Lipids (2012) 47:1001–1009
DOI 10.1007/s11745-012-3702-1

ORIGINAL ARTICLE

Comparison of Free Fatty Acids Composition of Cuticular Lipids of *Calliphora vicina* Larvae and Pupae

Marek Gołębiowski

Received: 11 March 2012 / Accepted: 11 July 2012 / Published online: 7 August 2012

Abstract The chemical characterization of the free fatty acid (FFA) fractions of the cuticular lipids of *Calliphora vicina* larvae and pupae was performed by separating the FFA fraction using high-performance liquid chromatography with laser light scattering detection (HPLC–LLSD) and quantitatively analyzing the FFA using gas chromatography–electron impact mass spectrometry (GC–MS). Thirty-two saturated and unsaturated FFA were identified and quantified in the insect lipids. Cuticular FFA profiles of *C. vicina* larvae and pupae were compared. Cuticular FFA of larvae and pupae accounted for 70.8 and 77.8 % of the total lipids, respectively. The cuticular lipids of *C. vicina* larvae contained 24 FFA ranging from 8:0 to 24:0, whereas the cuticular lipids of pupae contained 32 FFA ranging from 6:0 to 26:0. The cuticular lipids of the larvae contained 16 saturated, five monounsaturated, one diunsaturated, and two polyunsaturated FFA. The cuticular lipids of the pupae contained 18 saturated, nine monounsaturated, two diunsaturated, and three polyunsaturated FFA. The major cuticular FFA in *C. vicina* larvae and pupae was 18:1 (47.6 and 41.7 %, respectively). The highest amounts of total cuticular FFA were detected in larvae of *C. vicina* (1.7 mg/g of the insect body). The quantities of total cuticular FFA in pupae were smaller (1.4 mg/g of the insect body).

Keywords *Calliphora vicina* · Free fatty acids · HPLC–LLSD · GC–MS

M. Gołębiowski (✉)
Faculty of Chemistry, Institute for Environmental and Human Health Protection, University of Gdańsk, ul. Sobieskiego 18/19, 80-952 Gdańsk, Poland
e-mail: goleb@chem.univ.gda.pl

Abbreviations

TIC	Total ion current
SIM	Single ion monitoring
EI	Electron impact
BSTFA	*N,O*-Bis(trimethylsilyl) trifluoroacetamide
TMCS	Trimethylchlorosilane
$M^{+\cdot}$	Molecular ion
TMSi	Trimethylsilyl derivatives
FFA	Free fatty acids

Introduction

The cuticular lipids constitute the initial passive barrier to desiccation [1] as well as to fungal and bacterial infection [2], and they may reduce the penetration of toxins and chemicals [3]. Cuticular lipids are also involved in various types of chemical communication [4]. For example, insects use components of the surface lipids as contact pheromones when they encounter each other. The cuticular lipids in pheromones are hydrocarbons, short-chain unsaturated aldehydes, ketones, fatty acids, and acetate esters of short-chain unsaturated alcohols.

The insects belonging to the Calliphoridae, Sarcophagidae, and Muscidae families often colonize a human corpse [5], and are often very important elements in forensic investigation. For example, *Calliphora vicina* is considered as a synanthropic species and larvae of this species are used in criminal investigations to determine the post-mortem interval (PMI), i.e., the time from death to discovery of the corpse.

In view of the great importance of *C. vicina*, it was decided to identify and assign the components of the free fatty acid (FFA) mixtures found on the surface layers of the

cuticle in larvae and pupae. Susceptibility or resistance of various insect species to fungal infection may be caused by several factors. The most important of them are differences in the structure and composition of the exoskeleton. The exudation of ammonia and allantoin is very significant in cleansing the insects' microhabitat. The bacteriostatic effects of the heat generated by accumulations of actively feeding larvae, the presence of antifungal compounds in the cuticle, especially FFA [6], as well as the cellular and humoral defense reactions of insect are also important [7, 8]. Susceptibility to penetration by fungal pathogen depends on mechanical barriers which either prevent or reduce infection and chemical barriers which inhibit or kill the pathogen. For example, FFA possess the ability to kill or inhibit the growth of fungi and bacteria [6, 9–13]. The antibacterial and antifungal effects of FFA are frequently observed during bioassay of extracts from a variety of organisms [13–15]. The activity of each FFA is influenced by its structure, especially the length of the carbon chain and the presence, number, orientation, and position of double bonds, and the presence the hydroxyl group is important for the antibacterial activity of FFA [16]. Unsaturated FFA are more active than saturated FFA with the same carbon chain length [16, 17] and unsaturated FFA are more active against Gram-positive bacteria than Gram-negative [18]. Additionally, FFA with the double bonds in the *cis* orientation have greater antibacterial activity than FFA with double bonds in the *trans* orientation [10, 12].

The most popular techniques used for the identification and quantification of cuticular lipids are TLC [19], column chromatography [20], HPLC/LLSD [21], GC–FID [22], GC–MS-TIC [23, 24], GC–MS-SIM [25], and MALDI [26]. For analysis, relatively volatile compounds solid-phase microextraction (SPME) [27, 28] or solid injection (SI) [29] coupled to the GC or GC–MS are often used [30].

This paper describes the qualitative and quantitative comparisons of cuticular and internal FFA profiles of larvae and pupae. These results provide baseline data for further studies on the possible role of FFA in the resistance to microbial attack. The lipids of flies were separated into classes of compounds using high-performance liquid chromatography with laser light scattering detector (HPLC–LLSD). Qualitative and quantitative analyses were done by gas chromatography combined with mass spectrometry (GC–MS).

Methods and Material

Insects

A total of 140 larvae and 302 pupae of *C. vicina* were obtained from the Institute of Parasitology, Polish Academy of Sciences in Warsaw (Poland). The insects, raised from eggs laid on beef by adult flies, were reared at 28 °C with 70 % relative humidity and a 12:12 h photoperiod. Maternal generation was maintained in the same conditions. For experiments, 1-day-old larvae and freshly emerged pupae were used. All insects were quickly frozen and kept at −20 °C until being used.

Extraction of Lipids

The cuticular and internal lipids were extracted by methods described previously [24]. All larvae and pupae were first extracted separately by stirring in 20 ml of petroleum ether for 10 s (extracts 1L and 1P). All primarily extracted larvae and pupae were separately extracted for a second time in 20 ml of dichloromethane for 1 min (extracts 2L and 2P). Finally, the insects were extracted with 50 ml dichloromethane for 10 days (extracts 3L and 3P). The obtained extract was filtered (0.45 μm) and collected into a glass vial. A 1-ml aliquot of this extract was further taken and placed into a tared glass flask, and then evaporated under nitrogen in order to determine the dry mass of extracted lipids. For example, extracts 1L and 2L contained cuticular lipids, and extract 3L contained internal lipids of larvae.

High-Performance Liquid Chromatography with Laser Light Scattering Detector

The lipids were separated by methods described previously [31]. All lipid extracts (larvae and pupae) were separated using HPLC–LLSD. Separation in the normal phase (five replicates) was performed on a silica gel column (Econosil Silica 5 Micron, Alltech, 25 cm × 4.6 mm i.d.). Binary gradient elution with eluent A (hexane) and eluent B (15 % of acetone in dichloromethane) was applied with a linear gradient from A to B within 35 min. Total flow was maintained at 0.8 ml/min. The FFA fractions obtained by HPLC were silylated with 100 μl of a mixture of 99 % bis(trimethylsilyl)acetamide and 1 % chlorotrimethylsilane (Sigma Aldrich) for 1 h at 100 °C and then analyzed by GC–MS.

Gas Chromatography Mass Spectrometry

GC–MS analysis of each FFA fraction was performed with a Finnigan Mat SSQ 710 mass spectrometer coupled to a Hewlett–Packard 5890 gas chromatograph. Compounds were separated on a 30 m × 0.25 mm i.d., film thickness 0.25 μm, HP-5 capillary column. Helium was used as the carrier gas at a flow rate of 1 ml/min. The injector temperature was 320 °C and the column oven temperature cycle was 60 °C for 10 min, then 60–320 °C at 4 °C/min; the final temperature was then held for 20 min. Electron-impact

ionization (electron energy 70 eV, ion source temperature 200 °C) was used.

In order to quantitatively determine each of the analyzed FFA, GC–MS analysis was performed with an internal standard (19-methylarachidonic acid). The contents of the compounds in the analyzed samples were calculated from the chromatogram peak areas. The results were expressed as mean ± standard deviation of three GC–MS analyses.

Results

Total Lipids and Free Fatty Acids

The total quantity of cuticular and internal lipids larvae was 20.4 and 461.8 mg, which comprised 0.2 and 5.4 % of the total fresh weight of the biological material, respectively (Table 1). The percentage content of cuticular and internal lipids in pupae was similar. The total quantity of cuticular and internal lipids of pupae was 30.0 and 514.9 mg, which comprised less than 0.2 and 3.1 % of the total fresh weight of the biological material, respectively (Table 1). Cuticular FFA of larvae and pupae accounted for 70.8 and 77.8 % of the total lipids, respectively, whereas internal FFA of larvae and pupae made up 5.2 and 21.3 % of the total lipids, respectively.

Free fatty acid fractions obtained as a result of the HPLC–LLSD separations were then subjected to further GC–MS analysis. GC–MS in total ion current (TIC) mode was used to identify unknown components, and single ion monitoring mode (SIM) to verify recognized molecular ions. FFA in the cuticular and internal lipids of *C. vicina* larvae and pupae were identified on the basis of the characteristic ions: 73, 128, 132, 145, $M-15^{+}$, and $M^{+\cdot}$ (molecular ion).

The amount of cuticular FFA isolated from larvae was 1.7 mg/g. The results obtained from pupae were similar: 1.4 mg/g of the insect body. The total mass of internal FFA was greater in pupae (6.6 mg/g). This last quantity is about twice the average in larvae (2.8 mg/g).

Table 1 Quantitative summary of the experiment: numbers and masses of insect (*Calliphora vicina*); masses of lipids

Stages	Number of insects	Masses of insects (g)	Masses of lipids in extracts (mg)	Extracts	Masses of lipids (mg/g of the insect body)
Larvae	140	8.5	9.2 ± 0.1	1L	1.1
			11.2 ± 0.1	2L	1.3
			461.8 ± 5.5	3L	54.3
Pupae	302	16.6	11.6 ± 0.1	1P	0.7
			18.4 ± 0.2	2P	1.1
			514.9 ± 6.2	3P	31.0

1L and 1P, petroleum extracts (10 s), 2L and 2P dichloromethane extracts (5 min), 3L and 3P, dichloromethane extracts (10 days)

Cuticular Free Fatty Acids in Larvae

Cuticular lipids of larvae contained 24 FFA ranging from 8:0 to 24:0 (Table 2). Among cuticular FFA of larvae, 15 saturated, seven unsaturated with even-numbered, and two unsaturated with odd-numbered carbon chains FFA were present. The major FFA in *C. vicina* larvae were 18:1n-9 (47.6 %), 16:1n-9 (20.0 %), 16:0 (19.4 %), and 18:0 (9.4 %). FFA occurring in smaller quantities (from greater than 0.1 to 1 %) were 12:0 (0.4 %), 14:1n-9 (0.6 %), 14:0 (0.3 %), 17:1n-10 (0.8 %), 17:0 (0.6 %), and 18:2n-6 (0.9 %). The cuticular lipids also contained 14 FFA present in concentrations from traces to less than 0.1 %.

Cuticular Free Fatty Acids in Pupae

Table 2 lists the FFA contents calculated per gram of pupae body. Among 32 cuticular FFA of pupae, 18:1n-9 (41.7 %), 16:0 (19.3 %), 16:1n-9 (16.4 %), 18:0 (8.6 %), and 18:2n-6 (6.9 %) were the most abundant compounds. One fatty acid is present in a smaller amount (14:0, 2.4 %). Other FFA were detected in very small and comparable amounts from 0.7 % (26:1) to less than 0.1 % (e.g., 6:0). Cuticular lipids of pupae contained 18 saturated FFA, among which seven fatty acids are odd-numbered, and also 14 unsaturated FFA, among which three fatty acids are odd-numbered.

Comparison of Free Fatty Acids Composition of Cuticular Lipids of *C. vicina* Larvae and Pupae

The presence of six FFA with 20 carbon atoms in the chain, namely, 20:0, 20:1n-6, 20:2n-6, 20:3n-6, 20:4n-3, and 20:5n-3, in the cuticular lipids of pupae is very interesting. On the other hand, only four FFA with 20 carbon atoms in the chain were present in the cuticular lipids of larvae.

The following free acids present in the cuticular lipids of pupae were absent from the cuticular lipids of larvae: 6:0, 7:0, 19:1n-10, 20:3n-6, 20:2n-6, 22:1n-9, 26:1n-9, and 26:0. The percentage contents of cuticular 14:0 and 18:2n-6 in pupae were significantly higher than those of cuticular lipids in larvae and compromised 2.4 vis 0.3 % and 6.9 vis 0.9 %, respectively.

In cuticular lipids of larvae, the relative content of 16:1n-9 was higher than the relative content of 16:0. On other hand, in cuticular lipids of pupae, the relative content of 16:1n-6 was lower than the relative content of 16:0.

Internal Free Fatty Acids in Larvae

Internal lipids of larvae *C. vicina* contained 26 FFA ranging from 6:0 to 22:0 (Table 2). In the internal lipids 15

Table 2 Chemical composition of the fatty acids found in larvae and pupae of *Calliphora vicina*

FFA	Larvae		Pupae	
	Cuticular FFA	Internal FFA	Cuticular FFA	Internal FFA
Masses of free fatty acids (μg/g of fresh weight of insect)				
6:0	–	0.55 ± 0.06	0.13 ± 0.02	–
7:0	–	0.47 ± 0.07	0.14 ± 0.01	5.1 ± 0.5
8:0	0.28 ± 0.03	0.95 ± 0.08	0.48 ± 0.04	$1.3 \times 10^1 \pm 0.1 \times 10^1$
9:0	2.1 ± 0.2	0.91 ± 0.07	0.52 ± 0.04	4.7 ± 0.4
10:0	0.89 ± 0.06	0.51 ± 0.06	0.33 ± 0.03	0.78 ± 0.04
11:0	1.5 ± 0.1	1.01 ± 0.09	0.77 ± 0.09	Traces
12:0	7.4 ± 0.6	8.4 ± 0.7	2.4 ± 0.2	$1.6 \times 10^1 \pm 0.1 \times 10^1$
13:0	0.69 ± 0.06	1.36 ± 0.09	0.53 ± 0.04	$1.8 \times 10^1 \pm 0.2 \times 10^1$
14:1n-9	10.4 ± 0.8	$3.5 \times 10^1 \pm 0.3 \times 10^1$	7.8 ± 0.5	$6.3 \times 10^1 \pm 0.5 \times 10^1$
14:0	4.5 ± 0.4	$1.9 \times 10^2 \pm 0.2 \times 10^2$	$3.3 \times 10^1 \pm 0.2 \times 10^1$	$3.4 \times 10^2 \pm 0.3 \times 10^2$
15:1n-10	0.98 ± 0.07	–	0.92 ± 0.06	–
15:0	8.7 ± 0.4	$1.9 \times 10^1 \pm 0.1 \times 10^1$	5.8 ± 0.5	$1.8 \times 10^2 \pm 0.1 \times 10^2$
16:1n-9	$3.4 \times 10^2 \pm 0.2 \times 10^2$	$6.5 \times 10^2 \pm 0.5 \times 10^2$	$2.3 \times 10^2 \pm 0.2 \times 10^2$	$1.2 \times 10^3 \pm 0.1 \times 10^3$
16:0	$3.3 \times 10^2 \pm 0.2 \times 10^2$	$3.8 \times 10^2 \pm 0.2 \times 10^2$	$2.7 \times 10^2 \pm 0.2 \times 10^2$	$1.6 \times 10^3 \pm 0.1 \times 10^3$
17:1n-10	$1.4 \times 10^1 \pm 0.1 \times 10^1$	$6.5 \times 10^1 \pm 0.5 \times 10^1$	2.2 ± 0.2	$9.1 \times 10^1 \pm 0.8 \times 10^1$
17:0	10.3 ± 0.6	10.7 ± 0.6	8.8 ± 0.6	$1.4 \times 10^2 \pm 0.1 \times 10^2$
18:2n-6	$1.5 \times 10^1 \pm 0.1 \times 10^1$	–	$9.6 \times 10^1 \pm 0.6 \times 10^1$	–
18:1n-9	$8.1 \times 10^2 \pm 0.6 \times 10^2$	$1.5 \times 10^3 \pm 0.1 \times 10^3$	$5.7 \times 10^2 \pm 0.4 \times 10^2$	$2.7 \times 10^3 \pm 0.2 \times 10^3$
18:0	$1.6 \times 10^2 \pm 0.1 \times 10^2$	$4.4 \times 10^1 \pm 0.4 \times 10^1$	$1.2 \times 10^2 \pm 0.1 \times 10^2$	$3.2 \times 10^2 \pm 0.2 \times 10^2$
19:1n-10	–	4.2 ± 0.3	2.3 ± 0.2	–
19:0	0.36 ± 0.03	Traces	0.59 ± 0.05	–
20:5n-3	1.4 ± 0.1	$1.6 \times 10^1 \pm 0.1 \times 10^1$	3.9 ± 0.4	$1.3 \times 10^1 \pm 0.1 \times 10^1$
20:4n-3	0.96 ± 0.08	$1.6 \times 10^1 \pm 0.1 \times 10^1$	4.3 ± 0.4	$1.0 \times 10^1 \pm 0.1 \times 10^1$
20:3n-6	–	1.7 ± 0.2	0.36 ± 0.02	–
20:2n-6	–	0.34 ± 0.02	0.24 ± 0.02	–
20:1n-6	0.94 ± 0.06	1.16 ± 0.09	3.0 ± 0.2	–
20:0	1.5 ± 0.1	2.1 ± 0.1	6.0 ± 0.4	–
22:1n-9	–	–	1.4 ± 0.1	–
22:0	0.21 ± 0.03	0.48 ± 0.05	3.4 ± 0.2	2.3 ± 0.2
24:0	Traces	–	1.19 ± 0.08	2.2 ± 0.2
26:1n-9	–	–	$1.0 \times 10^1 \pm 0.1 \times 10^1$	–
26:0	–	–	1.5 ± 0.2	0.33 ± 0.03
Sum	1.7×10^3	2.8×10^3	1.4×10^3	6.6×10^3

Cuticular FFA, sum of FFA content in petroleum extract (1L and 1P) and dichloromethane extract (2L and 2P). Internal FFA, dichloromethane extract (3L and 3P). Data are presented as the mean ± standard deviation of three separate analyses performed on different samples

saturated, six monounsaturated, one diunsaturated, and four polyunsaturated FFA have been identified. Only three acids are present in high concentrations (more than 10 %): 16:0 (13.6 %), 16:1n-9 (23.2 %), and 18:1n-9 (53.6 %). Other acids amounted to from less than 0.1 to 6.8 %. Among saturated acids, the following are the most abundant: 16:0 (13.6 %), 14:0 (6.8 %), and 18:0 (1.6 %). Among unsaturated acids, the following are the most abundant: 18:1n-9 (53.6 %), 16:1n-9 (23.2 %), and 17:1n-10 (2.3 %). Internal lipids of larvae contained six FFA with 20 carbon atoms in the chain: 20:0, 20:1n-6, 20:2n-6, 20:3n-6, 20:4n-3, and 20:5n-3.

Internal Free Fatty Acids in Pupae

Internal lipids of pupae *C. vicina* contained 20 FFA ranging from 7:0 to 26:0 (Table 2). FFA occurring in the highest quantities were 16:1n-9 (18.2 %), 16:0 (24.2 %), and 18:1n-9 (40.9 %). Except for 16:1n-9 and 18:1n-9, which occurred in large amounts, the following unsaturated FFA

are present in smaller quantities: 14:1n-9 (1.0 %), 17:1n-10 (1.4 %), 20:5n-3 (0.2 %), and 20:4n-3 (0.2 %). Among saturated free acids, only C16:0 is present in large amount (24.2 %). Four saturated FFA are present from 2.1 % (17:0) to 5.2 % (14:0). Other saturated FFA occur in smaller quantities.

Comparison of Free Fatty Acids Composition of Internal Lipids of *C. vicina* Larvae and Pupae

The following acids present in the larvae lipids were absent in the pupae lipids: 6:0, 11:0, 19:1n-10, 19:0, 20:3n-6, 20:2n-6, 20:1n-6, and 20:0. On the other hand, only 13:0, 24:0, and 26:0 were absent from the internal lipids of larvae *C. vicina*.

In the internal lipids of larvae, the relative content of 16:1n-9 was higher than the relative content of 16:0. However, in the internal lipids of pupae, the relative content of 16:1n-6 was lower than that of 16:0. The same situation is observed in cuticular lipids of larvae and pupae.

Internal lipids of larvae contained six FFA with 20 carbon atoms in the chain but internal lipids of pupae contained only two of those compounds (20:4n-3 and 20:5n-3).

The percentage contents of internal 15:0 and 18:0 in pupae were significantly higher than those of internal lipids in larvae and compromised 5.2 vis 0.7 % and 4.8 vis 1.6 %, respectively.

Discussion

The major components of the cuticular lipid coating of insects are often hydrocarbons, such as *n*-alkanes, monomethylalkanes, dimethylalkanes, and alkenes [32], FFA [31], alcohols [33], aldehydes [34], wax esters [35], fatty acid methyl esters [32], and acylglycerols [36].

In our work, we stated that cuticular lipids of *C. vicina* larvae contained FFA from 8:0 to 24:0 and pupae from 6:0 to 26:0. Table 3 lists the FFA identified in insect cuticular lipids. For example, in the cuticular lipids the following FFA were identified: from 10:0 to 22:1 in *Locusta migratoria migratoriodes* adult [37], from 10:0 to 20:1 in *Schistocerca gregaria* adult [37], from 14:0 to 18:1 in *Frankliniella occidentalis* larvae [32], from 16:0 to 18:3 in *Acanthoscelides obtectus* female [21], and from 16:0 to 36:0 in *Apis mellifera* [38].

In our study, the major cuticular FFA were: 16:1n-9, 16:0, 18:2n-6, 18:1n-9 and 18:0 in pupae and 16:1n-9, 16:0, 18:1n-9 and 18:0 in larvae.

In the other insects species, the major FFA were 14:0 (40 %) in *Acyrthosiphon pisum* [42], 16:0 (25 %) in *L. migratoria migratoriodes* adult, (64 %) in *S. gregaria* adult, (63 %) in *F. occidentalis* larvae, (36 %) in *Fannia canicularis* adult [40], (38 and 30 %) in *Attagenus megatoma* 6.8- and 32-week-old larvae, respectively [43], 18:0 (58 %) in *A. megatoma* adult [41], (33 %) in *Lasioderma serricorne* larvae [44], (40 %) in *Dendrolimus pini* larvae [6], (49–52 %) in *Galleria mellonella* larvae [6] and (51 %) in *A. obtectus* female [21], 18:3 (28 %) in *Melanoplus sanguinipes* and *M. packardii* adult [36], 24:0 (29 %) in *A. mellifera* [38], and 28:0 (34 %) in *D. pini* exuviae [31]. On the basis of these results, we stated that two major FFA (16:0 and 18:1) were present in many insect species. Other major FFA are atypical (14:0, 24:0, and 24:0). In our study, the major compound in cuticular lipids of larvae and pupae, i.e., 18:1n-9, is typical.

Saturated, unsaturated, and even-numbered FFA ranging from 12 to 20 are typically found in many insect species [30, 33, 45], whereas the presence of FFA ranging from 5 to 11 and from 21 to 26 are unusual. In particular odd-numbered FFA and polyunsaturated FFA such as 15:1n-10, 17:1n-10, 20:5n-3, and 20:4n-3 identified in lipids of *C. vicina* are unusual. Only typical cuticular FFA ranging from 12:0 to 20:0 are present in cuticular lipids of larvae *F. occidentalis* [32], adult *F. cannicularis* [40], *L. bostrychophila* [34], and female *A. obtectus* [21].

In our study, the FFA (in the range from 5:0 to 11:0) 8:0, 9:0, 10:0, and 11:0 in larvae and additionally 6:0 and 7:0 in pupae were identified in the cuticular lipids. Similar cuticular lipids ranging from 5:0 to 11:0 were identified in the lipids of *D. pini* exuviae [31], *L. migratoria migratoriodes* adult, *S. gregaria* adult [39], and *A. pisum* [42]. The numbers of fatty acids in this range are not large: one compound in the cuticular lipids of *L. migratoria migratoriodes*, *S. gregaria*, and *A. pisum* and four FFA in *D. pini* exuviae.

Cuticular lipids of *C. vicina* larvae and pupae contained several odd-numbered FFA. Among this group the following acids are present: 9:0, 11:0, 13:0, 15:0, 17:0, and 19:0 in larvae and pupae and additionally 7:0 in pupae. All of these FFA are minor constituents. Those compounds were identified in the cuticular lipids of some insects. For example, 9:0, 11:0, 21:0, 15:0, 17:0, 19:0, and from 23:0 to 33:0 were present in *D. pini* exuviae [31]. The 15:0, 17:0, and 19:0 entities were identified in *S. gregaria* adult [39] and 15:0 and 17:0 were present in the cuticular lipids of *L. migratoria migratoriodes* adult [39].

Among odd-numbered and unsaturated acids only 15:1n-10 and 17:1n-10 in larvae and 15:1n-10, 17:1n-10, and 19:1n-10 in pupae of *C. vicina* are present in small amounts. These FFA (17:1 and 19:1) were also identified in cuticular lipids of *S. gregaria* adult [39]. Moreover, the presence of polyunsaturated and odd-numbered fatty acid (21:3) in cuticular lipids of *L. migratoria migratoriodes* was detected. However, its biological function needs additional research.

Table 3 The composition of the fatty acids found in lipids of insects

Insect species/references																			
FFA	Lm [39]	Sg [39]	Fo [32]	Fc [40]	Am [41]	Ap [42]	Am [38]	Ms [36]	Mp [36]	Lb [34]	Am [43]	Am [43]	Am [43]	Ls [44]	Cv [6]	Dp [6]	Gm [6]	Ao [21]	Dp [31]
$C_{5:0}$															Tr				
$C_{6:0}$															Tr				
$C_{8:0}$															Tr				Tr
$C_{9:0}$															Tr				0.1
$C_{10:0}$	1	4				2									Tr				0.1
$C_{11:0}$																			0.1
$C_{12:0}$	7	5			Tr	4		2	2						Tr				0.2
$C_{12:1}$		<1																	
$C_{13:0}$																			0.1
$C_{14:0}$	14	12	5	1	5	70		2	1				2	3	1.7		Tr		0.5
$C_{14:1}$	<1	<1																	
$C_{15:0}$	14	<1																	0.5
$C_{16:0}$	25	64	63	36	26	4	7	13	10	+	38	30	17	23	25.3	17.7	32.7–38.9	12.6	2.2
$C_{16:1}$	1	<1	4	16	3	2		3	4	+	4	5	19	4	22.6		Tr		
$C_{16:2}$													<1						
$C_{17:0}$	<1	<1																	0.4
$C_{17:1}$		1																	
$C_{18:0}$	14	12	12	18	10	4	7	6	5	+	16	11	3	9	1.6	8.8	14.5–6.6	7.3	1.7
$C_{18:1}$	<1	<1	17	25	22	5		21	23	+	17	26	58	33	23.2	39.8	49.2–51.9	50.9	
$C_{18:2}$	3	Tr		4	20	7		24	26	+		2		18	24.0	19.2	3.6–2.7	18.4	
$C_{18:3}$	<1	<3		Tr	2	2		28	28		2	3		1		14.6		10.7	
$C_{19:0}$		Tr																	0.4
$C_{19:1}$		Tr																	
$C_{20:0}$	5	Tr			1			Tr				<1	<1	3	1.7		Tr		4.7
$C_{20:1}$	<1	Tr															Tr		
$C_{21:0}$																			0.4
$C_{21:3}$	4																		
$C_{22:0}$	3				2		4	Tr			3	2		2		2			9.5
$C_{22:1}$	6																		
$C_{23:0}$																			0.1
$C_{24:0}$					9		29				15	14		<1					2.2
$C_{25:0}$																			0.3
$C_{26:0}$							12				3	5							6.7
$C_{27:0}$																			0.6

Table 3 continued

Insect species/references																		
FFA	Lm [39]	Sg [39]	Fo [32]	Fc [40]	Am [41]	Ap [42]	Am [38]	Ms [36]	Mp [36]	Lb [34]	Am [43]	Am [43]	Am [43]	Ls [44]	Cv [6]	Dp [6]	Gm [6]	Ao [21]	Dp [31]
$C_{28:0}$							11												34.1
$C_{29:0}$																			1.5
$C_{30:0}$							9												27.5
$C_{31:0}$																			0.4
$C_{32:0}$							9												5.2
$C_{33:0}$																			0.1
$C_{34:0}$							9												0.7
$C_{36:0}$							3												

Concentration given in % lipid total

+, compound present in the lipids; FFA, free fatty acids; Tr, traces; Lm, the migratory locust, *Locusta migratoria migratoriodes* adult; Sc, the desert locust, *Schistocerca gregaria* adult; Fo, the western flower thrips, *Frankliniella occidentalis* larvae; Fc, the lesser housefly, *Fannia canicularis* adult; Ame, the black carpet beetle, *Attagenus megatoma* larvae; Ac, the pea aphid, *Acyrthosiphon pisum*; Am, the honey bee, *Apis mellifera*; Ms, the migratory grasshopper, *Melanoplus sanguinipes* adult; Mp, the packard grasshopper, *Melanoplus packardii* adult; Lb, the booklouse, *Liposcelis bostrychophila*; Am, *Attagenus megatoma* 6.8- and 32-week-old larvae adult; Ls, the cigarette beetle, *Lasioderma serricorne* larvae; Cv, the blowfly, *Calliphora vicina* larvae; Gm, the wax moth, *Galleria mellonella* larvae; Dp, the pine-tree lappet moth, *Dendrolimus pini* larvae; Ao, the bean weevil, *Acanthoscelides obtectus* female; Dp, *Dendrolimus pini* exuviae

Free fatty acids with molecular weight from 20:0 to 26:0 (only even-numbered) are present in cuticular lipids of *C. vicina* larvae and pupae, but all of these compounds are minor constituents: in larvae from traces to 0.1 % and in pupae from less than 0.1 to 0.7 %. Cuticular FFA in range from 20:0 to 26:0 were detected in the cuticular lipids of *M. sanguinipes* adult [36], *G. mellonella* larvae [6], and *S. gregaria* adult [39]. Eight FFA even-numbered and ranging from 22:0 to 36:0 were present in cuticular lipids of *A. mellifera* [38] and three FFA ranging from 22:0 to 26:0 were identified in cuticular lipids of *A. megatoma* 6.8- and 32-week-old larvae [43] and also two compounds are present in *A. megatoma* larvae [41] and *L. sarricorne* larvae [44].

In our previous work [6] it was shown that the cuticular fatty acid profiles of *C. vicina* larvae (last-instar wandering larvae) differ from the profile of *C. vicina* larvae analyzed here. The major difference was the content of 18:1n-9 and 18:2n-6 in the cuticle. In our previous work, the relative content of 18:1 and 18:2 was 23.2 and 24.0 %, respectively, whereas in the present work, the relative content of 18:1n-9 and 18:2n-6 was 47.6 and 0.9 %, respectively. The concentrations of FFA of last-instar wandering larvae were circa eight times higher than those of the final instar larvae. Changes in the composition of cuticular lipids during development have been studied in only a few insects. For example, total cuticular lipid of *A. megatoma* increased from 4.1 to 13.7 μg/larvae for the 6.8- and 32-week-old larvae, respectively [43]. The amount of 16:0 and 18:0 decreased while 18:1 increased in the older *A. megatoma* larvae. The presence of 18:2 and 20:0 in 32-week-old larvae of *A. megatoma* is interesting, whereas these compounds were absent in the 6.8-week-old larvae. Additionally, the FFA profile in the adult insects is different: 18:3, 22:0, 24:0, and 26:0 were not found in the adult insects whereas they were present in both groups of larvae. A similar change in composition was also found during development of the *G. mellonella* larvae. The fatty acid profile in the VIIth larval instar of *G. mellonella* from 0–2 h to 8 days after molting is different [6]. In this case, the concentrations of 16:0, 18:0, 18:1, and 18:2 in the extracts of this larvae were found to increase significantly during this period. Evidently, the lipid composition is determined by sex, developmental stage, and age [43]. Moreover, the lipid composition depends on the living conditions, i.e., temperature, dryness, and available food [46, 47]. In this study, the number of FFA identified by GC–MS analysis of cuticular extracts of *C. vicina* larvae is higher than that reported in our previous work on this fly species. Besides changes in the composition of cuticular lipids during development, this observation may be consistent with the fact that in the present study we used more a sensitive mass spectrometer with lower limits of

detection and quantification. Both results (present and earlier) showed the similar qualitative fatty composition, although data were obtained using different extraction methods and GC–MS equipments.

Free fatty acids present in the cuticular surface of insect larvae and pupae could be inhibitory to germination and growth of conidia and penetration of the integument by entomopathogenic fungi. In our previous work [37] fungistatic effects of 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, and 20:1 were shown. The cuticular lipids of larvae and pupae *C. vicina* contained FFA in this range, with the exception of 18:3. Also medium- and short-chain fatty acids which are present in cuticular lipids of larvae and pupae of *C. vicina* were demonstrated to present toxicity to filamentous fungi, including some that had been isolated specifically from insect cuticle [48, 49]. FFA might be engaged in protecting larvae from fungal pathogens, whereas the presence of, for example, 15:0 stimulated *C. coronatus* growth [37]. The determination of the cuticular fatty acid profiles and information on which particular fatty acids support the fungal assault and which contribute to insect resistance to entomopathogenic fungi is of great importance and requires a better understanding of the physiological aspects of fungal growth and virulence factors.

In conclusion, the conducted analyses exposed qualitative and quantitative differences in FFA chemical composition of the examined developmental stages. The major differences are cuticular lipids of pupae contained 6:0, 7:0, 19:1n-10, 20:3n-6, 20:2n-6, 22:1n-9, 26:1n-9, and 26:0, which were not found in larvae; In the cuticular lipids of larvae, the relative content of 16:1n-9 was higher than the relative content of 16:0. On other hand, in cuticular lipids of pupae, the relative content of 16:1n-9 was lower than that of 16:0.

Differences in FFA chemical composition of larvae and pupae may be responsible for susceptibility or resistance to fungal infection. Experiments which are currently ongoing in our laboratories will show which of the identified compounds possess antimicrobial activity. Hopefully the results described here revealing the chemical composition of fatty acids of *C. vicina* will be an important contribution to further studies of the taxonomy and physiology of insects.

Acknowledgments I would like to thank Prof. Mieczysława I. Boguś for providing the insects to analyze. I would like to express my gratitude to Anna Grubba for her assistance. Financial support was provided by the Polish Ministry of Research and Higher Education for 2010–2013 (grant N N303 504238) and the University of Gdansk (grant DS 8110-4-0085-1).

References

1. Hadley NF (1994) Water relations of terrestrial arthropods. Academic, San Diego
2. St. Leger R (1991) Integument as a barrier to microbial infections. In: Binnington K, Retnakaran A (eds) Physiology of the insect epidermis. CSIRO, Australia, pp 284–306
3. Gilby AR (1984) Cuticle and insecticides. In: Bereiter-Hahn J, Matoltsy AG, Richards KS (eds) Biology of the integument. Invertebrates, vol 1. Springer-Verlag, Berlin, pp 694–702
4. Howard RW (1993) Cuticular hydrocarbons and chemical communication. In: Stanley-Samuelson DW, Nelson DR (eds) Insect lipids: chemistry, biochemistry and biology. University of Nebraska Press, Lincoln, pp 179–226
5. Bonacci T, Vercillo V, Brandmayr P, Fonti A, Tersaruolo C, Brandmayr TZ (2009) A case of *Calliphora vicina* Robineau-Desvoidy, 1830 (Diptera, Calliphoridae) breeding in a human corpse in Calabria (southern Italy). Legal Med 11:30–32
6. Gołębiowski M, Maliński E, Boguś MI, Kumirska J, Stepnowski P (2008) The cuticular fatty acids of *Calliphora vicina*, *Dendrolimus pini* and *Galleria mellonella* larvae and their role in resistance to fungal infection. Insect Biochem Mol Biol 38:619–627
7. Vilcinskas A, Götz P (1999) Parasitic fungi and their interactions with the insect immune system. Adv Parasitol 43:267–313
8. Park JW, Lee BL (2012) Insect immunology. In: Gilbert LI (ed) Insect molecular biology and biochemistry. Elsevier, Amsterdam, pp 480–512
9. Bergsson G, Arnfinnsson J, Steingrímsson Ó, Thormar H (2001) Killing of Gram-positive cocci by fatty acids and monoglycerides. APMIS 109:670–678
10. Kabara JJ, Swieczkowski DM, Conley AJ, Truant JP (1972) Fatty acids and derivatives as antimicrobial agents. Antimicrob Agents Chemother 2:23–28
11. Kabara JJ, Vrable R, Lie Ken Jie MSF (1977) Antimicrobial lipids: natural and synthetic fatty acids and monoglycerides. Lipids 12:753–759
12. Feldlaufer MF, Knox DA, Lusby WR, Shimanuki H (1993) Antimicrobial activity of fatty acids against Bacillus larvae, the causative agent of American foulbrood disease. Apidologie 24:95–99
13. Benkendorff K, Davis AR, Rogers CN, Bremner JB (2005) Free fatty acids and sterols in the benthic spawn of aquatic molluscs, and their associated antimicrobial properties. J Exp Mar Biol Ecol 316:29–44
14. Wille JJ, Kydonieus A (2003) Palmitoleic acid isomer (C16:1Δ6) in human skin sebum is effective against Gram-positive bacteria. Skin Pharmacol Appl Skin Physiol 16:176–187
15. Harada K-I, Suomalainen M, Uchida H, Masui H, Ohmura K, Kiviranta J, Niku-Paavola M-L, Ikemoto T (2000) Insecticidal compounds against mosquito larvae from *Oscillatoria agardhii* strain 27. Environ Toxicol 15:114–119
16. Zheng CJ, Yoo JS, Lee TG, Cho HY, Kim YH, Kim WG (2005) Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. FEBS Lett 579:5157–5162
17. Desbois AP, Lebl T, Yan L, Smith VJ (2008) Isolation and structural characterisation of two antibacterial free fatty acids from the marine diatom, *Phaeodactylum tricornutum*. Appl Microbiol Biotechnol 81:755–764
18. Galbraith H, Miller TB, Paton AM, Thompson JK (1971) Antibacterial activity of long chain fatty acids and the reversal with calcium, magnesium, ergocalciferol and cholesterol. J Appl Bacteriol 34:803–813
19. Maliński E, Hebanowska E, Szafranek J, Nawrot J (1986) The composition of the hydrocarbons of the larwae of the Khapra beetles, *Trogoderma granarium*. Comp Biochem Physiol 84B:211–215
20. Hebanowska E, Maliński E, Latowska A, Dubis E, Pihlaja K, Oksman P, Nawrot J, Szafranek J (1990) A comparison of

cuticular hydrocarbons of larvae and beetles of the *Tribolium destructor*. Comp Biochem Physiol 96B:815–819
21. Gołębiowski M, Maliński E, Nawrot J, Stepnowski P (2008) Identification and characterization of surface lipid components of the dried-bean beetle *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae). J Stored Prod Res 44:386–388
22. Nelson DR, Buckner JS, Fatland CL (1994) The composition of external lipids of adult whiteflies, *Bemisia tabaci* and *Trialeurodes vaporariorum*. Comp Biochem Physiol 109B:293–303
23. Buckner JS, Mardaus MC, Nelson DR (1996) Cuticular lipid composition of *Heliothis virescens* and *Helicoverpa zea* pupae. Comp Biochem Physiol 114B:207–216
24. Gołębiowski M, Boguś MI, Paszkiewicz M, Wieloch W, Włóka E, Stepnowski P (2012) The composition of the cuticular and internal free fatty acids and alcohols from *Lucilia sericata* males and females. Lipids 47:613–622
25. Gołębiowski M, Paszkiewicz M, Grubba A, Gąsiewska D, Boguś MI, Włóka E, Wieloch W, Stepnowski P (2012) Cuticular and internal n-alkane composition of *Lucilia sericata* larvae, pupae, male and female imagines: application of HPLC–LLSD and GC/MS-SIM. Bull Entomol Res 102:453–460
26. Vrkoslav V, Muck A, Cvacka J, Svatoš A (2010) MALDI imaging of neutral cuticular lipids in insects and plants. J Am Soc Mass Spectrom 21:220–231
27. Pasquale C, Guarino S, Peri E, Alonzo G, Colazza S (2007) Investigation of cuticular hydrocarbons from *Bagrada hilaris* genders by SPME/GC-MS. Anal Bioanal Chem 389:1259–1265
28. Villaverde ML, Girotti JR, Mijailovsky SJ, Pedrini N, Juárez MP (2009) Volatile secretions and epicuticular hydrocarbons of the beetle *Ulomoides dermestoides*. Comp Biochem Physiol 154B: 381–386
29. Turillazzi S, Sledge MF, Cremer S, Heinze J (2002) A method for analysing small-size specimens in GC–MS. J Insect Soc Life 4:169–175
30. Gołębiowski M, Boguś MI, Paszkiewicz M, Stepnowski P (2011) Cuticular lipids of insects as a potential biofungicides: methods of lipids composition analysis. Anal Bioanal Chem 399:3177–3191
31. Gołębiowski M, Boguś MI, Paszkiewicz M, Stepnowski P (2010) The composition of the free fatty acids from *Dendrolimus pini* exuviae. J Insect Physiol 56:391–397
32. Gołębiowski M, Maliński E, Nawrot J, Szafranek J, Stepnowski P (2007) Identification of the cuticular lipid composition of the Western Flower Thrips *Frankliniella occidentalis*. Comp Biochem Physiol 147B:288–292
33. Gołębiowski M, Dawgul M, Kamysz W, Boguś MI, Wieloch W, Włóka E, Paszkiewicz M, Przybysz E, Stepnowski P (2012) The antimicrobial activity of the alcohols from *Musca domestica*. J Exp Biol. doi:10.1242/jeb.073155
34. Howard RW, Lord JC (2003) Cuticular lipids of the booklouse, *Liposcelis bostrychophila*: hydrocarbons, aldehydes, fatty acids, and fatty acid amides. J Chem Ecol 29:615–627
35. Buckner JS, Hagen MM, Nelson DR (1999) The composition of the cuticular lipids from nymphs and exuviae of the silverleaf whitefly, *Bemisia argentifolii*. Comp Biochem Physiol 124B: 201–207
36. Soliday CL, Blomquist GJ, Jackson LL (1974) Cuticular lipids of insects. VI. Cuticular lipids of the grasshoppers *Melanoplus sanguinipes* and *Melanoplus packardii*. J Lipid Res 15:399–405
37. Boguś MI, Czygier M, Gołębiowski M, Kędra E, Kucińska J, Mazgajska J, Samborski J, Wieloch W, Włóka E (2010) Effects of insect cuticular fatty acids on in vitro growth and pathogenicity of the entomopathogenic fungus *Conidiobolus coronatus*. Exp Parasitol 125:400–408
38. Blomquist GJ, Chu AJ, Remaley S (1980) Biosynthesis of wax in the honeybee, *Apis mellifera* L. Insect Biochem 10:313–321
39. Oraha VS, Lockey KH (1990) Cuticular lipids of *Locusta migratoria migratoriodes*, *Schistocerca gregaria* (Acrididae) and other Orthopteran species-I. Polar components. Comp Biochem Physiol 95B:603–608
40. Kerwin JL (1984) Fatty acid regulation of the germination of *Erynia variabilis* conidia on adults and puparia of the lesser housefly, *Fannia canicularis*. Can J Microbiol 30:158–161
41. Baker JE (1978) Cuticular lipids of larvae *Attagenus megatoma*. Insect Biochem 8:287–292
42. Brey PT, Ohayon Lesourd M, Castex H, Roucache J, Latge JP (1985) Ultrastructure and chemical composition of the outer layers of the cuticle of the pea aphid *Acyrthosiphon pisum* (HARRIS). Comp Biochem Physiol A 82:401–411
43. Baker JE (1979) Developmental changes in cuticular lipids of the black carpet beetle, *Attagenus megatoma*. Insect Biochem 9:335–339
44. Baker JE, Sukkestad DR, Nelson DR, Fatland CL (1979) Cuticular lipids of larvae and adults of the cigarette beetle, *Lasioderma serricorne*. Insect Biochem 9:603–611
45. Lockey KH (1988) Lipids of the insect cuticle: origin, composition and function. Comp Biochem Physiol 89B:595–645
46. Szafranek B, Paszkiewicz M, Gołębiowski M, Stepnowski P (2011) Gas chromatographic analysis of plant and insect surface compounds: cuticular waxes and terpenoids. In: Gas chromatography/book 2. In Tech. ISBN 979-953-307-736-8
47. Espelie KE, Bernays EA (1989) Diet-related differences in the cuticular lipids of *Manduca sexta* larvae. J Chem Ecol 15:2003–2017
48. Saito T, Aoki J (1983) Toxicity of free fatty acids on the larval surfaces of two lepidopterous insects towards *Beauveria bassiana* (Bals.) Vuill. And *Poecilomyces fumoso-roseus* (Wize) Brown et Smith (*Deuteromycetes: Moniliales*). Appl Entomol Zool 18:225–233
49. Smith RJ, Grula EA (1981) Nutritional requirements for conidial germination and hyphal growth of *Beauveria bassiana*. J Invertebr Pathol 37:222–230