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Cuticular fatty acid profile analysis of three *Rhipicephalus* tick species (Acari: Ixodidae)

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Abstract Cuticular fatty acids (CFA) are important constituents of the arthropod exoskeleton, serving as structural and defense components, and participating in intra-species communication. Here we describe for the first time a comparative analysis of the CFA profiles of three tick species of the genus *Rhipicephalus*: *R. annulatus*, *R. bursa* and *R. sanguineus*. CFA profiles were determined for *R. bursa* and *R. sanguineus* grown both on rabbit or calf, and for *R. annulatus* grown on calf. CFA composition was compared for each species before and after ethanol treatment, for different hosts of each species, and between the different species. Our data suggest that adsorption of the host's fatty acids changes the apparent CFA composition. Ethanol treatment efficiently removed the unbound fatty acids from the ticks and revealed the actual composition. Comparison between ticks grown on rabbit versus calf showed significant difference in the relative abundance of fatty acids C14 and 9,12-C18:2 for *R. bursa*, and a difference in the relative abundance of C14 for *R. sanguineus*. Comparison of the CFA between the three species revealed significant differences in the abundance of fatty acids C16, 9,12-C18:2, 9-C18:1, C18 and C20. Our results show that while the host had a minor effect on CFA composition within each species, significant differences were observed in the CFA profiles of different species. We suggest that CFA profiles may be used to distinguish between related species. CFA analysis can also be used in studies of communication and defense mechanisms in ticks and other arthropods.

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Introduction

Body surface chemical components are known to play crucial roles in the biology of arthropods, not only building up protective layers on the cuticle surface to minimize water loss but also serving as barriers against microorganisms and taking part in chemical communication (Jackson and Baker 1970; Jackson and Blomquist 1976; Blomquist and Dillwith 1985; Lockey 1988; DeRenobales et al. 1991). Moreover, cuticular surface patterns have been shown to serve as useful tools for chemo-systematic analysis (Hunt 1986; Haverty et al. 1988; Estrada-Peña et al. 1992a, b). Consequently, the body surface components have been the subject of chemical investigations in more than a hundred insect species (DeRenobales et al. 1991), revealing a diversity of hydrocarbons, alkyl esters, sterols, alcohols, aldehydes, glycerides and fatty acids (Lockey 1988). The latter extractable free lipids, which originate in glandular epidermal cells, build up a special layer which covers the outer epicuticle and are anchored to it (Blomquist and Dillwith 1985; Lockey 1988). Total cuticular fatty acid profiles (CFA profile) are increasingly used as a chemotaxonomic tool for the identification and classification of bacteria and fungi for more than two decades (Andreev 1985; Karliński et al. 2007; O'Donnell 1994; Sasser and Wichman 1991; Whittaker et al. 2005). The body surface components of several arachnid groups were studied, among which are scorpions (Hadley and Jackson 1977; Toolson and Hadley 1977), spiders (Trabalon et al. 1996; Bagneres et al. 1997), ticks (Hunt 1986; Estrada-Peña and Dusbabek 1993a, b; Estrada-Peña et al. 1992a, b, 1996, 2004), astigmatid and oribatid mites, and termites (Leal and Kuwahara 1991; Kuwahara et al. 1995; Raspotnig and Krisper 1998; Haverty and Nelson 2007). There are approximately 74 known species of *Rhipicephalus* ticks. Among these, *R. sanguineus*, *R. bursa* and *R. annulatus* are some of the most common species in the Middle East (Camicas et al. 1998). To our knowledge, the cuticular fatty acid (CFA) composition of *Rhipicephalus* ticks has not been described, although fatty acid components of insects were found to be essential barriers against environmental influences and were found to be involved in intraspecific chemical communication (Jones 1954; Alberti et al. 1997; Norton et al. 1997). Since the outer waxy cuticular layer of *Ixodid* ticks is composed of a mixture of various lipid classes, such as free and esterified sterols, esters of long chain fatty acids and alcohols, and triacylglycerols (Hackman 1982), it can potentially be used to establish a species-specific CFA profile, which might be utilized as a chemotaxonomic tool for tick identification. The aim of the current study was to characterize the total CFA profile of three *Rhipicephalus* species, *R. annulatus*, *R. sanguineus* and *R. bursa*, with respect to different hosts, namely calf and rabbit, and to determine the utility of the CFA composition as a potential chemotaxonomic tool for species-specific identification.

Materials and methods

Reagents and materials

Dichloromethane, hexane, methanol, ethanol, methyl *tert*-butyl ether, hydrochloric acid 37 %, Bacterial Acid Methyl Ester Mix and sodium hydroxide pellets were purchased from

Sigma (BAME Mix, Supelco, SigmaAldrich, Israel). All glassware used was rinsed once with redistilled dichloromethane and dried before use.

Ticks growth and collection

Adult engorged female *Rhipicephalus* species, *R. bursa*, *R. sanguineus* and *R. annulatus*, were laboratory reared at the Kimron Veterinary Institute, Israel. Engorged ticks from each species grown on one of two different hosts (calf or rabbit) were collected. *R. sanguineus* and *R. bursa* ticks were collected from laboratory-grown infested rabbits (n = 20) and calf (n = 20). *R. annulatus* ticks were collected from calf. Ticks were grown in the KVI rearing facility in accordance with the KVI animal welfare committee. Ticks from each species and host were washed twice with 2.5 ml ethanol for 5 min, air dried and processed for CFA profiling. Two ethanol washes were performed to completely remove the free fatty acids originated from the host hair. As a negative control, the fatty acid profiles of calf and rabbit hair were determined and were compared with the tick CFA profiles.

Extraction procedure and preparation of fatty acid methyl esters

Freshly distilled dichloromethane (2.5 ml) was poured into 10.0 ml glass vials each containing 1 engorged adult female tick per species and host. The ticks in the glass vials were sonicated for 10 min at 43 kHz, after which the dichloromethane was transferred into a new glass tube and evaporated to dryness at 25 °C under a stream of N_2. Methyl esterification of total CFA was accomplished according to Sasser and Wichman (1991). Briefly, 1.0 ml of 4 M methanolic/NaOH solution was added to each tube containing dried cuticular extract and heated in a water bath at 100 °C for 30 min. The methylation of hydrolyzed fatty acids was accomplished by adding 2.0 ml of 3 M methanolic/HCl solution to the cooled tubes followed by heating at 80 °C for 10 min. Fatty acid methyl esters were extracted with 1.25 ml of a hexane: methyl tert-butyl ether (1:1) mix and subsequently washed with 0.3 M NaOH solution. The organic phase was transferred directly into a GC vial and injected into the GC/MS (Agilent 7890A Model equipped with an Agilent 5975C VL MSD mass spectrometry).

Analysis of total cuticular fatty acid methyl esters

Total cuticular fatty acid methyl esters were analyzed by GC/MS. The separation of fatty acid methyl esters was achieved using DB-5MS capillary column (30 meter length, 0.25 mm internal diameter, 0.25 μm film thickness). The temperature program was as follows: injector temperature, 230 °C; initial temperature, 180 °C for 3 min; gradient of 17 °C/min until 250 °C; gradient of 10 °C until 300 °C; hold time, 2 min. The mass spectrometer parameters were set as follows: source temperature, 230 °C; transfer line, 230 °C; positive ion monitoring; EI-MS (70 eV). Qualitative analysis was performed by comparing the pure mass spectrum of each eluting compound with those in the NIST 05 mass spectral library. In addition, the identity of each eluting fatty acid methyl ester was verified by comparing the mass spectrum and retention time with a corresponding standard mixture composed of 26 known fatty acid methyl esters (BAME Mix). Integration of the total ion chromatogram was performed using Agilent Chemstation data analysis software (Agilent Technologies®). Peak areas were converted to percentages of the total fatty acid fraction, excluding peaks with an area fraction below 1 %. This procedure standardized

runs by eliminating differences in sample volumes related to the size of tick specimens (Estrada-Peña et al. 1992a; Haverty and Nelson 2007).

Statistical analysis

Mean percentage abundance of total peak area was compared for each major fatty acid. Comparisons were made between the three species from the same host and within the same species under different hosts (Table 1), by utilizing a multiple comparison ANOVA test, followed by Tukey-test. The mean abundance values of the same species under different hosts were analyzed using student t test with a p value of <0.05 considered to be significant. The statistical tests were performed with GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla, CA, USA).

Results and discussion

In this study we report for the first time the total cuticular fatty acid (CFA) profile of engorged adult female ticks of three *Rhipicephalus* species: *R. bursa, R. sanguineus* and *R. annulatus.* We quantified only fatty acids with a relative total abundance of more than 1 %, due to the fact that fatty acids with lower abundance occurred at higher variability and often were below the detection limit for all species analyzed. Our analysis showed that a substantial fraction of the fatty acids found on the surface of unwashed ticks originated from the host's hair (Supplementary Fig. S1). The fatty acids associated specifically with the calf hair and detected on the cuticle of all unwashed *R. annulatus*, *R. sanguineus* and *R. bursa* ticks grown on calf, were capric acid (C10), lauric acid (C12), 2-hydroxy-palmitic acid (2-OH-C16), behenic acid (C22) and lignoceric acid (C24) (Supplementary Fig. S1). Moreover, 2-OH-C16, was the only fatty acid specifically associated with the rabbit hair, which was also detected in all unwashed *R. sanguineus* and *R. bursa* ticks (Supplementary Fig. S1). Therefore, additional ethanol treatment was taken in order to remove fatty acid residues associated with the host. The ethanol rinse was repeated twice and consisted of immersion of each tick in 2.5 ml ethanol for 5 min under mild shaking. The washing was highly efficient in removing free fatty acids associated mainly with the host's hair, as can be deduced by comparing the fatty acid chromatograms of the ticks before and after the washing step and the fatty acid profile and the corresponding host (Supplementary Fig. S1). Fatty acid analysis of the third consecutive washing step did not reveal any additional fatty acids; we therefore concluded that two ethanol washes were completely sufficient. The fatty acid composition and intensities of the ethanol fraction used to immerse ticks immediately after removal from the calf and rabbit were similar to the fatty acid profile of the calf hair and of the rabbit hair, respectively. To exclude the possibility of fatty acid contamination from the host's blood, blood isolated directly from engorged ticks grown on calf was analyzed under identical conditions to the tick's CFAs. A comparison of fatty acids profile obtained from calf blood isolated from engorged ticks to the CFA profile of the same ticks, revealed a completely different fatty acids profile (supplementary Figure S2). In addition to the prominent differences in the relative abundance of the fatty acids that were common to the tick's CFA and the calf blood (C16, 9-C18:1, 9,2-C18:2, C18), two fatty acids, namely 5,8,11,14-C20:4 (arachidonic acid) and C21(Heneicosanoic acid), were unique to the calf blood and completely absent from the CFA profile obtained from any of the tick species fed on calf (supplementary Figure S2). Consequently, a possible contamination of CFA's profile by the host's blood was ruled out.

Table 1 Cuticular fatty acid profile from three adult *Rhipicephalus* tick species (n = 20 for each tick species)

Fatty acid[a]	RT (min)[b]	*R. sanguineus* on			*R. bursa* on			*R. annulatus* on	Comparison of % fatty acid mean values with calf as a host[d]
		calf mean %[c] ± SD[e]	rabbit mean % ± SD	Unpaired *t* test: calf versus rabbit	calf mean % ± SD	rabbit mean % ± SD	Unpaired t test: calf versus rabbit	calf mean % ± SD	
C14	14.4	5.8 ± 0.9	2.2 ± 0.5	$p < 0.001$	5.3 ± 1.1	1.8 ± 0.5	$p < 0.0001$	4.5 ± 1.4	*Rs* versus *Ra*: $p < 0.01$
C16	18.2	42.2 ± 5.6	39.4 ± 2.7	NS	30.1 ± 2.5	28.5 ± 2.6	NS	33.2 ± 2.1	*Rs* versus *Rb*: $p < 0.001$ *Rs* versus *Ra*: $p < 0.001$
cis,cis-9,12-C18:2	21.6	1.9 ± 0.7	2.3 ± 0.8	NS	7.2 ± 2.5	15.7 ± 2.1	$p < 0.0001$	5.4 ± 3.2	*Rs* versus *Rb*: $p < 0.001$ *Rs* versus *Ra.*: $p < 0.001$
cis-9-C18:1	21.7	2.6 ± 0.4	2.9 ± 0.7	NS	8.0 ± 2.7	10.0 ± 3.7	NS	6.5 ± 2.1	*Rs* versus *Rb*: $p < 0.001$ *Rs* versus *Ra.*: $p < 0.001$
C18	22.3	41.1 ± 5.6	43.4 ± 3.6	NS	36.2 ± 4.2	33.9 ± 2.9	NS	51.1 ± 6.5	*Rs* versus *Rb*: $p < 0.05$ *Rs* versus *Ra.*: $p < 0.001$ *Rb* versus *Ra.*: $p < 0.001$
C20	26.3	3.1 ± 0.5	3.2 ± 0.4	NS	10.1 ± 3.5	10.2 ± 3.3	NS	<1	*Rs* versus *Rb*: $p < 0.0001$

Ra R. annulatus, *Rb R. bursa, Rs R. sanguineus*. *NS* not significant

[a] Fatty acids that co-elute as their corresponding methyl esters listed in order of their retention time. C14: myristic acid, C16: palmitic acid, cis,cis-9,12-C18:2: linoleic acid, cis-9-C18:1: oleic acid, C18: stearic acid, C20: arachidic acid

[b] *RT* retention time in minutes

[c] Mean (n) = mean percentage contribution to total area of all peaks with the total number of ticks in parenthesis

[d] One way ANOVA test followed by Tukey Test

The extraction of the total CFA required more harsh conditions using dichloromethane as an extraction solvent and a 10 min sonication step. Chromatographs of dichloromethane extracts of all investigated species irrespective of host constantly showed five major peaks, listed according to their retention times (RT): myristic acid (C14; RT = 14.4 min), palmitic acid (C16; RT = 18.2 min), linoleic acid (9,12-C18:2; RT = 21.6), oleic acid (9-C18:1; RT = 21.7) and stearic acid (C18; RT = 22.3 min), while arachidic acid (C20; RT = 26.3 min) was present only in *R. sanguineus* and *R. bursa* at levels above 1 % (Table 1; Fig. 1).

A comparison of the CFA profile obtained from *R. sanguineus* grown on two different hosts, revealed an identical fatty acid composition with comparable abundances of C16, 9,12-C18:2, 9-C18:1, C18 and C20 (Table 1; Fig. 1). Only C14 was significantly more abundant ($p < 0.001$) in CFA profile obtained from ticks fed on calf as compared to ticks fed on rabbit. The two most abundant CFA of *R. sanguineus* were C16 and C18 (Fig. 1d; Table 1). The CFA profiles of *R. bursa* fed on calf and on rabbit were identical regarding their fatty acid composition, however the abundances of C14 and 9,12-C18:2 were significantly higher in ticks fed on calf as compared to ticks fed on rabbit. The abundances of the major fatty acids C16, 9-C18:1, C18 and C20 were independent of the host. As was found for *R. sanguineus*, the most prominent CFAs of *R. bursa* were C16 and C18 (Table 1). *R. annulatus* feeds almost exclusively on cattle as a host, consequently the CFA profile of its components was determined exclusively for ticks fed on calf. Similar to *R. sanguineus* and *R. bursa*, the most abundant fatty acids were C16 and C18, whereas in contrast to the latter two species, C20 was less than 1 % of its total fatty acid components. A direct comparison of the fatty acid abundances of the three *Rhipicephalus* species using a multiple one way ANOVA test followed by Tukey-test, revealed that *R. sanguineus* was the most distinguished species in terms of fatty acid profile, showing a significantly different abundance pattern in comparison to both *R. bursa* and *R. annulatus* for fatty acids C16, 9,12-C18:2, 9-C18:1 and C18. *R. sanguineus* had a significantly higher abundance of C14 (5.8 %) as compared with *R. annulatus* (4.5 %), while C20 was less prevalent (3.1 %) in *R. sanguineus* as compared with *R. bursa* (10.1 %) (Table 1).

Interestingly, C18 and C20 were the only fatty acids enabling a clear distinction of *R. bursa* from *R. annulatus* based on their relative abundances (Table 1). The low abundance of C20 (<1 %) and the highest abundance of C18 (51.1 %) in the *R. annulatus* CFA profile, may be used as biomarkers to distinguish *R. annulatus* from *R. sanguineus* and *R. bursa*. Furthermore, the relative abundance ratios of the fatty acids determined to be host independent for both *R. sanguineus* and *R. bursa*, namely arachidic acid: stearic acid: oleic acid: palmitic acid, might potentially be used as a differentiation tool for the aforementioned species. The relative abundance ratio for the latter four fatty acids obtained for *R. sanguineus* and *R. bursa* after rounding off was 1:16:1:16 and 1:4.5:1:4 respectively.

In the early 90's Estrada-Peña et al. (1992a, b, 1993a, b) showed that different adult *Ixodidae* ticks exhibited a unique fingerprint profile of cuticular hydrocarbons and suggested that this can be used for species identification. However, more than 100 different cuticular hydrocarbons of various carbon chain length, saturation and branching degrees were identified for each species. Due to the large number of identified hydrocarbons and a relatively high variability of their occurrence, this technique may complicate the process of biochemical tick identification.

In the present study, we utilize the host independent CFAs palmitic acid, oleic acid, stearic acid and arachidic acid, as biomarkers for tick species identification (Table 1). Free fatty acids on the insect cuticle generally occur in homologous series of even carbon numbers which can be short or extensive, in most cases being located in the range between

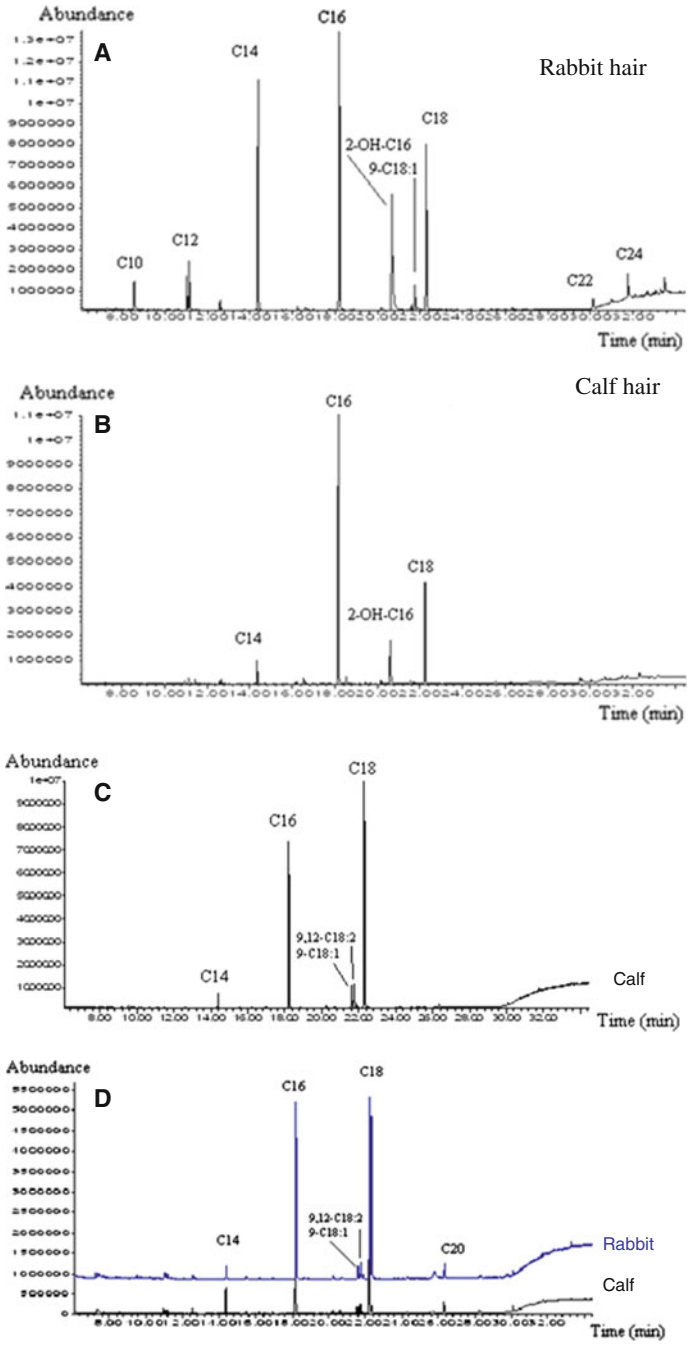

Fig. 1 Total ion chromatograms of CFA profile of calf hair (A), rabbit hair (B), adult female *Rhipicephalus annulatus* (C), *R. sanguineus* (D) and *R. bursa* (E) after washing step with ethanol. Time axis is given in minutes. Each peak in the chromatogram is designated with its corresponding fatty acid: C10, capric acid; C12, lauric acid; C14, myristic acid; C16, palmitic acid; cis,cis-9,12-C18:2, linoleic acid; cis-9-C18:1, oleic acid; C18, stearic acid; C20, arachidic acid; C22, behenic acid; C24, lignoceric acid

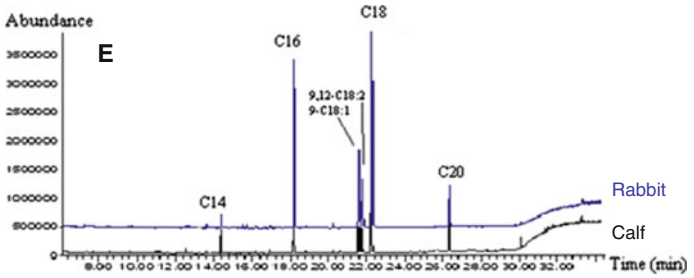

Fig. 1 continued

10 and 36 carbon atoms (Lockey 1988). In *Rhipicephalus* spp. C18-fatty acids of different saturation grade were shown to be most abundant, being frequently accompanied by significant levels of C16-, C14- and C12-fatty acids. This situation may also be true for other acari species and requires further investigation. For example, in the mite genus *Tortonia*, the CFA profile reflects large quantities of 9-C18:l-, 9,12-C18:2-, C18- and C16- fatty acids (Kuwahara et al. 1995) and therefore exhibits a similar fatty acid series, compared with *Rhipicephalus* spp. Moreover, free fatty acids with odd carbon numbers are not known from the arachnid cuticle and are possibly rare in insects as well. Our findings are in agreement with these observations. With regards to the effect of the host on the CFA composition, our data suggest that the composition of the host's free fatty acids needs to be considered. In this study, we applied a pre-treatment washing step and demonstrated that it altered the initial profile by removing host-originated fatty acids. Various factors such as sex, age, physiological state, climate and geographic origin might result in variations of fatty acids composition (Andreev 1985; Karliński et al. 2007; O'Donnell 1994; Sasser and Wichman 1991; Whittaker et al. 2005). Further investigations on the CFA chemistry of *Rhipicephalus* and other tick species is required in order to expand our knowledge on their relationships with their hosts and on inter-species variations, in the context of cuticular components.

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