Cuticular lipids of adults and puparia of the Australian sheep blowfly *Lucilia cuprina* (Wied.)

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ABSTRACT The presence of a strong contact component in the sex and ovipositing behavior of the sheep blowfly *Lucilia cuprina* Wied. prompted an investigation into the chemical composition of the cuticular wax of the adult male and female flies as well as that of the blowfly puparia. Thin-layer chromatography indicated that the lipids in all the waxes examined comprise hydrocarbons, nonglyceryl esters, triglycerides, free fatty acids, and hydroxy compounds, probably diglycerides and monoglycerides. Phospholipids were not detected. Straight- and branched-chain saturated compounds, the latter often predominating, are present in the hydrocarbon, free fatty acid, and ester fractions. Unsaturated molecules were absent.

The hydrocarbons resemble those of the cricket to some extent, but the absence of unsaturated compounds is in striking contrast to both the cricket and the cockroach. Pheromones may be present in the low molecular weight fatty acids obtained on brief extraction of the insects.

SUPPLEMENTARY KEY WORDS cuticular lipids · hydrocarbons · free fatty acids · esters · branched-chain · pheromones

In recent years the chemical composition of a number of insect cuticular lipids has been described, including those of the cricket *Anabrus simplex* Hald. (1), the cockroach *Periplaneta americana* L. (2), and the larva of the mealworm *Tenebrio molitor* L. (3). Such investigations have resulted in speculations about the role played by the waxes in resisting water loss by insects. More recently, Bartell, Shorey, and Browne (4) and Browne, Bartell, and Shorey (5) have demonstrated that both sex attraction and behavior leading to group oviposition of the Australian sheep blowfly *Lucilia cuprina* Wied. were pheromone-mediated and were more intense under conditions of close contact between flies.

The origin of these pheromones has not been determined. However, the presence of a strong contact component in each type of behavior suggests that pheromones are closely associated with the cuticle of the blowfly. The present investigation deals with the chemical composition of the wax of adult male and female flies and makes a comparison of this wax, the wax of the puparium, and that of the puparium in combination with the pupal cuticle. The pupal cuticle which is represented by the inner surface of the puparium, is of interest since it is likely to contain some lipid components in common with the exocuticle of the blowfly.

MATERIALS AND METHODS

All solvents used were A.C.S. reagent grade. Light petroleum refers to the fraction with bp 40–60°C. Ratios of solvent mixtures are on a volume basis.

Solvents were evaporated from extracts and solutions under reduced pressure at 30–35°C in a rotary evaporator.

Adult Blowflies

Adult flies were allowed to emerge from puparia in screened cages and were provided at all times with sucrose and water. On days 2 and 3 after emergence only, the flies were given a meal of ovine liver; on day 10 they were separated according to sex and then solvent-extracted.

Collection of Wax

(*a*) Adult male and female flies under carbon dioxide anesthesia were briefly immersed (approximately 3 min) in dichloromethane at 25°C (extracts designated "male short" and "female short" extracts).

(*b*) The above flies were reimmersed in dichloromethane and allowed to stand overnight at 5°C ("male long" and "female long" extracts).

(*c*) Blowfly puparia were briefly immersed (approximately 3 min) in dichloromethane at room temperature ("puparium extract").

(*d*) Pooled samples of empty puparia, including the pupal cuticle, were extracted in a Soxhlet apparatus with dichloromethane ("empty puparium extract").

Thin-Layer Chromatography

A preliminary examination of the waxes was carried out by TLC on Silica Gel G (E. Merck A. G., Darmstadt, West Germany). Standard lipids were obtained from commercial sources and were used without further purification. The TLC procedure used is that described by Kelley (6). The plates were developed in light petroleum–ether–acetic acid 70:30:1 to a height of 15 cm, air dried, and then developed in the same dimension to a height of 11 cm (a position just below the triglyceride spot) in ether–light petroleum–acetic acid 70:30:1.

Fractionation of Waxes

The methods used were based on those previously described (2, 7–9). Silicic acid (Mallinckrodt Chemical Works, St. Louis, Mo.) was screened to 100–200 mesh, washed with methanol and with ether, and dried for 12 hr at 110°C. Just before use the silicic acid was further heated to 110°C overnight. Silicic acid (10 g) slurried in light petroleum–ether 1:1 was added to a column (1 × 60 cm glass). After the packed column had been washed with ether (20 ml), acetone–ether 1:1 (30 ml), ether (20 ml), and light petroleum (500 ml), a wax extract (150–200 mg) in light petroleum (10 ml) was introduced on top of the column. Fraction 1, containing hydrocarbons only, was eluted from the column with light petroleum–ether 99:1 (100 ml). Compounds containing oxygen (fraction 2) were then washed from the column with ethanol (500 ml). After evaporation of the solvent, fraction 2 in light petroleum was shaken with alumina (The British Drug House Ltd., Poole, U.K.; Laboratory Reagent, "suitable for chromatography") for 10 min, and the alumina was washed with methanol–ether 1:9 (4 × 15 ml). Fatty acids were retained on the alumina and were methylated with dry methanolic HCl while still adsorbed (10). The esters were removed in ether and recovered by solvent evaporation under reduced pressure at room temperature. Material other than fatty acids, not retained on the alumina, was saponified with 0.5 N KOH in ethanol–water 1:1. The nonsaponifiable fraction was analyzed for sterol by the Schoenheimer–Sperry determination (11) after digitonin precipitation. The acidic fraction in dry ether was methylated with ethereal diazomethane prior to gas chromatographic examination.

Instrumental Analysis

(*a*) Melting points were determined on a Kofler block using a Reichert hot-stage microscope. Melting points are uncorrected.

(*b*) IR absorption spectra of wax fractions were obtained by use of an automatic recording Perkin–Elmer 337 grating spectrophotometer. Samples were examined in the range 4000–400 cm^{-1} as capillary films between rocksalt plates and as dispersions in potassium bromide discs.

(*c*) Gas chromatographic examinations of hydrocarbons and esters of fatty acids were carried out using a Packard series 7500 gas chromatograph with flame ionization detectors. The glass columns, 6 ft (1.8 m) long, were packed with the stationary phase (10% w/w) on Gas-Chrom P, 80–100 mesh (Applied Science Laboratories Inc., State College, Pa.). Hydrocarbons were separated on Apiezon L at 250°C and LAC-2-R-446 (polyester) at 150°C; esters were examined on Apiezon L at 220°C, and diethylene glycol succinate (DEGS) at 145°C. The carrier gas was argon at 50 ml/min. Samples of hydrocarbons were injected onto the columns with a warmed microsyringe, while samples of esters were applied in solution in acetone.

For identification, known mixtures of saturated *n*-hydrocarbons and methyl esters of straight-chain fatty acids (Applied Science Laboratories Inc.) were used to establish linear log (retention time) versus "carbon number" relationships on the appropriate polar and nonpolar stationary phases. Relative proportions of individual components were estimated by measurement of their peak areas with a planimeter.

Further Characterization of Components of Wax Fractions

The presence of unsaturated compounds in wax fractions was sought by bromination of the hydrocarbons and methyl esters in ether at 0°C followed by examination of bromination products by gas chromatography (2, 12).

Straight-chain hydrocarbons and methyl esters derived from wax fractions were separated from branched-chain components as inclusion compounds of urea, and the resulting products were examined by gas chromatography (13).

RESULTS

The extraction of blowflies, puparia, and empty puparia yielded pale brown, strong-smelling waxes. The percentage by weight of each extract obtained was as follows: male and female short extracts, 0.3% each; male and female long extracts, 0.5% each; puparium extract,

0.1%; and empty puparium extract, 4.3%. Preliminary examination of the waxes and of standard lipids by thin-layer chromatography suggested the presence of hydrocarbons, nonglyceryl esters, triglycerides, free fatty acids, diglycerides, and monoglycerides (Fig. 1). Absence of phospholipids from the extracts was shown by use of the Dittmer–Lester spray reagent (14). Sterols (positive Liebermann–Burchard test) were present in extracts of puparia and empty puparia but absent from the blowfly waxes. The IR spectra of the waxes showed absorption in the region 3500–2500 cm^{-1} (OH groups, both free and hydrogen-bonded), near 1740 cm^{-1} and 1705 cm^{-1} (carbonyl groups), and at 720 cm^{-1} (long alkyl chains).

The waxes were separated into hydrocarbons and compounds containing oxygen by chromatographic adsorption in silicic acid. The polar fraction was further separated into fatty acids and neutral compounds, including esters of fatty acids, by adsorption on alumina. The chemical nature of each fraction was confirmed by TLC and IR spectroscopy. In addition to the free fatty acids, other polar material, presumably polymeric, was adsorbed on alumina. This was not removed under the conditions used to obtain the acids. It comprised 20–30% of the polar fraction. The results are summarized in Table 1. The wax fractions were subsequently subjected to detailed analysis by gas chromatography.

Hydrocarbons

The hydrocarbon fractions from all extracts were semi-solid at room temperature. Hydrocarbons derived from blowflies melted in the range 40–42°C, while those from the puparia and empty puparia melted at 48–50°C. The IR spectra showed strong absorption at 1460 cm^{-1} and medium intensity bands near 720 cm^{-1} characteristic of methylene groups in long-chain compounds. There was no evidence of unsaturation.

Gas chromatography of a known mixture of *n*-hydrocarbons (C_{16}–C_{32}) on Apiezon L and LAC-2-R-446 (polyester) established a linear relationship between log (retention time) and chain length. The compositions of blowfly and puparium hydrocarbons on Apiezon L at 250°C, expressed as carbon numbers (15), are summarized in Table 2. The most abundant *n*-alkanes detected in all fractions were heptacosane (C_{27}) and nonacosane (C_{29}). Absence of unsaturation was confirmed by the failure of the fractions to react with bromine. Bromination produced no significant change in the gas chromatograms. Branched-chain compounds were found in every case and were among the most abundant components of fraction 1 derived from blowflies. They were distinguished from straight-chain hydrocarbons by the urea inclusion method (13): the area proportion of the gas-chromatographic peaks with integral carbon numbers increased for the fraction forming urea complexes and decreased for the fraction excluded from the urea.

Free Fatty Acids

The free fatty acids from all extracts were separated from other polar compounds by adsorption on alumina and isolated as their methyl esters. The IR spectra of the esters showed no sign of unsaturation. The light brown

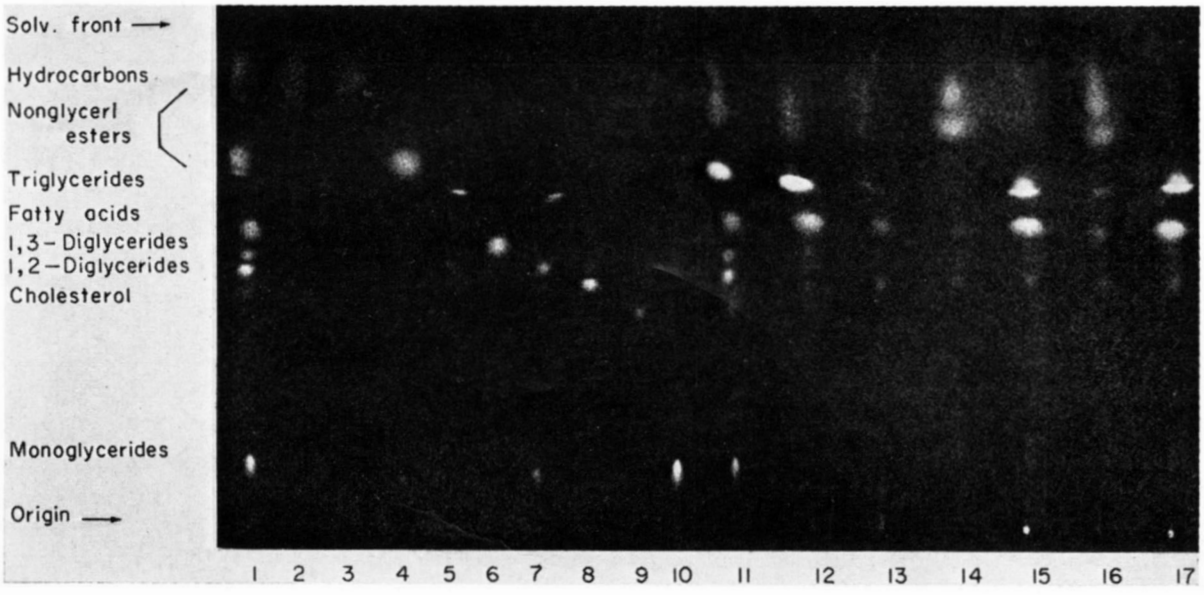

FIG. 1. TLC of wax extracts. *1* and *11*, standard lipid mixture; *2*, *n*-tetracosane; *3*, cholesteryl palmitate; *4*, methyl palmitate; *5*, tripalmitin; *6*, palmitic acid; *7*, natural dipalmitin; *8*, 1,2-dipalmitin; *9*, cholesterol; *10*, monopalmitin; *12*, empty puparium extract; *13*, puparium extract; *14*, male short extract; *15*, male long extract; *16*, female short extract; *17*, female long extract. Compounds were made visible with 2% dichlorofluorescein in methanol and photographed under UV radiation.

TABLE 1 LIPID COMPOSITION OF BLOWFLY CUTICULAR EXTRACTS

	Male Short	Female Short	Male Long	Female Long	Puparium	Empty Puparium
			wt %			
Hydrocarbons	59.2	65.7	7.2	7.6	33.3	14.1
Free fatty acids	8.8	5.4	43.8	33.7	15.6	7.0
Esters	15.8	16.1	34.0	50.0	25.6	60.0
Sterols (as cholesterol)	—	—	—	—	1.8	1.4
Total lipid accounted for*	83.8	87.2	85.0	91.3	76.3	82.5

* Remainder adsorbed on alumina with free fatty acids but not subsequently recovered.

TABLE 2 HYDROCARBON COMPOSITION OF CUTICULAR LIPID EXTRACTS BY GLC

Carbon Number	Male Short	Female Short	Male Long	Female Long	Puparium	Empty Puparium
				moles %		
$<C_{16}$	0.6	0.4	2.5	3.7	4.7	4.0
16.0	0.1	0.5	6.4	7.3	1.3	0.6
17.0	0.1	0.1	1.0	2.4	1.4	0.8
18.0	0.1	0.1	0.7	1.1	0.8	0.3
19.0	0.1	0.1	0.7	0.4	0.2	—
20.0	0.1	0.1	—	—	0.5	0.3
21.0	0.1	0.1	2.2	1.3	0.7	0.5
22.0	0.1	0.1	0.5	0.6	0.3	0.4
23.0	0.1	0.1	—	0.1	0.1	0.2
24.0	0.1	0.1	0.2	0.2	0.1	0.3
25.0	0.8	2.2	0.5	1.0	0.2	1.5
25.3	5.9	14.3	2.1	3.7	0.1	1.0
26.0	0.3	0.4	0.4	0.5	0.2	1.2
26.6	13.5	12.1	22.3	21.9	0.1	0.8
27.0	11.8	8.5	9.4	7.9	5.6	22.9
27.3	5.9	8.1	6.7	4.4	0.2	3.3
28.0	1.0	0.8	0.7	0.5	2.4	3.2
28.7	36.2	29.3	23.7	19.1	0.1	3.8
29.0	10.4	3.7	4.9	4.7	69.8	36.9
29.2	6.2	4.4	3.5	4.3	0.2	0.2
30.0	1.0	0.2	—	—	2.6	1.0
Minor unidentified peaks	5.5	14.3	11.6	14.9	8.4	16.8

GLC on Apiezon L, 250°C, argon flow 50 ml/min.
Minor amounts of $>C_{30}$ hydrocarbons were detected; their amounts have not been included in the calculation of percentages.

semisolid mixtures obtained were dissolved in acetone and were examined by gas chromatography on Apiezon L at 220°C. The compositions of the mixtures, expressed as carbon numbers, are summarized in Table 3. Absence of unsaturated esters was confirmed by the failure of all fractions to react with bromine. Branched esters were found to be present by the method described by Coles (13): after urea treatment the proportions of the GLC peaks with nonintegral carbon numbers increased. Similar results were obtained with the polar DEGS liquid phase. In every case the principal straight-chain saturated acids were stearic and palmitic acids. Branched acids were among the most abundant components in all fractions except those obtained from the male and female short extracts.

Esterified Fatty Acids

The light brown semisolid ester fractions obtained after removal of the free fatty acids showed absorption in the IR spectrum near 1740 cm^{-1} (ester carbonyl), 1460 cm^{-1} (methylene groups), 1370 cm^{-1} (methyl groups), and 720 cm^{-1} (long alkyl chains). Absorptions due to unsaturation were absent. These results are characteristic of long-chain saturated esters. The ester fractions were saponified, and the resulting fatty acids were gas-chromatographed as methyl esters (Table 3). Unsaturated compounds were absent; the major straight-chain saponification products were again stearic and palmitic acids; and branched-chain acids were again among the most abundant components in all fractions except those derived from the male and female short extracts.

TABLE 3 GLC OF METHYL ESTERS OF FREE AND ESTERIFIED FATTY ACIDS

Carbon Number	Male Short		Female Short		Male Long		Female Long		Puparium		Empty Puparium	
	Free	Esterified	Free	Esterified	Free	Esterified	Free	Esterified	Free	Esterified	Free	Esterified
						moles %						
<14.0	36.2	26.1	44.9	33.8	2.4	2.7	4.2	2.9	12.3	27.0	7.1	1.9
14.0	5.4	4.4	3.9	2.5	2.8	3.3	2.6	2.9	1.3	2.6	1.9	1.9
14.7	2.4	1.7	1.3	0.8	1.6	0.8	0.8	1.2	2.4	1.7	0.5	0.7
15.0	3.1	1.7	1.6	0.8	2.0	0.6	1.3	0.8	1.3	2.0	0.5	0.5
15.65	1.4	3.2	1.0	6.5	20.4	22.9	20.3	19.7	3.0	7.5	10.8	12.2
16.0	28.1	31.6	30.7	21.1	24.5	19.3	23.1	21.4	14.3	20.6	20.4	19.5
16.7	1.4	1.7	1.6	1.7	3.3	3.0	2.1	2.6	2.0	1.8	1.3	3.5
17.0	2.4	3.3	1.6	1.4	1.0	1.0	1.3	1.5	1.5	1.2	0.9	0.8
17.6	5.9	7.7	3.9	17.2	35.4	35.2	36.9	37.0	35.3	21.5	42.2	41.2
18.0	12.9	13.3	7.4	11.3	6.7	10.2	7.0	9.4	25.3	13.7	11.9	11.1
Minor unidentified peaks	0.8	5.3	2.1	2.9	—	1.0	0.4	0.6	1.3	0.4	2.5	6.7

GLC on Apiezon L, 220°C; argon flow 50 ml/min.
Minor amounts of $>C_{18}$ fatty acids were detected; their amounts have not been included in the calculation of percentages.

The nonsaponifiable fractions were examined for sterols by means of the Liebermann–Burchard reagent. Positive results were obtained only for fractions derived from the puparium and empty puparium (Table 1).

DISCUSSION

The hydrocarbons from the blowfly resemble those from the Mormon cricket *Anabrus simplex* Hald. (1) in that both types have many constituents and are saturated; they differ from the hydrocarbons of the cockroach *Periplaneta americana* L. (2) in that the latter are composed of a small number of compounds as well as a major unsaturated component. Hydrocarbons are the principal lipid derived from the puparium and the male and female long extracts. These are composed of straight-chain as well as branched-chain molecules containing both odd and even numbers of carbons. Straight-chain molecules are the major components obtained from puparia, while branched-chain compounds are the main blowfly hydrocarbons. The very large proportion of nonacosane (C_{29}) and the shortage of branched-chain alkanes in puparium extracts as compared with blowfly extracts seem to point to different biosynthetic pathways.

Esters comprise the major fraction in the wax from empty puparia as well as from the male and female long extracts. The higher percentage of esters in empty puparia than in the puparium extract is attributed to the wax of the pupal cuticle. Saponification of the ester fractions yields both straight-chain and branched-chain fatty acids. The free fatty acids were similar in composition to the esterified acids. Free acids were more abundant and esters less abundant in male than in female blowflies. The free and esterified fatty acids of the blowfly are, unlike those of the cricket and cockroach, completely saturated, a finding that can be related to the physical properties of the wax—thin and hard on the blowfly and the blowfly puparium, fluid on the cockroach and the cricket.

The complex mixture of blowfly lipids obtained may not be derived exclusively from the cuticle. Thus glycerides, which are commonly found in body tissues of insects, are likely to constitute an extracuticular component in male and female long extracts. However, the presence of this lipid class in other extracts, in particular the empty puparium extract, supports the idea that glycerides also occur in the insect cuticle.

Alcohols, acids, and esters are some of the classes of aliphatic organic compounds which have been shown to play an important role in the pheromone-mediated behavior of insects. Pheromones are usually characterized by low molecular weight ($< C_{20}$) and appreciable volatility (16). The short extracts of blowflies, which probably most closely represent the cuticular secretions of the insect, contain relatively large proportions of low molecular weight ($< C_{14}$) fatty acids both free and esterified. These volatile constituents, together with the corresponding neutral ester fractions from the waxes, are most likely to contain the pheromones responsible for the sex and ovipositing behavior of *Lucilia cuprina*.

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