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SUPPORTING INFECTIOUS DISEASE RESEARCH

Care and Maintenance of Phlebotomine Sand Flies

Version 2.0 Effective Date: 4/1/2023

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Foreword

Methods in Sand Fly Research is part of a comprehensive collection of new rearing and handling protocols for vector species of importance to human health was borne out of the Vector Biology Research Resources Workshop held in June 2015 at the National Institutes of Health with the generous support by BEI Resources. This effort was inspired by the BEI manual, *Methods in Anopheles Research*, started by Mark Benedict and widely expanded by Paul Howell, which has become the gold standard of mosquito rearing and manipulation protocols. It continues to be the go-to resource for laboratory-based scientists conducting basic research and public health entomologists from malaria endemic countries alike.

We would like to thank **David Bland, Paul Howell, Michael Levin, Kevin Macaluso, Claudio Meneses, Tobin Rowland, Saravanan Thangamani, and Margaret (Peggy) Wirth**, for sharing their techniques and expertise, and for putting together these protocols.

These protocols are intended as living, breathing documents with ample room for improvement based on a specific lab's capacity and infrastructure. They are intended as guidelines only, especially with regards to research involving vertebrate animals or biohazards, and arthropod containment, which require institutional approval tailored to individual laboratories.

We hope that the community can benefit significantly from the generation of this comprehensive set of new protocols and stimulate new work in vector biology and vector-borne diseases. Kristin Michel (kmichel@ksu.edu, Kansas State University) and Lyric Bartholomay (lyric.bartholomay@wisc.edu, University of Wisconsin-Madison)

To provide feedback on this or any of the vector resources protocols, please send an email to Contact@BEIResources.org.

This particular manual is a compilation of materials and experiences that have been adopted or developed over many years of rearing sand flies at the National Institutes of Health and Walter Reed Army Institute of Research. This manual is designed to familiarize newcomers to the wonderful world of rearing sand flies. There are many ways to accomplish successful sand fly rearing but here we share the methods that have been adopted by the two labs above.

We invite anyone wanting to contribute their methods or protocols to please do so. The aim of this manual is to become a one stop for researchers that are working with sand flies. We hope that this version is the first of many versions to be released as this will be a working manual in which the content can be changed or new protocols can be added at any time.

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SUPPORTING INFECTIOUS DISEASE RESEARCH

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Introduction

Phillip G. Lawyer

The importance of establishing and maintaining large laboratory colonies of phlebotomine sand flies was summarized by Safyanova (1964) as “necessary for the experimental study of their biology, behavior and mutual relations with disease agents and for the testing of new methods of vector control”. The WHO Scientific Working Group on Leishmanaisis (Anonymous 1977) added the following emphasis: “Colonies are valuable in work on vector potential, life cycles of *Leishmania* and transmission by bite. They are indispensable in genetic studies and in controlled observations on the physiology and behavior of sand flies, all of which are neglected subjects of high priority. Colonies are of particular value for screening insecticides.” However, until the early 1980s, fewer than a dozen closed colonies of about six species of sand fly were available to researchers for experimental use (Hertig & Johnson, 1961; Johnson & Hertig, 1961; Chaniotis, 1975; Gemetchu, 1976; Killick-Kendrick et al., 1977; Ward, 1977; Ready and Croset, 1980). Labor-intensive maintenance and low productivity limited their usefulness for leishmaniasis research, and still do for species that do not adapt well to laboratory conditions. As interest in leishmaniasis research has grown and rearing techniques have been refined, laboratory sand fly colonies have become more common, more robust and more useful, enabling many significant breakthroughs. Today there are more than 100 colonies representing 33 distinct species of sand fly in 24 laboratories throughout the world. Colonized sand flies are used as live vector models in a diverse array of research projects directed toward control of leishmaniasis and other sand fly associated diseases. There is no single, optimal procedure for rearing sand flies. Methods used by various workers are often a matter of choice rather than necessity, or they may vary depending on available resources or manpower. In this supplement we discuss common techniques used by researchers, with emphasis on those methods found most satisfactory for the species in colony at Walter Reed Army Institute of Research and National Institutes of Health. It should be noted, however, that not all species respond equally well to these methods and specific modifications must sometimes be made to accommodate the peculiarities of a particular sand fly species.

References

Anonymous. 1977. Report of the First Meeting, Scientific Working Group on Leishmaniasis. WHO Special Programme for Research and Training in Tropical Diseases, Geneva, 31 p.

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Gemetchu, T. (1976). The biology of a laboratory colony of *Phebotomus longipes* Parrot & Martin (Diptera: Phlebotomidae). *J. Med. Entomol.,* 12, 661-671.

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Killick-Kendrick, R., Leaney A.J., Ready P.D. 1977. The establishment, maintenance and productivity of a laboratory colony of *Lutzomyia longipalpis* (Diptera: Psychodidae). *J. Med. Entomol.,***13**, 429-440.

Ready, P.D. and H. Croset. 1980. Diapause and laboratory breeding of *Phlebotomus perniciousus* Newstead and *Phlebotomus ariasi* Tonnoir (Diptera: Psychodidae) from southern France. *Bull. Ento. Mol. Res.* **70,** 511-523**.**

Safyanova VM, 1964. Laboratory cultivation of sandflies (Diptera: Phlebotominae). *Bul. Wld. Hlth. Org.* **31**:573-576

Ward, R. D. 1977. The colonization of *Lutzomyia flaviscutellata* (Diptera: Psychodidae), A vector of *Leishmania mexicana amazonensis* in Brazil. *J. Med. Entomol.***14**, 469-476.

Chapter 1: Insectary Operation

1.1 General Overview of Sand Fly Insectary:

Claudio Meneses

The sand fly insectary at the Laboratory of Malaria and Vector Research (LMVR) of the National Institutes of Allergy and Infectious Diseases (NIAID-NIH) consists of two walk-in environmental chambers custom designed by Conviron (Model C1113). The chambers are controlled by a sophisticated computer system Conviron CMP-4030 v.6.10 which delivers the desired temperature and humidity needed to rear the sand flies. Small adjustments can be made daily to account for minor fluctuations in temperature and humidity levels.

Security
Biosafety issues are covered in the Arthropod Containment Guidelines (Benedict 2003) and are available free at the publisher's website (http://www.liebertonline.com/doi/pdf/10.1089/153036603322163475). Below is a brief description of general security that is in place at the sand fly insectary at LMVR.

The chambers have a negative air pressure system calibrated to 20 air changes per minute (ACPH) in a 0.15 inches of water column equivalent to 0.0037 kilopaskal (kPa) to minimize the possibility of escapee flies. All air ducts, air vents and floor drains in the insectary are covered with a fine screen (300 microns of opening size). Crevices and junctions of wall panels are sealed with caulk to prevent hiding places for sand flies. The door leading to the environmental chambers has an air curtain from Maxwell (model MASFO36-NT) on top to prevent sand flies from moving freely to the corridor and other areas of the laboratory.

We have a separate walk-in environmental chamber in a designated Bio-Safety Level (BSL-2) area where artificial infection of sand flies and experimental transmission of *Leishmania* to different hosts (mice and hamsters) takes place. This area is secured with a padlock. Once transferred to the BSL-2 area the sand flies are not allowed to return to the main insectary even if they were not exposed to *Leishmania* parasites. In both the rearing area and BSL-2 area we have two UV insect traps (model Mosquito indoor flying trap from Stinger), and two UV CDC light traps running on direct current (DC) voltage inside of the insectary to recapture possible escapees.

Environmental Security
The entire insectary, including all electrical outlets, is backed up by an emergency which minimizes the risk of losing colonies due to a prolonged power outage. There are environmental monitoring probes installed at strategic places in the insectary to monitor temperature and humidity parameters. The probes are incorporated to a remote alert system (Centron alarm from Rees Scientific) which sends out automated notifications to the laboratory personnel in case of any major change. We also have a team of two certified engineers on call 24/7 to assist the insectary with any mechanical issue that may occur.

Furniture
All insectary furnishings are either in metal, ceramic or plastic. Wood was avoided to prevent mold growth.

References
Benedict MQ (2003) Arthropod Containment Guidelines. Vector Borne and Zoonotic Diseases 3:63-98

1.2 Weekly Log

Tobin Rowland

The below log can be used to ensure task are completed every day. This chart can be adjusted to meet the needs of the laboratory

Weekly Log for Sand Fly Laboratory						**27 Oct 2014- 02 Nov 2014**		
ROUTINE	**Assigned**	**MON**	**TUE**	**WED**	**THU**	**FRI**	**SAT**	**SUN**
		27-Oct	28-Oct	29-Oct	30-Oct	31-Oct	1-Nov	2-Nov
Check temperature and RH in incubators								
Blood-feed adult flies*(M&TH)		BF			BF			
Change sugar on adult cages and ovipots								
Setup ovipots for hatch (>11-12 days post BM)								
Wash eggs with 1% clorox solution as needed								
Capture BF females to pots/tubs (>1 day)								
Clean adult cages prior to release of adult flies								
Release Eclosed Adults								
Prepare ovipots for capture (day before)								
Checked unhatched and newly hatched pots								
Feed larvae								
Transfer BF to ovipots as needed (5d post BM)								
Small Colonies		BF			BF			
Clean ovipots, wash lids, trays, screens								
Make New Sugar Solution w Antibotics								
Clean Laboratory								
AS NEEDED OR SCHEDULED		**Date**	**Notes/Initials**					
Safety Inspection		Monday						
Make Larval Food (2 week cycle)								
Wash Inside of Incubators (weekly)		Wed	Wash away sugar residue					
Heat Treat Incubators		Monthly						
Collect Rabbit Feces		M>W>F						
Make Ovi pots (as needed)		W or F						
Prepare adult cages for use (as needed)		Monthly	Alcohol clean, check/replace sleeves, screens, paper back					
Empty biohazardous waste								
Dispose of dead animals		Wed						
COLONY DATA of Female Sand Flies		**Numbers of Female Sand Flies**						
Captures		DATE:		DATE:		DATE:		TOTAL
		Potted	Left	Potted	Left	Potted	Left	Potted
L. longipalpis JACOBINA (**LLJB)**								
P. papatasi NORTH SINAI (**PPNS)**								
P. papatasi TURKEY (**PPTK)**								
PROJECT USE DATA		**Numbers of Sand Flies**						
Project/Name		DATE:	# SF's	Species/sex		Initials	Notes	

1.3 Trapping/Killing Escaped Sand Flies in the Laboratory

Tobin Rowland

Trapping
A mouth aspirator can be used to aspirate a sand fly that has escaped.
Model 612 Mouth Aspirator with HEPA filter (John W. Hock Company)

Figure 1.3.1: Mouth Aspirator with HEPA filter. Model 612 (John W. Hock Company) http://johnwhock.com/products/aspirators/mouth-aspirators/

Ultra violet or incandescent CDC light traps can be used to trap escaped flies. Place the light trap near the area where the sand fly was last spotted. Turn out all the lights and leave the area for 12 hours. Successful recapture can be enhanced by augmenting the trap with a CO2 source such as compressed CO2 or dry ice placed in a thermos. Sticky traps have been used in some labs but they are passive and rarely offer success in the laboratory setting.

Figure 1.3.2: CDC Light Trap. Miniature Light Trap Model 512 (John W. Hock Company) http://johnwhock.com/products/mosquito-sandfly-traps/cdc-miniature-light-trap/

Killing Escaped Sand Flies
There are several ways to kill escaped sand flies.

An electric fly swatter (Executioner®) purchased from Amazon.com (https://www.amazon.com/Executioner-Swat-Mosquito-Swatter-Zapper/dp/B000MU2MJA/ref=sr_1_1?ie=UTF8&qid=1468013882&sr=8-1&keywords=the+executioner+fly+swatter) is the easiest and most effective way to kill escaped flies.

Alcohol may also be sprayed on the fly to kill it (ensure there are no electrical outlets or open flames in the area). Sand flies can disappear quickly even in an insectary where the walls are painted white, success of killing an escapee depends on the response time.

References
Killick-Kendrick, R. 1987. Methods for the study of phlebotomine sand flies. pp. 473-497, in: W. Peters and R. Killick-Kendrick (eds.), The Leishmaniases in Biology and Medicine, Vol. 1. Academic Press, London.

Chapter 2 Sand Fly Biology

Tobin Rowland

2.1 General

Sand flies are small biting insects that are known vectors of several zoonotic diseases (Killick-Kendrick 1999). Sand flies are often mistaken for other biting flies. Unlike mosquitoes sand flies are strictly terrestrial in all stages of development. The life cycle of sand flies is relatively slow compared to other Diptera. The generation time is typically 1-3 months. Sand flies need a warm moist environment to survive. Both male and female sand flies require carbohydrates (sugar) as an energy source. Only the female sand flies bite and require a blood meal to produce eggs. Immature sand flies require organic material as nutrition for development.

2.2 Eggs

Sand fly eggs are small 0.3-0.5 mm long and 0.1-0.15 mm wide, elongated oval in shape and range in color from white when freshly deposited to brown or black. The surface of the egg contains chorionic sculpturing (Ferro et al. 1998). Burster spots appear 24 hour before hatching. Eggs are deposited singularly or in clusters.

Figure 2.2.1. *Phlebotomus papatasi* eggs containing burster spots. Photo: Edgar Rowton.

2.3 Larvae

Sand fly larvae range in size and color but are characterized as small and caterpillar-like. The color of the larvae varies by species but generally range from white to grey in color. As with the color the size of sand fly larvae vary by species and nutrition but can be up to 4mm long. Sand fly larvae have 4 instars. 1st instar larvae are small <1mm in length (Figure 2.3.1). The head capsule is dark (except within the first few hours of hatching). Lateral setae are present but extremely small. Two caudal setae are present and visible. 2nd instar larvae are larger <2mm and contain 4 caudal setae (Figure 2.3.2). 3rd instar larvae are larger than 2nd instars <3mm and contain 4 caudal setae (Figure 2.3.3). 4th instar larvae are even larger 4mm and contain 4 caudal setae, lateral seatae are more pronounced. 4th instar larvae also exibit a heavily sclerotized dorsal anal plate (Figure 2.3.4). Pupae resemble butterfly crysalis (3-4mm). The collapsed 4th instar exuvium can be seen at the caudal end. Pupae in early development appear whitish (Figure 2.3.5) then turn reddish brown to black as eclosion nears (Figure 2.3.6).

Figure 2.3.1. First instar larvae.

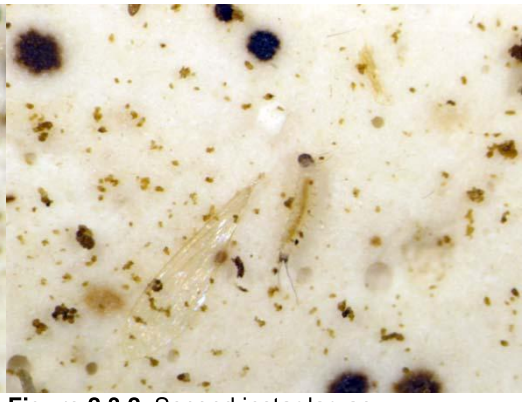

Figure 2.3.2. Second instar larvae

Figure 2.3.3. Third instar larvae

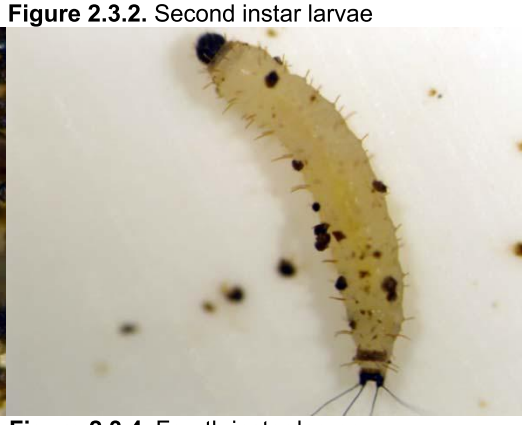

Figure 2.3.4. Fourth instar larvae

Figure 2.3.5. Early pupae

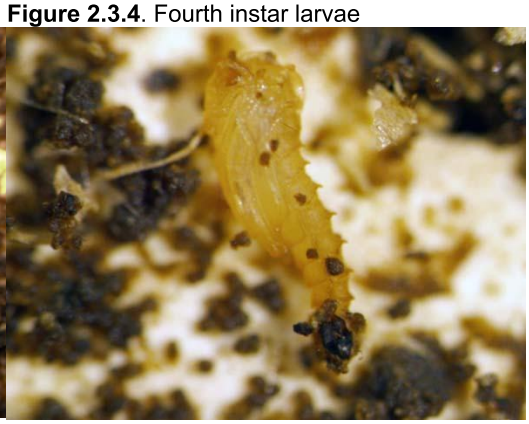

Figure 2.3.6. Late pupae

Photos: Tobin Rowland

2.4 Adults

Adult sand flies are distinct in that they are hairy and hold the wings in a “V” pattern when at rest (Killick-Kendrick 1999). Only the female sand fly requires a blood meal that is used as a protein source to produce eggs. Sand flies are generally considered to be weak flyer with flight patterns consisting of short hops. Sand flies are pool feeders with mouthparts consisting of 6 bladelike stylets. Male sand flies have clasping structures on the tip of the abdomen that is used for mating.

References

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Chapter 3 Sand Fly Rearing

Phillip G. Lawyer, Claudio Meneses, Tobin Rowland

Introduction

Rearing sand flies requires extreme dedication to maintain a colony. This often means working on the weekends and holidays. There are currently no methods to preserve sand fly eggs so production must be continuous. Care must be taken at every step in the rearing process so that healthy sand flies are produced for further generations and experiments. Problems often arise in sand fly laboratories when personnel become complacent or are no longer dedicated. Colony maintenance must take precedence over experiments.

Pertinent References for this chapter:

Modi, B. and B.R. Tesh. 1983. A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. J. Med. Entomol. **2**: 558-569
Modi, G.B. and E.D. Rowton. 1999. Laboratory maintenance of phlebotomine sand flies. Pp. 109-121. *In*: K. Maramorsch and F. Mahmood [eds.]. Maintenance of Human, Animal, and Plant Pathogen Vectors. Science Publishers, Enfield NH, USA.
Volf, P. and V. Volfoya. 2011. Establishment and maintenance of sand fly colonies. J. Vec. Ecology. 36:S1-S9

3.1 Rearing Conditions

Temperature and Humidity

In general colonies are maintained in reach-in incubators or in walk-in environmental rooms at 25 or 26 ºC and 80% relative humidity. However, temperature and humidity may vary depending on the species or life stage. For instance, a *Lutzomyia verrucarum* colony, which originated from the Andes mountain region of Peru, is maintained at 22 ºC because it does not thrive at higher temperatures. Adults of a *Phlebotomus argentipes* colony, originating from India, develop best at 26 ºC and RH higher than 80%. This higher RH is achieved by enveloping the adult cage in a plastic bag equipped with a wet sponge. Larval stages from most colonies mature faster at 26 ºC and adults survive longer at 25 ºC.

Light:Dark cycle

The incubators are set with a 12-hr dark:12-hr light photocycle but most colonies can be maintained in total darkness with no noticeable differences in feeding behavior, egg production or larval development. When incubators or environmental rooms are not available, cages and pots can be maintained at room temperature in plastic bags on bench tops or tables.

3.2 Adult Holding and Mating Cages

A variety of cages have been devised to contain adult sand flies ranging from fabric cages suspended over a wire frame to modified aquaria, to custom-made polycarbonate square-foot cages. In our insectaries we use custom-made polycarbonate cages fitted with removable back panels in front of which a piece of absorptive bench-top paper can be inserted to provide a suitable vertical resting surface for the flies (Figures 3.2.1- 3.2.3). The screen on top of the cage allows for ventilation and provides a surface on which sugar pads can be placed and through which the flies can obtain sugar meals (Figure 3.2.4). These sturdy cages are very durable and can be easily washed and sanitized. The screens, sleeves and backing can be replaced easily. Some of our cages have been in steady use for over ten years. Depending on the size of the colony, we use three sizes of cages, 8x8x8", 10x10x10" and 12x12x12".

Figure 3.2.1. Front view of polycarbonate cage

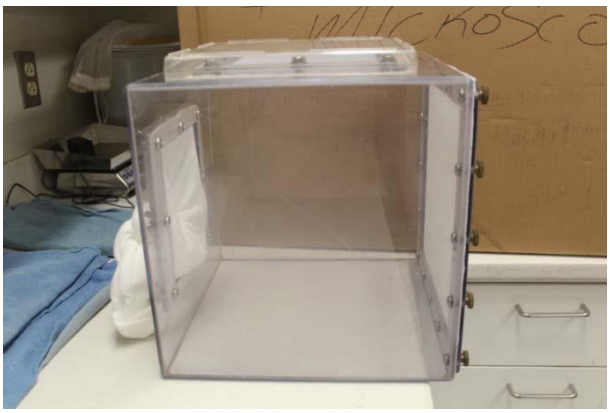


Figure 3.2.2. Side view of polycarbonate cage

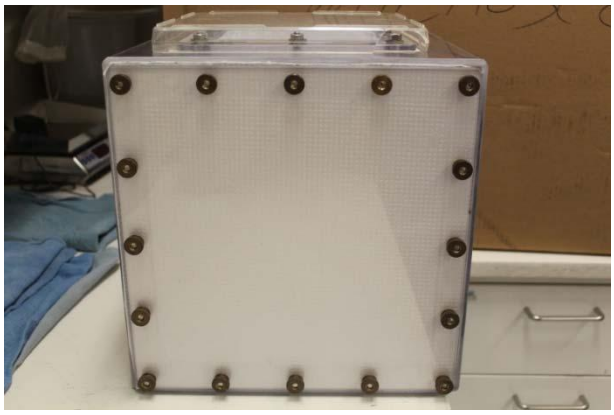


Figure 3.2.3. Rear view of polycarbonate cage showing removable back

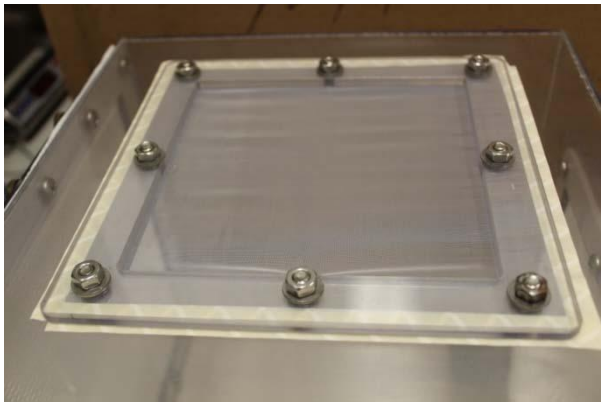


Figure 3.2.4. Top view of polycarbonate cage showing screen that is used to provide sugar meal to flies.

3.3 Mating, Sugar Feeding and Blood Feeding

Upon emergence, adult flies are released into holding/mating cages. A single large cage can easily contain up to 3,000 flies without significant overcrowding. Balls of cotton saturated with 30% sucrose in water are placed on the screen tops of the cages to provide a sugar meal for flight, energy and longevity (Figure 3.3.1). Mating occurs before, during and after feeding in most species and commences shortly after the females emerge. Two to five days after emergence, the females have developed blood hunger and are ready to feed.

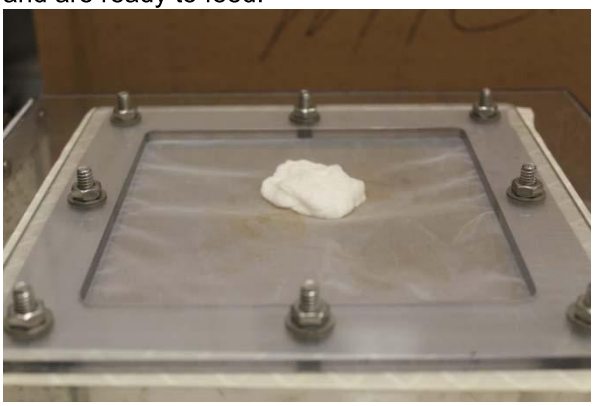


Figure 3.3.1. Sugar pad soaked with 30% sucrose solution placed on top of sand fly cage.

Blood Feeding

Only female sand flies take a blood meal. The females need the proteins present in the blood to lay eggs and to propagate the future generation. Various blood meal sources can be used depending on the availability of source animals and the preference of the flies. In the initial stages of colony establishment, the flies may refuse to feed on anesthetized rodents and may prefer a restrained rabbit or guinea pig. It may be necessary to try several blood-meal sources and feeding times until a suitable host and feeding time is found. We routinely use mice, hamsters and nude guinea pigs. Some labs use chickens, rabbits and artificial membrane feeders using rabbit blood. When using live animals, an animal-use protocol approved by the local Institutional Animal Care and Use Committee (IACUC) is essential. One day prior to blood feeding, the sugar balls are removed from the cage tops and the flies are starved for 24 hours, after which anesthetized animals such as mice or hamsters are placed on their backs, bellies shaved, inside the cage to provide a blood meal. Following the blood meal, the flies are left in the cage for another 24 hours to allow time for diuresis and for the fragile peritrophic membrane that surrounds the blood meal to harden (Figure 3.3.2). This also allows for further mating. Unnecessary handling prior to 24 hours may cause the peritrophic membrane to rupture, ultimately killing the fly.

Figure 3.3.2. Blood engorged females resting on the bench-top paper in a sand fly cage

The laboratory at NIH has an Institutional Animal Care and Use Committee (IACUC) approved animal protocol to use mice, hamsters and chickens as a source of blood to sand flies. We order 6-8 weeks old Swiss Webster mice (retired breeders) from Charles River Laboratories for colony feeding.

The mice are anesthetized on a mixture of Ketamine and Xylazine (10:1) and 50 micro liters are injected intraperitoneally per mouse. The mice remain anesthetized for approximately 45 minutes and once fully recovered from anesthesia the mice are returned to their respective cages in the animal facility. Our protocol allows 15 mice per week and at the end of the week they are euthanized in a CO2 chamber with 20% gas displacement set for 7 liters per min. We don't use the same group of animals for more than two consecutive feeds, this is important because it has been shown that the host produces antibodies for the saliva injected, reducing significantly the number of eggs laid by female flies if constantly fed on the same animal (Milleron et al. 2004). Because sand flies are slow feeders in addition to mice we offer anesthetized chickens to them. Two month old chickens (Gallus-Gallus Domesticus) are purchased from Charles River Laboratories and kept at our animal facility under constant care. The chickens are weighed and using a mathematical formula the cocktail of 1ml of Ketamine (100mg/ml), 2ml cerpomazine (10mg/ml) and 0.9% Sodium Chloride is calculated and injected intramuscularly to each bird. Each anesthetized chicken is restrained on a plexiglass board (Figure 3.7.1), custom fabricated by NIH mechanical design and fabrication shop and placed in the cage containing the flies for up to 45 minutes. We cover the cages with a black plastic bag while they are feeding in the insectary. We starve the flies by eliminating the sugars 12 hours preceding their feeding. This will make them avid to take a blood meal.

Ketamine/ Acepromazine Anesthesia for Chickens
Step 1: Mix 2ml Ketamine (100 mg/ml) with 1ml Acepromazine (10 mg/ml) and 2ml of 0.9% NaCl for injection to make a solution containing 40 mg/ml of ketamine and 2mg/ml of acepromazine.

Step 2: Calculate the dose to be administered by multiplying the chicken's weight in grams by using this formula: wt(g)*1 (kg)/1000 (g)*1 ml/40mg= volume (ml).

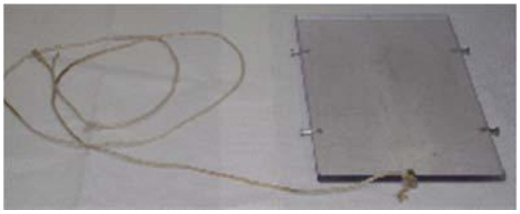


Figure 3.7.1. Restraint board for chickens.

Ketamine/ Acepromazine Anesthesia for Chickens
In addition or instead of the use of chickens as a blood meal source, several mammalian laboratory animals can be used for a blood meal source, including mice, hamsters and guinea pigs. Table 3.7.1 below lists anesthesia regimes that have been approved by the IACUC committee at NIH/WRAIR. Please note that adaptation of these regimes by other laboratories requires IACUC approval prior to use.

Table 3.7.1 Suggested anesthesia regimes for sand fly blood feeding

Animal	Ketamine	Xylazine	Route
Mouse	80-100 mg/kg	10 mg/kg	IP
Hamster	200 mg/kg	10 mg/kg	IP
Guinea Pig	40 mg/kg	5 mg/kg	IM

3.4 Oviposition

Twenty-four hours following blood feeding, engorged female flies are aspirated with a special vacuum aspirator into dry, 500-ml oviposition pots, 150-200 engorged females per pot along with about 50 males. Cotton balls soaked in 30% sugar are placed on the screen tops of the pots as an energy source and the pots are in plastic tubs (Rubbermaid®) and in an environmental incubator at 26 ºC and 75-80% relative humidity (RH). Sugar meals are replaced every other day. After five days the pots are soak in tap water to saturate the plaster oviposition surface. The flies will usually begin depositing eggs almost immediately. After 10 days oviposition should be complete and the adults are removed dead or alive from the pots with a vacuum aspirator.

The average number of eggs laid per fly depends on the species and nutrition but usually averages between 30 and 38 eggs per gravid female. Therefore, the expected number of eggs per 500-ml ovipot will be 5,000-7,000. Using a Waterpik®, the eggs are then rinsed from the pot into a soil sieve and then washed in 1% sodium hypochlorite solution for one minute, rinsed in tap water for another minute and placed back into the oviposition pot from which they came.

3.5 Immature Stages

Gravid females are captured with mouth or vacuum aspirators and placed in oviposition pots consisting of 500 ml straight-sided polypropylene Jars (Nalge Company, Rochester New York) modified by drilling six 7/8 inch holes in the bottom and then by pouring approximately 1” of dental plaster into the bottom of the pot, filling the holes and providing a porous oviposition surface that can be saturated as needed from the bottom up. Usually 150 females are placed in each jar and held for 7-10 days until they lay their eggs. The number of eggs laid per female varies according to species, rearing conditions, number of flies per pot, and size of the blood meal taken, but generally blood-fed females oviposit between 30-50 eggs each (range 1-100). Following oviposition, the dead flies are removed from the pot with vacuum-powered pipette aspirator and the surviving flies are either removed with the aspirator or release into the colony mating cage to start another gonotrophic cycle. Removal of the flies from the pots is critical to prevent excessive mold growth that might entrap the 1st instars, to reduce phorid mite infestations and to prevent the larvae from eating the adult fly carcasses and subsequently becoming infected with gregarines. With the adults removed, the eggs are then washed with a 1% sodium hypochlorite solution to remove gregarine cysts adhered to the exterior surfaces of the eggs. The washed eggs are then rinsed with clean tap water and placed back into the oviposition pots.

Eggs are checked daily for hatching.

3.6 Feeding and Soaking Larval Pots

Each pot is taken to a compound microscope for inspection. While inspecting and feeding the larval pots look for these factors: (1) pot density, (2) larval stage, differentiation, mobility, coloration and excretion (3) presence of mold, (4) presence of mites, (5) presence of bad odor coming from the jar and (6) free movement of the food. Each pot is treated independently and food is dispensed accordingly based on each factor. As the larvae hatch from eggs, an initial tiny amount like “salt and pepper” of food is given. The food will absorb moisture and change to a dark brown color. As the larvae develop more food is gradually added to the pot until late 4th instar larvae are seen. Before pupation larvae stop feeding so there is no need to feed them anymore. Feeding during late 4th instar and pupae can result in mold growth. The adults will emerge from the pots gradually then reach a peak at which time hundreds can be released from a single pot at one time. After the peak emergence the number of flies will slowly decrease until all adults have emerged. The time from first emergence to last emergence varies by species but generally lasts for one to two months (Ex. *L. longipalpis*). *Phlebotomus duboscqi* pots can have emergence that lasts for 3-4 months. The time to complete the life cycle of sand flies depends on the species as well as the nutrition, temperature and humidity. Drastic changes in temperature and humidity levels may accelerate or decelerate their cycle. In general, it takes 4-6 weeks to complete a sand flies life cycle (egg to adult). Other species may take as long as 6-8. Some species may go into diapause making the time to complete the life cycle even longer. As the pots are fed the bottoms of the pot are checked with the dorsal part of the hand for moisture. If the pots are dry and need moisture the pots are placed in a tray filled with about a half an inch of warm tap water for 2-10 minutes depending on the moisture level. Another important indicative of a dry jar is the aspect of the larvae and food coloration. If the larvae seem dry and lethargic it may be prudent to wet the pot. However, if the pot is too wet mold and other unwanted organisms may grow. To remove the moisture from an extremely wet pot sit the pot overnight in multiple layers of paper towel to absorb the excess of moisture.

References

Milleron et al. 2004. Negative effect of antibodies against maxadilan on the fitness of the sand fly vector of American visceral leishmaniasis. Am J Trop Med Hyg. Mar;70(3):278-85)

Sant’Anna, M.R.V., A. Nascimento, N. Alexander, E. Dilger,R.R. Cavalcante., H.M. Diaz-Albiter, P.A. Bates, and R.J.Dillon. 2010. Chicken blood provides a suitable meal for the sand fly Lutzomyia longipalpis and does not inhibit Leishmania development in the gut. Parasites and Vectors 3: 3.

Chapter: 4 Sand Fly Standard Operating Procedures (SOPs)

Tobin Rowland

Standard Operating Procedures are essential to maintain consistency and produce healthy colonies. It is important that every lab develop their own SOP to fit the needs of the laboratory. SOP's standardize procedures so that laboratory personnel perform tasks identically. As needs of the laboratory change it is necessary to revise SOP's periodically. Below we describe SOP's that are used in our laboratories for basic sand fly rearing procedures.

4.1 Preparation of Sugar Solution

Materials

- Gloves
- Lab coat
- Sugar
- Medium cotton balls
- 2000ml plastic container
- 500ml Pyrex container with stainless steel lid

Equipment

Refrigerator

Procedure

1. Mix 600ml of sugar in 1400ml of DI water in a 2000ml plastic container for a sugar concentration of 30%.

2. Pour 500mls of the 30% sugar solution into a 500ml Pyrex container.

3. Place the plastic container with the remaining 1500ml of the 30% sugar solution into the refrigerator to prevent fungal growth.

4. Gently place a handful of medium size cotton balls into the Pyrex container with the 30% sugar solution.

5. Allow adequate time for the cotton balls to fully absorb the solution (5 minutes).

6. Pick up one cotton ball and gently squeeze off the excess sugar solution over the Pyrex container.

7. Place the cotton ball onto a paper towel to absorb any remaining excess sugar solution.

8. Place the cotton ball on top of the screen of adult sand fly cages or ovipots containing adults.

9. Repeat steps 6-8 until all adult cages or ovipots contain cotton balls soaked with the 30% sugar solution.

10. Cotton balls must be replaced daily to prevent fungal growth.

11. Due to fungal growth old cotton balls from adult cages or ovipots must be discarded in the regulated medical waste container.

Antibiotics are routinely used for colony maintenance however depending on your research needs you may not want to use antibiotics. Antibiotics will inhibit metacyclogenesis in *Leishmania*. We use 10mls of Penicillin-Streptomycin with 10,000 units of penicillin and 10 mg streptomycin per ml into the 2000ml plastic container of sugar water.

4.2 Capturing Blood Fed Sand Flies into Oviposition Pots

Materials

Gloves
Lab coat
Screened ovipots

Equipment

Sand fly vacuum apparatus (Figure 4.2.1)

Procedure

1. Remove cage containing blood-fed sand flies from the incubator and place on the bench top next to the house vacuum.

2. Place a screened ovipot in the bottom housing of the sand fly vacuum apparatus.

3. Next place the top housing of the sand fly vacuum apparatus over the screened ovipot while ensuring the pointed tube protruding from the bottom of the top housing is inserted into the small hole in the screen (Figure 4.2.2).

4. Secure the top housing of the sand fly vacuum apparatus by looping the bungee cord attached to the bottom housing around the top housing and secure the bungee cord to the loop in the other side of the bottom housing.

5. Connect the vacuum hose of the sand fly vacuum apparatus to the house vacuum.

6. Turn the house vacuum on until there is a slight noise when a finger is placed over the end of the aspirator.

7. Insert the aspirator into the cage through the nylon sleeve.

8. Wrap the sleeve around the aspirator 4 times to ensure that adult sand flies cannot escape through the sleeve.

9. Grip the aspirator from the area where the sleeve is wrapped around it.

10. Move the aspirator towards a blood-fed sand fly until the fly is aspirated into the ovipot (Figure 4.2.3).

11. Continue aspirating blood-fed sand flies until there are 150 sand flies in the ovipot

12. Carefully detach the bungee cord.

13. Carefully remove the top housing of the sand fly vacuum apparatus while immediately placing a finger over the hole in the screen to prevent escape (sand flies have small mouthparts that cannot bite through gloves).

14. Carefully slide the screen on the ovipot until the hole is no longer accessible by the flies.

15. Roll the rubber band up the ovipot until it is above the hole ensuring that it does not come past the lip of the ovipot (this prevents escapes).

16. Repeat steps 2-15 until all blood-fed sand flies have been captured.

Figure 4.2.1. Vacuum apparatus.

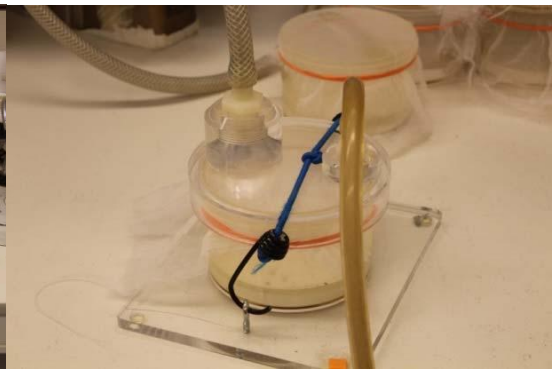


Figure 4.2.2. Close up of top and bottom housing of vacuum apparatus securing oviposition pot with pot.

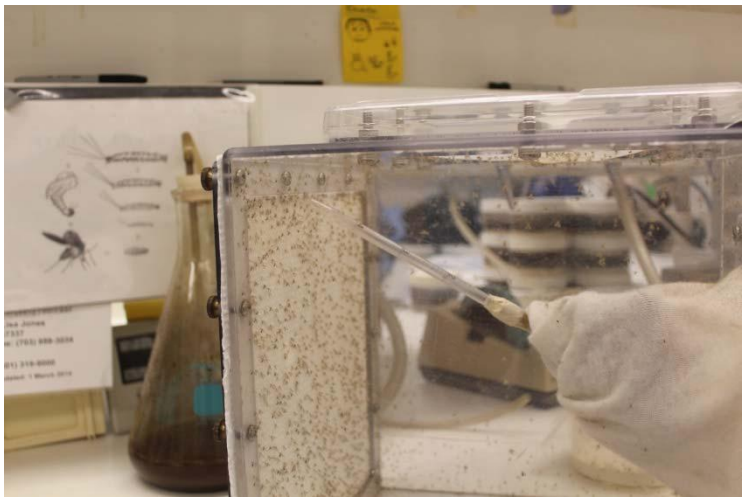


Figure 4.2.3. Aspiration of blood-fed females into oviposition pot using vacuum apparatus.

4.3 Adult Removal in Oviposition pots

Materials
Glass Pasteur pipette

Equipment
Vacuum aspirator

Procedure
Set up Ovipots for wash.
1. Select ovipots pots from incubator that have been captured for 7-10 days and in which the captured females have deposited their eggs and died to be washed/setup (Figure4.3.1).

2. Remove tape and cotton ball from the top of the screen.

3. Using the vacuum aspirator fitted with a 5” glass Pasteur pipette, aspirate any live flies that are flying around inside the pot through the pre-cut hole in the screen (Figure 4.3.2).

4. After ALL live flies have been aspirated, remove the rubber band from around the screen and remove the screen (Figure 4.3.3).

5. Aspirate the remaining dead flies from the sides and the plaster bottom of the container. To avoid aspirating eggs, ensure that the pipette does not touch the surface of the plaster (Figures 4.3.4 and 4.3.5).

6. To prevent cross contamination, ensure that the pipettes are changed between aspirations of different colonies of sand flies.

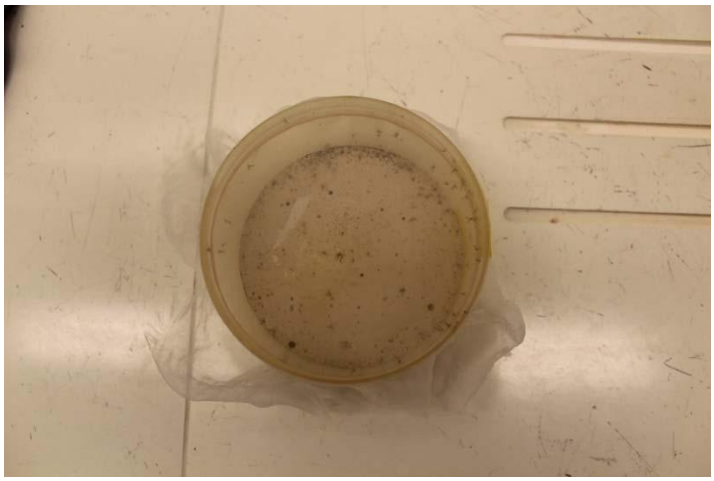


Figure 4.3.1. Screened oviposition pot containing eggs and dead adults

Figure 4.3.2. Vacuum aspirator with soapy water in the bottom for aspiration sand flies from oviposition pots.

Figure 4.3.3. Oviposition pot with screen off after live flies have been aspirated

Figure 4.3.4. Aspiration of dead sand flies

Figure 4.3.4. Oviposition pot containing eggs after all flies have been aspirated.

References

Modi, B. and B.R. Tesh. 1983. A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. J. Med. Entomol. **2**: 558-56

4.4 Egg Washing

Materials

Gloves
Lab coat
Color coded test sieves (0.0035” mesh)
3” nesting brass capture pan
Colored tape
6.15% Sodium Hypochlorite Solution (Clorox®)
Water bottle
Glass Pasteur pipette
1000ml beaker
500ml graduated cylinder
Distilled water
Timer
Lab towels

Equipment

WaterPik® oral irrigation apparatus

Procedure

1. Make the 1% Sodium hypochlorite solution by measuring out 162.6 ml of the 6.15% sodium hypochlorite stock solution (Clorox®) into the graduated cylinder and pouring it into the 1000-ml beaker.

2. Next measure out 837.4 ml of distilled water into the graduated cylinder and pour it into the 1000ml conical flask containing the 162.6ml of the sodium hypochlorite stock solution. New solution should be made on the day of the wash.

3. Mix gently by swirling the beaker.

4. Fill the Waterpik® reservoir with distilled water.

5. Turn the Waterpik® on and spray the inside of the pot to dislodge the eggs (Figure 4.4.1).

6. Next pour the egg/water suspension into the appropriate color-coded sieve. Ensure that the sieve is next to the sink so that the water can run into the drain. Repeat this step for all pots of the same species so that all eggs of a particular species on a particular setup date are collected together in the same sieve (Figure 4.4.2).

7. Place the sieve that now contains only the eggs into the nesting capture pan.

8. Gently pour the 1% sodium hypochlorite solution from the conical flask into the sieve until it is approximately half full. Overfilling will cause the eggs to spill out over the top (Figure 4.4.3).

9. Set the timer to 1 minute and press start.

10, After 1 minute has elapsed gently remove the sieve from the nesting capture pan.

11. Rinse the eggs by running tap water over them for 1 minute (set timer as before)

12. Tilt the sieve containing the eggs at a 45 degree angle and gently wash the eggs to the bottom portion of the sieve (Figure 4.4.4).

13. Once eggs are gathered at the bottom, hold the sieve containing the eggs at a 110 degree angle over the corresponding pot and squirt distilled water to dislodge the eggs from the sieve, and distribute them as evenly as possible back into the original ovipots that have been wiped clean and dry (Figure 4.4.5).

14. Place clean lids on the containers.

15. Place the corresponding color tape onto the lids and label with set up date and 1% Clorox. Example (Setup 6-2-10, 1% Clorox).

16. Repeat steps 12 thru 21 until all pots have been washed ensuring that the appropriate sieves are used for the corresponding species.

17. Allow the pot to dry on lab towels for 2 hours.

18. Remove lid and inspect the wetness of the plaster inside the pot. If the plaster is still soaking wet, or if there is standing water, allow ample time for the pot to dry.

19. Put pots into a white plastic tray and place the tray on the top shelf of incubator#3.

20. After this is complete wipe down the sink and counter top with sponges and paper towels to clean up remaining water and any Clorox® spillage.

21. Empty the water reservoir of the Waterpik® and pour the remaining 1% sodium hypochlorite (Clorox) solution down the drain.

Figure 4.4.1. Waterpik® dislodging eggs in oviposition pot

Figure 4.4.2. Pouring egg/water suspension into sieve

Figure 4.4.3. Pouring 1% sodium hypochlorite into sieve containing eggs

Figure 4.4.4. Rinsing eggs with water

Figure 4.4.5. Washed eggs placed back into oviposition pot to dry

References

Dougherty, M.J. and R.D. Ward. 1991. Methods for reducing Ascogregarina chagasi parasitaemia in laboratory colonies of Lutzomyia longipalpis. Parassitologia **33** (Suppl. 1): 185-191.

4.5 Counting the Number of Eggs

In order to maintain a healthy colony, it is prudent to keep consistency in the number of females placed in a pot to oviposit. This helps keep the number of amount of eggs somewhat standard and thus creates consistency in the number of larvae in each pot. Sand flies are reared in the same pot from egg to adult so egg counting is only necessary to maintain consistency or when your research requires knowledge of the number of eggs. There are 3 ways to count the number of eggs manually with a microscope, using an enlarged photo, or using computer software.

Manual Counting
To manually count the eggs in a sand fly pot simply place the pot under a dissecting microscope and count the number of eggs; be sure to look on the walls of the pots as sand flies will often lay their eggs on the walls. Prior to capturing the blood-fed females a grid can be etched into the plaster to make egg counting easier.

Enlarged Photo Counting
To use an enlarged photo simply place the pot on a flat surface and take a high quality photo of the pot. This method does not account for the eggs on the walls of the pot unless the pot is rinsed or washed to dislodge the eggs from the side. The photos can be enlarged with a grid overlay and the eggs can be counted on the photo (Figure4.5.1). A digital counter pen can be used to count and mark the eggs as they are counted.

Software Counting
Wash the eggs into a clean pot. Place the pot onto a flat surface and take a high resolution photo of the whole pot ensuring the eggs are in focus. Upload the high resolution photo into the software (We use ImageJ). Simply use the small pen tool to place a mark on each of the eggs using the mouse. The software will count all marks to give you a total number of eggs.

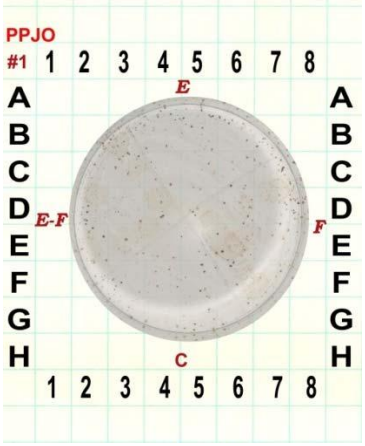


Figure 4.5.1 Photo of oviposition pot containing eggs with a grid overlay.

4.6 Larval Food Preparation

The larval food preparation is one of the most important factors in creating or maintaining a successful sand fly colony. Improperly prepared larval food can lead to malnourished larvae. Often time's improperly prepared larval food will halt the growth and lead to diapause in the larvae. Additionally, the food that is not eaten by the larvae will lead to mold and potentially mite infestations in the larval pots. There are several variations in the literature but here we describe the method that has worked best in our lab.

Materials

Rabbit feces
Rabbit chow
Plastic 5-gallon tub (18" x 12" x 9")
Water
Cat litter scoop
6" white plastic spatula
Polycarbonate larval food composting cabinet
18" x 26" x 3 "photographic developing trays
Gloves
Lab coat
Face mask

Procedure

1. Remove two 1- gallon sized bags of rabbit feces from the freezer and thaw at room temperature (approximately 4hours).

2. Empty the two bags of rabbit feces into a large white Rubbermaid® plastic tub (18" x 12" x 9").

3. Empty two 1- gallon sized bags of rabbit chow into the plastic tub with the rabbit feces.

4. Add 9 liters of warm water to the mixture of rabbit feces and rabbit chow.

5. Mix continuously until all of the water has been absorbed into the mixture.

6. Place approximately 3.5 scoops of the rabbit feces and rabbit chow mixture into each of the plastic trays [18" x 26" x 3"] and spread it out evenly so that no portion of the bottom surface of the pan is visible. (Approximately ¼ to ½ inch)

7. Lightly spray the surface of the rabbit feces and rabbit chow mixture inside the pans with water to make sure it is well saturated but not muddy (no standing water) (Figure 4.6.1).

8. Place the composting pans containing the rabbit feces and rabbit chow mixture onto the individual racks inside the larval food composting cabinet. There should be six pans of larval food mixture per cabinet.

9. Fill the pan in the bottom of the larval food composting cabinet with water.

10. Attach the door to the composting cabinet using the seven spring clamps (Figure 4.6.2).

11. Ensure that the two air vent holes in the cabinet door are opened ¾ of the way.

12. Clean off counter top and sweep the floor to ensure that all rabbit feces and rabbit chow have removed from the area.

13. Label each cabinet door with a piece of tape showing the date of setup, the date (1 week later) on which to “flip and mix” the composting mixture, and the date (2 weeks later) on which to harvest. Example: “Setup 8 June; Flip & mix 15 June; Harvest 22 June”

14. After 1 week of composting, remove the cabinet door and one-by-one remove each of the six trays and, using the large 6” white plastic spatula, flip the larval food over and mix it thoroughly. Spray the surface with water and place back into the composting cabinet.

15. After 1 additional week, remove the cabinet doors and place the treys in a hood to dry (Figure 4.6.3).

16. Once the composted food is completely dry (Figure 4.6.4) grind the food into a fine powder using a meat grinder or blender (Figure 4.6.5).

Figure 4.6.1. Mixture of rabbit feces and rabbit chow that has been soaked, placed in plastic trays and sprayed to begin composting.

Figure 4.6.1. Composting cabinet containing treys of prepared food with door closed.

Figure 4.6.3. Sand fly larval food that has completed the two week composting process. Notice the heavy white mold growth that is beneficial to the larvae.

Figure 4.6.4. Completed sand fly larval food that is dry and ready to be ground

Figure 4.6.5. Completed sand fly larval food that is ground using a meat grinder.

Reference

Modi, B. and B.R. Tesh. 1983. A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. J. Med. Entomol. **2**: 558-569

Volf, P. and V. Volfoya. 2011. Establishment and maintenance of sand fly colonies. J. Vec. Ecology. **36**:S1-S9

Young, D.G., P.V. Perkins and R.G. Endris.1981. A larval diet for rearing phlebotomine sandflies (Diptera: Psychodidae). J. Med. Entomol. **18**:446.

4.7 Food Coloration and Fungus Growth

Claudio Meneses

Different species of fungus will grow inside of the chamber. Some of them are extremely beneficial while others are harmful to the larvae. As a general rule, the final larval food product should consist of a dark brown powder resembling rich humus. If the final product is a yellowish or pale light brown powder, most likely a harmful fungus grew inside of the chamber or there was a lack of water along the preparation (Figure 4.7.1)

To be sure the freshly made food is nourishing the larvae it is recommend that you apply it into a trial pot first, containing recently hatched larvae (first instar). The pot should be reared with the same food to adult stage. When a batch of bad food is detected it should be immediately discarded and never be stored with the good food. The larval food is stored at -20C when not in use. If successive batches of bad larval food are produced in a short period of time, it is advisable to clean the entire food chamber. A combination of 1% bleach and 70% ethanol followed by three rinses with distilled water should disinfect the chamber. The same care should be applied to trays, spatula and box where the food is mixed.

Figure 4.7.1. Dish on the left containing a grey/light brown powder (bad) and dish on the right containing a dark brown powder (good)

4.8 Oviposition Pot Preparation

Sand flies oviposit and immature larvae develop in the same pot. Sand flies are strictly terrestrial but do require a moist environment develop. The method described below provides a habitat that allows for moister to be added to the plaster substrate in order to control the moisture level needed for oviposition and larval development.

Materials

Plaster of Paris (ortho Plaster)
Wide mouth 500ml clear polypropylene jars
Wide mouth 125ml clear polypropylene jars
Aluminum foil
2000-ml plastic beaker
¾-7/8 inch drill bit
Stainless steel spatula

Equipment

Electric drill

Procedure

1. Remove the lid from the jar and place the jar on the bench top upside down.

2. Holding the jar tightly, drill a hole in the center of the jar. Drill five more holes around the perimeter of the previous hole. If preparing 125ml jars, just drill one hole in the center.

3. Place aluminum foil on the bench top to cover the area needed.

4. Place drilled jars right-side up on top of the aluminum foil.

5. Put 1200-ml of plaster of Paris into a 2000-ml plastic beaker.

6. Slowly add 500 ml of water.

7. Stir vigorously with the metal stir bar or spatula for 2 minutes to ensure there are no clumps.

8. Pour plaster mixture into jar to a depth of approximately 3/4 inch from the bottom. Twist jar back and forth to ensure that the plaster completely fills the space on the underside of the jar. NOTE: When working with plaster of Paris, it is imperative that one work fast as the plaster hardens quickly, making it difficult to work with.

9. Repeat steps 5 thru 8 until all pots have been made.

10. Allow pots to sit on the bench top overnight. (The plaster produces heat and CO2).

References

Volf, P. and V. Volfoya. 2011. Establishment and maintenance of sand fly colonies. J. Vec. Ecology. **36**:S1-S9

4.9 Slide Mounting Phlebotomine Sand Flies

Phillip G. Lawyer, Edgar Rowton, and Kathrine Westbrooke

Various techniques have been use for mounting phlebotomine sand flies. Many workers have found water-soluble choral hydrate media such as gum chloral or Belese's fluid to be suitable for temporary mounts. While they are easy to use, they are not good for permanent preparations because they may shrink as the water evaporates, become crystallized and discolored. To make such mounts more permanent they can be ringed with fingernail polish to prevent moisture from being absorbed into the medium.
Permanent slide mounts can be made using Canada balsam. When using this medium, it is important to dehydrate the specimens by placing them for at least 5 minutes each in 70% alcohol, 90% alcohol, absolute alcohol and finally in xylene. If dehydration is incomplete the specimen will become clouded in the mountant. If specimens are in 80%-90% alcohol, they can be placed in cellosolve (ethylene glycol monoethyl ether) without having to use absolute alcohol and xylene. From the cellosolve they can be put directly into the Canada balsam mountant on the slide.

Some specimens require clearing before mounting to render important internal features more visible. Various media are used for this but we have found 10% lacto-phenol solution (Bioquip) works quite well. Place the specimens in lacto-phenol solution for at least an hour but preferably overnight. Specimens can even be stored for long periods of time in the lacto-phenol.

For making permanent slide mounts we have had good success with polyvinyl alcohol (PVA) mountant (Bioquip) or euparal. Place the specimen on a slide in a few drops of the medium and dissect it by removing the head, one wing, and the posterior (last 3-4) segments of the abdomen, which contain the spermathecae in the female and the external genitalia (claspers) in the male.

Use a cover glass that is suitable for the size of the specimen and make sure enough fluid is used to cover the area under the glass and to allow for some evaporation and shrinkage. Orient the body parts on the slide as shown in Figure 4.9.1. When the specimen is examined under a compound microscope, the body parts will be seen in reverse from right to left and top to bottom. Label the slide as shown in Figures 4.9.2.

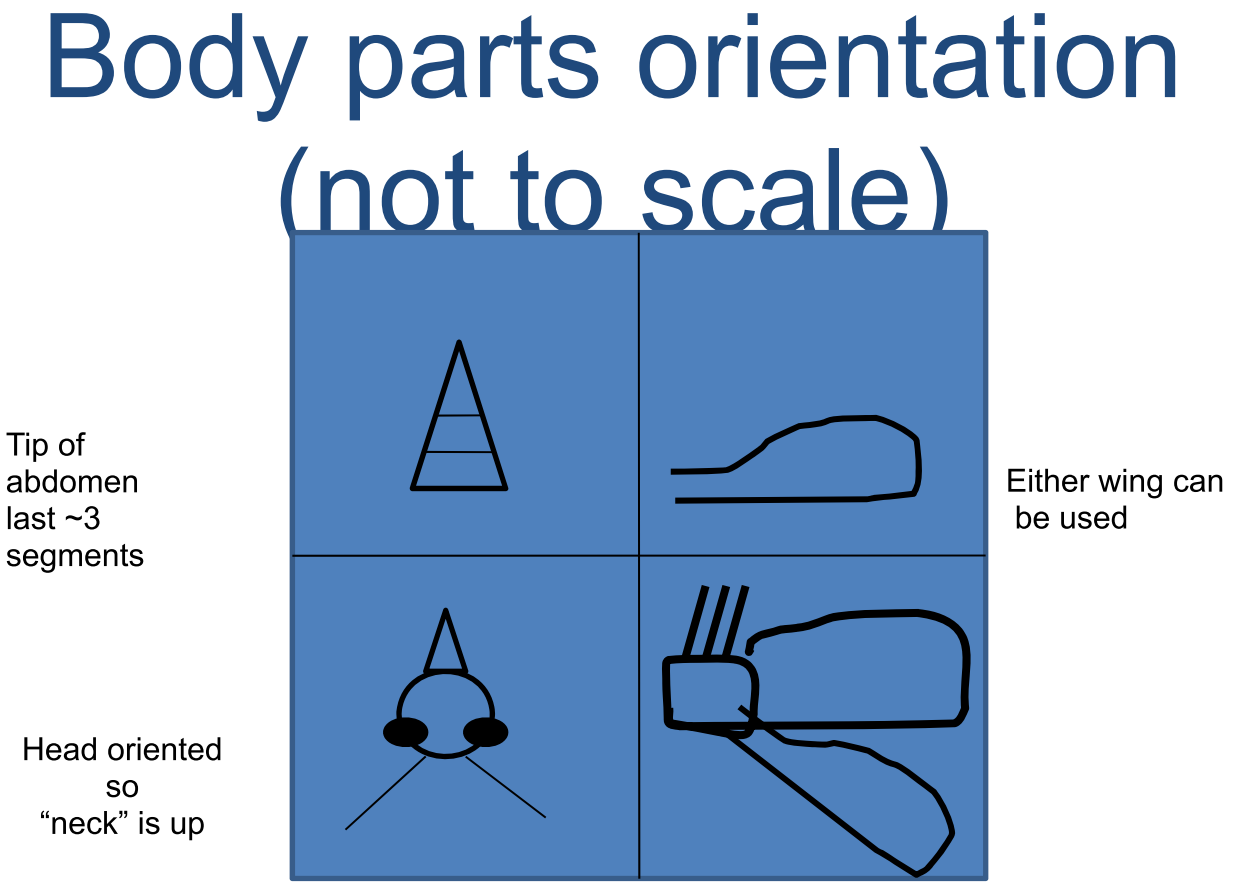


Figure 4.9.1. Orientation of sand fly body parts for slide mounting.

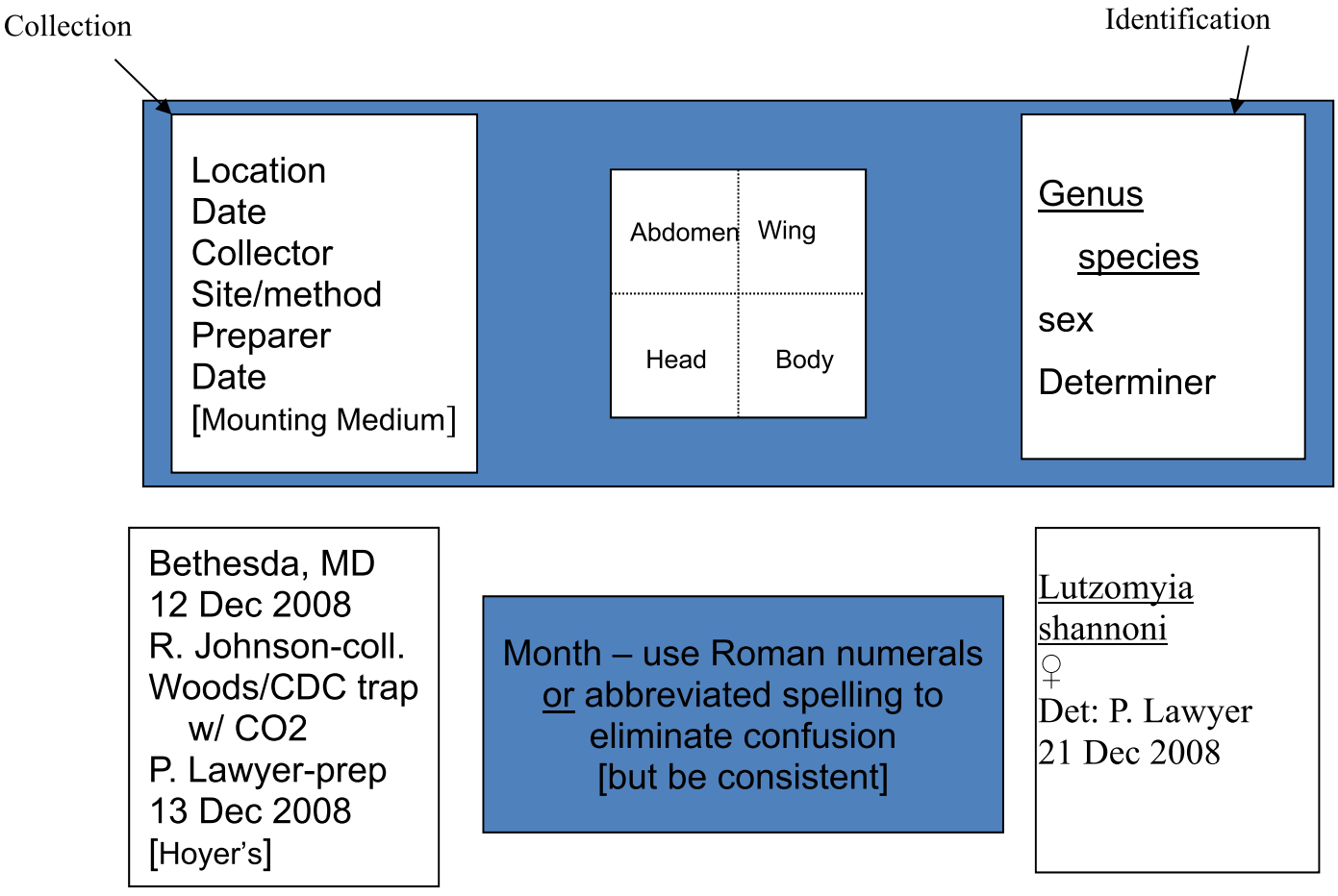


Figure 4.9.2. Proper labeling of slide-mounted specimens.

4.10 Dissection of Phlebotomine Sand Flies to Examine for *Leishmania*

Phillip G. Lawyer, Edgar Rowton, and Kathrine Westbrooke

Tools needed

- Dissecting pins – These can be made by inserting size 001 insect pins (BioQuip) into the end of a wooden applicator stick. Soften the end of the stick by soaking in water for an hour, then using a jeweler's forceps; drive the blunt end of the pin into the softened end of the applicator stick.
- Sterile microscope slides and cover glasses (18 x 18 mm)
- Soap solution in 15 ml cone-bottom tube for immobilizing the sand flies
- Beaker and fine-meshed screen
- Phosphate buffered saline (PBS,1x) or sterile saline for rinsing
- 1cc syringe of PBS
- Dissecting microscope
- Compound microscope

Procedure

1. Transfer flies to the tube of soap solution and shake gently

2. Pour contents of tube onto a fine-mesh screen stretched over the mouth of a large beaker and rinse with tap water until soap is gone.

3. Using a dissecting pin, lift the flies from the mesh screen and place them in a small Petri dish containing 1x PBS to rinse.

4. With the 1cc syringe, apply a drop of PBS onto the glass microscope slide

5. Place a freshly washed/rinsed sand fly in the drop of PBS on the microscope slide

6. With two dissecting needles (straight one in the left hand and bent one in the right hand), remove the sand fly's legs.

7. Steady the fly by piercing the thorax at the base of the wing with the straight pin and holding it against the glass. With the bent pin in the right hand, remove the sand fly's head.

8. Keeping the pin in the left hand in position to secure the fly, press the tip of the bent pin horizontally against the tip of the abdomen between segments 7 and 8 and pull firmly but slowly and steadily downward (toward you) until the gut comes out of the abdomen. If the end of the gut breaks off, place the bent segment of the pin horizontally across the anterior abdomen and squeeze the gut out of the abdomen like you would toothpaste out of a tube. Often the diverticulum (crop) breaks off and you must fish it out of the anterior abdomen.

9.Place a cover glass over the gut and examine the gut from one end to the other for parasites under a compound microscope.

4.11 Dissection of Phlebotomine Sand Flies for Salivary Glands

Phillip G. Lawyer, Edgar Rowton, and Kathrine Westbrooke

Tools needed.

- Dissecting pins – These can be made by inserting size 000 insect pins into the end of a wooden applicator stick. Soften the end of the stick by soaking in water for an hour, then using a jeweler's forceps, drive the blunt end of the pin into the softened end of the applicator stick.
- Sterile microscope slides and cover glasses (18 x 18 mm)
- Soap solution in 15 ml cone-bottom tube for immobilizing the sand flies
- Beaker and piece of fine-meshed screen
- Phosphate buffered saline (PBS,1x) or sterile saline for rinsing
- 1cc syringe of PBS
- Dissecting microscope

Procedure

1. Transfer flies to the tube of soap solution and shake gently

2 Pour the contents of the tube onto a fine-mesh screen stretched over the mouth of a large beaker and rinse with tap water until the soap is gone.

3. Using a dissecting pin, lift the flies from the mesh screen and place them in a small Petri dish containing 1x PBS to rinse.

4. With the 1cc syringe, apply a drop of PBS onto the glass microscope slide

5. Place a freshly washed/rinsed sand fly in the drop of PBS on the microscope slide

6. With two dissecting needles (straight one in the left hand and bent one in the right hand), remove the sand fly's legs. Steady the fly by piercing the thorax at the base of the wing with the straight pin and holding it against the glass. With the bent pin in the right hand, remove the sand fly's head.

7. When the head is pulled from the body, the salivary glands should come with it and will be visible at the back of the head.

8. Tease away the glands with the dissecting pins.

9. Aspirate the glands into a 1cc syringe with PBS and transfer to a small Eppendorf tube for storage.

Note: Sand Flies used for salivary gland dissection should be 7 days old in order to obtain the maximum amount of protein (Figure 4.11.1).

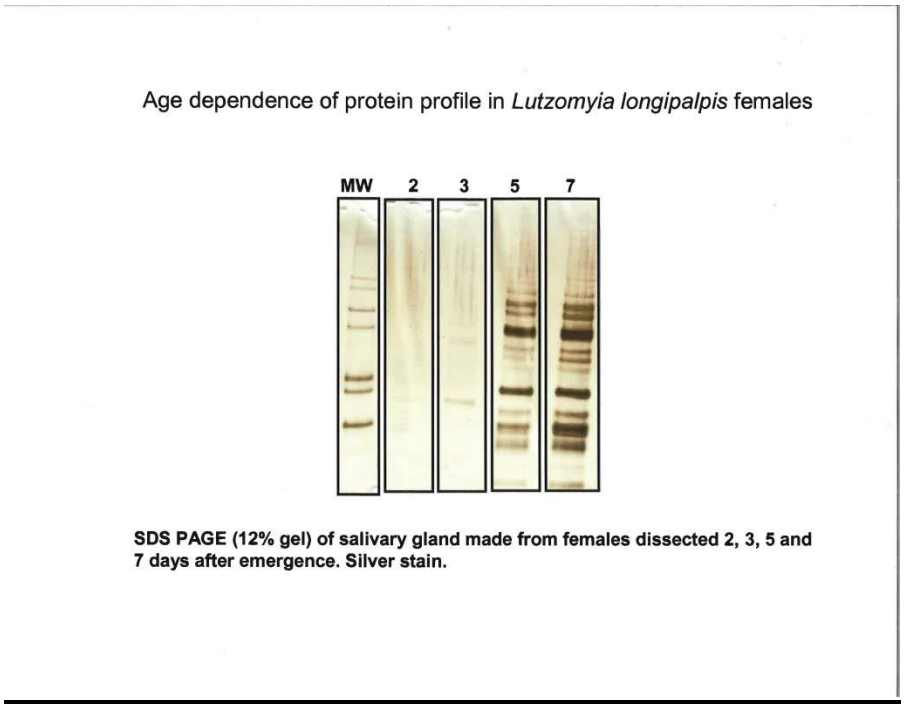


Figure 4.11.1. SDS Page gel showing protein profiles of *Lutzomyia longipalpis* salivary glands from sand flies 2,3,5, and 7 days post emergence. Photo: Jesus Valenzuela

4.12 Shipping Sand Fly Salivary Glands:

Claudio Meneses

Sand fly salivary glands can be shipped either in dry ice or as a dry pellet. In both methods the tube containing the glands is disrupted by ultrasonication (Brandson Sofiner 450) in 40 cycles (20 cycles to each side of the tube) for approximately 2 minutes. The tube is centrifuged for 3 minutes at 10,000g and the supernatant is collected and transferred to a clean nuclease free tube. Tubes can be concentrated, if needed, by merging their contents into one. The tube is accommodated in a box containing dry ice and rushed to its destination. Alternatively, the tube with supernatant can be placed in a speed vacuum centrifuge (model Eppendorf Vacufuge Plus) and spun down at 10,000g until a pellet is formed or until the liquid is completely died out. The centrifugation time will depend on the volume of supernatant to be dried. The dry tube containing the pellet is simply placed in a envelope and shipped out. There is no need to fill out complicated hazardous materials forms as required to ship dry ice goods since dry ice may pose a risk of explosion when it changes from solid to gaseous carbon dioxide in a seal tight container. (Protocol kindly provided by Dr. Jesus Valenzuela and Dr. Abdeladhim).

Note: Sand fly salivary glands also can be dissected, stored and shipped in RNA later. Although, they may look different in shape during dissection due to the acidic 5.2 pH of the RNA later solution.

Chapter 5 Identification

5.1 Recognition and Identification of Phlebotomine Sand Flies

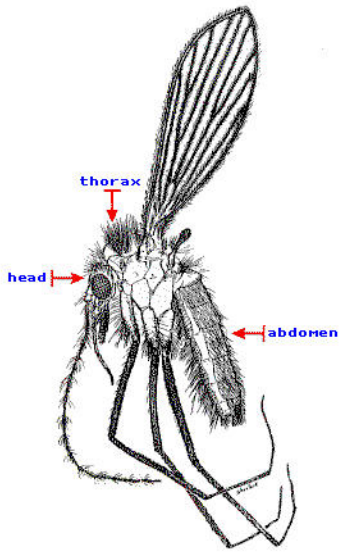
Phillip G. Lawyer, Edgar Rowton, and Kathrine Westbrooke

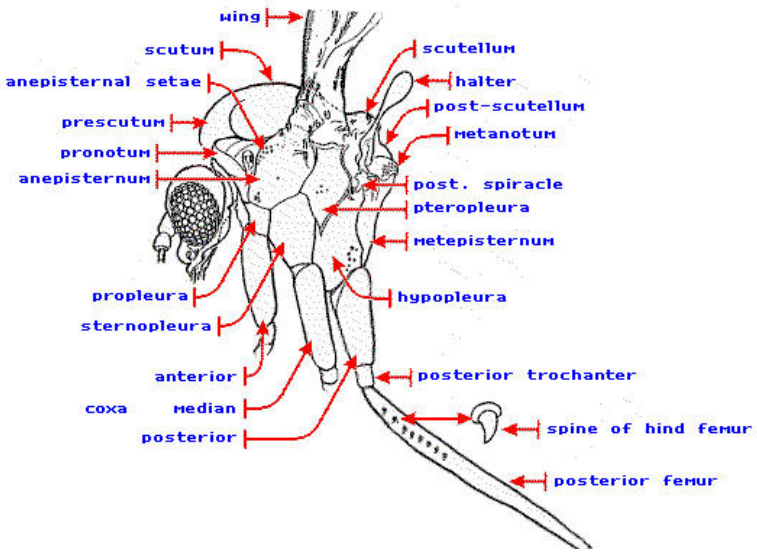
The purpose of this section is to familiarize you with the external and internal morphological features of phlebotomine sand flies to enable you to recognize them when you see them, separate the females from the males and, with the aid of various taxonomic keys, identify them to species.

1. IS IT A SAND FLY?

WITHOUT THE USE OF A MICROSCOPE

Sand flies are small (1-4 mm long), light brown to black in color and hairy insects with black eyes, long legs and almost erect wings.

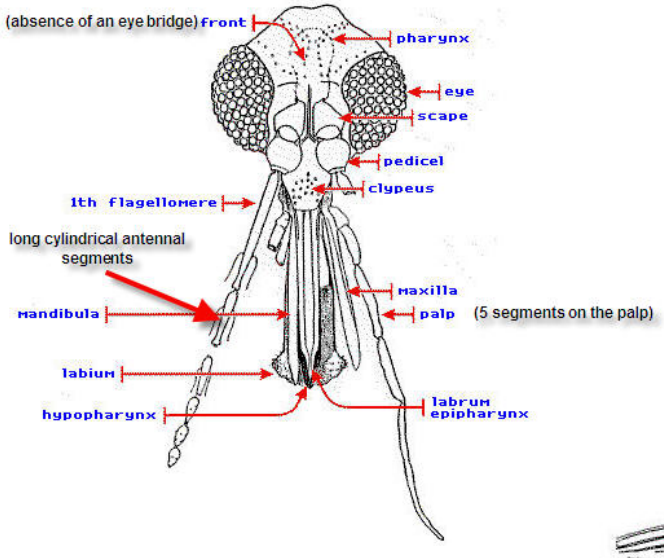


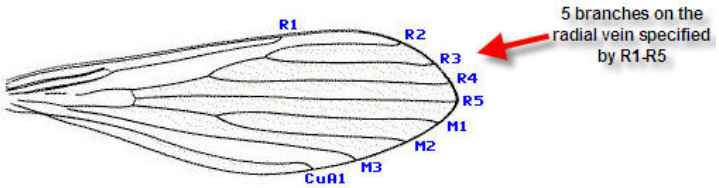


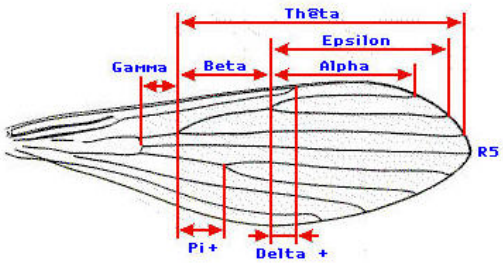
Lutzomyia longipalpis
In live specimens the wings are held in a V above the body as shown here.

The subfamily Phlebotominae is distinguished by

1. the absence of an eye bridge (eyes are separated)
2. presence of five-segmented palps
3. mouthparts at least as long as the head
4. antennal segments almost cylindrical
5. 5-branched radial vein on wing

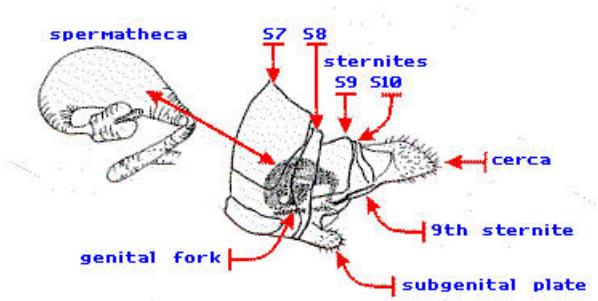




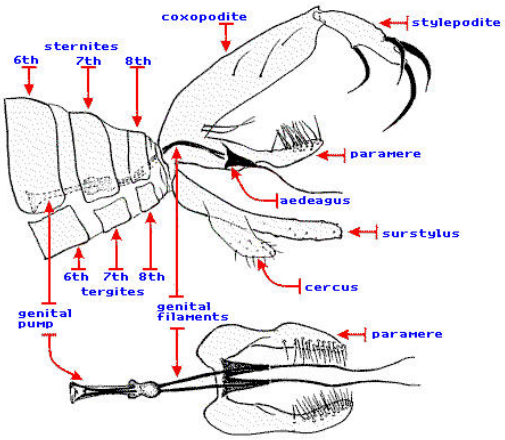


2. IS IT A MALE OR FEMALE?

If the posterior end of the abdomen looks blunt like this then it is a female. Females do not have external appendages on the tip of the abdomen. So, if you see any appendages…it's a male.



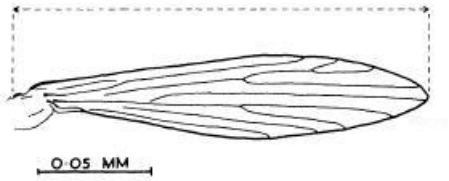
If the tip of the abdomen looks something like this then it is a male. The males have large terminalia (clasping structures) on the end of their abdomen. Not all male terminalia are as obvious as the examples below…often you'll need to look closely as they are often small, and folded shut like a pocket knife, especially in newly emerged males.



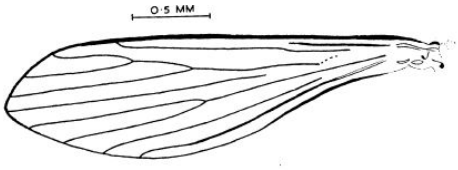
3. IS IT A FEMALE OF GENUS *PHLEBOTOMUS* OR *SERGENTOMYIA*?

USING THE DISSECTING MICROSCOPE

Wing Shape
Unless you slide mount the specimens the best way to separate female *Phlebotomus* from *Sergentomyia* is by wing shape.
Sergentomyia have narrow lanceolate wings (imagine that if you folded the wing in half from top to bottom the two halves would fit perfectly on top of each other).



Phlebotomus wings are broader and not symmetrical in comparing the top to the bottom of the wing.

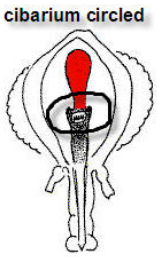


Body size and Integument color
Phlebotomus are usually larger bodied with a lighter integument (body color), while *Sergentomyia* are smaller with a darker cuticle. However, a given trap may have a variety of *Phlebotomus* and *Sergentomyia* spp. of different size and color so be careful when using these characters.

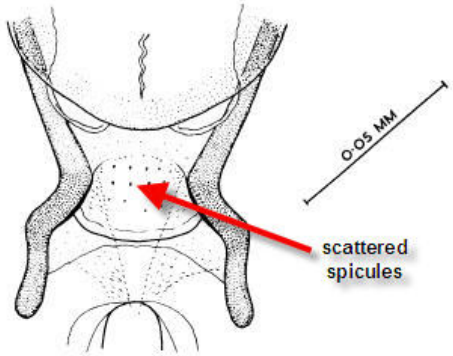
USING THE COMPOUND MICROSCOPE: SLIDE MOUNTING THE HEAD

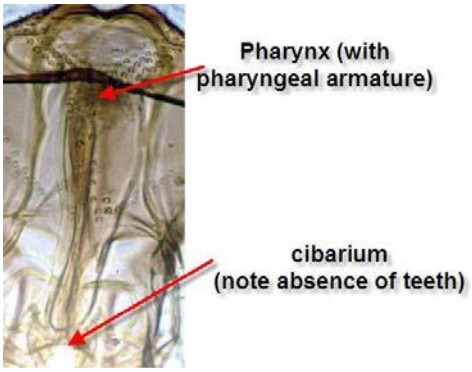
Occasionally you will come across a specimen with missing wings or with a wing that looks like it could be either *Phlebotomus* or *Sergentomyia*. One option is to slide mount just the head to identify to genus. Then the rest of the body can be used for DNA extraction.

Using the Cibarium and pigment patch for ID
The cibarium is an internal structure of the head that lies between the pharynx and the proboscis. The posterior part of the cibarium may have 2 or more horizontal teeth (=hind teeth) and vertical teeth (=fore teeth). The pigment patch represents the site of attachment of the posterior clypeus muscles on the dorsal wall of the cibarium.



In *Phlebotomus* the cibarium is unarmed (no teeth) or with scattered spicules without a pigment patch.





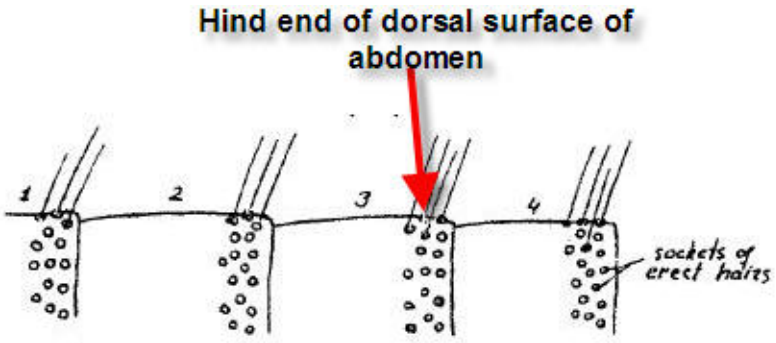
Cibarium of *Phlebotomus papatasi*

In *Sergentomyia* the cibarium has one or more rows of teeth (scarcely visible in *S. bailyi*) and the pigment patch is usually present.

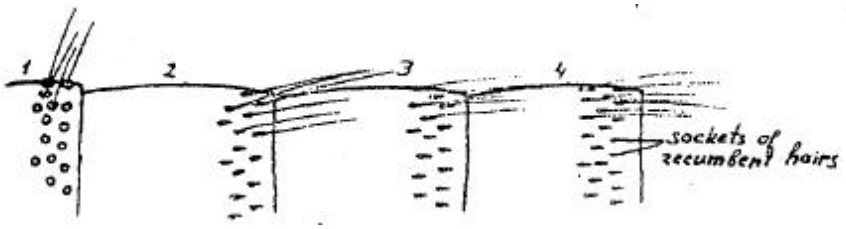
Cibarium of *Sergentomyia baghdadis* and *S. babu*

SLIDEMOUNTING THE ENTIRE BODY

Hairs and sockets on posterior margins of abdominal tergites
In *Phlebotomus,* hairs (setae) on the posterior margins of abdominal tergites 2-6 are erect, and their sockets are as large and round as on abdominal tergite 1.



In *Sergentomyia* generally, hairs (setae) on the posterior margins of abdominal tergites 2-6 are recumbent, and their sockets are much smaller than on abdominal tergite 1 and they are tear shaped. However, there are a few *Sergentomyia* species with erect hairs.

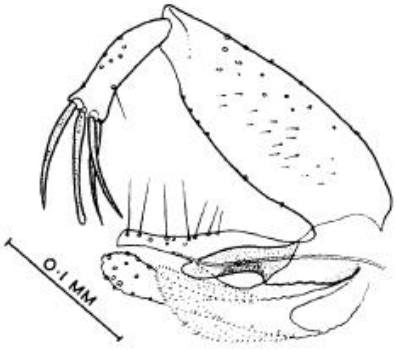


It is important to note that both *Sergentomyia* and *Phlebotomus* have large round sockets and erect hairs on abdominal tergite 1 and that anterior part of the tergite in both genera will have round sockets. Additionally," tergite" refers to only the dorsal (top) plate of the abdominal segment.

4. IS IT A MALE OF GENUS *SERGENTOMYIA* OR *PHLEBOTOMUS*?
WHAT SPECIES OF *PHLEBOTOMUS IS THE MALE*? (SOME COMMON EXAMPLES)

Old World male sand flies can be fairly easily identified to genus based on the arrangement and number of spines on their style (stylepodite). Because the style and spines are all part of the males external morphology, males can be identified using a stereomicroscope (dissecting scope) with a minimum magnification of 100X.

In male *Sergentomyia* the spines on the style are terminal and may vary in number from 4 to 5.

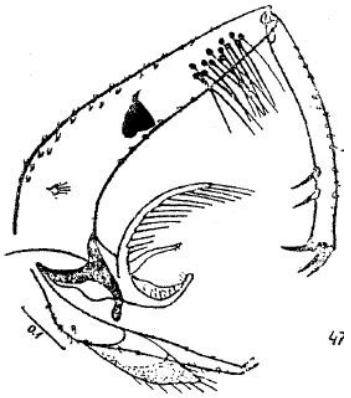


In *Phlebotomus* the style has 4 or 5 spines.
Following are three *Phlebotomus* species that may be found in the trap collections:
1. *P. papatasi*
2. *P. alexandri*
3. *P.sergenti*

Key characteristics for identifying males of these three species are below. Most *Phlebotomus* species are larger than *Sergentomyia* spp.

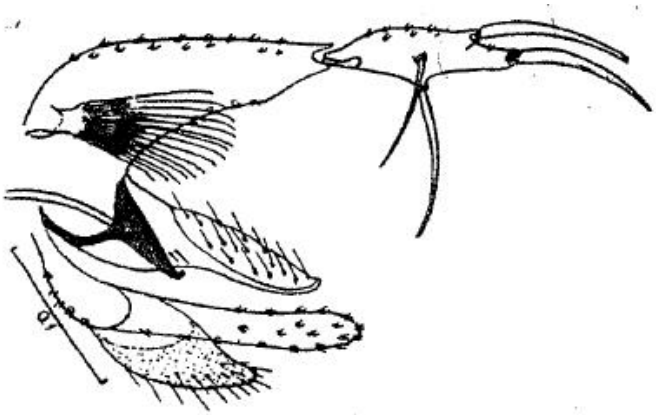
***Phlebotomus papatasi* (male)**

This is a big fly easily be identified with a dissecting microscope.



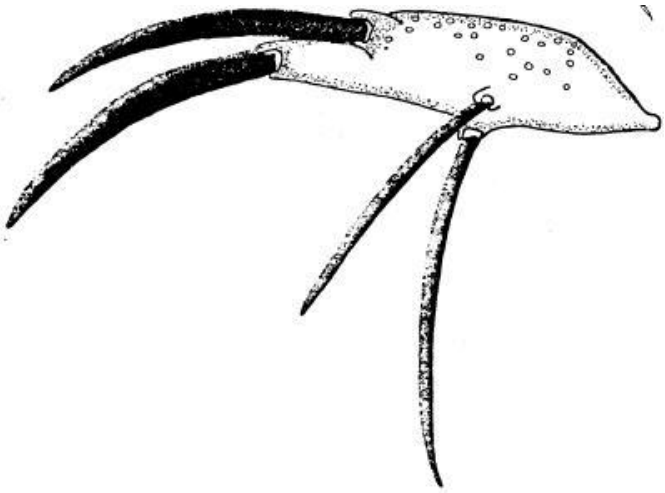
Basistyle long (0·37–0·63 mm.); basal process small, with few hairs. Dististyle long and cylindrical with 5 spatulate spines, 3 of them terminal. Paramere with 2 long dorsal processes. Surstyles with terminal spines . . . ***papatasi***

***Phlebotomus alexandri* (male)**
Note the offset alignment of the two terminal spines.

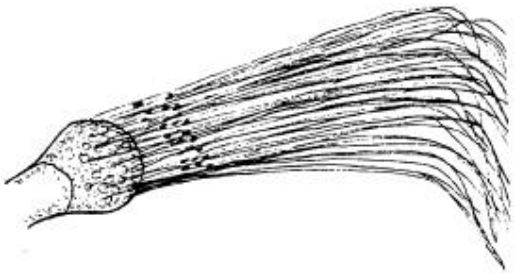


P. alexandri style

Photo of *P. alexandri* style



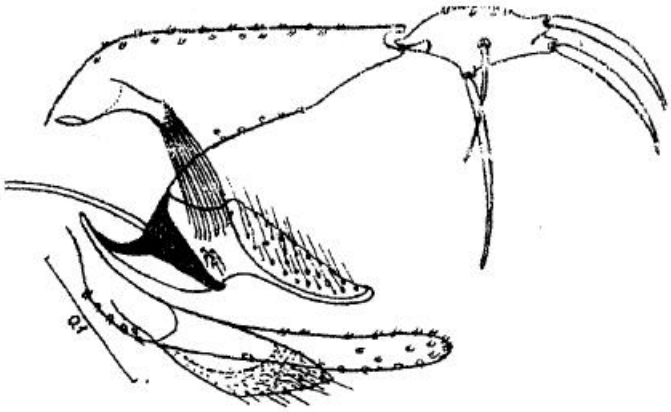
Another illustration of the style of *P. alexandri*

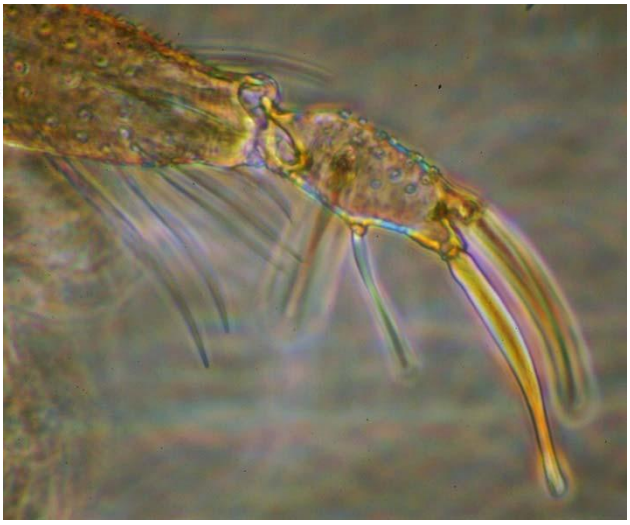


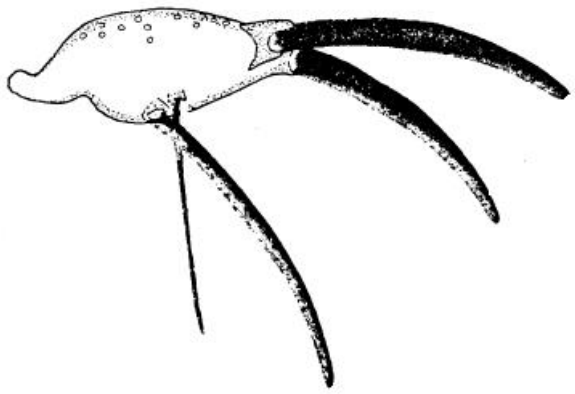
Basal process of *P. alexandri*

***Phlebotomus sergenti* (male)**

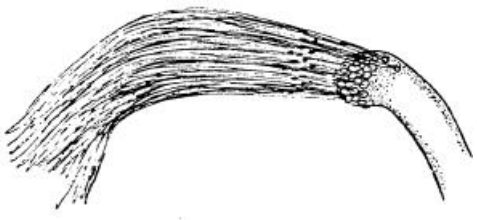
The arrangement of the style spines on *P. sergenti* is very similar to *P. alexandri,* but the two terminal spines are evenly aligned on *P. sergenti* while they are offset on *P. alexandri.*







Basal process of *P. sergenti*



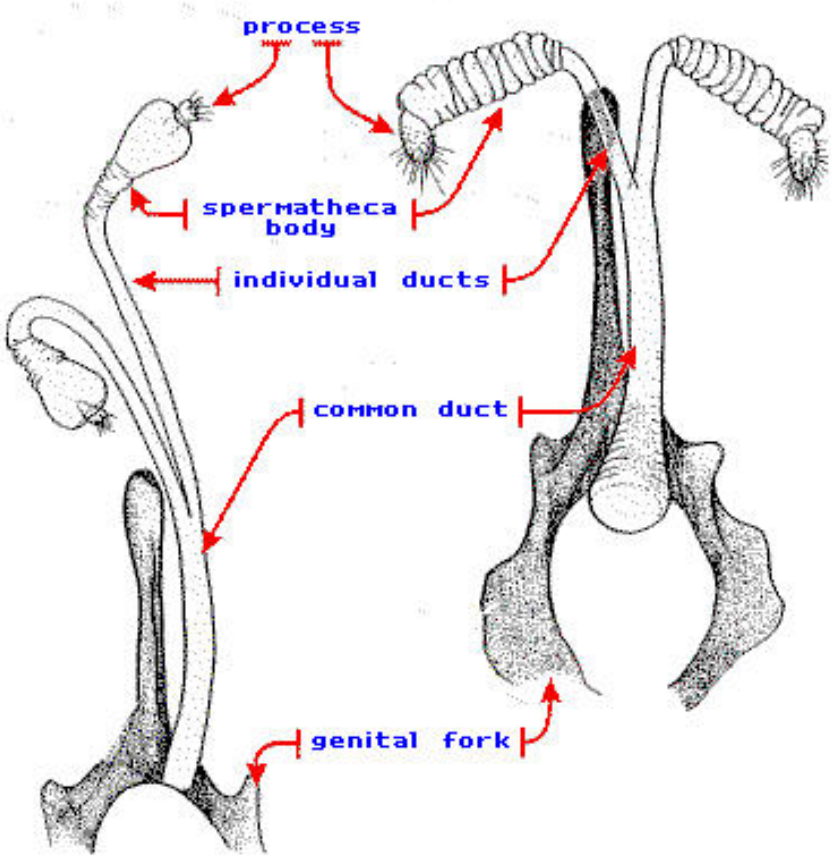
Another illustration of the style and spines of *P. sergenti*

5. What Species of Female *Phlebotomus*? (Some Common Examples)

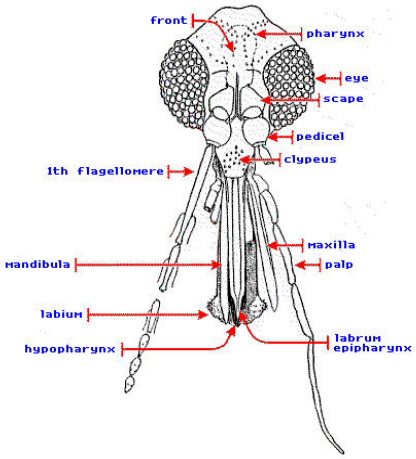
SLIDE MOUNTED FEMALES

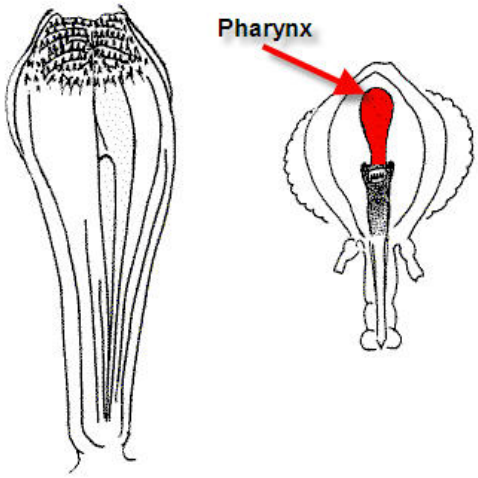
To identify female *Phlebotomus* to species we look at three main internal characters: (1) the spermatheca, (2) the pharynx, and (3) the cibarium. The females must be cleared and slide mounted for accurate identification.

1. **Spermatheca** - an organ of the female reproductive tract in insects. Its purpose is to receive and store sperm from the male, and it is usually the site of fertilization when the oőcytes are ready. The size, shape and other features of the spermatheca may vary depending on the species of sand fly.



2. **Pharynx**- an internal structure of the head. It is a posterior continuation of the cibarium and consists of two plates, one dorsal and one ventral. Usually it is bottle or lamp-glass shaped. The base of the pharynx may have spicules, scales, teeth, toothed lines or may be unarmed. Together, all these spicules and teeth are called the pharyngeal armature.

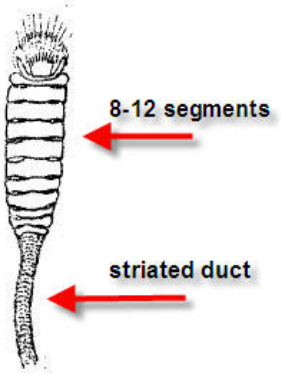


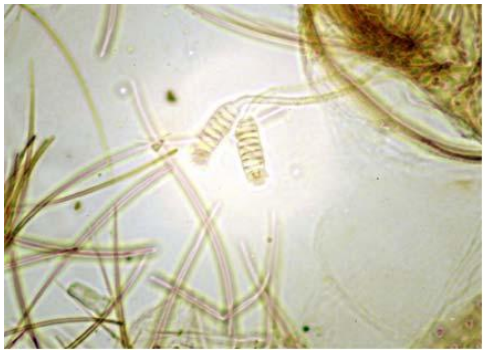


3. **Cibarium**- discussed previously in the section on differentiating *Phlebotomus* from *Sergentomyia.* The cibarium is difficult to use for species identification in *Phlebotomus*, but it may add additional evidence to the identification after using the spermatheca. As mentioned in the section on male identification, here are three species of *Phlebotomus* that are commonly found in the trap collections:
1. *P. papatasi*
2. *P. alexandri*
3. *P.sergenti*

The **spermatheca** tends to be the clearest character, but when in doubt use all three to verify the identity. A description of these key characters for each *Phlebotomus* female is found below. A brief description of the *Sergentomyia* spermatheca is also discussed.

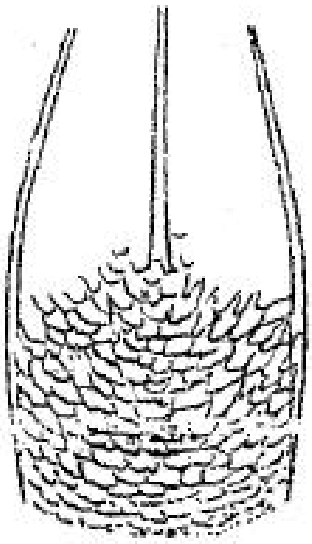
***Phlebotomus papatasi* (female)**
Spermatheca- cylindrical capsule consisting of 8-12 broad segments, those near the individual duct are slightly smaller; individual spermathecal duct striated.

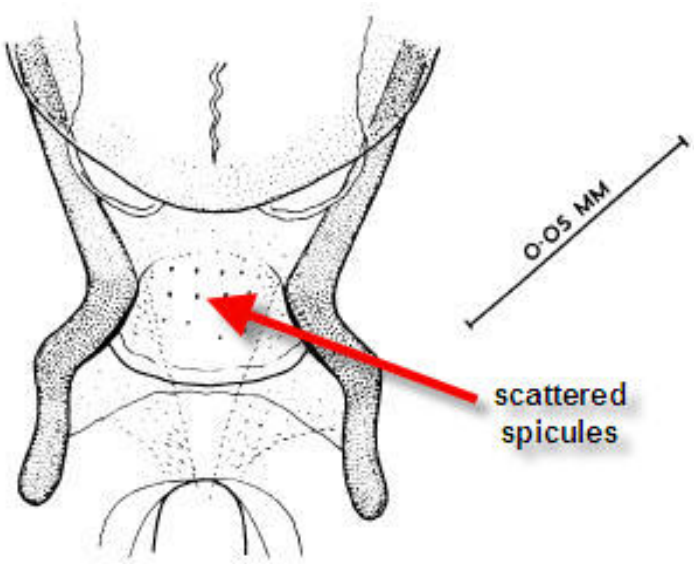




Pharynx- bottle shaped: armature occupying posterior half of wide part, composed of scale like teeth finely serrated at tips, densely arranged

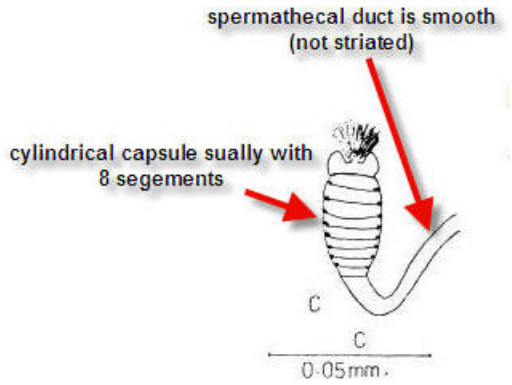
Cibarium- with many scattered minute ventral teeth and many lateral spicules

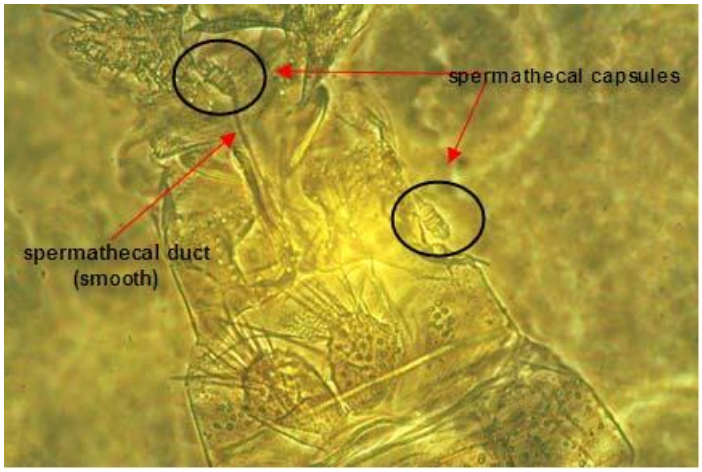




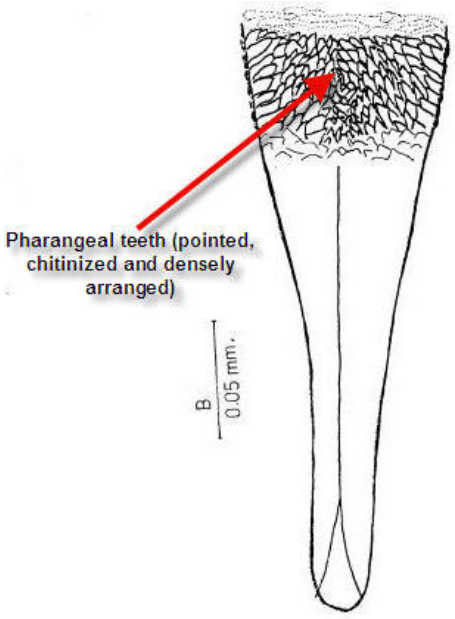
***Phlebotomus alexandri* (female)**

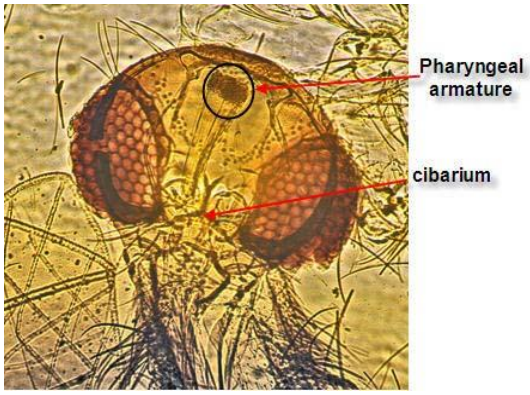
Spermatheca-spermathecal capsule cylindrical with 8 (6-10) short broad segments, terminal segment markedly enlarged; individual spermathecal duct smooth

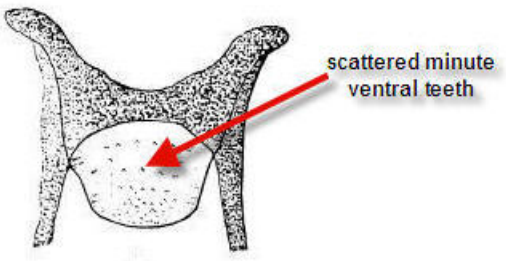




Pharynx- widens gradually posteriorly, with a straight base; armature occupying posterior 0.22 of pharynx, composed of numerous broad, smooth and pointed teeth that are well sclerotized and densely arranged with anterior teeth slightly separated, median teeth directed posteriorly, and lateral lateral teeth directed posterior-medially



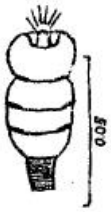




Cibarium- with numerous scattered minute ventral teeth, lateral spicules present

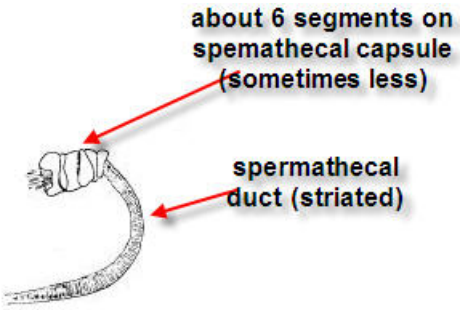
***Phlebotomus sergenti* (female)**

Spermatheca- spermatheca capsule short consisting of 4-6 segments, terminal segment enlarged, the segment nearest the individual duct small, spermathecal duct finely striated

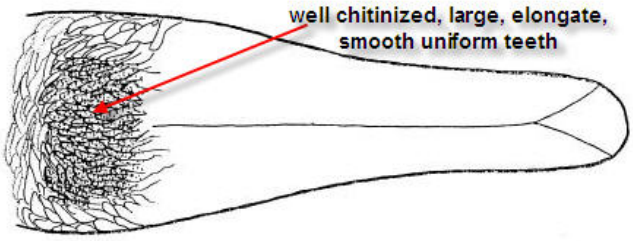


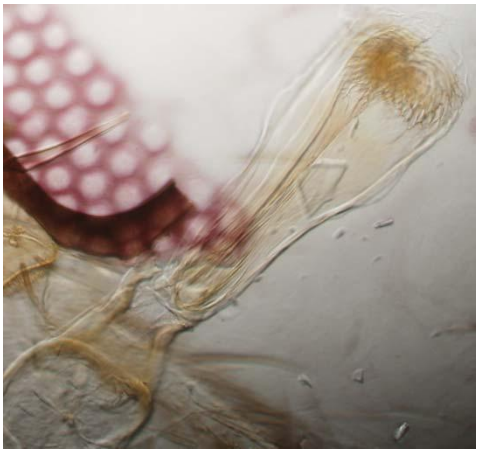
This illustration of the *P. sergenti* spermathecal capsule from the Afghan guide more accurately depicts the bulbous nature of the terminal (apical) annulation on the capsule.

This illustration of the *P. sergenti* spermathecal capsule is from the Iraq guide shows the striations on the duct. The capsule under desiccating conditions may collapse like this.



Pharynx- bottle shaped; armature occupying hind quarter of pharynx, composed of well-sclerotized, large, elongate, smooth and uniform teeth arranged medially, with punctuate ridges posteriorly.



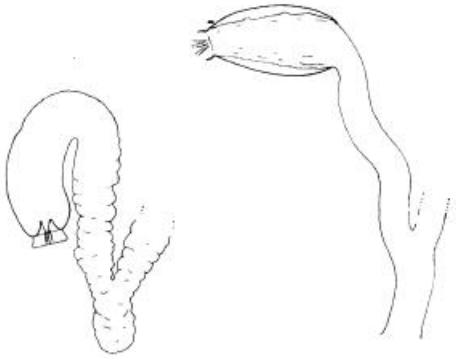


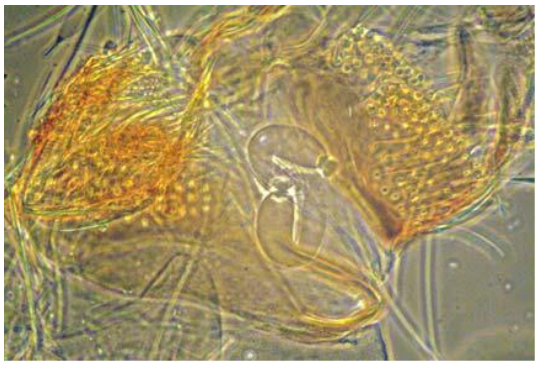
***Phlebotomus sergenti* (female) continued…**
Cibarium- with a few scattered minute ventral teeth, some lateral spicule

***Sergentomyia* spp.(females)**

Spermatheca- in most *Sergentomyia* the spermathecal capsule is not annulated.

They will look something like this:





Sergentomyia schwetzi

Pharynx-pharyngeal armature variable (I would not use this alone to identify)

Cibarium- previously discussed

Reference
El-Hossary S. 2006. Morphological characteristics for sandfly taxonomy [manual on the Internet]. Research and Training Center on Vectors of Diseases, Ain Shams University, Cairo, Egypt. Available from: afpmb.org/bulletin/vol26/181282.pdf.

5.2 Morphological identification/differentiation of Phlebotomus papatasi and Lutzomyia longipalpis

Tobin Rowland

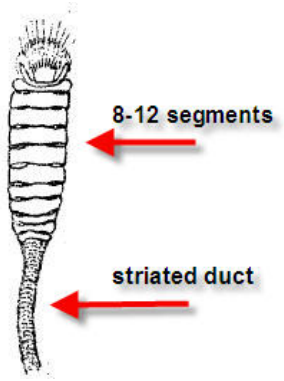
The two sand fly species currently available through BEI can easily be identified by the coloration of the fly. Phlebotomus papatasi is a light brown colored fly while Lutzomyia longipalpis is a dark almost black fly.

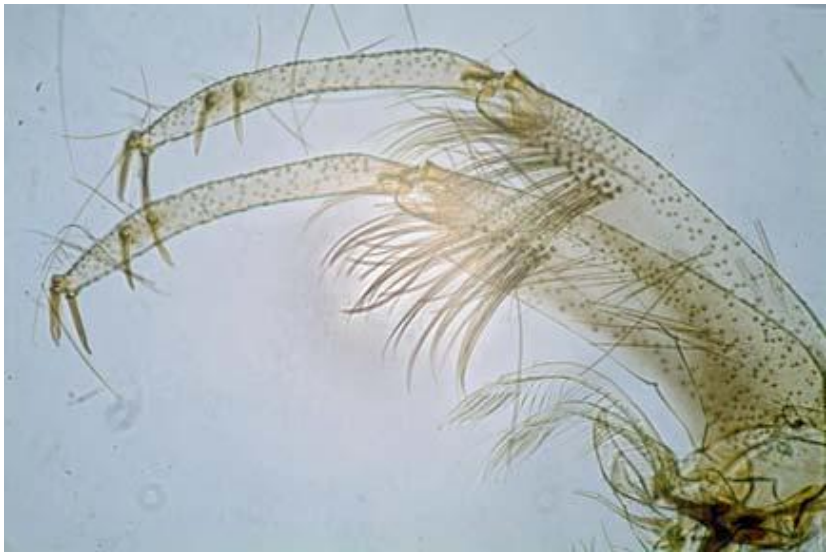
Dissections of both can be performed by removing the posterior (last 3) segments of the abdomen on a microscope slide in PBS under a dissecting scope. To make visualization of the male genatailia or the female spermathecal easier the fly can be placed in 10% lacto-phenol overnight prior to dissecting the abdomen. Place the fly on a slide in a few drops of polyvinyl alcohol or euparal for a permanent mount. Place a cover slip over the medium containing the last 3 segments of the abdomen (place carefully to ensure the segments do not drift to the edges). Place the slide on a compound microscope to visualize the spermatheca or male genatailia.

Male *Phlebotomus papatasi*

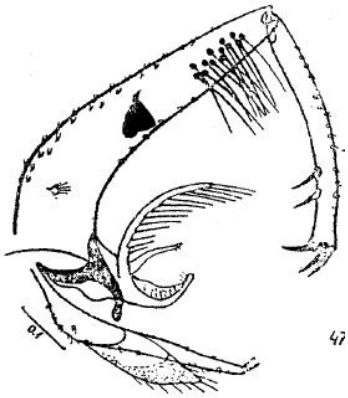
Female *Phlebotomus papatasi*

Female *Phlebotomus papatasi* spermatheca

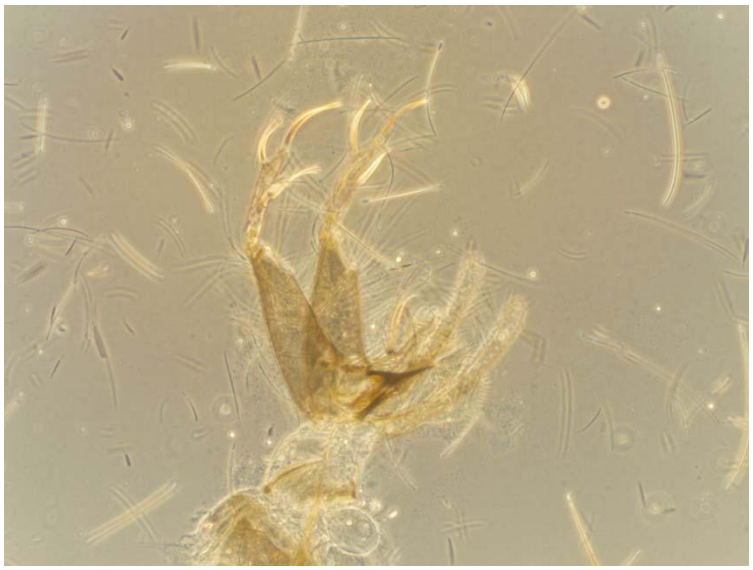




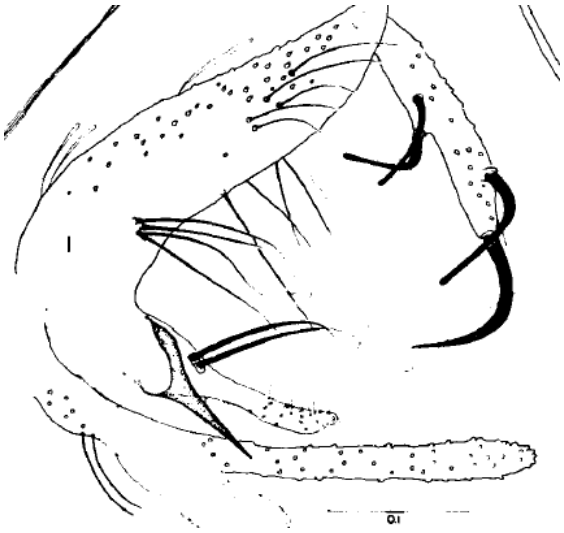
Male *Phlebotomus papatasi* terminalia



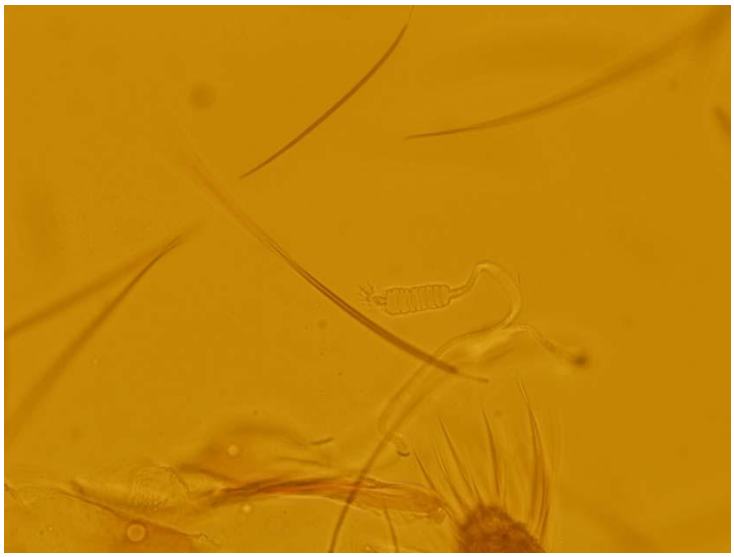
Male *Lutzomyia longipalpis*



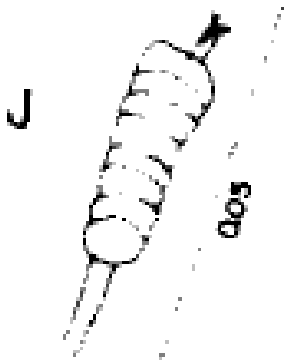
Male *Lutzomyia longipalpis* terminalia



Female *Lutzomyia longipalpis*



Female *Lutzomyia longipalpis* spermatheca



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Chapter 6 Resources

BEIResources Vector Resources
Sand flies can be acquired free of charge through BEI. Registration and account are required. Currently, *Phlebotomus papatasi* and *Lutzomyia longipalpis* are available. *Phlebotomus duboscqi*, *Phlebotomus sergenti*, and *Phlebotomus arabicus* will become available starting in 2016.
https://www.beiresources.org/Collection/28/Vector-Resources.aspx?f_vectorresources=Sand+Flies&page=1

Sand Fly Fellas Sand Fly Rearing Guide
Contains the following for sand fly rearing:
Forums
Videos
References
Contacts

https://www.vectorbase.org/forums/general-forums/methods-protocols-reagents/cd-sand-fly-fellas-sand-fly-rearing-guide

Walter Reed Biosystematics Unit/Sand Fly Identification Resources
Contains information and resources on:
Sand Fly Identification Keys
Sand Fly Morphology
Medically Important Sand Flies
Sand Fly Genera
Sand Fly Literature
Sand Fly Catalog
http://www.wrbu.org/VecIDResourcesSF.html

VectorMap
Contains information on sand fly collection records and distribution models
http://www.vectormap.org/sandfly.htm

United States Department of Agriculture Animal and Plant Health Inspection Service
The below link will take you to the USDA APHIS Permit page where information can be found to obtain an import permit for vectors.

https://www.aphis.usda.gov/wps/portal/aphis/ourfocus/animalhealth/sa_program_overview/!ut/p/a1/rVJJc4IwFP4tHnrEhEWWI66gUNs6tsKFCZElLRAMEeu_b2Q8dSq1M83tZb733rc8EIldCCvUkgxxQitUXOpQj5ZrR5HHUHEXG2sG3cfXuW96hrp2NAEIBGCysB3N8CCEmqlAdzp2poblQ-jq9_XDG8-G3_oXs7Hon3tPxmqpwGcNvIEQhLjiNc9BgOqcNBGmFU8qHhUkZoidH2CDInpkUUrxsekqVJESFVGeoILn3U_NaMZQGdE2YS1JTpepNSZ7EKiKKst7Q5diGaeSpui6ZMUWlFQVxxhZIyxjdFXRQ_MXFzoVPRNWoyugz-gO0ONkIEgaN1lYGtj8UfXyjnAV5k_8TIxFPJdIlVKw-8lugSTvh0NoizAv8X1ysPvHNAXTrKBxd9CBXcWqKSixJE1YwoY5bcS2ph6ekmG3c5jRFtTldlua6ln6eDGhOipaL_UbezD4AuUT_wY!/?1dmy&urile=wcm%3apath%3a%2Faphis_content_library%2Fsa_our_focus%2Fsa_animal_health%2Fsa_import_into_us%2Fct_organisms_and%2B_vectors

Failure to supply all applicable information can delay the processing of this application. **PLEASE TYPE OR PRINT CLEARLY**

No controlled material, organisms or vectors may be imported or moved interstate unless the data requested on this form is furnished and certified (9 CFR 94, 95, and 122).

According to the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number. The time required to complete this information collection is estimated to average .0166 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

The valid OMB control number for this information collection is 0579-0015, 0094, 0183, 0213, and 0245.

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
VETERINARY SERVICES
National Center for Import-Export, Products Program
4700 River Road, Unit 40
Riverdale, MD 20737-1231

APPLICATION FOR PERMIT TO:

IMPORT OR TRANSPORT CONTROLLED MATERIAL OR ORGANISMS OR VECTORS

1. MODE OF TRANSPORTATION *(Please "X"):*
☐ AIR ☐ SEA ☐ LAND ☐ ANY

2. U.S. PORTS OF ENTRY

3. IMPORTER *(Name, organization, complete address, telephone and fax number of individual who will receive and be responsible for the imported material)*

4. SHIPPER(s): *(Name and Address of producer/shipper)*

5. DESCRIBE THE MATERIAL TO BE IMPORTED *(Provide the following information, as applicable: Animal species and tissue of origin of animal product, country of origin of the animals from which the raw animal product was sourced, processing country, recombinant system and genetic inserts, antibody immunogens, stabilizers, nutritive factors of animal origin in media.) (COMPLETE VS FORM 16-7 for cell cultures and their products.)*

6. QUANTITY, FREQUENCY OF IMPORTATION, AND EXPECTED COMPLETION DATE *(estimate)*

7. PROPOSED USE OF MATERIAL AND DERIVATIVES *(Also, for animal pathogens or vectors, describe facilities/biosafety procedures)*

8. IF FOR USE IN ANIMALS, **SPECIFY** THE ANIMAL SPECIES

9. TREATMENT OF MATERIAL **PRIOR** TO IMPORTATION INTO THE U.S. *(Processing/purification methods, including time at specific temperatures, pH, other treatments, disease safeguards, etc.)*

10. METHOD OF FINAL DISPOSITION OF IMPORTED MATERIAL AND DERIVATIVES

I CERTIFY AS AUTHORIZED BY THE COMPANY/INSTITUTION THAT I REPRESENT, THAT THIS MATERIAL WILL BE USED IN ACCORDANCE WITH ALL RESTRICTIONS AND PRECAUTIONS AS MAY BE SPECIFIED IN THE PERMIT.

11. SIGNATURE OF APPLICANT

12. TYPED NAME AND TITLE

13. DATE

14. APHIS USER FEE CREDIT ACCOUNT NO. OR METHOD OF USER FEE PAYMENT *(for VISA or Mastercard include number and expiration date).*

VS FORM 16-3 (NOV 99)

Print Clear Form

VS Form 16-3 can be printed and mailed or filled out via e-permits. Please go to the website to fill out this form.

Supply List

Item	Vendor	item number
Mouth aspirators	John Hock Company	612
125 mil wide-mouth polycarbonate jars with vented lids – Nalgene®	VWR	16129-356
1-pint paper cans	Science Supplies	300
500 mil wide-mouth polycarbonate jars w/vented lids – Nalgene®, 16/case	VWR	16129-390
Autoclave tape/masking tape	Fisher Scientific	11-889-14
Bench top paper for cages	Kimtech Science	7546
Colored label tape	Fisher	Varies depending on color
Composting cabinet for making sand fly food– custom made	Percision Plastic	N/A
Cotton balls	Fisher Scientific	22-456-880
Dental dam	Henry Schein	101-0171
Erlenmeyer flasks and tubing for making aspirator (1000ml)	Fisher	07-250-098
Heavy duty rubberbands (several packages	VWR	500024-286
illuminators (fiber optic light with ring guide)	LW Scientific	ALPA-1502
Incubators/environmental chambers	Caron	Insect Growth Chambers
Minuten pins for dissecting needles	Bioquip	1208S
Nylon organdy fine-mesh screen – 1 bolt (100 yds)	Jo-Ann Fabric and Craft Store	6647242
Paper towels	Uline	S-17461
Pasteur Pipet	Fisher	13-678-20B
Plaster of Paris/dental plaster, 25-lb boxes	Henry Schein	1450025HS
Portable vacuum pumps for operating vacuum aspirators	Gast	
Rabbit Feces	Spring Valley Labs	N/A
Rabbit food	Quality Lab Products	5-P25
Sand Fly Cages	Percision Plastic	N/A
Sharpie marking pens	OfficeMax	10014156
Sodium hypochlorite (Chorox®)	Amazon	N/A
Soil sieves	Hogentogler	8407 (pan) 1309 (sieve)
Spatulas	Lowes	422618
Sponges	Fisher Scientific	14-417
Spray bottles	Fisher Scientific	01-189-100
Stockinette sleeve for 12" cages	Promed	855801-NS508

Sugar	**Netgrocer**	**Shoprite granulated sugar 4lb**
Trays for holding oviposition/rearing pots (white rubbermaid tub)	**Nationwide facility supplies**	**rcp3506 (tub), rcp3506 (lids)**
Tubing	**Fisher**	**14-169-7C**
Tubs for holding early stage larva pots and larval food	**B&H**	**CETP1417 (14X17) CETP1114 (11X14)**
Vacuum aspirator for multiple containers sizes –custom made	**Percision Plastic**	**N/A**
Vacuum aspirators for 500 ml wide-mouth oviposition pots – custom made	**Percision Plastic**	**N/A**
WaterPik Oral Irrigator	**Waterpik**	**WAT WP-60 WP-60**
Wooden applicator sticks	**Fisher Scientific**	**50-949-154**

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