PacMOSSI

Pacific Mosquito Surveillance Strengthening for Impact




INSECTICIDE RESISTANCE

MONITORING IN *AEDES* VECTORS IN THE PACIFIC

Participant Handbook for Short Course (Suva, Fiji)

Version 2 – December 2024

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Acronyms & Abbreviations

DC	discriminating concentration
cm	centimetres
h	hours
IRS	indoor residual spraying
m	metres
LLIN	long-lasting insecticidal net
PacMOSSI	Pacific Mosquito Surveillance Strengthening for Impact
PIC	Pacific Island Countries and areas
SOP	standard operating procedure
TIRS	targeted indoor residual spraying
WHO	World Health Organization

Glossary

The terms defined below have been extracted and/or adapted from a variety of sources including PacMOSSI and WHO documents. They may have different meanings in other contexts.

Principal vector	The species of mosquitoes mainly responsible for transmitting a specified pathogen in any particular circumstance Note: Different vector species may overlap seasonally or alternate in importance.
Secondary vector	Mosquito species thought to play a lesser role in transmission of a specific disease compared to the principal vector. Can be capable of maintaining disease transmission at a reduced level.
Insecticide	Chemical product (natural or synthetic) that kills insects. Ovicides kill eggs; larvicides kill larvae; pupacides kill pupae; adulticides kill adult mosquitoes Note: Prequalified insecticide products for vector control are listed by WHO at https://extranet.who.int/pqweb/vector-control-products
Insecticide mode of action	The way in which an insecticide affects the biological processes of an insect.
Insecticide susceptibility	Property of mosquitoes to be killed by exposure to a standard dose of insecticide. In WHO tube assays, a mosquito population is considered susceptible to an insecticide if 98% or more of test mosquitoes die after being exposed to a discriminating concentration of the insecticide.
Insecticide resistance	Ability of mosquitoes to survive exposure to a standard dose of insecticide. May be the result of behavioural or physiological adaptation in insects whereby traits are selected for that reduce the impact of insecticides and increase survival. In WHO tube assays, a mosquito population is considered resistant to an insecticide if 90% or less of test mosquitoes die (i.e., 10% or more survive) after being exposed to a discriminating concentration of the insecticide.
Behavioural resistance	Modification in mosquito behaviour that leads to avoidance of, or reduced contact with, insecticides.
Physiological resistance	Modification in mosquito physiology that leads to survival following exposure to insecticides.

Phenotypic resistance	Expression of the ability of mosquito population to survive insecticide exposure. Commonly characterised in susceptibility bioassays with discriminating concentrations of insecticides. These do not tell us anything about the underlying mechanism of resistance.
Mechanisms of resistance	The ways in which the impact of insecticides on insects is decreased to lower than expected. Can be due to changes to the insecticide target-site, the enzymes involved in insecticide metabolism or changes to the mosquito cuticle. Multiple mechanisms can be present in a single insect population.
Metabolic resistance	Changes in the amount or specificity of a metabolic enzyme so that it detoxifies an insecticide before it reaches the target site.
Genotypic resistance	Heritable mutations that confer physiological or behavioural resistance. In some cases, those genes can be identified using molecular assays. Examples include target site resistance, where alterations of the protein receptor targeted by the insecticide prevent the insecticide from binding and thereby reduces the effect the insecticide is expected to have on the mosquitoes.
Susceptibility bioassay	A bioassay in which a sample of mosquitoes is exposed to a discriminating concentration of an insecticide for a fixed period and the number of survivors is counted to quantify resistant mosquitoes.
Discriminating concentration for susceptibility bioassay	Concentration of an insecticide that, when a sample of mosquitoes is exposed to a surface treated with it for a standard period of time, reliably kills susceptible mosquitoes. Survivors are assumed to be resistant.
Resistance intensity bioassay	Strength of resistance in mosquitoes to insecticides, resulting from the level of expression of resistance phenotype(s). Resistance intensity is measured by testing the ability of mosquitoes to survive exposure to 5× and 10× a discriminating concentration of insecticide. Insecticide-based vector control is more likely to fail in areas of high intensity resistance.

Introduction to Course

Rationale

The Pacific region experiences recurring outbreaks of arboviral diseases. *Aedes* mosquitoes transmit diseases such as dengue, chikungunya and Zika. Most arboviral outbreaks are primarily vectored by *Aedes aegypti* and *Ae. albopictus*, although additional *Aedes* species (e.g., *Ae. hensilli, Ae. polynesiensis,* and *Ae. scutellaris*) may be involved.

Interventions against *Aedes* to prevent human disease often involve the use of insecticides, with application to surfaces or areas where adult mosquitoes commonly rest (e.g., indoor or outdoor residual spraying, or indoor space spraying) or to habitats that immature mosquitoes commonly inhabit (e.g., larviciding). The purpose is to reduce mosquito abundance and reduce the number of potentially infective bites received by humans.

For vector control interventions to be effective, *Aedes* vectors must be susceptible to the insecticides used by vector control programs. The most widely used method to measure mosquito susceptibility to insecticides is the WHO tube test. While this does not provide a definitive indication of whether an intervention will work or not, it serves as a useful proxy to inform the selection of insecticidal tools that are likely to impact on local mosquitoes if deployed appropriately.

Existing data for *Aedes* vectors in the Pacific indicate that insecticide resistance is present in some locations. However, there is limited information available for most countries and many insecticide classes, so the full extent of resistance across the region is not known. Additional monitoring is urgently required to inform decisions on effective *Aedes* vector control.

Resistance monitoring should be conducted with the most important local vector species because those are the species that interventions are expected to control. For that reason, some basic taxonomic ability is required to identify species locally and ensure adequate specimens are collected for resistance testing.

Overview

As part of the capacity building initiatives of the Pacific Mosquito Strengthening for Impact (PacMOSSI) consortium, short courses are conducted on key topics to support improved vector surveillance and control. This includes the current targeted training on monitoring of *Aedes* insecticide susceptibility in the Pacific. This participant handbook has been designed to serve as a reference for participants during and after the short course.

Goal

This short course has been designed to improve the capacity and capability of participants to generate, manage and use *Aedes* vector insecticide resistance data to ensure appropriate monitoring and intervention selection in their respective settings. The approach and content have been tailored to the needs of Pacific Island countries and areas (PICs) and developed to be suitable for women, men and, with support, people living with disabilities.

Objectives

By the end of the course, participants will be able to:

1. Explain insecticide resistance in *Aedes* vectors and the critical role of monitoring
2. Collect *Aedes* vector species from the field for resistance monitoring
3. Rear *Aedes* in low-capacity insectaries for use in WHO tube test
4. Conduct basic mosquito identification, distinguishing larvae by genus (e.g. *Aedes*, *Culex* and *Anopheles*) and adults by genus, species and sex (e.g. male, female).
5. Conduct standard *Aedes* insecticide susceptibility testing with WHO tube tests
6. Analyse and interpret insecticide resistance data from WHO tube tests
7. Understand the implications of resistance for selection of vector control interventions
8. Manage and report resistance data, including in Tupaia (or other platforms)

Modes

The course has been designed to be delivered in three modes, which are represented in this participant handbook with the following colour coding:

Classroom learning	Practical demonstration
Field-based activity	Hands-on participation

However, content may be delivered in a different mode to that specified depending on available time, facilities, or the number of participants.

Participants

Appropriate participants are staff of the Ministries of Health or other national and subnational health services or their development partners in PICs tasked with entomological surveillance and vector control. This may include women, men and, with support, people living with disabilities who are specific vector control personnel, Environmental Health Officers or community members.

Preparation

This short course has been designed to complement other PacMOSSI capacity building initiatives. Some theoretical knowledge is assumed for participants, based on completion of PacMOSSI online content including Module 5 on insecticide resistance and product efficacy.

Key resources

This *PacMOSSI Participant Handbook* provides a simplified overview of key technical content to be covered with links to relevant presentations and videos. The resources section includes further details. An electronic copy of this handbook is available by emailing pacmossi@jcu.edu.au.

The taxonomic guide *A morphological identification key to the mosquito disease vectors of the Pacific including medically important vectors* was developed as a basic guide to adult mosquito identification relevant to the 22 PICs targeted by the PacMOSSI program.

Content

This short course builds on content from the PacMOSSI online course. Modules can be accessed by registering via the PacMOSSI Online Course webpage. Other materials used in this course include PacMOSSI Standard Operating Procedures available in full on the PacMOSSI Resources webpage.
Extracts are also included in the Resources section at the end of this document.

The course also draws on materials from others sources, in particular the WHO Manual for monitoring insecticide resistance in mosquito vectors and selecting appropriate interventions.

The Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests and the accompanying video provide further details on the procedure used during the course.

Assessments

Participant competence assessment

- A pre-course self-assessment will be issued that evaluates participant competence in key skills domains that will be addressed during the training.
- Facilitators will then evaluate each participant in these skills domains throughout the course to inform any corrective directions and to issue course certificates once all skills have been demonstrated.

Course evaluation

- An end of course evaluation will capture feedback to inform any adjustment of course content or delivery for future courses.

Participant competence assessment

INSERT A TICK

	Component	PRE-COURSE Participant confident of competence	POST-COURSE Facilitator observed competence
1	Explain insecticide resistance in *Aedes* and the importance of monitoring in the Pacific		
2	Describe appropriate methods for collecting *Aedes* from the field for resistance testing		
3	Accurately differentiate between *Aedes* and other mosquito genera (immatures)		
4	Accurately differentiate between *Aedes* and other mosquito genera (adults)		
5	Accurately differentiate between male and female mosquitoes (adults)		
6	Demonstrate rearing of mosquitoes for adult WHO tube tests		
7a	Recall requirements and processes for ordering equipment for WHO tests		
7b	Demonstrate set up of equipment for WHO tube tests		
8a	Correctly run WHO tube tests		
8b	Accurately record information from WHO tube tests		
9	Appropriately analyse and interpret data from WHO tube tests		
10	Correctly use insecticide resistance data to inform vector control decisions		
11	Properly and completely enter insecticide resistance data into Tupaia (or other reporting system)		

Additional facilitator comments:

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Course Content

Topic 1: Review insecticide resistance in *Aedes* and importance of monitoring

Session mode	Classroom learning
Learning objectives	By the end of this session, participants will be able to: • Explain insecticide resistance in *Aedes* and the importance of monitoring in the Pacific
Resources	PacMOSSI Module 4 Unit 3, Module 5 Unit 1 WHO Manual for monitoring insecticide resistance in mosquito vectors and selecting appropriate interventions

Background

Insecticidal interventions used against *Aedes* dengue adult vectors include targeted indoor residual spray, spatial repellents, lethal ovitraps, outdoor residual spray, indoor space sprays, insecticide treated window screens and curtains, and outdoor truck-mounted space spraying. These interventions use a range of insecticides grouped into classes based on chemical structure and mode of action.

The mode of action describes how an insecticide works at its target site in the body of the mosquito. For example, the insecticide may kill the mosquito through being absorbed by its outer cuticle, or orally via ingestion. Knowing the mode of action of an insecticide is important to ensure effective and safe application of insecticides, and for managing insecticide resistance.

For vector control to be effective, the mosquitoes must be susceptible to the insecticide that is used. Insecticide resistance is the capacity of mosquitoes to survive insecticide treatments. Insecticide resistance can result from behavioural or physiological adaptations. If resistance is observed, another insecticide to which the vector is susceptible should be selected.

Examples of insecticides used for adult and immature *Aedes* control

Target site in the mosquito	Mode of action	Insecticide class	Example insecticides
Nerves and muscles	Sodium channel modulators	Pyrethroids	alphacypermethrin, deltamethrin, lambda-cyhalothrin, etofenprox, bifenthrin, transfluthrin, metofluthrin
Nerves and muscles	Acetylcholinesterase inhibitors	Organophosphates	pirimiphos-methyl
		Carbamates	bendiocarb
Mosquito midgut	Disruption of midgut membranes	*Bacillus thuringiensis var. israelensis*	*Bti*-based products

Strategies used to control *Ae. aegypti* and *Ae. albopictus* with adulticides (adapted from Ritchie et al. 2021)

Method	Target vector	Application target	Insecticides	Persistence
Entomological + epidemiological evidence of *Ae. aegypti* and dengue control, respectively				
Targeted indoor residual spray (IRS, TIRS)	endophilic *Ae. aegypti*	dark, shady areas indoors, inside wardrobes, under tables and beds, lower walls; surfaces treated to point of runoff.	synthetic pyrethroids, carbamates, organophosphates	depends on product: pyrethroids and carbamates up to 3-5 months; novel formulations (pirimiphos methyl) up to 7 months
Spatial repellent *	endophilic *Ae. aegypti*	inside human habitations	transfluthrin and metofluthrin	Formulation-dependent; 2-3 weeks established, 4 weeks under investigation
Evidence of urban *Ae. aegypti* control, limited evidence of impact on DENV transmission				
Lethal ovitraps (LOs)	gravid *Ae. aegypti*	multiple LOs set at premises	synthetic pyrethroids; adhesives	4-8 weeks
Outdoor residual spray; peri-focal spray; harbourage spray; barrier spray	*Ae. albopictus* and (to a lesser extent) endophilic *Ae. aegypti*	leaf litter and lower vegetation in shady, forested areas; outdoor container (tires, rubbish, etc.) dumps, bushy fence line	synthetic pyrethroids, carbamates, organophosphates	4-8 weeks depending on product formulations
Indoor space spray	*Ae. aegypti*	dark shady areas inside houses, rooms; must be applied repeatedly (i.e. 3 times weekly) (Gunning et al. 201	synthetic pyrethroids, carbamates, organophosphate	up to 1 week
Some evidence of urban *Ae. aegypti* control, no evidence of significant impact on DENV transmission				
Insecticide treated window screens	*Ae. aegypti*	windows and doors of house	synthetic pyrethroids	1+ year
Insecticide treated curtains	*Ae. aegypti*	windows and doors of houses	synthetic pyrethroids	ca. 1 year
Outdoor space spray: truck mounted and aerial	*Ae. aegypti, Ae. albopictus*	rapid widespread treatment of outdoor areas	primarily organophosphates; e.g. naled	<1 week

* There is no WHO recommendation for this intervention
CDC = Centers for Disease Control; AGO = autocidal gravid ovitrap; KD = knock-down; LO = lethal ovitrap; ULV = ultra-low volume spray.

Relevance of resistance

Resistance to some insecticide classes has been observed in *Aedes* vectors in the Pacific. This threatens to undermine the efficacy of some vector control interventions. Vector control programs in the Pacific can prevent the emergence and spread of resistance through the informed selection of interventions. This is known as insecticide resistance management. Knowledge of local vector resistance status is key to guiding evidence-based strategies.

It is also important to note that compounds within the same chemical class (e.g. pyrethroids) will all have the same mode of action. Rotating from one pyrethroid insecticide to another pyrethroid (i.e. switching to a different product that has the same class of active ingredient) has limited or no value in resistance management. Thus, when considering products to support resistance management plans, it is important to select different insecticides that are not in the same insecticide class or with the same mode of action.

- **Before** any insecticide-based vector control intervention is deployed, programmes should ensure that local vectors are susceptible to the relevant insecticide class planned for use and should establish a resistance management plan accordingly (see WHO guidance).
- **Once** the intervention(s) are in place, local vector susceptibility to the insecticide class(es) in use should be periodically monitored (such as once every 2 years) to proactively manage and detect any emergence of resistance early and act accordingly.

To avoid or manage resistance, strategies can be used which involve use of multiple insecticide products (of different classes, with different modes of action) in rotations, mixtures, mosaics or combinations. These is discussed further under Topic 9.

Monitoring for resistance

Laboratory tests are used to determine the **presence or absence** of resistance in local vector populations by exposure to fixed concentrations of insecticide class(es) used or planned for use in vector control interventions in the area. If resistance is confirmed, further investigations into the intensity of resistance or the mechanisms underpinning the resistance can be done.

This short course focuses on conducting resistance monitoring in the Pacific in accordance with the Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests. Key considerations for this approach to such monitoring have been summarised below.

Procedures exist to evaluate insecticide resistance in both adult and larval mosquitoes.

- If the vector control intervention under consideration targets adult mosquitoes (e.g. ITNs, TIRS, space spraying for *Aedes* control), insecticide resistance testing should be performed using adults.
- If the vector control intervention under consideration targets larval stages (e.g. chemical larviciding), insecticide resistance testing should be ideally conducted using larvae. Note: larval resistance testing is not covered in this course.

Uses of insecticide resistance monitoring data

It is imperative that insecticide resistance data are presented promptly to decision-makers. The data should be considered alongside intervention monitoring and evaluation data, epidemiological data and other relevant data to understand the impact of insecticide resistance on the effectiveness of disease control and to inform programmatic decisions.

Resistance data must be used to:

- Inform selection of appropriate vector control interventions.
- Trigger a change in vector control interventions when resistance is detected.
- Guide the introduction of novel or supplementary vector control interventions
- Inform resistance monitoring and management strategies in areas using insecticide-based vector control.

Priority aspects of resistance to monitor in common programmatic scenarios

Programmatic scenario	Programmatic needs	Priority
Introducing an insecticide-based vector control intervention for the first time	Select an intervention that is effective against the local vector population	To evaluate presence of resistance in the local vector population to the insecticides in the interventions under consideration, before any intervention is deployed
Changing vector control interventions or insecticide after resistance is detected		
Insecticide-based vector control is already in progress	Detect resistance as soon as it emerges to trigger a change of interventions	To evaluate presence of resistance in the local vector population to the insecticides in use

Summary of general recommendations for resistance monitoring of adult *Aedes* vectors (adapted from WHO, 2023)

Consideration	General recommendation
Species	✓ Prioritise testing known principal disease vectors first ✓ Consider testing secondary vectors if relevant to program decisions ✓ Test each vector species separately if possible (or collect, kill and identify the contents of each WHO tube at the end of the assay – see Annex 3)
Sampling	✓ A representative sample of mosquito specimens from the area targeted for insecticidal vector control should be used for testing. ✓ Collect eggs or larvae from several areas (largely in and around gardens and yards). If possible collect eggs or larvae from several areas that are > 50 m apart. This increases the genetic diversity of the collection, by reducing the chances of mosquitoes that are closely related.
Timing	✓ Monitor resistance when vector abundance is high to increase chance of collecting enough mosquitoes to test resistance to all relevant insecticides
Frequency	✓ Monitor every 1 or 2 years at all sentinel sites if possible, depending on usage of insecticides for domestic and public health and agriculture
Developmental stage	✓ Test adult stage of mosquitoes (unless extensive larviciding is undertaken or planned)
Adult age and physiological state	✓ Test unfed female adult mosquitoes that are 3–5 days of age and have been starved 6 h before the test* ✓ Test individuals in good physical condition (e.g. well nurtured, kept in uncrowded trays and cages)
Insecticides	✓ Test insecticides currently in use and being considered for use ✓ Test only one insecticide for each relevant class (e.g. one pyrethroid, one organophosphate) as results can be generalised for the class ✓ Don't test for resistance to insecticides no longer used unless this is well justified
Procedure	✓ Use the same test procedure (tube test, bottle bioassay) consistently to ensure comparability of results over time and geographic areas to identify changes or trends
Test conditions	✓ Temperature and humidity in the space used for testing should also be as consistent as possible between tests ✓ These conditions should be recorded with the test results to help with interpreting any discrepancies across test results
Further testing	✓ Assess physiological resistance first and if justified then do additional testing for resistance intensity or resistance mechanisms

Topic 2: Collect *Aedes* from the field for resistance monitoring

Session mode	Field-based activity
Learning objectives	By the end of this session, participants will be able to: • Describe appropriate methods for collecting *Aedes* from the field for resistance testing
Resources	PacMOSSI Module 5 Unit 1 PacMOSSI Standard Operating Procedure for conducting larval and pupal surveys for *Aedes*

Target primary vectors

In many regions endemic for vector-borne diseases, several vector species may be present concurrently, but their contribution to disease transmission may be different. Priority should be given to monitoring resistance of the species that play the greatest role in pathogen transmission. These species should be identified through entomological surveillance before starting insecticide resistance monitoring.

As part of resistance monitoring, the species being tested need to be clearly identified and recorded. A representative sample of the mosquitoes from the area targeted for insecticidal vector control should be tested for resistance.

Select resistance monitoring sites

Countries should aim to evaluate vector resistance to insecticides in each operational area where insecticide-based vector control interventions are deployed or under consideration for deployment. However, this may not be feasible due to resource constraints.

If this is the case, neighbouring areas with similar vector species composition, similar selection pressures (e.g. same interventions, similar agricultural practices) and insecticide resistance history can be grouped. Resistance can then be monitored at one site per grouping.

The priority should be on generating **high-quality data** (i.e. strictly following SOPs) at a **limited number of sites**, rather than a greater quantity of data from more sites at the risk of reduced quality.

Conduct field sampling

Resistance tests can be conducted with F0 (field-collected) or F1 (first laboratory generation) *Aedes* adults.

- **F0:** Mosquitoes collected directly from the field as eggs, larvae, pupae, or adults. These are wild specimens that have not undergone laboratory rearing.
- **F1:** The first laboratory generation, reared from F0 mosquitoes under controlled conditions to standardize age, size and environmental factors.

Efficient collection of field specimens

Collection of eggs (ovitraps) and larvae (container inspection) are the most efficient ways to collect large numbers of *Aedes* mosquitoes. These must be reared to adult, but this can be done in a basic space. Using F1 mosquitoes reduces variability and increases sample numbers but requires additional rearing time.

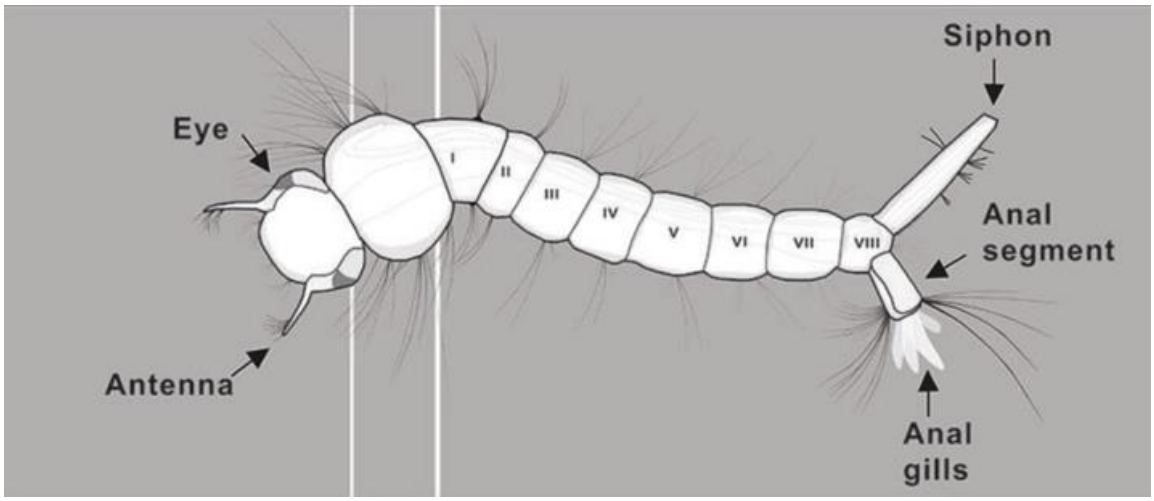
Operational notes:

- ✓ Collect larvae from the preferred larval habitats of the main vectors of transmission (i.e. urban and peri-urban containers, containing relatively clean water: natural and man-made), or eggs from representative locations.
- ✓ Ensure collection from diverse and geographically separate sites to increase genetic variability.
- ✓ Pool and rear together the wild mosquito samples to avoid having a high proportion of siblings in the same test sample.

Topic 3: Identify larvae

Session mode	Practical demonstration
Learning objectives	By the end of this session, participants will be able to: • Demonstrate rearing of mosquitoes for adult WHO tube tests
Resources	PacMOSSI Module 5 Unit 4 presentations PacMOSSI Standard Operating Procedure for maintaining *Aedes aegypti* and *Aedes albopictus* colony mosquitoes Operation of an insectary practical manual. *Aedes aegypti* rearing procedures and basic principles of biosafety

Larval mosquito anatomy



Identification keys

See:

- Belkin JN. 1962. The mosquitoes of the South Pacific (Diptera, Culicidae). Berkeley and Los Angeles: University of California Press, USA.
- Marks EN., Reye EJ (1982). An Atlas of Common Queensland Mosquitoes with A Guide to Common Queensland Biting Insects. Queensland Institute of Medical Research, Brisbane, 1982

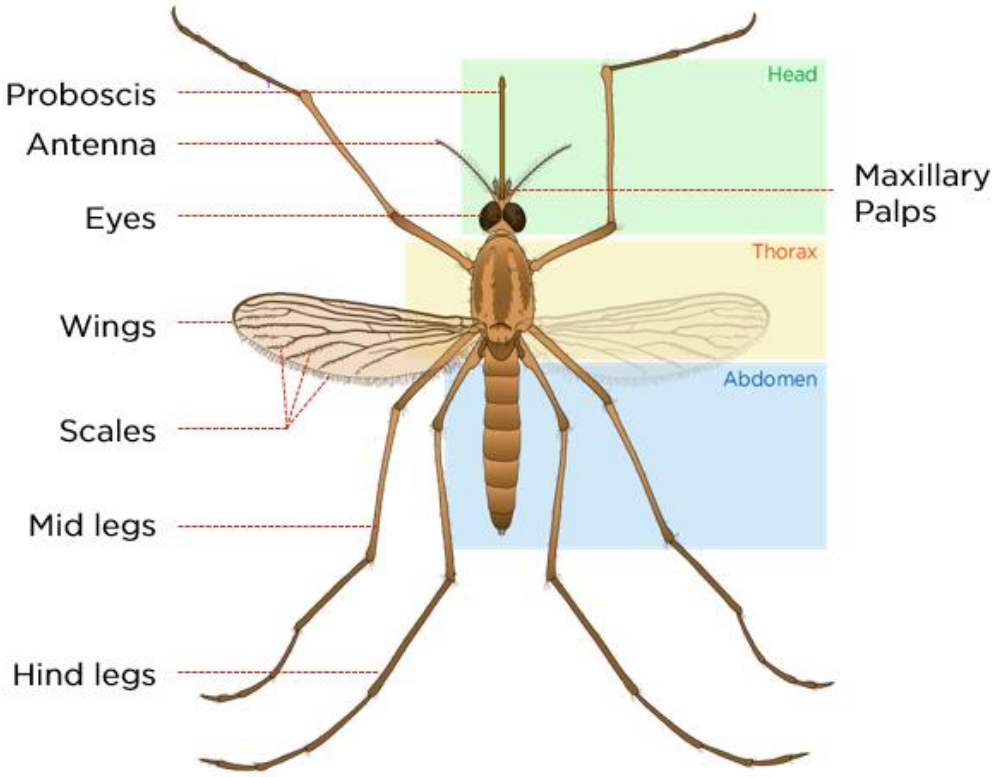
Topic 4: Distinguish between adult mosquito genus, species and sex

Session mode	Hands-on participation
Learning objectives	By the end of this session, participants will be able to: • Differentiate between mosquitoes and other insects • Accurately differentiate between *Aedes* and other mosquito genera • Accurately differentiate between male and female mosquitoes (adults)
Resources	PacMOSSI Module 3, Unit 4 A morphological identification key to the mosquito disease vectors of the Pacific including medically important vectors

Importance of knowing mosquito species

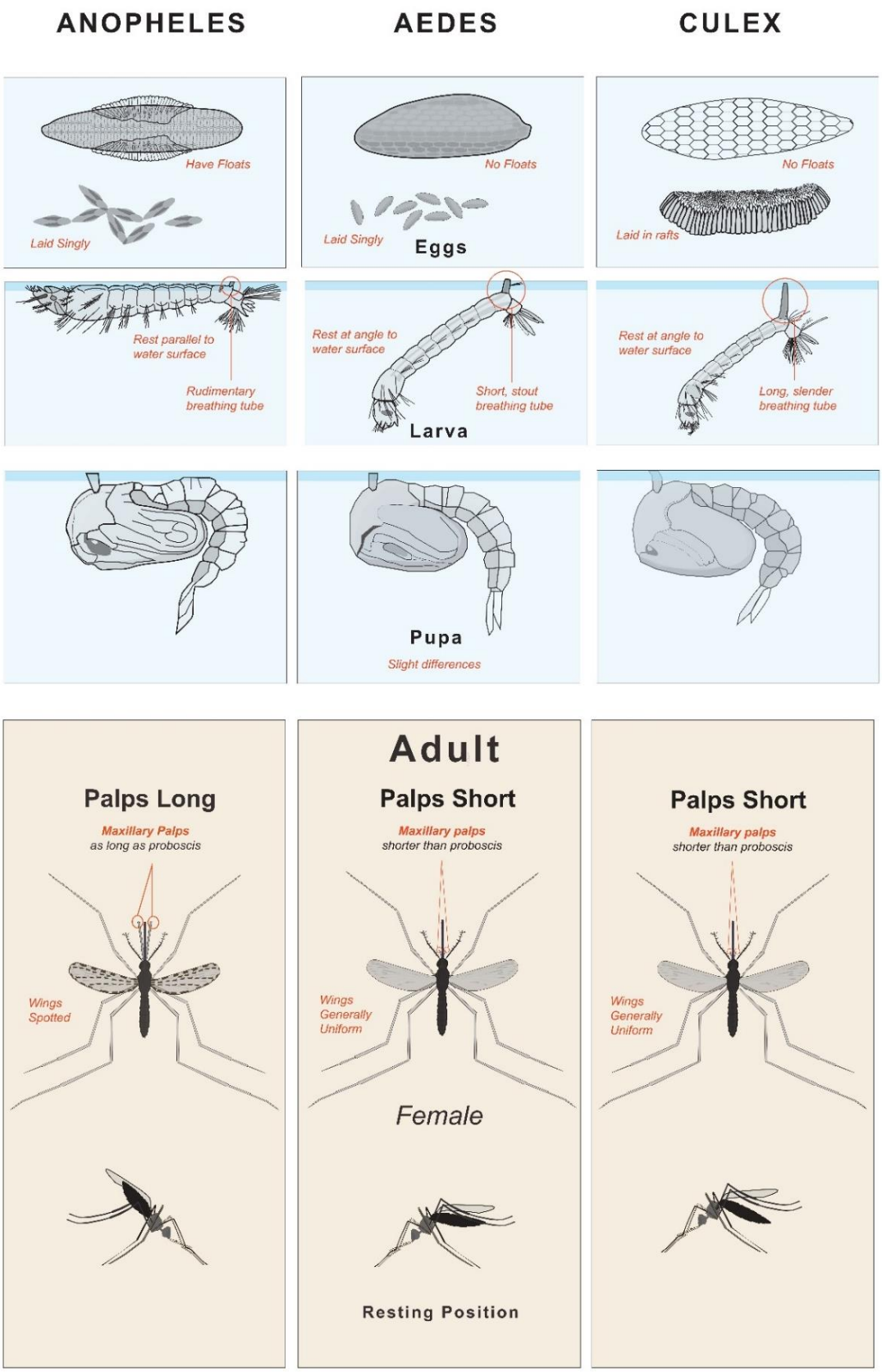
Resistance monitoring should be conducted against the main vectors of disease, and then in secondary vectors. The vector species occur concurrently in the field and after samples are collected, they need to be identified to species for testing purposes. Mosquito samples can be identified to species as larvae (usually done prior to testing) or as adults (may be done after testing).

Adult mosquito anatomy



Distinguishing between mosquito genera

(Source: GLOBE Worldwide Science Education)



Preparation of adult specimens

Specimens must be in good condition to allow for accurate identification. Older specimens, or those that have been damaged during capture, may have lost scales and other features that can aid in their identification.

If the specimens to be identified are still alive, manually aspirate those you wish to identify into a collection cup and seal. Kill them by planning in a freezer for a minimum of 30 minutes. (If ethyl acetate is available, this can alternatively be used).

Once the specimens are dead, use forceps to carefully place the first specimen for identification onto a Petri dish under a dissection microscope.

Determining mosquito species for mortality calculations

Species should be identified **after the resistance test** as follows.

- Separate mosquitoes into four groups:
    1. mosquitoes that died after exposure to the insecticide;
    2. mosquitoes that survived after exposure to the insecticide;
    3. control mosquitoes that died; and
    4. control mosquitoes that remained alive.
- Store specimens in 0.5 mL microcentrifuge tube with silica gel or in 70% ethanol. Pool and label for dead or alive, treated or control.
- Identify individual mosquitoes under the microscope (or using polymerase chain reaction if required).

Calculate mortality for individual species, considering their respective control mortality (i.e. correcting mortality with Abbott's formula, when needed).

Identification keys

See A morphological identification key to the mosquito disease vectors of the Pacific including medically important vectors

Topic 5: Rear *Aedes* in a low-tech field insectary

Session mode	Practical demonstration
Learning objectives	By the end of this session, participants will be able to: • Demonstrate rearing of mosquitoes for adult WHO tube tests
Resources	PacMOSSI Module 5 Unit 4 presentations PacMOSSI Standard Operating Procedure for maintaining *Aedes aegypti* and *Aedes albopictus* colony mosquitoes Operation of an insectary practical manual. *Aedes aegypti* rearing procedures and basic principles of biosafety

Mosquito rearing conditions

Mosquito rearing conditions can affect test results. This includes:

- ambient and water temperature
- water quality
- larval food
- larval crowding
- mosquito manipulation during rearing processes
- mosquito manipulation during the performance of resistance tests

To ensure comparability of test results over time, it is important to consistently adhere to the same mosquito rearing conditions and handling protocols. Ideally this would be:

- water temperature of 25 °C ± 5 °C
- ambient temperature of 27 °C ± 2 °C
- relative humidity of 75% ± 100%

To obtain sufficient mosquito samples for a test, wild-caught adult female mosquitoes or F1 offspring of wild-caught mosquitoes can be accumulated in cages as they emerge from pupae, or as they are collected from the field. While in the cages, mosquitoes should be provided with access to 10% sugar–water meal which should be removed about 6 h before conducting a test.

Handy tips for insectary rearing of wild-collected *Aedes* larvae

- ✓ **Water Quality:** Ensure that water in larval rearing containers is clean and free of contaminants. Rain/tank water or distilled/dechlorinated water works well.
- ✓ **Humidity:** Maintain moisture by covering adult cages with a wet towel.
- ✓ **Larval Feeding:** Provide larvae with a balanced diet of finely ground fish food, yeast, or algae.
- ✓ **Larval Density:** Immature larval stages should be kept in uncrowded trays to ensure sufficient growth and reduce mortality. Overfeeding can lead to poor water quality, so offer small amounts and remove uneaten food.
- ✓ **Space for Pupae:** Avoid overcrowding in rearing containers to reduce cannibalism. Ensure pupae have enough space to float freely and emerge as adults.
- ✓ **Regular Cleaning:** Clean all rearing containers and surfaces regularly to prevent the buildup of harmful microorganisms.
- ✓ **Monitor Immature Health:** Regularly check for signs of disease or abnormal behaviour, such as deformities or reduced activity, which could indicate poor rearing conditions.
- ✓ **Handling:** Use gentle techniques for handling and transferring to avoid injury. In particular, carefully handle adult mosquitoes before and during testing.

Specifics of adult mosquitoes to use in tests

Sex:

✓ **Female mosquitoes** should be used for resistance monitoring.

- Male mosquitoes should not be used because:
    - they are not epidemiologically relevant (as they do not transmit pathogens);
    - they are more susceptible to insecticides than females; and
    - vector control interventions often target female mosquitoes.

Age:

✓ **Mosquitoes aged 3-5 days old** should be used for resistance monitoring.

- Older mosquitoes have increased susceptibility to insecticides than younger ones (particularly when there is metabolic resistance, which may decline with mosquito age).

Physiological status:

✓ **Non-blood fed mosquitoes** should be used for resistance monitoring.

- Blood-fed mosquitoes have exhibited higher resistance than their unfed counterparts.

Test using adult female mosquitoes that are 3–5 days old and non–blood fed (F0 and/or F1)

Topic 6: Prepare and set up WHO tube tests for adult *Aedes*

Session mode	Classroom learning
Learning objectives	By the end of this session, participants will be able to: • Recall requirements and processes for ordering equipment for WHO tests • Demonstrate set up of equipment for WHO tube tests
Resources	PacMOSSI Module 5 Unit 2 presentations WHO Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests WHO Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests [video]

Procuring items for insecticide resistance testing of *Aedes*

Supplies for the WHO tube test are available from the Vector Control Research Unit (VCRU), School of Biological Sciences, Universiti Sains Malaysia (USM), Penang, Malaysia.

- **Consult the supplies catalogue**:
  https://cdn.who.int/media/docs/default-source/ntds/vector-ecology-mangement/catalogue_who-test-kits_29apr2024.pdf?sfvrsn=6782537a_36

- **Order form WHO susceptibility test kit:**
  https://cdn.who.int/media/docs/default-source/ntds/vector-ecology-mangement/order_form_7may2024_fillable.pdf?sfvrsn=a9fb662e_14

- **Place your order online:**
  https://inreskit.usm.my/

Insecticide-impregnated Whatman no. 1 filter papers or control papers come in boxes of eight papers each for a particular insecticide or control. There are different standard concentrations of insecticide used on the papers depending on the mosquito species being tested, as outlined in the SOP for the WHO tube test.

The range of insecticides for which impregnated test papers are available is expected to expand over time. Consult the WHO website and USM website to check the availability of papers and other relevant materials.

Insecticide discriminating concentrations (DCs) for WHO susceptibility bioassays with Aedes adult mosquitoes

Insecticide class	Active ingredient	Species for which DCs are validated	DC	Exposure period	Holding period	Carrier oil/ solvent/ surfactant
Pyrethroids	Alpha-cypermethrin	*Ae. aegypti*	0.05%[a]	1 h	24 h	Silicone oil
		Ae. albopictus	0.08%[a]	1 h	24 h	Silicone oil
	Deltamethrin	*Ae. aegypti, Ae. albopictus*	0.03%[a]	1 h	24 h	Silicone oil
	Lambda-cyhalothrin	*Ae. aegypti*	0.05%[a]	1 h	24 h	Silicone oil
		Ae. albopictus	0.08%[a]	1 h	24 h	Silicone oil
	Permethrin (40:60 cis:trans isomer ratio)	*Ae. aegypti, Ae. albopictus*	0.40%[a]	1 h	24 h	Silicone oil
Carbamates	Bendiocarb	*Ae. aegypti, Ae. albopictus*	0.20%	1 h	24 h	Olive oil
	Propoxur	*Ae. aegypti*	0.10%[b]	1 h	24 h	Olive oil
Organophosphates	Chlorpyrifos-ethyl	*Ae. aegypti, Ae. albopictus*	1.00%	1 h	24 h	Olive oil
	Pirimiphos-methyl	*Ae. aegypti, Ae. albopictus*	60 mg/m^2[a]	1 h	24 h	Acetone only
	Malathion	*Ae. aegypti*	1.50%[b]	1 h	24 h	Olive Oil
		Ae. albopictus	5.00%[a]	1 h	24 h	Olive Oil

[a] These DCs supersede the tentative concentration presented in *Test procedures for insecticide resistance monitoring in malaria vector mosquitoes* (second edition). Results obtained using the former tentative concentration should be validated against these new DCs.

[b] Impregnated papers no longer supplied by Universiti Sains Malaysia.

Storage of impregnated papers

Impregnated and control papers should be stored in their original plastic boxes that are sealed with tape at 4–8 °C in cool cabinets or refrigerators during and up to the end of the shelf-life period. Where feasible, use temperature data loggers to monitor storage conditions, as significant temperature fluctuations can compromise paper efficacy and result in unreliable assay outcomes. Test papers should never be exposed to direct sunlight or to temperatures higher than 8°C, except for short periods during use or shipment.

Shelf life under optimal conditions

Unused impregnated papers, if stored correctly, have a shelf life of 2-5 years, but this varies by insecticide class, as outlined below. During this period, the papers can be re-used in assays up to 5 times. The date of expiry of each batch of papers is given on their box. Papers should not be used after their expiry date.

Shelf life of papers

Class	Insecticide	Shelf life when stored at 4–8°C	Stability when stored 50°C for 2 weeks or 20°C for 8 weeks
Organochlorine	p,p'-DDT	5 years	Stable
Organophosphates	Malathion	3 years[a]	Stable[a]
	Pirimiphos-methyl	3 years[a]	-
Carbamate	Bendiocarb	3 years[a]	-
Pyrethroids	Alpha-cypermethrin	2 years	Stable
	Cyfluthrin	2 years	Stable
	Deltamethrin	2 years	Stable
	Etofenprox	2 years	Stable
	Lambda-cyhalothrin	2 years	Stable
Synergist	Piperonyl butoxide	3 years	Stable

Under WHO evaluation [a] Tentative (needs confirmation).

Between testing rounds

When a paper is used, the date of use should be written with a pencil on the edge of the paper on the untreated side. Papers already used 1-5 times can be stored for reuse between testing rounds. For this, they should be separated by aluminium foil, kept in their original plastic box, sealed with tape and stored in a cool container or refrigerator.

Between tests within the same testing round

For bioassays conducted over multiple days, impregnated papers can remain in exposure tubes to minimize handling and damage. Wrap each tube in aluminium foil and store it according to temperature guidelines. Before reuse, bring the wrapped tubes to room temperature for 1 hour before removing the foil.

Preparations for testing

Papers

Remove the box with impregnated papers from the cold cabinet or refrigerator one hour before testing and bring to room temperature while unopened. This avoids condensation of water on the surface of the papers, which can hydrolyse the insecticide.

Equipment

The WHO tube test kit consists of plastic tubes, labelled with different coloured dots:

- Holding tube: into which clean white paper is inserted, is identified by a **green** dot.
- Control tube: into which oil-/acetone-treated filter paper is inserted, is identified by a **yellow** dot.
- Exposure tube: into which the insecticide-treated paper is inserted, is identified by a **red** dot.

Start by labelling each green-, yellow- and red-dotted tube, according to the instructions outlined in the WHO SOP for WHO tube test and inserting each type of insecticide test paper accordingly.

Mosquitoes

This bioassay requires 150 non-blood-fed adult female mosquitoes aged 3–5 days. Mosquitoes need to be starved for 2 hours before using them in the test. Each tube should contain 25 female mosquitoes, or as close to 25 as possible, without exceeding 25 to avoid overcrowding.

To separate the females from the males in the cage, try placing a warm hand and/or breathing on the side of the cage to attract the females to one side before aspirating them out of the cage. Transfer the females into each holding tube through the filling hole on the tube sliding door using an aspirator. Once 25 mosquitoes have been transferred to each holding tube, continue to follow the steps as outlined in the WHO SOP.

Remember to note/record the time of assay start, temperature conditions etc.

Number of adult mosquitoes to use in WHO tube tests

Insecticide being used in test	Total No. mosquitoes per test	Control		Treatment	
		No. of mosquitoes per tube	No. of tubes	No. of mosquitoes per tube	No. of tubes
All insecticides	150	20-25	2	20-25	4

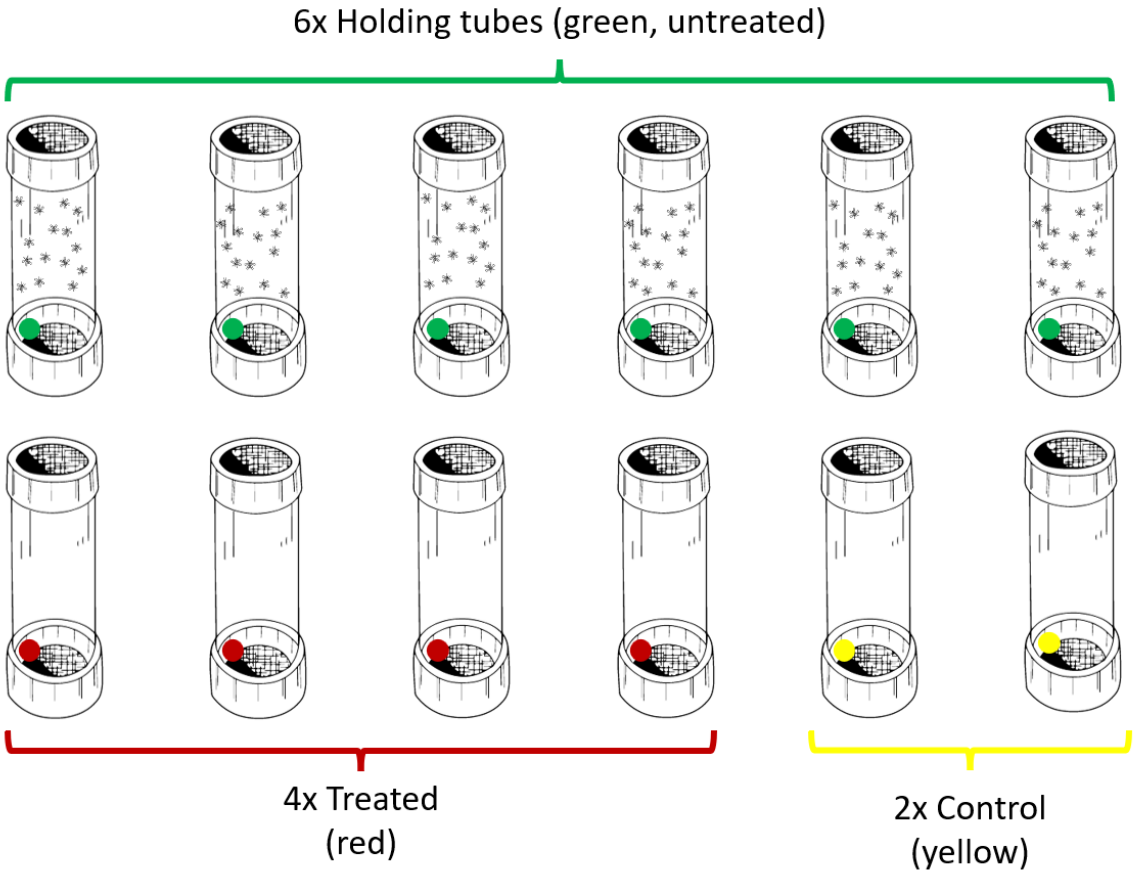
Topic 7: Run, record and interpret data from WHO tube tests for adult *Aedes*

Session mode	Classroom learning
Learning objectives	By the end of this session, participants will be able to: • Correctly run WHO tube tests • Accurately record information from WHO tube tests
Resources	PacMOSSI Module 5 Unit 2 presentations WHO Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests WHO Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests [video] PacMOSSI Excel sheet

Procedures for testing

The full SOP for the WHO tube test is available online here with a supporting video available here.

Overview of WHO tube test set up



Data recording and calculation of test results

During the test, data should be entered in paper-based or digital data recording forms. The end-point of the test is mosquito mortality 24 hours after 1 hour of exposure to the insecticide.

In each bioassay, mortality should be calculated separately for the test mosquitoes (i.e. those exposed to the insecticide) and the control mosquitoes. The number of mosquitoes killed at the end of the exposure period is then divided by the total number of mosquitoes initially exposed in the tubes and the result expressed as a percentage.

Mortality for mosquitoes exposed to treated papers (test) is:

$$\textit{Treatment mortality}\ (\%) = \frac{\textit{Number of test mosquitoes that died}}{\textit{Total number of test mosquitoes}} \text{ x}$$

Mortality for mosquitoes exposed to untreated papers (control) is:

$$\textit{Control mortality}\ (\%) = \frac{\textit{Number of control mosquitoes that died}}{\textit{Total number of control mosquitoes}} \text{ x}$$

Mortality-based criteria to validate tests

Criteria to validate susceptibility and intensity bioassays conducted with all insecticides

- control mosquito mortality 24 h post-exposure is ≤20% (i.e. in solvent/oil control).

Mortality adjustments when control mortality is high

Treatment mortality adjustments should be carried out as follows.

- If the control mortality is <5%, no correction of mortality is necessary.
- When control mortality is ≥5% and ≤20%, the observed mortality in insecticide-exposed mosquitoes must be corrected using Abbott's formula:

$$\textit{Corrected treatment mortality}\ (\%) = \frac{(\%\ \textit{treatment mortality} - \%\ \textit{control mortality})}{(100 - \%\ \textit{control mortality})} \text{ x}$$

Standard data collection forms to record the results of bioassays, both mortality and knockdown rates, are provided as Annex 3 and on the WHO website.

Checking the quality of the papers when unexpected results are obtained

In the event that unexpectedly high numbers of survivors are found following exposure to an insecticide that is expected to kill all test specimens (based on knowledge of local vectors and vector control interventions):

- If the papers were procured from USM, Malaysia, contact them to reconfirm the quality of the papers using the quality control samples kept in their storage.
- If possible, test papers against susceptible mosquito lab strain in a research laboratory following standard procedures with optimal number of mosquitoes.

Interpretation of bioassay results

Caution must be exercised when interpreting the results of bioassays.

- Using fewer than the optimal number of mosquitoes will increase uncertainty in test results and may lead to misclassification of resistance status of a vector population.
- Mosquito sampling, rearing techniques, handling, the quality of the impregnated papers and test ambient conditions may influence the results, leading to underestimation or overestimation of mosquito mortality.

For adulticides

Only tests conducted strictly following the relevant SOP should be considered for interpretation. When mortalities need to be corrected with Abbott's formula, test results should be interpreted only after mortalities have been corrected.

- **Confirmed resistance**: If mortality (corrected, if necessary) is <90%, provided that at least 100 mosquitoes were tested, the vector population can be considered resistant to the insecticide.
- **Possible resistance**: If mortality (corrected, if necessary) is ≥90% but <98%, the presence of resistance is possible but not confirmed. Test results should be confirmed by repeating the test with a new sample from the same mosquito population. (Note: Avoid using F1 of the tested mosquitoes.) If two tests consistently show mortality <98%, resistance is confirmed.
- **Susceptibility**: If mortality (corrected, if necessary) is ≥98%, the vector population can be considered susceptible to the insecticide.

Worksheet 1: Example calculations

Complete this example using the data provided

Results for individual tubes		Observed number of mosquitoes				% dead
		Introduced into each tube	Knocked down at 1 hour after exposure	Dead at 24 hours after 1 hour exposure	Alive at 24 hours after 1 hour exposure	Mortality at 24 hours after 1 hour exposure
	Tube	#	#	#	#	%
Exposed to insecticide-treated papers in tubes	Tube 1	25	20	24	1	
	Tube 2	21	18	21	0	
	Tube 3	25	23	23	2	
	Tube 4	20	15	17	3	
Exposed to untreated papers in tubes	Control 1	20	1	1	19	
	Control 2	24	2	3	21	

Final results for all tubes		Knocked down at 1 hour after exposure	Mortality at 24 hours after 1 hour exposure	Abbott's corrected mortality
		%	%	%
Exposed to insecticide-treated papers in tubes	All tubes			

Test result

The vector population tested is _____ to the insecticide tested

Topic 8: Manage and report *Aedes* resistance data, including input into Tupaia

Session mode	Hands-on participation
Learning objectives	By the end of this session, participants will be able to: • Properly and completely enter insecticide resistance data into Tupaia (or other reporting system)
Resources	PacMOSSI Module 6 Units 1,2,4 presentations

Information

Collecting mosquito surveillance data often requires a lot of human and financial resources and should be used to inform vector control activities. The more accurate, timely and user-friendly the surveillance data is, the more likely that it will be used to make appropriate and cost-effective vector control decisions.

Good data management is important to ensure there are good records of your mosquito data that can be accessed for the long term - even as staff in the department change. For this reason, it helps to manage data in a central electronic database that is managed by the department, not by one individual. Using electronic tools (or forms) with pre-set choices to select as you enter your data can help reduce mistakes from entering text manually. Electronic tools can also reduce or eliminate the need for analysing this data and manually creating graphs for reports as many of the tools create figures and tables for you automatically. Electronic tools can also facilitate quick and easy visualisation, reporting and sharing of the data.

Tools

There are several tools available to help you collect and manage your mosquito surveillance and insecticide resistance data. Here are some examples you might consider using:

- Tupaia Meditrak
- DHIS-2
- VectorSurv
- Epi Info Vector surveillance app
- MS Access
- Early Warning, Alert and Response System (EWARS)
- Go.Data
- ODK
- KoboToolbox

Each of these require different levels of technical support to establish and maintain them. You may need to seek assistance and advice on different options from your information management team.

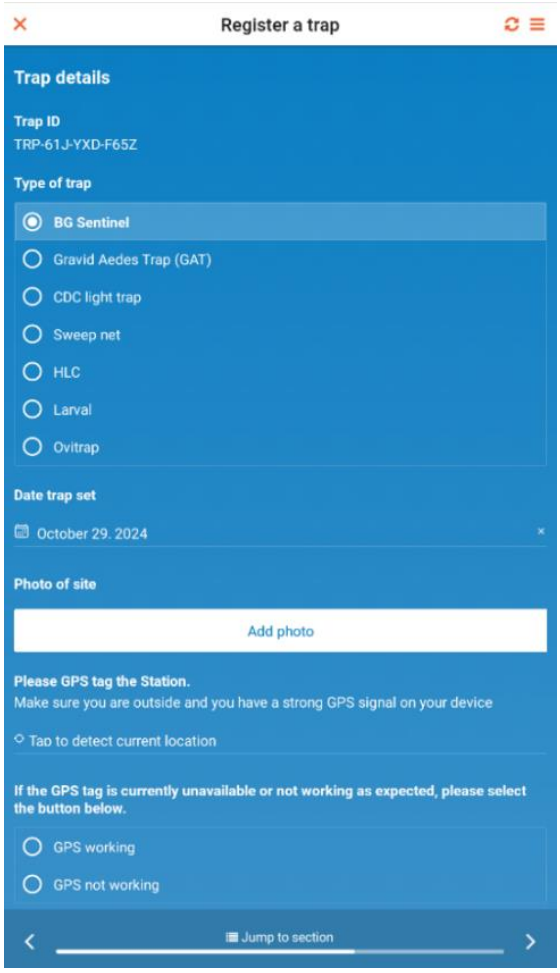
A data management platform designed and supported by the PacMOSSI consortium is Tupaia. Tupaia allows users to collect data using a smartphone or tablet through an app known as Tupaia MediTrak. The app can be used offline and has a suite of surveys to allow PICs to record data from vector surveillance and insecticide resistance testing activities. Once data is collected, and an internet connection established, it automatically updates customised data visualisations available on the Tupaia.org website.

Within Tupaia, two surveys are available for insecticide resistance testing:

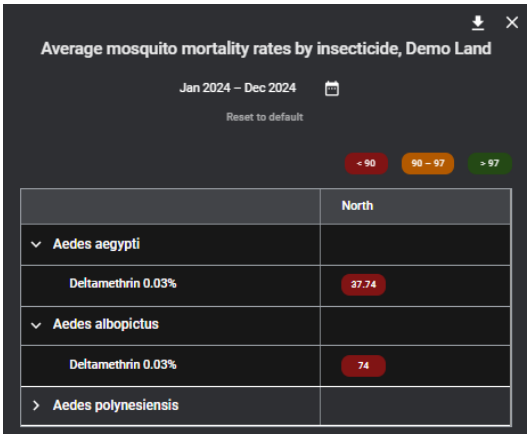
1. PacMOSSI IR Bioassay Setup & 60min Knockdown
2. PacMOSSI IR Bioassay 24h Mortality.

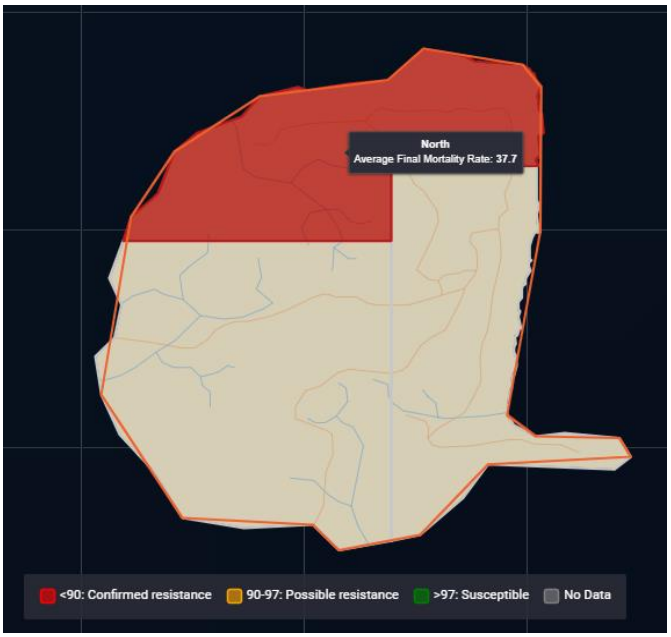
PacMOSSI IR Bioassay Setup & 60min Knockdown is designed for collecting data at the 60 minute stage of an IR test about test conditions including temperature, humidity, insecticide type and concentration, and the number of mosquitoes exposed within each test tube.

PacMOSSI IR Bioassay 24h Mortality is designed to be used at the 24 hour stage of an IR test to record counts of mosquitoes within each test tube according to the species, sex and mortality status. This survey also automatically calculates mortality rates based on the inputted counts.



Several data visualisations are available on Tupaia.org for IR testing, including counts of the number of surveys submitted by date, searchable survey response tables, a summary table of the average mortality rates according to species and insecticide, and interactive map overlays showing levels of insecticide resistance by species according to region.





Topic 9: Use resistance data to guide selection of insecticidal interventions against *Aedes*

Session mode	Classroom learning
Learning objectives	By the end of this session, participants will be able to: • Correctly use insecticide resistance data to inform vector control decisions
Resources	PacMOSSI Module 5 presentations Manual for monitoring insecticide resistance in mosquito vectors and selecting appropriate interventions Updated WHO guidance for monitoring resistance in mosquito vectors

Key principles for managing insecticide resistance

- Insecticides should be deployed to reduce unnecessary selection pressure.
- Avoid using a single class of insecticide everywhere and over consecutive years. Whenever possible, vector control programmes should diversify from pyrethroids to preserve the effectiveness of pyrethroids.
- IRM principles and methods should be incorporated as a core component of programme design.
- The agricultural sector should avoid using insecticide classes widely in use for public health and should collaborate with vector control authorities.
- Routine monitoring of insecticide resistance is essential to inform decisions on deploying insecticides.
- The short-term additional costs of IRM will be less than the long-term potential public health benefits and potential costs of arising insecticide resistance.

The main strategies for insecticide resistance management are: rotation, mixtures, mosaics and combinations. IRM strategies generally rely on using multiple insecticide classes with different modes of action. The rationale is that mosquitoes resistant to one insecticidal mode of action will be killed by exposure to a second unrelated insecticide with a different mode of action and thereby reduce the prevalence of resistance in the vector population to the first insecticide.

To be fully effective, the local mosquito population must be fully susceptible to all the insecticide classes used in the management strategy. The most effective management strategies are implemented before resistance appears.

Safe pesticide management

Pesticides are toxic substances and can pose risks to the environment and human health when they aren't managed according to recommended best practices. The management of pesticides, including insecticides, requires following regulatory controls and considers procurement, transport, storage, proper handling, equipment and safe disposal of insecticide products.

The following factors need to be considered when selecting and procuring insecticides:

- Choose a product that is effective against the target mosquito species
- Estimate quantities required, consider locations to be treated & shelf lives of products
- Ensure quality assurance processes are in place (i.e. how you will check that the insecticide is being applied safely and effectively)

Handling pesticides

Pesticides need to be handled with care as they can be toxic to those handling them under the wrong conditions. There are three ways in which pesticide poisoning may occur: ingestion, inhalation and absorption:

- **Ingestion**, or swallowing of pesticides can lead to oral toxicity. Accidental poisoning has occurred when pesticides were illegally stored in unmarked containers including drink bottles.
- The risk of **inhalation** (leading to inhalation toxicity) is minimised when pesticides are applied correctly as hazardous concentration levels are not usually achieved.
- Skin **absorption** of pesticides can lead to dermal toxicity, and is the most likely to threat to occur, particularly when mixing concentrates. The risk is greatly increased if you have dermatitis or any breaks in the skin.

To prevent these risks, the use of **PPE is essential** especially when mixing or handling the pesticide concentrate. PPE should be used in accordance with product manufacturer instructions.

Additional steps to ensure safe use of insecticides

- ✓ Read the label carefully.
- ✓ Exercise caution at all times, such as ensuring that eating, drinking and smoking while applying pesticides is strictly forbidden.
- ✓ Always wear protective clothing.
- ✓ Have good personal hygiene:
    - Spray operators should take off gloves and wash hands before eating, smoking or drinking any liquids.
    - Shower or bathe at the end of every day's work and change into clean clothes.
    - Wash your overalls and other protective clothing at the end of each working day and keep them separate from other clothes.
    - If the insecticide gets on your skin, wash off immediately.
    - Change your clothes immediately if they become contaminated with insecticides.
- ✓ Ensure insecticides are stored in a safe storeroom, free from moisture and heat and well-ventilated.
- ✓ Ensure equipment is well maintained.
- ✓ Ensure leftover insecticides are disposed of safely.
- ✓ Inform your supervisor immediately if you do not feel well.

Remember, safety is an attitude and is everyone's responsibility!

Worksheet 2. Scenarios of interventions and resistance

Consider the following questions and provide answers for your country. Discuss the situation with participants from other countries.

- ✓ Recent experiences of Aedes-borne diseases?
- ✓ Vector control focus – preventative or outbreak response?
- ✓ Resources available for vector surveillance and control?
- ✓ Insecticidal tools in use or being considered or planned for use?
- ✓ Active ingredients in insecticidal tools?
- ✓ Data on resistance in local vectors to relevant insecticide classes?
- ✓ Options for control if resistance is detected or emerges, or for resistance mitigation?

Resources

Annex 1. Key references

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Annex 2. Key technical considerations for developing a resistance monitoring plan

		Insert relevant information
Collect	Sentinel site location(s)	
	Target mosquito species	
	Target mosquito stage(s)	
	Collection method(s)	
	Number of specimens required (larvae)	
	Proposed dates of collections	
	Proposed time of collections	
Rear	Mosquito stage(s) to be tested	
	Location where eggs and larvae can be contained and reared to adults	
ID	Method of species identification	
	Stage(s) to be identified	Adult females
	Number of specimens to be identified	
Test	Location(s) where resistance tests will be performed	
	Resistance test method to be used	
	Insecticide papers to be used in tests	
	Controls papers to be used in tests	
	Proposed number of tubes with test papers	
	Proposed number of tubes with control papers	
	Target number of mosquitoes per tube	20-25
	Total number of mosquitoes needed per diagnostic concentration	120-150
Manage & use info	Paper forms available for data entry?	
	Excel file available for data entry?	
	How will data be summarised or visualised?	
	How will data be reported?	
	Who will data be used by to decide on vector control actions?	

Annex 3. WHO tube test form

Test date		Temperature	
Technician name		Humidity	
Mosquito collection location		Age of test females	
Mosquito species		Feeding status	
Insecticide tested		Exposure start time	
Insecticide concentration		Exposure end time	

Results for individual tubes		Observed number of mosquitoes				% dead
		Introduced into each tube	Knocked down at 1 hour after exposure	Dead at 24 hours after 1 hour exposure	Alive at 24 hours after 1 hour exposure	Mortality at 24 hours after 1 hour exposure
	Tube	#	#	#	#	%
Exposed to insecticide-treated papers in tubes	Tube 1					
	Tube 2					
	Tube 3					
	Tube 4					
Exposed to untreated papers in tubes	Control 1					
	Control 2					

Final results for all tubes		Knocked down at 1 hour after exposure	Mortality at 24 hours after 1 hour exposure	Abbott's corrected mortality
		%	%	%
Exposed to insecticide-treated papers in tubes	All tubes			

Test result

The vector population tested is _____ to the insecticide tested

Verified by supervisor **Date**

_____ _____

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