ZAP –ZIKA AIRS Project

Entomological surveillance protocol of immature *Aedes aegypti* mosquitoes (larvae/pupae)

1. INTRODUCTION

The entomological surveillance of the *Aedes aegypti* mosquito is carried out following the distribution and abundance of the different mosquito developmental phases. Consequently, it is very important to know mosquitoes' life cycle in order to implement successful surveillance activities.

Mosquito life cycle: Mosquitoes are Diptera, and according to current taxonomic classification, their taxonomical family is known as Culicidae. They are characterized by their complete metamorphosis, that is, their development involves transformation in different stages: **egg-larva-pupa-adult**.

During oviposition the female mosquitoes can deposit a large amount of eggs at one time. In general, this happens in bodies of water with different characteristics, and in some particular cases, it happens on solid substrates or surfaces with high humidity. In most species, the eggs hatch in two or three days. The larvae go through four phases of molting called "stages", which are morphologically similar, but have different sizes, as every time they molt into the next stage their size increases. The time larvae take to develop generally comprises a period of 7 to 10 days, during which they feed on organic material by filtering particles from fluid or by browsing on hard surfaces (Merritt et al. 1992). When the larva has fully developed, it stops feeding itself and converts into a pupa, a stage in which it remains for about two to three days, after which the adult individual emerges (Figure 1).

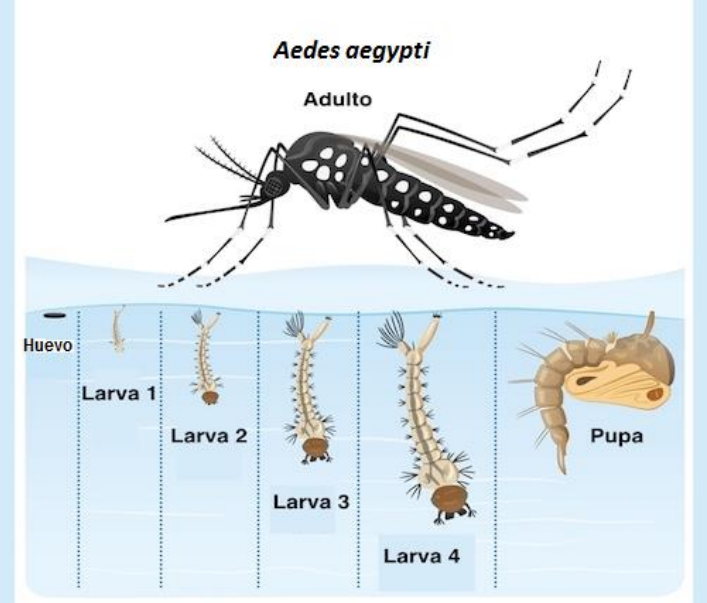

Figure 1. Life cycle of the *Aedes aegypti* mosquito. The different developmental stages of mosquitoes include egg, larva, pupa and adult. The development of a larva involves four different stages, in which the same individual becomes bigger as it grows, and exhibits a skin change or "molt". After the fourth larval stage, the individual transforms into a pupa, and afterwards into an adult. These developmental phases depend directly on the temperature of the environment (ideal temperature between 20Cº and 30 Cº) and the amount of food available for the individuals.

(Image Source: web site-Brasil- www.Tuasaude.com)

A few key ecological characteristics of the *Aedes aegypti* mosquitoes are worth noting:

Aedes aegypti is a species comprising mosquitoes that generally exhibit daytime habits. That is, they search for food sources (e.g. human blood) during the day and are well adapted to the environments built by men (mainly urban and semi-urban areas).

1. *Aedes aegypti females* prefer clean water to lay their eggs. The eggs are generally placed on wet surfaces in dark environments (not directly on the water).
2. Female *Aedes aegypti* individuals have successfully adapted to urban environments, and generally rest inside human households, showing short flights and, therefore, reduced movement. This indicates that *Aedes aegypti* mosquitoes can develop their entire life cycle inside human households or in their vicinity. It should be pointed out that in human households these mosquitoes easily find food and places to rest and lay eggs.

One important component of the entomological surveillance activities is the study of larvae and pupae in domestic or peridomestic breeding sites. Over the years, different types of breeding sites have been documented with high densities of immature *Aedes aegypti*. The most common examples are domestic or peridomestic recipients (See Figure 2).

Figure 2. Examples of places where *Aedes aegypti* mosquito species can develop:

A. Garden tools with rain water, B. Useless vacant lots inside urban or semi-urban areas, C. Plants in water or with accumulated rain water, D. Water tanks in domestic environments, E. Abandoned tires, F. Garbage cans for storing water. These types of sites representing ideal conditions for the development of immature mosquitoes are called, collectively, "breeding sites".

The study of *Aedes aegypti* breeding sites can vary due to various factors, such as: Sizes and types of households, habits of the local human population and the presence of an aqueduct system (places without a water supply are characteristic because local communities store water in garbage cans and containers that quickly become breeding sites).

1.1 Rational of larvae and pupae density determination:

Why is larvae density important	Why is pupae density important
- Inventory of the total breeding sites in a location. - A simple estimate of the number of larvae that a breeding site contains can provide significant information on the real role the breeding site plays in the location. - High larvae density is a good indicator of the most productive breeding site- types in a determined group of households or a locality. - The information related to larvae density IS NOT A GOOD PREDICTOR regarding the density of adult *Aedes aegypti* present in the study area. This is because the larvae mortality depends on the density of individuals present in a breeding site. If there is a very high larvae density, the immature population is subjected to a high mortality rate, which prevents relating larvae with abundance of adult mosquitoes.	-The presence of pupae in a breeding site indicates that site is ideal for the development of all the immature stages of the mosquitoes. This information contributes to determining which breeding site-types are the most productive for mosquitoes. The percentage each breeding site represents must be estimated in relation to the total pupae count in the same residency / locality. -**The estimate of pupae density in a breeding site IS THE BEST PREDICTOR of the adult density in the study area. This information has GREAT VALUE for estimating the adult mosquito population in the place, given that the development time from a pupa to its adult stage is just a few days.** -It is possible to obtain an **index of the number of *Aedes aegypti* adults/per person in the study area.** The ratio of adult mosquitoes per person is the base of the estimated transmission risk of the disease. -The presence of pupae can provide information when a control measure lost its residuality (example: larvicide) after it has been applied in the locality.

2. OBJECTIVES

2.1 To describe the significance of the study of larvae and pupae densities as part of an entomological surveillance system.
2.2 To standardize the procedures to conduct larvae and pupae surveys as components of the entomological surveillance system across ZAP countries.

3. MATERIALS

- Collecting nets (larvae screen) made with fine mesh
- Dipper with extendable handle (to use in big containers for sampling pupae)
- White plastic trays (to visualize mosquito larvae/pupae during field sampling); small or medium size (24.8 x 19.7cm)
- Plastic collecting tubes/containers (to transport live pupae to the lab)
- Mosquito breeding chambers (will be provided to countries)
- Disposable transfer pipettes
- Plastic turkey baster (as an additional suction tool to collect larvae and pupae)
- Small plastic tubes/vials to collect and conserve larvae
- Alcohol (95%-100%)
- Pencils
- Pens
- White paper labels (2 x 1 cm)
- Stereoscope
- Mosquito cages
- Clipboards
- Calculator
- Flashlights
- Entomological form

4. PROCEDURES

4.1 Steps for searching and determining larvae and pupae densities in households:

1. Provision of a "household card" for all households by vector control officers is recommended in areas and places where control activities are implemented. It is also important that the households are advised to keep the card in a safe place and present the card when asked by vector control or entomology technicians during their visit. The expectation is that the ZAP personnel will log in during each visit and record the activity

that was conducted in each of the households. This card must remain in each household in the possession of the inhabitants of the residence.

2. The search for larvae and pupae as part of the entomological surveillance will be carried out in 200 houses for each sentinel site. The way this number of houses is chosen will depend on where the ovitraps are placed. To search for larvae and pupae, one additional house will be chosen for each house with an ovitrap. It should be pointed out that one of the two houses chosen for searching larvae and pupae may be the same household that has the ovitrap. In this case, the other house for searching larvae and pupae can be in the area close to the ovitrap, preferably at a distance of 200 meters.

3. The selection of the 200 houses for the larvae/pupae search will follow a random selection process. Post random selection of houses, which house will be part of the entomological surveillance depend on the willingness of the residents to participate in the entomological surveillance program.

4. The staff in charge of searching for larvae and pupae must inspect each household, after obtaining permission from the residents every 10-15 days. The technicians will survey both the dwellings and the peridomicile area of the households, and inspect possible breeding sites or water containers to determine whether there are recipients with water, and if among the recipients with water there is presence of larvae or pupae of the *Aedes* mosquitoes.

5. Only the containers or sites with water should be entered in the inspection records (entomological form). A larvae and pupae count must be done for each type of breeding site or container. For containers with large volumes of water, see the specification in Figure 5 on how to calculate the number of pupae. The larvae density will only be provided as a gross estimation (none/1-100 individuals or more than 100 individuals). See data collection form in Annex 1.

6. Each recipient or water container must be carefully inspected, with the help of a flashlight and mesh or *larvae screen* (Figure 3) for collecting the immature individuals present in each sampling place. Small recipients may be transferred to white plastic trays (medium size), that the technicians can carry as part of the field material to facilitate the observation and capture of the biological material.

7. If the breeding site has a medium volume (<20 liters = less than 20 liters), the water can be sieved with larvae meshes or with a sieve for capturing immature individuals. Otherwise, pipettes can be used to pour the content of the breeding sites into the white trays for facilitating observation of the immature individuals.

8. In large recipients, a mesh is inserted to a depth of approximately 7.5 cm under the surface of the water, with constant circular movements around the perimeter of the container. This creates a descending vortex, which in turn generates a current that carries the larvae/pupae towards the center of the container and facilitates capturing them (Figure 4).

9. For large rectangular tanks, it is best to use the larvae mesh so that the corners are repeatedly inspected, as well as the volumes of water that is in the shade or partially

covered by a surface. A careful inspection must be carried out in these sites that offer ideal conditions for the development of immature mosquitoes.

10. The data collection in the forms (see Annex 1) must be completed for the entire household visited, recording all the inspected containers or recipients with water (whether positive or negative). The surveillance of larvae and pupae in the designated houses must be done every 10 to 15 days, during the time planned for surveillance in the study areas (or sentinel areas).

11. The larvae density per breeding site can be grossly calculated by estimating the number of larvae present in the container with each pass of the mesh. This procedure is repeated for several times and a gross estimation of the larvae density is recorded selection one of three options: “no larvae”; an amount from “1 to 100 larvae” or “more than 100 larvae” (See larval/pupal ZAP form).

12. A portion of the larvae material found in different households and breeding sites, may be preserved in tubes or vials with 95% alcohol, and duly labeled with the data of origin and type of breeding site in which the individuals were found. The amount (or proportion) of biological material that must be preserved in alcohol will depend on the taxonomic quality control system previously established by the entomological team, or the specific interests of the technical team (material for further training or conserve as part of entomological collections representatives of the local areas) .

13. The team should collect all the pupae from the breeding sites that have been sampled during the density estimation and transport them *alive* to the laboratory. Officials should transport the live pupae in plastic containers, properly labeled, with a low volume of the same water obtained from the breeding site. Once pupae are placed in the lab, the lab technicians should keep them under controlled conditions, until adults emerge and a proper confirmation of the species can be made (i.e. *Ae. aegypti*, *Ae. albopictus* or *Culex* mosquitoes). The information that complements the live pupae should be recorded carefully as of type of container/breeding site, exact house and person in charge (See larval/pupal ZAP form).

14. The scheme described in Figure 5 should be followed for estimating the number of pupae present in containers with different volumes.

Figure 3. Mesh or larvae screen for capturing material of immature individuals in recipients with water.

(Photo Source: Courtesy of Angel Gabriel Orellana, ZAP Project, Honduras).

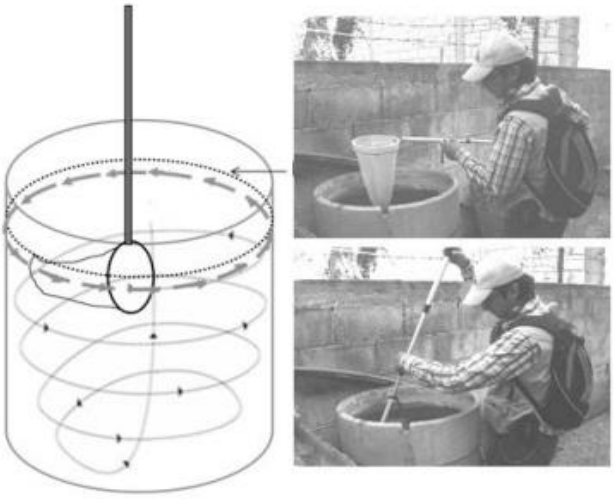

Figure 4. Circular movement around the tanks or containers with water for collecting immature *Aedes aegypti* individuals.

(Photo source: WHO, TDR, 2011. Operational guide for assessing the productivity of *Aedes. aegypti* breeding sites)

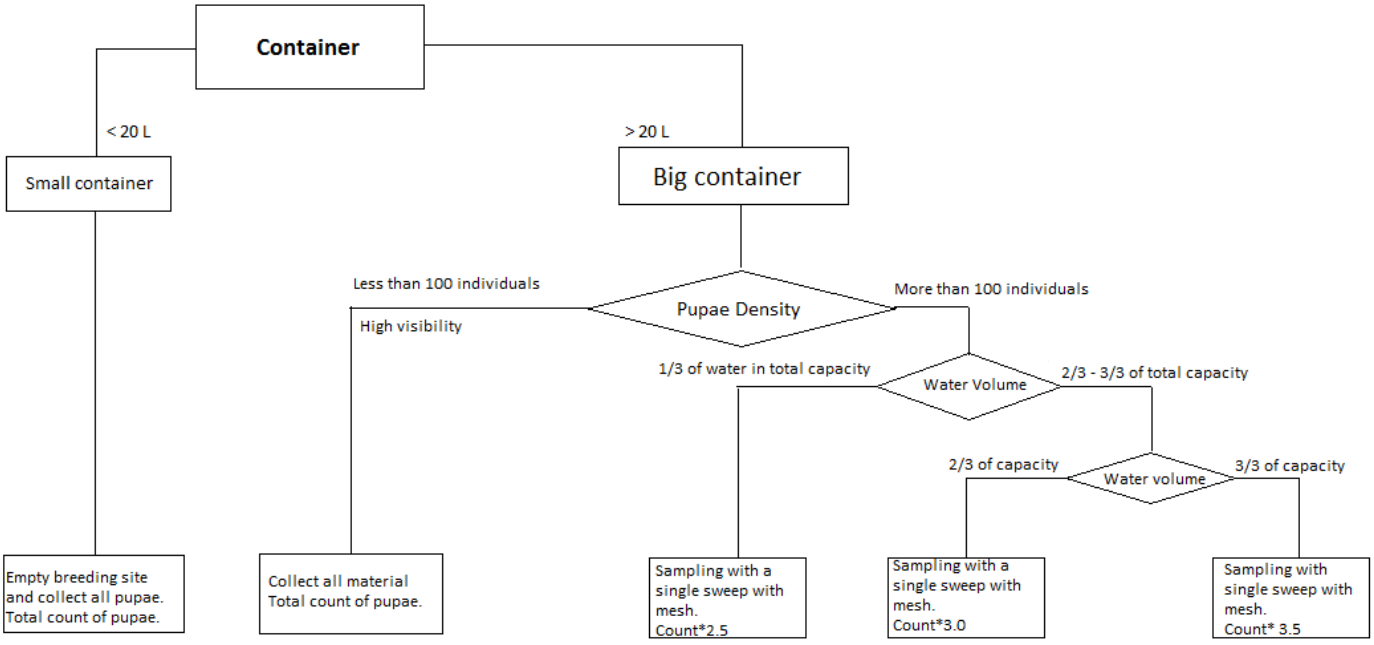

Figure 5. Strategy for collecting pupae in large containers (WHO, TDR, 2011).

4.2 Example for pupae density calculations

If a container or a breeding site has less than 20 liters of water (<20 L), collect all the pupae material by emptying the container, and use the mesh (or pipette) to collect the individuals. If the container has more than 20 liters of water (> 20 L) and it is not easy to empty its content, but there is good visibility and low pupae density (< 100 individuals), collect all the pupae with the mesh. If on the contrary, the container is big and has a high density of pupae (> 100 individuals), collect at least one sample by passing the mesh inside the breeding site (one sweep). Figure 5 shows the correction factor that must be used for estimating pupae density in the cases in which the breeding site only has 1/3, 2/3 or 3/3 of its total capacity filled with water. In these cases, the total number of pupae must be estimated using the correction factor, in accordance with the amount of water in the breeding site. Said correction factor (example: 2.5) is multiplied by the number of pupae collected with a simple sweep of the mesh/larvae screen. In this way, there will be a calculation for the pupae density in large containers with more than 100 individuals.

Example: If the official finds a water container with a capacity of more than 20 liters, and with an amount of water equivalent to 2/3 of its total volume, it is recommended to take a sample of the individuals (pupae) with the mesh or larvae screen, and then count the number of pupae captured with this single sweep of the screen, and then multiply the result with the corresponding correction factor (3.0 in this case). This will enable the official to calculate the equivalent to the pupae density in the breeding site. And all the pupae collected should be transported to the lab for corresponding rearing and identifying.

4.3 Steps for breeding pupae in the lab:

1. All pupae sampled during the density estimation procedure (Fig. 5) should be collected and transported to the laboratory. This means, while the technicians are sampling pupae in order to estimate their density, those individuals should be all collected in order to breed them in the lab. Use a separate rearing chamber for pupae collected from each breeding container.
2. Pupae can be maintained in plastic containers (mosquito breeders/rearing chamber) completely covered and with a very low volume of water (less than half the capacity of the plastic container). It is recommended to use the same water from the breeding site in which the individuals were collected. Alternatively, pupae can be kept in open containers inside a mosquito cage, to keep the newly emerged adults from escaping. Make sure to use an empty mosquito cage so the newly emerged adults can be properly observed.
3. Once in the lab, the newly emerged adults can be used for taxonomical confirmation of the species present in each container or breeding site (*Aedes aegypti*, *Aedes albopictus* or other mosquitoes). Please keep records of the species, the origin of the pupae, the type of container/breeding site, and the person responsible for the collection and species confirmation.
4. The information concerning the taxonomical confirmation of the species should be properly recorded in the entomological form (larval/pupal ZAP form).

4.4. How to preserve immature individuals in alcohol:

1. 95%-100% ethanol must be used to preserve larvae and pupae material in small tubes/vials. This will maintain the material in good conditions for future uses in training or for creating biological collections. Before placing the material in alcohol, remove as much residual water as possible, and pre-wash the biological material with 95% ethanol, so that afterwards the material can be deposited in a tube or vial containing only 95% ethanol. When biological material is preserved in alcohol that has water residues, a fast deterioration of the individuals will be observed (material

with dark spots of decaying tissue), which reduces the quality of the material intended for an entomological collection.

2. Check that the tube or vial is completely full of alcohol up to the top, and that the tap creates a hermetic seal to keep the alcohol from evaporating. Remember that unlabeled biological material loses all its value. Write a small paper-label with a pencil (with the location, breeding site type, household code, address, date, and person in charge). Carefully include this label inside the tube, together with the biological material, and seal the tube or vial. Depending on the size of the tubes or vials, it is better to have a few individuals (either larvae or pupae) per tube with alcohol. If more than five (5) individuals, larvae or pupae, are placed in a small vial with alcohol, when the individuals brush each other, the quality of the immature shapes will deteriorate, and even some important characteristics, such as setae/hairs in the body, could be lost.
3. When the field staff has doubts about the identity of the biological material found in the households such material should be conserved in a tube with alcohol and transported to the lab (i.e. when there is no certainty if the found larvae belongs to the species *Aedes aegypti*, the material must be packed in properly labeled tubes with alcohol, and delivered to the entomologist responsible for carrying out the taxonomic identification or confirmation of the material).

4.5 Filling out field forms for recording activities:

A careful record of the information must be kept for each visit to households included in the entomological surveillance or intervention programs (vector control). For the sample of immature individuals (larvae and pupae) there is a data collection form (ZAP larval/pupal form, see Annex 1) that includes important descriptors on type and characteristics of the breeding sites, number of larvae and pupae, and their general identification (mosquito genera). <u>This information is a valuable supplement for all the field activities, and the technical team should assign people to be in charge of filling out the forms, and a supervisor who can help resolve doubts and constantly ensure the information is rigorously recorded.</u>

4.6 Indicators from larvae/pupae surveillance

The surveillance activities must answer the following questions:

- How many positive houses are there per study area?
- How many recipients or potential breeding sites per household were found?
- How many positive breeding sites per household were recorded?
- Which is the most significant breeding site(s) in the study area?
- What is the pupae density in the positive breeding sites?

Finally, the percentage contribution of each breeding site compared to the total pupae count is calculated. This is done by taking the total of pupae that are found in a determined recipient category (or breeding site) and dividing it by the total amount of pupae in all the recipients of the area that resulted positive (with pupae). **This estimate will enable identifying the most important breeding sites in the study area.**

5. SPECIAL CONSIDERATIONS

- The technical teams of each country should prepare concise and informative material about the larvae/pupae surveys intended in different communities/municipalities in order to ask the local residents for their authorization to include their households in the entomological surveillance. Local residents should give their approval before the ento surveillance team is ready to start the sampling of larvae and pupae inside each of the households.

- In the event the residents of the chosen houses refuse to participate in the larvae/pupae surveys, the technical team must look for the next closest house, until obtaining all the needed houses for the larvae/pupae survey.

- When a residence is locked, the team must communicate with the residence contact and determine if the house can be accessed later or the following day. In case that any of the surveillance technicians cannot do his/her duty (due to disease, transportation problems, etc), the supervisor must assign a replacement immediately and give the respective location and formats to the newly assigned personnel. Specific instructions to control this kind of situation must be considered by the technical entomological surveillance team, under the coordination of the entomology manager and supervisors (Modified from Metholodogical guide for entomological surveillance with ovitraps, Mexico, 2015).

6. IMPORTANT DOCUMENTS

1. Manual compiled by ZAP. 2017. Draft for participants in training course. Good practices for the entomological monitoring of *Aedes aegypti*. Document prepared by ZAP. Regional workshop for directors in the ZAP entomology area. Bethesda. Africa Indoor Residual Spraying Project, Abt Associates Inc. May 2017.
2. Methodological Guide for entomological surveillance with ovitraps. 2015. Secretariat of Health, National Center of preventive programs and disease control. Office for the program of vector-transmitted diseases. Mexico. Internal document, 32 p. Available at:

http://www.gob.mx/salud/documentos/guia-metodologica-para-la-vigilancia-entomologica-con-ovitrampas (Consulted in May 2017).

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4. Natal, D. 2002. Bioecologia do *Aedes aegypti*. Biologico, Sao Paulo, v. 64, n. 2. P. 205-207. Jul./Dec.
5. Surveillance and Control of *Aedes aegypti* and *Aedes albopictus* in the United States. Document available online: https://www.cdc.gov/chikungunya/pdfs/Surveillance-and-Control-of-Aedes-aegypti-and-Aedes-albopictus-US.pdf (Retrieved on May 2017).
6. U.S. Department of Health and Human Services. 1980. (U. S. D. H. 1980). Biology and Control of *Aedes aegypti*. Vector Topics No. 4. Bureau of Tropical Diseases. Vector Biology and Control Division. Atlanta, Georgia.
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Annex 1. Larval/Pupal survey form

ZAP Larval / Pupal survey form

Geograpy Level 1 ____________________

Geography Level 2: ________________________

Neighborhood/District:______________________

Insert Your Country's MOH Logo Here

Serial No of the Survey form________________

Date

Ento tech ID/code _________________

Water source No.	Household number or code/Name of head of the house	Container type (See back)	Number of people in the household	Source of water			Shade status			Container location		Usage of water from the container				Intervention applied to this container?				Container covered?			Presence and density of Larvae			Pupal count					Number and type of mosquitos reared from pupae				
				Tap	Rain	Other	Fully	Partially	No shade	Inside house	Outside house	Daily	Frequently	Sometimes	Never	Use "√" to mark intervention (A=Abate; B=Bti;C=Fish;D=Other)	Date if Yes (√)	Water source cleaned? Yes(Y); Partially (P); No(N)	Date of cleaning	Completely	Partially	Not covered	None	From 1 - 100 individuals	More than 100 individuals	Total count (<20 L)	Total count, high visibility(>20 L)	Count*2.5 (1/3 total capacity>20 L)	Count*3.0 (2/3 total capacity; >20 L)	Count*3.5 (3/3 total capacity; >20 L)	# *Ae. aegypti*	# *Ae. albopictus*	# *Culex*	# *Anopheles*	# other mosquitos

Note: one row is for one mosquito breeding container/ type inspected. If more than one breeding/ water holding container is available in a compound more than row could be used per household.

Instructions on how to complete the Larval and pupal entomological monitoring forms ZAP

1. **Sr No:** Consecutive numbers for all the potential breeding sites. There may be several ones in a single home.
2. **Household No/Name of the head of the house**: Unique code for that house depending on the code system used in the country. Name of the point of contact in the household. The name needs to be recorded only once.
3. **Container type**: Choice of the type of container, potential or actual breeding site. From A to Z:
    A: Elevated tanks
    B: Elevated cisterns
    C: Drinking troughs
    D: Ground level barrels
    E: Ground level tanks
    F: Ground level cisterns
    G: Flowerpots
    H: Vases
    I: Aquatic plants
    J: Plastic containers
    K: Bottles (moveable)
    L: Rain catchments
    M: Sanitary facilities
    N: Non-Chlorinated swimming pools
    O: Decorative fountains
    P: Cement vases
    Q: Awnings
    R: Open tubes
    S: Tires
    T: Garbage cans
    U: Hollow trees
    V: Flowers
    W: Leaves
    Y: Wells
    Z: Others (specify)
4. **Source of water:** Where does the water stored in that particular container come from? (check the appropriate)
    Tap
    Rain
    Other
5. **Shade status**
    Fully

Partially
No shade

6. **Container location**: Where is the container located (check)
   Inside the house: containers located indoors, ie enclosed by four walls and a roof.
   Outside the house: containers located out of the main house, in peridomiciliary areas such as patios, backyards, open garages. Balconies and terraces are considered indoors, as they are part of the main house structure.
7. **Usage of the water from the container**: How often do people use the water in that container
   Daily
   Frequently: Twice or more than twice/week
   Sometimes: Once or less/week
   Never
8. **Intervention applied to this container?** Has been this container been subject of an intervention aimed directly or indirectly to do vector control?
      A: Abate (in sachets or granulated)
      B: Bti
      C: Fish
      D: Other
   Date if yes: If the exact date is unknown, the best estimate is fine
   Water source cleaned?
      Yes
      Partially (for example when only the walls are washed, not all water content eliminated, using chlorine on the walls, etc)
      No
   Date of cleaning (if the answer is Yes or Partially). If the exact date is unknown, the best estimate is fine.
9. **Container covered?** Was the container covered when the technician examined it?
   Completely
   Partially (check this even if there is a little opening in the cover)
   Not covered (check this if there is no cover at all)
10. **Presence and density of larvae:** Presence and estimates of the larval density
    None
    From 1 – 100 larvae
    More than 100 larvae
11. **Pupal count**: Counting or estimating the amount of pupae/container. When the count is partial, dip the larval net **only once**. For details see the back of the form, or the WHO protocol.
    Total count (<20 L): Count all pupae

Total count (> 20 L), high visibility: Count all pupae in conditions of high visibility and when there are <100 estimated pupae (according to the technician's criterion).
Count *2.5 (1/3 total capacity), >100 pupae: When the water in the containers is approximately 1/3, and the pupae density is high (>100 pupae).
Count *3 (2/3 total capacity), >100 pupae: When the water in the containers is approximately 2/3, and the pupae density is high (>100 pupae).
Count *3.5 (3/3 total capacity): When the water in the containers is approximately 3/3, and the pupae density is high (>100 pupae).

12. **Number and species of mosquitoes reared from pupae**: Normally where pupae are found, larvae too. If the larvae are all *Culex*, there is no need of counting the pupae; If the larvae are all *Aedes*, it is necessary to count and rear the pupae to adults to ensure they are *Aedes aegypti* or *Ae. albopictus*; If the larvae are *Culex* AND *Aedes*, it is necessary to count and raise to adults too.

    If the pupae are **evidently** not Aedes, Culex or Anopheles, it is not necessary to count or raise to adults. It is not necessary to report the sex, only the species.

    #Aedes aegypti: After raising in the insectary
    #Aedes albopictus: After raising in the insectary
    #Culex: After raising in the insectary
    #Anopheles: After raising in the insectary
    #Other mosquitoes: After raising in the insectary