Semiochemicals of the Scarabaeinae, IV*: Identification of an Attractant for the Dung Beetle *Pachylomerus femoralis* in the Abdominal Secretion of the Dung Beetle *Kheper lamarcki*

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Both sexes of the dung beetle *Pachylomerus femoralis* were observed to be attracted to the male dung beetle *Kheper lamarcki* when it exhibited typical calling behaviour including the release of a white flocculent sex-attracting secretion. Using GC-MS analysis and gas chromatography with electroantennographic (EAD) and flame ionization detection (FID) in parallel, methyl and ethyl propanoate, methyl and ethyl butanoate, methyl and ethyl pentanoate, and methyl and ethyl 4-pentenoate were identified as the constituents of the secretion that elicited electroantennographic responses in the antennae of male and female *P. femoralis*. In field tests, mixtures of these compounds were found to be about as attractive as horse dung. These esters appear to constitute an attractant for *P. femoralis*, enabling this species which does not form balls, to detect and utilize dung balls formed by *K. lamarcki*. *P. femoralis* has been observed to exploit food types as diverse as dung from rhinoceros and primates. It is therefore, possible that, on the other hand, this species utilizes different kairomones to detect various types of dung and that the coincidental presence of some or all of the components of one of these kairomones in the abdominal secretion of *K. lamarcki* is responsible for its attraction to calling *K. lamarcki* males.

Introduction

The peculiar pheromone secreting behaviour of the male dung beetle *Kheper lamarcki* (MacLeay), the chemical characterization of the proteinaceous carrier material constituting the bulk of the abdominal sex-attracting secretion produced by the secreting male, and the identification of the major volatile constituents present in the carrier material, have been described in earlier papers (Burger *et al.*, 1983, 1990). The genus *Kheper* is in general confined mainly to the hot Lowveld areas of the Natal and Transvaal provinces of South Africa, although *K. lamarcki* has a somewhat wider distribution, also being numerous in the central summer-rainfall areas of the Transvaal and the Orange Free State. *Pachylomerus femoralis* (Kirby) inhabits the northern parts of Natal and the Transvaal and its distribution overlaps with that of *K. lamarcki* in areas with sandy soil which appears to be a prerequisite for colonization by *P. femoralis* (Tribe, 1976). Whereas *K. lamarcki* is an industrious producer of dung balls, *P. femoralis* has never been observed constructing dung balls from dung taken from dung pats or middens. It has, however, been found rolling dung fragments which mostly have irregular shapes (Burger and Petersen, 1991). *P. femoralis* constructs burrows with a typical appearance in that they slope gently down into the ground. The excavated soil is often left at the mouth of the burrow in the shape of a ramp. Dung fragments or pieces of dung detached from a dung pat are rolled down the ramp by the insect and are butted into the excavated hole with its head.

Even before the attraction of *P. femoralis* to the fruit of the spineless monkey orange tree, *Strychnos madagascariensis*, was first observed in the Mkuzi Game Reserve in northern Natal (Burger and Petersen, 1991), it had been found that in captivity secreting *K. lamarcki* males are often disturbed by *P. femoralis*, whereas *K. nigroaeneus* Boheman did not seem to elicit similar interest from *P. femoralis*. During research aimed at the identification of the sex attractant secreted

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by male *K. lamarcki*, an antenna of *P. femoralis* was inadvertently used in one analysis and several highly volatile constituents of the abdominal secretion of *K. lamarcki* were found to elicit EAD responses in the antenna. The analysis was reproduced several times with other antennae of *P. femoralis*, whereafter the EAD-active constituents of the secretion were identified. The response of *P. femoralis* antennae to constituents of the abdominal secretion of another species was seen as an interesting phenomenon but its significance was only realized when it was later found that similar volatile esters are involved in the attraction of *P. femoralis* to a certain food source (Burger and Petersen, 1991). In this paper we wish to discuss the possibility that the attraction of *P. femoralis* to the abdominal sex-attracting secretion of *K. lamarcki* could have a semiochemical basis.

Materials and Methods

Gas chromatographic determinations were carried out with a Carlo Erba 4160 gas chromatograph with parallel flame ionization and electroantennographic detection (FID/EAD). The instrument was equipped with a 40 m x 0.3 mm glass capillary column coated with OV-1701-OH at a film thickness of 0.4 μm. Helium was used as carrier gas at a linear velocity of 28.5 cm/s at 40 °C, and a temperature programme of 4 °C/min from 40 ° to 250 °C was employed.

All solvents used for extraction purposes were of residue analysis grade (Merck) and all Pyrex glassware used in the handling of the the material was heated to 500 °C in an annealing oven to remove any traces of organic material. Spatulas, syringes, and other equipment used in handling the secretion, were cleaned by rinsing with dichloromethane of residue analysis grade.

The instrumentation used for gas chromatographic analysis with FID/EAD recording in parallel, and the preparation of the antennae of dung beetles for EAG and EAD recording have previously been described in full detail (Burger and Petersen, 1991). Mass spectra were recorded on a Carlo Erba QMD 1000 quadrupole mass spectrometer using the column and gas chromatographic conditions specified above.

Dung beetles were trapped annually during the first week of November in the Mkuzi Game Reserve in Northern Natal, using pitfall traps baited with horse dung (Tribe, 1976) and collected material was transported by air to Stellenbosch. On arrival, one hind leg was removed from each male *K. lamarcki* beetle. This prevented the dispersal of the secretion which, because it is not brushed off, collects on that side of the abdomen as a tuft of cottonwool-like material. The beetles were kept at subtropical temperatures in greenhouses, the floors of which were covered with about 15 cm of moist sandy soil.

When disturbed, male dung beetles immediately stop secreting the attractant to investigate, and may even disappear underground. Males observed to assume the secreting posture, were therefore fenced off from other beetles by using aluminium strips. In some cases the production of secretion continued for periods of up to 2 h. The secreted material was removed periodically from the incapacitated side of the beetle with ophthalmic forceps to prevent contamination with dust particles and the evaporation of the pheromone from the carrier material. The vial with material already collected, was kept cool in an ice-box. Tiny dust and dung particles, picked up from the abdomen of the insects by the emerging secretion, were carefully removed from the secretion viewed by a microscope. In a typical sample preparation, a few mg of the secretion collected from *K. lamarcki* was sonicated for 1 min with 50 μl of dichloromethane in a Reacti-Vial, whereafter the suspension was centrifuged at 3000 rpm for 10 min. In this solvent, the white carrier material was restricted to the upper layer of the solvent, while remaining dust particles were precipitated. The solvent, containing soluble organic material, was carefully removed from between these two layers of solid material with a 100-μl syringe, transferred to a 100-μl Reacti-Vial, and used for analytical work.

Synthetic compounds for comparison were obtained from either commercial sources or synthesized from authentic starting compounds and analyzed by capillary gas chromatography to ensure their purity. Bioassays were conducted by baiting pitfall traps with horse dung or with synthetic compounds as described previously (Burger and Petersen, 1991).

Results and Discussion

Gas chromatographic analysis with parallel FID/EAD recording of the volatile organic fraction extracted with dichloromethane from the abdominal secretion, produced the gas chromatogram shown in Fig. 1. Except for varying signal-to-noise ratios observed in the EAD traces of these analyses, reproducible results, even as far as the relative heights of the peaks in the EAD traces are concerned, were obtained. No EAD responses appeared in control analyses of the solvent and the antennae of male and female *P. femoralis* produced identical results. To avoid loss of highly volatile material from the dichloromethane extract, it was not concentrated by evaporation of the solvent. Splitless injection of the extract was, however, employed in order to obtain the highest possible loading of the capillary column. From the width of some of the peaks in the chromatogram it is clear that the column was, in fact, heavily overloaded with the major constituents. This had to be done in order to obtain an acceptable signal-to-noise ratio in the EAD trace shown in Fig. 1. Unfortunately this resulted in the FID responses of the earlier eluting EAD-active constituents being obscured by the very broad solvent peak, a problem which could have been solved by using a capillary coated with a thicker film of the same or another stationary phase. As expected, this hampered the identification of these constituents. By using suitable background-subtracting routines it was nevertheless possible to identify constituent 3 as methyl propanoate. This component and the others listed in Table I were identified by mass-spectral and retention-time comparison with authentic synthetic compounds. A relatively strong EAD response was observed at a retention time between those of ethyl butanoate (7) and methyl 4-pentenoate (9). No indication of a corresponding FID response is visible in this analysis (Fig. 1) and neither was any indication of the presence of a constituent at the corresponding retention time found in the total ion current trace (TIC) obtained

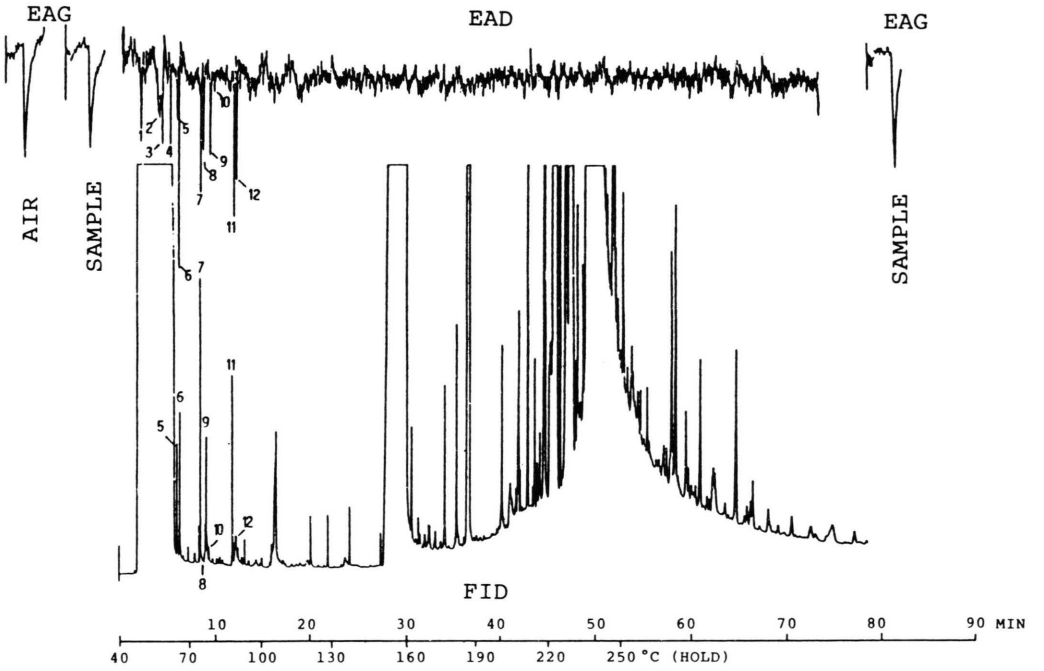

Fig. 1. Gas chromatogram, with FID and EAD recording in parallel, of an extract of the abdominal sex-attracting secretion of the male dung beetle *Kheper lamarcki*. A *Pachylomerus femoralis* female antenna was used as EAD-sensing element. Corresponding peaks in the FID and EAD traces are numbered consecutively. For peak identification see Table I. Glass capillary column coated with OV-1701-OH (40 m x 0.3 mm i.d., film thickness 0.4 μm); temperature programmed from 40° to 250 °C at 4 °C/min.

Table I. Constituents of the abdominal secretion of the male dung beetle *Kheper lamarcki* eliciting antennal responses in excised antennae of male and female *Pachylomerus femoralis* dung beetles.

Peak No. in Fig. 1	Component
1	unidentified
2	unidentified
3	methyl propanoate
4	unidentified
5	ethyl propanoate
6	methyl butanoate
7	ethyl butanoate
8	unidentified
9	methyl 4-pentenoate
10	methyl pentanoate
11	ethyl 4-pentenoate
12	ethyl pentanoate

in GC-MS analyses of the extract. Constituent 8 therefore remained unidentified, as did constituents 1, 2, and 4. Taking into consideration that 1-butanol and certain branched-chain esters were found to elicit EAD responses in *P. femoralis* antennae (Burger and Petersen, 1991), it is, however, possible that at least some of the unidentified constituents could be unsaturated or branched-chain alcohols and/or esters. For interest's sake EAD analyses were also done with the methyl and ethyl esters of the *E* and *Z* isomers of both 2-pentenoic acid and 3-pentenoic acid. It was found that all of these esters elicit EAD responses in *P. femoralis* antennae comparable to those produced by ethyl and methyl 4-pentenoate.

Solventless introduction of the abdominal secretion of *K. lamarcki* into the injector of the GC-MS system could possibly provide mass spectral evidence towards the identification of constituents 1, 2, and 4. With this in mind, attempts were made since 1989 to collect sufficiently large samples of abdominal secretion for this purpose. It is however difficult to induce calling behaviour in *K. lamarcki* in captivity and the quantities of material obtained were too small to provide additional information. In this regard it must be noted that in captivity typically fewer than about 5% of the dung beetles produced abdominal secretion during a few weeks after they had been transported to Stellenbosch. Some of them produced so little secretion that it was practically impossible to collect it. It must furthermore be taken into account that *K. lamarcki* only produces this secretion at relatively high temperatures (ca. 29 °C) and that the secretion is mostly produced at a very slow rate, so that it can only be collected at intervals of 15 to 30 minutes. The secretion is porous and as the volatile esters seem to be present in the secretion in very low concentrations, they are probably largely lost into the atmosphere at the temperatures at which the secretion is produced. The esters are therefore only found in material secreted by dung beetles capable of producing the secretion at an exceptionally high rate and at lower than normal temperatures, as has been observed on a few occasions. It is impossible to trap the volatiles directly from secreting males on headspace traps because calling behaviour cannot be induced in dung beetles kept in suitable vessels as is often done with other insects. Work on the unidentified constituents is being continued.

In several attempts to determine whether the EAD-active compounds are produced in the abdominal glands of the male insect, large samples of extracts of the glands were subjected to GC and GC-MS analyses. Using a solventless sample introduction technique (Burger *et al.*, 1990), samples of excised abdominal glands were also analysed for the presence of these compounds. No indication of the presence of any of the compounds listed in Table I could be found in any of these analyses. It must, however, be taken into consideration that the glandular material contains large quantities of heavy lipid material and that it was not feasible to introduce unlimited amounts of sample into the analytical capillary column. These experiments are therefore not absolutely conclusive in ruling out the presence of the EAD-active compounds in the abdominal glands.

Another possible explanation for the presence of the esters in the secretion is that the compounds are present in the horse dung on which the dung beetles were fed, and were adsorbed on the abdominal secretion. This possibility was investigated by carrying out headspace gas analyses of samples of horse dung. Solid phase micro extraction (Arthur *et al.*, 1992) was used as concentration technique and the volatiles were analysed gas chromatographically with FID/EAD recording in parallel. No indication was found that any of the EAD-active esters are present in the horse dung. In a control experiment the dung was spiked with

small amounts of methyl and ethyl pentanoate. The presence of these compounds in the spiked samples was readily detected by both detection techniques.

In field tests a mixture of the compounds listed in Table I and horse dung were about equally attractive to *Pachylomerus femoralis*. For reasons discussed in the previous paper in this series on the semiochemicals of the Scarabaeinae (Burger and Petersen, 1991), a statistical analysis of the results of field tests was not feasible. Some field tests were, however, carried out to compare the attractivity of the isomeric methyl and ethyl pentenoates. It was found that neither the Z nor the E isomer attracted any dung beetles in the absence of the saturated esters. Although they were not found in the abdominal secretion, the methyl and ethyl esters of the *Z* and *E* isomers of 2-butenoic acid (crotonic acid) were included in the field tests and likewise did not attract *P. femoralis*. In contrast to these results, the individual unadulterated saturated esters attracted small numbers of *P. femoralis*.

In the absence of sufficient behavioural information at this stage, it is difficult to explain the attraction of *P. femoralis* to the fruit of the spineless monkey orange (Burger and Petersen, 1991) and to the abdominal secretion of *K. lamarcki*. It can be argued that the available results and observations indicate that the EAD-active compounds are components of an attractant kairomone for *P. femoralis*. *K. lamarcki* produces the abdominal secretion only when in possession of fresh dung or a dung ball and *P. femoralis*, not being capable of forming dung balls, possibly employs the kairomone to find dung in a suitable shape for transportation to its burrow. The presence of a similar mixture of esters in the fruit of the spineless monkey orange tree, *Strychnos madagascariensis*, would therefore appear to be coincidental. The fleshy material which adheres to the seeds of this tree and which contains the attractant esters, dries out very quickly in the prevailing hot climate in northern Natal and therefore does not seem to be a worthwhile substitute for a dung ball. It is interesting to note that secreting *K. nigroaeneus* are normally not disturbed by *P. femoralis*. Male *K. nigroaeneus* have never been seen to release the abdominal secretion at dung pats, whereas the pheromone is often released by *K. nigroaeneus* males after a brood ball had been buried or even when not in possession of a dung ball (Edwards and Aschenborn, 1988). Developing a mechanism for the detection of a calling *K. nigroaeneus* male would therefore be futile from the viewpoint of *P. femoralis*.

On the other hand, because *P. femoralis* does not seem to prefer a specific type of dung, it is quite possible that this species employs different mechanisms and kairomones for the detection of different dung types. The coincidental presence of some or all of the constituents of one of these kairomones in the abdominal secretion of *K. lamarcki*, could therefore be responsible for the attraction of *P. femoralis* to secreting *K. lamarcki* males. This possibility will have to be investigated in further research on this phenomenon.

Acknowledgements

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