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The *Lutzomyia longipalpis* species complex: does population sub-structure matter to *Leishmania* transmission?

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Leishmania chagasi **causes visceral leishmaniasis and, to a lesser extent, atypical cutaneous leishmaniasis in Central and South America. Its main sand fly vector, *Lutzomyia longipalpis* s.l. (Diptera: Psychodidae) displays a complex population structure that might contribute to the observed clinical pleomorphism and to recent major urban epidemics. This article summarises our understanding on reproductive barriers and hybridisation among this vector's sibling incipient species. Identifying genes important for sand fly ecological adaptability and sand fly–*Leishmania* genetic co-variation could be helpful for interrupting *Leishmania* transmission.**

Genetic variability of *Lutzomyia longipalpis*

The human–*Leishmania* interaction ranges from asymptomatic infection, self-healing dermal lesions and mucosal destruction, to hepatosplenomegaly that is fatal if untreated. Disease severity and parasite-tissue tropism depend on an interplay (that is not yet understood fully) among genetic susceptibility and immune status of the host [1], *Leishmania* species [2], sand fly genetic variability [3] and (possibly) environmental factors [4]. *Lutzomyia longipalpis* s.l. is the main vector of *Leishmania chagasi*, which is also referred to as *Leishmania infantum* and is the cause of both visceral leishmaniasis (VL) and atypical cutaneous leishmaniasis (see Glossary) in Central and South America. This article summarises our understanding of the genetic variability of *Lu. longipalpis* and explores its possible links to genetic determinants of *Leishmania* transmission that might affect clinical presentation. It is argued that this will help us to begin to understand how ecological factors modify sand fly and vertebrate genetic co-variation patterns and affect their adaptation to man-made environments.

The taxonomic status of *Lu. longipalpis* s.l.

The taxonomic status and biogeography of *Lu. longipalpis* s.l. have been reviewed recently [5–7]. Here, we outline major steps in the discovery of sibling species within this single 'species' to illustrate the importance of a combination of approaches in understanding species complexes.

Although *Lu. longipalpis* was described originally in Brazil by Lutz and Neiva in 1912, it was only in a posthumous publication that Mangabeira (1969) detailed differences in the female and male morphologies between Northeast dry 'caatinga' and humid Amazonian populations, suggestive of different 'species' [8,9]. In 1983, Ward *et al.* provided the first support for the existence of *Lu. longipalpis* sibling species by crossing Brazilian populations with distinct male phenotypes [10]. However, with two exceptions [11,12], studies using multi-locus enzyme electrophoresis did not disclose fixed polymorphisms or strong deviations from random mating among *Lu. longipalpis* populations, suggestive of a species complex [7]. Furthermore, the relatively low genetic variation found within Central American and Brazilian populations supported either a single highly heterogeneous species or

Glossary

Atypical cutaneous leishmaniasis: Non-ulcerated benign skin lesion caused by *Leishmania chagasi* and first described in Honduras, Nicaragua and Costa Rica.
Caatinga: A dry, arid ecosystem in the Northeast of Brazil and Northern Minas Gerais State, characterised by 'caatinga' (white forest) vegetation: thorny shrubs, cacti and plants with thick-fleshed leaves and stems, which flower and die during the brief rainy season.
Clade: A lineage of organisms; a monophyletic group (i.e. a natural taxon).
Genotype: The collection of genomic alleles of an organism that result in the observable characteristics (phenotype) of the organism. Microsatellite-based genotype refers to the determination of both alleles for a number of microsatellite loci in a single individual organism.
Introgression: Gene flow among closely related species.
Lek: A mating arena in which males compete and court females.
Microsatellites: Highly polymorphic simple tandem-repeat nuclear sequences that show Mendelian inheritance and co-dominance and are useful as multi-locus population-genetic markers because they are considered virtually unselected.
Sibling species: Closely related 'sister' species.
Species: We have adopted the Dobzhansky and Mayr's biological species concept as modified by Coyne: 'species are groups of interbreeding natural populations that are substantially but not necessarily completely reproductively isolated from other such groups'.
Sympatric populations (or species): Populations (or species) occupying fully or partially overlapping habitats.

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incipient speciation [7]. More recently, mitochondrial and isoenzyme markers defined four *Lu. longipalpis* clades [13]. However, evidence from other markers indicates further sibling species within Brazil [7] (see later). Non-congruence among markers is common and isoenzymes do not always provide enough variation to study incipient speciation; in addition, mitochondrial DNA is prone to introgression among closely related species [14]. The rDNA repeat differentiated successfully among *Anopheles gambiae* s.l. sibling species and several *Phlebotomus* species [15–17] and it is surprising that has not been applied to *Lu. longipalpis* sibling species.

The role of reproductive isolation in restricting introgression among sibling species

Species formation involves a process of genetic differentiation that is maintained by reproductive isolation barriers: hybrid inviability (i.e. sterility) or non-random mating. There is evidence for two of these barriers in *Lu. longipalpis* s.l. First, populations from Costa Rica, Brazil and Colombia produced sterile male hybrids [11]; however, this was not investigated further. Second, there was strong sexual selection between two reproductively isolated populations at Sobral, Brazil, in which females recognised 'perfumes and love songs' (unique sex pheromone chemotypes and copulation songs, Boxes 1 and 2) produced specifically by conspecific males with concordant 'spot' morphology [18–24]. Striking differences in sex pheromones and love songs from different populations indicate strongly that sexual selection acts as a strong reproductive isolation barrier, shaping population sub-structure in *Lu. longipalpis* [25–29]. The pheromone induces neurophysiological responses and attracts both conspecific males and females to leks formed on near vertebrate host feeding [30,31], suggesting pheromones as attractive lures for sand fly vector-control measures.

'Speciation' genes responsible for pheromone synthesis remain to be identified but those involved potentially in determining *Lu. longipalpis* s.l. songs have been studied [27–29]. DNA sequence-polymorphism analysis of two love song genes, *period* and *cacophony* (Box 2), supports strongly 'species' defined by microsatellites and behavioural pre-zygotic isolation barriers [18–29].

Male phenotypes, genetic differentiation and cross-mating data are known for a few *Lu. longipalpis* s.l. populations. For example, males releasing the C_{16} 9MGB (9-methyl-germacrene B) (Lapinha and Sobral 1S) or 3MαH + αH (3-methyl-α-himachalene + α-himachalene) (Jacobina) produce pulse-type copulation songs and fail to inseminate the non-cognate C_{20} cembrene (Sobral 2S, Marajó or Natal) females, whose males produce a burst-pattern song [18–24]. Sand flies from these populations are also distinct genetically according to *period* and *cacophony* gene polymorphisms and microsatellite genotypes [25–29]. In addition, C_{16} sibling species (Jacobina, Lapinha and Sobral 1S) display different pulse patterns and 9MGB populations also differ in their pheromone amount [22–24]. Figure 1 illustrates the match between different pheromones and song patterns found in Brazilian *Lu. longipalpis* s.l. populations.

Box 1. Sex pheromone communication in sand flies

Sex pheromones are volatile chemicals (alkanes, alkenes, esters, aldehydes, ketones, fatty acids or terpenes) produced by either gender of a species to attract members of the opposite gender for mating. A sex pheromone can be a single compound or a mix (blend) from the same or different classes of compounds. Sex pheromones are a narrow communication channel, requiring significant biosynthetic control to produce the purity, correct stereochemistry and stoichiometric ratio of active compounds. Sex-pheromone communication is between an emitter (i.e. sex pheromone producer) and the receiver: an antennae containing transport proteins and dendritic membrane receptors that are attuned specifically to the capture and processing of the released sex pheromone. Stimulation of axons within the peripheral and central nervous system provides further specificity, leading ultimately to a behavioural outcome. Communication will only be effective when both the emitter and receiver are in the appropriate physiological state and in close proximity. Sex-pheromone communication acts as an efficient pre-zygotic mating barrier enabling conspecific mate discrimination and avoiding wasted mating attempts among non-compatible individuals [46]. *Lu. longipalpis* sex pheromones are terpenes made up of isoprenoid units and include 9MGB, 3MαH and two cembrene isomers that are unique novel compounds. They are used by some other insect groups in their defensive secretions (e.g. termites), by plants in floral volatiles and as defensive exudates but are rarely encountered in insect intraspecific communication [22]. *Lu. longipalpis* sex pheromones are produced by males from glands underlying the tergal cuticle of abdominal segment four or segments three and four [47]. The sex pheromone attracts conspecific females over 2 m in the laboratory and might attract females over greater distances in the field, where the presence of host odour synergises sex pheromone attraction [48].

Better defined candidate 'species' are the sympatric Sobral and El Paso populations in that gene-flow estimates among populations not separated by current geographic physical barriers provides the best test model for reproductive isolation [32]. It is worth noting that genetic differentiation for the *period* gene between the Sobral sympatric sibling species was greater than that reported between *bona fide* species *Lu. whitmani* and *Lu. intermedia* [33]. At

Box 2. Love songs in fruit flies and sand flies

During courtship, males of the fruit fly *Drosophila melanogaster* and several other species extend and vibrate one of their wings, producing a 'love song' that helps to 'turn on' the females [49]. This love song is usually highly species-specific and song differences are important in the sexual isolation of closely related *Drosophila* species. *Lu. longipalpis* males also produce acoustic signals and males from different populations from Brazil produce distinct songs during copulation [23,24] (Figure 1). These song differences probably contribute to the insemination failure observed in crosses between these populations, functioning as a potent reproductive-isolation mechanism [18]. In *Drosophila,* several genes that control features of the song have been identified [50]. One of these genes is *period (per)*, first identified by mutations affecting the circadian rhythms of *D. melanogaster* [51]. In the early 1980s, Kyriacou and Hall [52] showed that these same mutations affect one-minute rhythms that are found in *D. melanogaster* love songs. Later, it was also shown that *period* control the species-specific differences in love-song rhythms among closely related *Drosophila* species [53]. Another example of love-song gene is *cacophony (cac)*. A mutation in this calcium-channel gene affects several features of the *Drosophila* love song [54]. Fragments of both genes have been isolated in *Lu. longipalpis* s.l. and used as markers in population-genetic studies of the different Brazilian sibling species [27–29].

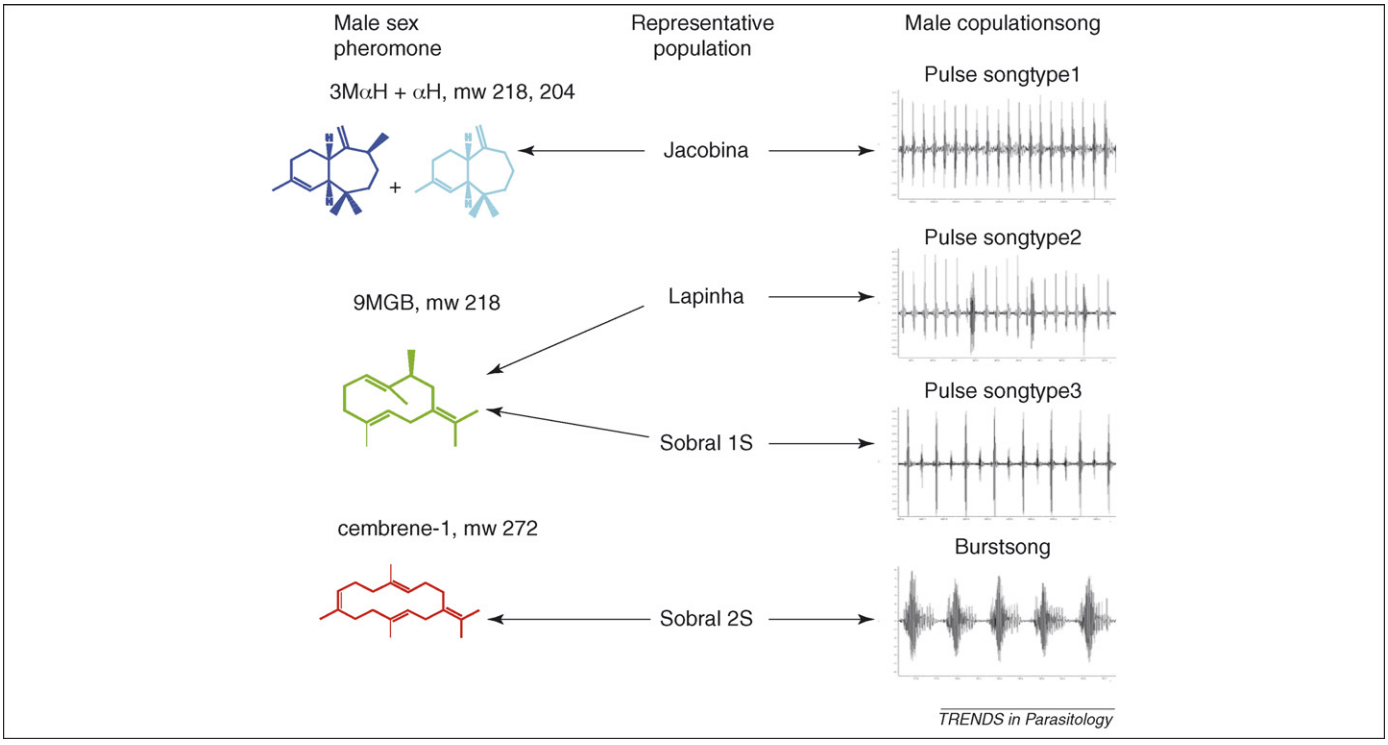

Figure 1. Male sex pheromone chemotypes and copulation songs in four Brazilian *Lutzomyia longipalpis* siblings. αH, α-himachalene; 3MαH, sesquiterpene 3-methyl-α-himachalene; 9MGB, the sesquiterpene (*S*) 9-methyl-germacrene B; 1S, males with one pair of pale patches in the 4th tergite; 2S, males with two pairs of pale patches in the 3rd and 4th tergites.

El Paso, a 9MGB population is sympatric with the named sibling species *Lu. pseudolongipalpis* Arrivillaga and Feliciangeli 2001 [12,34] and there was congruence between genetic and morphometric analyses [13,26], with males producing only 3MαH and not the 3MαH + αH unique to Jacobina [26]. It would be interesting to analyse the copulation song for this sibling species.

Introgression is a key feature of recent speciation [32]. It is likely that introgression has occurred in *Lu. longipalpis* s.l. because Brazilian populations with different pheromones [19–22] and copulation love songs [23,24] showed relatively low levels of isoenzyme divergence [7]. Indeed, only two out of the five microsatellite loci analysed in the Sobral sympatric sibling species showed strong differentiation (possibly closely linked to 'speciation' genes), whereas the other three might have introgressed [25], as was perhaps also the case for the *cacophony* gene [29]. The *Lu. longipalpis* s.l. microsatellite data resembles results for *An. gambiae* s.l. and indicates differential introgression rates along the genome [15,16]. Results obtained for the taxonomic status of *Lu. longipalpis* s.l. using different genetic markers possibly reflect not only different marker-mutation rates but also distinct chromosomal-marker locations, some of which are more prone to the molecular mechanisms of chromosome evolution that affect gene flow during introgression [15,16]. Indeed, Yin *et al.* reported chromosome rearrangements in an analysis of G-banding metaphase karyotypes of *Lu. longipalpis* sibling species [35].

Ecological adaptability, chromosome evolution and introgression

Lu. longipalpis s.l. and VL distribution coincide, with endemic foci showing wide ecological variation from Southern Mexico to Argentina [5,6]. This agrees with observed genetic differentiation among populations from different habitats [7]. Endemic regions are adjacent to forests inhabited by *Lu. longipalpis* and *Le. chagasi* reservoir sylvatic animals in which infection is benign. Endemic regions are characterised predominantly by semi-arid agricultural with human dwellings close to livestock that include infected dogs [5]. However, with progressive urbanisation within endemic regions, this sand fly is adapting to deforested drier areas, changing transmission patterns [36].

The most suggestive evidence of the epidemiological impact of population sub-structure of this sand fly was reported by Watts *et al.* [26] on a cluster of genotypically similar populations with similar pheromone and love songs that mapped to the Brazilian Northeastern region, contributing more than 90% of the cases of VL from the New World. However, the exact distribution limits of this 'species' are unknown and it is possible that other factors account for the higher number of cases of VL in this region.

How does complex insect-vector population sub-structure provide adaptation opportunities to new environments? Genes involved in multiple reproductive isolation mechanisms between sympatric *Drosophila pseudoobscura* and *Drosophila persimilis* populations map to species-specific inverted chromosomal regions [37]. In *Anopheles gambiae* s.l., microsatellite markers display allele differences also mapping within chromosomal inversions between the still evolving *An. gambiae* s.s. (more anthropophilic and adapted exquisitely to breed in man-made rain pools) and its sibling species *An. arabiensis* (more zoophilic and adapted to dry landscapes). Chromosomal inversions would protect adaptive genes effectively within

and around the break points from crossing over during meiosis, therefore enabling their accumulation within inversions [37]. *An. gambiae* s.s. are further subdivided into rRNA-defined M and S population forms in which rRNA genes and most nucleotide differences map to X-chromosome highly repeated centromeric regions that are also less prone to introgression [16]. Differential introgressive hybridisation across the genomes of adjacent or partially overlapping closely related species might pass adaptive traits (and their linked regions by hitch-hiking), as appears to have been the case, for example, with adaptability to drier habitats from *An. arabiensis* to *An. gambiae* s.s. [15]. However, transgenic-vector control requires reasonable introgression rates across vector sibling populations and, indeed, adaptive genes might be ideal targets themselves to help spread transgenes [38].

Drosophila and *Anopheles* studies on the mechanisms for adaptability might apply to *Lu. longipalpis*, although their different biology must be considered. In sand fly vectors, adaptive loci also include those encoding traits involved in host–parasite coevolution, as discussed later. Mapping loci to chromosomes in sand flies, however, would require alternative genome-wide or molecular karyotyping because intact sand fly polytene chromosomes are notoriously difficult to isolate [39].

Sand fly and *Leishmania* genetic variation

Sand flies are more than mere 'flying syringes', providing their gut-adapted *Leishmania* parasites with the necessary molecular interactions for *Leishmania* survival, differentiation, transmission and establishment into suitable vertebrate hosts. Most, if not all, of these sand fly–*Leishmania* interactions are specific and likely to be dependent on their shared endemic local landscape. *Leishmania*–sand fly gut-specific binding, downregulation of gut protease activity and degradation of peritrophic membrane chitin by upregulated activities of sand fly gut or *Leishmania* chitinase(s) would ensure survival within the sand fly gut [40]. Unknown sand fly gut molecules trigger *Leishmania* differentiation and secretion of a filamentous proteophosphoglycan-gel-like plug that blocks the stomodeal gut valve and also entangles and concentrates metacyclics at the biting site, aiding *Leishmania* transmission [40]. Recently, it has been suggested that adaptation of 'imported' Mediterranean *Le. infantum* to the New World involved specific binding between a *Leishmania* surface lectin and a *Lu. longipalpis* glycoprotein [41].

Furthermore, experimental animal models and epidemiological studies showed that sand flies facilitate *Leishmania* initial establishment in vertebrate hosts with a battery of sand fly salivary molecules co-opted by the parasite to downregulate the host's immune response at the biting site [42]. Initial attempts linking sand fly genetic variability to vectorial competence and *Le. chagasi* virulence focused on the salivary vasodilatory peptide maxadilan [3]. However, maxadilan's highly variable amounts and polymorphism did not correlate with vasodilatory or immunomodulatory functions or indeed with specific sibling species and maxadilan is no longer considered an ideal 'sand fly spit' vaccine [43]. Common salivary proteins among visceralising vector sand fly populations are being identified in an attempt to bypass genetic variability among sibling species [44].

Leishmania genetic variation appears to be determined largely by geographic isolation with only one example to date of a correlation between *Leishmania* genotype and clinical presentation [2]. Although yet to be explored in leishmaniasis, it is likely that *Leishmania* transmission, virulence and clinical outcome are influenced by coevolutionary interactions between specific *Leishmania* and sand fly genotypes, as has been suggested for malaria recently [45]. Parasitism would involve mutual manipulation between the host's and its parasite's genomes to achieve a largely non-virulent equilibrium or 'marriage of convenience' by 'protecting' the host from invasion by new more virulent pathogens that have not coevolved.

Concluding remarks

A meaningful understanding of *Lu. longipalpis* speciation is only possible using a combination of genomic markers and strong field studies of sympatric populations. In addition to sand fly landscape ecology-adaptive genes, those required for *Leishmania* survival, differentiation and transmission are obvious candidates for adaptive coevolution. Their dynamic interaction will determine vector biting rate, longevity and infectivity and, in turn, vectorial capacity, which is an important parameter for predicting basic case reproduction numbers when evaluating disease control measures.

It is challenging to apply evolutionary understanding to vector control strategies. However, post-genomics offers a 'multidisciplinary consortium approach' to understand sand fly ecological and parasite co-adaptation and: (i) define sand fly fast-adapting determinants linked to transmission risk factors responsive to climate and demographic changes; (ii) target the 'real' vector' by integrated vector control, including '*Leishmania*-unsupportive' engineered sand flies; and (iii) identify new coevolution transmission-blocking vaccine components.

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References

1 Blackwell, J.M. *et al.* (2004) Genetics and visceral leishmaniasis in the Sudan: seeing a link. *Trends Parasitol.* 20, 268–274
2 Mahdi, M. *et al.* (2005) Sudanese mucosal leishmaniasis: isolation of a parasite within the *Leishmania donovani* complex that differs genotypically from *L. donovani* causing classical visceral leishmaniasis. *Infect. Genet. Evol.* 5, 29–33
3 Lanzaro, G.C. and Warburg, A. (1995) Genetic variability in phlebotomine sandflies: possible implications for leishmaniasis epidemiology. *Parasitol. Today* 11, 151–154
4 Convit, J. *et al.* (2006) Inorganic particles in the skin of inhabitants of volcanic areas of Central America: their possible immunomodulatory influence in leishmaniasis and leprosy. *Trans. R. Soc. Trop. Med. Hyg.* 100, 734–739

5 Lainson, R. and Rangel, E.F. (2005) *Lutzomyia longipalpis* and the eco-epidemiology of American visceral leishmaniasis, with particular reference to Brazil: a review. *Mem. Inst. Oswaldo Cruz* 100, 811–827
6 Conn, J.E. and Mirabello, L. (2007) The biogeography and population genetics of neotropical vector species. *Heredity* 99, 245–256
7 Bauzer, L.G.S.R. *et al.* (2007) *Lutzomyia longipalpis* in Brazil: a complex or a single species? A Mini-review. *Mem. Inst. Oswaldo Cruz* 102, 1–12
8 Lutz, A. and Neiva, A. (1912) Contribução para o conhecimento das espécies do gênero *Phlebotomus* existentes no Brasil. *Mem. Inst. Oswaldo Cruz* 4, 84–95
9 Mangabeira, O.F. (1969) Sóbre a sistemática e biologia dos *Phlebotomus* do Ceará. *Rev. Bras. Malariol. Doencas Trop.* 12, 3–26
10 Ward, R.D. *et al.* (1983) Reproductive isolation between different forms of *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae), the vector of *Leishmania donovani chagasi* Cunha & Chagas and its significance to kala-azar distribution in South America. *Mem. Inst. Oswaldo Cruz* 78, 269–280
11 Lanzaro, G.C. *et al.* (1993) *Lutzomyia longipalpis* is a species complex: genetic divergence and interspecific hybrid sterility among three populations. *Am. J. Trop. Med. Hyg.* 48, 839–847
12 Lampo, M. *et al.* (1999) Occurrence of sibling species of *Lutzomyia longipalpis* (Diptera: Psychodidae) in Venezuela: first evidence from reproductively isolated sympatric populations. *Am. J. Trop. Med. Hyg.* 61, 1004–1009
13 Arrivillaga, J. *et al.* (2003) The taxonomic status of genetically divergent populations of *Lutzomyia longipalpis* (Diptera: Psychodidae) based on the distribution of mitochondrial and isoenzyme variation. *J. Med. Entomol.* 40, 615–627
14 Pesson, B. *et al.* (2004) Sandflies of the *Phlebotomus perniciousus* complex: mitochondrial introgression and a new sibling species of *P. longicuspis* in the Moroccan Rif. *Med. Vet. Entomol.* 18, 25–37
15 Besansky, N.J. *et al.* (2003) Semipermeable species boundaries between *Anopheles gambiae* and *Anopheles arabiensis*: evidence from multilocus DNA sequence variation. *Proc. Natl. Acad. Sci. U. S. A.* 100, 10818–10823
16 Stump, A.D. *et al.* (2005) Centromere-proximal differentiation and speciation in *Anopheles gambiae*. *Proc. Natl. Acad. Sci. U. S. A.* 102, 15930–15935
17 Depaquit, J. *et al.* (2002) ITS2 sequences heterogeneity in *Phlebotomus sergenti* and *Phlebotomus similis* (Diptera: Psychodidae): possible consequences in their ability to transmit *Leishmania tropica*. *Int. J. Parasitol.* 32, 1123–1131
18 Ward, R.D. *et al.* (1988) The *Lutzomyia longipalpis* complex: reproduction and distribution. In *Biosystematics of Haematophagous Insects* (Service, M.W., ed.), pp. 257–269, Systematics Association Special Volume 37
19 Hamilton, J.G.C. *et al.* (1996) 9-methyl-germacrene B, a novel homosesquiterpene from sex pheromone glands of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Lapinha. *Brazil. J. Chem. Ecol.* 22, 1477–1491
20 Hamilton, J.G.C. *et al.* (1996) 3-methyl-α-himachalene, sex pheromone of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Jacobina, Brazil. *J. Chem. Ecol.* 22, 2331–2340
21 Hamilton, J.G. *et al.* (2004) A fourth chemotype of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Jaíbas, Minas Gerais State, Brazil. *J. Med. Entomol.* 41, 1021–1026
22 Hamilton, J.G. *et al.* (2005) Analysis of the sex pheromone extract of individual male *Lutzomyia longipalpis* sandflies from six regions in Brazil. *Med. Vet. Entomol.* 19, 480–488
23 de Souza, N.A. *et al.* (2002) Copulation songs in three siblings of *Lutzomyia longipalpis* (Diptera: Psychodidae). *Trans. R. Soc. Trop. Med. Hyg.* 96, 102–103
24 Souza, N.A. *et al.* (2004) Analysis of the copulatory courtship songs of *Lutzomyia longipalpis* in six populations from Brazil. *J. Med. Entomol.* 41, 906–913
25 Maingon, R.D.C. *et al.* (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
26 Watts, P.C. *et al.* (2005) Male sex pheromones and the phylogeographic structure of the *Lutzomyia longipalpis* species complex (Diptera: Psychodidae) from Brazil and Venezuela. *Am. J. Trop. Med. Hyg.* 73, 734–743
27 Bauzer, L.G. *et al.* (2002) The *period* gene and genetic differentiation between three Brazilian populations of *Lutzomyia longipalpis*. *Insect Mol. Biol.* 11, 315–323
28 Bauzer, L.G. *et al.* (2002) Molecular divergence in the *period* gene between two putative sympatric species of the *Lutzomyia longipalpis* complex. *Mol. Biol. Evol.* 19, 1624–1627
29 Bottecchia, M. *et al.* (2004) Genetic divergence in the *cacophony* IVS6 intron among five Brazilian populations of *Lutzomyia longipalpis*. *J. Mol. Evol.* 58, 754–761
30 Jones, T.M. and Hamilton, J.G.C. (1998) A role for pheromones in mate choice in a lekking sand fly. *Anim. Behav.* 56, 891–898
31 Spiegel, C.N. *et al.* (2005) (1S, 3S, 7R)-3-methyl-α-himachalene from the male sandfly *Lutzomyia longipalpis* (Dipetra: Psychodidae) induces neurophysiological responses and attracts both males and females. *J. Insect Physiol.* 51, 1366–1375
32 Coyne, J.A. and Orr, H.A. (2004) *Speciation,* Sinauer Associates
33 Mazzoni, C.J. *et al.* (2006) Molecular polymorphism, differentiation and introgression in the *period* gene between *Lutzomyia intermedia* and *Lutzomyia whitmani*. *BMC Evol. Biol.* 6, 85
34 Arrivillaga, J.C. and Feliciangeli, M.D. (2001) *Lutzomyia pseudolongipalpis*: the first new species within the *longipalpis* (Diptera: Psychodidae: Phlebotominae) complex from La Rinconada, Curarigua, Lara State, Venezuela. *J. Med. Entomol.* 38, 783–790
35 Yin, H. *et al.* (1999) Metaphase kayotypes and G banding in sandflies of the *Lutzomyia longipalpis* complex. *Med. Vet. Entomol.* 13, 72–77
36 Oliveira, A.G. *et al.* (2006) Abundance of *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae) and urban transmission of visceral leishmaniasis in Campo Grande, state of Mato Grosso do Sul, Brazil. *Mem. Inst. Oswaldo Cruz* 101, 869–874
37 Ayala, F.J. and Coluzzi, M. (2005) Chromosome speciation: humans, *Drosophila* and mosquitoes. *Proc. Natl. Acad. Sci. U. S. A.* 102, 6535–6542
38 Marrelli, M.T. *et al.* (2007) Transgenic malaria-resistant mosquitoes have a fitness advantage when feeding on *Plasmodium*-infected blood. *Proc. Natl. Acad. Sci. U. S. A.* 104, 5580–5583
39 White, G.B. and Killick-Kendrick, R. (1975) Demonstration of giant chromosomes in the sand fly *Lutzomyia longipalpis* (Lutz and Neiva 1912). *Trans. R. Soc. Trop. Med. Hyg.* 69, 427–428
40 Kamhawi, S. (2006) Phlebotomine sand flies and *Leishmania* parasites: friends or foes? *Trends Parasitol.* 22, 439–445
41 Myskova, J. *et al.* (2007) A lipophosphoglycan-independent development of *Leishmania* in permissive sand flies. *Microbes Infect.* 9, 317–324
42 Kamhawi, S. (2001) The biological and immunomodulatory properties of sandfly saliva and its role in the establishment of *Leishmania* infections. *Microbes Infect.* 2, 1765–1773
43 Milleron, R.S. *et al.* (2004) Antigenic diversity of maxadilan, a salivary protein from the sandfly vector of American cutaneous leishmaniasis. *Am. J. Trop. Med. Hyg.* 70, 286–293
44 Anderson, J.M. *et al.* (2006) Comparative salivary gland transcriptomics of sand fly vectors of visceral leishmaniasis. *BMC Genomics* 7, 52
45 Lambrechts, L. *et al.* (2005) Host genotype by parasite genotype interactions underlying the resistance of Anopheline mosquitoes to *Plasmodium falciparum*. *Malar. J.* 4, 3
46 Cardé, R.T. and Haynes, K.F. (2004) Structure of the pheromone communication channel in moths. In *Advances in Insect Chemical Ecology* (Cardé, R.T. and Millar, J.G., eds), Cambridge University Press
47 Lane, R. *et al.* (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
48 Bray, D.P. and Hamilton, J.G. (2007) Host odour synergises the attraction of virgin female *Lutzomyia longipalpis* (Diptera: Psychodidae) to male sex pheromone. *J. Med. Entomol.* 44, 779–787
49 Hall, J.C. (1994) The mating of a fly. *Science* 264, 1702–1714
50 Gleason, J.M. (2005) Mutations and natural genetic variation in the courtship song of *Drosophila*. *Behav. Genet.* 35, 265–277

51 Konopka, R.J. and Benzer, S. (1971) Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 68, 2112–2116
52 Kyriacou, C.P. and Hall, J.C. (1980) Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuations in the male's courtship song. *Proc. Natl. Acad. Sci. U. S. A.* 77, 6729–6733
53 Wheeler, D.A. *et al.* (1991) Molecular transfer of a species-specific behaviour from *Drosophila simulans* to *Drosophila melanogaster*. *Science* 251, 1082–1085
54 Peixoto, A.A. and Hall, J.C. (1998) Analysis of temperature-sensitive mutants reveals new genes involved in the courtship song of *Drosophila*. *Genetics* 148, 827–838