The antifungal activity of the cuticular and internal fatty acid methyl esters and alcohols in *Calliphora vomitoria*

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SUMMARY

The composition of the fatty acid methyl ester (FAME) and alcohol fractions of the cuticular and internal lipids of *Calliphora vomitoria* larvae, pupae and male/female adults was obtained by separating these two fractions by HPLC–LLSD and analysing them quantitatively using GC–MS. Analysis of the cuticular lipids of the worldwide, medically important ectoparasite *C. vomitoria* revealed 6 FAMEs with odd-numbered carbon chains from $C_{15:0}$ to $C_{19:0}$ in the larvae, while internal lipids contained 9 FAMEs ranging from $C_{15:1}$ to $C_{19:0}$. Seven FAMEs from $C_{15:0}$ to $C_{19:0}$ were identified in the cuticular lipids of the pupae, whereas the internal lipids of the pupae contained 10 FAMEs from $C_{13:0}$ to $C_{19:0}$. The cuticular lipids of males and females and also the internal lipids of males contained 5, 7 and 6 FAMEs from $C_{15:0}$ to $C_{19:0}$ respectively. Seven FAMEs from $C_{13:0}$ to $C_{19:0}$ were identified in the internal lipids of females, and 7, 6, 5 and 3 alcohols were found in the cuticular lipids of larvae, pupae, males and females respectively. Only saturated alcohols with even-numbered carbon chains were present in these lipids. Only 1 alcohol ($C_{22:0}$) was detected in the internal lipids of *C. vomitoria* larvae, while just 4 alcohols from – $C_{18:0}$ to $C_{24:0}$ – were identified in the internal lipids of pupae, and males and females. We also identified glycerol and cholesterol in the larvae, pupae, males and females of *C. vomitoria*. The individual alcohols and FAMEs, as well as their mixtures isolated from the cuticular and internal lipids of larvae, pupae, males and females of *C. vomitoria*, demonstrated antimicrobial activity against entomopathogenic fungi.

Key words: antifungal activity, cuticular lipids, internal lipids, fatty acid methyl esters, alcohols, *Calliphora vomitoria*.

INTRODUCTION

Ectoparasites are of growing significance in contemporary veterinary medicine and human healthcare, therefore an understanding of their biology and biochemistry is fundamental. One of the most abundant, worldwide ecoparasitic fly species is *Calliphora vomitoria*, commonly found around houses and livestock facilities. Adults of *C. vomitoria* are attracted to feces and decomposing organic matter. Eggs are normally laid in batches in carrion or other waste material, but sometimes also on purulent wounds and excreta which may result in myiasis (Wall and Shearer, 2001). The high abundance of *C. vomitoria* in cadavers indicates the ecological significance of this necrophagous species in decomposition of human and animal vestiges and its usefulness in forensic cases including the sophisticated detection of morphine accumulation and metabolism (Bourel *et al*. 2001; Grassberger and Frank, 2004). The larvae of *C. vomitoria* are also used in the treatment of gangrene and wounds (maggot therapy), although less frequently than the larvae of *Lucilia sericata* or the closely related *Calliphora vicina*. On the other hand, an unhygienic habit of *C. vomitoria* gives rise to the mechanical transport of microorganisms that are potentially dangerous to humans and animals (Förster *et al*. 2007).

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One method of reducing noxious populations is the use of entomopathogenic microorganisms as insecticides (Oliveira *et al*. 2012). Bacteria, such as *Serratia* sp. and *Bacillus thuringiensis* or microsporidium *Octosporea muscaedomesticae*, can induce lethal effects in insect victims (Smallbridge *et al*. 1995; O'Callagan *et al*. 1996). Entomopathogenic fungi cause lethal infections of insects and can regulate their populations in nature by epizootics. Currently about 35 genera with more than 400 species of entomopathogenic fungi are known. Approximately 1800 associations between fungi and different insects have been recorded (Jankevica, 2004). Most pathogenic fungi have a broad host range, while fungi belonging to Entomophthorales are characterized by high selectivity (Bałazy, 2004). As a contact insecticide, entomopathogenic fungi invade their host through

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the cuticle, covered by an outermost lipid layer mainly composed of highly stable, very long chain structures (Pedrini *et al.* 2007).

There are a few natural insect defence mechanisms, including innate immunity and cuticular lipids, to counteract fungal infections. The cuticular surface of insects plays a primary role in biochemical and physiological functions, such as preventing insect desiccation (Yoder and Denlinger, 1990; Gibbs *et al.* 1997, 1998; Benoit and Denlinger, 2007). In many species, cuticular lipids also have communicative functions (Vásquez *et al.* 2008; Kühbandner *et al.* 2012). Antimicrobial activity of insects' cuticular lipids is frequently described (Kerwin, 1982; Gołębiowski *et al.* 2008*a*, 2012*c*, Urbanek *et al.* 2012). Susceptibility or resistance of various insect species to fungal invasion may result from several factors, including composition of the cuticular lipids. In particular, free fatty acids are responsible for resistance to fungal infection (Gołębiowski *et al.* 2008*a*). Cuticular fatty acids are toxic and fungistatic but also may be stimulatory. For example, palmitoleic acid enhances mycelial growth, but is toxic to conidia of *Erynia variabilis* (Kerwin, 1984). The toxic effects of palmitoleic acid can be mitigated by the presence of a sufficient concentration of oleic acid.

Larvae of *C. vicina*, closely related to *C. vomitoria*, are highly resistant to the entomophthoralean cosmopolitan soil fungus *Conidiobolus coronatus* (Gołębiowski *et al.* 2008*a*), known to be a potent entomopathogen (Boguś and Scheller, 2002; Domsch *et al.* 2007). Histological examination of *C. vicina* larvae exposed to sporulating *C. coronatus* colonies proved that conidia were unable to germinate on the fly cuticle, thus suggesting the presence of compounds inhibiting spore germination (Boguś *et al.* 2007). In fact, the cuticular fatty acid profile of *C. vicina* larvae significantly differs from the profiles of *Dendrolimus pini* and *Galleria mellonella*, which are highly susceptible to fungal infection. The major difference is the presence of $C_{14:0}$, $C_{16:1}$ and $C_{20:0}$ in the cuticle of *C. vicina* while these 3 fatty acids are absent in the cuticle of *D. pini* or present in trace amounts in *G. mellonella* cuticle (Gołębiowski *et al.* 2008*a*). *In vitro* cultivation of *C. coronatus* in the presence of these 3 fatty acids resulted in reduced sporulation, biomass of hyphae, ability to infect *G. mellonella* larvae and toxicity of metabolites released by the fungus into the culture medium (Boguś *et al.* 2010), proving a contribution of these fatty acids to the resistance of *C. vicina* larvae to fungal assault. Recent studies on *C. vomitoria* showed that crude extracts containing both cuticular and internal lipids showed no antifungal activity against *C. coronatus* efficiently killing *C. vomitoria* adults, but not larvae and pupae.

The aim of the present work was to reveal whether cuticular and internal alcohols as well as fatty acid methyl esters (FAMEs) of *C. vomitoria* larvae, pupae and adults demonstrate antimicrobial activity against 6 entomopathogenic fungal strains. This work presents qualitative and quantitative analyses of the cuticular compounds of larvae, pupae, males and females of *C. vomitoria*. Hydrocarbons of *C. vomitoria* have already been identified (Trabalon *et al.* 1992), so we focused on FAMEs, alcohols, glycerol and cholesterol profiles.

MATERIALS AND METHODS

Insects

Calliphora vomitoria (Diptera: Calliphoridae) were reared from eggs laid on beef by adult flies at 25 °C, 70% relative humidity and a 12:12 h photoperiod. The maternal generation was maintained under the same conditions. The insects were fed on beef. Approximately 7 days elapsed between hatching and puparium formation, and it took another 7 days for the adults to emerge. Cuticular and internal lipids were extracted from post-feeding third-instar larvae, freshly formed pupae and 6-day-old sexually mature adults.

Extraction of cuticular and internal lipids

Two solvents of different polarity – petroleum ether (Chempur Piekary Śląskie, Poland) and dichloromethane (Eurochem BGD Tarnów, Poland) – were used for the lipid extractions. Three extracts were obtained from each developmental stage. The insects were immersed in 50 mL of petroleum ether for 10 sec (extract I). Then the insects were placed in dichloromethane (50 mL) and left there for 5 min (extract II). The same insects then were transferred to dichloromethane for 10 days (extract III). All extracts were concentrated using a roto-evaporator. Analytical samples were concentrated under a stream of nitrogen. For each sample, 19-methylarachidic acid was added as internal standard.

HPLC–LLSD

Extract lipids were separated into classes of compounds using high performance liquid chromatography with a laser light scattering detector (HPLC–LLSD) and a normal-phase 250 × 4·6 mm analytical column with Econosil Silica (Alltech, particle size 5 μm). The mobile phase consisted of *n*-hexane (Solvent A) and dichloromethane containing 15% acetone (Solvent B). The gradient was programmed linearly from A to B within 30 min.

Derivatization

To transform a chemical compound into a more volatile product, the samples were dried under

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a stream of nitrogen and then 50 mL of a mixture of 99% bis(trimethylsilyl)acetamide and 1% chlorotrimethylsilane (Supelco) was added to 1 mg of each extract. The samples were heated in a heating block at 100 °C for 1 h and cooled prior to GC–MS analysis. Alcohols, glycerol and cholesterol were identified as trimethylsilyl (TMS) derivatives of these compounds, while FAMEs were analysed as native compounds.

Gas chromatography–mass spectrometry

GC–MS analysis was performed with a Hewlett -Packard 6890 gas chromatograph equipped with an Rtx-5 capillary column (J&W Scientific, 30 m × 0.25 mm i.d. × 0.15 μm film thickness) and SSQ 710 equipped with an HP 6890 GC. The program started at an initial temperature of 80 °C with an initial hold for 8 min, and was increased gradually to 310 °C at 4 °C min^{-1} with a final hold for 20 min. Helium was used as a carried gas (column head pressure 82 kPa, flow rate of 1 mL min^{-1}, operating at constant flow). Electron impact mode was performed at 70 eV. The temperature of the GC–MS interface was 310 °C. The ion source was maintained at 200 °C. Components were characterized by comparison of their individual mass spectra with standards.

Standards

In the HPLC analysis the following standards were used: *n*-tetracosane (Fluka AG), tricosanoic acid methyl ester (Applied Science Laboratories Inc.), eicosanoic acid (Fluka AG), hexadecanoic acid (Aldrich), 1-hexadecanol (Sigma) and cholesterol (Fluka AG). GC–MS analysis employed the following standards: 19-methylarachidic acid (Sigma), decanoic acid (Sigma), dodecanoic acid (Carl Roth RG), tetradecanoic acid (Fluka AG), hexadecanoic acid (Sigma), octadecenoic acid (Aldrich), octadecanoic acid (Sigma), eicosanoic acid (Fluka AG), tetracosanoic acid (Sigma) and n-alkane standards (Polyscience Corporation).

Determination of antifungal activity

The minimum inhibitory concentration (MIC) was determined using the broth dilution method according to the procedures recommended by the CLSI (Clinical and Laboratory Standards Institute). The following fungal strains were tested: *Beauveria bassiana* (Tve-N39), *B. bassiana* (Dv-1/07), *Lecanicillium lecanii, Metarhizium anisopliae, Paecilomyces fumosoroseus* and *Paecilomyces lilacinus*. Entomopathogenic fungi were obtained from the Institute of Plant Protection (Poznań, Poland). Fungal strains were cultured in Sabouraud

Table 1. Quantitative summary of the experiment: numbers and masses of insect; masses of lipids

Stages	Number of insects	Masses of insects (g)	Extracts	Masses of lipids (mg)
Larvae	53	2·2	I	0·9
			II	1·1
			III	158·4
Pupae	40	1·1	I	1·9
			II	13·1
			III	168·3
Male	52	0·9	I	1·9
			II	5·5
			III	33·4
Female	66	1·0	I	5·6
			II	13·6
			III	60·0

Glucose liquid medium (CARL ROTH GmbH) for 48 h at 25 °C with shaking (130 rpm). To determine the MICs, the suspensions of microorganisms in Sabouraud Glucose broth were adjusted with a spectrophotometer (Genesys 10uv, Thermo Electron Corporation) at $\lambda = 530$ nm to obtain initial inocula of *c.* 5×10^3 cfu mL^{-1}. Microorganisms placed on polystyrene 96-well plates were exposed to the investigated alcohols, FAMEs and their mixtures at appropriate concentrations (range: 2–1024 mg mL^{-1}) for 48 h at 25 °C. Mixtures of alcohols and FAMEs were prepared according to their chemical composition found in the extracts obtained from different living forms of *C. vomitoria*. The MIC was taken to be the lowest concentration of the tested compound at which observable growth was inhibited. Experiments were performed in triplicate on 3 different days.

RESULTS

Total lipids and their identification

The respective total quantities of cuticular lipids of larvae, pupae, males and females extracted with petroleum ether and dichloromethane (extracts I and II) were 2·0, 15·0, 7·4 and 19·2 mg, which correspondingly made up 1·3, 8·9, 22·2 and 32·0% of the total lipids. Table 1 lists the total amounts of internal lipids (extract III). The 3 fractions obtained from the HPLC-LLSD separations of FAMEs, alcohols and sterols were then analysed by GC–MS. Alcohols were identified on the basis of the characteristic ions of their silyl derivatives. The characteristic alcohol ions were at *m/z* 73, 75, 103 and $(M-15)^+$. The mass spectrum of the TMS ether of heptadecanol is illustrated by way of the example in Fig. 1. FAMEs were identified on the basis of the mass spectra obtained for native compounds. FAMEs have characteristic ions at *m/z* 74, 87 and $(M)^+$. The mass spectrum of hexadecanoic acid methyl ester is shown

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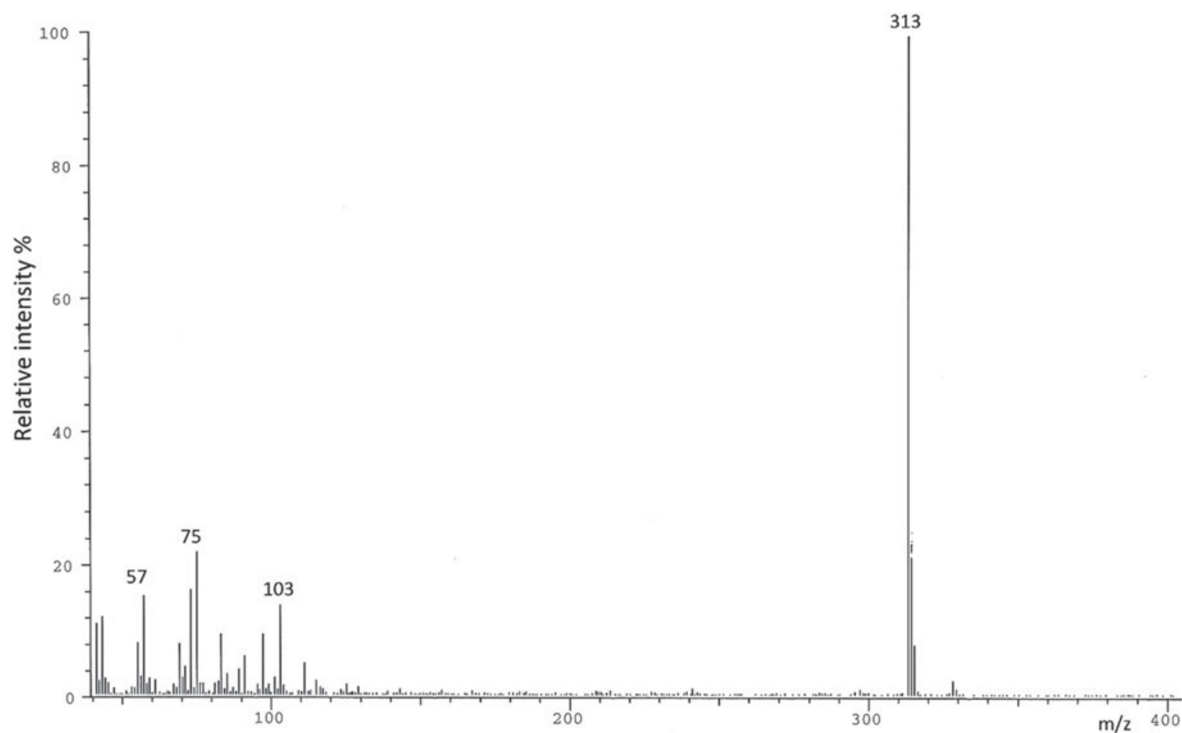

Fig. 1. Mass spectrum of the trimethylsilyl (TMS) ether of heptadecanol.

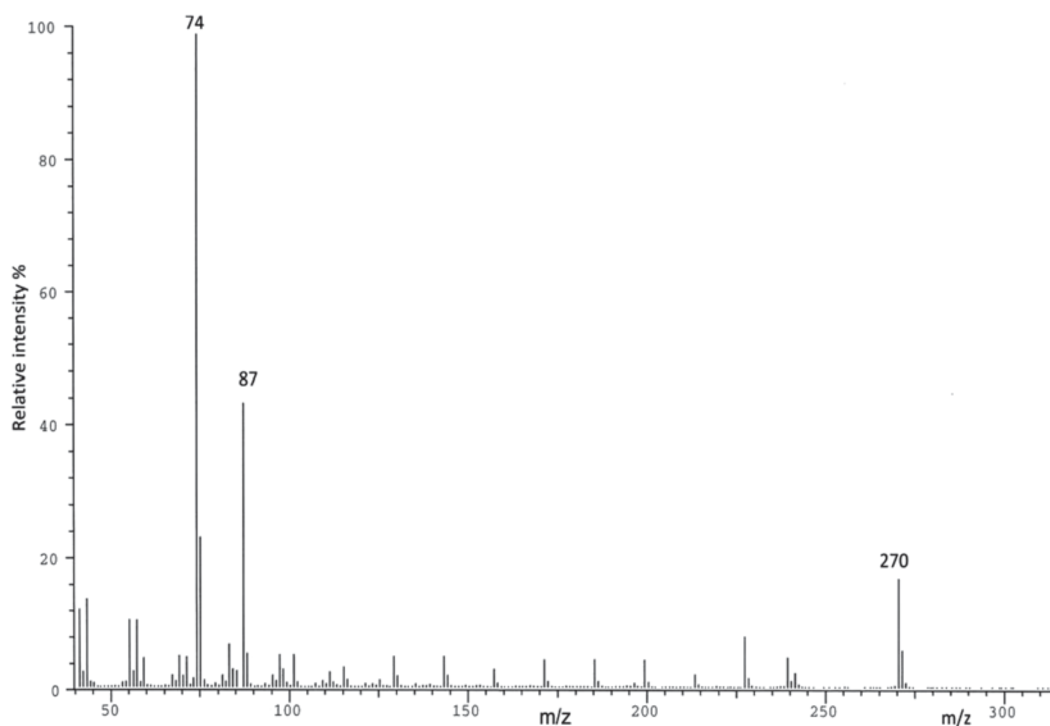

Fig. 2. Mass spectrum of hexadecanoic acid methyl ester.

in Fig. 2. The characteristic ions of the TMS ether derivatives of cholesterol were at m/z 129 (100%), 329 (87%), 368 (52%), 145 (38%), 121 (36%), 353 (32%) and 458 $(M)^+$ (Fig. 3), while those of the TMS ether derivatives of glycerol were at m/z 73, 103, 133, 147, 205 and 218 (Fig. 4).

Cuticular FAME composition of C. vomitoria

Table 2 lists the percentage contents of methyl esters in the cuticle of *C. vomitoria* as well as the methyl ester contents calculated per g of insect body. The total cuticular methyl ester content in *C. vomitoria*

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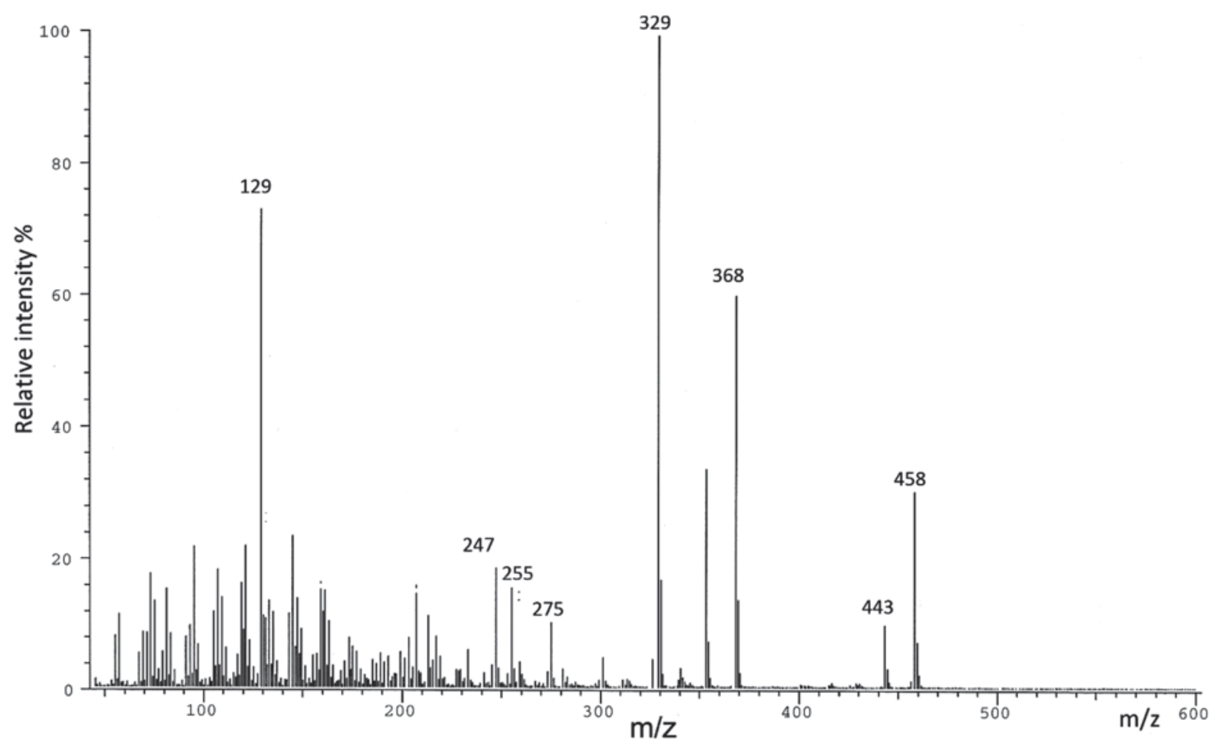

Fig. 3. Mass spectrum of the trimethylsilyl (TMS) ether of cholesterol.

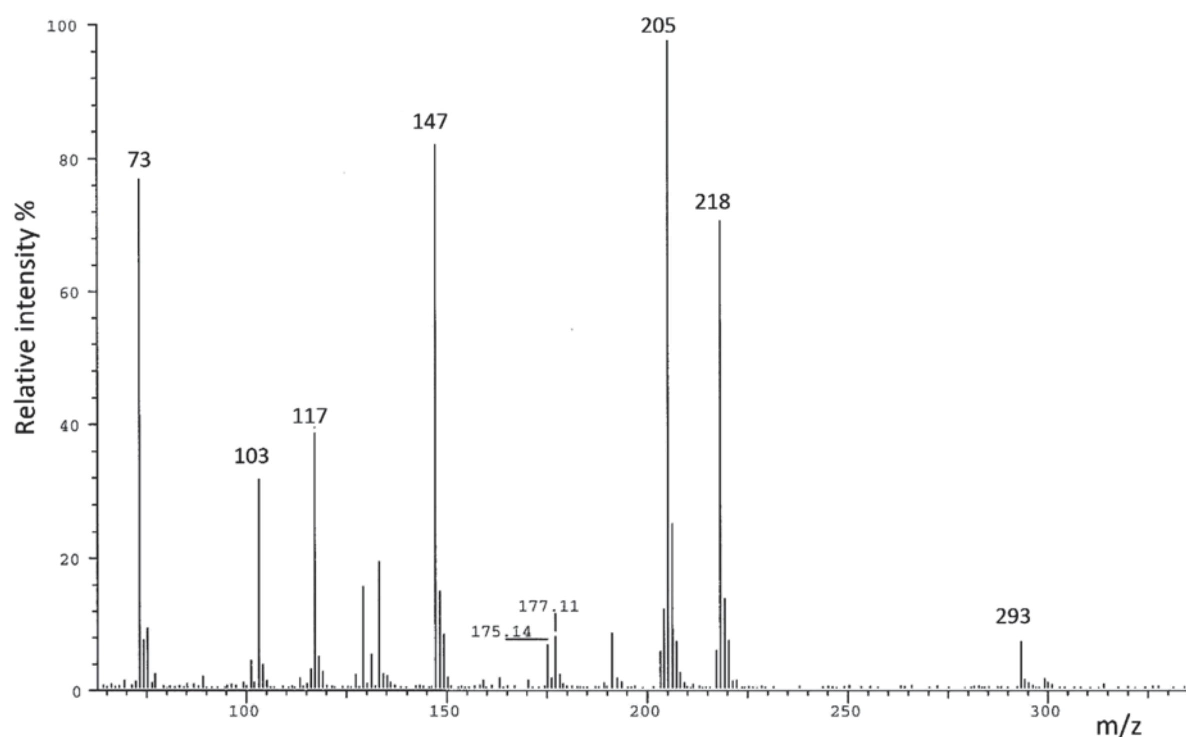

Fig. 4. Mass spectrum of the trimethylsilyl (TMS) ether of glycerol.

larvae was only 1·21 $\mu g\ g^{-1}$ of the insect body. Only 6 methyl esters (from C_{15} to C_{19}: 3 saturated and 3 unsaturated) were identified in the cuticular lipids of the larvae. The compounds present in the highest concentrations were $C_{17:1}$ (0·67 $\mu g\ g^{-1}$ of the insect body; relative content 55·4% of total methyl esters) and $C_{19:1}$ (0·41 $\mu g\ g^{-1}$ of the insect body; relative content 33·9% of total methyl esters). The remaining methyl esters, present in smaller quantities, were $C_{17:0}$ (5·0%), $C_{19:2}$ (3·3), $C_{15:0}$ (2·5%) and $C_{19:0}$ (traces).

FAMEs were the second largest group of compounds detected in pupal cuticular lipids (Table 2). They consisted of 7 compounds, with $C_{17:1}$ and $C_{19:1}$

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Table 2. Chemical composition of the cuticular FAMEs found in *Calliphora vomitoria*

	Content ($\mu g\ g^{-1}$)			Relative content (%)		
FAME	Extract I	Extract II	Sum of FAME	Extract I	Extract II	Sum of FAME
Larvae						
$C_{13:0}$	–	–	–	–	–	–
$C_{15:1}$	–	–	–	–	–	–
$C_{15:0}$	–	0·03 ± 0·01	0·03	–	2·5	2·5
$C_{16:0}$	–	–	–	–		
$C_{17:1}$	Traces	0·67 ± 0·05	0·67	Traces	55·4	55·4
$C_{17:0}$	Traces	0·06 ± 0·01	0·06	Traces	5·0	5·0
$C_{18:0}$	–	–	–	–		
$C_{19:2}$	–	0·04 ± 0·01	0·04	–	3·3	3·3
$C_{19:1}$	Traces	0·41 ± 0·04	0·41	Traces	33·9	33·9
$C_{19:0}$	Traces	Traces	Traces	Traces	Traces	Traces
Sum	Traces	1·21	1·21			
Pupae						
$C_{13:0}$	–	–	–	–	–	–
$C_{15:1}$	–	–	–	–	–	–
$C_{15:0}$	–	8·81 ± 0·67	8·81	–	3·9	3·9
$C_{16:0}$	–	Traces	Traces	–	Traces	Traces
$C_{17:1}$	0·19 ± 0·02	96·82 ± 8·76	97·01	31·7	42·5	42·5
$C_{17:0}$	Traces	14·05 ± 1·45	14·05	Traces	6·2	6·2
$C_{18:0}$	–	–	–	–	–	–
$C_{19:2}$	–	12·65 ± 1·24	12·65	–	5·6	5·5
$C_{19:1}$	0·41 ± 0·03	95·45 ± 7·55	95·86	68·3	41·9	42·0
$C_{19:0}$	Traces	Traces	Traces	Traces	Traces	Traces
Sum	0·60	227·78	228·38			
Male						
$C_{13:0}$	–	–	–	–	–	–
$C_{15:1}$	–	–	–	–	–	–
$C_{15:0}$	Traces	Traces	Traces	Traces	Traces	Traces
$C_{16:0}$	–	–	–	–	–	–
$C_{17:1}$	0·85 ± 0·06	7·10 ± 0·65	7·95	31·5	29·9	30·1
$C_{17:0}$	0·45 ± 0·05	2·76 ± 0·11	3·21	16·7	11·6	12·1
$C_{18:0}$	–	–	–	–	–	–
$C_{19:2}$	–	–	–	–	–	–
$C_{19:1}$	1·40 ± 0·13	13·87 ± 1·39	15·27	51·9	58·4	57·8
$C_{19:0}$	Traces	Traces	Traces	Traces	Traces	Traces
Sum	2·70	23·73	26·45			
Female						
$C_{13:0}$	–	–	–	–	–	–
$C_{15:1}$	–	–	–	–	–	–
$C_{15:0}$	0·42 ± 0·05	1·19 ± 0·11	1·61	0·4	0·6	0·5
$C_{16:0}$	–	Traces	Traces	–	Traces	Traces
$C_{17:1}$	24·48 ± 2·35	65·88 ± 4·70	90·36	21·7	34·9	29·9
$C_{17:0}$	35·62 ± 2·87	6·87 ± 0·43	42·49	31·5	3·6	14·1
$C_{18:0}$	–	–	–	–	–	–
$C_{19:2}$	8·83 ± 1·02	22·62 ± 2·18	31·45	7·8	12·0	10·4
$C_{19:1}$	42·94 ± 3·77	91·66 ± 9·69	134·60	38·0	48·5	44·6
$C_{19:0}$	0·64 ± 0·06	0·62 ± 0·07	1·26	0·6	0·3	0·4
Sum	112·93	188·84	301·77			

The data are presented as the mean ± standard deviation of three separate analyses performed on different samples; '–' not detected.

being the dominant components (42·5 and 42·0% respectively). Three other compounds were $C_{15:0}$ (3·9%), $C_{17:0}$ (6·2%) and $C_{19:2}$ (5·5%). Traces of 2 other FAMEs were also detected ($C_{16:0}$ and $C_{19:0}$).

Five FAMEs were identified in the cuticular lipids of male adults: $C_{19:1}$ (57·8%), $C_{17:1}$ (30·1%), $C_{17:0}$ (12·1%) and $C_{15:0}$ and $C_{19:0}$, traces of which were present (Table 2). The same FAMEs were identified in both cuticular extracts of males (extracts I and II).

$C_{17:0}$ (14·1%) and $C_{19:2}$ (10·4%) were present in relatively large amounts in addition to $C_{17:1}$ (29·9%) and $C_{19:1}$ (44·6%) in the lipids of females (Table 2). Three saturated FAMEs ($C_{15:0}$, $C_{16:0}$ and $C_{19:0}$) were present in smaller amounts from traces to 0·5%.

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Table 3. Chemical composition of the internal FAMEs found in *Calliphora vomitoria*

FAME	Content (μg g^{-1}) Extract III	Relative content (%) Extract III	Content (μg g^{-1}) Extract III	Relative content (%) Extract III
	Larvae		Pupae	
$C_{13:0}$	–	–	3·8 ± 0·46	0·1
$C_{15:1}$	17·94 ± 1·65	0·6	28·9 ± 2·21	0·9
$C_{15:0}$	85·56 ± 5·09	2·9	133·8 ± 9·43	4·1
$C_{16:0}$	13·71 ± 1·44	0·5	7·4 ± 0·54	0·2
$C_{17:1}$	1670·84 ± 152·73	57·1	1178·1 ± 125·56	35·8
$C_{17:0}$	110·42 ± 10·32	3·8	275·9 ± 17·59	8·4
$C_{18:0}$	Traces	Traces	8·9 ± 0·55	0·3
$C_{19:2}$	116·24 ± 9·45	4·0	220·3 ± 13·65	6·7
$C_{19:1}$	907·12 ± 78·23	31·0	1426·5 ± 162·19	43·3
$C_{19:0}$	4·11 ± 0·33	0·1	8·8 ± 0·76	0·3
Sum	2925·94		3292·40	
	Male		Female	
$C_{13:0}$	–	–	19·15 ± 1·68	0·8
$C_{15:1}$	–	–	–	–
$C_{15:0}$	3·49 ± 0·32	2·1	2·12 ± 0·27	0·1
$C_{16:0}$	Traces	Traces	–	–
$C_{17:1}$	58·58 ± 4·65	34·8	776·73 ± 56·32	34·1
$C_{17:0}$	14·90 ± 1·22	8·8	88·91 ± 6·55	3·9
$C_{18:0}$	–	–	–	–
$C_{19:2}$	–	–	234·43 ± 22·50	10·3
$C_{19:1}$	91·54 ± 7·66	54·3	1153·75 ± 110·31	50·6
$C_{19:0}$	Traces	Traces	4·25 ± 0·32	0·2
Sum	168·51		2279·34	

The data are presented as the mean ± standard deviations of three separate analyses performed on different samples; '–' not detected.

Internal FAME composition of C. vomitoria

Nine FAMEs – from $C_{15:1}$ to $C_{19:0}$ – were found in the internal lipids of larvae (Table 3). The FAMEs present in the highest concentrations were $C_{17:1}$ (1670·84 μg g^{-1} of the insect body; relative content 57·1% of total methyl esters) and $C_{19:1}$ (907·12 μg g^{-1} of the insect body; relative content 31·0% of total methyl esters). The other methyl esters were present in smaller quantities, from trace amounts to 116·24 μg g^{-1} of the insect body: $C_{15:1}$ (0·6%), $C_{15:0}$ (2·9%), $C_{16:0}$ (0·5%), $C_{17:0}$ (3·8%), $C_{18:0}$ (traces), $C_{19:2}$ (4·0%) and $C_{19:0}$ (0·1%). The internal lipids contained 2 even-numbered methyl esters ($C_{16:0}$ and $C_{18:0}$) and 1 unsaturated methyl ester ($C_{15:1}$), which were absent in the cuticular lipids. Other FAMEs were present in both cuticular and internal lipids of larvae.

Ten FAMEs were found in the internal lipids of the pupae (Table 3). Two of them ($C_{17:1}$ and $C_{19:1}$) were predominant, accounting for 35·8% and 43·3% of the total FAMEs. The remaining methyl esters were present in smaller quantities: $C_{13:0}$ (0·1%), $C_{15:1}$ (0·9%), $C_{15:0}$ (4·1%), $C_{16:0}$ (0·2%), $C_{17:0}$ (8·4%), $C_{18:0}$ (0·3%), $C_{19:2}$ (6·7%) and $C_{19:0}$ (0·3%).

The most abundant internal FAMEs in males and females were $C_{17:1}$ (34·8 *vs* 34·1%) and $C_{19:1}$ (54·3 *vs* 50·6%) (Table 3). Other FAMEs were present in smaller quantities, from trace amounts ($C_{16:0}$ and $C_{19:0}$) to 8·8% ($C_{17:0}$) in males and from 0·1% ($C_{15:0}$) to 10·3% ($C_{19:2}$) in females.

Cuticular and internal alcohol composition of C. vomitoria

The cuticular lipids of larvae contained 7 saturated alcohols only, with even-numbered carbon chains from $C_{12:0}$ to $C_{24:0}$ (Table 4). The total cuticular alcohol content in *C. vomitoria* larvae was only 3·11 μg g^{-1} of the insect body. The alcohols occurring in the highest concentrations were $C_{18:0}$ (25·7%), $C_{20:0}$ (28·6%) and $C_{22:0}$ (23·2%); those present in smaller quantities included $C_{12:0}$ (4·8%), $C_{14:0}$ (4·5%), $C_{16:0}$ (7·4%) and $C_{24:0}$ (5·8%). The internal lipids of the larvae contained only 1 saturated alcohol with an even-numbered carbon chain – $C_{22:0}$ (1·14 μg g^{-1} of the insect body) (Table 5).

The cuticular and internal lipids of pupae contained 6 and 4 alcohols respectively (Tables 4 and 5): $C_{18:0}$, $C_{20:0}$ and $C_{22:0}$ were the most abundant alcohols in these two developmental stages. Together, these compounds made up 88·0% of the alcohols present in cuticular lipids and 100% of those in the pupal internal lipids. Cuticular alcohols present in smaller quantities included $C_{14:0}$ (6·3%), $C_{16:0}$ (5·6%) and $C_{24:0}$ (traces). All the cuticular and internal alcohols of pupae were saturated and had an even number of carbon atoms.

The cuticular lipids of males contained 5 alcohols, present in small amounts (Table 4). The most abundant alcohol was $C_{22:0}$ (60·2%), but this made up only 0·80 μg g^{-1} of the insect body. The female

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Table 4. Chemical composition of the cuticular alcohols found in larvae of *Calliphora vomitoria*

	Content ($\mu g\ g^{-1}$)			Relative content (%)		
Alcohols	Extract I	Extract II	Sum of alcohols	Extract I	Extract II	Sum of alcohols
Larvae						
$C_{12:0}$	0·15 ± 0·02	–	0·15	5·8	–	4·8
$C_{14:0}$	0·14 ± 0·02	Traces	0·14	5·4	Traces	4·5
$C_{16:0}$	0·23 ± 0·02	–	0·23	8·9	–	7·4
$C_{18:0}$	0·58 ± 0·04	0·22 ± 0·02	0·80	22·5	41·5	25·7
$C_{20:0}$	0·75 ± 0·06	0·14 ± 0·02	0·89	29·1	26·4	28·6
$C_{22:0}$	0·55 ± 0·05	0·17 ± 0·02	0·72	21·3	32·1	23·2
$C_{24:0}$	0·18 ± 0·02	Traces	0·18	7·0	Traces	5·8
Sum	2·58	0·53	3·11			
Pupae						
$C_{12:0}$	–	–	–	–	–	–
$C_{14:0}$	0·09 ± 0·01	–	0·09	9·3		6·3
$C_{16:0}$	0·08 ± 0·01	–	0·08	8·2		5·6
$C_{18:0}$	0·35 ± 0·03	–	0·35	36·1		24·6
$C_{20:0}$	0·27 ± 0·03	0·24 ± 0·02	0·51	27·8	53·3	35·9
$C_{22:0}$	0·18 ± 0·02	0·21 ± 0·02	0·39	18·6	46·7	27·5
$C_{24:0}$	Traces	Traces	Traces	Traces	Traces	Traces
Sum	0·97	0·45	1·42			
Male						
$C_{12:0}$	–	–	–	–	–	–
$C_{14:0}$	–	–	–	–	–	–
$C_{16:0}$	0·09 ± 0·01	–	0·09	6·8	–	6·8
$C_{18:0}$	0·16 ± 0·02	Traces	0·16	12·0	Traces	12·0
$C_{20:0}$	0·28 ± 0·03	Traces	0·28	21·1	Traces	21·1
$C_{22:0}$	0·80 ± 0·07	–	0·80	60·2	–	60·2
$C_{24:0}$	Traces	–	Traces	Traces	–	Traces
Sum	1·33	–	1·29			

The data are presented as the mean ± standard deviation of three separate analyses performed on different samples; '–' not detected.

cuticular lipids contained only trace amounts of 3 alcohols.

The internal lipids of males and females contained only 4 alcohols (Table 5); all of those in the males were present in trace amounts. The most abundant alcohol in the internal lipids of the females was $C_{18:0}$ (53·4%; 7·32 $\mu g\ g^{-1}$ of the insect body).

Cuticular and internal glycerol and cholesterol in C. vomitoria

The cuticular and internal lipids of larvae, pupae, males and females contained cholesterol and glycerol (Table 6). The respective quantities of cuticular cholesterol obtained from larvae and pupae of *C. vomitoria* were 0·68 and 6·48 $\mu g\ g^{-1}$ of the insect body. There was a 10-fold higher concentration of cuticular cholesterol in the pupal lipids. However, a similar amount of internal cholesterol was identified in the larval and pupal lipids. The internal cholesterol content in larval and pupal lipids was 173·79 and 181·45 $\mu g\ g^{-1}$ respectively. A similar amount of cuticular cholesterol was identified in the male and female lipids. However, the content of internal cholesterol in females was significantly higher than that of the internal lipids in males: 232·19 and 164·29 $\mu g\ g^{-1}$ of the insect body respectively.

There was a 2- fold higher concentration of cuticular glycerol in larval lipids than in pupal lipids. On the other hand, the internal glycerol content in pupae was significantly higher than in larvae: 55·11 and 5·94 $\mu g\ g^{-1}$ of the insect body respectively. A 10- fold greater concentration of cuticular glycerol was determined in male lipids than in female lipids, but the glycerol contents in male and female internal lipids were similar.

Antifungal activity of FAMEs

The individual FAMEs displayed only weak activity, if any, against the entomopathogenic fungi tested. *Metarhizium anisopliae* and *P. fumosoroseus* were susceptible to almost all the compounds when exposed to a concentration of 1024 mg L^{-1}. The other fungal strains turned out to be resistant to the majority of FAMEs at the concentrations applied (2–1024 mg L^{-1}), although some growth reduction was observed with most of them at the highest concentrations (data not shown). The mixtures of FAMEs found in living forms of *C. vomitoria* were

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Table 5. Chemical composition of the internal alcohols found in *Calliphora vomitoria*

Alcohols	Content ($\mu g\ g^{-1}$) Larvae	Relative content (%)	Content ($\mu g\ g^{-1}$) Pupae	Relative content (%)
	Extract III	Extract III	Extract III	Extract III
$C_{12:0}$	–	–	–	–
$C_{14:0}$	–	–	–	–
$C_{16:0}$	–	–	–	–
$C_{18:0}$	–	–	1·27±0·11	17·2
$C_{20:0}$	–	–	1·76±0·15	23·8
$C_{22:0}$	1·14±0·08	100·0	4·37±0·33	59·0
$C_{24:0}$	–	–	Traces	Traces
Sum	1·14		7·40	
	Male		Female	
	Extract III	Extract III	Extract III	Extract III
$C_{12:0}$	–	–	–	–
$C_{14:0}$	–	–	–	–
$C_{16:0}$	–	–	–	–
$C_{18:0}$	Traces	Traces	7·32±0·61	53·4
$C_{20:0}$	Traces	Traces	3·70±0·35	27·0
$C_{22:0}$	Traces	Traces	Traces	Traces
$C_{24:0}$	Traces	Traces	2·69±0·28	19·6
Sum	Traces		13·71	

Data are presented as the mean±standard deviation of three separate analyses performed on different samples; '–' not detected.

somewhat more active than the individual compounds. Nearly all of the tested mixtures exhibited some activity against all the fungal isolates. The growth of *P. fumosoroseus* and *B. bassiana* (Tve-N39) was inhibited by the majority of extracts at a concentration of 1024 mg L^{-1}, while *B. bassiana* strain DV-1/107, *L. lecanii* and *M. anisopoliae* demonstrated a higher susceptibility (Table 7).

Antifungal activity of alcohols

The alcohols tested turned out to be very weak antifungal agents. They inhibited the growth of the test strains at a concentration of 1024 mg L^{-1} or were inactive at the concentrations applied (data not shown). Only $C_{12:0}$ and $C_{14:0}$ were more active against the entomopathogenic fungi in comparison with longer-chain alcohols (Table 7). Extracts containing alcohols also showed weak antimicrobial activity. The majority of the strains tested were resistant to the mixtures of internal alcohols extracted from larvae, pupae and females of *C. vomitoria* (data not shown). The activity of male internal alcohols and female cuticular alcohols were not tested as they were present only in trace amounts in the extracts. The remaining mixtures mostly suppressed fungal strains at a concentration of 1024 mg L^{-1}. Growth of *B. bassiana* (DV-1/107) and *M. anisopoliae* was inhibited by cuticular alcohols obtained from larvae and pupae at 512 mg L^{-1}, but only the cuticular alcohols obtained from larvae were effective towards *P. lilacinus* at this concentration (Table 7).

DISCUSSION

The composition of cuticular lipids typically includes various groups of compounds (Lockey, 1988; Buckner, 1993; Gołębiowski *et al.* 2011), often hydrocarbons (Lockey, 1980; Gołębiowski *et al.* 2007, 2012*b*; Fan *et al.* 2008) and fatty acids (Gołębiowski *et al.* 2010; Gołębiowski, 2012), but also aldehydes and ketones (Nelson *et al.* 1998), or wax esters, alcohols, FAMEs and sterols (Ikekawa *et al.* 1993; Buckner *et al.* 1994; Nelson *et al.* 1999; Gołębiowski *et al.* 2012*a*). The composition of lipids depends on various factors, primarily on the insect species, its developmental stage, environment and lifestyle (Mpuru *et al.* 2001). Each cuticular and internal compound of an insect has a specific role, governed by specific needs. The composition may also change with changing environmental pressures such as time of the year, or during ontogeny (Roux *et al.* 2006). Cuticular lipids can be defensive or fungistatic substances, but some of them are active as pheromones. For example, unsaturated fatty acids and FAMEs can affect the settling behaviour of *Liposcelis bostrychophila* and have been found to be repellent to ants (Dani *et al.* 1996; Green, 2009, 2011).

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Table 6. Glycerol and cholesterol contents in larvae and pupae of *Calliphora vomitoria*

Content ($\mu g\ g^{-1}$)

Compounds	Larvae			Pupae			Larvae	Pupae
	Extract I	Extract II	Sum	Extract I	Extract II	Sum	Extract III	Extract III
Glycerol	0·82±0·07	5·06±0·44	5·88	0·45±0·07	2·48±0·18	2·93	5·94±0·45	55·11±3·87
Cholesterol	0·08±0·01	0·60±0·07	0·68	0·21±0·02	6·27±0·57	6·48	173·79±18·23	181·45±18·45
	Male			Female			Male	Female
	Extract I	Extract II	Sum	Extract I	Extract II	Sum	Extract III	Extract III
Glycerol	–	46·94±4·49	46·94	–	4·56±0·37	4·56	59·63±5·33	54·47±4·17
Cholesterol	4·93±0·43	5·58±0·29	10·51	4·19±0·32	9·13±0·77	13·22	164·29±17·84	232·19±25·60

The data are presented as the mean ± standard deviation of three separate analyses performed on different samples; '–' not detected.

Although liquid chromatography has been used to analyse insect lipids (Kermasha *et al.* 1994; Jiann-Tsyh *et al.* 1995), gas chromatography, and gas chromatography hyphenated with mass spectrometry in particular, is the standard technique for this purpose (Nelson *et al.* 1998; Caputo *et al.* 2007; Jarrold *et al.* 2007; Ye *et al.* 2007; Buckner *et al.* 2009). GC–MS has become so commonly used because mass spectra can be obtained, which in contentious situations (similar retention times, traces of compounds) permit the unequivocal identification of compounds, which are analysed by comparing the molecular and fragment ions and using library spectra. Obviously, therefore, the *C. vomitoria* lipids were analysed primarily using GC–MS.

The compounds identified in *C. vomitoria* were FAMEs, alcohols, glycerol and cholesterol. Comparison of the total ion currents indicates that the type of solvent used and the different extraction time affect the composition of the lipid extracts.

FAMEs are minor constituents of cuticular lipids (Lockey, 1988). Methyl palmitate and methyl linolenate appear to act as pheromones for the honeybee and as a kairomone for the parasitic mite *Varroa jacobsoni* (Le Conte *et al.* 1990). FAMEs were detected in significant concentrations in the cuticle of *Acyrthosiphon pisum* and were primarily saturated (Brey *et al.* 1985). The dominant components of *A. pisum* consisted of $C_{19:2}$ (33%), $C_{15:0}$ (23%), $C_{19:1}$ (22%), $C_{19:3}$ (13%) and $C_{17:1}$ (9%). Other cuticular FAMEs were present in traces – $C_{17:0}$ and $C_{19:0}$. Only 3 FAMEs ($C_{17:0}$, $C_{19:1}$ and $C_{19:0}$) were identified in the cuticular lipids of both males and females of *Acanthoscelides obtectus* (Gołębiowski *et al.* 2008*b*).

The present study of the cuticular and internal lipids of *C. vomitoria* demonstrated a significant amount of $C_{17:0}$, $C_{17:1}$ and $C_{19:1}$ FAMEs. In addition, females had a high content of the methyl ester of $C_{19:2}$ (10·4% in cuticular lipids and 10·3% in internal lipids), whereas this compound was absent in males. Both saturated and unsaturated FAMEs were identified in the cuticular and internal lipids of larvae, pupae, males and females. Odd-numbered, saturated and unsaturated FAMEs are typically found in insect species. However, pupae and females of *C. vomitoria* contained 1 cuticular, even-numbered FAME ($C_{16:0}$) in trace amounts. Larvae and pupae of *C. vomitoria* contained 2 internal, even-numbered FAMEs ($C_{16:0}$ and $C_{18:0}$), whereas the male internal lipids contained only traces of $C_{16:0}$ methyl ester. Additionally, the presence of odd-numbered FAMEs ($C_{15:1}$ (0·9%) and $C_{13:0}$ (0·1%)) in the internal lipids of pupae is characteristic, although the $C_{15:1}$ ester (0·6%) was also identified in the internal lipids of larvae.

Since the FAMEs found in insects act mainly as semiochemicals (Le Conte *et al.* 1990), it was not surprising that the compounds did not display a strong antifungal potential. The antimicrobial activity of cuticular and internal FAMEs found in

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Table 7. Minimum inhibitory concentration (mg L^{-1}) of the mixtures of FAMEs, mixtures of alcohols and individual alcohols found in *Calliphora vomitoria*

Mixtures	*B. bassiana* DV-1/107	*B. bassiana* (Tve-N39)	*L. lecanii*	*M. anisopliae*	*P. fumosoroseus*	*P. lilacinus*
Internal FAME – larvae	512	1024	512	512	1024	1024
Cuticular FAME – pupae	512	1024	512	512	1024	1024
Internal FAME – pupae	1024	1024	512	512	1024	512
Cuticular FAME – male	512	1024	1024	512	1024	512
Internal FAME – male	1024	1024	512	512	1024	1024
Cuticular FAME – female	1024	512	512	512	1024	512
Internal FAME – female	1024	1024	512	1024	1024	1024
cuticular alcohols – larvae	512	1024	1024	512	1024	512
Cuticular alcohols – pupae	512	1024	1024	512	1024	1024
Cuticular alcohols – male	1024	1024	1024	512	512	1024
Individual alcohol $C_{12:0}$	512	1024	512	512	512	1024
Individual alcohol $C_{14:0}$	512	1024	1024	256	512	1024

C. vomitoria was not affected by either the source or the mode of isolation. Indeed, the composition of the isolated extracts was rather congruent. In all mixtures the most abundant FAMEs were $C_{17:1}$ and $C_{19:1}$.

The presence of alcohols in the cuticular lipids of some insects is well documented (Ohara and Lockey, 1990; Buckner *et al*. 1996, 1999; Jones *et al*. 1997). Short-chain unsaturated alcohols can be components of pheromones (Buckner, 1993). The composition of cuticular alcohols is very diverse. Some insect species contain only one or two cuticular alcohols (Buckner *et al*. 1994; Jones *et al*. 1997), while others contain a large number of alcohols (Ohara and Lockey, 1990).

In our study, we found the same cuticular alcohol level in pupae and males (1·42 *vs* 1·29%). However, 7, 6, 5 and 3 cuticular alcohols were respectively identified in the lipids of larvae, pupae, males and females. On the other hand, only 1 alcohol was identified in the internal lipids of larvae, and 4 alcohols were present in the internal lipids of pupae, males and females. Alcohols from $C_{12:0}$ to $C_{20:0}$ were present among the compounds identified in our study. The presence of alcohols in this range as major compounds was found only in lipids isolated from *Locusta migratoria migratoides* adults, *Schistocerca gregaria* adults (Ohara and Lockey, 1990) and *A. pisum* (Brey *et al*. 1985). However, even-numbered, saturated alcohols are compounds characteristic of insects. For example, only $C_{28:0}$, $C_{30:0}$, $C_{32:0}$ and $C_{34:0}$ alcohols were found in the lipids isolated from *Bemisia argentifolii* nymphs (Buckner *et al*. 1999), only 1 alcohol ($C_{30:0}$) was identified in a *Tenodera sinensis* female, *Tenodera angustipennis* male and *Stagmomantis carolina* female (Jones *et al*. 1997), and 2 alcohols ($C_{30:0}$ and $C_{32:0}$) were found in *Trialeurodes vaporariorum* adults (Buckner *et al*. 1994).

The antimicrobial activity of alcohols isolated from *M. domestica* has been reported (Gołębiowski *et al*. 2012*c*). These compounds demonstrated antibacterial activity against Gram-positive bacteria and weak activity against *Candida* sp., whereas Gram-negative bacterial strains turned out to be resistant. In the present work most of the individual alcohols as well as the alcohol extracts isolated from *C. vomitoria* demonstrated weak antimicrobial activity towards entomopathogenic fungi. The effectiveness of extracts seems to depend on the strain tested rather than on the source or mode of isolation. However, we also found that the cuticular extracts obtained from larvae and pupae were slightly more active. These are the only mixtures containing short-chain alcohols that exhibited stronger antifungal activity in the MIC assay performed for individual compounds.

Selective antibacterial and low antifungal activities of *C. vomitoria* cuticular alcohols and FAMEs indicate that these compounds may serve as additional factors supporting defence systems protecting this ectoparasite from microorganisms. Larvae of *C. vomitoria* and closely related *C. vicina* exposed to virulent colonies of *C. coronatus* remain unharmed (Boguś *et al*. 2007; Gołębiowski *et al*. 2013). *Callophora vicina* is known to produce, after an experimental challenge with bacteria, a series of potent antimicrobial substances namely defensin, diptericins, cecropins, proline-rich peptides, as well as alloferons showing amino-acid stretches similar to those of the antifungal protein isolated from *Sarcophaga peregrine* (Chernysh *et al*. 2002). Contact with *C. coronatus* activates cecropins and lysozyme (Boguś *et al*. 2007). Whether other elements of this weaponry are activated by contact with fungus still remains an open question.

In our study, glycerol was identified in the cuticle, as well as in the internal lipids of larvae, pupae, males and females. It occurred in the largest quantities in the internal lipids of pupae (55·11 μg g^{-1}) and in the cuticular lipids of males (46·94 μg g^{-1} of the insect body). The physiological role of the sexual differences in cuticular glycerol concentration

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(female cuticles contain 10·3 times less than male cuticles) is unknown. The reason for the 16- and 7·8-fold lower concentrations of cuticular glycerol, respectively detected in pupae and larvae, compared with males, also remains obscure. Additional experiments are needed to explain the developmental and sexual differences in the internal glycerol concentrations and the mechanism underlying the divergences in the internal/cuticular glycerol concentration ratios, which range from 1:1 in larvae, 1·3:1 in males, 11·9:1 in females to 18·8:1 in pupae.

Glycerol was detected as the main compound in the haemolymph and gland secretions of *Eudia pavonia, Saturnia pyri* and *Eupackardia calleta* caterpillars (Deml and Dettner, 1993). This compound usually acts as an antifreezing agent in the haemolymph of a number of diapausing insects (Somme, 1964, 1965). The properties of glycerol as a factor making insects tolerant towards low temperatures have been studied. Larvae of the flesh fly *Sarcophaga bullata*, exposed to −10 °C immediately after administration of glycerol, survived at high rates. However, when glycerol-fed larvae were kept for 2 days at 25 °C before exposure to −10 °C, survival rates were low (Yoder *et al*. 2006), indicating the complex nature of the mechanism underlying glycerol-induced cold tolerance. No data are available concerning the role of glycerol in the overwintering of *C. vomitoria*.

The final compound identified in the cuticular and internal lipids of *C. vomitoria* was cholesterol. No other sterol was identified apart from cholesterol. The presence of cholesterol has an important impact on insect development, especially internal cholesterol, which is an ecdysteroid precursor (Svoboda and Weirich, 1995). For example, in *Coleomegilla maculata*, Pilorget *et al*. (2010) examined whether and to what extent the type of food (corn pollen with a different sterol content – beta-sitosterol, cholesterol, ergosterol) would have an impact on the state of the insect. A strong correlation was found between the amount of administered sterols and population growth. The proportionately decreasing content of sterols in the diet over time and the growth of *C. maculata* larvae were also correlated (Pilorget *et al*. 2010). Although insects are unable to biosynthesize cholesterol (Ikekawa *et al*. 1993), the cholesterol they contain may be derived from their food (Böröczky *et al*. 2008), or they may convert phytosterols to cholesterol via the dealkylation of phytosterols. In our study, the high concentrations of cholesterol detected in the internal lipids of all developmental stages (with the highest values being in females) probably reflect the storage of lipids necessary for metamorphosis and the involvement of cholesterol in vitellogenesis. Note that internal cholesterol makes up 0·24–0·39% of the total extracted internal lipids in larvae, pupae and females, but is less than 0·04% in males. Assuming that lipids constitute a major metabolic store for the development of fly larvae to adults, large amounts of internal lipids in larvae and pupae cannot be surprising (Saunders, 2000). The physiological role of the observed developmental and sexual divergences in the concentrations of cuticular cholesterol remains to be discovered.

In conclusion, the methods applied enabled the cuticular and internal compounds of *C. vomitoria* to be identified and quantified. A total of 19 compounds were identified in the larvae, pupae and adult insects, including 10 FAMEs, 7 alcohols, cholesterol and glycerol. The alcohols and FAMEs displayed antimicrobial activity against entomopathogenic fungi.

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REFERENCES

Bałazy, S. (2004). Significance of protected areas for the preservation of entomopathogenic fungi. *Kosmos* **53**, 5–16.

Benoit, J. B. and Denlinger, D. L. (2007). Suppression of water loss during adult diapause in the northern house mosquito, *Culex pipiens*. *Journal of Experimental Biology* **210**, 217–226.

Boguś, M. I. and Scheller, K. (2002). Extraction of an insecticidal protein fraction from the pathogenic fungus *Conidiobolus coronatus*. *Acta Parasitologica* **47**, 66–72.

Boguś, M. I., Kędra, E., Bania, J., Szczepanik, M., Czygier, M., Jabłoński, P., Pasztaleniec, A., Samborski, J., Mazgajska, J. and Polanowski, A. (2007). Different defense strategies of *Dendrolimus pini*, *Galleria mellonella*, and *Calliphora vicina* against fungal infection. *Journal of Insect Physiology* **53**, 909–922.

Boguś, M. I., Czygier, M., Gołębiowski, M., Kędra, E., Kucińska, J., Mazgajska, J., Samborski, J., Wieloch, W. and Włóka, E. (2010). Effects of insect cuticular fatty acids on *in vitro* growth and pathogenicity of the entomopathogenic fungus *Conidiobolus coronatus*. *Experimental Parasitology* **125**, 400–408.

Böröczky, K., Park, K. C., Minard, R., Jones, T. H., Baker, T. C. and Tumlinson, J. H. (2008). Differences in cuticular lipid composition of the antennae of *Helicoverpa zea, Heliothis virescens*, and *Manduca sexta*. *Journal of Insect Physiology* **54**, 1385–1391.

Bourel, B., Fleurisse, L., Hedouin, V., Cailliez, J. C., Creusy, C., Gosset, D. and Goff, M. L. (2001). Immunohistochemical contribution to the study of morphine metabolism in Calliphoridae larvae and implications in forensic entomotoxicology. *Journal of Forensic Sciences* **46**, 596–599.

Brey, P. T., Ohayon Lesourd, M., Castex, H., Roucache, J. and Latge, J. P. (1985). Ultrastructure and chemical composition of the outer layers of the cuticle of the pea aphid *Acyrthosiphon pisum* (HARRIS). *Comparative Biochemistry and Physiology A* **82**, 401–411.

Buckner, J. S. (1993). Cuticular polar lipids of insects. In *Insect Lipids: Chemistry, Biochemistry and Biology* (ed. Stanley-Samuelson, D. W. and Nelson, D. R.), pp. 227–270. University of Nebraska Press, Lincoln, NE, USA.

Buckner, J. S., Nelson, D. R. and Mardaus, M. C. (1994). The lipid composition of the wax particles from adult whiteflies, *Bemisia tabaci* and *Trialeurodes vaporariorum*. *Insect Biochemistry and Molecular Biology* **24**, 977–987.

Buckner, J. S., Mardaus, M. C. and Nelson, D. R. (1996). Cuticular lipid composition of *Heliothis virescens* and *Helicoverpa zea* pupae. *Comparative Biochemistry and Physiology B* **114**, 207–216.

Buckner, J. S., Hagen, M. M. and Nelson, D. R. (1999). The composition of the cuticular lipids from nymphs and exuviae of the Silverleaf Whitefly, *Bemisia argentifolii*. *Comparative Biochemistry and Physiology B* **124**, 201–207.

Buckner, J. S., Pitts-Singer, T. L., Guédot, Ch., Hagen, M. M., Fatland, Ch. L. and Kemp, W. P. (2009). Cuticular lipids of female solitary bees, *Osmia lignaria* Say and *Megachile rotundata* (F.) (Hymenoptera: Megachilidae). *Comparative Biochemistry and Physiology* **153**, 200–205.

https://doi.org/10.1017/S0031182013000267 Published online by Cambridge University Press

Caputo, B., Dani, F. R., Horne, G. L., Fale, S. N., Diabate, A., Turillazzi, S., Coluzzi, M., Costantini, C., Priestman, A. A., Petrarca, V. and della Torre, A. (2007). Comparative analysis of epicuticular lipid profiles of sympatric and allopatric field populations of *Anopheles gambiae s.s.* molecular forms and *An. arabiensis* from Burkina Faso (West Africa). *Insect Biochemistry and Molecular Biology* **37**, 389–398.

Chernysh, S., Kim, S. I., Bekker, G., Pleskach, V. A., Filatova, N. A., Anikin, V. B., Platonov, V. G. and Bulet, P. (2002). Antiviral and antitumor peptides from insects. *Proceedings of the National Academy of Sciences USA* **99**, 12628–12632.

Dani, F. R., Cannoni, S., Turillazzi, S. and Morgan, E. D. (1996). Ant repellent effect of the sternal gland secretion of *Polistes dominulus* (Christ) and *P. sulcifer* (Zimmermann) (Hymenoptera: Vespidae). *Journal of Chemical Ecology* **22**, 37–48.

Deml, R. and Dettner, K. (1993). Biogenic amines and phenolics characterize the defensive secretion of saturniid caterpillars (Lepidoptera: Saturniidae): a comparative study. *Journal of Comparative Physiology B* **163**, 123–132.

Domsch, K. H., Gams, W. and Anderson, T.-H. (2007). *Compendium of Soil Fungi*, 2nd Edn. IHW Verlag, Eching, Germany.

Fan, Y., Eliyahu, D. and Schal, C. (2008). Cuticular hydrocarbons as maternal provisions in embryos and nymphs of the cockroach *Blattella germanica*. *Journal of Experimental Biology* **211**, 548–554.

Förster, M., Klimpel, S., Mehlhorn, H., Sievert, K., Messler, S. and Pfeffer, K. (2007). Pilot study on synanthropic flies (e.g. *Musca, Sarcophaga, Calliphora, Fannia, Lucilia, Stomoxys*) as vectors of pathogenic microorganisms. *Parasitology Research* **101**, 243–246.

Gibbs, A. G., Chippindale, A. K. and Rose, M. R. (1997). Physiological mechanisms of the evolution of desiccation resistance in *Drosophila melanogaster*. *Journal of Experimental Biology* **200**, 1821–1832.

Gibbs, A. G., Louie, A. K. and Ayala, J. A. (1998). Effects of temperature on cuticular lipids and water balance in a desert *Drosophila*: is thermal acclimation beneficial? *Journal of Experimental Biology* **210**, 71–80.

Gołębiowski, M. (2012). Comparison of free fatty acids composition of cuticular lipids of *Calliphora vicina* larvae and pupae. *Lipids* **47**, 1001–1009.

Gołębiowski, M., Maliński, E., Nawrot, J., Szafranek, J., Stepnowski, P. (2007). Identification of the cuticular lipid composition of the Western Flower Thrips *Frankliniella occidentalis*. *Comparative Biochemistry and Physiology B* **147**, 288–292.

Gołębiowski, M., Maliński, E., Boguś, M. I., Kumirska, J. and Stepnowski, P. (2008*a*). The cuticular fatty acids of *Calliphora vicina, Dendrolimus pini* and *Galleria mellonella* larvae and their role in resistance to fungal infection. *Insect Biochemistry and Molecular Biology* **38**, 619–627.

Gołębiowski, M., Maliński, E., Nawrot, J. and Stepnowski, P. (2008*b*). Identification and characterization of surface lipid components of the dried-bean beetle *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae). *Journal of Stored Product Research* **44**, 386–388.

Gołębiowski, M., Boguś, M. I., Paszkiewicz, M. and Stepnowski, P. (2010). The composition of the free fatty acids from *Dendrolimus pini* exuviae. *Journal of Insect Physiology* **56**, 391–397.

Gołębiowski, M., Boguś, M. I., Paszkiewicz, M. and Stepnowski, P. (2011). Cuticular lipids of insects as potential biofungicides: methods of lipids composition analysis. *Analytical and Bioanalytical Chemistry* **399**, 3177–3191.

Gołębiowski, M., Boguś, M. I., Paszkiewicz, M., Wieloch, W., Włóka, E. and Stepnowski, P. (2012*a*). The composition of the cuticular and internal free fatty acids and alcohols from *Lucilia sericata* males and females. *Lipids* **47**, 613–622.

Gołębiowski, M., Paszkiewicz, M., Grubba, A., Gąsiewska, D., Boguś, M. I., Włóka, E., Wieloch, W. and Stepnowski, P. (2012*b*). Cuticular and internal n-alkane composition of *Lucilia sericata* larvae, pupae, male and female imagines: application of HPLC-LLSD and GC/MS-SIM. *Bulletin of Entomological Research* **102**, 453–460.

Gołębiowski, M., Dawgul, M., Kamysz, W., Boguś, M. I., Wieloch, W., Włóka, E., Paszkiewicz, M., Przybysz, E. and Stepnowski, P. (2012*c*). The antimicrobial activity of the alcohols from *Musca domestica*. *Journal of Experimental Biology* **215**, 3419–3428.

Gołębiowski, M., Cerkowniak, C., Boguś, M. I., Włóka, E., Dawgul, M., Kamysz, W. and Stepnowski, P. (2013). Free fatty acids in the cuticular and internal lipids of *Calliphora vomitoria* and their antimicrobial activity. *Journal of Insect Physiology* **59**, 416–429.

Grassberger, M., Frank, C. (2004). Initial study of arthropod succession on pig carrion in a central European urban habitat. *Journal of Medical Entomology* **41**, 511–523.

Green, P. W. C. (2009). The effects of insect extracts and some insect-derived compounds on the settling behavior of *Liposcelis bostrychophila*. *Journal of Chemical Ecology* **35**, 1096–1107.

Green, P. W. C. (2011). Insect-derived compounds affect the behaviour of *Liposcelis bostrychophila*: effects of combination and structure. *Journal of Stored Product Research* **47**, 262–266.

Ikekawa, N., Morisaki, M. and Fujimoto, Y. (1993). Sterol metabolism in insects: dealkylation of phytosterol to cholesterol. *Accounts of Chemical Research* **26**, 139–146.

Jankevica, L. (2004). Ecological associations between entomopathogenic fungi and pest insects recorded in Latvia. *Latvijas Entomologs* **41**, 60–65.

Jarrold, S., Moore, D., Potter, U. and Charnley, A. K. (2007). The contribution of surface waxes to pre-penetration growth of an entomo-pathogenic fungus on host cuticle. *Mycological Research* **111**, 240–249.

Jiann-Tsyh, L., McKeon, T. A. and Stafford, A. E. (1995). Gradient reversed-phase high-performance liquid chromatography of saturated, unsaturated and oxygenated free fatty acids and their methyl esters. *Journal of Chromatography* **699**, 85–91.

Jones, T. H., Moran, M. D. and Hurd, L. E. (1997). Cuticular extracts of five common mantids (Mantodea: Mantidae) of the Eastern United States. *Comparative Biochemistry and Physiology B* **116**, 419–422.

Kermasha, S., Kubow, S. and Goetghebeur, M. (1994). Comparative high-performance liquid chromatographic analyses of cholesterol and its oxidation products using diode-array ultraviolet and laser light-scattering detection. *Journal of Chromatography* **685**, 229–235.

Kerwin, J. L. (1982). Chemical control of the germination of asexual spores of *Entomophthora culicis*, a fungus parasitic on dipterans. *Journal of General Microbiology* **128**, 2179–2186.

Kerwin, J. L. (1984). Fatty acid regulation of the germination of *Erynia variabilis* conidia on adults and puparia of the lesser housefly, *Fannia canicularis*. *Canadian Journal of Microbiology* **30**, 158–161.

Kühbandner, S., Sperling, S., Mori, K. and Ruther, J. (2012). Deciphering the signature of cuticular lipids with contact sex pheromone function in a parasitic wasp. *Journal of Experimental Biology* **215**, 2471–2478.

Le Conte, Y., Arnold, G., Trouiller, J. and Masson, C. (1990). Identification of a brood pheromone in honeybees. *Naturwissenschaften* **77**, 334–336.

Lockey, K. H. (1980). Insect cuticular hydrocarbons. *Comparative Biochemistry and Physiology* **65**, 457–462.

Lockey, K. H. (1988). Lipids of the insect cuticle: origin, composition and function. *Comparative Biochemistry and Physiology B* **89**, 595–645.

Mpuru, S., Blomquist, G. J., Schal, C., Roux, M., Kuenzli, M., Dusticier, G., Clément, J. L. and Bagnères, A. G. (2001). Effect of age and sex on the production of internal and external hydrocarbons and pheromones in the housefly, *Musca domestica*. *Insect Biochemistry and Molecular Biology* **31**, 139–155.

Nelson, D. R., Guershon, M. and Gerling, D. (1998). The surface wax composition of the exuviae and adults of *Aleyrodes singularis*. *Comparative Biochemistry and Physiology B* **119**, 655–665.

Nelson, D. R., Fatland, Ch.L., Buckner, J. S. and Freeman, T. P. (1999). External lipids of adults of the giant whitefly, *Aleurodicus dugesii*. *Comparative Biochemistry and Physiology* **123**, 137–145.

O'Callagan, M., Garnham, M. L., Nelson, T. L., Baird, D. and Jackson, T. A. (1996). The pathogenicity of *Serratia strains* to *Lucilia sericata* (Diptera: Calliphoridae). *Journal of Invertebrate Pathology* **68**, 22–27.

Ohara, V. S. and Lockey, K. H. (1990). Cuticular lipids of *Locusta migratoria migratoriodes, Schistocerca gregaria* (Acrididae) and other Orthopteran species – I. Polar components. *Comparative Biochemistry and Physiology B* **95**, 603–608.

Oliveira, I., Pereira, J. A., Lino-Neto, T., Bento, A. and Baptista, P. (2012). Fungal diversity associated to the olive moth, *Prays Oleae* Bernard: a survey for potential entomopathogenic fungi. *Microbial Ecology* **63**, 964–974.

Pedrini, N., Crespo, R. and Juárez, M. P. (2007). Biochemistry of insect epicuticle degradation by entomopathogenic fungi. *Comparative Biochemistry and Physiology* **146**, 124–137.

Pilorget, L., Buckner, J. and Lundgren, J. G. (2010). Sterol limitation in a pollen-fed omnivorous lady beetle (Coleoptera: Coccinellidae). *Journal of Insect Physiology* **56**, 81–87.

Roux, O., Gers, Ch. and Legal, L. (2006). When, during ontogeny, waxes in the blowfly (*Calliphoridae*) cuticle can act as phylogenetic markers. *Biochemical Systematics and Ecology* **34**, 406–416.

Saunders, D. D. (2000). Larval diapause duration and fat metabolism in three geographical strains of the blow fly, *Calliphora vicina*. *Journal of Insect Physiology* **46**, 509–517.

Smallbridge, C. J., Cooper, D. J. and Pinnock, D. E. (1995). The effect of the microsporidium *Octosporea muscaedomesticae* on adult *Lucilia cuprina* (Diptera: Calliphoridae). *Journal of Invertebrate Pathology* **66**, 196–197.

https://doi.org/10.1017/S0031182013000267 Published online by Cambridge University Press

Somme, L. (1964). Effects of glycerol on cold-hardiness in insects. *Canadian Journal of Zoology* **42**, 87–101.

Somme, L. (1965). Further observations on glycerol and cold-hardiness in insects. *Canadian Journal of Zoology* **43**, 765–770.

Svoboda, J. A. and Weirich, G. F. (1995). Sterol metabolism in the tobacco hornworm, *Manduca sexta*. A review. *Lipids* **30**, 263–267.

Trabalon, M., Campan, M., Clement, J. L., Lange, C. and Miquel, M. (1992). Cuticular hydrocarbons of *Calliphora vomitoria* (Diptera): relation to age and sex. *General and Comparative Endocrinology* **85**, 208–216.

Urbanek, A., Szadziewski, R., Stepnowski, P., Boros-Majewska, J., Gabriel, I., Dawgul, M., Kamysz, W., Sosnowska, D. and Gołębiowski, M. (2012). Composition and antimicrobial activity of fatty acids detected in the hygroscopic secretion collected from the secretory setae of larvae of the biting midge *Forcipomyia nigra* (Diptera: Ceratopogonidae). *Journal of Insect Physiology* **58**, 1265–1276.

Vásquez, G. M., Schal, C. and Silverman, S. (2008). Cuticular hydrocarbons as queen adoption cues in the invasive Argentine ant. *Journal of Experimental Biology* **211**, 1249–1256.

Wall, R. and Shearer, D. (2001). *Veterinary Ectoparasites: Biology, Pathology and Control*, 2nd Edn. Oxford: Wiley Blackwell.

Ye, G., Li, K., Zhu, J., Zhu, G. and Hu, C. (2007). Cuticular hydrocarbon composition in pupal exuviae for taxonomic differentiation of six necrophagous flies. *Journal of Medical Entomology* **44**, 450–456.

Yoder, Y. A. and Denlinger, D. L. (1990). Water balance in flesh fly pupae and water vapor absorption associated with diapause. *Journal of Experimental Biology* **157**, 273–286.

Yoder, Y. A., Benoit, J. B., Denlinger, D. L. and Rivers, D. B. (2006). Stress-induced accumulation of glycerol in the flesh fly, *Sarcophaga bullata*: evidence indicating anti-desiccant and cryoprotectant functions of this polyol and a role for the brain in coordinating the response. *Journal of Insect Physiology* **52**, 202–214.

https://doi.org/10.1017/S0031182013000267 Published online by Cambridge University Press