# Collecting, Rearing, Mounting and Shipping Mosquitoes

Thomas Gaffigan and James Pecor, 1997

The Walter Reed Biosystematics Unit, Division of Entomology, Walter Reed Army Institute of Research

## Introduction

The following protocols are used by personnel at the Walter Reed Biosystematics Unit for the collection, rearing, mounting and shipping of mosquitoes. It is provided as a quide and a training tool. Some of the techiques recounted here have been published, others have been modified, and some are innovations (see Belkin, J.N. y otros, 1967, Methodos para coleccionar, criar y preservar mosqitos, Contr. Am. Ent. Soc. 2:21-89 and Belkin, J.N., 1962, The Mosquitoes of the South Pacific (Diptera: Culicidae), Vol 1). Many techiques are adequately covered in numerous basic entomology texts such as Borror, Triplehorn and Johnson (1989) and are not considered here. Exuviae, a singular and plural word, is used here for the shed cuticle of larvae and pupae. These are also known as “skins” or “pelts.”

## Collection and specimen numbering

Adults and their associated immature exuviae are linked together using common collection and rearing numbers. By giving a unique number to each specimen this system avoids duplication of numbers and provides a consistent system for all collections. When the specimens are collected in the field, a collection number is assigned to an individual collection ( US 1; US 2; etc). When a larva or pupa is reared to the adult stage, a rearing number is assigned to both the adult and immature exuviae, e.g. US 1-1 and US 1-2 are individuals 1 and 2 from US collection number 1. Rearing numbers from 1 to 99 are used for an adult with both a larval and pupal exuviae preserved, while a three digit number (100- ) isused for adults with preserved pupal exuviae only. An individual number is assigned only after the associated adult has been mounted, not while the larva or pupa is still in a rearing vial. Progeny broods are identified by parentheses following the collection number, e.g. US 2(1) is female number 1 from US collection number 2. As individuals emerge from US 2(1) they are assigned individual numbers as above, e.g. US 2(1)-1, -2, etc.

## Collection of immatures

A two-sided collection form is used to record all pertinent data for each immature collection. The front is used to record data pertaining to the habitat (locality, ecology, etc.) while the reverse is used to record individual rearings and identifications. The front side of the form has four major sections, Locality, General Environment, Habitat of Immatures, and Adult Collections. The form should be filled out as completely as possible in the field at the time of collection, leaving nothing to memory. The collection number will serve to identify all individuals obtained from each collection. The country, state/province, nearest town, specific locality, date and collector should be filled out completely for every collection. Additional locality data points, (longitude/latitude, elevation, etc.) should also be filled out if known.

The “Habitat of Immatures” section offers a choice of possible collection sites for immature mosquitoes. Circle the larval habitat which most closely resembles the collection site sampled. The additional parameters listed, (light, type of water, distance from nearest home, etc.) are important for future reference and will be compared with data from other collections to develop a “species profile.” The other sections (General Environment and Adult Collections) can be filled out in part or entirety as appropriate.

Using a large-mouth dropper, larvae can be picked either from the habitat water surface or from water sampled with a dipper, and then transferred to a Whirl-Pak bag. Most plastic droppers have a long narrow tip which must enlarged to prevent injury to the immatures. If a glass dropper is used, the enlarged opening must also be heated sufficiently to smooth the rough edges left after breaking off the tip.

Where immatures are located in clumps of vegetation, one collecting technique is to sink the dipper almost to the rim next to the clump being sampled and allow the water to flow through the vegetation into the dipper. In this way water is drawn through the grass, carrying the immatures with it into the dipper. If possible, it is better to use a rectangular enamel pan for larval collections. It is much more efficient than a dipper, especially if used for collection of anophelines. Where considerable emergent grass is present, tramp down the grass in an area about one meter in diameter and quietly wait for the disturbed larvae to resurface. Muddying the water will also help make immatures visible by providing a background against which they are more easily seen.

Enclosed water habitats such as tree holes, cut bamboo and plant axils are usually collected with some version of a large suction device. We use a 4 oz.glass irrigation syringe, with a length of rubber tubing or a plastic dropper attached to the tip. Hand pumps and “turkey basters” have also been used, and some people prefer semirigid polyethylene tubing instead of flexible tubing. It is essential, regardless of what tool is used, to prevent the mixing of larvae from separate

collections. Since irrigation syringes are clear glass they allow visual confirmation that no immatures are present from a previous collection. Care must also be taken to rinse the bulb between collections as well. When all the water has been removed from a small habitat it is sometimes useful to add clean water to recover any additional individuals stranded by the receeding water level.

Whirl-Pak bags are used to transport immature collections. The collection number is written on the outside of the bag using a black wax pencil or an indelible marking pen. First, remove the top of the bag at the perforation. Larvae, pupae, and habitat water are then added to the bag with a wide mouth dropper until it is about 1/2 full. This allows enough air to maintain the larvae until they can be returned to the lab. Do not put too many larvae in a single bag. To seal the bag, grasp the ends of the wire and quickly twirl the bag around the wire until the bag becomes "pressurized." Tightly fold (crimp) the wires and a small portion of the bag towards the center to lock the bag closed. Keep the bags cool with wet cloth or paper towels inside a cooler. The bags can also be floated in water in a cooler to protect them during transport. Ice can be used to cool the water but should not come in direct contact with the bags. Do not keep the bag in your pocket as body heat is sufficient to kill the larvae. Larvae and pupae in air tight bags can survive travel over very rough terrain and for long distances if they are transported in this manner. Once the trip is complete, open the bags and allow them to return to room temperature slowly.

**Rearing of immatures**

Rearing of immatures in an empirical process and success depends on the experience of, and degree of care taken by, the individual researcher.

Immatures are reared to the 4th instar in 500 or 1000 ml plastic cups. Each cup will accommodate a single collection but multiple cups can be used if larval densities are too high. These cups work well for most mosquitoes. Some anophelines however, may require a greater surface area in the rearing container, and for these collections, enamel or photographic pans work well (approx. 8" X 10" x 2"). Black wax pencils are also satisfactory for marking these containers with the collection number and other notations. Tops may be used if you have fear of contamination, however cups and pans are best left open.

Because of normally occurring disease and parasite organisms and predators in habitat water, rainwater or dechlorinated tap water is preferred. Adding straw or dried leaves can enhance the success of rearing larvae. Fresh water and artificial feeding make it easier to monitor daily feeding requirements and water condition. One can often save a valuable collection by changing to new water if it becomes fouled. If a surface film develops in a rearing cup, it can be removed by drawing strips of paper toweling across the surface. Finely ground animal chow (e.g. guinea pig chow) or fish food (e.g. Tetra-Min) are used to feed larvae. A small amount of fine powder can be spread on the water surface or a few drops of mixture of water and a small amount of food can be added to the rearing container with a pipette. It is very easy to overfeed. The amount of food added depends on the number of larvae and their age. It is better to feed small amounts more often than just once a day and to carefully observe the results.

It is important to leave the larvae in the large pans as long as possible. Ordinarily larvae should be isolated for individual rearing only when pupae are observed. At this time the largest fourth instar larvae of what is presumed to be the same species should be removed.

Fourth instar larvae are isolated in 9 dram vials. The collection number should be written on the vial with a wax pencil and the vial partly filled with fresh water. Conveniently, an adequate amount of water for each 9 dram vial is contained in the 3 1/2 dram vials which are used as tops for preemergent pupae.

Isolated larvae are fed using a very small amount of a weak water-food mixture described above. Shake the slurry prior to use and let the large particles settle. Fill a pipette with the liquid and touch the water surface with the tip without any pressure on the bulb. This will usually leave only a small droplet of food. Pressure on the bulb can expel much more food than is necessary and will cause unequal application between vials. A single larva needs only a minute amount of food. Overfeeding of larvae in the small vials can result in loss of specimens or exuviae covered with food particles, bacteria or fungus. It will take only a few tries to tune this to your specific needs. The vials containing isolated larvae should be checked several times a day, and the water changed as needed.

After the larvae pupate, the larval exuviae is transferred as soon as possible to a 1/4 dram shell vial half full of reagent grade 80% ethyl alcohol using an small wooden stick (applicator stick). Soak the stick in water for at least 15-30 minutes prior to use. Maneuver the exuviae and stick so that the exuviae is supported along its entire length. Do not slide the exuviae up the side of the vial. Do not use a dropper as this dilutes the alcohol in the shell vial and definitely do not use forcepts. Do not pick up the head or tail only and leave the other end of the exuviae adhering to the surface tension as this will stretch and twist the exuviae. With the exuviae inside, put a stopper in the shell vial and then attach it to the 9

dram rearing vial with a rubber band. After the larva has pupated and the larval exuviae is removed, place an inverted 3 1/2 dram vial into the 9 dram vial as a cover. The 3 1/2 dram vial will drop about an inch down into the 9 dram vial. This vial should not get wet and it may be necessary to put a small rubber band around it to prevent it from entering the 9 dram vial too far. Also, each 31/2 dram vial is notched along the upper rim to permit air exchange. Note that more 9s are needed than 31/2s. This is because you need only to cap 9s which contain pupae and you will rarely have more than 50% of your isolations at any one time in this stage.

It is essential that larval and pupal exuviae not remain in the water longer than 8 hours. The exuviae begin to deteriorate almost immediately. Therefore, all rearing vials should be checked and exuviae removed a minimum of three times per day.

When the adult has emerged and gained enough strength to fly it must be transferred to a dry 9 dram vial. Grasp the 9 dram vial with the 3 1/2 lid and the adult in one hand. Hold a second dry 9 next to the one containing the specimen with the mouths of the 9s at the same height. Gently tap the vial with the adult to coax it up into the 3 1/2 top. Often adults are attracted to a light source. Therefore, a small desk lamp can be used to coax them to fly up into the smaller vial. Sometimes slightly tilting the vials helps but do not tilt them far enough to wet the 3 1/2. When the adult is up in the 3 1/2 gently lift the 3 1/2 and in one smooth motion, slide it onto a dry 9 dram vial. Extreme speed is not necessary. Recover the pupal exuviae with a wooden stick as above and place it in the same shell vial as the larval exuviae shed by that adult. Pupal exuviae are most easily picked-up with the applicator stick by slowly rolling the stick between the thumb and forefinger and approaching the exuviae from the underside of the abdomen. Touch the bottom side of the paddles and roll the abdomen on the stick. This maneuver will take some practice. Once again do not slide the exuviae up the side of the vial and do not use forceps or a pipette. If the specimen was collected as a pupa and there is no larval exuviae, you will need to get a new shell vial. Transfer the shell vial to the dry 9 dram vial attaching it to the outside with a rubber band. Write the collection number on the new 9 with a black wax pencil. If the cap should become wet, replace it. You will find after few tries that the 3 1/2 dram vial used as a cap is far superior to gauze attached with rubber bands, or similar methods. This method requires that dry vials be maintained to receive the emerged adults. Adults are very likely to become trapped in the smallest water droplets and become damaged.

Shell vials are labeled on the outside by affixing 1/4 inch masking tape, on which the specimen number is written in pencil or alcohol resistent ink. Labels placed inside of vials will damage the specimens, as will any air bubbles trapped inside the vials. First fill the vials as full as possible with 80% ethyl alcohol. Next place a stopper right side up on a firm surface. With a 23 guage or smaller hypodermic needle pierce the stopper so that the needle just enters the concavity on the bottom of the stopper. Leave the needle in place. Inclining the vial at a slight angle, insert the vented stopper into the shell vial and gently press it home. This process takes practice since the exuviae must not be near the top of the vial when the stopper is put on. The exuviae can easily be expelled through the needle or caught between the top and vial. If done properly, excess alcohol and air will escape through the needle leaving little or no air.

Labels for the 1/4 dram vials can be made as follows. Obtain a piece of sheet metal or plexiglass up to about 8 to 10 inches square(size is not critical). On it, scribe a series of lines parallel to each other and 1 1/2 “ apart . These will serve as cutting guides. Lay out strips of 1/4 “ masking tape perpendicular to the scribed lines. Using your finger press the tape down where it crosses the lines. Use a scalpel and cut all the strips of tape by running the blade along the scribed lines. Write the collection and rearing numbers on the tape labels using an alcohol resistent pen or a pencil. Use fine tipped forceps to lift the tape from the plate. The shell vials must be completely dry to ensure proper adhesion of the label. You may mail a small number of shell vials by wrapping a rubber band around them and packing in shock absorbent material( cotton,cellucotton, crumpled tissue paper, etc.). If you plan to ship vials in large quantity you should use the original vial carton for this purpose. Alcohol filled vials are fragile . They must be prevented from shifting in the box or coming in contact with the staples at the corners of the box. Tissue can be used to take up excess space and several layers of 3 X5 card over the staples is sufficient. Place thin foam rubber or layers of tissue over the vials to exert gentle downward pressure to prevent vertical movement, replace the top and tape the carton shut.

**Alcohol Preservation of Whole Larvae and Pupae**

Whole larvae and pupae are preserved by first killing them with very hot water. The hot water fixes proteins which prevents later darkening of the specimens. They are then placed in 80% ethyl alcohol which has been replaced at least twice to eliminate excess water.

**Preservation for Molecular Research**

For all stages, the best method for preservation for DNA and protein analysis is in liquid nitrogen. For protein analysis it

must be in liquid nitrogen. For preservation of DNA, 95-100% ethyl alcohol can be used very effectively without immediate need for refrigeration. Specimens are placed live into the alcohol followed by 1 or two changes of fresh alcohol. Specimens in alcohol should be placed at at least -70o when refrigeration is available. In alcohol the DNA remains in relatively good condition at room temperature for 1 or 2 months.

**Progeny Rearings**

The rearing of progeny from single females is of great value to: 1) obtain specimens of species rarely collected as larvae; 2) to use in demonstrating variation in a single brood and; 3) to provide associated morphological specimens for molecular studies.

Progeny series are obtained from egg rafts collected in the field or egg batches deposited by an individual gravid female. For induced oviposition in the laboratory, first obtain females by the usual collection methods. Blood engorged females are obtained and held in a moist environment for 72 hours. However, under very hot conditions the waiting period for egg development may be as little as 48 hours. Female Aedes and Psorophora can be isolated in a glass tube with moist cotton in the bottom. These females will often lay eggs spontaneously on the moist substrate. Female Culex and Anopheles usually must be "stressed" to induce oviposition. This is accomplished by removal of a wing. First anesthetize the female with ethyl acetate. Take care not to leave it in the tube any longer than it takes for the mosquito to quit moving. Quickly place the female under a dissecting microscope and remove a wing using two pairs of fine needle forceps, one place against the thorax, the other used to tear away the wing. The wing must be grasped at the very base and completely pulled off. If any of the wing remains there is a chance the mosquito will still be able to escape from the oviposition cup. Place the female in an small uncovered cup of water. It may be necessary to manipulate the specimen so that it is supported on the water by its legs, not on its back. Be sure the water is not too near the top of the cup. Oviposition will usually occur within an hour but sometimes up to 6 hours is needed. After oviposition do not mechanically disturb the cup and prevent air movement in the vicinity of the cup. Any such disturbance can cause the eggs to stick to the sides of the cup and dry out. If this happens, eggs can sometimes be coaxed away from the sides of the cups by a strong stream of water from a laboratory squirt bottle. To induce hatching it is sometimes necessary to add a little dried grass or a small amount of food to the water. Depending on the species and the temperature, hatching usually occurrs in 2 to 4 days.

**Mounting Adults**

Adults are killed using ethyl acetate. Glass killing tubes with the bottom 1/3 filled with oven dried plaster of paris are used. The plaster is saturated with the ethyl acetate, but not to the point of leaving excess liquid. It is much easier to mount specimens that have been killed with ethyl acetate than those that have been allowed to die in the rearing tubes. After placing the specimen in the killing tube place the tube on its side and gently tap the tube to cause to specimen to be resting on its side. As the mosquito, dies its legs will usually relax away from the body. In this position it is much easier to affix the specimen to a pin point.

To pin adults, attach pin points to No. 3 stainless steel insect pins. The preferred adhesive is Ambroid cement. Ambroid cement is a glue commonly found in hobby shops. Its usual solvent, acetone, evaporates much too quickly for use in mounting mosquitoes. Therefore, the Ambroid is first completely dried in thin sheets spread on a piece of glass, cut into thin strips and then redisolved in amyl acetate. Ambroid is superior to commonly used glues such as nail polish, which shrinks and becomes brittle in a relatively short time. Pin points are cut from from high surface 2-ply 100% rag Bristol board. This assures permanent firm attachment of the point to the pin shaft. The point is first moved to the top of the pin for unimpeaded access to the specimen. The point should only be moved by grasping the shaft of the pin directly under the point with a pair of forceps, and sliding it along the shaft until the point is against the pin head. The point is then run along the applicator rod of the Ambroid bottle to pick up a very small controlled drop of adhesive on the top side of the point. The specimen must be affixed to the point with some rapidity since the adhesive dries quickly. Too much adhesive will damage the specimen. The adhesive should be relatively thick so it does not spread easily on the point or on the specimen. Place the specimen on a flat surface, preferably under a dissecting microscope. The adult is then attached by inverting the pin/point and pressing the adhesive against the right pleuron of the adult with the legs pointing toward the pin. The pin is held gently against the specimen for three to five seconds and then reverted. At this point it may be necessary to adjust the position of the adult if the glue hasn't dried completely. To move the point back into position, again use the forceps to gently grasp the pin shaft above the point and slide it back down about 1/3 of the pin length from the top. Allow enough space above so that the pin can be grasped without damaging the specimen. Moving the point as described keeps the pin hole from enlarging and avoids damage problems caused by loose points. At this time a label is affixed to the pin and to the vial containing the exuviae. Both labels will have the same unique specimen number as described above. The same number is also written on the collection form with a preliminary identification and sex.

Mounting of Immature Exuviae and Whole Larvae

Immature exuviae are preserved in 80% ethanol. The following procedure serves to remove all water from the specimen since water is incompatible with permanent mounting media and will cause clouding of the preparation. The most difficult task in mounting exuviae, next to manipulating larval exuviae, is ensuring continued association of the specimen number with the specimen. If followed carefully, the system described below is not easily defeated.

1. Pine boards with rows of 19 holes are used to hold the shell vials. Nineteen is the number of plant industry watch glasses which will fit inside 6" diameter petri dish. Each of the watch glasses has a number etched backwards into the underside of the bottom (1 thru 19). These are arranged in numerical order starting with an outer ring and ending in the center.

2. A laboratory log book is set-up with lines numbered to correspond to the watch glasses in the petri dish. The label from the shell vials are pasted on the line for the watch glass into which the exuviae have been placed. Should anything be unusual, i.e missing exuviae, damaged or partial exuviae or extra exuviae, a notation is made next to the label at this time. The stopper is reinserted in the now empty vial and is replaced in the rack. This allows for rechecking if some structure is noted to be missing during the mounting procedure. It is best to retain the empty vials until the cover slips have been applied to the specimens.

3. The exuviae are emptied into the watch glasses. In some instances, where the vials were not completely full it may be necessary to add alcohol to the vial before attempting to dump the vial contents. Remove stopper and hold vial between thumb and forefinger using the latter as a stopper. Gently invert the vial several times and hold inverted to suspend the exuviae and allow them to sink towards the mouth. Then hold it over the watch glass and remove the finger so that the exuviae flows into the watch glass. Always have a squeeze bottle at hand to wash the exuviae off should it get hung up inside of the vial.

4. Using a Pasteur pipette gently remove as much alcohol as possible from the specimens. We usually empty about 6 or 7 dishes at a time and then refill them with 99% isopropyl or absolute ethanol. The removed alcohol should be put into a small beaker which can be emptied after all replacements are made. Using an intermediate container allows for the recovery of exuviae accidentally removed. Allow the exuviae to remain in the absolute alcohol for about 10 minutes.

5. Begin to prepare slides to receive the mounted specimens. Using a diamond pencil scratch the collection and rearing number of the specimen on the lower left corner of the slide. We use a system where each lot of specimens received is assigned a unique accession number. All specimens, collection records and correspondence pertaining to this lot receives the same number. This accession number is scratched in the lower right corner of each slide. The slides are cleaned with 80% alcohol to ensure that they are free of finger prints, dust, etc. All slides for the specimens from one petri dish are placed together in a 20 space aluminum slide tray. The slides are in the same order as the specimens in the dish. The slides are placed in the tray with the numbers on the top and towards the top of the tray so that they appear upside down. This is done to ensure that the specimens, when they are mounted, will be head down, while the numbers on the slide will be right side up. This is because the exuviae will be viewed using a compound microscope which inverts the image. A properly mounted specimen will appear head up in the microscope and the scratch numbers and labels will also be right side up as the specimen sits on the microscope stage.

6. Gently remove the alcohol and replace it with more 99% isopropyl or absolute ethanol. When adding the alcohol, do it gently. Do not squirt the alcohol forcefully. Exuviae can be ruined at any stage of the process. Leave in this bath for five minutes.

7. Remove the alcohol and replace it with Cellosolve (ethylene glycol monoethyl ether). Leave for five minutes.

8. Using a "lifter," gently transfer the larval exuviae to the slide. A lifter is made by hammering flat and bending, a thick wooden handled dissection needle. When picking up the larval exuviae orient the lifter so that the exuviae is supported along its entire length. Avoid lifting the exuviae when only partially supported. The end not supported will remain attached by surface tension to the Cellosolve and setae will be lost or the exuviae will be stretched or twisted and sometimes torn. Place the lifter almost in contact with the slide just to the left of center. Using either cellosolve or euparal essence gently wash the exuviae onto the slide. Usually only a single drop is needed. The pupal exuviae is handled in the same manner and mounted just to the left of center. It is useful to make a cardboard or plastic holder with the location of the respective exuviae marked on it to ensure consistent placement of the exuviae on each slide.

9. Manipulation and placement of larval exuviae. The larval exuviae should be arranged dorsal side up with the head directed away from the dissector. The mouth parts should be facing down. The exuviae splits roughly along the mid ventral line. It should not be twisted. If the exuviae is accordioned (the segments compressed together), an attempt should be made to stretch it. However, at the slightest sign of tearing, or if reasonable pressure produces no improvement, do not

persist. Turn the terminal segments so that the siphon is to the right and the tenth segment to the left. Arrange the large lateral hairs so that the branches of multi branched setae are spread and more or less perpendicular to the axis of the specimen. Arrange the larger thoracic setae into natural forward or lateral positions. All arranging is done while the specimen is still in Cellosolve or Euparal essence. Should the specimen start to dry add more liquid. If the exuviae are manipulated when too dry they will be torn and setae lost due to adhesion to the slide. Be sure the dorsal and ventral brushes are spread and that the anal papilae are straight and, as much as possible, not overlapping. If the larval exuviae is twisted take a minute to study the exuviae before straightening is attempted. It is imperative that there be enough liquid on the slide to float the exuviae when large adjustments are needed.

10. Pupal exuviae. The pupal exuviae is placed to the right of the larval exuviae. With the exuviae in a lateral position gently separate the abdomen, with the metanotum attached, from the cephalothorax. Position the abdomen-metanotum dorsal side up and arrange the float hairs at right angles to the midline. All setae should be more or less parallel to the midline. The cephalothorax splits along the mid dorsal ridge during emergence of the adult. A short posterior section still remains intact and must be separated before the cephalothorax can be spread. The cephalothorax is mounted so that the interior is against the slide and what was the mid ventral line is now medial and the mid dorsal line is now lateral. The trumpets should be directed laterally. The mouth parts and antenna cases are medial and straight with the leg cases symmetrically arranged on either side. At this point make sure that the specimens are positioned properly around the center of the slide. Using the rolled up corner of a kimwipe or an artists #3 paper shading stump, remove all excess liquid from around the specimens. Do not touch the specimens since as any movement of the specimen will cause damage. It is not necessary to attempt to remove liquid from the pupal abdomen as this will cause an air space to form which may not fill with mounting resin. Remove the slide from the microscope and carefully wipe around the specimen to dry the ring of liquid which usually extends out 25 mm from the specimen. Excess liquid will cause the initial application of Euparal to spread beyond the limits of a 15 mm cover slip and require extensive, time consuming cleanup after the cover slip is applied. Using thinned resin apply a small drop to the center of the slide between the exuviae. Enough Euparal should be used to extend just beyond the exuviae. It may take several minutes for the resin to fully spread. Place the slide back under the microscope and rearrange anything which may have moved. Begin to work on the next slide. After finishing the second slide go back to the first one and make sure both exuviae are properly arranged. Should they need attention at this point it may be necessary the apply some euparal essence to make the resin workable. One way is to dip the dissecting needle in essence so that only the smallest amount is transferred. It may be necessary to do this several times before enough solvent is applied. However, this is better than applying too much and softening the entire mount. Continue to work in this manner, rechecking the previous slide before going on to the next. After the slides have been cleaned, prior to mounting, they should be protected from dust with a cover. The best is a thin sheet of plexiglass which is slightly larger than the slide tray. Flexible covers, such as acetate sheets may be used, however, incidental contact may leave your specimens mounted on the under side of the cover.

11. Dry the slides over night, preferably in a drying oven at about 60oC. Early the next day the cover slips can be applied. This overnight drying holds the specimens in place when the coverslips are applied. The following method of coverslip placement cuts down on the number of air bubbles trapped under the cover slip. While holding the cover slip in a pair of forceps, apply a drop of Euparal to the top of the slip. One drop is usually enough. Apply 2-4 drops to the specimen depending on its size. Work quickly because the dried Euparal will begin to soften and allow the specimens to move under the pressure of the cover slip. Invert the cover slip so that a hanging drop forms and place it gently on the specimen. If just enough resin is used it will spread just to the edge of the cover slip and no cleanup will be necessary. Should cleanup be necessary use a camel hair brush and Euparal essence. First dip the tip of the brush into Euparol essence and then mop around the cover slip. Wipe the resin from the brush. Repeat the procedure as many times as necessary to produce a clean mount. Return the completed slides to the drying oven for about thirty days to continue drying. After this time they can be be transferred to slide boxes and stored horizontally with the mounted specimen facing up. The mounting media takes many years to thoroughly dry and mounts can suffer damage if stored vertically or one on top of the other in stacks. It is best to apply labels after the slides come out of the oven. Drying temperatures can adversely affect some self adhesive labels. For self sticking labels it is necessary to apply pressure to the entire surface of the label to ensure uniform adhesion. One method is to draw the the slide, label side down, across the edge of a sheet of cork. Examine the back of the label looking for a uniform pattern. Press against the label and see if the pattern changes. If it does, it is not yet completely applied and it must be rubbed down the cork again. Gummed labels, if properly applied, are better than self stick labels. Before application of the label the slide must be completely clean. Do not over wet the label, and be sure to press it down with some sort of blotting material to absorb all excess moisture.

12. Whole larvae and pupae. Because of their greater mass and water content, they require more preparation time

than exuviae. Mount only one specimen per slide. Follow the same procedures for cleaning and marking the slides as outlined in the exuviae mounting procedures. The following assumes that the specimen has been preserved in 80% ethyl alcohol.

a. Place the whole larva in in absolute ethanol or 99% isopropanol. Leave for 10 minutes.

b. Replace with absolute ethanol or 99% isopropanol and leave for 10 minutes.

c. Remove alcohol and replace with Cellosolve(ethylene glycol monoethyl ether). Leave for 15 minutes.

d. Whole larvae are mounted so they will appear in the standard head up position when viewed with a compound microscope. The specimen is centered on the slide, dorsal side up, and cut between the sixth and seventh abdominal segments. Setae are located near the posterior margin of the segment so care must be taken to cut on the intersegmental membrane. A No.3 scalpel with a No.10 blade is best. The cut can be made with either a slicing motion or by rocking the blade from heel to tip across the specimen. It is sometimes helpful to hold the scalpel away from vertical when making the cut to better see the intersegmental area and the actual cutting edge. It is possible to avoid cutting some long setae which may extend beyond the posterior margin of the segment by holding the scalpel blade just above the specimen and moving the blade into position from behind. The objective is to get the long setae to ride up the blade so they don't get cut. Position the terminal segments just below the rest of the specimen with the siphon to the right. Arrange all lateral setae in a natural position, approximately 90o to the midline of the specimen. Be sure the specimen is situated so that the mid dorsal line is in the center of the specimen. Spread all multi-branched setae. Do not break setae to achieve symmetrical positioning.

e. Cover the specimen with medium viscosity mounting media. Use a tray cover to exclude dust and leave to dry 24 hours. Large specimens such as Toxorhynchites and other predacious larvae usually need additional layers of resin prior to applying the cover slip. It is important that the cover slip be as level as possible. It may be necessary to use small pieces of glass made from microscope slides at the corners to level the cover slip. During the 1 to 2 months the whole larvae are drying in the oven it may be necessary to add mounting media under the cover slip to fill voids created by the evaporation of solvent from the resin.

**Shipment of Dead Mosquito Specimens**

Adults

When possible, adult mosquitoes should be mounted before shipment. Specimens should be mounted on paper points as described above. Adults should be pinned into a foam bottomed Schmidt box in rows, with all specimens properly labeled. Specimens with large or loose labels should have pins placed on either side of the labels to prevent rotation into adjacent specimens. Push the pins into the foam as far as possible to ensure a secure hold and then tape the lid of the box shut. NOTHING else should be placed inside the box. Cardboard over the pins is unnecessary and frequently causes considerable damage to the specimens. If fumigant is necessary it should be mixed with the packing material around the Schmidt box. NEVER put fumigant inside a specimen box for shipment. If more than one specimen box is to be included in a shipping container, they should be taped securely together, wrapped in brown paper (or equivalent) and placed in the center of an appropriate sized shipping container. The outer container must be large enough to allow for 3-4 inches of packing material on all sides (including top and bottom) of the specimen boxes. Styrofoam peanuts and wood excelsior are the most common packing materials used. However, if these are not available shredded paper or crumpled newspaper may be used. The object is to absorb shock and prevent the specimen boxes from shifting and coming in contact with the outer shipping container. If the package is to be mailed internationally it should have the necessary customs declaration form and the contents should be identified as "DEAD INSECTS FOR SCIENTIFIC STUDY--OF NO COMMERCIAL VALUE." Unmounted adults should be placed in pill boxes using a fine, light weight soft tissue or lens paper for cushioning. The paper should be cut slightly larger than the box to prevent settling and compacting of the specimens. The specimens should not touch each other and can be loosely packed between single sheets of paper. The pill boxes should be packed in a small box along with packing material to fill any excess space, and then packed as described above. Alcohol specimens.

Specimens shipped in vials or jars should be filled to the top with alcohol (80% ethanol) with all the air removed, as described above. When done properly there should be no air bubble inside the container. Each jar or vial should be wrapped to prevent contact of the containers. Cotton, tissues or cellucotton are excellent materials to pack around the containers since they also absorb alcohol should any of the containers break. The 1/4 dm. vials normally used to ship the exuviae of individually reared specimens should be packed in their original box with a slip of index card between each vial and index card at the corners to cover the exposed ends of the staples. Foam rubber or tissue should be placed over the vials and the boxes taped shut. Pack in an outer shipping container as described above.

**Shipment of Live Mosquito Specimens**

Eggs: Gently pack eggs between layers of moist filter paper in a small vial or other water tight container, making sure that the filter paper will not move during shipment. Package container as usual.

Larvae/pupae: Place larvae in a water-tight plastic bag bag and seal. Whirl -pak bags are excellent for this purpose. Be sure to leave an adequate air space above the water. Place bag inside a shipping container with adequate packing to prevent movement and to absorb shock during shipment.

Adults: Shipment of gravid females is most desirable. Place the specimens in a small container (e.g., a pint or quart cylindrical ice cream container) that has its open end covered with fine mesh netting or screen. Next place a wad of moist cotton and a few water-soaked raisins (soaked 1-2- hours in warm water) on top of the netting. Finally, place a lid over the top of the container, securing the moist cotton and raisins between the netting and the top. Package for shipping as usual.

The object of the above procedures, for eggs and adults, is to provide an environment with very high humidity without producing actual water droplets. Pupae will survive 2-3 days, eggs 5-6 days, larvae 7-10 days and adults as long as 15 days. In most cases delivery should be by air freight or package express companies to ensure that the material is delivered in 2-3 days.

Recipients of live material in the US must have a US Public Health Service entry permit and the shipper must comply with all local government and shipping company regulations.

The Walter Reed Biosystematics Unit | Museum Support Center, MRC-534 | Smithsonian Institution | 4210 Silver Hill Rd. | Suitland, MD 20746-2863 USA
Ph: 301-238-1077; FAX: 301-238-3168

Department of Entomology | Comm. Diseases & Immunology | Walter Reed Army Inst. of Research | 503 Robert Grant Avenue | Silver Spring, MD 20910-7500 USA