Establishment and maintenance of sand fly colonies

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ABSTRACT: Sand flies used to have a reputation for being difficult and labour-intensive to breed. Here we summarize our experience with establishment and maintenance of sand fly colonies and their use for infective experiments: techniques for collection and handling wild-caught females, rearing larvae and adults and experimental infections of sand flies by Leishmania using membrane feeding. In addition, we compare major life cycle parameters between various colonies maintained under standard laboratory conditions. The sand fly rearing is tricky but some species can be reared in large numbers with a minimum of space and equipment. Initiation of new colonies from endemic sites is a prerequisite for accurate studies on parasite-vector interaction but it is more difficult step than routine maintenance of colonies already established in laboratory for many generations. ***Journal of Vector Ecology* 36 (Supplement 1): S1-S9. 2011.**

Keyword Index: Phlebotomus, Lutzomyia, Leishmania, life cycle.

INTRODUCTION

Laboratory colonies are essential for studies on sand fly biology and sand fly-*Leishmania* interaction. A variety of techniques has been described for rearing these insects in laboratory conditions (reviewed by Hertig and Johnson 1961, Killick-Kendrick and Killick-Kendrick 1991, Modi 1997). A detailed bibliography on this subject is given by Killick-Kendrick et al. (1991).

Of about 700 known sand fly species, fewer than five dozen have been colonized in the laboratory so far, but only a few of them have been successfully reared continuously in large numbers to provide sufficient material for experimental work. Such work is essential for understanding sand fly physiology, epidemiology of leishmaniases, and parasite-vector-host relationships. Sand fly species and even colonies of different geographical origin vary in many biological and physiological parameters, including saliva composition (Warburg et al. 1994, Volf et al. 2000). There are also significant differences in susceptibility for *Leishmania* parasites. Certain species, for example *P. papatasi* and *P. sergenti,* are termed “restricted” (Kamhawi 2006) or “specific” (Myskova et al. 2007) vectors, as they allow the maturation and transmission of a single *Leishmania* species. The others tested so far seem to be permissive as multiple *Leishmania* species are able to mature within their gut (for review see Kamhawi 2006, Volf and Myskova 2007). However, for the majority of sand fly species, there are no data on susceptibility to *Leishmania* yet. Therefore, any new colony is a treasure and we like to encourage medical entomologists to colonize sand flies from nature.

The aim of this report is to summarize our experiences with the establishment and maintenance of sand fly colonies and their use in infective experiments: techniques for the collection and handling of wild-caught females, rearing larvae and adults, and experimental infections of sand flies by *Leishmania* using membrane feeding. In addition, we summarize data obtained by long-term maintenance of sand fly colonies in our laboratory and compare major life cycle parameters among various colonies under standard conditions.

COLLECTION IN THE FIELD

Sand fly adults of both sexes can be collected by several methods, either while resting during the day or foraging at night. Sampling from resting sites, like walls of houses, animal dwellings, caves, and tree holes, can be done by several forms of mouth aspirator; similarly, active catches with an aspirator can be done from bait animals or on human bait. For a review of sampling methods see Alexander (2000).

The dimensions of each component of an aspirator can be varied according to preferences of the collector. Sand flies are fragile and therefore the body of the aspirator should be wider than the opening. On the other side, aspirators are closed with a piece of fine mesh gauze or sinter filter. The plastic aspirators are light and strong but could damage sand flies because of static electricity. Therefore, the glass ones could be recommended for most studies in the laboratory. However, they are not commercially available and must be made by a glass blower. Working with a large number of sand flies, we favor a reservoir type, with the body wider than the entrance (Figure 1a). It consists of a tube sealed at one end by rubber, except for a narrow tube through which the sand flies enter. This makes it difficult for the insects to escape and helps capture more specimens without the necessity to transfer them into a cage or other type of container. Emptying the reservoir aspirator may involve dismantling it within a cage.

One potential problem is that sand flies shed large numbers of hairs which are inhaled by the collector and can cause respiratory problems. Also, resting sites involve dark and humid microhabitats with potential danger of fungal and bacterial infections, such as histoplasmosis or Q-fever caused by *Coxiella burnetti*. Therefore, some authors

introduced a filter to prevent the inhalation of dust, hair, and microorganisms (Warburg 1989) or introduced the condom barrier (Tang 1996). Hand-held power-operated aspirators are also commercially available (see for example, Higgs and Beaty 1996) and we advise they be used in the field for sand fly collections from crevices, stables, or even from traps.

Light traps are used extensively in field studies of sand flies. The most widely used type, battery-operated light-suction traps of the CDC type, can be left overnight to collect sand flies. The distance at which these traps are attractive to sand flies appears to be not great; Killick-Kendrick et al. (1985) estimated a maximum distance of only 2 m for *P. ariasi*. However, in resting or breeding places like stables, cellars, caves, or the entrance of burrows, sand fly population densities are high which results in good catches. As with mosquitoes, trapping methods using animal baits or carbon dioxide preferentially sample host-seeking females. Those involving light tend to yield a higher proportion of gravid females and males. However, in some sand fly species, like *Lutzomyia longipalpis* or *P. argentipes*, males are readily attracted to the hosts as mating sites (Lane 1993).

It is advisable to collect traps early in the morning and take out the sand flies with an aspirator as soon as possible before they start to suffer from heat and desiccation. In addition to sand flies, the light trap also collects various other insects and spiders, some of which may eat the sand flies or damage them. Similarly, overcrowding and leaving sand flies too long in the aspirator increases their mortality.

INITIATION OF THE COLONIES

Initiation of new colonies is a more difficult step than the routine maintenance of colonies already established in the laboratory for many generations. Therefore, it is advisable to get expertise by first maintaining an "easy" colony like *P. papatasi* or *L. longipalpis*.

Sand flies immobilized by cold or CO_2 are sorted under the stereomicroscope according to sex and physiological state and gravid females intended for the establishment of colonies are transferred into plaster-lined plastic pots used for larval rearing (see below). When this is not possible, blood-fed females visible to the naked eye could be selected and transferred from the traps using mouth aspirators. The size of the pot should consider the number of gravid females and prior to the placement of the sand flies the pot must be moistened with water. Pots are kept in plastic rectangular boxes with tight-fitting lids. High humidity is ensured by a layer of moistened filter paper or tissue in the bottom of the box. Females are provided with a sugar source such as a fresh apple slice or a cotton wool pad saturated in 10-50% sucrose solution or honey. For most species, the optimum temperature is between 24-28° C. Pots in boxes are also useful for the transporting of flies, but cooler conditions (15-20° C) during transportation is recommended.

Non-gravid and unengorged sand flies are first transferred into a suspended nylon cloth cage used for rearing the adults (see below) and offered a blood meal. Cages are enveloped in plastic bags, again with a piece of damp cotton wool or tissue on a Petri dish. Females can be fed on a variety of vertebrates, with the choice of animal dependent on its local availability and the species of sand fly. *L. longipalpis* or *P. papatasi* are opportunistic and readily feed on mice, while the others, like *Larroussius* or most *Adlerius* species prefer hamsters or rabbits. Anesthetized mice or hamsters are placed into the cage for about 1 h. The best results we obtained using anesthetization by combination of ketamin and xylazine (10 mg and 2 mg per 100 g body weight, respectively). The presence of sand fly males may improve the engorgement rate. Since females prefer feeding in the dark, the cage is covered with a dark tissue during feeding. After feeding, females are left undisturbed for 12-24 h, mainly for complete formation of the peritrophic matrix, which makes their gut less fragile. Details on formation of the peritrophic matrix and differences between various sand fly species are summarized by Sadlova and Volf (2009). After laying the eggs, females die or could be killed for species identification. Pots with eggs are then transported to the laboratory.

Catches often consist of mixed species and therefore the first laboratory generation must be sorted according to species. This step is time-consuming and laborious but in favorable conditions it can be done even with wild-caught sand flies before transportation. The technique we used is adapted from Killick-Kendrick and Killick-Kendrick (1991). Gravid females are tubed individually in small glass vials with a piece of folded filter paper inside. The mouth of the vial is closed with gauze and a plastic cap with a hole in the center. A tiny piece of cotton wool soaked in sucrose solution is put on the gauze and changed daily. The vials are kept in containers lined with filter paper or tissue moistened by distilled water. Females are encouraged to lay eggs by moistening the folded filter paper inside the vials with few drops of distilled water using the syringe with needle. With engorged females the filter paper inside vials is not moistened until after defecation when yellow, brown or black spots appear on filter paper or inner surface of vial.

Vials are checked for oviposition twice a day and females that laid eggs are removed (either dead or still alive). The terminal segments of females should be detached and placed in a drop of saline for immediate identification under the microscope (preferably by phase-contrast) or mounted in a drop of Berlese´s fluid and identified within a few days. The vial, labeled with date and identity of female, is kept for 2-4 days after the date of oviposition as during early period of embryonic development the eggs should not be handled. Then, eggs of the same species are transferred to plaster-lined pots using a fine brush or by washing with distilled water. Again, the diameter of the pot used depends on the number of eggs.

REARING THE COLONIES

Colonies are maintained either in a room with controlled temperature or in an incubator at 25 to 28° C.

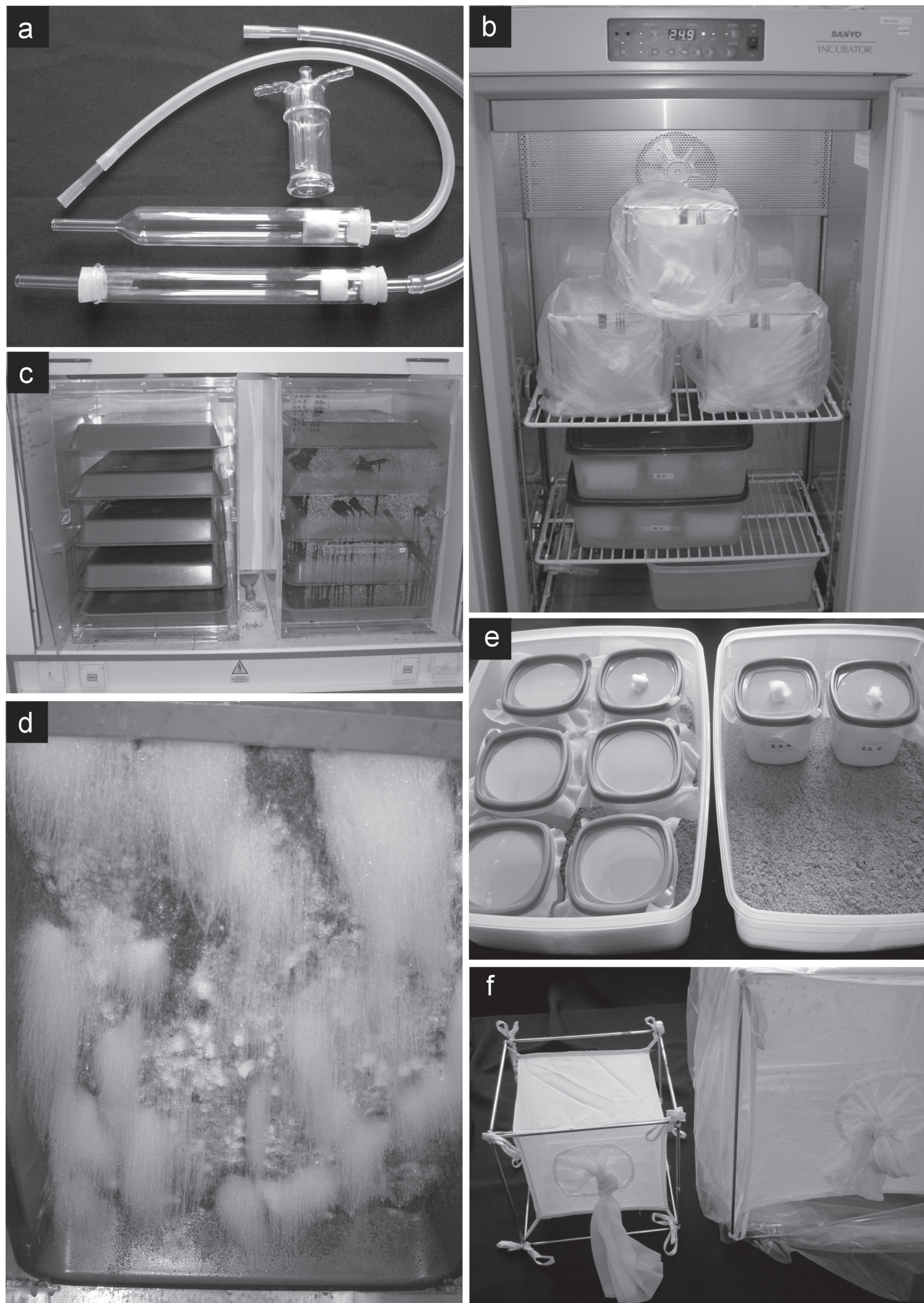

Figure 1. Equipment used for sand fly maintenance. **a:** two different types of glass aspirators and the glass feeder used for membrane feeding. **b:** boxes with larval pots and cages wrapped in plastic bags are maintained in incubator. **c:** two cabinets (one with front panel fitted) for fermentation of larval food. Up side down position of plastic trays ensures aerobic conditions during fermentation process. **d:** detail of larval food during composting process. Long, hair-like mycelia are evidence of optimal fermentation conditions. **e:** larval pots with a layer of plaster of Paris on sides and bottom are closed on the top with a snap cap and fine gauze. Pots are maintained in plastic boxes with a bottom filled with layer of fine sand. **f:** nylon cloth cages of different size are suspended on the steel frame (the larger cage here is enveloped in a plastic bag).

Most colonies in our laboratory are maintained at 25-26° C. Photoperiod is probably not a crucial factor but 14:10 (light:dark) is used in many laboratories. High humidity (70-95%) is ensured by wrapping cages in plastic bags with wet tissue or cotton wool inside (Figure 1b), the surrounding humidity in the insectary or incubator (60-70%) can be ensured by commercially available humidifiers.

For larval rearing we used different types of pots from a solid plastic with a big hole cut in the bottom and walls roughened with sand paper. The bottom is filled with a 1 cm thick layer of white plaster of Paris and, when set, a second very thin layer is plastered on the sides. Plaster ensures the humidity in the pot and provides a resting surface without water condensation. The pot is closed with a fine gauze and snap-cap or screw cap lid, the center of which is cut out (Figure 1e). The gauze should be fine enough to prevent the escape of larvae. Females are introduced through a small hole in the gauze using a aspirator (or tweezers if anesthetized) and the hole is then plugged with a cotton wool pad. We use three different types of pots, the smallest one (diameter 6 cm) for up to 20 gravid females, and the largest one (diameter 14 cm) for 100-150 gravid females. This corresponds to about 1 cm^2 of the bottom per female. Size of the pot is important mainly for larval development. Some colonies, like *L. longipalpis*, develop well in "crowded" pots while the others, like *P. arabicus*, prefer less-crowded conditions.

At 25-26° C, eggs are laid usually six to ten days post blood meal (Table 1). The length of the gonotrophic cycle is affected mainly by the speed of blood digestion, which varies within species (Volf and Killick-Kendrick 1994) and also depends on external factors, such as temperature (Benkova and Volf 2007) and blood meal source (Michalsky at al. 2007, Sant´Anna et al. 2010). In our colonies, *L. longipalpis* laid eggs usually on days 6-8 post blood meal, while *P. duboscqi*, *P. halepensis* and *P. tobbi* laid first eggs around day 9 post blood meal (Table 1).

Larvae hatch from eggs usually after another six to ten days. The day before hatching the egg tooth and caudal setae are visible in the egg as a dark spot and a dark line, respectively. Just before hatching is expected, a very small amount of food is sprinkled in several spots near the eggs.

Larval food is a composted mixture of rabbit faeces and rabbit pellets. Both feces and pellets are ground in an electric coffee mill, but the feces must be first air-dried. The resulting powder could be stored at -20° C for several months. The mixture of equal parts of ground feces and pellets is spread in a thin layer (less than 0.5 cm) inside plastic trays (for example, photographic trays) and saturated with distilled water. Trays with food are incubated in an upside down position at room temperature in acrylic-plastic cabinets for ageing. Another photographic tray serves as a reservoir filled with water to keep humidity near 100% (Fig 1c). Cabinets are placed in a hood to prevent odors and spores coming out into the laboratory. The front panel of the cabinet is tightly fitted and the mixture is composted under aerobic conditions for three to four weeks. Fermentation is controlled once a week and CO_2 or other gas metabolites are released during inspection of the fermentation process (Figure 1d). Then the food is dried in open air, scraped from the trays, and ground in an electric coffee mill. It should be free of fungal spores but full of mycelia and products of cellulolytic enzymes from fungi.

Food can be stored at -20° C for several months or at 4° C for several weeks. For some species, like *Lutzomyia longipalpis,* the food could be sterilized by autoclaving. Most *Phlebotomus* species that we maintain, however, prefer non-autoclaved food. The quality of larval food is a critical factor during early larval stages. For the 1st instar larvae, the food must be a very fine dust. This could be ensured by putting fine gauze over the mouth of a vial with food.

Pots with larvae are maintained in plastic boxes with a bottom filled with about 1 cm of fine sand dampened with distilled water (Figure 1e). For maintenance of colonies, sand is better than filter paper because of its higher water capacity. Sand, moreover, could be easily recycled as it is washed and sterilized before use. Shaking the sand layer (once a week) prevents fungal growth on the bottom of the container. Pots are checked at least three times a week (Monday, Wednesday, and Friday), and the food is replenished according to the number of larvae and their size. Excess food leads to fungal growth, its shortage to cannibalism and unequal development. The amount of food given to larvae and the moisture maintained are very important details during larval rearing. Before pupation, 4th instar larvae empty their guts, making them more opaque. By this time they do not need any additional food.

The larval period (four instars) usually lasts for about three weeks (see Table 1). *Lutzomyia longipalpis*, *P. tobbi,* and *P. perniciosus* had the shortest larval development, on average pupating 17-20 days after hatching, while *P. duboscqi* larvae developed for at least 28 days. Significant differences were observed between colonies of the same species differing in the geographical origin. In a *P. sergenti* colony originating from Turkey, the shortest interval of larval development was 18 days while for colony from Israel it was 23 days. Similar differences were found in *P. papatasi* colonies originating from Cyprus (32 days), Saudi Arabia (28 days), and Turkey (22 days) (Table 1). The pupal period lasts for about seven to ten days, with the pupal age distinguished by eye coloration. Dark eyes and wings are visible in pupae ready to emerge.

In some colonies, especially in those originating from temperate areas, 4th instar larvae may diapause. For this reason, it is very difficult to breed *P. ariasi* or *P. perfiliewi* in the laboratory. However, we experienced similar difficulties even with colonies originating from areas with warmer climates, namely with *P. simici* from Israel and *P. sergenti* from South-East Turkey. In the *P. sergenti* colony, some larvae were diapausing for up to nine weeks. In *P. simici*, a very long diapause was observed for the significant proportion of larvae and in some pots the generation time reached almost one year. The first *P. simici* adults emerged between days 69-93 and a second portion only after a long pause on days 197-335.

In colonies maintained for many generations at

Table 1. Sand fly colonies maintained in our laboratory in 1999 and 2009: average intervals are given for the whole year (with the range of average intervals for each month).

genus/subgenus	species	origin	maintained since	year of study	days post-blood meal			
					egg	1st instar larva	pupa	adult
Phlebotomus	*papatasi*	Cyprus	1994	1999	7,1 (6-8)	15,8 (14-17)	47,6 42-54)	56,1 (52-64)
		Saudi Arabia	1994	1999	7,3 (7-8)	14,8 (14-17)	42,9 (38-48)	50,7 (45-57)
		Turkey, Urfa	1998	1999	7,4 (6-9)	14,0 (13-16	35,5 (34-39)	41,9 (39-44)
		Turkey, Adana	2005	2009	7,2 (7-8)	13,8 (13-15)	37,1 (33-41)	43,5 (40-46)
	duboscqi	Senegal	1994	1999	9,1 (8-10)	17,7 (15-19)	45,8 (41-49)	54,5 (52-61)
				2009	8,1 (7-9)	18,3 (16-22)	50,6 (45-59)	58,4 (53-69)
Paraphlebotomus	*sergenti*	Turkey, Urfa	1998	1999	8,1 (7-9)	14,2 (12-18)	32,5 (30-37)	40,3 (38-45)
		Israel	2002	2009	7,3 (6-8)	14,0 (13-15)	37,1 (31-43)	47,8 (44-52)
Larrousius	*perniciosus*	Italy	1998	1999	9,3 (9-11)	17,0 (16-18)	37,6 (33-40)	48,1 (44-52)
		Spain	2007	2009	7,5 (6-11)	13,9 (12-16)	32,3 (30-35)	43,1 (41-47)
	tobbi	Turkey, Adana	2005	2009	9,6 (7-11)	15,5 (13-19)	33,6 (29-39)	42,3 (38-48)
		Turkey, Adana	2008	2009	8,3 (7-10)	14,3 (12-16)	30,9 (30-33)	39,1 (37-42)
Adlerius	*halepensis*	Jordan	1995	1999	9,1 (8-10)	16,2 (15-18)	38,2 (25-43)	48,4 (46-52)
	arabicus	Israel	2002	2009	7,6 (6-10)	13,5 (12-15)	33,9 (31-36)	45,5 (42-48)
Euphlebotomus	*argentipes*	India	2008	2009	7,0 (6-9)	13,4 (12-16)	35,0 (30-40)	41,3 (35-45)
Lutzomyia	*longipalpis*	Brazil	1991	1999	7,2 (6-8)	13,7 (12-15)	32,4 (28-37)	41,9 (37-47)
				2009	6,7 (6-8)	13,4 (13-15)	32,6 (29-36)	41,0 (38-44)

standard conditions, the generation time is very stable. In the *L. longipalpis* colony we maintained continuously since 1991, all life cycle parameters were almost the same in years 1999 and 2009 (Table 1), despite that the size of the colony doubled (from 5,050 to 11,070 egg-laying females per year). In such colonies, extended generation time is the first sign of problems. It might be caused by wrong larval food, mites, or other unfavorable conditions. The *Phlebotomus duboscqi* colony suffering from fungi and gregarines in 2009 had a longer generation time than the same colony thriving ten years ago (Table 1).

At least three times a week, emerged adults are released from pots into nylon cloth cages supported with a steel frame (Figure 1f). For the maintenance of colonies, larger cages (40x40 cm or 50x50 cm) are routinely used. Suspended cages prevent damage of adults due to crawling into splits and are removable for washing. Both sexes feed on sugar solutions. We prefer a brown sugar but white sugar or honey could be used as well. 50% sugar solution in distilled water is sterilized in a microwave oven and stored in 4° C for several weeks. A small piece of cotton wool soaked with a sugar solution is offered on a Petri dish and changed at least three times a week. Honey solution is changed daily as it is more sensitive to fungal growth.

Females are offered a blood meal on an anesthetized animal once or twice a week. For most colonies we use mice, but feeding preferences of various species differ. For example *P. papatasi*, *P. duboscqi*, *P. sergenti*, *P. arabicus,* and *L. longipalpis* colonies are routinely maintained on only mice. On the other hand, *Larroussius* (*P. perniciosus* and *P. tobbi*), *Euphlebotomus* (*P. argentipes*), and some *Adlerius* species (*P. simici* and *P. halepensis*) did not feed readily on murid rodents but prefer rabbits, golden hamsters and humans. Adaptation of such colonies to an alternative blood meal source is a long process which may take many generations. We had a good experience with hairless mice which are attractive even for colonies that refuse to feed on BALBc mice (e.g. *P. tobbi*).

Fed females are left undisturbed in a large cage for 24 h and then transferred into a small cage (20 x 20 cm) for defecation. When they are ready to lay eggs (for most species five to six days post-blood-feeding) they are transferred to moist oviposition pots using an aspirator. This two-step procedure prevents the early contamination of rearing pots by fungi. Selecting blood-fed females is laborious but it is necessary in colonies where only a small proportion of females take a blood meal. For large colonies with high feeding rate, a one-step procedure is frequently used. Females are left to defecate in the first cage and then all females, together with males, are transferred directly into the rearing pot. Such a procedure is advised especially for *Lutzomyia longipalpis*, where the feeding rate may reach almost 100% and the dark color of adults makes the selection of blood fed females difficult.

Cotton wool soaked with 50% sucrose is placed on top of the nylon screen of the rearing pot and changed at least every second day. Within the week of transfer, most females lay eggs and die. The dead females are removed with tweezers; the few surviving ones can be placed back into the big cage if necessary.

Usually, the most critical are the 3rd-5th generations, when the colony is adapting for laboratory conditions. By this time, even if females readily took blood and larvae are not diapausing, the generation time is 10-20 days longer than in the established colony. In some colonies the adaptation process was rapid, *P. papatasi* and *P. sergenti* colonies from Sanliurfa, Turkey, reached the "standard" larval development times in the 4th and 5th generation, respectively. On the other hand, some colonies, such as *P. arabicus* from Israel, had extended larval development times for more than eight to ten generations.

Colonies are in danger of infestation by mites, ascogregarines, pathogenic bacteria, and fungi. Some bacteria acquired by larvae from a larval food are transmitted transtadially to adults and may cause their death (Volf et al. 2002). Microorganisms in the gut may also interfere with experimental *Leishmania* infections (Schlein et al. 1985). Pots and larval food are easily contaminated by mites, most frequently by *Tyrophagus*. These storage mites are not predators but, if present in large quantities, can easily damage the colony eating the larval food and producing metabolites which stop larval development. We have a good experience with the biological control of *Tyrophagus* by predatory mites of the genus *Amblyseilus,* which are produced by Biola, Chelcice (Czech Republic). These *Amblyseilus* mites are commercially available for biological control of storage mites and are not dangerous even to 1st instar sand fly larvae.

Vertical transmission of ascogregarine infections could be reduced or even halted by washing the eggs in a 0.1% formol solution (Dougherty and Ward 1991) or in the series of disinfecting solutions used by Poinar et al. (1984). Briefly, eggs are washed down with distilled water from a rearing pot to filter paper in a Buchner funnel connected to a water pump. First, 70% ethanol is applied with the water pump on for 10 to 30 s until all excess liquid is removed. Afterwards, eggs are washed with 5.25% sodium hypochlorite (NaClO) with the water pump off for 3 min 30 s followed by an excess of all remaining liquid by turning the water pump on for 1 min. Subsequently, 10% sodium thiosulfate ($Na_2S_2O_3$) with the water pump off is used for 3 min 30 s. Finally, the eggs are properly washed with distilled water with the water pump on and washed down into a new clean pot. Using this method we effectively cleaned colonies of *L. longipalpis* from the ascogregarine *Psychodiela chagasi* (Votypka et al. 2009), the partial success was obtained in *P. sergenti* and *P. tobbi* infected by other *Psychodiella* species. Care should be taken to avoid cross contamination with these pathogens and cleanliness of cages and pots is a prerequisite for successful rearing.

EXPERIMENTAL INFECTIONS WITH *LEISHMANIA*

Leishmania-sand fly interaction and transmission of *Leishmania* promastigotes by phlebotominae sand flies were reviewed by Kamhawi (2006) and Bates (2007). For

experimental infection of sand flies with *Leishmania,* two methods can be used: feeding on anesthetized infected animals, and membrane feeding. Experimentally infected laboratory animals are usually mice of the susceptible strain (e.g., BALB/c) or golden hamsters (*Mesocricetus auratus*) intradermally inoculated into the ear and eventually into the footpad. The used of optimally aged females is important for the success of experiments. Very young flies (up to three to four days old for most species) do not feed, while the old ones (more than 10 days for most species) have a low survival rate after blood feeding.

Using an infected animal, the feeding rate (percentage of females that took blood) is usually high. However, the infection rate (percentage of blood-fed females that develop infections) is unpredictable as there are many variables that influence the infection. One of them is a local variation in the density of parasites in the skin. To maximize the infection rate, it is useful to expose the lesion site only and cover other parts of the animal's body with a thick cloth. Alternatively, sand flies are placed in plastic vials covered at one end with a nylon mesh and the ears of anesthetized mice are pressed against the mesh with specially designed clamps that hold the vial (Kamhawi et al. 2000, Kimblin et al. 2008). Even then, females may feed on different sites of the lesion with a different density of parasites. The method is used mainly in *Leishmania* species causing cutaneous lesions, like *L. major*, *L. tropica,* or *L. mexicana,* where high infection rates (around 50%) could be achieved if the method is optimized. Feeding sand flies on animals with visceral disease (*L. infantum* or *L. donovani*) usually results in infection rates around 5%, which are too low for most experimental purposes (Molina et al. 1994, Michalsky et al. 2007). Higher infection rates (28%) were achieved by xenodiagnosis of symptomatic dogs by Michalsky et al. (2007) and exceptionally high *Leishmania infantum* infection rates (56-78%) were observed by Gradoni et al. (1987) in *Phlebotomus perniciosus* fed on naturally infected dogs .

For membrane feeding, amastigotes or promastigotes are mixed with blood and offered to flies using a glass feeder. This method enabled us to standardize the concentration of parasites in the infective blood meal. If properly set, this method yields constant and very high infection rates of over 90%. It also enabled to manipulate the parasite numbers ingested which may be useful for studies of vector susceptibility.

Various designs of mosquito glass feeders can be used (Maroli 1985, Higgs and Beaty 1996), but we prefer a special one with a long body and outlets on the top (Figure 1a). This type is, however, more difficult for a glassblower to make. From many types of membranes tried, the best results were obtained using skin from 1-3 days old chicks (Ward et al. 1978). Parafilm™ membranes are not recommended for any sand fly species. Feathers are removed from the skin overlaying the breast region and the skin is carefully dissected away from the body. It is washed with 70% ethanol and sterile saline and then stored at -20° C. Just before use, the membrane is thawed, washed in sterile saline and laid over the opening of the feeder with the outer surface facing outwards. A strip of Parafilm secures the membrane. Parasites are suspended in the blood that fills the inner chamber of the feeder under sterile conditions. The feeder is clamped onto a stand, connected to the water bath with tubing. Water circulates through the feeder´s water jacket at a temperature of 37° C, one of critical factors during membrane feeding. The feeder is placed into the cage through the sleeve. Elastic bands help to prevent sand flies from escaping and secure the cage in the right position. Two or three feeders could be placed in a series.

Sterile rabbit blood (citrated or defibrinized) or human blood from a blood bank can be used. For promastigote-initiated infections, the blood must be heat-inactivated by incubation at 56° C for 30-40 min to destroy complement activity. Amastigotes may originate from different sources: cutaneous lesions of infected animals (most *Leishmania* species), spleen or liver (visceral leishmaniases), as well from *in-vitro* cultures of axenic amastigotes (Bates 1993) or macrophage-like cell lines (Tesh and Modi 1984). If desired, they can be cryopreserved in aliquots of culture medium with 5-10% DMSO, thawed and washed just before use. For details on preparation of *Leishmania* amastigotes, see Bates (1997). Promastigotes for sand fly infections should originate from a low subpassage and logarithmic phase of growth. Contrary to amastigotes, they can be very easily washed and counted by hemocytometer. Susceptibility of sand flies to *Leishmania* infections is dose-dependent and to mimic natural conditions the infective dose should not exceed 10^6 parasites per ml.

Feeding rates on the feeder are usually lower than on the animal. However, the presence of males in the cage, slight changes in the temperature of circulating water, covering the cages by a dark cloth and stimulation by human breath may increase feeding rates. The sugar meal may be also removed from the cage the day before infection to increase the feeding success. Females are left to feed for 1-3 h, than the feeder is removed taking a maximal care not to let any flies escape. At one day post-feeding, fed females are separated and counted for safety reasons. They are kept at 23-26° C with a free access to sugar. At the appropriate interval post-infection, females are examined for *Leishmania* infections. They are immobilized on ice or immersed directly in a detergent solution. Dissections are done in drops of sterile saline, but some workers prefer Locke´s solution. For details on dissection see Bates (1997). Those not familiar with dissections should practice first on uninfected flies.

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