

Cuticular hydrocarbons discriminate cryptic *Macrolophus* species (Hemiptera: Miridae)

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Abstract

Macrolophus pygmaeus is commercially employed in the biological control of greenhouse and field vegetable pests. It is morphologically undistinguishable from the cryptic species *M. melanotoma*, and this interferes with the evaluation of the biological control activity of *M. pygmaeus*. We analysed the potential of cuticular hydrocarbon composition as a method to discriminate the two *Macrolophus* species. A third species, *M. costalis*, which is different from the other two species by having a dark spot at the tip of the scutellum, served as a control. Sex, diet and species, all had significant effects in the cuticular hydrocarbon profiles, but the variability associated to sex or diet was smaller than among species. Discriminant quadratic analysis of cuticular hydrocarbons confirmed the results of previous molecular genetic studies and showed, using cross-validation methods, that *M. pygmaeus* can be discriminated from *M. costalis* and *M. melanotoma* with prediction errors of 6.75% and 0%, respectively. Therefore, cuticular hydrocarbons can be used to separate *M. pygmaeus* from *M. melanotoma* reliably.

Keywords: cuticular hydrocarbons, biological control, taxonomy

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Introduction

Several species in the genus *Macrolophus* (Hemiptera: Miridae) are efficient predators of vegetable crops pests (e.g. whiteflies, aphids and thrips) (Riudavets & Castañé, 1998; Hansen *et al.*, 1999; Montserrat *et al.*, 2000; Lucas & Alomar, 2002; Athanassiou *et al.*, 2003; Margaritopoulos *et al.*, 2003; Alomar *et al.*, 2006). Control strategies that incorporate *Macrolophus* species are based on both seasonal inoculative releases of commercially produced individuals and habitat management to conserve natural populations and enhance colonization (Alomar *et al.*, 2002). In order to avoid the

incorrect use of predator reservoir plants, it is essential to correctly identify the predator species.

Several *Macrolophus* species are morphologically similar or have highly variable taxonomic characters, which hampers correct identification and questions species-identity status (Josifov, 1992; Kerzhner & Josifov, 1999). This is particularly true for the two species that have been most cited as biological control agents in vegetable crops, *M. pygmaeus* (Rambur, 1839) and *M. melanotoma* (Costa, 1853) (the last one mostly cited as its junior synonym *M. caliginosus* Wagner, 1951), and has probably resulted in incorrect attribution of biological control by *M. pygmaeus* to *M. melanotoma* (see Martínez-Casales *et al.*, 2006 for a historical review). DNA methods have been employed recently to distinguish *M. pygmaeus* from *M. melanotoma* (Perdikis *et al.*, 2003; Martínez-Casales *et al.*, 2006). Cuticular hydrocarbon analysis could provide an additional method to separate morphologically similar species

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(Dall'Aglio-Holvorcem *et al.*, 2009; Bagnères & Wicker-Thomas, 2010).

The insect cuticle is coated with a thin lipid layer containing a high percentage of hydrocarbons (linear, branched, saturated and unsaturated), one of which main functions is to prevent desiccation and pathogen entrance into the body (Howard & Blomquist, 2005). Cuticular hydrocarbons are usually present in high amounts and are easy to extract by quickly rinsing the specimen in non-polar organic solvents (Blomquist *et al.*, 1987). In addition, most cuticular hydrocarbons are chemically stable and not very volatile (Martin *et al.*, 2009). The hydrocarbon blend is often species specific, and therefore it is a useful character in insect taxonomy (reviewed by Bagnères & Wicker-Thomas, 2010).

In contrast to other insect groups, there are relatively few studies of cuticular hydrocarbon taxonomy in Hemiptera. A recent publication reviews the hydrocarbons of blood-sucking bugs (Juárez & Fernández, 2007), and isolated studies compare cuticular hydrocarbons of *Orius* species (Anthicoridae) (Nakabou & Ohno, 2001) and aphids (Clements *et al.*, 2000). With regard to mirids, analysis of cuticular hydrocarbons has been reported only for *Lygocoris pabulinus* (L.) (Drijfhout & Groot, 2001).

The purpose of the present study is to determine if cuticular hydrocarbons can be used to distinguish adults of two *Macrolophus* species (*M. melanotoma* and *M. pygmaeus*) that cannot be distinguished easily by external morphological characters alone. Since diet and sex may affect insect cuticular hydrocarbon composition (Liang & Silverman, 2000; Thomas & Simmons, 2008; Guerrieri *et al.*, 2009), we controlled for the effect of diet (i.e. host plant and associated prey) and sex on cuticular hydrocarbons. *M. costalis* Fieber was included in the study as a reference species and to compare our results with a previous study on the taxonomic relationship of the three *Macrolophus* species based on DNA methods (Martínez-Cascales *et al.*, 2006).

Materials and methods

Insects

Chemical analyses were performed on adults of both sexes. Wild individuals were collected in the proximities of Cabriels (Barcelona, Spain) in the spring of 2008. *Macrolophus melanotoma* was collected from *Dittrichia viscosa* (L.) Greuter (Compositae), where it is abundant. *Macrolophus costalis*, which is readily distinguished by the dark spot on the scutellum, was collected on *Cistus albidus* L. (Cistaceae), where it is commonly found. *M. pygmaeus* was obtained from a >5-year-old laboratory colony originated from tomato fields, and maintained on tobacco plants and frozen *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) egg prey. This colony is refreshed annually with individuals collected in local tomato fields. Examination of the length and shape of the respiratory horns of eggs (Perdikis *et al.*, 2003) was used to confirm the identity of collected females of *M. melanotoma* and *M. pygmaeus*. Some of those individuals were frozen (-20°C) immediately or within seven days of being collected (in which case they were maintained in their respective diet) and are referred to as the 'field' individuals. Additionally, in order to evaluate the effect of diet on the cuticular hydrocarbon profile, 4th-instar *M. pygmaeus* nymphs taken from the colony, and the progeny of field-collected *M. melanotoma* and *M. costalis*, were reared individually on tobacco leaves and *E. kuehniella* eggs.

The resulting adults, referred to as the 'laboratory' individuals, were frozen when 3–6 days old and analyzed at the same time as the 'field' individuals described above.

Cages used for oviposition and nymphal development (7-cm diameter \times 3.5-cm high with a ventilated lid) had a layer of 0.5% agar and a tobacco leaf disc placed on top of it, with the abaxial surface facing upwards, and were kept at $25 \pm 2^{\circ}\text{C}$ and $85 \pm 5\%$ RH, under a 16:8 light:dark photo regime. Individuals were moved to new cages as needed and were provided with frozen *E. kuehniella* egg prey twice per week.

Chemical analyses

Individuals were taken out of the freezer one at a time, let to defrost and submerged in 20 μl of Hexane (HPLC grade, Sigma-Aldrich, Madrid, Spain) containing 50 ng of pentadecane (98% pure, Sigma-Aldrich, Madrid, Spain) in a conical-bottom hexane-rinsed glass vial. The use of internal standard allows minimization of any differences in injection volume and in the daily response of the equipment. Previous analyses indicated that pentadecane is not present in significant amounts in cuticular extracts of these species. Five minutes after immersion in the solvent the insect was removed and the solution was either analysed immediately or returned to the freezer (-20°C) for later analysis.

For chemical analyses the volume of the extract was reduced immediately before injection to 2–4 μl with a gentle nitrogen stream, and 1 μl was injected manually in a gas chromatograph (GC) in the splitless mode (split valve opened after 1 min). Samples were analysed in either a GC-FID or a GC-MSD (Agilent Technologies (Madrid, Spain) 6890NGC and 5973 Network quadrupole MSD), each equipped with a DB-5 column (30 m \times 0.32 mm ID \times 0.25 μm , Agilent Technologies) and run with the same temperature program: start at 60°C for 1 min, then increase to 320°C at $10^{\circ}\text{C min}^{-1}$, and maintain at this temperature for 25 min. Carrier gas was Helium at constant flow (1 ml min^{-1}), the injector was set a 250°C and the detector at 280°C .

A total of 119 insects were analysed by GC-FID, and these are the samples used in the statistical comparisons (ten individuals of each species, sex and diet (laboratory versus field), except for nine field *M. melanotoma* males). Two additional insects of each sex and species were analysed by GC-MSD, and these data were used in the chemical identification of the compounds. Straight-chain alkanes were identified by comparison of retention times and mass spectra of authentic standards (Fluka, alkane standard solutions 04070 ($\text{C}_8\text{--}\text{C}_{20}$) and 04071 ($\text{C}_{21}\text{--}\text{C}_{40}$), Sigma-Aldrich, Madrid, Spain). Retention indices (RIs) were calculated according to van den Dool & Kratz (1963). Linear and methyl branched alkanes were tentatively identified by comparison of their RI and the characteristic ion fragments with those reported in the literature (Blomquist *et al.*, 1987; Juárez & Blomquist, 1993; Carlson *et al.*, 1998; Juárez *et al.*, 2001; Gomes *et al.*, 2008; Mullen *et al.*, 2008; Dall'Aglio-Holvorcem *et al.*, 2009).

Chain length of the selected compounds ranged between C_{24} and more than C_{40} . Since our largest alkane standard was C_{40} , we fitted a curve to the straight chain alkane standards $\text{C}_{27}\text{--}\text{C}_{40}$ to extrapolate the retention times of C_{41} and C_{42} ($R^2=0.99$, $y=125.161-0.066 \times x+1.105 \times 10^{-5}x^2$, where y =estimated RT and x =chain length), and then estimated the RI of sample peaks 27 and 28 (table 1).

Table 1. Chemical structure of 28 cuticular hydrocarbons of three *Macrolephus* species deduced from the characteristic ions (after GC-MSD) and the retention indices (RI) relative to straight-chain hydrocarbons.

Peak number	RT (min)	RI	Tentative chemical structure	Diagnostic ions
1	22.1	2400	<i>n</i> -tetracosane	338
2	23.34	2557	11, 15-dimethylpentacosane	168, 211, 239
3	26.82	3069	hentriacontene	434 (M+)
4	27.2	3129	11-; 13-; 15-methylhentriacontane	168, 196, 224, 252, 280, 308
5	27.39	3157	11, 19-dimethylhentriacontane	168, 196, 295, 323
6	28.02	3248	triacontene	462 (M+)
7	28.1	3267	2-methyldotriacontane	421 (M-43), 449 (M-15)
8	28.58	3332	11-; 13-; 15-methyltriacontane	168, 196, 224, 252, 280, 308, 336
9	28.77	3355	mixture of di and trimethyltriacontanes	168, 196, 181, 211, 239, 252, 267, 323, 356
10	29.71	3465	2-methyltetracontane	449 (M-43), 477 (M-15)
11	29.98	3492	2, 24-dimethyltetracontane	168, 336, 365, 463 (M-43), 491 (M-15)
12	30.58	3552	11, 15-dimethylpentatriacontane	168, 211, 239, 280, 308, 379
13	30.81	3570	11, 15, 19-trimethylpentatriacontane	168, 239, 252, 309, 323, 393
14	31.43	3652	12, 16-; 12, 18-dimethylhexatriacontane	182, 253, 280, 308, 351, 379
15	31.68	3669	3-methylhexatriacontane	491 (M-29)
16	31.97	3692	4, 10-; 4, 12-dimethylhexatriacontane	168, 197, 225, 336, 364, 393
17	32.17	3711	4, 10, 14-trimethylhexatriacontane	168, 239, 336, 407
18	32.63	3729	11-; 13-; 15-methylheptatriacontane	168, 196, 224, 280, 308, 336, 364
19	33.03	3751	11, 15-; 11, 19-dimethylheptatriacontane	168, 239, 280, 295, 407
20	33.35	3771	11, 15, 19-trimethylheptatriacontane	168, 239, 280, 309, 351
21	33.61	3789	11, 15, 23-trimethylheptatriacontane	168, 224, 239
22	34.15	3837	unknown	
23	34.48	3849	12, 16-dimethyloctatriacontane	182, 407, 253, 336, 309
24	34.84	3868	unknown	182, 225
25	36.31	3953	11, 21-dimethylnonatriacontane	168, 280, 295, 323, 407, 435
26	36.7	3973	11, 15, 19-trimethylnonatriacontane	168, 239, 309, 379, 449
27	37.89	4072	12, 16-dimethyltetracontane	182, 253, 378, 449
28	40.85	4186	11, 15, 19-trimethylhentetracontane	168, 239, 280, 308

Statistical analyses

Percent relative abundance ((area of the target peak / area of the internal standard peak) × 100) was the parameter used in all the group comparisons. We chose those compounds that showed a high relative abundance combined with a moderate coefficient of variation, relative to other compounds, and that occurred consistently in at least one class (i.e. species, sex or diet). This resulted in the selection of 28 peaks, some of which were abundant in one sex or species but below threshold level in others. A zero value was assigned to undetected compounds. A Kolmogorov-Smirnov test was used to assess normality of the data.

We performed a multivariate analysis of variance (MANOVA) in order to study how the 28 cuticular hydrocarbons altogether vary with species, sex and diet. To measure the variability that could be explained by each of these factors, we computed the *Pillai-Bartlett statistic* (Hand & Taylor, 1987), which provides a measure of the ratio of effect variance to error variance. We performed principal component analysis (PCA), using the correlation matrix of untransformed data, to obtain uncorrelated scores, visualize aggregation patterns of the different insect groups, and to determine which compounds had the strongest effect in this pattern. To build a prediction model for the species, the main principal components (those adding at least 78% total variability) were used in a quadratic discriminant analysis (QDA), and the prediction error of discriminant functions was evaluated using a ten-fold cross-validation method. The statistical tests were performed with R software (R Development Core Team, 2008). Analysis codes of the prediction model are available as Supplementary Material.

Results

Representative chromatograms indicating the 28 diagnostic peaks (by order of elution) are shown in [fig. 1](#). Linear alkanes, from C₂₁ to C₃₅, were identified in the cuticular samples of the three species, but in very small quantities, and only one of them (C₂₄ or tetracosane, compound 1, [table 1](#)) was abundant enough to be included among the 28 selected compounds. The rest of the hydrocarbons consisted of unbranched monoenes and alkanes with one or more methyl branches ([table 1](#)). Ion fragmentation suggests that some peaks may contain more than one compound.

Normality assumption was accepted for all samples ($P > 0.0017$, Bonferroni-corrected). Significant differences in cuticular hydrocarbon profiles for diet, sex and species were observed (MANOVA, $P < 0.00001$; [table 2](#), [fig. 2](#)). The differences between sexes or diets were much smaller than those between species. Using the approximated F-statistic, we found that variability among species was almost four times higher than between males and females (26.8/6.9 = 3.87) and almost three times higher than between diets (26.8/9.1 = 2.91).

PC1 and PC2 explained 46.28% and 21.73% of the variability, respectively, and clearly segregated *M. melanotoma* from the other two species, whereas sex and diet differences were only apparent in *M. melanotoma* ([fig. 3](#)). Different compounds contributed in varying degrees to the PCs ([fig. 3](#)). Roughly the first half of the 28 compounds (2–15) were more abundant in *M. melanotoma* than in the other two species, and the second half (16–28) were less abundant in *M. melanotoma* than in the other species ([fig. 2](#)). Compound 7 (2-methyldotriacontane) is clearly characteristic of the *M. melanotoma* cluster ([fig. 3](#)) and is practically absent in the

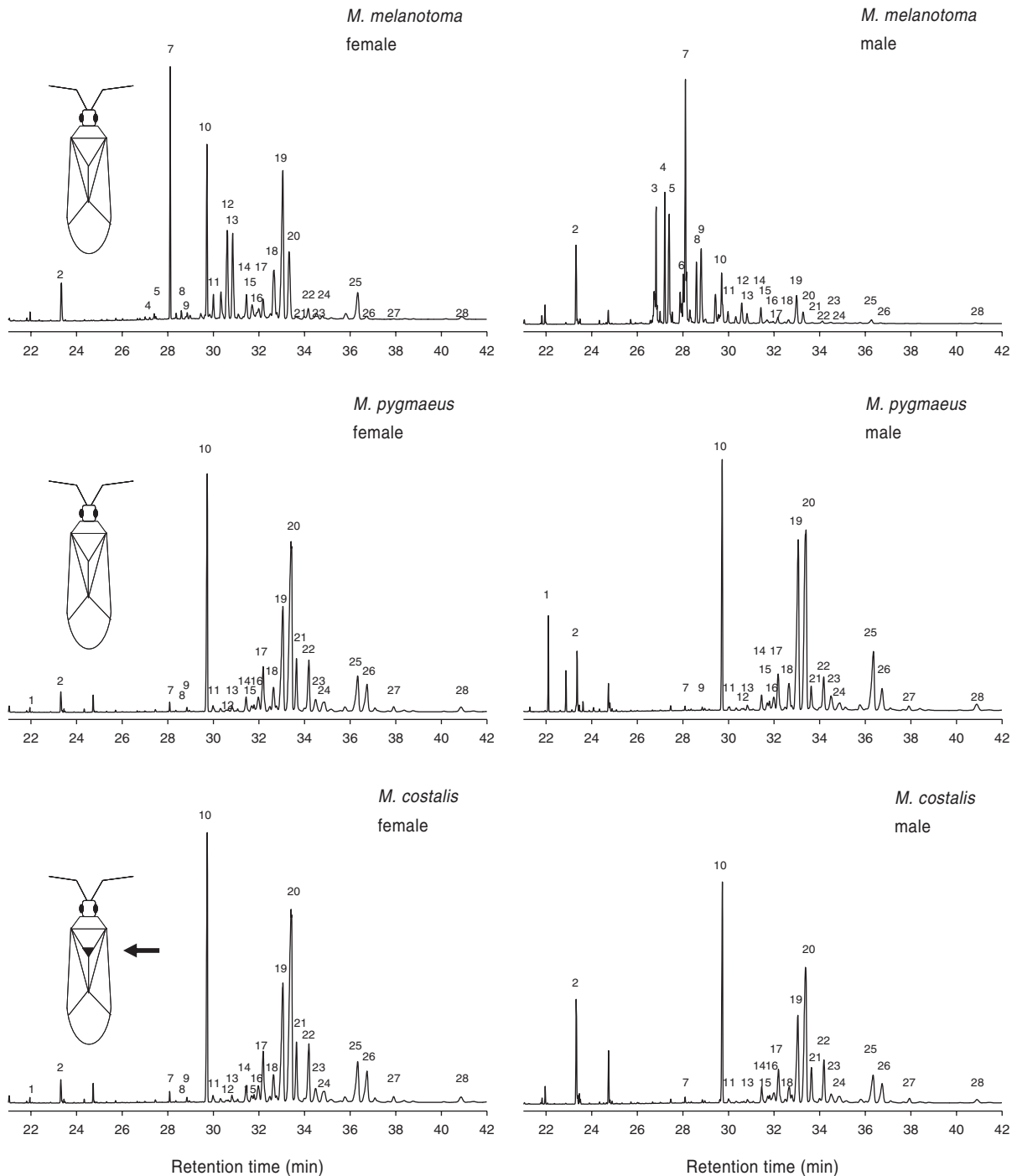


Fig. 1. Representative gas chromatograph traces of *Macrolophus* cuticular hydrocarbons. Each trace corresponds to a different single individual. The most representative peaks were numbered 1 to 28, corresponding with the column elution time. Notice the large difference between *M. melanotoma* and the other two species, as well as between *M. melanotoma* males and females. Arrow indicates the characteristic dark spot on the scutellum of *M. costalis*.

other two species (figs 1 and 2). Compounds 3 to 9 (but not 7) are less abundant in *M. melanotoma* females than males and contribute to separate the *M. melanotoma* female cluster, and compounds 11–15 are less abundant in *M. melanotoma* males than females and contribute to separate the *M. melanotoma*

male cluster (fig. 3). Compounds 17 and 20–28 characterize a cluster enclosing *M. pygmaeus* and *M. costalis*, with no clear distinction between species, sexes or diet conditions (fig. 3). Individuals reared in the laboratory in isolation with a homogeneous diet of tobacco and moth eggs had a higher

Table 2. Results from MANOVA using Pillai-Bartlett's statistic when analysing variability due to species, sex and diet for 28 cuticular hydrocarbons of *Macrolophus*.

	df	Pillai statistic	Approx. F	Numerator df	Denominator df	P-value
Species	2	1.790	26.806	56	176	<0.00001
Sex	1	0.689	6.910	28	87	<0.00001
Diet	1	0.747	9.198	28	87	<0.00001
Residuals	114					

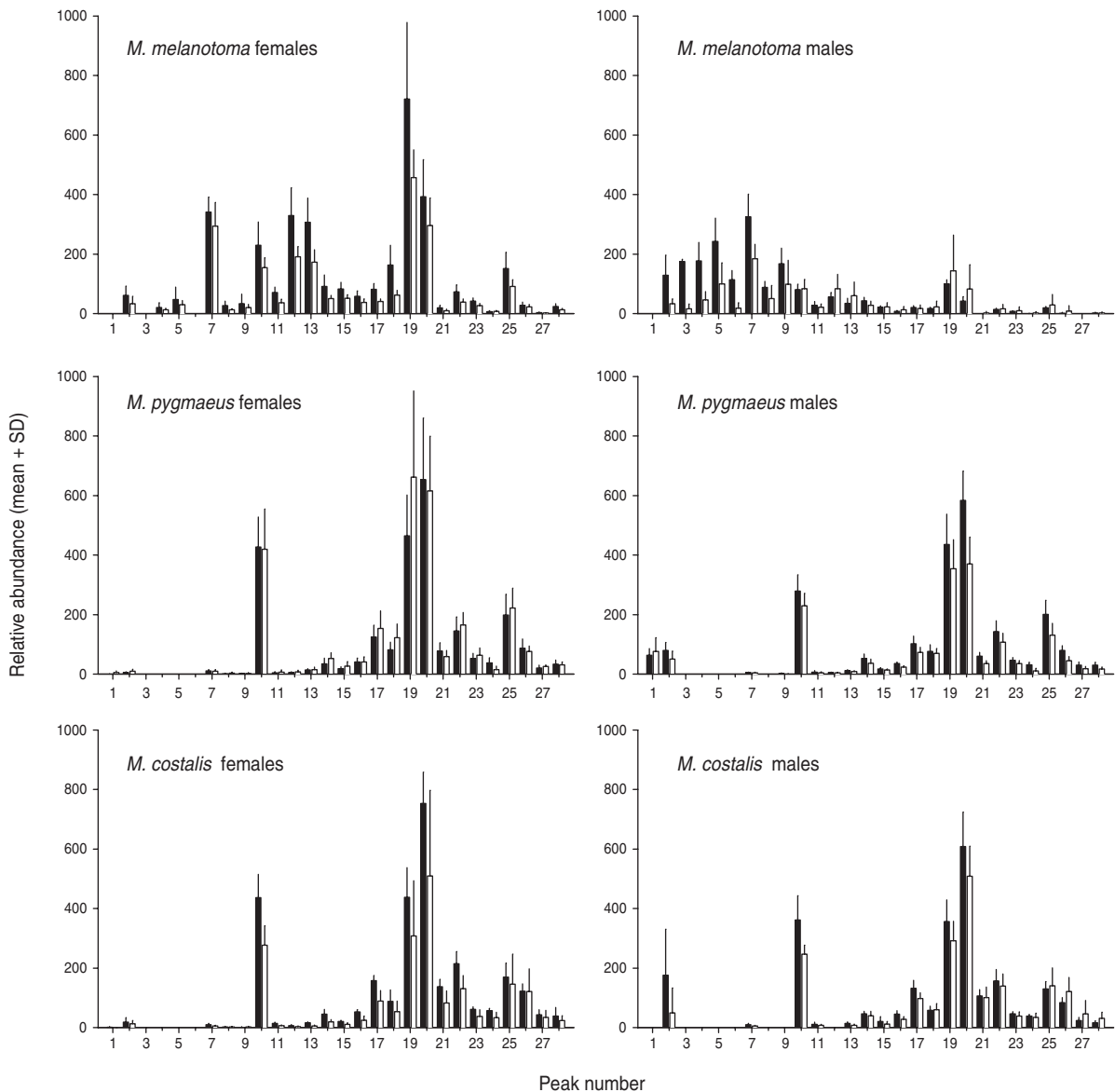


Fig. 2. Cuticular hydrocarbon abundance (relative to the internal standard) in males and females of three *Macrolophus* species. Field individuals (white bars) were fed on their natural diet; laboratory individuals (black bars) were reared in a standard tobacco and moth-egg diet.

quantity of some hydrocarbons than their 'field' relatives, but the species-specific cuticular hydrocarbon pattern was not altered (fig. 2), thus indicating a weak effect of diet.

With regard to the prediction model, a QDA based on PC1, PC2 and PC3 (which together explained 78% of total variability) was used to predict the species of each individual

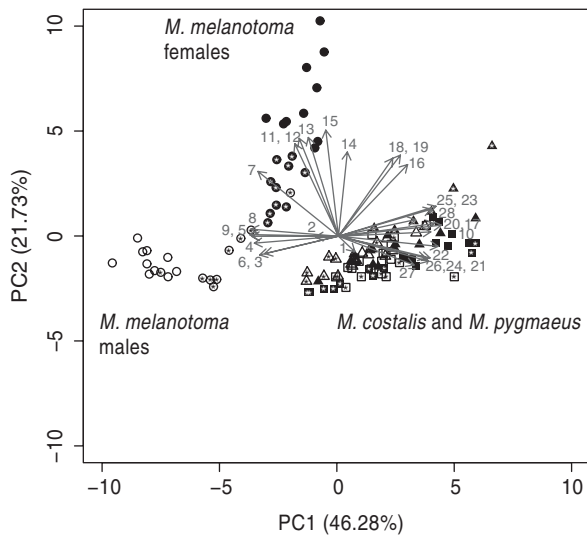


Fig. 3. Biplot showing individuals projected onto the plane defined by the first two principal components (PC), and cuticular hydrocarbons (vectors 1–28) representing directions and correlations in the same space. PC1 explains 46% of the variability of *Macrolophus* cuticular hydrocarbons and separates *M. melanotoma* males (○) from the females (●), and from the other two species (▲, *M. pygmaeus*; ■, *M. costalis*). PC2 explains 22% of the variability and separates *M. melanotoma* females from the males and from the other two species. Laboratory individuals are marked with an asterisk; field individuals are unmarked.

(see Supplementary Material files). QDA was able to correctly classify all *M. melanotoma* individuals, but five *M. costalis* individuals were classified as *M. pygmaeus* and two *M. pygmaeus* were classified as *M. costalis* (table 3). Therefore the global error was 5.88% when comparing these three species. To further assess the error prediction level, we first run a training and test method using three-quarters of the sample (90 individuals chosen at random) to build a QDA, and the remaining quarter of the sample (29 individuals chosen at random) to test the prediction accuracy, which resulted in a 3.5% global error. Next, we used a ten-fold cross-validation method which indicated a global prediction error of 6.75% (ranging from 0% to 25%), attributed to the separation between *M. pygmaeus* and *M. costalis*, given that the error rate when discriminating *M. melanotoma* from *M. pygmaeus* or *M. costalis* was 0% in all cases. We also performed QDA separately for each sex, reaching an improved global error of 1.7% in both cases (only one insect in each sex was erroneously classified; tables S1 and S2, Supplementary Materials). Thus, the model predicted species identity despite the variability contributed by sex and diet, which supports the previous observation that the differences among species outweigh those between sexes and diets. Scripts of the model are available in R as Supplementary Material and can be modified to include other species and compounds.

Discussion

Our study shows that the cuticular hydrocarbon profiles of *M. pygmaeus* and *M. melanotoma* are markedly different from each other and can be used as a phenotypic character to discriminate adults of these otherwise practically

Table 3. Prediction errors in the classification of three *Macrolophus* species based on a quadratic discriminant analysis (QDA) using the three main principal components of the cuticular hydrocarbons of males and females. The number in each cell represents the number of individuals of the true species (column) assigned to the three predicted species (rows). A total of 119 insects where analysed.

True species	Predicted species by QDA			Total
	<i>M. melanotoma</i>	<i>M. pygmaeus</i>	<i>M. costalis</i>	
<i>M. melanotoma</i>	39	0	0	39
<i>M. pygmaeus</i>	0	38	2	40
<i>M. costalis</i>	0	5	35	40
Total	39	43	37	119
Prediction error (%)	0	13.16	5.4	5.88

indistinguishable species. Inclusion of a third species, *M. costalis*, which has a cuticular hydrocarbon profile that is very similar to that of *M. pygmaeus*, but different from *M. melanotoma*, strengthens the validity of the cuticular hydrocarbon method for species separation. Our results agree with sequence variation of *mtDNA* where *M. melanotoma* and *M. pygmaeus* separate in different clusters, whereas *M. pygmaeus* and *M. costalis* group in the same cluster, and are therefore considered as sister species (Martínez-Cascales *et al.*, 2006). Crossing experiments between *M. pygmaeus* and *M. melanotoma* individuals collected from the same host plants as in this study produced no progeny (O.A. & C. C., personal observation), which is in accordance with the species status of the two taxa.

The prediction model results in 100% prediction of *M. melanotoma*, but the same model is less successful at separating *M. pygmaeus* from *M. costalis*, as the hydrocarbon profile of these two species are more similar to each other than to *M. melanotoma*. However, *M. costalis* has a dark spot on the scutellum that distinguishes it from *M. pygmaeus*, thus complete separation of all three species is possible. The same level of discrimination between *M. pygmaeus* and *M. melanotoma* than the one we have obtained is also achieved with *mtDNA* sequences (Martínez-Cascales *et al.*, 2006), so both methods are equally suitable to distinguish between the two species. Although there were sex differences in cuticular hydrocarbon profiles, making a separate prediction model for each sex reduces only slightly the prediction error for *M. pygmaeus* and *M. costalis*, and thus it is not necessary to analyse males and females separately. However, the two sexes are easily distinguished by eye, and so they could be analysed separately if finer discrimination is needed. Diet also had an effect on the cuticular hydrocarbons, but this effect was smaller than the differences among species and thus diet does not affect the ability of the model to predict species identity. Therefore, the differences among species outweigh those between sexes and diets, and the model will work despite these additional sources of variation.

To our knowledge, there is only one other report of cuticular hydrocarbons in mirids (Drijfhout & Groot, 2001; Drijfhout *et al.*, 2003). We did not find any of the reported *L. pabulinus* compounds in *Macrolophus* cuticular extracts, neither are they present in several haematophagous triatomide heteroptera (Juárez & Fernández, 2007); however, there are similarities between the cuticular hydrocarbons of triatomide

bugs and *Macrolophus*. For example, the majority of compounds in both groups of insects are mostly between 20 and 40 carbons, alkenes are few, and mono and methyl branched alkanes are frequent, often with positions in carbons 11, 13 and 15. In contrast to the pattern observed in triatomid bugs and *Macrolophus*, in three species of the antocorid bug *Orius*, straight-chain alkanes, alkenes and single mono-methylated alkanes between 25 and 33 carbons in length comprise the most abundant cuticular hydrocarbons (Nakabou & Ohno, 2001).

M. melanotoma males and females had very different cuticular hydrocarbon profiles, but the hydrocarbon profiles of males and females of the other two *Macrolophus* species were very similar. Sexual differences are also relatively small in the heteropterans *Oncopeltus fasciatus* (Lygaeidae) and *Rhodnius prolixus* (Reduviidae) (Jackson, 1983; Juárez et al., 2001). Whether sexual dimorphism of cuticular hydrocarbons plays a role in mediating sexual recognition in *Macrolophus* is unknown; however, there are many examples in other insects where this is the case (reviewed in Thomas & Simmons, 2008). In the mirid *L. pabulinus*, the leg hydrocarbons (Z)-9-pentacosene and (Z)-9-heptacosene are produced in sex-specific amounts and could be involved in mate location (Drijfhout & Groot, 2001; Drijfhout et al., 2003).

The effect of diet on cuticular hydrocarbon composition is well documented in social omnivorous insects such as ants and cockroaches (Liang & Silverman, 2000; Guerrieri et al., 2009). Although diet had a significant effect on the total quantity of cuticular hydrocarbons of *Macrolophus*, the species- and sex-specific patterns of cuticular hydrocarbons were not altered by the diet, suggesting that the cuticular hydrocarbon composition of *Macrolophus* is largely under genetic control and not strongly influenced by diet.

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