HOST-SEARCHING BY GONIOZUS NATALENSIS FEMALES ELICITED BY A SHORT-RANGE KAIROMONE IN THE FRASS OF ITS NATURAL HOST ELDANA SACCHARINA.

By

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PREFACE

The experimental work described in this thesis was carried out at the SASA Experiment Station, Mount Edgecombe, Private Bag XO2, Natal, from January 1989 to December 1990, under the supervision of Professor Norman Pammenter in the Biology Department University of Natal, Durban and Dr. James C. S. Allison at the Experiment Station.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

ACKNOWLEDGEMENTS

ABSTRACT

Petri dish and olfactometer tests demonstrated that *Goniozus natalensis* (Gordh) females exhibit a host-searching response upon contact and at a short distance by olfaction, to a kairomone in the frass of its natural host *Eldana saccharina* (Walker). The host-searching response was found to be elicited by *E. saccharina* frass from a range of substrates, namely: two host plants of *E. saccharina*, papyrus and sugarcane, and four media: sugarcane, papyrus, and cellulose based media and a synthetic medium containing no plant material. The host-searching response was not elicited by *Sesamia calamistis* (Hamps) sugarcane medium frass. The sexual state and age of *G. natalensis* females were found to influence the host-searching behaviour. Mated females showed the behaviour in the petri dish bioassays only after completing their preoviposition time of two to three days, whilst virgin females took longer, even though their preoviposition time was found to be the same. The response to male or female produced *E. saccharina* sugarcane frass was not statistically different, nor was there a statistically significant preference for either frass type, given the choice. Four way olfactometer tests showed that an *E. saccharina* sugarcane frass odour elicited a host-searching behaviour in mated two to three day old *G. natalensis* females. Various solvents were tested for their ability to isolate the kairomone from *E. saccharina* sugarcane frass. Chloroform proved to be the best solvent when tested in petri dish and olfactometer bioassays. The preliminary results of the GC/MS analysis of the chloroform extract of *E. saccharina* sugarcane frass are presented. The chemicals identified are compared with chemicals identified as host location kairomones for other insect parasitoid-host studies.

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An insect does not think; it reacts. The reactions are often highly stereotyped and cause the insects to perform appropriate behaviours that enhance species survival when appropriate stimuli are encountered. The reactions of insects to chemicals are so predictable that if man could learn enough he could literally make the insects jump through a hoop (Shorey, 1977).

To Dina

CHAPTER ONE

INTRODUCTION AND OBJECTIVES

I.1 INTRODUCTION

1.1.1. Background

In South Africa there are two known lepidopterous species whose larvae are sugarcane stalk borers, *Sesamia calamistis* Hamps (Lepidoptera: Agrotidae) and *Eldana saccharina* Walker (Lepidoptera: Pyralidae) (Carnegie and Smaill, 1980). *S. calamistis* is not regarded as a serious pest as it is rarely responsible for excessive sugarcane loss; the converse is true for *E. saccharina*.

Eldana saccharina is indigenous to Africa and surrounding islands (Walker, 1865). This pyralid borer has been recognised as a crop spoiler for *c*. 100 years (Carnegie, 1983). Girling (1972), Carnegie (1974), Atkinson (1980) and Atkinson *et al.* (1981) give summaries on the history of outbreaks by this pest.

Between 1939 and 1953 localised outbreaks occurred in the Umfolozi area . The pest then disappeared until 1970, when a severe but restricted outbreak occurred in the Hluhluwe area. In subsequent years the pest has spread in the sugarcane industry and by 1983 most low altitude sugarcane areas of South Africa and Swaziland have had to contend with *E. saccharina*. (See Fig 1.1. for areas in Southern Africa where *E. saccharina* has been reported.)

The infestation of sugarcane by *E. saccharina* from its natural host plants (see Atkinson, (1980) for a list of host plants which are largely sedges and include papyrus (*Cyperus papyrus* Linneaus)), is an unsettled problem. Girling (1978) suggested that this invasion was by a new population of *E. saccharina* with an altered host preference. Atkinson (1980) suggested this is not the case as his results showed that *E. saccharina* from sugarcane still preferred wild hosts. He hypothesised that disturbing the natural habitat of *E. saccharina* by draining wetlands and replacing wild hosts with sugarcane, along with modern farming practices that have made sugarcane nutritionally more

acceptable, caused local populations of *E. saccharina* to move into sugarcane, and these populations became self perpetuating and spread. Various physical and chemical stimuli may be involved in the process of host plant selection by ovipositing lepidopterous females (Damman and Feeny, 1988). Apparently these have been provided by sugarcane, with *E. saccharina* preferentially ovipositing on the trash (decaying leaf material) (Leslie, pers. comm.).

Fig. 1.2.a. and b. show the type of damage caused to a sugarcane stalk by *E. saccharina* larvae. Although it is difficult to determine exactly what loss *E. saccharina* damage causes, as is the case for most stalk borers (Sampson and Kumar, 1985a), there is an estimate of around 50% loss of sucrose by severe infestations (100 *E. saccharina*/ 100 stalks), (Thompson, 1983). From various trials it is estimated that on average for every one *E. saccharina* per one hundred sugarcane stalks throughout the season, 0.5 tons/hectare sugarcane is lost. Projecting this it is estimated that there was an average annual loss of R6 million rands for the period 1980/81 to 1985/86. Table 1.1 presents the recorded average number of *E. saccharina*/ hundred stalks for the period 1980/81 to 1985/86 and the resulting estimated tons of sugarcane and rands lost by the South African sugar industry (Moberly, pers. comm.).

Table 1.1. Estimated annual loss of sugarcane (tons) and rands in the South African sugar industry, due to *E. saccharina* (for the periods 1980/81 to 1985/86).

Season	Average *E. saccharina*/100 stalks	Tons cane lost (x 10^3)	Value in rands (x 1000 000)
1980/81	1.7	179	3.9
1981/82	2.7	351	7.0
1982/83	1.3	178	3.9
1983/84	2.9	334	10.1
1984/86	2.0	274	6.5
1985/86	1.8	247	6.2

Such estimates have warranted research into controlling *E. saccharina*, and since 1974 this research has been undertaken at the South African Sugar Association Experiment Station. Areas of research include:

    i)-Distribution and ecology of *E. saccharina*

    ii)-Crop management

    iii)-Biology of *E. saccharina*

    iv)-Sugarcane variety selection

    v)-Diet/host preference of *E. saccharina*

    vi)-Biological control

    vii)-Chemical control

Carnegie (1983) gives more detail on these areas of research. It is notable that insecticides have not shown positive results because *E. saccharina* is for most of its life cycle protected by the sugarcane plant, especially the mature crop, due to its cryptic nature and boring habit. The lack of insecticidal control has in all probability increased research into biological control. Fig. 1.3. illustrates the life cycle of *E. saccharina*. For more information on the biology of *E. saccharina* see Atkinson (1980, 1981), Farine (1983), and Sampson and Kumar (1985b). Although it is evident from the literature that biological control, which includes the use of micro-organisms, predators, and parasites does have advantages over the use of insecticides, their use is still common practice because they are more efficient in most cases (Wood *et al.*, 1983).

1.1.2. Biocontrol of *Eldana saccharina* with parasitoids

Parasitoids are the most important natural enemies of insects due to host specificity, effective searching abilities and good dispersal (Roush, 1979). Other sugarcane stalk borers have been successfully controlled using parasitoids e.g. *Lixophaga diatraeae* (Townsend) successfully controlled *Diatraeae saccharilis* (Fabricius) in various areas of the world (Goraya and Mohyuddin, 1983), and *Apanteles flavipes* (Cameron) has been used successfully against sugarcane stalk borers in a number of countries in the Western Hemisphere (Moyhuddin *et al.*, 1981).
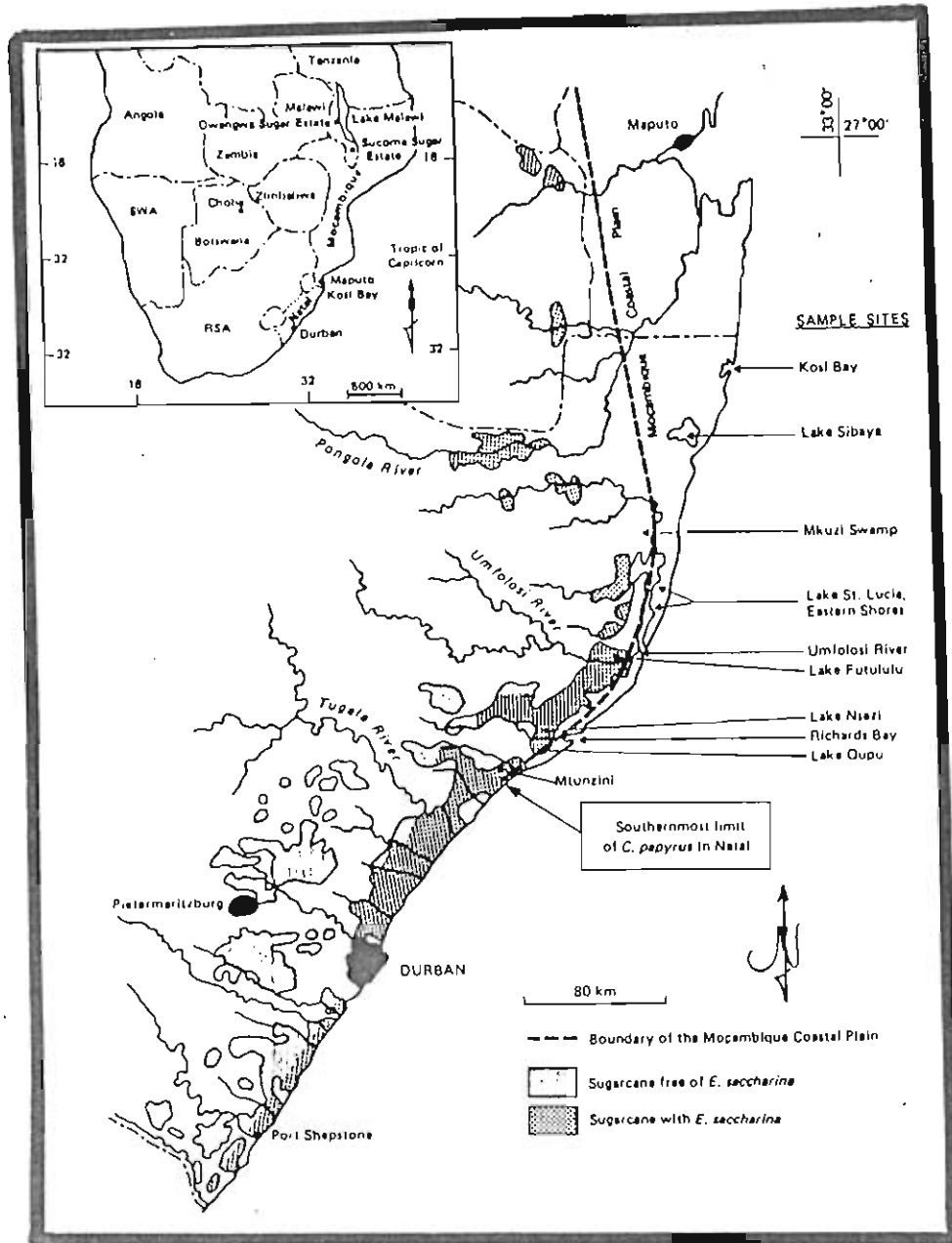
Fig. 1.1. A map illustrating areas of sugarcane and papyrus in Southern Africa where *E. saccharina* has been reported. The location of papyrus wetlands that have been sampled for *G. natalensis* and other parasitoids are indicated. Insert shows the locations in Botswana and Malawi where *G. natalensis* has been found. (Reproduced from Conlong *et al.*, (1988)).

Fig. 1.2.a. Photograph of the external view of a sugarcane stalk with *E. saccharina* borings.

b. Photograph of the internal damage caused to a sugarcane stalk by the stalk borer *E. saccharina.*
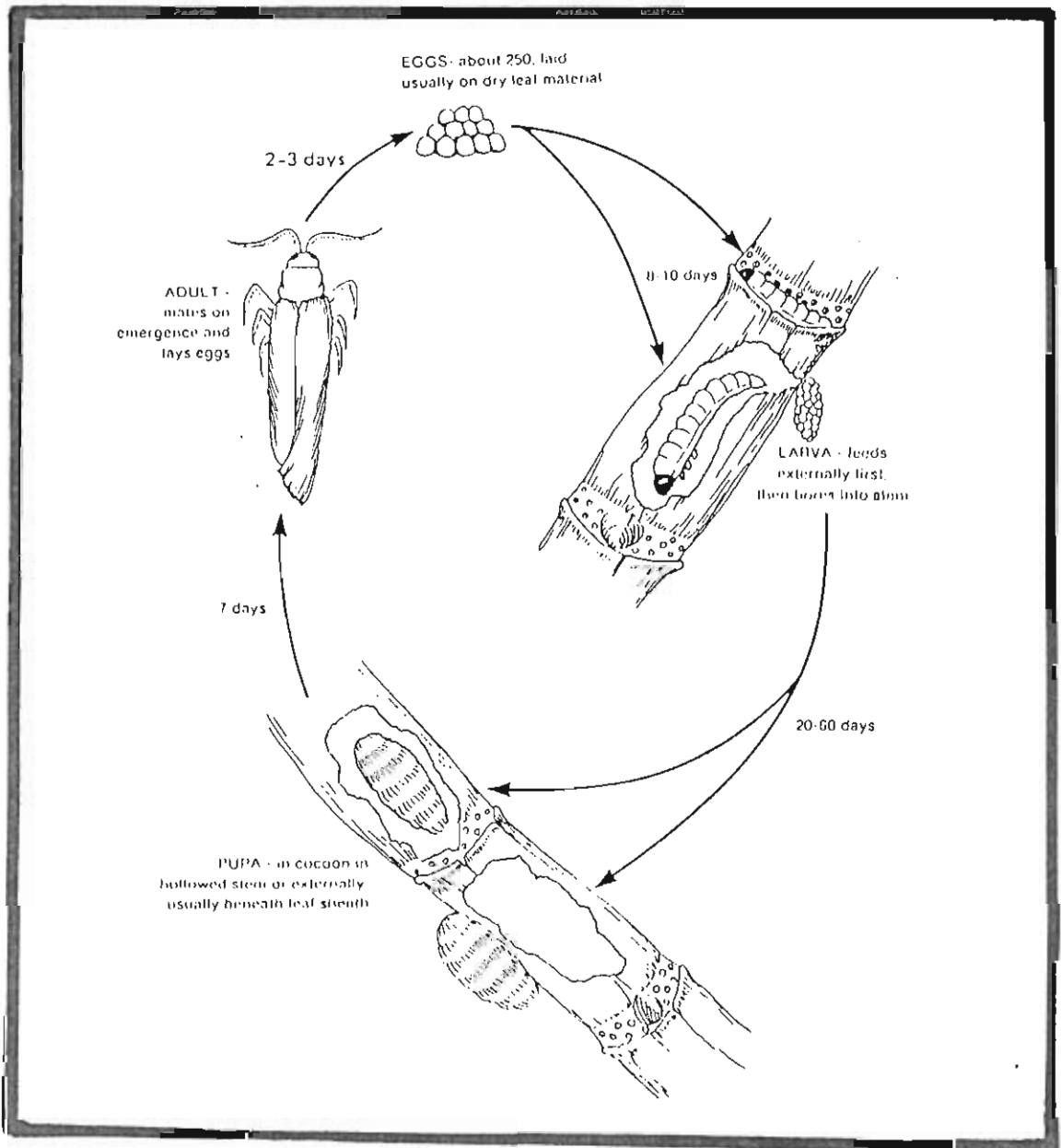
Fig. 1.3. Schematic representation of the life cycle of the stalk-borer *E. saccharina.*

To date the following exotic and local parasitoids have been investigated for use in combating *E. saccharina* in South Africa (Conlong, pers. comm.).

Egg parasitoids reared for testing against *E. saccharina* in S. Africa, and their origin.

*Trichogramma pretiosum (Riley)	U.S.A.; 1971
Trichogrammatoidea armigera (Nagaraja)	India; 1978
Trichogramma brazilensis (Ashmead)	Colombia; 1980
T. semifumatum (Perkins)	Colombia; 1980
*T. perkinsi (Girault)	Colombia; 1980
*T. australicum (= *T. chilonis*) (Girault)	Colombia, Taiwan; 1980, 1983, 1986, 1987
*Trichogrammatoidea eldanae (Viggiani)	Ivory Coast; 1980, 1981, 1984
*Trichogramma sp.	Ivory Coast; 1980
*Telenomus applanatus (Bin and Johnson)	Ivory Coast; 1980, 1981, 1982
Telenomus sp.	Bolivia; 1984, 1986
*Trichogramma evanescens (Westwood)	Germany, Switzerland; 1984
Trichogrammatoidea cryptophlebiae (Nagaraja)	Zebedela, S. Africa; 1990

*Species released into sugarcane fields

Larval parasitoids reared for testing against *E. saccharina* in S. Africa, and their origin.
Diptera

*Descampsina sesamiae (Mesnil)	Ghana, Nigeria; 1975, 1981, 1983, 1984
Sturmiopsis inferens (Townsend)	India; 1977
*Paratheresia claripalpis (Van der Wulp)	Brazil, Colombia,West Indies; 1978, 1985-87
*Metagonistylum minense (Townsend)	Brazil, Colombia, West Indies; 1978, 1985-87
Palpozenilla diatraeae (Townsend)	Bolivia; 1984, 1986
Lixophaga diatraeae	Trinidad; 1987
*Schembria eldana sp. n.	Natal, S. Africa; 1981-1985

*Released into sugarcane fields

Hymenoptera

Cotesia (=*Apanteles*) *flavipes* (Cameron)	Brazil, Pakistan, U.S.A.; 1978, 1983, 1985
*Allorhogas pyralophagus (Marsh)	Trinidad, U.S.A.; 1984, 1985
Iphiaulax kimballi (Kirkland)	U.S.A.; 1985
Rhaconotus rosliniensis (Lal)	U.S.A.; 1985
Macrocentrus prolificus (Wharton)	U.S.A.; 1985
Mallochia pyralidis (Wharton)	U.S.A.; 1985
*Goniozus natalensis (Gordh)	Malawi, Botswana, Natal; 1981-
*Apanteles sesamiae (Cameron)	Natal, S. Africa; 1981
*Orgilus bifasciatus (Turner)	Natal, S. Africa; 1986-
*Chriodes sp.	Natal, S. Africa; 1986-
Agathis sp.	Natal, S. Africa; 1981-
Iphiaulax sp.	Natal, S. Africa; 1981-

*Species released into sugarcane fields.

Pupal parasitoids reared for testing against *E. saccharina* **in S. Africa and their origin.**

Hymenoptera: Ichneumonidae

*Xanthopimpla stemmator (Thunberg)	Mauritius; 1984,1988

Hymenoptera: Eulophidae

Tetrastichus howardii (*ayyari*) (Olliff)	Philippines; 1988

*Species released into sugarcane fields

Conlong *et al.* (1984 and 1988) and Carnegie *et al.* (1985) give an overview of the rearing of these parasitoids and their effectiveness in the control of *E. saccharina*. At present the only parasitoids being reared for release into sugarcane fields are the two larval parasitoids, *G. natalensis* and *P. claripalpis*, and the pupal parasitoid *X. stemmator*. Wood *et al.* (1983) suggested that in biological programmes it is better to employ natural enemies of the target species. *G. natalensis* is the only natural enemy of *E. saccharina* amongst these three, and it has given better results in field releases than any other parasitoid tried (Conlong, pers. comm.). *G. natalensis* thus seemed the

most appropriate parasitoid to investigate. Fig. 1.4. illustrates the life cycle of *G. natalensis*. For more information on the biology of *G. natalensis* see Conlong *et al.*, (1988), and Gordh, (1986)

Female *Goniozus* searches for eldana larva in borin in sugarcane. If succes ful she stings and lays eggs.

Adults emerge from the cocoons after 10-14 days and mate.

After 10-14 days the grubs stop feeding, spin a cocoon and pupate on the remains of the host

Eggs hatch in 2-3 day The young grubs bite into the host larva an feed on the body fluic
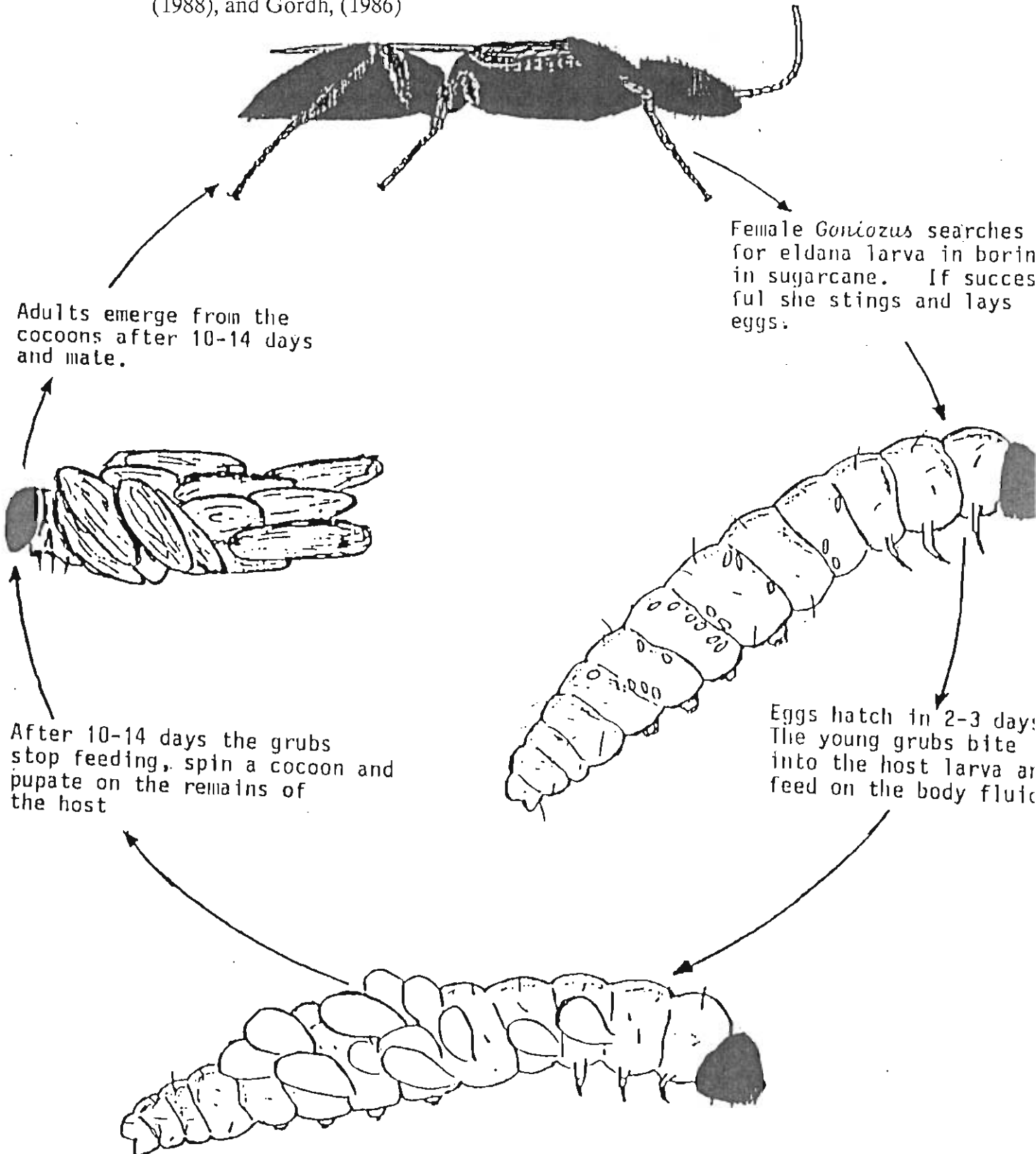
Fig. 1.4. Schematic representation of the life cycle of *G. natalensis*.

1.1.3. Chemical ecology

Chemical ecology became a rapid developing field of research around fifty years ago due to improvements in chemical technology (Nordlund, 1981b). It may be defined as the field of research into the interactions in natural communities that are based on a wide array of natural chemicals (Whittaker and Feeny, 1971).

As with any rapidly growing field of scientific endeavour, that is, "the attempt to make the chaotic diversity of our sense-experience correspond to a logically uniform system of thought," (Einstein, 1940), it has run into some growing pains, and the development of a suitable system of terminology has been, and probably will continue to be, a problem in chemical ecology (Nordlund, 1981b).

The work presented in this thesis pertains to the field of chemical ecology which may best be termed insect chemical communication. Shorey (1977) defines biological communication as "the release of one or more stimuli by one organism that alter the likelihood of reaction by another organism; the reaction is of benefit to the stimulus emitter, the stimulus receiver or both." According to Nordlund (1981b), chemical communication is generally accepted as a more restricted area of chemical ecology, unless a broad definition of communication is used, such as that of Shorey (1977), a definition so broad that according to Burghardt (1970) it renders the concept of communication meaningless. This criticism seems relatively harsh especially since Nordlund (1981b) restrains from giving a better definition, and Shorey (1977) acknowledged that the term communication has defied a definition that is satisfactory to most biological scientists.

1.1.4. Chemical communication

For the work presented in this thesis a definition of chemical communication based on that of Shorey (1977) should suffice. Chemical communication may be defined as the release of one or more chemical stimuli by an organism (emitter), which invokes a response in a receiving organism (receiver), which may be beneficial to to the emitter, the receiver, or both.

Around twenty years ago the study of insect chemical communication became a field of research for scientists from many disciplines due to a number of interacting factors: awareness of the vast ignorance on this subject, advances in microanalytical chemistry which allowed the identification and quantification of the minute quantities of chemicals involved, and an appreciation that the manipulation of these chemical communication systems may be a valuable tool in pest management (Shorey, 1977).

One of the many drawbacks in the terminology in this field is that some of the interactions described in this field may be open to debate as to whether the information transferred constitutes true communication (Blum, 1974; Burghardt, 1970). Nordlund (1981b) points out that this of little relevance because the interactions do occur, and the chemicals that mediate these interactions need to be classified irrespective of whether the interaction is true communication or not.

The chemicals that mediate interactions between organisms fall under the broad term semiochemicals which was first proposed by Law and Regneir (1971), (Gk. *semeon*, a mark or signal). The chemicals are further subdivided according to their function or effect in specific interactions between organisms (Whittaker and Feeny, 1971). There are two major subgroups, pheromones and allelochemics depending on whether the interactions mediated are intraspecific (i.e. between the same species) or interspecific (i.e. between different species) respectively (Nordlund, 1981b).

Pheromones have been defined as: a substance secreted by an organism to the outside that causes a specific reaction in a receiving organism of the same species. They are further classified according to the interaction mediated e.g. sex pheromone, alarm pheromone, and epideitic pheromone. (Nordlund, 1981b, Vinson *et al.*, 1975).

The term allelochemics, proposed by Whittaker (1970), refers to chemicals that are significant to organisms of another species different from the source, for reasons other than food as such. Allelochemics are further classified according to whether the chemical(s) invoke a response in the receiver that is adaptively favourable to the emitter (allomone), receiver (kairomone) or both (synomone). For further classifications and detail see Nordlund (1981a, b), and Nordlund and Lewis (1976).

The chemical ecology of interspecific interactions is extremely diverse and complex and is exemplified by the insect parasitoid-host relationship (Vinson, 1984a). Refer to Price *et al.* (1980) for a summary of the possible semiochemicals involved among interactions of three trophic levels. Parasitoids represent a very diverse group of organisms that are best represented in the class Insecta. There are over 100, 000 described species of parasitic Hymenoptera alone (Vinson, 1984b).

According to Vinson (1984a), the term parasitoid was first used by Reuter (1913) to distinguish organisms which are parasitic only during their immature stages from true parasites. A parasitoid may be classified as an organism which produces only one generation per host; only the immatures are parasitic, the adults are free living. The entire host is usually required by the immatures to meet their nutritional requirements for development, and thus the host is killed or rendered reproductively incompetent and in essence becomes a container for the developing parasitoid(s) (Vinson, 1984b).

Because only a limited number of progeny are produced per host, and the female parasitoid or her progeny must alter the host to meet environmental, physiological and nutritional needs, there is a tendency towards host specialization. Parasitoids therefore generally have to locate at least a few hosts of usually a small variety in order for subsequent generations to be ensured. This is further complicated by the fact that the female parasitoid upon emergence may find herself in a location where suitable hosts are absent (e.g. the right life stage such as a certain larval instar), or the host population may no longer be sufficient due to parasitoid attack or other biotic or

abiotic factors. Thus female parasitoids must have evolved means by which potential hosts are located successfully (Vinson, 1984b). The method required for successful parasitoidism has been divided into steps primarily for man's convenience in thought and communication (Vinson, 1981).

The processes required for successful parasitoidism were first subdivided into two steps by Salt (1935) and Laing (1937), they were later added to by Flanders (1953). Doutt (1964) combined these suggestions into four steps: host habitat location, host location, host acceptance and host suitability. Vinson (1976) reviewed these steps under the category host selection and added a fifth step namely host regulation. The host-selection behaviour of parasitoids are generally very similar (Nordlund et al., 1981b)

The steps have been reviewed in detail by various authors (Beevers et al., 1981; Lewis et al., 1975b, 1976, 1979; Nordlund et al., 1981b; and Vinson 1976, 1981, 1984a 1984b). The work presented in this thesis pertains to the host location process, for more detail on these various steps see the authors previously mentioned.

The process of host location and selection involves a series of stimuli to which the female parasitoid responds, each leading the parasitoid closer to the potential host. However, the steps of host selection are arbitrary, and a particular step may be absent or divided further depending on the parasitoid-host relationship in question (Vinson, 1984a and Lewis et al., 1975b).

A diagram of what is considered by various authors to be the basic pathways for host location and selection by parasitoids is presented in Fig. 1.5. (Lewis et al., 1975b; Lewis et al., 1976; Nordlund et al., 1981b; and Lewis and Nordlund, 1981). The thick lines show the processes that occur if the necessary stimuli are provided at each step. The thin lines show the alternative processes if stimuli for subsequent steps are absent. The transition (T_1) from inactivity to initial random movement is initiated by an innate

appetitive drive together with prevailing environmental conditions and physiological state of the parasitoid (Lewis *et al.*, 1975b). Jones (1986), suggested that although there had been no documentation till 1986 of a chemical causing arousal or elicitation in a resting parasitoid, their work suggested that chemicals may cause the transition. The parasitoid then makes a transition (T_2) from random movement and scans for desired habitats by orienting to olfactory, physical, and/or visual cues (S_2) associated with the plants or habitats where hosts occur. Upon detecting the stimuli indicating the presence of the host (S_3), the T_3 transition to a localised search within that habitat is released (Lewis *et al.*, 1975b). Frass, moth scales, decomposition products, or some other cue(s) associated with host presence have been found to elicit this response. Two or three substeps are usually involved in the search of host trails. Actual detection of the host results (T_4) in an orientation toward and approach to the host. In the case of a concealed host, perception of noise, movement or infrared radiation (S_4) stimuli may be the means of host detection. Upon reaching the host the female parasitoid examines it for acceptability for oviposition. Chemical, visual, and tactile cues (S_5 and S_6) have been demonstrated to be involved in releasing these responses (Lewis *et al.*, 1975b). With regard to larval parasitoids, whose hosts' are hidden e.g. stem-borers, the same chemical cues from the host that cause a host searching response may also act to elicit ovipositor probing, in which case decisions about acceptance of the host are made on the basis of the cues detected by the ovipositor (Jones, 1986). After ovipositing, parasitoids often innately make an intense examination of the area adjacent to the host, and then revert to searching for other hosts (Lewis *et al.*, 1975b).

The T_3 and T_{-3} transitions and the associated stimuli (S_3) between scanning of the habitat and more thorough investigation for the host i.e. host-searching/host location, illustrates that in order for female parasitoids to be retained and effectively search in a desired target area these (S_3) stimuli for the T_3 transition must be adequate, as periodic contact will be necessary or the T_{-3} transition will take place and the parasitoids will revert to general scanning and be lost from the area (Lewis *et al.*, 1975b).

These "S3" stimuli involved in host location may be chemical, physical and biological (Vinson, 1984a). However, chemical cues appear to be the key factors governing the host location process by parasitoids (Lewis et al., 1976). Numerous investigations, beginning with an observation by Thorpe and Jones (1937), have demonstrated that the behaviour of insect parasitoids is influenced by chemicals produced by their hosts.

The term kairomone, coined by Brown et al., (1970), is widely used to describe the chemicals which parasitoids use to locate their hosts. The term kairomone (Gk. kairos, opportunistic), is a term of chemical ecology that has probably received the most criticism (Nordlund and Lewis, 1976).

Kairomones are defined as chemical messengers that induce a behavioural or physiological response in an individual of another species which derives adaptive benefits (Brown et al., 1970). Pasteels (1982) argued that the definition implies that a kairomone is nonadaptive to the transmitter. If maladaptive it should, theoretically, be eliminated through natural selection. However Brown et al. (1970) pointed out, that a kairomone may benefit a population as a whole by regulating the population dynamics in a way that is adaptively favourable in the long term.

Often kairomones used by parasitoids for host location are incidental compounds utilised as cues by the receiver, or signal emissions intended as pheromones, allomones, or hormones for the legitimate receiver, but which are exploited by illegitimate parasitoid receivers (Nordlund and Lewis, 1976). Blum (1974) argues that "so called" kairomones appear to be pheromones and allomones that have "evolutionary back-fired", and as such do not "represent a class of chemical signals distinct from allomones and pheromones."

GENERAL INACTIVITY

T-1   T1 (S1)

RANDOM
MOVEMENT

T-2   T2 (S2)

SCANNING OF
HABITAT

T-3   T3 (S3)

INVESTIGATION OF
HOST TRAILS
WITHIN HABITAT

T-4   T4 (S4)

T8

POSTOVIPOSITION
BEHAVIOUR

Find and
Attack
Cycle

APPROACH
TO HOST

T5 (S5)

T7

OVIPOSITION

EXAMINATION
OF HOST

T6 (S6)
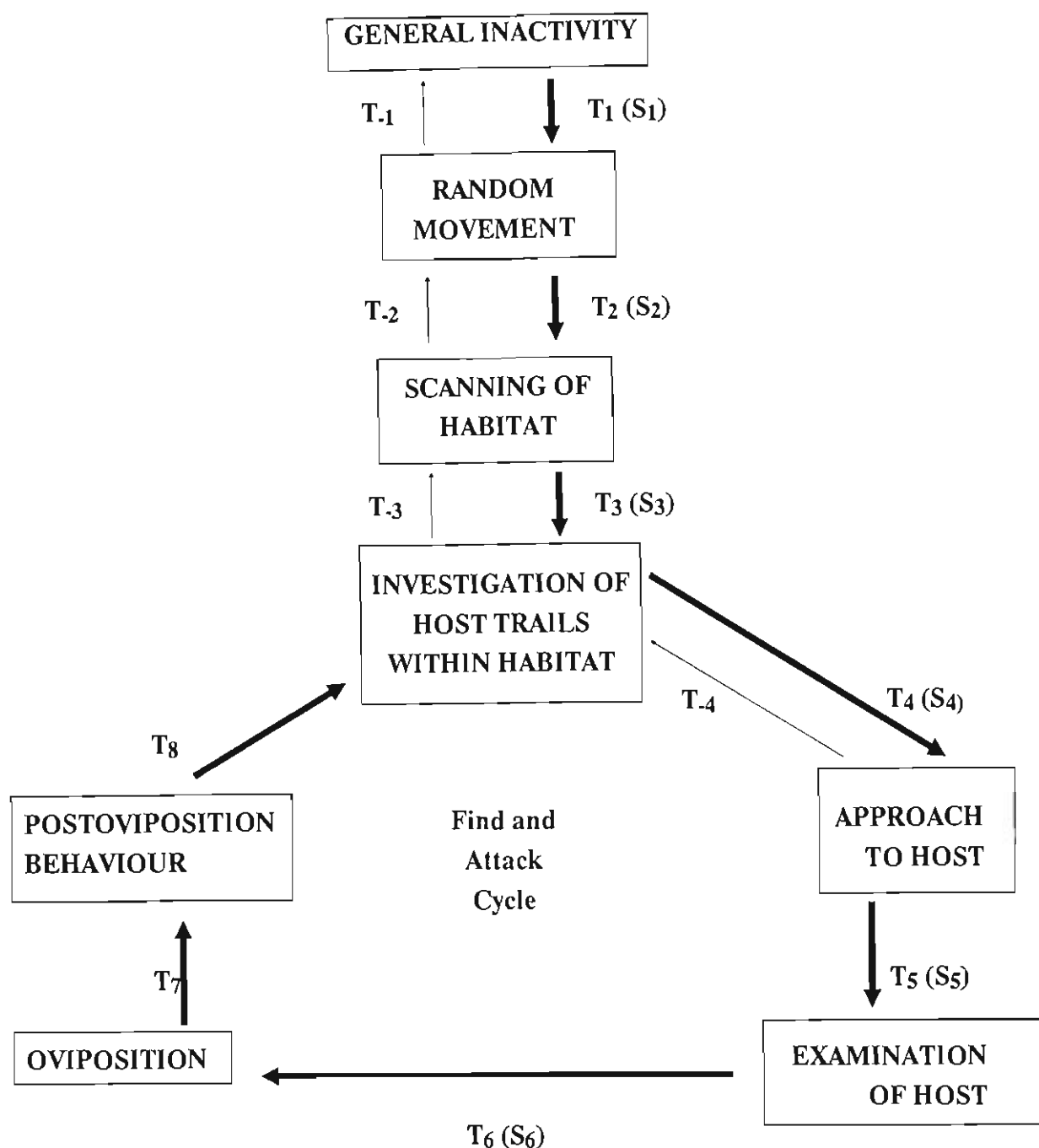
Fig 1.5. Basic sequence of host-finding activities by female parasitoids:

T_1 to T_8 and T_{-1} to T_{-4} = transitions among the behavioural acts.

S_1 to S_6 = stimuli releasing the indicated behavioural patterns.

S_2 = olfactory, visual, and physical cues associated with host plants or other characteristics of the habitat.

S_3 = primarily chemical cues from frass or other products associated with the presence of host insects

S_4 = olfactory, visual, auditory, and other chemical or visual cues from the host insect.

S_5 and S_6 = olfactory, tactile, auditory, and/or a combination of these cues from the host individual. (Lewis *et al.*, 1976b and Nordlund *et al.*, 1981b)

Nordlund and Lewis (1976) suggest that each specific interaction must be considered separately and these interactions are not mutually exclusive. Thus in some cases a chemical or group of plant chemicals may be a phagostimulant for a species, yet a deterrent for most others species. Thus the term kairomone is useful to distinguish the phagostimulant interaction from the deterrent interaction where the chemical or group of chemicals are classified as an allomone. This lead has been followed. Because the term kairomone is widely used, it is hoped that the term will be understood by most and present no problem in this communication to the scientific world.

When considering a biological control programme it must be realised that often man's activities create an environment so changed that natural regulating forces cannot operate normally. Therefore the challenge will be to develop techniques for augmenting and manipulating the performance of natural enemies (Lewis, 1981).

More than ten years ago scientists became increasingly aware of the importance of allelochemics, particularly kairomones, to the performance of parasitoids. Parasitoid efficiency was no longer considered to be dependent on the ability of the organism to randomly encounter hosts. The modification of the belief that parasitoid efficiency in a given environment is innately fixed and cannot be improved beyond predictable limits, which often render unsatisfactory control of the target species resulted in the desire and demand for the employment of kairomones in conjunction with inundative releases of entomophagous insects (Gross, 1981).

1.2 OBJECTIVES

The primary object of this research was to study an area of chemical communication between *E. saccharina* and *G. natalensis*, which it is hoped may prove beneficial in the control of *E. saccharina*.

Host location is regarded by various authors as the most important reason for the effectiveness of parasitoids, and this area provides good opportunities for manipulation by the kairomones involved. It was decided that the study of kairomones involved in host location by *G. natalensis* would be a worthwhile endeavour.

Lewis and Nordlund (1985) gave recommendations for the research, and major objectives in developing technology for integrating kairomones into biocontrol programmes. On the basis of their guidelines it was decided that this research would entail the development of bioassay techniques, which could illustrate the behavioural response of *G. natalensis* to behaviour-modifying kairomones, especially volatile kairomones from its hosts' frass (faeces). This would be followed by the isolation and identification of the most potentially useful kairomone observed.

This work is intended to serve as the preliminary research for identifying a useful behaviour-modifying kairomone. It is hoped that it will stimulate further research into this field, which may culminate in benefits from implementing behaviour-modifying chemicals into the biocontrol programme at the Experiment Station. The following authors may be referred to for the uses of host-searching kairomones in biocontrol programmes: Gross (1981); Gross *et al.* (1975); Gross *et al.* (1984); Gueldner *et al.* (1975); Lewis and Nordlund (1981 and 1985); Lewis (1981); Lewis *et al.* (1975a, 1975b, 1976, and 1979); Nordlund and Sauls (1981); Nordlund *et al.* (1977a, 1977b, 1981a, 1981b, and 1983).

CHAPTER TWO

CLOSE RANGE HOST LOCATION BY *G. NATALENSIS.*

2.1 INTRODUCTION

After a parasitoid has found a suitable habitat and before it can accept or reject a host, the host must be located (see Fig. 1.5.). The necessity for host habitat location before host location apparently does not apply to *G. natalensis* as *G. natalensis* females released into sugarcane fields (which as yet, are not recognised as host habitats) are able to locate and parasitise *E. saccharina*. Therefore, the study of host location without prior host habitat location did not pose a theoretical problem.

Host location is defined as the perception and orientation by parasitoids to their hosts from a distance by response to stimuli produced or induced by the host, or its products (Weseloh, 1981). It is agreed, as Weseloh (1981) suggested, that this definition, like most, is not perfect, as different mechanisms can be categorised under "host location" depending on one's point of view. An example he gives is that of the tachinid parasitoid *Cyzenis albicans* (Fallén) which lays its microtype eggs on damaged leaves of its host's host plant. This is due to an oviposition response caused by sugars in the sap; however the leaves need not be damaged by its host the winter moth (*Operopthera brumata* Linnaeus). Similarly we have found that *Paratheresia claripalpis* larviposition is stimulated by the sap of mechanically damaged sugarcane stalk tissue, and damaged sugarcane stalk from *E. saccharina* borings as well as the frass induce larviposition. However damaged papyrus or papyrus frass from *E. saccharina* do not (Eales, pers. comm.).

Various types of stimuli may be involved in host location and these may be divided into two broad groups, namely:

   i)-physical stimuli

   ii)-chemical stimuli

2.1.1. Physical stimuli

These have been documented for various parasitoids and include stimuli such as: sound, sight and even infra-red radiation (Weseloh, 1981). In the case of *G. natalensis* the location of *E. saccharina* larvae by physical stimuli such as sound, and infra red radiation seems unlikely as the 4th to 6th instar larvae are located within the host plant. Visual stimulus from the frass may be involved but only at a short distance, therefore chemical stimuli seem to be more probable for location from a distance.

2.1.2. Chemical stimuli

Mechanisms by which parasitoids use chemical stimuli to find their hosts may be divided into two categories, namely:

i)-short range chemoreception

ii)-long range chemoreception

Both are important in host location. As the terms imply long range chemoreception is to airborne chemical stimuli, which cause a response at distance from the source. Long range chemoreception thus can be equated to olfaction. Short range chemoreception involves the perception of the chemical stimulus only after physical contact with it in a solid or liquid form. The terms are arbitrary since even rather non volatile chemicals might vapourise to an extent which enable their detection by olfaction at a short distance. Hence it has been suggested that the chemical stimulus be classified by the method used, e.g. a wind tunnel would illustrate long range chemoreception (Weseloh, 1981). Haynes and Baker (1989) give a comparison of the long range chemoreception in a wind tunnel by female navel orange worm moths to the odour of their larval host, compared to the male moths' response to the sex pheromone emitted by the females.

The mechanism of chemoreception is interesting and various theories have been put forward. Altner and Prillinger (1980) give an excellent overview of the ultrastructure of chemo-receptors of invertebrates, and the theories that have been proposed. They

also outline how different sensilla morphology have been shown to be related to either short range or long range chemoreception.

Many more parasitoids have been shown to locate their hosts through short range chemicals in host related products rather than long range chemicals. However this is probably because the former are easily shown by relatively simple bioassays. Table 2.1. (modified from Weseloh,1981), lists a variety of host products that have been shown to contain such chemicals.

All that is needed do to demonstrate the presence of a short range kairomone is to show that the parasitoid changes behaviour when it contacts the chemical(s) of interest (usually obtained by extracting a host product with a solvent and comparing the response to the solvent as a control). The behavioural change is typically a distinct intense examination of the chemically impregnated substrate (Weseloh, 1981), which involves substrate sweeping with the antennae, increased turning movement etc. (Lewis *et al.*, 1976).

During the rearing of *G. natalensis* at the SASA Experiment Station it was noted that females intensively examined *E. saccharina* frass from sugarcane, papyrus, and the medium (sugarcane medium) used for culturing the host (Conlong, pers. comm.).

2.1.3. Hypothesis 1.

From the observations in the laboratory of the response of *G. natalensis* females to *E. saccharina* frass from sugarcane, papyrus and sugarcane medium, it was hypothesised that there is a kairomone in *E. saccharina* frass which is involved in host location by *G. natalensis* females.

Table 2.1. Parasitoids that locate their hosts by close-range chemoreception

PARASITOID	FAMILY	HOST	REFERENCES
ADULT HOST TRACES NEAR EGGS			
Ooencyrtus fecundus (Ferrière & Voegéle)	Encyrtidae	*Aelia germani* (Küst)	Lairachi and Voegele (1975)
Ibalia spp.	Ichneumonidae	*Sirex* sp.	Spradbery (1970a), Madden (1968)
Chelonus texanus (Cresson)	Braconidae	*Heliothis virescens* (Fabricius)	Vinson (1975)
Trissolcus viktorovi	Scelionidae	*Eurydema* spp.	Buleza (1973)
Trissolcus spp.,	Scelionidae	*Eurygaster*	Viktorov *et al.* (1975)
Telenomus chloropus (Thompson)		*intergriceps* (Puton)	
Trichogramma evanescens (Westwood)	Trichogrammidae	*Sitotroga* spp., *Heliothis zea* (Boddie)	Laing (1937), Lewis *et al.* (1975a) Jones *et al.* (1971, 1973)
T. achaeae (Nagaraja and Nagarkatti)	Trichogrammidae	*H. zea*	Lewis *et al.* (1975a)
T. pretiosum (Riley) *Trichoplusia ni* (Hübner)	Trichogrammidae	*H. zea*	Lewis *et al.* (1975a), Nordlund *et al.* (1977b)
HOST FAECES			
Lydella grisescens (Robineau-Desvoidy)	Tachinidae	*Ostrinia nubilalis* (Hübner)	Hsiao *et al.* (1966)
Lixophaga diatraeae (Townsend)	Tachinidae	*Diatraeae saccharilis* (Fabricius)	Roth *et al.* (1978)
Archytas marmoratus (Townsend)	Tachinidae	*Heliothis virescens*	Nettles and Burks (1975)
Spilocrytus extrematis (Cresson)	Ichneumonidae	*Hyalophora cercropia* (Linnaeus)	Marsh (1937)
Campoplex haywardi (Blanch)	Ichneumonidae	*Phthorimaea operculella* (Zeller)	Leong and Oatman (1968)
Bracon mellitor (Say)	Braconidae	*Anthonomus grandis* (Boheman)	Henson *et al.* (1977)
Orgilus lepidus (Muesebeck)	Braconidae	*Phthorimaea operculella*	Hendry *et al.* (1973)
Apanteles sesamilae (Cameron)	Braconidae	*Busseola fiusca* (Fuller)	Ullyet (1935)
A. chilonis (Munukata), *A. flavipes* (Cameron)	Braconidae	*Chilo suppressalis* (Walker)	Kajita and Drake (1969)
A. dignus (Muesebeck)	Braconidae	*Keiferia lycopersicella* (Walsingham)	Cardona and Oatman (1971)

-Table 2.1. Continued-

Cotesia (Apanteles) marginiventris (Cresson)	Braconidae	*Spodoptera frugiperda* (J. E. Smith)	Dmoch *et al.* (1985)
C. flavipes (Cameron)	Braconidae	*Diatraeae saccharilis*	Van Leerdam *et al.* (1985)
Macrogaster (Microplitis) croceipes (Cresson)	Braconidae	*Heliothis* spp.	Lewis (1970), Lewis and Jones (1971), Jones *et al.* (1971)
M. demolitor (Wilkinson)	Braconidae	*Heliothis zea Trichoplusia ni*	Nordlund and Lewis (1985)
Macrocentrus grandii (Goidanich)	Braconidae	*Ostrinia nubilalis*	Ding *et al.* (1989)
Microterys flavus (Howard)	Encyrtidae	*Coccus hesperidum* (Linneaus)	Vinson *et al.* (1978)
Trichomalus perfectus (Walker)	Pteromalidae	*Ceutorhynchus assimilis* (Payk.)	Dmoch and Rutkowska-Ostrowski (1978)
Epidinocarsis lopezi (De Santis)	Encyrtidae	*Phenacoccus manihoti* (Matile-Ferraro)	Langenbach and Van Alphen (1987)
Rhyssa persuasoria (Linneaus)	Ichneumonidae	*Sirex* spp.	Spradbery (1970b), Madden (1968)
Megarhyssa spp.	Ichneumonidae	*Sirex* spp.	Madden (1968)

HOST MANDIBULAR AND LABIAL GLAND SECRETIONS

Cardiochiles nigriceps (Viereck)	Braconidae	*Heliothis* spp.	Vinson (1968), Vinson *et al.* (1976)
Cotesia (Apanteles) marginiventris (Cresson)	Braconidae	*Spodoptera frugiperda*	Dmoch *et al.* (1985)
Venturia (Nemeritis) canescens (Gravenhost)	Ichneumonidae	*Anagasta kuehniella* (Zeller)	Corbet (1971,1973), Mudd and Corbet (1973)
Campoletis sonorensis (Cameron)	Ichneumonidae	*Heliothis* spp.	Wilson *et al.* (1974), Schmidt (1974)
Apanteles melanoscelus (Ratzeburg)	Braconidae	*Lymantria dispar* (Linneaus)	Weseloh (1976, 1977)
Macrocentrus grandii	Braconidae	*Ostrinia nubilalis*	Ding *et al.* (1989)
Pseudorhyssa sternata (Merrill)	Ichneumonidae	*Sirex* spp.	Spradbery (1968)
Temelucha interruptor (Gravenhost)	Ichneumonidae	*Rhyacionia buoliana* (Schiffermüller)	Arthur *et al.* (1964)

HOST - MARKING PHEROMONE

Opius lectus (Cameron)	Braconidae	*Rhagoletis pomonella* (Walsh)	Prokopy and Webster (1978)

HOST TRAIL

Bracon (Habrobracon) juglandis (Say)	Braconidae	*Anagasta (Ephestia) kuehniella* (Zeller)	Murr (1930)

2.2. MATERIALS

2.2.1. Insects.

G. natalensis used for the tests were obtained from: papyrus, sugarcane fields, and a laboratory culture which is described by Conlong *et al.*, (1988), and Graham and Conlong (1988). Fig. 1.1 shows the areas from which *G. natalensis* has been collected. Gordh (1986) gives a good description of male and female *G. natalensis* adults.

Mated *G. natalensis* females were produced by placing the females with males (2:1 ratio) in a jar for at least twenty-four hours. Virgin *G. natalensis* females were obtained by placing laboratory reared *G. natalensis* pupae into separate 25 ml vials (Polytop®), and collecting the virgin females from the individual vials each day. This was to prevent sibling mating observed in the laboratory culture (Conlong *et al.*, 1988). *E. saccharina* larvae were obtained from the laboratory culture described by Conlong *et al.*, (1988), and Graham and Conlong (1988).

2.2.2. Plants.

Papyrus was obtained from Tongaat, and NCo376 sugarcane was obtained from a field on the Experiment Station.

2.2.3. Media.

Cellulose, papyrus and sugarcane media were prepared by the method described by Graham and Conlong (1988), the media constituents are presented in the Appendix (Tables A, B, and C respectively). Synthetic medium was prepared by a similar method and the constituents are presented in the Appendix (Table D).

2.2.4. Frass.

All the types of frass collected were stored in 25 ml glass vials (Polytop®) at -20°C until tested. Frass was not stored for more than four consecutive weeks.

E. saccharina frass from sugarcane was collected from various Natal north coast sugarcane fields. And *E. saccharina* frass from papyrus was collected from various fields in Natal and Zululand. Fig 1.1 shows the various areas were *E. saccharina* may be found and its frass collected.

Sugarcane frass from male and female *E. saccharina* was produced in the laboratory by placing laboratory reared 4th and 5th instar larvae into mechanically preformed 0.5 cm i.d. x 0.5 cm deep borings in NCo376 sugarcane internodes. These were placed in 31 cm x 19 cm sealed Tupperware$^{®}$ containers which had a 17 cm x 8 cm 80 mesh wire vents in the lids. The larvae were reared at 27°C, r.h. 70% for fourty-eight hours and an 11 h light/ 13 h dark regime, after which they were placed into fresh internodes. Twenty-four hours later the larvae were killed by removing them from a boring and placing them in boiling water. The dead larvae were sexed (Atkinson, 1980) and the frass that they produced was partitioned according to sex.

Cellulose, papyrus and sugarcane medium frass were produced by placing 1st instar *E. saccharina* larvae from field-collected *E. saccharina* moths on the relevant media (see Appendix A), with the frass being harvested after the larvae had pupated.

Synthetic medium frass was produced by placing individual 4th and 5th instar sugarcane medium-cultured larvae onto the synthetic medium in 25 ml vials (Polytop$^{®}$). After fourty-eight hours the larvae were placed into clean vials containing fresh medium, and the frass was collected fourty-eight hours later. The larvae were cultured at 27°C, r.h. 70% and an 11 h light/ 13 h dark regime.

Sesamia calamistis sugarcane medium frass was produced by placing larvae collected from sugarcane fields onto sugarcane medium in 25 ml vials (Polytop$^{®}$). The following day the larvae were placed into jars containing fresh medium in a culturing room 27°C, r.h. 70% and an 11 h light/ 13 h dark regime. The frass was collected fourty-eight hours later.

Frozen frass used in the tests was removed from the refrigerator and thawed at room temperature, usually for two hours.

2.3 METHODS

All tests were carried out in a blinded room, with a temperature maintained at $26^{o}C$ $+/-2^{o}C$ to prevent phototactic and climatic bias. Translocation of parasitoids between containers and apparatus was achieved using a horse hair paintbrush wetted with distilled water, which successfully picked up the parasitoids once contacting their wings. This method was used to cause the least possible chemical contamination and stress to the insect during manipulation.

2.3.1. Petri dish test 1.

This test is similar to that described by Nordlund and Lewis (1985). The frass and plant materials tested were pressed onto a Whatman® no. 1 filter paper until a c. 0.5 cm spot was formed. The filter paper was turned over and placed into the lid of a 9 cm dia. petri dish. The parasitoid to be tested was placed into the bottom half of the petri dish, which was then placed into the lid. The dish was sealed by means of tape on opposite sides of the petri dish. In this way the parasitoid is exposed only to the chemicals from the test material, and any response observed is to chemical stimuli and not to physical stimuli. A 5 ml syringe was glued to the centre of the petri dish lid to serve as a handle so not to disturb the parasitoid by holding the sides. The dish was held in a 22.0 cm high x 26.5 cm wide white cardboard box through a 12.0 cm x 9.5 cm hole at the bottom, through which a hand could fit. The lid of the box was removed for observation.

The parasitoid was made to cross the central spot of test material by holding the dish vertically, causing the female to move upwards due to a negative geotactic response. By rotating the dish the female can be made to pass over the spot. The pass on which a female first responded to the test material was determined, with each female being allowed a maximum of three passes to respond to the test material, after which a no

response was scored. A positive response was scored if the female antennated (palpated antennae) intensively on the spot for more than five seconds. The Chi Square test was used to determine whether differences were statistically significant. Because the results of positive responses on passes two and three were less than five, the results of positive responses on passes one, two and three were pooled for analysis.

2.3.2. Petri dish test 2.

This test is similar to that described by Van Leerdam *et al.* (1985). The frass tested was placed within the circumference of a 1.5 cm i.d. rubber band on filter paper (Whatman® no. 1, 9 cm o.d.) which was placed into the lid of 9 cm i.d. petri dish, which had 6 cm on either side removed and covered with 80 mesh wire screen to allow a diffusion gradient. To standardise the tests 0.5 g of frass was used. Four female parasitoids were tested each time. The females were placed into the bottom half of the petri dish, which was placed into the lid of the the petri dish which contained the filter paper and frass encircled with a rubber band. The dish was sealed by means of tape on opposite sides of the dish.

To prevent excessive disturbance of the parasitoids by surrounding visual stimulus, the petri dish was placed within the white cardboard box described previously. Observations were made over the top of the box through the bottom of the petri dish. The number of visits and time of each visit was recorded for a 10 minute period. Four or five bioassays were done for each test. The t-test was used to determine any statistically significant differences for the average number of visits and average time of each visit observed in the bioassays.

2.3.3. Olfactometer test.

A four way airflow olfactometer first described by Vet *et al.* (1983) was used to test the olfactory response of G. *natalensis* to various odours. The olfactometer, shown in Fig. 2.1., is a slight modification of the one described by Vet *et al.* (1983).
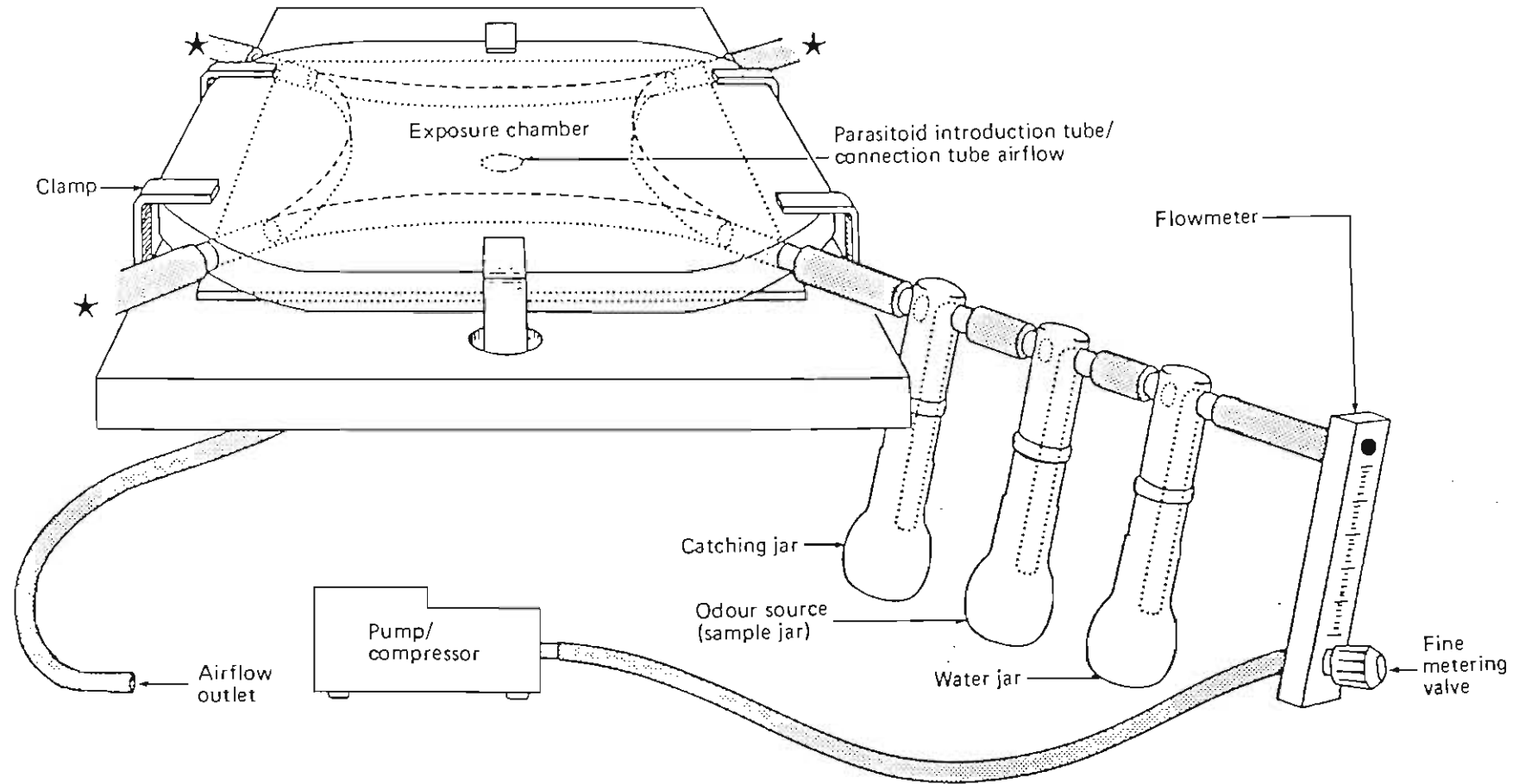
Fig. 2.1. Perspective view of the four way olfactometer. Only one arm is shown, the rest are represented by stars. (Modified from Vet *et al.* (1983)).

The depth of the exposure chamber is 13 mm instead of 10 mm, and the narrowest width across the exposure chamber was 108 mm instead of 110 mm. For further details of the structure of the olfactometer see Vet *et al.* (1983).

The olfactometer allows the formation of four distinct fields of odour in the exposure chamber due to the symmetrical design, and regulating each of the four airflows into the exposure chamber by means of an airflowmeter (Aalborg[®] FM 112-02G, mass flow meter).

Air was pumped through each arm by individual fish tank pumps (Elite[®] 800). The airflow rate required to create four distinct fields of odour was determined by a "smoke" test similar to that of Vet *et al.* (1983). By placing 5 ml of NH_4OH into the sample jar and 5 ml of HCl into the trapping jar of two opposite arms the best flow rate for the formation of four non-mixing fields was found to be 150 ml/min (see Fig. 2.2.), unlike the 300 ml/min for the olfactometer of Vet *et al.* (1983).

On each arm there were three glass vials (see Fig. 2.1.). The water jar contained distilled water over which the incoming air first passed, which served to create a uniform humidity in all four fields to prevent any bias for a particular field. The sample/odour jar contained the material responsible for the respective odour, the air passes over the material collecting the volatiles, which move into the respective field of the exposure chamber. This allows the entry of an odour from a solid or liquid material. In the case of a clean field no material was placed in the jar. Vet *et al.* (1983) use the term "odourless" field, but the term clean is favoured here because the air still has an odour. For the sugarcane frass odour 9.0 g of field collected sugarcane frass was placed in the sample jar. The catching jar caught any parasitoid that walked out of the exposure chamber and down the tube towards the odour source.

To prevent disturbing the parasitoid during the bioassays, the exposure chamber was observed at a distance via a monitor connected to a video camera which was mounted above the exposure chamber (see Fig. 2.3.).
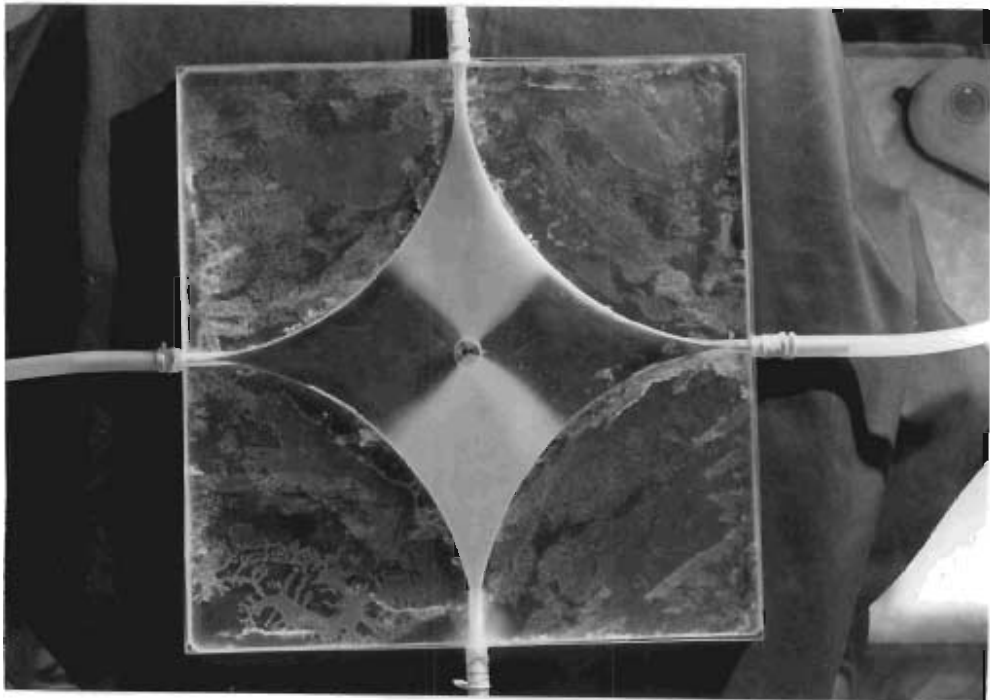
Fig. 2.2. Photographic representation of the four flow fields. NH4Cl "smoke" was pumped through two opposite arms of the apparatus with clean air being pumped through the remaining two arms. The flow rate through each arm was 150 ml/min.
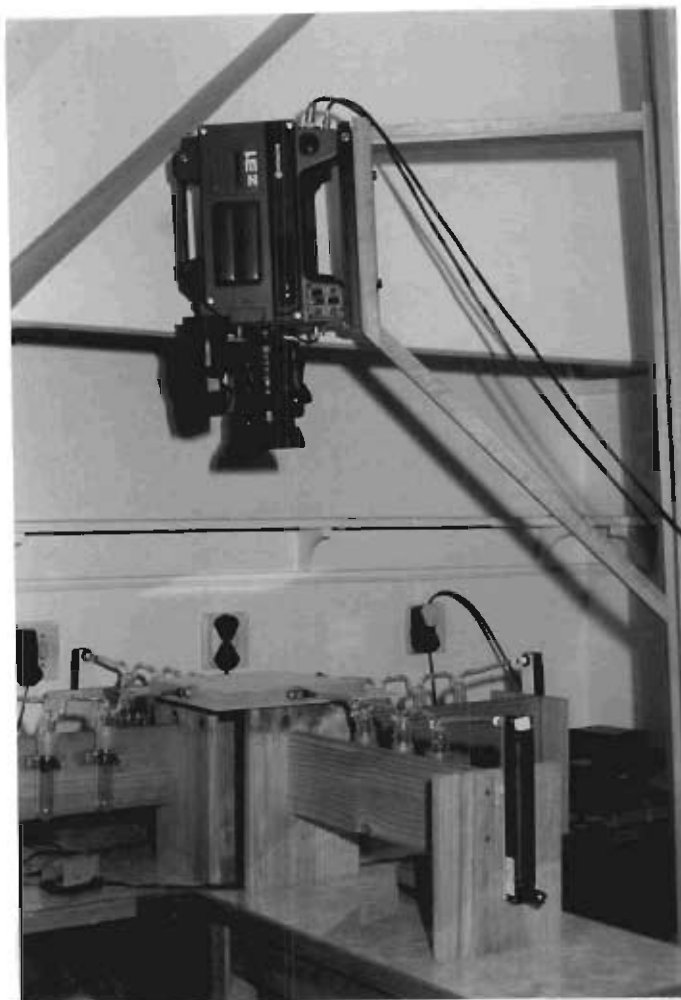
Fig. 2.3. Photographic representation of the video camera mounted above the four way olfactometer. This was connected to a monitor (see Fig. 2.4.) and video recorder from which observations and recordings of the bioassays could be made.

The bioassays were recorded when deemed necessary, using a video machine connected to the video camera. This enabled the playback of complex movements in the exposure chamber, which decreased the chance of incorrect observation. Tracing of a parasitoid's movement in the exposure chamber was done using a fine, slow-mode play back of the recordings, and tracing the movement onto a transparency placed over the monitor screen.

Fig. 2.4. indicates the nature of the view of the exposure chamber provided by the monitor. Parasitoids were tested individually as they were fairly aggressive towards each other, and chance contacts in the exposure chamber resulted in one female chasing the other away. The experimental odour fields were first set up in the exposure chamber by turning the four pumps on simultaneously. The outlet tube was then disconnected and the parasitoid was placed inside and the tube was then reconnected. This is an advantage of using pumps on each arm instead of a vacuum pump on the outlet as reported by Vet *et al.* (1983), because the odour fields once set up do not have to be disturbed for the parasitoid to be introduced into the exposure chamber. Most parasitoids tested moved directly upwards into the exposure chamber as they have a negative geotactic response. The parasitoid in the vertical entry tube was immediately exposed to a mixture of the four odours offered until reaching the exposure chamber floor, where it was exposed to an individual odour which depended on the sector it first moved into.

Once in the exposure chamber the parasitoid could move about freely over the floor, sides or ceiling of the exposure chamber. It had the choice to stay in an odour field, or leave it, sample adjacent fields and select which field to move in. This is an important advantage of this olfactometer over T- and Y- tube olfactometers used in the past (Monteith, 1985; Read *et al.*, 1970; Rotheray, 1980; Shahjahan, 1974). The ability to move and choose freely amongst odour fields is not possible in the T- and Y- tube olfactometers because distinct contiguous odour fields are not formed.
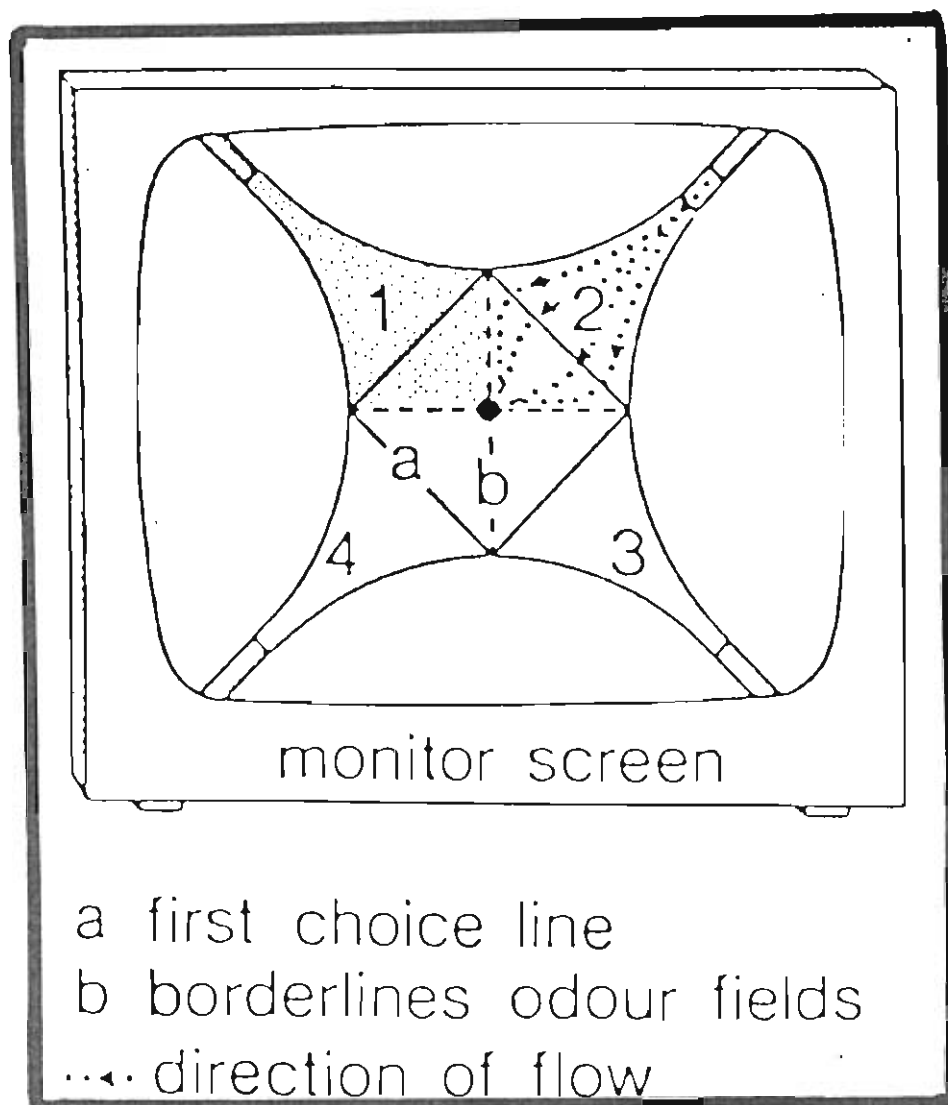
Fig. 2.4. Diagrammatic representation of the video monitors' view of the olfactometer exposure chamber indicating the boundaries, and direction of airflow in the four odour fields. The imaginary first choice line in each field is also shown. (Reproduced from Vet *et al.* (1983)).

Moreover at their junctions there is turbulence and mixing of odours which makes the chemotactic response of small parasitoids difficult, and if an arm is accidentally entered they are often behaviourally trapped (e.g. by a phototactic response) and thus the field finally chosen may be incorrect.

To mathematically score the observations of the bioassays the "first choice" and "final choice" described by Vet *et al.* (1983) was used. When a parasitoid crossed one of the lines of the arbitrary "first choice" square (Fig. 2.5.) it was recorded as having made a first choice for that odour field. A maximum of 10 min was allowed for a "final choice", which was recorded when the parasitoid either: i) left the exposure chamber and entered a trapping jar; ii) left the exposure chamber and remained out for 2 min; iii) the field in which it spent the most time if neither i) or ii) occurred. The Chi Square test was used to determine any significant differences in the number of observed first and final choices for the four airfields from what would have been expected if fields were randomly chosen.

Between each new test material the exposure chamber and arms of the olfactometer were washed with hot water and detergent, followed with distilled water and then swabbed with alcohol and allowed to dry. The controls before each new test material indicated that there was no bias for a field which had contained the previous test material.

During the bioassay of a test material the olfactometer was rotated, usually after five females had been tested. In the case of sugarcane frass after testing half the females the olfactometer was dismantled and cleaned and the sugarcane frass was placed into an adjacent arm. These procedures served to maintain a standardised system between and during the testing of materials. Results of controls proved that this had been achieved.

2.4 RESULTS AND DISCUSSION

2.4.1. Petri dish test 1.

In this test the characteristic positive response by *G. natalensis* females was the deceleration of the parasitoid before reaching the test material spot (*c.* 1-10 mm from the spot), with strong antennation (palpating of antennae) on the filter paper. Movement towards the spot involved more turns and a slower walking speed. Once contacting the spot movement ceased for two to five seconds (see Fig. 2.5.a), then the female moved around the spot intensively examining it with strong antennation (see Fig. 2.5.b), and occasionally stopping and sweeping her antennae on the spot (see Fig. 2.5.c). If a female moved off the spot she normally turned 180° within 1 cm of the spot and re-approached the spot in the manner previously described, even if it meant moving downwards against her negative geotactic response. Once contacting the spot again a similar examination occurred.

If left for long enough (ca. one minute) most of the females would bore into the spot (see Fig. 2.5.d), and a considerable number probed the spot with their ovipositor (see Fig. 2.5.e).

Waage (1978) started interpreting the often vague reported descriptions of parasitoid chemotactile behaviour in terms of orientation behaviour. The terms that he used adequately describe the characteristic searching response observed by *G. natalensis* females. He found that when a parasitoid contacts a chemical trace it usually slows down or stops (orthokinesis). As it examines the substrate with the antennae, it also increases its rate of turning, which may be in a random direction (klinokinesis) or orientated with respect to the chemical "patch" (klinotaxis). *G. natalensis* females therefore exhibited orthokinesis and klinotaxis (the turning movement was orientated to the patch, this was made evident by 180° turns when females lost contact with the spot).

Fig. 2.5.a. Photograph of a mated two to four day old *G. natalensis* female stopping ca. 1 cm from the test material (sugarcane frass) spot in the petri dish test 1 bioassay. (Magnification = 10 X).

Fig. 2.5.b. Photograph of a mated two to four day old *G. natalensis* female antennating intensively on the test material (sugarcane frass) spot in the petri dish test 1 bioassay. (Magnification = 10 X).

Fig. 2.5.c. Photograph of a mated two to four day old *G. natalensis* female sweeping her antennae across the test material (sugarcane frass) spot in the petri dish test 1 bioassay. In this photograph the left antenna is being swept across the spot which is followed by the sweeping of the right antenna (i.e. a side to side sweeping). (Magnification = 10 X).

Fig. 2.5.d. Photograph of a mated two to four day old *G. natalensis* female boring into the test material (sugarcane frass) spot after ca. one minute of examination in the petri dish test 1 bioassay. (Magnification = 10 X).
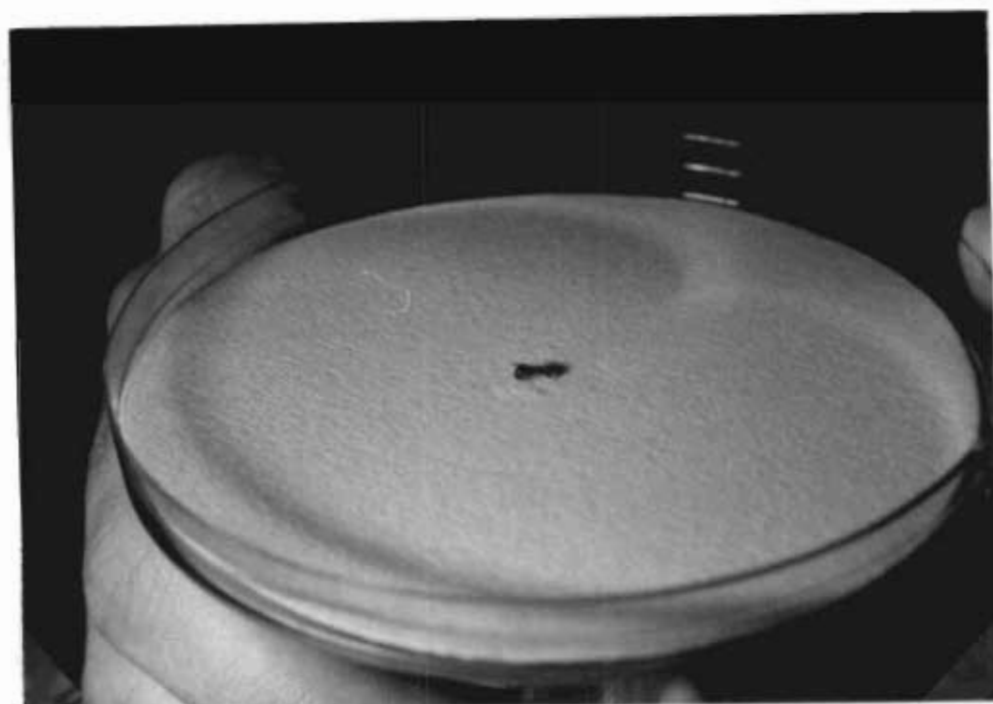
Fig. 2.5.e. Photograph of a mated two to four day old *G. natalensis* female probing the test material (sugarcane frass) spot with her ovipositor after ca. one minute and twenty seconds of examination in the petri dish test 1 bioassay. (Scale: 1 cm = 0.82 cm).

G. natalensis males showed little interest in *E. saccharina* frass. Bacon (1986) points out that chemo-receptors can be absent in one sex of a species. This is illustrated by Schneiderman *et al.* (1986) who showed that transplanting of the male antenna of the tobacco cornworm moth (*Manduca sexta*) to the female, causes the female to exhibit male specific behaviour to the pheromone released by the female. Alternatively it may be that the behavioural response is not part of the male genetic make-up (Bacon, 1986).

Table 2.2. presents the results obtained for the response of *G. natalensis* females of various ages and sexual state to materials of interest. Mated females two to four days old responded strongly to *E. saccharina* frass from sugarcane, papyrus umbels and the four media. These positive responses were the typical positive response, described previously.

[a]Mated *G. natalensis* females from papyrus, sugarcane and the laboratory culture did not show a significant difference in response to the sugarcane frass spot (P > 0.05). It was decided that because the laboratory reared parasitoids showed a similar response to the natural papyrus parasitoids, the laboratory reared *G. natalensis* would be used in the bioassays to represent natural parasitoids. This was done because the advantages of using the laboratory reared parasitoids (which include a larger consistent supply, and a greater knowledge of the insect being tested) outweighed the problems that might occur such as conditioning, and associative learning. The following results and discussion thus pertain to laboratory reared *G. natalensis*.

[b]Both sugarcane frass and papyrus frass from the field elicited host searching by mated two to four day *G. natalensis* old females. The response to the two types of frass were not significantly different (P > 0.05). The strong host searching response to frass from either plant is in accord with the ability of *G. natalensis* females to locate and parasitise *E. saccharina* in sugarcane, as is evident from the *G. natalensis* field releases by the Experiment Station.

[c]The preoviposition period for mated *G. natalensis* females is two to three days (Conlong *et al.*, 1988). Many hymenopteran parasitoids have a period after emergence during which host location is not of primary importance. This is advantageous as it is of no use for females to locate the host until they are ready to oviposit (Vinson, 1984a). This phenomenon is illustrated by *G. natalensis* females in this bioassay, where mated females less than one day old showed significantly fewer responses to sugarcane frass, than mated females two to four days old did (P < 0.001). The only two positive responses reported for the less than one day old females lasted more than five seconds, but the examination was not intense and the response lasted less than ten seconds in both cases.

[d]Virgin females two to four days old showed a poor response to sugarcane frass which is supported by petri dish test 2 results. Virgin females will parasitise *E. saccharina* larvae, but the progeny are male, unlike mated females whose progeny are mostly female (Conlong *et al.*, 1988). It is normal in hymenopterans for the haploid egg to result in male progeny and the fertilised diploid egg female progeny. The same virgins at an age of eight to ten days showed the characteristic positive response, which was significantly stronger than their response at a younger age (P = < 0.001). It was considered that the preoviposition period for virgin females may be longer than that of the mated females, which may explain why they only responded to the frass at an older age, but a small study showed the preoviposition time for virgin females was also two to three days. It is plausible that the location of a mate before locating a host is advantageous, and this behaviour obviously lasted longer in the virgin females.

[e]There was no significant difference in the number of responses of mated *G. natalensis* less than one to four days old to either frass from either male or female *E. saccharina* (P > 0.05). Conlong *et al.*, (1988) presented results of a laboratory experiment where *G. natalensis* females were exposed to artificial *E. saccharina* sugarcane stalk borings containing larvae. There was a larger parasitization of the female larvae. If frass from male larvae lacked the kairomone which elicits host searching then this may be

expected. However the present results suggest this is not the reason. It appeared that a smaller proportion of mated females responded to NCo376 laboratory produced sugarcane frass relative to the proportion that responded to sugarcane frass collected from the field. This may be due to some of the females tested on the NCo376 sugarcane frass being less than one day old and most mated females of this age did not respond to the field sugarcane frass[c].

[f-k]Mated female *G. natalensis* (two to four days old) showed significantly fewer positive responses to macerated tissue of papyrus umbels and sugarcane stalk, than to the *E. saccharina* frass from these plants ($P < 0.001$). Similarly there were significantly fewer positive responses to the four media than to the *E. saccharina* frass from these media ($P < 0.001$). Because the parasitoids showed strong searching responses to the different *E. saccharina* frass types but not to the substrates from which the frass was derived, hypothesis 1 (see section 2.2.), that there is a kairomone in *E. saccharina* frass which is involved in host location, was accepted.

[l]The number of positive responses by mated *G. natalensis* two to four day old females to *Sesamia calamistis* sugarcane medium frass were significantly less than to *E. saccharina* sugarcane medium frass ($P = <0.001$). This along with the results of the responses to *E. saccharina* frass from various substrates, but lack of response to the respective substrate suggests that the kairomone is produced by the *E. saccharina* larvae.

Table 2.2. Results of the response of *G. natalensis* females to test materials in petri dish test 1.

	(Test material) *Goniozus* origin [age]	Number of females with first response on pass:			Number of females with no response after three passes	Total number tested	Signifi -cance.
		1	2	3			
a	(Sugarcane frass from field) Sugarcane *G. natalensis*	25	1	0	10	36	
	Papyrus *G. natalensis*	10	4	4	11	29	NS
	Laboratory *G. natalensis* [All above 2-4 days old]	24	2	1	11	38	
b	Laboratory *G. natalensis* [2-4 days old] (Papyrus umbel frass from field)	20	2	0	8	30	NS
	(Sugarcane frass from field)	24	2	1	11	38	
c	(Sugarcane frass from field) Laboratory *G. natalensis* [2-4 days old]	24	2	1	11	38	***
	[< 1 day old]	2	0	0	28	30	
d	(NCo376 sugarcane frass) Virgin Laboratory *G. natalensis* [2-4 days old]	1	0	0	19	20	***
	[8-10 days old]	14	0	0	1	15	
e	Laboratory *G. natalensis* [< 1-4 days old] NCo376 sugarcane frass from: (Female *E. saccharina* larvae)	9	0	0	11	20	NS
	(Male *E. saccharina* larvae)	10	0	0	10	20	
f	Laboratory *G. natalensis* [2-4 days old] (Macerated NCo376 sugarcane)	0	0	0	20	20	***
	(Sugarcane frass)	24	2	1	11	38	
g	(Macerated papyrus umbels)	1	0	0	19	20	***
	(Papyrus umbel frass)	20	2	0	8	30	
h	(Sugarcane medium frass)	16	2	1	2	20	***
	(Sugarcane medium)	0	0	1	19	20	
i	(Papyrus medium frass)	14	2	0	4	20	***
	(Papyrus medium)	0	2	2	16	20	

-Table 2.2. Continued-

j	(Cellulose medium frass)	18	0	0	2	20	***
	(Cellulose medium)	0	0	0	20	20	
k	(Synthetic medium frass)	11	4	0	5	20	***
	(Synthetic medium.)	0	0	0	20	20	
l	(Sugarcane medium frass from:)						
	(*S. calamistis*)	1	0	0	19	20	***
	(*E. saccharina*)	16	2	1	2	21	
	(Control/ distilled. water)	0	0	0	20	20	

Letters **a-l** in the first column are referred to by superscripts [a-l] in the discussion.

Levels of significance are represented by asterisks:

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

NS = Not significant ($P > 0.05$)

2.4.2. Petri dish test 2.

This test was used to determine more quantitatively if there is i) a difference in response by mated females to male and female frass; ii) a preference for male or female frass; iii) a substantial difference in the response to *E. saccharina* frass by virgin females two to four days old relative to mated females of the same age.

The raw data of the results, and statistical analyses are given in the Appendix (Table E.). The results of the response of mated females (two to four days old) to male and female *E. saccharina* sugarcane frass are presented in Table 2.3. and Table 2.4. respectively. The average number of visits and average time of each visit for five bioassays are presented. There was no significant difference in the average number of visits ($P > 0.05$) and average time of each visit ($P > 0.05$) by mated females to sugarcane frass from either male or female *E. saccharina*.

Table 2.3. Response of mated two to four day old *G. natalensis* females to male produced *E. saccharina* NCo376 sugarcane frass.

Mean number of visits	7.8
Average time of each visit (s)	145.1

Table 2.4.: Response of mated two to four day old *G. natalensis* females to female produced *E. saccharina* NCo376 sugarcane frass.

Mean number of visits	7.4
Average time of each visit (s)	143.3

Table 2.5. presents the results of the response of mated females (two to four days old) to sugarcane frass from both male and female *E. saccharina* in the same petri dish (results of four bioassays). There was no significant difference in the number of visits ($P > 0.05$) and time of each visit ($P > 0.05$) by the *G. natalensis* females to sugarcane frass (either male or female) in this test. Thus there was no preference given a choice of frass from male or female *E. saccharina*.

Table 2.5. Results of the response of mated two to four day old *G .natalensis* females to male and female *E. saccharina* NCo376 sugarcane frass in the same petri dish.

	Male *E. saccharina* NCo376 sugarcane frass	Female *E. saccharina* NCo376 sugarcane frass
Mean number of visits	4.75	5.50
Average time of visits (s)	147.1	143.9

Table 2.6. presents the results of the response of virgin females (two to four days old) to sugarcane frass (results of five bioassays). The virgin females showed significantly less visits to the sugarcane frass relative to the number of visits by mated females to frass from male ($P < 0.001$) or female ($P < 0.001$) *E. saccharina* larvae; the average time of each visit by the virgin females was also significantly lower than the mated females average visiting time to male ($P < 0.001$) or female ($P < 0.001$) *E. saccharina* frass (Table 2.6. and Tables 2.3. and 2.4. respectively).

Table 2.6. Response of virgin two to four day old *G. natalensis* females to *E. saccharina* NCo376 sugarcane frass.

Mean number of visits	1.8
Average time of each visit (s)	24

2.5.3. Hypothesis 2.

Because the characteristic response of the females to *E. saccharina* frass in the petri dish tests indicated they detected the frass at a distance, (i.e. responded by olfaction before contacting the spot or frass), it was hypothesised that the short range kairomone was volatile and played a role in host location at a distance from the source.

2.6.4. Olfactometer test.

Many hymenopteran parasitoids use olfactory cues to locate their hosts (Vinson, 1981; Weseloh, 1981). Odour may provide a directional stimulus (Kennedy, 1977) enabling a parasitoid to make oriented movements to its source (Shorey, 1977). The possibility of the short range kairomone being volatile and causing a behavioural change at a distance was investigated using the olfactometer.

Table 2.7. presents the number of first choices and final choices made in the case of four clean airstreams (Test 1), and in the case of three clean airstreams and one sugarcane frass odour stream (Test 2). With four clean airstreams the number of first and final choices were not significantly different from what would have been expected if the field had been randomly chosen (P > 0.05), thus there was little bias for a particular field in the olfactometer and factors that may have caused bias had been satisfactorily standardised. In Test 2 the number of first and final choices for the sugarcane frass odour field was significantly greater than what would have been expected if fields had been randomly chosen (P < 0.05).

Table 2.7. The response of mated two to four day old *G. natalensis* **females to four** clean airfields in Test 1, and three clean airfields and an *E. saccharina* **sugarcane** frass odour airfield in Test 2.

TEST 1	CLEAN	CLEAN	CLEAN	CLEAN	N	Significance
First choice	10	9	10	7	36	NS
Final choice	11	8	9	8	36	NS
TEST 2	CLEAN	CLEAN	CLEAN	SUGARCANE	N	Significance
				FRASS		
First choice	1	2	5	16	24	*
Final choice	4	1	3	16	24	*

Levels of significance are represented by asterisks:

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

NS = Not significant ($P > 0.05$).

Figs 2.6.a.-p. are tracings of the movement of the parasitoids in the exposure chamber in Test 2. The field in the diagrams with the star next to it is the field with the sugarcane frass odour; the other three fields have clean air. Because the environment in the exposure chamber was artificial, no attempt was made to analyse the mechanism by which the females orientate to the odour by the behavioural searching patterns that were observed (e.g. anemotaxis or chemotaxis), nor have the results been extrapolated to what type of behaviour may be expected in the field . The diagrams serve only to show the patterns of movement. With reference to these patterns it is evident that within the host odour field (i.e. *E. saccharina* sugarcane frass), the turning rate was often much higher, in some cases the parasitoid moved into the arm. Due to the constraints of the size of the arena, and the possibility that a constrained searching pattern was observed, the turns and walking rates were not analysed. The turning response to chemicals emanating from *E. saccharina* frass was also observed in the petri dish test 1 bioassays when females neared the frass spot, at which stage they started turning and antennating on the substrate (but in a more uniform upwards direction due to the dish being vertical).

Fig. 2.6.a.-d. Tracings of the movement of individual two to four day old mated *G. natalensis* females in the four way olfactometer exposure chamber. The field with a star next to it is the sugarcane frass odour field, the other three are clean airfields.
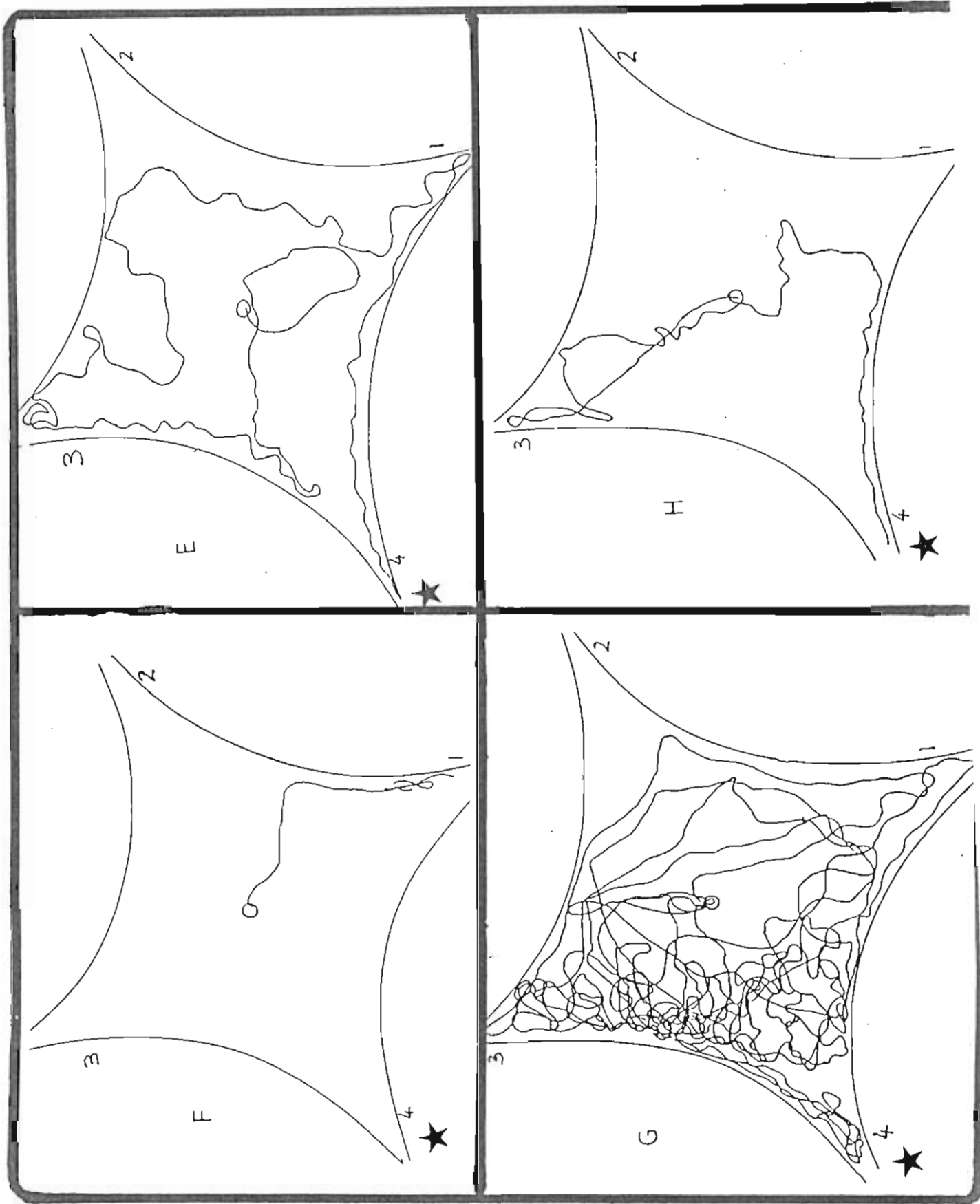
Fig. 2.6.e.-h. Tracings of the movement of individual two to four day old mated *G. natalensis* females in the four way olfactometer exposure chamber. The field with a star next to it is the sugarcane frass odour field, the other three are clean airfields.
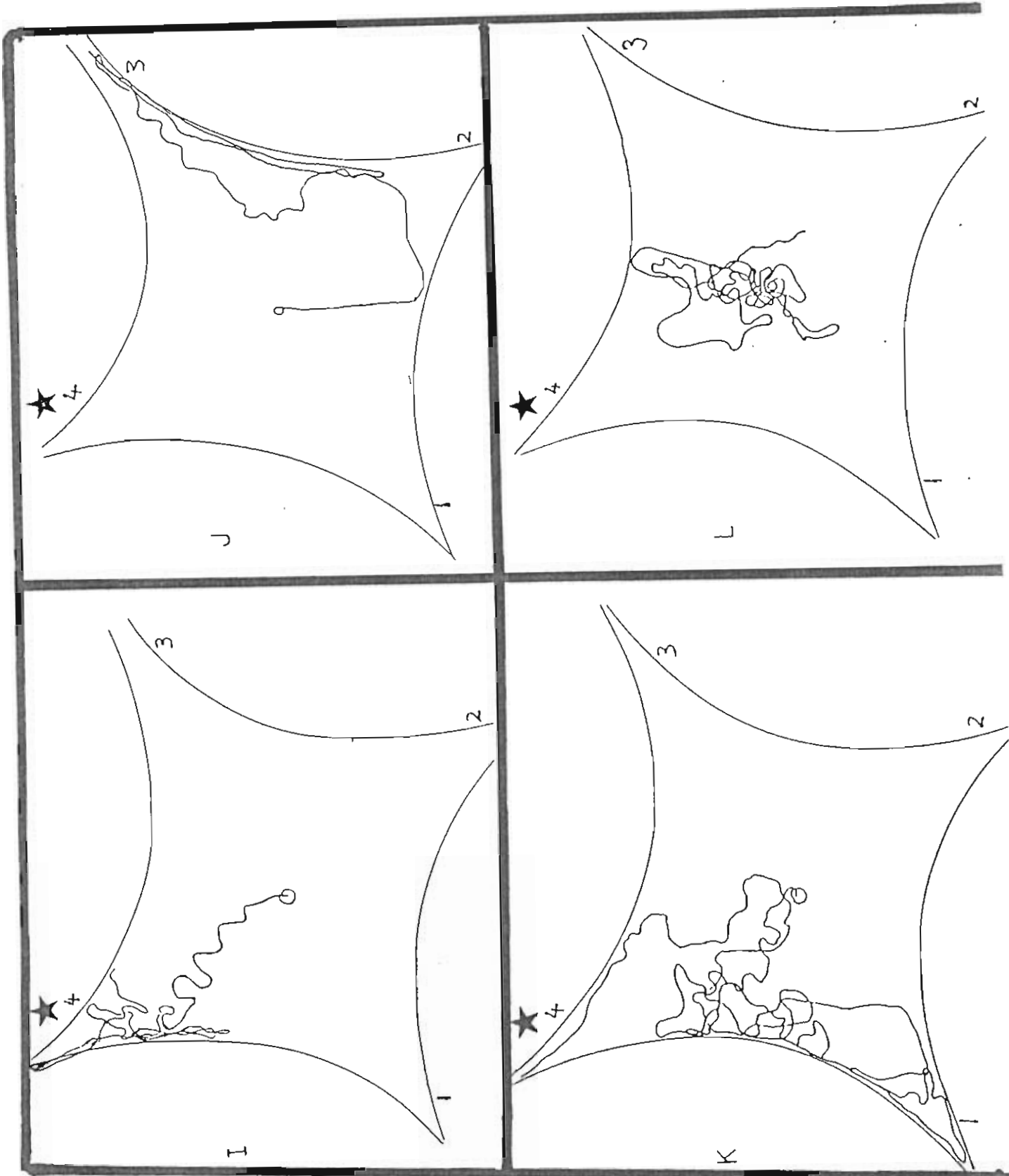
Fig. 2.6.i.-l. Tracings of the movement of individual two to four day old mated *G. natalensis* females in the four way olfactometer exposure chamber. The field with a star next to it is the sugarcane frass odour field, the other three
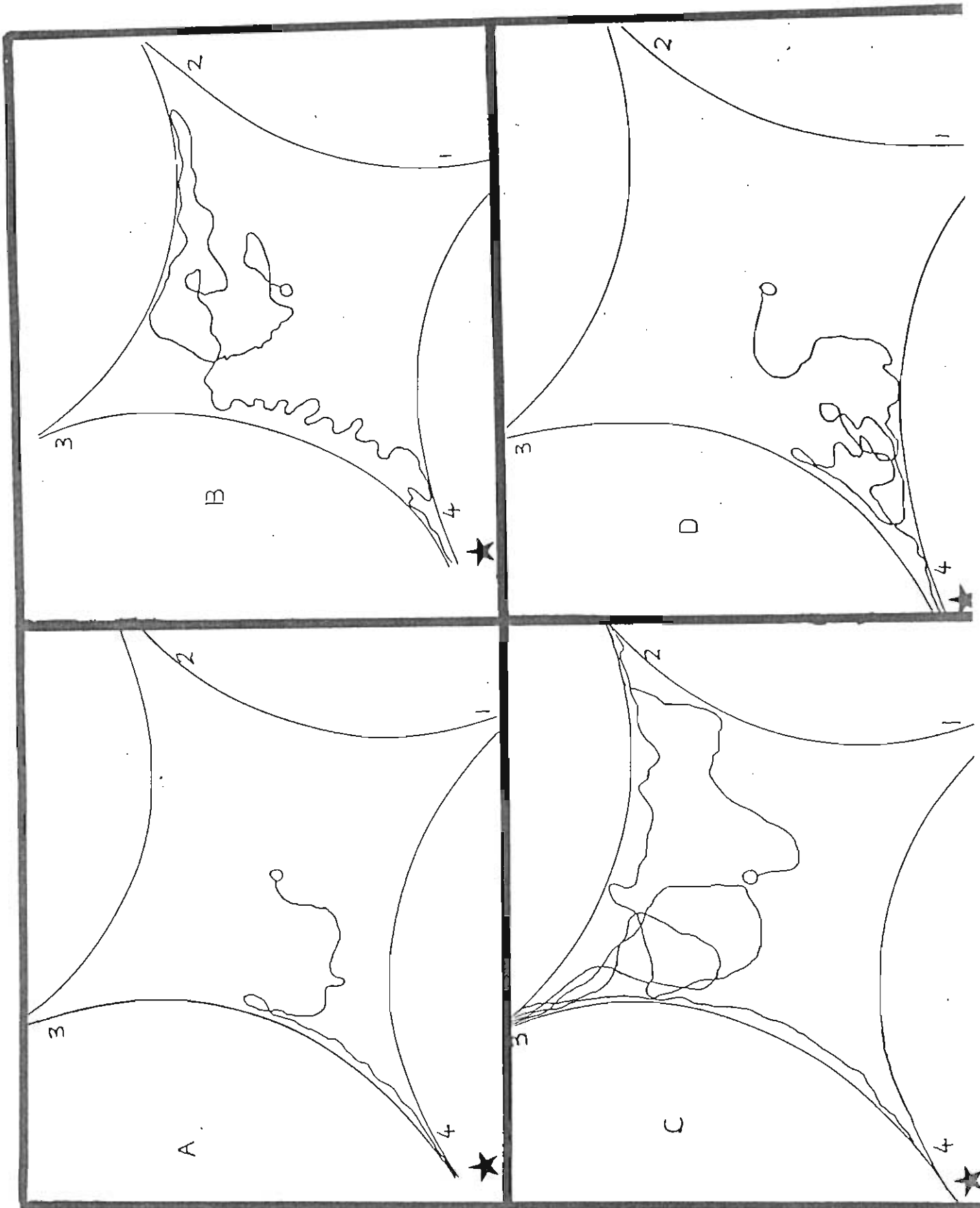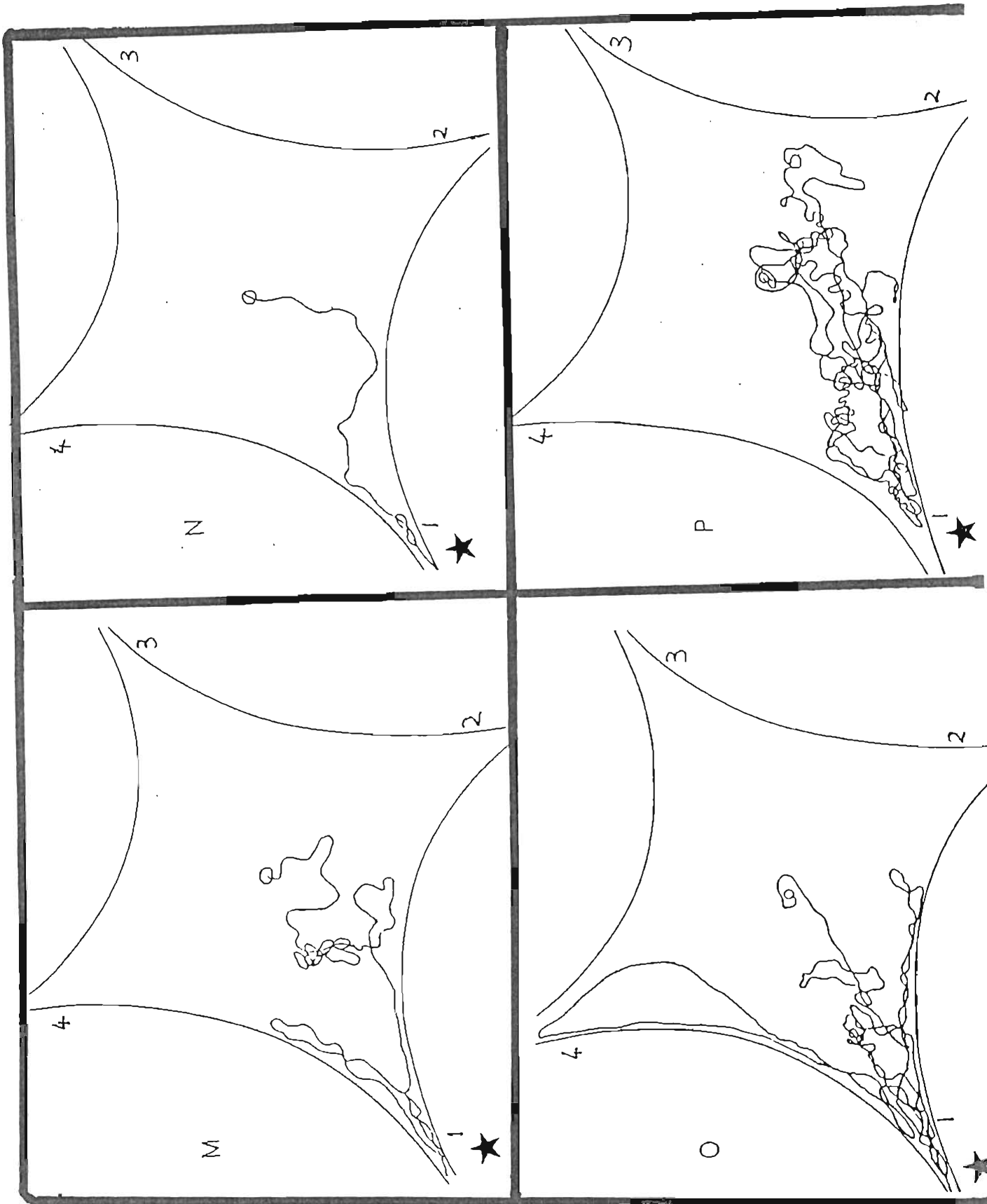
Fig. 2.6.m.-p. Tracings of the movement of individual two to four day old mated *G. natalensis* females in the four way olfactometer exposure chamber. The field with a star next to it is the sugarcane frass odour field, the other three are clear air fields.

The females in the olfactometer were often observed antennating on the exposure chamber substratum whilst walking and turning in the sugarcane frass odour field. These turning movements to chemicals emanating from frass would obviously increase the chance of a female contacting the host frass. However, this would only be advantageous close to the frass.

The observed host-searching responses in the olfactometer were assumed to be due to a short-range kairomone in the sugarcane frass. It was believed that long-range chemoreception (if any) would require additional investigation in a wind tunnel. Whatever the mechanism of orientation to the odour was, it was evident that in the sugarcane frass odour field a searching pattern was often elicited, and thus the hypothesis 2 (see section 2.5.3.), that the short-range kairomone in *E. saccharina* frass which elicited host-searching by *G. natalensis* females in the petri dish tests was volatile, and involved in host location at a distance from the source, was accepted.

2.6. CONCLUSIONS

Petri dish tests demonstrated that *G. natalensis* mated females would exhibit a searching response when exposed to chemicals of *E. saccharina* frass from sugarcane, papyrus umbels and four media. The sexual state and age of the females were found to be important for the elicitation of host-searching, as mated females would had to have completed their preoviposition time of two to three days, whilst virgin females needed even longer, even though their preoviposition time was the same.

No statistically significant difference in the response by *G. natalensis* females to male or female produced *E. saccharina* sugarcane frass was found, and there was no statistically significant preference for either frass type, given the choice. Olfactometer tests showed that a sugarcane frass odour elicited a host-searching behaviour in mated *G. natalensis* females, thus the kairomone elicited a searching behaviour at a distance from the frass.

It was evident from both the petri and olfactometer tests that there was a short range kairomone in *E. saccharina* frass which caused host-searching by *G. natalensis* females. This short range kairomone was present in *E. saccharina* frass from different plants and media, but not *S. calamistis* sugarcane medium frass. These results suggested that the kairomone was a product from the *E. saccharina* larvae. The next step was to identify the kairomone as its volatile nature made it potentially useful for manipulating *G. natalensis* in the field, as suggested by Lewis and Nordlund (1985).

CHAPTER THREE

ISOLATION OF THE KAIROMONE PRESENT IN *E. SACCHARINA* SUGARCANE FRASS.

3.1 INTRODUCTION

There are numerous techniques that may be employed to separate chemicals. When trying to isolate semiochemicals such as pheromones and kairomones it is important to realise that signals must be recognizable from the background noise of chemicals in the natural environment. Therefore they consist of either an unusual chemical, or more often a group of relatively common species-specific chemicals in an unusual combination (Francke, 1988).

If the latter is the case then very effective separation techniques may not isolate the group of required chemicals. Therefore solvents are often used for isolating the chemical or chemicals of interest, and even here the problem may arise, and a combination of solvents may be required.

Abdel-Kareim and Kozár (1988) found that in the case of scale insects the most commonly used solvents for isolating the chemical(s) of sex pheromones were diethyl ether, pentane and ethanol, yet chloroform proved to be the best solvent in the case of the scale insect they were studying.

This illustrates that there are no hard and fast rules as to which solvent should be used when trying to isolate a particular semiochemical. For this reason it was decided to try a spectrum of solvents from polar solvents through to non polar solvents.

3.2. MATERIALS

3.2.1. Solvents

The solvents used were: distilled water, acetone, methanol, ethanol, chloroform and hexane (all were HPLC grade).

3.2.2. Frass

The types of *E. saccharina* frass used were field sugarcane frass and synthetic medium frass (see Chapter 2, section 2.3. for more detail).

3.2.3. Insects

Laboratory mated two to four day old *G. natalensis* females were used for the bioassays (see Chapter 2, section 2.3 for more detail).

3.3. METHODS

Before being used all glassware was washed with hot water and extrapol, followed by distilled water and then acetone. The glassware was dried overnight in an oven at 80°C.

3.3.1. Extraction method

All glassware used was first cleaned with the solvent being used. Three grams of frass was mixed with 3 ml of solvent in a 25 ml beaker, the mixture was sonicated for 5 min in a Bransonic 52® (50/60 Hz) sonicator, placed into 25 ml glass centrifuge tubes and centrifuged at 3450 rpm in a benchtop centrifuge (Minor®) for 5 min. The supernatant was removed and stored at -4°C.

3.3.2. Petri dish test 1

The solvent extracts of field sugarcane frass, and the chloroform extract of synthetic medium frass were bioassayed by this method for kairomonal activity. 12 μl of the extract was placed onto the centre of Whatman® no. 1 filter paper, which was then used in the bioassay (see Chapter 2, section 2.3. for details of the bioassay method.). The solvent was evaporated down using nitrogen. The controls were 12 μl of the respective solvent, which were treated in the same manner.

3.3.3. Olfactometer test

The chloroform and methanol extracts of field sugarcane frass were bioassayed by this method for kairomonal activity. Two ml of the respective solvent extract was placed onto Whatman® no. 3 filter paper (9 cm dia.) and evaporated down with nitrogen in

a fume cupboard. The resulting filter paper and residue were used in the sample jar for the respective extract odour (see Chapter 2, section 2.4 for the method of bioassay). The controls were 2 ml of the respective solvent treated in the same manner.

3.4. RESULTS AND DISCUSSION

3.4.1. Petri dish test 1

The results of the response of mated 2 to 4 day old laboratory reared *G. natalensis* females to the odours of the distilled water, ethanol, methanol, acetone, chloroform and hexane extracts of field sugarcane frass and the respective solvent(s) as a control, are presented in Table 3.1. It is evident that only the chloroform and methanol extracts elicited a significantly stronger response than the controls (P = < 0.001 in both cases). The positive responses to the methanol extract, although lasting longer than five seconds, were less intense than the responses to the chloroform extract. Only the chloroform extract elicited boring into the spot and probing with the ovipositor. Thus chloroform appeared to be the only solvent tested which successfully isolated the short-range kairomone from the sugarcane frass.

The number of females that responded to the chloroform extract of *E. saccharina* synthetic medium frass were fourteen on the first pass, two on the second pass, none on the third pass, and four females did not respond after three passes. The chloroform extract of the synthetic medium frass from *E. saccharina* also elicited boring into the spot, and ovipositor probing. These results supported the idea that the kairomone was a product of the *E. saccharina* metabolism and not the plant's metabolism.

TABLE 3.1. Results of the response of mated two to four day old *G. natalensis* females to different solvent extracts of field sugarcane frass and the solvents (controls) in the petri dish test 1 bioassay.

Solvent used for extract (Laboratory *G. natalensis* [2-4 Days old])	Number of females with first response on pass:			Number of females with no response after three passes	Total number tested	Signifi--cance
	1	2	3			
Distilled water extract	0	0	0	20	20	NS
Distilled water (control)	0	0	0	20	20	
Acetone extract	1	0	0	19	20	NS
Acetone (control)	0	0	0	20	20	
Ethanol extract	2	0	0	18	20	NS
Ethanol (control)	0	0	0	20	20	
Methanol extract	7	10	3	0	20	***
Methanol (control)	1	2	0	17	20	
Chloroform extract	15	3	2	0	20	***
Chloroform (control)	2	0	0	18	20	
Hexane extract	2	3	0	15	20	NS
Hexane (control)	1	0	0	20	20	

Levels of significance are represented by asterisks:

* $P \leq 0.05$; ** $P \leq 0,01$; *** $P \leq 0.001$

NS = Not significant ($P > 0.05$).

3.4.2. Olfactometer test

The results of the response of mated two to four day old *G. natalensis* females in the olfactometer to the chloroform extract and methanol extract of field sugarcane frass as well as the respective solvents as controls are presented in Table 3.2. For both the controls no searching patterns were observed in the odour field of the respective solvent, and in both cases the number of first and final choices were not significantly different from what would have been expected if the field had been randomly chosen.

Therefore the solvents were not responsible for any positive results observed in the extracts. The methanol extract of sugarcane frass did not elicit searching patterns, and the number of first and final choices for this field were not significantly different to what would have been expected if fields had been randomly chosen (P > 0.05). On the other hand, the chloroform extract of sugarcane frass elicited a similar response to that caused by sugarcane frass odour (some of the more impressive searching patterns are illustrated in Fig 3.1.a.-d. Besides the searching behaviour the number of final choices for the chloroform extract odour field was also significantly greater than what would have been expected had the fields been randomly chosen (P = < 0.05).

Table 3.2. The response of mated two to four day old *G. natalensis* females to the odour of the chloroform extract of sugarcane frass in the olfactometer in relation to clean odour fields.

Test 1a	Clean	Clean	Clean	MeOH extract	N	Significance
First choice	6	9	8	9	32	NS
Final choice	10	8	6	8	32	NS

Test 1b	Clean	Clean	Clean	MeOH (control)	N	Significance
First choice	11	7	8	6	32	NS
Final choice	11	8	6	7	32	NS

Test 2a	Clean	Clean	Clean	CHCl3 extract	N	Significance
First choice	6	0	2	8	16	NS
Final choice	1	0	3	12	16	*

Test 2b	Clean	Clean	Clean	CHCl3 (control)	N	P
First choice	6	3	4	3	16	NS
Final choice	4	5	4	3	16	NS

The levels of significance are represented by asterisks:

* $P \leq 0.05$; ** $\leq P\ 0.01$; *** $P \leq 0.001$

NS = not significant (P0.05).
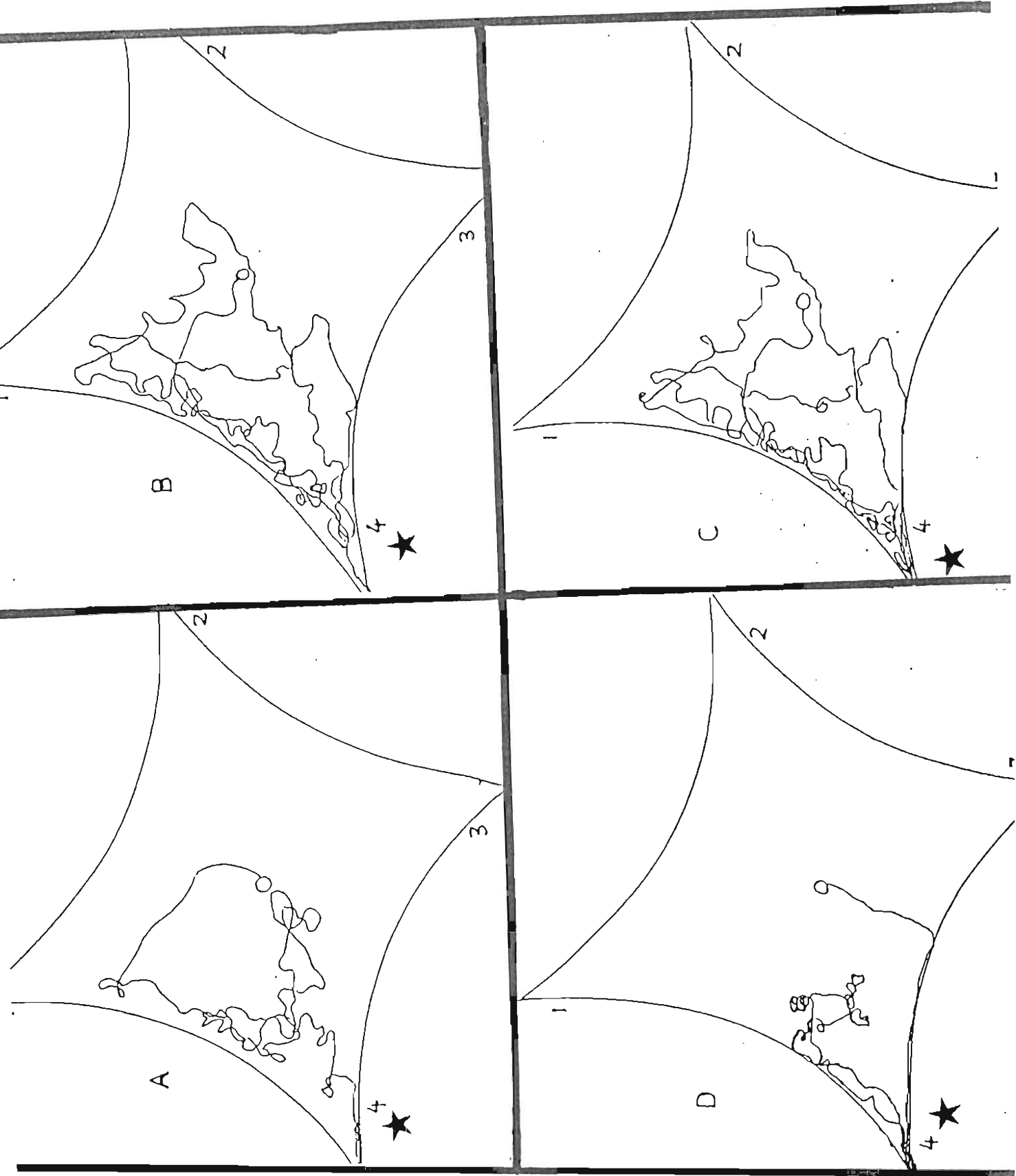
Fig. 3.1.a.-d. Tracings of the movement of individual two to four day old mated *G. natalensis* females in the four way olfactometer exposure chamber. The field with a star next to it is the sugarcane frass chloroform extract odour field, the other three are clean airfields.

3.5. CONCLUSIONS

Both petri dish and olfactometer tests showed that chloroform was the only solvent tested which successfully isolated the kairomone from *E. saccharina* frass. The chloroform extract of sugarcane frass elicited positive responses in the bioassays which were comparable to the responses observed to *E. saccharina* sugarcane frass and the other various types of *E. saccharina* frass that were bioassayed. It was for this reason that chloroform was chosen as the solvent to isolate the kairomone for identification. The chloroform extract of *E. saccharina* synthetic medium frass also elicited the host searching response in the petri dish bioassay. This suggested that the kairomonal chemical(s) did not arise from the plant, and the possibility that the kairomone was produced by the *E. saccharina* larvae seemed plausible. This was kept in mind during the identification of the chemicals in the chloroform extract.

CHAPTER 4

IDENTIFICATION OF THE CHEMICALS IN THE CHLOROFORM EXTRACT OF *E. SACCHARINA* FRASS.

4.1. INTRODUCTION

GC/MS combines high selectivity on the separation side and high sensitivity on the detector side (pico- to nanogram region). Although the data obtained for identifying chemicals is not as good as that from NMR, accurate identifications are possible using the data from standard compounds (Francke, 1988). GC/MS was used to analyse the chloroform extract of *E. saccharina* sugarcane frass. The Wiley library of standards made preliminary identifications possible.

4.2. METHODS

All glassware used was washed with hot water and extrapol, followed by distilled water, acetone and finally chloroform. The glassware was dried at 200°C overnight.

4.2.1. Sample preparation

Two grams of sugarcane field frass was weighed out into a 10 ml beaker. Four millilitres of HPLC grade chloroform were added. The mixture was sonicated for 5 min in a Bransonic 52® (50/60 Hz) sonicator. The mixture was placed into a 25 ml glass centrifuge tube and centrifuged at 3450 rpm in a benchtop centrifuge (Minor®) for 5 min. The supernatant was decanted into a 10 ml beaker. The chloroform of the supernatant was evaporated down to 2 ml in a fume cupboard using nitrogen (GC grade), this was then decanted into a 2 ml glass vial. The vial was sealed, packed in dry ice and taken to CSIR (Pretoria) for analysis.

4.2.2. GC/MS analysis

(Acknowledgements to Dr. A. Scheffer for analysis). The sample was analysed eight days after preparation by capillary gas chromatography-mass spectrometry on a 25 m x 0.2mm HP-5 capillary column installed in an HP 5890 GC coupled to a HP 5970 MSD. PBM (probability-based match) library searches were conducted on the Wiley library of mass spectra.

4.3. RESULTS AND DISCUSSION

The initial analysis of the chloroform extract of sugarcane frass showed that the sample was too dilute for identification of the chemicals present. Thus the sample was concentrated a further one hundred fold.

The gas chromatogram from the analysis of the concentrated sample is given in Fig. 4.1. Fig. 4.2. gives an exploded view of the chromatogram to show more clearly the first peak after the solvent front. Although numerous peaks are present the concentration of most of the chemicals were still too low and thus only the larger peaks were identified by PBM. These results are thus regarded as preliminary. Figs. 4.3.a-l. give the mass spectra of the larger peaks and their relevant retention times. The upper scan is the mass spectrum for the relevant GC peak, and the lower scan is the Wiley standard mass spectrum for the closest matched compound in the library, which is highlighted in the PBM results. Because of the low concentrations in some cases the isomeric form was not determined, and the match obtained to the Wiley library was poor (below 90%). The isomeric form especially e.g. the position of methyl isomers have been shown to be important factors for the kairomonal activity of chemical(s), and hence accurate identification is necessary. Unfortunately towards the end of the year the laboratory culture of *G. natalensis* was supressed by the mite *Tyrophagus putrescentiae* (Schrank). The cause of the infestation was probably due to an overcrowded contaminated medium. These mites are known to be attracted by secondary metabolites of yeast and fungi, both of which were contaminants of the diet used to rear *E. saccharina* and then the *G. natalensis*. The abundance of dead *E. saccharina* matter in the *G. natalensis* rearing trays, which was a consequence of the rearing method, provided an additional food source for the mites. Various methods were tried to control the mites including the use of menthol, double sided tape on benches and increased cleanliness. None of the methods tried provided satisfactory control, and the culture dwindled (Conlong and Gillespie, pers. comm.). For this reason the chemicals that have been identified were not bioassayed for kairomonal activity.
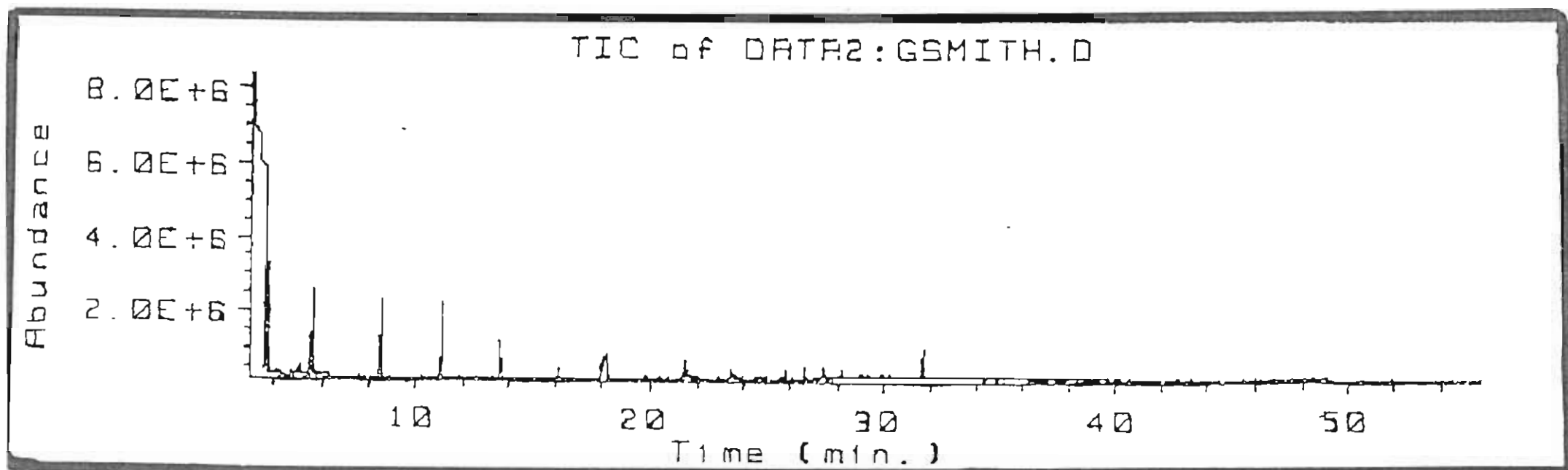
Fig. 4.1. Gas chromatogram of the chloroform extract of *E. saccharina* (Walker) sugarcane frass (25 m x 0.2mm (glass) ; HP-5; injection: 1μl (splitless); carrier gas (He) linear velocity 40 cm.s⁻¹; 30°C ___5°C/min___ 300°C).
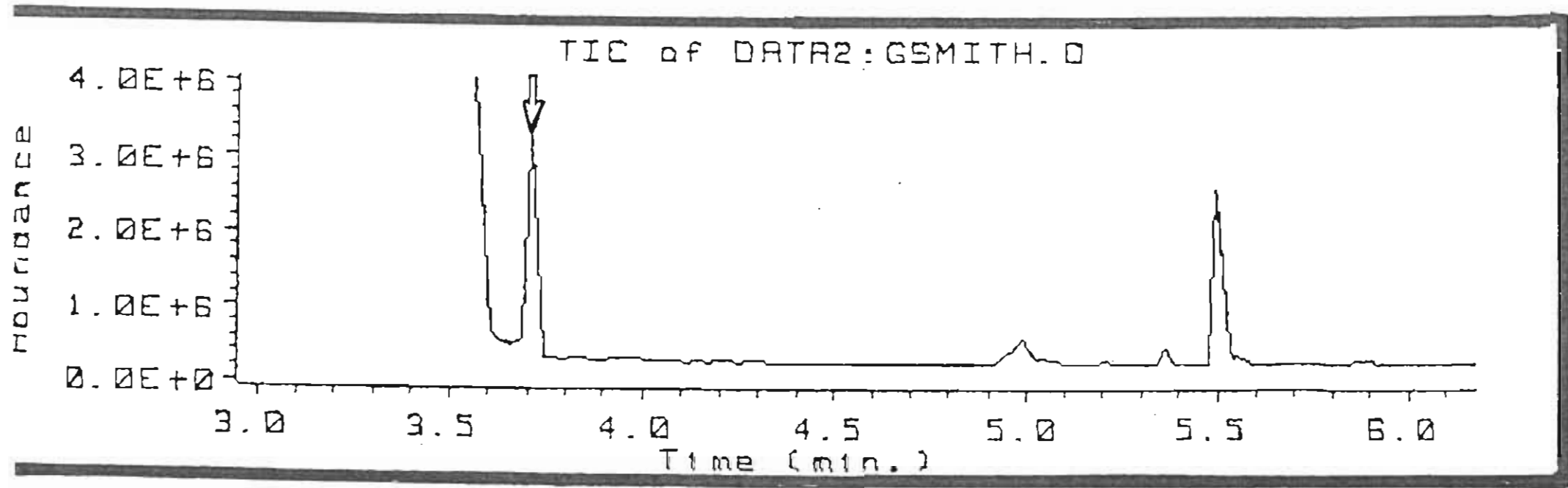
Fig. 4.2. Exploded view of the gas chromatogram (see Fig. 4.1.) illustrating the first peak after the solvent front (indicated with arrow).
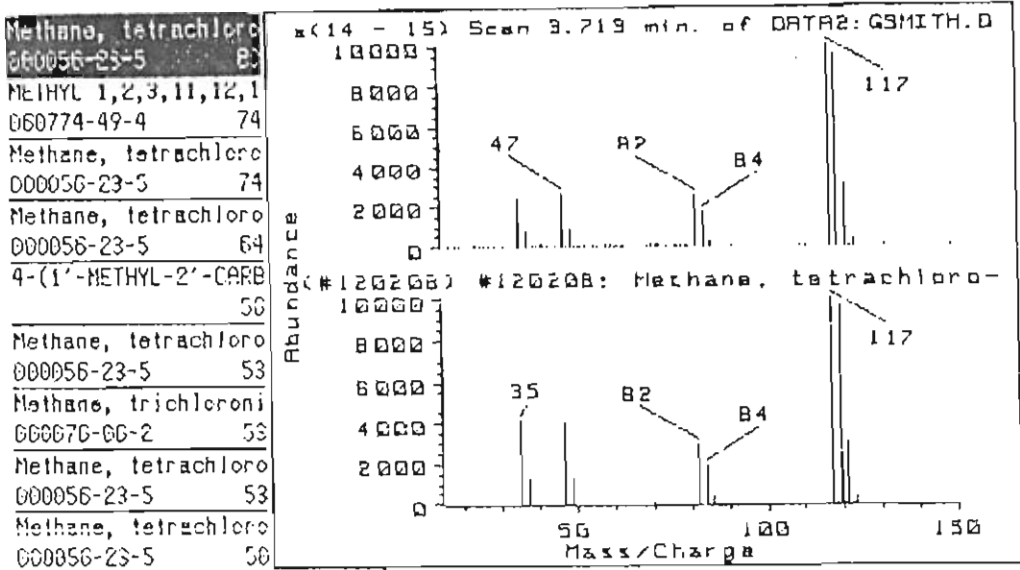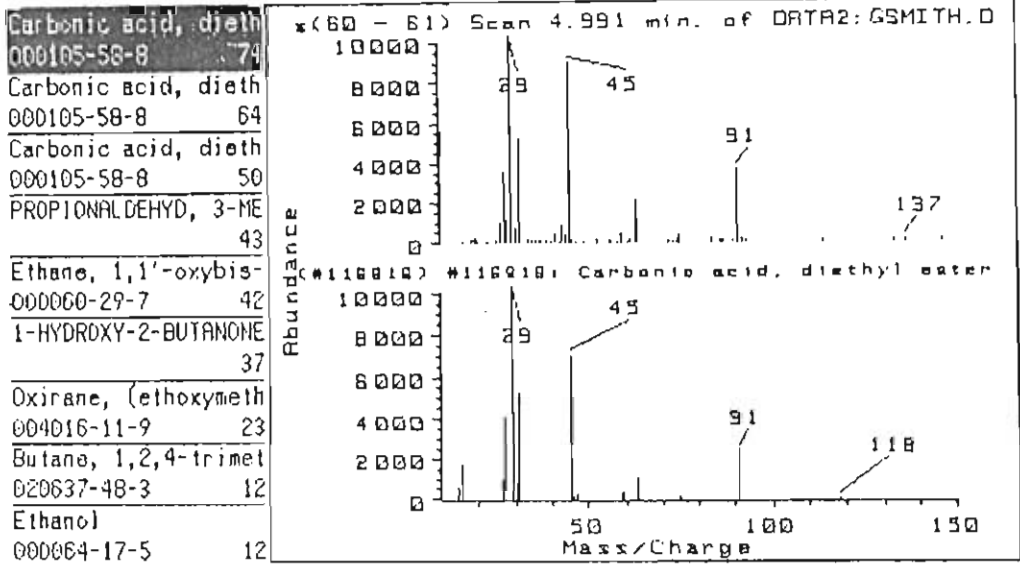
Fig. 4.3.a. Upper scan is the mass spectrum for the peak at 3.719 min (see Fig. 4.1.).
Lower scan is the Wiley standard mass spectrum of carbon tetrachloride.
b. Upper scan is the mass spectrum for the peak at 4.991 min (see Fig. 4.1.)
Lower scan is the Wiley standard mass spectrum of diethyl carbonate
(On the left of the figures the best probability base match (PBM) results of the upper
scan, with the Wiley library standards are given. The mass spectrum of the
highlighted standard is given in the the lower scan).

Ethene, tetrachloro- 000127-18-4	9
Ethene, tetrachloro- 000127-18-4	97
Ethene, tetrachloro- 000127-18-4	94
Ethene, tetrachloro- 000127-18-4	81
Ethene, tetrachloro- 000127-18-4	70
Ethene, tetrachloro- 000127-18-4	49
2,5-Furandione, 3,4- 001122-17-4	42
Phosphorodichloridot 002523-94-6	12
3,5-Dichloro-4-methy 076811-87-5	7

x(74 - 75) Scan 5.366 min. of DATA2:GSMITH.Q

#(121365) #121365: Ethene, tetrachloro-

Mass/Charge

c

Cyclotrisiloxane, he 000541-05-9	91
Cyclotrisiloxane, he 000541-05-9	72
Cyclotrisiloxane, he 000541-05-9	64
8-Methylisothiazolol 074801-78-8	52
Arsenous acid, tris(055429-29-3	38
Cyclotrisiloxane, he 000541-05-9	32
1H-Pyrrole, 1,1',1'' 018899-90-6	9
Benzene, 1,2,3-trime 000487-11-6	7

x(79 - 81) Scan 5.505 min. of DATA2:GSMITH.Q

#(44070) #44070: Cyclotrisiloxane, hexamethyl-

Mass/Charge

d

Fig. 4.3.c. Upper scan is the mass spectrum for the peak at 5.366 min (see Fig. 4.1.).
Lower scan is the Wiley standard mass spectrum of tetrachloroethene.

d. Upper scan is the mass spectrum for the peak at 5.509 min (see Fig. 4.1.)
Lower scan is the Wiley standard mass spectrum of cyclotrisiloxane (silicone oil).
(On the left of the figures the best probability base match (PBM) results of the upper scan, with the Wiley library standards are given. The mass spectrum of the highlighted standard is given in the the lower scan).
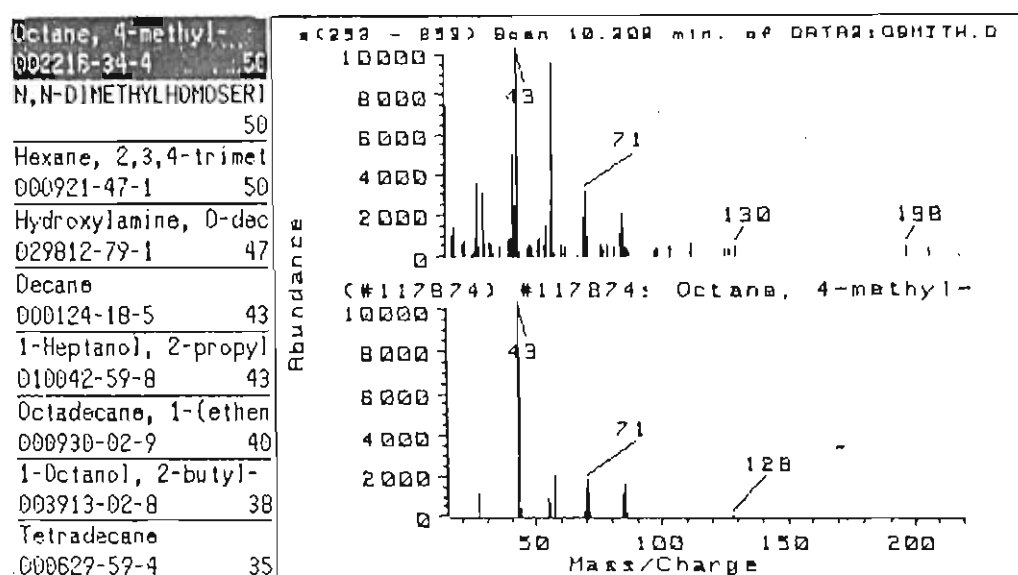
Fig. 4.3.e. Upper scan is the mass spectrum for the peak at 6.324 min (see Fig. 4.1.). Lower scan is the Wiley standard mass spectrum of xylene.

f. Upper scan is the mass spectrum for the peak at 10.308 min (see Fig. 4.1.) Lower scan is the Wiley standard mass spectrum of 4-methyl-octane.

(On the left of the figures the best probability base match (PBM) results of the upper scan, with the Wiley library standards are given. The mass spectrum of the highlighted standard is given in the the lower scan).
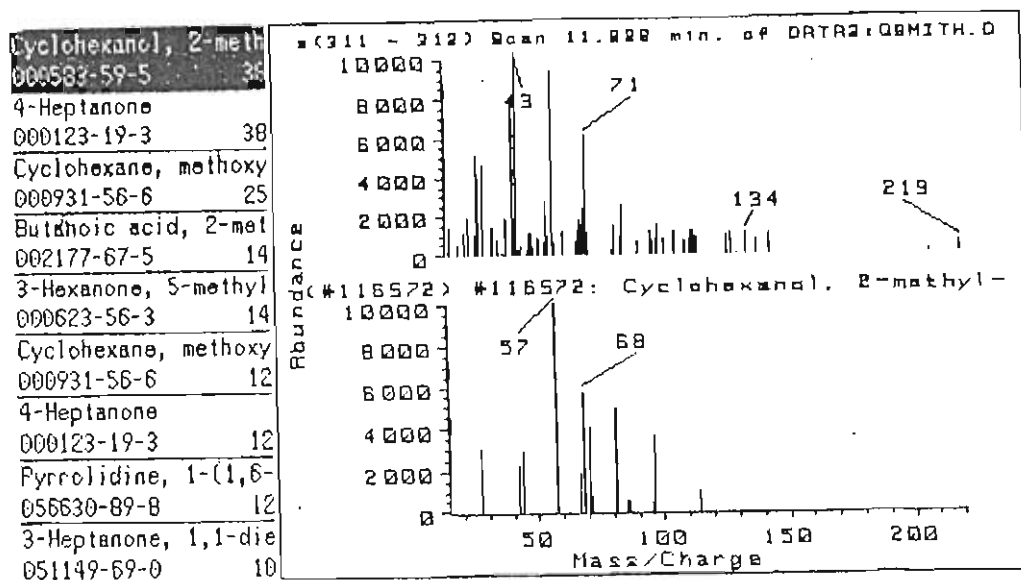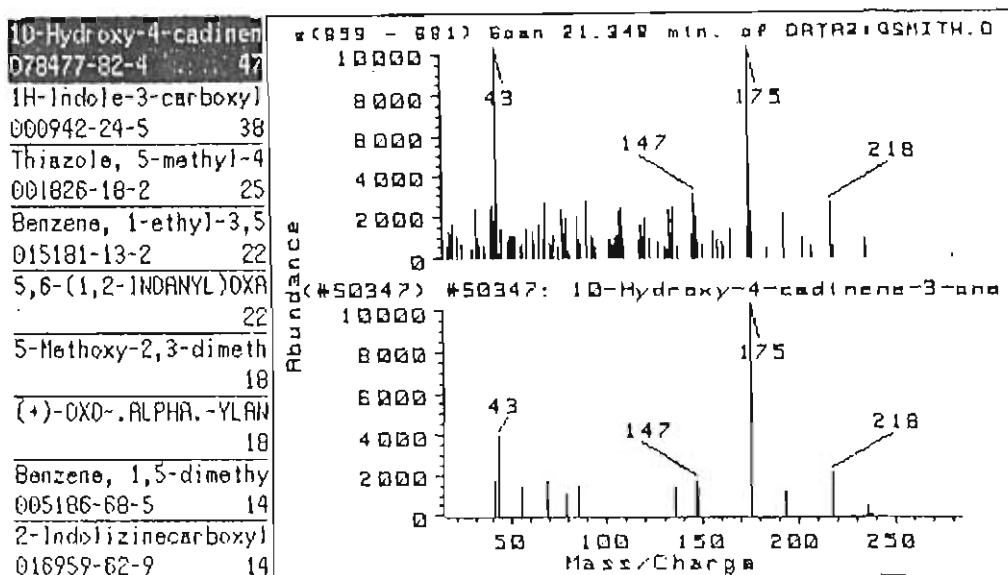
Fig. 4.3.g. Upper scan is the mass spectrum for the peak at 11.826 min (see Fig. 4.1.). Lower scan is the Wiley standard mass spectrum of 2-methylcyclohexanol.

h. Upper scan is the mass spectrum for the peak at 21.346 min (see Fig. 4.1.) Lower scan is the Wiley standard mass spectrum of 10-hydroxy-4-cadinene-3-one. (On the left of the figures the best probabillty base match (PBM) results of the upper scan, with the Wiley library standards are given. The mass spectrum of the highlighted standard is given in the the lower scan).
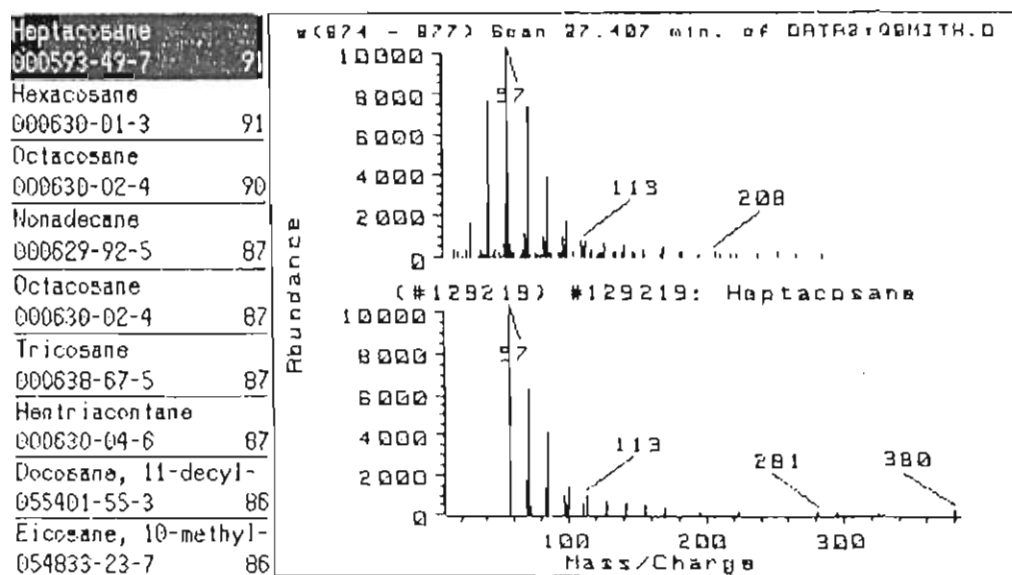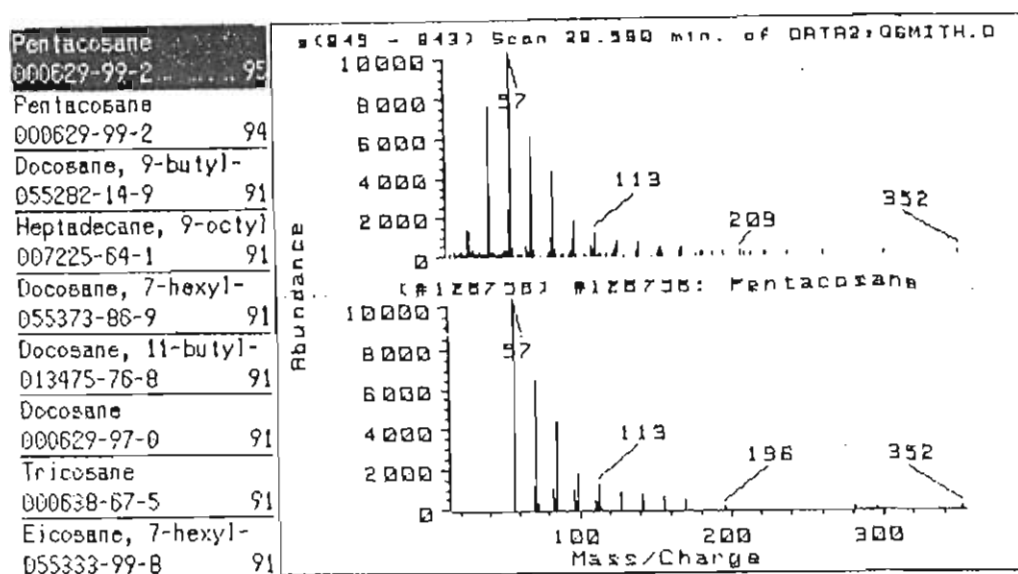
Fig. 4.3.i. Upper scan is the mass spectrum for the peak at 26.596 min (see Fig. 4.1.).
Lower scan is the Wiley standard mass spectrum of pentacosane.

j. Upper scan is the mass spectrum for the peak at 27.407 min (see Fig. 4.1.)
Lower scan is the Wiley standard mass spectrum of heptacosane.

(On the left of the figures the best probability base match (PBM) results of the upper scan, with the Wiley library standards are given. The mass spectrum of the highlighted standard is given in the the lower scan).
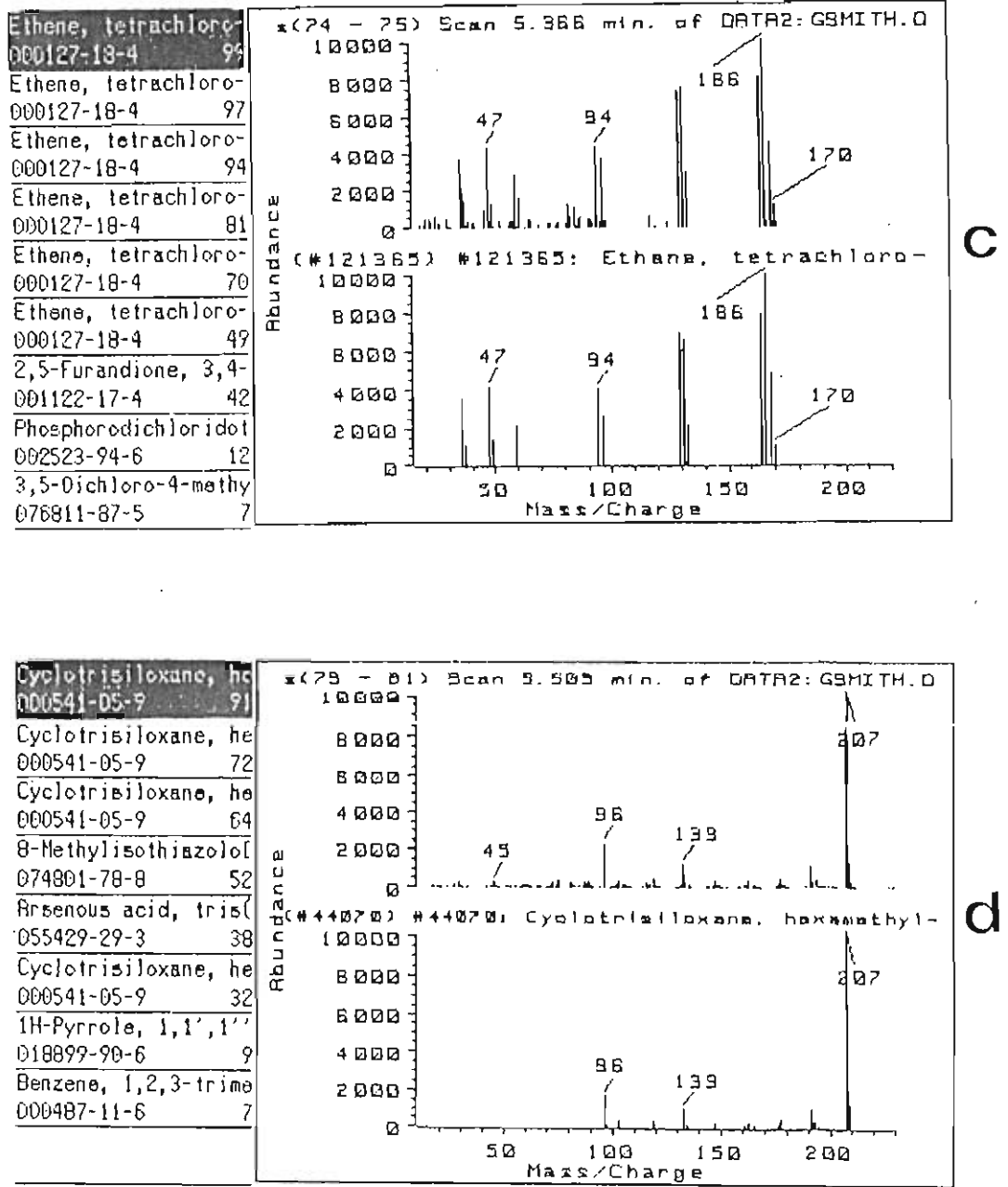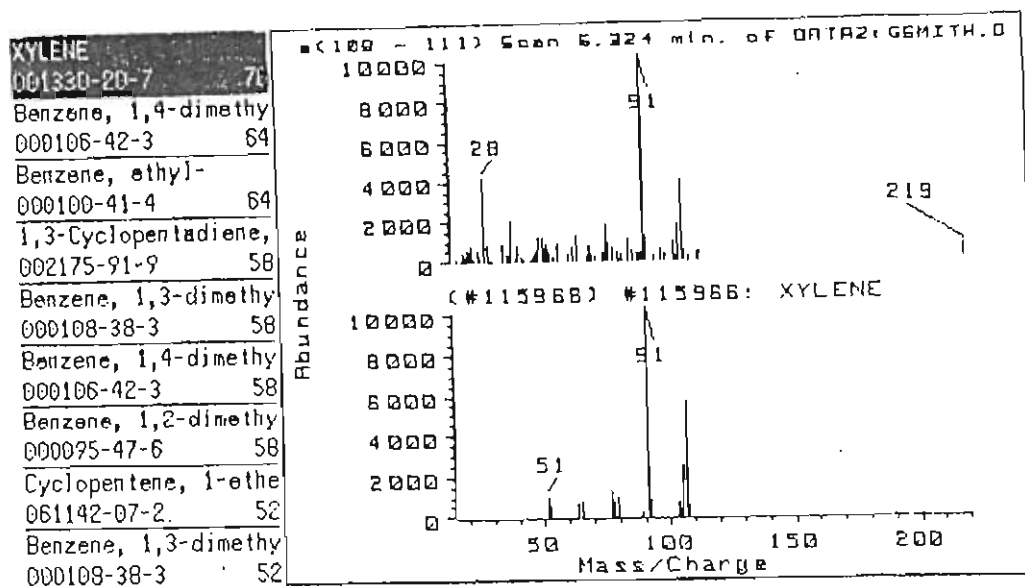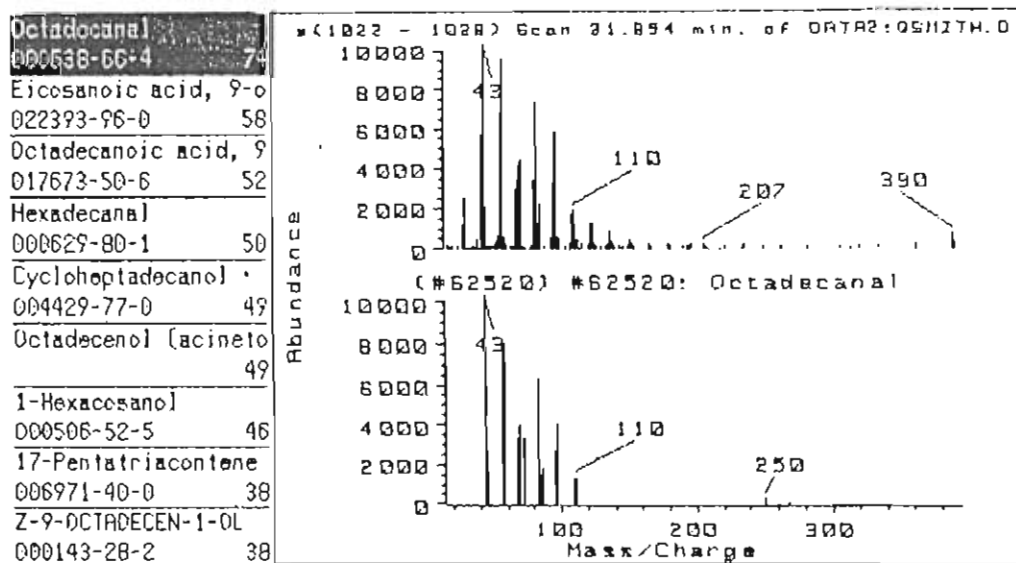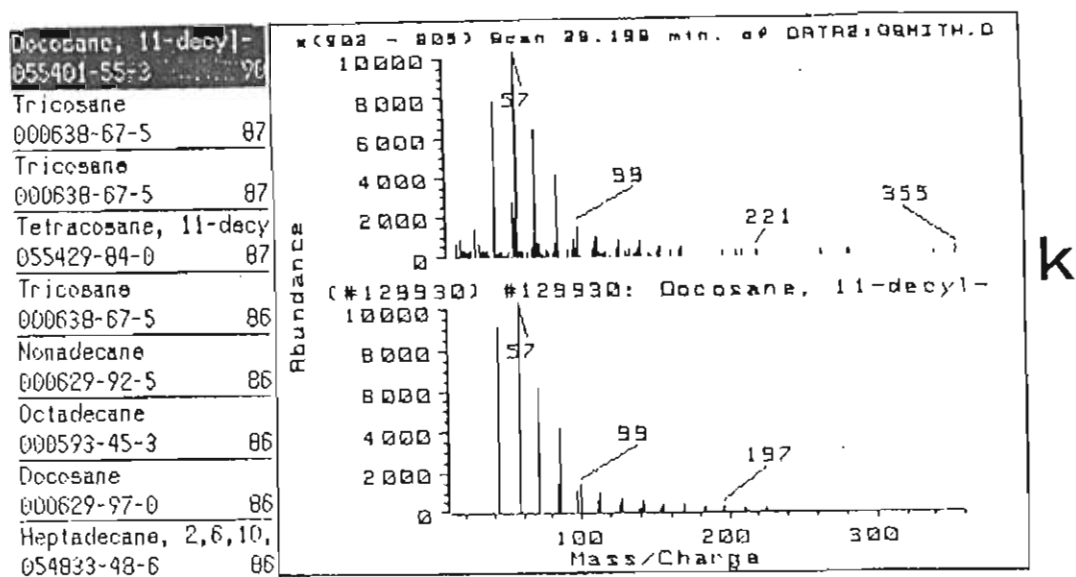
Fig. 4.3.k. Upper scan is the mass spectrum for the peak at 28.196 min (see Fig. 4.1.). Lower scan is the Wiley standard mass spectrum of 11-decyldocosane.

l. Upper scan is the mass spectrum for the peak at 31.654 min (see Fig. 4.1.) Lower scan is the Wiley standard mass spectrum of octadecanal.
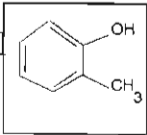
(On the left of the figures the best probability base match (PBM) results of the upper scan, with the Wiley library standards are given. The mass spectrum of the highlighted standard is given in the the lower scan).

These results and discussion serve to highlight the type of chemicals that have been shown to be host searching kairomones, especially those from host frass, and to compare these with these preliminary results. Hopefully the potential for identifying the chemical(s) of the *G. natalensis* host searching kairomone is made apparent. It is hoped that the concentrated chloroform extracts of sugarcane frass, papyrus frass, sugarcane medium frass, synthetic medium frass and *S. calamistis* frass that are presently being analysed will allow the chemical identification of the kairomone.

Table 4.1. gives a preliminary list of the results for chemicals that were identified in the chloroform extract of the *E. saccharina* sugarcane frass along with the PBM results of the comparison of the these compounds with the Wiley library standards. A PBM above 90% can be regarded as a very good match and in all probability the compound is the same as the Wiley library standard and has thus been identified correctly. The lower PBM results indicate that the match of the compound with the library standard was poor, and these identifications were not as reliable, however, this was in large part due to the low concentrations of these compounds and these results therefore give a relatively good idea of the type of chemical.

A homologous series of hydrocarbons was identified in the chloroform extract of *E. saccharina* sugarcane frass namely: 4-methyl octane (very poor match), heptacosane, pentacosane and 11-decyldocosane (possibly tricosane). Octadecanal, 2-methylcyclohexanol (possibly 4-heptanone), and 10-hydroxy-4-cadinene-3-one were also identified. These chemicals are regarded as potential candidates for the kairomone in *E. saccharina* frass. However, the analyses of the more concentrated samples mentioned previously, and subsequent bioassays will hopefully enable identification of the kairomone. It is likely that carbon tetrachloride, silicone oil, tetrachloroethene, and xylene were contaminants. The silicone oil possibly came from vacuum grease used on the nitrogen line. The other chemicals possibly came from contaminated glassware.

Table 4.1. Chemicals identified from the chloroform extract of *E. saccharina* (Walker) sugarcane frass, their relative retention times (see Fig. 4.1.) and PBM (see Figs. 4.3.a-l).
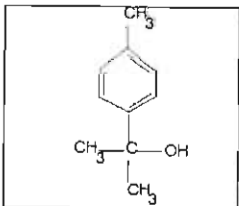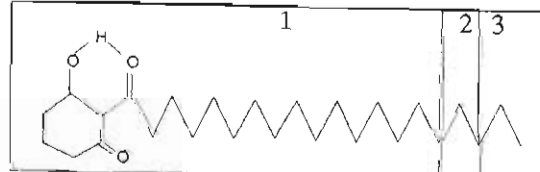
Chemical	Retention time	PBM	(see Fig. 4.3.)
Carbon tetrachloride CCl_4	3.719 min	88%	a
Diethyl carbonate $(C_2H_5O)_2C = O$	4.991 min	74%	b
Tetrachloroethene $Cl_2C = CCl_2$	5.366 min	99%	c
Cylotrisiloxane (silicone oil)	5.509 min	91%	d
Xylene	6.324 min	70%	e
4-methyl octane $CH_3(CH_2)_7CH_3$	10.308 min	50%	f
2-methylcyclohexanol	11.826 min	38%	g
or 4-heptanone $CH_3(CH_2)_2CO(CH_2)_2CH_3$		38%	
10-hydroxy-4-cadinene-3-one	21.346 min	47%	h
Pentacosane $CH_3(CH_2)_{23}CH_3$	26.590 min	95%	i
Heptacosane $CH_3(CH_2)_{25}CH_3$	27.407 min	91%	j
11-decyldocosane or Tricosane $CH_3(CH_2)_9CH(C_{10}H_{21})(CH_2)_{10}CH_3$ $CH_3(CH_2)_{21}CH_3$	28.196 min	90% 87%	k
Octadecanal $CH_3(CH_2)_{16}CHO$	31.654 min	74%	l

The source of behavioural chemicals depends on the nature and level of host selection under study (see Fig. 1.5.). Long range cues important in host location may emanate from the host, its food, shelter, or associated organisms. Short range cues important in host location usually come from the host. Furthermore the same source and chemical may act as pheromone in one context and a kairomone in another (Vinson, 1984a).

Table 4.2. gives a list of parasitoids and respective hosts, and the chemicals that have been found to be host searching kairomones in these cases; the sources of the chemicals are also presented. These substances belong to a broad group of chemicals termed hydrocarbons (containing hydrogen, carbon and oxygen). According to Lewis *et al.* (1976a), the fact that they serve as kairomones is not their "raison d'être". However, except for those that serve as pheromones for the host (sex pheromones, alarm pheromones etc.), the primary function for most of these chemicals is not known. For example Jones (1981) suggested that docosane, tricosane, tetracosane and pentacosane which were present in *Heliothus zea* female moth scales probably came from the host epicuticle, although the possibility that a specialised glandular secretion existed could not be ruled out. Furthermore, if such a secretion occurred, then the chemicals probably function as some type of pheromone for the host.

The saturated hydrocarbons that are part of the waxy layer of the cuticle serve as a water barrier, protecting the insect against desiccation. Their presence in the mandibular gland is not as clear, but they probably lubricate ingested food, and possibly serve as pheromones. In the haemolymph they may have been present as precursors for new cuticle formation. They are probably synthesised in the oenocytes. Their presence in frass was due, in part at least, to mandibular gland excretion and exuvia consumption (Lewis *et al.*, 1976a).

Table 4.2. Chemicals identified as kairomones (S_3 and S_4 stimuli see Fig. 1.5.).

Parasitoid	Host	Chemical	Source	Reference
Macrogaster (*Microplitis*) *croceipes*	*Heliothus zea*	13-methylhentriacontane (see below)	haemolymph, cuticle, frass	Jones *et al.* (1971)
Cardiochiles nigriceps	*Heliothus virescens*	11-methylhentriacontane 16-methyldotriacontane 13-methyltriacontane $CH_3(CH_2)_9CH(CH_3)(CH_2)_{19}CH_3$ $CH_3(CH_2)_{14}CH(CH_3)(CH_2)_{15}CH_3$ $CH_3(CH_2)_{11}CH(CH_3)(CH_2)CH_3$	mandibular gland	Vinson *et al.* (1975)
Orgilus lepidus	*Phthorimaea operculella*	heptanoic acid $CH_3(CH_2)_5COOH$	mandibular gland	Hendry *et al.* (1973)
Trichogramma evanescens	*Heliothus zea*	tricosane $CH_3(CH_2)_{21}CH_3$	haemolymph, adult cuticle	Jones *et al.* (1973)
Trichogramma pretiosum	*Heliothus zea*	hexadecanal (Z)-7-hexadecenal (Z)-9-hexadecenal (Z)-11-hexadecenal $CH_3(CH_2)_{14}CHO$ $CH_3(CH_2)_7CH=CH(CH_2)_5CHO$ $CH_3(CH_2)_5CH=CH(CH_2)_7CHO$ $CH_3(CH_2)_3CH=CH(CH_2)_9CHO$	pheromone gland	Lewis *et al.* (1982)
Vespula maculifrons (Buysson)	*Podisus fretus* and *P. maculiventris* (Say)	(E)-2-hexenal $CH_3CH_2CH_2CH=CHCHO$ α-terpineol linalool $CH_2=CHC(OH)(CH_3)CH_2CH_2CH=C(CH_3)_2$	pheromone gland	Aldrich *et al.* (1986)
Venturia canescens	*Cadra cautella* (Walker) *Plodia interpunctella* (Hübner)	1) 2-palmitoylcyclohexane-1,3-dione 2) 2-stearocyclohexane-1,3-dione 3) 2-arachidcyclohexane-1,3-dione	mandibular gland, frass	Nemoto *et al.* (1987)

In most cases the parasitoid was found to respond to a homologous series of hydrocarbons but one of these chemicals proved to be the most active. A particular methyl-isomer of a particular hydrocarbon often proved to be the most active compound. The chemicals that induced search behaviour in *Trichogramma evanescens* were determined by Jones *et al.* (1973). These parasitoids responded to docasane, tricosane, tetracosane, and pentacosane. Tricosane was found to be the most active component for *T. evanescens*. 11-decyldocosane (possibly tricosane), pentacosane and heptacosane were identified in the *E. saccharina* frass extract (see Table 4.1.).

A series of methyl isomers of hentriacontane namely 7, 9, 11, 13, and 15 methylhentriacontane were present in the frass of *Heliothus zea*. *Microplitis croceipes* responded more strongly to the 13-methyl isomers, and these were also found to be the most abundant in the frass (Jones *et al.*, 1971). In the frass of *H. virescens* a series of mono-methyl hentriacontanes, dotriacontanes, and tritriacontanes were identified. Bioassays for *Cardiochiles nigriceps* showed that the most active methyl position for hentriacontane was 11; 16 for dotriacontane; and 13 for triacontane. (Vinson *et al.*, 1975) A methyl isomer of octane was identified in the *E. saccharina* sugarcane frass extract although the identification of this peak was very poor (see Table 4.1.). A series of methyl isomers of hexadecanal from the abdominal pheromone gland were found to increase parasitisation of *H. zea* by females *T. pretiosum* (Lewis *et al.*, 1982). Octadecanal was identified in the *E. saccharina* sugarcane frass (see Table 4.1.). Hendry *et al.* (1973) reported that *Orgilus lepidus* responded optimally to heptanoic acid among a series of alkanoic acids ranging from acetic to decanoic acids which were identified in the host frass. This parasitoid must also probe for a hidden host, and the chemical elicited antennation and ovipositor probing. The authors reported the mandibular glands of the host as the probable source, but its function here was not determined. No alkanoic acids were identified in the *E. saccharina* sugarcane frass extract. Mudd and Corbet (1982) assessed the response of *Venturia canescens* to a series of 2-acyclohexane 1,3-diones isolated from the mandibular gland of the host

Ephestia kuehniella. The only ketone identified in the *E. saccharina* frass extract was 10-hydroxy-4-cadinene-3-one.

Jones (1986) looked at these results from the point of view of the nature of the possible acceptor sites on the antennae of the parasitoids for these various chemicals. He concluded that "parasitoids being at the third trophic level, thus having to locate not only an appropriate habitat (a plant species for example) but also a suitable host within the habitat, may be capable of detecting a wider range of chemicals than insects at the second trophic level. As a parasitoid may shift habitats phenologically there is even more reason for the parasitoid to be versatile at chemical detection. This versatility is supported by their sensory system organization. Olfactory sensilla of many hymenopteran parasitoids are of a general nature (few sensilla, but each with many neurones)". He highlighted the low levels that could cause the behavioural responses (ng and μg levels), and that often the response to dose has been shown to have a bell shaped curve. He suggested that acceptor sites might be specific for a certain methyl position on a hydrocarbon chain, as competitive inhibition was shown in the case of *M. croceipes* between the more active 13-methylhentriaconatone analogue and less active 15-methylhentriaconatone (Jones *et al.*, 1971). He also pointed out that the type of substituent on positions of a cyclic ring may alter the activity of a compound as was the case for *V. canescens* (Mudd and Corbet, 1982). On top of this he pointed out that chain lengths of hydrocarbons may alter the optimum methyl position, as was the case for *C. nigriceps*, and interestingly a mixture of varying chain lengths can cause a synergistic effect (Vinson *et al.*, 1975). This illustrates the problems that may be foreseen when trying to identify which chemical(s) cause a given behavioural response.

4.4. CONCLUSIONS

Although further analysis is needed for more accurate identification of the chemicals in the chloroform extract of *E saccharina* frass, these results are encouraging. Because the chemicals identified in the extract are similar to those identified for other host searching kairomones, there is a possibility that the kairomonal chemical(s) arise from the mandibular glands of the larvae, and/or the waxy layer of the cuticle, which was found to be the case in other studies previously mentioned.

GENERAL CONCLUSIONS.

The first objective was to develop suitable bioassay techniques which could illustrate the behavioural response of *G. natalensis* to behaviour-modifying kairomones from its hosts' frass. The petri dish and olfactometer bioassays successfully illustrated the presence of a short-range kairomone in various types of *E. saccharina* frass, which elicited the *G. natalensis* host-searching behaviour. These bioassays also provided some useful information as to what age and sexual state were required for a strong host-searching response by *G. natalensis* females to *E. saccharina* frass.

The next objective was to isolate the kairomone. This was achieved using a range of solvents. Chloroform proved to be the best solvent tested, and host searching to this sugarcane frass extract in both petri and olfactometer bioassays, was comparable to that elicited by fresh sugarcane frass. There is scope in this area for more research, as there are numerous techniques which may be employed to further isolate the kairomone from the chloroform.

The final objective was to identify the isolated kairomone. One of the techniques which has made the identification of minute quantities of volatile chemicals possible namely GC/MS (gas chromatography coupled to mass spectrometry), was employed for this purpose. GC work was done at the Station, but the lack of an MSD made the task of identification impossible. A sample was sent to CSIR for GC/MS analysis. The sample was unfortunately too dilute even for this powerful technique, thus

identification of the chemicals present was not satisfactory. Although the last objective was not completed, the results obtained are encouraging, and the lines for further analyses are open.

Results showed that host-searching by *G. natalensis* females was elicited by a short-range kairomone present in various types of *E. saccharina* frass. The kairomone was shown to be present in *E. saccharina* frass from sugarcane and papyrus. These results are supported by the fact that *G. natalensis* females released into Natal sugarcane fields have been able to locate and parasitise *E. saccharina* larvae even though sugarcane has not been recognised as a host habitat by *G. natalensis*. Research into the stimuli which enable *G. natalensis* to locate *E. saccharina* habitats such as papyrus and other sedges, could prove beneficial in determining why the parsitoid has been unable, as yet, to naturally colonise Natal sugarcane infested with *E. saccharina*.

It is hoped that further research on the short-range kairomone described in this thesis may provide a means to manipulate the efficiency of host location by *G. natalensis* females, enabling increased parasitisation and better control of *E. saccharina* in Natal sugarcane.

APPENDIX

Table A. Constituents of sugarcane medium.

Amount	Substance	Additional Information
250.0 g	Dried finely crushed sugarcane	Dried at 80°C overnight in a oven. Crushed in a mill.
100.0 g	Chickpea	
17.14 g	Brewers yeast	Take 200 ml of the water dissolve the yeast in this autoclave (30 min) before adding to the medium.
17.14 g	Casein	
9.14 g	Sodium propionate	
3.43 g	Ascorbic acid	
1.14 g	Calcium lactate	
0.06 g	Ferric citrate	
2.29 g	Tri-sodium citrate	
1.71 g	Multivitamins (without iron)	
0.17 g	Dithane	
2.0 g	Nipagin	
3.5 ml	Formaldehyde	
1.0 ml	Streptomycin	
35.0 ml	Methanol	
10 g	Agar	
1 l	Water	

Table B. Constituents of papyrus medium.

Amount	Substance	Additional Information
250.0 g	Dry finely crushed papyrus umbels	Dried at 80°C overnight in a oven. Crushed in a mill.
100.0 g	Chickpea	
17.14 g	Brewers yeast	Take 200 ml of the water dissolve the yeast in this autoclave (30 min) before adding to the medium.
17.14 g	Casein	
9.14 g	Sodium propionate	
3.43 g	Ascorbic acid	
1.14 g	Calcium lactate	
0.06 g	Ferric citrate	
2.29 g	Tri-sodium citrate	
1.71 g	Multivitamins (without iron)	
0.17 g	Dithane	
2.0 g	Nipagin	
3.5 ml	Formaldehyde	
1.0 ml	Streptomycin	
35.0 ml	Methanol	
10 g	Agar	
1 l	Water	

Table C. Constituents of cellulose medium.

Amount	Substance	Additional Information
250.0 g	Cellulose	
100.0 g	Chickpea	
17.14 g	Brewers yeast	Take 200 ml of the water dissolve the yeast in this autoclave (30 min) before adding to the medium.
17.14 g	Casein	
9.14 g	Sodium propionate	
3.43 g	Ascorbic acid	
1.14 g	Calcium lactate	
0.06 g	Ferric citrate	
2.29 g	Tri-sodium citrate	
1.71 g	Multivitamins (without iron)	
0.17 g	Dithane	
2.0 g	Nipagin	
3.5 ml	Formaldehyde	
1.0 ml	Streptomycin	
35.0 ml	Methanol	
10 g	Agar	
1 l	Water	

Table D. Constituents of synthetic medium (1% Nitrogen in dry mass (DM) with the correct amino acid balance found in 2nd instar *E. saccharina* larvae).

Amount	Substance	Additional Information
3.238 g	Casein	
2.63 g	Amino acid mix A (see next page)	Total Nitrogen = 1 g
0.86 g	Amino acid mix B (see next page)	(6.89 g x 0.145 (14.5% of the
0.021 g	Cysteine	DM of the adjacent substances
		is nitrogen))
3.0 ml	Glycerol	
1.0 g	Inositol	
8 g	Fructose	
12 g	Glucose	
20 g	Sucrose	
33.3 g	Cellulose	
0.015 g	Ferrous sulphate	
or		
0.012 g	Ferric orthophosphate	
4 g	Wesson's salts	
1.5 g	Vitamin mix	
0.5 g	Sorbic acid	
0.05 g	B carotene	
0.1 g	Palmitic acid	
0.1 g	Elaidic acid	
2.5 g	Propionic acid	
0.3 g	Stearic acid	Lipids, fatty acids and oils
1.0 ml	Linoleic acid	comprise 7% of the total DM.
0.5 g	Wheat germ oil	
2.0 g	Lecithin	
1.0 g	Cholesterol	
1.0 g	Choline Cl	
0.084 g	Streptomycin	Antibiotics mixed in 10 ml
0.172 g	Benlate	of distilled water and then
0.067 g	Dithane	added to the medium.

-Table D. Continued-

7 g	Agar	Mixed in 200 ml of boiling distilled water.

Total medium mass = 400 g
Total dry mass (DM) = 100 g
%DM = 25.0
%Fibre (i.e. agar and cellulose) = 10.0
%Sucrose = 5.0
%Reducing sugars = 5.0g

Amino acid mix A

Amount (g)	Amino acid
11.45	Aspartate
3.03	Glutamate
8.19	Serine
8.61	Glycine
6.80	Histidine (HCl)
13.05	Arginine (HCl)
5.17	Threonine
14.28	Alanine
7.14	Lysine (HCL)

Amino acid mix B

Amount(g)	Amino acid
6.26	Tyrosine
4.91	Valine
1.97	Methionine
3.62	Isoleucine
4.34	Leucine
1.94	Phenylalanine
2.40	Tryptophan
0.62	Cystine

Table E. Raw data and statistical analyses (t-test) for petri dish 2 results

RAW DATA OF RESULTS FOR NUMBER OF VISITS IN THE BIOASSAYS:					
Male frass	**Female frass**	**Male and Female frass**		**Virgin females**	
9	7	4	5	1	
8	8	3	6	3	
7	7	4	6	1	
7	8	8	5	2	
8	7			2	
5	5	4	4	5	N
7.800000	7.400000	4.750000	5.500000	1.80000	**MEAN**
8.000000	7.000000	4.000000	5.500000	2.00000	**MED.**
0.836660	0.547723	2.217356	0.577350	0.836660	s

RESULTS OF STATISTICAL ANALYSES (T-TEST) ON NUMBER OF VISITS:
Number of visits by mated females to:
Male frass verses Female frass

7.8	7.4	Means
0.7	0.3	Variances

$T = 0.8944274$ $P = 0.3972037$ d.f. $= 8$
Confidence limits on difference of means can be calculated as:
0.4000001 +/- T(d.f. $= 8$) x 0.4472136

Number of visits by mated females to:
Male and Female frass in the same petri dish (paired test)

4.75	5.5	Means
4..916668	0.3333334	Variances

$T = 0.5703518$ $P = 0.6083585$ d.f. $= 3$
Confidence limits on difference of means can be calculated as:
0.75 +/- T(d.f. $= 3$) x 1.314978

Number of visits by mated females to:
Male frass verses Number of visits by virgin females to frass

7.8	1.8	Means
0.7	0.7	Variances

$T = 11.33895$ $P = 3.218651 \times 10^{-6}$ d.f. $= 8$
Confidence limits on difference of means can be calculated as:
6 +/- T(d.f. $= 8$) x 0.5291496

-Table E. Continued-

Number of visits by mated females to:
Female frass verses Number of visits by virgin females to frass

7.4	1.8	Means
0.3	0.7	Variances

$T = 12.52196$ $P = 1.549721 \times 10^{-6}$ d.f. $= 8$

Confidence limits on difference of means can be calculated as:

$5.600001 +/- T(d.f. = 8) \times 0.4472143$

RAW DATA OF RESULTS FOR THE AVERAGE TIME (s) OF VISITS IN THE BIOASSAYS:

Male frass	Female frass	Male and Female frass		Virgin females	
125.6	129.2	90.75	122.2	26	
155.75	135.875	169	104.1667	11.6	
158.86	152.85	142	107.83	45	
125.17	149.264	186.6	241.2	18	
160.14	149.2			19	
5	5	4	4	5	N
145.104	143.278	147.088	143.849	23.9200	MED.
18.0722	10.1923	41.7979	65.3655	12.8426	MEAN

RESULTS OF STATISTICAL ANALYSES (T-TEST) ON AVERAGE TIME OF VISITS:

Number of visits by mated females to:
Male frass verses Female frass

145.104	143.2778	Means
326.604	103.8799	Variances

$T = 0.1968102$ $P = 0.8488841$ d.f. $= 8$

Confidence limits on difference of means can be calculated as:

$1.826187 +/- T(d.f. = 8) \times 9.278924$

-Table E. Continued-

Average time of visits by mated females to:
Male and Female frass in the same petri dish (paired test)

| 147.0875 | 143.8492 | Means |
| 1747.064 | 4272.65 | Variances |

$T = 0.1163287$ $P = 0.9147423$ d.f. $= 3$
Confidence limits on difference of means can be calculated as:
$3.238325 +/- T(d.f. = 3) \times 27.83772$

Average time of visits by mated females to:
Male frass verses Number of visits by virgin females to frass

| 145.104 | 23.92 | Means |
| 326.604 | 164.932 | Variances |

$T = 12.22222$ $P = 1.907349 \times 10^{-6}$ d.f. $= 8$
Confidence limits on difference of means can be calculated as:
$121.184 +/- T(d.f. = 8) \times 9.915053$

Average time of visits by mated females to:
Female frass verses Number of visits by virgin females to frass

| 143.2778 | 23.92 | Means |
| 103.8799 | 164.9321 | Variances |

$T = 16.27831$ $P = < 10^{-6}$ d.f. $= 8$
Confidence limits on difference of means can be calculated as:
$119.3578 +/- T(d.f. = 8) \times 7.332324$

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