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# Free fatty acids in the cuticular and internal lipids of *Calliphora vomitoria* and their antimicrobial activity

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ABSTRACT

The cuticular and internal lipid composition in *Calliphora vomitoria* larvae, pupae, and male and female adults was studied. The free fatty acid (FA) compositions of the lipids were chemically characterized using gas chromatography (GC) and gas chromatography-electron impact mass spectrometry (GC–MS).

Analyses of cuticular extracts from larvae, pupae, and male and female adults revealed that the carbon numbers of the acids ranged from $C_{7:0}$ to $C_{22:0}$, from $C_{8:0}$ to $C_{24:0}$, from $C_{7:0}$ to $C_{24:0}$ and from $C_{7:0}$ to $C_{22:0}$ respectively. The internal lipids of *C. vomitoria* larvae, pupae, male and female adults contained FAs ranging from $C_{8:0}$ to $C_{20:0}$, from $C_{9:0}$ to $C_{22:0}$, from $C_{8:0}$ to $C_{24:0}$ and from $C_{9:0}$ to $C_{22:0}$ respectively.

Nine FAs with odd-numbered carbon chains from $C_{7:0}$ to $C_{21:0}$ were identified in the cuticular lipids of the larvae. The internal lipids of *C. vomitoria* larvae contained 8 odd-numbered FAs ranging from $C_{9:0}$ to $C_{19:0}$. Eight odd-numbered FAs from $C_{9:0}$ to $C_{21:0}$ were identified in the cuticular and internal lipids of pupae, while nine such FAs were found in the cuticular lipids of male and female adults. The internal lipids of adult males and females respectively contained nine and seven odd-numbered FAs, while both larvae and pupae contained eight such compounds.

Eight unsaturated FAs were identified in the cuticular lipids of larvae, adult males and females and also in the internal lipids of females. Seven unsaturated FAs were identified in the cuticular lipids of pupae. The internal lipids of larvae, pupae and males contained 10, 11 and 12 unsaturated FAs respectively.

Developmental changes were found both in the amounts of extracted cuticular and internal FAs and in their profiles. Four cuticular FAs ($C_{7:0}$, $C_{9:0}$, $C_{10:0}$ and $C_{15:1}$), identified as being male-specific, were either absent in the female cuticle or present there only in trace amounts.

Cuticular and internal extracts obtained from larvae, pupae, adult males and females were tested for their potential antimicrobial activity. The minimal inhibitory concentrations of extracts against reference strains of bacteria and fungi were determined. Antimicrobial activity was the strongest against Gram-positive bacteria; Gram-negative bacteria, on the other hand, turned out to be resistant to all the lipids tested. Overall, the activities of the internal lipids were stronger. All the lipid extracts were equally effective against all the fungal strains examined. In contrast, crude extracts containing both cuticular and internal lipids displayed no antifungal activity against the entomopathogenic fungus *Conidiobolus coronatus*, which efficiently killed adult flies, but not larvae or pupae.



## 1. Introduction

Cuticular lipids of insects contain mainly hydrocarbons, wax esters, free fatty acids (FAs), alcohols, aldehydes, ketones and acylglycerols; the presence of triacylglycerols in the cuticle reported by Lockey (1988) was probably due to contamination by internal lipids. For extraction, two short immersions are preferable to one long immersion (Jackson and Blomquist, 1976) and are more effective for dissolving lipids from the cuticle. Accurate methods for determining the composition of insect cuticular and internal lipids are of considerable value and provide a better understanding of many aspects of insect physiology. Cuticular lipid compositions are usually studied using chromatographic and spectroscopic methods (Christie, 2003). The former techniques, based on retention parameters, include thin layer chromatography (TLC), gas chromatography (GC) and column chromatography (CC), while among the latter there are GC/MS in selected ion monitoring (SIM) and total ion current (TIC) modes (Gołębiowski et al.,

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2012b; Gołębiowski, 2012), as well as MALDI-TOF-MS and LC/MS. Compounds amenable to gas chromatography (GC) are analysed directly or after derivatization (mostly silylation), whereas the less volatile compounds have to be hydrolysed before analysis (Christie, 1989). Neutral lipids, such as wax esters or acylglycerols, can be analysed using electrospray, atmospheric pressure chemical ionization (APCI) or photoionization (APPI) mass spectrometry (Byrdwell, 2005). MALDI imaging has been used to identify neutral, highly hydrophobic compounds like hydrocarbons or wax esters (Vrkoslav et al., 2010). Volatile compounds, such as pheromones, can be qualitatively and quantitatively analysed by GC or GC/MS with SPME fibres (De Pasquale et al., 2007). Solid injection has been used to analyse these compounds in whole, small insects (Turillazzi et al., 2002) or in solid samples, such as body parts or glands.

The bluebottle fly *Calliphora vomitoria* is a common blow-fly found in most areas of the world. It is generally considered to have a rural distribution, where it prefers shady locations. Females of *C. vomitoria* lay their eggs where they feed, usually on rotting meat or faeces. On hatching, the larvae (maggots) immediately start feeding on the decomposing matter. When fully grown, they crawl away to a dry place to pupate. After 1 week to several months, depending on the season and geographic location, the adult insects eclose and the life cycle begins again (Davies and Ratcliffe, 1994). The large numbers of *C. vomitoria* maggots in cadavers give an indication of the species' ecological significance in the decomposition of human and animal remains; they are also useful in forensic cases, for example, for the sophisticated detection of morphine accumulation and metabolism (Grassberger and Frank, 2004; Bourel et al., 2001). In addition, these maggots are also used in the treatment of gangrene and wounds (maggot therapy), though less frequently than the larvae of *Lucilia sericata* or the closely related *Calliphora vicina*. Finally, there are abundant data on the passive transfer of animal and plant pathogens by *C. vomitoria* (Graham-Smith, 1913; van der Wolf et al., 2006; El Sherbini, 2010).

Depending on the developmental stage of the insect, the lipid composition of the cuticle changes and may perform completely different functions. Calliphorid larvae are focused primarily on nutrition. Their cuticle is not as rigid as in the puparium and imagines. Up to 90% of the larval cuticle is decomposed by enzymatic action during moulting. During pupation, the larval cuticle is transformed into the sclerotized puparium (Wolfe, 1954).

The main function of insect cuticular lipids is to restrict water transpiration from the insect (Gibbs, 1998; Hadley, 1994; Jackson and Baker 1970). Other such functions ensure chemical communication between species and between the instars of a single species (Howard, 1993). Unsaturated FAs and FA methyl esters can affect the settling behaviour of *Liposcelis bostrychophila* (Green, 2009) and have been found repellent to ants and *L. bostrychophila* (Howard et al., 1982; Dani et al., 1996; Green, 2011).

The antimicrobial activity of insect cuticular lipids has been described by a number of authors (Kerwin, 1982; Gołębiowski et al., 2008a, 2012c; Urbanek et al., 2012). The susceptibility or resistance of various insect species to fungal invasion may be due to a number of factors, including cuticular lipid composition. Free FAs in particular are responsible for resistance to fungal infection (Gołębiowski et al., 2008a). Cuticular FAs are toxic and fungistatic, but may also be stimulatory. For example, palmitoleic acid enhances mycelial growth but is toxic to the 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*, a species closely related to *C. vomitoria*, are very resistant to the cosmopolitan soil fungus *Conidiobolus coronatus* (Gołębiowski et al., 2008a), known to be a potent entomopathogen (Domsch et al., 2007; Boguś and Scheller, 2002). Histological examination of *C. vicina* larvae exposed to sporulating *C. coronatus* colonies showed 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 FA profile of *C. vicina* larvae differs significantly from that of *Dendrolimus pini* and *Galleria mellonella* (both these moth species are extremely 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*, whereas these three FAs are not present at all in the cuticle of *D. pini* or only in trace amounts in that of *G. mellonella* (Gołębiowski et al., 2008a). *In vitro* cultivation of *C. coronatus* in the presence of these three FAs reduced sporulation, hyphae biomass, the ability to infect *G. mellonella* larvae and the toxicity of metabolites released by the fungus into the culture medium (Boguś et al., 2010). This demonstrated that these FAs contribute to the resistance of *C. vicina* larvae to fungal attack.

The aim of the present work was to find out whether changes in the cuticular and internal FA profiles of *C. vomitoria* larvae, pupae and imagines are correlated with the sensitivity of *C. vomitoria* in its various developmental stages to *C. coronatus* infection. The antimicrobial potential of lipids extracted from *C. vomitoria* was tested using *C. coronatus* and three reference strains of Gram-positive bacteria, 3 reference strains of Gram-negative bacteria and four fungi pathogenic to humans.

## 2. Materials and methods

### 2.1. Insects

*C. vomitoria* maggots 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. Approximately 7 days elapsed between hatching and puparium formation and another 7 days for the adults to appear. For the experiments, post-feeding third instar larvae, freshly formed pupae and 6-day-old sexually mature adults were used. The insects in their various developmental stages were either used for lipid extraction or exposed for 18 h to fully grown, sporulating colonies of the entomopathogenic fungus *C. coronatus*. Ten flies (adult males and females separately), or 10 pupae, or 10 larvae were kept in one Petri dish containing a *C. coronatus* colony. Insects exposed for 18 h to sterile uninoculated Sabouraud agar medium served as controls. After exposure, the adults were transferred to clean Petri dishes with sugar and water, while the larvae and pupae were transferred to dishes containing water only. All the insects exposed to *C. coronatus* were maintained under appropriate growing conditions for 10 days, and their state was monitored daily. Exposure of the insects to the *C. coronatus* colony for 18 h was found to be the most efficient method resembling the natural infection process (Wieloch and Boguś, 2005). In order to avoid pseudoreplication, all fungi vs. insect assays were carried out using flies from different stocks incubated in three different chambers.

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

### 2.2. Microorganisms

*C. coronatus* (Entomophthorales), isolate number 3491, originally isolated from *Dendrolaelaps* spp., was obtained from the collection of Bałazy (Polish Academy of Sciences, Agricultural and Forest Environment Research Centre, Poznań). It was routinely



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