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# AGGREGATION OF THE SCARAB BEETLE *Holotrichia consanguinea* IN RESPONSE TO FEMALE-RELEASED PHEROMONE SUGGESTS SECONDARY FUNCTION HYPOTHESIS FOR SEMIOCHEMICAL

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**Abstract**—The pheromone system of the scarab beetle *H. consanguinea*, an agricultural pest native to India, was investigated by extracting abdominal glands of females with dichloromethane and ether and analyzing them by GC-MS. Indoor bioassays with the natural product separated on a silica gel column showed that males responded to the hexane–ether (80:20) fraction by displaying a clear sexual behavior. Although the indoor bioassay-oriented approach did not lead to the identification of the active compound(s), field tests of candidate chemicals—anisole, indole, and phenol—showed that beetles responded strongly to anisole. Males and females were captured in anisole-baited traps in a ratio close to 1:1. Nearly the same sex ratio was also observed in beetles captured over the time during the flight activity of the beetles in the field. Because no clear evolutionary basis exists for why competing females are attracted to the semiochemical, it was simply referred to as a "female-released pheromone."

**Key Words**—Coleoptera, Scarabaeidae, anisole, indole, phenol, aggregation behavior.

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## INTRODUCTION

Although we cannot rule out the existence of signals that arise *de novo*, perhaps most insect signals are postulated to have arisen from characteristics originally having other functions (Otte, 1974). For the evolution of chemical signals, either the compound or the sensory apparatus to detect these compounds may have preceded the evolution of functional communication. Thus, pheromones may have originated from hormones, plant allelochemicals, cuticular constituents, the insect's own defensive compounds, metabolic waste products, or some combination of these or other factors. The original functions of the chemicals may or may not be eventually lost. In the origin of chemical communication, there are several examples that seem to fit this secondary function hypothesis (reviewed in Haynes and Potter, 1995).

Recent studies on the chemical ecology of scarab beetles have led to the identification of sex pheromones of various species in the subfamilies Melolonthinae and Rutelinae (Leal, 1996). Interestingly, most of the chemicals utilized by the more advanced group (Rutelinae) are likely to be fatty acid derivatives, whereas the more primitive group (Melolonthinae) utilizes ubiquitous compounds per se or as an immediate precursor. Based on the facts that the sex pheromones of melolonthines are largely produced in huge amounts (Leal et al., 1993), enantiomeric purity is not critical (Matsuyama et al., 1994), and since most of them have antimicrobial activity (Leal and Kurata, 1994), it was suggested by Leal (1996) that these semiochemicals may have evolved from a primary defensive role.

Some melolonthine species, notably *Holotrichia* and *Phyllophaga* species, emerge at dusk and aggregate in a small time window. Females of *H. parallela*, for example, display a calling behavior only for ca. 15 min (Leal et al., 1993). It is conceivable that this behavior could also have advantages other than mate finding, such as minimizing the effect of a predator. In such cases, selection could favor nonspecific pheromones that would attract both sexes equally. We now report that a female-released pheromone of *H. consanguinea*, an economically important pest in India, is responsible for the aggregation of male and female beetles.

## METHODS AND MATERIALS

*Analytical Procedures.* Gas chromatography (GC) was carried out on a Hewlett-Packard 5890 II Plus instrument equipped with a split/splitless injector, electronic pressure control, a flame ionization detector, and an HP 3365 Series II Chemstation. High-resolution GC analyses were performed with polar and nonpolar capillary columns: HP-Innowax and HP-5MS (30 m × 0.25 mm ×

0.25 μm; Hewlett-Packard), respectively. These columns were operated at 50°C for 1 min, increased to 180°C at a rate of 4°C/min, held at this temperature for 1 min, increased again to 230°C at a rate of 10°C/min, and finally held at this temperature for 20 min. Low-resolution mass spectrometry (MS) was carried out either with an HP 5890 II Plus gas chromatograph linked to a mass selective detector MSD 5972 (Hewlett-Packard) or on an Hewlett-Packard gas chromatograph electron ionization detector GCD series. Chromatographic resolution was achieved on HP-5MS and HP-Innowax columns operated under the same conditions for GC.

*Extracts.* A kit containing all the materials necessary for the extractions of pheromone glands was shipped to India, where the pheromone was collected. Specially prepared solvents were used, a small portion being kept as a blank. Abdominal tips of calling females were excised with forceps, cleaned, and then washed with either dichloromethane or ether. Extracts of 10–20 females were transferred to ampules, flame-sealed, and shipped to Japan.

*Isolation of Pheromone.* In Japan, the extracts were analyzed by GC and GC-MS. An extract of 80 females equivalents (FE) was fractionated on a silica gel column (Wako C-200) by successively eluting with hexane–ether mixtures in the following order: 100:0, 95:5, 90:10, 80:20, 50:50, and 0:100. All the fractions were concentrated to 0.5 FE, and two thirds of each fraction was sent back to India for bioassay, whereas the remainder was used for chemical analysis.

*Indoor Bioassay.* The arena consisted of 75 × 60 × 60-cm wire gauge cages, containing a layer of moist soil (8 cm thick) on the bottom. Males collected in the field, when they were about to copulate, were released in each cage (45 per cage) one day before the bioassay. Fractions were transferred from the ampule to a filter paper that was placed inside the arena. Behavioral responses were observed from 19:00 to 21:30 hr.

*Field Experiments.* Evaluation of the candidate pheromones was conducted at the fields of the Agricultural Research Station, Durgapura-Jaipur, Rajasthan in the summers of 1994 and 1995. Funnel traps (Japan Tobacco, Inc., Tokyo), were baited with synthetic lures incorporated into plastic pellets (4–5 mm in diameter) made of polyethylene–vinyl acetate. These pellets were placed inside pellet holders (Fuji Flavor Co., Tokyo) and set 2 cm above the trap lip. Traps were positioned with the pheromone dispenser at 2.5 m above the ground. The candidate lures were replicated ($N = 2$) in randomized blocks with an intertrap distance of 10 m. Capture data were collected daily for at least three consecutive days and, after inspection, the traps were rerandomized. Capture data were transformed to log $(x + 1)$ before differences among means were tested for significance by ANOVA with JMP Software, Version 2 (Anonymous, 1989). Treatments followed by the same letters are not significantly different at the 5% level in the Tukey-Kramer honestly significant difference test.

## RESULTS AND DISCUSSION

GC and GC-MS analyses of the crude extract from abdominal glands of *H. consanguinea* females gave a very clean profile (Figure 1). Some of the peaks appeared also in the solvent when it was concentrated to the same extent

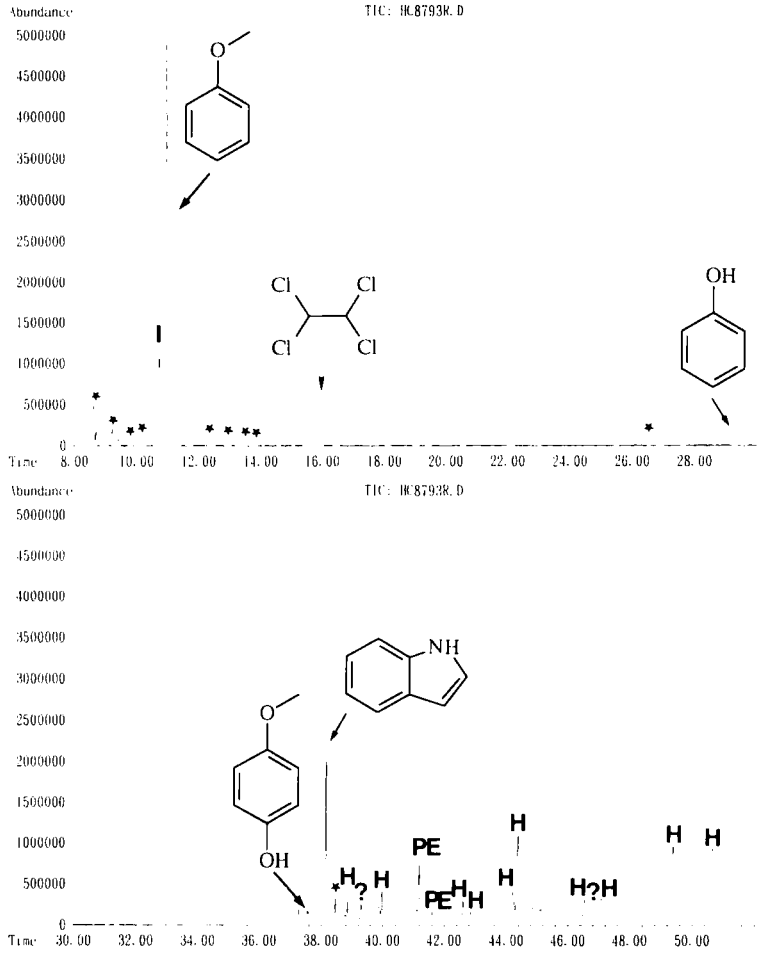


FIG. 1. Reconstructed total ion monitor profile of a crude extract sample from abdominal glands of *H. consanguinea* separated on a polar capillary column, HP-Innowax. The peaks labeled with an asterisk are due to solvent impurities. Cuticular hydrocarbons and phthalate esters are labeled with H and PE, respectively.

as the samples. One peak (I) was considered to be an impurity because it was observed only in one of 12 samples analyzed. Apart from the hydrocarbons, probably of cuticular origin, and phthalate esters, there were only a few relevant peaks. These were identified as methoxybenzene (anisole), 1,1,2,2-tetrachloroethane (an impurity from dichloromethane), phenol, 4-methoxyphenol, and indole.

Indoor bioassays revealed that the hexane–ether (80:20) fraction was very active. Males responded by attempting to take flight, orienting towards the pheromone source, aggregating on the source, and trying to copulate with each other. However, it was not possible to implement the bioassay for all the fractions under the same conditions. This was due to the fact that *H. consanguinea* is active for a very short period of time. Responses of one group to a certain fraction could not be compared to the responses of other individuals to a different fraction. This difficulty is intrinsic to the behavior of *Holotrichia* and *Phyllophaga* species. In order to overcome this barrier, bioassays to identify the active compound(s) in the pheromone system of *H. parallela* have been conducted in the field (Leal et al., 1992). This required a reasonable amount of sample and ultimately the full mixture could be identified only by assaying synthetic candidate compounds (Leal et al., 1993).

Because the indoor bioassay-oriented strategy could not lead unambiguously to the active compound(s) in a short period of time, we changed our strategy and assayed the insect response to candidate compounds in the field. The two major compounds found in the gland extracts, anisole and indole (Figure 1), were considered as possible attractants, notwithstanding the fact that indole is normally found in feces of scarab beetle males and females (Leal, unpublished data). Since phenol has been previously identified as a sex pheromone of another melolonthine species, *Costelytra zealandica* (Henzell and Lowe, 1970), it was also tested.

Field experiments showed that all the lures containing anisole were very attractive (Figure 2), whereas traps baited with indole and phenol (either alone or combined) did not capture significantly more beetles than control traps. Traps baited with caged virgin females did not capture significantly more than control traps, but this might be due to the fact that females of *Holotrichia* species hardly call when they are confined in a narrow cage (Leal, personal observation). Catches in traps baited at 3, 4.5, and 6 m were not significantly different.

Interestingly, the male-to-female ratio of the beetles captured was very close to 1:1. It has been previously observed that traps baited with synthetic sex pheromone of the Oriental beetle (and other species) also catch females (Leal et al., 1994). Nevertheless, the 1:1 ratio is very unusual. Recently, we have tested a previous hypothesis that males of the Oriental beetles caught in the traps released a semiochemical that would attract females. Traps baited with 30 males failed to attract any females in the field (Leal, unpublished data). On

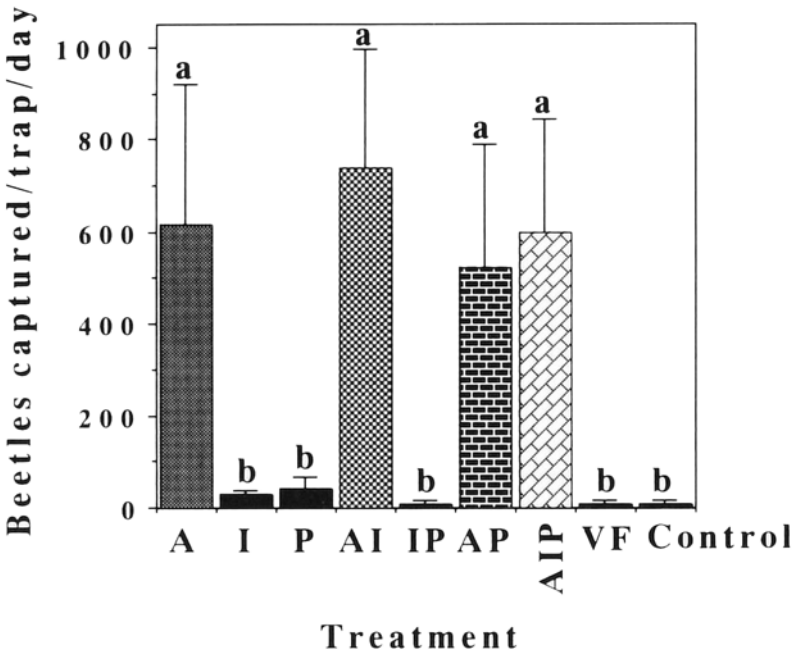


FIG. 2. Capture data of *H. consanguinea* in traps baited with candidate pheromones (A, anisole; I, indole; P, phenol), a combination of these compounds, virgin females (VF), and control traps.

the other hand, we also have evidence that female *Anomala octiescostata* can detect their own sex pheromone (Leal, unpublished data).

Recently, male-released pheromones have been identified from two scarab species in the subfamily Dynastinae, namely, *Oryctes monoceros* (Gries et al., 1994) and *O. rhinoceros* (Hallet et al., 1995). Because of male and female attraction to the semiochemicals, they were referred to as "aggregation" pheromones. However, there was evidence that chemical communication in *H. rhinoceros* involves a female-released sex pheromone in addition to the aggregation pheromone (Hallet et al., 1995).

In order to determine whether the male and female responses in *H. consanguinea* would be different during the calling period, we collected the capture data for both sexes at intervals of 5–10 min. Throughout the period of mating activity, the average ratio of three traps separated by 10 m was very close to 1 : 1 (Figure 3). Because the normal ratio in the field is also 1 : 1, we concluded that both males and females respond equally to the female-released compound.

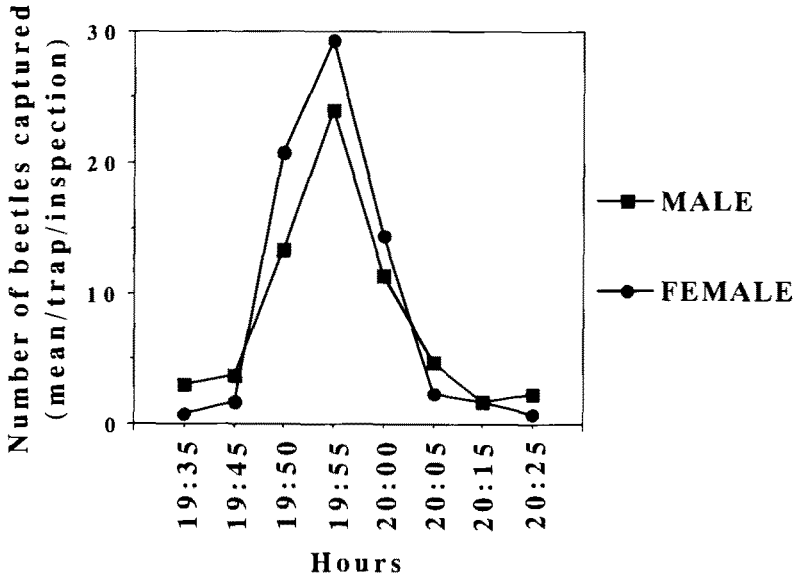


FIG. 3. Male and female responses of *H. consanguinea* as revealed by trap catches during the flight activity of July 12, 1995, in Durgapura. Throughout the period the male-to-female ratio was close to 1 : 1.

Sex pheromones have been considered by researchers to be those compounds that are emitted by individuals of one sex and that cause attraction of members of the opposite sex, resulting in the location of the emitter, and subsequently, mating (Baker, 1989; Tamaki, 1985; for a review of terminology, see also Nordlund, 1981). In that sense, anisole may be considered a sex pheromone because it is emitted by female beetles and males responded by displaying a clear sexual behavior. Males attracted to anisole tried to copulate with any individual (either male or female) in the vicinity. They also fluttered their wings when sitting on the trap lips or on the strings used to hang the traps. However, females were equally attracted to the same compound, and those that were not trapped displayed calling behavior on the traps.

The active compounds could be referred to as aggregation pheromones (sensu Borden, 1985): "substances that are produced by members of either and both sexes that induce members of both sexes to aggregate." It is not clear on an evolutionary basis why competing females are attracted to the female-released semiochemical. Thus, we rather prefer to refer to the semiochemical of *H. consanguinea* as a "female-released pheromone."

It is worth noting that aggregations of both sexes of melolonthines species

have been documented in other species as well. In the case of *H. serrata*, a species also native to India, it has been hypothesized that the chemical cues involved in the aggregation are not likely produced by only one of the two sexes to attract the opposite sex, because, if only females produce the cues to attract males and/or females, then the formation of pure male clumps could not be explained. Similarly, the formation of pure female clumps could not be explained if only males produced the pheromones to attract the males and/or females (Ganeshian and Kumar, 1993). We have no evidence for the formation of clumps of only one sex in *H. consanguinea*.

The adaptive basis of pheromone response by both sexes is not clear. It might not be selected for attracting females because it increases competition for mates. However, if the aggregation has any advantage other than mate finding, selection could be expected to favor the use of a pheromone that is not sex-specific. Due to our meager knowledge of the chemical ecology of this species, the advantage is obscure to us. It is intriguing, however, that such a phenomenon was found in a species that displays calling behavior in a very narrow window. Minimizing the period of calling and having, at the same time, a pheromone that attracts both males and females could be advantageous in escaping from a predator, for example.

These findings seem to shed more light on the secondary function hypothesis for the evolution of sex pheromonal communication in melolonthines (Leal, 1995). More recent findings support the conclusion that melolonthines largely utilize more "primitive" compounds as sex pheromones that may be also involved in other primary functions (Yarden et al., 1996; Leal et al., unpublished data), although one exception has been found. (*R*,*Z*)-7,15-Hexadecadien-4-olide has been identified as a sex pheromone of the Melolonthinae beetle *Heptophylla picea* (Leal et al., 1996).

Additional strong evidence for the secondary function hypothesis for the origin of sex pheromones in scarab beetles is the serendipitous discovery of the attraction of male *Cyclocephala lurida* (Scarabaeidae: Dynastinae) to grubs (Haynes et al., 1992). A recent study has been conducted (during a sabbatical leave of W.S.L. at Cornell University) to test whether the attraction to grubs was mediated by the same chemical(s) used by adults as sex pheromone. GC-EAD analyses of airborne volatiles and whole-body extracts from female beetles and whole-body extracts from grubs revealed that there is a common EAD-active component in the grubs and adult female extracts. That this EAD-active compound was involved in pheromonal communication was corroborated by a GC–behavioral bioassay (GC-BB). Males responded by gathering at the GC outlet soon after the EAD-active peak eluted from the GC, and some males displayed precopulatory behavior (Leal et al., unpublished data). The hypothesis for the ontogeny of the sex pheromone in this species is discussed elsewhere (Haynes and Potter, 1995).

If the chemical has preceded the evolution of functional communication in scarab beetles, it is possible that only later in the evolution a signal becomes sex-specific. *H. consanguinea*, for example, could be on an evolutionary ladder in which the signal is yet to be evolved as a sex-specific chemical cue.

We hope that this speculative hypothesis will stimulate further research on chemical communication of scarab beetles.

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