CHAPTER 3

Courtship Pheromones in Parasitic Wasps: Comparison of Bioactive and Inactive Hydrocarbon Profiles by Multivariate Statistical Methods

Abstract Cuticular hydrocarbons play a significant role not only in the regulation of cuticular permeability but also in chemical communication of insects. In the parasitoid *Lariophagus distinguendus* (Hymenoptera: Pteromalidae) the male courtship behaviour is mediated by a female-derived short-range sex pheromone. Previous studies have shown that the involved chemicals are already present in the pupal stage of both males and females. However, pheromone activity in males wear off shortly after emergence. Here we present evidence that the sex pheromone of *L. distinguendus* is composed of a series of cuticular hydrocarbons. Filter paper discs treated with the nonpolar fractions of cuticle extracts from freshly emerged males and females, 72-h-old females and yellowish pupae caused arrestment and stimulated key elements of the courtship behaviour in males whereas fractions of 72-h-old males were behaviourally inactive. Sixty-four hydrocarbons with chain length between C_{25} und C_{37} were identified in the fractions by gas chromatography-mass spectrometry (GC-MS). Methyl-branched alkanes with one to four methyl groups occurred as major components as well as traces of *n*-alkanes and monoenes. Principal component analysis (PCA) based on the relative amounts of the identified compounds revealed that cuticular hydrocarbon composition was clearly different between all five groups. By using partial least squares-discriminant analysis (PLS-DA), we determined a series of components by which active and inactive hydrocarbon profiles are discriminated and thus, may be responsible for the pheromonal activity of hydrocarbon fractions in *L. distinguendus*.

Key words Parasitoid, *Lariophagus distinguendus*, Pteromalidae, sex pheromone, cuticular hydrocarbons, Principal component analysis (PCA), Partial least squares-discriminant analysis (PLS-DA).

Introduction

Hydrocarbons are found on the cuticle of almost all insects. In addition to their primary function as water loss barrier to prevent desiccation (Lockey, 1988), cuticular hydrocabons are well-known to be involved in infochemical communication of insects (Howard, 1993; Blomquist et al., 1993, 1998; Howard and Blomquist, 2005). In social insects cuticular hydrocarbons contribute to the recognition of species, caste and nestmates (Blomquist et al., 1998) and may facilitate orientation towards the own nesting site (Steinmetz et al., 2003). Furthermore, cuticular hydrocarbons are involved in short-range sexual communication of insects enabling recognition of sexual mates, causing aggregation or acting as a courtship inhibitor to reduce the attractiveness of mated females (Blomquist et al., 1993; Ferveur, 2005).

Compared to other insect taxa, there are relatively few studies on parasitic wasps dealing with the composition of cuticular hydrocarbons with respect to their potential role as infochemicals. A number of investigations has focused on simple comparisons of qualitative and quantitative differences in hydrocarbon profiles of parasitoids depending on species, sex or the host used for development (Howard, 1992; Howard and Infante, 1996; Howard, 2001). However, only few studies included bioassays demonstrating behavioural activity of synthetic hydrocarbons or at least hydrocarbon fractions. In *Cardiochiles nigriceps* (Braconidae) elements of the male courtship behaviour are elicited by a series of female-specific alkadienes (Syvertsen et al., 1995). Recent studies on the pteromalids *Roptrocerus xylophagorum* (Sullivan, 2002), *Lariophagus distinguendus* (Steiner et al., 2005) and *Nasonia vitripennis* (Steiner et al., 2006) demonstrated that nonpolar hydrocarbon fractions from females arrested males and elicited courtship behaviour. Interestingly enough, in *L. distinguendus* pupae of both sexes also elicited male courtship behaviour. Freshly emerged males became behaviourally inactive for their male conspecifics 32 h after emergence. However, hitherto it is unknown which individual components contribute to the activity of biologically active extracts in the Pteromalidae.

Cuticular hydrocarbon profiles of insects are generally very complex consisting not seldom of more than hundred components, mainly saturated and unsaturated (one

to three double bonds) straight-chain and methyl-branched (one to four methyl branches) alkanes (Lockey, 1988). Studies on social insects showed that subtle quantitative differences in the relative composition of cuticular hydrocarbon profiles can be detected by analysing chemical data with multivariate statistical methods. By using this approach, it has been shown that cuticular hydrocarbon profiles of social insects are species- (e.g., Kaib et al., 1991; Page et al., 2002), caste- (e.g., Bagnères et al., 1990; Klochkov et al., 2005) and colony-specific (e.g., Butts et al., 1995; Lorenzi et al., 1997).

In the present study, we investigate the role of hydrocarbons as short-range sex pheromone in *L. distinguendus* more thoroughly by combining chemical analyses and behavioural bioassays. Firstly, we show that not only hydrocarbon fractions from females mediate male courtship behaviour but also those from freshly emerged males and pupae of either sex. Secondly, we compare the chemical composition of behaviourally active hexane fractions with inactive ones from older males by using principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) (Wold et al., 1989). The latter method is used to characterise those chemical components which strongly contribute to the discrimination of active and inactive hydrocarbon profiles. The results are discussed with respect to the putative function of these chemicals as constituents of the courtship pheromone in *L. distinguendus.*

Methods and materials

Insects Parasitoids were reared on larvae of the granary weevil *Sitophilus granarius* as described by Steidle and Schöller (1997) at a constant temperature of 25°C, a relative humidity of 60% and a photoperiod of 16:8 h (L:D). To obtain naïve parasitoids, single grains containing parasitoids about to emerge were transferred into 1.5 ml microcentrifuge tubes (Sarstedt, Nürnbrecht, Germany) and kept under rearing conditions. Emerging parasitoids were held in single-sex groups of 10 individuals in Petri dishes lined with moistened filter paper until used in the experiment.

General procedure for the bioassay The experiment was conducted in a bioassay chamber (10 mm diameter x 3 mm height) as described elsewhere (Ruther et al., 2000). Behavioural parameters were observed with a stereo microscope under illumination of a microscope light and recorded by using the computer software The Observer 3.0 (Noldus Information Technology, Wageningen, The Netherlands). Males were tested 2-3 days after emergence. One hour before being used in the bioassay, parasitoids were individualised in microcentrifuge tubes and kept under room temperature. The following behavioural parameters were considered as a criterion for the attractiveness of the tested odour samples: (1) Arrestment time: the time males stayed on the sample, (2) antennation time: the time males explored the sample by regular, alternating movements of the antennae, (3) wing-fanning behaviour: characteristic high frequency wing-fanning behaviour shown by males in the presence of the sex pheromone (Ruther et al., 2000).

Preparation of the hydrocarbon fractions Batches of ten individuals each of (1) freshly emerged females, (2) freshly emerged males, (3) 72-hour-old females, (4) 72-hour-old males and (5) yellowish pupae from *L. distinguendus* were extracted for 4 days with 60 µl dichloromethane at room temperature. Resulting extracts were concentrated under a gentle stream of nitrogen to 15 µl, applied to a 25 mg silica gel cartridge for solid phase extraction (IST, Mid-Glamorgan, UK) and eluted with 100 µl of hexane. The volume of each hydrocarbon fraction was concentrated under nitrogen to 20 µl (0.5 individual equivalent per µl) and stored at –80°C until used for bioassay and chemical analysis.

Bioassay: Activity of the hydrocarbon fractions The behavioural response of *L. distinguendus* males to filter paper discs (diameter 5 mm) treated with four individual equivalents of the five different hexane fraction types (see above) was investigated. After the solvent had evaporated for 15 min, single paper discs were offered to a male in the bioassay chamber and the arrestment time, antennation time and wing-fanning behaviour on the paper discs were recorded for 5 min. For control, filter paper discs were treated with pure solvent. Each parasitic wasp was tested only once. Parasitoids that did not respond to the filter paper disc by wing-fanning behaviour were released into another bioassay chamber containing an unmated *L. distinguendus* female. Males that did not show wing fanning in this

control experiment, were assumed to be unmotivated and discarded from statistical analysis. After five parasitoids had been tested, the paper disc was renewed. The bioassay chamber was cleaned regularly with ethanol and demineralised water to avoid contamination by the walking males. For each treatment, 10 parasitoids were tested.

Chemical analysis Hydrocarbon fractions (*N* = 10 for adults, *N* = 5 for pupae) were analysed by gas chromatography-mass spectrometry (GC-MS) on a Fisons GC 8060 equipped with a 30 m x 0.32 mm ID DB-5ms fused silica column (film thickness 0.25 µm) (J & W) and connected to a Fisons MD 800 quadrupole MS (Thermo Finnigan, Egelsbach, Germany). Helium was used as carrier gas, with an inlet pressure of 10 kPa. The temperature programme was started at 150°C, raising with 2°C/min to a final temperature of 280°C and held for 30 min. The column effluent was ionised by electron impact ionisation (EI) at 70 eV. One microlitre of each hexane fraction representing 0.5 individual equivalent was injected together with 25 ng of tetracosane as an internal standard. Relative retention indices (LRI) of methyl-branched and unsaturated hydrocarbons were estimated 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 LRI values with literature data (Carlson et al., 1998). Position of the double bonds of unsaturated hydrocarbons was determined by iodine-catalysed methylthiolation using dimethyl disulphide (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 analysis for the behavioural experiment was performed by using the software package Statistica release 4.5 (StatSoft, Tulsa, USA). Arrestment time and antennation time of males on filter paper discs treated with hexane fractions or solvent were analysed by Mann-Whitney *U* test. Number of males responding to the paper discs with wing-fanning behaviour were analysed by a 2 x 2 Chi²-test.

The quantitative composition of the five different hydrocarbon fraction types was evaluated by principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) using the software programme SIMCA-P 10.5 (Umetrics AB, Umeå, Sweden) (Wold et al., 1989; Eriksson et al., 2001). PCA and

PLS-DA were conducted as described in detail by Mumm et al. (2004) in order to extract and display the systematic variation in the data set consisting of 48 different hydrocarbon peaks (Table 1) (Eriksson et al., 2001). In PCA, so-called scores are obtained by projecting data observations onto model planes, which are defined by the extracted principal components.

Raw data (integrated peak areas) were normalised, i.e., peak areas of all analysed compounds (X variables) were summed and the relative amount of each variable was calculated. The normalised data were transformed to log (X + 0.00001). The constant 0.00001 was added to provide non-detectable components with a small non zero value (Sjödin et al., 1989). Transformed variables were then mean-centred, pareto scaled and represented as a matrix X. Pareto scaling gives each variable a variance equal to its standard deviation by dividing by the square root of the standard deviation of each column (Eriksson et al., 2001). The ellipse shown in score plots defines the Hotelling's T^2 confidence region (95%). The number of significant principal components was determined by cross-validation (Wold et al., 1989; Eriksson et al., 2001).

In PLS-DA the data set is modelled in a way similar to PCA, but in combination with a discriminant analysis. The objective of PLS-DA is to find a model that discriminates the X data according to the pheromone activity as good as possible (Eriksson et al., 2001). In contrast to PCA, PLS-DA is a supervised technique, so class memberships of the observations need to be predefined. Therefore, an additional Y matrix was made up with G columns containing the values 1 and 0 as dummy variables for either behaviourally active or inactive parasitoid groups respectively. In addition, we calculated the variable importance in the projection (VIP) which is a more numerical value describing the importance of the X variables, both for the X and the Y parts (Wold et al., 1993, 2001). Variables with VIP values larger than 1 are most influential for the model (Eriksson et al., 2001, Paolucci et al., 2004).

Results

Bioassay: Activity of the hydrocarbon fractions Hydrocarbon fractions from freshly emerged males and females as well as those from 72-h-old females and pupae caused arrestment and stimulated wing fanning in responding males when compared to the solvent control (Fig. 1a-c). In some cases, even more complex elements of the male courtship behaviour like antennal stroking and copulation attempts by the males were observed during the test period (data not shown). By contrast, males responded neither to hydrocarbon fractions from 72-h-old males nor to control paper discs treated with pure solvent.

Chemical and statistical analysis The 64 compounds identified in the hexane fractions were exclusively cuticular hydrocarbons with chain lengths between 25 and 37 carbon units (Table 1). Hydrocarbons of *L. distinguendus* were comprised of homologous series of *n*-alkanes (C_{25}-C_{33}), monomethyl alkanes (19-, 17-, 15-, 13-, 11-, 7-, 3-methyl), dimethyl alkanes (4,8-, 3,7- and 5,9-dimethyl), trimethyl alkanes (3,7,11-trimethyl), tetramethyl alkanes (3,7,11,15-tetramethyl) and monoenes with double bonds at position 9 or 7. Hydrocarbon profiles were dominated by methyl-branched alkanes with odd carbon chains. The most prominent compounds were 3,7,11,15-TetraMeC_{33}, 11,21-DiMeC_{33} and 13,17-DiMeC_{35}. Fractions that stimulated male courtship in the bioassays showed quantitative but no qualitative differences. In behaviourally inactive fractions of 72-h-old males, however, a series of hydrocarbons were missing.

A PCA was conducted based on the relative peak areas found in the five different types of hydrocarbon fractions. In this PCA model, 4 principal components were extracted explaining a total variation (R^2X) of 79%. A score plot of the first two principle components shows that the cuticular hydrocarbon composition is clearly different between all groups (Fig. 2). The samples of the 72-h-old male parasitoids were dissimilar to all other samples indicated by the projection on the left hand side of the plot. The first PC divides mainly samples of different age, whereas the second PC tends to separate the gender of the adult parasitoids.

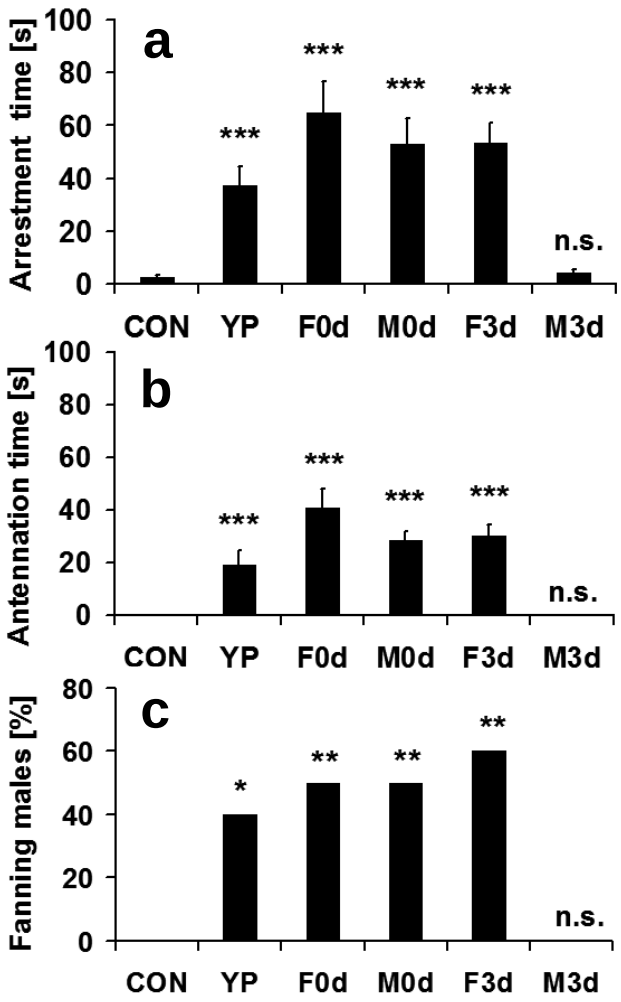

Fig. 1 Response of *L. distinguendus* males to filter paper discs treated with hexane fractions of cuticle extracts from freshly emerged females (F0d) and males (M0d), 72-h-old females (F3d) and males (M3d), yellowish pupae (YP) and solvent control (CON). (a) Mean arrestment time (±SE), (b) mean antennation time (±SE) and (c) percentages of males showing wing-fanning behaviour. Asterisks indicate significant preferences for fractions when compared to control (*** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$; n.s. = not significant). Mean arrestment times and antennation times were compared by Mann-Whitney *U* tests; wing-fanning behaviour was analysed by 2 x 2 chi-square tests ($N = 10$).

In order to elucidate which chemical compounds may be responsible for the pheromonal activity a PLS-DA was performed. Two classes were constructed consisting of parasitoids according to their pheromonal activity as shown in the bioassays, i.e. hydrocarbon fractions of freshly emerged males, freshly emerged females, 72-h-old females and pupae were joined in one class, the fraction of 72-h-old males represented the second class. PLS-DA resulted in a model with three

significant discriminant components with R^2X of 70.9%, R^2Y of 99.5% and Q^2Y of 98.7%. Q^2Y denotes the predictive power of the model, i.e., how class membership can be predicted by the model. The PLS score plot shows that the hydrocarbon fractions of 72-h-old male parasitoids which are not behaviourally active are clearly separated from the other active groups (Fig. 3a). The corresponding loading plot depicts which chemical variables contribute strongly to the separation of classes. Variables projected close to the dummy variables Y (shown as active and not active) contribute strongly to the class separation, thus have a high discriminatory power (Fig. 3b).

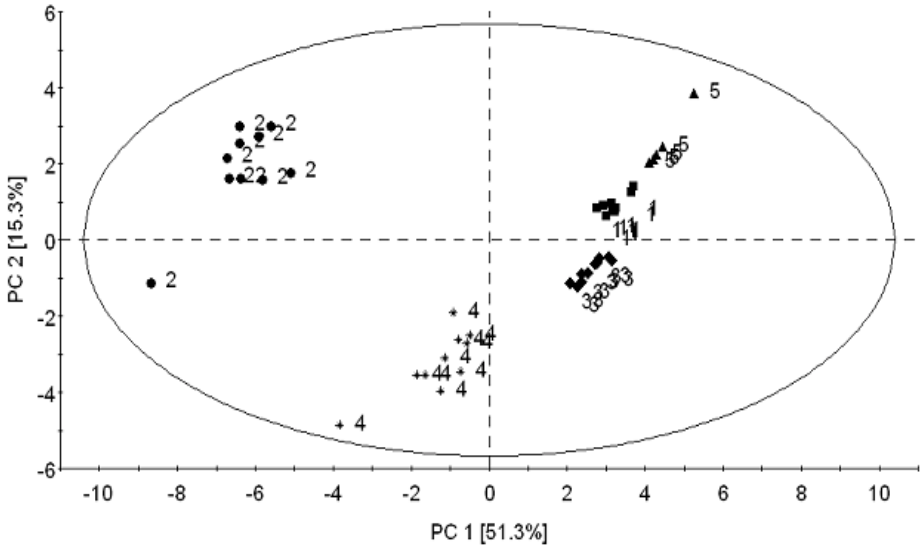

Fig. 2 Analysis of the cuticular hydrocarbon profiles from different *L. distinguendus* life stages**.** Score plot from principal component analysis (PCA) based on relative amounts of all analysed hydrocarbons shown in Tab. 1. 66.5% of the variance in the data is explained by the two first significant principal components, as judged by cross-validation. The ellipse shown in the score plot defines the Hotelling's T^2 confidence intervall (95%). 1 = freshly emerged males; 2 = 72-h-old males; 3 = freshly emerged females; 4 = 72-h-old females; 5 = yellowish pupae. Data were preprossed by log-transformation, mean centring and pareto scaling.

A more quantitative way to estimate the variable influence is described by the VIP-parameter. Chemical variables most important for resolving the behaviourally active parasitoid groups have VIP-parameter values above 1 (Eriksson et al., 2001). Compounds showing VIP-parameter > 1 are in descending order peak no. 10, 20, 23, 4, 14, 9, 17, 15 (data not shown). These compounds are marked with an asterisk in Fig. 3b.

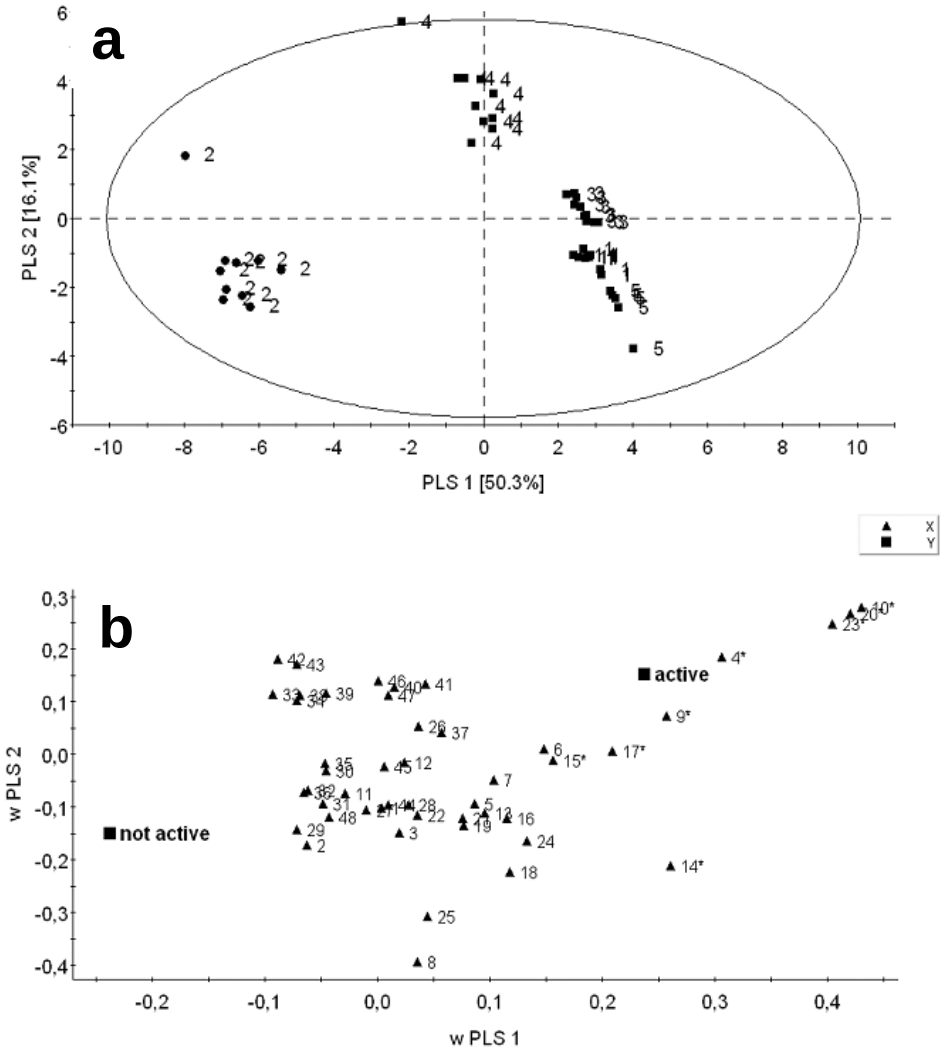

Fig. 3 Analysis of the cuticular hydrocarbon profiles from different *L. distinguendus* life stages**.** (a) Score plot and (b) loading plot from partial least squares-discriminant analysis (PLS-DA) based on relative amounts of all analysed hydrocarbons shown in Tab. 1. A total of 66.4% of the variance of the X variables and 98.5% of the variance of the dummy variable Y is explained by the two significant principal components, as judged by cross-validation. Y variables are shown as "active" or "not active". 1 = freshly emerged males; 2 = 72-h-old males; 3 = freshly emerged females; 4 = 72-h-old females; 5 = yellowish pupae; Box = no pheromone activity; Dot = pheromone activity. Data were preprossed by log-transformation, mean centring and pareto scaling.

Our PLS-DA model resulted in three significant PLS components. However, with G = 2 well separated classes one expects G – 1 significant PLS components (Eriksson et al., 2004). More components indicate presence of e.g. sub-clusters. In our model this is presumably caused by the samples of the 72-h-old females. The

hydrocarbon composition of these samples is slightly different from the composition of the freshly emerged parasitoids and pupae. This might be ascribed to the age of the parasitoids which apparently influences the composition of hydrocarbons (Fig. 2, Fig. 3a).

Discussion

The present study reports the characterisation of individual cuticular hydrocarbons with respect to their potential role as short-range sex pheromone in *L. distinguendus*. Behavioural experiments with hydrocarbon fractions from different life stages demonstrated that pheromone activity is not only present in females but also in freshly emerged males and immature stages of both sexes. In contrast, pheromone activity was absent in extracts from 72-h-old males. Chemical analysis by GC-MS revealed qualitative and quantitative differences in the hydrocarbon profiles of behaviourally active and inactive fractions of *L. distinguendus*. PLS-DA was applied to assess which hydrocarbons are of particular importance in separating the different life stages by pheromone activity. Methyl-branched alkanes (3-MeC27, 3,7-DiMeC27, 4,8-DiMeC28, 3-MeC29 and 13-MeC29) had the strongest impact in the discriminant analysis. However, also some alkenes (C27:1(9), C29:1(7)) had a high discriminating power.

During the past years, the number of studies on the role of cuticular hydrocarbons as infochemicals has grown immensely (reviewed by Howard and Blomquist, 2005). Evidence is increasing that cuticular hydrocarbons play a general role as courtship pheromones also in parasitoids mediating arrestment and more complex behavioural elements. However, only in a few studies individual bioactive cuticular hydrocarbons have been identified so far. Syvertsen et al. (1995) reported a series of female specific C_{25}-C_{35} (*Z*,*Z*)-alkadienes with pheromonal properties in the braconid wasp *Cardiochiles nigriceps.* In the ichneumonid parasitoid *Eriborus terebrans*, Shu and Jones (1993) reported a synergism between a polar female-derived sex pheromone and nonpolar components, most probably cuticular hydrocarbons. Apart from *L. distinguendus*, evidence for the function of cuticular hydrocarbons as courtship pheromones has been provided in two other pteromalids. Sullivan (2002) identified 28 compounds in female extracts of

Table 1 Relative composition of hexane fractions from different life stages of *L. distinguendus*. [a]LRI = Linear retention index, [b]DMDS = diagnostic ions after derivatisation with dimethyl disulphide, [c]Means ± SE.

No.	Compound	LRI[a]	Diagnostic ions	F0d[c]	M0d[c]	F3d[c]	M3d[c]	YP[c]
01	C25	2500	352	0.08±0.03	0.15±0.07	0.09±0.04	0.14±0.09	0.08±0.04
02	5-MeC25	2550	84/85, 308/309	0.07±0.04	0.27±0.15	0.10±0.04	0.39±0.19	0.17±0.13
03	5,9-DiMeC25	2582	337	0.09±0.03	0.08±0.04	0.03±0.02	0.05±0.03	0.04±0.01
04	C27:1(9)	2672	472, 173, 299 (DMDS)[b]	0.11±0.03	0.11±0.02	0.06±0.03	0.00	0.02±0.02
	+ 3-MeC26		380, 351					
05	4,8-DiMeC26	2691	379 (M-15), 351, 140/141, 280/281	0.00	0.01±0.01	0.00	0.00	0.00
06	C27	2700	380	0.87±0.17	1.02±0.15	0.36±0.09	0.10±0.05	0.15±0.12
07	11-MeC27	2733	168/169, 252/253	0.15±0.04	0.17±0.05	0.07±0.02	0.05±0.04	0.05±0.02
08	5-MeC27	2750	84/85, 336/337	0.06±0.01	0.08±0.01	0.00	0.01±0.01	0.01±0.00
09	3-MeC27	2773	364/365	7.47±1.35	5.04±0.94	1.76±2.16	0.03±0.01	0.98±0.98
10	3,7-DiMeC27	2808	393, 56/57, 379, 126/127, 308/309	1.44±0.31	0.97±0.20	0.29±0.14	0.00±0.00	0.13±0.13
11	squalene	2822	-	0.33±0.08	0.66±0.31	0.55±0.33	0.84±0.50	0.50±0.21
12	2-MeC28	2858	365	0.18±0.03	0.12±0.02	0.18±0.05	0.15±0.02	0.08±0.08
13	C29:1(9)	2873	500, 173, 327 (DMDS)[b]	0.23±0.08	0.21±0.07	0.05±0.02	0.06±0.05	0.05±0.02
14	C29:1(7)	2882	500, 145, 355 (DMDS)[b]	0.02±0.01	0.03±0.01	0.00	0.00	0.00
15	4,8-DiMeC28	2890	407 (M-15), 70/71, 379, 140/141, 308/309	0.06±0.02	0.03±0.01	0.03±0.02	0.00	0.01±0.01
16	C29	2900	408	1.15±0.21	1.25±0.26	0.28±0.09	0.40±0.62	0.33±0.20
17	13-MeC29	2931	196/197, 252/253	0.44±0.09	0.45±0.06	0.08±0.03	0.02±0.02	0.04±0.03
	+ 11-MeC29	2932	168/169, 280/281					
	+ 9-MeC29	2934	140/141, 308/309					
18	7-MeC29	2939	112/113, 336/337	0.05±0.01	0.06±0.01	0.00	0.02±0.03	0.01±0.01
19	6-MeC29	2950	84/85, 364/365	0.14±0.02	0.15±0.02	0.03±0.01	0.09±0.07	0.04±0.04

Table 1 continued.

No.	Compound	LRI[a]	Diagnostic ions	F0d[c]	M0d[c]	F3d[c]	M3d[c]	YP[c]
20	3-MeC29	2972	393	0.72±0.11	0.56±0.09	0.15±0.05	0.00	0.06±0.06
21	5,17-DiMeC29	2976	421 (M-15), 84/85, 379, 196/197, 266/267	0.14±0.03	0.12±0.01	0.03±0.01	0.04±0.01	0.02±0.01
22	C30	3000	422	0.04±0.01	0.13±0.04	0.05±0.06	0.05±0.04	0.05±0.01
23	3,7-DiMeC29	3010	421, 56/57, 406/407, 126/127, 336/337	0.30±0.05	0.25±0.04	0.05±0.02	0.00	0.02±0.02
24	5,X-DiMeC29	3033	421 (M-15), 84/84, 351	0.12±0.02	0.15±0.01	0.03±0.02	0.02±0.02	0.02±0.01
25	C31:1(9)	3063	528, 173, 355 (DMDS)[b]	0.56±0.23	0.57±0.16	0.02±0.02	0.06±0.02	0.06±0.06
26	C31	3100	436	0.51±0.07	0.80±0.07	1.31±0.36	1.10±1.53	0.87±0.63
27	15-MeC31	3132	224/225, 252/253	2.64±0.28	2.72±0.93	1.68±0.31	2.58±0.26	1.15±0.98
	+ 13-MeC31		196/197, 280/081					
	+ 11-MeC31		168/169, 308/309					
28	3-MeC31	3173	421	0.37±0.05	0.40±0.03	0.21±0.04	0.26±0.03	0.11±0.11
29	5,9-DiMeC31	3182	449(M-15), 84/85, 406/407, 154/155, 336/337	0.16±0.02	0.14±0.01	0.10±0.02	0.23±0.04	0.08±0.09
30	3,7,11-TriMeC31	3234	463 (M-15), 449, 126/127, 378/379, 196/197, 308/309	0.91±0.11	0.78±0.07	0.71±0.12	1.00±0.06	0.39±0.44
31	3,7,11,15-TetraMeC31	3257	492, 463, 126/127, 393, 196/197, 323, 266/267, 252/253	1.21±0.07	1.29±0.08	1.11±0.15	1.57±0.07	0.59±0.70
32	C33:1(9)	3277	556,173, 383 (DMDS)[b]	1.10±0.07	1.12±0.10	0.94±0.12	1.47±0.09	0.54±0.63
33	4,8-DiMeC32	3289	463 (M-15), 435, 140/141, 365	0.62±0.11	0.41±0.03	1.88±0.32	2.01±0.25	0.90±0.96
34	C33	3300	464	0.62±0.07	0.32±0.04	0.89±0.14	0.99±0.10	0.43±0.46
35	15-MeC33	3332	478, 224/225, 280/281	9.93±0.47	14.10±0.59	11.96±2.01	14.89±0.63	6.02±6.87
	+ 13-MeC33		478, 196/197, 308/309					
	+ 11-MeC33		478, 168/169, 337/338					
36	11,21-DiMeC33	3359	492, 168/169, 350/351, 196/197, 322/323	12.56±0.53	18.94±0.58	15.16±2.83	24.17±1.10	8.77±10.48
37	3-MeC33	3376	478, 449	3.10±0.30	2.41±0.15	2.01±0.23	1.46±0.13	0.79±0.88
38	5,9-DiMeC33	3382	492, 84/85, 435, 154/155, 364/365	1.42±0.23	1.53±0.09	2.84±0.20	3.00±0.09	1.24±1.53

Table 1 continued.

No.	Compound	LRI[a]	Diagnostic ions	F0d[c]	M0d[c]	F3d[c]	M3d[c]	YP[c]
39	3,7-DiMeC33	3406	492, 463, 126/127, 392/393	6.49±0.29	4.15±0.28	7.38±0.26	6.76±0.41	3.02±3.71
40	3,7,11-TriMeC33	3431	506, 477, 126/127, 407, 196/197, 336/337	6.25±0.31	4.05±0.31	6.80±0.45	4.18±0.52	2.45±2.92
41	3,7,11,15-TetraMeC33	3460	520, 491, 126/127, 421, 196/197, 351, 266/267, 280/281	18.37±1.14	11.07±0.61	18.68±1.48	10.13±0.99	6.38±7.93
42	C35:1(9)	3481	584, 173, 411 (DMDS)[b]	0.33±0.14	0.11±0.02	1.51±0.23	1.07±0.17	0.60±0.65
43	4,8-DiMeC34	3493	506, 70/71, 463, 140/141, 392/393	0.32±0.10	0.07±0.02	0.68±0.11	0.54±0.08	0.29±0.30
44	17-MeC35	3530	506, 252/253, 280/281	2.29±0.17	3.15±0.19	1.79±0.20	2.30±0.22	0.94±1.03
	+ 15-MeC35		506, 224/225, 308/309					
	+ 13-MeC35		506, 196/197, 336/337					
	+ 11-MeC35		506, 168/169, 364/365					
45	13,17-DiMeC35	3554	520, 196/197, 350/351, 266/267, 280/281	11.70±1.05	15.74±0.68	12.90±1.19	13.25±0.43	5.69±6.75
	+ 11,15-DiMeC35	3556	505 (M-15), 168/169, 378/379, 238/239, 308/309					
46	3-MeC35	3574	506, 477	1.78±0.13	1.17±0.05	2.52±0.19	1.59±0.11	0.89±1.11
	+ 5,9-DiMeC35	3578	520, 463, 393					
47	3,7,11,15-TetraMeC35	3644	548, 519, 126/127, 449, 196/197, 379, 266/267, 308/309	1.43±0.15	1.05±0.11	1.64±0.26	1.14±0.12	0.65±0.70
48	19-MeC37	3722	534, 280/281, 294/295	1.09±0.19	1.84±0.18	1.00±0.21	1.80±0.12	0.66±0.73
	+ 17-MeC37		534, 252/253, 322/323					
	+ 15-MeC37		534, 224/225, 350/351					
	+ 13-MeC37		534, 196/197, 378/379					
	+ 11-MeC37		534, 168/169, 407					

Roptocerus xylophagorum that consisted of aliphatic and methyl-branched alkanes with up to two methyl groups correlating best with the male response. Steiner et al. (2006) found numerous gender-related differences in *N. vitripennis* when comparing the composition of bioactive hydrocarbon fractions from females with inactive ones from males.

Our bioassays with hydrocarbon fractions from different life stages support recent investigations showing that *L. distinguendus* produces the sex pheromone already during pupal development (Steiner et al., 2005). The same phenomenon was previously reported in *Anisopteromalus calandrae* (Pteromalidae) (Yoshida, 1978) and *Apanteles glomeratus* (Braconidae) (Tagawa, 1977). Steiner et al. (2005) demonstrated that searching *L. distinguendus* males were arrested on parasitised grains containing females about to emerge. This pre-emergence pheromone release might increase the chance for females of being inseminated before having left the emergence site for searching hosts.

Interestingly enough, bioactive hydrocarbons are also present in both developing and freshly emerged males of *L. distinguendus* but then wear off soon. However, the mechanisms involved are not clear so far. One possible explanation is that the pheromone function of cuticular hydrocarbons has evolved secondarily from a primary role, e.g., the prevention of desiccation. Upon emergence the production of these compounds seems to be differently regulated in male and female parasitoids: females of *L. distinguendus* may be recognised by courting males with help of these compounds whereas males metabolise the hydrocarbons within 32 hours possibly to avoid molestation by courting competitors when searching for receptive females (Steiner et al., 2005). Another parasitoid species in which young males elicit courtship behaviour in male conspecifics is the ichneumonid *Itoplectis conquisitor*. Robacker et al. (1976) demonstrated that both extracts of freshly emerged males and females release sexual behaviour in older males. However, bioactive constituents of this multicomponent pheromone are aldehydes rather than hydrocarbons (Robacker and Hendry, 1977). Good evidence exists at least for *L. distinguendus* that developing males benefit from possessing the pheromone. Steiner et al. (2005) showed that searching males are not able to distinguish between grains containing female or male conspecifics that are about to emerge. Thus, authors suggested that developing males inside the grains might fool their

already emerged competitors by distracting them away from searching for actual females.

By applying discriminant analysis on the hydrocarbon composition of parasitic Hymenoptera, we identified a set of compounds that are highly important in discriminating the active from the inactive life stages and may be responsible for the pheromonal activity in *L. distinguendus*. However, since our findings are based exclusively on mathematical modeling, future studies will have to focus on behavioural experiments using synthetic reference compounds to verify the function of these hydrocarbons as courtship pheromone in *L. distinguendus*.

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