Journal of Chemical Ecology, Vol. 28, No. 3, March 2002 (© 2002)

IDENTIFICATION OF THE SEX PHEROMONE OF *Holotrichia reynaudi*

ANDREW WARD,[1,6] CHRIS MOORE,[2,*] V. ANITHA,[3] JOHN WIGHTMAN,[4] and D. JOHN ROGERS[5]

[1]*Farming Systems Institute*
Department of Primary Industries
PO Box 23, Kingaroy, Qld 4610, Australia

[2]*Farming Systems Institute*
Department of Primary Industries
665 Fairfield Road, Yeerongpilly, Qld 4105, Australia

[3]*International Crops Research Institute for the Semi-Arid Tropics*
Patancheru, Andhra Pradesh 502 324, India

[4]*International Pest Management*
Conondale Cottage, Lot 7 Stanley River Road
Maleny Qld 4552, Australia

[5]*Farming Systems Institute*
Department of Primary Industries
Meiers Road, Indooroopilly, Qld 4068, Australia

[6]*Current address: Department of Primary Industries and Fisheries*
PO Box 1346, Katherine, NT 0851, Australia

(Received December 20, 2000; accepted October 27, 2001)

Abstract—The male attractant pheromone of the scarab beetle *Holotrichia reynaudi*, an agricultural pest native to southern India, was extracted from abdominal glands of females with hexane and analyzed by gas chromatography–mass spectrometry. Field testing of the candidate chemicals, indole, phenol, and anisole, both alone and as binary mixtures, led us to conclude that anisole was the major component of the sex pheromone. Neither male nor female beetles were attracted to indole or phenol on their own. Similarly, when indole and anisole were combined, the attractiveness of the solution did not increase over that obtained with anisole alone. However, combination of phenol and anisole did alter the attractiveness of anisole, with fewer male beetles attracted to the binary mixture than to anisole on its own. The behavior of female beetles was not altered by any of the chemicals tested. Anisole is also the sex pheromone of *H. consanguinea*, making this the first known example of two melolonthine scarabs sharing the same pheromone.

* To whom correspondence should be addressed. E-mail: moorec@dpi.qld.gov.au

0098-0331/02/0300-0515/0 © 2002 Plenum Publishing Corporation

Key Words—Coleoptera, Scarabaeidae, Melolonthinae, *Holotrichia reynaudi*, anisole, indole, phenol, pheromone.

INTRODUCTION

In recent years, there has been a variety of studies examining the chemical ecology of white grubs injurious to agricultural crops. As a result, the sex pheromones of a number of species from the subfamilies Melolonthinae and Rutelinae have been successfully identified (Leal, 1998). These include the pheromones used by the melolonthine species *Costelytra zealandica* (Henzell and Lowe, 1970), *Holotrichia parallela* (Leal et al., 1992), and *Holotrichia consanguinea* (Leal et al., 1996).

Sex pheromones may play an important role in maintaining the reproductive isolation of closely related species. Despite this, beetles from two genera of rutelines have been found to share either the same pheromone or a complex of pheromones. Leal et al. (1994) observed that *Anomala albopilosa sakishimana* and *Anomala cuprea* shared the same pheromone complex. Similarly, Potter (1980) reported that *Cyclocephala lurida* (formerly *C. immaculata*) and *Cyclocephala borealis* appeared to share the same pheromones. In both cases, reproductive isolation was maintained by either temporal or spatial means. Although a number of rutelines have been observed sharing the same pheromones, no similar observations have been made in the melolonthines. This paper reports the first known record of the same phenomenon in the Melolonthinae.

Holotrichia is a genus of the melolonthine scarabs that occurs across the Indian subcontinent and through southeast and east Asia. A number of species, including *H. reynaudi* and *H. consanguinea*, are major agricultural pests in India. Leal et al. (1996) identified anisole as the pheromone used by *H. consanguinea*. In this paper, we report that *H. reynaudi* also uses anisole as a sex pheromone.

METHODS AND MATERIALS

Analytical Procedures. Gas chromatography–mass spectrometry (GC-MS) was carried out on a VG Trio-2000 instrument (VG Biotech, Altrincham, UK), equipped with a split/splitless injector and nonpolar DB5-MS column (30 m $\times$ 0.25 mm; 0.25 μm). The column was held at 40°C for 2 min, then raised to 260°C at 10°C/min, where it was held for 6 min.

Extracts. Abdominal glands were excised from four calling females and placed in a screw top vial containing 2 ml of solvent (hexane). The vial containing the glands was air freighted to Australia for analysis along with 2 ml of the original hexane, which acted as a blank.

Field Testing. Behavioral responses to the candidate pheromones, anisole, indole, and phenol were evaluated over two years at the International Centre for

Research in the Semi Arid Tropics (ICRISAT) near Hyderabad, in the southern Indian State of Andhra Pradesh. Indole and anisole were also tested at Mahbubnagar (approximately 100 km southwest of Hyderabad) in 2000. In 2000, field testing of anisole and indole was completed at ICRISAT over five nights, June, 7–8 and June, 12–14. In 2001, anisole and phenol were tested at ICRISAT over four nights, June, 4–7. The test site at ICRISAT, in both 2000 and 2001, was an area of approximately 1500 m^2 in which eight distinct patches of *H. reynaudi*'s host tree (*Zizyphus mauritiana*) were growing. The trees had a maximum height of approximately 2 m. Testing was carried out at Mahbubnagar over one night (June 16, 2000). The test site consisted of a U-shaped border to a commercial peanut field surrounded by another of *H. reynaudi*'s host trees, *Butea monosperma*.

At ICRISAT, in 2000, anisole, indole, anisole + indole, or a control (empty vial) was assigned to each patch of trees, giving two replicates of each treatment. Phenol was not tested until 2001, as it was not clearly evident in the initial samples (see Results and Discussion for details). The location of each chemical was rerandomized each night. The anisole, indole, and phenol were purchased as laboratory reagents in India (anisole from s.d. fine-chem pvt. Ltd., indole from Loba Chemie, Bombay; phenol from Qualigens Fine Chemicals, Glaxo India Limited) and were undiluted. Approximately 5 g of each of the candidate chemicals was placed in a 20-ml glass vial. This was approximately the quantity of anisole soaked onto sponges and placed into trees by J. N. Vijayvergia (personal communication) in on-farm beetle-management trials with *H. consanguinea* in Rajasthan. The glass vials were taped to the stem of the host tree approximately 10 min before the anticipated time of beetle emergence. In the case of the anisole + indole mixture, equal quantities of the two compounds were mixed together by weight. Each pheromone station was separated by at least 10 m. The attractiveness of each compound was assessed by counting the number of beetles attracted to each vial. The sex ratio was also recorded. A similar sampling procedure was adopted at Mahbubnagar. Beetle flights around the anisole and the anisole + indole trees during the period of attractiveness of the chemicals were too intense (>200 beetles) for accurate counts to be made. A funnel trap baited with anisole was also placed in the middle of the field at this site.

In 2001, field tests at ICRISAT compared the attractiveness of anisole, phenol, anisole + phenol and empty (clean) vials to *H. reynaudi* beetles. Beetle attraction was monitored with water traps, which consisted of plastic bowls 10 cm deep × 35 cm diam. Two 30-cm-high aluminum baffles, slotted to give a right angle fit, were attached to the top of each bowl. The glass vials, into which the test compounds were placed, were accommodated in the top center of the baffle. The bowl contained 4–5 cm of water + one drop of detergent. Data were analyzed by using ANOVA following a log $(x + 1)$ transformation. Means were separated using least significant differences.

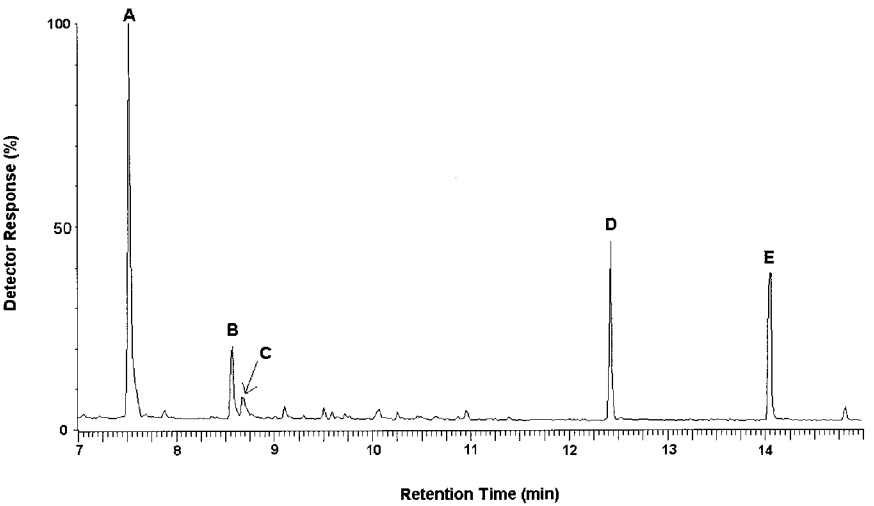

FIG. 1. GC-MS output from crude extracts taken from the abdominal glands of *H. reynaudi*. Peak A, anisole; B, dimethyltrisulfide; C, phenol; D, naphthalene; E, indole.

RESULTS AND DISCUSSION

GC-MS analysis of the crude extracts from the abdominal glands gave a clean profile (Figure 1). There were a number of relevant peaks. These were identified as indole (E), methoxybenzene (anisole, A), and phenol (C). They were the same as those identified by Leal et al. (1996) in the crude gland extracts taken from the closely related species, *H. consanguinea.* There were two additional peaks, naphthalene (D) appeared in both the solvent and the extract, suggesting that it was a contaminant. The other peak (B), was dimethyltrisulfide. As it was not possible to freight the samples in a frozen or chilled form, the gland tissues had started to decay by the time analysis was undertaken. This compound is a common component of volatiles emitted from animal wastes, biocultures, and anaerobically decomposing animal tissue (see, for example Niedziella et al., 2000). No record of the connection of this material with insect semiochemical phenomena has been found.

Early samples freighted to Australia, on which the synthetic formulation for field testing was based, had been insufficiently concentrated to reveal phenol in the original total-ion-current chromatograms. The samples had, however, been transported more carefully, and generation of mass chromatograms corresponding to phenol (*m*/*z* 94) and dimethyltrisulfide (*m*/*z* 126) from raw data acquired earlier from these samples revealed traces of phenol and the absence of dimethyltrisulfide. Although phenol has previously been identified as the pheromone used by

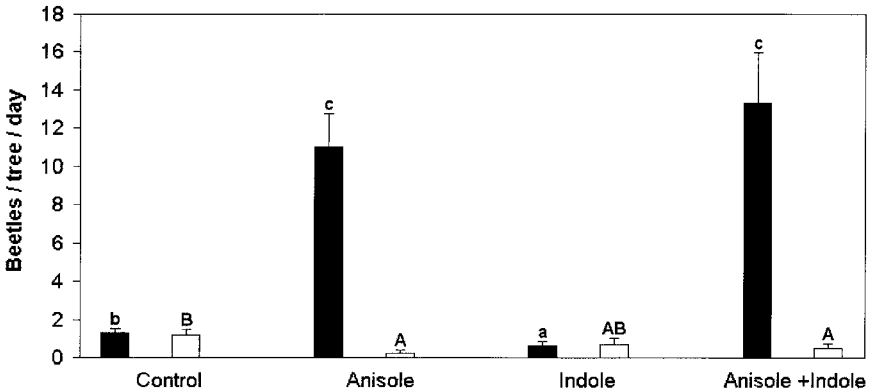

FIG. 2. Number of *H. reynaudi* beetles attracted to vials containing anisole, indole and a mixture of anisole + indole. Solid bars represent the number of male beetles and open bars represent the number of female beetles. Bars of the same color marked with the same letter are not significantly different at $P < 0.05$ using least significant differences.

Costelytra zealandica (Henzell and Lowe, 1970), field testing was, of necessity, confined to anisole and indole in 2000.

The results of the field testing at ICRISAT in 2000 demonstrated that anisole was highly attractive to male *H. reynaudi* (Figure 2). After emerging from the soil, male beetles flew towards the trees baited with vials containing anisole and the mixture of anisole + indole. More male beetles were attracted to these trees than to control trees or trees baited with indole alone [$F(3,36) = 64.97$, $P < 0.001$]. When the males reached the vials containing anisole, they flew around them and attempted to either copulate with the vials or with other male beetles. This activity commenced at about 19:10 hr (dusk) and continued for approximately 15 min before the beetles commenced feeding. By comparison, no male beetles were observed flying or displaying sexual behavior around the control vials or the vials containing indole alone.

In 2001, similar results were recorded with large numbers of male beetles being caught in the traps baited with anisole (Figure 3). Male beetles were also caught in the traps baited with anisole and phenol. However, the numbers were smaller than those in the traps baited with anisole alone [$F(3,60) = 11.11$, $P < 0.001$]. This observation was interesting because it makes little sense for a female beetle to produce a compound that repels members of its own species. Explanations for this may include that the concentration of phenol emitted from the vials was excessive and, therefore, masked the attractiveness of the anisole. No beetles were caught in the traps baited with phenol alone.

The behavior of the male beetles in the presence of anisole was similar to that observed in males seeking a mate. The normal pattern is for males to emerge

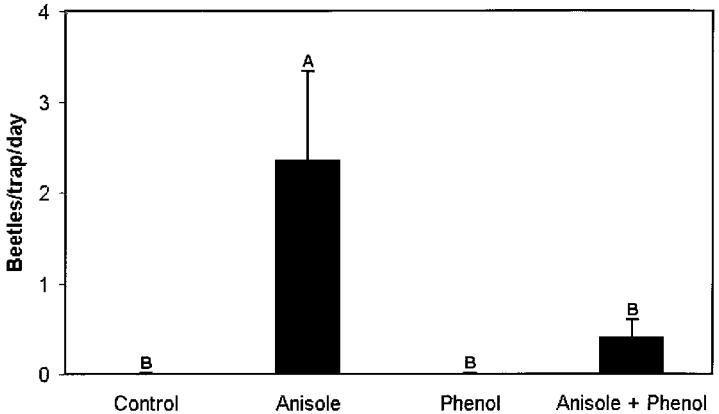

FIG. 3. Number of male *H. reynaudi* beetles attracted to vials containing anisole, phenol, and a mixture of anisole + phenol. No female beetles were caught. Bars marked with the same letter are not significantly different at $P < 0.05$ using least significant differences.

from the soil and fly around searching for calling females. If successful, copulation usually commences within five minutes of emergence from the soil. Males that are unable to find a mate continue to patrol in an attempt to find a calling female before settling to feed after approximately 15 min.

Although the number of females attracted to the candidate chemicals differed in 2000 [$F(3,36) = 3.55$, $P = 0.02$] (Figure 2), the number of females was low in all treatments. In 2001, no females were caught. Importantly, the behavior of female beetles was unchanged by the presence of the candidate compounds. In both the presence and absence of the candidate chemicals, female beetles emerged from the soil and commenced calling in an attempt to attract male beetles. Although mating usually commences within 5 min of emergence, females located close to the vials containing anisole were observed calling unsuccessfully for periods in excess of 10 min before ceasing, while males attempted to mate with either the vials containing anisole or each other less than 1 m away.

The field bioassay conducted at Mahbubnagar gave almost identical results to those obtained at ICRISAT. Large numbers of beetles (>200) were attracted to the trees baited with anisole, while low numbers of beetles were observed on the control and indole trees. Regular checks of the sex of the beetles flying around the trees baited with anisole showed that all of the beetles were male. Similarly, all of the beetles captured ($N = 47$) in the funnel trap baited with anisole were male.

Sex pheromones can be defined as compounds emitted by individuals of one sex that attract members of the opposite sex, resulting in the location of the emitter, and subsequently, mating (Baker, 1989; Tamaki, 1985). From our observations,

anisole can be regarded as a sex pheromone because it is released by females, and males show a clear sexual response to it. When vials containing anisole were placed on the trees, male beetles were observed orienting towards the vials after emerging from the soil. When they reached the vials, the beetles landed on or close to the vial and buzzed their wings vigorously before everting their genitalia and attempting to mate with either the vial or other male beetles in their vicinity.

Anisole has also been identified as the sex pheromone used by *H. consanguinea* (Leal et al., 1996), a species closely related to *H. reynaudi*, found in northern India. However, unlike *H. reynaudi*, where only male beetles were attracted to anisole, anisole was equally attractive to both male and female *H. consanguinea*, resulting in large aggregations of both male and female beetles. In northern India, this behavior is exploited for management of the pest, by spraying trees with insecticide prior to adult emergence (Yadava and Sharma, 1995), killing beetles of both sexes and reducing subsequent crop damage. More recently, this has been combined with the use of anisole to further concentrate the beetles and reduce the number of trees sprayed (Yadava, personal communication). On the basis of the data reported here, the prospects are limited for a similar approach with *H. reynaudi.*

From an evolutionary perspective, we believe that there are advantages for female beetles to release sex pheromones that attract members of the opposite sex only. Sex-specific pheromones may limit competition for males from other female beetles attracted by the semiochemical and, therefore, increase the probability of successful mating. This view is supported by Leal et al. (1996), who believe that, in the evolution of functional communication in scarab beetles, sex-specific signaling has evolved later than signals that attract beetles from both sexes. In this sense, *H. reynaudi* would be considered more advanced than *H. consanguinea* because the semiochemical used as a pheromone has developed a sex-specific function, attracting only male beetles.

The use of the same pheromone or complex of pheromones has previously been observed in closely related species from the subfamily Rutelinae. However, there are no similar reports in the melolonthines. Leal et al. (1994) observed that *Anomala albopilosa sakishimana* and *Anomala cuprea* shared the same pheromone. Similarly, Potter (1980) observed that *C. lurida* and *C. borealis* appeared to share the same pheromones. In both cases, reproductive isolation was maintained using either temporal or spatial means.

Although both *H. consanguinea* and *H. reynaudi* are endemic to India, there are no reports in the literature of them occurring sympatrically. As *H. reynaudi* is found in southern India and *H. consanguinea* is found in northern India, reproductive isolation is maintained through spatial separation, and as a result there is unlikely to be significant selective pressure for the pheromone system of either species to evolve further.

Acknowledgments—We thank the Australian Centre for International Agricultural Research for funding this work through project CS94/50 and Manaiah, Babu Rao and Md. Khaja for providing technical assistance.

REFERENCES

BAKER, T. C. 1989. Sex pheromone communication in Lepidoptera: New research progress. *Experientia* 45:248–262.

HENZELL, R. F. and LOWE, M. D. 1970. Sex attractant of the grass grub. *Science* 168:1005–1006.

LEAL, W. S. 1998. Chemical ecology of phytophagous scarab beetles. *Annu. Rev. Entomol.* 43:39–61.

LEAL, W. S., MATSUYAMA, S., KUWAHARA, Y., WAKAMURA, S., and HASEGAWA, M. 1992. An amino acid derivative as the sex pheromone of a scarab beetle. *Naturwissenschaften* 79:184–185.

LEAL, W. S., KAWAMURA, F., and ONO, M. 1994. The scarab beetle *Anomala albopilosa sakishimana* utilizes the same sex pheromone blend as a closely related and geographically isolated species *Anomala cuprea*. *J. Chem. Ecol.* 20:1667–1676.

LEAL, W. S., YADAVA, C. P. S., and VIJAYVERGIA, J. N. 1996. Aggregation of the scarab beetle *Holotrichia consanguinea* in response to female-released pheromone suggests secondary function hypothesis for semiochemical. *J. Chem. Ecol.* 22:1557–1566.

NIEDZIELLA, S., RUDKIN, S., and COOKE, M. 2000. Evidence for selectivity of adsorption of volatile organic compounds by a polydimethylsiloxane solid-phase microextraction fiber. *J. Chromatogr. A* 885:457–464.

POTTER, D. A. 1980. Flight activity and sex attraction of northern and southern masked chafers in Kentucky bluegrass. *Ann. Entomol. Soc. Am.* 73:414–417.

TAMAKI, Y. 1985. Sex pheromones, pp. 145–191, *in* G. A. Kerkut and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol 9. Pergamon Press, Oxford.

YADAVA, C. P. S. and SHARMA, G. K. 1995. Indian Whitegrubs and their Management. All India Coordinated Research Project on Whitegrubs Technical Bulletin 2. Indian Council of Agricultural Research, New Delhi, India, 26 pp.