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The Lifecycle of *Phlebotomus argentipes* (Diptera: Psychodidae) Sand Fly in a Newly Developed Colony in Bangladesh

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Abstract. Laboratory-established sand fly colonies provide important material for leishmaniasis research; however, the establishment and maintenance of such colonies can be complicated. In this study, a colony of *Phlebotomus argentipes* (*P. argentipes*) was established using wild-caught sand flies in Bangladesh by following standard procedures described in the published literature. The colony was initiated in a controlled laboratory environment. A total of 430 female *P. argentipes* sand flies were collected to develop the colony. Over multiple generations (F0 to F6), 1,034 *P. argentipes* sand flies laid eggs. The number of eggs that successfully emerged differed significantly between the wild-caught generation and other generations, except for the F1 and F6 generations. A significant difference was observed in the incubation period between wild-caught (F0) and laboratory-reared sand flies (F1 to F6). The mean mortality rate was highest in the first instar (13.9) and lowest in the pupae stage (1.9%). The development from pupae to adult sand flies was 98% successful. It was observed that the copulation was much more frequent after the females had taken a blood meal. This is the first successful attempt to colonize sand flies from Bangladesh in laboratory settings. The study's findings will contribute to a better understanding of the role of *P. argentipes* as a vector of *Leishmania* parasites in Bangladesh, as well as in the region. The colony can also be used for xenodiagnoses, insecticide resistance monitoring, and other experimental infections to generate the necessary evidence.

INTRODUCTION

Visceral leishmaniasis (VL), also known as kala-azar on the Indian subcontinent (ISC), is a fatal vector-borne disease caused by a protozoan parasite, *Leishmania donovani.*[1,2] The condition is highly clustered geographically and affects the poorest rural communities worldwide. Approximately 50,000–90,000 VL cases occur globally yearly.[3] Of these, the ISC and eastern Africa contribute more than 70% of the global VL cases.[4] The case fatality rates in Bangladesh, India, and Nepal are 1.5%, 2.4%, and 6.2%, respectively.[5] In Bangladesh, VL cases were reported sporadically in the 1970s, with a history of an outbreak in the Pabna district in the 1980s.[6] The number of VL cases started to increase in the early 1990s, and between 1994 and 2013, 329 deaths due to VL were reported to the Directorate General of Health Services, Bangladesh, from 37 endemic districts.[7] To eliminate the disease from ISC (Bangladesh, India, and Nepal) by 2015, a Memorandum of Understanding was signed by the respective Health Ministers in 2005 in the presence of the WHO. To achieve the elimination target, five regional strategies were defined, and countries agreed to implement them through their respective national programs. Integrated vector management (IVM) and active surveillance are among these strategies.[8] The causative parasite of VL is transmitted by female sand flies (*Phlebotomus* species and subspecies) that get infected by diseased individuals. Visceral leishmaniasis has an anthroponotic transmission cycle because humans are the only reservoir of the parasite, and no other mammalian host has shown sustained transmission in the ISC.[9–12] The only known vector for VL in Bangladesh is *Phlebotomus argentipes* (*P. argentipes*), and previous studies have found significantly higher densities of the species in the endemic areas.[13] Although a wide range of activities is considered to be included in IVM, indoor residual spraying with suitable insecticides has been undertaken as the mainstay vector control by the National Kala-azar Elimination Program. However, with the changing pattern of disease transmission, vector control methods, and activities, its efficacy and effectiveness are continuously under discussion, particularly with the increasing risk of developing resistance.[14] After the declaration from the WHO, as Bangladesh is the first country globally to eliminate VL as a public health concern, it remains a major challenge to sustain the elimination target in the post-elimination period.

A complete understanding of vector biology is essential for controlling any vector-borne disease, and it is achieved occasionally by studying laboratory colonies. Colonies of phlebotomine sand flies are indispensable for understanding sand fly physiology, the epidemiology of leishmaniasis, and the parasite–vector–host relationship. Such colonies are also vital for experimental studies of various biological aspects and for testing vector control methods for their efficacy. The bionomics of vector sand fly species (*P. argentipes*) was poorly studied in Bangladesh. The present study aimed to document the lifecycle of *P. argentipes* by developing a colony in the most endemic areas of Bangladesh.

MATERIALS AND METHODS

Study area.

An insectarium has been successfully set up at the premises of Surya Kanta Kala-azar Research Centre (SKKRC) in the Mymensingh District by Mymensingh Medical College and Hospital with the support from the Laboratory of Medical Entomology, National Centre for Microbiology, Instituto de Salud Carlos III (ISCIII), Majadahonda, Madrid, Spain, and Drugs for Neglected Diseases Initiative, Geneva, Switzerland.

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Sand flies were collected from the peri-domestic areas of houses of the village (central GPS point of the village: 24°36′18.6″N 90°21′41.7″E) of Trishal Upazila (subdistrict), Mymensingh, where VL cases have never been reported.

Collection of sand flies from the field.

In Bangladesh, the density of *P. argentipes* sand flies was found to be highest in the month of May, and no specific observations have been made on their lifecycle in field conditions.[1] Trained entomology technicians collected adult *P. argentipes* sand flies of both sexes from the inner walls of cattle sheds and nearby human dwellings using mechanical and manual aspirators. Sand flies were collected at 15 different time points from March 2016 to February 2017 between 5:30 PM and 7:00 AM. Collected sand flies were transferred to plastic pots with humid plaster of Paris. They were taken to the insectarium for colonization by following the method described by Molina et al.[15] The average number of sand flies available for colonization was 40 per week.

Identification of sand fly species.

Phlebotomus argentipes sand flies were collected on the field, and their speciation was performed in the laboratory using a phase-contrast stereo binocular microscope (Olympus model no. SZ2-ILST, Olympus, Tokyo, Japan) according to the taxonomic key described by Lewis.[16,17] However, any doubt about morphological identification was confirmed by dissection using the Killick-Kendrick[18] guideline to ensure species identification.

The housing of adults and feeding procedure.

The adult female sand flies were allowed to take blood meals on laboratory albino rats on the fourth day of their emergence after keeping them on 30% sucrose solution in the first 2 days and distilled water soaked in cotton on the third day. We anesthetized rats with ketamine (150 mg/kg) via the intramuscular route and then placed them inside the cage containing the sand flies for 1 hour. We closed the cell and covered it with a dark cloth to let the sand flies feed on the anesthetized animal.

Oviposition of sand flies.

The fed female sand flies, with a 25% ratio of male sand flies, were transferred to a clean plastic pot in a plastic box with filter paper soaked in distilled water on the bottom to adjust the humidity of the plaster and then kept for oviposition in an incubator. After the eggs were laid, all the sand flies were removed from the pot and kept in the incubator, which was maintained at a temperature of 27°C. Eventually, the pot was checked for hatching larvae.

Larval food preparation.

Larval food was a composted mixture of rabbit feces and pellets (mixed in equal parts). Briefly, rabbit feces were air-dried, and rabbit hairs were removed as much as possible. Both the feces and pellets were ground in an electric mill. We mixed the ingredients equally and spread the resulting powder inside a plastic box; distilled water was added. Boxes with larval food were incubated at room temperature and then allowed to undergo fermentation under aerobic conditions for 6–8 weeks. Fermentation was controlled once a week, and the mixture was stirred; we corrected the humidity if needed. After completing the fermentation process, the mixture was allowed to dry. Then, the meal was ground again in an electric mill. The feed was then distributed into plastic jars and stored at −20°C for several months until it was used.[15]

Larval and pupal rearing.

Larvae were checked under a stereo binocular microscope (Olympus model no. SZ2-ILST) manufactured in Japan to remove the fungi, if any, and they were allowed to be fed three times per week. Added food was given according to the larvae instar; the first instar needed less food than the fourth instar.[19] Although pupae were not fed, we provided less food during the pupal stages because some fourth-stage larvae were always in the pots. We transferred the recently emerged adults from the plaster pots to adult cages using the manual aspirator, labeled the cages, and placed a plastic petri dish containing cotton soaked in distilled water on top to adjust the humidity inside the cage. The cages were wrapped in transparent plastic bags to maintain a high and stable relative humidity. Sugar meals were replaced 4 days per week. Dead adult sand flies were removed using a manual aspirator before providing a new sugar meal.

Rearing conditions and safety measures.

Sand flies were kept in an incubator, maintaining a 12:12 (day/night) photoperiod at 27–28°C, with a relative humidity of 75%. Brown sugar was used for feeding purposes. Twenty randomly selected laboratory-bred sand flies of the first and second generations were tested for phlebo, flavi, and densoviruses using RNA polymerase chain reaction in the Laboratory of Arbovirus and Imported Viral Diseases, National Centre for Microbiology, ISCIII, Majadahonda, Madrid, Spain. The above-mentioned tests were performed to avoid any transmission of any unknown or known pathogen or virus to humans (laboratory personnel, study subjects, etc.).

STATISTICAL ANALYSES

The study entomologist checked the validity and consistency of the data at the SKKRC insectarium before data entry. We entered study data into Microsoft Excel version 2007 (Microsoft Corp., Redmond, WA).[20] A descriptive analysis was performed to see the stage-specific developmental time. Sand fly lifetime was calculated by adding larval time to death time. The stage-specific developmental time of the wild-caught sand flies was compared with other generations using *t*-tests. A stage-specific vertical life table was constructed based on live individuals and mortality. All statistical analyses were performed using STATA Version 13.0 (Stata Corp., College Station, TX).[21]

RESULTS

A total of 430 wild female-fed *P. argentipes* sand flies were collected from nonendemic villages of Trishal Upazila (subdistrict) to develop this insectarium. In this study, we analyzed a total of 1,034 sand flies that laid eggs in seven successive generations. Generation-specific numbers (the individual sand flies from each separated generation) of sand flies were 430, 101, 125, 59, 71, 120, and 128, respectively, for the F0 (wild-caught), F1, F2, F3, F4, F5, and F6 generations. Among the generations, the % mean (95% CI of % mean) of laid eggs was lowest in the wild-caught generation (10.8 [7.2–14.4]), and the highest was in the F3 generation (33.9 [26.0–41.8]). The number of eggs successfully reared to hatch sand flies was highest in the wild-caught generation (45.08% [34.8–56.8%]), followed by 37.4% (19.8–55.0%), 33.3% (20.1–46.5%), 29.0% (21.4–36.5%), 22.4%

TABLE 1
Productivity of *Phlebotomus argentipes* sand flies in a newly developed colony in Bangladesh

Generation	Total Sand Fly Eggs Laid	Total Eggs Laid	Mean (95% CI) Eggs Laid (*P*-Value)*	Total Emerged Sand Flies	% Mean (95% CI of % Mean) of Eggs Reared to Emerge (*P*-Value)*
F0 (wild)	430	4,407	10.8 (7.2–14.4)	1,987	45.08% (34.8–56.8%)
F1	101	2,128	22.5 (15.8–29.1; 0.003)	696	37.4% (19.8–55.0%; 0.388)
F2	125	3,158	28.3 (19.0–37.7; 0.001)	437	20.8% (10.0–31.5%; 0.002)
F3	59	1,932	33.9 (26.0–41.8; <0.0001)	316	17.6% (7.8–27.3%; 0.0003)
F4	71	1,917	26.1 (20.4–31.8; <0.0001)	489	29.0% (21.4–36.5%; 0.011)
F5	120	3,522	29.3 (24.4–34.3; <0.0001)	571	22.4% (9.3–35.5%; 0.006)
F6	128	3,046	29.0 (16.5–41.4; 0.002)	734	33.3% (20.1–46.5%; 0.122)
Total	1,034	20,110	25.6 (22.7–28.4)	5,230	29.4% (24.9–33.9%)

* *P*-value for wild-caught versus other generations.

(9.3–35.5%), 20.8% (10.0–31.5%), and 17.6% (7.8–27.3%) for F1, F6, F4, F5, F2, and F3, respectively. Eggs reared to hatch sand flies in the wild-caught generation differed significantly in all generations, except for F1 and F6 (Table 1).

The duration of pre-oviposition was highest in the wild-caught generation (mean days [95% CI] 9.6 [8.7–10.5]) and lowest in generation F5 (3.2 [2.8–3.7]). The mean pre-oviposition time of the wild-caught generation significantly differed from all other generations, except generation F4 (Table 2). Among all generations, the lowest incubation period was in the wild-caught generation (mean days [95% CI] 1.9 [0.0–3.7]), and the highest in the F1 generation (9.5 [7.3–11.7]). Table 2 shows a significant difference observed during the incubation period between wild-caught (F0) and laboratory-reared sand flies (F1 to F6). Differences in the mean larval duration of the wild-caught generation with all other generations were statistically significant (Table 2). The pupal time was highest in generation F3 (mean days [95% CI] 18.2 [7.4–29.0]), and it was lowest for the wild-caught generation (10.4 [7.0–13.8]). The differences in the pupal time duration between the wild-caught and other generations were significant only in the case of F4 (Table 2). For all generations, the emergence and death times are shown in Table 2. The emergence time was the highest in F3 (mean days [95% CI] 17.1 [9.0–25.3]), whereas for death time, the highest value was observed in F1 (mean days [95% CI] 25.4 [18.9–31.9]). Differences in the mean emergence and death time were not statistically significant in any of the generations. The longest lifetime of sand flies was observed in the F3 generation (mean days [95% CI] 89.9 [58.2–100]), and the lowest was observed in the wild-caught generation (70.7 [58.4–83.0]); the lifetime differences were not significant (Table 2).

A vertical life table was constructed on the basis of the lives of the individuals and mortality at each stage (instar) for seven successive generations (Table 3). Stage-specific mortality was based on data in the vertical life table, according to Southwood.[22]

The pupation of *P. argentipes* occurred mainly on the walls of the rearing pots, with few pupae remaining on the surface of larval food.

Sixty-six percent of eggs were unfertilized. The mean mortality rate was highest in the first instar (13.9%) and lowest in the pupae stage (1.9%). The development of pupae into adult sand flies was 98%. The percentages of male and female sexes among the emerging adult sand flies were 57% and 43%, respectively.

Copulation was much more frequent after the females were engorged with blood. Any copulation during blood feeding was not observed. The average longevity of adult sand flies was ~23.5 days. Sand flies of the F1 and F2 generations tested negative for phlebo, flavi, and densoviruses; therefore, none of them were infected with these arboviruses.

DISCUSSION

An insectarium is a controlled environment designed to breed and study insects. In a well-equipped insectarium, researchers can investigate sand fly biology, behavior, and disease transmission dynamics by manipulating environmental conditions, monitoring lifecycles, and influencing

TABLE 2
Developmental time (in days) of sand fly over seven generations in a newly developed colony of *Phlebotomus argentipes* in Bangladesh

	Developmental Time of Sand Fly (days), Mean (95% CI; *P*-Value)						
Generation	Pre-Oviposition Time	Incubation Time	Larval Time	Pupal Time	Emerge Time	Death Time	Lifetime of Each Generation
F0 (wild)	9.6 (8.7–10.5)	1.9 (0.0–3.7)	21.9 (18.6–25.2)	10.4 (7.0–13.8)	15.1 (11.1–19.1)	23.2 (18.3–28.1)	70.7 (58.4–83.0)
F1	5.9 (3.9–8.0; 0.0015)	9.5 (7.3–11.7; <0.0001)	28.5 (23.3–33.8; 0.0306)	14.9 (9.8–19.9; 0.1267)	16.8 (11.7–21.9; 0.5863)	25.4 (18.9–31.9; 0.5696)	85.6 (66.5–100; 0.1693)
F2	5.2 (3.9–6.5; <0.0001)	6.3 (4.7–8.0; 0.0006)	33.3 (27.3–39.2; 0.0013)	13.0 (8.7–17.3; 0.3187)	14.5 (10.5–18.6; 0.8230)	21.1 (16.3–25.9; 0.5250)	81.9 (65.6–98.2; 0.2466)
F3	3.8 (3.0–4.6; <0.0001)	7.5 (6.6–8.4; <0.0001)	32.3 (23.8–40.7; 0.0210)	18.2 (7.4–29.0; 0.1503)	17.1 (9.0–25.3; 0.6409)	22.3 (13.9–30.7; 0.8388)	89.9 (58.2–100; 0.2355)
F4	7.7 (5.5–9.9; 0.0914)	7.3 (6.4–8.3; <0.0001)	31.3 (28.0–34.7; 0.0002)	15.5 (11.5–19.6; 0.05)	14.5 (9.6–19.5; 0.8410)	23.9 (18.2–29.6; 0.8360)	85.3 (69.7–100; 0.1256)
F5	3.2 (2.8–3.7; <0.0001)	8.2 (7.6–8.8; <0.0001)	31.7 (26.5–36.9; 0.0018)	15.4 (10.0–20.7; 0.0989)	15.6 (10.4–20.9; 0.8685)	25.1 (19.5–30.8; 0.5797)	87.9 (67.7–100; 0.1230)
F6	3.6 (3.0–4.2; <0.0001)	8.6 (7.5–9.7; <0.0001)	27.7 (26.8–28.6; 0.0034)	13.8 (8.4–19.3; 0.2372)	13.6 (9.2–18.0; 0.5778)	23.5 (18.7–28.3; 0.9265)	78.6 (67.4–89.8; 0.3242)

TABLE 3
Life table and stage-specific mortality of *Phlebotomus argentipes* sand fly in a newly developed colony in Bangladesh

Generation	Statements	Eggs	First Instar	Second Instar	Third Instar	Fourth Instar	Pupae	Adults
F0 (wild)	Live individuals	4,407	2,225	2,141	2,089	2,041	2,007	1,987
	Mortality	2,182	84	52	48	34	20	
	Finite mortality	0.495	0.038	0.024	0.023	0.017	0.010	
	Apparent mortality (%)	49.51	3.80	2.43	2.30	1.70	1.00	
	Real mortality (%)	49.51	1.91	1.18	1.09	0.77	0.45	
F1	Live individuals	2,128	841	788	752	726	710	696
	Mortality	1,287	53	36	26	16	14	
	Finite mortality	0.604	0.063	0.045	0.034	0.022	0.019	
	Apparent mortality (%)	60.48	6.30	4.57	3.46	2.20	1.97	
	Real mortality (%)	60.48	2.49	1.69	1.22	0.75	0.66	
F2	Live individuals	3,158	582	501	477	462	448	437
	Mortality	2,576	81	24	15	14	11	
	Finite mortality	0.815	0.139	0.047	0.031	0.030	0.024	
	Apparent mortality (%)	81.57	13.92	4.79	3.14	3.03	2.46	
	Real mortality (%)	81.57	2.56	0.76	0.47	0.44	0.34	
F3	Live individuals	1,932	515	418	360	337	318	316
	Mortality	1,417	97	58	23	19	2	
	Finite mortality	0.733	0.188	0.138	0.063	0.056	0.006	
	Apparent mortality (%)	73.34	18.83	13.88	6.39	5.64	0.63	
	Real mortality (%)	73.34	5.02	3.00	1.19	0.98	0.10	
F4	Live individuals	1,917	808	654	579	521	492	489
	Mortality	1,109	154	75	58	29	3	
	Finite mortality	0.578	0.190	0.114	0.100	0.055	0.006	
	Apparent mortality (%)	57.85	19.06	11.47	10.02	5.57	0.61	
	Real mortality (%)	57.85	8.03	3.91	3.03	1.51	0.16	
F5	Live individuals	3,522	884	728	663	610	584	571
	Mortality	2,638	156	65	53	26	13	
	Finite mortality	0.749	0.176	0.089	0.079	0.042	0.022	
	Apparent mortality (%)	74.90	17.65	8.93	7.99	4.26	2.23	
	Real mortality (%)	74.90	4.43	1.85	1.50	0.74	0.37	
F6	Live individuals	3,046	1,082	889	837	797	766	734
	Mortality	1,964	193	52	40	31	32	
	Finite mortality	0.644	0.178	0.058	0.047	0.038	0.041	
	Apparent mortality (%)	64.48	17.84	5.85	4.78	3.89	4.18	
	Real mortality (%)	64.48	6.34	1.71	1.31	1.02	1.05	

Apparent mortality = (mortality/live individuals)*100; finite mortality = mortality/live individuals; real mortality = (stage-wise mortality/total eggs)*100.

reproduction. This specialized facility plays a crucial role in training future entomologists and health professionals, fostering a deeper understanding of vector-borne disease and the development and evaluation of vector control measures. A well-functioning insectarium for colonized sand flies is now a reality in Bangladesh. This is one of the very few facilities globally where sterile sand flies are being grown successfully. The proportion of colonized adult sand flies in laboratory conditions is impressive (85%). The reduction of larval mortality at the first instar should increase this proportion further. This colony has already made it possible to conduct some xenodiagnostic studies in Bangladesh to determine the infectiousness of post-kala-azar dermal leishmaniasis patients with regard to *P. argentipes.*[23,24]

In this study, we have found that the mean number of eggs laid was the lowest in the wild-caught generations. Previous studies reported that varying moisture levels could affect egg production and longevity.[25] Although it is not well documented, the change in their oviposition site, breeding site density, alternation in their natural habitat, and catchment-related stress could also be reasons behind this finding. However, paradoxically, the wild-caught generations have the highest mean of laid eggs from which sand flies emerged. Blood meal, a cool and moist habitat, and dark spaces are prerequisites for the sand fly eggs to survive.[1,25–27] For this reason, the sand flies were kept in an incubator with a relative humidity of ~75% and temperature between 27°C and 28°C. The continuous maintenance of optimal temperature and humidity in the insectarium may be the main reason behind this observation. Apart from that, the nutritional status of the female sand flies and less competition for resources might also play a pivotal role here.

This study clearly shows that albino rats attract female *P. argentipes* sufficiently so that they feed on blood.[28] Successful engorgement from albino rats suggests that *P. argentipes* might be an opportunistic feeder in nature. Further field and laboratory exploration are necessary to understand the host preferences of the local species. Blood feeding of the adult flies and food contamination with fungi and mites in the rearing vials were the key factors that acted as barriers in the laboratory colonization of *P. argentipes.* Killick-Kendrick et al.[29] described their inability to feed *P. argentipes* on hamsters. However, white mice were used for routine engorgement because they provided the highest percentage of engorgement, as described by Ghosh and Bhattacharya.[28] In our study, we also found the same result, although we were not comparing it with other animals. The best result was obtained by keeping the evening temperature at 24–28°C. Fungus and mite contamination of sand fly food has been detected by many workers.[18,30] In our experience, occasional mild fungus contamination of food generally took place 10–15 days after oviposition, and mite contamination took place during any stage of the rearing process. Both agents and organisms were responsible for relatively high rates of larval

mortality. The first and lowest maximum number loss was observed in the fourth instar stage. This may be attributed to several factors, including the increased sensitivity of the first instar to the quality of food provided and its reduced mobility, as well as fungal and bacterial infections and oscillations of the microclimatic conditions during handling.

One limitation of this study was that after feeding, we could not place each engorged sand fly in an individual pot to lay eggs and follow the whole lifecycle. However, our expert entotechnicians were closely monitored to ascertain the colonized species and ensure the targeted species (*P. argentipes* sand fly).

CONCLUSION

The colonization of *P. argentipes* was feasible under laboratory conditions in Bangladesh with proper maintenance practices and extreme care. Furthermore, the laboratory-reared individuals of *P. argentipes* will help us to better understand the role of this sand fly species as a vector of *Leishmania* parasites in the country. The colony can also be used for other purposes, such as xenodiagnosis, insecticide resistance monitoring, experimental infections, studies of the intravectorial cycle of the parasite (vectorial competence), molecular studies, teaching, and training.

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