Journal of Chemical Ecology, Vol. 26, No. 1, 2000

INTER- AND INTRA-POPULATION EFFECTS OF SEX AND AGE ON EPICUTICULAR COMPOSITION OF MEADOW GRASSHOPPER, *Chorthippus parallelus*

TOM TREGENZA*, STUART H. BUCKLEY,
VICTORIA L. PRITCHARD and ROGER K. BUTLIN

Department of Biology, University of Leeds
Leeds, LS2 9JT, United Kingdom

(Received November 4, 1998; accepted September 6, 1999)

Abstract - We analyze patterns of variation in cuticular lipids across and within 5 populations of the meadow grasshopper *Chorthippus parallelus*. This reveals considerable differences between the sexes and between populations, and differences in the pattern of sexual dimorphism between populations. The presence of sexual dimorphism and the extent of differences between populations suggests that divergence has been driven by sexual selection acting through the cuticle's role as a contact pheromone. However, those lipids which differ most between the sexes are not the same as those which vary the most between populations, suggesting that sexual selection alone is not responsible for driving divergence in cuticular composition. We also examine differences in cuticular composition with adult age, revealing that the proportion of all but one of the 14 lipid classes we identify changes significantly with age in at least one population. Overall the pattern of variation with age is fairly consistent across populations, with the proportion of shorter chain compounds increasing with age.

Key words - *Chorthippus parallelus*, cuticular hydrocarbons, cuticular lipids, gas chromatography, grasshopper, Orthoptera, pheromones, sexual dimorphism, sexual selection, age.

INTRODUCTION

The insect cuticle is composed of various polymeric structural components and its outer surface contains an array of long-chain compounds, notably lipids (Buckner, 1993). Although the primary role of these components is in preventing

*To whom correspondence should be addressed -gentbt@leeds.ac.uk

0098-0331/00/0100-257$18.00/0

desiccation, in many species they are also used for chemical signalling. Interest in this communication channel has led to much research on cuticular composition and has opened the door to the use of cuticular lipids as tools for taxonomy and for examination of divergence between populations (see Howard, 1993; Buckley et al., 1997). Many insect species are sexually dimorphic for cuticular composition (see Blomquist et al., 1993). However, relatively little attention has been paid to inter-population differences in the pattern of sexual dimorphism. Such variation has been found in *Drosophila melanogaster*, where the identity of the major cuticular hydrocarbon varies with population in females but not in males (Ferveur et al., 1996).

A second important aspect of cuticular composition which has received even less attention is how it changes with age. Differences between the cuticular hydrocarbons of subadult and adult stages have been studied in several insect orders including the Diptera (Goodrich, 1972), Coleoptera (Baker et al., 1979a, b), and Lepidoptera (de Renobales and Blomquist, 1983). However, the effect of age on cuticular composition in adults has been studied in only a few insects, mainly dipterans. Within the Orthoptera, to which our study animal belongs, the only species in which the effect of age has been studied are members of the *Schistocerca americana* complex (Grunshaw et al., 1990). A study of fifth instar larvae, sexually immature adults and mature adults showed that it was necessary to use mature adults to distinguish between five species (adults have an initial period when they will not mate). In *Schistocerca shoshone* there were significant differences in the blend of several *n*-alkanes between immature and mature adults, and mature individuals had a higher proportion of long chain components (Chapman et al., 1995). Although most studies investigating the effect of age on cuticular composition have identified changes, they are not ubiquitous. No age or sex differences were found in the adult cuticular hydrocarbons of two sibling Coleopteran species: *Diabrotica longicornis* and *D. barberi* (Golden et al., 1992) (although the blend of cuticular hydrocarbons does differ before and after the teneral stage).

Cuticular composition in Chorthippus parallelus. *Chorthippus parallelus*, the meadow grasshopper, inhabits grassland throughout Europe, where it is amongst the most common of the Orthoptera. It has become an important model system in the understanding of hybrid zones through study of a zone of contact between two divergent subspecies in the Pyrenees in southern France (Butlin & Hewitt, 1985a,b). The finding that assortative mating between subspecies is not abolished by preventing males from singing (Ritchie, 1990) has led to investigation of the possibility that an additional signalling channel in the form of contact pheromones is being exploited by this species (Buckley, 1998; Butlin, 1998). This in turn has led to examination of the chemical composition of the cuticle.

Neems and Butlin (1994) have shown that males and females of *C. paral-*

lelus differ in epicuticular composition, as expected if the epicuticle has a signalling function. However, it is not clear whether the pattern of sexual dimorphism found in their sites in the Pyrenees is typical for the species as a whole. As well as varying between the sexes, cuticular composition varies between populations across the hybrid zone (Neems and Butlin, 1994, 1995; Buckley, 1998), and between populations from around Europe (Butlin and Tregenza, 1998; Tregenza et al. submitted (a)), suggesting that the pattern of sexual dimorphism might also be subject to geographic variation. Such variation would open the way for future studies examining variation in sexual dimorphism between populations providing insights into the factors associated with signal character divergence, which ultimately will drive speciation.

Understanding the effect of age on cuticular composition in *C. parallelus* is important for a number of reasons. It allows possible confounding effects of age to be taken into account in studies of cuticular composition in this species. Additionally, identification of those compounds that change with age may allow future cross-species comparisons to investigate the role of particular cuticular compounds. For instance, if the same compound changes its representation in the cuticle with age in one species but not another, it may suggest different roles for the chemical in different species. Finally, if changes with age are sufficiently predictable, it may be possible to use cuticular composition to estimate the age of grasshoppers caught in the field.

Our aims were not to identify the particular compounds measured in our analyses, but rather to use quantitative analyses of gas chromatography profiles to compare individuals of different, sex, age and population. It would be useful for future work to know precisely which compound(s) each peak represents but this is not essential for examining the pattern of variation in cuticular composition. Little is known about the ecological roles of specific cuticular components identified in other Orthoptera. At present, identification of individual components would not appreciably improve our ability to interpret the biological significance of population and sex differences in cuticular composition.

METHODS AND MATERIALS

Adult grasshoppers were collected from locations in France, Germany and Spain (see below). They were returned to the laboratory and allowed to lay eggs in wet sand. Egg pods were placed in a cold room at 4°C and after a minimum 4-month winter diapause were removed and reared as described in Kelly-Stebbins & Hewitt (1972) but with *Dactylis glomerata* as a food plant. All individuals were reared under identical conditions, ruling out the possibility that differences in cuticular composition might result from differences in diet or other environmental effects. Males reached sexual maturity at around 2 days after

becoming adult, and females at 3 days. All grasshopper ages are given as days since reaching adulthood. Individuals were sampled with approximately even frequency over the age range 0-35 days old with fewer individuals in the range 35-55 days old. It is difficult to estimate how long adult grasshoppers live in the wild, however, in lowland areas there are typically several weeks at the end of the summer during which only adults can be found (pers. obs.), indicating that adults can survive for similar lengths of time in the wild as they do in captivity. Females lay multiple egg pods, and members of both sexes will readily re-mate throughout their lifetime in the laboratory.

Populations are numbered as in Butlin and Tregenza (1998) where locations are shown on a map of Europe. Populations 1 (44°21'N, 2°3'E) and 2 (45°11'N, 2°13'E) are both from sites in the Massif Central in Southern Central France, 92 km apart, near the villages of Villefranche and Tourniac respectively. Population 3 (48°43'N, 3°59'W) is from Brittany on the Northern French Coast near the town of Roscoff. Population 5 (42°50'N, 4° 29'W) is from Northern Spain 250 km to the west of a hybrid zone between the subspecies occupying the Iberian peninsula (*C. p. erythropus*) and the subspecies found in the rest of Europe (*C. p. parallelus*). Population 6 (49° 42'N, 10°48'E) is from a site near the city of Erlangen in Southern Germany. There are substantial differences between populations in terms of non-coding nuclear DNA (Cooper et al. 1997), mitochondrial DNA (Lunt et al., 1998), mate preference (Tregenza et al., submitted (a)), and male calling song and morphology of both sexes (Butlin and Tregenza, 1998; Tregenza et al., submitted (b)).

Individuals were kept in cylindrical plastic mesh cages, 25cm × 9cm with 0-4 other members of their own sex and population. Grasshoppers were never directly handled, and were moved using aspiration by mouth to avoid contamination of the cuticle. At varying ages, individuals were killed by freezing at -20°C. A hind femur was removed from each individual and stored separately in a gelatine capsule over silica gel at -20°C.

Cuticular hydrocarbons were extracted and analysed using the method of Neems and Butlin (1995). A single leg was immersed for 5 minutes in 500 µl of *n*-hexane (spectrophotometric grade, Aldrich Chemical Company, Gillingham, England). A 2 µl sample of the hydrocarbon extract was then injected into a Varian 3400 gas chromatograph, fitted with a 15 m x 0.32 mm DB1 (Durabond) capillary column with a 0.1 µm film thickness (J &W Scientific, 91 Blue Ravine Road, Folsom, CA 95630-4714, USA) and a flame ionisation detector. Helium was used as a carrier gas at a flow rate of 5 ml/min.

Analysis of the extract was carried out using a column temperature profile which began at 75°C rising immediately after injection to 220°C at a rate of 25°C/min. The temperature then rose to 260°C at a rate of 15°C/min before being held at 300°C for 10 minutes making a total run time of 26.45 minutes. The

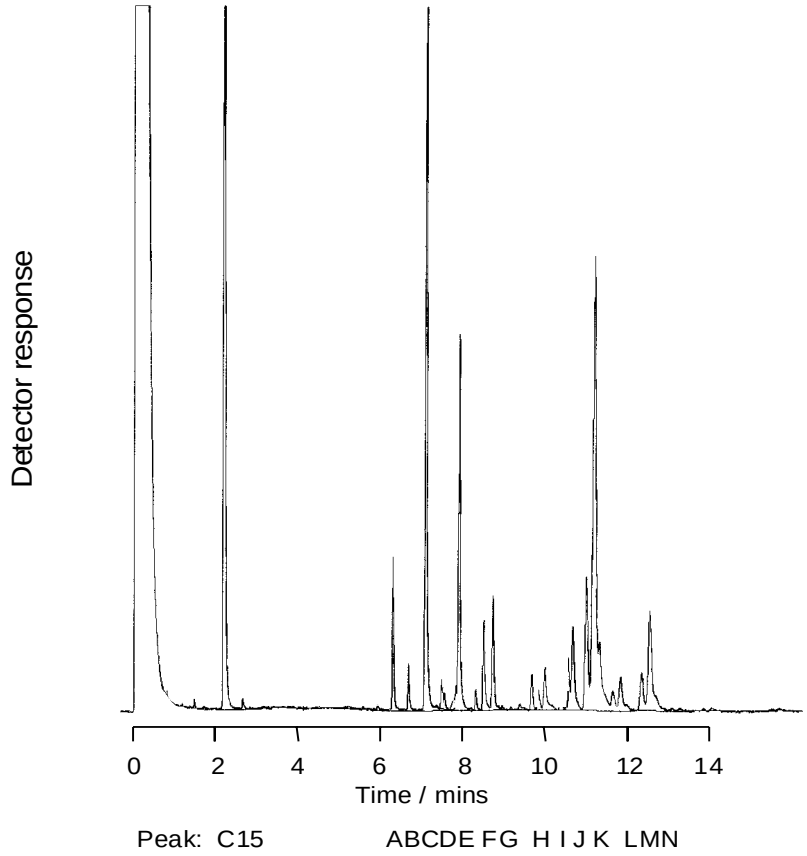


FIG. 1. A typical gas chromatograph trace from a 20 day old male *C. parallelus.* Letters are peak classifications used in analyses. C15 was added to the sample solvent as a standard but was not used in subsequent analyses.

injector also began at 75°C and rose immediately after the sample was introduced to 300°C.

Fourteen peaks were identified (labelled A-N), each of which occurred in >55% of individuals sampled (see Figure 1). All sampled individuals were used in statistical analyses. Varian Star Integrator software was used to calculate the retention time and total area of each peak for subsequent analysis.

RESULTS

Sex and Cuticular Composition. We carried out an initial analysis on the population 1, from southern France, for which we had the greatest sample size (N = 140). A MANOVA of sex versus peak area for the 14 peaks reveals that there

are significant differences between the sexes in the amounts of the various lipids represented by the peaks $F_{14,126} = 8.18$, $P<0.0005$. Furthermore, there are significant differences between the sexes in the total amount of hydrocarbons extracted from the cuticle (measured as mean total peak area) ($t = 2.879$, $df = 139$, $P = 0.0046$; mean total peak area for males (± SE) = 35200 ± 2750, for females = 25600 ± 1810). However, using untransformed peak areas is not the most sensitive way to analyse this data set, since there is some unavoidable experimental error due to variation in total sample quantities injected into the GC. Although this will not produce spurious results in the analyses above since the order of chemical analysis of individuals relative to sex and population was random, it does add noise to the data. Subsequent analyses control for total injection quantity by expressing each peak as the log(proportion of area of all peaks). For multivariate analyses using all peaks, log contrasts are employed (Aitchison 1986); each peak is divided by the area of peak K (the mean largest peak) which is then excluded from the analysis, thereby avoiding inflating degrees of freedom (since the sum of all simple peak proportions must be unity, there are only 13 independent proportions when there are 14 peaks). A MANOVA of sex versus log contrasts of the 14 peaks confirms the significant difference between the sexes: $F_{13,127} = 11.443$, $P<0.0001$.

There are two approaches to determining which peaks best separate the two sexes. The first is to perform univariate tests for differences in the mean values of each peak between males and females. The results of univariate ANOVA's of sex versus log peak proportions for all populations are given in Table 2, indicating which peaks differ more between the sexes than within the sexes. A second approach is to use discriminant function analysis to determine which peaks are most important in creating a function (a mathematical relationship between the peaks), which distinguishes between the sexes. Discriminant analyses produce a number of indications of the importance of each variable to discriminating between groups. One of the most useful of these for evaluating predictor variables is the '*F* to remove' value, which reflects the reduction in predictive power that would result if a variable were removed from the discriminant equation (Tabachnick and Fidell 1989). Table 3 gives *F* to remove values for each peak from separate discriminant analyses of each population, indicating which peaks are most useful in determining the sex of an individual from a particular population.

The two statistical approaches do not give the same results since the discriminant analysis indicates the unique contribution each peak makes to the separation between the two groups, whilst the univariate analysis treats each peak in isolation. If two different peaks are highly correlated with one another within individuals they will individually be less useful in distinguishing between sexes using a discriminant analysis approach, but their lack of independence will not

TABLE 1. PERCENTAGE OF TOTAL PEAK AREAS FOR EACH PEAK ACROSS BOTH SEXES IN ALL POPULATIONS.

	Percentage of total peak area									
Population	1		2		3		5		6	
Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Peak	72	69	36	51	48	49	53	67	72	48
A	3.4%	3.6%	4.6%	4.2%	5.4%	2.8%	4.0%	3.5%	5.5%	3.0%
B	0.7%	0.7%	0.9%	0.9%	1.0%	0.7%	0.8%	0.5%	0.9%	0.7%
C	14.6%	12.5%	16.5%	20.0%	22.5%	19.1%	14.8%	13.8%	20.5%	15.8%
D	0.7%	0.9%	0.9%	1.0%	1.2%	1.2%	1.1%	0.8%	1.1%	1.3%
E	8.9%	12.8%	8.1%	11.6%	8.1%	12.6%	7.8%	8.7%	8.2%	12.6%
F	2.8%	2.0%	2.3%	1.7%	1.1%	2.2%	1.9%	1.5%	0.9%	1.4%
G	2.9%	4.6%	2.5%	4.1%	2.5%	4.2%	2.5%	2.6%	2.2%	3.8%
H	2.2%	3.1%	3.2%	3.0%	3.0%	3.5%	2.8%	3.2%	4.6%	4.9%
I	3.5%	4.4%	3.5%	2.1%	2.7%	1.9%	2.4%	2.8%	3.4%	2.3%
J	7.8%	4.2%	7.3%	5.2%	8.7%	5.8%	9.3%	6.2%	8.9%	6.2%
K	37.7%	35.6%	35.5%	32.3%	31.8%	33.7%	35.4%	39.2%	31.9%	35.4%
L	2.8%	2.6%	2.8%	2.5%	2.6%	2.4%	2.9%	3.1%	2.8%	2.4%
M	2.5%	2.5%	2.4%	2.5%	2.2%	2.2%	3.8%	3.3%	2.0%	2.4%
N	9.5%	10.4%	9.5%	8.9%	7.3%	7.5%	10.6%	10.8%	7.2%	7.8%

TABLE 2. F VALUES FROM UNIVARIATE ANOVA'S FOR DIFFERENCES BETWEEN THE SEXES IN EACH INDIVIDUAL PEAK AS A PROPORTION OF ALL 14 PEAKS.

Population	1	2	3	5	6	All[a]
Peak	141	87	97	120	121	566
A	1.74	0.90	34.07[b]	2.30	37.56[b]	41.15[b]
B	0.39	0.44	13.17[b]	20.02[b]	12.06[b]	27.52[b]
C	8.86[b]	0.61	6.21	0.02	19.05[b]	13.91[b]
D	5.39	3.18	0.02	8.47[b]	4.45	1.70
E	30.03[b]	12.01[b]	74.07[b]	1.92	79.86[b]	98.60[b]
F	2.48	0.49	6.82	0.91	13.64[b]	16.60[b]
G	43.10[b]	33.75[b]	63.59[b]	1.46	59.01[b]	142.19[b]
H	3.60	1.00	4.47	2.20	1.78	8.74[b]
I	4.57	22.23[b]	2.07	1.12	10.90	8.04[b]
J	59.62[b]	11.28[b]	32.08[b]	31.92[b]	36.53[b]	141.00[b]
K	3.55	4.17	0.83	10.24[b]	6.58	0.59
L	1.28	1.26	2.30	0.86	3.76	3.27
M	0.09	0.42	0.01	3.09	9.09[b]	2.50
N	5.30	2.39	0.02	0.13	2.24	3.93

[a]*F* values from a single ANOVA combining all populations.
[b]Peaks differ significantly between the sexes at the Bonferroni adjusted minimum $P = 0.0036$ (adjusted from $P = 0.05$).

affect the univariate difference in each peak between the sexes. The opposite of this effect can be seen in those peaks which do not appear to differ using univariate analyses but which nevertheless contribute to the discriminant function separating the sexes. This type of difference is possible if two peaks are related to one another in different ways in the two sexes, and there is higher variance within these peaks than there is in the relationship between them. If two peaks are positively correlated in males and negatively correlated in females, individually they may not appear to differ between the two, but together they provide a powerful tool for distinguishing between the sexes.

The compounds which can be used to discriminate between males and females are equally variable in both sexes: jack-knife reallocation (which uses the discriminant function to determine the sex of each individual in turn) correctly classified a mean of 82% of both sexes across the 5 populations (Table 4). This indicates that for components which differ between the sexes, it is not the case that individuals of one sex are very similar whilst the other sex has considerable variation. A discriminant analysis by sex using all the populations together produces a function which is less reliable than the mean from analyses of the populations separately ('All' column, Table 4) despite the data set being much larger, indicating that there are differences between populations in the pattern of sexual dimorphism.

Population and cuticular composition. A MANOVA of population and

TABLE 3. '*F* TO REMOVE' VALUE FOR EACH PEAK FROM DISCRIMINANT FUNCTION ANALYSES BY SEX OF EACH POPULATION.

Population	1	2	3	5	6	All[a]
Peak	141	87	97	120	121	566
A	0.37	1.01	0.56	0.21	0.18	11.89[b]
B	1.04	0.33	0	6.47	0.39	7.43
C	1.89	5.92	0.27	8.38[b]	0.76	13.93[b]
D	1.8	0.55	0.02	0.03	0.05	3.53
E	2.24	2.89	3.78[b]	0.03	3.23[b]	1.48
F	0.86	0.53	2.8	0.35	0.03	0.06
G	7.6	19.91[b]	9.19[b]	5.78	2.32	31.73[b]
H	8.16[b]	0.26	1.12	3.4	0.07	0.82
I	13.09[b]	13.56[b]	0	3.35	0.96	6.66
J	48.19[b]	17.2[b]	6.79[b]	20.45[b]	10.91[b]	111.76[b]
K	8.36	11.29[b]	0.4	3.46	8.7	20.34[b]
L	0.27	5.91	0.69	8.54[b]	0.84	1.23
M	1.59	0	0.35	1.41	10.32[b]	4.30
N	1.23	3.84	0.11	0.69	0.16	1.58

[a] *F* values from a single ANOVA combining all populations.
[b] Peaks differ significantly between the sexes at the Bonferroni adjusted minimum $P = 0.0036$ (adjusted from $P = 0.05$).

TABLE 4. RESULTS OF JACKKNIFE REALLOCATION FROM DISCRIMINANT FUNCTION ANALYSIS[a]

Population	1	2	3	5	6	Mean[b]	All[c]
Percentage correctly classified							
Male	92%	83%	90%	65%	79%	82%	80%
Female	88%	75%	84%	74%	91%	83%	77%
Total	90%	78%	87%	69%	86%	82%	79%

[a]This shows the number of individuals correctly classified to their sex using GC results from the 5 different populations.
[b]Mean percentage correctly classified across all populations, weighted according to the number of individuals sampled in each population.
[c]Results from a discriminant analysis using all populations together.

sex versus log contrast peak proportions indicates that there are significant differences between populations: $F_{52,2109} = 10.91$, $P<0.0001$. There are also significant interactions between population and sex: $F_{52,2109} = 2.85$, $P<0.0001$, indicating that the pattern of differences between the sexes varies between populations. Samples from different populations had similar mean ages (ANOVA

TABLE 5. *F* VALUES FROM UNIVARIATE ANOVA'S FOR EACH PEAK INDEPENDENTLY.

Peak	Male 282	Female 284
A	9.39[a]	1.23
B	9.80[a]	6.51[a]
C	13.13[a]	7.67[a]
D	6.22[a]	5.50[a]
E	0.37	14.81[a]
F	0.87	0.41
G	3.75[a]	21.96[a]
H	4.99[a]	5.47[a]
I	8.44[a]	18.11[a]
J	4.13[a]	9.40[a]
K	9.79[a]	6.36[a]
L	0.45	5.16[a]
M	19.22[a]	10.41[a]
N	30.84[a]	18.13[a]

[a]Differ significantly between populations at the Bonferroni adjusted minimum $P = 0.0036$ (adjusted from $P = 0.05$).

of population versus age $F_{4,556} = 1.15$, $P = 0.33$) and age ranges (Bartlett's chi square test for homogeneity of variances $\chi^2 = 7.99$, $df = 4$, $P = 0.09$), indicating that differences between populations are not the result of age differences due to sampling error. To investigate whether the same peaks differ with population as differ with sex, we need to gain a picture of which peaks vary most between populations. Table 5 shows the results of univariate ANOVA's testing the significance of differences between the 5 populations in each peak. Significant F values indicate that there are differences between populations in the log(proportion) of a particular peak calculated separately for each sex. Because populations 1 and 2 come from very similar environments in southern France, we also conducted a separate MANOVA to look for differences in the pattern of sexual dimorphism between them. This reveals substantial differences, reflected in the interaction between sex and population in the MANOVA ($F_{13, 212} = 4.341$ $P < 0.0001$).

Age and Cuticular Composition Because of the clear differences between populations in cuticular composition, we initially consider only the largest population(1) in examining possible changes with age. Using population 1, and adding age as a covariate to a MANOVA of peak log contrasts reveals that cuticular lipids change with age (effect of age, $F_{13,126} = 15.315$, $P < 0.0001$). The nature of these changes can be investigated in more detail using univariate regressions for age versus each peak proportion, and treating the two sexes independently. This analysis is shown in Table 6, which gives the results of linear regressions of the log(proportion) of each peak against age, revealing which peaks change

TABLE 6. UNIVARIATE LINEAR REGRESSIONS ON EFFECT OF AGE ON LOG(PEAK PROPORTIONS) FOR THE TWO SEXES IN POPULATION 1.

	Males			Females		
Peak	r^2	t	slope[a]	r^2	t	slope[a]
A	0.01	0.67		0.00	0.55	
B	0.07	2.31		0.04	1.66	
C	0.10	2.76		0.49	8.01[b]	0.028
D	0.01	1.01		0.02	1.20	
E	0.01	0.82		0.01	0.71	
F	0.42	7.04[b]	-0.036	0.39	6.59[b]	-0.028
G	0.15	3.47[b]	-0.008	0.14	3.30[b]	-0.006
H	0.35	6.14[b]	-0.022	0.21	4.21[b]	-0.020
I	0.41	6.93[b]	0.008	0.00	0.01	
J	0.15	3.53[b]	0.007	0.05	1.85	
K	0.02	1.10		0.06	2.05	
L	0.03	1.54		0.03	1.50	
M	0.16	3.58[b]	0.007	0.01	0.83	
N	0.04	1.78		0.01	0.78	
Total[c]	0.58	9.84[b]	1728	0.29	5.29[b]	875

[a] Only the slopes of significant regressions are indicated: a positive slope indicates that the peak increases with age.

[b] Significant variation in peak log(proportion) with age, using the Bonferroni adjusted minimum $P = 0.0036$ (adjusted from $P = 0.05$).

[c] Results of a regression of total peak area versus age, again for each sex independently. The slope is the average increase in total area of all peaks per day from adult eclosion.

their proportions significantly with age, and whether they tend to become more or less abundant.

The total amount of compounds available for extraction from the cuticle increased with age in both sexes, with the increase occurring more steeply in males. Various non-linear models were applied to the data, but none provided a better fit than linear. Nevertheless, linear regression can only approximate the pattern of changes in total lipids extracted and in the relative proportions of the 14 identified components. This is exemplified by the fact that the regressions of age versus total lipids extracted give -249 as the intercept at age 0 for males and 8574 as the intercept for females. Since males cannot have negative quantities of lipids at eclosion this indicates that the pattern of increase in total hydrocarbons is somewhat non-linear, and also that males may start with lower quantities of cuticular lipids than females but catch up by around 20 days of age.

Regardless of the exact shape of changes in cuticular composition with age, it is clear from Table 6 that there are large differences between peaks in how they vary with age. There is a broadly similar pattern in peak variation in both sexes, with the proportion of peaks F, G, and H decreasing with age and peak C

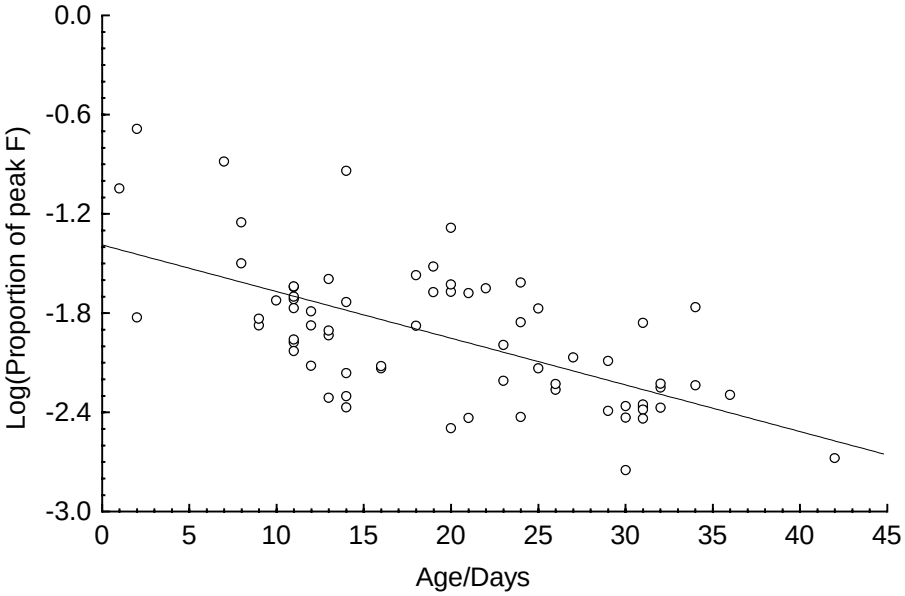
increasing, albeit non-significantly in males. This general similarity between the sexes in the effect of age is borne out by the finding that adding age as a covariate to the MANOVA of sex versus peak log contrasts does not increase the *F* value for the difference between the sexes (MANOVA without age covariate, $F_{13,127}$ = 11.44; with age covariate $F_{13,126}$ = 11.38). However, it is noteworthy that in males, the proportions of peaks I, J, and M increase significantly with age, whilst they do not do so in females. The pattern of variation in log(peak proportions) for 3 peaks is shown in Figures 2-4.

Consistency of Age Effects Across Populations. In order to investigate whether the changes in peak proportions with age seen in population 1 are repeated across other populations, we conducted regressions of peak log (proportions) against age for males and females separately in all populations. Figure 5 gives a summary of this analysis, showing the number of populations with significant regression slopes, either positive or negative for each peak. Significance is assessed at $P<0.05$ without Bonferroni adjustment since we are interested in the general pattern across populations. It is clear from this analysis that cuticular composition changes in broadly similar fashion across populations. Only one peak, G, increases with age in one population whilst decreasing in another. The remaining 67 significant increases or decreases are all in the same direction across populations and across sexes.

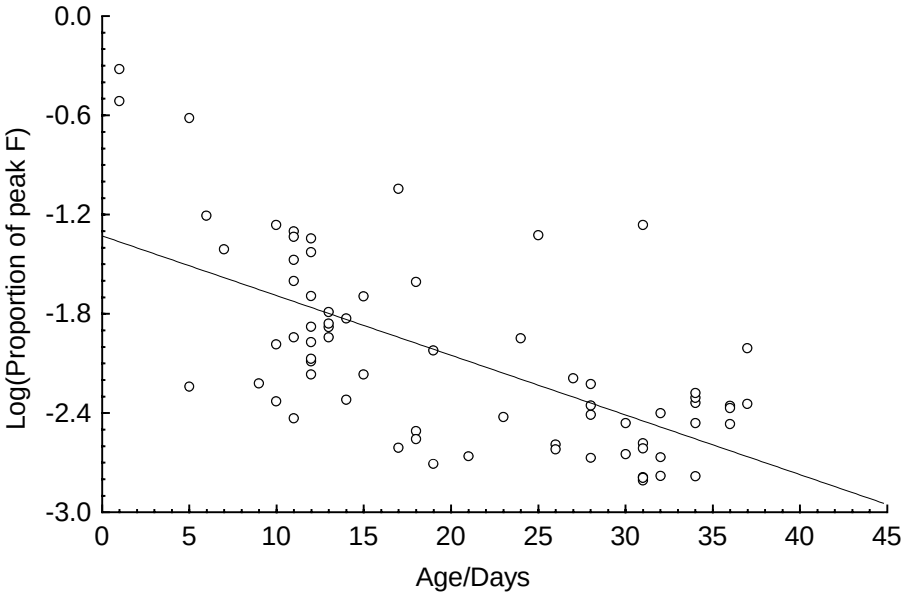
DISCUSSION

Sex, Population and Cuticular Composition. There are significant differences in cuticular composition in terms of the relative proportions of extracted lipids between males and females in all the populations studied, and between the populations themselves. Furthermore, significantly greater quantities of lipids are extracted from males despite their hind femurs being considerably smaller than those of females [84% as long and 84% as wide at the widest point (n = 832) (unpublished data)]. None of the extracted components is unique to either sex or to any particular population, but nearly all of the 14 peaks found in our chromatographs differ between the sexes in at least one population (Figure 2).

There are two classes of hypothesis likely to explain sexual dimorphism in cuticular composition and quantity of lipids, a) environmental selection or b) sexual selection. The former could drive dimorphism if the two sexes are exposed to different environmental conditions or have different life histories. For instance, females might tend to live lower down in the sward where it is more humid, and hence their cuticular composition may be adapted to lower desiccation stress. Sexual selection could drive differentiation either because the chemical composition of the cuticle is used as a contact pheromonal signal in sexual communication, or because the males' use of their hind femurs to pro-

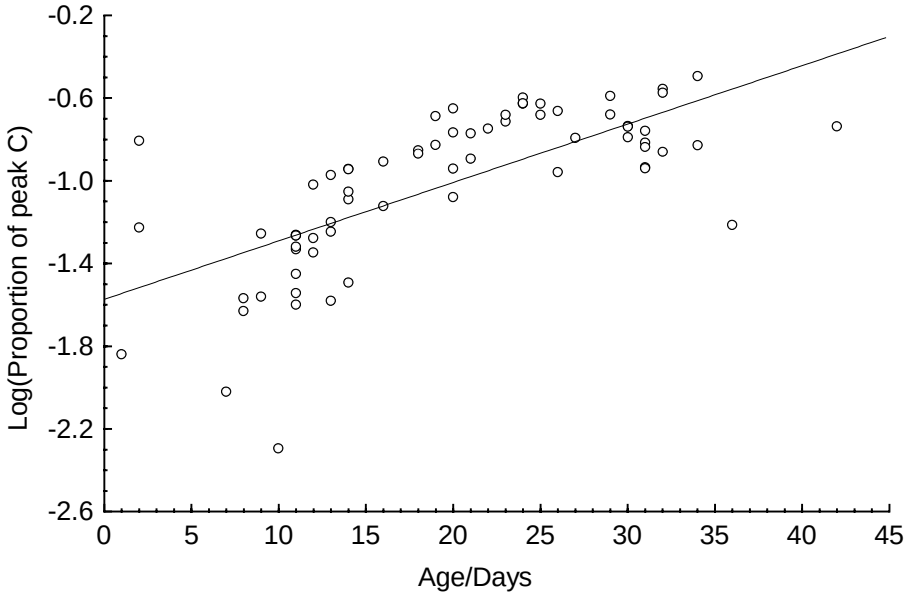


a) Females

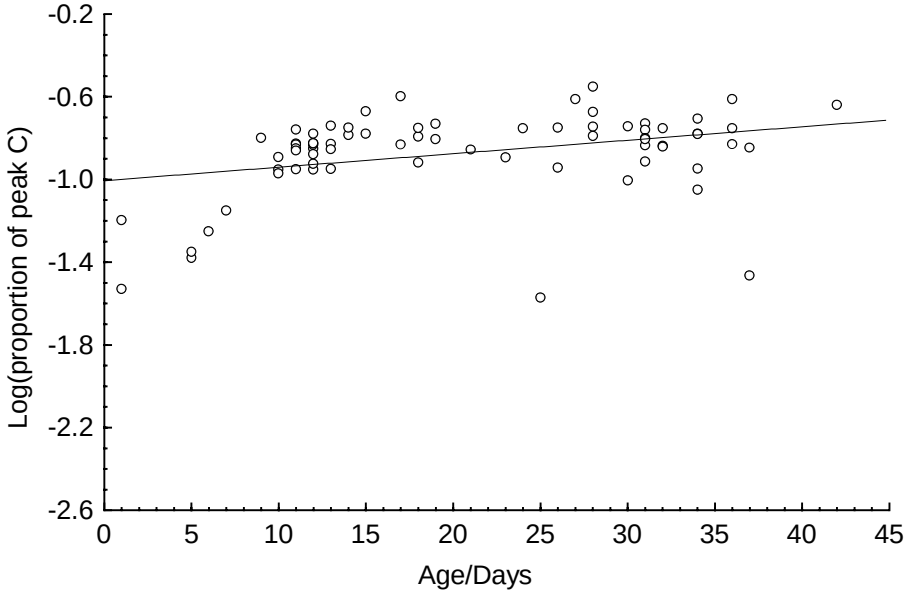


b) Males

FIG. 2. Changes in the log(proportion of peak F) with age in females (a) and males (b) from population 1. Both sexes show a significant decline in the proportion of this lipid. Regressions of total peak area versus age (not shown) reveal that even though the total amount of lipids extracted from the cuticle increases with age, the absolute value of peak F decreases with age: Males (r^2 = 0.07, F = 4.89, P = 0.03, slope = -12.6), Females (r^2 = 0.13, F = 9.80, P = 0.003, slope = -9.8).

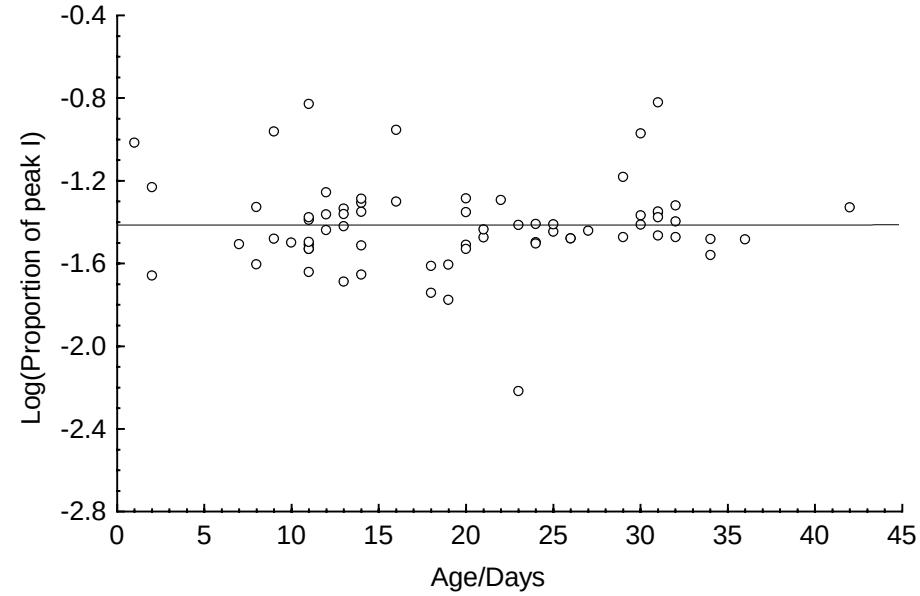


a) Females

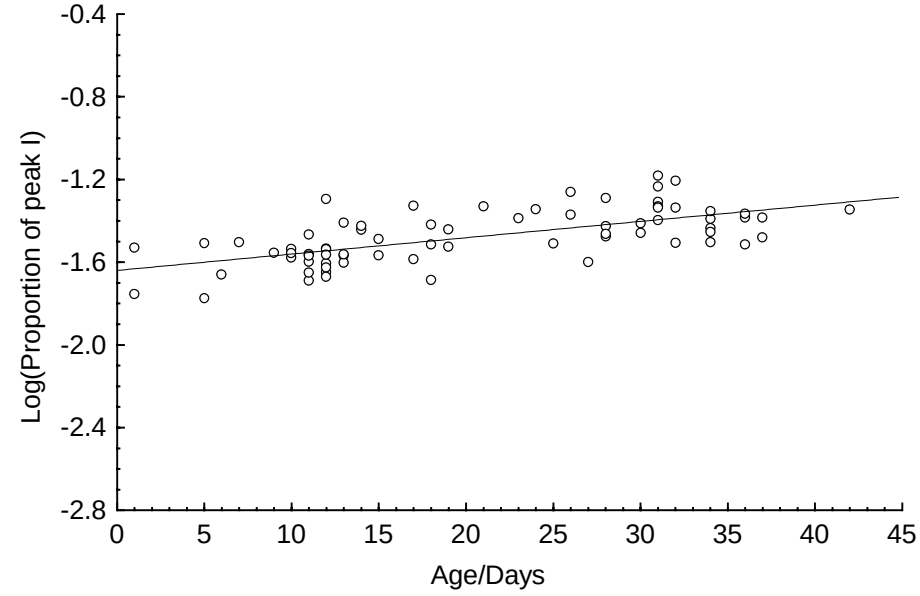


b) Males

FIG. 3. Changes in the log(proportion of peak C) with age in females (a) and males (b) from population 1. Females significantly increase the proportion of this lipid in their cuticle with age, whilst males show less increase. The total amount of peak C in the cuticle (not shown) increases with age in both sexes: Males ($r^2 = 0.49$, $F = 66.1$, $P < 0.001$, slope = 285), Females ($r^2 = 0.38$, $F = 41.9$, $P < 0.001$, slope = 252).



a) Females



b) Males

FIG. 4. Changes in the log(proportion of peak I) with age in females (a) and males (b) from population 1. Males significantly increase the proportion of this lipid in their cuticle with age, whilst females show no change. The total amount of peak I (not shown) increases with age in both sexes: Males ($r^2 = 0.66$, $F = 135$, $P < 0.001$, slope = 78), Females ($r^2 = 0.13$, $F = 10.2$, $P < 0.001$, slope = 32.9).

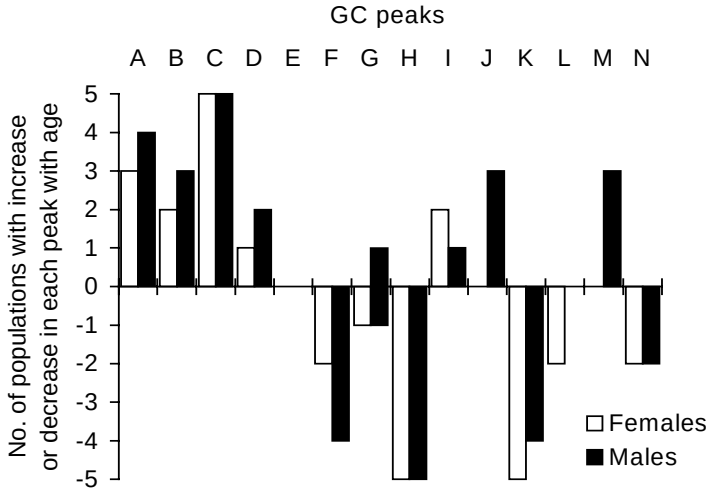


FIG. 5. Graph showing the number of significant regressions (at $P < 0.05$) of peak log (proportion) versus age across the 5 populations studied. Positive values indicate a significant increase in the proportion of a peak, negative values a decrease. For example, the log (proportion) of peak A increases significantly with age in females in 3 populations and in males in 4 populations, and does not decline with age in any population.

duce song (by rubbing a row of pegs on the inside of the leg against the hind wings) may require that part of the cuticle to be particularly abrasion resistant. However, this latter suggestion is militated against by Neems and Butlin's (1995) finding that similar gas chromatography (GC) profiles are obtained from male legs and the rest of the body. In addition, selection may act either directly or indirectly on the cuticle. Little is known about the biosynthetic pathways which produce cuticular compounds and it is possible that selection for other aspects of phenotype could incidentally affect the composition of the cuticle.

We can attempt to distinguish between hypotheses a) and b), by comparing the pattern of sexual dimorphism between populations. The environmental selection hypothesis predicts that the same compounds will tend to differ between the sexes in populations living in broadly similar environments. This is because natural selection pressures imposed by similar environments will also be similar, so the same patterns of differences between the sexes are predicted. Alternatively, if dimorphism is the result of sexual selection on a signal trait, different compounds may be sexually dimorphic in different populations. This is because (subject to constraints) such signals may be to a large extent, arbitrary, allowing them freedom to evolve in differing directions in independent populations. Furthermore, sexually selected traits can potentially evolve very rapidly, both because of the potential for co-inheritance of preferences and traits and because the strength of sexual selection may vary in relation to population density (Kaneshiro 1989).

Based on this reasoning, our results support the hypothesis that variation in cuticular composition is subject to sexual selection. There is considerable variation in the pattern of sex differences between populations living in similar environments. For instance, populations 1 and 2 are both from low altitude, rough grazing land in central France but have significantly different patterns of cuticular hydrocarbon dimorphism. High variation is also reflected both in the strong interaction between sex and population in the MANOVA and in the univariate tests of differences between the sexes in different peaks across populations.

A number of peaks stand out as being consistently different between the sexes. Using the discriminant function approach (Table 3), peak J is the most useful in distinguishing the genders in all populations except 2 and 3, in which peak J is the second most discriminating and peak G is the most discriminating. In an analysis using all populations together ('All' column in Table 3) G and J are the most discriminating by some margin. This pattern is confirmed by the univariate tests (Table 2), which show that J and G are the most consistently different between the sexes, J differing in all populations and G in all populations except 5. The consistency of sexual dimorphism in the lipids G and J is more convincing when one takes into account the extent of variation between populations (Table 5), which may tend to disrupt patterns of sexual differences.

If sexual selection were the only factor driving divergence between populations we might expect the same compounds to differ the most between populations and between sexes - which is not the case. It is possible that compounds which are not sexually dimorphic are used in sexual signalling, but for this to explain the lack of correlation in sexual and population differences would require that most or all of the compounds identified are used in sexual signalling. It is difficult to identify precisely which compounds are used in communication (Howard 1993), but it seems unlikely that so many different chemicals would be needed, given that most of them appear to be able to vary independent of the others. It seems likely that processes other than sexual selection, such as genetic drift, potentially accelerated by founder effects, have a role in causing divergence in cuticular composition between populations (Tregenza et al., submitted (a)).

Age and Cuticular Composition. It is clear from our analysis of the effect of age on cuticular composition that there are big differences between lipids in whether or not they change their representation in the cuticle as the insect ages (Table 6). In population 1 males, 6 out of the 14 peaks change significantly with age, in females, 4 peaks change significantly. Since we are investigating proportions, any increase in one lipid must be balanced by decreases in the representation of others. However, an analysis of total quantity of extracted lipid versus age reveals that both sexes significantly increase the total amounts of lipids in their cuticle as they age, with the increase almost twice as rapid in males.

Figures 1 to 3 show changes in the relative proportions with age of 3 different peaks, F, C and I. These figures illustrate the varying effects of age on different lipids. Peak F declines steadily in its proportional representation in the cuticle of both sexes. This is not simply the result of F remaining constant whilst other lipids increase their representation, since the overall quantity of F in the cuticle also decreases in both sexes. Peak C increases its proportional representation in both sexes (Figure 2), although it does so more rapidly in females than in males. In contrast, peak I increases significantly in males but shows no such increase in females. Such patterns are consistent with work by Gibbs et al. (1995) on the house fly, *Musca domestica*, showing that the melting point of lipids in the female cuticle decreased as the fly aged, whilst those in males showed no such change.

The majority of previous studies examining the effect of age on cuticular composition report that either short chain compounds or long chain compounds vary with age, rather than members of both classes. A distinction has been drawn between the two because short chain hydrocarbons (which typically have short GC retention times) are believed to be predominantly involved in chemical communication, whereas long chain hydrocarbons (which typically have long GC retention times) are believed to be predominantly involved in water retention (Toolson and Kuper-Simbron 1989). In a study of blowflies, *Calliphora vomitoria*, from 0-5 days old, Trabalon et al. (1992) found that younger individuals are characterised by the presence of cuticular hydrocarbons with chain lengths greater than 25 carbon atoms, whereas older individuals had more hydrocarbons of fewer than 25 carbon atoms. As mentioned previously, in the grasshopper *Schistocerca shoshone* mature individuals have higher proportions of long chain components than immatures (Chapman et al., 1995). Similarly, in the *Drosophila melanogaster* complex, immature adults of both sexes are characterised by long chain hydrocarbons (29-35 carbon atoms) which are replaced by short chain hydrocarbons (23-29 carbon atoms) as the individual matures. Simultaneously, the hydrocarbons become species specific and in certain species, sexually dimorphic (Wicker and Jallon, 1995). In *D. virilis* (Doi et al., 1996) and *Fannia pusio* (Muscidae) (Uebel et al., 1978) differences in age and sex are associated with differences in the presence/absence and blend of cuticular hydrocarbons. In *D. virilis*, Z-10-heneicosene, a male hydrocarbon absent at eclosion, appears first at 4 days post eclosion, and at 8 days accounts for more than half of all hydrocarbons. Z-11-pentacosene (a female sex pheromone eliciting male courtship) is present in similar amounts in both sexes at 4 days, but is three times more abundant in females at 8 days (Jackson and Bartlett, 1986).

Somewhat in contrast to these studies, our results show that both long- and short-chain compounds (determined according to their retention times, by analogy with other studies) vary with age (Figure 5). Although the general pattern across populations of *C. parallelus* is for the proportion of shorter chain com-

pounds to increase with age, not all the longer chain compounds show a corresponding decrease. There has been little discussion in the literature of why different size classes of cuticular lipids may decrease or increase with age. In some species changes are associated with the onset of sexual maturity. However, this is clearly not the case in *C. parallelus* since the vast majority of our samples were post-sexual maturity. It seems more likely that changes represent differential accumulation of certain compounds, either through differences in the rate of synthesis of particular components or differences in their rate of loss from the cuticle.

In comparing the effects of age across populations, it is clear that there are broadly similar changes both across populations and across sexes. When there was a significant change in the representation of a particular peak with age, in all but one case (peak G), the log (proportion) of the peak either increased or decreased in members of both sexes in all populations. Some peaks, such as C and H vary with age in all populations, whilst the majority show significant variation in some populations but not others. Similarly, there is significant variation with age in some peaks, such as J, L and M in one sex, but not the other. These sex differences are strikingly consistent: 6 populations show increases in peaks J and M in males, whilst none show increases in females, suggesting that the compound(s) associated with this peak may have different roles in the two sexes. There do not appear to be any striking patterns in those lipids which are affected by age compared to those lipids which separate the sexes.

The magnitude of the changes in certain peaks with age indicates that it might be possible to use gas chromatography to estimate the age of grasshoppers caught in the field. However, it is not known whether there are interactions between environment and cuticular composition such as those found in *D. mojavensis* (Markow and Toolson, 1990) which might make accurate age determination difficult. It would be relatively straightforward to investigate ecophenotypic effects such as temperature in the laboratory, if there were a pressing need for a tool for ageing wild-caught individuals.

Our analyses only consider the relative abundance of lipids with different gas chromatograph retention times. This is a widely used and straightforward approach, but it is worth bearing in mind that there may be aspects of variation in chemical composition which may vary with age which are not revealed by this approach. For instance, in *Fannia pusio,* the cuticular hydrocarbons of both sexes are virtually identical at emergence (Uebel et al., 1978), but with age begin to differ in the position of double bonds in their unsaturated hydrocarbons. These types of changes may not manifest themselves as differences in GC retention times so we are likely to be observing only a portion of changes occurring in cuticular composition with age.

Overall, our results reveal striking variation in the proportional composition of cuticular lipids between both populations and sexes and as individuals age.

This must be taken into account when investigating patterns of variation in cuticular composition. For instance, it would be possible to begin collecting grasshoppers for analysis across a transect in the Pyrenean hybrid zone at the beginning of the season and slowly move through the zone, collecting grasshoppers with progressively older average ages, which in itself might give the impression of a clinal change in cuticular composition.

Acknowledgements - We would like to thank Rachel Neems for assistance with the gas chromatography and two anonymous referees for comments on the manuscript.

REFERENCES

AITCHISON, J. 1986. The Statistical Analysis of Compositional Data. Chapman and Hall, New York.

BAKER, J.E., NELSON, D.R., and FATLAND, C.L. 1979a. Developmental changes in cuticular lipids of the black carpet beetles, *Attagenus megatoma*. Ins. Biochem. 9:335-359.

BAKER, J.E., SUKKESTAD, D.R., NELSON, D.R., and FATLAND, C.L. 1979b. Cuticular lipids of larvae and adults of the cigarette beetle, *Lasioderma serricorne*. Ins. Biochem. 9:603-611.

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

BUCKNER, J.S. 1993. Cuticular polar lipids of insects. pp. 227-270. in D.W. Stanley-Samuelson and D.R. Nelson, (eds.) Insect Lipids. University of Nebraska Press, Lincoln, Nebraska.

BUCKLEY, S.H. 1998. Cuticular hydrocarbons and the *Chorthippus parallelus* hybrid zone. Ph.D. diss. University of Leeds, Leeds.

BUCKLEY, S.H., TREGENZA, T., and BUTLIN, R.K. 1997. Speciation and signal trait genetics. Trends Ecol. Evol. 12:299-301.

BUTLIN, R.K. 1998. What do hybrid zones in general, and the *Chorthippus parallelus* zone in particular, tell us about speciation? pp. 367-378, in D.J. Howard and S. Berlocher (eds.) Endless forms: Species and Speciation. Oxford University Press, New York.

BUTLIN, R.K., and HEWITT, G.M. 1985a. A hybrid zone between *Chorthippus parallelus parallelus* and *Chorthippus parallelus erythropus* (Orthoptera: Acrididae): morphological and electrophoretic characters. Biol. J. Linn. Soc. 26:269-285.

BUTLIN, R.K., and HEWITT, G.M. 1985b. A hybrid zone between *Chorthippus parallelus parallelus* and *Chorthippus parallelus erythropus* (Orthoptera: Acrididae): behavioural characters. Biol. J. Linn. Soc.26: 287-299.

BUTLIN, R.K., and TREGENZA, T. 1998. Levels of genetic polymorphism: marker loci versus quantitative traits. Phil. Trans. Roy. Soc. Lond. B 353:1-12.

CHAPMAN, R.F., ESPELIE K.E., and SWORD, G.A. 1995. Use of cuticular lipids in grasshopper taxonomy: A study of variation in *Schistocerca shoshone* (Thomas). Biochem. System. Ecol. 23:383-395.

COOPER, S.J.B., IBRAHIM, K.M., and HEWITT, G.M. 1995. Postglacial expansion and genome subdivision in the European grasshopper *Chorthippus parallelus*. Mol. Ecol. 4:49-60.

DE RENOBALES, M., and BLOMQUIST, G.J. 1983. A developmental study of the composition and biosynthesis of the cuticular hydrocarbons of *Trichoplusia ni* (Lepidoptera: Noctuidae). Ins. Biochem. 13:493-502.

DOI, M., TOMARU, M., MATSUBAYASHI, H., YAMANOI, K., and OGUMA, Y. 1996. Genetic analysis of *Drosophila virilis* sex pheromone: Genetic mapping of the locus producing Z-(11)-pentacosene. Gen. Res. 68:17-21

FERVEUR, J-F., COBB, M., BOUKELLA, H., and JALLON, J-M. 1996. World-wide variation in *Drosophila melanogaster* sex pheromone: behavioural effects, genetic bases and potential evolutionary consequences. Genetica 97:73-80.

GIBBS, A. KUENZLI, M. and BLOMQUIST, G.J. 1995. Sex- and age-related changes in the biophysical properties of cuticular lipids of the housefly, *Musca domestica*. Arch. Ins. Biochem. Physiol. 29:87-97.

GOLDEN, K.L., MEINKE, L.J., and STANLEY-SAMUELSON, D.W. 1992. Cuticular hydrocarbon discrimination of *Diabrotica* (Coleoptera: Chrysomelidae) sibling species. Ann. Ent. Soc. of Am. 85:561-570.

GOODRICH, B.S. 1972. Cuticular lipids of adult and puparia of the Australian sheep blowfly *Lucilia cuprina* (Wiedemann) J. Lip. Res. 11:1-6.

GRUNSHAW, J.P., GUERMOUCHE, H., GUERMOUCHE, S., JAGO, N.D., JULLIEN, R., KNOWLES, E., and PEREZ, F. 1990. Chemical taxonomic studies of cuticular hydrocarbons in locusts of the *Schistocerca americana* complex (Acrididae: Cyrtacanthacridinae): chemical relationships between new world and old world species. J. Chem. Ecol. 16:2835-2858.

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

JACKSON, L.L., and BARTELT, R.J. 1986. Cuticular hydrocarbons of *Drosophila virilis* - comparison by age and sex. Ins. Biochem. 16:433-39.

KANESHIRO, K.Y. 1989. The dynamics of sexual selection and founder effects in species formation. pp. 279-296 in L.V. Giddings, K.Y. Kaneshiro, and W.W. Anderson (eds) Genetics of speciation and the founder principle. Oxford University Press, Oxford.

KELLY-STEBBINS, A.F., and HEWITT, G.M. 1972. The laboratory breeding of British Gomphocerine grasshoppers (Orthoptera: Acrididae). *Acrida* 1:233-245.

LUNT, D.H., IBRAHIM, K.M., and HEWITT, G.M. 1998. mtDNA phylogeography and post-glacial patterns of subdivision in the meadow grasshopper *Chorthippus parallelus.* Heredity 80:633-641.

MARKOW, T.A., and TOOLSON, E.C. 1990. Temperature effects on epicuticular hydrocarbons and sexual isolation in *Drosophila mojavensis*. pp. 315-331 in J.S.F. Barker, W.T. Starmer and R.J. McIntyre (eds.) Ecological and Evolutionary Genetics of *Drosophila*. Plenum Press, New York.

NEEMS, R.M., and BUTLIN, R.K. 1994. Variation in cuticular hydrocarbons across a hybrid zone in the grasshopper *Chorthippus parallelus*. Proc. Roy. Soc. Lond. B 257:135-140.

NEEMS, R.M., and BUTLIN, R.K. 1995. Divergence in cuticular hydrocarbons between subspecies of the meadow grasshopper *Chorthippus parallelus.* Biol. J. Linn. Soc. 54:139-149.

RITCHIE, M.G. 1990. Are differences in song responsible for assortative mating between subspecies of the grasshopper *Chorthippus parallelus* (Orthoptera: Acrididae)? Anim. Behav. 39:685-691.

TABACHNICK, B.G., and FIDELL, L.S. 1989. Using multivariate statistics. Second Edition. Harper and Row, New York.

TOOLSON, E.C., and KUPER-SIMBRON, R. 1989. Laboratory evolution of epicuticular hydrocarbon composition and cuticular permeability in *Drosophila pseudoobscura*: effects on sexual dimorphism and thermal-acclimation ability. Evolution 43:468-473.

TRABALON, M., CAMPAN, M., CLEMENT, J.L., LANGE, C., and MIQUEL, M.T. 1992. Cuticular hydrocarbons of *Calliphora vomitoria* (Diptera)-relation to age and sex. Gen.

Comp. Endo. 85:208-216.

TREGENZA, T., PRITCHARD, V.L., and BUTLIN, R.K. 2000. Patterns of trait divergence between populations of the Meadow Grasshopper, *Chorthippus parallelus.* Evolution. In Press.

TREGENZA, T., PRITCHARD, V.L., and BUTLIN, R.K. Submitted (b). The origins of premating reproductive isolation: testing hypotheses in the grasshopper *Chorthippus parallelus*.

UEBEL, E.C., SCHWARZ, M., MENZER, R.E., and MILLER, R.W. 1978. Mating stimulant pheromone and cuticular lipid constituents of *Fannia pusio* (Wiedemann) (Diptera: Muscidae). J. Chem. Ecol. 4:73-81.

WICKER, C., and JALLON, J-M. 1995. Hormonal-control of sex-pheromone biosynthesis in *Drosophila melanogaster*. J. Ins. Physiol. 41:65-70.