J Chem Ecol (2007) 33:825–838
DOI 10.1007/s10886-007-9265-6

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

Sven Steiner · Roland Mumm · Joachim Ruther

Received: 17 July 2006 / Revised: 22 November 2006 / Accepted: 27 November 2006 /
Published online: 1 March 2007

Abstract Cuticular hydrocarbons play a significant role in the regulation of cuticular permeability and also in the chemical communication of insects. In the parasitoid *Lariophagus distinguendus* (Hymenoptera: Pteromalidae), male courtship behavior is mediated by a female-produced sex pheromone. Previous studies have shown that the chemicals involved are already present in the pupal stage of both males and females. However, pheromonal activity in males decreases shortly after emergence. This pheromonal deactivation occurs only in living males, suggesting an active process rather than simple evaporation of bioactive compounds. Here, we present evidence that the sex pheromone of *L. distinguendus* is composed of a series of cuticular hydrocarbons. Filter paper disks treated with nonpolar fractions of cuticular extracts of freshly emerged males and females, 72-hr-old females, and yellowish pupae caused arrestment and stimulated key elements of courtship behavior in males, whereas fractions of 72-hr-old males did not. Sixty-four hydrocarbons with chain length between C_{25} and C_{37} were identified in the fractions by gas chromatography-mass spectrometry (GC-MS). Methyl-branched alkanes with one to four methyl groups were major components, along with traces of *n*-alkanes and monoalkenes. Principal component analysis, based on the relative amounts of the compounds, revealed that cuticular hydrocarbon composition differed among all five groups. By using partial least squares-discriminant analysis, we determined a series of components that differentiate bioactive and bioinactive hydrocarbon profiles, and may be responsible for pheromonal activity of hydrocarbon fractions in *L. distinguendus*.

Keywords Parasitoid · *Lariophagus distinguendus* · Pteromalidae · Sex pheromone · Cuticular hydrocarbons · Principal component analysis · PCA · Partial least squares-discriminant analysis · PLS-DA

S. Steiner (✉) · J. Ruther
Institut für Biologie, Freie Universität Berlin, Haderslebener Str. 9, 12163 Berlin, Germany
e-mail: svenst@zedat.fu-berlin.de

R. Mumm
Laboratory of Entomology, Wageningen University, Binnenhaven 7, 6709 PD Wageningen, The Netherlands

Introduction

Hydrocarbons are found on the cuticle of almost all insects. In addition to their primary function as a water loss barrier (Nelson and Blomquist, 1995; Gibbs, 1998), cuticular hydrocarbons are known to be involved in semiochemical 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 toward the 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 dealing with the composition of cuticular hydrocarbons with respect to their potential role as semiochemicals in parasitic wasps. A number of investigations have focused on comparisons of qualitative and quantitative differences in hydrocarbon profiles of parasitoids with respect to species, sex, or the host used for development (Howard, 1992, 2001; Howard and Infante, 1996). However, few studies have included bioassays demonstrating behavioral activity of synthetic hydrocarbons or hydrocarbon fractions. In *Cardiochiles nigriceps* (Braconidae), elements of male courtship behavior are elicited by a series of female-specific alkadienes (Syvertsen et al., 1995). Recent studies on the pteromalids *Roptrocerus xylophagorum* (Sullivan, 2002), *L. distinguendus* (Steiner et al., 2005), and *Nasonia vitripennis* (Steiner et al., 2006) have demonstrated that nonpolar hydrocarbon fractions from females arrest males and elicit courtship behavior. Interestingly, in *L. distinguendus*, pupae of both sexes also elicited male courtship behavior. However, 32 hr after emergence, males became behaviorally inactive to male conspecifics. To date, it is not known which components contribute to the biological activity of extracts in Pteromalidae.

Cuticular hydrocarbon profiles of insects are generally complex, often consisting of more than a hundred components, mainly saturated and unsaturated (one to three double bonds) straight chain and methyl-branched (one to four branches) alkanes (Lockey, 1988). Studies on social insects have shown that subtle quantitative differences in cuticular hydrocarbon profiles can be detected by analyzing chemical data with multivariate statistical methods. By this approach, cuticular hydrocarbon profiles of social insects have been shown to be 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 investigated the role of hydrocarbons as a short-range sex pheromone in *L. distinguendus* by combining chemical analyses and behavioral bioassays. First, we showed that hydrocarbon fractions from females, as well as from freshly emerged males and pupae of either sex, can mediate male courtship behavior. Second, we compared the composition of behaviorally active hexane fractions with inactive ones from older males by principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) (Wold et al., 1989). The latter method was used to characterize chemical components that contribute strongly to the differentiation of active and inactive hydrocarbon profiles. The results are discussed with respect to putative function of these chemicals as constituents of the courtship pheromone of *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 25°C, 60% RH, and a photoperiod of 16:8

(L/D). To obtain naive parasitoids, single grains containing parasitoids about to emerge were transferred to 1.5-ml microcentrifuge tubes (Sarstedt, Nümbrecht, 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 Procedures for Bioassay The experiment was conducted in a bioassay chamber (10 mm diameter×3 mm height) as described elsewhere (Ruther et al., 2000). Behavioral parameters were observed under a stereo microscope, with illumination by 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 d after emergence. One hour before being used in the bioassay, parasitoids were placed individually in micro-centrifuge tubes and kept at ambient temperature. The following behavioral parameters were recorded: (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, and (3) wing fanning behavior, characteristic high-frequency wing fanning shown by males in the presence of the sex pheromone (Ruther et al., 2000).

Preparation of Hydrocarbon Fractions Batches of 10 individuals each of: (1) freshly emerged females, (2) freshly emerged males, (3) 72-hr-old females, (4) 72-hr-old males, and (5) yellowish pupae from *L. distinguendus* were extracted for 4 d 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 microliter) and stored at −80°C until used for bioassay and chemical analysis.

Bioassay: Activity of Hydrocarbon Fractions The behavioral responses of *L. distinguendus* males to filter paper disks (5 mm diameter) treated with four individual equivalents of the five different hexane fraction types (see above) was investigated. After leaving the solvent to evaporate for 15 min, single paper disks were offered to a male in the bioassay chamber, and arrestment time, antennation time, and wing-fanning behavior to the paper disks were recorded over 5 min. For the control, filter paper disks were treated with solvent. Each wasp was tested only once. Males that did not respond to the filter paper disk by wing-fanning behavior were released into another bioassay chamber containing an unmated *L. distinguendus* female. Males that did not show wing fanning in this control test were discarded from the data set. After five parasitoids had been tested, the paper disk was renewed. The bioassay chamber was cleaned regularly with ethanol and deionized water to remove contamination by the walking males. Ten parasitoids were tested to each treatment.

Chemical Analysis Hydrocarbon fractions (N=10 for adults, N=5 for pupae) were analyzed by gas chromatography-mass spectrometry (GC-MS) on a Fisons GC 8060 equipped with a 30 m×0.32 mm DB-5ms fused silica column (film thickness 0.25 μm) 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 program was started at 150°C, raised by 2°C/min to a final temperature of 280°C, and then held for 30 min.

One microliter of each hexane fraction, representing 0.5 of an 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 coinjection of straight-chain hydrocarbons. Methyl-branched hydrocarbons were identified

by diagnostic ions resulting from the favored fragmentation at the branching points (Lockey, 1988; Nelson, 1993) and by comparing LRI values with literature data (Carlson et al., 1998). Double bond position was determined by iodine-catalyzed methylthiolation using dimethyl disulfide (Francis and Velant, 1981; Howard, 1993). Peak areas for each compound were calculated and related to total peak area for each run.

Statistical Analysis Statistical analysis for the behavioral experiment was performed with the software package Statistica release 4.5 (StatSoft, Tulsa, OK, USA). Arrestment time and antennation time of males on filter paper disks treated with hexane fractions or solvent were analyzed by Mann–Whitney *U* test. Numbers of males responding to the paper disks with wing-fanning behavior were analyzed by a 2×2 Chi^2 test.

The quantitative composition of the five different hydrocarbon fraction types was evaluated by PCA and PLS-DA by using the software program 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) 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 normalized, i.e., peak areas of all analyzed compounds (*X* variables) were summed and the relative amount of each variable was calculated. The normalized data were transformed to log (X+0.00001). The constant 0.00001 was added to provide nondetectable components with a small nonzero value (Sjödin et al., 1989). Transformed variables were then mean-centered, 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 modeled 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 pheromone activity, as well 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 behaviorally active group or nonactive parasitoid group, respectively. In addition, we calculated the variable importance in the projection (VIP), which is a 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 Hydrocarbon Fractions Hydrocarbon fractions from freshly emerged males and females, and those from 72-hr-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 male courtship behavior, such as antennal stroking and copulation attempts, were observed during the test period (data not

Table 1 Relative composition of hexane fractions from different life stages of *L. distinguendus*

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
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	unknown	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

Table 1 (continued)

No.	Compound	LRI[a]	Diagnostic Ions	F0d[c]	M0d[c]	F3d[c]	M3d[c]	YP[c]
	+ 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
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					

F0d=Fresh females, M0d=Fresh males, F3d=72-hr females, M3d=72-hr males, YP=Yellowish pupae

[a] LRI=Linear retention index

[b] DMDS=diagnostic ions after derivatization with dimethyl disulfide

[c] Means ± SE

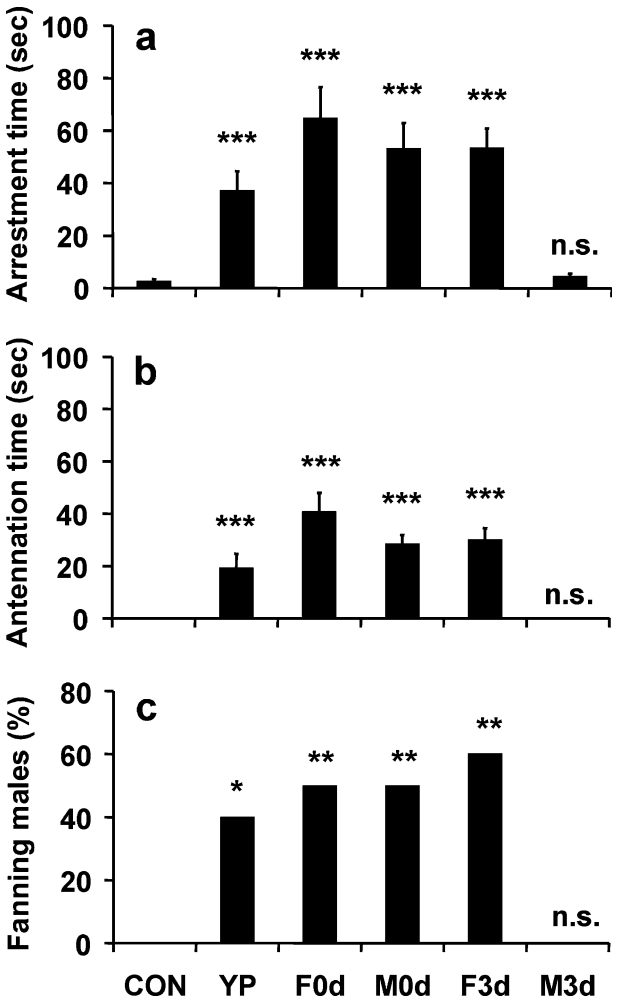

Fig. 1 Response of *L. distinguendus* males to filter paper disks treated with hexane fractions of cuticle extracts from freshly emerged females (F0d) and males (M0d), 72-hr-old females (F3d) and males (M3d), yellowish pupae (YP), and solvent control (CON). (a) Mean arrestment time (±SE), (b) mean antennation time (±SE), and (c) percentages of males showing wing-fanning behavior. 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 behavior was analyzed by 2×2 chi-square tests ($N=10$)

shown). In contrast, males did not respond to hydrocarbon fractions from 72-hr-old males or to control paper disks.

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. Additionally, squalene was found as a trace component in all life stages of both sexes. 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 bioassays showed quantitative but no qualitative differences. In behaviorally inactive fractions of 72-hr-old males, however, a series of hydrocarbons present in the active fractions was missing (Fig. 2).

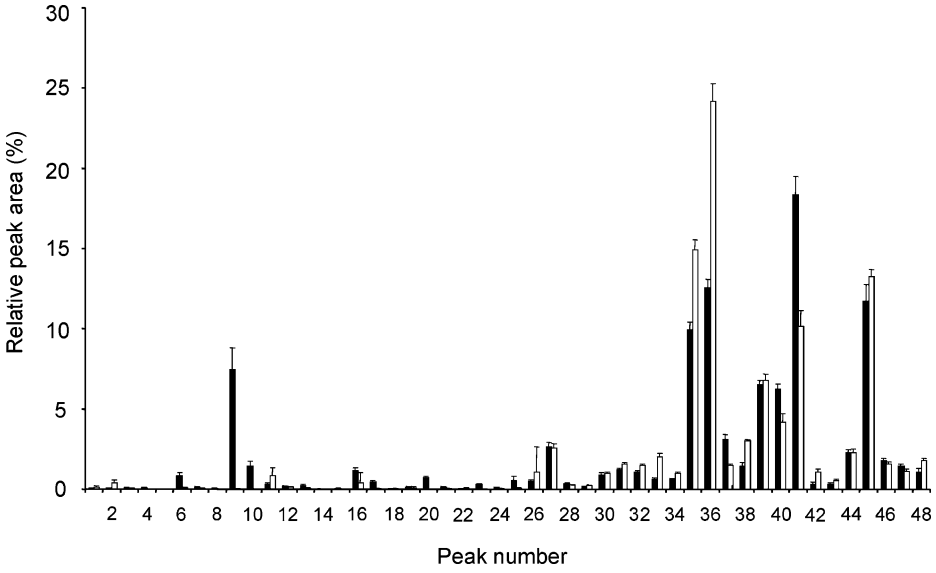

Fig. 2 Relative peak areas of cuticular hydrocarbons from freshly emerged *L. distinguendus* females (black bars; highest bioactivity) and 72-hr-old males (white bars, no bioactivity). Peak numbers correspond to those in Table 1

A PCA, based on relative peak areas found in the five different types of hydrocarbon fractions, was conducted. In this model, four principal components were extracted, explaining a total variation (R^2X) of 79%. A score plot of the first two principle components (PCs) showed that the cuticular hydrocarbon composition was different among all groups (Fig. 3).

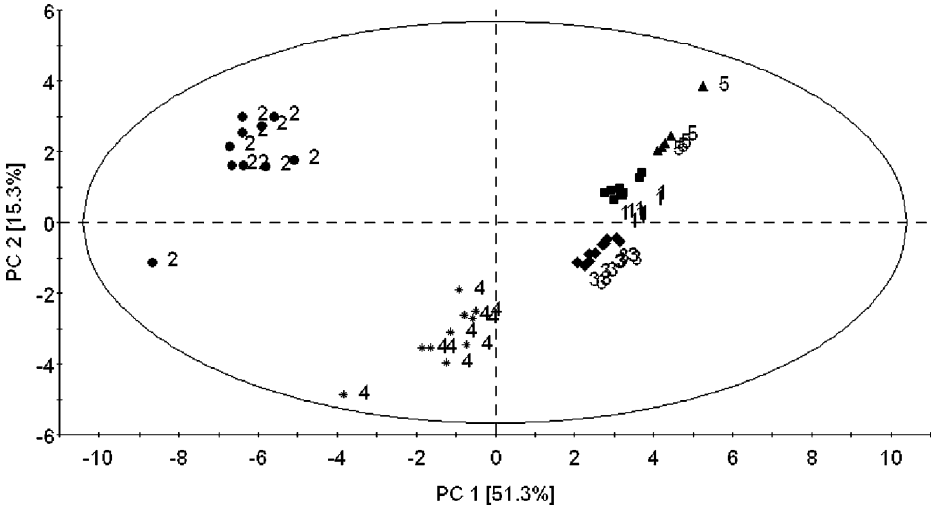

Fig. 3 Analysis of the cuticular hydrocarbon profiles from different *L. distinguendus* life stages. Score plot from principal component analysis (PCA) based on relative amounts of all analyzed hydrocarbons shown in Table 1; 66.5% of the variance in the data is explained by the two first significant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

The samples of 72-hr-old male parasitoids were dissimilar to all other samples, as indicated by the projection on the left hand side of the plot. The first PC mainly divided samples of different age, whereas the second PC tended to separate gender of adult parasitoids.

To elucidate which chemical compounds may be responsible for pheromonal activity a PLS-DA was performed. Two parasitoid classes were constructed based on pheromonal activity in the bioassays. Hydrocarbon fractions of freshly emerged males, freshly emerged females, 72-hr-old females, and pupae were combined in one class, and 72-hr-old males represented the second. 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 showed that the hydrocarbon fractions of 72-hr-old male parasitoids, which are not behaviorally active, are separated from the active groups (Fig. 4a). The corresponding loading plot depicts which chemical variables contribute strongly to separation of classes. Variables projected close to the dummy variables Y (shown as active and not active) contribute strongly to class separation, and thus have a high discriminatory power (Fig. 4b). A more quantitative way to estimate variable influence is described by the VIP-parameter. Chemical variables most important for resolving behaviorally active parasitoid groups have VIP-parameter values above 1 (Eriksson et al., 2001). Compounds showing VIP-parameter > 1 are, in descending order, peak numbers 10, 20, 23, 4, 14, 9, 17, 15 (data not shown). These compounds are marked with an asterisk in Fig. 4b.

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 can indicate presence of subclusters. In our model, the higher number of expected components was presumably caused by the samples from the 72-hr-old females. The hydrocarbon composition of these samples was slightly different from the composition of the freshly emerged parasitoids and pupae. This might be ascribed to the age of the parasitoids, which apparently influenced the composition of hydrocarbons (Figs. 3 and 4a).

Discussion

This study reports the characterization of individual cuticular hydrocarbons with respect to their potential role as a short-range sex pheromone in *L. distinguendus*. Behavioral experiments that used hydrocarbon fractions from different life stages demonstrated pheromonal activity from females and from freshly emerged males and immature stages of both sexes. In contrast, there was no pheromonal activity from extracts of 72-hr-old males. Chemical analysis by GC-MS revealed qualitative and quantitative differences in the hydrocarbon profiles of behaviorally 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, 3,7-DiMeC29, and 13-MeC29) had the strongest impact in the discriminant analysis. However, some alkenes (C27:1(9), C29:1 (7)) also had a high discriminating power. Apart from 3-MeC27, all chemicals that have been found to possess high discriminating power are minor components of the bioactive hydrocarbon profiles. Some of these compounds were totally absent in the profiles of (inactive) extracts from 72-hr-old males (Table 1, Fig. 2) and thus are good candidates for

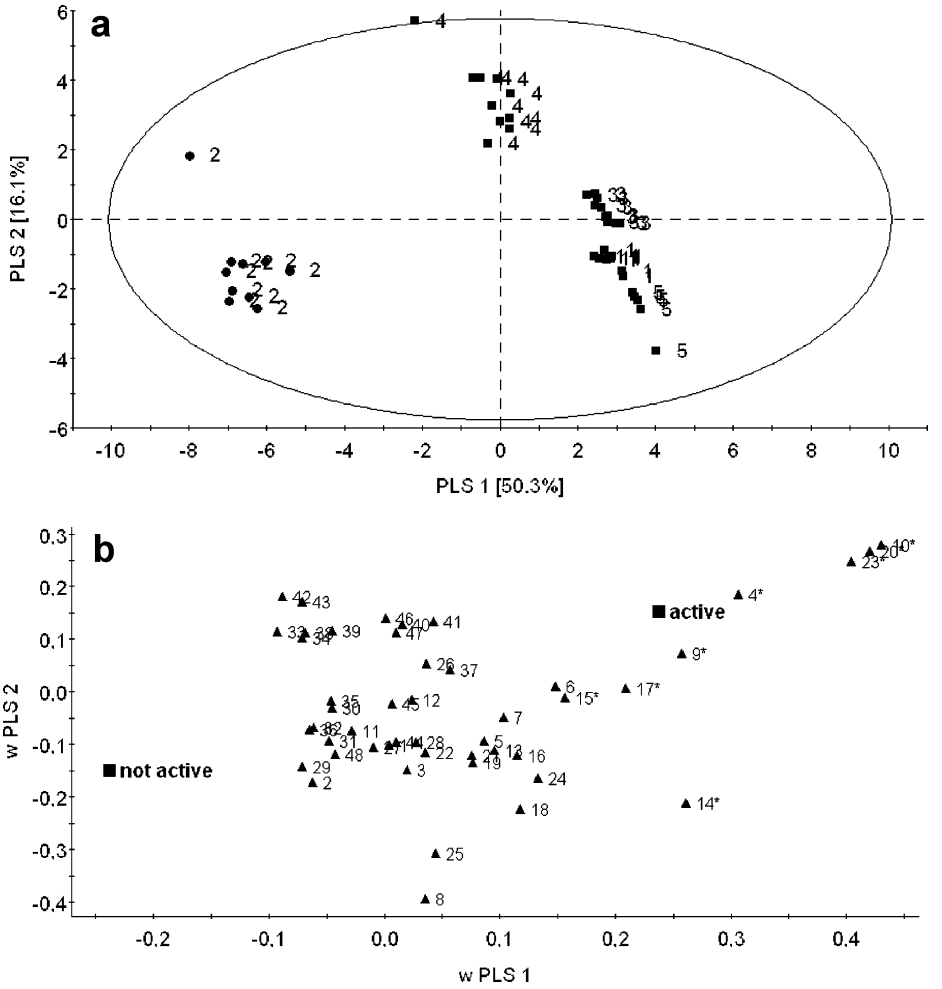

Fig. 4 Analysis of the cuticular hydrocarbon profiles from different *L. distinguendus* life stages. (a) Score plot and (b) loading plot from partial least squares-discriminant analysis (PLS-DA) based on relative amounts of all analyzed hydrocarbons shown in Table 1. A total of 66.4% of the variance 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-hr-old males; 3=freshly emerged females; 4=72-hr-old females; 5=yellowish pupae; box=no pheromone activity; dot=pheromone activity. Data were subjected to log-transformation, mean centering, and Pareto scaling

mediating pheromone activity in *L. distinguendus*. In recent years, the number of studies on the role of cuticular hydrocarbons as insect semiochemicals has grown immensely (reviewed by Howard and Blomquist, 2005). Evidence is increasing that cuticular hydrocarbons play a general role as courtship pheromones in parasitoids, by mediating arrestment and other, more complex behavioral elements. However, few studies have identified which cuticular hydrocarbons are bioactive. 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, consisting of aliphatic and methyl-branched alkanes with up to two methyl groups, in female extracts of *Roptrocerus xylophagorum* that correlated 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 sex pheromone during pupal development (Steiner et al., 2005). The authors demonstrated that searching *L. distinguendus* males were arrested on parasitized grains containing females about to emerge. A similar phenomenon has been reported in *Anisopteromalus calandrae* (Pteromalidae) (Yoshida, 1978) and *Apanteles glomeratus* (Braconidae) (Tagawa, 1977). This pre-emergence pheromone release may increase the chances of females being inseminated before leaving the emergence site to search for hosts.

Interestingly, bioactive hydrocarbons are also present in developing and newly emerged males of *L. distinguendus*. After emergence, males become less bioactive within 32 hr, suggesting the production and/or degradation of the bioactive hydrocarbons is regulated differently from that in female parasitoids, which remain bioactive to courting males. The mechanism of this deactivation is not yet understood. However, we demonstrated that only living males lose bioactivity; dead males stored under the same conditions had bioactivity for several days. This suggests that the loss of pheromonal activity in males is caused by an active process rather than simple evaporation of the bioactive compounds. This may be achieved by selective metabolism or translocation of individual hydrocarbons from the cuticle to inner regions of the insect. In termites, for example, radiolabeling experiments have demonstrated that topically applied hydrocarbons are transported to the hemolymph by the lipid carrier protein lipophorin (Sevala et al., 2000).

Developing *L. distinguendus* males may benefit from possessing the pheromone. Steiner et al. (2005) showed that searching males are unable to distinguish between grains containing female or male conspecifics that are about to emerge. They suggested that developing males inside the grains might fool their already emerged competitors by distracting them away from searching for actual females. Another parasitoid species in which young males elicit courtship behavior in male conspecifics is the ichneumonid *Itoplectis conquisitor*. Robacker et al. (1976) demonstrated that extracts of both freshly emerged males and females elicit sexual behavior in older males. However, bioactive constituents of this multicomponent pheromone are terpene aldehydes rather than hydrocarbons (Robacker and Hendry, 1977).

By applying discriminant analysis to the hydrocarbon composition of parasitic Hymenoptera, we identified a set of compounds that allow discrimation of bioactive from bio-inactive life stages and which may be responsible for pheromonal activity in *L. distinguendus*. As our findings are based on mathematical modeling, future studies will focus on behavioral experiments using synthetic reference compounds to ascertain whether these hydrocarbons function as courtship pheromone in *L. distinguendus*.

Acknowledgments This research was funded by the Deutsche Forschungsgemeinschaft (Ru 717/5-1). R.M. was funded by the European Commission contract MC-RTN-CT-2003-504720 “ISONET”.

References

BAGNÈRES, A. G., CLEMENT, J.-L., BLUM, M. S., SEVERSON, R. F., JOULIE, C., and LANGE, C. 1990. Cuticular hydrocarbons and defensive compounds of *Reticulitermes flavipes* (Kollar) and *R. santonensis* (Feytaud): Polymorphism and chemotaxonomy. *J. Chem. Ecol*. 16:3213–3244.

BLOMQUIST, G. J., TILLMAN-WALL, J. A., GUO, L., QUILLICI, D. R., GU, P., and SCHAL, C. 1993. Hydrocarbons and hydrocarbon-derived sex pheromones in insects: biochemistry and endocrine regulation, pp. 317–351, in D. W. Stanley-Samuelson and D. R. Nelson (eds.). Insect Lipids. Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln.

BLOMQUIST, G. J., TILLMAN, J. A., MPURU, S., and SEYBOLD, S. J. 1998. The cuticle and cuticular hydrocarbons: structure, function, and biochemistry, pp. 34–54, in R. K. Vander Meer, M. D. Breed, M. L. Winston, and K. E. Espelie (eds.). Pheromone Communication in Social Insects. Westview Press, Boulder.

BUTTS, D. P., CAMANN, M. A., and ESPELIE, K. E. 1995. Workers and queens of the European hornet *Vespa crabro* L. have colony-specific cuticular hydrocarbon profiles (Hymenoptera: Vespidae). *Ins. Soc.* 42:45–55.

CARLSON, D. A., BERNIER, U. R., and SUTTON, B. D. 1998. Elution parameters from capillary GC for methyl-branched alkanes. *J. Chem. Ecol.* 24:1845–1865.

ERIKSSON, L., JOHANSSON, E., KETTANEH-WOLD, N., and WOLD, S. 2001. Multi- and Megavariate Data Analysis: Principles and Applications. Umetrics Academy, Umeå.

ERIKSSON, L., ANTTI, H., GOTFRIES, J., HOLMES, E., JOHANSSON, E., LINDGREN, F., LONG, I., LUNDSTEDT, T., TRYGG, J., and WOLD, S. 2004. Using chemometrics for navigating in the large data sets of genomics, proteomics, and metabonomics. *Anal. Bioanal. Chem.* 380:419–429.

FERVEUR, J. F. 2005. Cuticular hydrocarbons: their evolution and roles in *Drosophila* pheromonal communication. *Behav. Genet.* 35:279–295.

FRANCIS, G. W. and VELANT, K. 1981. Alkylthiolation for the determination of double-bond position in linear alkanes. *J. Chromatogr.* 219:379–384.

GIBBS, A. G. 1998. Water-proofing properties of cuticular lipids. *Am. Zool.* 38:471–482.

HOWARD, R. W. 1992. Comparative analysis of cuticular hydrocarbons from ectoparasitoids *Cephalonomia waterstoni* and *Laelius utilis* (Hymenoptera: Bethylidae) and their respective hosts, *Cryptolestes ferrugineus* (Coleoptera: Cucujidae) and *Trogoderma variable* (Coleoptera: Dermestidae). *Ann. Entomol. Soc. Am.* 80:317–325.

HOWARD, R. W. 1993. Cuticular hydrocarbons and chemical communication, pp. 179–226, in D. W. Stanley-Samuelson and D. R. Nelson (eds.). Insect Lipids. Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln.

HOWARD, R. W. 2001. Cuticular hydrocarbons of adult *Pteromalus cerealellae* (Hymenoptera: Pteromalidae) and two larval hosts, Angoumois grain moth (Lepidoptera: Gelechiidae) and cowpea weevil (Coleoptera: Bruchidae). *Ann. Entomol. Soc. Am.* 94:152–158.

HOWARD, R. W. and BLOMQUIST, G. J. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol.* 50:371–393.

HOWARD, R. W. and INFANTE, F. 1996. Cuticular hydrocarbons of the host-specific ectoparasitoid *Cephalonomia stephanoderis* (Hymenoptera: Bethylidae) and its host the coffee berry borer (Coleoptera: Scolytidae). *Ann. Entomol. Soc. Am.* 89:700–709.

KAIB, M., BRANDL, R., and BAGINE, R. K. N. 1991. Cuticular hydrocarbon profiles: a valuable tool in termite taxonomy. *Naturwissenschaften* 78:176–179.

KLOCHKOV, S. G., KOZLOVSKI, V. I., and BELYAEVA, N. V. 2005. Caste and population specificity of termite cuticule hydrocarbons. *Chem. Nat. Compd.* 41:1–6.

LOCKEY, K. H. 1988. Lipids of the insect cuticle: origin, composition and function. *Comp. Biochem. Physiol. B* 89:595–645.

LORENZI, M. C., BAGNÈRES, A. G., CLÈMENT, J. L., and TURILLAZZI, S. 1997. *Polistes biglumis bimaculatus* epicuticular hydrocarbons and nestmate recognition (Hymenoptera, Vespidae). *Insectes Soc.* 44: 123–138.

MUMM, R., TIEMANN, T., SCHULZ, S., and HILKER, M. 2004. Analysis of volatiles from black pine (*Pinus nigra*): significance of wounding and egg deposition by a herbivorous sawfly. *Phytochemistry* 65:3221–3230.

NELSON, D. R. 1993. Methyl-branched lipids in insects, pp. 271–315, in D. W. Stanley-Samuelson and D. R. Nelson (eds.). Insect Lipids. Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln.

NELSON, D. R. and BLOMQUIST, G. J. 1995. Insect waxes, pp. 1–90, in R. J. Hamilton (ed.). Waxes: Biochemistry, Molecular Biology, and Function. W. W. Christie, The Oily Press, England.

PAGE, M., NELSON, L. J., FORSCHLER, B. T., and HAVERTY, M. I. 2002. Cuticular hydrocarbons suggest three lineages in *Reticulitermes* (Isoptera: Rhinotermitidae) from North America. *Comp. Biochem. Physiol. B* 131:305–324.

PAOLUCCI, U., VIGNEAU-CALLAHAN, K. E., SHI, H., MATSON, W. R., and KRISTAL, B. S. 2004. Development of biomarkers based on diet-dependent metabolic serotypes: characteristics of component-based models of metabolic serotypes. *OMICS J. Integr. Biol.* 8:221–238.

ROBACKER, D. C. and HENDRY, L. B. 1977. Neral and geranial: components of the sex pheromone of the parasitic wasp, *Itoplectis conquisitor*. *J. Chem. Ecol.* 3:563–577.

ROBACKER, D. C., WEAVER, K. M., and HENDRY, L. B. 1976. Sexual communication and associative learning in the parasitic wasp *Itoplectis conquisitor* (Say). *J. Chem. Ecol.* 2:39–48.

RUTHER, J., HOMANN, M., and STEIDLE, J. L. M. 2000. Female-derived sex pheromone mediates courtship behavior in the parasitoid *Lariophagus distinguendus*. *Entomol. Exp. Appl.* 96:265–274.

SEVALA, V. L., BAGNÈRES, A. G., KUENZLI, M., BLOMQUIST, G., and SCHAL, C. 2000. Cuticular Hydrocarbons of the dampwood termite, *Zootermopsis nevadensis*: caste differences and role of lipophorin in transport of hydrocarbons and hydrocarbon metabolites. *J. Chem. Ecol.* 26:265–789.

SHU, S. and JONES, R. L. 1993. Evidence for a multicomponent sex pheromone in *Eriborus terebrans* (Gravenhorst) (Hym.: Ichneumonidae), a larval parasitoid of the European corn borer. *J. Chem. Ecol.* 19:2563–2576.

SJÖDIN, K., SCHROEDER, L. M., EIDMANN, H. H., NORIN, T., and WOLD, S. 1989. Attack rates of scolytids and composition of volatile wood constituents in healthy and mechanically weakened pine trees. *Scand. J. For. Res.* 4:379–391.

STEIDLE, J. L. M. and SCHÖLLER, M. 1997. Olfactory host location and learning in the granary weevil parasitoid *Lariophagus distinguendus* (Hymenoptera: Pteromalidae). *J. Insect Behav.* 10:331–342.

STEINER, S., STEIDLE, J. L. M., and RUTHER, J. 2005. Female sex pheromone in immature insect males—a case of pre-emergence chemical mimicry? *Behav. Ecol. Sociobiol.* 58:111–120.

STEINER, S., HERMANN, N., and RUTHER, J. 2006. Characterization of a female-produced courtship pheromone in the parasitoid *Nasonia vitripennis*. *J. Chem. Ecol*. 32:1687–1702.

STEINMETZ, I., SCHMOLZ, E., and RUTHER, J. 2003. Cuticular lipids as trail pheromone in a social wasp. *Proc. R. Soc. Lond. B* 270:385–391.

SULLIVAN, B. T. 2002. Evidence for a sex pheromone in bark beetle parasitoid *Roptrocerus xylophagorum*. *J. Chem. Ecol.* 28:1045–1063.

SYVERTSEN, T. C., JACKSON, L. L., BLOMQUIST, G. J., and VINSON, S. B. 1995. Alkadienes mediating courtship in the parasitoid *Cardiochiles nigriceps* (Hymenoptera: Braconidae). *J. Chem. Ecol.* 21: 1971–1989.

TAGAWA, J. 1977. Localization and histology of the female sex pheromone-producing gland in the parasitic wasp, *Apanteles glomeratus*. *J. Insect Physiol.* 23:49–56.

WOLD, S., ALBANO, C., DUNN III, W. J., ESBENSEN, K., GELADI, P., HELLBERG, S., JOHANSSON, E., LINDBERG, W., SJÖSTRÖM, M., SKAGERBERG, B., WIKSTRÖM, C., and ÖHMAN, J. 1989. Multivariate data analysis: converting chemical data tables to plots. *Intell. Instrum. Comput.* 197–216.

WOLD, S., JOHANSSON, E., and COCCHI, M. 1993. PLS - partial least squares projections to latent structures, pp. 523-550, in H. Kubinyi (ed.). 3D QSAR in Drug Design, Theory, Methods, and Applications. ESCOM Science Publishers, Leiden.

WOLD, S., SJÖSTRÖM, M., and ERIKSSON, L. 2001. PLS-regression: a basic tool of chemometrics. *Chemom. Intell. Lab. Syst.* 58:109–130.

YOSHIDA, S. 1978. Behavior of males in relation to the female sex pheromone in the parasitoid wasp, *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae). *Entomol. Exp. Appl.* 23:152–162.