Characterization of copulatory courtship song in the Old World sand fly species *Phlebotomus argentipes*

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Acoustic communication in the form of courtship and mating songs are often involved in reproductive isolation between species of Diptera, such as Drosophila, mosquitoes and sand flies. The patterns of courtship songs in New World sand fly species evolve quickly under sexual selection; and therefore, represent an important trait that can be used as a marker to study the evolution of species complexes and may aid identification of sibling species with a complex. The ability to identify vector species within species complexes is of critical importance for effective and efficient vector control programs. Species-specific song patterns seem to contribute to reproductive isolation in New World sand fly species, suggesting that auditory communication signals may be widespread among these important vectors of leishmaniasis. The main goal of the present study was to characterize the copulatory courtship song of *Phlebotomus argentipes*, an important vector of visceral leishmaniasis in the Old World. *Ph. argentipes* males produce acoustic signals during copulation and two types of songs were observed. The one we called primary song is a ‘pulse song’ with similar length and amplitude to the previously observed ‘P1’ pattern recorded in Brazilian populations of *Lutzomyia longipalpis* s.l. The secondary song has ‘sine song’ characteristics and is quite different from any song produced by New World species. The discovery of this copulation courtship songs in *Ph. argentipes* supports the possibility that acoustic communication in sand flies might be more widespread than previously thought, including Old World species. Our results highlight the importance of further research on acoustic communication in the *Ph. argentipes* species complex and other Old World vectors of leishmaniasis.

Acoustic signaling represents one of several methods of insect communication and can be used as a defense mechanism in male-male competition and for male-female intra-specific recognition1,2. When associated with mating behavior, songs are frequently under sexual selection and thus can diverge quickly3–5. In *Drosophila* species, differences in acoustic signals are often associated with pre-mating reproductive isolation and represent sexual traits that result in restricted gene flow between closely related species6. Moreover, acoustic communication studies have played a key role in the identification of cryptic sibling species, and therefore, can provide species-specific traits for taxonomic studies when song are associated with reproductive success7,8.

*Lutzomyia longipalpis* s.l. Lutz & Neiva 1912 is known to consist of a number of cryptic species that are morphologically indistinguishable from each other9. Males of this species produce acoustic signals by flapping their wings. Usually acoustic signals associated with reproductive behavior are produced during pre-mating courtship, as in most *Drosophila* species, and these signals are important for reproductive success1. Unlike *Drosophila*, males of *Lu. longipalpis* s.l produce songs after copulation has started, e.g. once the male genital clasps the female genitalia8,10–12. Although not very common, copulatory courtship has been reported in some insect groups13–17. In the case of *Lu. longipalpis* s.l, copulatory courtship songs are likely to be involved in insemination success as many

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L. donovani is the vector of the Protist parasite, which was suggested to be a cue indicating differences among sibling species. In the burst-type, pulse-type and mix-type song variation observed suggests that the song varies considerably among Brazilian populations of *Lu. longipalpis* s.l with three patterns already identified: Burst-type, Pulse-type and Mix-type. The song variation observed suggests that the *Lu. longipalpis* species complex in Brazil consists of at least six cryptic species.

Males from other New World sandfly species also produce acoustic signals. The main vector of visceral leishmaniasis (VL) in the Central-West region of Brazil is *Lu. cruzi* and *Ph. argentipes*. Males of *Phlebotomus argentipes* s.s, known to produce a song during courtship. They produce two types of song: a primary song which is a pulse song, and a secondary song which has sine song characteristics (Fig. 1). Pulse songs consist of trains of uni- or polycyclic pulses with variable intervals that are produced by some (but not all) males. The primary copulatory courtship song varies considerably among Brazilian populations of *Lu. longipalpis* s.l with three patterns already identified: Burst-type, Pulse-type and Mix-type. The song variation observed suggests that the *Lu. longipalpis* species complex is the vector, *Ph. argentipes*.

### Results and Discussion

It is known that *Ph. argentipes* males, like *Lu. longipalpis* s.l. males, do not produce a courtship song similar to those that have been observed in *Drosophila* species. *Lutzomyia intermedia* is the only sand fly species that is known to produce a song during courtship.

The study presented here shows that *Ph. argentipes* males produce acoustic signals during copulation, which have similarities to the *Lu. longipalpis* s.l., *Lu. migonei* and *Lu. cruzi* copulation songs. Only males were observed to produce songs. They produced two types of song: a primary song which is a pulse song, and a secondary song which has sine song characteristics (Fig. 1). Pulse songs consist of trains of uni- or polycyclic sound pulses and sine songs are continuous, humming-like sounds. Figure 2 shows the song spectrograms of both types of songs (audio file: Additional File 1).

The primary song is composed of pulses with similar length and amplitude and shares the qualitative properties of the pulse-type song observed in some Brazilian populations of *Lu. longipalpis* s.l., the P1 pattern recorded previously in populations from Jacobina, Jequié and Cavunge (Bahia State). Each train is composed of 43 to 83 pulses (mean 58 ± 3.6), with a mean inter-pulse interval (IPI) of 54.9 ms (SEM ± 4.21), a length of 3.1 s (SEM ± 0.29) and a mean of frequency of 246.8 Hz (SEM ± 15.44) (Additional File 2; Fig. 1B). The secondary song follows immediately after the primary song and has a mean frequency of 313.1 Hz (SEM ± 7.36) and lasts approximately 2.3 s (SEM ± 0.21) presenting multiple harmonics that resemble the flight sound observed in several mosquito species. The *Ph. argentipes* secondary song presents more differences when compared to the New World species counterpart. Some male *Lu. longipalpis* s.l. also produce a secondary song, however, the pattern is quite different to the one observed in *Ph. argentipes*. The *Lu. longipalpis* s.l. secondary song is more of a pulse-like song with polycyclic pulses that are flanked by two primary songs, and it is not produced by every male. On the other hand, the secondary song of *Ph. argentipes* was produced by every male that we examined (n = 13). Both the primary song and secondary song are produced only once in each copulation interaction, unlike *Lu. longipalpis* s.l., where males can produce each song multiple times during the same copulation sequence.

Sexual signaling controls the exchange of sensory information between partners and plays a direct role in divergence and speciation. For example, in the *Drosophila montium* species subgroup the sine song frequency was suggested to be a cue indicating differences among sibling species. In the *Lu. longipalpis* species complex the same function may be performed by the pulse and burst pattern songs. *Phlebotomus argentipes* s.s. belongs to a species complex and although there are two nominotypical members, *Ph. annandalei* Sinton 1932 and *Ph. glucas* Mitra & Roy 1953, which can be distinguished by morphological characters, the full extent of the species complex is unclear. It would be interesting to analyze the songs of males from populations of the known species complex members as well as within *Ph. argentipes* s.s., particularly in areas of VL transmission, to evaluate the possibilities that acoustic signals are involved in reproductive isolation within this species.

The study of acoustic communication in vector insects, such as mosquitoes and sandflies, can provide a useful tool in vector control programs, such as the potential to design sound traps or for the assessment of male mating competitiveness in relation to control based on modified male release programs in the field.

### Conclusions

Our results show that *Ph. argentipes* males produce copulatory courtship songs. Two types of patterns are observed, a primary song similar to P1 subtype previously described in a sibling species of the *Lu. longipalpis* species complex, and a subsequent secondary sine song that has not been seen previously in *Lu. longipalpis*. Our
analysis represents the first report of the acoustic signals produced during copulation in *Ph. argentipes* and supports the idea that acoustic communication might be widespread in sandflies, including the Old World species. Future study is required to identify song patterns in other putative members of the *Ph. argentipes* species complex and to determine whether copulatory courtship song is important for sexual communication in Old World sandflies.

**Methods**

The *Ph. argentipes* specimens used in this study were obtained from a colony maintained at Keele University, UK, for more than 40 generations at 27°C, 95% RH, under a 12:12 light-dark photocycle. The colony originated from wild-stock collected near Pune, India, on the east side of India, in a region where there is no visceral leishmaniasis, also known as kala-azar. Recordings were performed according to Souza et al. The virgin male and a...
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Supplementary information

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**Author contributions**

A.S.A. and F.M.V. performed the experiments and analyzed the data. A.S.A. prepared figures. A.S.A., R.P.B., J.G.C.H. and F.M.V. wrote the main manuscript text. All authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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