CHAPTER 5

Characterisation of a Female-Produced Courtship Pheromone in the Parasitoid *Nasonia vitripennis*

Abstract Males of the parasitoid *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) show a characteristic courtship behaviour. We demonstrate that male arrestment and key behavioural elements of the courtship sequence are mediated by a female-derived contact sex pheromone. Males were arrested on paper discs treated with female extracts but not on those treated with male extracts. Male responsiveness was influenced by the surface to which female extracts were applied. Extracts applied to an extracted beetle elytron arrested males more strongly than those applied to filter paper of comparable size. However, more complex behavioural elements, such as head nodding and copulation attempts, were shown only when extracts were applied to extracted male cadavers, suggesting that tactile or visual cues synergise the male response. The chemicals involved are stable, of low volatility and nonpolar. Dead females arrested males and elicited courtship behaviour for at least 8 d. Males showed no sign of attraction to live females at a distance of 3 cm in an olfactometer. Fractionation of female extracts demonstrated that the activity was exclusively located in the nonpolar fraction. Analysis of the active fraction by gas chromatography–mass spectrometry revealed that cuticular hydrocarbons with chain lengths between 25 and 37 carbon units were present. Comparison of hydrocarbon profiles from males and females showed qualitative and quantitative differences. These results suggest that sex-specific cuticular hydrocarbons are the key signals mediating the male courtship behaviour in *N. vitripennis*.

Key words Parasitoid, *Nasonia vitripennis*, Pteromalidae, sex pheromone, courtship behaviour, cuticular hydrocarbons.

Introduction

Sex pheromones are assumed to play a major role in the sexual communication of parasitic wasps (Godfray, 1994; Quicke, 1997). Depending on the function of the pheromones, the range of activity varies: highly volatile compounds released by females are used by males for long-range orientation during mate finding (Eller et al., 1984; Swedenborg and Jones, 1992a,b; Swedenborg et al., 1994; McNeil and Brodeur, 1995; Jewett and Carpenter, 1999), whereas chemicals of relatively low volatility mediate male courtship behaviour at close range (Simser and Coppel, 1980; Mohamed and Coppel, 1987; Shu and Jones, 1993; Syvertsen et al., 1995; Sullivan, 2002).

Sex pheromones have been demonstrated in several families of the parasitic Hymenoptera, e.g., in Braconidae, Chalcididae and Ichneumonidae (reviewed by Kainoh, 1999). Within the Pteromalidae, little is known. The sex pheromones hitherto demonstrated in this taxon are exclusively those eliciting courtship behaviour at close range (van den Assem and Povel, 1973; Yoshida, 1978; Ruther et al., 2000; Sullivan, 2002). Studies on *Roptrocerus xylophagorum* (Sullivan, 2002) and *Lariophagus distinguendus* (Steiner et al., 2005) indicated that the active compounds are cuticular hydrocarbons. Interestingly, the female sex pheromone of *L. distinguendus* is already present in pupae of both sexes. However, males actively decompose the chemicals involved immediately after emergence (Steiner et al., 2005).

Nasonia vitripennis (Walker) (Hymenoptera: Pteromalidae) is a gregarious parasitoid attacking pupae of several fly species, including blowflies, flesh flies and houseflies. The parasitoid has been intensely investigated in studies addressing genetic (Reed, 1993), ecological (King and D'Souza, 2004), behavioural (Baeder and King, 2004), developmental (Rivers et al., 1999) and evolutionary (van den Assem and Jachmann, 1982) aspects of its biology. As with many other parasitoids, the mating system of *N. vitripennis* is characterised by protandry, i.e., males emerge first and females follow shortly after. When male density is low, single dominant individuals aggressively defend the escape hole in the host puparium against competitors to ensure that they mate with the emerging females. Inferior males may wander away and find other puparia in the vicinity. At high density, however, competition degenerates into scramble competition and the territorial

system breaks down (van den Assem et al., 1980a). Males of *N. vitripennis* perform a characteristic courtship behaviour that has been studied in detail (Barras, 1960; van den Assem and Vernel, 1979; van den Assem and Werren, 1994). When encountering a female, the male immediately mounts. Subsequently, the male shows regular movements of the wings. These movements are slight lifts rather than the high-frequency wing fanning as described from other pteromalids (e.g., Ruther et al., 2000). Wing movements may be accompanied by a characteristic head nodding consisting of rapid raising and lowering of the head in combination with chewing movements of the mouthparts. It has been hypothesised that head nodding serves to transfer a male aphrodisiac to the female (van den Assem et al., 1980b). As a result of the male courtship behaviour, the female signals sexual receptivity by lowering her antennae and synchronously raising the abdomen to expose her genitalia. Subsequently, copulation may occur. It has been suggested that male courtship behaviour in *N. vitripennis* is induced by a female-derived sex pheromone (van den Assem et al., 1980b), but the chemicals involved are unknown.

The present study was initiated to investigate more thoroughly the role of female sex pheromones in the sexual communication of *N. vitripennis*. We characterised the chemicals involved by studying their longevity, range of activity and polarity. We also analysed fractions eliciting behavioural activity. Finally, we investigated the possible influence of additional physical cues on male responsiveness and studied whether the pheromone is already present in the pupal stage.

Methods and materials

Insect rearing Laboratory cultures were kept at 25°C and 60% relative humidity with a daily light/dark cycle of 16:8 h. Thirty to 40 freshly emerged *N. vitripennis* were placed in Petri dishes with 50 freeze-killed puparia of the green bottle fly *Lucilia caesar* and kept there until their death. After a development time of 14–15 d, adult parasitoids from the next generation emerged and were collected every second day.

General methods for experiments To obtain virgin individuals for bioassays, parasitoid pupae were removed from host puparia 1–2 d before eclosion and sexed in the pupal stage according to the different-sized wing pads (van den Assem and Jachmann, 1999). Pupae were kept individually in 1.5 ml microcentrifuge tubes until emergence. Freshly emerged parasitoids were held for 1–2 d in single-sex groups of 10–15 individuals in Petri dishes lined with moistened filter paper and provided with one split raisin. One hour before experiments, parasitoids were transferred individually into microcentrifuge tubes. All experiments except for experiment 4 were performed in a bioassay chamber (10 mm diameter × 3 mm height) described elsewhere (Ruther et al., 2000). Behaviours were observed with a stereo microscope with illumination from a microscope light and recorded using The Observer 3.0 computer software (Noldus Information Technology, Wageningen, The Netherlands). Parasitoids were used only once in bioassays.

Experiment 1: Activity of pheromone extracts Ten freshly emerged females were killed by freezing (1 h, −20°C) and extracted for 30 min with 60 µl dichloromethane at room temperature. The resulting extracts were concentrated to one-third individual equivalent per microlitre under nitrogen. Two female equivalents of the extract were applied on a filter paper disc (diameter 5 mm, Melitta, Germany) and the solvent was allowed to evaporate for 15 min. The paper disc was offered to a male in the observation chamber and arrestment time on the disc as well as key elements of the courtship sequence (head nodding, copulation attempts) were recorded for 5 min. Paper discs treated with pure solvent or male extract were used as controls (N = 15 for each treatment). Extract samples were changed after every five parasitoids.

Experiment 2: Influence of the release matrix on male courtship behaviour This experiment was carried out to study whether male responsiveness was influenced by the matrix to which female extracts were applied. Extracts were prepared as described in experiment 1 and applied (two female equivalents) to (1) a piece (3 × 2 mm) of filter paper, (2) a piece (3 × 2 mm) of a solvent extracted elytra of the garden chafer *Phyllopertha horticola* (model insect cuticle) or (3) a solvent-extracted dead *N. vitripennis* male. Treated objects and solvent-treated control objects were presented to a male and arrestment time, head-nodding behaviour and copulation attempts were recorded for 5 min (N = 25 for each treatment).

Experiment 3: Stability of the biologically active chemicals This experiment investigated the time that dead females remained active to verify literature data suggesting that activity ceases within 2 d. For this purpose, freshly emerged females were killed by freezing and stored throughout the test period at a constant temperature of 25°C and a relative humidity of 60%. The cadaver was presented daily to a male during the first 6 d and finally 8 d after killing. Arrestment time on the cadavers, head-nodding behaviour and copulation attempts were recorded for 5 min. Solvent-extracted females were used as controls (N = 10 for each treatment).

Experiment 4: Test for attraction of males to females This experiment was performed in a static four-chamber olfactometer, which has been used successfully to study the long-range orientation of pteromalids (Steidle and Schöller, 1997; Ruther and Steidle, 2000). No airflow was generated. Ten freshly emerged virgin *Nasonia* females were placed in a Petri dish (5.5 cm diameter × 1.2 cm height) covered with gauze (mesh 0.5 mm) and transferred into one chamber of the olfactometer. The remaining three chambers contained empty Petri dishes as controls. The olfactometer was covered with a walking arena made from gauze (distance to the volatile source: 3 cm) and finally with a glass plate to prevent test animals from escaping. The olfactometer was placed on the bottom of a white bucket (29 cm diameter, 36 cm height) and illuminated from above (2050 lux). A single male was introduced in the centre of the walking arena and the time the parasitoid spent in the area above the four chambers was recorded for 10 min. Males that rested more than 5 min of the total observation time were assumed to be unmotivated and excluded from statistical analysis. Virgin females were replaced after every five males (N = 22).

Experiment 5: Production of the sex pheromone during pupal development To investigate whether *N. vitripennis* begins producing the sex pheromone during pupal development as shown for other pteromalids (Yoshida, 1978; Steiner et al., 2005), male and female pupae were dissected from host puparia and classified according to pigmentation. Behavioural responses of adult males to single male and female pupae from three stages (yellowish pupae, pupae with red mouthparts and totally melanised pupae) were observed for 5 min in a bioassay chamber. Extracted male and female pupae were used as controls. Pupae used as odour sources were tested only once (N = 20 for each pupal stage of both sexes).

Experiment 6: Activity of pheromone fractions Dichloromethane extracts of *N. vitripennis* females were fractionated by liquid chromatography and the resulting fractions were bioassayed. Batches of 10 freshly emerged females were extracted with 60 µl dichloromethane as described in experiment 1. The extracts were concentrated to 15 µl under nitrogen, applied to 25 mg silica gel cartridges for solid phase extraction (IST, Mid-Glamorgan, UK) and eluted sequentially with 120 µl each of hexane, 10% dichloromethane in hexane, dichloromethane and methanol. Four female equivalents of the fractions were applied onto extracted male cadavers and the behavioural responses of males (arrestment time, head nodding and copulation attempts) to the treated dead males were recorded for 5 min (N = 27 for each fraction). Extracted male cadavers treated with pure solvent were used as controls (N = 24 for each fraction).

Chemical analysis Hexane fractions from females and males (N = 7 for each sex) were prepared as described in experiment 6 and analysed by coupled gas chromatography–mass spectrometry (GC–MS) on a Fisons GC 8060 with a Fisons MD 800 quadrupole MS (Thermo Finnigan, Egelsbach, Germany). Analytical conditions were as follows: injector temperature 240°C, column 30 m × 0.32 mm ID DB-5 ms, film thickness 0.25 µm (J & W Scientific, Folsom, CA, USA), carrier gas: helium and inlet pressure 10 kPa. The temperature programme started at 150°C and increased 2°C/min to 280°C. One microlitre of each fraction, representing 0.5 wasp equivalent, was injected together with 25 ng of tetracosane as an internal standard. Linear retention indices of (LRI) methyl-branched and unsaturated hydrocarbons were calculated by co-injection of straight-chain hydrocarbons. Methyl-branched hydrocarbons were identified by diagnostic ions resulting from the favoured fragmentation at the branching points (Lockey, 1988; Nelson, 1993) and by comparing linear retention index values with literature data (Carlson et al., 1998). Position of the double bonds of unsaturated hydrocarbons was determined by iodine-catalysed methylthiolation using dimethyl disulfide (Francis and Velant, 1981; Howard, 1993). Peak areas for each compound were calculated and related to the total peak area for each run.

Statistical analysis Statistical analyses were performed with Statistica 4.5 scientific software (StatSoft, Hamburg, Germany). Mean arrestment times of males were analysed by Kruskal–Wallis H test followed by multiple U tests for individual comparisons. The time parasitoids spent in the areas above the four chambers of

the olfactometer (experiment 4) were analysed by a Friedman analysis of variance. Number of males responding by head nodding and copulation attempts to different odour sources were compared by using a 2 × 2 χ^2 test. Relative abundances of hydrocarbons in male and female hexane fractions were compared by a Mann–Whitney *U* test. Whenever multiple comparisons were carried out during statistical analysis, a sequential Bonferroni correction was applied (Sachs, 1992).

Results

Experiment 1: Activity of pheromone extracts Males spent more time on filter paper discs treated with two equivalents of a female extract than on discs treated with male extract or pure solvent (Fig. 1). Apart from this arrestment, however, no other behaviours related to courtship (e.g., head nodding or copulation attempts) were observed. Paper discs treated with male extracts did not cause arrestment in responding males when compared with solvent controls.

Experiment 2: Influence of the release matrix on male courtship behaviour Males were arrested on all objects treated with female extracts when compared with controls (Fig. 2). However, arrestment on treated beetle elytra was stronger than on filter paper of the same size. Male cadavers treated with female extracts arrested males more strongly than filter paper, whereas the difference between beetle elytra and male cadaver was not significant (U = 222, P = 0.079). When considering more complex behavioural elements such as head nodding and copulation attempts, only treated cadavers released male responses (head nodding: 28% response, χ^2 = 8.14, P = 0.004; copulation attempts: 48% response, χ^2 = 12.58, P < 0.001).

Experiment 3: Stability of the biologically active chemicals Females killed by freezing shortly after emergence and stored at 25°C and 60% relative humidity elicited responses from males for several days. During the first 8 d, males spent significantly more time on female cadavers when compared with controls (Fig. 3a). Head-nodding behaviour and copulation attempts were elicited up to the sixth day of the test period (Fig. 3b–c). This shows that the bioactive chemicals are relatively stable.

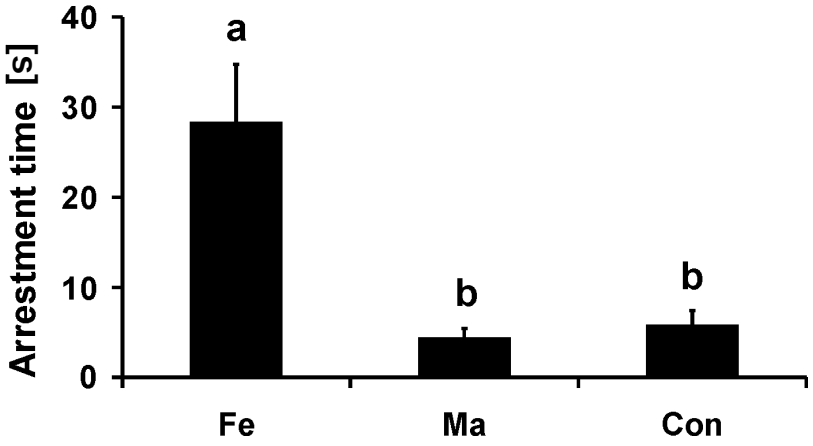

Fig. 1 Mean arrestment time (± SE) of *N. vitripennis* males on differently treated paper discs (Fe = female extract, Ma = male extract, Con = solvent control) during a 5 min observation period. Bars with different lowercase letters are significantly different at $P < 0.05$ (Kruskal–Wallis *H* test followed by multiple *U* tests; $N = 15$).

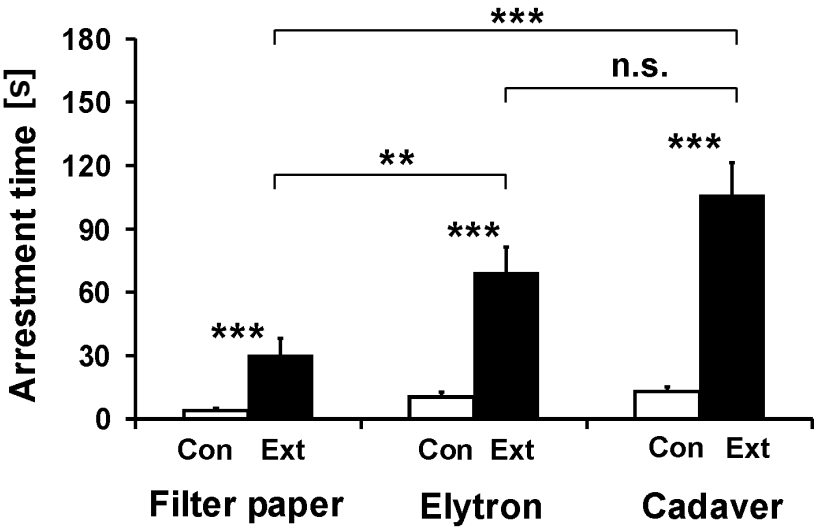

Fig. 2 Mean arrestment time (± SE) of *N. vitripennis* males on differently treated paper discs, extracted beetle elytra (model insect cuticle) and extracted male cadavers of *N. vitripennis* (Con = solvent control, Ext = treated with two equivalents of a female extract) during a 5 min observation period. Asterisks indicate significant differences (*** = $P < 0.001$, ** = $P < 0.01$, n.s. = not significant, Kruskal–Wallis *H* test followed by multiple *U* tests, $N = 25$).

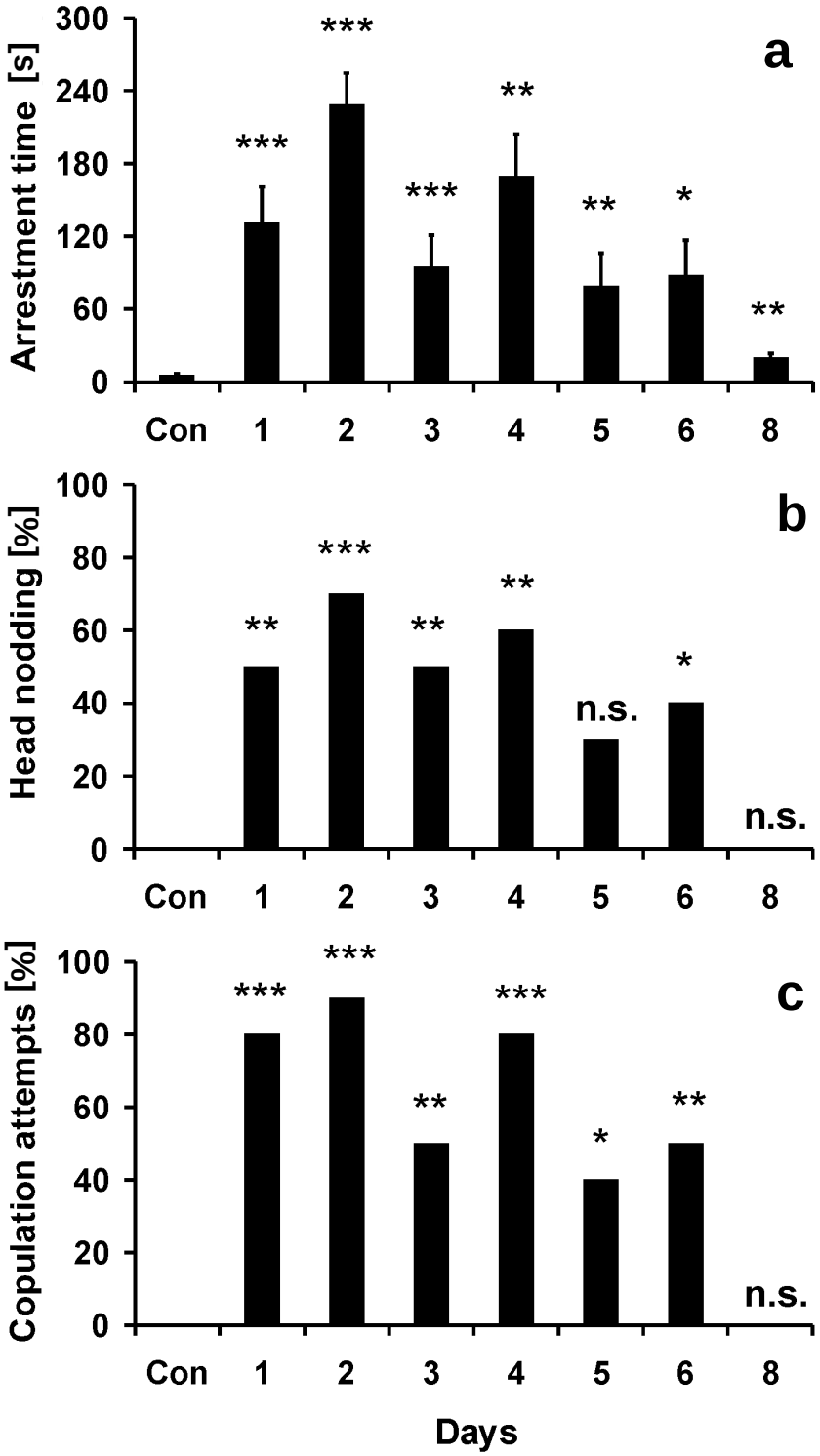

Fig. 3 Response of *N. vitripennis* males to female cadavers presented 1–6 and 8 d after killing. (a) Mean arrestment time (± SE), (b) percentages of males showing head nodding, (c) percentages of males showing copulation attempts. Asterisks indicate significant preferences for female cadavers when compared with control (*** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, n.s. = not significant). Mean arrestment times were compared by multiple Mann–Whitney *U* tests; head-nodding and copulation behaviour was analysed by 2 × 2 χ^2 tests ($N = 10$).

Experiment 4: Test for attraction of males to females In the four-chamber olfactometer, males did not spend more time in the test field above the 10 virgin females than in the control fields (χ^2 = 4.087, P = 0.252). This result indicates that females of this species do not produce a volatile sex pheromone that attracts males from a distance.

Experiment 5: Production of the sex pheromone during pupal development Male and female pupae of the three different stages elicited few behavioural responses from males. Males spent more time on totally melanised female pupae when compared with extracted female control pupae (Fig. 4). However, the arrestment time on pupae was smaller when compared with fully developed dead females (Fig. 3a). The arrestment on totally melanised male pupae did not differ significantly from the time spent on extracted male control pupae. Regardless of the sex, yellowish pupae and pupae with red mouthparts did not cause arrestment in responding males. Although head nodding was shown once and copulation attempts in two cases toward totally melanised pupae of either sex, these differences were not significant when compared with the respective control pupae, which elicited no responses at all (head nodding: 5%, χ2 = 1.030, P = 0.311; copulation attempts: 10%, χ2 = 2.110, P = 0.147).

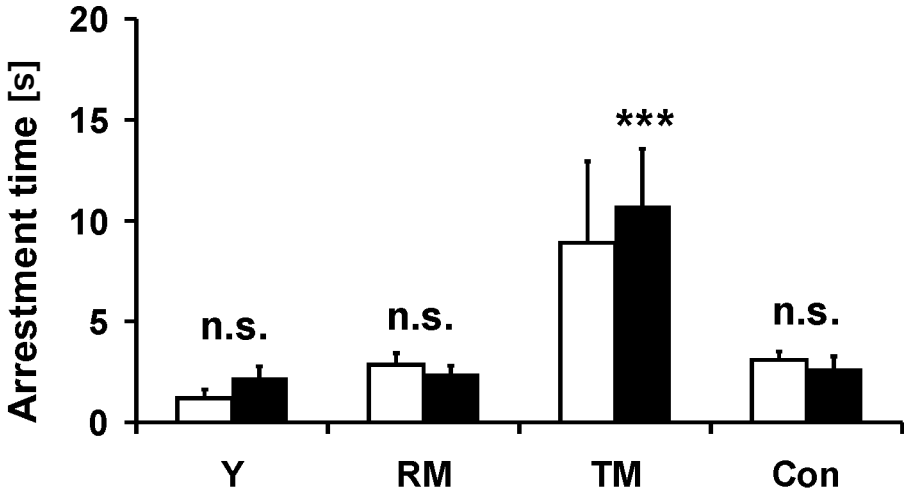

Fig. 4 Mean arrestment time (± SE) of adult *N. vitripennis* males on female (black bars) and male pupae (white bars) from different stages (Y = yellowish pupae, RM = pupae with red mouthparts, TM = totally melanised pupae, Con = extracted melanised pupae as control). Asterisks indicate significantly increased arrestment compared with control at P < 0.001 (Mann–Whitney U test, N = 20).

Experiment 6: Activity of pheromone fractions Only the hexane fraction of female extracts elicited arrestment and courtship behaviour (head nodding and copulation attempts) in males when applied to extracted male cadavers. The other fractions did not elicit behavioural responses (Fig. 5a–c). This shows that the arrestant pheromone is comprised of nonpolar compounds.

Chemical analysis Sixty-seven straight-chain, methyl-branched or monounsaturated hydrocarbons were identified in the hexane fractions of *N. vitripennis* (Table 1). The chain length varied between 25 and 37 carbon units. Most compounds occurred both in male and female parasitoids, but in most cases the relative abundances of the individual hydrocarbons were substantially different between sexes. In both sexes, the majority (> 80%) of hydrocarbons consisted of methyl-branched alkanes with one to four methyl groups. All compounds eluting before triacontane (peak 14) occurred in higher relative amounts in the hexane fractions from females than in those from males. The most striking sex-related differences among later-eluting hydrocarbons were found in homologous series of monomethylalkanes and dimethylalkanes. In hexane fractions from females, there were higher relative abundances of peaks resulting from the coelution of 9-, 11-, 13- and 15-methylalkanes (peaks 19, 36, 46) and of those belonging to 9,x-, 11,x-, 13,x- and 15,x-dimethylalkanes (peaks 22, 23, 39, 48, 52). Compounds with higher relative abundances in male fractions were some of the 5-methylalkanes and 7-methylalkanes (peaks 20, 21, 37, 47) as well as 3,x-, 5,x- and 7, x-dimethylalkanes (peaks 26, 40, 41, 44, 49). Alkanes with more than two methyl branches (peaks 30, 32, 42, 45) were more abundant in female extracts, whereas alkenes (peaks 16, 17, 33, 34) were predominantly found in extracts from males.

Discussion

The study demonstrates that arrestment and key elements of the male courtship sequence in *N. vitripennis* are mediated by a female-derived contact sex pheromone. Bioassays of fractions of cuticular extracts revealed that the active compounds are nonpolar. The pheromone components from cuticular extracts were only active at close range and were shown to be relatively stable because dead females elicited courtship in males for at least 6 d and caused arrestment for at

least 8 d. The loss of activity after this period might be explained with residues left by male responders on the surface of the cadavers during previous trials. The only compounds detectable in the biologically active hexane fraction were cuticular hydrocarbons. The relative composition of cuticular hydrocarbons from males and females showed numerous qualitative and quantitative differences. These findings suggest that sex-specific differences in cuticular hydrocarbon profiles form the chemical basis of signals mediating male courtship behaviour in *N. vitripennis*. Van den Assem et al. (1980b) assumed the presence of a sex pheromone in *N. vitripennis* after showing that male courtship behaviour toward dead females can be terminated by solvent extraction of the cadavers. However, they concluded that the chemicals involved were relatively unstable because dead females induced mounting responses in conspecific males for less than 2 d.

The primary function of insect cuticular hydrocarbons is to protect insects from desiccation (Lockey, 1988). However, numerous studies have demonstrated that these chemicals are also involved in orientation and recognition processes of insects, such as species, kin and sex discrimination, as well as chemical mimicry by social parasites or parasitoids to gain access to their hosts (reviewed by Blomquist et al., 1993; Dettner and Liepert, 1994; Singer, 1998; Howard and Blomquist, 2005). Carlson et al. (1999) analysed the cuticular hydrocarbon profiles of *N. vitripennis* and *Muscidifurax raptorellus* exuviae to develop a tool for discrimination of parasitoid species after these had left the host puparium. For comparison, the authors presented also some preliminary data on adult parasitoids which, however, did not allow for a systematic analysis of gender-specific differences. Our study reports also some additional components which have not been reported in the mentioned paper. This might indicate strain-specific variations or an influence of the host on the hydrocarbon profiles of the parasitoid, because Carlson et al. (1999) used house fly pupae to rear the parasitoids.

Only a few authors have studied the composition of cuticular hydrocarbons of parasitic wasps with regard to their possible role as contact pheromones. Syvertsen et al. (1995) found that some female-derived alkadienes arrested males of the braconid *Cardiochiles nigriceps*. As in the present study, pheromone activity was located in the hydrocarbon fraction of cuticular extracts of two other pteromalids, *R. xylophagorum* (Sullivan, 2002) and *L. distinguendus* (Steiner et al., 2005). In the

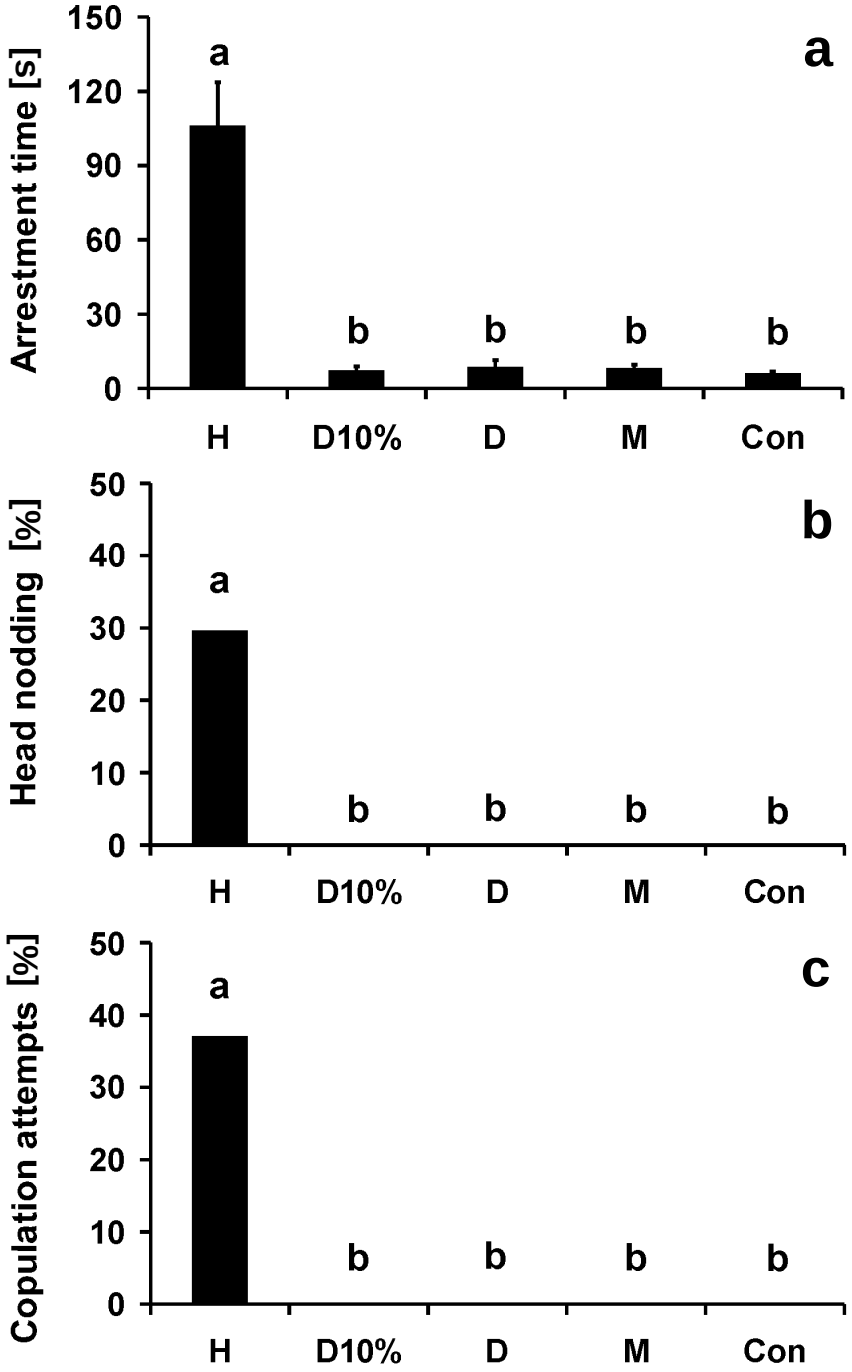

Fig. 5 Response of *N. vitripennis* males to extracted male cadavers treated with different fractions of female extracts (H = hexane, D10% = 10% dichloromethane in hexane, D = dichloromethane, M = methanol, Con = solvent control). (a) Mean arrestment time (± SE), (b) percentages of males showing head nodding, (c) percentages of males showing copulation attempts. Different lowercase letters indicate significant preferences at $P < 0.05$ (mean arrestment times were compared by Kruskal–Wallis H test followed by multiple U tests; head-nodding and copulation behaviour was analysed by multiple χ^2 tests; $N = 27$).

latter species, as well as in the closely related *Anisopteromalus calandrae* (Yoshida, 1978) and in the braconid *Cotesia glomerata* (Tagawa, 1977), the pheromone was already present in the early pupal stage. It has been suggested that this might enable mating of the females before they leave the site of emergence to search for new host patches (Steiner et al., 2005). Because male pupae and freshly emerged *L. distinguendus* males also contained the courtship pheromone, Steiner et al. (2005) assumed that developing males use the courtship pheromone to fool sexual competitors. The present study shows a different situation for *N. vitripennis*: contact pheromones appear in the cuticular lipids much later and only totally melanised female pupae are slightly attractive to males. Pheromone activity is fully developed in freshly emerged females but not in males. This explains why males of *N. vitripennis* are arrested on fly puparia containing females that are about to leave the host (King et al., 1969).

Interestingly, the response of *N. vitripennis* males to female extracts was influenced by the release substrate. Pheromone extracts applied to insect cuticles (extracted beetle elytra or male wasp cadaver) arrested males more strongly than those applied to pieces of filter paper. This might be because of differing adsorptive properties of the two matrices influencing the available amounts of the active compounds. Alternatively, physical cues associated with the insect cuticle might affect male responsiveness. More complex behavioural elements such as head-nodding or copulation attempts were shown only toward male cadavers treated with female extracts. In contrast, neither untreated male cadavers nor extracted female cadavers with reapplied male hydrocarbons elicited any responses in males (data not shown). This supports the hypothesis that visual and/or tactile cues synergise the male response to the female courtship pheromone. In other parasitic wasps, physical cues do not seem to play that crucial role because paper discs treated with pheromone extracts not only caused arrestment but also elicited elements of the courtship behavioural sequences (Takahashi and Sugai, 1982; Shu and Jones, 1993; Sullivan, 2002; Steiner et al., 2005).

Nasonia vitripennis females did not attract males from a distance. The present study revealed no apparent change in behaviour of males in the four-chamber olfactometer when exposed to virgin females at a distance of only 3 cm. This suggests that this species does not use a volatile sex pheromone, unlike some

Table 1 Relative composition of hexane fractions from freshly emerged *N. vitripennis* females and males. [a]LRI = Linear retention index; [b]DMDS = diagnostic ions after derivatization with dimethyl disulphide; [c]Means ± SE; [d]Relative amounts were compared by Mann-Whitney *U* test. Significantly higher abundances are shown in boldface (*N* = 7);[e]Not previously reported in *N. vitripennis* by Carlson et al. (1999).

No.	LRI[a]	Compound	Diagnostic ions	Female[c]	Male[c]	*P*-level[d]
1	2500	C25	352	**0.20 ± 0.04**[e]	0.06 ± 0.03[e]	0.013
2	2551	5-MeC25	351 (M-15), 84/85, 308/309	**0.17 ± 0.03**[e]	0.01 ± 0.01[e]	0.001
3	2700	C27	380	**0.48 ± 0.07**	0.17 ± 0.09[e]	0.025
4	2751	5-MeC27	379 (M-15), 84/85, 336/337	**0.18 ± 0.03**[e]	0.01 ± 0.01[e]	0.002
5	2773	3-MeC27	365 (M-29)	**0.15 ± 0.02**[e]	0.02 ± 0.01[e]	0.002
6	2800	C28	394	**0.07 ± 0.01**	0.02 ± 0.01	0.048
7	2900	C29	408	**3.6 ± 0.4**	1.1 ± 0.2	0.002
8	2931	13-MeC29	407 (M-15), 196/197, 252/253	**0.3 ± 0.03**	0.02 ± 0.01[e]	0.001
	2933	+ 11-MeC29	407 (M-15), 168/169, 280/281			
9	2936	9-MeC29	407 (M-15), 140/141, 308/309	**0.30 ± 0.04**	0.02 ± 0.01[e]	0.002
10	2941	7-MeC29	407 (M-15), 112/113, 336/337	**2.2 ± 0.2**	0.60 ± .12	0.002
11	2951	5-MeC29	407 (M-15), 84/85, 364/365	**0.40 ± 0.03**	0.15 ± 0.02	0.002
12	2973	3-MeC29	393 (M-29)	**0.21 ± 0.02**	0.04 ± 0.02	0.004
13	2981	5,17-DiMeC29	421 (M-15), 84/85, 379, 196/197, 266/267	**0.17 ± 0.02**[e]	0.06 ± 0.02[e]	0.009
14	3000	C30	422	**0.58 ± 0.05**	0.27 ± 0.04	0.003
15	3041	7-MeC30	421 (M-15), 112/113, 350/351	0.26 ± 0.01	**0.38 ± 0.04**	0.018
16	3075	C31:1(9)	528, 173, 355 (DMDS)[b]	0.22 ± 0.01[e]	**1.1 ± 0.18**[e]	0.002
17	3084	C31:1(7)	528, 145, 383 (DMDS)[b]	0.00	**0.16 ± 0.03**[e]	0.007
18	3100	C31	436	11.5 ± 1.1	10.5 ± 0.8	0.22
19	3128	15-MeC31	435 (M-15), 224/225, 252/253	**6.2 ± 0.3**	0.58 ± 0.22	0.002
	3129	+ 13-MeC31	435 (M-15), 196/197, 280/081			
	3132	+ 11-MeC31	435 (M-15), 168/169, 308/309			
	3135	+ 9-MeC31	435 (M-15), 140/141, 336/337			
20	3141	7-MeC31	435 (M-15), 112/113, 364/365	7.8 ± 0.2	**21.7 ± 1.0**	0.002

Table 1 continued

No.	LRI[a]	Compound	Diagnostic ions	Female[c]	Male[c]	*P*-level[d]
21	3150	5-MeC31	435 (M-15), 84/85, 392/393	2.5 ± 0.1	6.3 ± 0.4	0.002
		+13,17-DiMeC31	449 (M-15), 196/197, 294/295, 266/267, 224/225			
22	3157	11,15-DiMeC31	449 (M-15), 168/169, 322/323, 238/239, 252/253	0.90 ± 0.07	0.00	0.002
23	3163	9,21-DiMeC31	449 (M-15), 140/141, 350/351, 322/323, 168/169	0.81 ± 0.06[e]	0.00	0.002
24	3168	7,11-DiMeC31	449 (M-15), 112/113, 378/379, 182/183, 308/309	1.2 ± 0.09[e]	0.72 ± 0.16[e]	0.013
25	3174	3-MeC31	421 (M-29)	3.2 ± 0.3	2.7 ± 0.15	0.11
26	3181	5,x-DiMeC31	449 (M-15), 84/85, 406/407	0.97 ± 0.11[e]	3.2 ± 0.2[e]	0.002
		+ 7,x-DiMeC31	449 (M-15), 112/113, 378/379			
27	3191	unknown HC		0.34 ± 0.04	0.60 ± 0.05	0.003
28	3201	3,15-DiMeC31	449 (M-15), 435 (M-29), 238/239, 252/253	0.94 ± 0.04	0.38 ± 0.04[e]	0.002
29	3208	3,7-DiMeC31	449 (M-15), 435 (M-29), 126/127, 364/365, 435	0.31 ± 0.02	0.72 ± 0.04	0.002
30	3229	3,11,19-TriMeC31	449 (M-29), 182/183, 322/323, 252/253	1.00 ± 0.03[e]	0.52 ± 0.02[e]	0.0012
		+ 3,11,15-TriMeC31	449 (M-29), 182/183, 322/323, 308/309, 196/197			
	3234	+3,7,11-TriMeC31	463 (M-15), 449 (M-29), 126/127, 378/379, 196/197, 308/309			
31	3245	6-MeC32	449 (M-15), 98/99, 392/393	0.12 ± 0.01[e]	0.41 ± 0.04[e]	0.002
32	3258	3,7,11,15-TetraMeC31	477 (M-15), 463 (M-29), 126/127, 393, 196/197, 323, 266/267, 252/253	1.3 ± 0.08	0.08 ± 0.01[e]	0.0012
33	3277	C33:1(9)	556, 173, 383 (DMDS)[b]	0.09 ± 0.02[e]	0.62 ± 0.04[e]	0.002
34	3285	C33:1(7)	556, 145, 411 (DMDS)[b]	0.25 ± 0.02[e]	0.54 ± 0.04[e]	0.002
35	3300	C33	464	0.61 ± 0.07[e]	0.88 ± 0.08[e]	0.025
36	3327	15-MeC33	463 (M-15), 224/225, 280/281	8.5 ± 0.3	3.6 ± 0.2	0.002
	3328	+ 13-MeC33	463 (M-15), 196/197, 308/309			
	3331	+ 11-MeC33	463 (M-15), 168/169, 337/338			
37	3340	7-MeC33	463 (M-15), 112/113, 392/393	0.99 ± 0.07	3.9 ± 0.1	0.002
38	3350	5-MeC33	463 (M-15), 84/85, 420/421	1.1 ± 0.1[e]	1.3 ± 0.1[e]	0.34
39	3356	11,15-DiMeC33	477 (M-15), 168/169, 350/351, 239/240, 280/281	9.7 ± 0.2	1.7 ± 0.2	0.002
		+ 13,17-DiMeC33	477 (M-15), 196/197, 322/323, 266/267, 252/253			
		+ 15,19-DiMeC33	477 (M-15), 224/225, 294/295			
	3360	+ 11,21-DiMeC33	477 (M-15), 168/169, 350/351, 196/197, 322/323			

Table 1 continued

No.	LRI[a]	Compound	Diagnostic ions	Female[c]	Male[c]	*P*-level[d]
40	3368	7,19-DiMeC33	477 (M-15), 112/113, 406/407, 224/225, 294/295	3.7 ± 0.1	**8.3 ± 0.5**	0.002
	3372	+ 7,23-DiMeC33	477 (M-15), 112/113, 406/407, 168/169, 351/352			
41	3380	5,x-DiMeC33	477 (M-15), 84/85, 434/435	2.8 ± 0.1[e]	**5.8 ± 0.3**[e]	0.002
42	3388	11,15,23-TriMeC33	491 (M-15), 168/169, 364/365	**1.4 ± 0.1**	1.0 ± 0.1	0.025
43	3393	unknown HC		**1.1 ± 0.1**	0.33 ± 0.12	0.004
44	3405	3,17-DiMeC33	477 (M-15), 463 (M-29), 308/309, 238/239	0.00	**1.10 ± 0.1**[e]	0.002
		+ 3,15-DiMeC33	477 (M-15), 463 (M-29), 280/281, 266/267			
45	3460	3,7,11,15-TetraMeC33	505 (M-15), 491 (M-29), 126/127, 421, 196/197, 351, 266/267, 280/281	**1.6 ± 0.1**	0.41 ± 0.05[e]	0.002
46	3530	17-MeC35	491 (M-15), 252/253, 280/281	**3.4 ± 0.1**	1.8 ± 0.1	0.002
	3531	+ 15-MeC35	491 (M-15), 224/225, 308/309			
	3532	+ 13-MeC35	491 (M-15), 196/197, 336/337			
	3533	+ 11-MeC35	491 (M-15), 168/169, 364/365			
47	3542	7-MeC35	491 (M-15), 112/113, 420/421	0.08 ± 0.02	**0.47 ± 0.03**	0.002
48	3554	15,19-DiMeC35	505 (M-15), 224/225, 322/323, 294/295, 252/253	**8.5 ± 0.2**	2.0 ± 0.1	0.002
	3556	+ 13,17-DiMeC35	505 (M-15), 196/197, 350/351, 266/267, 280/281			
		+ 11,15-DiMeC35	505 (M-15), 168/169, 378/379, 238/239, 308/309			
49	3569	7,15-DiMeC35	505 (M-15), 112/113, 435/436, 280/281, 238/239	1.5 ± 0.1	**6.9 ± 0.3**	0.002
		+ 7,19-DiMeC35	505 (M-15), 112/113, 435/436, 252/253, 294/295			
		+ 7,23-DiMeC35	505 (M-15), 112/113, 435/436, 196/197, 350/351			
50	3579	5,17-DiMeC35	505 (M-15), 84/85, 462/463, 280/281, 266/267	3.5 ± 0.2	**4.9 ± 0.3**	0.006
	3585	+ 11,15,23-TriMeC35	519 (M-15), 168/169, 392/393, 238/239, 322/323, 196/197/364/365			
51	3722	+ 17-MeC37	519 (M-15), 252/253, 322/323	**0.34 ± 0.03**[e]	0.26 ± 0.02	0.048
		+ 15-MeC37	519 (M-15), 224/225, 350/351			
		+ 13-MeC37	519 (M-15), 196/197, 378/379			
52	3558	15,19-DiMeC37	533 (M-15), 224/225; 322/323, 294/295, 252/253	**2.1 ± 0.1**[e]	0.51 ± 0.03[e]	0.002
		+ 13,17-DiMeC37	533 (M-15), 196/197, 350/351, 266/267, 280/281			
		+ 11,15-DiMeC37	533 (M-15), 168/169, 406/407, 238/239, 308/309			
53	3770	7,x-DiMeC37	533 (M-15), 112/113, 462/463	0.21 ± 0.02	**1.25 ± 0.08**	0.002

other parasitic Hymenoptera (e.g., Lewis et al., 1971; Field and Keller, 1993; McNeil and Brodeur, 1995). This reflects the natural history and the mating strategy of *N. vitripennis*: the parasitoid is gregarious and protandrous males mate at the site of emergence with later emerging females. Thus, female-derived sex attractants are not necessary in this species. Males of the quasi-gregarious pteromalid *L. distinguendus* have been shown to use volatile cues other than sex pheromones for reaching or being arrested in the habitat of potential mates orienting toward the host-associated volatiles that females use for host finding. These chemicals are emitted from the larval faeces of the host *Sitophilus granarius* (Ruther and Steidle, 2000).

Future studies will address the question of whether *N. vitripennis* males also use host-associated cues for long-range orientation during mate finding and whether this strategy is a general feature among pteromalid wasps. Another challenge of future research is to identify the specific cuticular hydrocarbons that act as contact sex pheromones. However, the complexity of insect hydrocarbon profiles together with the nonavailability of synthetic reference compounds will complicate this undertaking.

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