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Antimicrobial activity in the egg wax of the tick *Amblyomma hebraeum* (Acari: Ixodidae) is associated with free fatty acids C16:1 and C18:2

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Abstract Untreated eggs of the tick *Amblyomma hebraeum* Koch (Acari:Ixodidae) exhibited antimicrobial activity (AMA) against Gram-negative but not Gram-positive bacteria; eggs denuded of wax by solvent extraction showed no AMA. The unfractionated egg wax extract, however, showed AMA against Gram-positive but not Gram-negative bacteria, as also shown by Arrieta et al. (Exp Appl Acarol 39: 297–313, 2006). In this study we partitioned the egg wax into various fractions, using a variety of techniques, analyzed their compositions, and tested them for AMA. The crude aqueous extract exhibited AMA. However, although more than 30 metabolites were identified in this extract by nuclear magnetic resonance analysis, none of them seemed likely to be responsible for the observed AMA. In the crude organic extract, cholesterol esters were the most abundant lipids, but were devoid of AMA. Fatty acids (FAs), with chain lengths between C13 to C26 were the next most abundant lipids. After lipid fractionation and gas chromatography/mass spectroscopy, free FAs, especially C16:1 and C18:2, accounted for most of the AMA in the organic extract. The material responsible for AMA in the crude aqueous extract remains unidentified. No AMA was detected in the intracellular contents of untreated eggs.

Keywords Ticks · *Amblyomma hebraeum* · Antimicrobial activity · Egg wax composition · Gram-positive bacteria · Gram-negative bacteria · Fatty acids · Antibiotic

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Introduction

Female ticks of the family Ixodidae imbibe an enormous blood meal during the 7–14 day feeding period, achieving a fed-to-unfed weight ratio of ~100 (Kaufman 2007). Over the next couple of months they lay a mass of eggs ultimately reaching 40–50 % of their engorged weight (Friesen and Kaufman 2002), and then they die. In a large species like *Amblyomma hebraeum*, the egg mass can approach 1,500 mg.

Under natural conditions, ticks lay their eggs on the ground under vegetation, where they are exposed to the rich soil microflora (Ment et al. 2010). How long the eggs spend there before hatching depends largely on the ambient conditions, including temperature, but *A. hebraeum* hatches within about 2 months when held at 26 °C at high relative humidity. Although in the wild, the humidity where the eggs are laid can be relatively high, the tiny eggs are subject to desiccation as well as to attack by soil bacteria and fungi. However, newly laid eggs are coated with a wax-like substance secreted by Gené's organ (Booth 1992). The wax is sticky, so the eggs adhere together in a mass. This tendency to stick together, along with the inherent impermeability of the wax to water, contribute to the eggs' resistance to desiccation.

Although most research has focused on the waterproofing function of the wax (Lees and Beament 1948; Booth et al. 1986; Schöl et al. 2001), the wax is also endowed with antimicrobial activity (AMA). Potterat et al. (1997) observed that although dead females (*Rhipcephalus (Boophilus) microplus*) following oviposition can be covered with fungi, only few of the laid eggs around them are infected. Arrieta et al. (2006) demonstrated the following in *A. hebraeum*: (1) Normal eggs inhibit the growth of Gram-negative (though not Gram-positive) bacteria. (2) Eggs denuded of wax by solvent extraction lost this AMA. (3) Surprisingly, when egg wax was extracted with chloroform/methanol, this material showed AMA against only Gram-positive bacteria. (4) The antimicrobial component was heat stable and resistant to proteinase K and pronase. (5) The antimicrobial material was also detected in Gené's organ, the egg waxing organ in ticks, but only from the time that oviposition begins (~10 days post-engorgement).

Esteves et al. (2009) demonstrated that the mRNA of microplusin, an antimicrobial peptide originally detected in the haemolymph of *Rhipicephalus (Boophilus) microplus*, is also found within the egg; levels of this mRNA were very low in recently oviposited eggs, but increased beginning around day 9 of oviposition, and attaining the highest level just prior to hatching. The same authors prepared an aqueous extract of material from the surface of the eggs, and fractionated it by reverse-phase-HPLC. Several adjacent fractions exhibited AMA against the Gram-positive bacterium *Micrococcus luteus*, and another fraction inhibited growth of the yeast *Candida albicans*; no fraction exhibited AMA against *E. coli* (Gram-negative).

In this study, we have attempted to identify components of the organic phase and aqueous phase of the egg wax of *A. hebraeum*, with a view toward determining the identity of the antimicrobial material.

Materials and methods

Tick feeding and egg collection

Both female and male *A. hebraeum* were fed together within a cloth-covered foam arena on the backs of rabbits, as described by Kaufman and Phillips (1973). Engorged, detached females were rinsed with water, dried with tissue paper, weighed and stored within mesh-

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covered plastic vials, and held in an incubator (darkness, 26 °C and ≥95 % RH). Laid eggs were collected every few days, weighed and frozen at −20 °C.

Extraction of egg wax

Based on the method of Arrieta et al. (2006), 3 ml chloroform/methanol (2:1, v/v) were added to each batch of eggs (~1 g in a glass vial) and vortexed for ~15 s. The supernatant was transferred to a clean glass vial (vial A), and 1 ml distilled water was added to the eggs. The egg-water suspension was vortexed for 15 s, and the subsequent aqueous supernatant was added to vial A. Two ml chloroform/methanol (2:1, v/v) were added again to the eggs, vortexed, and the supernatant added to vial A. After vortexing, the extracts were centrifuged for 10 min at 3,000 rpm to separate the phases. The lower phase was transferred to a new 5-ml pre-weighed glass vial (vial B). The upper phase was washed with 2 ml chloroform/methanol (2:1, v/v), vortexed, centrifuged again, and this lower phase was added to vial B. The combined lower phase solvents (organic phase) were dried under nitrogen stream in a fume hood. The dry weight was recorded and re-dissolved in 100 µl chloroform/methanol (2:1, v/v) for storage at −20 °C; this material is referred to below as "organic extract". The upper phase (aqueous phase) was lyophilized and stored at −80 °C until ready for use; this material is referred to below as "aqueous extract".

Antimicrobial spectrum of the eggs and egg wax extract in solid culture assay

Samples were tested against four Gram-negative bacteria (*Escherchia coli, Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Burkholderia vietnamiensis*) and four Gram-positive bacteria (*Staphylococcus epidermidis, Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis*). Each strain was suspended in 10 ml of Mueller–Hinton broth (MHB) (Difco) and incubated overnight at 37 °C. Under aseptic conditions, 0.1 ml of suspension was mixed with 4 ml soft agar (0.5 % agar in distilled water) and quickly poured and spread onto a Mueller–Hinton base agar plate (1.5 % agar with MHB).

Whole tick eggs and eggs denuded of wax were deposited directly onto agar plates overlaid with bacteria. The organic phase (10 mg/50 µl) or aqueous phase (2 mg/50 µl) was added to filter paper discs (6 mm), air dried for 15 min, and subsequently deposited onto the bacterial overlay plates. Plates were incubated for 48 h at 37 °C, and the inhibition zone was measured with calipers to the nearest 0.1 mm.

Lipid fractionation and determination of antimicrobial activity

As described by Lynch and Steponkus (1987), we fractionated total lipid extracts into (1) neutral lipid, (2) phospholipids, and (3) glycolipid plus fatty acid fractions by solid-phase extraction, using silica Sep Pak cartridges (Supelco). Organic extracts were first evaporated under N_2, re-dissolved in 1 ml chloroform, and transferred to the cartridge. Once the sample entered the packing, residual sample was washed into the column using 2 ml chloroform, followed by an additional 10–12 ml chloroform to elute neutral lipids. This was followed by addition of 15 ml acetone/methanol (9:1, v/v) to elute glycolipids plus fatty acids. Finally, phospholipids were eluted using 10 ml methanol. Fractions were dried under N_2, weighed, and re-dissolved in chloroform/methanol (2:1, v/v). Samples were applied to small paper discs, and then tested on *S. aureus* in a solid culture assay following solvent evaporation. The glycolipid/FA fraction (the one that showed AMA) was further analyzed for lipid composition by HPLC, and for FA composition by gas chromatography/mass spectrometry.

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High-performance liquid chromatography (HPLC) analysis

We analyzed samples by HPLC using an Agilent 1100 instrument equipped with a quaternary pump and an Alltech ELSD 2000 evaporative light-scattering detector (ELD). The column used was an Onyx monolithic Si (Phenomenex) and the method was based on that of Graeve and Janssen (2009). Solvent A consisted of isooctane:ethylacetate (99.8:0.2); solvent B was acetone:ethylacetate (2:1) with 0.02 % acetic acid; solvent C was isopropanol:water (85:15) with acetic acid and ethanolamine each at 0.05 %. Detector gas flow was 3.0 l/min and drift tube temperature was set at 60 °C. Lipid extract (5 ml) containing phosphatidyldimethylethanolamine as an internal standard was injected onto the column, and eluting peaks were analyzed using Agilent Chemstation software and quantified using calibration curves prepared with commercial lipid standards (Sigma-Aldrich, Avanti Polar Lipids).

Fatty acid methyl ester (FAME) quantification by gas chromatography—flame ionization detection (GC-FID) and gas chromatography—mass spectrum analysis (GC–MS)

Egg wax fatty acids were converted to their FAMEs according to the method of Myher et al. (1989) with minor modifications. Briefly, lipid extracts containing heptadecanoic acid as an internal standard were incubated with a solution of H_2SO_4 in methanol for 1–2 h. The reaction was then neutralized and FAMEs extracted with hexane. After passage through a Na_2SO_4 column to remove residual water, the resulting effluent was evaporated under a stream of N_2 and re-dissolved in 200 µl of hexane. Measurement of FAMEs in the lipid fraction was performed using GC-FID. Briefly, 2 µl of the latter was injected into an Agilent 6890 gas chromatography instrument equipped with a flame ionization detector. The column used was a J&W DB-23 column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies). FAMEs were identified and quantified by comparison to retention times and calibration curves of commercial standards (Sigma-Aldrich).

For measurement of FAMEs by GC–MS, analysis was performed on an Agilent 6890 N coupled to an Agilent MS-5975 inert XL mass selective detector in Electron Impact (EI) mode. Separation of fatty acids was achieved by injecting 2 µl of the FAMEs onto a J&W DB-5 column (30 × 0.25 mm × 0.25 µm, Agilent Technologies). A split injection (10:1) was performed with a constant carrier gas (helium) flow of 1 ml/min. Inlet temperature and transfer line temperatures were set at 250 and 280 °C respectively. Temperature programming was as follows: initial isotherm of 70 °C held for 1 min, raised to 76 °C at 1 °C/min, followed by 76–310 °C at a rate of 6.1 °C/min. The MS ion source temperature was 230 °C and the quadrupole temperature was 150 °C. Peak identification of fatty acids in the analyzed samples was carried out by comparison of the chromatogram with a mass spectral library (NIST) and against the retention times and mass spectra of Supelco 37 component FAME mix (Sigma-Aldrich, St Louis, MO, USA).

Antimicrobial activity of standard fatty acids and model fatty acid mixtures

Three saturated fatty acids (C15:0, C16:0 and C18:0) and three unsaturated fatty acids (C16:1, C18:1 and C18:2; Sigma Chemical Company) that were found in the FA fraction containing AMA were dissolved in chloroform/methanol (2:1, v/v) and tested individually in varying amounts: 0.05, 0.10, 0.25, 0.5, 1.0, 2.5 and 5.0 mg per disc, after solvent evaporation (Table 4).

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The FAs were also tested for AMA as a mixture corresponding to their relative amounts in the FA fraction of the egg wax (we call this a "model FA mixture"). The relative amounts were determined by GC/MS following lipid fractionation of the organic extract. The amounts detected from this procedure were, in descending order: C18:1 (70 μg), C16:0 (51 μg), C18:2 (34 μg), C18:0 (25 μg), C15:0 (10 μg), C16:1 (9 μg), C22:1(13) (6 μg), C14:0 (5 μg), C22:0 (3 μg), C23:0 (2 μg), C13:0 (2 μg), C20:0 (1 μg), (1 μg) and C24:0 (1 μg). The model FA mixtures were prepared from the first six of these FAs only, as shown in Table 5.

For both Tables 4 and 6, samples were tested against four Gram-negative bacteria (*E. coli, S. maltophilia*, *P. aerguinosa* and *B. vietnamiensis*) and four Gram-positive bacteria (*S. epidermidis, S. aureus*, *B. cereus* and *B. subtilis*) by exposing the discs to a solid culture assay.

Temperature and pH stability of the aqueous extract of the egg wax

The aqueous extract was lyophilized and re-dissolved in distilled water, and 50 μl aliquots (~2 mg dry weight) were pipetted into 1.5 ml polyethylene microcentrifuge tubes. Each aliquot was heated to 100 °C for 0, 10, 20, 30, 40, 50 or 60 min, centrifuged and held at 4 °C until the samples were tested for AMA. Another seven aliquots were prepared with the pH adjusted to 2, 4, 6, 7, 8, 10 or 12 and held at 4 °C overnight. They were then all adjusted to pH 7 (with HCl or NaOH) and tested for AMA in a solid culture assay.

Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of the aqueous phase extract

The micro-dilution broth method based on the method of Otvos and Cudic (2007) was used to determine the MIC and MBC. Four Gram-positive bacteria (*S. epidermidis*, *S. aureus*, *B. cereus* and *B. subtilis*) and four Gram-negative bacteria (*E. coli*, *S. maltophilia*, *P. aerguinosa* and *B. vietnamiensis*) were cultured overnight at 37 °C in MHB. Overnight cultures were transferred to fresh MHB and grown for a further 3.5 h prior to assay. MHB was dispensed into sterile 96-well polypropylene microtiter plates, and serial half-dilutions were made, beginning with 200 μl aqueous extracts (2 mg/ml for Gram-positive bacteria and 10 mg/ml for Gram-negative bacteria). Subsequently, 5 μl of a bacterial suspension was added to each well, except for control wells, which received 5 μl sterilized distilled water. The plates were incubated at 37 °C for approximately 18 h and absorbance was read at 600 nm. MICs were identified as the lowest concentration of sample that appeared to inhibit bacterial growth significantly.

Minimum bactericidal concentrations were determined according to the method of Nakajima et al. (2003). We first identified the three lowest concentrations from the 96-well microtitre plates that showed no visible bacterial growth on plates after incubation at 37 °C for 24 h. We then transferred 5 μl from each of these wells onto fresh wells containing MHB and incubated for a further 24 h at 37 °C. The lowest concentration among these samples that showed no bacterial growth was recorded as the MBC.

Tricine SDS–polyacrylamide gel electrophoresis (PAGE), native-PAGE analysis and gel overlay assay for AMA

To analyze the protein composition of the aqueous extract, we conducted a 12 % tricine SDS-PAGE analysis and a 12 % native-PAGE analysis. After electrophoresis, gels were stained with Coomassie Brilliant Blue G-250 for SDS-PAGE and Coomassie Brilliant Blue

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R-250 for native-PAGE. For the gel overlay assay, the freeze-dried aqueous phase was dissolved in distilled water and loaded onto a PD-10 desalting column (Amersham Biosciences, Canada). Two fractions, one containing most of the protein and the other being essentially protein-free, were eluted with water, collected and lyophilized. The lyophilized fractions were dissolved in distilled water ($\sim$2 mg/50 µl), subjected to 12 % native-PAGE overnight at 40 V and at 4 °C, and the gel was subsequently stained with Coomassie Brilliant Blue G-250. The gel was washed in distilled water, laid over an MHB agar plate, covered with soft agar containing bacteria in the logarithm growth phase, and the entire overlay assembly was incubated at 37 °C overnight. A clear, microorganism-free zone in the underlying agar would be interpreted as the location of antimicrobial substances in the gel.

Sample analysis using NMR spectroscopy

After passing through a PD-10 desalting column, the aqueous fraction showing AMA (this material is referred to in Table 8 as "whole") was lyophilized, re-dissolved in distilled water and centrifuged through a 10 kD centrifuge filter. The filtrate and filtrand were tested against *S. aureus*. Unfortunately we did not have a sufficient amount of filtrand to analyze by NMR, so instead we analyzed the whole material and the filtrate separately.

NMR sample preparation and spectroscopy

A total of 250 µl H_2O, 50 µl of 50 mM NaH_2PO_4 buffer (pH 7), 35 µl of D_2O and 15 µl of a standard buffer solution (3.73 mM DSS (disodium-2,2-dimethyl-2-silapentane-5-sulphonate and 0.47 % NaN_3 in H_2O) were added to the sample. The sample was vortexed for 1 min, sonicated for 30 min, and then transferred to a standard Shigemi microcell NMR tube for subsequent spectral analysis. All ^{1}H-NMR spectra were collected on a 500 MHz Inova (Varian Inc., Palo Alto, CA) spectrometer equipped with either a 5 mm HCN Z-gradient pulsed-field gradient (PFG) room-temperature probe. ^{1}H-NMR spectra were acquired at 25 °C using the first transient of the tnnoesy-presaturation pulse sequence. Spectra were collected with 256 transients using an 8 s acquisition time and a 1 s recycle delay.

NMR compound identification and quantification

All flame ionization detections were zero-filled to 64 k data points and subjected to line broadening of 0.5 Hz. The singlet produced by the DSS methyl groups was used as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. All ^{1}H-NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 6.0 (Chenomx Inc., Edmonton, AB), as previously described by Wishart et al. (2008) and Psychogios et al. (2011). Each spectrum was processed and analyzed by at least two NMR spectroscopists in order to minimize the chances for compound mis-identification and mis-quantification. We used sample spiking to confirm the identities of the assigned compounds. Sample spiking involves the addition of 20–200 µM of the suspected compound to selected *Clostridium* samples and examination to determine whether the relative NMR signal intensity changed as expected.

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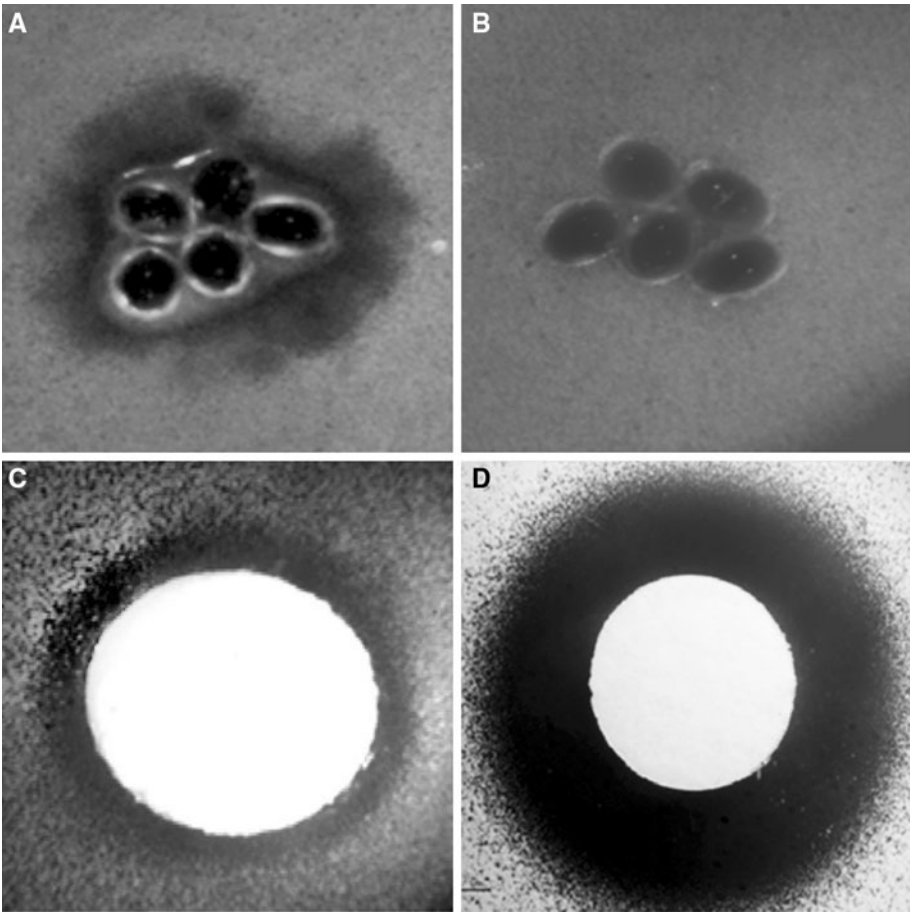

Fig. 1 Antimicrobial activity (*E. coli*) of the *A. hebraeum* eggs and egg wax extracts. **a** Intact eggs. The bacterial inhibition area appears as a dark zone surrounding the five eggs. **b** Eggs denuded of wax lost the inhibitory activity against *E. coli* (no dark zone). **c** The organic extract of the egg wax inhibited the growth of *S. aureus*. **b** The aqueous extract of the egg wax inhibited the growth of *S. aureus*

Results

Antimicrobial spectrum of the eggs and egg wax extract

Untreated eggs inhibited *E. coli* (Fig. 1a) and the other three Gram-negative bacteria tested, but not the gram-positive bacteria. The eggs denuded of wax lost the activity against *E. coli* (Fig. 1b) and the other three Gram-negative bacteria, and also showed no activity against Gram-positive bacteria (Table 1). The organic phase and aqueous phase extracts both had activity against *S. aureus* (Fig. 1c, d) and the other three Gram-positive bacteria, whereas they had no detectable effect on the growth of the four Gram-negative bacteria (Table 1). When the two extracts were combined, they still showed no effect on Gram-negative bacteria (results not shown). These results are consistent with previous results reported by Arrieta et al. (2006).

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Table 1 Spectrum of antimicrobial activity of egg wax extracts

Microorganism	Clear zone* (mm)		
	Aqueous phase	Organic phase	Ten whole eggs
Gram-positive			
S. aureus	13.4 ± 1.5	10.8 ± 0.5	0
S. epidermidis	15.0 ± 0.3	10.9 ± 0.4	0
B. cereus	15.4 ± 0.4	9.4 ± 0.7	0
B. subtilis	17.0 ± 1.2	10.3 ± 0.8	0
Gram-negative			
B. vietnamiensis	0	0	0.18 ± 0.06
P. aeruginosa	0	0	0.49 ± 0.24
St. maltophilia	0	0	0.26 ± 0.16
E. coli	0	0	0.63 ± 0.11

* Sterile paper discs impregnated with organic phase (equivalent to ~10 mg dry weight) or aqueous phase (equivalent to ~2 mg dry weight) were applied to the bacteria in solid culture, as were whole eggs. All plates were incubated for 48 h at 37 °C. Diameter of the clear zone (mean ± SEM; n = 4) includes diameter of paper discs (6 mm) or the 10 eggs

The question arises as to whether the AMA against Gram-positive bacteria may be intracellular, it being leached out during the extraction process, or whether the extraction process itself might convert the Gram-negative activity to Gram-positive activity. So we took 50 mg of whole eggs (either untreated or wax-denuded), and crushed them with forceps in a plastic dish. Small paper discs were pressed against the crushed material (either untreated or wax-denuded), in order to absorb/adsorb as much of it as possible. The discs were placed on agar plates containing either the Gram-negative *B. vietnamiensis*, or the Gram-positive *S. aureus*. We also tested 10 whole eggs, untreated and wax-denuded, as a control (two replicates of each). The untreated whole (but not crushed) eggs inhibited *B. vietnamiensis* (clear zone beyond the disc = 0.17 mm ± 0.04 SD, and 0 mm, respectively). The untreated whole eggs marginally inhibited *S. aureus* (clear zone beyond the disc = 0.045 mm ± 0.007 SD) but not the crushed eggs (clear zone beyond the disc = 0 mm).

Lipid composition of the organic extract

HPLC-ED analysis of the total lipid extract showed that the main components in the egg wax included cholesterol esters (CE), free cholesterol (FC), and fatty acids (FA). There were also detectable levels of phosphatidylcholine (PC), sphingomyelin (SM), triglyceride (TG), mono-glyceride (MG), phosphatidyl ethanolamine (PE) and phosphatidylserine (PS) (Table 2). We used GC–MS, to determine the FA composition of the organic extract, and the results are presented in Table 3.

Antimicrobial activity associated with the organic extract

After fractionation via Sep Pak cartridges (see "Lipid fractionation and determination of antimicrobial activity"), neutral lipids, glycolipids/fatty acids and phospholipids accounted for ~34, ~5 and ~3 %, respectively, of the dry weight of the egg wax extract. The remaining ~58 % consisted of unidentified (though probably non-lipid) material. When

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Table 2 Lipid composition of the egg wax of *A. hebraeum*, measured by HPLC-ELD

Retention time (min)	Amount (μg)	Relative amount in dried extract (μg/mg)	Lipid component
1.2	9.9	168.8	Cholesterol esters (CE)
4.2	–	–	Triglycerides (TG)
4.5	1.3	22.2	Free cholesterol (FC)
7.4	–	–	Monoglycerides (MG)
9.9	1.6	27.3	Fatty acids (FA)
14.1	–	–	Phosphatidylethanolamine (PE)
16.8	–	–	Phosphatidylinositol (PI)
20.6	–	–	Phosphatidylserine (PS)
25.8	0.23	3.9	Phosphatidylcholine (PC)
27.9	0.14	2.4	Sphingomyelin (SM)

assayed in solid culture for AMA, only the glycolipid/FA fraction showed inhibitory activity against *S. aureus* (results not shown).

Antimicrobial activity of the standard FAs and model FA mixture

The six main FAs plus C15:0 were tested individually against four Gram-positive and four Gram-negative bacteria (Table 4). There was no AMA associated with the C15:0, C16:0, C18:0 or C18:1 FAs up to the highest amount tested (5 mg per disc), on any of the bacteria (data not shown). The remaining FAs (C16:1, C18:2) showed AMA against most of the bacteria, with the Gram-positive *B. cereus* and *B. subtilis* being the most sensitive (AMA being expressed down to 0.05 mg per disc). The Gram-negative *B. vietnamensis* was particularly resistant, being affected by only C18:2 at the highest dose tested (5 mg per disc), and the Gram-negative *E. coli* was almost as resistant as *B. vietnamensis*.

Table 6 shows the AMA of the model FA mixture (see Table 5 for how the FA composition was arrived at). In general, the Gram-positives were more sensitive than the Gram-negatives. *E. coli* was affected only at the highest concentration tested (100 mg/ml), and *B. vietnamensis* was resistant even at this high concentration.

Antimicrobial properties of the aqueous extract of the egg wax

The aqueous extract was tested on *S. aureus* and proved to be extremely resistant to heat and changes in pH. When heated to 100 °C for up to 1 h, there was essentially no difference in AMA (Fig. 2). Even when the extract was autoclaved for 30 min at 121 °C, the aqueous extracts did not lose AMA. The aqueous extract likewise possessed great pH stability, there being no consistent differences in AMA between pH 2 through pH 12 (Fig. 2).

The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of the aqueous extract are shown in Table 7. Up to 10 mg/ml, the aqueous extract had no AMA on any of the Gram-negative bacteria (neither an MIC nor an MBC could be calculated from the range of concentrations tested). The MIC for most Gram-positive bacteria was ~0.25–0.5 mg/ml, and the MBC was ~0.25 mg/ml.

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Table 3 Fatty acid composition of the organic extract of the egg wax of *A. hebraeum* as measured by GC/MS

Retention time (min)	Identified FA	Amount in dried organic extract (μg/mg)	μg FA per μg total egg wax
6.4	C13:0	1.8	0.004
7.6	C14:0	2.2	0.005
8.9	C15:0	0.3	0.001
10.6	C16:0	49.1	0.104
11.1	C16:1	27.0	0.057
14.6	C18:0	9.5	0.020
15.2	C18:1	5.1	0.011
16.2	C18:2	0.8	0.002
17.7	C18:3	0.3	0.001
18.6	C18:4	–	–
19.4	C20:0	1.0	0.002
22.7	C20:4	0	0
24.5	C20:5	–	–
24.8	C22:0	0.7	0.002
25.6	C22:1	4.7	0.010
31.6	C24:0	0.9	0.002
32	C22:6	–	–
40.6	C26:0	3.4	0.007

The total weight of the egg wax in this sample, corresponding to the organic plus aqueous phases, was 470 μg

Tricine SDS-PAGE, native-PAGE and gel overlay assay on the aqueous extract of the egg wax

Tricine SDS-PAGE resolved the aqueous extract into six major bands (Fig. 3) between 10 and 27 kD. Native PAGE resolved the aqueous extract into four bands, two of them being rather broad.

The lyophilized aqueous fractions prepared according to "Tricine SDS–polyacrylamide gel electrophoresis (PAGE), native-PAGE analysis and gel overlay assay for AMA" were dissolved in distilled water (about 2 mg/50 μl), and tested against *S. aureus* in a solid culture assay. The protein fraction of the aqueous phase had no AMA, whereas the fraction consisting of small molecules inhibited the growth of *S. aureus* (results not shown). Then each of these fractions was loaded on a native-PAGE gel, overlayed with bacteria in soft agar, and cultured for 24 h at 37 °C. There were no discernable protein bands associated with the fraction having AMA, whereas the fraction that displayed bands of protein had no clear zone associated with any of the bands (Fig. 4). An additional technique to identify AMA in each specific protein in the proteinaceous fraction was tested (FPLC using a Mono Q column); this foray, however, was unsuccessful (data not shown).

Nuclear magnetic resonance (NMR) analysis on the aqueous extract of the egg wax

After passing through a PD-10 desalting column, the crude aqueous fraction was analyzed by NMR as described in "Sample analysis using NMR spectroscopy", which identified 36 compounds ("whole" in Table 8). Passing the whole fraction through a 10 kD centrifuge filter resulted in a filtrate and a filtrand. When tested against *S. aureus* using paper discs on

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Table 4 Antimicrobial activity of the standard fatty acids tested in solid culture (expressed as diameter of clear zone in mm, including the paper disc)

Fatty acid	Amount (mg/disc)	Gram-positive bacteria				Gram-negative bacteria			
		S. aureus	*S. epidermidis*	*B. cereus*	*B. subtilis*	*B. vietnamiensis*	*P. aeruginosa*	*St. maltophilia*	*E. coli*
C16:1	0.05	–	–	10.3 ± 1.6	11.5 ± 3.0	–	–	–	–
	0.10	–	–	10.7 ± 0.5	12.0 ± 0.9	–	–	–	–
	0.25	*	*	13.3 ± 0.4	13.3 ± 0.5	–	–	–	–
	0.50	10.3 ± 1.1	7.1 ± 0.0	15.4 ± 4.2	14.5 ± 5.6	–	–	–	–
	1.0	11.1 ± 0.7	7.8 ± 0.3	14.6 ± 0.4	13.9 ± 0.6	–	7.4 ± 0.2	–	–
	2.5	11.3 ± 0.8	8.6 ± 0.2	15.7 ± 0.8	14.7 ± 1.3	–	7.8 ± 0.2	–	–
	5.0	11.6 ± 1.9	9.5 ± 1.1	16.7 ± 5.8	14.8 ± 5.4	–	13.5 ± 1.7	8.4 ± 0.7	–
C18:2	0.05	–	–	–	*	–	–	–	–
	0.10	*	*	*	8.0 ± 0.5	–	–	–	–
	0.25	8.9 ± 0.2	10.3 ± 0.6	7.3 ± 0.3	9.1 ± 0.6	–	–	–	–
	0.5	10.4 ± 0.4	11.5 ± 2.0	10.8 ± 2.2	10.4 ± 1.1	–	–	7.7 ± 0.4	–
	1.0	9.9 ± 0.9	13.6 ± 0.9	11.5 ± 0.9	12.8 ± 0.7	–	9.0 ± 0.2	7.7 ± 0.5	–
	2.5	10.3 ± 1.4	13.8 ± 0.7	11.1 ± 0.1	13.1 ± 0.8	–	10.5 ± 0.8	8.4 ± 0.5	7.7 ± 0.4
	5.0	10.6 ± 0.7	13.5 ± 3.5	11.7 ± 0.8	13.8 ± 3.8	8.8 ± 0.7	11.1 ± 1.6	11.5 ± 2.3	9.2 ± 0.7

Diameter of the clear zone (mean ± SEM; n = 3) includes diameter of paper disc (6 mm)

The following FAs showed no AMA at any concentration tested, up to 5 mg/disc: C15:0, C16:0, C18:0, C18:1

– no clear zone observed

* marginal clear zone observed

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Table 5 How the 'model fatty acid mixture' was prepared

Concentration tested (mg/ml)		1	2	5	10	20	50	100
Fatty acid	µl	Milligrams of each fatty acid added to the paper disc						
C18:1	70	0.07	0.14	0.35	0.70	1.40	3.50	7.00
C16:0	51	0.051	0.102	0.255	0.51	1.02	2.55	5.10
C18:2	34	0.034	0.068	0.17	0.34	0.68	1.70	3.40
C18:0	25	0.025	0.05	0.125	0.25	0.50	1.25	2.50
C15:0	10	0.01	0.02	0.05	0.10	0.20	0.5	1.00
C16:1	9	0.009	0.018	0.045	0.09	0.18	0.45	0.90

Each of the six FAs shown in the left-hand column was dissolved in chloroform/methanol (2:1, v/v; 100 mg/ml), and serially diluted to the concentrations indicated in the first row of the table ("concentration tested, mg/ml"). For each concentration tested, the volumes of the fatty acid solutions indicated in the second column of the table ("µl") were mixed together. Then, the chloroform/methanol was evaporated off in a fume hood under a stream of nitrogen. The resulting dried material was then re-dissolved in 50 µl chloroform/methanol (for "concentrations tested "1–20 mg/ml"), or 100 µl chloroform/methanol (for "concentrations tested" 50 and 100 mg/ml). Each mixture was then applied to paper discs, 10 µl at a time, and air-dried in the fume hood between each 10-µl application. The discs were then exposed to a solid culture assay to test for anti-microbial activity as described in "Antimicrobial spectrum of the eggs and egg wax extract in solid culture assay". The absolute amounts (mg) of each fatty acid tested, at each concentration, are shown in the main body of this table

solid culture assay, the filtrand, but not the filtrate, showed AMA (results not shown). Unfortunately we could not get an NMR analysis on the filtrand alone, but the analysis of the whole fraction and filtrate revealed 36 compounds (Table 8). None of these looked remarkable in terms of specific AMA, although some could be toxic (e.g., the alcohols), although perhaps not at the low concentrations detected.

Discussion

The major function of the wax layer on the cuticle of terrestrial arthropods, including ticks, is to minimize water loss. Because the egg is so small, and hence the surface-to-volume ratio so high, the importance of the egg wax for retaining water in the egg cannot be overemphasized. The fact that the egg wax is sticky, and so causes the oviposited eggs to adhere together in a much larger mass, undoubtedly contributes to the water preservation strategy.

Because the eggs tend to be laid in relatively moist microhabitats, it is not surprising that the wax should also be endowed with anti-bacterial and anti-fungal agents. For example, Esteves et al. (2009) analyzed an aqueous wash of the eggs of *Rhipicephalus (Boophilus) microplus* by RP-HPLC. The resulting fractions were screened for anti-bacterial (*Micrococcus luteus*, *E. coli*) and anti-fungal (*Candida albicans*) activity. Several fractions inhibited *M. luteus* and one fraction inhibited *C. albicans*, but there was no inhibition of *E. coli*. In a previous study from this laboratory, we outlined some antimicrobial properties of the eggs of *A. hebraeum*, and demonstrated that the material is produced by Gené's organ, the egg-waxing organ in ticks (Arrieta et al. 2006). But the latter authors did not chemically characterize the antimicrobial material. Here we demonstrate that some FA components of the organic extract potentially contribute to the AMA. In addition, whereas the protein fraction of the aqueous extract showed no AMA,

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Table 6 Antimicrobal activity of model fatty acid mixture tested in solid culture (expressed as diameter of clear zone in mm, including the paper disc)

Concentration (mg/ml)	Gram-positive bacteria				Gram-negative bacteria			
	S. aureus	*S. epidermidis*	*B. cereus*	*B. subtilis*	*B. vietnamiensis*	*P. aeruginosa*	*S. maltophilia*	*E. coli*
1	–	–	–	–	–	–	–	–
2	–	–	*	*	–	–	–	–
5	*	*	6.7 ± 0.2	7.0 ± 0.4	–	–	–	–
10	8.6 ± 0.9	8.6 ± 0.6	9.2 ± 0.9	8.4 ± 0.3	–	–	–	–
20	11.8 ± 0.7	12.8 ± 0.5	9.6 ± 0.5	10.3 ± 0.4	–	8.2 ± 0.2	*	–
50	13.3 ± 1.2	12.6 ± 0.7	9.9 ± 0.4	11.0 ± 0.5	–	9.9 ± 0.5	7.7 ± 0.2	–
100	14.7 ± 0.8	14.6 ± 0.6	11.3 ± 0.4	11.6 ± 0.8	–	7.9 ± 1.1	9.7 ± 0.6	9.7 ± 0.9

Model fatty acid mixture was prepared as described in "Materials and methods"

Diameter of the clear zone (mean ± SEM; n = 3) includes diameter of paper discs (6 mm)

– no clear zone was observed

* marginal clear zone observed

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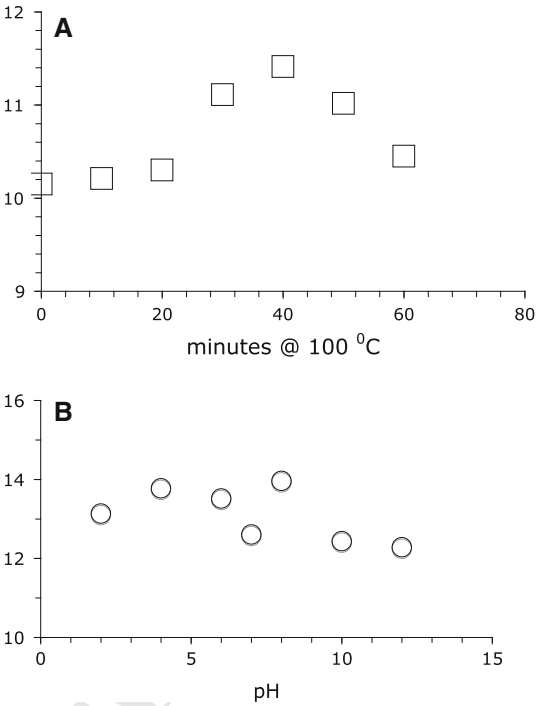

Fig. 2 Resistance to heat and extremes of pH of the aqueous extracts. Samples (2 mg/50 µl) were heated (**a**) or the pH adjusted (**b**), antimicrobial activity was tested in a solid culture assay, and clear zones were measured using calipers including the diameter of the paper discs (6 mm)

Table 7 Spectrum of antimicrobial activity of the crude aqueous extract of egg wax (each with three replicates)

Microorganism	MIC (mg/ml)	MBC (mg/ml)
Gram-positive		
S. aureus	0.25–0.5	0.25
S. epidermidis	0.5–1.0	0.50
B. cereus	0.25–0.5	0.25
B. subtilis	0.25–0.5	0.25
Gram-negative*		
B. vietnamiensis	>10	nt
P. aeruginosa	>10	nt
St. maltophilia	>10	nt
E. coli	>10	nt

The maximum concentration tested was 10 mg/ml

nt not tested

some component(s) of the "small molecule" fraction did, although they remain unidentified.

Booth (1992) conducted an analysis of egg wax lipids from *Rhipicephalus (Boophilus) microplus*, with details on their biosynthesis by Gené's organ. Although there are some differences in detail between the latter findings and ours, both waxes consist of a similar array of FAs. Moreover, Booth compared the FA composition of the tubular glands of Gené's organ with that of the egg wax. Although the alkane fraction of the wax matched that of the glands, there were some significant quantitative differences between the two sources, the equivalent chain length 25 alkane (the most abundant alkane in the gland) being almost 40 times higher (on a % composition basis) in Gené's organ than in the egg

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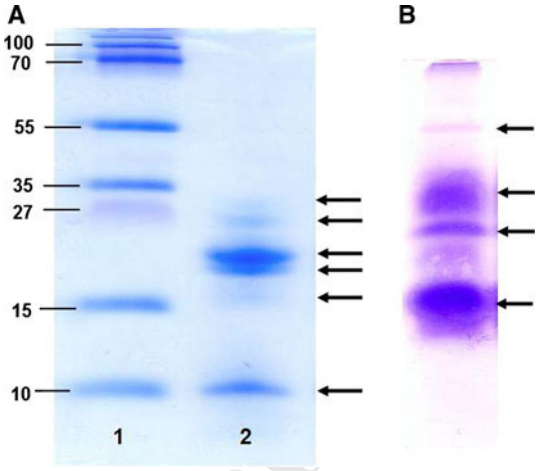

Fig. 3 Protein composition of the lyophilized crude aqueous extract re-dissolved in distilled water. **a** 12 % tricine SDS-PAGE. *Lane 1*: molecular weight markers. *Lane 2*: sample. **b** native PAGE

wax, and the equivalent chain length 32–35 being 3–12 times higher in the wax than in the tubular glands. Booth did not, however, report on the composition of the aqueous phase of his extract. A particularly important contribution by Booth was an analysis of the biosynthesis of egg wax lipids from the incorporation of ^{14}C-acetate in ovipositing ticks. A relatively small percentage of the total acetate was recovered from the egg wax (0.5–2.3 %), but of this, 78–87 % was incorporated into the non-polar fraction of the egg wax. The distribution of incorporated label differed significantly among the three major glandular cell types of Gené's organ: tubular, lobular and acinar accessory gland cells (Booth 1992).

Although in this study we found that cholesterol and cholesterol esters were the most abundant lipids, they had no AMA (data not shown). All of the AMA of the organic extract was associated with the FA fraction. The AMA of FAs has been reported previously. For example, FAs of 10–16 carbon atoms are optimal for inhibiting both Gram-positive and Gram-negative bacteria, with *cis*-unsaturated FAs yielding generally stronger activity (Desbois and Smith 2010), likely as a result of kinks that destabilize surrounding membrane structure. Our results on the standard FAs shown in Table 4 are broadly in accord with this, although C16:1 is the only FA showing strong activity. In general, longer chains appear to affect Gram-positive bacteria more strongly (Desbois and Smith 2010).

Desbois and Smith (2010) have reviewed potential mechanisms by which FAs may inhibit and kill bacteria. Because removing either the peptidoglycan layer of Gram-positive bacteria or the outer membrane of Gram-negative bacteria increases their susceptibility to FA activity, this suggests that the cytoplasmic membrane is the main site of activity. The electron transport chain could be disrupted by the interference of FAs with electron carrier or proton efflux enzymes. Likewise, oxidative phosphorylation could be uncoupled from proton pumping by a general disruption of membrane integrity, greatly undermining the ATP-generation system's efficiency. A more severe form of membrane disruption, in which larger pores are formed, or the membrane itself is dissolved by the FAs, could lead to cell lysis. Finally, reactive oxygen species formed by the degradation of FAs in the cell may be responsible for the observed toxicity.

An enigma that still remains is why whole eggs are active against Gram-negative but not Gram-positive bacteria, whereas the organic extract, presumably containing most, if not all, of the FAs is the converse? Equally puzzling are the results with the aqueous

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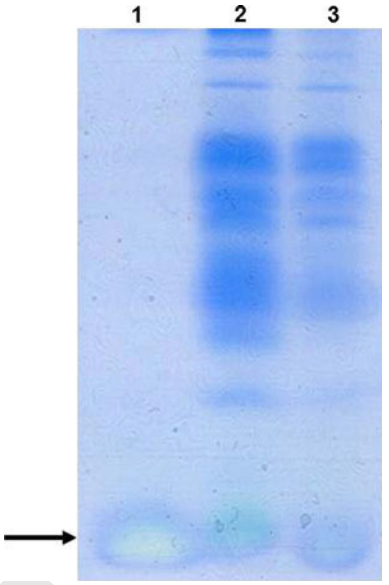

Fig. 4 Native-PAGE after PD-10 desalting column and gel overlay experiment. *Lane 1*: Fraction with small molecules collected from PD-10 desalting column (2 mg dry weight). *Lane 2*: fraction with protein collected from PD-10 desalting column (2 mg dry weight). *Lane 3*: Total sample before loading on PD-10 desalting column (2 mg dry weight). *Arrow* indicates the clear inhibition zone

extract. The aqueous extract, at only 2 mg per disc, was a more potent inhibitor (i.e. generally larger inhibition zone) of Gram-positive bacteria than the organic extract at 10 mg per disc (Table 1). It is certainly possible that the aqueous material diffuses more readily into the culture plate medium than do the FAs of the organic extract, and that this factor accounts for its apparently higher potency.

Might the Gram-positive AMA reside within the egg and the Gram-negative AMA in the wax, the extraction process somehow inactivating the Gram-negative toxin? Our experiments with whole and crushed eggs ("Antimicrobial spectrum of the eggs and egg wax extract") suggest otherwise. The crushed untreated eggs, as well as the crushed denuded eggs showed no AMA against Gram-negative or Gram-positive bacteria. These results suggest that the AMA against Gram-positive material found in the egg-wax extracts did not originate from within the egg itself. If it did, we would have expected the denuded crushed eggs to have inhibited *S. aureus* only, and the untreated whole crushed eggs to exhibit AMA activity against both *B. vietnamiensis* and *S. aureus*. What remains puzzling is why the egg wax of the crushed, untreated eggs did not inhibit *B. vietnamiensis*. Our overall, though still tentative, conclusion is that the extraction procedure itself changed the properties of the antimicrobial substance(s).

To date, few studies have investigated the relationship between AMA and the protein composition of the egg wax. Using RP-HPLC, Esteves et al. (2009) detected at least two water-soluble antimicrobial factors associated with the egg surface of *R. microplus*; one inhibited the Gram-positive *M. luteus*, and the other inhibited the yeast *Candida albicans*, but they did not identify the compounds responsible. Here, we have demonstrated that proteins contained within the egg wax of *A. hebraeum* seem to have no AMA ("Tricine SDS-PAGE, native-PAGE and gel overlay assay on the aqueous extract of the egg wax"); the source of these proteins is probably Gené's organ (Arrieta et al. 2006), but the functional significance of those proteins in the egg wax is unknown.

When the AMA-containing fraction (after elution from the PD-10 column) was applied to a 10 kD centrifuge filter, the retentate part exhibited the same AMA as the whole

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Table 8 Compounds present in the antimicrobial aqueous fraction, as measured by NMR

Compound	Concentration (μM)		Relative amount in dried extract (%)	
	Whole	Filtrate	Whole	Filtrate
2-aminobutyrate	178	29	0.32	0.11
3-indoxylsulfate	372	173	1.39	1.60
4-aminobutyrate	52	10	0.09	0.04
4-aminohippurate	175	90	0.60	0.76
Acetate	28	9.3	0.03	0.02
Acetone	8.1	12	0.01	0.03
Adenine	72	29	0.17	0.17
Alanine	355	154	0.56	0.60
Arginine	141	74	0.43	0.56
Asparagine	57	38	0.13	0.22
Betaine	24	17	0.05	0.09
Ethanol	108	20	0.09	0.04
Formate	20	23	0.02	0.05
Glucose	112	33	0.35	0.26
Glutamine	191	90	0.49	0.57
Glycerol	29	12	0.05	0.05
Glycine	140	73	0.18	0.24
Glycolate	498	72	0.66	0.24
Homoserine	121	74	0.25	0.39
Hypoxanthine	154	61	0.37	0.36
Inosine	393	131	1.85	1.53
Isoleucine	184	46	0.42	0.26
Isopropanol	11	6.4	0.01	0.02
Lactate	111.7	48	0.18	0.19
Leucine	692	272	1.59	1.55
Mannitol	68	36	0.22	0.28
Mannose	226	100	0.72	0.78
Methanol	38	85	0.02	0.12
Methionine	148	62	0.39	0.40
Phenylacetylglycine	176	88	0.60	0.74
Phenylalanine	1,068	343	3.09	2.47
Proline	717	300	1.45	1.50
Threonine	327	133	0.68	0.69
Tyrosine	620	288	1.97	2.27
Uracil	209	92	0.41	0.45
Valine	799	314	1.64	1.60

The aqueous extract of the egg wax was treated as described in "Sample analysis using NMR spectroscopy", resulting in a filtrate and filtrand after passing through a 10 kD centrifuge filter. Reliable detection limit is approximately 10 μM

fraction before centrifugation, and the filtrate part showed no AMA. Whichever components of the aqueous phase are responsible for AMA, at least we can eliminate those listed in Table 8.

In summary, we confirmed that the egg wax contains at least two antimicrobial components: FAs in the organic extract (Tables 4, 6) and no fewer than one heat-resistant and

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pH stable antimicrobial substance in the aqueous extract (Fig. 2; Table 7). Although this material has not yet been characterized further, it may be a small antimicrobial peptide.

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