International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2016; 3(5): 01-04

Received: 01-07-2016
Accepted: 02-08-2016

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Armature of genital atrium: An important tool of parity indicator and age grading in *Phlebotomus argentipes* (Diptera: Psychodidae) the vector of Indian Kala-azar

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Abstract
In Indian perspective, phlebotomine sand flies are considered as an insect of medical importance. Female phlebotomine sand flies, being haematophagy as well sheltering pathogenic parasite of genus Leishmania are very much responsible for transmission of Visceral leishmaniasis (VL) among its host. Along with the above mentioned features, it also bears an Armature of Genital Atrium (AGA) that has been exploited for age gradation, parity identification as well as exploring morphological variation in its sibling species, but yet has never been validated in case of *Phlebotomus argentipes* (Annandale and Brunetti) - a prompt and well established VL vector in Indian subcontinent. Therefore, experimental approaches including dissection and microscopic observation were performed for determining if morphological changes in AGA could successfully differentiate between laboratory reared parous and nulliparous *P. argentipes.* Parous female sand flies exhibited a diagnostic longitudinal crease in AGA that had developed after oviposition. Parity identification by observing longitudinal crease in AGA has its implications in age-determination via species establishment in wild-caught female sand flies. It will help in allocating epidemiologically viable sand flies' habitat following estimation of VL transmission risk compelled by them. The strategy will further help in implementing effective control strategy for the containment of vector population transmitting disease.

Keywords: Visceral leishmaniasis (VL), armature of genital atrium (AGA), age gradation, parity identification, *Phlebotomus argentipes*, sand flies

1. Introduction
Phlebotomus argentipes (Annandale and Brunetti) is an established vector of Visceral leishmaniasis (VL), a lethal disease in Indian subcontinent, particularly in Bihar that reportedly encounters 15-16 thousands VL cases per year [1, 2]. Among the available chemical strategies, Indoor Residual Spray (IRS) remained highlighted for successful control of vector population. Also, for successful IRS operations for vector control, one should have correct idea about IRS timing, which is exclusively based upon the availability of epidemiologically viable i.e. parous sand flies. Therefore, for this purpose age determination via parity identification is supposed to be the crucial criterion [3].
Depending upon the practical experiences, variety of approaches has been deployed for age-determination on the basis of overall body wear, fat body condition, and deposition of growth band in cuticle among insect population [4, 5]. In case of sand flies, age determination is supposed to be very tedious and unsatisfactory work due to small size of insect as well as its ovary that causes difficulty in determining the exact number of times female oviposited simply by counting the number of follicular relics in ovarioles. However, in case of mosquitoes (culicidae) and other dipteran insects, distinction of females' parity status on the basis of tracheolar coiling or compaction after release of eggs (Detinova's method) or number of follicular dilatations for confirming number of gonotrophic cycles passed in insects (Polovodova's method) had been successfully reported [6, 7] and validated due to its larger body size as well as size of ovary. The general techniques being deployed for age grading in sand flies includes the detection of physiological appearance of Ovaries (OV) and residual secretion in Accessory Gland (AG). In older female insects, AG appearance (nulliparous with translucent AG versus yellowish opaque granulated AG in parous) is poorly defined and applicable only in its few species. Previously conducted work on *Lutzomyia migonei* (Franca),

modification attained by Armature of Genital Atrium (AGA) after the discharge of mature eggs, had proposed a new and most appropriate tool for age grading with 97% success as compared to either OV (89%) or AG (58%) in female sand fly [8]. A longitudinal crease was formed along both side edges of AGA in parous female [6]; whereas longitudinal crease or lateral folds remained absent in nulliparous female sand flies [6, 7]. Genital atrium is a thin chitinous membranous structure, situated at the 8th tergite of female insects' abdomen containing an armature with small spines, visible between the arms of the furca [8, 9]. The spines of armature pointing towards the atrial opening assist the movement of eggs as they are being laid [10, 11]. Among these, the most remarkable modifications in the flanking armature are creased parallel folds or 'longitudinal crease' that appears after the release of eggs in case of parous female *L. migonei* [8]. However, nulliparous female sand flies are devoid of this arrangement and hence supposed to be an indicator of age-grading in female sand flies. As there was no previous documentation on AGA modification in response to parity indicator or age determination in *P. argentipes* therefore, microscopic examination was conducted in the present study to determine if morphological changes in the AGA could successfully differentiate between parous and nulliparous *P. argentipes*. Microscopic examination of AGA for revealing modifications attained by it during oviposition might prove to be a bench mark for age-determination via species establishment in wild-caught female sand flies.

Also, demarcation of sand flies based upon the parity might prove to be a significant approach in estimating VL transmission risk because only parous female sand flies are capable to transmit disease fulfilling other criteria for disease transmission [12, 8]. Hence, emphasize were made in the present study to distinguish between parous and nulliparous *P. argentipes* observing modifications attained by AGA post oviposition which will further help in designing and developing proper and efficient strategies for vector as well as disease control.

2. Materials and Methods

2.1 Sampling and Categorization of Sand flies

For present study, collection of *P. argentipes* [13, 14] and its colonization under the controlled environment with temperature of 28±2 ºC, relative humidity of 80±5% and photoperiod of 12:12 L: D [15] of insectorium at Vector Biology and Control Department of Rajendra Memorial Research Institute of Medical Sciences, Agamkuan, Patna [16, 17] has provided the baseline for the present study. Freshly emerged 40 female and 40 male *P. argentipes* were obtained from the colony after visual recognition and were categorized into two groups. Each group constituted 20 male and 20 female sand flies.

Group 1 (Blood and Sucrose fed sand flies)

Sand flies had been provided with 30% sucrose-meal after blood-meal from animal source in accordance with previously described technique [13, 14]. The rabbits exploited as an animal blood source for sand fly feeding were obtained from the animal house of Rajendra Memorial Research Institute of Medical Sciences (ICMR), Agamkuan, Patna (Bihar) in accordance with "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) that were followed for conducting research experiments involving animal. Protocols led by animal ethics committee of Indian Council of Medical Research (ICMR), Government of India, were also followed for conducting the present research work involving experiments using animal subject. All experiments had been conducted under the guidance of the Institutional ethical committee of RMRIMS (ICMR), Patna.

Group 2 (Sucrose fed sand flies)

Sand flies were provided with 30% sucrose-meal.

2.2 Confinement of Sand flies

After successful meal-supplementation, sand flies of each groups were confined in the plastered base oviposition pots 7 x 7 cm (height x diameter) annotated with all details viz., the date of feeding, date of confinement etc., and were kept for 5-6 days under the controlled environment of insectorium for mating and oviposition purpose [17].

After 6th- 7th days of confinement and post mortem of the adult sand flies of both groups, the oviposition pots were suspected for the eggs laid by the female individuals with the help of hand lens. Also, scrupulous observations of eggs in the oviposition pots were performed under the stereoscopic microscope (Carl Zeiss Stereoscopy Microscope, Austria; Model 426126). All the dead sand flies were removed from the plastered surface of oviposition pots with the help of fine needle piercing its wing and without harming its abdomen section. Each female sand fly was examined under the microscope for observing insemination condition as well as quality of their spermathecal contents. The samples of each experimented female sand fly were preserved for confirmatory evaluation of parity status as well as modifications attained by AGA post oviposition via dissection and microscopic observation of relevant tissues according to the previously established technique [8].

2.3 Dissection of female Sand flies

To obtain the furca of female sand flies for study purposes the last three segments of each female of each group were removed with the help of fine dissecting needles and then each separated segments were immersed separately into 10% potassium hydroxide, KOH (Merck®, Mumbai, India, 2014) solution for 24 hours in 10 ml eppendorf centrifuge vial. After 24 hours of immersion, separated segments were thoroughly washed with 10% acetic acid (Merck®, Mumbai, India, 2014) solution followed by water. After eluting each segments individually into 100µl of 1X Phosphate Buffer Saline (PBS) (Gibco®, New York, USA, 2013) these were transferred to the clean transparent glass slide for dissection under stereoscopic microscope (Labomed LX400, 10X, 40X). The dissection of segments were carried out by using standard procedure for isolating segments of the spermatheca with the help of fine needles within the genital atrium [18, 19]. The obtained specimens were then transferred onto the clean glass slide and mounted in Hoyer's media [prepared for laboratory use by mixing distilled water (50 ml), gum arabic (30 gm), chloral hydrate (200 gm) and glycerol (20 ml) as constituent] as described earlier in previous study [9].

After complete drying of specimen mounted on glass slide, it was observed under the stereoscopic microscope (Labomed LX400, 10X, 40X) and magnified images were obtained using phase contrast microscope (OLYMPUS U-CMAD3®,

manufactured in Japan) with digital camera (Photometric cool snap), attached with computer [20]. The software with specification Image pro-Express-6.0 was utilized for obtaining the final magnified images of specimen. Images of the morphological structures were created by capturing a series of individual images at 40X and 100X focal depths using oil immersion [19]. The focused portions of the captured images were then stacked together and calibrated with the image editor Adobe Photoshop™.

3. Results and Discussion

In order to perform microscopic experimental observation, two groups of sand flies were formed. First group containing adult sand flies, supplemented with sucrose and blood meal (playing significant role in follicle maturation as well as parity development) respectively, whereas adult male sand flies of another group were only provided with sucrose meal. Each female sand fly of both groups were dissected after observing insemination status and probed under the phase contrast microscope, for analysing modifications attained by their armature of genital atrium (AGA) post oviposition. Microscopic observation revealed the presence of furca, paired spermathecae and developing eggs (Figure 1) along with a peculiar structure i.e., longitudinal crease along the both side edges of armature of genital atrium of each 20 female sand flies (Figure 2) of first group i.e., blood and sucrose fed sand flies, confirming its parity status as positive. However in second group containing only sucrose fed adult sand flies, female sand flies exhibited negative parity or nulliparity, longitudinal crease or lateral folds were remained absent in female insects of this group (Figure 3). Thus, results establishes that blood meal positively implicate the oviposition rather than the sucrose meal in female *P. argentipes*. For present study, the microscopic assessment of obtained image of the dissected structure corroborated with the results of previous studies [3, 8, 19].

The genital atrium is a thin chitinous membrane, possessing armature with bands of spines. The AGA of female sand flies is located between arms of furca as illustrated in Figure 1. Points of spines are directed towards the opening of genital atrium [19]. The role of armatures of genital atrium is to assist the movement of eggs being laid during the oviposition in parous females sand flies [10, 11]. Presumably, the chitinous membrane becomes more stretched and spines move in back-forth manner to provide sufficient place for eggs during its discharging process from the body. Post-oviposition, chitinous membrane of genital atrium re-contracts its muscular shape but fails to restore its original/ normal shape [18, 19]. Therefore lateral fold or longitudinal crease appears at the edge of the AGA in parous female sand flies [19]. In case of unmated blood fed female or mated non-blood fed i.e., nulliparous female sand flies unable to lay eggs and hence, appearance of longitudinal creases in AGA remains insignificant among these population. The presence of longitudinal fold or crease at the edge of AGA is reliable and accurate method for age-grading as compared to the structural change of ovary/ovaries and accessory gland of parous female sand flies [3]. Also, longitudinal crease of AGA plays an important tool for differentiating nulliparous sand flies from the parous population [8].

Among the available strategies for controlling vector density as well as VL transmission, the allocation and identification of parous i.e., epidemiologically viable vector population remains ahead. The categorization of parous and nulliparous vector population acquire various significant approaches for harnessing the availability of parous population for cost effective and target specific Indoor Residual Spray (IRS). The study will also help in distinguishing very similar and identical species or sibling species of vector population at epidemiologically viable regime. Therefore, in this regard, this is an initial report and more detail study is required for the better understanding of taxonomy of *Phlebotomus argentipes*.

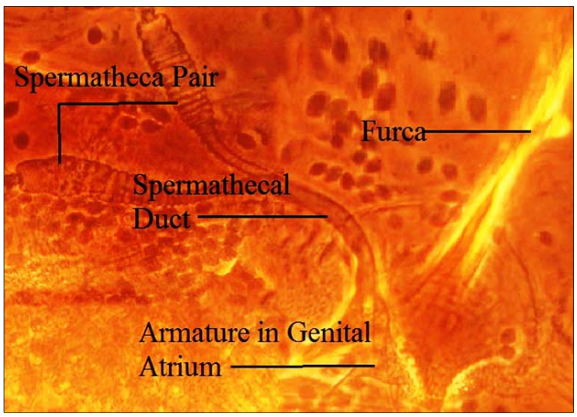

Fig 1: Figure allocating position of Furca, Paired Spermatheca attached with Spermathecal duct and Armature in Genital Atrium of female *Phlebotomus argentipes*.

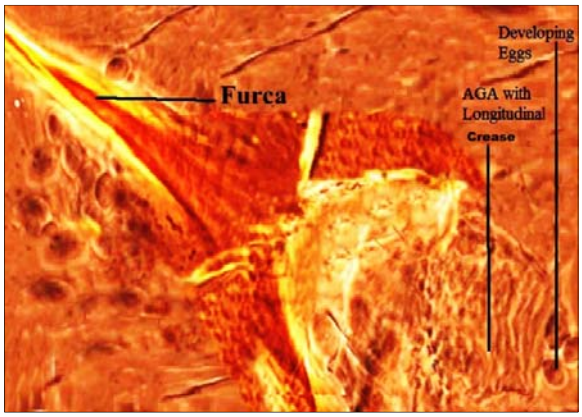

Fig 2: Magnified view of Armature of Genital Atrium (AGA) with longitudinal creases in parous *Phlebotomus argentipes*.

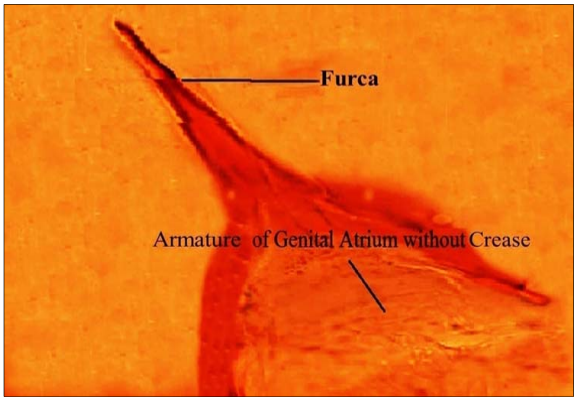

Fig 3: Magnified view of Armature of Genital Atrium (AGA) lacking longitudinal crease in nulliparous *Phlebotomus argentipes*.

4. Conclusions

From the study it can be concluded that the blood meal positively implicate the oviposition rather than the sucrose meal in female *P. argentipes*. Also identifying the modifications developed by parous female sand flies exhibiting a diagnostic longitudinal crease in AGA that had developed after oviposition has its implications in age-determination via species establishment in wild-caught female sand flies as well as estimating VL transmission risk compelled by them.

5. Acknowledgments

We cordially thank N.K Sinha, A.K. Mandal and S.A. Khan of Department of Vector Biology and Control, RMRIMS (ICMR), Patna, Bihar for their excellent technical support during the experimental session. We also express our gratitude towards Molecular Biology Department of RMRIMS (ICMR) for providing magnified images of dissected section of *P. argentipes* with the help of phase contrast microscope (OLYMPUS U-CMAD3®, manufactured in Japan) with digital camera (Photometric cool snap), attached with computer. Our thanks are due to the RMRI Institutional Ethical committee and the Animal Ethics Committee of Indian council of Medical Research, Government of India, for giving mandatory approval to the present research work, involving experiments using animals and rearing sand fly cultures in RMRIMS insectariums. Last but not least, we are also thankful to research publication committee of RMRIMS for considering the manuscript worth publication (with identity no-985/15).

6. Conflict of Interest

All the authors of this research article hereby declare that no conflict of interest exist among each others.

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