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Research Article

Ladybird beetle odour identified and found to be responsible for attraction between adults

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Abstract. The distinctive odour of the seven-spot ladybird beetle, *Coccinella septempunctata*, had not previously been identified unequivocally, although it was considered likely to arise from olfactorily potent pyrazines. The component responsible was located by human organoleptic evaluation of the gas chromatography effluent from ladybird volatiles and was fully characterized as 2-isopropyl-3-methoxypyrazine. Although insects may not be expected to have similar olfactory sensitivities to human beings, this compound was found to fulfil a pheromonal role in the attraction between adult *C. septempunctata*, typical of many ladybird species. Thus, in a behavioural assay, both males and females were attracted by amounts of this pyrazine equivalent to the ladybird volatiles.

Key words. Ladybird beetle; *Coccinella*; Coccinellidae; pheromone; attractant; pyrazine; predator.

Ladybird beetles, or ladybugs (Coleoptera: Coccinellidae), are important predators of pest aphids (Homoptera: Aphididae) and could be exploited more effectively as biological control agents by use of semiochemicals (behaviour-controlling chemicals), including pheromones [1, 2]. It is well known that some Coccinellids aggregate in the autumn [3], and any semiochemicals mediating such behaviour could be employed in strategies to preserve overwintering populations. Most studies on the chemical ecology of Coccinellids have concentrated on the chemistry associated with their aposematic colouration [4, 5]. Coccinellids with bright colours 'advertise' to predators that they possess a defence system, in this case a reflex bleed of haemolymph containing alkaloids with antifeedant and toxic activity together with volatile repellents. Recent reports on semiochemicals of Coccinellids are limited to studies on the sex pheromone of the two-spot ladybird, *Adalia bipunctata* L. [6]. Nonetheless, it is widely accepted that other semiochemicals comprising alkylmethoxypyrazines are commonly used as olfactory alerting signals by aposematic insects [7]. More specifically, 2-isopropyl-, 2-sec-butyl- and 2-isobutyl-3-methoxypyrazine (fig. 1; **I, II** and **III**, respectively) were suggested as components of the seven-spot ladybird, *Coccinella septempunctata* L., but tentative identifications only were achieved, using selected ion monitoring of volatile extracts and comparison of gas chromatography (GC) retention times [8]. In this study, the chemical

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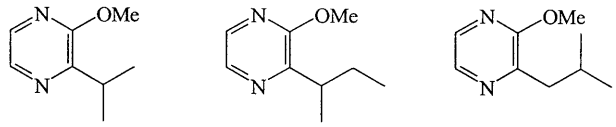

Figure 1. Alkylmethoxypyrazines tentatively identified from *C. septempunctata*.

identity of the aroma perceived by human beings and the chemical basis of pheromonally mediated ladybird aggregation were investigated.

Materials and methods

GC-organoleptic evaluation. Adult male and female *C. septempunctata* (ca. 1000), collected from the grounds surrounding IACR-Rothamsted and kept in ventilated sandwich boxes at 20 °C until required, were cooled with liquid nitrogen and extracted with freshly distilled chloroform (2 × 200 ml) for 24 h (48 h in total) at 25 °C. The combined extracts were dried using anhydrous magnesium sulphate, filtered and evaporated to ca. 5 ml. Volatiles were collected by vacuum distillation (0.03 torr) for 21 h at 25 °C, and the resulting extract was concentrated under a stream of nitrogen to 100 ml and stored in a tightly capped microvial at 20 °C. To determine which of the volatile components resembled, to human beings, the characteristic odour of Coccinellids, a panel of two judges (M.A.B., J.A.P.) was assembled to perform organoleptic evaluation. Prior to chromatographic separation, the odour of the entire vacuum distillate was assessed. Olfactory sampling of the GC effluent began 2 min after injection. Volatiles were separated on an AI 93 GC equipped with a cold on-column injector, a flame ionization detector (FID) and a 50 m × 0.32 mm i.d. HP-1 capillary column. The oven temperature was maintained at 40 °C for 2 min and programmed at 5 °/min to 100 °C, then at 10 °/min to 250 °C. The carrier gas was hydrogen.

Chemical analysis. Coupled GC-mass spectrometry (MS) was provided by a capillary GC column (50 m × 0.32 mm i.d. HP-1) fitted with an on-column injector directly coupled to the mass spectrometer (VG Autospec, Fisons Instruments, Manchester, UK). Ionization was by electron impact at 70 eV, 250 °C. The oven temperature was maintained at 30 °C for 5 min and then programmed at 5 °/min to 250 °C. Tentative identification by GC-MS was confirmed by peak enhancement on GC [9] with authentic 2-isopropyl-3-methoxypyrazine (97%) (Al-drich Chemical Company), using columns with different polarities: 50 m × 0.32 mm i.d. HP-1 (nonpolar) and 30 m × 0.3 mm i.d. SPB-35 (polar).

Olfactometer studies. *C. septempunctata* adults were collected from overwintering sites on a sandy ridge ca. 6 km south of Uppsala, Sweden. To avoid problems associated with sudden temperature changes, insects were collected only at temperatures above 0 °C, then brought to room temperature after an adaptation period of 3 h at 10–15 °C and kept in a glasshouse (18–22 °C) in cages infested with bird-cherry-oat aphids, *Rhopalosiphum padi* L., until used. The behavioural assays on *C. septempunctata* adults were carried out in a Perspex olfactometer, modified from that previously described [10] for use with walking insects. This comprised a weak airstream being directed towards the centre of the olfactometer from two drawn-out arms to which volatile sources were applied at the inlets. The arena was divided into two zones with a neutral zone in the centre. The olfactometer was surrounded by a white paper screen (30 cm high) to minimize the effect of visual stimuli in the environment. Both arms were supplied with moist filter paper (2 cm × 2 cm) to minimize differences in relative humidity. To avoid arena contamination, the bottom of the olfactometer was covered with white paper, which was changed between experiments. For each experiment, one *C. septempunctata* adult was introduced into the centre of the chamber, and its position was noted every 2 min for 20 min. Each experiment was replicated 5 or 10 times, and the results were analysed by paired *t* test; the number of visits into the treatment arm were compared with visits to the control arm. Stimuli comprised: (i) one *C. septempunctata* adult, either male or female; (ii) *C. septempunctata* vacuum distillate (0.04 insect equivalents containing 0.05 ng/μl 2-isopropyl-3-methoxypyrazine); (iii) 2-isopropyl-3-methoxypyrazine at 0.05 ng/μl. Treatments (ii) and (iii) were applied in 0.5-μl microcaps. If the insect did not move between two observations, the experiment was terminated and the data discarded.

Results and discussion

To characterize precisely the distinctive Coccinellid odour, volatiles from overwintering male and female *C. septempunctata* were collected by vacuum distillation and subjected to human organoleptic (olfactory) evaluation. In a system similar to that of coupled GC-electrophysiology [11], where GC-separated volatiles are passed over an insect antenna to locate areas of activity, the GC effluent was presented to the human nose for assessment of the components. One minor peak only from the vacuum distillate was found to be associated with the distinctive aroma of *C. septempunctata* (fig. 2)

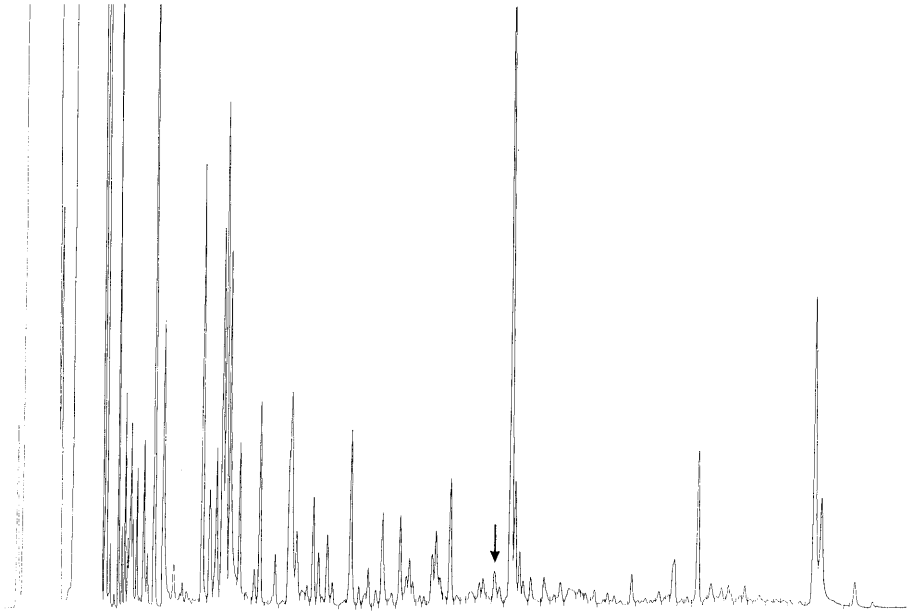

Figure 2. Gas chromatogram (50 m × 0.32 mm i.d. HP-1 column) of vacuum distillate from extract of *C. septempunctata* adults; arrow, peak at which organoleptic activity was detected.

and was tentatively identified, by coupled GC-mass spectrometry (fig. 3), as arising from 2-isopropyl-3-methoxypyrazine (**I**) by comparison with the published spectrum [12]. Identification was confirmed by peak enhancement on GC with authentic compound, using two capillary columns of different polarity. The authentic compound, at the concentration found in the vacuum distillate, was judged by the human panel to have the same aroma characteristics. This study provided the first unequivocal evidence that 2-isopropyl-3-methoxypyrazine is present in, and is responsible for, the characteristic odour of *C. septempunctata* adults.

Attention was then turned to the insect-insect interactions. The volatiles released by live male and female *C. septempunctata* were investigated in behavioural assays using an olfactometer and were shown to be significantly attractive to conspecific adults (table 1). The vacuum distillate isolated from a chloroform extract of *C. septempunctata* adults was equally attractive. At this point in the study, identification of pheromonally active components would normally involve GC analysis coupled with electrophysiological recordings from the insect antenna [11]. However, in this case, a compound highly active in human olfaction, i.e. the pyrazine **I**, was already available through the coupled organoleptic investigation. The compound, through having a nitrogen heteroatom, is likely to have a similarly high olfactory activity with other vertebrates and thereby be a useful signal for predatory species. In addition, such strong interactions with the human olfactory system could indicate a potential pheromonal role with the insects themselves. Thus, further olfactometer assays were made with the pyrazine **I**, at a concentration equivalent to that found in the vacuum distillate and indeed, somewhat surprisingly, it was found to account fully for the activity of the extract in attracting adult *C. septempunctata* (table 1). The behavioural activity appeared to take the form of an attractant-arrestant response, whereby the beetles were initially attracted towards the

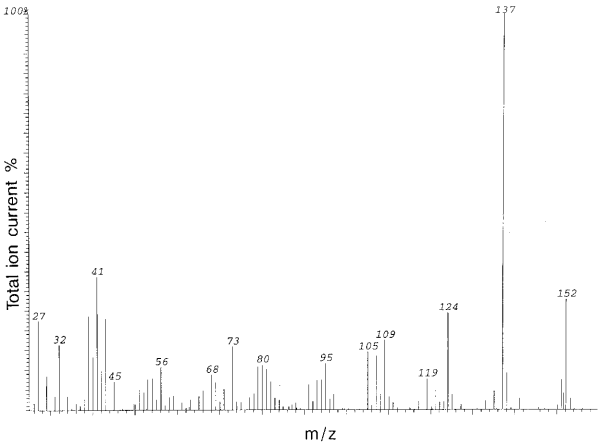

Figure 3. Mass spectrum from peak marked by arrow in figure 2.

Table 1. Responses of adult *C. septempunctata* L. in the olfactometer*.

Stimulus	Mean number of observations in		*P*	Replicates (*n*)
	treated arm	control arm†		
C. septempunctata (single adult)	5.8 (1.32)	2.9 (1.45)	<0.05	10
C. septempunctata vacuum distillate‡	6.8 (1.92)	3.2 (1.92)	<0.05	5
2-Isopropyl-3-methoxypyrazine§	8.4 (1.90)	1.6 (1.90)	<0.01	10

*Cumulative counts over 20 min (figures in brackets refer to standard deviation). †Control, solvent. ‡Tested at a level equivalent to 0.05 ng/μl 2-isopropyl-3-methoxypyrazine (0.5 μl applied). §Tested at 0.05 ng/μl (0.5 μl applied).

volatile source and thereafter exhibited reduced mobility. Organoleptic assessment of GC-separated components is a technique relatively unexploited in chemical ecology studies. It can nonetheless be extremely effective for locating volatiles characteristic of odours collected from biological systems [13]. Here, it proved to be particularly useful because, when heteroatoms are incorporated, human olfaction, on a body weight basis, can show similar sensitivity to that of insects [14]. However, if the technique is applied to the identification of insect and other semiochemicals generally, extreme caution must be exercised to ensure that the compounds detected by human olfaction are of significance to the target organism.

The results show that 2-isopropyl-3-methoxypyrazine can serve a dual function in Coccinellid chemical ecology, not only as an alerting signal to would-be predators but also as an aggregation pheromone component. The isolation and identification of only one alkylmethoxypyrazine in this study does not preclude the presence of analogues; studies elsewhere have provided evidence that related compounds may also be present in *C. septempunctata* (W. Francke, personal communication) The significant activity of 2-isopropyl-3-methoxypyrazine in attracting *C. septempunctata* adults in laboratory trials suggests that there is potential for its use in aphid pest control. However, further laboratory studies and field trials are required to determine whether the pheromone is active all year round or only at particular times of the year, e.g. during the summer months when aphid populations are likely to be at their highest, or when populations are low, usually prior to overwintering and/or at times of starvation.

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