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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

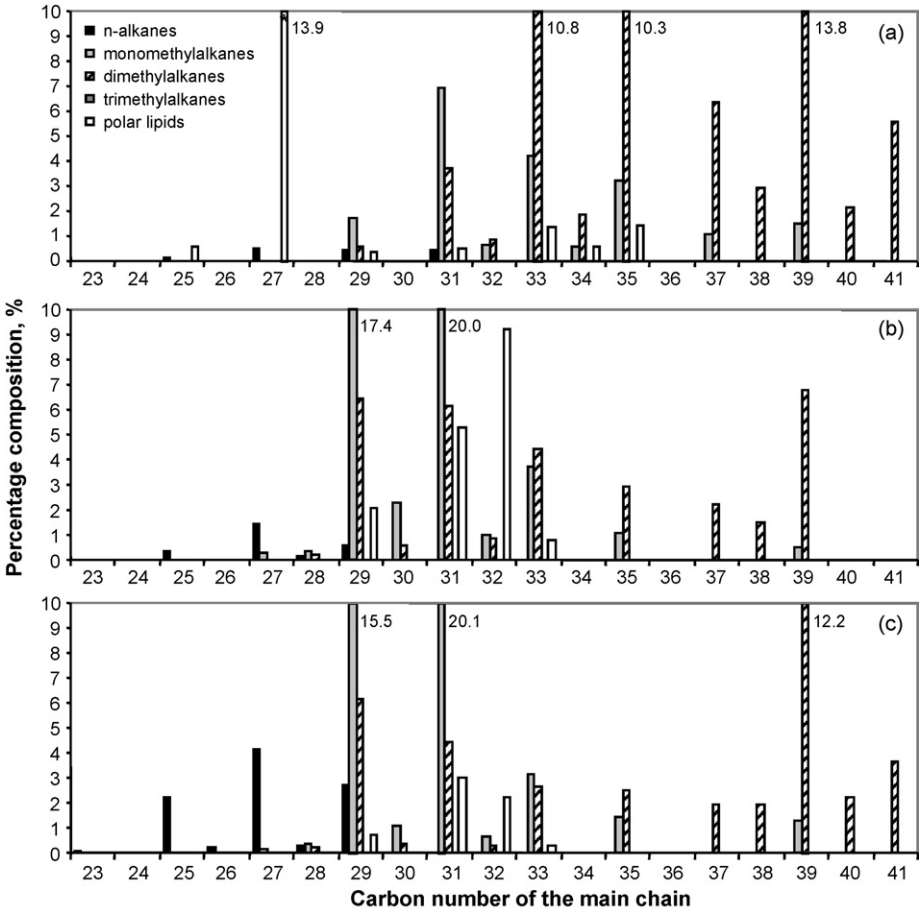

Fig. 1. Percentage composition of different lipid groups in the hexane washes of the antennae (a), forelegs (b), and whole body (c), of *H. zea*. Unidentified compounds (approximately 5% of the total area) are not included. Bars that are out of range are labeled with the percentage composition values. The bar for C27 (marked with an asterisk) on graph (a) represents cholesterol (13.5%), and 8- and 9-heptacosanone (0.4%).

($C_4H_9O_2^+$; measured: 89.0604 amu, calculated: 89.0603 amu). The high molecular-weight, even-mass fragment was shown to be an alkenyl fragment ($C_{25}H_{50}^+$; measured: 350.3919 amu, calculated: 350.3913 amu). The low abundance of the *m*/*z* 89 peak indicated a secondary alcohol ester. The high molecular-weight odd-mass fragments were easily explained by the loss of the possible alkyl fragments at the branching point of the secondary alcohol by α-cleavage. Thus a general structure of an ester of long-chain secondary alcohols (C25–C32) and short-chain acids (C2–C4) was proposed for the unknown compounds. One of these esters, 8-heptacosanyl butyrate, was synthesized (Scheme 1 in supplemental information) and the structure of the natural product was confirmed by the retention index on the Equity-5 column (*RI* 3105) and the EI mass spectrum (Fig. 1 in supplemental information).

Besides butyrates, we detected acetates on the foreleg of the male *H. zea* and acetates and propionates on the *H. virescens* foreleg (of both sexes). The chain-length of the alcohol varied between 25 and 32, and chains with an odd number of carbons were more abundant. The hydroxyl group was located at either carbon 6 or 11. Identification of a few of these esters was not possible due to their very low abundance.

3.3. Composition of the hexane extractable antennal lipids of the three moth species

The male antennal surfaces of *H. zea* and *H. virescens* were covered by a rather wide range (C29–C41) of mono- and dimethyl alkanes (Figs. 1a and 2b). The relative amounts of the monomethyl alkanes decreased with increasing chain-length, whereas dimethyl alkanes dominated in the higher molecular weight region (C35–C41). Oxygenated lipids, i.e. cholesterol and esters of secondary alcohols, were fairly abundant (20% for males, and 10% for females). In contrast, the major hydrocarbons on *M. sexta* antennae were dimethyl alkanes in the range of C33–C41, with by far the most abundant chain-length being C37 (Fig. 3a). The oxygenated lipids of *M. sexta* antennae were wax esters and their percentage composition was very low (1%). The relative amounts of *n*-alkanes in the antennal extracts of each investigated species were lower than in the corresponding whole-body extracts. The calculated amounts of cuticular lipids on the male antennae of *H. zea* and *H. virescens* were approximately 360 and 300 ng of lipids per antenna, respectively. In comparison, the male *M. sexta* antennae were found to be covered by approximately 7600 ng of lipids per antenna.

Sexual dimorphism was discovered in the chemistry of the antennae of *H. zea* and *H. virescens*. Although the hydrocarbon profiles of the different sexes of each species were very similar, the antennae of the males contained the secondary-alcohol esters in significantly higher concentrations than those of the females. The butyrates that were typical for the antennae of *H. zea* males, 7- and 8-pentacosanyl, and 8- and 9-heptacosanyl butyrate, were not detected on the female antennae. Interestingly, the corresponding ketones, 7- and 8-pentacosanone, and 9- and 8-heptacosanone, were found on the antenna of both sexes of *H. zea* (Table 1 in supplemental information). The ketones were not detectable on the *H. virescens* antennae. In general, the esters were more

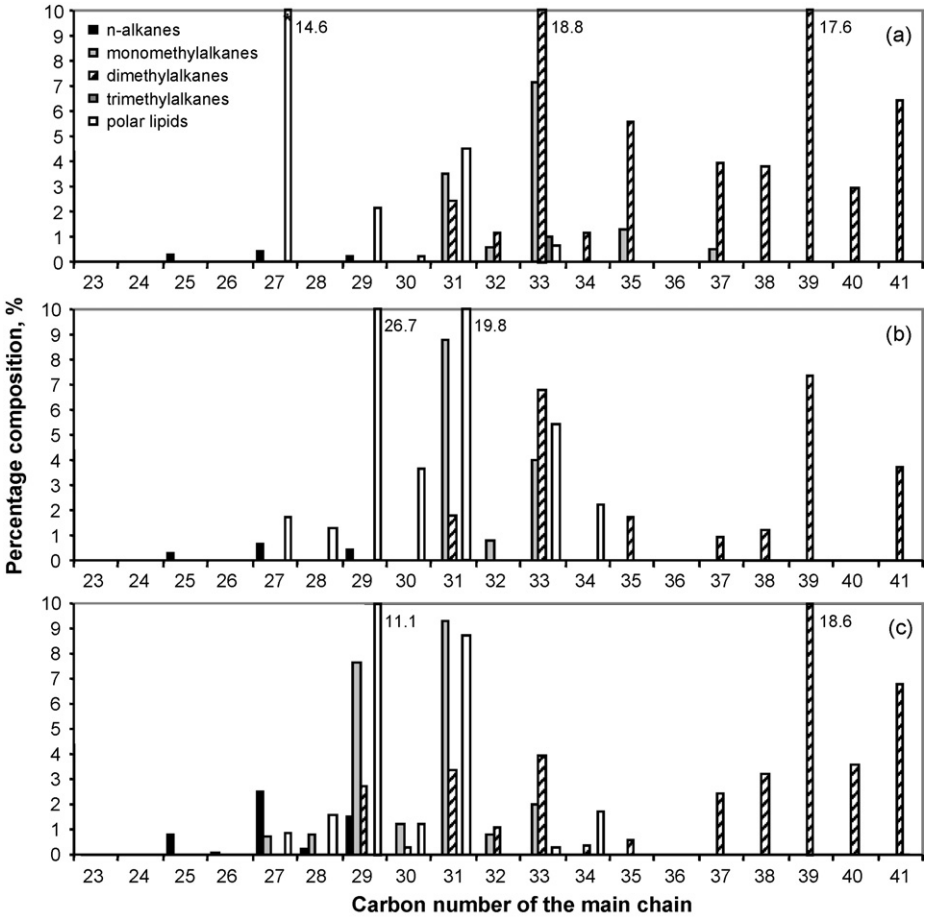

Fig. 2. . Percentage composition of different lipid groups in the hexane washes of the antennae (a), forelegs (b), and whole body, c), of *H. virescens*. Unidentified compounds (approximately 5% of the total area) are not included. Bars that are out of range are labeled with the percentage composition values. The bar for C27 (marked with an asterisk) on graph a) represents cholesterol.

abundant on the antennae of *H. virescens* than those of *H. zea*. A few of the butyrates were detected in the female antennal extracts, but they were never as abundant as in the male samples (Table 2 in supplemental information). Comparable differences between sexes in the antennal lipids of *M. sexta* were not found (Table 3 in supplemental information).

The cuticular lipid coatings of the antennae of the two heliothine species were not the same (Figs. 1a and 2a). The monomethyl alkanes on the *H. zea* antenna ranged from C29 to C39 with C31 being the most abundant chain-length. On the *H. virescens* antennae however, C29 and the higher molecular-weight monomethyl alkanes occurred only in traces, and the major monomethyl alkanes had a chain-length of C33. The distribution of the dimethyl alkanes in the antennal cuticle of *H. zea* was relatively even with C33, C35, and C39 being the more abundant chain-lengths. The antennae of male *H. virescens*, on the other hand, contained C33 and C39 dimethyl alkanes in significantly higher concentrations than other dimethyl alkanes. The branching pattern of dimethyl alkanes was slightly different in the two species (Tables 1 and 2 in supplemental information). Interestingly, we found two partially resolved GC peaks with identical MS spectra in the *H. zea* samples. Although not very likely, this indicates the existence of two possible diastereomers of 15,19-dimethyl pentatriacontane. Furthermore, trimethyl alkanes were detected in the peaks of monomethyl untriacontane, tritriacontane and pentatriacontane in the *H. zea* extracts. Although these trimethyl alkanes are likely to be the same as found by Nelson (2001), we have yet to confirm their identity. The trimethyl alkanes identified on the antennae of *H. virescens* were different, 7,17,21- and 5,17,21-trimethyl tritriacontane. The percentage composition of oxygenated lipids of the male antenna of the two heliothine species was similar, 13–15% for cholesterol and 6–7% for the secondary alcohol esters.

The antennae of the heliothine species exhibited a different lipid profile from that of the forelegs (Figs. 1 and 2). Whereas C33, C35, and C39 dimethyl alkanes were the main hydrocarbons on the *H. zea* antennae, C29 and C31 monomethyl alkanes dominated on the forelegs. The shift towards the lower molecular-weight monomethyl alkanes on the *H. virescens* forelegs was also apparent. The secondary alcohol esters were more abundant on the forelegs, more so in the case of *H. virescens* which exhibited a higher diversity of these compounds. Due to their low abundance, we were not able to identify the higher molecular-weight butyrates specific to the antennae of *H. zea* males. Ketones were not detected on the forelegs. In our samples, cholesterol was one of the major components of the antennal lipids of both species, but was detected only in traces on the forelegs. Hexane extracts of the forelegs and antennae of *M. sexta* were almost indistinguishable (Fig. 3). However, lower molecular-weight alkenes were found in traces on the forelegs but not on the antennae.

4. Discussion

The novelty of our results lies in the discovery of the esters of very short-chain acids (C2–C4) and long-chain secondary alcohols

(C25–C32) on the cuticle of the antennae and forelegs of *H. zea* and *H. virescens*. Oxygenated lipids in general are more abundant on the cuticle of larvae and pupae of these species (Buckner, 1993). Moreover, only a few papers have reported the existence of secondary alcohol esters on the cuticle of adult insects (Blomquist et al., 1972; Uebel et al., 1977; Pomonis et al., 1993; Finidori-Logli et al., 1996; Howard and Baker, 2003). Blomquist and Jackson (1973a) showed that both alkanes and secondary alcohols are incorporated into the cuticular esters of the grasshopper *Melanoplus sanguipines*, proving the biosynthetic origin of secondary alcohols and *de novo* esterification. Blomquist and Jackson (1973b) also demonstrated that secondary alcohols are not taken up from the diet by *M. sanguipines*. Also, Espelie and Bernays (1989) reported that enhanced quantities of secondary alcohols are bound on the cuticle of *M. sexta* larvae when fed on its host plants, tomato and potato, compared to artificial diet, though these authors did not see a correlation between plant lipids and the oxygenated cuticular compounds of the insect. *H. virescens* pupae have been shown to couple dietary acids with alcohols (Buckner, 1993). Furthermore, the cuticle of *M. sexta* pupae contains esters of oxoalcohols (Buckner et al., 1984) and acetate esters of long-chain and very long-chain methyl-branched alcohols (Nelson et al., 1990). We find it very interesting that the ketones that are corresponding oxidation products of the secondary alcohols forming the major butyrates on the male antenna of *H. zea* were detected in the antennal washes of both sexes. It is not clear whether an enzyme responsible for the esterification of secondary alcohols is located in the epidermal cells of the antenna and is not active in the case of the females or that the ester is selectively transported by lipophorin to the male antenna from the site of synthesis (Schal et al., 1998). Also, our *H. virescens* cuticular washes contained a larger amount and variety of esters than *H. zea*, although the insect was reared on exactly the same diet. This suggests that the possible enzyme could have different substrate specificities in the two species.

Cholesterol is one of the components of the free lipid layer of the insect cuticle originating from their food source (Lockey, 1988). When *M. sexta* larvae were fed on artificial diet, they contained cholesterol in 20% on their cuticular surfaces, whereas the proportions of this compound were very low after feeding on plant leaves (Espelie and Bernays, 1989). Our results show that the relative abundance of cholesterol was much higher in the hexane washes of the antennae than that of the forelegs or the whole body of the investigated heliothine species. When analyzing three consecutive hexane washes of the antennae of male *H. zea*, the last wash contained only traces of lipids, but a further wash with dichloromethane extracted more cholesterol and a small amount of triacyl glycerides, perhaps indicating an extraction of internal compounds (Lockey, 1988). Steinbrecht (1992) and Keil (1984) demonstrated that the inner surface of the pore tubules contains lipophilic as well as negatively charged (possibly proteinaceous) compounds. It is not unlikely that a portion of the cholesterol detected in our antennal washes could have come from inner surfaces, such as from those of the pore tubules.

Only a few papers have highlighted differences in cuticular lipids of body parts and have speculated about the relationship

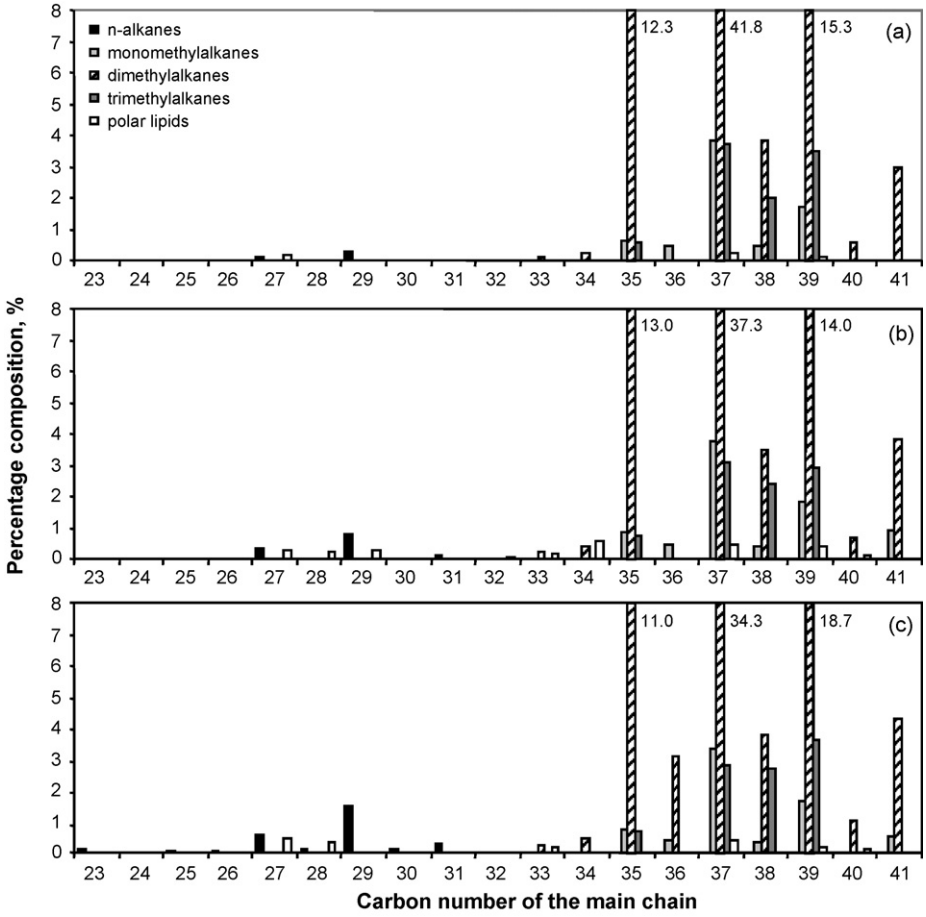

Fig. 3. Percentage composition of different lipid groups in the hexane washes of the antennae (a), forelegs (b), and whole body (c), of *M. sexta*. Unidentified compounds (approximately 5% of the total area) are not included. Bars that are out of range are labeled with the percentage composition values.

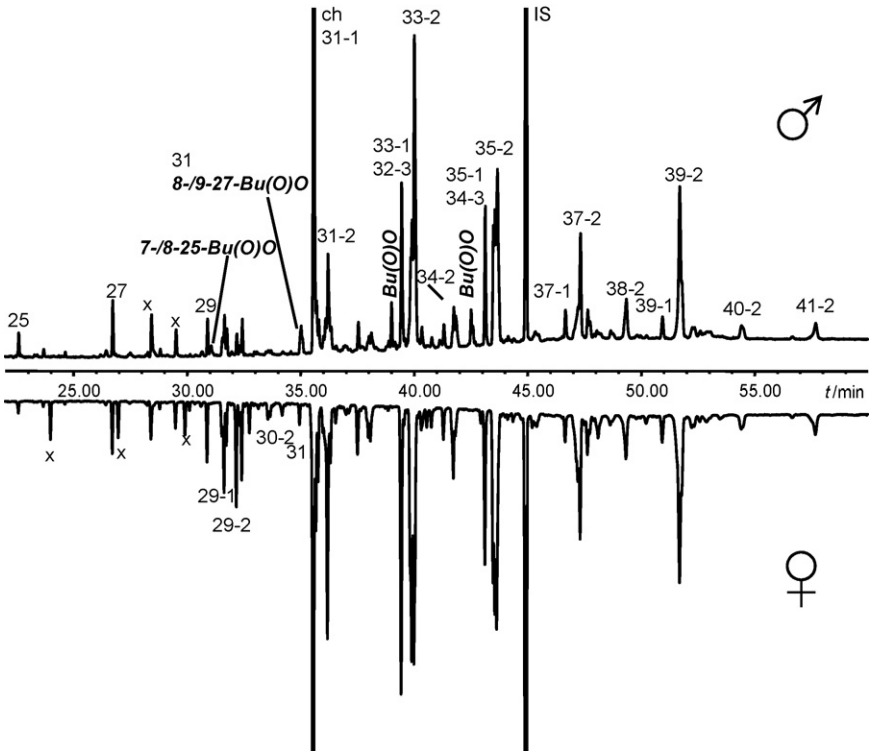

Fig. 4. Hexane washes of male (top) and female (bottom) *H. zea* antennae. Compounds in bold italics are male specific. Typical peak labeling of compounds in this figure and also in Fig. 5 is the following: x: impurity, 27: *n*-heptacosane, 31-1: unresolved monomethyl untriacontanes, 35-2: unresolved dimethyl pentatriacontanes, 8-/9-27-Bu(O)O: 8- and 9-heptacosanyl butyrates (unresolved), Bu(O)O: butyrate (not fully identified), ch: cholesterol, IS: 16-methyl hexatriacontane.

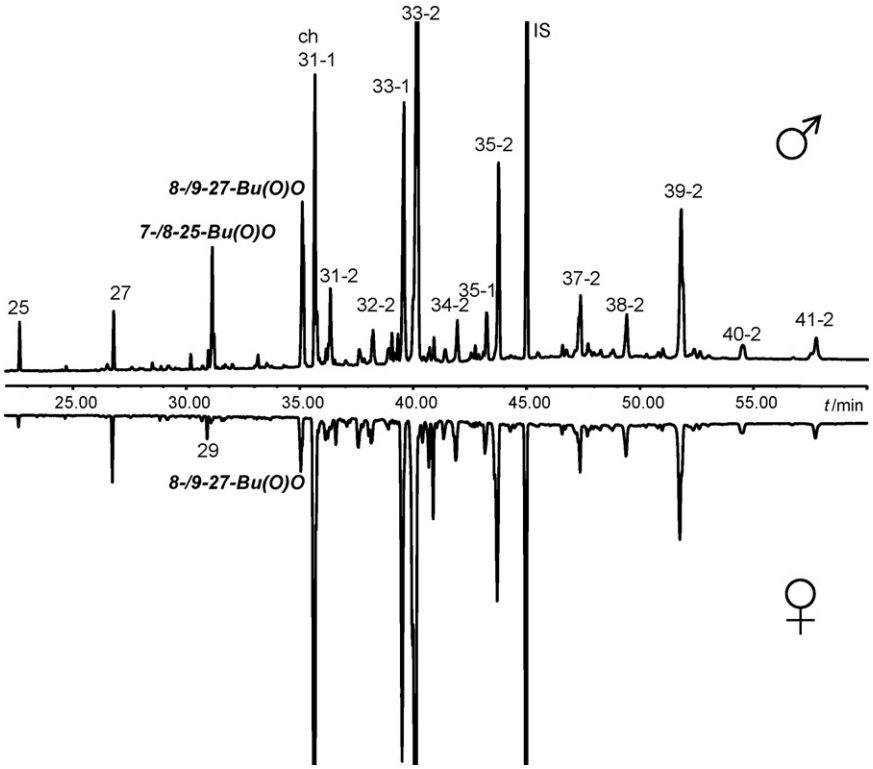

Fig. 5. Hexane washes of male (top) and female (bottom) *H. virescens* antennae. Compounds in bold italics are significantly more abundant on the male antenna than the female antenna. For peak labeling see Fig. 4.

between the function of the body part and the properties of its lipid coating (see references cited in Howard, 1993; Young et al., 2000; Arsene et al., 2002). We chose to use forelegs as a reference in our dipping experiments since they are the closest in shape and relative surface area to the antennae. Also, both legs and antennae are partially covered with scales. The shift toward higher molecular-weight alkanes and the high abundance of cholesterol in the antennal washes of *H. zea* and *H. virescens* suggests that there is a more highly regulated program of lipid coating of the antennae compared to that the forelegs. Thus, we now need to study the spatial heterogeneity of the different compound groups in order to understand how they might determine the physical-chemical adsorptive properties of the coatings of these body parts. Surprisingly, we saw no significant differences between the lipid coatings of the antennae and forelegs of *M. sexta*, or between the antennae of males and females. However, sexes seem to differ in the cuticular lipid composition of the legs (unpublished data).

The physiological role of the oxygenated lipids identified on the antennae of the heliothine moth species requires more investigation. Early works suggested that the existence of a densely packed monolayer of more polar lipids below the surface wax layer (Jackson and Baker, 1970; references cited in Lockey, 1988) could explain the temperature-dependant changes in the permeability of the cuticle to water. However, more recent studies have shown that permeability strongly depends on the composition and the thickness of the outermost surface lipid layer (Lockey, 1988; Gibbs, 2002). If cholesterol or the esters form a separate phase on the cuticle, they might create patches that are more polar in a bulk of non-polar surface thus affecting the interaction with water and polar organic molecules. Further work is required to determine the location of the oxygenated lipids on different body parts and the relevance of sexual dimorphism in the antennal coating of the heliothine species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2008.07.010.

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