Sexual Dimorphism in Scent Substances and Cuticular Lipids of Adult *Papilio protenor* Butterflies

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Z. Naturforsch. **67c**, 331–341 (2012); received July 21, 2011/February 13, 2012

Adults of *Papilio protenor demetrius* emit a faint odour; the male odour is notably stronger than that of the females. The extracts of whole individuals of each sex comprised 53 compounds regarded as cuticular lipid components, of which the 17 major compounds were straight-chain alkanes and alkenes with 23–31 carbon atoms, higher fatty acids, long-chain aliphatic ketones, squalene, and cholesterol. However, highly volatile compounds were not detected in the whole individual extracts. Eight of the 17 major compounds showed a significant sex difference in relative abundance per individual. Principal component analysis, using the major compounds as variables, revealed a marked sexual dimorphism in the chemical composition of cuticular lipids. From the extracts of 10 dissected individuals of each sex, 21 highly volatile compounds were identified in amounts of less than 200 ng/individual. Among them, linalool and 2,3-butanediol showed a significantly larger amount in males than in females, indicating that the adult odour is also sexually dimorphic. Moreover, both sexes shared several odoriferous compounds, such as heptanal, nonanal, methyl salicylate, benzyl alcohol, and benzoic acid. The faint odour of *P. protenor* adults, perceivable by the human nose, appears to originate from these volatile compounds.

Key words: Papilio protenor demetrius, Adult Scent, Cuticular Lipid

Introduction

Many insects demonstrate species specificity and/or sexual dimorphism in the chemical composition of secretions and cuticular lipids, by which particular compounds frequently function as chemical signals in intraspecific and interspecific communications (Steiger *et al*., 2011). Some butterfly species have a characteristic odour in the adult stage, which is usually specific to the males. Several compounds that contribute to the odour are believed to serve as sex pheromones for the mating behaviour. Many species in the Danaidae family use pyrrolizidine alkaloid derivatives (*e.g*., danaidone and viridifloric β-lactone) as aphrodisiac pheromones (Honda, 2008), while *Heliconius melpomene* (Heliconiidae) utilizes β-ocimene for antiaphrodisiac purposes (Schulz *et al*., 2008). In several *Pieris* species (Pieridae), citral, ferrulactone, and brassicalactone have been identified as aphrodisiac signals (Andersson *et al*., 2007; Yildizhan *et al*., 2009), while methyl salicylate and benzyl cyanide were found to be the antiaphrodisiacs (Andersson *et al*., 2000, 2003).

In contrast to odour, cuticular lipids have been little investigated in adult butterflies. The chemical composition has been determined in a limited number of species, such as the genera of *Pieris* (Arsene *et al*., 2002; Yildizhan *et al*., 2009) and *Colias* (Grula *et al*., 1980) in Pieridae, the genus *Lasiommata* in Nymphalidae (Dapporto, 2007), and the genus *Danaus* in Danaidae (Hay-Roe *et al*., 2007). The cuticular compounds identified in these species are long-chain aliphatic hydrocarbons, alcohols, ketones, carboxylic acids, esters, and sterols.

The Papilionidae, commonly called swallowtail butterflies, comprise more than 500 species (Aubert *et al*., 1999). Although they are the smallest group of the four major subdivisions of butterflies, Papilionidae have been intensely studied for various reasons, such as speciation, host shift, and mimicry. Adults of several papilionid species, particularly males, emit an odour detectable by the human nose. Because papilionid butterflies sharing the same wing colour are often sympatric,

they might depend on chemical signals for communication within and between species.

Papilio protenor is a Rutaceae-feeding papilionid species occurring from Southeast Asia to Japan. In Japan, this swallowtail species is categorized into two subspecies, *i.e.*, *sitalkes* Fruhstorfer living south of the Okinawa Islands and *demetrius* Stoll occurring from the Amami Islands north to the Mainland. Adults have overall black coloration and emit a faint odour, which is more conspicuous in males than in females. On the Nansei Islands including the Amami and the Okinawa Islands, *P. protenor* is sympatric with the related species possessing black coloration, *Papilio polytes* (Zakharov *et al.*, 2004; Shirôzu, 2006). *P. polytes* adults exhibit significant sex differences in both scent substances and cuticular hydrocarbons (Ômura and Honda, 2005). To consider the possibility of intra- and interspecific communication through chemical signals, it is necessary to investigate the chemical nature of *P. protenor* first. The aims of this study were (1) to identify the scent substances of the adults and (2) to determine whether the chemical composition of *P. protenor* differs between the sexes.

Material and Methods

Insects

Adults of *P. protenor demetrius* were obtained from our laboratory stock cultures that originated from females collected in Hiroshima Prefecture, Japan. Larvae were reared at 25 °C under a photoregime of 16 h light/8 h dark on fresh leaves of *Zanthoxylum ailanthoides* and *Citrus* spp. Within 24 h of eclosion, adults were sexed and kept individually in plastic containers (30 cm × 25 cm × 20 cm). They were fed with 20% aqueous sucrose solution 2 d after eclosion.

Extraction

Three days after eclosion, adults were frozen to death at –20 °C, and extraction with purified (twice distilled) dichloromethane for 3 min was performed according to the following protocol: (1) Ten male and 10 female adult butterflies were individually subjected to extraction with 2 mL of solvent each (whole-individual extracts). (2) To identify small amounts of volatile compounds, 10 individuals of each sex were dissected into three parts (forewings, hindwings, and body), and each part was soaked in 15 mL of the solvent (dissected-individual extracts).

All extractions were conducted at room temperature. The extracts were subsequently filtered, and concentrated to less than 1 mL *in vacuo* and then to 100 μL under a nitrogen stream at 10 °C. The concentrated extracts were stored at –20 °C until chemical analyses.

Dimethyl disulfide treatment

The major alkenes in the extract were derivatized to dimethyl disulfide (DMDS) adducts to determine the position of double bonds (Buser *et al.*, 1983). The reagent DMDS was purchased from Tokyo Chemical Industry (Tokyo, Japan). The extracts from three whole individuals of each sex were retrieved by column chromatography on 500 mg silica gel (6 nm, 70–120 mesh; Katayama Chemical Industry, Osaka, Japan), and the hydrocarbon compounds were obtained by eluting with 5 mL of *n*-hexane. The *n*-hexane fraction was concentrated at room temperature to 100 μL solution. Twenty μL of DMDS and 5 μL of 1% iodine solution in diethyl ether were added to the *n*-hexane solution. The mixture was sealed in an 1-mL glass tube and incubated at 40 °C for 12 h. Next, 100 μL of 5% aqueous $Na_2S_2O_3$ solution were added, and then 200 μL *n*-hexane were used for extraction. The extracts were dehydrated with anhydrous Na_2SO_4 and maintained at –20 °C until chemical analyses.

Chemical analyses

The crude and DMDS-treated extracts were analysed by gas chromatography-mass spectrometry (GC-MS) at 70 eV using a Shimadzu QP5000 mass spectrometer coupled with a Shimadzu GC-17A gas chromatograph (Shimadzu Corp., Kyoto, Japan). The whole-individual and DMDS-treated extracts were analysed with a J & W Scientific DB-1 fused-silica capillary column (0.25 mm I.D. × 15 m, 0.25 μm film thickness), using an injector at 280 °C, an interface at 280 °C, and the following oven temperature program: 50 °C (held initially for 1 min) to 280 °C (held finally for 10 min) at 10 °C/min. The dissected-individual extracts were analysed with a Varian CP-Wax 58 CB fused-silica capillary column (0.25 mm I.D. × 25 m, 0.20 μm film thickness), using an injector at 230 °C, an interface at 250 °C, and the following oven temperature program: 40 °C (held

initially for 2 min) to 200 °C at 5 °C/min. All samples were splitlessly injected using helium as the carrier gas. The Kovats retention index (RI) was calculated for each compound identified in the whole-individual extracts on the basis of the retention time (RT) of *n*-alkanes (C_{16}–C_{38}) under the same analytical conditions. Identification of the compounds was based on comparisons of the retention times and mass spectra with those of authentic standards unless otherwise noted. Authentic standards were purchased from Tokyo Chemical Industry and Sigma-Aldrich (St. Louis, MO, USA). Tentative identification was done by comparisons with RI data and mass fragmentation of other published data. Quantitative estimates of the individual compounds were based on comparisons of the peak intensities of the total-ion chromatogram with those of 100 ng pentacosane.

Statistical analysis

To assess sex differences in the chemical composition of the whole-individual extracts, we used the relative abundance (%) of the compounds, which was calculated by dividing the peak intensity of each compound by the sum of that of all compounds. The compounds showing more than 1% of the mean value in both sexes were regarded as the major compounds. For the major compounds, sex differences in the relative abundance were assessed by the Mann-Whitney *U* test. Moreover, principal component analysis (PCA) was carried out using the major compounds as variables (R version 2.8.1, R Project).

Results

Compounds in whole-individual extracts

Typical total-ion chromatograms of the whole-individual extract are shown in Fig. 1. The extract contained 53 compounds, in which long-chain aliphatic hydrocarbons were predominant but highly volatile compounds were absent (Table I). Among the 17 major compounds (Table II), six *n*-alkanes with carbon numbers of 23, 25, 27, 28, 29, and 31, respectively, two higher fatty acids (hexadecanoic and octadecanoic acids, respectively), one acyclic triterpene (squalene), and one sterol (cholesterol) were identified on the basis of comparison with authentic standards. Other major compounds were three alkenes with 25 and 27 carbon atoms, two higher aliphatic ketones, and two unknown compounds.

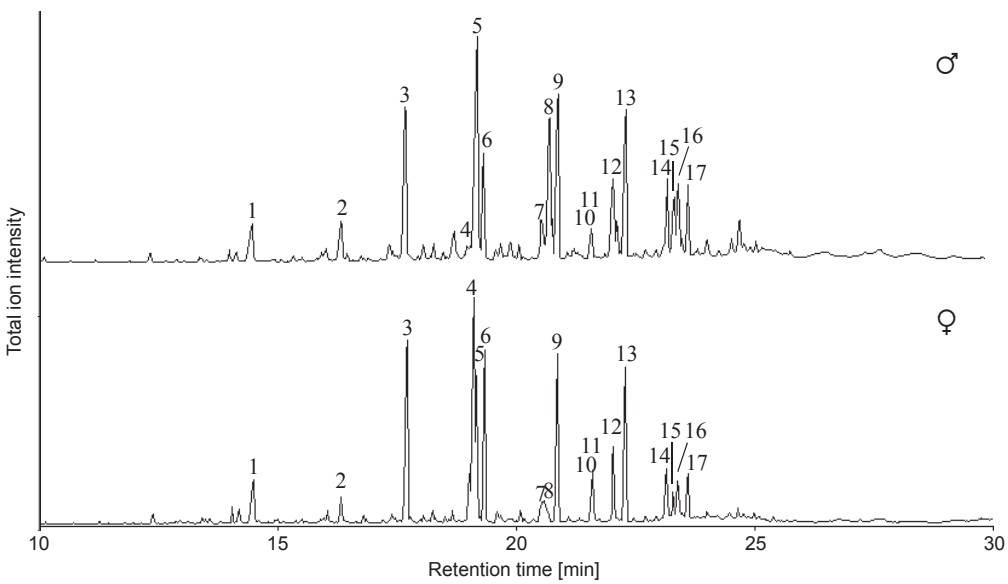

Fig. 1. Typical total-ion chromatograms obtained from crude extracts of whole *Papilio protenor* male and female adults. Chromatograms were run on a J & W Scientific DB-1 capillary column (0.25 mm I.D. × 15 m), programmed from 50 °C (held initially for 1 min) to 280 °C (held finally for 10 min) at 10 °C/min. Peak numbers correspond to compound numbers in Table II.

Table I. Relative abundance of 53 compounds in the whole-individual extract of *Papilio protenor* adults.

Compound	RI[a]	Relative abundance (%, mean ± SD)		Identification[b]	Diagnostic ions[c]
		Male (N = 10)	Female (N = 10)		
Tetradecanoic acid	1765	0.30 ± 0.12	0.55 ± 0.22	Std	
9-Hexadecenoic acid	1927	0.29 ± 0.14	0.69 ± 0.20	Std	
Hexadecanoic acid	1957	1.96 ± 0.39	3.13 ± 0.87	Std	
Eicosane	2000	0.18 ± 0.12	0.22 ± 0.07	Std	
Heptadecanoic acid	2044	0.22 ± 0.07	0.19 ± 0.09	Std	
Heneicosane	2100	0.15 ± 0.06	0.16 ± 0.06	Std	
9,12-Octadecadienoic acid	2111	0.30 ± 0.19	0.44 ± 0.18	Std	
9-Octadecenoic acid	2120	0.46 ± 0.21	0.86 ± 0.22	Std	
Octadecanoic acid	2152	1.69 ± 0.30	1.71 ± 0.45	Std	
Docosane	2200	0.44 ± 0.43	0.31 ± 0.12	Std	
7-Tricosene	2266	0.57 ± 0.40	0.65 ± 0.38	DMDS	43(B), 55, 69, 83, 97, 111, 125
Tricosane	2300	7.42 ± 1.96	6.71 ± 2.56	Std	
4,8,12,16-Tetramethylhepta-decan-4-olide	2310	0.41 ± 0.42	0.14 ± 0.09	Ref. (de Felício *et al.*, 2010)	43, 55, 71, 99(B), 114, 126, 324(M^+)
Eicosanoic acid	2348	0.60 ± 0.13	0.48 ± 0.17	Std	
7-Tetracosene	2376	0.34 ± 0.13	0.20 ± 0.10	DMDS	43(B), 55, 69, 83, 97, 111, 125
Tetracosane	2400	0.38 ± 0.14	0.37 ± 0.08	Std	
6,9-Pentacosadiene	2461	0.54 ± 0.16	1.15 ± 0.42	Ref. (Krokos *et al.*, 2001)	43(B), 55, 67, 81, 96, 110, 124, 348(M^+)
9-Pentacosene	2468	0.21 ± 0.12	3.65 ± 2.51	DMDS	43(B), 55, 69, 83, 97, 111, 125, 350(M^+)
7-Pentacosene	2482	8.28 ± 0.99	4.37 ± 3.11	DMDS	43(B), 55, 69, 83, 97, 111, 125, 350(M^+)
5-Pentacosene	2488	0.65 ± 0.32	0.47 ± 0.26	DMDS	43(B), 55, 69, 83, 97, 111, 125, 350(M^+)
Pentacosane	2500	5.40 ± 2.14	4.77 ± 2.12	Std	
11/13-Methylpentacosane	2536	1.04 ± 0.89	0.90 ± 0.31	Ref. (Lockey, 1991)	43, 57(B), 71, 85, 99, 113, 141, 168, 252
Docosanoic acid	2548	0.53 ± 0.18	0.32 ± 0.21	Std	
Hexacosane	2600	0.75 ± 0.29	0.81 ± 0.11	Std	
Unknown	2658	1.51 ± 0.40	1.24 ± 0.61		43(B), 57, 69, 83, 97, 111, 127, 281
6,9-Heptacosadiene	2662	0.72 ± 0.31	0.24 ± 0.22	Ref. (Suiter *et al.*, 1996)	43(B), 55, 67, 81, 96, 110, 124, 376(M^+)
8-Pentacosanone	2663	1.03 ± 0.35	0.89 ± 0.13	Ref. (Leonhardt *et al.*, 1991)	43(B), 57, 71, 85, 127, 143, 267, 282, 366(M^+)
9-Heptacosene	2668	0.35 ± 0.12	0.43 ± 0.20	DMDS	43(B), 55, 69, 83, 97, 111, 125, 378(M^+)
7-Heptacosene	2678	3.76 ± 1.64	0.57 ± 0.29	DMDS	43(B), 55, 69, 83, 97, 111, 125, 378(M^+)
5-Heptacosene	2686	0.83 ± 0.41	0.21 ± 0.11	DMDS	43(B), 55, 69, 83, 97, 111, 125, 378(M^+)
Heptacosane	2700	10.88 ± 2.41	13.25 ± 1.71	Std	
11/13-Methylheptacosane	2731	0.47 ± 0.19	0.42 ± 0.16	Ref. (Lockey, 1991)	43, 57(B), 71, 85, 99, 113, 141, 168, 252
Octacosane	2800	1.04 ± 0.27	1.19 ± 0.12	Std	
Squalene	2804	3.11 ± 1.39	4.16 ± 0.99	Std	
9/10-Heptacosanone	2865	4.23 ± 0.66	4.14 ± 0.70	Ref. (Yasui *et al.*, 2003; Böröczky *et al.*, 2008)	43(B), 57, 71, 141, 155, 171, 267, 281, 394(M^+)
7-Nonacosene	2875	0.80 ± 0.51	0.14 ± 0.04	DMDS	43(B), 55, 69, 83, 97, 111, 125
Nonacosane	2900	11.71 ± 1.25	14.62 ± 2.23	Std	
Unknown	2965	0.34 ± 0.10	0.31 ± 0.08		43(B), 57, 71, 141, 155, 281, 309
Triacontane	3000	0.46 ± 0.10	0.43 ± 0.16	Std	
Unknown	3034	3.87 ± 0.88	3.01 ± 0.69		43(B), 57, 71, 155, 169, 239, 253, 420

Table I continued.

Compound	RI[a]	Relative abundance (%, mean ± SD)		Identification[b]	Diagnostic ions[c]
		Male (N = 10)	Female (N = 10)		
Cholesterol	3063	1.77 ± 0.38	1.72 ± 0.54	Std	
9/10-Nonacosanone	3070	3.19 ± 0.92	2.85 ± 0.73	Ref. (Muckensturm *et al.*, 1997; Böröczky *et al.*, 2008)	43(B), 57, 71, 141, 155, 281, 295, 309, 422(M^+)
α-Tocopherol	3082	0.57 ± 0.40	0.33 ± 0.20	Std	
Hentriacontane	3100	4.47 ± 0.93	4.21 ± 1.23	Std	
Campesterol	3162	0.63 ± 0.25	0.51 ± 0.18	Std	
Dotriacontane	3200	1.00 ± 1.26	0.93 ± 0.87	Std	
β-Sitosterol	3267	0.93 ± 0.37	0.62 ± 0.21	Std	
Tritriacontane	3300	0.51 ± 0.42	0.46 ± 0.31	Std	
Tetratriacontane	3400	0.14 ± 0.13	0.07 ± 0.11	Std	
Pentatriacontane	3500	0.09 ± 0.04	0.36 ± 0.62	Std	
Hexatriacontane	3600	0.76 ± 0.79	0.19 ± 0.28	Std	
Heptatriacontane	3700	0.25 ± 0.24	0.45 ± 0.51	Std	
Octatriacontane	3800	1.33 ± 0.67	0.32 ± 0.44	Std	

[a] Retention index on a DB-1 column.
[b] Std, identification based on authentic standard; DMDS, tentative identification using DMDS treatment; Ref., the published data in parentheses.
[c] Diagnostic ions are shown in the components tentatively identified or unidentified. B and M^+ represent the base and molecular ions of the component.

Extracts from males contained large amounts of two alkenes (peaks 5 and 8). Because the molecular ions [M^+] were at m/z 350 and 378, respectively, peaks 5 and 8 were identified as pentacosene and heptacosene. DMDS treatment of the extract generated two new vicinal dithiomethyl ether derivatives with [M^+] and fragment ions at m/z 444 and 145 [$C_7H_{14}SCH_3^+$], and 472 and 145 [$C_7H_{14}SCH_3^+$], respectively. Therefore, peaks 5 and 8 were determined to be 7-pentacosene and 7-heptacosene, respectively. Extracts from females contained two major alkene compounds sharing an [M^+] ion at m/z 350 (peaks 4 and 5). Although the DMDS derivatives shared an [M^+] ion at m/z 444, there was a significant ion at m/z 173 [$C_9H_{18}SCH_3^+$] and the other at m/z 145 [$C_7H_{14}SCH_3^+$]. These results demonstrated that the major alkenes of the females were 9-pentacosene (peak 4) and 7-pentacosene (peak 5), respectively. The RI values of each of these alkenes correlated with those given in published data (Drijfhout and Groot, 2001; Geiselhardt *et al.*, 2009).

Peaks 7, 12, 14, and 16 were eluted with acetone from the column after eluting with *n*-hexane, indicating that these compounds had a higher polarity than hydrocarbons. The peak at RT 22.18 min (peak 12) showed a base ion at m/z 43 (100) and an [M^+] ion at m/z 394. Moreover, there were two pairs of fragment ions at m/z 155 (10) and 267 (4) for [$C_9H_{19}CO^+$] and [$C_{17}H_{35}CO^+$], and at m/z 141 (13) and 281 (5) for [$C_8H_{17}CO^+$] and [$C_{18}H_{37}CO^+$], respectively. The former pair showed maximal intensity at RT 22.175 min, and the latter pair at RT 22.183 min. These fragment ions indicated the presence of a carbonyl group at C-10 and C-9, and this peak (peak 12) was assigned to a mixture of 10- and 9-heptacosanones (Yasui *et al.*, 2003; Böröczky *et al.*, 2008). The peak at RT 23.55 min (peak 16) displayed an [M^+] ion at m/z 422 and some diagnostic ions at m/z 43 (100), 57 (75), 71 (55), 85 (21), 141 (11), 155 (11), 295 (4), and 309 (3). Among the diagnostic ions, one pair of the ions at m/z 155 ([$C_9H_{19}CO^+$]) and 295 ([$C_{19}H_{39}CO^+$]) reached maximal intensity at RT 23.550 min, while another pair of the ions at m/z 141 ([$C_8H_{17}CO^+$]) and 309 ([$C_{20}H_{41}CO^+$]) reached maximal intensity at RT 23.558 min. These results demonstrated that peak 16 was a mixture of 10- and 9-nonacosanones (Muckensturm *et al.*, 1997; Böröczky *et al.*, 2008). The RI value of each compound was identical to that in published data (Yasui *et al.*, 2003; Böröczky *et al.*, 2008). Peak 14 had some diagnostic ions at

Table II. Major compounds of cuticular lipids from *Papilio protenor* adults.

No.	Compound	RT [min][a]	RI[b]	Amount per individual (N = 10) [μg, mean ± SD]		Relative abundance (%, mean ± SD)	
				Male	Female	Male	Female
1	Hexadecanoic acid	14.63	1957	2.0 ± 0.5	2.0 ± 0.8	1.96 ± 0.39	3.13 ± 0.87**[c]
2	Octadecanoic acid	16.48	2152	1.8 ± 0.6	1.2 ± 0.7	1.69 ± 0.30	1.71 ± 0.45
3	Tricosane	17.81	2300	7.9 ± 3.0	5.0 ± 3.6	7.42 ± 1.96	6.71 ± 2.56
4	9-Pentacosene[d]	19.19	2468	0.2 ± 0.2	2.8 ± 2.8	0.21 ± 0.12	3.65 ± 2.51***
5	7-Pentacosene[d]	19.30	2482	8.8 ± 3.0	3.2 ± 2.5	8.28 ± 0.99*	4.37 ± 3.11
6	Pentacosane	19.45	2500	5.8 ± 2.7	3.7 ± 3.1	5.40 ± 2.14	4.77 ± 2.12
7	Unknown	20.68	2658	1.6 ± 0.7	0.9 ± 0.7	1.51 ± 0.40	1.24 ± 0.61
8	7-Heptacosene[d]	20.83	2678	4.2 ± 2.5	0.4 ± 0.3	3.76 ± 1.64***	0.57 ± 0.29
9	Heptacosane	21.00	2700	11.2 ± 3.1	8.9 ± 3.4	10.88 ± 2.41	13.25 ± 1.71*
10	Octacosane	21.70	2800	1.1 ± 0.5	0.8 ± 0.5	1.04 ± 0.27	1.19 ± 0.12
11	Squalene	21.73	2804	3.2 ± 1.6	3.1 ± 2.1	3.11 ± 1.39	4.16 ± 0.99*
12	9/10-Heptacosanone[d]	22.18	2865	4.4 ± 1.3	2.9 ± 1.7	4.23 ± 0.66	4.14 ± 0.70
13	Nonacosane	22.44	2900	12.2 ± 3.0	9.8 ± 4.0	11.71 ± 1.25	14.62 ± 2.23**
14	Unknown	23.31	3034	4.1 ± 1.5	2.2 ± 1.7	3.87 ± 0.88**	3.01 ± 0.69
15	Cholesterol	23.50	3063	1.9 ± 0.7	1.1 ± 0.7	1.77 ± 0.38	1.72 ± 0.54
16	9/10-Nonacosanone[d]	23.55	3070	3.5 ± 1.7	2.0 ± 1.5	3.19 ± 0.92	2.85 ± 0.73
17	Hentriacontane	23.75	3100	4.8 ± 1.9	3.2 ± 2.4	4.47 ± 0.93	4.21 ± 1.23

[a] Retention time on a DB-1 column (refer to the text for the analytical conditions).
[b] Retention index on a DB-1 column.
[c] Significant sex difference (Mann-Whitney U test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).
[d] Tentative identification.

m/*z* 43 (100), 57 (65), 71 (49), 85 (16), 141 (13), 155 (15), 169 (5), 183 (5), and 239 (7). Although the [M^+] ion of this compound was not obtained, the overall fragmentation pattern was similar to those of higher aliphatic ketones. The diagnostic ions of peak 7 were at *m*/*z* 43 (100), 57 (78), 69 (41), 83 (32), 97 (34), 111 (10), 127 (14), and 281 (4). Given that the diagnostic ions at *m*/*z* 127 and 281 were produced by α-cleavage, peak 7 appeared from a long-chain monoepoxide, probably 7,8-epo-pentacosane (Krokos *et al.*, 2001).

Of the major compounds, heptacosane and nonacosane predominated at nearly 10 μg/individual, followed by tricosane and 7-pentacosene. Several compounds showed a significant sex difference in relative abundance. 7-Pentacosene, 7-heptacosene, and peak 14 were significantly more abundant in males than in females at the level of $P < 0.05$, $P < 0.001$, and $P < 0.01$, respectively (Mann-Whitney U test). In contrast, females showed significantly higher values for hexadecanoic acid ($P < 0.01$), 9-pentacosene ($P < 0.001$), heptacosane ($P < 0.05$), squalene ($P < 0.05$), and nonacosane ($P < 0.01$) than males (Mann-Whitney U test). PCA revealed that males and females were distributed in discrete positions (Fig. 2).

Volatile compounds of the dissected-individual extracts

Because volatile compounds were negligible in the whole-individual extracts, the dissected-individual extracts, comprising the wings and body, were analysed within a retention time of 30 min on a CP-Wax 58 CB column. While forewings and hindwings had very similar chromatographic patterns, the body showed a pattern somewhat different from the wings in each sex (Fig. 3). The dissected-individual extracts contained five aliphatic hydrocarbons with 21–24 carbon atoms within the quantitative range of 200 ng to 4 μg per individual. Moreover, we identified 21 volatile compounds: three aliphatic alcohols and ketol, two aliphatic aldehydes, eight aliphatic acids, one terpenoid alcohol, two aromatic hydrocarbons, three oxygenated aromatic compounds, and two nitrogenous compounds (Table III). 1,2-Dichlorobenzene and diethylene glycol and its derivatives (*e.g.*, monomethyl, monoethyl,

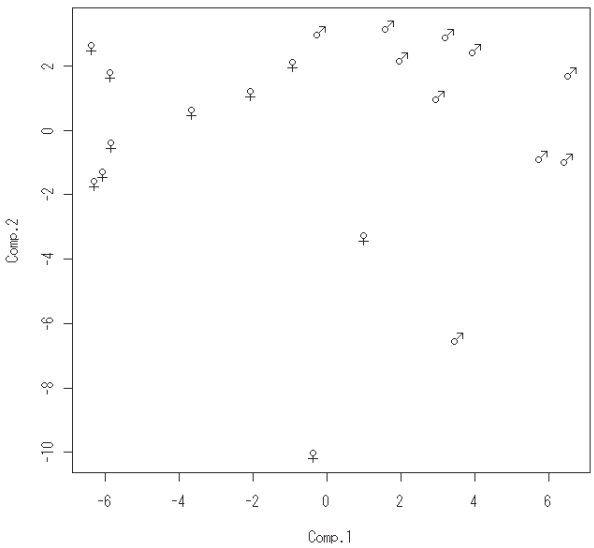

Fig. 2. Principal component analysis using the 17 major cuticular compounds of adult *Papilio protenor*. The 17 compounds were tricosane, pentacosane, heptacosane, octacosane, nonacosane, hentriacontane, 7-pentacosene, 9-pentacosene, 7-heptacosene, 9/10-heptacosanone, 9/10-nonacosanone, hexadecanoic acid, octadecanoic acid, squalene, cholesterol, and two unknown compounds.

and monobutyl ethers) were detected in the extracts at amounts of 50–500 ng/individual. These compounds were tentatively assigned to artifacts though we could not identify their sources.

Of the 21 volatile compounds, acetoin, 2-ethyl-1-hexanol, naphthalene, and nonanoic and undecanoic acids, respectively, were present at the relatively large quantity of >100 ng/individual, and were regarded as the major volatiles in both sexes. Interestingly, linalool and 2,3-butanediol were detected as the major volatiles from the males only (Table III). Moreover, two aliphatic aldehydes (heptanal and nonanal) and three oxygenated aromatic compounds (methyl salicylate, benzyl alcohol, and benzoic acid) were identified as minor scent substances (10–30 ng/individual) common to both sexes.

To assess a possible biased distribution in either wings or body, the wing/body ratio of quantities per individual was calculated for each compound. The ratio of <0.20 for acetoin indicated that the wings contained less than five times the amount of this compound than the body. In contrast, the ratio of >5.00 for linalool, 2,3-butanediol, and aliphatic acids with the carbon number of 5, 7, 8, 9, and 11 in the males, benzoic acid in the females, and acetamide in both sexes demonstrated that these compounds were concentrated mainly in the wings rather than in the body.

Discussion

The present study revealed that the whole-individual *P. protenor* extract consisted mainly of long-chain aliphatic compounds, including hydrocarbons, fatty acids, and ketones. Since the volatility of these compounds is very low at room temperature, most of them were regarded as cuticular lipid components (Lockey, 1988). The chemical composition of the cuticular lipids was sexually dimorphic, in that eight of the 17 major compounds displayed significant sex differences in relative abundance. Highly volatile compounds were not detected in the whole-individual extract, indicating that swallowtail adults possess some scent substances in negligible amounts. In the chemical analyses of the extracts from 10 individuals of each sex that had been dissected into three parts (forewings, hindwings, and body), we identified 21 volatile compounds in amounts of less than 200 ng/individual, together with five aliphatic hydrocarbons (C_{21}–C_{24} alkanes). Because other butterfly species have identical aliphatic hydrocarbons as cuticular substances (Hay-Roe *et al.*, 2007; Yildizhan *et al.*,

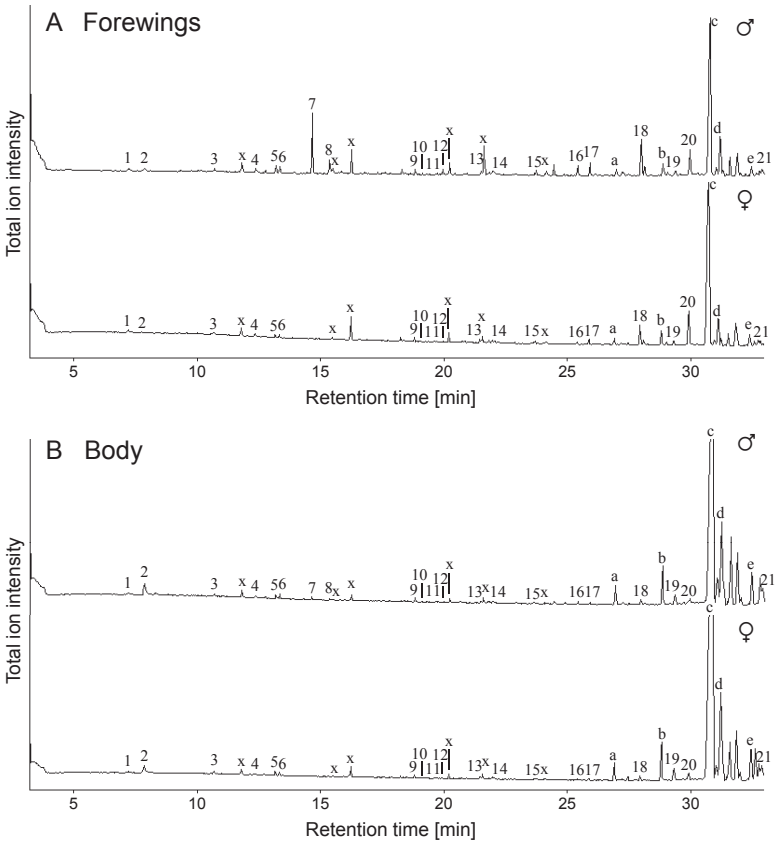

Fig. 3. Typical total-ion chromatograms obtained from crude extracts of the (A) forewings and (B) body of *Papilio protenor*. Chromatograms were run on a Varian CP-Wax 58 CB capillary column (0.25 mm I.D. × 25 m), programmed from 40 °C (held initially for 2 min) to 200 °C at 5 °C/min. Peak numbers correspond to compound numbers in Table III. Peaks labeled with letters are: a, heneicosane; b, docosane; c, tricosane; d, methyltricosane; e, tetracosane; and x, a possible artifact.

2009), these were also regarded as cuticular compounds of *P. protenor*. Among the volatile compounds, linalool and 2,3-butanediol displayed a significant male specificity, while heptanal, nonanal, methyl salicylate, benzyl alcohol, and benzoic acid were common to both sexes. The faint odour perceivable by the human nose seemed to originate from these volatile compounds.

To date, adult odours of three papilionid species have been investigated for their chemical composition. Male adults of *Atrophaneura alcinous* emit a strong odour, from which phenylacetaldehyde, 2-phenylpropanal, heptanal, 6-methyl-5-hepten-2-one, and linalool were identified (Honda, 1980). Phenylacetaldehyde, in particular, shows a distinctly male-biased distribution and predominant quantity (nearly 30 μg/male). In *Papilio machaon*, males have larger amounts of limonene, dodecane, and an unidentified sesquiterpene hydrocarbon than females (Ômura *et al.*, 2001). *Papilio polytes* has an odour qualitatively similar to that of *P. protenor*, because the major constituents are shared in similar quantities; notably, linalool is predominant in males but negligible in females (Ômura and Honda, 2005). Although several male-specific volatiles serve as close-range mating signals in particular species of Pieridae and Danaidae (Andersson *et al.*, 2007; Honda, 2008), these possible functions remain undetermined in Papilionidae. Further study is needed to clarify the semiochemical functions of linalool and 2,3-butanediol in *P. protenor*.

In addition to odoriferous compounds, *P. protenor* had several minor compounds with high

Table III. Distribution of volatile compounds of *Papilio protenor* adults.

No.	Compound	RT [min][a]	Average amount per individual [ng]									
			Male					Female				
			Fore-wings	Hind-wings	Body	Total	Wings/body[b]	Fore-wings	Hind-wings	Body	Total	Wings/body
1	Styrene	7.21	11	8	16	35	1.20	10	9	14	33	1.39
2	Acetoin	7.84	23	8	175	206	0.18	1	1	145	147	0.02
3	Heptanal	10.69	6	11	8	25	2.10	4	10	10	24	1.33
4	Acetic acid	12.33	22	38	20	80	2.98	11	26	14	51	2.58
5	2-Ethyl-1-hexanol	13.17	29	49	33	111	2.34	13	51	53	116	1.21
6	Nonanal	13.32	8	16	13	37	1.88	5	10	16	32	0.94
7	Linalool	14.63	120	15	10	146	13.61	N.D.[c]	N.D.	N.D.	N.D.	—
8	2,3-Butanediol	15.34	129	29	20	178	7.97	1	3	4	8	1.00
9	Naphtalene	18.80	33	48	46	127	1.77	23	36	50	110	1.19
10	Pentanoic acid	19.08	7	12	2	22	7.94	6	12	9	26	2.05
11	Methyl salicylate	19.71	4	6	6	16	1.73	2	11	6	18	2.27
12	Acetamide	19.92	26	24	6	55	8.97	4	8	2	13	7.01
13	Hexanoic acid	21.46	20	30	11	61	4.47	15	30	19	64	2.42
14	Benzyl alcohol	22.07	4	5	3	12	3.38	3	8	5	16	2.34
15	Heptanoic acid	23.72	18	22	8	48	5.06	8	12	9	29	2.26
16	2-Pyrrolidinone	25.39	34	34	19	87	3.67	7	12	9	28	2.02
17	Octanoic acid	25.88	35	43	12	90	6.61	17	26	12	55	3.72
18	Nonanoic acid	27.95	100	92	25	217	7.64	51	77	31	159	4.13
19	Decanoic acid	29.01	5	6	5	16	2.12	5	6	3	15	3.37
20	Undecanoic acid	29.92	52	72	21	146	5.87	83	99	40	221	4.57
21	Benzoic acid	32.72	4	4	3	11	2.36	7	12	2	21	8.97

[a] Retention time on a CP-Wax 58 CB column (refer to the text for the analytical conditions).
[b] Ratio of all wings per body in amount of component.
[c] Not detected (below 1 ng, if any).

polarity, *e.g.*, acetamide, 2-pyrrolidinone, acetoin, 2,3-butanediol, 2-ethyl-1-hexanol, and short-chain fatty acids. These compounds are also present in adults of other papilionid species (Ômura *et al.*, 2001; Ômura and Honda, 2005). Moreover, acetamide is frequently found in lepidopteran glands and is considered a nonspecific body substance (Attygalle *et al.*, 1987). Acetoin is specific to females in *P. polytes*, though it was male-specific in *P. protenor* (Ômura and Honda, 2005). Acetoin and 2,3-butanediol are also present in the secretions of the female European chafer *Rhizotrogus majalis* (Nojima *et al.*, 2003). The presence of short-chain fatty acids with 6–10 carbon atoms is described in the hairpencil scents of African danaine butterflies (Schulz *et al.*, 1993). Because short-chain fatty acids often exhibit strong antimicrobial activities (Smith and Grula, 1982), these compounds might serve as defensive substances.

Particular volatile compounds, such as linalool and 2,3-butanediol in the males, showed significantly larger amounts in the wings than in the body, suggesting that unidentified secretory organs are present mainly in the wings of *P. protenor*. A similar localization to the wings has been reported for linalool in *P. polytes* (Ômura and Honda, 2005) and for phenylacetaldehyde in *A. alcinous* (Honda, 1980). Indeed, characteristic microstructures, which are considered to be scent-producing organs, are found on the hindwings of male adults of *A. alcinous* (Honda, 1980). In contrast, the scent substances of *P. machaon* show a different distribution: linalool and geranylacetone are concentrated in the wings, while heptadienal and an unidentified sesquiterpene hydrocarbon predominate in the body (Ômura *et al.*, 2001). Among the volatile compounds of *P. protenor*, acetoin was limited to the body. Since the same distribution pattern was shown in *P. machaon* and *P. polytes* (Ômura *et al.*, 2001; Ômura and Honda, 2005), acetoin appears to be a commonly occurring compound in the body of papilionid butterflies.

The major constituents of the cuticular lipids of *P. protenor* were linear hydrocarbons with 23–31 carbon atoms, hexadecanoic and octadecanoic acids, long-chain aliphatic ketones with 27 and

29 carbon atoms, and cholesterol. Corresponding with this, linear alkanes with 23–29 carbon atoms, Δ7-alkenes with 25 and 27 carbon atoms, and hexadecanoic acid were also identified as the major substances of *P. polytes* (Ômura and Honda, 2005). In addition, the linear alkanes with 25, 27, and 29 carbon atoms are also the major cuticular hydrocarbons of *Pieris* and *Danaus* butterfly species (Arsene *et al.*, 2002; Hay-Roe *et al.*, 2007). Although sterols are minor constituents of cuticular lipids in some insects (Lockey, 1988), *P. protenor* adults were found to contain not only cholesterol but also campesterol and β-sitosterol. These sterols are present in the hairpencil secretion of *Idea leuconoe* (Danaidae) (Nishida *et al.*, 1996) and in the forewings of *Pieris* species (Yildizhan *et al.*, 2009).

Two volatile compounds (linalool and 2,3-butanediol) and eight major constituents of the cuticular lipids showed significant sexual differences in relative abundance. Consequently, like *P. polytes* adults (Ômura and Honda, 2005), *P. protenor* adults had a marked sexual dimorphism in the chemical composition of both the odour and cuticular lipids. However, *P. protenor* differed from *P. polytes* in the cuticular hydrocarbon profile, particularly in the relative abundance of Δ7- and Δ9-alkenes (Ômura and Honda, 2005). These results agree with previous results indicating that closely related butterfly species are distinguishable by their cuticular hydrocarbon profiles (Dapporto, 2007; Hay-Roe *et al.*, 2007).

In several orders of the Insecta, the body surface often includes close-range pheromones to mediate contact and copulation behaviours. Adult lepidopterans make physical contact with the partner's body during the mating process, in which their cuticular lipids conceivably act in close-range recognition. Indeed, such contact pheromones have been identified from body scales of a few moth species, including *Orgyia leucostigma* and *Anarsia lineatella* (Grant *et al.*, 1987; Schlamp *et al.*, 2005). Two closely related sulfur butterflies, *Colias eurytheme* and *C. philodice*, are well known to use male cuticular compounds as mating signals, in which three hydrocarbons (heptacosane, nonacosane, and 13-methylheptacosane) and three hexyl esters (hexyl myristate, palmitate, and stearate) mediate not only mating within the species but also reproductive isolation between the species (Grula *et al.*, 1980; Sappington and Taylor, 1990). Conceivably, some compounds present in the body surface of *P. protenor* may induce conspecific recognition and interspecific discrimination. Semiochemical functions of the cuticular lipids of *P. protenor* need to be investigated.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) to H. O. (No. 22780047).

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