SEX PHEROMONE STUDIES ON THE GRASS GRUB (COSTELYTRA ZEALANDICA (WHITE)) AND THE COMMON ARMYWORM (PSEUDALETIA SEPARATA).

by

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This thesis is dedicated to my
wife, Anne and my two children,
Marianne and Jane.

ABSTRACT

This thesis describes the identification of a sex pheromone of the grass grub beetle, Costelytra zealandica, and the attempts to use the sex pheromone directly to control grass grub infestations. The partial identification of a sex pheromone of the common armyworm moth, Pseudaletia separata, is also described.

The larval stages of the grass grub beetle cause extensive damage to pasture during the autumn-winter period and constitute New Zealand's major insect pest. Since the banning of DDT, the larvae have been treated by spraying pastures with one of a number of organophosphate insecticides but, in general, these materials have not given adequate control.

Attempts have been made, therefore, to develop alternative control procedures and to establish more selective and effective ways of using insecticides. Promising preliminary results have been obtained with other insects in using sex pheromones both as direct and indirect control agents. In the latter respect, a more judicious and effective use of the insecticide has been achieved, by using the properties of specificity and sensitivity of an insect's sex pheromone to detect, and to estimate, the insect population prior to the application of the insecticide.

The female grass grub beetle was shown to contain a sex pheromone which stimulated mating activity in males in the laboratory. Methods were developed to extract the sex pheromone and its properties in functional group tests were consistent with those of a phenolic compound. The active component was isolated by the means of high vacuum distillation onto dry ice followed by gas chromatography. The isolated material was then examined by high resolution mass spectroscopy and found to be phenol. On average each female beetle at

the time of mating contained about 1.5 µg phenol. Phenol itself not only stimulated mating activity in males in the laboratory but also attracted large numbers of young males in mating flights in the field. Field tests were also carried out with a number of substituted phenols but none of these was active.

An electroantennogram method was developed to measure the antennal response to phenol and a number of closely related phenolic materials. In all the tests, phenol gave the largest response. A comparison at the antennal level between phenol and a crude female extract indicated phenol was the sole pheromone.

Attempts were made to use phenol for grass grub control. The compound is cheap and readily available. Hence its direct use could provide an economically acceptable control method. Interestingly, this was one of the first opportunities to attempt to control an insect population with an unlimited supply of the sex pheromone.

There are two ways in which phenol might be used to prevent effective mating from taking place in the field. Firstly, the attractant properties might be used to out-compete females in the natural population. Secondly, sufficient phenol could be permeated into the atmosphere to possibly confuse the male in its search for the female. Both methods were evaluated in the field with grass grub infestations which ranged from 50 to 220 beetles per m^2. In the first approach the best attractant sources used were only about ten times more attractive than virgin females and, therefore, as they could not compete successfully with the large number of females in the natural population, control was not achieved. In the attempts to use the second approach it was possible to confuse male flight behaviour by releasing 5 grams/hour/hectare (5g/h/ha) of phenol over the trial sites. However, it was not possible to prevent males from locating females even with as much as 110 g/h/ha of phenol released

over the trial sites. Presumably, the beetle density of the infestations was such, that males could locate females by sight or simply by chance.

Larvae of the common armyworm can cause extensive damage to pastures and crops and although conventional insecticide methods give satisfactory control, the main problems are early detection and estimation of infestations. In this respect a knowledge of the sex pheromone used by the female moth should allow insecticides to be used more effectively.

The sex pheromone was extracted from the abdominal tips of female armyworm moths using methylene chloride. Functional group tests indicated that the sex stimulatory component was an unsaturated aliphatic acetate. Comparison of the R_f values and retention times of the active component with those of standard compounds on thin layer and gas chromatography (GC) respectively, suggested that a mono-unsaturated hexadecenyl acetate was involved. Of the mono-unsaturated hexadecenyl acetates tested for EAG activity, only cis-11-hexadecenyl acetate induced an intense antennal response. Furthermore, 0.1, 1.0, and 10.0 ng of this compound on glass rods induced strong sex stimulatory activity in males in laboratory behavioural tests.

The crude female extract was injected onto a number of different GC columns and several active fractions were collected from each column. This indicated that the female moth probably uses a multiple component pheromone system. The behaviour of one of the sex stimulatory components in the female extract, in all the chemical and concentration steps, was consistent with that of cis-11-hexadecenyl acetate.

In an attempt to verify that cis-11-hexadecenyl acetate was

a natural pheromone of the common armyworm moth a GC fraction of the crude female extract, eluting at the retention time of cis-11-hexadecenyl acetate, was collected and ozonized. One of the ozonolysis products of cis-11-hexadecenyl acetate is 11-acetoxyundecanal but this was not detected in the ozonized GC fraction, using the technique of mass fragmentography in which a mass spectrometer acts as a specific GC detector. It appears, therefore, that cis-11-hexadecenyl acetate is not present in female armyworm moths. When tested in the field, however, it attracted male moths into traps but was not as effective as virgin females.

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CHAPTER I

INTRODUCTION

1.1 Grass grub

The larval stages of the New Zealand grass grub, *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae), constitute the country's major insect pest because of the extensive damage caused to pasture during the autumn-winter period. Over the last 100 years or so, most of New Zealand's lowland tussock areas and much of its native forests have been replaced by improved pastures based on introduced grasses and legumes. These pastures form the basis of a highly efficient farming industry which provides about 80 percent of New Zealand's export earnings. Grass grub, which occurs throughout New Zealand, has adapted to this introduced environment to such extent that pasture damage has been estimated to cost between 30 and 60 million dollars annually (Fiay and Garrett, 1942; Harrison, 1967). Damage is most severe in the lighter soils of the Canterbury Plains and in the hill country in the Taupo-Rotorua and Taranaki areas, where stock treading of severely damaged pastures causes serious erosion problems.

Grass grub larvae are difficult to control with conventional insecticide methods due to the protection provided by the vegetation. For a time, control was achieved by the widespread use of DDT which was added to fertiliser and applied to pasture (Kelsey, 1951). This material had a long soil life which allowed good contact to be made between larvae and the insecticide. It is ironic, however, that this property of persistence which made DDT so useful, has led to its ban in New Zealand due to the build-up of biologically harmful

organochlorine residues in meat and dairy produce. Furthermore, widespread resistance to DDT had developed in a number of grass grub strains (Perrott and McGrath, 1966) which obviously limited its usefulness in control programmes.

A number of organophosphate insecticides have been substituted for DDT but limitations of these materials include cost of application, short residual life, and the necessity for a uniform distribution (Kain and Crabtree, 1972). Reductions in application cost can be achieved by spot treatment of heavily infested areas with insecticides(Kain and Atkinson, 1970) or injection of the insecticide in bands below the soil surface (Trought and Wood, 1970). The latter method has given satisfactory control but its use is limited to ploughable areas and not hill country where grass grub damage is a major problem.

There is increasing pressure from many sources, however, to restrict or ban the use of insecticides, especially those that are persistent. Consequently, there is an urgent necessity to develop safer alternative control methods which are specific to the insect target and without side effects on other life. Attempts to develop such methods for grass grub control have met with varying success. These have included the planting of resistant crops such as lucerne, various cultural practices, and biological control with predators, parasites, and insect pathogens. Chemicals such as sex pheromones which occur naturally in insects are also potentially useful as selective control agents.

In 1966, Kelsey reported the presence of a sex pheromone in the grass grub beetle but his studies, which were carried out with live virgin female beetles in the field, did not preclude the possibility of sight or sound being involved in the mating process. Preliminary laboratory behavioural tests carried out by the author,

however, established that a chemical alone was involved. This provided a basis for this study on the bioassay, isolation, identification, and use,of the sex pheromone of the grass grub beetle.

As all the attempts to mass rear the univolitine grass grub beetle in the laboratory had been unsuccessful, beetles were not available for study throughout the year. This study on the sex pheromone was restricted, therefore, to about a six week period in the spring when beetles were present in the field.

1.2 Common armyworm

Large areas of pasture and cereals, particularly maize, within the mid and northern areas of the North Island of New Zealand are attacked annually by the caterpillar of the armyworm moth, Pseudaletia separata. The insect is primarily a defoliator although early infestations may chew young maize silks causing poor pollination and seed set (Douglas et al., 1972).

The caterpillar can be satisfactorily controlled with a number of organophosphate insecticides (Kain et al., 1968) but sometimes crops have to be sprayed at least three times to control the pest adequately. A major problem in controlling armyworm in a crop such as maize, where the danger period is during and shortly after silking, is the early detection of an infestation. In this respect, the high specificity of sex pheromones has made them extremely valuable for early detection and estimation of an insect population. The use of sex pheromone traps in combination with insecticides should, therefore, result in a more effective control procedure. This thesis describes the attempts to identify one of the sex pheromone components in the female armyworm moth.

Prior to this study, the presence of a chemical pheromone in P. separata had not been demonstrated. Having established that a

chemical was used by the female moth to communicate with the male prior to mating, a laboratory behavioural bioassay was developed to monitor the presence of the active component in the various concentration procedures.

Work on sex pheromones is seasonal and the studies on the armyworm were mainly carried out with insects reared in the laboratory during the autumn-winter period, a time when it was not possible to work on the grass grub sex pheromone. The two studies were, therefore, carried out in parallel.

CHAPTER II

BIOLOGICAL AND CHEMICAL ASPECTS OF SEX PHEROMONES

2.1 Sex pheromone definition

It is well known that volatile organic chemicals with characteristic odours or tastes are widely used by insects to communicate with one another. This system of communication has reached its highest development in the social insects; ants, bees, wasps, etc., all of which communicate in the dark interiors of their nests. The term "pheromone" coined from the Greek "pherein" (to carry) and "horman" (to excite, stimulate) was proposed by Karlson and Luscher (1959) to describe the chemical substances involved in insect communication. Pheromones were defined as,

> "Chemicals secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or developmental process".

There are many possible ways for classifying responses of insects to pheromones. For instance, Wilson (1963) subdivided pheromones into "releaser" and "primer" substances. Releaser pheromones produce an immediate and reversible change on the behaviour of the recipient, on whose central nervous system the chemical substance apparently acts more or less directly. On the other hand, primer pheromones trigger off a chain of physiological changes in the recipient without causing immediate changes in behaviour. The most commonly used classification method is based on the response to the pheromone. Thus, chemicals that cause an insect to arrive at the vicinity of another insect of the same species and opposite sex and attempt to copulate have been called sex pheromones. The

terms "alarm", "aggregation", and "trail", are also used to describe the particular biological function of the pheromone.

The generality of sex pheromone communication among insects is indicated by the number of orders in which this behaviour is found. These include the Lepidoptera, Orthoptera. Diptera, Coleoptera, Hymenoptera, Neuroptera, Mecoptera and Homoptera.

Recently, a new term "parapheromone" (pheromone-like substance) has been introduced by Hummel and co-workers (1972) to describe chemicals that possess pheromonal activity, but are not identical in structure to the natural pheromone of a particular species. Compounds of this nature have been found for the pink bollworm moth, Pectinophora gossypiella (Green et al., 1969), the cabbage looper, Trichoplusia ni (Hummel et al., 1972) and the gypsy moth, Lymatria dispar (Adler et al., 1972b).

2.2 Identities of sex pheromones in the Coleoptera.

The sex pheromones of the Coleoptera, the largest order of insects, show some correlation of structure within families but not the close structural similarity associated with the Lepidoptera. Among the beetles, the group that has been most extensively studied is the bark beetles (Scolytids) which cause extensive damage to pine forests. The history and behaviour associated with bark beetle attractants have been reviewed by Silverstein (1970, 1971). Pheromone communication for bark beetles differs from that described for the Lepidoptera in that beetles bore into pine trees and produce a powdery frass of wood fragments and excrements which attract both males and females. The attractant substances have been called assembly or aggregation pheromones. The beetle pheromones that have been identified are shown in Table 2.1.

A mixture of three terpene alcohols constitutes the assembly pheromone in the frass of the male Ips confusus: (1), (-)-2-methyl-

Table 2.1 Insect Sex Pheromones of the Coleoptera

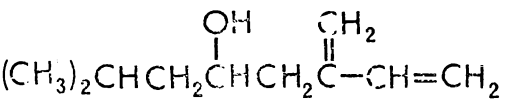
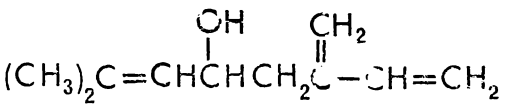
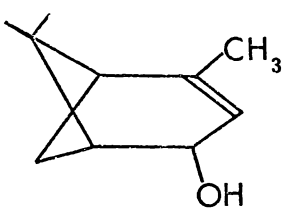
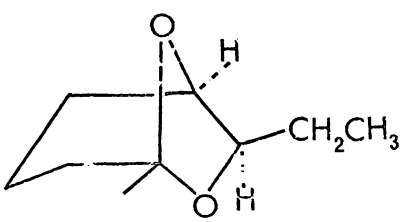
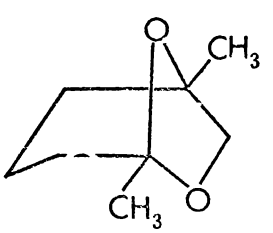
Compound	Insect
 (−)-2-methyl-6-methylene-7-octen-4-ol	Ips confusus (Le Conte)
 (+)-2-methyl-6-methylene-2,7-octadien-4-ol	I. confusus
 cis-(+)-verbenol	I. confusus
 exo-7-ethyl-5-methyl-6,8-dioxabicyclo [3.2.1] octane	Dendroctonus brevicomis Le Conte, western pine beetle
 1,5-dimethyl-6,8-dioxabicyclo [3.2.1] octane	Dendroctonus frontalis Zimmerman, southern beetle

Table 2.1 (contd)

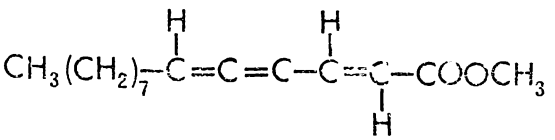
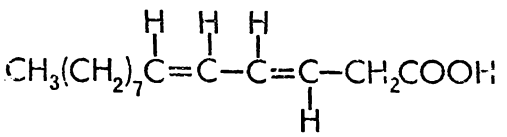
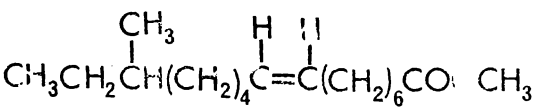
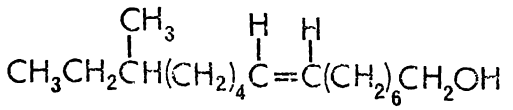
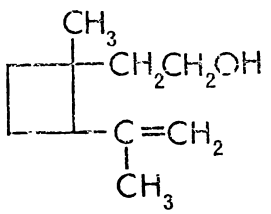
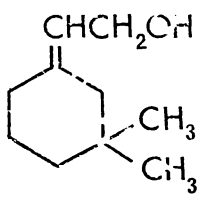
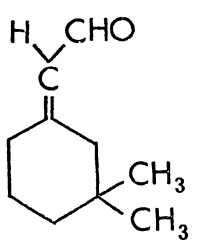
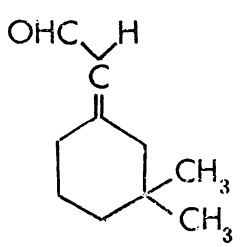
Compound	Insect
$CH_3(CH_2)_3COOH$ valeric acid	Limonius californicus (Mannerheim) sugarbeet wireworm
 (-)-methyl-trans-2,4,5-tetradecatrienoate	Acanthoscelides obtectus (Say) dried bean beetle
 trans,cis-tetradecadienoic acid	Attagenus megatoma (Fabricius) black carpet beetle
 (-)-methyl-cis-14-methyl-8-hexadecenoate	Trogoderma inclusum LeConte a grain beetle
 cis(-)-14-methyl-8-hexadecen-1-ol	T. inclusum

Table 2.1 (cont'd)

Compound	Insect
 cis-2-isopropenyl-1-methyl cyclobutaneethanol	Anthonomus grandis
 cis-3,3-dimethyl-cyclohexaneethanol	A.grandis
 cis-3,3-dimethyl-cyclohexaneacetaldehyde	A.grandis
 trans-3,3-dimethyl-cyclohexaneacetaldehyde	A.grandis

6-methylene-7-octen-4-ol, (II), (+)-2-methyl-6-methylene-2,7-octadien-4-ol and(III), cis-(+) verbenol (Silverstein et al., 1966). These compounds are not active by themselves but only in combination. It is of interest that two predators of Ips confusus also respond to the ternary mixture. Males and females of a closely related species Ips latidens are attracted to (I) and (I) + (II) but not the three compounds together, a factor which may play an important role in effecting reproductive isolation.

Bark beetles of the Dendroctonus species use a unique communication system in which pheromones are combined with host volatiles to bring about aggregation of both sexes at trees undergoing attack. Silverstein et al., (1968) identified a unique dioxabicyclo derivative in the frass of the female western pine beetle, D. brevicomis. The substance, called brevicomin, is attractive by itself but its activity in the field is enhanced by the host terpene hydrocarbon, myrcene (Bedard et al., 1969). The assembly pheromone of the southern pine beetle D. frontalis is a mixture of the host terpene, trans-verbenol and another dioxabicyclo derivative called frontalin (Kinzer et al., 1969). This compound has a close structural relationship to brevicomin and D. brevicomis is attracted to it (Vite and Pitman, 1969).

Four terpenes, two alcohols and two aldehydes, isolated from male boll weevils as well as their frass, caused an assembly of both sexes (Tumlinson et al., 1969). It is of interest that the male boll weevil is more attractive when fed on cotton, which naturally contains myrcene which could be the precursor in the insect biosynthesis of the pheromone mixture.

In addition to these terpene like substances there are a few alcohols, acids and methyl esters which have been identified as sex pheromones for coleopterous insects. For example, valeric acid for

the sugar beet wireworm (Jacobson et al., 1968), trans-3, cis-5-tetradecadienoic acid for the black carpet beetle (Silverstein et al., 1967) and two compounds, an alcohol, cis(-)-14-methyl-8-hexadecen-1-ol and its methyl ester, (-)-methyl cis-14-methyl-8-hexadecenoate, for another stored product beetle, Trogoderma inclusum (Rodin et al., 1969). The diversity of structure within the Coleoptera is well illustrated by the discovery by Horler (1970) who found that an allenic ester, (-)-methyl trans-2,4,5-tetradecatrienoate, functioned as a sex pheromone for the female dried bean beetle, Acanthoscelides obtectus.

The presence of sex pheromones in scarab beetles has been reported for a few species but their chemical nature is unknown. The species include Pachypus cornutus (Olivier) (Jeannel, 1960), the June beetle, Lachnosterna lanceolata (Say) (Travis, 1939), the cock chafers, Rhopaea magnicornis (Blackburn), R. verreauxi and R. morbillosa (Soo Hoo and Roberts, 1965) and the Japanese beetle, Popillia japonica (Newman) (Ladd 1970; Goonewardene et al., 1970a).

2.3 Identities of sex pheromones in the Lepidoptera.

The sex pheromones emitted by female moths have been reported in a number of recent reviews (Jacobson, 1970; Beroza, 1970, 1972; Roelofs, 1971; Ritter, 1971). Most of the sex pheromones of the Lepidoptera are fairly simple molecules which have been isolated in the neutral lipid fraction of gland extracts. Long chain acetates, alcohols, aldehydes, an epoxide, a methylbutanoate, and a hydrocarbon have been identified as sex pheromones in this order. Some of the compounds contain one or two centres of unsaturation and some have a branched aliphatic chain. A list of the insects and their pheromones which have been discovered by isolating the sex pheromones using the classical technique has been assembled in Appendix I.

By far the most productive method of discovering sex pheromones has been to screen compounds in field tests. This approach exploits the fact that a relatively large proportion of the sex pheromones are acetates of unsaturated alcohols with 12, 14 or 16 carbon atoms or simply the alcohols themselves. Roelofs and Comeau (1970) found sex pheromones for 37 lepidopterous species in field screening tests with 36 such compounds. Another means of finding sex pheromones which exploits this fact has been to use the electroantennogram (EAG) technique combined with gas chromatography (GC). Retention times of the sex pheromone are determined by injecting crude pheromone extracts on polar and non polar columns, collecting the effluent at 1-minute intervals and determining which fraction gives the maximum antennal response in EAG tests. The retention times define the number of carbon atoms and the degree of unsaturation in the aliphatic chain. Suitable candidate materials can then be screened in EAG tests and the appropriate compound giving maximum response is assumed to be the sex pheromone which is verified in field tests. These compounds may not be natural pheromones but simply closely related molecules i.e. parapheromones.

2.4 Sex pheromone production.

Detailed morphological and histological descriptions of female sex pheromone glands have been carried out mostly with lepidopterous insects. These glands are normally located in the intersegmental membrane between the eighth and ninth abdominal segments (Percy and Weatherston, 1971). When the female is at rest, the gland is not exposed to the air because the eighth and ninth segments are nomally retracted into the seventh segment. Immediately prior to mating, female moths of many species release the sex pheromone by stretching the abdomen outwards and actively protruding and retarding the scent gland. An illustration of the

"calling" behaviour of the female white butterfly, Pieris rapae is shown in Plate 2.1.

The sex pheromone glands, which are developed from the hypodermal cells, may be in the form of a protrusible scent ring or fold or a pair of lateral sacs. The glandular epithelium in this region is typically composed of large columnar to cuboidal cells with large, globular to oval nuclei and a granular cytoplasm.

The scent glands of insects within the Coleoptera have been studied for only a few species. Females of the scolytid beetles, Dendroctonus pseudotsugae, apparently produce their attractive odour in their Malpighian tubules in the hind gut region (Zethner-Møller and Rudinsky, 1967). In virgin females of the sugar beet wireworm, Limonius californicus, the sex pheromone is found primarily in the fifth and sixth abdominal segments (Lilly and McGinnis, 1968). The sex pheromone glands are also located in the female's abdomen in the beetles, Diabrotica balteata (Cuthbert and Reid, 1964), a number of Rhopaea species (SooHoo and Roberts, 1965) and Tenebrio molitor (Valentine, 1951). In the latter species, sex pheromone production and release by females is under the control of juvenile hormone produced in the corpora allata (Menon, 1970). This is also the case with the bark beetle, Ips confusus, in which juvenile hormone induced males to produce sex pheromone in the hind gut Malpighian tubule area (Borden et al., 1969). Hoyt et al., (1971) have suggested that the grass grub sex pheromone is produced by symbiotic bacteria in the colleterial glands in the female's abdomen.

Most of the sex pheromones that have been identified to date have been present in microgram (μg) or nanogram (ng) quantities. Very low quantities have been reported for the codling moth, 0.3 ng per insect (McDonough et al., 1969) and the pink bollworm moth, 2.0 ng per insect (Jones et al., 1966). Steinbrecht (1964) found

Plate 2.1 An illustration of the calling behaviour in the field of the female white butterfly, Pieris rapae. Females in the butterfly family use visual factors as well as chemicals for communication between the sexes.

that extracts of glands from newly emerged female silkworm moths contained about 1.5 µg of sex pheromone per female which is about the same order as that reported for the cabbage looper (Berger, 1966). Silverstein and co-workers (1967) found 0.5 µg per female for the black carpet beetle while the sugarbeet wireworm beetle contained 0.1 mg per insect (Jacobson et al., 1968).

Interestingly, as early as 1946, Kettlewell speculated that only a small quantity of sex pheromone is manufactured by a female moth at a time, the bulk of the material being kept in an inactive precursor state. This has proved to be correct with a number of insect species. For example, workers in Butenandt's laboratory (cited in Karlson and Butenandt, 1959) reduced an inactive silkworm moth extract with lithium aluminium hydride and generated sex pheromone activity. Presumably the precursor was present as an ester, aldehyde or ketone. McDonough and co-workers (1969) released alcohol pheromones by saponification while Roelofs and co-workers (1969) increased, by one hundred-fold, the acetate pheromone content in oriental fruit moth extracts by saponification and reacetylation. Bierl et al., (1970) increased the epoxide pheromone in the gypsy moth by epoxidation of hydrocarbons obtained from an extract of females.

A fascinating discovery was reported recently by Butler and co-workers (1972) who generated the female pheromone (an acetate) of the Zebra caterpillar, Ceramica picten, from the alcohol precursor in males of the same species.

Sex pheromone production is closely linked with the maturation of the female reproductive system. Obviously for eggs to be fertile, females must mate before oviposition takes place and males must not mate until they are capable of transferring sperm to the females. It appears from the present studies that female pheromone production

reaches a peak just prior to oviposition. For example, adults of the gum emperor moth, Antheraea eucalypti, begin to cut their way through their cocoons at about 2.0 to 3.0 p.m. and emerge after about half an hour. Within a few hours of emergence, the wing cases have hardened sufficiently for flight to take place and during the evening following emergence females begin to oviposit. Presumably, the sex pheromone is liberated more or less from the time the adult emerges to allow mating to take place before oviposition. The pheromone is definitely present in the female from this time for extracts of abdominal tips from fresh females are highly attractive to young males. This result is consistent with a report by Steinbrecht (1964) for the closely related silkworm moth. He detected the sex pheromone in female pupae 5 days before adult emergence and found maximum quantities at the time of emergence. Silkworm moth females are capable of mating within half an hour of emergence.

A similar situation was also found in this study with the common armyworm moth, Pseudaletia separata, where maximum pheromone production occurred about 5-6 days after final ecdysis just before oviposition took place. The male of this species did not respond to the sex pheromone until it was at least 5-6 days old when it was sufficiently mature. On the other hand the native New Zealand armyworm, Persectania aversa, commenced laying eggs within 2 days of emergence and mating took place within 1 day of emergence. Shorey et al., (1968a) noted that a rapid production of sex pheromone in females of seven noctuid moth species occurred within one day preceding emergence from the pupa. Many females of each of these species mate within two days and contain mature eggs within three days after emergence. Similar work on the stored beetle, Attagenus megatoma, by Burkholder (1970) indicated that females were not

attractive until at least 24 hours after emergence.

The influence of mating on sex pheromone production seems to depend on the frequency with which an insect mates. For example, if an insect mates only once, pheromone production in the female declines rapidly as is the case with the gypsy moth (Collins and Potts, 1932). In insects which mate more than once, pheromone production is maintained at a slightly lower level than that produced by the virgin female. This was the case in the silkworm moth (Steinbrecht, 1964), five noctuid moths (Shorey et al., 1968a), and the pink bollworm moth (Berger et al., 1964).

Data on the stored product beetle, Attagenus megatoma, indicated that the female is somewhat less attractive 1 day after mating and much less attractive 8 days after mating (Burkholder, 1970). Howell and Thorp (1972) reported that virgin female codling moths, Cydia pomonella, were about forty times as attractive as mated females which possessed almost no attractiveness.

2.5 Mechanism and distance of sex pheromone communication.

According to Dethier (1957) and Schwink (1958) when an insect responds to its sex pheromone it is stimulated to orient upwind i.e. it undergoes anemotactic behaviour, and it will continue to fly upwind as long as it receives olfactory stimuli. If it loses the odour, the insect makes a number of crosswind casts until it locates the odour again, and once more turns and flies upwind. This mechanism for the orientation of a flying insect to a distant odour has been tested in a flight tunnel using the pink bollworm moth, Pectinophora gossypiella, (Farkas and Shorey, 1972). These workers found that air movement is not necessary for orientation to a distant odour source. Males apparently sense the boundaries of the aerial trail extending from the source during their characteristic zigzag flights. This mechanism does not explain how the moth

distinguishes the "upwind" from the "downwind" direction, however.

Communication distances among insects in the field are highly variable and depend on the release rate of pheromone from the female, the threshold response of the male, and the rate of pheromone dispersion in the moving air mass (Sower et al., 1971). In general, the function of the sex pheromone is to bring insects close enough to see or touch one another. Early reports in the literature indicated male insects could be attracted from long distances by the female's scent. For example, 3800 m for the gypsy moth (Collins and Potts, 1932) and 2400 m for the ailanthus moth, Philosomia cynthia (Hecker, 1959). Later work with other insects, however, suggests much shorter distances. For example, 15 m for the banded cucumber beetle, Diabrotica balteata (Cuthbert and Reid, 1964); 27 m for the pasture cockchafer, Rhopaea magnicornis (SooHoo and Roberts, 1965); 60 m for the honey bee (Butler and Fairey, 1964); and 100 m for the cabbage looper (Kishaba et al., 1970). It is probable in the early experiments that males fortuitously reached points within a 100 m or so downwind of the female and then were attracted.

2.6 Sex pheromone reception.

The transfer of chemical information requires a chemical source, a transfer medium (air or water), and a receptor. In a review of insect olfaction in 1880, Hauser reported that males of Saturnia pavonia and Porthetria dispar never mated when deprived of their antennae. Since then, antennae have been implicated many times as the principal site of chemoreception in insects (Jacobson, 1965). The role of the antenna is well emphasized by differences between male and female species of moths in the families Lasiocampidae, Saturniidae and Bombycidae. Females in these families attract males from some distance. They have small threadlike antennae while

the males have much more complex, well developed plumous structures. This increased surface area presumably enhances the male olfactory sensitivity and allows the males to locate females from a considerable distance.

The external sense organs located on the antenna are called sensilla (Schneider, 1964). The sensillum is a specialized piece of cuticle with a minimum of three cells where at least one is a sensory neurone or primary receptor cell. Schneider (1969) described four types of sensilla (1) long, thick-walled hairs or pegs (sensilla trichodea) found in moths; (2) short, thin-walled hairs or pegs (sensilla basiconica); (3) plates (sensilla placodea) found in the bee and other Hymenoptera and (4) pit pegs (sensilla coeloconica) found in Lepidoptera, Hymenoptera and many other orders of insects. The sensilla have been examined under the electron microscope and the latter type can have between 10 and 30 receptor cells while the former types have between one and several receptor cells.

Three sensilla, the cuticular hairs, pegs and plates all have a pore-tubule system which connects the outside medium, air, with the hair lumen and the receptor dendrite i.e. the finely ramifying branches given off from a nerve cell. Schneider (1969) proposed that this canal system leads odour molecules from the outside to the dendritic membrane of the receptor cells. In tests with radioactive bombykol, the sex pheromone of the silkworm moth, researchers in Schneider's group (1968) showed that the antenna of the male silkworm moth functioned as an effective molecular sieve which was capable of absorbing odour particles from the air stream. Furthermore, a single molecule entering a pore in the sensilla stimulated a single cell response (Schneider, 1969).

Peripheral olfactory receptor cells (primary neurones) exhibit two types of electrical responses: a slow local receptor or generator

potential and travelling nerve impulses of the "on" or "off" type in which the nerve fibre gives either maximum response or no response (Schneider, 1969). The overall slow reaction in the antenna has been called the electroantennogram (EAG) (Schneider, 1957) and is composed of the receptor potentials elicited simultaneously in many olfactory sense cells. These potentials can be recorded by inserting one electrode near the tip of the antenna and another electrode at the base.

In order to gain some insight into the function of the olfactory receptor cell, extracellular recordings from single sensilla have been made (Morita and Yamashita, 1959; Boeckh et al., 1965). In these tests, simultaneous recordings of receptor potentials and nerve impulses were obtained by inserting the recording electrode into a hair sensillum of the antenna and the indifferent electrode in the haemolymph space of the antenna. Normally with this technique, generator potentials of neighboring neurones of the same sensillum are also obtained, but the impulse amplitudes of the cells are sufficiently different to differentiate between the cells. The impulse responses observed are normally of the phasic-tonic type i.e. the receptor cells respond to the stimulus with a transient phase of very high frequency followed by a steady state low frequency.

Studies of this nature have indicated that while all olfactory receptors respond to a spectrum of compounds, many are specialized for the detection of food and pheromones. Specialized receptor cells respond strongly and specifically to the sex pheromone and have been found in males of the silkworm moth, B. mori, the saturnid silkworm, Antheraea pernyii, and the drone of the bee, Apis mellifera (reviewed by Boeckh et al., 1965).

Receptor potentials are subject to a membrane process known as defacilitation and facilitation (Bullock, 1959). For example, when the EAG of B. mori was recorded using successive puffs of sex pheromone, the recorded potential showed a decreased amplitude with each successive stimulation. This is termed defacilitation and explains the behaviourial pattern of loss of male response to continued pheromone exposure.

In extracellular recordings with different stimuli, opposite polarities have been generated in the olfactory receptors. Excitatory odour stimuli, such as sex pheromones, caused an increase in nerve impulse frequency which was accompanied by a slow negative potential at the recording electrode. On the other hand, inhibitory stimuli induced a positive potential with a decrease in impulse frequency (Boeckh, 1965; Boeckh et al., 1965; Morita and Yamasnita, 1961; Yamada, 1966). With a view to understanding the mechanism of odour discrimination in the central nervous system of insects, Yamada (1971) recorded extracellular action potentials from single cells in the olfactory lobe of the American cockroach, Periplaneta americana. For this purpose a glass capillary electrode was inserted into the dorsal sensory neurone area of the olfactory lobe. Two main groups of secondary neurones were distinguished, odour specialists and odour generalists. The latter cells responded to a large variety of odours in an excitatory or inhibitory manner or not at all. The receptors had constant, overlapping, but highly individual reaction spectra. It is possible that the mechanism of odour encoding at the olfactory lobe involves linear combinations of every neurone's activity resulting in an odour coding pattern being received in the brain.

2.7 Sex pheromone perception.

There are a number of theories to describe the mechanism by which insects perceive odours. Most of these are unsubstantiated but two recent theories, the Dyson-Wright vibration theory (Wright, 1966) and the stereochemical theory of Moncrieff-Amoore (Amoore, 1964) have been subjected to experimental evaluation. The former theory attempts to correlate vibrational frequencies of a molecule in the far i.r. region (500-50 cm^{-1}) with the quality of odour, while the latter theory correlates the odour qualities of molecules with their size and shape. Blum and co-workers (1971) selected the alarm pheromone of Pogonomyrmex workers, 4-methyl-3-heptanone, to evaluate these theories. Deuteration of this molecule shifted the far i.r. absorption bands significantly but the activities of the deuterated ketones remained the same as their undeuterated precursors. On the other hand, 4-methyl-3-hexanone was as active as the natural pheromone, and the most active of the pheromonal surrogates were those that possessed a geometry and size similar to 4-methyl-3-heptanone. These results were consistent with the mechanism of olfaction where odour molecules fit into one of a number of olfactory receptors each of which is identified with a certain primary odour.

Roelofs and Comeau (1968, 1971 a,b) could not correlate molecular shape with the type of activity. They proposed an extension of this theory to describe the synergistic and inhibitory phenomena that they observed in the field with a number of compounds closely related to the sex pheromone of the red-banded leaf roller moth. Their data supported a mechanism of perception in which the sex pheromone, a chemical with the correct spatial arrangement of active sites, possessed the affinity and intrinsic activity for the receptor site proteins to elicit proper behavioural responses. Closely

related inhibitory compounds, which did not display intrinsic activity by attracting males, apparently possessed a strong affinity for the receptor sites and modulated the sensory input by causing central nervous system habituation. Synergistic chemicals were regarded as having a reduced affinity and no modifying effect on the receptor sites which allowed them to equilibrate readily with pheromone molecules. Thus, inhibitors were regarded as compounds which accelerated the rate habituation of receptor sites, while synergists modified the central nervous system input to slow the rate of habituation.

In the EAG studies carried out by Roelofs and Comeau (1971 b), the synergists and inhibitors elicited a range of responses of lower amplitude than that of the sex pheromone itself. This was consistent with the protein receptor model for pheromone perception. Recordings from single cells, however, in which the effect of synergists and inhibitors on nerve impulses could have been investigated, would have provided more basic and conclusive information for this theory. For example, Schneider (1969) demonstrated the inhibitory effect of the oil of cloves on single receptor cells in the male antenna of Antheraea pernyi, by the polarity reversal of the DC potential and the depression of nerve impulses during the period of stimulation.

In accordance with this theory and the mechanism of chemoreception, discussed in Section 2.6, the overall process of sex pheromone perception would involve the penetration of molecules through the pore-tubule system of the sensilla and into a thin layer of fluid to reach the dendritic endings of the receptor cells. Chemical transduction in the receptor membrane would then result in a generator potential to modulate the firing rate of the axons, which carry the nerve impulses directly to the central nervous system. The

mechanism of chemical transduction as outlined by Roelofs and Comeau (1971a) could involve flexible membrane proteins which could assume various conformational states. Interaction (hydrophobic, hydrophilic and electrostatic bonds) of a compound with certain segments of the proteins would effect conformational changes in the proteins as they"wrap around" the interacting molecule. These changes could then trigger the nerve impulse-producing mechanisms.

Recently, Ferkovich and co-workers (1973) measured the binding of the cabbage looper sex pheromone to soluble antennal protein and they found that the bound sex pheromone was enzymatically hydrolysed to the corresponding alcohol. The alcohol inhibits response to the sex pheromone in the field and perhaps enzymatic hydrolysis is the mechanism which regulates adaptation in the neurone and/or is a means of inactivating the pheromone to prepare the dendritic receptor membrane for subsequent stimulation.

2.8 Sex pheromone specificity.

In nature, interspecific mating with closely related insect species is not a normal occurrence for there is generally sufficient differences in courtship behaviour, circadian rhythms of activity, seasonal cycles, host plant selection, and geographical distribution to effect reproductive isolation (Marler and Hamilton, 1966). The high specificity of sex pheromones in attracting only one species is another mechanism by which cross mating is prevented. An example of this high specificity was demonstrated in studies with disparlure, the sex pheromone of the gypsy moth, and fifty closely related compounds. Disparlure was at least 100 times more attractive than any other compound tested. Moving a methyl group to an adjacent carbon, shortening or lengthening the chain by a methylene group or shifting the epoxide to an adjacent position considerably reduced

activity (Adler et al., 1972b).

Early physiological studies by Schneider (1962) indicated that sex pheromones were not completely species-specific, and that there was an overlap between related species. This is certainly the case for many sex pheromones of the Lepidoptera that have been identified. For instance, Berger and Canerday (1968) demonstrated in laboratory studies with five species of Plusiinae moths that cis-7-dodecenyl acetate stimulated sex pheromone responses. Subsequently, Roelofs and Comeau (1970) in field studies with this compound attracted eleven species of Plusiinae moths. In this same study, five moth species in the subfamily Hadeninae were attracted by cis-11-hexadecenyl acetate. In the Holomelina aurantiaca complex, eight sibling species were attracted by 2-methyl heptadecane (Roelofs and Garde, 1971). Differences in habitat preference and seasonal flight periods isolate some of these sibling species but secondary chemicals were also suggested by field studies.

The function of secondary chemicals in species isolation could be to either enhance the attraction of the sex pheromone for one species or negate the attraction for a second species. For example, Roelofs and Comeau (1971a) demonstrated in field tests with cis-11-tetradecenyl acetate, the sex pheromone of the red-banded and oblique-banded leaf roller moths, that dodecyl acetate acts as a synergist for the former insect and an inhibitor for the latter. A similar situation exists with the European corn borer and the red-banded leaf roller moths which ostensibly use the same sex pheromone and are both attracted to it (Klun and Robinson, 1970). Neither female species attracts males of the other species and presumably inhibitors are involved in the process. In a further example of this phenomenon, the Indian meal moth, Plodia interpunctella, and the almond moth, Ephestia cautella, both use the same sex stimulatory

compound, cis-9,trans-12-tetradecadienyl acetate, but in the presence of the former species, male attraction to females of the latter species is strongly inhibited (Ganyard and Brady, 1971).

Secondary chemicals present as host odours, as in the case for the bark beetles (Renwick and Vite, 1970), could also be important for reproductive isolation. The implication here is,that beetles using the same sex pheromone could achieve species isolation by mating on different host trees.

Inhibitory effects of geometrical isomers have been verified for several species of insects and may also play a role in interspecific communication. Roelofs and Comeau (1969, 1970), for example, reported the situation of two sibling species possessing similar seasonal and diurnal cycles in which one, Brytopha similis, was attracted to cis-9-tetradecenyl acetate and the other attracted to the trans isomer. The wrong isomer was not only unattractive, but actually inhibitory to males, so that neither species responded to the sex pheromone in the presence of the geometrical isomer.

Multiple pheromones may also be important in preventing cross-attraction in nature. For instance, the summer fruit tortrix moth is only attracted with a mixture of two compounds, cis-9-tetradecenyl acetate and cis-11-tetradecenyl acetate (Meijer et al., 1972). The multicomponent sex pheromones of the bark beetles reported by Silverstein's group (1966) and Renwick and Vite, (1970) and the cotton boll weevil (Tumlinson et al., 1969) illustrate the synergistic action of several compounds in the sex pheromone process.

2.9 Principles of bioassay methods.

Laboratory behavioural bioassays are based on sexual stimulation and compounds that perform this function do not necessarily attract insects in the field. Ideally, therefore, laboratory

bioassays should be complemented with field bioassays. In the case of the univolitine (1 flight/year) grass grub beetle, all attempts at mass rearing have failed. This meant that the sex pheromone studies were restricted each year to a 4 to 6 week period when the beetle was present in the field.

Shorey (1970) reviewed the important factors in the development of a reliable, quantitative laboratory bioassay for sex pheromones. In developing laboratory bioassay methods a knowledge of the insects' mating behaviour in the field is an obvious advantage. Thus, factors such as time of mating, duration of mating activity, and age of males and females when mating occurs are important.

In behavioural studies with the pyralid moth, Dioryctria abietella, mating behaviour was influenced by visual and possibly auditory stimuli in combination with the sex pheromone (Fatzinger and Asher, 1971; Fatzinger, 1972). On the other hand, Bartell and Shorey (1969b) showed that the sequence of the responses of male Epiphyas postvittana to the female sex pheromone depended on the pheromone concentration. Low pheromone levels induced antennal elevation while successively higher levels induced flight, upwind orientation, and copulatory movements in males.

Most insects have a diurnal rhythm or "internal clock", and mating, feeding, etc., take place at definite periods during the day. The importance of this factor was clearly demonstrated in the study on the grass grub beetle where consistent bioassay results could only be obtained in the laboratory for a few hours after dusk - a period when mating occurs in the field. With some insects it is possible, by altering their light-dark regime, to change their normal diurnal rhythm. This technique has been applied successfully in many sex pheromone studies.

The author has been able to use this technique with the two

armyworm species, Pseudaletia separata and Persectania aversa, that have been studied. These moths normally mate between 10.00 p.m. and 2.00 a.m. (Clearwater, 1972) but by altering their light-dark periods it has been possible to stimulate mating behaviour at more convenient times; for P. separata between 8.00 a.m. and 11.00 a.m. and for P. aversa between 10.30 a.m. and 1.00 p.m.

Insect sex pheromone studies have mainly been carried out with insects in the order Lepidoptera. This is probably due to the facts that moths generally show classical mating behaviour in the laboratory and can be mass reared which ensures a large and continuous supply of biological material throughout the year.

In laboratory bioassays of insect sex pheromones in the Lepidoptera, various behavioural responses have formed the basis of the method and have included: activation from rest of Trichoplusia ni males (Shorey et al., 1964), upwind orientation in an airstream containing the pheromone and approaches to the pheromone source by Prodenia litura and Agrotis ipsilon (Flaschentrager and Amin, 1950), dances of Bombyx mori (Karlson and Butenandt, 1959), and copulatory movements by Porthetria dispar (Block, 1960).

Many investigators have observed that male insect response to a pheromone source lasts for only short periods and decreases with repetitive tests. Ideally, therefore, behavioural bioassays for a group of insects should only be carried out once a day, for insects readily adapt to the presence of the sex pheromone. Shorey and Gaston (1964) demonstrated adaptation in the cabbage looper moth. At 60 minutes after a brief (30 sec.) exposure to the sex pheromone equivalent to 0.1 female, the moths required a ten-fold higher sex pheromone concentration to induce 50 percent of them to respond, than did males that had not been exposed previously. Adaptation to the sex pheromone was not as apparent in the elaterid beetle, L. calif-

ornicus (Lilly and McGinnis, 1968) for males continued to display strong sexual activity when the interval between tests was reduced to half an hour but not when reduced to 10 min.

Chemicals or extracts to be tested for activity may be presented to the insects in various ways, such as from a glass rod, (Onsager et al., 1968; Casida et al., 1963), from a medicine dropper contaminated with the sex pheromone (Berger, 1966; Butt and Hathaway, 1966) and from filter paper, cotton wick or other absorbent source (Barth, 1961; Gary, 1962; Ouye and Butt, 1962; Shorey, 1966).

Reports on the occurrence of sex pheromones within the order Coleoptera are not as widespread as those in the order Lepidoptera. Laboratory bioassays for beetles have mainly depended on aggregation to active extracts on filter papers and observations of sexual responses which normally take the form of copulatory attempts with one another. This was the case for the sugar beet wireworm, Limonius californicus, (Jacobson et al., 1968), the grain beetle, Trogoderma inclusum,(Rodin et al., 1969), the boll weevil, Anthonomus grandis, (Tumlinson et al., 1969) and the black carpet beetle, Attagenus megatoma, (Silverstein et al., 1967).

In studies with the cigarette beetle, Lasioderma serricorne, Burkholder (1970) found that males exhibited a strong response to the female odour in a Y-tube olfactometer. In this same study, males in a multiple choice olfactometer demonstrated a sequence of antennal elevation followed by extension of the pro- and mesothoracic legs. Leg extension preceded rapid zig-zag locomotion toward the pheromone source and finally copulatory attempts were made with nearby males.

Most of the bioassays of the bark beetle pheromones have been based on walking responses (Wood, 1970). In the bioassay used in the pheromone studies of Dendroctonus pseudotsugae, beetles walking

toward a light passed into a narrow air stream. If the air stream contained an attractive substance, beetles oriented upwind (Borden et al., 1968b). A slight variation of this method was used with Trypodendron lineatum (Borden et al., 1968a). The olfactometer consisted of a runway containing a perforated area underneath which the test substance was placed. Beetles, attracted initially to a microscope light, were observed for arrestment and turning reactions when they moved over the perforated area.

Bioassays based on sexual stimulation need to be used in conjunction with field tests for they do not necessarily lead to the sex pheromone used by the female in nature. For example, Meijer and co-workers (1972) isolated two unsaturated acetates from the female summer fruit tortrix moth both of which acted as sex stimulants in the laboratory. In the field, however, both compounds were inactive unless they were combined. Similar situations exist for the southern armyworm moth (Redfern et al., 1971), the fall armyworm moth (Jacobson et al., 1970a), and the pink bollworm moth (Jones and Jacobson, 1968). Thus, attraction in field bioassays is the ultimate criterion in the identification of sex pheromones.

A more objective laboratory bioassay method is the electroantennogram (EAG) method perfected by Schneider (1957). Since receptor cells on the antennae may respond to odours other than the sex pheromone (Schneider, 1969) the EAG by itself does not give information concerning the behaviour that will follow the response but it has proved extremely useful in complementing behavioural work (Roelofs et al., 1971b).

In some cases where the insect cannot be reared in the laboratory or it simply does not respond under artificial conditions, bioassays have to be carried out under field conditions. Complications in field bioassays may involve the absence or presence of secondary

chemicals (to act as synergists or inhibitors) and the rate of volatilisation of the pheromone (Kaee et al., 1973). Such factors can be critical in obtaining catches in baited traps. For example, Gaston and co-workers (1971) demonstrated that the number of cabbage looper moths caught went through a maximum as a function of the rate of evaporation of the pheromone.

2.10 Factors influencing electroantennograms (EAG's) in insects.

The electroantennogram (EAG) technique measures gross sensory responsiveness to odours at the antennal level. This has been used in sex pheromone studies mainly to complement laboratory behavioural studies. Measurements are made on either intact (Boeckh et al., 1965, 1970; Payne et al., 1970) or excised antennae (Roelofs and Comeau, 1971a, b; Schneider, 1957). In the latter case, the useful life span of the preparation ranged from 10 minutes to several hours while in the former case, measurements have been made for much longer periods without a deterioration in response.

The electrodes are normally placed between the base and the tip of the antenna and consist of either glass capillaries drawn to a fine point (20 μm) and filled with electrolyte, or tungsten wire electrolytically polished to a tip diameter of 1-3 μm (Hubel, 1957). The EAG measurements are generally obtained by pulsing purified air over pheromone-treated filter paper, held in a cartridge, and on to the antennal preparation. The signals from the antenna are normally amplified 200 to 300 times and then fed into a storage oscilloscope or high speed chart recorder. Adler (1971) indicated that in order to obtain reproducible EAG's the following conditions were important:

(1) the length and inside diameter of the cartridge holding the treated paper

(2) the distance from the outlet end of the cartridge to the antenna

(3) the approximate position of the treated paper in the cartridge

(4) the size and type of filter paper used

(5) the type of electrodes and electrolyte used and

(6) the flow rate of the air stream.

The variation in response with antennal length has been investigated for the red-banded leaf roller (Roelofs and Comeau, 1971b) and the cabbage looper moths (Payne et al., 1970). In the former study, maximum voltages were obtained by cutting the excised male antenna near the third distal segment and lower responses were obtained with shorter antennal lengths. On the other hand, Payne et al., (1970) obtained no response with the first flagellar segment of male cabbage looper antennae, presumably due to the lack of sensilla trichodea on that segment, but equal response from other segments along the antenna. These workers also found that while age, light intensity, and time of day, exerted a considerable influence on the behavioural response of male moths, the EAG responses were independent of these variables.

Normally, a maximum amplitude of response is reached as the quantity of pheromone on the paper is increased. This phenomenon may be due to antennal adaptation and/or the saturation of the air within the cartridge at high concentrations, which would mean that increased concentrations would have little effect on the number of molecules in each puff. Kaissling (in Schneider, 1969) obtained a curve without a plateau at high concentrations by standardising his results according to the number of molecules absorbed on the antenna and by eliminating problems due to antennal adaptation.

The shape of the EAG is determined by the rate at which the trace returns to the baseline. Various shapes have been observed with different chemicals and these may be important in terms of

their mode of action on the antennal receptors. For instance, the sex pheromone of the red-banded leaf roller moth exhibits a sharp spike and a moderately fast return to the baseline while the geometrical isomer, which inhibits response, does not produce as sharp a spike and the trace returns to the baseline at a much slower rate. Contrary to this situation, however, the EAG's of naturally occurring sex pheromones normally are characterised by a slow recovery back to the baseline.

Extensive use was made of the EAG technique in the identification of cis-9-dodecenyl acetate as the sex pheromone of the grape berry moth (Roelofs et al., 1971b). In this study, the GC retention times of the sex pheromone were determined by injecting crude extracts on to polar and non-polar columns, collecting the GC effluent at 1-minute intervals in capillary tubes and determining the tube (or tubes) which gave maximum antennal response. The EAG's of the tubes were measured by passing 1 ml of air through the tubes and into an air stream passing over the antenna. The identification of the pheromone depended heavily on the fact that closely related geometrical and positional isomers gave greatly reduced responses and the GC retention time of the acetate giving the maximum antennal response was precisely the same as that of the natural sex pheromone.

Provided model compounds are available, the great advantage of the GC-EAG procedure is that only small numbers of males and females are required. Furthermore, the EAG assay can be used to detect other compounds such as inhibitors and synergists (Roelofs and Comeau, 1971a) that have activity on the antennae but do not elicit behavioural responses in laboratory bioassays.

In general, the strongest EAG responses are obtained with compounds which correspond to the natural pheromone (Boeckh et al., 1965; Sturckow, 1965; Roelofs et al., 1971b; Adler et al., 1972b) while closely related positional isomers of the same carbon chain

length as the pheromone give stronger responses than other compounds with the same functionality.

For pheromones which are doubly unsaturated, EAG tests with mono-unsaturated compounds can sometimes be used to predict the position of the double bonds in the aliphatic backbone. For example, the natural sex pheromone of the almond moth is cis-9, trans-12,-tetradecadienyl acetate and maximum responses were obtained on the male almond moth antennae with both trans-12, and cis-9-tetradecenyl acetates (Beroza, 1972). Similarly, the silk worm moth pheromone is trans-10,cis-12-hexadecadien-1-ol and both trans-10-and cis-12-hexadecen-1-ol gave stronger responses than the respective geometrical isomers (Roelofs et al., 1971a).

Within the Coleoptera, EAG studies have been carried out for a few bark beetle species (Payne, 1970) and the scarab beetle, Popillia japonica (Adler and Jacobson, 1971; Adler, et al., 1972a). In the former study, EAG tests were carried out to test the responses of adult male and female Japanese beetles to their extracts. The heads of the beetles were removed and one glass capillary was inserted through the occipital foramen and the other into the hinged area between the large and the middle plates of the lamellated antenna. Beetles aged 1-2 days were used in the tests which indicated that both male and female adults had a larger EAG response to the male extract that to the female extract. The low responses of males to the female extract may have been associated with the fact that 1-2 day old females were used. Goonewardene et al., (1970a) found,for example,that females influenced trap catches at about 10 days after final ecdysis. In the study by Adler and co-workers (1972a), the magnitude of the EAG responses of the Japanese beetle to attractive and unattractive chemicals paralleled captures of the insects in field trials except that the individual chemicals e.g. phenethyl

propionate, phenethylbutyrate, methylcyclohexane propionate or eugenol, also elicited large responses.

2.11 General procedures for extracting, isolating, and identifying sex pheromones

The methods for isolation and identification of some of the lepidopterous sex pheromones have been reviewed recently by Jacobson (1970) and Beroza (1972). Most of the techniques discussed, however, can and have been used in sex pheromone studies of beetles.

Sex pheromone glands of insects are usually found in the abdominal segments and activity is lost soon after mating. Normally, therefore, virgin females are used and only the abdominal tips rather than whole insects are extracted. Such a step represents a worthwhile purification for Gaston and co-workers (1966) have shown that ether-extractable material in whole cabbage looper moths amounts to about 4%, whereas, ether-extractable material in the last two abdominal segments amounts to only 1% of the whole moth weight.

Extraction of sex pheromones by maceration of abdominal tips has been carried out with a variety of solvents. Lipophilic solvents have been used successfully to extract the sex pheromones of the gypsy moth (Bierl et al., 1972), the black carpet beetle (Silverstein et al., 1967), the western pine beetle (Silverstein et al., 1968) and a dermestid beetle (Rodin et al., 1969). More polar solvents, such as diethyl ether and methylene chloride, are probably more efficient solvents for extracting pheromones and have been preferred by many investigators. Roelofs and Feng (1967) found that extracts of the red-banded leaf roller moth prepared with these two solvents were equally attractive to males whereas extracts prepared with acetone, benzene, chloroform, methanol, or 95% ethanol were less attractive. Read (1968) extracted the sex pheromone of

the false codling moth with an initial ethanol maceration followed by the addition of water and partition into cyclohexane. In the isolation of the southern armyworm sex pheromone, however, (Jacobson et al., 1970a) methylene chloride was preferred to ethanol because ethyl esters formed during extraction with the latter solvent made subsequent purification steps difficult. A major problem in extracting pheromones by solvent maceration can be the co-extraction of lipids, sterols, etc., which mask or prevent the evaporation of the pheromone in bioassay tests. This was overcome in Read's study by column chromatography of the crude extract. A gain in activity of two orders of magnitude was also obtained during the first purification steps of the Indian meal moth pheromone (Dahm et al., 1971). Surface rinsing, rather than maceration with solvents, has also been used for pheromone extraction and may be a method of obviating masking problems, for fewer co-extracted impurities would be expected with this technique.

Sex pheromones have also been recovered from an air stream which is passed over virgin females and into a cold trap, a liquid, or through an adsorbent (Yamamoto, 1963; Jones et al., 1965; Silverstein and Rodin, 1966). Advantages of this method are that the only contaminants are other volatiles and the process can be carried out throughout the life of the insect. Silverstein has pointed out, however, that oxidation losses can occur and recovery by aeration is only feasible for compounds more volatile than methyl myristate. Pheromones have also been recovered by solvent extraction of filter papers on which virgin insects have been held (Brady et al., 1971a), from frass (Silverstein et al., 1966, 1968), and faecal material (Tumlinson et al., 1968).

Initial concentration steps are usually gross ones to remove the large bulk of co-extracted material. The methods include solvent

partition to give neutral, alkaline or acidic fractions, precipitation of inactive fats and waxes from methanol or acetone at -20°C, gel filtration, steam or vacuum distillation. The technique of precipitating fats and waxes has been used in isolation studies on the pink bollworm moth (Jones et al., 1966), the fall armyworm moth (Sekul and Sparks, 1967), the false codling moth (Read, 1968), the southern armyworm moth (Jacobson et al., 1970a), and the Indian meal and the almond moths (Kuwahara et al., 1971b). Gel filtration on Sephadex LH-20, a bead-formed dextran gel resistant to organic solvents, has been used for the Indian meal moth (Dahm et al., 1971) and the summer fruit tortrix moth (Meijer et al., 1972). In the latter study, high pressure liquid chromatography was used as the next concentration step and gave a fraction which had only two peaks on subsequent analyses by gas chromatography. Steam distillation was used as an initial purification method for the boll weevil (Tumlinson et al., 1968), while short path high vacuum distillation on to a cold finger filled with dry ice is an initial concentration method used consistently by Silverstein's group for bark beetle aggregation attractants.

Further purification of an active fraction can be achieved by column, thin layer, and paper chromatography. Thin layer chromatography gives a resolution of compounds which is comparable to paper chromatography and superior to column chromatography. Thin layer plates can be developed much faster than paper and can tolerate 10-100 times more material. In column and thin layer chromatography the usual adsorbents used are either silica gel, Florisil, or alumina. All these concentration steps result in pheromone loss due to adsorption effects. Gaston and co-workers (1966) showed, for example, that only 70 percent of the cabbage looper pheromone could be recovered from silica gel by elution with ether. Read

(1968) also had this difficulty and removed the pheromone activity from silica gel by steam distillation.

The final purification step normally involves the use of gas chromatography where an active component is collected on a polar stationary phase followed by re-injection and collection from a non-polar stationary phase. As insects are extremely sensitive to their sex pheromones. the presence of very small amounts of highly active impurities in the GC fraction is an ever present hazard which could lead to erroneous conclusions. Normally, therefore, the activity of the isolated component is checked on at least four different stationary phases of different polarity and selectivity (McDonough et al., 1972; Bierl et al., 1972). Gas chromatography on open tubular or capillary columns can also be used to determine purity.

Various methods have been used to trap sex pheromones in gas chromatograph effluents. Bierl and co-workers (1972), for example, use a gas chromatograph equipped with a flame ionisation detector for analysis and the less sensitive thermal conductivity detector for collection of fractions. The latter detector differs from the former in that it does not destroy the compound as it passes through the detector and it is relatively insensitive to back pressure at the detector exit. Thus, the thermal conductivity detector allows fractions to be collected as they pass from the detector exit while stream splitting has to be used with the destructive flame ionisation detectors. Bierl and co-workers (1972) trapped fractions in 700 µl of hexane or methylene chloride at -70°C after the effluent had passed through a cooled 90 cm Teflon coil attached to the detector exit. Roelofs and co-workers (1971b) trapped fractions in 30 cm long capillaries at the column outlet, while Tumlinson et al., (1969) bubbled the effluent through dichloromethane and Meijer et al., (1972)

collected fractions in glass capillaries cooled with liquid nitrogen.

Identification of minute quantities of the isolated pheromone has been achieved by use of mass, infra-red, ultraviolet and nuclear magnetic resonance spectroscopy. This information has been complemented by chemical transformations such as hydrogenation and micro-ozonolysis using a gas chromatograph combined with a mass spectrometer to identify the transformed products. Final confirmation of structure is achieved by synthesis and field testing for biological activity.

Normally, the small amount of pheromone isolated presents a major obstacle for identification purposes. Recent studies have shown, however, that considerable progress can be made despite this handicap by carrying out simple micro-chemical tests on crude extracts or active fractions to reveal the presence or absence of particular functional groups. Numerous investigators have determined the presence of unsaturation by loss of activity of the crude extract on hydrogenation, ozonolysis, or bromination. The presence of an acetate group has been determined by the loss of activity on saponification or reduction with lithium aluminium hydride and restoration of activity on acetylation of the neutral fraction. If the sex pheromone was a methyl ester, activity would be recovered by acidifying the alkaline solution and esterifying with diazomethane.

Beroza (1972) has pointed out that subtraction loops in a gas chromatographic system can also be used for functional group analysis. A subtraction loop is a short tube containing a reactive chemical on a gas chromatographic support that retains or 'subtracts' compounds with certain functional groups and allows other to pass. Thus, an o-dianisidine loop retains aldehydes, a benzidine loop aldehydes and ketones, a phosphoric acid loop epoxides, a zinc oxide

loop carboxylic acids and a boric acid loop primary and secondary alcohols.

Functional groups can also be determined by carrying out chemical reactions on thin layer plates. For example, Bierl et al., (1972) overspotted an active gypsy moth extract on a TLC plate with phosphoric acid and found after development of the plate and elution of the adsorbent, that the activity had disappeared. This indicated that the pheromone contained an epoxide group. Similar treatment of the active spot with aqueous semicarbazide hydrochloride did not affect the activity which indicated the absence of an aldehyde or ketone group in the pheromone. The polarity of the pheromone and the configuration of the double bond can also be derived from thin layer plates by comparing the chromatographic mobility of the pheromone with known compounds (Roelofs et al., 1971b; Bierl et al., 1972).

An indication of the size of the molecule, whether it is branched or a straight chain, and the functional groups possibly present, can be obtained from gas chromatographic retention times by the use of Kovats' retention indices (Ettre, 1964). These are obtained on several columns of different polarity.

A knowledge of the functional groups present in the pheromone can provide a means of generating more pheromone from an inactive chemical precursor in the extract. For instance, when functional group tests on the oblique-banded leaf roller (Roelofs and Tette, 1970) and oriental fruit moths (Roelofs et al., 1969) indicated the pheromones were acetates, the crude extracts were saponified and reacetylated. This resulted in a one hundred-fold increase in the sex pheromone for both moth species. This technique was also used for the Mediterranean flour moth (Kuwahara et al., 1971a) and the southern armyworm moth pheromones (Jacobson et al., 1970a).

In a different example, Bierl and co-workers (1972) obtained a ten-fold increase in pheromone content of the gypsy moth extract by epoxidising the precursor in an active hydrocarbon fraction. Beroza, in his review (1972), hypothesized on a number of possible precursors of pheromones containing a particular functional group and his table is reproduced below.

Table 2.1 Functional groups of attractants and possible precursors. Reproduced from Beroza (1972)

Functional group in attractant	Possible precursors
Epoxide	olefin
Ester	alcohol, phenol, carboxylic acid
Alcohol	ester, carboxylic acid, ketone aldehyde
Carboxylic acid	ester, alcohol, salt
Aldehyde	alcohol, carboxylic acid
Ketone	alcohol
Amine	amide
Methyl ether	phenol, alcohol
Methyl ester	carboxylic acid

A technique called mass fragmentography or single ion mass detection (Brooks and Middleditch, 1971; Gordon and Frigerio, 1972) is useful in identifying pheromones where the chemical behaviour of the pheromone, in various functional group tests and concentration steps, is consistent with that of a known compound. In this technique, the mass spectrometer (coupled to a GC) is focussed on one prominent, specific fragment ion so that only compounds in the GC effluent with a mass spectrum containing the focussed ion will be recorded by the ion monitor. In this capacity, the mass spectrometer can be used as a specific GC detector and the sensitivity of detection is at least 100 to 1000 times better than that of conventional GC methods based on flame ionisation and thermal conductivity. Compounds can,

therefore, be detected in the picogram range and this is very useful in pheromone studies where the active component is usually present in nanogram quantities.

2.12 Sex pheromones as control agents for insects

The use of sex pheromones for direct control is in its early stages. Promising preliminary results have been obtained with such insects as the boll weevil (Hardee et al., 1970), the cabbage looper (Shorey et al., 1967), the red-banded leaf roller (Roelofs et al., 1970), bark beetles (Vite and Pitman, 1970; Pitman, 1971) and the gypsy moth (Beroza and Knipling, 1972).

On the other hand, sex pheromones have been used successfully for a number of years as indirect control agents. For example, sex pheromone traps have been used to arrest the spread of the gypsy moth in North America by using insecticides in areas where moths have been found in traps (Beroza, 1970). Pheromones have also been used at international ports to provide early warning of possible entry of serious insect pests.

Various workers (Wright, 1964; Graham et al., 1966; Gaston et al., 1967; Shorey and Gaston, 1967; Shorey et al., 1967, 1968b) have recognised two possible ways of using sex pheromones in direct behavioural control programmes. One method depends on luring males to traps, points or zones where they can be removed from the population. The other method consists of confusing males with high levels of pheromones in the atmosphere. The objective of both programmes is based on disrupting communication between male and female insects to prevent effective mating from taking place.

Another possibility which has been suggested for insect control, but has not been tested, is the use of inhibitors to mask the natural female sex pheromone in the field. This phenomenon, associated with

chemicals which closely resemble the sex pheromone, has been well documented in recent scientific reports. For example, Roelofs and Tette (1970) found cis-11-tetradecenyl acetate to be the sex pheromone of the oblique-banded leaf roller moth. In the laboratory, the trans isomer elicited a stimulatory response in males but in field bioassays it acted as a very potent inhibitor when added in low percentages to the pheromone. A similar inhibition occurred with the geometrical isomers of the sex pheromones of the oriental fruit moth (Roelofs, et al., 1969), the cabbage looper moth (Berger, 1966), and the pink bollworm moth (Jacobson, 1969). Recently, tetradecyl acetate was found to be an inhibitor of hexalure, the pink bollworm moth synthetic sex pheromone (Beroza et al., 1971a).

The principles of insect suppression through the use of mass trapping with sex pheromones were developed by Knipling and McGuire (1966). They emphasised that pheromone trapping for insect control should only be attempted on a low population. Prior to this study, all attempts to control insect populations with this approach were unsuccessful. This was probably due to the fact that insufficient traps were used to out-compete the females in the high populations. For instance, Ambros (1938) used about 20,000 females of the nun moth, Porthetria monacha, in traps over an area of 2,000 ha and attracted 150,000 males. If it is assumed that the sex ratio in the field was 1:1, there must have been at least 150,000 females in the field competing against the 20,000 females in traps.

Beroza and Knipling (1972) have pointed out that to use sex pheromones effectively in control programmes a knowledge of behaviour, population density, and population dynamics of a given species is important. For example, the information needed in control programmes based on mass trapping, includes the size of the insect population, the ability of traps to compete successfully

with live females (which determines the number of traps required), and the growth rate of the insect population.

The validity of these principles has been confirmed by recent successful field-trapping studies on the boll weevil (Hardee et al., 1970) and the red-banded leaf roller moth (Roelofs et al., 1970). Promising results have also been obtained in control programmes with the bark beetle pheromones where beetles have been drawn to pre-selected resistant trees (Gara et al., 1965) or insecticide-sprayed trees (Pitman, 1971; Vite and Pitman, 1970).

In the boll weevil study, traps baited with males, placed in and around a field of cotton in a heavily infested area, captured more than 1,000 over wintered weevils per ha of cotton but failed to reduce populations because the weevils in the traps were unable to compete with the large number of native weevils. In a lightly infested area, however, a similar number of traps captured enough over-wintered weevils to suppress the population until dispersal began. To control a light infestation of red-banded leaf roller moths on an 8 ha site in an apple orchard, Roelofs and co-workers (1970) used sticky traps baited with a mixture of the sex pheromone and a synergist, dodecyl acetate. The traps were about twice as effective as females and 99% control was achieved with 1110 traps in a low population (1 moth/100 m^2) while only 48% control was obtained with 2,400 traps in a slightly higher population (16 moths/ 100 m^2).

Perhaps a greater chance of success of controlling high insect populations with sex pheromones is with methods based on confusion or inhibition. The chances of control would be improved even further if the insect's odour receptors became fatigued or saturated with the ever present pheromone. This phenomenon of adaptation has been observed in laboratory bioassays for a number of insects and

more particularly with male gypsy moths which become quiescent when exposed to high levels of the sex pheromone. This inhibitory effect of the sensory cells would be to no avail, however, if the final stage of mate-finding in the field, involved the use of sight as suggested by Doane (1968), Traynier (1968) and Shorey and Gaston (1970). This would be especially so with high population densities. Furthermore, there is the chance with insects held under inhibitory conditions by chemical stimuli, that they would adapt to this situation and depend more heavily on visual stimuli for communication purposes.

The feasibility of the confusion method has been demonstrated in laboratory studies for the black carpet beetle (Silverstein, 1971), and the European corn borer (Klun and Robinson, 1970), and on a limited scale in the field for the cabbage looper (Gaston et al., 1967; Shorey et al., 1967) and the gypsy moth (Stevens and Beroza, 1972). In their study on a 0.1 ha area with the cabbage looper sex pheromone, Shorey and co-workers (1967) calculated that approximately 10^{-10} g/l of air of the pheromone evaporated from 100 reservoirs each containing 17 mg of the synthetic material. This level of pheromone was sufficient to prevent completely the orientation of males to virgin females in traps on the plot. This quantity of pheromone amounted to less than 0.5 g/ha/night. In the gypsy moth trial carried out with light infestations on 16 ha plots, disparlure on hydrophobic filter paper pieces was distributed by air at the rate of 50 mg/ha. Released males were unable to locate virgin females for 6 days, and even 21 days after the treatment the number of males caught on the treated area was only one third of the number caught in the control area.

The mechanism of action in confusing males and the influence of population density on the effectiveness of the methods have not been established. For the gypsy moth inhibition trial, Beroza and

Knipling (1972) suggested a mechanism analogous to the over-flooding ratio proposed for mass trapping. They claim that as insufficient disparlure was used for habituation of the moth's receptors, the males were probably diverted to the pieces of filter paper which would have had a great attractant power for the males. Thus, confusion took place because of the numerical superiority of the pieces of filter paper over the females in the population. With such a mechanism, the confusion method would be more effective against low, than against high populations.

Some indication of the amount of pheromone needed to cause confusion in the field can be gained from a knowledge of the size of the insect population and the quantity of pheromone present in each female. Each female grass grub beetle contains an average of 1.5 µg phenol. If it is assumed that this is completely discharged during the mating period each day, [note that female silkworm and gypsy moths are apparently capable of releasing and re-synthesising their total pheromone content every few minutes (Shorey et al., 1968b)] this would mean that a population of 10,000 females, emerging over a hectare of pasture during the 20-minute mating period at dusk, would release approximately 0.015 g phenol into the atmosphere (i.e. ca.0.05 g/h).

Releasing phenol at a rate of 5 g/ha over this area would, therefore, reduce the chance of a male finding a female to 1 in 100. The efficiency of the technique depends largely on the average number of responses that the males make in their lifetime.

2.13 Sex pheromone release in the field

The critical nature of the sex pheromone release rate on attraction was demonstrated by Gaston et al., (1967) and Sharma et al., (1971). These workers showed that male moth catches with

pheromone baited traps went through a maximum as a function of pheromone release. In the latter study, the effective range of pheromone release was 0.03-1.0 µg per minute, and above this the activity decreased. In another study involving the red-banded leaf roller moth, the optimum rate of release of the sex pheromone was less than 1 µg per hour (Glass et al., 1970). These investigations clearly illustrate the degree to which pheromone release must be regulated in order to use the attractant properties of pheromones. Indeed, if mass trapping is to be used successfully as a control method, pheromone formulations have to be developed which are capable of out-competing virgin females many times over. None of the formulations that have been devised to date have succeeded in doing this, however.

In order to achieve the desired effect, pheromones have only to be released over the short period of the day when mating takes place. In practice this condition is difficult to achieve and is overcome by releasing the pheromone over the entire day during the mating cycle of the insect. Thus, there are lengthy periods throughout the day when the released pheromone is biologically ineffective. Various methods have been used to minimise the loss of pheromone during these periods and have mainly depended on selecting a substrate on which the rate of release of the pheromone has a low temperature dependance. Substrates that have been used have included sand (Wolf et al., 1967), cane fibre squares (Steiner et al., 1965), glycerides of lard (Hart et al., 1966), silicone greases, carbowax, glycerol monooleate (Daterman and McComb, 1970), polythene bags (Toba et al., 1969), rubber septa (Roelofs et al., 1971b), and polythene closures (Glass et al., 1970). Pheromone injected into the polythene closures permeated slowly through the polymer wall and retained activity for several months.

The sex pheromone of the gypsy moth, disparlure, has been combined with trioctanoin (a fat constituent) for slow release, and has remained active in the field at levels as low as 0.001 µg per trap for over three months (Beroza et al., 1971c). Furthermore, some of these formulations were slightly more attractive than virgin females.

The use of the terpene mixture, grandlure, which is attractive to both sexes of the boll weevil, has been restricted because it remains effective for very short periods in the field. Various formulations have been used to extend its activity in the field and have included mixtures of grandlure with firebrick, polyamide resin and cellulose acetate (McKibben et al., 1971). Recently, a formulation with Carbowax 1000 was devised which was about 80% as effective as males for several days (Hardee et al., 1972).

A further complication is the instability of some attractants in the field and this may be overcome by combination with antioxidants, ultraviolet absorbers or dilution with an inert solvent. For example, when antioxidants were combined with the cabbage looper sex pheromone, the activity was retained after 6 months' exposure in the field (Wolf et al., 1972).

CHAPTER III

THE BIOLOGY OF THE GRASS GRUB AND THE COMMON ARMYWORM

Grass grub

3.1 Introduction

The common grass grub is a native insect of New Zealand and can be found in all regions of the North and South Islands as well as the Chathams (Hoy, 1963). Given (1966) placed the grass grub in the following taxonomic position:

Order	Coleoptera
Family	Scarabaeidae
Subfamily	Melolonthinae
Tribe	Colpochilini
Genus	Costelytra Given
Species	zealandica (White)

The insect has adapted to a wide range of environmental conditions, for the North and South Islands span a latitude of 14 degrees and the climatic conditions range from subtropical in the north to temperate in the south. The increase in grass grub infestations has been favoured by the introduction of pastoral farming, which has meant the provision of permanent pasture in place of native vegetation which was dominated by forests and tussock grasslands (*Poa* spp. and *Notodanthonia* spp.). In the native vegetation, grass grub populations were probably held in check by natural enemies (Given, 1967).

The high producing introduced pasture species mainly affected

by grass grub larvae include, perennial ryegrass (Lolium perenne L.), cocksfoot (Dactylus glomerata L.) and white clover (Trifolium repens L.). Infestations are known to exceed 2000/m^2 and about 400/m^2 can cause major damage. The typical damage caused by the larvae is the drying off of pasture during the autumn - winter period, due to the severing of grass roots. Areas containing large larval numbers can be left completely bare as the grass dies and is blown away. Where the pasture has a closely knit mat of roots, it may be rolled up like a carpet. Where there is a lower amount of root growth, plants are left loose in the soil and may be easily lifted. Pasture in this condition can be readily damaged by stock and can lead to erosion problems especially in hill country areas.

In view of the economic importance of the pest and the unsatisfactory control obtained with conventional insecticide treatments, various alternative control methods have been investigated. Generally these have been unsatisfactory for one reason or another. For example, cultural methods and the planting of lucerne have given satisfactory control under favourable conditions (Kain and Atkinson, 1970) but such methods are not widely applicable in New Zealand due to the terrain and current agricultural practice. A number of bird species have been reported to feed on grass grub larvae (East, 1972) but as their numbers are small and their habitat preference fairly restricted they would not be expected to give adequate control. Attempts to establish biological control methods for grass grub have been unsuccessful due to the fact that it is a native insect (Hoy, 1955; Given, 1967).

Control of the Japanese beetle, Popillia japonica, a scarab which is closely related to grass grub, has been achieved in the United States with the bacterial infection, Bacillus popilliae

(Dutky). Dumbleton (1945) recorded a native bacillus, Bacillus (near) popilliae, in grass grub where the blood of the infected larvae had a milky appearance due to the presence of numerous spores of the bacillus. Hoy (1955) reported, however. that although the disease was widely distributed, the incidence of diseased larvae seldom exceeded 5 percent of the population. Grass grub larvae can also be infected with Bacillus popilliae (Dutky) but field liberations of the bacterium have failed to spread beyond the liberation site (Kelsey, 1966a). Fungal diseases have also been reported to infect grass grub larvae (Helson, 1965; Latch, 1965; Brown, 1966) but the incidence of these diseases is low.

3.2 Life cycle

The life cycle and biology of C. zealandica have been reviewed by Dumbleton (1942), Miller (1945), Kelsey (1951), Pottinger (1968) and East (1972). Grass grub is a univoltine insect but under drought conditions (East, 1972) and a high altitude environment, the life cycle may be extended to two years (Stewart and Stockdill, 1972). In all stages of the life cycle (Plate 3.1) except the adult, the habitat is subterranean. The adult beetle is brown, about 1 cm long and is commonly known as the brown beetle. The female beetle lays eggs about 7 - 18 cm deep in the soil from the end of spring to the beginning of December. The eggs are laid in clusters of 3 - 40 and after about two weeks, first instar larvae hatch (Kelsey, 1950).

In common with most scarabs, there are three larval stages which can be distinguished by head capsule size (Kelsey, 1970). Fully grown creamy-white larvae are about 1.8 cm long, crescent shaped, and feed on roots close to the soil surface. The third instar larvae are present from mid-autumn (April) through to late

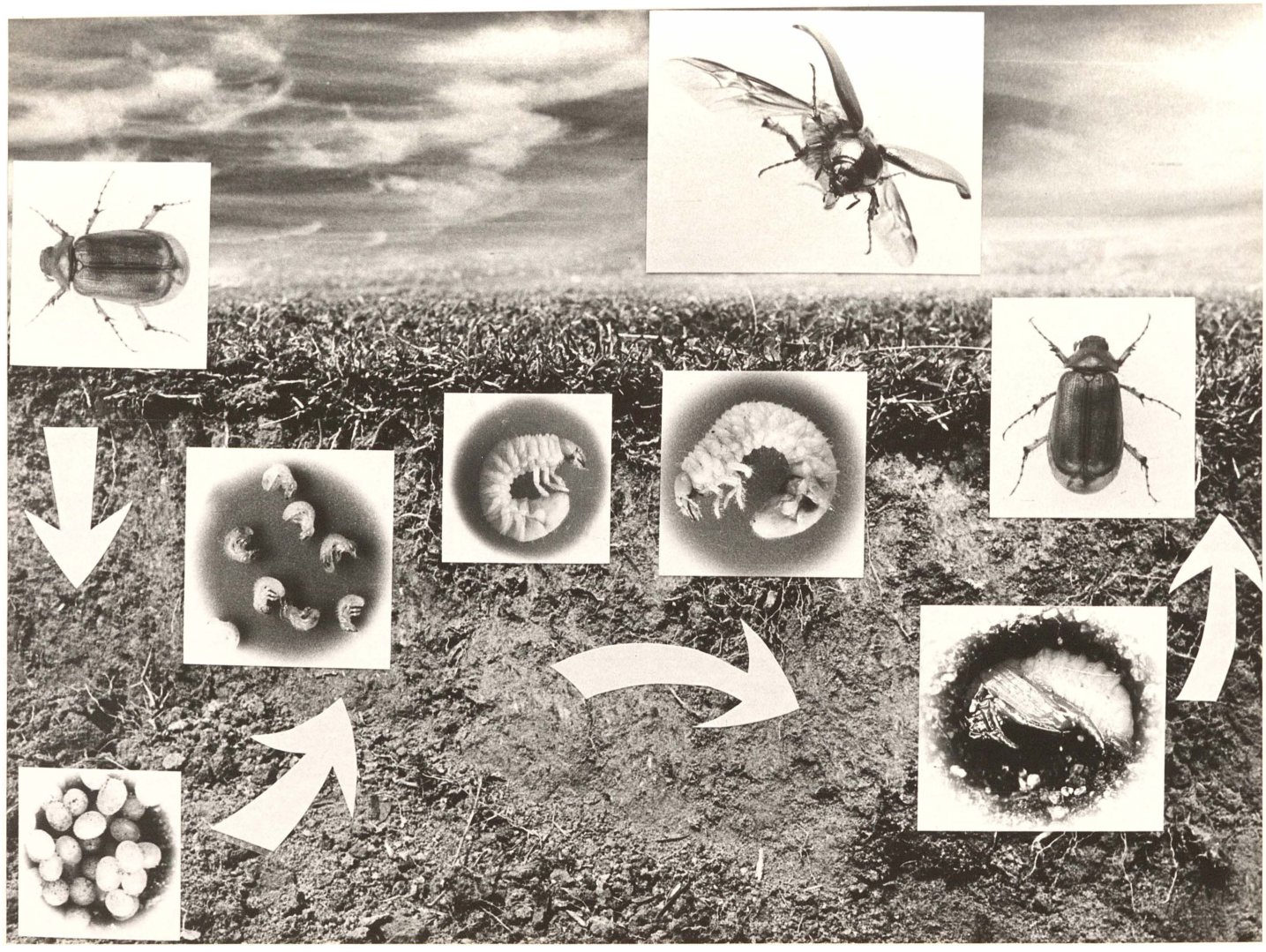


Plate 3.1 Stages in the grass grub life cycle superimposed on the soil profile indicating the depths in the soil that the stages occur.

June. From then onwards, fully grown larvae move down into the soil to depths of 10 - 25 cm, evacuate their gut contents and form an oval-shaped soil cell in preparation for pupation. Prior to pupation, the larvae build up large fat deposits which give them a yellow colouration. The prepupal and pupal stages extend over the September-October period. Adults first appear in the soil in late October and remain there, for 6 - 10 days or more as teneral adults, before emerging from the soil to fly and mate on the pasture on spring evenings. Helson (1967) found from light trap catches that peak beetle flights occur during November and December.

3.3 Adult Behaviour

Emergence, mating and flight behaviour have been reported by Kelsey (1968). He found that flights did not take place unless the air temperature exceeded 9.5°C and winds were below 10 kph. Flights commence at dusk on spring evenings and continue for about twenty minutes to half an hour. During this period, beetles fly in a random fashion in a dense swarm at heights of up to 3 m, with the great majority below 2 m.

The early flight period is dominated by males, (greater than 95 percent of the population) actively searching for females by making short low flights just above the pasture (Kelsey, 1951, 1968). Females, which emerge in the evening shortly after males, do not fly during this period but simply wait on grass stems or on the soil surface. Some females wait partly submerged beneath the soil surface with only their abdomens protruding. Under normal circumstances, female beetles mate within a few minutes of emerging from the soil and the oviposition site of the first cluster of eggs is close by in the soil. Males are attracted to females by a chemical sex pheromone (Kelsey, 1966b) which may be produced by symbiotic bacteria in the

colleterial glands of the female (Hoyt et al., 1971). The majority of beetles (in excess of 85%) emerge within one week and are mated usually within two days following the initial emergence (Kain, personal communication). Males and females are polygamous in their mating behaviour and under field conditions beetles live about 2 to 3 weeks (Fenemore, 1966).

The flight behaviour of adult females in relation to their reproductive condition has been clarified by the recent work of Kain, some of which is described in the words of East (1972). "Kain found that females are capable of laying four egg clusters, with the first cluster comprising over 80% of the total number of eggs laid. He found that most females did not feed prior to laying the first cluster but feeding was an essential prerequisite to subsequent ovipositions. His field observations confirmed that most females are mated close to the point of emergence and do not fly before the first egg cluster is laid. A single mating was found to be sufficient to fertilise all eggs, the female having the ability to store sperm from a single mating within the spermatheca for the rest of its short life. Kain observed that in the Waikato many females flew after they had laid the first egg cluster, with the sex ratio of flying beetles changing from predominantly males at the beginning of the flight season to predominantly females at the end. Females tended to fly at greater heights, in a more directional manner and later in the evening than males. Flight direction was into the wind or towards the setting sun on calm evenings."

Grass grub adults are polyphagous feeders but appear to favour willow, ornamental and fruit trees. Recently, Osborne and Hoyt (1968) demonstrated that flowers of the elder (Sambucus nigra L.) contained a chemical attractant for female beetles.

Common armyworm

3.4 Introduction

The common armyworm moth has been placed in the following taxonomic position:

Order	Lepidoptera
Family	Noctuidae
Subfamily	Hadeninae
Genus	Pseudaletia
Species	separata, Walker

Within the family Noctuidae, sex pheromones common to a number of species have been identified (Berger, 1966; Sekul and Sparks, 1967; Jacobson et al., 1970a). In general, females use unsaturated aliphatic acetates to attract males immediately prior to mating.

The insect is not a native of New Zealand and according to Franclemont (1951), its distribution ranges from the Amurland through China and Japan to the Phillippines, through eastern India to the Dutch Indies and thence to Australia, New Zealand and Fiji. The moths are capable of flights exceeding 1000 km (Johnson, 1969) and this may be the way the insect entered New Zealand.

Recently, in an effort to diversify New Zealand's farm produce, farmers have been encouraged to grow crops as an alternative for the more traditional forms of farming. In this respect, maize production, particularly in the Waikato region, has increased rapidly and as the army caterpillar is a major pest of maize, farmers have had to be particularly vigilant in assessing the levels of infestations in their crops.

A number of insecticides have proved to be effective in controlling the infestations (Kain et al., 1968) but the cost of

application of these materials can be significant, particularly if crops have to be sprayed several times. Very few investigations have been carried out on alternative control measures.

3.5 Life cycle

There has been no detailed information published on the life cycle of the common armyworm under New Zealand conditions. The life cycle is illustrated in Plate 3.2. The adult moth is about 2 cm long and each female can lay between 800-900 eggs. The female appears to have an oviposition preference for crops and on maize plants the oviposition sites include the inner leaf whorl, tassels, silks, and lower dried leaves. The adults live for about 2-3 weeks. Mating takes place in the evening between 10 p.m. and 2.0 a.m. (Clearwater, 1972) and females commence laying eggs when they are about 5-6 days old.

Light trap recordings in the Waikato region have indicated that the moth is present for about 9 months of the year from February until November with peak flights occurring any time from February until August (Kain et al., 1968).

The moths are capable of long distance flights with the flights of longest duration made by moths 3-4 days old. Hwang and How (1966) showed that the maximum flight activity occurred before the full development of the ovaries on about the fifth day after emergence.

Eggs normally take about a week to hatch and the caterpillars go through a number of instars before pupation takes place. Within the northern region of New Zealand, it is probable that there are more than three generations each year. The caterpillars are polyphagous feeders and as they approach maturity they become voracious feeders and do severe damage either at night or during dull moist day conditions. Under certain environmental conditions the cater-

Plate 3.2 Stages in the life cycle of the common armyworm.

pillars undergo phase polymorphism. For example, under over-crowding conditions the caterpillars change colour from brown to black and their behaviour becomes frenzied while their rate of development appears to increase. It normally takes a period of at least 4 weeks from the time eggs hatch to the formation of pupae. Brown pupae are formed in earthern cells and the insect probably overwinters in this form.

PART I

CHAPTER IV

LABORATORY HANDLING AND BIOLOGICAL TESTING OF GRASS GRUB

4.1 Introduction

A major obstacle in the identification of insect sex pheromones has been the minute amount of the pheromone usually present in insects and consequently it has been necessary to acquire 10^4-10^5 insects to obtain sufficient material for identification. In an extreme case, Butenandt and co-workers (1959), who identified the first sex pheromone - that of the silkworm moth, required 5×10^5 female moths and then only obtained 12 mg of pure material.

In order to obtain sufficient numbers, therefore, insects can be either collected in the field before adults emerge or mass reared in the laboratory. In the latter method especially, considerable skill is required to raise healthy colonies. Frequently the sexes must be separated before they mate because of the possible reduction in sex pheromone production after mating, and only the small part of the insect containing the sex pheromone glands is required for extraction purposes.

The rearing of grass grub adults from eggs in the laboratory has only met with limited success to date. Thus, in order to obtain a relatively large quantity of unmated biological material, it was necessary to use the established method of collecting the fully fed third instar larvae in the field and raising these through to adults in the laboratory.

The development of a consistent, reliable bioassay method is essential for the isolation and identification of a sex pheromone. The ideal bioassay method is one carried out under field conditions but this can impose severe restrictions on the investigation. These include short adult flight periods, limited duration of the insects' mating activity each day, prevailing weather conditions which may inhibit flight, and the large quantities of biological material required in each test. These difficulties can be overcome to some extent by developing suitable laboratory bioassay methods.

In a report on sex attraction in the grass grub beetle, Kelsey (1966) indicated that female beetles had the ability to attract adult males from distances of 200 m in the field and inferred that males were attracted to females by a windborne chemical sex pheromone. In the first year of this study, an attempt was made to quantitate this response in the laboratory and an olfactometer was developed which depended on upwind orientation of male beetles to the sex pheromone. Olfactometers of the "Y" choice type, in which insects have a choice at the "Y"-junction to fly up either the attractant arm or the unbaited (control) arm, have been used in a number of studies with moths (Guerra, 1963; Keys and Mills, 1968) and in one study with beetles (Burkholder, 1970).

In the second year of this study, a more direct bioassay was used. The method depended on observations of male copulatory activity and was based on a report by Onsager and co-workers (1968) who found that sexually excited elaterid beetles, L.canus, frequently mounted any small object encountered and performed copulatory actions. It was thought that such a method would allow a greater number of tests to be made each evening and provide a more direct, positive response when testing the activity of the many fractions obtained in developing the isolation procedure. In view of the short period that the adults

were available, such improvements in the bioassay procedure had obvious advantages.

4.2 Experimental

(a) Larval collection and laboratory handling procedures

Fully fed third instar larvae were turned to the surface, by means of a plough set to produce a 15 cm deep furrow, and placed in miniature ice-cube trays which were then sealed with polythene-covered hardboard lids. These methods are illustrated in Plates 4.1 and 4.2. The trays were placed in polythene bags containing damp sphagnum moss (to maintain a high humidity) and held in an incubator until pupation occurred. As pupae formed they were removed and separated into males and females in fresh ice-cube trays. As beetles emerged their sex was checked and they were placed in miniature ice-cube trays. A layer of plaster of Paris was used in the cubicle bottoms to reduce wing damage and mortality. The trays of beetles were sealed with polythene-covered hardboard lids and placed in polythene bags containing damp sphagnum moss. Beetles were held in a bioassay room and all the handling and observations of male beetles after they had been sexed were carried out in this room.

(b) The olfactometers used in the bioassay

The behaviour of grass grub beetles was observed in a temperature controlled room at 18-20°C which is slightly higher than the average air temperatures at dusk in spring (14-16°C) when grass grub mating flights take place. The bioassay room was illuminated with two 60 watt incandescent bulbs shining through two Ilford 900 filters having a transmittance above 620 nm. The intensity of the light was adjusted by a "Variac" transformer and approximated conditions at dusk. Light intensities were determined by a selenium photo

Plate 4.1 Collecting grass grub larvae in the field.

Plate 4.2 Sorting fully fed third instar larvae into ice-cube trays.

cell amplified by an E.I.L. "Vibron" electrometer.

The "Y"-choice olfactometer (Plate 4.3) was constructed from "Perspex" tubing 7.5 cm ID, each portion of the "Y" being 30 cm long. Flat "Perspex" tracks were placed at the centres of the tubes to enable the beetles to walk along the apparatus. Before each bioassay, male beetles were allowed to adjust to the lighting and temperature conditions of the test period by containing them in the straight portion of the "Y" behind removable wire mesh gates. The upstream gate was removed at 6.0 p.m. after a one and a half hour "conditioning period".

Traps were constructed at the end of each arm of the "Y" (Plate 4.4). To be trapped in either arm of the "Y", the beetles had to climb a wire gate situated just before each trap, and pass through a hole slightly larger than a beetle. The hole was placed 1 cm above the "Perspex" track in the centre of the wire gate. (Note: A prototype wire gate with two holes is shown in Plate 4.4).

Air was drawn through the apparatus by an electric hair drier controlled by a "Variac" transformer. The air velocity through each arm of the olfactometer was found to be the same and varied between 0.5 and 0.8 kph as measured by an A.E.I. Velometer. Because sex pheromones have a physiological activity at very low concentrations, vapours were removed from the room to prevent contamination by directing the air outlet of the hair drier towards an extract fan.

Each trial was conducted between 6.0 and 9.0 p.m. using male beetles 10-15 days after final ecdysis. Five female beetles to be used in each test were taken from sphagnum moss held at 10°C and crushed with the flat of a scalpel blade on to a small filter paper. The abdomens were opened completely and the bodies as well as the abdomen contents spread out on to filter paper. This was immediately

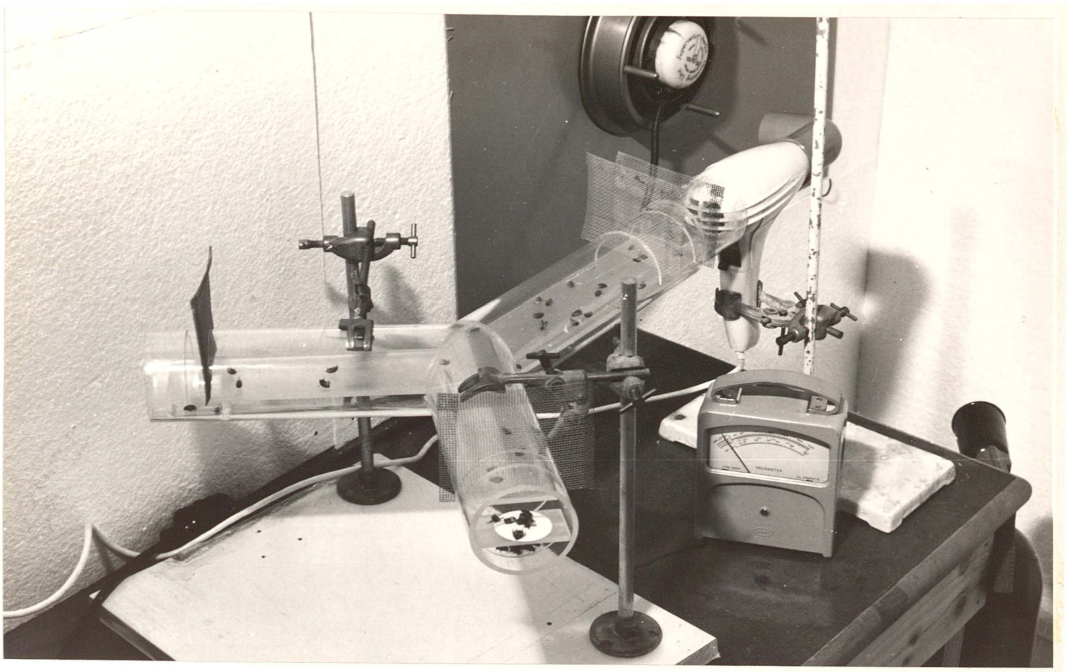


Plate 4.3 The "Y"-choice olfactometer used in the initial tests to demonstrate the presence of a sex pheromone in the female grass grub beetle.

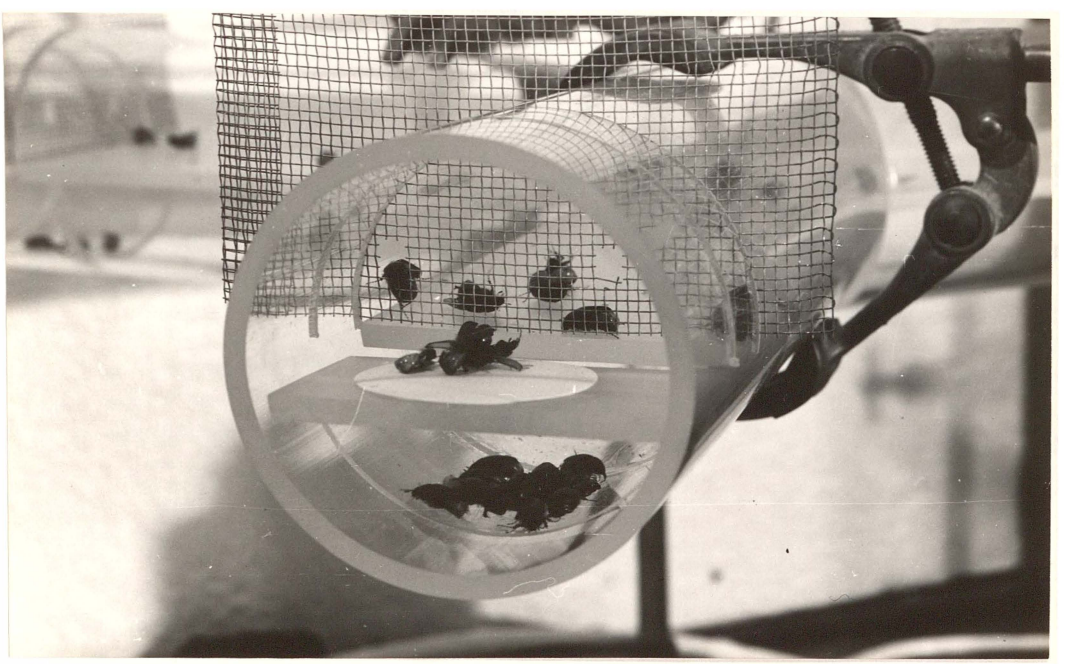


Plate 4.4 The prototype wire gate containing beetle-sized holes and the trap at the end of each arm of the "Y"-choice olfactometer.

placed in one arm of the "Y", the other arm being left empty, at the beginning of the "conditioning period" for males. The attractant source was alternated from one arm to the other during the tests. After each bioassay, the olfactometer was cleaned by immersion in a 5% aqueous solution of 'Decon 75' for a few hours.

The bioassay method based on observing male copulatory attempts comprised an olfactometer (shown in Plate 4.5) which was simply the top part of a 2.5 l Winchester bottle. An inverted filter funnel (12 cm dia.) served equally as well. Ten male beetles were dropped into each unit at about 1.0 p.m. and allowed to become accustomed to the lighting conditions of the bioassay room before testing commenced. The jars rested on a piece of plate glass covered with paper tissue which helped the beetles to move about and reduced the risk of contamination.

After each test, the olfactometer was cleaned by a 12 h immersion in a 5% aqueous solution of 'Decon 75'; rinsed with acetone, and dried at 160°C for a few hours.

4.3 Results and discussion

(a) Extension of the adult emergence period in the laboratory

In the first year of this study, 27,000 grass grub larvae were collected by eight men working over a period of about 3 weeks. The larvae were raised in the laboratory at 10°C, a temperature which corresponds to soil conditions in the Waikato region when pupation takes place. With this treatment an overall 70 percent mortality occurred, which meant that only 4,000 females were obtained from the initial collection. Obviously, this high insect death rate was a serious limitation to this study and in subsequent years improved handling techniques were developed. One of these consisted of lining the bottoms of the ice-cube trays with plaster of Paris.

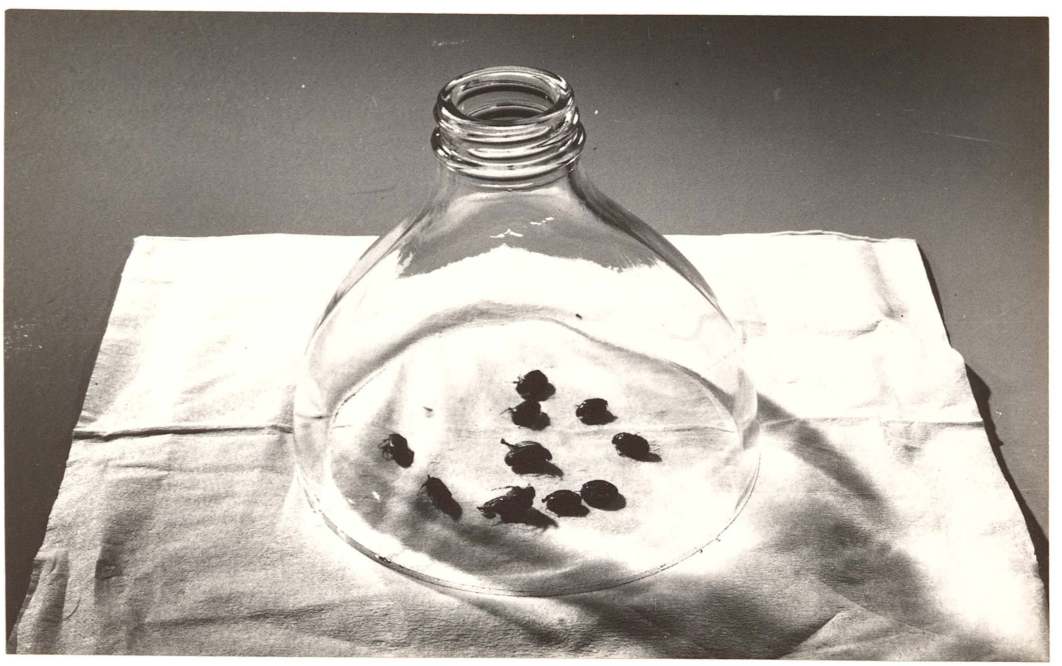


Plate 4.5 The bioassay apparatus used for observing male beetle copulatory attempts with paraffin dummies or filter paper treated with female extracts. Ten males were confined within each olfactometer which was placed on a piece of plate glass covered with a paper tissue.

Beetles were able to grip to this more readily than to the plastic and the improved grip allowed beetles to right themselves. This prevented the newly emerged adults (which usually came out of the pupal cases on their backs) from becoming stuck to the ice-cube tray bottoms as their wing cases hardened. This resulted in a high mortality of adults in the first year of the study.

A further limitation which became apparent from this initial work was that under the temperature conditions used, all the beetles emerged over a period of 2 - 3 weeks. This gave very little time for the study to be carried out effectively.

In the following year, these limitations were overcome to some extent by collecting a larger number of larvae (60,000) from heavily infested areas with much greater care than the previous year, and raising adults under various temperature conditions to regulate and extend the period of adult emergence.

Larvae were divided into three batches of varying size and adults from each batch raised under the three temperature conditions outlined in Table 4.1.

Table 4.1 Mortalities associated with different temperature conditions used in raising grass grub adults.

Treatment	Temperature °C	No of larvae	Viable beetles 10 days after final ecdysis	Mortality %
1	Larvae, pupae at 16, beetles at 8 - 10	5,400	1,840	66
2	Larvae at 16, pupae and beetles at 8 - 10	25,000	9,870	60.5
3	Larvae, pupae and beetles at 8 - 10	30,000	15,500	48

In treatment 1, the larvae and pupae were held at 16°C which was about 6°C above the soil temperature expected in the field. This increased temperature accelerated the rate of growth of the larvae and pupae, and beetles from this treatment emerged much earlier than beetles in the field. In treatment 2, only the larvae were raised at 16°C and the pupae and beetles were raised at the lower, more normal temperature of 8 - 10°C. Beetles from this treatment emerged later than treatment 1 beetles but earlier than those from treatment 3. This treatment roughly corresponded to the temperature conditions in the field. In all the treatments, as soon as beetles emerged they were transferred to a bioassay room held at 8 - 10°C.

The effect of the higher temperatures in spreading beetle emergence is illustrated by the fact that the peak of beetle emergence in the treatment 1 batch was 28 days ahead of that for the treatment 2 batch which in turn was 14 days ahead of the treatment 3 batch. Adults from treatments 1, 2, and 3 emerged over a period of 11, 15, and 21 days respectively, in a manner similar to that shown in Plate 4.6 for the treatment 3 beetles.

The appreciable difference in mortality between treatment 3 and the other treatments (48 percent compared with 66 and 60.5 percent) is probably explained by the fact that lower temperature conditions were used. The 48 percent mortality obtained in treatment 3 represented a considerable improvement over the 70 percent mortality obtained in the previous year. As a combined result of these three treatments, a total of 27,000 adults was available in the laboratory over a period of 6 - 8 weeks. This effectively doubled the period that the sex pheromone could normally be studied.

During this second year, the grass grub sex pheromone was isolated and identified, and in subsequent years, lower numbers of larvae

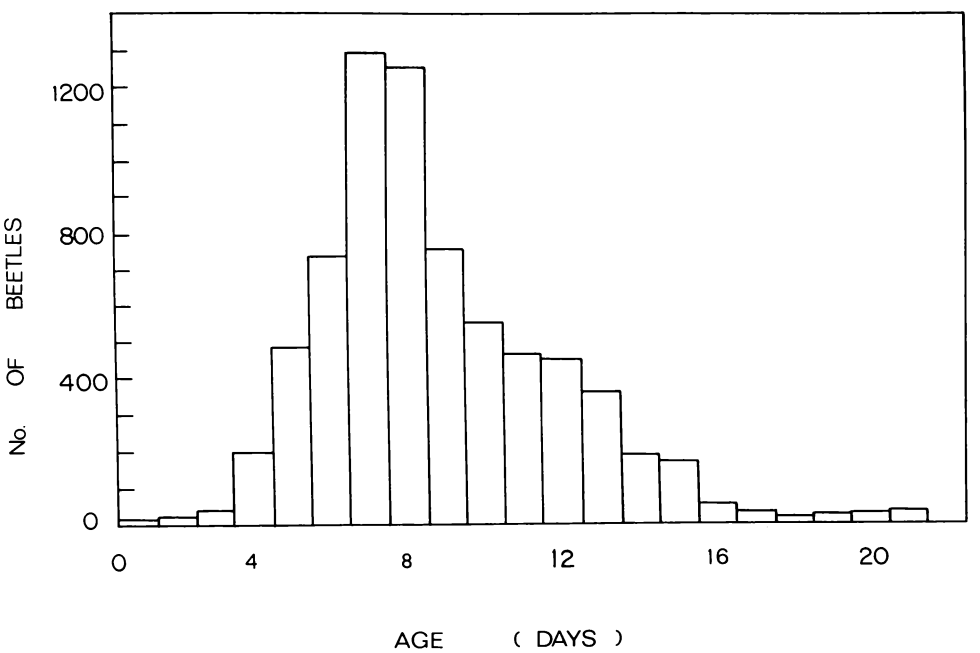


Plate 4.6 Emergence pattern of grass grub beetles raised from fully fed third instar larvae in the laboratory at 8-10°C throughout the larval, pupal and adult stages.

(in the order of 10,000 per year) were raised in the laboratory for use in field trials.

(b) Bioassay based on the Y-choice olfactometer.

Male responses were measured between 6.0 and 9.0 p.m. the period in the field when mating takes place. During this period the activity of the beetles within the olfactometer increased significantly; beetles became excited and some made attempts to fly. As the bioassay apparatus was primarily designed to measure insect flight behavioural responses, it was unfortunate that very few beetles actually took flight in the olfactometer. Responses were based on walking or mobile behaviour within the olfactometer, however. Male responses to virgin females of varying age are shown in Table 4.2.

The mobile response ratio is a direct measure of the response to the attractant by the males that have been stimulated to move through the holes in the wire gates. For a given test, a significant percentage mobile response was represented by a deviation from 50. A negative mobile response being less than 50 and a positive greater than 50. The overall response ratio is a general measure of the activity reasonably attributed to the attractant. A negative overall response is obtained when the number of beetles in the baited arm trap is equal to or less than the number of beetles in the control arm trap.

Tests were carried out with male beetles of the same age which varied between 10 and 15 days after final ecdysis. There appeared to be no relation between the age of male beetles and their response to the attractant within the age range utilized (10-15 days).

Variations in response were obtained within each female age group. For example, with females at 12 days after final ecdysis, in

Table 4.2 Male response to virgin females of varying age.

Female age (days after ecdysis)	Male age (days after ecdysis	No.of males	Males attracted	Males in control	%Mobile response (R_{mobile})*	%Overall response ($R_{overall}$)*
6	10	51	3	4	43	negative
6	11	24	1	4	20	negative
6	15	40	2	1	67	2.5
					Av. 43	Av. 1.0
8	15	30	6	8	43	negative
8	14	26	9	1	90	30
8	10	32	9	0	100	27
8	11	24	4	0	100	16
8	14	30	2	8	20	negative
					Av. 71	Av. 15
10	10	25	8	1	89	28
10	13	30	12	4	75	27
10		30	16	8	67	27
					Av. 77	Av. 27
12	12	25	6	6	50	negative
12	13	29	4	1	80	10
12	12	46	12	5	70	15
12	13	30	13	0	100	43
12	12	26	11	1	92	35
					Av. 78	Av. 21

Table continued on next page

Table 4.2 continued Male response to virgin females of varying age.

Female age (days after ecdysis)	Male age (days after ecdysis)	No.of males	Males attracted	Males in control	%Mobile response (R_{mobile})*	%Overall response ($R_{overall}$)*
14	12	27	10	3	77	26
14	12	30	11	8	58	10
14	13	37	21	5	80	43
					Av. 72	Av. 26
18	13	29	17	6	74	38
18	14	32	24	1	96	72
					Av. 85	Av. 55
22	11	40	8	2	80	15
22	11	40	4	1	80	7
					Av. 80	Av. 11

$$(R_{mobile})^* = \frac{\text{(No. beetles in baited arm trap)}}{\text{(Total no. beetles trapped)}} \times 100$$

$$(R_{overall})^* = \frac{\text{(No. beetles in baited arm trap} - \text{No. beetles in control arm trap)}}{\text{(Total no. beetles exposed)}} \times 100$$

different tests with virtually the same number of beetles, results were 6/6 (attracted/control) and 11/1. No explanation can be offered for results of this type. Statistical analysis of the percentage mobile response showed that variation between tests, with females of the same age, was greater ($P < 0.01$) than could be attributed to binomial variation. It is evident, therefore, that replication is an essential requirement in a bioassay of this nature.

Based on an analysis of transformed (inverse sine) percentage, the overall mean percentage mobile response was 77% with 95% confidence limits of 66.8% to 85.7%. Males reacted positively ($^{R}_{mobile} > 50$) to crushed female beetles whose age varied from 8 to 22 days in 17 of the 20 tests carried out. The similarity of the average mobile responses for each age group suggests that the pheromone is present in virgin females at the same level from about 8 days after final ecdysis until death. This does not eliminate the possibility, however, that a peak period of attraction does exist in live virgin females.

When crushed females were actually placed in the olfactometer males showed considerable sexual excitement by attempting to copulate with the crushed females as well as with other males. This result provided further evidence for the presence of a chemical sex pheromone in the adult female beetle.

Additional tests were carried out to test the attraction of males to males, females to females, and females to males. The results shown in Table 4.3 indicated that crushed females did not attract females while crushed males attracted neither males nor females.

While the data obtained in the Y-choice olfactometer supported the fact that the female beetle used a chemical sex pheromone, the

Table 4.3 Responses of males to males, females to females, and females to males

Male response to males

Male age (days)	No.of males	Males attracted	Males in control	% Mobile response	% Overall response
10	50	0	1	0	negative
10	33	2	0	100	6

Female response to females

Female age (days)	No.of females	Females attracted	Females in control	% Mobile response	% Overall response
10	29	0	1	0	negative
10	29	2	0	100	7

Female response to males

Male age (days)	Female age (days)	No.of females	Females attracted	Females in control	% Mobile response	% Overall response
10	10	45	3	1	75	4
10	10	40	0	1	0	negative
10	10	40	0	0		negative

method itself was not satisfactory for a number of reasons.

Firstly, the type of apparatus was originally designed to measure flight responses in moths and, as very few grass grub beetles actually took flight within the olfactometer, a walking response, rather than a flight response was measured. In preliminary studies carried out by the author, the apparatus was used successfully to measure flight responses of flesh flies, Sarcophaga milleri. These flies are strongly attracted to flowers of Euonymus japonicus and they gave quick, positive, clear-cut responses when the flowers were placed in one arm of the Y-tube olfactometer. Such a situation was not observed with the grass grub beetles, however. Their responses were sluggish with many beetles apparently wandering around aimlessly within the olfactometer. This was reflected in some of the results in Table 4.2 in which high percentages of males

were trapped in the control arm. This resulted in correspondingly low (<60) percent mobile responses.

Secondly, as replication of each treatment was essential, the bioassay method did not provide a decisive answer as to the presence or absence of biological activity. Thirdly, only one measurement could be made on the apparatus each evening. This was clearly inadequate in a study of this nature where beetles were only available for a few weeks each year and large numbers of fractions had to be tested for activity in the development of isolation procedures.

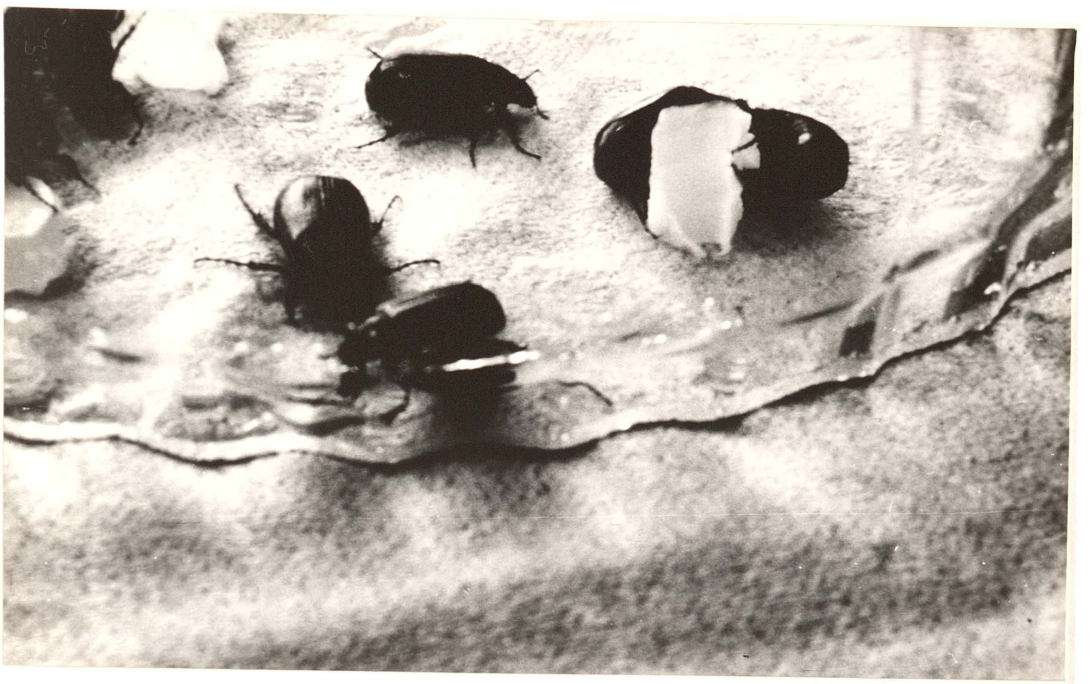
(c) Bioassay based on male copulatory attempts

Tests commenced about dusk and continued until about 9.0 p.m. These tests were carried out by spotting an extract on to a dummy of paraffin wax (m.p. 35°C) which was then dropped among the beetles. The dummy approximated the size of a beetle, but its size and shape did not appear to be critical. When a dummy contained an active fraction, beetles responded within a few minutes by attempting to copulate with it (shown in Plates 4.7) or with one another.

Male beetles also responded to extracts applied to pieces of Whatman No.1 filter paper. Behavioural responses were similar to those described, with males continually searching on, around, and under the treated papers.

A description has been given of the three different temperature conditions that were used to regulate the rate of beetle emergence in the laboratory. Beetles raised in the laboratory at 16°C throughout the larval and pupal stages (treatment 1) were regarded as being under 'forced' conditions, for they emerged about 3-4 weeks ahead of beetles raised under more normal conditions at 8-10°C throughout the larval and pupal stages (treatment 3).

The average percent responses of males from the three treatments to a standard ethereal solution of female extract are shown in



Plates 4.7 Copulatory responses of male beetles with paraffin dummies treated with active female extracts.

Table 4.4. The standard was stored at -10°C and 0.1 female equivalents of the standard was applied to the paraffin dummies for each test (1 female equivalent is 1 female abdomen extracted with solvent). In each test, 10-day old males were used in groups of ten.

Table 4.4 Effect of rearing conditions on male response

Beetle source	No. tests	Av. percent response	Variation in percent response
Treatment 1	6	30	0 - 50
Treatment 2	10	26	0 - 40
Treatment 3	93	34	0 - 90

Variable responses, which ranged from 0 to 90%, were obtained with beetles from the three treatments. Thus, some groups of beetles did not respond to the treated dummies at all. On average, however, about 30% of the beetles tested responded to the crude extract. Beetles did not respond to untreated dummies. Only a small number of tests (16) were carried out with the standard solution on beetles from treatments 1 and 2 but the variable responses obtained with males from treatment 2, in particular, suggested that beetles emerging late within a collection might not respond to the sex pheromone as well as those emerging before, or at, the emergence peak. This hypothesis, as well as the effect of age on male response, was tested with beetles from the treatment 3 batch. Groups of male beetles that had emerged before, at, and after the time of the emergence peak (shown in Plate 4.6) were selected. The groups were accordingly taken on the 4th, 8th, 12th, and 15th day after beetles from treatment 3 started emerging and beetles in these groups were classified as early, peak, post peak, and late, respectively. In all the tests, responses were measured against dummies containing 0.1 female

equivalents of the standard.

Percentage responses of males with age after final ecdysis are shown in Plate 4.8 for each group. Each response recorded is the mean of 6 replicated tests. Male beetles were not tested more than once. The response in individual tests varied from 0 to 90%. The variation between replicates was not greater than that attributable to binomial variation.

During the period when the late group beetles were 14 days old, work was concentrated on field tests and, consequently, this group was not tested. Initially it was intended to study the response of male beetles from each group at 6, 10, and 14 days after final ecdysis. However, when it was found that 6-day old males responded to the sex pheromone, 4-day old, and 2-day old beetles were also tested. These results are included in Plate 4.8.

Selected analyses from the early, peak, and post peak groups showed a difference ($P<0.01$) in male response with age within the beetle age range of 6-14 days. Thus, the response of 14-day old males was greater than that for 6- or 10-day old males. However, the mean responses obtained with 4-day old males were comparable to the 14-day old male responses and it appears that the difference above is not completely explained by an age effect.

There was no significant difference ($P<0.01$) in male response with beetles of the same age from the early, peak, and post peak groups but beetles from the late group, aged 6 and 10 days, gave a higher mean response than beetles from the earlier groups. This difference, however, was not apparent with 4-day old male beetles. It did appear, therefore, that beetles emerging late within the collection responded equally as well as, if not better than, those emerging before or at the emergence peak.

In the field, normally a period of 7-10 days or longer elapses

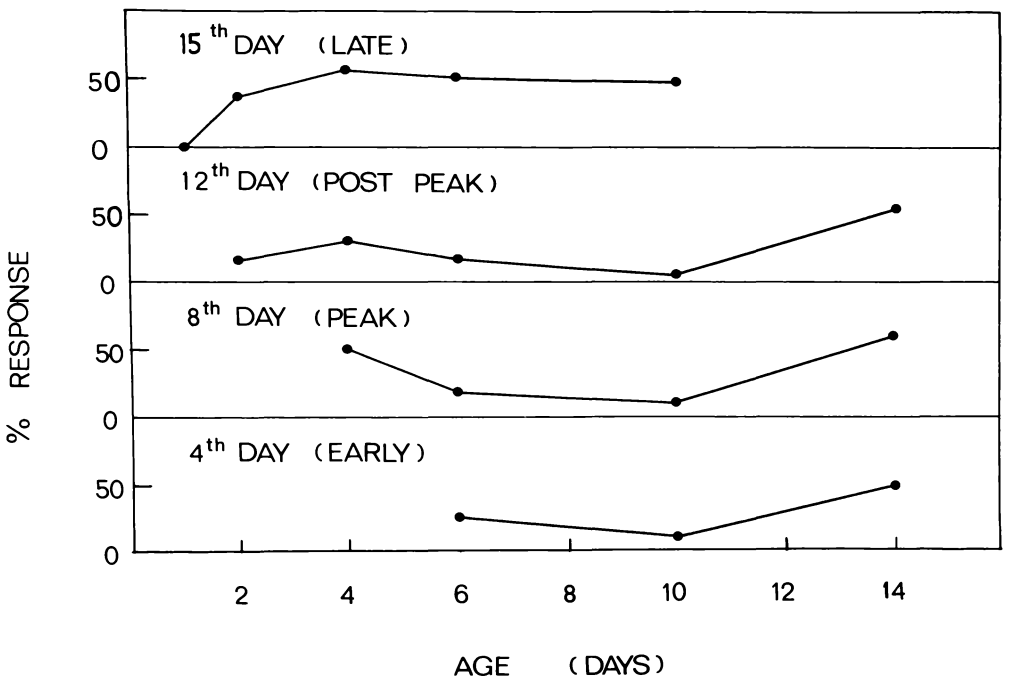


Plate 4.8 Variation in the percent copulatory responses of male beetles with age. Four groups of beetles were selected at different stages (termed: early, peak, post peak and late) throughout the 21-day emergence period of beetles raised in the laboratory at 8-10°C from fully fed third instar larvae.

between final ecdysis and primary beetle emergence from the soil. Mating is believed to take place as soon as the female beetle emerges from the soil. Presumably then, the male beetle takes 7 days or so to mature before mating takes place under normal circumstances. It can be seen from Plate 4.8, however, that male beetles aged 2 days after final ecdysis responded to the sex pheromone, even though males are definitely too immature to mate at this age.

A total of ninety three tests was carried out on the treatment 3 beetles with the standard solution. In approximately 8% of these tests, no response was induced in the male beetles and in 20% of the tests only one copulatory attempt was observed. The breakdown of the tests into categories ranging from 0-100% responses is shown in Table 4.5.

Table 4.5 Breakdown of the tests with the treatment 3 beetles into categories based on the magnitude of the behavioural response

%Response	0	10	20	30	40	50	60	70	80	90	100
%Tests, above responses obtained	8	20	14	13	11	9	11	6	4	4	0

The bioassay method based on observing male copulatory activity was adopted throughout this study. It provided a quick method to determine activity which meant that tests could be easily repeated when required. Verification of an active fraction was normally based on obtaining positive responses in each of three separate tests.

4.4 Conclusions

Grass grub adults were obtained by collecting fully fed third instar larvae in the field and raising these under high humidity conditions to adults in the laboratory. In the first year of the study, a 70% mortality of the initial collection took place but in

subsequent years this was reduced to about 50% by using improved handling techniques.

The short period for studying the sex pheromone was extended by raising some of the larvae and pupae at elevated temperatures. Even though these higher temperatures resulted in somewhat higher mortalities, the accelerated rate of growth meant that beetles were available in the laboratory for about a month before beetles emerged in the field. This effectively doubled the period that the sex pheromone could be studied.

A Y-choice olfactometer was used in the initial experiments and the walking response of male beetles to crushed females placed in one arm of the "Y" was measured. Males responded positively to the crushed female beetles in 17 of the 20 tests carried out and all the data supported the fact that the female used a chemical to attract the male. The method had a number of limitations, however, and included (1) the long length of time to perform the bioassay which meant that only one test could be carried out each evening (2) the necessity for replication of each treatment as clear-cut responses were generally not obtained and (3) the absence of flight activity within the olfactometer.

In subsequent years, a bioassay method based on observing male copulatory activity proved to be more satisfactory than the former method, for it gave more direct responses and repetition could be carried out easily. The responses obtained were somewhat variable and ranged between 0 and 90%. On average, 34% of the males tested responded to the crude female extract. The effects on male response of age, and time of emergence within a beetle collection, appeared to be minimal. Males aged 2 days old responded to the sex pheromone even though they were definitely too immature to mate.

CHAPTER V

EXTRACTION, ISOLATION, AND IDENTIFICATION OF THE GRASS GRUB SEX PHEROMONE

5.1 Introduction

The classical approach for identifying pheromones involves solvent extraction of active components, followed by purification steps monitored by bioassay. The final purification step normally involves the use of a gas chromatograph where the active component (or components) is separated on the column and trapped as it emerges in the column effluent. The collected material is usually obtained in mg or µg quantities and identified with modern techniques such as mass and nuclear magnetic resonance spectrometry together with infra-red and ultraviolet spectrophotometry.

In the initial experiments with the female grass grub beetle, attempts were made to locate the site of pheromone production so that only the "active region", rather than the whole insect, could be extracted with solvent. These experiments were followed by functional group tests on an active ethereal extract. The tests were carried out on a microscale and were based on the loss or retention of activity after various chemical treatments were applied to the active extract. This information, together with that on the behaviour of the active component in a number of concentration steps, formed the basis for the isolation of the pheromone.

The laboratory behavioural bioassay based on observing male copulatory activity with treated paraffin dummies was used throughcut the study. Final verification of the identity of a sex pheromone, however, depends on its attractiveness in field tests for

it has been shown that compounds which stimulate sexual activity in insects in the laboratory do not necessarily attract them in the field. This phenomenon was discussed in Section 2.9.

5.2 Experimental

(a) Production site of the grass grub sex pheromone

Frozen sections were made of 10-day old virgin female beetles on a 'Spencer' sledge microtome fitted with a "peelcool" freezing stage. As each section was cut, it was transferred immediately on to filter paper in a petri dish and then stored in the deep freeze at -10°C until the bioassay was carried out in the evening. The microtome knife was carefully wiped clean with acetone between each cut.

The initial work to establish where the sex pheromone was produced in the female beetle, consisted of cutting each beetle into 5 transverse sections of equal thickness. When sections from 5 beetles were combined on filter paper and bioassayed, two of these sections produced male responses of 70% and 80% respectively. Male beetles did not respond to any of the other three sections. Male response to active sections was similar to that described for active paraffin dummies. The active sections correspond to the first few segments of the female abdomen.

In an attempt to pinpoint the active area, transverse sections of equal thickness in individuals were cut between two distinct anatomical features, e.g. 5 beetles were cut into 3 transverse sections between the end of the thorax, at the point where the last pair of legs arose, and a point mid-abdomen. The corresponding sections were combined and bioassayed. Beetle responses of 20% and 60% respectively were obtained with the 2 sections which correspond to the first three abdominal segments. Beetles did not

respond to the third section. In another experiment, 5 beetles were cut into two horizontal sections and these were bioassayed. Males did not respond to the ventral section but 50% responded to the dorsal section. This experiment was repeated with another group of female beetles and again the dorsal side was active and the ventral side inactive. From these experiments it was established that the sex pheromone was produced dorsally mainly within the first three abdominal segments.

(b) Extraction of the grass grub sex pheromone

A crude extract was obtained by surface rinsing the first few abdominal segments of 10-day old female beetles with diethyl ether. In the initial experiments surface rinsing was used in preference to maceration for it was considered that the latter method was more likely to co-extract impurities which could mask the biological activity of the extract. Reference was made to this masking phenomenon in Section 2.11.

Active fractions were consistently obtained with diethyl ether surface rinses and this method of extraction was used throughout this study. Male responses to an extract of this type varied between 10 and 90 percent but normally when 20 to 30 percent of the males responded to the dummy this was regarded as a good response. Indeed, any attempt by males to copulate with a treated dummy was indicative of the active fraction, for males never responded to untreated dummies. Males responded to dummies treated with 0.01 and 0.001 female equivalents of the crude extract but good responses were obtained with either 0.1 or 1.0 female equivalents (discussed in Chapter 4) and all subsequent bioassays were carried out at one of these levels.

(c) Partitioning of ethereal extracts against aqueous buffers

Before any attempts were made to develop an isolation procedure,

a number of partitioning experiments were carried out on the crude ethereal extract to obtain some idea of the nature of the pheromone.

Saturated sodium bicarbonate - An ethereal extract (10 ml) containing 20 female equivalents was washed with saturated sodium bicarbonate (3 x 5 ml). The ether phase was tested for activity and gave a 20% response in males when 0.1 female equivalents was used on a paraffin dummy. This compared favourably with the 30% response obtained with the ethereal extract before washing. No activity was found in the sodium bicarbonate soluble phase after neutralisation with ice-cold 0.1M HCl and washing with diethyl ether. Thus the active component was insoluble in saturated sodium bicarbonate. This experiment was repeated and a similar result obtained. This indicated that the pheromone was not a strong acid.

10 percent sodium carbonate - An ethereal extract (5 ml) containing 10 female equivalents was washed with 10% sodium carbonate (3 x 2 ml.). The active component remained in the ether phase. Responses of 40% and 60% were obtained in duplicate tests at the 0.1 female equivalent level.

1 Molar potassium hydroxide - An active ethereal extract (2 ml) containing 4 female equivalents was washed with 1M potassium hydroxide (3 x 1 ml). The ether phase, when tested at 0.1 female equivalents on paraffin dummies, produced no response in male beetles. The aqueous phase was covered with ether (5 ml) and neutralised to pH 6.5 with ice cold 0.1M HCl. (The reason for neutralising in this manner is discussed below). The ether phase when tested at 0.1 female equivalent level was active causing a 20% response in males. A repeat of this experiment showed again that the active component was soluble in 1M potassium hydroxide. After neutralisation, the ether phase gave a 40% male response. These base

wash experiments show that the active component is acidic.

Acidic buffers - Preliminary work with acidic washes of active ethereal extracts suggested that the acidity was destroyed by acids. Thus a series of acidic buffers was prepared by mixing 0.2M disodium hydrogen phosphate with 0.1M citric acid in various proportions (Vogel, 1959). Partitioning experiments were carried out to ascertain the pH at which activity was lost. In these experiments aliquots of the active ether extract (5 ml) containing 10 female equivalents were washed with the acidic buffers (3 x 2 ml) and the ether phase tested for activity. The results of these experiments, shown in Table 5.1, indicated that the activity was destroyed by washing the ethereal extract with acidic buffers with a pH less than 4.8.

Table 5.1 Effect of acidic buffers on the activity of a crude female extract

pH of buffer	% Response (0.10E)
7.0	40
6.0	30
5.3	10
4.8	20
4.6	0
4.0	0

Subsequently it was found that phenol was present in the female beetle and functioned as a sex pheromone for the male beetle. The result obtained in the partitioning experiment above was inconsistent with the known behaviour of phenol, however. For example, ethereal solutions containing 10-20 µg phenol were shaken with the acidic buffers down to pH 2.0 and the phenol level in the diethyl ether was unaffected. Possible explanations for this apparent contradiction are:

(1) the condensation of an aldehyde, possibly present in

the female extract, with phenol under acidic conditions. Accordingly, mixtures of acetaldehyde (100 µg) and phenol (20 µg) in diethyl ether were shaken with acidic buffers down to pH 2.0, but the phenol remained unaffected.

(2) an acid sensitive component additional to phenol is required to induce sexual activity. Against this, however, phenol alone stimulated copulatory activity in males in laboratory tests.

(3) the inconsistent behaviour of males in the laboratory bioassay. This is the most likely explanation as it has been shown that in about 30% of the tests, eight of the ten beetles in each olfactometer failed to respond to the crude female extract.

(d) Functional group tests on the crude ethereal extract

Chemical tests on the crude extract were carried out to determine the functional groups present in the pheromone.

Diazomethane - Excess diazomethane in diethyl ether was added to the ethereal extract (2 ml) containing 4 female equivalents. After 2 hours, the ether phase was tested for activity and was inactive. An unsuccessful attempt was made to restore activity by treatment with 0.1 ml of 1M methanolic potassium hydroxide. This result suggested the presence of a phenolic group.

Lithium aluminium hydride - Excess lithium aluminium hydride was added to the ethereal extract (2 ml) containing 4 female equivalents. After half an hour, the ether extract was assayed and found to be inactive. At the time, this result was interpreted as indicating the presence of a carbonyl group, but when considered retrospectively it is obvious that an ether insoluble lithium salt of phenol was formed.

(e) Short path high vacuum distillation

Extracts of female abdomens were concentrated to 0.5 ml by rotary evaporation at 25°C and 70 mm-Hg and then sublimed on to a condenser cooled with dry ice in a short path distillation apparatus at 0.001 mm-Hg. The temperature was maintained by a constant temperature bath and the pressure by means of an oil diffusion pump backed by a mechanical pump.

In order to establish optimum conditions for the distillation, temperatures were varied and the activities of the sublimate and sublimand determined after a set period of time. The results of these experiments are shown in Table 5.2 below.

Table 5.2 Effect of vacuum distillation temperature on the volatility of the pheromone

Distillation conditions	No. females extracted	% Male response	
		sublimate	sublimand
30°C, 0.001 mm Hg, 3 h	160	10	20
30°C, 0.001 mm Hg, 3 h	500	70	80
50°C, 0.001 mm Hg, 3 h	1000	40	0

This short path high vacuum distillation procedure carried out at 50°C on the crude extract (665 mg) from 1000 females gave a satisfactory initial purification step for the weight decrease in the sublimand (60 mg) indicated at least a 10-fold increase in the concentration of the active component.

(f) Thin layer chromatography

Thin layer chromatography was carried out on plates (5 x 20 cm) coated with Kieselgel G 254 at a thickness of 0.5 mm. An aliquot (5 µl) containing 5 female beetle equivalents of an active sublimate fraction was spotted on to a plate and eluted with diethyl ether:

hexane (1:9). The adsorbent material was divided into 5 equal sections measured between the point of application and the solvent front. Each section was washed with diethyl ether (1 ml) and tested for activity. Responses of 60% and 30% respectively were obtained with the lower sections. The top three sections were not active.

This experiment was repeated using chloroform as the eluent. When the solvent front had travelled 14 cm, the plate was removed from the tank and the adsorbent material divided as before. Only the area between 0.5 mm and 2.0 cm from the point of application was active. This produced a 30% response in male beetles.

Thus the active component was strongly adsorbed on silica gel which indicated its polar character.

(g) Paper chromatography

Paper chromatography was used by Jacobson and co-workers (1968) in the identification of valeric acid as the sex pheromone of the elaterid beetle, Limonius californicus. In this study on the grass grub beetle, paper chromatography was carried out with strips (5 x 20 cm) of Whatman No.1 filter paper. An ethereal extract (70 µl) of vacuum sublimate containing approximately 10 female equivalents was spotted separately on to three paper strips. These papers were eluted with water, ethanol, and a mixture of diethyl ether and ethanol (1:1) respectively. When the solvent fronts travelled about 16 cm the papers were removed and, after drying, were each cut into approximately 2.5 cm sections. In the case of the filter paper eluted with water, the activity was found in the area between R_f 0.50 and 0.67. With the ethanol and diethyl ether/ethanol systems the activity travelled further up the paper and was found between R_f 0.76-0.86 and R_f 0.67-0.79 for the respective solvents. This activity was tested by simply dropping the filter sections among the male beetles. Beetles responded immediately by attempting to copulate with one another on top of the filter paper and by actively searching

over and under the active areas. Prior to this testing procedure, unsuccessful attempts were made to wash the active component from the filter paper with diethyl ether. This retention of activity on the filter paper after a diethyl ether wash suggested that the active component was strongly hydrogen-bonded to the cellulose. From these results on paper (and thin layer plates)it was apparent that the properties of the active component were consistent with those of a phenol.

(h) Gas liquid chromatography

Preliminary experiments were carried out on a semi-preparative scale gas chromatograph, in order to obtain further information on the nature of the pheromone and to determine the applicability of this technique in this study. The initial work was carried out concurrently with the tests already described and before the polar, phenolic character of the active component had been ascertained.

The gas chromatograph used was an F and M model 402 equipped with dual flame ionisation detectors. A number of general purpose polar and non-polar stationary phases were used initially. Both the polar stationary phases, viz., Carbowax 20M and DEGS (diethylene glycol succinate) and the non polar phases, viz., Apiezon-L and UCW-98 are extensively used in gas chromatography for a wide range of applications. These were used in the hope that at least one of them would be suitable for the grass grub sex pheromone.

The four column packings used in the initial study were: (1) 10% Carbowax 20M on 60-80 mesh Gas Chrome P, (2) 2% DEGS on 60-80 mesh Gas Chrome Z (3) 2% Apiezon L on 60-80 mesh Silocel and (4) 4% UCW-98 on 60-80 mesh Gas Chrome P. Of the three solid supports used, only Gas Chrome Z was a silanized support. Neither of the other two support materials was deactivated by silanizing

i.e. treating with dimethyldichlorosilane or hexamethylsilazane before applying the stationary phase. The column materials were packed into four separate glass U-tubes (1.5m x 4mm ID) and conditioned for at least a day, in a gas chromatograph oven, at the maximum operating temperatures for the respective stationary phases.

An active vacuum sublimate from 90 female abdomens was dissolved in diethyl ether (20 ml) and concentrated to 1 ml with a rotary evaporator. The attractant material (10 µl) was injected on to one of the above columns and the gas chromatograph operated under temperature-programme conditions. The column oven temperature was held initially at 95°C for four minutes and was raised at 5°C per min to a maximum temperature of 240°C. The nitrogen carrier gas flow was 40 ml/min and the flash heater temperature 250°C. Similar conditions were used for all four columns.

The gas chromatogram of the vacuum sublimate on the 2% Apiezon L column is shown in Plate 5.1. Similar chromatograms were obtained for the other three columns. Fractions were collected from these columns in glass U-tubes held at the exit port by means of a teflon sleeve which allowed the U-tube to butt directly on to the copper exit port (Plate 5.2). The "U" was immersed in dry ice-methanol at -70°C in a micro Dewar flask. The gas chromatograph was fitted with a stream splitter which was adjusted to give a 10:1 split of the effluent to the collecting unit and detector respectively. Four fractions were collected from each of the four columns and when bioassayed were all found to be inactive.

This inactivity could have been due to a number of factors. Firstly, the pheromone may have been retained on the columns. At the time, the phenolic nature of the pheromone had not been determined and while phenol separations have been achieved with stationary phases such as Apiezon L and silicone oils, the solid support

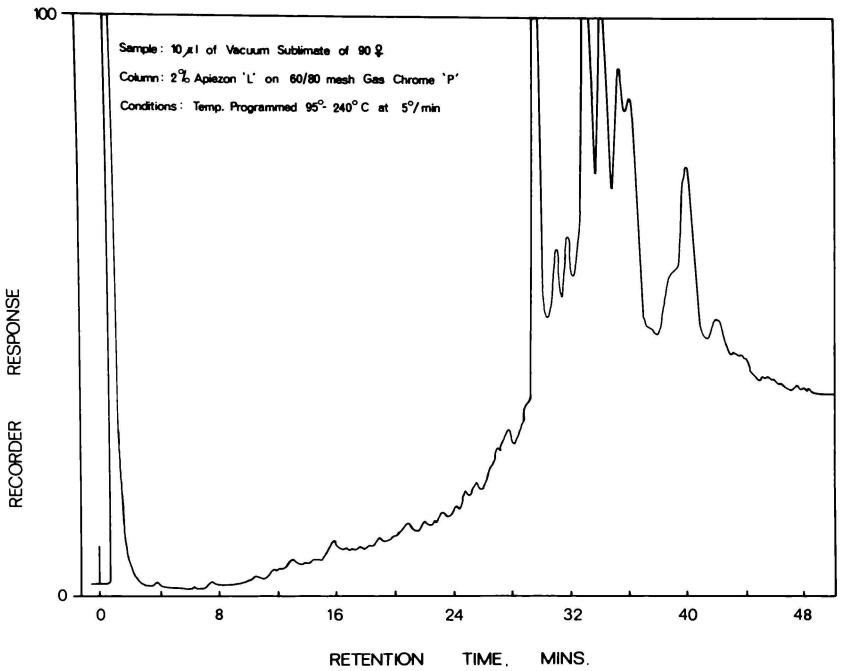


Plate 5.1 Gas chromatogram of an active vacuum sublimate on a 2% Apiezon L column. The effluent from such a column failed to induce a response in male beetles.

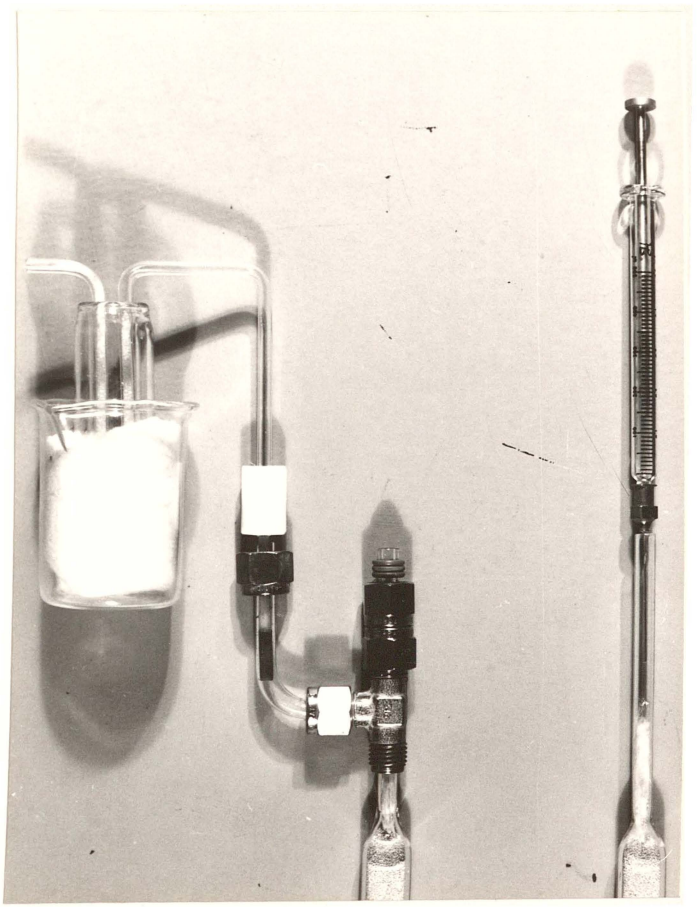


Plate 5.2 The apparatus used to trap fractions from gas chromatographic columns.

normally has to be silanized. Polar compounds such as phenol can be strongly adsorbed to the active sites of unsilanized support and retained on the column. Secondly, the pheromone was not expected to be a compound as volatile as phenol and, therefore, it could have emerged with the solvent front (which was not collected) or more likely, not have been trapped in the glass U-tube at all. In subsequent work, the U-tube was packed with glass wool to improve its trapping efficiency and even then, it only trapped about 17% of the phenol injected into the column.

When the phenolic nature of the sex pheromone had been determined in the paper chromatography experiments, gas chromatography was recommenced and a column was prepared which was suitable for phenol separations. The column consisted of 2% Atlox G 1292 emulsifier on 40-70 mesh Silocel. This material was recommended by Dr D. B. Wills, of Ivon Watkins-Dow Limited, who kindly forwarded a sample of Atlox G 1292 to me. Dr Wills had used the material for the separation of chlorinated phenols and butylated hydroxy anisoles.

At about the same time as these GC experiments were being carried out, field bioassays with a number of phenolic materials indicated that phenol itself attracted male beetles. The GC conditions were selected, therefore, to give a separation of phenol if it happened to be present in the female extract. An active vacuum sublimate injected on to the Atlox column under isothermal conditions at 120°C gave essentially one peak as shown in Plate 5.3. All the co-extracted fats, waxes, etc., shown in Plate 5.1 were retained at the inlet and this facilitated the collection of relatively pure fractions. The active compound collected from this column had a retention time of 5.2 minutes which corresponded precisely with that of phenol. A 10 µl sample containing 5 female equivalents of an active vacuum sublimate from 500 females (30 mg) applied to the 2% Atlox G 1292 column, in-

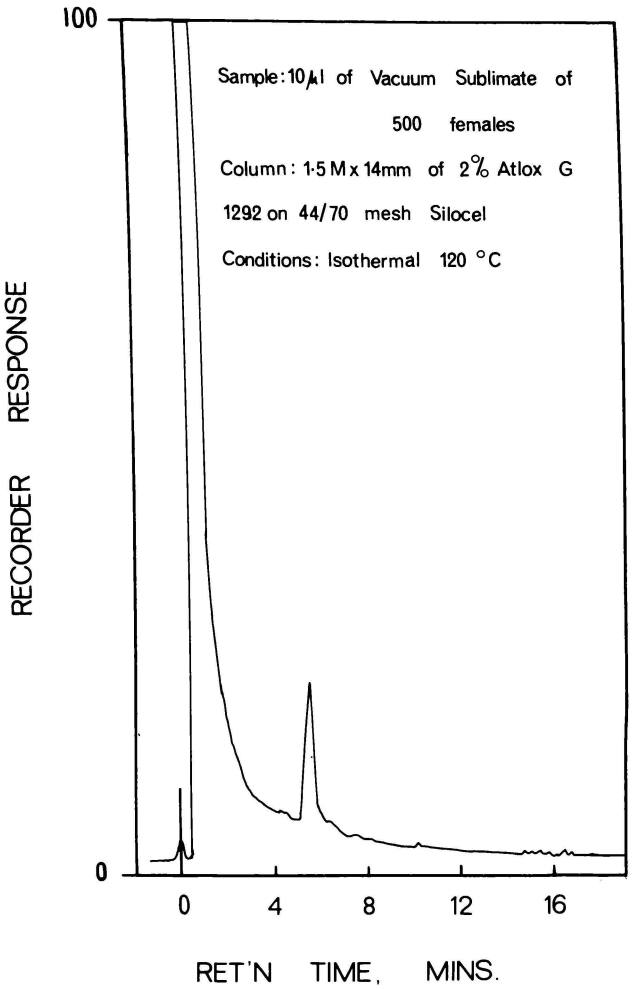


Plate 5.3 Gas chromatogram of an active vacuum sublimate on a 2% Atlox G 1292 column. The major component is phenol.

Plate 5.4 Field attraction of male beetles. Aqueous phenol was used in the jar or phenol was dissolved in the trap-water.

dicated that each female contained between 1.0 and 1.5 µg phenol. This step resulted, therefore, in a fifty-fold increase in the concentration of the active component.

The separating efficiency of the Atlox stationary phase for phenols may be related to the fact that it is an emulsifier and, therefore, it readily coated or 'wetted' the solid support and deactivated the active sites.

(i) Isolation and identification of phenol as the sex pheromone

The sex pheromone was isolated from 1500 virgin female beetles by surface washing their abdomens with diethyl ether. The ether extract was concentrated to 0.5 ml by rotary evaporation at 25°C and 70 mm-Hg, and then sublimed on to a condenser cooled with dry ice, in a short path distillation apparatus at 50°C and 0.001 mm-Hg for 3 hours. The biologically active sublimate in diethyl ether was then subjected to semi-preparative gas liquid chromatography, using a glass U-tube (1.5 m x 14 mm) of 2% Atlox G 1292 on 40-70 mesh Silocel at 120°C and a nitrogen carrier flow rate of 40 ml/min. A number of 10 µl injections of the vacuum sublimate was applied to the column and approximately 130 µg of the compound which had a retention time precisely the same as phenol was collected. Collection experiments with phenol itself using the trapping method described gave a 17% recovery.

The collected fraction was dissolved in diethyl ether and examined by high resolution mass spectroscopy. The mass spectrum (Fig. 5.1) was consistent with that of phenol in diethyl ether, and the presence of phenol was confirmed by measuring the masses of the ions at (mass to charge) m/e 94, 66, 65, and 39. These had the expected compositions of C_6H_6O, C_5H_6, C_5H_5 and C_3H_3. In addition, it was established by use of the "defocussing" technique that the

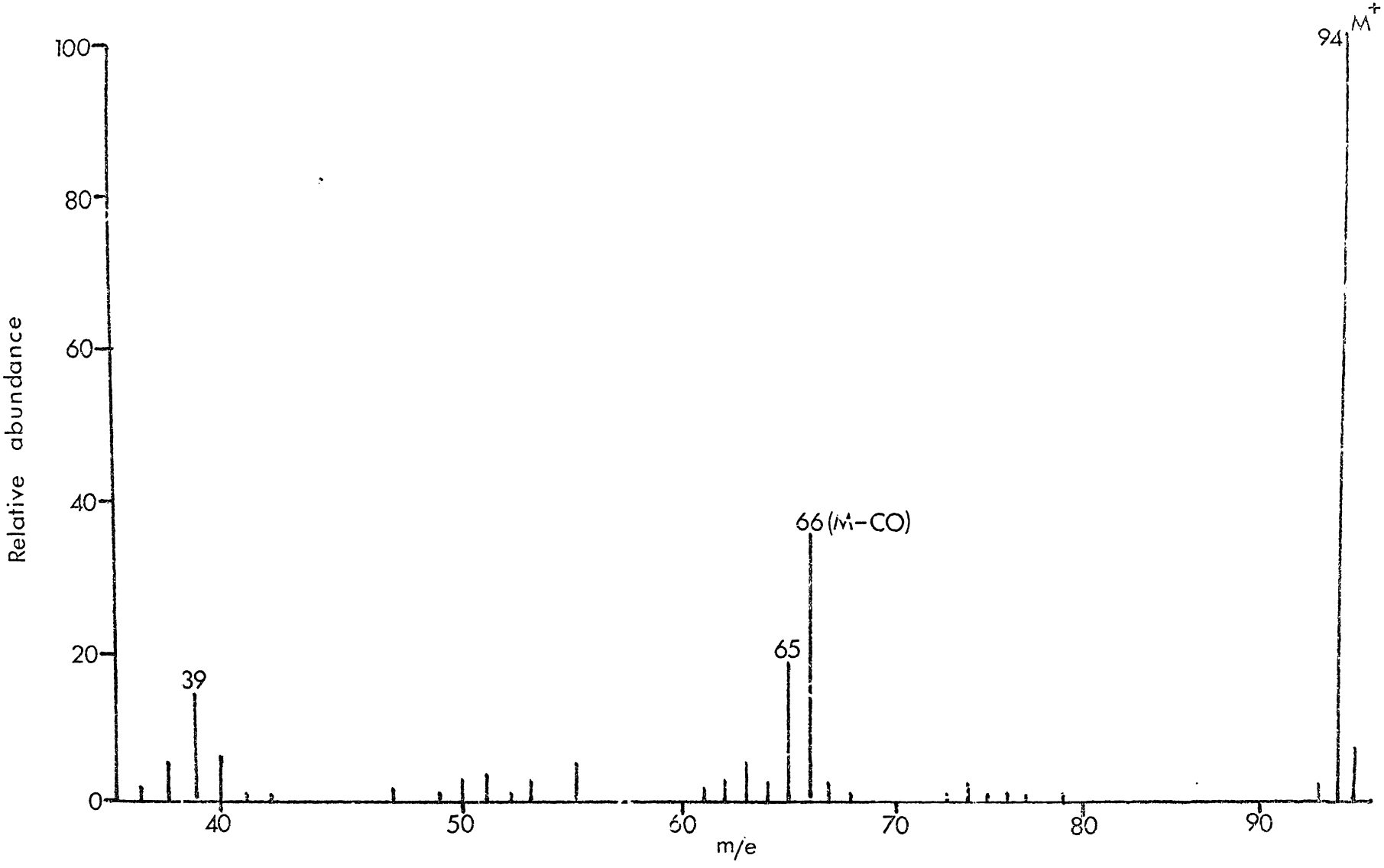


Figure 5.1 Mass spectrum of phenol.

following transitions had taken place: 94 → 66, 93 → 65, 66 → 65 and 65 → 39. There was no evidence that the ion $C_6H_6O^+$ had arisen from a precursor of higher molecular weight and, therefore, it was concluded that the compound under examination was phenol.

Analysis by gas liquid chromatography of ethereal extracts of female abdomens indicated that each beetle contained between 1.0 and 1.5 µg of phenol, which corresponds to approximately 20 parts per million by weight. No phenol was detected in ethereal extracts of male abdomens.

(j) Laboratory and field bioassay of phenol

Laboratory bioassays were carried out as described earlier. Male beetles responded within a few minutes to paraffin dummies treated with 0.1, 1.0, and 10.0 µg phenol respectively by attempting to copulate with the dummies as well as one another. Eighty percent of the males responded to each amount of phenol on the dummy.

Attempts in subsequent seasons to repeat these excellent responses with phenol and crude female extracts failed to induce greater than 20% copulatory attempts in males. In all the field tests, however, phenol has consistently attracted male beetles in mating flights. Furthermore, in experiments with aqueous phenol on filter paper, males that landed on the paper frequently showed sex stimulatory activity by raising their elytra and attempting to mate with males nearby.

It is possible that, in order to induce sex stimulatory attempts in males in the laboratory, the phenol release rate from a substrate must fall within a fairly narrow range. The release rate is an important factor in the field in determining field attraction of males to phenol and, therefore, it is also likely to be important in laboratory tests. Indeed, it may well be more critical than in

the field in view of the close vicinity of males to the attractant source in the laboratory tests. Thus a possible explanation for the inconsistency of phenol in the laboratory tests with males is that the rates of release obtained from the paraffin dummies were above or below the range for sex stimulatory activity.

Field activity of phenol was tested in several ways. The traps consisted of open tins containing approximately two litres of water (Plate 5.4) with the attractant suspended in jars (110 ml) above the tin. Interestingly, when 10 mg of pure phenol was used in the jar, very few beetles were caught in the trap but a number of beetles were observed to drop short of the trap on the downwind side. Obviously the concentration of phenol in the air was important and determined the attractiveness of the bait. Thus with pure phenol the concentration was too high around the trap and beetles were not drawn completely to it. The release rate was reduced by mixing phenol with water at various concentrations. Pure phenol mixed with water at concentrations varying from 500 to 10 ppm was found to be attractive to the male beetle. Two litres of the phenol-water mixtures were placed in open tins and tested at dusk when beetle flights occurred. On one such evening, a total of 71 males and no females was caught in seven traps each containing approximately 100 ppm of the phenol-water mixture. On the following evening, 222 males and 19 females were caught in the same traps. Control traps containing water, placed alongside each baited trap, did not catch any beetles.

Thus it appeared that phenol functioned as both a sex attractant and a sex stimulant for the male grass grub beetle.

(k) Variation in phenol content with age and mating of the female beetle

The variation in the phenol content of females with age was determined by extracting batches of 100 females with 3 x 10 ml of

diethyl ether. The extract was concentrated to 10 ml and analysed for phenol by gas chromatography on a 2% Atlox G-1292 column.

The results (Table 5.3) indicated that phenol was not present in the female beetle until about 4 days after final ecdysis. This level gradually increased to about 1.5 µg per female when beetles were aged 8-10 days after final ecdysis. It is of interest that this is the age when females initially mate in the field. In these experiments the females retained the maximum phenol level for at least 13 days after final ecdysis and presumably would have done so until mating took place. The phenol level in mated females dropped to 0.7 µg/female.

Table 5.3 Variation in phenol content with age of the female beetle

Female age, days after final ecdysis	0.1	2	4	6	8	10	12	13
Phenol, µg per 100 female equivalents	0	0	25	95	160	155	95	145

(1) Activity of substituted phenols in field bioassays

A number of moth species (Jacobson et al., 1970b; Adler et al., 1972b) have been shown to respond to compounds closely related to the sex pheromone and so field tests were carried out with a few simple phenolic compounds to test their activity against the male grass grub beetle. The following compounds were tested: 1-naphthol, 2-naphthol, 2-phenylphenol (sodium salt), 3-aminophenol, 4-aminophenol, 2-methylphenol, 4-methylphenol, guaiacol, vanillin, thymol, carvacrol, catechol, resorcinol, quinol, pyrogallol, 1,2-dihydroxy-3-allylbenzene, and 1,2-dihydroxy-4-allylbenzene. The last two compounds were synthesised using the method of Perkin and Trikojus (1927). Each chemical was tested in duplicate as an undiluted reagent (0.25-0.5g) or in aqueous solution (50 ppm w/v).

The field tests were carried out on three successive evenings when beetle flights occurred but none of the compounds attracted grass grub beetles.

5.3 Discussion

Prior to this study, the beetle sex pheromones that had been identified included terpene and terpene-like compounds for a few bark beetles, valeric acid for the sugar beet wireworm and trans-3, cis-5,-tetradecadienoic acid for the black carpet beetle. It was quite unexpected, therefore, that phenol should perform this function in the grass grub beetle. Recently, however, 1:6 dichlorophenol has been identified as the sex pheromone of the lone star tick (Berger, 1972).

The presence of phenol in a beetle was not unexpected for it had previously been shown to function as a defence substance in the myriapod, O.coarctata (Monteiro, 1961), the tenebrionid beetle, Z.rugipes (Tschinkel, 1969) and a number of carabid beetles (Schildknecht et al, 1968). It is also of interest that the phenolic compound, methyl eugenol, has been used successfully in combination with methylcyclohexane propionate, phenethyl butyrate, (Beroza, 1970; Ladd, 1971) and host volatiles (Goonewardene et al., 1970b) as an attractant for both sexes of the scarab beetle, Popillia japonica. This insect is closely related to the grass grub beetle. Mathison (personal communication) also reported that phenol attracts another scarab, the rhinoceros beetle, Oryctes rhinoceros. It may be shown in the future, therefore, that volatile phenolic compounds play an important role in the life cycle of a number of scarab beetles.

The presence in the female grass grub beetle of phenol which is highly toxic to most organisms raised a number of questions as

to its origin and storage. A possible metabolic origin of phenol is phenylalanine which is an essential amino acid in the diet of insects (Gilmour, 1961). In the process of hardening and darkening (sclerotization) of the cuticle of insects, phenylalanine is oxidized to tyrosine and a number of phenolic intermediates which include dopa and dopamine. Modification of tyrosine or one of these intermediates by decarboxylation, deamination, and demethylation could produce phenol.

A number of studies on the sclerotization process have revealed that phenols can be stored as the innocuous glucoside and when required can be liberated by the action of the enzyme, β-glucosidase (Brunet and Kent, 1955; Brunet, 1967; Winteringham, 1965; Smith, 1955). This mechanism of phenol storage was investigated for the female grass grub beetle. Tests were carried out with emulsin (β-glucosidase) on aqueous extracts of female beetles aged 2-4 days old. This age range was selected as the phenol level was at a minimum and if phenylglucoside was the precursor it may have been present at a maximum level in young beetles. No experimental support for this method of storage was obtained, however, for phenol was not generated in the female extract with β-glucosidase.

Hoyt and co-workers (1971) suggested that the colleterial glands, which are associated with the female's reproductive organs, may play an important role in pheromone production in grass grub. They showed, for example, that a tyrosine medium innoculated with symbiotic bacteria, found in the colleterial glands, contained phenol after five days. They inferred that this could be the mechanism of phenol production in the beetle.

The colleterial glands in the female beetle are situated in the last few abdominal segments and while it does not necessarily follow, it is reasonable to suggest that, if phenol was produced in

this region it would be released nearby. If so, this would be inconsistent with work on the site of pheromone production described in Section 5.2 (a), where it was shown that the pheromone appeared to be produced within the first three abdominal segments on the dorsal side of the female.

Studies of the aromatic defense substances in a member of carabid and tenebrionid beetles have revealed that phenols and quinones are produced by specialized exocrine glands and stored in sac-like reservoirs. The gradual build-up of phenol in the female grass grub beetle from about 4 days onwards suggests that the pheromone is stored in a similar manner.

The level of phenol in the virgin female beetle which increased to a maximum of 1.5 µg per insect was about three times greater than that reported for the black carpet beetle sex pheromone and one hundred times less than that of the sugar beet wireworm.

The phenol level in the mated female beetle was about half that in the virgin female and this is in agreement with work on other insects which mate more than once. In these cases the sex pheromone is maintained in the mated female at a significant, but somewhat lower level than that in the virgin female.

Throughout the development of the isolation procedure, individual bioassay results were not always reliable, therefore, each significant isolation step was repeated at least two or three times. This inconsistency of the behavioural tests may provide one explanation for the results obtained in the partitioning experiments with acidic buffers. Another possible explanation is that the female grass grub beetle, like many other insects that have been studied, uses multiple pheromones one of which is acid sensitive. On the bulk of the evidence presented in this and following chapters, however, it appears unlikely that a multiple pheromone system is

involved.

5.4 Conclusions

Sectioning experiments on female grass grub beetles aged 10 days old indicated that the sex pheromone activity was produced dorsally within the first three abdominal segments.

The sex pheromone was extracted from the females' abdomens by surface washing with diethyl ether. The behaviour of the pheromone in a number of functional group tests and concentration steps was consistent with that of a phenolic compound. The isolation procedure used on a crude ethereal extract of female abdomens consisted of high vacuum distillation onto a condenser filled with dry ice followed by gas liquid chromatography of the vacuum distillate. The stationary phase selected retained the majority of co-extracted impurities at the top of the column and this facilitated the isolation of pure fractions. The gas chromatogram consisted essentially of one component whose retention time was identical with that of phenol. The presence of phenol in the female beetle was subsequently confirmed by high resolution mass spectrometry.

Phenol, which could not be detected in male beetles, was shown to stimulate males in the laboratory but the excellent responses obtained in the initial tests could not be repeated in subsequent seasons. In contrast to this situation, phenol consistently attracted young males in mating flights in the field and this is the ultimate criterion in the identification of a pheromone.

The phenol level in female beetles increased to a maximum level of 1.5 µg per insect at about 8-10 days after final ecdysis. This is about the length of time the female is present in the soil before mating takes place.

Field tests were carried out with eighteen phenolic compounds

as undiluted reagents and in aqueous solution but only phenol itself attracted male grass grub beetles.

CHAPTER VI

ELECTROANTENNOGRAM RESPONSES OF THE GRASS GRUB BEETLE TO PHENOL AND RELATED COMPOUNDS

6.1 Introduction

During the course of this study, phenol consistently attracted male grass grub beetles in mating flights in the field but reliable results were not obtained with it in laboratory behavioural studies. This suggested that, in order to induce strong sex stimulatory activity in males in the laboratory, either the rate of phenol release was critical or an additional phenol was involved.

The electroantennogram (EAG) was developed, therefore, in an attempt to resolve this issue. It has been shown in all the EAG studies with sex pheromones that the strongest responses are obtained with compounds which correspond to the natural pheromone. This phenomenon was discussed in Section 2.9. Thus, if a multiple component pheromone system was used by the female grass grub beetle, the crude female extract should produce a stronger EAG response than phenol alone.

Normally the EAG method has been used to complement laboratory behavioural studies but preliminary EAG studies carried out by the author, with the excised antenna of the gum emperor moth, Antheraea eucalypti, revealed a number of advantages of this assay method over behavioural bioassay methods. The EAG provided a quick, reliable, objective bioassay which could be carried out at all periods throughout the day.

The EAG method has been used in sex pheromone studies of the closely related scarab, Popillia japonica (Adler and Jacobson, 1971).

The relationship between field attractiveness of a number of chemicals and the magnitudes of the EAG response in P.japonica adults has also been investigated (Adler et al., 1972). The aim of the latter study was to screen possible attractants for the beetle in the field.

In the present grass grub beetle study, antennal response to a variety of phenolic compounds was studied. This provided a comparison with the response to phenol and helped check for inhibitors, since as Boeckh et al., (1965) had shown, inhibitory stimuli induced a positive potential in the antenna, rather than a negative potential exhibited by the sex pheromone.

6.2 Experimental

Beetles aged 7-10 days after final ecdysis were used in all the tests. They were raised in the laboratory using techniques previously described in Section 4.2a. For electrical recording, the beetle was placed on its side in a cork cavity about the size of a beetle and held in position with 'Sellotape'. The antenna was exposed to facilitate the insertion of the electrodes. In order to select suitable electrodes for the EAG measurements, the impedance of the antennal fluid with a number of different electrode materials was measured. The impedance for glass capillaries filled with saline was 2 megohms while that for gold, platinum, tungsten, and nickel electrodes was 3, 20, 35, and 100 megohms respectively. In view of the lower impedance with the glass capillaries these were used throughout the study. One of these was inserted into the third antennal lamella and the other towards the distal end of the scape (Plate 6.1). To assist in the insertion of the latter electrode the cuticle was pierced with the tip of a tungsten wire, electrolytically polished to a tip diameter of 5-10 μm (Payne, 1970). The glass

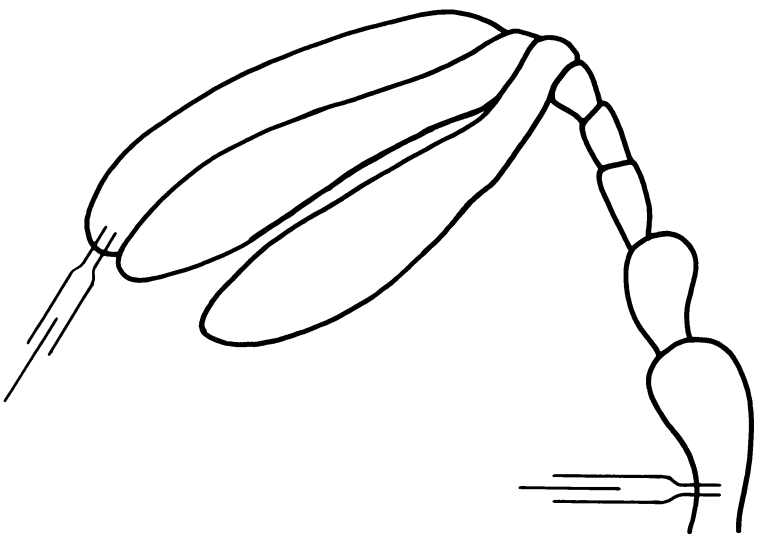
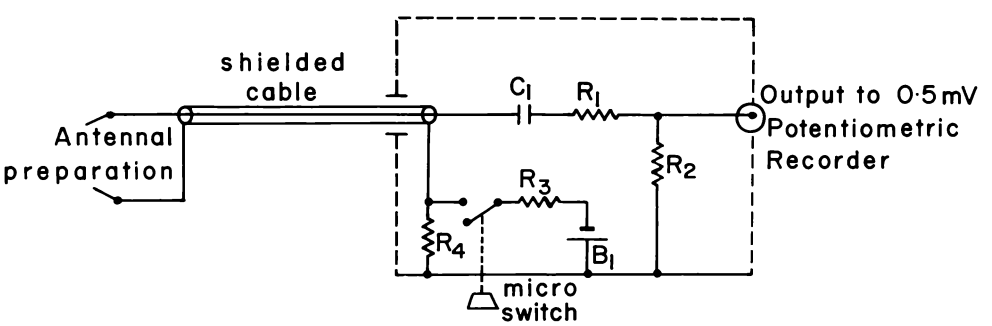


Plate 6.1 Position of glass capillaries in the male grass grub beetle antenna.



C_1	0·1 - 1μF	R_3	13 KΩ
R_1	1 meg Ω	R_4	10 Ω
R_2	2 meg Ω	B_1	1·35V mercury cell

Plate 6.2 Coupling box circuit diagram.

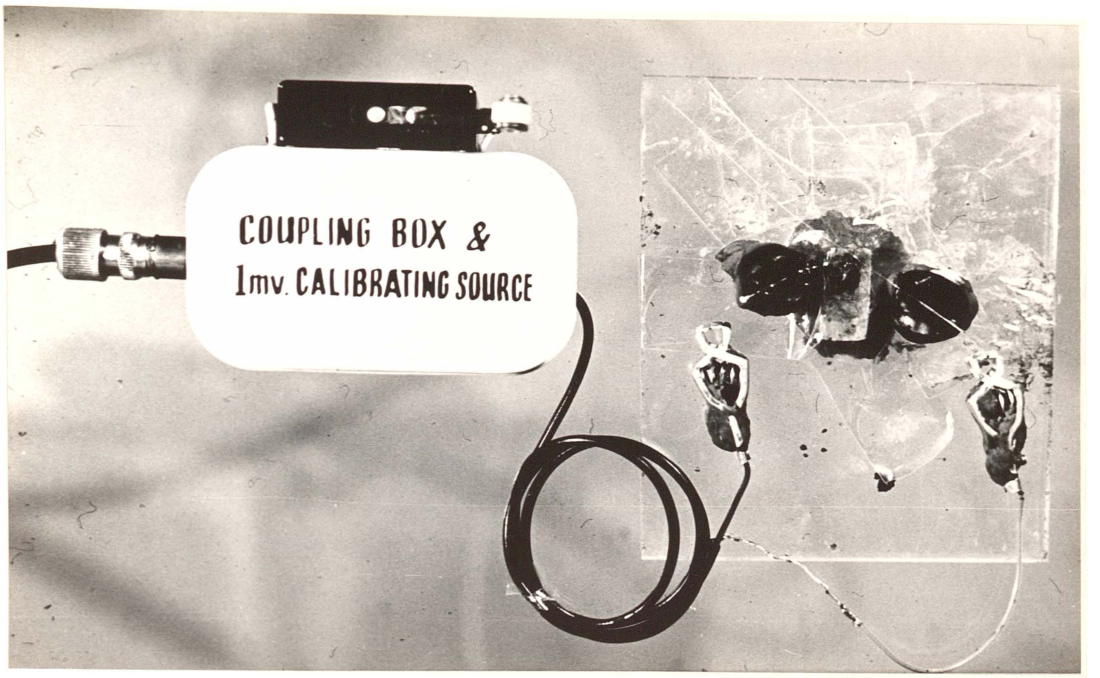


Plate 6.3 The antennal preparation and electrical connection to the coupling box and 1 mV calibrating source. The glass capillaries filled with saline were held on Picien wax mounted on plasticine. The beetle was held in a cork cavity with 'Sellotape'.

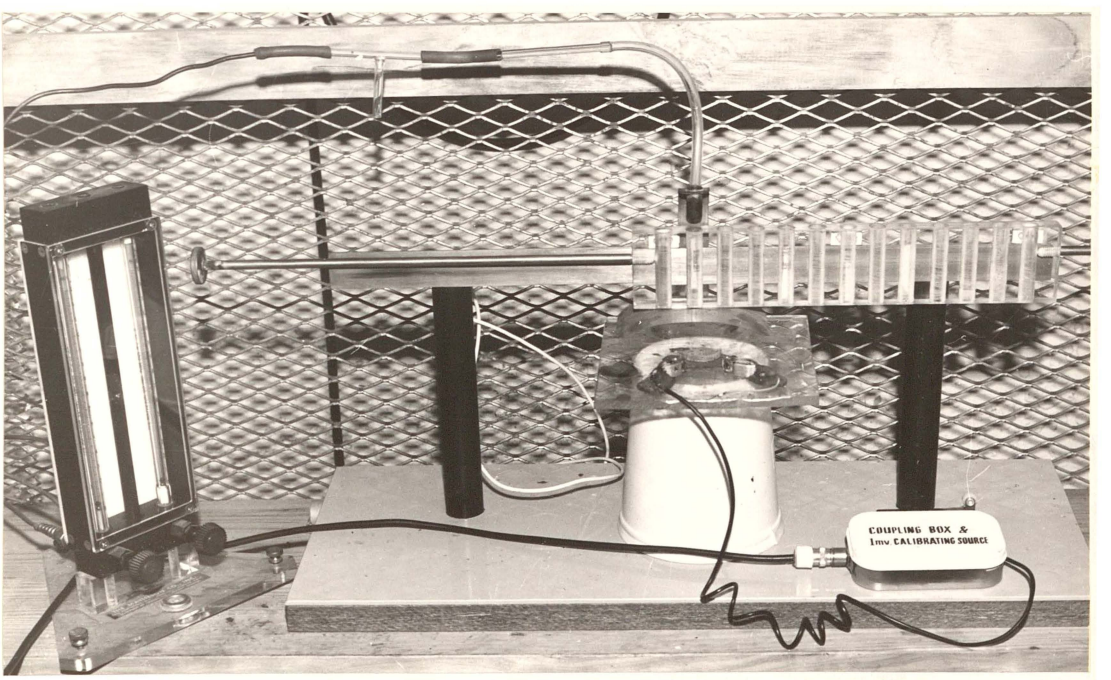


Plate 6.4 The apparatus used for EAG measurements inside a Faraday cage.

capillaries were drawn by hand to a tip size approximately 20 µm from 5 µl 'Microcap' capillaries heated in a microburner. Before insertion, these were filled with 1M sodium chloride and sealed on to a piece of Picien wax mounted on plasticine. The plasticine provided sufficient flexibility for the insertion of the electrodes while the Picien wax gave rigidity to the electrode system minimising the displacement of the electrodes due to antennal movements. The insertion of the electrodes was carried out at a 40X magnification using a stereomicroscope.

The electrical connection was made with 0.25 mm platinum wire through a shielded lead to a coupling box and a Bausch and Lomb strip chart recorder with a response of 0.5 mV full scale deflection. The coupling box circuit diagram is shown in Plate 6.2. In addition, the EAG's from each beetle were calibrated with a 1 mV source which could be placed in series with the electrodes by means of a microswitch. The antennal preparation and electrical connection to the coupling box and 1 mV calibrating source is shown in Plate 6.3.

Each odour sample to be tested was placed on a piece of Whatman No.1 filter paper contained in a glass tube. This was inserted into a hole in the perspex holder, shown in Plate 6.4, and moved into position directly below the air supply. A metal plate (5 x 10 cm) with a hole (1 cm dia.) in the centre was held beneath the holder to reduce contamination from adjacent glass tubes. The antennal preparation was placed below the hole in the path of the air stream. Samples were tested inside a 2 m^3 Faraday cage to minimise electrical interference, and a 2-second puff of air, regulated at 700 ml/min was passed over the antenna.

6.3 Results and discussion

(a) Antennal response of males to phenol

The shapes of the EAG's recorded from an antenna of a male grass grub beetle in response to given amounts of phenol on filter paper are shown in Figure 6.1. For this male beetle, the EAG response to a puff of air with no phenol on the filter paper (a) was 150 μV. The air response varied from beetle to beetle and ranged from 30 to 250 μV. All the EAG results presented are net responses i.e. EAG response to the treated filter paper minus the corresponding air response for the particular beetle. The observed responses were similar in shape to those recorded for the Japanese beetle (Adler et al., 1972a). The EAG response to stimulation typically consisted of a rapid (within 0.5 sec) negative change in potential at the recording electrode, relative to the indifferent electrode, followed by a slow return to the baseline (within 4-5 sec). The amplitude of the rapid initial negative change in potential was used as a quantitative measure of the strength of the EAG.

The response to a 10-fold phenol dilution series which ranged from 0.01 to 1000 μg phenol on paper was measured on seven male antennae. An unequal number of measurements was made at each level and this is indicated (n-values) on Figure 6.2. The curve indicates that antennal response rose to a maximum with about 10-20 μg phenol on the filter paper and diminished below 0.01 μg phenol. While this was the case in general, a few male beetles did respond to as little as 0.001 μg phenol on the paper.

Male response to phenol varied considerably within and between antennae and the within antenna variation was greater for the more responsive antennae. With the exception of one antenna, the response of each antenna diminished with time. This effect,

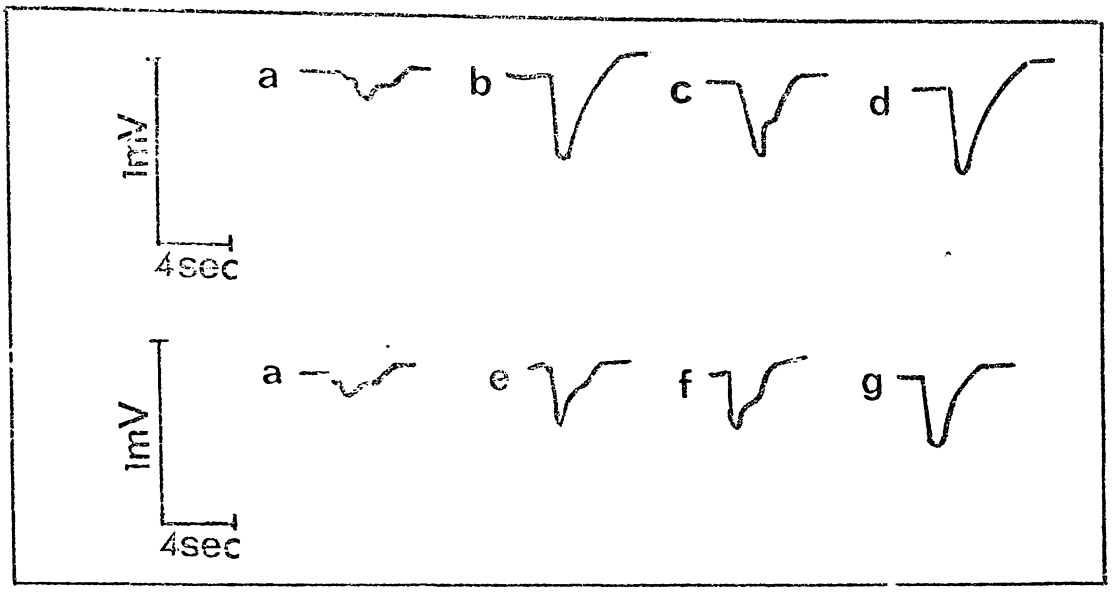


Figure 6.1 Shapes of the antennal response in a male grass grub beetle antenna to (a) air, (b,c,d) 1 µg phenol tested at 20, 100 and 630 secs resp., after the initial air response, (e,f,g) a female extract equivalent to 1 µg phenol tested at 180, 245 and 280 secs resp., after the initial air response.

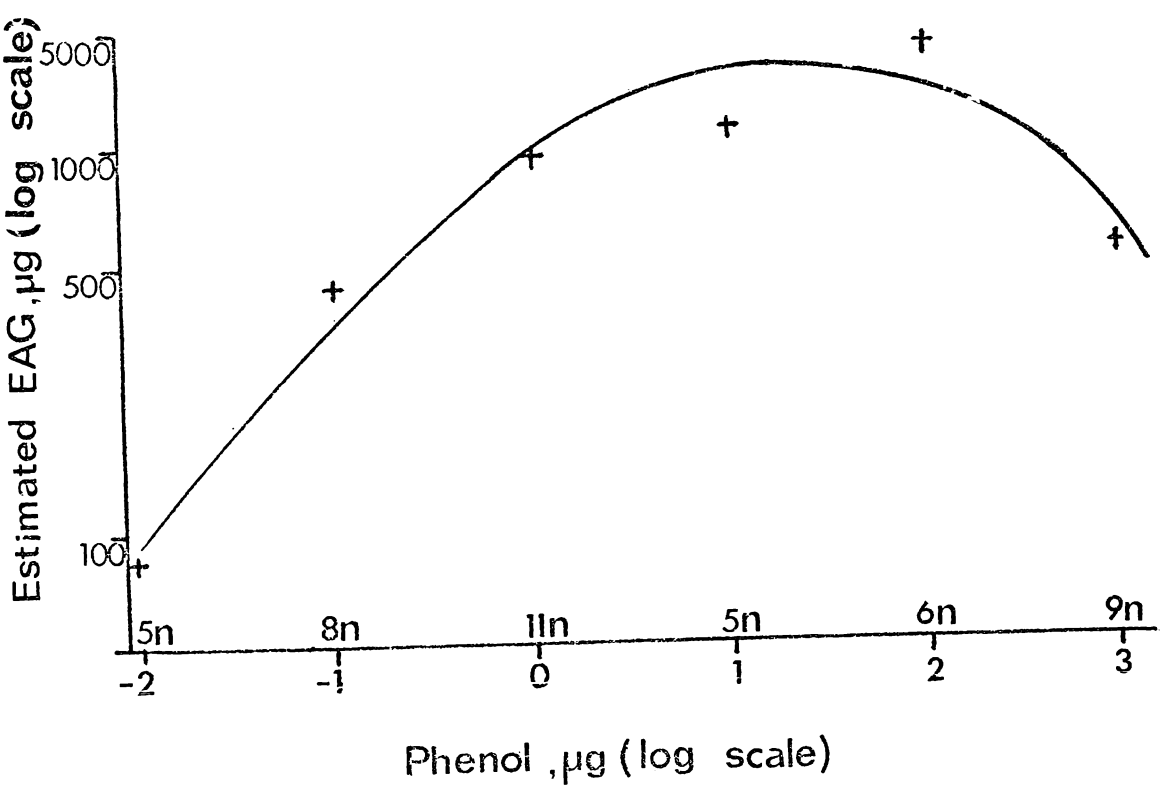


Figure 6.2 Estimated EAG amplitudes of response for 10-fold increases in phenol weights from 0.01-1000 µg on paper.

presumably due to fatigue or adaptation was indicated by a sluggish response with considerably reduced amplitude. The results presented were obtained before the antenna showed obvious signs of diminished activity. Dilution series were always presented in sequentially increasing amounts to minimise adaptation, and the period between each test was at least 2 min. A 1 mV pulse was applied to the recording electrode for calibration purposes before and during each test sequence.

After adjusting for the unbalanced replication and correcting for the time trend effect by using a limited time range for each antenna, least squares estimated means were obtained for each phenol level and are shown in Figure 6.2. There was insufficient data to warrant testing the variation in shape of the curve from antenna to antenna, but the quadratic component was statistically significant ($P<0.01$) compared with the average of the variances of the estimated means.

(b) Antennal response of males to substituted phenols

EAG studies carried out by other workers have indicated that while insect antennae respond to compounds closely related to the sex pheromone, they are usually less sensitive to them. A number of substituted phenols possessing vapour pressures comparable with phenol (Table 6.1) were selected, therefore, and tested for activity.

Table 6.1 Vapour pressures at room temperature (20°C) of the substituted phenols used in the EAG tests.

Compound	V.p. (mm Hg)	Compound	V.p. (mm Hg)
Phenol	0.53	2-Ethylphenol	0.18
2-Methylphenol	0.27	3-Ethylphenol	0.12
3-Methylphenol	0.12	4-Ethylphenol	0.07
4-Methylphenol	0.11	2-Isopropylphenol	0.08

The tests were carried out on four male antennae and the results for one of these is shown in Table 6.2

Table 6.2 EAG responses of a male antenna to phenol and alkyl substituted phenols

Compound	EAG (μV) for phenols on paper		
	0.1 μg	1.0 μg	10 μg
Phenol	620	750	1650
2-Methylphenol	0	450	670
4-Methylphenol	130	90	690
2-Ethylphenol	0	320	1000

These results were typical of the pattern of response for each antenna i.e. for a given weight of compound, phenol itself elicited a higher response than any other phenol tested. Thus the responses to 10 μg of each of the phenols on paper indicated that the response to phenol itself (1650 μv) was half as much again as that to 2-ethylphenol (1000 μV) and nearly three times as large as those to 2-methyl-, and 4-methylphenol (670, and 690 μV respectively). At the 1.0 μg level, however, the differences were not as large. As expected, the antenna was always more sensitive to phenol than to the substituted phenols. This is illustrated in Table 6.2 where at the 0.1 μg level, the antenna did not respond to either 2-methyl or 2-ethylphenol while a 620 μV response was recorded for 0.1 μg phenol itself. The shapes of the antennal responses to some of these substituted phenols are shown in Figure 6.3.

With the exception of phenol at the 0.1 and 1.0 μg levels, the measurements were not repeated. At the 0.1 μg phenol level, the antennal response varied between 500 and 780 μV (5 measurements) and 320 and 1200 μV (5 measurements) at the 1.0 μg phenol level. Other compounds which gave lower responses than phenol included: 3-methylphenol, 4-ethylphenol, 2-isopropylphenol, 2,3,5-trimethylphenol and

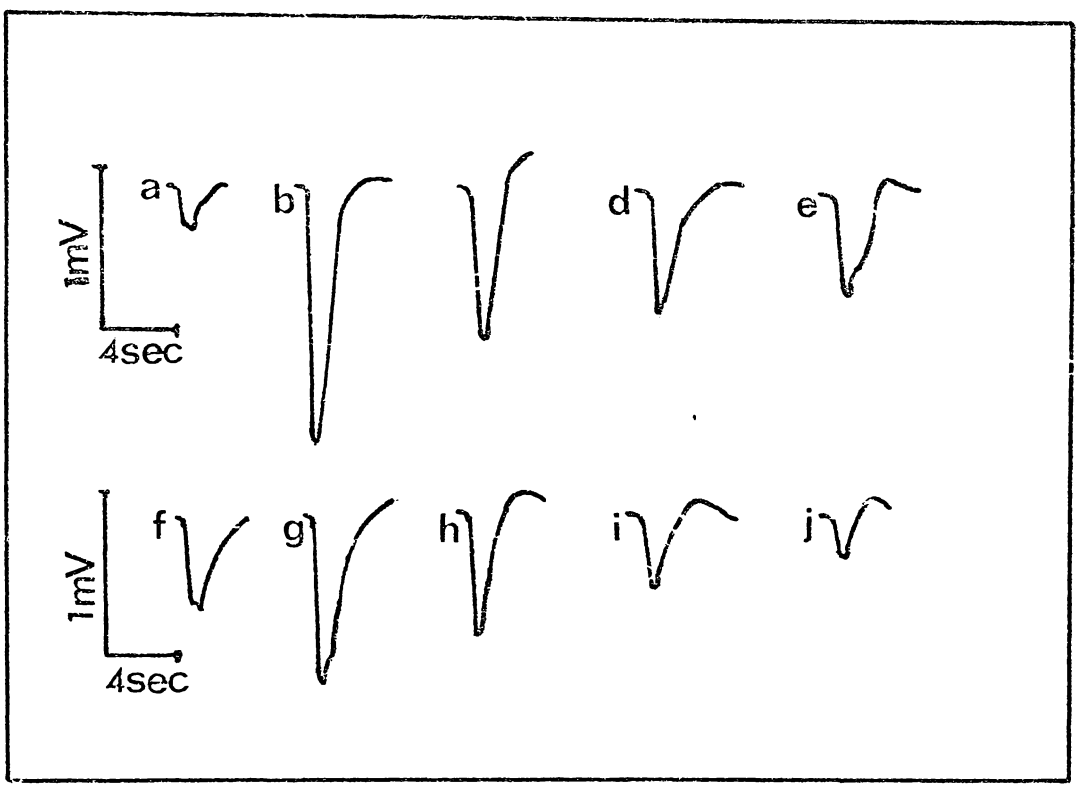


Figure 6.3 Shapes of the antennal response in a male grass grub beetle antenna to (a) air, (b,c) 10 µg and 1 µg phenol resp., (d) 10 µg 2-methylphenol, (e) 10 µg 3-methylphenol, (f) 10 µg 4-methylphenol, (g) 10 µg 2-ethylphenol, (h) 10 µg 4-ethyl phenol, (i) 10 µg 2-isopropylphenol and (j) 10 µg 2-hydroxybenzyl alchohol.

2-hydroxybenzyl alcohol. These phenols have been tested in the field and only phenol itself was found to be active (Section 5.2(1))

The possible masking effects of closely related phenols were examined by combining a few cresols and brominated phenols with phenol itself and measuring any depression or polarity reversal in the antennal response. This latter phenomenon was reported by Boeckh et al., (1965) in studies on the repellant action of oil of cloves on the antennal response of Antheraea pernyii. In the tests with male grass grub beetles, however, all the phenols tested induced a negative potential at the recording electrode.

The EAG responses obtained with phenol (at two levels on paper, viz., 10 and 100 µg) mixed with varying quantities of other phenols are shown in Table 6.3.

Table 6.3 EAG response of a male antenna to mixtures of phenol and substituted phenols.

Phenolic mixtures	EAG's (µV)
10 µg phenol	540*
" + 10 µg 2-methylphenol	750
" + 10 µg 3-methylphenol	750
" + 1000 µg 2-methylphenol	520
" + 1000 µg 2-bromophenol	720
100 µg phenol	700
" + 100 µg 2-bromophenol	390
" + 100 µg 3-bromophenol	500
" + 100 µg 4-bromophenol	500

*Response varied between 250 and 950 µV in 8 measurements on the antenna.

The results of the tests, which were not repeated on other antennae, suggested that the male antennal response to phenol was not substantially depressed by the addition of substituted phenols. The brominated phenols were selected in the expectation of a possible

blocking effect of the bromine atom on the antennal receptors.

It may not be possible to detect such effects with the EAG technique, however, for in a paper published after this work was completed, Roelofs and Comeau (1971b) showed that trans-11-tetradecenyl acetate, which inhibited the attractiveness of the cis isomer in the field, induced a good EAG response in the red-banded leaf roller moth.

(c) Comparison of the male antennal response to phenol and a female extract.

A crude female extract prepared by surface washing the abdomens of females with diethyl ether was analysed for phenol by gas chromatography and the volume of the extract adjusted to give a phenol concentration of 50 ppm (w/v). An aliquot of this extract (20 µl) containing 1 µg phenol was then applied to filter paper and its activity at the antennal level compared with 1 µg phenol from a 100 ppm (w/v) standard. The order of testing was randomised and the treatments were tested three times on each of three male beetle antennae. The results of this comparison are shown in Table 6.4.

Table 6.4 Comparison of the male antennal response to phenol and a female extract.

	Average male response (µV)								
Beetle	1	2	3	Mean	4	5	6	Mean	Overall mean
Female extract[a]	50	80	298	143	167	168	77	138	140
Phenol (1 µg)	54	98	351	170	164	257	162	194	182
MSD 5%[b]				38				68	37

[a] An aliquot was taken containing 1 µg phenol.

[b] Minimum difference for significance at 5% probability level.

The comparison was made initially with three beetles (numbers 1-3) and the difference of 27 between the means of the average responses to the female extract and phenol was not significant at the 5% probability level. The average responses for two of these three beetles were low (<100 μV) and, therefore, the comparison was repeated with a further three beetles (numbers 4-6). These results indicated that there was no significant difference ($P<0.05$) in antennal response between the female extract and phenol.

The overall analysis did suggest, however, that the male antenna was more responsive to phenol ($P<0.05$) than to the crude female extract. This difference may be associated with the co-extracted fats, etc., which would be expected to reduce the rate of phenol release and thereby reduce the amount of phenol dispersed over the antenna. The shapes of the antennal response curves were similar (Figure 6.1) for the two treatments.

(d) Antennal response of females to phenol

All the sex pheromones extracted from female moths have elicited strong EAG responses in male but not in female antennae. However, in the cases of the queen butterfly, Danaus gilippus berenice (Schneider and Seibt, 1969) and some bark beetles (Payne, 1970) both sexes have responded to the same extent to the sex pheromone. In the latter case both sexes aggregate to the pheromone.

It was found that phenol induced a reaction in the female grass grub beetle. For example, the average responses (air responses subtracted) from three female antennae to 0.01, 1.0, 10, 100, and 1000 μg phenol on paper were 25, 35, 150, 150, and 150 μV respectively.

Thus 10 μg phenol gave a response in the female antenna comparable to that obtained for 1 μg phenol in the male antenna in Table 6.4. In field experiments, very few females have been attracted to phenol.

6.4 Conclusions

Maximum antennal responses in the male grass grub beetle were obtained with 10-20 µg phenol on filter paper. Normally the activity of phenol diminished with levels less than 0.01 µg but in some cases beetles responded to as little as 0.001 µg phenol.

In the EAG tests with a number of alkyl substituted phenols, all the compounds induced negative potentials in the antennae. As expected, male antennae were less sensitive to these compounds than to phenol and the magnitude of the response was always less than the corresponding weight of phenol. Attempts to use the technique to investigate possible inhibitory effects of phenol analogues, by measuring a polarity reversal or depression of EAG activity, were unsuccessful.

Comparable EAG responses were obtained with a crude female extract and phenol alone when compared on an equal phenol weight basis. It appears, therefore, that the female beetle does not use a multiple component pheromone system.

Phenol induced a slight antennal response in female beetles but the magnitude of the response was at most one tenth that for males.

CHAPTER VII

FIELD TRIALS WITH PHENOL FOR GRASS GRUB CONTROL

7.1 Introduction

The identification of phenol as the sex pheromone of the female grass grub beetle opened up one of the first possibilities of using a sex pheromone to control an insect with an unlimited supply of the pheromone. Furthermore, if such direct control by phenol was possible, the method would probably be economic because of the low cost of this compound.

Sex pheromones are by necessity volatile chemicals and if they were simply used alone in the field they would remain effective for a very short time. Thus, in order to use sex pheromones in control programmes, they have to be formulated in a manner to release the pheromone at a controlled, optimum rate for the entire mating period of the insect concerned. This condition is especially critical for the attractant methods and to a lesser extent for the 'confusion' method (discussed in Sections 2.12 and 2.13) where too high a release rate will be an unnecessary waste of pheromone.

In order to evaluate the possible behavioural methods of grass grub control with phenol, therefore, it was important to develop a means of dispensing phenol at low, uniform release rates for the entire adult mating period of 3-4 weeks. In this respect a paper by Osborne and Hoyt (1970) was significant. These workers reported a thermosetting phenol-formaldehyde resin, Durez 12687 (manufactured by Durez Chemical Co., Akron, Ohio, U.S.A.), as being highly attractive to male grass grub beetles and stated that phenol was an active component in the resin. Subsequently, it was

found that the addition of water to the resin to form a paste produced a ten-to one hundred-fold increase in the potency of the resin (Osborne, personal communication).

Durez resin has many advantages in that it is potent, cheap, readily available and can be easily formulated with insecticides. It has been found, however, that the release of phenol is drastically reduced when the powder is applied to pasture. Attempts were made, therefore, to develop a cheap means of dispensing phenol which could be spread onto pasture in aerial topdressing programmes and which would retain activity for the entire adult mating period, under all weather conditions. In order to assist in the development of suitable dispensers, a method of measuring phenol release rates in the laboratory was used.

When suitable dispensers had been developed, field trials were carried out to evaluate the potential of phenol in grass grub control. Prior to this, however, preliminary field work was carried out to assess the rates of release for optimum attraction, the distance of attraction, and the activity of various phenol formulations compared to females in the natural population.

7.2 Laboratory studies

(a) Determination of phenol release rates.

Release rates of phenol were determined by passing moist air slowly over the substrate for a definite period of time and trapping the phenol in 100 ml of 0.5% sodium bicarbonate or water. The substrates e.g. wood cubes, filter paper circles, or powders, were held at 16°C in a glass tube (1m x 4.5 cm ID). The apparatus held 20 papers (4.2 cm dia) placed on their edge, and allowed the surface of all the substrates to be exposed to the carrier gas. Weathered substrates taken from the field were held at 16°C for a few hours

before the determination of release rates. Wood cubes were analysed in batches of 20 or 50 depending on the release rate of phenol. Phenol release rates from pasture were obtained by placing a Winchester bottle with the bottom removed on a piece of treated turf. Air was then drawn through the bottle and into 50 ml of 0.5% sodium bicarbonate contained in a wash bottle attached to the Winchester.

Phenol was analysed by either gas chromatography (GC) or the standard colormetric method of analysis developed by Welcher (1963). In the GC method, phenol was extracted from the aqueous solution with diethyl ether (3 x 15 ml) and the combined diethyl ether phase reduced to 5 ml by passing a gentle stream of air over the ether held in a water bath at 40-50°C. A 5 µl aliquot of the sample solution was injected on to a GC column which consisted of 2% Atlox G-1292 emulsifier on 40-70 mesh Silocel. The column was operated under isothermal conditions at 150°C and a nitrogen flow rate of 40 ml/min. This method was used when the total amount of phenol exceeded 100 µg for the results of repeated recovery experiments, shown in Table 7.1, indicated that low recoveries were obtained below this level.

Table 7.1 Recoveries of phenol using the GC method.

Amount of phenol applied to 0.5% sodium bicarbonate (µg)	Percent recoveries
10^4	100, 100, 100
10^2	100, 97, 97
5 x 10	50, 50, 50
2 x 10	20, 15, 15

The colorimetric method depends on the reaction between 4-aminodimethylaniline and phenol under acidic conditions. The

reaction product when treated with calcium hypochlorite produces a blue colouration due to the formation of an indophenol dye (α-Naphthol blue). In this method the aqueous bicarbonate solution was treated with a few drops of 4-aminodimethylaniline sulphate in dilute sulphuric acid and then titrated with 0.1% calcium hypochlorite solution until the blue colouration appeared. The blue dye was extracted into chloroform (10 ml.) and the optical density measured at 600 nm. This method, which was used for phenol levels below 100 µg, had a phenol identification limit of 2 µg and gave 100% recoveries down to the 5 µg phenol level.

(b) Phenol dispensers

(i) substrates

It was found that when powders containing phenol were applied to pasture the rate of phenol release was considerably reduced. This effect is illustrated in Table 7.2 for Durez powder and a powder containing 1% phenol on lime.

Table 7.2 Effect on the phenol release rate of the application to pasture of powders containing phenol.

Formulation	Surface of application	Time after application (h)	Phenol release rate (µg/h)
100 mg Durez 12687	Filter paper	0.1 - 4.0	37.5
"	" "	24.0 - 28.0	28.5
"	Pasture	0.1 - 4.0	3.0
"	"	24.0 - 28.0	0
100 mg 1% phenol on lime	Filter paper	0.1 - 4.0	6.5
"	" "	24.0 - 28.0	5.5
"	Pasture	0.1 - 4.0	2.0
"	"	24.0 - 28.0	0

The phenol release from both these formulations was reduced, compared to that from filter paper, as soon as the powders were

applied to the pasture. For example, with the Durez powder which contains 1.5-2.0% phenol, the phenol release rate dropped ten-fold from 37.5 to 3.0 µg/h and after 24 hours, phenol could not be detected over the pasture. A three-fold drop occurred for the phenol on lime (6.5 to 2.5 µg/h) and again phenol could not be detected over the pasture 24 hours after application. This reduction is probably related to soil moisture effects as well as phenol adsorption on soil particles, soil organic matter, and the foliage.

Attempts were made, therefore, to develop suitable cheap dispensers that could be applied to pasture and retain activity for the 3-4 weeks of the adult mating period.

Wood was selected initially as a possible dispensing medium because of its ability to absorb liquids and the expected strong H-bonding adsorption effects between cellulose and phenol. Such effects would be likely to assist the retention of phenol on weathering. Kauri wood was selected as it was reasonably porous, possessed very little odour and could be cut easily. This latter property was important in preparing the thousands of 0.6 cm^3 wooden cubes used. Phenol was applied at three rates (1, 10, and 100 mg/cube) in diethyl ether to batches of 2,000 - 3,000 cubes and allowed to soak for about a week. After removal of the diethyl ether by rotary evaporation, the cubes were coated with a chemically resistant epoxy polymer, 'Durepon', which assisted in retaining high levels of phenol. When the cubes were uniformly coated, an absorbent material, powdered firebrick, was added to prevent the cubes from clumping together on drying. In each coat approximately 50 mg Durepon/adduct (2:1) and 120 mg firebrick were applied to each wooden cube.

The coated wooden cubes, similar to those shown in Plate 7.1, were used in the pilot scale field trials in the second year of this study to evaluate the 'confusion' method of grass grub control.

Plate 7.1 Beetles killed with fensulphothion-treated wood cubes. The cubes were treated with phenol and then coated with an epoxy polymer mixed with insecticide.

Plate 7.1 illustrates the knockdown effect of insecticide treated wooden cubes. A few simple experiments of this type were carried out but were unsuccessful as phenol formulated on wooden cubes was not a sufficiently potent attractant.

Phenol was also incorporated into emulsion paint at 1% and 5% by weight and the mixture coated onto blue metal chip or coarse pumice particles. This method was unsatisfactory as the phenol disappeared within one week on weathering. A similar result was obtained when phenol (1% and 5% by weight) was added to polyethylene glycol-4000 in methylene chloride and coated onto 30-60 mesh firebrick. Phenol incorporated into paraffin wax (0.1, 1.0, and 5.0% by weight) was retained within the body of the wax and was not released after a few days weathering.

(ii) Derivatives

As all the substrates onto which phenol was incorporated were unsatisfactory, alternative methods of dispensing phenol were studied. One such method was to synthesize non volatile phenol derivatives which would liberate phenol slowly on hydrolysis by moisture. The aim was to use these materials in field trials and the basic requirements of these derivatives were that they could be readily prepared in good yield from relatively cheap starting materials. Various phenyl esters were selected on the basis of expected ease of hydrolysis and the following compounds were synthesized:

(1) Phenylstearate - this was prepared (90% yield) by heating phenol (23.5 g) under reflux with stearyl chloride (75.5 g) for half an hour on a water bath. The solid product, obtained by pouring the contents onto ice-water, was re-crystallized three times from ethanol-water and had a m.p. 50.5-51.5°C, reported m.p. 52°C (Huntress and Mulliken, 1941). The IR spectrum of the compound showed no hydroxyl group absorption and a strong absorption band at 1740 cm^{-1} consistent

with a carbonyl bond of an aryl ester. A portion of phenylstearate (100 mg) was applied uniformly to 20 filter papers (4.2 cm dia.) and allowed to stand in the laboratory for several days but phenol was not detected above the papers on analysis. Attempts to induce breakdown under acid conditions by combining phenylstearate with stearic acid (1:1) were also unsuccessful.

(2) Phenylglucoside - this material was regarded as a possible precursor of phenol in the grass grub beetle and so there was a dual purpose for it's preparation. It was prepared in low yield (10%) by treating acetobromoglucose (20 g) (Redemann et al., 1942) with excess sodium phenate (7 g) in methanol (50 ml). This was followed by hydrolysis of the deposited phenyltetraacetyl glucoside by shaking with saturated barium hydroxide solution. The crystalline product had a m.p. 170-175°C, reported m.p. 174-175°C (Heilbron et al., 1965). The IR spectrum showed a broad hydroxyl band between 3600-3100 cm^{-1} and strong aromatic absorption at 1600 and 1495 cm^{-1}.

Even though the compound broke down readily to phenol, it was not considered to be a suitable candidate for widespread use in field trials because the synthesis was time consuming and expensive.

(3) Phenylhydrogensuccinate - this material was prepared by fusing phenol (9.4 g) with succinic anhydride (10 g) in a sealed flask. A quantitative yield was obtained and the crystals were re-crystallized from ethanol-water and had a m.p. 94-96°C, reported m.p. 98°C (Heilbron et al., 1965). The IR spectrum was consistent with the above compound. It showed broad hydroxyl absorption at 3400-3000 cm^{-1}, two strong carbonyl bands at 1750 and 1700 cm^{-1} and aromatic bands at 1590 and 1490 cm^{-1}. Attempts to prepare phenylhydrogenphthalate and phenylhydrogenmaleate by this method failed. Phenylhydrogen-succinate (1 g) applied to 20 filter papers degraded slowly to phenol

on standing in the laboratory and the results are shown in Table 7.3.

Table 7.3 Degradation of phenylhydrogensuccinate on standing in the laboratory.

Time after application (days)	Phenol release rate (µg/h)
1	12
8	32
13	46
21	330
28	330
32	330

The results indicated that phenol was produced at an increasing rate over a period of 3 weeks and was then released at a steady rate for a period greater than 4 weeks. The degradation was the result of hydrolysis of the phenyl ester group and the increased rate of hydrolysis was presumably the result of auto catalysis due to the formation of succinic acid. The sodium salt of phenylhydrogensuccinate degraded very readily to phenol and was completely hydrolysed within a week.

Field experiments with 1% phenylhydrogensuccinate on wood shavings applied to pasture were not encouraging, however, for phenol could not be detected above the pasture 4 days after application.

(4) Diphenylsuccinate - this compound was prepared by heating thionyl chloride (2 ml) and phenylhydrogensuccinate (2 g) under reflux for half an hour. The excess thionyl chloride was removed by distillation and phenol (1 g) added to the reaction product and refluxed for half an hour. A white powder was obtained after recrystallisation from ethanol-water and had a m.p. 118-120°C, reported m.p. 121°C (Huntress

and Mulliken, 1941). In the IR spectrum both the hydroxyl absorption at 3400-3000 cm^{-1} and the carbonyl absorption at 1700 cm^{-1}, present in the phenyl hydrogen succinate spectrum, were absent. Carbonyl absorption of an aryl ester was present at 1750 cm^{-1} together with aromatic bands at 1590 and 1495 cm^{-1}. This compound did not degrade to phenol.

(5) Phenol - hexamethylene tetramine adduct - a white crystalline product (m.p. 118°C) was deposited in quantitative yield on the addition of hexamethylene tetramine (14 g) to liquefied phenol (10 g). The product, a 1:1 adduct did not degrade to phenol.

A number of other derivatives obtained off the shelf did not degrade to phenol and these included: salicylic acid, phenyl salicylate, phenyl phthalate, aluminium-, calcium-, and zinc,- salts of phenylsulphonic acid.

(iii) Encapsulation in PVC tube

A suitable means of dispensing phenol was obtained by filling polyvinyl chloride (PVC) tube with liquefied phenol (9:1, phenol:water), sealing the ends of the tube and allowing phenol to permeate through the polar polymer wall. In weathering experiments with 30 m of this dispenser (0.7 mm wall thickness), phenol was released at 3 g/day for a period exceeding 4 weeks. The obvious advantages of this dispenser were protection from rain, soil adsorption, and soil moisture effects. This method was used to dispense phenol in the initial large scale field trials at the Takapau Research Area to test the 'male confusion' control method.

7.3 Preliminary field studies

(a) Male beetle attraction to phenol released at different rates

The importance of pheromone release on attraction has already been discussed (Section 2.13). In the field, the rate of release of

pheromone from a substrate will be somewhat variable and affected by environmental conditions such as wind velocity and temperature as well as the type of surface and the surface area from which the pheromone evaporates. In the initial grass grub field studies in 1970, attempts were made to determine the phenol release rates which attracted the largest number of beetles into traps. Such information was essential if effective control methods based on mass trapping were to be developed.

(i) Trial layout

Tests were carried out on three successive evenings. Phenol release rates varied between 0.5 and 10 µg/h. Eight replicates of each release rate were used. The traps were open pans (30 x 23 x 10 cm) containing 2 L of water with the attractant suspended over the water in 110 ml jars (Plate 5.4). The traps were laid out at least 20 m apart in groups of three or four in a line across the direction of the prevailing wind. Each group was at least 50 m apart.

The release rates were obtained by using different amounts of phenol (100 to 1,000 µg) on 4.2 cm circles of either No. 1, 3, or 17 Whatman filter paper. The treated papers were placed in the jars about 3-4 hours before flights commenced and the approximate release rates, from similarly treated papers, were measured in the laboratory 3-4 hours after the phenol was applied.

(ii) Results and discussion

In Table 7.4 the mean number of beetles caught per trap for each phenol release rate is shown. As the trials were carried out on a low beetle population (10-20 beetles/m^2) the number of beetles attracted was low.

Table 7.4 Mean number of beetles attracted to different phenol release rates on a low population area

Phenol release rate (µg/h)	Mean number beetles per trap		
	Evening 1	Evening 2	Evening 3
0.5	41	6	-
1.0	50	23	-
3.0	-	22	9
6.0	-	12	11
10.0	-	15	30
MSR (5%)	1.8	3.1	1.8

The beetle trap numbers were analysed on logarithmic transformed data. For one treatment to be significantly better than another at the 5% probability level, the ratio of the mean catches for the two treatments had to exceed the minimum significant ratio (MSR). For example, on evening 2, the mean catch of 6 beetles by the 0.5 µg/h treatment was significantly less, at the 5% probability level, than the mean catch of 23 for the 1 µg/h treatment, since the ratio 23/6 (=3.9) was greater than 3.1, the MSR value. Furthermore, there was no significant difference ($P < 0.05$) between the mean catch for the 1 µg/h treatment and the mean catches for any of the higher phenol release rates because the MSR values were all less than 3.1.

On the second evening, therefore, a phenol release rate in the order of 1 µg/h gave optimum catches while rates 6 to 10 times higher than this did not give significantly greater catches. In fact, at these higher rates the number of beetles trapped actually decreased. On the first evening, only two rates, viz., 0.5 and 1 µg/h were used and there was no significant difference ($P < 0.05$) between these two treatments. This situation was reversed on evening 3, however, when

an increase in release rate from 6 to 10 µg/h resulted in a significantly greater ($P<0.05$) number of beetles caught.

This change in optimum release rate from one evening to the other illustrates another difficulty in developing effective control methods based on mass trapping. Normally at dusk when mating flights take place, conditions are calm with the average wind velocity dropping to less than 0.1-0.2 kph. On these calm evenings the lower release rates would be expected to be more effective while the presence of a slight breeze would probably cause an increase in potency of the higher release rates. In studies on the banded cucumber beetle, Cuthbert and Reid (1964) found that fluctuations in the number of males approaching traps appeared to be related to changes in direction or velocity of slight breezes blowing during the test.

The type of surface and the surface area also have a significant effect on the release of pheromone, and, therefore, its attractiveness. This was demonstrated in two different field tests with Durez resin. In the first test, a comparison in activity was made between 4 mg Durez powder, 4 mg Durez powder dissolved in acetone and added to filter paper, and 100 µg phenol on filter paper. (This amount of phenol was selected as 4 mg Durez contained 100 µg phenol). The total number of beetles attracted in the field to 8 replicates of each treatment were 342, 154, and 158 respectively. As expected, statistical analysis showed no significant difference ($P<0.01$) between the latter two treatments (phenol release rates 0.5 and 1.0 µg/h), but a significant difference ($P<0.01$) between these treatments and the Durez powder (phenol release 1.0-2.0 µg/h). Thus, the activity of the powder (and the phenol release rate) were considerably reduced by simply dissolving Durez powder in acetone

which on drying gave a film of resin rather than a powder. A similar result was described by Fenemore et al., (1972).

In the other test which illustrated this effect, a comparison in activity was made between the same quantity of Durez paste (200 mg) applied to red cotton mesh and red baffles placed over traps. (A baffle consisted of a vertical strip (10 cm wide) of red painted aluminium-coated insulating paper which was suspended over the trap). Eight replicates of each treatment, spaced 10 m apart at random in a 2 ha paddock, indicated that Durez paste on the mesh was at least twenty times less effective than that on baffles ($P<0.001$). An average of 23 and 556 beetles per trap were caught with the respective treatments. Presumably in the case of the mesh, phenol was adsorbed on the fibre which substantially modified the rate of phenol release.

(b) Distance of attraction

The distance of attraction to the female sex pheromone for a number of scarab species has varied between 10 and 30 m. Soo Hoo and Roberts (1965), for example, found that in a steady breeze marked males of a number of Rhopaea species flew upwind towards caged females from about 30 m. Studies on the sex stimulatory effect of isoamylamine on the male June beetle (Travis, 1939) revealed a distance of attraction of 10-15 m. Females of the Japanese beetle, Popillia japonica, were observed to attract males from at least 20 m. In this same study, Ladd (1970) found that 5.4, 2.5, and 0.8% of the marked males released, were recaptured at distances of 100, 200, and 300 m respectively from caged females.

Attempts were made to determine these parameters for the male grass grub beetle by releasing marked beetles at various distances downwind from baited traps. A combination of males caught in traps on previous evenings and males reared in the laboratory was used in

the tests. The males were marked with 'Day-Glo' fluorescent powder (Turner and Gerhardt, 1965) and placed in tins of soil, in batches of 100 beetles. The tins were placed in the field about half an hour before dusk.

(i) Results and discussion

Low numbers of marked beetles (< 10%) were trapped from both short (1.5 m) and long (30-60 m) distances and it was obvious from the results (Table 7.5) that such attractant formulations would be of little value in mass trapping programmes.

Table 7.5 Distance of attraction and male recoveries in release-recapture experiments

Attractant formulation and phenol release rate	Distance of marked males (m)	No. males escaped	Percent recoveries
Wooden cubes, 3 µg/h	1.5	80	2
	3.0	80	8
Durez powder, 10 µg/h	1.5	70	4
	3.0	65	3
	30.0	70	1
Wooden cubes, 15 µg/h	3.0	70	6

The distance of attraction was also assessed by observing the position downwind of a trap from which males in the natural population flew direct to the trap. Another useful method to determine distance of attraction was to make observations of beetles flying acrosswind, downwind of the trap. Beetles responding to the sex pheromone would make an abrupt change in direction and fly upwind towards the trap. These assessed distances of attraction (Table 7.6) varied between 3 and 30 m.

Table 7.6 Assessed distances of attraction to various phenol release rates

Attractant formulation	Phenol release rate (µg/h)	Assessed distance of attraction (m)
4 mg Durez powder	1.5	3-4
20 "	8	25-30
25 "	10	15
200 µg phenol on No.3 filter paper	1-2	10

(c) Attraction trials

These trials were carried out to investigate the potential of a control method based on male annihilation. The aim was to assess any reduction in grass grub numbers in the close vicinity of a trap releasing phenol at an optimum rate for attraction. At the commencement of these field trials in 1970 this optimum rate was not known and so it was decided to carry out trials on plots using a number of different release rates of phenol.

(i) Trial layout

Trials were carried out on plots (5 m^2) and these were spaced at least 10 m apart. The attractant sources mainly consisted of varying numbers of wooden cubes treated with phenol and coated several times with epoxy resin as outlined in Section 7.2b(i). None of the cubes was replaced during the entire adult flight period, but in the case of the Durez powder this was replaced on three occasions.

The attractant formulations were suspended above a water trap placed at the centre of each plot and each evening the effectiveness of a particular attractant source was assessed from the number of beetles caught.

A 1 m dia. circle of pasture at the centre of the plot was treated with the organophosphate insecticide, 'Dursban', at a rate

of 5 kg active ingredient (a.i.)/ha. This treated circle was covered with 2.5 cm soil to facilitate burrowing of attracted beetles and to provide protection for the insecticide, against weathering. This area was selected for the insecticide treatment since it was known that beetles were not always attracted directly to the source but landed at varying distances from the source.

(ii) Sampling

The beetle numbers on each plot were assessed from the third instar larvae and pupae numbers obtained by taking twenty, 225 cm^2 x 20 cm deep spade samples and hand sorting. Treatment effects were assessed by sampling the plots for the following generation eggs, first and second instar larvae. These were sorted using the floatation technique developed by Kain at Ruakura. In order to assess the effect of the attractant in a close vicinity of the source, ten, 225 cm^2 x 20 cm deep samples were taken from an annular area between 0.5 and 1.2 m from the centre and twenty, 225 cm^2 x 20 cm deep samples from the annular area between 1.2 and 2 m from the centre.

(iii) Results and discussion

The attractant sources consisted of different numbers of wooden cubes treated with either 1, 5, 10, 100 mg phenol, or Durez powder. All the attractant sources were held in a glass jar above a water trap as outlined in Section 7.3a. The results of the trials are shown in Table 7.7.

In all the treatments, very small numbers of males were attracted into traps. This is particularly evident when the number of males within a short range of the sources is considered. For example, in treatment 7, where the initial male beetle population within 2.0 m of the source was of the order of 2,000, only 475 males (24%) were attracted to the trap. If the distance of attraction is considered

Table 7.7 Effect of various phenol release rates from single traps on the resultant larval population close to the attractant source

Attractant formulation	Estimated beetle numbers within 2 m of attractant source	% Males attracted within 2 m of attractant source	Larvae/m^2 inner annular area	Larvae/m^2 outer annular area
1. 1 x 1 mg phenol on wood cube	480	59	50	65
2. 5 x "	700	22	60	40
3. 1 x 10 mg phenol	700	31	130	85
4. "	880	12	85	250
5. 2 x "	1250	9	100	120
6. 1 x 100 mg phenol	500	56	8	50
7. "	2000	24	40	85

Table 7.7 continued on following page

Table 7.7 continued Effect of various phenol release rates from single traps on the resultant larval population close to the attractant source

	Attractant formulation	Estimated beetle numbers within 2 m of attractant source	% Males attracted within 2 m of attractant source	Larvae/m^2 inner annular area	Larvae/m^2 outer annular area
8.	2 x 100 mg phenol on wood cube	2300	10	235	215
9.	5 x "	630	33	30	115
10.	10 x "	1600	13	400	215
11.	10 mg Durez powder	750	65	110	110
12.	"	1200	71	90	65

to be about 5 m rather than 2 m, which is probably more likely, then the percentage of male beetles attracted to the traps would be less than one-fifth of the figures in Table 7.7.

None of the formulations of phenol on the wooden cubes attracted more than 60% of the total number of males present within a 2 m radius of the attractant source. On the other hand, the two replicates of the Durez powder attracted 65 and 71% of the total male population within a 2 m radius of the source. All these catches would be inadequate for grass grub control, however, for at least 80% of the total male population would have to be destroyed merely to maintain grass grub numbers at the level of the previous generation.

The problem of males dropping on the downwind side of the attractant source which was releasing phenol at a high rate, was highlighted in treatment 10. This treatment had the highest rate of phenol release and males attracted to this source dropped short of the insecticide-treated area and mated with females. On a number of evenings when major flights took place, beetles on this plot were spread over an area on the leeward side of the trap to a distance of about 5 m.

The results could not be statistically analysed due to the insufficient replication of the treatments. It did appear, however, that very little reduction in larval numbers took place in the close vicinity of the attractant source with any of the treatments. On control plots the number of larvae re-infesting the plots was about double the parent beetle infestation and on most of the treated plots, with the exception of treatments 2, 5, 6, and 7, the larval population increases were of this magnitude. For treatments 2, 5, and 6, the larval populations were at about the same density as the original beetle population. Some reduction in larval numbers appeared to take

place with treatment 7, but, in view of the low number of beetles trapped with this attractant formulation, this effect was probably due to sampling variation.

These trials indicated that none of the attractant formulations competed successfully with virgin females in the natural population. Mating even took place very near to (within 0.5-1.2 m) all of the attractant sources. It was obvious from these preliminary trials that superior attractant formulations had to be developed before mass trapping programmes could be contemplated.

(d) Activity of Durez paste versus virgin females versus other phenol formulations

The increased potency of the Durez paste over the dry powder has been mentioned. This phenomenon is probably related to two factors. Firstly, the addition of water to the dry resin liberates phenol which is adsorbed onto the polymer backbone. This was demonstrated in the laboratory, for example, when a five-fold increase in phenol release rate was obtained, viz., 40 to 195 µg/h (average of three tests), with the addition of 1.0 ml water to 100 mg dry Durez powder. Secondly, the paste is normally exposed by brushing it onto a surface. In this form, the paste covers a large surface area and dries quickly thus allowing a greater surface area of the resin to be exposed. The attraction of males to Durez paste painted onto a baffle is illustrated in Plate 7.2.

In Section 7.3(a) it was stated that about 4,500 beetles were attracted to eight traps each baited with 200 mg Durez paste on one evening. Obviously the potency of this formulation was such that it was potentially useful for mass trapping. Field trials were carried out, therefore, to compare the attractiveness of Durez paste with that of a virgin female beetle and a number of other phenol formulations.

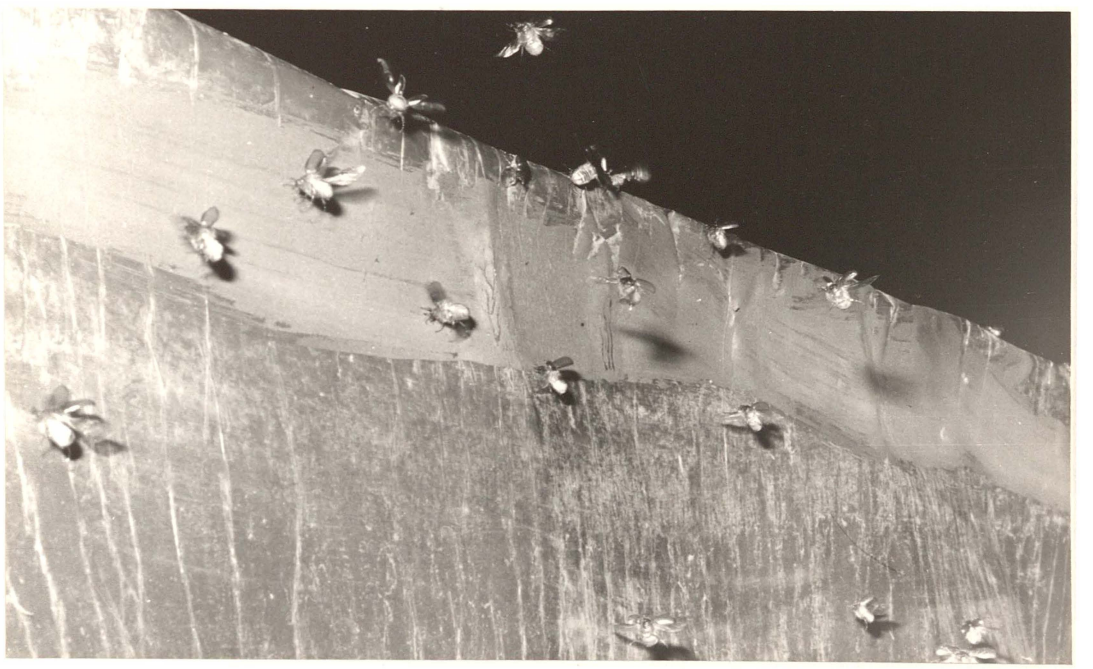


Plate 7.2 Field response of male grass grub beetles to Durez paste applied to a red baffle.

(i) Trial layout

Eight replicates of each treatment were tested at random over a 5 ha area in groups of three, four, or five in a trial layout similar to that described in Section 7.3(a). The Durez paste, prepared by mixing 0.8 g of the powder with water (4 ml), was applied about half an hour before the flights commenced to 2.5 x 35 cm horizontal strips of red-painted aluminium-coated insulating paper placed over water traps.

In exposing females as lures in traps, attempts were made to maintain them in conditions as close to natural as possible. Virgin female traps each contained five beetles held in soil in honey pots which were covered with gauze. Beetles were allowed to emerge naturally and an average of three beetles emerged from the soil each evening. An illustration of the female trap is shown in Plate 7.3 but a larger water trap (50 x 35 x 10 cm) similar to the one shown in Plate 7.4 was used in all the tests.

In attempts to equal, and possibly surpass, the attractiveness of Durez, using a number of other phenol formulations, a continuous wick trap was developed. The attractant source consisted of either an aqueous phenol solution or an aqueous phenol-hexamethylene tetramine mixture held in a plastic tube (35 x 2.5 cm dia.) with a slit in the top for the wick. This consisted of Whatman No.1 or No.3 filter paper and normally extended 5 cm above the slit in the plastic tube. The trap design is illustrated in Plate 7.4. The aqueous solution in the tube remained for about three to six hours and as the wick traps were baited between 1.0 and 2.0 p.m. their condition at dusk varied between wet, damp, and dry.

(ii) Results and discussion

In the initial trials, the activity of Durez paste (100 mg/trap) was compared with that of virgin female beetles and the results are

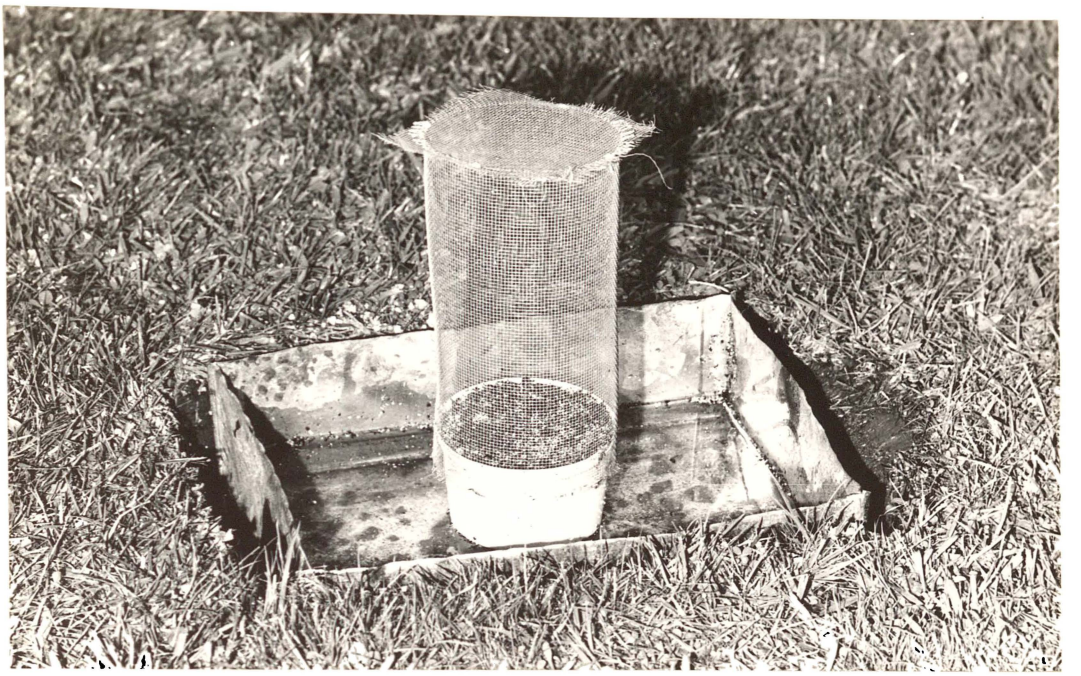


Plate 7.3 A virgin female trap containing five beetles in a honey pot filled with soil.

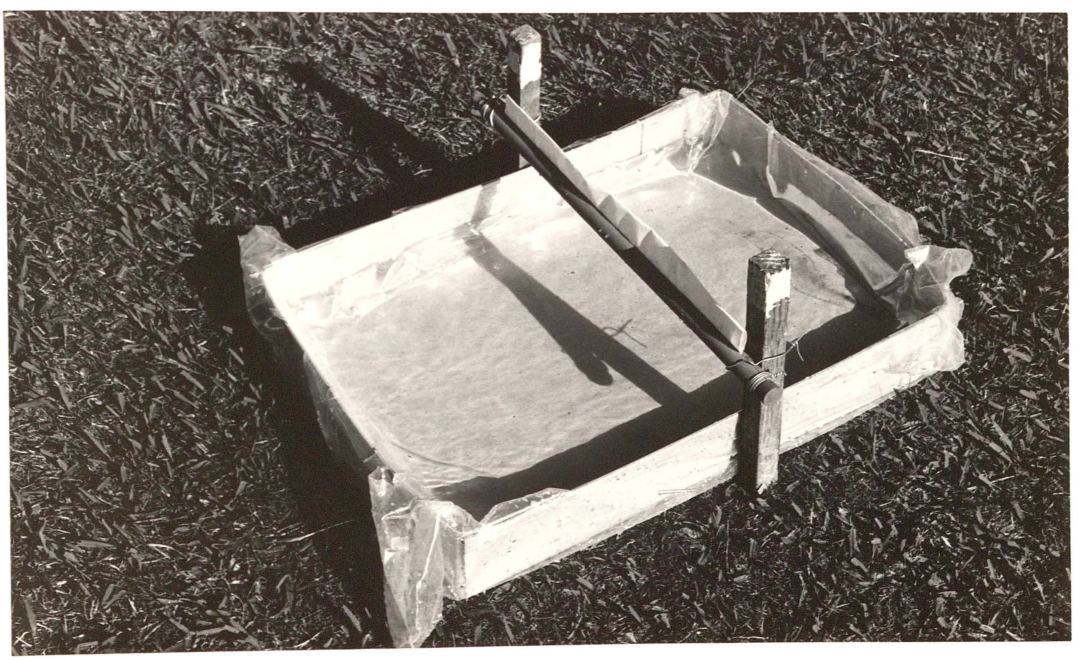


Plate 7.4 Continuous wick trap used to compare the activity of aqueous phenol formulations with Durez paste.

shown in Table 7.8

Table 7.8 Comparison of attractiveness of Durez paste and virgin female beetles throughout the flight season

Attractant	Av. males caught/trap	Durez/female ratio	Flight period and infestation
Durez paste, 100 mg/trap	150	350:1	4-5 days prior to initial major flight, 50-100 beetles/m^2
Females (3 x 8)	1.2		
MSR (5%)	1.7		
Durez paste, 100 mg/trap	1,106	450:1	2 days prior to initial major flight, 200-250 beetles/m^2
Females (3 x 8)	7.4		
MSR (5%)	1.6		
Durez paste, 100 mg/trap	682	20:1	4 days after initial major flight, 200-250 beetles/m^2
Females (3 x 8)	104		
MSR (5%)	1.9		
Durez paste, 100 mg/trap	82	7:1	5 days after initial major flight, 200-250 beetles/m^2
Females (3 x 8)	35		
MSR (5%)	1.7		

Early in the flight season, about 4-5 days prior to the major flights when few females were present in the field, an average of 150 beetles was caught in the Durez traps. On the other hand, the three females which emerged in each virgin female trap only attracted about 1.2 beetles/trap. Thus Durez paste significantly out-competed each female by over three hundred times. A similar result was

obtained on a higher beetle infestation two days prior to the major flight when the Durez out-competed the females by four hundred and fifty to one.

In these initial tests, the female beetles attracted less than one male each which suggested that they were not as attractive as females in the natural population. If this were the case, then this would result in an inflated value for the potency of Durez paste.

This potency of Durez was not maintained during the major flight period, however, when a greater number of females was present in the pasture to act as competition. On two evenings during the major flights, Durez paste attracted significantly greater numbers of males than females but the relative attractiveness was reduced by about a factor of ten.

In the initial attempts to equal the effectiveness of Durez paste with other phenol formulations, 0.1% aqueous phenol and a mixture of 0.1% aqueous phenol and 0.5% hexamethylene tetramine were compared with 50 mg Durez paste. The mean numbers of beetles per trap for these three treatments were 326, 126, and 41 respectively with an MSR (5%) of 6.1. Thus 0.1% aqueous phenol applied continuously to a wick was about eight times ($P<0.05$) more attractive than 50 mg Durez paste. The presence of hexamethylene tetramine would have been expected to reduce the rate of release of phenol from the wick. Consequently smaller catches resulted, which were not significantly different from those with Durez paste.

In the next series of trials, the influence of wick height and filter paper absorbancy were investigated. Aqueous phenol (0.1%) was used in traps containing Whatman No.3 filter paper at

two wick lengths 1-2 cm and 5 cm. The mean number of beetles attracted per trap for these treatments was 68 and 191 respectively with an MSR (5%) of 3.3. As expected, the wicks with the larger surface areas were considerably more attractive, but the mean catches for the two treatments were not significantly different at the 5% probability level.

The effect of filter paper absorbancy was investigated by comparing the attractiveness of 0.1% aqueous phenol dispensed from Whatman No.1 and the thicker Whatman No.3 filter paper wicks (5 cm high). Traps containing 100 mg Durez paste were also used to give an overall comparison. The mean numbers of beetles attracted per trap for these three treatments were 208, 191, and 1,390 respectively with and MSR (5%) of 3.3. Thus the thicker No.3 filter paper had very little effect on the potency of 0.1% aqueous phenol but both treatments were six to seven times less effective than Durez paste.

In further attempts to equal the potency of Durez paste, the attractiveness of a series of aqueous solutions containing 1.0, 0.1, 0.01 and 0.001% phenol was determined and compared with 100 mg Durez paste. The mean numbers of beetles per trap for the five treatments were 20, 239, 51, 22, and 1,330 respectively with an MSR (5%) of 2.8. Thus, for this phenol dilution series, optimum attraction was attained with the 0.1% aqueous phenol wick but this was five and a half times less effective than the Durez paste. The low catches associated with the 1.0, 0.01, and 0.001% probably indicated that the phenol release was too high from the 1.0% solution and too low at the lower concentrations. A further series of trials was carried out, therefore, with aqueous solutions which varied about the 0.1% phenol level. The mean numbers of beetles/trap attracted to the 0.05, 0.08, 0.1, and 0.3% aqueous phenol

solutions and 100 mg Durez paste were 270, 250, 220, 180, and 1100 respectively with an MSR (5%) of 2.7. The catches associated with these aqueous phenol solutions were still four to six times lower than the average obtained with the Durez paste.

Other attempts to modify the release of phenol from paper included: 0.01% aq. phenol mixed with 0.04% aq. hexamethylene tetramine, 0.05% aq. phenol mixed with 0.2% aq. hexamethylene tetramine, and 0.1% aq. phenol mixed with 0.2% aq. hexamethylene tetramine. These formulations were compared with 100 mg Durez paste and the mean numbers of beetles per trap obtained were 150, 150, 170, and 350 respectively with an MSR (5%) of 1.6.

Thus, on average, the Durez traps were always several times more attractive than any of the traps containing the aqueous phenol formulations. This result could be explained by the presence of a synergist in Durez or more simply by the manner in which phenol was released from the paste.

The rate of phenol release from Durez paste applied to 3 x 30 cm strips of painted aluminium-coated insulating paper was measured in the laboratory. The Durez paste was formed by the addition of 0.5 ml water to 100 mg powder and the experiment was carried out in triplicate. An hour after the application of the paste to the strips, the phenol release rates were 49, 53, and 60 µg/h and in the following hour the release rates were 48, 51, and 62 µg/h. This uniform rate of release of phenol is probably the reason for the potency of the paste in the field.

This uniform, optimum rate of phenol release was difficult to reproduce in the field with the aqueous phenol formulations because the filter paper wicks dried at varying rates. In eleven instances, however, the filter paper wicks were as attractive as the Durez paste but the overall consistency of the catches obtained with the

paste resulted in significantly greater catches being obtained. Nevertheless, the fact that large numbers of males could be attracted to the aqueous phenol formulations indicated that phenol itself could be as potent as the Durez paste, provided the rate of release was at the optimum level. This result illustrated once again the critical nature of the phenol release rate on male attraction.

Although the Durez paste consistently attracted greater numbers of males than the aqueous phenol solutions on filter paper, the behavioural responses to the two treatments were precisely the same. Before alighting on the paper, males hovered over the top of it and the males that landed on the treated filter paper frequently raised their elytra and attempted to copulate with nearby males. A typical response to the sex pheromone in the field is shown in Plate 7.2.

7.4 Conclusions from the preliminary studies

It was shown that when powders containing phenol were applied to pasture, phenol was not released twenty four hours after application. This effect, presumably due to soil adsorption and soil moisture, obviously meant that such methods could not be used in grass grub control programmes.

Various attempts were made to develop alternative methods of dispensing phenol. The most satisfactory method was encapsulation of liquefied phenol in PVC tube. This dispenser both protected the phenol from weathering, and allowed it to be continuously released in the field, for a period greater than four weeks, via permeation through the polymer wall. Promising results were obtained in the laboratory with a synthesized material, phenylhydrogensuccinate, which slowly degraded to phenol but the initial results could

not be reproduced under field conditions.

The distance of attraction of marked male beetles to phenol released at rates which varied from 0.5 to 10 µg/h was in the order of 15 m. However, as the percent recovery of the marked beetles was less than ten percent, such attractant sources would be unlikely to be effective in grass grub control.

This was verified in a number of attraction trials in which no reduction in resultant larval numbers took place within a 1.2 m radius of a series of attractant sources. It was apparent, therefore, that none of the attractant sources was competing successfully with females in the natural population. Superior attractant formulations were needed before mass trapping could be contemplated.

Such a material appeared to be available in the form of Durez 12687, particularly in paste form. Tests were carried out with Durez paste to compare its attractiveness to a number of other phenol formulations, and to virgin female beetles. Early in the male flight season, the paste out-competed females by about four hundred times but this result was not maintained during the major male flight period when the paste was only about ten times more attractive than virgin female beetles.

Attempts to equal and possibly surpass the potency of the Durez paste with a number of phenol formulations were unsuccessful. In fact, the paste was consistently five to six times more effective than the best phenol formulations devised. This was probably associated with the optimum, uniform phenol release rate produced by the resin which could not be readily reproduced with the other phenol formulations used. The potency of the resin was influenced by its physical nature as well as the surface and surface area from which the resin was exposed.

The behavioural responses of male beetles on mating flights to Durez paste and phenol on filter paper were precisely the same and consistent with that expected of a sex pheromone.

7.5 Population model for the theoretical control of grass grub mating with sex pheromone traps

Knipling and McGuire (1966) postulated insect population models and calculated the theoretical effects of using sex pheromones for insect control. They concluded that insect populations can be controlled and probably eradicated if the ratio of traps to insects, i.e. the overflooding ratio, is high enough. Because of the number of traps required, the approach can be considered practical only when dealing with light infestations or with attractant formulations which are considerably superior to virgin females in the natural population. Roelofs and co-workers (1970) used the basic equations proposed by Knipling and McGuire to calculate the theoretical control of mating by trapping males of the red-banded leaf roller moth.

The information needed for traps to be used effectively for insect control was discussed by Beroza and Knipling (1972) and includes:

(1) An assessment of the attractant power of the pheromone in traps as opposed to the attractant power of competing virgin females in the natural population.

(2) The number of traps required relative to the size of the population.

(3) The growth rate of the insect population which determines the degree of control needed to reduce numbers.

Additional information such as the emergence profile and average life span of the insect are also required to construct a

population model. Sufficient data on the biology and population growth of grass grub were available from this study and other research work to allow a population model to be drawn up for the theoretical control of grass grub mating with sex pheromone traps.

Under field conditions the life span of the male grass grub beetle is about 2 weeks while that of the female is 2-3 weeks during which time it lays between 30-40 eggs. A hypothetical model of grass grub growth at Takapau, drawn by Kain (1972), is reproduced in Figure 7.1. The reproductive capacity of grass grub is fairly low but populations increased steadily (2- to 5-fold annually) to post proportions at about 300-400 grass grub larvae per m^2. At this stage, fluctuations occurred about a given level depending on food availability and climatic conditions. Normally the populations did not fluctuate greatly due to the absence of parasites and predators and the subterranean habitat of the larvae which protected them from environmental conditions. According to Kain (1972), a population collapse can be induced by high soil temperatures usually associated with severe droughts and extremely high soil moisture levels during winter.

In his paper, Kain (1972) indicated that the majority of grass grub larvae in pasture are confined to colonies and the density of the larvae within these colonies does not increase markedly as populations increase. Thus, populations increase by the formation of new colonies rather than the build-up of larval density within colonies. Kain illustrated this by suggesting that the best stage to treat grass grub is when populations are low so that annual increases in numbers and pasture damage from year to year are minimal.

To construct the theoretical model for an isolated grass grub population, the following assumptions were made. Male beetles were

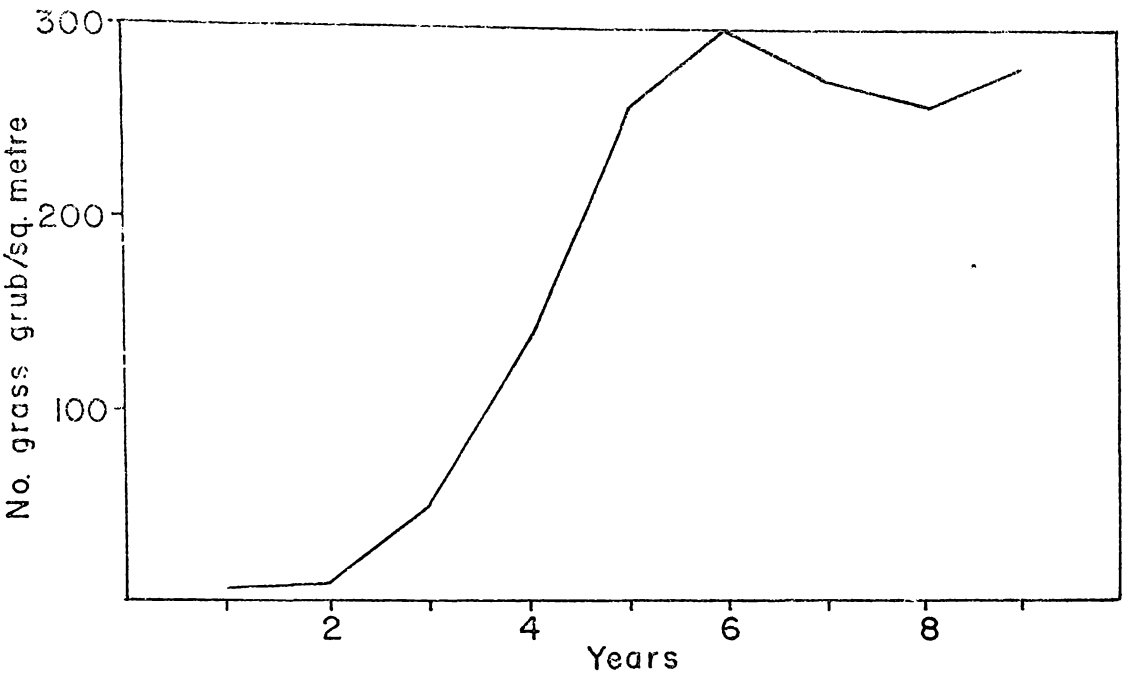


Figure 7.1. Hypothetical model of grass grub reproductive capacity. Reproduced from the model of Kain, 1972.

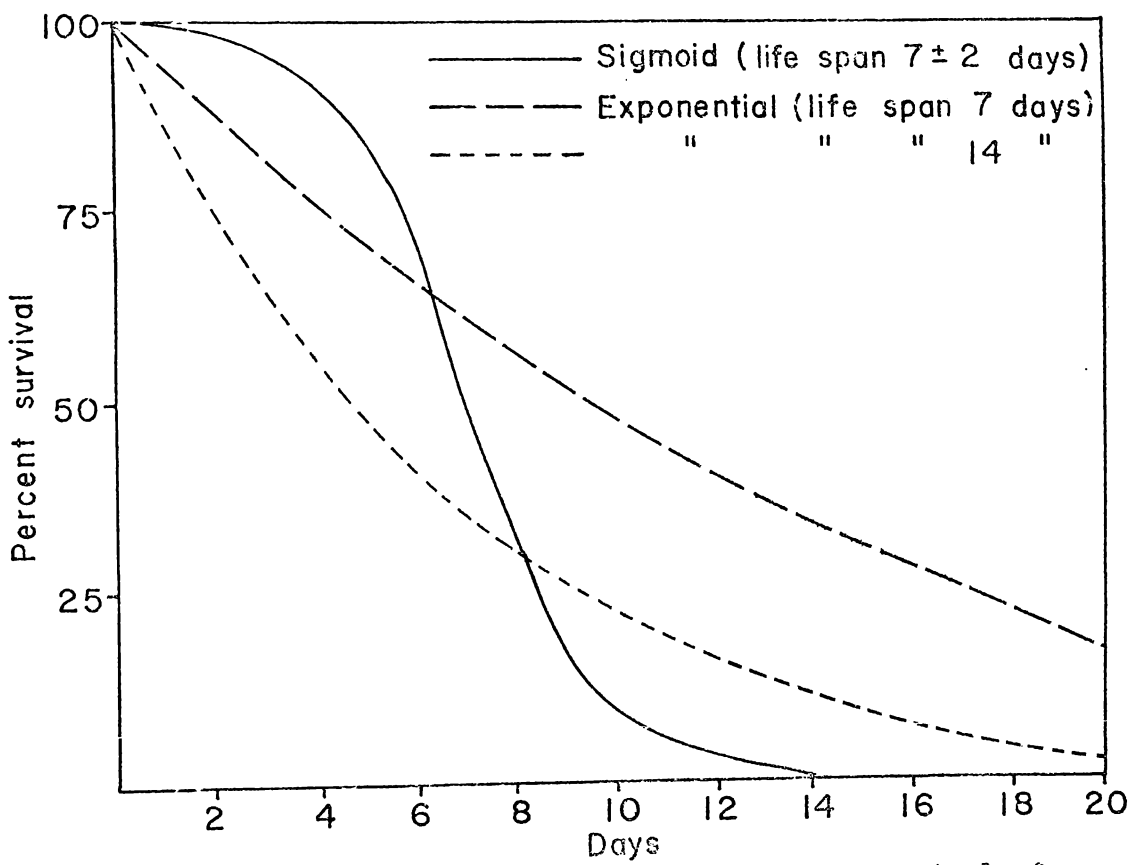


Figure 7.2. Survival rate curves used in theoretical control of mating calculations.

considered to be polygamous and to not mate with a female more than once every 24 hours. It was also assumed that females mated only once before oviposition and then left the population on feeding flights. A further assumption was that females lived longer than males after primary soil emergence. Males and females were considered to live 7 and 14 days respectively after soil emergence but the effect, on the theoretical control of mating, of males having a life span of 14 days was also investigated. Both a constant daily survival rate(7 or 14%) which resulted in an exponential survival curve, and a sigmoidal survival curve (Figure 7.2) were used in the calculations. The latter curve simulates natural populations better than the former but only slight differences in theoretical control were obtained with models based on either of these curves (Table 7.9). A similar result was calculated in the model proposed by Roelofs' group.

Table 7.9 Control of mating with female/competition ratios (r) = 0.6 and 10 using exponential and sigmoid survival rates. Parameters used in the calculations included a 7-day life span for males and a 14-day life span for females in a natural emergence profile

r value	Survival rate	Percent control
0.6	Exponential	83
0.6	Sigmoid	79
10.0	Exponential	13
10.0	Sigmoid	13

The emergence profile for a batch of 20,000 beetles raised in the laboratory was used to construct the model. Beetles in this batch were raised from fully fed third instar larvae at 10°C which approximated the soil temperatures when beetles emerged naturally in the field. Males and females in this batch emerged over 21 days

in a profile (Figure 7.3) which was modified slightly to give equal numbers of males and females. Normally in the field the emergence period for beetles extends over 21 to 28 days. In order to make the model relate more closely to a low grass grub infestation in the field, it was assumed that the 20,000 beetles occupied one hectare of pasture (i.e. beetle density was 2 beetles per m^2).

It was also assumed that a five-fold increase in population took place annually. With such an increase, 80 percent of the incipient population would have to be killed each year merely to keep the total population from increasing. The reproductive potential of each generation would have to be reduced more than 80 percent to achieve population reduction.

The basic equations used (Table 7.10) were the same as those adopted by Roelofs et al., (1970) for the theoretical control of the red-banded leaf roller moth. A calculation subroutine repeated day after day gives the daily number of matings. These are added until the generation is complete and the number of males is infinitesimal. Percent control was calculated from the following equation:

$$\% \text{ control} = \frac{\text{No.females} - \text{No.matings}}{\text{No.females}} \times 100$$

Table 7.10 Equations used to calculate the number of matings on Day i when pheromone traps compete with the female. Taken from the model of Roelofs et al., (1970)

$S_i = V_i + (S_{i-1} - F_{i-1})d$

$R_i = M_i + F_{i-1}d$

$P_i = S_i \quad (AN + S_i)$

$F_i = P_i R_i$

Table 7.10 continued on next page

Table 7.10 continued

Definition of the symbols

V_i : number of females emerging on day i.
M_i : number of males emerging on day i.
P_i : probability of a male to mate with a wild female on day i.
S_i : total number of virgin females on day i.
R_i : total number of males on day i.
F_i : number of matings on day i.
d : survival rate, or fraction of the total population surviving another day.
AN : relative attractiveness of a trap, as compared to that of a wild female.
N : number of traps.

The theoretical number of traps needed to out-compete the female population was written into the model for the red-banded leaf roller. Roelofs et al., state that:

"The amount of competition for any female population is the number of traps (N) times relative trap attractiveness (A) and the percentage of control for any size population depends upon the ratio (r) of the total female population (T) to trap competition (A) x (N), or

$$r = \frac{T}{(A)x(N)}$$

For example, if each pheromone trap is equal to 2 wild females (A = 2) and we place 3000 traps (N = 3000) in a population of 3000 ♀ (T =3000), then

$$r = \frac{3000}{(2)(3000)} = 0.5$$

We expect the same percentage of control of mating in every situation where r = 0.5, such as placing 2000 traps (N = 2000), each with the attractiveness of 1 wild female (A = 1), in a

population of 1000 ♀, so

$$r = \frac{1000}{(1)(2000)} = 0.5$$

The percentage of control of mating for a given set of parameters can be plotted against r and the resulting curves will apply to any size population and trap potency".

The theoretical percentage of control plotted against three female/competition ratios ((r) = 0.6, 2 and 10)) are illustrated in Figure 7.4. To obtain these r values it was assumed that 100 traps per ha were used and these traps had a relative trap attractiveness of 167, 50, and 10 respectively. Males and females were assumed to emerge naturally with an exponential survival rate based on a 7-day or 14-day life span for males and females respectively.

The effect on control by using different life spans for males and females with an exponential survival rate was also calculated (Table 7.11).

Table 7.11 Control of mating with a female/competition ratio (r) = 10 for various average life spans for males and females based on an exponential survival rate.

Ave.female life span (days)	Ave.male life span (days)	Percent control
7	7	28
14	7	13
14	14	17

As expected, the model predicts a greater control of mating with a shorter female life for then the female has a lower chance of mating. In contrast to this, very small effects on control of mating were predicted by extending the life span of males from 7 to 14 days.

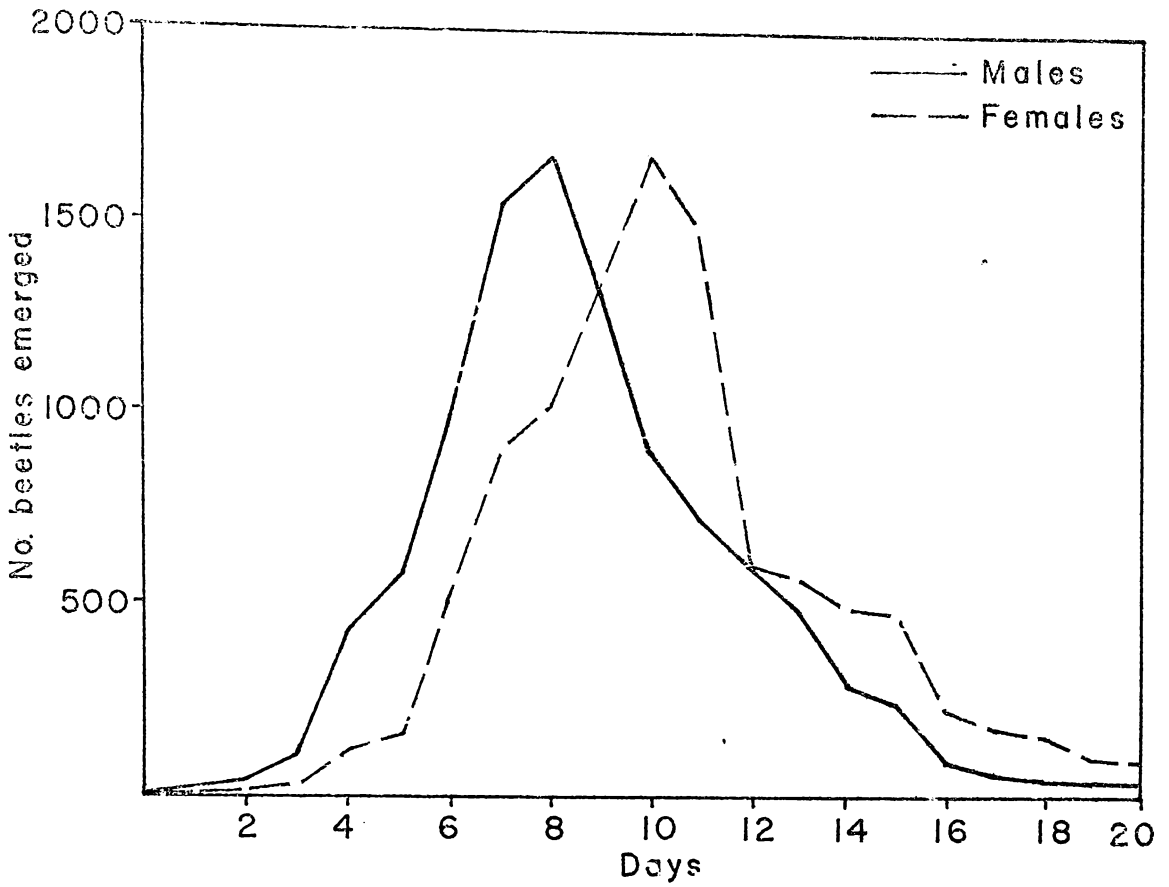


Figure 7.3. Emergence profiles for males and females exhibiting 2-day protandry used in theoretical control of mating calculations. Populations were 10,000 males and 10,000 females.

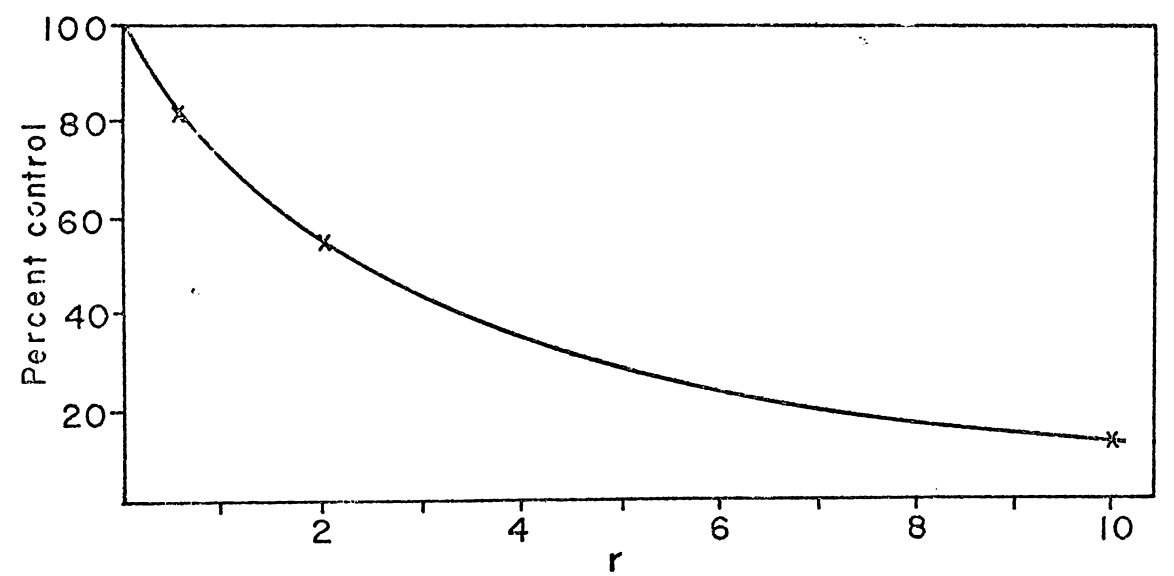


Figure 7.4. Theoretical percentage of control plotted against total female/competition ratios (r) for an ave. life span of 7 days for males and 14 days for females, a natural emergence profile and an exponential death rate.

7.6 Mass trapping field trials with Durez paste

Introduction

In 1972, Fenemore and co-workers reported on field trials carried out with traps, baited with Durez powder, arranged in two grid patterns on 4 ha and 1 ha sites in Canterbury. These workers found that while large numbers of beetles (approximately 60,000) were attracted to the traps, the numbers were insufficient to affect the following generation. It was subsequently found by Osborne and Hoyt (personal communication) that Durez mixed with water to form a paste was a far more potent attractant than the powder itself - a phenomenon which has already been discussed in Section 7.3d.

In view of this increased potency, together with the economic importance of grass grub and the urgency to develop alternative control methods, it was decided to carry out field trials with Durez paste in the Waikato region during the 1972 flight season. Dr G. O. Osborne kindly supplied several kg of Durez resin. His group at Lincoln College also planned to carry out further mass trapping trials with the paste in the Canterbury region. It was felt that some duplication of effort was desirable in view of the univolitine nature of grass grub. This restricts the period for trials to a few weeks each year, which is a severe limitation on progress. Furthermore, differences in beetle behaviour have been observed in the North and South Islands of New Zealand. For example, Kelsey (1968) stated that in Canterbury, (South Island) adult flight is dominated by males and very few females actually fly throughout the flight season. On the other hand, in the Waikato, (North Island) Kain has found a similar situation in the early flight period but adult flights later in the season mainly consisted of female beetles.

Two different approaches were used in the mass trapping trials discussed below. In the first, water traps were arranged in a grid pattern with the Durez paste painted on the top of a baffle over each trap. In the second approach it was hypothesized that a line source of attractant would be more efficient at attracting beetles than a number of attractant sources. Thus, Durez paste was painted on the top of a continuous baffle about 40 cm high formed in a circle of 15 m radius. The circular layout was selected to overcome the difficulty of changes in wind direction from one evening to another.

Experimental

The mass trapping trial with the traps arranged in a grid pattern was carried out about 30 km south of Te Awamutu on a hill country farm which has had a history of grass grub damage over many years. Two 0.25 ha sites were selected, about 30 m apart, which had fairly heavy, uniform infestations of grass grub larvae.

Sixteen traps spaced at 10 m square were placed on each site. The traps (Plate 7.5) consisted of wooden frames (1 m^2 in area) set at ground level with black polythene lining the bottoms and sides to hold a depth of 5-7 cm water. Dark red baffles (15 cm high) were suspended across the traps. Each evening, half an hour before beetle flights took place, the baffles on the treated site were wiped clean and fresh Durez (50-150 mg per trap) was applied in a 5 cm band across the top of the baffle. To increase the efficiency of the attractant, the pasture around the traps on both sites was maintained as low as possible by allowing sheep to graze in between the traps.

In order to assess likely beetle numbers, twelve plots (1 m^2) were selected at random within each site and 20 soil cores

Plate 7.5 The type of trap used in mass trapping trials with Durez paste. The paste (50-150 mg) was applied across the top of the baffle each evening prior to beetle flights.

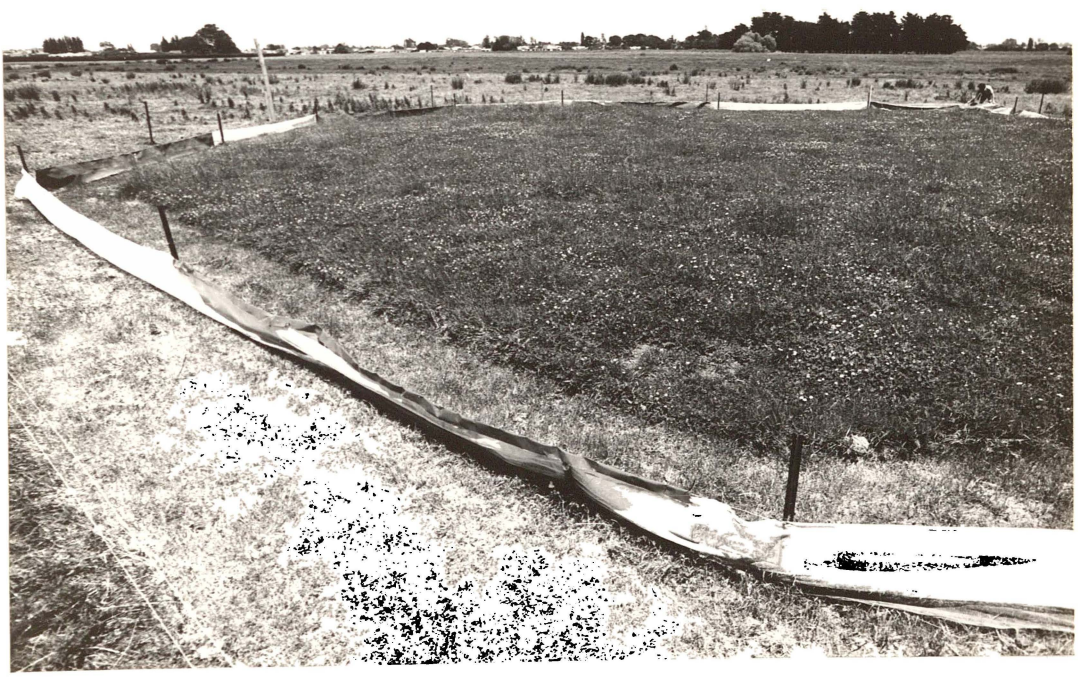


Plate 7.6 Circular layout of the baffle used in mass trapping field trials to evaluate the effectiveness of Durez paste as a line source of attractant.

(10.2 cm dia.) were taken to a depth of 20 cm from each of the 24 plots at the fully fed, third instar/pupal stages. To assess the effect of the treatment, a further 20 soil cores were taken from each of the 24 plots after beetle flights at the egg/first instar larval stages.

In the second trial to test the efficiency of the attractant arranged as a line source, a site was selected which contained a medium (ca. 50-100 beetles/m^2) grass grub infestation. The control and treated sites were each about 1.5 ha in area and were spaced about 75 m apart. The treatment was applied to the northern site. A baffle about 40 cm high made from aluminium-coated insulating paper and painted dark red was placed in a circle of 15 m radius (shown in plate 7.6) on both the treated and control sites. Within the circle, the pasture was mowed once a week for the duration of the trial which extended from 20 October to 19 November 1972.

One of the problems in testing the effectiveness of a treatment on low infestations is the difficulty in obtaining sufficient larval numbers from soil cores for meaningful statistical analysis. This is especially the case with samples taken at the egg to first instar larval stage. To obviate this problem, twelve plots (1 m^2) were located on known infested areas on both the control and treated sites. The effectiveness of the treatment was measured by taking 20 soil cores (10.2 cm dia.)to a depth of 20 cm from each plot before beetle flights at the pupal stage and again at the resultant egg to first instar larval stages.

To measure the effectiveness of the treatment during the flight period, ten virgin female traps (Plate 7.3) with controls alongside, were placed at random on both the control and treated sites.

Prior to the commencement of the trial, "Fensulphothion" at

2 kg a.i./ha was applied in a 1 m band at the inner base of the baffles on both sites. This insecticide treatment was repeated 10 days later (at 5 kg a.i./ha) immediately prior to the major flights.

Results and discussion

(a) Traps arranged in a grid pattern

The control site contained an average of 230 third instar larvae/pupae per m^2, while the treated site, about 30 m east of the control, contained an average of 220 third instar larvae/pupae per m^2, with a minimum significant ratio (MSR, 5%) of 1.6.

The traps were operated from 19 October 1972, the evening after beetle flights commenced until 13 November 1972 which was about a week after the major flights took place. During this period the traps on the control site caught about 1500-2000 beetles while those on the treated site caught over 290,000 beetles. The magnitude of the catch is illustrated in Plate 7.7 where the size of the beetle catch was compared with that of a lamb. Each male beetle weighs about 50 mg and a total weight of 14 kg of beetles was caught (the weight of the lamb was in the order of 50 kg). Thus, an average of about 18,000 beetles per trap was caught on the treated site. If it is assumed that the influence of the attractant sources extended 10 m on all sides except upwind, then the sphere of influence was an area about 50 x 40 m (0.2 ha). Such an area would have contained about 450,000 beetles and assuming a 1:1 male to female ratio this would mean that approximately 220,000 males were present in the initial population. This is at least 60,000 males less than the number trapped. These figures suggest, therefore, that control on the treated site should have been obtained. This was not the case, however, for the average

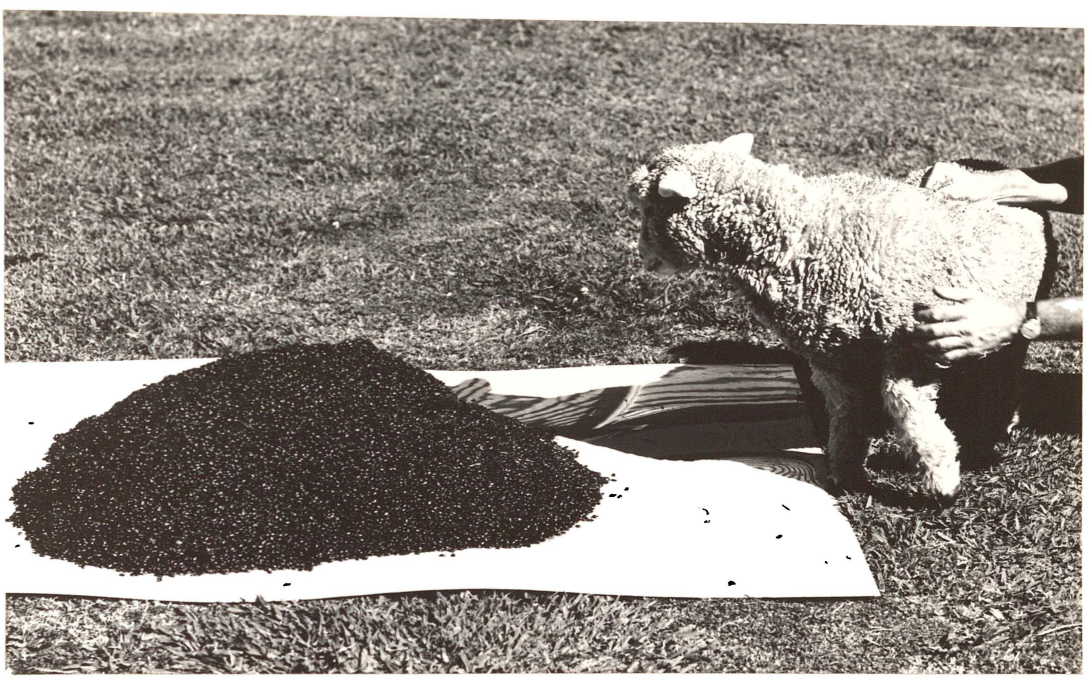


Plate 7.7 Beetles trapped from a 0.25 ha site in 3 weeks with 16 traps baited with Durez paste.

first instar larval numbers on the control and treated sites were 1020 and 920 respectively with an MSR (5%) of 1.5 which indicated no significant difference in larval numbers between the two areas.

It was observed during the progress of the trial that as soon as major flights took place, extensive mating took place on the treated site. This meant the baited traps were not competing effectively against the females in the natural population. It was also observed that baited traps were attracting large numbers of beetles onto the treated site from a group of small willow trees in a gully about 10-15 m downwind. The tops of these trees were slightly above the ground level of the treated area. Unfortunately this phenomenon invalidated any conclusions that could be drawn from this trial concerning the effectiveness of Durez baited traps in grass grub control. Nevertheless, it does illustrate the difficulty in using such methods in undulating hill country with its associated gullies, trees, and orographical effects. Ideally, therefore, trials of this nature should be carried out on flat land without any disturbing influence such as nearby trees.

Although the above trial failed to resolve the issue, the expected effectiveness of Durez as a control agent for grass grub can be postulated from the theoretical model outlined earlier in Section 7.5, together with the results obtained in Section 7.3(d), on the relative attractiveness of Durez compared to the female beetle.

The density of traps used on the treated site above was equivalent to 178 per ha. Early in the flight season Durez was shown to be about 400 times more effective than females but only about 10 times more effective during the major flight period.

Thus, early in the flight season with a population of say, 10,000 females per ha (equivalent to 2 beetles per m^2) an r value of 0.15 (10,000/400 x 178) would be achieved. This r value would be expected to give in excess of 90% theoretical control of mating from the graph in Figure 7.4. Now the actual beetle population on the treated site was about 100 times greater, i.e. 200 beetles per m^2, which would give an r value of 15. Such a figure would give less than 10% control of mating and would explain the lack of success of the trial. Obviously, with the much lower catches of males in the Durez traps during the major male flight period, very little grass grub control could be achieved with such a heavy infestation of beetles.

These calculations illustrate the importance of using mass trapping techniques on low infestations. If it is assumed that the traps were 10 times more effective than females throughout the entire flight period, then an r value of 10 is obtained for a population of 2 beetles per m^2 using the traps in a 10 m square grid pattern. Even with this extensive trapping programme the expected control of mating from Figure 7.4 would be less than 20% which is far below the level of 80% control required merely to maintain the population at 2 beetles per m^2.

The result of this trial was consistent with that of Fenemore et al., (1972) who also found that Durez traps had no effect on mating behaviour.

(b) Attractant arranged as a line source

Soil sampling prior to the beetle flights on the control and treated sites indicated that 145 and 150 pupae per m^2 were present on the control and treated sites respectively with an MSR (5%) value of 1.3. These figures were obtained on grass grub colonies within the sites. The true beetle density over the sites was much lower,

because of the non uniform distribution of larvae.

Each evening, about half an hour before beetle flights, Durez powder from the previous evening was removed from the baffle and fresh Durez in quantities which varied between 5, 10, 20, and 40 g was applied in a 5 cm band along the top of the baffle.

The results of male catches by virgin females on the control and treated sites are shown in Table 7.12.

Table 7.12 Total males attracted to ten virgin female traps on the control and treated sites for the period 20 October-4 November 1972

Site	Female traps	Control traps	Ratio	
Control	1282	430	3:1	$\chi^2_{(1)} = 423$ $(P<0.01)$
Treated	627	259	2.4:1	$\chi^2_{(1)} = 152$ $(P<0.01)$

On the treated site significantly greater numbers of males ($P<0.01$) were caught by the females than the control traps alongside. This indicated that Durez applied as a line source was having little effect on the mating activity. Interestingly, however, twice as many males were caught on the control site as on the treated site (1812 compared to 886). Also, the ratios of the female trap catches to control trap catches on the control and treated sites, viz., 3:1 and 2.4:1, were significantly different ($\chi^2 = 4.9$, $P<0.05$). This suggested that male behaviour on the treated site was somewhat modified by Durez. Effective mating was not prevented, however. The average egg/first instar larval numbers on the treated ($520/m^2$) and the control ($335/m^2$) had an MSR (5%) of 1.9. This difference was not significant.

7.7 Male confusion field trials

(a) 1970 flight season

In 1970, the first year in which attempts were made to use phenol to control grass grub populations, field trials were carried out on a pilot scale to test the feasibility of the male confusion methods. The preliminary experiments were designed to establish whether the mating behaviour of the male grass grub beetle could be confused or possibly inhibited and, if so, what quantity of phenol was required to achieve this effect.

(i) Trial layout and soil sampling

The trials were carried out on 5 x 5 m plots containing beetle numbers which ranged from 50-100 per m^2. Phenol was released at different rates over each plot by using different weights of phenol (1, 10, or 100 mg) on wood cubes (Plate 7.1) and spacing these cubes on the plots at 40 and 90 per m^2. With the exception of one treatment, none of the treatments was repeated. A monitor trap containing 10 mg Durez powder was placed at the centre of each plot and trials commenced as soon as males were caught in these traps.

Treated plots were situated at least 100 m apart and the control plots were at least 300 m from treated plots. Each control plot was at least 10 m apart. The numbers of third instar larvae and pupae on each plot were assessed by taking 20 x 225 cm^2 spade samples at random to a depth of at least 20 cm and hand sorting each sample. A few weeks after mating took place soil samples were taken for the resultant eggs and first instar larvae, by taking 20 x 225 cm^2 spade samples from each plot. The samples were sorted by means of the floatation technique developed by Kain at Ruakura. In this, eggs and larvae are separated from soil with aqueous magnesium sulphate (s.g. 1.3) and sieved.

(ii) Results and discussion

The minimum rate of phenol released over each plot and the effect of these treatments on the resultant larval populations is detailed in Table 7.13.

Table 7.13 Number of pupae and resultant first instar larvae on control and treated plots in the preliminary male confusion trials

Treatment	Pupae/m^2	First instar larvae/m^2	Minimum phenol over plots during trial ($\mu g/h/m^2$)
Six control plots	Ave. 55	Ave. 129	0
Plot 1. 100 mg phenol cubes at 90/m^2	110	46	180
Plots 2 and 3. 10 mg phenol cubes at 90/m^2	70 and 80	150 and 46	55
Plot 4. 10 mg phenol cubes at 40/m^2	56	52	25
Plot 5. 1 mg phenol cubes at 90/m^2	150	32	10

During the flight season it was not possible to observe all the plots and so observations were confined to Plot 1 over which the highest rate of phenol was released. Each cube on this plot contained 100 mg phenol and received five coats of an epoxy resin as described earlier. The cubes used on Plots 2, 3 and 4 had three coats of the epoxy resin while the cubes on Plot 5 were uncoated. For the 100 mg phenol cubes, the release rate initially was in the order of 150 $\mu g/h$/cube but this dropped to 2 $\mu g/h$/cube after 3 weeks' weathering. Thus a phenol level of at least 180 $\mu g/h/m^2$ was

maintained over Plot 1 throughout the trial period.

In the early beetle flights, males were observed to fly upwind towards Plot 1 and drop at distances of about 5 metres downwind. As the amount of phenol released over the plot dropped with time, the number of males moving onto the plot appeared to increase and by the time major flights took place a large number of beetles was observed hovering over the plot. Under normal circumstances, males on mating flights actively search for females waiting quietly on grass stems, the soil surface, or partly beneath it with only their abdomens protruding. Males on Plot 1, however, did not seem capable of orienting to females for they were often observed to approach within a few centimetres of females and then fly off after an unsuccessful search. Males did not become quiescent or fatigued under the influence of the phenol but on the contrary, actively searched over, under and near the cubes where in most cases females were not present.

On a few evenings during the major male flight period, beetles on Plots 1, 2 and 3 were sampled after the flight and the number of unmated males, females and copulating pairs recorded. These results, together with the number of males attracted to the centre jar of Durez are shown in Table 7.14. The Durez in the centre jars was replaced on three occasions during the major male flight period.

Table 7.14 Numbers of unmated males and females and copulating pairs together with male attraction to the "super-female" on Plots 1-3 during major male flights

Plot	Day	No.unmated males	No.unmated females	No.copulating pairs	No.males attracted to centre trap
1.	1	0	8	3	0
	2	7	16	2	0
	3	8	5	2	0
	7	45	20	8	0
	8	6	4	0	0
	9	10	9	0	0
2.	1	0	0	0	1
	2	2	0	0	0
	3	0	0	0	0
	6	15	8	3	0
	7	3	1	0	0
	8	0	0	0	0
	9	28	5	0	50
	11	12	4	2	21
3.	2	5	3	0	2
	3	5	0	1	2
	7	46	20	4	3
	9	4	1	0	11
	11	36	13	0	15

As shown in Table 7.13, Plots 1,2 and 3 had larger amounts of phenol released over them than Plots 4 and 5 and, therefore, if the treatments were ineffective on the former plots, no effect could be expected on the latter plots. Mating took place on all the plots and Durez attracted males on all the plots except Plot 1. The centre jar containing Durez was regarded as a "super-female" for when placed in the field by itself catches of 100-200 beetles were readily recorded. This is an order of magnitude greater than the catches

normally recorded for each female in virgin female traps. Thus if males could not locate the "super-female" in the centre of the plot it was deduced that males could not locate virgin females on the plot. In such a situation, mating on the plot would therefore depend on visual factors or a simple chance meeting of males and females, rather than chemical attraction. Thus, as males continued to search for females on Plot 1, male confusion rather than male inhibition appeared to be occurring. The probability of a male finding a female by chance or by sight on Plot 1 was high because of the high beetle density (100-200 per m^2). Even so, the results in Table 7.14 indicated that on any one night on this plot (and also Plots 2,3) a higher number of females was unmated than mated. These females may have mated on subsequent nights, however.

The number of beetles attracted to the centre jar on each plot, on an evening when a major male flight took place, was assessed in relation to the quantity of phenol released from the centre jar and the surrounding wooden cubes on the plots (Table 7.15). The area of the top of the jar was 19 cm^2 and so the release rates were compared on this basis.

Table 7.15 The influence of various quantities of phenol surrounding the trap on the potency of Durez powder

Phenol release rate from centre jar (19 cm^2) containing 10 mg Durez	Phenol release rate from cube over plots/19 cm^2	No. of beetles attracted to centre jar
4 µg/h	0.02 µg/h	ca. 400
"	0.1 "	ca. 50
"	0.3 "	20
"	0.6 "	11
"	0.6 "	11
"	6.0 "	0

It was of considerable interest to find that beetles were not attracted to the centre jar when the phenol release around the jar exceeded that from within the jar. Also, the Durez in the centre jar became more effective as the rate of phenol release around the jar decreased. These results provide further evidence that the active component in Durez is phenol.

Treatment effects were assessed by measuring the reduction in resultant egg and first instar larval numbers between the treated and control plots. The results (Table 7.13) indicated that the control population increased by approximately double the initial beetle infestation (55 to 129/m^2) while the larval populations on the combined treated areas dropped to about two-thirds the original beetle infestation (93 to 65/m^2). This difference was significant ($P<0.01$). The treatments could not be analysed separately as they were not replicated.

These initial attempts indicated, therefore, that provided a sufficient phenol level was used above the pasture (>180 µg/h/m^2) male beetles could be confused but not inhibited in their search for females. Reduced mating activity also appeared to take place on the treated plots. These results were regarded as a promising basis for further extensive field trials to evaluate the potential of phenol in grass grub control provided a satisfactory method could be found of dispensing phenol at suitable levels for the entire adult flight period.

(b) 1971 flight season

(i) Trial layout and soil sampling

The trials were carried out at the Takapau Research Area in Hawke's Bay as the facilities and manpower were available there for large scale grass grub field trials. Two sites about 5 km apart

were selected, one with a high grass grub population and the other with a low population. Two plots (64 x 64 m) spaced greater than 100 m apart were located within each site. Each plot was matched as far as possible for similarity in grub numbers, damage and history. On the low population plots, six sub-plots (4.6 m^2) were placed on known pockets of infestation. These were set out at random on the high population plots.

Phenol was released over the pasture at a reasonably controlled rate by sealing it into 3.2 mm PVC tubes and allowing it to permeate slowly through the polymer wall. A total of 3 km of tube containing approximately 25 kg of phenol:water (9:1) was placed on each of the treated plots. Lengths of tube (64 m) were spaced 1 m apart over each of the treated plots. The layout of the field trials is illustrated in Plate 7.8.

In order to assess likely beetle numbers, four hundred(10.2 cm dia.)soil cores were taken at random to a depth of 20 cm from each site at the pupal stage. During the flight season soil samples were taken daily, at random, from each sub-plot. At the finish of the flight season, twelve soil samples were taken from each sub-plot to assess treatment effects by determining the resultant eggs and first instar larvae.

To record flight behaviour, a mechanical rotary trap (Plate 7.9) sampling at three heights (0.2, 1.7, 2.7 m) was placed on each plot. On the low population plots these were placed on known infested areas.

Treatment effects during flight were assessed with ten traps placed on each plot each baited with five virgin female beetles. The females were held in soil at ground level and allowed to emerge naturally each evening. Control traps were placed alongside each

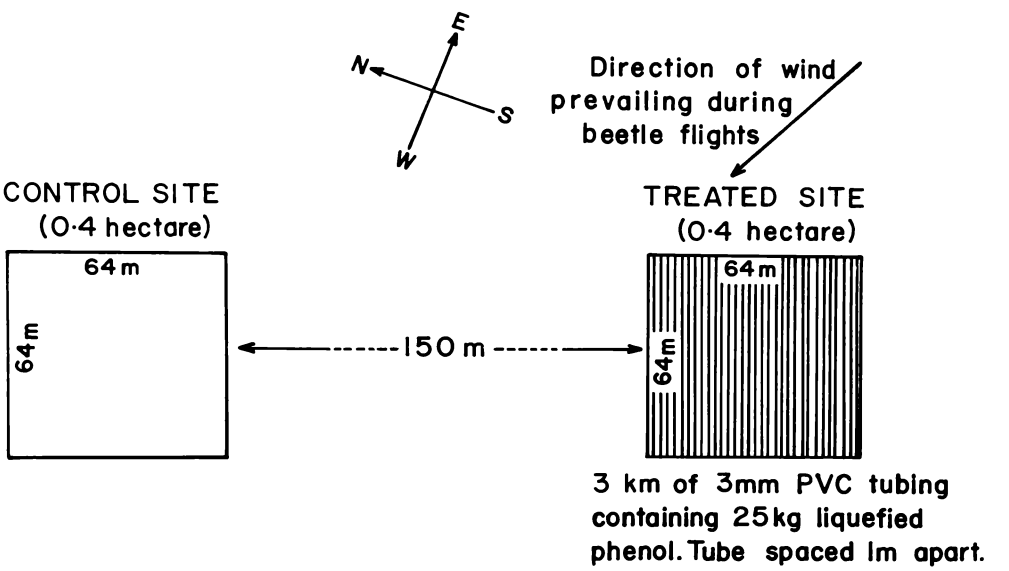


Plate 7.8 Field trial layout at the Takapau Research Area testing the male confusion principle during the 1971 beetle flight season.

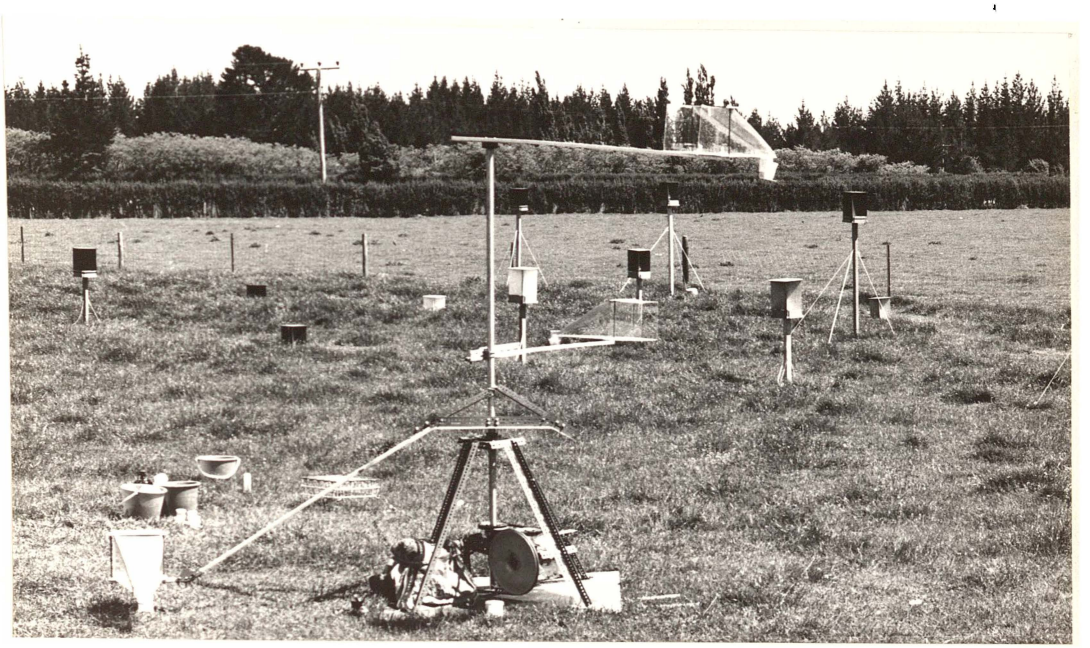


Plate 7.9 Mechanical rotary trap used to record flight behaviour during the 1971 and 1972 grass grub flight seasons.

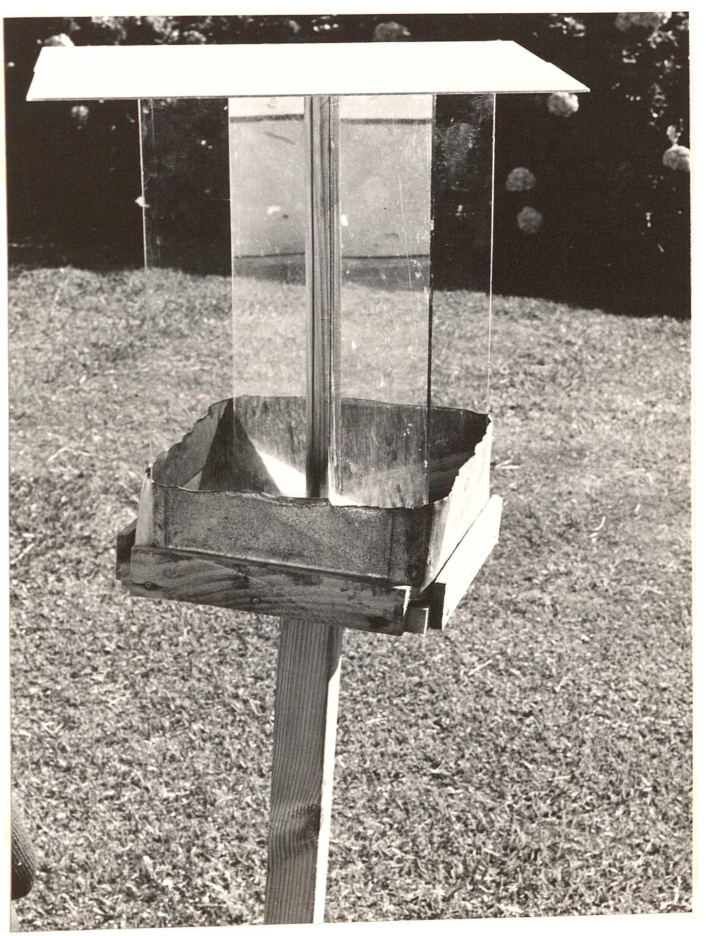


Plate 7.10 Glass baffle trap used to assess directional flight behaviour during the 1971 and 1972 grass grub flight seasons.

baited trap.

Directional flight within each site was measured with eight glass baffle traps (Plate 7.10), four at ground level and four at 1.0 m above the ground. These traps were placed at random around the site about 9 m from the fence line.

(ii) Results and discussion

Prior to the beginning of the trial, the control and treated plots on the low population site contained 36.7 and 24.8 pupae per m^2, respectively. On the high population site, the control and treated plots contained 140 and 161 pupae per m^2, respectively.

On both sites, male flights were observed to commence on October 21, 1971 and the phenol tubes were laid out the following day. On the low population site, major flights took place between November 4 and 9 and the flight season was finished by November 15. Major flights on the high population site took place between November 7 and 15 and the beetle flights extended to November 25, 1971.

At Takapau, grass grub flight is usually dominated by males throughout the entire season. The percentage of the total males caught in the rotary traps at three heights is shown in Table 7.16.

Table 7.16 Percentage of total males caught in rotary traps at different heights on low and high populations

Treatment (height)	Low population		High population	
	Control	Treated	Control	Treated
Low	81.3	77.1	76.1	73.2
Medium	11.9	13.6	17.6	18.6
High	6.8	9.3	6.3	8.2
Total	1185	875	2143	1912
	$\chi^2_{(2)} = 6.2$ ($P<0.05$)		$\chi^2_{(2)} = 6.3$ ($P<0.05$)	

A significantly lower proportion ($P<0.05$) of male beetles was caught in the lower rotary traps on the treated plots on both the high and low population sites. This difference was more marked in the baffle trap catches on the low population site (Table 7.17). On the high population site, differences were not significant, a result which remains unexplained.

Table 7.17 Total male beetles caught in baffle traps over the 1971 flight season

Treatment (height)	Low population		High population	
	Control	Treated	Control	Treated
0	496	153	1042	949
1m	486	317	937	737
	$\chi^2_{(1)}$ 40.7 ($P<0.001$)		$\chi^2_{(1)}$ 4.7 ns	

To assess whether the treatment was disrupting normal communication between males and females, the attractiveness of females on the control and treated plots was compared and the results are shown in Table 7.18.

Table 7.18 Male beetles attracted to virgin female traps over the flight season

Treatment (trap)	Low population		High population	
	Control	Treated	Control	Treated
Female traps	1026	317	1018	848
Control traps	361	209	415	493
Percentage caught in female traps	76.5 (52-94)*	60 (38-81)*	71 (50-100)*	63 (47-75)*
	$\chi^2_{(1)}$ = 33 ($P<0.001$)		$\chi^2_{(1)}$ = 19 ($P<0.001$)	

* Day-to-day range in percentages.

On the low beetle population sites, the females on the control plots attracted 76.5% of the total males caught in the female traps plus the control traps alongside. This was significantly different ($P<0.001$) from the 60% of males attracted to virgin females on the treated plot which meant that males on this plot were not locating females as effectively as normal. A similar result was obtained with the virgin female traps on the high population sites. Males on the treated plots, however, were not completely disoriented, for the traps containing females attracted significantly more males than the control traps alongside (low population $\chi^2_{(1)} = 22$, $P<0.001$; high population $\chi^2_{(1)} = 93$, $P<0.001$). Thus, at the phenol release rate used, males were still capable of locating females.

The fact that males could be completely disoriented was confirmed by using a higher concentration of phenol in the air on an area adjacent to the high population treated plot. Phenol was released over a 0.1 ha area by covering the bottoms of eight open trays (54 x 40 x 10 cm) with liquefied phenol. The trays were spaced approximately 9 m apart and phenol was readily detectable over the area. Seven female traps, with controls alongside, were placed equidistantly between the phenol trays.

The test was carried out on a calm evening when a major flight took place. Female traps on the control area caught 197 males while, on the phenol area, they attracted only 14 males. Control traps on the control and phenol areas caught 30 and 18 males respectively.

Massive attraction of male beetles on to the treated plots was not observed to take place and this was verified by the soil sampling results in Table 7.19 in which the male/female beetle ratios for the control and treated plots were similar throughout the trial.

Table 7.19 Male/female ratio obtained from soil samples for [illegible] throughout the 1971 flight season

Treatment (dates)	Low population		High population	
	Control	Treated	Control	Treated
Oct. 22-28	43/52	68/60	45/31	50/38
Oct. 29-Nov. 4	97/129	116/163	224/181	169/149
Nov. 5-11	64/84	106/109	227/182	141/128
Nov. 12-18	3/30	7/20	23/32	1/6
Nov. 19-Dec. 1	Sampling terminated			

In order to measure the effectiveness of the treatment at preventing mating, a comparison was made between the rates at which females were mated in the sub-plots on the control and treated plots. The results (Plate 7.11) indicated that there was no difference on the control and treated plots for both the high and low populations. Thus, all the females on the treated plots were mated at the same time as those on the respective control plots. This was confirmed by the soil sampling results shown in Table 7.20 where a similar number of eggs/first instar larvae were found on the treated and respective control plots for the low and high populations.

Table 7.20 Average number of teneral beetles and resultant eggs/first instar larvae and sub-plots

	Low population		High population	
	Control	Treated	Control	Treated
No. beetles/m^2	105	139	171	175
No. eggs and first instar/m^2	514	484	603	475

One of the problems associated with the use of sex pheromones in the field is the wasteful liberation of pheromone during the day when it is biologically ineffective. In the case of the grass

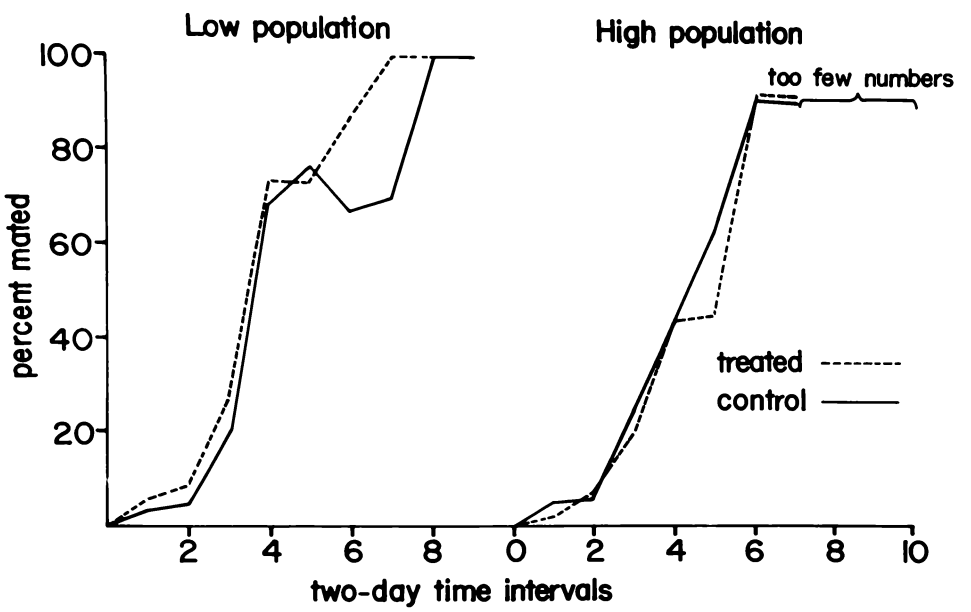


Plate 7.11 Percentage females mated on control and treated plots at 2-day intervals during the 1971 field trials.

grub beetle, phenol needs to be present for only about half an hour at dusk when mating takes place.

In these trials the average phenol loss over the flight season of 4 weeks was 200 g/day/ha. During the hour at dusk, the average amount of phenol released was 5 g/h/ha. With the exception of three evenings, when the rate dropped to 1 g/h/ha, the phenol liberated at dusk varied between 3.5 and 10 g/h/ha.

It did appear, therefore, that on both low and high grass grub populations, mating activity was not reduced by releasing phenol over the pasture at a rate of 5 g/h/ha. With higher phenol release rates, male flight was completely confused. This provided a basis for further work on this aspect of grass grub control. It is clear that prevention of normal chemical communication may not give significant control because of the high probability of chance mating in the field.

(c) 1972 flight season

Field trials were carried out during the 1972 flight season to evaluate the effect on the mating activity of grass grub beetles of releasing larger quantities of phenol over the pasture than that used during the 1971 flight season.

(i) Trial layout and soil sampling

The trial was carried out a few kilometers from the Ruakura Research Centre on a farm with a moderate infestation of grass grub. Two sites were selected, each with an area of 0.25 ha, and spaced at least 150 m apart. This separation was considered to be sufficient to prevent the treatment (applied to the western site) from having any influence on the control site. Twelve plots (1 m^2) were placed on known infested areas on each site.

In order to assess likely beetle numbers and possible treatment

effects, twenty soil cores (10.2 cm dia.) were taken to a depth of 20 cm from the twenty plots at the third instar-pupal stage and again at the subsequent egg-first instar larval stage.

To record flight behaviour, twenty glass baffle traps were used on both the treated and control sites. Ten were placed at ground level and ten, 1 m above the ground. Within each site, directional flight was measured by locating fourteen baffle traps (seven high, seven low) within 3 m of the outer boundaries. The other six traps (three high, three low) were located at random around the centre of each site.

Treatment effects during flight were assessed with ten female traps placed on each site, each baited with five virgin female beetles. The females were held in soil at ground level and allowed to emerge naturally each evening. Control traps were placed alongside each baited trap.

In order to obtain a higher release of phenol than that used during the 1971 flight season, a different phenol dispenser was used. This consisted of plastic tubs containing 1 kg of phenol: water (9:1). Each tub, placed in the soil to a depth of 15 cm, was located at 10 m intervals over the treated site in a 5 x 5 grid pattern. During the day, the lids were on the tubs and phenol sublimed onto the bottom surface of the lid. At least half an hour before flights commenced, the lids were removed to allow sufficient time for phenol to vaporize and diffuse into the atmosphere. The bottom of the lid was also exposed during the flight and replaced each evening after the flight had taken place. The quantity of phenol released over the pasture during the flight period was determined by measuring the difference in weight of five tubs when the lids were removed for a few hours about dusk.

(ii) Results and discussion

Before the trial, the average number of third instar larvae and pupae on the control and treated sites were 100 and 100 per m^2 respectively with an MSR (5%) of 1.3. Male flights were observed on October 18 and the trial commenced the following evening. Major flights took place between November 1-7 and the flight season was completed by November 15, 1972.

The baffle trap results for males (Table 7.21) followed the same pattern as in the Takapau trials during the 1971 flight season in that a significantly greater proportion of males ($P<0.001$) flew higher over the treated site than the control. In fact, about three times as many males were caught in the baffle traps on the treated site than on the control which was probably a reflection of the confused male flight behaviour over the treated plot.

Table 7.21 Total male beetles caught in baffle traps over the 1972 flight season

Treatment (height)	Control site	Treated site
0	390	717
1 m	150	765

$\chi^2_{(1)} = 89.8$ ($P<0.001$)

The baffle trap catches for females (Table 7.22) also showed that a greater proportion of females flew above 1 m over the control site plot. This was consistent with the known behaviour of female beetles in the Waikato region. About twice as many female beetles were caught in the baffle traps on the treated site than on the control. This suggested that a greater proportion of females flew higher over the treated site than the control. Such a phenomenon may have been consistent with the observations of

Kelsey (1968) who suggested that female beetles took flight if they remained unmated for some time after the initial soil emergence.

Table 7.22 Total female beetles caught in the baffle traps over the 1972 flight season

Treatment (height)	Control site	Treated site
0	51	78
1 m	208	456

$\chi^2_{(1)} = 2.95$ ($P<0.10$)

The results of the virgin female beetle catches are shown in Table 7.23.

Table 7.23 Male beetles attracted to virgin female traps over the 1972 flight season

Treatment	Control site	Treated site
Female traps	1786	397
Control traps	349	344
Percentage caught in female traps	84 (20-100)*	54 (40-59)*

$\chi^2_{(1)} = 272$ ($P<0.001$)

* day to day range in percentages

A total of 2135 males was caught in the female and control traps on the control site while only 741 males were caught in similar traps on the treated site. This result is probably related to the confused male flight behaviour on the treated site. A significantly greater proportion of males was attracted to virgin females on the control site than on the treated site. This is consistent with confused flight behaviour. Statistical analysis

of the female trap catches and the controls alongside revealed a significant difference ($\chi^2_{(1)} = 3.65$, $P<0.06$). This meant that male flight behaviour was still not completely confused in spite of the high rate of phenol release. The female trap/control trap difference occurred on 3 out of 26 evenings during which major male flights took place. On these evenings, females on the treated site attracted one and a half times as many males as the control traps alongside. On the other hand, females on the control site attracted 10-15 times as many males as the control traps.

During the hour at dusk, an average of 28 g phenol was released over the trial area. This phenol release rate was fairly consistent throughout the flight season but did vary from 10 to 60 g/h. The phenol release rate used, was about twenty times higher than that used in field trials during the 1971 flight season.

The mechanism of action in confusing males and the influence of population density on the effectiveness of the methods were discussed in Section 2.12. If it is assumed that each female beetle releases its entire phenol supply (1.5 µg) during the hour at dusk, then the 28 g/h of phenol released from the tubs over the 0.25 ha site is roughly equivalent to 80×10^6 females/ha i.e. 8,000 females/m^2. The female density on the treated site was 50/m^2. If it is assumed that the phenol from the 25 tubs emanated from 8,000 point sources/m^2, then the chance of a male locating a female on the treated site was 1 in 160.

Although male flight over the treated site was confused, mating was still observed to take place. The mean number of resultant eggs/first instar larvae on the control and treated sites were 450 and 280 respectively with an NSR (5%) of 1.64. While the numbers on the treated site were about 40% lower than those on the control,

the difference was not significant at the 5% probability level. In any case, a reduction of 40% in the larval population is too low for an economic control. As discussed earlier, an 80% reduction is required merely to maintain a population at the same level as the previous year. In view of this result, with the large quantities of phenol used for such a small effect, there appears very little prospect of using phenol as a direct behavioural agent for grass grub control.

7.8 Conclusions from mass trapping and male confusion field trials

The theoretical calculations on low beetle infestations outlined in the population model, suggested that grass grub control by mass trapping becomes possible only when the attractant is of the order of hundreds of times more effective than the female. Durez paste appeared to be a phenol formulation which partly fulfilled this requirement. Hence the effectiveness of Durez as a direct behavioural control agent was tested in field trials.

In these trials, Durez-baited traps were arranged in a grid pattern. Over a quarter of a million male beetles were trapped during the flight period. Mating was observed to take place on the plots between the traps, however, and no reduction in the resultant larval numbers took place on the treated site. Thus, with a trap density of 64 per ha and a grass grub population around 200 beetles per m^2, Durez paste did not compete successfully with virgin female beetles in the natural population, particularly when the major male flights commenced. The trial was carried out on a hill country farm and large numbers of males were attracted onto the treated site from nearby trees. This phenomenon illustrated a difficulty in using attraction methods in undulating hill country with its associated gullies, trees, and orographical effects.

It was hypothesized that the potency of the attractant would be enhanced if it was presented in the field as a line source rather than a number of point sources. Evidence from the number of males attracted to virgin females on such control and treated sites suggested that male flight behaviour on the treated site was somewhat modified by the attractant. Extensive mating was observed on the treated site, however, and no reduction in resultant larval numbers took place. Thus the potency of Durez paste as a line source was not sufficient to allow it to out-compete the females in the natural population.

In the initial field trials to test the male confusion method of control, phenol was dispensed from wooden cubes placed on the pasture. Results indicated that if the phenol level was above 1.8 g/h/ha, male beetles could be confused but not inhibited in their search for females. It was also shown that the potency of Durez powder in a jar could be reduced by releasing different quantities of phenol around the jar. When the phenol release rate around the jar exceeded that from the powder within the jar, the potency of the powder was nullified. These results provided further evidence that the active component in Durez is phenol.

In the following season, phenol released over the pasture at a higher rate (5 g/h/ha) caused males on the treated site to fly higher than those on the control. This amount of phenol was insufficient to completely disorientate or confuse male flight behaviour and mating activity on the treated site was not reduced. An even higher release of phenol (110 g/h/ha) was used in the subsequent flight season and the effects on flight behaviour established the previous season were reproduced. Although flight behaviour was confused, males on the plot were able to locate females and there was no reduction in larval numbers on the treated site. It appeared,

therefore, that phenol is of little value as a control agent for grass grub especially with populations in the order of 100-250 beetles per m^2.

PART II

CHAPTER VIII

SEX PHEROMONE STUDIES ON THE COMMON ARMYWORM MOTH

8.1 Introduction

The majority of sex pheromone studies that have been carried out have been with lepidopterous insects. The reasons for this are probably two-fold. Firstly, moths can be reared fairly readily in the laboratory and, therefore, they can be made available in large numbers throughout the year. Secondly, moths normally exhibit strong sex stimulatory activity in the laboratory which allows reliable bioassay procedures to be developed.

Males of many species in the family Noctuidae have been attracted in the field to specific mono-unsaturated alcohols or acetates (Roelofs and Comeau, 1970). These workers showed, for example, that five species of moth in the subfamily Hadeninae, to which P.separata belongs, were attracted to cis-11-hexadecenyl acetate. The study by Roelofs and Comeau in which sex pheromones were discovered for 37 lepidopterous species was carried out simply by using standard compounds in traps.

Roelofs' group also used a comparison method to identify sex pheromones. They compared the behaviour of crude natural pheromone extracts to that of standard compounds in functional group tests, concentration steps, and EAG tests. Suspected pheromones were then synthesized and tested in the field for activity. This procedure was outlined in Sections 2.10 and 2.11 and is basically the approach adopted in this study on the common armyworm moth.

8.2 Experimental

(a) Maintenance of the culture

To initiate the armyworm population, moths were collected from light traps and mated females allowed to lay eggs in the folds of fluted paper held in buckets. In the initial attempts to raise adults in the laboratory, the larvae were fed on tufts of ryegrass in cardboard boxes (30 x 35 x 10 cm) covered with terylene sheer. When the caterpillars were fully developed they were transferred to containers lined on the bottom with pumice, and fed with cut grass until pupation took place. The pupae were sexed on the basis of the genital apertures (Clearwater, 1971) and the males and females were placed in separate boxes. With this rearing method the overall mortality was in the order of 90% which meant that only a few thousand moths were reared in the laboratory at any one time. The mortality rate was fairly constant throughout the early instar stages but increased significantly during the late instar period when the larvae became infected with a viral disease.

In the second year of this study, in an effort to obviate this disease, fully fed larvae and pupae were collected in the field and raised to adults in the laboratory. Approximately 15,000 insects were collected and yielded about 10,000 moths. The 30% loss in insect numbers was due to parasitism in the field-collected larvae.

The method was slow and painstaking and in the third year of this study on the armyworm moth (1972 season), attempts were made to mass rear larvae, from eggs, under field conditions. To do this, young maize plants at about the silking stage were covered with tents made from terylene sheer (Plate 8.1) and moths were introduced into the tents and allowed to oviposit on the maize. About 2-3 days prior to the introduction of the moths, the tents were sprayed with

Plate 8.1 Terylene tent used for mass rearing armyworm in the field.

Plate 8.2 Army caterpillars within the terylene tent.

pyrethrum oil to remove possible parasites.

Approximately 5,000 moths were introduced into six tents at varying stages throughout the summer. Eggs were laid in extremely large numbers and the resultant larvae exhausted the food supply under each tent within a few weeks. The caterpillar density under each tent was approximately 250 per maize plant. When the caterpillars exhausted the food supply, the tent was extended to include fresh maize. This technique was not successful, however, for the majority of caterpillars did not move towards the fresh food supply. Consequently the caterpillars went into a phase polymorphism state. In this condition the caterpillars become more active, use a lot of their stored energy reserves, and are more susceptible to disease. Indeed, a great majority of the larvae became infected with the viral disease that had been observed in laboratory populations in previous years. In subsequent experiments in a controlled environmental laboratory (D.S.I.R. Palmerston North), the caterpillar density was reduced to five per maize plant and larval mortality has been minimal.

A number of parasites were also found to be a problem in rearing larvae under field conditions even though fairly stringent precautions were taken against them. A parasite that was particularly troublesome was a braconid fly, Apanteles rufricus, which oviposits in the respiratory pores of early instar larvae. Other parasites included a tachinid and two ichneumonid wasps, Netelia sp and Amblyteles rodatorius.

Despite the presence of parasites, large numbers of developed larvae were obtained (Plate 8.2) but these eventually died due to the viral infection mentioned earlier. The number of moths obtained with this technique was disappointing for only about 3,000 moths were reared in the third year of this study. Overall, only

about 10,000 female moths were available throughout the three years of this study.

(b) Laboratory behavioural bioassay

The bioassay apparatus (Plates 8.3 and 8.4) was a modification of that described by Cardé and Shorey (1969). The olfactometers were made from 2.5 l clear Winchester bottles with the bottoms removed. The Winchesters were inverted and the moths were confined between plastic gauze, placed over the open end, and metal gauze retained on the inside by the neck of the bottle. Fresh air was blown through each olfactometer at a rate of 25-30 l/h by means of a forge blower placed outside the laboratory. The air temperature was maintained at 17 ± 1°C by blowing air over a thermostatically controlled radiant element placed in the air outlet pipe from the forge blower. A closed-air system was maintained for each olfactometer by leading the air from each olfactometer to an exhaust tube. Test materials were placed on the metal gauze through a hole (2 cm dia.) drilled in the side of the bottle just above the neck. The temperature in the bioassay room was also controlled and varied between 16 and 18°C.

Male moths were placed in the bioassay room as soon as they emerged and were conditioned to the 12h:12h light:dark cycle used in the room. The light period commenced about 3.30 p.m. and finished at 3.30 a.m. Red filtered light from two 40 W incandescent bulbs at low intensity (ca. 100 lx) was used during the dark period. Male moths were used in groups of ten in the olfactometers and were fed with 8% sucrose solution.

The sex pheromone was exposed to male moths either as crushed female abdominal tips on metal gauze squares or more usually as a concentrated extract on the end of a glass rod. Moth flight

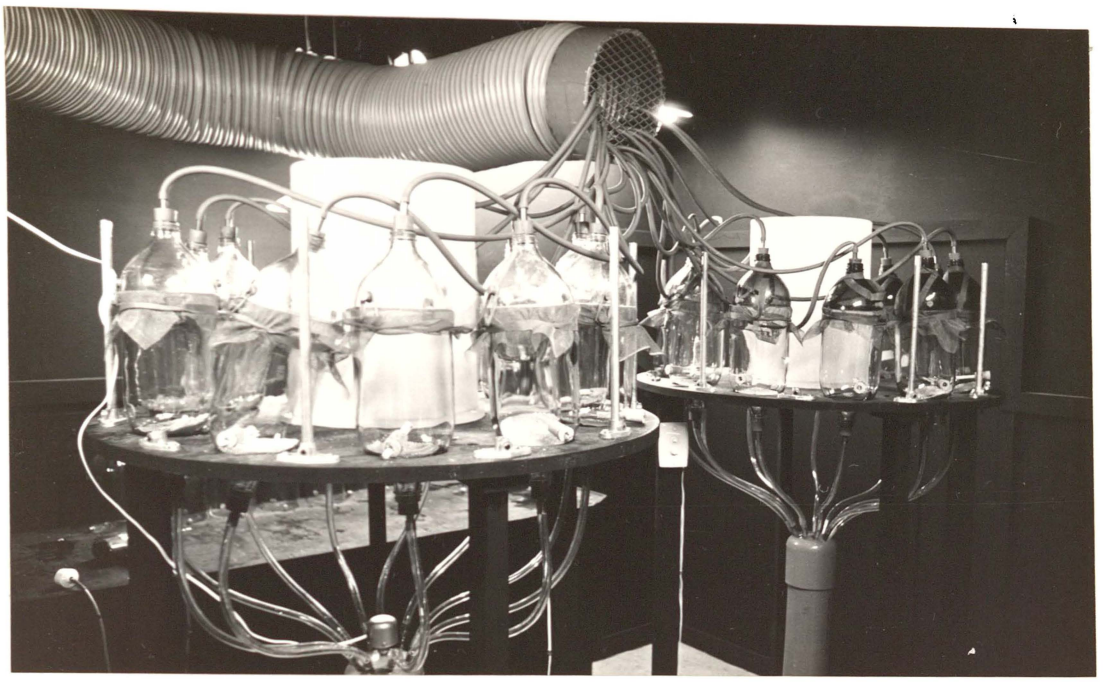


Plate 8.3 General view of the olfactometers used for sex pheromone studies of the common armyworm.

Plate 8.4 Close-up view of an olfactometer.

activity increased during the period from 8.30-10.00 a.m. and testing was mainly carried out during this period. On exposure to the sex pheromone, receptive males became excited, took flight and dropped down around the glass rod. Highly stimulated males extended their claspers and attempted to copulate with the rod and with one another. Normally males were tested once each day.

Groups of males aged from 1-12 days after final ecdysis were tested against females aged from 1-8 days. Three female abdominal tips of each age group were smeared over small wire gauze squares and exposed to males of different ages in the olfactometers. Males were observed for both flight activity (FA) and copulatory activity (CA). The results are shown in Table 8.1.

Table 8.1 Variation of male behavioural response and female pheromone production with age

Expt. 1.

Female age (days)	Percent male response with male age		
	1-2 days	4-5 days	6-8 days
1-2	0	0	0
4-5	0	0	0
7-8	0	0	40FA, 10CA* ** 40FA, 30CA

Expt. 2.

	5-6 days	8-9 days	10-12 days
3-4	0	0	0
5-6	50FA,20CA	75FA,50CA	100FA,50CA
6-8	150FA,50CA	0	40FA

* FA = flight activity; CA = copulatory activity

** tested 20 mins after initial test

The results indicated that virgin females did not produce the sex pheromone until they were 5-6 days old while males did not give the complete mating response until they were at least 5-6 days old. The results relating to the female pheromone production were consistent with the known behaviour of females which commence oviposition about 5 days after final ecdysis. Presumably mating takes place in the field just prior to oviposition.

(c) Preliminary extraction and concentration methods

To establish a suitable solvent system for extracting the pheromone, female abdominal tips in batches of at least one hundred were surface rinsed or macerated with either methylene chloride or benzene and tested for activity. The results are shown in Table 8.2.

Table 8.2 Behavioural responses to the sex pheromone extracted with methylene chloride and benzene

Extraction method and female equivalents tested	Percent behavioural response
Benzene surface rinse, 10♀ E	20 FA, 10 CA
" , 0.5♀ E	40 FA, 10 CA
Benzene macerate, 10♀E	20 FA, 10 CA
Methylene chloride surface rinse, 1♀E	100 FA, 50 CA
" , "	100 FA, 40 CA
Methylene chloride macerate, 1♀ E	40 FA, 10 CA
" , 0.1♀ E	30 FA, 20 CA

Weak sex stimulatory activity (10-20% copulatory responses) was induced with both the methylene chloride and benzene macerates presumacly due to co-extracted impurities which reduced the rate of volatilisation of the pheromone or partially inhibited behavioural response. On the other hand, strong sexual activity

(40-50% copulatory responses) was induced in males when the female abdominal tips were surface rinsed with methylene chloride. This extraction procedure was adopted throughout the study. In general, the strongest responses were induced in males which had not been tested previously.

(d) Pheromone heat stability and functional group tests

The stability of the pheromone to heat was tested with a benzene extract containing 3 female equivalents. The extract did not lose its activity after it had been taken to dryness and heated to 100°C for 4 hours. Clearly, the pheromone is reasonably heat stable.

An ethanol extract (1 ml) containing 3 female equivalents was treated with a small quantity of Adams catalyst and hydrogen slowly bubbled through the solution for 3 hours. The extract was inactivated by this treatment which probably indicated the presence of unsaturation in the pheromone.

An ethanol extract containing 3 female equivalents was treated with 2 ml of 1N ethanolic-potassium hydroxide and refluxed for 1 h on a water bath. The ethanol solution was inactive when bioassayed. The solution was extracted with light petroleum (4 ml) after the addition of water (2 ml) and after reducing the light petroleum extract to 0.5 ml, acetyl chloride (0.3 ml) was added and the solution warmed for about a minute. Water (4 ml) and light petroleum (2 ml) were added and the petroleum extract washed with 10% sodium bicarbonate and tested for activity. This reacetylated phase induced 30% CA and 100 FA in males. These results are consistent with the sex pheromone being an unsaturated acetate.

(e) Electroantennogram responses to a series of standard acetates

Since preliminary studies suggested the armyworm moth sex

pheromone was an unsaturated aliphatic acetate, standard compound studies were made. Dr Martin Jacobson of the United States Department of Agriculture kindly supplied C_{12}-C_{16} unsaturated aliphatic acetates prepared in his laboratory.

The EAG tests were carried out with both excised and intact antennae of male moths. In the former case, the tip of the antenna was snipped off and the antenna, with the sensory side facing upwards, held on paraffin wax with each end placed in a drop of 1 M sodium chloride solution. Electrical contact was made with the salt solution through platinum wire and the measurements made on the apparatus described in Chapter 6.

A series of dodecenyl acetates were prepared as 100 p.p.m. standards in light petroleum and included: 1-; 11-; cis-3-, -6-, -6-, -7-, -8-, -9-; and trans-10-dodecenyl acetates. These compounds were tested at the 1 µg level on paper but gave no response on the antenna. Likewise, a series of tetradecenyl acetates which included: 1-; 13-; cis-4-, -9-, -10-, -11-; trans-9-tetradecenyl acetates and cis-9, trans-12-tetradecadienyl acetate did not give an antennal response. The results obtained with a series of hexadecenyl acetates (HDA's) tested at the 1 µg level on paper are shown in Table 8.3.

Only cis-11-HDA gave strong EAG responses. For example, the average response to 1 µg cis-11-HDA in eight measurements on 4 antennae was 700 µV. The shapes of the EAG's of the natural pheromone and cis-11-HDA (Figure 8.1) were similar but broader EAG's were obtained with the natural pheromone. This could suggest that a multiple component pheromone system is used by the female armyworm moth. Female antennae did not respond to any of the acetates.

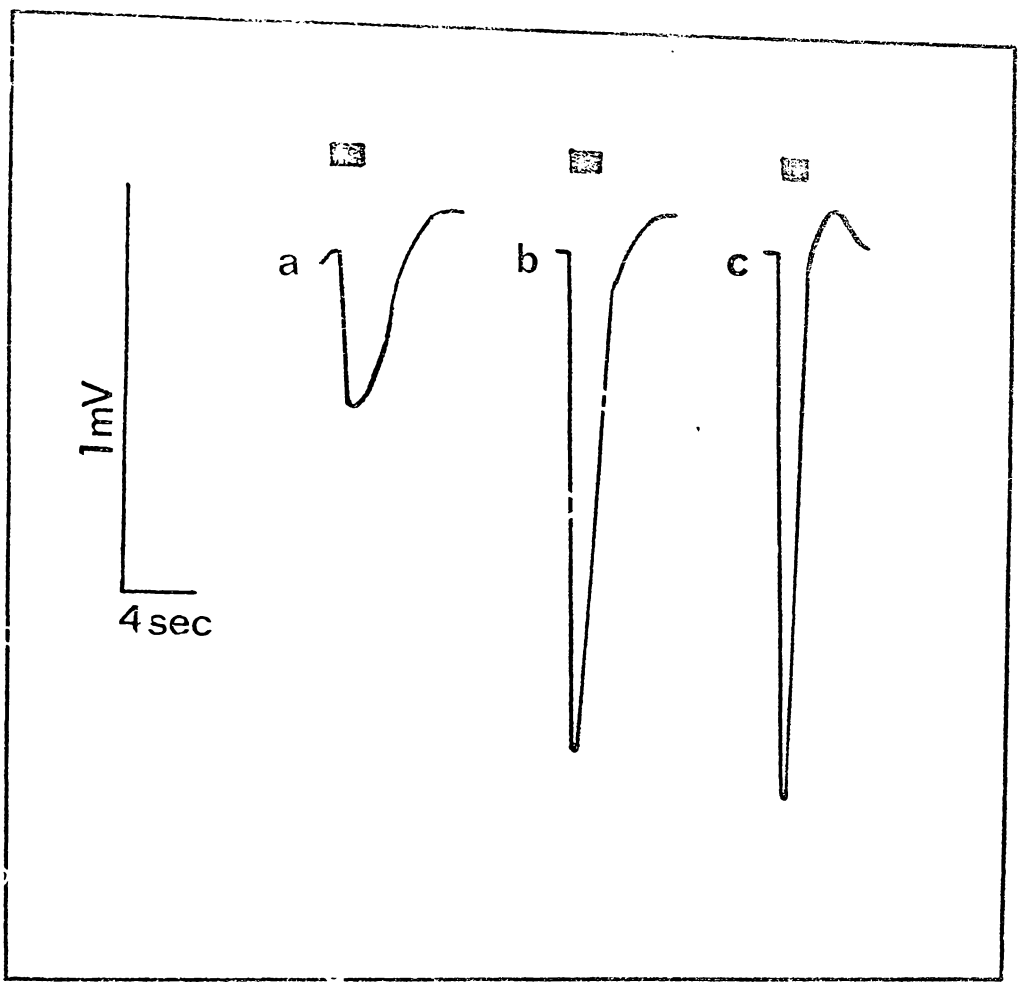


Figure 8.1 EAG responses of an excised male antenna of P.separata to (a) an air pulse, (b) 3 female equivalents of a crude extract and (c) 1 µg cis-11-HDA. Bars over the traces indicate the duration of the air pulse over the antenna.

Table 8.3 EAG responses (μV) to a series of hexadecenyl acetates (1 μg) on paper for two male antennae

Compound	Antenna 1	Antenna 2
1-hexadecenyl ac.	0	0
cis-4- "	0	0
cis-6- "	0	0
cis-7- "	0	0
cis-8- "	0	0
cis-9- "	0	0
cis-10- "	0	0
cis-11- "	835	555
cis-13- "	85	0
0.2 ♀E (crude ext)	1025	385

Tungsten electrodes were used in the EAG tests with intact antennae on live moths. The electrodes were electrolytically polished to a tip diameter of 2-5 μm (Hubel, 1957) and were then protected with glass down to the tip. To do this, a 10 μl glass micropipette was placed over each tungsten electrode and sealed onto it by drawing the glass capillary down to a fine point by heating it for a few seconds in a microburner.

Moth antennae were exposed in a manner similar to that described by Payne and co-workers (1970). The EAG measurements were made by blowing air, regulated at 1.5 l/min, over paper containing one of the standard acetates. The signal from the antenna was fed into a preamplifier and displayed on an oscilloscope. The response amplitudes were measured directly from the oscilloscope.

The tests carried out with the standard acetates on the excised antennae were repeated with the intact antennae and the results were essentially the same in that, at the 1 μg level, the antennae only responded to cis-11-HDA. All the HDA's were also tested at the 10 μg level and a similar result was obtained.

The average responses to 0.001, 0.01, 0.1, 1.0, and 10.0 µg of cis-11-HDA on paper were 0, 0.01, 0.2, 0.7, and 2.0 mV respectively. Seven measurements were made for each weight of compound on each of two antennae. Thus, under the conditions used, male antennae did not respond to less than 100 ng of cis-11-HDA. Unlike the situation with the excised antennae, the EAG tests on intact antennae with 1 female equivalent of crude extract did not induce a response in the antennae. If cis-11-HDA is a natural pheromone, therefore, it is present at levels less than 100 ng per insect.

The excised antennae were more sensitive in the EAG tests than the intact antennae on live moths. No explanation can be offered for such a result. The tests on the intact antennae were probably more reliable, however. For instance, there was less variation from antenna to antenna with live moths and the responses to air alone were minimal. Large air responses (Figure 8.1) were obtained with the intact antennae and these were probably related to mechanical movements of the antennae when exposed to the air pulse. This mechanical movement of the excised antennae was a constant problem in the EAG tests and was one of the reasons for developing the alternative method with the live moths.

(f) Laboratory behavioural responses to a series of standard acetates

In the initial tests the standard acetates were tested in laboratory bioassays either as 0.1 or 1 µg on Whatman No.1 filter paper or 10 µg on the end of a glass rod. The results are shown in Table 8.4. Five of the dodecenyl acetates tested, viz., trans-7 and cis-5, -7, -9, and -11, induced flight responses in males while cis-7-dodecenyl acetate, at the 10 µg level, also induced weak copulatory attempts. Among the tetradecenyl acetates tested,

Table 8.4 Initial laboratory behavioural tests with unsaturated aliphatic acetates

Compound	Percent behavioural response
Dodecenyl acetates (DDA)	
1 µg, 1-dodecenyl ac.	0 FA, OCA
1 " , cis-3- "	60 FA, OCA
" , cis-4- "	0 FA, OCA
" , cis-6- "	0 FA, OCA
" , cis-7- "	30 FA, OCA
" , cis-8- "	0 FA, OCA
" , cis-9- "	10 FA, OCA
", 11- "	10 FA, OCA
" , trans-7- "	30 FA, OCA
" , trans-10-"	0 FA, OCA
10 µg, cis-7- "	80 FA, 10CA
Tetradecenyl acetates (TDA)	
1 µg, cis-4-tetradecenyl ac.	0 FA, OCA
" , cis-9- "	40 FA, OCA
" , trans-9- "	30 FA, OCA
" , cis-10- "	10 FA, OCA
" , cis-11- "	0 FA, OCA
" , 13- "	50 FA, OCA
0.1 µg, cis-9, trans-12-tetradecadienyl ac. (TDDA)	20 FA, OCA
" , "	50 FA, 10CA
1 µg, "	40 FA, 20CA
10 µg, "	50 GA, 40CA
Hexadecenyl acetates (HDA)	
0.1 µg, cis- 4-hexadecenyl ac.	0 FA, OCA
" , cis- 6- "	" , "
" , cis- 7- "	" , "
" , cis- 9- "	" , "
" , cis-11- "	20 FA, OCA

Table 8.4 continued on page 202

Table 8.4 continued

Compound	Percent behavioural response
Hexadecenyl acetates (HDA)	
0.1 µg, cis-13-hexadecenyl ac.	0 FA, OCA
1.0 µg, 1- "	0 FA, OCA
" , trans-2- "	0 FA, OCA
" , cis-4- "	0 FA, OCA
" , cis-6- "	" , "
" , cis-7- "	" , "
" , cis-8- "	" , "
" , cis-9- "	" , "
" , cis-10- "	" , "
" , cis-11- "	30 FA, 10CA
" , cis-13- "	0 FA, 0CA
10 µg, cis-6- "	" , "
" , cis-7- "	20 FA, OCA
" , cis-8- "	80 FA, OCA
" , cis-9- "	0 FA, OCA
" , cis-10- "	" , "
" , cis-11- "	40 FA. OCA
" , cis-13- "	0 FA, OCA
10 µg, cis-11-HDA plus 10 µg, cis, trans-9, 12-TDDA	40 FA, 20CA
"	60 FA, 30CA

cis-9-, -10; trans-9, and -13- induced flight activity while the doubly unsaturated compound, cis-9, trans-12-TDDA, induced both flight response and weak to medium copulatory attempts in males. This compound functions as a sex pheromone in the southern armyworm moth (Jacobson et al., 1970) the Indian meal moth and almond moth (Brady et al., 1971b; Kuwahara et al., 1971b)and the Mediterranean flour moth (Kuwahara et al., 1971a).

Among the hexadecenyl acetates tested cis-7, -8, and -11, induced flight responses in males. The compound, cis-11 HDA, which produced strong EAG responses did not induce copulatory attempts in males with 100 and 1000 ng on paper or 10 µg on a glass rod. However, when this compound was tested in combination with cis-9, trans-12-TDDA on separate glass rods, medium to strong copulatory activity took place with both rods. The responses obtained with these compounds at the 10 µg level were not as intense or prolonged as those obtained with some gas chromatography fractions of the crude female extract.

In all the initial behavioural tests with cis-11 HDA, 100 ng was the lowest level used and in view of the EAG result which indicated that the expected level of cis-11-HDA in each female moth (if present) would be less than 100 ng, it was decided to test cis-11-HDA in the 0.01-10 ng range. The results are shown in Table 8.5

Table 8.5 Laboratory behavioural responses to ng levels of cis-11-HDA on glass rods

Weight cis-11 HDA (ng)	Percent behavioural response
0.01	100FA, 0CA
0.1	30FA, 0CA
"	100FA, 0CA
1.0	100FA, 10CA
"	100FA, 10CA
"	100FA, 20CA
10.0	60FA, 20CA
"	100FA, 20CA
"	100FA, 40CA
"	90FA, 50CA

At the 0.01 and 0.1 ng levels, cis-11-HDA only induced flight responses in males while at the 1.0 and 10.0 ng levels medium-($<50\%$ CA) copulatory responses were obtained. When this result

was obtained all the MDA's were tested again at the 10 ng level. Two of the MDA's, viz., cis-4-, and -8-induced weak (< 20% CA) copulatory attempts in males. From the above results it appeared that, if cis-11-MDA was the natural pheromone, it was present at a level between 1 and 10 ng per insect.

(g) Column chromatography

Six hundred female abdominal tips were macerated with methylene chloride (3 x 20 ml) and centrifuged to separate the solvent from the residue. The extract was then bioassayed at the 0.1 female equivalent level and induced 20% copulatory activity and 30% flight activity in males. The concentrated extract (0.9 g) was applied to a Florisil column (40 g, 19 x 2.2 cm ID) and eluted with 200 ml of each of the following solvents: 50-60°C light petroleum; diethyl ether: light petroleum (1:6); diethyl ether: light petroleum (1:1); diethyl ether; and acetone. Sixteen fractions were collected and concentrated on a rotary evaporator but none of the fractions was active in behavioural tests. This indicated that the activity was strongly adsorbed on to the Florisil which is a very strong adsorbent. A similar result was obtained in the thin layer chromatography experiments where some of the activity was retained at the point of application and active fractions were difficult to remove from the adsorbent.

(h) Thin layer chromatography

Thin layer chromatography was carried out with a number of C_{12}-C_{16} unsaturated acetates and the crude female extract on plates coated with Kieselgel G and Kieselgel G impregnated with 30% silver nitrate (35 g Kieselgel G, 15 g silver nitrate, and 70 ml water). The latter technique has been used for resolving geometrical isomers and unsaturated aliphatic acetate homologues (Roelofs et al., 1971b).

The range of R_f values for a number of closely related acetates on a silver nitrate impregnated plate is shown in Table 8.6. The acetates were spotted in a line along the bottom of a plate (20 x 20 cm) and were eluted with diethyl ether:benzene (1:9) for an hour. After drying, the plate was sprayed with 0.2% dichlorofluorescein in ethanol and exposed to 254 nm UV light. The compounds appeared as yellow spots on a violet background.

Table 8.6 R_f values for a number of unsaturated acetates on a 3% silver nitrate - silica gel plate eluted with diethyl ether:benzene (1:9)

Compound	R_f range
cis-7-DDA ($C_{12}^{=}$)	0.47-0.53
cis-9-TDA ($C_{14}^{=}$)	0.51-0.57
trans-9-TDA ($C_{14}^{=}$)	0.59-0.65
cis-11-HDA ($C_{16}^{=}$)	0.56-0.62
cis, trans-9, 12-TDDA ($C_{14}^{2=}$)	0.40-0.45

It is obvious from this table that the geometrical isomers of 9-TDA were easily resolved and as expected the doubly unsaturated cis-9, trans-12 TDDA was more strongly adsorbed than the singly unsaturated compounds. The length of the aliphatic chain also had an influence on adsorption; the lower homologues had lower R_f values than the higher homologues.

Attempts were also made to resolve a series of HDA's with the unsaturated group at different positions along the aliphatic chain. Some of these compounds had markedly different R_f values (Table 8.7) while others, for example, cis-9 and cis-11-HDA were similar. Geometrical isomers were readily resolved, however.

Table 8.7 R_f values for a series of unsaturated HDA's on a 30% silver nitrate silica gel plate eluted with diethyl ether:benzene (1:9)

Compound	R_f range
trans-2-HDA	0.80 - 0.86
cis-4- "	0.75 - 0.80
trans-4- "	0.82 - 0.86
cis-7- "	0.57 - 0.63
cis-9-	0.64 - 0.69
cis-11- "	0.66 - 0.72

The HDA's were also spotted separately on a silica gel plate and eluted with diethyl ether:benzene (1:9) but all the compounds had R_f values in the range 0.31 - 0.32 and, therefore, could not be resolved with silica gel alone.

Thin layer chromatography was also carried out on the crude female extract with 30% silver nitrate impregnated silica gel. In the initial experiment, 2.5 female equivalents were spotted at the bottom of one half of a plate and 100 µg cis-11-HDA spotted onto the other. The plate was eluted with benzene:light petroleum (2:3) and the side containing the standard was sprayed with 0.2% dichlorofluorescein in ethanol and the spot detected with 254 nm UV light. The adsorbent containing the crude extract was divided into six areas and the fractions removed by steam distillation. The steam distillates were washed into light petroleum and bioassayed. The area on the plate within the R_f range 0.17-0.25 induced 20% copulatory activity in males. This area corresponded precisely with that of cis-11-HDA.

A further experiment was carried out with diethyl ether: benzene (1:9) as the eluting solvent. The area around the point of application induced 30% flight response in males while the area

in the R_f range 0.69-0.72 induced 50% flight response and 30% copulatory response in one set of males and 40% flight response and 10% copulatory response in a different set. The copulatory attempts were weak responses, however, which indicated that a considerable amount of activity was retained on the adsorbent.

Different methods were used to remove the fraction from the adsorbent, viz., steam distillation, and elution with diethyl ether from the adsorbent packed in pasteur pipettes. Strong copulatory attempts could not be induced with any of the fractions, however. The weakly active areas all had R_f's which were similar to those of cis-11-HDA which indicated that the natural pheromone was probably closely related to this compound.

(i) Gas chromatography

In this study the GC retention times of the sex pheromone were determined by injecting crude methylene chloride extracts on to polar and non polar columns, collecting the GC effluent at various intervals in glass U-tubes cooled to -70°C with dry ice/methanol, and determining the fractions which stimulated behavioural responses in males.

The crude extract was obtained by surface washing 500 female abdominal tips with methylene chloride (3 x 20 ml), concentrating the extract to 1 ml, and injecting 10 µl onto each column i.e. 5 female equivalents. In Table 8.8. the retention times of cis-11-HDA on a number of GC stationary phases are shown, together with those of collected fractions which stimulated copulatory and/or flight activity in male moths.

All the columns were 1.5 m glass U-tubes and were operated isothermally with a nitrogen carrier gas flow rate of about 40 ml/min. On all the columns the cis-11-HDA eluted over a period of a few

Table 8.8 Retention times of the pheromone activity of the crude female extract and cis-11-HDA on polar and non polar stationary phases

GC conditions	Retention times (mins)		Percent behavioural response to GC fractions
	cis-11-HDA	GC fraction	
1. 10% OV-1, 160°C	28(26-30)	6-14 24-41*	20CA, 40FA 40CA, 100FA
2. 10% OV-1, 170°C	17(15.5-18.5)	10-14* 14-18* 22-26	60CA, 100FA 40CA, 100FA 50FA
3. 10% OV-1, 170°C	17(15.5-18.5)	13-15* 15-17* 17-19	20CA, 50FA 50CA, 100FA 20CA, 10FA
4. 10% Atlox G-1292, 200°C	21(20-22.5)	14-18* 18-22	30CA, 100FA 100FA
5. 10% HI-Eff 8BP, 150°C	16.8(15.5-20)	12-16 16-20* 20-24	20CA, 40FA 20CA, 50FA 40CA, 100FA
6. 10% HI-Eff 8BP, 150°C	16.8(15.5-20)	18-22* 22-26* 26-30	30CA, 60FA 30CA, 50FA 50FA
7. 3% HI-Eff 8BP, 180°C	5.5(4.5-6.5)	4-8* 10-12 12-14	50CA, 100FA 20CA, 40FA 50FA

* indicates the fractions which stimulated the most intense sexual responses in males

minutes and [illegible] is indicated by the figures in the brackets in Table 8.8.

On the non polar 10% OV-1 column, operated at 160°C and 170°C, the fractions of the crude female extract which induced the strongest copulatory activity in males corresponded in retention times to those of cis-11-HDA. Other fractions, collected at shorter and longer retention times, also induced copulatory attempts and/or flight responses in males. This indicated that a multiple component pheromone system was used by P.separata females.

A similar situation existed for the polar HI-Eff columns. On the other hand, the active fraction collected from the polar 10% Atlox G 1292 column was a few minutes shorter than that of cis-11-HDA. On all the columns, with the exception of 10% Atlox G 1292 at 200°C and 3% HI-Eff 8BP at 150°C, copulatory activity was induced with more than one fraction. The most potent fractions were obtained from the HI-Eff 8BP (polyester) columns which suggested a higher degree of purification was achieved with this stationary phase.

Gas chromatograms of a crude female extract (ca. 10♀E) on 10% HI-Eff 8BP on 100-120 mesh Embacel at 170°C (Nitrogen flow = 40 ml/min) and 10% SE-30 on 100-120 mesh silanized Embacel at 200°C (Nitrogen flow=40 ml/min) are shown in Plates 8.5 and 8.6 respectively. Similar chromatograms were obtained on both columns. On the polar HI-Eff column cis-11-HDA eluted between 13.6-15.2 min with a peak maximum at 14.8 min. This corresponds with peak A, in Plate 8.5, which had a retention time of 15.1 min. The corresponding peak on the 10% SE-30 column had a retention time of 12.8 min which was 1 min less than that of cis-11-HDA. On the addition of 100 ng cis-11-HDA to the crude female extract, peak A and cis-11-HDA could not be resolved on the 10% SE-30 column despite the fact that this column had an

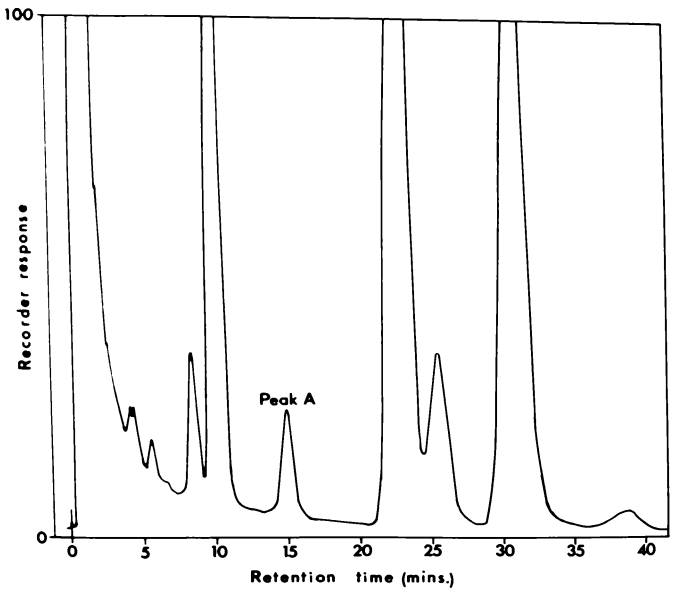


Plate 8.5 Gas chromatogram of 10 female equivalents of a crude extract on 10% HI-Eff 8BP at 170°C (N_2 = 40 ml/min)

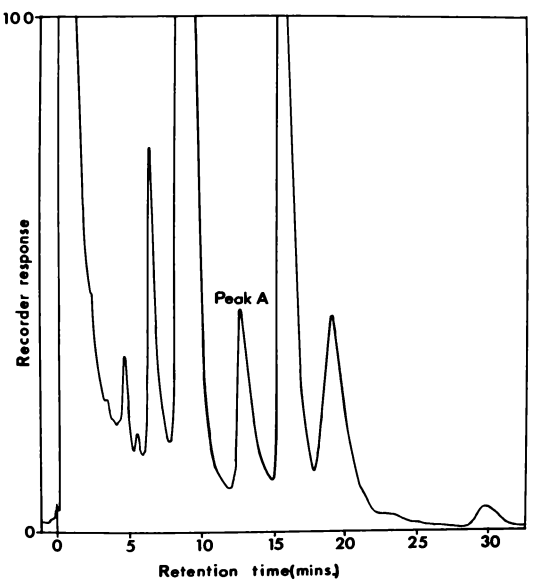


Plate 8.6 Gas chromatogram of 10 female equivalents of a crude extract on 10% SE-30 at 200°C (N_2 = 40 ml/min).

efficiency of 2400 theoretical plates. The size of peak A indicated that about 100 ng was present in each female and this was at least an order of magnitude higher than the level of cis-11-HDA expected in each female from the EAG and behavioural tests. It did appear, therefore, that peak A mainly represented a co-extracted impurity.

Attempts were made to separate a series of hexadecenyl acetates on both the 10% HI-Eff-8BP column at 180°C and the 10% SE-30 column at 200°C. The results are shown in Table 8.9.

Table 8.9 Retention times of a number of cis unsaturated hexadecenyl acetates on polar and non-polar stationary phases

Position of unsaturation	10% HI-Eff 8BP on 100-120 mesh Embacel at 180°C, N_2 = 35 ml/min	10% SE-30 on 100-120 mesh Embacel at 200°C N_2 = 40 ml/min
4	14.5	13.4
6	18.0	12.7
7	14.0	12.0
8	19.4	12.6
9	14.4	12.2
10	15.0	12.3
11	14.7	12.7
13	19.8	14.1

On the 10% HI-Eff 8BP column at 180°C it was not possible to separate cis-4-, -7-, -9-, -10 and -11- hexadecenyl acetates from one another. All these compounds had retention times around 14-15 min and were well separated from cis-6-,8-,and -13- hexadecenyl acetates which had retention times around 18-20 min. The efficiency of this column was approximately 2000 theoretical plates.

With the exception of cis-4- and -13- hexadecenyl acetates, which had retention times between 13 and 14 mins, all the other hexadecenyl acetates injected onto the non polar 10% SE-30 column

had retention times in the 12-13 min range.

(j) Mass fragmentography and microozonolysis

It appeared from gas chromatography experiments that one of the natural sex pheromone components had retention times similar to those of cis-11-HDA on a number of polar and non polar stationary phases. An attempt was made, therefore, to detect the presence of cis-11-HDA in the crude female extract using the technique of mass fragmentography combined with microozonolysis on a fraction of the crude female extract collected from a GC column at the expected retention time of cis-11-HDA.

In the technique of mass fragmentography (discussed in Section 2.11) the mass spectrometer is used as a specific GC detector by focussing it on an ion which is characteristic of the compound concerned. When the mass spectrometer is used in this manner, known compounds can be detected at the picogram level.

The mass spectrum of cis-11-HDA is shown in Figure 8.2. The molecular ion at m/e 282 is not present and the parent ion at m/e 222 (282-60) results from the loss of an acetate group. The relative intensity of the ions at m/e 222 and 223 is characteristic of hexadecenyl acetates. A further characteristic ion of the acetates is protonated acetic acid at m/e 61.

The mass spectrum of cis-11-HDA was very similar to that of any C_{16} olefin apart from the peak at m/e 61. Mass spectral analysis does not distinguish the double bond position in an aliphatic chain, however, due to the migration of the double bond upon ionization.

The mass spectrometer was focussed at m/e 222 and cis-11-HDA injected onto a 1.0 m column of 1% OV-101 operated isothermally at 180°C. The gas chromatograph was coupled to a Varian Mat CH-5 mass spectrometer through a Watson-Biemann separator and the retention

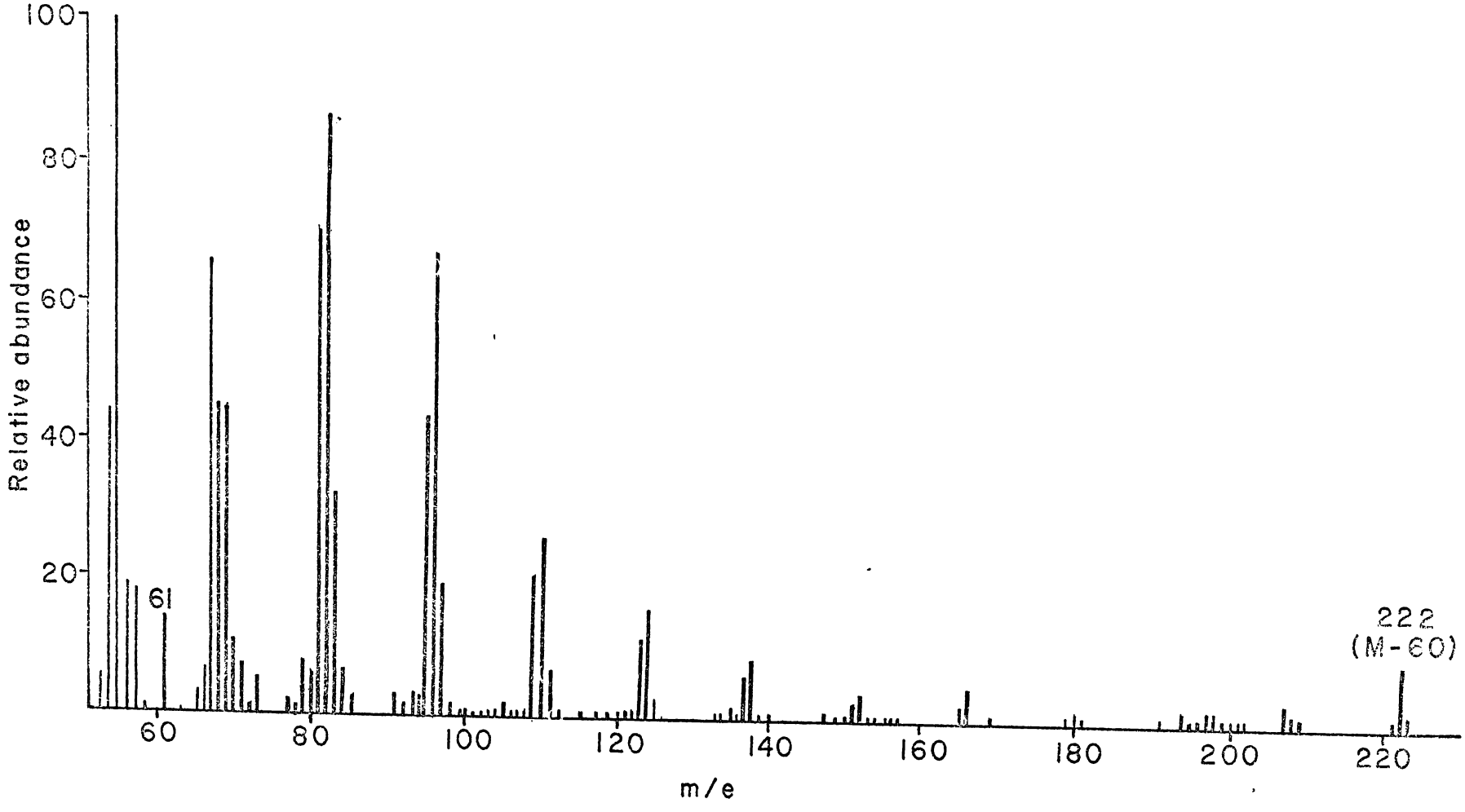


Figure 8.2 Mass spectrum of cis-11-hexadecenyl acetate.

time of cis-11-... on the column, as recorded by the ion monitor, varied between ... and 2.6 min. Using this technique, it was possible to detect as little as 100 picograms of cis-11-HDA.

A crude female extract was prepared by surface washing 100 female abdominal tips in 10 ml methylene chloride and 1-2 µl of this extract (i.e. 0.01 ♀E) was injected into the gas chromatograph. The mass spectrometer monitored ions of m/e 222 at retention times which varied between 2.5-2.6 mins. These retention times were consistent with that of cis-11-HDA (Figure 8.3). Single ion monitoring of the crude female extract injected into the GC was also carried out at m/e 221 and 223. The relative intensities of these ions (detected at a retention time of 2.5 min) compared to that of m/e 222 were the same as in the mass spectrum of cis-11-HDA. It was important to carry out the relative intensity measurements at m/e 221 and 223 because a higher alkane with a retention time similar to that of cis-11-HDA could have given a group of ions at m/e 221, 222 and 223. In the case of the alkane, however, there would be substantially more ionisation at 221 and 223 than at 222. A similar result was obtained with the mass spectrometer focussed at m/e 61 (Figure 8.4). The relative intensities of the ions at m/e 60 and 62 were low as in the mass spectrum of cis-11-HDA.

The 2 µl injections of crude female extract contained 0.01 ♀E and based on the response of the ion monitor to a series of cis-11-HDA standards, there was about 10 ng of an hexadecenyl acetate in each female moth. A crude extract (2 µl) of male moths (10 males/ml) was injected onto the GC column but no peaks at the retention time of cis-11-HDA were observed for m/e 222 or 61 (Figures 8.3 and 8.4)

The next step was to determine whether the position of the unsaturated group in the aliphatic backbone was at C_{11} as in cis-11-HDA.

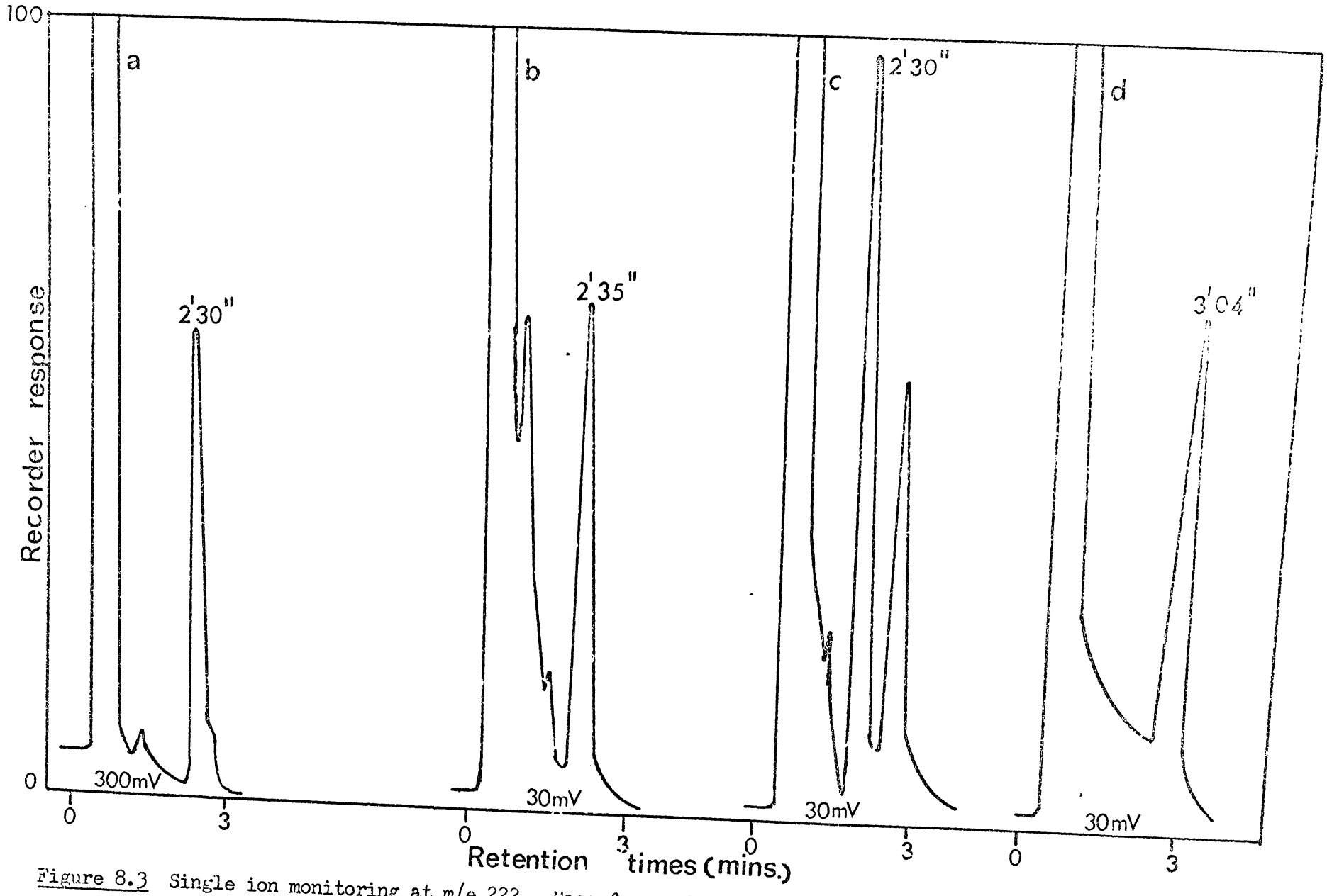


Figure 8.3 Single ion monitoring at m/e 222. Mass fragmentograms of (a) 1 ng cis-11-HDA, (b) 0.1 ng cis-11-HDA, (c) 2 µl crude extract containing 10 female abdominal tips/ml, (d) 2 µl crude extract containing 10 male abdominal tips/ml.

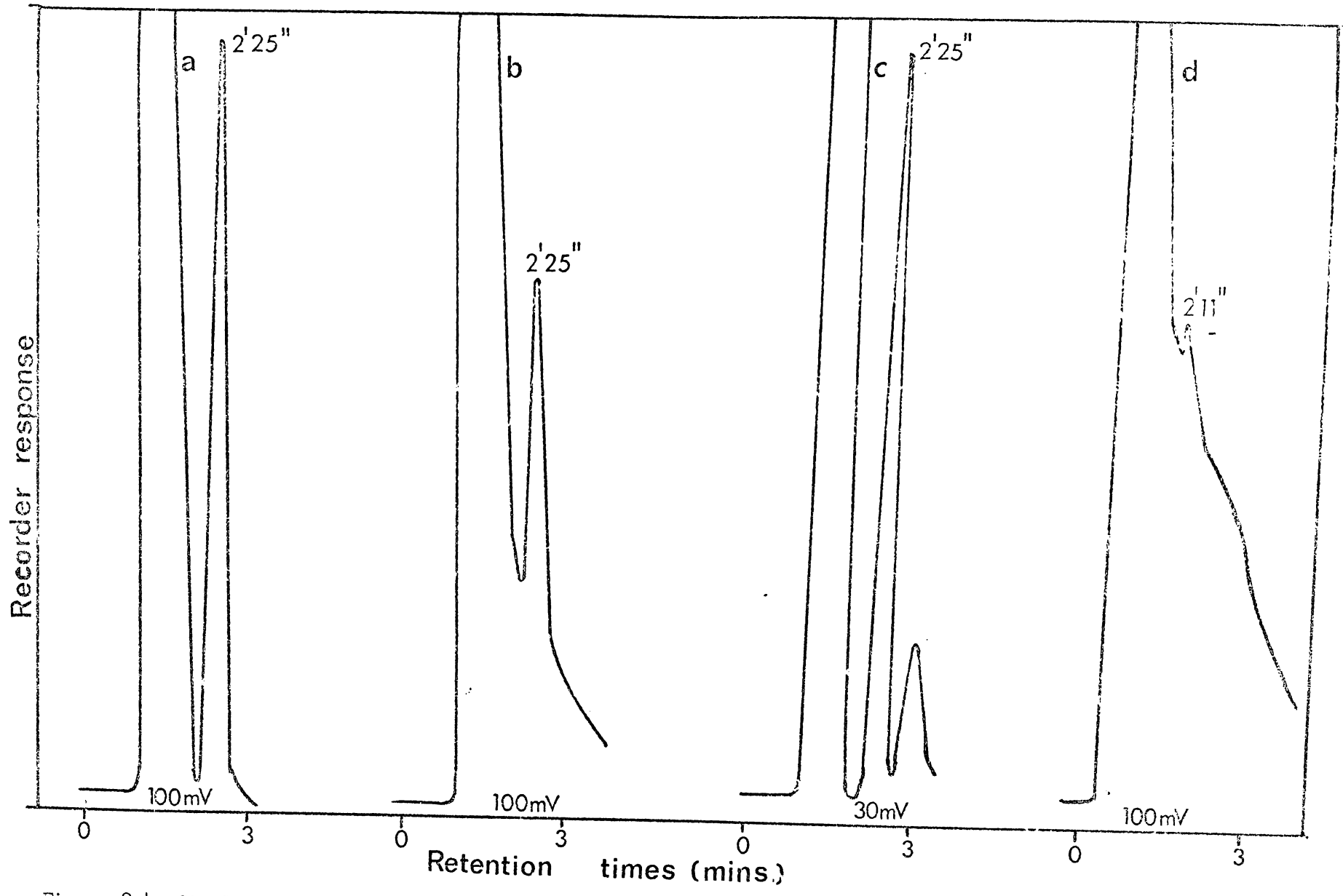


Figure 8.4 Single ion monitoring at m/e 61. Mass fragmentograms of (a) 1 ng cis-11-HDA, (b) 0.02 ng cis-11-HDA, (c) 2 µl crude extract containing 10 female abdominal tips/ml and (d) 2 µl crude extract containing 10 male abdominal tips/ml

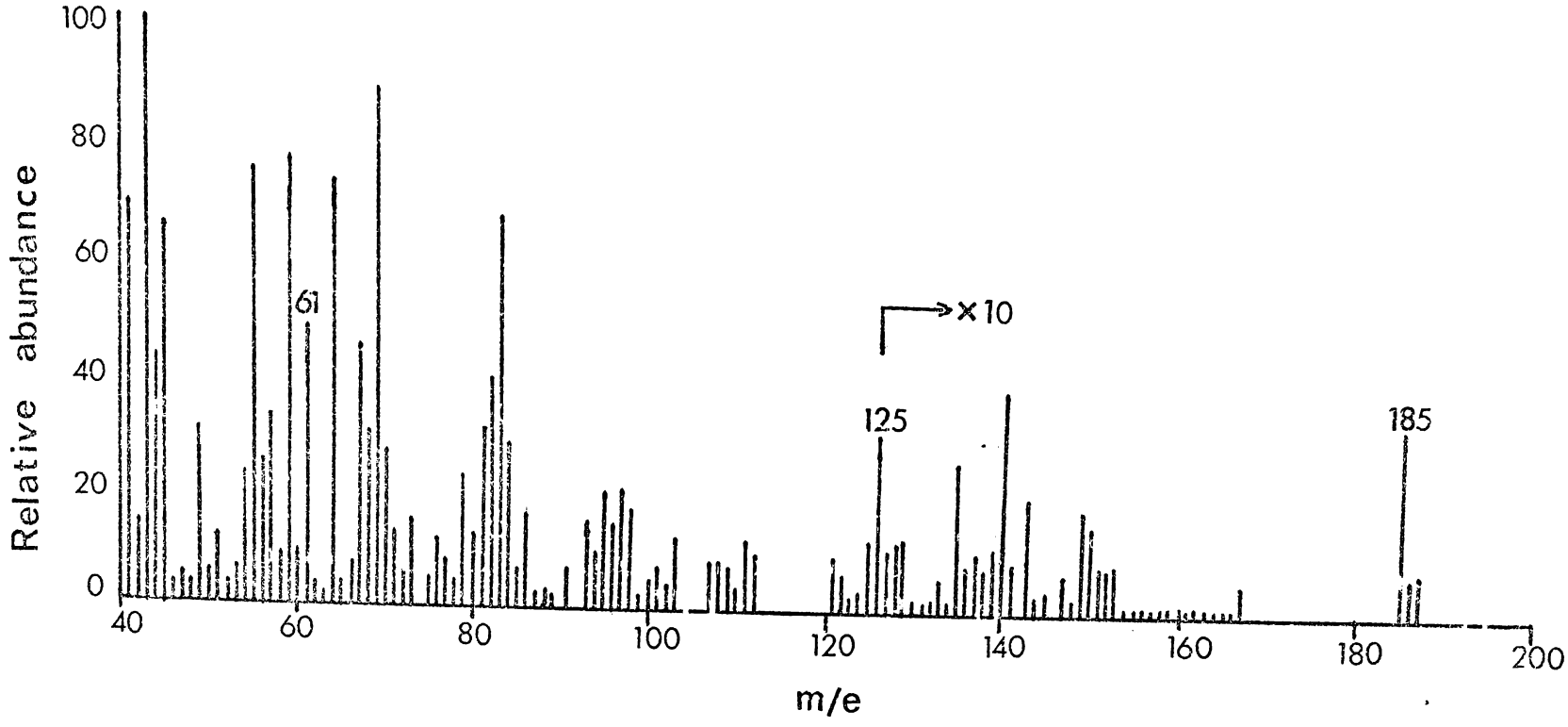


Figure 8.5 Mass spectrum of 11-acetoxyundecanal.

The ozonolysis products of cis-11-HDA are pentanal and 11-acetoxyundecanal. The mass spectrum of the latter compound is shown in Figure 8.5. The molecular ion at m/e 228 is not present. The parent ion at m/e 185 (228-43) is the result of β-cleavage of the aldehyde group (i.e. CH_2CHO) of the molecular ion. Other characteristic ions occur at m/e 125 (185-60, loss of acetate group), and 61.

Microozonolysis of cis-11-HDA was carried out in methylene chloride using essentially the method of Beroza and Bierl (1967). The ozonide was degraded with tetracyanoethylene (1 mg), rather than triphenyl phosphine, as no interference peaks were obtained in the gas chromatograms with the former compound. With 1.0 ppm solution of cis-11-HDA in methylene chloride, approximately 30% yield of 11-acetoxyundecanal was obtained on microozonolysis.

The crude female extract (5 x 10 µl of 10♀E/ml) was injected onto a 10% HI-Eff 8BP column operated at 225°C, and the fraction eluting between 4-6 min was trapped in a glass U-tube at room temperature (the trap design was discussed in Section 5.2h). This column was selected because large quantities of co-extracted impurities were retained at the column inlet. The retention time of cis-11-HDA under the above conditions was 4.5 min. It was also found that 10 ng quantities of cis-11-HDA injected onto the column could be recovered quantitatively with the trapping technique used.

The combined collected fractions were then dissolved in 50 µl methylene chloride and ozonized. A 3 µl sample of the ozonized solution was injected onto a 1% OV-101 column held at 160°C in a gas chromatograph which was coupled to the mass spectrometer. The mass spectrometer focussed at m/e 185 did not detect a compound at a retention time corresponding to that of 11-acetoxyundecanal. Thus 11-acetoxy-

undecanal was not generated from the collected GC fraction of the crude female extract and it appears that cis-11-HDA is not present in the female moth at the level suspected. This was an extremely disappointing result.

(k) Field tests with cis-9, trans-12-tetradecadienyl acetate and cis-11-hexadecenyl acetate used alone and in combination

Field tests were carried out within a maize crop with a reasonably low infestation of armyworm moths for the crop had been sprayed with insecticide two weeks before the tests. Both cis-9, trans-12-TDDA and cis-11-HDA were tested alone and in combination. Two microlitres of each chemical was injected into polythene caps (Roelofs and Comeau, 1970) and onto rubber septa (Roelofs et al., 1971b). The caps or septa were placed in traps made from 1 pint pots lined on the inside with glue ("Stikem"). The traps were suspended from maize plants about 2 m above ground level and five replicates of each treatment were used for 9 days in the field.

The activity of the attractant formulations was compared with that of virgin females which were held in groups of three in traps. These traps were constructed from 2 pieces of plastic pipe (6 x 10 cm dia.) lined on the inside with "Stikem" and joined to an inner chamber (2.5 x 10 cm dia.). Female moths were held within this chamber between gauze partitions and were fed with 8% sucrose solution which was held in a jar containing a wick. An illustration of the trap is shown in Plate 8.7.

The results of the field tests are shown in Table 8.10.

An average of 5 males per trap was caught with cis-11-HDA used alone on rubber septa, and this compared favourably with the 5 males trapped per virgin female over the 9-day period. None of the other attractant formulations was effective. Indeed, contrary to expectations cis-9, trans-12-TDDA appeared to inhibit response in the field

Plate 8.7 A moth trap baited with three virgin female armyworm moths.

Table 8.10 Male moth catches in a 9-day period with virgin females, cis-11-HDA and cis, trans-9,12-TDDA used alone and in combination

Compound	Dispenser	Total males attracted
cis-11-HDA	rubber septs	26
"	polythene caps	2
cis,trans,-9,12-TDDA	rubber septa	0
"	polythene caps	0
cis-11-HDA + cis,trans,-9,12-TDDA	rubber septa	1
" + "	polythene caps	0
30 virgin females, replaced once		152

to cis-11-HDA. Phenomena of this nature have been observed by other workers (Carde et al., 1973; Berisford and Brady, 1973) and have been discussed in Section 2.7.

8.3 Discussion

Normally in the identification of insect sex pheromones, insect numbers in the order of ten to a hundred thousand have been required. Recent studies have shown, however, that reasonable progress can be made with fewer insect numbers by carrying out simple tests on crude extracts to reveal the presence or absence of functional groups (Bierl et al., 1972; Meijer et al., 1972) and by comparing gas chromatograph-electroantennogram data of the natural pheromone with that of synthetic standards (Roelofs et al., 1971b).

These approaches were used in this study of the common armyworm sex pheromone and use was also made of a combined gas chromatograph-mass spectrometer. The mass spectrometer was used as a specific GC detector and in this form it can detect known compounds at the picogram level. This is apparently the first time this technique has been applied to insect sex pheromone studies. Obviously such methods are only applicable in cases where the properties of the sex pheromone

are consistent with a known compound.

The usefulness of the technique was demonstrated in this study where approximately 10 ng of cis-11-HDA was suspected to be present in each female moth. This meant that only 0.01 female equivalents of the crude extract was injected onto the GC column at any one time. The ability to detect extremely low levels of a compound was particularly important in this study because of the difficulty in rearing large quantities of female moths.

With the single ion monitoring technique, problems did occur with interference peaks from GC phases, solvents, etc., and care had to be taken to select and purify suitable materials to obviate this problem.

Many sex pheromones within the Lepidoptera have been identified as unsaturated aliphatic acetates and, in general, one or two sex pheromones have been reported for each species. Recently, George and McDonough (1972) detected seven sex pheromones in GC fractions of extracts of both the female codling moth, Laspeyresia pomonella and the zebra caterpillar, Ceramica picta. All the pheromones induced copulatory activity in males. A multiple pheromone system also appeared to be used by the female armyworm moth, for a number of GC fractions from the one column induced both copulatory and flight responses in male moths.

The armyworm moth belongs to the subfamily Hadeninae and Roelofs and Comeau (1971) discovered that cis-11-HDA attracted five species of moths in this subfamily. It was not altogether surprising, therefore, to find that the same compound induced copulatory activity in male P.separata moths in laboratory bioassays. Furthermore, cis-11-HDA attracted male armyworm moths in the field but it was not as effective as virgin females.

Of the ten hexadecenyl acetates available for laboratory screening purposes, only cis-11-HDA induced strong EAG responses in male

antennae. There appeared to be a very good chance, therefore, that this compound was probably one of the natural pheromones used by the female moth. The initial experiments using the single ion monitoring technique confirmed the presence of an hexadecenyl acetate in a crude female extract. Subsequently ozonolysis of a GC fraction of the crude female extract was carried out in an attempt to determine the position of the unsaturated group along the aliphatic backbone. The ozonolysis product of cis-11-HDA is 11-acetoxyundecanal and no evidence was found for the presence of this compound. The failure of this experiment may have been associated with the impurities in the collected GC fraction which could have interfered with the microozonolysis technique. All the evidence prior to the ozonolysis experiment suggested that one of the sex pheromone components was cis-11-HDA. On the basis of the ozonolysis result, however, it appears that cis-11-HDA may not be a natural sex pheromone but a "parapheromone".

The hexadecenyl acetates which were not available for screening included: 3-, 5-, 12-, 14-, and 15- hexadecenyl acetates. Perhaps one of these will prove to be one of the natural sex pheromones.

8.4 Conclusions

The female armyworm moth was shown to produce a sex pheromone about 5-6 days after final ecdysis. Likewise male moths did not respond to the pheromone until they were at least 5-6 days old.

Functional group tests on crude female extracts, together with the behaviour of the sex pheromone in thin layer and gas chromatography, indicated that the properties of one of the sex pheromone components were consistent with those of cis-11-hexadecenyl acetate. Electroantennogram tests were carried out with a series of dodecenyl, tetradecenyl, and hexadecenyl acetates and only cis-11-hexadecenyl acetate induced strong antennal responses in male moths. Significant

antennal responses were not induced in female antennae with any of the acetates, however.

In the laboratory behavioural studies with the standards only cis-7-dodecenyl acetate, cis-9,trans-12-tetradecadienyl acetate, cis-4-, cis-8- and cis-11-hexadecenyl acetates induced copulatory activity in males. Intense sexual activity was induced with nanogram quantities of the latter compound.

The technique of mass fragmentography was used in an attempt to confirm the presence of cis-11-hexadecenyl acetate in female moths at a level of about 10 ng per insect. Females did not appear to contain this compound, however. A number of fractions collected from different GC columns induced both copulatory activity and flight responses. It appears, therefore, that the female uses a multiple component pheromone system.

Males were attracted to cis-11-hexadecenyl acetate in the field. Large numbers of moths were not caught in the traps, however. When cis-11-hexadecenyl acetate was combined with cis-9,trans-12-tetradecadienyl acetate and used in field traps, the latter compound acted as an inhibitor. This combination of acetates had previously been shown to stimulate copulatory attempts in males in laboratory behavioural tests.

CHAPTER 9

CONCLUSIONS

The female grass grub beetle, Costelytra zealandica (White) (Coleoptera: Scarabaeidae), is shown to contain a chemical sex pheromone which stimulates mating behaviour in males in the laboratory and the field. The sex pheromone was extracted by surface rinsing female abdominal tips with diethyl ether and the various concentration steps were monitored with a simple laboratory bioassay based on male copulatory attempts with treated paraffin dummies. The sex pheromone was isolated by high vacuum distillation of the ethereal extract followed by gas liquid chromatography. Identification of phenol as the active component was deduced by the properties exhibited in functional group tests, thin layer, paper, and gas chromatography. The structure was confirmed by high resolution mass spectrometry.

The phenol content of virgin female beetles varied with age and reached a maximum of 1.5 µg per female at 8-10 days after final ecdysis. Phenol applied to dummies at 0.1, 1.0, and 10.0 µg respectively, stimulated copulatory activity in males. Phenol released at low rates in the field attracted male beetles in mating flights from distances of 10-30 m.

The electroantennogram was used to compare the activity of phenol with closely related phenols. These compounds had no masking effect on the antennal response. A comparison in activity between phenol and a crude female extract, indicated phenol as the sole pheromone.

Various phenol formulations and phenol derivatives were prepared to extend the activity of the pheromone under field conditions. Phenol encapsulated in PVC tube retained its activity for the duration

of the beetle flights of 3-4 weeks. Continuous phenol wick traps were superior to virgin females in attracting males but were about 5-10 times less effective than Durez 12687 paste.

The fact that phenol is cheap and readily available provided a unique opportunity to evaluate this pheromone for grass grub control. Field trials based on mass trapping and male confusion methods with beetle populations ranging from 50-150 beetles per square metre indicated that phenol was of little use as a direct behavioural agent for grass grub control. In the former method over a quarter of a million male beetles were trapped but, as the attractant did not successfully compete with females in the natural population, these numbers were insufficient to reduce the resultant larval population. In the later method, male flight behaviour over the 0.25 ha areas was disoriented by releasing phenol at 28 g/h but effective mating was not prevented, presumably due to the high density of beetles present which enabled males to locate females either by sight at short range or simply chance.

Sex pheromone studies were also carried out with the armyworm moth, Pseudaletia separata, and intense sexual excitement was induced in males in laboratory bioassays with female extracts. Female moths did not produce the sex pheromone until about 5-6 days after final ecdysis and males needed to be about 5-6 days old before they gave the full sexual response.

Functional group tests indicated the sex pheromone to be an unsaturated aliphatic acetate and the properties of the sex pheromone in thin layer and gas chromatography were similar to those of cis-11-hexadecenyl acetate. Thirty one unsaturated acetates with an aliphatic chain length of 12, 14 and 16 carbons were tested for laboratory sex stimulatory activity and EAG responses. Strong EAG responses were only obtained with cis-11-hexadecenyl acetate. In the laboratory behavioural

tests, intense sexual activity was induced in males with either 1 or 10 ng cis-11-hexadecenyl acetate on glass rods. Weak to medium sexual responses were also induced with cis-7-dodecenyl acetate, cis-9, trans-12-tetradecadienyl acetate, cis-4-, and cis-8-hexadecenyl acetates.

Both cis-11-hexadecenyl acetate and cis-9, trans-12-tetradecadienyl acetate were field tested alone and in combination. The former compound when tested by itself, attracted male moths into traps but, contrary to expectations, moth response was inhibited when the latter compound was combined with it.

Using the technique of mass fragmentography in which a mass spectrometer acts as a specific GC detector, it was possible to detect 100 pg of cis-11-hexadecenyl acetate. This compound was not detected in extracts of female moths, however. The female armyworm moth appears to use a multiple component pheromone system.

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APPENDIX I

Lepidopterous sex pheromones found by classical techniques

Compound	Insect	Reference
cis-7-dodecenyl acetate	Trichoplusia ni cabbage looper moth	Berger (1966).
trans-7-dodecenyl acetate	Argyroploce leucotreta false codling moth	Read et al., (1968).
cis-8-dodecenyl acetate	Grapholitha molesta oriental fruit moth	Roelofs et al., (1969).
cis-9-dodecenyl acetate	Paralobesia viteana grape berry moth	Roelofs et al., (1971b)
cis-9-tetradecenyl acetate	Spodoptera eridania southern armyworm moth	Jacobson et al., (1970a)
"	Adoxophyses orana summer fruit tortrix moth	Meijer et al., (1972).
cis-9,trans-12-tetradecadienyl acetate	S.eridana	Jacobson et al., (1970a)
"	Plodia interpunctella Indian meal moth	(Brady et al., (1971b) (Dahm et al., (1971)
"	Cadra cautella almond moth	Kuwahara et al., (1971a)
"	Ephestia kuehniella Mediterranean flour moth	Brady et al., (1971a).
cis-11-tetradecenyl acetate	Argyrotaenia velutinana redbanded leafroller moth	Roelofs and Arn (1968).

Appendix I continued

Compound	Insect	Reference
cis-11-tetradecenyl acetate	Choristoneura rosaceanna obliquebanded leafroller moth	Roelofs and Tette (1970)
"	Ostrinia nubilalis European corn borer moth	Klun and Brindley (1970)
"	A.orana	Meijer et al., (1972)
trans-10,cis-12-hexadecadien-1-ol	Bombyx mori silkworm moth	Butenandt et al., (1959)
undecanal	Galleria mellonella greater wax moth	Roller et al., (1968)
cis-5-decenyl-2-methyl butanoate	Nudaurelia cythera cythera pine emperor moth	Henderson et al., (1973).
cis-7,8 epoxy-2-methyl octadecane	Porthetria dispar (L.), gypsy moth	Bierl et al., (1970).
2-methylheptadecane	Holomelina aurantiaca (Hbn.) complex, various tiger moths	Roelofs and Carde (1971).
2-cis,6-trans-7-methyl-3-propyl-2,6-decadien-1-ol	Laspeyresia pomonella (L.) codling moth	McDonough et al., (1972).