

A Fourth Chemotype of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Jaúbas, Minas Gerais State, Brazil

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ABSTRACT *Lutzomyia longipalpis* (Lutz and Neiva) is a species complex of *Lutzomyia pseudolongipalpis* (Arrivillaga and Feliciangeli) and at least three other as yet undefined siblings. Isozyme and mitochondrial studies of allopatric populations across Central and South America have suggested the presence of four “clades” that have been hypothesized to have arisen mainly because of geographical isolation mechanisms. Parallel studies of sexual behavior as well as cross-mating and genetic analysis, of both allopatric and sympatric populations, suggest at least four sibling species that do not seem to correspond to the defined four “clades.” In an effort to understand this apparent discrepancy, sympatric populations of *L. longipalpis* from a single South American country, Brazil, are being studied. In Brazil, three putative species can be identified by their male-produced sex pheromones: (S)-9-methylgermacrene-B, 3-methyl- α -himachalene, and a cembrene. We report here that analysis by coupled gas chromatography–mass spectrometry shows that *L. longipalpis* from Jaúbas, Minas Gerais State, Brazil, occurs as two sympatric sex pheromone chemotypes. One chemotype is the cembrene type previously recorded in a *L. longipalpis* population from Sobral, Ceará State, Brazil, and the other is a new cembrene isomer not previously observed in *L. longipalpis*. The finding of this new chemotype strongly suggests that the *L. longipalpis* species complex in Brazil consists of four members rather than the three previously recognized and confirms previous analysis of genetic variation that had suggested the presence of a complex in Brazil.

KEY WORDS *Lutzomyia longipalpis*, sex pheromone, diterpene, cembrene, species complex

Lutzomyia longipalpis is the main vector of *Leishmania chagasi/infantum* (Cunha and Chagas) (Kinetoplastida: Trypanosomatidae), the causative agent of visceral leishmaniasis in South and Central America. However, although it is recognized as a species complex, no consensus has been established on the number or delineation of species. Based on isoenzyme analysis (Lanzaro et al. 1993, Arrivillaga et al. 2003), cytogenetic characters (Yin et al. 1999), and mitochondrial DNA (Uribe Soto et al. 2001, Arrivillaga et al. 2002, 2003), four possible “species” or “clades” have been characterized within this taxon. Furthermore, Arrivillaga et al. (2003) concluded that there was only one species in Brazil, although their data showed that two “clades” were present. Detailed isoenzyme examination has indicated that *L. longipalpis* in Brazil is highly polymorphic (Mukhopadhyay et al. 1998, Mutebi et al. 1999, Azevedo et al. 2000). Thus, it was assumed that the two Brazilian “clades” represent a single genetically heterogeneous “species” (Arrivillaga et al. 2003).

These observations contrast with results from cross-mating experiments (Ward et al. 1983, 1988, Souza et al. 2002a), copulation song analysis (Souza et al. 2002b), and genetic analysis using nuclear markers (Bauzer et al. 2002a, b, Bottecchia et al. 2002, Maingon et al. 2003), which together constitute compelling evidence for multiple species of *L. longipalpis* in Brazil and indicate that sexual selection is involved in maintaining species isolation. The Brazilian “species” can be recognized and defined by their sex pheromones (Lane et al. 1985, Phillips et al. 1986, Hamilton et al. 1999b, c). However, it should be emphasized that verification of the biological concept of species as reproductively isolated populations by cross-mating studies is needed for a number of the pheromone-defined populations. Male *L. longipalpis* produce terpene sex pheromones that attract females across several meters (Morton and Ward 1989, Kelly and Dye 1997), and along with copulation songs, represent typical nongeographical prezygotic reproductive barriers (well described in *Drosophila* flies and *Ostrinia* moths; Futuyama 1998, Coyne and Orr 1998, Thomas et al. 2002). Three different biochemical phenotypes (chemotypes) of *L. longipalpis* occur in Brazil and each consists of distinctly different sex pheromones. The two homosesquiterpenes are 3-methyl- α -himachalene (mw 218), found in a population from Jacobina, Bahia State, and (S)-9-methylgermacrene-B (mw 218),

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found in populations from Lapinha Cave, Minas Gerais, Montes Claros, Minas Gerais, and Sobral, Ceará (Hamilton et al. 1996a, b, 1999b, c). The third sex pheromone is a diterpene (mw 272), partially characterized as a cembrene isomer (diterpene; mw 272), found only in the northeastern states of Brazil in populations from Sobral, Ceará; Santarém and Ilha de Marajó, Pará; Natal, Rio Grande do Norte; and Maceió, Alagoas (J.G.C.H., unpublished data).

The sex pheromones are produced in glandular tissue that underlies the cuticle (Lane and Ward 1984, Lane and de Souza Bernardes 1990) in either abdominal segment 3 or segments 3 and 4, and give rise to one-spot (1S) or two-spot (2S) morphology. However, there is no relationship between spot morphology and sex pheromone type; for example, the 1S males from Lapinha and Sobral produce (S)-9-methylgermacrene-B, whereas the 1S males from Marajó and Santarém produce cembrene. The 2S males from Jacobina produce 3-methyl- α -himachalene, and the 2S males from Sobral produce cembrene. Despite this general lack of correlation between sex pheromone and spot morphology, there are some areas of Brazil where different sex pheromone-producing populations (chemotypes) occur sympatrically, and the males do have different spot morphology. One-spot males from Sobral, for example, produce (S)-9-methylgermacrene-B, whereas the 2S males produce cembrene. A similar site occurs in Januária, MG, where the 1S *L. longipalpis* were reported to possess a methylsesquiterpene, and the 2S form, a diterpene (Ward et al. 1990). These sympatric populations are useful because the different chemotypes can be readily identified by visual inspection before biochemical analysis and thus allow us to conduct experiments to understand the roles of both pre- and postmating barriers in maintaining speciation within the *L. longipalpis* complex.

Recently one of us (R.P.B.) collected *L. longipalpis* in a site where previous collections had shown the presence of both 1S and 2S male sandflies. The objective of this study was to determine the pheromone type of these two sympatric populations.

Materials and Methods

L. longipalpis were collected in Jaíbas, Minas Gerais State, Brazil (15°3'20" S, 43°8'50" W) on the nights of 16 and 17 April 2003 (ambient temperature, 27–30°C). Males were collected using both CDC light traps placed in chicken coops overnight and by direct aspiration of males from the chicken and sides of the chicken coop between 1800 and 2100 hours. Two locations 5 m apart within the Jaíbas site were sampled; 1S and 2S males in approximately equal proportions were found at each location. No intermediate spot forms (Ward et al. 1988) were found. In total, 81 1S and 137 2S *L. longipalpis* were collected along with several other species (*L. ischnacantha*, *L. renei*, *L. forattinii*, *L. sallesi*, and *L. quinquefer*). Sandflies were kept alive in Baraud cages and returned to the laboratory, where they were killed by placing them in a –20°C freezer

for 20–30 min. All male *L. longipalpis* were tentatively identified by examination of external morphological features and separated from females and other species. Individuals were placed in single, flame-cleaned Pasteur pipette ampoules, covered with 30 μ l of hexane (Spectroscopic grade; EM Science, Gibbstown, NJ), and flame sealed. Samples were stored at –20°C until analysis. These tentative identifications were confirmed by thorough morphological examination after chemical analysis.

Analysis. The hexane extracts of 15 individual male Jaíbas 1S and 17 individual Jaíbas 2S males were analyzed by gas chromatography–mass spectroscopy (GC-MS) (Hamilton et al. 1999a) on a Hewlett-Packard 5890 II+ gas chromatograph with an HP-5MS capillary column (Agilent Technology, Stockport, United Kingdom). After the hexane extract was removed for GC-MS analysis, the bodies were preserved in 70% ethanol before morphological examination.

Standards containing commercially available n-alkanes (C₁₂–C₂₁; 10 ng/ μ l; Sigma Aldrich, Dorset, United Kingdom) were used to provide relative retention time/scan data. Extracts of *L. longipalpis* from Sobral 2S, Santarém, and Marajó males were also used to provide comparative retention time and mass spectral data.

Confirmation of Species Identity. To confirm the tentative species identification of male *L. longipalpis* made in the field after GC-MS analysis of the hexane extract, all bodies were preserved in ethanol and were mounted individually on glass slides for detailed morphological examination and species identification (Young and Duncan 1994).

Results and Discussion

The results show that *L. longipalpis* in Jaíbas, Brazil, occur as two sympatric populations, both of which produce diterpene sex pheromone compounds. The major and minor diterpene sex pheromone compounds produced by the Jaíbas 2S population have the same retention times and mass spectra as those diterpenes found in the Sobral 2S, Santarém (2S), and Marajó (1S) *L. longipalpis* populations (Ward et al. 1988, Hamilton et al. 1999a, Maingon et al. 2003). In comparison, the major and minor diterpene sex pheromone compounds produced by the Jaíbas 1S population are different, with later retention times and unique mass spectra, from any other *L. longipalpis* population previously studied. Detailed morphological examination of all individual *L. longipalpis* after biochemical analysis confirmed that both the Jaíbas 1S and 2S populations were *L. longipalpis*. Therefore, in addition to the previously recorded diterpene and two methylsesquiterpene *L. longipalpis* chemotypes, the Jaíbas 1S population is a new chemotype. The chemical presumably has a similar role, i.e., to provide a species isolation mechanism (Jones and Hamilton 1998), as the terpene chemicals in the other members of the complex and provides an early barrier to crossmating that would be reinforced by the courtship/

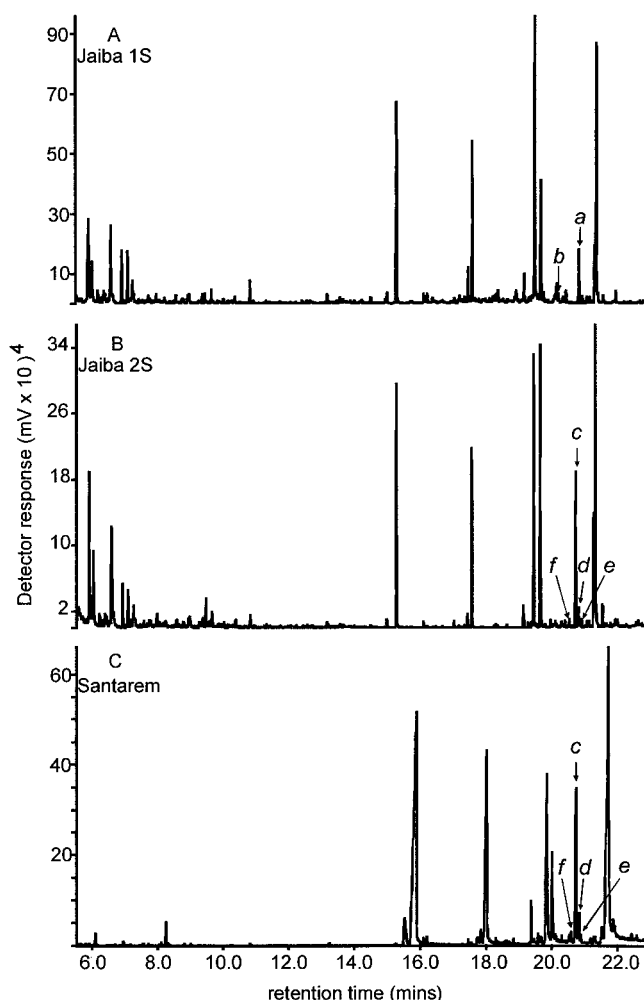


Fig. 1. Total ion current (TIC) chromatograms of individual male *L. longipalpis* from (A) Jaibas (1S population), (B) Jaibas (2S population), and (C) Santarém population. Peaks with the same letter in different chromatograms are the same compound as confirmed by retention time and mass spectral comparisons.

population songs that are present within the *L. longipalpis* species complex (Souza et al. 2002b).

The chromatograms for the two Jaibas populations and the Santarém control population are given in Fig. 1. The retention time (Rt) of the major diterpene component of the Jaibas 1S population is at 20.88 min (peak *a*; 89%; the percentage value refers to the relative abundance of the peak as a proportion of the total terpene content present in the extract). Another diterpene compound was present as a minor component, i.e., an abundance of $\approx 10\%$ of the total diterpene content (peak *b*; Rt = 20.20 min; 10%). Trace amounts (incomplete spectra) of diterpenes were seen at Rt = 19.75, 19.87, and 20.74 min.

The retention times of the major diterpene components of the Jaibas 2S and Santarém 2S populations are at 20.74 min (peak *c*; 79%). Minor diterpene compounds were present at 20.54 (peak *f*; 3.5%), 20.85 (peak *d*; 13%), and 20.90 min (peak *e*; 4%). A diterpene

was also present in trace amounts at 20.55 min. The chromatograms of the Jaibas 2S, Santarém 2S, Marajo 1S, and Sobral 2S populations are superimposable, indicating a very high level of similarity.

The mass spectra of the principle (most abundant) diterpene compounds of the Jaibas 1S, Jaibas 2S, and Santarém 2S populations are shown in Fig. 2. The Jaibas 2S and Santarém 2S spectra are substantially different in shape and ion composition from the Jaibas 1S spectra. The Jaibas 2S and Santarém 2S spectra are characterized by a base peak (most abundant ion) at mass/charge ratio (m/z) 257 and a very strong peak at m/z 109. In comparison, the Jaibas 1S spectrum has a base peak at m/z 119. The 257 m/z ion is present, and although relatively strong, its abundance is reduced to $\approx 50\%$. The Jaibas 2S and Santarém 2S spectra are the same as the spectra of the major diterpenes found in both Marajo 1S and Sobral 2S *L. longipalpis* populations. This compound from Sobral 2S males has been

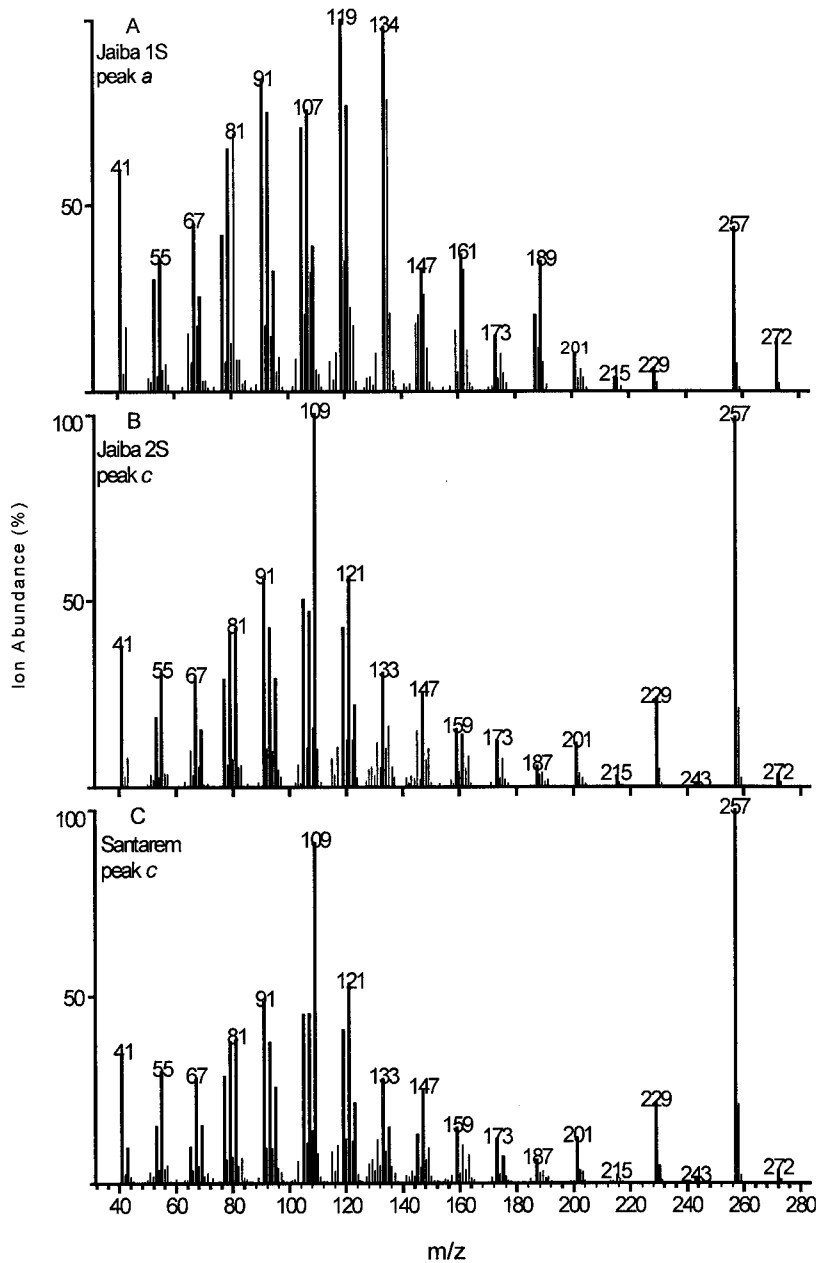


Fig. 2. Electron impact mass spectra of (A) peak *a*, the major diterpene component of the extract from male Jaibas 1S *L. longipalpis*, (B) peak *c*, the major diterpene component of male Jaibas 2S *L. longipalpis*, and (C) Santarém, *L. longipalpis*.

partially characterized as a novel monocyclic cembrene isomer (J.G.C.H., unpublished data). Similarly, the minor diterpene mass spectra profiles are the same for the Jaibas 2S, Santarém 2S, Marajo 1S, and Sobral 2S populations. Thus, the Jaibas 1S major diterpene is different from those seen previously.

There was no evidence of the presence of (*S*)-9-methylgermacrene-B or 3-methyl- α -himachalene in either the Jaibas 1S or 2S population.

A diterpene was first identified in a population of *L. longipalpis* from Morada Nova, Ceará State, Brazil

(Lane et al. 1985), and subsequently from Sobral (2S population) Ceará, Santarém Pará, and Marajo Pará (Phillips et al. 1986). Diterpenes have also been found in two other species of South American sandflies, *L. pessoai* and *L. lenti* (Hamilton and Ward 1994, Hamilton et al. 2002). In both cases, the diterpenes are believed to be cembrenes, although their mass spectral details are substantially different from those found in the Jaibas 1S and 2S *L. longipalpis*. It is likely that these compounds are all cembrene structural isomers.

Although the demonstration of four chemotypes of male sex pheromones in Brazilian *L. longipalpis* populations does not in itself imply that these are sibling species, both the role of these courting signals in establishing prezygotic mating barriers and the reportedly higher frequency of ethological isolation of sympatric versus allopatric species in *Drosophila* strongly suggests that they may prove to be true *L. longipalpis* "species" or "semispecies," depending on the extent and direction of their relative gene flow, rather than only "races." Recently and in this context, Maingon et al. (2003) used nuclear microsatellite markers to show significant genetic differentiation ($F_{ST} = 0.229$; $P < 0.001$) and correspondingly negligible gene flow ($N_e m = 0.84$) between Sobral 1S and 2S phenotype *L. longipalpis* males that produce distinctly different sex pheromones. This result combined with song analysis (Souza et al. 2002b), cross-mating studies (Ward et al. 1988, Souza et al. 2002a), and *per* and *cac* gene polymorphisms (Bauzer et al. 2002a, b, Bottecchia et al. 2002) led to the conclusion that, as shown by the Sobral populations, the Brazilian *L. longipalpis* fauna was indeed a species complex. Speciation within Brazil is further supported by the presence of two clades ("cis-Andean" at the Brazilian side of the Venezuelan Roraima mountain formation and the "Brazilian" elsewhere in Brazil) as reported by Arrivillaga et al. (2003).

The new chemotype described here suggests that, in Brazil, the *L. longipalpis* species complex consists of at least four reproductively isolated chemotypes rather than the two or three previously recognized (Arrivillaga et al. 2003, Maingon et al. 2003). While further work on the extent of gene flow between these chemotypes by microsatellite and cross-mating studies is in progress, the importance of undertaking a study of the factors that act as reproductive isolation barriers when discriminating between "clades" and allopatric populations cannot be overemphasized. The study of sympatric populations provides a useful platform for understanding the molecular basis for speciation in *L. longipalpis*. Comprehending the status of the *L. longipalpis* species complex in South and Central America is of great importance in appreciating the epidemiology, transmission, and ultimately in directing our resources to the amelioration or control of leishmaniasis.

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References Cited

- Arrivillaga, J. C., D. E. Norris, M. D. Feliciangeli, and G. C. Lanzaro. 2002. Phylogeography of the neotropical sand fly *Lutzomyia longipalpis* inferred from mitochondrial DNA sequences. *Infect. Genet. Evol.* 48: 1–13.
- Arrivillaga, J. C., J. P. Mutebi, H. Pinango, D. Norris, B. Alexander, M. D. Feliciangeli, and G. C. Lanzaro. 2003. The taxonomic status of genetically divergent populations of *Lutzomyia longipalpis* (Diptera: Psychodidae) based on the distribution of mitochondrial and isozyme variation. *J. Med. Entomol.* 40: 615–627.
- Azevedo, A.C.R., F. A. Monteiro, P. H. Cabello, N. A. de Souza, M. G. Rosa-Freitas, and E. F. Rangel. 2000. Studies on populations of *Lutzomyia longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae) in Brazil. *Mem. Inst. Oswaldo Cruz.* 95: 305–322.
- Bauzer, L.G.S.R., J.S.M. Gestó, N. A. Souza, R. D. Ward, J.G.C. Hamilton, C. P. Kyriacou, and A. A. Peixoto. 2002a. Molecular divergence in the *period* gene between two putative sympatric species of the *Lutzomyia longipalpis* complex. *Mol. Biol. Evol.* 19: 1624–1627.
- Bauzer, L.G.S.R., N. Souza, R. D. Ward, C. Kyriacou, and A. A. Peixoto. 2002b. The *period* gene and genetic differentiation between three Brazilian populations of *L. longipalpis*. *Insect Mol. Biol.* 11: 315–323.
- Bottecchia, M., N. A. Souza, and A. A. Peixoto. 2002. Molecular polymorphism in the IVS6 region of the *cacophony* gene in *Lutzomyia longipalpis* from Sobral (Ceara-Brazil). *Entomol. Vectores.* 9 (suppl. 1): 23.
- Coyne, J. A., and H. A. Orr. 1998. The evolutionary genetics of speciation. *Phil. Trans. Roy. Soc. Lond. B.* 353: 287–305.
- Futuyma, D. J. 1998. *Evolutionary biology*, 3rd ed. Sinauer, Sunderland, MA.
- Hamilton, J.G.C., and R. D. Ward. 1994. Chemical analysis of a putative sex pheromone from *Lutzomyia pessoai* (Diptera: Psychodidae). *Ann. Trop. Med. Parasit.* 88: 405–412.
- Hamilton, J.G.C., G. W. Dawson, and J. A. Pickett. 1996a. 9-Methylgermacrene-B, a novel homosesquiterpene from sex pheromone glands of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Lapinha, Brazil. *J. Chem. Ecol.* 22: 1477–1491.
- Hamilton, J.G.C., G. W. Dawson, and J. A. Pickett. 1996b. 3-Methyl- α -himachalene; sex pheromone of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Jacobina, Brazil. *J. Chem. Ecol.* 22: 2331–2340.
- Hamilton, J.G.C., R. P. Brazil, E. D. Morgan, and B. Alexander. 1999a. Chemical analysis of oxygenated homosesquiterpenes: a putative sex pheromone from *Lutzomyia longipalpis* (Diptera: Psychodidae). *Bull. Entomol. Res.* 89: 139–145.
- Hamilton, J.G.C., A. M. Hooper, K. Mori, J. A. Pickett, and S. Sano. 1999b. 3-Methyl- α -himachalene confirmed, and the relative stereochemistry defined, by synthesis as the sex pheromone of the sandfly *Lutzomyia longipalpis* from Jacobina, Brazil. *Chem. Commun.* 355–356.
- Hamilton, J.G.C., H. C. Ibbotson, A. M. Hooper, K. Mori, J. A. Pickett, and S. Sano. 1999c. 9-Methylgermacrene-B confirmed by synthesis as the sex pheromone of the sandfly *Lutzomyia longipalpis* from Lapinha, Brazil, and the absolute stereochemistry defined as 9S. *Chem. Commun. (Camb)* 2335–2336.
- Hamilton, J.G.C., R. P. Brazil, D. Campbell-Lendrum, C. R. Davies, D. W. Kelly, F.A.C. Pessoa, and R. G. deq Ueiroz. 2002. Distribution of putative male sex pheromones among *Lutzomyia* sandflies (Diptera: Psychodidae). *Ann. Trop. Med. Parasit.* 96: 83–92.
- Jones, T. M., and J.G.C. Hamilton. 1998. The role of pheromones in mate choice in a lekking sandfly *Lutzomyia longipalpis*. *Anim. Behav.* 56: 891–898.
- Kelly, D. W., and C. Dye. 1997. Pheromones, kairomones and the aggregation dynamics of the sandfly *Lutzomyia longipalpis*. *Anim. Behav.* 53: 721–731.
- Lane, R. P., and R. D. Ward. 1984. The morphology and possible function of abdominal patches in males of two forms of the leishmaniasis vector *Lutzomyia longipalpis*

- (Diptera:Phlebotominae). Cahiers O.R.S.T.O.M. Série Entomol. Méd. Parasit. 22: 245-249.
- Lane, R. P., and D. de Souza Bernardes. 1990. Histology and ultra structure of pheromone secreting glands in males of the phlebotomine sandfly *Lutzomyia longipalpis*. Ann. Trop. Med. Parasitol. 84: 53-61.
- Lane, R., A. Phillips, D. H. Molyneux, G. Procter, and R. D. Ward. 1985. Chemical analysis of the abdominal glands of two forms of *Lutzomyia longipalpis*: site of a possible sex pheromone? Ann. Trop. Med. Parasitol. 79: 225-229.
- Lanzaro, G. C., K. Ostrovska, M. V. Herrero, P. G. Lawyer, and A. Warburg. *Lutzomyia longipalpis* is a species complex: genetic divergence and interspecific hybrid sterility among three populations. Am. J. Trop. Med. Hyg. 48: 839-847, 1993.
- Maingon, R.D.C., R. D. Ward, J.G.C. Hamilton, H. A. Noyes, N. De Souza, S. J. Kemp, and P. C. Watts. 2003. Genetic identification of two sibling species of *Lutzomyia longipalpis* (Diptera: Psychodidae) that produce distinct male sex pheromones in Sobral, Ceará State, Brazil. Mol. Ecol. 12: 1879-1894.
- Morton, I. A., and R. D. Ward. 1989. Laboratory response of female *Lutzomyia longipalpis* sandflies to a host and male pheromone source over distance. Med. Vet. Entomol. 3: 219-223.
- Mukhopadhyay, J., K. Ghosh, C. R. Azevedo, E. F. Rangel, and L. E. Munstermann. 1998. Genetic polymorphism of morphological and biochemical characters in a Natal, Brazil, population of *Lutzomyia longipalpis* (Diptera: Psychodidae). J. Am. Mosq. Control Assoc. 14: 277-282.
- Mutebi, J. P., B. Alexander, I. Sherlock, J. Wellington, A. A. Souza, J. Shaw, E. F. Rangel, and G. C. Lanzaro. 1999. Breeding structure of the sandfly *Lutzomyia longipalpis* (Lutz & Neiva) in Brazil. Am. J. Trop. Med. Hyg. 61: 149-157.
- Phillips, A., R. Ward, L. Ryan, D. H. Molyneux, R. Lainson, and J. J. Shaw. 1986. Chemical analysis of compounds extracted from the tergal "spots" of *Lutzomyia longipalpis* from Brazil. Acta Trop. 43: 271-276.
- Souza, N. A., E. F. Rangel, and A. A. Peixoto. 2002a. Reproductive isolation between three Brazilian populations of *Lutzomyia longipalpis* (Diptera, Psychodidae, Phlebotominae). Entomol. Vect. 9(suppl. 1): 62.
- Souza, N. A., R. D. Ward, J.G.C. Hamilton, C. P. Kyriacou, and A. A. Peixoto. 2002b. Copulation songs in three siblings of *Lutzomyia longipalpis* (Diptera: Psychodidae). Trans. Roy. Soc. Trop. Med. Hyg. 96: 102-103.
- Thomas, Y., M. T. Bethenod, L. Pelozuelo, B. Frérot, and D. Bourget. 2002. Genetic isolation between two sympatric host-plant races of the European corn borer *Ostrinia nubilalis* Hubner. Evolution. 57: 261-273.
- Uribe Soto, S. I., T. Lehmann, E. D. Rowton, I. D. Velez, and C. H. Porter. 2001. Speciation and population structure in the morphospecies *Lutzomyia longipalpis* (Lutz & Neiva) as derived from the mitochondrial ND4 gene. Mol. Phylogenet. Evol. 10: 84-93.
- Ward, R. D., A. L. Ribeiro, P. D. Ready, and A. Murtagh. 1983. Reproductive isolation between different forms of *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae), the vector of *Leishmania chagasi* Cunha and Chagas, and its significance to kala-azar distribution in South America. Mem. Inst. Oswaldo Cruz. 78: 269-280.
- Ward, R. D., A. Phillips, B. Burnet, and C. B. Marcondes. 1988. The *Lutzomyia longipalpis* complex: reproduction and distribution, pp. 257-267. In Service, MW (ed.), Biosystematics of haematophagous insects. Systematics Association. Clarendon Press, Oxford, United Kingdom.
- Ward, R. D., I. A. Morton, R. P. Brazil, S. Trumper, and A. L. Falcão. 1990. Preliminary laboratory and field trials of a heated pheromone trap for the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae). Mem. Inst. Oswaldo Cruz. 85: 445-452.
- Yin, H., J. P. Mutebi, S. Marriott, and G. C. Lanzaro. 1999. Metaphase karyotypes and G-banding in sandflies of the *Lutzomyia longipalpis* complex. Med. Vet. Entomol. 13: 72-77.
- Young, D. G., and M. A. Duncan. 1994. Guide to the identification and geographic distribution of *Lutzomyia* sandflies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). Mem. Am. Entomol. Inst. 54: 1-881.

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