Methods in *Anopheles* Research

2015 Edition

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Preface

This training manual represents the latest incarnation of materials I began developing years ago for the instruction of personnel working with me who were given the dicey proposition of culturing *Anopheles* mosquitoes. Originally, it was entitled "Raising Mosquitoes for Fun and Profit," and then it became "*Anopheles* Mosquitoes for Insectary Personnel: An Introduction to Cleanliness, Biology, Culture, Genetics, and Productivity" which had the nifty graphic that you see here. As is perhaps inevitable, the latest incarnation doesn't have the same fun name as the original, but we have broadened the contributing authors list and refined the content into something that we hope will better serve the needs of a larger audience. We also hope it will provide the basis for a manual that is continually growing in content and quality. Therefore, the complete manual has no version or edition number. New sections or revisions may appear on the web at any time.

We acknowledge the contributions of many who have cultured mosquitoes and published their experiences such as Trembley (1944) and Gerberg (1994). We are extremely indebted to the continuously useful two edition set of books by Clements on mosquito biology from which we have unashamedly extracted extensive information (1992; 1999). We highly recommend their purchase by any laboratory culturing and studying mosquitoes.

This inaugural version was stimulated by the needs of the first of many (we hope) courses entitled "Advanced Techniques in *Anopheles* Culture" conducted at the Centers for Disease Control and Prevention in Atlanta, GA USA in 2007. No doubt its quality will be improved by comments and response from the attendees.

Previously, it was shaped by many people who listened to my sermons, forgave my pride (in mosquito culture!?), tolerated my boring lectures, and commented on ways to improve the course including the following:

Safia Ali	Sarah Jordan	Wolfgang Schmeid
Hervé Bossin	Bart Knols	Osama Seidahmed
Gena Lawrence	Dwight Mount	Sharon Soliban
Genelle Grossman	Matt Murphy	Theresa Stevens
Andrew Hammond	Doug Nace	Janis Thailayil
Michelle Helinski	Pamela 'P2' Patterson	Julian Turk
Rebecca Hood	Cristina Rafferty	Tyrone Williams

Without Liz Wilkins and Paul Howell, this content would not be possible. They worked to collect techniques, test them, and edited the sections to a uniform style. They are super people to work with and I cannot say enough good about their efforts on this *and* in the insectary where they have demonstrated skill applying the principles described here. Where you see authors as 'MR4 staff,' that's Paul, Liz, and me.

We invite researchers to contribute chapters to supplement what is presented here and to offer corrections and refinements. This manual is unashamedly full of prejudice and biases due to certain experiences. These very qualities will make it personal and enjoyable.

May it serve well those working to reduce malaria and other vector-borne diseases by the study of their hosts.

May, 2007 Mark Q. Benedict CDC, Atlanta USA

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Trembley HL (1944) Mosquito culture technique. Mosquito News 4:103-119

Preface to Second Edition

When Mark Benedict and the MR4 Vector Activity Team launched the first edition of **Methods in *Anopheles* Research** in 2007, they never imagined its enormous success. This manual has become one of the most accessed items in the MR4 Vector Activity website, providing practical techniques to the intrepid researcher tasked with the "dicey" activity of rearing and investigating anopheline mosquitoes. It has become a valuable resource not only for the scientist conducting basic research but also for public health entomologist from malaria endemic countries and members of the vector control industry.

The first edition of **Methods in *Anopheles* Research** was "the latest incarnation" of training materials that Mark had developed for his personnel over many years. In the preface of that version, he invited other researchers to contribute protocols and to make corrections. And they did. Several minor revisions were made and a few new techniques were added periodically over the following couple of years. As the manual became better known, the number of suggested additions increased. Because the new material did not fit well in the original chapters, we realized that we would need to make more than minor changes and could no longer consider it the same edition. This second edition of the **Methods in *Anopheles* Research** reflects those modifications. Many of the previous chapters remain much the same, new ones have been added to accommodate the new procedures and a few have been rearranged. It still remains a work in progress as Mark requested in the first edition, and we continue to urge researchers to critique and to contribute.

We thank all the people in the last several years that have made comments and suggestions and are especially grateful to those who offered their techniques including the following:

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I would like to give special recognition to the invaluable contributions of Paul Howell and Alice Sutcliffe, whose diligence and persistence made this second edition possible.   Finally I would thank all the users who have made this such a popular download and I hope that the manual remains a valuable resource for those working to reduce malaria and other vector-borne diseases.

Sincerely,

Ellen M. Dotson, Principal Investigator for MR4 Vector Activity, CDC Atlanta GA, USA
April, 2011

Chapter 1: Insectary Operation

1.1 Equipping and Operating an Insectary

Mark Benedict

Introduction

Mosquito insectaries vary widely in their sophistication and cost. Fortunately, the requirements for good mosquito culture are easily met and can be achieved by both simple (inexpensive) and complex (expensive) means. In the following section, I will present very personal prejudices and experiences to guide you. My objective is to convey approaches that are sufficiently useful, safe, and - where possible - inexpensive. Additional ideas can be found in Gerberg (1994), and many of those below are also found in Benedict (1997).

Temperature

Constant temperature is the most important environmental criterion. Immature mosquitoes are typically cultured at a water temperature of 26-27°C. There are several ways in which this can be accomplished.

- Stand-alone incubators. These are a good choice particularly where temperature experiments are planned or space cannot be dedicated to a mosquito insectary. Care should be given to the shelf spacing and sizes so that the space can be occupied efficiently with the trays you plan to use for mosquito culture. The disadvantage is that floor space is not efficiently used relative to some other systems, and the probability of equipment failures multiplies with each additional unit. This approach does provide a good way to divide work spaces for different individuals, stocks or species.

- Walk-in incubators are often used for larger facilities. These are usually equipped with rust-resistant shelving giving greater flexibility for space use. They also allow entry of carts to transport materials and should not have raised thresholds.

- Air-conditioned rooms. Dedicated rooms for mosquito culture require a commitment of the spaces for this activity. Ideally they are designed with water-resistant wall- and floor-coverings such as tile or a monolithic material. These provide the greatest flexibility but often mean that all insectary activities will be performed in the hot humid environment. They often contain screened enclosures to separate activities or stocks.

- Heater tapes (Dame et al. 1978) and shelves. Means have been devised to heat trays of larvae by placing heating elements under the trays or shelves. This is a flexible method that provides comfort for personnel. However, the equipment must be devised on an ad hoc basis, and trays must be covered to prevent water loss and evaporative cooling if the room is not humidified.

Relative Humidity

In all of the designs listed under temperature above, humidity is necessary only for adults. 80% relative humidity to maintain adults is an often-mentioned value that possibly requires more experimental support. None-the-less, one should plan on being able to reach this level. Excessively high humidity is harmful to adult mosquitoes and must be prevented. The only benefit of high humidity to immatures is to prevent water loss and evaporative cooling, both of which can be prevented by covering containers with an impermeable cover. Keep in mind that if the humidity is not high or the trays are not covered, evaporative cooling of the water will require you to have an air temperature significantly higher than that of the desired water temperature. In rough order of descending space capacity, means to generate humidity are:

- Steam injection into the central ventilation
- Greenhouse-type misting humidifiers
- Centrifugal room humidifiers
- Evaporative coolers
- Home-type steam humidifiers
- Boiling water steam generators e.g. in stand-alone incubators
- Evaporation

Consistent humidification is a chronically difficult goal due to the cycling of temperature control systems and the intrinsic unreliability of humidity creation and control systems with which I have had experience. In order to develop a simple and reliable system, I recommend you answer the following questions:

1. Do you need to humidify the immatures culture space? If you do, a larger capacity active system will be required. It is preferable for worker comfort and ease to simply humidify only the adult holding area and cover the immatures' containers.

2. Can I hold the adults I have in a relatively small area? If you are using small cages, cups etc. a very simple and effective solution is to use a glass or Plexiglas case in which cups of water containing sponges are placed on the shelves. Such a passive system is effective, inexpensive and foolproof.

3. Do I need to physically segregate large numbers of adults? In this case, multiple systems, subdivided rooms, or incubators will be required.

Two of the above systems are inexpensive and self-regulating: evaporation in a sealed space and evaporative coolers. Both of these methods will attain sufficient humidity and require no controls.

Centrifugal room humidifiers and misting systems suffer the problem that droplet sizes are often too large resulting in puddles and/or reservoirs of water in which microbes can grow. Of the high capacity systems, steam injection into the ventilation system has been most reliable for us. If possible, the ductwork must be constructed of stainless steel since the high humidity will quickly rust it.

Lighting
Many insectaries use a 12:12 light:dark schedule. This is easily accomplished using a simple light timer. Most laboratories also try to achieve gradual dimming and lightening to stimulate natural behavior. This feature can be purchased with many incubators but is less easily accomplished in dedicated rooms. In the latter case, a control system, dimming light fixtures and bulbs must be used. These should be capable of changing from full light to none in approximately 30 min.

Security
Biosafety issues resulting from escape have been covered adequately in the Arthropod Containment Guidelines (Benedict 2003) and will not be addressed here.

Physical security
Appropriate means should be in place to prevent casual interference by untrained persons with mosquito culture. The variety will vary from electronic keypads, locked doors, to no deliberate means at all. Simple location of the insectary in the basement or back of a building may be sufficient. If you are handling transgenic or exotic species, restricted access is recommended by the Arthropod Containment Guidelines (Benedict 2003).

Environmental security

Environmental alarm systems should be in place to protect valuable stocks and experimental materials. One should ask, "How much would my program suffer if the temperature in this room (or incubator) were excessively cold or hot resulting in the death of all the mosquitoes it holds?" This will place a value on an alarm system. Therefore, it should be a priority to install a monitoring and alarm system that *notifies insectary staff in real time* if conditions are not suitable. We have found it is not sufficient for facilities maintenance staff to receive such alarms. This system has saved stocks numerous times and has used pagers, cell phones, and Blackberries for notification.

Comfort of personnel

This should be strongly considered during the design of the insectary. Few enjoy working in a hot humid insectary, and this can be reduced by subdividing adult and larval holding areas, using lower humidity or relying on incubators.

Furniture

Rust-proof metal, fiberglass or plastic furnishings are preferable. Shelving should be easily adjustable and stand-alone units should be equipped with wheels. As is discussed in the chapter on cleanliness and general maintenance, one's ability to clean equipment – and beneath it – is essential. All furnishings must be suitable for being wet frequently.

Supplies and culture equipment

Following is a list of typical supplies needed to equip a small insectary. The sources for much of this material will be different depending on your location and many substitutions are possible. Though some sources are local, the URLs will provide information to give you an idea of what is described. We indicate Fisher Scientific as a source for many items, but these products are widely available. Many practices and innovative devices are found around the world, so collect good ideas and device solutions in all the insectaries that you visit.

Mosquito Rearing Equipment/Supplies	Source example	Model example	URL
8 oz. white plastic containers (for pupae)- approx 250ml, used in food service	US Plastics	81134	www.usplastic.com
BugDorm1 adult cage (option 1)	MegaView Science	DP1000	www.megaview.com.tw www.bioquip.com
12x12x12 Metal cage (option 2)	BioQuip	1450B	www.bioquip.com
Larval rearing trays	BioQuip	1426B	www.bioquip.com
Plexiglas covers for trays	Fabricate locally		
2 ml amber latex pipette bulbs	FisherScientific	S32324	www.fishersci.com
Plastic disposable pipettes (trim end, attach bulb and use as a pupae picker)	FisherScientific	13-711-7	www.fishersci.com
Stainless steel mesh strainer (to filter larvae and pupae)	Local source	SDM-05	www.atlantafixture.com
Tubes for mixing yeast e.g. 15 ml disposable	FisherScientific	05-538-51	www.fishersci.com

10 ml pipettes	FisherScientific	13-678-12E	www.fishersci.com
Sucrose (to make 10% sugar solution for adults)	Local source		www.fishersci.com
Large cotton balls	FisherScientific	07-886	www.fishersci.com
colored tape (to label and discriminate stocks-choose 1 color per stock)	FisherScientific	15-901-15(color code)	www.fishersci.com
Larval diet e.g. Drs. Foster and Smith Koi Staple Diet	Drs. Foster and Smith	Koi Staple Diet	www.drsfostersmith.com
'dash, pinch, smidgen' stainless steel measuring spoons	Local source		
Mouth aspirator[1]	John Hock Co.	412	www.johnwhockco.com
Feather-tip forceps	Bioquip	4748	www.bioquip.com
2 liter clear plastic pitchers with volume markings	Local source		
Filter paper sheets	FisherScientific	09-803-5E	www.fishersci.com
Qorpak tubes or similar	Qorpak	3891P	www.qorpak.com
500 ml wash bottles	FisherScientific	02-897-11	www.fishersci.com
Waterproof felt tip markers e.g. 'Sharpie'	Local source	13-379-1	http://new.fishersci.com

Table 1.1.1. Some useful insectary supplies and manufacturers.

Common Entomological supply sources:
- John Hock Company   www.johnwhockco.com
- Watkins and Doncaster   www.watdon.com
- BioQuip   www.bioquip.com
- Educational Science Co.   www.educationalscience.com
- MegaView   www.megaview.com.tw

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[1] An aspirator can be constructed from a section of rigid transparent plastic tubing 20 cm long with an inside diameter of about 15 mm. One end of the tube is covered with fine cloth netting or metal gauze and then inserted into a piece of rubber hose/tubing 50-60 cm)

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1.2 Cleanliness and General Maintenance

MR4 Staff

Introduction

Cleaning and general maintenance of insectaries can easily fall to the bottom of the list of things to do. However, daily light cleaning and routine deep cleaning help prevent serious problems such as infection and predation. Following are some reasons a clean environment has a major impact on mosquito culture and research results.

Promotion of cleanliness and sterility

Reduction of infections and pests

Most insectary infections are fungal, protozoan, or bacterial and are routinely transmitted via water or air (see **Chapter 2.2**). While it is not practical to completely eliminate these pathogens from the environment, it is possible to reduce their prevalence. A primary infection may not be lethal or significantly debilitate the mosquitoes, but it may produce conditions that are favorable to the development of secondary infections that are lethal. Often fungal infections may be chronic in nature, diminishing the immune status of larvae resulting in a secondary, lethal bacterial infection. Microbial control can also lead to a reduction in biogenic toxins.

Minimizing insect pests in the insectary can also be crucial to maintaining healthy stocks. Insect pests, such as predatory roaches and ants, are of greatest concern in an insectary as they can easily consume a colony of adult mosquitoes overnight. Larger pests such as rodents introduce waste products that harbor pathogens in the rearing environment. The easiest way to minimize pests in the insectary is to reduce or eliminate the conditions that attract them: food, accessible water, and harborages (shelter). Clean conditions alone are usually insufficient to prevent all pest problems. In this case, baits and traps can be used, but be sure they do not contain insecticides to which your mosquitoes will be exposed (see below).

Achieving sanitary conditions

At a given period of log phase growth of a microbial population, the titer of organisms is proportional to the titer at the beginning. Therefore, minimizing microbial growth by any means can significantly reduce the capacity for growth.

Insectaries often use equipment and solutions that cannot be autoclaved or otherwise fully sterilized, nor are facilities for gas or irradiation sterilization practical. Nonetheless, measures must be taken that provide a reduction in microbial contamination. Heat killing on surfaces and rearing equipment can be done by boiling, autoclaving, or baking. Exposing fluids, tools and containers to even a sub-sterilizing level of heat can allow fewer microbes into your environment. Autoclaving is most effective, but liquids that contain components destroyed by autoclaving can be partially decontaminated by an elevated heat process such as pasteurization or filtration. Many of these treatments and practices are similar to those practiced in restaurants: sanitary, but not sterile conditions are the goal.

Worker health and morale

A clean, pleasant-smelling, uncluttered insectary is healthier and more desirable to work in for long periods of time, and an uncomfortable and smelly insectary is one reason people are not eager to remain. Moreover, an abundance of molds and dust are likely to irritate asthmatics and those with allergies.

Techniques for Achieving Clean and Sanitary Conditions

Insectary workers must recognize that sanitation – in addition to sterilization – is an effective way to promote consistent mosquito health. We have listed many options for achieving sanitary conditions, and

some or all of these can be employed in any laboratory. The consistent, combined use of these is essential.

Chemicals including bleach, gases and solvents

Chlorine bleach (sodium hypochlorite) is commonly used to sterilize plastic containers, countertops, and floors. Ethanol also has some sterilization effect on bacteria and fungi, but be careful not to expose mosquitoes directly to ethanol as it will kill them instantly. Hydrogen peroxide is another common and useful chemical that is compatible with many materials for sterilization purposes. Finally, ethylene oxide sterilization can be useful if facilities are available.

Cold temperatures including freezing and refrigeration

Unless special precautions are taken to protect the organisms, freezing will kill many microbes. Even those that will survive cold/freezing to some extent may be reduced in number or their growth-rate diminished. Both larval and adult diets should be stored in a refrigerator or freezer.

Desiccation

Extremely low humidity, especially in combination with elevated heat, reduces the abundance of many microbes. Therefore, plastic rearing containers and other equipment dried and stored in a dry place are likely to harbor fewer microbes than those dried and stored inside a humid insectary. Drying ovens provide low humidity and high heat and are useful for sanitizing equipment that cannot withstand autoclaving.

Detergents

Hand-washing with soap is more effective than just using water since detergents break down cell membranes and kill microbes in the process. Similarly, detergents will kill microbes and loosen microbial food sources such as grease and dirt in the insectary better than water alone. While excessive detergent residues might also kill mosquitoes, surfaces that are cleaned with detergents and rinsed thoroughly will harbor fewer microbes. If you are in doubt about the toxicity of a detergent, perform a simple dose response mortality test with L1s using realistic concentrations that might exist as when containers are not completely rinsed.

Filtration

Ultra-filtration will remove fungi and bacteria from solutions. However, this method is usually only useful for small volumes of solutions due to the cost.

Heat

Heat-treatment via autoclaving is standard for total sterilization. Therefore equipment should be selected with this in mind. As mentioned above, drying ovens reduce microbes and may be compatible with equipment that cannot be autoclaved. Brief immersion in hot water is a measure that provides some benefit, and it can be made available in even the most basic insectaries.

Irradiation: gamma, X-ray, UV, photons

Many types of irradiation educe the abundance of microbes. At first glance, such methods as listed might not appear to be appropriate for an insectary. However, they might be used in the ventilation system (UV) or for sanitizing rearing containers. UV rays from sunshine will even kill some microbes.

Figure 1.2.1. Any household water-heating pot designed for kitchens can work well in the insectary for sanitizing equipment. Immersing equipment between uses addresses two problems simultaneously: It will clean instruments to protect the transfer of infection and will also kill any larvae/pupae that were accidentally left behind on the tool.

Starvation

Few microbes can survive indefinitely without minerals or complex organic compounds. Cleanliness in the insectary generally reduces such sources.

Specific procedures to enhance sanitation

Air filters

Central air-conditioning air filters are effective only if they are changed regularly (**Figures 1.2.2-1.2.4**). The demand and their performance depend on the cleanliness of the air entering the filter in the first place, so routine floor cleaning and dusting have a double benefit.

Recirculating filters utilizing activated charcoal, particulate meshes, and HEPA are relatively inexpensive and readily available (**Fig 1.2.4**). Consider installation of these in addition to the filtration provided by the air-conditioning system to reduce the number of free-floating particles in the insectary. As with air filters, these filters are only effective if they are changed regularly.

Figure 1.2.2. Air filters are great for mosquitoes and people if they are changed regularly. Air distribution systems can normally be adapted easily to include filtration.

Figure 1.2.3. Dirty air filters are useless or even harmful. Filters should be checked and changed regularly.

Figure 1.2.4. (Left)

Stand-alone HEPA air filtration units are readily available and useful, especially in confined spaces.

Humidifier selection and maintenance

Many humidifiers contain a water reservoir that never empties completely. This means that even though deionized or even sterile water may enter the humidifier, airborne particles that fall into the reservoir will introduce sufficient material to establish microbial growth. These microbes will then conveniently ride on the water droplets into and onto everything they reach. Steam generators are a better choice for insectary design. Routine cleaning of any water system should be done e.g. by flushing with bleach or according to the manufacturer's recommendations.

Larval diet

Keeping the larval food frozen will not sterilize it, but it will prevent microbial growth and decay during storage. Process only a small amount of food and aliquot it into smaller portions. Store at -20°C until needed. We recommend keeping any unused food in a refrigerator to reduce contamination since the growth rate of microbes is temperature-dependent. When using liquid food, keep it in the refrigerator once mixed and minimize the amount of time it is out at room temperature. If you pre-mix larval slurry, make only as much as you can use in 2-3 days to prevent microbial growth *in situ*. Refrigerate the food overnight and discard it if it's left out regardless of how 'good' it smells.

Replace the food container between batches. If this is not possible, clean the container with a detergent soap and thoroughly dry in a warm oven. Likewise, soaking a plastic food container overnight in bleach is good for reducing pathogens. At a minimum, wash with a brush, detergent, and hot water. Rinse thoroughly in clean water and dry.

Never combine batches of old and newly prepared food. Mixing preparations could inadvertently disseminate microbes that were growing in the older food to the fresh batch.

Adult sugar water

Many laboratories place sugar-water-soaked cotton pads on cages. These require replacement at intervals, in part due to microbial growth. When working with sugar water, keep your hands clean. This is especially necessary when replacing old with new sugar. For example, if changing cotton sugar pads, think: "Did I just pick up a moldy cotton ball and stick my fingers in the fresh sugar water to get another?" In this example, a solution is to use one hand to remove the old cotton balls, the other for the fresh ones. Wearing a glove on the 'clean' hand is a good reminder.

Another important measure of mold prevention is to make sure that the feeder you are using is sanitized. Cotton balls can be autoclaved and stored in sealed containers. An open bag of cotton in a humid insectary is a great settling ground for mold spores, so keep them sealed until use. Feeders of different sorts, vials or screen covers, can be soaked in bleach and dried prior to re-use. They should also be stored in a closed container prior to use. NOTE: Bleach oxidizes steel very quickly. If you plan to use bleach for sanitizing, choose metals such as aluminum or stainless steel.

Finally, autoclave sugar water. Once the container is opened, it begins accumulating microbes. A cup of sugar water stored in the refrigerator and reused for weeks becomes increasingly contaminated. If you have a cup of cotton balls in sugar water, discard it weekly and start each week with a clean container, new sugar water, and new cotton balls. Also, you can use a preservative such as methylparaben in the sugar water at low concentrations to reduce microbial growth. See culture section, **Chapter 2.4.7**, for ideas on sugar feeders that lessen mold problems.

Mosquito containers

In order to prevent mold growth on mosquito containers, discard dead mosquitoes from used containers as soon as possible. Dead mosquitoes in containers can shed potential primary and secondary pathogens. Even if you autoclave materials, the microbes may have produced toxins before autoclaving or cleaning that will cause problems. For this reason, try to remove dead mosquitoes from active rearing containers as much as possible. If a pathogen killed a mosquito that is dead in a rearing pan, when the dead carcass decays it will probably release more pathogens into the water.

Autoclave as many types of containers as possible. This is the best way to eliminate microorganisms. Select autoclavable or disposable containers over reusable ones that cannot be sterilized effectively. Or, use disposable containers for no more than one generation (**Figure 1.2.5**)
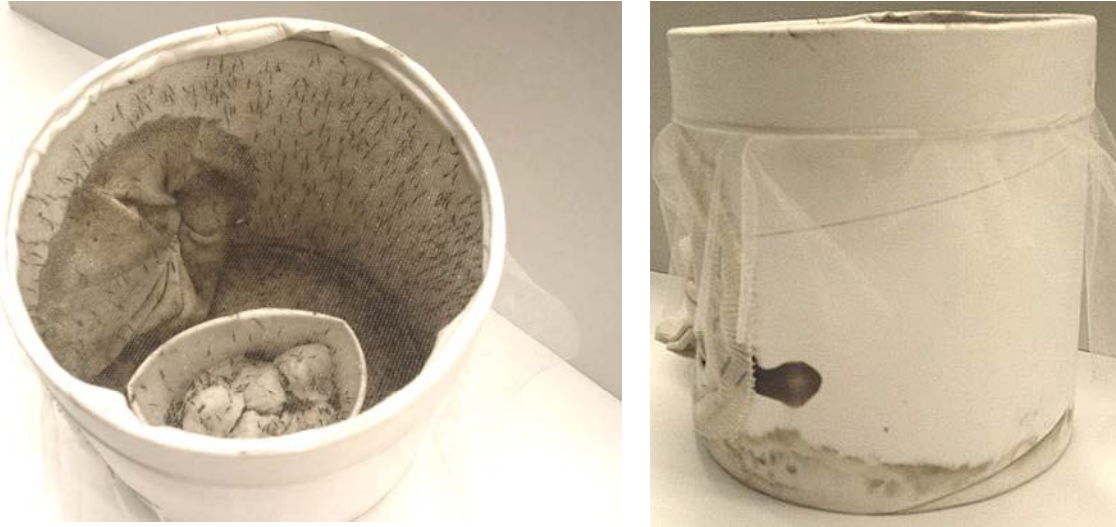
Figure 1.2.5. This disposable, paper mosquito carton was reused several times. Notice the mold on the walls and floor. Dripped sugar water and blood in a warm, humid insectary are perfect for growing microbes. Paper cartons should be considered disposable.

Dry thoroughly

Wet pans and cups allow microbes to multiply, so thoroughly drying them before reuse reduces the total number of microbes. Plastic containers and covers are especially important to keep dry between uses. Also, since many mosquito eggs cannot survive drying, this is an additional way to prevent contamination between stocks. Stack cups and trays in a way that promotes thorough drying.

Clean containers as soon as possible after use

Clean containers as quickly as you can. If you have to leave them, stack them in a dry place. Placing them in water for several days before cleaning, even with detergent, is a good way to allow the film of food, sugar water, dead adults etc. to support microorganisms. If at any time you notice a slimy feel on containers, you are observing microbial growth and should change your methods to prevent it.

Dry and store outside of insectary

The insectary is a convenient place to store supplies, but the high heat and humidity also make it a good place to grow microbes. Also, stacks of storage items can offer harborage for pests. Store as little in the insectary as possible. Store as few disposable items in the insectary as possible. Move boxes of cotton balls, cups etc. away from high heat and humidity areas when not needed.

Physical cleanliness

Walls

It may not be intuitive that keeping walls clean would have an effect on mosquito populations, but wiping walls regularly with warm soapy water kills fungi and microbes and removes food sources. To make cleaning the walls as painless as possible, keep walls accessible for cleaning by using racks that can be easily moved or are on wheels. This also benefits mopping floors and moving mosquitoes etc.

Figure 1.2.7. The mildew and mold growth on the walls and the floor of this insectary can stress mosquitoes causing them to be more susceptible to infections and less able to respond to changing conditions.

Floors

Wipe up spills and eliminate leaks to keep floors as dry and unfriendly as possible. For these purposes, it may be helpful to equip the insectary with a wet-dry vacuum cleaner, making sure that the vacuum cleaner is not used elsewhere to vacuum toxins such as under furniture where insecticides have been sprayed. For all of the reasons above, and especially in relationship to desiccation, don't let water accumulate on floors, in containers or on counters.

Even though detergent may not be necessary to make the floor appear clean, it does have an anti-microbial effect and should be used for routine mopping.

Shelves and counters

Remove unused equipment and supplies. Unused materials in the insectary make it more difficult to clean around and beneath. Items stored in the insectary will likely begin to accumulate molds: Cardboard is especially poor in moist environments as it holds water, molds, and provides harborages for cockroaches and other arthropods.

Keep shelves uncluttered, dusted, and free of spills, especially sugar water and food sources. Removal of dust is also important as it is highly organic. It carries mold and bacterial spores and therefore circulation of dust by air in the lab spreads potential sources of infection. When you wipe up a spill, you are not only removing the spill. You are removing the spores of the organisms that grow in the spill, those carried by the pests attracted to the spill, etc.

Use as many sealable storage containers as possible. Tupperware-types are good and withstand bleaching; however, you can't autoclave them. Avoid cardboard, paper and wood. Use instead, plastic, metal, or glass which are easier to sterilize with bleach or heat.

Keep items sealed until use. Open one bag of cotton balls or one box of cups at a time. Keep covered except when in use. Consider putting everything into covered containers such as plastic closet boxes or shoe-boxes.

Figure 1.2.8. (Left) This is an example of well cleaned working insectary with appropriate storage. Items prone to mold spores from the environment (in this case sugar-soaked cotton balls) are placed in Tupperware containers to shield them. Minimum amounts of daily use items are stored on the shelves. Storage items are placed outside the insectary environment. Using rolling carts with brakes in the insectary can be a great way to make cleaning easy and efficient. Also, in case of emergency, it is easy to move items in or out of the insectary.

Figure 1.2.9. **(Right)** Clutter in the insectary renders the cluttered items dirty as well as the environment harmful for mosquitoes. Any cardboard surfaces such as the one on the bottom of this shelf can and will hold mold and fungal growth.

Pest control

Sentinels for cleanliness

Infestations of many insect pests occur due to inadequate cleanliness in the insectary. Periodic cleaning and routine trapping of insects will eliminate food sources thereby reducing their numbers. Cockroaches (**Figures 1.2.11** and **1.2.12**) typically occur in areas where excess food has been spilled and there are numerous harborage sites. They are known to passively carry several different pathogenic bacteria on their carapaces that they spread while they move about. Cockroaches will also catch and consume living mosquitoes. Book lice, also known as paper lice, are cosmopolitan insects that live in dark, humid conditions (**Figure 1.2.13**). They are typically associated with excess food spillage or starchy paper goods on which they feed. Unlike cockroaches, book lice are not known to cause harm to mosquitoes, however their appearance may equate to unclean conditions in the insectary. Once established, they are difficult to eliminate however turning off the humidity and raising the temperature during a temporary shut-down in the insectary may eliminate large numbers of them. Excess diet should also be disposed of, autoclaved, or kept at 4ºC to kill off lice.

Figure 1.2.11. *Blatella germanica* (German cockroach)

Figure 1.2.12. *Periplaneta americana* (American cockroach)

Figure 1.2.13. *Liposcelis corredens* (Book louse)

Regular preventative trapping

Ensure that the insectary is monitored for the presence of rodents, ants, and cockroaches. Ants particularly can destroy a cage of mosquitoes overnight. Furthermore, both ants and cockroaches can spread microbes and leave feces in the insectary. The MR4 has used both Maxforce ant granules and Maxforce roach killer bait gel without evidence of harm to the colonies. Routine distribution of outdoor ant baits around the perimeter of the insectary building may be a useful preventative measure.

Figure 1.2.10. Anticipating the introduction of pests before they are seen can save an insectary from an overnight, unforeseen invasion. Ant baits such as the one shown here are important to place around doorways and other entry points. Otherwise, an ant colony can move in and decimate mosquito stocks overnight.

Reduce food sources

Spilled sugar water and food is difficult to control. One method of prevention is to dispense them only over a counter top that gets cleaned daily. Otherwise, make sure any spills are cleaned up as quickly as possible. Larval food is especially protein and fat-rich so ants and roaches thrive on it. Dead mosquitoes can also be food sources for ants or roaches so clean old cages as soon as possible. Dirty rearing pans are a food rich source for cockroaches so the cleaning as soon as possible applies to the pans as well.

Trash cans are also well known food sources for pests. If you dispose of old sugar soaked cotton balls in the trash, for example, make sure the trash is removed from the insectary daily.

Ultimately, the cleaner your insectary, the healthier your mosquitoes will be. Attention to sanitation methods makes a huge difference in mosquito health management.

1.3 Scheduling and Regulating Your Work Load

MR4 Staff

Develop and maintain a schedule

Rearing multiple stocks and strains of mosquitoes or using large numbers of mosquitoes for experiments and stock maintenance can be very difficult without thought for scheduling and planning. The first rule is never to endanger the colony by using too much material for experiments. Once a strain is lost, it is lost forever. You should ensure that your colony is sufficiently large to support current experimental work and the colony's future generations. If colonies are reared in a haphazard manner, it is difficult to know when or if you will have new material available for experiments. However, if the insectary is operated in a controlled and consistent manner, it will be easy to produce enough material without risking a colony, and following strict standards and schedules makes it effortless to say with assurance when you will have material at the needed stage. Some suggestions toward achieving this are outlined below.

Decide on discrete or overlapping generations

There are two general approaches for stock maintenance, each of which has particular advantages: discrete and overlapping generations. The discrete approach produces sufficient material for the next generation which is placed in a fresh cage - there is no mixing between generations. The overlapping approach produces material which is placed in a cage with adults of the previous generation. so progeny from the cage could result from either generation. The MR4 almost exclusively uses discrete generations. Each generation of adults is bloodfed the first and only time for stock and experimental use if there are sufficient numbers of progeny. A second blood-feeding is performed only to produce experimental material and/or a backup if needed. It is most efficient to label all trays indicating whether they are the primary stock, experimental material or a backup.

If contamination is detected in stocks cultured by the discrete method, previous generations provide a backup generation that may provide pure material if contamination occurs. On the other hand, stocks that are difficult to bloodfeed or produce few progeny may be best maintained by pooling all the available material in a cage(s) and culturing by overlapping generations.

We are aware of no studies of the differences in genetic changes or selection that might occur in either mode. However, it seems intuitive that maintaining stocks by the discrete method would select individuals that reproduce early with little effect of greater longevity.

Establish a single schedule of activities

Insectaries are more efficient if there are fixed days for specific tasks such as egging and blood feeding. If experiments require material reared on a different schedule, the individual researcher should be responsible for keeping their experimental materials separate from the general flow of the insectary schedule. Having a strict schedule also makes it easier to share chores between technicians as duties can be assigned routinely for certain days.

Keep the environmental conditions fixed in the insectary

To ensure predictable development of mosquitoes in the insectary, temperature, and to a lesser extent humidity, must be controlled. Uncontrolled fluctuations in temperature or humidity will cause colonies to develop faster or slower, affect fecundity and can cause mortality in extreme cases.

Follow culturing density standards

Similarly, if colonies go underfed or are grown in a more crowded/less crowded density than normal; your mosquitoes will more than likely not be at the stage you had anticipated for your schedule. There are several simple methods for quantifying larvae and eggs though many people can estimate closely enough

by eye with experience. Because not all stocks have the same hatching rates, quantitative methods for eggs will require adjustment.

Feed larvae appropriately and consistently

All trays of larvae should be observed carefully daily and fed and/or the density adjusted because these practices affect the success of colony maintenance more than any others. There are several indicators to determine whether you are feeding too much or too little in Chapter 2.

Suggested Schedule 1: a three-week cycle beginning on a Friday

Below is an example schedule based on a typical strain of *An. gambiae* reared at constant 80% RH, 27°C under the conditions detailed in the culture section of Chapter 2. You will have to make modifications to this depending on the specific strains you culture and the availability of labor and blood source. Each method referenced is described at length in Chapter 2.

Friday: Blood-feed adult females. The mosquitoes should be a minimum of two days post-emergence for the best results. In many cases, 4-7 days post-emergence is optimal, but do not wait longer for the first feeding as mortality will endanger your primary stock and/or opportunity to re-feed.

Saturday: No attention required.

Sunday: No attention required.

Monday: Insert the egging dish into the cage.

Tuesday: Remove the egg dish from the cage. Bleach the eggs and store them in a humid sealed cup overnight.

Wednesday: Rinse eggs into pans for hatching and feed.

Thursday: No attention required.

Friday: Split the larvae into pans based on the number you will need but keeping in mind proper densities. Add yeast to a final concentration of 0.02% w/v and a very small amount of the larval diet you will use.

Saturday: No attention is required.

Sunday: Feed the larvae a volume of ground diet based on their size and density. If there are too many larvae in the pan, thin or split into more trays to ensure no crowding occurs.

Monday through Wednesday: Continue splitting/thinning and feeding the pans daily as needed. It is best if the density at this point is the same as the final density; crowding slows development.

Wednesday through Friday: Pupae should be collected daily and transferred to a cup with clean water and placed into a new cage with a sugar source. If you chose to allow adults to emerge in the tray for later transfer, cover trays at this point. If you are working with a strain that remains in pupal form for 48 hours or more, you may want to collect pupae every other day. However, you will need to feed the larva daily. Most Anophelines have a higher proportion of male pupae developing on the first day so if you are collecting only 100 for stock you should check to make sure you have a good number of females before discarding any remaining larvae.

Friday of the following week: Bloodfeed the adults to initiate the cycle again.

If you find that the adults are beginning to die before you blood-feed on Friday, alternate the schedule between a generation of bloodfeeding on Monday and then Fridays. This way, every other weekend will be work-free. This makes a 2 1/2 week schedule; better for mosquitoes but not as convenient for mosquito culturists.

Suggested Schedule 2: a three-week cycle beginning on Monday

This follows the schedule above, but shifted. This schedule will probably result in pupation over the weekend so it may not be as convenient.

Monday: Blood-feed adult females.

Tuesday: No attention required.

Wednesday: No attention required.

Thursday: Collect eggs.

Friday: Remove the egg dish and bleach the eggs.

Saturday: Hatch larvae.

Sunday: No attention required.

Monday: Feed and split/thin larvae.

Tuesday through Thursday: Thin and feed pans as needed.

Friday through Sunday: Collect pupae or adults and feed larvae every day.

Monday following week: Blood-feed to reinitiate the cycle.

Both schedules are laid out in calendar form in **Table 1.3.1**.

		Mon	Tue	Wed	Thu	Fri	Sat	Sun
Week 1	Schedule 1					blood		egg
	Schedule 2	blood			eggdish	bleach	hatch	
Week 2		**Mon**	**Tue**	**Wed**	**Thu**	**Fri**	**Sat**	**Sun**
	Schedule 1	bleach	hatch		split/thin		split/thin	
	Schedule 2	split/thin		split/thin	feed	feed	feed	feed
Week 3		**Mon**	**Tue**	**Wed**	**Thu**	**Fri**	**Sat**	**Sun**
	Schedule 1	feed	feed	Feed	pupation	pupation	pupation	pupation
	Schedule 2	pupae	pupae	Pupae	pupae			
Week 4		**Mon**	**Tue**	**Wed**	**Thu**	**Fri**	**Sat**	**Sun**
	Schedule 1					blood		
	Schedule 2	blood			eggdish	bleach	hatch	

Table 1.3.1. Calendar layout of two schedules as described above.

Planning experiments: Working backward from the deadline

Whether you are coordinating materials for feeding or simply determining if you can complete an experiment before a holiday, it is helpful to plan beginning with the deadline date and work backward to the present using a schedule such as the one presented here. Failure to plan ahead could result in the experimental material you reared for three weeks being ready on a weekend when you are not at work. You will need to modify the schedule to the actual time periods you experience with your colonies in your laboratories. An example of how to plan is given in **Table 1.3.2**.

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1	2	3	4	5	6	7
						blood
8	9	10	11	12	13	14
		egg dish	bleach egg	hatch egg		feed
15	16	17	18	19	20	21
check	feed	check	feed	feed	feed	feed
22	23	24	25	26	27	28
pupae	1 day old	2 days old	3 days old	4 days old		

Table 1.3.2. In this example, the researcher needs 4-day old mosquitoes for an infection experiment on Thursday the 26th (yellow highlight). By working backwards on a calendar, one can see that bloodfeeding must occur on Saturday the 7th. For convenience, they may wish to bloodfeed on Friday and collect eggs on Tuesday.

1.4 Maintaining Stock Purity

MR4 Staff

Introduction

Any lab that cultures more than one stock must prevent contamination. Stock identity is determined ultimately by genetic composition; therefore, stocks that are contaminated are of little value, especially if their only known distinguishing characteristic was origin location. Physical isolation in different rooms is often used to prevent contamination but this has limits as the number of stocks increases. Therefore, keeping stocks pure ultimately depends on conscientious methodical attention to detail when making labels, transferring pupae and adults, putting egg dishes into cages, etc. Moreover, if your strains are not phenotypically defined, it may be impossible to determine that they are contaminated later.

Diligent exercise of precautionary methodology is the only way you will prevent contamination. This can be augmented by using phenotypically marked stocks when possible. Recessive markers are the best choice since contamination is more readily detected. The best advice is to stay conscious, careful and follow routines designed to avoid contamination.

Ways to avoid contamination:

There is no substitute to consistent attention to detail, but the following are some ways stocks can become contaminated with suggestions for avoiding them.

Use carefully decontaminated materials

Cause: Pupae and larvae easily get stuck in devices and are very difficult to see at a glance. When switching to another stock, it is easy to not notice the contaminant and transfer from strain to strain (**Figures 1.4.1 and 1.4.2**).

To prevent: visually examine tools and rinse in hot water between handling each stock. If you keep only a couple of stocks, separate, clearly marked tools should be kept for each. Use white and transparent containers when possible and white countertops.
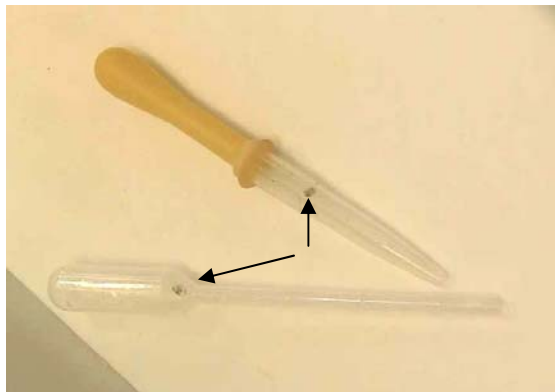
Figure 1.4.1. Hand held pupa pickers with a single pupae stuck in the apparatus, shown by arrows.
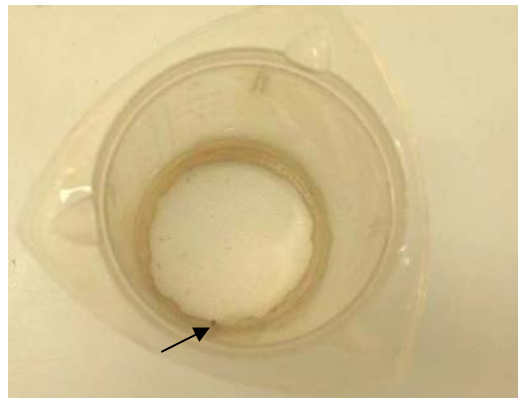
Figure 1.4.2. Larval strainer with a single larva stuck in the apparatus, shown with arrow.

Cause: Eggs in water can easily spill or splash onto the lid of a pan or cup (**Figure 1.4.3**). Reusing the same lid or cup for another stock without decontamination can lead to egg transfer.

To prevent: use fresh lids and cups that are decontaminated by desiccation, washing, and/or autoclaving, and consistently return the same lid to each container.
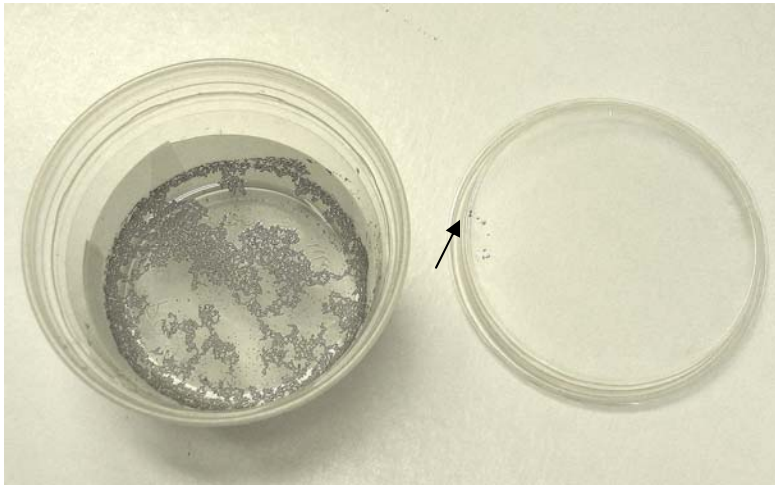
Figure 1.4.3. This is a common cause of contamination. The eggs have splashed onto the lid. Accidental mixing of lids at this point can cause transfer of eggs that could go easily unnoticed. Keep all lids exclusive to the cup or pan you are working with assuming contamination has occurred.

Cause: Mosquitoes are put into the wrong container *e.g.* pupae into adult cages.

To prevent: consistently use a different color of tape/marker color for each stock (**Figure 1.4.4**). For small numbers of stocks, this allows color-coding pans and cages without writing labels. Using different colors makes it difficult to not notice mixing of strains. Give stocks distinct names.

Cause: Free flying adult mosquitoes are a contamination concern. For example, a mosquito can be flying by or biting your hand when you are placing something inside a mosquito cage or blowing in mosquitoes. A gravid female can lay eggs in any pans that are left uncovered. Even covered pans can sometimes have enough of a gap for a mosquito to slip inside and lay eggs, thereby contaminating the entire cohort.

To prevent: routinely trap free mosquitoes in light traps and make every effort to prevent escapes (**Figure 1.4.5**). Inspect trays daily for pupae. If adults are allowed to emerge from the culture tray before transfer to adult cages, covers must be securely fastened.

Figure 1.4.4. Different colors of tape and/or different colors of markers make it obvious to see the difference between stocks at a glance. They also make it simple to locate material rapidly.

Figure 1.4.5. Light traps, such as the one pictured here, are good for trapping any loose adult mosquitoes. Flying mosquitoes are a serious source of contamination in an insectary and a risk for escaping into the outside environment.

1.5 Insectary Manager Responsibilities

Mark Benedict

"To provide authenticated, high-quality mosquito reagents, training and information to the research community of today and the future, in a timely and professional manner."

Introduction

We begin this section with the mission statement for the MR4 vector activities. In order to accomplish this, the following list of responsibilities was developed as guidelines for the MR4 insectary manager. While the details are specific for the MR4 vector activities at the CDC in Atlanta, it provides a useful guide for supervisors employing a manager to oversee daily operations and in the development of a job description. With little modification, this has served us well to describe the core activities of the manager.

Insectary Facilities

Environment

The Vector Repository Manager (VRM) shall ensure that...

1. Environmental conditions in insectaries are constantly maintained at 80°F (±1.5 ^{o}F). Relative humidity will be controlled to be in the range of 80% (± 10%) 365 days a year without interruption. Lighting is controlled such that a 30 minute sunrise and sunset occur, in between which times, the fluorescent lights will be on continually. The total darkness between the end of sunset and the beginning of sunrise is 12 hours.

2. The environmental conditions, except for lighting, are continually monitored by CDC maintenance staff and changes should be made to settings to achieve the above only after consultation and approval by the VRM.

3. Environmental conditions including lighting are continually and independently monitored by the MR4 staff. This is achieved by sensors that are located in all three insectaries and capable of notifying MR4 staff of conditions that are outside of the permissible range within 10 minutes regardless of whether staff are in the MR4 facility, at home, or traveling as necessary to ensure that at least one staff is aware of the problem.

4. Pest insect control is continually performed to ensure essential absence primarily of ants and cockroaches. This is achieved in a way that no harm occurs to the insect colonies either directly or by contamination with toxicants transported by pests. In the event that other pests are observed (e.g. mice), control is enacted as needed, but again with highest regard for the health of the repository insects. Modifications of the facility are considered that physically reduce entry points, breeding sites, and harborages.

5. Insect pest control around the perimeter of the building to reduce external sources is considered and exercised if needed.

6. Neither CDC personnel, nor local municipalities conduct insect control in the vicinity of the insectary facilities.

7. Properly operating mosquito traps or other killing devices operate continuously and are monitored for catches in all insectaries. These should be capable of trapping primarily *Anopheles*, but also *Aedes* and *Culex* species.

8. Cleanliness is maintained in all insectaries and support areas. While hospital cleanliness is neither attainable nor necessary, a consistent effort should be made to improve the level of cleanliness. In part this will require labor, but use of materials and furnishings that do not rust and are easily cleaned will be helpful. Only cleaning compounds that are non-toxic to the mosquitoes are used, but these should be used to reduce cleaning maintenance when possible. Moreover, the CDC maintenance

staff is instructed to maintain the cleanliness of the floor and other areas within their responsibility. The VRM is responsible for cleanliness but is not to become the custodian.

9. Air filtration is installed and maintained properly to reduce the level of odors, fungi, dust, hair etc. Installing additional equipment or modifying existing equipment is considered to improve the air quality. Mold growing on mosquitoes and the insectary walls can be reduced by consistent attention to eliminating spores. Centralized UV sterilization of the air may be feasible.

10. Ensure that documentation of the maintenance of the emergency generator is available and being maintained. Notify the PI in the event of any planned power outage.

Infrastructure Improvements

The VRM shall ensure that...

1. Sign-holders are installed that contain information about specific courses of action to take in the event of various environmental anomalies. These will be located either near the alarms and/or by each doorway.

2. Signage is current and attractively maintained.

3. All infrastructure and environmental changes are consistent with the MR4 objectives. Furthermore, these changes are approved by all insectary users.

Infrastructure Maintenance

The VRM shall ensure that...

1. Hallways are kept clear of trash, boxes, unused carts, old equipment etc.

2. All lights function. The maintenance personnel should be notified in the event of lights burning out and other electrical problems.

3. Timers are properly set and maintained.

4. Hallway and insectary walls are kept clean and free of un-necessary notes, tape, scuffs, holes, tacks etc.

Insectary Supplies

The VRM shall ensure that...

1. Consumables required for the operation of the insectary are maintained at sufficient levels that shortages do not occur. The supply should be supplemented long before the need becomes critical. Allowance should be made for shipping delays and incorrect or incomplete orders.

2. Establish minimum levels of supplies at which orders will be placed.

3. Maintain inventory information sufficiently to ensure above.

4. Consumables are safe, and have no characteristics that are an immediate threat to the mosquito stocks.

5. Alternative consumables are considered for use. Materials that save time and/or money are sought and tested.

6. Maintain the cleanliness and order of the storage areas.

7. Mosquito food and blood sources are safe and of an adequate amount to ensure that shortages do not occur.

Other

The VRM shall insure that...

1. Office supplies necessary for the timely shipment and documentation of MR4 reagents is ensured.

2. Materials to produce documentation for MR4 reagents are of high quality and of adequate amounts.

3. Shipping materials are of good supply, quality, and suitability.

4. Computer consumables such as CD/Rs, diskettes, paper etc. is of an adequate supply to produce documentation, file archives, communication etc.

Mosquito Authentication

The VRM shall ensure that...

1. Only authenticated materials are shipped from and maintained by the MR4.

2. Authentication methods are developed that are reproducible with reasonable ease both within the repository and by requesters.

3. Materials required for authentication are protected from accidental contamination or loss and can be produced on demand using independent means.

4. Documentation is sufficient to enable requesters to authenticate materials independently.

Preservation and Production

The VRM shall ensure that...

1. Levels of all MR4 stocks are sufficient to ensure a constant supply of material for all MR4 activities.

2. Non-MR4 personnel who maintain MR4 stocks are informed about the requirements for the environment in the insectary and procedures to follow to ensure that the stocks are maintained without contamination or loss. This must be done without imposing upon them or requiring significant alteration of the existing procedures.

3. No MR4 stocks become contaminated or lost. This is very important.

4. Sufficient duplication of stocks is implemented to ensure an independent supply that provides insurance against accidental loss. This may be in the form of on-site maintenance in separate facilities, or a backup stock in another laboratory from whom the material could be obtained if necessary that would notify the VRM in the event of loss. Records of recipients of stocks should be referred to as a final source of stocks.

5. DNAs of stocks are prepared as proposed and distributed to the ATCC and additional backup stocks are maintained at the CDC.

6. Sentinel adults are monitored for unusually reduced life span.

7. The PI is notified promptly by voice and e-mail in the event of any stock contamination, reduction in supply, or unusual culture conditions.

8. Improvements to culture methods are considered if these can save time and/or money.

9. A current log is available on the web describing the condition of the stocks at all times including all authentication.

Distribution

The VRM shall ensure that...

1. Shipments of mosquitoes are made at first availability of the requested material.

2. Contents of shipments are correct, contain appropriate documentation, and are properly packaged.

3. Packaging is of a consistently high quality, is labeled with computer-imprinted labels, and environmental conditions of containers are suitable to ensure viability of the product.

4. Improved incubation and storage methods are investigated to both prolong the life of laboratory material and longevity in transit.

5. The recipient is notified of the anticipated shipment date, actual shipment, and tracking information. This may be done by e-mail, phone, mail, or FAX. A record should be kept for all stages.

6. Receipt of a request for materials is promptly acknowledged.

7. Shipments are made only to authorized requesters.

8. The PI is notified of all intentions to ship mosquitoes before shipment is made.

Documentation and Records

The VRM shall ensure that...

1. Monitoring of all environmental conditions is documented. This means that records of humidity, temperature, and lighting are consistently stored and readily available for the entire 24 hours, 7 days per week, 365 days of the year.

2. Both mosquito culture anomalies and nominal conditions are documented and recorded.

3. Records of all requests and shipments are made in a database format. This database should include at least:

       a. Date of request

       b. Record of confirmation

       c. Anticipated shipping date

       d. Actual shipping date

       e. Carrier and tracking number

       f. Record of receipt

4. Nominal stock levels and quality should be documented consistently. These records should be publicly available on the web.

5. Changes to SOPs should be documented.

6. All versions of the handbook should be permanently stored in hard and digital form with date and version number

7. Alterations of the handbook should be coordinated with the requirements of the ATCC.

8. An annotated version of the handbook indicating the reasoning behind the changes should be available.

9. Digital and hardcopy forms of the product information sheets are current and also available on the web.

10. All forms are current.

11. All standard operating procedures are detailed sufficiently in hard and digital copy so that a successor knows what to do in every situation. These procedures should be diligently maintained and bound in a clearly divided notebook. Contents should contain SOPs, but also include (for example):

       a. What to do when the alarms go off

       b. Nominal environmental parameters

       c. Where records are stored and how they are backed up

    d. What to do when nobody is here on the weekend and there is water leaking

    e. What to do when a request for a stock comes and the PI is not available to review the request

    f. How to authenticate a DNA sample or mosquito stock

    g. Who the current contacts are at ATCC with whom to communicate regarding bioinformatics

    h. Information required for quarterly and annual reports is consistently recorded and made available to the PI.

    i. Number of shipments

    j. Most-requested materials

    k. Summaries of destinations

    l. Summaries of material arriving unusable

    m. Summaries of replacement requests

12. Web information is correct and understandable. This will be accomplished by:

    a. Coordinating with the ATCC bioinformatics personnel

    b. Producing all data in database form so that it can easily be sorted, searched, and stored.

    c. Acquiring new information, photographs, and technologies to make the MR4 web site more useful and interesting.

    d. Informing ATCC of changes needed in catalogues, forms, product information sheets etc. that are available on the WWW.

Budgets and Financial Management

In coordination with the Branch Program Specialist, the VRM is expected to ensure that:

1. Supplies and equipment budgets for the repository are managed so as to best provide items needed for the smooth operation of the repository.

2. Budgets are not over or under-spent

3. Orders are received and billed correctly

4. Items are not charged to the VR budget without approval by the VRM or PI.

Supervision of Personnel

While the ultimate responsibility for the conduct of personnel supervised by the VRM is with the PI, the VRM is expected to:

1. Ensure that supervised personnel are aware of their responsibilities

2. Be trained to perform all tasks

3. Ensure that tasks are performed promptly

4. Receive safety and security training

5. Make the PI aware of any problems with managed personnel including:

    a. Consistently poor technical performance

    b. Failure to comply with safety or security requirements

    c. Conflicts with other employees

    d. Difficulties responding to requests from the VRM

    e. Time and attendance problems.

1.6 Trapping Adults in the Insectary

Mark Benedict and Paul Howell

Introduction

Research on laboratory cultured mosquitoes has elucidated many processes including mechanisms of insecticide resistance, vector-parasite interactions, and speciation. One of the primary aims of any insectary operation is to minimize the release of free-flying mosquitoes during day-to-day operations. Not only are free-flying haematophagous insects a nuisance, but they can pose both a public health and cross-contamination concern (Tabachnick 2006). Released insects could escape into the environment and become established potentially leading to increased disease transmission (Scott 2005). Loose mosquitoes flying in a rearing room may be inadvertently blown into a cage containing a different strain/species while removing pupae cups. Alternatively, gravid females could lay eggs in any pans that are left uncovered. Even covered pans can sometimes have enough of a gap for a mosquito to slip inside and lay eggs, thereby contaminating the entire cohort. The purity of the strain being used is paramount to quality research. Cross-contamination between strains can lead to the nullification of desired genotypes/phenotypes which will alter research results.

Responsibility for containment lies solely with the researcher. USDA/APHIS requires BSL2 facilities to maintain safeguards to prevent the escape of vectors into the environment. Therefore, additional containment and trapping methods should always be considered when designing or updating an insectary. Guidelines for containment can be found online in the Arthropod Containment Guidelines (http://www.liebertonline.com/toc/vbz/3/2).

Training on how to remove items from a cage, such as sugar pads or pupae emergence cups, should be provided to help reduce the number of insects released. Reducing the number of times a cage is entered is also recommended. Using long-life sugar feeding devices (**Chapter 2.4.9**) or introducing all larvae and pupae via a single emergence cup are excellent ways to reduce the number of times a cage is opened. If any insects are accidentally released, efforts should be made to immediately track and destroy them.

In some instances, capturing all released insects may not be possible. Employment of secondary, active trapping devices is highly recommended to ensure free-flying insects are captured expeditiously. There are several products available to the public, some of which are listed in **Tables 1.6.1** and **1.6.2**. Traps should be checked on a routine basis to determine the number of insects being captured and that they are operating properly.

It is also important that mosquito traps that burn propane NOT be used indoors, nor should systems that release insecticide be used in the insectary.

Figure 1.6.1. An example of a UV light trap

Table 1.6.1: Commercially available UV light traps

Product name	Website
NoSquito® (Stinger) Indoor Trap MA06	Numerous web vendors
Mega-Catch Alpha	http://www.megacatch.com/?gclid=CJ2Vz7md0agCFYPD7Qodd0ldgw
DynaTrap	Numerous web vendors
Viatek Mini Mosquito Trap	http://www.viatekproducts.com/ProductDetail.jsp?LISTID=800001B4-1174417771
Indoor Natural Attractant Insect Trap	http://www.hammacher.com/Product/78397
CDC Light Trap	Numerous web vendors
Rush Hampton 61001	Numerous web vendors

Table 1.6.2 Commercially available electrocution devices**

Flowtron PV-75A	http://www.mosquitozapper.com
Flowtron BK-15	http://www.mosquitozapper.com
Stinger UV-15	http://kaz.com/kaz/insect-control/
Stinger FP15-CR	http://kaz.com/kaz/insect-control/
Black & Decker BZ15	Numerous web vendors

** These traps may include octenol or other odiferous baits. Although not lethal to insects, the scent may be disagreeable to workers over time.

References

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Scott TW (2005) Containment of Arthropod Disease Vectors. ILAR Journal 46(1): 53-61.

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Chapter 2 : *Anopheles* Laboratory Biology and Culture

2.1 Behavior and Physiology of Anophelines in the Laboratory

Adapted from (Clements 1992)

Introduction

Behavior and physiology are important to understand in making decisions in the insectary. The way the mosquitoes behave will affect choices of food, blood, egging, insectary supplies, insectary space demanded and much more. Additionally, understanding more about the differences between your stocks can be used to give clues of possible contamination along with the morphological and molecular authentication methods discussed in Chapter 4. These tips can also be practical in understanding why mosquitoes are not thriving or behaving as predicted.

Eggs

Culex, *Aedes*, and *Anopheles* eggs are laid in different patterns and observing the patterns on egg collection can be one way of catching a cross-genus contamination event early. *Culex* eggs are laid in discrete rafts of attached eggs by individual females. The eggs are tapered (**Figure 2.1.1**) and tend to drift to the edges of containers and remain there. *Anopheles* eggs are unattached and lie in stellate patterns horizontally on the water surface (**Figures 2.1.2** and **2.1.5**). Exochorion 'floats' on the eggs aid in keeping them at the surface. *Aedes* lay their eggs unattached to one another above the water but do not have floats (**Figure 2.1.3** and **2.1.5**).

Aedes eggs survive drying well (**Figure 2.1.4**) though the amount of time they can be kept dry prior to hatching varies with the species and conditions of storage. Some strains can be kept dry as much as 6 months prior to hatching. *Anopheles* and *Culex* eggs do not survive extended drying and should be kept moist and in a humid atmosphere prior to hatching. The amount of time that can pass before hatching *Anopheles* or *Culex* eggs varies. If an insectary has *Aedes* and *Anopheles* or *Culex*, it is best to always allow the *Aedes* eggs to dry before hatching to minimize contamination by the more sensitive strains.

Figure 2.1.1. *Culex* eggs less than 24 hours post oviposition. Eggs are cemented together forming an egg-raft that floats on the surface of the water.

Figure 2.1.2. *Anopheles* eggs 30 hours post deposition. Clear floats are visible on sides of eggs. The non-melanized egg (center) will not hatch.

Larvae hatch from the blunt underside.

Figure 2.1.3. *Aedes albopictus* eggs 48 hours post oviposition on seed germination paper.

Figure 2.1.4. *Aedes aegypti* eggs 2 weeks post oviposition stored under insectary conditions.
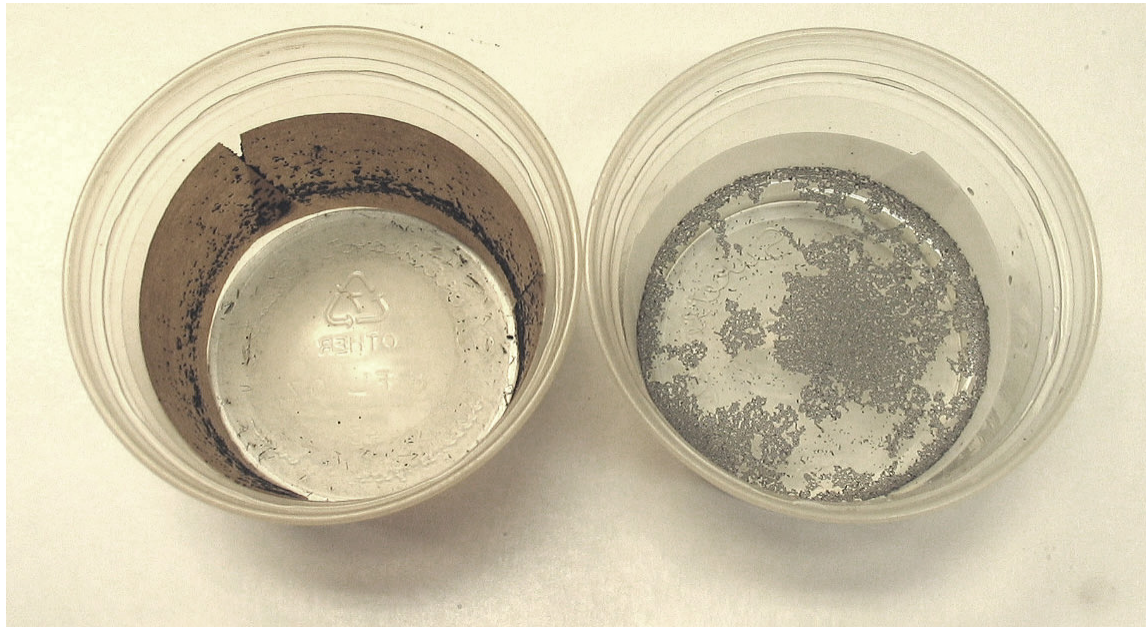
Figure 2.1.5. Egg cups removed from cages 24 hours after insertion. *Aedes* eggs (left) were laid on seed germination paper with only a small amount of water in the bottom to keep the paper wet. *Anopheles* eggs (right) are laid on the surface of water and will spread across the water surface. In smaller numbers, they accumulate at the edge of the water.

When mosquito eggs are laid, they are white. They normally darken and harden within a few hours. The rate at which they change color and harden depends on the strain and temperature. *Anopheles* eggs that fail to melanize or sink do not hatch.

Larval Feeding

In the wild, mosquito larvae survive in a large variety of habitats. The food types in these habitats are largely the same as that in the insectary in that they contain microorganisms, detritus (particulate organic matter), biofilm, and other organic matter such as dead invertebrates. A major source of nutrients for mosquito larvae comes from plant material that has been already degraded by fungi or bacteria.

Important in choosing a food is to note the method and location of feeding for the particular strain you are using. Many *Anopheles* and *Culex* use the feeding mode **collecting-filtering** which is feeding by removing particles that are suspended in the water column or at the water surface. For *Aedes*, **collecting-gathering** is a more common method of feeding which involves first causing materials that have settled or are attached to surfaces to resuspend and then ingesting them from the resuspension mixture. Other methods of feeding include **scraping** (removal and ingestion of the biofilm and protists on the surface of submerged plants and other surfaces), **shredding** (biting off small fragments of plants or dead matter), and **predation** (eating other insects). Much of the differences seen in feeding preferences can be associated with the differences in mouthparts and head structures (**Figures 2.1.6** – **2.1.8**). More detailed information of the various structures can be found in Clements' The Biology of Mosquitoes.

Figure 2.1.6 *Aedes* head and mouthparts.

Figure 2.1.7 *Anopheles* head and mouthparts.

Figure 2.1.8 *Culex* head and mouthparts.

Even though some *Culex* and *Anopheles* share the same method of feeding, the location of the feeding can be different. Anophelines tend to feed at the air/water interface or on the bottom (**Figure 2.1.9**) while *Culex* and *Aedes* typically feed throughout the water column (**Figure 2.1.10**).

Figure 2.1.9 *Anopheles* larvae feed at the water surface and bottom, but not in the column.
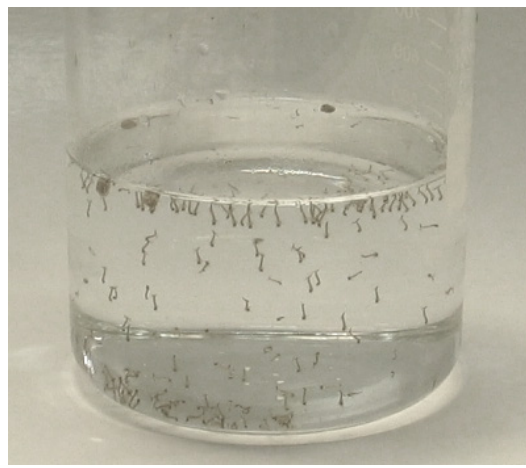
Figure 2.1.10 *Culex* (pictured) and *Aedes* larvae feed throughout the water column.

Larval mouthparts are complex and suitable for a form of filter feeding and limited 'chewing.' The mouthparts are well developed but differ among strains. Parts associated with feeding are "teeth" for both biting and chewing, curved setae which bring food particles from the water to the mouth, and other brushes and combs around the mouth to bring in food. The brush filaments and mandibles are suited to the type and location of feeding. For example, collector-gatherers are adapted to resuspending settled particles.

Anophelines live at the air/water interface. Typically they are seen lying just below the interface, dorsum up. This is also where they feed as water surfaces are covered with an organic microlayer in the wild. At the surface which their head rotated 180 degrees, they beat the mouthpart brushes and create currents which bring particles toward the mouth.

Collector-filterers such as anophelines have lateral palatal brushes at the mouth that are thought to function as paddles rather than as filters as previously thought. However, in the paddling, the movement of the brushes delivers water concentrated with larger particles toward the mouth.

The size of the particle that larvae can ingest increases with the size and age of the larva. Factors such as size and age should be taken into consideration when determining which larval food to use. Also, as larvae grow, the amount of food they will eat increases by as much as 5 times what they ate in the first instar.

Growth and Development

Intrinsic Effects

Mosquito larvae have four stages. The body size changes continually while the head capsule increases (mainly) only at molts i.e. saltatorily. Thus, the instar is best determined by the head capsule size (Timmermann and Briegel 1993). Performing some measurements on the head capsule of your species to determine the range of values that could be observed in any stage is a good idea if working with an exact stage is important for your research project. A series of photographs of stages of larval life might make it easier for staging to be apparent by eye until you become familiar with your particular stocks and strains. Larval stages for *An. gambiae* are shown in **Figure 2.1.11**.

Generally, males develop faster and are smaller adults than females. Males also typically spend less time in the pupal stage before emerging than females (Haddow et al. 1959; de Meillon et al. 1967). The degree of sexual size dimorphism varies between stage and species. For example, though the adult size differs quite widely from male to female in *Anopheles* and *Aedes*, the pupal size differences are not as apparent in *Anopheles* as in *Aedes* (**Figures 2.1.12 and 2.1.13**).

Extrinsic effects

Temperature

Temperature is the most important and easily controlled extrinsic factor affecting growth rates of larvae. The effect of temperature on the growth of mosquito larvae has been studied extensively. Specific for each species, there is a temperature range in which development can occur. Within this range, growth and development vary dramatically with the temperature fluctuations. For this reason, it is important to control temperature to achieve predictable culture.
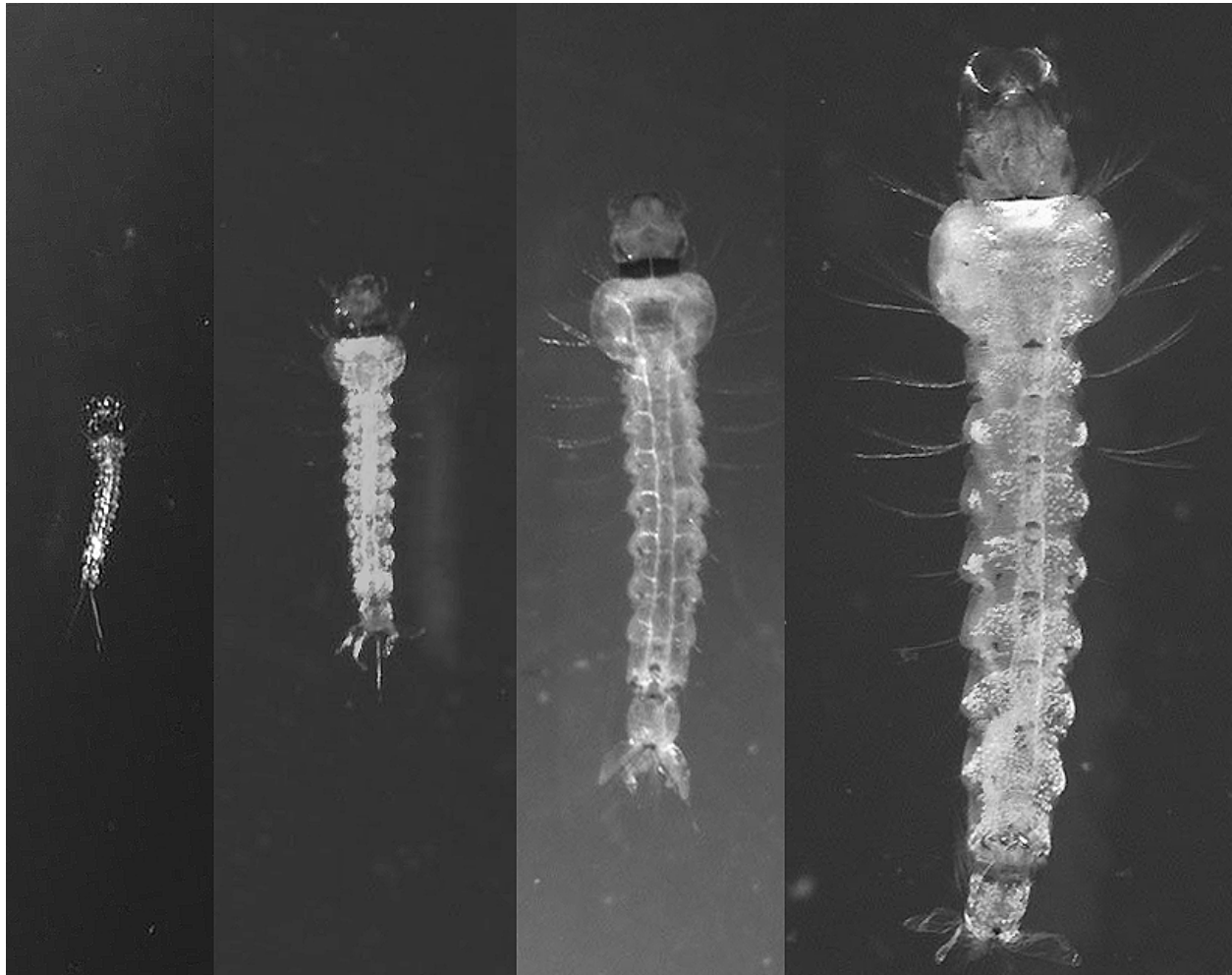
Figure 2.1.11 From left to right, *Anopheles gambiae* larvae 24 hours post hatch (1st instar or L1), 2 days post hatch (2nd instar or L2)**,** 5 days post hatch (3rd instar or L3) and 6 days post hatch (4th instar or L4). All were photographed at the same magnification.

Figure 2.1.12. *Anopheles gambiae* pupae: two females (bottom right) and one male (top left corner). Size difference is not obvious by eye.

Figure 2.1.13. *Aedes aegypti* pupae: two males (right) one female (left). Size disparities are apparent.

Nutrition

The amount of available food significantly affects larval growth. Underfeeding can cause as much delay as overfeeding but will likely be evident later, especially in the adult stage. Much has been written about Dietary Restriction (DR) in mice and flies and is reviewed in Chapter 3 for its contributions to longevity and fecundity. In short, DR causes the animals to live longer but age faster having negative influence on fecundity and tolerance for environmental fluctuations and infection.

Larval Density

Achieving the right density is very important in growth and development. The most common problems associated with over crowding are: longer development time, reduced pupation and eclosion, and a decrease in pupal weight. See Chapter 2 Culture section for more on proper density for anophelines.

Effect of Larval Health on Adults

Adults from larvae that were crowded are typically smaller and less fecund. The ultimate size of an adult mosquito will be based on genetics in combination with the environmental conditions experienced through development. Studies have shown that larvae that are reared in crowded conditions had negative effects on weight at emergence, quantity of the blood meal and overall fertility. Poor larval conditions cannot be totally overcome by good diet or care in later stages, therefore careful attention to larval conditions determines high overall quality of production.

Environmental effects on rhythms

Studies show a link between environmental factors and ecdysis. These studies are limited to certain species and conditions; however, the evidence supports the shifting of ecdysis under temperature changes, light/dark cycles, and larval stress such as salinity. For example, researchers found that in continual darkness, with variable temperature cycles, the larval-pupal ecdysis was more likely to occur during the warm phase.

The time of day/night the ecdysis will occur is species-dependent. It is thought that the trigger to molt is switched on and off based on a daily rhythmic activity cycle of 24-hour intervals that is exhibited by many organisms, or a circadian rhythm. This is not universal among all mosquitoes. Examples of some found not to have such a rhythm are *An. quadrimaculatus* (Nayar and Sauerman 1970) and *Ae. aegypti* (Haddow et al. 1959). The best rule of thumb is if your insectary has problems with ecdysis being temporally irregular or extended over several days, experiment with your conditions to correct the problem. Light and temperature are the key factors.

Adult feeding

Plant Juices

Both adult male and female mosquitoes will drink plant juices as an energy source (see review in (Foster 1995). Plant sugar is the major food resource for mosquitoes. In the wild, the most common source is floral nectar, but other sources exist such as damaged fruit (**Figure 2.1.14**) or vegetative tissue. With these different meals, the mosquito would be receiving largely sucrose, fructose, or glucose, depending on the source. Other sources such as maltose or melibiose are seldom found in mosquitoes. Amino acids needed for ovary development can be found in some nectar, but the concentrations are not high enough to replace a blood meal. Natural sugar sources can have a wide range of amounts of sugar from none to 50% w/v, though 20-50% w/v is the normal range. Mosquitoes have been seen ingesting crystallized sucrose by liquefying it with saliva.

Figure 2.1.14. *Anopheles gambiae* taking sugar meal from a tangerine. Female at bottom, center is drinking juice. Two males have been attracted.

Figure 2.1.15. Sugar-fed *Anopheles farauti*. Plant juices are stored in the crop rather than the midgut. Different species have been observed to ingest 29-62% of their unfed weight in sugar. In many tests, certain types of sugar sources have been shown to be important in increasing the life span of females that are frequently blood fed over those who were given only blood.
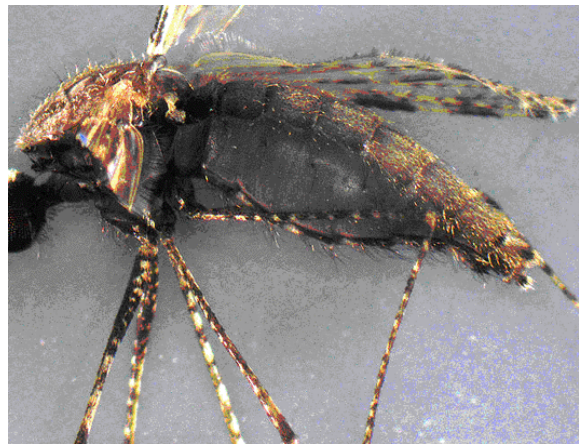
Blood

Only females take blood meals. The blood is their resource for protein needed in ovary development. There is data that substantiates blood as also providing a source of energy since blood fed females have been shown to survive longer than females given only water. It is very common for a mosquito to take as much as 2-4 times their weight of blood in a single meal. Females can also excrete clear to reddish fluid while bloodfeeding in order to concentrate the protein as much as 2 fold (**Figure 2.1.16**), a process called diuresis.

Mouthparts Used in Feeding

Mosquitoes have mouth parts conducive to taking up liquids. Females have more complex mouthparts because they must probe flowers and pierce skin. The mouthparts vary among the types of mosquitoes, especially among those who have different necessary functions such as those that take blood meals versus those that do not.

Figure 2.1.16. *Anopheles stephensi* mosquito taking a blood meal. Notice the blood moving through the proboscis. The mosquito is undergoing a process called diuresis by which she concentrates the blood.

(Photo contributed by James Gathany, CDC, used with permission.)

Phases of Blood-feeding

- **Exploration**. After a mosquito lands on its host, it goes through an exploratory phase before penetrating the skin. *Anopheles* and *Aedes* remain stationary for a period of time after landing on a human. Host movement during this time causes the mosquito to leave. Without movement, the mosquito explores the skin either in the area of landing or sometimes moving around.

- **Probing**. Prior to penetration, the mosquito goes through a process of probing in which it touches its labium (not the proboscis) to the skin surface many times to decide where to penetrate with the proboscis. The mosquito then stabilizes by straightening the legs just prior to penetration.

- **Penetration**. Proboscis is inserted into the skin.

- **Imbibing.** This process begins when the palps stop vibrating from the penetration process.

- **Withdrawal**. This action takes about 3 seconds. A complete withdrawal can occur or only a partial one with re-insertion. After final withdrawal, the female usually flies away quickly.

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2.2 Infections in Mosquito Cultures

James J. Becnel and Paul Howell

Introduction

Fortunately, there are few naturally occurring pathogens that become established in mosquito colonies. There are, however, numerous microbes living within an insectary which, under normal conditions, are not considered **pathogenic** (capable of causing disease) but may have deleterious effects when an insect colony is stressed. Environmental stressors include larval overcrowding, unstable heat or humidity, poor quality diet and overfeeding.

Infections in the insectary are spread through one of the following routes: diet, injury, infestation of the egg, or environment. Dietary routes include feeding insects a contaminated diet or the cannibalism of expired, infected larvae. Larval injury creates an opening for many water-borne pathogens. Insect eggs can be either internally (viruses) or externally (fungi or microsporidia) infected.

Environmental routes are non-specific but can include the following:

Introduction of pathogens by wild strains- Wild or newly acquired insects can carry pathogens which do not affect them in the wild, but when introduced in a newly stressed atmosphere, these pathogens can become opportunistic. Also, a newly acquired laboratory colony can introduce a chronic infection to an insectary.

Airborne entry- Several fungi, bacteria, and protists can be introduced on airborne particles.

People- Some of the more common bacterial contaminants in insect colonies are considered normal human fauna like *Escherichia coli*. These can be passed to the water by touching the water or food while feeding.

Surfaces- Contaminated or poorly cleaned surfaces and equipment can harbor large numbers of opportunistic microbes.

Knowing what a healthy colony looks and smells like can be the easiest way to detect an infection. Water that is malodorous, cloudy, has persistent bubbles or contains excessive foreign matter may be detrimental to an insect colony (see **Chapter 2.4.5** for more on water quality). In the insects, signs and symptoms can include changes in normal size or color, deformity, lengthened duration of rearing, reduced longevity of adults, decreased fecundity, and decreased fertility rates. Excess mortality, short life-spans, low reproductive rates, or the presence of fungi on the cuticle are some of the early signs of a possible infection in a colony.

Infected larvae typically display one of the following: deformation (**Figure 2.2.1** and **2.2.2**), or discoloration of the larvae as well as abnormal larval behavior. In pupae, typical signs of infections include elongation of early pupae, failure to emerge (**Figure 2.2.3**), or selective emergence of one sex.

Figure 2.2.1. *Anopheles stephensi* larvae that failed to complete metamorphosis due to infection.

Figure 2.2.2. *Anopheles gambiae* showing deformation and signs of infection in the larval state.

Figure 2.2.3. Infected *Anopheles stephensi* that died during ecdysis.

Commonly reported infections in mosquito colonies

There are two forms of infections in a colony: chronic and acute. Chronic infections may persist for several generations and never be fully apparent but result in poor quality insects, such as infections with fungi and protozoa. Conversely, acute infections quickly spread through a colony and lead to high mortality rates. Pathogens that have been implicated as agents of acute infections include bacteria, viruses, and some fungi. Although the following is not a complete list, it covers the most reported pathogens and if possible, includes a description of the microbe or the appearance of the infected insect.

Viruses

The two types of viruses routinely found to be pathogenic to mosquitoes are defined as occluded or non-occluded (Federici 1985). Although not routinely found in insectary populations, there have been viral infections reported in laboratory colonies of *An. stephensi.* One of these reports showed high mortality rates in the colony associated with this infection (Bird et al. 1970). At least two other types of viruses have been isolated from mosquitoes with only one being reported as detrimental to development of the larvae (Jenkins 1964).

Occluded Viruses (Becnel and White 2007)

These viruses form proteinaceous crystal occlusions within the mosquito.

Baculoviridae

Deltabaculovirus: This Dipteran-specific NPV is the only member of this family that is commonly associated with feral mosquito larvae. These viral particles infect the larval midgut epithelium resulting in a stunted appearance, delayed growth and death. Infected larvae often have white cysts or nodules throughout the midgut and gastric cecae. These virions are highly virulent and cause mortality within 72-96 hours after the initial infection. Transmission of the virus is enhanced with a high concentration of Mg^{2+} cations in the larval habitat (Becnel 2007).

Reoviridae

Cypovirus: These are also referred to as cytoplasmic polyhedrosis viruses (CPV). Infections with CPVs are typically benign in nature but can cause larval mortality when present in high numbers. Infections are easily seen in L3 or L4 larvae as opaque sections limited to the gastric caeca and/or posterior stomach. Transmission of these viruses is enhanced in the presence of divalent cations or when larvae are stressed.

Non-Occluded Viruses (Becnel and White 2007)

These viruses form paracrystalline arrays of virions in infected cells.

Iridioviridae

Mosquito Iridescent Virus (MIV): These are cosmopolitan in nature in field collected larvae. Infections can be detected by placing larvae in a dark pan and scanning them with a fluorescent light. Infected larvae will have iridescent patches of turquoise, green or orange depending on the specific infecting virus. Infections are usually localized in the fat body or epidermis. Larvae infected in early instars are highly susceptible. Infections in later instars are typically passive allowing for transovarial transmission. Sub-lethal infections often result in reduced fecundity and longevity in the stock (Becnel 2007).

Parvoviridae

Mosquito Densovirus (MDV): Infections with the viruses are typically subtle in nature. Infected larvae will become lethargic, loose their body color, have a contorted appearance, or appear whitish in color before expiring (Becnel and White 2007) Infections are widespread except in the midgut epithelium. Many of these viruses have been isolated from anopheline mosquitoes and from established cell lines.

Bacteria

Although several bacteria are known to be pathogenic to mosquito larvae, relatively few of them occur naturally in an insectary setting. Infections in the rearing containers are often caused by the inadvertent introduction of bacteria from the skin of an insectary employee or through the addition of contaminated water or food. In Africa, *Enterobacteria* infections were seen in the haemolymph of insect larvae (Muspratt 1966). In advanced stages of disease, black spores were visible inside the larvae and eventually the larvae displayed a "milky" coloration and swollen appearance **(Figure 2.2.4)**.

Figure 2.2.4 Infected *Anopheles stephensi*. Note the milky swollen appearance indicative of an infection.

Escherichia coli common human fauna. *E. coli* bacteria have been found to be pathogenic to early instar *Culex* mosquitoes (Jenkins 1964).

Serratia marcescens commonly found growing in standing water. During rearing, it will grow to form a reddish film on the bottom of the pan. This bacterium is not known to be lethal under normal conditions, however the red pigment prodigiosin has been found to have larvicidal properties (Patil et al 2011).

Pseudomonas fluorescens ubiquitous flagellated bacterium. *P. fluorescens* is a commonly isolated bacterium from soil and water sources, and it has been shown to be lethal to mosquito larvae. Pseudomonads are known to cause extensive larval mortality due to their production of toxic substances (Jenkins 1964).

Leptothrix buccalis common water bacterium isolated in fresh and polluted water sources and found to be highly lethal to *An. maculipennis.* With this infection, the larva maintains the disease through eclosion, but death does not occur until sometime after emergence (Jenkins 1964).

Streptococcus spp common human fauna. These bacteria can rapidly grow in the warm insectary conditions and will attach to larvae in large numbers. The bacteria invade the integument (insect's hard outer coat) and cause internal damage leading to mortality in the L3 or L4 stage (Kramer 1964).

Treatment of bacterial infections

Antibiotics such as Penicillin-Streptomycin-Fumigillin (PSF) (Invitrogen 15240-062) can be used in larval culturing (de St. Jeor and Nielsen 1964). However, it is recommended that before full scale implementation, trials are conducted to determine the dosage required to eliminate the infection without killing too many larvae. Additionally, you can feed antibiotics to adults in the sugar meal (Touré et al. 2000).

In the insectary

To ensure that an infection does not spread to another colony, sterilize all rearing trays by either autoclaving them or soaking them in a 5-10% household bleach solution for 24 hours. Wipe down all counters with a 5-10% bleach solution and replace any larval rearing diets in the rearing room.

Fungi

Fungi have been considered one of the most pathogenic organisms that can infect mosquitoes. A large number of fungi have been isolated from mosquitoes, both wild and laboratory reared, recently reviewed by (Scholte et al. 2004). Most fungal infections are transmitted by free-floating spores.

Coelomomyces spp This is the one of the most widely studied fungi that infects mosquitoes. Infections typically occur in early larval instars, and infected larvae rarely pupate or emerge. The infection can be detected by locating "buds" or lumped structures in the anal gills of the larva (**Figures 2.2.5 and 2.2.6**). If infection is suspected, dissecting the larvae should reveal hyphae emanating from several tissues including the malpighian tubes and muscles. The hyphae will be branched, multinucleate, and clavate (club) shaped. Sporongia, the reproductive portion, will develop into dark spherical spores. Although this is highly pathogenic, it is self limiting and usually only 1 or 2 generations will be affected due to the need of an intermediary host to complete its life cycle (Madelin 1965), (Scholte et al. 2004). In Africa, these fungi have been often reported to be isolated from the ovaries and fat bodies of females only (Hazard 1973). Authors have reported that larvae infected with *Coelomomyces* fungi often have a yellow, orange, or brownish color due to a number of sporangia that have developed internally (Kramer 1964).

Leptolegnia spp. These are typically encountered in wild isolates. They are highly pathogenic, especially to L1 and L2 larvae. In *An. gambiae* it was shown that 100% of larvae were killed within 72 hours post infection (Scholte et al. 2004).
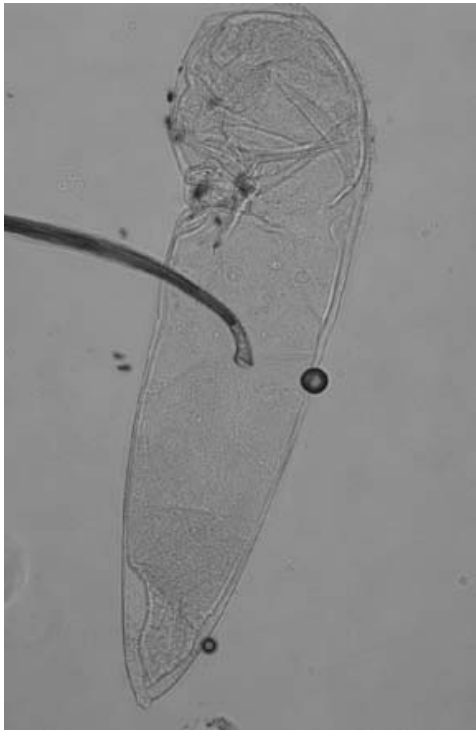
Figure 2.2.5. Normal anal gill of larva of *Anopheles gambiae*.
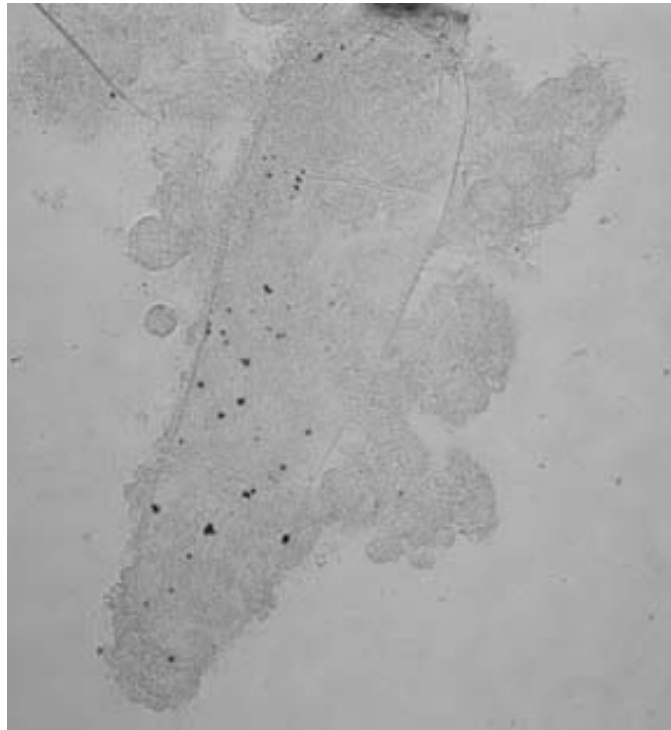
Figure 2.2.6. Infected anal gill of larva of *Anopheles gambiae.*

Other locations of notable fungal development are in the spermathecae and terminalia (**Figures 2.2.7** and **2.2.8**).
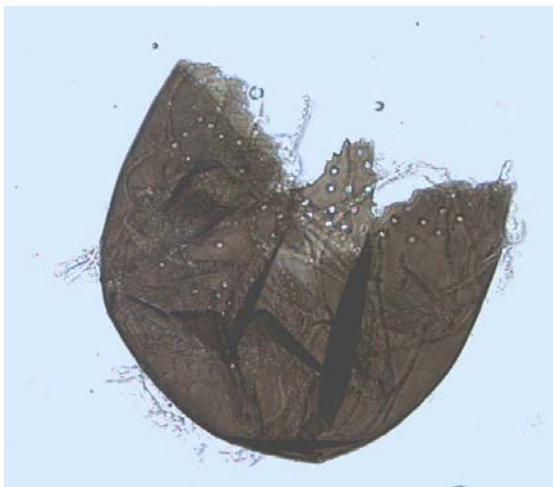
Figure 2.2.7 Fungal hyphae growing within a spermathecae of *Anopheles quadriannulatus*.

Figure 2.2.8 Fungal mass on the terminalia of a male *Anopheles dirus.*

Entomophthora spp. Whereas *Coelomomyces* infects larvae, *Entomophthora* typically infects only adult mosquitoes. Spores are transmitted after the sexually mature conidiophore ruptures through the adult cuticle. The easiest method for detecting *Entomophthora* infections is to remove a recently deceased

adult, place them on a black piece of paper and cover with a Petri dish. In 24-48 hours inspect the paper; if a white ring is visible around the mosquito, then the colony is infected. The white ring is formed by the forcible release of conidia into the atmosphere (Scholte et al. 2004).

Aspergillus spp. As a cosmopolitan fungus, these can be readily isolated from almost any surface. *Aspergillus* is better known as the causative agent behind bread mold. Like *Entomophthora*, *Aspergillus* infects by penetrating the adult cuticle. Although the exact reason behind its lethality is unknown, it has been shown to cause mortality and reduced egg production in adult mosquitoes. Experimental research has also shown that larvae infected by *Aspergillus* will mature and emerge; however, adults will have shorter lives and produce fewer offspring (Nnakumusana 1985). *Aspergillus* colonies typically have a dark green to black color and will quickly cover any surface where they are growing.

Smittium spp. A recently reported fungus that can cause larval paralysis (personal communication). It is not commonly encountered but has been reported to be highly lethal to *Anopheles* larvae compared to aedines.

Treatment

Antifungal agents like PSF and fumigillin can be used in either adult diets or in the larval rearing pan. As with antibiotics, before any full scale implementation, trials should be conducted to determine the dosage required to eliminate the infection without killing large numbers of the larvae or adults.

In the insectary

Isolate the infected colony and employ one-time use only cages until the condition improves. If you routinely maintain colonies in permanent cages, you will want to purchase some temporary cages until you can completely clean the old cages. Surface sterilization of cages is typically ineffective since most fungi produce spores that are resistant to desiccation or chemical treatment. However, soaking cages in a strong bleach solution overnight may help. Additionally, dispose of all diets, even unopened ones, which may have been the source of the infection. Clean the insectary with an antifungal agent.

Protists

Although these are rarely associated with mortality within a laboratory colony, their presence can mean impending problems. Often parasitism with protozoans results in reduced mobility in mosquito larvae. Larvae often take on a whitish color and their abdomens swell due an increase in spores internally, eventually leading to deformation. Usually, infections are not evident and are transmitted through embryos.

Protists can be introduced from many sources including rearing water, spores in the air, a previously infected colony, or from wild material. Protozoan infections can occur as internal or external infections. *Microsporidians* are typically responsible for internal infections while *Pertrichidians* are responsible for external infestations.

Internal infections

Lankesteria culicis (*Gregarina culicis*) This protozoan is often isolated from the gut and malpighian tubules in adult mosquitoes. Aedine colonies are a popular source, but this protozoan has also been isolated from a few other genera. Larvae can become infected after ingesting spores in the rearing water. Mature forms are then released back into the larval water during the adult emergence stage. *L. culicis* is not considered highly pathogenic unless found in high numbers (Jenkins 1964).

Amblyosporidae (Amblyospora and Parathelohania). These protists are often isolated from wild material. *Parathelohania* microsporidians are almost exclusively parasites of anopheline mosquitoes. They are transovarially transmitted and require a copepod intermediate host therefore infections will be limited in an insectary (Becnel and Andreadis 1999). Infections are principally seen within the fat bodies, and mortality occurs before pupation. In Africa, infections were associated with L4 male larvae resulting in sex specific mortality (Hazard 1973).

Brachiola algerae (Nosema algerae) This is considered one of the most important infections in an insectary. As with other protists, these are often found infecting the tissues associated with larval fat bodies, ovaries, midguts, and gastric systems in mosquitoes. Infections with this parasite are rarely reported from insectaries. Intra-insectary transmission is reported often from colony to colony (Savage and Lowe 1970). In *An. gambiae,* infections with *Brachiola* have caused a reduction in egg production (Jenkins 1964). Infections are transmitted via contaminated surfaces, especially eggs, therefore surface sterilization of eggs can be used to break the transmission cycle. It is a chronic infection that will sweep through a colony until all individuals are infected (J. Becnel personal communication).

Vavraia spp (Plistophora). These were formally recognized as *Brachiola* but have since been moved into a new genus. As in *Brachiola*, *Vavraia* parasitize the malpighian tubules and ovaries and will continue to infect other organs over time. However, mortality is usually negligible. Transmission of this parasite is thought to occur through the embryos. The main effect of parasitism is *Vavraia's* ability to interfere with ookinete development of *Plasmodium* parasites within the midgut (Jenkins 1964), (Kramer 1964)). Infections within laboratory colonies have been reported from minimal to critical, and some infections have been said to spontaneously disappear.

External infections

Epistylis spp. These protists are recognized as **epibionts** (An organism that lives on the body surface of another) of mosquito larvae. Although they attach themselves to larvae, they do not cause any disease within the animal. These generally will appear as a fuzzy coating around the larvae. Mortality from infestation with *Epistylis* is caused by either physically blocking the larva from feeding or by overwhelming the larva and reducing its normal movements. Heavily infested larvae may have trouble reaching the surface to breathe or their siphons may be blocked by attached protists (Larson 1967).

Vorticella spp. As in *Epistylis*, these are considered epibionts of mosquito larvae and appear as a fuzzy coating around the larvae (**Figures 2.2.9 and 2.2.10**) (Schober 1967). Although they are frequently encountered in nature, they have been isolated in laboratory colonies (Armstrong and Bransby-Williams 1961; Hati and Ghosh 1961; Larson 1967).

Figure 2.2.9 *Vorticella*-infested *Anopheles stephensi* L4.

Figure 2.2.10 *Vorticella*-infested anal gills of *Anopheles stephensi* L4.

Treatment

There are no formal treatments for most protozoan infections. Some researchers have had some success in treating *Vorticella* infestations with mepacrine hydrochloride (Jupp and Smith 1986). Surface sterilization of eggs can also help to eliminate microsporidian infections (see **Chapter 2** Culture section for technique).

In the insectary

Infected colonies may need to be replaced or isolated to ensure the infection does not spread to other insect colonies. Some microsporidian infections are self-limiting and will resolve themselves within 1-2 generations. However, others are persistent due to transmission through the embryos. Routine disinfection of pans and surfaces should limit the number of resistant spores. Epibionts are frequently associated with poor rearing conditions. Therefore, efforts should be made to keep rearing water as clean as possible and any infected larvae should be removed promptly. For *Vorticella* treatment, it is possible to reduce the number of protists affecting a colony by adding a 1% bleach solution to the rearing water in a 1:20-1:30 ratio.

Nematodes

Although these rarely occur within insectaries, they can be introduced into an insectary from field-collected material. Mermithids, the main pathogen from this group, are worms that infect mosquito larvae and typically cause high mortality before pupation (Kalucy 1972).

Acarids

Acarids are mites (see **Figure 2.2.11**) from several genera that often attach themselves to the abdomen or thorax of an adult mosquito. Light infestations are not fatal; large infestations, however, can lead to mortality (Jenkins 1964).

Figure 2.2.11. Shown is a mite that was found persistently in the insectary, typically in egg dishes and in the bottom of cages. Harm from this mite has not been observed.

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2.2.1 PCR assay to detect Microsporidian infections in mosquito colonies

Charles Vossbrinck, Neil Sanscrainte, James Becnel, and the MR4 Staff.

Although protist infections are rarely associated with mosquito mortality within a laboratory colony, their presence can mean impending problems. Protists can be introduced from many sources including rearing water, spores in the air, a previously infected colony, or from wild material. Microsporidians are typically responsible for internal infections resulting in reduced mobility in larvae and a decrease in colony production.

Although infections are typically systemic, they are typically localized in the abdominal region. This is due to the route of infection. After ingestion, the pathogen spreads from the midgut into the fat body resulting in a swollen, milky appearance in infected larvae due to the abundance of protists inhabiting the tissue.

Because the spores are concentrated in the abdomen and host DNA will inhibit the PCR, we recommend performing the assay on extracted DNA from pooled samples (3-5 works best) of dissected abdomens. Whole abdomens are removed using a scalpel and pooled into a homogenization tube. Most commercial DNA extraction kits will work, however it is important that after homogenizing the tissues in a microcentrifuge tube, you must place the samples in a dry heat block at 95-100°C for 15 minutes prior to DNA extraction. This heating step is necessary for rupturing the Microsporidian spores.

PCR Assay

Prepare PCR Master Mix for one 25µl PCR reaction. Add reagents in the order presented.

1	Reagent
16.9 µl	Distilled H2O
5.0 µl	5X PCR buffer
1.0 µl	dNTP (2mM concentration)
0.1 µl	$MgCl_2$ (25 mM concentration)
1.0 µl	Amby18F (4.2pmol/ul) [CAC CAG GTT GAT TCT GCC]
1.0 µl	Amby1492R (4.2pmol/ul) [GGT TAC CTT GTT ACG ACT T]
0.1 µl	GoTaq DNA polymerase (5U/ µl)
25 µl	**Total (To each 25 µl reaction add 1.0 µl template DNA)**

Table 2.2.1.1 F and R indicate forward and reverse orientation

PCR cycle conditions

94°C - 3min x 1 cycle
(94°C - 45s , 50°C - 1min , 72°C - 1.5min) x 35 cycles
72°C - 10min x 1 cycle
4°C hold

Run samples on a 1.5% agarose EtBr, or other intercalating agent like Gel Red, gel. Infected mosquitoes will have an ~1250-1400bp band, the length of which is determined by the microsporidian species infecting the colony (**Figure 2.2.1.1**). In this assay we used *Vavraia culicis* as a positive control. Note we often encounter the ~250bp band when mosquito DNA is present as seen in lanes 3, 4, and 6.
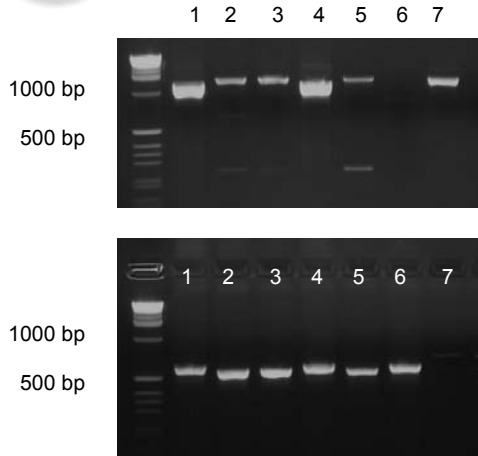
Figure 2.2.1.1. Top row: 1kb ladder, lane 1, 50,000 spores and 4 abdomens dissected, lane 2, 100,000 spores and 4 abdomens, lane 3, 200,000 spores and 4 abdomens, lane 4, 500,000 spores and 4 abdomens, lane 5, 1 million spores and 4 abdomens, lane 6, 4 abdomens, lane 7, 500,000 spores. Bottom row, general anopheline ITS2 PCR (**Chapter 7.2.1**) with the same samples.
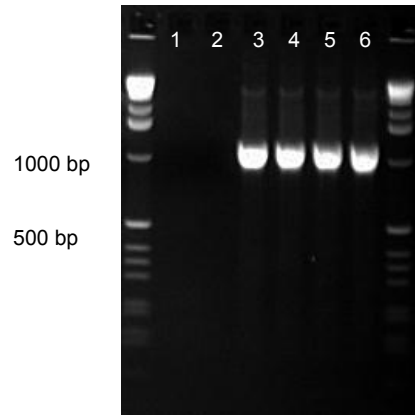
Figure 2.2.1.2 1kb ladder, lanes 1-2 uninfected laboratory colonies, lanes 3-5 are infected samples from a laboratory colony, lane 6, positive control sample.

The MR4 Vector Activity would like to thank Dr. James Becnel for the genomic DNA and preserved samples of *Vavraia culicis* and *Edhazardia aedis* which were used to optimize this assay.

96 well PCR sample preparation template

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2.2.2 PCR Assays to Detect Entomopathogenic Fungi in Mosquito Colonies

MR4 Vector Activity

Although several fungi are known to be highly pathogenic to mosquitoes, only a few are routinely encountered in the laboratory (see **Section 2.2**). These fungi tend to persist as latent, sub-lethal infections that can cause reductions in fecundity and longevity within the colony and can even interfere with *Plasmodium* infections (Blanford et al 2005).

Since pathogenic fungi come from different classes, there are no universal PCR assays that can be performed to determine if a colony is infected as in the case of Microsporidians (see **Section 2.2.1**). Several fungi are routinely implicated as entomopathogens in the field, however only a few are capable of reproduction within the confines of the insectary. Fungi such as *Coelomomyces* require an intermediary host to complete their life cycle and are typically self-limiting while others like *Entomophthora*, *Lagenidium* and *Smittium* can be transmitted within the confines of the insectary and usually result in moderate to high mortality rates. Here we present assays designed to detect those fungi which are capable of reproduction within an insectary and known to be pathogenic to mosquitoes.

Prior to the PCR reaction, you will need to homogenize samples. Samples previously homogenized for Microsporidian analysis can be used for this assay otherwise please refer to **Section 2.2.1** for instructions on how best to prepare samples for this assay.

Table 2.2.2.1: Common fungal pathogens

Species	Stage mortality is commonly observed in	Notes
Culicinomyces	larval	
Lagenidium	larval	Common laboratory pathogen
Leptolegnia	larval	Affects early instars
Pythium	larval	Opportunistic pathogen/affects early instars
Saprolegnia	larval	Appears as a "cottony" film on host[1]
Conidiobolus	adult	Opportunistic pathogen
Entomophthora	adult	Predominantly transmitted from adult to adult
Smittium	adult	Found mainly in the digestive tract

Adult Infections

Smittium spp.

Adapted from AM Rizzo and KL Pang (2005)

Although previous research has shown that some *Smittium* cultures are lethal to mosquito larvae (Sweeney 1981), the current belief is that many members of this genus are actually endocommensal to aquatic invertebrates (Gottleib and Lichtwardt 2001). Regardless of their pathogenic status, these fungi typically establish themselves in the abdomen and could interfere with digestion or reproduction. This PCR assay is designed to specifically amplify a region within the small subunit (SSU) rRNA gene for *Smittium* spp. (Rizzo et al 2005).

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[1] A "cottony" appearance can also be indicative of a microsporidian infection (see **Section 2.1**)

Table 2.2.2.2: Prepare PCR Master Mix for 1 - 25µl PCR reaction. Add reagents in the order presented.

1	Reagent
15.6 µl	Distilled H2O
5.0 µl	5X PCR buffer
1.0 µl	dNTP (2 mM concentration)
1.0 µl	TR3F (10 pmol/µl) [GGC ACT GTC AGT GGT GAA ATA C]
1.0 µl	TR4R (10 pmol/µl) [GAT TTC TCT TAC GGT GCC AAG CA]
0.3 µl	$MgCl_2$ (25 mM)
0.1 µl	Taq DNA polymerase (5 U/ µl)
24 µl	**Total (To each 24 µl reaction add 1 µl template DNA)**

PCR cycle conditions
94°C - 2min x 1 cycle
(94°C – 1min , 55°C – 1.5m , 72°C – 1.5m) x 35 cycles
72°C - 10min x 1 cycle
4°C hold

Run samples on a 1.5% agarose EtBr gel. Positive samples will yield an approximately 200bp band.
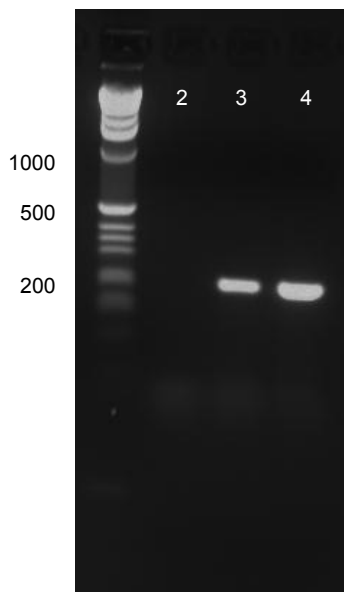
Figure 2.2.2.1 (left). Lane 1 1kb ladder, lane 2, gDNA from *Anopheles gambiae* G3 colony (MRA-112), Lane 3, DNA extracted from *Smittium culicis*, Lane 4 DNA extracted from four mosquito abdomens infected with *Smittium culicis*.

Figure 2.2.2.2 (below). Lanes 1&14 1kb ladder, lanes 2-5, DNA extracted from mosquito abdomens spiked with fungi, lanes 6-9, DNA extracted from G3 (MRA-112), lanes 10-13, DNA extracted from *L. giganteum.*

Larval Infections

Lagenidium, Saprolegnia, Leptolegnia, and *Pythium*

Adapted from Peterson and Rosendahl (2000) and Daniel Usry (MR4)

Although *Pythium, Saprolegnia* and *Leptolegnia* infections are rare in the insectary, *Lagenidium giganteum* infected larvae are often encountered in the field and could represent a route of contamination into an insectary. Due to the need to form zoospores to become infective, these would present as short term infections that might resolve themselves without intervention. This assay is designed to amplify a portion the large ribosomal subunit (LSU rDNA) from various *Oomycetes* (Peterson and Rosendahl 2000). Due to cross binding between LSU-0025 and *An. gambiae* gDNA, a novel forward primer was designed and is listed in the table below.

Table 2.2.2.3: Prepare PCR Master Mix for 1 - 50µl PCR reaction. Add reagents in the order presented.

1	Reagent
9.9 µl	Distilled H_2O
5.0 µl	5X PCR Buffer
2.0 µl	dNTP (2 mM concentration)
1.0 µl	LSUCDCF (10 pmol/ul) [GAA CTT TGA AAA GAG AGT TAA AGA]
1.0 µl	LSU-1170R (10 pmol/ul) [GCT ATC CTG AGG GAA ATT TCG G]
2.0 µl	MgCl2 (25 mM)
0.13 µl	Taq DNA polymerase (5 U/ µl)
21 µl	**Total (to each 21 µl reaction, add 1µl of extracted DNA)**

* Primers are originally from van der Auwera et al 1994.

PCR cycle conditions
96°C - 1min x 1 cycle
(95°C – 1min , 59°C – 1min , 72°C – 2min) x 30 cycles
72°C - 7min x 1 cycle
12°C hold

Run samples on a 1.5% agarose EtBr gel. The various species will have differing sizes of bands ranging from 800-1000 bp depending on the species (**Figure 2.2.2.2**).

96 well PCR sample preparation template

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2.3 Modifying Fecundity, Longevity and Size

Paul Howell and Liz Wilkins

Introduction

Maintaining proper nutrition throughout larval development will have positive effects for the entirety of a mosquito's life. Optimization of nutrition, photoperiod, competition, and temperature will result in healthier larvae and a more productive colony. If any of these is suboptimal, smaller and generally less vigorous mosquitoes will result. Large size has been often associated with increased fecundity and longevity in many different species (Blackmore and Lord 2000, Briegel 1990, and Takken et al. 1998). Here we present some general information to assist in establishing rearing protocols for anopheline mosquitoes.

Larval Diet and Nutrition

Increased fecundity and longevity result partly from reserves accumulated during immature stages. Several factors can reduce these reserves and result in poor quality adults. Some studies indicate that diets high in protein are superior in producing larger, more fecund mosquitoes. Increases in egg clutch size have been positively associated with high protein diets (Akoh et al. 1992, Lang 1978). Conversely, sub-optimal diets resulted in the production of smaller adults which were less likely to seek out a blood meal (Klowden et al. 1988). A high protein diet also reduced immature development times in *Toxorhynchites splendens* (Amalraj et al. 2005). Therefore the quality of the diet can have significant effects on colony production and growth.

Not only is the quality of diet fed to larvae important, but the quantity is also significant. Reisen et al. (1984) illustrated the relationship between the concentration of food and larval development to pupation. Overfeeding larvae will often lead to high larval mortality (Reisen 1975). However, the surviving larvae will develop rapidly and result in large adults (Lillie and Nakasone 1982; Reisen et al. 1984). Conversely, underfed larvae will result in the production of smaller adults. In *Cx. pipiens fatigans*, underfeeding leads to a reduction in the number of ovarian follicles (Ikeshoji 1965; Arrivillaga and Barrera 2004). Therefore, measured amounts of a larval diet should be supplied to ensure optimal growth and fecundity.

Dietary Restriction (DR) has been studied for its effect on longevity extensively in flies and mice. Though the results are not completely in agreement, the general conclusions are compatible. By and large, in animals and insects, one of the most successful ways to extend longevity is through DR (Burger and Promislow 2004). For many species of animals, restricting diets yields animals that are leaner and have increased longevity (Hopkin 2003). These same animals, however, are typically less fertile and become infected or sick more easily. DR animals are much slower than their better-fed counterparts (Hopkin 2003). DR-affected animals live longer but show signs of age much more quickly (Hopkin 2003; Burger and Promislow 2004). Longevity benefits are easily reversed with the introduction of any stressors to the environment as the DR animals are extraordinarily susceptible, especially to infection and sickness. In those cases, the better nourished animals will have the greater longevity (Hopkin 2003). A balanced diet at the beginning of life has the greatest benefit on overall lifetime longevity (Rasnitsyn and Yasyukevich 1988; McCay et al. 1989).

In flies, DR increased the life span of all females including fertile or sterile flies. Dietary manipulation generally has a greater and longer effect on females than males, though in a few studies, the opposite is true (Burger and Promislow 2004).

Alternately, it has also been reported that in some animals, the quality of the diet is the more important factor in increased growth and longevity than the amount (McCay et al. 1989). Most generally, in the wild, reproduction and speed are the most important elements for survival, and both are severely hindered by DR (Hopkin 2003). So, for a wild animal or insect, the best bet for reproduction and evasion of predators is to be big and fat (Hopkin 2003)!

Some dietary supplementation can be beneficial in a few insect genera. Folic acid supplementation increased egg production in the screwworm *Cochliomyia hominivorax*, even in the presence of a reducing agent (Crystal 1964). The addition of nordihydroguariaretic acid (NDGA) led to increased longevity in adult *Ae. aegypti* mosquitoes (Richie et al. 1986). The MR4 staff has observed significant increases in longevity by addition of methylparaben to the sugar meal, though the effect is probably one of reducing microbial growth.

Larval Density

In addition to larval diet, the density at which larvae are cultured will affect the size and quality of adult mosquitoes. Overcrowded larvae often become smaller, short lived adults. *Ae. sierrensis* larvae reared under high densities resulted in the production of small adults that had reduced life spans when compared to a field population (Hawley 1985). Similar results have been reported in *Cx. tarsalis* (Reisen et al. 1984), *Wyeomyia smithii* (Bradshaw and Holzapfel 1992) *Ae. aegypti* (Bedhomme et al. 2003), and *An. stephensi* (Reisen 1975). *An. gambiae* larvae reared in crowded conditions resulted in increased larval development times and small adult body size; however, no appreciable effect on longevity was noted (Takken et al. 1998; Gimnig et al. 2002). This effect was also seen in *An. arabiensis*, but the size-limiting effects of crowding could be overcome by adding maize pollen to the rearing water (Ye-Ebiyo et al. 2003). When *An. gambiae* was reared in the same container with *An. arabiensis* at high densities, the effects of overcrowding were not seen in *An. gambiae.* An associated reduction in lifespan of *An. arabiensis* was noted (Schneider et al. 2000).

Increased larval density has also been linked to sex-specific larval mortality. In *Cx. quinquefasciatus* and *An. stephensi*, larvae reared at high densities suffered excess male mortality (Reisen 1975; Suleman 1982). It has been hypothesized by one of the authors that this skewed sex ratio may be due to greater male susceptibility to stressors than females due to their smaller size and reserves (Reisen 1975).

Overcrowding also results in increased larval development times. In *Cx. tarsalis*, overcrowding increased development times from 10 days to 14 days (Reisen et al. 1984); in *An. gambiae,* overcrowding resulted in an average of an additional 1-2 days (Gimnig et al. 2002).

Finally, overcrowding affects the fecundity of females. In *Wy. smithii*, lifetime fecundity can be linked to pupal mass whereby larger females produced more eggs over a lifetime than smaller ones (Bradshaw and Holzapfel 1992). Similarly, in *An. stephensi,* it was found that females originating from crowded pans produced fewer eggs than those from less crowded environments (Reisen 1975). Therefore, rearing animals at suitable densities e.g. 1 L4 per 1-2 ml of water, will result in long lived and more fecund mosquitoes than if reared at sub-optimal conditions.

Temperature

Although not as obvious as nutrition and larval density, ambient temperature can dramatically effect mosquito production. Most mosquito larvae are reared around 25-27°C which is similar to the environment from which they are isolated. High temperatures can be lethal. In *An. albitarsis* and *An. aquasalis*, colder temperatures delayed embryo eclosion by 2 days or more (de Carvalho et al. 2002). Additionally, there was a reported reduction in the hatch rate of *An. albitarsis* when reared at 21°C. In *Ae. albopictus*, larvae reared at 26°C pupated faster than those reared at 22°C; however, fewer larvae completed ecdysis at the higher temperature (Alto and Juliano 2001). This trend has also been reported in *Cx. tarsalis* (Reisen et al. 1984) and *An. sergentii* (Beier et al. 1987). In addition to reduced ecdysis rates, adults from temperature stressed environments can have reduced longevity. In *An. gambiae* (Afrane et al. 2006), *An. superpictus* (Ayetkin et al. 2009) and *Cx. tarsalis* (Reisen et al. 1984), adults derived from heat stressed larval regimens were found to have life spans reduced by several days. Interestingly, in *Ae. dorsalis*, mosquitoes from a high temperature regimen had a reduced number of ovarian follicles (Parker 1982) while *An. gambiae* mosquitoes derived from the same environment had increased fecundity when compared to adults from a lower temperature region (Afrane et al. 2006). Finally, as temperatures increased, it was found that both the larval head capsule size and adult wing size decreased in *An. merus* (le Sueur and Sharp 1991). Wing size and shape were also affected by increased temperatures in *An. superpictus* (Ayetkin et al. 2009). Therefore careful maintenance and

monitoring of the correct temperature in the rearing environment is necessary in order to produce consistent-sized mosquitoes.

Photoperiod

Photoperiod is defined as the amount and schedule of light versus darkness. Even less studied than temperature, the effect of photoperiod can dramatically affect longevity in adult mosquitoes. Many insectaries operate on a 12 hour day/night cycle since it is convenient for workers and similar to average conditions. *Cx. pipiens* reared under a short photoperiod had smaller ovarian follicles than those reared under a long photoperiod (Oda and Nuorteva 1987). This was attributed by the author to the mosquitoes preparing for diapause, which can be induced by shortening the photoperiod.

Reducing the photoperiod has also been found to be positively associated with an increased lifespan in *Anopheles.* In both *An. crucians* and *An. quadrimaculatus*, larvae reared under a short photoperiod regimen produced adults that lived longer than those reared under a long photoperiod (defined as greater than 15 hours of daylight) (Lanciani 1993; Lanciani and Anderson 1993).

The length of photoperiod chosen, therefore, should reflect a balance between what is convenient for the insectary staff and what is necessary to maintain a healthy colony.

Adult diet

Though not as critical as other effectors mentioned above, adult diet can also have an affect on the longevity and fecundity of colonized vectors (reviewed in Foster 1995). *Ae. aegypti* fed sucrose supplemented with nodihydroguariaretic acid lived longer than those reared on sucrose alone (Richie et al. 1986). *An. gambiae* mosquitoes fed sucrose plus blood lived longer than those fed just sucrose or blood alone. However, mosquitoes given only blood were more fecund than those fed sucrose and blood (Gary and Foster 2001). *An. gambiae* fed sucrose supplemented with 0.2% w/v methylparaben lived longer than controls fed on sucrose alone (Benedict et al. 2009). Supplementation with uric and ascorbic acids lead to an overall increase in fecundity over the lifetime of a female in *An. gambiae* (DeJong et al 2007). In general, however, sucrose solutions are suitable for regular maintenance of laboratory colonies and care should be taken before adding supplements to the adult diet.

The rearing environment, therefore, can have dramatic effects on laboratory colonies. Subtle changes in any of the above mentioned areas could result in declining fecundity or longevity and lead to colony collapse. Optimization of some of these areas could result in a healthier colony however. It is suggested that testing should be done on a sample population before implementation of any changes as results may vary between species, colonies and laboratories.

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2.4 Anopheles *Culture*

2.4.1 Collecting *Anopheles* Eggs

MR4 Staff

Introduction

For colony maintenance, we blood feed females only once in their lifetime, between 3 and 7 days post-emergence (see Chapter 1, Rearing Schedule). After subsequent feedings, anophelines will lay eggs again, which is helpful for maintaining a colony and having plenty of experimental material. After bloodfeeding, allow 2-3 days for embryo development prior to egging. Certain species may take more time (*An. dirus* - 4 days), so you may have to modify your schedule according to the needs of your particular colony.

Among the water types we have used, we have observed no effect on oviposition. While there are almost certainly measurable differences that would be important in mass rearing facilities, an excess of eggs beyond what is required for stock keeping is almost always obtained, and efficiency is not an issue.

Most eggs are laid at night, and egg dishes are typically removed the day following their insertion. There are several ways to collect eggs. One method is to fill a cup with clean water to about 1 cm depth and line the edges with filter paper such that half of the paper is submerged (**Figure 2.4.1.1**). The filter paper prevents the eggs from sticking to the dry plastic sides of the cup when it is sloshed.

Other methods include allowing mosquitoes to lay eggs on wet filter paper supported by wet cotton or sponges which are then floated in water the next day, filter paper funnels partially submerged in water, and dark bowls lined with filter paper (**Figure 2.4.1.2-Figure 2.4.1.4**).

Regardless of the method used, after collecting eggs, remove all dead adults to help prevent carrying infections into the new larva pans. Adults can be removed individually with forceps or filtered out by washing the egg/adult mix through a screen and collecting the eggs beneath.

Figure 2.4.1.1. Egg collection cup containing standing water to a depth that is approximately half way up the filter paper liner.
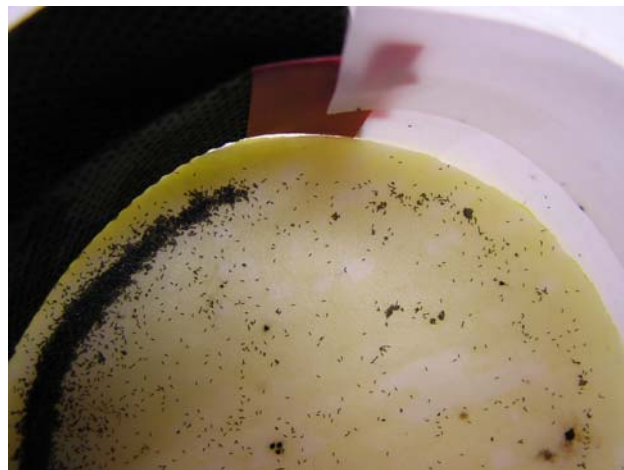
Figure 2.4.1.2. Filter paper on a damp sponge in a plastic dish. The sides are lined with either white or black paper, and it is apparent that *An. gambiae* and *arabiensis* prefer resting on the latter for oviposition.

Figure 2.4.1.3. Filter paper funnel partially submerged in water.

Figure 2.4.1.4. Dark bowl lined with filter paper.

2.4.2 Bleaching *Anopheles* Eggs

MR4 Staff

Introduction

Many laboratories find that surface-cleansing of eggs with bleach is a helpful routine procedure to minimize the growth of microsporidians (Alger and Undeen 1970; Robert et al. 1993). Mortality due to these pathogens is sometimes observed only after several generations of culture without surface cleansing. The following method describes a method to 'surface sterilize' eggs and also allows for concentrating eggs on a filter paper disk. This only works well if eggs are collected in water or on a surface from which they are easily removed.

Materials
- Filtration device (A Buchner funnel is an option, but take care to clean carefully if used for several stocks since eggs will leak around the edge of the paper). **Figure 2.4.2.1** shows an example of a Nalgene 500 ml disposable filter modified by carefully breaking off the upper chamber and removing the membrane. It can be reused indefinitely.
- 1% v/v household bleach in wash bottle
- Sterile water in wash bottle
- A vacuum source equipped with trap

Protocol
1. Remove adults from surface by filtering through screen or with forceps.
2. Center filter paper on platform with vacuum applied.
3. Lightly wet the filter paper with sterile water and ensure that the filter paper is held securely by the vacuum.
4. Slowly pour water/eggs onto the center of the disk so that eggs do not spill outside of the depression (**Figure 2.4.2.2**).
5. Remove vacuum.
6. Add the bleach solution to the entire depression for up to 1 minute. Remove bleach by applying vacuum.
7. Add water until depression is full. Apply vacuum.
8. Wash with water two times. Apply vacuum until enough surface water remains to keep eggs moist without allowing them to run off the sides of the paper. Eggs can typically be stored in a humid container on filter paper for up to 24 hours before hatching.

Figure 2.4.2.1 Modified chamber attached to vacuum.

Figure 2.4.2.2 Pour eggs gently into the center of the filtration device. Use bleach solution to remove final eggs from the sides of the container.

References

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2.4.3 Hatching *Anopheles* Eggs

MR4 Staff

Introduction

Anopheles eggs are usually hatched as soon as they are mature – normally about two days after they are laid. Methods to dry and store *Anopheles* eggs for a couple of weeks have been developed (Bailey et al. 1979), but these are useful primarily in larger production settings. Eggs are usually hatched at a high density and given a diet consisting of small particles such as live baker's or brewer's yeast. While excessive crowding can stunt their growth irreversibly, culture at too low a density often results in excessive microbial growth and larval death.

This protocol starts with eggs that have been collected and possibly bleached and are sufficiently mature to hatch immediately. See the sections on Collecting (**2.4.1**) and Bleaching *Anopheles* Eggs (**2.4.2**) for more information.

Materials

- ▪ 2 % w/v active (live) baker's or brewer's yeast in purified water. Mix and use only that day.
- ▪ water wash bottle
- ▪ mosquito tray
- ▪ 5 ml pipet
- ▪ 50-100 ml bottle
- ▪ mosquito culture water

Procedure

1. For each egg batch, add deionized water to a rearing pan so that water just completely covers the bottom of the pan. Add more if your trays will be uncovered (not recommended). Avoid adding too much water as this will increase larval mortality (Timmermann and Briegel 1993).

2. Add a yeast suspension to each pan to a final concentration of 0.02% (**Figure 2.4.3.1**). For example, in the pan shown in **Figure 2.4.3.1**, we add 300 mL of water and 2 mL of a 2% w/v yeast solution. The pan shown will rear approximately 300 L4s at a nice density. Swirl until the yeast has dispersed throughout the pan. Place the pan on the shelf or rack where it will be kept.

3. Hold the egging paper by the edge to avoid touching the eggs (**Figure 2.4.3.2)** and gently rinse the eggs into the pan (**Figure 2.4.3.3**). Ensure that no eggs stick to your fingers as they can easily be accidentally transferred to the next pan. Be careful that eggs do not splash into an adjacent pan or onto the water bottle. After rinsing the eggs into the pan and before rinsing the next strain, clean your fingers using a paper towel. Be careful not to disturb or move the pan at all after adding the eggs. Disturbing the pan can cause the eggs to stick to the sides of the pan above the water where they will dry and not hatch. If you accidentally jar the pan, gently rinse the eggs down from the side of the pan into the rearing water.

4. Make sure the pans are clearly labeled with strain name and date of hatching.

5. Cover the pan to prevent contamination that could occur from accidental splashing when rinsing eggs into an adjacent pan and oviposition by loose females that can contaminate the stock.

6. *Anopheles* eggs will generally hatch immediately or within 24-48 hours after placement in water. On the day after placing the eggs in water, without disturbing the pans, gently uncover and scan to see if there are L1s present. They are very small and hard to see in many genera (**Figure 2.4.3.4)**. If you do not see any L1 larvae, carry the pan into a well lit area and check again. For more on screening hatching of individual eggs microscopically, see Chapter 3.

7. Allow one day between placing the eggs in water and splitting or thinning.

Figure 2.4.3.1. Add yeast slurry to pan with just enough water to cover the bottom.

Figure 2.4.3.2. Hold egg paper in such a way that your fingers do not touch the eggs.

Figure 2.4.3.3. Gently rinse eggs into pan. Cover and do not disturb for 24 hours.
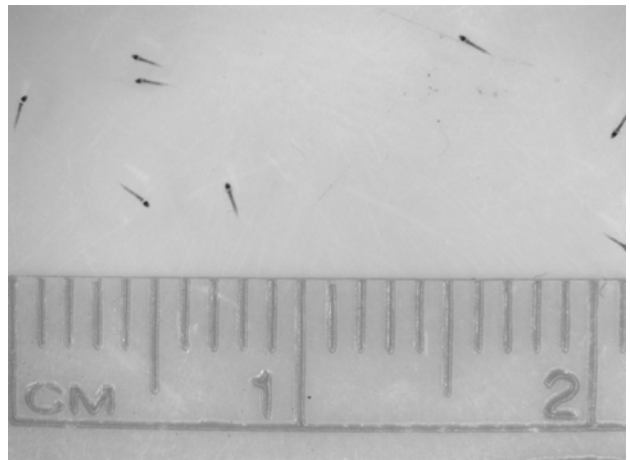
Figure 2.4.3.4. 24 hours after rinsing eggs into tray, check for hatch (stock G3 *An. gambiae* sensu stricto shown).

Notes

Many labs line the trays with a strip of filter paper. This prevents the eggs from becoming stranded and drying out if the water is sloshed. This technique is useful if you are adding the eggs to the tray and then moving it to the shelf where the eggs will hatch. When using this method, discard the paper when hatching is complete.

An efficient method for food preparation is to pre-package 1 g of yeast in 50 ml disposable Falcon tubes and store them at -20°C until needed. Simply remove as many tubes as are needed that day, mix with water, and use immediately.

References

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2.4.4 Determining Egg Hatch Rates

MR4 Staff

Introduction

Egg hatching rates vary between stocks depending on intrinsic fertilization rates, semisterility due to crossing type or presence of chromosomal aberrations and the methods used to handle eggs after oviposition. Egg hatch rates >80% are typical. Counting hatched larvae is not a proxy for determining the hatch rate as mortality may occur in the L1 stage and these larvae are very difficult to detect. Typically, anopheline eggs that sink to the bottom of the pan do not hatch; however, they should be inspected and included in hatch rate data.

Unhatched eggs fall into several classes which may be of interest to record: (1) Unmelanised eggs are often observed but will inevitably fail to hatch; (2) Unhatched, melanised eggs in which no indication of an embryo can be seen; (3) Unhatched, melanised eggs in which a developing embryo is seen but never hatches; (4) Unhatched, melanised eggs in which an embryo is alive but has not hatched.

The last category is problematic. In some species, hatch is very synchronous, but in others – *An. gambiae* – it occurs over several days even when eggs are moist (Lehmann et al. 2006). We have often observed egg batches in which most eggs have hatched a day earlier, but the activity of counting the eggs stimulates further hatch. These various types and timings of hatching should be taken into account when collecting and interpreting hatching data.

Materials

- Filter paper
- Wash bottle containing water
- Fine probes

Equipment

- Stereoscope
- 2-place denominator (counter)

Determining hatch rates for eggs collected from individual females

1. Assuming eggs have been collected in tubes, these should have been lined previously with filter paper (See **Chapter 3.9**). If the eggs are not all at the edges, carefully touch the center of the water. The oil on your fingers will usually cause the eggs to move to the side. If not, tease them to the edge with a probe.

Figure 2.4.4.1. An unhatched (left) and hatched egg (right) from the same female, laid on the same day. It is apparent the lower one has hatched because of the dislocated operculum. Un-melanised eggs do not hatch.

2. Very slowly and smoothly slide the papers up the side of the tube and transfer to a rigid, movable, flat surface. A small piece of Plexiglass (10 X 20 cM) is ideal for this.

3. Count the eggs *in situ* under a stereoscope recording hatched vs. unhatched on the denominator. You may need to prod or burst the egg with a probe to determine whether it is hatched. Typically the operculum of hatched eggs will be dislodged somewhat (**Figure 2.4.4.1**). If only a sample of eggs is needed for rate information, counting 50 eggs is sufficient. Otherwise, count all the eggs.

Determining hatch rates for *en masse* egg collections

In this case, the container from which eggs are taken may or may not be lined with filter paper. If it is, skip to step 2.

1. Slide a small piece of filter paper (approx 2 X 6 cm) down the side of the tray above the eggs to be collected. When the paper begins to wet, slowly slide it behind the eggs until the paper is well-submerged.

2. Slowly slide the paper on which the eggs are resting up the side of the tray until it is above the water. The eggs should adhere to the paper as it is raised.

3. Transfer the paper to the counting board as described above and count.

References

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2.4.5 Estimating the Number of Eggs and Larvae

MR4 Staff

Introduction

In order to maintain mosquito colonies, a sufficient number of individuals most be produced within a specific time. The most efficient way to accomplish this is to consistently rear a fixed number of larvae per tray and volume of water and diet. This prevents waste of resources, creates healthier and more fecund colonies, and you can reliably predict when you will have material available for experiments. Crowding the early stages in anopheline larvae is detrimental and should be avoided in most species (exceptions include *An. quadrimaculatus* and *An. freeborni* which seem to require higher densities in the early instars). If you cannot consistently estimate the appropriate number of larvae, to prevent over-crowding in the early instars you can either (1) measure the number of eggs or (2) the number of larvae per pan (Dame et al. 1978).

In the following sections, we suggest two general approaches by which repeatable numbers of larvae can be placed in trays.

Estimating the number of eggs

- If one is culturing large amounts of a single stock and the egg hatching rate is consistent, volumetric estimation of the number of eggs to place in each tray is a feasible approach. Since larvae are more susceptible to damage when handled individually in the early stages, Dame et al. (1978) suggested that this is the best method. They collected *An. albimanus* eggs, bleached them, (see Bleaching *Anopheles* Eggs, **Chapter 2.4.2**) and immediately floated them on 28°C water for 30 hours. Afterward, the eggs were strained through a screened bottom cup (100 micron pore size) and allowed to dry by drawing air over the eggs at a rate of 600-700m/sec for 30 minutes (Dame et al. 1978). Eggs were then measured into a severed 1ml disposable pipette for dispensing. It was reported in *An. albimanus* that .085ml of eggs was equal to 5000-6000 eggs.

- For implementation, one would choose some small volume container into which semi-dry eggs would be placed using a fine paint brush. After filling a predetermined volume, the eggs would be removed and counted. An average and standard deviation could be determined. Use of glassine weighing papers and methods to reduce static electricity will improve this method.

- Depending on the sophistication and needs of the system, one could also devise photographic and image analysis methods to count eggs in a monolayer on paper. However, this would not result in specific amounts for dispensing as the above method does.

Estimating the number of larvae

If one is working with numerous stocks, it is difficult to dry and measure eggs. Hence it is more common to dispense a known number of larvae into rearing pans for culturing. Use L2s as they are less delicate than L1s.

- The simplest yet most time-consuming and accurate method is to individually count larvae into the rearing pan. Using a pipette, aspirate and count several larvae and place the drop in the bottom of the pan until the desired number has been reached (e.g. 300 larvae per tray).

- A faster but less accurate method is to use a photographic scale (Rutledge et al. 1976). Place an unknown number of larvae into a Petri dish. Place the dish near the photographs of counted larvae at the same stage (**Figure 2.4.5.1**) and determine which one contains a similar number of larvae. Adjust the number of larvae until the desired density is achieved.

100

200

300

400

500
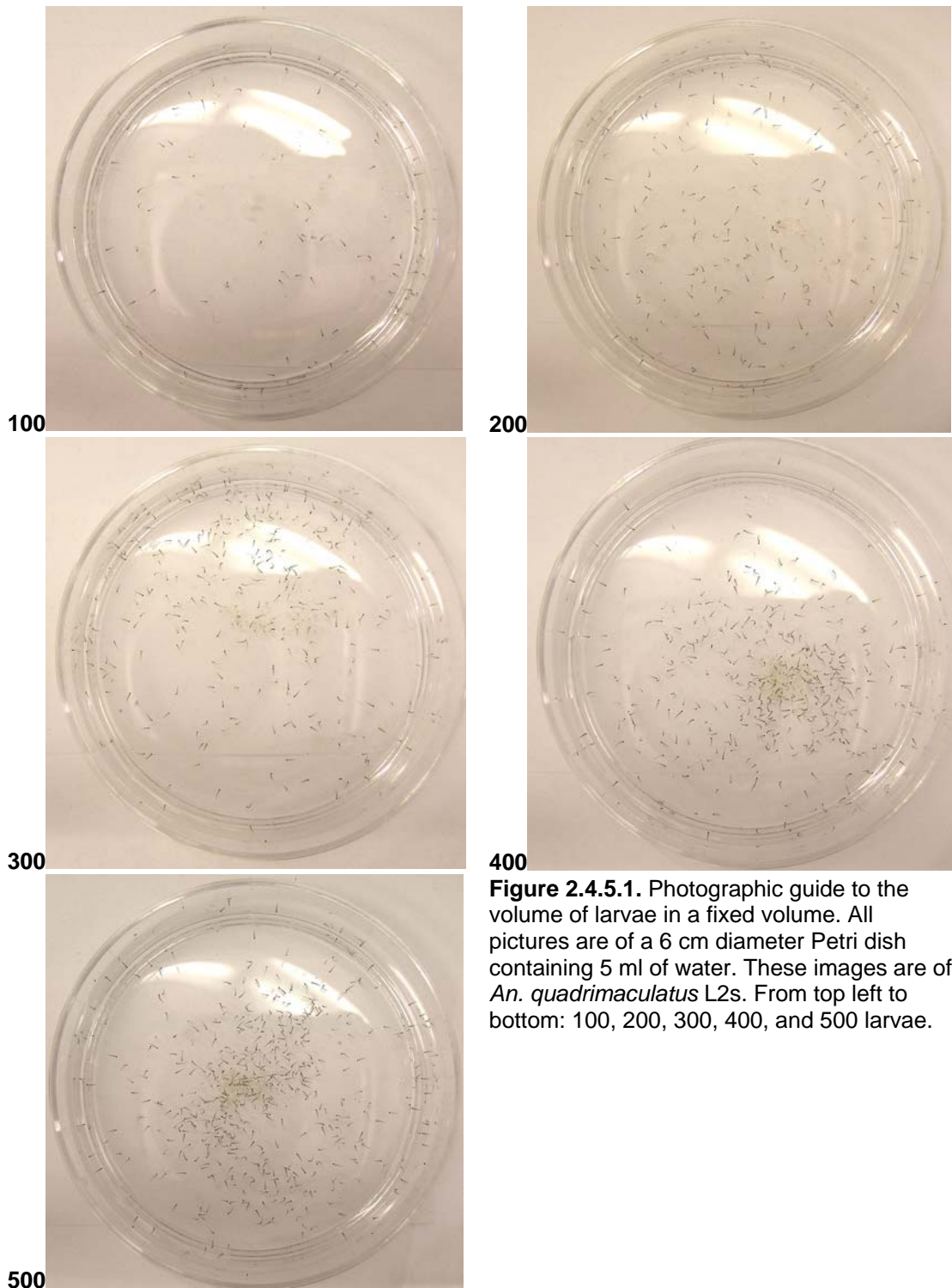
Figure 2.4.5.1. Photographic guide to the volume of larvae in a fixed volume. All pictures are of a 6 cm diameter Petri dish containing 5 ml of water. These images are of *An. quadrimaculatus* L2s. From top left to bottom: 100, 200, 300, 400, and 500 larvae.

- More recently, a graphical display was developed for culicid larvae, which is similar in nature to the photographic method above. A dish of larvae is compared to the pictograph from which the density is estimated (Carron et al. 2003).

- Finally, one could pour the concentrated larvae into a known volume of water and mix them until they were uniformly dispersed (Gerberg et al. 1968, 1994). From the known volume 20-25 1ml aliquots are removed so as to create an estimate of larvae per ml from which aliquots can be dispensed into new rearing pans.

References

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2.4.6 *Anopheles* Larval Culture

MR4 Staff

Introduction

Except in rare cases, mosquito larval culture is septic, and the diet consists of both added food and the microbial growth that results. Reliable diets probably provide good nutrition directly and also promote a microbial environment that nourishes larvae. The relationship between microbial growth, larval consumption, water volume and larval density is complex, but the following recommendations are typical for *Anopheles* culture.

Studies have shown that above a weight threshold, the number of eggs laid per female is in fairly direct proportion to their adult weight. Adult size is ultimately determined by larval size, and that on larval culture. So, it is logical that larger larvae yield higher numbers of eggs per female – a desirable outcome in most laboratory cultures.

Consistent use of successful practices developed and proven in each lab should be adhered to faithfully.

Culture water and the environment

When larval culture fails, laboratorians often suspect something is wrong with the water. In spite of this concern, many different sources of water have been used successfully - in combination with good rearing techniques: chlorinated/fluoridated municipal supply, deionized, untreated deep well, and distilled water. Water source is probably not a critical factor for most species unless it contains high levels of toxic chemicals. If in doubt, try changing to a more purified form to see if conditions improve. The consistency of the source should be considered in making the choice as this will determine long-term success.

Mosquito rearing water is a rich environment in which to grow microbes. Therefore, 'more microbes in, even more microbes out' is a good way to think of larval culture. Use of almost any treated water will reduce microorganisms relative to natural sources. However, some chemical treatment methods used in municipal supplies may not be compatible with larval mosquitoes. Chloramine is reportedly of particular concern for invertebrate culture. The rule of thumb is to test the water source with a small number of larvae before using it on a larger scale. If you are concerned about chlorine residues, water from a hot water source that is allowed to cool, or chlorinated water that is allowed to sit overnight should be adequate. For critical applications where physiologically uniform water is required, we use reverse/osmosis deionized UV sterilized water to which we add 0.3 g / liter artificial sea salts such as Instant Ocean™.

A water temperature of 27°C is suitable for rearing most anophelines. The room or incubator temperature should be adjusted to achieve this depending on whether the trays are covered or not. Uncovered trays will have a water temperature significantly below the air temperature due to evaporative cooling. Trays placed higher in a room will often be at temperatures significantly above that of lower ones.

Diets and preparation

Diet can be provided as a water slurry or dry powder. The former offers ease of measuring while the latter is simple and places the diet – at least initially - at the surface where much feeding occurs. Any powdered food can be provided either way, but if a slurry is fed, ensure that it is well suspended before each feeding.

0.02% w/v baker's or brewer's yeast (final concentration) is perfect for the day of hatching and 48 hours post-hatching (see Hatching *Anopheles* Eggs, Chapter 2). These are often inexpensive and readily available. Their high nutritional content and size make them a good choice.

For later stages, switch to either a powdered larval food or a slurry consisting of larger particles. Different types of food may superficially appear suitable for larval feeding, but to ensure high quality, measure survival from egg hatch to eclosion at least once with any new food source before routine use. Larval culture affects adult longevity and fecundity in the long term (see Modifying Fecundity, Longevity, and Size), so using the best larval food available will save you time in the future.

One well-tested larval food is finely ground Koi Staple Diet from Drs. Foster and Smith, though TetraMin flake food is widely used alternative. For large scale production where cost may be a greater consideration, inexpensive and readily available diets such as Farex baby food, hog chow, dog chow, and liver powder have also been used. Since *Anopheles* feed primarily at the water surface (see Behavior and Physiology of Anophelines in the Laboratory), food that floats on the water surface is ideal. Grinding food and sifting for a small size can ensure the food will be of a small enough particle-size for the larvae to ingest.

Koi pellets and similar pellet and flake diets can be prepared in a grinding mill or blender and sifted through a 250 micron sieve (**Figure 2.4.6.1**). When fed as a powder, this will remain temporarily on the surface. Such finely ground food is suitable for feeding from L2 to pupation. If you sift food, the larger particles of food that did not go through the sieve can be saved for feeding L4s or more tolerant strains such as *Aedes*. Larval food should be stored at -20°C until ready for use if possible to prevent microbial growth.

When feeding, disperse dry food evenly across the top of the water (**Figure 2.4.6.2**). It may be dispensed from a salt shaker, some other simple improvised device or shaken from a tiny weighing spoon.

Figure 2.4.6.1. Preparing food. Use a general laboratory grinder (pictured here), a grinding mill or a household coffee grinder. To ensure food is small enough for the earliest stages, use a sieve with holes no larger than 250 microns in size.

Figure 2.4.6.2. Check pans daily and assess for feeding and density. Adjust the density by splitting or thinning to about 200-300 larvae in a typical tray.

Density

It has been shown experimentally that eclosion rates diminish as density in the pan increases (Timmermann and Briegel 1993). High larval density has also found to distort sex ratios by favoring males over females in *An. stephensi* (Reisen and Emory 1977). Larval vigor irreversibly restricts adult health so attention to this factor cannot be over-emphasized. Larval crowding stresses larvae and is implicated in some larval infections.

A reasonable density for most L3-4 anophelines is 1 larva per ml with the water level 0.5-1 cm in depth. When it is impractical to estimate the exact density in the early (L1-2) stages, larvae are usually cultured at a high density. For this reason, the rearing schedule (Chapter 1) is designed for thinning progressively in stages. Some species such as *An. quadrimaculatus* and *An. freeborni* may culture more successfully at a higher density in the early instars than is typical for *An. gambiae*.

Feeding

At a constant temperature and given an appropriate amount of diet, the time from hatch to pupation should be predictable within one day from generation to generation. If pupation is delayed more than a day or two, any of the following could be responsible: temperature is too low, inadequate food was given at some stage, the density was too high, or excessive food was given in the early stages. Poor culture results in disparate developmental rates leading to pupation over the course of several days. This results in a great deal of extra work for the technicians. Ideally, almost all pupae form over 2 to 3 days.

Examine the pans daily (even when you do not feed) to ensure that the larvae are developing as expected and the density is appropriate. If you notice great differences in sizes of larvae between or within one tray of a cohort at any time, you likely have them too crowded and/or they are underfed. Not surprisingly, the amount of food provided daily must increase as the larvae develop and as their density increases. However, there is a limit to the amount of food and larvae you can place in one tray, so we recommend adhering initially to the density guidelines above and modifying only the amount of diet.

Underfed trays will contain larvae that die, are slow-growing or are variable in their development rate. In extreme cases, unusually long fecal pellets will be observed due, presumably, to re-ingestion of feces. L4s that are overcrowded and/or underfed will be small and have little fat body accumulation.

Overfeeding is common and is indicated by numerous observations that precede larval death.
1. *Foul smell*. If you smell a foul odor when you remove the cover, you're feeding too much. A healthy organic odor is normal. However, what is considered healthy is admittedly dependent on personal aesthetics!.
2. *Excessive turbidity*. Yellowish to greenish-colored water is fine and often appears in later stages of rearing (referred to as *gelbstoff*). However, if the water is turbid, feed less or not at all until the water clarifies. If turbidity persists, filtering the larvae out from the old culture water and at least a partial water change may be necessary. Greater turbidity is tolerable during the L3 and L4 stages whereas L1s and L2s are more sensitive. You will develop judgment regarding how much turbidity is appropriate.
3. *Excessive surfactants*. When the water in the pan is agitated, bubbles that form should burst rapidly. If they persist, bacterioneuston has formed an excessive surface microlayer which is not healthy for *Anopheles* larvae. Check for these by sloshing the water gently. Larvae exposed to water with high levels of surfactants often do not survive and re-feeding of the adult stock may be necessary. If bubbles persist, filtering the larvae out from the old culture water or dragging a tissue over the surface and at least a partial water change are recommended. If this is observed routinely, the culture conditions must be changed.

Overfeeding and microbes in the larval culture can cause irregular scum to form on the bottom of the trays. These pans should be scrubbed with a detergent, rinsed thoroughly and autoclaved or bleached. It is important to remember that in almost all cases, a thin layer will form on the bottom of the pan from waste materials and settled diet. It is a problem only when the layer becomes gelatinous or jelly-like in appearance, irregular in its reflectance, or contains bubbles. Change the container and reduce the feeding rate.

More signs of poor larval health related to density and feeding rates

The slowest -growing larvae and those cultured in turbid water often develop melanic nodules in the abdomen and thorax or black patches on the cuticle. These are both bad signs, and the individuals that have these should be discarded - they seldom survive. See the section on minimizing infections for photos of infected larvae. Sub-optimal larval rearing conditions can also result in missing setae or those that are covered with black film (probably fungus). This can often be observed in the slowest-growing larvae even under good conditions, but if the condition is prevalent, a change in your methods is warranted.

When food is overly abundant, *Vorticella* reproduce to such an extent that they cover the larvae and give them a fuzzy appearance. This is sometimes evident even without microscopic examination.

Pupae that are not curled into the typical 'comma' shape but have a horizontally extended abdomen will not emerge. If you observe this among the first-forming pupae, it may not be too late to rescue the remaining larvae by changing the culture conditions.

The metamorphic transitions are the most sensitive stages to the effects of poor larval health. This can be observed as failure of larva to pupate or pupae to emerge as adults. One should observe >95% of adults have emerged from pupa cups under good conditions. The effects of poor larval/pupal conditions are often evident in a short adult life span, and males are especially sensitive to this effect.

Day	Stage	Amount of diet
1	Hatching L1	60 mg yeast
2	L1/L2	No additional diet needed
3	L2	60 mg L3/4 diet
4	L2/3	No additional diet needed
5	L3	120 mg L3/4 diet
6	L3/4	120-300 mg L3/4 diet
7	L4	120-300 mg L3/4 diet
8	L4/pupae	120-300 mg L3/4 diet

Table 2.4.6.1. Approximate food amounts to be fed to the L1-L4 larvae per day assuming L3 and L4 densities of 1 larva/ml. The table assumes younger larvae are at a higher density – at least 2 X.
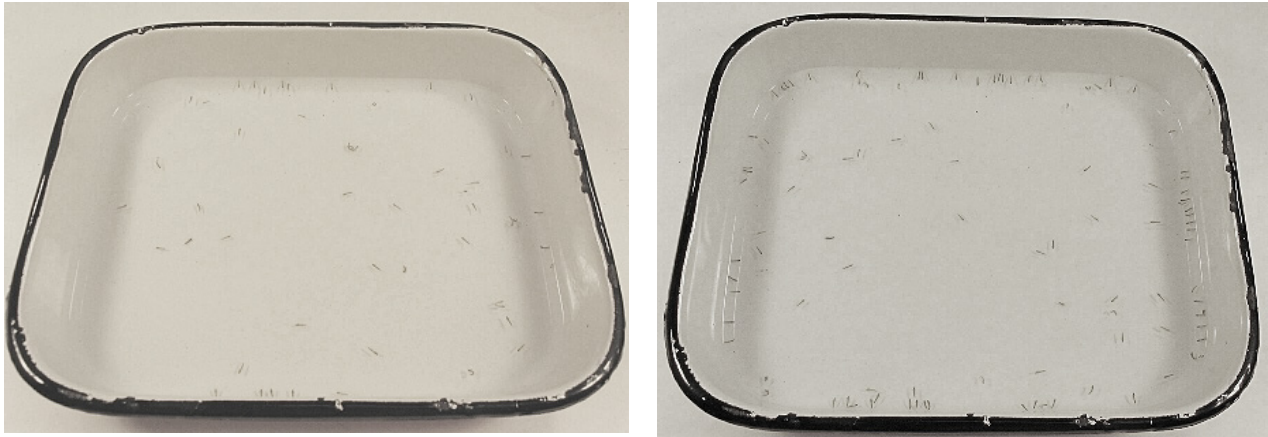
Figure 2.4.6.3. An example of a density reference photo of 50 (left) and 100 (right) L4 *An. albimanus.* Creating a photograph of the correct density in the pan size that you use may be helpful for technicians in routinely estimating a good density by eye.

References

Reisen WK, Emory RW (1977) Intraspecific competition in *Anopheles stephensi* (Diptera Culicidae) II. The effects of more crowded densities and the addition of antibiotics. Can. Ent. 109:1475-1480

Timmermann SE, Briegel H (1993) Water depth and larval density affect development and accumulation of reserves in laboratory populations of mosquitoes. Bull. Soc. Vector Ecol. 18:174-187

2.4.6.1 Standardized Larval Culture

Mark Q. Benedict & Jeremie R.L. Gilles

Introduction

Culturing anopheline larvae reliably is often problematic, due primarily to the difficulties in achieving a good balance between the number of larvae and the amount of food. The following is a method for culturing anopheline larvae from the L1 stage to pupa that does not require judgment regarding the number of larvae or the amount of diet to be provided. While we developed and tested this method most extensively on *An. arabiensis,* we have also used it on *Ae. albopictus* with good results. We typically obtained 80% L1 to pupa survival (Gilles et al 2011).

This method is not intended for routine or large-scale rearing because it is somewhat labor-intensive to initiate. However, when a staff member is new, the species is potentially difficult to culture, good conditions have not been determined or uniform quality mosquitoes are needed for experimental purposes, this is a reasonable approach.

Note that the diet we suggest is one that was developed specifically for *An. arabiensis.*(Damiens et al 2012). We have also tested it with *Ae. albopictus* and by the standards of survival and growth rate, it is very good for that species as well. However, we have also used the (now unavailable) Koi diet with similar results, so we suspect that the specific diet chosen is not critical. The MR4 has had similar rearing success using Koi Staple Diet from Drs. Foster and Smith as it did with the now unavailable diet previously mentioned. However, we do recommend grinding it as fine as is possible. Larger particles of food are slower to be digested.

We suggest two different culture conditions, either one of which you may want to continue using depending on the results.

Materials

- 90 mm polystyrene Petri dishes with lids
- dropper for transfer of larvae
- 2% w/v tuna/beef liver powder/vitamin larval diet in sterile water (store refrigerated and prepare only as much as can be used within two days; powder should be stored frozen)
- large white tray (or similar)
- incubator or room where dishes can be held at 27-28 °C
- P-1000 pipettor with tips (will likely be necessary to cut a bit of the ends off of the tips so that they do not clog)
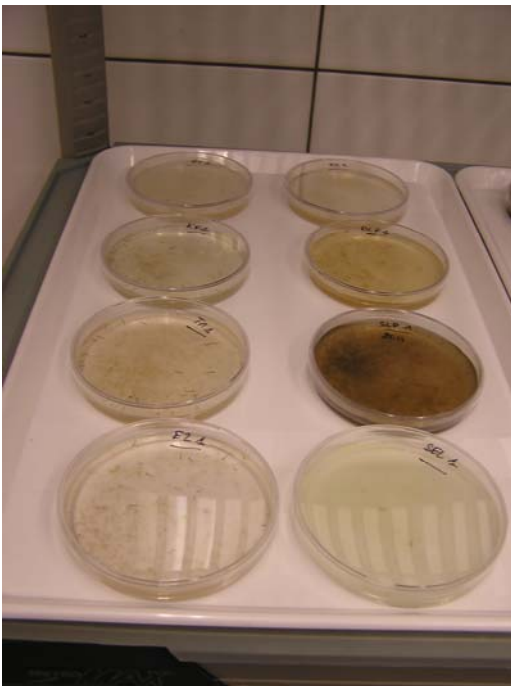
Figure 2.4.6.1.1 Trays of larvae set up as described here. While time is required to set them up, it can pay off when predictability is needed.

Procedure

1. After bleaching eggs (see MR4 protocol **2.4.2 Bleaching Anopheles Eggs**), hold on damp filter paper until the day of hatching.

2. On the day of hatching place eggs in 500 ml of water in a white plastic 9" x 12" pan. No food is necessary because they will not feed immediately after hatching.

3. As the larvae hatch (this will normally begin immediately), pipet and count larvae into Petri dishes. Placing the dish on a white background will allow you to count the larvae easily in the dish.

4. When the appropriate number has been placed in the dish, add food and fill the dish with water until it contains 30 ml. We recommend using the following two combinations of food and number of larvae: 640 µl of food and 16 or 32 larvae. You may wish to weigh the dish beforehand and determine the final amount of water based on this. Shake or vigorously stir the food between every pipetting to ensure the solids are evenly distributed.

5. Replace the Petri dish cover and place in incubator.[1]

6. Add 640 µl larval slurry every other day.[2]

7. Collect pupae as they form.

8. As pupae form, you can continue providing food at the same rate. Once it begins, pupation should be complete within 2-3 days and L4s are hardy, so the excess food should not have a harmful effect.

References

J. R. L. Gilles, R. S. Lees, S. M. Soliban, and M. Q. Benedict. 2011. Density-dependent effects in experimental larval populations of *Anopheles arabiensis* (Diptera: Culicidae) can be negative, neutral, or over compensatory depending on density and diet levels. *Journal of Medical Entomology*, 48: 296–304.

D. Damiens, M. Q. Benedict, M. Wille, and J. R. L. Gilles. 2012. An inexpensive and effective larval diet for *Anopheles arabiensis* (Diptera: Culicidae): Eat like a horse, a bird or a fish? *Journal of Medical Entomology*, in press.

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[1] We have observed that if transparent dishes are placed on a black surface and exposed to fluorescent light, a noticeable temperature increase occurs in the dishes.
[2] We measured no significant difference in the development rate and survival when larvae were fed 320 µl daily vs. 640 µl on alternate days. To make measuring simpler, you might wish to use a 1% slurry.

2.4.6.2 Species specific rearing guidelines: Protocols for various other anophelines.

MR4 Staff

Introduction

Anopheles albimanus (Wiedemann)

Anopheles albimanus is one of the primary vectors of vivax malaria in Central America, South America, and the Caribbean. It is easily recognized by its large white bands on the distal metatarsi. In the wild it is typically found in more permanent bodies of water.

MR4 rearing protocol

a. Day 1:   Hatch eggs in 350 ml water with 1.5 ml of 2% yeast slurry. We will hatch in three pans and thin out of each over time.

b. Day 2:   Thin pans as needed, an appropriate number of larvae per 9in X 12in pan should not exceed 300-500. Feed 60 mg powdered koi food as needed and refill water to approximately 400-500 ml.

c. Day 3:   Feed larvae 60-100 mg powdered koi food as needed depending on container size and clarity of water. If water is starting to appear cloudy, feed less.

d. Day 4:   Thin larvae to 400 per pan. Feed 60-120 mg powdered koi food. Replenish rearing water to a total volume of 350-500 ml.

e. Day 5:   Feed 60-120 mg powdered koi food. Add 50 ml water.

f. Day 6:   Check larvae and feed or add water if necessary.

g. Day 7:   Strain larvae, feed 120-200 mg powdered koi food and refill water to 500 ml.

h. Day 8:   Feed larvae 120-250 mg powdered koi food.

i. Day 9:   Feed larvae 120-200 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.

j. Day 10:   Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.

k. Day 11:   Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed.

l. Day 12:   Pick pupae. Add pupae to existing cage. Feed larvae as needed.

m. Day 13:   Pick pupae and feed larvae as above.

n. Day 14:   If enough adults are present, discard leftover pupae and larvae. Remove spent pupae cups to prevent adults from drowning in cup.

Figure 2.4.6.2.1 *Anopheles albimanus* note the large white bands on the metatarsi.

Anopheles atroparvus (van Theil)

Anopheles atroparvus is a member of the cryptic species *An. maculipennis* complex and was historically considered responsible for falciparum malaria transmission across most of Western Europe. Larvae are typically found in brackish areas or in riverine settings. Unlike most anophelines, *An. atroparvus* has been reported to over-winter. It is a rather non-descript anopheline with no prominent markings.

MR4 rearing protocol

a. Day 1: Hatch eggs in 350 ml water with 1ml of 2% yeast slurry. We typically split a large egging into two pans to start with.
b. Day 2: Check eggs for hatch. Add 50 ml water and 60mg powdered koi food.
c. Day 3: Thin pans to approximately 500-600 larvae, add 60-100 mg powdered koi food and fill water to approximately 500 ml.
d. Day 3: Feed 50-100 mg powdered koi food as needed depending on container size and number of larvae.
e. Day 4: Check larvae. If needed, add water and diet (60mg at most)
f. Day 5: Thin larvae down to 400-500 per pan. Feed 60-120 mg powdered koi food. Keep water volume between 400-600 ml.
g. Day 6: Check larvae and feed if necessary. Add water and food it needed.
h. Day 7: Feed larvae 120-200 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.
i. Day 8: Feed larvae 120-250 mg powdered koi food.
j. Day 9: Thin larvae to 300-400 per pan. Feed 60-120 mg powdered koi food. Keep water volume between 400-600 ml.
k. Day 10: Feed larvae 120-250 mg powdered koi food.
l. Day 11: Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.
m. Day 12: Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed.
n. Day 13: Pick pupae. Add pupae to existing cage. Feed larvae as needed.
o. Day 14: Pick pupae and feed larvae as above.
p. Day 15: Pick pupae and feed larvae as above.
q. Day 16: If enough adults are present, discard leftover pupae and larvae. Remove spent pupae cups to prevent adults from drowning in cup.

NOTE: Larvae survive better if not disturbed in the early instars, hatch in no more than 2 pans and keep dense early on. Watch for scumming in pans as larvae will succumb quickly. Pupae can take up to 36 hours to eclose.

Anopheles dirus (Payton and Harrison)

The primary member of the *Anopheles dirus* cryptic species complex, *An. dirus* is considered a vector of several malaria parasites across Southeast Asia. It is easily recognized by its white tibial knees on the metatarsi and speckled tarsi. In nature, *An. dirus* is typically found in semi-permanent to temporary habitats.

MR4 rearing protocol

a. Day 1: Hatch eggs in 350 ml water with 1.5 ml of 2% yeast slurry. We typically hatch a large egging in two 9x12 pans.

b. Day 2: Thin larvae to 400 per pan and feed 60mg powdered koi food. Larvae are light in color and may be difficult to see so use caution when thinning.

c. Day 3: Thin pans as needed. An appropriate number of larvae per 9in X 12in pan should not exceed 300-400. Feed 60 mg powdered koi food as needed depending on container size and number of larvae and condition of rearing water.

d. Day 4: Feed larvae 100-150mg powdered koi food. If water is beginning to become cloudy or discolored, strain larvae out and replace water up to 500ml.

e. Day 5: Feed 60-120 mg powdered koi food. Add 50 ml of fresh water.

f. Day 6: Feed larvae if necessary, if water is discolored, has a distinct odor, or is heavy in particulate matter – strain larvae and replace water.

g. Day 7: Feed larvae 120-200 mg powdered koi food.

h. Day 8: Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.

i. Day 9: Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed.

j. Day 10: Pick pupae. Add pupae to existing cage. Feed larvae as needed.

k. Day 11: Pick pupae and feed larvae as above.

l. Day 12: If enough adults are present, discard leftover pupae and larvae. Remove spent pupae cups to prevent adults from drowning in cup.

NOTE: Pupae will be extremely light colored for up to 24 hours, then darken before eclosion which can take up to 36 hours.

Anopheles farauti s.s. (Laveran)

Anopheles farauti is one of the primary vectors of Bancroftian filariasis and malaria across the Oceania region. It is the primary member of the *An. farauti* complex and is found from Papua New Guinea to the Solomon Islands. *An. farauti* prefers brackish coastal waters but can expand its range to inhabit man-made containers and mangrove swamps. It is a dark colored mosquito with speckled tarsi and prominent white bands on the palps.

MR4 rearing protocol

a.   Day 1:   Hatch eggs in 350 ml water with 1.5 ml of 2% yeast slurry. We typically hatch large eggings in two pans.

b.   Day 2:   Thin pans to 400-500 larvae, replenish water and feed 60mg powdered koi food.

c.   Day 3:   Feed 60-100 mg powdered koi food as needed depending on container size, condition of water, and number of larvae. If needed, up to 50ml of fresh water can be added.

d.   Day 4:   Thin larvae to 400 per pan. Feed 60-120 mg powdered koi food. Replenish rearing water to 500ml.

e.   Day 5:   Feed 60-120 mg powdered koi food.

f.   Day 6:   Strain larvae and replace rearing water. Feed 100-150mg powdered koi food.

g.   Day 7:   Thin larvae to 300-400 per pan. Feed larvae 120-200 mg powdered koi food.

h.   Day 8:   Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.

i.   Day 9:   Feed larvae 120-200 mg powdered koi food.

j.   Day 10:   Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.

k.   Day 11:   Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed.

l.   Day 12:   Pick pupae. Add pupae to existing cage. Feed larvae as needed.

m.   Day 13:   Pick pupae and feed larvae as above.

n.   Day 14:   If enough adults are present, discard leftover pupae and larvae. Remove spent pupae cups to prevent adults from drowning in cup.

NOTE: larvae will exhibit a unique "U" shaped curve during resting. This is a normal behavior and not indicative of an infection.

Figure 2.4.6.2.2. L4 larvae showing distinctive "U" resting position.

Anopheles freeborni (Aitken)

Anopheles freeborni was considered one of the primary vectors of malaria in the Western United States. It typically is found in marshy areas, rice growing fields, and other semi-permanent bodies of water. Unlike other anophelines, *An. freeborni* has been found to over-winter underground. *An. freeborni* is a rather non-descript anopheline with no prominent markings.

MR4 rearing protocol

a.   Day 1:   Hatch eggs in 350 ml water with 1.5ml of 2% yeast slurry. As this is a proficient egg producer, we will start by hatching into three pans.

b.   Day 2:   Thin pans to 500-600 larvae, feed 60mg powdered koi food.

c.   Day 3:   Feed 60 mg powdered koi food as needed, if adding food also add 50ml fresh water.

d.   Day 4:   Thin larvae to 400-500 per pan, feed 60-100mg powdered koi food.

e.   Day 5:   Feed 60-120 mg powdered koi food. Keep water volume between 350-500 ml.

f.   Day 6:   Check larvae and feed if necessary.

g.   Day 7:   Feed larvae 120-200 mg powdered koi food.

h.   Day 8:   Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.

i.   Day 9:   Thin larvae to 300 per pan. Feed 60-120 mg powdered koi food. Keep water volume between 350-500 ml.

j.   Day 10:   Feed larvae 120-250 mg powdered koi food.

k.   Day 11:   Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.

l.   Day 12:   Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed.

m.   Day 13:   Pick pupae. Add pupae to existing cage. Feed larvae as needed and change water if it is starting to cloud.

n.   Day 14:   Pick pupae and feed larvae as above.

o.   Day 15:   If enough adults are present, discard leftover pupae and larvae. Remove spent pupae cups to prevent adults from drowning in cup.

NOTE: It is best to keep early instars at a higher density than regular anophelines, if they are too thin in the early instars the colony will suffer. Pupae will take up to 36 hours to eclose.

Anopheles funestus (Giles)

This is one of the primary vector of falciparum malaria across sub-Saharan Africa. It is the primary member of the *An. funestus* cryptic species complex. This species prefers to oviposit in permanent to semi-permanent sites and is known to prefer sites with algal growth. We rear this strain on a mixed diet of powdered Spriulina and koi fish food. Adults range in size in the wild but at the MR4 we find this to be a small anopheline.

MR4 rearing protocol

a.	Day 1:	Hatch eggs in 350 ml water with 50 mg powdered *Spirulina* and 50mg powdered koi food.
b.	Day 2:	Check eggs for hatch.
c.	Day 3:	Do not split or thin pan the original pan. Feed 25 mg powdered koi food, 50 mg powdered *Spirulina* and add 50-100 ml water. Larvae survive better when crowded and kept in slightly cleaner conditions in the early instars
d.	Day 4:	Split larvae to 300-500 per pan. Feed 50-100 mg powdered koi food and 50 mg powdered *Spirulina*. Keep water volume between 350-500 ml.
e.	Day 5:	Feed 50-100 mg powdered koi food and 50 mg powdered *Spirulina*. Keep water volume between 350-500 ml.
f.	Day 6:	Check larvae and feed if necessary.
g.	Day 7:	Strain larvae and refill with fresh water. Feed larvae 50mg *Spirulina* and 100 mg powdered koi food.
h.	Day 8:	Feed larvae 100 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding. A 200 micron mesh filter works best.
i.	Day 9:	Thin larvae to 300 per pan. Feed 50 mg powdered koi food and 50 mg powdered *Spirulina*. Keep water volume between 350-500 ml.
j.	Day 10:	Feed larvae 100 mg powdered koi food.
k.	Day 11:	Feed larvae 50 mg powdered koi food and 100 mg powdered *Spirulina*. If the water is starting to foul, strain larvae and replace water before feeding.
l.	Day 12:	Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed.
m.	Day 13:	Pick pupae. Add pupae to existing cage. Feed larvae as needed. Strain water and replace as it will start to foul quickly due to the diet.
n.	Day 14:	Pick pupae and feed larvae as above.
o.	Day 15:	If enough adults are present, discard leftover pupae and larvae. Remove spent pupae cups to prevent adults from drowning in cup.

NOTE: It is best to house this strain in a minimum of 12 x 12 x 12 inch cage. Blood-feed 2 times, every other day for first egg batch. Only one blood-meal is needed for each subsequent egg batch. Pupae will take up to 36 hours to eclose.

Anopheles minimus s.s (Theobald)

An. minimus is one of the primary vectors of malaria across Southeast Asia. As with most other species, it is a member of a cryptic species complex and is distantly related to *An. funestus*. Like *An. funestus*, *An. minimus* prefers permanent to semi-permanent oviposition sites. Adults range in size in the wild but at the MR4 we find this to be a small anopheline.

MR4 rearing protocol

a.	Day 1:	Hatch eggs in 350 ml water with 50mg powdered koi food.
b.	Day 2:	Check eggs for hatch.
c.	Day 3:	Do not split or thin pan the original pan. Feed 25 mg powdered koi food and add 50-100 ml water. Larvae survive better when crowded and kept in slightly cleaner conditions in the early instars
d.	Day 4:	Split larvae to 300-500 per pan. Feed 50-100 mg powdered koi food. Keep water volume between 350-500 ml.
e.	Day 5:	Feed 50-100 mg powdered koi food. Keep water volume between 350-500 ml.
f.	Day 6:	Check larvae and feed if necessary.
g.	Day 7:	Strain larvae and refill with fresh water. Feed larvae 100 mg powdered koi food.
h.	Day 8:	Feed larvae 100 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding. A 200 micron mesh filter works best.
i.	Day 9:	Thin larvae to 300 per pan. Feed 50 mg powdered koi food. Keep water volume between 350-500 ml.
j.	Day 10:	Feed larvae 100 mg powdered koi food.
k.	Day 11:	Feed larvae 50 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.
l.	Day 12:	Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed.
m.	Day 13:	Pick pupae. Add pupae to existing cage. Feed larvae as needed. Strain water and replace as it will start to foul quickly due to the diet.
n.	Day 14:	Pick pupae and feed larvae as above.
o.	Day 15:	If enough adults are present, discard leftover pupae and larvae. Remove spent pupae cups to prevent adults from drowning in cup.

Notes: Larvae survive best if not disturbed after hatching until the late L2, early L3 instar**.** Feed sparingly, larvae survive better if the larvae are not overfed early on. We also find that the larvae prefer to be crowded in the early instars. Pupae will take up to 36 hours to eclose.

Anopheles quadrimaculatus s.s. (Say)

An. quadrimaculatus was once considered a vector of malaria in the Eastern United States. It is a large mosquito that prefers permanent bodies of water. In the more northern parts of its range it can overwinter, however in more southern clines it will breed year round.

MR4 rearing protocol

a.	Day 1:	Hatch eggs in 350 ml water with 1.5ml of 2% yeast slurry.
b.	Day 2:	Thin larvae to 500-600 per 9x12 pan. Feed 60mg powdered koi food.
c.	Day 3:	Feed 50-100 mg powdered koi food as needed depending on container size and number of larvae. Add 50ml fresh water.
d.	Day 4:	Thin larvae to 400-500 per pan. Feed 60-120mg powdered koi food.
e.	Day 5:	Feed 60-120 mg powdered koi food depending on the amount of food already in the pan and the condition of the water. If the water is starting to foul, replace to a volume between 350-500 ml.
f.	Day 6:	Check larvae and feed if necessary.
g.	Day 7:	Feed larvae 120-200 mg powdered koi food.
h.	Day 8:	Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.
i.	Day 9:	Thin larvae to 300 per pan. Feed 60-120 mg powdered koi food. Keep water volume between 350-500 ml.
j.	Day 10:	Feed larvae 120-250 mg powdered koi food.
k.	Day 11:	Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.
l.	Day 12:	Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed.
m.	Day 13:	Pick pupae. Add pupae to existing cage. Feed larvae and replace rearing water as needed.
n.	Day 14:	Pick pupae and feed larvae as above.
o.	Day 15:	If enough adults are present, discard leftover pupae and larvae. Remove spent pupae cups to prevent adults from drowning in cup.

NOTE: It is best to keep early instars at a higher density than regular anophelines, if they are too thin in the early instars the colony will perish. Pupae will take up to 36 hours to fully eclose.

Anopheles sinensis (Wiedemann)

An. sinensis is one of the most widely distributed vectors across Asia. It is incriminated in *vivax* malaria transmission in parts of it range while in other parts it is mostly zoophilic. *An. sinensis* is opportunistic and will oviposit in most water bodies but does show a preference for rice fields.

MR4 rearing protocol

a.	Day 1:	Hatch eggs in 350 ml water with 1ml of 2% yeast slurry.
b.	Day 2:	Check eggs for hatch, feed 50mg powdered koi food and add 50ml fresh water.
c.	Day 3:	Check larvae, add 50-100ml of fresh water and 60-100 mg of ground koi food.
d.	Day 4:	Split and thin pans as needed. An appropriate number of larvae per 9in X 12in pan should range between 400-500. Feed 1ml ground koi slurry and 50-100 mg powdered koi food as needed depending on container size and number of larvae.
e.	Day 4:	Check larvae.
f.	Day 5:	Check larvae, add 50-100ml of fresh water and 60-100 mg of ground koi food.
g.	Day 6:	Thin larvae to 400 per pan and feed 50mg powdered koi food.
h.	Day 7:	Feed larvae 120-200 mg powdered koi food.
i.	Day 8:	Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.
j.	Day 9:	Thin larvae to 300 per pan. Feed 60-120 mg powdered koi food. Keep water volume between 350-500 ml.
k.	Day 10:	Feed larvae 120-250 mg powdered koi food.
l.	Day 11:	Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.
m.	Day 12:	Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed.
n.	Day 13:	Pick pupae. Add pupae to existing cage. Feed larvae as needed.
o.	Day 14:	Pick pupae and feed larvae as above.
p.	Day 15:	If enough adults are present, discard leftover pupae and larvae. Remove spent pupae cups to prevent adults from drowning in cup.

NOTE: the larval period can last up to 14 days before pupation begins. The duration of pupation may last from 3-6 days. Also, we typically egg in a dark dish lined with white paper.

Anopheles stephensi (Liston)

An. stephensi is one of the more important vectors of malaria across Southeast Asia and into the Middle East and Africa. It is highly anthropophilic and can be found in wells and other man-made bodies of water as well as agricultural areas and temporary water sources. This species is considered very easy to infect and will produce large numbers of sporozoites. Note, it is similar in appearance to *An. gambiae* and can be distinguished by looking at the number of "wing spots" from the humeral to the pre-apical region of the wing as well as the "speckled" appearance of its legs. *An. stephensi* mosquitoes will have more white in the apical region compared to *An. gambiae*. We rear this species in a similar manner to *An. gambiae*.

MR4 rearing protocol

a.   Day 1:   Hatch eggs in 350 ml water with 1.5 ml of 2% yeast slurry.

b.   Day 2:   Split and thin pans as needed. An appropriate number of larvae per 9in X 12in pan should not exceed 300-400. Feed 50 mg powdered koi food.

c.   Day 3:   Feed 50-100 mg powdered koi food as needed depending on container size and number of larvae.

d.   Day 4:   Thin larvae to 300 per pan. Feed 60-120 mg powdered koi food. Replenish rearing water to a total volume of 350-500 ml.

e.   Day 5:   Feed 60-120 mg powdered koi food. Add 50ml of fresh water.

f.   Day 6:   Check larvae and feed if necessary.

g.   Day 7:   Feed larvae 120-200 mg powdered koi food.

h.   Day 8:   Feed larvae 120-250 mg powdered koi food. If the water is starting to foul, strain larvae and replace water before feeding.

i.   Day 9:   Pick pupae. Feed larvae as needed. Amount of food should decrease once pupation is observed.

j.   Day 10:   Pick pupae. Add pupae to existing cage. Feed larvae and clean water as needed.

k.   Day 11:   Pick pupae and feed larvae as above.

l.   Day 12:   If enough adults are present, discard leftover pupae and larvae. Remove spent pupae cups to prevent adults from drowning in cup.

NOTE: This species will take up to 36 hours to fully eclose. Also, they will diurese extensively during blood-feeding so make sure to put a pad under the cage.
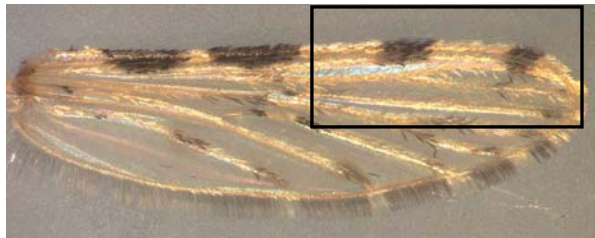
Figure 2.4.6.2.3 Wing of *An. stephensi*, note the small apical spot.
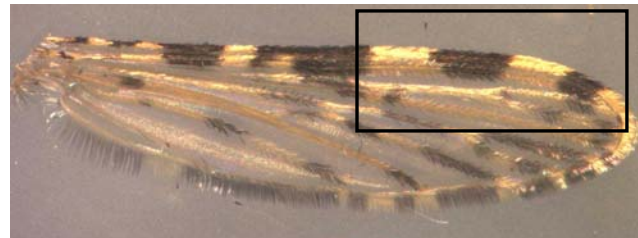
Figure 2.4.6.2.4 Wing of *An. gambiae*, note the larger apical spots

2.4.7 Separating Larvae and Pupae

MR4 Staff

Introduction

Four features used alone or in combination to separate pupa from larva are: 1) buoyancy 2) size 3) activity and 4) appearance. Pupa of most species should be separated from larva daily; otherwise, adults will emerge. Some species require a longer pupal development and can be separated every other day.

There are several larva/pupa separation methods that use equipment varying in sophistication. All will not work equally well for all species and must be tested. While pipetting will select pupae of all species based on appearance, the most uniformly effective *en masse* method for all species is size separation using a glass plate pupa separator (Fay and Morlan 1959). Another device based on the size differences is that of McCray (1961) but this does not allow on-the-fly adjustment as readily as does the Fay and Morlan device.

Separate by appearance

The simplest, but most time consuming method involves using a "pupa picker" to individually manually remove pupae from the rearing pan and place them into an emergence cup. This method is appropriate only when small numbers of pupae are present. Examples of some utensils are shown in **Figure 2.4.7.1**.

Figure 2.4.7.1. Examples of utensils useful for manual pupae separation or "hand picking". On the left is a piece of wire mesh glued to a metal spatula and is used to scoop pupae. The middle implement is made of a 1,000 μl disposable pipette tip, the tip of which has been trimmed off and fitted with a 2 ml pipette bulb. The pupa picker on the right is a disposable pipette especially good for avoiding contamination as it is transparent. Rinsing these in hot water often is necessary to prevent contamination.

Separation by differential buoyancy of pupae and larvae

Larvae are negatively buoyant whereas pupae are positively buoyant. Pupal and larval activity counteracts this useful difference, but chilled water can be used to reduce this.

Swirling

One quick method to try is swirling larvae and pupae a cup: larvae will accumulate in the middle on the bottom and the pupae at the sides of the cup. Using a pipette such as one shown in **Figure 2.4.7.1**, remove the larvae from the middle or the pupae from the sides.

Swirling with chilled water

A variation is to use ice water to stun the mosquitoes allowing them to be separated in water without their behavior interfering (Weathersby 1963).

1. Collect the pupae and larvae together in a strainer and wash them into a small bowl (**Figure 2.4.7.2**).

2. Add ice-cold water free of ice (**Figure 2.4.7.3**).

3. Quickly swirl and wait until separation occurs (**Figure 2.4.7.4-5**).

4. Gently pour the pupae into a sieve and transfer to emergence cups containing culture temperature water.

Notes

A separatory funnel or Imhoff cone can also be used with the ice water method (**Figure 2.4.7.6**) (Hazard 1967). This is a good method for large numbers of a single strain.

Ice water increases larval mortality in some strains/species so the time of ice water exposure should be minimized. Larvae typically only survive this treatment 2 or 3 times, so this is most useful when large numbers are cultured and picking is limited to 2 to 3 days.

Carbon dioxide anesthesia has also been used for the purpose of immobilizing pupae and larvae for separation, though we have not tried it (Lin and Georghiou 1976). This method could be similar to ice water in its usefulness.

Figure 2.4.7.2. Place pupae and larvae in a small volume of water.

Figure 2.4.7.3. Pour in ice water leaving enough room for swirling and pouring.

Figure 2.4.7.6.

Chilled water method as shown to left but using an Imhoff cone. Larvae are on the bottom and pupae at the top of the funnel.
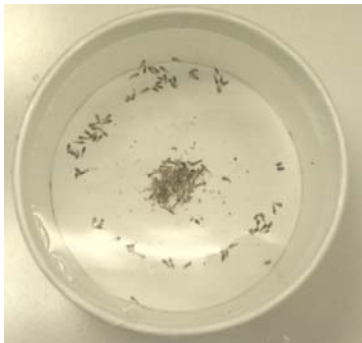
Figure 2.4.7.4. Swirl gently and then allow separating. Larva will accumulate in the bottom middle of the cup and pupae will float at the top and around the sides.

Figure 2.4.7.5. Side view of larval/pupal separation in ice water.

Separation by larger size of pupae

Fay-Morlan Separator

A custom-built glass-plate pupa separator (**Figure 2.4.7.7**) is good for large numbers of one strain and has minimal harmful effects. Two roughly parallel glass plates are adjusted with knobs to control the spacing of the upper and lower portions of the glass. By controlling the space you can trap the pupae (larger diameter) and allow the larvae (smaller) to flow through into a tray. Then the glass plates are loosened and the pupae flushed into a separate pan. This method can also be used to separate the sexes of pupae when male and female size differs considerably though it is not usually possible with *Anopheles* mosquitoes since both sexes are roughly the same size in the pupal stage (see Chapter 2, Physiology section). Care must be taken not to overload the device or it will become congested with pupae and the flow of larvae will be prevented. The risk of contamination is the main drawback to this method as larvae and pupae may get caught on the edges and be inadvertently transferred to another stock. Rinsing thoroughly between strains can prevent such transfer.

Figure 2.4.7.7. Custom-built pupa separator relying on the size difference between pupa and larva for separation (produced by the John Hock Company).

Figure 2.4.7.8. The McCray gate separator. The closest gate to the viewer has a narrower gap than the farthest gate; this is set up to separate dimorphic pupae and larvae.

Gate Separator

A smaller machine is the McCray Separator (1961). The separator shown utilizes a series of pre-cut aluminum plates that can be adjusted to catch pupae of varying sizes; however these cannot be adjusted during use - only between **(Figure 2.4.7.8)**. It is especially good for separating pupae that have distinct size dimorphism such as *Ae. aegypti*. The contents of the larval rearing pans are poured into the upper chamber of the sluice where the plate gap is set to catch the largest pupae first. By applying a gentle stream of water, the smaller size pupae and larvae are washed down to the lower chamber which is set at a slightly narrower gap to catch any smaller pupae. From here, the larvae are flushed down into a new rearing pan where they can be fed and left to pupate. After all the larvae have been rinsed through, remove the aluminum dividers and rinse pupae into a new pan and transfer to a cage for emergence. As with the Fay-Morlan Separator, care must be made to ensure that all the pupae have been washed from the apparatus to avoid contamination when handling several different stocks.

Figure 2.4.7.9. *An. gambiae* larvae and pupae in the process of separation based on activity and buoyancy. Pupae are floating to the surface.

Gentle vortex: Separate by both activity and buoyancy

The vortex method not using chilled water ranges from great to poor depending on the mosquito strain. A variation is to collect pupae and larvae in a strainer and washing them into a Florence flask with fresh water (**Figure 2.4.7.9**). Swirl as you fill to the top. Most of the larvae will dive and most of the pupae will rise to the neck. When separation is maximal, pour the pupae off. This method is seldom 100% effective, so some hand-picking is required. This can also work with an Imhoff funnel (as shown in **Figure 2.4.7.6**) in which case the larvae are drained from the bottom. Cool water will improve the separation, but take care that repeated chilling is avoided as much as possible to prevent mortality.

References

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Lin CS, Georghiou GP (1976) Tolerance of mosquito larvae and pupae to carbon dioxide anesthesia. Mosq News 36:460-461

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2.4.8 *Anopheles* Adult Caging

MR4 Staff

Introduction

Cages can be quite simple and are often improvised from easily obtained materials. The type of cage you will use for adults will depend on amount of mosquitoes, availability of cage material, security required and behavioral constraints of the stock. The size of your cage must be large enough to promote or allow mating. Following are some caging options.

Before purchasing or building cages, consider the number of adults that will be housed, ease of cleaning, and whether you prefer to dispose of them or reuse them. Purpose-built improvised cages are often as useful as commercial options.

Paper cans

Cylindrical paper containers are popular in insectary usage as they come in different sizes, are inexpensive and are disposable (**Figure 2.4.8.1**). A relatively small number (*e.g.* about 10-50 females and males) of *An. gambiae*, *An. stephensi*, and *An. albimanus* adults will mate well in pint (~1/2 liter) paper cups but can hold up to 100 adults without crowding, so this is a good container for many genetic crosses and small stocks. For a colony of 500-1,000 adults, a 3.8 liter (1 gallon) size container is more appropriate.

You can create a hole in the screen plugged with cotton for introducing mosquitoes or use tube-gauze stapled into a hole cut in the side to permit the introduction of cups for pupae or egg collection (**Figure 2.4.8.1**). Using mesh netting on the top allows blood- and sugar feeding. Because of dripping blood and sugar water, paper containers will grow fungi in the bottom and must be disposed after use.

Figure 2.4.8.1. Paper cartons of two sizes, pint (left) gallon (right), showing top entry (left) and side entry (right) solutions.
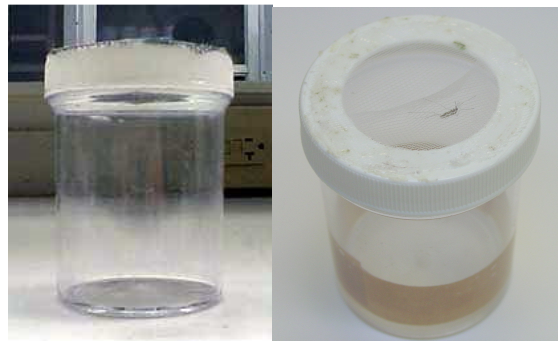
Figure 2.4.8.2. Left: Small vial perfect for single pair matings or single female oviposition. Right: Single female placed in small vial with water and egging paper for oviposition.

Single-pair matings work well in small (approx 120 ml) plastic vials (Qorpak No. 3891, 6.75 cm deep, 4.5 cm diameter) such as the one shown in **Figure 2.4.8.2**. It has been modified by cutting a hole in the top and covering with mesh for easy feeding and aspirator entry. Using small containers for single pair mating can delay mating from the typical 1 or 2 days after emergence to 6-7 days. These small vials are also useful for collecting eggs from individual females. To egg with this container, simply fill to about 1 cm depth with water and line with two filter paper strips that extend half way under water (**Figure 2.4.8.2**).

Metal-frame cages

Metal cages are good alternatives since they are autoclavable and therefore easily sterilized and reusable. One metal cage developed by Savage and Lowe (1971) consists of a single-piece bent-to-shape aluminum sheet covered with tube-gauze (Medical Action Industries White T-1 Tubegauze ® #58205) (**Figure 2.4.8.3**). This cage is appropriate for small colonies of up to 500 adults if the colony mates well in a cage of this size. Removing the mesh netting between uses and autoclaving the metal portions make this a great choice for avoiding infection in colonies. The netting can be cleaned and reused or discarded. The cage measures 10" x 6" x 8.25" (LxWxH) and is made from 14 gauge aluminum (1/16" thickness).

Another example of a larger design is shown in **Figure 2.4.8.4** (BioQuip, www.bioquip.com) and works better for colonies of the same number but that need more space for mating. It does require cleaning between uses. BioQuip also distributes a less expensive plastic version which can stain but is less expensive than the metal version seen in **Figure 2.4.8.4**. With either of these choices, introducing cups and bloodfeeding is easy.

Figure 2.4.8.3. Savage cage, perfect for small colonies that mate well in small spaces.
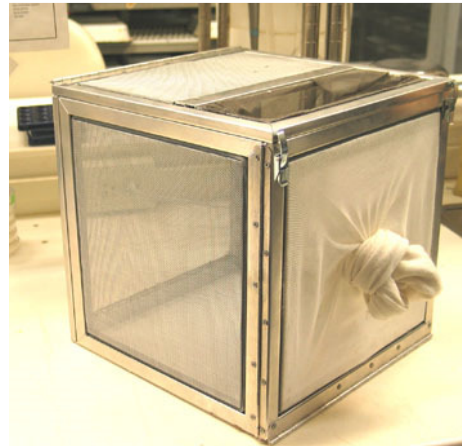
Figure 2.4.8.4. Autoclavable cage especially useful for colonies that mate poorly and larger numbers**.**

The best cage solutions have plenty of resting space for the mosquito. Though having such space does not seem to be important for mating, it is preferred by some species and therefore assumed important for 'comfort'. With *An. arabiensis* mosquitoes, if a resting space is offered, usually more than 90% of the mosquitoes will be inside that space (assuming it is large enough) at daylight times, see **Figure 2.4.8.6**. The use of red boxes has been published as preferred resting sites over some alternative trapping devices in anophelines (Goodwin 1942). Thus, if mosquitoes choose red resting boxes in the wild, they will most likely work well inside your cages. Red, most notably, and some shades of blue have been shown to be preferred to black or other colors for *Anopheles* resting (Nuttall and Shipley 1902) and are therefore good color choices.

Figure 2.4.8.6. *An. arabiensis* adults crowding into a single resting tube in daylight hours.

References

Goodwin MN (1942) Studies on artificial resting places of *Anopheles quadrimaculatus Say*. In: Emory Univ. Field Sta., Newton, GA

Nuttall GHF, Shipley AM (1902) Studies in relation to malaria. The structure and biology of *Anopheles*. Amer. Journal Hyg. 2:58-84

Savage KE, Lowe RE (1971) A one-piece aluminum cage designed for adult mosquitoes. Mosq. News. 31:111-112

2.4.9 *Anopheles* Adult Diet

MR4 Staff

Introduction

6% glucose, 10% v/v corn syrup, 10% w/v sucrose, and dilute honey are common sugar sources for adult male and female mosquitoes. These can be provided using soaked cotton balls lying on top of a cage if the mesh is non-absorbent (e.g. nylon rather than cotton). This requires ensuring that the cotton stays wet enough for the mosquitoes to drink the sugar. Sugar pads normally need to be changed no less than every week because mold spores and fungi grow well on exposed sugar pads. 0.2% methylparaben added to a sugar source can extend the time before the sugar source begins to collect mold spores that are harmful to mosquitoes without causing early mortality or reduced longevity (Benedict 2009). In this case, sugar sources can be left unchanged for 30 days as long as they stay wet.

Cleanliness of sugar water and cotton balls is of the utmost importance. After a bag of cotton balls is opened, mold spores can settle on them immediately. Cotton balls can be sterilized by autoclaving and should be stored in sealed containers.

One way to avoid having to moisten sugar pads is to use a hanging feeder that holds soaked cotton (**Figure 2.4.9.1**). This example consists of an inverted sample vial (Fisherbrand Polystyrene Sample Vial (20ml) Cat. No. 03-341-13) hung in the cage. A hole has been punched in the cap (at the bottom) to allow mosquitoes access to the soaked sugar pad. The vial hangs from a bent wire inserted into the vial base by heating the wire and forcing it through the plastic. Two large cotton balls are soaked with sterilized sugar solution and then placed in the vial. This orientation has the advantage that *Anopheles* will seldom lay eggs in it, and it will remain moist for 1 week without attention. If using such a source, the sugar vials and covers should be bleached between use and stored in a closed container to prevent mold spores from accumulating on them.

Figure 2.4.9.1. Inverted hanging sugar vial.

Another alternative is to fit the opening in the cap (as shown in **Figure 2.4.9.2**) of the inverted feeder with a piece of porous plastic large enough for the mosquitoes to feed through but small enough that water does not drip. The porous plastic center in the photograph to the left was obtained from Small Parts Inc., catalog no. SPE-040-20. This modification allows for pouring the sugar water directly into the feeder with no need for cotton. Using this hanger in combination with sugar water containing methylparaben solution should require no attention for about 30 days.

Figure 2.4.9.2. Cap of feeder with a porous plastic insert.

References

Benedict MQ, Hood-Nowotny RC, Howell PI, Wilkins EE (2009) Methylparaben in *Anopheles gambiae s.l.* sugar meals increases longevity and malaria oocyst abundance but is not a preferred diet. J. Insect Phys. 55: 197-204.

2.4.10 Bloodfeeding : Membrane Apparatuses and Animals

MR4 Staff

Blood Feeding Strategies:

Blood-feeding is one of the most problematic activities of anopheline culture. Even when ethical rules permit the use of a live animal, their care and housing are expensive and time-consuming. In spite of this, many scientists have concluded that there is no equal to a live animal regarding attraction, satiation, and egg production. There are many reasons why animals are employed as a blood source: animals provide a constant source of blood which is at the proper temperature, the animal provides necessary stimuli for feeding, and it is not necessary to handle blood directly. However, these must be weighed against the cons which include the potential for animal biting, accidental disease transmission, and the procurement, maintenance, and care of animals.

There has been one reported success of using an artificial blood meal to produce eggs in *Aedes*; however, it has never been reported successful in anophelines (Kogan 1990).

One full blood meal is enough to mature oocysts in most well-cultured anophelines. The length of feeding time needed to ensure females are fed to repletion or the type of blood necessary will depend on the mosquito you are using. Usually 3-5 day old females feed most readily. However, some colonies of *An. atroparvus* and *An. minimus* feed more avidly at 6-9 days.

Keeping in mind the amount of blood that a single animal can safely provide, it is best to maximize the number of cages you are feeding at one time to reduce the amount of total time the animal is restrained and/or anesthetized. Also, feed no more frequently nor more mosquitoes than are necessary to generate the number of progeny you need.

In some instances a mosquito colony may not feed on the animal provided. Sugar-starving females for 5-12 hours prior to blood feeding can increase success rates in some hesitant strains. However, removing sugar jeopardizes males and should be used only when necessary or on separated females. If starvation does not help bloodfeeding rates and the females are of optimum age, then there may be other factors affecting the feeding rate:

A new species of animal. Mosquitoes can be selected to feed on one animal source by propagating the colony only with those mosquitoes that fed a particular species. It may take a few generations before the mosquitoes readily accept a new host. In the effort to increase feeding, increase the feeding time and the frequency. Supplement with another blood source if critical to maintenance of the colony.

Infection in the colony. Mosquitoes infected with fungi (Scholte et al. 2006) may not respond to a host or be unable to imbibe properly. Some infections will stall the digestion process resulting in a reduction in egg production. See Chapter 2 for information about preventing infections.

Improper feeding time: This is important in both newly acquired strains and material brought from the field. The new mosquito colony may be accustomed to feeding at a specific time or under certain conditions. Wild mosquitoes feed in darkness, so lighting is a factor that should be considered. With a new or wild colony, try to determine when the mosquitoes are actively searching for a blood meal and schedule feeds for that time if possible. If they prefer to feed at night, cover the cages with a dark shroud for several hours before feeding and turn out the lights while offering the bloodmeal. Also, the length of time you bloodfeed may be too short. Some colonies require time near the blood source before they will take a meal.

Use of Live Animals

Colonizing mosquitoes involves locating a source of blood for the propagation of the colony. Live animals were utilized more often than not in the past; however, their use has diminished due to the implementation of strict guidelines governing the use of animals in a research setting. Likewise,

Institutional Review Boards (IRBs) disallow any use of live animals when deemed unnecessary or if an alternate, less intrusive method for feeding is known. It is best to check with your local IRB before considering blood sources to determine what blood sources and conditions are allowed. If you decide to use live animals, obtaining an approved protocol from a colleague in your country is a good start. The following are a good list of sources for rules and regulations for the U.S. to consider when writing a protocol.

The **Public Health Services Policy** on humane care and use of laboratory animals is provided by the NIH as a resource in caring for animals in a research setting.
http://grants.nih.gov/grants/olaw/references/phspol.htm

The **Animal Welfare Act** sets rules for how animals are to be used.
http://www.nalusdAn.gov/awic/legislat/awAn.htm

The **Guide for the Care and Use of Laboratory Animals** gives standard criteria used by the Public Health Services (PHS) in the production of the PHS Policy.
http://www.nap.edu/readingroom/books/labrats/chaps.html

The **Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)** is a body that accredits local institutions if the facilities meet the standardized requirements above. http://www.aaalac.org/

The **World Organization for Animal Health (OIE)** is an international agency that also sets standards for the use of animals in research. http://www.oie.int/eng/en_index.htm

Institutional Animal Care and Use Committee (IACUC) is another resource for the use of any animal in a laboratory setting. This group also has some links to other government resources as well as an extensive online library. http://www.iacuc.org/

Institute for Laboratory Animal Research (ILAR) provides information and publications on the use and care of several animals common to laboratory settings. http://dels.nas.edu/ilar_n/ilarhome/

There are a few types of animals which are more commonly used in a research setting. They are chosen for many reasons and are all considered small animals which can be easily procured.

Lagomorphs-rabbits (**Figure 2.4.10.1**)

Rodents-mice, rats, and guinea pigs

Chickens

Birds are a special case and there are additional resources for their use located at the websites below:

http://www.nmnh.si.edu/BIRDNET/GuideToUse/index.html

http://www.mbc.edu/osp/docs/IACUC_Proposal_Sheet.doc

http://www.fiu.edu/~dsrt/animal/birds_lab.htm

Medical schools may have research animals that they plan to destroy after their experiments have ended. This can be a good source for single use feeding (i.e. the animal will be euthanized after feeding). Avoid using any animals that were infected with any pathogen if you use animals from a medical school.

Figure 2.4.10.1. Use of a rabbit for mosquito feeding utilizing a combination of drugs for anesthesia and pain under an IRB-approved protocol. Restraining the animal ensures uninterrupted feeding for the mosquitoes and safety for the rabbit should it awaken unexpectedly. Shaving the belly prior to feeding makes the skin easily accessible.

Membrane feeding

Due in part to the restrictions on the use and difficulty of live animals in a research setting, artificial membrane methods for feeding have been developed. Designs all have two basic features: a heating element and a membrane to hold the blood. The heating element is necessary to maintain the blood at a reasonable temperature at which mosquitoes will imbibe, typically between 35- 40°C. The membrane should be relatively thin so mosquitoes can pierce it easily but sturdy so that spilling does not occur.

Membrane types

There are several materials that have been used in membrane feeding. Natural membranes are claimed to work best (e.g. animal skin membranes), but they are can be difficult to procure and can usually only be used once (Novak et al. 1991). Other membranes used with some success include: Parafilm M®, collagen sheets, latex membranes e.g. gloves or dental dam, sausage casings (natural or synthetic), Baudruche membranes (Joseph Long Inc, N.J.), and condoms (lambskin or latex).

Membrane Feeders

Several different methods have been developed for feeding mosquitoes, some simple, some are more complex. A short list of some reported examples from the literature are:

Latex or lambskin condoms: Fill the condom with heparinized blood. Tie it closed and soak it in a warm water bath (~40°C) for 30 minutes to 1 hour to fully heat the blood. Condoms have been reported to have varying levels of success.

Tseng feeder (Tseng 2003): This employs a Parafilm packet filled with blood then wrapped in a wire mesh casing. It is best to heat the blood before pouring it into the Parafilm packet.

Mishra feeder (Mishra et al. 2005)*:* A Petri dish is wrapped with Parafilm on the outside leaving a small pocket in the center of the bottom. Warm water is poured into the Petri dish and a small amount of blood is injected into the Parafilm. This unit can be placed directly on the cage.

Mourya feeder (Mourya et al. 2000): A 10 mm hole is drilled into a sheet of acrylic. Parafilm is then stretched over the sheet and warmed blood added into the 10 mm hole. A beaker of warm water placed on the top of the hole maintains the temperature.

More Complex Designs for Membrane Feeders

*Glass feeder*s: Although there are several models of these, most are built in the same manner: An outer area contains circulating warm water and an inner chamber where the blood is poured, see **Figure 2.4.10.2**. A nice reference to several styles is given by Kasap et al. (2003).

Figure 2.4.10.2. Example of a membrane feeder using dental dam and an electric pump and heater to circulate warm water.

Electric units: These models e.g. Hemotek (**Figures 2.4.10.3-2.4.10.5**) employ an electric heating element to maintain the temperature of the blood meal. These are very reliable and easy to maintain (Hagen and Grunewald 1990; Cosgrove et al. 1994).

Figure 2.4.10.3. The Hemotek system offered by Discovery Workshops (mail@hemotek.co.uk)

Figure 2.4.10.4. The electric heating unit with a membrane feeder attached to the bottom.

Figure 2.4.10.5. The components that make up the membrane feeder. Clockwise from top, a membrane (in this case Parafilm™; the manufacturer's membranes are recommended), a completely assembled membrane unit ready to be attached to the heating unit, the metal membrane unit without a membrane, and the "O" ring used to attach the membrane.

Switching from a live animal to a membrane system can be difficult if a colony is accustomed to feeding on a live animal. There are some methods commonly employed to increase success rates when using a membrane feeder.

Starvation: sugar-starving females 5-12 hours can be helpful in increasing desire to take a blood meal; sugar-starving can cause mortality in males and should be limited if the males are not first removed.

Lighting: most successful membrane feeds are conducted in the dark; you can either cover the cages being fed or turn off the lights.

Stimuli: gently rubbing the membrane on your arm will transfer human volatiles needed for phago-stimulation in mosquitoes.

ATP: adding this chemical to the blood has been shown to improve membrane feeding with some strains of *Aedes* and recently *An. gambiae* (personal communication F. Catteruccia lab).

Blood sources

A reliable source of blood for membrane feeding must be found. Although blood can be drawn from a human volunteer or from a laboratory animal, the use of either type of blood may require special permission through an IRB or an IACUC (Institutional Animal Care and Use Committee). This typically involves a review as well as a trained specialist to perform the venipuncture procedure.

Blood can sometimes be obtained from a slaughter house (an *abattoir*). If properly treated (either collected in a heparinized vial or provided some anti-coagulant agent and stored at 4°C), these sources of blood have been used successfully for up to 2 months. Heparinized or citrinated animal blood can also be procured from specialized vendors such as Lampire, HemoStat, and Bioreclamation.

Recent work has shown that it is possible to use packed red blood cells resuspended 1:1 with 0.09% saline and ATP when feeding *An. gambiae* however egg production was lower when compared to the use of whole blood (Sara Mitchell, W. Robert Shaw, Evdoxia Kakani, Emily Lund, Francesco Baldini, Ainhoa Mariezcurrena and Flaminia Catteruccia).

Personal safety while using a membrane feeding apparatus is critical since all blood should be handled as if it were infected. Proper training in the use of Personal Protective Equipment (PPE) such as latex gloves, lab coat, and face-shield should be provided to all personnel handling the blood or performing the feedings. Personnel should receive appropriate instructions according to their institutions rules.

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2.5 Basic Anopheles *Mendelian Genetics*

Mark Benedict

Introduction

It is difficult to culture stocks on a long-term basis without some knowledge of their genetics. After all, what ultimately distinguishes stocks and species is their genetic constitution, not the name that we assign to them. The following are aspects of *Anopheles* genetics that are very relevant to understanding the day-to-day stability and integrity of stocks. Exceptions can be applied to every generalization made here. However, the following will ground you in *Anopheles* genetics.

As a basis for the modern discussions of anopheline phylogenetics and genomics, the existing reviews are still very useful (Kitzmiller and Mason 1967; Kitzmiller 1976).

In this chapter, we provide the minimum background to understand anopheline genetics and exercises using anopheline characters to help make the information relevant. Answers are provided after the questions. If you have no understanding of Mendelian genetics, study the basic genetic principles of diploid organisms in a basic text before proceeding. You should particularly understand Punnett squares and basic probability.

Genetic Glossary

Allele: Variant forms of genes

Coupling: Also called 'cis.' Two alleles being referred to are on the *same* homolog. In contrast, see 'repulsion..

Diploid: Having two sets of chromosomes.

Expressivity: The degree or Extent of expression of the phenotype. This addresses the issue e.g. not of what proportion of individuals have freckles, but how *many* or *how large* the freckles are. Or for anophelines not, do they have a red stripe, but how intense and clear is it? In contrast, see 'penetrance.'

Gene: The basic unit determining heritable expression. (There are other kinds of heritable expression, but gene covers 99.9% of them.)

Genotype: The allelic constitution of an individual, but sometimes applied to tissues or cells.

Haploid: Having one set of chromosomes.

Hemizygote: Usually refers to the state of having only one copy of a gene located on the X chromosome in males (which have only one X).

Heterozygote: The condition in which a diploid individual has two different alleles of a gene.

Homolog: One member of a pair of chromosomes.

Homozygote: The condition in which a diploid individual has two apparently identical alleles of a gene.

Linkage group: May temporarily be the genes or loci that are experimentally identified as not segregating independently from one another, but ultimately refers to the collection of everything located on one chromosome. The number of linkage groups identified experimentally may be greater than the actual number of chromosomes. (Think of contigs in a DNA sequence. One usually starts with several, but as the sequence in the gaps is obtained, multiple contigs coalesce into one.

Locus: A general term for a place on the chromosomes. It may be a region, a base-pair, or functionally defined.

Paracentric inversion: A rearrangement of the chromosome in which a portion of a homolog is flipped and the centromere is NOT included in the flipped region. Important because they often occur naturally and areuseful as phylogenetic and population genetic tools.

Penetrance: A qualitative (low vs. high) or numerical value that refers to the Proportion of individuals in a population that express the phenotype that definitively identifies a particular genotype *when observed*. For example, in *An. gambiae*, c+ / c females have a red stripe on the larval dorsum *generally*. Something like 5% of c+ / c females do not appear to have a red stripe. So one could say that red stripe is about 95% penetrant. In contrast, see 'expressivity.'

Pericentric inversion: A rearrangement of the chromosome in which a portion of a homolog is flipped and the centromere is included in the flipped region. Virtually all inversions of this sort in anophelines are induced by irradiation.

Phenotype: The expressed manifestation of a genotype.

Quantitative vs. discrete traits: Traits whose expression varies primarily in degree. For example: plant height, intensity of flower color, malaria parasite encapsulation. Discrete traits can be classified into classes e.g. white eye vs. wild eye, collarless vs. collarless+, ebony vs. ebony+.

Repulsion: Also called 'trans'. Two alleles being referred to are on the *opposite* homologs. In contrast, see 'coupling.'

Trait: Rough term meaning the same as a 'character'. (Sounds more scientific than 'a thingie!') It is a functional description of some distinct behavior, form, color etc. Used the same as the way we would use it in common speech.

Allelic relationships

Complete dominant: In a heterozygote, only the dominant allele is expressed, and the recessive allele is not. For example: c+ is completely dominant over c. *Stripe*+ is dominant over *st* in *An. albimanus*.

Codominant: In a heterozygote, the phenotypes associated with both of two alleles present are observed. For example: microsatellite alleles are usually referred to as codominant markers because the repeat size of both alleles in a heterozygote can be observed. Another example would be enzyme electromorphs.

Epistasis: The phenotype of the expression of one gene eliminates the expression of another gene i.e. the phenotype of gene A prevents expression of gene B. white anophelines have no pigment associated with the stripe and collarless genes.

Gene interaction: The phenotype that is observed is different from that associated with gene A or gene B, but is a result of their combined effect. For example: *An. gambiae* that have *pink-eye* mutations generally have white or pink eyes. *An. gambiae* that have *red-eye* mutations have red eyes. When they have both *pink-eye* and *red-eye* mutations, the have 'pumpkin' colored eyes. Neither gene alone can produce this effect.

Partially dominant or semi-dominant: In a heterozygote, the dominant allele is expressed to a lesser degree than in a homozyous dominant individual. For example: *A. albimanus ebony* heterozygotes are intermediate in darkness to either homozygote

The Facts of Life

Female Monogamy & Male Polygamy

To generalize the observations of numerous studies, *Anopheles* females mate only once. The sperm with which the male inseminates the female are stored in the spermatheca and are sufficient to fertilize several batches of eggs. Under typical insectary conditions, the sperm in the spermathecae are never depleted.

Males, on the other hand, will mate several females if given the opportunity. One male can fertilize approx. 6-10 females. But again, males are seldom depleted of sperm in typical insectaries due to the limited number of virgin females and the presence of competing males. The genetic significance of these

facts is that progeny from one female can almost always be considered as resulting from the mating of one male. The significance of single-male mating is that among the progeny from a single female (i.e. a 'family'), at most four alleles will be observed.

Chromosome Number

All anophelines have a haploid number of 3: there are two autosomes (chromosomes not involved in sex determination) named chromosome 2 and 3, and sex chromosomes named X or Y. These can be observed in mitotic spreads of brains and testes (**Figures 2.5.1 and 2.5.2**). However, salivary glands and/or ovaries may contain polytenized chromosomes in various species.

Sex determination

Sex determination appears superficially similar to humans and *Drosophila melanogaster*. Females are XX and males are XY. The X chromosomes contain a region or regions of euchromatin, in which most expressed genes are located, and highly condensed heterochromatin which contains highly repetitive DNA and presumably few expressed genes. These regions are fairly distinguishable in chromosome preparations.
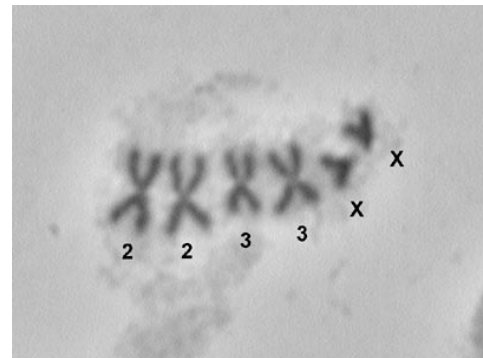
Figure 2.5.1. Female brain metaphase chromosomes of A. gambiae s.s.

Recombination

Recombination frequency is roughly proportional to distance on the chromosomes over most of the genome. Its frequency is similar in males and females in some species (e.g. *An. gambiae, An. albimanus*) but is higher in females in others (*An. quadrimaculatus*). Though observations have been made of differences between the sexes, there is insufficient knowledge to predict in which species a difference will be observed.

Effects of sex on expression

Sex-limited traits

Traits that are expressed in only one of the sexes. Most obvious are sexual morphologic characters like ovarian development. To use the same example again, why do only females express the red stripe since both males and females can have the c+ / c genotype? This observation is expressed by saying that red stripe is a sex-limited trait. This should not be confused with sex-linked traits. It has nothing to do with the location of the gene.

Sex-influenced traits

Traits that can be observed in both sexes, but the *kind or degree* of expression is influenced by the sex. For example: I lied for heuristic purposes. Sometimes red stripe *can* be observed in c+ / c males. However it is so faint relative to females that depending on where one draws the line between

Figure 2.5.1. Male testes metaphase chromosomes of A. gambiae s.s. *While the Y is of variable size in this species, this stock has a very small heterochromatic Y.*

individuals that have a 'red stripe' and those that do not, you could say they don't show red stripe. Regardless, red stripe is so much fainter in males so that even if we do not consider it sex-limited, it is strongly sex-influenced. Traits of this class may also be determined by autosomal genes.

Sex-linked traits

Except for maleness, which is associated with the presence of a Y chromosome, sex-linked traits are determined by genes on the X chromosome.

Linkage Relationships

Autosomal linkage or inheritance

Problem 1: Use the Punnett Square to describe the frequency of gametes, genotypes, and phenotypes when two *collarless* (Mason 1967) heterozygotes mate: The "*c+*" allele of this autosomal gene is fully dominant over "*c.*" Fill in the genotypes and phenotypes in the blanks. Answers can be found at the end of this section.

		Female Gametes	
		1a. *c+* proportion = …………….	1b. c proportion = …………….
Male Gametes	1c. *c+* proportion = …………….	1e. proportion = ………………..	1f. proportion = ………………….
	1d. c proportion = …………….	1g. proportion = ………………..	1h. proportion = ……………….

Note that among the three <u>genot</u>ypic classes of progeny, females and males will occur at equal frequencies in each.

Determine the <u>genot</u>ypic frequencies

1i. c+ / c   _____

1j. c+ / c+   _____

1k. c / c   _____

Determine the <u>pheno</u>typic frequencies

1l. c+   _____

1m. c   _____

Sex linkage

Problem 2: Again, use a Punnett Square to illustrate the pattern. This example uses the sex-linked marker *white* (*w*) which is located on the X chromosome (Benedict et al. 1996). The w+ allele is fully dominant over the w allele. The cross is between a heterozygous female and a hemizygous male who carries the w+ allele. Fill in the pheno- and genotypes including sex.

		Female Gametes	
		2a. w+ proportion = …………….	2b. w proportion = …………….
Male Gametes	2c. w+ proportion = …………….	2e. w+ / w+ proportion = …………….……Sex = ………	2e. w+ / w proportion = …………………..Sex = ………
	2d. Y proportion = …………….	2e. w+ Y proportion = …………………Sex = ………	2e. w Y proportion = ………………..Sex = ………

Note that among the progeny, females and males occur at different frequencies in each eye-color phenotypic class.

Determine the genotypic frequencies

2i. w+ / w female   _____

2j. w / w female   _____

2k. w+ / w+ female   _____

2l. w / Y (male)   _____

2m. w+ / Y (male)   _____

Determine the phenotypic frequencies

2n. w+ female   _____

2o. w female   _____

2p. w+ male   _____

2q. w male   _____

Inheritance of two linked autosomal genes

Problem 3: In this case, we consider two loci that are linked. We will use the Punnett Square to describe the pattern using *collarless* and *Dieldrin resistance* (Rdl) (Davidson 1956; Davidson and Hamon 1962) assuming 15% recombination. Rdl^R is fully dominant. The recombinant gametes of either complementary class will be formed at equal rates. This example is set up as a typical testcross in which a double-heterozygous female is crossed to a homozygous male that has the recessive alleles for both markers.

		Female Gametes			
		Non-recombinant		Recombinant	
		3a. Rdl^R c+ proportion = ………………	3b. Rdl^S c proportion = ………………	3c. Rdl^R c proportion = ………………	3d. Rdl^S c+ proportion = ………………
Male Gametes	3e. Rdl^S c proportion = ………………	3g = ………….	3h = ………….	3i = ………….	3j = ………….
	3f. Rdl^S c proportion = ………………	3k = ………….	3l = ………….	3m = ……….	3n = ………….

Determine the genotypic frequencies.

3o. Rdl^R c+ / Rdl^S c   _____ This is one 'parental' class

3p. Rdl^S c / Rdl^S c   _____ This is the other 'parental' class

3q. Rdl^R c / Rdl^S c   _____ This is one 'recombinant' class

3r. Rdl^S c+ / Rdl^S c   _____ This is the other 'recombinant' class

Determine the phenotypic frequencies

3s. Rdl^R c+   _____ parental

3t. Rdl^S c   _____ parental

3u. Rdl^R c   _____ recombinant

3v. Rdl^S c+   _____ recombinant

Notice that in a testcross, the genotypic and phenotypic frequencies are identical. The frequency of all genotypes can be determined conclusively. In contrast, a backcross to one of the parents may or may not be a testcross.

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Problem answers

1a-d: all 0.5

1e-h: all 0.25. This is calculated as the product of the gamete frequencies of that row and column.

1i: 0.5 (0.25 + 0.25 since there are two sources of these genotypes)

1j: 0.25

1k: 0.25

1l: 0.75 (since c+ is fully dominant, this is 0.25 + 0.5)

1m: 0.25

2a-d: all 0.5

2e and f: both 0.25 and females

2g and h: both 0.25 and males

2i: 0.25

2j: 0

2k: 0.25

2l: 0.25

2m: 0.25

2n: 0.5

2o:0

2p:0.25

2q:0.25

3a and b: 0.425

3c and d: 0.075

3e and f: both 0.5

3g and h: 0.2125

3i and j: 0.0375

3k and l: 0.2125

3m and n: 0.0375

3o and p: 0.425

3q and r: 0.075

3s and t: 0.425

3u and v: 0.075

2.6 Basic Anopheles *Population Genetics*

Mark Benedict

Introduction

Most stocks kept in insectaries are of value because they contain one or more interesting alleles that are either fixed (pure-breeding) or polymorphic in the populations. On the other hand, some laboratories keep a single wild-type stock, and no other stocks are present in the laboratory. In the latter case, the main concern is rarely contamination with a different species, but changes in allele frequency, excessive inbreeding etc. In that case, the relative fitness of various alleles will determine whether one or another is lost or becomes fixed over time.

The following information is primarily directed toward the issues of maintaining alleles in polymorphic populations, and of the fate of alleles in contaminated stocks. As in the other genetics chapter, we present some simple problems that are similar to those encountered in the insectary. These will assist you in planning crosses, isolating mutations etc. Make plenty of drawings and your own Punnett squares to figure these out.

Before you read this section, you should be very comfortable with the contents of the chapter on the basic Mendelian genetics of *Anopheles* and the background to that chapter.

Hardy-Weinberg Equilibrium and the Binomial Equation

You're probably familiar with the assumptions of Hardy-Weinberg Equilibrium: a hypothetical model that predicts the frequency and stability of allele frequencies in populations given certain assumptions. According to its principles, the fundamental dynamics of the fate of alleles introduced into populations can be predicted by some fairly simple equations and applications of probability. While these predictions assume a few facts to be true that may not be, for Mendelian traits in laboratory stocks of mosquitoes, these assumptions approximate reality sufficiently that you can predict probable mating, allele, and geno- and phenotypic frequencies for planning experiments.

Two conclusions flow from their principle (quoted from (Strickberger 1968). As we are using the terminology, it would be more proper to substitute "allele" for "gene" in the following description.):

1. Under conditions of random mating in a large population where all genotypes are equally viable, gene frequencies of a particular generation depend upon the *gene* frequencies of the previous generation and not upon the *genotype* frequencies.

2. The frequencies of different genotypes produced through random mating depend only upon the gene frequencies.

The best-known method for determining allele and genotype frequencies in a randomly mating population is a simple binomial equation (considering only two alleles per gene or locus):

$p^2 + 2pq + q^2$ which is the same as $(p+q)^2$ and these must equal 1

The variables 'p' and 'q' are the frequencies of either of two alleles where $p + q = 1$. The frequency of the " p p " (homozygous) genotype is simply " p^2 " and the frequency of " q q " genotype is " q^2 ." The frequency of the heterozygous genotype is " 2 p q ."

Problem 1: *red eye* (*r*) is recessive to wild type (*r+*, (Beard et al. 1994). In a cage of randomly mating mosquitoes, you observe 4 out of 100 individuals that have the *red eye* (*r r*) phenotype.

If p = the allelic frequency of *r* and q = the allelic frequency of *r+*, what are the allelic frequencies in this population? Substitute the value you know into the binomial equation and solve for the other variable.

1a. frequency p = ………………..

1b. frequency q = ………………..

Using the binomial equation, what are the expected frequencies of *r+ r+* and *r+ r* genotype individuals?

1c. Proportion *r+ r+* (i.e. q^2) = ………………..

1d. Proportion *r+ r* (i.e. 2pq) = ………………..

You have now estimated the frequencies of both the *alleles* and *genotypes* based on a quick determination of the number of *red eye* individuals. The simple binomial equation allows you to make useful conclusions about the population!

Since we are assuming that individuals of all genotypes mate with one another randomly with no preference, we can estimate the probability of various *mating*s by simply multiplying the genotypic frequencies.

Problem 2: From the above cage of adults, you selected only wild eye-color males and virgin females and mated them to one another. You have collected eggs from 21 of these females and obtained 20 families of larvae.

Among what proportion of families do you expect to observe *red-eye* progeny? In the table, first circle the matings that will produce *red-eye* progeny. (Use the Punnett square as an aid to calculate the expected mating frequencies. There is a little trick here. You have to *think*!)

		Proportion Females (hint: these proportions must equal 1)	
		2a. proportion heterozygotes (*r+ r*) = ………………..	2b. proportion homozygotes (*r+ r+*) = …………………..
Proportion Males	2c. proportion heterozygotes (*r+ r*) = …………………….	2e. proportion = ………………..	2f. proportion = ………………..
	2d. proportion homozygotes (*r+ r+*) =………………………	2g. proportion = ………………..	2h. proportion = ………………..

Approximately <u>how many families</u> will contain *red eye* progeny?

2i. number of families = …………………

You may be wondering, "What is the probability that if I have 20 families I won't get *any* that have *red-eye* individuals just by chance?" Simple probability can be used to estimate the probability of getting various numbers of families. (Usually, one is interested in knowing how many families you need to isolate to ensure you'll get at least one that you desire; in this case a *r+r* X *r+r* mating.)

As a heuristic device to determine the value, let's think of the recurrence of the worst case: What is the probability of isolating one family and it will NOT have any *red-eye* individuals? It is simply…

1 minus the probability that one DOES contain *red eye* which = 2j. …………………

The probability of isolating 2 families, neither of which contain any *red eye* progeny is simply the probability of isolating the one above times the probability of isolating a second whose probability is EQUAL TO isolating the first family. (NOTE that these are independent events.)

p X p = p^2

2k. …………………..

So if we take p to the 2nd power for two families, we take it to the 'nth' power for 'n' families. So for 20 families, the probability of NOT seeing any families containing *red eye* is simply

2l. ……………………

<u>Problem 3:</u> *collarless* is a very common polymorphism in *Anopheles gambiae s.l.(Mason 1967).* The *c* allele is recessive to *c+* and therefore both " *c+ c* " and " *c+ c+* " individuals have identical phenotypes. Suppose you wanted to purify a pure-breeding " *c+ c+* " stock. You go to the G3 (an old wild stock from The Gambia) stock tray and out of 100 larvae, you find that 90 are phenotypically *c+*. You inbreed the *c+* phenotype individuals.

In the following exercise, we will determine the probability that you will get <u>at least</u> one family that was the result of a mating between two " *c+ c+* " individuals if you obtain 30 families from the c+ individuals mated to one another.

First, calculate the allele frequencies in this population using the binomial equation.

If p = the allelic frequency of *c* and q = the allelic frequency of *c+*

3a. p^2 = ………………...

3b. p = …………………

3c. q = …………………

Using the binomial equation, what are the expected frequencies of *c+ c+* and *c+ c* genotype individuals?

3d. proportion *c+ c+* (i.e. q^2) = ………………..

3e. proportion *c+ c* (i.e. 2pq) = ……………….

Remember, as in the previous example where individuals have been removed from a population, we're inbreeding ONLY the *c+* phenotype individuals so you must adjust the frequencies of the above *c+* phenotypes before proceeding to reflect the fact that they now make up all (= 1) of the individuals being considered.

Proportion *c+ c+* (i.e. q^2) + Proportion *c+ c* (i.e. 2pq) = 1

		Proportion Females	
		Heterozygotes (*c+ c*) 3f. proportion 2pq = ………………..	Homozygotes (*c+ c+*) 3g. proportion q^2 = ………………..
Proportion Males	Heterozygotes (*c+ c*) 3h. proportion 2pq = ………………..	3j. proportion =………………...	3k. proportion = ………………..
	Homozygotes (*c+ c+*) 3i. proportion q^2 = ………………..	3l. proportion = ………………..	3m. proportion = ………………..

We asked, "If you isolate 30 families, what is the probability that you will get <u>at least</u> one family that was the result of a mating between two ' *c+ c+* ' individuals?" This is the converse of asking, "If you isolate 30 families, what is the probability that ALL will be from matings between <u>something other than</u> *c+ c+* types?"

3m: p^{30} = ………………..
3n: and the likelihood of getting at least 1 family is $1 - p^{30}$ or ………………..

Fitness Effects in Polymorphic Populations
Finally, we consider polymorphic alleles in populations where the fitness of the various alleles is not equal. Fitness in the genetic sense refers simply to the relative reproductive success of an allele or a genotype. Mutant alleles are usually assumed to have reduced fitness relative to wild-types, but this is not always so. Perhaps the *collarless* phenotype is less fit than wild-type. If so, what happens to the frequency of that phenotype and the *collarless* allele frequencies over time? Other alleles whose fitness we might want to consider in the insectary are recessive lethals, insecticide resistance alleles, and parasite susceptibility alleles.

Alleles can have a positive or negative fitness depending on the environment.

Fitness can be expressed as an adjustment variable 's' to the allele frequency. For example, a neutral allele with no effect on fitness has a relative fitness of ' s = 0 ' such that the allelic frequency adjustment would be q (1- s) or q (1 - 0) or simply q. On the other hand, a lethal has a fitness of 1 so that q (1- s) or q (1 - 1) becomes simply 0 (zero).

Alleles with fitness of <1 will tend to become less frequent in populations. However, they do not necessarily disappear. The practical application is to <u>ensure frequent enrichment</u> of individuals in polymorphic stocks whose genotype is known to carry reduced fitness alleles.

Obviously, how this is applied to the calculation of genotypic and phenotypic frequencies depends on the dominance relationship and whether the fitness effect is on zygotes or gametes, but suffice to say that generally the <u>rate of effect on allele and phenotypic frequencies is proportional to the fitness</u>. This is illustrated in the following table from Strickberger (1968):

Table 31–6
Number of generations necessary for a given change in *q* of a deleterious recessive gene under different selection coefficients

Change in gene frequency		Change in frequency of homozygotes		No. generations for different s values						
From q_0	*To* q_n	*From* q_0^2	*To* q_n^2	s = 1 (*lethal*)	s = .80	s = .50	s = .20	s = .10	s = .01	s = .001
.99	.75	.980	.562	} 1	5	8	21	38	382	3,820
.75*	.50	.562	.250	}	2	3	9	18	176	1,765
.50	.25	.250	.062	2	4	6	15	31	310	3,099
.25	.10	.062	.010	6	9	14	35	71	710	7,099
.10	.01	.010	.0001	90	115	185	462	924	9,240	92,398
.01	.001	.0001	.000001	900	1,128	1,805	4,512	9,023	90,231	902,314
.001	.0001	.000001	.00000001	9,000	11,515	18,005	45,011	90,023	900,230	9,002,304

* The change in gene frequency is most rapid when q_0 = .67.

Several things should be observed from this table:

- Observing the vertical axis trend, as the overall frequency of an allele decreases, the <u>rate</u> of decline in the allele (gene) frequency also decreases. Why? Since selection is occurring on homozygotes, the probability of individuals having two of the reduced fitness alleles becomes smaller as the probability of appropriate matings to produce these declines.

- Observing the horizontal axis trend, as the fitness effect diminishes, the number of generations required for a given change in allele frequency to occur becomes greater. This is rather intuitive. We expect a lethal to have a more rapid effect on the frequency of the lethal allele than say an allele for laying an average of 110 vs. 111 eggs.

The take home messages of this discussion of fitness are the following:

- You can expect lethals to be maintained in laboratory populations at low frequencies unless they are balanced by something or artificially selected.

- Conversely, you can expect alleles with weak effects on fitness to be maintained at consistent frequencies.

- Genetic rearrangements such as inversions and translocations that cause semi-sterility and/or are homozygous lethal will decline in frequency and possibly become extinct unless deliberately selected for or balanced.

- Recessive alleles that have reduced fitness relative to wild-type are very persistent even in the absence of positive selection.

- Inbred populations tend toward fixation of the alleles with the strongest effects on fitness.

References

Beard CB, Benedict MQ, Primus JP, Finnerty V, Collins FH (1994) Eye pigments in wild-type and eye-color mutant strains of the African malaria vector *Anopheles gambiae*. J Heredity 86:375-380

Mason GF (1967) Genetic studies on mutations in species A and B of the *Anopheles gambiae* complex. Genetical Res, Cambridge 10:205-217

Strickberger MW (1968) Genetics. Macmillan, New York

Answers

1a: 0.2 (this is the square root of the frequency of " r r " individuals)

1b: 0.8

1c: 0.64

1d: 0.32

2a: 0.33

2b: 0.67

2c: 0.33

2d: 0.67

2e: 0.1089

2f: 0.211

2g: 0.211

2h: 0.4489

2i: about 2

2k: 0.79

2l: 0.0997 or odds of about 1 in 10

3k: 0.25

3l: 0.25

3j: 0.23

3n: 7.9×10^{-5}

3o: 0.999

3a: 0.10

3b: 0.316

3c: 0.684

3d: 0.47

3e: 0.43

3f: 0.48 (2pq X 1.111)

3g: 0.52

3h: 0.48

3i: 0.52

3m: 0.27

Chapter 3: Specific *Anopheles* Techniques

3.1 Embryonic Techniques

3.1.1 Microinjection Methods for *Anopheles* Embryos

Mark Benedict

Introduction

We present two methods that have been successful (and a variation of Method 1 using oil). The first was developed by John R. (Randy) Clayton for injection of *An. gambiae* embryos, and it has been used to obtain high frequency egg hatching and EGFP transient expression rates as described by Grossman et al., 2001 (Grossman et al. 2001). This method is similar to that used by Dave O'brochta's group at the Univ. Maryland. They successfully transformed *An. gambiae* using a similar method by covering the embryos with halocarbon oil prior to injection (Kim et al. 2004). Using oil should provide better visibility of the needle flow rate.

The second method is fast and requires less judgment than those above. It was developed by Hervé Bossin and Mark Benedict for *An. arabiensis* and *An. gambiae*. However, it should be useful for many mosquito species. Anecdotally, mosquito species vary in the ease with which they can be microinjected. Both of the methods above have been used successfully with *gambiae* s.l. which is supposed to be one of the more difficult to inject.

We recommend mounting the injection needle in a fixed position and moving the slide holding the aligned embryos using the stage controls to the appropriate place for injection. This allows one to use a rather simple needle positioner mounted on or by the microscope.

Anopheles embryos cannot be dechorionated, so use of rigid needles and firm positioning of the embryos is necessary. Quartz glass injection needles are by far preferable to aluminosilicate or borosilicate needles. These require higher pulling temperatures than the other glasses and therefore a laser needle-puller must be used.

Materials:

- Mated adult females bloodfed 3-5 days post-eclosion.
- Clean water in a wash bottle
- Pipettor e.g. P20
- Fine paint brushes[1] and forceps
- Filter paper
- 2X Na phosphate Injection buffer (see below for preparation; requires KCl and di- and mono-basic sodium phosphate)
- Minimum fiber filter paper e.g. (Whatman 1450-090, 'Hardened circles')
- Eppendorf Microloader tips (no. 5242-956-003)
- Ultrafree-MC filters (no. UFC30HV00)
- Quartz glass capillaries, 1 mm OD, 0.7 mm ID X 10 cm length (e.g. Sutter no. QF100-70-10)

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[1] Select the brushes carefully from among the finest at an art or craft store. Sable brushes are excellent and more expensive, but regardless, a very fine pointed tip is essential.

- Double-sided adhesive tape. 3M type 415 has been tested for vertebrate toxicity and is a good choice (Method 1 only)

- 22 x 22 glass or plastic coverglasses with a strip of pre-cut double-sided tape attached (Method 1 only)

- 25 mM NaCl (or 10-50 mM range for testing, Method 1 only)

- Millipore (or other) mixed cellulose ester membranes (e.g. HAWP02500- US or HAWP03700, Method 2 only)[2]

Equipment

- Either a compound or high-quality dissecting microscope can be used for injections. It is preferable if it can be dedicated to this purpose.

- Sutter P-2000 Micropipette puller or similar device[3]

- Needle positioner and holder

- pH meter

- Eppendorf Femtojet or similar device equipped with a foot pedal

- Dissecting scope and illuminator for embryo alignment

Solutions

This recommendation is for *Drosophila melanogaster* from Bill Engels lab, but it seems suitable for mosquitoes. Prepare two 0.1 M solutions of monobasic and dibasic sodium phosphate. Mix the two and adjust pH with one or the other to pH 6.8-7.8. Prepare a solution of 0.5 M KCl in purified water.

2X injection buffer is:

> 0.2 mM Na phosphate
>
> 10 mM KCl

Filter sterilize and store at room temperature or lower.

Starting procedures common to both methods

1. Prepare the capillaries by flushing them several times with purified water followed by ethanol to remove lint and glass chips. Blot the remaining ethanol and flame the capillaries briefly to remove all liquid. It is convenient to store them in a covered glass culture tube.

2. Immediately before use, thaw the DNA and filter through a 0.2 micron Millipore Ultrafree-MC filter filter to remove particulates. This latter measure (suggested by D. O'brochta) is simple and effective. Store on ice until use.

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[2] Any non-fibrous membrane that is very thin, hydrophilic and does not contain detergent would probably work for this e.g. Southern blotting membrane. If in doubt regarding the presence of wetting agents etc. rinse the membrane well before use. These types of membranes are perfect because when wet, they adhere closely to a microscope slide so that the embryos don't slide beneath the membrane, and they are about the same thickness as an embryo so visualization is easy.

[3] The program we use for the P-2000 is: HEAT: 650, FIL: 4, VEL: 40, DEL: 150, PUL: 157. However, conditions necessary to produce suitable needles may differ on your device and may require slight change, even from day to day.

3. Harvest eggs 60-96 hours after females are bloodfed by placing 10 to 15 females in a transparent cylindrical container (~40 ml) open at one end and covered with rubber dental dam at the other (**Figure 3.1.1.1**). Alternatively, eggs may be collected on damp filter paper in a small Petri dish[4].
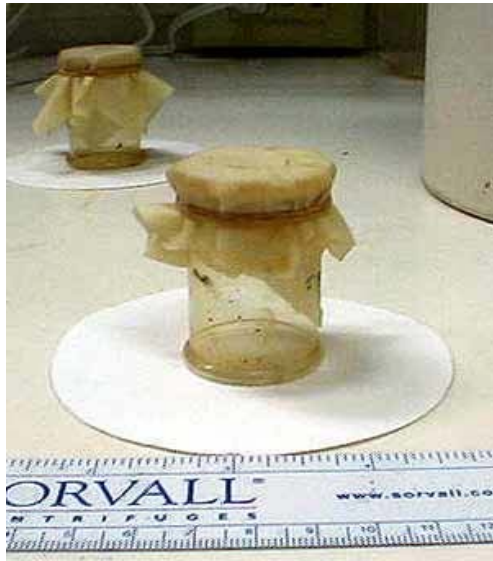
Figure 3.1.1.1. Cylindrical container (~40 ml) containing 10-15 previously bloodfed females open at one end and covered with rubber dental dam at the other

Figure 3.1.1.2. Container with females resting over a single water-filled well on a cobalt blue ceramic depression plate. Embryos deposited in single depression shown (inset).

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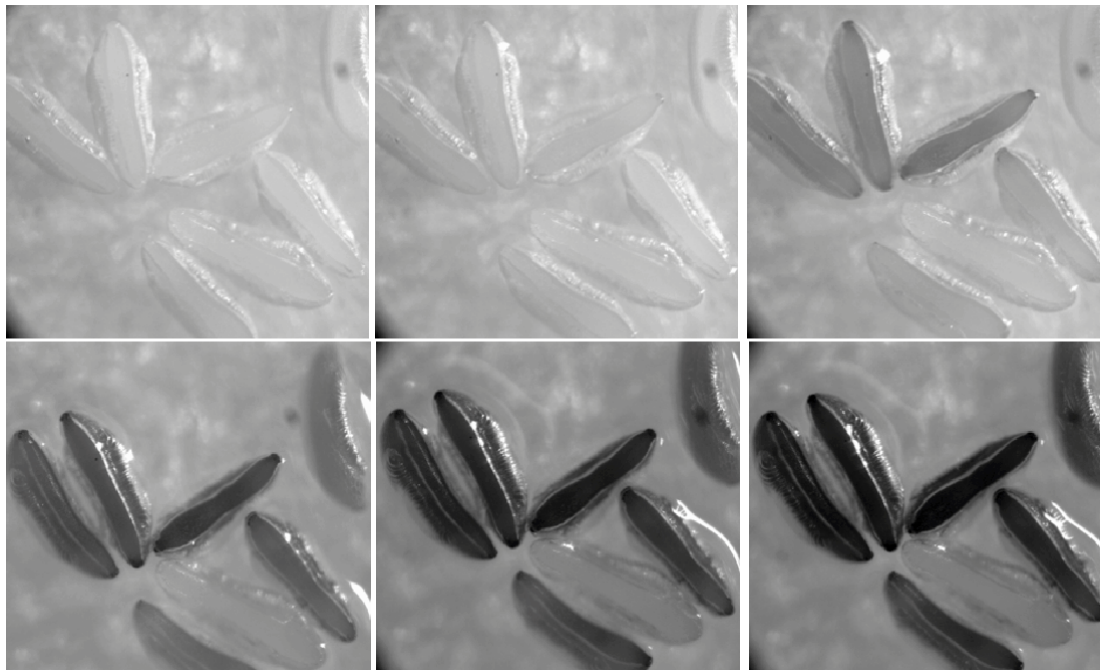
[4] Cooling the eggs will extend their useful time, but MQB's experience has been that this also reduces survival.

Figure 3.1.1.3. Egg darkening at 20 min intervals (top left to bottom right) beginning approximately 15 m after oviposition and incubated at room temperature (~22°C). Most of the darker eggs in the lower two left panels are suitable for injection. Note that one egg did not darken. It will not hatch. When eggs have fully darkened (as in the lowest right panel) but are often still at the blastoderm stage, they are more difficult to inject (photographs courtesy of G. Labbe, Oxitec, used with permission). Non-melanized eggs cannot be handled without suffering mortality.

4. Slide the container over a single water-filled well on a depression plate such that the females can access the water (see **Figure 3.1.1.2**). Cover to darken for 30 minutes and then remove the females.

5. Age the new embryos for at least 30 minutes at insectary conditions (28°C; 80% humidity) or at room temperature. After this time, the eggs should be medium gray (**Figure 3.1.1.3)**.

Method 1:

The distinctive feature is that embryos are injected under saline, the concentration of which is determined empirically to balance internal pressure. The balance pressure must be such that the turgidity is sufficient for needle penetration yet low enough that oozing and needle backflow are minimized. The salt concentration can be adjusted as needed to achieve this for each species.

1. At room temperature (~ 24°C), transfer eggs from the depressions to a glass slide with a fine paintbrush and align with the dorsal (flattened, concave) surface facing up.

2. Align the anterior ends of 25-35 eggs in 25 mM NaCl against a strip of reduced-fiber filter paper.

3. Remove the filter paper by tugging it away sharply, so as not to disturb the alignment of the embryos.

4. Allow eggs to desiccate slightly.

5. Press a taped coverslip gently against the eggs' dorsal surface and immediately invert. Getting the embryos to stick to the tape is the most difficult part of this procedure. They cannot be too wet - they will not stick - or too dry - in which case they die.

6. Cover with a solution of 25 mM NaCl (or Halocarbon oil) to prevent drying and place eggs in a humid box at room temperature until injection. Eggs are appropriate for injecting around 2 hours after deposition when they have are medium dark. Choosing eggs for injection is discussed in **Figure 3.1.1.3**.

7. Immediately prior to injection, add more 25 mM NaCl to the coverslip. A large volume surrounding the embryos is desirable as it reduces distortion of the image. Attach the coverslip to a glass slide with a bit of double-stick tape along the edge and place on the stage.

8. Inject embryos on the ventral surface, near the posterior end, with the embryo turned at an angle of about 15-25 degrees. The horizontal angle of the needle can vary, but should be roughly within 30 degrees vertical from the plane of the stage. Take care to avoid injection into the periplasmic space. Instead, inject immediately anterior to the periplasmic space and posterior to the egg floats. Injections should be carried out at 100X magnification.

9. Immediately after all of the embryos on a slide are injected, remove the slide from the scope and and place the coverslip carrying the eggs into a cup of reverse-osmosis/deionized sterilized (RO/DI) H_2O at room temperature to recover.

10. When all injected coverslips from a cohort have been aligned and injected, placed all coverslips in a cup of 50 ml RO/DI H_2O under insectary conditions to hatch. It is not necessary that the eggs float for hatching.

11. Hatching will begin in approximately 48 hours after which the larvae can be handled as described in the chapter on family culture.

Method 2:

This method differs in that embryos are aligned against a thin membrane and injected semi-dry. No adhesive tape is necessary.

1. Cut pieces of membrane with a scalpel or razor blade at an approximately 45° angle so that the posterior edge for injection will be perpendicular to the needle. A cleanly cut edge is desirable.

2. Cut a piece of filter or blotter paper smaller than the height of the slide. You may wish to stack a couple of pieces to provide a larger water reservoir.

3. Assemble the membrane and filter paper as shown (**Figure 3.1.1.4**) and wet with water so that all paper is wet and the membrane is moist but not dripping.

4. Using a brush, transfer 30-50 embryos to the edge of the membrane.

5. Distribute them as shown (**Figure 3.1.1.5**) with the narrower posterior end toward the bottom. When aligning the embryos, roll them over so that the ventral side (convex) is upward, and they will nest nicely in the 90° niche between the membrane and slide.

6. Orient all in the same direction. As you work, keep the papers moist by adding small volumes (10 µl) of water to the blotter paper. You should maintain a meniscus of water around the eggs, but do not wet excessively causing the eggs to become dislocated.

7. When you have filled the edge of the membrane with eggs (~50), transfer to the scope for injection. Keep in mind that when using this technique, the needle will not be submerged in liquid, so keep sufficient back-pressure on the needle to keep it cleared. Frequently check the needle flow by withdrawing the needle and 'inject' into air. You should see a small droplet appear or run back up the needles into a larger droplet that often hangs on the needle shaft. Add small volumes of water to the blotter paper as the eggs dry during injection.

8. After injection rinse the eggs off into a Petri dish using water and incubate as in Method 1.
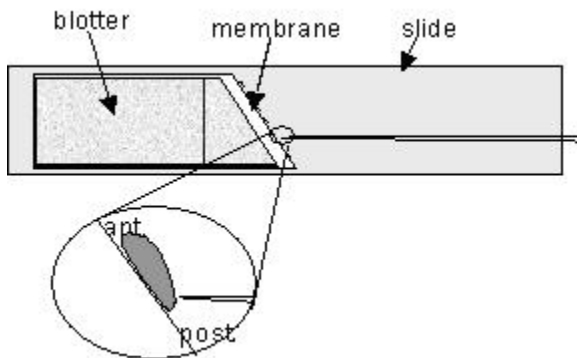
Figure 3.1.1.4. Diagram of microinjection method using membranes as opposed to the adhesive double-stick tape.
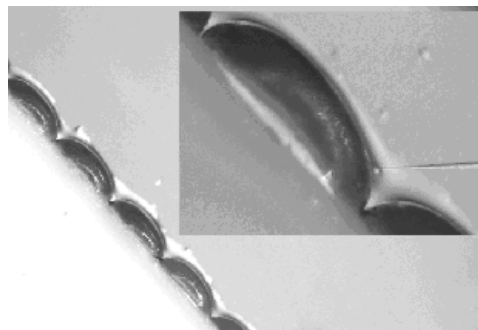
Figure 3.1.1.5. Eggs aligned for injection with a closeup of the needle approaching perpendicular to the injection area (photograph courtesy of G. Labbe, Oxitec).

Other things you might want to know

Q: Do you remove the chorion before injection as has been described in *Drosophila*?

A: No. Endochorion removal in anophelines has not been accomplished. This is why the quality of the needles and turgor of the eggs is crucial.

Q. What does a good *An. gambiae* injection look like?

A: Larval hatch rates vary between 10% and 50% using either method. The most probable cause of this variation is physical wounding of the embryo during injection. If the needle does not slide easily through the chorion of the egg during injection then something is wrong. It is the ease of penetration that allows continuous injection without needle clogging or breakage.

Under good conditions, the needle will slide in and out of the egg with little effort. Slight resistance to penetration is apparent when entering the egg and a small volume of yolk can sometimes be seen flowing into the tip of the capillary, only to be expelled immediately during injection. Although visibility is worse injecting under aqueous solution rather than halocarbon or mineral oil, a slight clearing of the yolk is often seen, even through the dark chorion. Injected eggs sometimes recoil and bulge briefly and slightly when a sufficient volume has been released into them and this is also a good sign as long as a minimal amount of yolk escapes from the wound site.

Q: I don't have a laser needle puller. Will this method work with boro- or aluminosilicate needles?

A: In principle, there is no reason why this method would not work with a softer glass but with frequent needle replacement; however, an attempt at this has not been published. Quartz needles may simply allow a larger degree of error on the part of the person injecting. Aluminosilicate glass needles are preferable to borosilicate because of their greater hardness.

Q: Have you used a chorion hardening inhibitor?

A: No. Many inhibitors have been tested of the prophenoloxidase activation cascade (pNpGB, benserazide, PTU), but we have found nothing that clearly resulted in an increase in embryo injectability.

Q: Do you bevel your needles?

A: No. Non-beveled quartz is hard and sharp enough so that needles can be pulled and used immediately.

Q: How do you prepare your DNA for injection?

A: DNA was prepared with a Qiagen Endo-Free kit and resuspended in injection buffer. It was then stored at -80°C until use.

Q: What do you feed your hatching larvae?

A: We feed L1 larvae two drops of 2% w/v baker's yeast on day two post-injection and another two drops on day four. Beyond day four, we feed as appropriate with our standard food mixture of finely ground Koi Floating Blend.

Q: I thought anopheline eggs floated when they hatched. Aren't your injected embryos submerged when they hatch in Method 1?

A: Yes. While *Anopheles* eggs typically do float, submerging eggs post-injection does not seem to have a strong effect on mortality relative to floating controls. In addition, this method avoids the large degree of mortality which was inflicted when attempting to remove the eggs from the adhesive surface of the tape.

Q: How hard do you press the coverslip down on the embryos when you're picking them up in Method 1?

A: Delicately but firmly (!?). Just hard enough to see that the eggs have come into contact with the tape and bulge slightly.

Q: What happens if I inject the embryos earlier than you describe?

A: Younger embryos are difficult to inject due to sensitivity to handling. Simply moving them early in development kills them.

References

Grossman GL, Rafferty CS, Clayton JR, Stevens TK, Mukabayire O, Benedict MQ (2001) Germline transformation of the malaria vector, *Anopheles gambiae*, with the piggyBac transposable element. Insect Mol Biol 10:597-604

Kim W et al. (2004) Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae): effects on susceptibility to *Plasmodium*. J Med Entomol 41:447-455

3.1.2 Anopheles Embryo Fixation

MR4 Staff

Introduction

The following embryo fixation method is suitable for preparing *An. gambiae* embryos for immunostaining and may be suitable for other anophelines or even genera of mosquitoes. It was developed and used by Yury Goltsev (2004) and further tested by John Yoder (2006). After fixation, embryos of the proper developmental stage must be selected from the pool for analysis. Removal of the relatively impermeable chorion of *An. gambiae* requires a method different from that used for *Drosophila melanogaster*.

Solutions

- purified water e.g. distilled or reverse-osmosis/deionized
- 25% household bleach diluted in purified water
- heptane
- 9% formaldehyde in purified water, adjust to pH 7 with NaOH.
- methanol

Materials

- glass vials e.g. scintillation vials
- 100 micron nylon mesh or similar (for device in **Figure 3.1.2.1**)
- Pasteur pipettes

Procedure:
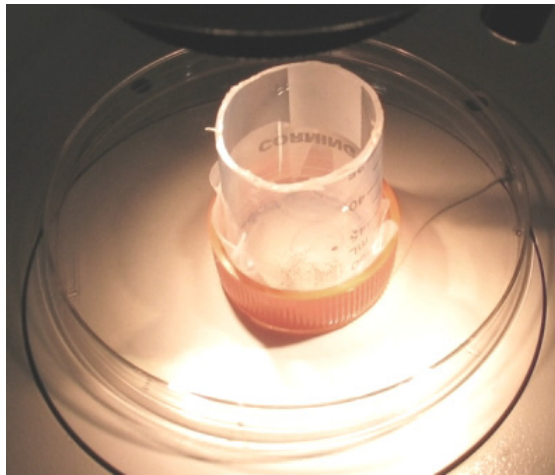
Figure 3.1.2.1. Eggs must be contained in a manageable mesh container in which solutions can be added and removed by draining and rinsing. Shown is an example of a possible container improvised from a 50 ml disposable tube that was cut. The lid clamps the mesh and has a hole to allow solutions to pass.

1. Remove egging cup containing newly laid embryos from mosquito cage and hold at 20°C. Embryos can be collected for about 3 hours and then held until the desired developmental stage.

2. Rinse eggs into a fine mesh basket (e.g. 100 micron nylon mesh) with deionized water. Place mesh with eggs into an empty Petri dish under a stereo microscope. An example of a possible container for eggs is shown in **Figure 3.1.2.1**.

3. While watching the embryos through the microscope, gently add bleach solution to the egg container until the eggs are floating. This step washes away the exochorion. Swirl gently 1-2 times; remove the mesh container when approx 50% of the eggs sink.

4. Rinse eggs and mesh container thoroughly with deionized water.

5. Place eggs in a new Petri dish containing purified water while you "test crack" a sample of the embryos. This step is necessary to ensure that the exochorion was removed during the bleaching step. If this layer is not removed, the fixatives will not permeate the embryo and fixation will fail.

    a. Aliquot approx. 25 test embryos into a scintillation vial. Remove the water with a pipette and add 5 ml heptane.

    b. Incubate at room temperature for 5 minutes while occasionally swirling gently.

    c. Add 5 ml of methanol and vigorously swirl once to mix. Place scintillation vial on its side under the stereoscope and watch for cracking (**Figure 3.1.2.2).** It is normal for the embryos to seep out of their chorion. If the embryos crack, discard these test eggs and proceed with the protocol. If they do not, longer bleaching is needed.
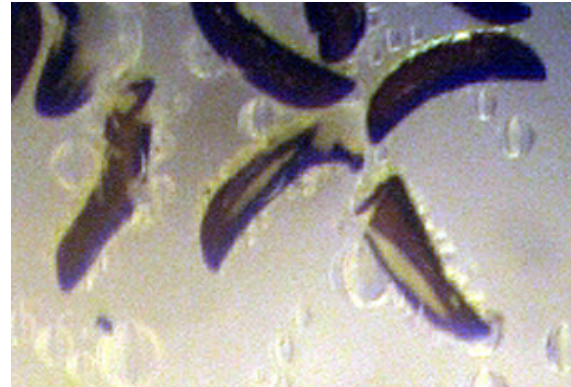
Figure 3.1.2.2. Examples of eggs cracking properly. Cracking is an indication that the process is proceeding correctly, and you can continue. Discard these test eggs.

6. Rinse embryos into a new scintillation vial with deionized water. Remove as much water as possible using a Pasteur pipette.

7. Add 5 ml heptane and shake 3-4 times gently by hand to mix. Remove as much remaining water as possible. Add 5 ml formaldehyde. Shake on rotary platform for 25 minutes on a medium speed setting. Eggs will accumulate between layers as shown in **Figure 3.1.2.3**.

8. Remove formaldehyde phase only (leaving heptane phase) using a fresh pipette. Replace with a large volume of deionized water. Briefly shake 3-4 times gently by hand and remove only the water phase (leaving heptane phase). Add 10ml of fresh deionized water.

9. Shake on platform an additional 30 minutes on a medium speed setting.

10. Remove only water phase (leaving heptane phase). Fill vial to the top with boiling deionized water. Incubate for 30 seconds.

11. Remove hot water phase (leaving heptane phase) and replace with ice-cold deionized water.

12. Place vial on ice for 10 minutes.

13. Remove water phase using a glass pipette.

14. Remove as much of the heptane phase as possible.

Figure 3.1.2.3. Non-damaged eggs will accumulate at the interface between two layers.

15. Add 5ml of fresh heptane. Remove as much water as possible.

16. Add 5ml methanol and swirl vigorously once, place scintillation vial on its side under stereo scope to watch for cracking, a sign the fixation has been successful to this

point.

17. Let stand 15-20 minutes.

18. Remove as much of both phases as possible. Rinse with 5 ml methanol twice removing any excess liquid, then add 5 ml fresh methanol.

19. At this point, the embryos can be stored at -20°C in methanol for several months.

20. The endochorion must be manually peeled away using fine needles and double stick tape before staining.

References

Goltsev Y, Hsiong W, Lanzaro G, Levine M (2004) Different combinations of gap repressors for common stripes in *Anopheles* and *Drosophila* embryos. Dev Biol 275:435-446

Yoder JH, Carroll SB (2006) The evolution of abdominal reduction and the recent origin of distinct Abdominal-B transcript classes in Diptera. Evol Dev 8:241-251

3.1.3 Establishing Cell Lines from *Anopheles* spp. Embryonic Tissues

Ulrike Munderloh

Materials

Anopheles eggs

Mosquitoes are commonly reared at ~28°C; other temperatures are suitable, but will influence the timing of egg production and embryonic development. Female mosquitoes are provided a blood meal from a suitable host in the afternoon of day 0. The afternoon/evening of day 2, a dish with clean water is placed in the cage, to allow females to deposit eggs over night.

Eggs aged 24-36 hrs are collected using a transfer pipette, strip of screen, or filter paper, and added to a 35 mm diameter Petri dish containing 70% ethanol with a drop of Tween 80 (e.g., Sigma-Aldrich catalog Nr. P4780). The eggs will sink, and should be agitated by swirling the dish. The ethanol is replaced with 0.5% benzalkonium chloride (e.g., Sigma-Aldrich catalog Nr. 09621) with a drop of Tween 80, and the dish again agitated for 5 min. The benzalkonium chloride is removed, and the eggs are rinsed 2-3 times in sterile, distilled water. 50~100 eggs are transferred to a new dish containing 0.2 ml of culture medium supplemented with 10-20% fetal bovine serum (FBS, heat-inactivated), 5-10% tryptose phosphate broth (TPB), and a mixture of penicillin (50-100 units/ml) and streptomycin (50-100 µg/ml; e.g., Invitrogen catalog Nr. 15140-122) and fungizone (0.25 – 0.5 µg/ml; e.g., Invitrogen catalog Nr. 15290-018).

Media

We have used Leibovitz's L-15 medium successfully, as well as a modification thereof, L-15B, diluted to ~300 mOsm/L using sterile cell culture grade water (Munderloh and Kurtti 1989; Munderloh et al. 1999). Other media may be substituted, such as RPMI1640, Medium 199, Eagles' MEM, or Ham's F12 (e.g., from Invitrogen, http://www.invitrogen.com/site/us/en/home/Applications/Cell-Culture/Mammalian-Cell-Culture.reg.us.html) with 10% - 20% FBS (Invitrogen or Sigma) and 5-10% TPB (Becton Dickinson, catalog Nr. 260300), but should be tested for their ability to sustain primary and established cell lines. The pH of the medium should be adjusted to 7.0 to 7.2 using either sterile 1-N NaOH or 1-N HCl, as needed. If the medium pH drifts up beyond 7.8, it may be useful to add a buffer such as HEPES (e.g., Invitrogen catalog Nr. 15630) or MOPS (e.g., Sigma-Aldrich catalog Nr. M1442) at ~25 mM concentration

Methods

Eggs are crushed by applying gentle downward pressure using a sterile glass or plastic plunger from a 3 or 5-ml syringe, the flattened end of a sterile glass rod, or similar device. Crushed eggs and tissues are collected with a 2-ml pipette, and transferred to a 5.5 cm^2 flat-bottom tube (Nunc, catalog Nr. 156758) in 1-2 ml of complete medium containing antibiotics and antifungal solution as above, and the tubes are tightly capped. Cultures are incubated flat side down at 28-31°C. Use of a CO_2 incubator is not necessary and not recommended.

Cultures are fed approximately once a week by removing as much of the medium as possible without aspirating any tissue fragments or cells and replacing it with 2 ml of fresh medium. Antibiotics/antifungals should be included in the medium for the first few weeks, and can be omitted subsequently. If it is desired to continue using antibiotics, the antifungal component should be omitted. A mixture of penicillin and streptomycin is preferable over gentamycin as the latter may adversely affect mosquito cell lines in the long term.

The progress of the cultures is best monitored using an inverted phase contrast microscope. During the first days after adding the embryonic fragments, most tissue clumps will remain non-adherent, and organs such as guts and Malpighian tubules should show active peristaltic movements. Within days to weeks, cells should be migrating out from the torn tissue ends, and will often anchor to the bottom of the tube. Commonly, hollow balls consisting of a "monolayer" of cells surrounding a fluid-filled interior, will be seen

sprouting from embryonic fragments. Once cells in a primary culture have replicated sufficiently, a portion (~1/2 to 1/3) of the tissues can be removed by pipetting, and transferred to a new tube to set up a subculture. It is advisable to keep the other portion of cells in the parent flask or tube, as it is common that they will remain vigorous even if the subculture should fail.

This process is repeated many times until cultures can be subcultured or split on a regular basis, and the culture is considered established. During this process increasingly larger culture vessels will be used, e.g., 12.5-cm^2 flasks, then 25-cm^2 flasks, etc. Although the first several subcultures are usually made by transferring 30 – 50% of the cells to a new culture vessel, with time it is advisable to "push" a cell line by using higher dilutions of 1:10 or more. Some mosquito cell lines can be diluted up to 100-fold. Seeding subcultures at relatively high densities (dilutions of 1:2 or 1:3) will depress cell replication and slow growth, often resulting in cultures of poor condition.

It is not uncommon that a single primary culture will give rise to sublines displaying differing morphologies. Some sublines may continue growth as adherent cells, and others may become established as suspension cultures. In particular, the "hollow ball" or "vesicle" phenotype frequently develops, and may become fixed. Sublines with particular, desired characteristics can also be selected by culture manipulation (e.g., adherent lines can be developed by continuously discarding non-adherent cells during medium changes). Once cells are growing reliably, it is a good idea to try to reduce the amount of FBS, and TPB. Established mosquito cell lines commonly grow quite well with only 5% of FBS. Adding a lipoprotein supplement (such as the one from Rocky Mountain Biologicals, Missoula, MT, or the CellPro-LPS from Fisher Scientific), if available, can further reduce the requirement for FBS, and reduce cost.

These methods can equally be applied to other mosquito species, keeping in mind the length of time required for embryonic development. Eggs should be at least at their half point before hatching, all the way up to just before hatching. Although open culture vessels can be used, such as small Petri dishes or multi-well plates, they require a humidified atmosphere as well as a CO_2 incubator when media containing bicarbonate are employed. Also, open culture vessels are far more susceptible to contamination than closed ones.

Additional references: (Munderloh et al. 1982) (Mazzacano et al. 1991)

References

Mazzacano CA, Munderloh UG, Kurtti TJ (1991) Characterization of a New Continuous Cell Line from the Flood Water Mosquito, *Aedes vexans*. Cytotechnology 5:147-154

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Munderloh UG, Kurtti TJ, Maramorosch K (1982) *Anopheles stephensi* and *Toxorhynchites amboinensis*: aseptic rearing of mosquito larvae on cultured cells. Journal of Parasitology 68:1085-1091

3.2 Eye Color Mutant Screening

Mark Benedict

Introduction

Anopheles mosquitoes undergo induced color change (called homochromy) based on perception of the background against which they are cultured.[1] When larvae are reared on either a dark black or white background, they become pigmented dark or pale respectively as shown by the pair of *An. albimanus* larvae in **Figure 3.2.1**. The degree of darkening depends in part on the length of time the larvae have been cultured in a black container and the degree of fat body development. Therefore, larvae cultured during their entire development in a dark container at a low density show this change most dramatically. This color change depends on the normal eye pigmentation and, presumably, on the proper function of any pigmentation and signaling pathways involved in the response.

The method simply requires culturing the larvae from at least the second stage in black or dark-colored containers that are illuminated. (Larvae cultured in darkness will develop typical pale pigmentation.) The source of illumination does not appear to be critical. Occasional transfers of a few minutes to white containers for feeding or correcting the density does not interfere with the effect.

At the L3 or L4 stage, larvae are scanned *en masse* in the dark tray in a well-illuminated location for those that appear lighter in color. They are usually quite apparent as demonstrated by the two larvae in **Figure 3.2.2**, but purposely seeding a sample of dark larvae with a few that are pale will demonstrate the degree of effect that can be expected. These individuals are transferred to a dish for microscopic examination. Leaving the larvae undisturbed during examination provides better visualization since the dorsal side coloration is a more consistent indicator of general color. After this initial selection, it is also helpful to transfer the larvae to a white tray and scanning for pale individuals. Usually, no more than approximately 25 larvae per thousand cultured in this way require individual examination.

Figure 3.2.1. *Anopheles albimanus* larvae reared on a black background (top) and a white background (bottom).

Figure 3.2.2. Eye color mutants are easier to detect on a dark background as they will not change pigmentation and will appear much lighter in color.

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[1] Before beginning a large screen, it is advisable to culture a thousand or so larvae of the species of choice in dark containers. While all individuals of most species change color, some laboratory stocks have a low frequency of individuals that do not.

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3.3 Determining the sex of Anopheles *larvae and pupae*

MR4 Staff

Introduction

There is often an experimental need to separate the sexes before they emerge e.g. in order to preserve unmated status of females, to obtain material for molecular analysis, or to determine male/female larval ratios. *Anopheles spp.* differ from many other mosquitoes in that there is often no easily discernable difference in the female/male larval or pupal size or pigmentation. Some, but not all, anophelines L4 females can be identified based on the generally darker color and larger size. Here we present three methods for determining the sexes based on larval and pupal characteristics.

Larval sex determination: Option 1

An early method for sexing *Anopheles* larvae based on the form of the imaginal antennal lobes has been reported (Jones 1956), but the graphics in the manuscript can be difficult to interpret – particularly in copies. Here we offer a refinement of the method and new images developed for *An. gambiae*. (Note: This method is not very useful with *An. stephensi* because the imaginal disks are difficult to see.) The best results are usually obtained with 2nd day L4s as the pre-antennal lobe is almost fully formed. All observations and photographs were made on a stereoscope, and it is important to use the dark-field setting.

Figure 3.3.1. Cartoon of a 'sandwich' slide which works well to position and immobilize a larva for viewing without causing injury.

Constructing the viewing slide:

Materials

- Standard glass microscope slide

- 0.3 - 0.5 mm thick plastic spacer e.g. a thin plastic laboratory ruler cut into 1 X 1.5 cm pieces. The thickness must be selected to support a coverslip over the gap so that a larva is held firmly but not crushed. A stack of plastic coverglasses may be stacked and glued together to obtain the appropriate thickness.

- Epoxy glue

Construction and use

1. Clean the slide with ethanol and dry.

2. Apply a small drop of epoxy glue to the plastic spacers.

3. Glue the spacers onto the slide 0.8 - 1cm apart from each other and allow to cure.

4. Place a larva, dorsum upward, between the spacers. Add sufficient water to fill the gap, and place a coverglass on top such that it bridges the spacers. The pre-antennal lobes can be seen between the imaginal eyes while viewing the dorsal side of the head. In males, the lobe is large, circular and easier to see. The female's lobe is smaller and it is only easily seen in the second and third days of the fourth instar. Males are typically a bit easier to identify than females (see **Figures 3.3.2** and **3.3.3**).
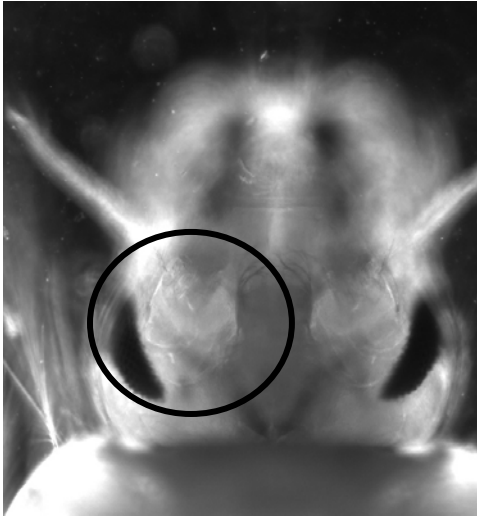
Figure 3.3.2. L4 *Anopheles gambiae* male. Region of interest for sex determination is circled.
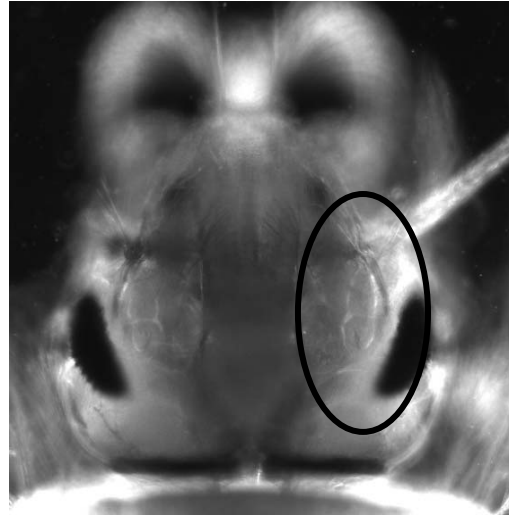
Figure 3.3.3. L4 *Anopheles gambiae* female. Region of interest for sex determination is circled.

Larval sex determination: Option 2

In *An. gambiae* and *arabiensis,* the "Red stripe" character can be visualized in L3s through mid pupae (Benedict et al. 2003) and provides a character that can be used to positively identify females with high certainty. When the *collarless* alleles (see Morphological Characteristics, Chapter 4) are heterozygous (c+ / c), a red stripe is evident on the female dorsum. The *collarless* trait is polymorphic in most colonies and wild populations and appears to have little if any effect on vigor. In **Figure 3.3.3**, the dorsum of this L4 larva has both white and red pigment characteristic of a c+ / c heterozygous female. While the *presence* of the red stripe can be used to select females with high certainty, the *absence* of the red stripe does not necessarily indicate a male in a polymorphic population. Though this method does not allow one to distinguish males, a cross between a homozygous c+ / c+ and c / c individuals would create F1 heterozygotes in which both sexes could be distinguished with high certainty. X-chromosome markers could also be used in a genetic scheme to produce progeny whose sex could be determined as early as the L1 stage. (Strains suitable for such crosses are available from the MR4.)

Figure 3.3.3. Red stripe character indicates a female larva.

Pupal sex identification

Anopheles pupae are much simpler to sex than larvae. The pupa should be lying on its side semi-dry in order to see the genitalia easily, and it may be necessary to use a small brush or forceps to gently lift the paddles.

1. Using a pipette, gently transfer 1 pupae to either a depression well plate or a piece of damp filter paper. If using a depression well plate, remove as much water as possible so that the pupa is lying on its side.

2. Under a stereoscope, observe the prominent genitalia for comparison with **Figure 3.3.4**.

References

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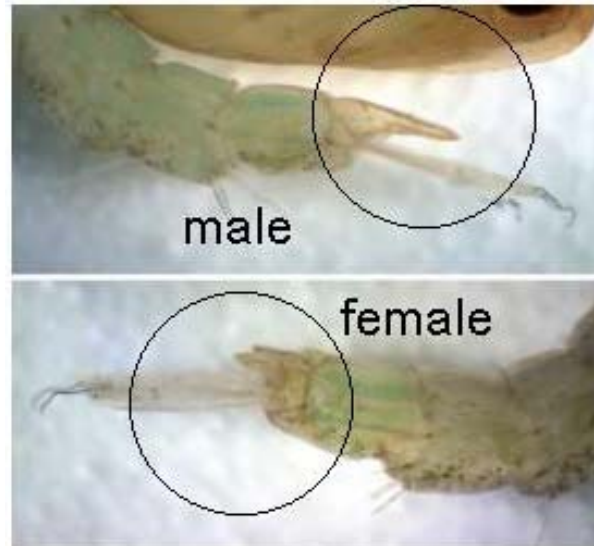
Figure 3.3.4. The terminalia of the pupae are very distinctive. However, the paddles can easily get in the way making it difficult to distinguish. Gently poking the pupa will usually make them change position to reveal the terminalia.

3.4 Mosquito Anesthesia

Mark Benedict and Paul Howell

Introduction

Adult mosquitoes can be immobilized by chemical anesthesia or by chilling. In the following, we describe simple methods and apparatuses to accomplish this. Be aware that in excess, all of these methods result in mortality and must be tested before routine use. Generally, highest survival is obtained with the minimum exposure sufficient to immobilize the mosquitoes. A common indicator of stress due to anesthesia short of lack of recovery is tarsi falling off.

CO_2 and N_2

Carbon dioxide and nitrogen gas are both useful for anesthesia and are very safe for human exposure. They can be supplied as compressed gas, as vapor from sublimation of dry ice or evaporation of liquid nitrogen. CO_2 has the distinct advantage over nitrogen that, its density being greater than air, it pools in trays where mosquitoes are placed. Furthermore, its smell is distinct and unpleasant making detection simple. Nitrogen has no odor and regulation of the amount and its presence is more difficult to determine. However, given the apparent disadvantages of N_2, I have heard reports that nitrogen anesthesia is preferable to maintain some behaviors e.g. for forced copulation (for which see McCuiston and White 1976).
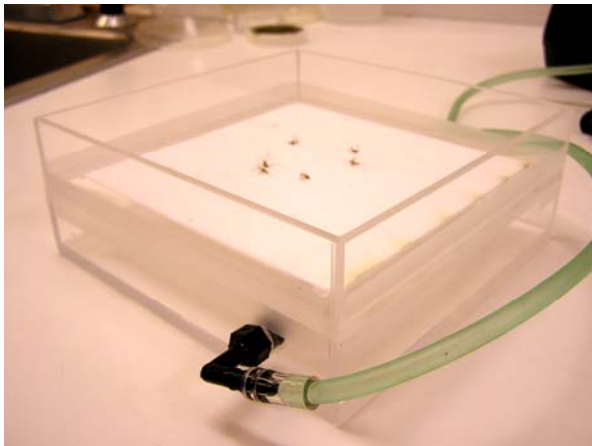
Figure 3.4.1. This improvised chamber provides sufficient area for as many mosquitoes as is safe to keep anesthetized at one time.

Shown is a small chamber made for anesthesia of mosquitoes (**Figure 3.4.1**). It is constructed of Plexiglas and has a white plastic platform made of porous polyethylene (e.g. Small Parts no. SPE-040-20) through which gases can pass. The gas is fed into the lower portion from which it oozes up onto the mosquitoes lying on the tray. The raised walls of the upper portion help retain a reservoir of CO_2. This same container can be used for nitrogen anesthesia. Similar simple improvised anesthesia containers can be constructed using fine nylon mesh and plastic containers at hand.

A simple modification is to bubble the gases through water to humidify them. A device that will perform this can be made from a flask and rubber stoppers. We have no information indicating whether this measure increases longevity and/or recovery, but it is a prudent measure.

Ethyl Ether and Chloroform

Vapors of both of these are useful for anesthesia when used safely (see safety measures at the end of this section). Their volatility and combustibility combined with their effects on humans make them a second choice to CO_2 and N_2. However, they are very portable and require little equipment in use. Moreover, their effects are generally longer-lasting than those of CO_2 and N_2 meaning that more working time with an immobilized mosquito can be achieved. Chloroform kills mosquitoes more readily.

Both can be administered by pouring the minimum effective amount (e.g. 1 ml) onto an absorbent material such a sponge or cotton wool. The container holding this should be air tight and sufficiently large to introduce a mosquito holding tube. Mosquitoes are blown into the tube which is then placed in the chamber until the adults are knocked down. The tube is then removed and the adults poured out.

Triethylamine

This chemical is used for *Drosophila* quite successfully because it is extremely safe. It is available for this purpose from Carolina Biological Supply as "FlyNap." Our limited experience with this is that it 'anesthetizes' *Anopheles* mosquitoes irreversibly. In this regard, it is similar to chloroform but would be a good choice when extended immobilization but not recovery is acceptable. Normal biological activities of several types during anesthesia have been confirmed in *Culex* (Kramer et al. 1990), but we are not aware of similar observations of *Anopheles.*

Chilling

Figure 3.4.2. A small Peltier cold table on which damp filter paper is placed to conduct cold and prevent mosquitoes from sticking to the condensation.

All mosquitoes with which I have had experience will withstand some degree of chilling on ice followed by resting on a near-freezing surface. Shown is a small chilling table that can be used for this purpose. For mosquitoes it is usually necessary to cover the cold surface with a thin piece of *damp* paper to prevent the mosquitoes from sticking to the condensate on the bare platform. Being slightly damp makes the paper adhere uniformly to the plate and increases heat transfer. Even this must be changed frequently as it becomes sticky. Entire cages or cups of mosquitoes can be placed briefly in a freezer in order to knock them down and then transferred to a chilled surface, but make the time as short as possible since most anophelines will not survive total freezing.

Ether and Chloroform Safety

Exposure to ether and chloroform should be minimized by keeping containers sealed, dispensing minimum amounts, and using them for the shortest possible durations of time.

Diethyl ether should be stored in a flammable storage cabinet or an explosion proof refrigerator not longer than 3 - 6 months. This cabinet should not be used to store oxidizing agents. Explosive peroxides can form with long term storage, so purchase and store only enough for immediate needs. The occupational exposure limit is 400 ppm as a time-weighted average (TWA, 8 hour exposure) and it has a short term limit exposure (15 minutes) of 500 ppm. Keep the anesthesia chamber closed as much as possible. Review the specific MSDS of the manufacturer you purchase ether from for any additional handling and storage recommendations, as well other relevant health and safety information. Some manufacturers recommend that you do not open unless contents are at room temperature or below, and that after opening the container, any unused ether be discarded or disposed of after 2-3 days. Only dispense ether in a chemical fume hood. Avoid agitation and sparks during all phases of use.

Chloroform is considered a known animal carcinogen with unknown relevance to humans. Its occupational exposure limit for an 8-hour TWA exposure is 10 parts per million (ppm). However, exposures to chloroform should never exceed 50 ppm at any time. This is referred to as OSHA's ceiling occupational exposure limit. Chloroform has a low odor threshold of 85 ppm, so by the time you smell chloroform the ceiling concentration would have already been exceeded. Anesthetizing procedures

should be performed in an area with good ventilation, preferably a chemical fume hood or other form of local exhaust enclosure.

Employees using this chemical should be trained to recognize the acute and chronic health effects associated with an over-exposure that can occur by inhalation, absorption through the skin and by ingestion. Selection of gloves is of particular importance since permeation of some nitrile gloves can occur within as little as 3 minutes. Contact the glove manufacturer for specific selection recommendations. Employees should always be informed of glove limitations and trained accordingly even for incidental use. Chloroform should not be stored with caustics. Review the MSDS for additional safe handling, storage and disposal information.

Acknowledgments

Thanks to Paul Vinson and Cheryl Connell of the CDC Office of Health and Safety for safety advice.

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3.5 Protocol for 96 Well DNA Extraction
Clare Holleley and Alice Sutcliffe

Introduction
This protocol describes the expansion of a common genomic DNA extraction method (salting-out method) to a 96-well platform adapted for *Anopheline* mosquitoes. DNA extraction of individual insects has traditionally been conducted by grinding specimens separately in 1.5ml tubes and can be a very time-consuming process, especially when studies require large collections or sample sizes. Expansion of the salting-out method to 96-well PCR plates dramatically reduces the amount of time it takes to perform a large number of extractions. Simultaneous 96-well insect maceration is achieved using a commercially available bacterial colony replicator tool (**Figure 3.12.1**). The novel application of this tool as a maceration device coupled with the salting-out procedure described below drastically improves the efficiency of individual genomic DNA extraction without having a large impact on DNA yield or extraction failure. This protocol has been adapted for mosquitoes from the original protocol used in *Drosophila* (Holelley 2007). Using this protocol, we obtained average yields of 6.15µg and 5.65µg per mosquito by processing live and desiccated mosquito samples respectively. This compares to yields of 5.90µg and 3.50µg we obtained in our laboratory using the DNA extraction protocol described by Collins *et al* (1987).

Materials
Forceps

Incubator or thermocycler

48 or 96-pin bacterial replicator (See **Figure 3.12.1**)

microplates and plastic caps

Centrifuge (capable of holding 96-well microplates)

Bunsen burner, striker, and gas source

Reagents
10mM Tris-HCl (pH 9.0 at 25°C)

50mM KCl

Triton® X-100

5M Potassium Acetate

Proteinase K (Roche, 03 115 887)

100% Isopropanol

70% ethanol

TE buffer 0.01 M, pH 7.4

Reagent Preparation
Thermophilic DNA polymerase 1X buffer

1. Add 46.95ml deionized water, 1250 µl 1M KCl, 500µl 1M Tris-HCl (pH 9.0 at 25°C) and 50µl Triton X-100. This is a 10X solution.

2. Dilute 10X buffer to yield 1X buffer by adding one part 10X buffer to 9 parts sterile water

3. Prepare aliquots of 10X buffer for storage at -20°C and store 1X buffer at 4°C for short term use.

Bacterial replicator sterilization:

1. Place bacterial replicator in ethanol for 5 minutes ensuring the entire length of the pins is immersed.

2. Heat replicator over Bunsen burner or similar until ethanol has evaporated for sterilization (5-10 seconds).

3. Set bacterial replicator aside to cool to room temperature before use in next steps.

OPTIONAL: Other methods for sterilization include autoclaving or 2 hours of UV light.

Mosquito preparation:

1. Prepare a master mix of buffer as shown in **Table 3.12.1**.

2. Aliquot 50µl of master mix into each well of a 48 or 96-well microplate.

3. Using forceps, place one mosquito in each well cleaning forceps between samples.

4. Carefully place sterilized, room temperature bacterial replicator into microplate (containing samples).

5. Carefully grind mosquito samples for 10 minutes by moving replicator up and down and/or rocking replicator from side to side. It is important that this is not done too vigorously as this can cause the bottom of the well to break or samples to be splashed, contaminating adjacent wells. When extracting DNA from mosquitoes and other insects, it is particularly important to thoroughly macerate the insect to allow cell lysis.

6. Place plastic caps onto microplate and incubate 12-15 hours at 55°C.

	Number of samples		
	96	48	1
1X DNA polymerase buffer	5000µl	2500µl	50µl
Proteinase K	20µl	10µl	0.2µl

Table 3.12.1. Master mix volumes for 96, 48 or one 25µl PCR reactions. Amounts for larger master mixes have been adjusted upwards to be for 50 and 100 reactions compensate for imprecise measurements

Genomic DNA extraction

1. Remove microplate from thermocycler and cool to room temperature before proceeding.

2. Add 25µl of 5M potassium acetate to each well containing sample.

3. Reseal with plastic caps and briefly vortex.

4. Centrifuge at 4100rpm and room temperature for 15 minutes to pellet cell debris.

5. In a new microplate, add 60µl of 100% isopropanol to each well.

6. Carefully remove the supernatant (approximately 60µl) from each well and add to the new microplate containing isopropanol. Avoid dislodging cell pellet.

7. Seal with new plastic caps and invert 30 times to mix. Microplate containing cell pellets can be discarded.

8. Centrifuge at 4100rpm for 15 minutes at room temperature.

9. Carefully remove and discard supernatant. The DNA is should be visible at the bottom of each well and should not be dislodged or removed. The pellet may appear purple in color.

10. Add 50µl of 70% ethanol to each well. Replace caps and invert 20 times to wash DNA.

11. Centrifuge at 4100rpm for 15 minutes at room temperature.

12. Carefully remove and discard supernatant as before.

13. Allow remaining ethanol to evaporate from microplate by leaving uncovered at room temperature for 30 minutes or until no ethanol remains.

14. Resuspend DNA in 50µl of TE buffer to each well.

15. Seal microplate with new plastic caps and incubate at 65°C for 1 hour tapping periodically.

16. DNA samples can be stored at -20°C or -80°C.

Figure 3.12.2 shows the result of 23 *An. gambiae s.s.*, 23 *An. arabiensis* and 23 desiccated mosquito samples analyzed with the *Anopheles gambiae* authentication PCR (Wilkins et al. 2006) and 23 *An. quadrimaculatus* samples analyzed with the *Anopheles* ITS2 Amplification (Beebe and Saul 1995), using 1µl of template genomic DNA extracted with this protocol.

Figure 3.12.1. Example of a 96-bacterial replicator tool used for maceration of mosquito samples and reservoir.
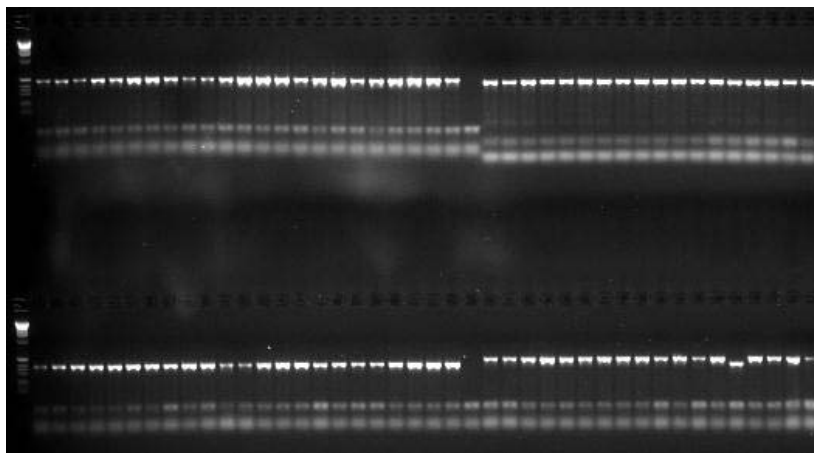
Figure 3.12.2. Lanes 1, 50, 51, 100 1kb ladder, lanes 25, 49, 75 and 99 control wells. Lanes 2-24 An. gambiae s.s., lanes 26-48 An. quadrimaculatus, lanes 51-74 An. arabiensis and lanes 76-79 desiccated mosquito samples. Bands are species specific. 5µl of sample loaded and run on a 2% agarose EtBr gel.

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3.6 Determination of Lipid, Glycogen and Sugars in Mosquitoes

Adapted from (Van Handel 1985a; Van Handel 1985b; Van Handel and Day 1988; Kaufmann and Brown 2008)

Introduction by C. Kaufmann

This method determines the lipid, glycogen, and sugar content of a single insect. It has the advantage that reasonable results can be achieved with only a few numbers insects, which may be of great significance in the field.

The separation of lipid, glycogen and sugar is rather important because vast amounts of ingested sugar, as given via a feeding solution in the laboratory or sugar sources like nectar and honeydew that are ingested in nature, can interfere with the lipid analysis (blue/violet coloring of the vanillin test). Also, the hot anthrone test does not differentiate between the ingested sugar within the crop and the carbohydrate that is already transferred to its storage form glycogen.

The homogenization with sodium sulfate solution will help to co-precipitate the glycogen after the addition of the chloroform-methanol solution, in which the lipids and sugar dissolve; the addition of water allows the separation of the sugar and lipid (sugar in the upper phase and lipid in the lower phase). Be careful when handling the chloroform and acid.

Note that for larger insects, you should use different values of chloroform/methanol (2.8 ml instead of 1.6 ml) and water (2 ml instead of 0.6 ml); the rest is the same.

Materials
1. Glass centrifuge tube
2. Glass grinding pestle or stir rod
3. Heating block at 90-110°C
4. Erlenmeyer flask
5. Centrifuge

Reagents
1. Sulfuric acid (95-98%)
2. Anthrone
3. Anhydrous glucose
4. Sodium sulfate
5. Methanol
6. Commercially available vegetable oil (e.g. soybean oil)
7. Vanillin
8. Phosphoric acid (85%)
9. Chloroform

Solutions
1. 2% sodium sulfate (NaSO$_4$) solution
2. chloroform/methanol mixed 1:1 (v/v)
3. Vanillin-phosphoric acid reagent
   a. Dissolve 600 mg vanillin in 100 mL DI hot water.
   b. Add 400 mL 85% phosphoric acid.
   c. Store in the dark. Stable for several months but discard if it darkens.
4. Anthrone reagent
   a. Add 385 mL sulfuric acid (95-98%) to 150 mL DI
   b. Dissolve 750 mg anthrone

    c.   Store at 4°C. Stable for several weeks.

Standards

1. Lipid: 100 mg per 100 mL of a commercial vegetable oil (e.g. soy bean oil) in chloroform
   a) In triplicate, add 50, 100, 200 and 400 µl of solution to glass tube.
   b) Place in heating block at 90-110°C to evaporate the solvent.
   c) Add 0.2 mL of sulfuric acid and heat for 10 min at 90-110°C
   d) Add vanillin reagent to 5 mL level and mix.
   e) Remove from heating block and allow to cool.
   f) Allow reddish color to develop; this will take approximately 5 min and will be stable up to 30 min.
   g) Determine OD at 625 nm and plot µg lipid vs. OD for calibration line.
2. Sugar and glycogen: 100 mg per 100 mL of anhydrous glucose in deionized water.
   a) In triplicate, add 25, 50, 100, 150 and 200 µl of glucose solution to glass tube
   b) Add anthrone reagent to 5 mL level and mix.
   c) Heat for 17 minutes at 90-110°C.
   d) Remove from heating block and allow to cool.
   e) Determine OD at 625 nm and plot µg glucose vs. OD for calibration standard.

Extraction of Lipid, Glycogen and Sugar Fractions from Mosquito

1. Add mosquito to glass centrifuge tube.
2. Add 0.2 mL sodium sulfate solution.
3. Homogenize mosquito in solution until no identifiable parts remain (glass rod or other utensil).
4. Wash glass rod into centrifuge tube with two x 0.8 mL volumes of chloroform/methanol solution.
5. Centrifuge (3000 rpm, 1 min).
6. Transfer supernatant to clean centrifuge tube. Retain pellet for glycogen analysis.
7. Add 0.6 mL DI water to supernatant. Mix.
8. Centrifuge (3000 rpm, 1 min).
9. Separate top fraction (water/methanol) for sugar analysis.
10. Bottom portion (chloroform) holds the portion for lipid analysis.

Lipid Analysis

1. Place portion for lipid analysis in a tube with a marking at the 5 mL level.
2. Place in heating block at 90-110°C to evaporate the solvent.
3. Add 0.2 mL of sulfuric acid and heat for 10 min at 90-110°C
4. Add vanillin reagent to 5 mL level and mix.
5. Remove from heating block and allow to cool.
6. Allow reddish color to develop; this will take approximately 5 min and will be stable up to 30 min.
7. Determine OD at 625 nm

Sugar Analysis

1. Place portion for sugar analysis in a tube with a marking at the 5 mL level.
2. Place in heating block at 90-110°C to evaporate the solvent down to 0.1-0.2 mL.
3. Add anthrone reagent to 5 mL level and mix.
4. Heat for 17 minutes at 90-110°C.
5. Remove from heating block and allow to cool.
6. Determine OD at 625 nm

Glycogen Analysis

1. Add anthrone reagent to 5 mL level and mix.
2. Heat for 17 minutes at 90-110°C.
3. Remove from heating block and allow to cool.
4. Determine OD at 625 nm.

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3.7 Anopheles *Mating*

3.7.1 Mating : General considerations

Paul Howell

Introduction

Mating in most anopheline mosquitoes occurs during the early evening and is believed to occur primarily in swarms. *Anopheles* male mosquitoes aggregate just before dusk and commence swarming at the onset of sunset. Swarming males use their erect antennal fibrillae to detect a nearby female mosquito's wing beat frequencies (Nijhout and Sheffield 1979; Ikeshoji et al. 1985; Leemingsawat 1989; Clements 1992). In *Toxorhynchites* it was found that males will actually harmonize their wing beat with females as they near, possibly as a form of species recognition, before mating commences (Gibson and Russell 2006). In many species, copulation is initiated in flight with males and females meeting within the swarms (Clements 1992). Once a male anopheline has grasped a receptive female, it reorients itself so it is in the venter-to-venter position allowing the reproductive organs to meet. After coitus commences, the male moves into an end-to-end position with the female as the pair falls (Charlwood and Jones 1979). Copulation may continue for a short period of time after alighting, but in most genera it is a very quick process which ceases before the pair reaches the ground.

Newly emerged anophelines are not sexually mature. Male mosquitoes require about 24 hours before their terminalia have rotated and their fibrillae are mature enough to become erect and detect females (Clements 1992). Female mosquitoes, however, typically need 48-72 hours before they become receptive to males - usually prior to blood feeding in the wild. *Anopheles* males can mate several times, but females become refractory to re-insemination and re-mating is rare (Villarreal et al. 1994; Yuval and Fritz 1994). In *An. culicifacies*, it was found that a proportion of colonized females had multiple inseminations, but this was attributed to the laboratory setting and not a natural behavior (Mahmood and Reisen 1980).

In the laboratory, it is often not feasible to maintain a colony in a large enough cage to promote natural swarming behavior. Instead, selection of a stenogamous colony - one that breeds in a small cage - is performed. During colonization, only a proportion of individuals will respond to the novel environment, and a genetic 'bottleneck' occurs - loss of heterozygosity. Norris et al. showed that even a newly colonized strain has an extreme loss of heterozygosity compared to field samples from the same area (Norris et al. 2001). Often, fixation of particular alleles is a quick inevitable process and cannot be remedied without the introduction of new field material.

Although little is known about cues that are needed to stimulate mating within the laboratory, some experiments have been done to develop methods to improve colony mating. The addition of a simulated sunrise and sunset has been shown to have a positive effect on colonization efforts – presumably due to improved mating (Charlwood and Jones 1980; Panicker and Bai 1980). There are also reports of researchers utilizing a low watt colored light prior to the dark period to stimulate mating (Pan et al. 1982; Villarreal et al. 1998). Cage size also has an impact on the success of a colony. Some species will not mate within a small cage due to some unknown parameter, such as eurygamy, and require larger cages to complete mating (Pan et al. 1982; Marchand 1985). Marchand (1985) and Peloquin (1988) utilized an artificial sky and horizon to promote mating.

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3.7.2 Forced Copulation

MR4 Staff

Introduction

Adults from several *Anopheles* - *An. dirus*, *An. funestus*, and *An. darlingi* - have proved difficult species from which to establish a *stenogamous* laboratory colony. The main obstacle has been to stimulate mating. In the late 1950's, McDaniel and Horsfall (1957) developed an induced copulation technique to produce *Aedes* mosquitoes that are intractable in the insectary. Their method was based on reported observations on the mating behavior of European mantids which decapitated their mates prior to copulation. When the male mantis was decapitated, the suboesophageal ganglion was severed thereby overcoming the innate inhibition of copulatory muscles in the male (Baker 1964).

It is sometimes necessary to use the induced, or 'forced,' copulation technique in order to initiate and even to maintain a colony or to obtain matings with rare or specific individuals. Often, colonies initiated by forced copulation will eventually become stenogamous. Forced mating has been applied to several mosquito genera. Specific adaptations increase the success rate for mating of anophelines (Baker et al. 1962), (Ow Yang et al. 1963). Caravaglios found that males did not need to be decapitated and could be simply held with a small suction pipette, mated, and then returned to the colony without harm (Caravaglios 1961). Conditioning the males by placing them in a 15°C room for 12-24 hours was found to increase copulation rates (Baker et al. 1962).

Materials

- Minutien pins mounted on small wooden sticks (10-20)
- Ethyl ether
- 50 ml Falcon tubes modified to hold mosquitoes (or similar)
- Vacuum source with modified tip to prevent mosquitoes from being damaged
- Glass container with tight fitting lid (staining jar)
- Cotton balls

Method - modified from the *An. maculatus* technique (Ow Yang et al. 1963)

Success depends on the preparation of high-quality males and females. Both sexes should be of an appropriate age that reflects when the species mates. Males should be at least 72 hours old and females at least 48-72 hours old to ensure the reproductive organs have fully matured.

1. Two to four hours prior to mating, bloodfeed 3-4 day old females to repletion. Using bloodfed females is not absolutely necessary, but it has been reported that the engorged abdomen of the female makes forced copulation more successful (McCuiston and White 1976). Moreover, this ensures that at least one of the requisites for obtaining progeny has been accomplished!

2. Separate males from females and place them in separate containers.[1]

3. Gently aspirate approximately 10 blood fed females into an anesthetizing container (**Figure 3.7.2.1**). Before proceeding, see Mosquito Anesthesia section (**Chapter 3.4**). The anesthetizing container can be made from a 50 ml Falcon tube with the tip removed and both ends covered in mesh held in place

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[1] In some *Anopheles* species, it is has been found that "seasoning" the males by placing them at approximately 15-20° Fahrenheit at least 12 hours prior to copulation increases the mating success rate (Ow Yang CK, Sta Maria FL, Wharton RH (1963) Maintenance of a laboratory colony of *Anopheles maculatus* Theobald by artificial mating. Mosq News 23:34-35). This is not true for all species.

with rubber bands or any container that can withstand ether. In a glass container with a lid, place 10-20 cotton balls in the bottom and pour in 5-10 ml of ether. Nitrogen or carbon dioxide gas can be used as well to anesthetize the females with little to no affect on the rate of successful copulation (Fowler 1972).

Figure 3.7.2.1. An example of an anesthetizing container.

Figure 3.7.2.2. Proper vacuum pressure aspiration of a male prior to pinning.

4. Prepare un-anesthetized males by attaching them to a fine pipette attached to a mild vacuum, the tip of which is just large enough to hold the male by the thorax without damaging it (**Figure 3.7.2.2**). The optimal place to capture a male is on the mesonotum. However, if the vacuum is weak you can capture the male by slipping the pipette over the male's abdomen. Caution should be used when capturing males in this manner as the vacuum may damage the male's claspers.

5. Once the male is captured, gently pierce the side of the thorax with a minutien pin mounted on a small wooden stick (**Figures 3.7.2.3 and 3.7.2.4**) e.g. the stick from an oral cotton swab could be modified with a pin for this purpose. It will be necessary to support the male against a firm surface to enable the pin to penetrate. Prepare 5-10 males at a time in this manner. Use only males that are still moving for matings.
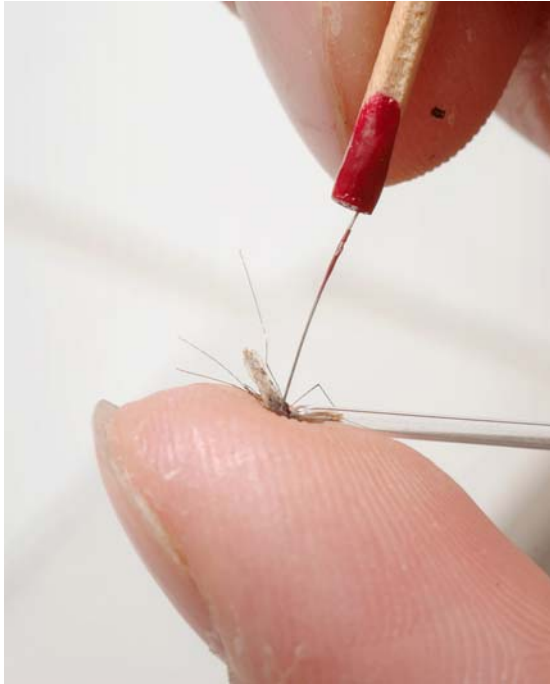
Figure 3.7.2.3. Holding male with vacuum pressure, gently stab the male through the thorax taking care to not crush the head.

Figure 3.7.2.4. Properly pinned male.

6. Place one container of 10 females into the anesthetizing chamber and leave it for 6-10 seconds (depending on the species and strength of ether).

7. Watch the females closely, and once they have all fallen from the sides, remove them from the chamber. Do not leave females in the ether too long or they will not recover from the treatment.

8. Gently disperse the females onto a piece of filter paper and position ventral side up. Take a mounted male, pinch off the head and hind-tarsi (**Figure 3.7.2.5**), and then gently stroke the abdomen of the male over the female's abdomen to stimulate the claspers to open (Figure **3.7.2.6**).

9. Place the male at a 45-90° angle venter-to-venter with the female until the male clasps the female (**Figure 3.7.2.7**). Leave clasped for 1-2 seconds then pick up both using the male on the pin. If mated successfully, they will remain attached for several seconds.[2] Successful mating has usually occurred if they remain attached for 3-5 seconds (**Figure 3.7.2.8**).

10. Place male and female together into a new cage to allow female to recover from the anesthesia.

11. Ensure that the females are recovering from the anesthesia by gently blowing into the recovery cage. If females are not waking up within 10 minutes, anesthetize more lightly.

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[2] It is possible to reuse males to mate more than one female.

Figure 3.7.2.5 Male with head and legs removed.

Figure 3.7.2.6 Positioning of the male prior to stroking the females abdomen

Figure 3.7.2.7 Pairing of male and female, note the male is near a 90°angle to the female

Figure 3.7.2.8 If pairing is successful, you are able to lift the female using the male.

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3.7.3 Pair Matings

MR4 Staff

Background

Pair mating (or single-pair mating) is most commonly used when knowledge of the genetic makeup of both parents is important. The isolation of specific phenotypes within *Anopheles* mosquitoes is useful in determining vector population species/genetic composition (Rabbani et al. 1976). Phenotypes such as insecticide resistance as well as other novel genetic mutations can be quickly isolated and purified using pair mating (Collins et al. 1986).

Beside forced copulation (**Chapter 3.7.2**), mating between a particular male and female can be obtained by free-mating in small numbers in small containers. Allowing a male and a female to mate freely is less invasive and time consuming than the method of forced copulation. Neither method is efficient, however.

Benedict and Rafferty (2002) reported a method for obtaining reasonable frequencies of free matings. They observed that mating did not occur until later than is typical, so we recommend keeping the pair in the mating tube for 7 or 8 days.

Materials

- Qorpack tubes with modified lids, or similar
- Cotton balls
- 10% sucrose solution
- Rack for holding the single pair mating tubes
- Aspirator

Procedure

1. Ensure the lighting in the space in which the mosquitoes will be held is adjusted to obtain sunset and sunrise periods to entrain the larvae before adulthood.

2. After emergence, place a male and female in an individual rearing container (**Figure 3.7.3.1**) and leave for 5-8 days. Additional females may be added if desired – 5 would be a reasonable maximum.

3. Maintain the adults by placing a sugar pad on top of the tube and keeping it wet.

4. Obtain eggs as described in Chapter 3.9 - Family Culture.

Figure 3.7.3.1. This particular choice of tube that we use for family oviposition is modified from a chamber purchased through Qorpack (Bridgeville, PA. No. 3891). Many similar tubes are suitable. The bottom may be covered with a filter paper disk to absorb fluids.

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3.7.4 *An. gambiae* and *arabiensis* Mating Status Determination in Preserved Females

K.R. Ng'habi and Greg Lanzaro

Introduction

The *An. gambiae* mosquito has a karyotype consisting of two pairs of autosomes (Chromosome 2 and 3) and one pair of sex chromosomes (Chromosome X and Y). The Y chromosome constitutes ~10% of the whole genome and contains a male determining factor which, when present in a XX/XY system, induces male development (Clements 1992). Y-chromosome linked DNA fragments have been characterized and Y-chromosome specific PCR markers have been developed (Krzywinski et al. 2004; Krzywinski et al. 2005). Previously, detection of mating success among females relied on microscopic dissection of female ovaries or examination of sperm in the female spermatheca. This method is reliable and robust but with the limitations that it is time consuming, labor intensive, and requires fresh specimens. A simple and rapid method to determine the mating status of dried female *An. gambiae* is therefore useful in order to analyze large sample sizes within a short period of time.

Prepare PCR mixture for 96, 48 or 1 50µl PCR reactions[1]. Add reagents in the order presented.

96	48	1	Reagent
3.73 ml	1.87 ml	38.8 µl	Sterile water
18.63 ml	9.31 ml	5 µl	*Taq* 10X PCR Buffer with $MgCl_2$ (1.5 mmol/L)
3.73 ml	1.87 ml	0.2 µl	dNTP (2.5 mmol/L)
931.2 µl	465.6 µl	0.25 µl	S23 (F, 25pmol) [CAAAACGACAGCAGTTCC]
232.8 µl	116.4 µl	0.25 µl	S23 (R, 25pmol) [TAAACCAAGTCCGTCGCT]
116.4 µl	58.2 µl	0.5 µl	*Taq* polymerase
27.354 ml	13.677 ml	45 µl	Total (To each 45µl add 5µl of DNA template)

Table 3.7.4.1. F and R indicate forward and reverse primers, respectively. DNA extractions of males may also be used as positive controls.

96	48	1	Reagent
3.73 ml	1.87 ml	38.8 µl	Sterile water
18.63 ml	9.31 ul	5 µl	*Taq* 10X PCR Buffer with $MgCl_2$ (1.5 mmol/L)
3.73 ml	1.87 ml	0.2 µl	dNTP (2.5 mmol/L)
931.2 µl	465.6 µl	0.25 µl	128125I (F, 25pmol) [GGCCTTAACTAGTCGGGTAT]
232.8 µl	116.4 µl	0.25 µl	128125I (R, 25pmol) [TGCTTTCCATGGTAGTTTTT]
116.4 µl	58.2 µl	0.5 µl	*Taq* polymerase
27.354 ml	13.677 ml	45 µl	Total (To each 45µl add 5µl of DNA template)

Table 3.7.4.2. F and R indicate forward and reverse primers, respectively. DNA extractions of males may also be used as positive controls.

PCR cycle conditions

94^oC/3min x I cycle
(94^oC/20sec, 55^oC-64^oC/30sec and 72^oC/1 min)* x 35 cycles
72^oC/10min x 1 cycle
4°C hold

---

[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns compensate for imprecise measurements.

Run samples on a 3.0 % agarose EtBr gel for visualization. *The primers have different amplification temperatures but within this range should work.
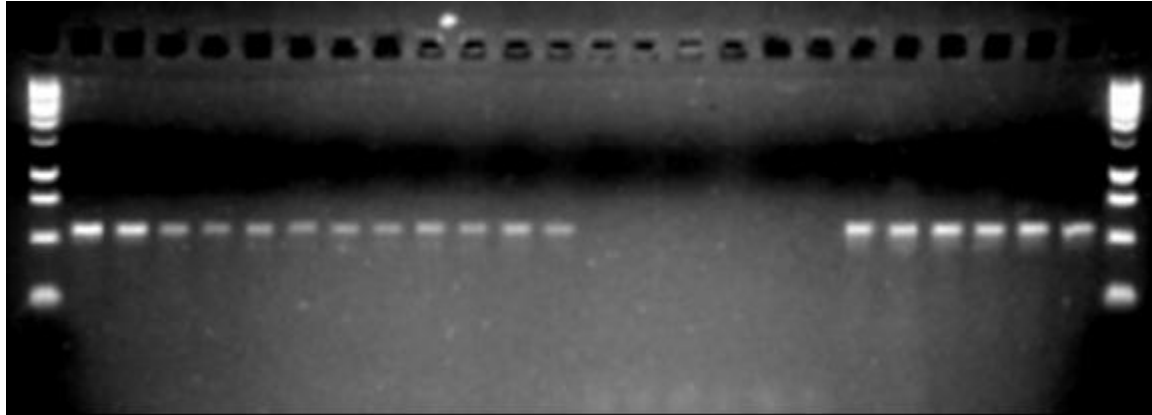
Figure 3.7.4.1: Agarose gel electrophoresis showing amplification of Y chromosome sequences in *An. arabiensis* (primer 128125I) males and mated females. There was no amplification in virgin females. Lanes 1 and 26, ladder, lanes 2-13 mated, lanes 14-19 unmated, lanes 20-25 males. (Ng'habi et al. 2007), used with permission.
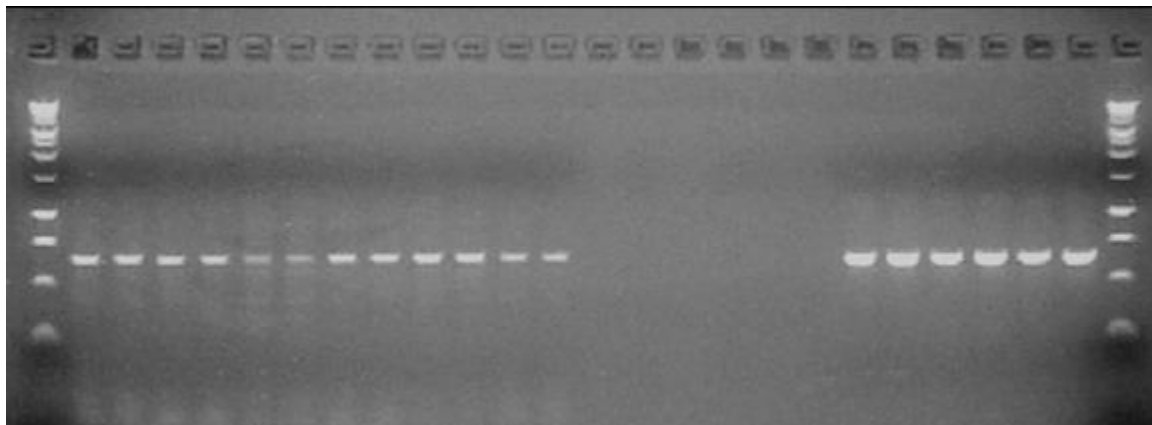
Figure 3.7.4.2: Agarose gel electrophoresis showing amplification of Y chromosome sequences in *An. gambiae s.s.* (Primer S23) males and mated females. There was no amplification in virgin females. Lanes 1 and 26, ladder, lanes 2-13 mated, lanes 14-19 unmated, lanes 20-25 males. (Ng'habi et al. 2007), used with permission.

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96 well sample preparation template

3.8 Protocol for Forcing Female Anophelines to Oviposit

John Morgan

Introduction

This forced oviposition technique has proved extremely useful when collecting *Anopheles* species from field material where large numbers of families are required, e.g. for association mapping and microarray gene expression analyses. However, the method will be of use for any work benefitting from improved synchronicity of egg-laying. If this method is used with mosquitoes collected from the field, it is essential that all mosquitoes be removed from the tube and preserved before dispatching. On no account should live mosquitoes be transferred using this method because of the potential health hazard they present. To date, this protocol has been used to obtain eggs from wild-caught resting *Anopheles gambiae s.s.*, *A. arabiensis* and *A. funestus*.

The forced laying takes place within 1.5 ml Eppendorf tubes which should be prepared as follows.

Materials

- 1.5 ml Eppendorf tubes
- Whatman no.1 filter paper
- Dissecting needles(seeker)
- Forceps

In the Field

1. Collect resting blood-fed females using an aspirator and transfer to a paper storage cups with a flexible gauze cover. Provide mosquitoes with 10% sugar solution by placing moistened cotton wool on top of the gauze.

2. Maintain mosquitoes for 3-5 days to allow them to become gravid. During this period they should be maintained at ≈25°C and RH >70%.

Preparation of Oviposition Containers

3. Carefully pierce the Eppendorf tube with 2 holes in the cap and also 2 holes near the base; a seeker produces holes of the appropriate size (**Figure 3.8.1**).

4. Cut isosceles triangles of Whatman no 1 filter paper approximately 1cm wide at base and 2cm long.

5. Insert the paper onto the side of the Eppendorf tube with forceps (**Figure 3.8.2**).

Technique

6. Moisten the filter paper with water and tap out any excess water. The amount of water on the paper is integral to this protocol. (**Figure 3.8.3**).

7. Position the filter paper so that the condition of the mosquito and the presence of eggs can be determined without the need to open the tube.

8. Using an aspirator, gently introduce a gravid mosquito into each Eppendorf tube (**Figure 3.8.4**).

9. Label the tube with a unique identifier.

10. Enclose the tubes in a sealable plastic bag if necessary to maintain humidity and check the tubes daily to ensure the filter paper remains moist.

11. When the eggs are seen (**Figure 3.8.5**), carefully remove the female parent with forceps and preserve individually over silica gel. A convenient method for storing dry individuals on silica gel is to use pierced numbered 0.2ml color-coded PCR tubes.

12. Cohorts of sample tubes are enclosed with silica gel in labeled snap closure plastic bags. Groups (e.g. daily collections) are then enclosed in larger snap bags and transported in sealed plastic boxes.

13. Tubes containing eggs should be kept cool in a sealed plastic bag, monitoring the moisture level before dispatch, transportation or synchronized emerging.

14. For most purposes it will be preferable to place egg papers into individual containers for hatching in order to maintain iso-female families and to assess quality and size of families (**Figure 3.8.6**) see also (**Chapter 3.9 Family Culture**).
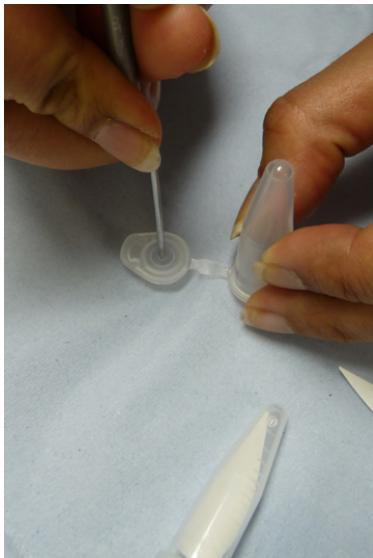
Figure 3.8.1 Preparing the tube

Figure 3.8.2 Inserting the filter paper triangle

Figure 3.8.3 Moistening the filter paper

Figure 3.8.4 Aspirating the female into the tube

Figure 3.8.5 Checking for oviposition

Figure 3.8.6 Single family rearing

Reference

Morgan JC, Irving H, Okedi LM, Steven A, Wondji CS (2010) Pyrethroid resistance in an *Anopheles funestus* population from Uganda. PLoS ONE 5(7):e11872. doi:10.1371/journal.pone.0011872

3.9 Family Culture

MR4 Staff

Introduction

Family – or single family - culture is useful for many experimental plans or for the development of new strains. It is particularly important when the mother's genetics must be known, in genetic crossing, and when genotypic frequencies are of interest. If specific knowledge of the father's genetics is important to studies, pair matings must be used (**Chapter 3.7.3**) or inferred from progeny analysis. Otherwise, *en masse* -mated females can be isolated for individual egging. It some cases, the genetics of the mother will be determined based on the genotypes/phenotypes of her progeny.

Another application of family culture is to establish wild colonies. In species complexes such as *An. gambiae, An. funestus* and *An. dirus* where sympatric forms co-exist, it is essential to isolate the species of interest (Mpofu et al. 1993). By utilizing family rearing techniques, individual females are separated so that eggs from a single pair are segregated, thus allowing the establishment of pure-breeding lines that can be transformed into laboratory colonies.

It is best to culture individual families in the dish in which the eggs were laid for the first couple of days to avoid moving fragile eggs/larvae. The Qorpak vial shown in **Figure 3.9.1** are suitable for 100 or fewer progeny, but larger numbers should be collected in larger cups to prevent early larval mortality. The larvae should be progressively transferred to larger containers and water volumes as they develop. It is not uncommon to culture a family in three different containers before pupation to ensure good survival.

A common error is to conclude that one's family culture method is suitable even in the absence of hatching data based on total egg hatch. Because L1s may die and decay rapidly, their presence can only be known based on a count of the *total number of eggs that hatched*. Particularly for frequency and survival data this is essential. Do not rely on counts of larvae even one day after hatching for quantitative data.

Obtaining eggs from reluctant females

Low rates of oviposition often hinder successful family culture. Several methods have been developed that have been shown to increase oviposition within the laboratory. The use of a dark oviposition dish is more attractive than a clear or white dish in *An. quadrimaculatus* (Lund 1942), *An. gambiae* (Huang et al. 2005), and *An. arabiensis* (MQB pers. comm.). In *An. albimanus,* wild caught blood fed females that were allowed to oviposit in a 5 dram vial laid more eggs than those allowed to oviposit in a large cage (Bailey and Seawright 1984). The complete removal of one wing of a gravid female that is lightly anesthetized will promote oviposition soon thereafter, though this is time consuming for the technician. This method is a last resort because mortality results.

Rearing schedule for individual families

If you are starting with bloodfed material, begin with schedule at day 4.

Day 1- Blood feed females.

Day 2- No attention is required.

Day 3- No attention is required.

Day 4- Transfer gravid females to vials lined with filter paper (for example: Qorpack Bridgeville, PA. No. 3891 containing strips of filter paper cut to size, **Figure 3.9.2**) and containing 1-2 cm of water.

Day 5- Remove the females from their vials.

Day 6- add 2 drops of a 2% w/v yeast slurry to each vial.

Day 7-count hatch rate and transfer larvae to a larger container (**Figure 3.9.2**) containing 0.02% w/v final concentration of yeast (see Determining Egg Hatch Rates, Chapter 2).

Day 8-observe, but feeding is usually not needed

Day 9 through pupation-feed ground fish food or other larval diet. Monitor water quality carefully in these smaller pans as it is easier to accidentally over feed such a small number of larvae. Transfer to larger containers as needed.
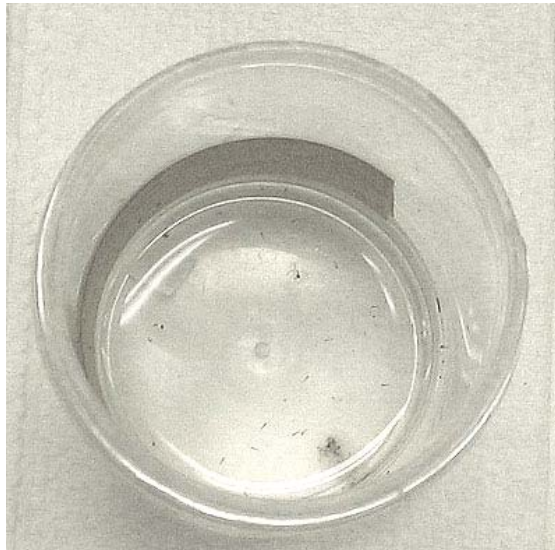
Figure 3.9.1. This particular individual family oviposition tube is modified from a chamber purchased through Qorpack Bridgeville, PA. No. 3891.

Figure 3.9.2. Examples of family rearing trays.

References

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3.10 *Picogreen Quantification of DNA from a Single Mosquito*

Craig Wilding

Introduction

DNA extractions are typically quantified through spectrophotometry, often using a NanoDrop. A single mosquito typically yields 300ng DNA (from silica gel preserved samples – see Wilding *et al.*, 2009). Following standard extraction methods DNA is eluted/re-dissolved in 100µl i.e. at a concentration of ≈ 3ng/µl. However, spectrophotometry is accurate only within a dynamic range of 5–90 ng/µl DNA (Sambrook & Russell 2001). Thus, NanoDrop, or other spectrophotometric methodologies are unsuitable for measurement of typical extractions from mosquitoes.

Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen) is an ultra sensitive fluorescent nucleic acid stain for quantifying concentrations of double-stranded DNA (dsDNA) in solution. Quant-iT assays can detect down to 25 pg/ml of dsDNA (50 pg dsDNA in a 2 ml assay volume) or 250 pg/ml dsDNA (50 pg in a 200µl assay volume) using a fluorescence microplate reader. In addition to the much higher sensitivity of PicoGreen, the assay is unaffected by co-purifying compounds such as RNA, protein and common salts. Using PicoGreen Wilding *et al.* (2009) showed that concentration readings of mosquito DNA extracted using Qiagen DNeasy were reproducible whilst reproducibility was poor on a NanoDrop since the concentrations were outside the dynamic range of the spectrophotometer. Where DNA concentration is important for downstream processes e.g. Next Generation Sequencing or SNP-chip (Affymetrix or Illumina) then concentrations should be measured using PicoGreen and not spectrophotometric methods.

Quant-iT assays can be undertaken individually using the Qubit® Fluorometer (Invitrogen catalogue number Q32857) and Quant-iT™ dsDNA High Sensitivity Assay Kit (Q33120) or microplate reader set to excite at 480 nm and read emission at 530 nm. Below is a methodology for Quant-iT PicoGreen DNA quantitation using a Varioskan Flash spectral scanning multimode reader (ThermoFisher, Basingstoke, UK).

Items required

- Quant-iT PicoGreen (Invitrogen catalogue number P7589 (1ml vial) or P11496 (10 vials of 100 µL each). Kit also contains 20 x TE and lambda DNA standard (100 µg/ml in TE)
- Varioskan Flash spectral scanning multimode reader
- 96-well flat-bottomed plates (black are the best choice, clear the next best whilst white plates may increase the background)
- USB stick for data collection

Make sufficient 1x TE - 200µl per sample is required.

Add 99µl TE and 1µl sample to each well of a flat bottomed 96 well plate. Keep 5 wells spare for the standards. In these 5 wells place:

1. 20µl DNA standard (see PicoGreen manual for preparation) + 80µl TE
2. 10µl DNA standard + 90µl TE
3. 5µl DNA standard + 95µl TE
4. 1µl DNA standard + 99µl TE
5. 100µl TE

Make PicoGreen solution. 100µl is needed per sample. PicoGreen is provided at 200x concentration. Thus, for 1 sample add 0.5µl PicoGreen to 99.5µl TE. Make excess to allow for pipetting errors. Wrap tube in foil to protect from light.

2-5 minutes prior to reading add 100µl PicoGreen solution to each well (with multichannel if necessary). Read with excitation wavelength of 480nm and emission wavelength of 520nm.

Subtract the background reading (from 100µl TE well) from all sample readings then produce a fluorescence standard curve. Use the calculated curve equation to calculate sample concentrations.

References

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3.11 Microinjection of adult *Anopheles gambiae*

KaraJo Sprigg, Kristin Michel

Introduction

Delivery of double-stranded RNA in adult *Anopheles gambiae s.s.* is most often achieved through direct injection into the hemocoel. This protocol gives a detailed description of such a method first introduced by Blandin et al, in 2002. Additionally, specific aspects that in our experience are key to good survival (>90% 24 h/after injection) are emphasized. Using mosquitoes 2-3 days post emergence seems to work best in our hands.

Materials

CO_2	Local provider
Fine Forceps	Fine Science Tools, Dumont #5 Standard Inox, 11251-20
Glass Capillaries	Borosilicate, Drummond; 3.5" #3-000-203-G/X
Mineral Oil	Fisher Scientific BP2629-1 or comparable
Needles	26 Gauge, 0.5 inch, beveled, Fisher Scientific, 1482028, or comparable
Paint Brush	Fine tip; local supermarket
Syringes	1ml, Fisher Scientific, 1482028, or comparable

Equipment

CO_2 Flow regulator	Any flow regulator for CO_2 with a range of at least 1-14L/min would work. We use the "flowbuddy" system and closed one of the outlets (Genesee Scientific, Benchtop Flowbuddy Flow Regulator, 789099) http://www.flystuff.com/anesthesia.php)
CO_2 pad	Genesee Scientific, Flypad, Standard Size, 789060
Injector	Drummond Nanoject II, 3-000-204 + footswitch, 3-000-026; http://www.drummondsci.com/products/oo_01.html
Microscope lamp	A cold light source is preferred. We use Zeiss, KL 1500 LCD, with a 2-branched Goose-neck light guide.
Needle puller	Sutter instruments Model P-97, http://www.sutter.com/products/product_sheets/p97.html
Stereomicroscope	Any basic dissecting stereomicroscope will do. We use Zeiss, Stemi 2000

Pulling injection needles

Borosilicate capillaries can be pulled into injection needles using the Sutter Instruments needle puller Model P-97. The program we use is "H= *420**, P=150, V=150, TIME=133". The heat setting (H) is dependent on the specific heat filament that is used, and needs to be determined using the ramp test according to the manufacturer's instructions. Pulled needles are sealed at the tip and can be stored in a 10mm diameter petri dish secured into a strip of modeling clay. **Figure 1** shows the borosilicate capillaries after pulling. Good injection needles have a fine opening and an inflexible tip. Injection needles with a wide opening cause substantial tissue damage, while needles with a very flexible tip do not easily penetrate the body wall.
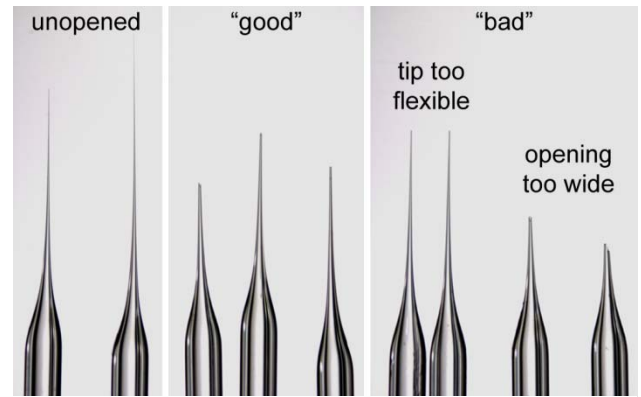
Figure 1: Injection needles.

Injection setup

Injections are performed with a hand-held needle connected to a volume dispenser. We use the Nanoject II (Drummond), which dispenses up to 69nl per injection. For larger volumes, one can either dispense several times (up to 3x works in our hands), or switch to a different system (e.g. Hamilton dispensers).

To prepare the injection needle, clip off the flexible part of the tip of a pulled borosilicate capillary using fine forceps (**Figure 2**). Fill a 1 ml syringe with mineral oil and place a 26 gauge needle on the syringe. Place the needle into the end of the clipped borosilicate capillary ("injection needle") and fill the capillary with oil. There should be no bubbles in the capillary, otherwise the injection volume will be imprecise.

Figure 2: Clip the tip of the borosilicate capillary with forceps.

Assemble the Nanoject injector head with the oil-filled injection needle according to manufacturer's description. Place the collet onto the back of the injection needle and then push the large black O-ring onto the micropipette. Place the injection needle onto the wire plunger so that it "sits" in the white spacer O-ring (**Figure 3**). Tighten the collet and empty the oil until the plunger is fully extended. Set up the remaining parts of the Nanoject II

Figure 3: Injection needle placed on the Nanoject.

including foot pedal. Front fill the injection needle with the solution to be injected. We use the Nanoject II set to 69nl injection and to "fast" dispense. Settings are described on the back of the control box as well as in the Nanoject II user Manual.

Mosquito Anesthesia

To immobilize the mosquitoes for injection, one can use a chill plate or CO_2 (see **Chapter 3.8**). We routinely use CO_2 and the flow buddy system with a CO_2 pad (Flystuff.com). To prevent static that could lead to loss of legs and decrease survival, moisten the CO_2 pad with water prior to use. Initial knockdown of mosquitoes should be done at a relatively high flow of CO_2 (10 l/min) for about 30 sec. During this time, hold the pad over the mosquito cup until all the mosquitoes have been anesthetized. Turn the flow buddy down to 3-5
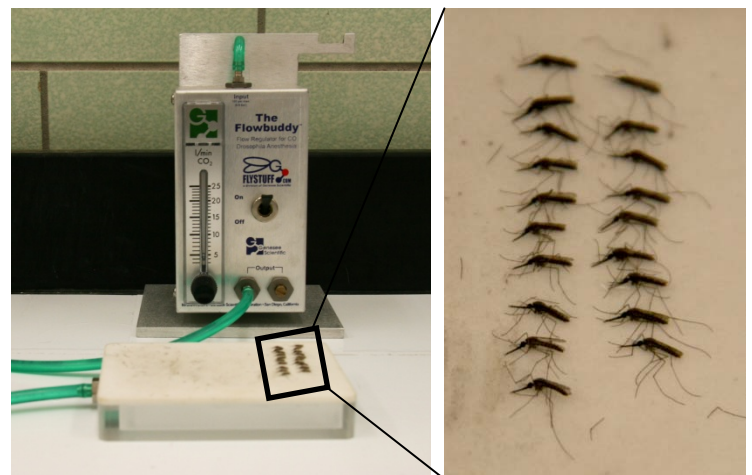
Figure 4 : CO2 anesthesia and mosquito line-up for injections

l/min and lightly dump the mosquitoes onto the flow buddy pad. Using forceps, grip the mosquitoes' legs and place them on their side. The final setup is shown in **Figure 4**. Best survival rates are obtained, if CO_2 exposure is limited to 15-20 minutes at flow rates of 2-5 l/min.

Mosquito injections

Mosquitoes are injected on the CO_2 pad under the dissecting microscope while holding the Nanoject in the dominant hand and a paintbrush in the other. As gently as possible insert the needle below the wing base above the 2nd epimeron/episternum (**Figure 5**),. Dispense liquid using the foot paddle. We visually confirm that all liquid is injected into the mosquito and usually discard any mosquito from which liquid oozes back out. Using the paintbrush, gently brush the mosquito off the injection needle. Continue injecting all mosquitoes on the pad – groups of 35 work the best in our hands. Once finished, dump the mosquitoes back into the rearing cup, using the paintbrush when needed. Mosquitoes should be returned to their normal rearing conditions as soon as possible after injection. Gentle handling throughout the procedure is paramount to survival. Good indicators are (i) all mosquitoes keep their legs, and (ii) mosquitoes are awake and resting either on the netting or sides of the cup.
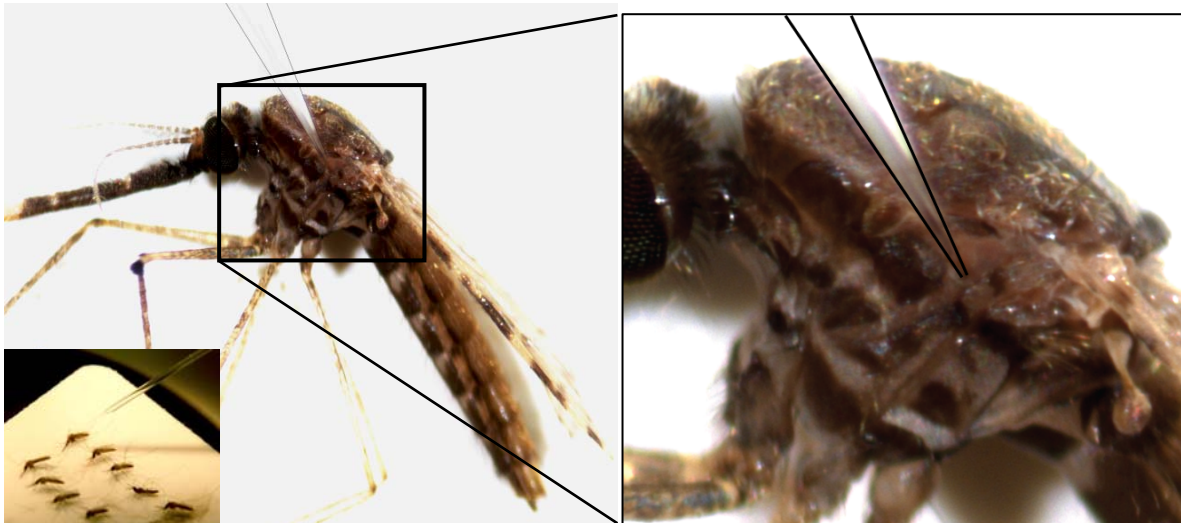
Figure 5: **Mosquito injection into the lateral part of their thorax**

Typical survival after injection:

Survival is affected by a variety of factors. In general, adult females 2-3 days post emergence, have the best survival rates, which decreases with increased age. A blood meal, given within 12 hours of injection, usually negatively affects survival. Males always show decreased survival compared to females of the same age.

Performed by a trained person, typical survival of 2-3 d old female *An. gambiae* s.s. 24h post injection should be >90% and should remain so for the next few days. First timers usually kill anywhere between 20-100% of mosquitoes, but
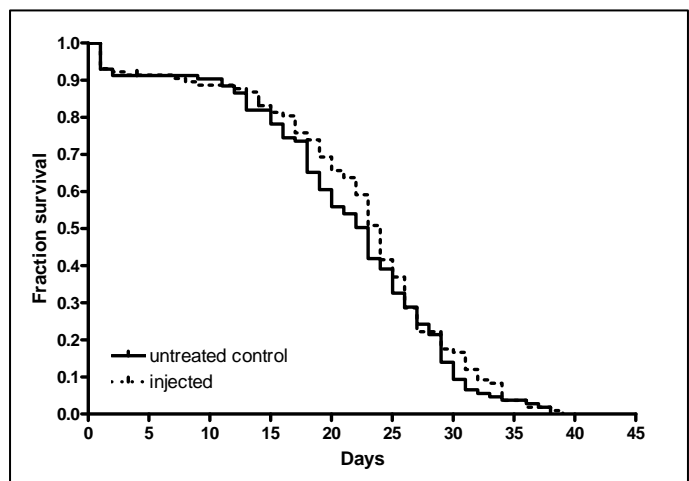
Figure 6: **Typical survival curves of injected and untreated adult female *An. gambiae s.s.*** mosquitoes were injected 3 days post eclosion.

survival rates usually improve quickly with a few practice sessions. We found that the key to good survival is a good injection needle (see **Figure 1**), minimal penetrance of the needle into the mosquito body during injection, and limiting the exposure to CO_2 to 15 minutes per mosquito batch. Steady hands are a plus, so people try to keep their caffeine intake to a minimum on injection days. A typical survival curve is shown in **Figure 6**. If handled properly, injected mosquitoes survive at rates similar to those of uninjected mosquitoes.

References:

Blandin S, Moita LF, Kocher T, Wilm M, Kafatos FC, Levashina EA (2002) Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the Defensin gene. EMBO Rep 3: 852-856

3.12 Hemocyte Collection Protocol for *Anopheles*

Ryan Shepherd and Dr. Aparna Telang (Adapted from Qayum and Telang, 2011)

Background

Some of our deadliest human diseases are caused by pathogens that are vectored by mosquitoes: *Aedes aegypti* vectors viruses that cause Dengue and *Anopheles gambiae* vectors a protist that causes malaria. Physiological mechanisms behind the ability of mosquitoes to vector or resist pathogens are being actively studied. While vertebrates have both innate (general against many pathogens) and adaptive immunity (one that is specific and shows memory of previous infections), studies thus far indicate that insects exhibit innate immunity only. Insect hemolymph plays a major role in this immunity and has humoral and hemocytes as cellular responses. In this research communication, we provide a protocol to collect hemolymph and prepare hemocytes from *Anopheles* mosquitoes. This protocol is modified from previously published methods for *Ae. aegypti* (Qayum and Telang, 2011).

Materials
- Dissection microscope
- Microinjector
- Needle puller
- Glass needles (borosilicate with filament, length: 10cm, OD 1.0mm, ID 0.78mm)
- Glass depression-well slides
- Glass microscope slides and cover slips
- Glass etching tool
- HEMA3 staining set (Fisher Scientific)
- Deionized water
- 70% ethanol
- Ice container
- Forceps and micro-scissors
- Perfusion solution (from Castillo et al, 2006)
- Shurn/Mount water base glue

Needle Pulling Settings (for a Sutter Instruments, Model P-87)[1]
- Heat ramp +5
- Pull: 45
- Vel: 75
- Time: 175
- Pressure: 580

Perfusion Solution

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[1] Note: we typically obtain needles of about 50mm in overall length with shanks of 3.5-4mm

- 60% Schneider's Medium (Sigma Aldrich)
- 10% fetal bovine serum
- 30% citrate buffer (anticoagulant) [98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 4.5)

METHODS

Initial Setup

1. Pull a set of glass needles. Pre-pulled needles can also be purchased (e.g. Tritech Research). Break the tip to create needle opening.
2. Prepare perfusion solution: We make a stock and then 1 mL aliquots (store at -20°C). Each aliquot should only be used once.
3. Prepare glass slides (1 per mosquito): score a 1 cm diameter circle at one end of each slide using a glass-etching tool. Glass slides with two etched circles can also be purchased (Fisher Scientific).
4. Backfill the microinjector tubing and syringe with white mineral oil according to manufacturer instructions.
5. Insert a prepared needle into the microinjector needle holder and backfill it with white mineral oil according to manufacturer instructions.
6. Place 15 adult females into small cages or cup containers that can be completely submerged in ice on all sides. This will ensure that all mosquitoes will be exposed to the cold.
7. Cold anesthetize mosquitoes for about 8 minutes or until they are immobilized.

Hemocyte Collection

1. Set the microinjector to pick up 10 µl of perfusion solution into the glass needle and inject 1.5-2µl of solution. Inject solution into the abdomen of the mosquito between the seventh and eighth segment. The mosquito should be held lightly with forceps during the injection. After injection, the abdomen should look "filled" or bloated. This step is done to dislodge adhering hemocytes from tissues so that all hemocytes are free in hemolymph. About 4 mosquitoes can be injected using the designated microinjector setup. Excess volume of perfusion solution should remain in the needle to prevent mineral oil from being injected.
2. Injected mosquitoes should be placed in a separate cup on ice to recover for 7 minutes. Another 3-4 mosquitoes can be injected during this time.
3. After 7 minutes of recovery, the wings, legs, and tip of the abdomen (at the eighth segment) of each mosquito should be removed one at a time using micro scissors. This should be done in a separate recovery cup on ice. Removing the legs and wings reduces the likelihood of scales being transferred to the collection slides. Do not cut legs and wings too close to the thorax as this may create other openings for hemolymph to escape. Hemolymph should only be collected through the cut abdominal opening.
4. Wash an etched slide with 70% Ethanol and dry it with a kimwipe. Place one prepared and recovered mosquito on the outer edge of the etched circle. Take up 6µl of perfusion solution into the microinjector and inject this volume into the lateral side of the mesothorax. Deliver most but not the entire volume of the solution to prevent mineral oil from being injected. There should be a small amount of solution left in the tip of the needle.

5. Once the needle is removed, position the mosquito so that its abdominal opening is at the edge of the 1 cm circle. Hold the mosquito between the tips of the forceps and gently squeeze the abdomen to encourage the flow of hemolymph onto the etched circle area.
6. Allow collected hemolymph to dry on slides for about 10 minutes or until it looks dry.
7. Stain each slide separately with HEMA3 (Hillyer et al., 2005): Dip slide in the Fixative 5 times for 1 second each; Dip slide into Solution I 3 times for 1 second each; and lastly, dip slide into Solution II 3 times for 1 second each. Wash only the back of the slide with DI water. The front should be blot dried outside the etched circle in order to prevent loss of hemocytes.
8. Apply mounting glue around the outer edge of etched circle and place a cover slip over this area. Apply the coverslip gently so that the glue spreads to the entire area under the cover slip. Allow stained and prepared slides to dry for a minimum of 3 hours or let dry overnight. Slides should be kept in the dark during this time to ensure the tissues stain well. Hemocytes should be imaged soon after preparation because the stain intensity tends to deteriorate about 2 weeks after collection.

Representative Data

We used previously published criteria for classifying hemocytes (Castillo et al., 2006). **Figures 3.12.1-3.12.4** show representative images of each hemocyte type. **Table 3.12.1** shows hemocyte numbers obtained from 3 separate replicates of one-week old *Anopheles dirus* females.

Table 3.12.1. Hemocyte population profile from one-week old *Anopheles dirus* females shows that oenocytoids make up the largest percentage of total hemocytes in this species.[2] Hemocyte numbers overall are expected to be higher in younger females.

	Total hemocytes	Prohemocyte	Granulocyte	Oenocytoid
Average	395.1	46.5	11.5	337.1
Std Dev	205.2	23.3	4.7	187.8

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[2] We collected hemocytes from a single replicate of one-week old adult *Anopheles gambiae* females (n=10) and observed that granylocytes made up the largest percentage of total hemocytes (data not shown). This is consistent with previously published data on *An. gambiae* (Castillo et al., 2006).

Figure 3.12.1: Granulocyte	**Figure 3.12.2:** Oenocytoid
Figure 3.12.3: Prohemocyte	**Figure 3.12.4:** Prohemocyte (enlarged)
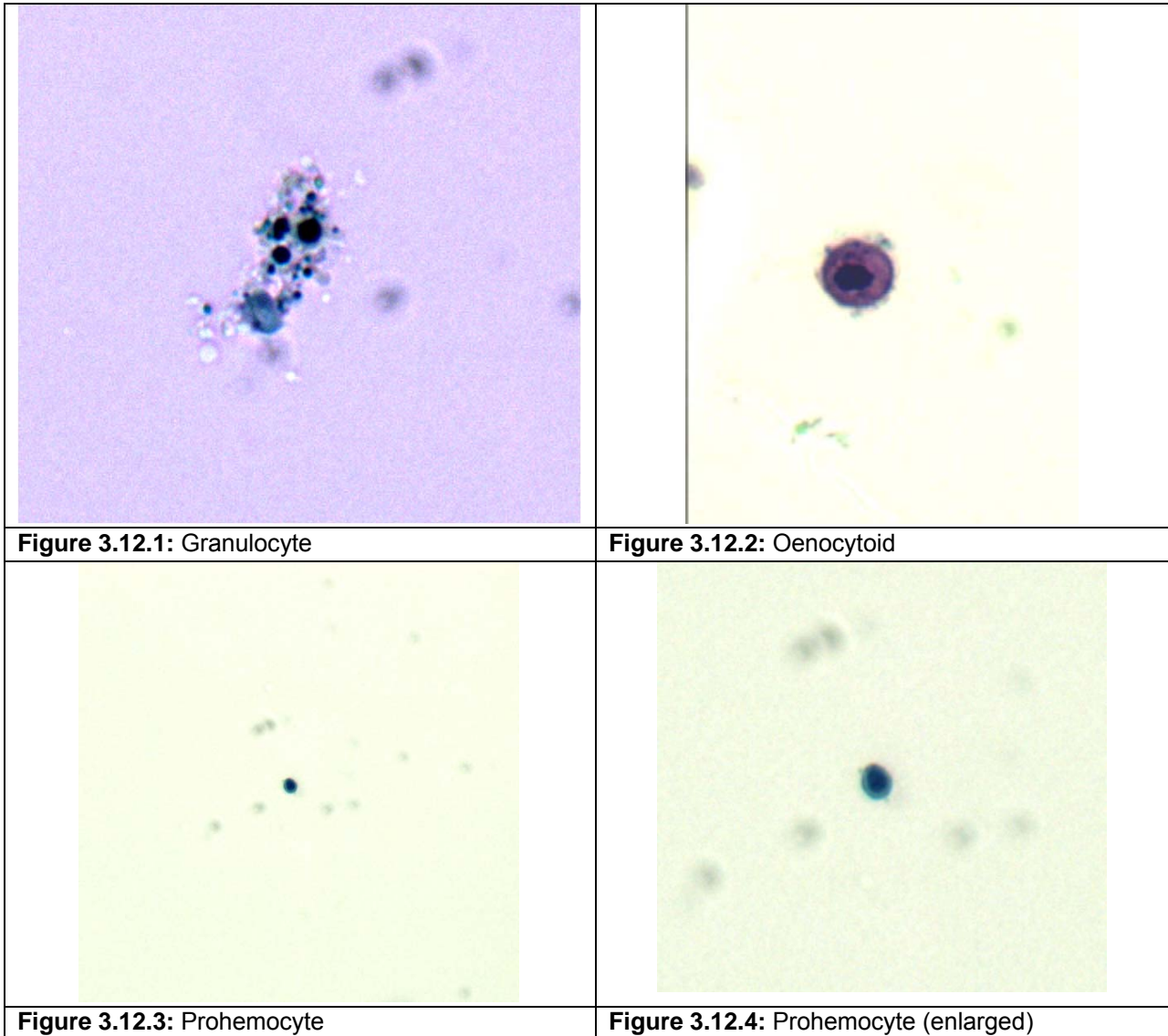
Figure 3.12.1: Light microscopes images (400x magnification) taken of HEMA3 fixed, stained hemocytes.

References

Castillo, J.C., Robertson, A.E., & Strand, M.R. (2006). Characterization of hemocytes from the mosquitoes *Anopheles gambiae* and *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* **36:** 891-903.

Hillyer, J.F., Schmidt, S.L., Fuchs, J.F., Boyle, J.P., & Christensen, B.M. (2005). Age-associated mortality in immune challenged mosquitoes (*Aedes aegypti*) correlates with a decrease in haemocyte numbers. *Cellular Microbiology* **7:** 39-51.

Qayum, A.A., Telang, A. (2011). A protocol for collecting and staining hemocytes from the Yellow Fever mosquito *Aedes aegypti*. JoVE. 51. http://www.jove.com/index/Details.stp?ID=2772, doi: 10.3791/2772.

Chapter 4 : Stock Authentication

4.1 Stock Authentication by Morphological Characteristics

MR4 Staff

Introduction

It is common for laboratories to rear several different species and/or stocks of the same species in one insectary. Often it is difficult to determine whether a colony has been contaminated, especially when the stocks appear identical. A PCR method to distinguish four anopheline species based on their 28S ribosomal subunit was developed as a quality control method to ensure contamination had not occurred between colonies (Kent et al. 2004). Although this method is highly specific, it can be very costly performing several PCR assays to detect a rare contaminant, so it is desirable to develop simple direct methods to verify colony purity.

Morphological discrimination of adults

It is not necessary to have extensive knowledge of mosquito identification to develop methods to keep stocks in order. A simple method to confirm identity is to develop 'local authentication standards' based on morphological characteristics of adults. These standards are not meant to distinguish your mosquito from all of those in the world but rather to distinguish the ones *you* maintain from one another. Therefore the standards are 'local'. The features can be described in very general language e.g. large adults, white knees, grey. Although these methods are not useful for members of cryptic species complexes like *An. gambiae*, it does work well when several different species of different appearance are maintained.

The local authentication standard consists simply of a chart that lists useful morphological characters that individually, or in some combination, distinguish all the species you keep. Its creation is simple. Remove several male and female members of each species and stun them in the freezer for a few minutes or anesthetize them by some other method. Place them side-by-side under a dissecting scope and scan prominent morphological landmarks (see below) to see if any differ. After selecting some candidate feature(s), scan larger numbers to ensure all individuals have the characteristic and it can be seen even in older individuals in which e.g. the scales may have rubbed off. An example of such a local standard is shown in Table 1.

Common Morphological Characteristics

- **Protarsi**: In *An. gambiae* and *An. farauti* you will usually find three white bands on the distal end of the protarsus. This characteristic is not seen in *An. dirus*, *An. freeborni*, or *An. quadrimaculatus*. **Figures 4.1.1-3**.

- **Metatarsi**: Unique, species specific, white banding patterns are often seen in *An. dirus* (white banding on the femur-tibia joint) and *An. albimanus* (prominent broad white bands on metatarsi). **Figures 4.1.4-6**.

- **All tarsi**: In some species the legs will appear spotted or speckled under magnification. This can be seen in *An. stephensi*, *An. dirus*, and *An. farauti*.

- **Abdominal banding patterns**: The ventral side of the abdomen can look very similar between species e.g. *An. stephensi* and *An. gambiae*. However, among others, there are various sizes of bands seen (e.g. *An. freeborni* have narrow transverse banding while *An. quadrimaculatus*, *An. atroparvus*, and *An. minimus* have wide transverse abdominal banding). **Figures 4.1.7-9**.

- **Halteres**: We have found that coloration of these structures is a good separation technique for a few strains. *An. dirus* and *An. farauti* both have halteres that are black ventrally and white dorsally. **Figures 4.1.10-12**.

- **Anterior wing margin**: Although the specific banding pattern is highly distinct, often the presence or absence of dark scaling on the wing margin is enough to distinguish between 2 species. **Figures 4.1.13-15**.

- **Palps**: Most anophelines have some banding on their palps. The number or width of bands or the lack of bands can be very diagnostic. **Figures 4.1.16-18**.

Protarsi

Figure 4.1.1. *An. gambiae.*

Figure 4.1.2. *An. farauti.*

Figure 4.1.3. *An. quadrimaculatus.*

Metatarsi

Figure 4.1.4. *An. gambiae.*

Figure 4.1.5. *An. albimanus.*

Figure 4.1.6. *An. dirus.*

Abdominal pigment

Figure 4.1.7. *An. gambiae.*

Figure 4.1.8. *An. dirus.*

Figure 4.1.9. *An. albimanus.*

Halteres

Figure 4.1.10. *An. gambiae.*

Figure 4.1.11. *An. farauti* (dorsal side).

Figure 4.1.12. *An. farauti* (ventral side).

Wings
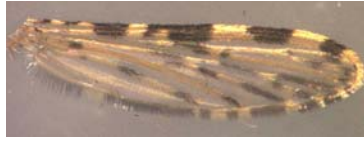
Figure 4.1.13. *An. gambiae.*

Figure 4.1.14. *An. albimanus.*
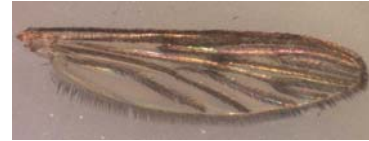
Figure 4.1.15. *An. quadrimaculatus.*

Palps

Figure 4.1.16. *An. gambiae.*

Figure 4.1.17. *An. quadrimaculatus.*

Figure 4.1.18. *An. dirus.*

Once you have made a checklist of which traits each species has, simply tabulate the results to see how they differ (**Table 4.1.1**). For example, if you have *An. stephensi* and *An. gambiae,* the two can be difficult to separate with most features but can be separated by the heavily spotted tarsi on *An. stephensi.* Likewise, *An. farauti* and *An. stephensi* can be separated from one another based on the presence of a ventrally black haltere as seen in *An. farauti.*

	STECLA	ORLANDO	F1	GA/AR/ME/QD	FAR1	STE-2
Character	albimanus	quadrimaculatus	freeborni	gambiae complex	farauti	stephensi
liberally spotted tarsi	N	N	N	N	Y	Y
haltere black ventrally	N	N	N	N	Y	N
prominent broad white band distal on metatarsi	Y	N	N	N	N	N
sooty dark wings	N	Y	N	N	N	N
prominent dark anterior margin wing pigment	Y	N	N	Y	Y	Y
mottled abdominal pattern similar to camouflage	Y	N	N	N	N	N

Table 4.1.1: A simple table of a few adult morphological characteristics useful to distinguish different species in laboratory settings.

Useful traits of immatures

Although not as useful as adult characters, there are some unique phenotypes that vary and are easily observed. The use of these phenotypes in conjunction with adult characteristics ensures strain purity.

Larval / pupal stripe: *Anopheles* larvae and pupae often have a unique white (**Figure 4.1.19**) or red (**Figure 4.1.20**) stripe on their dorsum (French and Kitzmiller 1964; Mason 1967), and its pattern and intensity varies. The *An. freeborni* F1 colony uniformly carries a white stripe phenotype which is especially noticeable in the pupal stage. The red stripe characteristic in *An. gambiae* is typically sex-limited to females. Not every *gambiae* stock will have this phenotype (*e.g. An. arabiensis* from Sudan do not express this whereas our *An. arabiensis* from Tanzania do) and the variation itself is a useful observation.

Figure 4.1.19. *An. freeborni* white stripe larvae (bottom) shown for comparison with *An. quadrimaculatus* (top).

Figure 4.1.20. *An. gambiae* larvae shown with (bottom) and without (top) red stripe characteristic.

Larval color: Mutants with differing larval color have been widely reported from *An. stephensi* in India as well as *An. quadrimaculatus* and *An. albimanus* (Seawright et al. 1985). Often these are genetic, but they also may be linked to diet. Culturing a pure-breeding variant-color colony can make separation of that colony from others quite easy (**Figure 4.1.21**).

Eye color mutations: Eye color mutants can be separated from wild-eye larvae based on their inability to melanize when reared in a dark or black pan (See Eye-Color Mutant Screening). Most larvae detect their environment and darken. Eye color mutants cannot discern their backgrounds so they will not melanize (**Figure 4.1.22**).

Collarless trait: Some larva will have a "collar" on the dorsum of the abdomen and some will not (**Figure 4.1.23**) (Mason 1967). Many wild strains are polymorphic for this trait. However, choosing those that either have the trait or do not to continue a colony can make it easy to quickly note a contamination event.

Larval postures when resting on the bottom: These are not definitive by any means. However, some species rest differently when compared side by side. *An. farauti* has a "U" shape, *An. gambiae* rest in an "L" shape, and some *An. atroparvus* larvae will appear to rest in a "?" manner (**Figure 4.1.24**). Other behaviors are distinct: *An. minimus* larvae cluster around the edge of the pan while very few will venture into the center. Once you learn what is customary for your strains, watch for changes.
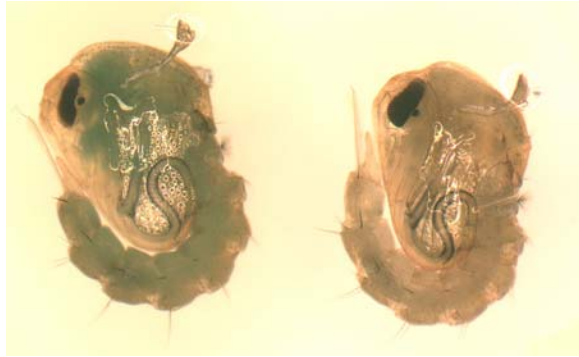
Figure 4.1.21. Examples from two *An. stephensi* strains carried simultaneously in an insectary, GREEN1 (left) and STE2 (right). GREEN1 was selected for a green mutation from the wild type strain. The green mutant is likely that of Suguna (1981).

Figure 4.1.22. *An. gambiae* (ASEMBO1) larva reared in a white pan (bottom) and a black pan (top) demonstrating the melanization capabilities of this wild-type strain.

Figure 4.1.23. *Collarless* trait shown on dorsum of *An. gambiae*. Compare the white pigment to collarless-minus individuals shown in **Figure 4.1.22**.

Figure 4.1.24. Curled larval resting posture seen commonly in a disturbed pan of *A. farauti*.

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Kent RJ, West AJ, Norris DE (2004) Molecular differentiation of colonized human malaria vectors by 28S ribosomal DNA polymorphisms. Am J Trop Med Hyg 71:514-517

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Seawright JA, Benedict MQ, Narang S (1985) Color mutants in *Anopheles albimanus* (Diptera: Culicidae). Ann Entomol Soc Am 78:177-181

Suguna SG (1981) The genetics of three larval mutants in Anopheles stephensi. Indian J Med Res 73:120-123

4.2 Authentication by PCR

4.2.1 *Anopheles gambiae* Thioester-Containing Protein (TEP1) PCR Assay

MR4 Staff

Introduction

Recent work has shown a relationship between the presence of a TEP1 mutation with an increase in *P. berghei* parasite mortality in *An. gambiae* mosquitoes (Blandin et al. 2004). An SNP-based PCR assay was developed to detect the mutation in *An. gambiae* mosquitoes. Several MR4 stocks are distinguished by being pure-breeding for either allele.

PCR assay for the TEP1/16 mutation in *An. gambiae*

Prepare PCR Master Mix for 96, 48 or 1 25 µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	0.5	Reagent
785 µl	517.5 µl	7.85 µl	6.7 µl	sterile H_2O
500 µl	250 µl	5.0 µl	2.5 µl	5X Taq PCR Buffer
250 µl	125 µl	2.5 µl	1.25 µl	dNTP (2.5 mM mix)
250 µl	125 µl	2.5 µl	0.5 µl	TEP1 (F, 10pmol/µl) [AAAGCTACGAATTTGTTGCGTCA][2]
250 µl	125 µl	2.5 µl	0.5 µl	TEP1R (R, 10pmol/µl) [ATAGTTCATTCCGTTTTGGATTACCA]
250 µl	125 µl	2.5 µl	0.5 µl	TEP16 (R, 10pmol/µl) [CCTCTGCGTGCTTTGCTT]
100 µl	50 µl	1.0 µl	0.5 µl	$MgCl_2$ (25 mM)
15 µl	7.5 µl	0.15 µl	0.05 µl	Go-Taq DNA polymerase (5U/µl)
2.4 ml	**1.2 ml**	**24 µl**	**12.5 µl**	**Total (To each 24 ul reaction add 1 µl template DNA)**

Table 4.2.1.1. F and R indicate forward and reverse orientation. Use 1 µl DNA template. If performing the ½ volume assay, use 0.5µl template DNA.

PCR cycle conditions
94°C/5min x 1 cycle
(94°C/30sec, 52°C/30sec, 72°C/45sec) x 40 cycles
72°C/5min x 1 cycle
4°C hold

Run samples on a 1.5% agarose gel stained with EtBr, or other intercalating agent like SYBR Green or Gel Red, and load 10µl of sample. Run gel for at least 1.5 hours at 120V to separate the TEP1 bands. This PCR will yield fragments of 372 bp for homozygous susceptible and 349 bp for homozygous refractory, respectively. Heterozygous samples will have both bands.

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.
[2] Note: the TEP1 forward primer differs from that published in Blandin et al 2004. The sequence shown above is correct based on a personal communication from the author.

Figure 4.2.1.1. The TEP1 assay performed on a homozygous and heterozygous samples. Lane 1, 1kb ladder.

References

Blandin S, Shiao SH, Moita LF, Janse CJ, Waters AP, Kafatos FC, Levashina EA (2004) Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. Cell 116:661-670

96 well sample preparation template

4.2.2 *Anopheles arabiensis* ND5 Molecular Authentication

Liz Wilkins

Introduction

Anopheles arabiensis is a member of the *An. gambiae* s.l. complex. *An. arabiensis* strains from different regions have been found to be variable in the region of the mtDNA dehydrogenase gene subunit 5 (ND5) (Temu and Yan 2005). This region yielded the single nucleotide polymorphisms (SNPs) necessary to create a PCR to distinguish two laboratory colonies of *An. arabiensis*: KGB which originated from Zimbabwe about 1975, and *A. arabiensis* Dongola which originated from Sudan in 2004. The two are morphologically indistinguishable in the larval or adult stages and both are susceptible to insecticides making molecular distinction necessary. Part of the ND5 region was amplified and sequenced from each strain, and SNP sites were used to create primers using the intentional mismatch primer (IMP) (Wilkins et al. 2006) method of design at the SNP sites. This protocol is useful in distinguishing these two strains one from the other.

PCR authentication for the members of the *Anopheles arabiensis* complex

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions. Add reagents in the order presented.

96	48	1	0.5	Reagent
1185 µl	592.5 µl	11.85 µl	5.95 µl	sterile H_2O
500 µl	250 µl	5.0 µl	2.5 µl	5X PCR Buffer
200 µl	100 µl	2.0 µl	1.0 µl	dNTP (2.5 mM mix)
100 µl	50 µl	1.0 µl	0.5 µl	DF (F, 25pmol/µl) GATAAAGCAATAATTTTCTTTAAAGCG
100 µl	50 µl	1.0 µl	0.5 µl	KR (R, 25pmol/µl) GGTGCAAATTTTGAATTTGATTTACAA
100 µl	50 µl	1.0 µl	0.5 µl	IPCF (R, 25pmol/µl) GCATGAGTTAATAAATGAAAAAAAGC
100 µl	50 µl	1.0 µl	0.5 µl	IPCR (R, 25pmol/µl) CTATAACTAAAAGTGCCCAAATTC
200 µl	100 µl	2.0 µl	1.0 µl	$MgCl_2$ (25mM)
15 µl	7.5 µl	0.15 µl	0.05 µl	Go-Taq DNA polymerase (5U/µl)
2.5 ml	**1.25 ml**	**25 µl**	**12.5 µl**	**Total (To each 25 µl reaction add 1 µl template DNA)**

Table 4.2.2.1. F and R indicate forward and reverse orientation. If performing the ½ volume assay, use 0.5µl template DNA instead.

PCR Cycle conditions

95°C/5min x 1 cycle
(95°C/30sec , 58°C/30sec , 72°C/30sec) x 30 cycles
72°C/5min x 1 cycle
4°C hold

Run samples on a 1.5% agarose gel stained with EtBr, or other intercalating agent like SYBR Green or Gel Red. Primers create an internal positive control of 350 bp and a species specific fragment of 260 bp for Dongola and 130 bp for KGB (**Figure 4.2.2.1**).

Figure 4.2.2.1. Lane 1 1Kb marker, lane 2 *An. arabiensis* KGB, lane 3 *An. arabiensis* Dongola, Lane 4 1Kb marker.

References

Temu EA, Yan G (2005) Microsatellite and mitochondrial genetic differentiation of *Anopheles arabiensis* (Diptera: Culicidae) from western Kenya, the Great Rift Valley, and coastal Kenya. Am J Trop Med Hyg 73:726-733

Wilkins EE, Howell PI, Benedict MQ (2006) IMP PCR primers detect single nucleotide polymorphisms for *Anopheles gambiae* species identification, *Mopti* and *Savanna* rDNA types, and resistance to dieldrin in *Anopheles arabiensis*. Malar J 5:125

96 well PCR sample preparation template

4.2.3 *Anopheles gambiae* white gene

Liz Wilkins, Alice Sutcliffe, Paul Howell

Introduction

The white gene of *Anopheles gambiae* is located on the X chromosome and known for intron variation, making it a good marker for differentiating closely related species (Mukabayire et al. 2001). In laboratories where several wild stocks from the same sub-species and origin are reared concurrently, it can be difficult to ensure that contamination events between the stocks has not occurred. The MR4 cultures two pairs of such closely related stocks, MOPTI and MALI, ZAN/U and PIMPERENA. An 800 bp segment of intron 3 of the white gene ((Besansky et al. 1995); GenBank accession number U29485) was amplified using primers WG2 GAGCATCATTTTTTGCTGCG and WG5 CGTGGTTATCGTATCAAAAG as published by (Mukabayire et al. 2001) and sequenced to discover single nucleotide polymorphisms (SNPs) between the stocks (**Figure 4.2.3.2.**). Primers were designed using the intentional mismatch primer (IMP) method (Wilkins et al. 2006) at these sites for a stock specific authentication method.

PCR authentication for stock specific SNP sites in the white gene

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1]

96	48	1	Reagent
1085 µl	542.5 µl	10.85µl	Distilled H2O
500 µl	250 µl	5.0 µl	5X GoTaq PCR Buffer
200 µl	100 µl	2.0 µl	dNTP (2.5 mM concentration)
100 µl	50 µl	1.0 µl	UFOR (25 pmol/ µl) ATT ATC TGA TGA AGC TTG GAG TCT TTT
100 µl	50 µl	1.0 µl	UREV (25 pmol/ µl) ATG AAA ATA AGG AGC TTC CTG GCA T
100 µl	50 µl	1.0 µl	PIMPR (25pmol/ µl) TCA ATG ACA TGA CGT TAT AAT CTG TCT TT
100 µl	50 µl	1.0 µl	MOPR (25pmol/ µl) CTG TTG TCT TAC AGT AGG GTT ATG T
100 µl	50 µl	1.0 µl	MOP2R (25pmol/ µl) AAC GTA CGA CGT ATG ATC TAA CTG A
100 µl	50 µl	1.0 µl	MALR (25pmol/ µl) CTC ATA TTC AAG GAT GAA CAC AAT AC
100 µl	50 µl	1.0 µl	MgCl2 (25 mM)
15 µl	7.5 µl	0.15 µl	Go-Taq DNA polymerase
2.5 ml	**1.25 ml**	**25 µl**	**Total (To each 25 µl reaction add 1 µl template DNA)**

Table 4.2.3.1. Add reagents in order presented.

PCR cycle conditions

94°C/5min x 1 cycle
(94°C/30sec , 56°C/30sec , 72°C/30sec) x 35 cycles
72°C/10min x 1 cycle
4°C hold

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 reactions to compensate for imprecise measurements.

Figure 4.2.3.1 Lane 1 1kb ladder, lane 2

Run samples on a 1.5% agarose gel stained with EtBr,or other intercalating agent like SYBR Green or Gel Red. (**Figure 4.2.3.1**). Primers create fragments of 478 universal, 413 mopti, 350 mopti & zan/u, 292 mali, 116 pimperena

Figure 4.2.3.2. Specific SNP sites in the white gene used to create this assay. Numbering based on accession number U29485.

```
                   11591       11767       11826       11885
SNP sites
U29485      TTACTATGAC GATTTCTTGT GCTTGTTAGA GACGCTTTAC
Mopti       TTACTATGAC GATTTCTTGT GTCTGTTAGA GACACTTAAC
Mali        TTACTATGAC GATGTCTTGT GCTTGTTAGA GACGCTTTAC
Pimperena   TTACAATGAC GATTTCTTGT GCTTGTTAGA GACGCTTTAC
Zan/u       TTACTATGAC GATTTCTTGT GTCTGTTAGA GACGCTTTAC
```

References

Besansky NJ, Bedell JA, Mukabayire O, Hilfiker D, Collins FH (1995) The white gene of *Anopheles gambiae*: structure and sequence of wild type alleles. submitted

Mukabayire O, Caridi J, Wang X, Toure YT, Coluzzi M, Besansky NJ (2001) Patterns of DNA sequence variation in chromosomally recognized taxa of *Anopheles gambiae*: evidence from rDNA and single-copy loci. Insect Mol Biol 10:33-46

Wilkins EE, Howell PI, Benedict MQ (2006) IMP PCR primers detect single nucleotide polymorphisms for *Anopheles gambiae* species identification, *Mopti* and *Savanna* rDNA types, and resistance to dieldrin in *Anopheles arabiensis*. Malar J 5:125

96 well PCR sample preparation template

4.2 Authentication by PCR

4.2.4 *PCR assay to detect the insertion/deletion haplotypes of the* APL1A *gene in* Anopheles gambiae s.s.

Michelle Riehle, Jiannong Xu, Brian Lazzaro, Susan Rottschaefer, Boubacar Coulibaly, Madjou Sacko, Oumou Niare, Isabelle Morlais, Sekou Trare, and Kenneth Vernick

Resistance to *Plasmodium spp.* infections by *An. gambiae* is associated with alleles located within the *Plasmodium* Resistance Island (PRI) (Riehle et al 2007). Of these, the leucine-rich repeat proteins encoded by *APL1* family are associated with resistance to *Plasmodium* (Mitri et al 2009). Within *APL1* there are three paralogous members; *APL1A, APL1B,* and *APL1C. APL1A* has been found to be protective against the human malaria parasite, *P. falciparum* (Mitri et al 2009). *APL1A* itself has been shown to have two haplotypes, *APL1A*[1] and *APL1A*[2], which also influence oocyst loads within the mosquito (Riehle et al 2008). This assay was developed to detect the insertion/deletion variants.

PCR (adapted from Riehle et al 2008)

Prepare PCR Master Mix for 96, 48 or 1 25 µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
1590 µl	795 µl	15.9 µl	sterile H_2O
250 µl	125 µl	5.0 µl	5X Taq PCR Buffer
100 µl	50 µl	1.0 µl	dNTP (2.5 mM mix)
100 µl	50 µl	1.0 µl	APL1F (10 pmol/µl) [GCT GGA TCC CAA CTA GTG CTG TT]
100 µl	50 µl	1.0 µl	APL1R (10 pmol/µl) [AGT AAA GCA GCG GGC AGT TTG C]
10 µl	5.0 µl	0.1 µl	Go-Taq DNA polymerase (5U/µl)
2.25 ml	**1.125 ml**	**24.0 µl**	**Total (To each 24 µl reaction add 1 µl template DNA)**

Table 4.2.4.1. F and R indicate forward and reverse orientation. Use 1 µl DNA template.

PCR cycle conditions

94°C/1min x 1 cycle
(94°C/30sec , 55°C/30sec , 72°C/45sec) x 30 cycles
72°C/7min x 1 cycle
10°C hold

Run samples on a 1.5% agarose EtBr, or other intercalating agent like GelRed, gel, load 10µl of sample.

Results: Mosquitoes that possess the APL1A[1] deletion will have a 663bp product while APL1A[2] individuals will have an 854bp product. We have also found in APL1A[1] a second, slightly smaller product in some colonies as seen in **Figure 4.2.4.1**

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.
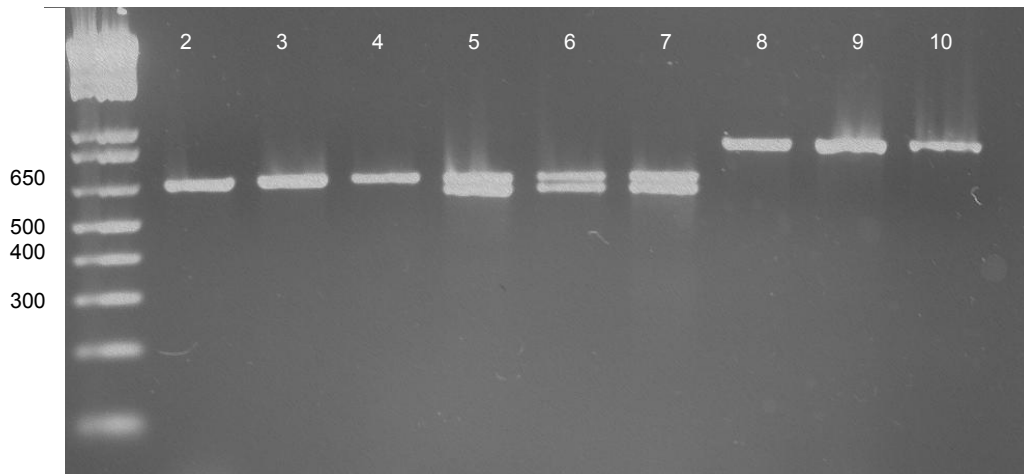
Figure 4.2.4.1. The *APL1A* assay performed on a homozygous and heterozygous samples. Lane 1, 1kb ladder, lanes 2-4 MALI NIH, lanes 5-7 RSP, lanes 8-10 KISUMU1

96 well sample preparation template

References

MM Riehle, K Markianos, L Lambrechts, A Xia, I Sharakhov, J Koella, and KD Vernick (2007). A major genetic locus controlling natural *Plasmodium falciparum* infection is shared by East and West African *Anopheles gambiae*. Malaria J 6:87.

C Mitri, JC Jacques, I Thiery, MM Riehle, J Xu, E Bischoff, I Morlais, SE Nsango, KD Vernick and C Bourgouin (2009). Fine pathogen discrimination within the APL1 gene family protects *Anopheles gambiae* against human and rodent malaria species. PLoS Pathog 5(9): e10000576.

MM Riehle, J Xu, BP Lazzaro, SM Rottschaefer, B Coulibaly, M Sacko, O Niare, I Morlais, SF Traore, and KD Vernick (2008). *Anopheles gambiae* APL1 is a family of variable LRR proteins required for Rel1-mediated protection from the malaria parasite, *Plasmodium berghei*. PLoS ONE 3(11): e3672.

4.2.5 *Anopheles* 28S species ID (Kent et al 2004)

Introduction

In many instances, laboratory colonies of different species are often reared in the same environment, often due to a lack of available or suitable rearing space. As they are different species, it is assumed that they will not interbreed and therefore remain distinct however they can be intermixed accidentally. In these instances, the contamination event may go unnoticed and the more fecund species may dominate and eventually replace its competitor. Often this isn't noticed until research results have changed. Kent et al (2004) designed a simple PCR assay based on polymorphisms in the mitochondrial DNA to discriminate between various species held in an insectary. Although most species can be discerned visually, some like *An. gambiae* and *An. stephensi* may not be initially noticed as they are similar in appearance at a quick glance. The MR4 has adopted this PCR and added in two different species, *An. sinensis* and *An. albimanus*, for which primers were designed and are listed below.

PCR authentication for the differentiation of *An. sinensis*, *An. farauti*, *An. stephensi,* and *An. albimanus*

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	0.5	Reagent
1478 µl	739.2 µl	15.4 µl	7.7 µl	sterile H_2O
480 µl	240 µl	5.0 µl	2.5 µl	5X GoTaq PCR Buffer
96 µl	48 µl	1.0 µl	0.5 µl	dNTP (2.5 mM mix)
96 µl	48 µl	1.0 µl	0.5 µl	$MgCl_2$ (25mM)
48 µl	24 µl	0.5 µl	0.25 µl	UN400F (F, 10pmol/µl) [TCTGAATAGAGAGTCAAATAGTACG]
48 µl	24 µl	0.5 µl	0.25 µl	StephensiR (R, 10pmol/µl) [GGGAGCGGCCGCCAAGC]
48 µl	24 µl	0.5 µl	0.25 µl	FarautiR (R, 10pmol/µl) [CGGAATCACGCGCTGTCGG]
48 µl	24 µl	0.5 µl	0.25 µl	ALB400R (R, 10pmol/µl) [CGCTACCGTCTGCCCACCG]
48 µl	24 µl	0.5 µl	0.25 µl	SIN400R (R, 10pmol/µl) [GGCCCCCTTCTGCGTGACAG]
9.6 µl	4.8 µl	0.1 µl	0.05 µl	Go-Taq DNA polymerase
2.5 ml	1.25 ml	25 µl	12.5 µl	Total (To each 25 µl reaction add 1 µl template DNA)

Table 1. Use 1 µl DNA template Add water to compensate for this volume if all are not included. If performing the ½ volume assay, add 0.5µl template DNA instead.

PCR cycle conditions

95°C/5min x 1 cycle
(95°C/60sec , 60°C/90sec , 72°C/120sec) x 25 cycles
72°C/5min x 1 cycle
12°C hold
Run samples on a 2% agarose EtBr gel; load 10 µl sample.

Primers create fragments of 329 bp for An. albimanus, 235 bp *An. stephensi*, and 179 bp *An. farauti,* and 112 bp for *An. sinensis*.(**Figure 1**).
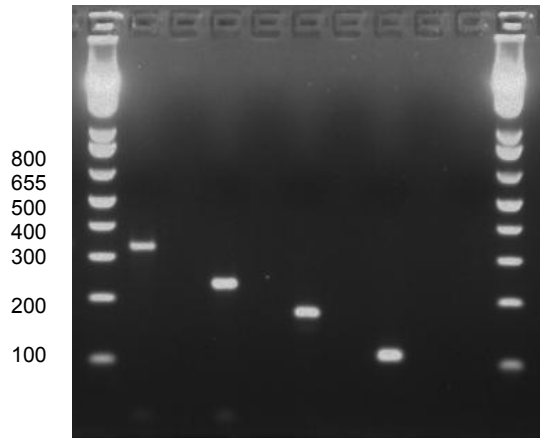
Figure 4.2.5: Lane 1, 1kb ladder, Lane 2: An. *albimanus,* Lane 4: An *stephensi,* Lane 6: *An farauti; Lane 8: An. sinensis,* Lane 11 1kb ladder

References

Kent RJ, West AJ, Norris DE (2004) Molecular differentiation of colonized human malaria vectors by 28S ribosomal DNA polymorphisms. *Am. J. Trop. Med. Hyg.*71: 514-517

Chapter 5 : Insecticide Resistance Monitoring

5.1 Insecticide Resistance Bioassays

5.1.1 Larval Insecticide Resistance Assays

MR4 Staff

Introduction

Insecticide-susceptibility defined stocks require routine quality control measures to determine whether a contamination event has occurred. Exposure is also conducted to ensure that the level of resistance has not changed. Larval exposures are simple to conduct since they are a non-flying stage and exposure levels are consistent in the aquatic environment. There are numerous variations for larval exposures, but the MR4 uses fixed time and dose treatments of L4s routinely because of their ease. Exposure of other stages and even embryos is also possible using similar methods.

In each of these assays, L4s are subjected to a known concentration of an insecticide for a fixed time period (French and Kitzmiller 1963). Because some mortality may occur even among resistant individuals, if the purpose is selection for a resistant colony, a large cohort should be tested to perpetuate the colony.

Experience with a particular insecticide will indicate how long the exposure should be and what time periods are appropriate for your application. The exact time and concentration necessary for causing mortality or boosting insecticide resistance is best determined utilizing dose-response curves.

Some typical discriminating concentrations and treatment times for L4s (solvent).

- DDT: 0.4ppm DDT (ethanol) for 24 hours.

- Permethrin: 1ppm permethrin (ethanol) for 24 hours.

- Propoxur: 20ppm propoxur (acetone) for 1 hour.

- Dieldrin: 1ppm dieldrin (ethanol) for 1 hour.

- Malathion: 1ppm malathion (ethanol) for 24 hours.

Materials[1]

- Concentrated insecticide from which stock dilution will be prepared (1000 X the final treatment concentration is recommended)

- Glass graduated cylinders

- Volumetric flask

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[1] Many of the insecticides will be formulated in solvents that dissolve plastics - especially acetone - so glass bottles and pipettes are recommended.

- Glass pipettes

- Pipetting device

- 100 ml glass bottles with screw tops for insecticide stock storage

- Dedicated treatment containers. These can be assigned to a particular insecticide, or if they are made of glass or metal, they can be cleaned thoroughly with solvent for reuse. Polypropylene food cups and other disposable containers are good for treatments. Treatment containers should not be mixed with colony maintenance trays.

- Solvents for dilution of insecticide (see above)

- Stainless steel or nylon strainer fine enough to collect L4s.

Protocol

Exposure:

1. Prepare the stock solution of insecticide.[2]

2. Prepare the insecticide dilution in water at the desired concentration.

3. Collect early stage L4s ensuring that sufficient numbers for a backup are retained in the event of an unexpected result or accident. For example, MQB once accidentally washed resistant larvae with acetone! Resistance was not observed. Do not expose pharate pupae. They are often more resistant than L4s.[3]

4. Drain water off of larvae using a fine mesh. Stainless steel kitchen handheld strainers or improvised devices are suitable.

5. Transfer the larvae to the empty treatment pans by tapping the screen containing larvae sharply on the side of the treatment tray or by washing them in. Attempt to add as little water with the larvae to the pans as possible (**Figure 5.1.1.1**) to avoid insecticide dilution. Aspirate out excess water.

6. Add insecticide to the pans using a volume similar to that used for larval culture (~ 1 ml / L4) (**Figure 5.1.1.2**). After a few minutes, susceptible larvae can often be recognized by unusual twitching movement. Typically, these larvae will not recover and will be classified as moribund (French and Kitzmiller 1963).

7. For all assays that are longer than 1 hour, feed larvae to ensure mortality is due to the insecticide and not starvation.

8. At the end of the exposure, strain off the larvae and wash thoroughly with water before returning to a culture tray.

---

[2] For routine use of insecticides at the same concentration, we have found it convenient to formulate insecticides at 1000 X the concentration desired. This makes formulation of the final dilution simple (1 ml per liter) and keeps the solvent concentration sufficiently low that it alone does not cause mortality.

[3] A susceptible reference stock is often necessary to ensure that a lethal dose has been delivered. For routine authentication purposes, this may be a stock of a different species.

9. Determine mortality at fixed time intervals (**Figure 5.1.1.3**). It is often helpful to sink dead larvae by stirring and poking. Affected larvae are usually unable to return to the surface.[4]

Figure 5.1.1.1. L4 larvae in a dedicated treatment cup. Most of the water has been removed using a plastic pipette.
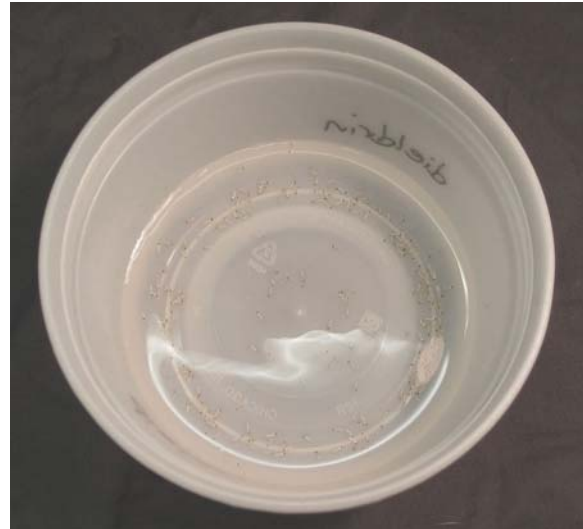
Figure 5.1.1.2. 200ml of a 1ppm dieldrin solution with approximately 200 L4 larvae. Since this is a 1 hour treatment, no food has been added.

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[4] There is often excessive concern that insecticides used for treating larvae will kill susceptible stocks in the insectary due to carryover e.g. on trays, gloves, larvae. While this is possible and should be considered, calculation of realistic amounts of insecticide contamination can show that carryover amounts are usually too low to cause mortality even among susceptible individuals.

Figure 5.1.1.3. 2 hours post treatment, the pan on the left contains the resistant population which is evenly dispersed throughout the container while the pan on the right contains the susceptible control. Note the clumped L4 larvae on the bottom of the pan.

References

French WL, Kitzmiller JB (1963) Time in concentration: a simple technique for the accurate detection of resistance to insecticides in mosquito larvae. In. World Health Organization, Geneva, WHO/MAL401

5.1.2 WHO Paper Testing

MR4 Staff

Introduction

This test involves the use of a specially designed plastic container lined with insecticide impregnated papers. Adult mosquitoes are aspirated into the containers forcing exposure to the insecticide for a fixed time period. The limitations reported for this type of assay include expensive test kits and the inability to detect low frequency resistance in a population (Brogdon 1989). At the WHO website you can find the Insecticide Resistance Monitoring PDF file in which they list how the assays should be run and how to determine the proper discriminating dose for the species you are handling. The necessary supplies can be obtained from the WHO using the link: http://www.who.int/whopes/resistance/en/. Examples of the WHO tube are shown in **Figure 5.1.2.1**.
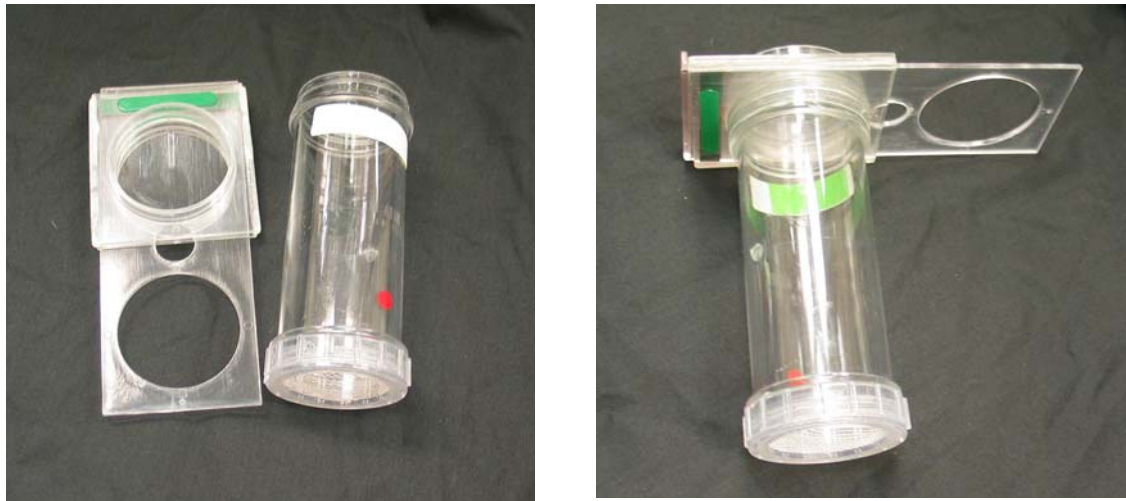
Figure 5.1.2.1. WHO paper insecticide resistance testing tube for adult mosquitoes.

References

Brogdon WG (1989) Biochemical resistance detection: an alternative to bioassay. Parasitol Today 5:56-60

5.1.3 Guidelines for Evaluating Insecticide Resistance in Vectors using the CDC Bottle Bioassay

William Brogdon and Adeline Chan

PREFACE

Insecticide resistance in a vector population is initially detected and characterized by using some sort of bioassay to determine whether a particular insecticide is able to control a vector at a given time. Ideally, this fundamental question should be answered before a particular insecticide is chosen and procured for vector control.

The Centers for Disease Control and Prevention (CDC) bottle bioassay is a surveillance tool for detecting resistance to insecticides in vector populations. It is designed to help determine if a particular formulation of an insecticide is able to control a vector at a specific location at a given time. This information, combined with results of bioassays using synergists and those of biochemical and molecular assays, can assist in determining which insecticide should be used if resistance is detected.

The aim of this document is to provide a practical laboratory guideline that describes how to perform and interpret the CDC bottle bioassay. Information for resistance testing can also be obtained from the CDC website at http://www.cdc.gov/malaria.

We hope you find this tool useful in the support of vector control programs.

Sincerely,

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ACKNOWLEDGEMENT

INTRODUCTION

Bioassays allow for the detection and characterization of insecticide resistance in a vector population. This guideline will describe the Centers for Disease Control and Prevention (CDC) bottle bioassay, a tool for detecting resistance to insecticides. The information provided by this bioassay, combined with results of bioassays using synergists and those of biochemical and molecular assays, can also assist in determining mechanisms associated with resistance.

The CDC bottle bioassay relies on time mortality data, which are measures of the time it takes an insecticide to penetrate a vector, traverse its intervening tissues, get to the target site, and act on that site. Anything that prevents or delays the compound from achieving its objective — killing insects — contributes to resistance. Information derived from the CDC bottle bioassay may provide initial evidence that an insecticide is losing its effectiveness. This methodology should be considered for routine use even before an insecticide is considered, and procured, for vector control.

The CDC bottle bioassay can be performed on vector populations collected from the field or on those reared in an insectary from larval field collections. It is not recommended to use mosquitoes that have emerged from eggs laid in the insectary.

A major advantage of this bioassay is that different concentrations of an insecticide may be evaluated. Furthermore, the technique is simple, rapid, and economical compared to other alternatives. The CDC bottle bioassay can be used as part of a broader insecticide resistance monitoring program, which may include the World Health Organization (WHO) paper-based bioassay, and biochemical and molecular methods.

The CDC bottle bioassay can be used for any insect species. For the purposes of this guideline, mosquitoes will be used as an example.

Material and Reagents

- 250 ml Wheaton bottles with screw lids (**Figure 5.1.3.1**). Each bioassay typically requires five bottles: four for replicates and one for control;
- Graduated disposable plastic pipettes that can measure 1ml; or micropipetters and tips;
- Aspirator apparatus for collecting mosquitoes;
- Containers for transferring/holding mosquitoes;
- Bottles for stock solutions. These can be amber-colored or foil-wrapped if clear bottles are used (100–1,000ml depending on the user's choice of stock solution volume);
- Timer capable of counting seconds;
- Permanent markers for labeling bottles, caps, and pipettes;
- Masking tape for labeling bottles, caps, and pipettes;
- Disposable gloves;
- Sheets, pens, and pencils for data recording.

Reagents

- Insecticide(s) to be tested (technical grade or formulations);
- Acetone or technical grade absolute ethanol.

NB: Use safety procedures as recommended by your institution when handling insecticides (e.g., procedure gloves, laboratory coat).

Figure 5.1.3.1: Material and reagents for the CDC Bottle bioassay

Initial considerations: Diagnostic dose and diagnostic time

The first step in standardizing the CDC bottle bioassay is to determine the diagnostic dose and the diagnostic time. The diagnostic dose is a dose of insecticide that kills 100% of susceptible mosquitoes within a given time. The expected time for the insecticide to achieve this objective is called the diagnostic time. Those are the reference points against which all other results are compared. Resistance is assumed to be present if a significant portion of the test population survives the diagnostic dose at the diagnostic time.

The diagnostic dose and the diagnostic time should be defined for each insecticide, each region, and each vector species that is monitored. The diagnostic dose and the diagnostic time are validated using a susceptible population of vectors collected from the field. Once the diagnostic dose and the diagnostic time for a species from a given location have been determined, these parameters should be used for testing that particular vector population from that location from that time on. Use of the same parameters is required to detect changes in the response of the population over time (e.g., number of test mosquitoes surviving after an exposure time that originally killed 100% of the test population). Detailed information about diagnostic doses, diagnostic times, and calibration of the CDC bottle bioassay is given in Appendix 2.

Diagnostic doses and diagnostic times have been determined for mosquitoes from many geographical regions. **Table 5.1.3.1** shows diagnostic doses and diagnostic times applicable to *Anopheles* and *Aedes* mosquito populations. The diagnostic doses and the diagnostic times for anophelines shown below were agreed upon for use on anophelines collected in South America as part of the Amazon Malaria Initiative (AMI). These doses and times, as well as those listed for *Aedes,* are well within the range of diagnostic doses and diagnostic times for use worldwide. Therefore, the diagnostic doses and the diagnostic times in **Table 5.1.3.1** serve as sample reference points for the main insecticides used globally. Diagnostic doses and diagnostic times for other insect species may still need to be determined.

Table 5.1.3.1: Sample diagnostic doses and diagnostic times for *Anopheles* and *Aedes* mosquitoes.

Insecticide	Insecticide concentration per species (µg/bottle)		Diagnostic time (minutes)
	Anopheles	*Aedes*	
Bendiocarb	12.5	12.5	30
Cyfluthrin	12.5	10	30
Cypermethrin	12.5	10	30
DDT	100	75	45
Deltamethrin	12.5	10	30
Fenitrothion	50	50	30
Lambdcyhalothrin	12.5	10	30
Malathion	50	50	30
Permethrin	21.5	15	30
Pirimiphos-methyl	20	--	30

In summary, determining the diagnostic dose and the diagnostic time is the first step to standardize the CDC bottle bioassay. This step should be done at the national or regional level in a given country or region to allow for comparability among different laboratories over time. Once the diagnostic dose and diagnostic time are agreed upon for a particular insecticide and mosquito species, there is no need to redo this exercise until evidence of high levels of resistance in this species is documented.

Preparation of stock solutions

The bottles used for the bioassay need to be coated inside with the diagnostic dose of the insecticide under evaluation. As can be seen from **Table 5.1.3.1**, the diagnostic dose is a determined amount of insecticide per bottle. Therefore, if 12.5µg of deltamethrin is to be added to a testing bottle, it would be advisable to have a stock solution with 12.5µg/ml, which means that 1 ml of the solution would contain the desired amount of insecticide to be added to the bottle. This is equivalent to saying that it is practical to make stock solutions with concentrations that can be easily correlated to the dose needed to coat the bottles.
To make insecticide stock solutions, dilute the appropriate amount of insecticide (technical grade or formulation) in acetone or technical grade ethanol. Examples of quantities of technical grade insecticide needed to prepare 100 ml, 500 ml, and 1,000 ml of stock solutions are shown in Table 5.1.3.2. Technical grade insecticide may be solid or liquid and need to be of good quality and not be expired. It is important to label the stock solution bottle with the name of the insecticide, concentration, and date of preparation. Examples of preparation of stock solutions from technical grade and formulations are shown in Box 1. Once the stock solution is made, it can be stored in the refrigerator (4°C) in light-proof bottles (amber-colored bottles or foil-wrapped if clear) for future use. At the CDC, refrigerated stock solutions of many

insecticides have been used for 2–3 years without degradation of activity. It is recommended to take the stock solutions out of the refrigerator at least 1 hour before running the bioassay to allow them to come to room temperature before use. The stock solution should be gently swirled before use to mix it.

Table 5.1.3.2: Quantities of technical grade insecticide required for preparation of different volumes of stock solution.

Insecticide	Weight (mg) of technical grade insecticide needed per volume of stock solution *(Anopheles)*			Weight (mg) of technical grade insecticide needed per volume of stock solution *(Aedes)*		
	100 ml	**500 ml**	**1,000 ml**	**100 ml**	**500 ml**	**1,000 ml**
Bendiocarb	1.25	6.25	12.5	1.25	6.25	12.5
Cyfluthrin	1.25	6.25	12.5	1	5	10
Cypermethrin	1.25	6.25	12.5	1	5	10
DDT	10	50	100	7.5	37.5	75
Deltamethrin	1.25	6.25	12.5	1	5	10
Fenitrothion	5	25	50	5	25	50
Lambdcyhalothrin	1.25	6.25	12.5	1	5	10
Malathion	5	25	50	5	25	50
Permethrin	2.15	10.75	21.5	1.5	7.5	15
Pirimiphos-methyl	2	10	20			

Box 1: Examples of stock solution preparation.

1. Preparing stock solutions from technical grade insecticide
Assume a 100% deltamethrin technical grade insecticide. To obtain a concentration of 12.5 µg/bottle, dissolve 12.5 mg the insecticide in enough acetone or absolute ethanol to make 1 liter of total solution. Each 1 ml of this solution will contain 12.5 µg of the insecticide. Stock solutions of varying volumes (for example 100 ml) can be made for the convenience of the user as long as the proportion of insecticide and solvent remains constant.

2. Preparing stock solutions from concentrations other than technical grade
To calculate the volume of a formulation to be added to the solvent to reach the desired concentration in the stock solution, it is necessary to consider the concentration of the active ingredient in the formulation. To do that, simply divide the desired amount of milligrams needed for 1 liter of the stock solution by the concentration in the formulation available. This will give the volume of the formulation needed to make 1 liter of stock solution. The formula:

$$\frac{\text{Milligrams of technical grade}}{\text{\% of active ingredient in formulation}}$$

Example:
Using a formulation of 10% deltamethrin, calculate how much is needed to obtain 12.5 mg.

$$\text{Volume needed} = \frac{12.5 \text{ mg}}{0.10} = 125 \text{ mg of the 10\% formulation}$$

So, 125 mg of the 10% concentration of deltamethrin will need to be added to enough acetone or absolute ethanol to make 1 liter of total solution. By doing so, each 1 ml of the stock solution will contain 12.5 µg of deltamethrin.

Mosquito handling

Female mosquitoes to be used in the bioassay can be collected as adults from the field (of mixed age and physiological status) or as adults of a known age reared from field larval collections. Use of mosquitoes that have emerged from eggs laid in the insectary is not recommended. If field-collected adults are used, their physiological status (i.e., unfed, blood fed semi-gravid, gravid) should be recorded on the result sheet. Female mosquitoes should be fed only with 10% sugared solution the day before testing. It is recommended that a minimum of 100 mosquitoes, divided among four replicate bottles, should be tested for an insecticide at a given concentration. When it is not possible to collect this number of mosquitoes on a single occasion, results of multiple bioassays over a few days may be pooled to achieve the recommended sample size, 100 mosquitoes. In either case, each bioassay must include a control bottle with 10–25 mosquitoes.
Some field collections may contain different species. Therefore, species must be identified either before or after the bioassay is conducted to validate its results (Box 2). To determine the species composition of mosquito collections before the bioassay, it is possible to "knock down" (anesthetize) mosquitoes with ethyl acetate.

Box 2: Guidelines for situations where different mosquito species exist in sample collections.

In those situations where different mosquito species exist, it is recommended that species be identified, either before or after the CDC bottle bioassay. If a predominant species is detected (i.e., more than 95% belong to one single species), consider this the species tested, and the results of the CDC bottle bioassay can be considered adequate for the predominant species.

If no particular species represents at least 95% of the mosquitoes being tested, account for this heterogeneous population. To achieve this:

1. Identify the species and sort before the bioassay using ethyl acetate. Conduct separate bioassays for each, or predominant, species; or
2. Start the bioassay without pre-identification if this is not possible (lack of expertise with mosquito "knock down," or presence of closely related or cryptic species). If there are surviving mosquitoes at the diagnostic time, stop the bioassay and separate live from dead mosquitoes. Identify the species for both live and dead mosquitoes, and consider them separately for analysis.

Procedures for cleaning and drying bottles before coating

1. Wash the bottles with warm soapy water and rinse thoroughly with water at least three times. Tap water can be used for this step;
2. Place bottles in an oven (50°C) for 15–20 min or until they are thoroughly dry before using them;
3. If there is no oven, leave bottles to dry completely at room temperature or in the sun, with the caps off. In humid situations, bottles can be left to dry with caps off overnight or longer;
4. To assure that the cleaning procedure is adequate, introduce some susceptible mosquitoes into a sample of recently washed and dried bottles. Mosquitoes should not die right away. If they do, repeat the washing and drying procedure.

Marking of bottles

1. Since the bottles will be reused, consider using a piece of masking tape on the bottles and caps for marking them instead of writing directly on the bottles and caps (**Figure 5.1.3.2**). This may facilitate the cleaning of the bottles after the bioassay is completed;
2. Mark one bottle and its cap as control;
3. Mark the other four bottles and caps with the replicate number (1–4) and the bioassay date;
4. If more than one type of insecticide or more than one concentration of the insecticide is being tested at the same time, also label the bottles and their caps with the insecticide name and concentration;
5. Mark both the cap and the bottle so that bottles are associated with their respective caps. This is vitally important because the inside of the entire bottle will be coated, including the inside of the cap.

Figure 5.1.3.2: Labeling bottles and caps

Bottle coating

1. Make sure that bottles and caps are completely dry;
2. Remove caps from the bottles;
3. If using disposable pipettes, label one pipette as 'solvent only' for the control bottle, and another pipette as 'insecticide solution' for the test bottles;
4. Add 1 ml of acetone/ethanol to the control bottle and put the cap back on tightly;
5. Add 1 ml of the stock insecticide solution to the first test bottle and put the cap back on tightly. This step is to be done if the stock solution has the same concentration per ml as is desired for the bottle (**Table 5.1.3.1**);
6. Repeat **Step 5** with the other three test bottles;
7. Swirl the contents inside the bottle so that the bottom is coated (**Figure 5.1.3.3**);
8. Invert the bottle and swirl to coat the inside of the cap (**Figure 5.1.3.4**);
9. Place the bottle on its side for a moment to let the contents pool. Gently rotate while rocking the bottle gently so that the sides all the way around are coated;
10. Repeat this for all the test bottles (**Figure 5.1.3.5**);
11. Remove the caps and continue rolling bottles on their side until all visible signs of the liquid are gone from inside and the bottles are completely dry (**Figure 5.1.3.6**);
12. Leave bottles on their sides and cover with something that will keep them protected from light;
13. If bottles are not used right away, store bottles in a dark place (such as a drawer) with the caps off to avoid moisture build-up. If shipping pre-coated bottles, ship the bottles with the caps on. More information on the storage of coated bottles is given on page 11 of this chapter.

Figure 5.1.3.3: Coating the bottom of the bottle

Figure 5.1.3.4: Coating the top of the bottle

Figure 5.1.3.5: Coating the sides of the bottle

Figure 5.1.3.6: Removing caps and rolling the bottles on their sides

CDC bottle bioassay method

General considerations

1. Use a filter in the aspirator to avoid inhaling mosquitoes or insect fragments;
2. Blow gently to expel the mosquitoes into the bottles. If you blow too hard, the mosquitoes can be damaged by hitting the sides of the bottle and killed before the insecticide has a chance to do so;
3. Be careful not to touch the inside of the bottle with the aspirator, as this may contaminate the aspirator;
4. Remember that the number of mosquitoes in the each of the test bottles does not need to be equal;
5. Determine species composition of mosquitoes either before or after the bioassay is conducted (Box 2).

Bioassay procedure

The bioassay can be performed with the bottles in an upright position or with the bottles lying on their sides. The important thing is to be consistent and follow the same procedure each time.

The steps:
1. Using an aspirator, introduce 10–25 mosquitoes into the control bottle. It is not necessary to count the mosquitoes; the exact number does not matter;
2. Introduce 10–25 mosquitoes into each test bottle; again, the exact number does not matter (**Figure 5.1.3.7**);
3. Start a timer. Be sure to examine the bottles at Time 0 and count the number of dead and/or live mosquitoes;
4. If you find dead mosquitoes at Time 0, make a note of them on the form (**Appendix 3**);
5. Record how many mosquitoes are dead or alive, whichever is easier to count, every 15 minutes until all are dead, or up to 2 hours (**Figure 5.1.3.8**). It is not necessary to continue the bioassay beyond 2 hours;
6. Record these data on the reporting form (**Appendix 3**);
7. Graph the total percent mortality (Y axis) against time (X axis) for all replicates considered together using a linear scale;
8. Remember that mortality at diagnostic time is the most critical value because it represents the threshold between susceptibility and resistance. Refer to **Table 5.1.3.1** for diagnostic doses and times for commonly used insecticides;
9. Take into consideration mortality in the control bottle at 2 hours (end of the bioassay) when reporting the results of the bioassay (See validity of the data section below). Use Abbot's formula to correct results if the mortality at 2 hours in the control bottle is between 3% and 10%. You may need to discard the bioassay results if mortality in the control bottle at the end of the test was >10%.

Mosquitoes are considered dead if they can no longer stand. See Box 3 for more information.

A timer could be started for each bottle, but it is sufficient to start one timer when the first or last bottle receives its mosquitoes. It is, however, important to be consistent and follow the same timer start procedure each time. Mosquitoes alive at the diagnostic time (**Table 5.1.3.1**) represent mosquitoes resistant to the insecticide being tested. These mosquitoes may be transferred to a sleeved carton for further analysis (e.g., molecular or biochemical assays). Mosquitoes flying at the end of the bioassay in the control bottle may need to be killed to get an accurate count. Mosquitoes can be killed by freezing or stunning them.

Box 3: Notes about mortality criteria.
- "Dead" mosquitoes are mosquitoes that cannot stand.
- It helps to gently rotate the bottle while taking the count.
- Immobile mosquitoes that slide along the curvature of the bottle can be easily categorized as dead.
- It is easier to count the number of dead mosquitoes in the first readings of the bioassay, and it is easier to count the number of live mosquitoes when few remain alive.
- In the end, the percentage of dead mosquitoes at the diagnostic time (dead mosquitoes/ total of mosquitoes in the assay) is the most important value in the graph.

Figure 5.1.3.7: Transferring mosquitoes into insecticide-coated bottles
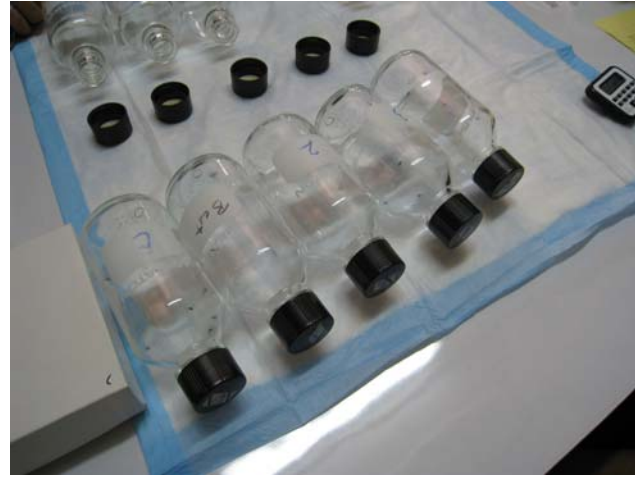
Figure 5.1.3.8: CDC bottle bioassay in progress

Handling of coated bottles

More than one batch of mosquitoes can be run in a single bottle in one day. However, the main limiting factor for reusing previously coated bottles is moisture build-up with successive introductions of mosquitoes, especially in humid conditions. If the bottles are to be reused on the same day, it is necessary to leave some time (2–4 hours, longer if in a humid climate) between the bioassays for the bottles to dry out (with caps off) before introducing more mosquitoes. If the bottles are to be reused the following day, bottles with caps off can be left to dry overnight protected from direct light. **It is prohibited to dry bottles in the oven after they have been coated with insecticide.**

If the bottles are not to be used soon after coating them with insecticide, it is recommended to let them dry with their caps off. When the bottles are dry, they should be stored in a dark place (such as a drawer) with their caps off. Depending on the insecticide used, bottles can be stored from 12 hours to 5 days in this manner. The length of time bottles can be stored depends on the insecticide. Resmethrin- and Naled-coated bottles do not store well, so they should be used immediately after being prepared. Organophosphate-coated bottles should be used within 24 hours. To check if a stored bottle is still adequate, it is possible to put some mosquitoes known to be susceptible in the bottle. If they die in the expected time frame (within the diagnostic time), the bottle can still be used. Bottles can be coated in a central laboratory and shipped for use in the field. During transport, bottles should have their caps on.

Mosquito preparation for testing to determine mechanisms of insecticide resistance

Resistance is assumed to be present if a portion of the test population survives the diagnostic dose at the diagnostic time. Mosquitoes that survive the bioassay can be used for testing to identify mechanisms of resistance using enzymatic assays or molecular methods. Surviving mosquitoes may be easily released from bottles into a sleeved holding carton to separate them from those killed during the CDC bottle bioassay. Mosquitoes that will be further tested using enzymatic assays should be stored frozen. Mosquitoes to be used for molecular studies can be frozen, dried, or stored in 70% (or higher) ethanol. In addition, it may be necessary to use products like RNALater® (Applied Biosystems [Ambion], Foster City, California) to preserve samples for measurement of RNA levels associated with up-regulated enzyme mechanisms.

Validity of the data

With practice, the mortality of mosquitoes in the control bottle at 2 hours (end of the bioassay) should be zero. In most cases, mortality of up to 3% in the control bottle may be ignored. In cases where mortality is 3%–10% in the control bottle at 2 hours, it is possible to either use Abbot's formula to correct the findings (see Box 4), or discard results and repeat the bioassay. If mortality in the control bottle is greater than 10% at the end of the bioassay, the results of this particular run should be discarded, and the CDC bottle bioassay should be repeated. If a particular mosquito collection is essentially irreplaceable and the bioassay cannot be repeated, Abbot's formula can be considered even when control mortality is >10%.

Box 4: Abbott's f**ormula.**

Corrected mortality = $\dfrac{\text{(mortality in test bottles [\%] - mortality in control bottle [\%]) x 100}}{\text{(100\% - mortality in control bottle [\%])}}$

For example: If mortality in test bottles is 50% at diagnostic time and control mortality is 10% at 2 hours, the corrected mortality is [(50%-10%) / (100%-10%)] x 100 = 44.4%

Note: In cases of 100% mortality in test bottles, Abbott's formula has no effect. For example: [(100% - 10%) / (100% - 10%)] x 100 = 100% corrected mortality

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Interpretation of results

As with other resistance bioassays, data from the CDC bottle bioassay using test mosquitoes need to be compared with data from susceptible mosquitoes or from a population that will serve as baseline. Resistance thresholds for each insecticide can be determined by calibrating the CDC bottle bioassay (**Appendix 2**). Calibration entails determining the diagnostic dose and the diagnostic time for a particular species in a given region, which correspond to the dose and time at which all of susceptible mosquitoes die (**Figure 5.1.3.9**). If test mosquitoes survive beyond this threshold, these survivors represent a proportion of the population that has something allowing them to delay the insecticide from reaching the target site and acting. In other words, they have some degree of resistance. In the example shown in **Figure 5.1.3.9**, all mosquitoes that died before the diagnostic time when exposed to insecticide-coated bottles were susceptible. Test mosquitoes surviving beyond the diagnostic time threshold were assumed to have some degree of resistance. In the example, only 23% of the test population was susceptible. Recommendations for interpretation of bioassay data are shown in Box 5. The most important information is the mortality at the diagnostic time, but the bioassay is carried out beyond the diagnostic time to evaluate the intensity of resistance.

Box 5: Interpretation of data for management purposes.

WHO recommendations for assessing the significance of detected resistance:
- 98%–100% mortality at the recommended diagnostic time indicates susceptibility;
- 80%–97% mortality at the recommended diagnostic time suggests the possibility of resistance that needs to be confirmed;
- <80% mortality at the recommended diagnostic time suggests resistance.

Note: Where <95% mortality occurs at the diagnostic time in bioassays that have been conducted under optimum conditions and with a sample size of >100 mosquitoes, then resistance can be strongly suspected.

Figure 5.1.3.9: Determination of resistance threshold.
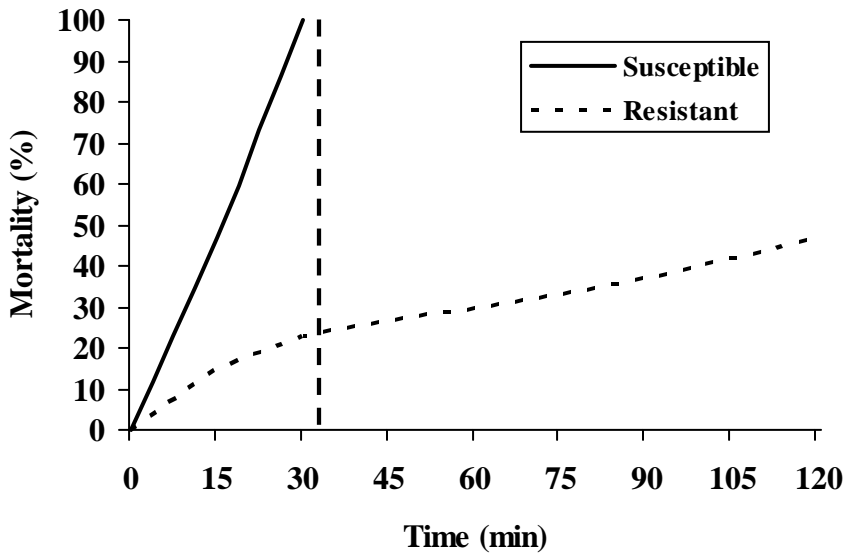
Resistance surveillance

Background

Although resistance data are often collected as part of vector control programs, this is often not done as regularly as it should be in a true resistance surveillance effort. Surveillance requires the regular collection and interpretation of epidemiological data to support changes in public health programs. It is important to consider the CDC bottle bioassay an instrument to collect information to support an insecticide resistance surveillance system. Resistance data are most valuable when collected over time to allow for comparisons and for monitoring of trends.

It is important to consider how information collected as part of an insecticide resistance surveillance system will be used. Most malaria control programs carefully assess the efficacy of their vector control program by, for example, plotting incidence of malaria cases or by counting adult mosquitoes or larval collections at sentinel sites. The integration of insecticide resistance data and other kinds of malaria-related data needs to be taken into consideration before proposing and implementing remediation strategies for insecticide resistance.

Features of resistance emergence

Several genetic, biologic, and operational factors influence the development of insecticide resistance. In many respects, resistance is a complex problem, with different outcomes possible in a particular area, depending on the influence of diverse factors on initial conditions. Even so, certain factors affect resistance development throughout the world. Major resistance characteristics are discussed below, showing why each manifestation of resistance is potentially unique and therefore must be evaluated on case-by-case basis.

Focal nature of resistance

Vector control personnel frequently assume that resistance in a particular species occurs throughout their control area, but insecticide resistance can be focal. In Guatemala, sampling sites for *Anopheles albimanus* only a few kilometers apart varied not only by presence or absence of resistance, but also by level of resistance and by dominant mechanism responsible for resistance. Generally speaking, areas of ongoing vector control activities tend to have higher levels of resistance; when resistance levels in adjacent areas are compared, levels may be higher in areas of more intensive mosquito control.

Resistance and disease control

In some cases, vector control strategies in a given area may not be affected by the level of insecticide resistance. For example, a control program may be able to control only 75% of the vector population. In these cases, an insecticide resistance level lower than 10% will likely not affect disease control efforts. In such a situation, it would be sufficient to increase surveillance and monitor the level and frequency of resistance but no change in control strategies would be needed.

Guiding principles

In general terms, resistance surveillance should be conducted in areas where disease transmission is a concern and where insecticide-based control measures are contemplated, ideally before purchase of insecticide. In addition to constraints imposed by economic resources, the number of sites that can be sampled is highly dependent on the size of the area contemplated for insecticide use. Due to the potential focal nature of resistance, efforts must be made to choose spatially distributed sites in the area of interest, if possible. Areas 20 km or more apart should not be assumed to have similar resistance patterns. Another means of deciding on surveillance sites is to focus on those areas of active disease transmission. Even if only one or a few sites can be monitored, this is far preferable to having no surveillance sites. In addition, efforts should be made to operate sites for at least a few years, since comparative data are the most meaningful information.

Ideally each site should be monitored once a year. Where control efforts are seasonal, it may be useful to monitor at the beginning and at the end of the control season. This does not apply to situations such as the use of insecticide-treated bednets, where the insecticide exposure is year round. If several vectors in the area are seasonal, the resistance testing schedule should be adjusted to the species of interest.

It is also important to consider that it will be necessary to try to identify resistance mechanisms once resistance is detected with the CDC bottle bioassay, whether using the CDC bottle bioassay with synergists, or biochemical and/or molecular methods. Decisions on which insecticide to change to will depend upon the specific mechanism(s) of resistance.

Finally, some countries have found it useful to centralize preparation of bottles and administrative organization of surveillance. A central reference laboratory can provide support for technical assistance and quality assurance. It can also serve as a reference laboratory for training, provision of supplies, species identification, and enzymatic assays and molecular methods for determination of resistance mechanisms.

CDC bottle bioassay and synergists

Background

The CDC bottle bioassay using bottles that were coated with a single insecticide provides information on insecticide resistance to that particular insecticide in adult vectors. These data may provide early evidence that an insecticide is losing its effectiveness.

Once resistance is detected, or at least suspected, one must decide what to do next and which other compounds are likely to still be effective and not compromised by cross resistance. This requires knowledge of the resistance mechanism(s) in place; information usually acquired using either biochemical (microplate) assays or molecular methods. A rapid and inexpensive alternative to assess resistance mechanisms is to use the CDC bottle bioassay with synergists. Synergists are enzyme inhibitors of insecticide detoxification enzymes. Synergists are available for the metabolic detoxification enzymes: esterases, oxidases, and glutathione s-transferases.

Synergists act by abolishing the apparent resistance observed in the CDC bottle bioassay if a detoxification enzyme plays a role in that particular resistance mechanism (**Figures 5.1.3.10a** and **5.1.3.10b**). Data for resistant and susceptible populations are shown (**Figure 5.1.3.10a**). Once a synergist is used on the resistant population, one of three things might happen (**Figure 5.1.3.10b**):

   a. Resistance to the insecticide is abolished (time-mortality line A), which suggests that the mechanism related to that synergist is playing a role in the insecticide resistance observed;
   b. Resistance to the insecticide is partially abolished (time-mortality line B). This suggests that the mechanism related to that synergist is involved in the resistance, but it is not the only mechanism involved in this particular case;
   c. Resistance to the insecticide is unaffected (time-mortality line C). This indicates that the mechanism related to that synergist is not involved in the resistance.

It is also possible to determine if a target site mechanism, such as the presence of the kdr gene (sodium channel mutation) or insensitive acetylcholinesterase, is involved. This is done by using the synergists in combination. Their combined use will not abolish the resistance in the bioassays when a target site mechanism is present. It is crucial in areas where pyrethroids and/or DDT are used to evaluate the relative role of detoxification and target site mechanisms involved in a particular incidence of resistance. A target site mechanism confers DDT–pyrethroid cross-resistance, while a detoxification mechanism may or may not. Knowledge of the resistance mechanism involved is required to select a replacement insecticide.

Figures 5.1.3.10a and 5.1.3.10b. Effects of synergists on resistant vector populations. Figure 5.1.3.10a shows data for a population of resistant vectors compared to a susceptible population. Figure 5.1.3.10b shows the three possible outcomes of synergist exposure (Line A — Resistance to the insecticide is abolished; Line B — Resistance to the insecticide is partially abolished; and Line C — Resistance to the insecticide is unaffected).

Fig. 5.1.3.10a.
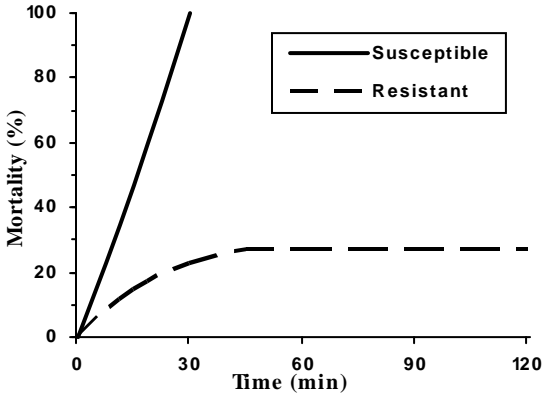
Fig. 5.1.3.10b.
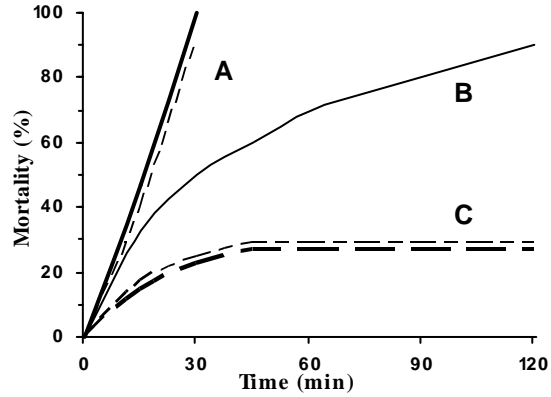
Figure 5.1.3.11. Performing the CDC bottle bioassay with synergists.
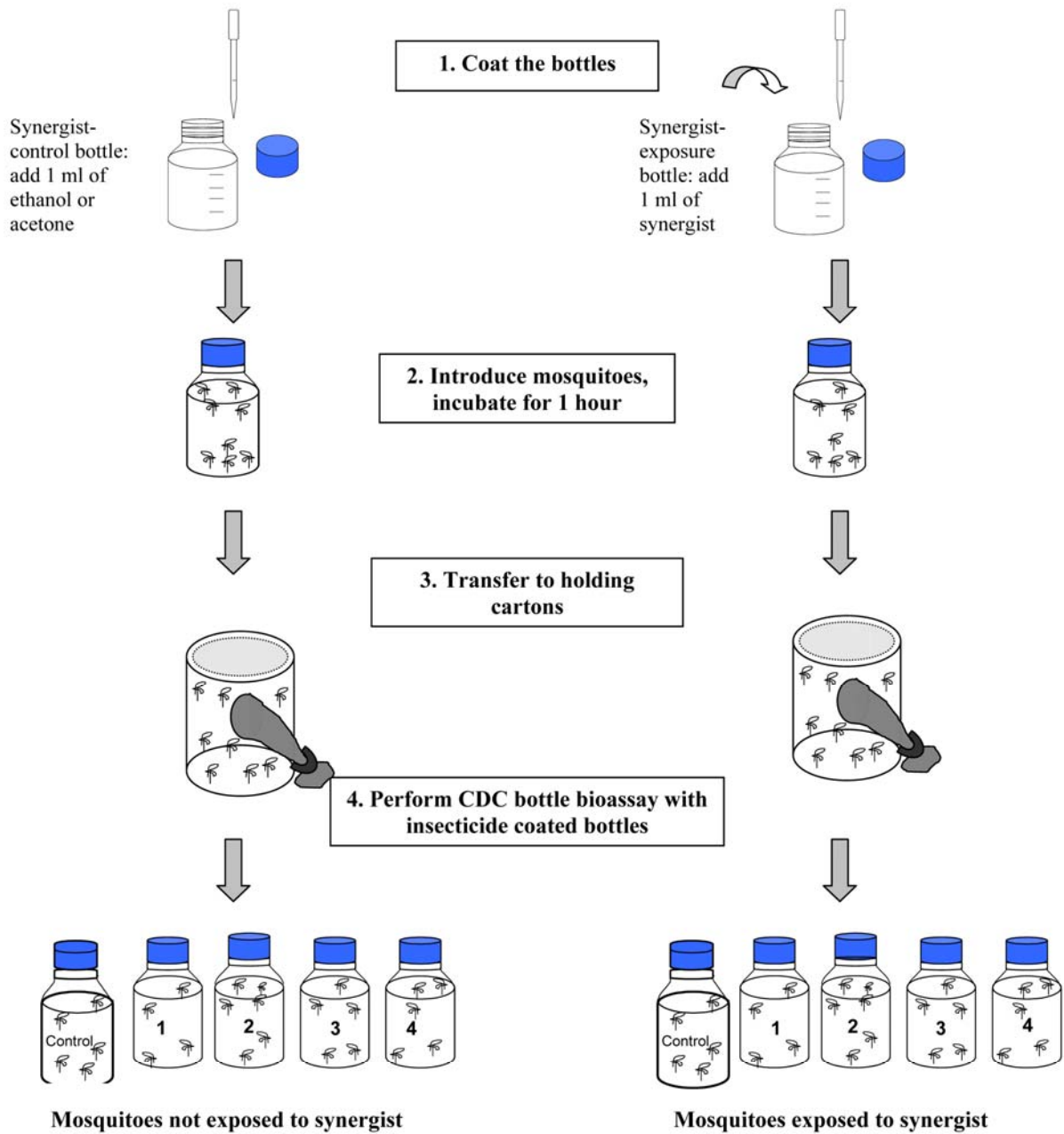
Use of synergists

Commonly used synergists in conjunction with the CDC bottle bioassay:
- Piperonyl butoxide (PBO), which inhibits oxidase activity;
- S.S.S-tributlyphosphorotrithioate (DEF), which inhibits esterase activity;
- Ethacrynic acid (EA), diethyl maleate (DM), and chlorfenethol (CF), which inhibit glutathione transferase activity.

It is also possible to use a combination of the above synergists. Testing mosquitoes with synergists is a two-step procedure. Mosquitoes are first exposed to the synergist(s) for 1 hour and then tested for insecticide resistance using the CDC bottle bioassay. A schematic representation of performing a bioassay with synergist(s) is shown in **Figure 5.1.3.11**.

Preparation of bottles for synergist bioassays

To use the bioassay with synergists:
1. Prepare the synergist stock solution by diluting the appropriate amount of synergist in acetone or technical grade ethanol to be able to coat the bottles with the concentrations shown in Table 3. To make these stock solutions, use the same procedure used for making insecticide stock solutions. In brief, dilute the appropriate amount of synergist in acetone or technical grade ethanol. To get a concentration of 400 µg/bottle of piperonyl butoxide, dissolve 400 mg in enough acetone or absolute ethanol to make 1 liter of solution. Each 1 ml of this solution will contain 400 µg of piperonyl butoxide.
2. Mark one bottle and its cap as the synergist-control bottle (without synergist);
3. Mark a second bottle and its cap to be the synergist-exposure bottle;
4. Add 1 ml of acetone or ethanol to the synergist-control bottle and put the cap back on tightly;
5. Add 1 ml of the synergist stock solution to the synergist-exposure bottle and put the cap on back tightly;
6. Coat the bottles, remove the caps, and let the bottles dry (**Figures 5.1.3.3 – 5.1.3.6**)
7. Prepare two test sets of bottles to run the CDC bottle bioassay.

Table 5.1.3.3: Synergist concentrations used in the CDC bottle bioassay.

Synergist	Synergist concentration (µg/bottle)
Chlorfenethol	80
Diethyl maleate	80
Ethacrynic acid	80
Piperonyl butoxide	400
S.S.S-tributlyphosphorotrithioate	125

CDC bottle bioassay with synergist

To run the CDC bottle bioassay with synergists:
1. Introduce equal numbers of mosquitoes into the synergist-control bottle and into the synergist-exposure bottle (about 125 mosquitoes in each bottle);
2. Keep the mosquitoes in the bottles for 1 hour to allow the synergist to act;
3. After the 1-hour exposure is completed, transfer the mosquitoes to two holding cartons, one for the synergist-control mosquitoes and another for the synergist-exposed mosquitoes. This makes it easier to transfer mosquitoes into the insecticide-treated bottles;
4. Perform the CDC bottle bioassay using one set of insecticide-coated bottles (one control and four test bottles) for the synergist-control mosquitoes and another set (one control and four test bottles) for the synergist-exposed mosquitoes;
5. Compare the data for the two populations of test mosquitoes.

Interpretation of bioassays with synergists

Pages 15-16 provide information on how to interpret the results of the CDC bottle bioassay using synergists. Resistance that cannot be attributed to one of the detoxification mechanisms after all synergists have been used is likely to be due to a target site mechanism, such as *kdr* (sodium channel mutation) or insensitive acetylcholinesterase.

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Appendix 1. Frequently asked questions (FAQs)

1. What happens if there are not enough mosquitoes for a complete bioassay?
When the number of mosquitoes captured in the field is insufficient for a full bioassay (four coated and one control bottles), you can reduce the number of bottles to be tested, but each bioassay must **ALWAYS** be run with a control until the required number is completed. If the testing takes place over a long period of time, use recently coated bottles if necessary. See expected lifetime of coated bottles in the guideline. Except in the case of organophosphate-coated bottles, coated bottles can be use multiple times over several days until the bioassay is completed, as long as moisture build-up from aspiration does not become excessive.

2. Should some bottles be designated solely as control bottles?
No, some bottles should not be designated as control bottles. Bottles should randomly be assigned as test or control bottles. This will provide an additional quality control to the adequacy of the washing procedure.

3. What if there are no susceptible mosquitoes available for CDC bottle bioassay calibration?
The diagnostic dose and diagnostic time for a particular species in a given area are similar. Use the diagnostic dose and the diagnostic time published in this guideline or consult the authors of this guideline or other users with experience in the method for that particular vector. Note that the value of the CDC bottle bioassay lies in showing changes over time in the characteristics of vector populations. Therefore, a baseline is useful even if some individual mosquitoes show resistance when the initial baseline is established.

4. Can male mosquitoes be used for the control bottle?
No, males should not be used for the control bottles. Some resistance mechanisms are sex- linked, and one can be misled by using males in the control. In addition, most mosquitoes collected will be females.

5. How can mosquitoes be introduced into the bottle without letting other mosquitoes escape?
Some people have found it useful to employ a piece of cotton wool held against the aspirating tube at the top of the bottle as the mosquitoes are being introduced into the bottles. As the aspirator is withdrawn after the mosquitoes are introduced, the cotton wool can be used to close the bottle top, until the bottle cap is put in place. In our experience, a swift decisive puff of air will introduce mosquitoes without loss. Attempting to introduce mosquitoes into a bottle more than once may allow some to escape. This sometimes happens if the user attempts to put exactly the same number of mosquitoes into each bottle, which is not necessary.

6. What happens if there are fed and unfed mosquitoes among the field-collected mosquitoes to be used in the bioassay?
A collection of mosquitoes from the field may contain female mosquitoes in various physiological states, e.g., fed and unfed mosquitoes. There are two ways that this can be dealt with. First, mosquitoes can be randomly selected. Alternatively, mosquitoes can be held for one or two days for the blood meal to be digested and then used for the bioassay.

Appendix 2. Diagnostic doses and CDC bottle bioassay calibration

It is assumed that resistance is present if a diagnostic dose, proven and validated against a susceptible insect population, is survived by members of a test population at a predetermined diagnostic time. The diagnostic dose and diagnostic time are optimal parameters for detecting insecticide resistance. A diagnostic dose that is too low will not kill susceptible mosquitoes during the bioassay, providing a false-positive result for resistance. On the other hand, a diagnostic dose that is too high will kill resistant mosquitoes during the bioassay, masking resistance.

For some insect vectors from some geographic regions, diagnostic doses and diagnostic times for several insecticides have already been determined. It is recommended that countries in these regions use the already established parameters to allow them to compare data across countries or within regions. However, if this information is not available, the diagnostic dose and the diagnostic time will need to be defined for each insecticide, in each region, and for each main vector species that is to be monitored. To determine the diagnostic dose and the diagnostic time for use in the CDC bottle bioassay, the assay will have to be calibrated.

Calibration assay

The assay is calibrated by first selecting the testing population and possible lengths of test time, and then by determining possible diagnostic doses, given preferred diagnostic times.

Population: The first step is to select a susceptible vector population to use as a baseline. If such a population is not available, it is possible to use the vector population from the area where the chemical vector control measures are to be applied. This will be the reference point against which all future populations can be compared.

Diagnostic time: For practical reasons, the diagnostic time should be between 30 and 60 minutes.

Diagnostic dose: The diagnostic dose will be a dose of insecticide that can kill 100% of mosquitoes sometime between 30 and 60 minutes and that is below the saturation point. To determine possible diagnostic doses, first prepare bottles with a range of different concentrations of insecticides per bottle, as outlined in the guideline. Using each of these different bottles, run separate CDC bottle bioassays on 25 mosquitoes of the susceptible population to determine upper limit of the diagnostic dose, which is the saturation point. The saturation point can be defined as a concentration above which no additional decrease in the time required to kill 100% of the mosquitoes with an increase in insecticide concentration. See a more detailed explanation on how to determine the saturation point below.

It may be necessary to run additional sets of concentrations around that range until the optimal diagnostic dose is determined. For example, starting with 10 µg/bottle, increase concentration with increments of 5 µg/bottle, and continue to a final concentration of 200 µg/bottle. If no clear saturation point can be determined, run more assays using bottles with <10 µg/bottle and/or >200 µg/bottle, with increments of 5 µg. If the saturation point still cannot be determined, more assays may be run with bottles using smaller increments of insecticide.

Interpretation of calibration data

Graphing the results of the calibration assay will show that the time-mortality line becomes straighter, steeper, and moves toward the Y-axis as the insecticide concentration increases (**Figure 5.1.3.4**). This means that by increasing insecticide concentration the time-mortality line will reach a point where increasing the concentration of insecticide will not kill all mosquitoes any faster. In the example below, 15

µg/bottle is the toxicological saturation point for insecticide entering the mosquito and reaching its target. Increasing the concentration to 25 µg/bottle does not cause the insecticide to penetrate the mosquito, reach the target site, and kill the mosquito any faster. Therefore, 15 µg/bottle is the saturation point and the maximum concentration to use as the diagnostic dose. Otherwise, there is a risk that resistant mosquitoes will be killed by doses higher than the saturation point and then be recorded as susceptible, i.e., false negatives for resistance.

A slightly smaller concentration compared to the toxic saturation point will kill mosquitoes in an amount of time perhaps more convenient for the user (e.g., 30 to 60 minutes). So, it is possible to choose a lower diagnostic dose that kills 100% of mosquitoes within 30 to 60 min. It must be understood that several different pairs of diagnostic doses and diagnostic times will give interpretable results, but it is necessary to consistently use the same diagnostic dose and diagnostic time for that particular insecticide on that vector in future assays over long periods of time to allow for comparability. Otherwise, the method will not allow assessment of changes in resistance over time for that species.

Figure 5.1.3.4: Determining diagnostic doses and diagnostic times.
In the example shown below, 15 µg/bottle is the saturation point because higher doses did not decrease the time for 100% of susceptible mosquitoes to be killed. A concentration of <5 µg/bottle takes more than 60 minutes to kill 100% of susceptible mosquitoes, which means that those would take a long time to be killed by this dose. The doses between 5 and 15 µg/bottle (shaded area) are in the usable range for detecting resistance, and the diagnostic time for each of these concentrations will be the time at which 100% of mosquitoes were killed. So, for example, a diagnostic dose of 10 µg/bottle and diagnostic time of 45 min could be selected.
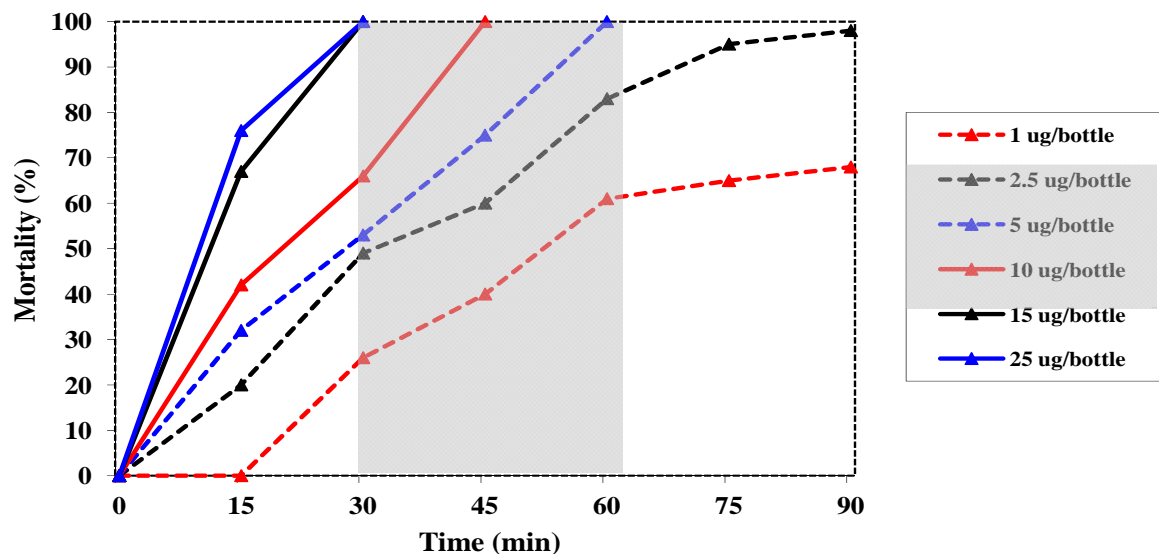
Appendix 3. CDC bottle bioassay data recording form

Date: _____ Mosquito species: _____

Insecticide: _____

Diagnostic dose: _____ Diagnostic time: _____

Location of mosquito collection: _____

Time (min)	Bottle 1		Bottle 2		Bottle 3		Bottle 4		All test bottles			Control		
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Total dead	Total	% dead	Total dead	Total	% dead
0														
15														
30														
35														
40														
45														
60														
75														
90														
105														
120														
Total in bottle														

Comments: _____

_____

5.1.4 WHO Cone Assay

Adeline Chan (Adapted from WHO)

The WHO cone bioassay is designed to measure the knock down (KD) and mortality of susceptible mosquitoes when exposed to treated long-lasting insecticide-impregnated net (LLIN) materials. The methodology presented here is adapted from the "WHO guidelines for testing of long-lasting insecticidal mosquito LLIN" (2005).

General considerations
- Susceptibility of the *Anopheles* strain **-** The susceptibility of the *Anopheles* strain to be used in the assay should be confirmed at least twice a year using standard WHO susceptibility test kits or with the CDC Bottle bioassay.
- Each WHO test unit can hold 4 test cones. Each cone (5 mosquitoes each) is considered a replicate, so you should have a total of 10 replicates at the end of each LLIN sample assay. A total of 50 mosquitoes will be used per LLIN sample. An alternative modification to this is to run 8 replicates (total of 40 mosquitoes). Each sample LLIN will be tested three times in the same unit: Step A with four cones, Step B with 4 cones and Step C with 2 cones
- Insecticide contamination of the equipment and LLINs used for the negative controls is a major issue in this assay. Every effort must be made to avoid contaminating the equipment and LLINs used for the negative controls. Negative controls should be performed on an untreated LLIN prior to starting the LLIN test for each day. After completing an assay with one sample, the test unit is removed for washing. For each successive assay, a clean WHO test unit and aspirator will be used.
- Negative controls will be run to validate the test results. Since there are no untreated polyethylene LLINs, untreated polyester LLINs should be used as negative control materials when testing both the Olyset and PermaNets. Negative controls should be performed before proceeding to test the LLIN samples to decrease the possibility of insecticide contamination. Run one negative control per operator per day. For example if there are 3 operators that will be testing the LLINs on a particular day, each operator will run one negative control for that day. Each negative control will be for all LLIN samples that will be tested by that operator on that day. The untreated LLIN sample can be reused as the negative control until such time when unacceptable mosquito mortality is seen. Replace with a new untreated LLIN sample when necessary.

Equipment and Materials

1. WHO cone test units - each test unit consists of:
    a. One wood block (21.5 cm x 28 cm)
    b. One large Kimwipe (or clean paper towel)
    c. One Teflon plastic sheet (25 cm x 25 cm)
    d. Four WHO standard cones
    e. One bioassay plastic frame (25 cm x 25 cm)
    f. Four medium binder clips to secure unit
2. Four timers capable of counting seconds
3. Cotton balls or wadding
4. Aspirators
5. Containers for transferring/holding mosquitoes
6. Permanent markers
7. Label tape
8. Disposable gloves
9. 10% sugar solution

Other Materials

1. LLIN samples (25 cm x 25 cm) from untreated LLINs (25 cm x 25 cm) - for negative controls
2. LLIN samples (25 cm x 25 cm) taken from **Position 2** from the each test LLIN
3. 2-5 day old unfed female mosquitoes from a susceptible *Anopheles* colony

Sample Preparation

1. After examination of the LLINs for durability, samples will be cut from each side of a LLIN in 5 different positions as shown in **Figure 5.1.4.1**.
2. Select an area of the LLIN in the approximate positions as shown in **Figure 5.1.4.2a** that does not have holes.
3. Although the area of the sample LLIN to be tested will be 25 cm X 25 cm, it is necessary to cut samples larger than 25 cm X 25 cm. The LLIN will be stretched over the PVC frame when the samples are cut and therefore the actual size of the samples will shrink after they are cut. In addition it is important to ensure that there is enough LLIN material to fit on the WHO cone bioassay unit for testing.
4. A cardboard template 30 cm X 30 cm, can be used as a guide when cutting the samples (**Figure 5.1.4.2.1**). To ensure that there will be enough LLIN cut around the cardboard template leaving a generous amount of material around the edge, as in **Figure 5.1.4.2.2**. **Figure 5.1.4.2.3** shows an example of the size of the hole in the LLIN in comparison to the cardboard template after the sample was removed.
5. Record the LLIN barcode identification from the LLIN and position from where the LLIN test sample was cut (1, 2, 3, 4 and 5) on a piece of marking tape. For example IAA-1, IAA-2, IAA-3, IAA-5 and IAA-5.
6. Staple the identification marking tape to a corner of the LLIN (**Figure 5.1.4.2.4)**;
7. Wrap the five labeled samples from each LLIN together in aluminum paper to protect it from light that may degrade the insecticide.
8. Place the wrapped LLIN samples in individual polyethylene bags and label the bags with the date, and the barcode of the LLIN from which it was cut.

Figure 5.1.4.1: Collection of LLIN samples for the determining insecticide content with WHO cone bioassay and with chemical methods.
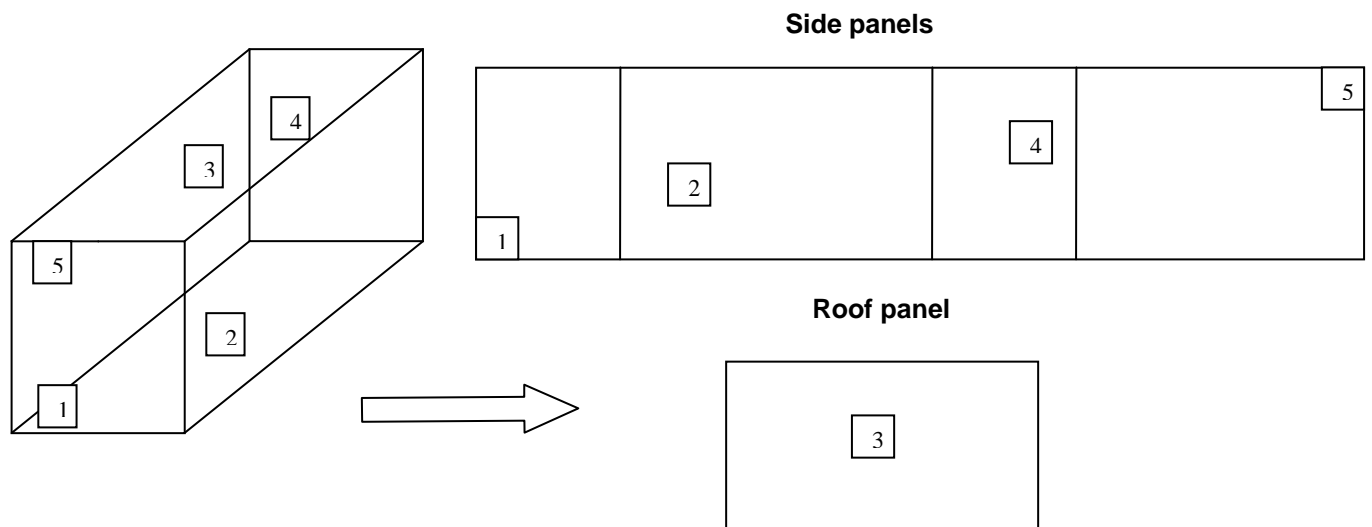
Figure 5.1.4.2.1: 30 cm x 30 cm cardboard template used in guiding the sample removal.
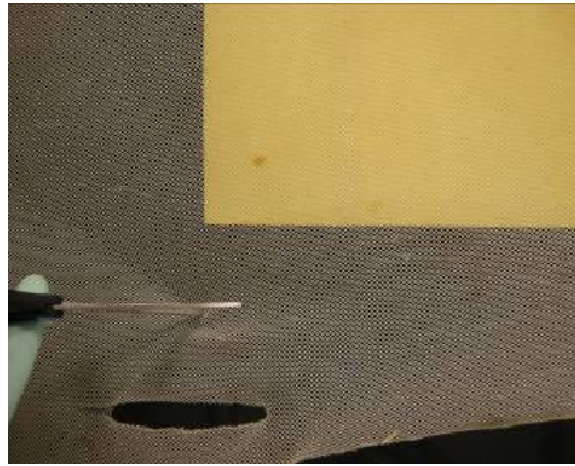
Figure 5.1.4.2.2: Cutting the sample ensuring that there is a generous amount of material left around the template.

Figure 5.1.4.2.3: Comparison of the size of the template to the size of the sample removed from the LLIN.

Figure 5.1.4.2.4: Identification of the LLIN. In this example, it is done by stapling masking tape with the barcode number to a corner of the LLIN sample.

Instructions for the Cone Bioassay

These are instructions for the 10-replicate procedure. The bioassays should be carried out at 25 ± 2°C and 75 ± 10%RH. If this is not possible, average temperature and relative humidity at the time of the assay should be recorded.

1. To assemble the bioassay unit, lay down the following pieces in order (**Figure 5.1.4.3**):
    a. Wood block
    b. 1 Large Kimwipe (or clean paper towel)
    c. 1 Teflon plastic sheet
    d. 1 LLIN sample
    e. Four WHO standard cones
    f. 1 bioassay plastic frame
    g. Use four medium binder clips to secure the following components together: the Kimwipe, plastic sheet, LLIN sample, cones and the plastic frame. This is necessary as it keeps mosquitoes in WHO cones and stops the mosquitoes from escaping. Be sure to keep the net sample flat but do not stretch the sample when assembling the bioassay unit (**Figure 5.1.4.4.1**).
2. Mark the mosquito-holding containers to ensure that the mosquitoes from each assay are placed in the corresponding carton. 3 holding containers (A, B and C) will be needed for each assay.
3. Set four timers, one timer for each cone, for 3 minutes
4. Bioassay Replicate:
    a. Aspirate five female mosquitoes in each cone (total 20 female mosquitoes) and plug each cone with a cotton ball. Blow gently to expel the mosquitoes into the cones and the holding containers. If the mosquitoes are blown too hard, they can be damaged against the sides of the cones/containers, killing them before the insecticide has a chance to do so (**Figure 5.1.4.4.2**).
    b. Press Start on the timers after each cone is filled.
    c. Aspirate mosquitoes from cones after 3 minutes and place in the corresponding holding container (**Figure 5.1.4.4.3**).
    d. Record knockdown (KD) rates (mosquitoes that are dead and/or lying at the bottom of the container) at 60 minutes on the data recording sheet (**Appendix 1**)
5. After KD is recorded for each replicate, place the mosquito holding containers in a humid area (at 27±2°C, 80±10% RH), and provide a cotton ball soaked in a 10% sugar solution. Ensure that no ants have access to the cages.
6. Record mortality after 24 hours.
7. Replace the cones, plastic sheet, plastic frame, and aspirator with a clean set of equipment after each LLIN sample is tested

Cleaning the equipment

1. After each LLIN sample, the Kimwipe/paper towel must be discarded. Wash the plastic sheets, cones, plastic frame and aspirator in soapy water and rinse thoroughly at least three times with tap water.
2. Dry equipment completely before using the equipment again.

7. Four medium binder clips to secure unit

6. Bioassay plastic frame

5. Four WHO bioassay cones

4. One LLIN sample

3. One plastic sheet (25 x 25 cm)

2. One large paper towel (or Kimwipe)

1. Two wooden blocks
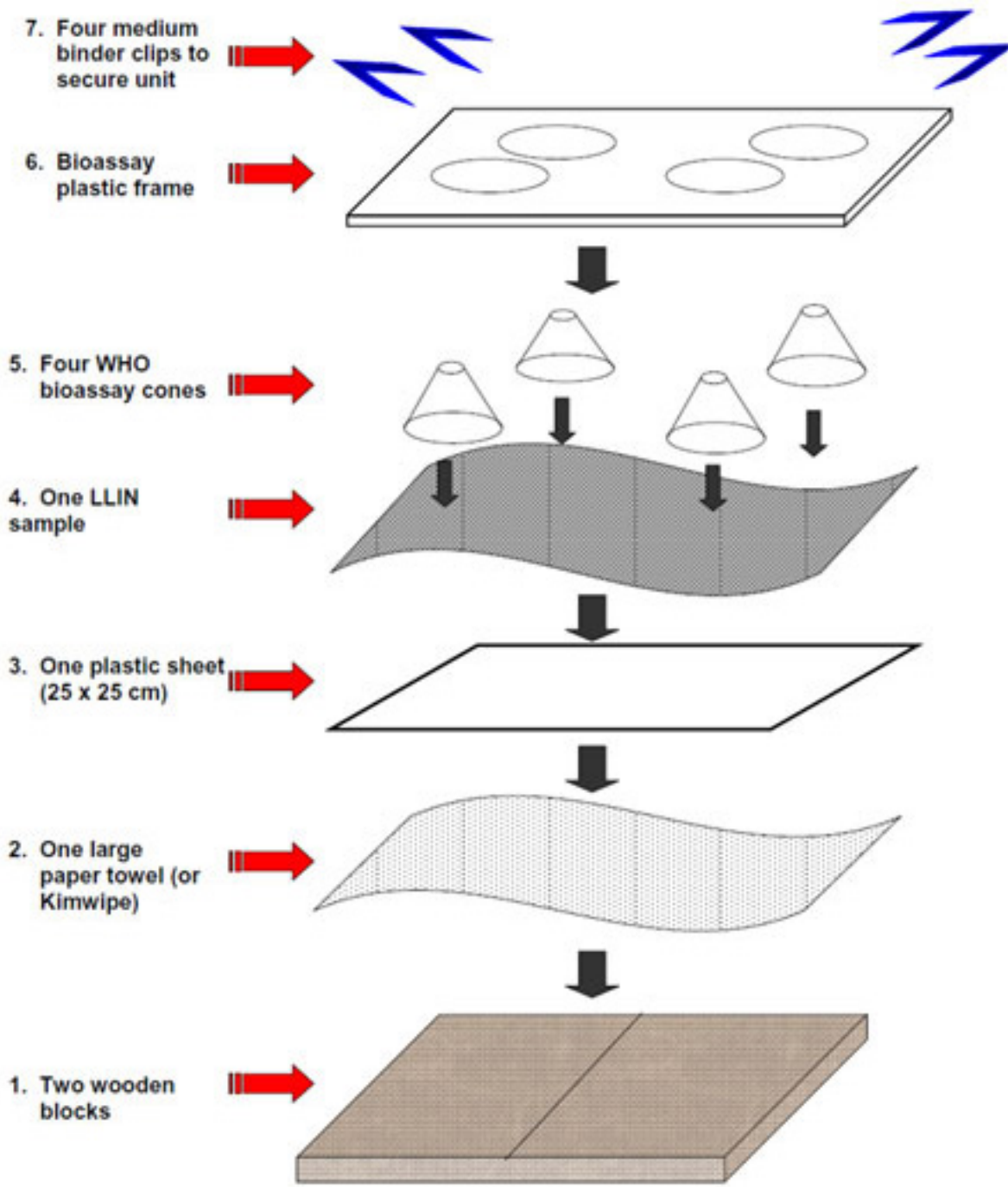
Figure 5.1.4.3: Schematic diagram for assembling the WHO cone bioassay.

Figure 5.1.4.4.1: The completed set-up for an individual WHO cone bioassay.
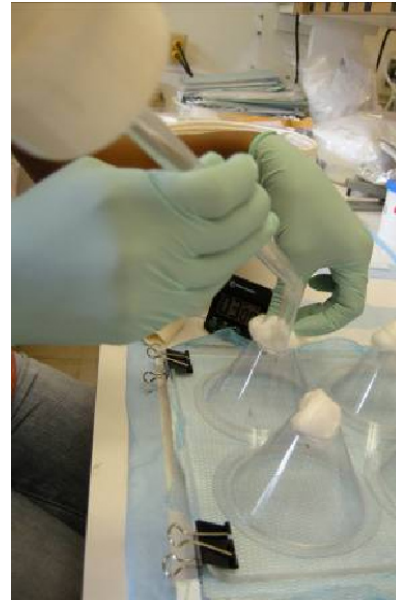
Figure 5.1.4.4.2 (above): Gently aspirate 5 female mosquitoes into the cone then quickly plug with a cotton ball to prevent mosquitoes from escaping.
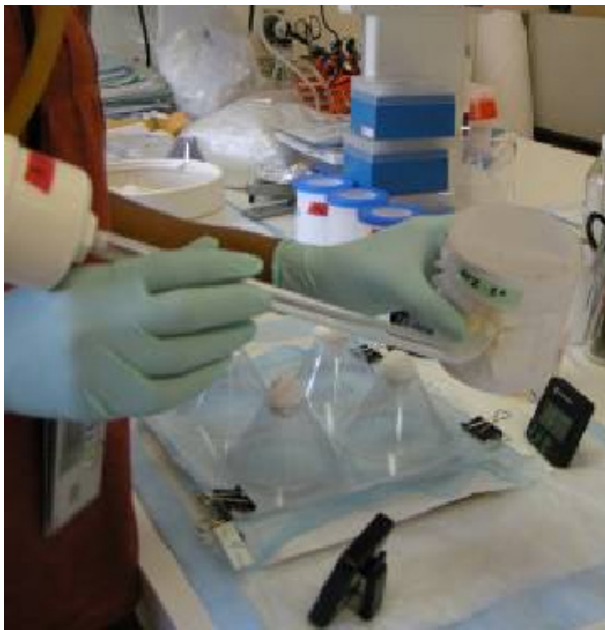
Figure 5.1.4.4.3 (left): After 3 minutes, remove the females and gently transfer them to a marked resting container.

Analysis and Interpretation

With experience there should be little or no mortality in the negative control bioassay.
Guidelines for adjustment of data are:
- If the control knockdown or mortality is < 5% no adjustment of the data is necessary
- If the control knockdown or mortality is 5-20%, use Abbott's formula to adjust the data (**Figure 5.1.4.5**).
- If the control knockdown or mortality is > 20%, discard the results and repeat the assay(s)

Both the proportion of mosquitoes knocked down at 60 minutes (KD60) and the percentage mortality after 24 hours should be calculated. The percentage control knockdown data is used to adjust the KD60 and

the percentage control mortality is used to adjust the 24 h mortality data. Examples of analysis and interpretation of the data with and without control mortality is shown in **Appendix 2**.

Criteria for dead/knocked-down versus alive

Mosquitoes able to fly, stand upright at the bottom of the cage or on the walls of the cage are counted as alive. Note that some mosquitoes may be able to fly up and immediately fall back to the bottom of the cage. If these are unable to stand upright, they should be counted as dead/knocked down.

The WHO recommendation for definitions of effectiveness of LLINs based on bioassay results are as follows (Kilian et al. 2008):
- Minimal effectiveness: KD60 ≥75% or mortality ≥ 50%
- Optimal effectiveness: KD60 ≥ 95% or mortality ≥ 80%

**Note that the minimal effectiveness is a substitute for the tunnel test.

Figure 5.1.4.5 Abbott's Formula

Corrected mortality = $\dfrac{(\%\text{mortality in test bottles} - \%\text{ mortality in control bottle}) \times 100}{(100\% - \%\text{ mortality in control bottle})}$

For example: if mortality in test bottles is 50% and control mortality is 10%, the corrected mortality is $[(50\% - 10\%) / (100\% - 10\%)] \times 100 = 44.4\%$

Note: In cases of 100% mortality in test bottles, Abbott's formula has no effect. For example: $[(100\% - 10\%) / (100\% - 10\%)] \times 100 = 100\%$ corrected mortality

References:

Kilian A, Byamukama W, Pigeon O, Atieli D and Phan C. 2008. Long-term field performance of a polyester-based long-lasting insecticidal mosquito LLIN in rural Uganda. Mal. J. 7:49

WHO Guidelines For Laboratory and Field Testing of Long-Lasting Insecticidal Mosquito LLIN" WHO/CDS/WHOPES/GCDPP/2005.11

Appendix 1:

WHO Cone Bioassay - Data Record Sheet for LLIN monitoring

Date_____   Operator Name: _____

Mosquito Strain_____Temperature:_____, % RH:_____

LLIN sample	Knockdown	24 Hours After Test	
	60 min	**Dead**	**Total Number**
Control – Test A			
Test B			
Test C			
Total control:			
LLIN Code:_____			
A			
B			
C			
Total:			
LLIN Code:_____			
A			
B			
C			
Total:			
LLIN Code:_____			
A			
B			
C			
Total:			
LLIN Code:_____			
A			
B			
C			
Total:			
LLIN Code:_____			
A			
B			
C			
Total:			

Appendix 2: Example of analysis and interpretation of data.

Data sheet 1 is an example of cone bioassays carried out on several LLIN samples and where there was no negative control mortality. **Data sheet 2** shows the data from same LLIN samples but where there was an 8% knockdown in the control at 60 minutes and 12% mortality at 24 hr.

The WHO recommendation for definitions of effectiveness of LLINs based on bioassay results are as follows (Kilian et al. 2008):

- Minimal effectiveness: KD60 ≥75% or mortality ≥ 50%
- Optimal effectiveness: KD60 ≥ 95% or mortality ≥ 80%

Table 5.1.4.6.1 and **5.1.4.6.2** shows the unadjusted data and data adjusted (Abbott's formula) to take into account control knockdown and mortality. LLIN sample KAB 02 for example can be considered minimally effective when there was no control mortality. When adjusted for control mortality, KAB 02 could no longer be considered minimally effective. If negative adjusted KD60 and mortality is obtained (e.g. HAB 02), the LLIN is considered to no longer have any insecticidal effectiveness.

Table 5.1.4.6.1 Analysis and interpretation of data without control mortality

Year	LLIN Code	LLIN Type	Location	KD60 (%)	Mortality, (%)	Optimal effectiveness	Minimal effectiveness
1	**KAA 02**	Type 1	Village A	100	100	yes	yes
1	**KAB 02**	Type 1	Village B	69.6	55	no	yes
1	**LAC 02**	Type 1	Village B	96.3	55	yes	-
1	**HAA 02**	Type 2	Village C	21.1	96.2	yes	-
1	**HAB 02**	Type 2	Village D	4	10	no	no

Table 5.1.4.6.2 Data adjusted with Abbot's formula using 8% control knockdown and 12% control mortality.

Year	LLIN Code	LLIN Type	Location	Adjusted KD60 (%)	Adjusted Mortality, (%)	Adjusted optimal effectiveness	Adjusted minimal effectiveness
1	**KAA 02**	Type 1	Village A	100	100	yes	yes
1	**KAB 02**	Type 1	Village B	67	48.9	no	no
1	**LAC 02**	Type 1	Village B	96	51.6	yes	-
1	**HAA 02**	Type 2	Village C	14.9	95.7	yes	-
1	**HAB 02**	Type 2	Village D	-4.3	-2.3	no	no

Data Sheet 1: Example of a data sheet for one day of testing on several LLIN samples with no mortality in control assay

WHO Cone Bioassay - Data Record Sheet for LLIN monitoring -

Date: 3 Sept 2009   Operator Name: _John Doe_

Mosquito Strain: _An. gambiae_ (Kisumu) Temperature: 29 °C, % RH: 82%

LLIN sample	No. Knockdown	No. 24 Hours AfterTest	
	60 min	**Dead**	**Total Number**
Control – Test A	0	0	20
Test B	0	0	20
Test C	0	0	10
Total: Control	**0**	**0**	**50**
LLIN Code: KAA 02			
A	20	20	20
B	20	20	20
C	10	10	10
Total: KAA 02	**50**	**50**	**50**
LLIN Code: KAB 02			
A	18	16	22
B	19	14	24
C	5	1	10
Total:	**42**	**33**	**56**
LLIN Code: LAC 02	3	5	24
A	3	4	20
B	1	2	10
C	**7**	**11**	**54**
Total: LAC 02			
LLIN Code: HAA 02	3	4	22
A	5	7	20
B	3	3	10
C	**11**	**14**	**52**
Total: HAA 02			
LLIN Code: HAB 02	0	2	20
A	0	1	22
B	1	2	8
C	**1**	**5**	**50**
Total: HAB 02	**7**	**11**	**54**

Data Sheet 2: Example of a data sheet for one day of testing on several LLIN samples with 4% mortality in control assay

WHO Cone Bioassay - Data Record Sheet for LLIN monitoring

Date: 3 Sept 2009   Operator Name: _____John Doe_____

Mosquito Strain: _An. gambiae_ (Kisumu) Temperature: 29 °C, % RH: 82%

LLIN sample	Knockdown	24 Hours After Test	
	60 min	**Dead**	**Total Number**
Control – Test A	2	2	20
Test B	1	2	20
Test C	1	1	20
Total: Control	4	6	50
LLIN Code: KAA 02			
A	20	20	20
B	20	20	20
C	10	10	10
Total: KAA 02	**50**	**50**	**50**
LLIN Code: KAB 02			
A	18	16	22
B	19	14	24
C	2	1	10
Total:	**39**	**33**	**56**
LLIN Code: LAC 02			
A	23	15	24
B	20	12	20
C	9	4	10
Total: LAC 02	**52**	**31**	**54**
LLIN Code: HAA 02			
A	3	4	22
B	5	7	20
C	3	3	10
Total: HAA 02	**11**	**14**	**52**
LLIN Code: HAB 02			
A	0	2	20
B	0	1	22
C	1	2	8
Total: HAB 02	**1**	**5**	**50**

5.2 Introduction to Microplate Enzyme Activity Assays

William G. Brogdon

Introduction

In this section, preparations of the mosquito homogenates for microplate enzyme analysis are described. Enzyme-specific assays are presented in the following sections.

The detection of enzyme activities relevant to insecticide-resistance are often performed using microplate assays. These can be conducted with either pupae or adults. Since mosquitoes must be destroyed, it should only be used to sample the population to determine if a specific mechanism is present and at what frequency it occurs. This method does, however, allow you to detect underlying resistance mechanisms that may not be detected using bioassays. A disadvantage of these assays is that there is no way to reliably detect heterozygotes for many enzymes. These assays should always be performed with a susceptible control so baseline values can be used for comparison. Suitable samples can be obtained from among the MR4-held stocks.
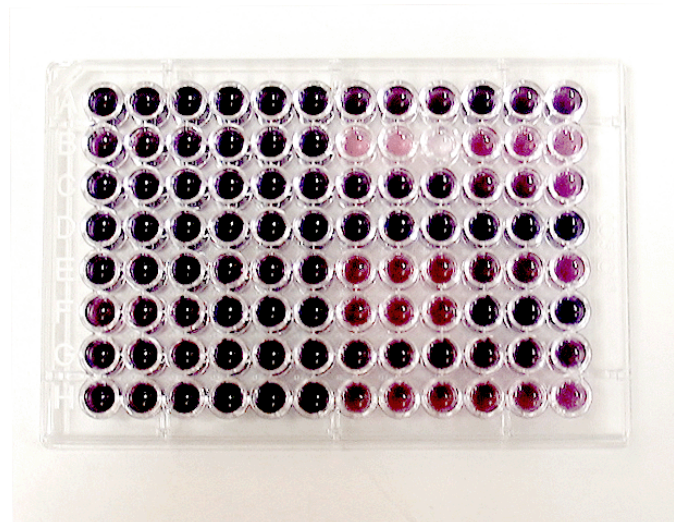
Figure 5.2.1. An example of an oxidase enzyme bioassay with elevated enzyme levels (possible resistance) indicated by the darker colors.

Specimens for biochemical analysis must be freshly killed and immediately used or frozen for later testing. These samples may be mosquitoes used to perform insecticide bioassays (**Section 5.1.3**). Otherwise no other chemicals should be used for killing as they may interfere with the microplate assays. Whole mosquitoes may be frozen and stored at -20°C for up to 7 days. Thereafter, -80°C or colder storage is advised. Samples may be stored only briefly in the refrigerator (4° C).

Alcohol-persevered specimens **cannot** be used for biochemical analysis. Alcohol will reduce or eliminate the enzyme activities that these procedures are measuring.

You may wish to perform total soluble protein analysis on your samples. This enables size-correction when comparing different species or different "broods" of the same species which allows you to correct for higher enzyme levels due only to their size. The standard curve is usually determined using bovine serum albumin. Procedures for how to do this are provided by the manufacturer of the protein detection system used or are widely available and will not be discussed further.

Collecting and interpreting data

A plate-reading spectrophotometer will be used to collect data at the appropriate absorbing wavelength (described in the protocol for each assay). The software for these instruments will probably include a number of data handling features that may be especially useful.

Following is a brief introduction to data analysis. A susceptible population shows an upper absorbance range limit for susceptibility in terms of activity. Individuals with levels above that threshold are less susceptible (**Figure 5.2.2**).

The susceptible population (white bars) is normally distributed with regard to enzyme activity. The upper range limit at e.g. 570 nm (in this esterase assay) is 0.9. This becomes the resistance threshold. The

population shown in black is a hypothetical field population containing individuals with elevated activity (possibly corresponding to resistance) causing the distribution to be skewed to the right. Most field populations will contain both resistant and susceptible individuals. When this method is performed in conjunction with bioassays, the levels of resistance observed in the bioassay should correspond to the enzyme activity. If a higher frequency of resistance is observed in the bioassay than that suggested by the enzyme assay, a different resistance mechanism may be responsible.
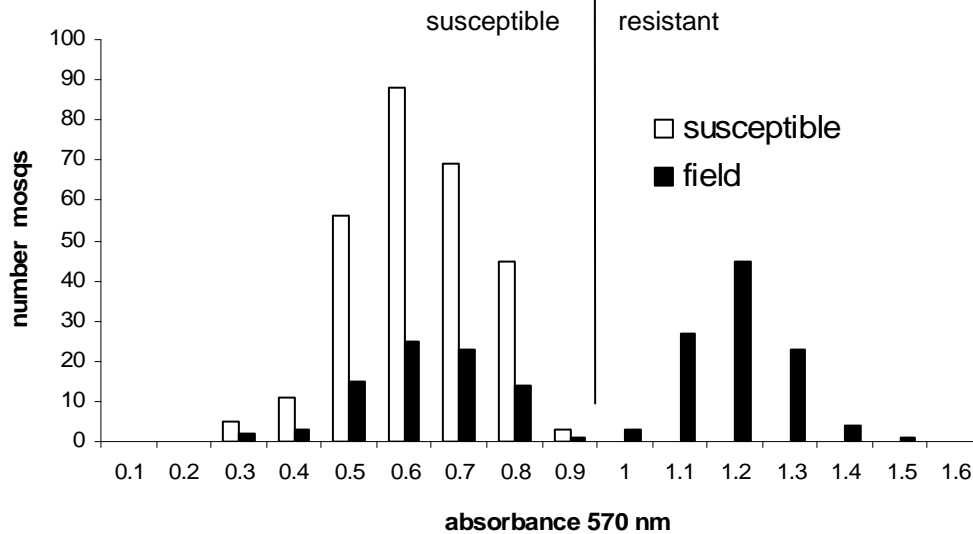
Figure 5.2.2. The absorbance of individuals of two different populations. Note that the reference susceptible population contains no individuals having an absorbance > 0.9. In contrast, the field population contains not only individuals with absorbance levels similar to those of the susceptibles but also many with much higher absorbance levels. One may conclude that the latter individuals represent insecticide-resistant mosquitoes if similar proportions are confirmed by bioassays.

It is sometimes useful to directly compare the absorbances obtained from two different enzyme assays of two populations when different mechanisms may be responsible for resistance observed in a bioassay. This also allows one to identify the presence of resistant individuals using a smaller sample. Such a representation is shown in **Figure 5.2.3**.

Materials:

- 1.5 ml tubes (e.g. Kontes)

- Disposable or reusable pestles for above tubes

- Pipettors and tips

- Multichannel pipettors

- Forceps

- Freezer (used to kill or anesthetize samples)

- Timer (digital counter capable of counting seconds)

- Microplates

- Microplate-reading spectrophotometer

- Analytical balance

- Dibasic potassium phosphate

- Monobasic potassium phosphate

- Bottles of various sizes to store chemical solutions

- Graduated cylinders

- pH meter

- Laboratory stirrer
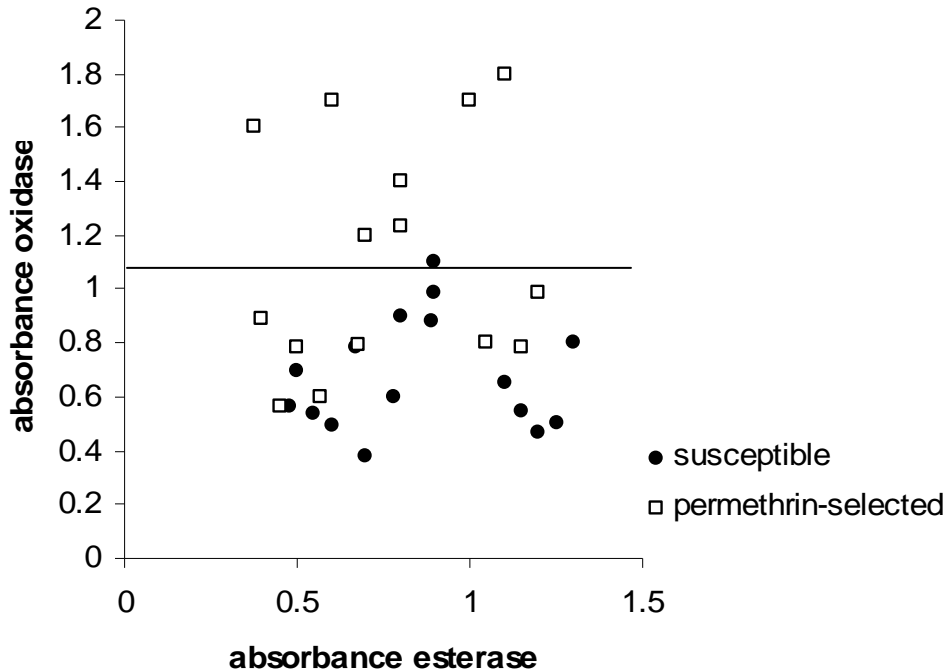
Figure 5.2.3. Oxidase and esterase absorbances of individual mosquitoes have been plotted for two populations in an XY graph. In this example, permethrin exposure has been applied and has increased the level of resistance observed in bioassays. The upper limit of oxidase activity in the unselected population is indicated by the horizontal line. Many individuals in the permethrin-selected stock now have elevated levels of oxidase though the level of esterase has not been affected.

Reagent Preparation:

0.1 M Potassium Phosphate Buffer [KPO_4]

1. Place 800 ml purified water in a glass beaker on a stirrer

2. Add 6.6 g dibasic potassium phosphate

3. Add 1.7 g monobasic potassium phosphate

4. Adjust to pH 7.2 using one of the above.

5. Store at room temperature

6. Adjust to 1000 ml final volume

<u>0.25M Sodium Acetate Buffer [NaOAc]</u>

1. Place 900 ml purified water in a glass beaker on a stirrer.

2. Add 83 ml 3M sodium acetate.

3. Adjust to pH 5 with glacial acetic acid.

4. Adjust to 1000 ml final volume.

5. Store at room temperature.

Note: 3M NaOAc can be purchased or made by dissolving 408. 1g of NaOAc in 800 ml of water. Once dissolved, adjust the final volume to 1 liter

Protocol for mosquito preparation:

1. Kill the mosquitoes by placing them in a **freezer for at least 10 minutes**. Mosquitoes revive after brief exposures to freezing temperatures and may escape.[1]

2. Homogenize 1 adult or pupa in 100 µl of KPO_4 buffer in a grinding tube.

3. Dilute to 1000 µl final concentration with KPO_4.

4. OPTIONAL: To increase the number of assays that can be performed from each mosquito, aliquot 500 µl of the homogenate into separate tubes and dilute each to 1000 µl.

Loading homogenates into microplates

1. At room temperature (or on ice if desired), load 100 µl aliquots of homogenate into the microplate wells in triplicate on the same plate for each enzyme assay. Use a new pipette tip for each sample. Load the first mosquito sample three wells across (A 1-3) and the next mosquito in the wells directly below the first (B 1-3). Continue down the plate until you reach the bottom, then shift right to the next three vacant set of columns and continue at the top working downward. Wells A 4-6 should contain your 9th mosquito if you have followed this pattern.

2. Load the positive and negative controls into the last 6 wells on the plate.

3. See specific assays for detection of various activities in the following sections. These should be performed immediately.

Notes on using multi-channel pipettors

▪ Tips must be firmly attached by strong quick pressure of the pipettor.

▪ Use your gloved fingers to individually check the tightness of the tips before attempting to load the pipettor with reagent.

▪ Particular care must be taken to directly observe that each tip has loaded with the same volume as others.

▪ Tight-fitting tips and **slow deliberate** loading are essential to obtaining accurate and precise data.

References:

http://www.cdc.gov/ncidod/wbt/resistance/assay/microplate/index.htm

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[1] See alternatives in **Section 3.4 Mosquito Anesthesia**. Killing mosquitoes with insecticide may drastically affect resistance enzymes levels measured in the assays.

5.2.1 Microplate Insensitive Acetylcholinesterase Assay

William G. Brogdon

Introduction

Insensitive acetylcholinesterase (AChE) has been associated with resistance to carbamates and organophosphates. Propoxur is used in this assay to inhibit the activity of the sensitive (i.e. susceptible) AChE, allowing the detection of the altered enzyme when it is present: The number of alleles of insensitive AChE is greater as the yellow color darkens. It may be possible to clearly distinguish homozygous resistant, heterozygous and homozygous susceptible individuals by their discrete absorbance classes.

Materials

- Acetone

- Acetylthiocholine iodide (ATCH)

- Dithiobis (2-nitrobenzoic acid), (DTNB)

- 0.25 M KPO_4 buffer prepared as described in Microplate Enzyme Assays Introduction

- Propoxur, technical grade

Reagent preparation

ATCH

1. Dissolve 75 mg acetylthiocholine iodide (ATCH) and 21 mg propoxur in 10 ml acetone.

2. Add 90 ml 0.25 M KPO_4 buffer.

3. Store at 4° C for up to 3-4 days.

DTNB (Ellman's reagent)

1. Dissolve 13 mg Dithiobis (2-nitrobenzoic acid), (DTNB), in 100 ml 0.25 M KPO_4 buffer.

2. Store at 4° C for up to 3-4 days.

Protocol

1. To the plate containing the mosquito homogenates (see Microplate Enzyme Assays Introduction), add 100 µl of KPO_4 to the negative control wells.

2. Add 100 µl ATCH to each well.

3. Add 100 µl DTNB to each well.

4. Read plate immediately (T_0) with microplate reader at 414 nm.

5. Read plate at 10 minutes (T_{10}).

6. Subtract the T_0 reading from the T_{10} reading and use this for your statistical analyses.

5.2.2 Microplate Glutathione S-Transferase Assay

William G. Brogdon

Introduction

Elevated glutathione s-transferase (GST) activity has been associated with resistance to DDT. Users should be familiar with the contents of Microplate Enzyme Assays Introduction before proceeding.

Materials

- Acetone

- Purified water

- Reduced glutathione (e.g. Sigma G4251)

- 0.25 M KPO_4 buffer (prepared as described in Microplate Enzyme Assays Introduction)

- Brown glass storage bottles

Reagent preparation

GST solution

1. Dissolve 61 mg reduced glutathione in 100 ml KPO4 buffer.

2. Store at 4° C for up to 3-4 days

cDNB solution

1. Dissolve 20 mg 1-chloro-2,4'-dinitrobenzene (cDNB) in 10 ml acetone.

2. Add 90 ml 0.25 M KPO_4 buffer.

Protocol

1. To the plate on which you have previously added the 100 µl of mosquito homogenates (see Microplate Enzyme Assays), add 100 µl of 0.25 M KPO_4 buffer in three negative control wells on the lower right corner of the plate.

2. To each well, add 100 µl reduced glutathione solution.

3. To each well, add 100 µl cDNB solution.

4. Read plate immediately (T_0) with microplate reader using 340 nm filter.

5. Read plate at 5 minutes (T_5).

6. Subtract the T_0 reading from the T_5 reading and use this for your statistical analysis.

5.2.3 Microplate Nonspecific Esterase Assay

William G. Brogdon

Introduction

Measures levels of non-specific α - and ß-esterases present. These enzymes have been implicated in resistance organophosphates and pyrethroids. Users should be familiar with the contents of Microplate Enzyme Assays Introduction before proceeding.

Materials

- Acetone
- α - or ß-naphthyl acetate (e.g. Sigma, N6875)
- 0-dianisidine tetrazotized (e.g. Sigma, D9805)
- α - or ß- napthol (e.g. Sigma, S477753)

Reagent Preparation

Esterase activity stock solution

1. Dissolve 50 mg α - or ß-naphthyl in 10 ml acetone depending on the assay you have chosen.
2. Add 90 ml 0.25 M KPO_4.
3. Place 1 to 1.5 ml aliquots of solution in microfuge tubes and freeze. Use a lightproof storage container.[1]

Standard

1. For positive controls, dilute the esterase stock 1:35 (i.e 35 µl ß-naphthyl stock, 1.2 ml KPO_4 buffer) and 1:70 (i.e. 17.5 µl ß-naphthyl stock, 1.2 ml KPO_4 buffer).

α or ß-naphthyl acetate

1. Dissolve 56 mg α or ß-naphthyl acetate in 20 ml acetone
2. Add 80 ml 0.25 M KPO_4
3. Can be stored at 4° C. Bring back to room temperature before use as it may come partially out of solution when cold.

Dianisidine solution

1. Dissolve 100 mg 0-dianisidine tetrazotized in 100 ml purified water immediately before use.
2. Store at 4° C in a light-proof bottle. Check color of dianisidine before use as it will degrade. Color should be pale yellow. If color is amber, discard and make fresh.

Protocol

1. If previously frozen, remove esterase activity stock solution from the freezer and thaw.
2. If previously refrigerated, bring the naphthyl solution to room temperature. Solid may come partially out of solution when cold. If this has happened, swirl until dissolved.

---

[1] You can re-freeze the extra stocks if you keep them protected from light e.g. by wrapping the container in foil or placing in a dark box.

3. To the plate containing the mosquito homogenates (see Microplate Enzyme Assays Introduction), add 100 µl of KPO$_4$ to the negative and positive sample wells.

4. Positive Control= α or ß-naphthol. Add 100 µl of the appropriate control to each of three wells on each plate you run as a positive control.

5. Add 100 µl α or ß-naphthyl acetate to each well.

6. Incubate at room temperature for 10 minutes.

7. Add 100 µl dianisidine to each well

8. Incubate 2 minutes

9. Read at 620 nm if using α -naphthyl or at 540 nm if using ß-naphthyl.

5.2.4 Microplate Oxidase Assay

William G. Brogdon

Introduction

Elevated oxidase levels are associated with resistance to many classes of insecticide. Users should be familiar with the contents of Microplate Enzyme Assays Introduction before proceeding.

Materials

- Pure methanol
- Sodium acetate
- Glacial acetic acid
- Purified water
- TMBZ or TMBZ[2HCl]
- > 3% hydrogen peroxide (H_2O_2)
- 0.25 M KPO_4 buffer prepared as described in Microplate Enzyme Assays Introduction
- Cytochrome-C from bovine heart
- Brown glass storage bottles

Solution Preparation

0.25 M Sodium Acetate Buffer pH 5.0

1. To 800 ml of purified water in a beaker, add 0.25 moles of NaAc and dissolve by stirring.
2. Adjust pH to 5.0 with acetic acid.[1]
3. Store at room temperature.

TMBZ solution

1. Dissolve 20 mg 3,3',5,5'-Tetramethyl-Benzidine Dihydronchloride* (TMBZ [2HCL] or TMBZ) in 25 ml methanol.[2]
2. Add 75 ml 0.25 M Na Acetate, pH 5.0 buffer (prepared above).
3. Store for up to a few days at 4°C. If this reagent turns light blue, discard and make a fresh batch.

3% hydrogen peroxide

Hydrogen peroxide is available in many concentrations. Prepare a 3% solution in purified water using what is available.

Oxidase positive control stock

1. Add 10 mg Cytochrome-C to 100 ml 0.25 M Na Acetate buffer, pH 5.

---

[1] It is very important the pH is exactly 5.0.

[2] TMBZ [2HCl] will dissolve if left to sit for a few minutes. TMBZ will dissolve if swirled under hot water from the tap. Do not heat with an open flame or on a hot pad. Do not shake vigorously to aid in dissolving.

2. Prepare two dilutions of the above for positive controls: 1:55 (i.e. 22 µl stock, 1.2 ml KPO$_4$ buffer) and 1:110 (i.e. 11µl cytochrome-stock, 1.2 KPO$_4$ buffer).

3. Use fresh.

Protocol

1. To the plate containing the mosquito homogenates (see Microplate Enzyme Assays Introduction), add 100 µl of KPO$_4$ to the negative and positive sample wells.

2. Add 100 µl of the cytochrome-C positive control to three wells at the lower right side of the plate – undiluted and the two dilutions prepared above.[3]

3. Add 200 µl TMBZ solution to each well.

4. Add 1 drop (or 25 µl) 3% hydrogen peroxide (H$_2$O$_2$) to each well.

5. Incubate for 5 minutes.

6. Read with microplate reader at 620 nm.

---

[3] Cytochrome-C is very photo labile. Make sure it is the last chemical you add to your oxidase plates before adding the TMBZ and H$_2$O$_2$.

5.3 Insecticide Resistance Allele Assay by PCR

5.3.1 Knockdown Resistance - *Anopheles gambiae*

Introduction

Knockdown Resistance, or KDR, is a commonly occurring permethrin resistance mutation found throughout Africa. Currently there are two main PCR assays to detect the West and East African forms (Martinez-Torres et al. 1998; Ranson et al. 2000). An RT-PCR based assay can be found in **Chapter 8.5.1.3**.

***PCR authentication for KDR resistance in* An. gambiae**

Prepare PCR Master Mix for 96, 48 or 1 25 µl PCR reactions. Add reagents in the order presented.

96	48	1	Reagent
1710 µl	855 µl	17.1 µl	sterile H_2O
250 µl	125 µl	2.5 µl	10X PCR Buffer
100 µl	50 µl	1.0 µl	dNTP (2.5 mM mix)
100 µl	50 µl	1.0 µl	AgD1 (2.5 pmol/µl) [ATAGATTCCCCGACCATG]
100 µl	50 µl	1.0 µl	AgD2 (2.5 pmol/µl) [AGACAAGGATGATGAACC]
100 µl	50 µl	1.0 µl	AgD3 (2.5 pmol/µl) [AATTTGCATTACTTACGACA]
100 µl	50 µl	1.0 µl	AgD4 (2.5 pmol/µl) [CTGTAGTGATAGGAAATTTA]
30 µl	15 µl	0.3 µl	$MgCl_2$ (25 mM)
12.5 µl	6.25 µl	0.125 µl	Taq DNA polymerase (5 U/µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total**

Table 5.3.1.1. PCR for West African KDR resistance mechanism.

Prepare PCR Master Mix for 96, 48 or 1 25 µl PCR reactions. Add reagents in the order presented.

96	48	1	Reagent
1710 µl	855 µl	17.1 µl	sterile H_2O
250 µl	125 µl	2.5 µl	10X PCR Buffer
100 µl	50 µl	1.0 µl	dNTP (2 mM mix)
100 µl	50 µl	1.0 µl	AgD1 (2.5 pmol/µl) [ATAGATTCCCCGACCATG]
100 µl	50 µl	1.0 µl	AgD2 (2.5 pmol/µl) [AGACAAGGATGATGAACC]
100 µl	50 µl	1.0 µl	AgD4 (2.5 pmol/µl) [CTGTAGTGATAGGAAATTTA]
100 µl	50 µl	1.0 µl	AgD5 (2.5 pmol/µl) [TTTGCATTACTTACGACTG]
30 µl	15 µl	0.3 µl	$MgCl_2$ (25 mM)
12.5 µl	6.25 µl	0.125 µl	Taq DNA polymerase (5 U/µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total**

Table 5.3.1.2. PCR for East African KDR resistance mechanism.

PCR Cycle sequence (for both East and West versions):

94°C/5min x 1 cycle
(94°C/1min, 48°C/2min, 72°C/2min) x 40 cycles
72°C/10min x 1 cycle
4°C hold

Run samples on a 2% agarose EtBr gel; load 5 µl sample.
Primers create fragments of 293 internal control, 195 resistant, 137 susceptible. (**Figure 5.3.1.1**).

Figure 5.3.1.1. East African PCR. Lane 1, 1 kb marker, 2, resistant, 3, susceptible, and 4, heterozygous.

References

Martinez-Torres D et al. (1998) Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae s.s.* Insect Mol Biol 7:179-184

Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH (2000) Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol 9:491-497

96 well sample preparation template

5.3.2 Kdr – Knockdown resistance in *Anopheles gambiae*

MR4 Staff, Lynn Huynh

Introduction

Resistance to pyrethroids in *Anopheles gambiae* is commonly associated with a single base-pair mutation in the voltage-gated sodium channel referred to as knockdown resistance (KDR). In Africa there are two forms of this mutation, the west African form which results from a leucine to phenylalanine substitution (TTA/TTT) (Martinez-Torres et al. 1998) and the east African form which results from a leucine to serine substitution (TTA/TCA) (Ranson et al. 2000). A variation on the method of Huynh et al. (2007) uses the intentional mismatch primer method described by Wilkins et al. (2006).

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96 rxn	48 rxn	1 rxn	Reagent	
680 µl	340 µl	6.8 µl	sterile H$_2$O	
500 µl	250 µl	5.0 µl	Taq 5X PCR Buffer (containing 15 mM MgCl$_2$)	
250 µl	125 µl	2.5 µl	dNTP (2.0 mM mix)	
50 µl	25 µl	0.5 µl	MgCl$_2$ (25 mM)	
200 µl	100 µl	2.0 µl	IPCF: (F, 2.5 pmol/µl) [GATAATGTGGATAGATTCCCCGACCATG]	
200 µl	100 µl	2.0 µl	AltRev: (R, 2.5 pmol/µl) [TGCCGTTGGTGCAGACAAGGATG]	
200 µl	100 µl	2.0 µl	EAST	WT: R, (5.0 pmol/µl) [GGTCCATGTTAATTTGCATTACTTACGAaT**A**]
200 µl	100 µl	2.0 µl		East: F, (2.5 pmol/µl) [CTTGGCCACTGTAGTGATAGGAAAaT**C**]
20 µl	10 µl	0.2 µl	GoTaq DNA polymerase (5 U/µl)	
2.5 ml	**1.25 ml**	**23 µl**	**Total (To each 23 µl reaction add 2 µl template DNA)**	

96 rxn	48 rxn	1 rxn	Reagent	
880 µl	440 µl	8.8 µl	sterile H$_2$O	
500 µl	250 µl	5.0 µl	Taq 5X PCR Buffer (containing 15 mM MgCl$_2$)	
250 µl	125 µl	2.5 µl	dNTP (2.0 mM mix)	
50 µl	25 µl	0.5 µl	MgCl$_2$ (25mM)	
200 µl	100 µl	1.0 µl	IPCF: (F, 2.5 pmol/µl) [GATAATGTGGATAGATTCCCCGACCATG]	
200 µl	100 µl	1.0 µl	AltRev: (R, 2.5 pmol/µl) [TGCCGTTGGTGCAGACAAGGATG]	
200 µl	100 µl	1.0 µl	WEST	WT: (R, 25.0 pmol/µl) [GGTCCATGTTAATTTGCATTACTTACGAaT**A**]
600 µl	300 µl	3.0 µl		West: (F, 8.8 pmol/µl) [CTTGGCCACTGTAGTGATAGGAAATgT**T**]
20 µl	10 µl	0.2 µl	GoTaq DNA polymerase (5 U/µl)	
2.5 ml	**1.25 ml**	**23 µl**	**Total (To each 23 µl reaction add 2 µl template DNA)**	

Table 5.3.2.1. East and west resistance alleles are distinguished in two separate reactions. Use the two primers listed and either the East or West primer along with the WT primer at the appropriate concentration. Lower case nucleotides indicate the intentional mismatch in the primer sequences. Nucleotides in bold are located at site of SNP (where applicable). F and R indicate forward and reverse orientation. Add 2 µl DNA template per reaction.

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

PCR cycle conditions

95°C/5min x 1 cycle
(95°C/30sec, East 57°C/30sec **OR** West 59°C/30sec, 72°C/30sec) x 35 cycles
72°C/5min x 1 cycle
4°C hold

Run samples on a 2% agarose EtBr gel; load 5 µl sample.

All successful reactions should contain a band of 314 bp. (**Figure 5.3.2.1**). In addition, a band of 214 bp indicates the susceptible (wild type) allele and one of 156 bp the resistant allele.
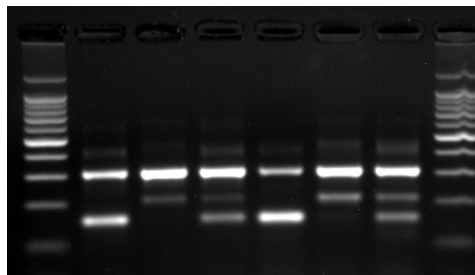
Figure 5.3.2.1. Gel electrophoresis of knockdown resistance assay, east and west samples from separate PCRs. The Lane 1 contains a 100 bp ladder marker, Lane 2, east African homozygous resistant (RSP-ST), Lane 3, east African homozygous susceptible (G3), Lane 4, west African heterozygous (RSP-ST X G3), Lane 5, west African homozygous resistant (AKRON), Lane 6, east African homozygous susceptible (G3), Lane 7, east African heterozygous (AKRON X G3), Lane 8, 100 bp ladder marker

References

Huynh LY, Sandve SR, Hannan LM, Van Ert M, Gimnig JE (2007) Fitness costs of pyrethroid insecticide resistance in *Anopheles gambiae*. In: Annual Meeting of the Society for the Study of Evolution, Christchurch, New Zealand

Martinez-Torres D et al. (1998) Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae s.s*. Insect Molecular Biology 7:179-184

Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH (2000) Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol 9:491-497

Wilkins EE, Howell PI, Benedict MQ (2006) IMP PCR primers detect single nucleotide polymorphisms for *Anopheles gambiae* species identification, *Mopti* and *Savanna* rDNA types, and resistance to dieldrin in *Anopheles arabiensis*. Malar J 5:125

5.3.3 Dieldrin Resistance - *An. gambiae* and *An. arabiensis v.2*

Introduction

Due to the ban on the use of dieldrin as an insecticide, resistance to dieldrin (Rdl) is not prevalent in the wild; however, several laboratory colonies are maintained that have this mutation. The Rdl mutation has been found to be associated with cross-resistance to newer insecticides that are currently being employed (Brooke et al. 2000; Kolaczinski and Curtis 2001). Two PCR assays have been developed to detect the Rdl mutation; one in *An. gambiae* (Du et al. 2005) and one in *An. arabiensis* (Wilkins et al. 2006). An RT-PCR based assay can be found in **Chapter 8.5.1.5**. Due to issues with differentiating resistant and susceptible alleles in *An. arabiensis* and the lack of a susceptible band in *An. gambiae*, these assays were updated with new primers to allow for better discrimination of allele types.

An. gambiae (Du et al. 2005) Ala296Gly

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions. Add reagents in the order presented.

96	48	1	0.5	Reagent
1460 µl	730 µl	14.60 µl	7.3 µl	sterile H_2O
500 µl	250 µl	5.0 µl	2.5 µl	5X GoTaq PCR Buffer
100 µl	50 µl	1.0 µl	0.5 µl	dNTP (2.5 mM mix)
100 µl	50 µl	1.0 µl	0.5 µl	RDLF (F, 25 pmol/µl) [AGTTTGTACGTTCGATGGGTTA]
100 µl	50 µl	1.0 µl	0.5 µl	RDLRV2 (R, 25 pmol/µl) [GTGACAACGGATTACTTAGAGC]
100 µl	50 µl	1.0 µl	0.5 µl	WTRDL (16 pmol/µl) [AGACAGTAGTTACACCTAATGCG]
100 µl	50 µl	1.0 µl	0.5 µl	DF1RDL (F, 25 pmol/µl) [AATGCTACACCAGCACGTGTTGG]
30 µl	15 µl	0.3 µl	0.15 µl	$MgCl_2$ (25 mM)
15 µl	7.5 µl	0.15 µl	0.05 µl	Go-Taq DNA polymerase (5 U/µl)
2.5 ml	**1.25 ml**	**25 µl**	**12.5 µl**	**Total**

Table 5.3.3.1. F and R indicate forward and reverse orientation. Use 1 µl DNA template. If performing the ½ volume assay, use 0.5µl template DNA instead.

An. arabiensis (Wilkins et al. 2006) Ala296Ser

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions. Add reagents in the order presented.

96	48	1	0.5	Reagent
1455 µl	727.5 µl	14.55 µl	7.3 µl	sterile H_2O
500 µl	250 µl	5.0 µl	2.5 µl	5X PCR Buffer
100 µl	50 µl	1.0 µl	0.5 µl	dNTP (2 mM mix)
100 µl	50 µl	1.0 µl	0.5 µl	RDLF (F, 25 pmol/µl) [AGTTTGTACGTTCGATGGGTTA]
100 µl	50 µl	1.0 µl	0.5 µl	RDLRv2 (R, 25 pmol/µl) [GTGACAACGGATTACTTAGAGC]
100 µl	50 µl	1.0 µl	0.5 µl	AARDL (F, 25 pmol/µl) [GCTACACCAGCACGTGaT**T**]
100 µl	50 µl	1.0 µl	0.5 µl	RDLSS (R, 25 pmol/µl) [CAAGACAGTAGTTACACCTAAaG**C**]
30 µl	15 µl	0.3 µl	0.15 µl	$MgCl_2$ (25 mM)
15 µl	7.5 µl	0.15 µl	0.05 µl	Go-Taq DNA polymerase (5 U/µl)
2.5 ml	**1.25 ml**	**25 µl**	**12.5 µl**	**Total**

Table 5.3.3.2. In primer sequence, lower case nucleotides indicates the intentional mismatch, nucleotides in bold are located at site of SNP (where applicable); F and R indicate forward and reverse orientation. Use 1 µl DNA template. If performing the ½ volume assay, use 0.5µl template DNA instead.

PCR Cycle conditions for both assays

94°C/3min x 1 cycle
(94°C/1min -0- **XX**°C/1min -0- 72°C/1min) x 30 cycles
72°C/7min x 1 cycle
4°C hold

An. gambiae cycles at 53°C annealing temperature while *An. arabiensis* cycles at 58°C annealing temperature. Run samples on a 1.5% agarose gel stained with EtBr, or other intercalating agent like SYBR Green or Gel Red, and load 10 µl sample.

An. gambiae

Primers create fragments of: control band 345 bp, *An. gambiae* resistant 252 bp, and *An. gambiae* susceptible 136 bp (**Figure 5.3.3.1**)
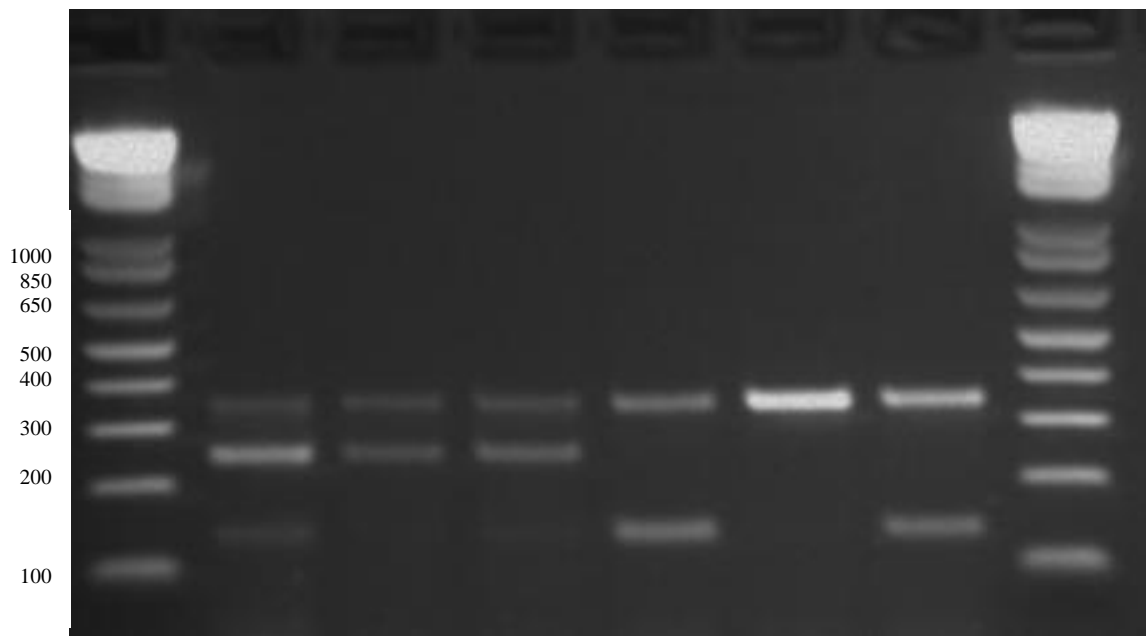
Figure 5.3.3.1. Lane 1 1kb ladder, lanes 2-4 PIMPERENA (resistant), lanes 5-7 MALI NIH (susceptible)

An. arabiensis

Primers create fragments of: internal control band 340 bp, *An. arabiensis* resistant 242 bp, and *An. arabiensis* susceptible 139 bp (**Figure 5.3.3.2**)
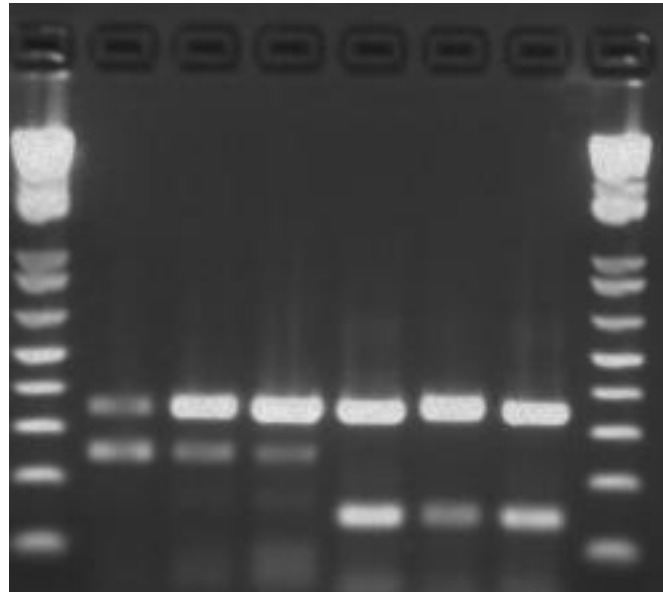
Figure 5.3.3.2. Lane 1, 1Kb ladder, lanes 2-4 SENN (resistant), lanes 5-7 DONGOLA (susceptible), lane 8 1Kb ladder

References

Brooke BD, Hunt RH, Coetzee M (2000) Resistance to dieldrin + fipronil assorts with chromosome inversion 2La in the malaria vector *Anopheles gambiae*. Medical and Veterinary Entomology 14:190-194

Du W et al. (2005) Independent mutations in the Rdl locus confer dieldrin resistance to *Anopheles gambiae* and *An. arabiensis*. Insect Molecular Biology 14:179-183

Kolaczinski J, Curtis C (2001) Laboratory evaluation of fipronil, a phenylpyrazole insecticide, against adult *Anopheles* (Diptera: Culicidae) and investigation of its possible cross-resistance with dieldrin in *Anopheles stephensi*. Pest Management Science 57:41-45

Wilkins EE, Howell PI, Benedict MQ (2006) IMP PCR primers detect single nucleotide polymorphisms for *Anopheles gambiae* species identification, *Mopti* and *Savanna* rDNA types, and resistance to dieldrin in *Anopheles arabiensis*. Malar J 5:125

96 well sample preparation template

	1	2	3	4	5	6	7	8	9	10	11	12
A	○	○	○	○	○	○	○	○	○	○	○	○
B	○	○	○	○	○	○	○	○	○	○	○	○
C	○	○	○	○	○	○	○	○	○	○	○	○
D	○	○	○	○	○	○	○	○	○	○	○	○
E	○	○	○	○	○	○	○	○	○	○	○	○
F	○	○	○	○	○	○	○	○	○	○	○	○
G	○	○	○	○	○	○	○	○	○	○	○	○
H	○	○	○	○	○	○	○	○	○	○	○	○

5.3.4 ACE-1 Resistance in *An. gambiae*

MR4 Staff

Introduction

Insensitive acetylcholinesterase (AChE) is a resistance mechanism associated with tolerance to carbamate and organophosphate insecticides. Mutations within AChE genes in Dipterans are widespread and have varying effects on the tolerance levels to insecticides. This is of great importance due to the increased interest in utilizing bendiocarb as a potential treatment for bed-nets and increased resistance levels to common insecticides across Africa. (Weill et al. 2004) isolated a unique mutation found in both old and new world vectors in the ace-1 allele. From this a PCR-RFLP was designed based on the G119S mutation isolated from *An. gambiae.* An RT-PCR based assay can be found in **Chapter 8.5.1.4**.

Anopheles spp. (Weill et al. 2004)

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions. Add reagents in the order presented.

96	48	1	Reagent
1635 µl	817.5 µl	16.35 µl	sterile H_2O
500 µl	250 µl	5.0 µl	5X GoTaq PCR Buffer
100 µl	50 µl	1.0 µl	dNTP (2.5 mM mix)
125 µl	62.5 µl	1.25 µl	MOUSTDIR1 (25 pmol/µl) [CCGGG**N**GC**S**AC**Y**ATGTGGAA]
125 µl	62.5 µl	1.25 µl	MOUSTREV1 (25 pmol/µl) [ACGAT**M**ACGTTCTC**Y**TCCGA]
15 µl	7.5 µl	0.15 µl	Taq DNA polymerase (5 U/µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total (To each 25 µl reaction add 1 µl template DNA)**

Table 5.3.4.1.

PCR Cycle conditions

93^oC/5min x 1 cycle
(93^oC/1min -0- 53^oC/1min -0- 72^oC/1.5min) x 35 cycles
72^oC/10min x 1 cycle
4^oC hold

Restriction enzyme digest

Add 1µl *AluI* restriction enzyme, 2 µl of H20, and 2µl of buffer to 15 µl PCR product from above reaction. Allow to incubate at 37°C for 8-24 hr. For shorter times, incomplete digests could be a problem.

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red.

Anopheles spp.

Primers create a 194 bp amplicon, after restriction enzyme digest homozygous resistant individuals will have 120bp and 74bp fragments (**Figure 5.3.4.1**).

Figure 5.3.4.1 *Ace*-1 resistance PCR-RFLP. Lane 1, 1kb ladder, lanes 2-4 *An. gambiae* homozyogous for *ace*-1 resistance mutation, lane 5 homozygous *An. arabiensis* negative for the *ace*-1 mutation.

References

Weill M et al. (2004) The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. Insect Mol Biol 13:1-7

96 well sample preparation template

5.3.5 An Intentional Mismatched Primer - PCR (IMP-PCR) assay to detect ACE-1 resistance in *An. gambiae*

MR4 Staff

Introduction

Insensitive acetylcholinesterase (AChE) is a resistance mechanism associated with tolerance to carbamate and organophosphate insecticides. Mutations within AChE genes in Dipterans are widespread and have varying effects on the tolerance levels to insecticides. (Weill et al. 2004) isolated a unique mutation found in both old and new world vectors in the *ace*-1 allele. Using the IMP technique (Wilkins et al 2006) a new, single-step PCR assay was developed eliminating the lengthy and expensive RFLP step previously needed to detect the resistance mutation. An alternative RT-PCR based assay can be found in **Chapter 8.5.1.4**.

Anopheles gambiae s.s

Prepare PCR Master Mix for 96, 48 or 1 10µl PCR reactions. Add reagents in the order presented.

96	48	1	Reagent
500 µl	250 µl	5.0 µl	sterile H_2O
200 µl	100 µl	2.0 µl	5X GoTaq PCR Buffer
75 µl	37.5 µl	0.75 µl	dNTP (2.5 mM mix)
15 µl	7.5 µl	0.15 µl	$MgCl_2$ (25 mM)
50 µl	25 µl	0.5 µl	CDCACEF (8 pmol/µl) GGT GGA CGT GTG TGG CTC
50 µl	25 µl	0.5 µl	CDCACER (8 pmol/µl) CTA CCG TAG CGC AAG GTT C
50 µl	25 µl	0.5 µl	CDCWT (10 pmol/µl) TGT GGA TCT TCG GCG tC**G**
50 µl	25 µl	0.5 µl	CDCG119SR (25 pmol/µl) CGG TGC CGG AGT AGA AtC **T**
10 µl	5.0 µl	0.1 µl	Taq DNA polymerase (5 U/µl)
1 ml	**500 µl**	**10 µl**	**Total (To each 20 µl reaction add 1 µl template gDNA)**

Table 5.3.5.1. F and R indicate forward and reverse orientation. Lower cases represent intentional mismatches while bold letters indicate SNPs.

PCR Cycle conditions

94°C/5min x 1 cycle
(94°C/30sec , 61°C/1min , 72°C/1min) x 35 cycles
72°C/7min x 1 cycle
10°C hold

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red.
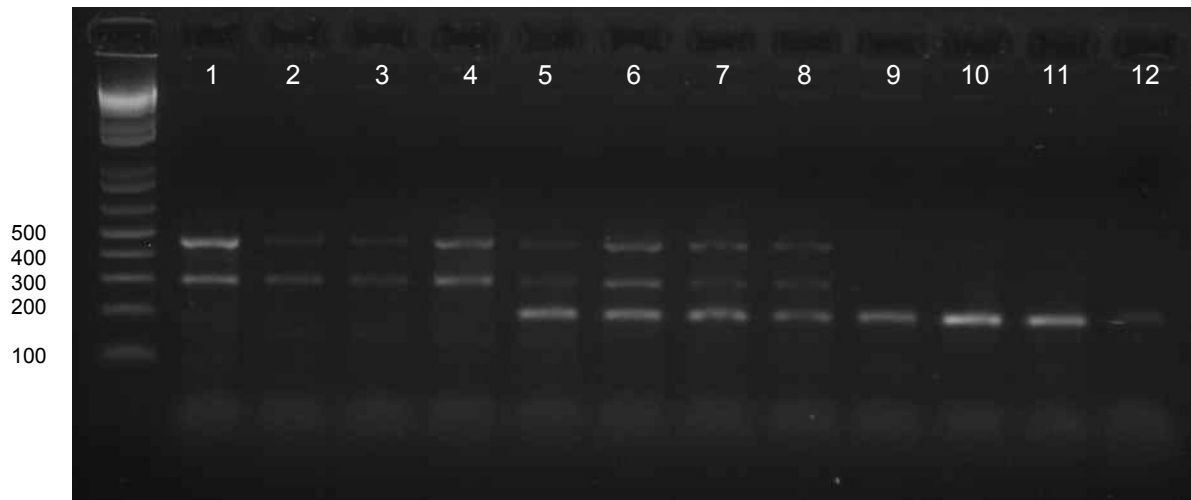
Figure 5.3.5.1: Primers create a 456bp universal band, 288bp for resistant individuals, and 196bp for susceptible individuals. Lanes 1-4, *An. gambiae* AKRON, lanes 5-8 heterozygous individuals, lanes 9-12, *An. gambiae* ASEMBO1

96 well sample preparation template

References

Weill M et al. (2004) The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. Insect Mol Biol 13:1-7

Wilkins EE, Howell PI, Benedict MQ (2006) IMP PCR primers detect single nucleotide polymorphisms for *Anopheles gambiae* species identification, *Mopti* and *Savanna* rDNA types, and resistance to dieldrin in *Anopheles arabiensis*. Malar J 5:125

5.4 Mechanical aspirator (MA) for transferring mosquitoes from surfaces treated with active ingredients.

John Morgan

Introduction

This mechanical aspirator (MA) was developed for easy to use and provide an alternative to the mouth aspirator when transferring mosquitoes from the treated surfaces of cone assays to paper cups. Operators are thus prevented from being exposed to inhalation of novel and known compounds being used in the trial. The operational methods are adapted from the WHO guidelines presented in WHO_CDS_NTD_WHOPES_GCDPP_2006.3_eng, p 13 – 15.

Construction
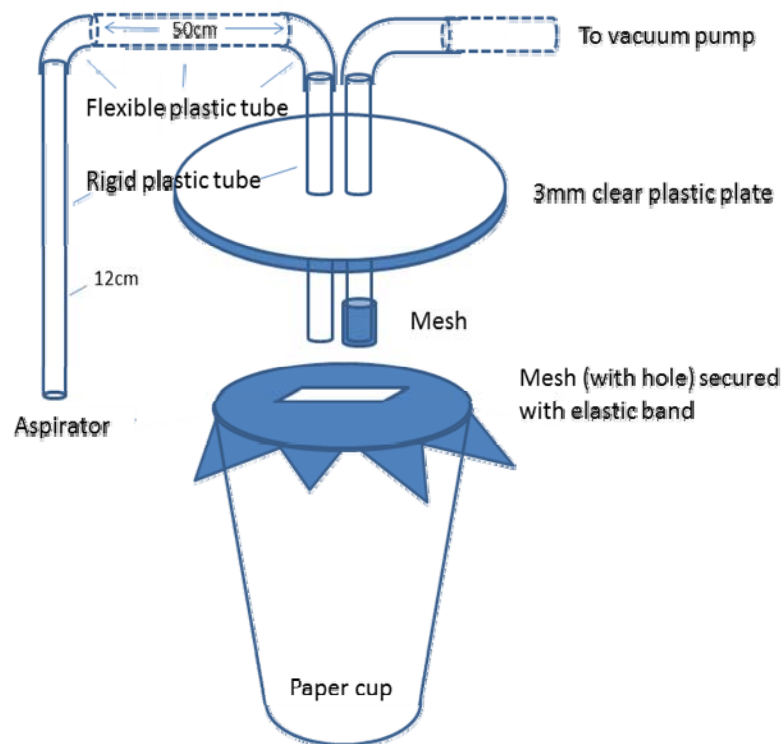
Not to scale

Operation

Two persons are required to operate the MA. With the vacuum pump in operation the first operator inserts the two tubes through the hole in the gauze and presses the plate firmly to the gauze stretched over the top of the paper cup. This creates a vacuum in the cup and draws air in through the aspirator tip. The second operator directs the tip of the aspirator to the mosquitoes being tested in the cone assay allowing the mosquitoes to be drawn up and into the cup. The gauze covering the entrance to the vacuum tube prevents the mosquitoes from being drawn in the pump. Once all the mosquitoes are in the cup the plate and tubes are carefully removed and a cotton wool plug inserted in the hole in the gauze to prevent escapes. The mosquitoes are provided with sugar solution and monitored for knockdown and mortality as explained in the WHO protocol.

Additional notes

Prior to using the MA for assays, tests should be conducted with different levels of the vacuum to establish a pressure sufficient to draw the mosquitoes into the cup without damaging the mosquitoes or causing mortality.

Problems of contamination of the aspirator between samples need to be carefully addressed. A control assay using mosquitoes on an untreated surface should be made every 10 assays. Each compound should have separate aspirators. At the end of the assay the cup and gauze is discarded and all tubing and plastic washed with Decon™ as recommended by WHO.

References

World Health Organization. 2006. Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets. Ed. M. Zaim. WHO/CDS/NTD/WHOPES/GCDPP/2006.3

Chapter 6 : Dissection Techniques

6.1 General Dissection Buffers

Marc Klowden

Background

When dissecting mosquitoes for examination or for studying the functions of their isolated tissues, a physiological saline should be used to prevent them from drying out and to maintain these tissues in a reasonably normal state. These solutions will ideally mimic the composition of hemolymph. However, because insect hemolymph can often vary considerably during the life cycle in response to feeding and reproduction, insect tissues generally show a wide tolerance to variations in composition, and the saline solutions that have been formulated are sufficiently generic to enable them to be used for most species.

Saline solutions suitable for dissection

(Hayes 1953)

Component	g/l
NaCl	9.0
$CaCl_2$	0.2
KCl	0.2
$NaHCO_3$	0.1

(Ephrussi and Beadle 1936)

Component	g/l
NaCl	7.5
KCl	0.35
$CaCl_2$	0.21

Saline solutions suitable for long-term tissue survival:

(Beyenbach and Masia 2002). Used for adult Malpighian tubules.

Component	g/l
NaCl	8.76
KCl	0.25
$CaCl_2$	0.19
$MgCl_2$	0.09
Hepes	5.96
Glucose	0.90
$NaHCO_3$	0.15

Adjust to pH 7 with 1M NaOH

(Onken et al. 2006). Used for larval midgut tissue.

Component	g/l
NaCl	2.48
KCl	0.22
$MgCl_2$	0.06
$CaCl_2$	0.55
$NaHCO_3$	0.42
Succinic acid	0.59
Malic acid	0.67
L-proline	0.58
L-glutamine	1.33
L-histidine	1.35
L-arginine	0.57
Glucose	1.80
Hepes	5.96

Adjust to pH 7 with 1M NaOH

References

Beyenbach KW, Masia R (2002) Membrane conductances of principal cells in Malpighian tubules of *Aedes aegypti*. J Insect Physiol 48:375-386

Ephrussi B, Beadle GW (1936) A technique of transplantation for *Drosophila*. Am. Nat. 70:218-225

Hayes RO (1953) Determination of a physiological saline solution for *Aedes aegypti (L.)*. J. Econ. Entomol. 46:624-627

Onken H, Moffett SB, Moffett DF (2006) The isolated anterior stomach of larval mosquitoes (*Aedes aegypti*): voltage-clamp measurements with a tubular epithelium. Comp Biochem Physiol A Mol Integr Physiol 143:24-34

6.2 Rapid Larval Midgut Extraction

Marco Neira, Dmitri Boudko, Leslie VanEkeris, Paul Linser

Introduction

Once mastered, this technique allows the researcher to quickly separate the midgut from the rest of the body, and is therefore suitable for procedures which require the pooling of large amounts of tissue, such as protein and/or RNA extraction.

Materials

- Dissection dish (a Petri dish that has its bottom coated with a fine layer of Sylgard® (silicone, Dow-Corning Corporation, Midland, Michigan)

- Stereoscope

- Fine forceps

- Microdissection scissors

- Dissecting needles or minutien pin mounted on a long wooden stick

- Clean microscope slides and cover slips

Protocol

1. Place larvae on ice for 10-15 minutes in order to immobilize them.

2. Transfer each larva to a dissection dish containing 70% ethanol.

3. Pin the head capsule to the bottom of the dish using a minutien pin (sometimes placing the larva ventral-side up can facilitate this task).

4. Using sharp microdissection scissors, clip-off abdominal segments 8-10.

5. Use the sides of two sets of forceps to carefully 'squeeze' the gut out of the larval body. Start by applying pressure at the head/thorax junction (**Figure 6.2.1 A**), and work your way across the rest of the thorax and the abdomen using a stepwise motion. The gut should progressively protrude out of the distal end of the abdomen (**Figure 6.2.1 B**).

6. After the gut has been extracted, it might need to be carefully cleaned of attached fat-body and/or large tracheal trunks. Clean guts should immediately be transferred to the appropriate reagents for protein or RNA isolation.
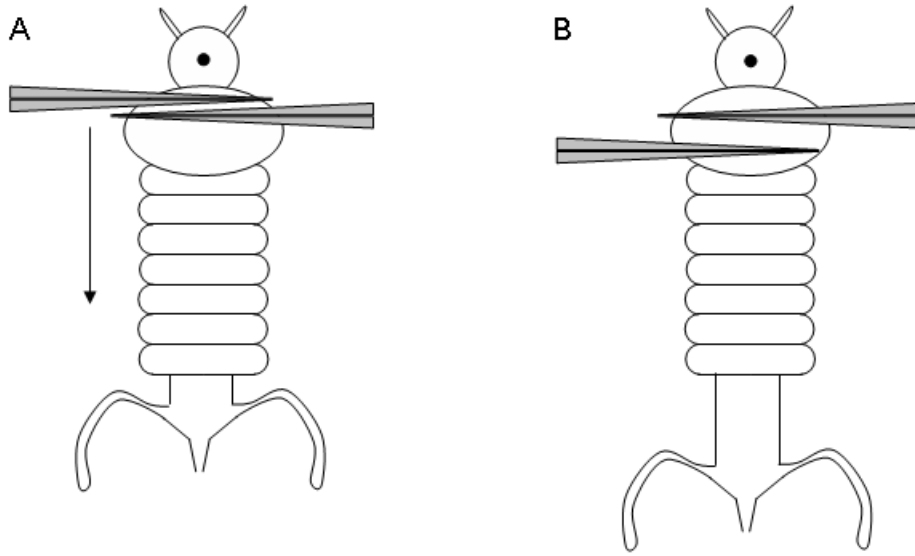
Figure 6.2.1. Larval mosquito midgut extraction.

6.3 Larval Midgut Vivisection

Marco Neira, Dmitri Boudko, Leslie VanEkeris, Paul Linser

Introduction

This technique is used for studies that require direct access to the internal organs of a live larva, such as electrophysiological measurements. It can also be used to perform whole-mount immunostaining of internal organs, in which case the specimen should be dissected in fixative solution instead of hemolymph substitute.

Materials

- Dissection dish (a Petri dish that has its bottom coated with a fine layer of Sylgard® (silicone, Dow-Corning Corporation, Midland, Michigan)

- Stereoscope

- Fine forceps

- Dissecting needles or minutien pin mounted on a wooden applicator stick

- Clean microscope slides and cover slips

Hemolymph substitute

42.5mM NaCl, 3mM KCl, 0.6mM $MgSO_4$, 5mM $CaCl_2$, 5mM $NaHCO_3$, 5mM succinic acid, 5mM malic acid, 5mM L-proline, 9.1mM L-glutamine, 8.7mM L-histidine, 3.3mM L-arginine, 10mM dextrose, 25mM Hepes, pH 7.0

Fixative Solution

To make fixative solution for whole-mounts, mix equal volumes of 4% paraformaldehyde in 0.1M sodium cacodylate buffer

Variant 1:

1. Place larvae on ice for 10-15 minutes to immobilize them.

2. Transfer each larva to a dissection dish containing Ringer's solution, hemolymph substitute, or another isotonic medium.

3. Place the larva dorsal-side up, and pin the head capsule to the bottom of the dish using a minutien pin.

4. Using sharp microdissection scissors, clip-off abdominal segments 8-10.

5. Using fine forceps, carefully grab the integument of seventh abdominal segment and pull it just enough to straighten the larva's body (**Figure 6.3.1 A**).

6. While keeping the body straight, use microdissection scissors to cut across the lateral side of the abdomen and thorax, and then across the dorsal side of the thorax, as shown by the dashed arrows in **Figure 6.3.1 A**.

7. Move the dorsal integument aside to expose the internal organs. Use minutien pins to secure the exoskeleton to the bottom of the dish.

Variant 2:

1. Place larvae on ice for 10-15 minutes in order to immobilize them.

2. Transfer each larva to a dissection dish containing Ringer's solution, hemolymph substitute, or another isotonic medium.

3. Place the larva dorsal-side up, and pin the head capsule and the last abdominal segment to the bottom of the dish using minutien pins.

4. Insert microdissection scissors at the head/thorax junction and cut the integument following the mid-dorsal line of the body (between the two main dorsal tracheal trunks). Then, cut the integument across the dorsal side of the thorax as shown by the dashed arrows in **Figure 6.3.1 B**.

5. Using fine forceps, grab the dorsal integument and pull it gently towards the sides (**Figure 6.3.1 B**), exposing the internal organs. Use minutien pins to secure the exoskeleton to the bottom of the dish. **Figure 6.3.2** shows the final result of this kind of dissection.
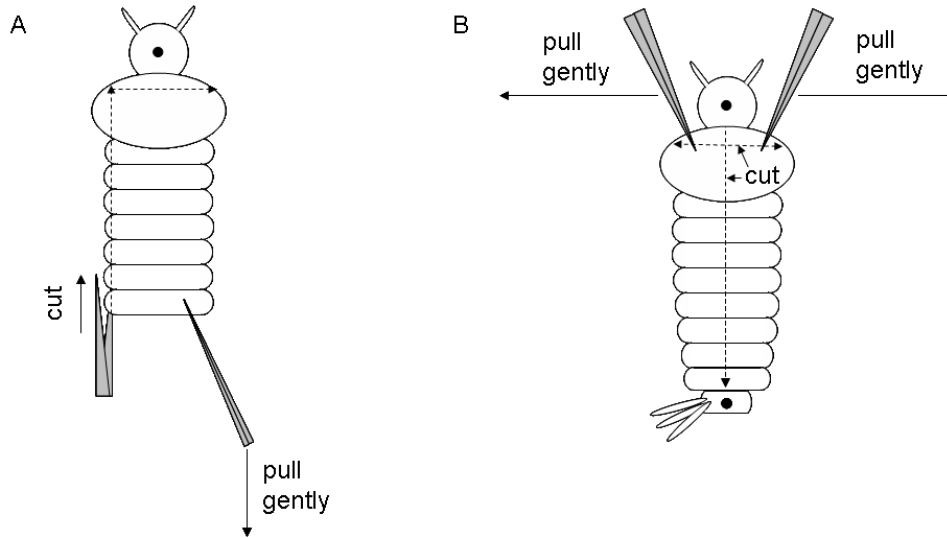
Figure 6.3.1. Larval mosquito vivisection. **A**) variant 1; **B**) variant 2
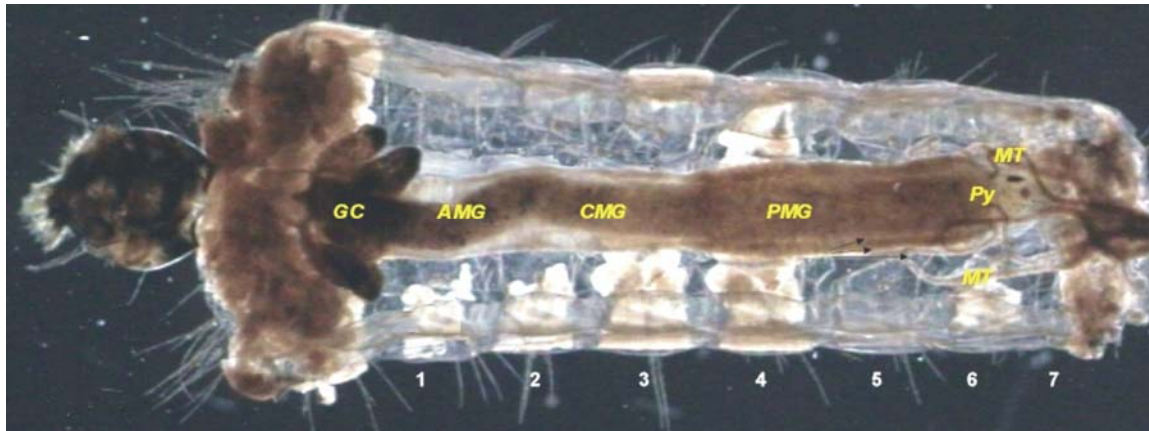
Figure 6.3.2. Final result of larval vivisection. AMG: anterior midgut; CMG: central midgut; GC: gastric caeca; MT: Malpighian tubes; PMG: posterior midgut; Py: Pylorus. Numbers indicate abdominal segments. Figure modified from (Boudko et al. 2001). Used with permission.

References

Boudko DY, Moroz LL, Linser PJ, Trimarchi JR, Smith PJ, Harvey WR (2001) In situ analysis of pH gradients in mosquito larvae using non-invasive, self-referencing, pH-sensitive microelectrodes. J Exp Biol 204:691-699

6.4 Adult Male Testes Dissection

MR4 Staff

Introduction

Male reproductive organs can provide valuable data about population age and mating history (Mahmood and Reisen 1982; Huho et al. 2006). Testes size has been associated with male age in *An. stephensi* in which it was found that longer testes were seen in sexually mature individuals while shorter ones were found in sexually immature or older mosquitoes (Mahmood and Reisen 1982). Additional studies in *An. gambiae* have found that differences in the appearance of the male accessory glands (MAG) were related to the age of the mosquito (Huho et al. 2006). Mosquitoes that were greater than 4 days old typically had no visible clear area in their MAG and large sperm reservoirs while mosquitoes that were younger than 4 had a small, transparent area on the edge of their MAG and small sperm reservoirs. Adult male testes are also a good source of metaphase chromosomes (French et al. 1962). The dissection technique described below should successfully remove all the reproductive organs.

Materials

- Stereoscope
- Compound microscope
- Fine forceps
- Dissecting needles or minutien pin mounted on a long wooden stick
- Clean microscope slides and cover slips
- PBS solution (0.01M, pH 7.2)

Protocol

1. Aspirate males into a container and anesthetize by gently chilling them at -20ºC for 5-7 minutes.

2. Place a drop of PBS on a clean slide

3. Under the stereoscope, gently grasp the male by the thorax with a pair of forceps and place ventral side up with the abdomen resting in the PBS.

4. Take a fine tip needle or forceps and gently remove the claspers of the male by piercing them and gently pulling them away while holding the thorax with forceps (**Figure 6.4.1**).

5. Using the dissecting needles, gently remove the extraneous tissues isolating the male reproductive organs then cover with a clean cover slip.

6. Examine both the testes and MAG to determine their dimensions and presence or absence of a clear zone (**Figures 6.4.2-9**).

References

French WL, Baker RH, Kitzmiller JB (1962) Preparation of mosquito chromosomes. Mosq News L1 - 2927pdf 22:337-383

Huho BJ, Ng'habi KR, Killeen GF, Nkwengulila G, Knols BG, Ferguson HM (2006) A reliable morphological method to assess the age of male *Anopheles gambiae*. Malar J 5:62

Mahmood F, Reisen WK (1982) *Anopheles stephensi* (Diptera: Culicidae): changes in male mating competence and reproductive system morphology associated with aging and mating. J Med Entomol 19:573-588
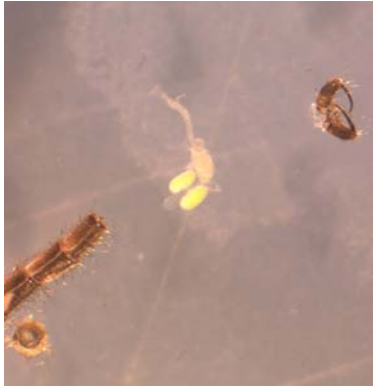
Figure 6.4.1. Dissected male *Anopheles gambiae* mosquito. The MAGs are visible as the yellow sacs near the terminalia.
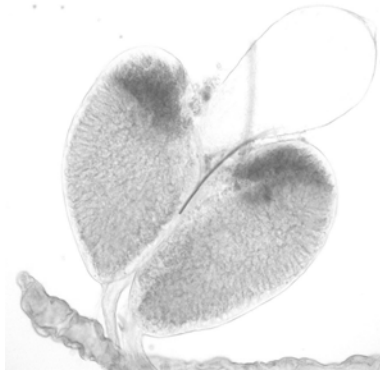
Figure 6.4.2. MAG of a mosquito that is <4 days old. Nnote the clear zone on the edge of the MAG.

Figure 6.4.3. MAG of a mosquito that is > 4 days old. Note the absence of a clear zone.
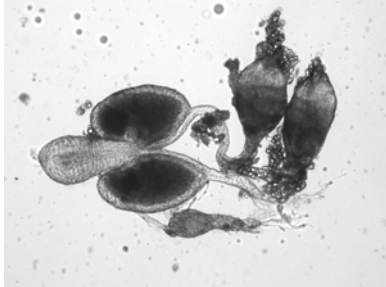
Figure 6.4.4. MAG and testes of a 1 day old *An. gambiae* mosquito.
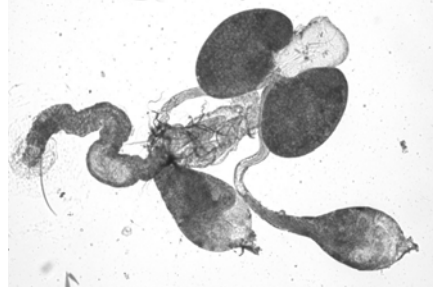
Figure 6.4.5. MAG and testes of a 5 day old *An. gambiae* mosquito.

Figure 6.4.6. MAG and testes of a 7 day old *An. gambiae* mosquito.
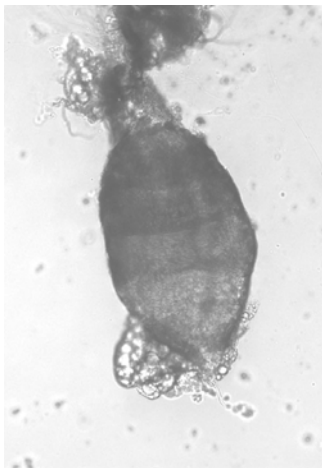
Figure 6.4.7. Testis of a 1 day old *An. gambiae* mosquito.

Figure 6.4.8. Testis of a 5 day old *An. gambiae* mosquito.
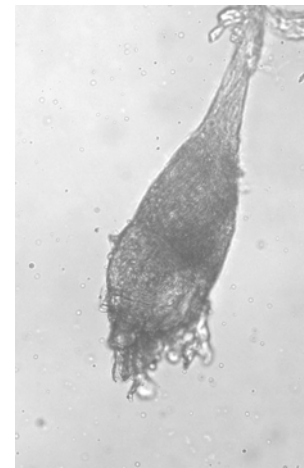
Figure 6.4.9. Testis of a 7 day old *An. gambiae* mosquito.

6.5 Dissecting Plasmodium-*Infected Mosquitoes*

6.5.1 Midgut

MR4 Staff

Introduction

Experimental *Anopheles* infections may be performed to determine the length of sporogony for various *Plasmodium* species or to establish susceptibility or refractoriness to *Plasmodium* infections. Dissection of the midgut to observe oocysts should be performed 5-7 days after infection occurs. The precise number of days that is best will vary with parasite species. Some examples of variability are shown in **Table 6.5.1.1** (WHO 1975). An alternate method by Looker and Taylor-Robinson can be found in Methods in Malaria Research (Looker and Taylor-Robinson 2004).

Species	Days to oocysts
P. vivax	4-7
P. ovale	3-4
P. falciparum	3-7
P. malariae	3-11

Table 6.5.1.1. Adapted from WHO Manual on Practical Entomology, 1975.

Materials

Stereoscope

Dissecting needles

Microscope

Forceps

PBS or saline solution

Microscope slides

Mercurochrome

Cover slips

Chloroform

Detecting the presence of *Plasmodium* oocysts in the midgut:

1. Anesthetize the females (see section on Mosquito Anesthesia).

2. Place a drop of PBS on a clean microscope slide.

3. Place a female in the drop of PBS ventral side up with the terminalia in the center of the drop.

4. Grasp the female by the thorax using forceps.

5. Lay a dissecting needle across the 7th abdominal segment (without slicing the abdomen) and gently detach the terminalia.

6. While still grasping the thorax with forceps, gently pull the terminalia away from the abdomen using the dissecting needle. Pull very slowly to ensure that the midgut does not detach from the terminal end (**Figure 6.5.1.1**).

7. If the midgut detaches, gently cut the edges of the 1st abdominal segment and gently pull the cuticle over the contents.

8. Remove the Malpighian tubes as well as any other accessory tissue and debris leaving only the midgut.

9. On a new microscope slide, place a small drop of mercurochrome.

10. Place the dissected midgut in the stain and cover with a clean cover slip.

11. Visualize under a compound microscope. Depending on the model, you may have to use phase contrast in order to identify the oocysts.

What you will see:

In most mosquitoes you will find varying numbers of encapsulated and unencapsulated (normal) oocysts. Encapsulated oocysts appear as disk-shaped structures in the midgut (small melanized particles) (**Figure 6.5.1.2, right panel**). Normal oocysts appear as large spherical objects, usually slightly lighter in coloration and attached to the apical surface (**Figure 6.5.1.2, left panel**). There are certain strains of mosquitoes that have been selected to highly express an immune response to *Plasmodium* infection (L3-5, *An. gambiae*) or to be completely susceptible to infection (4ARR, *An. gambiae*).

Figure 6.5.1.1. Dissection of midgut (shown inside circle). Cut away other portions to avoid confusion under the microscope.
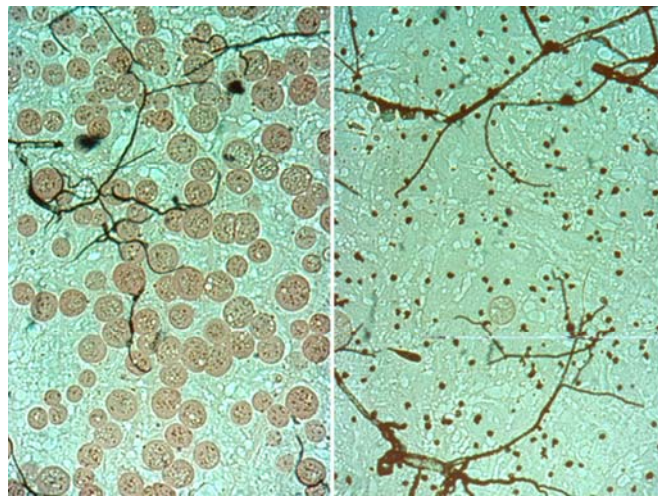
Figure 6.5.1.2. Left panel shows wild-type or normal unencapsulated oocysts. Right panel shows all encapsulated oocycsts with the exception of one normal oocyst toward the center (Collins et al. 1986).

References

Collins FH et al. (1986) Genetic selection of a Plasmodium-refractory strain of the malaria vector *Anopheles gambiae*. Science 234:607-610

Looker M, Taylor-Robinson AW (2004) Methods in Malaria Research, 4th edn. MR4/ATCC, Manassas, VA

WHO (1975) Manual on practical entomology in malaria. Part II. In. World Health Organization, Geneva, p 191pp.

6.5.2 Salivary Glands

MR4 Staff

Introduction

Dissection of the salivary glands should be done 10-18 days post-infection. The precise number of days that is best will vary with species. Some examples of variability are shown in **Table 6.5.2.1** (WHO 1975). An alternate method by Looker and Taylor-Robinson can be found in Methods in Malaria Research (Looker and Taylor-Robinson 2004).

Species	Days to sporozoites
P. vivax	8-10
P. ovale	10-15
P. falciparum	10-18
P. malariae	15-21

Table 6.5.2.1. Adapted from WHO Manual on Practical Entomology, 1975.

Materials

- Stereoscope
- Dissecting needles
- Microscope
- Forceps
- PBS or saline solution
- Microscope slides
- Giemsa stain
- Cover slips
- Chloroform, ethyl ether or ethylacetate

Protocol

Detecting the presence of sporozoites in the salivary glands.

1. Anesthetize adult females (see 3.8 Mosquito Anesthesia).
2. Place a drop of PBS on a clean microscope slide.
3. Place a female on her side with her thorax in the PBS solution.
4. While firmly holding the female with forceps or a needle, gently lay another needle across the neck near the head and slowly pull the head away (**Figure 6.5.2.1**).
5. Gently detach the salivary glands from the head.
6. If the salivary glands remain within the thorax, using the needle, apply gentle pressure to the thorax towards the mesonatum end and squeeze the glands out.
7. At this stage you can either process the slides for staining or perform a brief inspection to determine if sporozoites are present.

Brief inspection

Cover the dissected salivary glands with a cover slip and inspect the glands under a microscope for the presence of the threadlike sporozoites. Gentle pressure on the glands will help rupture the tissues freeing the sporozoites into solution.

Staining

Let glands fully dry on the slide. Once dried, fix with methanol for 1 minute, rinse with water, then stain with Giemsa stain for 40 minutes. Rinse again and air dry before inspecting under a microscope.

What you will see

In uninfected salivary glands, you will see a tissue layer with no visible threadlike sporozoites (**Figure 6.5.2.2**). In infected mosquitoes, you will see the threadlike parasites both in the tissues or, if the gland is ruptured, in the field surrounding the tissues (**Figure 6.5.2.3**). Giemsa staining helps estimate low parasite loads by increasing resolution of the sporozoites within the glands.

Figure 6.5.2.1. Dissection of salivary glands.
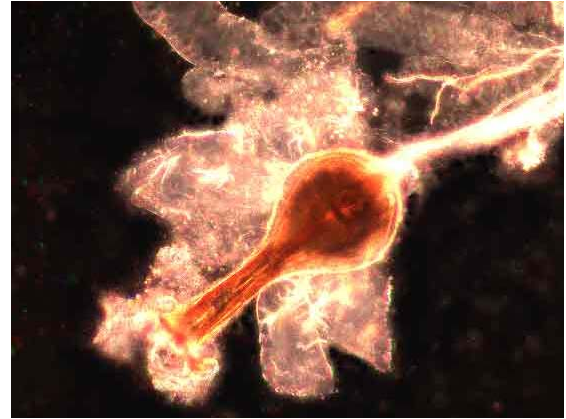
Figure 6.5.2.2. Stained, uninfected salivary glands.
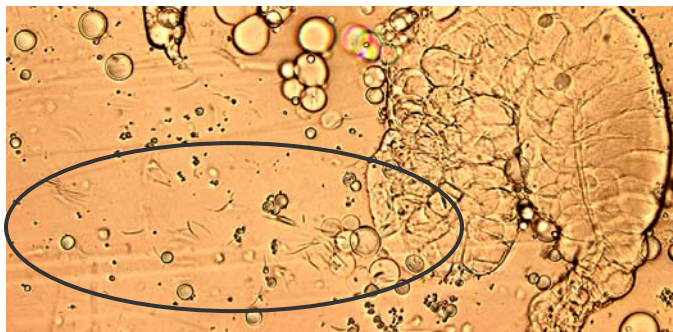
Figure 6.5.2.3. Stained, infected salivary glands. Note the thin, threadlike sporozoites (circled) of *P. vivax* spilling from the salivary gland.

References

Looker M, Taylor-Robinson AW (2004) Methods in Malaria Research, 4th edn. MR4/ATCC, Manassas, VA

WHO (1975) Manual on practical entomology in malaria. Part II. In. World Health Organization, Geneva, p 191pp.

6.6 Examination of Ovaries by Tracheal Distension to Determine Parity

Francis Atieli

Introduction

Parity is used to determine the age structure of a feral population, and it can also be used to ascertain the net reproductivity of a colony (Githeko et al. 1993). Parous mosquitoes are those that have taken a blood meal and oviposited at least once. Nulliparous mosquitoes have never oviposited. Net reproduction rate of a colony can be determined by dissecting several females to determine the number of parous individuals within the population.

A more advanced technique was developed by Detinova (1962) that assesses how many egg batches have been developed by an individual female (reviewed by Hoc and Wilkes 1995). In this method, as embryos develop within the ovaries they stretch the ovariole sheath. After oviposition, these sacs which contain remnants from oogenesis shrink and develop into permanent dilatations. After each subsequent feeding, a new embryo will form anterior to the previous dilatation. A mosquito with three dilatations would be labeled "3 parous". Caution should be taken when examining for dilatations as resorbed embryos will also form dilatations. Likewise in some species such as *An. atroparvus*, dilatations may not be formed at all (Service 1993).

The simplest technique for determining parity is to examine the tracheoles within the ovaries (Kardos and Bellamy 1961). As the ovaries expand after the primary blood meal, the tracheoles that are associated with them are permanently distended (Hoc and Charlwood 1990). Therefore, in nulliparous females the tracheoles are tightly wound coils called 'skeins.' Parous females will have tracheoles that have distended. Note that in younger females there may be a mixture of both skeins and 'extended' tracheoles; if any distended tracheoles are present, you can assume that the female is parous and has fed and laid eggs at least one time.

Materials

- Microscope slides
- PBS or another physiological dissecting solution
- Forceps
- Dissecting needles
- Stereoscope
- Compound Microscope (200 X magnification)

Technique

1. Gently anesthetize adult females.
2. Place a drop of PBS on a clean microscope slide.
3. Under the stereoscope, gently grasp female by the thorax with forceps, and place ventral side up with her abdomen in the PBS.
4. While viewing the specimen under the stereoscope, take a fine tip needle or forceps and gently remove the 7th and 8th abdominal segments of the female by grasping them and pulling away slowly.
5. Locate the ovaries; they will appear as a pair of white oval objects attached to the removed segments (**Figure 6.6.1**). Dissect away the accessory tissues and isolate the ovaries.
6. Transfer ovaries to a new slide and allow to air dry.

7. Multiple pairs of ovaries can be mounted on a single microscope slide, but ensure that all the samples have dried before viewing them under a microscope.

8. View under a compound scope at 200 X magnification. Locate the tracheoles and determine if the specimen is nulliparous (**Figure 6.6.2**) or parous (**Figures 6.6.3** and **6.6.4**).

Figure 6.6.1. Dissection of female terminalia under stereoscope. The ovaries are circled. Terminalia and segments VII and VIII are visible in the upper right hand corner.

Figure 6.6.2. An example of a tightly coiled tracheole called a "skein" is shown inside the circle. This is a nulliparous female.
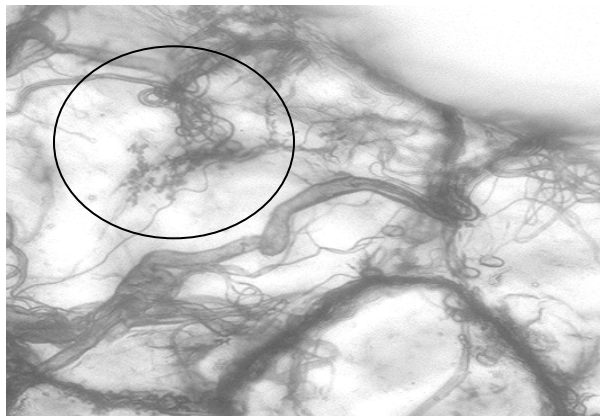
Figure 6.6.3. Inside the circle is the loose structure of the tracheoles after oogenesis. This is referred to as the extended state. This female is parous.
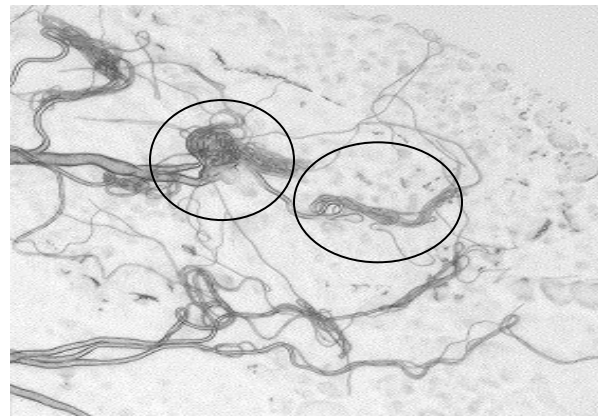
Figure 6.6.4. Both types of tracheoles are seen in this photograph. The circle on the left side shows a tightly coiled skein while the circle on the right shows an extended tracheole. Even though both are present in this example, this female would be considered parous.

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6.7 Dissecting Spermathecae to Determine Insemination Status

MR4 Staff

Introduction

In order to monitor colony mating activity and to assess wild-mosquito mating status, it is often necessary to determine whether females are inseminated. The least complicated method to determine this is to dissect spermathecae and determine whether spermatozoa are present. The spermatheca is located near the terminalia inside segment VIII and can often be seen through the cuticle while viewing the ventral side. It is a spherical, fenestrated dark-brown organ which has a golf-ball-like appearance. After gently removing the terminalia and segment IX, the spermathecae will be exposed along with the malpighian tubules, ovaries, and digestive tract. Then the spermathecae can be easily removed and viewed under magnification, and insemination status is usually readily apparent.

Materials:

- Fine forceps
- Dissecting needles or minutien pin mounted on a long wooden stick
- Clean microscope slides and cover slips
- PBS solution
- Dropper

Equipment:

- Stereoscope
- Compound microscope with dark field capacity

Procedure:

1. Anesthetize or kill the adult females.

2. Place a drop of PBS on a clean slide.

3. Gently grasp the female by the thorax with a pair of forceps and place ventral side up with the abdomen resting in the PBS under the stereoscope.

4. Gently remove the terminalia of the female by grasping them and pulling away slowly using a fine tip needle or forceps.

5. Locate the spermatheca within the 8th segment and terminalia section removed previously. It should appear as a dark sphere that may or may not be surrounded by accessory tissues (**Figure 6.7.1**). Dispose of the mosquito carcass and remaining tissues.

6. Gently lower a cover slip onto the spermatheca using a needle (to avoid rupturing the spermathecae). On the underside of the slide, circle the area surrounding the spermathecae using a permanent marker. The marking will make it much simpler to locate the tiny organ. With experience, you will determine how much PBS to use to prevent rupturing the spermatheca.

7. Under 100X magnification on a compound microscope, look for movement of the long thread-like spermatozoa within the spermathecae (**Figure 6.7.2**). They will appear as fine concentric threads within the spermathecae and are often seen rotating as a cluster. If the spermatheca ruptured after placing the cover slip, scan the surrounding field for the spermatozoa. Uninseminated females will have a fairly transparent spermatheca (**Figure 6.7.3**).
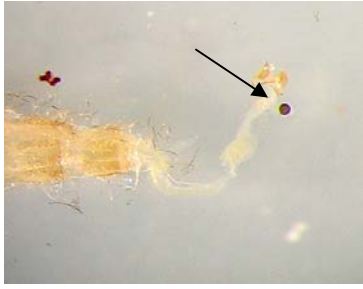
Figure 6.7.1. Golf ball shaped spermatheca.

Figure 6.7.2. 100X magnification of a full spermatheca. The bright ring is the bundle of sperm.
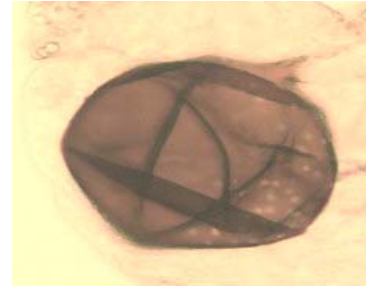
Figure 6.7.3. Uninseminated female spermatheca.

6.8 A. gambiae s.l. *Ovarian Polytene Chromosome Preparation*

Anthony Cornel[1]

Introduction

The highest quality *Anopheles gambiae* s.l. polytene chromosomes are prepared from nurse cells in eggs at Christophers' III stage (Clements 1992) of ovarian development. Chromosomes of adequate polytenization also occur in salivary gland cells of late fourth stage larvae which are useful in larval ecological studies. *Anopheles gambiae* s.l. has a diploid chromosome complement of 2n = 6 with one pair of heteromorphic X and Y sex chromosomes and two pairs of autosomes numbered as 2 and 3 (see section on Anopheles Mendelian Genetics).

A typical polytene chromosome squash will produce a spread of arm configurations known as X, 2R, 2L, 3R and 3L (**Figure 6.8.1**). The entire Y and portions of the X cannot be visualized because they are heterochromatic and under-replicated. Drawings depicting the banding patterns of *An. gambiae* s.l. polytene chromosomes are available to identify the divisions and subdivisions and locations of paracentric inversions (Coluzzi et al. 2002; Holt et al. 2002).

Wherever possible, multiple methods have been described from which you can choose depending on laboratory equipment and the needs and quality of chromosome preparation required. The most crucial step to obtain excellent chromosomes is to recognize the appropriate half gravid state of the female and late 4th stage larvae from which to dissect. Thereafter, the most crucial steps are the tapping procedures to spread the chromosomes into recognizable arms. It will take a little while and practice to achieve the appropriate skills, and I suggest you spend a few days learning these skills with someone who is both familiar with chromosome preparation techniques and *An. gambiae* s.l. chromosome banding patterns.

Solutions

- Anesthetic (ethyl ether, triethylamine, CO_2, -20°C freezer space)
- Modified Carnoy's fixative (three parts pure (100%) ethanol and one part glacial acetic acid). Ethanol absorbs water from the atmosphere so ensure that the ethanol is pure, as water in fixative compromises quality of spreads.
- Sigmacote® - (Sigma-Aldrich – St. Louis MO, USA)
- Propionic acid - prepare 5% and 50% in water.
- 2% lacto-aceto orcein - prepare this solution by adding slowly 2% by weight of synthetic orcein powder to a solution of 1 part glacial acetic acid and 1 part pure lactic acid under constant stirring (use a magnetic stirrer). Remove un-dissolved orcein particulates by filtering the solution through Whatman 3MM paper. The solution can be stored indefinitely at room temperature.
- Ethanol – 70%, 90% and 100% in water.

Materials

- Anesthetizing mosquito chamber: 45 ml Falcon® tubes, razor blade, 925 mµ mesh or smaller screening, glue, rubber bands, dental rubber latex sheeting (Super Dam- Patterson Brand), regular household sponge, desiccator.
- 4°C refrigerator
- -20°C freezer

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- 0.13- 0.17 mm thick cover glasses of dimensions 22L X 22W mm

- Frosted 1mm thick microscope slides 75 x 25 mm in size.

- Beaker

- Aluminum foil

- Forceps

- Pasteur pipettes

- Absorbent paper (Whatman 3MM paper)

- Dissecting needles and minutien pins

- Dissecting Microscope

- 1.5 ml screw cap polypropylene vials

- Phase contrast compound microscope with 10X, 40 or 60 X and 100X objective lenses.

- Digital imaging system
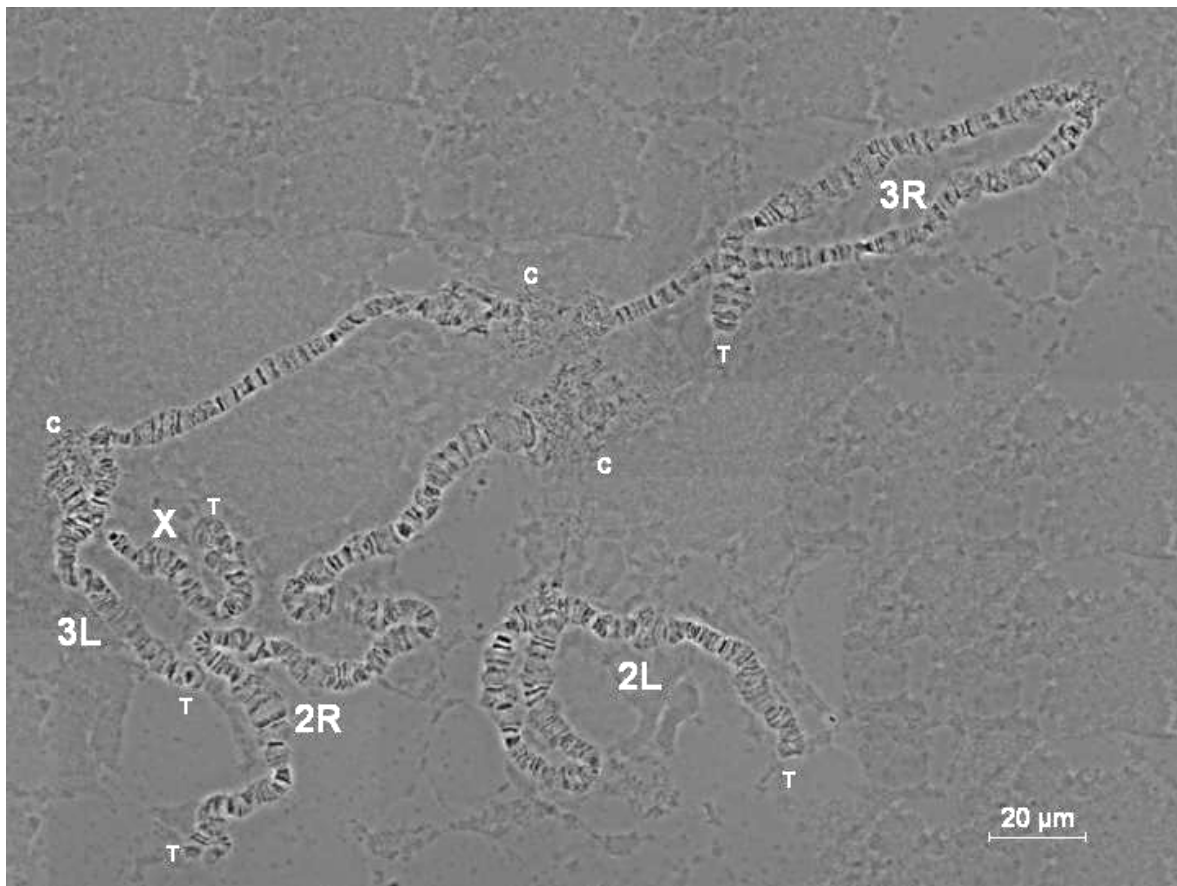
Figure 6.8.1. Chromosome squash showing the five reasonably well spread chromosome arms X, R, L, 3R and 3L of *An. gambiae sensu stricto*. Magnification X 600 under phase contrast. T = Telomere; C = Centromere. Specimen from Eau et Foret, Cameroon 2006.
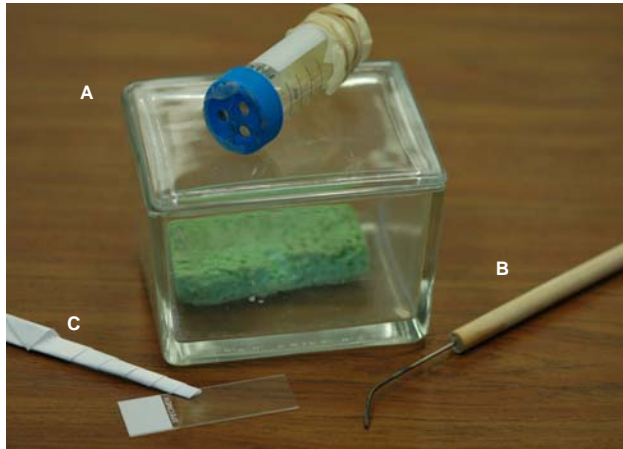
Figure 6.8.2. Selection of items useful for preparing *An.gambaie* s.l. polytene chromosomes. A - Anesthetizing chamber; B - mattress needle with end curved downwards for tapping; C – rolled up absorbent paper for drawing up small volumes of fluids on microscope slides.

Procedure:

1. Siliconized cover-glasses are not crucial for temporary squashes but they are if cover-glasses need to be removed for preparation of permanent mounts and *in situ* hybridization. Prepare siliconized cover-glasses by fully immersing them indiviually in Sigmacote® in a beaker or other container made of glass (repel silane solutions from other companies can be used as well). Place the beaker in a fume hood, and cover tightly with foil. Use gloves when handling Sigmacote®. Leave overnight. The next day, lift each coverslip by its corner with forceps and dip into a beaker of water and then 100% ethanol to clean. Place each cover-glass on a lint free surface to dry, and thereafter store them in a box until required.

2. Blood feed adult females between the hours of 8 to 10:00 am. Remove the fully blood fed mosquitoes, and hold them in a separate cup or cage in the insectary and supply with 10% sucrose solution. Quarter pint ice cream carton cups with mosquito-proof netting on the top are ideal. When an insectary is not available or if you are in the field collecting blood fed females resting inside dwellings, holds the mosquitoes in cups inside or in the shade of a tree and keep humid by covering with a moist cloth.

3. Place 1 ml of modified Carnoy's solution into each 1.5 ml polypropylene vial. Ovaries from one mosquito will be placed into each vial. Anticipate the number of mosquitoes to be dissected to ensure sufficient numbers of vials are prepared in advance. Place the vials at 4°C.

4. Select half gravid females 18-33 hours after blood feeding. Ovaries will develop to the half gravid stage at different rates depending on the mosquito strain and the temperature and humidity at which they are held. Generally at 26°C ovaries take about 20 hours and at 30°C ovaries take 16- 18 hours to reach the half gravid stage. Half gravid females (ovaries at Christopher III stage) will appear similar to the image in **Figure 6.8.3.** When the ovaries take up less than 3/5 of the abdomen they are generally too young. When the ovaries extend beyond 3/5 of the abdomen and the developing eggs have a greenish tinge under sunlight they are too old for cropping. Identifying half gravid females with ovaries at the appropriate age for harvesting chromosomes will take some practice, and you should err on the side of harvesting a little young at Christopher's II stage if you are unsure. Polytenization begins in Christopher's II stage and the chromosomes at this stage will be thin, brittle and have less well defined bands than at Christopher's III stage but can be scored by an experienced person after light tapping.

5. Anesthetize the mosquitoes. This can be done in various ways depending on availability of equipment and chemicals (see section on Mosquito Anesthesia). The least invasive and easiest is to aspirate half gravid females into a container and hold at -20°C for five minutes to knock the mosquitoes down. Do not hold them for longer to kill them. Removal of ovaries is best done when the mosquitoes are still alive as chromosomes degenerate very quickly after death. Place the mosquitoes back into the freezer for a few minutes if they recover before dissecting. If no freezer is available then anesthetize the mosquitoes with ethyl ether, triethylamine or CO_2 using an anesthetizing chamber (**Figure 6.8.2**). Make the chamber by cutting the end of a 45 ml Falcon® tube and covering the cut end with two sheets of rubber latex sheeting with slits just large enough to insert the end of an aspirator blowing mosquitoes into the chamber. Drill holes into the cap of the Falcon® tube and cover the holes with

mosquito proof mesh so the mosquitoes do not escape but vapor can pass through. Place the Falcon® tube chamber, with mosquitoes in, into a dessicator saturated with CO_2 gas or a sponge soaked with the anesthetizing chemical (ethyl ether or triethylamine). Wait until the mosquitoes are knocked down before dissection.

Figure 6.8.3. Examples of adult females at various stages of ovarian development. A – too young, B at correct age and C too old. OV indicates the developing ovaries while BL indicates undigested blood meal. Photographs taken by Marshall Johnson.

6. In conditions where no anesthetizing facilities are available, such as in the field, knock out the mosquitoes by shaking the cup or container they are in from side to side for about 30 seconds. The mosquitoes will be stunned by hitting against the sides, but it takes some experience to judge how vigorously to shake the container without damaging the mosquitoes.

7. Removing ovaries can be done with or without a dissecting microscope depending on your eyesight and dexterity. Without a microscope - which is by far the quickest way - gently hold the head, thorax and base of abdomen of the mosquito between the tips of your thumb and index finger. Grab the last two segments of the abdomen with forceps. Gently squeeze the base of the abdomen and simultaneously pull on the last two abdominal segments with the forceps to extract the ovaries. In this way, only the ovaries will pull out. Immediately place the ovaries into the vial containing modified Carnoy's solution. Do not let the ovaries dry out after dissecting. After each dissection place a label (write label with a pencil, not a pen) with ovaries into the vial. The remaining head and thorax can be discarded or preserved in a separate vial for other purposes such as for DNA extraction. If preserved, label the remaining carcass with an accession number corresponding to that of the ovary.

8. To remove ovaries under a microscope, pierce the side of the mosquito with a minutien pin and on a clean microscope slide pull the last two apical abdominal segments with a forceps or needle until the ovaries have extruded. Immediately place the ovaries into modified Carnoy's.

9. Dissected ovaries fixed and preserved in modified Carnoy's for more than 24 hours typically provide the best preserved and clean chromosome spreads, especially if required for *in situ* hybridization. Ovaries preserved in modified Carnoy's can be stored for many months at 4ºC or for years at -20ºC.

10. Placing the whole abdomen in modified Carnoy's also produces readable spreads but the chromosomes can have a washed out appearance after longer storage due to presence of more water in the whole abdomen versus ovaries alone. It is not recommended to fix the entire adult in Modified Carnoy's solution as then too much water enters the preservation solution.

11. To make the chromosome squashes, remove the ovaries from the vials with a pair of forceps and place them into a drop of modified Carnoy's (approximately 25 µl) on a dust- and grease-free microscope slide. Quickly separate half the ovules or follicles from one ovary, and return the rest of the ovaries to the vial to use as back-up for later preparations. You have to do this quickly before the modified Carnoy's solution dries out on the slide as no spreads can be recovered from dry ovules.
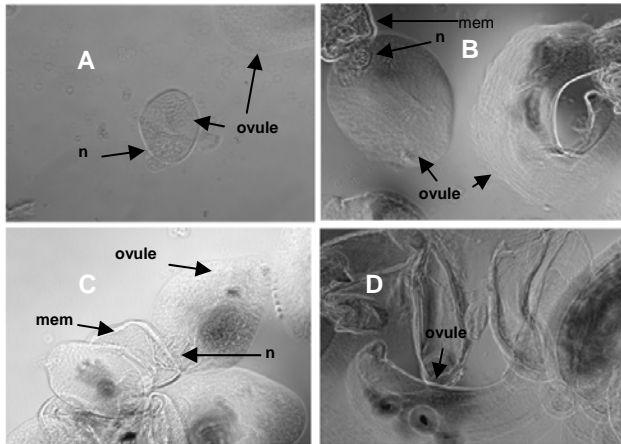
Figure 6.8.4. Examples of ovules after 5 minutes in 5% propionic acid that were dissected at various stages of development. A – ovule at early Christopher's II stage which is too young for harvesting, Note for comparison the edge of an ovule of appropriate age and size in the top right hand corner; B and C – ovules at Christopher's III stage of development which are at the correct age and size for producing good chromosome squashes. In image B the ovule to the right is too old. In both B and C the ovules have popped out of their surrounding membranes (mem) and the nuclei of nurse cells are enlarged (n); C- ovule too old. All images taken at 100 X mag. under phase contrast.

12. Drop about 50 µl of 50% propionic acid onto the ovules and leave them for about 3 minutes until they have cleared and swollen to about twice their original size. Consult **Figure 6.8.4** for further guidance to visualize appropriately aged ovules. Once again do not let the ovules dry out.

13. Under a dissecting microscope carefully separate the ovules from each other and gently squeeze each ovule (follicle) out of its surrounding membrane. Remove connecting tissues, trachea and membranes by sliding them away from the ovules and draw up the waste tissues with the tip of tightly rolled up absorbent paper.

14. From my experience, staining the chromosomes is unnecessary particularly in these days of improved microscope optics. Staining with 2% lacto-aceto orcein is optional and this step should be done after the ovules have been separated and cleaned (after completion of step 13). Do this by adding a drop of the stain to the ovules in the 50% propionic acid. Create an even-staining mixture around the ovules by stirring gently with a needle. Leave this for about 4 minutes for the ovule cells to absorb the stain. Gently absorb the excess stain with a piece of tightly rolled up absorbent paper and add a drop of 50% propionic acid to the ovules which by now should have a pale pink hue to them. Proceed to step 15.

15. Place a further drop of 25µl of 50% propionic acid onto the clean ovules.

16. Wipe a cover-glass with a clean sheet of lint free paper – lens cleaning tissue or KimWipe® (Kimberley-Clark- Roswell GA) and place on top of the ovules. Ensure the cover-glass is dust and grease free.

17. Carefully set the microscope slide on a piece of absorbent paper on a flat surface.

18. Various people use different tapping techniques to make chromosome squashes, but essentially all begin by tapping quite firmly to break open the nuclear membranes and spreading out the chromosomes followed by harder tapping or pressing to flatten the chromosomes. I prefer to first gently tap the cover-glass with a mattress needle about 8 to 10 times (end bent downwards as depicted in **Figure 6.8.2**). While tapping with the needle the cover-glass will shift around a little which helps to shear the nuclear membranes and spread the chromosome arms out. View the slide at 100X magnification under phase contrast in a compound microscope to determine if more tapping to spread chromosomes is required.

19. Once the first round of tapping has been completed, remove excess propionic acid by gently pressing absorbent paper over the cover-glass edges. Place a new dry piece of absorbent paper over the cover-glass, and press firmly downwards with a thumb directly over the cover-glass. Do not move the cover-glass laterally while thumb pressing as the chromosomes will roll and be unrecognizable. Scan the slide at 100X magnification to get an overall perspective if the chromosomes are flat enough. If they are not sufficiently flat then give them a further thumb press. Chromosome spreads can also be flattened by holding the slide at about 70°C for 5 to 10 seconds on a slide warmer. Don't overheat the chromosomes as the banding will fade. View again at 100X magnification and select the best chromosome compliment to examine at higher power.

20. On occasions you may wish to view the spreads under oil at 600 to 1,000X magnification and then decide to go back to a lower magnification that does not require oil. The oil can be absorbed relatively easily from the surface of the cover-glass with tightly rolled up absorbent paper. Oil cannot be removed effectively from an unsiliconized cover-glass which is another reason why I recommend you routinely coat cover-glasses (refer to step 1).

21. On occasions the slide will begin to dry up (air creeps in between the cover-glass and microscope slide) while you are examining the spreads under the microscope. Simply add a small drop of 50% propionic acid to the edge of the cover-glass and the acid will, by capillary action, draw under the cover-glass.

22. If results are required immediately, chromosomes can be prepared from freshly dissected ovaries by first dissecting them out of the female mosquito in 5% propionic and then transferring them into a drop of 50% propionic acid on a clean microscope slide. Continuing with the process as described from steps 11 to 20.

23. Interpretable chromosome spreads, but of lesser quality, can be extracted from ovaries preserved for less than 1 hour in modified Carnoy's solution. This comes in useful in the field when on the spot decisions are required to know which *An. gambiae* s.l. member or chromosomal forms of *An. gambiae* occur in an area. Use the same procedure as described from steps 11 to 20.

24. Permanent records of chromosome preparations can be kept either by taking photographs or by making permanent mounts. Digital cameras attached to the microscope and computer make capturing images much easier than the past when emulsion films were used. If permanent preparations are still required then proceed to the next step.

25. Remove the cover-glass by holding the microscope slide in one corner with a forceps and holding in liquid nitrogen until the bubbling stops. Take it out of the liquid nitrogen and immediately place the microscope slide on a flat surface and briskly pry the cover-glass off with a razor blade form one corner. Immediately begin dehydrating the preparations placing the slides into an ethanol series of 70%, 90% and 100% for 5 minutes each at 4°C. Air dry the slide and add a drop of mounting medium used for histology onto the chromosomes and cover with a cover-glass (does not have to be siliconized). Leave to dry and ring the cover-glasses with sealant according to recommended mounting medium instructions.

References

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Holt RA et al. (2002) The genome sequence of the malaria mosquito *Anopheles gambiae*. Science 298:129-149

6.9 An. gambiae *s.l. Salivary Gland Chromosome Preparation*

Anthony Cornel[1]

Introduction

Chromosome preparations from larval salivary glands first require a clean dissection of the salivary glands from L4 larvae. Fourth stage larvae of appropriate age are at about two to three hours before pupal trumpets can be seen developing in the thorax under the cuticle. Thereafter, histolysis of the salivary glands renders them useless for this purpose.

Solutions

- Modified Carnoy's fixative (three parts pure (100%) ethanol and one part glacial acetic acid). Ethanol absorbs water from the atmosphere so ensure that the ethanol is pure, as water in fixative compromises quality of spreads.

- Propionic acid - prepare 5% and 50% in water.

- 2% lacto-aceto orcein - prepare this solution by adding slowly 2% by weight of synthetic orcein powder to a solution of 1 part glacial acetic acid and 1 part pure lactic acid under constant stirring (use a magnetic stirrer). Remove un-dissolved orcein particulates by filtering the solution through Whatman 3MM paper. The solution can be stored indefinitely at room temperature.

- Ethanol – 70%, 90% and 100% in water.

Materials

- 0.13- 0.17 mm thick cover glasses of dimensions 22L X 22W mm

- Frosted 1mm thick microscope slides 75 x 25 mm in size.

- Beaker

- Aluminum foil

- Forceps

- Pasteur pipettes

- Absorbent paper (Whatman 3MM paper))

- Dissecting needles and minutien pins

- Dissecting Microscope

- 1.5 ml screw cap polypropylene vials

- Phase contrast compound microscope with 10X, 40 or 60 X and 100X objective lenses.

- Digital imaging system

Procedure

1. Dissect salivary glands by placing a larva in a drop of 5% propionic acid on a microscope slide. Sever the abdomen from the thorax and insert a needle from the rear of the thorax along the mid dorsal surface just underneath the cuticle up to just inside the head. Rub another needle over the inserted

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needle to abrade and break the larval cuticle along the mid dorsal line. Carefully open up the thorax and separate the glands from the connecting tissue but not from their ducts that lead to the head. Gently pull the head away from the thorax and the glands should come out still attached via the ducts.

2. Place the head and attached glands in a drop of modified Carnoy's on a microscope slide for fresh preparations or in a vial of modified Carnoy's for later squashing.

3. For fresh preparations, under the dissecting microscope separate the salivary glands from the head and discard the head.

4. Place another drop of modified Carnoy's onto the salivary glands to fix for a minute.

5. Drop about 50 µl of 50% propionic acid onto the salivary glands and leave them for about 3 minutes until they have cleared and swollen to about twice their original size.

6. Under a dissecting microscope carefully separate the glands from each other. Remove connecting and fatty tissue and trachea if any are still attached to the glands by sliding them away and draw up the waste tissues with the tip of tightly rolled up absorbent paper.

7. Staining with 2% lacto-aceto orcein is optional and this step should only be done after the glands have been separated and cleaned. Do this by adding a drop of the stain to the glands in the 50% propionic acid. Create an even staining mixture around the glands by stirring gently with a needle. Leave this for about 4 minutes for the gland cells to absorb the stain. Gently absorb the excess stain with a piece of tightly rolled up absorbent paper and add a drop of 50% propionic acid to the glands which by now should have a pale pink hue to them. Proceed to step 8.

8. Place a further drop of 25µl of 50% propionic acid onto the clean glands.

9. Wipe a cover-glass with a clean sheet of lint free paper – lens cleaning tissue or KimWipe® (Kimberley-Clark- Roswell GA) and place over the glands. Ensure the cover-glass is dust and grease free.

10. Carefully set the microscope slide on a piece of absorbent paper on a flat surface.

11. Various people use different tapping techniques to make chromosome squashes but essentially all begin by tapping quite firmly to break open the nuclear membranes and spreading out the chromosomes followed by harder tapping or pressing to flatten the chromosomes. I prefer to first gently tap the cover-glass with a mattress needle about 8 to 10 times (end bent downwards). While tapping with the needle the cover-glass will shift around a little which helps to shear the nuclear membranes and spread the chromosome arms out. View the slide at 100X magnification under phase contrast in a compound microscope to determine if more tapping to spread chromosomes is required. Once the first round of tapping has been completed, remove excess propionic acid by gently pressing absorbent paper over the cover-glass edges. Place a new dry piece of absorbent paper over the cover-glass and press firmly downwards with a thumb directly over the cover-glass. Do not move the cover-glass laterally while thumb pressing as the chromosomes will roll and be unrecognizable. Scan the slide at 100X magnification to get an overall perspective if the chromosomes are flat enough. If they are not sufficiently flat then give them a further thumb press. Chromosome spreads can also be flattened by holding the slide at about 70°C for 5 to 10 seconds on a slide warmer. Don't overheat the chromosomes as the banding will fade. View again at 100X magnification and select the best chromosome compliment to examine at higher power.

12. On occasions you may wish to view the spreads under oil at 600 to 1,000X magnification and then decide to go back to a lower magnification that does not require oil. The oil can be absorbed relatively easily from the surface of the cover-glass with tightly rolled up absorbent paper. Oil cannot be removed effectively from an unsalinized cover-glass which is another reason why I recommend you routinely salinize cover-glasses (refer to step 1).

13. On occasions the slide will begin to dry up (air creeps in between the cover-glass and microscope slide) while you are examining the spreads under the microscope. Simply add a small drop of 50%

propionic acid to the edge of the cover-glass and the acid will, by capillary action, draw under the cover-glass.

14. Salivary glands attached to the head can be preserved up to several weeks and longer for later chromosome preparation. I recommended dissecting and preserving the salivary glands still attached to the head via the salivary gland ducts for handling purposes. It is much easier to grab the head with forceps than the smaller salivary gland. Furthermore, there is a much higher likelihood of getting chromosomes out of intact salivary glands rather than broken ones because only few salivary gland cells produce polytene chromosomes.

15. Permanent records of chromosome preparations can be kept either by taking photographs or by making permanent mounts. Digital cameras attached to the microscope and computer make capturing images much easier than the past when emulsion films were used. If permanent preparations are still required then proceed to the next step.

16. Remove the cover-glass by holding the microscope slide in one corner with a forceps and holding in liquid nitrogen until the bubbling stops. Take it out of the liquid nitrogen and immediately place the microscope slide on a flat surface and briskly pry the cover-glass off with a razor blade form one corner. Immediately begin dehydrating the preparations placing the slides into an ethanol series of 70%, 90% and 100% for 5 minutes each at 4°C. Air dry the slide and add a drop of mounting medium used for histology onto the chromosomes and cover with a cover-glass (does not have to be siliconized). Leave to dry and ring the cover-glasses with sealant according to recommended mounting medium instructions.

Chapter 7 : Taxonomy and Systematics

7.1 Methods for Collecting and Preserving Mosquitoes

Theresa Howard, Ralph Harbach, and Yvonne Linton

INTRODUCTION

The primary purpose of this section is to provide uniform methods for the collection, preservation and rearing of material for the project. The emphasis here is on methods suited for obtaining *Anopheles* mosquitoes for taxonomic studies. Essential for this type of study is a large amount of uniformly prepared material with all the stages individually associated, general information on bionomics and conspicuous environmental factors, and a sample from as many habitats as possible in the study areas. Important considerations in selecting the methods and techniques adopted here have been simplicity and suitability for use under field and laboratory conditions and standardisation and simplification of records and labelling to minimise errors and to save time.

COLLECTION RECORDS

COLLECTION FORM. A standard form (see attached) for recording all the data pertaining to a collection has been developed for the project. The form must be filled out in the field as completely as possible and the remainder added in the laboratory. A pencil should be used for all entries. All measurements should be indicated in the metric system. There is a minimum of writing to be done (only in lined open spaces) on the form, the rest is to be done by circling or underscoring appropriate words or statements or by placing check marks or other signs in appropriate columns.

COLLECTION OF ADULTS

EQUIPMENT. The basic equipment and supplies needed for the collection of adults are (1) aspirators, (2) plastic vials, (3) covered cups (4) cages and (5) flashlight (torch).

CAPTURING. Resting, landing or biting mosquitoes are readily collected with an aspirator one or a few at a time and then transferred to individual collection vials or covered cups. It is always preferable to capture mosquitoes with an aspirator but it may not be practicable when very large numbers are encountered. It is also possible to place a tube directly over a resting or biting specimen but to do this numerous tubes may be needed

KILLING AND STORING. The most satisfactory killing tubes are charged with ethyl acetate but other killing agents may be used (e.g. chloroform). The killing tubes should be used exclusively for mosquitoes, and should ideally contain Plaster of Paris to absorb the killing fluid (strips of dry absorbent tissue paper or paper towelling may be used, but when these strips get damp they must be replaced with fresh dry ones). The specimens must be removed from the killing tubes within a few minutes after being introduced.

ISOLATING FOR OVIPOSITION. A number of species of mosquitoes which are very common as adults are very seldom encountered as larvae and pupae and the immature stages and breeding sites of some of these are poorly known. To obtain the immature stages of these it is necessary to isolate live individual females collected in the field, induce them to oviposit and to rear all the stages from the eggs (see the section on **PROGENY REARINGS**). It is also frequently desirable to identify cryptic species by chromosomes, enzymes or DNA using this technique for obtaining material. The progeny broods can be divided for study using different techniques.

COLLECTING AND RECORDING. Collect mosquitoes in houses and outdoor sites selected for study. Record all the data for every collection on the standard record form (see **Appendix 7.1.1 & 7.1.2**). First enter the locality and general information in the top section of the form. Usually several collections will be made in one locality. A separate collection number is assigned to every collection in a specific site, of a specific type on a specific host or bait, and at a specific time of capture. A collection of adults for progeny rearings must be assigned a number distinct from any general adult collection made at the same time and place. Record all the appropriate items on the collection form. If hourly collections are made to study biting activity, separate cups must be used for each hour of collection, appropriately labelled, and the species and specimens collected should be recorded on an hourly biting record (see **Appendix 7.1.3 & 7.1.4**).

COLLECTION OF IMMATURE STAGES

EQUIPMENT. The basic equipment and supplies needed for the collection of immature stages are: (1) dippers or pans, (2) aquatic and dip nets, (3) collection bags or vials, (4) plastic pipettes and (5) plastic, enamel or porcelain sorting bowls.

COLLECTING AND RECORDING. Because of the much greater percentage of species that can be collected as immature stages as compared to adults the emphasis in taxonomic surveys should be placed on the collection of immature stages, which can then be reared with relative ease in the laboratory to provide definite association of both sexes and all stages.

The immature stages should be collected with great care to prevent injury and should be provided with a sufficient volume of water and fine debris from the original breeding site to insure adequate food supply for rearing. Larvae and pupae are removed from the breeding site with the appropriate tool such as a dipper, aquatic net, dip net, or pan. All immature stages of *Anopheles* are placed with ample water from the breeding site into a plastic cup, sorting bowl or pan until the desired number is obtained. Large debris is removed from the container and the sediment (if present) is allowed to settle. The larvae and pupae are then transferred to a plastic bag (twirlpak®) or vials for transport to the laboratory. These bags or vials must be carefully marked on the outside with the collection number.

TRANSPORTING COLLECTIONS. The various containers with immature stages collected in the field should be handled very carefully. Usually no difficulty is encountered and little or no mortality occurs if some care is taken in transporting live mosquitoes in field vehicles. First, the containers should be placed away from direct sunlight and excessive heat; wet towels may be placed over and around the containers when high temperatures are encountered. Second, drive carefully avoiding high speeds, sudden stops and excessive bouncing. Third, transport larvae and pupae over long trips or rough terrain by floating collection bags (these are preferred over vials for this reason) in water contained in a cool box or large plastic bag. The water absorbs the shock of rough travel.

INDIVIDUAL REARINGS. Individual rearings are made from either pupae (pupal rearings) or larvae (larval rearings), which have been isolated in individual plastic vials and marked with the country identifier and collection number. Each individually reared specimen must be provided with an individual rearing number, which will identify the stages of each individual.

FACILITIES AND EQUIPMENT. A large cool room with electricity, running water and several tables or laboratory benches serves very well as a simple laboratory for mosquito rearing. The room should be ant-proof but if it is not, the legs of the tables or benches should be placed in cans of oil. Air-conditioning is a convenience for the workers but is not necessary or even desirable for mosquito rearing.

A laboratory or a collapsible stereoscopic field microscope is extremely useful. The basic equipment and supplies required for rearing and processing are: (1) pipettes and medicine droppers, (2) aspirators, (4) plastic, enamel or porcelain collecting bowls, (5) plastic rearing vials, (6) applicator sticks, (7) glass vials

and lids, (8) 80% and 95% ethanol, (9) labels and (10) pencils, grease pencils. All the equipment should be for use exclusively in the laboratory and should not be taken into the field.

CARE OF COLLECTIONS AND SORTING. Immediately after a field trip all the collections must be checked carefully. Labels on all containers should be made plainly visible and legible.

Set up immature collections by emptying contents of collection bags and vials into separate bowls. Label each bowl with the same number marked on the collection bag or vial. The sorting of the immature stages and their separation for individual rearings and preservation can now be carried out. The sooner this is done the better, and this should always be completed within 24 hours of capture. Work with one collection at a time going through the whole process before turning to the next collection. If more than one collection bag or vial contains immature stages from the same collection, assemble them all together.

Generally, more than one species is found in a single collection of immature stages even when collections are made from individual breeding sites, but as a rule one species is dominant in a particular collection and the others are frequently represented by few specimens or by younger larval instars.

First, all the pupae are transferred one to a plastic vial in about 2 cm (somewhat less than one inch) of fresh clean water (always use rainwater or distilled water). The plastic vials are marked with the collection number (grease or wax pencil is preferred but paper labels can be used). These will be later processed as indicated below under EMERGENCE VIALS. As a rule, all the pupae present in a collection should be isolated individually unless the collection consists primarily of pupae and the total number exceeds 10. If it is obvious that several species are represented among the pupae, a larger number should be isolated individually (see EMERGENCE VIALS below).

Second, the fourth-instar larvae are picked up one by one and placed into separate plastic vials. Isolate all mature larvae in the collection, or a maximum of 10 per species if the collection is very large. The individual vials are filled with distilled water or rainwater to a height of about 2 cm (somewhat less than one inch), marked with the collection number in grease pencil and set aside to be processed as indicated below under PUPATION VIALS. The remaining larvae in each collection, normally up to a maximum of 20, are set aside for killing and preservation (see WHOLE LARVAE in the section on **KILLING AND PRESERVATION**), and any larvae left over are retained in the bowl and removed for individual rearing as they become fourth instars. In case it is not practical or desirable to take care of a large numbers of collections or rearings, all the larvae remaining after isolation of individuals should be killed and preserved (NEVER DISCARD ANY MATERIAL ONCE COLLECTED). Great care should be taken to thoroughly rinse sorting pans and pipettes between the processing of different collections to eliminate contamination.

WASHING CONTAINERS. All containers used for collecting, sorting and rearing must be washed thoroughly before they are used again. First, wipe off the grease pencil markings (if used) from the dry bowls and vials with a piece of cotton or paper. Containers that are reasonably clean may be merely rinsed several times in clean fresh water. Aspirators should also be cleaned periodically and the netting replaced on the plug.

PUPATION VIALS. Vials containing isolated larvae, marked with collection numbers, should be examined twice a day for pupation, preferably in early morning and late afternoon. Check through all the vials and set aside all those containing larval skins before further processing. Remove the larval skin with an applicator stick (without dragging it along the side of the rearing vial) and transfer it into a storage vial with 80% alcohol and attach it with an elastic band to the rearing vial containing the pupa. Place a lid on the rearing vial to prevent escape of the adult mosquito after it emerges from the pupa. This marked vial is now set aside to be processed as indicated under REARED ADULTS in the section on **KILLING AND PRESERVATION** below.

EMERGENCE VIALS. Vials containing isolated pupae should be examined twice a day, preferably in early morning and late afternoon. Check all the vials and set aside all those with emerged or drowned

adults for processing. To remove the viable emerged adult loosen the lid and carefully slip in and replace it by the mouth of an inverted plastic holding vial. Tap on the side of the pupal container to induce the mosquito to enter the holding vial and quickly stopper it with the original lid. Label this holding vial with the same number that appears on the rearing vial containing the pupal skin. Remove the pupal skin with an applicator stick (without dragging it along the side of the vial) and place it in the vial of 80% ethanol with the associated larval skin. Completely fill the vial with ethanol to exclude air and attach it to the plastic holding vial containing the adult mosquito. This vial will now be processed as indicated below under REARED ADULTS. The entire process is repeated for all the pupal vials.

All vials containing dead pupae and drowned, partially emerged adults stuck to the water should be discarded. These specimens are not useful for taxonomic, but if they are alive they may be preserved for enzyme or DNA analysis.

HOLDING VIALS. Reared adults for taxonomic study should be kept for at least 24 hours before being killed and processed as indicated in the section on REARED ADULTS. Many adults may die before they are processed, in which case they will more difficult to mount on points on pins for study.

The individual holding vials require relatively little attention. Check at least twice a day and process dead specimens immediately or as soon as possible, following the directions in the section on REARED ADULTS. After the required 24-hour holding period, process the live specimens following the same directions.

PROGENY REARINGS

Gravid females collected in the field will generally oviposit within 3 to 4 days if they will lay eggs at all. Females should be retained in a cool damp environment and provided with 5-10% sucrose solution in saturated wads of cotton. The cotton wads should be moistened or replaced once or twice daily. On the third day following capture, each female is lightly anaesthetised with ethyl acetate or knocked down in a freezer (about 1 minute) for the purposes of removing a wing. This is accomplished by grasping each wing with a forceps and gently pulling until one of the wings is torn from the thorax. The anaesthetised female is then placed on the surface of water (containing a hatching stimulus) in a small cup. Females stressed in this manner usually oviposit soon after being placed on the water. Some gravid females will refuse to lay eggs and these can be preserved for enzyme or DNA studies. Once larvae become late first or early second instars, they should be transferred to a larger bowl or pan and fed at least once per day by sprinkling powdered food onto the surface. A portion of fourth-instar larvae (pre-pupae) should be removed and reared individually for taxonomic study as described in the section on **INDIVIDUAL REARINGS**. Remaining larvae can be preserved for enzyme or DNA analysis.

KILLING AND PRESERVATION

EQUIPMENT AND SUPPLIES. The following equipment and supplies are needed for killing and preserving mosquitoes: (1) killing tubes (preferably with ethyl acetate), (2) aspirators, (3) forceps, (5) labels, (6) 80% and 95% ethanol, (7) applicator sticks or scoop, (8) glass vials with lids, (9) beaker or pan for hot water.

FIELD-COLLECTED ADULTS. Adult mosquitoes collected in the field are usually killed, identified and preserved immediately on return to the laboratory. The method of preservation will depend on the type of study they will be used for. Some blood-fed adults will be retained and set-up to obtain progeny broods (see PROGENY REARINGS).

REARED ADULTS. All reared adults after being held for 24 hours in plastic vials (see HOLDING VIALS) should be killed and processed as indicated below. Parent females in **PROGENY REARINGS** should be processed immediately after oviposition following the procedure required for the type of study to be undertaken (i.e. morphology, enzymes or DNA).

Process all the adults (dead or alive) from individuals that have been held for the length of time (24 hours) together at some convenient time in the afternoon or evening. Numbers are assigned to individual specimens at this stage, and the necessary information is recorded on the appropriate collection and rearing form (see **Figures 7.1.1 and 7.1.2)**. For example, an adult female with larval and pupal skins is given the next available number on the form, perhaps 12-2, indicating that this is the second specimen reared from collection 12. The first specimen from this collection would have been labelled 12-1. The number needs to be written on 2 labels, one for the adult and one for the vial containing the associated skins. Pre-printed labels may be used to avoid errors and increase efficiency.

To speed the process, have at hand 2-5 killing tubes to be used serially. Loosen the lid of a holding vial and holding the vial upside down slip its mouth over that of a killing tube. The adult will be knocked down by the ethyl acetate within seconds and will fall into the killing tube. To hasten knockdown, tap on the vial. Remove the vial and hold a thumb over the mouth of the killing tube until the specimen ceases struggling. Place the label inside the killing tube, stopper the tube and set it aside. Proceed in the same manner with 1-4 other adults and place the killing tubes in sequence. After all the tubes are used, return to the first one and process it as indicated in the next paragraph. After this step is finished kill another adult with the emptied tube and place it in sequence after the others. Continue in this manner until all the adults are processed. Time the processing to allow the adult to remain in a tube about 5 minutes, but not longer than 10 minutes by using the appropriate number of killing tubes.

Transfer the adult and its label from the killing tube to a clean white card. Using fine forceps pick up the adult by one leg and place it on an elevated surface (e.g. empty slide box) with a white background. Orient the specimen with its left side facing down. This should be accomplished without grasping the specimen with the forceps. Move the specimen to the edge of the surface with its legs projecting beyond the edge. Insert a heavy paper point an appropriate distance from the head of an insect pin and place a tiny droplet of ambroid$^{®}$ cement or other suitable glue on the upper apical angle of the point. Holding the pin so that the point is upside down, gently touch the droplet of the glue to the thorax (right side) of the mosquito. Final orientation of the mosquito on the upper surface of the point is with the left side up, head facing left and the legs extended toward the pin. This orientation protects the specimen from damage and corresponds to the preferred orientation of illustrations in taxonomic publications. Place the label with the collection and rearing number on the pin and store the specimen in an appropriate insect storage box.

WHOLE LARVAE. It is essential to preserve an adequate sample of whole larvae of every species from every field collection and from all progeny rearings (see CARE OF COLLECTIONS AND SORTING in the section on **INDIVIDUAL REARINGS**). To be useful for taxonomic purposes, whole larvae must be killed and preserved carefully so that the body shape and all structures, particularly setae, are retained. The larvae set aside for killing and preservation (identified by the collection number) are first transferred with a dropper to a small cup or bowl with fresh clean water as a washing procedure. If much debris or sediment is still present, additional serial transfers should be made until it is eliminated. Remove as much water as possible from the cup or bowl using a fine pipette. Next heat a beaker or pan of water is about 60ºC (140ºF) and pour the hot water into the cup or bowl. As soon as the larvae float up to the surface, the water is removed with a fine pipette and replaced with a quantity of 80% ethanol. After 5 minutes the larvae are transferred with a lifter (do not use forceps) to a vial with 80% ethanol. Completely fill the vial with 80% ethanol to remove all air and cap it tightly. No more than 20 larvae should be placed into a single vial as the water contained in the bodies of the larvae will significantly dilute the concentration of a small quantity of ethanol and jeopardise preservation. Prepare a paper label in pencil and tape it to a vial. The label may be placed inside the vial if the larvae are separated from the label by a small wad of cotton. Larvae to be used for DNA studies should ONLY be killed by placing them into a vial of 95% ethanol.

SKINS. The most valuable material for taxonomic purposes is the associated larval and pupal skins from individual rearings and the corresponding adults. The greatest care must be taken in processing these. Labels should not be enclosed in vials with skins unless these are separated by a small wad of cotton. It

is far better to tape a label to the outside of the vial, or to write the specimen number on a piece of tape and wrap it around the vial in a lightly overlapping manner to enhance adhesion.

PRESERVATION AND MOUNTING TECHNIQUES

WHOLE LARVAE

1. Kill larvae in hot water (not boiling), remove promptly with a lifter. Store in small vial containing 80% ethanol (ethyl alcohol). For material to be used for DNA studies, larvae should be killed by placing directly into 95% ethanol – DO NOT kill in hot water.
2. Transfer specimens from alcohol to cellosolve for 15 minutes or more (dark specimens can be stored in cellosolve for 8 hours or overnight).
3. Lift the specimen from cellosolve and place on the centre of glass microscope slide with the dorsal side up.
4. Drop a small amount of Euparal on the specimen. Mount specimen dorsal side up with the head pointing down; arrange head, thorax and abdomen in natural position, then cut the abdomen between segment VI and VII (with a fine scalpel blade). Place the terminal segments with siphon to the left in culicine larvae or segment X to the right in anopheline larvae (see **FIGURE 7.1.3**).
5. Place more Euparal on the specimen and check the arrangement of setae and larval position, then carefully cover the specimen with a 22 mm rectangular cover glass.
6. Dry in an oven at 45⁰-55⁰C for 4 weeks or more.

LARVAL AND PUPAL EXUVIAE

The fourth-instar larval and pupal exuviae from an individually reared adult should be mounted on the same slide.

1. Store in 80% alcohol.
2. Transfer the specimens into cellosolve for 15 minutes.
3. Lift the specimen from cellosolve placing it on a glass microscope slide, the larval exuviae on the left and pupal exuviae on the right (pointing head down and dorsal side up).
4. Drop a small amount of Euparal on the specimens. Arrange and spread the body and setae of larval exuviae into better position, then separate the pupal cephalothorax just cephalad of the wing, leaving the metanotum attached to the abdomen. Open the cephalothorax, mount it ventral side up and place it below the metanotum (see **FIGURE 7.1.3**).
5. Add more Euparal, check the position of the exuviae then cover the specimens with a 15 mm round cover glass.
6. Dry in an oven at 45⁰-55⁰C for 4 weeks or more.

ADULTS
1. After emergence adults should be held for at least 24 hours before killing.
2. Kill in a killing tube charged with ethyl acetate or chloroform[1] (ethyl acetate is preferred because it keeps specimens relaxed longer).
3. Apply a small amount of Ambroid® cement[2] or other glue to the tip of a paper point and affix the specimen on the right side of the thorax with the legs toward the pin (see **FIGURE 7.1.3**).

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[1]CAUTION! Chloroform and Ethyl Acetate are toxic and dangerous to breathe. These chemicals are stored in liver   tissues and may cause health problems if used frequently. Always use in well ventilated areas.

[2] Ambroid® cement should be thinned down with amyl acetate.

Pinned specimens should be kept in boxes. Seal each box in a self-seal or taped closed plastic bag. If possible include a sachet of Silica Gel in the bag to keep specimens dry. The bag should prevent the specimens from being eaten by beetles, cockroaches, mites and other insects.

LABELING (see **FIGURE 7.1.3**)

> Label the slide with 2 labels:
>> Left label contains:
>>> Country, collection number
>>> Province, date collected
>>> Location
>>> Collector's name
>>> Habitat
>>> Slide number
>>
>> Right label contains:
>>> Genus
>>> Subgenus
>>> Species
>>> Person making the determination

Label the adult with 2 labels. Each label should be ¼" x ½" in size or smaller.
>> Upper label contains:
>>> Country, province
>>> Location
>>> Collection Number
>>> Date/Year
>> Lower label contains:
>>> Genus, subgenus and species
>>> Sex
>>> Person making the determination

STORING, PACKING AND SHIPPING

STORING. All preserved adults and all supplies used for the preservation of adults must be stored in a dry pest-proof box to protect them from mold and insects. A simple cardboard shipping box sealed in a plastic bag or beem capsules in individual sealed plastic bags with a drying agent placed inside the plastic bags or air-tight wood, pest-proof box can be used to store the material. The vials with ethanol should be kept in a cool place. The vials should be packed neatly in cardboard containers of a convenient size or wrapped together in paper towelling in bundles of 20 to 50. Vials containing alcohol should have the tops tightly sealed to prevent evaporation and be packed in small cardboard boxes. Microscope slides should be stored in slide boxes with the cover-slip parallel to the surface of the storage shelf to reduce the risk of slippage in cases where the mounting media is not 100% dry.

PACKING AND SHIPPING. Material should be shipped as soon as possible for final processing and mounting. Do not let large quantities accumulate but send it in small parcels. Be sure to include the collection forms with the preserved specimens.

It is very important to pack the material very carefully or it may be completely ruined. Use a sturdy corrugated cardboard box lined with appropriate packing material. All the material to be shipped must first be carefully packed in small cardboard boxes as indicated in the section above. Check every box for loose containers and fill in the unused space with cotton or crumpled paper so that nothing will move when the box is shaken but do not pack too tightly. Next seal the individual boxes with tape. Place all the boxes in the shipping container and fill all the spaces between them tightly with crumpled paper so that nothing moves and the container is completely filled. Enough packing material should be placed on top of the boxes so that when the lid is put on a slight pressure will be needed to keep it down. Now close the shipping container and seal the top with tape. The container can then be covered with wrapping paper if required.

If pinned specimens in Schmitt or other boxes are shipped, a corrugated cardboard container lined with 2 inches of packing material should be used. Make certain that all the pins are inserted tightly in the box. Remove all the foreign material such as drying agents or naphthalene flakes from the box. If large labels have been used, insert pins on each side of each label to prevent it from swinging in transit. Microscope slides should be secured in slide boxes with strips of paper or foam rubber to prevent movement during shipment. All packages should be shipped by air, preferably by AIR PARCEL POST. All packages should be plainly marked "Preserved material for scientific study, no commercial value."

Figure 7.1.1.

Comprises 2-letter country identifier and
individual collection locality number
e.g. IN12 (12th collection site in Indonesia)

Collection No.	Nearest Town	Date
Country	Specific Locality	Time
Province	Latitude/Longitude	Collector(s)
Second Administrative Division	Elevation m	Organisation

COLLECTION TYPE
- Immature
- Resting -
  - House
  - Animal Shelter
  - Cave
  - Tree Hole
  - Vegetation
  - Other: _____
- Biting/ Landing -
  - Human
  - Animal: _____
- Net
- Light Trap: _____
- Bait Trap
- Swarming
- At Light
- Other: _____

TERRAIN
- Mountain
- Hill
- Valley
- Plateau
- Plain

DISTANCE FROM HOMES
- _____ m

SKY
- Clear
- Partly Cloudy
- Overcast
- Fog
- Mist
- Light Rain
- Heavy Rain

SHADE
- None
- Partial
- Heavy

HOST
- Human
- Horse
- Cow
- Other: _____

ENVIRONMENT
- Rain Forest
- Evergreen Forest
- Deciduous Forest
- Cloud Forest
- Coniferous Forest
- Scrub/Bush
- Savanna
- Prairie
- Island
- Swamp
- Salt Marsh
- Beach
- Mangrove
- Orchard - Plantation
- Cultivated Field: _____
- Bamboo Grove
- Urban
- Village
- Other: _____

ENVIRONMENTAL MODIFIERS
- Primary
- Secondary

- Agriculture
- Pasture
- Grove/Plantation: _____
- Other: _____

WIND
- None
- Light
- Gusts
- Strong

HEIGHT ABOVE GROUND
- _____ m

LARVAL HABITAT
- Pond - Lake
- Ground Pool
- Swamp
- Marshy Depression
- Stream Margin
- Stream Pool
- Rock Pool
- Seepage - Spring
- Ditch
- Well
- Artificial Container
- Hoof Print
- Rut
- Rice Field
- Mangrove
- Other: _____

ALGAE
- Filamentous
- Green
- Blue-Green
- Brown
- Other: _____

ALGAL DENSITY
- None
- Scarce
- Moderate
- Abundant

DIMENSIONS OF SITE
- _____ m X _____ m

- Depth _____ m

WATER:
- Permanent
- Temporary

WATER MOVEMENT
- Stagnant
- Slow
- Moderate
- Fast

SALINITY
- Fresh
- Brackish

TURBIDITY
- Clear
- Coloured
- Turbid
- Polluted

PHYSICAL FACTORS
- pH
- Conductivity
- Temperature (°C)
- TDS

AQUATIC VEGETATION
- Submerged
- Floating
- Emergent
- Submerged and Floating
- Submerged and Emergent
- Floating and Emergent
- All Types

QUANTITY OF AQUATIC VEG.
- None
- Scarce
- Moderate
- Abundant

Mark all collection information
clearly and as fully as possible

REMARKS

Any additional information that may be useful
i.e. how many whole larvae preserved in ethanol

Figure 7.1.2.

The same country identifier and number as on the main collection sheet - these forms are usually printed back-to-back

Individual specimen number - each specimen will have a unique number comprising the Collection Number and this number e.g. IN12-1

Which life stage reared or obtained - may only be an adult collection

Sex of individual specimen

Collection Number				Country
Number	Le	Pe	Sex	Identification / Notes
-1		✓	♂	
-2	✓	✓	♀	

Figure 7.1.3.

THAILAND: 07953
Chumphon. 6 Sep78
Ban Kon Thae
Coll: Kol
HBT: water jar
Slide # F-8204

Aedes
(Stegomyia)
aegypti

Det. RR

LARVA

THAILAND: 07819-1
Chiang Mai. 4 Jul 78
Doi Inthanon
Coll: Harrison
HBT: banana axil
Slide # F-7084

Malaya
jacobsoni

Det. RR

LARVAL AND PUPAL EXUVIAE

THAILAND: Chon Buri
Huai Kuum
Coll. 09022   1979

THAILAND: Chon Buri
Huai Kuum
Coll. 09022   1979

An.(Cel.) dirus ♀
Det. RR

An.(Cel.) dirus ♀
Det. RR

ADULT

APPENDECIES 7.1.1 – 7.1.4

Appendix 7.1.1 & 7.1.2: Collection Forms - pages 13-14

Appendix 7.1.3 & 7.1.4: Biting Collection Record - pages 15-16

(When printing - print page 14 on the reverse of page 13 (additional rearing forms can be printed separately) and 16 on the reverse of 15)

Collection No.	Nearest Town	Date
Country	Specific Locality	Time
Province	Latitude/Longitude	Collector(s)
Second Administrative Division	Elevation m	Organisation

COLLECTION TYPE
- Immature
- Resting -
    - House
    - Animal Shelter
    - Cave
    - Tree Hole
    - Vegetation
    - Other: _____
- Biting/ Landing -
    - Human
    - Animal: _____
- Net
- Light Trap: _____
- Bait Trap
- Swarming
- At Light
- Other: _____

TERRAIN
- Mountain
- Hill
- Valley
- Plateau
- Plain

DISTANCE FROM HOMES
    m

SKY
- Clear
- Partly Cloudy
- Overcast
- Fog
- Mist
- Light Rain
- Heavy Rain

SHADE
- None
- Partial
- Heavy

HOST
- Human
- Horse
- Cow
- Other: _____

ENVIRONMENT
- Rain Forest
- Evergreen Forest
- Deciduous Forest
- Cloud Forest
- Coniferous Forest
- Scrub/Bush
- Savanna
- Prairie
- Island
- Swamp
- Salt Marsh
- Beach
- Mangrove
- Orchard - Plantation
- Cultivated Field: _____
- Bamboo Grove
- Urban
- Village
- Other: _____

ENVIRONMENTAL MODIFIERS
- Primary
- Secondary

- Agriculture
- Pasture
- Grove/Plantation: _____
- Other: _____

WIND
- None
- Light
- Gusts
- Strong

HEIGHT ABOVE GROUND
    m

LARVAL HABITAT
- Pond - Lake
- Ground Pool
- Swamp
- Marshy Depression
- Stream Margin
- Stream Pool
- Rock Pool
- Seepage - Spring
- Ditch
- Well
- Artificial Container
- Hoof Print
- Rut
- Rice Field
- Mangrove
- Other: _____

ALGAE
- Filamentous
- Green
- Blue-Green
- Brown
- Other: _____

ALGAL DENSITY
- None
- Scarce
- Moderate
- Abundant

DIMENSIONS OF SITE
    m X   m

Depth   m

WATER:
- Permanent
- Temporary

WATER MOVEMENT
- Stagnant
- Slow
- Moderate
- Fast

SALINITY
- Fresh
- Brackish

TURBIDITY
- Clear
- Coloured
- Turbid
- Polluted

PHYSICAL FACTORS
- pH
- Conductivity
- Temperature (ºC)
- TDS

AQUATIC VEGETATION
- Submerged
- Floating
- Emergent
- Submerged and Floating
- Submerged and Emergent
- Floating and Emergent
- All Types

QUANTITY OF AQUATIC VEG.
- None
- Scarce
- Moderate
- Abundant

REMARKS

Collection Number				Country
Number	**Le**	**Pe**	**Sex**	**Identification / Notes**
Collection Number				Country
Number	**Le**	**Pe**	**Sex**	**Identification / Notes**

MOSQUITO BITING COLLECTION RECORD

Coll. No. _____   Date _____

Host _____   Collector _____

Location _____

Weather _____   Temp. (W/D) _____

Habitat _____

Collection: Inside   Outside   Other

SPECIES	2400-0059	0100-0159	0200-0259	0300-0359	0400-0459	0500-0559	TOTAL
TOTAL							

MOSQUITO BITING COLLECTION RECORD

Coll. No. _____   Date _____

Host _____   Collector _____

Location _____

Weather _____   Temp. (W/D) _____

Habitat _____

Collection: Inside   Outside   Other

SPECIES	1800-1859	1900-1959	2000-2059	2100-2159	2200-2259	2300-2359	TOTAL
TOTAL							

7.2 Ribosomal DNA PCR Assays

7.2.1 Amplification of the Second Internal Transcribed Spacer Region (ITS2) in Anophelines

MR4 Staff

Introduction

In many insect genera, often the amplification and sequencing of the ITS2 region is useful in order to detect intraspecific differences. The ribosomal DNA region is made up of 3 functional rDNA genes separated by two spacer regions: the ITS1 and ITS2 regions. In many anopheline species it has been found that the ITS2 region is more informative, especially in designing PCR assays to distinguish members of cryptic complexes. Universal primers designed by Beebe and Saul (1995) and have been shown to be suitable in a number of anopheline species.

PCR assay for amplifying the ITS2 region in anopheline mosquitoes (Beebe and Saul 1995)

Prepare PCR Master Mix for 96, 48 or 1 25 μl PCR reactions[1]. Add reagents in the order presented.

96	48	1	0.5	Reagent
1555 μl	777.5 μl	15.55 μl	7.8 μl	sterile H_2O
500 μl	250 μl	5.0 μl	2.5 μl	GoTaq 5X PCR Buffer with $MgCl_2$
100 μl	50 μl	1.0 μl	0.5 μl	dNTP (2.5 mM mix)
150 μl	75 μl	1.5 μl	0.75 μl	ITS2 A primer (1 pmol/μl) [TGT GAA CTG CAG GAC ACA T]
150 μl	75 μl	1.5 μl	0.75 μl	ITS2 B primer (1 pmol/μl) [TAT GCT AAA ATT CAG GGG GT]
30 μl	15 μl	0.3 μl	0.15 μl	$MgCl_2$ (25 mM)
15 μl	7.5 μl	0.15 μl	0.05 μl	Go-Taq DNA polymerase (5 U/μl)
2.5 ml	**1.25 ml**	**24 μl**	**12.5 μl†**	**Total (To each 24 ul reaction add 1 μl template DNA)**

† Add 0.5μl template DNA if performing the ½ volume PCR reaction.

PCR Cycle conditions

94°C/4min x 1 cycle
(94°C/30sec, 53°C/40sec, 72°C/30sec) x 35 cycles
72°C/10min x 1 cycle
4°C hold

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red. Band sizes will vary by species (**Figure 7.2.1.1**).

---

[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

Figure 7.2.1.1. Lane 1, 1kb ladder, lane 2, *An. gambiae*, lane 3, *An. arabiensis*, lane 4, *An. freeborni*, lane 5, *An. sawadwongporni*, lane 6, *An. albimanus*, lane 7, *An. quadrimaculatus*, lane 8, *An. stephensi*, lane 9, *An. minimus*

References

Beebe NW, Saul A (1995) Discrimination of all members of the *Anopheles punctaulatus* complex by polymerase chain reaction-restriction fragment length polymorphism analysis. Am J Trop Med Hyg 53:478-481

96 well sample preparation template

7.2.2 PCR amplification of expansion segments within the 28S ribosomal DNA in *Anopheles* mosquitoes

Introduction

The most common molecular systematic and phylogenetic assays for anophelines target the ribosomal DNA (Krzywinski and Besansky 2003). Ribosomal DNA (rDNA) is a tandem-repeated array comprised of three subunits (18S, 5.8S, and 28S) and several intergenic spacer regions (reviewed in Hwang and Kim 1999). Although the spacer regions evolve faster than the transcribed regions, the 18S and 28S subunits are comprised of several expansion segments which have varying levels of interspecific polymorphism (Hancock et al. 1988). The large subunit (28S) is the largest of the three units and has been found to show more interspecific polymorphism within its domains compared to the small subunit (18S) (Hwang and Kim 1999). Here we present PCR assays for three of the domains located within the 28S subunit.

PCR primers for specific expansion segments and their associated product sizes

Domain	Primer sequence	Tm	Approx. Product size	Reference
D2 forward	GTG GAT CCA GTC GTG TTG CTT GAT AGT GCA G	60	560 bp	Porter
reverse	GTG AAT TCT TGG TCC GTG TTT CAA GAC GGG	60		
D3 forward	GAC CCG TCT TGA AAC ACG GA	50	415 bp	Baldwin
reverse	TCG GAA GGA ACC AGC TAC TA	50		
D7 forward	CTG AAG TGG AGA AGG GT	42	480 bp	Friedrich
reverse	GAC TTC CCT TAC CTA CAT	42		

Table 7.2.2.1. Primers are presented 5' to 3'. Product sizes may vary between species due to expansion of the domains.

PCR amplification of the expansion segments of the 28S subunit in anophelines

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions. Add reagents in the order presented.

96	48	1	Reagent
1.49 ml	745 µl	14.9 µl	sterile H_2O
500 µl	250 µl	5.0 µl	5X PCR Buffer
100 µl	50 µl	1.0 µl	dNTP (2 mM mix)
100 µl	50 µl	1.0 µl	Forward primer (25 pmol/µl)
100 µl	50 µl	1.0 µl	Reverse primer (25 pmol/µl)
100 µl	50 µl	1.0 µl	$MgCl_2$ (25mM)
10 µl	5.0 µl	0.1 µl	Taq DNA polymerase (5U/µl)
2.4 ml	1.2 ml	24.0 µl	**Total**

Table 7.2.2.2. Use 1 µl of DNA template per reaction.

PCR Cycle conditions

95°C/5min x 1 cycle
(95°C/30sec , **Tm**[1]°C/30sec , 72°C/45sec) x 30 cycles
72°C/5min x 1 cycle
4°C hold

---

[1] **Tm**, Information for specific annealing temperatures is shown in Table 7.2.2.1 for each of the expansion segments.

96 well PCR sample preparation template

Run samples on a 1.5% agarose gel stained with EtBr, or other intercalating agent like SYBR Green or Gel Red, and load 10 µl of the sample. Primers create a single band that is specific for each domain as listed in table 7.2.2.1 (**Figure 7.2.2.1**).
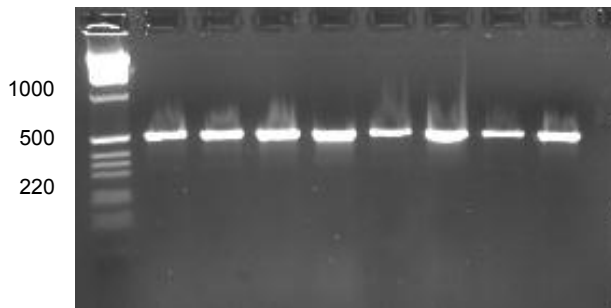
Figure 7.2.2.1. D7 expansion segment: lane 1 1Kb marker, lanes 2-9 *An. subpictus* samples.

References

Baldwin JG, Frisse LM, Vida JT, Eddleman CD, Thomas WK (1997) An evolutionary framework for the study and developmental evolution in a set of nematodes related to *Caenorhabditis elegans*. Mol Phylog Evol 8: 249-259.

Friedrich M, Tautz D (1997) An episodic change of rDNA nucleotide substitution rate has occurred during the emergence of the insect order Diptera. Mol Biol Evol 14: 644-653.

Hancock JM, Tautz D, Dover GA (1988) Evolution of the secondary structures and compensatory mutations of the ribosomal RNAs of *Drosophila melanogaster*. Mol Biol Evol 5: 393-414.

Hwang UW, Kim W (1999) General properties and phylogenetic utilities of nuclear ribosomal DNA and mitochondrial DNA commonly used in molecular systematics. Korean J Parasit 37: 215-228.

Krzywinski J, Besansky NJ (2003) Molecular systematics of *Anopheles*: from subgenera to subpopulations. Ann Rev Entomol 48: 111-139.

Porter CH, Collins FH (1996) Phylogeny of Nearctic members of the *Anopheles maculipennis* species group derived from the D2 variable region of 28S ribosomal RNA. Mol Phylog Evol 6: 178-188.

7.2.3 General PCR to amplify a portion of the 18S rDNA subunit in anophelines

MR4 Vector Activity

Introduction

The rDNA is a multicopy (>100) locus useful for phylogenetic studies due to its rapid, concerted evolution. It is employed to distinguish between cryptic species since nucleotide differences will quickly fix after gene flow between two populations ceases.

PCR assay for the amplification of the 18S subunit region

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions. Add reagents in the order presented.

96	48	1	Reagent
1.54 ml	770 µl	15.4 µl	Distilled H2O
500 µl	250 µl	5.0 µl	5X PCR buffer
150 µl	75 µl	1.5 µl	dNTP (2mM concentration)
100 µl	50 µl	1.0 µl	18SForDros (25pmol/ul) [GAG GGA GCC TGA GAA ACG GCT AC]
100 µl	50 µl	1.0 µl	18SRevDros (25pmol/ul) [CCT TCC GTC AAT TCC TTT AAG TTT C]
100 µl	50 µl	1.0 µl	MgCl2 (25 mM)
10 µl	5.0 µl	0.1 µl	Taq DNA polymerase (5U/ µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total (To each 25 µl reaction add 1.0 µl template DNA)**

Table 7.2.3.1. F and R indicate forward and reverse orientation. DNA extraction negative control to be included in addition to PCR reaction mix negative control.

PCR Cycle conditions

94°C/5min x 1 cycle
(94°C/1min , 54°C/1min , 72°C/1min) x 35 cycles
72°C/7min x 1 cycle
4°C hold

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red. Primers create an approximately 900 bp band (**Figure 7.2.3.1**).
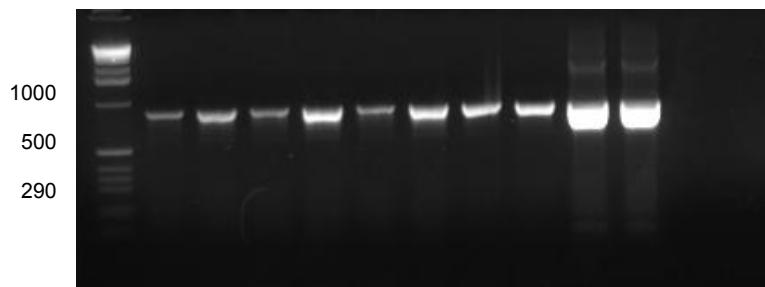
Figure 7.2.3.1. Lane 1 1Kb marker, Lane 1, 1kb ladder, lanes 2-9 *An. subpictus*, lanes 10-11 *An. gambiae.*

96 well PCR sample preparation template

	1	2	3	4	5	6	7	8	9	10	11	12
A	○	○	○	○	○	○	○	○	○	○	○	○
B	○	○	○	○	○	○	○	○	○	○	○	○
C	○	○	○	○	○	○	○	○	○	○	○	○
D	○	○	○	○	○	○	○	○	○	○	○	○
E	○	○	○	○	○	○	○	○	○	○	○	○
F	○	○	○	○	○	○	○	○	○	○	○	○
G	○	○	○	○	○	○	○	○	○	○	○	○
H	○	○	○	○	○	○	○	○	○	○	○	○

References

Beebe NW, Foley DH, Cooper RD, Bryan JH, and Saul A. 1996. DNA probes for the *Anopheles punctulatus* complex. Am J Trop Med Hyg. 54: 395-398.

7.3 Mitochondrial DNA PCR Assays

7.3.1 General PCR for amplification of Cytochrome c Oxidase I and II in anophelines

Introduction

Mitochondrial DNA (mtDNA) is one of the most commonly studied regions in insect systematics due to its high rate of homoplasmy (Caterino et al 2000). Within the mtDNA there are several segments of which only a few are routinely examined: cytochrome c oxidase I (COI), cytochrome c oxidase II (COII), and cytochrome b (cytb – see Chapter 7.3.2).

	Primer sequence	**Reference**
COI forward	GGA GGA TTT GGA AAT TGA TTA GTT CC	Beard 1993
COI reverse	GCT AAT CAT CTA AAA ATT TTA ATT CC	
COII forward	TCT AAT ATG GGA GAT TAG TGC	Simon 1994
COII reverse	ACT TGC TTT CAG TCA TCT AAT G	

Table 7.3.1.1. Primer sequences and references used in these assays.

Prepare PCR Master Mix for 96*, 48* or 1 25µl PCR reactions. Add reagents in the order presented.

96	**48**	**1**	**Reagent**
1.24 ml	620 µl	12.40 µl	sterile H_2O
500 µl	250 µl	5.0 µl	5X PCR Buffer (go taq Promega)
200 µl	100 µl	2.0 µl	dNTP (25 mM mix)
150 µl	75 µl	1.5 µl	Forward primer (600 nM/ µl)
150 µl	75 µl	1.5 µl	Reverse primer (600 nM /µl)
250 µl	125 µl	2.5 µl	$MgCl_2$ (25mM)
10.0 µl	5.0 µl	0.1 µl	Taq DNA polymerase (5U/µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total**

Table 7.3.1.2. Add 1 µl of template gDNA to each reaction. DNA extracted negative controls are included in addition to PCR reaction mix negative control.

PCR Cycle conditions

95°C/3min x 1 cycle
(95°C/30sec , 55°C/30sec , 72°C/45sec) x 35 cycles
72°C/10min x 1 cycle
4°C hold

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red. You will expect the following fragment sizes: COI – 700bp, COII – 730 bp (**Figure 7.3.1.1.**).
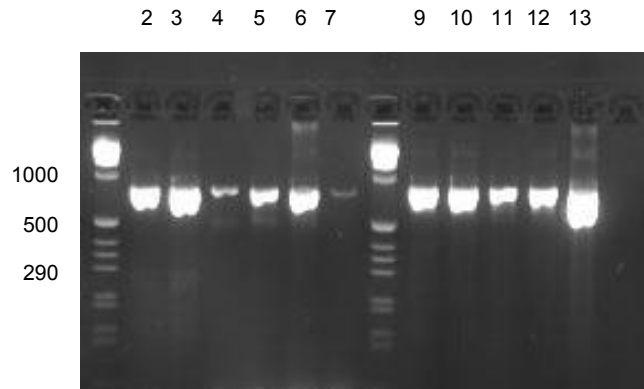
Figure 7.3.1.1. Lane 1, 1kb ladder, lanes 2-6 COI amplicon for *An. subpictus*, lane 7 negative control, lane 8 1kb ladder, lanes 9-13 COII amplicon for *An. subpictus*, lane 14 negative control.

96 well PCR sample preparation template

References

Beard CB, Hamm DM, and Collins FH. 1993. The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. Insect Mol Biol 2: 103-124.

Caterino MS, Cho S, and Sperling FAH. 2000. The current state of insect molecular systematic: a thriving tower of Babel. Ann Rev Entomol. 45: 1-54.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, and Flook P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc Am. 87: 651-701.

7.3.2 General PCR to amplify the cytochrome B region in anophelines

MR4 Vector Activity

Introduction

Although most phylogenetic studies involve one of the cytochrome oxidase genes (COI and COII) or one of the D domains in the 28S subunit of the rDNA, the cytochrome B (cytb) gene has been used extensively by vertebrate scientists (Simmons 2001). The utility of mitochondrial DNA (mtDNA) for phylogenetic studies is based on its lack of recombination, maternal inheritance, rapid evolution, and intraspecific polymorphisms (Avise et al. 1987).

PCR assay for the amplification of the cytochrome B region

Prepare PCR Master Mix for 96, 48 or 1 50µl PCR reactions. Add reagents in the order presented.

96	48	1	Reagent
3.32 ml	1.66 ml	33.2 µl	sterile H_2O
1.0 ml	500 µl	10.0 µl	5X PCR Buffer
200 µl	100 µl	2.0 µl	dNTP (2 mM mix)
200 µl	100 µl	2.0 µl	cytbF (25 pmol/ µl) GGA CAA ATA TCA TTT TGA GGA GCA ACA G
200 µl	100 µl	2.0 µl	cytbR (25pmol/ µl) ATT ACT CCT CCT AGC TTA TTA GGA ATT G
60 µl	30 µl	0.6 µl	$MgCl_2$ (25mM)
20 µl	10 µl	0.2 µl	GoTaq DNA polymerase (5U/µl)
5.0 ml	**2.5 ml**	**50 µl**	**Total** (To each reaction add 1.0 µl of DNA)

Table 7.3.2.1. F and R indicate forward and reverse orientation.

PCR Cycle conditions

94°C/5min x 1 cycle
(94°C/30s , 50°C/30s , 72°C/1min) x 35 cycles
72°C/10min x 1 cycle
4°C hold

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red.
Primers create an approximately 459 bp band (**Figure 7.3.2.1**).
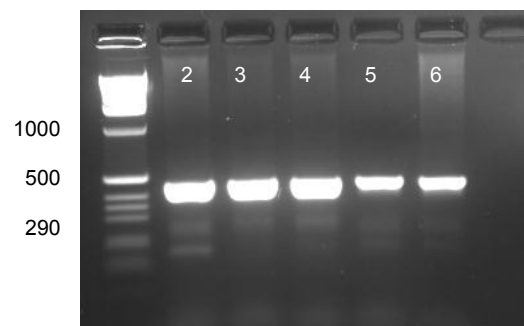
Figure 7.3.2.1. Lane 1 1Kb marker, lane 2 *An. atroparvus*, lane 3 *An. gambiae*, lane 4 *An. arabiensis*, lane 5 *An. funestus*, lane 6 *An. pharoensis*.

96 well PCR sample preparation template

References

Avise JC, J Arnold, RM Ball, E Bermingham, T Lamb, JE Neigel, CA Reeb, and NC Saunders. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Ann Rev Ecol Syst. 18: 489-522.

Krzywinski, J, RC Wilkerson, and NJ Besansky. 2001. Evolution of mitochondrial and ribosomal gene sequences in Anophelinae (Diptera: Culicidae): Implications for phylogeny reconstruction. Mol Phyl Evol. 18 (3): 479-487.

Simmons RB and SJ Weller. 2001. Utility and evolution of cytochrome *b* in insects. Mol Phyl Evol. 20 (2): 196-210.

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8.1.1 Single-step molecular identification of *Plasmodium* spp. in Anophelines
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Chapter 8 : Field Techniques

8.1 Plasmodium *Detection by PCR in Mosquitoes*

8.1.1 Single-step Molecular Identification of *Plasmodium* spp. in Anophelines

Alice Sutcliffe, Jessica Kissinger

Introduction

The standard method for molecular identification of *Plasmodium* spp. relies on ribosomal RNA gene targets in the 18S subunit and requires a nested PCR (Snounou *et al.* 1993) which can be time consuming, expensive and difficult to optimize (Mixson-Haden *et al.* 2010). Through data mining of *P. vivax* and *P. falciparum* genomes we have identified species specific targets, Pvr47 and Pfr364 respectively, which occur in 14 to 41 copies and can be used in a single or multiplex, non-nested PCR platform with high sensitivity (Demas *et al.* 2011). The single and multiplex PCR utilize the same *P. vivax* primer set but require an alternate primer pair for *P. falciparum*. The present protocol is based on the Qiagen® DNeasy® purification of total DNA from insects method, and identifies *P. falciparum*, *P. vivax* and mixed infections of these two species in individual mosquitoes. Alternative DNA extraction methods can likely be substituted but have not been tested with this protocol.

DNA Extraction

The Qiagen® DNeasy® blood and tissue kit (www.qiagen.com, cat. No. 69504 or 69506) minimizes DNA extraction time allowing this protocol to be completed in a minimum of approximately three hours. The Qiagen® DNeasy® purification of total DNA from insects method, beginning on page 4 was used with the following modifications:

> Step 8: Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided in kit), and pipet **100 µl** Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for **5 min**, and then centrifuge for 1 min at ≥6000 x g (8000 rpm) to elute.

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent	
1030 µl	515 µl	10.3 µl	sterile H$_2$O	
500 µl	250 µl	5.0 µl	5X GoTaq PCR Buffer	
300 µl	150 µl	2.5 µl	dNTP (2.5 mM mix)	
400 µl	200 µl	4.0 µl	MgCl$_2$ (25mM)	
100 µl	50 µl	1.0 µl	*P. vivax*	Pvr47F(F, 25pmol/µl) [CTGATTTTCCGCGTAACAATG]
100 µl	50 µl	1.0 µl		Pvr47R (R, 25pmol/µl) [CAAATGTAGCATAAAAATC**Y**AAG]
		OR		
100 µl	50 µl	1.0 µl	*P. falciparum*	Pfr364 (F, 25pmol/µl) [CCATTTTACTCGCAATAACGCTGCAT]
100 µl	50 µl	1.0 µl		Pfr364 (R, 25pmol/µl) [CTGAGTCGAATGAACTAGTCGCTAC]
20 µl	10 µl	0.2 µl	Go-Taq DNA polymerase (5units/ µl)	
2.5 ml	**1.25**	**25 µl**	**Total (To each 24 µl reaction add 1 µl template DNA)**	

---

[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

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8.1 *Plasmodium* Detection by PCR in Mosquitoes
8.1.1 Single-step molecular identification of *Plasmodium* spp. in Anophelines
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Table 8.1.1.1. F and R indicate forward and reverse orientation.

PCR cycle conditions
95°C/2min x 1 cycle
(95°C/30sec , *P. vivax* 54°C/30sec **OR** *P. falciparum* 57°C/30sec, 72°C/30sec) x 35 cycles
72°C/5min x 1 cycle
4°C hold

Run samples on a 2% agarose EtBr gel; load 5 µl sample.

Primers will create fragments of 333 bp for *P. vivax* (**Figure 8.1.1.1**) and 700 bp for *P. falciparum* (**Figure 8.1.1.2**).
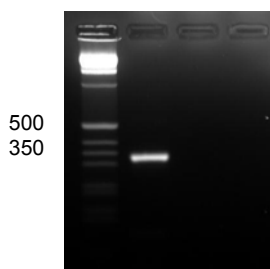
Figure 8.1.1.1. Lane 1 1kb ladder, lane 2 *P. vivax*, lane 3 negative control (mosquito DNA only), lane 4 blank control
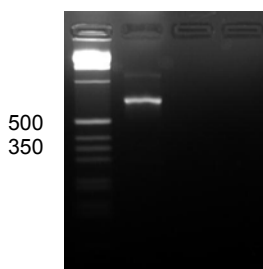
Figure 8.1.1.2. Lane 1 1kb ladder, lane 2 *P. falciparum*, lane 3 negative control (mosquito DNA only), lane 4 blank control

Multiplex PCR Identification of *Plasmodium vivax* and *Plasmodium falciparum*
Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[2] Add reagents in the order presented.

96	48	1	Reagent
1120 µl	560 µl	11.2 µl	sterile H_2O
500 µl	250 µl	5.0 µl	5X GoTaq PCR Buffer
40 µl	20 µl	0.4 µl	dNTP (2.5 mM mix)
400 µl	200 µl	4.0 µl	$MgCl_2$ (25mM)
60 µl	30 µl	0.6 µl	Pvr47F(F, 25pmol/µl) [CTGATTTTCCGCGTAACAATG]
60 µl	30 µl	0.6 µl	Pvr47R (R, 25pmol/µl) [CAAATGTAGCATAAAAATC**Y**AAG]
100 µl	50 µl	1.0 µl	Pfr364 AltF (F, 25pmol/µl) [CCGGAAATTCGGGTTTTAGAC]
100 µl	50 µl	1.0 µl	Pfr364 AltR (R, 25pmol/µl) [GCTTTGAAGTGCATGTGAATTGTGCAC]
20 µl	10 µl	0.2 µl	Go-Taq DNA polymerase (5units/ µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total (To each 24 µl reaction add 1 µl template DNA)**

Table 8.1.1.2. F and R indicate forward and reverse orientation.

---

[2] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

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8.1.1 Single-step molecular identification of *Plasmodium* spp. in Anophelines
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PCR cycle conditions
95°C/2min x 1 cycle
(95°C/30sec, 60°C/30sec, 72°C/30sec) x 35 cycles
72°C/5min x 1 cycle
4°C hold

Run samples on a 2% agarose EtBr gel; load 5 µl sample.

Primers will create fragments of 333 bp for *P. vivax* and 220 bp for *P. falciparum* (**Figure 8.1.1.3**).
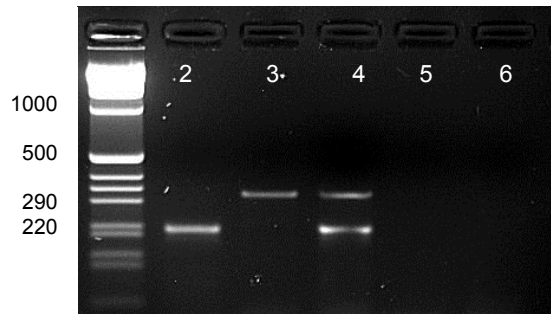
Figure 8.1.1.3. Lane 1 1kb ladder, lane 2 *P. falciparum*, lane 3 *P. vivax*, lane 4 *P. falciparum* and *P.vivax*, lane 5 negative control (mosquito DNA only), lane 6 blank control

References

Demas A, Oberstaller J, DeBarry J, Lucchi NW, Srinivasamoorthy G, Sumari D, Kabanywanyi AM, Villegas L, Escalante AA, Kachur SP, Barnwell JW, Peterson DS, Udhayakumar V, Kissinger JC (2011) Applied gemomics: data mining reveals species-specific malaria diagnostic targets more sensitive than 18S rRNA. J Clin Microbiol 49:2411-18

Mixson-Hayden T, Lucchi NW, Udhayakumar V (2010) Evaluation of three PCR-based diagnostic assays for detecting mixed *Plasmodium* infection. BMC Res Notes 61:315-20

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High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction.
Mol Biochem Parasitol 61:315-20

8.2 Plasmodium *Sporozoite ELISA*

Robert Wirtz, Melissa Avery, Mark Benedict, Alice Sutcliffe

Introduction

Enzyme-linked immunosorbent assays (ELISAs) were developed to detect *Plasmodium falciparum*, *P. vivax*-210, and *P. vivax*-247 circumsporozoite (CS) proteins in malaria-infected mosquitoes. The sensitivity and specificity of the ELISAs are based on the monoclonal antibodies (Mabs) used. The ELISAs detect CS proteins, which can be present in the developing oocysts, dissolved in haemolymph, and on sporozoites present in the haemocoel or in the salivary glands. A positive ELISA on a mosquito does not establish that species as a vector, and ELISA results may not be synonymous with salivary gland sporozoite dissections.

ELISAs can be carried out on fresh, frozen, or dried mosquitoes. If specimens are to be dried, they must be processed quickly and kept dry (stored with desiccant) to prevent microbial growth that can result in high background values. Before collection of the mosquitoes is initiated, consideration should be given to the possibility of conducting other tests (e.g., molecular, host blood meal, etc.) that may require different storage conditions or extraction buffers. Voucher specimens should also be collected and saved.

The "sandwich" ELISA is begun by adsorption of the capture Mab to the wells of a microtiter plate (**Figure 3.3.1**). After the capture Mab has bound to the plate, the well contents are aspirated and the remaining binding sites are blocked with blocking buffer. Mosquitoes to be tested are ground in blocking buffer containing IGEPAL CA-630, and an aliquot is tested. Positive and negative controls are also added to specific plate wells at this time. If CS antigen is present (depicted as diamond in Fig. 1.B) it will form an antigen-antibody complex with the capture mAb. After a 2-hour incubation at room temperature, the mosquito homogenate is aspirated and the wells are washed. Peroxidase-linked Mab is then added to the wells, completing the formation of the "sandwich" (Fig. 1.C). After 1 hour, the well contents are aspirated, the plate is washed again and the clear peroxidase substrate solution is added (Fig 1.D). As the peroxidase enzyme reacts with the substrate, a dark green product is formed (Fig 1.D), the intensity of the color is proportional to the amount of CS antigen present in the test sample.

Results are read visually or at 405-414 nm using an ELISA plate reader 30 and/or 60 minutes after the substrate has been added.

The assay involves 2 steps:

1. Screening phase where the ELISA is used to identify positive samples (Worksheet 1).

2. Quantification ELISA - where the ELISA positive samples from the initial screening are retested to: a) confirm positives and b) quantify the amount of CS protein per sample (Worksheet 2).

If it is necessary to test for all three Plasmodium species (Pf, Pv210 and Pv247) these tests can be run concurrently on 3 plates or if there is not enough material, the 3 assays may be run consecutively.

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Figure 8.2.1. The "sandwich" ELISA for detection of *Plasmodium falciparum* and *P. vivax* circumsporozoite proteins.

A. Anti-sporozoite monoclonal antibody adsorbed to the plate
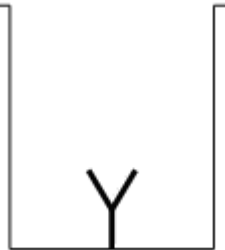
B. Blocking buffer added to prevent non-specific binding
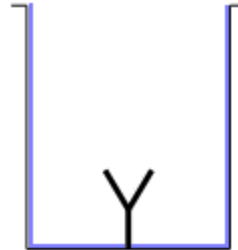
C. Mosquito triturate added to the well.
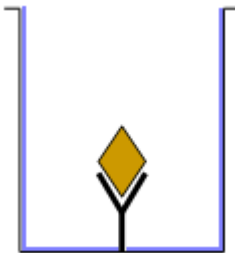
D. Peroxidase-linked anti-sporozoite monoclonal antibody added.
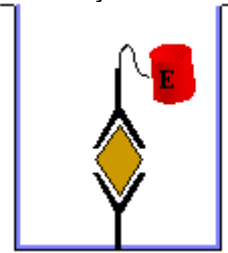
E. ABTS substrate added to the wells.

If CS antigen Present

.
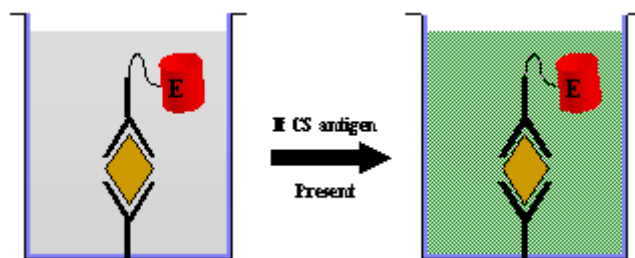
Sporozoite ELISA Solutions, Capture and Conjugate

PHOSPHATE BUFFERED SALINE (PBS), pH 7.2: Use stock laboratory PBS OR Dulbecco's PBS (Sigma #D5773); adjust pH if necessary. Add 0.01 gm phenol red or 100μl of phenol red stock solution (1gm/10ml water)/L PBS. Store 4°C; shelf life is 2 weeks.

BLOCKING BUFFER (BB): Shelf life is 1 week at 4°C; BB may be frozen. Use only ELISA grade Sigma casein.**BB** – blocking buffer: Use only ELISA grade casein (Sigma C7078). Shelf life is 1 week at 4°C; BB may be frozen.

Boiled casein (BB):	½ liter	1 liter
PBS, pH 7.4	450 ml	900 ml
casein	2.5 g	5.0 g
0.1 N NaOH	50 ml	100 ml
phenol red*	100 μl	200 μl

1. Bring to a boil the 0.1 N NaOH and slowly add the casein (Sigma C7078).

2. After casein is dissolved, allow to cool and slowly add the PBS, adjust the pH to ~7.4 with HCl, and add the phenol red.

*A stock solution of phenol red (1g/10ml water) eliminates the need to weigh out small amounts of this material. Phenol red added to BB is only used as a dye to aid in visualization and is optional.

Grinding Buffer and Mosquito Sample Preparation: Shelf life at 4°C is 1 week.

> Grinding solution for approximately one plate:
> 1. Combine 25ml of BB and 125µl of Igepal CA-630
> 2. Mix well to dissolve the Igepal CA-630 in the BB.
> (Note: IGEPAL CA-630 (Sigma I3021) replaces NONIDET P-40, no longer available from SIGMA-ALDRICH. If available, NONIDET P-40 can be used.)
>
> To Grind:
> 1. Place the mosquito, head-thorax only, of no more than 10 pooled mosquitoes in a labeled 1.5ml micro centrifuge grinding tube
> 2. Add 50µl of grinding buffer.
> 3. Grind well
> 4. Rinse the pestle with two 100µl volumes of Grinding Solution , catching the rinses in the tube containing the mosquito triturate. **Final volume will be 250μl**. Test or freeze for later use.
> 5. Rinse the pestle in PBS-Tween twice; dry with tissue to prevent contamination between mosquitoes.

Wash Solution (PBS-Tween): PBS plus 0.05% Tween 20. Add 0.5 ml Tween 20 to 1 liter of PBS. MIX WELL. Store at 4°C. Shelf life is 2 weeks.

Monocolonal Antibodies (mAb) Capture and Conjugate: Monoclonal antibodies (mAb) capture and conjugate received from KPL will be lyophilized. Label will list the amount of glycerol water to be added. Glycerol water is a 1:1 mixture of distilled water and glycerol. Glycerol water allows for storage at -20°C without freeze-thawing. This step only needs to be performed when a new vial of capture or conjugate needs to be reconstituted.

Notes and Troubleshooting for Sporozoite ELISAs

1. Do not add sodium azide to solutions as it is a peroxidase inhibitor. We no longer add thimerosal to the solutions, as this is mercury-based and presents problems with proper disposal.

2. To fill each of the 96 wells on a plate with 50µl requires 4.8ml. It is convenient to make up 5.0ml of each mAb solution and 10.0ml of substrate (100µl/well) per plate.

Species	mAb	µg/50µl/WELL	µg/5ml	µl STOCK/5ml PBS
Pf	capture	0.200	20.0	40
Pv - 210	capture	0.025	2.5	5
Pv - 247	capture	0.025	2.5	5
				µl STOCK/5ml BB
Pf	peroxidase	0.050	5.0	10
Pv - 210	peroxidase	0.050	5.0	10
Pv - 247	peroxidase	0.050	5.0	10

3. Cover plate during incubations to prevent evaporation. Incubate plate in the dark (especially for incubation of peroxidase conjugate and substrate solution). An efficient way of doing this is to use a small cardboard box lid or to line the lid of a pipette tip box with aluminum foil.

4. ELISAs can be carried out on fresh, frozen or dried mosquitoes. If specimens are to be dried, they must be processed quickly and kept dry (store with desiccant) to prevent microbial growth that can result in high background values in the ELISAs. Once identified to species, mosquitoes should be pooled in groups of maximum 10 and stored at -20°C or dried over silica gel until they can be processed. Store the triturate at -20°C until tests are to be performed.

5. Negative controls: Triturate laboratory reared, known uninfected female mosquitoes (same as test species if possible) in 50µl BB:IG-630, dilute with 150µl BB (total volume 200µl) and place 50µl from each into negative control wells 1B-1H for initial testing or wells 1A-1H for conformational testing.   To determine the negative cut off value for initial testing calculate mean on these 7 negative controls and retest any mosquito with a value two times the mean.

6. Phenol red added to BB is only used as a dye to aid in visualization and is optional.

7. Only high, lab grade paper towels should be used while performing CS-ELISA testing, particularly to "bang" the ELISA plate on, to remove excess solutions. Use of brown paper towels and kitchen paper towels should be avoided as the result is high background values. It is thought that fibers from these towels adhere to the plates and interfere with detection. If high, lab grade paper towels are not available, perform this step over the sink or ensure careful removal of all solutions with a vacuum system for each required step.

8. Do not vortex samples as this can lead to high background and inaccurate absorbance values. Short centrifugation can but used to settle body parts and allow pipetting of a clean sample. This centrifugation will not pellet sporozoites and they will remain suspended in the supernatant.

9. Wipe the bottom of the ELISA plate with alcohol to remove debris and oils from ELISA plate before reading. Fingerprints, oils and debris on the plate can lead to inaccurate absorbance values.

10. BB should be placed in wells without negative mosquito controls, positive controls, or samples as "filler".

11. The Corning® 96 well clear round bottom PVS (soft plate) cannot be reliably used with certain, especially newer, models of plate readers. This is due to distortion of the plate upon entering the reader carriage or a poor fit of plate in the reader carriage. This can be solved by using Costar 96-Well EIA/RIA plates (Round well; high binding), available from www.fishersci.com see supplies list).

Dilutions for Positive Control Antigens for CS-ELISA

Serial dilutions are easily prepared by one of the two following methods:

1. All working concentrations required can be prepared and labeled ahead of time. When addition of positive controls to wells of ELISA plate is required, 50μl of each solution is added. This method is suited for inexperienced pipetters.

    Label microfuge tubes with solution number and prepare sequentially according to chart. Remaining volumes can be stored at -20ºC and used again when necessary.

Plasmodium species	Solution Number	Volume of Positive Control Antigen	Volume of Blocking Buffer (μl)	Antigen Concentration (pg/50μl)
P. falciparum	Stock	Lyophilized Pf	1000	500,000
	I	20μl of stock	1000	5,000
	*II	10μl of I	500	100
	*III	500μl of II	500	50
	*IV	500μl of III	500	25
	*V	500μl of IV	500	12
	*VI	500μl of V	500	6
	*VII	500μl of VI	500	3
	*VIII	500μl of VII	500	1.5
	*IX	0 (Blank)	500	0 (Blank)
P. vivax 210	Stock	Lyophilized Pv210	500	500,000
	A	10μl of stock	1000	5,000
	B	20μl of A	500	200
	*C	200μl of B	800	40
	*D	500μl of C	500	20
	*E	500μl of D	500	10
	*F	500μl of E	500	5
	*G	500μl of F	500	2.5
	*H	500μl of G	500	1.25
	* I	500μl of H	500	0.6
	*J	0 (Blank)	500	0
P. vivax 247	Stock	Lyophilized Pf	1000	227,500
	*A	20μl of stock	1000	4,450
	*B	500μl of I	500	2,275
	*C	500μl of II	500	1,140
	*D	500μl of III	500	570
	*E	500μl of IV	500	285
	*F	500μl of V	500	140
	*G	500μl of VI	500	70
	*H	0 (Blank)	500	0 (Blank)

* Amounts used in ELISA plate wells for the quantitative assays

2. The highest concentration to be used on ELISA plate is prepared ahead of time. When addition of positive controls to wells of ELISA plate is required, serial dilutions are made directly on the plate. This requires very accurate and advanced pipetting skills.

    Positive controls (be sure to label all vials):
    ### *P. falciparum*:
    STOCK: Add 1000µl BB to lyophilized positive control (5µg) (do not use glycerol:water or water alone) to give 10,000pg/µl BB.
    Vial I = Transfer 20µl (200,000pg) of STOCK to 1000µl BB to for 100pg/µl BB.
    Vial II = Transfer 10µl (1000pg) of Vial I to 500µl BB for 2pg/µl or 100 pg/50µl BB

    Begin in well 2A as wells 1A-1H should be reserved for 8 negative control mosquitoes when retesting. Freeze the stock solution and Vials I and II for continued use.
    Use 100µl **Vial II** in well 2A and add 50µl BB to wells 2B through 2H. Pipette 50µl from well 2A and add to well 2B. Continue this serial dilution through well 2H and discard 50µl from well 2H so each well contains 50µl of BB + diluted positive control.
    Concentrations of positive controls are 100, 50, 25, 12, 6, 3, 1.5 and 0pg/50µl BB (starting with 2A and finishing with 2H)
    Run standard curve in triplicate (wells 2A-4A to 2H-4H) as well as 8 negative control mosquitoes (wells 1A-1H) when retesting.

    ### *P. vivax*-**210**:
    STOCK = Add 500µl BB to lyophilized positive control (5µg) (do not use glycerol:water or water alone) to give 10,000pg/µl BB.
    Vial A = Transfer 10µl (100,000pg) of STOCK to 1,000µl BB for 100pg/µl BB.
    Vial B = Transfer 20µl (2,000pg) of Vial A to 500µl BB for 4pg/µl BB.
    Vial C = Transfer 200µl (800pg) of Vial B to 800µl BB for 0.4pg/µl or 40 pg/50µl BB.

    Begin in well 2A as wells 1A-1H should be reserved for 8 negative control mosquitoes when retesting. Freeze the stock solution and Vials I and II for continued use.
    Use 100µl **Vial C** in well 2A and add 50µl BB to wells 2B through 2H. Pipette 50µl from well 2A and add to well 2B. Continue this serial dilution through well 2H and discard 50µl from well 2H so each well contains 50µl of BB + diluted positive control.
    Concentrations of positive controls are 40, 20, 10, 5, 2.5, 1.25, 0.6 and 0pg/50µl BB (starting with 2A and finishing with 2H)
    Run standard curve in triplicate (wells 2A-4A to 2H-4H) as well as 8 negative control mosquitoes (wells 1A-1H) when retesting.

    ### *P. vivax*-**247**:
    STOCK = Add 1000 µl BB to lyophilized positive control (4.55ug) (do not use glycerol:water or water alone) to give 4,550pg/µl BB .
    Vial 1 = Transfer 20µl (91,000pg) of STOCK to 1,000µl BB to give 4,550pg/µl or BB.

    Begin in well 2A as wells 1A-1H should be reserved for 8 negative control mosquitoes when retesting. Freeze the stock solution and Vials I and II for continued use.
    Use 100µl **Vial 2** in well 2A and add 50µl BB to wells 2B through 2H. Pipette 50µl from well 2A and add to well 2B. Continue this serial dilution through well 2H and discard 50µl from well 2H so each well contains 50µl of BB + diluted positive control.
    Concentrations of positive controls are 4450, 2275, 1140, 570, 285, 140, 70 and 0pg/50µl BB (starting with 2A and finishing with 2H)
    Run standard curve in triplicate (wells 2A-4A to 2H-4H) as well as 8 negative control mosquitoes (wells 1A-1H) when retesting.

Sporozoite ELISA Directions

Please be sure to read the notes preceding and following these directions before beginning.

1. Fill out the top portion of the appropriate Sporozoite ELISA worksheet. Mark the ELISA plate in order to maintain correct plate orientation.

2. Prepare a working solution of mAb capture by adding **PBS** to the reconstituted capture mAb based on the volumes by species listed below. Vortex gently.

Species	mAb	μg/50μl/WELL	μg/5ml	μl STOCK/5ml
Pf	capture	0.200μg/50μl	20.0μg	40μl stock+ 5ml **PBS**
Pv-210	capture	0.025μg/50μl	2.5μg	5μl stock+ 5ml **PBS**
Pv-247	capture	0.025μg/50μl	2.5μg	5μl stock+ 5ml **PBS**

3. Place 50μl of mAb solution made in step 2 in each well of the ELISA plate. Use a separate plate for each sporozoite species.

4. Cover plate and incubate for **0.5 HOUR** room temperature.

0.5 HOUR

5. Aspirate well contents and bang plate upside down on paper towel 5 times, holding sides only. Note: If aspiration system is not available, bang plate on sink edge into the sink and then again on paper towels. **Do not use brown paper towels**.

6. Fill wells with 200μl BB

7. Cover plate, leaving space between well and top of lid. Incubate for **1 hour** at room temperature.

1 HOUR

8. Aspirate well contents and bang plate upside down on paper towel 5 times holding sides only.

9. Load samples and controls into the plate (initial and quantitative tests).
-Use 9a when **first testing** the samples to determine if there are positives
-Use 9b when **retesting, confirming and quantifying** any positives from the initial/first test.

    9a. **Initial test** see Worksheet 1 (plate #1, typically Day 1):
        i. Add 50μl of positive controls to well A1. See the notes on 6 and 7 for positive control dilutions. (Pf = Vial II, Pv210= Vial C, Pv247= Vial 2)
        ii. Add 50μl of negative control(s) to wells B1-H1.
        iii. Add 50μl of mosquito triturate per well to remaining wells.
        iv. Cover and incubate **2 hours**
    b. **Quantification test** see worksheet 2 (plate #2, typically Day 2):
        i. Add 50μl of negative control(s) to wells A1-H1.
        ii. Add 50μl last vial dilution of positive control to wells A2, A3, and A4 and 50μl of each serial dilution to wells B2-H2, B3-H3 and B4-H4. See pages 7 and 8 for positive control dilutions. (Pf = Vial II, Pv210= Vial C, Pv247= Vial 2)
        iii. Add 50ul of mosquito triturate per well to remaining wells.
        iv. Cover and incubate **2 hours**

Please note, steps 10-12 can be performed just before the end of the 2 hour incubation

2 HOURS

10. Prepare substrate by mixing Substrate A and Substrate B at a 1:1 ratio. A full 96-well plate will be 5ml of Substrate A + 5ml of Substrate B

11. Prepare a working solution of mAb conjugate by adding **BB** to the reconstituted capture mAb based on the volumes by species listed below. Vortex gently.

Species	mAb	ug/50 μl/WELL	μg/5ml	μl STOCK/5ml
Pf	peroxidase	0.050μg/50μl	5.0μg	10μl stock + 5ml **BB**
Pv-210	peroxidase	0.050μg/50μl	5.0μg	10μl stock + 5ml **BB**
Pv-247	peroxidase	0.050μg/50μl	5.0μg	10μl stock + 5ml **BB**

12. Check enzyme activity by mixing 5μl of the mAb conjugate made in step 11 with 100μl of the substrate made in step 10 in a separate tube. Vortex gently. There should be rapid color change indicating that the peroxidase enzyme and the substrate are functional.

13. Aspirate well contents bang plate upside down on paper towel 5 times holding sides only.

14. Wash wells two (2) times with 200μl of PBS-Tween, aspirating and banging plate 5 times with each wash.

15. Add 50μl of peroxidase conjugate solution made in step 11 to each well.

16. Cover and incubate **one (1) hour**

1 HOUR

17. Aspirate well contents and bang plate upside down on paper towel 5 times holding sides only.

18. Wash wells 3 times with 200μl of PBS-Tween, aspirating and banging plate 5 times with each wash.

19. Add 100μl substrate solution per well.

20. Cover plate and incubate **thirty (30) minutes.** Handle plate carefully to avoid splashing.

0.5 HOUR

21. Read visually, or at 405-414nm

22. Cover plate and incubate an additional **thirty (30) minutes** if testing Pv-210 or Pv-247 species, for a total incubation time of 1 hour.

0.5 HOUR

23. Read visually, or at 405-414nm

WORKSHEET 1: ELISA Template for Screening (Initial test)

ELISA Plate No:_____   **DATE:** _____

Capture mAb: Lot #_____   Peroxidase-mAb: Lot #:_____

Positive control: Lot#_____

NEG

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Pos**											
B	**Neg**											
C	**Neg**											
D	**Neg**											
E	**Neg**											
F	**Neg**											
G	**Neg**											
H	**Neg**											

_____ 1) Coat PVC plate with **50μl** capture mAb.

→ **0.5 hr incubation**

_____ 2) Aspirate wells; fill wells with **200μl** blocking buffer (BB).

→ **1 hr incubation**

_____ 3) Aspirate wells; Add **50μl** mosquito triturate and positive control

→ **2 hr incubation**

_____ 4) Aspirate and wash two times with 200μl PBS-0.05% Tween 20.

_____ 5) Add **50μl** peroxidase-mAb. Mix substrate (1:1).

→ **1 hr incubation (in the dark)**

_____ 6) Aspirate and wash 3 times with 200μl PBS-0.05% Tween 20.

_____ 7) Add **100μl** substrate: _____ a) Enzyme check; _____ b) 100μl/well.

→ **0.5 hr incubation (in the dark)**

_____ 8) Read absorbency 405nm.

Analysis:
Samples which have OD values above the cut-off (cut-off = 2 X mean OD of negative samples)
are considered positive and should be followed up with quantitative testing.

WORKSHEET 2: ELISA Template for Quantitative Testing (Quantification Test)

ELISA Plate No:_____   DATE: _____

Capture mAb: Lot #_____   Peroxidase-mAb: Lot #:_____

Positive control: Lot#_____

	NEG	POS. CONTROL										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

_____ 1) Coat PVC plate with **50μl** capture mAb.

→ **0.5 hr incubation**

_____ 2) Aspirate wells; fill wells with **200μl** blocking buffer (BB).

→ **1 hr incubation**

_____ 3) Aspirate wells; Add **50μl** mosquito triturate and positive control

→ **2 hr incubation**

_____ 4) Aspirate and wash 2 times with 200μl PBS-0.05% Tween 20.

_____ 5) Add **50μl** peroxidase-mAb. Mix substrate (1:1).

→ **1 hr incubation (in the dark)**

_____ 6) Aspirate and wash 3 times with μlPBS - 0.05% Tween 20.

_____ 7) Add **100μl** substrate: _____ a) Enzyme check; _____ b) 100 μl/well.

→ **0.5 hr incubation (in the dark)**

_____ 8) Read absorbency 405nm.

Analysis: Compare to standard curve from positive control antigen stocks to estimate CS antigen in each sample. From this estimate, calculate the equivalent number of sporozoites for that sample by chart comparison.

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8.3 Molecular Identification of Mammalian Blood Meals from Mosquitoes

Christen M Fornadel, Rebekah J Kent, Douglas E Norris

Introduction

Identification of blood meals is an important step in understanding mosquito ecology. The following protocol was developed to differentiate between a select group of potential mammal host bloods in engorged anophelines, but may be adapted or expanded to suit particular needs (i.e. most often an expanded or altered host list). This protocol was designed for use on genomic DNA extractions of mosquito abdomens.

Initial Blood Meal Identification PCR (Kent and Norris 2005; Kent et al. 2007)

This multiplexed PCR produces species-specific fragments of varying sizes amplified from the cytochrome b gene, encoded in the mitochondrial genome. Host DNA is detectable up to 24-30 hours post feeding.

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent	Product size (bp)
1.83 ml	915 µl	18.3 µl	sterile H2O	
250 µl	125 µl	2.5 µl	Taq 10X PCR Buffer with MgCl2	
100 µl	50 µl	1.0 µl	dNTP (final concentration of 100 µM of each dNTP)	
50 µl	25 µl	0.5 µl	UnRev1025 (50 pmol/µl) [ggttg[t/g]cctccaattcatgtta]	
50 µl	25 µl	0.5 µl	Pig573F (50 pmol/µl) [cctcgcagccgtacatctc]	453
50 µl	25 µl	0.5 µl	Human741F (50 pmol/µl) [ggcttacttctcttcattctctcct]	334
50 µl	25 µl	0.5 µl	Goat894F (50 pmol/µl) [cctaatcttagtacttgtacccttcctc]	132
50 µl	25 µl	0.5 µl	Dog368F (50 pmol/µl) [ggaattgtactattattcgcaaccat]	680
50 µl	25 µl	0.5 µl	Cow121F (50 pmol/µl) [catcggcacaaatttagtcg]	561
20 µl	10 µl	0.2 µl	Taq DNA polymerase (5 U/µl)	
2.5 ml	**1.25 ml**	**25 µl**	Total	

For DNA template use up to 3 µl DNA sample (from abdomen extraction eluted in 50µl dH$_2$0)

PCR Cycle conditions

95°C/5min x 1 cycle
(95°C/60sec , 56°C/60sec , 72°C/60sec) x 40 cycles
72°C/7min x 1 cycle
4°C hold

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 reactions to compensate for imprecise measurements.

Small Blood Meal PCR and Enzyme Digest (Fornadel and Norris 2008)

For samples that fail to produce a reaction product with the multiplexed PCR the following small blood meal PCR/enzyme digest may be used. This assay allows identification of host source from partially digested blood meals (out to 60 hours post feeding), as well as from partially degraded DNA extractions. The PCR was designed to produce a small 98bp amplicon from the mammals tested above, which can then be incubated with specific restriction enzymes to determine host source.

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions[1]. Add reagents in the order presented.

96	48	1	Reagent	size (bp)
1.83 ml	915 µl	18.3 µl	sterile H2O	
250 µl	125 µl	2.5 µl	Taq 10X PCR Buffer with MgCl2	
100 µl	50 µl	1.0 µl	dNTP (final concentration of 100 µM of each dNTP)	
50 µl	25 µl	0.5 µl	UnRev1025 (50 pmol/µl) [ggttgtcctccaattcatgtta]	
50 µl	25 µl	0.5 µl	UniForA (50 pmol/µl) [tccaaacaac[a/g][a/c]agcataatatt]	98
20 µl	10 µl	0.2 µl	Taq DNA polymerase (5 U/µl)	
2.3 ml	**1.15 ml**	**23 µl**	Total	

For DNA template use 2 µl DNA sample (from abdomen extraction eluted in 50µl dH$_2$0)

Save 6 µl of the PCR products to run as undigested controls on a 3% agarose gel alongside the digested amplicons.
Note: The master mix can be tripled for a total reaction volume of 78 µl. This will allow one to perform up to 4 digests of the amplified product with enough undigested sample leftover as a control.

PCR Cycle conditions

95°C/5min x 1 cycle
(95°C/60sec , 55°C/60sec , 72°C/60sec) x 40 cycles
72°C/7min x 1 cycle
4°C hold

Restriction Enzyme Digests

The following digests can be performed in whichever order/combination provides the best efficiency for the samples under study.

Host	Cut position	Enzyme	Recognition Seq.
Human	59	Fnu4HI	GC^N_GC
Cow	54	BanII	G_RGCY^C
Dog	24	MspI	C^CG_G
Goat	43	NsiI	A_TGCA^T
Pig	54	SpeI	A^CTAG_T

Restriction Digest Reaction

Per reaction (1)	Reagent
7.5 µl	sterile H2O
2.5 µl	Taq 10X PCR Buffer with MgCl2
1-2 U	Enzyme
15 µl	PCR product
.025 µl only for SpeI digests	BSA 100X
~25 µl	**Total**

All digests are carried out at 37°C for at least 3hrs but can be left overnight.

96 well PCR sample preparation template

References

Fornadel CM, Norris DE (2008) Increased endophily by the malaria vector *Anopheles arabiensis* in southern Zambia and identification of digested blood meals. Am J Trop Med Hyg 79:876-880

Kent RJ, Norris DE (2005) Identification of mammalian blood meals in mosquitoes by a multiplexed polymerase chain reaction targeting cytochrome B. Am J Trop Med Hyg 73:336-342

Kent RJ, Thuma PE, Mharakurwa S, Norris DE (2007) Seasonality, blood feeding behavior, and transmission of *Plasmodium falciparum* by *Anopheles arabiensis* after an extended drought in southern Zambia. Am J Trop Med Hyg 76:267-274

8.4 Species Complex Authentication by PCR

8.4.1 *Anopheles gambiae* complex (Scott et al.)

MR4 Staff

Introduction

The *Anopheles gambiae* complex is comprised of 7 cryptic species: *An. gambiae s.s, An. arabiensis, An. bwambe, An. melas, An. merus, An. quadriannulatus* A and B, some of which are sympatric. Distinguishing the members of the complex was first done based on karyotype and chromosomal inversions (Coluzzi et al. 1979). Recently, however, easier PCR based assays have been developed that distinguished several of the members based on species-specific single nucleotide polymorphisms (SNPs) in the intergenic spacer region (IGS) (Scott et al. 1993; Fettene and Temu 2003; Besansky et al. 2006; Wilkins et al. 2006). A good overview and the fine points of this assay has been published (Cornel and Collins 1996). Alternatives to this assay can be found in **Chapters 8.4.3** and **8.5.1.1**.

PCR authentication for the members of the *Anopheles gambiae* complex (Scott et al. 1993)

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
835 µl	417.5 µl	8.35 µl	sterile H_2O
500 µl	250 µl	5.0 µl	GoTaq 5X PCR Buffer with $MgCl_2$
250 µl	125 µl	2.5 µl	dNTP (2.5 mM mix G,A,T,C)
100 µl	50 µl	1.0 µl	$MgCl_2$ (25 mM)
100 µl	50 µl	1.0 µl	UN (F, 25 pmol/µl) [GTGTGCCCCTTCCTCGATGT]
100 µl	50 µl	1.0 µl	AR (R, 25 pmol/µl) [AAGTGTCCTTCTCCATCCTA]
100 µl	50 µl	1.0 µl	GA (R, 25 pmol/µl) [CTGGTTTGGTCGGCACGTTT]
100 µl	50 µl	1.0 µl	ME (R, 25 pmol/µl) [TGACCAACCCACTCCCTTGA]
200 µl	100 µl	2.0 µl	QD (R, 25 pmol/µl) [CAGACCAAGATGGTTAGTAT] OR
200 µl	100 µl	2.0 µl	QDA (R, 25 pmol/µl) [CATAATGAGTGCACAGCATA])
15 µl	7.5 µl	0.15 µl	Taq DNA polymerase (5 U/µl)
2.5 ml	**1.25 ml**	**24 µl**	**Total (To each 24 ul reaction add 1 µl template DNA)**

Table 8.4.1.1. F and R indicate forward and reverse orientation. It may improve specificity to leave out primers for species that do not occur in the area of sample collection. If removing a primer, replace primer volume with an equal volume of sterile water. Use the QDA primer instead of the QD primer in areas where *An. merus* and *An. quadriannulatus* are sympatric. Use 1 µl DNA template.

PCR cycle conditions

95°C/5min x 1 cycle
(95°C/30sec , 50°C/30sec , 72°C/30sec) x 30 cycles
72°C/5min x 1 cycle
4°C hold

---

[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red; load 5 µl sample (**Figure 8.4.1.1**). Primers create fragments of 153bp (QD) or 415bp (QDA) *An. quadriannulatus*, 464bp/466bp *An. melas/merus*, 390bp *An. gambiae*, 315bp *An. arabiensis*.

References

Besansky NJ, Collins FH, Townson H (2006) A species-specific PCR for the identification of the malaria vector *Anopheles bwambae*. Ann Trop Med Parasitol 100:277-280

Coluzzi M, Sabatini A, Petrarca V, Di Deco MA (1979) Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. Trans Roy Soc Trop Med Hyg 73:483-497

Cornel AJ, Collins FH (1996) PCR of the ribosomal DNA intergenic spacer regions as a method for identifying mosquitoes in the *Anopheles gambiae* complex. In: Clapp JP (ed) Methods in Molecular Biology: Species Diagnostic Protocols: PCR and other nucleic acid methods. Humana Press, Totowa NJ, pp 321-332

Fettene M, Temu EA (2003) Species-specific primer for identification of *Anopheles quadriannulatus sp. B* (Diptera: Culicidae) from Ethiopia using a multiplex polymerase chain reaction assay. J. Med. Entomol. 40:112-115

Scott JA, Brogdon WG, Collins FH (1993) Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am J Trop Med Hyg 49:520-529

Wilkins EE, Howell PI, Benedict MQ (2006) IMP PCR primers detect single nucleotide polymorphisms for *Anopheles gambiae* species identification, *Mopti* and *Savanna* rDNA types, and resistance to dieldrin in *Anopheles arabiensis*. Malar J 5:125

1000

500

390

290

Figure 8.4.1.1. An. gambiae species ID. Lane 1, *An. gambiae*, Lane 2, *An. arabiensis*, Lane 3, 1 kb ladder.

96 well PCR sample preparation template

8.4.2 *An. gambiae* Ribosomal DNA Type (Fanello et al.)

MR4 Staff

Introduction

The Fanello et al. (2002) method for differentiating between the rDNA types of *An. gambiae* uses a restriction enzyme, *Hha I*, to digest the PCR product from the Scott et al. assay (1993) discussed in *Anopheles gambiae* complex authentication section. The *HhaI* enzyme will specifically digest the S form leaving 2 discernable bands while the M form will only have 1 discernable band. A small 23 bp band is also created, but it is not seen on standard agarose gels. An alternate PCR based method is presented in **Chapter 8.4.3**.

PCR discrimination of M and S members of the* Anopheles gambiae *complex (Fanello et al. 2002)

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
1235 µl	617.5 µl	12.35 µl	sterile H_2O
500 µl	250 µl	5.0 µl	GoTaq 5X PCR Buffer
250 µl	125 µl	2.5 µl	dNTP (2.5 mM mix)
100 µl	50 µl	1.0 µl	UN (F, 25 pmol/µl) [GTGTGCCCCTTCCTCGATGT]
100 µl	50 µl	1.0 µl	GA (R, 25 pmol/µl) [CTGGTTTGGTCGGCACGTTT]
100 µl	50 µl	1.0 µl	AR (R, 25 pmol/µl) [AAGTGTCCTTCTCCATCCTA][2]
100 µl	50 µl	1.0 µl	$MgCl_2$ (25 mM)
15 µl	7.5 µl	0.15 µl	Taq DNA polymerase (5 U/µl)
2.4 ml	**1.2 ml**	**24 µl**	**Total (To each 24 ul reaction add 1 µl template DNA)**

Table 8.4.2.1. F and R indicate forward and reverse orientation. Use 1 µl DNA template.

PCR cycle conditions

94°C/5min x 1 cycle
(94°C/30sec -o- 50°C/30sec -o- 72°C/30sec) x 30 cycles
72°C/5min x 1 cycle
4°C hold

Restriction enzyme digest

Add 0.5 µl *HhaI* restriction enzyme to 10 µl PCR product from above reaction. Allow to incubate at 37°C for 3-24 hr. For shorter times, incomplete digests could be a problem.

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red. Primers create fragments of 397 bp for M and 225 and 110bp for S forms (**Figure 8.4.2.1**).

---

[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.
[2] Arabiensis primer not necessary if certain of working with gambiae strain.

Figure 8.4.2.1. Gel electrophoresis of M/S rDNA assay.
Lanes 2-5 and 6-9 contain M and S PCR products
respectively. Lanes 1 and 10 contain 1kb ladder marker.
Photo from (Wilkins et al. 2006); used with permission.

References

Fanello C, Santolamazza F, della Torre A (2002) Simultaneous identification of species and molecular forms of *Anopheles gambiae* complex by PCR-RFLP. Med Veterinary Entomol 16:461-464

Scott JA, Brogdon WG, Collins FH (1993) Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am J Trop Med Hyg 49:520-529

Wilkins EE, Howell PI, Benedict MQ (2006) IMP PCR primers detect single nucleotide polymorphisms for *Anopheles gambiae* species identification, *Mopti* and *Savanna* rDNA types, and resistance to dieldrin in *Anopheles arabiensis*. Malar J 5:125

96 well PCR sample preparation template

8.4.3 Combined *An. gambiae* complex and ribosomal DNA type assay for M/S discrimination

Liz Wilkins

Introduction

The Wilkins et al. (2006) method of *Anopheles gambiae* complex discrimination is based on species-specific single nucleotide polymorphisms (SNPs) in the intergenic spacer region (IGS) (**Chapter 8.4.1**). We have added primers to this method to simultaneously elucidate the Ribosomal DNA type. These additional primers also incorporate the intentional mismatches into the primers (Intentional Mismatch Primers (IMPs)) to increase the specificity (Wilkins et al. 2006). An RT-PCR based method is also available in **Chapter 8.5.1.1**.

PCR authentication for the members of the *Anopheles gambiae* complex (Wilkins et al. 2006) with additional primers for Ribosomal DNA type

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	0.5	Reagent
910 µl	455 µl	9.1 µl	4.55 µl	sterile H$_2$O
500 µl	250 µl	5.0 µl	2.5 µl	5X GoTaq PCR Buffer
250 µl	125 µl	2.5 µl	1.25 µl	dNTP (2.5 mM mix)
30 µl	15 µl	0.3 µl	0.15 µl	MgCl$_2$ (25mM)
100 µl	50 µl	1.0 µl	0.5 µl	IMP-UN (F, 25pmol/µl) [GCTGCGAGTTGTAGAGATGCG]
100 µl	50 µl	1.0 µl	0.5 µl	AR-3T (R, 25pmol/µl) [GTGTTAAGTGTCCTTCTCCgT**C**]
100 µl	50 µl	1.0 µl	0.5 µl	GA-3T (R, 25pmol/µl) [GCTTACTGGTTTGGTCGGCAtG**T**]
100 µl	50 µl	1.0 µl	0.5 µl	ME-3T (R, 25pmol/µl) [CAACCCACTCCCTTGACGaT**G**]
200 µl	100 µl	2.0 µl	1.0 µl	QD-3T (R, 25pmol/µl) [GCATGTCCACCAACGTAAAtC**C**]
100 µl	50 µl	1.0 µl	0.5 µl	IMP-S1 (R, 25pmol/µl) [CCAGACCAAGATGGTTCGcT**G**]
100 µl	50 µl	1.0 µl	0.5 µl	IMP-M1 (R, 25pmol/µl) [TAGCCAGCTCTTGTCCACTAGTtT**T**]
15 µl	7.5 µl	0.15 µl	0.05 µl	Go-Taq DNA polymerase
2.5 ml	**1.25 ml**	**25 µl**	**12.5 µl**	**Total (To each 25 µl reaction add 1 µl template DNA)**

Table 8.4.2.1. Lower case nucleotides indicate the intentional mismatch in the primer sequences. Nucleotides in bold are located at site of SNP (where applicable), F and R indicate forward and reverse orientation. Use 1 µl DNA template. Improved specificity can be achieved by eliminating primers for species not found in the sampling area. Add water to compensate for this volume if all are not included. If performing the ½ volume assay, add 0.5µl template DNA instead.

PCR cycle conditions

95°C/5min x 1 cycle
(95°C/30sec , 58°C/30sec , 72°C/30sec) x 30 cycles
72°C/5min x 1 cycle
4°C hold

---

[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red; load 5 µl sample. Primers create fragments of 636 *An. quadriannulatus,* 528 *An. merus*, 463 *An. gambiae*, 387 *An. arabiensis,* 333 *An. gambiae* M*,* 221 *An. gambiae* S. (**Figure 8.4.2.1**).
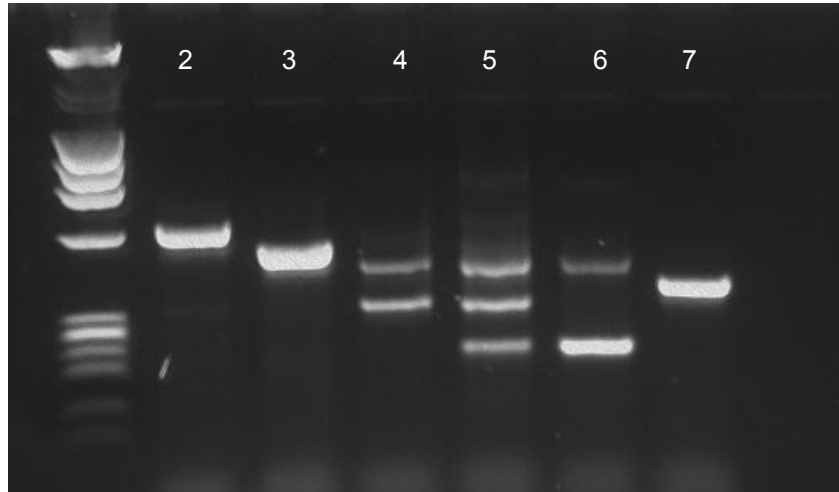
Figure 8.4.2.1. Lane 1, ladder, lane 2 *An quadriannulatus*, lane 3 *An merus*, lane 4, *An gambiae* M rDNA form, lane 5 *An gambiae* MS hybrid, lane 6 *An gambiae* S rDNA form, lane 7 *An arabiensis*.

References

Wilkins EE, Howell PI, Benedict MQ (2006) IMP PCR primers detect single nucleotide polymorphisms for *Anopheles gambiae* species identification, *Mopti* and *Savanna* rDNA types, and resistance to dieldrin in *Anopheles arabiensis*. Malar J 5:125

8.4.4 *Anopheles funestus* Complex

Lizette Koekemoer

Introduction

The *Anopheles funestus* group consists of at least eleven species: *An. funestus* Giles, *An. vaneedeni* Gillies and Coetzee, *An. rivulorum* Leeson, *An. rivulorum*-like, *An. leesoni* Evans, *An. confusus* Evans and Leeson, *An. parensis* Gillies, *An. brucei* Service, *An. aruni* Sobti, *An. fuscivenosus* Leeson and an Asian member *An. fluviatilis* James. These species are not all sympatric. Originally, distinguishing the members of the group was mainly based on karyotyping (Green and Hunt 1980; Green 1982). Recently, however, easier PCR based assays have been developed that distinguished the most common members of the group. The PCR assay presented is based on species-specific single nucleotide polymorphisms (SNPs) in the internal transcribed spacer region 2 (ITS2) (Koekemoer et al. 2002; Cohuet et al. 2003).

PCR authentication for the members of the *Anopheles funestus* group (Koekemoer et al. 2002; Cohuet et al. 2003)

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
300 µl	150 µl	3.0 µl	sterile H_2O
250 µl	125 µl	2.5 µl	Taq 10X PCR Buffer with $MgCl_2$
250 µl	125 µl	2.5 µl	dNTP (2 mM mix)
150 µl	75 µl	1.5 µl	$MgCl_2$ (25mM)
200 µl	100 µl	2.0 µl	UV (F, 33 pmol/µl) [TGT GAA CTG CAG GAC ACA T]
200 µl	100 µl	2.0 µl	FUN (R, 33 pmol/µl) [GCA TCG ATG GGT TAA TCA TG]
200 µl	100 µl	2.0 µl	VAN (R, 33 pmol/µl) [TGT CGA CTT GGT AGC CGA AC]
200 µl	100 µl	2.0 µl	RIV (R, 33 pmol/µl) [CAA GCC GTT CGA CCC TGA TT]
200 µl	100 µl	2.0 µl	PAR (R, 33 pmol/µl) [TGC GGT CCC AAG CTA GGT TC]
200 µl	100 µl	2.0 µl	RIVLIKE (R, 33 pmol/µl)[CCG CCT CCC GTG GAG TGG GGG]
200 µl	100 µl	2.0 µl	LEES (R, 33 pmol/µl) [TAC ACG GGC GCC ATG TAG TT]
50 µl	25 µl	0.5 µl	Taq DNA polymerase (5 U/µl)
2.5 ml	**1.25 ml**	**24 µl**	**Total (To each 24 ul reaction add 1 µl template DNA)**

Table 8.4.4.1. F and R indicate forward and reverse orientation. Volume can be reduced to 12.5 ul for economy. DNA extraction negative control to be included in addition to PCR reaction mix negative control. If all primers are not needed, complete the total volume with water.

PCR cycle conditions

94°C/2min x 1 cycle
(94°C/30sec , 45°C/30sec , 72°C/40sec) x 36 cycles
72°C/5min x 1 cycle
4°C hold

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

Run samples on a 2.5% agarose gel stained with EtBr, or other intercalating agent like SYBR Green or Gel Red; load 10 µl sample. Primers create fragments of 587bp *An. vaneedeni*, 505bp *An. funestus*, 411bp, *An. rivulorum*, 313bp *An. rivulorum*-like (West Africa), 252bp *An. parensis* and 146bp *An. leesoni* (**Figure 8.4.4.1**).

Figure 8.4.4.1. Amplified fragments using the species-specific polymerase chain reaction for the identification of members of the *Anopheles funestus* group. Lanes 1 and 9, 100-basepair DNA size marker ladder; lane 2, *An. vaneedeni*; lane 3, *An. funestus*; lane 4, *An.rivulorum*; lane 5, *An. rivulorum-like*; lane 6, *An. parensis*; lane 7, *An.leesoni*; lane 8, negative control. The sizes of the fragments of the ladder are 1,000, 800, 700, 600, 500, 400, 300, 200, and 100 bp.

References

Cohuet A, Simard F, Toto JC, Kengne P, Coetzee M, Fontenille D (2003) Species identification within the *Anopheles funestus* group of malaria vectors in Cameroon and evidence for a new species. Am J Trop Med Hyg 69:200-205

Green CA (1982) Cladistic analysis of mosquito chromosome data (*Anopheles* (Cellia) Myzomyia. J Heredity 73:2-11

Green CA, Hunt RH (1980) Interpretations of variation in ovarian polytene chromosomes of *Anopheles funestus* Giles, *An. parensis* Gillies, and *An. aruni*. Genetica 51:187-195

Koekemoer LL, Kamau L, Hunt RH, Coetzee M (2002) A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. Am J Trop Med Hyg 66:804-811

96 well PCR sample preparation template

8.4.5 PCR Discrimination of the *Anopheles funestus* Complex

Liz Wilkins, MR4 Staff

Like the Koekemoer et al.(2002) assay, the MR4 method of *Anopheles funestus* complex discrimination is based on species-specific single nucleotide polymorphisms (SNPs) in the second internal transcribed spacer region (ITS2). However, it also incorporates intentional mismatches into the primers (Intentional Mismatch Primers (IMPs)) to increase the specificity (Wilkins et al. 2006).

PCR authentication for the members of the *Anopheles funestus* group

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
1000 µl	500 µl	10.0 µl	sterile H_2O
500 µl	250 µl	5.0 µl	Taq 5X PCR Buffer with $MgCl_2$
100 µl	50 µl	1.0 µl	dNTP (2 mM mix)
200 µl	100 µl	2.0 µl	$MgCl_2$ (25mM)
100 µl	50 µl	1.0 µl	MR4UV (F, 5 pmol/µl) [CCG ATG CAC ACA TTC TTG AGT GCC TA]
100 µl	50 µl	1.0 µl	MR4FUN (R, 5 pmol/µl) [CTC GGG CAT CGA TGG GTT AAT CAT G]
100 µl	50 µl	1.0 µl	MR4VAN (R, 5 pmol/µl) [AAC TCT GTC GAC TTG GTA GCC GAA C]
100 µl	50 µl	1.0 µl	MR4RIV (R, 5 pmol/µl) [AAT CAG GGT CGA ACG GCT TGC CG]
100 µl	50 µl	1.0 µl	MR4PAR (R, 5 pmol/µl) [GCC CTG CGG TCC CAA GCT AGA TT]
100 µl	50 µl	1.0 µl	MR4RIVLIKE (R, 5 pmol/µl) [CTC CCG TGG AGT GGG GGA TC]
100 µl	50 µl	1.0 µl	MR4LEES (R, 5 pmol/µl) [GAC GGC ATC ATG GCG AGC AGC]
10 µl	5 µl	0.1 µl	Taq DNA polymerase (5 U/µl) –MR4 uses GoTaq, Promega
2.5 ml	**1.25 ml**	**25 µl**	**Total (To each 25 µl reaction add 1 µl template DNA)**

Table 8.4.5.1. F and R indicate forward and reverse orientation. DNA extraction negative control to be included in addition to PCR reaction mix negative control. If all primers are not needed, complete the total volume with water.

PCR cycle conditions

94°C/4min x 1 cycle
(94°C/30sec , 58°C/30sec , 72°C/45sec) x 30 cycles
72°C/7min x 1 cycle
4°C hold

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red; load 10 µl sample

---

[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

Primers create fragments of 496bp *An. vaneedeni*, 424bp *An. funestus*, 346bp, *An. rivulorum*, 241bp *An. rivulorum*-like (West Africa), 176bp *An. parensis* and 93bp *An. leesoni* (**Figure 8.4.5.1**)

References:

Koekemoer LL, Kamau L, Hunt RH, Coetzee M (2002) A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. Am J Trop Med Hyg 66:804-811

Wilkins EE, Howell PI, Benedict MQ (2006) IMP PCR primers detect single nucleotide polymorphisms for *Anopheles gambiae* species identification, *Mopti* and *Savanna* rDNA types, and resistance to dieldrin in *Anopheles arabiensis*. Malar J 5:125

Figure 8.4.5.1 Lane 1 1kb ladder, lane 2 *An. funestus*, lane 3 *An. rivulorum*, lane 4 *An. parensis*, lane 5 *An. leesoni*.

96 well PCR sample preparation template

8.4.6 *Anopheles minimus* Species Complex

MR4 Staff

Introduction

Anopheles minimus is a complex comprised of three members: species A (*minimus* sensu stricto), C (*harrisoni*), and E. *Anopheles minimus* and *An. harrisoni* occur sympatrically throughout Southeast Asia and are implicated in malaria transmission while species E is limited to the Ryuku Islands of Japan (Garros et al. 2006). An original method for distinguishing the various members was determining the presence of a pale humeral spot; however this method has proven unreliable in use (Sungvornyothin et al. 2006). A PCR-RFLP (restriction fragment length polymorphism) designed utilizing SNP differences in the ITS2 regions has been developed to distinguish complex members of *An. minimus* (Van Bortel et al. 2000).

PCR authentication for the members of the *Anopheles minimus* complex (Van Bortel et al. 2000)

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
1555 µl	777.5 µl	15.55 µl	sterile H_2O
500 µl	250 µl	5.0 µl	GoTaq 5X PCR Buffer with $MgCl_2$
100 µl	50 µl	1.0 µl	dNTP (2.5 mM mix)
30 µl	15 µl	0.3 µl	$MgCl_2$ (25 mM)
150 µl	75 µl	1.5 µl	ITS2 A primer (1 pmol/µl) [TGT GAA CTG CAG GAC ACA T]
150 µl	75 µl	1.5 µl	ITS2 B primer (1 pmol/µl) [TAT GCT TAA ATT CAG GGG GT]
15 µl	7.5 µl	0.15 µl	Go-Taq DNA polymerase (5 U/µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total (To each 24 ul reaction add 1 µl template DNA)**

Table 8.4.6.1. Prepare PCR Master Mix for 96, 48 or 1 25 µl PCR reactions. Add reagents in the order presented. Use 1 µl DNA template.

PCR cycle conditions

94°C/4min x 1 cycle
(94°C/30sec, 53°C/40sec, 72°C/30sec) x 35 cycles
72°C/10min x 1 cycle
4°C hold

RFLP Procedure
Per reaction well:

2 µl	sterile H_2O
2 µl	Restriction Enzyme buffer
1 µl	*Sau* 96I restriction enzyme (GGNCC cutting site)
15 µl	PCR product
20 µl	**Total**

Incubate at 60°C for 2 hours.

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red. The PCR-RFLP procedure will yield the following products: *An. minimus* 220+200bp and *An. harrisoni* 300+220bp products.

References

Garros C, Van Bortel W, Trung HD, Coosemans M, Manguin S (2006) Review of the *Minimus* Complex of *Anopheles*, main malaria vector in Southeast Asia: from taxonomic issues to vector control strategies. Trop Med Int Health 11:102-114

Sungvornyothin S, Garros C, Chareonviriyaphap T, Manguin S (2006) How reliable is the humeral pale spot for identification of cryptic species of the *Minimus* Complex? J Am Mosq Control Assoc 22:185-191

Van Bortel W, Trung HD, Roelants P, Harbach RE, Backeljau T, Coosemans M (2000) Molecular identification of *Anopheles minimus s.l.* beyond distinguishing the members of the species complex. Insect Mol Biol 9:335-340

96 well sample preparation template

8.4.7 *Anopheles quadrimaculatus* Species Complex (Cornel et al.)

MR4 Staff

Introduction

Though malaria is no longer endemic in the United States, the competent vector *An. quadrimaculatus* is still present. It has been determined that the *An. quadrimaculatus* complex is made of at least five members: *An. inundatus, An. diluvialis, An. maverlius, An. smaragdinus,* and *An. quadrimaculatus* (Narang et al. 1990). A PCR based on SNPs in the internal transcribed spacer region has been developed for *An. quadrimaculatus* that can distinguish the various members of the complex (Cornel et al. 1996).

PCR authentication for the members of the *Anopheles quadrimaculatus* complex (Cornel et al. 1996)

Prepare PCR Master Mix for 96, 48 or 1 25 µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
450 µl	225 µl	4.5 µl	sterile H_2O
250 µl	125 µl	2.5 µl	10X Perkins Elmer PCR Buffer
100 µl	50 µl	1.0 µl	dNTP (2 mM mix)
200 µl	100 µl	2.0 µl	$MgCl_2$ (25 mM)
160 µl	80 µl	1.6 µl	AQU (F, 1 pmol/µl) [CGACACAGCTCGATGTACAC]
100 µl	50 µl	1.0 µl	AQA (R, 1 pmol/µl) [TCCGTAGGAGGCTGCATTTT]
460 µl	230 µl	4.6 µl	AQB (R, 1 pmol/µl) [CACACTACACAACACGCTTT]
230 µl	115 µl	2.3 µl	AQC (R, 1 pmol/µl) [TACCCCGGCCTTGTAGCAAA]
540 µl	270 µl	5.4 µl	AQD (R, 1 pmol/µl) [ATGCAAAAGGTGGTGTTGTG]
12.5 µl	6.25 µl	0.125 µl	Taq DNA polymerase (5U/µl)
2.5 ml	**1.25 ml**	**25 µl**	**Total (To each 24 ul reaction add 1 µl template DNA)**

Table 8.4.7.1. F and R indicate forward and reverse orientation. Use 1 µl DNA template. If all primers are not used, complete the volume with water.

PCR Cycle conditions

94°C/2min x 1 cycle
(94°C/1min, 50°C/2min, 72°C/2min) x 25 cycles
72°C/10min x 1 cycle
4°C hold

Run samples on a 2% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red; load 5 µl sample. Primers create fragments of 319bp *An. quadrimaculatus*, 227bp *An. smaragdinus*, 293bp *An. inundatus* or *An. diluvialis*, and 141bp *An. maverlius*.

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

References

Cornel AJ, Porter CH, Collins FH (1996) Polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. J Med Entomol 33:109-116

Narang SK, Seawright JA, Kaiser PE (1990) Evidence for microgeographic genetic subdivisions of *Anopheles quadrimaculatus* species C. J Am Mosq Control Assoc 6:179-187

96 well sample preparation template

	1	2	3	4	5	6	7	8	9	10	11	12
A	○	○	○	○	○	○	○	○	○	○	○	○
B	○	○	○	○	○	○	○	○	○	○	○	○
C	○	○	○	○	○	○	○	○	○	○	○	○
D	○	○	○	○	○	○	○	○	○	○	○	○
E	○	○	○	○	○	○	○	○	○	○	○	○
F	○	○	○	○	○	○	○	○	○	○	○	○
G	○	○	○	○	○	○	○	○	○	○	○	○
H	○	○	○	○	○	○	○	○	○	○	○	○

8.4.8 *Anopheles quadrimaculatus* Species Complex (Levine et al.)

Mark Benedict

Introduction

Though malaria is no longer endemic in the United States, the competent vector *An. quadrimaculatus* is still present. It has been determined that the *An. quadrimaculatus* complex is made up of at least five members: *An. inundatus, An. diluvialis, An. maverlius, An. smaragdinus,* and *An. quadrimaculatus* (Narang et al. 1990). A PCR based on SNPs in the internal transcribed spacer region was developed for *An. quadrimaculatus* that can distinguish the various members of the complex (Cornel et al. 1996), however a later modification was developed that uses primers that have similar 60°C annealing temperatures and the concentrations recommended are the same as one another (Levine et al. 2004). Fragment sizes are also slightly more distinct (**Figure 4.2.6.1**).

PCR authentication for the members of the *Anopheles quadrimaculatus* complex (Cornel et al. 1996)

Prepare PCR Master Mix for 96, 48 or 1 25 µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
380 µl	190 µl	3.5 µl	sterile H_2O
250 µl	125 µl	2.5 µl	10X Promega Taq PCR Buffer w/o $MgCl_2$
250 µl	125 µl	2.5 µl	dNTP (2 mM mix each G,A,T,C)
250 µl	125 µl	2.5 µl	$MgCl_2$ (32 mM)
250 µl	125 µl	2.5 µl	AquadU-2 (F, 10 pmol/µl) [GTGCGACACAGCTCGATG]
250 µl	125 µl	2.5 µl	AquadQ-2 (R, 10 pmol/µl) [CCGTAGGAGGCTGCATTTTA]
250 µl	125 µl	2.5 µl	AquadS-2 (R, 10 pmol/µl) [GAACACACTACACAACACGCTTT]
250 µl	125 µl	2.5 µl	AquadD1-2 (R, 10 pmol/µl) [AGGCCCATGTACTCCGTAGG]
250 µl	125 µl	2.5 µl	AQD (R, 10 pmol/µl) [ATGCAAAAGGTGGTGTTGTG]
20 µl	10 µl	0.5 µl	Taq DNA polymerase (5U/µl)
2.4 ml	**1.2 ml**	**24 µl**	**Total (To each 24 ul reaction add 1 µl template DNA)**

Table 8.4.8.1. F and R indicate forward and reverse orientation. Use 1 µl DNA template. If all primers are not used, complete the volume with water.

PCR cycle conditions

95°C / 2min x 1 cycle
(95°C / 30s, 57°C / 30s, 72°C / 30s) x 30 cycles

4°C hold

Run samples on a 2% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red; load 5 µl sample. Primers create fragments of 321 bp for *An. quadrimaculatus*, 233 bp for *An. smaragdinus*, 353 bp for *An. diluvialis*, 365 for *An. inundatus* and 141bp for *An. maverlius*.
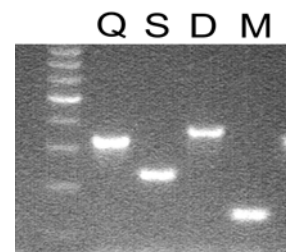
Figure 8.4.8.1. PCR products obtained from amplification of *A. quadrimaculatus s.s.* (Q), *A. smaragdinus* (S), *A. diluvialis* (D) and *A. maverlius* (M)

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

References

Cornel AJ, Porter CH, Collins FH (1996) Polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. J Med Entomol 33:109-116

Levine RS, Peterson AT, Benedict MQ (2004) Distribution of members of *Anopheles quadrimaculatus* Say s.l. (Diptera: Culicidae) and implications for their roles in malaria transmission in the United States. J Med Entomol 41:607-613

Narang SK, Seawright JA, Kaiser PE (1990) Evidence for microgeographic genetic subdivisions of *Anopheles quadrimaculatus* species C. J Am Mosq Control Assoc 6:179-187

96 well sample preparation template

8.4.9 SINE based assay for discrimination of *Anopheles gambiae* M and S molecular forms.

Federica Santolamazza, Emiliano Mancini, Alessandra della Torre

Introduction

The PCR diagnostic approach here proposed is based on the specific and irreversible insertion of a 230 bp transposable element (SINE200) in the M-form (and its absence in S-form), thus allowing an unambiguous, simple and straightforward recognition of M and S forms (even without preliminary species-specific PCR identification in areas where exclusive sympatry with *A. arabiensis* is found) (Santolamazza *et al.*, 2008). However, it is important to keep in mind that the M-form specific SINE insertion is a character linked to the IGS-SNPs defining the M- and S-forms along most of their range, but with a different evolutionary history (i.e. its origin and rapid fixation in M-form) (Santolamazza *et al.*, in press).

It is also interesting to note that, although the S-form amplicon is identical to those of *A. melas* and *A. quadriannulatus*, the 26 bp deletion reported for *A. arabiensis* allows to propose the use of the novel approach to discriminate *A. gambiae* from *A. arabiensis* specimens without preliminary species identification in large areas of sub-Saharan Africa where *A. gambiae* molecular forms and *A. arabiensis* are the only species of the complex present. The *S200* X6.1 locus is located only about 1 Mb from IGS-region.

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
1860 µl	930 µl	18.6 µl	Distilled H2O
500 µl	250 µl	5.0 µl	5X PCR buffer
100 µl	50 µl	1.0 µl	dNTP (2mM concentration)
10 µl	5 µl	0.1 µl	200X6.1F (25pmol/ul) TCG CCT TAG ACC TTG CGT TA
10 µl	5 µl	0.1 µl	200X6.1R (25pmol/ul) CGC TTC AAG AAT TCG AGA TAC
30 µl	15 µl	0.3 µl	MgCl2 (25 mM)
10 µl	5 µl	0.1 µl	Taq DNA polymerase (5U/ µl)
2.52 ml	**1.26 ml**	**25 µl**	**Total (To each 25 µl reaction add 1 µl template DNA)**

Table 8.4.9.1. Use 0.5 µl DNA template.

PCR cycle conditions
94°C/5min x 1 cycle
(94°C/30sec -o- 54°C/30sec -o- 72°C/30sec) x 35 cycles
72°C/10min x 1 cycle
4°C hold

The resulting products were analyzed on 1.5% agarose gels stained with EtBr, or other intercalating agent like SYBR Green or Gel Red, with low and high molecular weight bands corresponding to fragments containing or lacking the targeted *SINE200*, respectively (see **Figure 8.4.9.1**).

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

Figure 8.4.9.1. Lane 1: *An. quadriannulatus* (279 bp), lane 2: *An. melas* (279 bp), lane 3: *An. arabiensis* (223 bp), lane 4: ladder 100bp (BIOLINE HyperLadder IV), lane 5: S-molecular form (279 bp), lane 6: M-molecular form (479 bp), lane 7: heterozygote M/S (479 bp and 279bp).

References

Santolamazza F, Mancini E, Simard F, Qi Y, Tu Z, della Torre A (2008) Structure and insertion polymorphisms of SINE200 retrotransposons in genomic islands of speciation of *Anopheles gambiae* molecular forms. *Malar J,* 7:163.

Santolamazza F, Caputo B, Calzetta M, Vicente JL, Mancini E, Petrarca V, Pinto J, della Torre A (2011) Comparative analyses reveal discrepancies among results of commonly used methods for *Anopheles gambiae* molecular form identification. *Malar J*: in press.

96 well PCR sample preparation template

8.4.10 Identification of *Anopheles arabiensis* from *An. gambiae* and its two molecular forms from degraded DNA templates or museum samples.

Federica Santolamazza, Alessandra della Torre, Adalgisa Caccone

Introduction
This method is based on the amplification of a ribosomal DNA fragment smaller than that amplified by other PCR-RFLP approaches for the identification of M and S *A. gambiae* molecular forms (Favia et al. 2001; Fanello et al., 2002) and, thus, performs better than these on degraded DNA templates, such as those from old museum collections and from poorly preserved field-collected samples). Moreover, this method does not only efficiently discriminate the two *A. gambiae* forms, but also clearly identify *An. arabiensis*.

Prepare PCR Master Mix for 96, 48 or 1 15µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
1040 µl	520 µl	10.4 µl	sterile H_2O
150 µl	75 µl	1.5 µl	Taq 10X PCR Buffer with $MgCl_2$
50 µl	25 µl	0.5 µl	dNTP (2.5 mM mix)
50 µl	25 µl	0.5 µl	IGS441 For (25 pmol/µl) [TGG TCT GGG GAC CAC GTC GAC ACA GG]
50 µl	25 µl	0.5 µl	IGS783 Rev (25 pmol/µl) [CGT TTC TCA CAT CAA GAC AAT CAA GTC]
100 µl	50 µl	1.0 µl	$MgCl_2$ (25 mM)
10 µl	5 µl	0.1 µl	Taq DNA polymerase (5 U/µl)
1.45 ml	**725 µl**	**14.5 µl**	**Total (To each 14.5 µl reaction add 0.5 µl template DNA)**

Table 8.4.10.1. Use 0.5 µl DNA template.

PCR cycle conditions
94°C/5min x 1 cycle
(94°C/30sec -o- 58°C/30sec -o- 72°C/30sec) x 30 cycles
72°C/7min x 1 cycle
4°C hold

Restriction enzyme digest

After amplification, one unit of *Mse* I and 1× buffer 2 (New England Biolabs, Beverly, MA) is added to 15 µl of the PCR product. Digestion is carried out for at least three hours at 37°C followed by separation of the bands by electrophoresis on a 1.5% agarose gel. The number and size of the digested fragments allow the simultaneous differentiation of *An. arabiensis* and *An. gambiae* M and S. The S form does not have any *Mse* I restriction site. The M form and *An. arabiensis* have *Mse* I sites located in different positions

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns to compensate for imprecise measurements.

Figure 8.4.10.1. Figure shows lane 1: 1-kb ladder (Gibco-BRL, Gaithersburg, MD), lane 2: the restriction profiles obtained for *An. gambiae* M- form (181bp and 107 bp), lane 3: *An. arabiensis* (253bp and 35 bp), lane 4 *An. gambiae* S-form (288 bp), lane 5 and 6 are *An. gambiae* S-form (288 bp) from 1970.

References

Santolamazza F, della Torre A, Caccone A (2004) A new PCR-RFLP method to identify *Anopheles arabiensis* from *An. gambiae* and its two molecular forms from degraded DNA templates or museum samples. Am J Trop Med & Hyg, 70: 604–606.

Favia G, Lanfrancotti A, Spanos L, Siden-Kiamos I, Louis C (2001) Molecular characterization of ribosomal DNA polymorphisms discriminating among chromosomal forms of *Anopheles gambiae* s.s. Insect Mol Biol, 10:19-23.

Fanello C, Santolamazza F, della Torre A (2002) Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. Med Vet Entomol, 16: 461-464.

96 well PCR sample preparation template

8.5 Real-Time PCR Assays

8.5.1 Vector Population Monitoring Tool Using Real-Time PCR

Chris Bass, Martin Williamson, John Vontas, Hilary Ranson, Martin Donnelly and Lin Field

Introduction

The 'Vector Population Monitoring Tool' (VPMT) consists of a suite of high-throughput assays that can be used to screen mosquito disease vector populations for a number of traits. These include:

- *Anopheles gambiae s.l.* species identification **(Section 8.5.1.1)**

- Detection of infection with *Plasmodium* parasites **(Section 8.5.1.2)**

- Detection of insecticide resistance mechanisms. To date this includes:

  1) Detection of knock-down resistance (*kdr*) mutations **(Section 8.5.1.3)**

  2) Detection of insensitive acetylcholinesterase (*iAChE*) **(Section 8.5.1.4)**

  3) Detection of resistance to dieldrin (*rdl*) **(Section 8.5.1.5)**

The assays that constitute the VPMT have been designed to be high-throughput and require very small amounts of starting material (DNA) which can be extracted from mosquitoes that have been stored in a wide variety of ways (e.g. in ethanol, isopropanol, dried, frozen). This is to ensure that they can be used for assaying field collected mosquitoes without the need for a cold chain to preserve the specimens. In the case of the species identification assays, a single mosquito leg can be used as template for PCR without the need to first extract DNA.

The assays presented in this section are based on TaqMan SNP genotyping. The TaqMan assay is a PCR method employing oligonucleotide probes that are dual-labeled with a fluorescent reporter dye and a quencher molecule. Amplification of the probe-specific product causes cleavage of the probe, generating an increase in reporter fluorescence as the reporter dye is released away from the quencher. By using different reporter dyes, cleavage of allele-specific probes can be detected in a single PCR. The 'closed-tube' nature of the TaqMan platform means there is no requirement for post-PCR processing and consequently assays are simple to perform and rapid to run.

Ordering Reagents

Primers: These are standard oligonucleotides and are available from a range of suppliers.

Probes: Protocols described here use 2 types of fluorescently labeled probes:

TaqMan® MGB™ Probes: available from Applied Biosystems. Each probe is labeled with a 5′ reporter dye (e.g. Vic or Fam) and also carries a 3′ non-fluorescent quencher and a minor groove binder (MGB) at the 3′ end. The minor groove binder provides more accurate allelic discrimination by increasing the T_M between matched and mismatched probes. Store aliquots in aluminum foil.

LNA Probes: These probes employ locked nucleic acid (LNA) modified nucleotides which have a similar effect to the MGB moiety. They can be purchased from Sigma or from Thermo Scientific. These are usually delivered lyophilized, with the yield given in nanomoles. Prepare aliquots and store each aliquot wrapped in aluminum foil/out of light. The LNA probe used in the species identification assay described in this guide is labeled with Cy5. (note: Sigma class the Cy5 label as non-standard so the probe is more expensive than standard). When ordering: Type: mix DNA/LNA, backbone: PO, 5-Modification: Cy5, 3-Modification: BHQ2; [Cy5] AC+A+T+AG+GATGGA+G+A+AGG [BHQ2]. Thermo scientific uses LA, LC, LG, LT for LNA bases, so the sequence of the probe above should be copied in as acLALTLAgLGatggaLGLALALAgg. Fill out the modifications part of the table as follows: at 5'-end: CY5, at 3'-end: Black Hole Quencher 2.

PCR Mastermix: All the assays have been optimized using SensiMix *NoRef* DNA kit (Cat # QT505) from Quantace.

Assay Cost

Assay cost will vary from country to country so the following should be used as a general guide and is an approximate price, correct as of 2008. In each case costs were calculated in US $ by using retail prices from US websites. These prices do not include promotional or negotiated discounts or the cost of delivery. All primer/probe/PCR master-mix costs are from ordering at the largest available scale (substantial cost savings are made by ordering at the largest available scales). Cost calculations include all the plastics required to set up the reactions (PCR tubes/filter tips etc.).

- Species ID (2 plex assay) US$ 0.75/specimen

- Species ID (3 plex assay) US$ 1/specimen

- *Plasmodium* detection assay US$ 0.75/specimen

- *Kdr* assay US$ 1.5 (this is the combined cost of running two assays (*kdr-w*+*kdr-e*)

- iAChE assay US$ 0.75/specimen

- *Rdl* assay US$ 1.5 (this is the combined cost of running two assays (*A296G*+*A296S*) /specimen

Real-time PCR machines

The assays described in this guide were originally developed using a Rotor-Gene 6000™ (Corbett Research). We have also trialed several of the assays using a Chromo4 and Mini-Opticon (Bio-Rad). In the case of the latter machines, both clear and white PCR tubes/plates were used successfully with slightly improved results using the white tubes/plates. Users of alternative real-time machines may find a small amount of optimization is required to achieve optimal results.

General advice and Troubleshooting

Make aliquots of all reagents to avoid repeated freeze-thawing. This is especially important for the fluorescently labeled probes.

Examine both the fluorescent traces and scatter plots when scoring samples.

Use the auto-scale function of the real-time PCR machine software **with caution** as this can artificially elevate the fluorescent trace resulting in mis-scoring.

If further assay optimization is required, the annealing temperature, the concentration of probes in the reaction, and number of temperature cycles are good parameters to alter in attempts to enhance sensitivity or reduce non-specific signals.

If mosquito individuals are to be tested with the *Plasmodium* detection assay, DNA should be extracted from the head/thorax of mosquito specimens to avoid detecting *Plasmodium* stages in the blood meal/gut in addition to sporozoites in the salivary glands.

Always include controls in assay runs. We recommend that these include one or more no-template controls, and in the case of the *An. gambiae s.l.* species ID and *Plasmodium* assays, a positive control for each probe (e.g *An. gambiae s.s*, *An. arabiensis* and *An. quadriannulatus* for the 3 plex species ID assay and *P. falciparum* and *P. vivax* for the Plasmodium assay). In the case of the *kdr*, iAChE and *rdl* assays, a control template for each genotype should be included (e.g., when running the *kdr-w* assay, include a *kdr-w* homozygous sample, a wild-type homozygous sample, and a *kdr-w* heterozygous sample). We have found that including these controls greatly facilitates the interpretation of results and generally speeds up the scoring of unknown samples.

Assay Controls

The following table details the control genotypes currently available from the **Malaria Research and Reference Reagent Resource Center** (**MR4**) www.**mr4**.org. We have also created additional plasmid and whole genome amplified controls for these assays available from the authors on request.

Anopheles gambiae s.l. species identification **(Section 8.5.1.1)**		
MRA-495	*Anopheles arabiensis*	Genomic DNA from *An. arabiensis* KGB
MRA-142	*Anopheles gambiae*	Genomic DNA from *An. gambiae*, G3 strain
MRA-761G	*Anopheles quadriannulatus*	Genomic DNA from *An. quadriannulatus*, SKUQUA strain
Detection of infection with *Plasmodium* parasites **(Section 8.5.1.2)**		
MRA-102G	*Plasmodium falciparum*	Genomic DNA from *P. falciparum* 3D7
MRA-341G	*Plasmodium vivax*	Genomic DNA from *P. vivax* ONG
Detection of knock-down resistance (*kdr*) mutations **(Section 8.5.1.3)**		
MRA-762	*Anopheles gambiae* KISUMU	Wild-type susceptible homozygous control
MRA-334	*Anopheles gambiae* RSP	Kdr L1014S (Kdr-e) homozygous
Detection of insensitive acetylcholinesterase (*iAChE*) **(Section 8.5.1.4)**		
MRA-762	*Anopheles gambiae* KISUMU	Wild-type susceptible homozygous control
MRA-913	*Anopheles gambiae* AKRON	Wild-type resistant control
Detection of resistance to dieldrin (*rdl*) **(Section 8.5.1.5)**		
MRA-115	*Anopheles gambiae* IN22C+	Wild-type resistant homozygous control (contained within IN22C+ strain, only c+ individuals)
MRA-762	*Anopheles gambiae* KISUMU	Wild-type susceptible homozygous control
MRA-495	*Anopheles arabiensis* KGB	Wild-type susceptible homozygous control
MRA-764	*Anopheles arabiensis* SENN	Wild-type resistant homozygous control

Authors also have the following plasmid controls available:

Wild-type (susceptible homozygous control), *Kdr* L1014S (*kdr-e*) homozygous and *Kdr* L1014F (*kdr-w*) homozygous

Wild-type (susceptible homozygous control), and the G119S homozygous

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8.5.1.1 An. gambiae s.l. *species complex ID assay*

Chris Bass, Martin Williamson, John Vontas, Hilary Ranson, Martin Donnelly and Lin Field

Introduction

Two alternative assays have been developed. The first (Bass et al. 2007) (2-Plex Assay) uses two probes to distinguish between the main malaria vectors *An. gambiae s.s.* and *An. arabiensis* as one group and *An. quadriannulatus, An. melas* or *An. merus* as a second group. This assay is suitable for use on real-time PCR machines that have two detection channels (such as Biorad's Mini Opticon). This assay can be used alone to discriminate vector from non-vector (in regions where *An. merus/melas/bwambae* are not present) or in combination with an existing TaqMan assay (Walker et al. 2007) to further distinguish *An. arabiensis* from *An. gambiae s.s.*.

The second assay (Bass et al. 2008) (3-Plex Assay) is an enhancement of the first assay and uses three probes to distinguish between *An. arabiensis*, *An. gambiae s.s.* and *An. quadriannulatus/ merus/melas/bwambae* as a group. This assay requires a real-time PCR machine that has a least three detection channels. Traditional PCR assays can be found in **Chapters 8.4.1** and **8.4.3**.

2-Plex Assay

96	48	1	Reagent
500 µl	250 µl	5.0µl	sterile H_2O
1 ml	500 µl	10.0 µl	SensiMix DNA kit (Quantace)
160 µl	80 µl	1.6 µl	primer ComF (800 nM) GCTTGGTGGTTTGTCCG
160 µl	80 µl	1.6 µl	primer ComR (800 nM) CTGTGTCGACGTGGTCCC
40 µl	20 µl	0.4 µl	probe AG/AA (200 nM) 6FAM- GACCAAGACGAGC
40 µl	20 µl	0.4 µl	probe AQ/AM (200 nM) VIC- GACCAAGACGCGC
1.9 ml	**950 µl**	**19 µl**	**Total (To each 19 µl reaction add 1-2 µl genomic DNA)**

2-Plex Assay PCR cycle conditions

95°C/10 min x 1 cycle; (95°C/15sec, 50°C/20sec, 72°C/20sec) x 45 cycles
Measure fluorescence at the end of each cycle

3-Plex Assay

96	48	1	Reagent
630 µl	315 µl	6.3µl	sterile H_2O
1.25 ml	625 µl	12.5 µl	SensiMix DNA kit (Quantace)
200 µl	100 µl	2.0 µl	primer Uni F (800nM) GTGAAGCTTGGTGCGTGCT
200 µl	100 µl	2.0 µl	primer Uni R (800nM) GCACGCCGACAAGCTCA
50 µl	25 µl	0.5 µl	LNA probe Aa+(200nM)[Cy5]AC+A+T+AG+GATGGA+G+A+AGG [BHQ2]
20 µl	10 µl	0.2 µl	TaqMan MGB probe (80 nM) Ag + VIC-TGGAGCGGaACAC
50 µl	25 µl	0.5 µl	TaqMan MGB probe (200nM) Aq + 6FAM-TGGAGCGGgACAC
2.4 ml	**1.2 ml**	**24 µl**	**Total (To each 24 µl reaction add 1-2 µl genomic DNA)**

3-Plex Assay PCR cycle conditions

95°C/10 min x 1 cycle
(95°C/25sec, 66°C/60sec) x 40 cycles
Measure fluorescence at the end of each cycle

Interpreting Results and Examples: 2-Plex Assay

Interpreting results is carried out by examining the levels of FAM and VIC fluorescence during PCR. A substantial increase in FAM fluorescence during PCR indicates an *An. gambiae s.s.* or *An. arabiensis* specimen while a substantial increase in VIC fluorescence indicates an *An. quadriannulatus*, *An. melas* or *An. merus* specimen (**Figure 8.5.1.1.1.**). An increase in both dyes would indicate a hybrid or a contaminated sample.

To help identify species the software that accompanies the real-time PCR machine may allow endpoint fluorescence values for the two dyes to be automatically corrected for background and plotted against each other in bi-directional scatter plots (**Figure 8.5.1.1.2.**).
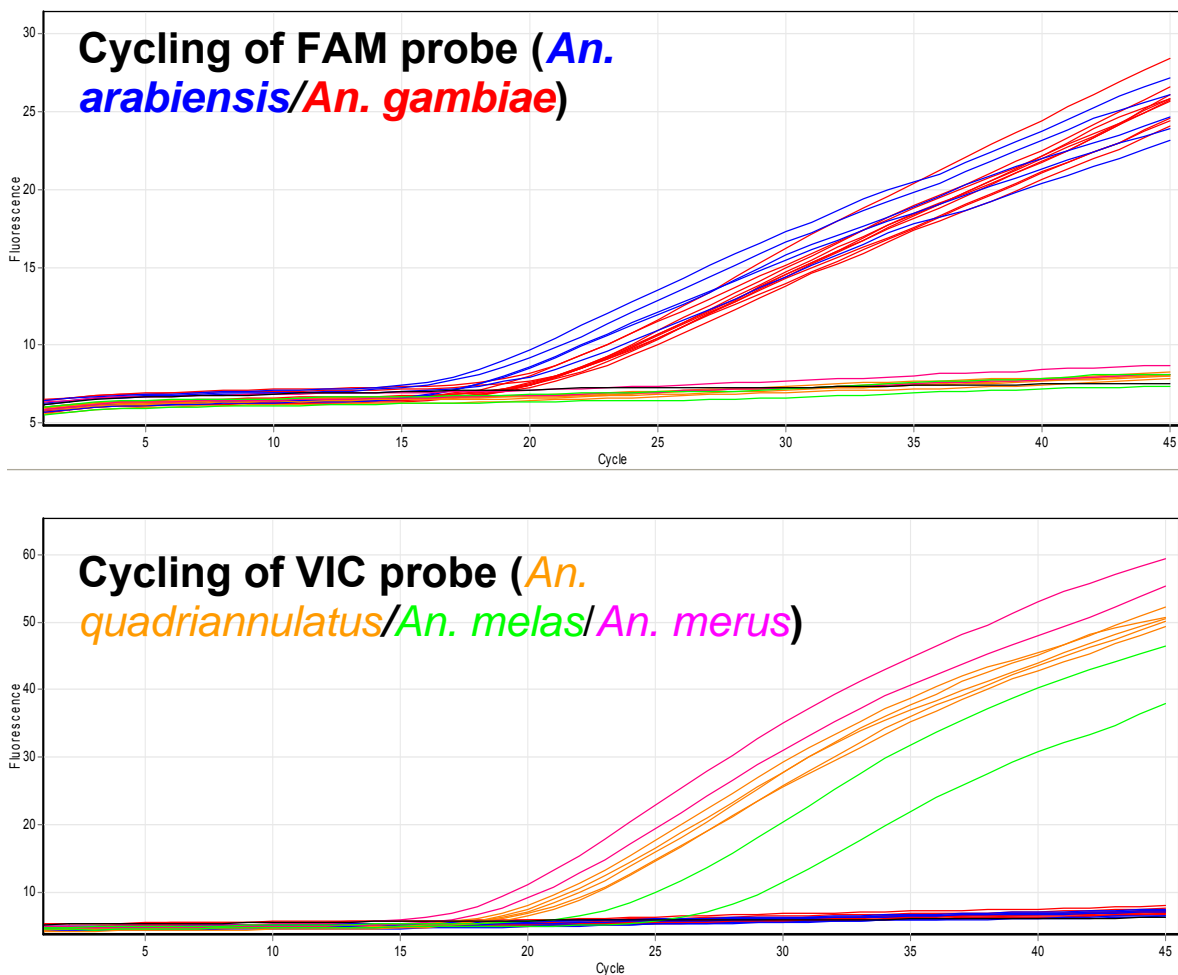
Figure 8.5.1.1.1 Species identification using the 2-plex TaqMan assay designed to distinguish the principal vector species *An. gambiae s.s.* and *An. arabiensis* from the other members of the complex. In this example two or more specimens of *An. gambiae s.s.*, (red trace) *An. arabiensis*, (blue trace) *An. melas* (green trace), *An. merus* (purple trace) and *An. quadriannulatus* (orange trace).
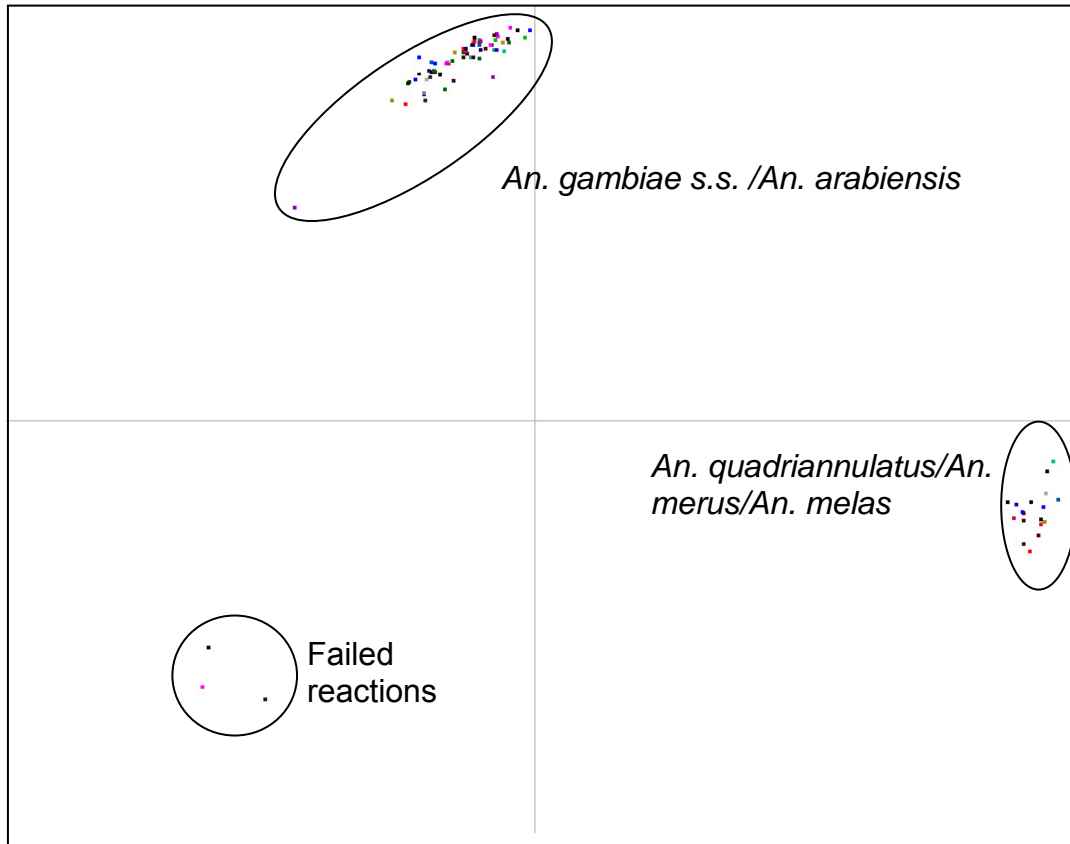
Figure 8.5.1.1.2. Scatter plot analysis of TaqMan fluorescence data. In this example real time PCR was carried out using the newly developed TaqMan assay designed to distinguish the principal vector species *An. gambiae s.s.* and *An. arabiensis* from the other members of the complex on 96 samples. Fluorescence values of the FAM labeled probe specific for *An. gambiae s.s.* and *An. arabiensis* were then plotted against the VIC labeled probe specific for *An. quadriannulatus, An. melas* and *An. merus*.

Interpreting Results and Examples: 3-Plex Assay

A substantial increase in Cy5 fluorescence (probe Aa) during PCR identifies an *An. arabiensis* specimen, an increase in VIC fluorescence (probe Ag) identifies an *An. gambiae s.s.* sample, and an increase in 6FAM fluorescence (probe Aq) identifies *An. quadriannulatus*, *An. melas* or *An. merus* specimen (**Figure 8.5.1.1.3.**). An increase in two or more of the dyes would indicate a hybrid or a contaminated sample.
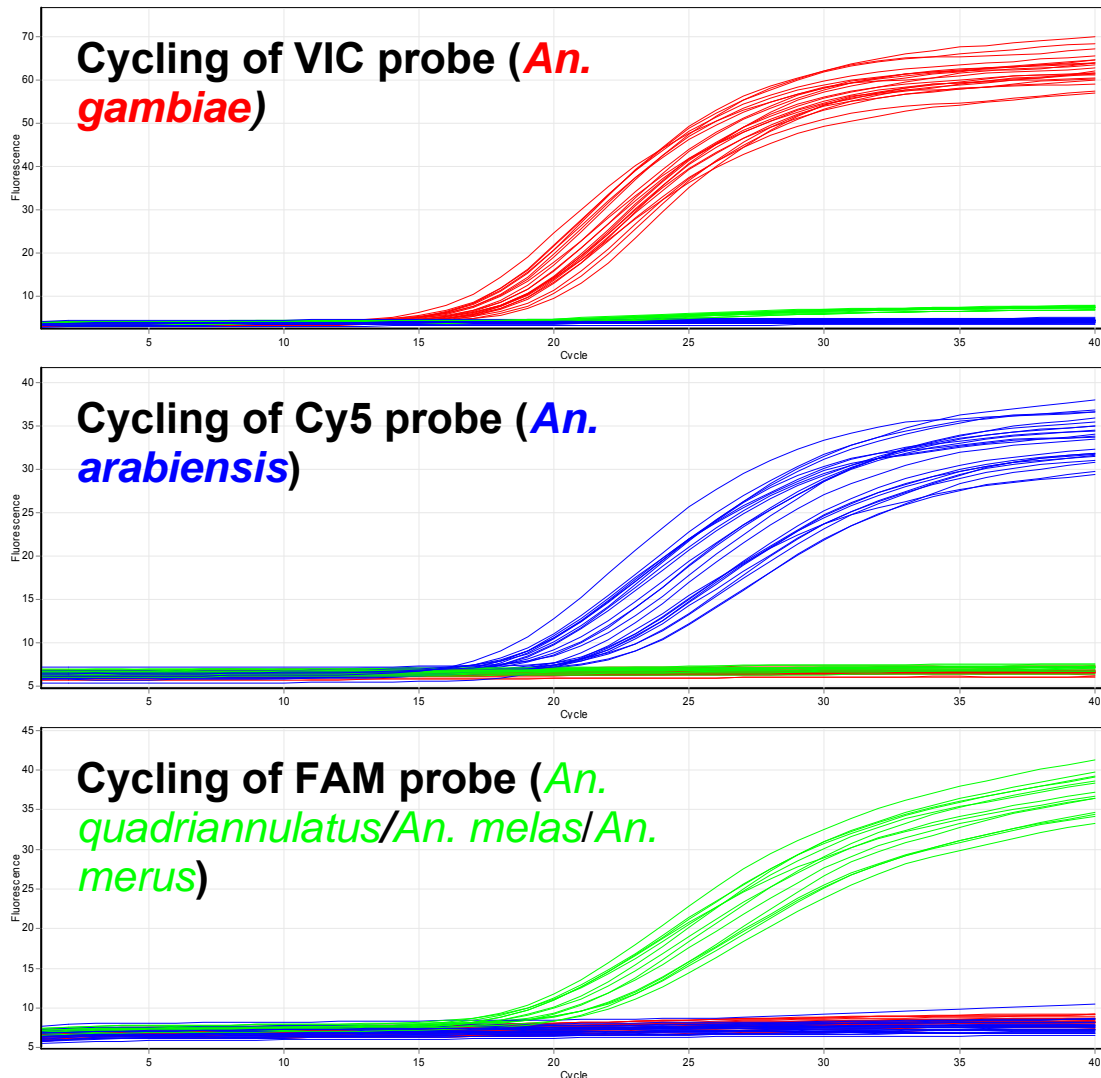
Figure 8.5.1.1.3. Species identification using the multiplex real-time PCR assay. 20 or more specimens of *An. gambiae s.s.* (red trace), *An. arabiensis* (blue trace) and *An. quadriannulatus/An. melas*/*An. merus*. (green trace).

Assay Notes and Troubleshooting

The 3-plex species ID was initially run on a Corbett rotorgene PCR machine with an annealing/extension time of 60°C. At this temperature the probes Aa and Aq (Cy5- and 6FAM-labelled) showed specific amplification of *An. arabiensis* and *An. quadriannulatus*/*An. melas*/*An. merus* respectively. However while the probe Ag (VIC-labelled) also gave sensitive detection of *An. gambiae s.s.,* when *An. quadriannulatus*, *An. melas* or *An. merus* DNAs were tested a low level 'background' fluorescence signal was observed, presumably from non-specific binding of this probe (see figure 4 below). This could be eliminated by increasing the annealing/extension temperature to 66°C and lowering the final probe concentration in the PCR from 200 to 80 nM (**Figure 8.5.1.1.3.**).

However, when this assay was run recently on an alternative PCR machine (Chromo4, Bio-Rad) using the modified conditions and white PCR tubes the same low level 'background' was seen once more. Increasing the annealing/extension temperature to 67°C and lowering the final probe concentration in the

PCR from 200 to 50 nM showed a degree of improvement but did not completely eliminate the background signal. In practice this background does not inhibit scoring as an *An. quadriannulatus*, *An. melas* or *An. merus* specimen shows a strong signal in the Cy5 channel while a true *Anopheles gambiae s.s.* individual only shows signal in the VIC channel.
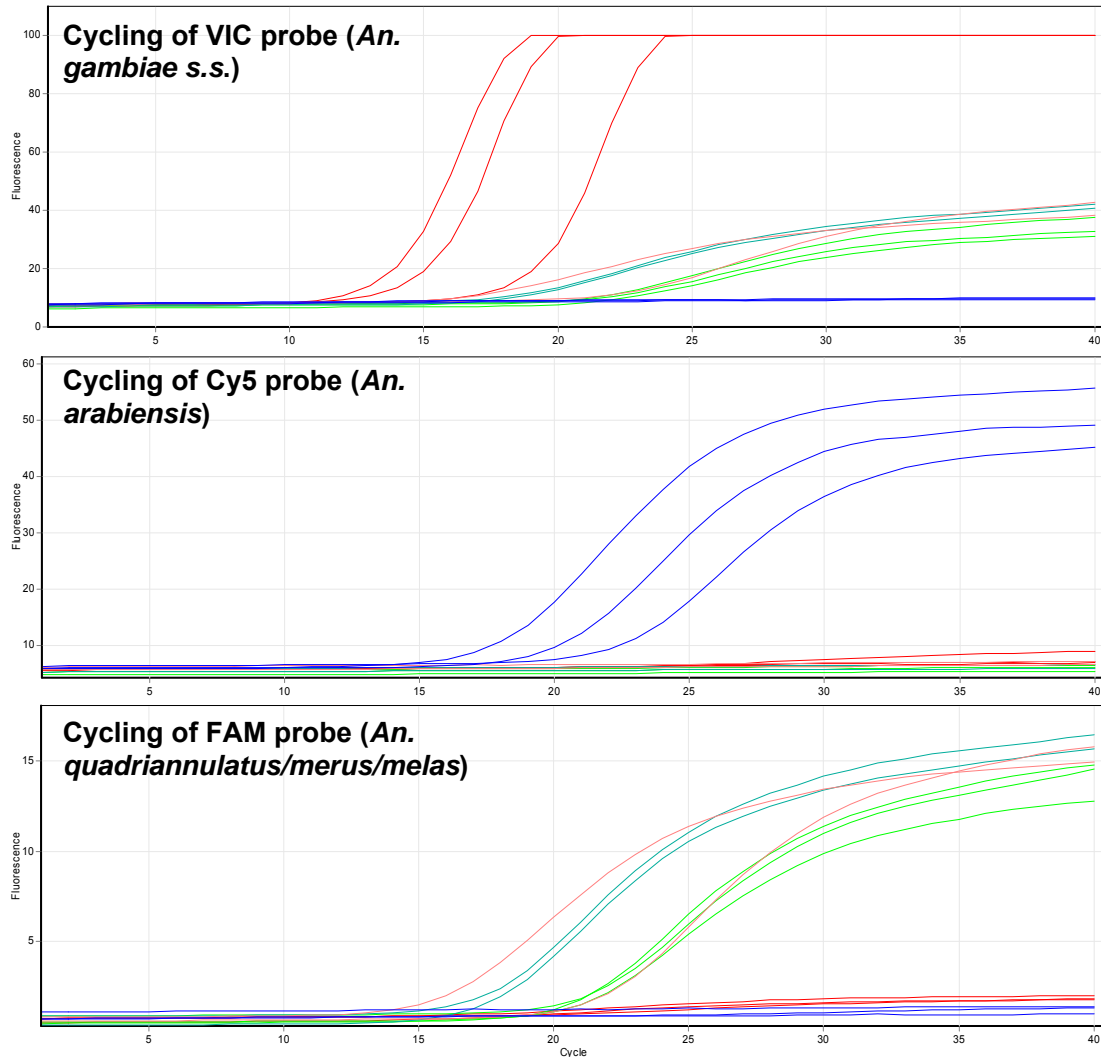
Figure 8.5.1.1.4. Species identification using the multiplex real-time PCR assay. When the assay is run at 60°C a background signal from the VIC probe is observed when using *An. quadriannulatus*, *An. melas* or *An. merus* DNAs. *An. gambiae s.s.* (red trace), *An. arabiensis* (blue trace) and *An. quadriannulatus/An. melas/An. merus* (green, pink and light blue traces).

References

Bass C, Williamson MS, Field LM (2008) Development of a multiplex real-time PCR assay for identification of members of the *Anopheles gambiae* species complex. Acta Trop 107:50-53

Bass C, Williamson MS, Wilding CS, Donnelly MJ, Field LM (2007) Identification of the main malaria vectors in the *Anopheles gambiae* species complex using a TaqMan real-time PCR assay. Malar J 6:155

Walker ED et al. (2007) Identification of field caught *Anopheles gambiae s.s.* and *Anopheles arabiensis* by TaqMan single nucleotide polymorphism genotyping. Malar J 6:23

8.5.1.2 Plasmodium *detection assay*

Chris Bass, Martin Williamson, John Vontas, Hilary Ranson, Martin Donnelly and Lin Field

Introduction

This assay detects all four malaria-causing *Plasmodium* species and discriminates *P. falciparum* from *P. vivax*, *P. ovale* and *P. malariae*. This method is not inhibited by the storage of mosquito specimens by drying or in ethanol or isopropanol (Bass et al. 2008). The assay is suitable for use on real-time PCR machines that have two or more detection channels. This assay will detect stages of *Plasmodium* in the blood meal/gut of the mosquito; therefore, to ensure only sporozoites within the salivary glands are detected, DNA should be extracted from head-thorax only and the abdomen removed prior to extraction. An alternate technique can be found in **Chapter 8.1**.

Assay conditions

96	48	1	Reagent
480 µl	240 µl	4.8 µl	sterile H_2O
1.25 ml	625 µl	10.0 µl	SensiMix DNA kit (Quantace)
200 µl	100 µl	1.6 µl	primer PlasF (800nM) GCTTAGTTACGATTAATAGGAGTAGCTTG
200 µl	100 µl	1.6 µl	primer PlasR (800nM) GAAAATCTAAGAATTTCACCTCTGACA
20 µl	10 µl	0.6 µl	TaqMan MGB probe (200 nM) Falcip+ 6FAM-TCTGAATACGAATGTC
50 µl	25 µl	0.4 µl	TaqMan MGB probe (200 nM) OVM+ VIC-CTGAATACAAATGCC
2.2 ml	**1.1 ml**	**19 µl**	**Total (To each 19 µl reaction add 1-2 µl genomic DNA)**

NOTE: In tests of this assay using the Rotor-Gene 6000™ (Corbett Research) no loss of sensitivity was observed for half volumes of reagents (10µl total volume).

Assay PCR cycle conditions
95°C/10 min x 1 cycle; (95°C/10sec, 60°C/45sec) x 40 cycles
Measure fluorescence at the end of each cycle

Interpreting Results and Examples

The *Plasmodium* assay uses two probes, the first labeled with 6FAM detects *P. falciparum* and the second, labeled with VIC, detects *P. vivax/P. ovale/P. malariae.* A substantial increase in FAM fluorescence during PCR indicates the presence of *P. falciparum* whilst a substantial increase in VIC fluorescence indicates the presence of *P. vivax*, *P. ovale* or *P. malariae* (**Figure 8.5.1.2.1**). An increase in both dyes would indicate a mixed infection.

To help with species assignment, the Rotor-Gene software allows endpoint fluorescence values for the two dyes to be automatically corrected for background and plotted against each other in bi-directional scatter plots (**Figure 8.5.1.2.2**).
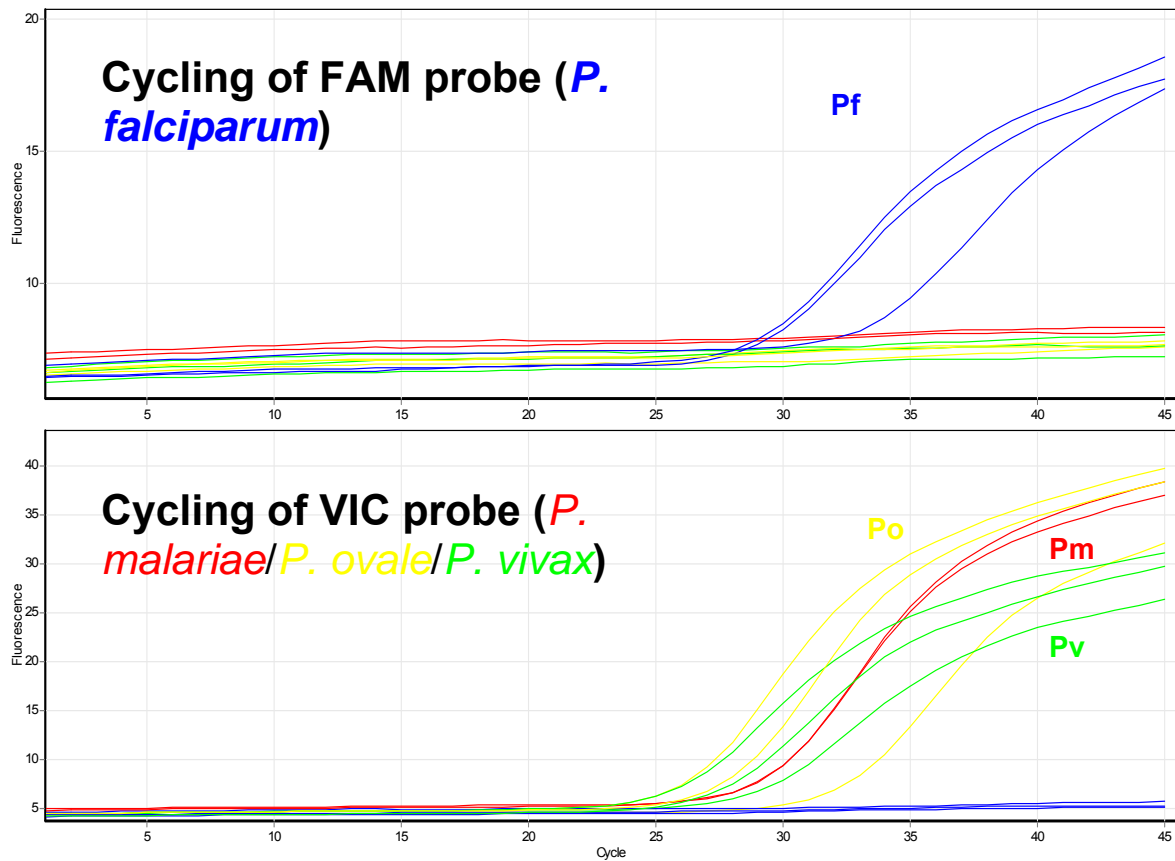
Figure 8.5.1.2.1. Detection of *Plasmodium* species by TaqMan assay. In this example two or more specimens of *P. falciparum* (blue trace), *P. vivax* (green trace), *P. ovale* (yellow trace) and *P. malariae* (red trace) were tested.
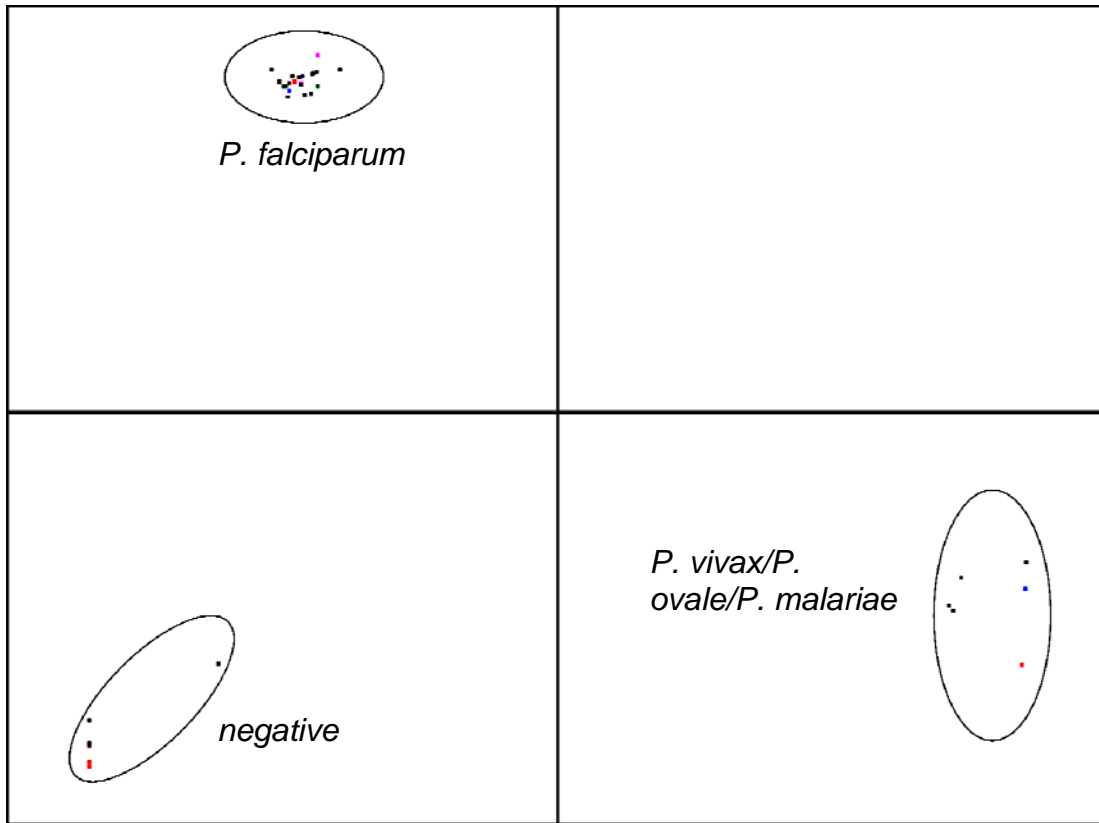
Figure 8.5.1.2.2. Scatter plot analysis of TaqMan fluorescence data. In this example the TaqMan assay was carried out on ~30 *Plasmodium* genomic DNA samples and five no template negative controls. Fluorescence values of the FAM labeled probe specific for P*. falciparum* were plotted against those of the VIC labeled probe specific for *P. vivax, P. ovale* and *P. malariae*.

Notes and Troubleshooting

The *Plasmodium* detection assay was originally optimized using purified *Plasmodium* genomic DNA as template and carrying out 45 cycles of PCR. When tests were carried out on blood fed mosquitoes using a standard 'quick and dirty' DNA extraction, a small amount of non-specific fluorescence was sometimes seen after 40 cycles (see figure 7 below). For this reason we recommend restricting the number of cycles in PCR to 40.
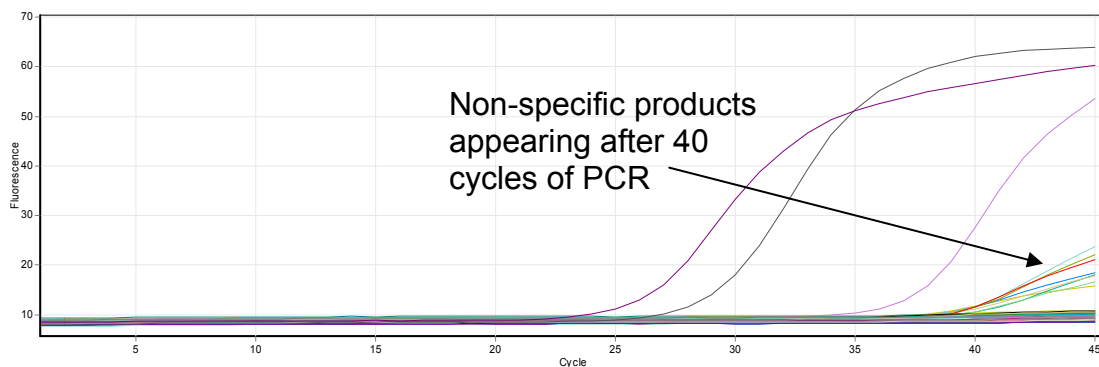
Figure 8.5.1.2.3. Cycling of VIC labelled probe specific for *P. ovale*, *P. vivax*, and *P. malariae*.

References

Bass C, Nikou D, Blagborough AM, Williamson MS, Vontas J, Sinden RE, Field LM (2008) PCR-based detection of *Plasmodium* in *Anopheles* mosquitoes: a comparison of a new high-throughput assay with existing methods. Malaria J 7:177

8.5.1.3 Knock down resistance (kdr) assays

Chris Bass, Martin Williamson, John Vontas, Hilary Ranson, Martin Donnelly and Lin Field

Introduction

Two separate assays have been developed for the detection of *kdr-w* (L1014F) or *kdr-e* (L1014S) (Bass et al. 2007). This assay is suitable for use on real-time PCR machines that have two or more detection channels. Alternate PCR assays can be found in **Chapters 5.3.1** and **5.3.2**.

Assay conditions for *kdr-w* (L1014F)

96	48	1	Reagent
500 µl	250 µl	5.0 µl	sterile H_2O
1.0 ml	500 µl	10.0 µl	SensiMix DNA kit (Quantace)
160 µl	80 µl	1.6 µl	primer kdr-forward (800nM) CATTTTTCTTGGCCACTGTAGTGAT
160 µl	80 µl	1.6 µl	primer kdr-reverse (800nM) CGATCTTGGTCCATGTTAATTTGCA
40 µl	20 µl	0.4 µl	TaqMan MGB probe (200 nM) WT VIC-CTTACGACTAAATTTC
40 µl	20 µl	0.4 µl	TaqMan MGB probe (200 nM) KdrW 6FAM-ACGACAAAATTTC
1.9 ml	**950 µl**	**19 µl**	**Total (To each 19 µl reaction add 1-2 µl genomic DNA)**

Assay conditions for *kdr-e* (L1014S)

96	48	1	Reagent
500 µl	250 µl	5.0 µl	sterile H_2O
1.0 ml	500 µl	10.0 µl	SensiMix DNA kit (Quantace)
160 µl	80 µl	1.6 µl	primer kdr-forward (800nM) CATTTTTCTTGGCCACTGTAGTGAT
160 µl	80 µl	1.6 µl	primer kdr-reverse (800nM) CGATCTTGGTCCATGTTAATTTGCA
40 µl	20 µl	0.4 µl	TaqMan MGB probe (200 nM) WT VIC-CTTACGACTAAATTTC
40 µl	20 µl	0.4 µl	TaqMan MGB probe (200 nM) KdrE 6FAM-ACGACTGAATTTC
1.9 ml	**950 µl**	**19 µl**	**Total (To each 19 µl reaction add 1-2 µl genomic DNA)**

Assay PCR cycle conditions

95°C/10 min x 1 cycle; (95°C/10sec, 60°C/45sec) x 40 cycles
Measure fluorescence at the end of each cycle

Interpreting Results and Examples

Both kdr assays use two probes, the first specific for the wildtype allele is labeled with VIC, and the second, specific for the mutant allele (*kdr*-w or *kdr*-e), is labeled with FAM. In either assay, a substantial increase in VIC fluorescence indicates a homozygous wildtype, a substantial increase in FAM fluorescence indicates a homozygous mutant, and a, usually intermediate, increase in both signals indicates a heterozygote (**Figure 8.5.1.3.1-2**). Individuals homozygous for the *kdr*-e mutation display no increase in VIC or FAM fluorescence in the *kdr*-w assay and vice versa. To help score the genotypes, the Rotor-Gene software allows endpoint fluorescence values for the two dyes to be automatically corrected for background and plotted against each other in bi-directional scatter plots (**Figure 8.5.1.3.3.**).
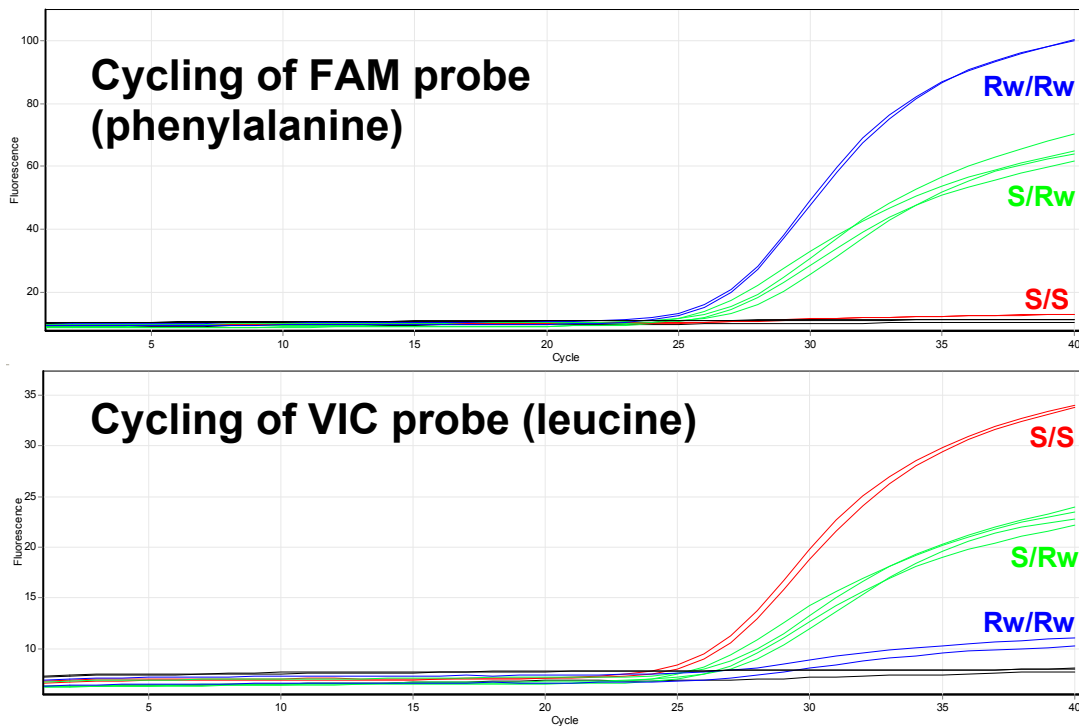
Figure 8.5.1.3.1. Real-time TaqMan detection of *kdr*-w (L1014F)S: Wild type allele (L1014), Rw: Resistant allele, West African mutation (L1014F).
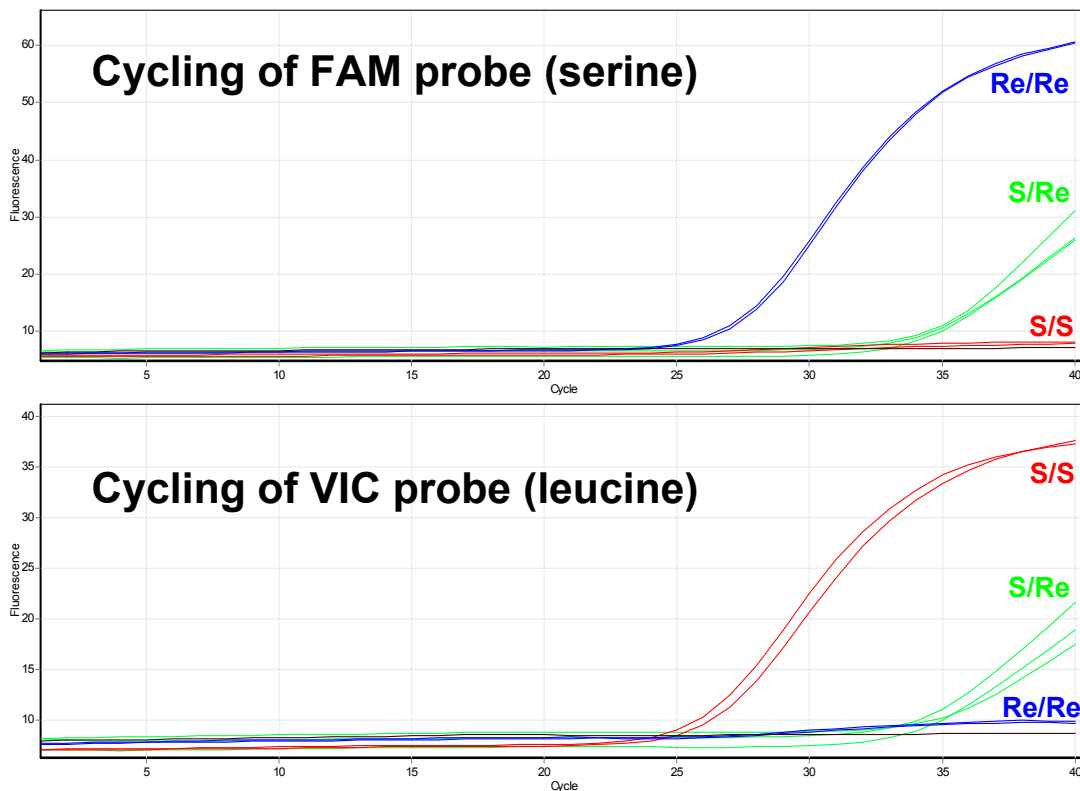
Figure 8.5.1.3.2. Real-time TaqMan detection of *kdr*-e (L1014S) S: Wild type allele (L1014), Re: Resistant allele, East African mutation (L1014S)
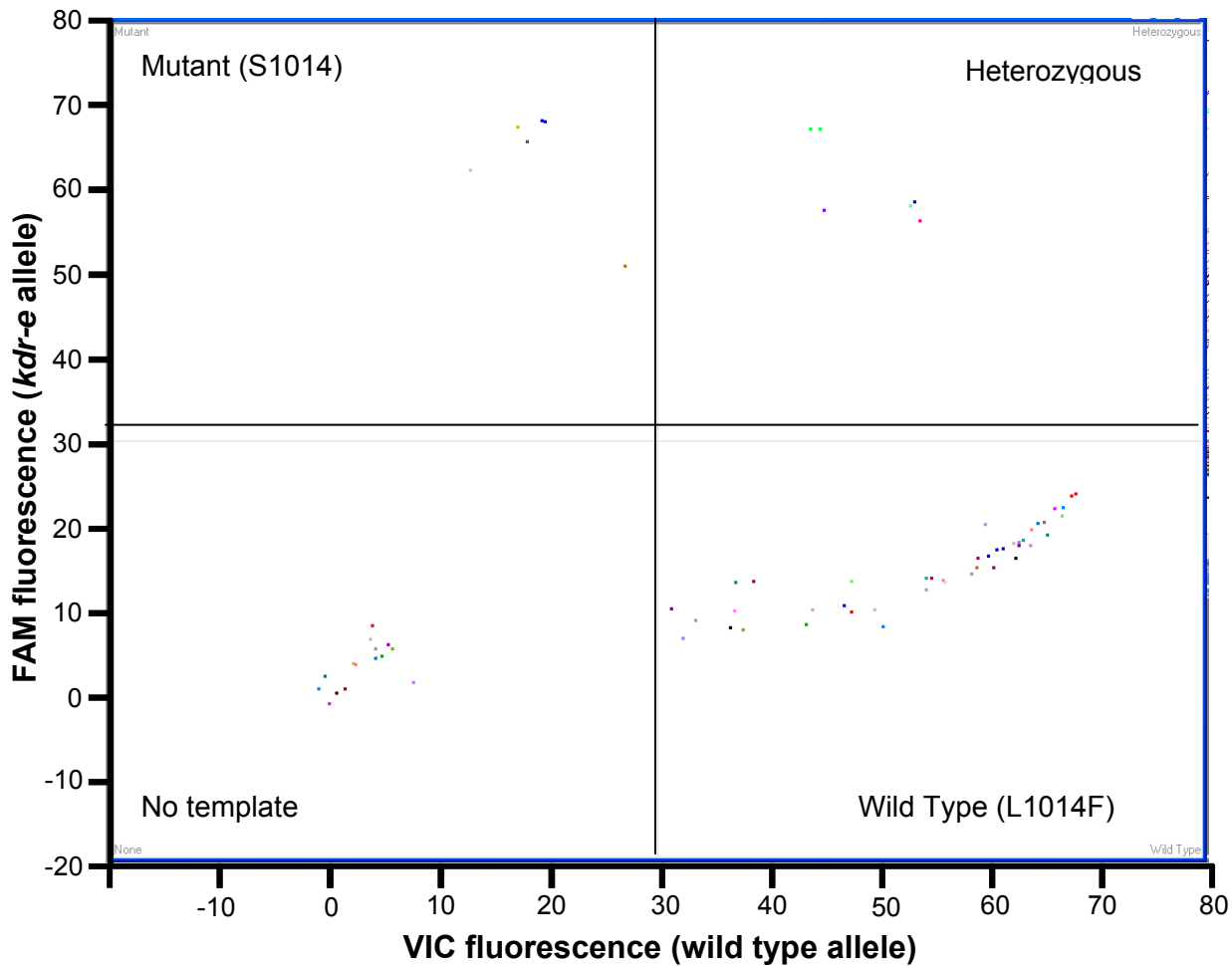
Figure 8.5.1.3.3. Scatter plot analysis of TaqMan fluorescence data In this example real time PCR was carried out using the east *kdr* assay on ~70 samples then fluorescence values of the FAM labeled probe specific for the *kdr*-e mutation were plotted against the VIC labeled probe specific for the wild type allele.

Notes and Troubleshooting

If separate runs have been performed on the same samples for *kdr-e* and *kdr-w*, when interpreting the results it is useful to have two copies of the real-time PCR machine software open on the computer at once. In one copy open the *kdr-w* run and in the other open the *kdr-e* run. The same sample can then viewed in each assay by moving between the two runs/copies of the software. This helps to rapidly assign each sample a genotype. For example, sample one is examined in the kdr-W run and shows a signal in the FAM channel and not in the VIC channel and when examined in the kdr-E run shows a signal in neither channel. Sample one is therefore scored kdr-W homozygous. Sample two is examined in the kdr-W run and shows an intermediate signal in the VIC channel but no signal in the FAM channel, when examined in the kdr-E run it shows an intermediate signal in both the FAM and VIC channels. Sample two is therefore scored kdr-E heterozygous. Alternatively if the east and west assays were run together on the same samples the same approach can be followed using a single copy of the software.

In most of our experiments using wild-caught mosquito specimens, we have found that interpreting the results of the kdr TaqMan assays is relatively straightforward. However in some instances when using 'quick and dirty' DNA extraction protocols, we have sometimes seen background signals which have

made assigning genotypes more difficult. Difficulty in scoring genotypes is often associated with very low signals due to the poor or low yield of DNA obtained.

In our experience, distinguishing between Rw/Rw and S/Rw genotypes can seem less obvious than scoring other genotypes because a Rw/Rw genotype shows a very low level signal in the VIC channel rather than a completely flat line (as with a no-template control). In addition the signal strength from the FAM and VIC probe can be different. Running controls of the two genotypes is very helpful and allows for a direct comparison with unknown samples. Further examples of RwRw and S/Rw genotypes (without the autoscaling) illustrating these points are included below in **Figure 8.5.1.3.4**.
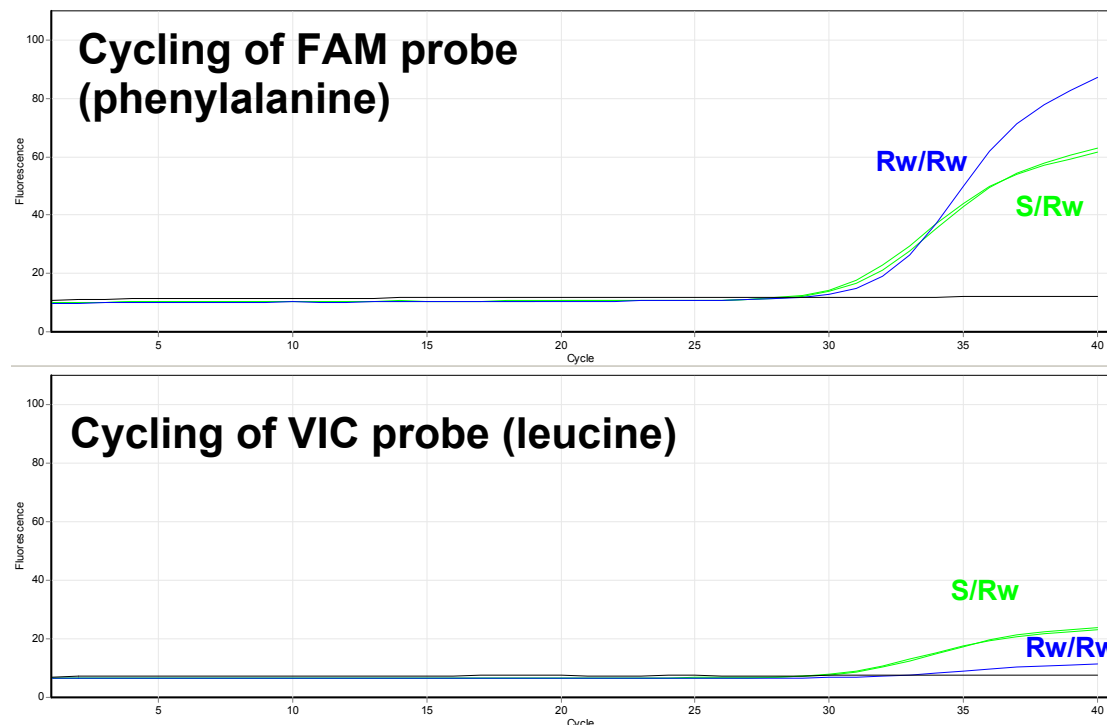
Figure 8.5.1.3.4. Real-time TaqMan detection of kdr-w (L1014F). Blue trace, R/Rw: Resistant allele, Green trace S/Rw, Black trace, blank. Note the autoscale function was not used in this example and the signal from the VIC probe is lower than the FAM probe. In addition note that the Rw/Rw signal in the VIC channel is above that of the blank shown in black.

Use of Assay on single legs

In our experiments to date when we have run the assay on DNA extracted from a single leg we have obtained mixed results (possibly due to the low quality/concentration of DNA extracted) and therefore we cannot recommend the TaqMan kdr assays for this approach.

References

Bass C et al. (2007) Detection of knockdown resistance (kdr) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. Malar J 6:111

8.5.1.4 Insensitive acetylcholinesterase (iAChe) assay

Chris Bass, Martin Williamson, John Vontas, Hilary Ranson, Martin Donnelly and Lin Field

Introduction

This assay detects the G119S mutation in the gene *ace-1* which encodes the acetylchoinesterase enzyme. This assay is suitable for use on real-time PCR machines that have two or more detection channels. A PCR based assay can be found in **Chapter 5.3.4**.

Assay conditions

96	48	1	Reagent
500 µl	250 µl	5.0 µl	sterile H_2O
1.0 ml	500 µl	10.0 µl	SensiMix DNA kit (Quantace)
160 µl	80 µl	1.6 µl	primer ACE1-F (800nM) GGCCGTCATGCTGTGGAT
160 µl	80 µl	1.6 µl	primer ACE1-R (800nM) GCGGTGCCGGAGTAGA
40 µl	20 µl	0.4 µl	TaqMan MGB probe (200 nM) Ace1G119 VIC-TTCGGCGGCGGCT
40 µl	20 µl	0.4 µl	TaqMan MGB probe (200 nM) Ace1S119 6FAM-TTCGGCGGCAGCT
1.9 ml	**950 µl**	**19 µl**	**Total (To each 19 µl reaction add 1-2 µl genomic DNA)**

Assay PCR cycle conditions

95°C/10 min x 1 cycle; (95°C/10sec, 60°C/35sec) x 40 cycles
Measure fluorescence at the end of each cycle

Interpreting Results and Examples

The iAChE assay uses two probes, the first specific for the wild-type allele is labeled with VIC and the second, specific for the mutant allele (S119), is labeled with FAM. A substantial increase in VIC fluorescence indicates a homozygous wild-type, a substantial increase in FAM fluorescence indicates a homozygous mutant and a, usually intermediate, increase in both signals indicates a heterozygote (**Figure 8.5.1.4.1**).

A
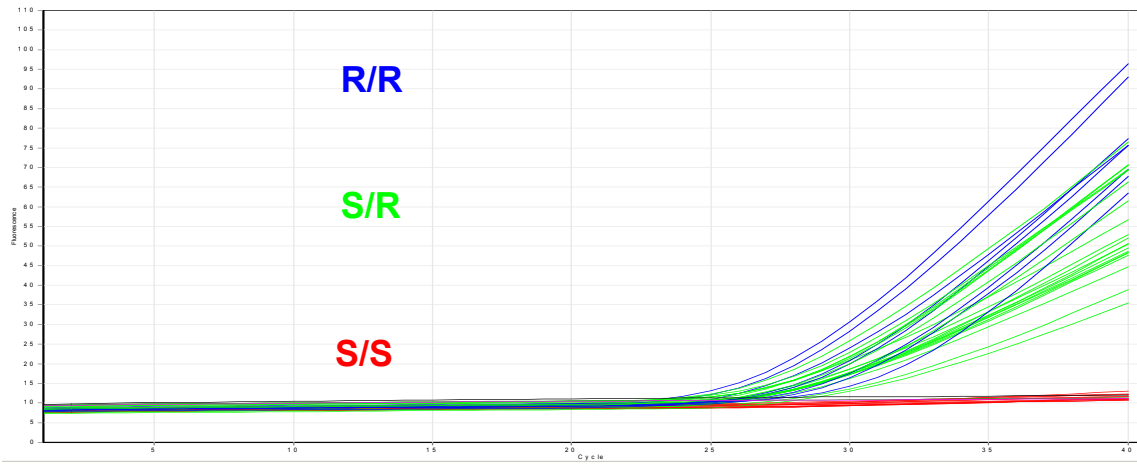
B
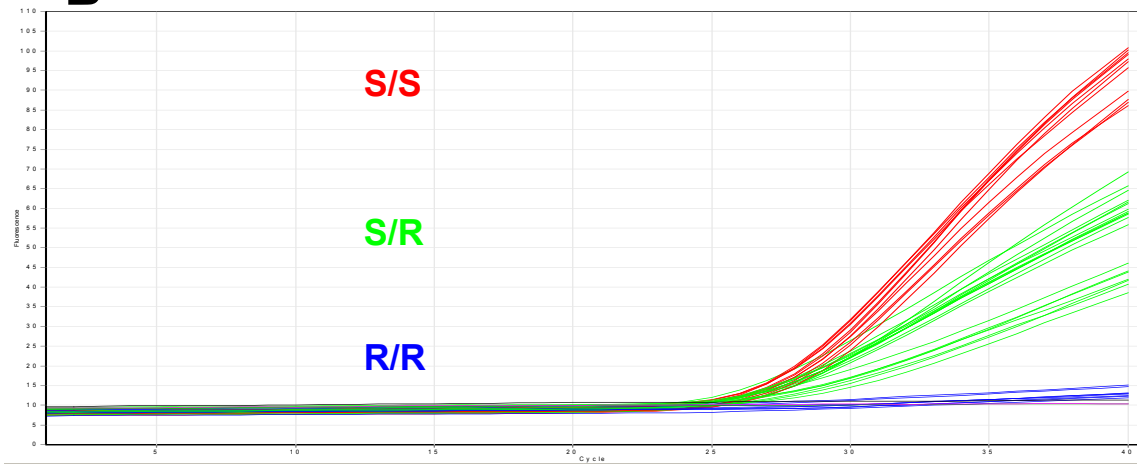
Figure 8.5.1.4.1. Real-Time TaqMan detection of G119S. A) Cycling FAM probe (Serine) B) Cycling VIC probe (Glycine).

8.5.1.5 Resistance to dieldrin (rdl) assay

Chris Bass, Martin Williamson, John Vontas, Hilary Ranson, Martin Donnelly and Lin Field

Introduction

Two TaqMan assays have been developed, one for each of the two alternative mutations found *in An. gambiae s.s.* (A296G) and *An. arabiensis* (A296S). This assay is suitable for use on real-time PCR machines that have two or more detection channels. An alternate PCR based assay can be found in **Chapter 5.3.3**.

A296G Assay conditions

96	48	1	Reagent
500 µl	250 µl	5.0 µl	sterile H_2O
1.0 ml	500 µl	10.0 µl	SensiMix DNA kit (Quantace)
160 µl	80 µl	1.6 µl	primer GlyRdlF (800nM) TCATATCGTGGGTATCATTTTGGCTAAAT
160 µl	80 µl	1.6 µl	primer GlyRdlR (800nM) CGACATCAGTGTTGTCATTGTCAAG
40 µl	20 µl	0.4 µl	TaqMan MGB probe (200 nM) WT1 VIC-ACGTGTTGCATTAGG
40 µl	20 µl	0.4 µl	TaqMan MGB probe (200 nM) Gly 6FAM-ACGTGTTGGATTAGG
1.9 ml	**950 µl**	**19 µl**	**Total (To each 19 µl reaction add 1-2 µl genomic DNA)**

A296G Assay PCR cycle conditions

95°C/10 min x 1 cycle; (95°C/10sec, 62°C/45sec) x 40 cycles. Measure fluorescence at the end of each cycle.

A296S Assay conditions

96	48	1	Reagent
500 µl	250 µl	5.0 µl	sterile H_2O
1.0 ml	500 µl	10.0 µl	SensiMix DNA kit (Quantace)
160 µl	80 µl	1.6 µl	primer SerRdlF (800nM) TCATATCGTGGGTATCATTTTGGCTAAAT
160 µl	80 µl	1.6 µl	primer SerRdlR (800nM) TCGTTGACGACATCAGTGTTGT
40 µl	20 µl	0.4 µl	TaqMan MGB probe (200 nM) WT2 VIC-TTACACCTAATGCAACACG
40 µl	20 µl	0.4 µl	TaqMan MGB probe (200 nM) Ser 6FAM-CACCTAATGAAACACG
1.9 ml	**950 µl**	**19 µl**	**Total (To each 19 µl reaction add 1-2 µl genomic DNA)**

A296S Assay PCR cycle conditions

95°C/10 min x 1 cycle; (95°C/10sec, 60°C/45sec) x 40 cycles. Measure fluorescence at the end of each cycle.

Interpreting Results and Examples

Both *rdl* assays use two probes, the first specific for the wild type allele is labeled with VIC and the second, specific for the mutant allele (S296 or G296), is labeled with FAM. In either assay, a substantial increase in VIC fluorescence indicates a homozygous wild type, a substantial increase in FAM fluorescence indicates a homozygous mutant and a, usually intermediate, increase in both signals indicates a heterozygote (**Figures 8.5.1.5.1** and **8.5.1.5.2**).
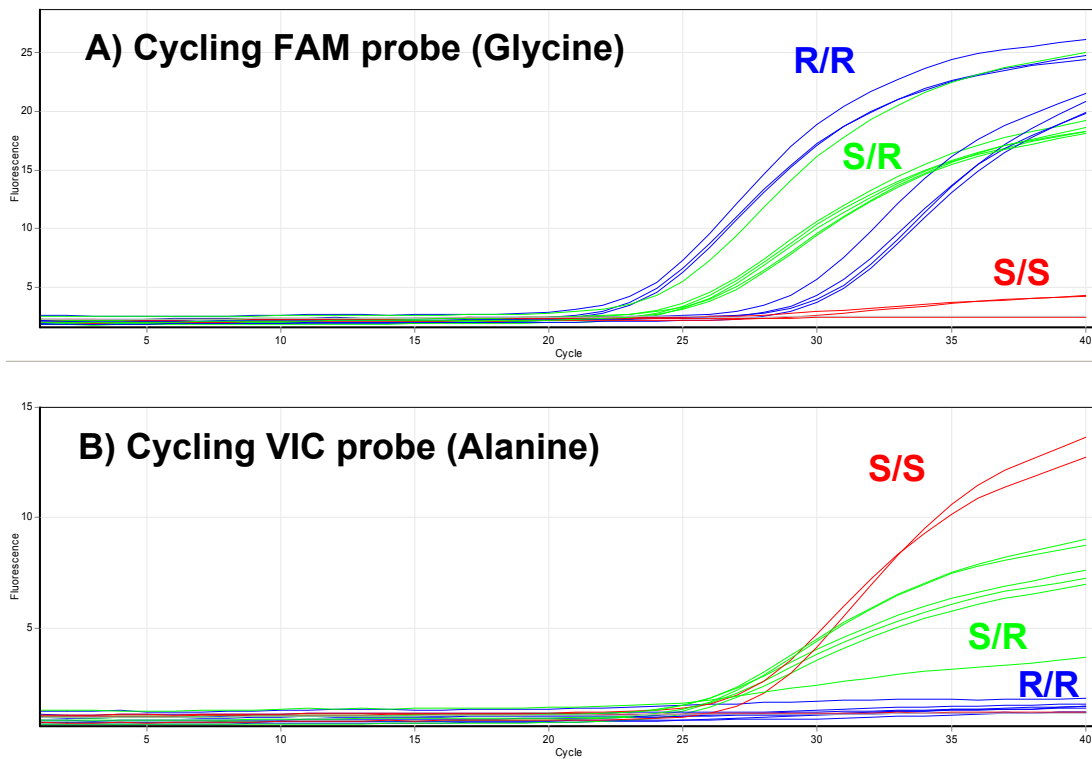
Figure 8.5.1.5.1. Real-time TaqMan detection of A296G
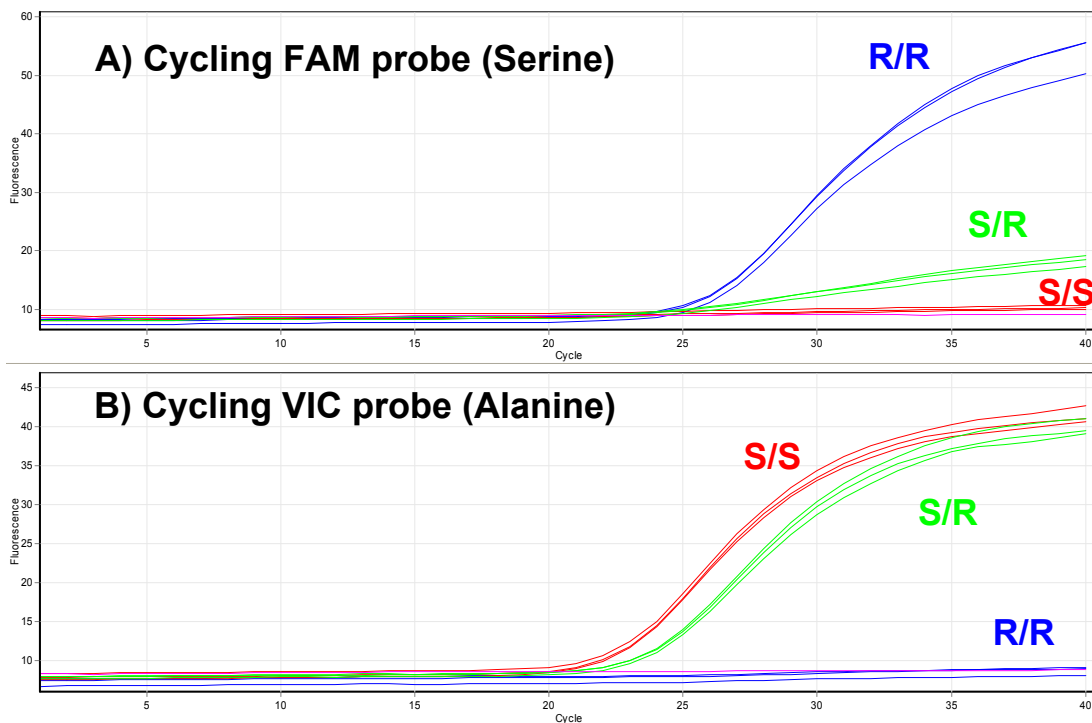
Figure 8.5.1.5.2. Real-time TaqMan detection of A296S

8.6 Molecular Karyotyping PCR Assays

8.6.1 *Anopheles gambiae* 2La inversion (White et al.)

Bradley White and Nora Besansky

Introduction

The 2La inversion in *An. gambiae* is associated with tolerance to aridity and indoor resting behavior. This inversion may also be involved in the incipient speciation between the Mopti and Savanna chromosomal forms (White et al. 2007). Traditionally, polytene chromosomal banding patterns were used to differentiate between the 2La and 2L+[a] forms. However, due to the complex procedures needed to produce these, few laboratories have trained personnel. In order to better understand the effects of karyotype on phenotype, a PCR-based method was developed based on the published sequence of *An. gambiae.* This method has been shown to be robust in identifying the inversion within all members of the *An. gambiae* complex.

PCR authentication for the 2La inversion in the *Anopheles gambiae* complex (White et al. 2007)

Prepare PCR Master Mix for 96, 48 or 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
1185 µl	592.5µl	11.85µl	Distilled H2O
500 µl	250 µl	5.0 µl	5X PCR buffer
200 µl	100 µl	2.0 µl	dNTP (2.5 mM concentration)
100 µl	50 µl	1.0 µl	23A2 (R, 25 pmol/ µl) CTC GAA GGG ACA GCG AAT TA
100 µl	50 µl	1.0 µl	27A2 (F, 25 pmol/ µl) ACA CAT GCT CCT TGT GAA CG
100 µl	50 µl	1.0 µl	DPCross 52L+ (F, 25pmol/ µl) GGT ATT TCT GGT CAC TCT GTT GG
200 µl	100 µl	2.0 µl	MgCl2 (25 mM)
15 µl	7.5 µl	0.15 µl	Go-Taq DNA polymerase (5U/ µl)
2.4 ml	**1.20 ml**	**24 µl**	**Total (To each 24 µl reaction add 1 µl template DNA)**

Table 8.6.1.1. F and R indicate forward and reverse orientation

PCR cycle conditions

94°C/2min x 1 cycle
(94°C/30sec , 60°C/30sec , 72°C/45sec) x 35 cycles
72°C/10min x 1 cycle
4°C hold

Run samples on a 2% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red. (**Figure 8.6.1.1**). Primers create fragments of 492 and 207bp for 2La and 2L+[a] arrangements, respectively.

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 reactions to compensate for imprecise measurements.
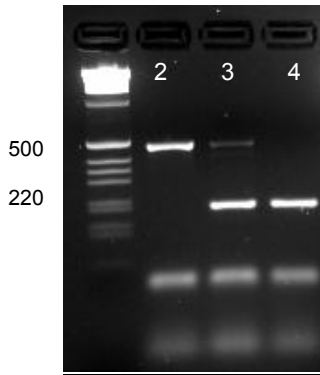
Figure 8.6.1.1 Lane 1 1kb ladder, lane 2 2La/a homozygous *An. gambiae*, lane 3 2La/+[a] hybrid, lane 4 2L+[a]/+[a] homozygous.

Troubleshooting:

Lowering the annealing temperature (to 58°C or 55°C) may effectively improve PCR amplification under some conditions.

96 well PCR sample preparation template

References

White BJ et al. (2007) Molecular karyotyping of the 2La inversion in *Anopheles gambiae.* Am J Trop Med Hyg 76:334-339

8.6.2. Anopheles gambiae s.s. *2Rb Inversion PCR Assay*

Neil Lobo, Djibril Sangare, Allison Regier, Kyanne Reidenbach, David Bretz, Maria Sharakhova, Scott Emrich, Sekou Traore, Carlo Constantini, Nora Besansky, and Frank Collins

Introduction

Alternative arrangements of chromosome 2 inversions in *Anopheles gambiae* are important sources of population structure, and are associated with adaptation to environmental heterogeneity. Molecular characterization of inversion breakpoints provides insight into how they arose, and provides the basis for development of molecular karyotyping methods useful in future studies. Sequence differences between alternative 2R*b* arrangements were exploited in the design of a PCR diagnostic assay, which was evaluated against the known chromosomal banding pattern of laboratory colonies and field collected samples from Mali and Cameroon. The molecular diagnostic was reliable when applied to laboratory colonies, but its accuracy was lower in natural populations. The complex repetitive sequence flanking the 2R*b* breakpoint region may be prone to structural and sequence-level instability resulting in variable results in wild populations not tested. The 2R*b* molecular diagnostic has immediate application in studies based on laboratory colonies, but its usefulness in natural populations awaits development of complementary molecular tools.

PCR authentication for the 2Rb inversion in *Anopheles gambiae* (Lobo et al. 2010)

Prepare PCR Master Mix for 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	0.5	Reagent
1.46 ml	730 µl	14.6 µl	7.8 µl	Distilled H2O
500 µl	250 µl	5.0 µl	2.5 µl	5X PCR buffer
100 µl	50 µl	1.0 µl	0.5 µl	dNTP (2mM concentration)
100 µl	50 µl	1.0 µl	0.5 µl	b-For (5 pmol/ µl) CGG GAG CAA AGA TAA GTA GCA
100 µl	50 µl	1.0 µl	0.5 µl	b+ Rev (2.5pmol/ µl) CCG GAT AAT CGA CGC TCT AC
100 µl	50 µl	1.0 µl	0.5 µl	b-Rev (5 pmol/µl) AAC CCT ACC ATA TAC CAG TAC CAA CG
30 µl	15 µl	0.3 µl	0.15 µl	MgCl2 (25 mM)
10 µl	5.0 µl	0.1 µl	0.05 µl	Taq DNA polymerase (5U/ µl)
2.4 ml	**1.2 ml**	**24 µl**	**12.5 µl**	**Total (To each 24 µl reaction add 1 µl template DNA)**

Table 8.6.2.1. If performing the ½ volume assay, use 0.5µl template DNA instead.

PCR cycle conditions

94°C/2min x 1 cycle
(94°C/30sec , 58°C/30sec , 72°C/45sec) x 35 cycles
72°C/10min x 1 cycle
4°C hold

Run samples on a 1.5% agarose gel stained with EtBr or other intercalating agent like SYBR Green or Gel Red.

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 reactions to compensate for imprecise measurements.
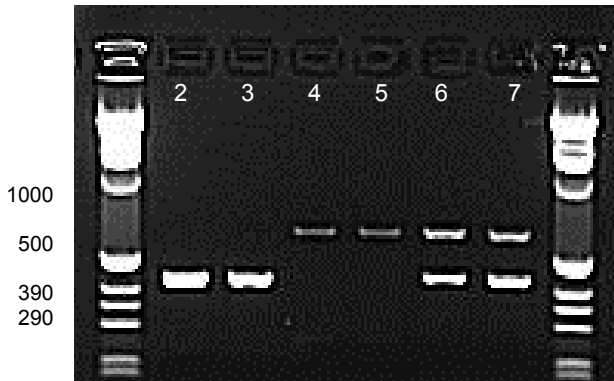
Figure 8.6.2.1 Lane 1, 1kb ladder, lanes 2-3 are 2Rb/b (inverted), lanes 4-5 are 2R+b/+b (standard), and lanes 6-7 are 2Rb/+b (heterozygous).

96 well PCR sample preparation template

References

Lobo NF, Sangare DM, Reiger AA, Reidenbach KR, Bretz DA, Sharakhova MV, Emrich SJ, Traore SF, Constantini C, Besansky NJ, and Collins FH (2010). Breakpoint structure of the *Anopheles gambiae* 2Rb chromosomal inversion. Malaria J. 9: 293.

8.6.3. Anopheles merus *2Ro Inversion PCR Assay*

Paul Howell, Daniel Usry, Igor Sharakhov, and Maryam Kamali

Introduction

Molecular characterization of inversion breakpoints provides insight into how they arose, and provides the basis for development of molecular karyotyping methods useful in future studies. Many of these inversions have been found to be species-specific, such as Xag in *An. gambiae*. To date, the 2Ro inversion has only been found in *An. merus*, a salt-water breeding vector found along the east coast of Africa. Based on recent chromosomal work done (Kamali et al 2012), a new molecular diagnostic assay was developed to detect the distal end of the 2Ro inversion. Although this assay is reliable when applied to laboratory colonies, it is untested with natural populations.

PCR authentication for the 2Ro inversion in *Anopheles merus*

Prepare PCR Master Mix for 1 25µl PCR reactions.[1] Add reagents in the order presented.

96	48	1	Reagent
1.31 ml	655 µl	13.1 µl	Distilled H2O
400 µl	200 µl	4.0 µl	5X PCR buffer
80 µl	40 µl	0.8 µl	dNTP (2mM concentration)
50 µl	25 µl	0.5 µl	2RoF (10 pmol/ µl) CCG TAG AAG TGA ACT CGC GTC
50 µl	25 µl	0.5 µl	2RoInvR (10 pmol/ µl) TGA CAG CTG ATA GCC TTC ACG
50 µl	25 µl	0.5 µl	2RoStdR (10 pmol/µl) GCT CCA CTG GCT AGC TTT CC
50 µl	25 µl	0.5 µl	MgCl2 (25 mM)
10 µl	5.0 µl	0.1 µl	Taq DNA polymerase (5U/ µl)
2.0 ml	**1.0 ml**	**20 µl**	**Total (To each 20 µl reaction add 1 µl template DNA)**

Table 8.6.3.1. If performing the ½ volume assay, use 0.5µl template DNA instead.

PCR cycle conditions

94°C/5min x 1 cycle
(94°C/60sec , 58°C/60sec , 72°C/120sec) x 40 cycles
72°C/5min x 1 cycle
4°C hold

Run samples on a 1.5% agarose EtBr, or other intercalating agent, gel. *An. gambiae* will give one of two band sizes, either 360 or 400 bp while *An. merus* will yield a band of 315 bp.

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[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 reactions to compensate for imprecise measurements.
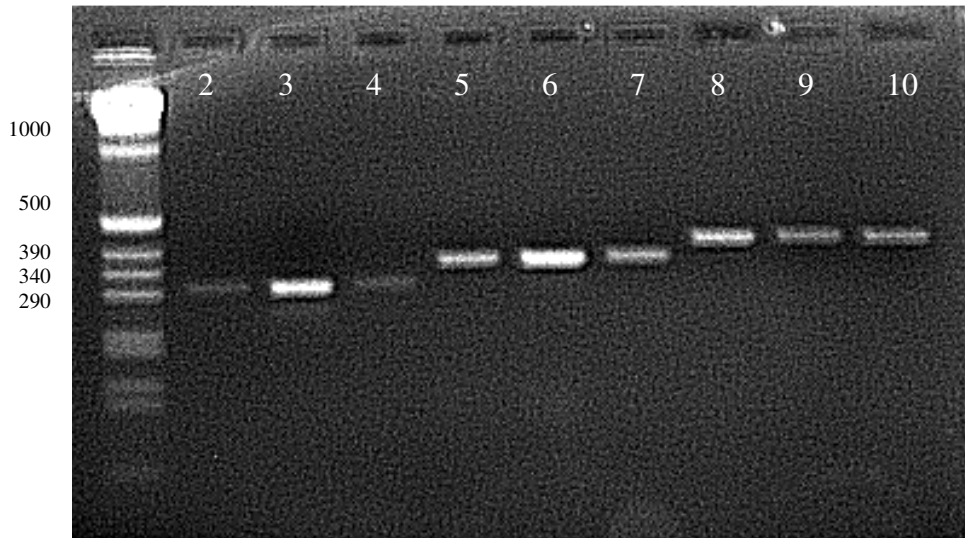
**Figure 8.6.3.1**. Lane 1: 1kb ladder, lanes 2-4 *An. merus*, lanes 5-7 *An. gambiae* (ASEMBO1 strain), lanes 8-10, *An. gambiae* (MOPTI strain)

96 well PCR sample preparation template

References

Kamali M, Z Tu, IV Sharakhov (2012). A new chromosomal phylogeny supports the repeated origin of vectorial capacity in malaria mosquitoes of the *Anopheles gambiae* complex. PLoS Pathogens 8(10): e1002960. doi: 10.1371/jourla.ppat.1002960

Loop-Mediated Isothermal Amplification (LAMP) for species discrimination between *Anopheles gambiae* and *Anopheles arabiensis*.

Mariangela Bonizzoni, Yaw Afrane and Guiyun Yan

Introduction

This protocol describes the application of the Loop-Mediated Isothermal Amplification (LAMP) technique for the rapid identification of *Anopheles gambiae* and *Anopheles arabiensis* mosquitoes. LAMP is a one-step nucleic acid amplification performed under isothermal condition using a DNA polymerase with strand displacement activity. It requires two sets of primers (B3 and F3, FIP and BIP) that recognize six separate regions within the target DNA, conferring high sensitivity and specificity to the amplification (Tomita et al., 2008). Template for the reaction is mosquito DNA extracted from either a leg or the whole body. Different procedures for DNA extraction can be applied adding versatility to the LAMP protocol (Bonizzoni et al., 2009). Additionally, the amplification products can be visualized directly by eye (Figure 1). However, the amplified products are not suitable for further downstream application such as cloning. When compared to the conventional rDNA-polymerase chain reaction (PCR) technique (Scott et al., 2003), the sensitivity and specificity of the LAMP method for species discrimination between *An. gambiae* and *An. arabiensis* ranged from 0.93 to 1.00 (Bonizzoni et al., 2009).

Materials

- Sterilized and clear 1.5 ml and 0.2 ml tubes
- Incubator or thermocycler
- Centrifuge for 1.5 ml and 0.2 ml tubes
- Vortex
- Ice and ice box
- Loopamp Amplification kit (Eiken Chemical Co.,[1] Tokyo, Japan) composed of 2X Reaction Mix [Tris-HCl ph 8.8 40mM, KCl 20mM, $MgSO_4$ 16 mM, $(NH_4)_2SO_4$ 20mM, Tween20 0.2%, Betaine 1.6 M, dNTPs 2.8 mM each] and *Bst* DNA polymerase
- Loopamp Florescence Detection Reagent (Eiken Chemical Co., Tokyo, Japan)

OPTIONAL: UV lamp (wavelength at ~300nm)

Table 8.7.1 Primers used for the LAMP mediated assay

Primer name	Sequence (5' to 3')
F3 gambiae	ACG TAA CAC TAG TGA GCT TGT C
B3 gambiae	CCA CCT CGA CAC ACG ACG
FIP gambiae	GGT GTG TAA GCT TAC TGG TTT GGT GCG TGC CTC GTT CTC GA
BIP gambiae	ATA AGT TAA TCC GTT TGG GCC GGT AAC CGA ACA TGG TCA ACA ACA
F3 arabiensis	AGG ACA CTT AAC ACT AAT GAG C
B3 arabiensis	CTC GAC ACA CGA CCT GTT
FIP arabiensis	CGA GCA TGT GTA AGC TTA CTG GTT CTC GAC TTG ATT GTC TTG ATG
BIP arabiensis	AGT TGT ATA AGT TGA CCC GTT TGG CAA CCG AAC ATG GTC AAC ACC

Preparation of reagents

1. Take out LAMP reagents from the freezer at -20°C and thaw them at room temperature. Once thawed, keep all reagents in ice throughout the experiment.
2. Prepare the master mix in ice by adding the followings (amounts indicated are for one reaction): 12.5 µl of 2X reaction mix, 40 pmol of FIP, 40 pmol of BIP, 5 pmol of F3, 5 pmol of B3, 1 µl of *Bst* DNA polymerase, 1 µl of Fluorescent Detection Reagent, distilled water up to 23 µl.
3. Mix the master mix either by inverting the tube or by vortexing 1 second 3 times. More intense vortexing can inactivate the polymerase. Briefly spin down.
4. Aliquot 23 µl of master mix into 0.2 ml clear tubes.
5. Add 2 µl of DNA (5-10 ng). Mix by pipetting or tipping, do not vortex. Briefly spin down to avoid the presence of air-bubbles.

NOTE: prepare two separate master mixes for the *An. gambiae* and *An. arabiensis* primer sets and test each DNA separately with the two master mixes. LAMP assay also works when reducing reaction volumes by half (12.5 µl final volume) and by two-thirds (8.3 µl final volume) if keeping the primer concentration at 40 pmol for FIP and BIP and 5 pmol for F3 and B3 (**Figure 8.7.2**).

Reaction and visualization of the product

1. Incubate the 0.2 ml tubes containing the master mix and the DNA at 63°C for 60 minutes
2. Inactivate the *Bst* polymerase at 80°C for 5 minutes
3. Inspect samples by eye and/or under UV

Figure 8.7.1 shows the results of the LAMP method for *Anopheles gambiae* and *Anopheles arabiensis* species discrimination visualized by eye (A, B) or by UV (C, D). LAMP reaction was conducted using *An. arabiensis* (A, C) or *An. gambiae* (B, D) specific primers. Tubes 1-3, 8: *An. arabiensis* DNA; tubes 4-6, 9: *An. gambiae* DNA. Tubes 7, 10 negative control with water as template. Positive samples that result from a successful LAMP amplification are shown with a turbid yellow liquid with a white precipitate when inspected by eye (tubes 1-3 in panel A and tubes 4-6 in panel B) or with intense white when inspected under UV (sample 8 in panel C and sample 9 in panel D). Absence of LAMP amplification is evidenced by a clear orange-like liquid when inspected by eye (tubes 4-7 in panel A and tubes 1-3, 7 in panel B) or opaque white under UV (Tubes 9,10 in panel C and tubes 8, 10 in panel D).
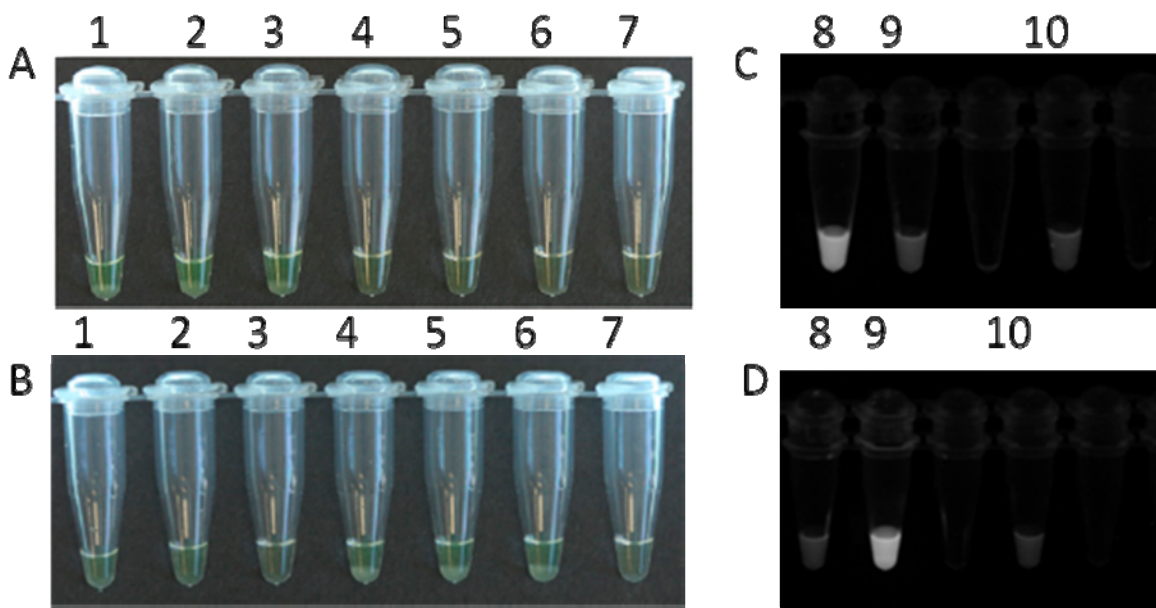
Figure 8.7.2 shows the effect of reducing LAMP reaction volumes, from 25 µl to 12.5 µl and 8.3 µl, on the ability of LAMP method to diagnose *An. gambiae*. In each case, template was *An. gambiae* DNA*; An. arabiensis* and *An. gambiae* specific primers were used in tube 1 or 2, respectively.
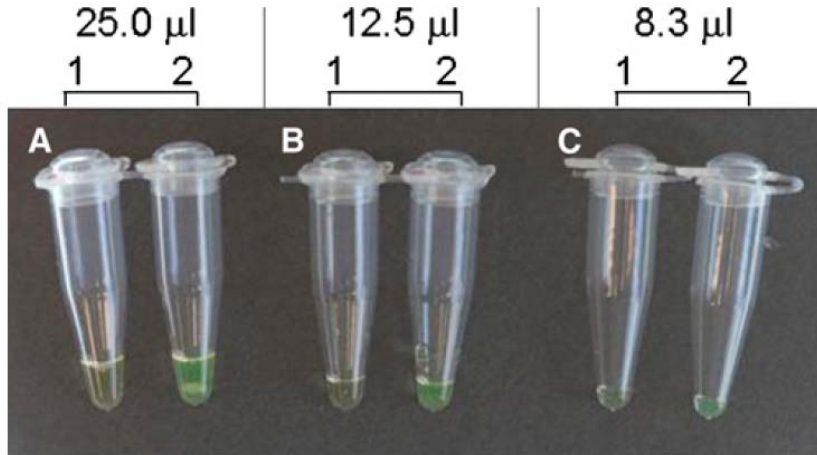
References

Tomita N, Mori YY, Kanda H, Notomi T (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nature Protocols *3:* 877–882.

Bonizzoni M, Afrane Y, Yan G (2009). Loop-Mediated Isothermal amplification (LAMP) for rapid identification of *Anopehels gambiae* and *Anopheles arabiensis* mosquitoes. The American Journal of Tropical Medicine and Hygiene 81: 1030-1034.

Scott JA, Brogdon W, Collins FH (1993). Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. The American Journal of Tropical Medicine and Hygiene 49: 520–529.

Chapter 9 : Guidance for Obtaining Live Material

9.1 Permits & Regulations

Paul Howell

Introduction

Obtaining insect vectors for research purposes is often a lengthy process involving the procurement of permits and the development and certification of an adequate space to contain them. Over the last few years, several rules and regulations have changed concerning the importation of insect vectors into the United States as well as transfer of vectors within the country. Although there are no reports of a vector escaping from a laboratory into the wild in North America, the potential exists for escapees becoming established as evidenced by the inadvertent introduction of *An. gambiae* into Brazil in the early 20th century (Soper and Wilson 1943).

Interstate Transfer of Insects

Over the last decade, the rules for transferring insects within the United States have changed. Some states may not allow the importation of exotic vectors that could become established if they escaped such as *Aedes albopictus* or *Ae. aegypti*. States such as California, Florida, Texas, and Hawaii often have more strict rules for the transfer of exotic vectors that could potentially become established due to the hospitable climate in these states. To determine if there are any state specific regulations for the interstate transfer of materials to your location, contact your local Department of Agriculture, Health, or Natural Resources. Aside from state specific regulations, the USDA Veterinary Services requires notification for any transfer of live insects between institutions.

Additionally, the interstate transfer of live, infected adults (either animal or human pathogen) or transgenic insects containing a *PiggyBac* transposable element will require additional approvals and permits (see **Permits and Approvals** section).

Table 9.1.1. Guidance for the interstate transfer of mosquitoes within the United States.

Permit name	Dead material, cell lines	Living Material						
		Colony, uninfected	Colony, infected (non human parasite)	Colony, infected (human parasite)	Colony, transgenic*	Colony, infected (non human parasite), transgenic*	Colony, infected (human parasite) and transgenic*	Wild, possibly infected
APHIS 2000					X*	X*	X*	
CDC USPHS 0.753								X
USDA APHIS VS 16-3	X	X	X			X		X
USDOT HAZMAT			X†	X†		X†	X†	

* Transposable elements originating from known plant pests are regulated by the USDA – BRS. Currently, mosquitoes that posses a *PiggyBac* TE are required to have an APHIS 2000 permit before any shipment occurs.

†The interstate transfer of live insects that are infected with human pathogens is regulated under 49 CFR 173.196 and all shipments must be approved by the U.S. Department of Transportation. Information for garnering approval for these shipments can be found at the link listed below. Note, all shipments must be packaged according to guidelines for Category A infectious substances (see **Packaging Regulations** Section).

Importation of Insects into the United States

As of 2010 the USDA will no longer allow hand-carrying of any samples into the United States. All materials, either living or dead, will require at a minimum one USDA-APHIS permit and must be shipped using a commercial carrier service such as Federal Express. This is in response to a GAO mandate that all material entering the United States be sent using a commercial carrier so that there are physical records showing that the materials have been received by the appropriate party. As with the interstate transfer of insects, various permits may be required for the importation of insects (**Table 9.1.2**). The importation of live, infected adults requires additional approvals from the U.S. Department of Transportation and is not covered in this chapter at this time.

Table 9.1.2. Guidance for the importation of mosquitoes into the United States.

Permit name	Dead material, cell lines	Living Material						
		Colony, uninfected	Colony, infected (non human parasite)	Colony, infected (human parasite)	Colony, transgenic*	Colony, infected (non human parasite), transgenic*	Colony, infected (human parasite) and transgenic*	Wild, possibly infected
APHIS 2000					X*	X*	X*	
CDC USPHS 0.753				X			X	X
USDA APHIS VS 16-3	X	X	X	X	X	X	X	X

* Transposable elements originating from known plant pests are regulated by the USDA – BRS. Currently, mosquitoes that posses a *PiggyBac* TE are required to have an APHIS 2000 permit before any shipment occurs.

Exportation of Insects out of the United States.

Due to the varied nature of permits needed by recipients, it is best that international recipients check with their respective Secretariats or Departments of Agriculture, Natural Resources, or whichever body governs the importation of live animals to determine if permits are needed. The European Union as well as Japan currently do not require import permits for insect vectors however some nations do. Other nations, such as Austria, may have increased local regulations at their respective quarantine stations which may cause delays in processing shipments. Certain documents should always accompany an international shipment, these include: recipients import permit (if applicable), Shippers Export Declaration (SED), a commercial invoice stating what is contained within the package, and in some cases a letter stating that the material is free of any known etiologic agent (Sanitary Certificate).

Permits and Approvals (updated July 2011)

APHIS 2000

Currently the importation or transfer of vectors containing a *PiggyBac* TE must be accompanied by an APHIS 2000 permit. Vectors containing TEs that are not derived from known plant pathogens are not required to have this permit. A listing of known plant pathogens are listed under 7 CFR 340 and can be found at the Bloomington Stock Center website (see below under permit information).

CDC – USPHS 0.753

USPHS 42 CFR Part 71.54 states (a) A person may not import into the United States, nor distribute after importation, any etiologic agent or any arthropod or other animal host or vector of human disease, or any exotic living arthropod or other animal capable of being a host or vector of human disease unless

accompanied by a permit issued by the Director. As of 2010, the CDC Etiologic Agent Import Permit Program (EAIPP) states that they will only issue a permit for "living vectors containing, or suspected of containing an etiologic agent". Importations of live eggs from long-established laboratory colonies or dead or preserved materials no longer require a permit from the CDC.

Per the CDC EAIPP: To facilitate clearance of materials that do not require a U.S. PHS Import Permit, it is recommended that each shipment of this material be accompanied by a signed statement, on official letterhead, from the person responsible for the shipment of this material with the following information:

(1) A description of the material;

(2) A statement that this material meets one of the above criteria (e.g., this material is not known or suspected to contain an etiological agent, host, or vector of human disease); and,

(3) Verification that it has been packaged, labeled, and transported in accordance with all applicable regulations. Note that other permits may be required (e.g., USDA).

Information on these permits is given below. Shipments without a USDA-APHIS permit are subject to being held at customs for an extended period of time or returned to the sender depending on the carrier being used.

USDA APHIS VS 16-3

Currently, a VS 16-3 permit must accompany any shipment of vectors, either live or dead. Prior to the issuance of a VS 16-3 permit, applicants' laboratories must be inspected by a USDA Veterinary Service employee. We recommend obtaining a minimum clearance of BSL2.

US DOT Hazardous Materials Approval

Although not a formal permit, shipments of live, infected adults within the United States are regulated under 49 CFR 173.196 and require approval from the U.S. Department of Transportation, Hazardous Materials Safety Administrator.

Information at a glance:

United States Department of Agriculture APHIS 2000

Information on how to apply as well as PDF forms are available at:

http://www.aphis.usda.gov/brs/pdf/2000.pdf

An excellent resource for filling out the APHIS 2000 permit can be found at the Bloomington Stock Center website:

http://flystocks.bio.indiana.edu/Regulatory/import_transgenic.htm.

United States Public Health Services form CDC 0.753, Application for Permit to Import Biological Agents or Vectors of Human Disease into the United States

Information on how to apply as well as PDF forms are available at:

http://www.cdc.gov/od/eaipp/

Unites States Department of Agriculture APHIS Permit VS 16-3

A site visit by a USDA official is required before issuance of a VS 16-3 permit. This site visit is not free, fees are listed at the website as well as information on how to apply, fee schedule, and PDF forms are available at:

http://www.aphis.usda.gov/animal_health/permits/

United States Department of Transportation Approval

Information on how to apply for approval can be found at the following website:

http://phmsa.dot.gov/portal/site/PHMSA/menuitem.ebdc7a8a7e39f2e55cf2031050248a0c/?vgnextoid=c1
0cd0dfb2e87110VgnVCM1000009ed07898RCRD&vgnextchannel=612328df28887110VgnVCM1000009
ed07898RCRD&vgnextfmt=print

Shipping Rules

Domestic shippers have implemented several varying rules as to which types of insects can be transferred and to where. Below is a list of the 4 most common shippers in the United States and their regulations as they apply to the transfer of insect vectors.

The United States Department of Transportation, as well as other agencies have created regulations that pertain to the shipment of insect vectors, both infected and uninfected. The following regulations should be viewed in order to help decide if an insect can be shipped. All of these regulations can be viewed at http://www.gpoaccess.gov/cfr/index.html

- Public Health Service 42 CFR Part 72 and 42 CFR Part 71-Foreign quarantine, importation of vectors. The USPHS CFR specifically deals with the direct importation and transfer of vectors of human disease.

- Department of Transportation 49 CFR Parts 171-178. These regulations pertain to the packaging of Hazardous Materials including specifications on types of containers and their proper use.

- Department of Agriculture 9 CFR Parts 102 and 120. These regulations are used by the USDA in order to determine if the vector that is being imported can be a vector of animal disease. Although there are several other parts to this CFR, these seem to be the most applicable.

Federal Express (www.fedex.com)

FedEx allows the transfer of insect eggs, including mosquitoes, from institute to institute only without any clearance. Clearance to ship adult insects, however, must be gained by contacting the Packaging Department (800-633-7019, option 5) and then your local Sales Representative (call Live Animal Desk at 800-405-9052 to determine who is your local representative). The Sales Representative will then contact the Legal Department to obtain a Waiver of Liability, once this is granted your institution is on file as being permitted to ship live adult insects.

Information at a glance:

Live Animal Desk   800-405-9052

http://www.fedex.com/us/government/intl/termsandconditions.html#liveanimals

Packaging Department   800-633-7019 option 5

United Parcel Service (www.ups.com)

UPS does not currently allow the transfer of mosquitoes, defined as obnoxious insects on their website, even if they are classified as research insects.

Information at a glance:

http://www.ups.com/content/us/en/resources/prepare/guidelines/animals.html

DHL Worldwide Express (www.dhl.com)

DHL does permit the transfer of live insects (non-venomous) throughout the U.S. Importation into the U.S. must be accompanied by a USPHS Import Permit and 24 hour notice must be given to DHL Customs at the airport stating where the package originated, what the contents are, is the material infected, and where the material originated from (wild or laboratory collected material). DHL can then expedite the shipment through customs once it arrives in the U.S.

Information at a glance:

Quarantine and Customs   718-995-0001

http://www.dhl-usa.com/resources/Prohibited_Restricted_Commodities.pdf

United States Postal Service (www.usps.com)

The USPS will accept shipments of live animals only if cleared by their Expedited Services Office. Contact your local District Representative to determine if they will accept your package. You can locate the numbers for your local representative at the website address listed below.

Information at a glance:

http://www.usps.com/send/waystosendmail/extraservices/specialhandlingservice.htm

Packaging Regulations

When importing or transferring materials, it is recommended that non-motile forms are shipped since there is little possibility of these forms escaping into the wild if the package is accidentally opened (Benedict 2003). Packaging information can be obtained from the International Air Transportation Association (IATA) under CR 61-66. Requirement 62 directly deals with insects used for research purposes (see addendum) and outlines packaging and labeling requirements necessary for shipments. If adult forms are to be sent, it is recommended that animals be triple packaged to ensure that if one container is compromised, the others will contain the material inside. All packages should have the following information prominently displayed on them: Shippers information, Recipients information, genus and species of all materials being sent and the quantity of animals being sent. Alternately, you can ship materials using the IATA Biological Material Packaging Instructions 650 (PI 650).

International Air Transportation Association (www.iata.org)

This association develops guidelines used by most major airlines and international shippers for the transportation of various materials including live animals. These guidelines are often used by the USDA and US Fish and Wildlife Services for enforcement issues.

Information on shipping hazardous materials (research insects are included in this heading)

https://iataonline.com/Store/Products/Product+Detail.htm?cs_id=9515%2D48&cs_catalog=Publications

Live animal regulations (Requirement 62)

https://www.iataonline.com/Store/Products/Product+Detail.htm?cs_id=9105%2D33&cs_catalog=Publications

If the shipment of live, adult mosquitoes is necessary, it is recommended that they be packaged and labeled as Category A infectious substances (UN 2814). Regulations associated with this can be found online from various sources.

http://www.mayomedicallaboratories.com/specimen-transport/intltest/infectious.html

References

Benedict MQ (2003) Arthropod Containment Guidelines. Vector Borne and Zoonotic Diseases 3:63-98

Soper FL, Wilson DB (1943) *Anopheles gambiae* in Brazil: 1930-1940. In. Rockefeller Foundation, New York