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Mate recognition in the two-spot ladybird beetle, *Adalia bipunctata*: role of chemical and behavioural cues

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Abstract

On encountering a mature female, a male of the two-spot ladybird, *Adalia bipunctata* (L.), first palpated her elytra with his maxillary palps, then mounted her, extruded his penis and mated. Copulation never occurred between active males but males copulated with dummies bearing male elytra as frequently as with dummies with female elytra of their own species. Similarly, males attempted mating with immobilised conspecifics of both sexes. However elytra washed in chloroform failed to stimulate mating. Analysis of the chloroform extracts of the elytra revealed that male and female ladybirds are coated by the same blend of hydrocarbons among which 9- and 7-methyl tricosane are dominant. Our results are consistent with a role of these cuticulars hydrocarbons in species recognition and show that behaviour, in particular movement, is necessary for discrimination between males and females.

Keywords: *Adalia bipunctata*; Coccinellids; Mate recognition; Mating behaviour; Cuticular hydrocarbons

1. Introduction

Males of the two-spot ladybird beetle, *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae), do not show a functional response to increase in aphid abundance and consume markedly fewer aphids than females. In terms of movement, males are less active than females and do not respond to an increase in prey abundance by a change in searching behaviour. Males appear to mainly search for females. Studies on ladybird behaviour (Obata, 1987a, b, 1988; Majerus, 1994; Osawa, 1994; Hodek and Honek, 1996; Hemptinne et al., 1996) and the anatomy of their sense organs (Barbier et al., 1992; Jourdan et al., 1995) have led several authors to hypothesize the existence of a sexual pheromone. In addition, conspecific mate recognition is thought to be primarily a function of females (Majerus, 1997).

The mating behaviour of two-spot ladybirds has been intensively studied and a lot of attention has been devoted to mate choice (Muggleton, 1979; Majerus et al., 1982; Kearns et al., 1990, 1992; Tomlinson et al., 1995). The results, however, are very variable and inconclusive. Mate choice presupposes ladybirds can appreciate differences between individuals. There is, therefore, a need to have a better understanding of how ladybirds recognise one another.

This paper reports the results of a study on the mating behaviour of males of the two-spot ladybird, which extends and builds on our previous research (Hemptinne et al., 1996) by investigating the role of chemical(s) present on the elytral surface. The results appear to indicate that both behavioural and chemical cues are implicated in mate recognition.

2. Material and methods

2.1. The insects

The stock cultures of two and ten-spot ladybirds, *A. bipunctata* and *A. decempunctata* (L.) were established with individuals collected in the vicinity of Gembloux

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(Belgium) seven years ago and replenished every year by the addition of ladybirds collected from hibernation sites. Both species were reared at 20 ± 1°C and a photoperiod of 16 h light and 8 h darkness. Groups of approximately forty adults were kept in 5 litre plastic boxes, which also contained a piece of corrugated filter paper and wet paper tissue to increase the surface area and provide a source of drinking water. Every other day the ladybirds were fed an excess of pea aphids, *Acyrthosiphon pisum* (Harris). Once a week they were transferred to clean containers to stimulate egg laying.

The two-spot ladybirds used in the experiments were obtained by rearing fourth instar larvae from the stock culture in isolation in 5 cm diameter Petri dishes. These larvae were supplied with an excess of pea aphids until they pupated. After emergence from the pupae the adults were also kept in isolation in 5 cm Petri dishes, fed aphids every other day and transferred to clean dishes once a week. Only virgin ladybirds of a particular age were used in the experiments, and individual beetles were only used once. This standardisation of the adults gave considerably less variable results than when ladybirds from the stock cultures were used.

The sex of the adults was determined under a binocular microscope by examining the shape of the posterior edge of the last abdominal sternite, which is hemispherical in females and notched in males (Hodek, 1973).

2.2. *Identification of the source of the initial cues used in mate recognition and standardisation of beetles*

Our previous study (Hemptinne et al., 1996) indicated that mate recognition depended on physical contact between beetles and mainly involved cues on the female elytra. To confirm this the searching behaviour of males in the presence of females was recorded in detail in order to determine whether males encountering females initially make contact mainly with their elytra.

The readiness to mate appears to be dependent on the age of the beetles, particularly that of the females. Therefore, it was decided to only use females of a particular age in order to reduce the variation in the results. This was done by determining the readiness with which females of different ages mated with 20–30 day old males. Two, 3, 4 and 20–30 day old females were kept individually in 5 cm Petri dishes and each provided with 50 pea aphids. They were allowed two hours to acclimatise to these conditions and then a previously isolated male, kept in the same conditions, was induced to walk on to a triangular piece of filter paper and then transferred to a Petri dish containing a female. This was repeated from 5 to 16 times depending on the age of the females. The number of pairs that mated and their behaviour were noted.

2.3. *Mating behaviour*

2.3.1. *(i) female-male interactions*

In order to determine the mating behaviour in detail sixteen 20–30 day old two-spot females were each put into a 9 cm diameter Petri dish lined with filter paper. They were each provided with 50 pea aphids. Sixteen males of the same age as the females were treated similarly. They were allowed two hours to acclimatise to the experimental conditions then at 12.00 h males were induced to walk onto triangular shaped pieces of filter paper and then each randomly transferred to one of the Petri dishes containing a female. A pen camera, Toshiba AC-M240, coupled to a JVC BR-S925E Time Lapse video cassette recorder, was used to film and record their behaviour.

2.3.2. *(ii) male-male interactions*

The experimental set-up was the same as in the study of the female-male interactions. In this case, males replaced the females and the experiment was repeated twenty times.

2.3.3. *(iii) response of males to elytra of two-spot ladybirds*

A two-spot male was picked up from its Petri dish, by applying suction via plastic capillary tubing to its pronotum, and randomly transferred to a 3 cm diameter glass Petri dish at the centre of which was a little pellet of Blu Tak® bearing a fresh pair of female elytra. In order to reduce bleeding and the chance of the surface of the elytra becoming contaminated with defensive alkaloids females were placed for 15 min in a deep freeze prior to the removal of their elytra. All dissections were performed under a microscope and only uncontaminated pairs of elytra were selected. The size and structure of the Blu Tak® pellet and attached elytra were similar to a two-spot ladybird. The males were allowed to encounter the dummy three times and their behaviour at each encounter was recorded and classified according to the escalating mating sequence identified in section (i), i.e.,: 1: Contact, 2: Palpates, 3: Mounts, 4: Extrudes genitalia, 5: Mates. This was repeated nineteen times. Similarly twenty-three males were offered a dummy bearing conspecific male elytra.

2.3.4. *(iv) response of males to the elytra of ten-spot ladybirds*

As in the above experiment, a two-spot male was given access to dummies, but in this case the elytra were from a female or a male ten-spot ladybird. This was repeated, twenty and nineteen times, respectively.

2.3.5. *(v) response of males to washed and painted elytra of female two-spot ladybirds*

As in the above experiment but the dummies bore female elytra that had been washed for either 5 mins,

18 h or 24 h in chloroform; or female elytra that had been washed for 24 h in chloroform and then painted with the chloroform extract from two female elytra. The extract was obtained by immersing two elytra in 5 ml of chloroform for 5 mins. It was applied to the surface of the washed elytra by means of an Hamilton® microsyringe while viewed under a microscope in order to avoid loss of the extract. The elytra so treated were exposed to a flow of nitrogen for at least 20 mins and then dried at 30°C to evaporate the solvent before they were presented to males. The males' responses were recorded.

2.3.6. (vi) response of males to immobile conspecific ladybirds

A male was carefully lifted from its rearing dish on a triangular shaped piece of filter paper and randomly introduced into a 5 cm Petri dish at the centre of which was a freshly killed conspecific male or female. Both sexes were killed by keeping them at −20°C for 24 h, then returned to room temperature for about an hour before sticking each of them down individually in a living position at the centre of a Petri dish. The adhesive was a small droplet of a water soluble glue (Gloy®). This was repeated 16 times for each sex. In addition, the response of males to immobilised living males or females was recorded. These ladybirds were made lethargic by keeping them at 5°C for a short period and then each was stuck down in a living position at the centre of a Petri dish using a small droplet of the water soluble adhesive. When the glue had dried a male was placed in each dish and its responses recorded. This was repeated thirteen times with males and fifteen times with females.

In all the above experiments (Mating behaviour: i to vi) each ladybird was only used on one occasion as were the different dummies.

2.4. Statistics

The behaviour was recorded in the form of proportions and analysed by χ^2 tests (Siegel, 1956) or Fisher exact tests (Sokal and Rohlf, 1995) when the expected frequencies were smaller than 5. Two tailed binomial tests (Siegel, 1956) were used to compare the proportions of males respectively encountering the elytra or the rest of the body of conspecifics, or mating with living or immobilised males and females of their species. The null hypotheses were that the probabilities of occurrence of both events were equal (H_o: $p_1 = p_2 = 0.5$).

2.5. Chemical analysis

2.5.1. (i) identification of the chemicals

For identification purposes, the cuticular lipids were extracted from 51 elytra of 7 day old ladybirds in 1 ml pure chloroform for 24 h at room temperature. The crude chloroform extract was recovered and concentrated to 100 μl under a gentle stream of nitrogen.

The GC-MS (Gas Chromatography-Mass Spectrometry) investigations were performed on a Hewlett-Packard HP 5989 Mass Spectrometer coupled to a HP 5890 Serie 11 gas chromatograph equipped with a HP-5 (crosslinked 5% phenyl-methylpolysiloxane) column (30m × 0.25 mm I.D.; film thickness 0.25 μm). The operating conditions were fixed as follows: split-splitless injector at 275°C; carrier gas: helium at 0.9 ml min^{-1}; temperature programme: from 50°C to 140°C at 20°C min^{-1} then from 140°C to 290°C at 5°C min^{-1} with a final hold of 30 min at 290°C. The mass spectra were recorded in the electron impact mode at 70 eV (Source Temperature: 200°C, scanned mass range: 35 to 600 amu).

The detected peaks were identified by their retention data and their characteristic fragmentation patterns. The identification of straight-chain hydrocarbons and free fatty acids were confirmed by co-injections of pure references. Their mass spectra were finally compared with those of the NBS 75K.L and WILEY 138.L computer MS libraries. The structural assignments of internally methyl-substituted hydrocarbons was performed on the basis of the prominent mass ions C_nH_{2n+1} and C_nH_{2n} (McCarthy et al., 1968) formed by the α-cleavage at the branch position(s). The 2-methylalkanes were fragmented to give a characteristic strong M-43 ion (loss of an isopropyl group) and a less intense M-15 peak (loss of a methyl group). For the 3-methylalkanes, a characteristic M-29 peak (loss of an ethyl group) together with a smaller M-57 fragment (loss of a secondary butyl group) were the diagnostic ions.

2.5.2. (ii) measurements of the chemicals

The total lipids (i.e. epi- and endocuticular extractible material) of 16 elytra were extracted as indicated above. This was repeated 4 times. Fifty μl of a chloroform solution of n-nonadecane (62 μg ml^{-1}) was added as internal standard. The molecules of interest were measured by gas chromatography on a Carlo Erba 5060 Mega fitted with a cold on-column injector and a FID detector mantained at 295°C. A fused silica OPTIMA 1 (30m × 0.32 mm I.D.; film thickness 0.35 μm) from Macherey-Nagel (Germany) was used for the analysis. The temperature programme was identical to that of the GC-MS analytical procedure. For diagnostic purposes, the relative retention times of the different peaks were calculated with n-tricosane as reference and compared to the GC-MS chromatographic data and to the retention times of n-alkane homologos injected in the same conditions.

Table 1
Frequencies with which males of *Adalia bipunctata* encountered the elytra relative to the rest of the body of conspecific females and males

	Female	Male
Elytra	13	16
Rest of the body	3	4
Binomial test	0.005**	0.003**
Fisher exact test	0.7336 NS	

NS: not significant. **: $P < 0.01$.

3. Results

3.1. Source of the initial cues used in mate recognition and standardisation of beetles

Males on encountering another ladybird were much more likely to make contact with its elytra than any other part of its body, and this is independent of the gender of the encountered ladybird (Table 1). Therefore, if this ladybird uses a contact pheromone for mate recognition then it is likely to be on the elytra.

Two day old females were never observed to mate. The proportion of encounters with older females that resulted in mating was significantly greater than with younger females (Table 2: χ^2 for 2 and 3 day old females versus 4 and 20–30 day old females = 9.93, 1 df, $P < 0.01$). This is associated with a change in the behaviour of the females. Two day old females walked away as soon as a male started to palpate their elytra; some even became aggressive and attacked the males. This response was significantly less frequently observed when the females were 4 days old or older (Table 2). In contrast, they immediately stopped walking when encountered by a male and slowly raised the apex of their abdomen when the male started to palpate them (Table 2: χ^2 for 2 and 3 day old females versus 4 and 20–30 day old females = 15.32, 1 df, $P < 0.001$). That is, if a female stops after being encountered by a male she will be mated.

Table 2
The incidence of mating recorded when males of *Adalia bipunctata* were presented with conspecific females of different ages (in days)

	Female age (days)			
	2	3	4	20–30
Mating				
No	5	3	5	1
Yes	0	3	9	15
Reaction of female				
Stop	0	2	9	15
Moves	5	4	5	1

The reaction of these females when touched by a male are also presented.

3.2. Mating behaviour: male- female and male-male interactions

On meeting a mature female, males reacted very quickly. In an interval of about 10 sec, they climbed onto the back of the female, extruded their genitalia, aligned themselves and attempted copulation. The sequence of behaviour following an encounter is: 1: Contact, 2: Palpates the elytra of the encountered beetle for up to 5 seconds; 3: Mounts—climbs onto the back of the encountered beetle and examines the surface with its palps; 4: Extrudes his genitalia; 5: Mates. Males continued palpating the elytral surface of females throughout phases 2 to 4. When a male extruded his genitalia, the two parameres were constantly active and continuously palpated the outer edge of the elytra or the genital aperture of the female.

The proportion of males that palpated and mounted males was not significantly different from that observed when they encountered a female. However, the proportion that subsequently extruded their penis and mated was lower in homosexual than heterosexual pairs (Table 3).

3.3. Response of males to:

3.3.1. Conspecific elytra

The incidence of mating with dummies bearing elytra was not significantly affected by the gender of the elytra (Table 4). This response is different from that observed with living conspecifics (cf. Table 3). The speed of response to dummies with male or female elytra was also similar and in both cases attempted matings occurred mostly at the first encounter. Nevertheless, the duration of the copulation attempts with female dummies lasted longer than those with male dummies (Table 4).

3.3.2. Washed conspecific elytra

Female elytra washed in chloroform for 5 min elicited fewer attempted matings than unwashed elytra, and the number of attempted matings decreased with washing time. Males did not attempt to mate with elytra that had been washed for 24 h in chloroform. That is, the chloroform removed the chemicals that make elytra attractive to males. When washed elytra were painted with elytral extract they again became as attractive to males as unwashed elytra (Table 5).

3.3.3. Heterospecific elytra

There were significantly fewer attempted matings with dummies bearing female ten-spot than two-spot elytra (Table 6). There was, however, no significant differences in the response of males to dummies bearing male ten and two-spot elytra. Overall, the response of the males to dummies with female and male elytra, was weaker

Table 3
Numbers of males of *Adalia bipunctata* that reached the different stages in the mating sequence after encountering conspecific females or males

Mating sequence	Females	Males	χ^2 test (1 df)
Contact	16	20	
Palpates	16	14	0.52 NS
Mounts	16	14	0.52 NS
Extrudes genitalia	15	2	9.12**
Mates	15	0	13.71***
Test of homogeneity (4 df)	20.51***		

NS: not significant; **:$P < 0.01$; ***:$P < .0.001$.

Table 4
Numbers of males of *Adalia bipunctata* that reached the different stages in the mating sequence, length of attempted matings and frequencies of attempted matings at the first and subsequent encounters with dummies bearing conspecific female or male elytra

	Dummy bearing female elytra	male elytra	χ^2 tests (1 df)
Mating sequence			
Contact	19	23	
Palpates	19	23	0.00 NS
Mounts	19	23	0.00 NS
Extrudes genitalia	19	17	0.44 NS
Mating attempt	18	14	0.88 NS
Length of attempted mating			
< 30 s	0	2	
> 30 s	18	12	
Fisher exact test	0.03*		
Mating			
at first encounter	14	10	
at a subsequent encounter	4	4	
Fisher exact test	0.08 NS		

NS: not significant; *:$P < 0.05$; ***:$P < 0.001$.

Table 5
Numbers of males of *Adalia bipunctata* that reached the different stages in the mating sequence after encountering elytra of conspecific females washed in chloroform for three different times or chloroform washed elytra painted with elytral extract

Mating sequence	Elytra washed for				Elytra + extract(5)	χ^2 tests (1 df)	
	0 min (1)	5 min (2)	18 h (3)	24 h (4)		(1) and (4)	(1) and (5)
Contact	19	21	27	16	21		
Palpates	19	21	27	16	21	0.00 NS	0.00 NS
Mounts	19	18	19	15	20	0.07 NS	0.01 NS
Extrudes genitalia	19	12	11	4	16	4.93*	0.34 NS
Mates	18	8	6	0	15	4.93*	0.34 NS
Test of homogeneity (1) to (4) (12 df)	23.31*				15	11.79***	0.36 NS

NS: not significant; *:$P < 0.05$; ***:$P < 0.001$.

in the case of heterospecific than conspecific dummies (Table 6).

3.3.4. Movement

Males showed very similar responses when they encountered freshly killed conspecific females and males. That is, the proportions palpating, mounting, extruding genitalia and mating were the same (Table 7). Also, males did not appear to differentiate between living immobilised conspecific males and females (Table 8).

Table 6
Numbers of males of *Adalia bipunctata* that reached the different stages in the mating sequence after encountering elytra of conspecific female or male, elytra of 10-spot female or male

Mating sequence	Female			Male		
	10-spot	2-spot	χ^2 tests (1 df)	10-spot	2-spot	χ^2 tests (1 df)
Contact	20	19		19	23	
Palpates	20	19	0.00 NS	19	23	0.00 NS
Mounts	16	19	0.21 NS	15	23	0.21 NS
Extrudes genitalia	6	19	3.89*	8	17	1.14 NS
Mates	3	18	7.42**	5	14	1.96 NS
Tests of homogeneity (4 df)	13.07*			3.11 NS		

NS: not significant; *:$P < 0.05$; **:$P < 0.01$.

Table 7
Numbers of males of *Adalia bipunctata* that reached the different stages in the mating sequence after encountering freshly killed conspecific males or females

Mating sequence	Freshly killed female	male	χ^2 tests (1 df)
Contact	16	16	
Palpates	16	12	0.31 NS
Mounts	16	12	0.31 NS
Extrudes genitalia	15	12	0.18 NS
Mates	15	11	0.34 NS
Test of homogeneity (4 df)	0.49 NS		

NS: not significant.

Table 8
Number of matings observed when males of *Adalia bipunctata* were presented with living immobilised males or females

Mating	Immobilised male	female
No	2	0
Yes	11	15
Binomial test	0.005**	0.001***
Fisher exact test	0.2063 NS	

NS: not significant. **:$P < 0.01$; ***:$P < 0.001$.

3.4. Identification of the chemicals on the surface of the elytra

GC-MS and GLC investigations of ladybirds cuticular lipids have led to the detection of at least 80 different components. The most important substances (2 alkaloids, 3 fatty acids, 2 fatty acid ethyl esters and 46 hydrocarbons) were identified. Numerous other minor constituents representing each less than 0.1% of the total hydrocarbons have not yet been identified.

The principal alkaloid found in elytra lipids was adaline. Another N-containing compound has also been detected in all extracts, adalinine (2-pentyl-2-propanone-6-piperidone), a new piperidine alkaloid recently described (Lognay et al., 1996). Due to the split-splitless injection at 275°C some thermodegradation of the two alkaloids occurred. Therefore, several by-products have been systematically observed at short retention times.

The cluster of unresolved chromatographic peaks eluted between C_{21} and C_{23} n-alkanes has been attributed to a mixture of 9*cis*, 12*cis* octadienoïc acid (linoleic), 9*cis* octadecenoïc (oleic) and octadecanoïc (stearic) acids respectively. Minute amounts of ethyl esters of linoleic and oleic fatty acids have also been identified. In order to facilitate the identification of hydrocarbons potentially co-eluted with the fatty acids, short extractions (2 × 1 min) were performed. In this way only surface alkaloids and hydrocarbons have been recovered with very small quantities of other fatty materials.

As shown in Tables 9 and 10 the monomethyl substituted hydrocarbons, among which the internally branched molecules (from C_{22} to C_{33}), were prevalent (51.7 ± 3.4%) within the hydrocarbon profile. The principal molecule of elytral extract belongs to this family. It was represented by an isomeric mixture of 9- and 7-methyl tricosane (28.5 ± 2.0% and 13.8 ± 1.0% respectively). Superior homologues have been detected in lower proportions. They were found as isomeric mixtures the chromatographic resolution of which decreased as a function of the branch position. In this way 2-, 3- and 5- methylalkanes were fully separated, 9- and 7- isomers were partially resoluted, 11- and 13- methyl-substi-

tuted molecules co-eluted regardless of the chain lengths. Therefore, these products could not be quantified separately.

The terminally branched 2- and 3- methyl alkanes represented only 1.7 ± 0.2% and 0.8 ± 0.1%. The n-alkane series from C_{21} to C_{29} accounted for 24.5 ± 2.5% of the total with n-tricosane as the major peak (7.6 ± 1.4%). Six di-substituted methylalkanes with odd numbered carbon backbone could be recognised: C_{23} (two different molecules), C_{25}, C_{29}, C_{31} and C_{33}. They represented 5.5 ± 1.2% of the total. The C_{25}, C_{29}, C_{31} and C_{33} dimethylalkanes possessing isoprenoid spacing of the methyl groups i.e in the positions 9, 13-, 11, 15- and 13,17- have been detected as isomeric mixtures.

A single unsaturated molecule has been observed in a low proportion (0.6 ± 0.3%). It has been tentatively attributed to n-hentriacontene. Due to its very low level, the recovery and the purification of this minor compound could not be undertaken. Therefore the localization of the double bond by other spectrometric investigations has not been realized.

From a quantitative point of view, the hydrocarbons removed from the elytra after an extraction of 24 h varied from 16 to 25 μg per insect with a mean value of 19 μg.

To evaluate sexual differences in cuticular lipid, the surface chemicals were recovered after short time extractions (2 × 1 min) of male and female elytra and analyzed following the aforementioned conditions. The chromatographic profiles for both sexes were identical and the total weight of hydrocarbons was 11.3 ± 1.7 μg (n = 5) and 12.8 ± 2.6 μg (n = 18) for the male and the female respectively (F = 1.361; 1 and 21 df; P = 0.256).

4. Discussion

In contrast to young females, 20 to 30 day old females readily mated. On encountering such females, a male first palpated her elytra with his maxillary palps, then mounted her, extruded his penis and mated. Contrary to the claim of Jourdan et al. (1995) antennae did not appear to be involved in mating. Males of *A. bipunctata* did not stop and "watch" a female before touching her as has been described by Obata (1987a) for another ladybird beetle, *Harmonia axyridis* Pallas. Our results agree with the casual observations reported by Majerus (1994) and are very similar to that reported for other beetles (e.g. Ward, 1981).

The results presented confirm those of Hemptinne et al. (1996) that males usually encounter the elytra of females first and do not attempt to mate with active males. However, unlike in our previous study, males tried to mate as frequently with dummies bearing male or female elytra and with immobilised males and females. It is not easy to explain this difference unless the morphology of the elytra is taken into consideration. The elytral surface of *Semiadalia undecimnotata* is

Table 9
Cuticular hydrocarbons extracted from elytra of *Adalia bipunctata*

Hydrocarbons	CN[1]	RRT[2]	Diagnostic ions (m/z)			Percentage[3]
			M^+	$[M-CH_3]^+$	Others	Mean ± SD
nC_{21}	21	0.854	296			6.9 (0.3)
nC_{22}	22	0.928	310			0.5 (0.1)
9-me-C_{22}	23	0.958	324		140/141-210/211	1.6 (0.1)
n-C_{23}	23	1.000	324			7.6 (1.4)
11-Me-C_{23}	24	1.028	338	323	168/169-196/197	Σ 11 and 9-Me-C_{25} =
9-Me-C_{23}	24	1.028	338	323	140/141-224/225	28.5 (2.0)
7-Me-C_{23}	24	1.030	338	323	112/113-252/253	13.8 (1.0)
2,5-diMe-C_{23}[4]	25	1.051	352	338	309-84/85	1.2 (0.3)
5,15-diMe-C_{23}[4]	25	1.060			84/85-140/141-238/239-294/295	1.8 (1.0)
nC_{24}	24	1.069	338			0.4 (0.1)
9-Me-C_{24}	25	1.095			140/141-238/239	1.5 (0.1)
2-Me-C_{24}	25	1.112	352	337	309	1.0 (0.1)
nC_{25}	25	1.136	352			3.9 (0.6)
13-Me-C_{25}	26	1.159	366	351	196/197	
11-Me-C_{25}	26	1.159		351	168/169-224/225	Σ X-Me-C_{25} =
9-Me-C_{25}	26	1.159		351	140/141-252/253	1.8 (0.2)
7-Me-C_{25}	26	1.159		351	112/113-280/281	
2-Me-C_{25}	26	1.178	366	351	323	0.3 (0.0)
3-Me-C_{25}	26	1.183	366	351	337, 308	0.8 (0.1)
nC_{26}	26	1.200	366			0.3 (0.6)
2-Me-C_{26}	27	1.240	380	365	337	0.4 (0.1)
nC_{27}	27	1.262	380			3.9 (1.1)

Table 9
Continued

Hydrocarbons	CN[1]	RRT[2]	Diagnostic ions (m/z) M^+	$[M-CH_3]^+$	Others	Percentage[3] Mean ± SD
13-Me-C_{27}	27	1.284		379	196/197-224/225	
11-Me-C_{27}	27	1.284		379	168/169-252/253	Σ X-Me-C_{27} =
9-Me-C_{27}	27	1.284		379	140/141-280/281	0.3 (0.1)
7-Me-C_{27}	27	1.284		379	112/113-308/309	
5-Me-C_{27}	27	1.294			84/85-337/338	0.2 (0.1)
nC_{28}	28	1.324	394			0.2 (0.0)
nC_{29}	29	1.384	408			0.7 (0.3)
13-Me-C_{29}	30	1.398		407	196/197-252/253	
11-Me-C_{29}	30	1.398		407	168/169-280/281	Σ X-Me-C_{29} =
9-Me-C_{29}	30	1.398		407	140/141-308/309	0.4 (0.1)
11,15 diMe-C_{29}[5]	31	1.424		421	168/169-238/239-224/225-294/295	0.2 (0.1)
C_{31}unsaturated	31	1.497	434		97,83,69,55	0.6 (0.3)
15-Me-C_{31}	32	1.560		435	224/225-252/253	
13-Me-C_{31}	32	1.560		435	196/197-280/281	Σ X-Me-C_{31} =
11-Me-C_{31}	32	1.560		435	168/169-308/309	0.2 (0.0)
9-Me-C_{31}	32	1.560		435	140/141-336/337	
11,15 diMe-C_{31}	33	1.585		449	168/169-322/323-252/253-238/239	Σ diMe-C_{31} =
13,17 di Me-C_{31}	33	1.585		449	196/197-224/225-266/267-294/295	0.7 (0.3)
13-Me-C_{33}	34	1.763		463	196/197-308/309	Σ X-Me-C_{33} =
11-Me-C_{33}	34	1.763		463	168/169-336/337	1.0 (0.2)
11,15 diMe-C_{33}	35	1.798			168/169-350/351-252/253-280/281	Σ diMe-C_{33} =
13,17 diMe-C_{33}	35	1.798			196/197-322/323-252/253-266/267	1.7 (0.7)

(1) Total carbon number; (2) Relative retention time (nC_{23} as reference); (3) Weight % of the total detected molecules (Mean ± SD of four determinations); (4) Tentative interpretation; (5) Mixture of isomers.

marked by glands with curved trichoid seta (Barbier et al., 1992), which Faustini et al. (1981) thought might play a role in the mutual attraction of the sexes. The elytra of both sexes of the two-spot ladybird have very similar glands (Pasteels and Hemptinne, unpubl.) that secrete long strands of a wax-like material. These glands are more numerous along the lateral edge of the elytra. The only visible difference between males and females is that there are slightly fewer glands in males. As during their experiment Hemptinne et al. (1996) only presented an elytron to males for a very short time, it is possible that the signal delivered by male elytra could have been too weak to elicit sexual behaviour.

Elytra washed in chloroform failed to trigger mating. Scanning electron micrographs of elytra revealed that the wax-like material is completely removed after washing for 24 h in chloroform (Pasteels and Hemptinne, unpubl.). However, if washed elytra were painted with a chloroform extract of female elytra then they recovered their attractiveness for males. Chloroform removed a blend of 46 hydrocarbons among which the internally branched molecules ranging from C_{22} to C_{33} were dominant. These substances appear to play a role in the mating behaviour.

The above results indicate that both males and females have the same hydrocarbons on their elytra. That is, contrary to what was proposed by Hemptinne et al. (1996), males do not appear to recognise females by means of a contact pheromone present on their elytra. It is more likely that these hydrocarbons are used in species recognition. This hypothesis is supported by the very few matings provoked by the elytra of the closely related ten-spot ladybird, *A. decempunctata*. However, the experimental males were virgin and 20–30 days old. Their frustration was probably great enough to account for the few matings recorded. In addition, these two species are known occasionally to mate in the field (Majerus, 1994, 1997).

How do males discriminate between males and females? Males responded similarly to freshly killed males and females, and to living but immobilised males and females as they do to normal mature females. Therefore movement appears to be an important cue in the mating process. Mature females stop as soon as they are touched by a male and then slightly elevate the tip of their abdomen, which appears to indicate a readiness to mate. Young females behaved differently: they ran away or tried to dislodge the male as previously recorded by

Majerus (1994). As females aged they refused to mate less frequently and the incidence of copulation increased accordingly. Reluctance to copulate is inversely related to the maturity of the ovary (Obata, 1988), which in turn is positively correlated with age in well fed ladybirds (Hemptinne, 1989).

This study has revealed that the standardisation of the beetles is of paramount importance. The variability observed in mate choice experiments is likely to be a consequence of not only using male and female ladybirds of a particular age. Although the beetles used in the experiments reported here had no previous experience of mating and had been deprived of mates for a considerable period of time, especially the males, nevertheless, there was no evidence that either sex exercised any choice. If there is mate choice then it is most likely exercised by the female as her response appears to initiate mating. Whether mate choice occurs or not, however, will have to be determined using standardised beetles, but less frustrated ones than used in this study.

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