Semiochemicals of the Scarabaeinae, III*: Identification of the Attractant for the Dung Beetle *Pachylomerus femoralis* in the Fruit of the Spineless Monkey Orange Tree, *Strychnos madagascariensis*

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Z. Naturforsch. **46c**, 1073–1079 (1991); received April 15, 1991

Semiochemicals, Insect Attractant, Dung Beetle, Scarabaeinae, Electroantennographic Detection

Following the observation that the non-ball-rolling dung beetle *Pachylomerus femoralis* (Kirby) rolled the flesh-covered seeds from the fruit of the spineless monkey orange tree, *Strychnos madagascariensis* Poiret, an investigation into the chemical attractants of this fruit was made. Using headspace gas chromatography with electroantennographic and flame ionization detection in parallel, followed by GC-MS analysis, 1-butanol, methyl butanoate, ethyl 2-methylpropanoate, ethyl butanoate, butyl ethanoate, ethyl 2-methylbutanoate, propyl butanoate, butyl propanoate, methyl hexanoate, butyl 2-methylpropanoate, and butyl butanoate were identified as the constituents of the attractant. It was found that *P. femoralis* beetles were attracted to a mixture of the synthetic compounds about as strongly as to the fresh fruit. The beetles were also equally attracted to the fruit and to horse dung in areas where ripe fruit was not available. In areas permeated with the smell of the ripe fruit the horse dung retained its attractiveness, whereas no beetles were caught in traps baited with fruit.

Introduction

Both species of the African dung beetle genus *Pachylomerus* are found in southern Africa, but do not overlap in distribution. *P. femoralis* inhabits wetter areas, occurring from the northern parts of Natal, through northern Transvaal and into north-eastern Botswana. *P. opaca* Lansberge is smaller than *P. femoralis* and inhabits the drier north-western Cape and southern Namibia [1]. *P. femoralis* is a large dung beetle (*ca.* 40 × 25 mm) with highly developed prothorax and forelegs which led Halffter and Matthews [2] to question whether this genus had lost the ability to roll dung balls. According to Tribe [1] the typical behaviour of *Pachylomerus* in the field is to construct an unbranched tunnel within 300 mm of a dung pat by digging with the foretibiae and clypeus and then turning around in the burrow and pushing out the loosened soil or sand, using the prothorax as a shovel. The excavated soil is used to build a ramp which leads to the dung pat. The burrow is provisioned with dung by the beetle making several trips to and from the dung. Irregular pieces of dung are then either butted with the head or rolled to the burrow entrance and pushed inside with the head. *P. femoralis* is very aggressive, defending both a large section of the dung pat and the ramp against any intruder [1].

During the past 10 years annual field trips were undertaken to Mkuzi Game Reserve in northern Natal to collect material for research on the abdominal sex attractant produced by male beetles of the genus *Kheper*. As large numbers of *P. femoralis*, the largest dung beetle in the reserve, were often found together with *Kheper lamarcki* (M'Leay) and other *Kheper* species on dung middens, it was possible to observe some of the peculiarities in the behaviour of this insect. Although we have never observed the formation of dung balls from dung taken from a dung pat or midden by *P. femoralis*, this species has on a few occasions been found rolling dung fragments along the roads of the reserve. The dung fragments mostly had irregular shapes, not having been formed into balls, and are relatively small in comparison to the size of the enormous dung balls that the slightly smaller *K. lamarcki* usually constructs. Another peculiarity is that on the few occasions that this ball-rolling behaviour had been observed, several *P. femoralis*

* For the preceding paper in this series see B. V. Burger, Z. Munro, and W. F. Brandt, Z. Naturforsch. **45c**, 863 (1990).

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0939–5075/91/1100–1073 $ 01.30/0

were found rolling dung fragments along the roads of the reserve during the late afternoon, whereas the behaviour was not repeated on the following days. It therefore seems possible that the ball rolling behaviour is induced by metereological conditions. During previous visits to Mkuzi there was no ripe fruit on the spineless monkey orange trees (*Strychnos madagascariensis* Poiret) that are found in certain parts of the reserve. However, in November 1989 a *P. femoralis* was observed rolling one of the seeds of a spineless monkey orange. It appeared to be a rare phenomenon, but subsequent work revealed this behaviour to be quite common in this insect.

The spineless monkey orange tree, also known as the yellow or black monkey orange tree in certain parts of South Africa, is found in large numbers in those parts of the Mkuzi Game Reserve with light sandy soil. It bears fruit with the shape, size and colour of an orange, but with an extremely hard shell. Each fruit contains 10 or more hard seeds to which a thin layer of soft flesh tightly adheres. The seeds have irregular shapes and an average diameter of about 20–30 mm and are interspaced with thin layers of fleshy material which is eaten by animals having strong enough teeth and jaws to fracture the shell of the fruit. The fruit has a strong pleasant flavour similar to that of the mango, but as the edible parts form a relatively small proportion of the fruit, it has not been commercially exploited for human consumption. The fruit usually start ripening towards the middle of November in the northern parts of Natal. In a preliminary test using 12 seeds, two were removed by *P. femoralis* within 10 min and in another experiment 24 *P. femoralis* beetles were caught in two pitfall traps baited with seeds of the fruit, whereas only one *P. femoralis,* in addition to several dung beetles from other species, was caught in nine traps baited with horse dung.

These observations led to an investigation of the chemical basis for the attraction of *P. femoralis* to the fruit of *S. madagascariensis*. In this paper we wish to report the results of experiments in which the constituents of the effluvium of the ripe fruit were isolated by headspace gas chromatography, and the compounds eliciting beetle antennal responses in an electroantennographic detector (EAD) characterized by gas chromatography-mass spectrometry (GC-MS).

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) [3]. The instrument was equipped with a 40 m × 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 2 °C/min from 40 °C to 250 °C was employed.

The instrumentation used for parallel FID/EAD is illustrated in Fig. 1. Using a 4-way junction, the column effluent was diluted with helium at a flow-rate of 15 ml/min and the diluted effluent split in a 1:1 ratio between the FID and EAD. One outlet of the splitter was connected to the FID and the other was used to introduce the diluted column effluent into a stream of humidified air flowing over the antenna at a linear velocity of 15 cm/s.

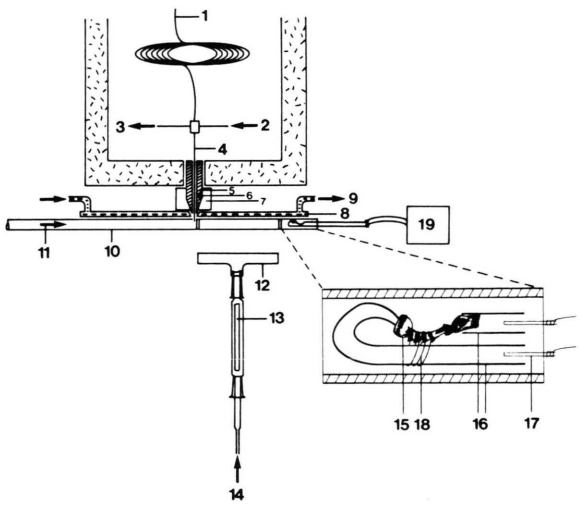

Fig. 1. Top view of a gas chromatograph equipped with an effluent splitter for flame ionization detection (FID) and electroantennographic detection (EAD) in parallel: 1 = capillary column; 2 = make-up gas at 15 ml/min; 3 = FID; 4 = fused silica capillary; 5 = aluminium block; 6 = heater; 7 = insulation; 8 = heat shield; 9 = water at 18 °C; 10 = glass tube, 5 mm i.d.; 11 = humidified air at 15 cm/s; 12 = T-piece for recording of EAG; 13 = paper strip impregnated with sample; 14 = air puffed over paper strip and antenna; 15 = antenna; 16 = glass capillaries with saline solution; 17 = Ag/AgCl electrodes; 18 = antenna tied to the lower capillary with 0.1 μm polyester thread; 19 = amplifier.

The insects were too strong to tether and thus the antennae were removed and used instead. An antenna was inserted lengthwise into the air duct as this arrangement produced a less noisy baseline than positioning the antenna sideways in the air stream, a position in which the club of the antenna is subjected to the effect of the turbulent mixing of the column effluent and ambient air. The antenna was protected against heat radiation from the gas chromatograph by a heat shield cooled with water at 18 °C. Electrical responses in the antenna were recorded *via* Ag/AgCl electrodes placed in pipettes filled with saline solution [4] containing NaCl (7.5 g/l), $CaCl_2$ (0.21 g/l), KCl (0.35 g/l), and $NaHCO_3$ (0.2 g/l), as well as polyvinylpyrrolidone K 90 (Fluka, 40 g/l) to increase its viscosity [5]. Antennal responses were amplified with a Murphy Developments AMS-025 amplifier, using a time constant of 12 s and were recorded on an Omni-Scribe recorder. A section of the air duct between the tip of the capillary column and the antenna could be replaced by a T-piece (Fig. 1) for the introduction of air or whole spineless monkey orange headspace gas into the air flowing over the antenna in order to test the system by recording EAG responses. Mass spectra were recorded on a Carlo Erba QMD 1000 quadrupole mass spectrometer using the column and gas chromatographic conditions specified above.

The volatile organic components of the fruit of *S. madagascariensis* were trapped on short open tubular capillary traps (70 mm coated with 15 µm of PS-255) for qualitative analysis using headspace analytical methods described by Grob and Habich [6], and Burger *et al.* [7, 8]. A 1 l round-bottomed flask, cleaned by heating at 500 °C in a muffel oven to remove all traces of organic material, was fitted with a screw cap having a PTFE-lined septum. Two glass capillaries (0.5 mm i.d.) were inserted into the flask through the septum. Nitrogen, purified by passing it through a column of activated charcoal, was introduced into the flask through the longer of these capillaries reaching to about 10 cm from the bottom of the flask. The second, shorter capillary was used to conduct the nitrogen from the upper part of the flask to an open tubular capillary trap to which it was connected with shrinkable PTFE tubing.

Qualitative analyses of the aroma components of the spineless monkey orange were carried out using the seeds from one fruit. The material was placed in the flask at 22 °C and left to equilibrate for 30 min, whereafter 30 ml of the headspace gas was pushed through the capillary trap at a flow rate of 5 ml/min with purified nitrogen. The connection of the trap to the capillary column, desorption of the volatiles, and GC and GC-MS analyses were carried out as described in full detail in a recent paper on the application of capillary traps in headspace gas analysis [8]. The traps used for qualitative determinations contained an immobilized film of the apolar phase PS-255 at a film thickness of 15 µm.

Quantitative determinations of the organic volatiles released into a slow stream of nitrogen flowing over the contents of a spineless monkey orange, was carried out using the recently developed ultra-thick film traps having an apolar polysiloxane rubber lining with a thickness of 145 µm [7]. The seeds with flesh attached were removed from one fruit and placed at one end of a wind tunnel consisting of a glass tube with an internal diameter of 80 mm. Nitrogen, purified by passing it through an activated charcoal filter, was introduced at this end of the tube at a linear velocity of 0.16 km/h. The volatiles were sampled by sucking 2 ml of the headspace gas through two ultra-thick film traps connected in series to an outlet situated 300 mm downstream from the fruit. Sampling was started 6 min after the nitrogen flow had been turned on, and was continued for 6 min at a flow rate of *ca.* 0.33 ml/min. The compounds desorbed from the first trap were quantified using a mixture of the synthetic EAD-active constituents of the fruit as external standards. The second trap was subjected to a similar procedure in order to detect and quantify the breakthrough, if any, of the volatiles to the second trap.

Synthetic compounds for comparison purposes were obtained from commercial sources, or were synthesized from authentic starting compounds and analyzed by capillary gas chromatography to ensure their purity.

Bioassays were conducted by baiting pitfall traps [9] with horse dung, the fleshy parts of the fruit of *S. madagascariensis* or with synthetic compounds. The synthetic compounds were dispensed from 1.8 ml screw-cap septum vials by replacing their septa with filter-paper wicks dipping into 200 to 400 µl quantities of solutions of the synthetic

compounds in hexane. The vials were suspended about 15 cm above the pitfall traps.

Results and Discussion

Gas chromatographic headspace analysis is the preferred method for the determination of the aroma constituents of food and beverages. If the volatiles retained on a concentration trap is thermally desorbed, it is possible to detect constituents that could otherwise be obscured by the solvent peak when using conventional extraction methods. The headspace analytical method developed by Grob and Habich [6] in which the volatiles, trapped off-line on an open tubular capillary trap, and desorbed in the injector of the chromatograph, requires very little expertise and no additional hardware, except for a short length of shrinkable PTFE tubing to connect the trap to the capillary column. Activated charcoal is widely used as adsorbent in packed as well as in open tubular capillary traps, but it has been found that thermal desorption of the trapped volatiles leads to the formation of artifacts [6, 10]. A high concentration of moisture may also result in incomplete trapping of less volatile and less polar compounds on such traps [8]. Artifact formation can be avoided by using open tubular traps containing a thick film of an apolar stationary phase (FTs). However, the more volatile constituents of a headspace gas sample may have low breakthrough volumes on these traps, *i.e.* breakthrough could already occur after a relatively small volume of the headspace gas had passed through the trap. These traps are therefore not suitable for the quantitative determination of highly volatile headspace constituents. They could, however, be ideal for qualitative determinations, as the less volatile constituents that are mostly present in low concentrations in headspace gas samples, can be selectively concentrated without overloading the capillary column with the more volatile ones. The identification of the attractive constituents in the spineless monkey orange aroma was carried out with a FT having a film thickness of 15 μm. During the final stages of the investigation ultra-thick film traps (UFTs) became available [7] and these were employed for the quantitative determination of the active constituents.

The compounds that could possibly be responsible for the attraction of *P. femoralis* to the spineless monkey orange were distinguished from the other inactive volatiles using FID and EAD in parallel. In an attempt to extend the life of the antennae used in such a biological detector, the air flowing over the detector was cooled to 15 °C. This, however, resulted in droplets of condensed water forming in the air duct. This water impedes the rapid passage of polar compounds through the air duct and can be expected to result in the prolonged saturation of the olfactory sensilla with the major active constituents to such an extent that minor active constituents, eluting just after an EAD-active peak, might not be observed. The problem in EAD is to deliver the column effluent into the air flowing over the antenna by a fused silica capillary that is heated to as near to its end as possible, in order to avoid condensation of the less volatile compounds in the tip of the capillary, while at the same time ensuring that the air flowing over the antenna remains saturated with water to avoid dehydration of the antenna. It was found that using a water-cooled insulator between the air duct and the gas chromatograph was more effective in keeping the temperature of the air in the duct constant and shielding the antenna against heat radiation than asbestos and other insulating materials used in earlier experiments. The *P. femoralis* antennae could then be used for more than 8 h.

The FID/EAD analyses, an example of which is shown in Fig. 2, gave remarkably reproducible results, even as far as the relative peak sizes in the EAD trace are concerned. Male and female antennae produced identical responses. The constituents giving rise to EAD responses that coincided precisely with peaks in the FID trace, were identified by GC-MS analysis, followed by retention time comparison with authentic synthetic samples using FID and EAD in parallel. The compounds giving EAD responses are listed in Table I. It is interesting to note that there is a preponderance of compounds having C_4 units amongst the EAD-active compounds. Many other constituents related to the EAD-active compounds, such as 1-hexanol, butanoic acid, hexanoic acid and a large number of saturated and unsaturated esters from 2-methylpropyl ethanoate to butyl dodecanoate that are present in the fruit [11] gave no, or very weak, EAD responses.

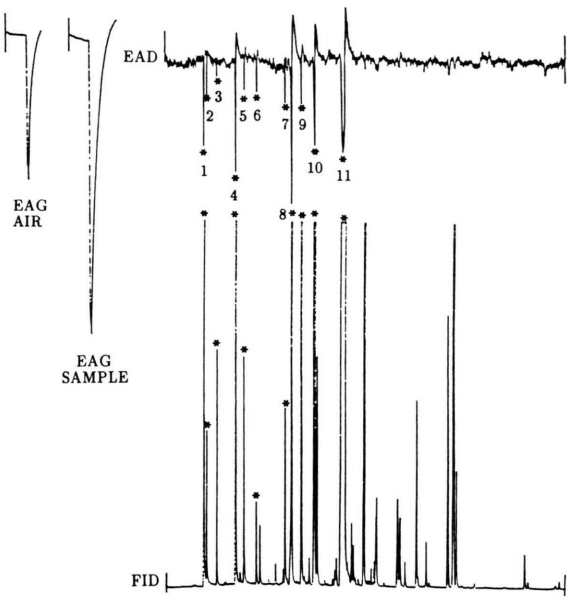

Fig. 2. Gas chromatogram with FID and EAD recording in parallel of the headspace gas volatiles from fruit of the spineless monkey orange tree, *Strychnos madagascariensis*, concentrated under static conditions on an open tubular capillary trap coated with 15 µm of PS-255. A *Pachylomerus femoralis* female antenna was used as EAD-sensing element. Corresponding peaks in the FID and EAD traces are marked with an asterisk and are numbered consecutively. For peak identification see Table I. Glass capillary column coated with OV-1701-OH (40 m × 0.3 mm i.d., film thickness 0.4 µm); temperature programmed from 40 to 220 °C at 2 °C/min.

Table I. Constituents of the headspace gas of the fruit of the spineless monkey orange tree, *Strychnos madagascariensis*, that elicited antennal responses in excised antennae of the dung beetle *Pachylomerus femoralis*.

Peak No. in Fig. 2	Component	Amount (µg/ml headspace gas)[a]
1	1-butanol	0.06
2	methyl butanoate	0.005
3	ethyl 2-methylpropanoate	0.02
4	ethyl butanoate	0.38
5	butyl ethanoate	0.02
6	ethyl 2-methylbutanoate	0.01
7	propyl butanoate	0.04
8	butyl propanoate	0.06
9	methyl hexanoate	0.05
10	butyl 2-methylpropanoate	0.26
11	butyl butanoate	2.04

[a] Headspace gas samples were withdrawn from a stream of nitrogen flowing at a linear velocity of 0.16 km/h over the contents of one monkey orange at a point 300 mm downstream from the fruit.

The qualitative analyses were carried out using a static procedure in which headspace gas in equilibrium with the fruit was sampled. The results of these determinations clearly cannot be expected to provide information on the composition of the aroma of the fruit under conditions in the field, for example when there is a slight breeze. For determinations aimed at the quantitative imitation of the EAD-active bouquet of the fruit, the organic volatiles were therefore determined in a current of air flowing at approximately 0.16 km/h over 20 seeds and fleshy parts of a spineless monkey orange. The volatiles were trapped on an ultra-thick film trap [7] using a sample volume and headspace gas flow rate at which only a small percentage of the 1-butanol broke through to a second trap connected in series to the first. A gas chromatogram obtained in one of these analyses is shown in Fig. 3 and the quantitative results are included in Table I.

As no dung beetles were caught in pitfall traps baited with fruit such as banana and paw-paw, banana was used as dispensing medium for small quantities of a mixture of the synthetic EAD-active compounds in preliminary field tests. In comparison to fresh seed or dung, a synthetic mixture containing the EAD-active constituents in a quantitative ratio corresponding to the figures in Table I, elicited very little response. The reason for this result appeared to be that the synthetic compounds were absorbed very rapidly by the banana and were released so slowly that the typical pleasant smell of the spineless monkey orange could not be detected after a short while. A higher release rate was obtained by dispensing the solution from a small vial *via* a filter-paper wick. This method cannot be expected to release the compounds in the same ratio in which they are present in the mixture, as the more volatile compounds will evaporate faster than the others, resulting in a steadily increasing concentration of the less volatile constituents accumulating on the tip of the wick. However, in spite of the continuously changing quantitative composition of the attractant plume released in this manner, 8 and 14 *P. femoralis* were caught within 2 h in two pitfall traps baited with the synthetic material, whereas 5 and 32 dung beetles were found in traps baited with spineless monkey orange. It was furthermore found that the individual compounds and mixtures of two or three of the compounds were also attractive to *P. femo-*

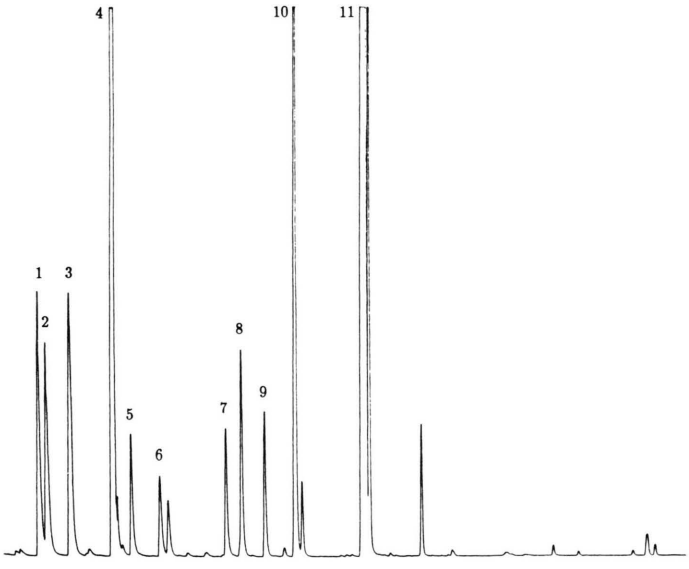

Fig. 3. Gas chromatogram of the volatiles quantitatively trapped from 1 ml of headspace gas from the fruit of the spineless monkey orange tree, *Strychnos madagascariensis*, sampled under dynamic conditions from a stream of nitrogen flowing over the contents of the fruit at a linear velocity of 0.16 km/h. Numbering of compounds and gas chromatographic conditions as in Fig. 2.

ralis, 3 insects being found in a trap baited with a 1:1 mixture of methyl and ethyl butanoate and 8 insects in a trap baited with a 4:4:1 mixture of butyl 2-methylpropanoate, butyl butanoate and butyl propanoate.

In a series of field tests carried out to obtain results for statistical analysis, some interesting observations were made with regard to the attraction of *P. femoralis* to traps baited with horse dung and spineless monkey orange. At the time when these tests were carried out in November 1990, the spineless monkey orange season had reached its peak and large numbers of fruit, some of them opened by monkeys, were lying beneath the trees. In areas with a large population of these trees, the air was permeated with the smell of the fruit and as anticipated, no dung beetles were caught in traps baited with the fruit, whereas more than 30 *P. femoralis* were found in traps in the same area baited with horse dung. It must be noted that the area where this test was carried out, lies in and around the rest camp where there is considerable traffic and human activity and which is not frequented by large herbivores. Dung of the type preferred by *P. femoralis* is therefore not available in this area. In contrast, *P. femoralis* appeared to be attracted more strongly to the fruit than to horse dung in areas where there is much animal activity and where *S. madagascariensis* trees are scarce or the ripe fruit is not available. The tests mentioned in the introduction were carried out in such an area. In spite of fierce competition for food on relatively small quantities of horse dung placed in the field, no dung ball formation or rolling of balls or fragments of dung from these dung sources by *P. femoralis* was observed. In contrast, practically all the *P. femoralis* attracted to the contents of spineless monkey oranges started frantically rolling seeds either immediately on arriving at the fruit or after inspecting the fruit for a few seconds. A few examples of dung beetles attempting to push seeds into existing holes in the ground were observed.

In the areas where these tests were carried out, large differences were found in the numbers of beetles caught in traps baited with either dung or fruit. One trap, for example, contained 38 *P. femoralis*, whereas not a single beetle of this species was found in a trap situated 100 m from the first one and baited with the same material. Due to dense vegetation it was impossible to observe the movements of animals in the areas where the tests were carried out and to determine how far a trap was situated from, for example, a rhinoceros dung midden containing an enormous quantity of dung, or from the nearest spineless monkey orange tree. Under such circumstances, changes in the direction of air movement can have a pronounced effect on the results of the tests. It has furthermore to be taken into consideration that these dung beetles can cover distances of several kilometers at speeds

of more than 30 km/h. Under these conditions it was found to be impossible to obtain quantitatively reliable data. It is clear that tests will have to be carried out over longer periods and that a method will have to be developed to release the synthetic material at a more accurately controlled rate before a final conclusion about the activity of the synthetic material can be reached. However, the results obtained so far indicate that the attractiveness of the synthetic mixture for *P. femoralis* is at least comparable to that of the fresh spineless monkey orange.

The attraction of *P. femoralis* to fruit is not a unique phenomenon. In a recent paper on the behavioural evolution of non-ball-rolling dung beetles, Halffter and Halffter [12] have mentioned the attraction of dung beetles to carrion and rotting fruit and in Mkuzi Game Reserve a few of the small golden-brown dung beetles, *Proagoderus aureiceps* (d'Orbigny), were also found in many of the traps baited with spineless monkey orange. It will be interesting to find out whether the presence of derivatives of short-chain fatty acids, such as butanoic acid, is the common factor in the attraction of certain dung beetle species to food sources other than dung.

Acknowledgements

Support of this work by the University of Stellenbosch and the Foundation for Research Development, Pretoria, is gratefully acknowledged. The authors are indebted to the Natal Parks Game and Fish Preservation Board for permission to study and collect dung beetles in the Mkuzi Game Reserve. We thank Mr. G. D. Tribe, Plant Protection Research Institute, Rosebank, Cape Town, for reading the manuscript.

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