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Transitions in cuticular composition across a hybrid zone: historical accident or environmental adaptation?

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Two subspecies of the grasshopper *Chorthippus parallelus* form a hybrid zone in the Pyrenees. Transitions across the zone, including changes in mating signals and reproductive isolation have been intensively studied. Cuticular pheromones have been identified as likely mate recognition signals. Since the major role of the cuticle is in waterproofing, environmental adaptation of cuticular composition has the interesting potential to generate assortative mating as an incidental by-product. We describe the pattern of variation in cuticular hydrocarbon blend in four transects through the hybrid zone. We find no evidence for a previously observed displaced cline in one blend component. There were differences between subspecies but these varied among transects and were small compared with variation between transects. We examined environmental variation within one transect and found a correlation between vegetation and cuticular composition, suggesting that environment influences the constitution of the cuticle, and hence natural selection may interact with mating signals in this species. © 2003 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2003, **78**, 193–201.

ADDITIONAL KEYWORDS: *Chorthippus parallelus* – cuticular hydrocarbons – cuticular lipids – grasshopper – hybrid zone – Orthoptera – reproductive isolation – sensory drive – speciation.

INTRODUCTION

Hybrid zones occur when genetically distinct groups of individuals meet and mate, resulting in at least some offspring of mixed ancestry (Harrison, 1990). Hybrid zones have been found in many taxa, including amphibians, birds, butterflies, grasshoppers, lizards, and mammals (Barton & Hewitt, 1985, 1989; Hewitt, 1988, 1989; Harrison, 1990). A transect through a hybrid zone will typically reveal coincident clines in allele frequencies or quantitative characters that differ between the two hybridizing taxa. However, there are examples of zones where clines are displaced relative to one another (Barton, 1993). One such example is the hybrid zone in the Pyrenees between subspecies of the meadow grasshopper *Chorthippus parallelus* (Orthoptera: Acrididae). The origin of the *C. parallelus* zone is believed to lie in secondary contact after the last glaciation around 9000 years ago (Hewitt, 1993), so it is expected to show the typical pattern of coincident clines. However, cline centres for morphological traits (Butlin, Ritchie & Hewitt, 1991), loci influencing hybrid male sterility (Virdee & Hewitt, 1994; Butlin, 1998), and a chromosomal marker (Ferris *et al.*, 1993) are significantly displaced from one another and from clines in most other characters. Such displacement is interesting because distinguishing among possible explanations for non-coincidence, such as asymmetrical selection or patchy colonization, can lead to a better understanding of the interactions between the hybridizing taxa.

An additional displaced cline, this time in the blend of cuticular hydrocarbons has recently been reported (Neems & Butlin, 1994). In a transect at Col de la Quillane in the eastern Pyrenees, a cline in one male hydrocarbon peak (a single compound or group of related compounds identified by gas chromatography) was displaced by 17 km to the north of the clines for most other characters, into the range of *C. p. parallelus.* Displacement in this character is particularly intriguing because of the potential for cuticular hydrocarbons to function as mating signals and hence to generate assortative mating. The primary

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function of insect cuticular hydrocarbons is in the prevention of desiccation and studies of dipterans *Drosophila pseudoobscura* (Toolson & Kuper-Simbron, 1989) and *Phlebotomus argentipes* (Kamhawi *et al.*, 1992) have shown that cuticular hydrocarbons evolve in response to humidity. However, they have also been shown to function in mate recognition in a variety of insect species (Cobb & Ferveur, 1996a). There is indirect evidence suggesting that the cuticular hydrocarbons of *Chorthippus parallelus* function in the mating system as contact chemical signals, from ablation experiments (Ritchie, 1990), hydrocarbon removal experiments (Buckley, 1998; Butlin, 1998) and from the relationship between female cuticular composition and pattern of assortative mating between populations (Tregenza *et al.*, 2000).

The aim of this study was to examine in more detail the pattern of variation in cuticular hydrocarbons through the Col de la Quillane and in three additional transects in other areas of the Pyrenees. Specifically, we sought to confirm the presence of a displaced cline and to investigate the possibility that variation in cuticular composition may be the result of adaptation to local environmental conditions.

MATERIAL AND METHODS

FIELD COLLECTIONS

Fifty-seven sites were studied in the summers of 1994, 1995, and 1996 in transects at four cols in the Pyrenees (Fig. 1). Eleven sites were sampled at Col du Pourtalet (western Pyrenees: nine sites for females, Fig. 2), 14 sites at Col du Beret (central Pyrenees: Fig. 3), 12 at Col du Tosas (eastern Pyrenees: 10 sites for females, Fig. 4) and 20 at Col de la Quillane (Fig. 5). The first three of these transects traverse ridges from one valley to another but are orientated differently from the Col de la Quillane transect and from each other. In Col du Pourtalet the direction is the most similar to Col de la Quillane, as it is a near linear transect running from north to south. Col du Beret is more complex topographically, and the transect runs from north-west to south-east in two curves. The Col du Tosas transect also runs approximately from north-west to south-east but differs from the other cols in that the transition between subspecies occurs in the opposite direction. In this area, there is an enclave of *C. p. parallelus* populations to the south of the main Pyrenean mountain chain (Butlin *et al.*, 1992). The transect through the Col de la Quillane differs from the other three, which cross the transition between the two subspecies. In the Col de Quillane we only examined an area within the range of *C. p. parallelus.* Ten sites were chosen in the immediate area where a displacement in cuticular component peak C was observed by Neems & Butlin (1994), while five sites were located north and a further five south of this area. In all transects, hind legs were collected from individuals of both sexes giving a total of 796 legs from males and 758 from females (7–24 and 6–25 legs per site in males and females, respectively). Legs were stored individually in gelatine capsules over desiccant at 0°C in the field and at –20°C on return to the laboratory.

CUTICULAR HYDROCARBONS AND STRIDULATORY PEGS

Cuticular hydrocarbons were extracted from individual legs and analysed using the methods of Neems &

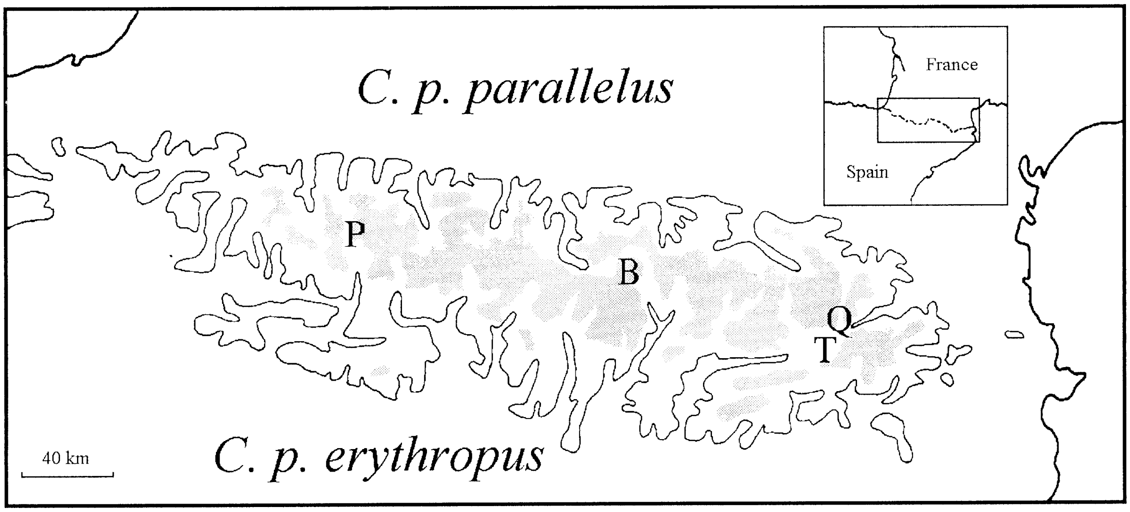

Figure 1. Map showing location of transects.

Butlin (1995). Briefly, gas chromatography of a hexane extract from a single leg produces a chromatogram in which 13 peaks could be identified reliably, 11 of which were included in the analysis for consistency with Neems & Butlin (1994, 1995). Each peak consists of one, or a few related lipids, which may not all be hydrocarbons, although this is expected to be the dominant class of compounds (Grunshaw *et al*., 1990). For the purposes of our analysis it is not necessary to identify the specific compounds in each peak, although this is an obvious area for future research. The area under each peak is proportional to the amount of each compound (or group of compounds) in the extract.

The number of pegs on the stridulatory file of the hind femur of each male was counted using a light microscope. This has previously been shown to be the best single trait with which to distinguish between the two subspecies of *C. parallelus* (Butlin & Hewitt, 1985). For the purposes of the analyses in this paper, samples with mean peg number greater than 122.5 were considered to be *C. p. erythropus* (the Spanish subspecies) and samples with mean less than 122.5 were considered to be *C. p. parallelus* (the northern European subspecies). Only three sites had mean peg numbers between 120 and 125.

VEGETATION AT COL DE LA QUILLANE

The environment was characterized using vegetation analysis. *C. parallelus* is always found in meadow habitats but these range from marshy to dry and from closely grazed to tall grass with scrub. Quadrat analysis of vegetation cover was used as an integrated measure of medium term environmental conditions. It is possible that food plants have a direct effect on cuticular hydrocarbon composition through use of plant secondary compounds in their biosynthesis, but this is not usually the case (Blomquist & Jackson, 1979).

Vegetation was sampled at all 20 grasshopper collection sites. At each site, five 1 m^2 quadrats were chosen at random within the grasshopper sampling area of about 20 m × 20 m. In each quadrat, the plants present were identified to species level where possible (Fitter, Fitter & Farrer, 1984; Grey-Wilson & Blamey, 1995; Grey-Wilson, 1995). Percentage cover was scored by eye for each species to the nearest 5%, allowing a total cover of greater than 100% where there was more than one layer. Vegetation was identified and scored by SHB at all sites within a period of 21 days. Sites were not sampled in either a north–south or south-north sequence. Sixty-seven plant species were present only in a single quadrat and were not included in the analysis. Of the remaining 119 species all but 11 were identified to species level.

STATISTICAL ANALYSIS

All analyses were carried out using Genstat5, Release 3 (Genstat5, 1993). Separate analyses were carried out for males and females, which are known to differ in cuticular composition (Tregenza *et al*., 2000). We used univariate analysis of variance to test for differences in individual peaks (using $\log_{10}$ [proportion of the total hydrocarbon extract in a specific peak]). Multivariate analysis of variance (MANOVA) was used to analyse overall patterns of variation in vegetation and hydrocarbon blend (using log contrasts ($\log_{10}$ [proportion of peak X/proportion of reference peak]: (Aitchison, 1986)). Discriminant function analyses were used to reduce the dimensionality of both hydrocarbon and vegetation data sets.

We used two approaches to examine the relationship between cuticular hydrocarbon blend and habitat at Col de la Quillane. First, we examined correlations between site mean discriminant scores derived independently from the two data sets. Secondly, we calculated between site similarity matrices, using the Euclidean measure of similarity (Genstat5, 1993: 622–628) for each data set, and tested for a correlation between matrices using Mantel tests. To examine the dependence of the overall correlation between these matrices on individual hydrocarbon peaks, plant species, or sample sites we repeated the whole analysis leaving out variables or samples one at a time. We did not use canonical correlation analysis because the units of data in the two sets were different: quadrats vs. individuals. Therefore, some method of summarizing data at the level of sites was needed. If individual variables were summarized at this level, the number of sites was insufficient relative to the number of variables for a meaningful analysis.

RESULTS

PATTERNS OF CUTICULAR HYDROCARBON VARIATION IN RELATION TO HABITAT

Col du Beret, Col du Pourtalet and Col du Tosas

Figures 2–5 show the sites examined in our four transects and also show patterns of variation in peg number and the proportion of cuticular component peak C in males, for comparison with Neems & Butlin (1995) who reported a displaced cline in this character in the Col de la Quillane. At Col du Pourtalet (Fig. 2) and Col du Tosas (Fig. 4), the number of stridulatory pegs increased in a pattern similar to the smooth clines previously described at Col de la Quillane and Col du Pourtalet from approximately 100 pegs in *C. p. parallelus*, to 150 in *C. p. erythropus* (Butlin & Hewitt, 1985a; Butlin *et al*., 1991). A different pattern was observed at Col du Beret (Fig. 3) where a pocket of

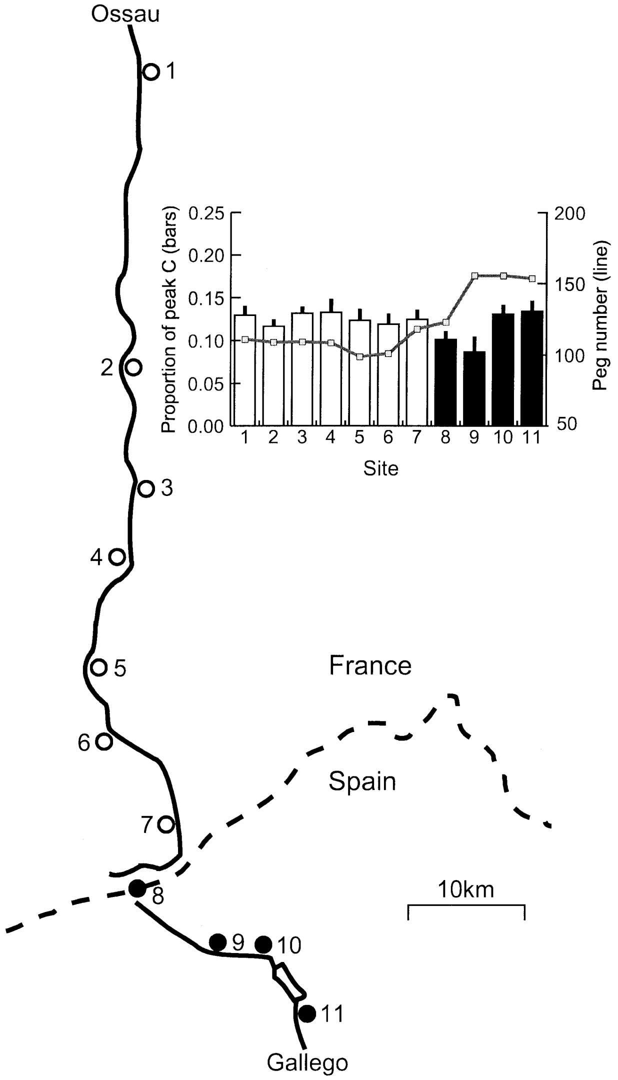

Figure 2. Sample sites at Col du Pourtalet, the proportion of hydrocarbon peak C in males (mean ± SE) and the mean number of stridulatory pegs in males at each site. *C. p. parallelus* sites are indicated by white circles/bars, and *C. p. erythropus* sites are indicated by black circles/bars. The solid lines are rivers and the dashed line is the international border. There are differences between sites within subspecies (males $F_{9,168} = 2.1$, $P < 0.05$; females $F_{7,142} = 4.9$, $P < 0.01$), but no differences in proportion of Peak C between subspecies in this Col (males $F_{1,9} = 1.3$, NS; females $F_{1,7} = 1.7$, NS). Multivariate analyses do reveal differences between subspecies (males $F_{10,159} = 29.8$, $P < 0.01$; females $F_{10,133} = 20.8$, $P < 0.01$), and between sites within subspecies (males $F_{90,1089} = 5.7$, $P < 0.01$; females $F_{70,782} = 6.1$, $P < 0.01$).

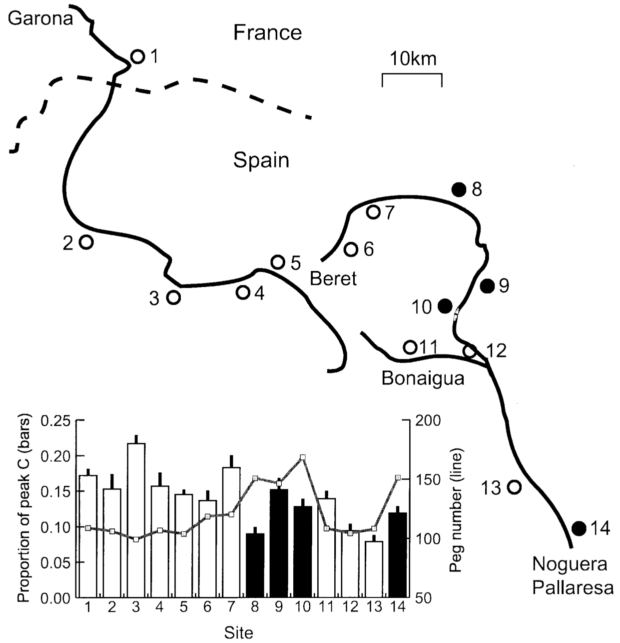

Figure 3. Sample sites at Col du Beret presented as in Fig. 2. There are differences between sites within subspecies (males $F_{12,153} = 10.7$, $P < 0.01$; females $F_{12,162} = 13.7$, $P < 0.01$), but no differences in proportion of Peak C between subspecies in this Col (males $F_{1,12} = 0.5$, NS; females $F_{1,12} = 0.4$, NS). Multivariate analyses do reveal differences between subspecies (males $F_{10,144} = 14.3$, $P < 0.01$; females $F_{10,153} = 3.3$, $P < 0.01$), and between sites within subspecies (males $F_{120,1132} = 7.0$, $P < 0.01$; females $F_{120,1202} = 7.6$, $P < 0.01$).

C. p. parallelus exists at sites 11–13, south of the centre of the cline in peg number, which is positioned, as expected, at the Col du Beret itself (sites 6 and 7). This pattern is consistent with a previous observation of an exceptional low mean peg number for a collection near site 13 (Butlin *et al.*, 1992).

The steep, displaced cline in proportion of peak C, expected from the previous observations at Col de la Quillane (Neems & Butlin, 1994), was not observed in any of these three transects (male data shown in Figs 2–4). The proportion of peak C did differ significantly among sites within subspecies in all three transects (see legends to Figs 2–4). However, there were no significant differences in the proportion of peak C between subspecies in either sex in any of the three transects (maximum $F_{1,7} = 1.7$, N.S.). In all three sites males had a greater proportion of peak C than females as observed by Neems & Butlin (1995), and as in five out of six European populations examined by Tregenza *et al.* (2000).

Although the pattern of cuticular hydrocarbon variation did not conform closely to that expected from previous data, significant differences between subspecies in the 11-component blend did exist within each of the three transects (see legends to Figs 2–4). Analysing data from all three transects together reveals sig-

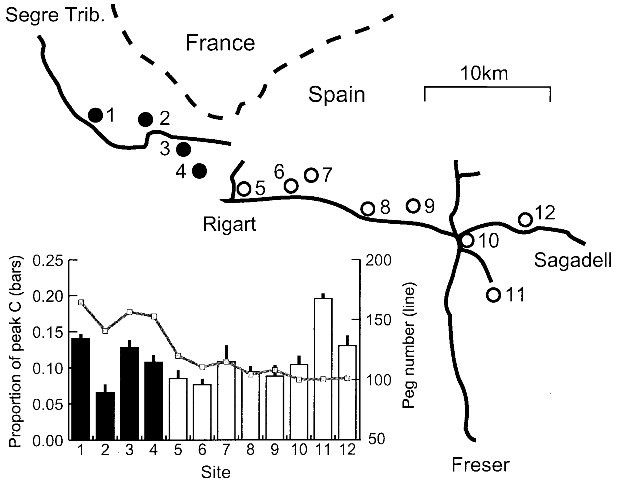

Figure 4. Sample sites at Col du Tosas, presented as in Fig. 2. There are differences between sites within subspecies (males $F_{10,152} = 9.6$, $P < 0.01$; females $F_{8,103} = 5.8$, $P < 0.01$), but no differences in proportion of Peak C between subspecies in this Col (males $F_{1,10} = 0.3$, NS; females $F_{1,8} = 1.6$, NS). Multivariate analyses do reveal differences between subspecies (males $F_{10,143} = 22.4$, $P < 0.01$; females $F_{10,94} = 18.4$, $P < 0.01$), and between sites within subspecies (males $F_{100,1036} = 7.0$, $P < 0.01$; females $F_{80,605} = 4.1$, $P < 0.01$).

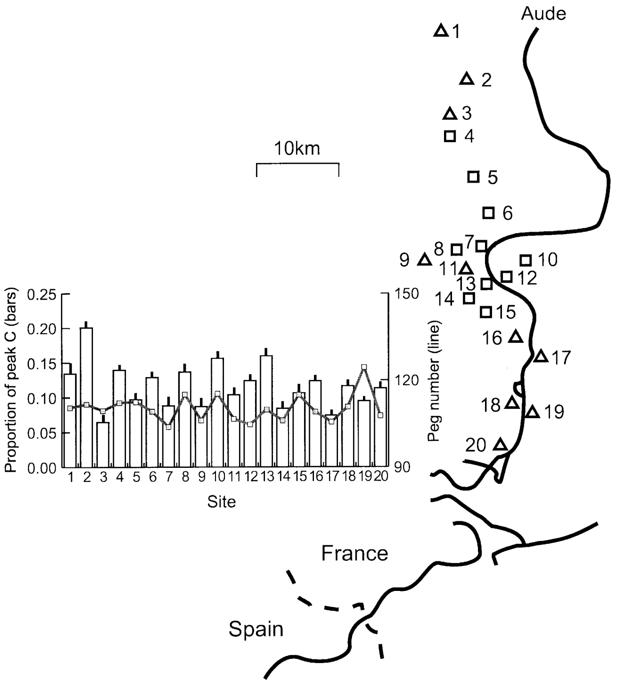

Figure 5. Sample sites at Col de la Quillane, presented as in Fig. 2. This transect differs from those shown in Figs 1–3, since we concentrated on the area within the range of *C. p. parallelus* where a displaced cline had previously been reported (Neems & Butlin, 1995). Triangles/squares indicate sites with high/low scores on male cuticular composition discriminant axis 1 (see Fig. 6).

nificant differences between cols (males $F_{20,928} = 57.0$, $P < 0.01$; females $F_{20,796} = 38.9$, $P < 0.01$) and between subspecies (males $F_{10,464} = 29.3$, $P < 0.01$; females $F_{10.398} = 14.6$, $P < 0.01$), and also a significant subspecies by col interaction (males $F_{20,928} = 20.41$, $P < 0.01$; females $F_{20,796} = 12.54$, $P < 0.01$) indicating that patterns of hydrocarbon variation differ between these independent areas.

Col de la Quillane

The pattern of male cuticular hydrocarbons described by Neems & Butlin (1994) – a cline in peak C which was displaced 17 km to the north of the hybrid zone centre (which is approximately 3 km south of site 20) into the range of *C. p. parallelus* – was not reproduced in our data (Fig. 5). The expected steep cline explained about 50% of among site variation in the Neems & Butlin (1994) data set and would have split the 20 sites into two groups (between sites 8 and 9). However, in our data the effect of this grouping was not significant ($F_{1,18} = 1.90$, $P > 0.1$). Indeed, there is no evidence of a clinal transition in our data set, although there were differences in overall male and female cuticular hydrocarbon blends among the 20 sites (MANOVA: males, $F_{190,2308} = 8.49$, $P < 0.01$; females, $F_{190,2593} = 7.35$, $P < 0.01$).

Discriminant function analysis was used to reduce the dimensionality of the vegetation and hydrocarbon data. Using percentage cover of plant species per quadrat with sites as the grouping function, the first five axes explained 81.9% of the between site variation. A similar proportion of variation in cuticular composition (males 83.2%, females 81.5%) was explained by the first three axes of a discriminant analysis of these data. The first discriminant axis for cuticular composition defines two distinct groups of sites, especially for males (Fig. 6), but the geographical distribution of these groups follows no obvious pattern (Fig. 5). Male and female axes represent very similar aspects of blend variation (correlation between loadings of individual chromatogram peak areas on the male and female axes, $r = 0.92$). Furthermore, this axis of blend variation appears to be significant throughout the Pyrenean populations of *C. parallelus*. Both male and female axes are closely related to the primary axes resulting from discriminant function analysis of collections from the other three cols together (correlations between loadings: males, $r = 0.71$; females, $r = 0.89$). In all cases, this axis contrasts the first six peaks in the chromatogram (short-chain hydrocarbons) with the last four peaks (longer-chain hydrocarbons). By analogy with *Schisto-*

cerca (also an acridid grasshopper), the hydrocarbons probably fall in the range from 14 to 40 carbon atoms (Grunshaw *et al.*, 1990).

The relationship between cuticular hydrocarbons and habitat was assessed by calculating correlations between the mean values per site for the discriminant scores based on cuticular hydrocarbon blend with the mean values per site for the discriminant scores based on vegetation (Table 1). This revealed a strong positive correlation, consistent across the sexes, between the first discriminant axis for hydrocarbon blend and the fourth axis for vegetation. Although not individually statistically significant, correlations between the third vegetation axis and both the first and second hydrocarbon blend axes were also suggestive and consistent between the sexes.

These axes were further investigated by examining correlations between them and the percentage cover of individual plant species. Of the 10 plant species with the highest absolute correlation coefficients with axis 4, five are associated with wet habitats (*Carex* sp., $r = -0.55$; *Petasites fragrans*, $r = -0.55$; *Epilobium hirsutum*, $r = -0.54$; *Lotus pedunculatus*, $r = -0.50$; and *Cirsium palustre*, $r = -0.43$), and five with a range of habitats (*Urtica dioica*, $r = -0.59$; *Mentha* sp., $r = -0.55$; *Plantago major*, $r = -0.54$; *Galium mollugo*, $r = -0.45$; *Geranium molle*, $r = -0.44$). Apparently, negative scores on the 3rd axis are associated with moist habitats but there are clearly less obvious environmental variables as well. The correlation between hydrocarbon blend and vegetation axis four is consistent with the suggestion (Neems & Butlin, 1995) that high relative abundances of longer-chain compounds (high scores on cuticular composition axis 1) are associated with drier environments because of their better waterproofing properties.

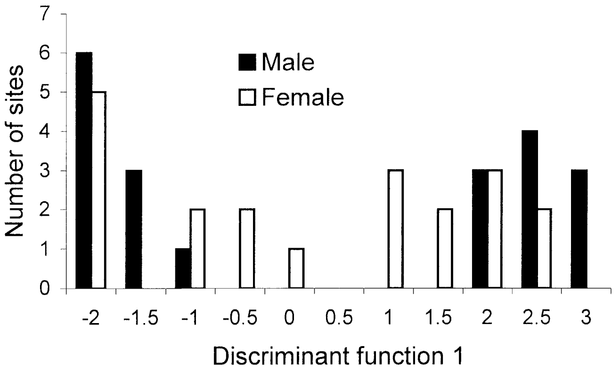

Figure 6. Distribution of Col de la Quillane male and female site mean scores on discriminant function 1 for cuticular composition.

The relationship between cuticular hydrocarbon blend and the environmental variation measured by vegetation was also analysed by calculating correlations between similarity matrices derived from each of the two data sets. This provides a measure of association that uses all of the variables in each data matrix simultaneously. A significant correlation was observed for male cuticular hydrocarbon blend with vegetation measured as percentage cover per quadrat (standardized Mantel coefficient from 10 000 randomizations, $Z = 0.29$, $P = 0.03$) or as mean percentage cover per site ($Z = 0.29$, $P = 0.02$). However, there was no significant correlation between the similarity matrices for female cuticular hydrocarbons and either the percentage cover per quadrat ($Z = 0.12$, $P = 0.26$) or the mean percentage cover per site ($Z = 0.14$, $P = 0.20$). This is surprising given the congruence between results for male and female hydrocarbon blends in the discriminant function analyses (Table 1). It may reflect the generally lower variation among sites in female blends.

Table 1. Correlations between the first three discriminant scores for cuticular hydrocarbons (males and females) and the first five discriminant scores for vegetation (mean values per site) for the 20 sites sampled at Col de la Quillane. $^{**}P < 0.01$ (remains significant in both sexes after Bonferroni correction). Abbreviations: CHA = cuticular hydrocarbon axis; ASV = among site variance explained by axis

			Vegetation axis				
CHA	Sex	ASV	1 (41.3%)	2 (16.9%)	3 (9.4%)	4 (8.8%)	5 (5.4%)
1	M	(59.4%)	0.143	0.073	– 0.337	0.666**	– 0.049
	F	(45.9%)	0.012	0.103	– 0.338	0.726**	– 0.014
2	M	(15.0%)	0.115	0.038	0.352	0.001	0.178
	F	(19.8%)	0.358	0.083	0.273	0.009	0.134
3	M	(8.8%)	– 0.026	– 0.369	– 0.368	0.082	0.318
	F	(15.8%)	– 0.162	0.065	– 0.103	– 0.189	– 0.254

The sensitivity of the correlation between male hydrocarbon blend and vegetation to individual hydrocarbon peaks, plant species, sites or quadrats was examined by recalculating the similarity matrices after removal of variables or samples and repeating the Mantel test. Hydrocarbon peaks were removed one by one from the cuticular hydrocarbon data set, and the effect of removal on the correlation coefficient (with similarities based on percentage plant cover per quadrat) was used to indicate the extent to which the peak contributed to the correlation. No individual peak had a large effect (range of Z-values 0.261–0.326). When individual sites were removed, only one (site 3) had a large effect ($Z = 0.116$ without this site, range after removal of other sites 0.256–0.372). Removal of individual quadrats never reduced the Mantel coefficient below 0.266, confirming that the effect of site 3 is not the result of a single atypical quadrat. Finally, removal of individual plant species showed that the six species with the largest effect (*Potentilla reptans, Rubus fructicosus, Stachys palustris, Filipendula ulmaria, Poa annua,* and *Equisetum* sp.; Z ranging from 0.247 to 0.267) were all more abundant at site 3 than in the rest of the sites. The cover of four of these six species was also strongly correlated with discriminant function axis 3 ($r = -0.47$ to -0.68), indicating that the two methods of analysis have detected similar patterns although none of these species is strongly correlated with axis 4.

Further matrix correlations were used to check whether the significance of the correlation between similarity matrices for male cuticular hydrocarbons and vegetation could be an incidental effect of spatial autocorrelation in both data sets. A matrix for the geographical distances between sites was not significantly correlated with either the similarity matrix for male or female cuticular hydrocarbons ($Z = -0.03$ and -0.01, respectively; $P > 0.1$ in both cases) or the similarity matrix for the percentage cover of plant species per quadrat ($Z = -0.18$, $P > 0.1$).

DISCUSSION

By examining cuticular hydrocarbon blend across three new transects in the Pyrenees we have confirmed the existence of an overall difference between subspecies in the Pyrenees. Our transects differ in many environmental features that might have confounded previous studies in which the two subspecies were sampled from the northern and southern slopes of the mountains. In particular, in the Col du Tosas *C. p. erythropus* occurs to the north-west of *C. p. parallelus* populations. This is also true at Col du Beret, partly because of the complex topography of the area and partly as a result of the enclave of *C. p. parallelus* populations in the Noguera Pallaresa valley (Fig. 3). The formation of this enclave was probably a result of movement of *C. p. parallelus* across the Col du Bonaigua (west of site 11, Fig. 3). This col is higher than the Col du Beret (south of site 6, Fig. 3) and close to the altitudinal limit of the species. There are currently no *C. parallelus* in the steep dry valley from site 11 to site 12, and very few in the high col between sites 4/5 and 11, but there may have been in the past, allowing *C. p. parallelus* to colonize parts of the Noguera Pallaresa valley that were free of *C. p. erythropus*.

There was no evidence that the steep, displaced cline in proportion of peak C reported by Neems & Butlin (1995) was repeated in the three additional transects and it was also not replicated in our new set of samples from Col de la Quillane. There was substantial variation in cuticular composition among sites within the transect, suggesting that the misleading pattern observed previously could have been due simply to chance. Such errors are more likely when all cuticular components (peaks) are analysed separately. Combined with our finding that individual peaks do not dominate the pattern of variation within and among subspecies, this highlights the value of using multivariate analyses of these types of data.

Significant correlations between environment (as measured by vegetation analysis) and cuticular hydrocarbon blend within the Col de la Quillane suggest the existence of an association with the environment. As with all correlations, it is possible that both factors vary clinally for independent reasons, and that the observed correlation is a coincidence, but this seems unlikely. This relationship was significant by both methods of analysis employed: correlation of discriminant functions and comparison of similarity matrices. The principal variation in cuticular composition, throughout the Pyrenees as well as within the Col de la Quillane, seems to be in the relative contributions of short and long chained compounds. However, the environmental variation detected through analysis of plant cover in quadrats is not easy to interpret. It is clear that none of the discriminant axes based on vegetation composition separates the sample sites at Col de la Quillane into two discrete groups even though the cuticular blend variation is discontinuous. This suggests that although the environmental features captured by axis four explain a significant amount of variation in hydrocarbon blend, other factors must also be at work. Although the Mantel correlations between habitat and hydrocarbon blend identify a single site as a major contributor, this site was not identified as unusual on the basis of hydrocarbon data alone and its removal does not affect the strong correlation between hydrocarbon discriminant axis 1 and vegetation axis 4 (without site 3: $r = 0.646$ in males, $r = 0.694$ in females). Overall, the pattern of hydrocarbon variation and the contributions of plant species to vegetation axis 4 suggest that adaptation to variable desiccation stress is at least partly responsible for the observed phenotype–environment association.

The environmental effect on cuticular hydrocarbon blend could be an adaptive, genetic response to long-term habitat differences or it could be an ecophenotypic response to the current conditions because the grasshoppers were sampled directly from the natural habitat. Another study of cuticular hydrocarbon blend variation in populations of *C. parallelus* from across Europe detected significant genetic variation in hydrocarbon blend with all individuals reared under standard conditions in the laboratory (Tregenza *et al*., 2000). In other systems, both types of adaptation have been observed. Ecophenotypic control occurs in *Drosophila mojavensis* with an increase in long-chain relative to short-chain hydrocarbons in flies reared at 34°C rather than 17°C (Markow & Toolson, 1990). Temperature during the pupal stage influences this ratio in *D. pseudoobscura* (Toolson, 1982) but, in this species, a progressive change in hydrocarbon blend across generations in response to humid laboratory culture conditions suggests genetically based adaptation (Toolson & Kuper-Simbron, 1989). A similar change has been observed in *Phlebotomus argentipes* (Kamhawi *et al*. (1992). Genetic control has been demonstrated directly in *Drosophila melanogaster* and its sibling species (Cobb & Ferveur, 1996a) and in the grasshopper *Melanoplus sanguinipes* (Gibbs, Mousseau & Crowe, 1991). In both *Melanoplus sanguinipes* and *Schistocerca shoshone* associations between hydrocarbon blend and environmental temperature have been detected (Gibbs & Mousseau, 1994; Chapman, Espelie & Sword, 1995).

Cuticular hydrocarbon blend variation among European populations of *C. parallelus* is associated with variation in the levels of assortative mating between populations (Tregenza *et al*., 2000). Combined with behavioural studies of male response to dead females with and without hydrocarbons (Buckley, 1998; Butlin, 1998), and ablation experiments (Ritchie, 1990), this indicates a role for cuticular hydrocarbon blend in mate choice, as in other insects (Cobb & Ferveur, 1996b). Studies of premating isolation between populations of *D. mojavensis* (Etges, 1992; Brazner & Etges, 1993; Stennet & Etges, 1997) reveal substantial effects of larval rearing substrates on cuticular hydrocarbons, and correlated effects on premating isolation. Additionally, selection experiments indicate that adaptation to larval host plants influences premating isolation between lines (Etges, 1998). Cuticular hydrocarbons in *C. parallelus* may provide a further example of an interaction between separate functions of the same trait: signalling and waterproofing. Where populations diverge in response to selection on the waterproofing function, this may incidentally induce assortative mating between populations and evolutionary changes in the signalling system within populations: an example of the process of sensory drive (Endler, 1992) and a potential cause of speciation.

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