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Original article

Surface lipidome of the lone star tick, *Amblyomma americanum*, provides leads on semiochemicals and lipid metabolism

Robert Renthal[a,b,*], Kim Lohmeyer[c], Lígia M.F. Borges[d], Adalberto A. Pérez de León[c]

[a] Department of Biology, University of Texas at San Antonio, San Antonio, TX, 78249 USA
[b] Department of Biochemistry and Structural Biology, University of Texas Health Science Center, San Antonio, TX, 78229 USA
[c] USDA-ARS Knipling-Bushland U.S. Livestock Insects Research Laboratory, and Veterinary Pest Genomics Center, Kerrville, TX, 78029 USA
[d] Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Goiás, Brazil

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ABSTRACT

Lipids extracted from the surface of the lone star tick, *Amblyomma americanum*, were analyzed by high resolution mass spectrometry. Prior to lipid extraction, the adult ticks were either unfed or fed on cattle, and the fed ticks were in groups either containing males and females together, or containing only males or females. Cholesteryl esters were found on the surfaces of fed females, and they may provide a more complete description of the composition of the mounting sex pheromone. Dihydrocholesteryl esters were detected on the surfaces of unfed males and females, suggesting a possible role in survival during host-seeking. Dehydrodeoxyecdysone, found on fed females, could be a component of the genital sex pheromone. The most abundant polar surface lipids detected were acylglycerides. High levels of sphingolipids and glycerophospholipids on males fed separately might be derived, in part, from sperm development. A high level of a 20:4 fatty acid, presumably arachidonic acid, was found on the surface of fed females, indicating that it may be a component of the genital sex pheromone. A high level of docosenamide was found on the surface of fed females. Wax esters were found on the surfaces of fed ticks but not on unfed ticks. These esters could be involved in elasticity of the cuticle of engorged females or in wax coating of eggs. N-acylethanolamines were found on the surfaces of male and female ticks fed together, and on male ticks fed separately, but were absent or at low levels on females fed separately and on unfed ticks. This pattern suggests a possible role as a metabolic coordination primer pheromone.

1. Introduction

The lone star tick, *Amblyomma americanum* (Acari: Ixodidae) is a vector of bacterial pathogens affecting humans, domestic animals, and wildlife (Childs and Paddock, 2003). Among the bacteria transmitted by *A. americanum* is *Ehrlichia chaffeensis*, the agent of human ehrlichiosis, a potentially fatal infectious disease (Goddard and Varela-Stokes, 2009). A successful strategy for controlling vector-borne diseases has been to limit vector populations. Detailed knowledge of vector signaling chemicals (semiochemicals) may provide a basis for new methods to control tick feeding and mating, thereby decreasing disease transmission. Previous studies identified components of volatile pheromones released by *A. americanum* females (Berger, 1972) and the males of some *Amblyomma* species (Apps et al., 1988; Lusby et al., 1991; Schoni et al., 1984). Non-volatile substances are used by ticks as short range pheromones, including a mounting sex pheromone (MSP) and a genital sex pheromone (GSP) (Sonenshine, 2004). MSPs are known to consist of a mixture of cholesterol esters (Hamilton et al., 1989; Phillips and Sonenshine, 1993; Sobbhy et al., 1994), and GSPs contain a mixture of fatty acids and ecdysterone (Allan et al., 1991; Sonenshine et al., 1985). However, the chemical description of *A. americanum* MSPs and GSPs may be incomplete. Since the time that these pheromones were initially described, new methods of mass spectrometric analysis have been developed (Xian et al., 2012) that could provide a more detailed characterization of non-volatile contact pheromones. Complex mixtures of neutral molecules are separated by nano-flow high performance liquid chromatography (nano-HPLC) and, after electrospray ionization, directly injected into a high resolution mass spectrometer. Fourier transform mass analyzers can determine the exact chemical formulas of thousands of components in a mixture during a single analysis. In

Abbreviations: FFT, female ticks fed together with male ticks; MFT, male ticks fed together with female ticks; FFS, female ticks fed separately from male ticks; MFS, male ticks fed separately from female ticks; FU, unfed female ticks; MU, unfed male ticks; MSP, mounting sex pheromone; GSP, genital sex pheromone; MS^2, second stage of tandem mass spectrum; CE, cholesteryl ester; PC, phosphatidylcholine; SM, sphingomyelin; DHCE, dihydrocholesteryl ester

* Corresponding author at: Department of Biology, University of Texas at San Antonio, 1 UTSA Circle, San Antonio, TX, 78249, USA.
E-mail address: Robert.Renthal@UTSA.edu (R. Renthal).

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addition, selected components can be fragmented by collision-induced dissociation to provide information about chemical structure, often narrowing the identification of unknown components to a single compound or a few isomers.

From a wide range of previous studies, it is well established that solvent washes collect cuticular hydrocarbons and contact pheromones from the surfaces and surface-connected cavities of arthropods. In addition to extracts of cuticular lipids, solvent washes of ticks may also include extracts from dermal glands, dorsal fovea, genital pore, genital aperture, preoral canal, and anus. Solvent extraction of tick cuticle has contributed to phylogenetic analysis (Estrada-Peña et al., 1994), volatile pheromone identification (recently summarized in Carr and Roe, 2016), and insight into resistance to tick fungal infections (Kirkland et al., 2004; Ment et al., 2013). Large-scale lipid analyses of insects have provided unique insights by comparing males and females, different metabolic states, and different tissues (Carvalho et al., 2012; Guan et al., 2013). However, there have been no previous high resolution lipidomic analyses of surface solvent extracts from ticks.

The experiments described in this paper were designed to detect low-volatility semiochemicals by comparing ticks in different feeding states. Studies of *A. americanum* feeding and mating behavior indicate that the onset of mating occurs in after about 5 days of feeding (Gladney and Drummond, 1970a). Females tend to remain in the same location until full engorgement, but, after feeding, males move around on the host seeking females (Gladney and Drummond, 1970b). The presence of fertile males has an effect on female feeding (Darrow et al., 1976). Thus, comparisons were made between fed and unfed ticks, and between males and females fed together or separately, with the expectation that these groupings might aid in the discovery of semiochemicals involved in feeding and mating. We undertook a comprehensive lipidomic analysis of solvent washes of *A. americanum* ticks. The results described in this paper offer new leads on additional components of the *A. americanum* MSP and GSP pheromones, and also uncover areas of lipid metabolism that suggest new targets for tick control.

2. Materials and methods

2.1. Ticks

The *A. americanum* ticks used in these experiments were the same as in a previously published study (Renthal et al., 2017). Briefly, the ticks were fourteenth generation adults from a cattle-reared colony established at the Knipling-Bushland U.S. Livestock Insects Research Laboratory in 2006 and were 45–50 days post molt from the nymphal stage. Ticks were maintained off host in 25 mm x 95 mm (8-dram) shell vials with a cloth mesh stopper, in climate-controlled aquaria at 27 ± 2 °C, 86% relative humidity, and a photoperiod of 12:12 light:-dark. Six different groups of fifteen adult ticks each were prepared for lipid extraction: females fed together with males (FFT), males fed together with females (MFT), females fed separately from males (FFS), males fed separately from females (MFS), unfed females (FU), and unfed males (MU). Ticks in the fed groups were fed within stockinette sleeves attached to the shaved sides of a single stanchioned calf. To reduce stress, the calf was stanchioned with a companion calf and was monitored daily to ensure overall good health. The calf was approximately 12–18 months old, approximately 160 kg and was fed a standard grain ration and given water ad libitum. Stanchioned calves interact with animal caretakers several times a day and are provided with music, daily leg and foot joint hydrotherapy, as well as daily brushing. Ticks were allowed to feed for 5 days to ensure that they had properly attached and begun to feed, but had not reached the rapid engorgement phase. At the end of the 5 day period, the ticks were removed from the host with soft forceps and lipids were extracted. Care was taken to use a clean pair of forceps for removal of ticks from each group to avoid cross contamination.

2.2. Surface lipid extraction

Live ticks were transferred from ventilated plastic holding tubes to solvent-washed 20 mL glass vials and anaesthetized with CO_2. Even after the vials were thoroughly flushed with CO_2, some of the ticks were still quite active, so the vials were placed on ice, and the chilled glass prevented the ticks from escaping. To each vial was added 5 mL of pentane from a freshly opened bottle (Burdick & Jackson HPLC, GC and pesticide grade, Honeywell, Muskegon, MI). The ticks were gently swirled in the pentane periodically during a span of five minutes at room temperature. Then the pentane was removed with a glass pipette and transferred to solvent-washed 5 mL V-vials (Wheaton, Millville, NJ). The pentane was evaporated in a fume hood with a gentle stream of N_2 over a period of about 10 min, and the vials were closed with Teflon-lined caps. The dried samples were immediately stored at -20 °C until analyzed by mass spectrometry. A solvent blank was also prepared using an identical procedure.

2.3. Mass spectrometry

Each sample, containing lipids extracted from fifteen ticks (i.e. FFT, MFT, etc.), was analyzed in triplicate on a Q Exactive mass spectrometer (Thermo Fisher, San Jose, CA) with a PicoChip column and Nanospray source from New Objective (Woburn, MA), as previously described (Renthal et al., 2014).

2.4. Data analysis

Phospholipids and acylglycerols were identified and quantified using CoMet software (Progenesis/Waters, Milford, MA). CoMet analysis of positive ion data sets gave chromatographic peak areas, in units of ion counts, for identified lipids. For three technical replicates of each of the six different feeding groups (FFT, MFT, FFS, MFS, FU, MU), we pooled the chromatographic areas of glycero- and sphingolipids into the following groups. Sphingolipids: ceramide, cerebroside, sphingomyelin; lysoglycerolipids: lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylinositol, lysophosphatidylserine; glycerophospholipids: phosphatidylcholine, phosphatidylethanolamine, phsophatidylinositol, phosphatidylserine. Acylglycerols were pooled as monoacylglycerols, diacylglycerols, and triacylglycerols. Summed chromatographic areas were normalized to the total ion current for the chromatogram.

Free fatty acids were identified from the Thermo raw negative ion mass spectra of three technical replicates, after using msConvert (Kessner et al., 2008) with peak picking to convert to mzXML format. Custom software searched the precursor ions in the MS^2 spectra for $(M-H)^-$ exact masses of fatty acids containing 12 to 25 carbons and 0 to 5 double bonds, within the range of the mass error of the spectrometer's calibration.

Cholesterol esters were identified and quantified using CoMet, as described above for phospholipids and acylglycerols. Dihydrocholesterol esters were identified from the positive ion mass spectra by a similar method to that described above for free fatty acids, except only one data set was analyzed. Custom software searched the MS^2 spectra for the $C_{27}H_{47}$ dihydrocholesteryl ion, which is the base peak in the spectra of the dihydrocholesteryl esters, and then collected the precursor ion *m/z* and intensity. Intensities of dehydrodeoxyecdysone were obtained by searching the mzXML files of three technical replicates for the precursor ion at *m/z* 464.338. Custom software was also used to search the mzXML files of three technical replicates for 12- to 27-carbon fatty acyl amides containing 0–3 double bonds, 20- to 76-carbon wax esters containing 0–6 double bonds, and N-acylethanolamides of 12- to 38-carbon fatty acids with 0–6 double bonds. For the wax esters and N-acylethanolamides, after we identified the major components by searching for molecular ions, we used Xcalibur software (ThermoFisher, Waltham, MA) to obtain selected ion chromatograms. The areas of the chromatographic peaks corresponding to the specific molecular components were then integrated for

quantification.

Statistical parameters (one-way ANOVA and Tukey-Kramer HSD) were calculated using JMP Pro 13 software (SAS, Cary, NC). Data analysis was aided by ChemCalc (www.chemcalc.org), Metlin (metlin.scripps.edu), and mMass (mmass.org) software.

3. Results and discussion

3.1. Steroids

3.1.1. Cholesteryl esters

Cholesteryl esters (CEs) were identified from the MS^2 fragmentation spectra by the presence of the cholesteryl cation at *m/z* 369.352, which was usually the base peak. In the MS^2 spectra, the fatty acyl groups generally became neutral fragments, so they were identified by difference from the molecular ions. The molecular ions were exclusively observed as ammonium adducts. The CE chromatographic retention times were approximately linear with number of acyl chain carbon atoms, as was previously noted (Butovich, 2009). Each CE with unsaturated acyl chains formed a separate linear series, eluting slightly earlier than the corresponding saturated chains. The relative amounts of the CEs that were identified are shown in Table 1 as detector ion current areas. The most abundant CE, containing a doubly unsaturated 18 carbon acyl chain, was observed in fed females. The double bond isomer cannot be determined from the available fragmentation data, but it is likely to be a single isomer, because it elutes as a single chromatographic peak. The amount of 18:2 CE extracted from the surfaces of females fed separately was more than twice the amount extracted from male surfaces (significantly different from males fed together with females, p-value = 0.02; and from males fed separately, p-value = 0.01). For many acyl chains of less than 30 carbons (28:1, 26:1, 23:0, 22:1, 21:0, 20:1, 18:3, 18:2, 18:1, 16:1), the amounts of CEs on females fed separately were significantly higher than the amounts on males and, in some cases, on females fed together with males. *Amblyomma* ticks mate just after feeding, so the presence of higher levels of CEs on fed females supports their role as components of the mounting sex pheromone (MSP) (Sonenshine, 2004). If CEs that are present on both males and females comprise the MSP, then the male must have a mechanism to distinguish females from other males, either by threshold detection or by coincidence detection of CEs plus other substances. The levels of these CEs were generally higher on females fed separately than on females fed together with males. This suggests the possibility that females feeding in contact with males produce less MSP than females fed separately from males. Some of the CEs found primarily on females fed separately were present at relatively low levels (e.g. 23:0, 22:1, 21:0). It is possible that a mixture of female-specific lower abundance CE components, combined with higher abundance CE components, constitutes the MSP. Evidence for this possibility comes from bioassays on *Rhipicephalus microplus* males that were reported to show responses to CE mixtures containing minor components at 100-fold higher sensitivity than CE mixtures lacking the minor components (de Bruyne and Guerin, 1998).

Unfed females generally had very low levels of CEs, indicating that the CEs are derived from feeding. However, the presence of CE acyl chains with lengths greater than 30 is unusual in vertebrates (Duffin et al., 2000; Furland et al., 2003; Gallego et al., 2018; Liebisch et al., 2006), so it is not likely that the very long chain CEs are directly obtained from bovine blood without modification by the ticks. If bioassays show that a full MSP response in *A. americanum* requires the long chain CEs, future experiments might explore the biosynthesis of very long acyl chain CEs in *A. americanum*, with the objective of finding inhibitors that could suppress mating. Very long chain fatty acids (22 carbons or greater) are synthesized by fatty acyl elongases. There are more than fifty elongase-like genes in the *Ixodes scapularis* genome (Gulia-Nuss et al., 2016), and we found highly similar sequences in two *A. americanum* expressed sequence tags (JZ172812 and JZ169829).

Table 1
Relative amounts of extracted cholesteryl esters.

Acyl chain[a]	Mean detector ion current area[b]						
	FFT	MFT	FFS	MFS	FU	MU	CI[c]
38:0	0^{B}	0^{B}	0^{B}	0.31^{B}	2.86^{A}	0.81^{B}	0.80
37:0	0^{B}	0^{B}	0^{B}	0^{B}	1.04^{A}	0.78^{A}	0.20
36:4	0.71^{A}	0^{B}	0.43^{AB}	0^{B}	0^{B}	0.17^{AB}	0.29
36:2	1.65^{A}	2.43^{A}	1.83^{A}	2.37^{A}	0.05^{B}	0.21^{B}	0.60
36:1	3.92^{BC}	9.81^{A}	4.05^{BC}	5.71^{AB}	0.16^{C}	0.52^{C}	2.02
36:0	0.61^{A}	2.04^{A}	0.61^{A}	0.31^{A}	2.37^{A}	2.01^{A}	2.01
34:2	1.32^{AB}	1.55^{A}	1.59^{A}	1.35^{A}	0.05^{B}	0.40^{AB}	0.58
34:1	7.52^{BC}	21.4^{A}	9.55^{B}	9.84^{B}	0.15^{C}	0.85^{C}	3.92
33:0	0^{B}	0^{B}	0^{B}	0^{B}	1.24^{A}	0.38^{B}	0.24
32:2	1.52^{A}	1.25^{A}	1.27^{A}	1.10^{A}	0.14^{B}	0.90^{AB}	0.35
32:1	3.74^{A}	9.73^{A}	6.06^{A}	8.31^{A}	0.59^{A}	1.57^{A}	4.64
32:0	0^{B}	0^{B}	0^{B}	0^{B}	1.44^{A}	1.48^{A}	0.17
30:2	1.00^{A}	0.66^{A}	0.56^{A}	0.22^{A}	0.83^{A}	0.37^{A}	0.44
30:1	12.0^{A}	12.7^{A}	17.7^{A}	9.86^{AB}	0.39^{C}	2.53^{BC}	3.72
30:0	0^{B}	0^{B}	0^{B}	0^{B}	1.96^{A}	1.81^{A}	0.56
28:1	9.41^{B}	9.68^{B}	14.6^{A}	8.71^{B}	0.93^{C}	6.14^{B}	2.07
26:1	4.16^{AB}	4.99^{AB}	10.1^{A}	3.86^{B}	1.08^{B}	2.16^{B}	2.80
26:0	0.62^{B}	2.42^{A}	0.49^{B}	2.30^{A}	2.07^{A}	1.32^{AB}	0.63
25:0	1.04^{A}	1.80^{A}	1.72^{A}	1.76^{A}	0.61^{A}	0.54^{A}	0.71
24:0	4.07^{B}	7.06^{A}	2.63^{B}	7.74^{A}	2.38^{B}	2.05^{B}	0.98
23:0	0.46^{B}	0.44^{B}	2.22^{A}	0.33^{B}	0.21^{B}	0.20^{B}	0.73
22:1	1.91^{B}	0^{B}	6.36^{A}	0^{B}	0.27^{B}	0.84^{B}	0.99
22:0	7.37^{A}	5.62^{AB}	6.15^{AB}	4.26^{ABC}	0.75^{C}	2.59^{BC}	1.64
21:0	2.05^{B}	0.84^{B}	4.38^{A}	0.20^{B}	0.77^{B}	0.66^{B}	0.86
20:1	2.30^{B}	0.66^{B}	13.7^{A}	0^{B}	0.98^{B}	3.14^{B}	2.27
20:0	12.6^{AB}	11.6^{AB}	14.2^{A}	8.22^{ABC}	0.52^{C}	5.93^{BC}	3.67
18:3	6.11^{B}	2.98^{C}	12.0^{A}	1.56^{CD}	0.20^{D}	0^{D}	1.04
18:2	36.2^{AB}	24.2^{BC}	52.1^{A}	21.0^{BC}	6.05^{C}	13.6^{BC}	11.0
18:1	33.4^{A}	24.4^{B}	38.3^{A}	24.6^{B}	7.56^{C}	17.1^{B}	3.81
18:0	28.5^{A}	23.3^{A}	20.9^{AB}	23.7^{A}	5.50^{C}	11.2^{BC}	4.76
16:1	3.02^{AB}	1.81^{BC}	3.90^{A}	0^{D}	1.45^{C}	1.41^{C}	0.59
16:0	26.9^{A}	22.4^{A}	27.0^{A}	25.1^{A}	5.8^{B}	13.7^{B}	3.65
15:0	3.27^{A}	2.74^{A}	2.65^{A}	3.20^{A}	0.71^{B}	0.38^{B}	0.69
14:0	28.7^{AB}	25.5^{AB}	24.3^{B}	32.3^{A}	4.33^{C}	9.78^{C}	3.29
12:0	6.85^{A}	6.39^{A}	7.39^{A}	6.27^{A}	0^{B}	0.66^{B}	1.67

Notes: a. In X:Y acyl chain notation, X = number of carbons and Y = number of double bonds. b. Detector ion current areas × 10^{-8}, mean of three technical replicates. Column headers indicate different feeding groups: FFT, females fed together with males; MFT, males fed together with females; FFS, females fed separately; MFS, males fed separately; FU, females unfed; MU, males unfed. Capital letter superscripts are from Tukey-Kramer HSD analysis; different letters in a particular row indicate significantly different levels of cholesteryl ester in the corresponding feeding groups. c. 95% confidence intervals for entries in row are mean ± indicated value.

Males fed together with females had about two-fold higher levels of 36:0 and 34:1 CEs than fed females. In contrast, these CEs were at much lower levels in males that were fed separately, suggesting a possible signaling role in mating.

3.1.2. Dihydrocholesteryl esters

Dihydrocholesteryl esters (DHCE) were present in unfed males and females, detected by the base peak at *m/z* 371.367 for the dihydrocholesteryl cation in the MS^2 spectra. DHCEs were at 3–4 times lower levels in fed ticks compared with unfed ticks (differences significant in *t*-test at $P < 0.0001$) (Fig. 1). All the DHCE acyl chains detected were saturated and they were exceptionally long, up to 44 carbons. No DHCEs were detected with chains shorter than 31 carbons. The much higher levels of DHCEs in unfed ticks suggest a possible role in survival between feeding events.

3.1.3. Ecdysone

20-hydroxyecdysone (ecdysterone) has been found in some ticks as a component of the genital sex pheromone (GSP) (Sonenshine et al., 1985), and *A. americanum* males were shown to respond to it (Allan et al., 1991). Nevertheless, we were unable to detect in our extracts any

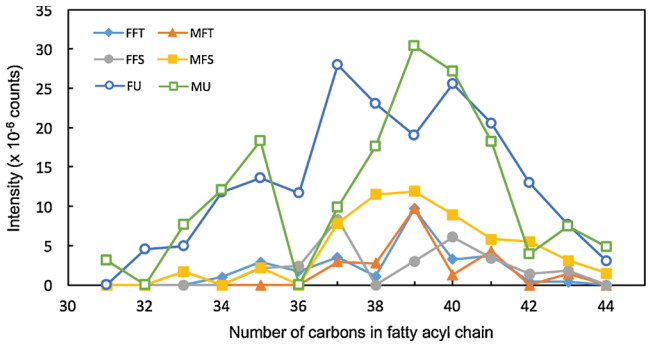

Fig. 1. Relative amounts of extracted dihydrocholesteryl esters. Long chain dihydrocholesteryl esters are shown by carbon chain lengths, compared in six different feeding conditions: FFT, females fed together (diamonds); MFT, males fed together (triangles); FFS, females fed separately (filled circles); MFS, males fed separately (filled squares); FU, unfed females (open circles); MU, unfed males (open squares). Each point is an extract from 15 ticks. Only saturated carboxyl chains were detected. Intensity scale shows MS^2 precursor intensity.

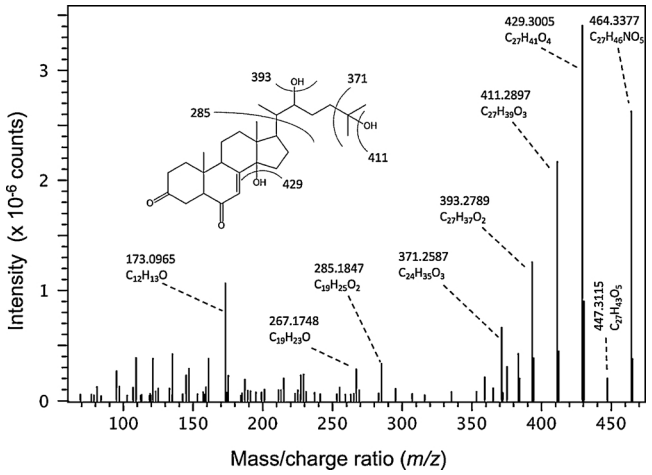

Fig. 2. Dehydrodeoxyecdysone mass spectrum. MS^2 spectrum of *m/z* 464.338 precursor ion, from unfed females. Structure of a possible isomer, 3-dehydro-2-deoxyecdysone, shown with identification of fragments consistent with spectrum.

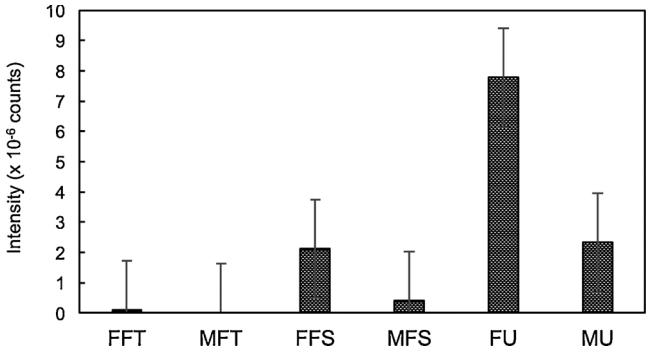

Fig. 3. Relative amounts of extracted dehydrodeoxyecdysone. Tick groups are: FFT, females fed together; MFT, males fed together; FFS, females fed separately; MFS, males fed separately; FU, unfed females; MU, unfed males. Intensity scale shows MS^2 precursor intensity. Error bars show the 95% confidence interval.

20-hydroxyecdysone, either with H^+ or NH_4^+ adducts as molecular ions, or from expected dehydration products that might have appeared as apparent molecular ions or as base peaks in MS^2 spectra. However, when we searched for base peaks of several known ecdysteroids (Blais et al., 2010), we detected the NH_4^+ adduct of $C_{27}H_{42}O_5$ at *m/z* 464.338. This formula and the main fragments observed in the MS^2 spectra are consistent with an isomer of dehydrodeoxyecdysone (Fig. 2). The specific isomer cannot be determined from the spectrum. The levels of dehydrodeoxyecdysone are more than five times higher in fed females compared with fed males (Fig. 3), but the differences were not significant in the Tukey-Kramer HSD test, due to the large measurement errors at the overall low levels of the steroid. It would be interesting if *A. americanum* females use dehydrodeoxyecdysone as part of the GSP. The previously reported experimental results showing a GSP response in *A. americanum* males to 20-hydroxyecdysone (Allan et al., 1991) would be expected if the dehydrodeoxyecdysone isomer is, for example, 3-dehydro-2-deoxy (depicted in Fig. 2) and if the male tick's receptor for dehydrodeoxyecdysone recognizes only the oxygen atoms at the 3, 6, 14 and 22 positions, which match oxygen atoms in 20-hydroxyecdysone. An argument could be made against the involvement of dehydrodeoxyecdysone in the GSP in view of the similar or higher levels of dehydrodeoxyecdysone in unfed ticks compared with female ticks fed separately (Fig. 3). The level of dehydrodeoxyecdysone in unfed females was significantly higher than fed ticks and unfed males (Tukey-Kramer HSD p-values at 0.0025 or less). However, it was previously shown that unfed male ticks produce and can detect the attractant sex pheromone (ASP), but they do not respond to it until after feeding (Sonenshine, 2004). Thus, dehydrodeoxyecdysone might have a similar response pattern. The question could be settled by a more detailed chemical analysis to confirm the structure and determine the isomeric configuration of the extracted dehydrodeoxyecdysone, and by behavioral testing.

3.2. Glycerophospholipids, sphingolipids, and acylglycerides

Surface glycerophospholipids, sphingolipids, and acylglycerides, extracted from ticks in different feeding conditions, were identified from MS^2 spectra. The detector ion current areas of identified lipids were pooled into groups and are shown in Figs. 4 and 5 as percent of total ion counts (roughly the percent of total extracted material). Triglycerides were very abundant, in the range of 10–15% of the total ion counts, except for unfed females (Fig. 4). Males fed separately (MFS) had substantially higher levels of sphingolipids, lysoglycerophospholipids, and glycerophospholipids than ticks in other feeding conditions (Fig. 5) (significantly higher, with p-values < 0.0001 in the Tukey-Kramer HSD test for all pairs except MFT-MFS sphingolipids, for which the p-value was 0.0002). One possible explanation is that the MFS extracts include material from the seminal vesicle, which connects to the genital aperture (Matsuo and Mori, 2000) and thus would be extractable by soaking whole ticks in solvent. After feeding, males begin

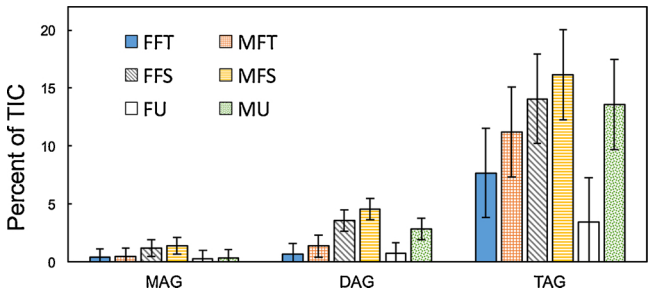

Fig. 4. Relative amounts of extracted acylglycerols. MAG: monoacylglycerols; DAG: diacylglycerols; TAG: triacylglycerols. Each bar is the average of three measurements from a pooled extract from 15 ticks, shown as the chromatogram peak area as a percent of the area of the total ion chromatogram (TIC). Error bars show the 95% confidence interval. Tick groups are: FFT, females fed together (solid fill); MFT, males fed together (crosshatch); FFS, females fed separately (diagonal lines); MFS, males fed separately (horizontal lines); FU, unfed females (unfilled); MU, unfed males (stippled), as indicated on the figure inset.

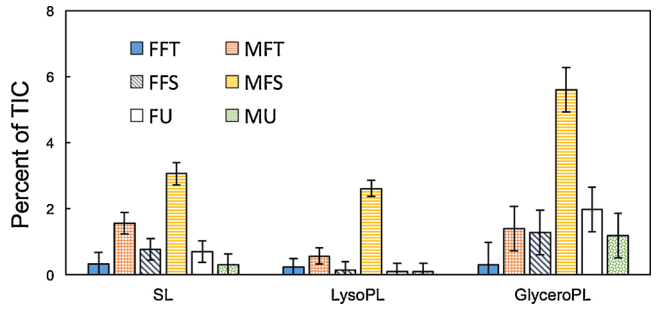

Fig. 5. Relative amounts of extracted glycerophospholipids and sphingolipids. Each bar is the average of three measurements from a pooled extract from 15 ticks, shown as the chromatogram peak area as a percent of the area of the total ion chromatogram (TIC). Error bars show the 95% confidence interval. SL: sphingolipids, including sphingomyelin and ceramide; LysoPL: lysoglycerophospholipids, including lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, and lysophosphatidylinositol; GlyceroPL, glycerophospholipids, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. Tick groups are: FFT, females fed together (solid fill); MFT, males fed together (crosshatch); FFS, females fed separately (diagonal lines); MFS, males fed separately (horizontal lines); FU, unfed females (unfilled); MU, unfed males (stippled), as indicated on the figure inset.

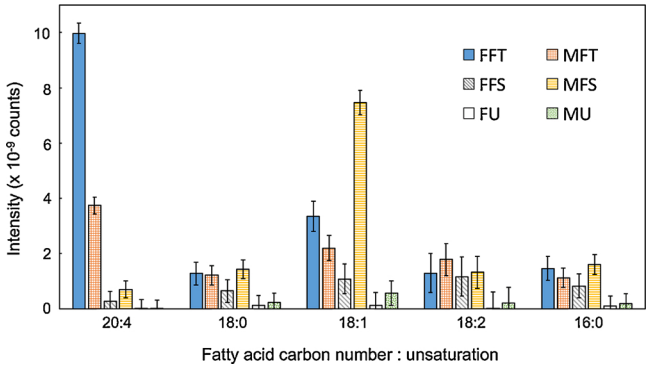

Fig. 6. Relative amounts of extracted fatty acids. Fatty acids with even numbers of carbon atoms. Each bar is the average of three measurements from an extract from 15 ticks, shown as the MS^2 precursor intensities. Error bars show the 95% confidence interval. Tick groups are: FFT, females fed together (solid fill); MFT, males fed together (crosshatch); FFS, females fed separately (diagonal lines); MFS, males fed separately (horizontal lines); FU, unfed females (unfilled); MU, unfed males (stippled), as indicated on the figure inset.

developing sperm, and prospermia collect in the seminal vesicle. This material would contain high levels of membrane sphingolipids and glycerophospholipids. However, sperm in males feeding in the presence of females would likely be inaccessible to extraction, either because they are sealed off by the preliminary stages of spermatophore formation, or because the males have already mated. Thus, lower sphingolipid and glycerophospholipid levels would be observed on males fed together with females. This could be tested in future experiments by analyzing lipid extracts of dissected seminal vesicles from males fed separately and fed in the presence of females.

Bovine blood was the exclusive food source for the ticks in this study. Bovine blood has a sphingomyelin to phosphatidylcholine ratio (SM/PC) of approximately 1 (calculated either on a weight or mole basis) (Nelson, 1967). This is due to the unusually high SM content of bovine erythrocytes, in which SM is the only choline-containing phospholipid (Hanahan et al., 1960). Thus, an increase in the SM/PC ratio after blood feeding would be expected if, on a short time scale, lipids are directly passed from the tick's gut to the cuticle surface. The SM/PC ratio in unfed females is 0.19, and in unfed males is 0.15. In male and female ticks fed separately, the ratios were not much changed from the unfed ratios: 0.12 for females and 0.14 for males. In male and female ticks fed together, the SM/PC ratios actually decreased: 0.06 for females and 0.04 for males. Thus, the results indicate that phospholipids derived from a blood meal are either metabolized before being transported to the cuticular surface and to exocrine glands, or sphingomyelin is transported to the surface much less than phosphatidylcholine. Hussein and Kamal (1977) reached similar conclusions from examining hemolymph lipids of *Argas arboreus* and *Dermacentor andersoni* ticks after feeding, suggesting that the ticks alter the composition of the host blood lipids via metabolic and/or transport mechanisms, although the host and tick SM/PC ratios were different from our observations of *A. americanum* feeding on cattle.

A previous report identified arachidonylmonoacylglycerol in salivary glands of fed females (Fezza et al., 2003). Our extracts of both fed females and fed males showed low levels of arachadonylmonoacylglycerol, as determined by the exact mass of the molecular ion $(M + NH_4)^+$ and the close match of the fragmentation pattern compared with the experimental spectrum in the Metlin database.

3.3. Free fatty acids

Free fatty acids were identified in negative ion spectra by their exact masses. Peak detector ion currents for the main even-numbered fatty acids are shown in Fig. 6. Previous studies identified a mixture of 14- to 20-carbon fatty acids in the genital tract of female *Dermacentor* ticks as part of a genital sex pheromone (GSP) (Sonenshine et al., 1985). Bioassays indicated that *A. americanum* females have a GSP (Allan et al., 1991), but a weak response was observed to solvent extracts of the anterior reproductive tract. We found that the most abundant free fatty acid in female ticks fed together with males (FFT) was 20:4, presumably arachidonic acid (Fig. 6). The level of 20:4 fatty acid in the FFT group was higher than in all other groups (significantly higher, with p-values < 0.0001 in the Tukey-Kramer HSD test). If arachidonic acid is the main constituent of the *A. americanum* genital sex pheromone, the weak bioassay response to genital tract extracts might be explained by its chemical instability, because arachidonic acid is easily oxidized (Porter, 1986). Our results do not indicate the anatomical source of the free fatty acids. Previous studies of the salivary gland lipids of *A. americanum* (Shipley et al., 1993, 1994) detected large amounts of arachidonic acid esterified to glycerol in phospholipids and triglycerides, but free arachaidonic acid was detected only in unfed females. Therefore, the high level of arachidonic acid we found in external extracts of fed females is unlikely to be derived from the salivary glands.

Males fed separately (MFS) had high levels of an 18:1 free fatty acid, presumably oleic acid (significantly higher than in all other groups, with p-values < 0.0001 in the Tukey-Kramer HSD test). For the same reasons as discussed above for the high levels of cholesteryl esters and phospholipids in MFS ticks, these fatty acids could be part of a pheromone signal for mating, or constituents of male accessory gland secretions.

3.4. Other extracted substances

3.4.1. Docosenamide

Females fed together with males had high levels of docosenamide (22:1 amide). The identification was made both by the exact mass of the molecular ion $(M+H)^+$, and also by the fragmentation pattern, which closely matched the experimental pattern of 13-docosenamide in the Metlin database. Females fed together with males had significantly higher amounts of docosenamide compared with in other feeding

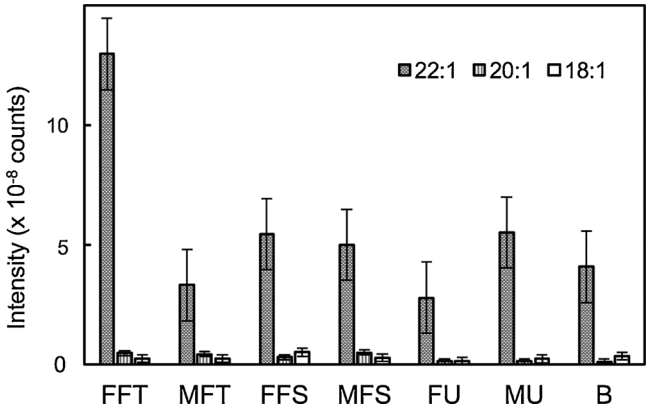

Fig. 7. Relative amounts of extracted fatty acylamides. Intensities (MS^2 precursors) of docosenamide (22:1), eicosenamide (20:1) and oleamide (18:1) extracted from surfaces of ticks fed under different conditions, compared with solvent blank, B. Inset identifies bar shading. Tick groups: FFT, females fed together; MFT, males fed together; FFS, females fed separately; MFS, males fed separately; FU, unfed females; MU, unfed males. Error bars show the 95% confidence interval.

conditions (Tukey-Kramer HSD test p-values < 0.0001), which showed only background levels (Fig. 7). This substance has not been previously reported in ticks. It would be interesting if docosenamide is used by female ticks to manipulate the host physiology, since it is known to be present in cerebrospinal fluid of sleep-deprived cats (Cravatt et al., 1995). However, docosenamide was not found at high levels in fed males or in females fed separately, implying that it is not involved in host manipulation. Further work will be necessary to confirm that docosenamide is not a contaminant, since it is also a component of plastic film manufacturing (Prichard, 1998). However, docosenamide was present in only one of seven samples that were processed identically (Fig. 7). In particular, the FFT and MFT ticks were all in contact with the same materials and the same hosts, yet docosenamide was only found at high levels in the females. The background level of docosenamide from plastics may be estimated from the blank, which was in the range of 4×10^8 counts (Fig. 7). Commercial sources of docosenamide typically contain 10% of other amides (see gzcardlo.com). The levels of two other acylamides, eicosenamide and oleamide, which are also likely to be in plastics, are also shown in Fig. 7. Eicosenamide (20:1) levels of FFT, MFT and MFS were not distinguishable (Tukey-Kramer HSD test p-values nearly 1.0), and none of the oleamide levels, including the blank, were distinguishable (Tukey-Kramer HSD test p-values all > 0.05 and most values close to 1.0). This suggests that the high level of docosenamide in FFT is biological rather than due to contamination.

3.4.2. Wax esters

Relatively high amounts of five wax esters were identified in fed ticks (Fig. 8). The identifications were made by searching for H^+ and NH_4^+ adducts and then examining the MS^2 spectra for the acylium and protonated acid fragments (Iven et al., 2013). The molecular ion intensities were in the same range as cholesteryl esters (Table 1). In some cases the wax ester molecular ions were mixtures of isomers, so the wax esters are reported in Fig. 8 by the total number of carbon atoms and total unsaturation (e.g. 32:1). The MS^2 spectra showed a range of even-numbered 8- to 16-carbon saturated fatty acyl groups and 22-, 23- and 24-carbon mono-unsaturated alcohols. For 34:1 and 36:1 wax esters, fed females had significantly higher levels than fed males (p-values < 0.0001 for 34:1 wax esters and < 0.01 for 36:1 wax esters in all Tukey-Kramer HSD pair-wise comparisons). In unfed ticks, the wax esters were at very low levels or undetectable. The differences between fed and unfed ticks were highly significant (p-values < 0.0001 for 32:1, 34:1 and 36:1 for all pairs, and < 0.02 or < 0.03 for 38:1 or 40:1

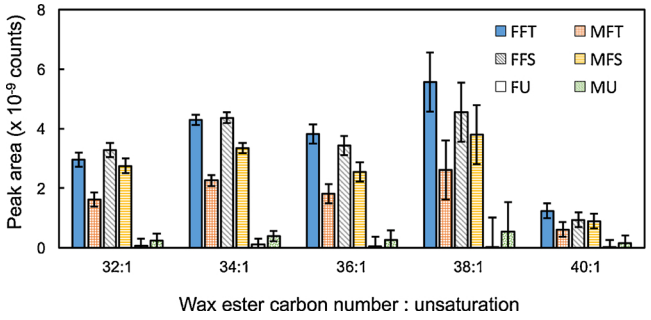

Fig. 8. Relative amounts of extracted wax esters. Chromatographic peak areas of most abundant wax esters extracted from surface of ticks fed under different conditions. Error bars show the 95% confidence interval. Tick groups are: FFT, females fed together (solid fill); MFT, males fed together (crosshatch); FFS, females fed separately (diagonal lines); MFS, males fed separately (horizontal lines); FU, unfed females (unfilled); MU, unfed males (stippled), as indicated on the figure inset.

respectively, for all pairs except unfed males and males fed together with females, which were not significantly different). Wax esters are generally thought to be used by arthropods for preventing water loss (Lockey, 1988). However, this function would seem to be more important in unfed ticks than in fed ticks, since unfed ticks may need to protect themselves from dehydration for a long period of time before finding a host for a blood meal. Furthermore, studies of purified wax esters (Patel et al., 2001) indicate that the mono-unsaturated wax esters found on *A. americanum* ticks are in a liquid state (i.e. above their melting transition temperatures) at the host's body temperature. Above their melting transition temperature, wax esters are more permeable and thus are not effective at protecting arthropods from desiccation (Patel et al., 2001). Therefore, we conclude that the wax esters on fed *A. americanum* ticks do not play a role in protection against dehydration. Instead, the higher levels in fed females suggest possible roles in engorgement or egg-laying. The wax esters could contribute to the elasticity of the cuticle in engorged females (Kaufman et al., 2016). Alternatively, the wax esters could be from the initial stages of egg wax synthesis in the developing Gené's organ as the female feeds in preparation for oviposition (Dos Santos et al., 2018; McCamish et al., 1977). These alternatives could be tested in future experiments by characterizing wax esters in the cuticle of engorged *A. americanum* females, and on the surface of *A. americanum* eggs.

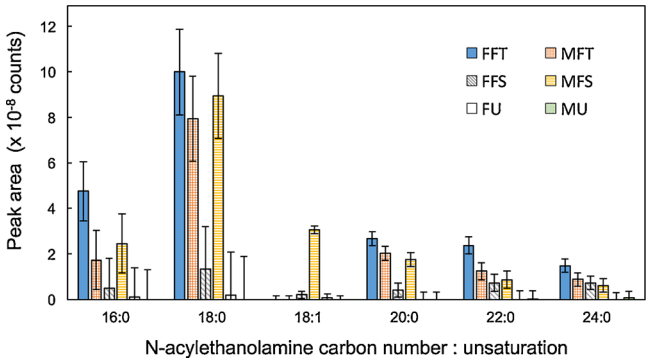

Fig. 9. Relative amounts of extracted N-acylethanolamines. Chromatographic peak areas of most abundant N-acylethanolamines extracted from surfaces of ticks fed under different conditions. Error bars show the 95% confidence interval. Tick groups are: FFT, females fed together (solid fill); MFT, males fed together (crosshatch); FFS, females fed separately (diagonal lines); MFS, males fed separately (horizontal lines); FU, unfed females (unfilled); MU, unfed males (stippled), as indicated on the figure inset.

3.4.3. N-acylethanolamines

Fed ticks had significant levels of N-acylethanolamines (Fig. 9), which were detected by searching for the ethanolammonium ion at *m/z* 62.06, which is the base peak in the mass spectra. Males and females fed together, as well as males fed separately, had N-stearoylethanolamine (18:0) at about two-fold or higher levels than the 16:0, 18:1, 20:0, 22:0 and 24:0 N-acylethanolamines. By contrast, females fed separately and unfed males and females had very low or undetectable levels of N-acylethanolamines. Females fed together with males had significantly higher amounts of 18:0 N-acylethanolamine than females fed separately (p-values at the 0.0001 level in the Tukey-Kramer HSD test). Males either fed together with females or fed separately had significantly higher amounts of 18:0 N-acylethanolamine than females fed separately (p-values at the 0.0016 level or less). N-acylethanolamines are well-known in vertebrates as ligands for cannabinoid and TRPV1 receptors. However, ticks lack these receptors. Previously, N-acylethanolamines were identified in *A. americanum* salivary glands (Fezza et al., 2003), and it was postulated that the N-acylethanolamines are involved in the tick's manipulation of host sensory and immune responses. However, the strikingly different levels of N-acylethanolamines in males and females fed separately suggests a possible additional role involving chemical signaling between ticks. N-acylethanolamines are known to be produced in response to environmental signals and they activate nuclear receptors that regulate fat metabolism (Folick et al., 2015; Ratnappan et al., 2014). N-acylethanolamines might act as primer pheromones to coordinate fat metabolism in a group of feeding ticks. The low level of N-acylethanolamine in females fed in the absence of males could indicate there is a mechanism to delay full metabolic development in feeding females until males are present. Also, the levels of 18:1 N-acylethanolamine were significantly higher in male ticks fed separately than in any other feeding group (p-values < 0.0001 for all comparisons), suggesting a unique signaling role for this substance.

If some of the N-acylethanolamines we detected were extracted from saliva, we might expect also to have detected prostaglandins, which are known to be present in tick saliva and have been measured by mass spectrometry in tick saliva extracts (Oliveira et al., 2011). We searched for expected molecular ions and typical base peak ions of PGE2, and although some of these ions were found, none of the MS^2 spectra matched known PGE2 fragmentation patterns. Therefore, we conclude that our pentane extraction method does not efficiently extract prostaglandins from whole ticks, unlike methods that use acidification of the extraction medium (Oliveira et al., 2011; Unger et al., 1971). By contrast, we did detect neutral lipids known to be in tick saliva, such as arachidonoylmonoglyceride (see Glycerophospholipids, sphingolipids, and acylglycerides section above), as well as N-acylethanolamines.

4. Conclusions

1) Cholesteryl esters with a wide range of acyl chains were found to be present at much higher levels on the surfaces of fed females than on fed males. These compounds may provide a more complete description for the composition of the mounting sex pheromone (MSP).
2) Dihydrocholesteryl esters were detected at higher levels on the surface of unfed males and females, suggesting a possible role in survival during host-seeking.
3) Dehydrodeoxyecdysone was found at higher levels on fed females than fed males, suggesting it may be the ecdysone derivative that is known to be a component of the genital sex pheromone (GSP).
4) The most abundant polar surface lipids were acylglycerides.
5) Males fed separately had much higher levels of sphingolipids and glycerophospholipids than females or than males fed together with females, suggesting the lipids may derive in part from sperm development.
6) A high level of a 20:4 fatty acid, presumably arachidonic acid, was found on the surface of fed females, indicating that it may be a component of the genital sex pheromone (GSP).
7) A high level of docosenamide was found on the surface of fed females.
8) Wax esters were found on the surfaces of fed ticks but not on unfed ticks. The properties of the esters, their absence on unfed ticks, and their higher levels on fed females suggest the possibility that these substances are involved in elasticity of the cuticle of engorged females or in wax coating of eggs.
9) N-acylethanolamines were found on the surfaces of male and female ticks fed together, and on male ticks fed separately, but were absent or at low levels on females fed separately and on unfed ticks. This pattern suggests a possible role as a metabolic coordination primer pheromone.

Ethical statement

Tick rearing on cattle was conducted according to the standards of procedure for tick rearing and animal care as reviewed and approved by the Knipling-Bushland U.S. Livestock Insects Research Laboratory IACUC, Rearing Adult and Nymphal Three Host Ticks, SOP #017-02.

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