SEMIOCHEMICAL MEDIATION OF OVIPOSITION BY THE PHLEBOTOMINE SANDFLY *LUTZOMYIA LONGIPALPIS* (DIPTERA: PSYCHODIDAE)

THESIS SUBMITTED IN ACCORDANCE WITH THE REQUIREMENTS
OF THE UNIVERSITY OF LIVERPOOL
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

By Martin James Dougherty

December 1994

I DEDICATE THIS THESIS TO MY FAMILY

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ABSTRACT

The phlebotomine sandfly *Lutzomyia longipalpis* (Lutz & Neiva, 1912) is the vector of visceral leishmaniasis in south and central America. Little is known about the environmental, physical and semiochemical factors mediating oviposition for sandflies and in the laboratory most females die after laying one batch of eggs. This phenomenon hinders studies on leishmaniasis transmission and limits our ability to colonise sandflies. Previous studies have shown that *L. longipalpis* uses an oviposition pheromone that attracts gravid females to eggs. In addition, oviposition attractants and stimulants were shown to be present in extracts of rabbit faeces, colony frass and larval rearing medium. The objectives of the present study were to isolate and identify the compounds used by gravid sandflies when choosing an oviposition site. The role of these compounds as behavioural cues was investigated to gather information that would allow the development of an oviposition monitoring trap.

Behavioural bioassays and chromatographic investigations showed the oviposition pheromone was produced in the accessory glands and secreted onto the eggs during oviposition. Egg extract was fractionated by high performance liquid chromatography (HPLC) and the fractions were tested in an oviposition bioassay; a single compound was shown to induce oviposition attraction. When gravid flies were exposed to the active compound in individual oviposition tubes, they oviposited earlier and laid more eggs than control flies. The compound was identified as dodecanoic acid using coupled gas chromatography mass spectrometry and chemical derivatization. When tested in an oviposition bioassay the synthetic compound induced the same oviposition response from gravid *L. longipalpis* as the HPLC isolated pheromone fraction and whole egg extract.

Extracts from the air entrainment of rabbit and chicken faeces attracted gravid flies in an oviposition bioassay and stimulated cells in the ascoid sensillum on the antennae of *L. longipalpis*. A continuous recording technique combining gas chromatography and single sensillum activity revealed two electrophysiologically active compounds present in the faecal extracts. These compounds were identified as hexanal and 2-methyl-2-butanol. When used together in the oviposition bioassay the synthetic compounds elicited the same oviposition attraction and electrophysiological response from gravid flies as did the total volatile components of rabbit or chicken faeces. Both combined extract of rabbit food and oviposition pheromone and the combined synthetic compounds had a synergistic effect on sandfly egg laying, greatly increasing the number of eggs laid and resulting in a highly focused oviposition attraction. Individually tubed flies exposed to the combined extract were 3.5 times more likely to survive oviposition and laid 2.5 times more eggs than control flies. A laboratory oviposition trap baited with a combined extract caught 62% of gravid *L. longipalpis* over a 72 hour period compared to 8% in an un-baited trap.

It would appear that the synthetic semiochemicals closely mimic the cues that *L. longipalpis* encounter in the wild during the search for a suitable oviposition site. The initial attraction to an oviposition site may be caused by the perception of the volatile compounds from organic matter. This attraction is then synergised by the presence of the oviposition pheromone and environmental cues and oviposition follows.

ACKNOWLEDGEMENTS

I would like to thank Professor Richard Ward and Dr. Gordon Hamilton for encouraging me to register for a higher degree and then providing an ideal environment for a student. Their advice, counsel and friendship has made my Ph.D. a positive experience. Thanks are due to friends and colleagues at the Liverpool School of Tropical Medicine for making my time there so enjoyable and for their enthusiasm in such stimulating surroundings. I am indebted to Pauline Ambrose whose dedication to the sandfly colonies enabled me to spend more time researching, Dr. Phillip McCall for help with the entrainment apparatus and Dr. Glenn Dawson for help with chemical derivatizations. Professor Harold Townson and Dr. Steven Ward gave me encouragement and further insight into my research as members of my Ph. D. panel.

Dr. Patrick Guerin had the foresight that started the collaboration with Neuchâtel University, Switzerland and led the way to the development of electrophysiological recordings from sandflies. I am grateful to him and his colleagues for making my trips to Neuchâtel such beneficial, exciting and friendly experiences. I would like to thank Michéle Vilmant (Neuchâtel) for scanning electronmicrographs of *L. longipalpis* antennae.

Financial support for this research was provided by: Jean Clayton Fund of the Liverpool School of Tropical Medicine; British Council - Swiss National Science Foundation Joint Research Programme; UNDP/ World Bank/ WHO Special Programme for Research and Training in Tropical Diseases (Director's Initiative Fund); The Roche and Hasselblad Foundations; The Wellcome Trust, London.

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LIST OF COMMON ABBREVIATIONS

AC	Alternating current.
a.m.u.	Atomic mass unit.
ANOVA	Analysis of variance.
Bp	Boiling point
CI	Chemical ionisation.
CO_2	Carbon dioxide.
CS	Centistrokes.
DC	Direct current.
diam.	Diameter.
EE	Egg equivalents
EI	Electron ionisation.
EPC	Electronic pressure control.
EtOH	Ethanol.
eV	Electron volt.
FID	Flame ionisation detector used in gas chromatography.
GC	Gas chromatography.
GC#	A HPLC generated fraction monitored by GC.
GC-MS	Coupled gas chromatography mass spectrometry.
GC-SSR	Gas chromatography linked single sensillum recording.
H_2SO_4	Sulphuric acid.
HPLC	High performance liquid chromatography.
HPTLC	High performance thin layer chromatography.
Hz	Frequency.
i. d.	Internal diameter.
LC#	Fraction generated by HPLC
M^+	Molecular ion generated by EI.
$M+H^+$	Molecular ion generated by CI.

ME	Male equivalents.
Mp	Melting point
Mw	Molecular weight
N_2	Nitrogen gas.
NIST	National Institute of Standards and Technology.
P	Probability.
PDA	Photo diode array detector.
PMP	Poly-methyl-pentene (larval rearing pot).
r. h.	Relative humidity.
R_f	Ratio of distance moved by a particular solute to that moved by the solvent front, measurement used in TLC.
Rt	Retention time.
SE	Standard error.
SIM	Single ion monitoring.
SSR	Single sensillum recordings
TIC	Reconstructed total ion current.
TLC	Thin layer chromatography.
TME	Trans methyl esterification
TTL	Transistor - transistor logic.
v/v	Volume to volume ratio.
V	Volts.
W	Watts.
Z11-16: Al	Type of classification of moth pheromones. Z = conformation of double bond (Z=cis / E=trans); 11 = position of double bond; 14 = carbon number; Al = aldehyde (other functional groups include OH, alcohol and AC, acetate).
σ	Density (kg m^{-3}).
λ	Wavelength (M).

INTRODUCTION

1.1 Sandflies

Sandflies are the vectors of pathogenic micro-organisms sandfly fever is one of several viruses transmitted by the flies and the bacterium *Bartonella bacilliformis* is the causative agent of Bartonellosis in South America. The most important micro-organisms that sandflies transmit are species of the genus *Leishmania,* protozoan parasites which are the causative agents of leishmaniasis. World-wide 12 million people are thought to be infected with this disease (Anon, 1990) and a further 20 million are considered to be at risk of infection (Ashford *et al.,* 1992). Sandflies are Dipteran and belong to the family Psychodidae; sub family phlebotominae. There is often confusion with the trivial name "sandfly" as flies of the Ceratopogonidae (West Indies) and Simuliidae (Australia) are also called sandflies. First described by Philippo Bonanni in Rome 1691, Phlebotomine sandflies are principally identified by the external genitalia of the male, the spermatheca of the female and the pharyngeal and cibarial armatures of both sexes. Attempts have also been made at classifying sandflies by a variety of other morphological structures (Ready *et al.,* 1980). A computerised standard identification for Latin American phlebotomine sandflies was proposed by Bermudez *et al.* (1991). These workers used 90 descriptors (morphological variables) and the definition of the different states (modality) of these descriptors, to classify all 386 New World sandfly species. The standard description system had the flexibility to be developed into computer software that would enable a rapid screening and identification of sandfly species.

There are six genera of sandflies, with *Brumptomyia, Lutzomyia* and *Warileya* in the New World and *Chinius, Phlebotomus* and *Sergentomyia* in the Old World (Anon, 1990). Their distribution stretches from central and

western Europe to northern China, south Africa and Australia in the Old World and from southern Canada to northern Argentina in the New World.

1.2 Sandfly biology

The biology, ecology and taxonomy of sandflies are reviewed in the literature by Abonnenc (1972), Martins *et al.*, (1978), Lewis (1973, 1974, 1982), Killick Kendrick (1978, 1990), Ward (1985, 1989), Lewis and Ward (1987), Peters and Killick-Kendrick (1987), WHO (Anon, 1990), Lane (1993) and most recently by Young and Duncan (1994).

Adult sandflies are small (1.5-2.5 mm) and hairy in appearance, they have long legs compared to body size and erect wings, in Brazil a local name for sandfly is "Asa de palha" or "wings of straw". Sandflies have an unusual flight pattern and hop over short distances, generally less than 1 m. With mark-release-recapture techniques, Killick-Kendrick *et al.* (1984) found that *Phlebotomus ariasi* in the South of France covered distances of up to 2 kilometres over a period of a few days. Morrison *et al.* (1993) found that 3% of recaptured *Lutzomyia longipalpis* had travelled a distance of 0.5 km, while few marked flies were recaptured at the release site. This dispersal behaviour of peridomestic *L. longipalpis* more closely matched that of Old World sandfly species, rather than the sylvan Neotropical sandflies and was in contrast to the findings of earlier work by Chaniotis *et al.*, (1974) and Dye *et al.*, (1991). Adult sandflies are crepuscular and most species rest during the day in damp dark places. Adult sandflies obtain a sugar meal which is thought to be from plants (Lewis and Domoney, 1966; Young *et al.*, 1980; Schlein and Warburg, 1986), although there is evidence that the honeydew secretion of aphids is also ingested (Killick-Kendrick and Killick-Kendrick, 1987a; Moore *et al.*, 1987 and MacVicker *et al.*, 1990; Wallbanks *et al.*, 1991: Cameron *et al.*, 1993). Additionally, it is known that sandflies exhibit plant preferences, with

significantly greater numbers found on beans and maize than on surrounding primary and secondary scrub, at an endemic focus of leishmaniasis in Peru. A significant relationship was also observed between the presence of two species of aphid feeding on the plant alfalfa and the presence of sandflies (Cameron *et al.*, 1994a; Cameron *et al.*, 1994b). Tesh *et al.* (1992) showed that the aphid alarm pheromone, trans-β-farnesene, was a sugar feeding stimulant for both and female *L. longipalpis*.

Only females take a blood meal, they are opportunistic and mainly feed during the scotophase, while if disturbed some species will feed during the day. A feeding aggregation pheromone has been found for the sandfly *Phlebotomus papatasi*, this pheromone was thought to attract other females to a "groom free" feeding site, an area on the host at which the animal cannot physically displace the insect (Schlein *et al.*, 1984). One or more blood meals are required for oogenesis and females lay eggs 3-5 days after feeding (Magnarelli *et al.*, 1984; ElNaiem *et al.*, 1992). Although autogeny has been recorded for some species, most require a blood meal for maturation of viable eggs (Johnson, 1961; Brazil *et al.*, 1991). Copulation generally takes place on or in proximity to the blood meal host. In some species mating "leks" have been described, with females in copula at these sites (Lane *et al.*, 1990; Dye *et al.*, 1991). At emergence, males are incapable of inseminating females as their genitalia must first rotate through 180°, this generally occurs within twenty four hours.

Little is known about the biology of the immature stages of sandflies. There are four larval instars which are rarely located in the wild, while adults may be abundant in the same area (Killick-Kendrick, 1987; Bettini *et al.*, 1986; Bettini, 1987). Larval development takes between 20-30, or even as long as 100 days, depending on environmental conditions. In areas with a seasonal climate fourth instar larvae may diapause in cold

conditions or in the dry season eggs may undergo aestivation (Ready *et al.*, 1980; Ward, 1985). In the laboratory fourth instars tend to migrate vertically upwards just before pupation, this behaviour is more evident when the rearing pots are very wet (Killick-Kendrick *et al.*, 1977). This activity is thought to be a response to inundation and may be controlled by humidity levels. Mukhopadhyay *et. al.* (1990) found that the immature stages of *P. argentipes* were able to migrate vertically up the walls of houses to a height of 90 cm in time of flood, when the water receded the larvae descended. For immature stages that develop in rodent burrows, this vertical migration at the time of pupation would allow the adult to emerge from the pupal skin and get to the surface without injury (Johnson and Hertig, 1961). At the final moult pupae attach themselves, at the posterior end, to the eclosion surface. *Lutzomyia gomezi,* when reared in crowded pots will migrate vertically and pupate synchronously in small groups of 5 or 6. The fourth instar larvae moult into pupae that are initially pale, but tan to brown or black and eclosion of adults occurs 1-2 days later. Due to the lack of identified oviposition sites in the wild, nothing is known about the natural predators of sandfly larvae. In laboratory colonies, maintained in the tropics, mites and ants can be serious pests and a water moat has been used for protection.

1.3 Leishmaniasis

Only species and subspecies of the *Lutzomyia* and *Phlebotomus* genera, within the phlebotominae, feed on mammals and are the only vectors of leishmaniasis. Lainson and Shaw (1987) classified *Leishmania* in the protozoan order Kinetoplastida, family Trypanosomatidae with digenetic forms, with a rounded amastigote living and dividing in the macrophages of the vertebrate host and a flagellate promastigote in the alimentary canal of the insect vector.

The transmission among vertebrate hosts is by the bite of an infected phlebotomine sandfly, the obligate invertebrate host. In humans, the various species of *Leishmania* parasite give rise to a variety of clinical symptoms called leishmaniasis. The syndromes afflict various human populations and are characterised by parasite species, their vectors and zoonotic reservoir. If the parasite invades macrophages at the bite site, cutaneous lesions ensue (cutaneous leishmaniasis), this is debilitating and disfiguring. Sometimes, if not treated serious secondary infections can complicate treatment. Some species of parasite are more able to invade macrophages of the buccal and pharyngeal tissues and the disease is then termed mucocutaneous leishmaniasis. It is possible for cutaneous leishmaniasis to be metastatic and form diffuse cutaneous leishmaniasis. The presence of parasites in the pharynx of the sandfly may make blood feeding difficult and the sandfly may then attempt to feed many times on one host, leading to multiple lesions. The fatal form of the disease if not treated is visceral leishmaniasis (Kala-azar), here the parasite invades macrophages that are found in the viscera including the spleen, liver and bone marrow. In India and Africa it is possible to develop multiple cutaneous lesions after kala-azar has been treated, the disease is then termed post-kala-azar dermal leishmaniasis. Reviews on *Leishmania* and leishmaniasis are found in; Lumsden and Evans (1979), Molyneux and Ashford (1983), Chang and Bray (1985), Hart (1987), WHO (Anon, 1984), Peters and Killick-Kendrick (1987) and WHO (Anon, 1990).

1.4 Sandflies and visceral Leishmaniasis in Latin America

Lutzomyia longipalpis has a wide distribution in Latin America from Mexico (20°N) to the north of Argentina (28°S) (Martins *et al.*, 1978). This species of sandfly occurs in semi-arid areas in a domestic and peridomestic environment. The ecology and disease relationships have been reviewed by Lainson and Shaw (1979) and Young and Lawyer

(1987). *Lutzomyia longipalpis* have been found with natural infections of *Leishmania* and epidemiological and laboratory transmission evidence confirmed this sandfly as the vector of the American visceral leishmaniasis (AVL) parasite, (Lainson *et al.*, 1977; Lainson and Shaw, 1979; Ryan *et al.*, 1984; Lainson *et al.*, 1985; Young and Lawyer, 1987; Lainson 1989; ElNaiem *et al.*, 1994). However, Travi *et al.* (1990) found evidence that *L. evansi* was a vector of AVL in Colombia, whilst in a different ecological niche than *L. longipalpis*.

In the New World the parasite causing visceral leishmaniasis is *Leishmania chagasi*. There is some controversy over the classification of this parasite, with some workers regarding *Le. chagasi* as an introduced strain of Old World visceral parasite *Le. infantum* (Killick-Kendrick *et al.*, 1980 Lainson *et al.*, 1981; Schnur *et al.*, 1981). Other workers classify the parasite as precinctive to the Americas (Young and Lawyer, 1987; Lainson and Shaw, 1987). Classification is further complicated by *Le. chagasi* in some locations being dermatropic instead of the more normal visceratropic parasite (Ponce *et al.*, 1991). This may relate *Le. chagasi* to *Le. infantum* that also demonstrates this variable tissue parasitology (Rioux *et al.*, 1980), or alternatively it may be related to a high level of nutritional status of the human population in the areas with dermatropic *Le. chagasi*. Lanzaro *et al.* (1994) proposed that the salivary enzymes of the sandfly altered the entry of the parasite into the macrophages and determined if an infection was visceral or cutaneous. These workers isolated two strains of *L. longipalpis* by their salivary enzymes, showing a difference between flies from a visceral focus and an atypical cutaneous one.

The disease is most prevalent in rural areas and is thought to be greatly under-reported (Vieira *et al.*, 1990). Cases are found from Mexico in the north to southern Brazil (Lainson, 1983; Molyneux and Ashford, 1983; Lainson, 1989 and Vieira *et al.*, 1990). In Latin America, 1.6 million people

are considered to be at risk of infection and over 16,000 cases are reported annually (Ashford *et al.*, 1992). The disease is zoonotic and the hosts are the dog, the 'savannah fox' *Lycalopex vetulus* (Deane and Deane, 1954) and the 'crab eating fox' *Cerdocyon thous* (Lainson *et al.*, 1969; Lainson and Shaw, 1979; Silvera *et al.*, 1982; Lainson *et al.*, 1990). In the sylvatic cycle the fox is the vertebrate host and *L. longipalpis* the invertebrate host. Parasites are introduced into a domestic cycle when foxes scavenge from human dwellings or raid chicken coops and dogs may then become infected (Lainson and Shaw, 1979; Ward, 1985). The close proximity of chicken coops to human dwellings in combination with the anthropophillic nature of the sandflies can further aid transmission of the disease. Sixty six percent of all visceral leishmaniasis cases occur in Brazil and in particular in the north-east, central-east and south-eastern regions and endemic foci have recently been reported in the states of Roraima, Teresina and Sâo Luís (Vieira *et al.*, 1990).

1.5 Insect communication

Insects communicate with sound, vision, tactile and chemical stimuli (olfaction and gustation). The more insect communication is studied the more complex the systems seem to be (Roitberg, 1992). It is important to recognise and study the various modes of communication used by pest insects, if aspects of their biology are to be used against them in the development of traps and lures. Pickett (1991) stated that:

> "....pheromones (chemical communication) must be considered with other behavioural cues and that, above all, a complete knowledge of the chemical ecology of the target pest is required."

Burghardt (1970) gave a wide ranging yet precise definition of communication as:

> ".....the phenomenon of one organism producing a signal that, when responded to by another organism confers some

advantage (or statistical probability of it) to the signaller or his group.....".

This definition concluded that:

".....communication must involve intent and thus be adaptive.....".

Some workers (Dawkins and Krebs, 1978; Lloyd, 1983) would contend that communication does not have to be of benefit to the sender, this definition would then encompass more of the chemical interactions used by insects. Chemicals released by an organism may be used by predators or parasites for host location and there would be no benefit to the emitter.

It may be possible to use chemicals that are important as behavioural cues, in combination with other modes of perception, against *L. longipalpis* and develop a greater understanding of their biology and ecology.

1.6 Insect chemical communication

If the chemicals that *L. longipalpis* uses for communication are to be deployed against them, in the form of a trap, or when developing a greater understanding of the biology of the sandfly, it is important to establish a system of terminology which can be used to describe the chemo-ecological interactions.

The use of chemicals which convey information between organisms has been described by Brown *et al.* (1970) and Whittacker and Feeny (1971). Norlund and Lewis (1976) and Norlund (1981) used terminology to describe chemical communication between interspecific and intraspecific organisms and developed the term "semiochemical" which they used to describe information conveying chemicals and toxins. Dicke and Sabelis (1988) classified chemicals that convey information as info-chemicals, a sub-class of semiochemicals and used three parameters to describe them; whether the interaction is inter or intra-specific; the cost and benefits that

fall to each organism; the identity of the producer and receiver. An info-chemical is a compound that in a natural context conveys information in an interaction between two individuals, evoking in the receiver a behavioural or physiological response (Table. 1.1).

A further group of info-chemicals, apneumones, have been described, this term is used when the semiochemical is derived from non-living organic material. Another way of classifying semiochemicals, not dependant on the parameters used above, is to describe the behaviour induced in the individual receiver insect upon perception of chemical stimuli (Dethier *et al.*, 1960).

Arrestant

> "A chemical which causes an insect to aggregate in contact with it, the mechanism of aggregation being kinetic or having a kinetic component, by slowing linear progression or increasing the turning rate."

Locomotor stimulant

> "A chemical which causes, by a kinetic mechanism, insects to disperse from a region more rapidly than if the area did not contain the chemical, by increasing speed of locomotion or decreasing the rate of turning."

Attractant

> "A chemical which causes insects to make orientated movements towards its source."

Repellent

> "A chemical which causes insects to make orientated movements away from its source."

Table. 1.1 Classification of semiochemicals, after Dicke and Sabelis (1988)

Description communication specificity	benefit to sender	benefit to receiver	Classification	Example
Pheromone: An info-chemical that mediates an interaction between two organisms of the same species.	✓	–	(+,–) pheromone	The pine beetle over produces aggregation pheromone on a healthy tree to mimic a high level of infestation, keeping other females away (Raffa and Berryman, 1983).
	–	✓	(–,+) pheromone	Recruitment of male bark beetles by females on the tree, also attracting competing females (Birch, 1984; Raffa & Berryman, 1983).
	✓	✓	(+,+) pheromone	Sex attractant pheromone of sandflies, increasing the likely hood of both organisms passing genes on to the next generation (Hamilton *et al.*, 1994).
Allelochemical: An info-chemical that mediates an interaction between two organisms that belong to different species	✓	–	allomone	Immature stages of sandflies produce a defensive compound, used against predators (Dougherty and Hamilton 1995).
	–	✓	kairomone	Mosquitoes use host odours and other factors to locate a blood meal (Takken, 1991).
	✓	✓	synomone	Attraction of insects to pollinate flowers (Harborne, 1988; Bertin, 1989).

Feeding, mating or oviposition stimulant

> "A chemical which elicits mating, feeding or oviposition in insects."

Deterrent

> "A chemical which inhibits feeding or oviposition when present in a place where insects would, in its absence, feed or oviposit."

A kinetic mechanism is one which is related to or caused by motion.

It is important to note that one chemical can have several effects on behaviour and the communicated meaning may change depending on the perceiving insects age, physiological state, receptor specificity and other non-chemical stimuli. Kennedy (1977; 1983) introduced a classification that not only dealt with chemical communication, but was also more wide ranging and could involve other non-chemical stimuli. This integrated approach described the action of semiochemicals along with other complex modes of communication and behavioural cues. The system was based on the various locomotory ways in which an insect responded to a stimulus as follows: a) kinesis, a non-directed response which may involve stopping, slowing or increasing the rate of linear progression; b) increasing or decreasing the rate of turning; c) taxis, the orientation of an insect toward a source that can either be positive (attraction) or negative (repulsion); d) anemotaxis, upwind orientated flight, with the insect turning the body angle, stimulated by wind direction and velocity; e) optomotor anemotaxis, upwind orientated flight stimulated by vision.

1.7 Perception of semiochemicals by insects

Insects face diametrically opposed problems in the perception of semiochemicals. There appears to be a need for a finely tuned receptive system that can perceive minute quantities of specific compounds, such as sex pheromones, a semiochemical used to increase the likelihood of

mating. In combination with this, there also appears to be a necessity for a broadly tuned chemoreceptive system that can discriminate between acceptable and unacceptable environments and hosts. This has been achieved through the evolution of specialised sensilla that are either tuned to specific compounds or can perceive the "general" chemical background. The perception of volatile semiochemicals (olfaction) is predominantly carried out by sensilla on the antennae of insects, although contact semiochemicals (gustation) may be perceived by receptors on various parts of the body, such as the oral cavity, mouthparts, ovipositor and tarsi (Stadler, 1984; Mustaparta, 1984). These sensilla fall into two broad categories, either generalist or specialist depending on the degree of specificity of receptor molecules on the dendritic membrane of the neurone. The specialist receptors include those perceiving pheromones, the sensilla of the lepidopterans can discriminate between geometric, positional and enantiomeric isomers (Ritter, 1988). The specificity of these sensilla is however not absolute and closely related compounds may stimulate them when present in quantities far higher in magnitude than the real pheromone (Mustaparta, 1984). In some insect species, the stereoisomer of a pheromone may inhibit a behavioural response. It is still unclear if this phenomenon is due to inhibition at the receptor site level, or if a second cell type is being stimulated.

Biosynthesis of pheromones in the Lepidoptera and Coleoptera has been found to be highly conserved, with a similar or identical biosynthetic pathway used by many insect species. The outcome is that often intraspecific chemical communication is carried out with a blend of semiochemicals, it is the different quantities and ratios of the compounds released that allow intraspecific identification at the receptor site level. The Heliothine moths use a blend of between 4 and 9 compounds as sex attractant pheromones, although only 2 of these compounds have to be present to elicit a full response. The principal pheromone is cis-11-

hexadecanal (Z11-16:Al) and there are two minor pheromone components cis-9-tetradecanal (Z9-14:A1) and cis-9-hexadecanal (Z9-16:Al), the receptors to these compounds have been highly conserved through evolution. The ratio of principal pheromone to the minor product is different for *Heliothis virescens* (Z11-16:Al/Z9-14:Al) were one female equivalent would yield 16:1 ng respectively and *H. zea* (Z11-16:Al/Z9-16:Al) which would yield 16:0.1 ng respectively. *H. assulta* (Z9-16:Al/Z11-16:Al) uses the same principal pheromone as *H. zea*, however with Z9-16:Al as the major quantitative component, at a ratio of 15:1 ng per female respectively (Mustaparta, 1993). Variations in ratios and quantities of pheromone released allows these moths to locate members of the same species.

Insects that use a pheromone blend usually have a sensillum specifically tuned to each of the compounds and they are present in relative percentages that mimic the quantitative ratio of the chemicals in the blend. This is typified by the perception of the two component sex pheromone blend of *H. virescens*. The aldehyde receptors sensitive to the two major sex pheromone components are present on the antennae in the same ratio as their biological importance (Z-11-16:Al - 58% and Z-9-14:Al - 27%), when tested by bioassay (Almass and Mustaparta, 1990).

Concentration effects have been found to be important when behaviour is mediated by semiochemicals. There is evidence of dose-dependant reversal of response in mosquito oviposition. Normally, *Ae. aegypti* is attracted to hexanoic acid for oviposition, this effect was reversed, repelling the gravid mosquitoes when the acid was released at higher rates, above a critical concentration (Knight and Corbet, 1991).

The generalist receptor cells are commonly used as gustatory receptors and are often important in the identification of hosts (Isman, 1992). The food sources of insects present a very complex chemical odour and in response they have developed receptive cells which are relatively non-specific. In this way the insect is able to develop a chemical search image or "template" that codes for either acceptable or unacceptable food, hosts and oviposition sites (Mitchell *et al.*, 1990). Hymenopteran parasitoids use this system of positive reinforcement in the sophisticated learning behaviour that they exhibit in response to a blend of host odours and volatile compounds from damaged plants (Turlings *et al.*, 1990; Tumlinson *et al.*, 1992). Eventually, after oviposition attempts and positive experiences, the wasps can utilise a few compounds as markers for ideal hosts. In haematophagous insects, host location is also associated with blends of highly complex odours from which the insect must discern a suitable (Den Otter and Van Der Goes Van Natters, 1993; Takken 1991). A review of the sensilla of haematophagous insects that are sensitive to host odour was given by McIver (1987). Three main findings were reported: a) females of species of mosquito and blackfly that were completely autogenous, had fewer olfactory chemosensilla than those requiring a blood meal; b) there was a correlation between the number of labial chemo- and mechanosensilla and the feeding behaviour of an insect, the greater the variation of food sources, the more numerous the sensilla; c) there was a positive correlation between the number of chemosensitive antennal neurones and the distance travelled by an insect to a host, the further the distance, the greater the number of neurones.

The exact mechanism for the perception of a compound at the sensilla of an insect is currently a highly active research field. The basic model is as follows;

1. Adsorption of the pheromone molecule to the surface of a sensillum;
2. Diffusion down a pore tubule or through cuticle to the dendritic membrane;
3. Binding to a membrane-bound protein receptor;
4. Activation of the receptor;

   Increased impulse frequency due to depolarisation
5. Increased membrane permeability ;

   Decreased impulse frequency due to hyperpolarisation
6. Early inactivation of the stimulus molecule.

This sequence of events would lead to action potentials and the receiving of a signal via the antennal nerve at the antennal lobe of the deutocerebrum in the insect brain. From here the information is passed into the calyx of the corpus pendunculatum for integration with other sources of information. It is thought that only a few semiochemical molecules need to be present on the dendritic membrane for signal transmission (Kaissling, 1974; Kaissling and Thornson, 1980; Vogt and Riddiford, 1986).

It is the net result of perception of threshold levels of both positive and negative stimuli that brings about a behavioural action. The neural inputs are described as 'labelled lines' in the case of perception of a single substance, such as a sex pheromone. Where the signal is a complex one, with a large number of receptors of over-lapping sensitivities, information from the neurones is transmitted via an 'across-fibre patterning'. Here a theoretical chemical search image or template exists at the level of integration of the primary neurones (Stadler, 1984). The physiological state of the insect may alter the perception of semiochemicals. *Ae. aegypti*

uses lactic acid as an attractant when locating a host for a blood meal (Takken, 1991). This mosquito has two sensilla that can perceive lactic acid, one is excited by it the other inhibited. After the mosquito has fed, a hormone is released from the fat body during oogenesis. This hormone increases the threshold sensitivity of the excitable neurone, making it 10 times less sensitive. When lactic acid is perceived by the mosquito after a blood meal, the net result is repellent (Davis *et al.*, 1987).

1.8 Chemical communication used by *Lutzomyia longipalpis*

L. longipalpis males occur in two morphological forms, some with a single pair of pale patches on tergite four and others with an additional pair on tergite three (Mangaberia, 1969). It was first thought that these pale patches were the markers associated with two possible sibling species. Cross mating experiments revealed that mating barriers did exist, but the number of pale patches was not a reliable marker for identification of sibling species (Ward *et al.*, 1983). It was shown that 1 spot males were sometimes unable to generate progeny with 1 spot type females from a different geographical location. To further confuse the issue, some 2 spot males were able to inseminate 1 spot type females. Upon closer investigation of the pale patches, pheromone releasing pores were observed and underlying glandular tissue of the type III class was found to be associated with a sex pheromone (Lane and Ward, 1984; Lane *et al.*, 1984; Phillips *et al.*, 1986; Ward, 1986; Boufana *et al.*, 1986; Ward *et al.*, 1989; Boufana 1990; Lane and Bernades 1990; Hamilton *et al.*, 1994). Initial chemical characterisations of the contents of the sex pheromone glands showed that the pheromones fell into two broad classes of compounds, a C_{20} (diterpene) and a C_{15} or C_{16} (sesquiterpene or homosesquiterpene) (Lane *et al.*, 1984; Phillips *et al.*, 1986; Hamilton and Ward, 1991). The pre-zygotic isolation of the sibling species was between the C_{15}/C_{16} and C_{20} compounds, although structural variations within the two groups may

further divide the complex. A further detailed investigation of the C_{16} sex pheromone has shown the compound to be a homogermacrene (Hamilton *et al.*, 1995). The pheromone compound has been found to be novel and is currently being synthesised. Munns *et al.* (1989) and Munns *et al.* (1990) went some way to show genetic differences in *L. longipalpis* populations and Lanzaro *et al.* (1993) revealed the existence of post-zygotic barriers confirming the presence of a species complex.

The sex pheromone is thought to attract females to males at the lekking site on or near the host. At this site males space themselves out equidistant apart and vigorous wing fanning occurs, in conjunction with parading and aggression. Mature males also establish dominance hierarchies (Jarvis and Rutledge, 1992). The combination of sex pheromone and kairomone host odours were a strong attractant to female *L. longipalpis* (Morton and Ward, 1989a; Nigam and Ward, 1991; Hamilton 1992). Also, Morton and Ward (1989b) showed that female sandflies were attracted to a combination of host odour and sex pheromone in a wind tunnel over a distance of 2.4 m. However, when a preliminary field trial was undertaken using hexane extract of sex pheromone glands on a heated pad in chicken coops, females were not attracted and males were repelled (Ward *et al.*, 1990). It is known that attractant pheromones in high concentrations can have an opposite effect and may become repellent (Knight and Corbet, 1991). Hamilton and Ramsoondar (1994) showed that male and female sandflies were attracted to petri dishes that had been handled by humans. Furthermore, they found that the flies exhibited preferences for different individuals used in the investigation. It has been demonstrated in the laboratory that both male and female sandflies are attracted to the volatile entrained components of hamster odour (Oshaghi *et al.*, 1994).

Similar pheromone releasing papules, to those found in *L. longipalpis*, were observed in an electron microscopy survey of several *Lutzomyia* and one *Brumptomyia* species by Gallati (1990). Fifteen species were found to possess the papules, and Lane and Bernardes (1990) found three further species. Ward *et al.* (1993) undertook a study of further specimens and to-date 52 New World sandfly species are known to have pheromone disseminating structures. The morphology of the papules is diverse with at least nine different structures. A gas chromatography linked mass spectral investigation of the tergal glands of *L. pessoai*, showed the presence of a $C_{20}H_{32}$ (cembrene structure) pheromone and was the first example of a sex pheromone in phlebotomine species other than *L. longipalpis* (Hamilton and Ward, 1994).

Another mode of communication that has been studied in *L. longipalpis* is sound. It is thought that male sandflies attract and encourage females with pulses of wing vibration (Ward *et al.*, 1988). Males from various sibling species have a different "courtship song", as the inter-pulse frequency of the wing generated sound is different for sibling populations.

The antennae of both sexes of *L. longipalpis* consists of 16 segments, the scape, pedicel and 14 flagellar segments. Volatile semiochemicals could be perceived via olfactory sensilla on the antennae of the sandfly, which bear a range of 14 different sensilla. The most likely location for receptors is within the large ascoid sensillum, (Boufana, 1990). Ascoids are paired structures borne laterally on antennal segments 3-15 of both sexes, they are transparent with many pores in the sensillum wall. The ascoids of males are longer than those of females, and variations in ascoid length and distribution have been used for taxonomic purposes, especially in the genus *Lutzomyia* (Lane, 1993). Ascoids on the antennae of females are more highly innervated than those of males, with thicker and more

numerous dendrites. Although ascoid morphology has been studied, neuronal activity associated with this olfactory sensillum has not been recorded. Another possible site of chemo-reception is the Newstead organ, which is found on the third segment of the palps. This bulb shaped organ is placed into contact with the host during feeding, and may be used as a "generalist" host recognition sensillum (Lane, 1993).

1.9 Oviposition strategies and site selection mechanisms

Oviposition is the act of depositing eggs, and the oviposition site selected by a female is usually characteristic for the species. The site chosen is of great importance in the survival of the egg and the availability of food for the emerging progeny, and for most insects it is the choice of the female that controls these factors. The selection of a site is controlled by a general attraction to a particular area followed by a set of specific stimuli that determine the precise location for oviposition. Mosquitoes are known to use visual cues, such as colour, optical density and reflectance, when searching for a suitable oviposition site (Bentley and Day, 1989). Other long range attractants include volatile compounds such as fatty acid esters, and terpenes such as carvacrol and citral (Bentley and Day, 1989). The perception of these stimuli would lead to the choosing of an area as acceptable for oviposition, but it is not until further specific stimuli are fulfilled that oviposition occurs

1.9.1 Semiochemical factors affecting oviposition. Two main strategies have been adopted by insects when controlling the density of eggs at an oviposition site. Some species will lay eggs over a dispersed area or in individual hosts if they are parasitic. These include the parasitic Hymenoptera (Turlings *et al.*, 1993), the parasitoids discriminate between parasitised and non-parasitised hosts and will oviposit in the latter to reduce competition for food resources if the host was

hyperparasitised. The problem of overcrowding at the oviposition site is one that many insects have overcome by utilising an oviposition deterring pheromone. Many phytophagous insects use this type of semiochemical due to the limited food resources for their young. *Pieris brassicae* females can detect a volatile pheromone on the eggs that have been laid on cabbage leaves. The detection of this pheromone will cause the females to continue the search for an alternative oviposition site without laying any eggs in the proximity (Visser and Minks, 1982). Alternatively with some insect species there seems to be an ecological advantage in ovipositing in groups or clusters. Female *Lucilia cuprina* produce a pheromone that attracts other females to oviposit in the same area on the host. It is thought that the sheep blowfly larvae can better utilise the tissue of the host in larger rather than smaller numbers (Barton Browne *et al.*, 1969). The blackfly *Simulium damnosum* exhibits aggregated oviposition that is controlled by an oviposition attractant pheromone. In this case, the individual female fly would put its eggs at an advantage by attracting other females to lay eggs on top of its own as this would lessen the possibility of desiccation (McCall *et al.*, 1994). However, it should be noted that with the *Simulium*, a greater risk of suffocation and fungal infection has been recorded with aggregated oviposition (Imhof and Smith, 1979; Hywel-Jones and Ladle, 1986). There are also advantages to be gained with aggregated oviposition by lessening the chances of predation (Manning 1979). Stamp (1980) found that butterfly species that oviposited communally developed synchronously at a faster rate than individual larvae. This resulted in a synchrony at emergence thus optimising the choice for mate selection.

Another group of semiochemicals that control behaviour at oviposition are stimulants and deterrents. A stimulant may be used in combination with an oviposition attractant, for example some mosquitoes use close range specific stimuli that would include tactile, microclimatic, apneumones and

an oviposition pheromone (Bentley and Day, 1989), after attraction to an oviposition site. These stimuli would be the last behavioural cues a mosquito needs before oviposition. Alternatively, many phytochemicals deter mosquito oviposition, for example, the volatile oil (1,8-cineole) of aster *Hemizonia fitchi* deters oviposition by *Ae. aegypti* (Klocke, *et al.*, 1987). Other terpenes such as citronella and geraniol have also been shown to be oviposition deterrents (Saxena and Sharma, 1972). These compounds mark a specific area as not suitable for oviposition.

Pheromones from the eggs of *Cx. quinquefasciatus* have been shown to attract ovipositing females (Bruno and Laurence 1979; Sakaibara *et al.*, 1984; Laurence *et al.*, 1985; Laurence and Pickett 1985; Hwang *et al.*, 1987; Otieno *et al.*, 1991). A full review of the chemical ecology of behavioural aspects of mosquito oviposition was given by Bentley and Day (1989). Ovipositing *Ae. atropalpus* are also attracted to an oviposition attractant produced by immature stages (Kalpage and Brust 1973). Soman and Reuben (1970), showed that gravid *Ae. aegypti* females were attracted to water containing immature stages of the same species. Compounds from bacterial cultures of *Pseudomonas aeruginosa* were found to be an oviposition attractant for *Ae. aegypti* (Ikeshoji *et al.*, 1975 & 1979). *Cx. quinquefasciatus* will preferentially oviposit in pools with hay infusion, high levels of methane and furfural (Hazard *et al.*, 1967). Hay infusion also increased the number of eggs laid by this mosquito.

1.9.2 Physical and environmental factors affecting oviposition.

The physical characteristics of an oviposition site are very important criteria for most insects (Bursell, 1974; Engelmann, 1984, Thompson, 1988; Bentley and Day, 1989). These factors include temperature, humidity, photoperiod and the physical nature of the oviposition surface.

The temperature ranges over which insects will oviposit are generally limited, the extent of the range and optimal temperature being species dependant (Engelmann, 1970). If *Musca domestica* females lay eggs within the temperature range favouring this species, many more eggs are laid than if the female oviposits in hotter or colder environments (Fletcher *et al.*, 1990). The level of humidity is critical as even at 80% r.h. only 15% of the eggs will survive. Low r.h. generally has a negative effect on oviposition, with insects preferring more moist environments. Water loss from insect eggs is critical at the time of oviposition. The chorion of eggs is not water proof and at the time of oviposition water loss from the eggs is limited by a mono-layer lipid. This gives an end-point temperature over which the lipid layer degenerates and water loss is rapid. The critical temperature for *Rhodnius prolixus* (Hemiptera) is 38°C (Beament, 1946).

The circadian rhythm that governs the timing of oviposition is often under the control of the photoperiod and light intensity. The periodicity of oviposition for *Rhagoletis mendax* has a very strong correlation throughout the day to both ambient temperature and light intensity, laying eggs when both of these factors decreased below a maximum threshold level (Smith and Prokopy, 1981). Insect oviposition behaviour can be classified as: a) diurnal (during the day); b) crepuscular (during dawn or twilight); c) nocturnal (during the night).

Mosquitoes use visual cues to locate suitable oviposition pools prior to coming into contact with the substrate. The brightness of an oviposition pool is an important factor in the oviposition of *Cx. restuans* and *Anopheles gambiae* (Bentley *et al.*, 1982; McRrae, 1984). Furthermore, Russo, (1980) showed that *Ae. vexans* had strong preferences for oviposition among sand grains of a particular diameter. A review of techniques and traps used for sampling mosquito egg populations was given by Service (1993). Many of

the investigations utilised both physical and semiochemical properties of the oviposition site for specific mosquito species.

1.10 Sandfly oviposition

Female *L. longipalpis* lay eggs in microhabitats, such as cracks and crevices of chicken coops and cow stables, sites which are rich in organic nutrients (Young *et al.*, 1926; Lewis and Kirk, 1954; Ward, 1974;). Sandfly eggs are laid under conditions of stable humidity (Bettini *et al.*, 1986; Bettini and Mellis, 1988). Females are known to be gonotrophically concordant and pass through several gonotrophic cycles (Guilvard *et al.*, 1980; Dye *et al.*, 1987), laying up to 100 eggs but a more normal number is 40. However, ElNaiem *et al.* (1992c) found both field and laboratory evidence that *L. longipalpis* did take multiple blood meals. Difficulties in mimicking natural environmental conditions in the laboratory resulted in high mortality rates during oviposition and in most species very few females surviving past the first gonotrophic cycle (Killick-Kendrick *et al.*, 1977; Chaniotis, 1986; ElNaiem, 1991). As parasite transmission depends on the female ovipositing and taking a second or even third blood meal (ElNaiem *et al.*, 1994) this problem not only hampers colonisation but also limits studies of *Leishmania* and arboviruses. ElNaiem (1991) found the physiological state of female flies very important for oviposition. It is known that the quality of blood meal will affect oogenesis in sandflies. The number of mature oocytes developed by *L. longipalpis*, when given an equal weight of blood meal form various hosts, was maximum with rodent blood and minimum with human or other primate blood (Ready, 1979). The number of oocytes matured depended not on weight of blood meal but on the amount of protein present in the blood.

1.10.1 Physical factors mediating sandfly oviposition. The behaviour of sandflies when choosing an oviposition site is controlled by complex interactions between environmental and physiological factors. It

is known that temperature, humidity and photoperiod all play a role in the selection of oviposition sites by sandflies (Foster *et al.*, 1970; Ready, 1976; Chaniotis, 1986). Temperature and humidity are paramount for the successful colonisation of sandflies, and the speed of development of all stages of the life cycle are affected by changes in these two parameters (Ward, 1989). Ward, (1989) found the optimal temperature for colonisation of tropical sandflies to be between 24 - 30°C, whereas, *P. ariasi* had to be maintained at a temperature of 10°C (Killick-Kendrick and Killick-Kendrick, 1987b). ElNaiem (1991) initially found a strong correlation between temperature and oviposition, but when the mean number of eggs was calculated for females that actually oviposited, there was no correlation with temperature. More females actually oviposited, rather than the females laying more eggs, showing that the effect of temperature was an "all or nothing" response, mainly stimulating the initiation of oviposition. ElNaiem (1991) found no correlation between humidity and the number of eggs actually oviposited. It is accepted that high humidities are beneficial for sandfly oviposition and workers who maintained a high humidity increased the colony output by 185% (Killick-Kendrick *et. al.*, 1977). It has to be noted that at a constant 100% humidity many females die because they become trapped in the moisture.

Schlein *et al.* (1990) found that rough surfaces such as soil and pebbles enhanced oviposition in sandflies. In the laboratory Ward (1974) showed that more eggs were laid by gravid *L. longipalpis* in the fold that had been used to simulate a crevice, than on a straight strip of paper. When *L. longipalpis* was given the choice between a test site containing artificial crevices and an open control site, significantly more eggs were laid in the crevices. Moreover, when individual flies were placed alone into tubes with either a folded or flat piece of paper, the flies exposed to the folded paper laid significantly more eggs (ElNaiem and Ward, 1992a). This behaviour was associated with a thigmotropic response, and it is known

that sandflies probe an oviposition surface with their ovipositor before egg laying. ElNaiem (1991) stated that:

> "...*Lutzomyia longipalpis* is greatly influenced by physical factors characterising the oviposition surface."

1.10.2 Semiochemical factors mediating sandfly oviposition. A further major influencing factor in oviposition site selection by gravid *L. longipalpis* is the presence of an oviposition pheromone associated with eggs. The oviposition pheromone is an attractant and/or stimulant and was first reported by ElNaiem and Ward (1990). Gravid flies given the choice in an oviposition pot between an area previously exposed to eggs and one that had not, preferentially laid eggs on to the former area. ElNaiem and Ward (1991a) found that when eggs were placed into grooves on the plaster of Paris base of the oviposition pot, significantly more eggs were laid in this area than control groves with no eggs present. When the eggs were washed in a cocktail of solvents, the attraction was lost. One - 2 day old eggs were as attractive to gravid flies as 5 -6 day old eggs (ElNaiem and Ward, 1991). ElNaiem and Ward (1991b) showed the presence of oviposition attractants and stimulants in colony frass, larval rearing medium and rabbit faeces. Gravid females were attracted to these materials in an oviposition choice chamber bioassay. When rabbit faeces were washed in solvents of differing polarity, but the physical integrity of the material maintained, the flies were not attracted. This demonstrated the presence of chemical attractants in these materials. Females kept individually in oviposition vials oviposited earlier and survived oviposition in greater numbers than control flies when they were exposed to an aqueous extract of rabbit faeces. Schlein *et al.*, (1990), found that cow manure promoted oviposition in the sandfly *L. papatasi*.

However, an initial chemical investigation of the pheromone found several compounds present in a hexane extract of *L. longipalpis* eggs, two

were identified as cholesterol and squalene. When synthetic squalene and cholesterol were used in a bioassay they neither attracted nor stimulated gravid sandflies to oviposit (ElNaiem *et al.*, 1991).

1.11 Objectives of the present work

ElNaiem (1991), ElNaiem and Ward (1990, 1991, 1992b) and ElNaiem *et al.* (1991) showed the presence of chemical attractants and/or stimulants associated with the eggs of *L. longipalpis* and organic material, such as rabbit faeces. The object of this present study was to identify the compounds used by gravid sandflies when choosing an optimal oviposition site. Additionally, the natural source and role of these compounds as behavioural cues was studied to develop a basic model that describes the oviposition process for this sandfly.

It may also be possible to use the chemical attractant cues to monitor sandfly population levels, or use them against the insect in the form of an oviposition trap. The semiochemicals could be used to mimic, in the laboratory, the natural behavioural cues that gravid flies would encounter in the wild when choosing an oviposition site. It is hoped that this will aid colonisation. The transmission of the *Leishmania* is dependant on completion of at least one gonotrophic cycle (Guilivard *et al.*, 1980; Dye *et al.*, 1987; ElNaiem *et al.*, 1994) and often female sandflies die the first time they oviposit. If this problem could be surmounted using chemical behavioural cues it would be highly beneficial in the study of disease transmission.

MATERIALS AND METHODS

2.1 Maintenance of insects

2.1.1 Adult sandflies. *L. longipalpis* used in the investigations were originally collected from Jacobina, Bahia State, Brazil and Curarigua, Lara State, Venezuela and reared over approximately 73+ generations and 12+ generations respectively, according to the method of Modi and Tesh (1987). Adult flies were kept in 12X12X12 cm Barraud cages, sealed in plastic bags with a piece of laboratory paper towelling wetted with distilled water, to maintain a high level of humidity (95% r.h.). Female flies used in the oviposition bioassays were blood-fed on Syrian hamsters anaesthetised with a 12 mg ml^{-1} solution of sodium pentobarbitone. Hamsters were maintained in a state of anaesthesia for over 1 h, to ensure that a high proportion of females blood-fed. Only females that had obviously blood-fed (distended red abdomen) were then isolated with an equal number of males at 27±1 °C and a photoperiod of 12:12 (light:dark), for 4 days to allow copulation, complete defecation and oogenesis. Sandflies used for electrophysiology experiments were 2-3 days post blood meal. As provision of a sugar meal increases the number of eggs laid per sandfly (Ready, 1979; ElNaiem, 1991), all flies were allowed access to a solution of super saturated sucrose, soaked onto a pledge of cotton wool placed on a plastic vial cap inside the Barraud cage.

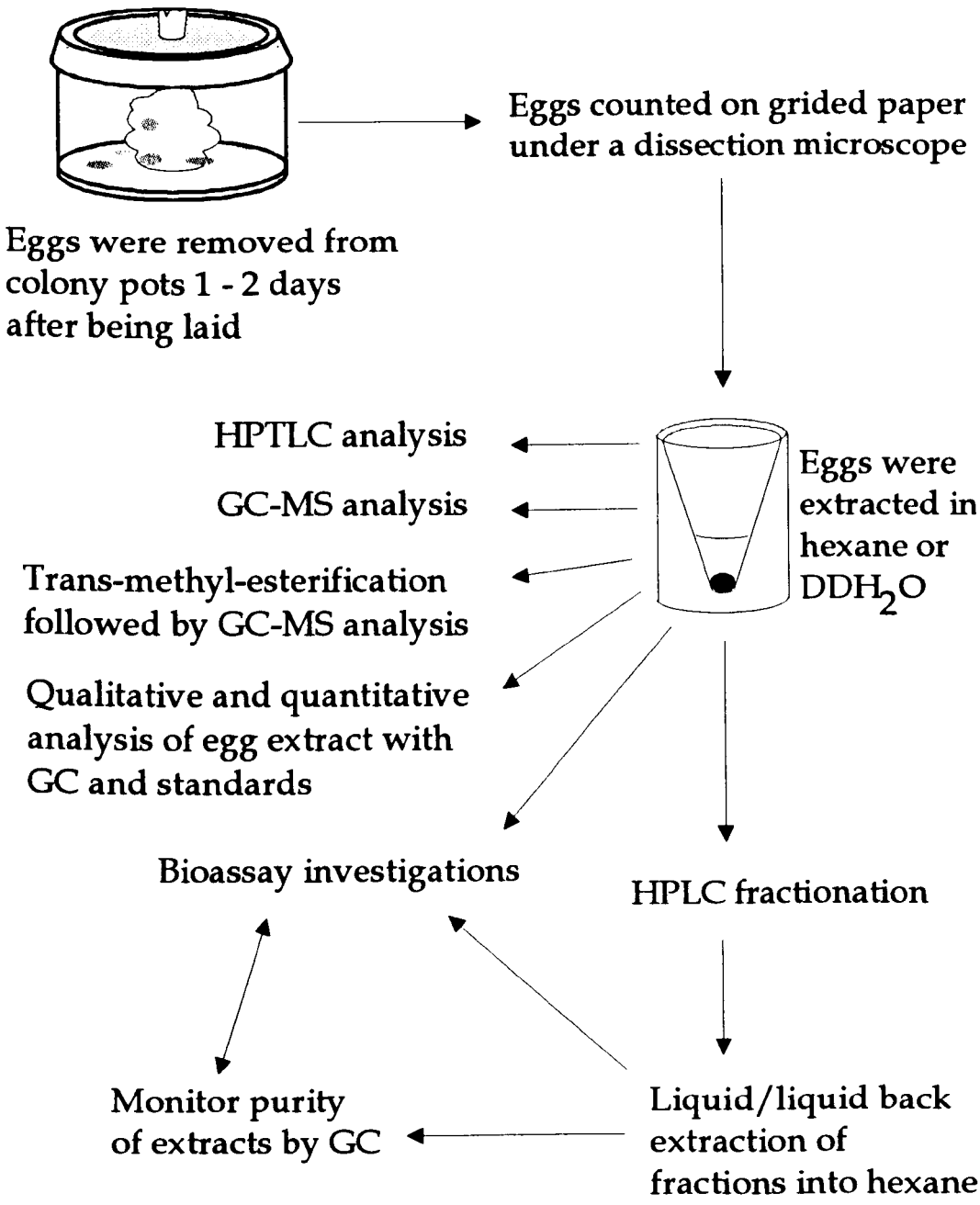
2.1.2 Immature stages. Larval instars were maintained in polymethylpentene (PMP) rearing pots (11 cm diam.), which in the base had 20 (0.5 cm diameter) holes drilled at regular intervals. Plaster of Paris was then poured into the pot to a depth of approximately 1 cm which produced a solid base, continuous with the outside. The larval pots were kept in plastic Tupperware boxes on dampened filter paper discs (30 cm diam., Grade 1, Whatman, BDH, UK), this maintained high humidity in

the boxes and kept the plaster of Paris damp. The immature stages were fed on an equal mixture, by dry weight, of *Daphnia*, rabbit faeces, fine sand and potting compost.

2.2 Biological origin of the oviposition pheromone

2.2.1 Sample preparation. Eggs used to prepare extracts were 1 to 2 days old and were collected from pots which had been re-plastered and autoclaved prior to oviposition. A fine brush was used to collect eggs which were then counted on clean filter papers. All glassware was acid cleaned in chromic acid (BDH Chemicals, UK) for 24 h and washed in double distilled deionised water, before being baked for 24 h at 250 °C. To prepare the non-polar egg extract, 1000 eggs were placed in a 2 ml microvial (Alltech, UK) and left for 24 h at -70°C in 100 µl of pesticide grade hexane (BDH Chemicals, UK). All solvents were of pesticide residue analysis grade, unless otherwise stated. Accessory gland extracts were prepared from 50 gravid females (100 accessory glands), blood-fed four days previously. Glands were excised under a stereoscopic microscope and the tissue transferred to a 2 ml microvial using a micro-pipette and extracted in 100 µl of hexane as described above. The remaining female bodies were also similarly extracted, to provide material for a negative control. A polar extract was prepared from 1000 eggs placed in a 2 ml microvial and extracted in 100 µl of double distilled, de-ionised H_2O at 3 °C for 24 h, 100 accessory glands and the remainder of the female bodies were also similarly extracted, the experimental protocol is summarised in Fig. 2.1. The preparation of material for high performance thin layer chromatography (HPTLC) and gas chromatography (GC) analysis used the same protocol as described above. Five hundred *L. longipalpis* eggs and 50 excised female accessory glands were extracted in 20 µl of hexane. For GC analysis five whole male, whole female and female flies without accessory glands were extracted in 40 µl of hexane.

Fig. 2.1 Diagrammatic representation of the extraction technique for the eggs of *L. longipalpis*, showing the subsequent use of the various extracts.



2.2.2 Bioassay to test for oviposition attraction. The bioassay was an adaptation of that used by ElNaiem and Ward (1991). Bioassay chambers were modified PMP larval rearing pots, which had been re-plastered and autoclaved. Only eggs laid in the test and control sites were counted. Modifications included the use of two oviposition areas, 2 cm^2, (test and control), with lightly demarcated perimeter grooves on the plaster of Paris base.

After loading the oviposition areas in the bioassay chambers with the test and control solutions, gravid females were introduced and left for 3 days under normal insectary conditions. The females were provided daily with a small pledge of cotton wool, soaked with a super saturated solution of sucrose, placed on top of the oviposition chamber.

Six bioassays were conducted, each with 10 replicates, using 20 females per replicate. In each oviposition chamber a 10 µl aliquot of test extract was pipetted onto the plaster of Paris base at the test site. The control of 10 µl of hexane was applied to the control site. The test consistently gave an equivalent of 100 eggs, accessory glands from 5 females or 5 female bodies without accessory glands. Bioassays performed were: A) Non-polar egg extract versus hexane control; B) non-polar accessory gland extract versus hexane; C) polar egg extract versus water control; D) polar accessory gland extract versus water; E) non-polar extract of females without accessory glands versus hexane; F) polar extract of females without accessory glands versus water. The bioassay design gives a matched pair of results, that are best analysed with non-parametric statistics. The Wilcoxon matched pair signed rank test was used to compare the number of eggs laid at the test and control sites. All statistical analysis was carried out using Minitab statistical software (Minitab version 9 for Windows, Celocom UK).

2.2.3 High performance thin layer chromatography (HPTLC). High performance TLC plates were 10 cm x 20 cm coated with a 200 μm thick layer of silica (Whatman Lab Supplies, UK). The mobile phase was a 1:1 chloroform/hexane solution (BDH Chemicals, UK), described by Kates (1986). HPTLC plates were prepared by baking at 200 °C for 24 h and then cleaned by running twice in the solvent system. Aliquots of egg and accessory gland extract, (20 μl = 25 female equivalents), were applied to the plates with 2 μl capillary tubes. Five standards (each of 20 μg) were applied to the plate. Standards applied were cholesterol, cholesterol linolenate, cholesterol stearate, squalene and caryophyllene oxide (Sigma Chemicals, UK). Plates were visualised with ultra violet light at 254 nm, followed by charring with concentrated H_2SO_4/EtOH solution (1:1, v/v) and baking at 110 °C for 10 min. R_f values were calculated for insect derived material and standards to allow comparison.

2.2.4 Gas Chromatography (GC). Gas Chromatography was performed on a Shimadzu GC-15A (Dyson Instruments, UK), fitted with a Grob split/splitless injection system, used in the splitless mode, with a 0.6 min sampling time and a flame ionisation detector (FID). The column used was a fused silica DB-1 capillary column, (30 m, 0.32 mm i. d. and 0.25 μm film thickness; J & W. Scientific, UK). The temperature program was: 45 °C initial, increased at 15 °Cmin^{-1} to 250 °C, held for 4 mins, increase at 10 °Cmin^{-1}, and then held at 350 °C for 4 mins. The carrier gas was helium at a flow rate of 2 ml min^{-1}, the injector and detector temperatures were 250 °C and 350 °C respectively. Peak integration was carried out with the Shimadzu CR5A Chromatopac (Dyson Instruments, UK). Only non-polar extracts were analysed by GC. Samples included extracts of eggs, female accessory glands, whole males, whole females and females without accessory glands and were prepared as described above. Before being analysed by GC, extracts were placed on ice and reduced in

volume under N_2 to 1 µl (1 µl = 500 egg equivalents, EE).

2.3 Isolation of the oviposition pheromone

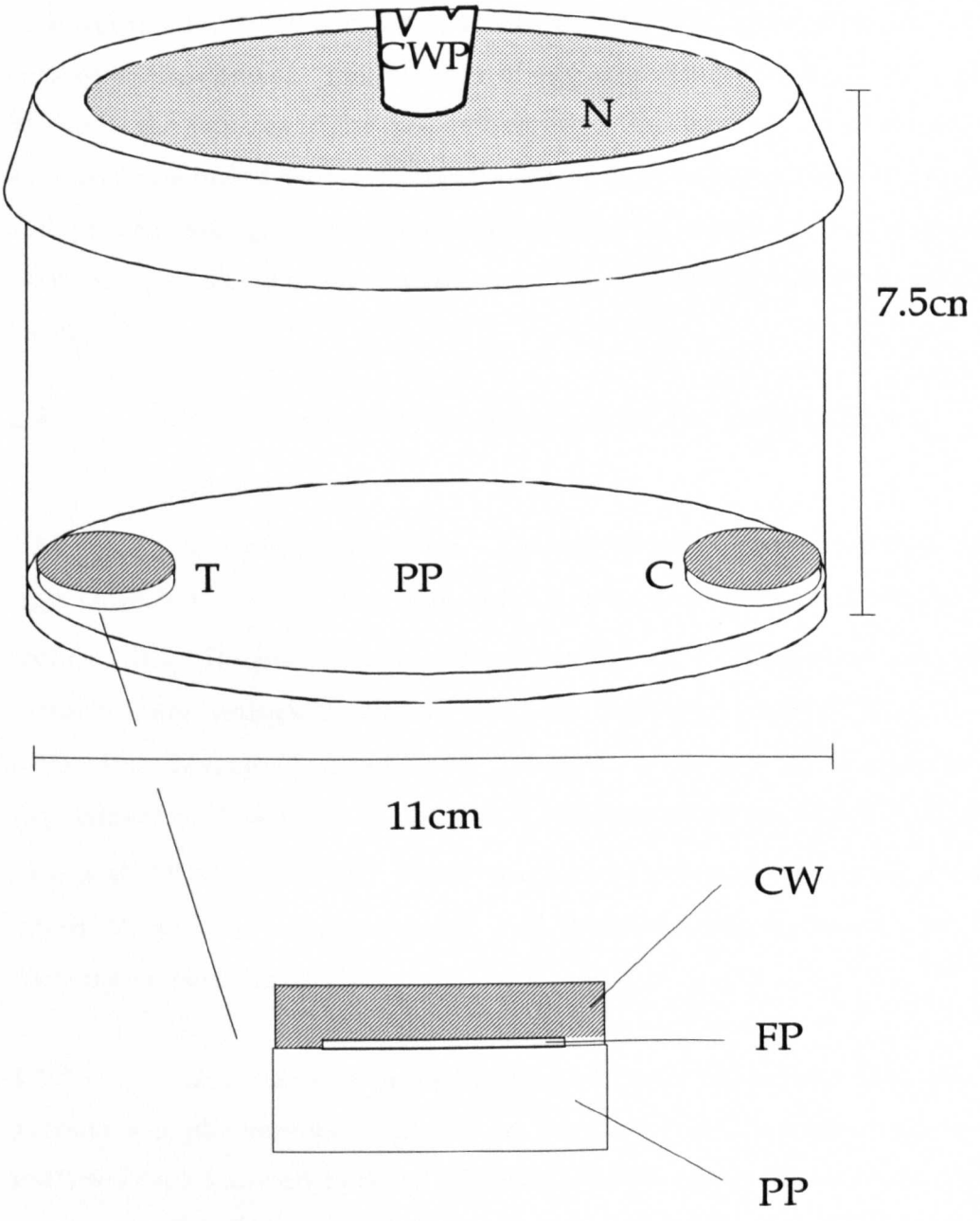
2.3.1 Sample preparation. Extracts were prepared from 1,200 eggs by placing them in a 2 ml microvial with 100 ml of hexane. The sealed vials were left for 48 h at -20 °C to allow complete extraction and minimise loss of volatiles. This method of extraction provided a greater quantitative recovery of compounds than the one used previously and was adopted as standard.

2.3.2 High Performance Liquid Chromatography (HPLC). Egg extract (100 µl) was placed on ice and reduced in volume to 10 µl under a stream of nitrogen. Samples were fractionated on a Waters HPLC system; 600E gradient pump, U6K injector and a 991 photo-diode array (PDA) detector (Waters, UK). Data was acquired and analysed using a NEC APC IV personal computer, running Waters 991a software (Waters, UK). Separation was carried out on two Resolve C18 analytical columns (Whatman, UK) connected in series (3.9×600 mm) heated to 30°C by a Waters steel column heater. The mobile phase was acetonitrile/isopropanol (HPLC grade, Rathburn Chemicals, UK) (80:20) maintained isocratically with a flow rate of 1 ml min^{-1}. Solvents were degassed with helium (BOC Gases, UK) at a flow rate of 33 ml min^{-1}. Calibration of instruments and monitoring of separations were carried out using caryophyllene oxide standards (Sigma Chemicals, UK). The HPLC generated fractions were collected manually in a 10 ml separation funnel. The fractions were separated from the mobile phase by a liquid/liquid separation. Seven mls of hexane and 2 mls of ultra pure water were added to the fractionated eluent and shaken vigorously. The hexane fraction was pipetted into a microvial on ice and the volume was reduced to 80 µl under N_2. (HPLC generated fractions = LC#).

2.3.3 Gas chromatography. GC analysis was carried out using the method described previously (section 2.2.4). Before bioassay, samples were injected in 1 µl aliquots (reduced in volume under N_2 so that 1 µl = 500 EE) from the initial unmodified extract and from the various HPLC fractions after the liquid/liquid back extraction. The number of egg equivalents used in each bioassay was calculated and the quantities of pheromone were compared to a caryophyllene oxide standard (GC analysed fractions = GC#).

2.3.4 Bioassay to test for oviposition attraction. The bioassay method used was an adaptation of the one used previously (section 2.2.2). An 11 cm diameter PMP larval rearing pot had two 2.5 cm filter paper discs placed opposite each other on a plaster of Paris base. The paper discs had pads of cotton wool, 3 cm diameter by 0.5 cm thick, placed on top which prevented direct access of gravid flies to the test and control samples (Fig. 2.2). Cotton wool has been found to be an acceptable sandfly oviposition surface (ElNaiem, 1991). One of the filter paper discs was termed the test disc and had 10 µl of one of the five HPLC fractions absorbed on to it. There was some loss of material from the fractionation and the liquid/liquid separation. Taking into account the recovery efficiency, the number of egg equivalents used in each bioassay was 145±3.75. The other filter paper disc was termed the control and had absorbed onto it hexane from a liquid/liquid separation of HPLC eluent without any of the egg extract fractions. The hexane was reduced in volume, as above and used in 10 µl aliquots. Twenty gravid flies were introduced into each pot for oviposition and eight replicates were performed for each fraction. The flies were left in the dark for 3 days and then the number of eggs on the test and control sites were counted. The results were analysed using the Wilcoxon matched pair signed rank test. The relative oviposition attraction of each HPLC fraction to the whole extract was tested by Kruskal-Wallis analysis.

Fig. 2.2 Diagrammatic representation of the bioassay apparatus used to test for oviposition attraction of gravid *L. longipalpis* to the various HPLC generated fractions, to isolate the active pheromone component from hexane egg extract.



CWP = cotton wool plug; N = netting used to contain flies; T = test site; C = control site; CW = cotton wool pad used as suitable surface for oviposition; FP = filter paper onto which was placed either the test solution of the solvent control; PP = plaster of Paris base used to maintain a moist humid environment in the PMP pot.

2.3.5 Bioassay to test for oviposition stimulation. Following the method of ElNaiem and Ward (1992b), flies were placed individually into oviposition tubes 24 h after a blood meal. There were three groups of thirty flies one for each treatment. The filter paper disc at the bottom of each oviposition tube was treated with either HPLC generated L4, L2 or hexane as the control. The number of egg equivalents used per tube (145 EE) were the same as in the oviposition attraction bioassay. The following data were recorded daily: mean number of eggs laid per female; number of flies ovipositing; onset of oviposition after the blood meal; the general survival up to day 5 of the experiment. The experiment was repeated four times.

2.4 Structural characterisation of the oviposition pheromone

2.4.1 Sample preparation. The egg extracts were prepared with eggs collected from the Jacobina and Curarigua colonies, as described in section 2.3.1. The extracts were stored for 72 h at -20 °C to allow complete extraction and reduce the loss of volatiles. The samples were transported from the Liverpool School of Tropical Medicine to Rothamstead Experimental Station on an ice/NaCl freezing mixture, which had been frozen at -70 °C over night. The extracts were reduced in volume, on ice, under N_2 to 30 µl. Subsequently 2 µl injections (approximately 80 EE) were made onto the GC-MS.

2.4.2 Gas chromatography linked mass spectral analysis of oviposition pheromone. Extracts of Jacobina and Curarigua eggs were analysed on a Hewlett Packard GC-MS (HP 5890 series II+ chromatograph - mass selective detector 5972A, Hewlett Packard, UK). The GC-MS was in electron impact (EI) mode, with a source temperature of 180 °C, a transfer line temperature of 280 °C and an ionisation energy of 70 eV. The samples

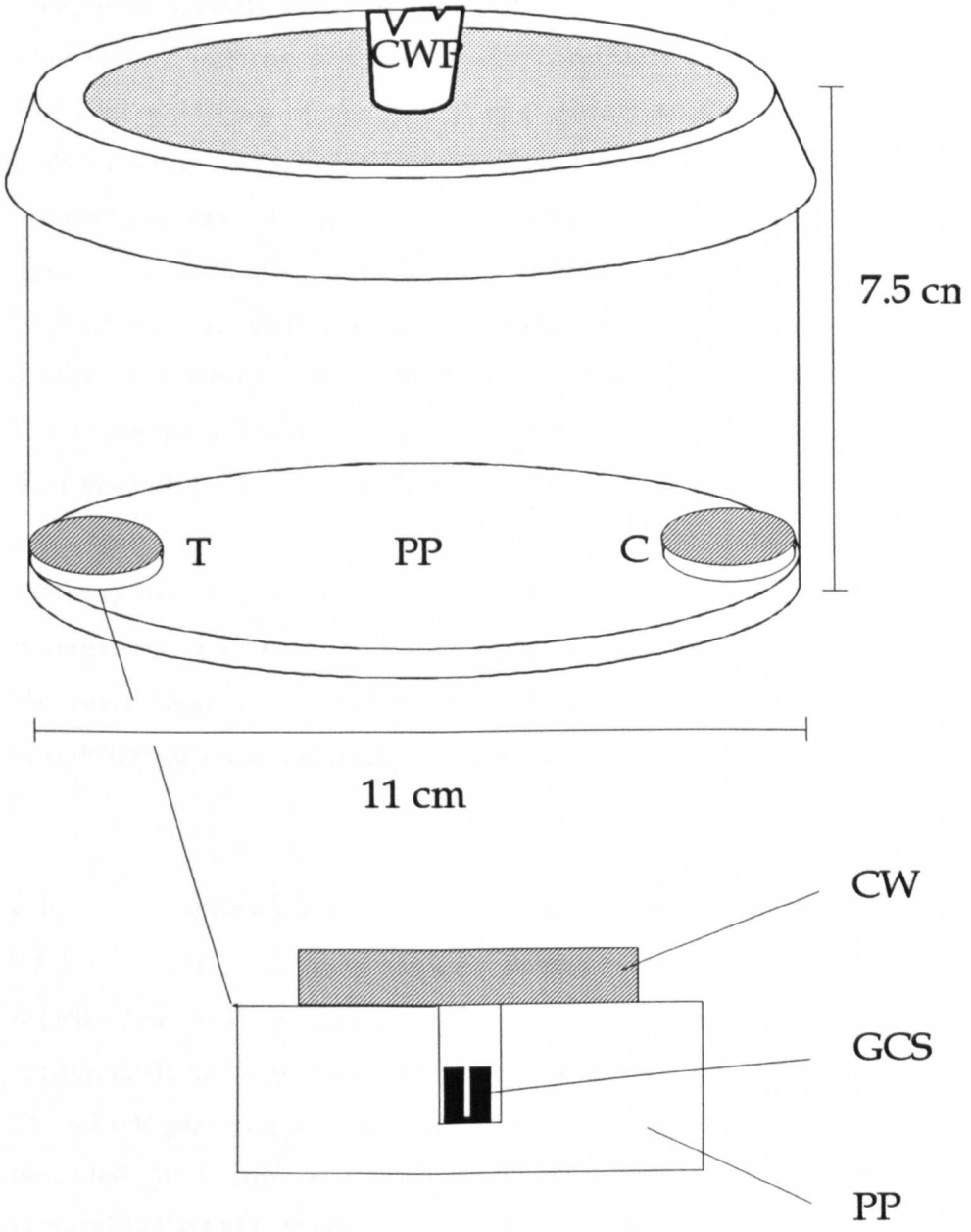
of egg extract were analysed on two columns, a non polar HP-5MS (Hewlett Packard, UK; 30 m, 0.25 mm i.d., film thickness 0.25 μm, phase ratio 250 and 5% methyl silicone) and a polar DB-Wax (J. W. Scientific, UK; 30 m, 0.25 mm i. d., film thickness 0.25 μm). The HP-5MS column was used with two temperature programs for both egg extracts, a fast elution temperature programme, (initial 35 °C for 4 min then increased at 15 °C min^{-1} up to 250 °C, held for 2 min and then increased at 10 °C min^{-1} up to 310 °C and held for 2 min) and a slow elution temperature programme, (initial 50 °C for 4 min then increased at 5 °C min^{-1} up to 310 °C). The DB-Wax column was used with one temperature program for both egg extracts, (Initial 50 °C for 4 min then increased at 10 °C min^{-1} up to 220 °C). The electronic pressure control (EPC) injector was in splitless mode (0.6 min sample time), at a temperature of 250 °C for the fast and slow temperature programs and 180 °C for the polar column. Helium was the carrier gas under constant pressure, with an initial linear velocity of 1 m sec^{-1} at 50 °C. The pheromone peak was initially relocated in the GC-MS total ion chromatogram by locating it relative to a hydrocarbon series and by comparison of chromatogram profiles obtained by GC and then GC-MS. The molecular ion, base peak and fragmentation ions of the putative pheromone in each egg extract was determined. A library search was undertaken using the mass spectra of the pheromone, to try and match it with that of known products (National Institute of Standards and Technology library, ChemStation, version C. 00. 007, Hewlett Packard, UK). A synthetic compound (100 ng in 1 μl, 2 μl injected), which closely matched the pheromone peak in the library search, was analysed on both columns using the same conditions and temperature programmes as above (section 2.4.2). This gave comparative retention times and mass spectral data.

2.4.3 Trans-methyl-esterification of the pheromone and synthetic compound. To confirm the structure of the proposed pheromone in hexane egg extract, both extract and synthetic compound were chemically modified with a free acid esterification with diazomethane in ether. The reaction products were analysed using the GC-MS (EI mode) with the temperature programme and conditions described in section 2.4.2 for the DB-Wax column. The spectra of the trans-methyl-ester (TME) products were library searched to confirm structural detail.

To confirm the molecular weight of the pheromone, the TME reaction products of hexane egg extract and synthetic compound were analysed by GC-MS in chemical ionisation (CI) mode. The injector temperature was 180 °C and the transfer line was heated to 280 °C. The source temperature was 180 °C and the ionising gas was methane. Helium was the carrier gas at an initial linear flow rate of 1 ml min^{-1}. The DB-Wax column was used and the temperature programme was; initial 50 °C, then 10 °C min^{-1} up to 250 °C.

2.4.4 Bioassay to test for oviposition attraction. The bioassay was an adaptation of the method used previously in section 2.3.4. Cored GC septa (Dyson Instruments, UK), heat sealed at one end were placed into holes opposite each other (10 cm apart) in the Plaster of Paris base of a PMP larval rearing pot (Fig. 2.3).

Fig. 2.3 Diagrammatic representation of the bioassay apparatus used to test for oviposition attraction of gravid *L. longipalpis* to hexane egg extract and synthetic oviposition pheromone.



CWP = cotton wool plug; N = netting used to contain flies, on which the pledge of cotton wool with saturated sugar solution was placed; T = test site; C = control site; CW = cotton wool pad used as suitable surface for oviposition and to stop gravid flies from coming into contact with the test solution; GCS = gas chromatograph septa, sealed at one end, absorbed into it either the test solution or the solvent control; PP = plaster of Paris base used to maintain a moist humid environment in the PMP pot.

Both test and control septa were covered with a pad of cotton wool to prevent access by the flies. A solution of the chemical to be tested was placed into the test septum drop-wise. The control septum had an equal volume of solvent alone dropped into the core. The following samples were tested; 400 ng of the synthetic candidate pheromone (in hexane so that 1 μl = 100 ng of synthetic); egg extract so that 100 egg equivalents were present; and a negative control of solvents only. 400 ng of synthetic compound was approximately the concentration found in 100 sandfly eggs. Ten replicates were carried out for the above treatments, each with 20 females. The data was not normally distributed when tested with the Anderson-Darling normality test (Minitab for Windows, Version 9). Therefore the differences in the number of eggs laid at the test and control sites was statistically analysed using the Wilcoxon matched pair signed rank test. To test for differences between the treatments used in the bioassay, the number of eggs laid at the test site for each material was log transformed and an analysis of variance (ANOVA) was performed. This treatment was used rather than non-parametric ANOVA because the variability of each test treatment was not the same.

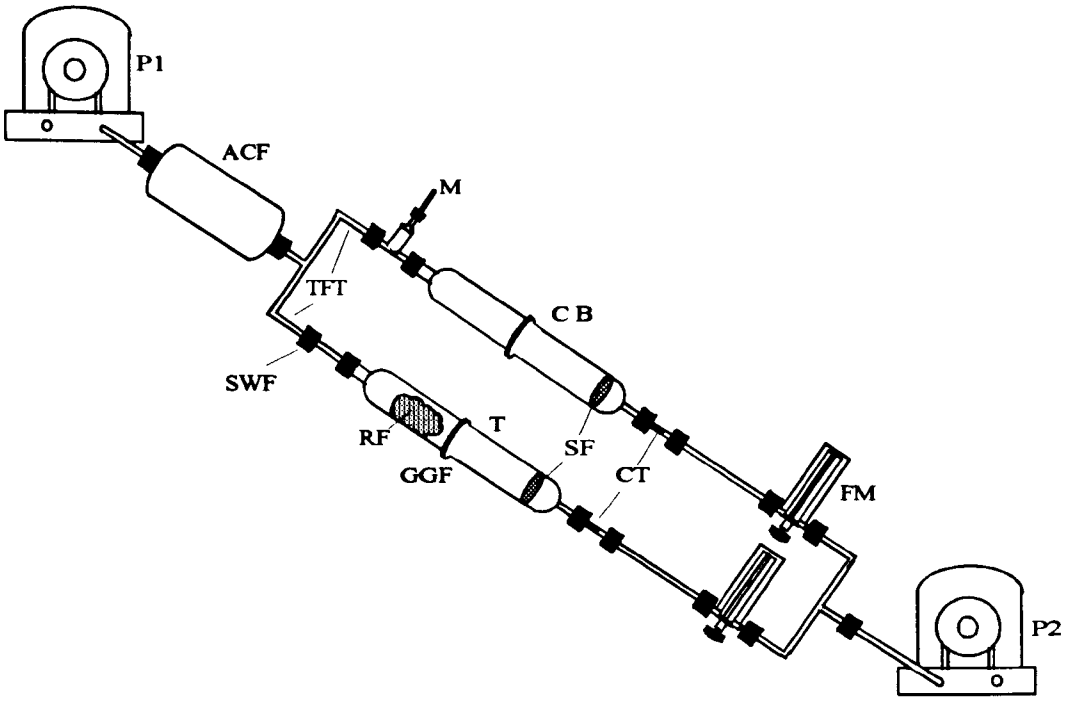
2.5 Isolation of volatile faecal oviposition attractants

2.5.1 Sample preparation. Rabbit faeces were obtained from a pet rabbit fed on commercially available rabbit food, supplemented occasionally with green vegetables. The entrainment system used a pump P1, which pumped air into the system in greater volume than pump P2 removed it. This was indicated by a positive pressure within the apparatus that was measured with a manometer M (Fig. 2.4).

Volatile components of the faeces were entrained onto 25 mg of Super Q adsorbent (80-100 mesh; Alltech, USA) packed into 4 cm diameter glass collection traps using the method of Turlings *et al.* (1991). The volatile

collection system used air purified over an activated charcoal filter which blew (1.5 L min^{-1}) over 10 g of rabbit faeces in a closed Pyrex container. After 2 h the collection traps was rinsed with 200 µl of dichloromethane (BDH Chemicals, UK). The extract was reduced in volume for electrophysiology to 5 µl (standard concentration) by letting it stand at room temperature and for bioassay to 5 µl on ice under N_2. A blank control was prepared by carrying out an entrainment in the empty Pyrex vessel, CB, to ensure there was no contaminants in the system .

Fig. 2.4 Diagram of the apparatus used to collect the volatile compounds from rabbit and chicken faeces.



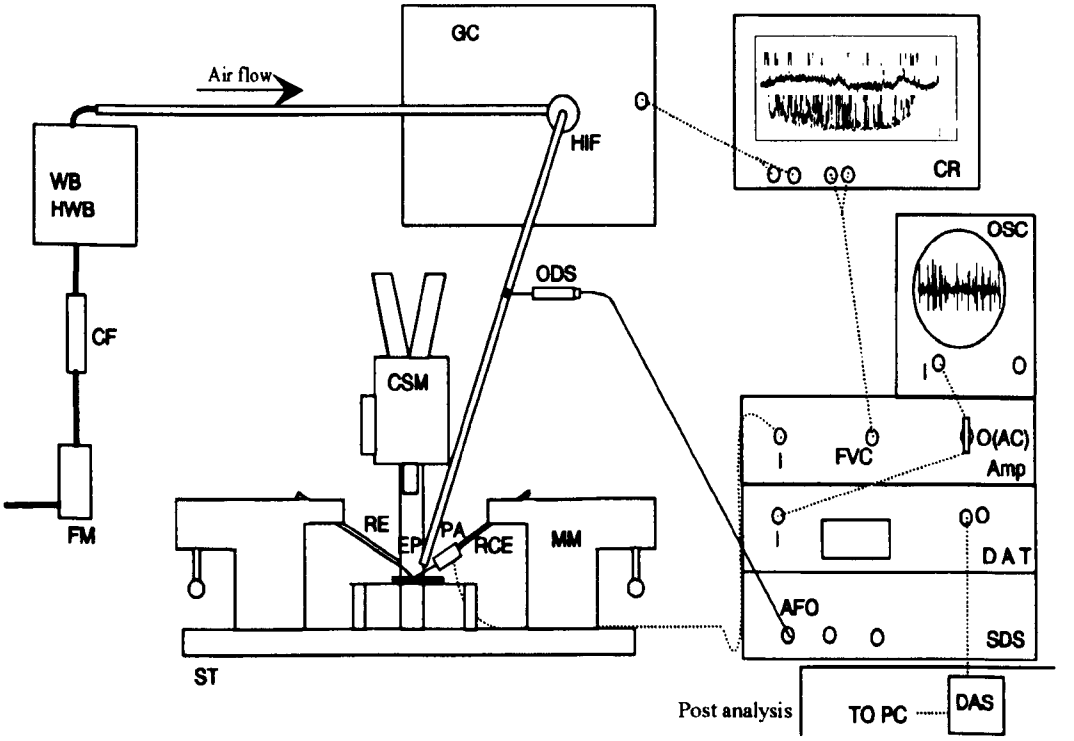
P1 = pump one, pushing air into the system; ACF = activated charcoal filter; TFT = Teflon tubing; SWF = swage lock fittings; M = line to simple manometer, used to measure the slight positive pressure; RF = rabbit faeces; T = test bottle; CB = control blank; GGF = ground glass fittings, to ensure air tight junction, seal was held with a bracket; SF = silica frit, to ensure no particulate material contaminated the collection trap; CT = collection trap, glass vial packed with Super Q adsorbent (Whatman, UK); FM = flow meter, two independent meters were used to ensure the same flow rate through both arms of the system; P2 = pump two, pulling air from the system.

L. longipalpis sex pheromone extracts were prepared by placing dissected tergal glands from 20 males into an extraction vessel with 100 µl of hexane (BDH Chemicals, UK), the extract was then reduced in volume so that 1 µl contained 1 male equivalent (ME) (Hamilton *et al.*, 1994).

2.5.2 Electrophysiological bioassay to identify faecal oviposition attractants. Each sandfly was anaesthetised with CO_2 and the legs removed with micro scissors under a dissection microscope. The removal of the insects legs reduced the occurrence of spurious muscle potentials on the electrophysiological recordings. The insect was then mounted on double sided sticky tape on a microscope slide, with wings, thorax and abdomen pressed gently into place. A black background (insulating tape) was used as ascoid sensilla were transparent under the incident cold light source employed.

The tungsten electrodes were mounted on Leitz micromanipulators. Under a Leica M32 (Switzerland) combistereo microscope the reference electrode was inserted in the occipital sulculus of the sandfly, which provided good electrical contact and also kept the head stationary. The antenna was laid out on a second layer of double sided sticky tape and carefully embedded in the glue segment by segment, using a human eye lash held in an all-purpose scalpel blade holder. The second layer of sticky tape went some way to offset the relative position of the antenna on the head of the insect, against the rest of the body. The tungsten recording electrode, with an electrolytically etched tip of 1 µm, was gently introduced at an angle of 120° into the base of the ascoid until spontaneous cellular activity was heard on the audio monitor of the main amplifier (Fig. 2.5).

Fig. 2.5 Diagram of the equipment used for single sensillum recordings from the ascoid sensillum on the antennae of *L. longipalpis*, stimulated by either the syringe delivery method or the gas chromatograph.



FM = flow meter, use to control the rate of flow of air over the electrophysiological preparation; CF = charcoal filter assembly; HWB = heated water bath, to heat air and control the level of humidity; WB = water bottles, bottles of water placed into the water bath, through which the air bubbles so becoming humidified; Air flow = show the direction of the air passing through the glass tubing; GC = gas chromatograph, used to separate the complex mixture of compounds in rabbit faeces; HIF = heated interface, heated line that transferred the eluent of the GC into the atmosphere of the air flow assembly; CSM = combi stereomicroscope; ST = still table; RE = reference electrode; RCE = recording electrode; EP = electrophysiological preparation; MM = micromanipulator; PA = pre-amplifier; Amp = amplifier; O = output; I = input (non-inverting input for the amplifier); O(AC) = output- alternating current signal; FVC = output of frequency - voltage conversion; DAT = digital audio tape; SDS = stimulation delivery system; AFO = air flow output; ODS = odour delivery syringe; OSC = oscilloscope; CR = chart recorder, synchronous recording from the amplifier and the GC. Post analysis, DAS = digital analogue conversion board; PC; personal computer running SAPID software.

Recorded signals were passed through a preamplifier (10^{12} Ω input impedance) into a universal AC/DC amplifier (UN-03, Syntech, The Netherlands) and recorded on one channel of a digital audio tape recorder (Biologic, DTR 1200, France). Signals were simultaneously visualised on an oscilloscope (Tektronix, LR 37158, USA). Hard copies of recorded data were obtained with the plot option of the spike analysis programme for insect data, SAPID (Smith *et al.*, 1990). For this the recordings were played back from the DTR 1200 and fed into a 386 IBM compatible personal computer via a DAS 16 analogue/digital board (digitising rate 10 KHz). Discrimination of the different cell types in the multicellular recording from the ascoid sensillum was carried out by eye using spike amplitude, frequency and shape as criteria.

The electrophysiological preparation (EP) was maintained in a charcoal scrubbed airflow at 1 m/s and 100 % r.h. at 26 °C±1 by circulating the air through water filled gas-wash bottles in a heated water bath. The exit of the glass tube (6 mm i.d.) conducting the air-flow was approximately 0.6 cm from the sandfly antenna. Stimulation was achieved with a charcoal filtered air stream applied to a 5 ml polypropylene syringe containing the stimulus. A solenoid valve in the stimulus delivery assembly (ST-05, Syntec, Netherlands) permitted displacement of 2 ml of the syringe content in 1 s into the humidified air stream, through a septum covered hole in the glass delivery tube at 20 cm from the outlet. In each stimulation the recorded pre-stimulus period was equal to the recorded stimulation period. In order to reveal specific cell types that were responding to chemical stimuli, a double successive form of stimulation was employed. In this case the ascoid was stimulated for 1 s with compound A and then the stimulation source was switched to compound B for one second. Any change in the cell types responding to the two compounds was then evident. The 5 volt TTL (transistor-transistor logic) signal generated from the ST-05, when stimulation occurred, was recorded

on the right channel of the DTR-1200 and used as the "point of stimulation" signal for subsequent analyses of responses. The biological extracts and the following synthetic chemicals, some of which are active semiochemicals for other insects, were at first employed to study the specificity of the receptors located in the ascoid sensillum: carbon dioxide, hydrogen sulphide, copane, R-(-)-β-pinene, R-(+)-α-pinene, S-(-)-α-pinene, S-(+)-β-pinene, β-caryophyllene, α-terpene, R-(+)-limonene, elemene, farnesene, α-terpinene, β-elemene, terpinolene, trans-methylisoeugenol, carvacrol, α-terpineol, linalool, trans-dihydrocarvone, β-ionone, γ-bisabolone, geranyl acetate, citral, bormyl acetate, hexylacetate, benzylacetate, 5,6-hexadecyl acetate, linaylacetate, cis-3-hexenyl pyruvate, lactic acid, octanoic acid, 10 undecanoic acid, γ-valerolactone, benzaldehyde, E-2-hexen-1-al, trans-2-nonenal, methanol, 1-octen-3-ol, hexanol, 2-methyl-amino-methanol, hexylamine, 1-methylnapthalene, methylsalicylate, phenol, 2-methoxy butylpyrazine. Except for methanol, CO_2 and hydrogen sulphide, all the chemicals (procured from commercial sources and >95% purity) were dissolved in dichloromethane and tested at 1 mg in the stimulus delivery syringe (unless otherwise stated). A 100 µl aliquot of the synthetic stimulation solution was applied to a 4 by 1 cm filter paper strip. The extracts of biological origin were used at concentration of 20 µl of male gland extract (20 male equivalents) and 1 µl of concentrated faeces extract. One drop of paraffin oil was applied to reduce evaporation of the test compound. Separate syringes were used for each stimulus and a delay of at least 30 min was allowed for evaporation of the compound within the syringe. Three standards hexanal, 2-methyl-2-butanol and α-(+)-pinene were used to stimulated the ascoid sensillum at doses of 1 mg, 100 µg and 10 µg, and the former two compounds were also combined in equal quantities of 1 µg, in the odour delivery syringe. These three compounds and benzaldehyde were used in double successive stimulations. In all experiments the stimuli were tested on at least two ascoid sensila on the antennae of a minimum of eight flies.

2.5.3 Gas chromatography linked single sensillum recordings (GC-SSR). Olfactory receptors of the ascoid sensillum which had been characterised as responding to some synthetic chemicals and biological extracts were subsequently employed to locate any active products among the many constituents of the odours entrained from the faecal material. Components of these biologically active extracts were separated by high resolution capillary gas chromatography on a Carlo Erba chromatograph (HRGC 5160 Mega Series) equipped with an on-column injector and a FID.

Samples of rabbit faeces odour (2 µl of standard concentrate) were injected on column at 40 °C, onto a 30 m DB-Wax capillary column (J, & W. Scientific), 0.25 mm i.d., 0.25 mm film thickness, with the FID at 230 °C. The column temperature programme was: initial 40 °C for 5 min then increased at 8 °C min^{-1} up to 240 °C. The carrier gas was H_2 at a flow rate of 1 ml min^{-1}. The column effluent was split (glass Y splitter) 75% being sent to the FID and, 25% (longer arm) to the ascoid sensillum via a heated transfer line (250 °C) in the side wall of the GC. The conditioned air-stream (1 ms^{-1}), described above, swept the effluent to the sensillum preparation 30 cm away. Column effluent was thus simultaneously monitored by the FID and any responding receptor in the ascoid sensillum, in order to locate active component(s) of the extract being analysed. Any change in the activity of the preparation was monitored by applying α-(+)-pinene, as a standard stimulus before and after each chromatogram.

All spikes from what usually amounted to multicellular recordings (AC signal) were sorted from background noise with a level discriminator incorporated into the UN-03 amplifier, and the sum of the frequencies of all firing cells was continuously converted to a voltage (time constant of the frequency voltage converter: 1 s). This signal was printed on a

multichannel chart-recorder simultaneously with the FID response. An electrophysiological response was indicated by a sudden change in the overall activity of the olfactory cells being recorded.

2.5.4 Gas chromatography linked mass spectral identification of faecal oviposition attractants. Extracts analysed by GC-SSR to locate active constituents were subsequently concentrated 100 times and analysed on the same GC column phase by GC-MS (Hewlett Packard 5890 series II chromatograph - mass selective detector 5971A) to identify the active compounds. Extract (2 µl) was injected on-column onto a DB-Wax capillary column (dimensions as in section 2.5.3), connected via 1 m of deactivated fused-silica capillary to the MS (source temperature 180 °C; ionisation energy 70 eV). Helium was used as carrier gas under constant pressure (velocity 0.3 ms^{-1} at 40 °C) and separation was achieved with the same temperature programme as in GC-SSR. Active components of the extracts isolated by GC-SSR were relocated in GC-MS by placing them relative to a hydrocarbon profile and by comparison of the chromatogram profiles obtained in GC-SSR and GC-MS. Identification of an electrophysiologically active peak in an extract was first based on the match of it's mass spectrum with that of a known product stored in a computer-based library of the GC-MS. Furthermore, using the single ion monitoring facility (SIM) of the mass selective detector, the presence of a biologically active compound in an extract was searched for at the retention time of the synthetic analogue by several of its characteristic fragment ions.

2.5.5 Gas chromatography analysis of volatile faecal oviposition attractants. Analysis of the faeces odour extract was carried out on polar and non-polar column phases, to compare the retention time of the proposed olfactory stimulants with those of synthetic compounds identified in section 2.4.4. The chromatograph (AI GC 93, AI Scientific,

UK) was fitted with a Grob split/splitless injection, used in the splitless mode and an FID (injector with 0.5 min sampling time at 180 °C, FID at 220 °C). Two fused-silica capillary columns, a DB-1 (non-polar) and a DB-Wax (polar) were used (30 m, 0.25 mm i.d., 0.25 μm film thickness; J. & W. Scientific, USA). The temperature programme employed for both columns was: initial 35 °C for 5 min then increased at 8 °C min^{-1} up to 80 °C, held for 5 min and then increased at 10 °Cmin^{-1} to 240 °C. The carrier gas was helium at a flow rate of 1.4 ml min^{-1}. Integration was carried out with the Varian Star Workstation and integrator board (A2 release, Varian Instruments, UK). Hexanal (10 ng) and 2-methyl-2-butanol (10 ng) were injected on each column and then 1 μl of the rabbit faeces (standard concentration) was injected. The retention time of the synthetic standards and the two predominant early eluting peaks in the faecal odour extracts were compared on each column, over four injections.

2.5.6 Bioassay to test volatile faecal oviposition attractants. The bioassay method used was the same as for the synthetic oviposition pheromone in section 2.4.4. The samples tested included, entrained volatiles of rabbit and chicken faeces, hexanal, 2-methyl-2-butanol, hexanal/2-methyl-2-butanol, dimethyl disulphide, hexanal/dimethyl disulphide, α-(+)-pinene and a negative control of solvents only. Concentrated (100 times) rabbit and chicken faeces volatile extracts and 600 ng of standards were used in each test. The combined mixture of hexanal and 2-methyl-2-butanol was also used at a quantity of 200 ng and α-(+)-pinene was used at quantity of 1 mg. Ten replicates were carried out for each test material and twenty gravid flies were used in each replicate. Only eggs laid on the test and control pads were counted. The data was not normally distributed as indicated by the Anderson-Darling normality test, therefore the difference between the number of eggs laid at the test and control sites was statistically analysed using the Wilcoxon

matched pair signed rank test. To test for differences between the treatments used in the bioassay, the number of eggs laid at the test site for each material was log transformed and ANOVA was performed.

2.6 Effect of combined semiochemicals on sandfly oviposition

2.6.1 Sample preparation. Faeces were air dried, homogenised in a coffee grinder and 2 g of the homogenate were added to 5 ml of each of the following solvents in a 10 ml extraction beaker: double distilled deionized water, isopropanol, diethyl ether and hexane (BDH). Extraction was carried out overnight at -5 °C, the water extract was kept at 3 °C. The extracts were reduced in volume under nitrogen and placed in a 2 ml vial (Alltech). This gave an equivalent concentration of approximately 0.4 mg of organic material per 1 µl. The oviposition pheromone of *L. longipalpis* was extracted as in section 2.3.1. The pheromone was used at a concentration of 100 egg equivalents per 10 µl of hexane. A combined extract was prepared, containing 10 µl of *L. longipalpis* oviposition pheromone and 5 µl of diethyl ether extract of rabbit food.

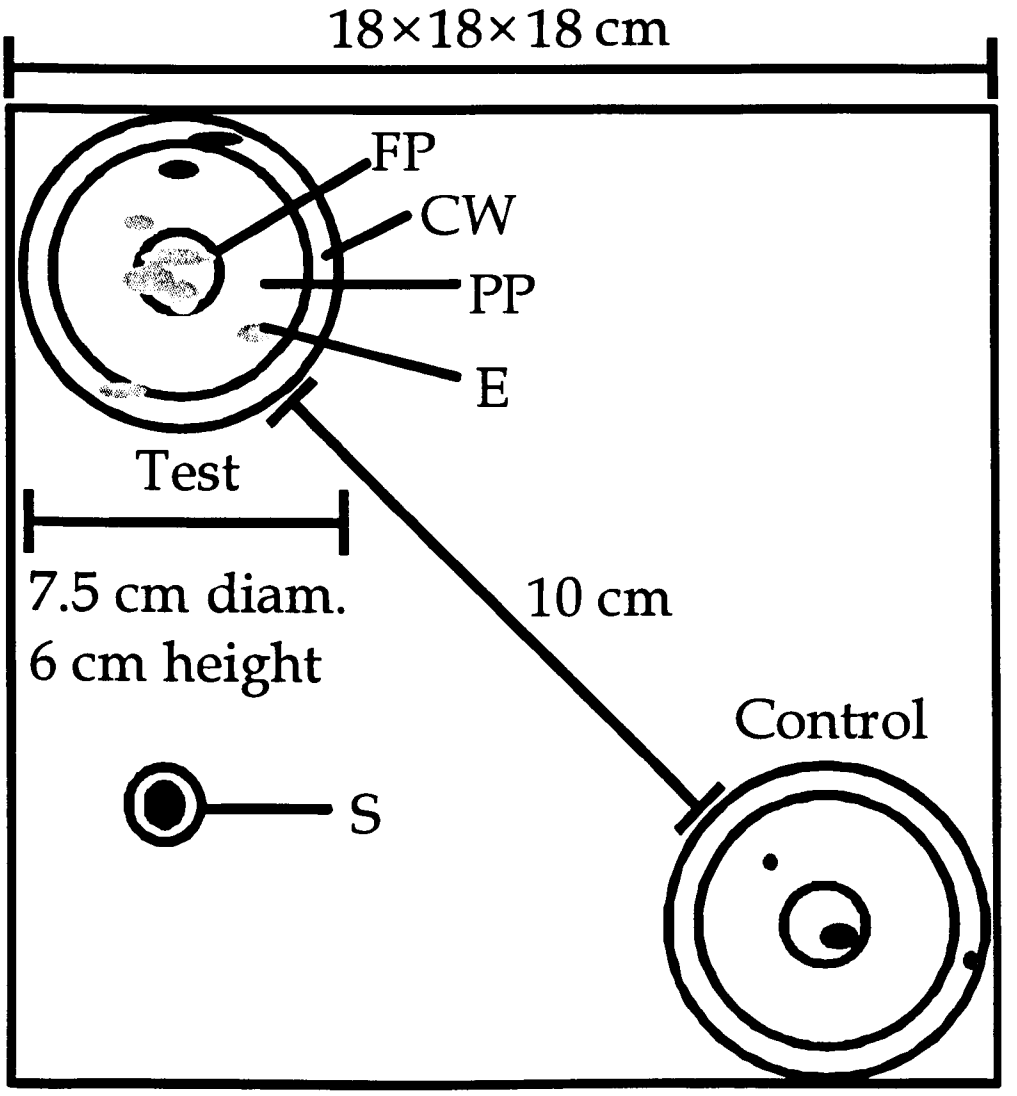
2.6.2 Bioassay to determine optimal extraction solvent for rabbit faeces. The bioassay was an adaptation of the method used by ElNaiem and Ward (1991). Two 2.5 cm diameter filter paper discs (Whatman Lab Supplies), were placed opposite each other on the plaster of Paris base of a PMP pot. To determine if the semiochemicals were acting as contact stimuli, 3 cm diameter by 0.5 cm thick pads of cotton wool were placed on top of the filter papers. This prevented the flies from gaining physical contact with the test and control extracts. In each experiment one filter paper disc was termed the test and impregnated with 10 µl of test extract. The other disc was termed the control and impregnated with 10 µl of the corresponding extraction solvent.

Ten gravid females were placed into each PMP pot and allowed access to super saturated sugar solution on a pledge of cotton wool. The flies were left in the dark for three days, then eggs on the test and control sites were brushed off the cotton wool pad and counted. Each experiment was repeated six times. The results were analysed using the Wilcoxon matched pair signed rank test.

2.6.3 Bioassay to compare rabbit faeces with undigested dietary components. Diethyl ether was used to make test extracts from rabbit faeces, rabbit food and hay. The method of extraction and bioassay were as described above and six replicates were performed. The results were analysed using the Wilcoxon matched pair signed rank test.

2.6.4 Bioassay to determine the effect of combined odour cues from known semiochemical attractants and stimulants on oviposition. The second bioassay was carried out in an 18x18x18 cm Barraud cage (Fig. 2.6). Inside the cage were placed two 7.5 cm diameter PMP pots, with ten 0.5 cm diameter holes drilled in the base, which was lined with a 1 cm thick layer of plaster of Paris, wetted with distilled water to maintain a humid environment. The inner wall of the each pot was lined with a 1 cm layer of cotton wool. Preliminary studies had shown that cotton wool was necessary to provide a thigmotropic stimulus for the sandflies. In the centre of the plaster of Paris base was a 2.5 cm diameter filter paper disc. The disc in the test pots was treated with 10 µl of test solution and the disc in the control pot was treated with 10 µl of extraction solvent or solvents. Three test solutions were used in the bioassay; diethyl ether extract of rabbit food, *L. longipalpis* oviposition pheromone and a combined extract of rabbit food and oviposition pheromone.

Fig. 2.6 Diagrammatic plan view of the Barraud cage bioassay, showing the position and dimensions of the PMP oviposition pots, the three oviposition media and the distribution of eggs within the pots.



Test = PMP pot with the test extract on the filter paper disc; Control = PMP pot with the control solvent on the filter paper disc; FP = 2.5 cm diameter filter paper disc; CW = 1 cm thick layer of cotton wool lining on the base of the pot; E = eggs of *L. longipalpis*; S = pledget of cotton wool soaked in saturated sucrose solution.

A negative control with extraction solvents alone was also conducted. Each experiment was repeated six times. Ten gravid *L. longipalpis* were introduced into the bioassay cage and left in the dark for three days. The pots were removed and the number of eggs on each media (plaster of Paris, cotton wool or filter paper) was recorded. The number of eggs on each oviposition medium, for the test pots, was compared using ANOVA after a log transformation of the data. The total number of eggs in the test and control pots were recorded and compared using the Wilcoxon matched pair signed rank test. A comparison of the number of eggs laid in response to each of the test extracts was carried out using ANOVA, after a log transformation of the data.

2.6.5 Bioassay to determine the effects of combined semiochemicals on oviposition of individually tubed flies. Two groups of 30 gravid flies were placed in individual oviposition tubes, using the method described by ElNaiem and Ward (1992). One group was termed the test and the filter paper on the bottom of the tube was treated with combined extract as described in section 2.6.1 and 2.6.4. The other group was termed the control and had only solvents on the filter paper. The flies were kept in a dark, humid environment and the mean number of eggs laid per female, how many flies oviposited, when the flies started to oviposit and the general survival, were recorded daily for five days. The experiment was repeated 4 times and Mann-Whitney analysis was used to statistically compare the mean numbers of eggs laid by each female, the other parameters were analysed using χ^2.

2.6.6 A laboratory oviposition trap. Given the targeted oviposition observed in the previous bioassay an attempt to trap gravid flies using a modified Barraud cage bioassay was made. The plaster of Paris base in the test and control PMP pots was coated with a thin layer of dimethyl-polysiloxane (Sigma Chemical Company), to trap the attracted

sandflies. The filter paper discs on the base of the test and control pots was treated as described in section 2.6.1 and 2.6.4, with the combined extract as the test solution The Barraud cage was 60x60x60 cm, this greatly increased the distance for potential attraction. Fifty gravid females and 50 males of a similar age were introduced into the bioassay cage and left in the dark. The flies were allowed access to a sugar meal and after three days the numbers of flies trapped, dead in the cage and still alive were recorded. The experiment was replicated 4 times.

2.6.7 Bioassay to test the effect of combined synthetic oviposition attractants. The bioassay method used was the same as in section 2.4.4. The solutions to be tested were added drop wise to the test septa whilst an equal volume of clean solvent was added to the control septa. The samples used included; combined synthetics consisting of dodecanoic acid (400 ng / 100 EE), hexanal (200 ng) and 2-methyl-2-butanol (200 ng); entrained rabbit faeces volatiles (2 µl of 100 times concentrated extracted solution); hexanal and 2-methyl-2-butanol (200 ng of each); a negative control of solvents only. The synthetic compounds tested in the bioassay were present in quantities at which they would naturally occur in attractant samples of entrained rabbit faeces volatiles or sandfly eggs. Eight replicates were undertaken for every sample with 20 females in each. The analysis of the data was the same as in section 2.4.4.

RESULTS

3.1 Biological origin of the oviposition pheromone

3.1.1 Bioassay to test for oviposition attraction. The bioassay results are summarised in Table 3.1. The oviposition response of *L. longipalpis* to hexane extracts of eggs and accessory glands, showed a highly significant difference between the number of eggs laid at the test and control sites. Both hexane and aqueous extracts of female bodies without accessory glands failed to induce gravid flies to lay more eggs at the test site compared to the control, as did aqueous extracts of both egg and accessory glands.

Table 3.1 The oviposition response of gravid *L. longipalpis* to aqueous and hexane extracts of eggs, accessory glands and whole female *L. longipalpis* without accessory glands.

Extract	Solvent	Oviposition response		P
		Test Mean±SE	Control Mean±SE	
Egg	Hexane	95.4 ± 13.79	20.8 ± 8.68	0.006
Egg	Water	22.0 ± 8.16	26.2 ± 4.76	0.186*
AG	Hexane	98.8 ± 12.31	26.4 ± 11.54	0.006
AG	Water	23.6 ± 6.26	24.3 ± 4.90	0.610*
Female without AG	Hexane	25.9 ± 6.49	26.5 ± 6.98	0.799*
Female without AG	Water	25.7 ± 5.60	23.5 ± 4.20	0.262*

AG = Accessory gland, Solvent = Solvent with which extract was made. The number of eggs laid at the test and control sites were compared using by Wilcoxon matched pair signed rank test. P = Probability, * = Non-significant.

3.1.2 High performance thin layer chromatography. Analysis showed chemicals present in both egg and accessory gland samples that were of similar polarity to each other (Table 3.2). Cholesterol (lane 1) was present in both, as was a compound more polar than cholesterol. Two other pairs of matching spots were at R_f = 0.51 (AG) and R_f = 0.50 (egg) these were slightly less polar than caryophyllene oxide (lane 2). At R_f = 0.62 (AG) and R_f = 0.66 (egg), two spots were exhibited of similar polarity to cholesterol stearate (lane 3) and cholesterol linolenate (lane 4),

Table 3.2 R_f values of standards and extracts of accessory glands and eggs calculated from the HPTLC plate.

Sample	Polar				Non-polar
1) Cholesterol	0.12				
2) Caryophyllene oxide		0.48			
3) Cholesterol stearate				0.67	
4) Cholesterol linolenate				0.69	
5) Squalene					0.72
6) Accessory gland	0.12 0.03		0.51	0.62	
7) Egg	0.12 0.06		0.5	0.66	

3.1.3 Gas Chromatography analysis. Retention times of major peaks found in the GC analysis of hexane extracts of accessory glands, eggs and females without accessory glands are shown in Table 3.3. All chromatograms had a similar pattern to the traces with peaks 3, 4 and 5 common to all the extracts (Fig. 3.1). Male *L. longipalpis* had two major

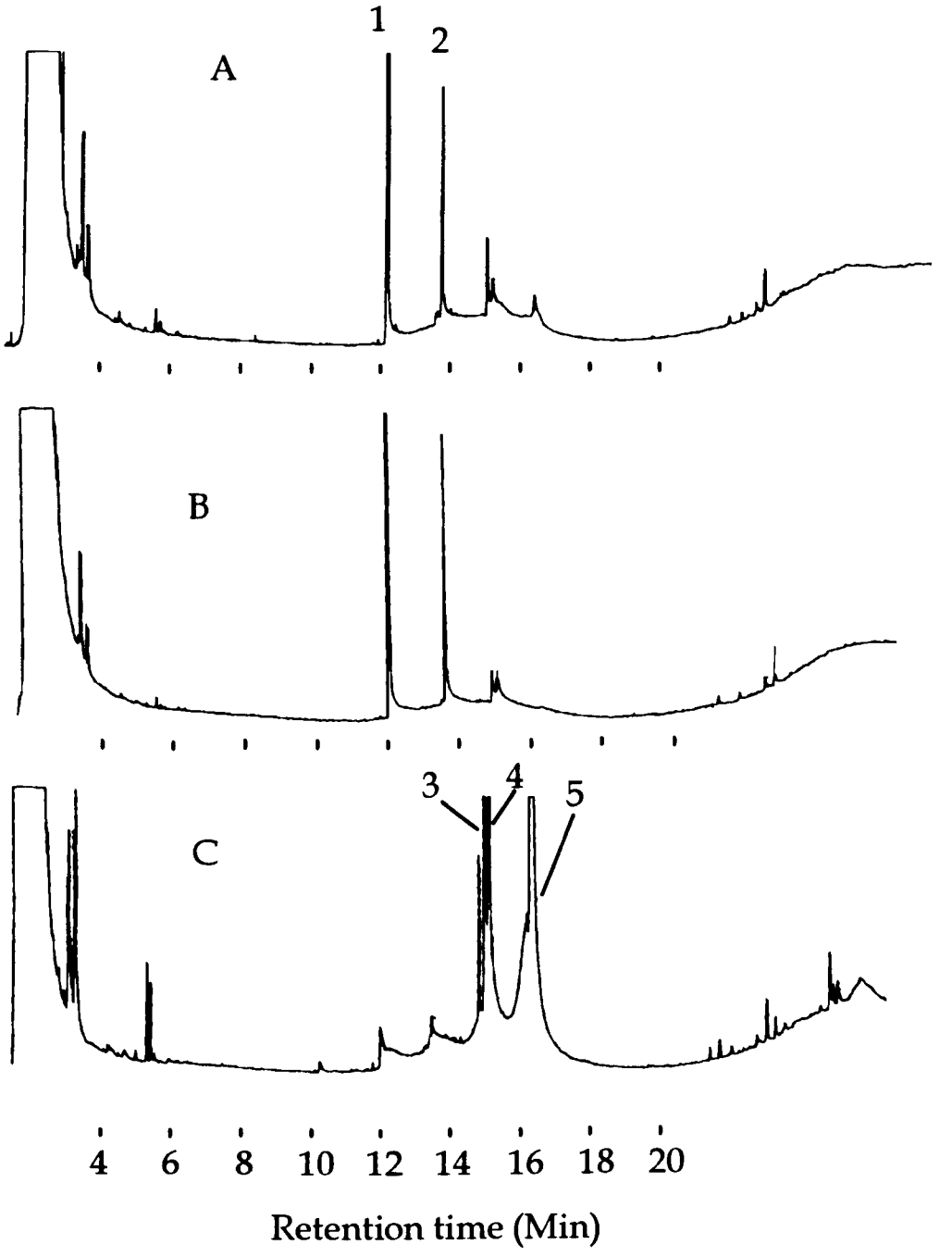
peaks at a retention time (Rt) of 11.05 mins and 11.51 mins these are known to originate from the tergal glands, (Hamilton *et al.*, 1994). Whole female extract had major peaks at Rt = 11.89 and Rt = 13.51, which were not present in the whole male samples. These two peaks (1 and 2 on the chromatogram) were found in female accessory gland and in egg extract, but were greatly reduced in extracts of females with excised accessory glands.

Table 3.3 Gas chromatograph retention times for extracts of egg, accessory gland, whole female and whole male samples.

Retention time (Rt = Minutes)	11	12	13	14	15	16
Egg	11.86		13.52	14.82 14.99		16.21
AG	11.89		13.50	14.84 14.99		16.20
Whole female	11.88		13.34 13.50	14.86	15.00	16.2
Female without AG	-		-	14.85 14.98		16.18
Whole male	11.05 11.51			14.85 14.99		16.19
Corresponding number on chromatogram (Fig. 3.1)	(1)		(2)	(3/4)	(4)	(5)

AG = Accessory gland

Fig. 3.1 Chromatograms of hexane extracts of (A) accessory glands, (B) eggs, and (C) females with accessory glands excised. Peak numbers correspond to those in Table 3.3.

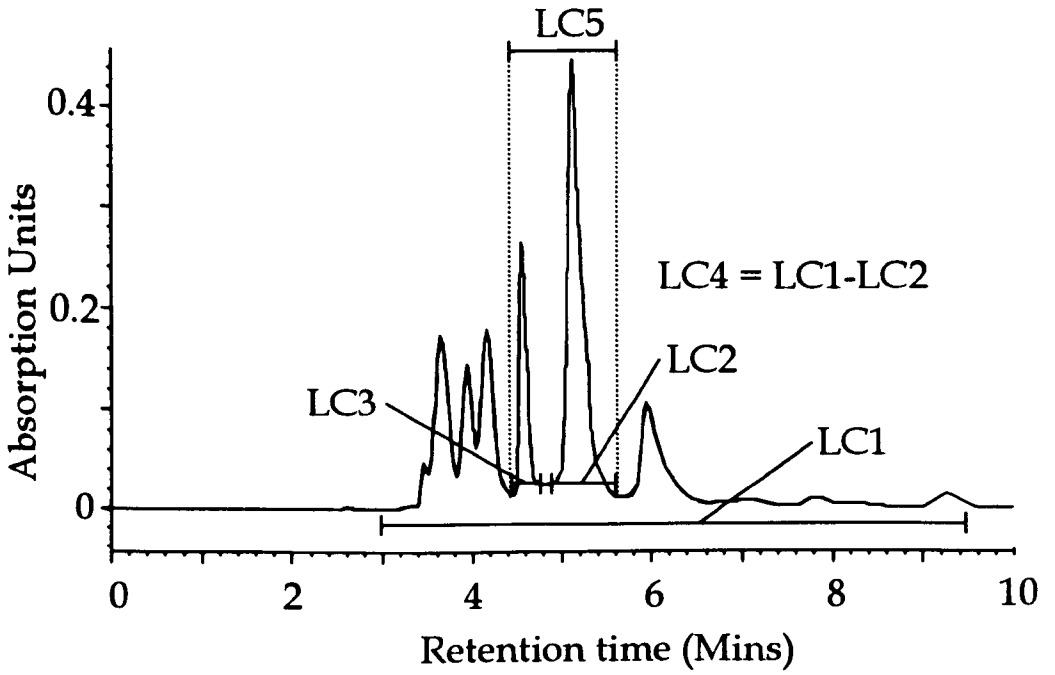


3.2 Isolation of the oviposition pheromone

3.2.1 High performance liquid chromatography fractionation.

Analysis resolved crude hexane extract of *L. longipalpis* eggs into a number of peaks which could be fractionated for bioassay and GC analysis. Throughout the investigation HPLC generated fractions were termed LC# and GC analysed fractions were termed GC#. A typical HPLC chromatogram is shown in Fig. 3.2.

Fig. 3.2 Maximum wavelength chromatogram from HPLC analysis of hexane extract of *L. longipalpis* eggs, showing the fractions isolated for GC and bioassay analysis.

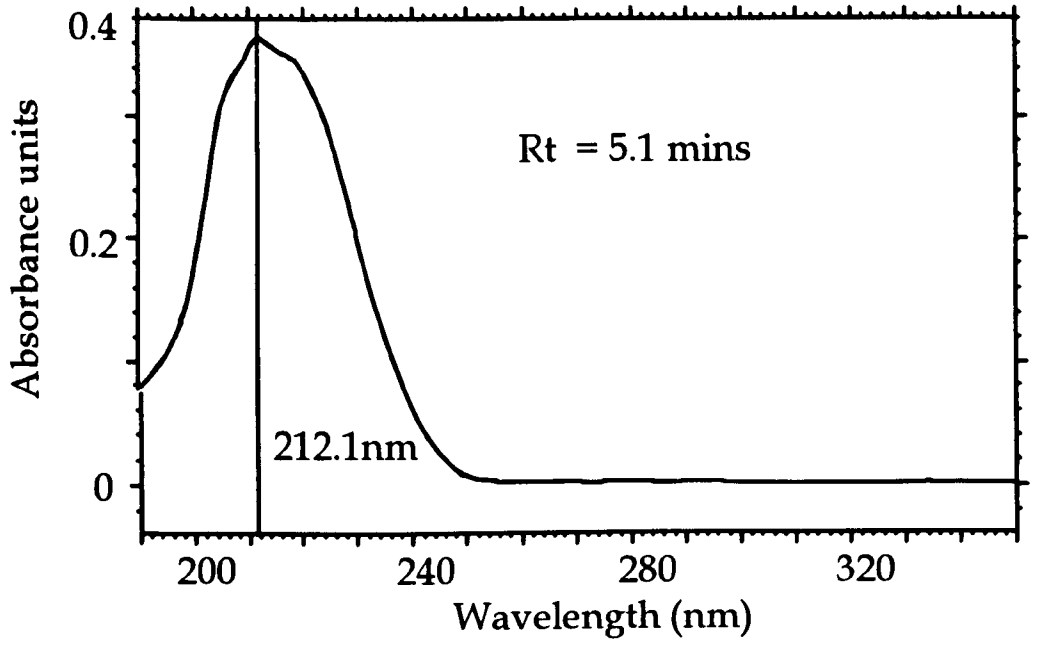


LC = HPLC generated fraction. LC5 = combined fraction of peaks LC2 and LC3, LC4 = whole extract with fraction LC2 removed.

The chromatogram had a characteristic large peak (LC2) at Rt = 5.10 min. The λ max. (maximum absorbance) for this compound was 212.1 nm and the UV spectrum can be seen in Fig. 3.3., dodecanoic acid had a λ max. of 212 nm. LC3 eluted at Rt = 4.56 min (λ max. 208 nm), there were 4 smaller

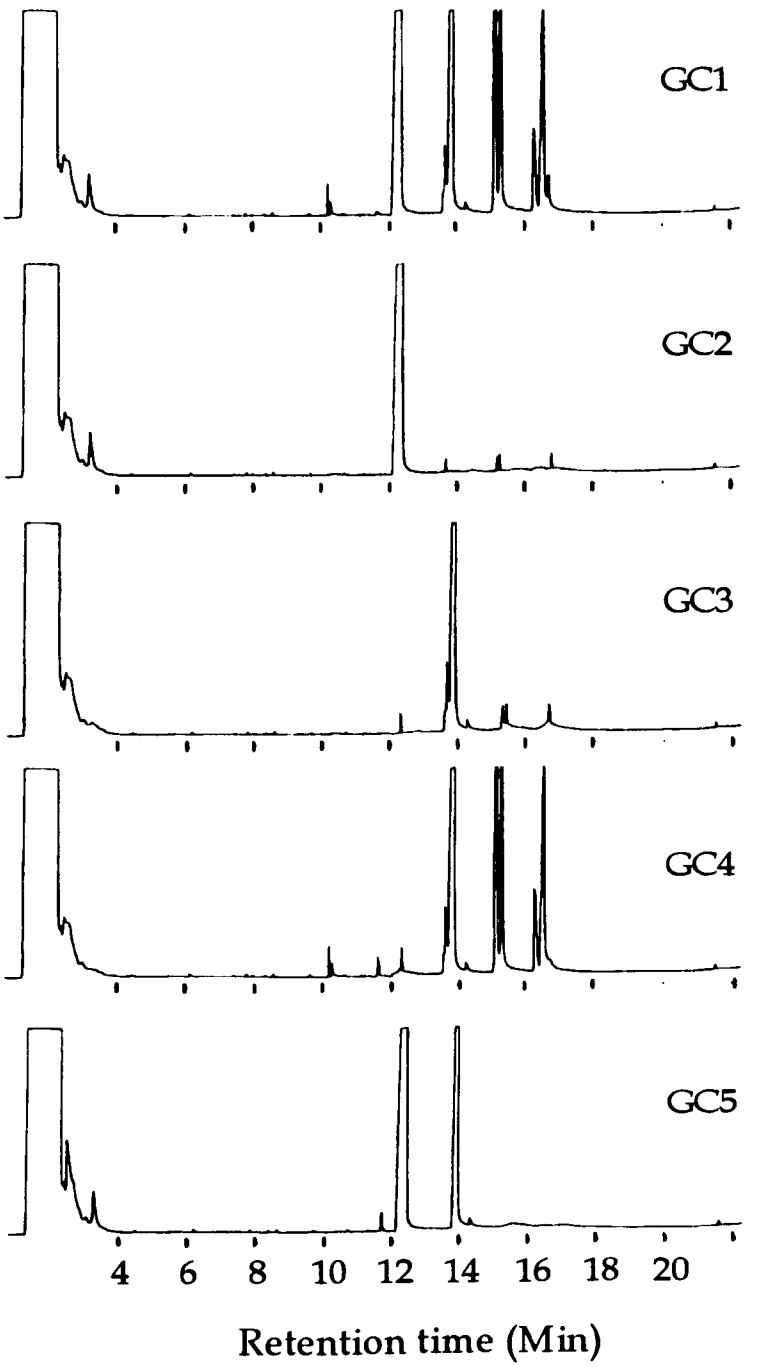
peaks between Rt = 3.46 to 4.17 min and Rt = 5.95 to 10 min. Five fractions were collected for bioassay and GC analysis. These were: LC1 (whole HPLC eluent of hexane egg extract), LC2, LC3, LC4 (whole extract minus LC2) and LC5 (LC2 and LC3 combined) (see Fig. 3.2). The caryophyllene oxide standard eluted with a Rt = 5.13 min.

Fig. 3.3 Ultraviolet spectrum of HPLC F2, with a primary absorbance at 212.1 nm.



3.2.2 Gas chromatography analysis. Analysis showed that HPLC collected fractions were free from major contamination (Fig. 3.4). Peaks observed with GC analysis of whole egg extract (GC1) were correlated with those in the HPLC chromatogram (LC1). LC2 was GC2 (Rt = 12.36 min) and LC3 was GC3 (Rt = 13.89 min). The correlation of HPLC fractions with specific peaks on the GC was necessary before a gas chromatography coupled mass spectral investigation could be undertaken.

Fig. 3.4 Chromatograms from capillary gas chromatography of fractionated hexane extract of *L. longipalpis* eggs, isolated by HPLC and used in the bioassays.



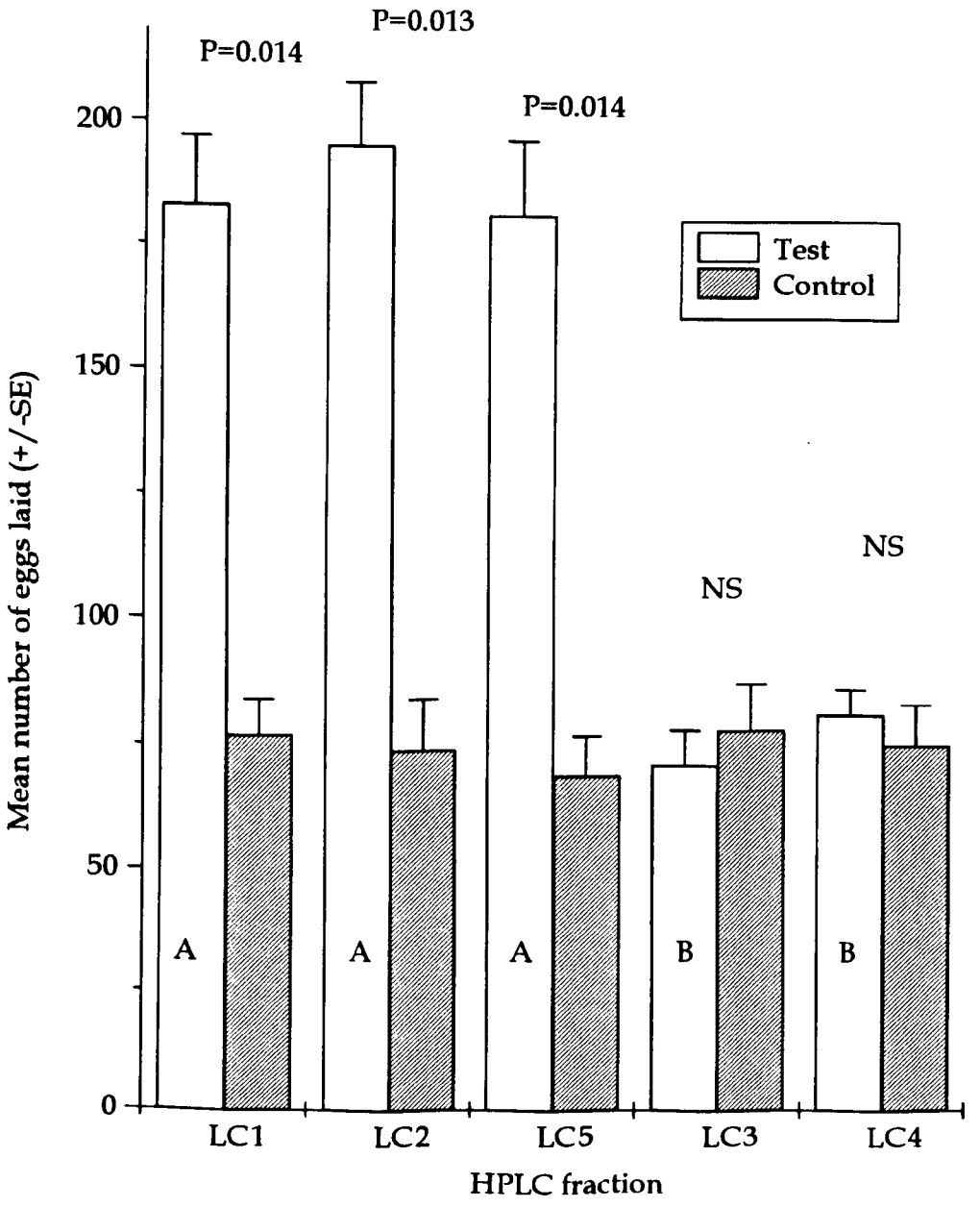
GC = various HPLC generated fractions that were analysed with the GC. GC1 = Whole egg extract (LC1); GC2 = LC2; GC3 = LC3; GC4 = LC4; GC 5 = LC5.

When compared with caryophyllene oxide standard (Rt = 12.49 min), there was 12.75 μg of LC2 in the crude extract of 1200 eggs, with a recovery of 97.23% for bioassay. Loss of material may be due to the fractionation and liquid/liquid separation processes. There was 7.65 μg of LC3 in the crude extract and a 98.7% recovery for bioassay. LC2 and LC3 had similar GC retention times to the two peaks that were found in both egg and accessory gland extracts described in section 3.1.3

3.2.3 Bioassay to test for oviposition attraction. Bioassay results are shown in Fig. 3.5. LC1, LC2 and LC5 induced positive oviposition responses from gravid *L. longipalpis* and significantly more eggs were laid at the test sites than the controls. LC2 produced an oviposition response not significantly different from the whole egg extract LC1 (P=0.33), when tested separately against a control. LC3 and F4 did not produce significant differences in the number of eggs laid at the test and control sites. LC5 did produce a positive oviposition response, but this was not greater than LC1 (P=0.819) or LC2 (P=0.521).

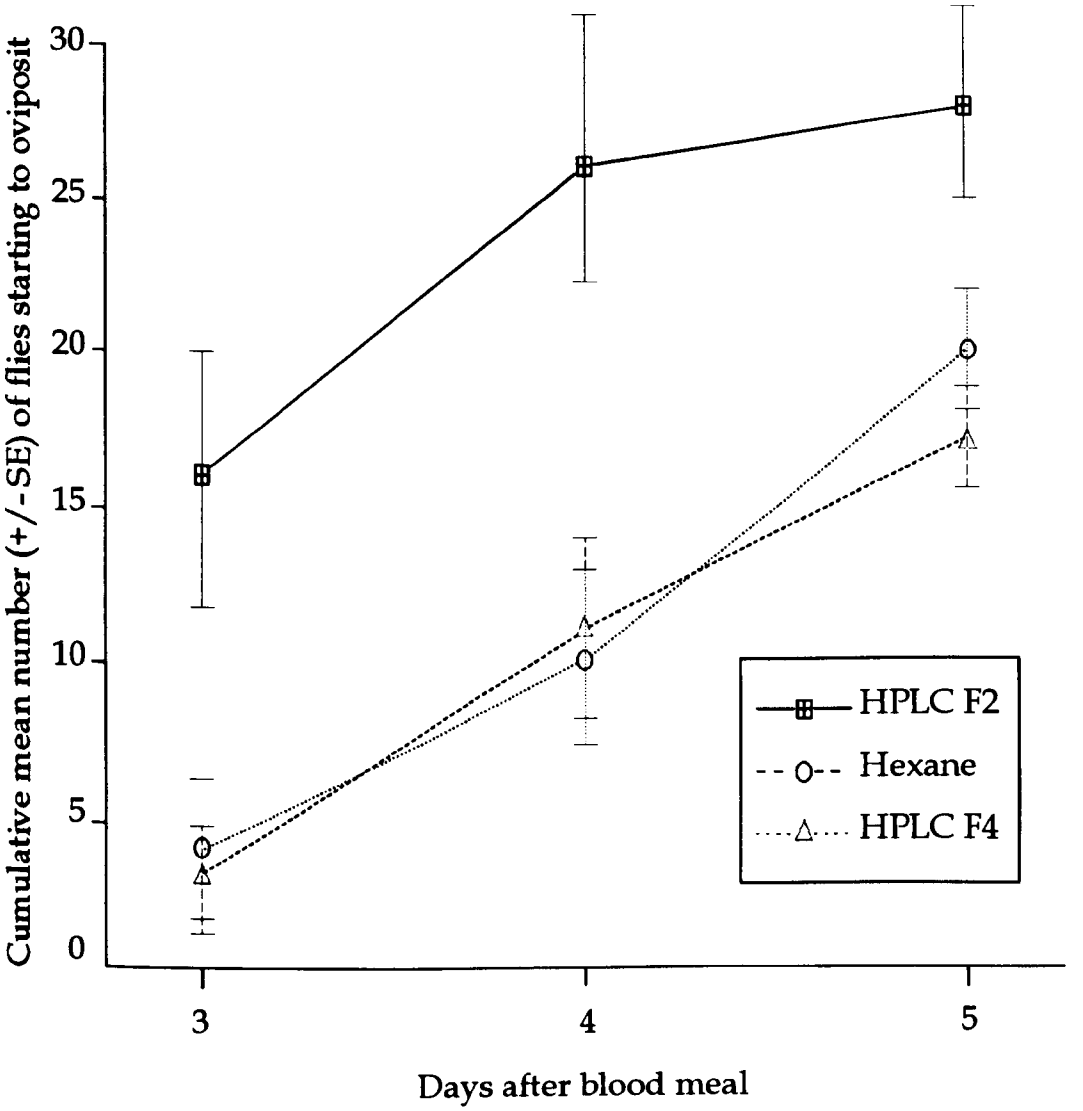
3.2.4 Bioassay to test for oviposition stimulation. Gravid flies exposed to either LC4, LC2 or the hexane control survived oviposition in comparable but low numbers, averaging 6.9 (23%), 6 (20%) and 9 (30%) respectively. A mean of 3 (10%) flies exposed to LC2 did not lay eggs compared to 9 (30%) for both the hexane control and LC4. The time of onset of oviposition may be seen in Fig. 3.6. The flies exposed to LC2 oviposited earlier than flies exposed to hexane or LC4. In addition, gravid flies exposed to LC2 produced a mean of 46.9±10.5 eggs per female, in contrast to the flies exposed to hexane and LC4, which produced significantly lower mean numbers of eggs (P=0.0001) of 20.7±3.1 and 20.5±4.4 respectively.

Fig. 3.5 Oviposition response of gravid *L. longipalpis* to HPLC generated fractions of whole egg extract.



The number of eggs laid at the test and control sties were compared using Wilcoxon matched pair signed rank test. P = probability, NS = non-significant. Treatments with the same letter are not relatively significantly different (Kruskal-Wallis analysis). Fraction numbers F1, F2, etc. correspond to fractions seen in Fig. 3.2.

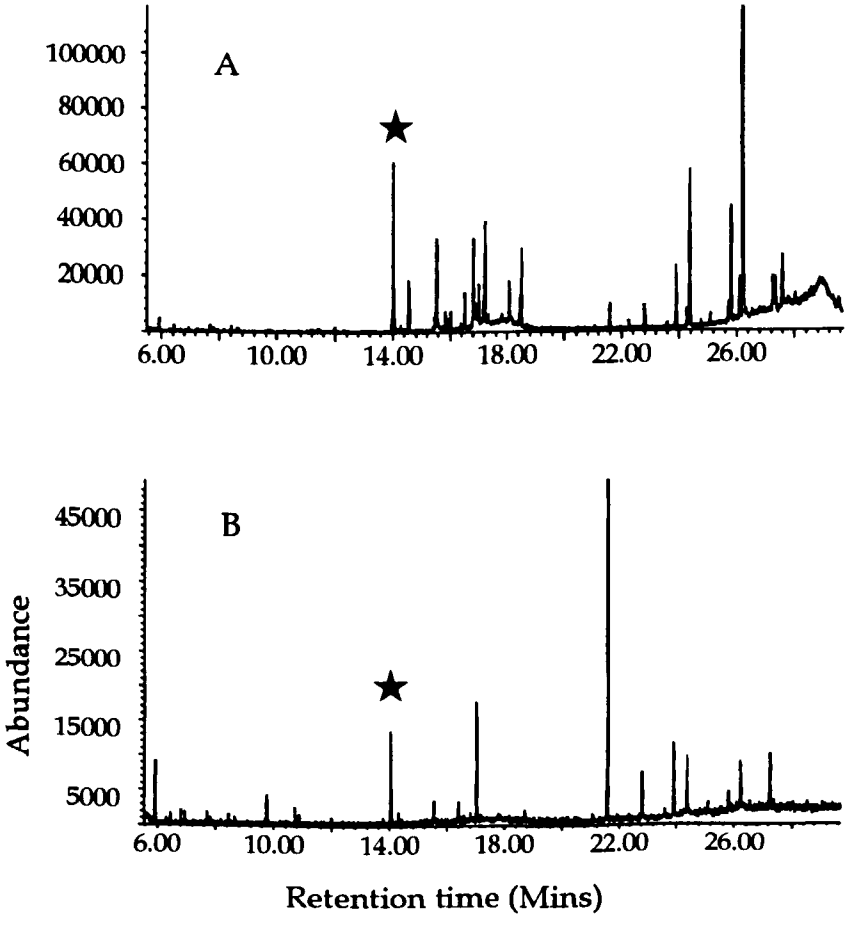
Fig. 3.6 The effect of the hexane control, HPLC fraction 4 and fraction 2 on the period of time taken by individually tubed *L. longipalpis* females to oviposit after a blood meal.



3.3 Structural characterisation of the oviposition pheromone

3.3.1 Gas Chromatography linked Mass Spectrometry (EI) of oviposition pheromone. The total ion chromatograms (TIC) of hexane egg extract of *L. longipalpis* originally collected from Jacobina, Brazil and Curarigua, Venezuela are shown in Fig. 3.7.

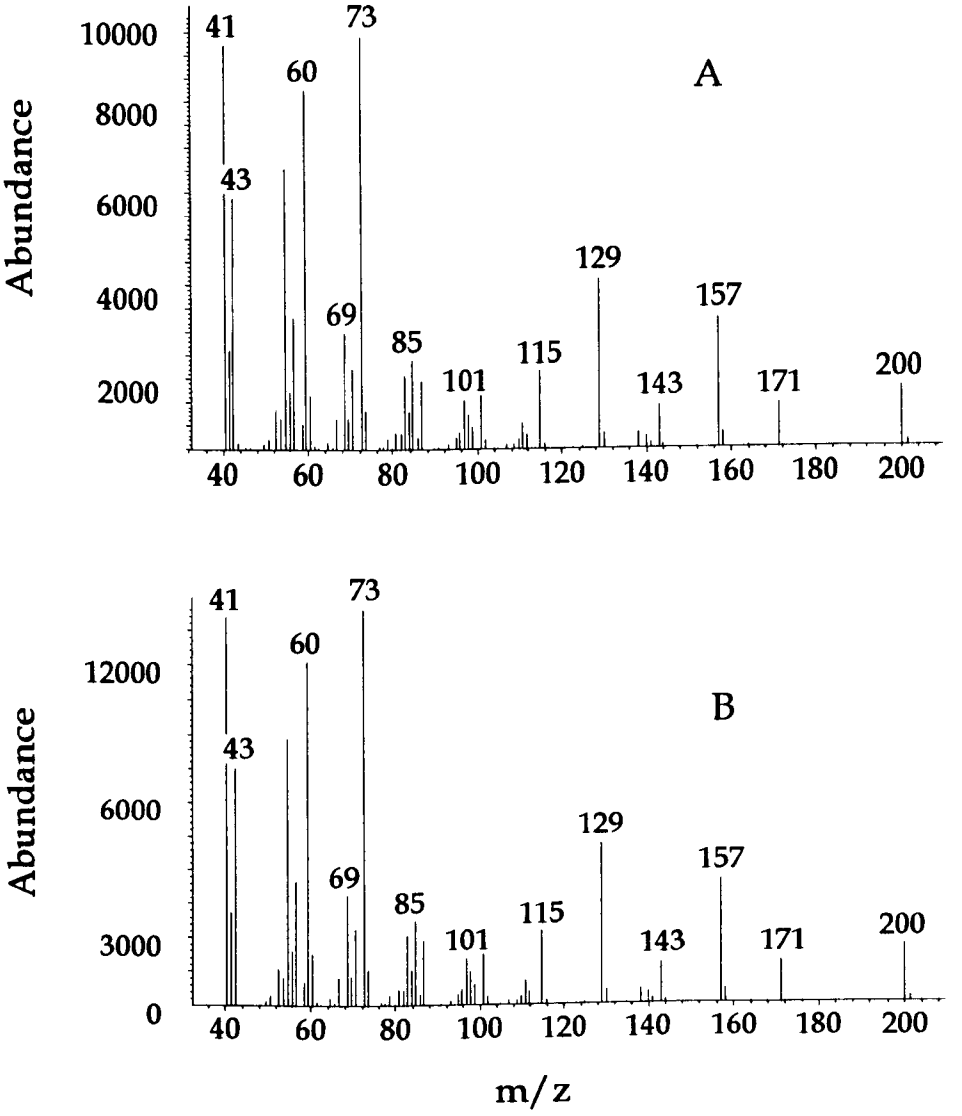
Fig. 3.7 Total ion chromatograms (TIC, EI) of hexane extracts of *L. longipalpis* eggs, from colonies originally collected at Jacobina, Brazil (A) and Curarigua, Venezuela (B).



Each chromatogram represents 80 egg equivalents and the two peaks indicated by ★ are the pheromone peaks.

The chromatograms were characterised by several peaks, common to both egg extracts. The pheromone peak eluted at Rt = 14.04 min for Jacobina and Rt = 14.03 min for Curarigua with the fast temperature (fast TP) and Rt = 26.31 min and Rt = 26.26 min respectively with the slow temperature programme (slow TP), on the HP-5MS column

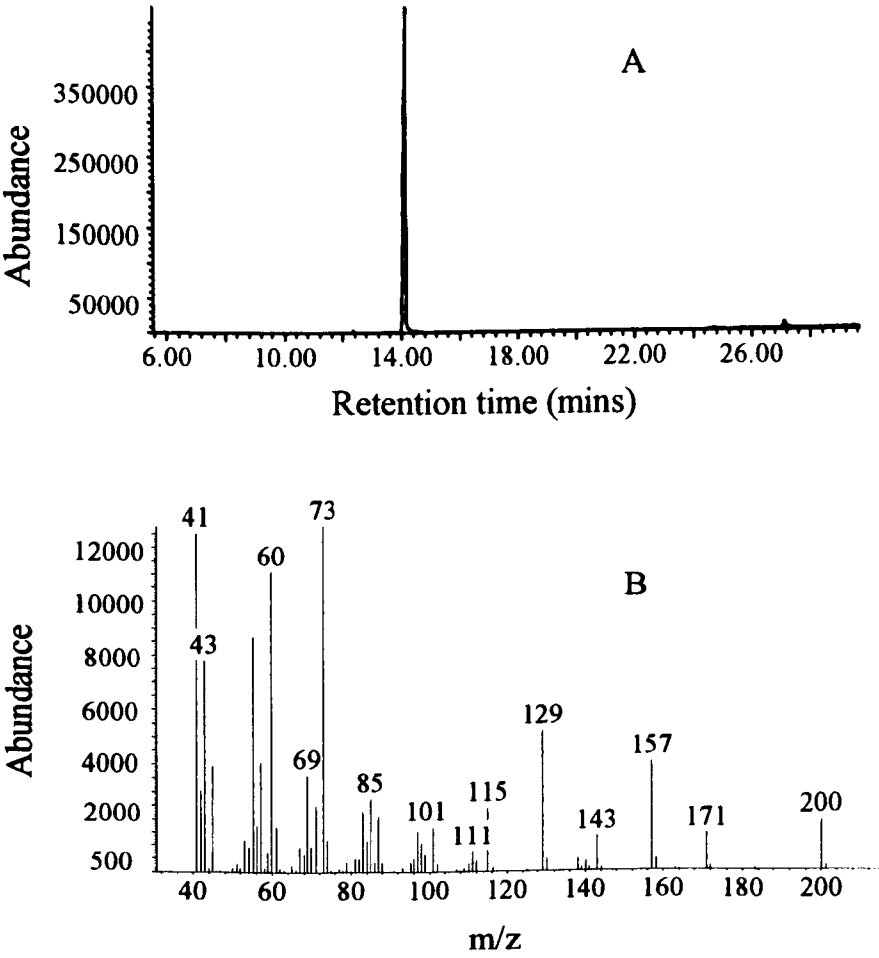
Fig. 3.8 EI mass spectra of the pheromone peak indicated in Fig. 3.7 as ★, in hexane extracts *L. longipalpis* eggs, from Jacobina, Brazil (A) and Curarigua, Venezuela (B).



m/z = mass to charge ratio.

On the DBwax column the Rt of the pheromone peaks increase to 23.20 (Jacobina) min and 23.17 min (Curarigua) respectively. The mass spectra of the pheromone peak for the two extracts of *L. longipalpis* eggs are shown in Fig. 3.8. The spectra indicated a compound with a molecular weight of 200 a.m.u. (M^+ = 200) and a base peak of 73 a.m.u. When a library search was undertaken, there was a 97% match for Jacobina pheromone with the compound dodecanoic acid. The Curarigua spectrum gave a 96% match with dodecanoic acid.

Fig. 3.9 TIC (EI) of synthetic dodecanoic acid (A). Mass spectra of dodecanoic acid peak (B).



3.3.2 Gas chromatography linked mass spectral analysis (EI) of synthetic compounds. Dodecanoic acid eluted at 14.09 min (fast T. P.) and 26.25 min (slow T. P.) on the HP-5MS column. With the DBwax column the Rt = 23.17 min. The TIC and mass spectra for the synthetic dodecanoic acid are shown in Fig. 3.9. The mass spectra confirmed the known molecular weight of dodecanoic acid at 200 a.m.u. ($M^+ = 200$) with a base peak at 73 a.m.u. There was a 96% match between the synthetic dodecanoic acid and NIST library search. The spectra and retention times of synthetic dodecanoic acid matched that of the pheromone peak in both the Jacobina and Curarigua, *L. longipalpis* samples. When compared to the dodecanoic acid standard, each egg from the Jacobina sample yielded approximately 3 ng.

3.3.3 Methyl-esterification of pheromone and synthetic compounds and confirmation of molecular weight. The mass spectra of Jacobina pheromone methyl-ester (ME) and synthetic dodecanoic acid ME can be seen in Fig. 3.10. The spectra showed the molecular weight of dodecanoic acid ME to be 214 a.m.u. this matched the molecular weight of the methyl esterified pheromone. The dodecanoic acid ME produced a peak that eluted with a Rt = 16.34 min, had an M^+ of 214 a.m.u. and a base peak at 74 a.m.u. Jacobina pheromone ME gave a peak at Rt = 16.37 min, an M^+ of 214 a.m.u., a base peak of 74 a.m.u. and ions that closely matched the synthetic dodecanoic acid ME. When the EI spectra was library search, the Jacobina pheromone ME and synthetic dodecanoic acid ME gave a 95% and 96% match respectively. Spectra of Jacobina pheromone ME and dodecanoic acid ME obtained by CI are shown in Fig. 3.11. CI gave molecular ions of $M+H^+$ of 215 a.m.u. for both products, confirming the a MW of 214 a.m.u.

Fig. 3.10 EI mass spectra of the Jacobina pheromone ME (A) and synthetic dodecanoic acid ME (B).

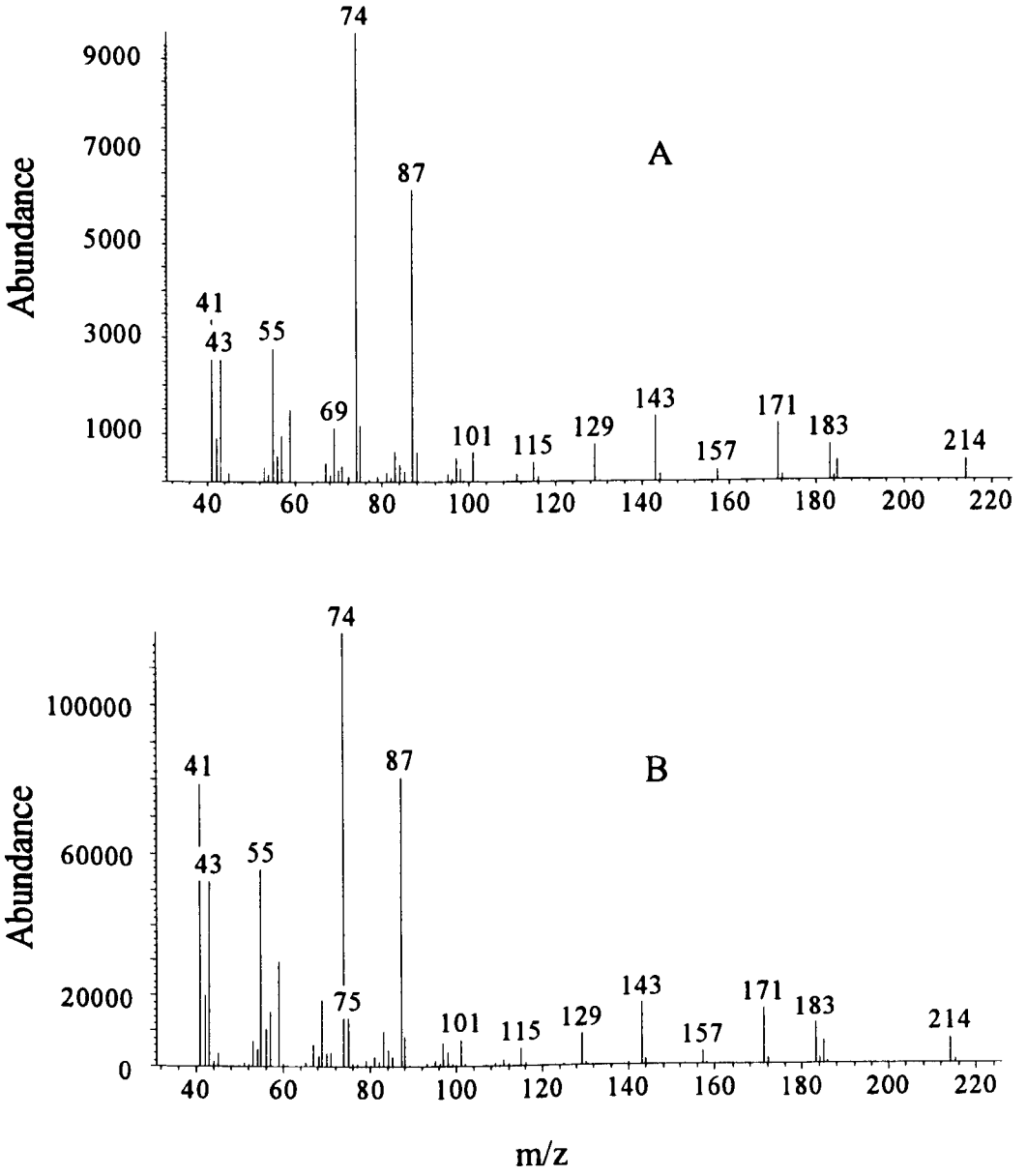
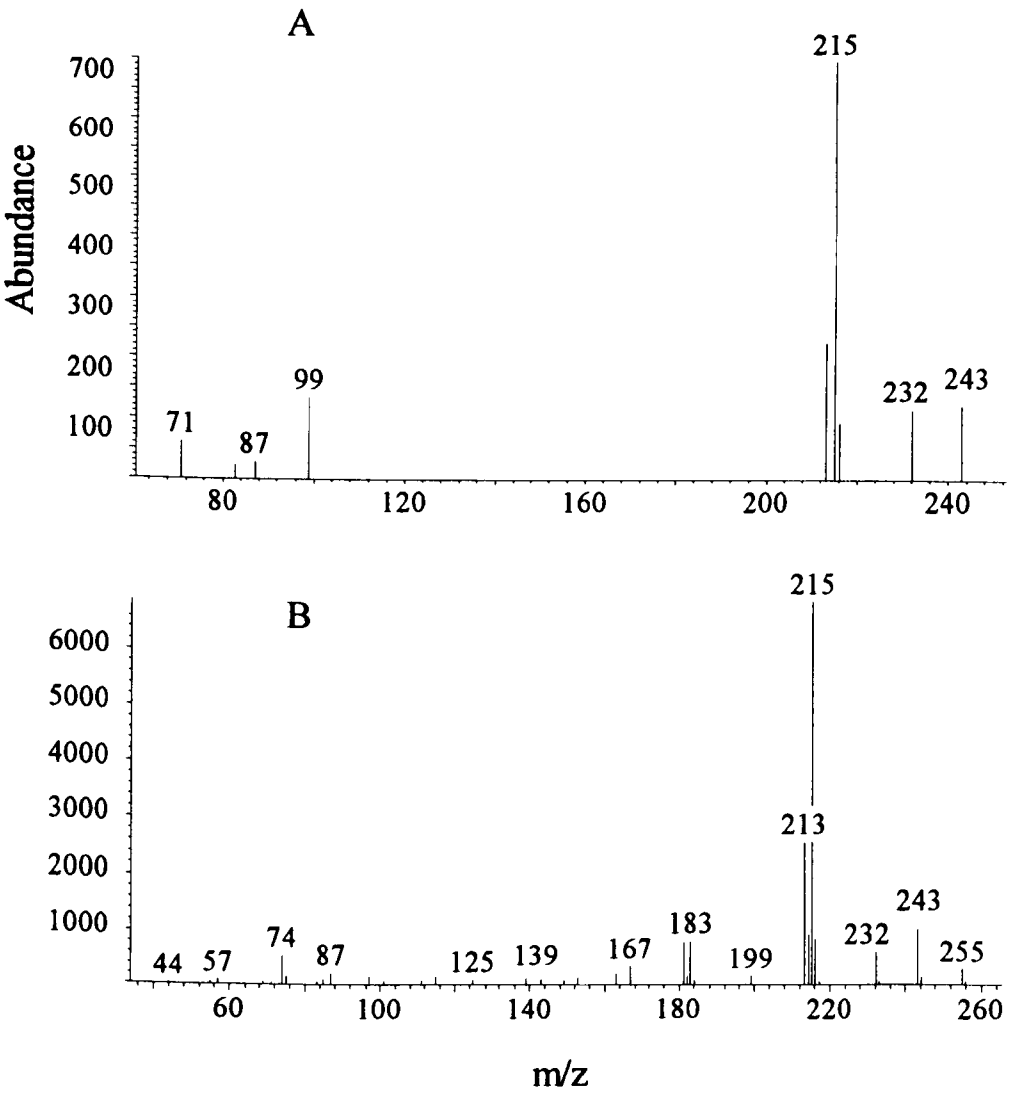
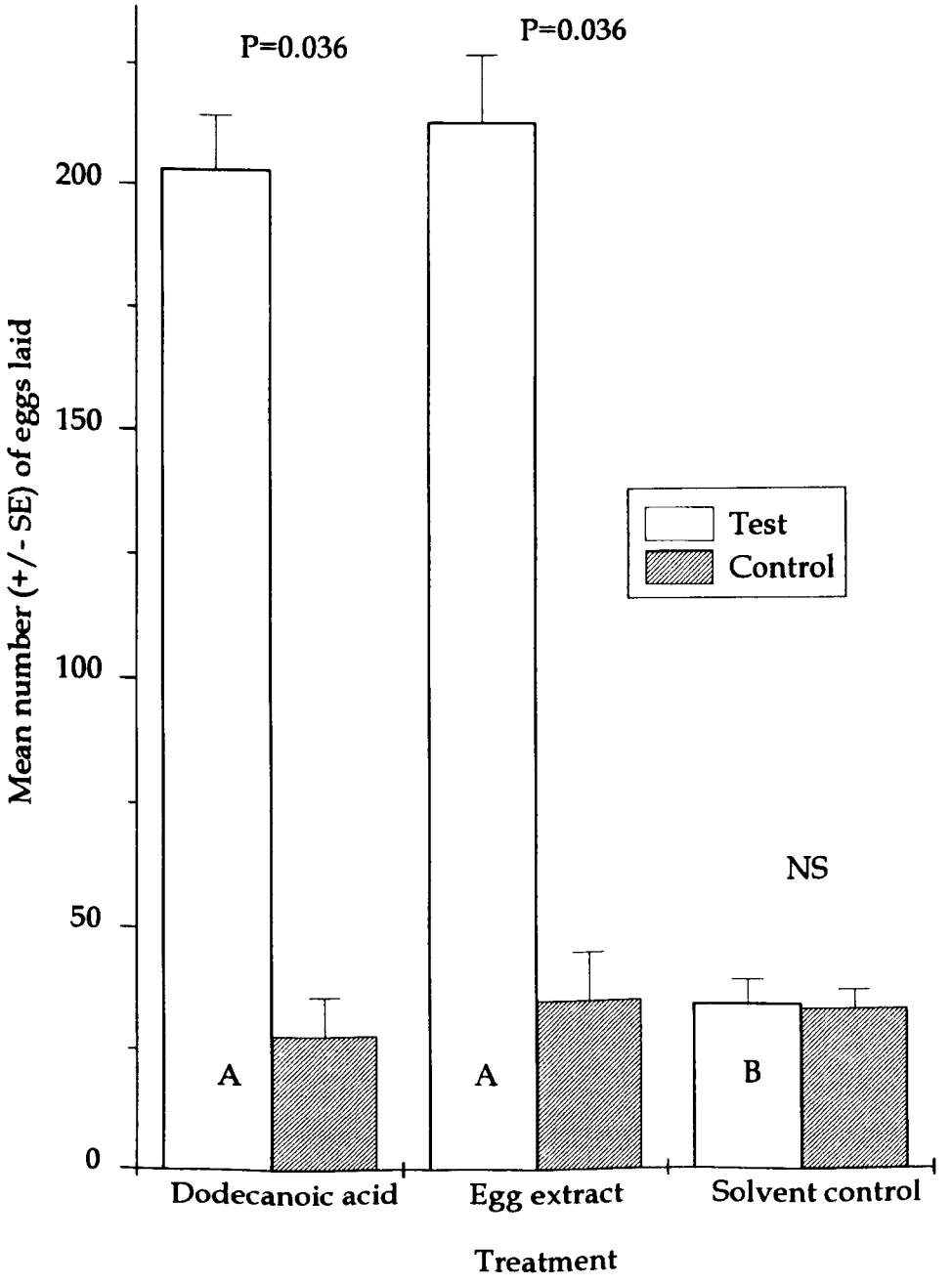


Fig. 3.11 CI mass spectra of Jacobina pheromone ME (A) and synthetic dodecanoic acid ME (B).



3.3.4 Bioassay to test for oviposition attraction. The results of the bioassay are shown in Fig. 3.12. Both whole egg extract and dodecanoic acid produced a targeted oviposition response from gravid *L. longipalpis,* with significantly more eggs being laid at the test site than control. There was no significant difference between the numbers of eggs laid at the test sites for the whole egg extract or dodecanoic acid. The solvent control did not produce an oviposition response and the number of eggs laid at the test site was significantly less than the other two samples.

Fig. 3.12 The number of eggs laid by gravid *L. longipalpis* in response to whole egg extract, synthetic dodecanoic acid and a control of solvents only.



The number of eggs laid at the test and control sites were compared using Wilcoxon matched pair signed rank test. P= probability. NS = Non significant. Treatments with same letter are not relatively significantly different (ANOVA).

3.4 Isolation of faecal oviposition attractants

3.4.1 Electrophysiological responses to biological extracts. An electronmicrograph of the ascoid sensillum is shown in Fig. 3.13. The overall spontaneous activity within the sensillum was complex, with numerous cells firing. Clear cut responses were obtained to the extracts of biological origin which were tested (Fig. 3.14). The response to faecal volatiles was characterised by the inhibition of the cell producing the large amplitude spike (type 1), decreasing from its normal spontaneous frequency of 9.1±2.3 Hz to zero. When stimulation stopped this cell type returned with a rebound, increasing from its normal spontaneous frequency of 9.1±2.3 to 19.3±2.61 Hz. By contrast cell types 2 and 3a, producing smaller spike amplitudes increased their firing rates, with rabbit faeces, cell type 2 from 27.8±4.9 to 91.8±8.9 Hz and cell type 3a from 26.9±3.98 to 49.5±2.9 Hz; for chicken faeces, cell type 2 from 26.0±3.7 to 77.3±9.9 Hz and cell type 3a from 26.0±4.83 to 49.0±6.42 Hz. The tergal gland extract, containing the sex pheromone, stimulated a cell (type 4) from 26.0±5.6 to 115.25±8.3 Hz. The cell was characterised by a small amplitude spike showing a strong negative component.

3.4.2 Electrophysiological responses to synthetic volatiles. Eight compounds elicited a response from olfactory receptors in the ascoid sensillum; R-(+)-α-pinene, R-(-)-α-pinene, S-(+)-β-pinene, S-(-)-β-pinene, α-terpinene, benzaldehyde, hexanal and 2-methyl-2-butanol (Fig. 3.15). Hexanal and benzaldehyde activated cell type 2 and mimicked the faeces odour extract by inhibiting cell type 1. Hexanal was the most active synthetic tested, increasing firing from 13.9±2.7 to 93.7±8.1 Hz, it was the only product to activate a receptor at 100μg dose at source under paraffin oil. Benzaldehyde also activated cell type 2, but the threshold was somewhat higher as the increase in spike frequency was only from 14.3±3.8 to 74.8±7.8 Hz, with a 1 mg source.

Fig. 3.13 Scanning electronmicrograph of the ascoid sensillum (A) on antenna segment 14 of a female *L. longipalpis*. Ascoids are set proximally on the lateral face of segments 3-15; the ascoid is approximately the same length as the segment here.

The bars (upper right and lower left) = 5 μm. Other large sensilla include, SCH = sensillum chaeticum, and ST = sensillum trichodeum (after Boufana 1990).

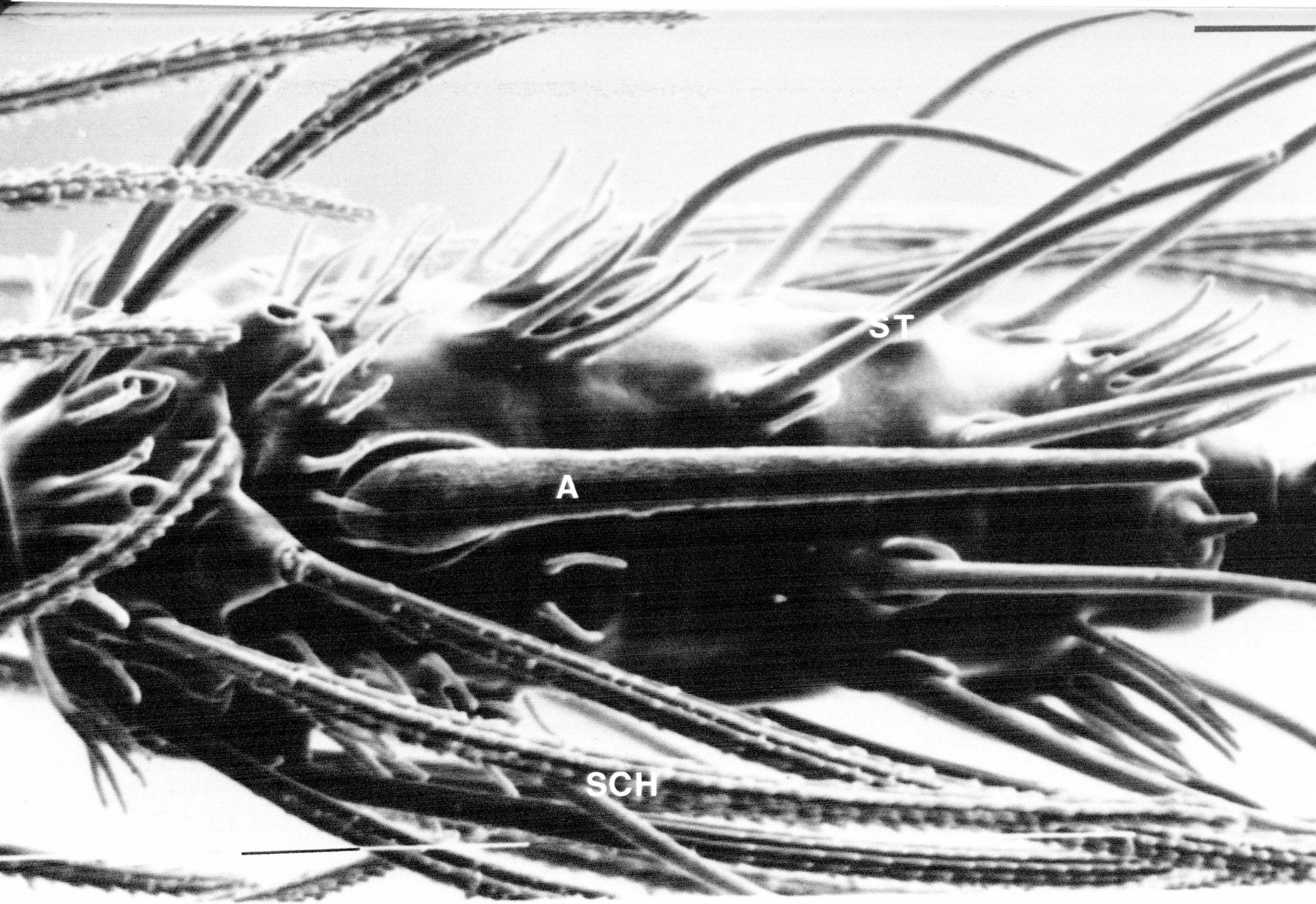
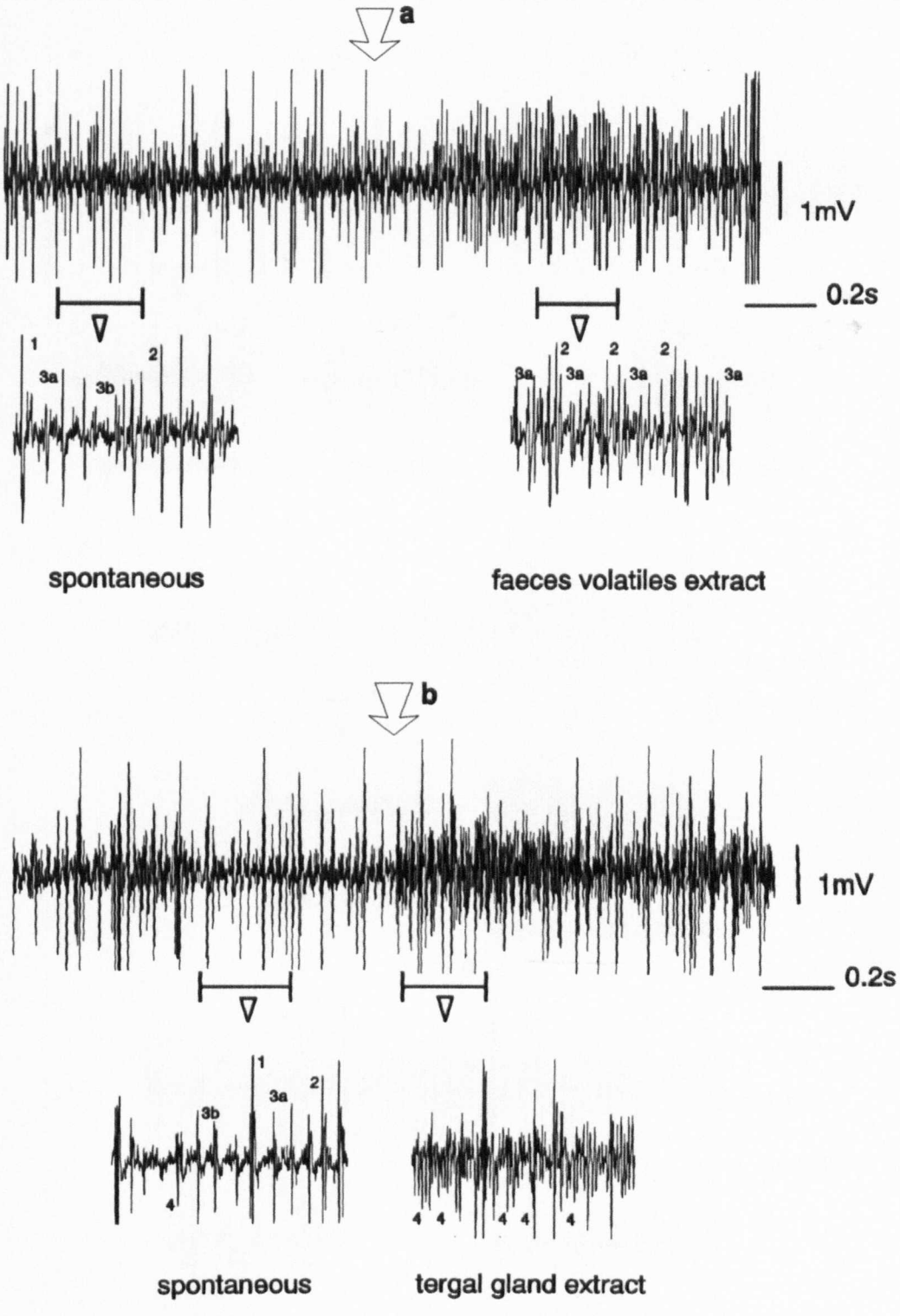
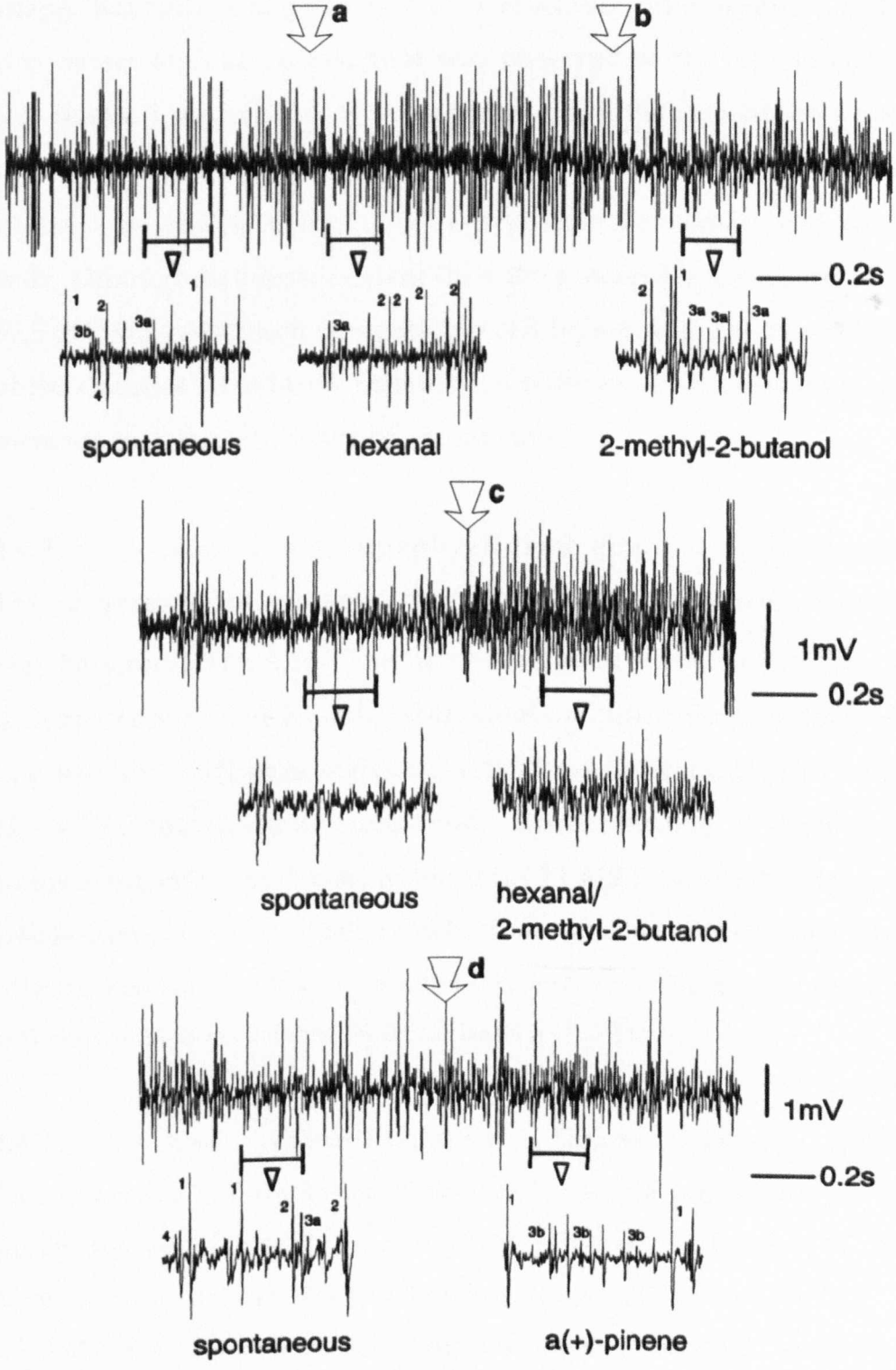

ST
A
SCH

Fig. 3.14 Electrophysiological responses of olfactory cells within an ascoid sensillum, on the antenna of a female *L. longipalpis,* to faeces volatile extract (a) and to a male sandfly tergal gland extract (b).



Spikes with the same number are characteristic of a particular cell type. Hollow arrows on the upper traces in a and b show the point of stimulation which lasted 1 s, lower traces are expanded 200 ms sections of the upper. Horizontal bars represent a period of 0.2 s and vertical bars represent 1 mV.

Fig. 3.15 Electrophysiological responses of olfactory cells within an ascoid sensillum on the antenna of a female *L. longipalpis,* to double consecutive stimulation with hexanal (a), 2-methyl-2-butanol (b), to a 1:1 mixture of hexanal plus 2-methyl-2-butanol (c) and R(+)-α-pinene (d).



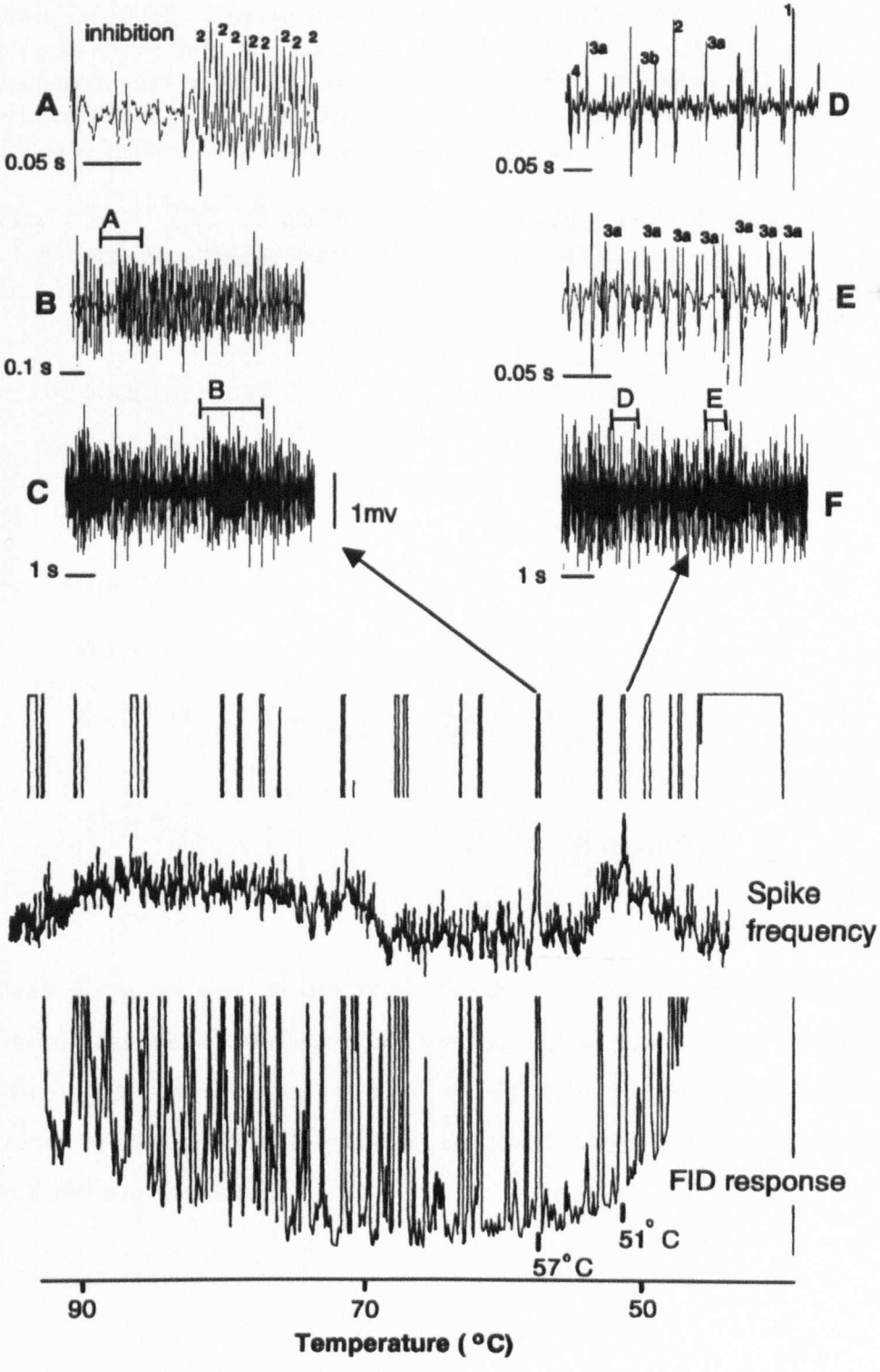
Hollow arrows on the upper traces indicate the point of stimulation which lasted 1 s; lower traces are expanded 200 ms sections of the upper. Horizontal bars represent a period of 0.2 s and vertical bars represent 1 mV.

2-Methyl-2-butanol activated cell type 3a from a base frequency of 29.3±4.9 to 84.4±6.3 Hz, with a 1 mg source. The R-(+)-α-pinene and its isomers stimulated a cell type (3b) characteristically similar to 3a in spike shape, but with a slightly smaller amplitude. The stimulation threshold was rather high as no response was observed to the pinene isomers with less than a 1 mg source. R-(+)-α-pinene gave the greatest response in cell type 3b from 26.6±4.2 to 74.9±6.7 Hz and S-(-)-β-pinene the smallest from 24.0±4.3 to 51.4±4.16 Hz. The α-terpinene also stimulated the type 3b cells, although to a lesser extent than the pinene isomers from 26.3±6.34 to 51.5±3.7 Hz. Although recognisable cell type 4 was not stimulated by any of the chemical standards. Stimulation with air alone caused no significant increase in spike frequency of ascoid cells.

3.4.3 Gas chromatography linked single sensillum recordings. The chromatogram and spike frequency conversion trace for rabbit faeces may be seen in Fig. 3.16. The first active peak eluted at 51°C and matched the retention time of 2-methyl-2-butanol on both column phases employed (see below). This peak activated a cell type 3a from 24.3±2.7 to 51.3±2.54 Hz, as did the synthetic compound. During elution of the second active component, cell type 1 shut down from 11.3±2.7 to 2.1±1.1 Hz, as with the whole extract. This peak matched the elution Rt of hexanal on both column phases employed (see below) and as with the synthetic product activated cell type 2 from 26.0±1.3 to 84.1±8.6 Hz.

3.4.4 Gas chromatography linked mass spectrometry identification of electrophysiologically active compounds. Fig. 3.17 shows the TIC for rabbit faeces volatile extract and Fig. 3.18 shows the mass spectra of the electrophysiologically active compounds. GC-MS analysis of rabbit and chicken faeces extracts confirmed the presence of 2-methyl-2-butanol (M^+ 88) and hexanal (M^+ 100) as major components in the early part of the chromatogram.

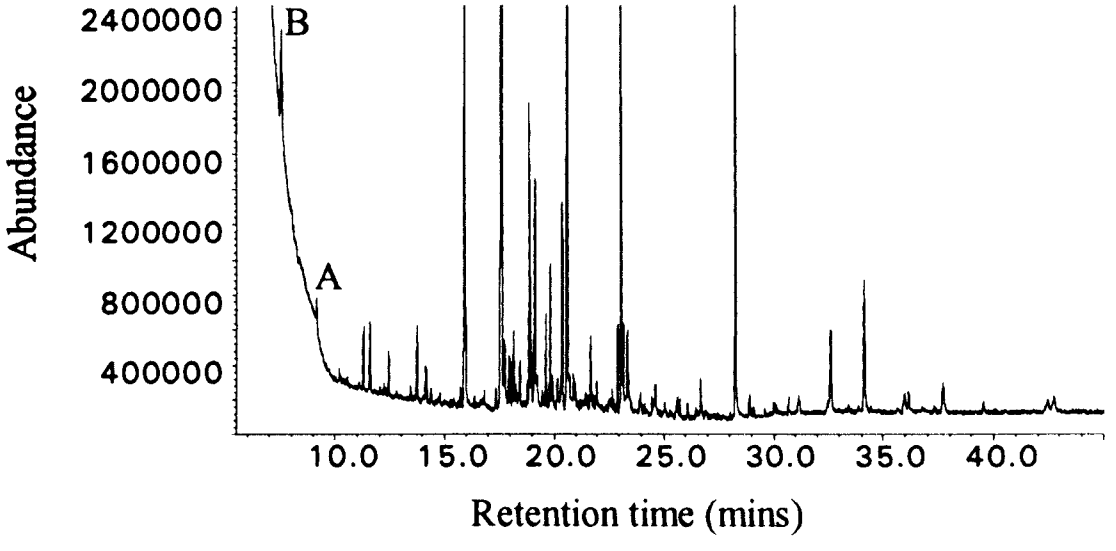
Fig. 3.16 Analysis of rabbit faeces volatiles by gas chromatography coupled electrophysiological recording from an ascoid sensillum of a female *L. longipalpis*.



The bottom trace is the FID response. Spike frequency is the summed frequency of all firing cells (after frequency to voltage conversion of the spike train). The

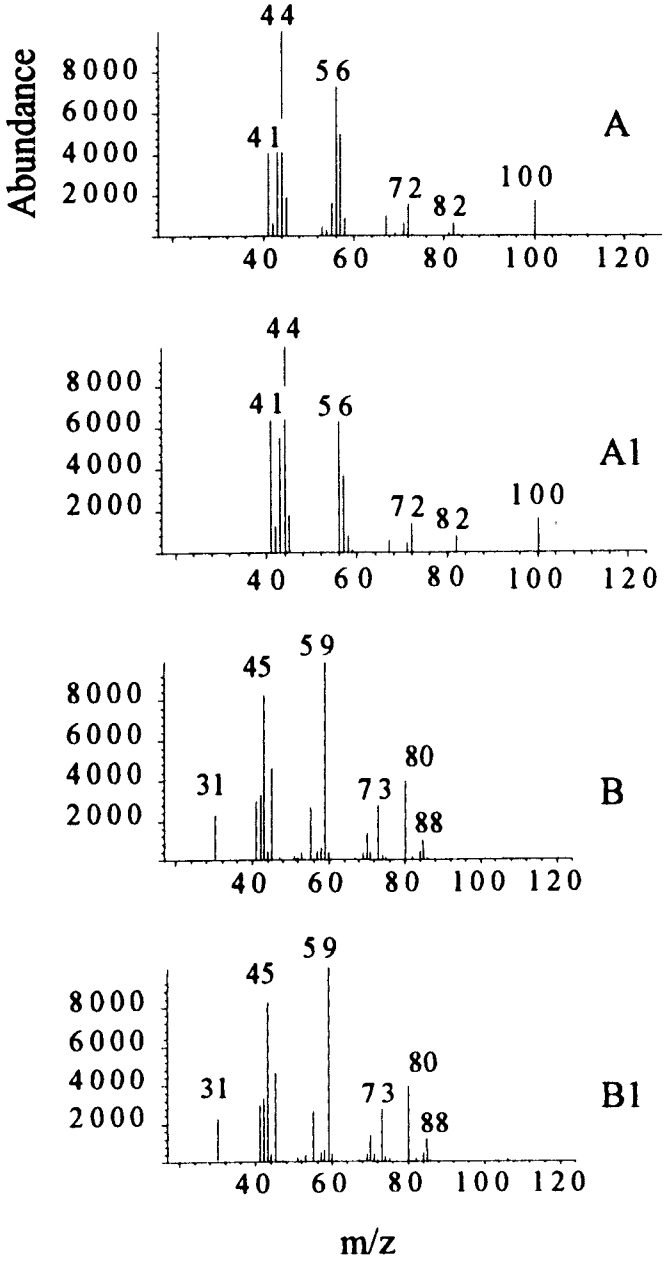
AC signal (upper trace) was stored on a digital audio tape for subsequent analysis of responding cells during elution of active peaks. The first active component of the extract eluted at 51°C (2-methyl-2-butanol) and caused an increase in cell activity (F), above the background level(D), which was clearly audible on the audio channel of the amplifier; cell type 3a was selectively stimulated (E). The second active peak eluted at 57°C (hexanal). A burst of muscle potentials preceded the increase in activity of the ascoid cells, which followed with a second clearly audible volley of spikes (C) that accompanied elution of the peak. Detailed analysis of the second burst indicated that cell type 2 was selectively activated (B) while cell type 1 was inhibited (A).

Fig. 3.17 TIC of rabbit faeces volatile extract from GC-MS. The identified electrophysiologically active peaks are 2-Methyl-2-butanol (B) and hexanal (A).



Peak location was made with reference to a series of alkanes injected under the same conditions as the biological extracts in GC-SSR and GC-MS. The mass spectra and Rt of the electrophysiologically active constituents in faecal extracts matched those of synthetic 2-methyl-2-butanol and hexanal.

Fig. 3.18 Mass spectra of the peaks found at A and B in Fig. 3.18 and known to be electrophysiologically active, compared to the spectra identified by the library search.



A = spectra of peak A, A1 = spectra of synthetic hexanal, B = spectra of peak B, B1 = spectra of synthetic 2-methyl-2-butanol.

Synthetic dimethyl disulphide had a similar retention time to hexanal and was found in chicken faeces but not rabbit faeces volatiles. Whilst, the pinene isomers were not present in the faeces extracts.

3.4.5 Gas chromatography analysis. On the non-polar phase, the first active peak eluted at 4.28 min and 2-methyl-2-butanol at 4.287 min. The second active component eluted at Rt = 10.02 min and hexanal at Rt = 10.02 min. The match between the active peaks and the two synthetics was equally high on the polar phase: active peak one at Rt = 9.632 min and 2-methyl-2-butanol at Rt = 9.631 min, whereas active peak two eluted at Rt = 11.763 min and hexanal at Rt = 11.770 min. The two active peaks constituted major components in the early part of the chromatogram on both phases, with 2-methyl-2-butanol at 60 ng/ml and hexanal at 40 ng/ml of concentrated extract.

3.4.6 Bioassay to test for oviposition attraction. Volatiles from rabbit and chicken faeces, produced an oviposition response from gravid flies and significantly more eggs were laid at the test site than the control (Table 3.4). There was no relative significant difference between the number of eggs laid at these test sites for rabbit and chicken faeces. The control had no effect on oviposition choice. Hexanal evoked an oviposition response that yielded significantly more eggs at the test site than the control, whereas 2-methyl-2-butanol did not. However, hexanal alone did not produce an oviposition response equivalent to either the rabbit or chicken faeces volatile extract. When hexanal was applied together with the 2-methyl-2-butanol, significantly more eggs were laid at the test site than the control. Furthermore, there was no relative significant difference in the number of eggs laid at the test sites in response to the combined synthetics and rabbit or chicken faeces volatiles.

Table 3.4 Oviposition response of *L. longipalpis* to test septa treated with entrained volatiles of rabbit faeces or chicken faeces, hexanal, 2-methyl-2-butanol, dimethyl disulphide and 1:1 mixtures of hexanal plus 2-methyl-2-butanol and hexanal plus dimethyl disulphide.

Material to be bioassayed	Mean number of eggs Laid ± SE		P	Comparison of test sites
	Test	Control		
Rabbit faeces volatiles	224.3 ± 13.8	42.7 ± 11.7	0.006	a
Chicken faeces volatiles	185.5 ± 15.5	37.7 ± 7.9	0.007	a
Solvent control	31.8 ± 14.5	35.2 ± 10.8	0.41*	c
Hexanal	126.4 ± 13.5	39.4 ± 9.6	0.006	b
2-methyl-2-butanol	27.5 ± 3.25	34.4 ± 7.3	0.221*	c
Dimethyl disulphide	34.6 ± 8.62	42.3 ± 12.2	0.262*	c
Hexanal/ 2-methyl-2-butanol	212.4 ± 13.6	38.6 ± 10.6	0.006	a
Hexanal/ Dimethyl disulphide	127.3± 15.2	41.4 ± 9.2	0.011	b

Numbers of eggs laid at the test and control sites were compared using Wilcoxon matched pair signed rank test (* = Non significant). A comparison between treatments (vertical) was carried out using ANOVA; treatments with the same letter are not relatively significantly different. For concentrations see section 2.5.2.

A 400 ng mixture of 2-methyl-2-butanol and hexanal (1:1) produced a positive oviposition response at the test site (197 ± 14.2 eggs at the test and 40.7 ± 8.2 at the control, P=0.001) to the same extent as an aliquot of faeces volatiles containing comparable amounts of these two compounds. Dimethyl disulphide neither attracted gravid females for oviposition on its own nor altered the efficacy of hexanal when used in combination at the test septa. Pinene attracted gravid flies for oviposition only when present on the test septa in high quantities i.e. 1 mg (98.3±9.7 eggs at the test and 33.6±8.81 at the control, P=0.041) and the response was poor compared to hexanal used in a much lower quantity.

3.5 The effect of combined semiochemicals on sandfly oviposition

3.5.1 Bioassay to determine the optimal extraction solvent for the semiochemicals in rabbit faeces. The diethyl ether extract induced the greatest oviposition response from the gravid females (Table 3.5).

Table 3.5 Number of eggs laid by *L. longipalpis* in response to extracts of rabbit faeces made with water, isopropanol, diethyl ether and hexane.

	No. of eggs laid on the $3cm^2$ cotton wool pad (Mean ± SE)		
Extraction solvent	Test	Control	P
Water	68.83 ± 14.47	12.00 ± 6.34	0.005
Isopropanol	80.17 ± 16.46	17.33 ± 5.61	0.005
Diethyl ether	92.20 ± 24.00	16.67 ± 5.58	0.004
Hexane	9.50 ± 6.06	5.17 ± 2.49	0.200*

The number of eggs laid at the test and control sites were compared using Wilcoxon matched pair signed rank test. P = Probability, * = Non significant.

There was no relative significant difference between the three solvent extracts which were found to be active. The only solvent to give a non-significant result, when compared with the control was hexane.

3.5.2 Bioassay to compare rabbit faeces with undigested dietary components. Diethyl ether extracts of rabbit faeces, rabbit food and hay caused gravid females to lay significantly more eggs at the test site than the control (Table 3.6.). The rabbit food extract induced the greatest oviposition response at the test site and hay extract the least, although there was no relative significant difference between the three extracts.

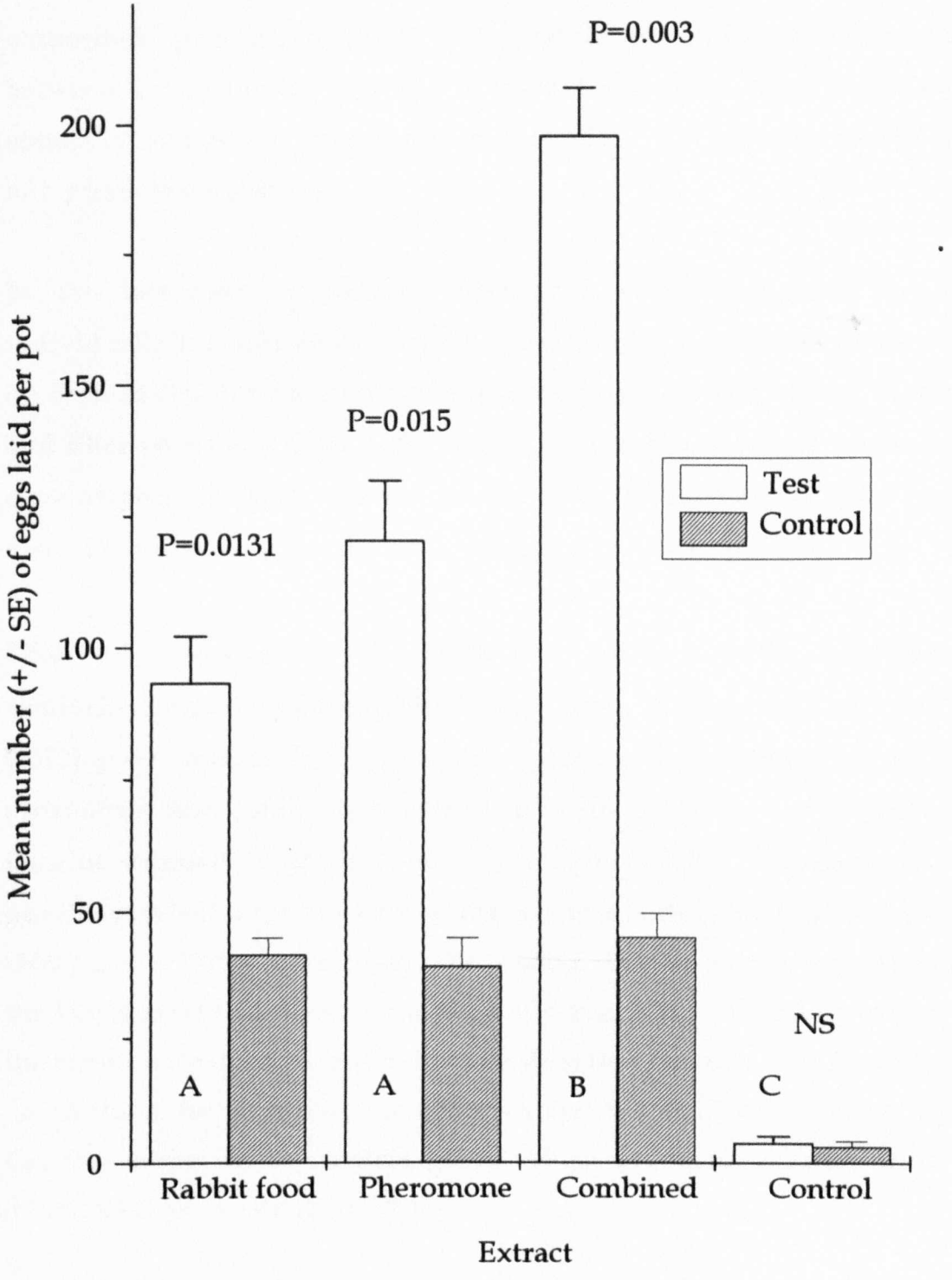
Table 3.6 Number of eggs laid by *L. longipalpis* in response to diethyl ether extracts of rabbit faeces, rabbit food and hay.

Extracted Material	No. of eggs laid on the $3cm^2$ cotton wool pad (Mean ± SE)		P
	Test	Control	
Rabbit faeces	81.80 ± 36.00	12.83 ± 7.47	0.0051
Rabbit food	96.00 ± 34.40	11.17 ± 9.06	0.0049
Hay	77.17 ± 20.06	14.00 ± 4.15	0.0051

Numbers of eggs laid at the test and control sites were compared using Wilcoxon matched pair signed rank test. P = Probability.

3.5.3 Bioassay to determine the effect of combined odour cues, from known oviposition attractants and stimulants, on oviposition. When tested individually, extracts of rabbit food and oviposition pheromone resulted in a significant difference between the mean number of eggs laid in the test and control pots (Fig. 3.19).

Fig. 3.19 Number of eggs laid by *L. longipalpis* in response to extracts of rabbit food, oviposition pheromone, a combination of the above extracts and a control of solvents only.



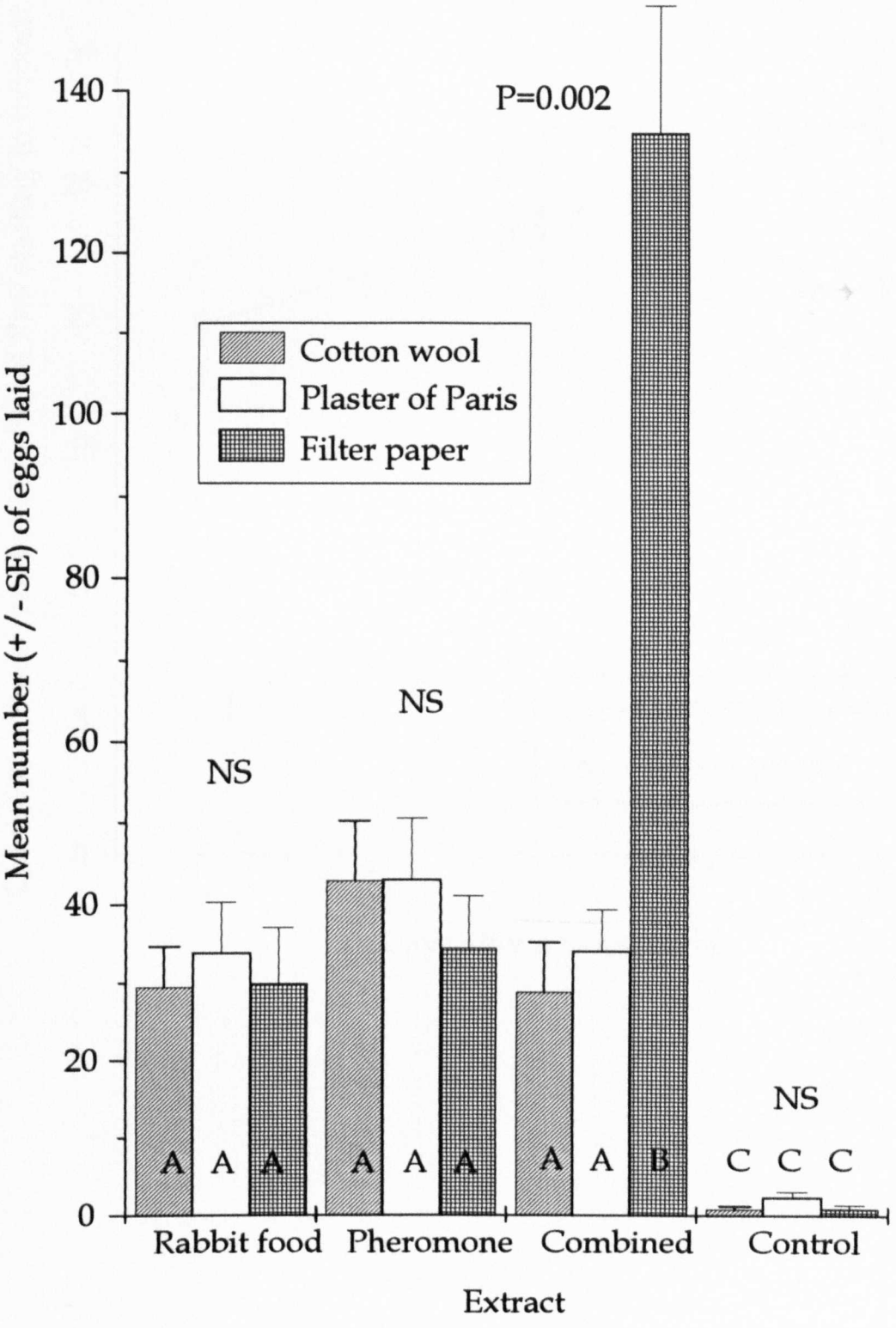
Numbers of eggs laid at the test and control sites were compared using the Wilcoxon matched pair signed rank test. P = Probability, NS = Non significant. Test treatments with the same letter are not relatively significantly different (ANOVA).

However, the mean number of eggs laid in response to combined extracts of rabbit food and oviposition pheromone was significantly greater than the number of eggs laid in response to either the rabbit food extract or oviposition pheromone alone. No significant difference was found between the number of eggs laid in the test pots when either rabbit food extract or oviposition pheromone were applied. The negative control did not promote oviposition.

In the test pots, oviposition pheromone and rabbit food extracts individually induced an oviposition response that was evenly distributed on each of the three oviposition substrates: cotton wool, plaster of Paris and filter paper disc (Fig. 3.20). When the combined extract was used a clear oviposition preference was shown by the females for the filter paper disc, the site on to which the semiochemicals had been absorbed.

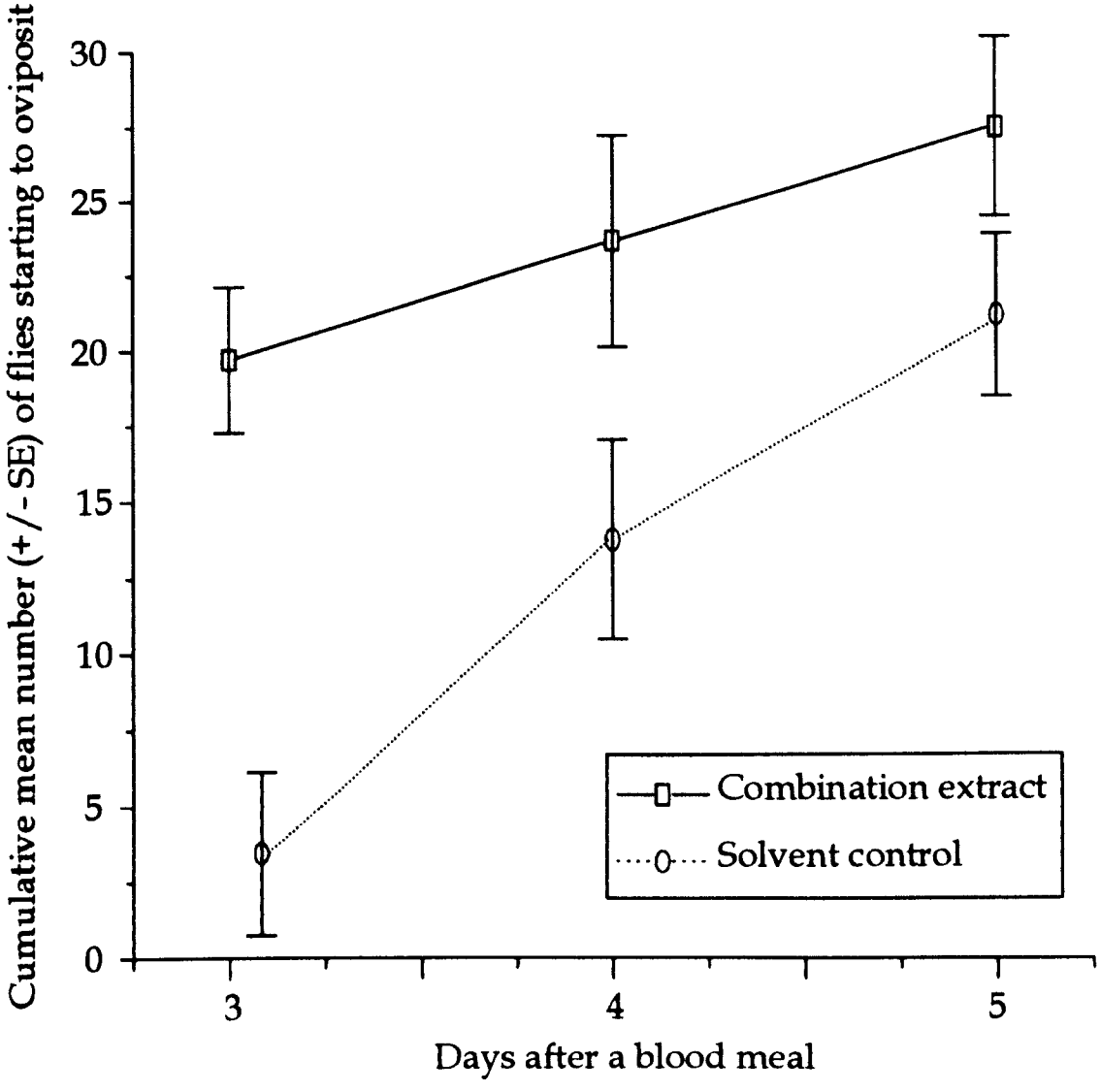
3.5.4 Bioassay to determine the effects of the oviposition semiochemicals on individually tubed flies. Significantly more ($P = 0.012$) gravid females laid eggs when exposed to the combined oviposition pheromone and rabbit food extract, a mean of 27 (90%) compared to females exposed to the solvent controls 20 (66.7%). Furthermore, the general survival rate of test group females was better than control flies (21 (70%) and 6 (20%) respectively, $P = 0.002$). The latent time period from the blood meal to oviposition is shown in Fig. 3.21. The flies exposed to the combined extract oviposited earlier than flies exposed to only solvents. In addition, test group flies produced a mean of 46.77 (SE±9.79) eggs per fly, in contrast to the control group which produced a mean of 18.25 (SE±4.96) eggs per fly ($P=0.0001$).

Fig. 3.20 Effect of extracts of rabbit food, oviposition pheromone, the above extracts combined and a control of solvents only, on the choice of oviposition media by *L. longipalpis* in the test pots of the Barraud cage bioassay.



Columns with the same letter are not relatively significantly different (ANOVA), P = Probability, NS = Non significant.

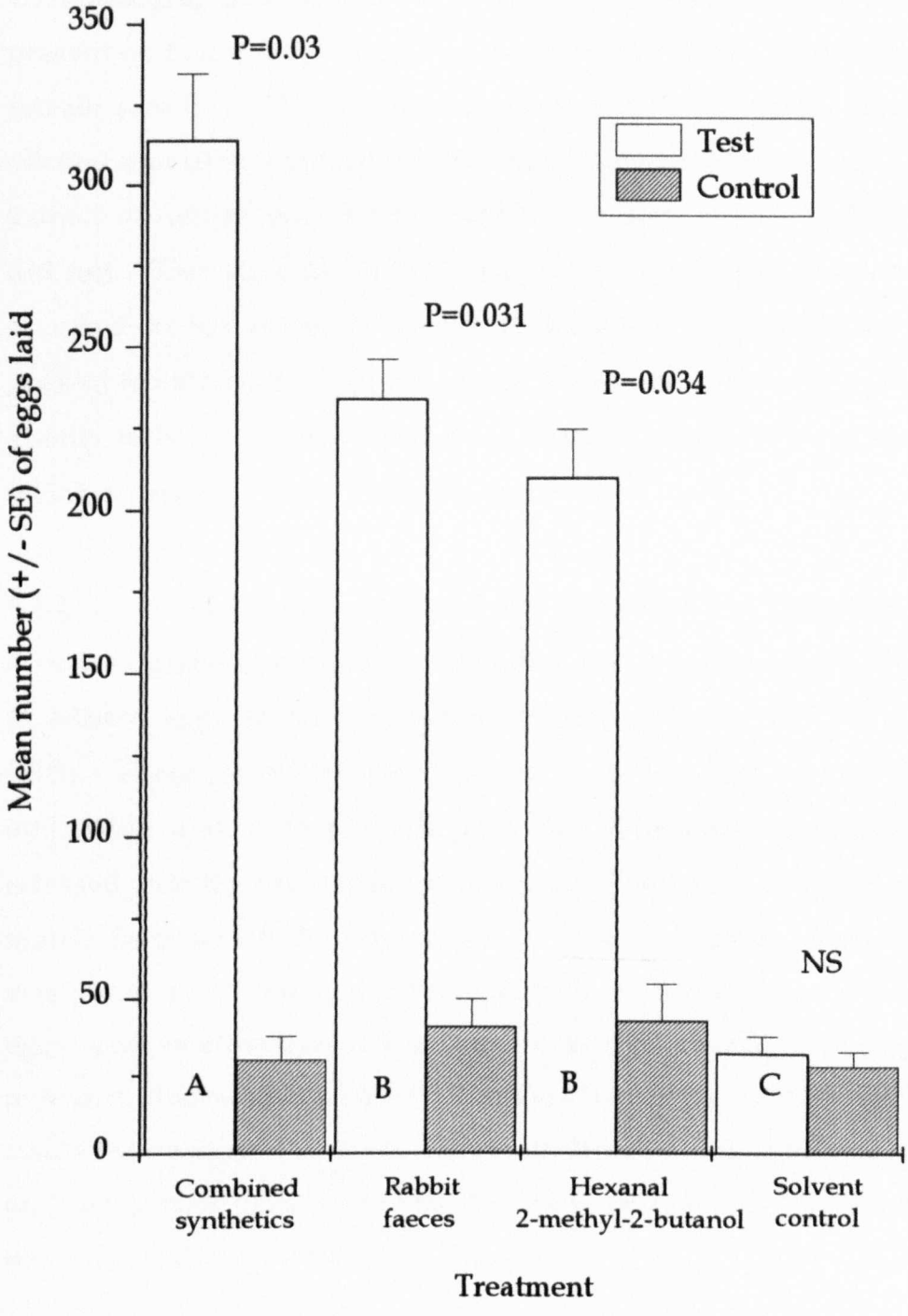
Fig. 3.21 The effect of the combined extracts of oviposition pheromone and rabbit food on the period of time it takes individually tubed *L. longipalpis* to oviposit after a blood meal.



3.5.5 A laboratory oviposition trap. The oviposition trap caught a mean of 18.6 (62%) females in the test pot and 2.4 (8%) in the control pot. A mean of 6 (20%) females were alive at the end of the experiment and 3 (10%) were dead on the bottom of the cage. A mean of 2.4 (8%) males were trapped in the test pot and 1.8 (6%) in the control pot. On average six (20%) males were dead on the bottom of the cage at the end of the experiment and 19.8 (66%) were still alive.

3.5.6 Bioassay to show the effect of combined synthetic oviposition attractants. Rabbit faeces volatiles, hexanal, 2-methyl-2-butanol and the combined synthetic oviposition attractants (hexanal and 2-methyl-2-butanol), produced a positive oviposition response with females laying significantly more eggs at the test site when compared to the control (Fig. 3.22). The negative control of solvents only did not cause females to lay more eggs at the test site compared to the control. When the number eggs laid at the test sites were compared statistically, significantly more eggs were laid at sites containing the combined synthetic oviposition attractants compared to sites loaded with other test treatments.

Fig. 3.22 The number of eggs laid by gravid *L. longipalpis* in response to rabbit faeces volatiles extract, hexanal/2-methyl-2-butanol, dodecanoic acid/hexanal/2-methyl-2-butanol (combined synthetics) and a control of solvents only.



The number of eggs laid at the test and control sites were compared using the Wilcoxon matched pair signed rank test. P = probability, NS = Non significant. Test treatments with the same letter are not relatively significantly different (ANOVA).

DISCUSSION

4.1 Biological origin of the oviposition pheromone

Chromatographic and bioassay evidence suggested that the semiochemical present on *L. longipalpis* eggs was produced by the accessory glands of the female sandfly. The hexane extract of female accessory gland material elicited a positive oviposition response from gravid flies, though a hexane extract of female bodies from which accessory glands had been excised did not. The GC and HPTLC analysis revealed two compounds which occurred in the accessory glands of female *L. longipalpis* and were also present in extracts of eggs and whole female flies. These compounds were greatly reduced in concentration in extracts prepared from females from which accessory glands had been removed.

4.1.1 The accessory gland and pheromones. Secretions from the accessory gland of the sandfly include a sticky substance which were used to adhere eggs to an oviposition substrate (Davis 1967; Wu and Tesh 1989). These secretions were applied on to the eggs as they were being oviposited and it is possible that the oviposition pheromone is also released onto the egg surface at this time. The secretion may form a stable matrix from which the pheromone is slowly released. Evidence for this was given by ElNaiem and Ward (1991), who found that 5 or 6 day-old-eggs were as attractive as 1 or 2 day-old-eggs, although it is important to note that, dodecanoic acid with a molecular weight of 200 a.m.u. would be stable over a period of time. However, the complex chorionic sculpturing on *L. longipalpis* eggs (Leite and Williams, 1991; Ward, 1974), may increase the evaporative process.

The presence of oviposition deterring pheromones, in sticky secretions used to adhere eggs to an oviposition substrate, have been shown for the sorghum shoot fly *Atherigona soccata* (Raina 1981). A water soluble glue

produced in the accessory glands, was used to stick eggs to the leaves of Sorghum. The glue forms a stable matrix from which the pheromones were released. Unlike the sandfly oviposition pheromone, the oviposition pheromone of *Cx. quinquefasciatus* is produced in the eggs before oviposition. This was determined by dissection of various parts of the mosquito body and quantifying the amount of pheromone present in each sample (Laurence and Pickett, 1985). The pheromone was released slowly from the apical droplet of the egg raft into the air above the water.

The accessory glands of many female insects have several secretory areas, the functions of which are unknown, the role of the glands was reviewed by Gillot (1989). One function that has been ascribed to the accessory gland secretions of *Musca domestica* is the production of enzymes that break down the acrosomal membrane of sperm (Leopold, *et al.*, 1978).

4.1.2 Pheromone glands. Noirot and Quennedy (1974) classified exocrine glands as follows; class I and II are secretory but have no duct to connect them to the outside. Class III glands posses a duct to the exterior and generally are grouped together to form a glandular unit. The glandular tissue which produced and secreted the oviposition pheromone of *L. longipalpis*, whilst being present in the accessory glands, has not been investigated. Further work including sectioning accessory glands for light and transmission electron microscopy is required. The gland which releases the sex pheromone of *L. longipalpis* was described by Lane and Bernades (1990) and Boufana (1990). The organ consists of several identical, vacuolated secretory units which open independently into a reservoir with a duct that leads into the surface papule.

4.2 Isolation of the oviposition pheromone

The results of the bioassays showed that fraction LC2 (GC2) from the extract of *L. longipalpis* eggs was responsible for the observed pheromonal activity. LC2 caused gravid females to both oviposit earlier and lay significantly more eggs than control flies. The nature of the stimulation observed was different from that described by Dethier *et al.* (1960), the presence of the active compounds did not "elicit" an oviposition response. However, the pheromone did "facilitate" oviposition activity, with gravid flies ovipositing earlier and laying more eggs than control flies. None of the other HPLC fractions of sandfly eggs either attracted gravid females for oviposition nor altered the activity of the pheromone peak.

The use of HPLC enabled larger quantities of egg extract to be fractionated than would preparative GC. The fractionation allowed the HPLC peaks to be located on the gas chromatogram, which was necessary before a GC- MS analysis and molecular characterisation of the active components could be undertaken. The liquid/liquid back extraction was a highly efficient way of recovering the HPLC fractions back into hexane for bioassay and GC analysis. This ensured that a minimum amount of solvent was applied to the test and control discs.

4.3 Structural characterisation of the oviposition pheromone

The peak identified as the biologically active pheromone component of hexane extract of *L. longipalpis* eggs was found to be dodecanoic acid. The peak was isolated from eggs of flies from Jacobina (Brazil) and Curarigua (Venezuela). When synthetic dodecanoic acid was analysed, using the GC-MS in EI mode, the Rt and spectra matched the unknown pheromone peak of Jacobina and Curarigua eggs. The molecular weight of the pheromone was confirmed at 200 a.m.u. using GC-MS in CI mode and

matched dodecanoic acid, with each egg having an average of approximately 3 ng. This means a hexane extract of 1200 sandfly eggs could potentially contain a maximum of approximately 4.4 μg of dodecanoic acid. The structure of dodecanoic acid may be seen in Fig. 4.1. The methyl esterification confirmed the presence of dodecanoic acid in hexane extract of Jacobina eggs.

The bioassay of dodecanoic acid produced an oviposition response at the test site that was significantly different to the control, as did the whole egg extract. When analysed by ANOVA there was no significant difference between the number of eggs laid at the test site of the bioassay when dodecanoic acid or egg extract was present. The control of solvents only, did not produce a positive oviposition response at the test site when compared to the controls.

4.3.1 Dodecanoic acid. Dodecanoic acid is a common compound, found in a variety of sources and is an industrial oil obtained from *Cuphea* species, where the fatty acids are recovered for further processing (Thompson *et al.*, 1990). This is the first observation suggesting that dodecanoic acid has semiochemical properties. The compound is readily metabolised by plant microsomes and bio-transformations by fungi, causing the rancid flavour of over-ripe coconuts (Kinderlerer, 1992).

Fatty acids are obtained in the blood meal by haematophagous insects and phytophagous insects synthesise them in the fat body. Initial bio-transformations involve the production of even numbered, saturated fatty acids by the condensation of 2-carbon units. The most common fatty acid used as a food reserve is palmitic acid or hexadecanoic acid. In times of non-feeding this compound is mobilised by a hydrolysis with a lipase enzyme. The compound then forms lipoprotein complexes which enter the haemolymph, upon entering muscle they are hydrolysed and the fatty

acid progressively oxidised to produce acetyl-coenzyme A (Chapman, 1985). The role of dodecanoic acid on the sandfly egg may be more complex than just a semiochemical one. The compound could form part of the water proof coating which covers the chorion of the egg (Chapman, 1985). Also, 1st instars often ingest their egg case and the fatty acid may give nourishment to the newly emerged larvae.

4.3.2 Oviposition pheromone synthesis. It is interesting to note that the oviposition pheromone of *Cx. quinquefasciatus* (Laurence and Pickett, 1982) (erythro-6-acetoxy-5-hexadecanolide) and *Cx. tarsalis* (Staratt and Osgood, 1972) (1,3-diglycerides of acetylated hydroxy fatty acids) may have a synthesis starting point from hexadecanoic acid. The hydroxylation of this compound is normal for mobilisation of the food source, the product is then a methanolysis step away from the active lactone oviposition attractant (Bentley and Day, 1989). Hexanoic acid is an oviposition attractant for *Ae. aegypti*, but the tertiary methylated compound also increased egg counts in a field trial in Kenya (Knight and Corbet, 1991). The oviposition pheromone of *L. longipalpis*, dodecanoic acid, may be used for food storage, this is supported by the fact that the compound was isolated in the greatest quantity from the accessory glands, an organ of storage and metabolic activity (Gillot, 1989). Many pheromones of the Lepidoptera are even numbered carbon chains with a terminal oxygen, i.e. unsaturated acetates, aldehydes, alcohols or ketones. The two commonest carbon lengths are C_{12} and C_{14} with both mono- and dienes. This is highlighted by the fact that desaturases for dodecanoic and tetradecanoic acids have been isolated from many insects (Kelly, 1993).

The presence of a single pheromone component in the hexane extract of sandfly eggs, is not atypical. The oviposition pheromone of *Cx. quinquefasciatus* is a single component (Laurence and Pickett, 1982). The sex pheromone of *L. longipalpis* from Jacobina, Brazil was also found to be

a single component in the hexane fraction of male tergal pheromone gland extract (Hamilton *et al.*, 1994).

4.3.3 Bacterial odour effects on sandfly oviposition. It is recognised that some oviposition pheromone-like activity has been associated with the presence of volatiles from bacterial activity, as with *Lucilia cuprina* Wiedmann (Emmens and Murray 1983). Accessory gland extract of female *L. longipalpis* elicited an oviposition response and samples of female bodies without these glands did not. This suggested that the phenomenon of attraction to bacterial odour was not occurring, although other, as yet unidentified, volatile compounds from microbial activity may used by sandflies in locating an optimal oviposition site. Radjame (per. com. 1994) found that *P. papatasi* females were attracted for oviposition to several bacterial cultures including *Bacillus licheniformis, B. sphaericus* and *B. firmus*. When bodies of *P. papatasi* adults were allowed to decompose, they became attractant to gravid females. This phenomenon could be explained by the oviposition pheromone being released from the bodies, or more probably by the products of bacterial and fungal decomposition that act as oviposition attractants (Killick Kendrick, per. comm. 1994).

It is important to note that dodecanoic acid is hydroxylated by microsomal activity (Manson *et al.*, 1994) and undergoes catalytic oxygenation by plant microsomes (Salun *et al.*, 1992). The aldehyde, ketone and lactone products of these and other bio-transformations should be tested in the sandfly oviposition bioassay, to reveal if natural products of dodecanoic acid are oviposition attractants.

Fig. 4.1 The chemical structure of dodecanoic acid.

Common name of dodecanoic acid is Lauric acid. The molecular weight is 200 a.m.u. and the compound is extracted from coconut oil and the seeds of the plant *Holoptela intergrifolia*. It is a white crystalline powder with a slight odour of Bay oil.

4.4 Isolation of faecal oviposition attractants

This is the first electrophysiological investigation on olfactory receptors of phlebotomine sandflies and confirms the ascoid sensillum on the antennae of *L. longipalpis* as a significant olfactory organ. Receptor cells in this sensillum responded to behaviourally active odour extracts of faeces from rabbits and chickens, and to a male tergal gland extract. Two constituents of these faecal extracts, 2-methyl-2-butanol and hexanal were identified as olfactory stimulants by GC-SSR. In oviposition bioassays these two synthetic volatiles combined were as active as an aliquot of faeces odour containing equivalent amounts of these products. Although R-(+)-α-pinene attracted gravid flies in the oviposition bioassay it was not detected in faecal extracts. Furthermore, when compared to either hexanal or a 1:1 mixture of hexanal/2-methyl-2-butanol, the oviposition response from the gravid flies was not as strong when pinene was applied to the test septa, even though present at a quantity of 1 mg. However, R-(+)-α-pinene has been shown to be an attractant for a number of insects, particularly the boll weevils and bark beetles (Vanderwel and Oehlsclager, 1987).

4.4.1 Electrophysiological responses to faecal volatiles and synthetic compounds. Despite the complexity of the multicellular response recorded from the sensillum, double successive stimulation clearly showed that separate cells were being activated by hexanal and 2-methyl-2-butanol. A mixture of these two products evoked an overall electrophysiological response similar to the extract of faecal volatiles.

The neuronal response from cells in the sandfly ascoid was similar in complexity to that obtained from olfactory cells of the tsetse fly *Glossina morsitans morsitans* (Den Otter and Van Der Goes Van Natters, 1993). The tsetse fly has four neurones in the funiculi sensillum which both respond to and are inhibited by a range of compounds including phenols, 3-alklyphenols and 4-methylphenols. It is the combined inhibition and stimulation of these neurones that causes the tsetse fly to orientate towards a host for blood feeding.

A number of other volatiles were found to activate cells within the ascoid sensillum i.e. R-(+)-α-pinene and some of its isomers, α-terpinene and benzaldehyde. None of these compounds were detected by GC-MS in the faeces volatile extracts. However, it is possible that the simple terpenes play a role in the orientation of sandflies to plants where these insects are known to take sugar meals in the wild, and even exhibit plant preferences (Cameron and Davies, 1993). Mosquitoes also use plant nectar as an energy source, and terpene-sensitive olfactory receptors have been identified on the antennae of *Cx. pipens* (Bowen, 1992).

Benzaldehyde activated the same olfactory cell as hexanal and likewise inhibited cell type 1. Since hexanal and benzaldehyde both posses a carbonyl group it is not surprising that they both activate the same cell. However, other aliphatic aldehydes failed to activate olfactory receptors in the electrophysiology experiments. This would tend to suggest that

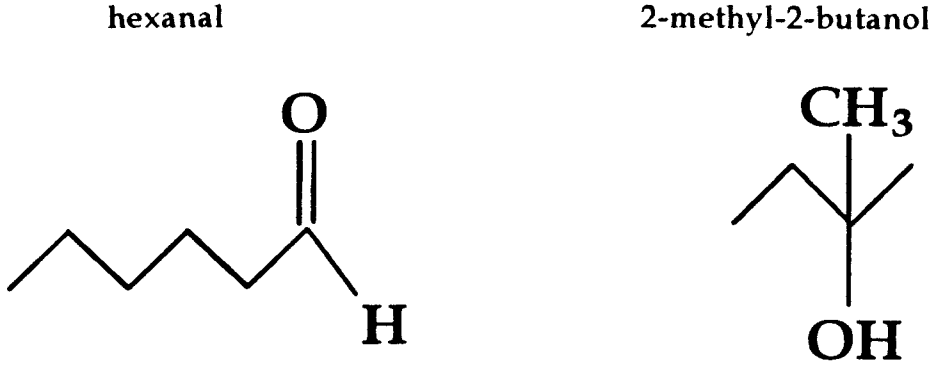
reception of benzaldehyde is of some significance for the sandfly. Benzaldehyde has been identified as a host-odour olfactory stimulant for *Amblyomma variegatum* (Steullet and Guerin, 1994), and this product is a well known aromatic constituent of flower blossoms (Knudsen *et al.*, 1993). Further work is needed to establish the role in affecting sandfly behaviour, if any, of benzaldehyde on its own and in combination with the other identified attractants.

4.4.2 Electrophysiology of known sandfly attractants. Although α-terpineol did not stimulate cells of the ascoid sensillum, it has been shown to be an attractant for *P. papatasi* (Wilson *et al.*, 1989a). It is known that other "olfactory like" sensilla are present on the antenna of the sandfly which could serve to perceive other semiochemicals, or alternatively, this compound may be important to *P. papatasi* during its life-cycle but not to *L. longipalpis*. Other compounds identified as attractants for *Phlebotomus* species are dibutyl succinate, dimethyl disulphide and mixtures of these two compounds (Wilson *et al.*, 1989b and Wilson *et al.*, 1991). Dimethyl disulphide was detected in the extract of chicken faeces volatiles, but when bioassayed it did not attract gravid *L. longipalpis* for oviposition, nor did it enhance the activity of hexanal.

Cell type 4 was stimulated by the tergal gland extract of *L. longipalpis* males. This species is a homosesquiterpene type phlebotomine from Jacobina, Brazil, producing a one component sex pheromone (Hamilton *et al.*, 1994). This is supported by the electrophysiology responses because only one olfactory cell was stimulated by tergal gland extract. The identification of the neurone that responds to the sex pheromone should enable an electrophysiological investigation of the *L. longipalpis* species complex.

4.4.3 Hexanal and 2-methyl-2-butanol. Hexanal and the methylated butan-2-ol are found as constituents of flower blossom odour, green leaf volatiles (Knudsen, 1993) and are common compounds in the environment (Fig. 4.2). 2-Methyl-2-butanol (amyl alcohol), has not previously been described as playing a semiochemical role in a chemo-ecological interaction, although butyl alcohol is attractant to the screw worm, *Chrysomya bezziana* (Mookherjee *et al.*, 1992). It is interesting to note that some *Bacillus* species when growing on meat products produce isoamyl alcohol or 3-methyl-1-butanol. This volatile compound is an anti-cyanobacteria agent and a phytotoxic (Wright, 1991). Because of the close association of the sandfly breeding sites to animal pens and faecal material, a bacterial source for this compound cannot be ruled out and further work is needed to clarify this point. Hexanal was identified as an attractant produced by males of the flesh fly *Sarcophaga bullata* to attract females of the species in a laboratory bioassay over a distance of 45 m and is attractant to soldier bugs, *Rhagonchya fulva*, (Mookherjee *et al.*, 1992).

Fig. 4.2 The structures of the two compounds found to be active in both the electrophysiology and the oviposition bioassays.



Hexanal has a trivial name of caproaldehyde, 2-methyl-2-butanol has a trivial name of amyl alcohol.

4.5 The effect of combined semiochemicals on *Lutzomyia longipalpis* oviposition

Oviposition attraction was observed to a range of organic extracts, this mirrored the wide distribution of the organic compounds that sandflies can perceive when choosing an ideal oviposition site. The Barraud cage bioassay showed for the first time that gravid *L. longipalpis* can be induced to oviposit in an adult rearing cage. Any one of the extracts had to be present for oviposition to occur in the Barraud cage bioassay. There was a synergistic effect between the oviposition pheromone and the rabbit food extract on the behaviour of the gravid flies, with enhanced egg laying and post oviposition survival. Also, oviposition was targeted towards the test filter paper disc when the combined extract was applied.

The perception of several semiochemicals that have a greater effect on insect behaviour than the individual components, is well studied. *Tomicus minor* (pine shoot beetle) used a pheromone and host odours to target more accurately host and mate location (Lanne *et al.*, 1987). The oviposition pheromone of *Cx. quinquefasciatus* has an additive effect on stagnant water when combined as an oviposition attractant (Mordue *et al.*, 1992 and 1993), becoming both electrophysiologically (by electroantenogram) and biologically more active than the individual components.

4.6 Synthetic oviposition semiochemicals

The transmission of Leishmania may be dependant on the sandfly passing through at least one complete gonotrophic cycle and ElNaiem *et al.* (1994) found a higher probability of parasite transmission at the third blood meal rather than the second. As the synergistic effect of the semiochemicals enables more flies to survive oviposition, it may be possible to study the transmission of leishmaniasis with a greater number of flies that pass

through the first gonotrophic cycle. It should be possible to introduce these compounds into everyday colony maintenance of *L. longipalpis*, increasing the efficiency of oviposition and so the fecundity of the colonies, making the difficult task of rearing these insects easier. The presence of dodecanoic acid, 2-methyl-2-butanol and hexanal mimicked the presence of whole extract of eggs and rabbit faeces, there was no significant difference between these two test preparations. This does not mean that all the semiochemicals mediating the behaviour of *L. longipalpis* have been isolated. Other compounds may be inducing behaviour that was not observed due to the nature of the bioassays used. Sandflies have other sensilla on their antennae, mouth parts and ovipositor (Boufana, 1990). It is possible that gravid flies also perceive semiochemicals with these sensilla, which would not have been observed with the GC-SSR of the ascoid.

4.7 Oviposition attraction or stimulation

4.7.1 Extracts of organic origin. ElNaiem and Ward (1992b) used individual extracts of faecal material and found that flies were attracted to the semiochemical source in an olfactometer bioassay. In this present investigation flies were attracted to organic extracts in a volume 0.216 m^3 with no air movement. The flies were also attracted to organic extracts and synthetic compounds in the PMP bioassay, giving targeted oviposition on the test sites. The behavioural action of the flies would fulfil the criteria laid down by Dethier (1960) to describe the faecal material as an oviposition attractant. The compounds could also be described as apneumones, as the semiochemical source is non-living and organic, whilst accepting that there was a possibility of bacterial involvement. As rabbit faeces extract caused ovipositing flies to lay their eggs earlier, ElNaiem and Ward (1992b) described the extract as an oviposition stimulant. However, in this investigation there was a synergistic oviposition stimulation and female flies laid more eggs, laid

earlier and survived in oviposition in greater numbers than control flies. This seems to indicate that the stimuli used in the bioassay more closely mimicked the optimal semiochemical, environmental and thigmotropic cues that control oviposition in the wild.

4.7.2 The oviposition pheromone. When exposed to the oviposition pheromone flies laid eggs earlier and in greater numbers but did not have a greater survival rate than control files. The semiochemical effect could be described as stimulatory. The oviposition pheromone also attracted gravid flies over short distances, as did the synthetic oviposition pheromone. It is important to note that as no wind tunnel experiments have been conducted, the oviposition attraction observed in the bioassays may in fact function as an arrestant. The PMP pot bioassay was in a reasonably small arena (11 cm diam, 9 cm height). The only evidence to suggest that true attraction is being observed is the large Barraud cage bioassay, the arena was much larger 0.216 m^3 and flies had to enter into a small PMP pot to oviposit. In conclusion it would appear that the combined oviposition pheromone and faecal extract are both attractant and stimulant for gravid *L . longipalpis.*

4.8 Aggregated sandfly oviposition

4.8.1 Sandfly aggregation in the wild. Attempts to locate sandfly larval breeding sites in soil samples have only shown sparsely scattered immature stages, (Hanson 1961; Thatcher 1968; Rutledge and Mosser 1972). However, Bettini *et al.*, (1986), using emergence traps, were successful in collecting 27,405 emergent flies from a 25 m^2 breeding site in one year and the sampling area was only one twenty seventh of the total area. Further investigation is therefore needed to clarify the apparent disparity between the results from these two sampling techniques and to investigate the oviposition pheromone and apneumones in the field.

4.8.2 Why do sandflies aggregate their young? Gravid *L. longipalpis* were attracted to extracts of organic substances. Oviposition in organic material would provide a readily available source of food for sandfly larvae, which would be beneficial for the survival of their progeny (Manning, 1979). If a particular area has already been chosen as a suitable oviposition site by a number of females, a gravid sandfly could save energy by accepting it as an area for egg laying, as long as the other oviposition criteria are fulfilled. The phenomenon of aggregated oviposition would cause larvae to be present in large numbers in a small area. It is known that the sheep blowfly *Lucilia cuprina* are better at utilising host tissue when present in greater numbers (Barton Browne *et al.*, 1969) and females of this species aggregate for oviposition. Sandfly larvae live in soil types such as friable loams and may be able to maintain their environment when present in greater numbers. In the laboratory sandfly instars are more able to control fungal growths, by eating the developing hyphae, when they are present in greater numbers. If the fungi are not controlled, the caudal setae become entangled in the hyphal mat and they die. Sandfly pupae have recently been shown to posses a defensive compound that is used in combination with a flicking movement, when they are challenged (Dougherty and Hamilton 1994). It has been postulated that if large number of instars pupated together, the collective nature of the defensive response would give a greater benefit than if the pupae were dispersed. Furthermore, for organisms that live together in large numbers the chances of individual predation are lessened (Manning, 1979).

4.8.3 Cost - benefits and control of sandfly oviposition attraction. The cost - benefits of the semiochemical interaction are difficult to elucidate as little is known about sandfly oviposition and in particular about the predators of the juvenile stages. Using the criteria of Dicke and Sabelis (1988), it would appear that the oviposition pheromone

is a +,+ pheromone, with benefits for both the female that produces the pheromone emanating eggs and gravid flies wanting to oviposit. There must be a point when the number of eggs at a site is a maximum for the efficient rearing of the larvae and it is possible that at high concentrations the pheromone causes females to continue to search for an oviposition site.

The *Ae. aegypti* oviposition attractant, hexanoic acid, was repellent to gravid females at concentrations only slightly higher than those used for attractancy (Knight and Corbet, 1991). Other factors may be detrimental to larval aggregations, one is the increased risk of disease and parasites spreading through the population (Manning 1979). *L. longipalpis* is parasitised by an aspetate gregarine, *Ascogregarina chagasi*, this parasite is known to decrease female fecundity and longevity in laboratory flies, but its effect in the wild are largely unknown (Wu and Tesh 1989)

4.8.4 Sandfly oviposition and genetic variation. Chemical variation of the oviposition pheromone among sibling species of *L. longipalpis* has not been studied in this investigation. However, the pheromone isolated from flies collected at Curarigua (Venezuela) and Jacobina (Brazil) was the same, dodecanoic acid. It is to be noted that these flies belong to the same sibling group when they were analysed by their sex pheromones, (Hamilton, pers. com.). ElNaiem (1991) used eggs from Jacobina and L'Aguila (Colombia) and found cross sensitivity in bioassay between these two populations. Once again these flies were from the same pheromone group and it would be interesting to investigate the cross sensitivity of flies that are from the homosesquiterpene and diterpene populations, the two pheromone groups that are known not to cross inseminate.

Another area that deserves further investigation is the specificity of the organic attractants. *L. longipalpis* is attracted to two compounds 2-methyl-2-butanol and hexanal, these compounds may be specific to the

environment that this sandflies lives in. Other sandflies may perceive different chemicals that are better markers for ideal larval breeding sites in their environment.

4.9 Oviposition trap development

4.9.1 Oviposition traps. Loor and DeFoliart (1969) used oviposition traps to monitor the egg laying of *Ae. triseriatus*. The ovitrap was baited with organic debris consisting of 75% dry and 25% green oak leaves. The egg monitoring trap of *Cx. quinquefasciatus* (Yasuno *et al.*, 1973) was enhanced by using 2 litres of 1% yeast infusion in clay pots. The synthetic oviposition pheromone for *Cx. quinquefasciatus* was successfully used in a trapping experiment in Kenya (Otieno *et al.*, 1988). Kairomones from the host plant of *Athergonia soccata*, the sorghum shoot fly, have been used to re-direct shootfly oviposition to a non-host plant, on which the larvae are not viable. This resulted in the management of this pest insect (Unnithan *et al.*, 1987).

4.9.2 Sandfly oviposition trap. The adapted Barraud cage bioassay is the first demonstration that semiochemicals can be used to trap gravid *L. longipalpis* in the laboratory. In the long term it may be possible to use oviposition traps to monitor populations of sandflies or collect eggs in the wild. A greater knowledge of sandfly breeding sites including the physical, environmental and visual cues would have to be gathered before an oviposition trap could be deployed.

An important issue that needs to be addressed in the area of chemical ecology is the long range attraction of gravid *L. longipalpis* to the oviposition site. A wind tunnel experiment may reveal the contribution of the semiochemicals, identified thus far, to the general attraction of gravid sandflies.

4.10 Concluding remarks: a model for the mechanism of sandfly oviposition

This investigation suggests that sandfly oviposition site selection is mediated through a step-wise progression of stimuli. Using the criteria of Kennedy (1977; 1983) the sandflies would perceive the chemical constituents of the oviposition substrate from a distance, as yet unknown, with an initial anemotaxis followed by a positive taxis towards the semiochemical source. Finally at closer range the oviposition pheromone synergises the attractant and stimulant cues and a negative kinesis occurs with the flies settling on the oviposition site, when the oviposition criteria have been fulfilled oviposition occurs. It is important to note that the physical nature of the substrate, environmental micro-habitat and possibly visual cues are important factors and the sandfly may probe with its ovipositor until these criteria are fulfilled.

It is possible that the pheromone acts over a limited range, with the female being directed to oviposition sites by long range volatile chemicals from the organic substrate and then physical characteristics of the oviposition substrate. In this situation the pheromone would be the final phase in the oviposition site selection mechanism. Schlein *et al.*, (1990) found that *P. papatasi* chose optimal oviposition sites by physical and chemical stimuli in the oviposition substrate. Furthermore it is known that *Culex* females select oviposition sites by environmental, physical, chemical and pheromonal cues (Maire 1989; Bentley and Day 1989). It would appear that *L. longipalpis* females are capable of recognising a set of common compounds which, in its ecological niche, designate an ideal site for larval rearing. Through evolution the sandfly has evolved specific receptors that perceive these compounds and enable it to orientate towards their source, where it is then stimulated to oviposit.

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APPENDICES

List of Papers published from this thesis

Dougherty, M. J., Ward, R. D. and Hamilton, J. G. C. (1992) Evidence for the accessory glands as the site of production of the oviposition pheromone of *Lutzomyia longipalpis*. *Journal of Chemical Ecology*, **18:** 1165-1175.

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