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Vertebrate excreta based semiochemical influencing oviposition & neonates' survival in *Phlebotomus argentipes*- Visceral Leishmaniasis vector in Indian subcontinent.

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Abstract
Present study deals with the comparative evaluation of attraction of female *Phlebotomus argentipes* (Diptera: Psychodidae), Indian Visceral Leishmaniasis vector towards the vertebrate excreta viz., cow's manure and rabbit's faeces through the 3-way bioassay choice chamber. Also, its stimulatory effect on females' oviposition as well as growth and development of forthcoming generation was studied through bioassay experiment. From the study, it was observed that 71.42% female *P. argentipes* preferred the surface treated with rabbit's larval rearing medium (RLM) over the cow's larval rearing medium (CLM) i.e., 28.57% for egg deposition purpose, while the best survival support for the developing stages of sand flies leading to the higher number of adult emergence i.e., 95.39% was observed in case of cow's larval rearing medium, as compared to those in case of rabbit's larval rearing medium with 46.21% adult emergence. These studies will definitely catalyze our present knowledge regarding the sand fly's behavior especially related to oviposition followed by strengthening of sand fly colony in controlled condition.

Keywords: Attraction, Stimulatory effect, Excreta, Faeces, Oviposition, Survival.

1. Introduction
Kala-azar or Visceral Leishmaniasis (VL) is a major health problem responsible for morbidity & mortality causing serious economic losses leading to hampering of the socio-economic development of many nations during 80's [1]. According to the current scenario, 1.6 million new cases of leishmaniasis emerge annually world-wide, of which an estimated 500 000 are visceral leishmaniasis [2]. Among the Old World (Eastern Hemisphere), VL is found in parts of Asia (particularly the Indian subcontinent and southwest and central Asia), the Middle East, Africa (particularly East Africa), and southern Europe. Overall, VL is found in focal areas of >60 countries. Most (>90%) of the world's cases of VL occur in the Indian subcontinent (India, Bangladesh, and Nepal), East Africa (Sudan, South Sudan, and Ethiopia), and Brazil [2]. It is caused by the protozoan parasite *Leishmania donovani* (Kinetoplastida: Trypanosomatidae) and transmitted from man to man by the tiny insect vector sand fly *Phlebotomus argentipes* (Diptera: Psychodidae) [3].
VL elimination strategy involves vector control which is the fore-most priority by the specialists working on the control aspect of VL. For this purpose chemical pesticides had always been in the central focus for the control of sand flies. Continuous use of chemical based insecticide is not a permanent solution as sand flies' population had developed resistance against it [4, 5, 6, 7, 8]. With resistance to insecticides, sand flies adapt their behavior to ensure their survival and reproduction. Thus, there is an urgent need for exploring & developing various alternative techniques for controlling vectors without creating much environmental nuisance. It could become easier by understanding the bionomics and behaviour of VL vector i.e., sand fly, much better.
The hematophagous female sand fly population plays key role in finding & choosing a suitable site for proper & successful oviposition, which is supposed to be a challenging task for them as they lack parental care [9]. Oviposition substrate stimulates a thigmotropic response in gravid flies [10]. Substrate treated with 'Frass'- an organically enriched larval rearing medium

containing vertebrate faeces remains, hatched eggs particulate, dead remains of larvae, etc, acts as semiochemicals serving as an oviposition attractant & stimulant for *L. longipalpis* [10, 11, 12]. Adult female detect these semiochemicals by odor receptors on the antennae, as well as by contact chemoreceptors on tarsi, mouthparts and antennae. Different cues offered from the diversified arena of colony include egg, larva, habitat, microbes, infusions and plant produced volatiles which are detected by the female insects influencing their oviposition behavior [9]. Finding & choosing the suitable site with humid & damped soil, organically enriched with humus & bacteria, for oviposition by the female sand flies, is programmed under the influence of odour released by the organic environment around it. The odours released from the semiochemical substance along with the concentrations of active substances of decaying organic matter of oviposition surface to which insects are exposed influence eggs deposition by them [13].

This further ensures the proper egg-deposition by female insects [14] and hence regulation of their generation. Also the decaying substance from organic matter nurtures the feeding larvae ensuring their proper growth and development [15]. Distribution of insect-larvae is also followed by the feeding as well as oviposition-site selection by the female population. The excavation of breeding site is a very tedious work for controlling the pathogenic vector-borne diseases. Therefore, knowledge regarding feeding & oviposition behavior becomes an essential criteria for understanding the insects' bionomics as it is directly related to offspring production [16]. But unfortunately there has been very little work done on the behavioral aspect of insects especially in case of sand fly as the oviposition behavior is one of the most neglected aspects of the bionomics to be studied in case of sand fly [17]. Also, lack of well-established sand fly colony limits our knowledge regarding this aspect. Hence the study was undertaken to strengthen the sand fly colony for better understanding of different behavioral aspect including testing the susceptible status of sand fly against the different insecticides for adopting proper planning strategy for sand fly control.

2. Materials & Methods

2.1 Study Period

In a way of dealing the oviposition response associated with semiochemicals, the attraction of ovipositing female *P. argentipes* were tracked & monitored in controlled condition during the month of April 2014, while the stimulatory response of insect were evaluated during the month of July 2014 to August 2014. For screening attraction & stimulatory response of ovipositing female *P. argentipes* towards oviposition surface treated with vertebrate excreta producing odorant leading to the survival of its larval stages, rabbit & cow were selected as vertebrate for source of excreta during the experimental session.

2.2 Preparing Larval Rearing Medium

Larval rearing medium being chief experimental material containing animal excreta was prepared by adopting following technique.

Rabbit larval rearing medium (RLM) was prepared by collecting rabbit's feces from the animal house of RMRIMS (ICMR), Agamkuan, Patna (Bihar), India. These feces were finely ground, sieved & kept in an air- tight container, after complete drying in presence of sun for about a week. Similarly, for cow larval rearing medium (CLM) preparation, aged cow dung was collected from the nearby cattle shed of RMRIMS (ICMR), Agamkuan, Patna, and kept under sun light for about a week, after complete drying it was also finely ground, sieved, and kept in an air- tight container.

2.2.1 Screening the attraction & stimulatory response of female insects towards the animal excreta for oviposition.

For comparative evaluation of attraction & stimulatory response of ovipositing female *P. argentipes* towards the vertebrate excreta, 3-way bioassay choice chamber set was prepared, containing 3 oviposition pots 7×7 cm (height: diameter) i.e., (OP1), (OP2) and (OP3) with their plastered surface, joined with centrally placed insertion chamber (IC), with the help of 3 hard, transparent joining tubes 9×2.5 cm (length× diameter) i.e., (JT 1), (JT 2) and (JT 3) respectively arranged together as represented pictorially in **Figure 1**.

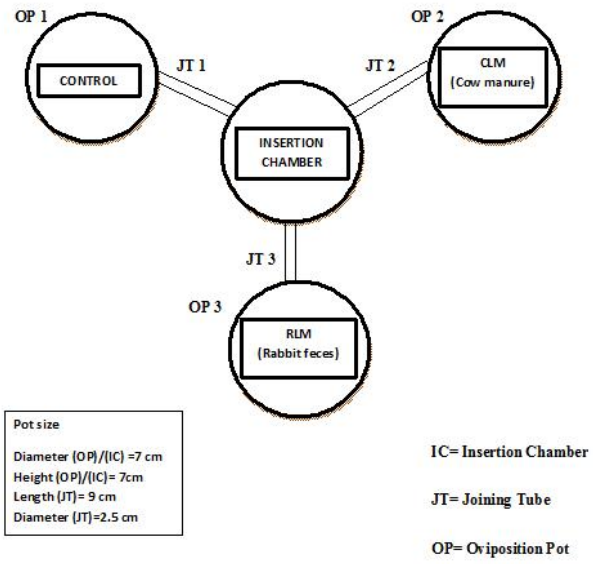

Fig 1: Arrangement of Oviposition Pots (OPs) & Insertion Chamber (IC) in 3-way bioassay choice chamber set for comparative attractancy evaluation of female *P. argentipes* towards the vertebrate excreta leading to successful egg-deposition.

Round filter papers (d=7.5 cm), individually treated with aqueous solution of CLM, RLM & distilled water were placed onto the plastered surface of oviposition pots which were accordingly labeled as CLM, RLM & CONTROL respectively.

Fresh batch of 20 blood-fed female sand flies were released into the IC through the aspiration technique, & cotton was gently plugged over it after the successful insertion of insects into the IC. The hourly attraction movement & position of *P. argentipes* in each section of experimental arrangement, occurring in response to the odor released from the vertebrate excreta was tracked & recorded up till 6 hours from the time of confinement. The data are summarized in Table 1.

After recording the hourly movement of insects towards the treated surface, the set was kept for 5-6 days under controlled condition of 28 ± 2 ºC; 80 ± 5% RH, in complete darken environment for the purpose of successful egg deposition by the females. After the sixth day, post-mortem of insects, data of insects' attraction towards the OP containing excreta treated filter paper, was recorded by visually counting the number of dead insects. While the number of eggs deposited by the female individual, on the surface of each OP's representing stimulatory response of *P. argentipes*, were visually observed under the stereoscopic microscope (Carl Zeiss Stereoscopy Microscope, Austria; Model no- 426126) & obtained data was recorded.

The whole experiment was replicated thrice. Every time the data of insects found in the IC as well as the JT's were ignored for the result saturation purpose.

2.2.2 Evaluating the role of animal excreta in the neonates' survival leading to adult emergence

Oviposition jars were prepared by cutting the 50 ml centrifuge tube of Tarson® Ltd. company up to 6 cm height, also 1 cm hole was cut on its lid (d=4 cm). Base of these jars was filled with the mixture of Plaster of Paris (Calcium Sulphate Hemi-hydrate water molecule, $CaSO_4$), larval rearing medium & water (H_2O), mixed in 2:1:1 ratio. The scraps of POP were removed & cleaned with cotton after complete hardening & drying of its base. The oviposition jars with surface containing cow's larval rearing medium & rabbit's larval rearing medium were labeled as CLM & RLM oviposition jars respectively as illustrated in **Figure 2**.

Fig 2: Bioassay Experimental pots for the demonstration of vertebrate excreta stimulating female sand flies' oviposition as well as survival of developing stages of *P. argentipes.*

Fresh batch of sand flies were used for conducting the experiment. These were reared at the controlled condition of 28 ± 2 ºC ; 80 ± 5% RH and 12:12 (L: D) hours of photoperiod maintained in the closed insectarium of Rajendra Memorial Research Institute of Medical Sciences (ICMR), Agamkuan, Patna, Bihar, India, by following the earlier reported protocol [18, 19]. Single fed female & male sand fly were collected from the insectariums of RMRIMS (ICMR), Patna & confined in each CLM & RLM labeled oviposition jars of experimental sets through gentle sucking technique with the help of aspirator tube & then cotton wool were plugged on its lid immediately after successful confinement of adults. The jars were vertically kept on the damp & moisten cotton cloth in a darker room with temp 28 ± 2^0 & 80 ± 5 RH for the purpose of successful mating under controlled conditions. After the 6^{th} day of confinement, the dead insects were removed from the plaster surface with the help of needle, without disturbing the position of eggs. Number of eggs laid in each experimental oviposition jar was recorded post-visual counting with the help of Stereoscopic Microscope (Carl Zeiss Stereoscopy Microscope, Austria; Model no - 426126).

After observing & counting the number of eggs laid by the female insects, the eggs were washed with the distilled water for removal of unnecessary & unwanted materials from the oviposition jars. The jars were then kept on the damp cloth for the regular & continuous supply of moisture & humidity. The oviposition jars were monitored daily for the eggs' hatching & immediately anti-fungal treated larval-food [19] were sprinkled over the neonates soon after their emergence from the eggs deposited by the insects. The number of eggs' hatching in each jar of each category viz., CLM, RLM was again recorded after visual counting under the stereoscopic microscope. The jars were monitored daily for the transformation of larvae to the matured adult form via non-feeders pupal stage & larval-food were regularly supplied to the developing stages of insect. The adults emerged out leaving behind the puparium in the oviposition jars were then transferred into the Barraud cages of the insectariums of RMRIMS post counting & recording the number of emergence of adult insects. The recorded data for female insects' oviposition, eggs hatching as well as number of adults emerged out after crossing its developmental stages were recorded. Overall 15 replicates of experiment were conducted with 15 fed females & 15 males individually isolated in separate oviposition jars, labeled as CLM & RLM respectively for result saturation purpose.

For statistical hypothesis testing of the variance between the egg depositions, eggs hatching & adult emergence from the eggs laid by the insect population in the two groups of experimental sets of bioassay experiment, T-test technique was adopted.

3. Results

In a way of evaluating hourly attraction & position of *P. argentipes* in each section of experimental arrangements up till 6 hours of confinement of insect, overall 60 female sand flies were released during the experimental session & observed comparatively higher number of insects being attracted towards the surface of oviposition pots (OPs) treated with animal excreta. The value of higher attraction percentage fluctuates between the OPs treated with CLM and RLM. However presence of insects in other sections of the arrangements i.e., insertion chamber (IC) and Joining Tube (JTs) were also recorded. Data of attraction of insect are illustrated in Table 1.

Table 1: Hourly attraction movement & position of *P. argentipes* (in %) at each section of experimental arrangement, occurring in response to the odor released from the vertebrate excreta.

	Insertion Chamber (IC)	Oviposition Pots (OPs)			Joining Tubes (JTs)
		Control	Cow's manure (CLM)	Rabbit's feces (RLM)	
1st hour reading	53.33	8.94	2.32	32.15	3.26
2nd hour reading	9.38	9.93	28.24	46.27	6.18
3rd hour reading	15.00	10.62	42.55	26.39	5.38
4th hour reading	24.8	-----	68.01	7.00	------
5th hour reading	----	17.30	23.38	43.43	15.89
6th hour reading	12.38	-----	53.35	11.05	23.22

Through the 3-way bioassay choice chamber experiment it was observed that 71.42% Female *Phlebotomus argentipes* preferred the substrate treated with rabbit's larval rearing medium (RLM) over the cow's larval rearing medium (CLM) i.e., 28.57% for egg deposition onto which the number of eggs deposition by the female insects were recorded as 62.33% & 37.66% respectively as illustrated in Table 2 & Figure 3.

Table 2: Attractiveness of female *P. argentipes* towards the oviposition surface treated with vertebrate excreta stimulating their oviposition.

	No. of Dead Insects in OP's	Female's attraction % age	Mean attraction	No. of eggs deposited by female individuals	% age egg deposition	Mean Egg Deposition
Control	F=0	00	00	00	00	00
Cow's Manure	F=12	28.57%	6	232	37.66%	116
Rabbit's feces	F=30	71.42%	15	384	62.33%	192

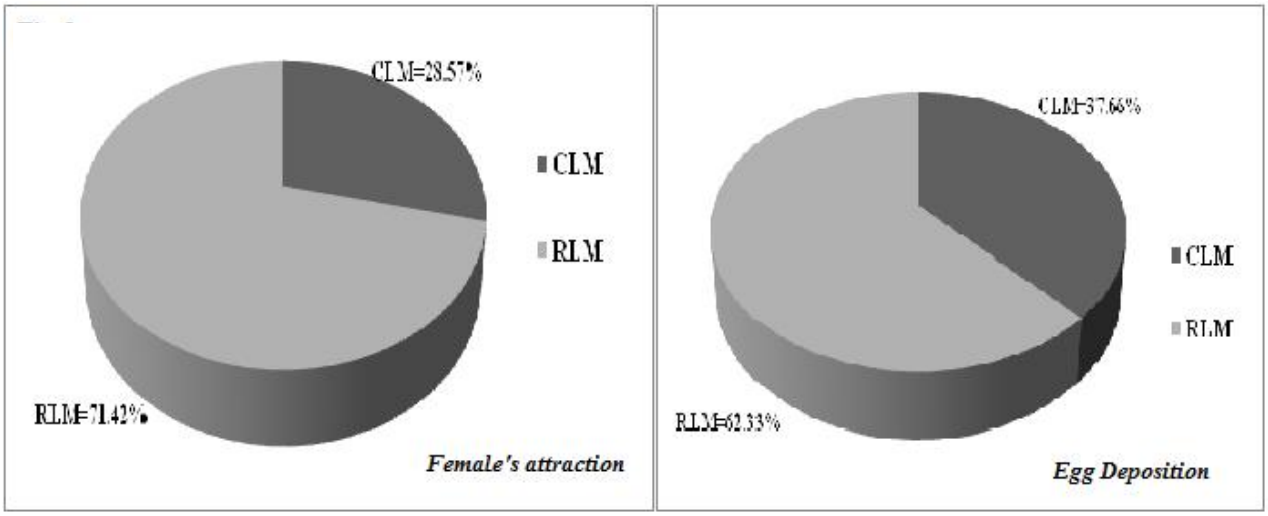

Fig 3: Graphical representation of results of experimental demonstration of the female sand flies attraction towards the oviposition surface treated with vertebrate excreta stimulating their oviposition behavior.

Through the bioassay experiment, 792 & 521 eggs deposited by the female insects were counted on the oviposition surface treated with RLM & CLM respectively. About 60.31%, of the total eggs deposited on the oviposition surface treated with RLM evidenced the stimulatory responses of female insects for successful oviposition over the 39.68% in case of CLM. Despite being less preferred by female *P. argentipes* for oviposition, CLM provided best survival to the insect's developing stages post hatching of eggs i.e., 97.12%, 33.73 ± 38.55 over the RLM in which 65.4%, 34.53 ± 35.69 hatchings were recorded. This definitely affected the adult sand flies' emergence after crossing developmental stages, that were recorded as 95.39%, 53.13 ± 40.45 & 46.21%, 24.4 ± 36.29 in case of CLM & RLM respectively as illustrated in Table 3 & Figure 4.

Table 3: Comparative observation of vertebrate excreta influencing sand flies' oviposition behavior as well as survival of neonates of *P. argentipes* through bioassay experiment.

	No. of Eggs Deposited	No. of Hatching	Mean ±St. Dev for hatching	% age Hatching	No. of Adults Emerged	Mean ± St. Dev for emergence	% age of Adult Emergence
CLM	521	506	33.73 ± 38.55	97.12%	497	53.13 ± 40.45	95.39%
RLM	792	518	34.53 ± 35.69	65.4%	366	24.4 ± 36.29	46.21%

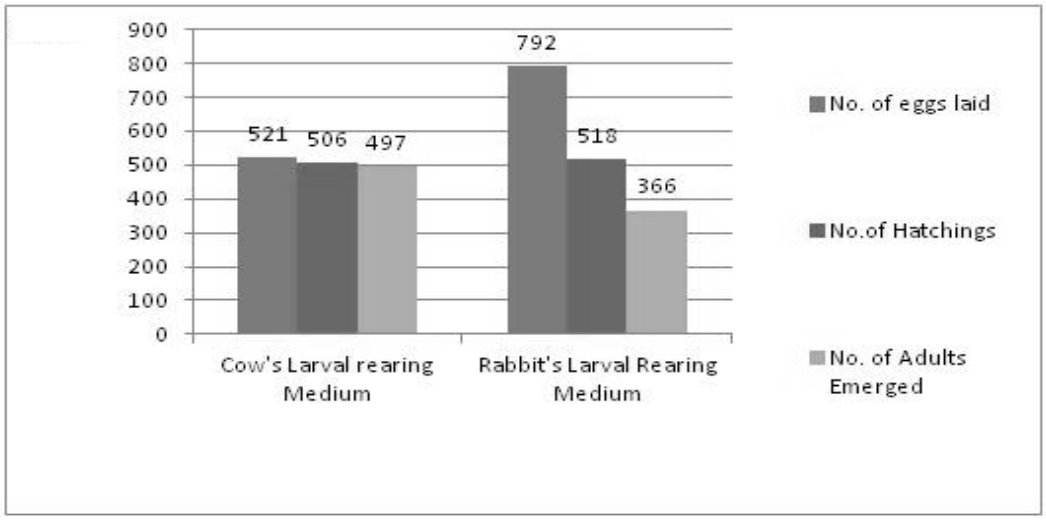

Fig 4: Graphical Representation of observations for bioassay experiment for evaluation of vertebrate excreta stimulating sand flies' oviposition as well as survival of developing stages of *P. argentipes.*

Applying T-test, the data of eggs' deposition & adult emergence from the eggs laid by the female insect onto the oviposition surface labeled as CLM & RLM, was found to be statistically difference at 5% level of significance $P<0.05$ indicating the presence of variation in the observation.While the data of eggs hatching onto the CLM & RLM was observed to be statistically insignificant at 95% level of significance $P>0.05$, indicating the absence of variation in the observation. Hence, the result of RLM proved to be very promising for the assured larger number of eggs deposition by the female insects, while the result of the survival of larval stages leading to the adult emergence had been satisfactorily observed in case of CLM.

4. Discussion

Previously conducted experiments on *P. argentipes* evidenced the presence & release of odor from the organic substances of pre-existing colony that potentially attracts female insects for the purpose of successful egg-deposition as well as the regulation of its generation [17, 11] had paved the way for present study.

For our present comparison based study, rabbit and cow were selected for the source of animal excreta, as rabbit's faeces in form of larval rearing medium is regularly used for culturing colony of *P. argentipes* in closed environment of insectariums at RMRIMS Patna, whereas cow's manure facilitates opportunity for their outgrowth in open condition. Along with that, nature of faeces eliminated off from the animal's body also served as selection criterion for our experimental need. The nitrogen content of cow's manure & rabbit's faeces is 0.49% & 0.25% respectively [20]. Rabbit's excreta containing low nitrogen percentage and low moisture (75%) facilitates larval rearing medium to be served as hot manure. However, cow's larval rearing medium containing cow's manure possessing comparatively high nitrogen level, & high moisture retaining capacity (80%), is considered as cold manure [21].

During the 6 hours of insect confinement in the experimental arrangement, values of higher attraction percentages of *P. argentipes* (Table 1), oscillates between the surface treated with CLM and RLM elucidate the pulling of insects towards the odor released from animal excreta. Though having no excretal treatment, on surface of OP labeled as 'Control', presence of insects were observed. The odor released from the surface of excreta-treated OPs, joined together with the help of JTs could only mediate insects' movement towards the untreated surface. Hence, odor of organic substance brings behavioral movement in insects.

Through the comparative evaluation of oviposition response of female *P. argentipes* towards the presence of aged cow manure & rabbit feces for the oviposition (Table 2), it was observed that females prefer larval rearing medium containing rabbit's faeces over the cow's manure for egg deposition purpose. On other hand, larval rearing medium comprising cow's manure witnessed comparatively higher number of adult's emergence followed by survival of its larvae & pupae. The result clearly suggests that surface treated with CLM being less preferred by the female sand flies for egg-deposition purpose, on to which smaller no. of eggs were recorded, provides advantage of higher maturation to the deposited eggs & hence emergence of adult insects. On other hand surface treated with RLM potentially attracts the female sand flies for egg deposition purpose, on to which higher no. of eggs was observed, correlates with the previous findings conductaed on *P. argentipes* [18]. Higher egg-deposition compensates the less hatching of eggs & adults' emergence. As the maximum productivity & higher yield of sand fly in the controlled condition was targeted, following the evaluation of attraction of insects towards the larval rearing medium, the result of cow's larval rearing medium were found to be very supportive & promising.

Due to compact nature, cow manure holds large amount of water facilitating necessary environment to the bacterial colony that decomposes down & gives off its nutrients slowly [22, 23]. The results of our study also corroborate with the hypothesis of incorporation of cow's larval rearing medium with a huge amount of live and complex microbial community, generating volatile compounds [24] faster eliciting oviposition stimulation among the sand flies, though after natural autoclaving. Sterilization of faeces & manure do hamper the bacterial growth but sterilized feces did not remain sterile at the end of assay [15] ensuring the re-emergence of bacteria enhancing the important microbial community growth that produces chemical cues serving as an attractant and/or stimulant for oviposition [14].

Hence in nutshell, we summarize that rabbit faeces being hot manure behave as an attractant as well as stimulant for female insects for egg deposition purpose while cow manure i.e., cold manure, supports the bacterial growth enhancing the bacteria-

mediated cues for the neonates essential for their surveillance & hence emergence of adult insects in huge amount.

Minimal larval mortality with huge amount of adult insects' emergence is foremost criterion for strengthening of sand fly colony & therefore it can be achieved by making use of both types of manure in optimal concentrations.

5. Conclusion

In addition to offering oviposition cues to the gravid insects for strengthening colony, the semiochemical compounds might be used to attract the vectors for push-pull control strategies using odor- baited traps. For this purpose, present study might be helpful for accompanying the effect of laboratory oviposition trap that had been developed using extracts of whole faeces along with oviposition pheromone in case of *L. longipalpis*, for attracting gravid females in huge amount at a particular place [14].

In conclusion, there are still lots of unexplored secrets about the behavior of this important vector that are really needed to be revealed with the help of strong and well-established *P. argentipes* colony. Hence, in a way of quench our thirst for knowledge regarding behavior of the targeted insect, the study was undertaken whose results are very much promising for strengthening the sand fly colony. Further, strengthened colony of *P. argentipes* can be exploited for testing the susceptible status of sand fly against the different insecticides leading to the adoption proper & effective planning strategy for sand fly control.

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7. Competing financial interest

All authors hereby declare that there is no actual conflict of interest among each others.

8. Ethical Issue

Not Applicable regarding this study.

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