Chapter 13

Sand fly sex/aggregation pheromones

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Abstract

Sand flies are an ancient group of Diptera estimated to contain 1000 species. Approximately 70 of these transmit pathogens (viruses, bacteria and protists), which cause human and animal diseases. The most important are the *Leishmania* parasites, transmitted to humans and animals, during blood feeding by female sand flies, and which cause diseases that can be fatal or disfiguring. Sand flies are known to use volatile chemicals produced by plants to locate sugar meals, host odours to locate a blood meal, and chemicals from decaying vegetation and other sources to identify oviposition sites. In a limited number of cases, male sand flies also produce volatile chemicals (sex/aggregation pheromones) that are attractive to females and other males. The presence of sex/aggregation pheromones is well documented in *Lutzomyia longipalpis sensu lato,* the South American vector of *Leishmania infantum*, in which they were first identified 40 years ago. During this time, a range of behavioural and chemical methodologies have been applied to their study in the laboratory and the field. The presence of sex/aggregation pheromones has also been suggested in a small number of other New and Old-World vectors, but the evidence is incomplete, as it is either solely chemical, i.e. without supporting behavioural evidence or behavioural evidence is available, but there is no supporting chemical evidence. Within the *Lu. longipalpis s.l.* species complex, the sex/aggregation pheromones provide a taxonomic guide to the members of the complex. There are four different known chemical types (five members of the complex), and one of these, the most geographically widespread, has been synthesised in bulk quantity. The synthetic pheromone, co-located with insecticide, has been shown to significantly reduce numbers of sand flies, and leishmania infection in dogs, the reservoir of human infection, and could significantly impact the number of human cases.

Keywords: phlebotomines, leishmaniasis, vector control, *Leishmania infantum*, (*S*)-9-methyl-germacrene-B, 3-methyl-α-himachalene, sobralene

13.1 Introduction

13.1.1 Sand flies and leishmaniasis

Sand flies belong to an ancient Dipteran family, the Psychodidae. There are six Psychodid subfamilies, and only the Phlebotominae and the Sycoracinae have biting mouthparts capable of piercing animal skin and feeding on blood. The Phlebotominae, commonly known as sand flies, has nearly 1000 species, which primarily feed on mammals, birds and reptiles. Around 70

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of these species are important to humans because they can transmit pathogens, which cause important medical and veterinary diseases (Lane, 1993; Munstermann, 2019). By comparison, the Sycoracinae has 45 known species, three of which are known to feed on amphibians.

The Phlebotominae have an elongated and more fragile structure than the other physically shorter and broader, and more robust Psychodid subfamilies. These sand flies have a hairy appearance, their bodies are 1.5-3.5 mm long, with large black eyes and long, stilt-like legs. Their hairy wings are held erect in a 'V' shape, at an angle of 40° over the body, when the fly is resting or blood feeding. The subfamily Phlebotominae, which are found around the world in tropical, sub-tropical and temperate zones, are subdivided into six genera, *Phlebotomus* (Afrotropical, Palearctic, Indo-Malay), *Chinius* (China), *Sergentomyia* (Afrotropical, Asia), *Lutzomyia*, *Warileya* and *Brumptomyia* (Neotropical). However, as new genera have been identified, and as many subgenera within *Lutzomyia* have been raised to generic status, there are now 23 proposed genera (Galati, 2003).

Sand flies are obligate haematophages, and two genera in particular, *Lutzomyia* and *Phlebotomus,* are the natural vectors of most of the pathogenic organisms transmitted by sand flies to humans and animals. These pathogenic organisms include the intracellular protozoan parasites *Leishmania* (*Leishmania*) spp. (Kinetoplastida: Trypanosomatidae), phleboviruses (Bunyavirales: Phenuiviridae) and bacterium *Bartonella bacilliformis*, which cause Leishmaniasis (visceral, cutaneous and muco-cutaneous), sand fly fever or vesicular stomatitis and bartonellosis, respectively. Leishmaniasis causes an estimated loss of 2.4 million disability-adjusted life years (DALYs) (Hotez, 2018), and is considered the most important of these diseases because of its impact on humans and animals. This burden, estimated to be the ninth largest among individual infectious diseases (Hotez *et al.*, 2004, 2006), is carried predominantly by the urban and rural poor (Alvar *et al.*, 2006; Donato *et al.*, 2020).

Leishmaniasis is a complex disease, in which the clinical manifestations of infection in humans depends on the species of *Leishmania* parasite, the host immune response (Hong *et al.*, 2020) and the sand fly vector (Lestinova *et al.*, 2017; Warburg *et al.*, 1994). Visceral leishmaniasis (VL), also known as kala-azar, is characterised by irregular bouts of fever, weight loss, enlargement of the spleen and liver, and anaemia, and has a 95% case fatality in the absence of treatment (Martins-Melo *et al.*, 2014; WHO, 2017; 2020b). Between 50 000 to 90 000 new human VL cases are reported each year (Alvar *et al.*, 2012; WHO, 2017), with an estimated actual occurrence of between 200 and 400 thousand cases. Visceral leishmaniasis occurs predominantly in ten countries: Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan and Sudan (Alvar *et al.*, 2012; Burza *et al.*, 2018).

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis, and although not fatal, it is characterised by significant morbidity. Skin lesions, which lead to life-long scars, can be seriously stigmatising, when present on exposed parts of the body. About 95% of CL cases occur in the Americas, the Mediterranean basin, the Middle East and Central Asia (WHO, 2020b). Over 200,000 cases are reported annually, but it is estimated that between 700,000 to 1.2 million new cases occur worldwide (Alvar *et al.*, 2012; WHO, 2017).

Mucocutaneous leishmaniasis (MCL), also known as espundia, is a development of CL (Hong *et al.*, 2020), which leads to partial or total destruction of mucous membranes of the nose, mouth and throat. Over 90% of MCL cases occur in Bolivia, Brazil, Ethiopia and Peru. It has been difficult to estimate the numbers of cases of MCL or post-kala-azar dermal leishmaniasis because of lack of data (Alvar *et al.*, 2012).

Leishmaniasis is considered a neglected tropical disease (NTD), because of underreporting, lack of access to diagnosis and lack of access to inexpensive and effective treatments (Alvar *et al.*, 2006, 2012). Control relies upon multiple approaches, including diagnosis, treatment and vaccines, detection, control of reservoir hosts, epidemic response, vector control and education (WHO, 2010). These strategies have met with varying degrees of success, however, Leishmaniasis remains a severe and potentially growing problem in many areas because of urbanisation, population disruption, increase in peridomestic habitation and changing climate patterns (WHO, 2010).

13.1.2 Sex pheromones

Volatile chemicals used for communication between (inter-) and within (intra-) species are known as semiochemicals, and sex pheromones used within species are a subclass of these chemicals. Semiochemicals include pheromones, kairomones, allomones and synomones, and examples can be found from across the animal kingdom (Cardé and Millar, 2009). Pheromones are used in intraspecific communication, the definition is 'a chemical or a mixture of chemicals that is released to the exterior by an organism that causes one or more specific reactions in a receiving individual of the same species' (Shorey, 1976). Pheromones mediate a wide variety of responses, and arguably the best examples are mate attraction in the Lepidoptera, trail following by ants or the regulation of larval development to workers or queens in honeybees (Wyatt, 2019).

Sex pheromones of Lepidoptera are volatile chemicals, usually active in very small quantities, made up of a very specific blend of biosynthetically related hydrocarbons, 10-18 carbons in length. Typically, these pheromones have 1-3 double bonds, a terminal acetate, alcohol, or aldehyde, and are produced by females from specialised abdominal glands, to attract males, sometimes over very long distances. Since the pioneering work that led to the chemical identification of the first sex pheromone in the Lepidoptera (Butenandt, 1959), the blend of chemicals comprising the sex pheromone of many species has been identified. Sex pheromones are long-range attractants (over tens to hundreds of metres), and when coupled with the specificity of the antennal receptors (Foster and Dugdale, 1988) constitute a narrow, stable communication channel (Leary *et al.*, 2012), which act as a species-isolating barrier (Cardé and Haynes, 2004).

Although male-produced sex pheromones are found in several insect orders, these are often referred to as aggregation pheromones, to distinguish them from female-produced sex pheromones. This terminology can be confusing as it tends to obscure a principal function of the pheromone, which is to facilitate the co-location of females and males for mating (Cardé, 2014), and could also be confused with other aggregation pheromones, such as the defensive aggregation pheromone of aphids, the 'mass attack' aggregations of some Coleopterans, the attachment pheromones in hard ticks (Rechav *et al.*, 1977), or the recently identified swarming aggregation pheromone in mosquitoes

(Mozūraitis *et al.*, 2020). Therefore, to encapsulate the multi-purpose nature of the activity of male-produced pheromones in sand flies, these have been called sex/aggregation pheromones, i.e. they are produced by males and are attractive to both conspecific females and males. Most evidence for the presence of a sex/aggregation pheromone in the Phlebotominae comes from *Lutzomyia longipalpis s.l.* There is also some evidence for the presence of sex/aggregation pheromones in other sand fly species, but in most cases it is incomplete. Within *Lu. Longipalpis*, many aspects relating to the biology and ecology of sex/aggregation pheromone are poorly understood.

13.2 Identification of sex/aggregation pheromones in sand flies

Evidence for the presence of sex/aggregation pheromones in sand flies has come from both behavioural and chemical experiments, in both laboratory and field settings. Behavioural evidence has come from both observational and manipulative experiments, and chemical evidence includes an array of chromatographic techniques allied with advanced structural analysis and synthesis.

13.2.1 Behavioural evidence

The identification of the *Lu. longipalpis* sex/aggregation pheromone started with observations of the behaviour of male sand flies, and the potential source of pheromone production (Lane and Ward, 1984). Two morphological forms of *Lu. longipalpis*, males with either one or two pale patches on abdominal tergites 4 or 3 and 4 (1 spot or 2 spot), were originally described (Mangabeira Filho, 1969). It was thought that these 'spots' were markers, which could be used to differentiate between members of a *Lu. longipalpis* species complex, however, subsequent cross mating studies (Ward *et al.*, 1983, 1988) and pheromone analysis (Hamilton *et al.*, 2005), have shown that this is not the case. Nonetheless, in some localities, e.g. Sobral (Ceará State) and Jaíba (Minas Gerais State), differences in spot morphology allow us to conveniently differentiate between different sympatric sex/aggregation producing populations.

The role of the spots as a site for pheromone production was initially suggested after scanning and transmission electron microscopy examination suggested a glandular function (Lane and Ward, 1984; Ward *et al.*, 1993). The confirmation that chemicals produced by male *Lu. longipalpis* had pheromonal activity started with simple cage experiments, in which a hexane extract of whole male sand flies, as well as abdominal sections containing the excised spot(s), was placed on filter paper resting on the abdomen of an anaesthetised hamster in a cage with 30 female sand flies. Controls were filter paper disks with hexane only placed on an adjacent hamster. The number of times that females alighted on the filter paper in a given period of time was recorded, and activity was concluded if there were significantly more contacts on the test filter paper compared to the control (Ward, 1986). These experimental designs were simple to stage, but suffered from several drawbacks, including potential interaction between the sand flies within the cage, difference in, and confounding effect of, the attractiveness of hamsters and pseudo-replication. However, these experiments were useful for providing information on the potential biological activity of the extracts. An improved experimental design, in which two cages containing either a test or control stimulus (live males, extract of males, extract of abdomens) were connected via tubing in a Y-shape to a third cage, in which live females were placed. Air drawn through the system by a small fan

placed in the cage, in which the females were released, provided a directional dimension to the sand fly response. The resulting distribution of females after a period of time was then recorded (Ward *et al.*, 1989). This methodology allowed full separation of the test and control stimuli from each other, however, potential interactions between females (and males), pseudo-replication, the presence of live males and live hamsters in close proximity to the release point of the females, could all have had a confounding effect on outcomes.

Wind-tunnels are a useful way to measure response of individual insects to odours, and have been used with success for a wide range of flying haematophagous insects (Kainoh, 2011; Knudsen *et al.*, 2018). However, sand flies are small and believed to be weak fliers (Killick-Kendrick *et al.*, 1984), and they move towards their destination in a series of short hops. To overcome the effects of the static electricity, which can limit sand fly movement in an apparatus typically constructed from Perspex panels, the wind-tunnels used to test sand fly response to potential sex/aggregation pheromones have been made out of netting, and thus provide a surface on which the sand flies may alight (Morton and Ward, 1989). Using this type of wind-tunnel it was shown that female *Lu. longipalpis* were attracted to pheromone extract and host odour over 2.4 m. A similar approach was adopted, using a netting tube suspended within a frame enclosed within a Perspex tube, to measure the response of female and male *Lu. longipalpis* to synthetic sex pheromone (Spiegel *et al.*, 2005) or host odour (Dougherty *et al.*, 1999) (Figure 13.1A). A convenient alternative to the more sophisticated wind tunnel approach, but which overcomes the limitations of the cage bioassays, is the use of a glass Y-tube olfactometer (Figure 13.1B) (Bray and Hamilton, 2007). The glass and other components can be easily cleaned to remove potential contaminating odours, and sand flies can be introduced individually so that the experiments can be repeated many times to make them statistically robust. This apparatus can also be modified to introduce odour extracts or odour from live animal sources (Nevatte *et al.*, 2017), while at the same time removing potential confounding stimuli (acoustic/visual, etc.) The main limitation of the Y-tube olfactometer is that it is limited to the demonstration of upwind anemotaxis only.

Observational experiments are important in providing initial evidence for the presence of sex/aggregation pheromones in species of sand flies other than *Lu. longipalpis*. There is observational evidence for the presence of a male-produced sex/aggregation pheromone in *Phlebotomus argentipes* in Sri Lanka (Lane *et al.*, 1990), in which it was noted that male *P. argentipes* swarm on their host and beat their wings in short pulses. The authors concluded that *P. argentipes* may use a pheromone, as this behaviour is similar to the lekking behaviour of *Lu. longipalpis s.l.* (Lane *et al.*, 1990). Similar observations were made on *P. argentipes* at animal hosts in India (Palit *et al.*, 1993) and *P. orientalis* in Ethiopia, where males were observed to aggregate, dance and wing flutter (Ashford, 1974). Wing-fluttering in *Lu. longipalpis* and *P. argentipes* (Araki *et al.*, 2020) during copulation is related to acoustic communication, and is strongly associated with post-copulatory species isolation (Vigoder *et al.*, 2020). Wing fluttering, prior to copulation, may also be related to distribution of pheromone (Jones and Hamilton, 1998).

In subsequent manipulative cage bioassays, female *P. argentipes* were shown to respond positively to hexane extracts of male sand flies by an increased number of contacts with filter paper disks containing the male extract (Kumar *et al.*, 2012). The presence of host odour also increased the

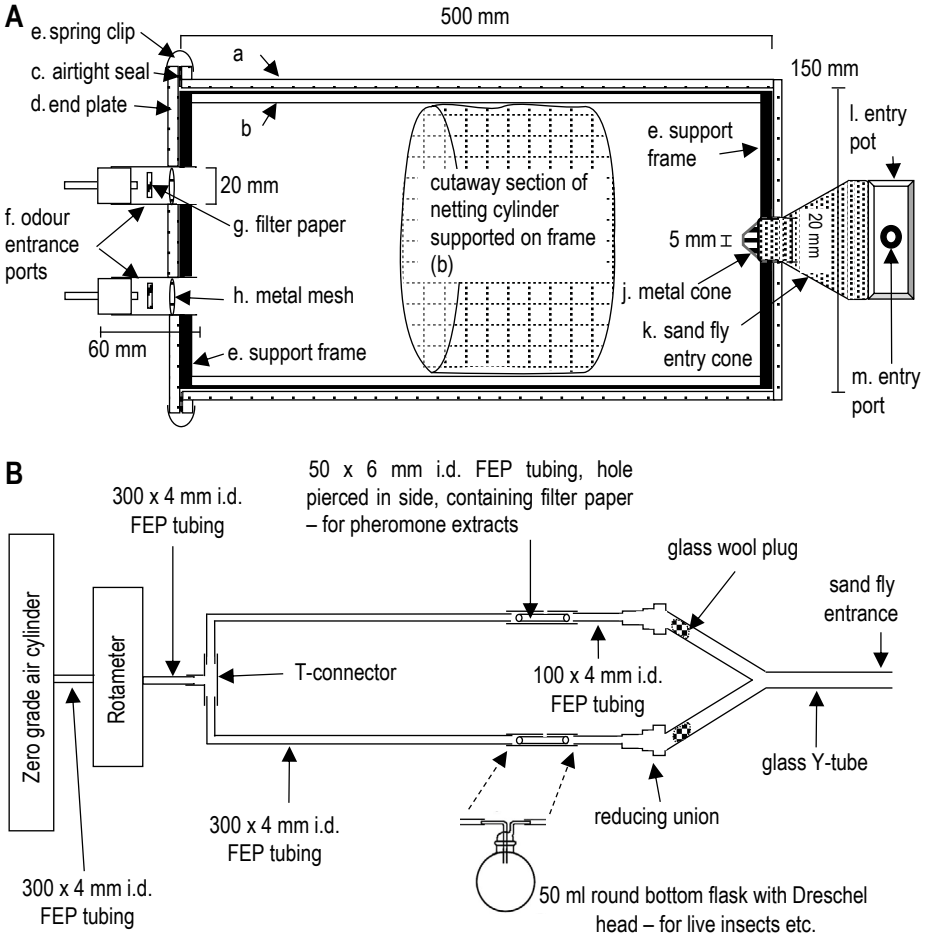


Figure 13.1. Schematic diagrams of two different types of olfactometers, (A) wind tunnel and (B) Y-tube, used to study sand fly response to volatile chemicals, including sex/aggregation pheromones. (A) (a) A Perspex tube forming the body of a wind tunnel olfactometer, 500 mm long x 150 mm diameter (i.d.), (b) Perspex frame supporting netting cylinder inside Perspex tubing, (c) airtight seal between endplate and Perspex cylinder, (d) removable end plate, to allow access to interior of olfactometer for cleaning and removal of sand flies, (e) spring clips around the end plate and end of Perspex tubing holds two components together firmly during use, (f) odour entrance ports (×2) where the air from compressed gas cylinders enters the olfactometer via Perspex tubing (60 mm × 20 mm i.d.). (g) filter paper, for placing solutions containing compounds of interest, (h) metal mesh fit to prevent the escape of sand flies, (j) metal mesh cone attached to the end plate of netting frame, allowing entrance of sand flies and restricting their exit, (k) entry cone milled from single block of Perspex, (l) pot for holding sand flies prior to entry into olfactometer, and (m) port for placing sand flies into holding pot. Redrawn from Dougherty *et al.* (1999). (B) Glass Y-tube shown on right of diagram with Teflon tubing connections to 50 ml round bottom or larger flasks (up to 5 L), which can be used to hold sand flies or other sources of odour. Flasks are connected via Teflon tubing to compressed air supply (zero grade) and rotameter to adjust airflow. Airflow typically set at 120 ml/min. Individual male or female sand flies are introduced at the entry point in the stem of the olfactometer, and their position in either arm (or no response) noted after 2 or 3 minutes (Nevatte *et al.*, 2017).

number of contacts made by the female sand flies on the filter paper discs containing the male sand fly extract (Kumar *et al.*, 2012). There is similar evidence that the effect of a male sex/aggregation pheromone in *Lu. longipalpis* is synergised by the presence of host odour (Bray and Hamilton, 2007). Female *P. argentipes* also showed an anemotactic response to extracts of males in Y-tube olfactometer experiments, however, the response was dependent on the age of the sand flies, and also on the presence of host odour (Yaman, 2016). Similarly, *P. papatasi* females were attracted to the headspace volatiles of small groups of males, as well as of males and females together, but they were not attracted to females in Y-tube olfactometer experiments (Chelbi *et al.*, 2011). Field experiments showed that small groups of males and females, held together in small netting cages, were attractive to females and males, whereas large groups of males and females together were repellent at close range, but increased the proportion of females caught, compared to males overall, suggesting long-range attraction of females (Chelbi *et al.*, 2011). The presence of a sex/aggregation pheromone has also been behaviourally demonstrated in male *Migonemyia migonei* and *Lutzomyia cruciata*, in which females of each species were found to be significantly attracted to hexane extracts of conspecific male abdomens in Y-tube olfactometer experiments (Costa, 2016; Serrano *et al.*, 2016).

13.2.2 Chemical analysis of sex/aggregation pheromones in *Lutzomyia longipalpis s.l.*

Although there is strong behavioural evidence for sex/aggregation pheromone in *Lu. Longipalpis*, and partial evidence in several other species, only in *Lu. longipalpis* and *Lu. cruciata* is there both chemical and behavioural evidence for the presence of a sex/aggregation pheromone. In several other New World *Lutzomyia* species, and some Old World *Sergentomyia* species, there is evidence for the presence of chemicals, which could be sex/aggregation pheromones, but the behavioural evidence for their biological activity is lacking.

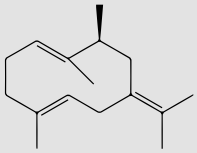
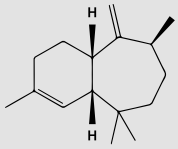
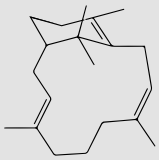
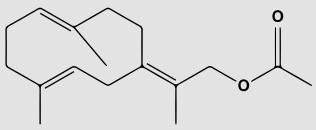
In *Lu. longipalpis*, chemical analysis of the tergal gland extracts, using gas chromatography coupled mass spectrometry (GC/MS), showed the presence of two different types of compounds in populations from different parts of Brazil. These were described as either farnesene/homofarnesene-like or diterpene-like (Lane and Ward, 1984; Lane *et al.*, 1985; Phillips *et al.*, 1986). Compounds present in the extracts were shown to have molecular weights of 204 and 218 or 272 g/mol, and were characterised as terpenes, a class of natural products constructed, in this case, from either three or four 5-carbon (C5) isoprene units.

Fractions containing the major, minor and mixtures of components of the pheromone gland extract from a population of *Lu. longipalpis* sand flies from Jacobina (Ceará State, Brazil) were prepared by high pressure liquid chromatography (HPLC), and bioassays showed that the fraction containing only the major component was responsible for the same amount of biological activity as that which had been observed in the whole extract. The minor components were not biologically active (Hamilton *et al.*, 1994), and led to the conclusion that the major component of the extract is the sex/aggregation pheromone.

Studies have suggested that there are at least five different pheromone types representing cryptic species of *Lu. longipalpis* in South and Central American countries, based on qualitative and quantitative differences in their sex/aggregation pheromones (Hamilton *et al.*, 1996c, 2005; Hamilton and Ward, 1994). The main components of the pheromone gland extracts of several *Lu. longipalpis* populations have been characterised in detail. These populations, which represent the different pheromone types, are named after the area where they were initially collected.

The sex/aggregation pheromone of one member of the complex from Lapinha (Minas Gerais State, Brazil) has been shown to be the novel C16 monocyclic methylsesquiterpene (mw 218), (*S*)-9-methylgermacrene-B (Hamilton *et al.*, 1996b; Hamilton *et al.*, 1999c; Kurosawa and Mori, 2000) and another from Jacobina, the novel C16 bicyclic methylsesquiterpene (mw 218), 3-methyl-α-himachalene (Hamilton *et al.*, 1996a, 1999b; Mori *et al.*, 2000; Spiegel *et al.*, 2005; Tashiro *et al.*, 2000). These two compounds are unusual, in that they have an additional CH_3 (methyl) group, probably added in the early biosynthesis of the molecule (Hamilton *et al.*, 1999a), and have not been found in nature before (Table 13.1).

Table 13.1. Confirmed and tentative structures of sex/aggregation pheromones of the New World sand flies *Lutzomyia longipalpis s.l.* and *Lutzomyia cruciata*.

Common name[1]	Formula	mw	Structure	Source
(*S*)-9-methylgermagrene-B[a] CAS RN: 183158-38-5	$C_{16}H_{26}$	218		*Lu. longipalpis s.l.*
3-methyl-α-himachalene[2]	$C_{16}H_{26}$	218		*Lu. longipalpis s.l.*
Sobralene[3]	$C_{20}H_{32}$	272		*Lu. longipalpis s.l.*
Germacrene-B acetate[4]	$C_{17}H_{26}O_2$	262		*Lu. cruciata*

[1] The common name, molecular formula, molecular weight, structure and sand fly source of known sex/aggregation pheromones.
[2] Structure confirmed by GC/MS, NMR, synthesis and bioassay.
[3] Structure assigned by GC/MS and NMR.
[4] Structure assigned by GC/MS and IR.

Interestingly, males from Lapinha produce approximately twice as much (*S*)-9-methylgermacrene-B as those from the Sobral (*S*)-9-methylgermacrene-B-producing populations. The Lapinha males are also significantly larger. These results led to speculation that the Lapinha pheromone type represented a different member of the *Lu. longipalpis* complex (Hamilton *et al.*, 2005), and recent analysis of molecular correlates (SNPs and CNVs) in the chemosensory genome confirms that these populations have significant genetic differences (Hickner *et al.*, 2021).

Two further members of the *Lu. longipalpis* species complex each produce a different diterpene (C20, mw 272) compound. The structure of one of these compounds, found in the Sobral population with glandular areas on tergites 4 and 3 (2S), is a novel bicyclic pentadeca-*E*-3,4-*Z*-8,9-triene structure, and has been called Sobralene (Palframan *et al.*, 2018, 2019) (Figure 13.2). The chemical structure of the fourth pheromone type of *Lu. longipalpis* from Jaíbas (Minas Gerais, Brazil) has not been elucidated. Previously, both diterpenes were considered to be cembrene isomers (Hamilton *et al.*, 2004), but the elucidation of the Sobralene structure in the Sobral 2S population means that a cembrene structure for the Jaíbas population is unlikely.

Sesquiterpenoids and diterpenoids are enormously diverse classes of natural products, derived from a 15-carbon precursor, farnesyl diphosphate (FPP), and a 20-carbon precursor, geranylgeranyl diphosphate (GGPP), respectively. Within the coniferous and angiosperm plants, which produce a vast array of terpenes, there are closely related terpene synthase (TPS) enzymes that make significantly different terpenes, as well as highly divergent enzymes, which make the same terpenes. In coniferous plants, diterpene and sesquiterpene synthase enzymes are significantly different in sequence and biochemical function (Martin and Bohlmann, 2005). The structural and phylogenetic relationship between the TPS enzymes in the *Lu. longipalpis* complex remain to be clarified, however six of the seven enzymes of the mevalonate-pathway, plus the enzymes involved in sesquiterpenoid biosynthesis, have been found in (*S*)-9-methylgermacrene-B-producing *Lu. longipalpis* (González-Caballero *et al.*, 2014), and Sobralene is believed to be derived via the taxadiene synthase (TXS) catalysed cyclisation cascade from GGPP (Palframan *et al.*, 2019).

13.2.3 Chemical analysis of sex/aggregation pheromones in other sand fly species

Terpenes have also been found in other species of sand flies, and although their function has not been determined through rigorous behavioural analysis, as they have been found in hexane extracts made from males, it is likely that they are male sex/aggregation pheromones. In addition, for the most part, they have only been partially characterised, as full structural determination requires enough chemical to carry out nuclear magnetic resonance (NMR) experiments, followed by *de novo* synthesis and confirmatory behavioural experiments. Male *Lutzomyia pessoai* (Hamilton and Ward, 1994) (suspected vector of *Leishmania braziliensis* in Southern Brazil) were found to produce a diterpene (C20; mw 272), with a mass spectrum similar to that of the *Lu. longipalpis* diterpene population from Sobral, Brazil. Male *Lu. lichyi* were found to produce two compounds with mass spectra consistent with both a primary and tertiary methylsesquiterpene alcohol (Hamilton *et al.*, 1999a). Male *Lu. lenti* was found to produce a series of diterpenes, the most abundant of which (ca. 70%) was significantly different to the diterpene in the Sobral *Lu. longipalpis* population. Thus, the

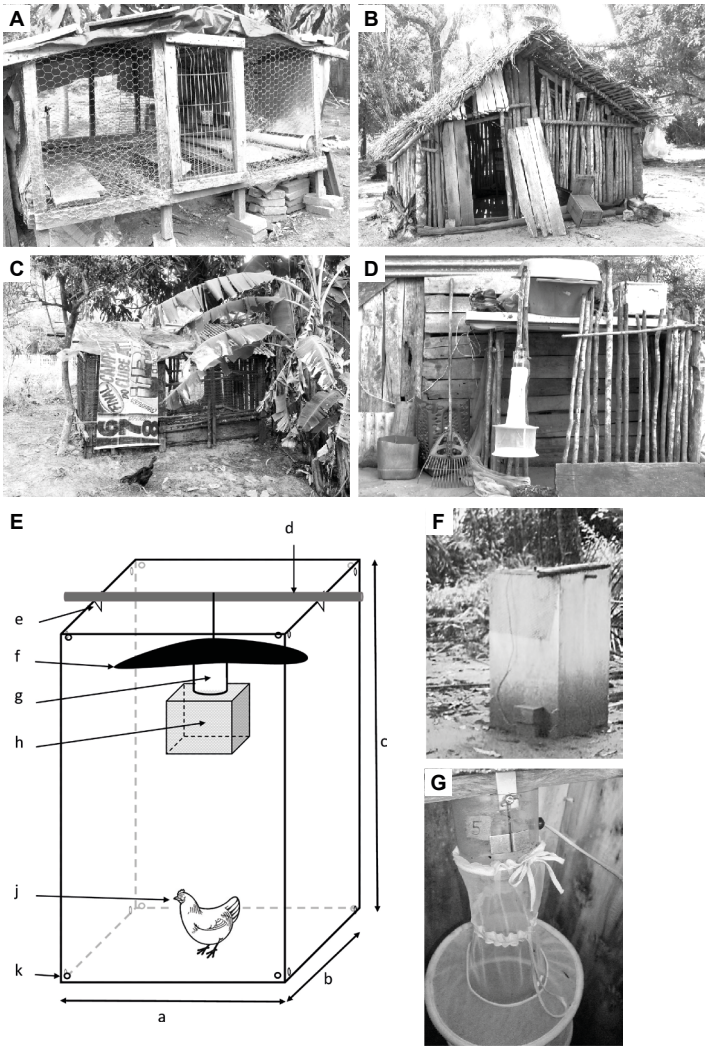


Figure 13.2. The real chicken sheds of Brazilian householders, illustrating the range of sizes, from larger more permanent structures (A and B) to smaller semi-permanent structures (C and D), as well as the methods and materials, used in their construction. The primary purpose of the chicken sheds is to provide a safe overnight roost for the occupants. Experimental chicken sheds were used to standardise field experiments with the synthetic sex/aggregation pheromone 9-methylgermacrene-B. (E) Diagram of experimental chicken shed constructed from 4×10 mm thick plywood panels. (a) 55 cm, (b) 55 cm, (c) 105 cm, (d) wooden rod resting in (e) notches cut in the middle of the top edge of the wooden panels, supporting a modified CDC style light trap, (f) lid of the CDC trap, (g) motor and fan of CDC trap, (h) Barraud cage suspended under the CDC trap to collect sand flies, (j) chicken placed in experimental chicken shed to provide host odour, (k) 10 mm diameter holes drilled in the corners of wooden panels, nylon zip tie fasteners are passed through the holes and used to tie adjacent panels together. (F) The experimental chicken shed *in situ* in the yard of a householder in Araçatuba, Brazil. (G) The interior of the experimental chicken shed showing a CDC light trap and attached Barraud cage. The metal lid has been removed to enable a clear view of the apparatus.

mass spectra and retention times of all three of the diterpenes in *Lu. lenti* from Southern Brazil, and both *Lu. longipalpis* populations (Jaíba 1S and Sobral 2S), are significantly different from each other, indicating that they are all different diterpenes. While male *Lutzomyia cruzi*, which is closely related to, or part of, the *Lu. longipalpis* species complex, produces 9-methylgermacrene-B, it has not been established whether it is the *R* or *S* isomer (Brazil and Hamilton, 2002). The most abundant terpene compound found in male *Lutzomyia pseudolongipalpis* from Curarigua in Venezuela, also considered to be part of the *Lu. longipalpis* species complex, is 3-methyl-α-himachalene. This is the same compound that is produced by male *Lu. longipalpis* from Jacobina, Brazil. However, Jacobina males also produce a related compound, the sesquiterpene himachalene (C15; mw 204), and it represents ca. 15% of the total terpene extract, but this compound is absent from *Lu. pseudolongipalpis*. There is also evidence that *Lu. cruciata*, which is distributed throughout Central America and the Southern United States, and which is a vector of *Leishmania mexicana*, an etiologic agent of cutaneous leishmaniasis, produces germacrene-B acetate (ca. 60%), along with a number of other sesqui- and diterpene compounds (L. Cruz-Lopez *et al.*, personal communications) (Table 13.1).

13.3 The potential of sex/aggregation pheromones for use in control and monitoring

Considerable effort has been directed towards the development of practical applications for pheromones, primarily in the agricultural sector, where pheromones are widely used in four major ways: (1) population monitoring using traps baited with pheromone; (2) mass trapping using a large number of high-capacity trapping devices; (3) pheromone combined with insecticide (lure-and-kill); and (4) mating disruption by permeating an area with pheromone (Ujváry, 2001). Additional approaches, which aim to manipulate insect pest populations include: manipulating interactions between insect pheromones and other semiochemicals; manipulating populations of pests, their predators and the plants on which they feed; and using pheromones and other semiochemicals to repel pests or attract natural enemies (Smart *et al.*, 2014).

The pheromone communication system serves as a primary basis of premating (prezygotic) reproductive isolation among species. Chemical communication channels that are distinctive at the species level permit the co-existence of many species in the same habitat or region (Cardé and Haynes, 2004). The practical consequence of this narrow communication channel is that each species has its own unique pheromone, which may be a single chemical or group of chemicals. Therefore, the ecology and behaviour of each insect must be understood, in addition to preparing a synthetic copy of each pheromone for application in the field. Additional barriers to application are encountered as pheromones must comply with national registration requirements prior to application, and this can impose significant cost restrictions.

Amongst the phlebotomine sand flies, a substantial amount of work has been directed towards the use of the synthetic sex/aggregation pheromone to target one member of the *Lu. longipalpis* species complex. Only (*S*)-9-methylgermacrene-B, the pheromone of the most geographically widespread member of the *Lu. longipalpis* species complex (Spiegel *et al.*, 2016), has been synthesised in bulk. The compound was originally synthesised to confirm its structural identity (Hamilton *et al.*,

1996b, 1999c; Kurosawa and Mori, 2000). However, from an applied perspective, these multistep syntheses are impractical, as they are too expensive to complete in bulk. An alternative semi-synthesis, using an available plant-derived intermediate, germacrone, produced the pheromone in four synthetic steps, and in bulk (Hooper *et al.*, 2006; Krishnakumari *et al.*, 2004). Although the product of the synthesis is a racemic mix, i.e. it contains both the *S* and *R* forms of the compound, the *R* form does not inhibit the activity of the pheromone (Hamilton *et al.*, 1999c). Laboratory studies showed that the synthetic compound was attractive, and subsequent studies showed that it was attractive in the field (Bray *et al.*, 2009). Following on from this preliminary work, a lure and kill strategy for using the synthetic pheromone was developed, which relied on available knowledge of the ecology of *Lu. longipalpis* in the peridomestic environment. Chicken sheds, and other animal shelters, are known to be aggregation sites for *Lu. longipalpis*, but chicken sheds are not ideal locations for experimentation because of their diverse construction styles and materials (Figure 13.2). To overcome this, we prepared a standardised experimental chicken shed, so that we could carry out experiments with pairs of sheds, one of which could be treated as the test and the other the control, and thus allow us to use appropriate robust experimental designs. The experimental chicken sheds were constructed from plywood to a simple plan (Bray *et al.*, 2010), and a chicken from the household flock was placed in the shed overnight to provide host odour, which was believed to synergise the attractiveness of the host odour (Bray and Hamilton, 2007) (Figure 13.2).

In a series of experiments carried out in Campo Grande (MS, Brazil), we showed that when experimental chicken sheds were treated with insecticide, the numbers of both male and female *Lu. longipalpis* caught dropped significantly, but when synthetic pheromone was added to the insecticide treated sheds, female and male sand flies continued to be attracted and killed. The synthetic pheromone (50 µg) was placed in small polythene sachets (lures), which in turn was placed beside a CDC light trap, used for one night and then discarded. These experiments showed that the synthetic pheromone overcame the disruptive effect of the insecticide on *Lu. longipalpis* leks (Bray *et al.*, 2010). An important argument against using residual insecticide as a vector control tool against *Lu. longipalpis* (Ministério da Saúde, 2016), is that using the insecticide kills male *Lu. longipalpis*, and therefore stops the establishment of leks, and the further recruitment of males and females, potentially diverting them to untreated sites, and thereby increasing rather than reducing the risk of human infection (Kelly and Dye, 1997; Kelly *et al.*, 1997).

To use the pheromone in a practical way, we developed a lure that could release the pheromone for longer than a single night. Based on the design of commercially available lures (Russell-IPM Ltd., UK), we developed a lure containing 10 mg of pheromone, which could remain attractive to female and male *Lu. longipalpis* for up to three months under field conditions (Bray *et al.*, 2014). To investigate the potential of the combined pheromone and insecticide on numbers of *Lu. longipalpis* and *Le. infantum* infection in dogs, the reservoir host for human infection, we carried out a large-scale, stratified randomised control trial, in the Araçatuba region of western São Paulo State, Brazil. The trial, which ran between July 2012 and May 2016 (45 months), had three arms; pheromone + insecticide (lure and kill), insecticide impregnated dog collars (positive control (Gavgani *et al.*, 2002)) and placebo control. The trial involved 33 municipalities and nine districts of Aracatuba (42 clusters in total within an area of 11,250 km^2), the testing of 4,918 dogs and the recruitment of 1,454

seronegative dogs into the trial, which were followed up for a median of 15.2 months. To carry out the intervention, real chicken sheds and roosts in arm one, were treated with insecticide following Brazilian MoH protocols (Ministério da Saúde, 2016), and a pheromone lure was added. In arm 2, dogs were treated with insecticide-impregnated dog collars (Scalibor, MSD Saúde Animal, São Paulo, Brazil). In the placebo control arm, chicken sites were sprayed with water, and an empty lure added. For arm 1, the households were visited every three months, and the insecticide resprayed and lures replaced. For arm 2, dog collars were replaced every six months, and for arm 3, the sites were revisited every six months (Courtenay *et al.*, 2019). The study showed that the pheromone + insecticide intervention provided 52% (95% confidence interval (CI) 6.2%, 74·9%) protection against parasite infection, reduced tissue parasite loads by 53% (95% CI 5.4%, 76.7%), provided 13% (95% CI 0%, 44.0%) protection against anti-*Leishmania* antibody seroconversion, and reduced household female sand fly abundance by 49% (95% CI 8.2%, 71.3%). Comparison of the two interventions (pheromone and dog collar) showed no statistically consistent differences in their efficacies. Reductions in sand fly numbers were predominantly found where insecticide was located (chicken and dog sleeping sites), with no evidence of insecticide-induced repellence on humans or dogs (Courtenay *et al.*, 2019).

The entomological aspects of these results were further explored in a trial carried out in Governador Valadares (Minas Gerais, Brazil), a city endemic for VL. Instead of applying the insecticide at chicken roosting sites, the insecticide was applied to a 2.6 m^2 area of external wall, e.g. house or boundary wall. When 5 times more pheromone was used, 5 times more female sand flies were caught, and the intervention reduced household female *Lu. longipalpis* numbers by up to 70%. Importantly, the numbers of females in nearby untreated houses were also reduced by 24% (Gonçalves *et al.*, 2021). The reduction in females in nearby houses is likely a consequence of females travelling up to 30 m to a pheromone source (González *et al.*, 2020), and demonstrates the potential beneficial community effect of using a pheromone based lure and kill approach for sand fly/leishmania control.

The attractiveness of the pheromone is not linearly related to the amount released. As the quantity of pheromone increased, the attraction of males and females increases asymptotically, so that increasing the amount of pheromone 10 times from 20 to 200 mg led to a trap catch increase of 4 times, whereas increasing the amount of pheromone from 200 to 1000 mg resulted in no corresponding significant trap catch increase (Bell *et al.*, 2018). Interestingly, this study also showed that when competing sources of pheromone were placed close together, the sand flies did not relocate from one site to the other, and illustrates that the role of the pheromone is to maintain the aggregation, as well as to establish it. Thus, *Lu. longipalpis* that are attracted to an area treated with insecticide are likely to stay in that area, rather than to be dispersed by the repellent effect of the insecticide (Cutolo *et al.*, 2018).

Synthetic sex pheromone is continually released from the lures, but how this compares with actual release dynamics from real males is unclear. Laboratory-based studies have suggested that pheromone release is not continuous over time, and was greatest during the first hour after males were first placed together, during a period when wing fanning was most intense. Afterwards, pheromone release diminished, presumably as gland reserves were depleted (González *et al.*, 2017). The presence of females also significantly increased pheromone release, and indicates that

males respond to the presence of females, potentially, by increased mating attempts when more pheromone is released (González *et al.*, 2017). It is likely that the amount of pheromone produced by a real lek increases in the early part of the evening as the first males arrive, and this production is maintained as females arrive. However, as pheromone resources are depleted in the cohort of males that arrived early, only the arrival of new males maintains pheromone output of the lek. Modelling of the response of female and male *Lu. longipalpis* to different host odours, using field data, indicates that when the synthetic pheromone is present, it is the most attractive odour source in the peridomestic environment, and can attract 53% of host-seeking female *Lu. longipalpis*. The synthetic pheromone, thus, out-competes alternative attractive odours from humans and dogs, thereby reducing risk of *Le. infantum* transmission (Retkute *et al.*, 2021).

13.4 Conclusions and future work

The phlebotomine sand flies contain a globally important group of vectors of human and animal diseases. Between 1990 and 2016 the global burden (DALYS) of VL dropped by 71% to 708,000, whereas for CL/MCL it increased by 118% to 273,000 (Hay *et al.*, 2017). The WHO recognises that vector control is an important element in reducing the burden of disease globally (WHO, 2020a). Sex pheromones are widely used to monitor and control a wide range of insects of agricultural importance, but by comparison, little is known about the chemical ecology of sand flies, and specifically, about their sex pheromones. Although behavioural evidence exists for their presence in some important vector species, little is known of their chemical identity. Only in *Lu. longipalpis s.l.*, the Latin American vector of *Leishmania infantum*, has one of the sex/aggregation pheromones of the species complex been identified, characterised, synthesised and demonstrated to have potential as a vector control tool (Courtenay *et al.*, 2019; Hamilton, 2008; Retkute *et al.*, 2021). Much work, however, remains to be done to understand the integration of the sex/aggregation pheromones within other odour based communication channels, including those relating to host finding (Ortiz *et al.*, 2020; Rebollar-Tellez *et al.*, 1999) and oviposition site location (Dougherty *et al.*, 1993; Kowacich *et al.*, 2020) as well as within visual (Mellor and Hamilton, 2003; Mellor *et al.*, 1996) and acoustic ecology (Vigoder *et al.*, 2020).

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