BUREAU OF SUGAR EXPERIMENT STATIONS

QUEENSLAND, AUSTRALIA

FINAL REPORT

SRDC PROJECT BS17S

ASSESSMENT OF THE POTENTIAL OF SEX PHEROMONES AS STRATEGIC LURES FOR THE CONTROL OF CANEGRUBS

by

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SUMMARY

Sex pheromones were shown to be emitted by females of *Antitrogus consanguineus*, *A. parvulus* and *L. picticollis*. Attraction of males of *L. picticollis* to females of *A. consanguineus* shows that the compound or compounds involved are similar in those two species. Detection of pheromones was probably related to the morphology of the adult antennae. One compound was present in emissions of adult female *L. negatoria*, but this compound could not be identified.

1.0 INTRODUCTION

Sixteen species of melolonthine canegrubs are endemic to eastern Australia, where the larvae feed on the roots of grasses (Allsopp and Chandler, 1989). They are important pests of sugarcane, destroying roots and thus depriving the plant of moisture, nutrients and mechanical support (Allsopp and Hitchcock, 1987). Since 1982, canegrubs have been responsible for an average annual loss of cane valued at $2.4M and growers have treated an annual average of 31 600 ha with insecticides at a cost of $2.4M. The cost of insecticide treatment for canegrub control has increased significantly over the last 7 years with the replacement of low-cost organochlorines by more expensive controlled-release products. However, without insecticides, the level of losses would rise dramatically. The Australian sugar industry is now almost completely dependent on controlled-release chlorpyrifos (SuSCon Blue) for canegrub control. Alternative control systems must be developed.

Sex pheromones are substances released by animals which affect the behaviour of individuals of the same species. They can attract members of the opposite sex from several kilometres in some species or over only short distances in other species. Sex pheromones have been identified in two scarab species, Japanese beetle (McGovern *et al*, 1973) and New Zealand grassgrub (Henzell and Lowe, 1970), and are known to occur in other scarabs (Soo Hoo and Roberts, 1965; Lilly and Shorthouse, 1971; Domek and Johnson, 1988).

Such attraction offers potential for reducing populations of adult beetles by trapping or, by indicating flight periods, allowing better timing of applications of knockdown chemicals such as Mocap. Additional benefit could be obtained by incorporating a suitable insecticide into the attractant. After contact, this would kill not only the attracted beetle but, if correctly dosed, contaminate and kill any subsequent sex partner. If such chemicals can be identified, they could be used, either on their own or mixed with other attractants (Ladd *et al*, 1981), to reduce insecticide usage for canegrub control by eliminating adults before they complete oviposition.

There is considerable interest in the community in development of pest management strategies which involve environmentally 'soft' pesticides. The development of sex pheromones as a control strategy for canegrubs would be environmentally acceptable and would provide an alternative to persistent pesticides.

The main objectives of this research project were:

1. to detect and identify the female sex pheromone produced by cane beetles.

2. to investigate the use of pheromones to disrupt reproduction of cane beetles.

To achieve the first objective it was necessary to demonstrate the presence of pheromones in the field. Additional evidence of the involvement of pheromones in beetle behaviour was obtained from a study of antennal morphology.

In the laboratory, equipment was assembled to enable the analysis of samples of air from around cane beetles, collected in the field and from laboratory-reared insects.

2.0 RESEARCH METHODS

2.1 Demonstration of pheromone presence

The presence of pheromones was demonstrated by determining the attractancy of unmated males and females of *Antitrogus consanguineus* (Blackburn), *A. parvulus* Britton, *Lepidiota negatoria* Blackburn and *L. picticollis* Lea. Adults were exposed in individual Catch-can Japanese beetle traps (Trécé Inc., Salinas) during the flight period of the target species. Traps were hung 1 m above the ground on steel rods and placed 10 m apart in randomised block designs. Each block included an unbaited trap. Unmated adults were reared in individual containers in the laboratory from field-collected larvae. Adults were confined in perforated containers placed in the same position in the trap as the standard Trécé lure. Catches were counted three days after the initial exposure.

Experiment 1 tested *A. consanguineus* from 17-20 September 1989. Experiment 2 tested *A. parvulus* from 7-10 November 1989. Experiment 3 tested *A. consanguineus* and *L. picticollis* from 14-17 September 1990. Experiment 4 tested *L. negatoria* from 22-29 December 1990. Experiment 3 used three replicates, while other experiments used five replicates.

As none of the raw data sets were normally distributed ($P < 0.01$, Shapiro-Wilk test for normality) and were not normalised by transformation, they were analysed using the non-parametric Friedman two-way analysis of variance (Conover, 1980). All analyses used STATISTIX 3.1 (Analytical Software, 1989).

2.2 Antennal morphology

Differences in the behaviour of canegrub adults are likely to be reflected in their antennal morphology. To assess this, antennae of both sexes of *Antitrogus parvulus* and *Lepidiota negatoria* were examined using scanning electron microscopy.

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2.3 Cane beetles

Air from around the following beetle species was sampled and analysed in this project:

Antitrogus consanguineus, *A. parvulus* (both Bundaberg and Childers strains), *Lepidiota negatoria*, *L. picticollis*, *L. frenchi* Blackburn, and *Dermolepida albohirtum* (Waterhouse).

Laboratory-reared beetles were obtained from larvae collected in the field at Bundaberg and Meringa and from other research projects at the BSES laboratories in Brisbane. They were kept in moist soil in screw-topped glass jars and fed once a month with pieces of cane stalk. Once the larvae began to pupate they were disturbed as little as possible. As the pupae became more beetle-like they were monitored closely so that the beetles were obtained as they were just emerging. For some beetle species this is the time of greatest pheromone release.

2.4 Adsorbent traps, sampling and analysis

The adsorbent used was Tenax TA, a porous polymer material, based on 2,6-diphenyl-p-phenylene oxide, 20-35 mesh (Alltech Australia Pty Ltd). The unconditioned Tenax was packed into a glass column and connected to a vacuum pump via a Büchner flask. The bed of Tenax was washed by drawing methanol and hexane through the tube, prior to conditioning as recommended by the manufacturer. The conditioned Tenax was transferred to a clean, screw-capped test-tube with a Teflon-lined cap.

Traps were prepared by packing 0.05 to 0.10 g of conditioned Tenax into Pyrex glass tubing, 5 to 7 cm long, 6 mm diameter. The adsorbent was held in place with a disc of Nytrel-Ti (Polyamide 66) cloth and a disc of stainless steel mesh, placed at both ends of the Tenax bed. The ends of the trap tube were closed with plastic caps and each trap stored in a screw-capped test-tube. Exposed traps were stored in a freezer and forwarded to the Brisbane laboratory in dry ice. In Brisbane, traps were stored in a freezer at -70°C.

For air sampling in the laboratory, beetles were placed in glass or plastic containers and provided with wire structures on which to climb and call. Air was drawn through the system using a water vacuum pump or an electric pump. A portable pump, Gilian Air Sampling System Model # 513AUP-DK (Selby-Anax), was used in the field. An adsorbent trap, containing Tenax, was connected in the airflow path between the enclosure and the pump.

For sampling, the following combinations were used:

- males and females in one enclosure, mixed
- females only
- males only
- males and females in one enclosure but separated from each other by a wire mesh screen

Samples collected included calling females and mating pairs. Field samplings were carried out at Bundaberg and Meringa.

Several organic solvents were tested for the extraction of adsorbed substances from the Tenax, including hexane, acetone, ethanol, heptane and diethyl ether. Hexane was found to be the most suitable. Problems were encountered with impurities where hexane concentration was required, so a rotary-film evaporator was used to distil the hexane.

Several extraction methods were tested, involving use of an ultrasonic bath, use of a vibrating spatula or a Vibramix, vigorous shaking and a Soxhlet-type extraction.

Where a micro-Soxhlet system (Quick cat. no. X/14 MU) was used, Tenax from the exposed trap was transferred to the sample holder and 10 mL of hexane placed in the receiving tube. Extraction was carried out for 24 hours using an electric heating mantle.

The hexane extract was concentrated using the rotary-film evaporator. Extracts were stored in glass vials with Teflon-lined caps in a freezer at -70°C prior to the analysis.

In the laboratory, much of the first year of the project, up to early October 1989, was occupied with setting up the analytical equipment, developing methods for sampling the air around beetles, and developing analytical methods. Initially, an existing Hewlett-Packard 5830A gas chromatograph, fitted with dual flame ionisation detectors, was converted to capillary column operation using a SGE Unijector kit (code no. UNIK10). The instrument was also modified by fitting a heated exit port and a liquid nitrogen cold-trap, to obtain samples of compounds separated by the chromatography column. This proved to be extremely difficult to set up and the problem was not helped when the gas chromatograph became inoperable in August 1989. As the instrument was well beyond its support life, the only alternative was to replace it.

Application was made to SRDC for permission to purchase a new gas chromatograph in conjunction with another project (BS3S). This was approved but the new gas chromatograph was not delivered until January 1990. While awaiting delivery of the new instrument, a Model 3700 gas chromatograph was borrowed from Varian. This did, however, cause some problems as the instrument had to be converted to capillary operation. As BSES did not own the instrument, no further modifications were able to be carried out.

The replacement gas chromatograph was a Varian Model 3300, fitted with a flame ionisation detector and a thermal conductivity detector. Injectors were split/splitless for columns 0.10 to 0.53 mm and a Universal injector for packed columns or 0.53 mm on-column injection. When the new gas chromatograph was received, time was again needed to modify analytical methods as all three instruments had different inlet systems.

Initially, data were acquired using the dedicated terminal of the Hewlett-Packard 5830A gas chromatograph. With the purchase of the Varian Model 3300 gas chromatograph, a computer-based data handling system was used (Delta Chromatography Data System,

Version 4.00, Digital Solutions Pty Ltd, Queensland). This was run on an Epson PCAX2 computer with EGA colour monitor. This software had the advantage that the chromatograms from the sample extracts could be overlaid in different colours for comparison of peaks.

Four capillary columns were used: RSL-150 (polydimethylsiloxane) and RSL-300 (polyphenylmethylsiloxane), both 10 m long x 0.53 mm diameter with film thickness 1.2 μm (Alltech Associates); DB-5, 15 m long x 0.53 mm diameter (J and W Scientific); Carbowax-20M, 10 m long x 0.53 mm with film thickness 1.33 μm (Hewlett-Packard, HP-20M).

A small number of samples were taken from exposed tissue, possibly associated with the calling process. These were collected, using cotton buds, from *L. frenchi* females in the pre-mating calling pose in December 1989 at Meringa. Glands were also dissected from *L. frenchi* females at Meringa and laboratory-reared *D. albohirtum* beetles.

Extracts of cotton buds and glands were prepared using solvents for direct analysis by gas chromatography.

3.0 RESULTS

Demonstration of pheromone presence: Male *A. consanguineus* were attracted to unmated female *A. consanguineus* in experiment 1 ($\bar{x}$ per trap = 3.40, SE = 0.93), but not to unmated males or unbaited traps (T = 10.00, df = 2, P = 0.0067). In experiment 3, male *A. consanguineus* were again attracted to unmated females ($\bar{x}$ per trap = 4.33, SE = 1.20), but not to unmated females of *L. picticollis*, unmated males of both species, or unbaited traps (T = 12.00, df = 4, P = 0.017).

Male *A. parvulus* were attracted to unmated female *A. parvulus* in experiment 2 ($\bar{x}$ per trap = 3.20, SE = 1.07), but not to unmated males or unbaited traps (T = 10.00, df = 2, P = 0.0067).

Male *L. picticollis* in experiment 3 were significantly attracted to unmated female *L. picticollis* ($\bar{x}$ per trap = 2.67, SE = 0.67) and unmated female *A. consanguineus* ($\bar{x}$ per trap = 0.67, SE = 0.33). They were not attracted to unmated males of both species or unbaited traps (T = 10.86, df = 4, P = 0.0282).

In experiment 4, no *L. negatoria* were attracted to either unmated female or unmated male *L. negatoria*.

Antennal morphology: Males of *A. parvulus* have seven large lamellae, the inner surfaces of which have about 150 000 cup-shaped placoid sensilla. Females have fewer and smaller lamellae, no placoid sensilla and few basiconic sensilla. Both sexes of *L. negatoria* have similar 3-lamellate antennae with placoid sensilla and two types of basiconic sensilla.

Cane beetle sampling: A large number of samples on Tenax traps were obtained from six beetle species over the 3-year period of the project. However, only one set of samples showed a compound which appeared to be associated with the female of the species. These came from a sampling of *Lepidiota negatoria*, carried out at Bundaberg in October 1989, and the chromatograms obtained are shown in Figures 1 and 2.

The sample from *L. negatoria* was sent to the Agricultural Chemistry Branch of QDPI for further analysis, using gas chromatography-mass spectrometry, in an effort to identify the unknown compound. Unfortunately the compound did not match any of the substances in the reference library. There was insufficient sample to allow additional analyses and it was not possible to obtain further samples from this species in late 1990. The total ion current chromatogram obtained and the mass spectrum of the compound of interest are shown in Figures 3 and 4.

The samples collected on cotton buds from the exposed tissue possibly associated with the 'calling' process of *L. frenchi* female beetles showed no significant compounds upon analysis.

Samples of glands taken from *L. frenchi* females at Meringa revealed no significant compounds when solvent extracts were analysed. No significant compounds were detected in gland extracts from female *D. albohirtum* beetles which had been reared in the laboratory.

4.0 DISCUSSION

In the field experiments with beetle traps, attraction of males to unmated females in *Antitrogus consanguineus*, *A. parvulus* and *Lepidiota picticollis* demonstrates that females of these species release sex pheromones. The attractancy of male *L. picticollis* to unmated females of *A. consanguineus* indicates that this chemical or mixture of chemicals is similar in these two species. The apparent absence of attraction in *L. negatoria* may indicate that females of this species use pheromones to attract males over only short distances such as in feeding trees. This is in line with the sensilla differences which suggest that male *A. parvulus* use pheromones to locate distant females, whilst in *L. negatoria* the antennal sensilla are used to locate feeding trees and proximate females. *L. negatoria* may also have more complicated cues for the release of pheromones, cues which were not met under the conditions of the experiment.

Observations of homosexual behaviour in *A. consanguineus* also indicate that females of this species use the pheromone to attract males over long distances. Over short distances, chemical cues are apparently not important, allowing males to misidentify other nearby males and copulate with them.

Detailed descriptions of these differences are given in Appendix 1.

Cane beetle sampling: A large number of samples on Tenax traps were obtained from six beetle species over the 3-year period of the project. However, only one set of samples showed a compound which appeared to be associated with the female of the species. These came from a sampling of *Lepidiota negatoria*, carried out at Bundaberg in October 1989, and the chromatograms obtained are shown in Figures 1 and 2.

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Observations of homosexual behaviour in *A. consanguineus* (Appendix 1) also indicate that females of this species use the pheromone to attract males over long distances. Over short distances, chemical cues are apparently not important, allowing males to misidentify other nearby males and copulate with them.

The number of chemical analyses which could be carried out was restricted largely by the seasonal availability of beetles. Some of the canegrub beetles are short lived, with a life of only one to two days in the field, and all species fly for short periods only during spring and summer. Rearing beetles from field-collected larvae is time consuming as most species have a two-year life cycle.

Although the field experiments with beetle traps and studies of antennal morphology indicated the presence of pheromones in the sexual behaviour of the beetle species, none of the laboratory sampling experiments yielded extracts with compounds unique to female beetles. Laboratory-reared beetles were sampled as soon as they emerged, however, beetles may only release pheromones after they have developed to a certain stage. In addition, other behaviour cues, such as the need to fly and feed, may have to be met before sex pheromones are produced.

We are still largely ignorant of these requirements and thus our laboratory rearing and collection techniques may not have been suitable. This aspect may be more important in the *Lepidiota negatoria/Dermolepida albohirtum* group, which appears to have a complex mating behaviour following flights to feeding trees. In contrast, *Antitrogus* spp. and *L. picticollis* females rarely fly, do not feed and appear to produce pheromones on emerging from the soil. These factors may explain why the presence of pheromones was successfully demonstrated in the field for these beetles species but not with laboratory-reared insects which had emerged one to two days before sampling.

Unfortunately, there was insufficient sample of the extract from *L. negatoria*, collected in the field, to enable QDPI to make a positive identification of the compound which appeared to be unique to the female of the species. The substance did not match any of the compounds in their mass spectrometer library. It was not possible to obtain further samples for analysis due to the seasonal availability of this species.

Overall, this study has indicated that sex pheromones are involved in cane beetle behaviour and has demonstrated the need for a better knowledge of the behaviour of adult beetles to allow collection of samples at the most appropriate times for positive pheromone identification.

5.0 RECOMMENDATIONS

As a result of this project, it is suggested that the following recommendations be considered to build on knowledge and expertise already gained:

1. carry out detailed studies of cane beetle mating behaviour under field conditions;

2. use this information to attempt to mimic field conditions in the laboratory and then collect further samples for analysis;

3. use a gas chromatograph fitted with a mass spectrometer as the initial separation and detection technique, rather than gas chromatography alone, as used in this project, with samples sent elsewhere for characterisation by mass spectrometry.

6.0 ACKNOWLEDGMENTS

We thank Norm McGill, Greg Sullivan, Tom Morgan and Darren Brady for their technical assistance and the sugarcane farmers who allowed trapping on their properties.

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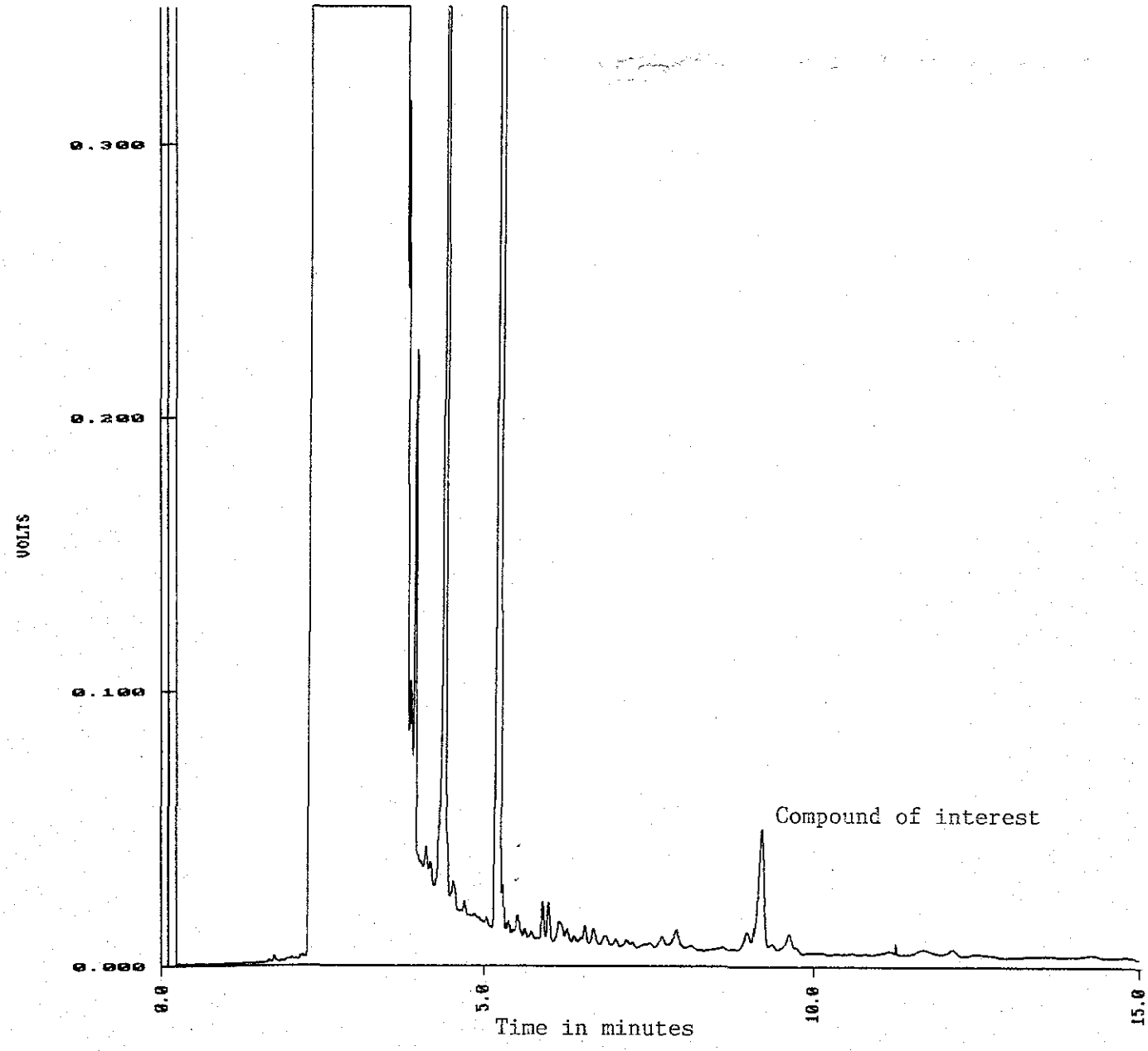

Figure 1 Chromatogram of sample from female Lepidiota negatoria beetle

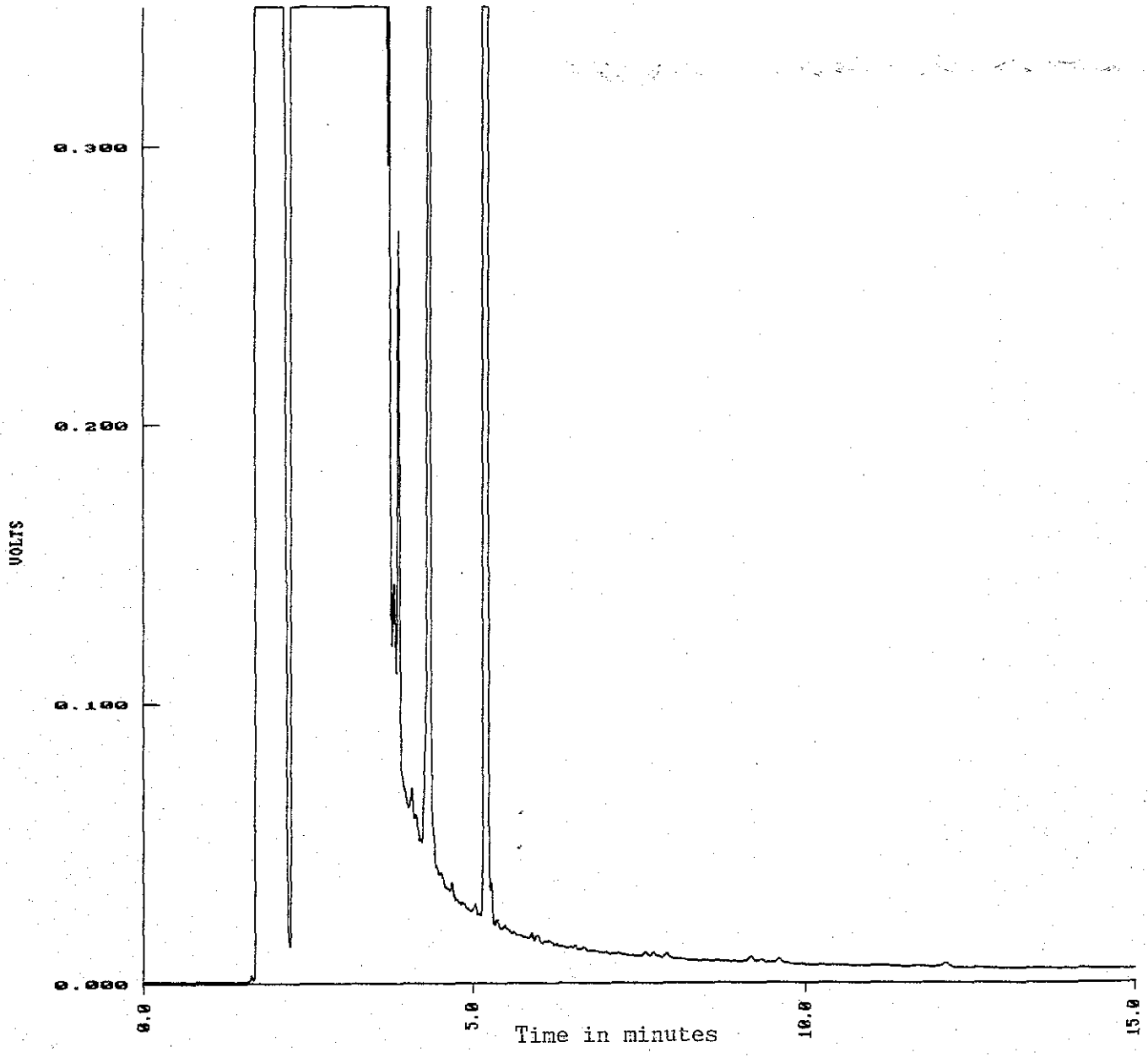

Figure 2 Chromatogram of control for Lepidiota negatoria samples

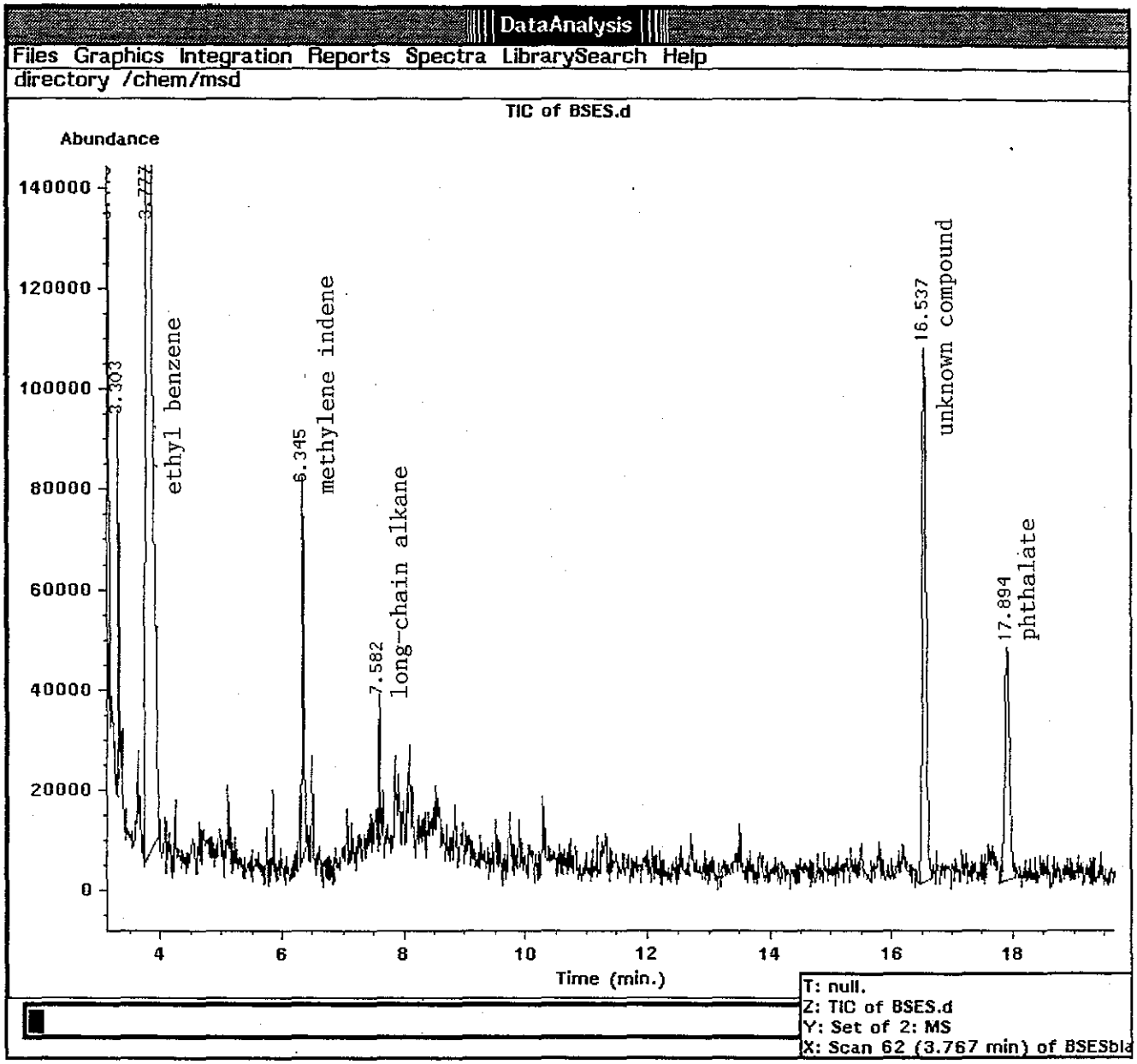

Figure 3 Total ion current chromatogram for Lepidiota negatoria sample

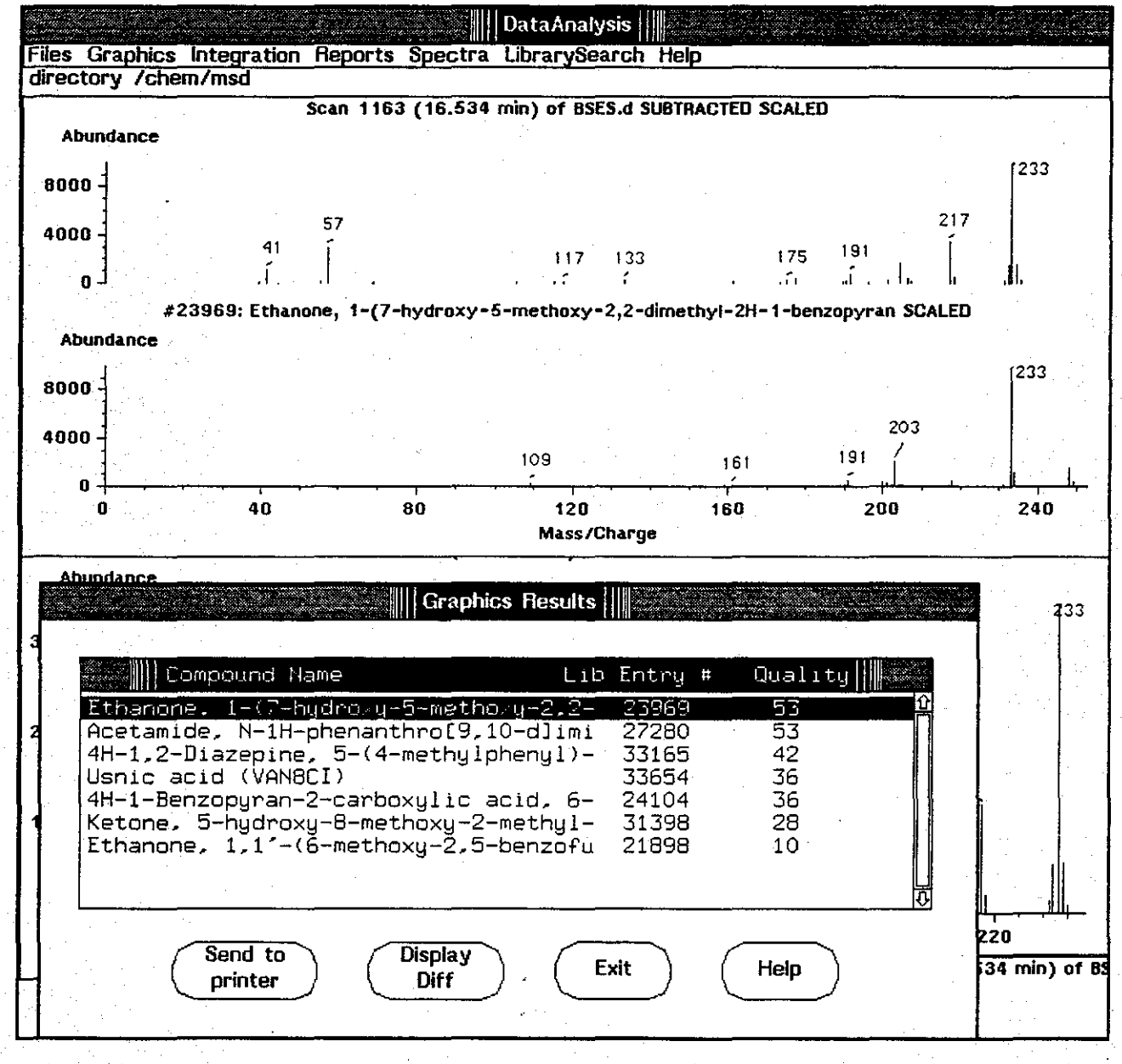

Figure 4 Mass spectrum of compound of interest from Lepidiota negatoria sample

APPENDIX 1 PUBLICATIONS RESULTING FROM THIS PROJECT

J. Aust. ent. Soc., 1990, 29: 261-266

SEXUAL DIMORPHISM IN THE ADULT ANTENNAE OF *ANTITROGUS PARVULUS* BRITTON AND *LEPIDIOTA NEGATORIA* BLACKBURN (COLEOPTERA: SCARABAEIDAE: MELOLONTHINAE)

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MALE-MALE COPULATION IN ANTITROGUS CONSANGUINEUS (BLACKBURN) (COLEOPTERA: SCARABAEIDAE)

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Abstract
Copulation between males of the melolonthine Antitrogus consanguineus is reported. This has implications in the understanding of pheromone-mediated behaviour.

Larvae of the melolonthine Antitrogus consanguineus (Blackburn) feed on the roots of sugarcane in south-eastern Queensland (Bull 1972). Adult males have large 5½-lamellate antennal clubs, while antennal clubs of females are shorter, more rounded and 3½-lamellate (Britton 1978). Unmated females attract males, presumably by a pheromone (Allsopp unpubl. data).

We observed male-male copulation in A. consanguineus five times. The first pair was amongst 20 males confined in a container. None had mated, as we had reared them in individual containers from field-collected third instars. Within 5 min of placing them together in the container, the pair was copulating.

The other times were within three groups of 10-30 males collected in a light trap during September and October 1990. Pairs of males were copulating when we counted the beetles the morning after capture. No females were present in any of these captures.

In all cases the posture of the copulating pair was the same as that of a copulating male-female pair (Fig. 1). Only the large antennal clubs of the lower male distinguished the pair from a normal mating pair. The aedeagus of the upper male was inserted into the genital capsule of the lower male. The upper male's parameres were below those of the lower male, with the tip of the upper male's parameres level with the base of the lower's parameres. Dissection showed that the lower individuals were always males in all respects; none were gynandromorphs similar to a female of Golofa tersander Burmeister with external male characters (Ratcliffe 1989).

This homosexual behaviour indicates that females of A. consanguineus use the pheromone to attract males over long distances. Over short distances chemical cues are apparently

not important, allowing males to misidentify other nearby males and copulate with them. The observations also bear out predictions from sexual selection theory that males of species with low male parental investment should be indiscriminate in mating relative to females (Daly and Wilson 1982).

Acknowledgment
We thank the Sugar Research Council for their financial support through project BS17S.

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SCIENTIFIC NOTE

EVIDENCE FOR FEMALE SEX PHEROMONES IN *ANTITROGUS CONSANGUINEUS* (BLACKBURN), *A. PARVULUS* BRITTON, AND *LEPIDIOTA PICTICOLLIS* LEA (COLEOPTERA: SCARABAEIDAE)

Sixteen species of endemic melolonthine canegrubs are important pests of sugarcane in eastern Australia (Allsopp and Chandler 1989). Larvae destroy roots, depriving plants of moisture, nutrients, and mechanical support. Canegrubs are presently controlled with insecticides, but alternative control measures are being sought. Pheromones offer potential for reducing populations of adult beetles through trapping or, by indicating flight periods, allowing better timing of insecticide applications. Sex pheromones have been identified in two phytophagous scarabs, *Popillia japonica* Newman (Tumlinson *et al.* 1977) and *Costelytra zealandica* (White) (Henzell and Lowe 1970), and are thought to occur in other species (Soo Hoo and Roberts 1965; Lilly and Shorthouse 1971; Domek and Johnson 1988).

To test for pheromones, I exposed unmated males and females of *Antitrogus consanguineus* (Blackburn), *A. parvulus* Britton, and *Lepidiota picticollis* Lea in individual Catch-can Japanese beetle traps (Trécé Inc., Salinas) during flight periods of the target species. The unmated adults were reared in individual containers in the laboratory from field-collected larvae. Adults were confined individually in perforated vials within each trap in the same position as the standard Trécé lure. Traps were hung 1 m above ground on steel rods 10 m apart in randomized block designs. Each block included an unbaited trap. Catches were counted 3 days after exposure.

Experiment 1 tested *A. consanguineus* starting 17 September 1989, experiment 2 tested *A. parvulus* starting 7 November 1989, and experiment 3 tested *A. consanguineus* and *L. picticollis* starting 14 September 1990. Experiment 3 used three replicates; other experiments had five replicates.

As none of the raw data sets were normally distributed ($P < 0.01$, Shapiro-Wilk test for normality) and were not normalized by transformation, they were analyzed using the nonparametric Friedman two-way analysis of variance (Conover 1980). All analyses used STATISTIX 3.1 (Analytical Software 1989).

Male *A. consanguineus* were attracted to unmated female *A. consanguineus* in experiment 1 ($\bar{x}$/trap = 3.40, SE = 0.93), but were not caught in unbaited traps or traps baited with unmated males ($T = 10.00$, df = 2, $P = 0.0067$). In experiment 3, male *A. consanguineus* were again attracted to unmated conspecific females ($\bar{x}$/trap = 4.33, SE = 1.20), but not to unmated females of *L. picticollis*, unmated males of both species, or unbaited traps ($T = 12.00$, df = 4, $P = 0.017$).

Male *A. parvulus* were attracted to unmated female *A. parvulus* in experiment 2 ($\bar{x}$/trap = 3.20, SE = 1.07), but not to unmated males or unbaited traps ($T = 10.00$, df = 2, $P = 0.0067$).

In experiment 3, male *L. picticollis* were not attracted to unmated males of *L. picticollis* or *A. consanguineus* or to

unbaited traps, but were attracted to unmated females of both species (T = 10.86, df = 4, P = 0.028). Significantly (P < 0.05) more males were attracted to unmated female L. picticollis ($\bar{x}$/trap = 2.67, SE = 0.67) than to unmated female A. consanguineus ($\bar{x}$/trap = 0.67, SE = 0.33).

Attraction of males to conspecific unmated females in A. consanguineus, A. parvulus, and L. picticollis demonstrates that females of these species release sex pheromones. Attraction of male L. picticollis to unmated females of A. consanguineus indicates that the compound or mixture of compounds is similar in these two species. Lepidiota and Antitrogus are closely related genera within the Melolonthini. Identification of the compounds is being attempted.

Demonstration of pheromone presence confirms my suggestion (Allsopp 1990) that males of A. parvulus use pheromones to locate females. I based this on the sexual dimorphism in the number, shape, and size of lamellae, and in the number and shape of sensilla in A. parvulus antennae. A. consanguineus and L. picticollis also have sexually-dimorphic antennae.

Observations of homosexual behaviour in A. consanguineus (Allsopp and Morgan in press) indicated that females of this species use pheromones to attract males over long distances. Over short distances chemical cues are apparently not important, allowing males to misidentify other nearby males and copulate with them.

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