Behav Ecol Sociobiol (2007) 61:811–821
DOI 10.1007/s00265-006-0312-z

ORIGINAL ARTICLE

Population differentiation in female sex pheromone and male preferences in a solitary bee

Nicolas J. Vereecken · Jim Mant · Florian P. Schiestl

Received: 21 August 2006 / Revised: 7 November 2006 / Accepted: 8 November 2006 / Published online: 19 December 2006

Abstract Population differentiation in female mating signals and associated male preferences can drive reproductive isolation among segregated populations. We tested this assumption by investigating intraspecific variation in female sex pheromone and associated male odour preferences among distant populations in the solitary bee *Colletes cunicularius* (L.) by using quantitative gas chromatography and by performing field bioassays with synthetic blends of key sex pheromone compounds. We found significant differences in sex pheromone blends among the bee populations, and the divergence in odour blends correlated positively with geographic distance, suggesting that genetic divergence among distant populations can affect sex pheromone chemistry. Our behavioural experiments, however, demonstrate that synthetic copies of allopatric female sex pheromones were cross-attractive to patrolling males from distant populations, making reproductive isolation by non-recognition of mating signals among populations unlikely. Our data also show that patrolling male bees from different populations preferred odour types from allopatric populations at the two sites of bioassays. These male preferences are not expected to select for changes in the female sex pheromone, but may influence the evolution of floral odour in sexually deceptive orchids of the genus *Ophrys* that are pollinated by *C. cunicularius* males.

Keywords *Colletes cunicularius* · Population differentiation · Odour preferences · *Ophrys* orchids · Pollination by sexual deception

Communicated by R. F. A. Moritz

N. J. Vereecken (✉)
Behavioural and Evolutionary Ecology,
Free University of Brussels CP 160/12,
Avenue F.D. Roosevelt 50,
1050 Brussels, Belgium
e-mail: nicolas.vereeecken@ulb.ac.be

J. Mant · F. P. Schiestl
Plant Ecological Genetics, Institute for Integrative Biology,
ETH Zürich CHN, Universitätsstrasse 16,
CH-8092 Zurich, Switzerland

Introduction

Divergence in courtship signals has often been referred to as a prominent force promoting reproductive isolation among evolutionary lineages, which may ultimately lead to speciation (West-Eberhard 1983; Andersson 1994; Panhuis et al. 2001). The major mechanisms commonly raised for driving allopatric divergence in mating cues are (1) stochastic processes (Fisher 1930; Lande 1981) and (2) adaptation to local environments (see Boughman 2002 for a review). These evolutionary forces are not mutually exclusive and might even act in concert, thereby promoting adaptive population divergence over time (Schluter 2000). In spite of many reports on the nature of courtship signals across the animal kingdom, the extent to which population differentiation in mating cues affects species recognition has received little empirical support and remains a key issue in evolutionary biology (Andersson 1994).

Most solitary bee species display patchy distribution patterns throughout their range as an outcome of the spatial heterogeneity of their nesting sites and foraging resources (O'Toole 1994; Tscharntke and Brandl 2004)—which may include only particular floral rewards (oligolectism; Eickwort and Ginsberg 1980; Simpson and Neff 1981)—and an overall restricted ability to forage over great distances (see e.g. Gathmann and Tscharntke 2002). These constraints may restrict continuous gene flow among distant populations and

thus lead to evolutionary divergence, especially under spatially varying selection regimes (Jordan 1905; Wright 1943; Antonovics 1971; Hedrick et al. 1976).

As in many insects, mate recognition in solitary bees is mediated by chemical cues, so-called sex pheromones (reviewed by Ayasse et al. 2001), although other mating cues can also be involved (e.g. Larsen et al. 1986; Candolin 2003). One of the few solitary bees for which sex pheromone data are available is *Colletes cunicularius* (L.), which can be found in early spring throughout the Euro-Siberian region (Noskiewicz 1936) when few other solitary bee species are active (Michener 1974; Larsson and Tengö 1989; Mader 1999). The chemical basis to mate location and recognition by patrolling *C. cunicularius* males has recently been unravelled by Mant et al. (2005a), after earlier studies by Bergström and Tengö (1978), Cane and Tengö (1981) and Borg-Karlson et al. (2003). The species-specific mate attraction mechanism in *C. cunicularius* females has been shown to include, as an early step in the males' sexual stimulation and inspection flights, emission of the long-range (>1 m) attractant linalool (3,7-dimethyl-1,6-octadien-3-ol; Borg-Karlson et al. 2003), a highly volatile and ubiquitous monoterpene alcohol (Knudsen et al. 1993; Raguso and Pichersky 1999; Knudsen and Gershenzon 2006). Subsequent short-range (<10 cm) mate attraction and copulation attempts are triggered by cuticular hydrocarbons (CHCs) located on the female body surface. A functional dissection of behaviourally active compounds identified by gas chromatography with electroantennographic detection, in addition to behavioural bioassays, has pinpointed a set of three (*Z*)-7 alkenes of 21, 23 and 25 carbons chain length as key compounds of the female sex pheromone in this bee species (Mant et al. 2005a).

To date, population variation (i.e. dialects) in female sex pheromone signals and associated preferences have only been reported from moths (see e.g. Klun et al. 1975; Miller and Roelofs 1980; Löfstedt et al. 1986; Hansson et al. 1990; Toth et al. 1992; Kawazu et al. 2000; McElfresh and Millar 2001) and from *Drosophila* flies (see e.g. Jallon and David 1987; Markow 1991; Stennett and Etges 1997; Etges and Ahrens 2001). In most cases, geographic isolation of populations along with genetic drift have been shown to foster the evolution of population-specific signals, usually consisting of quantitative "variation on a theme" (i.e. identical key sex pheromone compounds in different relative amounts; Löfstedt et al. 1986; Hansson et al. 1990; Toth et al. 1992; Löfstedt 1993). Field tests performed with synthetic blends of female sex pheromone compounds have demonstrated population differentiation in compound detection and odour preferences in male moths (see Hansson et al. 1990 and references therein; Toth et al. 1992). Although the topic of sex pheromone differentiation and its potential role in reproductive isolation and allopatric speciation is one of high interest in evolutionary ecology, very few studies have addressed this issue in hymenopterans, especially in solitary bees (reviewed by Ayasse et al. 2001).

In this study, we investigated differences in female mating signals in *C. cunicularius* by performing comparative chemical analyses of the female sex pheromone from five populations from Austria, France, Italy and Switzerland (Fig. 1). Additionally, we tested the hypothesis of population-specific odour preferences of *C. cunicularius* males by performing bioassays with synthetic imitations of population-specific blends of key compounds for mate attraction in two natural populations in Austria and Switzerland. Specifically, we ask the following questions: (1) Does the sex

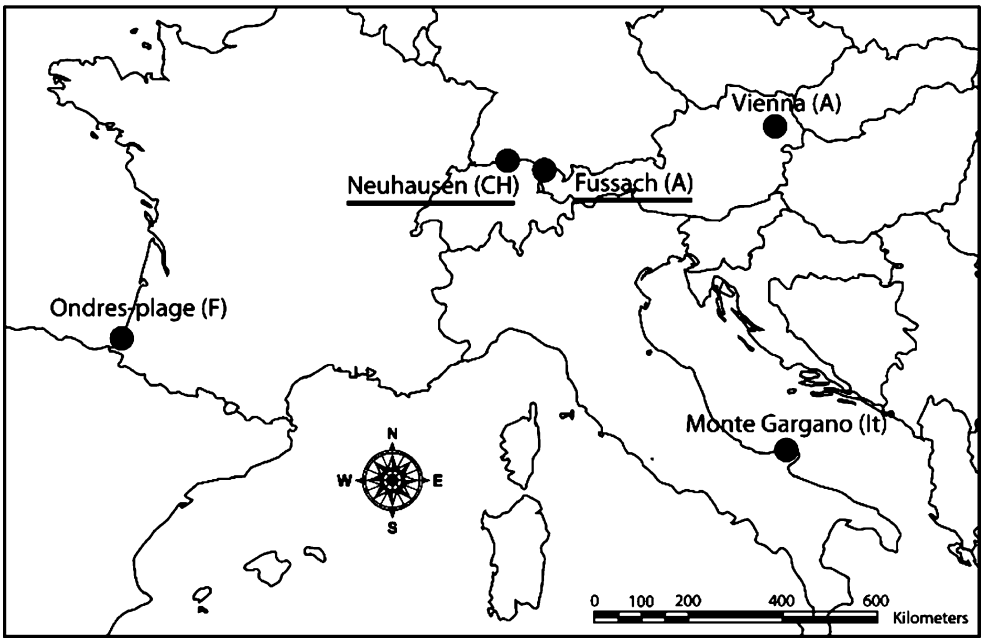

Fig. 1 Sampling localities of *C. cunicularius* and *Ophrys exaltata*. The bees were sampled at Ondres-plage (*F*), Neuhausen (*CH*), Fussach (*A*), Vienna (*A*) and Monte Gargano (*It*). The sites of bioassays are *underlined in black*

pheromone signal of *C. cunicularius* females vary among distant populations? (2) Do *C. cunicularius* males have population-specific odour preferences?

Materials and methods

Sample collection

Virgin *C. cunicularius* females were collected in early spring in geographically distant populations at Fussach (A; n=33), Vienna (A; n=12), Neuhausen (CH; n=56), Ondres-plage (F; n=20) and Monte Gargano (It; n=16; Fig. 1). Virgin females are easily detected after emergence when a cluster of sexually aroused males forms around them. All attractive *C. cunicularius* females were caught with a hand net, stored individually in chilled plastic cups (Eppendorfs) and instantaneously killed by freezing. Epicuticular waxes of the bees were sampled by extracting the body of individual female bees in 400 µl hexane (high performance liquid chromatography grade) for 1 min. All extracts were stored at −20°C. Before GC analyses, 100 ng n-octadecane was added as internal standard to all samples.

Chemical analyses

All samples were analyzed by gas chromatography (GC) on a Hewlett Packard 6890N GC equipped with a HP-5 capillary column (30 m×0.32 mm×0.25 µm). The injector temperature was kept at 300°C. One microlitre aliquots of the extracts was injected splitless at 50°C (1 min) followed by a programmed increase of oven temperature to 300°C at a rate of 10°C/min; helium was used as the carrier gas. Compounds were identified by comparison of retention times with authentic standard compounds. Additionally, selected samples were analyzed with a GC with a mass selective detector (MSD—Hewlett Packard G1800 A), and MS spectra compared with those of known reference substances (Mant et al. 2005a). The absolute amounts of the 40 identified compounds were calculated by the internal standard method as described by Mant et al. (2005a). Relative proportions (%) were calculated by summing up the absolute amounts of all compounds; absolute amounts of individual compounds were then divided by the sum and multiplied by 100.

Preparation of synthetic blends

The mean relative and absolute amounts of all compounds found in epicuticular extracts of *C. cunicularius* females (Table 1) were used to prepare three synthetic blends designed to mimic relative proportions of key sex pheromone compounds found in natural extracts of virgin *C. cunicularius* females from Fussach (A), Monte Gargano (It) and Neuhausen (CH; Table 2). Not all behaviourally active compounds (see Mant et al. 2005a) could be used to prepare the synthetic blends, as only 12 active compounds [including the major compounds for mate attraction described by Mant et al. (2005a)] were available to us in synthetic form. GC runs of the synthetic blends (synthetic compounds mixed with hexane) were made before bioassays to check the relative amounts of compounds in the blends. Each synthetic mixture tested contained equal absolute amounts of active compounds in different ratios (Table 3). This method allowed focusing on ascertaining the impact of individual hydrocarbon profiles (and not absolute amounts of compounds) on the short-range attractiveness of the blends towards mate-searching males.

Behavioural experiments

Bioassays were performed in late March and early April in natural populations at Fussach (A) and Neuhausen (CH; Fig. 1) where thousands of *C. cunicularius* males were patrolling for emerging females on restricted nesting/emergence sites. The density of bees in each site was stable over the days of observations, although higher at Neuhausen (CH) than at Fussach (A). Behavioural responses of male bees towards dummies (black cylindrical

Table 1 Mean absolute amounts (µg) of compounds recorded in natural extracts of individual virgin *C. cunicularius* females

	Natural extracts[a] (Mean absolute amounts in micrograms ± SE)					
	Fussach (A)	Vienna (A)	Neuhausen (CH)	Ondres-plage (F)	Monte Gargano (It)	χ^2—F values[b]
All compounds	3.776±1.697 (a)	3.854±3.031 (a)	8.542±3.477 (b)	2.604±0.816 (c)	10.724±3.563 (d)	18.888*
Active compounds	3.130±1.628 (a)	2.987±3.053 (a)	7.728±3.134 (b)	1.201±0.390 (c)	9.881±3.290 (d)	92.006**
Non-active compounds	0.645±0.283 (a)	0.867±0.421 (a)	0.813±0.429 (a)	1.402±0.427 (b)	0.842±0.307 (a)	6.342**

Different letters inside the parentheses indicate significant differences.

[a] Pairwise comparisons among groups for each class of compounds (same line) using (1) non-parametric Mann–Whitney U-test (with Bonferroni correction for all compounds and active compounds, α=0.005) or (2) ANOVA post-hoc LSD test [for non-active compounds, α=0.005 (df=4)]

[b] Overall comparisons for each class of compounds (same line) using (1) non-parametric Kruskal–Wallis test (for all compounds and active compounds, α=0.05 (df=5; *P<0.05, **P<0.01) or (2) ANOVA (for non-active compounds, α=0.005 (df=4; *P<0.005, **P<0.001)

Table 2 Mean relative amounts (%) of compounds recorded in cuticle extracts of individual virgin *C. cunicularius* females

Active compounds	Natural extracts[a] (Mean relative amounts in % ± SE)					
	Fussach (A)	Vienna (A)	Neuhausen (CH)	Ondres-plage (F)	Monte Gargano (It)	χ^2 Values [b]
Alcohols						
1. Linalool	3.721±0.638 (a,d)	4.560±0.744 (a)	1.318±0.198 (b,c)	1.369±0.236 (c,e)	2.801±0.882 (d,e)	29.859 **
Aldehydes and esters						
2. Hexadecanal (and isopropyle)	0.350±0.039 (a)	0.241±0.039 (a)	0.532±0.033 (b)	0.019±0.015 (c)	0.128±0.019 (d)	83.704 **
3. Eicosanal	0.891±0.076 (a)	0.847±0.105 (a,b)	1.070±0.042 (b)	0.425±0.037 (c,d)	0.485±0.059 (d)	30.404 **
4. Tetracosanal	0.168±0.055 (a,d)	0.441±0.135 (b)	0.106±0.014 (a)	0.000±0.000 (c)	0.064±0.015 (d)	60.949 **
5. Dodecyltetradecanoate (and decylhexadecanoate)	0.297±0.061 (a,c)	0.468±0.222 (a)	0.314±0.024 (a)	0.012±0.008 (b)	0.300±0.089 (c)	47.281 **
Unsaturated hydrocarbons						
6. (Z)-7-Heneicosene	2.761±0.335 (a)	1.533±0.751 (b)	6.069±0.325 (c)	14.481±1.902 (d)	5.828±1.215 (c,e)	62.468 **
7. (Z)-7-Tricosene	1.673±0.286 (a)	1.113±0.576 (a,b)	0.822±0.096 (b)	7.803±1.681 (c)	5.202±1.349 (c)	45.628 **
8. (Z)-7-Pentacosene	2.091±0.235 (a)	2.310±1.143 (b)	1.911±0.176 (a)	2.390±1.048 (a,b)	16.291±2.051 (c)	32.566 **
9. (Z)-9-Tricosene	1.878±0.612 (a)	1.054±0.564 (a)	2.385±0.336 (b)	6.754±1.759 (a,b)	1.128±0.445 (a)	25.054 **
10. (Z)-9-Tetracosene	0.311±0.063 (a,b)	0.494±0.253 (a,b)	0.305±0.026 (a)	0.956±0.383 (a,b)	0.379±0.046 (b)	8.210 *
11. (Z)-9-Pentacosene	7.425±1.069 (a)	1.984±0.527 (b)	8.966±0.684 (a)	3.702±1.022 (b)	9.693±1.309 (a)	34.990 **
12. (Z)-9-Heptacosene	4.006±0.390 (a)	2.938±0.647 (a)	6.679±0.523 (b)	1.020±0.181 (c)	3.785±1.079 (a)	56.575 **
13. (Z)-9-Nonacosene	5.323±0.455 (a)	4.837±0.929 (a)	4.602±0.317 (a)	0.533±0.087 (b)	0.720±0.117c (b)	39.545 **
14. Z-9-Hentriacontene	2.323±0.299 (a)	2.219±0.483 (a,b)	1.440±0.221 (b)	0.373±0.060 (c)	0.831±0.247 (c)	76.038 **
15. (Z)-8,(Z)-20-Nonacosadiene	0.597±0.105 (a,c)	0.341±0.106 (a,e)	0.636±0.052 (c)	0.000±0.000 (d)	0.312±0.082 (e)	58.964 **
16. (Z)-8,(Z)-20-Hentriacontadiene	2.419±0.310 (a)	2.553±0.668 (a)	2.456±0.176 (a)	0.173±0.078 (b)	0.887±0.302 (c)	60.840 **
17. (Z)-11-Nonacosene	1.010±0.334 (a)	1.523±0.445 (b)	1.347±0.389 (a)	0.000±0.000 (c)	1.478±0.398 (b)	60.111 **
Saturated hydrocarbons						
18. Heneicosane	6.142±0.712 (a)	3.911±0.747 (b)	6.142±0.312 (a)	17.740±1.868 (c)	6.668±0.916 (a)	38.957 **
19. Tricosane	18.257±0.992 (a)	11.755±1.188 (b)	24.871±0.746 (c)	21.621±0.951 (d)	20.113±1.447 (a,d)	49.764 **
20. Pentacosane	8.677±0.421 (a)	8.891±0.930 (a)	11.279±0.450 (b)	8.490±0.555 (a)	10.053±0.935 (a)	25.649 **
21. Heptacosane	5.869±0.326 (a)	7.582±1.096 (a)	6.650±0.344 (a)	4.177±0.285 (b)	3.660±0.347 (b)	40.408 **
Unknown compound						
22. A14	0.035±0.019 (a)	0.106±0.048 (a,b; c,d)	0.034±0.008 (b)	0.000±0.000 (c)	0.065±0.011 (d)	45.278 **
Non-active compounds						
23. Dodecosane	0.435±0.027 (a)	0.306±0.032 (b)	0.478±0.014 (a,d)	0.635±0.024 (c)	0.687±0.177 (d)	43.076 **
24. Tetracosane	0.34 ±0.024 (a)	0.339±0.042 (a,b)	0.358±0.011 (b)	0.348±0.022 (a,b)	0.476±0.033 (c)	17.507 *
25. Hexacosane	1.162±0.450 (a,c)	9.498±1.161 (b)	0.274±0.014 (a)	0.202±0.022 (c)	0.217±0.017 (c)	38.820 **
26. Octacosane	0.186±0.034 (a)	0.198±0.060 (a,b)	0.178±0.018 (a)	0.079±0.027 (b)	0.178±0.049 (a)	10.915 *
27. Nonacosane	3.128±0.208 (a)	0.888±0.375 (b)	2.754±0.189 (a)	1.716±0.158 (c)	1.672±0.208 (c)	46.248 **
28. (Z)-3-Tricosene	0.479±0.062 (a)	0.397±0.080 (a)	0.373±0.027 (a)	0.824±0.066 (b)	0.392±0.064 (a)	29.574 **
29. (Z)-5-Tricosene	0.426±0.075 (a,d)	0.214±0.053 (a,b)	0.211±0.015 (b)	0.102±0.087 (c)	0.399±0.058 (d)	41.633 **
30. (Z)-5-Pentacosene	0.966±0.096 (a)	0.679±0.138 (a,b)	0.749±0.218 (b)	0.333±0.056 (c)	0.078±0.078 (d)	52.890 **
31. (Z)-7-Heptacosene	1.921±0.297 (a,c)	1.296±0.252 (a)	1.490±0.152 (a)	0.649±0.158 (b)	2.366±0.339 (c)	27.042 **
32. (Z)-7-Nonacosene	1.461±0.184 (a)	3.103±0.537 (b)	0.861±0.090 (c)	0.377±0.067 (d)	0.173±0.037 (e)	73.489 **
33. C—Unknown	0.016±0.011 (a)	0.050±0.025 (a)	0.053±0.010 (b)	0.000±0.000 (a)	0.117±0.027 (c)	82.367 **
34. D—Unknown	0.479±0.028 (a)	0.448±0.059 (a,c)	0.466±0.041 (a,c)	0.793±0.080 (b)	0.395±0.020 (c)	27.822 **
35. E—Unknown	0.210±0.020 (a)	0.563±0.317 (a,b)	0.237±0.018 (a)	0.441±0.047 (b)	0.277±0.051 (a)	25.848 **
36. F—Unknown	0.862±0.329 (a)	1.389±0.369 (b)	0.157±0.015 (a)	0.000±0.000 (c)	0.218±0.051 (a)	57.105 **
37. M—Unknown	3.001±0.452 (a)	1.237±0.354 (a)	0.143±0.011 (b,d)	0.514±0.070 (c)	0.224±0.054 (d)	45.870 **
38. S—Unknown	0.341±0.042 (a,c)	1.597±1.170 (a)	0.171±0.008 (b,c)	0.403±0.066 (a)	0.195±0.022 (c)	34.047 **
39. Y—Unknown	0.367±0.048 (a)	0.132±0.065 (b)	0.394±0.023 (a)	0.000±0.000 (b)	0.293±0.042 (a)	64.485 **
40. Z—Unknown	0.334±0.051 (a)	0.463±0.201 (a,b)	0.248±0.021 (a)	0.543±0.068 (b,c)	0.772±0.103 (c)	36.825 **

Different letters inside the parentheses indicate significant differences.

[a] Pairwise comparisons among groups for each compound (same line) using non-parametric Mann–Whitney *U*-test, $\alpha=0.005$

[b] Overall comparisons for each compound (same line) using non-parametric Kruskal–Wallis test, $\alpha=0.05$ ($df=4$; $*P<0.05$, $**P<0.01$)

Table 3 Mean relative amounts (%) of behaviourally active compounds in cuticle extracts of individual virgin *C. cunicularius* females vs synthetic blends used for the bioassays

Active compounds	Fussach (A)		Monte Gargano (It)		Neuhausen (CH)	
	Natural extract	Synthetic blend	Natural extract	Synthetic blend	Natural extract	Synthetic blend
1. (Z)-7-Heneicosene	4.59	3.9	7.25	6.95	7.19	6.67
2. Heneicosane	9.63	11.64	6.62	8.59	7.09	7.9
3. (Z)-9-Tricosene	5.49	5.35	0.85	0.93	2.65	2.39
4. (Z)-7-Tricosene	1.73	7.21	6.85	7.47	1.06	1.22
5. Tricosane	28.1	29.82	24.32	25.96	30.89	31.3
6. (Z)-9-Pentacosene	16.33	13.91	12.75	10.32	10.71	11.42
7. (Z)-7-Pentacosene	3.49	3.15	20.14	17.2	2.41	2.81
8. Pentacosane	10.8	7.89	11.7	11.64	13.7	13.37
9. (Z)-9-Heptacosene	5.59	5.22	3.64	4.17	8.12	8.32
10. Heptacosane	5.73	5.24	4.12	4.95	7.87	8.48
11. (Z)-9-Nonacosene	5.35	4.53	0.83	1.07	5.5	4.57
12. (Z)-8-(Z)-20-Hentriacontadiene	3.18	2.12	0.95	0.73	2.8	1.54
Total (%)	100	100	100	100	100	100
Sum of absolute amounts (μg)	2.897	2.5	9.054	2.5	7.423	2.5

plastic beads, 4×5 mm, mounted on an insect pin) scented with synthetic blends were taped using a voice recorder during 3 min and classified into two categories: (1) number of approaches [hovering in front of the dummy at close range (<10 cm) without any contact with the odour source] and (2) number of contacts (from a short pounce to a copulation attempt with the scented dummy). Odour sources were presented individually for each test (i.e. each scented dummy was used only once). A female-equivalent amount of 2.5 μg of each synthetic blend was applied on each dummy with a Hamilton glass syringe (100 μl; see blend composition in Table 3). The dummy was then placed in a male patrolling area after the solvent had evaporated. Controls (dummies treated with solvent only and placed in a male patrolling area after the solvent had evaporated) were tested independently for their attractiveness after every 5th test. All bioassays were conducted between 10 A.M. and 3 P.M.—when *C. cunicularius* males' patrolling activity was at peak. As males of *C. cunicularius* have been shown to patrol fairly localized regions on the nesting/emergence site (Peakall and Schiestl 2004), test spots were changed after each bioassay in both populations to test the responses of different males to synthetic odour blends.

Statistical analyses

Means and standard errors (SE) of absolute (μg/ml) and relative amounts (%) of all identified compounds were calculated for all natural extracts. When transforming data did not yield normal distributions and variances were not homogeneous, we used a non-parametric Kruskal–Wallis (K-W) test for multiple independent comparisons of absolute and relative amounts of compounds among bee populations. Mann–Whitney (M-W) *U*-tests were performed for a posteriori pairwise comparisons of (1) the total amounts of compounds and (2) relative amounts of each compound among populations. A standard Bonferroni correction was used for pairwise comparisons among bee populations; the level of significance (α) was set to 0.005 (α=0.05 divided by the number of comparisons, i.e. α=0.005).

Multivariate analyses of population variation in cuticular hydrocarbons (relative amounts, in %) of *C. cunicularius* females were performed by canonical discriminant function (CDF) analysis, as the data did not contain significant outliers and given that this multivariate method is robust even when the homogeneity of variances assumption is not met (Brosius 2002). CDF analysis was performed with all behaviourally active compounds. To test for differences in male bee responses to synthetic blends, a one-way analysis of variance (ANOVA; with LSD post-hoc test) was used. All these statistical tests were performed with the SPSS 11.5 package (Brosius 2002).

The spatial structuring of the female sex pheromone in *C. cunicularius* was investigated by performing a Mantel (1967) test, as implemented in GenAlEx 6 (Peakall and Smouse 2005a,b) based on individual-by-individual Euclidean distances in relative amounts of chemical compounds vs geographical distance among populations. Random permutations (n=99) were used to test for significant correlation between divergence in odour compound profiles (active compounds) and geographical distance among sample populations.

Results

Odour differences among bee populations

Our results show that all natural extracts of virgin *C. cunicularius* females of each population investigated contained all 40 odour compounds identified by Mant et al. (2005a). Overall significant differences were found among *C. cunicularius* populations in absolute amounts of (1) sum of all compounds (K-W test, $P<0.005$), (2) sum of active compounds sensu Mant et al. (2005a) (K-W tests, $P<0.005$) and (3) sum of non-active compounds (ANOVA $F_{(4,132)}=6.342$, $P=0.0001$). Within all populations, except Ondres-plage (F), significantly higher absolute amounts of behaviourally active compounds were found compared to non-active compounds (Table 1).

A CDF analysis performed with all behaviourally active cuticular hydrocarbons recorded in solvent extracts of attractive *C. cunicularius* females allowed us to resolve the five bee populations into weakly overlapping groups (Fig. 2). This CDF analysis rejects the null hypothesis of homogeneity of covariance matrices (Box's $M=538.135$, $P<0.001$; small Wilks' λ values: $W\lambda1=0.025$; $W\lambda2=0.115$ and associated $P1$ and $P2<0.001$). The high discriminatory ability of the canonical discriminant functions 1 and 2 (plotted in Fig. 2) provides evidence for the importance of the independent variables (i.e. all behaviourally active odour compounds, including compounds 6–8 in Table 2) to the discriminant analysis. Canonical correlation values close to 1 ($Cc1=0.884$; $Cc2=0.822$) associated with the two CDFs plotted in Fig. 2 further account for the significant contribution of the first two canonical discriminant functions to the resolving of all five *C. cunicularius* populations into weakly overlapping groups. The two CDFs plotted in Fig. 2 account for 76.4% of the overall variance among groups, which further indicates their great discriminatory ability in the model (81.0% of cross-validated grouped cases were correctly classified). Overall, more than 50% of all cross-validated samples were assigned correctly to their population by the two CDFs [Fussach (A), 51.5%; Vienna (A), 50.1%; Neuhausen, 98.2%; Ondres-plage, 85%; Monte Gargano, 81.3%].

A Mantel test performed with all active compounds revealed a significant positive correlation between divergence in chemical compounds profiles and geographical distance among populations ($y=0.0001x+0.1649$; $P<0.05$; $r=0.654$; Fig. 3).

Behavioural experiments

Results from bioassays carried out at Fussach (A) and Neuhausen (CH; Fig. 1) indicate that male bees from both the Austrian and the Swiss populations were able to discriminate between the three synthetic odour blends with different relative amounts of behaviourally active cuticular hydrocarbons (Fig. 4). We also found that the three synthetic blends triggered different levels of attraction in *C. cunicularius* males and that synthetic blends designed to mimic sex pheromones from allopatric populations were significantly more attractive to patrolling males than synthetic copies of sympatric sex pheromones at both sites of bioassays [i.e. the Fussach (A) and Monte Gargano (It)

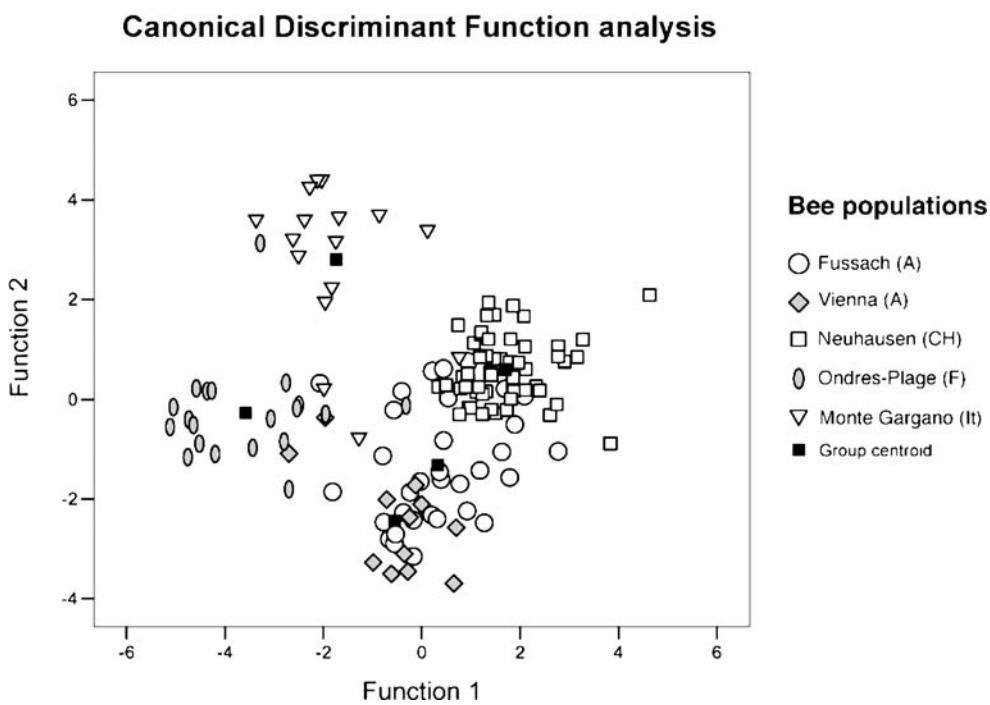

Fig. 2 Population differentiation in cuticular hydrocarbons in virgin *C. cunicularius* females. Canonical discriminant function (*CDF*) plot of all behaviourally active compounds (relative proportions, in %) found in epicuticular extracts of the female bees. Functions 1 and 2 account for 76.4% (48.4 and 28.0%, respectively) of the total variability among populations

blends were significantly more attractive than the Neuhausen (CH) blend at Neuhausen (CH), whereas the Monte Gargano (It) and the Neuhausen (CH) blends were significantly more attractive than the Fussach (A) blend at Fussach (A)] (Fig. 3). The differences in total responses between the two sites of bioassays are due to the higher bee density in Neuhausen (CH) than Fussach (A).

Discussion

Sex pheromone "dialects"

In this paper, we report "dialects" in both absolute and relative amounts of compounds of the female sex pheromone in the wild bee *C. cunicularius*. Multivariate analyses performed with relative amounts of behaviourally active compounds identified in solvent extracts of virgin *C. cunicularius* females allowed us to place the five populations sampled into weakly overlapping clusters (i.e. population-specific dialects) within a multidimensional "olfactory landscape" (Fig. 2). Similar cases of sex pheromone dialects have been found in the turnip moth *Agrotis segetum*, for which females from segregated populations use population-specific blends consisting of different relative amounts of the same behaviourally active compounds (see e.g. Löfstedt et al. 1986; Hansson et al. 1990; Toth et al. 1992). In *Drosphila mojavensis*, where cuticular hydrocarbons are involved in mate recognition, Stennet and Etges (1997) and Etges and Ahrens (2001) have provided evidence for population-specific patterns of long-chain cuticular hydrocarbons. Collectively, these results suggest that polymorphism in sex pheromone systems among segregated populations might concern a wide array of insect taxa.

Our results also show significant spatial structure in the female sex pheromone in this bee species, i.e. that odour samples from neighbouring populations, presumably encompassing the genetically most similar individuals, cluster together in the olfactory landscape (Figs. 2 and 3). Although this finding strongly suggests isolation-by-distance and a prevalent genetic basis of differences in patterns of behaviourally active compounds, the impact of environmental components such as changes in larval rearing substrates on variation in chemical signals cannot be ruled out a priori. Indeed, persuasive fits between shifts in larval diet and the resulting differences in cuticular hydrocarbon profiles in adults have already been documented in ants (see e.g. Liang and Silverman 2000) and in *Drosophila* (see e.g. Jallon and David 1987; Markow and Toolson 1990; Stennett and Etges 1997) where changes in CHC profiles have been shown to affect dramatically species recognition and, in some cases, to result in premating isolation among lineages adapted to different foraging resources (Koepfer 1987a,b; Etges 1992; Etges and Ahrens 2001). The genetic basis of population differences in sex pheromone signals has, however, been recently supported by Watts et al. (2005) in the tropical fly *Lutzomyia longipalpis*. These authors combined data on sex pheromone chemistry and phylogeography from multiple populations and showed that spatial genetic structure was detected and that increased genetic differences among populations were positively correlated with increased differences in sex pheromone chemistry. Likewise, analyses performed by Dapporto et al. (2004) on CHC profiles in the paper wasp *Polistes dominulus* have shown that island and mainland populations sampled in the Tyrrhenian region formed separate clusters. As suggested by Dapporto et al. (2004), part of the explanation for this finding could be that the similarities in proportions of CHCs might reflect the closer genetic relatedness among individuals inhabiting populations of islands vs the mainland. In *C. cunicularius*, future studies on genetic structure of populations and their relatedness may help to elucidate whether population differentiation in sex pheromones are primarily determined by genetic and/or environmental factors.

Odour preferences in *C. cunicularius* males

It has long been argued that population divergence in secondary sex traits and associated mate preferences has the potential to lead to the establishment and evolution of pre-zygotic isolating barriers among segregated populations (Andersson 1994; West-Eberhard 1983). For example, in *Drosophila mojavensis*, premating isolation has been described as a consequence of significant differentiation in both courtship (chemical) signals and mate preferences for these traits among populations (Krebs and Markow 1989; Etges 1992). Similarly, Roelofs et al. (2002) have demonstrated the occurrence of shifts in the structure of sex pheromone components in *Ostrinia* moths. Along with the existence of rare males that might track these changes and respond to the new pheromone blend, such changes may lead to the evolution of an *Ostrinia* species with distinct sex pheromone signal.

Our study shows that patrolling *C. cunicularius* males from both sites of bioassays perceive subtle differences

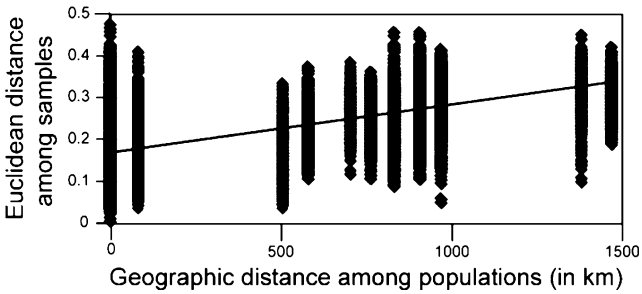

Fig. 3 Mantel's correlogram of Euclidean distance among chemical samples in active compounds plotted by spatial distance among sample sites (Mantel's r=0.654; P<0.05 with 99 permutations)

among odour samples that consist of identical key compounds but differ in relative amounts (Fig. 4), a phenomenon that was already shown in other non-*Apis* bees such as *Andrena nigroaenea* (Schiestl and Ayasse 2000), *Lasioglossum malachurum* (Ayasse et al. 1999) and *Osmia rufa* (Ayasse and Dutzler 1998; Ayasse et al. 2000). Our study provides a multiple-population comparison of male odour preferences in a solitary bee and shows that patrolling *C. cunicularius* males are attracted to sex pheromone dialects from "exotic" (i.e. allopatric) populations, which suggests the sharing of mate recognition cues (sensu Paterson 1985) among segregated populations in this solitary bee species.

Many studies on sexual selection have largely documented that individuals often recognize and prefer to mate with individuals from "local" (or proximate) vs "exotic" populations (see e.g. Andersson 1994 and references therein; Boake 2002; Wong et al. 2004 and references therein). By contrast, our bioassays show that patrolling *C. cunicularius* males prefer odour types different from those found in their own population (i.e. "exotic" sex pheromones). We suggest two explanations for this "exotic effect": (1) As *C. cunicularius* is a gregarious solitary bee for which males search for mates in a restricted area of their nesting/emergence site (Peakall and Schiestl 2004), preferences for "exotic" pheromone signals in patrolling males may be innate and promote outbreeding by avoiding sibling mating, should the opportunity arise. The recourse to odour-based preferences for females to which males are probably less related has rarely been found before in bees (but see Smith and Ayasse 1987; Smith and Breed 1995), yet other similar cases have been found in flies (reviewed by Boake 2002), female crickets (Simmons 1989) as well as in mammals (Potts and Wakeland 1993; Clarke and Faulkes 1999), where it has also been advocated that such instances of odour-based mate choosiness might mirror optimal outbreeding. (2) Alternatively, odour-based discrimination may reflect learning abilities of patrolling *C. cunicularius* males, which prefer odour cues dissimilar to those they have encountered during earlier mating attempts (e.g. Wcislo 1992 and references therein; Ayasse et al. 2000). Under strong male–male competition for access to emerging, virgin females and given that females in this solitary bee species are monandrous (Bergström and Tengö 1978), such odour preferences might help to avoid futile mating attempts by successively directing males towards virgin females they have not yet encountered.

Collectively, as our results show, population divergence in female mating signals and associated male preferences in *C. cunicularius* is unlikely to lead to speciation (i.e. premating isolation by non-recognition of female secondary sex traits), as synthetic copies of allopatric female mating signals were shown to be cross-attractive to patrolling *C. cunicularius* males from distant populations (Fig. 4). Besides, the odour preferences for "exotic" blends found in males of *C. cunicularius* (Fig. 4) are unlikely to select for extreme deviation from the median female sex pheromone blend

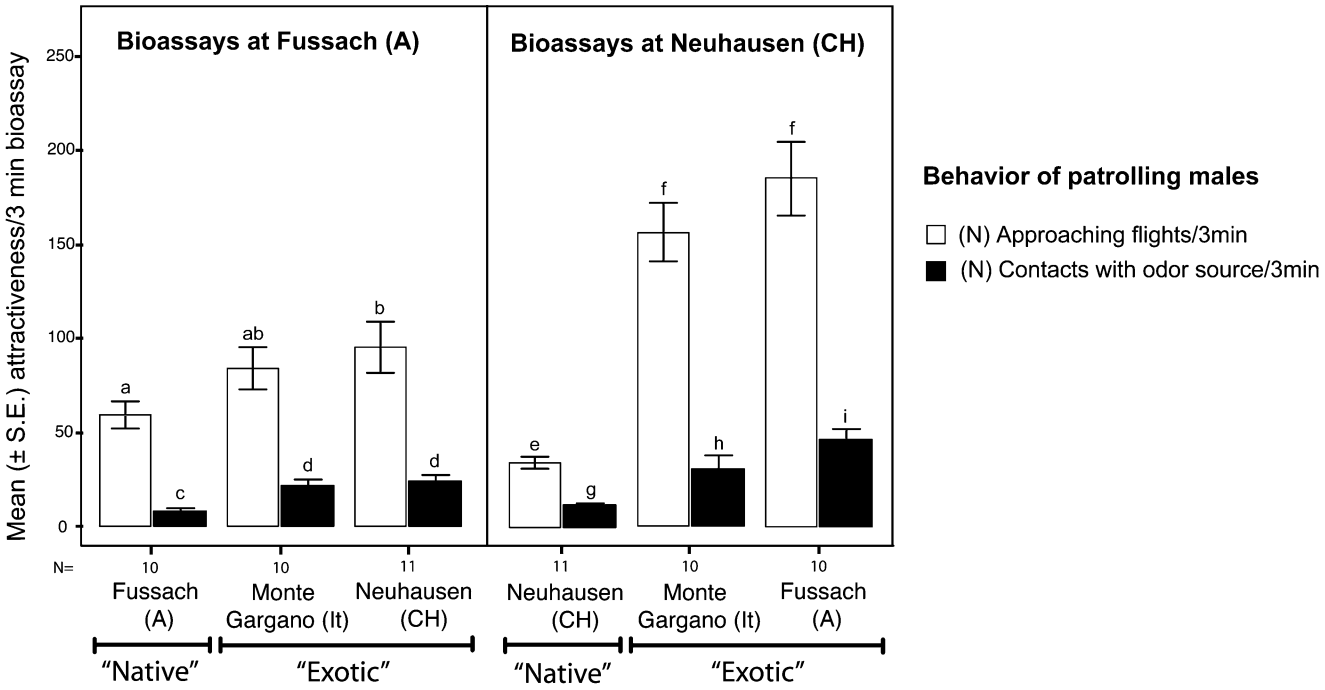

Fig. 4 Comparative level of attractiveness of the synthetic sex pheromone trio [Fussach (*A*), Monte Gargano (*It*) and Neuhausen (*CH*)] when assayed individually at Fussach (*A*, *left*) and Neuhausen (*CH*, *right*) on patrolling males of *C. cunicularius*. One-way ANOVA with LSD post-hoc test (α=0.05). Different *superscript letters* on *top of error bars* indicate significant differences; the number of replicates are listed underneath the columns

within populations because females are the limiting sex and hence, little subjected to male-mediated selection. However, mate preferences in male bees such as those reported here for *C. cunicularius* might transfer into selection on floral traits in those orchids that imitate mating signals of female hymenopterans for pollination and that are limited in their reproductive success by access to pollinators (see e.g. Schiestl 2004).

Evolutionary implications for *Colletes–Ophrys* mimicry systems

Insect communication signals are sometimes imitated by other organisms, which exploit the behaviour of the duped species (Dettner and Liepert 1994; Maynard Smith and Harper 2003 and references therein). A well-known case of this sort involves orchids of the genus *Ophrys* whose flowers mimic chemical, visual and tactile stimuli of virgin female bees (the model) and are pollinated exclusively by sexually aroused males of the respective species (the dupe or operator; reviewed by Schiestl 2005). Among the puzzling diversity of *Ophrys* species specialized on different pollinator species, at least four *Ophrys* species have evolved to mimic *C. cunicularius* mating cues (Mant et al. 2005b and references therein). One of them is *Ophrys exaltata* Tenore, which emits a species-specific floral odour blend consisting of compounds identical to those employed by *C. cunicularius* females to attract their mate (Mant et al. 2005a). In this orchid species, population differentiation in the floral odour was found to be stronger in floral odour compounds involved in pollinator attraction (active compounds) as compared to non-active compounds (Mant et al. 2005b), which implied pollinator-imposed selection mediated by population-specific preferences of male bees for mating cues. Our study demonstrates population-specific preferences of pollinators, the requirement for such a scenario, and thus supports the findings of Mant et al. (2005b).

Although it is often predicted for Batesian mimicry systems that mimics (i.e. orchids) are selected for signal refinement to optimally match the signal emitted by their model (i.e. virgin female bees; Fisher 1930; Turner 1988 and references therein; Stowe 1988), our finding of an "exotic effect" predicts that the orchids should evolve "exotic" odour bouquets (yet remaining within the boundaries of the communication channel to ensure attractiveness to the male pollinators), which slightly differ from the female bees within populations, and thus, be preferred by *C. cunicularius* males. These differences between orchid and female bee signals might, however, only be detectable through multivariate comparisons of orchid odour and female sex pheromone, using large sample sizes, which has not been employed in any orchid-pollinator study so far. We are currently investigating odour bouquets of orchids and virgin female bees from multiple populations to test, at the population scale, whether population differentiation in male bee preferences drives associated divergence in odour between female bees and the *Ophrys* species they pollinate.

Acknowledgement We sincerely thank C. Schulz and W. Francke (University of Hamburg, Germany) for providing us with synthetic compounds. J. Alcock (Arizona State University, USA) is gratefully acknowledged for having provided helpful comments on an earlier version of the manuscript. D. Genoud (Tarnos, France), G. Mahé (Saint-Nazaire, France), C.E. Hermosilla (La Rioja, Spain), G. Hölzler (Vienna, Austria) and A. Müller (ETH Zürich, Switzerland) shall also be warmly thanked for their useful help in collecting specimens. G. Caruso (Cambridge University, UK) provided us with generous help for the preparation of Fig. 1. We thank two anonymous referees for their valuable comments. The experiments comply with the current laws of the country in which they were performed. NJV was financially supported by the Belgian "Fonds pour la formation à la Recherche dans l'Industrie et l'Agriculture" (F.R.I.A.) and by a "Directeur Moiny" studentship delivered by the Order of Leopold ASBL (Belgium); JM and FPS were funded by the Swiss National Fund (SNF; project 3100-0681).

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