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Effect of age and sex on the production of internal and external hydrocarbons and pheromones in the housefly, *Musca domestica*

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Abstract

The epicuticular and internal waxes of male and female houseflies were examined by capillary gas chromatography-mass spectrometry at closely timed intervals from emergence until day-6 of adulthood. New components identified included tricosan-10-one, 9,10-epoxyheptacosane, heptacosen-12-one, a series of odd-carbon numbered dienes from C31 to C39, several positional isomers of monoenes including (*Z*)-9- and 7-pentacosene and a number of methyl- and dimethylalkanes. (*Z*)-9-tricosene appears in internal lipids prior to appearing on the surface of the insect, suggesting that it is transported in the hemolymph to its site of deposition on the epicuticle. The large increases in the amount of (*Z*)-9-tricosene in females from day-2 until day-6 is compensated for by a concomitant decrease in (*Z*)-9-heptacosene. The C23 epoxide and ketone only appear in females after the production of (*Z*)-9-tricosene is induced, and are only abundant in epicuticular waxes, suggesting they are formed after (*Z*)-9-tricosene is transported to the cells which are involved in taking them to the surface of the insect. Mathematical analysis indicated that the time shift between internal production and external accumulation in females is more than 24 h. The divergence between male and female lipid production occurs at an early stage, when insects are less than one day old. © 2001 Published by Elsevier Science Ltd.

Keywords: Cuticular hydrocarbons; Sex-pheromone production; Internal vs external lipids; Selective transport; GC-MS; Mathematical modeling; Housefly *(Musca domestica*)

1. Introduction

The sex pheromone of the female house fly, *Musca domestica* (L.) consists of (*Z*)-9-tricosene (Carlson et al., 1971), cis-9,10-epoxytricosane, (*Z*)-14-tricosen-10-one (Uebel et al., 1978) and a complex mixture of methylalkanes (Uebel et al., 1976; Nelson et al., 1981), which function as short range attractants, sex recognition factors, and arrestants (Adams and Holt, 1987). These pheromone components are modified epicuticular lipids. Extensive work has been performed to understand the shift in the chain lengths of hydrocarbons to produce pheromone at both the endocrine and biochemical levels (Adams et al. 1984a,b, 1995; Blomquist et al. 1984b, 1987; Tillman-Wall et al., 1992) and to gain an understanding of the mechanism of hydrocarbon formation (Dillwith and Blomquist, 1982; Dillwith et al. 1981, 1982; Reed et al. 1994, 1995; Mpuru et al., 1996). In maturing female houseflies, ovarian-produced ecdysteroids cause a modification in hydrocarbon production such that (*Z*)-9-tricosene becomes a major component (Adams et al., 1984). In addition to accumulating on the surface of the female, a portion of the (*Z*)-9-tricosene is converted to the corresponding epoxide and ketone (Blomquist et al., 1984a; Ahmad et al., 1987). This modification of cuticular profiles, changing the hydrocarbon and oxygenated derivatives, makes the female attractive to males and is a key factor in the reproduction of some dipteran and hymenopteran species (Uebel et al. 1976, 1978; Trabalon et al. 1992, 1994; Finidori-Logli et al., 1996; Paulmier et al., 1999).

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Several studies indicate that hydrocarbons are synthesized in oenocytes. The location of oenocytes varies widely among insects (Romer, 1991). Even in insects whose oenocytes are associated with the epidermis, it appears that hydrocarbons are transported through the hemolymph, with lipophorin serving as a shuttle (Chino, 1985; Schal et al., 1998a,b; Sevala et al., 2000). The female housefly begins producing (*Z*)-9-tricosene about two days after emergence (Dillwith et al., 1983), with relatively large amounts building up internally followed by deposition on the surface of the insect (Adams et al., 1995). Thus, the change in the chain length of the major alkenes, and the presence of oxygenated derivatives of (*Z*)-9-tricosene (epoxide, ketone) provides a unique opportunity to study the transport of these pheromone components.

Analysis of the cuticular hydrocarbons (Nelson et al., 1981) of male and female insects showed a complex mixture of normal, unsaturated, and methyl-branched components. This analysis was performed with packed columns, which are not as efficient as modern capillary columns. In addition, complete analyses were not performed on the oxygenated cuticular components. In this manuscript, we present a more complete analysis of the hydrocarbons of both male and female insects at various ages and identify a number of new hydrocarbons and oxygenated components. Comparison of the internal waxes with those found on the surface of the insect revealed interesting patterns and provided insight into the location of the synthesis and transport of both hydrocarbons and oxygenated components.

2. Materials and methods

2.1. Insects

Pupae of Fales 1958 strain T-II houseflies were supplied by the Biology Section at S.C. Johnson and Sons, Racine, Wisconsin, USA. Males were separated from females within the first six hours post-emergence under carbon dioxide anaesthesia. Houseflies were maintained ad libitum on sucrose and low-fat powdered milk (1:1 w/w) and tap water at 24 to 27°C with a 12h light/12h dark photoperiod. Samples consisted of five replicates of five individuals at different ages, i.e. pupae, pharates, and various times post-emergence for each sex: 0, 12, 24, 36, 48, 60, 72 (3 days), 96 (4 days), 120 (5 days), and 144 (6 days) h. Dead insects were kept at −80°C until thawing for lipid extraction.

2.2. Lipid extraction

External (epicuticular) lipids were extracted from each group of five insects as previously described (Dillwith et al., 1983) except that the three washes were done with 3 ml rather than 10 ml of hexane. Resulting extracts were pooled and concentrated to 1 ml under a gentle stream of nitrogen before addition of 800 ng of *n*-nonadecane (*n*-C19) as internal standard prior to GC and GC-MS analysis.

For extraction of internal lipids, insects that had already been washed with hexane to remove external hydrocarbons, were immersed in 3 ml of chloroform/methanol (1:2 v/v) for 1 h in an ultrasonic bath with ice, before extracting the internal lipids by the procedure of Bligh and Dyer (1959). The chloroform layer was evaporated under a gentle stream of nitrogen and the lipids taken up in 1 ml hexane to which 800 ng of *n*-C19 was added as an internal standard.

2.3. Gas chromatography

For quantification, an aliquot of each external and internal sample (2 μl with the purge off for 0.5 min) was analysed on a HP-5 column (30 m×0.32 mm ID, 0.25 μm film thickness) on a HP 5890 GC equipped with FID and interfaced with an HP 3393A computing integrator. Oven temperature was held at 150°C for 5 min then increased at a rate of 5°C/min up 320°C, where it was held for 10 min. Both the injector and detector were maintained at 325°C. Helium was the carrier gas. Five replicates for each time and sex were analysed. Peak areas were analysed in Excel spreadsheet and processed as described below.

2.4. Fractionation

Prior to GC-MS analyses, the five replicates for each time-point were pooled into one sample (total of 44 samples). The samples were then fractionated into saturated and unsaturated components on a Silica gel minicolumn (Aldrich, 60Å, 70–230 mesh) that was impregnated with 20% (w/w) silver nitrate (Still et al., 1978; Dillwith et al., 1983). The 44 unsaturated fractions were derivatized by DMDS for GC-MS analyses (Francis and Veland, 1981; Carlson et al., 1989).

2.5. Gas chromatography-mass spectrometry (GC-MS)

Analyses were conducted by GC-MS, before and after DMDS derivatization, on a Hewlett-Packard 5890 GC Series II equipped with a CP-Sil-5CB WCOT apolar capillary column (Chrompack, 25 m, 0.25 mm ID) using a split/splitless injector at 280°C. Oven temperature was programmed from 70 to 150°C at 30°C/min and from 150 to 320°C at 5°C/min, and held at 320°C for 5 min (15 min for DMDS samples). The carrier gas was helium (2 bars). The interface to the mass spectrometer, a HP 5989A MS Engine, and a HPUX Chemsystem control unit, was maintained at 300°C. The MS was operated in the electron impact mode at 70 eV and scanned from m/z

35 to 600 with about one scan per second, with source temperature held at 240°C and quadrupole at 100°C.

2.6. Quantitative analyses

After introduction into the Excel spreadsheet, each of the 116 peak areas was readjusted using a correcting FID coefficient (Bagnères et al., 1991a,b). Corrected areas were then expressed as relative proportions (%) and quantities (Q) in μg/mg of insect. Averages and standard deviations were calculated with the five replicate % and Q.

2.7. Principal component analysis

Principal Component Analysis (PCA) was performed with Statgraphics® Plus for Windows (v.4.0) and the Uniwin Plus extension (v.3.01). The complete PCA matrix was formed using the 220 samples (ranks) and their 116 peak percentages (columns). Various PCAs were performed including various combinations (e.g. all individuals, only males only females, only internal samples, or only external samples). In the present paper only two analyses will be described in order to illustrate the main divergence (male versus female and internal versus external).

Various data sets were analysed with the principal component method, taking the percentage of each product as a variable. The principal axes were computed from the correlation matrix. In the following analyses, the coordinates of the observations were considered as new variables, since they are a linear combination of the initial variables.

2.8. Modeling the delay between external and internal hydrocarbon production

Analysis of internal and external production of hydrocarbons showed that points on the main factorial plane (Axes 1 and 2) were ordered along the first axis (F1) according to age. Points corresponding to external samples were shifted toward the negative coordinates, clearly indicating that the same products were observed on the surface of the insect slightly after their internal counterpart. Based on this finding a mathematical expression relating the F1 coordinates to the age of the insects was developed. The logistic function was successfully adjusted to both external and internal samples:

$$x = F1(t) = a/[1 + b\ Exp(ct)]$$

Other functions were tried but they did not fit the data better than the logistic function. It was then easy to obtain a graphical estimate of the delay between the two series. The logistic function can be inverting as follows:

$$t = (1/c)Ln[9a - x)/bx]$$

It was then possible to calculate the production time for hydrocarbon assemblage summarized by its x=F1-coordinate.

2.9. Modeling the divergence between male and female production of hydrocarbon

Plotting of male and female extracts again indicated a time relation between production of hydrocarbons along F1. Points corresponding to male components remained on a positive coordinate on the second axis F2, whereas female points became negative coordinates. Calculations were undertaken to determine exactly when this shift took place. Since female F2-coordinates were found to increase, then decrease and eventually increase again, it was decided to fit a third order polynomial:

$$x = F2(t) = at^3 + bt^2 + ct + d$$

The first optimum of this function was taken as the point of divergence point between males and females. However since the curve representing the fitted polynomial did not pass through the observation points correctly, a fourth order polynomial was attempted:

$$y = F2(t) = at^4 + bt^3 + ct^2 + dt + e$$

The new fit was tested with respect to the previous one using the Fisher's F for embedded models:

$$F = (SS4 - SS3)/(RSS3/(n - 4 - 1))$$

where:

SS3=Sum of squares for the 3rd degree polynomial model;
SS4=Sum of squares for the 4th degree polynomial model;
RSS3=Residual some of squares for the 3rd degree polynomial model;
n=number of observations.

3. Results

3.1. Mass of components

On a wt/wt basis the total amount of internal and external hydrocarbons was fairly similar in males and females. Total hydrocarbons increased in both sexes both internally and externally from emergence until 36 h post-emergence. Thereafter fluctuations occurred with lows ranging from 0.3–0.6 μg/mg of insect weight at emergence and highs ranging from about 0.7 to just over 1.6

Table 1
Identities of components and percentage contributions of each in lipid extracts from external and internal extracts of day 1 and day 4 male and female houseflies

GC peakno.	ECL	Compound identity	Day 1				Day 4			
			Male		Female		Male		Female	
			Ext	Int	Ext	Int	Ext	Int	Ext	Int
0	19.45	Unk	0	0	0	0	0	0.09	0	0.14
1	19.54	16:1 FA	0	0	0	0	0	0.03	0	0
2	19.63	16:0 FA	0.08	0.15	0.09	0.14	0.04	0.37	0.02	0.35
2a	19.72	Unk	0	0.05	0	0	0	0.18	0	0.12
3	20.00	Unk	0.08	0.10	0	0.03	0	0.14	0	0.08
4	20.32	Unk	0	0	0.04	0.05	0.09	0.07	0	0.01
5	20.41	Unk	0	0.16	0	0.05	0	0.32	0	0.24
5a	20.55	Unk	0	0.04	0	0	0	0.05	0	0.04
5b	20.69	9-C21:1 Hyd	0	0.03	0	0.01	0	0.03	0	0
6	20.71	Unk	0	0	0	0	0	0	0.52	1.15
8	21.00	*n*-C21 Hyd	0.10	0.63	0.11	0.65	0.07	0.32	0.54	1.23
9	21.18	Unk	0	0.40	0	0.73	0	0.20	0	0.30
10	21.40	18:2 FA	0	0.44	0	0.64	0	0.19	0	0.60
11	21.73	18:1 FA	0	0	0	0.01	0	0.02	0.21	0.38
11a	21.79	18:0 FA	0	0	0	0	0.01	0	0	0
12	22.00	*n*-C22 Hyd	0.05	0.30	0.04	0.25	0.03	0.27	0.21	0.51
13	22.14	Unk	0	0	0	0.01	0	0.02	0	0
15	22.64	Unk	0	0.06	0	0.06	0	0.03	0.12	0.02
16	22.72	9-C23:1 Hyd	0	0.16	0.02	0.23	0.01	0.12	11.9	25.0
17	22.78	Unk	0	0.09	0.01	0.19	0	0.06	0.33	1.51
17a	22.84	Unk	0	0	0	0.04	0	0	0	0.02
18	23.00	*n*-C23 Hyd	1.20	3.14	1.02	4.02	2.19	2.85	12.5	21.0
19	23.10	Unk	0	0.56	0	1.01	0	0.14	0	0.50
20	23.17	Unk	0	0.72	0	1.20	0	0.26	0	0.97
22	23.72	Unk	0.02	0.03	0.01	0.13	0	0.02	0.05	0.07
23	24.00	*n*-C24 Hyd	0	0.38	0.11	0.36	0.01	0.26	1.03	0.18
24	24.10	Unk	0.30	0	0	0	0.17	0	0	0
25	24.41	10-C23:1 One	0	0	0	0	0	0	1.74	0
26	24.61	C23:0 Epox+Unk	0.05	0.26	0.04	0.39	0	0.11	5.61	0.34
26a	24.69	9-C25:1 Hyd	0.04	0.14	0	0.25	0.07	0.07	0	0.23
28	24.82	10-C23:0 One	0.10	0.48	0	0.25	0.18	0.17	2.97	0.23
29	24.86	7-C25:1 Hyd	0	0	0.12	0.16	0	0	0	0
30	25.00	*n*-C25 Hyd	6.57	6.67	0	7.26	15.9	7.65	3.24	2.06
32	25.51	3-MetC25 Hyd	0.12	0.59	5.20	0.61	0.31	0.57	0.32	0.47
33	25.63	Unk	0.01	0	0.11	0.02	0	0	0	0
34	25.64	9-C26:1 Hyd	0.30	0.56	0	0.45	0.22	0.47	0.05	0.08
35	25.81	Unk	0	0	0.19	0	0	0	0.06	0
36	26.00	*n*-C26 Hyd	0.54	0.45	0	0.54	0.38	0.26	0.15	0.20
39	26.33	Unk	0.06	0	0.49	0.02	0.01	0	0.30	0.08
40	26.53	Unk	0.06	0.16	0.07	0.39	0.01	0.02	0	0
41	26.57	Unk	0.06	0.18	0	0.49	0	0.16	0	0.55
43	26.75	9-C27:1 Hyd	22.1	36.4	0.08	25.5	48.2	59.5	6.73	7.17
44	26.84	8-C27:1 Hyd	1.01	1.54	11.7	1.50	0.79	0.55	0.27	0.20
45	26.88	7-C27:1 Hyd	0.10	0.06	0.76	0.81	0	0	0	0.04
46	27.00	*n*-C27 Hyd	15.2	6.84	0.33	7.83	11.8	5.44	6.16	1.66
47	27.17	Unk	0	0.03	15.63	0	0.01	0.01	0	0
48	27.32	9-, 11- and 13-MetC27 Hyd	1.12	0.85	0	1.36	0.01	0.06	2.90	1.84
49	27.41	7-MetC27 Hyd	0.40	0.86	1.26	0.49	0	0	0.23	0.26
50	27.51	5-MetC27 Hyd	0.14	0.09	0.46	0.17	0	0	0.42	0.30
51	27.63	5,9-, 7,11-, 7,13-, 11,15-diMet C27 Hyd+4-MetC27 Hyd	0.53	0.24	0.17	0.38	0.08	0.02	1.37	0.57

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Table 1 (*continued*)

GC peakno.	ECL	Compound identity	Day 1				Day 4			
			Male		Female		Male		Female	
			Ext	Int	Ext	Int	Ext	Int	Ext	Int
52	27.73	3-MetC27 Hyd	0.92	0.78	0.58	0.87	0.68	0.74	1.61	0.96
53	27.81	2-MetC27 Hyd	0.31	0	0.91	0.25	0	0	0.98	0.61
55	28.00	*n*-C28 Hyd	0.41	0.19	0.34	0.22	0.11	0.09	0.37	0.19
56	28.08	3,7-, 3,9-, 3,11- and 3,13-diMetC27 Hyd	0.15	0.04	0.43	0.18	0	0	1.17	0.87
56a	28.13	8-, 10-, 11-, 12-, 13- and 14-MetC28 Hyd	0	0	0.26	0	0	0	0	0
57	28.32	4-MetC28 Hyd+12-C27:1 One	0.47	0.33	0	0.35	0.04	0.05	1.33	0.82
58	28.39	x,y-C29:2 Hyd+C27 Epox	0	0	0.54	0	0.05	0	0.04	0.05
59	28.63	2-MetC28 Hyd	1.04	0.31	0	0.52	0.57	0.03	0.61	0.14
60	28.67	10-C29:1 Hyd	0.15	0	1.17	0	0.94	0	0	0
61	28.75	9-C29:1 Hyd	6.76	8.51	0	9.98	8.85	11.1	3.18	2.59
62	28.81	8-C29:1 Hyd	0	0	0.14	0	0	0.03	0	0.01
63	28.90	7-C29:1 Hyd	0	0	0	0	0.60	0	0	0
64	29.00	*n*-C29 Hyd	2.63	0.92	0	1.20	0.63	0.27	5.93	2.56
65	29.33	9-, 11-, 13- and 15-MetC29 Hyd	3.90	2.71	3.18	3.19	0.15	0.26	5.39	3.86
66	29.43	7-MetC29 Hyd	1.19	0.79	4.49	0.82	0.01	0.02	1.23	1.04
67	29.53	5-MetC29 Hyd	0.22	0.09	1.25	0.08	0	0	0.42	0.31
68	29.65	4-MetC29 Hyd	2.47	1.04	0.22	0.99	0.31	0.07	2.06	1.08
69	29.76	3-MetC29 Hyd	2.22	1.08	2.58	1.23	0.35	0.14	2.34	4.06
70	29.78	Unk	0	0	2.62	0	0	0	0.35	0.25
70a	29.95	Unk	0.04	0	0	0	0	0	0	0
71	30.00	3,7-, 3,9-, 3,11- and 3,13-diMetC29 Hyd+*n*-C30 Hyd	0.31	0.11	0.08	0.20	0.13	0.10	1.69	1.43
72	30.30	8-, 10-, 11-, 12-, 13- and 14-MetC30 Hyd	0.65	0.65	0.43	0.63	0.01	0.10	0.59	0.67
73	30.45	4-MetC30 Hyd	0.02	0	0.84	0	0.56	0	0	0
74	30.48	3-MetC30 Hyd	0	0	0.07	0	0	0	0	0
75	30.62	2-MetC30 Hyd+10-C31:1 Hyd	2.24	0.69	0.07	0.39	0.23	0.08	0.70	0.37
76	30.66	x,y-C31:2 Hyd	0	0.20	2.37	0.13	0.44	0	0	0
77	30.74	9-C31:1 Hyd	1.47	1.16	0.22	1.91	0.20	0.24	1.14	1.05
78	31.00	*n*-C31 Hyd	0.79	0.42	1.95	0.52	0.88	0.46	1.27	0.61
79	31.32	9-, 11-, 13- and 15-MetC31 Hyd	5.01	4.40	1.06	4.27	0.53	0.97	1.49	1.50
80	31.54	7-MetC31 Hyd	0.12	0.08	5.81	0.04	0	0.02	1.05	0
81	31.65	4-MetC31 Hyd	2.07	1.61	2.51	1.31	0.20	0.17	1.0	0.96
82	31.78	3-MetC31 Hyd	1.12	0.65	2.51	0.65	0.38	0.24	1.02	0.62
83	32.00	*n*-C32 Hyd	0.21	0.13	1.34	0.27	0.05	0.07	0.06	0.16
84	32.28	x,y-C33:2 Hyd	0.55	0.35	0.30	0.35	0.13	0.08	0.11	0.08
85	32.44	Unk	0.42	0.09	0.65	0.21	0.33	0	0	0
86	32.67	9-C33:1 Hyd	1.15	1.06	0.49	1.01	0.08	0.15	0.14	0.16
87	32.80	Unk	0.44	0.49	1.47	1.29	0.36	0.75	0.38	0.36
88	33.00	*n*-C33 Hyd	0.15	0.18	0.83	0.11	0.21	0.15	0.03	0.02
89	33.28	9-, 11-, 13- and 15-MetC33 Hyd	1.74	1.64	0.20	1.85	0.22	0.49	0.29	0.23
90	33.58	11,21- and 13,23-diMetC33 Hyd+4-MetC33 Hyd	1.28	0.91	2.12	0.90	0.03	0.21	0.31	0.33
91	33.87	3-MetC33 Hyd	0.11	0	1.44	0	0.02	0	0.02	0
92	34.00	*n*-C34 Hyd	0.08	0.08	0.10	0.09	0	0.02	0	0
93	34.27	11-, 12- and 13-MetC34 Hyd	0.27	0.22	0.08	0.21	0	0.04	0.05	0

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Table 1 (*continued*)

GC peakno.	ECL	Compound identity	Day 1				Day 4			
			Male		Female		Male		Female	
			Ext	Int	Ext	Int	Ext	Int	Ext	Int
94	34.48	x,y-C35:2 Hyd	0.18	0.10	0.30	0.08	0.12	0	0	0.01
95	34.66	12,16- and 13,17-diMetC34 Hyd+9-C35:1 Hyd	0.22	0.17	0.22	0.10	0	0	0.14	0
96	34.87	x-C35:1 Hyd+Unk	0.13	0.01	0.39	0.16	0.01	0.09	0	0
97	35.00	*n*-C35 Hyd	0.08	0	0.21	0.04	0	0	0	0
98	35.15	Unk	0.18	0	0.13	0	0	0	0.38	0
99	35.40	11-, 13-, 15- and 17-MetC35 Hyd	1.22	0.92	0.19	1.06	0.06	0.31	0.16	0.16
100	35.54	9,13-, 11,21- and 13,23-diMetC35 Hyd	0	0	1.63	0	0	0	0.34	0
101	35.64	Unk	0.73	0.55	0	0.54	0.01	0.15	0.07	0.17
102	36.00	Unk	0	0	0.80	0	0.01	0	0	0
103	36.21	Unk	0.20	0.08	0.07	0.11	0	0.10	0.14	0.01
103a	36.33	Unk	0.14	0	0.35	0	0	0	0	0
104	36.41	x,y-C37:2 Hyd	0.77	0.52	0.08	0.11	0.34	0.25	0.10	0.01
104a	36.54	x-C37:1 Hyd	0.07	0	1.22	0.57	0	0	0	0.12
104b	36.70	x,y-C37:2 Hyd	0.04	0	0.46	0	0	0	0	0
105	37.00	*n*-C37 Hyd	0.05	0	0.17	0	0	0	0	0
106	37.21	11-, 13-, and 15-MetC37 Hyd	0.52	0.32	0.29	0.58	0.45	0.28	0.24	0.02
107	37.50	11,21- and 13,23-diMetC37 Hyd	0.66	0.53	0.89	0.48	0	0.22	0	0.07
108	37.77	Unk	0.16	0	0.85	0	0.06	0.06	0.10	0.03
109a	38.25	Unk	0.03	0	0.16	0	0	0	0	0
110	38.54	Unk	0.37	0.34	0.82	0.23	0	0.09	0	0.04
110a	39.11	x,y-C39:2 Hyd	0.15	0	0.09	0	0.03	0	0	0
111	39.25	11- and 12-MetC39 Hyd	0	0	0	0	0.05	0	0	0.03
112	39.59	11,21- and 13,23-diMetC39 Hyd	0.68	0.62	0.21	0.67	0	0.18	0	0.08

μg/mg on day 3. Males had slightly more external hydrocarbons than females at all times studied.

3.2. New components

GC-MS analyses of total waxes from pupae, pharates, and adult males and females from emergence to day 6 showed that saturated and unsaturated hydrocarbons were the most abundant components with lesser amounts of epoxides, ketones and free fatty acids (Table 1). The relative proportion of many components varied considerably with age. Table 1 presents data for day 1 and day 4 females and males and for internal and external components. This report confirmed the presence of most components identified in earlier studies (Uebel et al., 1976; Nelson et al., 1981). Only the new findings will be described below.

Free fatty acids accounted for less than 2% of the total components at all times examined. They were more abundant in internal extractions and consisted of palmitic, palmitoleic, stearic, oleic and linoleic acids.

One series of hydrocarbons not detected in this study comprised 4,8-, 4,10- and 4,12-dimethylalkanes with even-numbered carbon chains. In the earlier study, these products had ranged from trace to 0.4% of total hydrocarbons in day 4 females. Strain differences could account for this discrepancy.

Several new hydrocarbons were identified. The saturated *n*-alkanes C34, C35 and C37 were not detected in the earlier study although their presence was implicit. Unlike the previous study which found no C25 unsaturated components, this study detected small amounts of pentacosene with double bonds in both the *Z*-9 and *Z*-7 positions. Also identified were several positional alkene isomers (9- and 7-pentacosene as well as 9-, 8-, 7-heptacosene and 10-, 9-, 8-, 7-nonacosene). The major isomer for each of these alkenes was the *Z*-9 isomer. A series of alkadienes (31:2, 33:2, 35:2, 37:2 and 39:2) were also

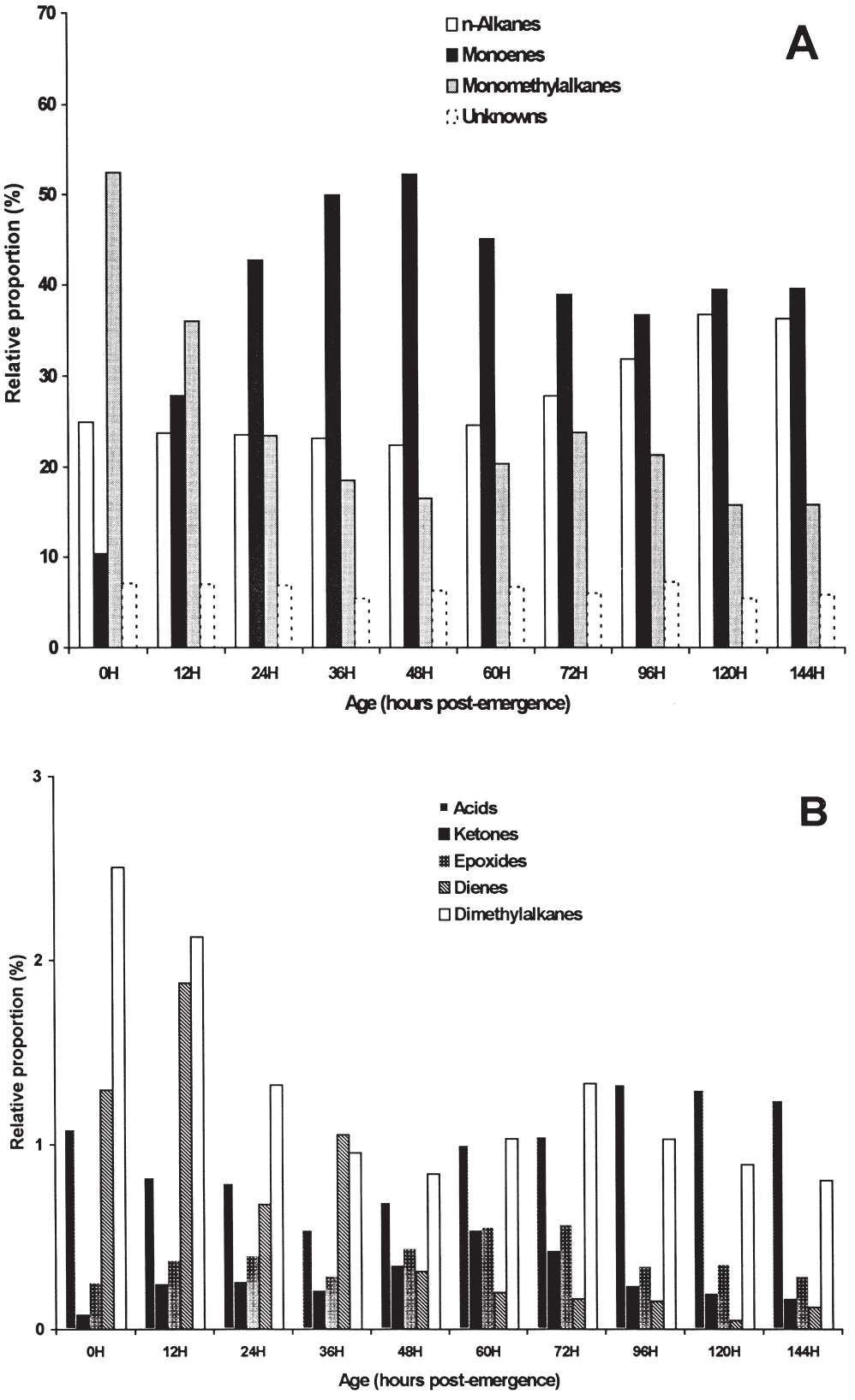

Fig. 1. Effect of age on the relative proportion of major wax components (A) and minor wax components (B) in the internal extracts from female houseflies. Lipids were extracted and analysed as described in Materials and methods.

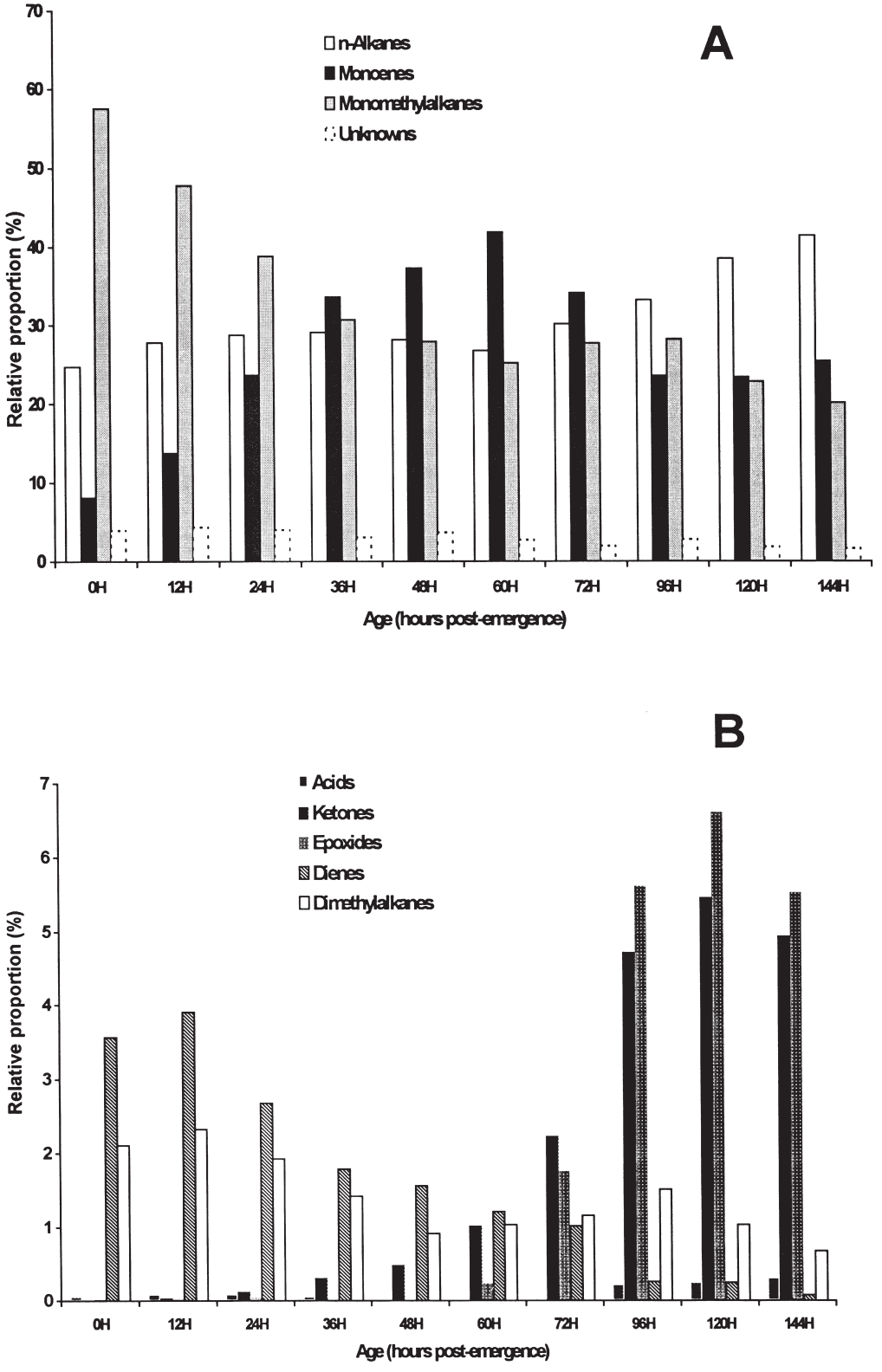

Fig. 2. Effect of age on the relative proportion of major wax components (A) and minor wax components (B) in the external extracts from female houseflies. Lipids were extracted and analysed as described in Materials and methods.

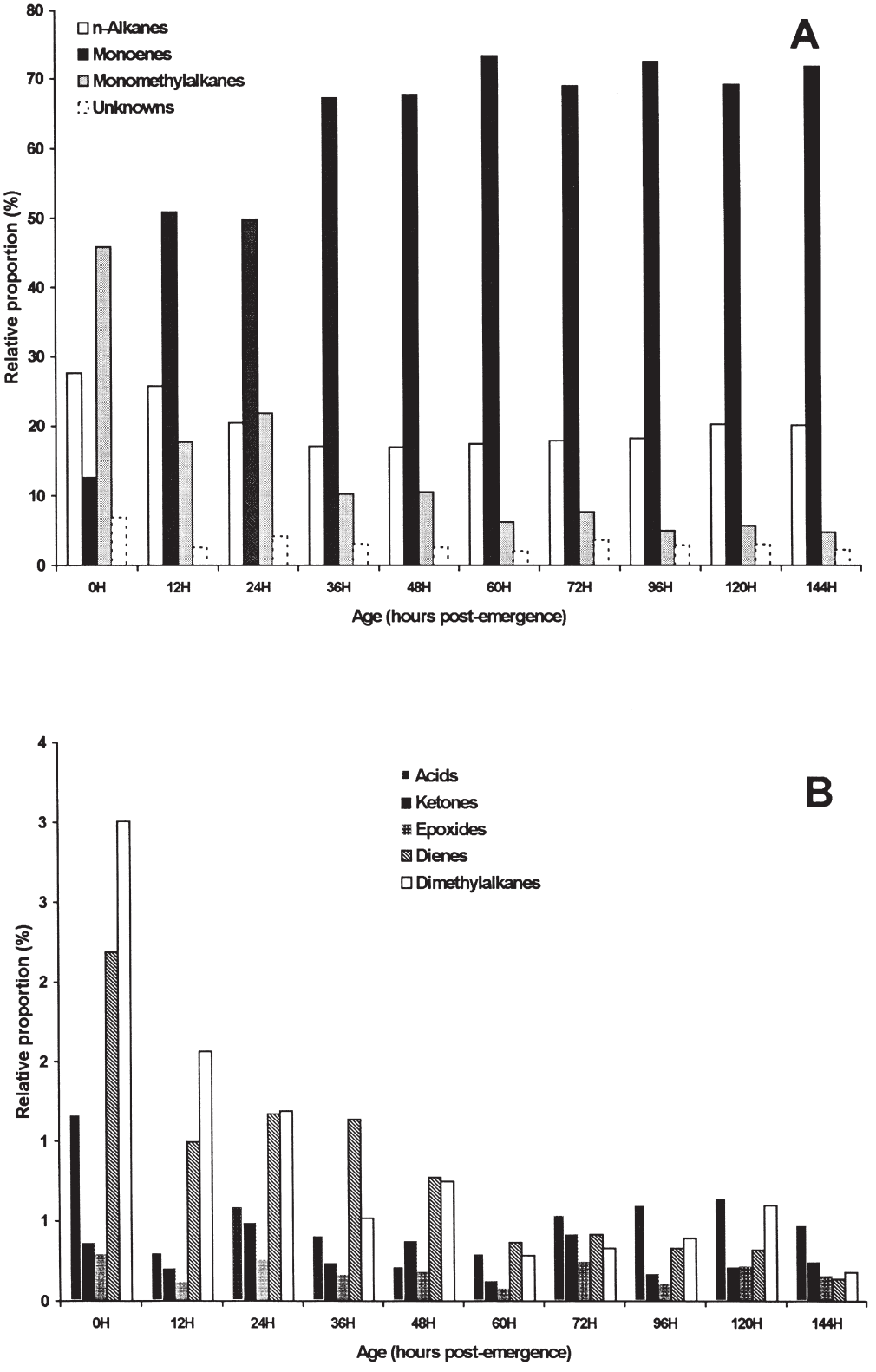

Fig. 3. Effect of age on the relative proportion of major wax components (A) and minor wax components (B) in the internal extracts from male houseflies. Lipids were extracted and analysed as described in Materials and methods.

identified, but amounts were too small to allow DMDS derivatization and localization of double bonds.

Monomethyl-branched hydrocarbons not reported before included 4-methylheptacosane, 11- and 13-methyloctacosanes, 11- and 13-methyltriacontane, 11-methyltetratriacontane and 15-methylheptatriacontane. New dimethyl branched hydrocarbons included 11,21- and 13,23-dimethyltritriacontane as well as 9,13-, 11,21- and 11,23-dimethylpentatriacontane.

New oxygenated components identified herein

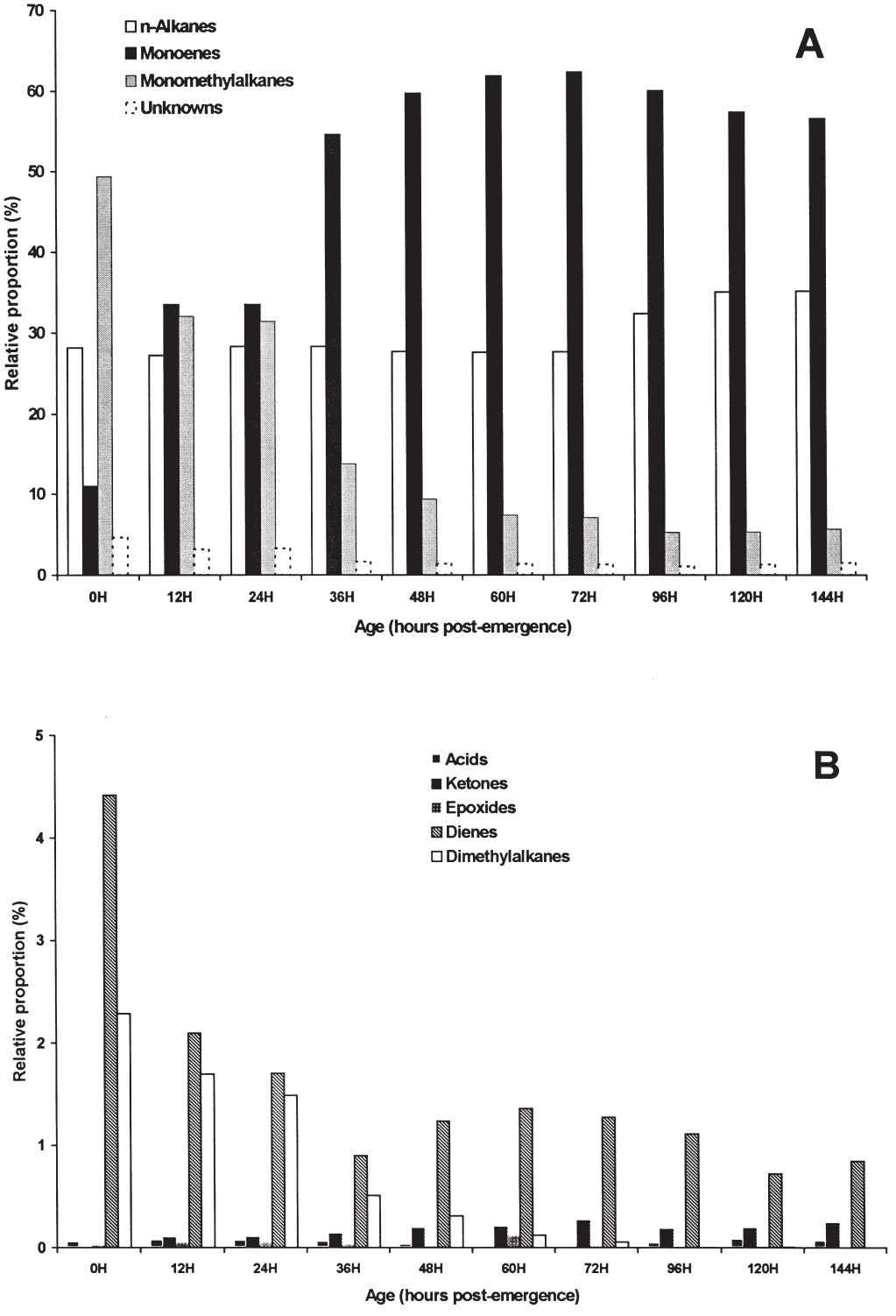

Fig. 4. Effect of age on the relative proportion of major wax components (A) and minor wax components (B) in the external extracts from male houseflies. Lipids were extracted and analysed as described in Materials and methods.

included tricosan-10-one, heptacosen-12-one (most likely the (*Z*)-18-isomere) and 9,10-epoxyheptacosane (likely the *cis* isomer). The saturated C27-ketone must be under one of the nonacosenes (certainly the 8- or 7-). Interestingly, these compounds, as well as *cis*-9,10-epoxytricosane and (*Z*)-14-tricosen-10-one were observed only in external lipids. Again, the quantities of the unsaturated oxygenated compound were too low (≤1%) to allow determination of the double bond.

3.3. Changes with age and sex

Marked changes were observed in internal and external waxes as houseflies progressively aged. Initially accounting for more than 50% of total components in internal lipid extracts from females (Fig. 1A), monomethylalkanes decreased until 48 h, then increased slightly at 60 and 72 h and finally decreased to only 15% of the total compounds at day 6. The contribution of *n*-alkanes increased after 48 h. Monoenes increased from about 10% at emergence to more than 50% at vitellogenesis (48 h) and then decreased down to about 37% at 96 h post-emergence. Throughout the period investigated, unknowns contributed between 4 and 7% of the total compounds. As shown in Fig. 1B, fatty acids, ketones, epoxides, dienes and dimethylalkanes were all present in relatively small amounts in internal lipid extracts from females. The distribution of the external hydrocarbons in females (Fig. 2A) mirrored that of internal hydrocarbons. In contrast, epoxides and ketones, which comprised less than 0.6% of the internal waxes at all times investigated, became significant components in external waxes, increasing from less than 0.5% at 48 h up to over 5% for both epoxides and ketones at 120 h (Fig. 2B).

In males, the percentage of monomethylalkanes and dimethylalkanes in the internal (Fig. 3A) and external (Fig. 4A) hydrocarbons decreased dramatically with age. In contrast, the amount of monoenes increased from less than 15% at emergence to over 55% at 36 h. Thereafter monenes remained high. Very small amounts of fatty acid, ketone and epoxide were observed at all times both internally and externally in males (Fig. 3B and Fig. 4B).

The important role of hemolymph in transporting hydrocarbons is illustrated by Fig. 5, which compares the relative proportions of internal and external (*Z*)-9-tricosene and (*Z*)-14-tricosen-10-one as a function of age in female houseflies. Increasing amounts of (*Z*)-9-tricosene were observed internally after 48 h. This was followed by increasing amounts on the surface of the insect. No (*Z*)-14-tricosen-10-one was observed internally but it appeared externally in very small amounts at 48 h and increased up to day 5. A similar trend may have occurred for *cis*-9,10-epoxytricosane, but since this component co-eluted with another component, it was more difficult to obtain a clear picture. The absence of the epoxides and ketones internally, compared to the presence of sig-

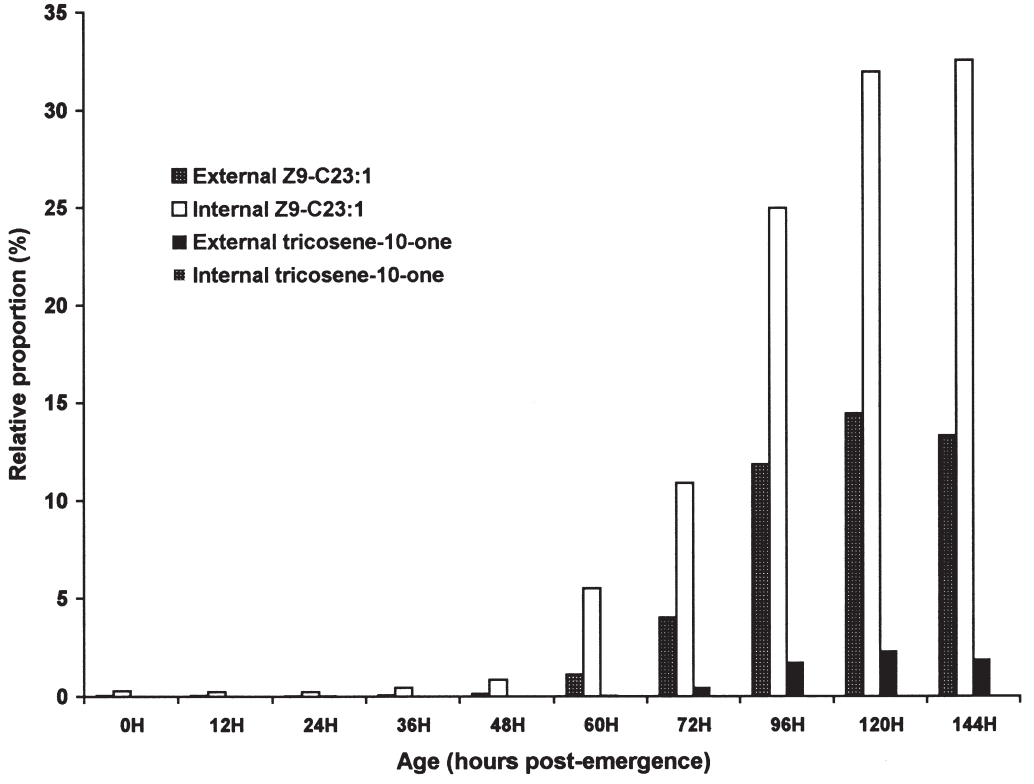

Fig. 5. Effect of age on the relative proportion of internal and external (*Z*)-9 tricosene and tricosen-10-one in female houseflies. Lipids were extracted and analysed as described in Materials and methods.

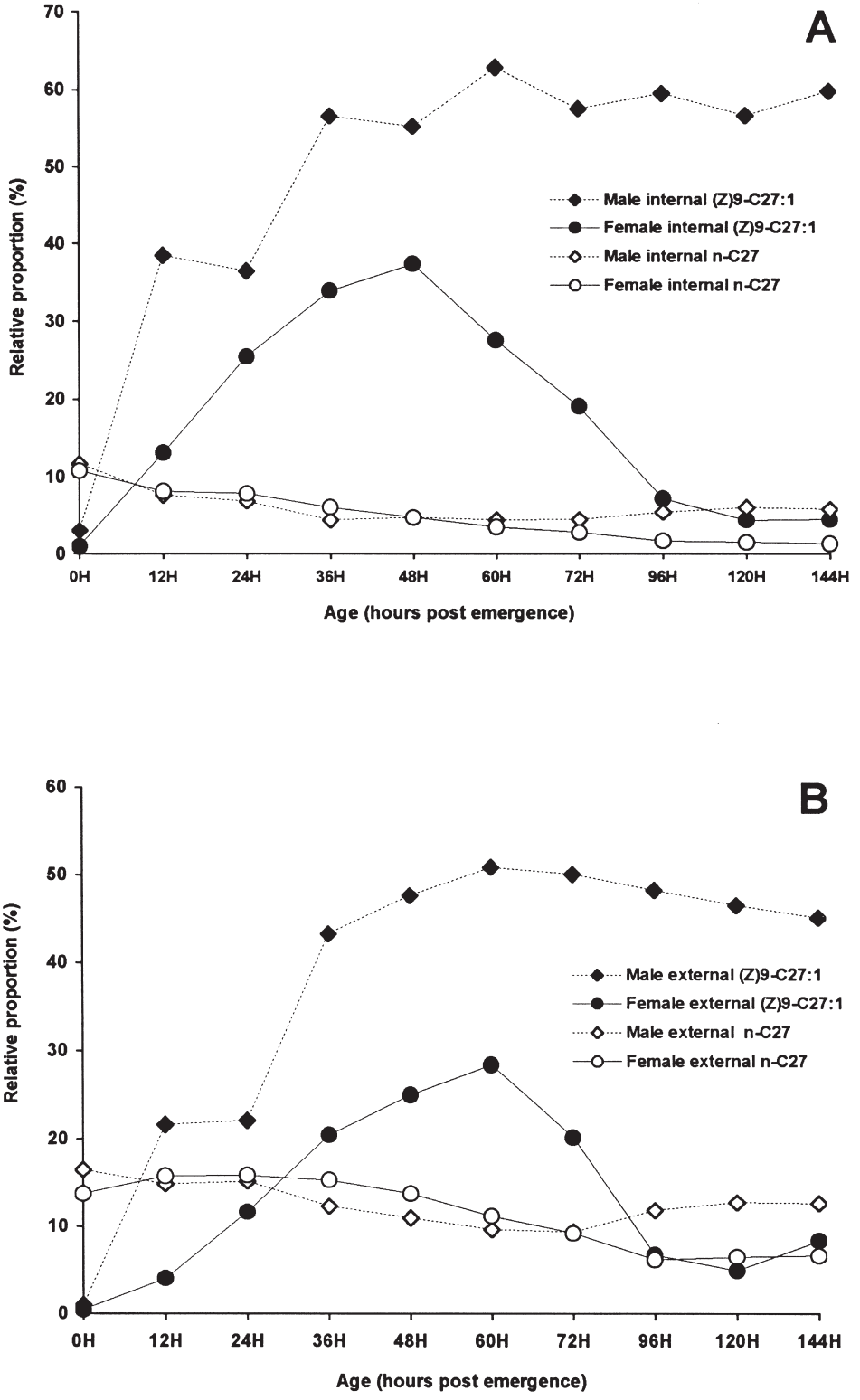

Fig. 6. Effect of age on the relative proportions of male and female *n*-heptacosane and (*Z*)-9-heptacosene in internal and external lipids of the male and female houseflies. Lipids were extracted and analysed as described in Materials and methods.

nificant amounts externally, suggests that their precursor, (*Z*)-9-tricosene, is produced in epidermal cells, enters the hemolymph, and then is transported to the surface. A portion of the (*Z*)-9-tricosene may be converted to the epoxide and ketone during transport to the surface.

The large increases in (*Z*)-9-tricosene (Fig. 5) in females after 48 h were compensated for by a dramatic decrease in (*Z*)-9-heptacosene both internally (Fig. 6A) and externally (Fig. 6B). In contrast in males, (*Z*)-9-heptacosene increased from very low levels at emergence and became the major component both internally and externally by 36 h.

3.4. Estimation of the time shift between external and internal lipid production

Principal components analysis of external and internal production of males from emergence to day 6 showed two parallel curves with a time-shift between the two curves. The internal curve lead the external curve differently during development. The first axis (F1, 29.3% of variance) separated the extracts based on age, and the second axis (F2, 16.1%) separated the internal (top) and external (bottom) lipids (Fig. 7A). On day 4 (96H) we can see stagnation on F1 coming with an inverse shift on F2 when adult flies reach maturity.

The data plot shown in Fig. 7B was obtained using the different points on F1, i.e. the axis accounting for most of the divergence between external and internal lipid production. Fig. 7C shows the fitted curves for internal (x_{in}) and external (x_{ex}) production.

The logistic curves were: $x_{in}=18.94/(1+2.72\ \mathrm{Exp}(-1.0998\ t))$, for internal production, and $x_{ex}=18.25/(1+9.3\ \mathrm{Exp}(-1.1009\ t))$ for external production. Using a mid-range value of 12 for the *x*-coordinate (note that a constant of 12 was added to all centered coordinates), the following times were obtained: $t_{in}=1.41$ days and $t_{ex}=2.62$ days.

Thus the time shift between external and internal lipid production was: 2.62 days−1.41=1.21 days, i.e. 29 h.

3.5. Estimation of the divergence time between males and females.

PCA projection of male and female internal extracts at various ages indicated that male extracts evolve linearly, whereas a female shift appears at an early stage following a negative curve (Fig. 8A). The confidence ellipse at 12 h for females shows a large individual variability. Males develop quicker with very little change being observed after 36 h. The first axis (F1, 29.5% of variance) separated the groups mainly by time. The second axis (F2, 17%) shows the shift of the female extracts after the onset of vitellogenesis (48 h). When the second principal axis was fitted with a cubic polynomial, the following equation was obtained:

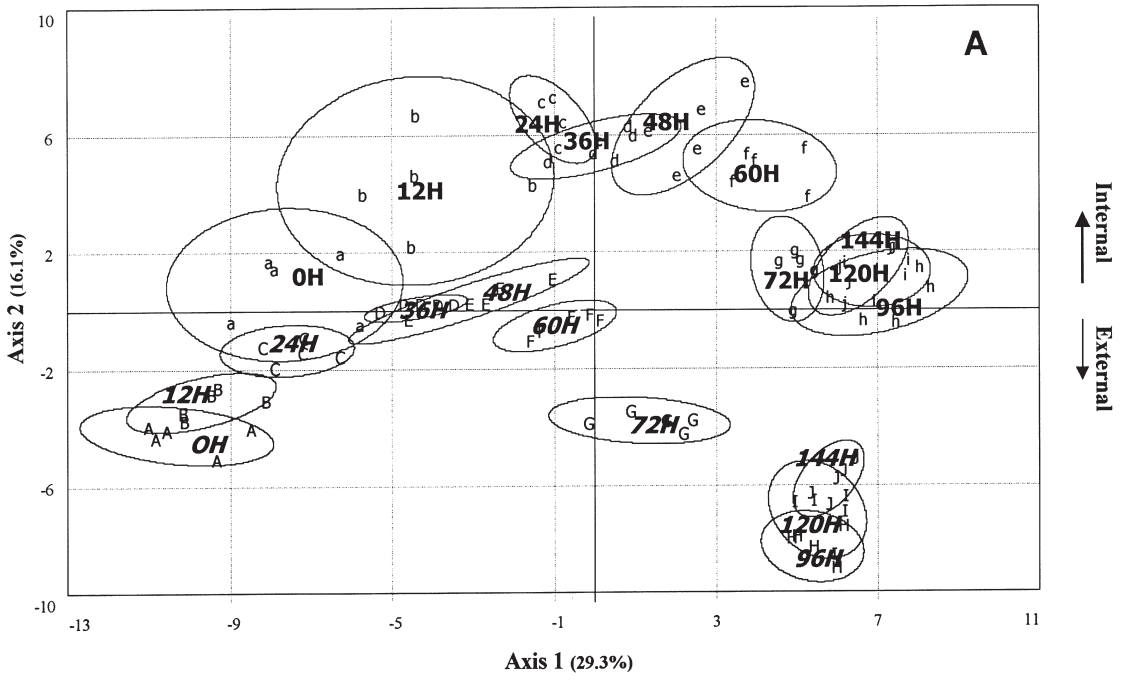

Fig. 7. Principal component analysis (A) of the 116 variables (relative proportions of HCs) for 100 samples from 50 *M. domestica* females after emergence (50 external lipid extracts: from A to J; and their corresponding 50 internal lipid extracts: from a to j; with 5 individual samples for the 10 different times), ellipses of confidence (per time group) are provided with a risk of 5%. Fitting of a logistic equation expressing the relation between the time (horizontal axis) and the first principal component F1 (vertical axis) with, in (B): real data plot; in (C): fitted curves for internal and external lipid production.

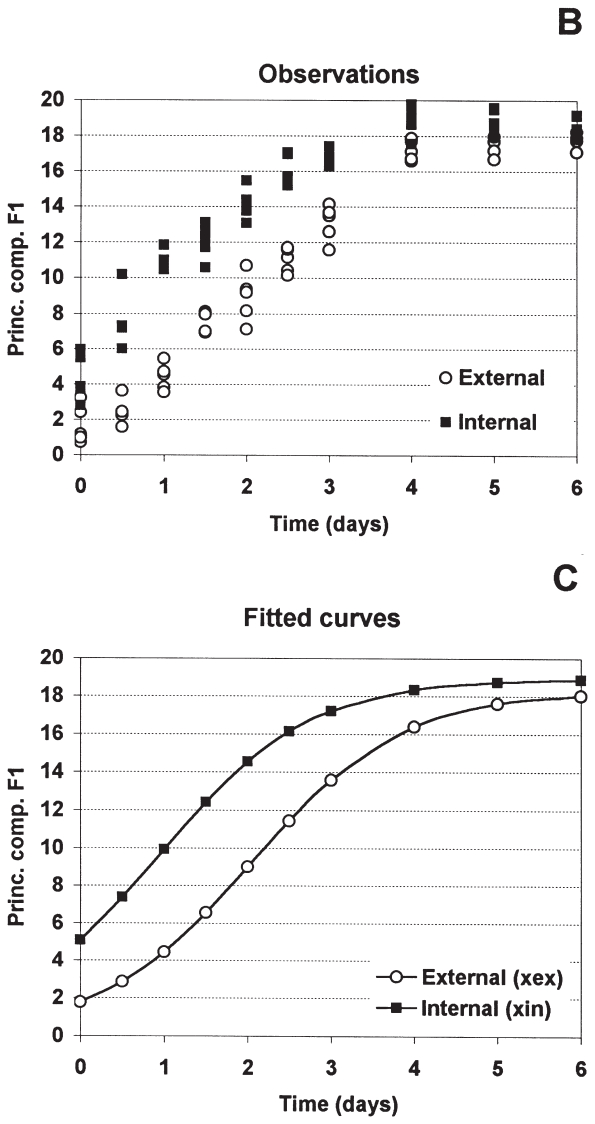

Fig. 7. (*continued*)

$$x = F2(t) = 0.279t^3 - 2.223t^2 + 1.774t + 1.842$$

with a coefficient of determination $R2=0.9152$.

To improve this result, fitting to a fourth order polynomial was made resulting in the following equation:

$$y = F2(t) = 0.0918t^4 + 1.3722t^3 - 6.2646t^2 + 6.65t + 0.9716$$

the corresponding coefficient of determination being $R2=0.9418$. Since comparison between the two models resulted in a highly significant difference: F=14.116 (for 1 and 45 degrees of freedom), the fourth order polynomial equation was chosen. The first derivative of the equation was:

$$dy/dt = -0.3672t^3 + 4.1166t^2 - 12.5292t + 6.65.$$

This derivative is null for $t=0.669$ day, i.e. approximately 16 h. This value corresponds to the highest coordinate in F2, which can be considered as the divergence production time between the two sexes.

4. Discussion

This study documents a number of additional components in the waxes of the housefly, all present in small amounts. The absence of pentacosenes in earlier reports implied a tremendous specificity in the enzymes that regulated alkene chain length. Whereas (*Z*)-9-heptacosene predominates in males at all times and in females prior to vitellogenesis, (*Z*)-9-tricosene predominates in vitellogenic females. In the present study, relatively small amounts of pentacosenes was found at all given time. This clearly argues that regulation of alkene chain length is highly specific but not absolute.

Likewise, the presence of tricosan-10-one, 9,10-epoxyheptacosane and heptacosen-12-one shows that the cytochrome P450 that normally epoxidizes and hydroxylates (*Z*)-9-tricosene to the C23 epoxide and ketone (Ahmad et al., 1987) can also hydroxylate *n*-tricosane (which is then presumably oxidized to the ketone) and use (*Z*)-9-heptacosene as substrate. The much larger amounts of C23 epoxide and ketone compared to the lower amounts of C27 epoxide and ketone suggest that the specificity is certainly toward the shorter chain alkene.

The change in the chain length of the alkenes from predominantly C27 and longer to predominantly C23 as the female becomes vitellogenic is primarily due to ecdysteroids (Adams et al., 1984; Blomquist et al., 1987) produced in the ovaries. These ecdysteroids apparently repress the synthesis of specific elongases (Tillman-Wall et al., 1992) that elongate monoenoic fatty acyl-CoAs of 24 carbons to 28 carbons and longer. This results in the accumulation of 24:1-CoAs, which are then converted to (*Z*)-9-tricosene by a two-step reaction involving a cytochrome P450 (Reed et al. 1994, 1995; Mpuru et al., 1996).

The divergence in male and female wax production occurs at an early stage. If less than one day is sufficient to discriminate male and female cuticular composition, it means that control of wax production starts at the pre-vitellogenesis stage. This indicates that corpora allata and juvenile hormone play an indirect role in ovarian maturation and sexual receptivity in dipterans (Trabalon et al., 1987). However, ecdysteroids play the most important role in regulation of pheromone production from day 2 through day 4 in *M. domestica* females (Blomquist et al., 1992; Adams et al., 1995). Nevertheless it is difficult to correlate metabolic pathways and/or transport of different components with the early (first few hours) sexual divergence of components since this depends on relatively minor differing proportions of the same compounds.

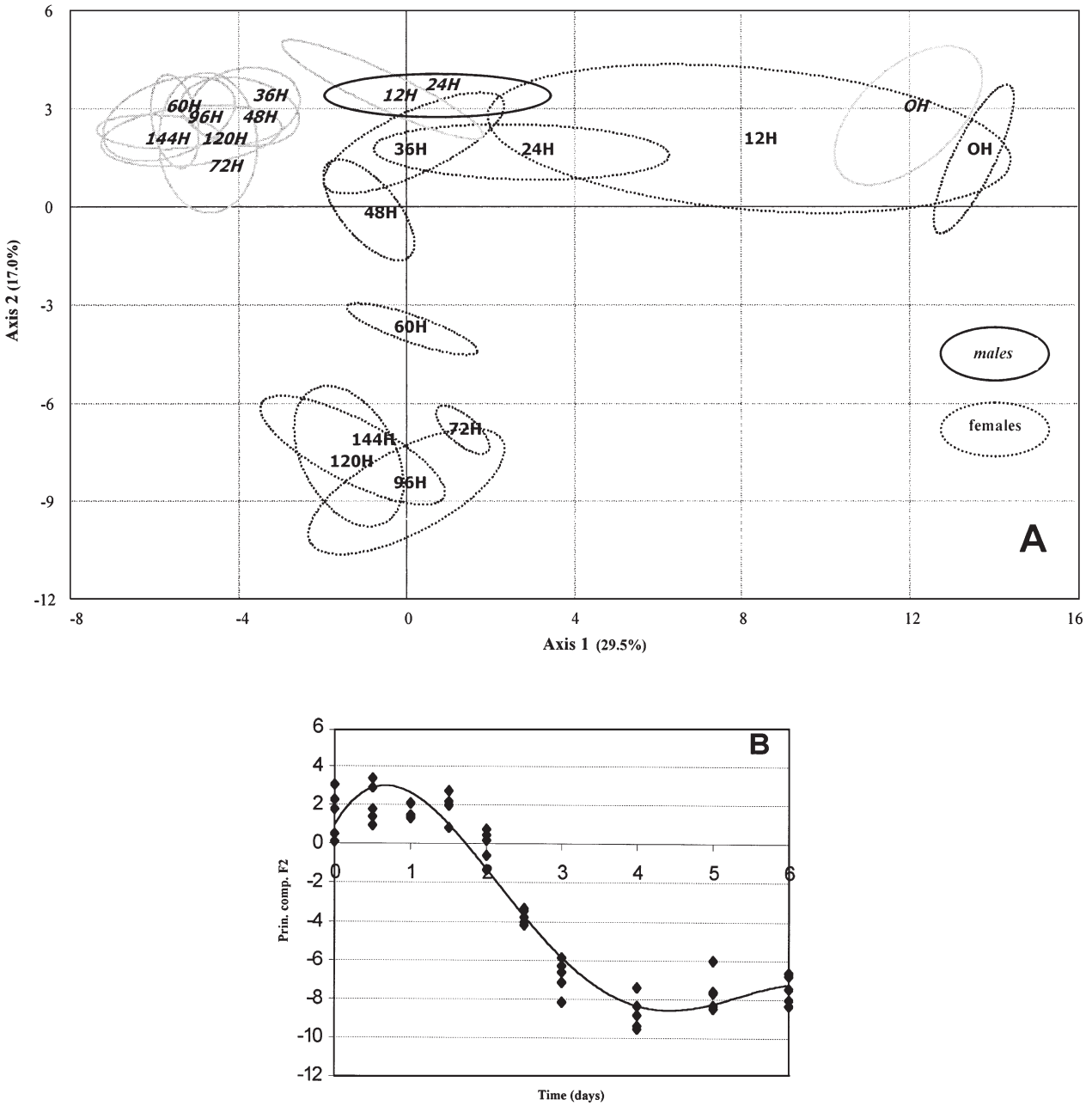

Fig. 8. Principal component analysis (A) of the 116 variables (relative proportions of HCs) for 100 internal lipid extracts from 50 males (5 individuals for the 10 times) and 50 females (5 individuals for the 10 times) of *M. domestica*), ellipses of confidence (per time group) are provided with a risk of 5%. In B: Fitting of a 4th order polynomial (*y*) expressing the relation between the time (horizontal axis) and the second principal component F2 (vertical axis).

The role of hemolymph in transporting cuticular hydrocarbons and hydrocarbon pheromones in insects has only recently become fully appreciated. Older models of hydrocarbon formation showed epidermal cells (oenocytes) synthesizing and transporting hydrocarbons directly to the surface (Hadley, 1984). In the housefly, the role of hemolymph is most clearly seen when (*Z*)-9-tricosene production is initiated. (*Z*)-9-tricosene first accumulates in the hemolymph, and then after a number of hours, is observed on the surface of the insect. The result of the modeling of the delay between internal and external composition gave a good timing for transport from the oenocytes and their transport onto the epicuticle. Surprisingly, the delay is longer than expected. More than 24 h are necessary for transportation and regulation of that transport. The delay appears to decrease with age, which probably means that the biosynthesis stops after the vitellogenesis.

The results presented herein show that the C23 epoxide and ketone are absent internally in the insect, but

accumulate on the surface. This finding suggests that hydrocarbon in the hemolymph is taken by epidermal cells. The cytochrome P450 that converts the (*Z*)-9-tricosene to the epoxide and ketone (Ahmad et al., 1987) is probably located in these cells, where a portion of the alkene is metabolized to the epoxide and ketone as they pass through to the surface of the insect. These data also suggest that the synthesis of (*Z*)-9-tricosene occurs in cells different from those that convert the alkene to the epoxide and ketone. The possibility that oxidative processes occur on the surface of the insect appears unlikely since microsomal cytochrome P450s have been implicated in the conversion of (*Z*)9-23:1 Hyd to epoxide.

Another clear example of the transport of cuticular hydrocarbon and pheromone in the hemolymph is the tiger moth, *Holomelina aurantiaca*. The adult female simultaneously synthesizes short-chain hydrocarbon that serve as volatile sex pheromone components and long-chain hydrocarbons that are deposited on the epicuticle (Schal et al., 1998a). All hydrocarbons are loaded onto a hemolymph high-density lipophorin. 2-Methylheptadecane and related pheromone homologs of similar chain length are specifically deposited into pheromone glands that open and emit the pheromone near the ovipositor. Long-chain hydrocarbons, on the other hand, appear on the epicuticular surface. In this insect the profile of "internal" hydrocarbon is different from "hemolymph" hydrocarbon because specific hydrocarbons are sequestered in an internal gland. This, however, is much different from most reports, which show that internal and epicuticular hydrocarbons are relatively similar (Schal et al., 1998b). In contrast to the "spatial" (tissue) dissociation of unique hydrocarbons in the tiger moth, we see a "temporal" dissociation of unique hydrocarbons in the female housefly, as they first appear in the hemolymph and later on the surface of the insect.

Acknowledgements

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