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RESEARCH ARTICLE

Antimicrobial activity of alcohols from *Musca domestica*

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SUMMARY

Information on the stimulatory and inhibitory effects of cuticular alcohols on growth and virulence of insecticidal fungi is unavailable. Therefore, we set out to describe the content of cuticular and internal alcohols in the body of housefly larvae, pupae, males and females. The total cuticular alcohols in larvae, males and females of *Musca domestica* were detected in comparable amounts (4.59, 3.95 and 4.03 μg g^{-1} insect body, respectively), but occurred in smaller quantities in pupae (2.16 μg g^{-1}). The major free alcohol in *M. domestica* larvae was $C_{12:0}$ (70.4%). Internal alcohols of *M. domestica* larvae were not found. Among cuticular pupae alcohols, $C_{12:0}$ (31.0%) was the most abundant. In the internal lipids of pupae, only five alcohols were identified in trace amounts. The most abundant alcohol in males was $C_{24:0}$ (57.5%). The percentage content of cuticular $C_{24:0}$ in males and females (57.5 and 36.5%, respectively) was significantly higher than that of cuticular lipids in larvae and pupae (0.9 and 5.6%, respectively). Only two alcohols were present in the internal lipids of males in trace amounts ($C_{18:0}$ and $C_{20:0}$). The most abundant cuticular alcohols in females were $C_{24:0}$ (36.5%) and $C_{12:0}$ (26.8%); only two alcohols ($C_{18:0}$ and $C_{20:0}$) were detected in comparable amounts in internal lipids (3.61±0.32 and 5.01±0.42 μg g^{-1}, respectively). For isolated alcohols, antimicrobial activity against 10 reference strains of bacteria and fungi was determined. Individual alcohols showed approximately equal activity against fungal strains. $C_{14:0}$ was effective against gram-positive bacteria, whereas gram-negative bacteria were resistant to all tested alcohols. Mixtures of alcohols found in cuticular lipids of larvae, pupae, males and females of *M. domestica* generally presented higher antimicrobial activity than individual alcohols. In contrast, crude extracts containing both cuticular and internal lipids showed no antifungal activity against the entomopathogenic fungus *Conidiobolus coronatus*, which efficiently kills adult house flies.

Key words: cuticular lipids, internal lipids, insects, HPLC-LLSD, GC-MS.

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INTRODUCTION

The house fly, *Musca domestica*, is a well-known cosmopolitan pest, both on farms and in homes. This species is always found in association with humans or human activity. The house fly has a complete metamorphosis with distinct egg, larvae/maggot, pupal and adult stages. Warm summer conditions are generally optimal for the development of the house fly. The complete life cycle of *M. domestica* lasts from 7 to 10 days; however, under suboptimal conditions the life cycle may require up to 2 months.

The control of *M. domestica* is vital to human health and comfort in many areas of the world. The most important impacts of *M. domestica* are damages caused by the potential transmission of pathogens (viruses, bacteria, fungi, protozoans and nematodes). Pathogenic organisms are picked up by flies from garbage, sewage and other sources of filth, and then transferred on their mouthparts, through their vomitus, feces and contaminated external body parts to human and animal food.

The more commonly used control measures for house flies are traps and insecticides, but in some instances integrated fly control has been implemented. The use of biological control in fly management is still at a relatively early stage. The sex pheromone (Z)-9-tricosene is formulated with sugar as a commercially available fly bait for local population suppression, as well as an enhancement for population monitoring. Ultraviolet light traps can be used to assess population levels, but also serve as a non-chemical control technique that can be applied in both agricultural and non-agricultural confined spaces. Natural biological suppression of the house fly results primarily from the actions of certain chalcidoid wasps (Hymenoptera: Pteromalidae), many species of which are associated with the house fly around the world. Among the most important are *Muscidifurax* and *Sphalangia* spp. Ichneumonids and other parasitoids, as well as some predatory insects [especially histerids (Coleoptera: Histeridae) and staphylinids (Coleoptera: Staphylinidae)], also contribute to fly mortality.

The potential application of fungal pathogens as control agents against insect pests is also well documented (Roberts and Hajek, 1992; Clarkson and Charnley, 1996; Khachatourians, 1996; Shah and Pell, 2003; Faria and Wraight, 2007). Unlike other insect-pathogenic microorganisms, which must be ingested to initiate disease (e.g. viruses, bacteria, nematodes and protozoans), entomopathogenic fungi such as *Metarhizium anisopliae*, *Conidiobolus coronatus* and *Beauveria bassiana* mostly invade target hosts by penetrating through their cuticle. Susceptibility or resistance of various insect species to fungal invasion may be caused

by several factors, including differences in the structure and composition of the exoskeleton and the presence of antifungal compounds in the cuticle (Vilcinskas and Götz, 1999). It is believed that the epicuticular lipid profile of insect hosts may be one of the pivotal factors determining insect susceptibilities or resistance to fungal attack (Gillespie et al., 2000).

The outermost insect surface or epicuticle is covered by a lipid-rich layer, usually composed of hydrocarbons (Gołębiowski et al., 2012a) together with variable amounts of alcohols (Buckner, 1993), fatty acids (Gołębiowski et al., 2007) and wax esters (Nelson et al., 1998). Entomopathogenic fungi have the ability to degrade insect cuticular lipids with hydrocarbons, the preferred components capable of supporting fungal growth (Napolitano and Juárez, 1997). Although several aspects of the interaction between entomopathogenic fungi and insect host cuticular hydrocarbons and fatty acids have been examined (Pedrini et al., 2007; Boguś et al., 2010), there is no information concerning the other surface compounds.

Free fatty alcohols are not commonly found in epicuticular lipids of insects, although high molecular weight alcohols were identified in honeybees (Blomquist et al., 1987). It was found that honeybees produced alcohols of 17–22 carbon atoms (Donze et al., 1998). In the epicuticle of the blood-sucking bug *Triatoma infestans*, two female-specific fatty alcohols, docosanol (C22) and eicosanol (C20), were found (Cocchiararo-Bastias et al., 2011). Long-chain alcohols have also been reported in the defensive secretions of the scale insects *Bemisia tabaci* and *Trialeurodes vaporariorum* (Byrne and Hadley, 1988).

Information on both stimulatory and inhibitory effects of cuticular alcohols on growth and virulence of insecticidal fungi is unavailable. This paper describes the content of cuticular and internal alcohols in the body of housefly larvae, pupae, males and females. The surface lipids of flies were separated into classes of compounds using high performance liquid chromatography equipped with a laser light scattering detector (HPLC-LLSD). Qualitative and quantitative analysis was achieved by gas chromatography (GC) and gas chromatography combined with mass spectrometry (GC-MS).

Isolated compounds were tested according to their potential antimicrobial activity. We have selected three representative strains each of gram-positive bacteria, gram-negative bacteria and five strains of fungi. Activity against reference strains was determined for individual alcohols as well as for their mixtures found in cuticular lipids of larvae, pupae, males and females of *M. domestica*. Antimicrobial activity of alcohol mixtures was considered according to their composition.

MATERIALS AND METHODS

Insects

Musca domestica Linnaeus 1758 raised from eggs laid on diet by adult flies were reared at 28°C with 70% relative humidity and a 12 h:12 h light:dark photoperiod. The maternal generation was maintained under the same conditions. House flies were reared on a mixture 1:1 of standard rodent chow (LSM and LSK, Fodder Manufacture, Motycz, Poland) dissolved in water. For experiments, 1-day-old larvae, freshly emerged pupae and 6-day-old sexually mature adults were used. The insects were either used for lipid extractions or exposed for 18 h to fully grown and sporulating colonies of the entomopathogenic fungus *Conidiobolus coronatus*. In a Petri dish containing a *C. coronatus* colony, 10 flies (males and females separately), 10 pupae or 10 larvae were maintained. The insects exposed for 18 h to sterile uninoculated Sabouraud agar medium (SAM) served as controls. After exposure, the adults were transferred to clean Petri dishes with sugar and water, whereas larvae were transferred to dishes with fresh diet. Pupae were transferred to clean Petri dishes without food. All insects exposed to *C. coronatus* were kept at proper growing conditions for 10 days and their condition was monitored daily. Exposure of tested insects to the *C. coronatus* colony for 18 h was found to be the most efficient method of resembling the natural infection process (Wieloch and Boguś, 2005). To avoid pseudoreplication, all assays of fungi *versus* insects were performed with flies from different stocks incubated in three different chambers.

A culture of the wax moth *Galleria mellonella* was maintained and reared in temperature and humidity controlled chambers (30°C, 70% relative humidity) in constant darkness on an artificial diet (Sehnal, 1966). Fully grown larvae were collected before pupation, surface sterilized, homogenized and used as supplement in fungal cultures.

Microorganisms

Conidiobolus coronatus (Zygomycetes), isolate number 3491, originally isolated from *Dendrolaelaps* spp., was obtained from the collection of Prof. Bałazy (Polish Academy of Sciences, Research Center for Agricultural and Forest Environment, Poznań, Poland), and was maintained in 90 mm Petri dishes at 20°C with cyclic changes of light (12 h:12 h light:dark) on SAM with the addition of homogenized *G. mellonella* larvae to a final concentration of 10% wet mass. Addition of homogenized *G. mellonella* larvae has been shown to enhance SAM cultures of *C. coronatus*. The levels of mycelial growth, conidia production and virulence were good in hundreds of successive transfers (Wieloch, 2006), suggesting a stimulatory effect of insect proteins, carbohydrates and lipids on *C. coronatus* growth and insecticidal properties. Seven-day-old cultures were briefly washed with sterile water to harvest conidia. The number of harvested conidia was determined under a microscope with the use of a hemocytometer. One-hundred-microliter portions of suspension each containing ~50 conidia were used for inoculations.

Bacterial (*Bacillus subtilis* ATCC 6633, *Rhodococcus equi* ATCC 6939, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13882 and *Proteus vulgaris* ATCC 13315) and fungal (*Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231, *Candida lipolytica* PCM 2680 and *Candida tropicalis* PCM 2681) reference strains were obtained from The Polish Collection of Microorganisms (Polish Academy of Sciences, Wrocław, Poland). Reference strains of bacteria were inoculated in Mueller–Hinton II broth 24 h before performing the minimal inhibitory concentration (MIC) test, and incubated at 37°C with 150 r.p.m. shaking. Fungal strains were cultured at the temperature 25°C with 150 r.p.m. shaking for 48 h by the time the MIC was tested.

Extraction of cuticular lipids

The cuticular lipids of *M. domestica* were obtained by immersing insects in 40 ml of petroleum ether for 10 s (extract I) and in 40 ml of dichloromethane for 5 min (extract II). The same insects were transferred to dichloromethane for 10 days (extract III). Extracts I and II contained cuticular lipids and extract III contained internal lipids. The extracts were evaporated on a rotary evaporator at a temperature not exceeding 40°C. Table 1 lists the number of insects, as well as the masses of the extracts. Extraction was carried out according to previously published methods (Gołębiowski et al., 2008a).

Derivatization of extracts

The lipid fractions obtained by HPLC were silylized with 100 µl of a mixture of 99% bis(trimethylsilyl)acetamide and 1%

Table 1. Quantitative summary of the experiment, showing numbers and masses of insects, and masses of lipids

Stage	Number of insects	Insect mass (g)	Extract	Lipid mass (mg g^{-1} insect body)
Larvae	100	1.1	I	0.96
			II	0.29
			III	48.73
Pupae	1288	21.0	I	0.08
			II	0.08
			III	33.81
Male	654	5.9	I	5.52
			II	3.26
			III	8.70
Female	468	6.8	I	3.17
			II	0.80
			III	17.38

I, petroleum extract (10 s); II, dichloromethane extract (5 min); III, dichloromethane extract (10 days).

chlorotrimethylsilane for 1 h at 100°C. Silylized samples were analyzed by GC-MS.

HPLC-LLSD

The lipid extracts were separated using HPLC-LLSD. The detector evaporation temperature was 45°C, and the carbon dioxide pressure was 0.1–0.2 MPa. The separation in the normal phase was performed on a silica gel column (Econosil Silica 5 Micron, 25 cm × 4.6 mm, Alltech, Nicholasville, KY, USA). The mobile phase consisted of hexane (Solvent A) and dichloromethane with the addition of 15% acetone (Solvent B). The gradient was programmed linearly from 100% A to 100% B within 30 min. Total flow was maintained at 0.8 ml min^{-1}.

GC-MS

Electron-impact (EI; 70 eV) mass spectra were obtained on an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) coupled with an SSQ-7100 mass spectrometer (San Jose, CA, USA). GC analysis was performed on an Rtx-5MS column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness; Restek, Warsaw, Poland). The temperature was programmed from 80°C (held for 8 min) to 320°C (held for 5 min) at a rate of 10°C min^{-1}. Each extract (1 μl) was injected (injector temperature, 320°C), using helium as the carrier gas. MS conditions were as follows: interface temperature, 320°C; ion source temperature, 220°C; mass range, 40–650 a.m.u.

Antimicrobial activity assays

Antimicrobial activity was determined for individual alcohols and mixtures of alcohols found in larvae, pupae, males and females of *M. domestica*. The MIC was determined using a microbroth dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI). For bacterial strains, the Mueller Hinton Broth II (MHB II) (Fluka Analytical, Sigma-Aldrich, Steinheim, Germany) and initial inoculums of 5×10^5 CFU ml^{-1} were applied, whereas Sabouraud glucose 2% broth (Carl Roth, Karlsruhe, Germany) and initial inoculums 10^3 CFU ml^{-1} were used for tested fungi. Polypropylene 96-well plates (Becton Dickinson, Le Point de Claix, France) were incubated for 18 h at 37°C (bacteria) or 48 h at 25°C (fungi). The MIC was taken as the lowest alcohol concentration at which a noticeable growth was inhibited. The experiments were performed in triplicate.

The antifungal potential of crude extracts obtained from *M. domestica* against the entomopathogenic fungus *C. coronatus* was screened using the disc diffusion method recommended by the CLSI. Different concentrations of each tested extract dissolved in DMSO (five dilutions for each extract; concentration range 1 μg–2.6 mg per disc) were applied onto sterile discs (5 mm diameter, Whatman filter paper no. 42) presoaked in DMSO (Sigma-Aldrich). Discs were placed onto Petri dishes containing SAM supplemented with *G. mellonella* homogenate and inoculated with *C. coronataus* as described above. Inoculated plates were incubated at 20°C for 7 days, after which plates were checked for the presence of inhibition zones. Each test was performed in triplicate.

RESULTS

Susceptibility of *M. domestica* to fungal infection

Exposure of *M. domestica* adults to the sporulating *C. coronatus* resulted in prompt death of nearly all adults: 93±3% of tested males and 87±6% of females died around the termination of the 18 h exposition to fungal culture. The lack of resistance of *M. domestica* males and females to *C. coronatus* infection was confirmed using a pairwise *t*-test: we did not notice any significant differences in the susceptibility of males and females. In contrast, house fly pupae and larvae were totally resistant to fungal infection (Table 2).

Composition of alcohols from *M. domestica*

In order to obtain total amounts of the cuticular lipids, two short extractions with petroleum ether and dichloromethane were applied. The cuticular lipids of larvae, pupae, males and females amounted to 1.25, 0.16, 8.78 and 3.97 mg g^{-1} of the insect body, respectively. The amount of internal lipids obtained by long dichloromethane extraction was considerable (48.73, 33.81, 8.70 and 17.38 mg g^{-1} of the insect body, respectively; Table 1).

Table 2. Susceptibility of *Musca domestica* to fungal infection

Developmental stage / treatment	Number of insects	Mortality (%)
Adult females		
Control	20	0
Exposed to *C. coronatus*	59	87±6
Adult males		
Control	20	0
Exposed to *C. coronatus*	60	93±3
Pupae		
Control	20	0
Exposed to *C. coronatus*	60	0
Larvae		
Control	20	0
Exposed to *C. coronatus*	29	0

Insects were exposed to sporulating *Conidiobolus coronatus* colonies as described in the Materials and methods. The insect susceptibility to fungal infection is expressed as a percentage of mortality in tested populations.

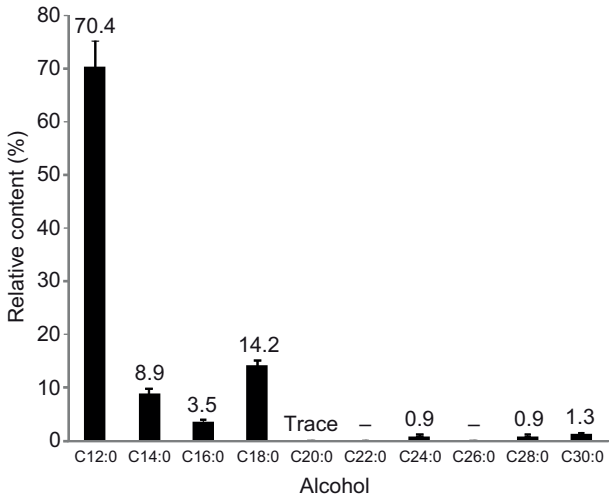

Fig. 1. Composition of alcohols in the cuticular lipid extracts of *Musca domestica* larvae. Values are means ± s.d.

The cuticular alcohols of larvae contained eight saturated even-numbered carbon-chain alcohols ranging from $C_{12:0}$ to $C_{30:0}$ (Fig. 1). The major free alcohol in *M. domestica* larvae was $C_{12:0}$ (70.4%). The alcohols occurring in smaller quantities (from 1 to 15%) were $C_{14:0}$ (8.9%), $C_{16:0}$ (3.5%), $C_{18:0}$ (14.2%) and $C_{30:0}$ (1.3%). The cuticular lipids also contained three alcohols present in concentrations <1%: $C_{20:0}$ (trace), $C_{24:0}$ (0.9%) and $C_{28:0}$ (0.9%). The total cuticular alcohol content in the cuticular lipids (petroleum ether and dichloromethane extracts) was 4.59 $\mu g\,g^{-1}$ of the insect body. Internal alcohols of *M. domestica* larvae were not found. Table 3 lists the alcohol contents calculated per gram of insect body in larvae.

Among cuticular alcohols of pupae, $C_{12:0}$ (31.0%) was the most abundant compound (Table 4, Fig. 2). Other alcohols were detected in comparable amounts from 3.7% ($C_{14:0}$) to 14.4% ($C_{16:0}$). Only $C_{22:0}$ was present in trace amounts. In the internal lipids of pupae, only five alcohols were identified, and these occurred in trace amounts.

Eight alcohols were present in the cuticular lipids of males. The most abundant alcohol was $C_{24:0}$ (57.5%) (Table 5, Fig. 3). The percentage content of cuticular $C_{24:0}$ in males and females (57.5 and 36.5%, respectively) was significantly higher than that in cuticular lipids in larvae and pupae (0.9 and 5.6%, respectively). The major alcohol found in larvae and pupae ($C_{12:0}$) was also present in the cuticular lipids of males, and had a high percentage content (27.8%). Three alcohols of males present in smaller quantities were $C_{18:0}$ (10.1%), $C_{20:0}$ (3.0%) and $C_{16:0}$ (1.5%). Three alcohols were also present in trace amounts ($C_{14:0}$, $C_{26:0}$ and $C_{28:0}$). Only two alcohols were in the internal lipids of males ($C_{18:0}$ and $C_{20:0}$, in trace amounts).

Similar profiles of alcohols were identified in the cuticular and internal lipids in males and females. The most abundant cuticular alcohols in females were $C_{24:0}$ (36.5%) and $C_{12:0}$ (26.8%) (Fig. 4). Other alcohols were present in smaller quantities from 1.5% ($C_{14:0}$) to 11.9% ($C_{22:0}$) and two alcohols were present in trace amounts ($C_{26:0}$ and $C_{28:0}$). Only two alcohols ($C_{18:0}$ and $C_{20:0}$) were detected in comparable amounts in the internal lipids of females (3.61±0.32 and 5.01±0.42 $\mu g\,g^{-1}$ of the insect body, respectively). Table 6 lists the alcohol contents calculated per gram of insect body in females.

The total cuticular alcohols in females, males and larvae of *M. domestica* were detected in comparable amounts (4.03, 3.95 and 4.59 $\mu g\,g^{-1}$ of the insect body, respectively). The total cuticular

Table 3. Chemical composition of the alcohols found in larvae of *Musca domestica*

	Content ($\mu g\,g^{-1}$)			
Alcohol	Extract I	Extract II	Sum of cuticular alcohols	Extract III
$C_{12:0}$	1.18±0.08	2.05±0.14	3.23	–
$C_{14:0}$	0.10±0.02	0.31±0.02	0.41	–
$C_{16:0}$	0.16±0.02	Trace	0.16	–
$C_{18:0}$	0.23±0.02	0.42±0.02	0.65	–
$C_{20:0}$	–	Trace	Trace	–
$C_{22:0}$	–	–	–	–
$C_{24:0}$	Trace	0.04±0.01	0.04	–
$C_{26:0}$	–	–	–	–
$C_{28:0}$	Trace	0.04±0.01	0.04	–
$C_{30:0}$	Trace	0.06±0.01	0.06	–
Sum	1.67	2.92	4.59	–

Data are presented as means ± s.d. of three separate analyses performed on different samples.

Table 4. Chemical composition of the alcohols found in pupae of *Musca domestica*

	Content ($\mu g\,g^{-1}$)			
Alcohol	Extract I	Extract II	Sum of cuticular alcohols	Extract III
$C_{12:0}$	0.33±0.03	0.34±0.03	0.67	–
$C_{14:0}$	0.04±0.01	0.04±0.01	0.08	–
$C_{16:0}$	Trace	0.31±0.03	0.31	–
$C_{18:0}$	0.10±0.01	0.11±0.01	0.21	Trace
$C_{20:0}$	0.06±0.01	0.07±0.01	0.13	Trace
$C_{22:0}$	Trace	Trace	Trace	Trace
$C_{24:0}$	0.06±0.01	0.06±0.01	0.12	Trace
$C_{26:0}$	0.13±0.01	0.14±0.02	0.27	Trace
$C_{28:0}$	0.15±0.01	0.12±0.01	0.27	–
$C_{30:0}$	Trace	0.10±0.05	0.10	–
Sum	0.87	1.29	2.16	Trace

Data are presented as means ± s.d. of three separate analyses performed on different samples.

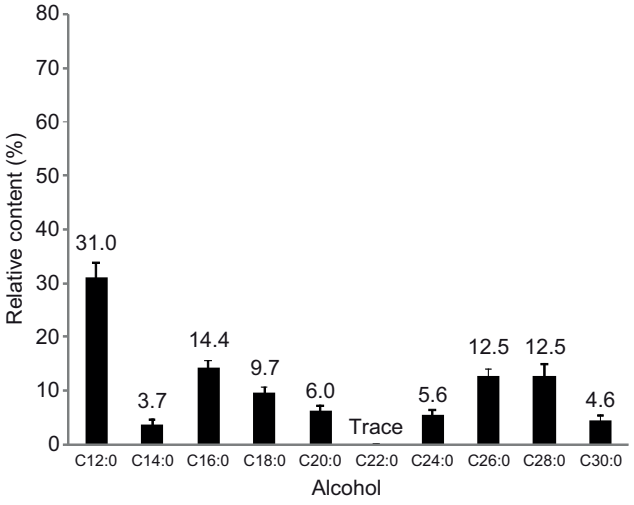

Fig. 2. Composition of alcohols in the cuticular lipid extracts of *M. domestica* pupae. Values are means ± s.d.

Table 5. Chemical composition of the alcohols found in males of *Musca domestica*

Alcohol	Content ($\mu g\,g^{-1}$) Extract I	Extract II	Sum of cuticular alcohols	Extract III
$C_{12:0}$	0.71±0.05	0.39±0.03	1.10	–
$C_{14:0}$	Trace	Trace	Trace	–
$C_{16:0}$	Trace	0.06±0.01	0.06	–
$C_{18:0}$	0.23±0.03	0.17±0.02	0.40	Trace
$C_{20:0}$	–	0.12±0.02	0.12	Trace
$C_{22:0}$	–	–	–	–
$C_{24:0}$	Trace	2.27±0.19	2.27	–
$C_{26:0}$	Trace	Trace	Trace	–
$C_{28:0}$	Trace	Trace	Trace	–
$C_{30:0}$	–	–	–	–
Sum	0.94	3.01	3.95	Trace

Data are presented as means ± s.d. of three separate analyses performed on different samples.

alcohols occurred in smaller quantities in pupae ($2.16\,\mu g\,g^{-1}$ of the insect body). No internal alcohols were found in larvae, and these only occurred in trace amounts in males and pupae. Only in females were internal alcohols detected in high amounts ($8.62\,\mu g\,g^{-1}$ of the insect body). The cuticular alcohols of pupae contained 10 saturated even-numbered carbon chain alcohols ranging from $C_{12:0}$ to $C_{30:0}$, whereas $C_{30:0}$ in females, $C_{22:0}$ and $C_{30:0}$ in males, and $C_{22:0}$ and $C_{26:0}$ in larvae were absent.

Comparison of petroleum with dichloromethane extract

Seven and eight alcohols were present in the petroleum ether extract (I) and in the dichloromethane extract (II) of larvae, respectively. The dichloromethane extract generally had more alcohols than the petroleum ether extract. The respective quantities were $2.92\,\mu g\,g^{-1}$ and $1.67\,\mu g\,g^{-1}$ of the insect body. $C_{20:0}$ (alcohol) was present only in the dichloromethane extract. The following alcohols were qualitatively predominant in the petroleum ether extract: $C_{12:0}$ (70.7%), $C_{18:0}$ (13.8%) and $C_{16:0}$ (9.6%). In the dichloromethane extract, the following alcohols were the most abundant: $C_{12:0}$ (70.2%), $C_{18:0}$ (14.4%) and $C_{14:0}$ (10.6%). In the petroleum ether extract, three alcohols were presented in trace amounts ($C_{24:0}$, $C_{28:0}$ and $C_{30:0}$). In the dichloromethane extract, two alcohols were presented in trace amounts ($C_{16:0}$ and $C_{20:0}$).

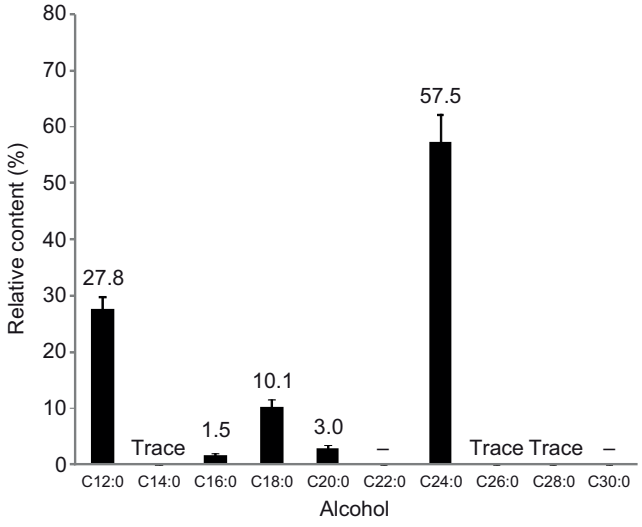

Fig. 3. Composition of alcohols in the cuticular lipid extracts of *M. domestica* males. Values are means ± s.d.

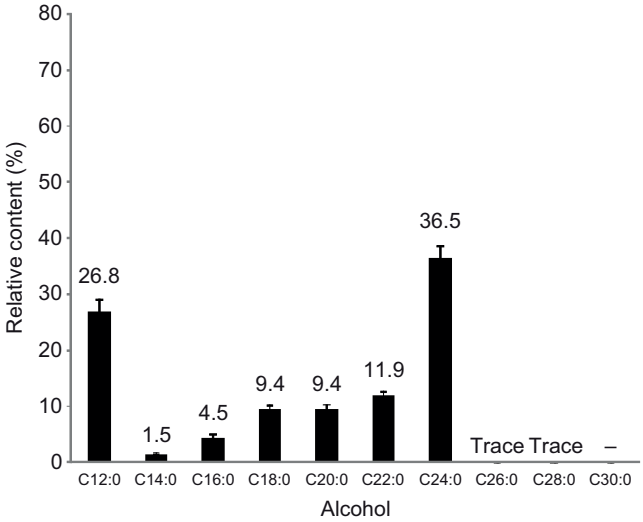

Fig. 4. Composition of alcohols in the cuticular lipid extracts of *M. domestica* females. Values are means ± s.d.

The most abundant alcohol in both pupae extracts (petroleum and dichloromethane) was $C_{12:0}$. The respective contents were 37.9 and 26.4%. In the petroleum ether and dichloromethane extracts of pupae, the relative contents of $C_{14:0}$ (4.6 and 3.1%), $C_{18:0}$ (11.5 and 8.5%), $C_{20:0}$ (6.9 and 5.4%), $C_{22:0}$ (both in trace amounts), $C_{24:0}$ (6.9 and 4.7%), $C_{26:0}$ (14.9 and 10.9%) and $C_{28:0}$ (17.2 and 9.3%) alcohols were similar. The relative contents of $C_{16:0}$ and $C_{30:0}$ in the petroleum ether extract were less than in the dichloromethane extract (trace *versus* 24.0% for $C_{16:0}$ and trace *versus* 7.8% for $C_{30:0}$).

The alcohol occurring in the highest concentrations in petroleum ether extract of males was $C_{12:0}$ (75.5%). The relative content of this compound in dichloromethane extract was 13.0%. There was a significant difference in the relative content of $C_{24:0}$ in both extracts. This alcohol was present in the highest concentrations in dichloromethane extract (75.4%), but in the petroleum ether extract occurred in trace amounts. In both cuticular extracts of males, three

Table 6. Chemical composition of the alcohols found in females of *Musca domestica*

Alcohol	Content ($\mu g\,g^{-1}$) Extract I	Extract II	Sum of cuticular alcohols	Extract III
$C_{12:0}$	0.57±0.05	0.51±0.04	1.08	–
$C_{14:0}$	Trace	0.06±0.01	0.06	–
$C_{16:0}$	0.18±0.02	–	0.18	–
$C_{18:0}$	0.20±0.02	0.18±0.01	0.38	3.61±0.32
$C_{20:0}$	0.15±0.01	0.23±0.02	0.38	5.01±0.42
$C_{22:0}$	–	0.48±0.03	0.48	–
$C_{24:0}$	–	1.47±0.08	1.47	–
$C_{26:0}$	–	Trace	Trace	–
$C_{28:0}$	–	Trace	Trace	–
$C_{30:0}$	–	–	–	–
Sum	1.10	2.93	4.03	8.62

Data are presented as means ± s.d. of three separate analyses performed on different samples.

alcohols were present in trace amounts ($C_{14:0}$, $C_{26:0}$ and $C_{28:0}$). The $C_{20:0}$ alcohol present in the dichloromethane extract (4.0%) was absent in the petroleum ether extract. The percentage contents of other alcohols in petroleum ether and dichloromethane extracts were very distinct: $C_{16:0}$ (trace *versus* 2.0%), $C_{18:0}$ (24.5 *versus* 5.6%).

The most abundant cuticular alcohols in females were $C_{12:0}$ in petroleum extract (51.8%) and $C_{24:0}$ in dichloromethane extract (50.2%). Four alcohols present in the dichloromethane extract ($C_{22:0}$, $C_{24:0}$, $C_{26:0}$ and $C_{28:0}$) were absent in the petroleum ether extract. In contrast, $C_{16:0}$ occurred only in petroleum ether extract (16.4%). Other alcohols were present in both extracts: $C_{14:0}$ (trace *versus* 2.0%), $C_{18:0}$ (18.2 *versus* 6.1%) and $C_{20:0}$ (13.6 *versus* 7.8%).

Antimicrobial activity

The activity of individual alcohols varied significantly depending on the tested strain (Table 7). In general, long-chain alcohols were less active in comparison to shorter-chain compounds. The alcohol 1-tetradecanol ($C_{14:0}$) turned out to be very effective against gram-positive bacteria (*B. subtilis*, *R. equi* and *S. areus*). This compound also presented antifungal activity in tests with the use of *C. albicans*, *C. tropicalis*, *C. lipolytica* and *A. niger*. Its activity against gram-negative bacteria (*E. coli*, *K. pneumoniae* and *P. vulgaris*) was minor, similar to the activity of all remaining alcohols. Tested alcohols presented approximately equal activity against reference strains of fungi. $C_{10:0}$ and $C_{12:0}$ were the most active antifungal compounds and presented some antibacterial potency against gram-positive bacteria. The remaining alcohols exhibited minor antibacterial activity. *Rhodococcus equi* turned out to be the most susceptible bacterial strain – most tested alcohols (with the exception of $C_{20:0}$, $C_{26:0}$ and $C_{30:0}$) presented certain activity against these bacteria. Gram-negative bacteria were resistant to all alcohols at tested concentrations (data not shown).

Antimicrobial activity of alcohol mixtures was determined for all representative strains of fungi and *R. equi* as the most susceptible bacterial strain. A mixture of alcohols found in the cuticular lipids of females presented the highest activity against *R. equi* and *C. lipolytica*. Cuticular alcohols of larvae acted the strongest against the above-mentioned strains and *C. tropicalis*, whereas cuticular alcohols of males were the most efficient against *C. tropicalis* and *C. lipolytica*. *Candida albicans* and *A. niger* were the more resistant fungal strains. All extracts acted on *C. albicans* with the same strength, whereas *A. niger* was slightly more susceptible to female and larval lipids (Table 8).

In contrast to data presented in Tables 7 and 8, diffusion disc tests performed with the use of lipids extracted from larvae, pupae and adults of *M. domestica* (extracts I, II and III, as depicted in Table 1) showed no antifungal activity against the entomopathogenic fungus *C. coronatus*. No inhibition zones were seen around discs containing both cuticular and internal lipids. In addition, mycelia grew over not only substrate but also discs (data not shown).

DISCUSSION

Thin layer chromatography, together with HPLC and column chromatography, are standard methods in lipid class analysis. GC and GC-MS are often the methods used for identification of insect lipids (Lockey, 1988; Buckner, 1993; Gołębiowski et al., 2011). In the present study, the use of HPLC-LLSD and subsequent GC-MS for lipid analysis allowed us to obtain more accurate results than direct analysis by GC-MS. It was found that under optimized chromatographic conditions, closely eluting peaks did not interfere with the alcohols. LLSD is often used for plant lipid class analysis (Gołębiowski et al., 2010a) and can also be used for detection of insect lipids (Gołębiowski et al., 2008b; Gołębiowski et al., 2010b). In other words, LLSD is the universal detector for all substances less volatile than mobile phase, which is used in HPLC. In the present study, the GC analysis for quantities of underivatized *n*-alcohols was not reliable because of peak broadening. Therefore, the lipid samples reacted with a mixture of 99% bis(trimethylsilyl) acetamide and 1% chlorotrimethylsilane and were heated for 1 h at 100°C, and the resulting alcohol trimethylsilyl ether was analyzed by GC-MS. Alcohols were identified on the basis of silyl derivative ions $[M-15]^+$ and *m*/*z* 103 (Evershed, 1992).

Early analyses of cuticular lipids of insect species indicated that cuticular alcohols vary in qualitative and quantitative composition. In insect cuticular lipids, the following alcohols have been identified: $C_{22:0}$ to $C_{32:0}$ in *Melanoplus sanguinipes* and *Melanoplus packardii* adults (Soliday et al., 1974), $C_{28:0}$ to $C_{34:0}$ in *Bemisia argentifolii* nymphs (Buckner et al., 1999), $C_{24:0}$ to $C_{34:0}$ in *Heliothis virescens* pupae and *Aleyrodes singularis* exuviae (Buckner et al., 1996; Nelson et al., 1998), $C_{26:0}$ to $C_{34:0}$ in *Helicoverpa zea* pupae (Buckner et al., 1996), $C_{22:0}$ to $C_{34:0}$ in *A. singularis* adults (Nelson et al., 1998), $C_{8:0}$ to $C_{22:0}$ in *Acyrthosiphon pisum* (Brey et al., 1985), $C_{32:0}$ and $C_{34:0}$ in *Bemisia tabaci* adults (Buckner et al., 1994), $C_{30:0}$ and $C_{32:0}$ in *Trialeurodes vaporariorum* adults (Buckner et al., 1994), $C_{12:0}$ to $C_{34:0}$ in *Locusta migratoria migratoriodes* adults (Oraha and Lockey, 1990) and $C_{12:0}$ to $C_{32:0}$ in *Schistocerca gregaria* adults (Oraha and Lockey, 1990) (Table 9). In the present study, the cuticular lipids of *M. domestica* males and females contained alcohols from $C_{12:0}$ to $C_{28:0}$ and those of larvae and pupae contained alcohols from $C_{12:0}$ to $C_{30:0}$.

The major free alcohols that have been found in insects are: $C_{14:0}$ (16.0%) in *L. m. migratoriodes* adults (Ohara and Lockey, 1990), $C_{15:0}$ (18.8%) in *S. gregaria* adults (Ohara and Lockey, 1990), $C_{16:0}$ (35%) in *A. pisum* (Brey et al., 1985), $C_{18:0}$ (55.2 and 48.8%) in

Table 7. Minimal inhibitory concentrations (MICs) obtained for individual alcohols in 7 microbial reference strains

	MIC (mg ml^{-1})						
Alcohol	*Rhodococcus equi*	*Staphylococcus aureus*	*Bacillus subtilis*	*Candida albicans*	*Candida tropicalis*	*Candida lipolytica*	*Aspergillus niger*
$C_{10:0}$	2	>2	2	1	0.512	1	2
$C_{12:0}$	2	1	2	1	0.512	1	1
$C_{14:0}$	0.064	0.128	0.128	2	2	1	1
$C_{16:0}$	1	2	>2	2	>2	1	2
$C_{18:0}$	2	>2	>2	2	>2	2	2
$C_{20:0}$	>2	>2	>2	2	>2	2	>2
$C_{22:0}$	2	>2	>2	2	2	2	2
$C_{24:0}$	2	>2	2	1	1	1	1
$C_{26:0}$	>2	>2	>2	1	2	1	>2
$C_{28:0}$	2	>2	>2	1	2	1	2
$C_{30:0}$	>2	>2	>2	2	2	1	>2

Table 8. Minimal inhibitory concentrations (MICs) of five microbial reference strains obtained for mixtures of alcohols found in *Musca domestica* larvae, pupae, males and females

	MIC (mg ml^{-1})				
Alcohol mixture	*Rhodococcus equi*	*Candida albicans*	*Candida tropicalis*	*Candida lipolytica*	*Aspergillus niger*
Female	0.512	2	1	0.512	1
Male	2	2	0.512	0.512	2
Pupae	1	2	1	1	2
Larvae	0.256	2	0.512	0.512	1

Lucilia sericata males and females, respectively (Gołębiowški et al., 2012b), 14.6% in *L. m. migratoriodes* adults and 12.6% in *S. gregaria* adults (Ohara and Lockey, 1990), $C_{22:0}$ (32%) in *A. pisum* (Brey et al., 1985), $C_{24:0}$ (34%) in *M. sanguinipes* adults and 48% in *M. packardii* adults (Soliday et al., 1974), $C_{26:0}$ (36%) in *M. sanguinipes* and *M. packardii* adults (Soliday et al., 1974), 73% in *H. virescens* pupae (Buckner et al., 1996), <60% in *A. singularis* exuviae and <20% in *A. singularis* adults (Nelson et al., 1998), $C_{28:0}$ (16%) in *H. virescens* and *H. zea* pupae (Buckner et al., 1996), $C_{30:0}$ (74%) in *H. zea* pupae (Buckner et al., 1996), $C_{32:0}$ (36.9%) in *B. argentifolii* nymphs (Buckner et al., 1999), <35% in *A. singularis* exuviae and <70% in *A. singularis* adults (Nelson et al., 1998) and $C_{34:0}$ (36.1%) in *B. argentifolii* nymphs (Buckner et al., 1999). All major alcohols had an even number of carbon atoms, except $C_{15:0}$, which was present in *S. gregaria* adults (Ohara and Lockey, 1990). In the present study, the major free alcohols were: $C_{12:0}$ (70.4%) and $C_{18:0}$ (14.2%) in *M. domestica* larvae, $C_{12:0}$ (31.0%) and $C_{16:0}$ (14.4%) in pupae, $C_{12:0}$ (27.8%) and $C_{24:0}$ (57.5%) in males, and $C_{12:0}$ (26.8%) and $C_{24:0}$ (36.5%) in females.

Alcohols found in insects' lipids mainly contain even-numbered carbon chains. For example, only even-numbered alcohols occurred in the lipids of *B. argentifolii* nymphs (Buckner et al., 1999), *Tenodera sinensis* females, *T. angustipennis* males, *Stagmomantis carolina* females (Jones et al., 1997), and *Bemisia tabaci* and *T. vaporariorum* adults (Buckner et al., 1994). Likewise, in the present study, only even-numbered alcohols were identified in the cuticular and internal lipids of larvae, pupae, and males and females of *M. domestica.* In some insect species, even-numbered alcohols

Table 9. Composition of alcohols found in lipids of insects

	Insect species												
Alcohol	Ba[a]	Ts, Ta, Sc[b]	Hv[c]	Hz[c]	Ase[d]	Asa[d]	Ms[e]	Mp[e]	Ap[f]	Bt[g]	Tv[g]	Lm[h]	Sg[h]
$C_{8:0}$	–	–	–	–	–	–	–	–	17	–	–	–	–
$C_{12:0}$	–	–	–	–	–	–	–	–	5	–	–	1.7	1.7
$C_{13:0}$	–	–	–	–	–	–	–	–	11	–	–	–	–
$C_{14:0}$	–	–	–	–	–	–	–	–	–	–	–	16.0	4.4
$C_{15:0}$	–	–	–	–	–	–	–	–	–	–	–	6.3	18.8
$C_{16:0}$	–	–	–	–	–	–	–	–	35	–	–	12.3	13.8
$C_{17:0}$	–	–	–	–	–	–	–	–	–	–	–	5.0	4.4
$C_{18:0}$	–	–	–	–	–	–	–	–	–	–	–	14.6	12.6
$C_{19:0}$	–	–	–	–	–	–	–	–	–	–	–	0.8	0.6
$C_{20:0}$	–	–	–	–	–	–	–	–	–	–	–	5.5	4.1
$C_{21:0}$	–	–	–	–	–	–	–	–	–	–	–	4.2	4.3
$C_{22:0}$	–	–	–	–	–	<5	3	1	32	–	–	12.9	8.1
$C_{23:0}$	–	–	–	–	–	–	–	–	–	–	–	1.0	1.1
$C_{24:0}$	–	–	8	–	<5	<5	34	48	–	–	–	3.7	2.8
$C_{25:0}$	–	–	–	–	<5	–	Trace	–	–	–	–	1.0	1.6
$C_{26:0}$	–	–	73	4	<60	<20	36	36	–	–	–	3.6	3.2
$C_{27:0}$	–	–	–	–	<5	–	Trace	Trace	–	–	–	0.7	1.8
$C_{28:0}$	5.9	–	16	16	<5	–	9	4	–	–	–	2.3	2.6
$C_{29:0}$	–	–	Trace	2	<5	–	5	2	–	–	–	–	0.9
$C_{30:0}$	21.1	4.7, 5.1, 2.6*	2	74	–	<5	12	5	–	–	1.3*	2.9	1.6
$C_{31:0}$	–	–	–	Trace	<5	10	–	–	–	–	–	–	–
$C_{32:0}$	36.9	–	Trace	4	<35	<70	Trace	Trace	–	3.2*	31.9*	2.8	1.7
$C_{33:0}$	–	–	–	–	–	–	–	–	–	–	–	–	–
$C_{34:0}$	36.1	–	Trace	Trace	<5	<5	–	–	–	33.3*	–	1.4	–

Ba, *Bemisia argentifolii* (nymphs); Ts, *Tenodera sinensis* (females); Ta, *T. angustipennis* (males); Sc, *Stagmomantis carolina* (females); Hv, *Heliothis virescens* (pupae); Hz, *Helicoverpa zea* (pupae); Ase, *Aleyrodes singularis* (exuviae); Ase, *Aleyrodes singularis* (adults); Ms, *Melanoplus sanguinipes* (adults); Mp, *Melanoplus packardii* (adults); Ap, *Acyrthosiphon pisum*; Bt, *Bemisia tabaci* (adults); Tv, *Trialeurodes vaporariorum* (adults); Lm, *Locusta migratoria migratoriodes* (adults); Sg, *Schistocerca gregaria* (adults).

[a]Buckner et al., 1999.
[b]Jones et al., 1997.
[c]Buckner et al., 1996.
[d]Nelson et al., 1998.
[e]Soliday et al., 1974.
[f]Brey et al., 1985.
[g]Buckner et al., 1994.
[h]Ohara and Lockey, 1990.
*Concentrations are given in percent of lipid total.

predominate and alcohols with an odd number of carbon atoms are present in smaller quantities. For example, cuticular lipids of *H. virescens* pupae contained only one odd-numbered alcohol ($C_{29:0}$) in trace amounts (Buckner et al., 1999). And in the cuticular lipids of *H. zea* pupae, only two alcohols are present: $C_{29:0}$ (2%) and $C_{31:0}$ in trace amounts (Buckner et al., 1999). A similar relative content of the alcohol $C_{29:0}$ has been found in the cuticular lipids of adult *M. sanguinipes* and *M. packardii* (5 and 2%, respectively) (Soliday et al., 1974). Moreover, extracts from *M. sanguinipes* have been shown to contain traces of two odd-numbered saturated alcohols ($C_{25:0}$ and $C_{27:0}$) and *M. packardii* has been shown to contain only one alcohol in traces ($C_{27:0}$) (Soliday et al., 1974). In the cuticular lipids of *A. pisum* (Brey et al., 1985) and *L. m. migratoriodes* and *S. gregaria* adults (Oraha and Lockey, 1990), odd-numbered alcohols shorter than $C_{20:0}$ were observed. The relative content of the alcohol $C_{13:0}$ was 11% in *A. pisum* (Brey et al., 1985). *Locusta migratoria migratoriodes* and *S. gregaria* adults contained three shorter odd-numbered alcohols: $C_{15:0}$ (6.3 and 18.8%, respectively), $C_{17:0}$ (5.0 and 4.4%, respectively) and $C_{19:0}$ (0.8 and 0.6%, respectively) (Ohara and Lockey, 1990).

In the present study, the alcohols $C_{12:0}$ to $C_{30:0}$ were identified. However, the alcohols $C_{31:0}$ to $C_{34:0}$ are also present in most insects. Alcohols with molecular weight >C30 occurred in cuticular lipids of *B. argentifolii* nymphs (Buckner et al., 1999), *H. virescens* and *H. zea* pupae (Buckner et al., 1996), *A. singularis* exuviae and adults (Nelson et al., 1998), *M. sanguinipes* and *M. packardii* adults (Soliday et al., 1974), *B. tabaci* adults, *T. vaporariorum* adult (Buckner et al., 1994), and *L. m. migratoriodes* and *S. gregaria* adults (Oraha and Lockey, 1990). In *B. argentifolii* nymphs, alcohols with a molecular weight >C30 are the major alcohols. The relative contents of $C_{32:0}$ and $C_{34:0}$ alcohols were 36.9 and 36.1%, respectively. In contrast, the cuticular lipids of *H. virescens* pupae and *M. sanguinipes* and *M. packardii* adults contained alcohols with molecular weight >C30 in trace amounts.

Alcohols from $C_{22:0}$ to $C_{34:0}$ are typically found in many insect species, but the presence of alcohols with molecular weight <C22 are seldom met. Alcohols with molecular weight <C22 are present in cuticular lipids of *L. m. migratoriodes* adults, *S. gregaria* adults (Oraha and Lockey, 1990) and *A. pisum* (Brey et al., 1985). Among alcohols with low molecular weight, the following were identified: $C_{8:0}$ to $C_{16:0}$ in *A. pisum* (Brey et al., 1985), $C_{12:0}$ to $C_{21:0}$ in *L. migratoria migratoriodes* and *S. gregaria* adults (Ohara and Lockey, 1990), and $C_{12:0}$ to $C_{20:0}$ in larvae, pupae, males and females of *M. domestica* (present study).

Most insects contain a small number of alcohols. For example, in the cuticular lipids of *Tenodera sinensis* (females), *T. angustipennis* (males) and *Stagmomantis carolina* (females), only one alcohol ($C_{30:0}$) was identified (Jones et al., 1997). Two alcohols were present in the cuticular lipids of *B. tabaci* adults ($C_{30:0}$ and $C_{32:0}$) and *T. vaporariorum* adults ($C_{30:0}$ and $C_{32:0}$) (Buckner et al., 1994). And the following insect species contained from four to nine alcohols: *B. argentifolii* nymphs, *H. virescens* pupae, *H. zea* pupae, *A. singularis* exuviae and adults, *M. sanguinipes* adults, *M. packardii* adults and *A. pisum* (Brey et al., 1985). However, greater numbers of alcohols were identified in the lipids of *L. m. migratoriodes* adults (19 alcohols) and *S. gregaria* adults (19 alcohols) (Ohara and Lockey, 1990). In our work, eight, 10, eight and nine alcohols were present in the cuticular lipids of larvae, pupae, males and females, respectively.

The antimicrobial activity of components of cuticular lipids isolated from insects has been previously reported. It was found that cuticular fatty amides may contribute to *Liposcelis bostrychophila*'s tolerance to entomopathogenic fungi (Lord and Howard, 2004). Caprylic acid, found on the surface of *Heliothis zea* and *Spodoptera frugiperda* larvae, possesses the ability to inhibit the germination of *B. bassiana*. Other free fatty acids present on the surface of *H. zea* and *S. frugiperda* also present mycostatic activity towards fungi (Smith and Grula, 1982). The current state of knowledge on diverse, species-specific effects of free fatty acids on the growth and virulence of insecticidal fungi is reviewed by Boguś et al. (Boguś et al., 2010).

Mixtures of alcohols present in cuticular lipids of *M. domestic*a turned out to be effective against tested microbial strains. The gram-positive bacteria, *R. equi*, was the most susceptible to cuticular lipids found in larvae. This extract contained a high amount of $C_{14:0}$, which was the most active individual alcohol against all tested gram-positive bacteria. The least active compound against *R. equi* was the extract isolated from males, where $C_{14:0}$ was present only in trace amounts. The compound 1-tetradecanol seems to play an important role in the effectiveness against gram-positive bacteria. However, the activity of alcohols in females was slightly stronger in comparison to that in pupal extract, despite the lower amount of $C_{14:0}$. Females' alcohols contained considerably higher amounts of alcohols $C_{18:0}$ to $C_{24:0}$, and perhaps some synergistic action of these weak antimicrobials is responsible for the stronger activity. According to the literature, linoleic and oleic fatty acids inhibit the growth of gram-positive bacteria and fungi, whereas gram-negative bacteria are resistant to these compounds (Dilika et al., 2000). Similarly, alcohols didn't show any activity against gram-negative bacteria. Antifungal activity was presented by all tested compounds and extracts. In most cases, the activity of mixtures was higher in comparison to that of individual alcohols, which suggests some synergy in action. A synergistic effect between oleic and linoleic acid was confirmed for *Micrococcus kristinae* and *Staphylococcus aureus* (Dilika et al., 2000). Our results suggest synergistic action between components of cuticular lipids against *C. lipolytica*. The MICs obtained for females, males and larvae alcohols were lower than any MIC for individual compounds. A different situation was observed for *C. albicans*: the activity of mixtures was equal or even slightly lower compared with alcohols. The activity against *C. tropicalis* was determined mainly by the content of $C_{12:0}$ and/or $C_{24:0}$, which were the most potent alcohols against this fungus. Correspondingly, the activity against *A. niger* of alcohols isolated from larvae was greater than that from males and pupae extract. Alcohols extracted from females presented activity comparable with larval mixtures. However, female extracts did not contain high amounts of either $C_{12:0}$ or $C_{24:0}$. In this case, $C_{18:0}$–$C_{22:0}$ seem to play crucial role in antimicrobial effectiveness as they were the most abundant alcohols.

Primary aliphatic alcohols from C_6 to C_{13} tested for their antifungal activity against *Saccharomyces cerevisiae* showed high fungicidal potential of undecanol and decanol (Kubo et al., 2003). The antifungal action of amphipathic medium-chain alcohols is mediated by biophysical processes and comes mainly from their ability as nonionic surfactants to disrupt the native membrane associated function of the integral proteins. This inhibition pattern in yeast is not specific to only alkanols, but also applies to alkenals and fatty acids (Kubo et al., 2003). However, it should be pointed out that none of the reference bacteria strains and medically important fungi we have tested here are pathogenic for *M. domestica*. A variety of microorganisms, including *S. aureus*, *Pseudomonas* sp., *Proteus* sp., *Escherichia* sp., *Klebsiella* sp., *A. niger* and *A. flavus*, which are highly pathogenic for humans, have been previously isolated from healthy house flies (Omalu et al., 2011;

Banjo et al., 2005; Nazni et al., 2005). It seems that antimicrobial properties of alcohols and other cuticular compounds, together with the innate immunity mechanisms, efficiently protect *M. domestica* from the infection by common pathogens inhabiting similar habitats as those of the house fly. In contrast, the same compounds fail to protect adult flies against the entomopathogenic fungus *C. coronatus* 'equipped' with effective weaponry composed of proteolytic, chitinolytic and lipolytic enzymes that degrade the insect cuticle, as well as toxic metabolites that disable the victim's immune system (Wieloch et al., 2011). Similarly, Kontoyiannis and Lewis (Kontoyiannis and Lewis, 2010) reported high mortality of fruit flies infected with other Zygomycetes representatives – *Rhizopus*, *Mucor* and *Cunninghamella* species – contrasting with the resistance of *Drosophila* to *Aspergillus* and *Candida* species. In studying *Drosophila* pathogenesis caused by various fungal species, the authors learned that an intact Toll signaling pathway, the crucial element of innate immunity, is not sufficient to protect fruit flies against Zygomycetes. This finding contrasts with *Aspergillus* and *Candida* infections and shows complexity of insect victim–fungal pathogen relationships.

More experiments are needed to decipher the reason for the high susceptibility of adult flies *versus* the total resistance of larvae and pupae to the *C. coronatus* infection. Comparing developmental fluctuations in the concentration of cuticular alcohols, the contribution of these compounds seems doubtful. Assuming that adult flies are licking all substrates, it seems probable that during 18 h of exposition to fungal colonies, insects ingest fungal spores, which quickly germinate inside the gut. Growing hyphae release toxic metabolites (Samborski, 2000; Boguś and Scheller, 2002; Wieloch et al., 2011), which may promptly kill the flies. Whether larvae burrowing in the fungal colony during the exposure ingest it as well remains unknown. In contrast, fungus can invade pupae only *via* the cuticle.

The role of cuticular fatty acids in the resistance of insects to fungal infection has been widely discussed. According to our results, the composition of cuticular alcohols seems to be no less important. It would be worthwhile to continue the research and focus on the synergistic action between components of cuticular lipids against various microbial strains including multidrug-resistant bacteria.

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