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Composition of the surface hydrocarbons from the vitelline membranes of dipteran embryos

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Abstract

Hydrocarbons were the major lipid class extracted by hexane from the vitelline membrane surface of dechorionated eggs of the house fly, *Musca domestica*, the New World screwworm, *Cochliomyia hominivorax*, the secondary screwworm, *Cochliomyia macellaria*, the green bottle fly, *Phaenicia sericata*, the sheep blow fly, *Lucilia cuprina* and the Mexican fruit fly, *Anastrepha ludens*. The length of time the embryos must be exposed to hexane with or without a small amount of alcohol in order to attain permeability was species-dependant. Long-chain *n*-alkanes comprised the major lipid class removed from vitelline membranes of all species except *A. ludens* where 2-methylalkanes were the major class. The range in size by the total number of carbon atoms in the hydrocarbons was: C23–C49 in *C. hominivorax*, C27–C33 in *C. macellaria*, C24–C35 in *L. cuprina*, C25–C36 in *M. domestica*, C25–C33 in *P. sericata* and C21–C51 in *A. ludens*. The major hydrocarbon component, expressed as percent of the total hydrocarbons, was *n*-nonacosane (C29) in *C. hominivorax* (40%), *C. macellaria* (43%), *L. cuprina* (38%), *M. domestica* (39%) and *P. sericata* (60%). However, in *A. ludens*, 2-methyloctacosane (32%) was the major hydrocarbon. Unsaturated hydrocarbons, monoenes (16%) and dienes (11%), were abundant only in *A. ludens*. Since prior studies indicated that the length of time the embryos must be exposed to hexane with or without a small amount of alcohol in order to attain permeability is species dependant, we suggest that the differences in hydrocarbon composition may contribute to this variation in lipid extractability.

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Keywords: Hydrocarbons; Methyl-branched alkanes; Alkenes; Alkadienes; Mass spectrometry; Diptera; *Musca domestica*; *Cochliomyia hominivorax*; *Cochliomyia macellaria*; *Phaenicia sericata*; *Lucilia cuprina*; *Anastrepha ludens*

1. Introduction

Cryopreservation of insects is becoming a major focus in maintaining genetic diversity, preserving genotypes from genetic research programs, stockpiling insects used for biological control programs until needed for release and for combating genetic

drift in mass rearing programs (Leopold, 1991, 1998). Cryopreserved insects can be used to reestablish rearing programs, to help mass rearing programs operate on a more continuous level of production and to markedly lower insect maintenance costs by eliminating the need to maintain specific lines of germplasm by continuous rearing.

Protocols developed for cryopreservation of almost all cells, tissues and embryos require that the freezable cell water be replaced with a chemical cryoprotectant such as ethylene glycol, dimeth-

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yl sulfoxide or glycerol. Insects depositing their eggs into harsh environments provide the eggs with elaborate eggshells and membrane barriers to contend with changes in temperature, moisture and possible microbial attack (Margaritis, 1985). These barriers must be removed to accommodate the requirements for successful cryopreservation. Insect egg shells or chorions, especially those of muscid, tephritid and calliphorid flies, are easily removed with an aqueous sodium hypochlorite treatment. In *Drosophila melanogaster* Meigen eggs, Zalokar (1971) and Margaritis (1985) observed that an impermeable layer remained even though treatment with sodium hypochlorite dissolved the chorion down to the innermost chorionic layer. This layer is synthesized by the ovarian follicle cells along with the vitelline membrane at the beginning of eggshell assembly (Margaritis, 1986).

Treatment of the dechorionated eggs with an organic solvent, such as pentane, hexane, heptane or octane, has been used to remove a lipid barrier that prevents the efflux of water and the influx of cryoprotectants (Zalokar, 1971; Leopold et al., 1995). The length of time the embryos must be exposed to hexane, with or without a small amount of isopropanol, in order to attain permeability, is apparently species dependant (Wang et al., 2000; Leopold et al., 2001; Rajamohan et al., 2003). Hexane was used as the standard solvent in these studies, and our preliminary work shows that it extracts hydrocarbons (the major lipid class), wax esters and acylglycerols from the vitelline membranes of *Musca domestica* L., *Phaenicia sericata* (Meigen) and *Lucilia cuprina* Wiedemann (Leopold and Nelson, 1997).

Because permeabilization of insect embryos is a critical step in the development of cryopreservation procedures, and because the eggshell is an important barrier to desiccation of the embryo during development, we wanted to understand more fully the chemical nature of the vitelline membrane lipid layer. Wang et al. (2000) pointed out that inclusion of isopropyl alcohol during the extraction of the vitelline membrane lipids in hexane increased the permeability of screwworm embryos but it also increased mortality. In this study, we characterized the individual components of the major lipid class, the hydrocarbons, of six species: *M. domestica*, *P. sericata*, *L. cuprina*, the New World screwworm, *Cochliomyia hominivorax* (Coquerel), the secondary screwworm, *Cochlio-*

myia macellaria (Fabricius) and the Mexican fruit fly, *Anastrepha ludens* (Loew). Lipid classes were determined by thin-layer chromatography, with standards, and the hydrocarbon components were analyzed by gas chromatography-mass spectrometry.

2. Methods

2.1. Insects

Eggs from the house fly, secondary screwworm and green bottle fly were obtained from our laboratory colonies which have been in colonization for over 100 generations. The eggs from screwworms, sheep blow flies and Mexican fruit flies were obtained from laboratory colonies maintained at the USDA Midwest Livestock Insects Laboratory in Lincoln, NE, CSIRO Entomology Laboratory in Canberra, Australia and USDA Plant Protection Laboratory, Edinburg, TX, respectively. The eggs from the screwworms and the sheep blow flies were transported in 70% ethyl alcohol. The 70% alcohol was also tested for presence of hydrocarbons to determine whether ethyl alcohol had a leaching effect on the eggs. No significant levels of hydrocarbons were found.

2.2. Lipid extraction

All glassware, glass wool and metal pins, etc. were rinsed with chloroform (CHCl_3) before use. All plastics, hand lotion, etc. were avoided. Samples consisted of approximately 0.5–0.75 g of eggs. Eggs were dechorionated with a 50% bleach solution, washed with water for 3 min and then placed into isopropyl alcohol for 30 s. The alcohol was suctioned off the eggs and the eggs were allowed to air dry for approximately 1 min before placing in a 10-ml buret on top of glass wool. Hexane was added to the biuret containing the dechorionated eggs. This mixture was allowed to set for 1 min, then drained through the glass wool to collect the solvent containing the vitelline membrane surface lipids. With this method, no putative internal yolk lipids, such as triacylglycerols were found to be extracted.

2.3. Thin-layer chromatography

Lipid classes were first determined by thin-layer chromatography (TLC). A portion of the CHCl_3

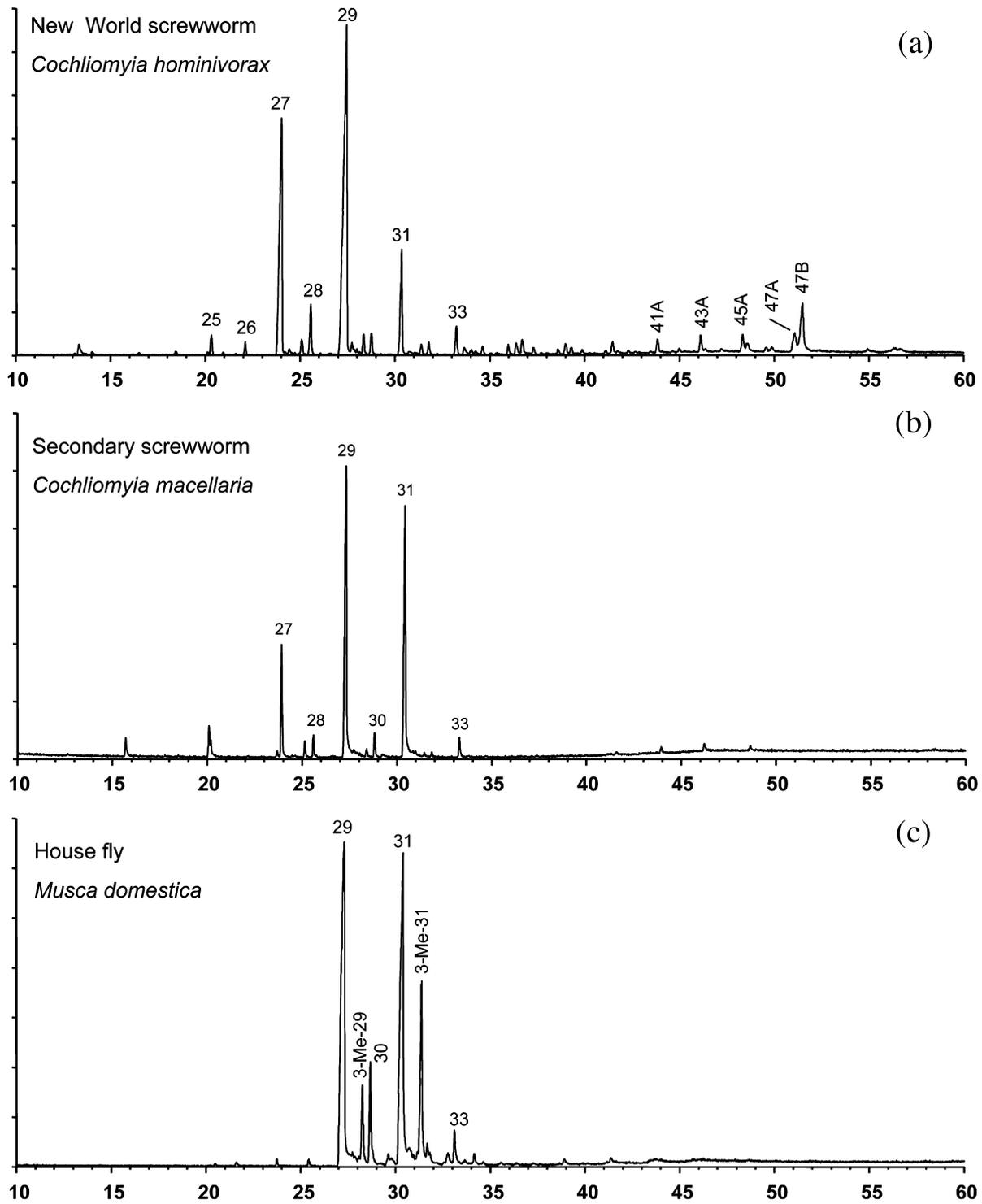


Fig. 1. GC-MS traces of hydrocarbons from the vitelline membrane surface of: (a) New World screwworm, *C. hominivorax*; (b) secondary screwworm, *C. macellaria*; (c) house fly, *M. domestica*; (d) green bottle fly, *P. sericata*; (e) sheep blow fly, *L. cuprina*; and (f) Mexican fruit fly, *A. ludens*.

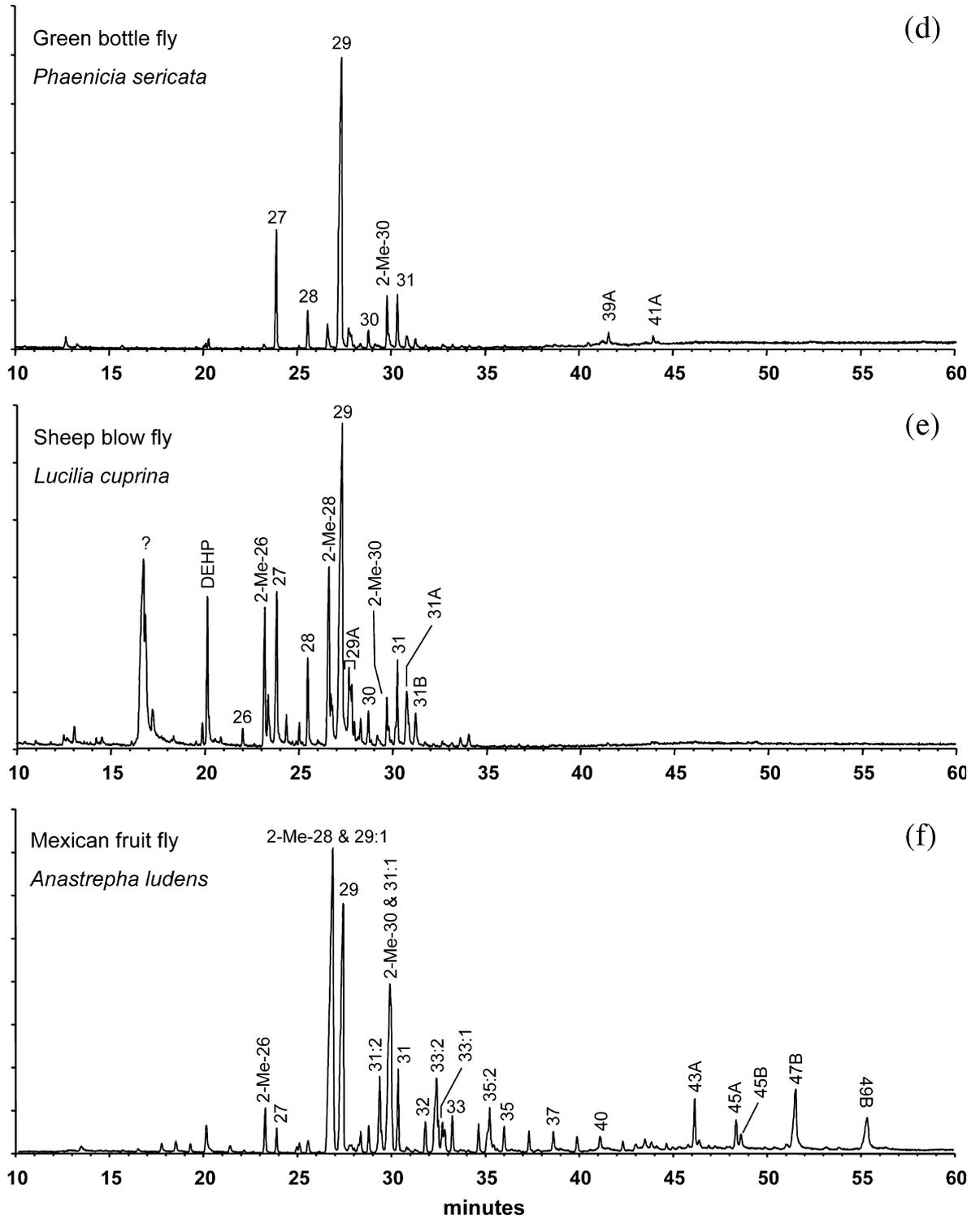


Fig. 1 (Continued).

Table 1
Percent composition of the hydrocarbon classes on the vitelline membrane

Hydrocarbons	<i>Cochliomyia hominivorax</i>	<i>Cochliomyia macellaria</i>	<i>Musca domestica</i>	<i>Phaenicia sericata</i>	<i>Lucilia cuprina</i>	<i>Anastrepha ludens</i>
<i>n</i> -Alkanes	81	91.1	76.9	84.5	62.3	28.3
2-Methylalkanes	nd	nd	0.4	7.2	18.4	34.4
3-Methylalkanes	3	2.8	14.6	0.9	3.0	1.2
Monoenes	nd	nd	t	nd	nd	16.0
Dienes	nd	nd	nd	nd	nd	11.4
Methylalkanes	17	6.1	8.1	6.9	16.3	9.1

Nd=not detected; t=trace amount.

extract and a mixture of standard lipids were spotted side-by-side on high-performance silica gel plates with hexane/diethyl ether/formic acid (80:20:1 v/v) as the developing solvent. The locations of lipid bands were visualized by charring plates after spraying with a solution of 5% concentration sulfuric acid in 95% ethanol, allowing the ethanol to evaporate, heating at 150 °C for 10 min, and then at 250 °C for 10–20 min.

2.4. Hydrocarbon analysis

The total underivatized samples were analyzed by GC-MS (gas chromatography-mass spectrometry) on a HP 5890A gas chromatograph equipped with a pressure programmable cool on-column injection port and an autoinjector (Nelson et al., 2002). The column consisted of a 1-m retention gap connected to a 12.5 m×0.2 mm capillary column of cross-linked dimethylsilicone Ultra 1 (HP) (Agilent Technologies, Wilmington, DE, USA) and was coupled to a HP 5970B quadrupole mass selective detector. The carrier gas was helium. The initial column temperature was set between 150 °C and 200 °C, then programmed to reach 320 °C, at a rate of 3 or 4 °C/min and finally held at 320 °C for 20–120 min. The mass range scanned was 50 to 800 amu at a rate of 1.1 scans/s. An aliquot of 1 µl in chloroform was injected. Mass spectra of the hydrocarbons were interpreted as previously described (Blomquist et al., 1987; Nelson, 1993; Bernier et al., 1998; Carlson et al., 1998; Schulz, 2001; Nelson et al., 2003).

The total ion current (TIC) data were analyzed using a computer spreadsheet program in which the dose–response was adjusted using a three-component standard curve prepared using *n*-alkane standards as described (Nelson et al., 2002). The equation component from 0 to 3.1 ng was linear,

the next equation component was polynomial was from 3.1 to 100 ng and the third component was again linear above 100 ng. The formula in the spreadsheet selected the equation component to be used based on the peak area of the total ion current of the GC-MS peak being measured to calculate the femtomoles and/or nanograms that the peak represented. The standard mixture was run on the GC-MS system each day before and after the samples.

3. Results

Analysis of the total hexane extract by thin-layer chromatography (data not shown) demonstrated that the major lipid class was the hydrocarbons. Bands corresponding to traces of cholesterol esters and/or wax esters, triacylglycerols, free fatty acids and/or long-chain alcohols and cholesterol and/or diacylglycerols may have been present, but further analysis to verify the identity of these compounds was not pursued.

Analysis of the total underivatized samples by GC-MS showed that, except for *A. ludens*, the other five dipteran species had relatively simple hydrocarbon profiles of the vitelline membranes, and that the dominant hydrocarbon class was the *n*-alkanes (Fig. 1 and Table 1). The peak designations in Fig. 1 are abbreviated. The numbers marking the peaks represent the number of carbon atoms in the backbone of the molecule, i.e. 2-Me-26 indicates 2-methylhexacosane. The letters A, B and C indicate 1, 2 and 3 methyl branches, respectively. A prime symbol indicates that one of the methyl branches was near the end of the carbon chain, on carbons 2, 3 or 4. The 2-methylalkanes were the major hydrocarbon class in *A. ludens* (Table 1). *A. ludens* was also distinguished from the other species by the presence of alkenes (16%) and alkadienes (11%) (Table 1). No alkadienes

were detected in any of the other species and only a trace amount of alkenes were detected in *M. domestica*.

Except for the unsaturated hydrocarbons in *A. ludens*, the hydrocarbon components marked in Fig. 1 were methyl-branched alkanes in all the species. There were some subtle differences between the species when major components were examined closely for the presence of co-eluting or unresolved minor components. In addition to the alkenes and alkadienes, *A. ludens* also differed from the other species in that a methyl-branched alkane was found in minor amounts eluting on the tailing shoulder of the major *n*-alkanes with an odd number of carbons. There was no observable peak shape to indicate its presence. The compound appeared to be a B' component with a mass

spectrum similar in appearance to that of a 3,*X*-dimethylalkane but with a shorter retention time. The components were concluded to be 2,*X*-dimethylalkanes from their mass spectra. A series of mass spectra are presented for the components in peaks 28A' (2-methyloctacosane) to 28B' (2,*X*-dimethylnonacosane) (Fig. 2) from *A. ludens*.

The major hydrocarbon component of *A. ludens* was 2-methyloctacosane (Fig. 2a) in which nonacosene (29:1) co-eluted (Fig. 2b). From approximately 30 scans taken as the peak eluted, the mass spectra of nonacosene was only apparent in the last six scans of the peak although the *m/z* 406 molecular ion was present in all scans. The next peak to elute was nonacosane (peak 29; 17%, Table 2) (Fig. 2c). From 20 scans taken as the peak eluted, 2,6-dimethyloctacosane was evident

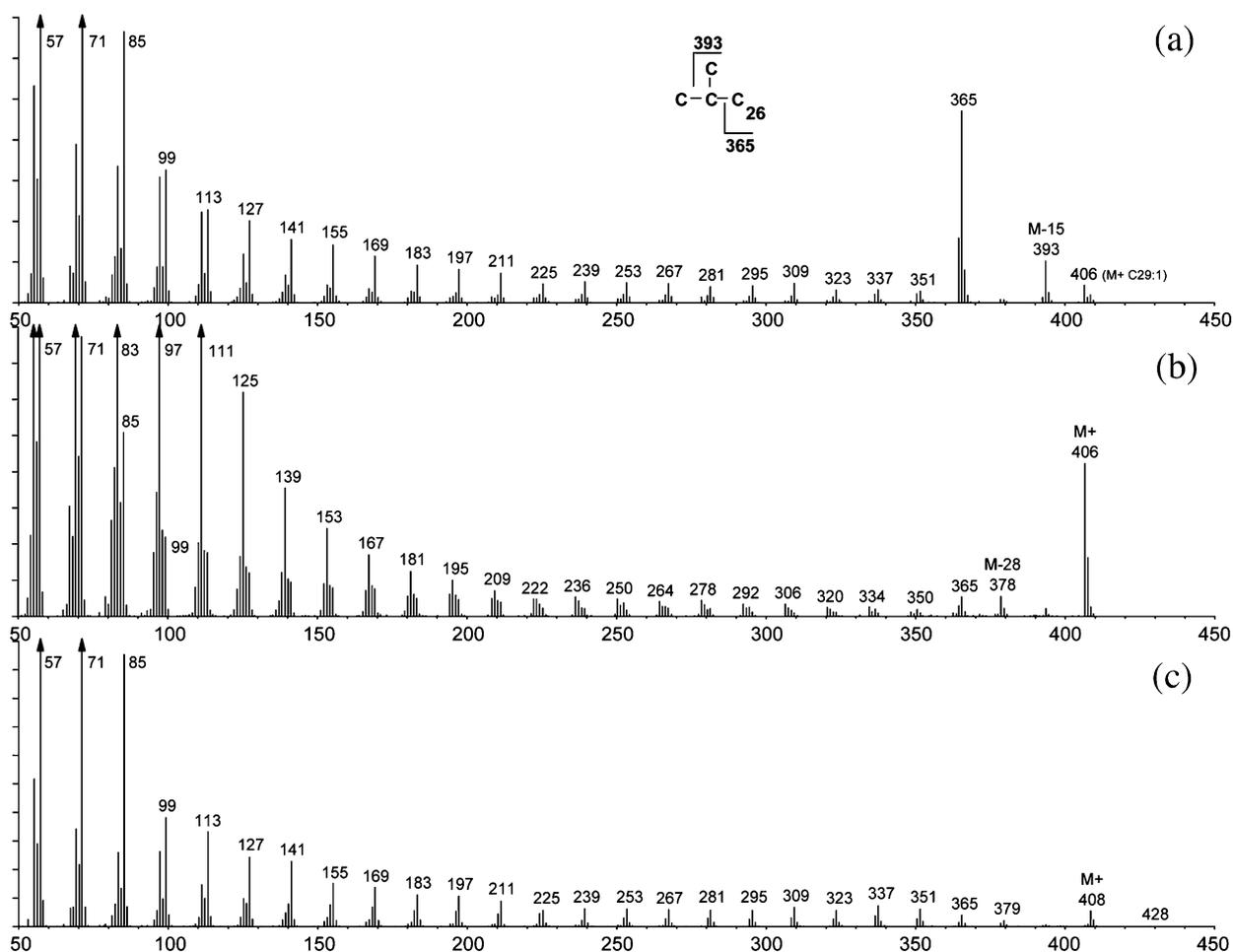


Fig. 2. Electron impact mass spectra of hydrocarbons from the vitelline membrane surface of the Mexican fruit fly, *A. ludens*: (a) 2-methyloctacosane; (b) *n*-nonacosene; (c) *n*-nonacosane; (d) 2,6-dimethyloctacosane; and (e) 2,6- and 2,4-dimethyloctacosanes.

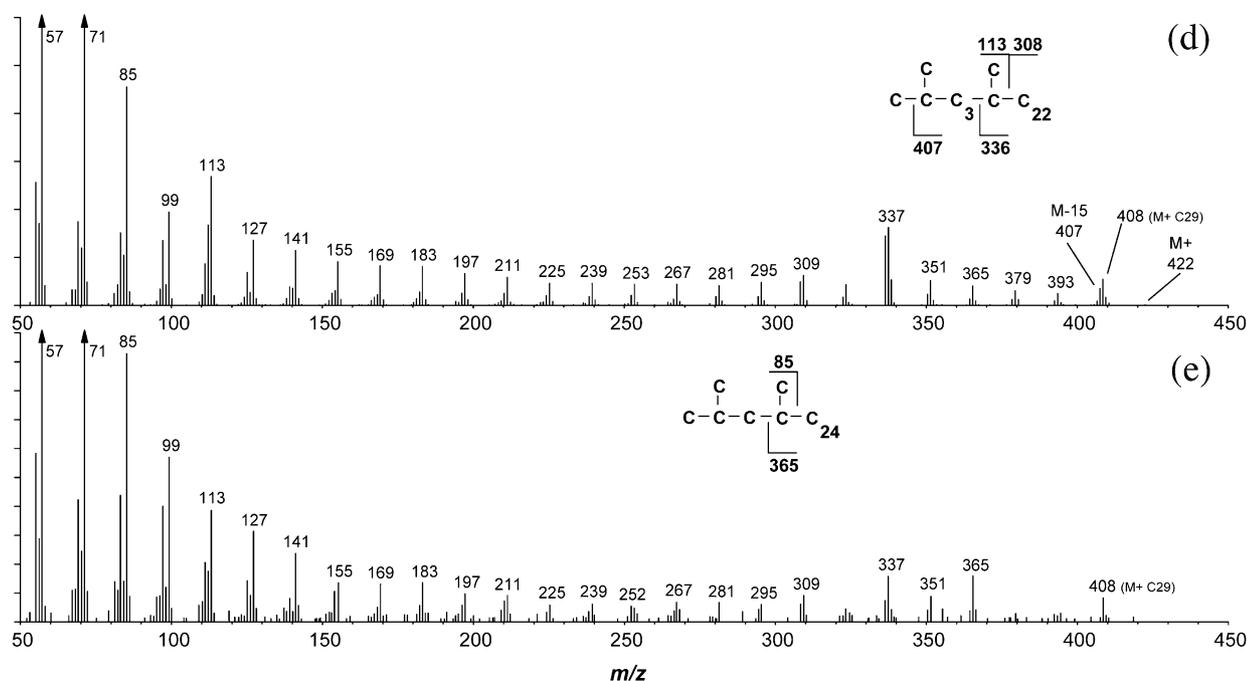


Fig. 2 (Continued).

in the last three scans (Fig. 2d). In an overloaded analysis, a second isomer of 28B', 2,4-dimethyloctacosane, was indicated in the last scan by the ion at m/z 365 (Fig. 2e). The ion at m/z 408 is the molecular ion for *n*-nonacosane (Fig. 2d and e). A similar series of components was observed for 30A' to 30B' where 31:1 eluted on the tailing shoulder of 2-methyltriacontane and 2,6-dimethyltriacontane was found in the tailing shoulder of hentriacontane. A possible 2,4-dimethyl isomer was not detected.

Alternate structures can be proposed for 2,*X*-dimethylalkanes with an even-numbered carbon backbone which gives the same mass spectral fragment ions of diagnostic intensity (Schulz, 2001; Nelson et al., 2002, 2003). The alternate structure for 2,6-dimethyloctacosane is 2,22-dimethyloctacosane. However, we have named the structures assuming that the methyl groups are added early during carbon-chain elongation as has been shown for the biosynthesis of methyl-branched alkanes in the house fly (Dillwith et al., 1982) and of methyl-branched alcohols in the tobacco hornworm, *Manduca sexta* Linnaeus (Nelson and Fatland, 1992).

Additional ions of diagnostic intensity occur when a single methylene separates two methyl

branch points. The mass spectra contain an ion or ions that may be designated as arising from cleavage of the carbon-carbon bond on either side of the methylene between the two methyl branch points. In the case of 2,4-dimethyloctacosane, the only apparent ion is at m/z 365 (Fig. 2e) because other diagnostic fragment ions are masked by the presence of the 2,6-isomer. This type of fragmentation has been seen in both di- and trimethyl-branched alkanes in which two of the methyl branch points are separated by a single methylene (Thompson et al., 1981; Haverty et al., 1990, 1996; Nelson, 2001; Nelson et al., 1980, 2001, 2002, 2003). The mass spectrum of synthetic 13,15-dimethylheptacosane confirmed the occurrence of such a putative fragmentation (Thompson et al., 1981).

Another subtle difference between the species, again involving dimethylalkanes (B') co-eluting with the major *n*-alkanes, was observed in the hydrocarbons from *L. cuprina*. In this insect, the methyl-branched alkanes were found as minor components eluting on the leading shoulder of the major *n*-alkanes with an odd number of carbons, rather than on the tailing shoulder. Again there was no observable disturbance or shouldering to the peak shape to indicate their presence. The

Table 2
Percent composition (\pm S.D.) of the hydrocarbons from the vitelline membrane surface^a

Peak no. ^b	<i>Cochliomyia hominivorax</i> % Comp. \pm S.D.	<i>Cochliomyia macellaria</i> % Comp. \pm S.D.	<i>Musca domestica</i> % Comp. \pm S.D.	<i>Phaenicia sericata</i> % Comp. \pm S.D.	<i>Lucilia cuprina</i> % Comp. \pm S.D.	<i>Anastrepha Ludens</i> % Comp. \pm S.D.
21						0.1 \pm 0.1
22						t
23	0.2					0.3
24	0.3	t		t	0.9 \pm 0.2	
25	1.3 \pm 0.2	t	0.1 \pm 0.1	t	2.0 \pm 0.4	0.2
25A	0.2					
3-Me-25	0.1 \pm 0.1					
26	0.8	t		0.1 \pm 0.2	1.7 \pm 0.3	0.2 \pm 0.1
2-Me-26		t?		0.5 \pm 0.1	5.5 \pm 1.3	2.2 \pm 0.7
2,12-diMe-26						
27	26.8 \pm 2.9	8.7	0.4 \pm 0.1	12.7 \pm 1.4	9.6 \pm 0.5	1.0 \pm 0.2
27A	1.1 \pm 0.4	t		t	1.6	
7-Me-27	0.3 \pm 0.2	t				
5-Me-27	0.4 \pm 0.3	t				
2-Me-27						0.3
11,15-diMe-27						
3-Me-27	1.3 \pm 0.4	1.5 \pm 0.2		0.3 \pm 0.2	1.4	0.5 \pm 0.1
28	2.7 \pm 0.5	2.2 \pm 0.2	0.4 \pm 0.1	4.1 \pm 0.6	4.1 \pm 0.2	0.5
3,7-diMe-27		t				
28A	t				0.3 \pm 0.2	
4-Me-28	t	t				
2-Me-28				2.6 \pm 0.3	10.1 \pm 0.7	
2-Me-28 & 29:1						31.5 \pm 2.5
2,14- & 2,12-diMe-28					t	
29	39.7 \pm 2.1	42.6 \pm 1.0	38.7 \pm 0.7	59.7 \pm 2.8	37.6 \pm 2.2	17.3 \pm 0.7
2,6-diMe-28	t?		t		t?	
29A	1.3 \pm 0.5	1.2 \pm 0.1	0.6 \pm 0.1	1.1 \pm 0.4	2.8 \pm 0.8	0.2
9-Me-29		t	0.2 \pm 0.1	0.7 \pm 0.4	1.7 \pm 0.3	0.2
7-Me-29	0.5 \pm 0.3	0.8 \pm 0.1	0.4 \pm 0.1	0.9 \pm 0.3	1.9 \pm 0.2	0.2
5-Me-29	0.5 \pm 0.2	0.4 \pm 0.1	0.3 \pm 0.1	0.5 \pm 0.1	1.2	0.2
2-Me-29				t		
29B	0.3 \pm 0.1	t	0.3 \pm 0.1			0.2 \pm 0.1
30:1						0.2 \pm 0.1
3-Me-29	1.2 \pm 0.3	0.8 \pm 0.1	3.8 \pm 0.5	0.6 \pm 0.1	1.5	0.7 \pm 0.1
30	1.0 \pm 0.1	2.7 \pm 0.3	3.7 \pm 0.2	1.8 \pm 0.3	2.2 \pm 0.4	0.7 \pm 0.2
3,7-diMe-29			0.4 \pm 0.1			
30A ^c	t		0.1 \pm 0.1	t	0.6 \pm 0.2	
31:2						4.8 \pm 1.3
4-Me-30	t		0.6 \pm 0.1			
2-Me-30			0.4	4.6 \pm 0.2	2.8 \pm 0.7	
2-Me-30 & 31:1						13.4 \pm 1.0
30B			t?			
3-Me-30	t		0.5 \pm 0.2			
2,14- & 2,12-diMe-30			0.2		0.4	
31	4.9 \pm 0.9	31.7 \pm 0.6	31.0 \pm 1.7	4.2 \pm 0.3	3.7 \pm 0.3	2.3 \pm 0.4
30B'?			0.2			
31A	0.6	0.4 \pm 0.2	0.4 \pm 0.1	0.9 \pm 0.5	2.4 \pm 0.6	0.2
9-Me-31			0.3 \pm 0.1			
7-Me-31	0.2 \pm 0.1	0.4 \pm 0.1	0.3 \pm 0.1		0.6	
32:2						0.2
5-Me-31	0.2	t	0.3			
31B	0.2 \pm 0.1		0.4 \pm 0.1	0.6 \pm 0.4	1.6 \pm 0.3	
3-Me-31	0.6	0.6 \pm 0.1	10.2 \pm 1.0	t		
32:1						0.1

Table 2 (Continued)

Peak no. ^b	<i>Cochliomyia hominivorax</i> % Comp. ±S.D.	<i>Cochliomyia macellaria</i> % Comp. ±S.D.	<i>Musca domestica</i> % Comp. ±S.D.	<i>Phaenicia sericata</i> % Comp. ±S.D.	<i>Lucilia cuprina</i> % Comp. ±S.D.	<i>Anastrepha Ludens</i> % Comp. ±S.D.
32	0.5±0.2	0.7±0.2	0.8±0.1	0.5±0.4	0.4±0.1	0.8±0.2
3,11- & 3,7-diMe-31			0.4±0.1			
32A ^c	t?			t		
33:2						4.3±0.2
4-Me-32	t					
2-Me-32				0.3±0.2		
32B			t		0.3±0.1	
33:1			0.9±0.1			1.7±0.1
33	1.0±0.2	2.5	1.4±0.1	0.5±0.3	0.3	1.0±0.2
33A	0.7±0.1		0.3±0.2	t	0.4±0.1	0.1
34:2						0.1±0.1
33B	0.5±0.3			t	0.5±0.3	
3-Me-33	0.2±0.1	t	0.5±0.1			
34	0.6±0.3	t	0.2	t		0.6±0.2
3,7-diMe-33	t?		0.1±0.1			
34A	t					
34B	t					
35:2						1.7±0.5
35:1						0.3
35	0.6±0.3	t		t		0.7±0.2
35A	0.7		0.2±0.1			
13,17-diMe-35	1.0±0.3					
5,xx-diMe-35	t					0.1
36	0.5±0.3	t		t		0.5±0.1
36A	t					
37:2						0.1
36B	t					
37:1						0.1±0.1
37	0.2±0.1	t		t		0.6±0.1
13- & 11-Me-37	0.5±0.1	t	0.4±0.1	t		
13,17-diMe-37	0.5	t				
38	0.2±0.1	t		t		0.4±0.1
38A	t	t				
39:2						0.1
39:1						0.1
39	0.1±0.1	t		t		0.6±0.1
39A	0.5±0.1	0.5±0.1	0.4±0.2	1.0±0.2		
39B	0.2	t				
40	0.2±0.1	t		t		0.2±0.1
40A	0.1±0.1	t		t		
41:2						0.1
41:1						0.3
41	0.1±0.1	t		t		0.4±0.1
41A	0.5±0.1	0.8±0.1		0.6±0.1		0.3±0.1
41B	t	t		t		0.1±0.1
42	0.1±0.1	t				0.1±0.1
42A	0.2±0.1	t				0.1
43:2						0.1
43:1						0.1
43A	0.6±0.1	1.1±0.1		0.3±0.1		1.1±0.2
13,17-diMe-43	0.2	t				0.3
12-Me-44	t	t				t
45:2						0.1
45:1						t
13- & 11-Me-45	0.5±0.1	0.6		t		0.6±0.1
45B	0.4±0.1	t		t		0.5

Table 2 (Continued)

Peak no. ^b	<i>Cochliomyia</i>	<i>Cochliomyia</i>	<i>Musca</i>	<i>Phaenicia</i>	<i>Lucilia</i>	<i>Anastrepha</i>
	<i>hominivorax</i>	<i>macellaria</i>	<i>domestica</i>	<i>sericata</i>	<i>cuprina</i>	<i>Ludens</i>
	% Comp. ± S.D.	% Comp. ± S.D.	% Comp. ± S.D.	% Comp. ± S.D.	% Comp. ± S.D.	% Comp. ± S.D.
46A	0.4 ± 0.2					
46B	0.4 ± 0.2					t
13-Me-47	0.7 ± 0.2	0.6				0.2 ± 0.1
13,21-diMe-47	2.9 ± 0.4	1.0				3.1 ± 0.1
48B						t
13,21-diMe-49						1.6 ± 0.3

^a Percentage composition was calculated from the integrated area data, adjusted for system response, from GC-MS analysis and rounded to the nearest tenth. The amount of trace components, less than 0.1%, are indicated by 't'. Where no standard deviation is given, it was less than 0.1 when rounded.

^b The peak numbers have been abbreviated: the number is the number of carbon atoms in the backbone of the molecule. For example, 26 indicates hexacosane, 2-Me-26 indicates 2-methylhexacosane. Where the methyl branch positions could not be determined the number of methyl branches based on the relative retention of the compound was used: A = monomethylalkane; B = dimethylalkane; C = trimethylalkane; A' = monomethylalkane with the methyl branch on carbon 2, 3 or 4; B' = dimethylalkane with one of the methyl branches on carbon 2, 3 or 4. A '?' means the component eluted at a position corresponding to the peak no. but could not be identified as that component from the mass spectra. The 2,X-dimethylalkanes eluted on either the leading or tailing edges of the adjacent *n*-alkane depending on the value of 'X', but were minor components of the *n*-alkane peak. Their percentage is a best estimate from the peak shape or is a 't' if no estimate could be made.

^c In the house fly, overload GC-MS analysis showed that peaks 30A and 32A also contained 29C' (the 3,9,13- and 3,7,11-isomers) and 31C' (3,7,11-isomer), respectively, on the tailing shoulder. The screwworm also may have similar unidentified C components plus 33C, but not terminally branched.

components were a series of 2,X-dimethylalkanes with greater separation of the methyl branches, which caused them to elute earlier than would their 2,6- and 2,4-isomers which would elute on the tailing shoulder of the *n*-alkane. The components identified were 2,12-dimethylhexacosane (26B') (Fig. 3a), 2,12- and 2,14-dimethyloctacosanes (28B') (Fig. 3b) and 2,12- and 2,14-dimethyltriacontanes (30B') (Fig. 3c). For 30B', a 2,16-dimethyltriacontane structure would form the same fragment ions at *m/z* 224 and 252 as the 2,14-isomer. Therefore, the two could not be distinguished based on their mass spectra.

The A' series of methylalkanes apparently consists of the 4-methylalkane, the 2-methylalkane and the 3-methylalkane eluting in that order. The identity of the 4- and 3-methylalkanes was established based on their mass spectra. No definitive mass spectra were obtained to confirm the presence of 2-methylalkanes. However, comparing the change in intensity of the *m/z* 70 ion, characteristic of 4-methylalkanes, from scan to scan indicated that 2-methylalkanes were probably present in minor amounts.

Very long-chain methyl-branched hydrocarbons have been found in many arthropods. However, in this study, such large hydrocarbons were identified in significant amounts only in *C. hominivorax* (a series of methyl-branched alkanes of 42–49 total

carbons) and *A. ludens* (a series of methyl-branched alkanes of 44–51 total carbons). Possibly, minor amounts were present in *C. macellaria*, but this was not verifiable by adequate mass spectra.

4. Discussion

Water loss in insect eggs after oviposition can produce fatal damage and, in some species of Muscinae, even slight reductions in ambient humidity can prevent nearly all hatching (Hinton, 1960). The existence of a 'waxy' layer located at the inner surface of the endochorion was reported by Beament (1946) and Slifer (1949) to impart resistance to water loss in *Rhodnius prolixus* Stal and *Melanoplus differentialis* (Thomas), respectively. Within this lipid layer of the species analyzed in this study, we found qualitative and quantitative differences in the hydrocarbon fraction, the major lipid class. Prior evidence for such differences in water loss between species has been demonstrated by heating the eggs to temperatures that presumably melt the components of the lipid layer. Davies (1948) found that the permeability of *Lucilia* (= *Phaenicia*) *sericata* eggs increased if they were heated to 38 °C, while King and Koch (1963) showed that desiccation increased only when *D. melanogaster* eggs were heated to 45 °C.

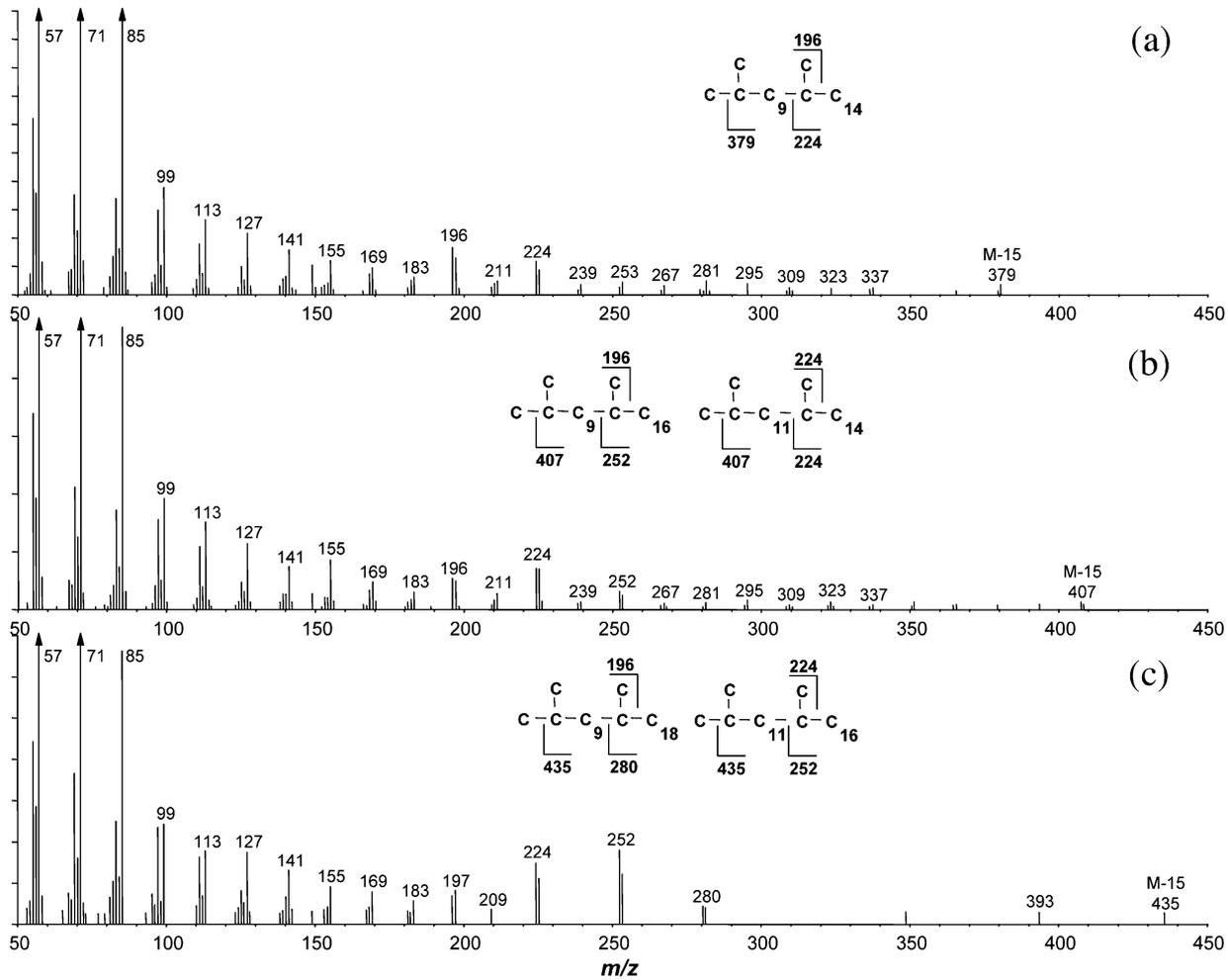


Fig. 3. Electron impact mass spectra of hydrocarbons from the vitelline membrane surface of the sheep blow fly, *L. cuprina*: (a) 2,12-dimethylhexacosane; (b) 2,12- and 2,14-dimethyloctacosanes; and (c) 2,12- and 2,14-dimethyltriacontanes.

In both artificial membrane and insect studies, surface lipids formed a better water-proofing layer as solids than as liquids (Gibbs, 1998; Rourke and Gibbs, 1999). The water-proofing properties are believed to correlate with phase changes of the cuticular lipids and saturated and unsaturated hydrocarbons may melt separately leading to liquid–solid phase separation (Gibbs, 2002). Hydrocarbons are the major components of the surface lipids of house flies. Surface lipids of the female house fly melted at a lower temperature as the female matured due to an increase in methyl-branched and unsaturated hydrocarbon components (Gibbs et al., 1995). In mixtures of pure hydrocarbons, melting temperatures decreased by as much as 30 °C depending on the position of the

methyl branch and by as much as 50 °C depending on the position of unsaturation (Gibbs and Pomonis, 1995). If present, polar lipids such as wax esters also will decrease the melting temperature of the cuticular lipids (Patel et al., 2001). Anecdotal evidence also indicates that vitelline membrane permeability differences exist between *M. domestica* and *C. macellaria* and that of *A. ludens*. *M. domestica* and *C. macellaria* eggs resist desiccation when allowed to remain in the open air, while those of *A. ludens* quickly dry out and collapse (RAL, unpublished observations).

The major differences in the vitelline membrane hydrocarbon composition exist between species may be correlated to the preferred oviposition site of a particular insect. The muscid and calliphorids

analyzed in this study deposit eggs either in relatively exposed areas or on sites that may be highly hyperosmotic (e.g. garbage, wounds, decaying flesh) while *A. ludens*, inserts its eggs into fresh fruit. Furthermore, the eggs of *Lyperosia irritans* L. do not hatch at 80% relative humidity, but are deposited into a moist environment, and in this way avoid even modest desiccation before hatching (Hinton, 1960).

Differential permeability of the eggshell has been recognized in a number of insects (Margaritis, 1985) but it is unclear whether those insects, where this has been observed, have waterproofing layers on the vitelline membranes of their eggs similar to the insects of our study. Interestingly, the permeabilized vitelline membranes of the flies examined in this study have similar molecular size exclusion limits. Isopropanol (MW 60.1) readily penetrates the vitelline membranes of these insects while trehalose or sucrose (MW 342.3 for both) does not (Leopold and Atkinson, 1999; Wang et al., 2000, 2001; Leopold et al., 2001). Dechorionated eggs of *A. ludens* treated with isopropanol, but not with hexane, will lose water when placed into hyperosmotic solutions and will allow slow penetration of chemical cryoprotectants having a MW of approximately 60, such as ethylene glycol. However, similarly-treated eggs of the house fly will not allow penetration of ethylene glycol (RAL, unpublished observations).

Hydrocarbons are also the major component of the cuticular surface lipids of many Diptera whereas wax esters are the major components of many Homoptera (Lockey, 1988; Nelson and Blomquist, 1995). *n*-Alkanes were identified as major components in some Diptera and in bees (Carlson and Bolten, 1984). However, *n*-alkanes were not the major hydrocarbons in cockroaches, honey bees and tsetse flies (Carlson, 1988), but were in the giant honey bee *Apis dorsata* Fabricius (Carlson et al., 1991). The hydrocarbons on the cuticular surface of the unmated female house fly consisted of *n*-alkanes (50%), methyl-branched alkanes (37%) and *n*-alkenes (14%) (Nelson et al., 1981). However, only trace amounts of *n*-alkenes were detected in the hydrocarbons from the vitelline membranes of the female house fly.

The results for the vitelline membrane alkanes of *A. ludens* were similar to those reported for the cuticular surface hydrocarbon composition of adult female *A. ludens* as well as for other adult tephritids; the Mediterranean fruit fly, *Ceratitidis capitata*

(Wiedemann), the Caribbean fruit fly, *Anastrepha suspensa* (Loew) and the melon fruit fly, *Dacus cucurbitae* Coquillet (Carlson and Yocom, 1986). However, the oriental fruit fly, *Dacus dorsalis* (Hendel) and the natal fruit fly, *Ceratitidis rosa* Karsch, had little or only trace amounts of *n*-alkanes. The major methyl-branched alkanes on the vitelline membrane of *A. ludens* were 2-methylalkanes (34%; Table 1), mainly 2-methyltacosane (~31%; Table 2). This composition of saturated hydrocarbons was similar to that found on the cuticular surface of the female adult (Carlson and Yocom, 1986). Long-chain alkadienes were reported in the cuticular surface lipids of *A. ludens* but the presence of alkenes was not reported (Carlson and Yocom, 1986).

In summary, *n*-alkanes formed the major hydrocarbon group in the lipid layer associated with the vitelline membrane of all flies analyzed except for *A. ludens* where methyl-branched alkanes were most prominent. *n*-Alkenes also were a major portion of the hydrocarbon fraction of *A. ludens* vitelline membrane extracts, but were basically non-existent on egg membranes of the other flies examined in this study. We suggest that these differences may be an accommodation for the preferred site for oviposition and the likelihood for desiccation to occur.

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