CHAPTER 2

Female Sex Pheromone in Immature Insect Males - A Case of Pre-Emergence Chemical Mimicry?

Abstract Sexual selection by competition for mates is a formidable force that has led to extraordinary adaptations in males. Here we present results suggesting a novel case of pheromone mimicry in males of *Lariophagus distinguendus*, a parasitic wasp of beetle larvae that develop in stored grain. Females of *L. distinguendus* produce a pheromone even before they emerge from a grain. Males are attracted to the parasitised grain and wait for females to emerge. Males emerging later than others are under enormous selection pressure since females mate only once. We show evidence that developing males fool their earlier emerging competitors by mimicking the female pheromone. Males exposed to pupae of either sex exhibit typical courtship behaviour. Searching males are not only arrested by grains containing developing females but spend as much time on grains containing developing males. Hence, by distracting their competitors away from receptive females late males may increase their own chance to mate with these females. After emergence, males decompose the active compounds within 32 h probably to decrease molestation during their own search for mates. Chemical analyses of active pheromone extracts and bioassays using fractions demonstrate that the active compounds are among the cuticular hydrocarbons.

Key words Chemical mimicry, parasitoid, sex pheromone, mate competition, *Lariophagus distinguendus*.

Introduction

Fitness of insect males is generally correlated with their mating success. Therefore, male mate competition characterises several insect mating systems (Thornhill and Alcock, 1983; Godfray and Cook, 1997). Intraspecific sexual mimicry is one male strategy to increase the chance of mating. Males may fool their sexual competitors by mimicking females of their own species with respect to morphology, behaviour or pheromone chemistry (Thornhill, 1979; Peschke, 1987; Field and Keller, 1993).

Lariophagus distinguendus (Förster) (Hymenoptera, Pteromalidae) is a polyphagous ectoparasitoid parasitising larvae and prepupae of several stored product infesting beetles (Steidle and Schöller, 1997). For parasitisation, the female penetrates through the shell of the infested grain and lays one egg onto the outside of the host and the hatching larva feeds upon the host (Hase, 1924). Normally, a single parasitoid emerges from each grain. Mating occurs in the vicinity of the emergence sites. Like in several other pteromalids (King et al., 1969; Yoshida, 1978; van den Assem et al., 1980b; Sullivan, 2002), a female-derived, hitherto unknown sex pheromone induces male courtship behaviour and arrestment in *L. distinguendus* during encounters with females (Ruther et al., 2000). However, it is not yet known whether emerged males can already detect the sex pheromone during development of the females in the grains, which would increase their chance of locating virgin mates.

As in many other parasitoids of gregarious hosts, the mating system of short-lived *L. distinguendus* males is characterised by local mate competition (LMC), associated with protandry (i.e., males emerging before females), a female-biased sex ratio (about 2/3 females) and a high degree of sibling mating (Quicke, 1997). Protandry in *L. distinguendus* is due to differing developmental times of the two sexes rather than to the oviposition tactic of the females (who might influence the sex ratio by laying unfertilised eggs). Developmental time of males under optimal conditions is 1.5 days shorter than that of females (unpublished data), suggesting that selection favours early development in males. Male–male competition under LMC conditions is restricted to a given patch and males emerging later than others (e.g., because of a later oviposition date or delayed development due to suboptimal microclimatic conditions) are under a strong selection pressure since most hymenopteran parasitoid females including *L. distinguendus* normally mate only

once (Ruther et al., 2000). Furthermore, males may mate with several females within short time periods (unpublished data). Thus, late-emerging males are likely to encounter predominantly already mated females (Quicke, 1997). Therefore, all traits distracting the earlier emerged competitors from mate finding should increase the chance of mating for the later males.

In the present study, we investigated whether males of *L. distinguendus* are already able to detect the sex pheromone during pupal development. We demonstrate that not only females but also males produce the courtship pheromone during pupal development and that developing males, by mimicking female odour, distract their earlier emerged competitors from searching for actual females. Furthermore, we investigated whether males actively decompose the pheromone after emergence. Finally, we chemically characterised the courtship pheromone by analysing whole body extracts from *L. distinguendus* and assaying fractions for behavioural activity.

Methods and materials

Insects and general methods *L. distinguendus* was reared on larvae of the granary weevil *Sitophilus granarius* (Curculionidae), as described elsewhere (Steidle and Schöller, 1997). For all experiments, parasitoids were sexed directly after emergence and held in groups of at most 20 individuals in Petri dishes outfitted with moistened filter paper. One hour before the bioassays, parasitoids were individually transferred in 1.5 ml polyethylene reaction tubes. Unless otherwise noted, males were tested 1–2 days after emergence. All observations were done at room temperature using a stereo microscope under illumination of a microscope light. Behavioural recordings were done using the computer software The Observer 3.0 (Noldus, Wageningen, The Netherlands). The following behavioural parameters were defined: (1) wing fanning: males were allowed to have at most three antennal contacts with the sample. At least one typical high-frequency wing-fanning response within theses contacts was considered to be a positive reaction. (2) Antennation time: the time during which males showed typical antennation behaviour (regular, alternating contact with both antennae) was recorded. (3) Allocation time: the time during which males stayed on a sample was

recorded. In all experiments, individuals used as responders or potential pheromone sources were tested only once.

Experiment 1 A possible male response to pupae of either sex was investigated in this experiment. For this purpose, parasitoid pupae were excised from infested grains and categorised according to stepwise melanisation (Yoshida, 1978) into the following stages: (1) white pupae (8 days old), (2) yellowish pupae (9–11 days), (3) pupae with red eyes (11–13 days), (4) pupae with red mouthparts (13–15 days), (5) partially melanised pupae with black heads and thoraces (14–16 days) and (6) totally melanised pupae (15–17 days). Single pupae from the different stages were exposed to males in a bioassay chamber (10 mm diameter × 3 mm height) for 5 min as described elsewhere (Ruther et al., 2000) and male wing-fanning response was considered as a criterion for the presence of the sex pheromone. After the experiments, pupae were kept individually in reaction tubes and sex of emerging parasitoids was determined. Between 16 and 36 replicates were done for each pupal stage of both sexes. Males that did not respond to pupae were transferred to another bioassay chamber containing an unmated female. Males that still did not respond in this control experiment were assumed to be unmotivated and were excluded from statistical analysis.

Experiment 2 The intention of this experiment was to demonstrate that the courtship pheromone is present in freshly emerged adults of both sexes and to investigate the effect of parasitoid age on pheromone presence. Single unmated individuals of both sexes at four different ages (freshly emerged, 8, 24, 32 h) were presented to one 48-hour-old male in a bioassay chamber and wing-fanning behaviour shown by the older male was interpreted as evidence for the existence of the pheromone in the younger wasp. Each test was replicated 30 times. Other experimental details were as described in experiment 1.

Experiment 3 This experiment investigated whether the loss of pheromone activity in males is due to active decomposition or passive evaporation. For this purpose, 20 freshly emerged males and females were killed by freezing at −20°C for 1 h and stored for 72 h at a constant temperature of 25°C and a relative humidity of 75%. Simultaneously, 20 males and females were kept alive under the same conditions for 72 h and killed by freezing shortly before the experiments. The wing-fanning

response of individual males to dead conspecifics of the four categories (males/females stored dead/alive) was observed in a bioassay chamber.

Experiment 4 This experiment addressed the question of whether males are arrested by grains containing developing male and female conspecifics and whether they exhibit the courtship element wing fanning on these grains. In a triple-choice bioassay, single grains of the following three types were presented to individual males in a glass arena (3.5 cm diameter × 1.5 cm height) covered by a glass plate: (1) undamaged grain, (2) grain infested with *S. granarius* and (3) grain infested with *S. granarius* and parasitised by male or female *L. distinguendus*. Weevil larvae in the infested grains were between 21 and 28 days old and at the optimal age for parasitisation by *L. distinguendus*. The developing parasitoid males and females in the parasitised grains were between 17 and 18 days old and shortly before emergence. Wing-fanning behaviour, allocation time and antennation time of responding males on each type of grain was recorded for 10 min. The position of the grains was rotated clockwise after every insect to avoid bias due to side preferences. For sex determination of developing parasitoids, parasitised grains were transferred individually into polyethylene reaction tubes and held under rearing conditions until emergence. The experiment was replicated 21 times for female and 20 times for male parasitoids.

Experiment 5 This experiment was performed to exclude the possibility that wing-fanning behaviour and arrestment of the responding males on the parasitised grains in experiment 4 were due to pheromone residues left behind by females during oviposition. For this purpose, experiment 4 was repeated with one modification: the infested and parasitised grains (treatment 3 in experiment 4) were tested 1 day after parasitisation, that is, the developing parasitoids inside the parasitised grains were freshly hatched larvae rather than pupae about to emerge as in experiment 4. Given that ovipositing females release pheromone residues during oviposition, the parasitised grains in this experiment should induce arrestment or wing fanning in responding males. This experiment was replicated 28 times without distinguishing between grains containing developing male or female parasitoids.

Experiment 6 This experiment was done to study whether the courtship pheromone is extractable by dichloromethane from active parasitoids and to ensure biological activity of extracts used for chemical analysis. Fifteen individuals each of

(1) freshly emerged males, (2) freshly emerged females, (3) 72-hour-old males and (4) 72-hour-old females were killed by freezing at −20°C for 1 h and extracted for 4 days with 90 µl dichloromethane at room temperature. The resulting extracts were concentrated to 30 µl under a stream of nitrogen. Four microlitres representing two individual equivalents were applied on a filter paper disc (diameter 5 mm) and the solvent was allowed to evaporate for 15 min. One single paper disc with extract of either (1), (2), (3), (4) or solvent control was introduced together with a male into an observation chamber and allocation time on the disc and wing fanning (*N* = 20 replicates for each type of extract) were observed for 5 min. Filter discs were renewed after every five males.

Chemical analysis Extracts from freshly emerged and 72-hour-old parasitoids were prepared as described in experiment 6 (*N* = 5 for each type of extract) and analysed by coupled gas chromatography–mass spectrometry (GC–MS). One microlitre representing one-third individual equivalent of each extract was injected together with 50 ng of tetracosane as an internal standard. Analytical separations were performed on a Fisons model 8060 GC and mass spectra were obtained on an MD 800 quadrupole mass spectrometer (Thermo Finnigan, Egelsbach, Germany), with analytical conditions as follows: injector temperature: 240°C. Column: 30 m × 0.32 mm ID DB-5ms fused silica column, film thickness 0.25 µm (J & W). Carrier gas: helium, inlet pressure 10 kPa. Temperature programme: start at 150°C with a rate of 2°C/min to 300°C. Identification of the straight-chain hydrocarbons was done by comparisons of mass spectra and retention times with those of authentic reference compounds. Branched hydrocarbons were identified by diagnostic ions resulting from the favoured fragmentation at the branching points (Lockey, 1988; Nelson, 1993). Position of the double bonds of unsaturated hydrocarbons was determined by iodine-catalysed methyl-thiolation using dimethyl disulphide (Francis and Velant, 1981; Howard, 1993). Relative retention indices of branched and unsaturated hydrocarbons were estimated by co-injection of straight-chain hydrocarbons (van den Dool and Kratz, 1963). For quantification of selected hydrocarbons, peak areas of these compounds were compared with those resulting from co-injection of 50 ng of the internal standard and individual amounts in the extracts were calculated as tetracosane.

Experiment 7 Extracts of *L. distinguendus* females were fractionated by adsorption chromatography. Resulting fractions were tested for biological activity. Fifty females

were extracted with 300 µl dichloromethane as described in experiment 6. The solvent was evaporated under a stream of nitrogen and extracted components were resolved in 50 µl hexane. The hexane extract was applied to a 500 mg silica gel cartridge for solid phase extraction (IST, Mid-Glamorgan, UK) and eluted with 2 ml of (1) hexane, (2) 10% dichloromethane in hexane, (3) dichloromethane and (4) methanol. The fractions were concentrated to 100 µl each under nitrogen and four female equivalents of each fraction were tested as described in experiment 6 (N = 15 replicates). Paper discs treated with a mixture of pure solvents were used as control.

Statistical analysis Numbers of males responding by wing fanning to the different developmental stages of pupae (experiment 1) and adults (experiment 2), dead conspecifics (experiment 3), grains (experiments 4 and 5) or differently treated paper discs (experiments 6 and 7) were compared by using a chi-square test of homogeneity, followed by a series of Bonferroni-corrected 2×2 chi-square tests for individual comparisons (Sachs, 1992). Allocation and antennation times on grains in experiments 4 and 5 were analysed using a Friedman analysis of variance (ANOVA), followed by Wilcox–Wilcoxon test for multiple comparisons. In experiment 6 allocation times on test and control paper discs were compared by a Mann–Whitney U test. In experiment 7 allocation times on differently treated paper discs were analysed by a Kruskal–Wallis H test followed by a Nemenyi test for separation of means (Sachs, 1992). Mean absolute amounts of selected hydrocarbons in extracts from parasitoids differing in sex and age were compared by one-way ANOVA and subsequent least significant difference (LSD) tests. Statistical analyses were done using Statistica 4.5 scientific software (StatSoft, Hamburg, Germany).

Results

Experiment 1 Males responded to pupae of all developmental stages by wing fanning, indicating the presence of the courtship pheromone (Fig. 1a). There were no significant differences in the male response to male and female pupae of the same age. Comparison of the different stages within one sex revealed no differences in female pupae (χ^2 = 4.071, df = 5, critical value for P < 0.05 is 11.07)

whereas in male pupae the response to the red-mouthparts stage was significantly reduced compared to pupae with melanised heads and totally melanised pupae (χ^2 = 15.850, *df* = 5, *P* < 0.001).

Experiment 2 Both freshly emerged male and female parasitoids elicited wing fanning in older males (Fig. 1b). Whereas female pheromone activity remained on a constant high level throughout the test period (χ^2 = 3.025, *df* = 3, critical value for *P* < 0.05 is 7.82), male pheromone activity decreased significantly with increasing age (χ^2 = 50.606, *df* = 3, *P* < 0.0001). Thirty-two hours after emergence, males did not elicit any wing fanning in male conspecifics.

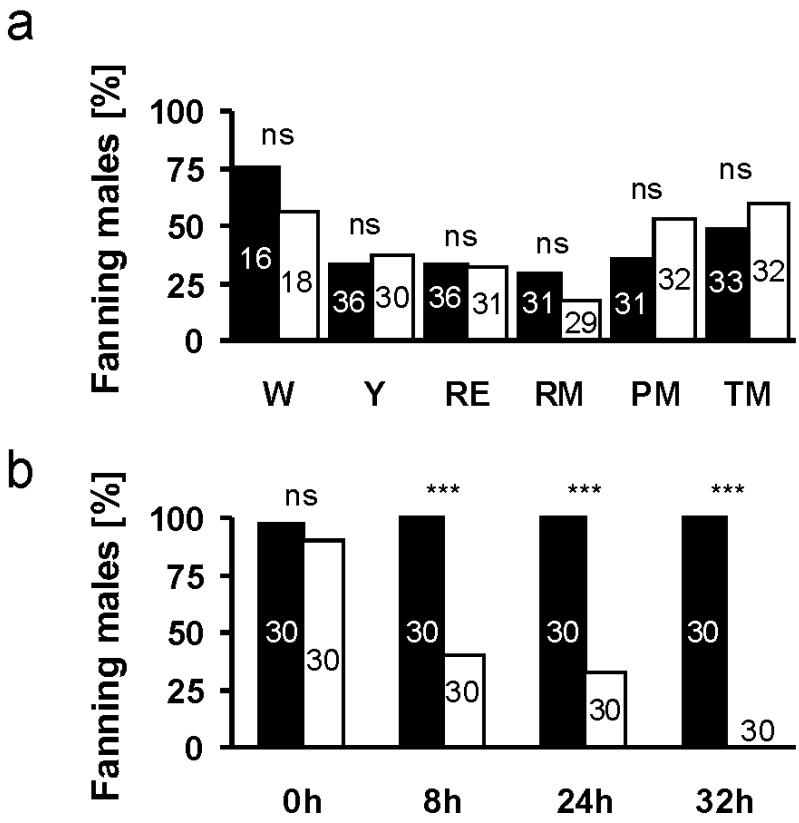

Fig. 1 Percentages of *Lariophagus distinguendus* males responding by wing fanning to (a) pupae of different developmental stages and (b) adult parasitoids at 0, 8, 24 and 32 h after emergence. W = white pupae, Y = yellowish pupae, RE = pupae with red eyes, RM = pupae with red mouthparts, PM = partially melanised pupae with black heads and thoraces, TM = totally melanised pupae; black bars = female pupae, white bars = male pupae; numbers in bars = number of replicates; ns = not significant; asterisks indicate significant differences between male and female individuals at *P* < 0.001 (chi-square test).

Experiment 3 Regardless of sex, freshly emerged parasitoids that had been killed and stored for 72 h at 25°C elicited wing-fanning behaviour in responding males (Fig. 2). This suggests a high degree of stability and a low volatility of the involved chemical(s). In parasitoids that upon emergence were stored alive under the same conditions until 72 h, only females elicited wing fanning, whereas males did not. This suggests that the loss of pheromone activity in males is caused by active decomposition processes in living males rather than by passive evaporation.

Fig. 2 Percentages of *L. distinguendus* males responding by wing fanning to dead female (black bars) and male (white bars) conspecifics stored dead or alive for 72 h (for details see text). Different lowercase letters indicate significant differences at $P < 0.001$ (Bonferroni-corrected multiple chi-square test; $N = 20$).

Experiment 4 Allocation and antennation times of male parasitoids were drastically increased on parasitised grains when compared to undamaged grains or grains infested by weevil larvae (Fig. 3a and b). Males spent about 20% of the total bioassay time on the parasitised grains. This indicates that male parasitoids are able to distinguish wheat grains containing developing conspecifics of either sex from intact grains and weevil-infested grains. Furthermore, only grains containing parasitoids elicited wing-fanning behaviour (Fig. 3c). In contrast, male parasitoids did not distinguish between undamaged and weevil-infested grains. A comparison of the male response to grains containing male or female conspecifics revealed no differences (allocation time: $U = 195.5$, $P = 0.705$; antennation time: $U = 202.0$, $P = 0.835$; wing fanning: $\chi^2 = 0.21$, $P = 0.647$). These results show that male wasps are able to detect grains containing parasitoids but are not able to differentiate between grains containing male or female conspecifics.

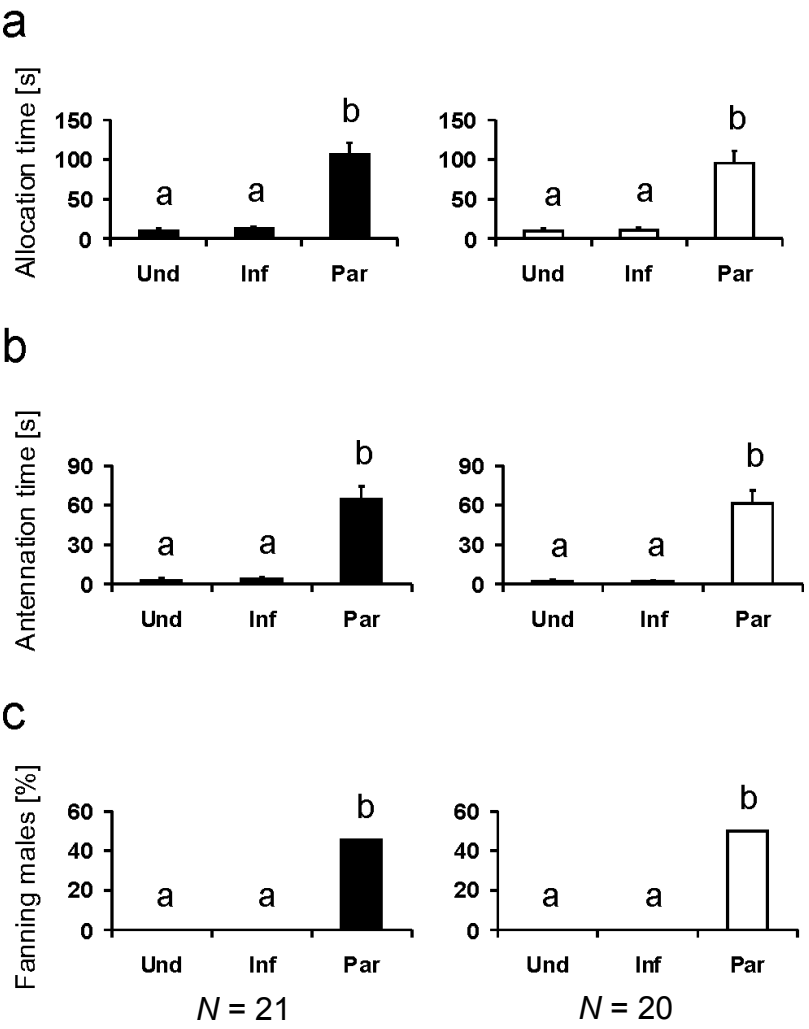

Fig. 3 Response of *L. distinguendus* males to different grains in triple-choice bioassays. (a) Mean allocation time (± SE), (b) mean antennation time (± SE) and (c) percentages of males fanning on undamaged grains (Und), grains infested by granary weevil (Inf) and infested grains parasitised by *L. distinguendus* (Par) during a 10 min observation period. Black bars = grains containing 17- to 18-day-old female parasitoids, white bars = grains containing 17- to 18-day-old male parasitoids. Different lowercase letters indicate significant differences at $P < 0.01$ (mean allocation and antennation time analysed by Friedman ANOVA followed by Wilcox–Wilcoxon test for multiple comparisons; percentages of fanning males analysed by Bonferronicorrected multiple χ^2 tests).

Experiment 5 Wing-fanning behaviour, allocation time and antennation time of male parasitoids were not significantly increased on parasitised grains 1 day after parasitisation when compared to undamaged grains or grains infested by weevil larvae (Fig. 4a–c). These results demonstrate that the response shown by males in experiment 4 cannot be explained by pheromone residues left behind by females during oviposition.

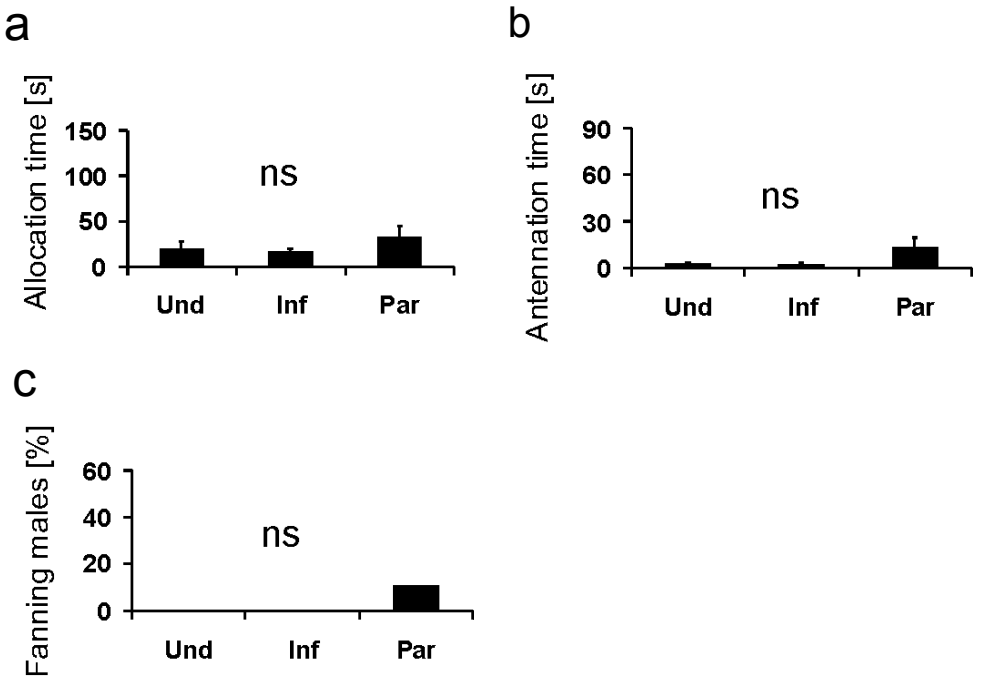

Fig. 4 Response of *L. distinguendus* males to different grains in triple-choice bioassays. (a) Mean allocation time (± SE), (b) mean antennation time (± SE), (c) percentages of males fanning on undamaged grains (Und), grains infested by granary weevil (Inf) and infested grains parasitised by 1-day-old female or male *L. distinguendus* (Par) during a 10-min observation period; ns = not significant (mean allocation and antennation time analysed by Friedman ANOVA; percentages of fanning males analysed by Bonferroni-corrected multiple chi-square tests; *N* = 28).

Experiment 6 Paper discs treated with extracts from freshly emerged males, freshly emerged females, and 72-hour-old females initiated wing fanning and arrestment in responding males when compared to solvent controls (Fig. 5a–c). Occasionally, they performed the whole courtship sequence on the treated paper discs. In extracts from 72-hour-old males no pheromonal activity was detectable (Fig. 5d).

Chemical analysis A total of 72 compounds were identified in the whole body extracts from *L. distinguendus*, the majority of which were hydrocarbons (Fig. 5, Table 1). Major components were methyl-branched hydrocarbons with one to four methyl groups. Saturated aliphatic hydrocarbons and monounsaturated straight-chain hydrocarbons occurred as minor compounds. The chain length of the

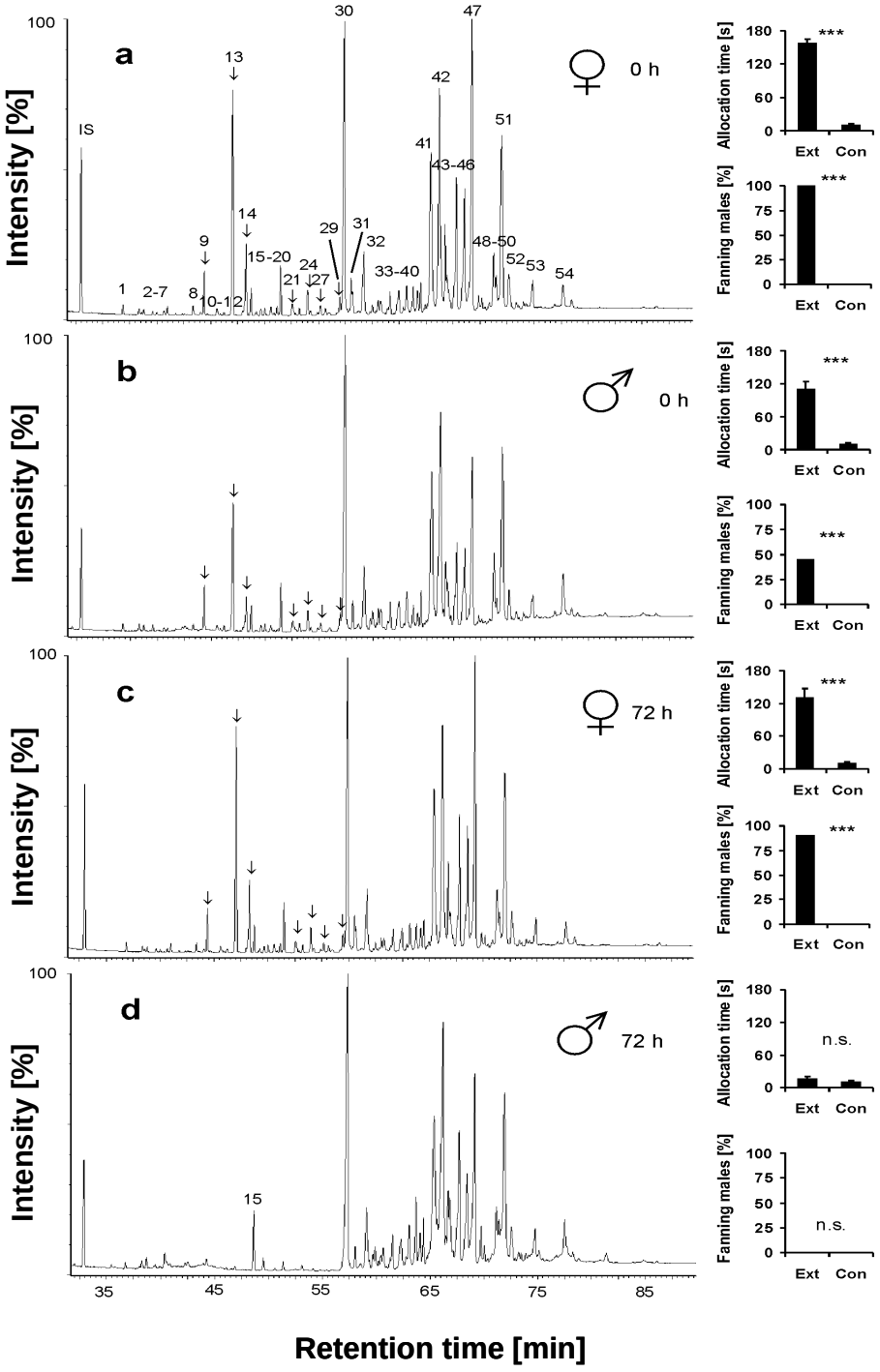

Fig. 5 Left side: Total ion current chromatograms of dichloromethane extracts from different groups of *L. distinguendus* [(a) freshly emerged females, (b) freshly emerged males, (c) 72-hour-old females, (d) 72-hour-old males]. The peak numbers correspond to those in Table 1. *IS* Internal standard. Arrows indicate compounds correlating strongly with pheromonal activity. Right side*:* Response of *L. distinguendus* males to filter paper discs treated with the corresponding extracts (Ext = extract, Con = solvent control). Mean allocation time (± SE) and percentages of males fanning on the paper discs during a 5 min observation period; ns = not significant, asterisks significant at $P < 0.001$ (mean allocation time analysed by *U* test; percentages of fanning males analysed by chi-square test; $N = 20$ for each experiment).

hydrocarbons varied between 25 and 37 carbon units. Furthermore, cholesterol was extracted from the parasitoids as a major component. All identified hydrocarbons are typical constituents of extracts from insect cuticles (Lockey, 1988; Nelson, 1993). Comparative chemical analyses of extracts from freshly emerged and 72-hour-old males and females revealed the presence of a series of hydrocarbons with retention times between those of the internal standard tetracosane and cholesterol only in females and fresh males but not in old males (Table 2). This pattern correlates strongly with pheromone activity described for experiment 6.

Experiment 7 After fractionation of an active pheromone extract by adsorption chromatography only the hexane fraction elicited wing fanning and caused arrestment in the responding males whereas all other fractions were inactive (χ^2 = 31.765, *df* = 3, *P* < 0.001; Fig. 6a and b). This suggests that the courtship pheromone of *L. distinguendus* is composed of cuticular hydrocarbons because the hexane fraction only contained these nonpolar substances (results not shown).

Discussion

In agreement with other studies on pteromalids (King et al., 1969; Yoshida, 1978), this study demonstrates that females of *L. distinguendus* produce a sexual pheromone as early as during pupal development. Searching males are able to detect the active compounds, which are released by developing females inside the parasitised grains and obviously diffuse through the thin shell of the grains. This pheromone arrests males on grains containing developing females and induces male antennation and wing fanning. It can be excluded that this effect is due to chemical residues left behind by ovipositing females of the former generation because allocation time of males was not increased on infested grains 1 day after parasitisation. The release of pheromones during pupal development might increase the chance for females to be inseminated immediately after emergence. This might be of particular importance, since females of *L. distinguendus* do not produce long-range pheromones (Ruther et al., 2000) and are probably more difficult to locate after having left the emergence site to search for new host patches. For males, on the other hand, it is crucial to encounter receptive females earlier as competitors because *L. distinguendus* females mate only once. Thus, by

searching for grains containing females about to emerge they increase their chance of encountering virgin females.

Further experiments demonstrate that male wasps perform fanning behaviour not only in the presence of female wasps but also in the presence of male pupae and freshly emerged males. Grains containing developing males stimulate the same degree of arrestment and courtship in adult males as grains with developing females. Even complete courtship sequences and copulation attempts with grains containing both developing male and female parasitoids were observed occasionally. These findings suggest that also males of *L. distinguendus* possess the female courtship pheromone both before and after emergence. As the presence of a female sex pheromone in freshly emerged males has been shown also in *Itoplectis conquisitor* Say (Hymenoptera: Ichneumonidae) (Robacker et al., 1976) this might be a general phenomenon.

What are the possible explanations for the observed response of *L. distinguendus* males to chemical cues from their rivals developing in the grains? Given that these males are not simply making mating mistakes, as known from male insects responding to visual cues when reliable chemical cues are absent (e.g., Wang et al., 1996; Harari et al., 2000), there are two alternative hypotheses cogitable: (1) benefits for the pheromone-carrying males might arise after or (2) already before emergence. In many other insects (Thornhill and Alcock, 1983), the limited number of receptive females causes male–male competition. In this context, adult males of some insect species have been demonstrated to deceive conspecific competitors by mimicking female behaviour or chemistry to steal matings or avoid intrasexual aggression (Thornhill, 1979; Peschke, 1987; Field and Keller, 1993). After emergence, males of *L. distinguendus* might benefit from the release of female pheromones for three reasons: (1) males might be sexually immature upon emergence and distract older competitors away from females until reaching sexual maturity; (2) young males might benefit from the female pheromone by distracting older males in situations of direct competition for females; and (3) young males might use the sex pheromone to reduce male–male aggression as demonstrated, for example, in studies on staphylinid beetles (Peschke, 1987). However, our own data hitherto support none of these three scenarios of post-emergence benefits. Freshly emerged males are immediately fertile and have the same mating chances when directly competing for females against older males (unpublished results).

Table 1 Composition of dichloromethane extracts from adult *L. distinguendus*. Numbers correspond with peak numbers in Fig. 4. Asterisks indicate compounds that correlate stringently with pheromone activity. LRI = linear retention index.

No.	LRI	Compound
1	2500	C25
2	2550	5-MeC25
3	2572	3-MeC25
4	2582	5,9-DiMeC25
5	2600	C26
6	2608	3,7-DiMeC25
7	2672	C27:1(9) + 3-MeC26
8	2691	4,8-DiMeC26
9*	2700	C27
10	2733	11-MeC27
11	2741	7-MeC27
12	2750	5-MeC27
13*	2773	3-MeC27
14*	2808	3,7-DiMeC27
15	2822	squalene
16	2858	2-MeC28
17	2873	C29:1(9)
18	2882	C29:1(7)
19	2890	4,8-DiMeC28
20	2900	C29
21*	2931 2932 2934	13-MeC29 + 11-MeC29 + 9-MeC29
22	2939	7-MeC29
23	2950	6-MeC29
24*	2972	3-MeC29
25	2976	5,17-DiMeC29
26	3000	C30
27*	3010	3,7-DiMeC29
28	3033	5,X-DiMeC29
29*	3063	C31:1(9)
30	3075	cholesterol
31	3100	C31
32	3132	15-MeC31 + 13-MeC31 + 11-MeC31
33	3173	3-MeC31
34	3182	5,9-DiMeC31
35	3209	3,7-DiMeC31
36	3234	3,7,11-TriMeC31
37	3257	3,7,11,15-TetraMeC31
38	3277	C33:1(9)
39	3289	4,8-DiMeC32
40	3300	C33
41	3332	15-MeC33 + 13-MeC33 + 11-MeC33
42	3359	11,21-DiMeC33
43	3376	3-MeC33
44	3382	5,9-DiMeC33
45	3406	3,7-DiMeC33
46	3431	3,7,11-TriMeC33
47	3460	3,7,11,15-TetraMeC33
48	3481	C35:1(9)
49	3493	4,8-DiMeC34
50	3530	17-MeC35 + 15-MeC35 + 13-MeC35 + 11-MeC35
51	3554 3556	13,17-DiMeC35 11,15-DiMeC35
52	3574 3578	3-MeC35 5,9-DiMeC35
53	3644	3,7,11,15-TetraMeC35
54	3722	19-MeC37 + 17-MeC37 + 15-MeC37 + 13-MeC37 + 11-MeC37

Furthermore, male–male aggression in situations of direct competition is commonly not shown in *L. distinguendus* (van den Assem et al., 1980a). Therefore, it is more likely that benefits occur pre-emergence. As mentioned above, males emerging later than others should be strongly disadvantaged in mate competition, since they predominantly encounter already mated females. The production of female courtship pheromones by males before they emerge from grains might decrease

this disadvantage by distracting already emerged competitors away from actual females. Thereby the probability of mating with these females themselves might increase. To our knowledge, this would be the first example of chemically mediated pre-emergence female mimicry in an insect.

Most examples of female mimicry have been discussed in terms of alternative mating strategies exhibited by a subset of disadvantaged males (Wyatt, 2003). In contrast, chemical mimicry in *L. distinguendus* during a certain stage appears to be obligatory for males rather than a facultative mating strategy performed only by some of them. The question is whether a tactic that is used by all males can be evolutionarily stable. For *L. distinguendus* this might be the case because the benefit for males is high when they emerge late (i.e., avoidance of zero fitness and increasing the chance to mate at all) compared to physiological and ecological costs (i.e., the danger of a reduced number of matings because of being fooled) when they emerge earlier. Experiment 3 shows that males but not females actively decompose the courtship pheromone, suggesting that it becomes detrimental for males to carry female odour after emergence. The compounds involved are both stable and of low volatility since paper discs treated with pheromone extracts remain active for weeks, eliciting wing fanning, arrestment and in some cases the complete courtship sequence. Hence, without active decomposition the pheromone would remain active the whole lifetime of the males and molestation by competitors would probably disturb their own efforts to find females. In fact, older males were regularly observed trying to court and mate with freshly emerged males but not with those having already decomposed the pheromone. Since females mate only once, the question arises why they do not decompose the pheromone after mating, to avoid molestation by courting males during host seeking. A possible explanation is that for females it pays to maintain the chance for a second mating if depleted of sperm, thereby maintaining the ability to produce female progeny. This situation might arise because females do not seem to perform mate choice and also mate with older males that do not transfer many sperms (unpublished data).

Although it is expected that the majority of parasitic hymenoptera use sex pheromones at close range (Quicke, 1997), there are relatively few detailed studies about pheromone chemistry (e.g., Swedenborg and Jones, 1992; Syvertsen et al., 1995). The existence of close-range pheromones in Pteromalidae has been

Table 2 Mean amounts per individuum (± SE, *N* = 5) of selected compounds correlating with biological activity of dichloromethane extracts from adult *L. distinguendus* differing in sex and age. Means with different lowercase letters are significantly different within each line at *P* < 0.05 (one-way ANOVA, LSD test). Peak numbers correspond with those in Fig. 5.

		Freshly emerged		72-h-old		one-way
No.	**Compound**	**female**	**male**	**female**	**male**	**ANOVA**
9	C27	21.1 ± 2.9a	27.4 ± 6.6a	15.9 ±1.0a	1.0 ± 0.1b	*F* = 9.481 *d.f.* = 3.16 *P* < 0.001
13	3-MeC27	143.4 ± 13.7a	60.2 ± 11.9b	35.8 ± 4.1b	0c	*F* = 42.783 *d.f.* = 3.16 *P* < 0.001
14	3,7-DiMeC27	30.1 ± 3.3a	11.8 ± 2.0b	8.8 ± 1.5b	0c	*F* = 37.991 *d.f.* = 3.16 *P* < 0.001
21	13-MeC29 + 11-MeC29 + 9-MeC29	9.5 ± 1.7a	6.7 ± 1.3a	4.1 ± 0.4b	0c	*F* = 14.033 *d.f.* = 3.16 *P* < 0.001
24	3-MeC29	12.9 ± 1.9a	6.0 ± 1.2b	6.0 ± 0.8b	0c	*F* = 19.598 *d.f.* = 3.16 *P* < 0.001
27	3,7-DiMeC29	5.8 ± 1.0a	2.8 ± 0.4b	2.7 ± 0.4b	0c	*F* = 16.423 *d.f.* = 3.16 *P* < 0.001
29	C31:1(9)	4.4 ± 1.5a	3.5 ± 0.8a	3.0 ± 0.7a	0b	*F* = 4.200 *d.f.* = 3.16 *P* < 0.05

demonstrated previously in several studies (King et al., 1969; Yoshida, 1978; van den Assem et al., 1980b; Sullivan, 2002). In *Roptrocerus xylophagorum* Ratzeburg (Hymenoptera: Pteromalidae), sex pheromone activity was localised in the hydrocarbon fraction (Sullivan, 2002). However, it was not possible to finally establish the chemical structures of the active chemicals. In the present study, comparative chemical analyses of whole body extracts strongly indicate that the sex pheromone of *L. distinguendus* is composed of a series of long-chain, apolar hydrocarbons of low volatility that correlate with male responsiveness. This result is supported by the bioassays using fractionated pheromone extracts and showing that only the apolar hexane fraction was behaviourally active. As expected, this active fraction contained all hydrocarbons found also in the raw extracts (results not shown). Thus, pteromalid wasps appear to be another group of insects where cuticular hydrocarbons play an important role as infochemicals. Further examples

comprise the use of hydrocarbons for species, kin and sex discrimination in solitary and social insects and the mimicry of hydrocarbon profiles 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).

It is possible that the sex pheromone in *L. distinguendus* has evolved from hydrocarbons having a common function in immature stages of either sex but is differentially regulated by hormones and metabolising enzymes upon emergence. In this case, the putative function of the pheromone for later emerging males in pre-emergence chemical mimicry might have been evolved secondarily, favoured by the ecological conditions of the tritrophic system.

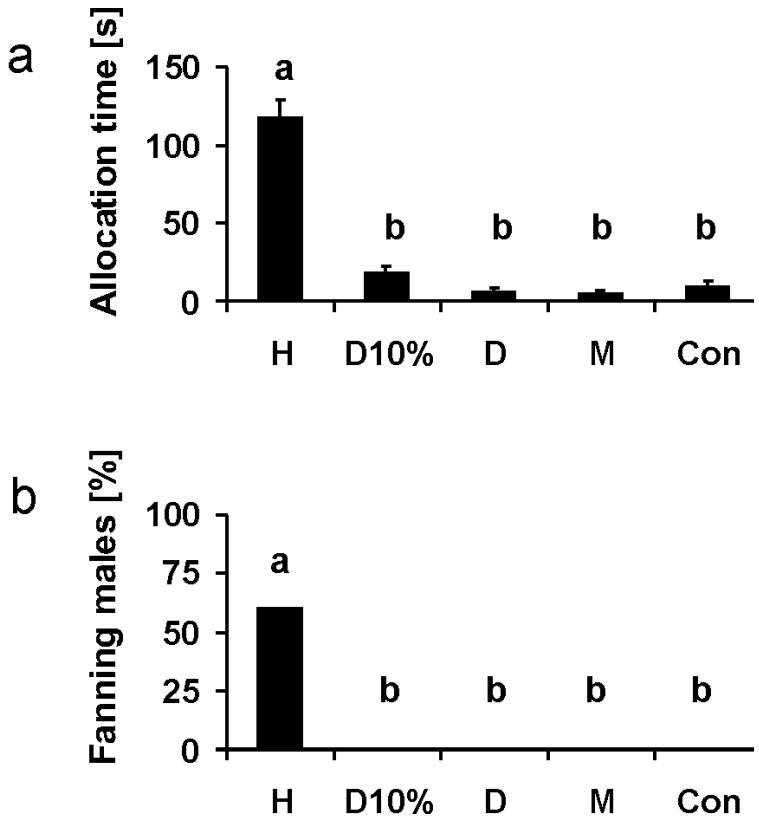

Fig. 6 Response of *L. distinguendus* males to filter paper discs treated with different fractions of a female extract (H = hexane, D10% = 10% dichloromethane in hexane, D = dichloromethane, M = methanol, Con = solvent control). (a) Mean allocation time (± SE) and (b) percentages of males fanning on the paper discs during a 5 min observation period. Different lowercase letters indicate significant differences at $P < 0.05$ (mean allocation time analysed by Kruskal–Wallis H test followed by Nemenyi test for multiple comparisons; percentages of fanning males analysed by Bonferroni-corrected multiple chi-square tests, $N = 15$).

Future studies will address the potential fitness consequences for *L. distinguendus* males resulting from the described phenomenon. The present study can open the door for pheromone identification of further parasitic hymenopterans and for investigating the question of whether the putative pre-emergence chemical mimicry is a common feature in parasitic wasps.

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