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Differences in cuticular lipid composition of the antennae of *Helicoverpa zea*, *Heliothis virescens*, and *Manduca sexta*

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ABSTRACT

Analyses of the hexane washes of antennae, forelegs and whole bodies of *Helicoverpa zea*, *Heliothis virescens*, and *Manduca sexta* revealed notable differences in the components of the cuticular coatings of each species. Most striking were the differences between the cuticular coatings of male and female antennae of both *H. zea* and *H. virescens*. Novel esters of short-chain acids (C2–C4) and long-chain secondary alcohols (C25–C32) were identified in the hexane washes of the male antenna and forelegs of *H. zea* and *H. virescens*. These compounds were found in only small amounts or were completely absent on the female antennae of both species. In *H. zea*, butyrates of 7- and 8-pentacosanol and 8- and 9-heptacosanol were found, whereas, in the foreleg extracts of *H. virescens*, acetates and propionates were detected in addition to butyrates. While cholesterol is a major component of antennal washes (10–15%), only traces were found in the foreleg extracts. Although the composition of the cuticular coating of *M. sexta* differed greatly from that of the other two species, the extractable coatings of the antennae of male and female *M. sexta* were nearly identical.

1. Introduction

The major components of the cuticular lipid coating of adult insects are often hydrocarbons, among which methyl-branched alkanes are the most abundant (Lockey, 1988). Nevertheless, a wide range of more polar lipids have been identified as constituents of the cuticle, including fatty acids, alcohols, aldehydes, ketones, wax esters, and esters of primary and secondary alcohols (references cited in Buckner, 1993).

Little is known about the distribution and the function of various compound groups on the surface of adult insects. Two characteristics of the free cuticular lipids have been investigated thoroughly in insect species: the melting temperature and the critical temperature at which the cuticle becomes more permeable to water (Noble-Nesbitt, 1991). While in recent years substantial efforts have been made to prove that the correlation between these two physical parameters is true over a wide range of insect species (Gibbs, 2002), the transport of organic molecules (pheromones, plant volatiles, pesticides) through the cuticle has gained less attention (Theisen et al., 1991; Steinbrecht, 1992). Moreover, it has become apparent that lipid classes may not mix when they form the outer epicuticular layer (Gibbs, 2002) resulting in separate phases with different chemical and physical properties.

Since the role of the epicuticular lipids on the olfactory sensilla has never been studied, an investigation to analyze the surface lipids of the antenna of selected moth species was conducted. Two heliothine species, *Helicoverpa zea* (Boddie) and *Heliothis virescens* (Fabricius), and the hawkmoth, *Manduca sexta* (Linnaeus) were chosen due to the extent to which their pheromone systems (Klun et al., 1980a, 1980b; Teal and Tumlinson, 1986; Tumlinson et al., 1989; Baker et al., 2004) and olfactory mechanisms (Matsumoto and Hildebrand, 1981; Vickers et al., 1991; Mustaparta, 1996; Vickers et al., 2005) have been studied. The pheromone systems of these moths are similar in that the male antenna holds thousands of trichoid sensilla that contain neurons finely tuned to the components of the female produced pheromone blend. Further, unsaturated 16-carbon aldehydes play a major role in the pheromone of each species. This report describes the cuticular coatings of the antennae and the forelegs of *H. zea*, *H. virescens*, and *M. sexta* and includes the identification of esters of short-chain

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acids and long-chain secondary alcohols that contribute to sexual dimorphism in the surface chemistry of the *Heliothis* antennae. The differences between the cuticular lipid profile of the heliothine and *M. sexta* antennae, and differences between the antennae and the forelegs are also described.

2. Methods

2.1. Rearing

H. zea and *H. virescens* larvae from our lab colony (eggs came originally from the Department of Entomology Insectary at The North Carolina State University) were fed on Lepidopteran diet (Southland Products Inc.). The pupae were sexed and kept separately in 20 × 20 aluminum mesh cages at 16:8 L:D cycle (7:00 a.m.–11:00 p.m. day) at 25 °C until emergence. *Manduca sexta* pupae were obtained from the Department of Entomology Insectary at The North Carolina State University. Male and female pupae were kept in separate mesh cages (same as above) at 25 °C in dark before emergence.

Freshly emerged insects were fed with an 8% sugar solution. Adults were taken out of the cage within a day after emergence and kept at 8–10 °C in glass beakers containing an aluminum mesh lining. Adult moths were 2–5 days old when used for the experiments.

2.2. Dipping experiments

The antennae and forelegs of live insects were cut using stainless steel scissors and placed in hexane (Omnisolv, EMD) in a 1 mL micro-reaction vessel with a cone-shaped interior (Supelco), with the cut end of the respective body part oriented above the meniscus level to avoid extraction of internal compounds. Ten antennae or 2 forelegs of *H. zea*, and 12 antennae or 2 forelegs of *H. virescens* were rinsed in this manner with three aliquots of 50 μL of hexane for 2 min for each aliquot. The three extracts were combined and the solvent was evaporated under a gentle stream of nitrogen at 40 °C. The residue was redissolved in 20 μL of an internal standard solution containing tridecane (10 ng/μL) and 16-methylhexatriacontane (50 ng/μL). In case of *M. sexta* two antennae or forelegs were rinsed with three aliquots of 150 μL of hexane for 2 min for each aliquot. The three extracts were combined and treated as described above. The residue was redissolved using 20 μL of an internal standard solution containing tridecane (10 ng/μL) and octatriacontane (50 ng/μL).

Whole bodies were also extracted. For the heliothine species three aliquots of 500 μL of hexane were used, whereas for *M. sexta* the volume was three times 2 mL. Extraction time was the same as for the body parts. The fractions were combined and 100 μL of the extract was evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was redissolved as described above.

2.3. Chemical analysis

Samples were analyzed for quantification purposes using an Agilent 6890 GC-FID system equipped with an Equity-5 column (30 m × 0.2 mm × 0.2 μm; Supelco, Bellefonte, PA). Selected samples were analyzed on an identical column in an Agilent 6890N GC coupled with a 5973N MSD system in EI mode (+70 eV) to identify components. For all the analyses the oven temperature program was 50 °C (1 min) −20 °C/min −210 °C −3 °C/min −320 °C (25 min). The temperature of the injector was held at 280 °C, the FID in the GC and the transfer line in the GC–MS were kept at 300 °C. Samples were injected splitless (0.75 min) and run at an average linear flow velocity of 25 cm/s.

Identification of the compounds was based primarily on their MS spectra (NIST05, Masslib) and their Kovats indices (Van Den Dool and Kratz, 1963; Kovats, 1965) when analyzed on the Equity-5 column. Position of the methyl branching of mono-, di-, and trimethylalkanes was determined using characteristic even- and odd-mass fragments of their mass spectra (Nelson, 2001) as well as calculating retention indices (Carlson et al., 1998). A few samples were analyzed in positive CI mode using isobutane to determine the molecular mass of unknown compounds. High-resolution MS measurements were performed at the Proteomics and Mass Spectrometry Core Facility at The Huck Institutes of the Life Sciences of The Pennsylvania State University.

Quantification of compounds was based on their peak area values obtained from our data acquisition and analysis software (Chemstation, Agilent). The output values were corrected with the relative response factors to calculate percentage composition of the identified compounds. Since standards were not available for every compound, response factors were estimated. The response factor of *n*-alkanes was calculated based on the linear relationship found between their chain-length and their response factor. For methyl-branched alkanes the relative response factor of the main chain was used. Response factors of oxygenated compounds were estimated using a purchased or synthesized standard for each compound group. The FID response was linear in the concentration range of the compounds we analyzed.

3. Results

3.1. General

Using the methods described above we were able to detect and quantify lipids in the retention index range of 2100–4200 (Tables 1,2, and 3 in supplemental information). The sum of peak area of the identified peaks was approximately 95% of the total area. Major components in all three species were mono- and dimethyl alkanes with an odd-numbered chain, whereas *n*-alkanes, trimethyl alkanes, and oxygenated lipids were less abundant (Figs. 1–3). The only exception to this was the hexane extract of the *H. virescens* foreleg in which oxygenated lipids were the major components (Fig. 2b). *H. zea* and *H. virescens* were fairly similar with regard to the overall composition of cuticular lipids as well as the heterogeneity of body parts. Whereas the antennal lipid profiles of the heliothine species were significantly different from those of the forelegs and whole body washes (Figs. 1 and 2), the cuticular lipid coatings of *M. sexta* body parts examined in this study were nearly identical to each other (Fig. 3). Furthermore, sexual dimorphism was observed in the antennal lipids of the heliothine species (Figs. 4 and 5), but not in those from *M. sexta*.

3.2. Identification of secondary alcohol esters of short-chain acids

A series of isomeric compounds was found to be significantly more abundant on the male antennae of *H. zea* and *H. virescens* compared to the female antennae of these species (Figs. 4 and 5). These isomeric compounds exhibited a base peak at *m*/*z* 71 paired with a peak at *m*/*z* 89 in their MS spectra. The higher molecular-weight region of the spectrum contained an even-mass fragment in low abundance as well as characteristic odd-mass fragments. No molecular ion was observed either in either the EI or the CI mode.

The HR-MS analysis of the peak at *m*/*z* 71 revealed the presence of the alkyl ($C_5H_{11}^+$; measured: 71.0862 amu, calculated: 71.0861 amu) and the acyl fragment ($C_4H_7O^+$; measured: 71.0495 amu, calculated: 71.0497 amu), which led us to propose a butyrate ester structure. This was supported by the *m*/*z* 89 fragment being the protonated butyric acid elimination ion