will allow for the chlorine to evaporate. Be sure to use only clean containers, free of soap residue. As an alternative, boiled and cooled water can also be used.
- Once the eggs have been activated or placed in trays of water, label each tray with the name of the mosquito strain (the name usually corresponds to the geographical origin of the biological material), date collected, and hatching or activation date (Figure 20).
- If the reason for activating the *Ae. aegypti* eggs corresponds to the need to obtain biological material for insecticide susceptibility tests, then calculate the quantity of adults and larvae required for these bioassays before deciding on the number of eggs that should be hatched. Cut the oviposition papers, getting rid of any excess paper that does not contain eggs, before placing them on the tray

Figure 20. Use of plastic trays

Plastic trays used in the activation of eggs and rearing of the immature phases of *Aedes aegypti*. A) The covers have been modified to add a fine mesh that protects the trays from other insects and enables air flow. B) All trays should have corresponding labels and covers.



Source: ZAP El Salvador and ZAP Guatemala Entomology laboratories.

- Add powdered food. The larval food should be macerated and dispensed as a very fine powder. The recommendations on the amount of food needed for immature Culicidae can be found in the following sections of this chapter.
- Cover the trays containing eggs with a fine mesh or screen to prevent contamination of the trays by other female mosquitoes that might have escaped their cage. The mesh cover also reduces the risk of recently emerged adults leaving the trays during periods of time in which the staff are not working (weekends or holidays, when the working hours are reduced) (Figure 20).
- All the viable eggs (containing developed embryos) should hatch within a period of 24 - 48 hours.
- Once the first-stage larvae have hatched, the paper towels with the egg shells should be removed. The idea is to keep the trays clean, and removing any paper remnants will provide more space for the larvae to move freely. Remove the paper very carefully because first-stage larvae are very small and could be easily taken away with the paper.
- Add a pinch of fresh food (macerated fine powder) without saturating the water surface. Cover the trays again and maintain larvae trays daily (the next section gives recommendations on the rearing of *Ae. aegypti* larvae).
- Even though *Ae. aegypti* eggs can be activated in any type of container, it is preferable to use a medium to large square or rectangular plastic tray with a fine mesh cover. Once the larvae hatch, they should be kept in the same container to prevent excessive handling of them if they were to be transferred to other containers. Ideally, the rearing trays are cleaned daily by removing organic waste from the bottom and surface of the water.

2.1.4 STORAGE OF *AE. AEGYPTI* EGGS

Because *Ae. aegypti* eggs can remain dormant once embryonic development is complete, it is feasible to conserve oviposition obtained from the live colonies for future use (months after collection dates). To guarantee that eggs remain viable, the oviposition surfaces (paper towels, wood, or fabric) should remain damp during embryonic development (48 hours after oviposition). Once the embryonic development is completed, the egg will enter into a dormant state and the egg batches can be preserved for future use. If such care is not followed, the insectary personnel might store unviable eggs, which will jeopardize the success of future bioassays.

Following is a list of recommendations for preserving *Ae. aegypti* eggs under insectary conditions:

- After oviposition and for a period of 48 hours, egg batches should be kept in moist conditions, and be examined using a magnifying glass or stereoscope to ensure the membranes have not collapsed. (A collapsed egg means that the membrane encasing the embryo has lost its turgor, causing the egg to die.) The egg strips or paper strips (papers to which eggs are adhered) can be stored in plastic bags with hermetic seals or plastic containers with hermetic lids. Recipients should be labeled.
- Each paper strip should be duly marked with the corresponding *Ae. aegypti* strain and date of collection. Conserving eggs under insectary or laboratory conditions may be carried out successfully over the course of weeks or months, and the insectary staff must rigorously assess the time in which the oviposition remains viable under local microclimate conditions (temperature and humidity). For this purpose, it is recommended that portions of the stored egg papers be activated regularly (every one, three, and five months) to confirm the viability of the stored strain or to correct the storage conditions if mistakes are detected.
- At the time of activating eggs originating from stored material, ensure that the material matches the oldest date, so that continuous monitoring of the stored egg batches is maintained. With such supervision, the insectary staff will know precisely how long it is possible to conserve *Ae. aegypti* oviposition in their local microclimate conditions.
- Paper strips containing eggs should not be stored for longer than six months. Such material will have low or minimal hatching rates.
- During storage, the paper strips should be inspected occasionally to rule out the presence of mold, collapsed eggs, or insect pests inside the containers or storage bags. Even when the containers have hermetic seals, the handling of these containers depends on the training and care taken by the insectary staff.
- If damaged paper strips are found, they should either be activated or removed from the storage containers to be destroyed (see Chapter III for biosafety guidelines). If mold or fungus is detected on the paper strips, the storage containers should be immediately cleaned with a bleach solution (5–10% sodium hypochlorite solution) and be completely dried before reusing.

Chapter II

2. BREEDING AND MAINTENANCE OF *AEDES AEGYPTI* LARVAL PHASE MOSQUITOES

2.2.1 BIOLOGY AND ECOLOGY

The Culicidae and *Ae. aegypti* larval development takes place in an aquatic environment. Once the larvae hatch a four-stage cycle (I–IV, or L1–L4) begins, wherein the larvae are easily distinguishable by size (Figure 21). At each stage of development, a complete metamorphosis can be detected, because every time a larva moves from one stage to another, the exoskeleton molts, and the skin (exuvia) can be observed in the breeding trays.

Figure 21. Larval Stages

Aedes aegypti larval stages

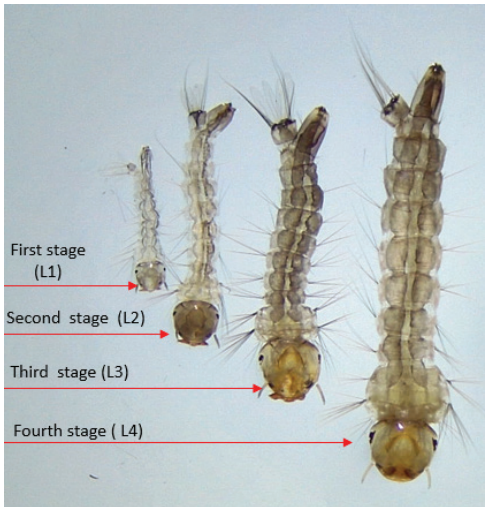


Image source: ZAP El Salvador, El Paisnal Entomology laboratory.

As with the majority of holometabolous insects, larval phase development has four stages noted either as Roman numerals I through IV or with the letter L and an Arabic number (L1, L2, L3, and L4). The larval phase is the period of greatest feeding and growth; in particular, larval stages L1-4 develop rapidly, while stage L4 is prolonged. During the larval stages, the individuals constantly search for food, which they need to accumulate the energy reserves that will permit the development of the pupa phase and, subsequently, the adult phase. In the final aquatic phase, the pupae are very active; during this period, the individuals do not ingest any food, but instead take in only oxygen, through breathing trumpets, until the transformation into the adult phase is complete [10]. The amount of time each individual remains at L4 stage depends in large part on external factors such as the temperature, humidity, available food, larval density of the breeding tray, and the presence or absence of predatory organisms [11].

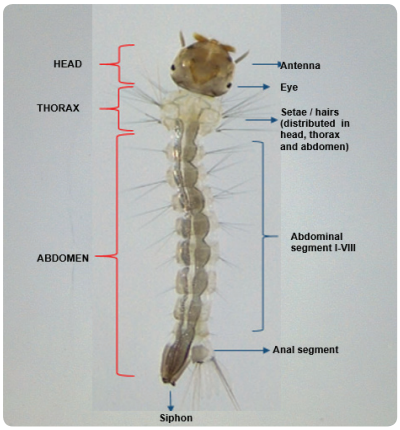
2.2.2 MORPHOLOGY

Ae. aegypti larvae have three distinct regions: the oval head, thorax, and a nine-segment abdomen. The rear (anal) segment has four lobular gills for osmotic regulation and a short siphon in the shape of a barrel with a single pair of sub-ventral tufts (hairs) to breath at the surface of the water [7, 12]. The larvae periodically steer themselves to the surface to breathe by way of posterior spiracles located on the siphon or respiratory tube [7].

Additional morphological characteristics include a narrow head, a pair of chitinized structures (in the shape of a hook) on the side lobes of the thoracic segment, and pecten teeth on the eighth abdominal segment arranged in a single row and in the shape of a trident [8]. Figures 22 and 23 show the general morphology of *Ae. aegypti* larvae; additionally, Annex 1 demonstrates important morphological characteristics to properly distinguish the species *Ae. aegypti* from *Ae. albopictus*, considering that these two species exhibit similar morphologies and often share urban and rural environmental habitats.

Figure 22. Morphology

Dorsal view of an *Aedes aegypti* mosquito larva.



Source: ZAP El Salvador, El Paisnal Entomology laboratory.

Figure 23. *Aedes aegypti* Larvae

Photo of a single *Aedes aegypti* mosquito in larval stage L3.

Image source: C. Torres Gutierrez, ZAP Entomologist.

The different larval stages are distinguishable by the larvae's size, which increases with each stage. The larvae eat constantly and increase in size after every molting and change in the larval phase, releasing the exuviae in the process. The shed exuviae or exoskeleton is visible in the water, especially when the larvae are confined to a breeding tray. At first, the *Ae. aegypti* larvae measure only 1–2 mm long; in the following three stages (L2, L3, and L4), measurements of 2.9 mm, 4.3 mm, and 7.2 mm in length have been recorded, respectively [13]. It should be noted that 80–90% of growth and biosynthesis occurs during the final phase (L4) of larval development, which allows for the subsequent development from pupa to adult. If the larvae have not accumulated the energy needed for the subsequent stages of metamorphosis, the fourth larval stage might be prolonged for weeks, until the transition from the pupa phase is complete, or, alternatively, the larvae die. The nourishment accumulated during the larval phases will be significant in determining the size that an individual will reach adulthood [14].

The resting position of the larvae inside the water column is almost vertical, with a siphon facing the surface of the water, since respiration or oxygen intake takes place through spiracles located in this

structure. With regard to their movement in water, the larvae exhibit very active behavior by moving quite rapidly, in a serpentine pattern. In addition, the *Ae. aegypti* larvae are photophobic, meaning that when they are exposed to a source of light, the organisms will move in the opposite direction.

2.2.3 BREEDING AND HANDLING OF *AE. AEGYPTI* LARVAL PHASES

The most critical variables in successfully rearing Ae. aegypti mosquito larvae are:

- The amount and quality of their food
- Larvae density per rearing tray
- Daily maintenance of the breeding trays

Maintaining an optimal environment for larvae includes sufficient nutrition, stable photoperiod in the insectary room, low density of larvae per volume of water, and ideal temperature. These facilitate the development of healthy larva and the establishment of a productive colony. If any of these parameters is not regulated, the resulting insects will be smaller and generally less vigorous. More detailed information is provided below as key points regarding the maintenance of *Ae. aegypti* colonies:

FEEDING THE LARVAE

During its larval phase, a mosquito's diet consists mainly of microorganisms present in the aquatic ecosystems (bacteria, fungus, protozoa) and organic matter (of plant or animal origin) available in different strata of the water column. Larvae generally feed on organic matter dissolved in the water column, or attached to mineral or plant surfaces. Larvae exhibit different feeding habits, generally known as "filtering," "foraging," "scraping," or "chewing"; each habit involves various degrees of activity. A distinctive feature of feeding during the larval phases is the constant movement of the oral brushes, which allow the entry of small particles into the oral cavity of these organisms. *Ae. aegypti* larvae have a shredding behavior which means they bite off small particles of organic matter and even dead organisms of their own species [15].

For the purposes of maintaining a colony of *Ae. aegypti* under insectary conditions, the most practical solution is to use concentrated food, originally manufactured to feed pets or breed fish. There are many foods of industrial origin that are used under laboratory conditions to feed mosquito larvae; among them are yeast, concentrated foods for pets (dogs, cats, rodents), and other complementary products such as fishmeal and bovine liver powder. Some commercial brands that are commonly used in the maintenance of mosquito colonies are:

- TetraMin® (manufactured by Tetra), nutritional composition: protein 46%, minimum fat content 7%, fiber 2% max., moisture 8% max., phosphorus 1.4% min., ascorbic acid 100 mg/Kg min.
- Truchas 50%® (manufactured by Solla), nutritional composition: protein 50% min., minimum fat content 13%, fiber 4% max., ash 13% max., moisture 13% max.
- Mojarra 38%® (manufactured by Solla), nutritional composition: protein 38% min., minimum fat content 4%, fiber 4% max., ash 12% max., moisture 13% max.
- Rodentina® (manufactured by Agrinal), nutritional composition: protein 23% min., minimum fat content 6%, fiber 5% max., ash 8% max., moisture 13% max.

The diet should be designed to provide all the components needed for optimal larval growth, including a high protein and carbohydrate count and low proportion of fat, as well as complex B vitamins (especially biotin) and minerals (especially calcium) [16]. Providing food with a high concentration of protein increases the likelihood that the *Ae. aegypti* larvae develop successfully, even under insectary conditions. It has been documented that organisms fed with high protein content grow to be larger adults [14, 17].

Different types of food might seem adequate for feeding larvae. However, to ensure a high yield in the established mosquito colonies, it is recommended that the vital parameters such as the developmental time between the immature and adult phases and the adult sizes, obtained from laboratory breeding, be systematically evaluated with each of the chosen or available foods. This will determine the food resource that best sustains a productive mosquito colony and from which healthy and fertile organisms can be obtained for multiple public health uses (see Figure 24).

Figure 24: *Aedes aegypti* Larvae

Aedes aegypti larvae in breeding trays.

Image source: ZAP Guatemala Entomology laboratory.

LARVAL DIET PREPARATION

Procedure to prepare the food:

- Grind any solid food croquettes or pellets, enough for a week, using an electric grinder or manual mortar. The solid food should be ground into uniformly fine particles that are like a fine powder.
- Filter the ground food through a sieve to eliminate remaining large particles. This finely ground food is ideal for feeding larvae in stages L1 and L2; the larger particles that did not pass through the sieve can be saved to feed the later stages (L3 and L4).
- Once the food has been ground, but before the larvae are fed, sterilize the food (if possible) using an autoclave or ultraviolet (UV) light.
- Separate the food into sufficient quantities for feeding during a week. Store the food in a 4°C refrigerator or in a -20°C freezer until ready to use.
- Store the food for no longer than a week. Dispose of the remainder after this period to prevent microbial contamination.
- Weigh the food quantity according to the stage and number of larvae in each tray (see following sections).
- When adding the food to the breeding tray, disperse it evenly on the surface of the water. Use a salt shaker or a small clean spoon to do this. (Keep the implement for this exclusive use.)

FOOD DOSAGE DURING LARVAL PERIOD

Not only is the type of food important to maintain the mosquito larvae, so is the amount of food provided to the breeding tray daily. To maintain a healthy colony of mosquitoes, the breeding trays should be cared for continuously in such a way that a daily feeding and cleaning routine of all the larvae trays is established.

In order for the larvae in the breeding trays to effectively utilize the food, there should be enough food for the entire number of specimens. The amount of food to be added to a breeding tray (usually plastic or metallic, of varying sizes) should be precise and based on the quantity of larvae in the tray. When the amount of food added is insufficient, the larvae's life cycle will be extended and mortality may increase. In contrast, when the amount of food added is sufficient, the development time will be consistent and within the expected average (6–10 days) [3, 18].

It should be noted that during larval phase development (from L1 to L4), the amount of food needed to maintain a healthy colony at a consistent pace may increase. That is, the amount of food necessary to feed early-stage larvae (L1 and L2) is clearly less than the amount needed for larvae in their third and fourth stages (L3 and L4), when their size has increased and they can ingest more organic matter [7].

When excessive amounts of food are added to the breeding tray, the particles can accumulate on the surface of the water, creating a film that prevents the larvae from breathing properly. Moreover, the excess particles may support the growth of microorganisms that infect the larvae, mainly those in the earliest stages, which are more susceptible to the quality of the aquatic environment [7].

It is important that the staff members in charge of the mosquito breeding comply with feeding and cleaning activities of the breeding trays in a consistent manner, every day, and preferably at the same time. The cleaning of the breeding trays mainly consists of removing the skins or exuvias that result from the molting process. It also includes the removal of dead larvae that fall to the bottom of the tray, and excess organic matter from the bottom and surface of the water.

With regard to the ideal amount of food necessary for larvae, important information can be found in references such as Consoli and de Oliveira [19], who describe the amount needed in milligrams per mosquito larva (Table 1). Using this as a reference, it is possible to calculate how much food is needed for a tray holding 100 larvae, for example: 100 larvae, approximately 3 days old, would need an amount of food equivalent to 0.4 x 100 = 40 mg.

Table 1. Food doses for mosquito larvae according to their age (days)

Hatching days	Amount of food mg/ larva
1	0.2
2	0.3
3	0.4
4 - 7	0.6

Source: Consoli R, and de Oliveira R; 1994 [19]

According to PECET records [20], the following specifications for breeding *Ae. aegypti* larvae in an insectary were successful: larvae kept in 26 x 14.5 x 7 cm plastic trays holding a volume of 1000 ml of dechlorinated water and 30 larvae per tray. Each tray was fed with 30 mg of commercial food known as Truchas 50% (See the section about feeding larvae). Feeding and cleaning the trays containing immature larvae was performed daily, at the same time every day. The adults obtained featured a sex ratio equivalent to 1:1. Individual development to reach the adult phase took at least 10 days and a maximum of 11 days.

A document produced by CENAPRESE [18] contains additional considerations of the amount of food and the recommended larval density for insectary colonies.

LARVAL DENSITY

The density or number of larvae per breeding unit (trays or water containers) has an important effect on the development of the mosquitoes, since each individual competes for the food resources and requires a certain amount of space to adequately develop. In simple terms, when breeding trays contain an excessive amount of larvae per tray, the mortality of the individuals will increase rapidly and the efficiency of the mosquito colony will diminish, resulting in a reduced number of adults at the end of each cycle.

It has been demonstrated experimentally that the rates of survival decrease as the density in the breeding tray increases [21], and it has also been documented that a high density of larvae distorts the proportion of *Ae. aegypti* sexes, favoring males over females [22].

The most common problems associated with overcrowding are: longer development cycle, decrease in egg hatching and the development of pupae, weight loss of the pupae, and increased risk of infection from microorganisms that can kill developing mosquitoes. The loss of larval vigor negatively and irreversibly affects the health of an adult mosquito, so attention to space is greatly important. The ideal density to obtain mosquitoes with long and fruitful lifespans is one fourth-stage larva per milliliter of water [23]. Since water depth is not as important for *Aedes* mosquitoes as it is for *Anopheles* mosquitoes, it has been demonstrated that a depth of 2 cm of water is adequate for the survival of *Aedes* larvae [14]. When it is not feasible to estimate the exact density of the early stages (L1–2), since larvae are generally bred at high densities and *Aedes* species tend to cluster in the corners or along the sides of the tray making the counting difficult, then it is advisable to add more breeding trays to ensure that larval density per tray is adequate. Furthermore, two larvae per milliliter is considered an adequate measurement to maintain colonies of healthy individuals [7].

CAUSES OF DEATH IN IMMATURE LARVAE

If unusual behavior is observed in the immature mosquitoes contained in the tray, for example, very slow movement of larvae, or pupae and larvae that remain at the bottom of the tray for extended periods, the insectary staff should act quickly to determine the reason for this behavior. The most common reasons are containers with detergent or soap residues that are acting as contaminants and the presence of microorganisms that cause infection in the external and internal larval tissues. Either can cause the death of the immatures.

COLONIZATION OF FIELD MATERIAL

When attempting to form a colony from material collected in the field, it is preferable to begin with eggs or larvae from a known geographic location. It is important that the geographic origin of a new strain of organisms be registered and that the individuals brought to the insectary to establish a colony originate from different neighborhoods or dwellings to ensure that the new mosquito strain is genetically diverse and does not include the oviposition or larvae generated by only one or only a small number of females. Ideally, a colony representing a specific geographic point should include descendants of various females, or eggs or larvae from multiple females.

Once the material is transferred to the laboratory, a description of the geographical origin should be duly recorded (locality, neighborhood, house, etc.). This material should remain completely separate from other mosquito strains, especially when the insectary has other strains of the same species or when local facilities have only one room for the rearing of immatures.

The next step involves the separation of the different stages of larvae (early stages like L1 and L2 should be placed in separate trays from the older stages L3 and L4). Sorting individuals per developmental stage will facilitate the breeding procedures and synchronize the time in which adults are obtained. Once the individuals are separated by developmental stages in duly marked trays, daily food should be provided and the trays kept clean to facilitate the observation and separation of the biological material.

When larvae are collected in the field for transfer to the laboratory to establish colonies from a known geographical origin, it is common to find in the sample individuals that represent a different genus or species of Culicidae (e.g., *Aedes* and *Culex* larvae). Therefore, one of the most important steps after separating the collected material according to the developmental stage is the taxonomic confirmation of the material. Ideally, some L4 larvae will be used to perform preliminary observations; however, the identification should take place by observing immature and adult phases. Any specimen that does not coincide with the expected species should be removed immediately.

When larvae are collected in the field and transferred to a laboratory, it is best to use water from their natural breading sites for the first few days in the lab to avoid sudden changes and mortality of the wild specimens.

Chapter II

3. PUPA STAGE

2.3.1 BIOLOGY AND MORPHOLOGY

The pupa is the stage of a mosquito's life cycle that follows the larval stage and precedes the adult stage. In contrast with larvae, pupae do not feed; their only function is to protect the specimen that is transforming into an adult. *Ae. aegypti* pupae are very sensitive to vibration and visual stimuli, to which they respond by moving rapidly. As such, it is common to see the individuals moving through the breeding trays with great agility [4].

The pupae resemble a comma and have two main sections, the cephalothorax (a fused head and thorax) and the abdomen (Figure 25) [4, 12]. A pupa's cephalothorax includes a pair of eyes and a pair of respiration tubes called "breathing trumpets," which break through the surface of the water in order to take in oxygen [4]. At the terminal portion of the abdomen is a pair of broad swimming paddles, which help the pupae move in the water. These swimming paddles are wider and overlapping in females; in males, the structures are thin and separated [24].

Figure 25: Characteristics of the pupa

Characteristics of the *Ae. aegypti*.pupa

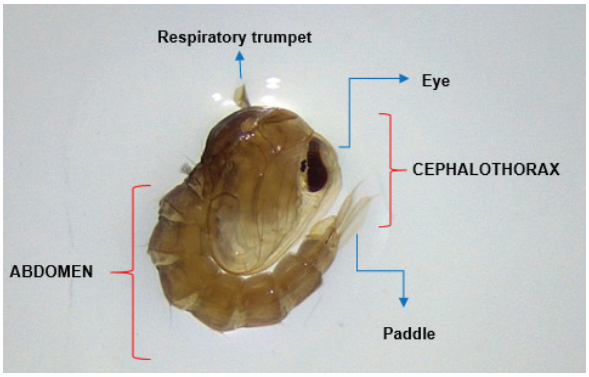


Image source: ZAP El Salvador, El Paisnal Entomology laboratory

When pupae are inactive, they remain on the surface of the water due to their high buoyancy, which assists in the emergence of the imago (an individual in the adult phase). In contrast to the majority of pupae from other holometabolous insects, the mosquito pupae actively move in an aquatic environment mainly as a reaction to external stimuli such as vibrations in the water column or changes in the light intensity.

Pupae development lasts 2–3 days on average, depending on the water temperature. As previously mentioned, when the water temperature and room temperature of an insectary room increases, the insect development cycles tend to accelerate. The amount of time necessary for larval development decreases when local temperature increases [25]. Once the adult has completed its development cycle, the mature individual is ready to emerge or break out: it exerts pressure on the dorsal section of the pupa's cephalothorax, creating an opening from which emerges an adult mosquito, characterized by its soft exoskeleton [5]. After emerging, the adult rests on the water surface for a few hours, while its exoskeleton hardens and flight muscles strengthen so that it is ready to continue its life cycle out of water.

2.3.2 HANDLING PUPAE UNDER INSECTARY CONDITIONS

The pupae should be removed from the breeding trays every day to prevent the uncontrolled emergence of adults. Once the adults emerge and are ready to fly, they can easily escape, thereby creating uncontrolled conditions in the insectary rooms. There are various methods available to separate the pupae. The simplest one consists of using disposable Pasteur pipettes to transfer the pupae, one by one, from the breeding trays to covered and labeled recipient containers on a different shelf or directly inside clean adult cages in the insectary room. The manual separation of Aedes pupae can be time consuming because the pupae generally cluster in corners or along the borders of the breeding trays, and the staff members must develop skills to precisely collect the individuals without causing them physical harm.

The ideal procedure is to remove the pupae from the breeding trays as soon as they have been identified (during maintenance duties of the insectary each morning). Once they have been placed in a water container and separated from other immature specimens, they should be placed in clean medium- to large-sized cages (depending on resources available), duly marked with the name of the mosquito strain or geographic origin and the date they were added to a cage. Introducing the pupae to a cage will ensure that the emergence of the adults occurs in a controlled manner.

Below is a summary of steps recommended to ensure that the pupae separation is performed correctly:

- Use the pipette to collect a small amount of immature specimens and then release them into a clear area of the tray. Once they spread out, pupae will start moving and capturing them will be much easier. If the opening of the pipette is too small it might damage the larger pupae; as such it is recommended to cut the end of the plastic pipette to make the opening wider and efficiently capture pupae without injuring them (Figure 26).

Figure 26. Pupa separation

Separation of *Aedes aegypti* pupae.

Image source: ZAP El Salvador, El Paisnal Entomology laboratory.

Figure 27. Emergence chamber

Pupa can be placed in emergence chambers.

Source: Mosquito Control and Research Unit, University of West Indies, ZAP Jamaica..

- Once the pupae have been separated and transferred to separate containers, the staff can either (1) introduce them in emergence chambers (see Figure 27) or (2) place them inside plastic or metallic mosquito cages, until the adults emerge. All biological material added to the cages or emergence chambers should be labeled with the geographical origin of the mosquito strain, the species, and the date. Maintaining a clear record of the age of the adult individual in a mosquito colony is very important so that the specimens can be used in biological testing (e.g., to assess mosquito susceptibility of mosquitoes to insecticides).
- If the biological material has been unattended or neglected, and the staff forgot to mark the material with dates of pupae collection and adult emergence, the material cannot be used in any type of biological study. Not knowing an adult's age precludes the material from being used in laboratory tests or insecticide bioassays – having unclear information about specimens' age makes it impossible for the staff to determine if the mosquitoes died from old age or the effects of insecticide exposure.

Chapter II

4. ADULT PHASE

2.4.1 BIOLOGY AND ECOLOGY

The adult mosquito, also called imago, is the developmental stage in which the reproductive and dispersal cycles occur. The emergence of an adult *Ae. aegypti* usually occurs around twilight, when the adults exit through their pupal exuviae and begin flying in search of a dry and safe place. The first 24 hours after emergence is known as the "teneral" period, which is a very important time for the mosquito's physiological development. During this period, the exoskeleton hardens, flight muscles strengthen, and most organs, including the sexual organs, finish maturing [7, 14]. By the time the teneral phase ends, the adult is fully mature and ready to fly and mate. In males, maturation of the genitalia, which include the eighth abdominal segment through the tenth segment, can take 18 to 24 hours (during the teneral period), during which their genital organs permanently rotate 180° [5, 26]. Once the genitals have rotated, males are considered mature and capable of copulating with females.

Once the males and females have passed through the teneral phase, both sexes are mature enough to copulate. The males generally emerge before the females so copulation usually takes place before a female begins her search for blood sources. The *Ae. aegypti* species is classified as anautogenous because it relies on a blood meal in order to develop its eggs [27]. Adult males are usually smaller than their female counterparts [5].

Copulating and feeding activities often occur simultaneously because males are also attracted by the chemical and visual signals of vertebrates, as well as by the frequency of the females' wing movement [4]. Under natural conditions, mating can take place in flight or on various surfaces; however, under laboratory conditions, mating frequently occurs when a female is in a resting position either on a vertical or horizontal surface. During mating, the male anchors the end of female's abdomen with his terminalia (a pair of structures that resemble hooks) and inserts his reproductive organ (aedeagus) into the genital chamber. A female's bursa copulatrix fills with a male's sperm, which passes to the spermathecae in a short period of time (1–2 minutes). The sperm is stored in the spermathecae and used during oviposition to fertilize the eggs; the fertilization takes place in the females' internal sexual organs moments before oviposition [4]. Generally, a single coupling with a fertile male is sufficient for a female to receive enough male sperm to fertilize all the eggs produced and deposited during her life cycle [4].

The average life expectancy of adult females varies from 10 to 35 days under controlled conditions [28] and of males from three to six days [7], although these estimates depend largely on the temperature and relative humidity established in controlled experiments. In his book about *Ae. aegypti* biology, Christophers [5] documents experiments where the lifespan of adults of this species can vary widely and depends on multiple factors such as temperature and humidity, and especially the availability of food. The dispersal of adult individuals of this species varies under natural conditions and is influenced by a variety of factors that include the sex of the mosquito, the density of available blood sources, the availability of oviposition

locations, the abundance of plants in human dwellings, the climate (e.g., wind force, humidity, temperature, and precipitation) as well as the composition and configuration of the ecological landscape [29, 30, and 31]. There are numerous scientific records regarding the flight capacity of *Ae. aegypti*, although most publications indicate that mosquitoes of this species disperse within a limited radius, generally 100 to 200 m, as long as basic conditions for their crucial development, such as food sources, and resting and oviposition places, are met. When they are not available, the mosquitoes have been recorded as capable of flying as far as 500 m, even 800 m, from their adult emergence sites [32, 33, and 34].

2.4.2 MORPHOLOGY

The body of an adult *Ae. aegypti* is composed of a head, thorax, and abdomen. The adult phase is easily distinguishable by their dark bodies, with light-colored markings, and the proboscis, or mouth parts, in a characteristic cylindrical shape (proboscis). The adult *Ae. aegypti* have a pair of wings and bodies with characteristic silvery-white markings as lines on their thorax and white bands on their legs (the base of the tarsal segments) and on abdominal segments. As with most insects, the mosquitoes have three pairs of legs. The three pairs (anterior, middle, and posterior) flow from the lateral sections of the thorax. Leg length may differ among the different pairs, but the posterior pair are usually the longest. The thoracic spots found on the dorsal surface of these mosquitoes are the most common and appear in groups of silvery scales in a half-moon shape, or curved line, one on each side of the anterior half of the dorsal part of the thorax; between the half moons, two straight silvery lines pass parallel (made of silvery-white colored tiny scales), following the line of the body until almost reaching the scutellum. This ornamentation is known as the "lyre shape" that is commonly mentioned in textbooks (Figures 28 and 29).

Figure 28. Morphology

Basic morphology of an adult *Aedes aegypti*.

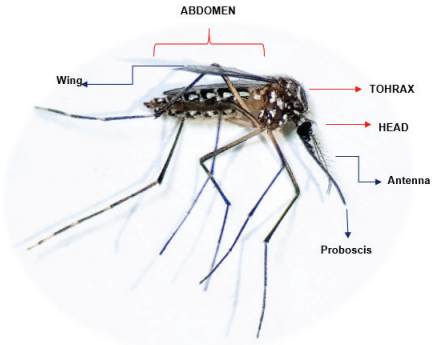


Image source: Katia López, ZAP Honduras.

Figure 29. Male and female *Ae. aegypti*

Male (left) and female (right) of *Ae. aegypti* species

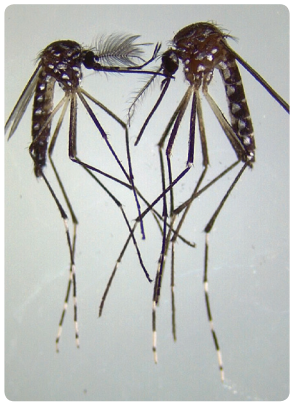


Image source: ZAP El Salvador, El Paisnal Entomology laboratory

The *Ae. aegypti* males and females are similar in appearance, except for the difference in their size and the shape of their antennae; the males have feathery and pubescent antennae, while females have filiform antennae with little pubescence (Figure 29). In addition, the maxillary palps are similar in length to the proboscis (long palps) in males, while the females have shorter palps, less than half the length of the proboscis. Another characteristic of the adult females (when observed with an stereoscope) is their acute abdomen apex. By closely examining the surface of the lateral thorax, the presence of post-spiracle setae can be seen, although they do not have spiracle (or pre-spiracle) setae. It is important to note that the size of adult mosquitoes depends on a combination of genetic material (which determines all physiological processes in insects) and the environment in which the mosquitoes develop. For example, it has been

demonstrated that adults obtained from larvae bred in laboratory conditions in trays with a high density of individuals breed small-sized adults (reduced size). As such, these female adults will ingest a smaller amount of blood than a large-sized adult, and will also deposit fewer eggs. Therefore, it is clear that the care, larval density, and the amount of food supplied during the immature phases have a direct effect on the physical and physiological characteristics of the adults [35].

See morphological differences between *Ae. aegypti* vs. *Ae. albopictus* species in a supplementary visual aid displayed in Annex 1.

2.4.3 REARING AND HANDLING ADULT MOSQUITOES

In order to rear adult mosquitoes, one can use cages of different sizes. The cage sizes most commonly used are 30.5 cm^3 for an approximate specimen population of 250 - 500 [18, 19]. The mosquito population must be adjusted to the size of the cage. For example, for a 30.5 cm^3 size cage, the total population should be around 500 specimens, with a ratio of 250 males to 250 females. This facilitates the colony's reproductive cycle within the insectary conditions [18]. However, other authors [36] recommend a ratio of 3 females to 1 male to optimize the cage space of a 30 cm x 30 cm x 30 cm cage. With this ratio, you would obtain a vertical surface density of 0.5 mosquitoes/cm inside the cage. The increased density of adult mosquitoes within the cage would also positively influence the number of eggs obtained from a given colony as long as successful methodologies for feeding the females within laboratory conditions are applied.

On the other hand, it is possible and even recommended to maintain a smaller number of adults within each cage (150–300 mosquitoes per 30 cm x 30 cm x 30 cm cage with a 1 female to 1–2 males ratio). This would facilitate the interior cleaning procedures for the cage, and increase the success during the feeding process. However, the density of adults per cage should be established after careful observation of the mosquito development in local conditions, and in accordance with the number of available employees in the insectary and the deadline for when a given number of mosquitoes must be obtained for use in biological tests or other scientific procedures. All these factors vary in every situation, and depend on existing resources in each country or institution that supports the insectary facility.

Mosquito cages must be efficiently attended on a daily basis, with care to prevent the escape of the individuals. As mentioned in Chapter I of this document, all mosquitoes that escape the cage must be eliminated. The staff will select the most appropriate disposal measures based on their resources. No mosquito captured inside an insectary can be introduced back to any cage of the colony. Only adult mosquitoes that have emerged from reared pupae can be used for adult colony formation of *Ae. aegypti*. All the cages should be properly marked with the following information:

- Origin of the *Ae. aegypti* strain, i.e., indicate the place where the material was collected. If the strain corresponds to a standard strain like Rockefeller or New Orleans (strains that have remained for years inside insectaries and are used as the control group in bioassays as 100% susceptibility to insecticides is expected), cages must be so marked.
- The dates in which new pupae were entered into mosquito cages and dates of adult emergence.
- The name of the person responsible for each cage or each strain.
- Any other relevant information needed to establish the identity of the strain and to allow the reared mosquitoes to be used in bioassays.

Ideally, adult cages are the source of new egg batches that will facilitate or limit the successful growth and maintenance of a mosquito strain under controlled conditions. One of the key factors for adult colony maintenance is the density of specimens in the cage. High mosquito densities could cause an increase in mortality and low fertilization of female specimens. Even though mosquitoes of the species *Ae. aegypti* are easily maintained in laboratory colonies, one should remember that they are living organisms, with specific biological cycles that are easily altered by artificial conditions kept in insectary rooms, for example, lack of food, constant changes in the photoperiod (light cycles vs dark cycles), and overcrowded cages that generate stress and mortality in live colonies.

Maintaining *Ae. aegypti* colonies under insectary conditions requires a specific infrastructure to maintain the ideal conditions of temperature, humidity, and food availability (larval food and sugar solution and blood sources for adult individuals). The decision to maintain mosquito colonies must correspond to very clear objectives of basic and applied research, or for training purposes by local health authorities or academic institutions. Maintaining a colony of hematophagous insects, for reasons that correspond to each individual institution, also means that there is a risk that such insects will escape from their cages into the outside environment and increase the local mosquito population in the geographic region where the insectary is located. For this reason, the establishment of mosquito colonies should always follow guidelines and national regulations that require the supervision and allocation of appropriate human resources for this type of space and manual labor.

FEEDING

- **Feeding and Nutrition:** According to Consoli and de Oliveira [19], "the energetic metabolism of the vast majority of Culicidae mosquitoes, both females and males, depends on the ingestion of carbohydrates, usually from plant substances or juices (flower nectar, fruit juices and plant sap). For adult mosquitoes' bodies, glycogen and triglycerides accumulation is a determining factor for both their vital energy and their longevity. In the majority of Culicidae females, the hematophagous habit is based on the need for protein or essential amino acids for egg development. In the same way, feeding based on blood contributes to the females having a longer life span than males."

  Plant sugars are the principal food source for mosquitoes. In nature, the most common sources of sugars are flower nectar, but there are other sources such as ripe fruit or vegetative tissues [37, 38]. When mosquitoes ingest carbohydrates, these substances are directed to the central diverticulum (part of the digestive system), from where they slowly pass to the stomach. In this way, carbohydrate digestion occurs at a slow rate. This mechanism allows the females to maintain an empty stomach in order to ingest blood. Some specific carbohydrates considered important in mosquito nutrition include glucose, sucrose, maltose, and fructose [19].

  In the case of *Ae. aegypti* females, the amino acids needed for the development of the ovaries can be found in some nectars, but the concentrations are not high enough to replace the protein obtained from blood ingestion.

  In laboratory conditions, the general recommendation is to make a sugar solution available to mosquito colonies, at most times. An alternative carbohydrate source for mosquito colonies are fruit portions of oranges, bananas, watermelon, or other juicy fruits with high sugar content. The concentrations of sugar solutions can be 5% to 15%, with the most common concentration being 10%. Solutions can also be prepared with honey in the same concentrations [38].
  Basic steps for making a solution are the following:

  - On an electronic balance, weigh 10 g of white sugar.

- Add 90 ml of distilled or boiled water.
- Mix well until the sugar crystals are completely dissolved.
- Label the bottle with the date of preparation and the initials of the person responsible for making the solution.
- Keep this solution refrigerated (4°C) to avoid bacterial growth and prepare a new solution every week.

The best way to deliver the sugar solutions to live colonies is through a cotton swatch or cotton balls moistened with the solution and placed atop the mosquito cages in a way that gives all the individuals access to it through the pores of the cage wall. An alternative is to place the moist cotton in a plastic container, leaving a portion of it uncovered, and to hang it from the upper side of the cage, so the cotton holder remains fixed to the top the cage. If the device with the soaked cotton is placed in the lower part of the cage, it is possible that the females will lay their eggs on this cotton. If this happens, it will be very difficult to isolate the eggs from the cotton fibers.

Keep the cotton supplies in tight containers and away from high humidity. Change the soaked cottons once introduced in the mosquito cages daily..

Blood Feeding: The vast majority of Culicidae species have hematophagous females, as blood is the essential resource that provides the protein content required to complete the oogenesis or egg development in the ovaries, and to complete the development of a complete batch of eggs. Every oviposition is preceded by one or more events of blood ingestion.

One of the challenges to rearing mosquitoes in the laboratory is finding a way to administer a blood meal for adult females on a frequent basis so that the reproductive cycle of the species is met. Finding a method to deliver blood to live colonies is key if the goal is to maintain live colonies consistently.

There are various sources of blood that include the use of vertebrates such as cattle, rabbit, guinea pig, mice, sheep, and birds. A conventional feeding method has been the direct use of these animals (or their blood). However, their use must be coordinated with international ethical regulations that rule the maintenance of bioteriums (animal facilities) with proper scientific rigor involved [39]. These ethical conditions are necessary for the maintenance of mosquito colonies as well, to ensure that all procedures follow good scientific practices, and comply with international standards that dictate avoiding overcrowding, cruelty, or stress to live organisms. For this reason, there are also artificial feeding methods that use vertebrate blood provided from blood banks or from livestock research facilities. Institutions that legally obtain and store medical supplies represent a useful resource for obtaining small volumes of blood through standard practices and under supervision of local authorities in each country.

Direct feeding method: In the past, insectary personnel would volunteer to feed mosquito colonies by exposing one of their arms inside the mosquito cages. This practice is no longer used. Instead, the recommendation is that the insectary employees employ direct feeding methodologies using laboratory or bioterium animals, or, as a more current alternative, artificial feeding (using only the blood and not the actual animals). Whatever the choice, the insectary and the staff must comply with sustainable and bioethical practices that also follow the standard and international guidelines (cited in Chapter III of this manual).

Direct feeding consists of using live animals for hematophagous mosquito colonies. With this method, one shaves/plucks and immobilizes the source of blood (mammal or bird) and places it inside the cage for 15 to 30 minutes [40]. This type of direct feeding should be done with animals that are sedated. When the source of blood is mice or small animals, this can be achieved with anesthetics or with devices

that restrict the animal's movement (Figure 30). For mammals, the use of a veterinary prescription for an anesthetic is recommended. In this way, the mosquito feeding is facilitated and the time in which the laboratory animal is exposed to mosquito bites is limited [40]. When live immobilized animals are used, it is of utmost importance to use anesthetics that are prescribed by a professional veterinarian because the dosages and type of anesthetic might change with the animal used.

Figure 30. Direct feeding

Direct feeding of *Aedes aegypti* exposing a lab mouse

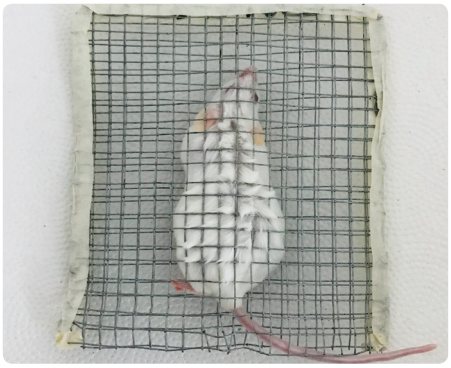


Image source: ZAP El Salvador Entomology laboratory, El Paisna

- **Artificial feeding methods:** Artificial feeding techniques that use only small volumes of vertebrates' blood for the mass breeding and maintenance of mosquito colonies have great advantages over the use of live animals as a blood source in terms of productivity and convenience.

  Recent studies have shown that when mosquitoes feed on human blood, their feeding success, fertility, and oviposition rates are much greater than that of mosquitoes from colonies fed with bovine or poultry blood. However, procuring blood sources to maintain mosquito colonies depends on a country's or institution's resources. Ideally, small quantities of blood should be obtained from specialized blood banks or livestock facilities that follow standard and legal procedures. In some countries, the procurement of human blood is limited [41]. Thus, educational institutions and public health laboratories should consider institutions that host livestock facilities and research for the legal procurement of pathogen-free blood.

  Artificial feeding systems differ in the techniques, in terms of membranes and mechanisms, they use to maintain blood so they can supply an adequate volume of blood to the mosquito colonies. Procedures involve the use of artificial membranes at a temperature similar to the body of endothermic vertebrates (organisms capable of regulating and maintaining their body temperature at 37°C) [42].

  The artificial feeders usually consist of bell-shaped glass devices that function as blood receptors. They are covered by an artificial membrane (synthetic or animal sourced, such as laboratory parafilm or intestines) that allow the circulation of hot water from an analog bath (Figure 31, 32, and 33). The analog bath, or "water bath," consists of an electric water tank that keeps the water at a constant temperature (38 ± 1°C) and pumps hot water through a system of latex hoses attached to the water tank. The circulating water maintains the blood at a warm temperature so the female mosquitoes in the colony can feed on the blood contained in the glass device or other artificial feeder. The feeder is located above the container or cage in which the female mosquitoes are kept (Figures 31, 32, and 33) [44].

  The blood given to the mosquitoes from artificial feeders can be of human or animal origin. Generally, the blood is supplied in conjunction with an anticoagulant substance that does not have negative effects on the mosquitoes. The most common anticoagulant is Heparin [43]. It is important to note that once the blood is obtained from a clinical blood bank, via legal means, it needs guaranteed refrigerated transport until its

Figure 31. Bell-shaped glass devices

Bell-shaped glass devices with blood and a membrane (parafilm), used as an artificial feeder..

Image source: A. https://www.m.ehime-u.ac.jp/school/parasitology/eng/Thai0108-E.htm; B. National Institute of Health (Instituto Nacional de Salud, INS), Bogotá, Colombia.

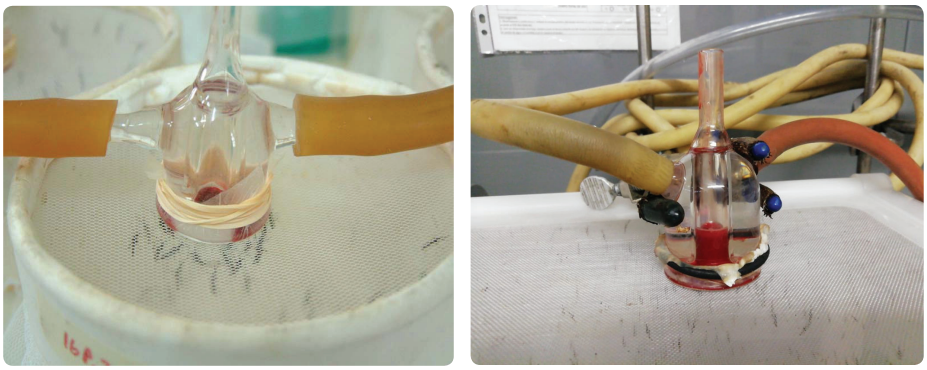


Figure 32. Artificial feeder Colombia

Artificial feeder used in Culicidae mosquito colonies

Image source: INS, Bogotá, Colombia

Figure 33. Artificial feeder Jamaica

Artificial feeder used in Culicidae mosquito colonies at the Mosquito Control and Research Unit, University of West Indies, Jamaica.

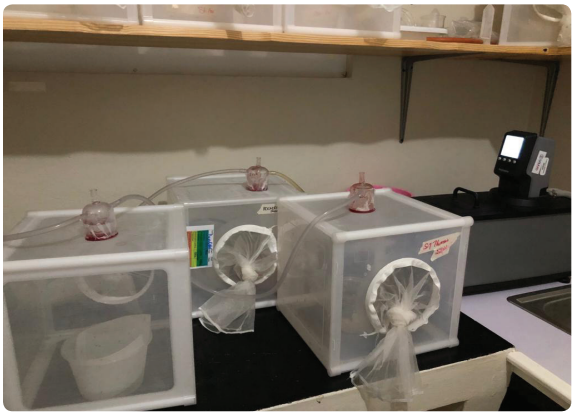


Image source: ZAP Jamaica.

arrival at the laboratory or insectary. The institution also needs to use an anticoagulant to ensure that the blood will be available for the female mosquitoes.

- **Use of animal-sourced membrane:** An alternative to the use of bell-shaped glass devices for the artificial feeding of *Ae. aegypti* colonies is the use of membranes of animal origin, such as vertebrate's intestines or membranes obtained from the skin of animals such as rabbits or poultry [45]. Figure 34 shows a Spanish product called "edible collagen gut," a membrane that can be filled with blood with an anticoagulant and placed atop a mosquito cage to feed the females of the colony. This collagen gut, once filled with blood, is similar in shape and appearance to an edible sausage.

Figure 34. Artificial membrane

Artificial membrane of animal origin

Image source: Photo by C. Torres Gutierrez. Courtesy of the Ministry of Health, Jamaica, 2017.

The obvious disadvantage to using an animal membrane is that it cannot keep the blood at the ideal temperature by itself; glass containers filled with hot water must be placed beside the animal membrane to keep the blood warm.

While the artificial feeding option is both a modern and efficient way to maintain mosquito colonies, financial resources are not always available to obtain the necessary equipment (analog bath and glass bell-shaped devices). An alternative that is within reach for all institutions is the bottle method, developed by the Program for the Study and Control of Tropical Diseases (PECET) of the University of Antioquia in Medellin, Colombia. It uses a glass bottle filled with hot water, with cotton or sterile gauze soaked in human blood supplied by a clinical blood bank (Figure 35).

Materials for the bottle method:

- Glass bottle with a screw top, refractory (resistant to heat) with 250 ml capacity
- 250 ml of hot water (40°C)
- Cotton pads (preferably rectangular, sterile)
- Disposable 3 ml syringe
- Blood with anticoagulant (resource obtained from a clinical blood bank)
- Adhesive tape (surgical tape)

Procedure

- Attach the cotton pad around the bottle with the adhesive tape, as if you were placing a bandage on the bottle.
- Fill the bottle with hot water (40°C) and close the bottle.
- Using a syringe, uniformly distribute 3 ml of blood on the cotton pad as demonstrated in Figure 35.
- Place the bottle in the mosquito cage and keep it in the cage for 30 minutes.

The advantage of using blood supplied by a clinical blood bank is that the blood can be preserved in a refrigerator (at 4°C) for one week. This enables the mosquito colonies to be fed at least twice per week, as recommended.

Figure 35. Bottle method

Feeding *Aedes* females with the bottle method.

Image source: ZAP Guatemala Entomology laboratory. Photo by Ricard Busquets. The original method was developed by PECET of the University of Antioquia, Medellin, Colombia.

- **Preparation of the colony prior to blood feeding:** According to Christophers [5], both virgin and fertilized female *Ae. aegypti* will be receptive to blood ingestion for 18 to 24 hours after their emergence.

  When staff intend to feed mosquito colonies with blood, regardless of the method chosen (direct or artificial feeding), they should adequately prepare the mosquitoes to ensure that the females are alert and hungry when the blood source is delivered. It is recommended to remove the source of the sugar solution or soaked cotton at least 3–4 hours before the feeding occurs. The colonies may respond differently to the feeding techniques depending on the local temperature and humidity. However, it is essential to consider the species' behavior to facilitate the delivery of the blood meal to the females. For example, *Ae. aegypti* is a diurnal species, which means it is most likely to feed during daylight hours, if females have been correctly prepared (the sugar solution has been withdrawn). In this case, and under local insectary conditions, the feeding system should be organized during the day. In contrast, colonies of *Culex* species feed nocturnally, so their blood meal should occur at night. These conditions can be created inside the insectary room by maintaining the room without light during the feeding process.

  Additionally, it is advisable to excite the females in the colonies through close contact of the human staff to the cages where specimens are kept. It is recommended that the staff get close to the cages and breathe out several times, so that the carbon dioxide they exhale is perceived by the *Aedes* females. The caged insects will recognize both the chemical signal of the released carbon dioxide and also the body temperature of the humans standing next to the cages. The staff member should provoke the females via his/her respirations and presence for a few minutes before the feeding is administered.

  Whatever the method chosen to feed mosquito colonies, the procedure used should be consistent. The ideal frequency for administering a blood meal to female mosquitoes is twice a week, at the same time of the day, and it is recommended that the colonies be kept in a quiet room for a minimum of 30 minutes while the females feed.

  The best procedure is when the staff follow a rigorous timetable for every practice and maintenance task needed in a mosquito colony. **The successful maintenance of a mosquito colony depends upon the organization, consistency, and rigorousness of the procedures that are carried out.**

- **Recommended actions after a blood feeding:** After every feeding event, the staff responsible for the feeding should register the number of engorged females by observing the distended abdomen (an obvious sign of blood ingestion) in every cage of specimens that have received the blood meal. This is to create a careful record and count of the number of females with full abdomens, and in this way obtain enough information to evaluate the impact of each feeding method on the respective colony.

  To avoid handling organisms with a distended abdomen, fed *Aedes* females should be kept in the same cage at complete rest for at least 24 hours. If it is absolutely necessary to transfer the females to another cage or container, the engorged females should be handled only 24 hours after the successful feeding. The cages where blood feeding has occurred should always have cotton soaked with a 10% glucose solution readily available and replaced daily.

  After the blood ingestion, *Ae. aegypti* females begin the process of digestion and the corresponding development of eggs within their reproductive tissues (ovarioles). This period is called the gonadotrophic cycle, which generally occurs within 2–3 days after the female has ingested blood and reached the maximum of its abdominal capacity. In cases when females do not attain a sufficient blood intake with the first feeding, a second feeding is necessary. Only when females are fully engorged they will receive the necessary amount of protein for the development of a complete batch of eggs. The gonadotrophic cycle is by definition the period between the blood ingestion and the oviposition [5].

The duration of each gonadotrophic cycle in *Aedes* females depends strictly upon the environmental temperature, ideally 27°C. The cycle tends to be shorter as the temperature rises [28]. This information is important for estimating the moment that the eggs will be deposited by the females kept in a colony, and when the staff may collect them. In general, after a group of females has been successfully fed (by whatever method previously mentioned), the time frame for oviposition is 48–72 hours. It is recommended that all the factors are documented by the insectary staff, including: the temperature at which the colony was successfully fed, and the time when the first, second, and later oviposition events took place. These records will establish the productivity of the colony according to the local microclimate conditions [18]. It is important to note that the temperature is the most important environmental factor that regulates the females' appetite, as well as the cycle in which the digestion and egg development occurs in the system of each individual female.

- **Instructions to obtain egg batches from a mosquito colony:** Forty-eight hours after females are successfully fed the staff should introduce a plastic container (transparent, white or black) to which an oviposition substrate has been added. The oviposition substrate can be a piece of white cloth (pellon), resistant paper towel, lab filter paper, or even coffee filter paper. The amount of water should be minimal so that the females do not drown during the oviposition. The container is introduced in the cage for 48 hours (Figure 36).

Figure 36. Container used to collect eggs

Container used to collect eggs from cages with fed females

Image source: ZAP El Salvador, El Paisnal Entomology laboratory

After the oviposition, the embryos have not completely developed and need additional time to mature before they hatch. To allow for embryo development, the substrate is kept in the container (ovitrap) for 48 hours, thus maintaining moisture in the paper strip without the water covering the eggs. During this process, it is recommended to keep the oviposition vessel and substrate under observation and protected from possible pest/insects. After this period, the substrate is removed and allowed to dry in a protected area, under strict supervision, to avoid insects that prey on the eggs (see chapter I). When the egg substrates are almost completely dry, they are stored in closed containers and properly labeled [18] (with the name of the strain, date of collection, and corresponding generation of the strain). See the section regarding storing eggs in laboratory conditions.

Chapter II

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III. Biosafety in Entomology Laboratories

Chapter III

1. INTRODUCTION

The World Health Organization (WHO) published the third edition [1] of the "Biosafety Handbook in Laboratories," stressing the importance of incorporating basic biological safety concepts and practices to ensure the risk-free handling of biological agents and/or chemicals.

According to the WHO [1]: "Laboratory biosafety is the term used to describe the containment principles, technologies and practices that are implemented to prevent unintentional exposure to pathogens and toxins, or their accidental release." And "laboratory biosecurity refers to institutional and personal security measures designed to prevent the loss, theft, misuse, diversion or intentional release of pathogens and toxins."

The biosecurity principles to be applied in laboratories include the universal precaution code for laboratory work referring to personal protective measures for all personnel, implementation of the use of barriers to avoid direct contact with materials harmful to human health, and the disposal of contaminated material to reduce the risk to workers' health, the environment, and the community.

On the basis of this framework, potential risks to the health of humans working in entomology laboratories, where vector control strategies are developed with basic research techniques, come from inadequate handling of reagents or chemical substances. These workers can contaminate the environment by directly disposing of biological or chemical waste. Implementation of basic biosecurity standards will reduce the risks and ensure the quality of work for the laboratory staff, and for the community at large.

To be successful, any biosecurity measure implemented in an entomology laboratory should be based on a specific risk analysis that considers local conditions, characteristics of the facility, and type of activities conducted in each space. The risk evaluation should consider all the arthropod species being tested/reared, the external environment, and the level of risk in the event of a possible release, among others.

This chapter presents the basic tools to ensure compliance with international guidelines for biosafety and biosecurity. It is structured as follows: risk assessment, biosafety and biosecurity, best practices for entomologists, safety and infrastructure equipment, and laboratory waste management.

Chapter III

2. RISK ASSESSMENT

3.2.1 MAIN FACTORS TO CONSIDER IN THE RISK ASSESSMENT

A key step in complying with the international guidelines on biosafety is doing a risk assessment, which should be carried out systematically, as needed. The risk assessment is the most basic approach for determining the appropriate level of biosecurity that a laboratory or insectary requires if it is to avoid a possible adverse event, such as the release of the organisms or reagents being used in the facility.

According to the WHO, the main factors to consider in the risk assessment fall into two categories:

- The risks of working with infectious agents
- The risks of laboratory procedures

A qualitative assessment is recommended and all aspects of procedures and facilities should be identified (see below), taking into account the current international guidelines such as the Biosecurity Guide in Microbiology and Biomedicine Laboratories (BMBL) [2], WHO Biosecurity Manual, and Guide to Arthropod Containment by the American Committee on Medical Entomology of the American Society of Tropical Medicine and Hygiene (ASTMH) [3].

The variables to consider are the following:

- Classification of risk groups associated with laboratory work described in the BMBL guidelines [2]. Such groups include Group 1–4, depending on the degrees of risk at the individual level vs. community level.
- Risk factors for human health from the direct effects of bites, workspace infestation, or others, and indirect morbidity due to transmitted pathogens [1, 2].
- Potential risks of the physical laboratory facilities that could affect workers' health [1, 2].
- Personal protective equipment (PPE) according to the type of work required in the lab.
- Criteria for effective arthropod containment [3].

3.2.2 ARTHROPOD CONTAINMENT

According to the ASTMH's Arthropod Containment Guide: "arthropod risk assessment is primarily a qualitative judgment and several factors must be considered in combination: the agents transmitted, whether the arthropod is or may be infected, the mobility and longevity of the arthropod, its reproductive potential, biological containment, and epidemiological factors influencing transmission in the proposed location or region at risk." Arthropod vectors of infectious agents can be assigned to the following discrete categories:

- Category 1: Arthropods known to be pathogen-free
- Category 2: Exotic and indigenous arthropods that contain specific pathogens
- Category 3: Arthropods containing unknown infectious agents or whose status is uncertain
- Category 4: Arthropods containing recombinant DNA molecules

A supplementary guide for questions to identify risk factors for each facility within categories 1 and 2 is in Annex 2.

The lab personnel or supervisor must carry out the risk assessment at the beginning of laboratory activities and on an annual basis to verify the conditions according to the scope of work. The laboratory director must ensure that the assigned personnel has received the necessary training to recognize the potential risks of their activities and implement the appropriate measures for each level of biosecurity that corresponds to the local installation, work proceedings, and scope of the vector studies.

Chapter III

3. BIOSAFETY IN LABORATORIES

The Centers for Disease Control and Prevention established four levels of biosafety that define safe conditions for working with biological agents in research laboratories [2]. According to the biosafety level, there are recommended measures for the design and construction of the laboratory; means of containment commensurate with the work involving infectious agents; and equipment, practices, and operating procedures for the defined risk groups.

3.3.1 LEVELS OF BIOSAFETY IN LABORATORIES

The WHO biosafety manual [1] classifies the levels of biosafety according to the type of laboratory, as follows:

- **Basic Laboratory - Biosafety Level 1 (BSL-1):** Refers to basic teaching and research laboratories that use biological agents of low pathogenic potential with basic microbiological practices and techniques. This is the basic level of protection and is appropriate for agents that do not cause disease.

  The biosafety considerations do not require special practices, nor an isolated area, safety cabinets, or special containment facilities, usually applicable to conventional research laboratories. However, the areas need to be marked with symbols signaling the location where live organisms are being kept, and where the biological hazards or waste are placed, the use of basic PPE, and the installation of safety equipment, among other requirements.

- **Basic Laboratory - Biosafety Level 2 (BSL-2):** Refers to laboratories with primary care, diagnostic, and research services. It applies to laboratories that work with agents that pose moderate hazards to the personnel and the environment.

  BSL-2 builds upon the recommendations of BSL-1 and also includes [2]:

  - Laboratory personnel must have specific training in the handling of pathogens and in the management of infectious agents and associated procedures.
  - Laboratory areas have restricted access.
  - All procedures that might create infectious aerosols or splashes are carried out in a biological safety cabinet or other physical containment equipment.
  - Procedures are in place to minimize the creation of aerosols and splashes.
  - Laboratory personnel wear protective clothing.
  - Careful management of needles and other sharp objects is prioritized.
  - Laboratory equipment is routinely decontaminated.
  - Laboratory has a strict protocol for the storage of biological agents.

- **Containment Laboratory - Biosafety Level 3 (BSL-3):** "Applicable to clinical, diagnostic, teaching, research or production facilities where work is performed with indigenous or exotic agents that may cause serious or potentially lethal disease through the inhalation route of exposure" [2]. The facilities classified under this level involve the handling of pathogens that represent a risk of transmission to the personnel, but a low risk of transmission to the community. As part of the laboratory practices, all personnel must be trained in handling pathogenic agents, wear protective clothing, use appropriate microbiological techniques and biological hazard signaling, and be constantly supervised.
- **Maximum Containment Laboratory - Biosafety Level 4 (BSL-4):** This level of biosafety is required for facilities that work with "dangerous and exotic agents that pose a high individual risk of aerosol-transmitted laboratory infections and life-threatening disease that is frequently fatal, for which there are no vaccines or treatments, or a related agent with unknown risk of transmission" [2]. Such laboratories should have restricted access and incorporate the use of airtight entry chambers, the use of specialized PPE, shower exits, and special waste disposal according to WHO recommendations [1]. It is recommended that the BSL-4 laboratory be monitored or controlled by national health authorities, enforce controlled access to the laboratory, and follow strict scientific methodologies.

  For the effective implementation of biosafety and biosecurity standards, it is essential, to designate a manager or supervisor who constantly verifies the biosafety processes in the work areas and the development of a continuous education program for all personnel.

3.3.2 BIOSECURITY LEVEL IN ENTOMOLOGY LABORATORIES

This section describes the biosafety and biosecurity measures for BSL-1 and BSL-2 levels that are to be implemented in entomology laboratories, taking into consideration the criteria in the WHO biosafety manual [1] and the ASTMH arthropod containment guide [3]. Depending upon the type of research conducted, the defined activities, and the associated risks, basic entomology laboratories can be defined as BSL-1 or BSL-2. Such laboratories focus primarily on Ae. aegypti research for public health, and employ basic microbiology techniques with low or moderate risk to the workers.

When using pathogen-free vectors that are already present in the local geographical areas, regardless of active disease transmission, the entomology laboratory would be included within BSL-1 and the arthropod containment level 1 (ACL-1) [3], in accordance with the criteria established by the ASTMH Arthropod Containment Guide.

However, when studying individual specimens of Ae. aegypti collected in the adult phase for laboratory studies during an outbreak of some disease, it is recommended to classify the laboratory as BSL-2, and the ACL as 2 as well (ACL-2), again according to the ASTMH Arthropod Containment Guide. All entomology laboratories should implement special practices and stricter containment measures to protect their personnel.

BSL-2 is based on the practices, procedures, containment equipment, and requirements of BSL-1 facilities; however, it is stricter in physical containment and special practices when working with adult mosquitos collected in the field.

To ensure that a particular laboratory adequately complies with the established biosafety standards, it is necessary to implement practices, evaluation tools, and, in general, various measures, to enable staff to understand the potential risks and to implement all appropriate actions during their work routines, with constant training on the use of safety equipment and regular supervision.

Chapter III

4. STANDARD PRACTICES FOR ENTOMOLOGY LABORATORIES

- Entry into the laboratory is restricted and each entry must be marked and controlled by the lab technicians in compliance with the general standards. The visitors or external persons entering the laboratory must be accompanied by internal staff. Cleaning personnel must be trained on the potential risks and consequences of coming into contact with live arthropods and, if applicable, with infectious agents.
- The entomology technicians, entomologists, and lab technicians must be trained in laboratory practices and work procedures to minimize occupational hazards and arthropod release.
- There are standard procedures for handling needles and other sharp objects and these are strictly applied to all working areas.
- All biological material entering the laboratory must be registered, and the personnel handling it should be wearing PPE.
- Laboratory personnel must wash their hands before and after handling biological material.
- The use of PPE and application of hygiene practices is compulsory in work areas. The use of gloves is obligatory for handling insecticide and chemical products. The gloves must not be used to open laboratory doors. Gloves should always be discarded after use.
- A cleaning and disinfection plan (or calendar) must be established for each work area including the insectary's equipment and tools.
- To eliminate any possible contamination, work surfaces must be decontaminated at the end of each day or when a spillage or splatter occurs.
- Periodic checks and cleaning of the entire laboratory, furniture, and insectary rooms must be done to avoid insect pests or undesired accumulation of clutter.
- Sharp objects used during vector research must be disinfected daily and/or before use. Select the appropriate disinfection method that is safe for the personnel and that ensures the quality of work.

3.4.1 BEST PRACTICES FOR THE INSECTARY

- Mosquito cages and larval trays should be placed on shelves in an orderly way. The insectary room should have strict procedures to avoid accidental contact with or release of specimens.
- The use of primary containers such as mosquito cages should be controlled to use only functional cages, sealed with mesh of an appropriate size to prevent the insects from escaping. The transfer of live mosquitoes from one container to another should be done with care, using sealed and unbreakable containers [3].

- All biological material must be sterilized in an autoclave before being disposed of.
- Mosquito maintenance supplies must be stored inside the insectary, in a designated area under closed cabinets. A closed storage room, and cabinets with fitted doors or drawers are recommended [3].
- The mosquito larvae diet must be kept in sealed containers [3].
- All biological material should be labeled according to strain/origin, date of collection, developmental phase, type of mosquito colony, and any other relevant information. The labels must be attached to the container to avoid confusion during the research activities or bioassays.
- The risk assessment may show that a medical examination program for entomology technicians should be considered as part of the biosecurity protocols.

3.4.2 SPECIAL PRACTICES

- The blood used at the insectary is a potential source of pathogens that can be transmitted when lab workers handle this material. Therefore, whenever possible, the use of sterile blood or blood from known sources with a pathogen-free certification is highly recommended. The use of animal or human blood whose disease status is uncertain should be avoided [3].
- When feeding mosquitoes by directly exposing mammals or other animals to insectary cages, the laboratory should comply with all considerations and regulations of animal husbandry. A number of procedures must be followed to comply with the guidelines of the laboratory animal welfare unit of each country. Laboratory animals that are to be used in scientific research need special care, caging, and sanitation.
- A plan for solving an emergency event or an undesired mishandling of biological material must be in place during laboratory activities that involve the delivery of blood or a live blood source to caged mosquitoes. Among these, primary containers (cages) should be robust enough to prevent the escape of mosquitoes during the feeding procedure [3].
- Mosquitoes that escape must be killed and discarded by mechanical vacuum cleaners.
- If there is an accidental release of live insects, or an accident that involves the health of the staff, an incident report should be written and submitted to local authorities. When there is an accidental release of arthropods infected with pathogens, the reporting should follow emergency measures. A mitigation plan should be prepared as part of the laboratory's or insectary's biosecurity policies and should be followed in such an emergency.
- Incident reports must describe the room where the accident occurred. They should give a detailed account of the activities that led to the release of live specimens or other matter. A warning sign should be placed in the insectary and building, indicating the corresponding information of the incident or release, until it is resolved [3].
- Potentially infectious lab materials, such as syringes, scalpels, and glass slides, must be placed in a strong, leak-proof container during their collection, handling, processing, storage, or transportation within a facility [2].
- Animals and plants that are not associated with the work being done must not be allowed in the laboratory [2].

- When collecting adult mosquitoes from field areas, entomology technicians must wear mandatory PPE, and have a dedicated space inside the insectary in which the field material can be kept under observation once transferred to the laboratory, with visible labels informing of the type of specimens collected.

3.4.3 BIOASSAYS BEST PRACTICES

Insecticide-impregnated papers are commonly used to determine a mosquito population's susceptibility to an insecticide. The standard protocol involving insecticide-impregnated papers were set by the WHO; some of its recommendations are the following:

- When conducting bioassays that involve the use of insecticide solutions or insecticide-impregnated papers, staff must wear PPE (laboratory coats, latex gloves, and protective lenses). See Annex 3 for a list of insecticides used by the ZAP Project.
- Inventory the boxes containing insecticide-impregnated papers, detailing the type and dates of the insecticides (unexpired, used boxes, and expired/discarded boxes), trade names, etc.
- Store boxes of impregnated paper at 4°C, by type, and by the expiration date of each box, and note the number of times the material contained is used. Use the materials based on the first-expired first-used principle. Mark on the boxes the date every time papers are used.
- Obtain additional information regarding the regulation of chemical products, including disposal requirements and guidelines that are each country's national policy.

Chapter III

5. SAFETY EQUIPMENT (PRIMARY BARRIERS)

To minimize the laboratory personnel's risk of exposure to arthropod bites, this section provides further recommendations on PPE and biosafety protocols.

3.5.1 PERSONAL PROTECTIVE EQUIPMENT

Laboratory safety tools include the protective equipment to be used by personnel in each work area:

- Lab coats or uniforms must be used during the work day. Such uniforms must not be used outside the laboratory. This clothing will be removed and left in a designated area in the laboratory.
- Ideally, lab coats are washed and decontaminated by the institution that runs the insectary. If this option is not available, the clothing should be washed at home and handled with caution.
- Protective clothing used in the laboratory must not be stored in the same closets as streetwear [1].
- Personal clothing should minimize the exposed skin area since this could increase the risk of attracting and being bitten by escaped mosquitoes. For example, skirts, shorts, open shoes, sandals, and T-shirts are not recommended [3]. Closed shoes must be worn inside the laboratory.
- Protective goggles must be worn in the bioassay area to avoid direct contact with insecticide or chemicals in the event of a splash.
- Disposable latex gloves should be used when hands could have contact with potentially infectious materials or contaminated surfaces or equipment, and when handling insects, chemicals, or other infectious items.
- Gloves must be used, as required, in each laboratory work area, according to the following practices [2]:
  - Change the gloves when they are contaminated, when their integrity is compromised, or whenever necessary.
  - Remove the gloves correctly and then dispose of them in the corresponding container, generally in disposable red colored bags.
  - Gloves must not be washed or re-used.
  - Workers must wash their hands immediately after finishing work with hazardous materials and before leaving the laboratory.
  - Hand-washing protocols must be rigorously observed.
- Depending on the type of laboratory work, the local risk assessment determines if other PPE is required, such as airway protection masks.

3.5.2 SAFETY SYSTEMS AND DEVICES

Safety systems that serve as support and emergency response tools (i.e., fire extinguishers, emergency eyewash stations, fire alarm) in the case of an emergency are recommended within the entomology laboratory.

It is important to keep the safety system in good condition, with a maintenance plan and labeled according to the safety signage guidelines of each country.

For efficiency in the case of an emergency, entomology technicians, especially the person responsible for the laboratory, are required to be specially trained. (See Annex 4, Safety Systems Verification Guide). Training should cover drill practices, equipment use, formation of emergency brigades, emergency response procedures, and other local laboratory requirements.

- **Fire Extinguishers:** Fire extinguishers should be used only for minor laboratory fires. Fire extinguishers contain an extinguishing agent to control specific types of fire. (See Annex 5, Fire Classification and Types of Fire Extinguishers.)

  Since there are different types of fire, classified according to solids, liquids, gases, or metals, the appropriate extinguishing agent must be used in each case of fire. Agents include water spray or sand, powder, multipurpose powder, foam, or CO2 [4].

  Fire extinguishers should be located near areas of potential fire risk. Every laboratory area (insectary, biotesting, and taxonomy) should have a fire extinguisher on the vertical wall near evacuation points at a visible height and easily accessible. They should be correctly marked with valid international safety symbols.

- **Emergency Eye Wash:** An insectary should have a rapid and effective eye decontamination system, consisting of two sprays to provide low-pressure drinking water for washing eyes or face, and a drainage basin. Such washbasin should be fixed to the floor or wall and have a standing (pedal) or elbow operation [4]. One additional sink should be located in the bioassay area where chemicals or insecticides are handled.

  Water should not be applied directly to the eye surface but rather to the bridge of the nose. This is more effective for the extraction of chemicals. Eyelids should be open to enable the complete washing of the eyes. Apply the wash for approximately 15 minutes. In severe cases, the worker should go immediately to the nearest health center.

- **Fire Alarm:** Fire alarms are designed to alert staff to a potential fire. The laboratory must be equipped with a smoke detection system and the entomology technicians must be trained in their use and location of the alarm in the event of an emergency.

3.5.3 SAFETY MEASURES

- No infected material is to be disposed of in the sewer. Physical barriers are recommended, as appropriate. Reused PPE (lab coats) should be checked for contamination before leaving the insectary [3].
- Any biological material requested should be tracked by means of a corresponding record and report [5].
- Primary barriers: For the adult phase of mosquitoes, arrange a cage area with a small-hole mesh (1 mm^2) to prevent escape [6]. The cages and hatcheries should be labelled with the species being bred and the possible biological risks.
- All spills, accidents, and actual or potential exposures to infectious materials are to be communicated to the laboratory supervisor. A written record of such accidents and incidents must be maintained [1].
- Corridors and circulation zones must be designed to allow for the free access and movement of people. Avoid holding meetings or storing objects in areas that prevent people from passing through. When circulating within the laboratory, move with care and without interrupting staff who are working [4].
- In the bioassays area, keep all information related to the care and handling of insecticides readily available [4].
- Entomology technicians should be trained on special hazards and are required to follow instructions on established safety practices, procedures, and policies [3]. Provide additional training as needed when there are procedural or policy changes and keep records of all the training.
- Maintain a first aid kit equipped with medicines that correspond to the chemicals used in the laboratory and possible staff health concerns.

3.5.4 SIGNAGE

- In accordance with laboratory conditions, clear signage with arrows should mark evacuation routes and emergency exits to be used in emergencies.
- The emergency system must have its corresponding labelling and technical labels.
- In the area where biological material is handled, label the laboratory as such and use international signage for the biological hazard. (See Annex 6, WHO guidelines on hazard symbols)
- Place safety decals in areas where insecticides and reagents are stored, and near the electrical supply, among others.
- Post signs for each laboratory work area: Insectary, Bioassays, Taxonomy, and Offices.
- Place labels on the laboratory waste management containers by category. (See chart on final disposal of residues)
- Label biological material in the insectary.
- The dimensions and colors of each label must comply with local regulations.

Depending upon the local risk assessment, other labeling criteria may be determined.

3.5.5 EMERGENCY CONTACT TELEPHONE NUMBERS

The laboratory must maintain an updated database of contacts that could provide assistance and quick support in the case of an emergency.

El laboratorio debe mantener una base de datos actualizada de contactos que puedan dar auxilio y prestar servicio de apoyo en caso de emergencia.

Tabla 2. Basic contacts list

Institutional Contact		Contact Information	
Type of institution	Contact Name or number	Address	Telephone/Email
Designated medical hospital for emergency response			
Designated alternative hospital			
General fire department (address)			
National police (local station)			
International Red Cross			
Laboratory safety company			
U.S. Embassy and Regional Security Officer (RSO)			
Electric energy company			

Note: Depending upon the country, the list could include additional emergency numbers.

Chapter III

6. INFRASTRUCTURE

An entomology laboratory has the following areas: insectary, bioassays, taxonomy, and administrative offices. Space is allocated according to the work being done in each area. To minimize work-related risk, the following measures should be taken.

3.6.1 INSECTARY

The insectary is the physical space within the laboratory used for the breeding and handling of mosquito colonies for research purposes and vector control. This space should maintain the following conditions:

- During the design phase, incorporate containment measures such as safety capsules and window protection to minimize the risk of mosquito escape.
- Insectary doors: The recommended entry/exit for the insectary is through a double-door lobby with mesh/net that prevents mosquito escape [3].
- Safety capsule: This is the space between the two entrance doors of the insectary; it divides the external area from the internal. A fan extractor in it absorbs any mosquitos that might be free in this area [5].
- In regions with a warm climate (temperatures between 28° and 30°C), one can establish facilities with natural photoperiods, installing windows with translucent glass to allow sunlight to enter, but prevent light rays from falling directly into the interior [5].
- Assign a designated area within the insectary for each mosquito colony: larval phase and adult breeding.
- All ventilation outlet ducts including doors must be protected with mosquito netting.
- Hydraulic and sanitary installation to provide drinking water access to all the areas is indispensable for various routine procedures within the laboratory, as well as for the cleaning of material, equipment, and work areas.
- The insectary must have a washbasin that is designed with appropriate pipes that prevent mosquito escape.
- It is preferable to not have windows in an insectary. If the space already has windows installed, they must be sealed with mosquito netting to prevent mosquitoes from escaping.
- The insectary should be designed, built, and maintained to facilitate cleaning, preferably with interior walls and low ceilings painted in a light color so that free mosquitoes can be easily located; bright finishes that are resistant to chemical disinfectants and light-colored floors [3].
- Floor drains should be modified to prevent the escape of mosquitoes. The traps should be filled with a chemical that will kill escaped mosquitoes of all stages [3].

3.6.2 BIOASSAYS AREA

The bioassays area of the laboratory is for the evaluation of mosquito susceptibility to insecticides. The following conditions should be maintained:

- The walls should be a light color, preferably white.
- Entry should be restricted, with access for laboratory personnel only.
- Work tables should be resistant to solvents, and water-repellent.
- There must be a washbasin for work use and disinfection of the area.
- The area must have a refrigerator exclusively for the storage of insecticides and other chemicals that require cooling. All refrigerated materials should have a corresponding safety label.
- All equipment must be spaced in such a way as to allow for cleaning access.
- Spaces between work benches, cabinets, and equipment must be easily accessible for cleaning [2].
- Chairs used in the laboratory work area must be covered with a non-porous material that can be easily cleaned and decontaminated with the appropriate disinfectant [2].
- The bioassay area should have an autoclave, to sterilize instruments before they are used.
- There should be a supply area with the appropriate safety labels, for reagent and chemical storage.
- The bioassay area must have an eyewash facility in case a chemical splashes into a worker's eye.
- There are no specific requirements for the ventilation systems; however, the planning of new installations should consider mechanical ventilation systems that provide an airflow toward the interior space, without recirculation to spaces outside the laboratory [2].

3.6.3 TAXONOMY AREA

The laboratory should designate an area for mosquito dissection and taxonomical determination of specimens, to examine them or to study the mosquitoes' morphology and tissues. So that this area is safe, the following conditions are recommended:

- Sturdy, fixed tables for mosquito identification and use of microscope and stereoscopes.
- Identify a container for the disposal of sharp objects, and have a waste management disposal plan for final disposal.
- Keep the areas clean after the work is concluded.
- Cover microscopes and stereoscopes immediately after use to prevent their deterioration.

The monitoring of compliance with the facility measures will ensure the safety of the laboratory's work and its personnel. The director must inspect the facility at least once a year to ensure that modifications and maintenance have not compromised the safety features [3]. If there are changes in work practices, agents,

Chapter III

7. COMPREHENSIVE WASTE MANAGEMENT PLAN

The comprehensive waste management plan is meant to identify and classify the types of waste generated in the laboratory areas, and to establish procedures for their management, storage, and final disposal.

3.7.1 SOLID WASTE CLASSIFICATION

The different activities conducted in the entomology laboratory areas were analyzed, allowing for the identification of the waste that could be generated and its classification, which are presented in detail in Table 3.

Table 3. Identification of laboratory solid waste per work area

Physical area	Hazardous Waste			Non-hazardous waste
	Infectious biological	Sharp cutting	Chemicals	
Biotesting area	Cotton with blood, tips	Needles, pipettes, scalpel blades, slides, vials.	Insecticide waste (impregnated papers) Organophosphorus Pyrethroids Carbamates Insecticide	Paper Cardboard Glass Plastic gloves Paper towel
Insectary	Substrates with eggs Pieces of cotton and blood Paper with biological material	N/A	N/A	Paper Cardboard Gloves Netting Mosquito net Suction tubes
Taxonomy	N/A	Coverslips and slides	N/A	Paper towel Cardboard
Office	N/A	N/A	N/A	Paper Cardboard Plastic

Note: N/A: Not Applicable

3.7.2 WASTE MANAGEMENT

The management plan includes the activities of source segregation, storage, and the final waste disposal.

SEGREGATION AT THE SOURCE

Source segregation is the fundamental basis for proper waste management. It consists of the initial separation of different types of waste, starting a chain of activities and processes whose efficiency depends upon the initial appropriate classification of the waste [7].

To facilitate waste segregation, the entomology technicians must have waste management training that includes the classification, correct container management, and full responsibility for waste management activities.

CONTAINER CHARACTERISTICS

Waste should be classified and separated as soon as it is created, and deposited in the relevant container, which is kept closed.

Tabla 4. Color codes for waste containers

Classification	Recyclable	Common	Biological and infectious	Sharp and cutting	Chemical
Waste	Plastic Cardboard	Gloves Nets Mosquito Net Suction tubes	Substratum with eggs Cotton pads with blood. Paper with biological material	Needles, pipettes, scalpel blades, slides, vials	Insecticide waste Organophosphates Pyrethroids Carbamates Packaging
Color/Type of container	Grey	Green	Red	Red guardian type	Red
Symbol		Label with: non-hazardous, ordinary, and/ or inert			Labelling of hazardous chemical waste
Graphical representation					

Note: The container colors could vary based on the national regulation in each country. This chart has been adapted from [8].

The waste storage containers must meet the following requirements:

- Plastic, rigid, waterproof, lightweight, and of adequate size for the amount of waste generated in each laboratory environment.
- No internal edges and with handles that facilitate handling during waste collection.
- The infectious waste containers must have lids and handling items for their proper closure [8]. Each container must have a plastic bag in the corresponding color.
- Wide opening for easy bag change.
- Labeled with symbol for the type of waste contained.

For wastes with pointed or sharp edges, the container must meet the following requirements:

- Rigid high-density polypropylene or other polymer that does not contain PVC. They can be recyclable containers known as "Safety Guardians" [8].
- Resistant to rupture or perforation by sharp items.
- With a narrow mouth cap, so that when closed, it is completely airtight.
- Containers must be labeled with symbols and the corresponding residue type.

3.7.3 INTERNAL WASTE MOVEMENT

Each area's waste is deposited in its corresponding container and must have a bag of the same color as that of the container. Disposable bags should be doubled outwards covering the edges to prevent contamination of the container.

The waste collection must be done by trained personnel using PPE. A weekly schedule should be established to carry out this activity.

When the bags are removed from the container, they are to be sealed using a plastic strip or cord to ensure proper sealing. All the bags must be labelled according to the classification of the waste and transported by wheelbarrow to the temporary storage site.

The waste that is removed from the laboratory must maintain a label on the disposable bag with the following information: area, waste type symbol, quantity by weight (kg), area manager, person who delivers it, collection date, entity responsible for waste management, observations, and other items. (See Annex 7, Labelling format for the disposable bags.)

To maintain hygiene in the work areas, the cleaning staff collect recyclable and common waste every day.

- **Biological Infectious Waste:** Biological material, such as mosquitoes, napkins, and bloody cotton, deposited in the container for "Biological waste" should be removed weekly. The person responsible for waste management should be trained to carry out this work in a proper and safe manner.
- **Sharp and Cutting Waste:** With respect to sharp, cutting waste, the containers should be removed from the work areas to the temporary storage area when they are up to 3/4 parts full or have been accumulating for a maximum of three weeks. The containers must be labeled with biosecurity pictograms, origin, date of collection, and other information. (See Annex 8, Labelling format for sharp cutting waste.)

- **Chemical Waste:** Most of the chemical residues and insecticides come from the bioassay area, which generates a large quantity of insecticide-impregnated papers, which are used in the resistance tests. A container with a safety label must be assigned for the disposal of this material, and the use of gloves is obligatory.

  When one works with bases, acids, alcohols, dyes, or other reagents, it is necessary to dispose of them properly. Use laboratory-located containers intended for this purpose [9].

  The chemical waste disposal report must detail the chemical group, concentration, safety sheet, and safety measures, among other things.

  All insecticide-contaminated material should be disposed of in red bags and red containers, labelled with the chemical or insecticide group used for its subsequent proper disposal. The security label is placed on the outside of the bag with a permanent marker, easy to read for those who handle the waste.

3.7.4 TEMPORARY STORAGE

The laboratory must have a temporary storage area that complies with the following recommendations for maintaining the hygiene and security of waste management:

- Restrict access to laboratory waste management personnel.
- Post a "Waste Area" label and signs that communicate the danger level.
- Organize the storage space to arrange spaces for each type of waste and maintain an ample walkway for waste movement.
- Dedicate a space with a faucet for washing containers and establish a management plan for the water drainage.
- For the best waste management control, keep a scale and record each waste material's weight.
- Organize a pest control plan for the waste storage area to avoid proliferation of insect pests.
- Locate the warehouse in an area where there is no risk of flooding. It should be in a roofed area, ventilated, easily accessible, and with waterproof flooring that facilitates disinfection and cleaning.
- Be equipped with extinguishers in accordance with the local rules and regulations.
- Have a lateral and posterior containment perimeter in the case of spillage.
- Mount safety signage and adequate lighting.
- Disinfect and dry hazardous waste containers every week, and wash and dry non-hazardous waste containers every 15 days. However, this may vary according to the each laboratory's regulations and procedures.

Table 4. Management plan and disposition of paper impregnated with insecticides

Type of waste	Environmental threat of these activities	Mitigation measures	Responsible person(s) for the monitoring	Monitoring indicator	Method of monitoring	Frequency of monitoring
Used impregnated papers		Maintain a log of acquisitions and inventory. Secure the storage installation. Store safely. Arrange appropriate incineration (850°C)		Purchase and inventory records are maintained. Workers use PPE. Operations facilities are located appropriately. All insecticide management records are reviewed and maintained.		
Unused impregnated papers		Maintain a log of unused papers. Ensure that storage facilities and PPE are appropriate for the active ingredient used and in accordance with approved SOPs. Store with safety measures. Dispose of by means of thermal treatment in a suitable incinerator.				
Expired impregnated papers		Maintain a log of unused papers. Ensure that storage facilities and PPE are suitable. Secure safely and separately from the stock. If the country where the insectary is located does not have satisfactory incineration technology for this category of waste, export it.		The incinerator must have environmental permits and authorization to handle this type of waste. Monitor the quality of atmospheric emissions and ashes.		

Type of waste	Environmental threat of these activities	Mitigation measures	Responsible person(s) for the monitoring	Monitoring indicator	Method of monitoring	Frequency of monitoring
Plastic boxes of impregnated papers		Maintain a record of purchases and inventories. Ensure that the storage facilities and PPE are both appropriate for the active ingredient used and in compliance with the approved SOPs. Store safely in accordance with the approved SOPs. Decontaminate boxes with detergent. Arrange for recycling or incineration.				
Wooden boxes with impregnated papers		Maintain a record of procurement and inventories. Ensure that the storage facilities and PPE are both appropriate for the active ingredient used and in compliance with the approved SOPs. Store safely in accordance with the approved SOPs. Decontaminate boxes with detergent. To diminish the boxes' waste effluents, rinse and incinerate in a designated incinerator (850°C).				

3.7.5 FINAL DISPOSAL

To comply with the biosafety and environmental health protection measures, it is essential to establish within the waste management plan a monitoring system of the final disposal of each type of waste that is generated in the laboratory.

The external collection, transport, treatment, and final disposal of non-hazardous and hazardous waste shall be carried out by specialized cleaning companies that are contracted to provide these services. Such companies must be authorized by the competent environmental authorities [7].

Table 5 provides recommendations for the mechanisms to use for the final treatment of each type of waste. It also lists the disposal and technical compliance requirements; these, however, can vary with local conditions.

3.7.6 USE OF PERSONAL PROTECTION EQUIPMENT TO MANAGE WASTE

The personnel involved in handling solid waste must use PPE, especially in the transport of infectious biological solid waste and sharp, cutting material.

Tabla 5. Final Waste Treatment Mechanisms

Type of waste	Final disposal	Requirement
Recyclable	National recycling companies	Environmental authorization for recycling of plastic, cardboard, and other waste.
Common	Municipal sanitary landfill	Authorization from the Mayor's office for the final waste disposal.
Infectious, biological	Incineration (thermal treatment)	Environmental authorization to incinerate the laboratory waste. Environmental ash management and evaluation of atmospheric emissions of the incinerator.
Sharp items	Incineration (thermal treatment)	Environmental authorization to incinerate the laboratory waste. Environment ash management and evaluation of atmospheric emissions of the incinerator.
Chemicals	Incineration (thermal treatment)	Environmental authorization to incinerate the laboratory waste. Environment ash management and evaluation of atmospheric emissions of the incinerator.
Reagents and chemical packaging	Triple washing Perforation Recycling	Environmental authorization for handling of the insecticide packages.

Note: Every waste management service company must comply with local environmental legislation.

- It is recommended to use the following PPE; Closed shoes; Safety gloves that are resistant to sharp objects; Complete uniform to prevent splashes on the skin; Protective goggles.

Chapter III

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Annexes

Annex 1

MAIN TAXONOMICAL FEATURES FOR *AEDES AEGYPTI* AND *AEDES ALBOPICTUS*

A. MAIN TAXONOMICAL FEATURES FOR *AEDES AEGYPTI* AND *AE. ALBOPICTUS* LARVAE IDENTIFICATION

Aedes aegypti

Scales of the comb of the VIII abdominal segment in the form of a trident, and located in a horizontal row.

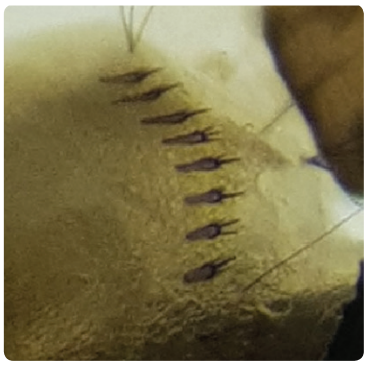


Image Source: Courtesy of Katia Lopez, ZAP project.

Aedes albopictus

Scales of the VIII abdominal segment in the form of a spine with sharp long ends and located in a horizontal row.

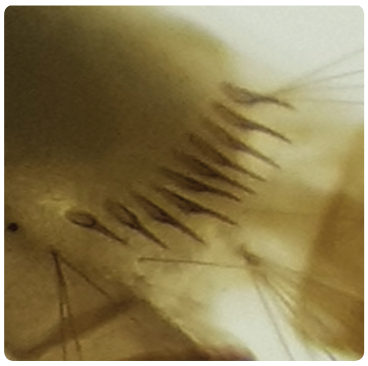


Image Source: Courtesy of Katia Lopez, ZAP project.

Aedes aegypti

Large and visible chitinized hooks (hardened structures) on both sides of the thorax.

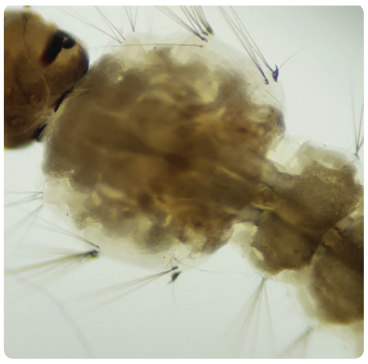


Image Source: Courtesy of Katia Lopez, ZAP project

Aedes albopictus

Small and inconspicuous chitinized hooks on both sides of the thorax.

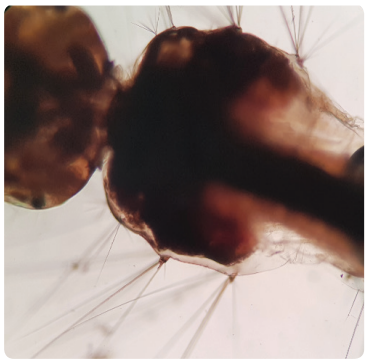


Image Source: Courtesy of Katia Lopez, ZAP project.

Aedes aegypti

The pecten teeth on siphon have three well-defined pointed denticles. Pecten teeth (b) are better visualized in a compound microscope (20x-40x).

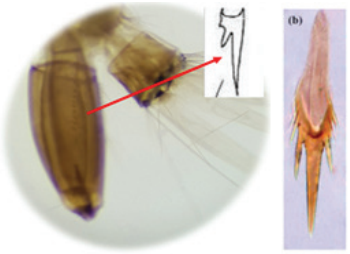


Image Source: Courtesy of Katia López, ZAP project; Farajollahi & Price (2013).

Aedes albopictus

The pecten teeth on shipon have less defined denticles. Pecten teeth (b) are better visualized in a compound microscope (20x-40x).

Image Source: Courtesy of Katia López, ZAP project; Farajollahi & Price (2013).

Aedes aegypti

Head with hair C-7 simple.

Image source: Farajollahi & Price (2013).

Aedes albopictus

Head with hair C-7 double or triple.

Image source: Farajollahi & Price (2013).

Consulted Literature: Farajollahi, A., & Price, D. C. (2013). A rapid identification guide for larvae of the most common North American container-inhabiting *Aedes* species of medical importance. *Journal of the American Mosquito Control Association,* 29(3), 203-222.

B. MAIN TAXONOMICAL FEATURES FOR *AEDES AEGYPTI* AND *AE. ALBOPICTUS* ADULT IDENTIFICATION

Aedes aegypti

White scales on the chest forming a lyre: two longitudinal midlines and two curved lines on each outer side of the chest.

Image Source: Courtesy of Katia Lopez, ZAP project

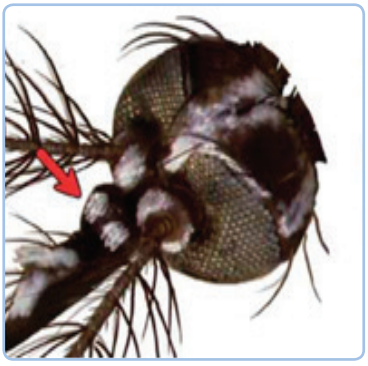
Aedes albopictus

White scales on the chest forming a longitudinal midline

Image Source: Courtesy of Katia Lopez, ZAP project.

Aedes aegypti

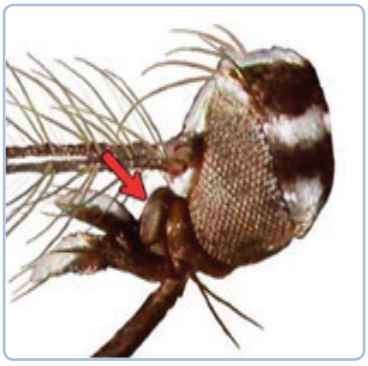
Clypeus with white scales



Fuente de la imagen: Rueda (2004)

Aedes albopictus

Clypeus without white scales



Source of Image: Rueda, L. M. (2004).

Literature Consulted: Rueda, L.M. 2004. Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with Dengue Virus Transmission. Zootaxa 589: 1 - 60. Magnolina Press

Annex 2

KEY FACTORS CORRESPONDING TO BIOSAFETY LEVELS 1 AND 2

The below questions can be used for the purpose of the risk assessment in a laboratory or insectary

Arthropods known for being free of pathogens	Arthropods containing unknown infectious agents or whose status is uncertain: diagnostic samples
Are the insect species (mosquito vector) already established in the location where the insectary is placed?	In case of escape, what are the infectious agents that can be transmitted by the vectors? What are the risks for the local community?
If the vector species is exotic, in the event of escape, is it likely to be established temporarily or permanently in the municipality where live colonies are maintained? Detailed the potential risks.	In the event of escape of mosquitoes that carry an infectious agent what are the risks to the laboratory (or insectary) staff?
Does the arthropod have a known or characterized insecticide-resistant genotype or phenotype? Detail all the information available on the subject of insecticide susceptibility for each species that will be housed in the insectary.	Describe characteristics of the insect vectors and potential infectious agents that might be transmitted by their bite. List the potential health risks for the laboratory/insectary personnel and for the local municipality. Check the CDC Center's Arbovirus Catalog: https://wwwn.cdc.gov/arbocat/VirusBrowser.aspx
In the event of escape, can the vector be controlled or eliminated with conventional methods of control? Detail the consequences	In the event of escape, can the vector be controlled or eliminated with conventional methods of control? Consider the available information on insecticide susceptibility to common insecticides.
If mosquito vectors are infected with infectious agents, are such pathogens already documented in the municipality where the insectary is located? Or are the infectious agents endemic in the locality of research?	
In the case of local or exotic vectors, can the vector release cause any risk to the health of humans and animals?	
In the event of an escape of insects housed in the insectary, can these organisms transmit infectious agents to humans or animals? Describe all potential risks.	

Reference: Adapted from the Arthropod Containment Guide [3]

Annex 3
CLASSIFICATION OF INSECTICIDES

Classification of the insecticides included during ZAP's entomological surveillance when testing the susceptibility of local *Aedes aegypti* populations to insecticides used in public health interventions

Insecticide Classification	Brief Description	[1]Insecticide	[2]WHO Classification	[3]EPA Classification	[4]Health risks
Organophosphates	Act on the acetylcholinesterase enzyme. These enzymes degrade the neurotransmitter acetylcholine. As the insecticide inhibits this enzyme, the levels of the neurotransmitter (acetylcholine) will remain high in the receptor of a neuron, hence it generates permanent stimulation.	Temephos	U: it is improbable that severe danger could arise with normal use	III. Caution	Occupational risk only (for handlers of the product).
		Malathion	III. Slightly hazardous	III. Caution	Harmful if swallowed. It can alter the immune system, liver, adrenal glands, blood cells, sense organs, peripheral neuropathy, and induce behavioral changes.
		Pirimiphos-methyl	III. Slightly hazardous	II, III: Warning, caution	Harmful if swallowed.
Carbamates	Designed to inhibit the normal breakdown of the enzyme acetylcholinesterase. The carbamates have relatively high toxicity for mammals.	Bendiocarb	II Moderately hazardous	II. Warning	Harmful when skin contact occurs. Toxic if inhaled or swallowed.
		Propoxur	II Moderately hazardous	I, II, III: Danger, Warning, Caution	Potential eye irritant, hepatotoxicity, and nervous system toxicity.

1 Commercial brand
2 World Health Organization. Manual on the development and use of FAO and WHO specifications for pesticides, 1st edition. FAO plant production and protection paper, 173. FAO, Rome, 2002.
3 United States Environmental Protection Agency (EPA): Toxicity per type of insecticide.
4 Manual de Plaguicidas de Centroamérica, Universidad Nacional Costa Rica http://www.plaguicidasdecentroamerica.una.ac.cr/

Insecticide Classification	Brief Description	[5]Insecticide	[6]WHO Classification	[7]EPA Classification	[8]Health risks
Pyrethroids	These insecticides are classified as Type I and Type 2, in accordance with their toxic effects and chemical structures. Act on the central nervous system and peripheral nervous system. Knockdown effects: Pyrethroids block or disable the sodium channels in nerve cells, but some tend to dissociate from their target sites (especially Type I), causing mosquitoes to survive. While the Type I can keep channels open for milliseconds, Type II can do so for seconds.	Permethrin	II Moderately hazardous	II, III: Warning, Caution	Light eye and skin irritant capacity, harmful if inhaled or swallowed.
		Deltamethrin	II Moderately hazardous	II, III: Warning, Caution	Light eye and skin irritant capacity, harmful if inhaled or swallowed.
		Lambda-Cyhalothrin	II Moderately hazardous	II. Warning	Light eye and skin irritant capacity, harmful if skin contact occurs, toxic when swallowed, very toxic if ingested.
		alpha-Cypermethrin	II Moderately hazardous	No Consensus	Mild eye irritant, toxic if swallowed, respiratory tract irritant, health risk if prolonged exposure via swallowing.
		Etofenprox	U: it is improbable that it will cause acute danger with normal use	III. Caution	Light skin irritant capacity
Growth inhibitors	Prevents metamorphosis by acting as a juvenile hormone that inhibits insect development	Methoprene	U: improbable to cause acute danger with normal use	IV. Does not require labelling	
		Diflubenzuron	No serious danger	III. Slightly toxic	
Biological Larvicide	The Bti bacterium produces a protein crystal which is toxic only to mosquito and black fly larvae. These crystals are ingested by insect larvae when feeding. In the alkaline environment of the susceptible insect's digestive system, the crystals are dissolved and converted into toxic protein molecules that destroy the walls of the insect's stomach (GDG, 2014)*	Bacillus thuringiensis (Bt)	Unlikely to pose any hazard to humans or other vertebrates, or to the great majority of non-target invertebrates. Safe for use in aquatic environments.		Light eye irritation.

5 Commercial brand

2 World Health Organization. Manual on the development and use of FAO and WHO specifications for pesticides, 1st edition. FAO plant production and protection paper, 173. FAO, Rome, 2002.

3 United States Environmental Protection Agency (EPA): Toxicity per type of insecticide.

4 Manual de Plaguicidas de Centroamérica, Universidad Nacional Costa Rica http://www.plaguicidasdecentroamerica.una.ac.cr/

*GDG Environment, Ltd. 2014. Everything you should know about Bt

Annex 4

LIST FOR GENERAL SAFETY AND HYGIENE CONDITIONS

Verification list for general safety and hygiene conditions

Responsible person(s): _____

Date: _____

I. Work Places (Stations)	Good Condition	Regular	Poor Condition	Observations
1.1 Are the work spaces adequate for the tasks that are expected ?				
1.2 Do the work spaces comply with these minimum requirements?				
a) Space				
b) Location				
1.3 In general, are the laboratory work areas in orderly and clean conditions?				
1.4 Are there containers for every type of waste that is generated in the laboratory or insectary areas?				
1.5 Is the quantity of waste containers in the work area sufficient for the necessities of the company?				
1.6 Are the hallways free of objects (clutter)?				
1.7 Do the floors have drainage systems with grids, strainers, or any other secure means that allow for maintenance and avoids the liquid stagnation?				
1.8 Is the insectary area organized and the work areas duly labeled according to the biological material being handled?				
1.9 Do the insectary doors and windows have mosquito nets or adequate barriers to prevent mosquito escape?				
1.10 Is the furniture in the insectary adequate for the conditions of high temperature and humidity, and resistant to frequent cleaning?				
1.11 Does the insectary have biosecurity measures in place and physical barriers to avoid the escape of insects?				
1.12 Is there a sink available for work in the laboratory?				
1.13 Are the countertops and desk surfaces clean and decontaminated?				
1.14 Are the chemicals and insecticides kept in cool temperatures?				

I. Work Places (Stations)	Good Condition	Regular	Poor Condition	Observations
1.15 Are the insectary's shelves adjustable and equipped with wheels? Are they tightened well?				
1.16 Is the insectary's thermo-hygrometer in good condition? Is there maintenance and control of the temperature and relative humidity? Are there paper forms to record hese variables on a daily basis?				
1.17 Is the drain of the sink covered with mesh or filter to avoid exit of biological material?				
1.18 Does the laboratory's autoclave have an airtight seal (hermetic closing)? Is it working properly?				
1.19 Do the refrigerators where chemicals, insecticides, and biological material are stored operate at a suitable temperature, properly labeled, and with hygiene measures?				

II. Access and Safety	Yes	No	N/A	Observations
2.1 Is there restricted access to the laboratory/ insectary? Does the laboratory entrance comply with the corresponding label requirements?				
2.2 Are there safety labels indicating that the lab access is restricted?				
2.3 Does the laboratory have a written registration system to document all workers when entering?				
2.4 Are there labels for the personal protection equipment materials required to work in the laboratory?				
2.5 Are there labels indicating any risk to the staff while working inside the laboratory/insectary?				
2.6 Are the laboratory waste containers labeled?				
2.7 Do the facilities have an emergency lighting system?				
2.8 Are the evacuation routes defined and clearly marked?				
2.9 Are the biological risk and laboratory waste containers properly marked with their corresponding symbols?				
2.10 Are the laboratory work areas clearly defined: bioassays, taxonomy, insectary, and administrative offices?				
2.11 In the case of an emergency, are the emergency evacuation routes properly signposted?				
2.12 Does the chemical storage area meet the corresponding symbology and label requirements?				

III. Safety systems and devices	Yes	No	N/A	Observations
3.1 Are the fire extinguishers strategically located in the areas of potential risk?				
3.2 Are the extinguishers kept refilled and up to date?				
3.3 In the case of fire emergencies, is the staff trained in using the fire extinguishing equipment?				
3.4 Are they visibly located and well-marked?				
3.5 Are the fire extinguishers easily accessible, free of obstacles, so that there is free access to reach them?				
3.6 When they are used, are they reloaded immediately?				
3.7. Is there an external entity in place that is responsible to inspect the company's fire extinguishers? Name of the Company				
3.8 Are the extinguishers inspected: _____ weekly ____ biweekly _____ monthly ____ quarterly ____ half-yearly ____annually				
3.9 Are the fire extinguisher's operating instructions legible and in clear sight?				
3.10. Do they keep a documented log of fire extinguisher inspections?				
3.11. Are the fire extinguishers sufficiently filled, pressurized, and free of dirt?				
3.12. Are there signs indicating the prevention and danger of fire?				
3.13. Does each fire extinguisher have its proper labelling for its location?				
3.14 Are the eyewash facilities kept in good condition and tested for their continued efficiency?				
3.15 Are appropriate medicines kept in the first-aid kit?				

IV. Personal Protection Equipment	Yes	No	N/A	Observations
4.1 ¿Are there gloves available in different sizes and made of the appropriate materials in each work area (insectary, bioassay areas, taxonomy, administrative area)?				
4.2 Does each worker keep his/her uniform or lab coat in good condition?				
4.3 Is there a visible symbol warning against the use of lab coats, gloves, and other personal protection items outside the laboratory?				
4.4 Are the gloves utilized correctly and as per work requirements?				
4.5 Are there protective work goggles available in the bioassay and taxonomy areas?				

Note: The checklist should be adapted according to the local conditions of each laboratory

Annex 5
TYPES OF FIRES AND EXTINGUISHERS

Types of fires		Fire extinguishing agent	Do Not Use
Class A	Solid materials (wood, paper, rags, etc)	Type A Extinguisher (water, polyvalent powder)	Normal powder
Class B	Gases and vapors (butane, acetylene, etc.)	Extinguisher Type B (polyvalent powder)	Water, carbon dioxide, foam, special powder
Class C	Equipment and electrical appliances	Type C Extinguisher (carbon dioxide)	Water, sand, foam, various powders
Class D	Light metals (magnesium, lithium, sodium, titanium, aluminum)	Special powder or dry sand	Water, carbon dioxide, foam, special powder, normal powder

Reference: Obtained from the Department of Occupational Safety's Fire prevention and extinction guide [10].

Annex 6
WHO INTERNATIONAL BIOHAZARD SYMBOL

BIOHAZARD

WHO 0494

ADMITTANCE TO AUTHORIZED PERSONNEL ONLY

Biosafety Level: _____

Responsible Investigator: _____

In case of emergency call: _____

Daytime phone: _____ **Home phone:** _____

Authorization for entrance must be obtained from the Responsible Investigator named above.

Source: World Health Organization. 2006. Biorisk management. Laboratoy biosecurity guidance. WHO/CDS/EPR/2006.6.

Annex 7

FORMAT OF LABELS FOR DISPOSABLE WASTE BAGS

Hazardous Waste Labelling

Entomology Laboratory:

Date	Area		Person Responsible for the Area	
Safety Symbol	Type of Waste			
	Infectious Biological	Sharp and cutting	Chemical Waste	Insecticide Waste
Quantity (kg)				
Responsible person for Handling				
Observations				

Note: Symbol depending on type of waste.

Reference: Comprehensive management plan for common waste, recyclables, chemicals, and special wastes, created by the Material Sciences Laboratory. University of Antioquia, Medellin, Colombia

Annex 8

FORMAT OF LABELLING FOR SHARP AND CUTTING WASTE CONTAINERS

Format of labelling for sharp and cutting waste containers

Entomology Laboratory:

Sharp and cutting waste

To be handled with caution, close hermetically.	Date: Origin: Person Responsible: Quantity (kg)
	

Reference: Comprehensive management plan for common waste, recyclables, chemicals, and special wastes, created by the Material Sciences Laboratory. University of Antioquia, Medellin, Colombia

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