ASPECTS OF THE BIOLOGY OF *Phlebotomus argentipes* (DIPTERA: PSYCHODIDAE) IN KURUNEGALA DISTRICT

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ABSTRACT

The sand fly species *Phlebotomus argentipes* is reported to be the suspected primary vector of leishmaniasis in Sri Lanka transmitting *Leishmania donovani* which causes visceral leishmaniasis. Thus, studies about some aspects of the reproductive biology of *P. argentipes* and the performances of immature stages are highly important for adopting control measures. Accordingly, sand flies were captured from the wild using five CDC light traps set in pre-identified places in the Kurunegala district. Gravid and fully engorged females were sorted out and they were directly placed for oviposition in plaster of Paris lined pots. The unengorged ones were allowed to feed on a mouse and placed for oviposition in similar pots. The rate of oviposition, egg hatching, and adult emergence was determined by making daily observations. Moreover, the duration of eggs, each larval instar, pupae, and adult longevity were recorded. According to the observations the life cycle of *P. argentipes* lasted about 3 to 4 months. The eggs were laid 10-13 days after the blood meal, and they hatched after 12-16 days. Similarly, each second instar, third instar, fourth instar and pupae spent 12-16 days for the emergence. However, the pupal duration was 8-10 days. Furthermore, the rate of oviposition was 15.8 per female and egg hatching and adult emergence was 62.52% and 52.32% respectively. The longevity of the emerged adults was 8-15 days. The information presented on *P. argentipes* in this study is highly important to initiate and maintain a sand fly colony under laboratory conditions for the implementation of successful control measures.

Keywords: Biology, Fecundity, Fertility, Life-history parameters, *Phlebotomus argentipes*

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INTRODUCTION

The most common sand fly species: *Phlebotomus argentipes* is the proven vector of *Leishmania donovani* which causes visceral leishmaniasis in the Indian sub-continent had also been reported from Sri Lanka earlier (Lewis and Killick-Kendrick, 1973; Swaminath *et al*., 2006). Molecular studies have revealed the presence of *Leishmania* DNA in female sand flies confirming the potential of *P. argentipes* as a vector of leishmaniasis (Gajapathy *et al*., 2013). People are subjected to leishmaniasis with the infection of *Leishmania* promastigotes through the bite of an infected sand fly (Da Silva and Sacks, 1987). There are two life stages of *Leishmania sp.* namely, procyclic and metacyclic that dwell in the midgut of the sand fly. The metacyclic stage is the most virulent and disease-inducing form (Sacks *et al*., 1985).

Among 700 known sand fly species, very few of them had been successfully reared in large numbers within laboratories. Such experimental work is essential for studying physiology, and epidemiology of leishmaniasis and parasite vector relationships of sand flies. Sand fly species and even colonies of dissimilar geographical origin differ in many biological and physiological parameters, including saliva composition (Warburg *et al*., 1994; Volf *et al*., 2000). Even though a variety of rearing techniques were described earlier, a detailed bibliography on this matter was given by Killick-Kendrick and Killick-Kendrick (1991). Others have tested hitherto that numerous *Leishmania* species are capable to mature within their midgut (Kamhawi, 2006; Volf and Peckova, 2007). However, for most sand fly species, there are no data on susceptibility to *Leishmania* sp. yet. Hence, establishing and maintaining a new colony is essential for studying the biological aspects: reproductive biology, life cycle parameters, performances of different developmental stages and behavioural aspects of sand flies under laboratory conditions.

METHODOLOGY

Sampling was carried out using five CDC light traps to collect live samples of female and male sandflies of *P. argentipes*. They were set up overnight (4.30 pm - 06.30 am) at pre-identified breeding sites in Bamunakotuwa, Wariyapola, and Narammala areas in the Kurunegala district. Attracted sand flies were collected with a glass aspirator and they were transferred into plastic vials individually. These vials were freezed in a refrigerator for an hour to immobilize them (Volf and Volfova, 2011). Afterward, immobilized *P. argentipes* adults were sorted under the low-power stereo microscope using the previous knowledge of taxonomic identification.

Sorted gravid females were used to initiate a new colony of *P. argentipes* and they were transferred into plaster of Paris lined plastic oviposition pots. Oviposition pots were made using transparent plastic containers (400 mL) and the bottom was filled with a thick layer (1 cm) of white plaster of Paris as the plaster ensures humidity in the pot and provides a resting surface without condensation of water. The pot was closed with a fine net having a small hole at the centre. Females were introduced through the hole in the net, and it was plugged with a wet cotton wool pad. The cotton wool pad was replaced twice a day. Affording to the size of the pot, 5 gravid females were placed in one oviposition pot. The pots were moistened with water before the introduction of gravid females. Pots were kept in rectangular plastic trays. Higher humidity was ensured using a layer (2 cm) of fine sand at the bottom of the tray. Gravid females in each pot were provided with a fresh slice of apple as the sugar source.

Rearing cages were made with a metal frame of 30 x 30 x 30 cm and covered with a white-coloured nylon mesh (0.1 mm). In the middle of one side has an opening (20 cm diameter) with a 30 cm long mesh tube. Trapped non-gravid and unengorged sand flies were transferred into rearing cages through the mesh tube. A captured mouse in a small

wire mesh cage was introduced into the rearing cage and the opening was tied to prevent the escape of sandflies. The mouse was kept for about 1 hour inside the cage and the cage was covered with a black-coloured polythene bag to enhance the feeding of sand flies. After feeding, females were left undisturbed for 24 hours. Fully engorged females were transferred into oviposition pots using an aspirator. The females were killed and subjected to species identification once they have finished laying eggs after 1-3 days.

The eggs of the same species were relocated in plaster of Paris lined pots after a purification procedure to wash the eggs. Prepared 1% sodium hypochlorite solution was gently poured into the pot and after 1-minute eggs were rinsed with normal tap water in the same pot. The first instar larvae that emerged from the hatched eggs were fed with a pre-prepared fine powder of larval feed (Volf and Volfova, 2011). Similarly, the second, third and fourth instar were fed with the same feed *ad libitum*. Larval feed was a composted mixture of rabbit faeces, animal pellets and a small amount of decaying leaf litter. Air-dried rabbit faeces, animal pellets and decaying leaf matter were finely ground using a mortar, sieved and saturated with distilled water in the trays to decompose under aerobic conditions for 3-4 weeks. Subsequently, the food was dried in the open air, scraped from the trays, and again finely ground into a powder to be used as the larval feed. Finally, the fourth instar larvae moult into pupae and adult sand flies emerged in the same larval-rearing pots. Subsequently emerged sand flies were collected, and they were subjected to the same procedure for the maintenance of the sand fly colony.

All the adult rearing cages, larval rearing or oviposition pots were kept inside the adjusted insect rearing room. The laboratory was maintained at high humidity levels (60 % - 80 %) and low-temperature levels (26 ^{0}C - 28 ^{0}C) during the experimental period. The room temperature and humidity were checked hourly using a thermometer and hygrometer. Soaked gunny bags were hung inside the laboratory and they were drenched thrice a day to

maintain moist conditions uninterruptedly. A layer of fine sand was spread on the floor and plastic trays which included larval rearing pots or oviposition pots were placed on this wet layer of sand.

Estimation of the reproductive performance and life history parameters of *Phlebotomus argentipes*

The rate of oviposition, egg hatching, and adult emergence was determined by making daily observations. The rate of oviposition was calculated as the number of eggs laid per female and the rate of egg hatching was defined as the percentage number of eggs hatched from the number of eggs laid. Moreover, the percentage of adult emergence was defined as the percentage number of adults that emerged from the number of eggs laid. The duration of the eggs, each larval instar, pupae, and adult longevity was also recorded by making daily observations.

RESULTS AND DISCUSSION

Phlebotomus argentipes belongs to the family Psychodidae and according to the literature it is reported to be the suspected primary vector of leishmaniasis in Sri Lanka. As such, studies about some aspects of the reproductive biology of *P. argentipes* and the performances of immature stages are highly important for adopting control measures. Thus, a colony of *P. argentipes* was maintained in the insectary at the university of Kelaniya using the wild-caught sand flies from the areas of Bamunakotuwa, Wariyapola and Narammala. Sand flies can be captured using mouth aspirators from their resting sites during the daytime or using light traps during nighttime (Volf and Volfova, 2011). However, during the current study, they were caught using the CDC type light traps. When the light trap was examined for the captured insects, there were some mosquitoes, spiders and moths in addition to the sand flies. Most of the captured sand flies were fully engorged females and some were gravid and non-gravid females while the rest were males.

However, light traps which had been kept near the entrance of burrows had resulted in high catches of sand flies (60-80). Thus, it is sensible to set up traps near the caves, entrance of burrows, resting places and breeding places like stables as it increases the catch. Furthermore, it is good to collect the trapped sand flies early in the morning, unless they may suffer from heat and desiccation (Volf and Volfova, 2011).

When using the mouth aspirators there is a possibility to inhale dust, fungal spores, microorganisms, and hairs shed by the sand flies and that may cause respiratory problems (Volf and Volfova, 2011). Hence, Warburg (1989) introduced a filter to be used in mouth aspirators to prevent the inhalation of such harmful matter. Moreover, it is better to use a glass aspirator as the plastic ones would damage the sand flies due to static electricity (Volf and Volfova, 2011). Correspondingly, overcrowding and retaining the sand flies for a long period inside the aspirator may increase their mortality.

During the current study, captured sand flies were immobilized by freezing. In addition to freezing, CO_2 gas can be used to immobilize sand flies and sort out the desired species (Killick-Kendrick, 1999). Also, gravid, fully engorged and un-engorged females were used to initiate the new colony of sand flies in the laboratory. Initiation of the colony using captured gravid and fully engorged females is easier than using unengorged females. When unengorged females are used, they should be transferred to a rearing cage to supply a blood meal. Blood meal can be provided with the use of a mouse or a rabbit. Anesthetization of the animal may facilitate sand flies to drain blood without any disturbance. Furthermore, it was observed that the blood feeding rate was slow in the absence of male sand flies, and it was high in the presence of male sand flies. A similar observation was reported by Volf and Volfova (2011). The presence of males may stimulate the females to drink blood. Since the sand flies prefer to feed during the nighttime, covering the rearing cage with black-coloured paper or polyethene while

feeding also increases the efficiency of blood feeding. Once the blood feeding is complete, females should be left undisturbed until the complete formation of the peritrophic matrix which makes their gut, less fragile (Sadlova and Volf, 2009).

The life cycle of sand flies is relatively slow compared to the other dipterans and the present study shows that the life cycle extends 3-4 months under laboratory conditions which is almost like natural conditions. Each gravid female laid an average of 15.8 eggs, 10-13 days after the post-blood meal, and they were very tiny about 0.4 mm in length and about 0.2 mm wide elongated oval-shaped brown-coloured eggs (Figure 1). However, according to the study conducted under strict laboratory conditions by Wijerathna *et al*. (2020), the life cycle of *P. argentipes* is completed within 36-50 days with an average oviposition rate of 10.03 eggs per female and the eggs are laid 6 days after the post blood meal. Bestowing to the current study, after 12-16 days first instar larvae emerged, and they were white-coloured worm-like very tiny (< 1.0 mm) microscopic organisms (Figure 2a). The head capsule was darker and two caudal setae were present. The larval duration of the first instar lasted for about 12-16 days and they moult into tiny, microscopic (< 2.0 mm) second-instar larvae (Figure 2b). They possessed a white-coloured body, darker head capsule and four caudal setae. After another 12-16 days tiny, visible third instar larva which had four caudal setae emerged and the third instar duration was 12-16 days. Fourth instar larvae were about 4 mm in length and had sclerotized plates on the dorsal surface. The duration of the fourth instar stage was about 12-16 days and pupated. The pupae were pinkish, and the pupal duration was about 8-10 days (Table 1 and Figure 3). Before pupation, fourth instar larvae empty their guts thus making them opaquer and by this time they had not been fed. However, Wijerathna *et al*. (2020) showed that the larval durations of the first, second, third and fourth instars were 5-7, 4-5, 3-4, and 6-9 days respectively while the pupal duration was 8-10 days. The duration of the life cycle, egg hatching, and

larval development in the current study show a contrasting result with that of the study conducted by Wijerathna *et al*. (2020). This may be because the current study tried to concentrate more on giving natural conditions in the laboratory than using artificial conditions.

Table 1. Different life stages, life history parameters and characteristic features of *Phlebotomus argentipes.*

Life stage	Mean length (mm)	Duration of life stage (days)	Specific features
Eggs	0.40 ± 0.04	12- 16	Oval-shaped, Brown colour
1st instar larva	0.80 ± 0.04	12- 16	Whitish worm-like body Dark head capsule, 2 caudal setae
2nd instar larva	1.70 ± 0.06	12- 16	Dark head capsule, 4 caudal setae
3rd instar larva	2.50 ± 0.07	12- 16	4 caudal setae, (no specific features)
4th instar larva	4.00 ± 0.13	12- 16	Light brown body, Sclerotized plates on the dorsal surface, 4 caudal setae
Pupa	5.30 ± 0.22	8- 10	Pinkish

Furthermore, the percentage of eggs hatched was 62.52% and the adult emergence was 52.32%. According to Wijerathna *et al*. (2020), the hatching rate was 76.57% and the pupation success was 46.10%. This distinct result may be due to the changes in the humidity and temperature levels maintained in the two studies. However, the lifespan of the adults showed a similar result. Consequently, longevity was 8 to 15 days in the current study while it was 7-8 in males and 9 -11 in females in the study conducted by Wijerathna *et al*. (2020).

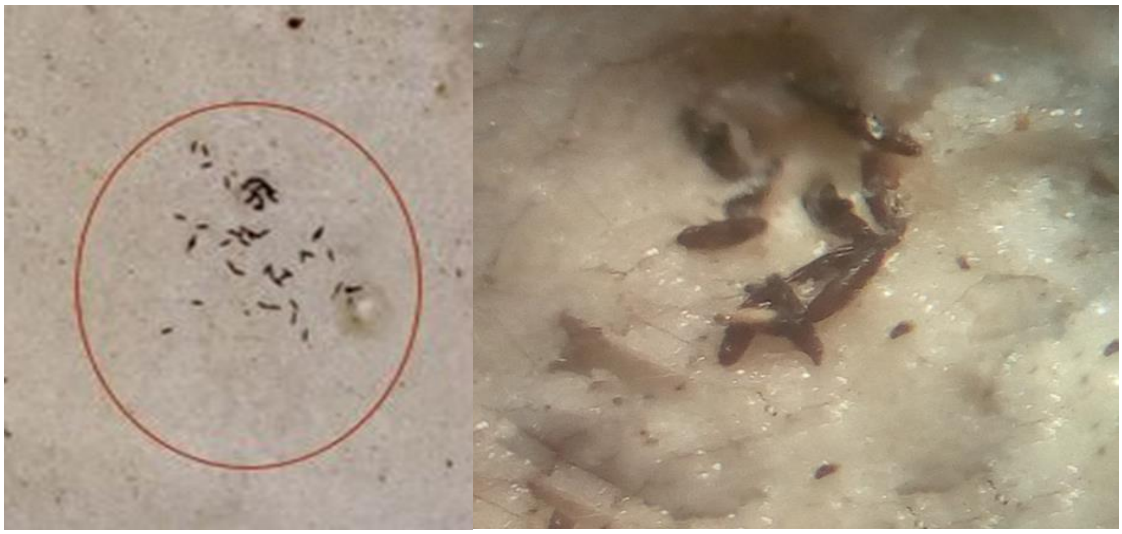
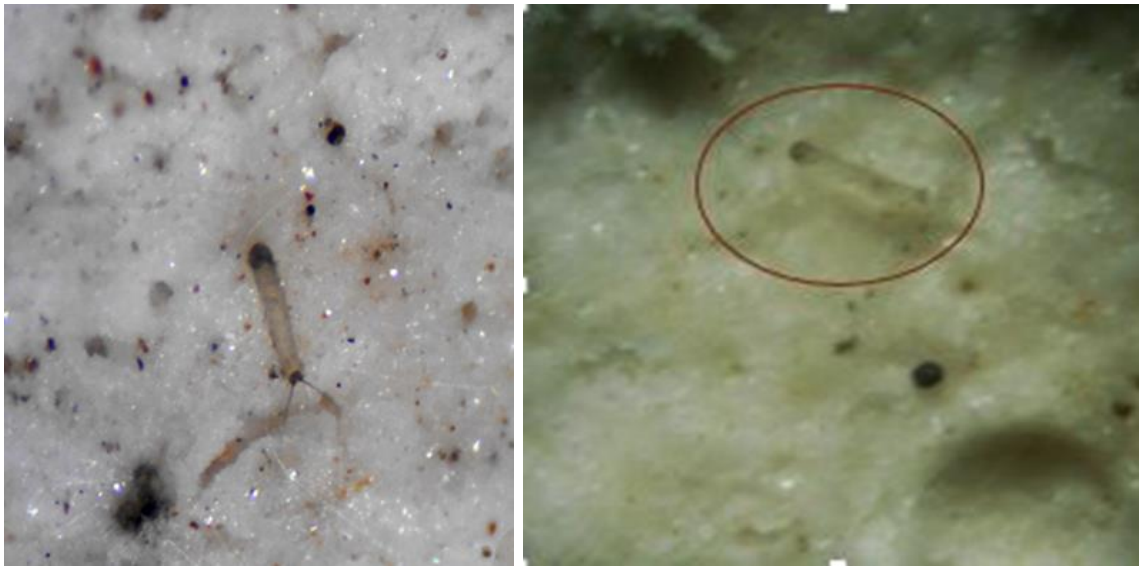


Figure 1. Eggs of *Phlebotomus argentipes*



(a) (b)

Figure 2. (a) First instar larva (b) second instar larva of *Phlebotomus argentipes*

Figure 3. Pupa of *Phlebotomus argentipes*

Temperature, humidity, and photoperiod are important factors that should be considered in maintaining a sand fly colony. Temperature between 25 to 28 ^{o}C, and high humidity of 70 to 95% at rearing cages and 60 to 70% at the insectary should be maintained (Volf and Volfova, 2011). Although during the current study water-soaked gunny bags were used to maintain the humidity at the insectary, a commercially available humidifier could be used to maintain the humidity easily. Photoperiod is not that much important in maintaining the colony. It can be maintained at 12:12 - 14:10 (Light: Dark). The adults were fed a slice of apple during the present study. Apart from that, cotton wool soaked in a 50% sucrose solution could be used. (Volf and Volfova, 2011). However, the length of the gonotrophic cycle may vary depending on the blood meal source (Michalsky *et al.*, 2007; Sant'Anna *et al*., 2010). Additionally, the speed of blood digestion and temperature influence the length of the gonotrophic cycle (Volf and Killick-Kendrick, 1996; Benkova and Volf, 2007). Furthermore, the amount of feed given to larvae is important as excess feed will lead to fungal growth owing to a reduction of water quality and lack of feed will lead to uneven growth and cannibalism. The feed is prepared using a mixture of rabbit faeces and rabbit food. However, the amount of feed given is increased proportionately with the growth of the larvae.

Occasionally, the colonies get infected with some pathogenic bacteria, ascogregarines and mites through the larval feed. Meanwhile, some pathogenic bacteria can be transmitted to adults transtadially and cause their death (Volf *et al*., 2002). The mites do not act as predators, but the metabolites produced by them after the digestion of feed would halt the larval development (Schlein *et al*., 1985). However, the infections in eggs can be controlled or avoided by washing the eggs in a formol solution of 0.1% (Dougherty and Ward, 1991). Poinar *et al*. (1984) proposed a series of disinfecting solutions to purify the eggs and prevent infection by ascogregarines. Accordingly, the eggs

are taken to a filter paper by washing the pot with distilled water. Then the filter paper is placed in a Buchner funnel and washed with 70% ethanol for 30 seconds. Next 5.25% sodium hypochlorite followed by sodium thiosulphate is used to wash the eggs for about 3.5 minutes each and finally distilled water is used. This ensures that no ascogregarine infections are present in the eggs of *P. argentipes*.

When few gravid females are placed for oviposition in a single oviposition pot, there is a chance for the first laboratory generation to contain mixed species. Hence, the first generation should be sorted out to the species level to maintain a pure colony. However, this method is laborious and time-consuming. Thus, Killick-Kendrick and Killick-Kendrick (1991) proposed a technique that would prevent a mixture of species in the first laboratory generation. Accordingly, gravid sand flies were placed in small glass vials individually and a small piece of folded filter paper was introduced to them. The opening was covered with a piece of gauze. The filter paper was kept moist by adding a few drops of distilled water using a syringe and cotton wool soaked in a sucrose solution is placed on the gauze. Moistening the filter paper encourages the females to lay eggs while sugar solution serves as the energy source for the sand flies to acquire energy that is necessary to maintain body functions. However, moistening the filter paper inside the vial should not be done until yellow, brown, or black colour spots are visible on the filter paper or glass vial due to the defecation by females. The glass vials are arranged in containers lined with moist filter paper to maintain the required humidity. The glass vials are examined for the presence of eggs twice a day and if the eggs are present the female is killed and subjected to species identification. Subsequently, the vials are labeled with the name of the species and the date of oviposition and left without any disturbance for 2-4 days. This is because the eggs should not be handled during the early period of embryonic development. After about four days eggs of the same species are transferred to plaster of

Paris lined rearing pots by washing with distilled water or using a fine brush. The number of eggs transferred to a single pot should be proportionate to its diameter. Hence, the described procedure prevents the mixing of different species in the first laboratory generation and avoids the tedious sorting of species.

CONCLUSIONS

Humidity is a crucial factor that should be considered in maintaining a *P. argentipes* colony under laboratory conditions. Reproductive performance and life-history parameters such as rate of oviposition, egg hatching, adult emergence, adult longevity, larval and pupal durations fluctuate greatly depending on the conditions provided in the laboratory.

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