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Antimicrobial activity in the egg wax of the African cattle tick *Amblyomma hebraeum* (Acari: Ixodidae)

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Summary

Eggs of the tick *Amblyomma hebraeum* Koch (Acari: Ixodidae) inhibited the growth of *Escherichia coli* and *Serratia marcescens* (Gram-negative bacteria) in solid culture, but not the growth of *Staphylococcus epidermidis,* and only marginally the growth of *Bacillus subtilis* (Gram-positive bacteria). When egg wax was extracted with chloroform/methanol (2:1), the extract contained antibacterial activity, but the denuded eggs did not. When assayed against bacteria in liquid culture, the aqueous phase inhibited the growth of *S. epidermidis.* However, the activity against *E. coli* was lost during extraction. The antimicrobial component of the aqueous phase was heat stable (100°C for 10 min), resistant to proteinase K (15 min @ 55^0C) and to pronase (30 min @ 37^0C). The antibacterial activity in the aqueous phase increased the permeability of the cell membrane of susceptible bacterial cells within 30 min. However, lysis of the cells was detected by optical density measurements (OD 600 nm) only after 1.5 h. The most evident cytological changes observed by transmission electron microscopy were a thickening of the cell wall and the appearance of numerous electron lucent areas within the cytoplasm of treated bacteria. Gené's organ, the egg-waxing organ in ticks, grows enormously during the first 16 days post-engorgement, and gains antimicrobial activity by day 10 (when oviposition begins). This suggests that Gené's organ is the major source of the antibacterial substance in the egg wax. The vitellogenic hormone in *A. hebraeum*, 20-hydroxyecdysone, when injected into recently engorged females, did not stimulate growth of Gené's organ or precocious secretion of antimicrobial activity.

Keywords: *Amblyomma hebraeum*; ixodid ticks; antimicrobial activity, egg wax; Gené's organ.

Introduction

Antimicrobial substances have been identified in numerous arthropods, including ticks. A defensin was isolated from the haemolymph of *Dermacentor variabilis* one hour post inoculation with the bacteria *Borrelia burgdorferi* and *Bacillus subtilis*. This defensin is also active against *Escherichia coli* and *Staphylococcus aureus* (Johns et al., 1998, 2001). A 4 kDa peptide, with high homology to a scorpion defensin, was purified from the haemolymph of the argasid tick *Ornithodoros moubata*. This heat resistant defensin is present in the haemolymph even without challenging them with bacteria (van der Goes van Naters-Yasui et al., 2000). Although gene expression of this defensin is apparent in unfed ticks it increases substantially following the blood meal (Nakajima et al., 2001). Kopacek et al. (1999) isolated a lysozyme from the gut of *O. moubata*. A haemoglobin fragment in the gut of the ixodid tick, *Boophilus microplus* inhibits the growth of the Gram-positive bacterium, *Micrococcus luteus*, two filamentous fungi (*Aspergillus nidulans* and *Neurospora crassa*) and the yeast, *Candida albicans* (Fogaca et al., 1999). Nakajima et al. (2003b) reported that a haemoglobin fragment in the gut of *O. moubata* also exhibits antimicrobial activity. Further instances of antimicrobial peptides have been reported in recent years (eg: Fogaca et al., 2004, for *B. microplus*; Lai et al., 2004a,b, for *Amblyomma hebraeum*; Yu et al., 2005, for *Ixodes sinensis*).

Ticks lay their egg mass on the ground where they are exposed to the rich soil microflora. To date, however, not much has been published on antimicrobial substances associated with the eggs of arthropods. Marchini et al. (1997) reported an antibacterial peptide in the eggs of the Mediterranean fruit fly *Ceratitis capitata* that inhibits the

growth of Gram-negative and Gram-positive bacteria. Gorman et al. (2004) reported upregulation of six antimicrobial protein genes following infection of eggs of the tobacco hornworm *Manduca sexta* with killed *Serratia marcescens* (a Gram-negative insect pathogen).

The literature on antimicrobial substances in tick eggs is also minimal. Potterat *et al.* (1997) observed that while some dead females of *B. microplus* were covered with fungi, the eggs next to them remained uninfected. They extracted a sterol amide (which they named 'boophiline') from whole *B. microplus* females that inhibited growth of *E. coli* and *B. subtilis*, as well as growth of the filamentous fungi *Cladosporium cucumerinum* and *Candida albicans*. Preliminary assays by the latter authors also detected the presence of an antifungal compound in an egg extract of *B. microplus*, possibly also boophiline.

Female ixodid ticks protects their eggs from desiccation by covering them with a waxy substance secreted by Gené's organ (Lees and Beament, 1948; Schöl et al., 2001). Booth (1992) suggested that the waxy secretion may also have other functions, including constituting a physical barrier against microorganisms. To date, most physiological studies on Géné's organ have focused on demonstrating waterproofing by the wax secretion (Lees and Beament, 1948), on the organ's morphology, histology and ultrastructure (Booth *et al*, 1984, Booth, 1989, Schöl et al, 2001), on determining the composition and general biosynthesis pathway of the lipid secretion (Booth, 1989, 1992), on the ultrastructure and physiology of the retractor muscles (Booth et al, 1985) and on the pharmacological control of egg wax secretion (Booth *et al*, 1986). Kühn et al. (1996) detected a sialic acid specific lectin in various tissues of *I. ricinus*, including the

invaginations of Géné's organ. They suggested that this lectin recognizes foreign substances and pathogens in the haemolymph and thus is an important component of the tick's immune system. Its association with Géné's organ suggested that it may also serve to protect the eggs. In the present study we show that the egg wax of *A. hebraeum* contains an antimicrobial substance(s), and we have begun to define some of its properties and effects.

Materials and Methods

Tick feeding and collection of eggs

Female and male *A. hebraeum* were fed together in a cloth-covered foam pack on the backs of rabbits (Kaufman and Phillips, 1973). Engorged females detach spontaneously, usually within about 10 days. Engorged females were rinsed with water, dried with tissue paper, weighed and stored in the colony incubator (darkness, 26˚C and high relative humidity). Under these conditions, oviposition normally begins about 10-12 days post-engorgment, and continues for about 3-4 weeks (Friesen and Kaufman, 2002). Only engorged ticks over 1000 mg were used in this study (normal engorged weight in this species spans the range of ~1000-3000 mg). Laid eggs were stored in the colony incubator within mesh-covered plastic vials. Because oviposition normally occurs over 3-4 weeks, the eggs within each batch were mixed with a spatula prior to experimentation in order to homogenize the age distribution of the egg mass. Because freezing the eggs would simplify the design of some experiments, and would eliminate the risk of eggs hatching during an experiment, eggs used in this study were frozen at -20˚C. Preliminary

experiments (Table 1) demonstrated that frozen eggs had very similar antimicrobial activity in solid culture as non-frozen eggs.

Extraction of egg-wax

We adapted the method of Folch et al. (1957). One ml of a mixture of chloroform and methanol (2:1, v/v) was added to batches of eggs (100 mg each) and vortexed for 1 min. The supernatant was transferred to a clean tube with a glass pipette. Distilled water (0.5 ml) was added to the eggs and the suspension was vortexed for 1 min. This supernatant was collected and vortexed briefly with the first solvent mixture. The solvent mixture was centrifuged for 1 min at 1000 rpm to separate the phases. The organic phase was transferred into 2-ml pre-weighed glass ampoules (Wheaton, New Jersey, USA) and the aqueous phase and interphase extracts transferred to pre-weighed microfuge tubes. The solvents were evaporated under a nitrogen stream (organic) or lyophilized overnight (aqueous and interphase), and the dry weights of the extracts were recorded. The organic extract was resuspended in 0.1 ml of chloroform methanol (2:1) to minimize lipid hydrolysis from exposure to air (Hamilton and Hamilton, 1992). The samples were stored at -80˚C until ready for analysis.

Solid culture diffusion assay for antimicrobial activity of eggs and egg-wax extracts

The following bacterial species and fungal species were tested: four Gram-negative species (*Escherichia coli* B5, *Serratia marcescens, Proteus vulgaris* and *Pseudomonas aeruginosa*); three Gram-positive species (*Staphylococcus epidermidis, Bacillus subtilis* and *Micrococcus luteus)*; and the yeasts *Candida albicans* and *Saccharomyces cerevisiae*. One colony of each strain was suspended in 10 ml of Mueller Hinton (MH) broth (Difco, Maryland, USA) and incubated overnight at optimal growth

temperature (37°C for all species, except for *B. subtilis* and *M. luteus*, which were grown at 30°C). Optical density (OD) measurements were taken at 600 nm, and each suspension was aseptically diluted in MH broth to obtain an $OD_{600\ nm}$= 1.0, while being held on ice. Within 30 min, 0.1 ml of this diluted suspension was mixed with soft agar to overlay the base agar plates (1.5% agar). Soft agar was made as follows. Sterile test tubes were filled with 4 ml molten 0.6% soft agar (in distilled water) and kept at 45˚C. Under aseptic conditions, 0.1 ml of each bacterial suspension was added to a soft agar aliquot, the tube was vortexed and the contents quickly poured and spread onto a MH base agar plate. The final cell count in the soft agar was 1-5 X 10^7 colony forming units ($CFU.ml^{-1}$ ($OD_{600\ nm}$ ~ 0.1).

Whole tick eggs, eggs denuded of wax, and filter paper discs (5 mm) impregnated with 50 µl of egg wax extract (and then air dried for 15 min), were deposited on MH agar plates overlaid with bacteria, and incubated for 48 h at 37˚C or 30°C. Sterile untreated paper discs and paper discs dipped in solvent and then air dried were plated as controls. The clear zone around the eggs was measured with calipers or with an ocular micrometer fitted to a dissection microscope at 9X magnification. Measurements of the clear zone surrounding the paper discs included the disc diameter.

Bacterial growth inhibition assay in liquid culture

In our initial experiment to test for antimicrobial activity in the aqueous and organic extracts, one colony of *S. epidermidis* was inoculated into tubes containing 10 ml MH broth and incubated overnight. Aliquots of the overnight cultures were diluted 1:100 in 5 ml fresh MH broth. Because the solvents of the organic phase were assumed to be toxic to bacterial cultures, in this experiment sterile paper strips were impregnated with a

volume containing 10 mg of the aqueous or the organic egg wax extracts. After the solvents had evaporated, the paper strips were introduced to the culture tubes. Bacterial suspensions were then added to each tube, the tubes placed in a tube rotor, and $OD_{600\ nm}$ was recorded at time 0, then at each hour for 3 h and then at 20 h.

To study the basic mechanism of action (bacteriostatic, bacteriocidal or bacteriolytic) of the antibacterial substance in the aqueous extract, liquid cultures of *S. epidermidis* were started as described above ($OD_{600\ nm}$ ~ 0.01-0.03), and $OD_{600\ nm}$ was recorded every hour until the culture reached the logarithmic growth phase (OD= 0.2 - 0.3). At this point, about 10 mg of the dried aqueous extract dissolved in 200 µl of MH broth were added to the culture tubes which were then vortexed briefly. The same volume of sterile broth was added to control tubes. ODs were recorded every 30 min after addition of the extract. Viable cell counts were performed at the same time points by transferring 100 µl of each culture to a sterile microfuge tube. The samples were then serially diluted $10^{-4} – 10^{-13}$ in MH broth. One hundred microliters of each dilution were plated onto MH agar plates in duplicate. After incubation for 48 h at 37 ˚C, only those plates with about 30-300 colonies were used to calculate CFU at each time point.

Protease and heat treatments

The aqueous extract was tested in liquid culture against *S. epidermidis* after exposure to proteinase K (75 µg ml^{-1}, 15 min @ 55˚C), or pronase (50 µg ml^{-1}, 30 min @ 37˚C), or after boiling for 10 min. Lysozyme (10 mg ml^{-1}) treated in the same way served as a positive control. To inactivate the proteases before adding the aqueous phase to the cultures, the samples were boiled for 5 min.

Transmission electron microscopy (TEM)

Samples (1 ml) of untreated bacterial cells, and cells 30 min and 2 h after the addition of the aqueous extract, were prepared for TEM. Cells were pelleted by centrifugation (3000 rpm) and fixed in glutaraldehyde (2% in 0.1M sodium cacodylate buffer) for 18 h. The cell pellets were washed with phosphate buffered saline (PBS) three times for 10 min, and treated with osmic acid (1% in 0.1M cacodylate buffer) for 2 h, followed by three 15-min washes with PBS. Samples were dehydrated in a series of ethanol solutions (20, 30, 40, 60, 80, 90 and 100%, 15 min each). The samples were then placed in propylene oxide for 15 min. A mixture of propylene oxide and Spurr's low viscosity resin (1:1) was added to the samples for 24 h. Finally, the samples were embedded in Spurr's low viscosity resin for 72 h and sectioned with a diamond knife on a Reichert-Jung Ultracut microtome. Sections were collected on copper grids and stained with uranyl acetate (1% in distilled water) and Reynold's lead citrate (0.01 % in 0.1N NaOH).

Live/Dead cell staining

S. epidermidis cells were treated with the aqueous phase of the egg wax extract as described earlier, and stained with the LIVE/DEAD BacLight kit (Molecular Probes, Oregon). The kit contains two nucleic acid stains: SYTOX-9 (penetrates intact cells and fluoresces green) and propidium iodide (does not penetrate intact cells and fluoresces red). Thus normal cells fluoresce green and damaged cells, or those showing enhanced permeability, fluoresce orange-red or red. A mixture of SYTOX-9 and propidium iodide was prepared (1:1, v/v) and diluted 1:500 in PBS. One ml samples of the treated (30 min or 2 h exposure to about 10 mg of the aqueous extract) and untreated bacterial

suspensions were centrifuged for 10 min at 10,000 rpm, and the cell pellets were incubated for 15 min in the dark at room temperature with 0.2 ml of stain mix. A droplet of each sample preparation was mounted with immersion oil on glass microscope slides and the cells were observed by confocal fluorescence microscopy.

Antimicrobial activity of various tissues

Just prior to dissection, engorged ticks were rinsed in water, dried with tissue paper, and immobilized on Petri dishes with cyanoacrylate glue. The ticks were then cooled in a refrigerator for 30 min (anaesthesia and to reduce the risk of puncturing the gut during dissection; Kaufman, 1991). Ticks were then flooded with ice-cold modified TC Medium 199 (TC 199, Sigma, without antibiotics, plus 2.1 g MOPS buffer (Sigma) and 2.1 g NaCl per litre; pH was adjusted to 7.2), dissected from the dorsal surface and the following tissues collected: ovary, oviducts, Gené's organ, salivary glands, trachea, and gut. Gené's organ is a complex structure, comprising a number of secretory lobes and an array of retractor muscles (Schöl et al., 2001). In this study, the tissue collected included the secretory lobes and muscles. All tissues were washed three times in modified Tissue Culture Medium 199 to remove haemolymph (a potential source of contaminant antimicrobial peptides). Tissue samples were stored at -20˚C in microfuge tubes containing 35 µl modified TC 199. Just prior to use, frozen tissues were rapidly thawed in a 37˚C water bath. Two conditions were assayed: tissues dissected before day 4 post engorgement and those dissected after day 10 post engorgement. Gené's organ was also tested for antimicrobial activity on days 0, 2, 4, 6, 8, 10, 12, 16 and 20 post engorgement. For each sample, the width of the clear zone was measured at four points around the circumference and the readings averaged. Antimicrobial activity was determined as the

average width (in mm) of inhibition per mg tissue sample. Sterile paper discs containing 50 µl of modified TC 199 served as control.

Injections of the tick vitellogenic hormone, 20-hydroxyecdysone (20E)

The hormone (20E; Sigma) was dissolved in 70% ethanol and stored at -20˚C as a 4 mg ml^{-1} stock solution. On the day of injection it was diluted to working concentration in modified TC 199 (0.2 mg ml^{-1}). Engorged ticks were injected through the coxa of the 4^{th} leg on the day of engorgement (day 0) with 20E (4 µg g^{-1} body weight) in a delivery volume of 20 µl g^{-1} body weight, with a 50-µl Hamilton™ syringe. Injected ticks were kept in the colony incubator for 2, 4, or 6 days. Because 20E is metabolized quickly (Weiss and Kaufman, 2001), those ticks kept for more than 2 days received a booster injection (4 µg g^{-1} body weight) every 2 days. Thus, ticks kept for 2, 4 or 6 days received 1, 2 or 3 injections, respectively. On days 2, 4 or 6 respectively, Gené's organ was removed from each tick, weighed, and tested for antimicrobial activity against *S. epidermidis* in solid culture, as described earlier. Uninjected ticks and ticks injected with 3.5% ethanol in modified TC 199 (vehicle for 20E; 20 µl g^{-1} body weight) served as controls.

Statistical analysis

Unless otherwise stated, all data are reported as mean ± SEM (n). Means were compared using a Student's t-test.

Results

Antibacterial activity of the intact eggs

Eggs inhibited the growth of the Gram-negative bacteria *E. coli* (Fig. 1B) and *Serratia marcescens* (Table 1), but not that of *Staphylococcus epidermidis* (Fig 1A), and only very marginally that of *B. subtilis* (Table 1), both Gram-positive bacteria. In a separate experiment, when the eggs were deposited on *E. coli* cultures only, width of the inhibition zone increased progressively with egg number per batch (one egg = 0.20 ± 0.01 mm; five eggs = 0.38 ± 0.03 mm; 10 eggs = 0.57 ± 0.04 mm and 20 eggs = 0.73 ± 0.07 mm; n = 6 for all groups). Eggs no longer inhibited the growth of *E. coli* when the egg wax was removed (Fig. 1C).

Antibacterial activity of egg-wax extracts

The total weight of dried residue in the aqueous and organic phases of the egg-wax extracts was 23.4 ± 6.9 mg g^{-1} eggs (n = 20). In contrast to the results shown with whole eggs, none of the organic and aqueous extracts inhibited the growth of the Gram-negative bacteria tested (*E. coli, S. marcescens*, *Proteus vulgaris* and *Pseudomonas aeruginosa)* even after combining the organic and the aqueous residues (Table 2). The wax extracts were active only against Gram-positive bacteria (*S. epidermidis, B. subtilis* and *M. luteus)*. The organic phase (Fig. 1D) produced a stronger inhibition of *S. epidermidis* than the aqueous phase (Fig. 1E). The extracts were inactive against the yeasts *C. albicans* and *S. cerevisiae* (Table 2).

The organic extract did not inhibit bacterial growth in liquid culture, perhaps because the material is too hydrophobic to diffuse through the aqueous medium. The growth of the Gram-negative *E. coli* was not inhibited by any of the extracts (data not shown). Only the aqueous extract (10 mg dry weight) inhibited the Gram-positive *S.*

epidermidis. Inhibition was noticeable within 1 h of incubation, and by 20 h, growth was inhibited by about 94% (Fig. 2).

Basic mechanism of action of the antimicrobial component(s) in the aqueous extract

Following addition of the extract (10 mg), viable cell counts began to decrease within 1 h and OD (total cell count) started to decrease within 1.5 h (Fig. 3). Viable cell counts dropped from 8.3×10^8 to 6.6×10^6 CFU ml^{-1} by 2 h. Control and solvent control cultures grew normally (Fig. 3). The drop in both viable cell count and total cell count strongly suggests a bacteriolytic action by the aqueous extract (Madigan et al., 2002). Further evidence that the antimicrobial substance at least increases the permeability of *S. epidermidis* cells comes from the experiment using LIVE/DEAD staining. By 30 min of exposure to the aqueous extract, many cells exhibited orange fluorescence, indicating that some propidium iodide had penetrated those cells; by 2 h, essentially all the cells exhibited red fluorescence, whereas control cultures remained impermeable to propidium iodide, exhibiting green fluorescence throughout the experiment (data not shown). However, when bacterial cells were observed under TEM, their cell walls were noticeably thicker, but the cell membranes and cell walls were not obviously disrupted. The most evident cytological change observed was the appearance of numerous electron lucent zones within the cytoplasm (Fig. 4).

Protease and heat treatments

The aqueous phase did not lose its antibacterial activity after being boiled for 10 min, or following treatment with proteolytic enzymes (proteinase K or pronase; Fig. 5). Lysozyme, a bacteriolytic protein serving as a positive control for demonstrating protease activity was inactivated with proteinase K (Fig. 5).

Antimicrobial activity of various tick tissues

All tick tissues had at least some antimicrobial activity, with the exception of Gené's organ before day 4, and the 'closed gut' samples (only the serosal surface exposed to bacteria) before day 4 and after day 10. When the gut contents and mucosal surface were exposed ('opened gut'), bacterial growth was inhibited (Table 3).

Because Gené's organ lacked antimicrobial activity on the 4th day post-engorgement, but possessed it by day 10, we tested Gené's organ for antimicrobial activity in solid culture at various times post engorgement. First, Fig. 6A shows that the weight of Gené's organ increased enormously as a function of time post-engorgement, beginning at less than 2 mg on days 0-2, but growing steadily thereafter, plateauing at about 50 mg on day 16. Second, antimicrobial activity appeared in Gené's organ some time between days 8-10, just prior to the onset of oviposition (Fig. 6B). Both antimicrobial activity and egg mass laid increased in parallel thereafter. Prior to oviposition, no Gené's organ showed any antimicrobial activity. With one exception, all ticks represented in Fig. 6B which had begun oviposition also showed antimicrobial activity in Gené's organ.

Although Gené's organ grew enormously during the post-engorgement period, and although it began displaying antimicrobial activity at the time of engorgement, 20E (4 μg g^{-1} body weight) did not stimulate precocious growth nor appearance of antimicrobial activity in 39 preparations (data not shown).

Discussion

We assume that the antimicrobial activity of the egg wax is an adaptation to protect the eggs from soil microflora. Identity of the antimicrobial substance(s) described here remains unknown. Several possibilities are: a lipid component of the egg wax, an antimicrobial peptide, or perhaps a lectin as described by Kühn et al. (1996). Although the antimicrobial substance within the egg wax may well be a lipid, many antimicrobial peptides are amphiphilic, with up to 50% hydrophobic residues (Hancock, 2001), and so could potentially be incorporated into a wax. We think that the antimicrobial substance in the organic phase of the extract may be the same as that of the aqueous phase, primarily because both the organic and the aqueous extracts showed a similar spectrum of activity against all the microorganisms tested (Table 2). A complete characterization of the two extracts would be required to decide on this, however.

The most surprising result in this study was the change in antibacterial activity following wax extraction. Intact eggs inhibited the growth of two Gram-negative species, but after extraction, the material from both the organic and aqueous extracts inhibited the growth of the Gram-positive bacteria only. Until the chemical identity of the antimicrobial substance reported here is known, one can only speculate as to what caused the switch in activity. There are at least two possibilities. (1) First, the antimicrobial activity against Gram-negatives may have been inactivated during the extraction. For example, strong organic solvents can denature proteins. Thus, antimicrobial activity in the haemolymph of *O. moubata* was lost on extraction with phenol (van der Goes van Naters-Yasui et al., 2000). A change in pH (not controlled during our experiment) could also cause loss of activity. For example, the peptide, hebraein (recently identified in the haemolymph of *A. hebraeum*) has substantially higher antimicrobial activity at pH 6.2

than at pH 7.6 (Lai et al., 2004b). The extraction may have caused aggregation of the compound and the aggregates may be inactive or might not be able to penetrate the bacterial cell wall. Hydrolysis or oxidation of lipid components could have abolished the antimicrobial activity. These possibilities remain to be tested experimentally. If the antimicrobial substance against Gram-negative bacteria was indeed inactivated, it implies that the egg wax contains at least two antimicrobial components: a large amount active against Gram-negative bacteria, and a small amount active against Gram-positive bacteria. If this were so, with the anti-Gram-negative material inactivated during wax extraction, the smaller amount of anti-Gram-positive material would become apparent following its concentration in the organic phase. (2) Secondly, a single antimicrobial substance (anti-Gram-negative) could have been modified during the extraction in such a way that it became active only against Gram-positive bacteria.

Mechanism of action of the antimicrobial component(s) of the aqueous phase

One can get clues about the mode of action of an antimicrobial substance from the speed of its effect. The aqueous extract did not seem to kill bacteria as rapidly as many antimicrobial peptides isolated from insects are reported to do. A noticeable growth inhibition was observed only after 1.5 h, when all the extract obtained from 1 g of eggs (about 10 mg) was applied to the culture. Many insect defensins have an almost immediate lytic effect on bacteria. For example, a 1-min exposure of recombinant defensin to exponentially growing *M. luteus*, was sufficient to lyse all cells in the culture (Cociancich et al., 1994). However, slow acting antimicrobial peptides do exist; the proline-rich drosocin (anti- Gram-negative) required 24 h to inhibit the growth of

exponentially growing *E. coli* (Bulet et al, 1993). A defensin isolated from *O. moubata* required over 30 min to inhibit the growth of *M. luteus* (Nakajima et al., 2003a).

The slow killing kinetics, together with evidence suggesting a bacteriolytic effect (Fig. 3), argues against the antimicrobial substance in the aqueous extract being a membrane-active peptide with a mode of action similar to that of most insect cecropins and defensins. The cecropins and defensins exhibit rapid killing kinetics because they act as membrane pores, causing membrane depolarization and a resulting decrease in cytoplasmic ATP and inhibition of respiration (Otvos, 2000). The results from LIVE/DEAD staining do suggest that the antimicrobial substance in the aqueous extract caused a change in the permeability characteristics of the bacterial cell membrane, as there was slow penetration of propidium iodide into the *S. epidermidis* cells. However, in addition to this effect on the cell membrane permeability, the cell wall was also affected, showing significant thickening in treated compared to control cells (Fig. 4). The relationship between cell wall thickening and eventual lysis of the bacterial cells (Fig. 3) is not clear, and will require more detailed mechanistic studies once the antimicrobial substance has been identified.

The most noticeable cytological effect of exposure of *S. epidermidis* to the aqueous extract was the formation of what appear to be numerous electron lucent areas within the cytoplasm (Fig. 4). Nakajima et al. (2003b) observed similar clear areas near the periphery of the cell in *M. luteus* cells treated with a tick defensin isolated from *O. moubata*. They interpreted these as areas where cytoplasm leaked from the cell.

Stability to protease and heat treatments

The antimicrobial substance in the aqueous phase of the tick egg wax extract was stable to heat and protease treatment (Fig. 5). Notwithstanding these results, however, the antimicrobial substance might still be a protein. For example, the antifungal peptide, drosomycin, is remarkably resistant to proteases, heat and extremes of pH (Fehlbaum *et al*, 1994).

Antimicrobial activity of various tissues of the engorged tick

Antimicrobial activity was associated with at least several tissues within the tick (Table 3). This widespread activity could be due to diverse substances. It is interesting that the gut contents, but not the serosal surface, showed antimicrobial activity. Similar observations have been reported for other tick species. The argasid tick *O. moubata*, uses a fragment of mammalian haemoglobin as an antimicrobial peptide; this fragment was isolated from the gut (Nakajima et al., 2003b). Fragments of haemoglobin with antimicrobial activity were also isolated from the midgut of the ixodid tick *D. variabilis,* but no other antimicrobial peptides or proteins were found in the gut lumen, even after attempts to upregulate their expression by exposing the midgut to several bacterial species or to peptidoglycan, a bacterial cell wall component (Sonenshine et al., 2005). This suggests that the midgut antimicrobial activity in this tick originates from haemoglobin fragments only, and not from expression of other antimicrobial proteins (Sonenshine et al., 2005). However, blood feeding does upregulate the expression of defensins in the haemolymph of *O. moubata* (Nakajima et al., 2002).

Gené's organ dramatically increased in size until day 16 post-engorgement (Fig. 6A), but it did not inhibit bacterial growth until egg laying began (about day 10; Fig. 6B). The other tissues tested (ovary, oviduct, gut, salivary gland and trachea), however,

showed a similar level of antimicrobial activity throughout the post-engorgement period (Table 3). Together, these results suggest that antimicrobial activity in Gené's organ is under physiological control, and timed specifically to correspond with the onset of oviposition. In this context it is interesting that the antimicrobial peptide, hebraein, is absent from haemolymph on the day of engorgement, but is present by day 4 (Lai et al., 2004b). If the antimicrobial substance reported here is a peptide, it is unlikely to be hebraein because of the temporal difference just mentioned (day 4 for hebraein; day 10 for Gené's organ) and because hebraein has a wider antimicrobial spectrum (anti-Gram-positive, anti-Gram-negative and anti-fungal; Lai et al., 2004b). It is also unlikely to be ixosin, the recently described antimicrobial peptide isolated from the salivary gland of *Ixodes sinensis*, because ixosin also has a wider antimicrobial spectrum (*S. aureus*, *E. coli*, and *C. albicans* (Yu et al., 2005). As mentioned earlier, we do not assume that the antimicrobial activity found in all the tissues listed in Table 3 is due to the same material as found in the egg wax. However, because the activity in Gené's organ appears only around the time that oviposition begins, it seems reasonable to suggest that at least this antimicrobial substance may be the same as in the egg wax.

Considering its importance for maintaining egg viability, it is surprising that we still know relatively little about the function(s) of Gené's organ and control of its secretion. The gland is composed of three defined sections: tubular, lobed and acinar; all three sections appear to contribute components of the wax (Booth, 1992). Not surprisingly, the gland shows the ultrastructural hallmarks of a lipid-secreting tissue (abundance of smooth endoplasmic reticulum and prominent Golgi bodies with numerous budding membrane-bound vesicles and lipid droplets (Booth et al., 1984; Booth, 1989).

However, the egg wax also contains some protein (equivalent to about 16 nmol total amino acid per mg egg wax; Arrieta, 2004). This observation is supported by earlier data that the luminal contents react histochemically to protein stains (Lees and Beament, 1948; Chinery, 1965). The glandular epithelium is also rich in ribosomes and rough endoplasmic reticulum, suggesting the synthesis of a secretion containing protein or lipoprotein (Booth et al., 1984; Booth, 1989; Schöl et al, 2001). These observations leave open the possibility that the antimicrobial component of the egg wax may be a peptide.

Most antimicrobial peptides isolated from insects are induced only following bacterial challenge. The ceratotoxins (antimicrobial peptides isolated from the eggs of the Mediterranean fly), are an exception (Marchini et al., 1997). The antimicrobial substance presented here is also expressed independently of specific bacterial challenge. Moreover, the vitellogenic hormone, 20E, neither stimulated growth of Gené's organ nor secretion of the antimicrobial substance. However, 20E is rapidly metabolized when introduced to the haemocoel by bolus injections, 85-99% disappearing within 4-7 hours (Weiss and Kaufman, 2001), so it is possible that the injection protocol we used (bolus injections) may not have been appropriate for stimulating growth of Gené's organ. Alternative procedures that avoid widely fluctuating 20E titre profiles should be tested before concluding that Gené's organ is refractory to ecdysteroids.

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Figure 1. Antimicrobial activity of *A. hebraeum* eggs and egg-wax extracts. (A) There was no inhibition zone around the eggs exposed to *S. epidermidis* (Gram-positive) and (B) There was a distinct inhibition zone around the eggs exposed to *E. coli* (Gram-negative). (C) Inhibition of *E. coli* did not occur when the wax was removed from the eggs. Measurement of the inhibition zones were made after the plates were incubated @ 37°C for 48 h. Antimicrobial activity of egg wax extract applied to filter paper discs: (D) organic phase and (E) aqueous phase.

Figure 2. Bacterial inhibition assay in liquid culture. The aqueous or organic phase of an egg wax extract was added to tubes containing 5 ml sterile MH broth. These tubes were subsequently inoculated with *S. epidermidis* (time 0) and bacterial growth was measured by optical density at 1, 2 and 3 h, and then at 20 h. Solvent controls showed similar bacterial growth as the MH broth alone (control). SEMs are shown wherever they exceed the size of the symbol; n=4 for all treatments.

Figure 3. Antimicrobial activity of an aqueous egg wax extract. The aqueous extract was added (equivalent to 10 mg dry weight) to a log phase culture of *S. epidermidis* at 2.5 h (arrow) and optical density (open squares) and viable cell count ($CFU.ml^{-1}$; open diamonds) were measured every 30 min for 2 hours. SEMs are shown wherever they exceed the size of the symbols; n=3 for all treatments except viable cell count (n=1).

Figure 4. Ultrastructure of *S. epidermidis* cells treated with 10 mg of the aqueous egg wax extract per ml of bacterial suspension (A) for 30 min. and (B) for 2 h. (C) Control cells; 30 min, and (D) Control cells 2 h. Electron lucent zones (solid arrow)

and thickened cell walls (dashed arrow) were observed in treated bacteria. Magnification of all images is 56,000 X.

Figure 5. Effect of heat and protease treatments on the antibacterial activity of the egg wax aqueous extract. Aqueous phase (10 mg dry weight per ml) incubated with (Δ) proteinase K (75 µg ml^{-1}; 15 min @ 55°C, (*) pronase (50 µg ml^{-1}; 15 min @ 56°C, or (□) heated (10 min @ 100°C) were tested in liquid culture against *S. epidermidis*. (X) Lysozyme control (10 mg ml^{-1}) and (○) lysozyme plus proteinase K were used to demonstrate sensitivity of lysozyme activity to protease digestion.

Figure 6. Development of Gené's organ (GO; open squares), appearance of antimicrobial activity against *S. epidermidis* in Gené's organ (open circles) and progress of oviposition (open triangles) following engorgement. SEMs are shown wherever they exceed the size of the symbol; n = 5 or 6. NB: for visual clarity on the graph, the plotted weights of Gené's organ are 6 X the actual values. Thus the actual average values range from 1.4 mg on day 0 to 49.4 mg on day 16.

Table 1. Comparison of antimicrobial activity of fresh and frozen eggs of *A. hebraeum.*

Bacteria	Clear zone† (mm) fresh eggs n=4	Clear zone† (mm) frozen eggs n=4	p
Gram-negatives			
Escherichia coli	0.28 ± 0.02	0.31 ± 0.02	0.06
Serratia marcescens	0.11 ± 0.01	0.09 ± 0.02	0.7
Gram-positives			
Bacillus subtilis	<0.09 ± 0.02*	<0.10 ± 0.02*	0.8
Staphylococcus epidermidis	0	0	N/A

* These cultures exhibited an incomplete clear zone.

† Clear zones (mean ± SEM) around batches of five fresh and frozen eggs were measured in a solid culture diffusion assay (see Materials and Methods).

Table 2. Spectrum of antimicrobial activity of the egg wax extracts.

Microorganism	Clear zone † aqueous phase (mm)	Clear zone † organic phase (mm)	Clear zone † aqueous plus organic phases (mm)
Gram-negatives			
Escherichia coli	0	0	0
Serratia marcescens	0	0	0
Proteus vulgaris	0	0	-
Gram-positives			
Staphylococcus epidermidis	16.2 ± 3.0	23.9 ± 2.9	30.3 ± 4.1
Micrococcus luteus	9.2 ± 3.4	12.0 ± 1.1	21.0 ± 3.3
Bacillus subtilis	10.2 ± 2.0	13.5 ± 4.6	-
Yeasts			
Candida albicans	0	0	-
Saccharomyces cerevisiae	0	0	-

† Sterile paper discs impregnated with the organic and aqueous extracts (each 10 mg dry weight) were assayed against six bacterial and two fungal species in solid culture. All plates were incubated for 48 h @ 37°C, except for *B. subtilis*, *M. luteus* and the yeasts, which were incubated at 30°C. Diameter of clear zone (mean ± SEM; n=7 for all groups) includes diameter of the paper disc.

- (not tested)

Table 3. Antimicrobial activity of tissues dissected from *A. hebraeum.*

Sample	Tissue weight (mg)	Clear zone † (mm)	Ratio of clear zone to tissue weight (mm/mg)
Tissues collected before day 4 post-engorgement			
Gené's organ	2.1 ± 0.6	0	0
Oviduct	7.1 ± 2.2	1.8 ± 0.8	0.25
Ovary	11.1 ± 4.6	0.8 ± 0.3	0.07
Gut (intact) *	16.3 ± 5.5	0	0
Gut (opened) ‡	9.7 ± 4.8	2.3 ± 1.9	0.24
Salivary gland	13.7 ± 4.2	1.3 ± 0.3	0.09
Trachea	4.1 ± 1.9	0.7 ± 0.1	0.17
Tissues collected after day 10 post-engorgement			
Gené's organ	37.1 ± 4.1	3.2 ± 1.5	0.09
Oviduct	8.4 ± 3.3	1.5 ± 0.9	0.18
Ovary	21.9 ± 6.0	1.6 ± 1.0	0.07
Gut (intact)	15.0 ± 1.9	0	0
Gut (opened)	16.2 ± 4.9	3.1 ± 0.6	0.19
Salivary gland	6.4 ± 2.2	0.8 ± 0.1	0.12
Trachea	4.8 ± 1.0	0.5 ± 0.1	0.10

† Inhibition zones formed around tissues after 48 h incubation on agar plates with *S. epidermidis* at a cell concentration of 1 X 10^8 CFU/ml. Samples of tissues were weighed, washed and plated before day 4 or after day 10 post-engorgement (mean ± SEM; n=4).

* Gut contents not exposed to the culture.

‡ Gut contents exposed to the culture.

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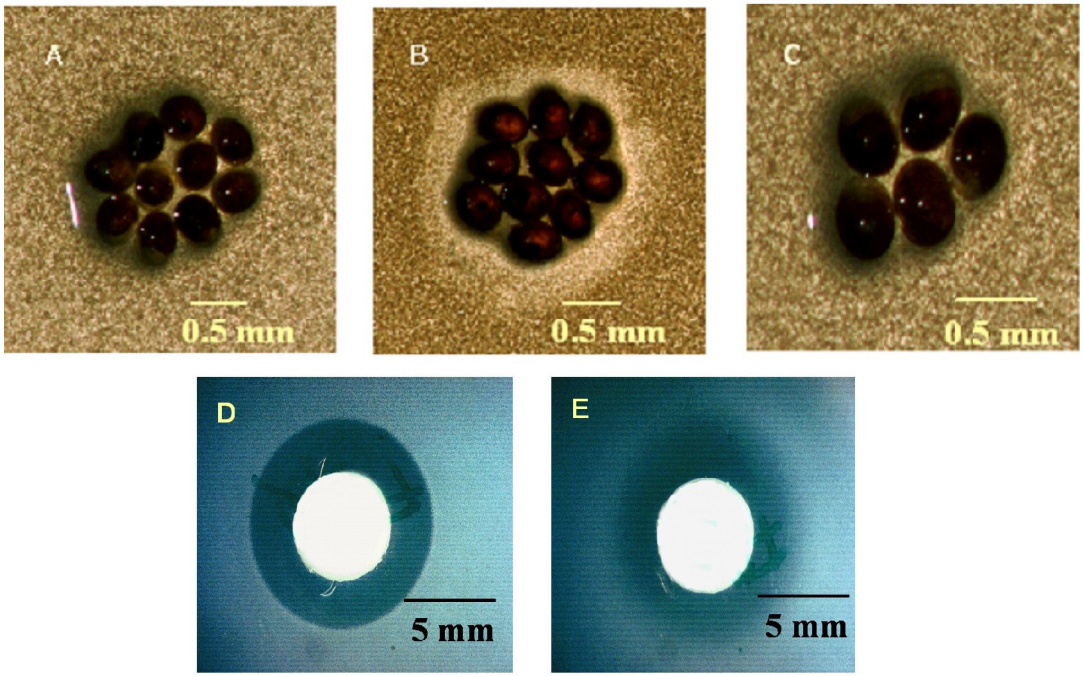

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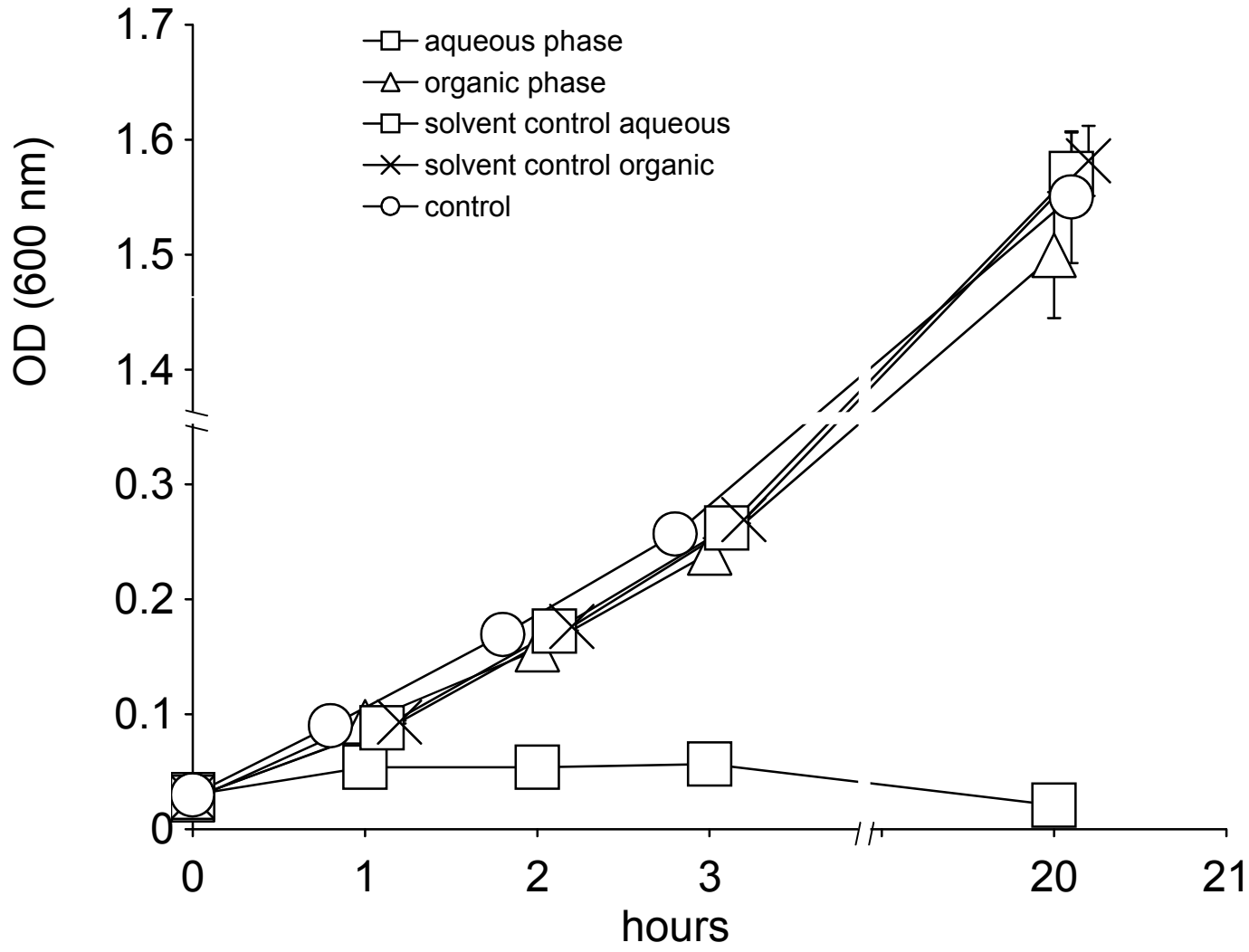

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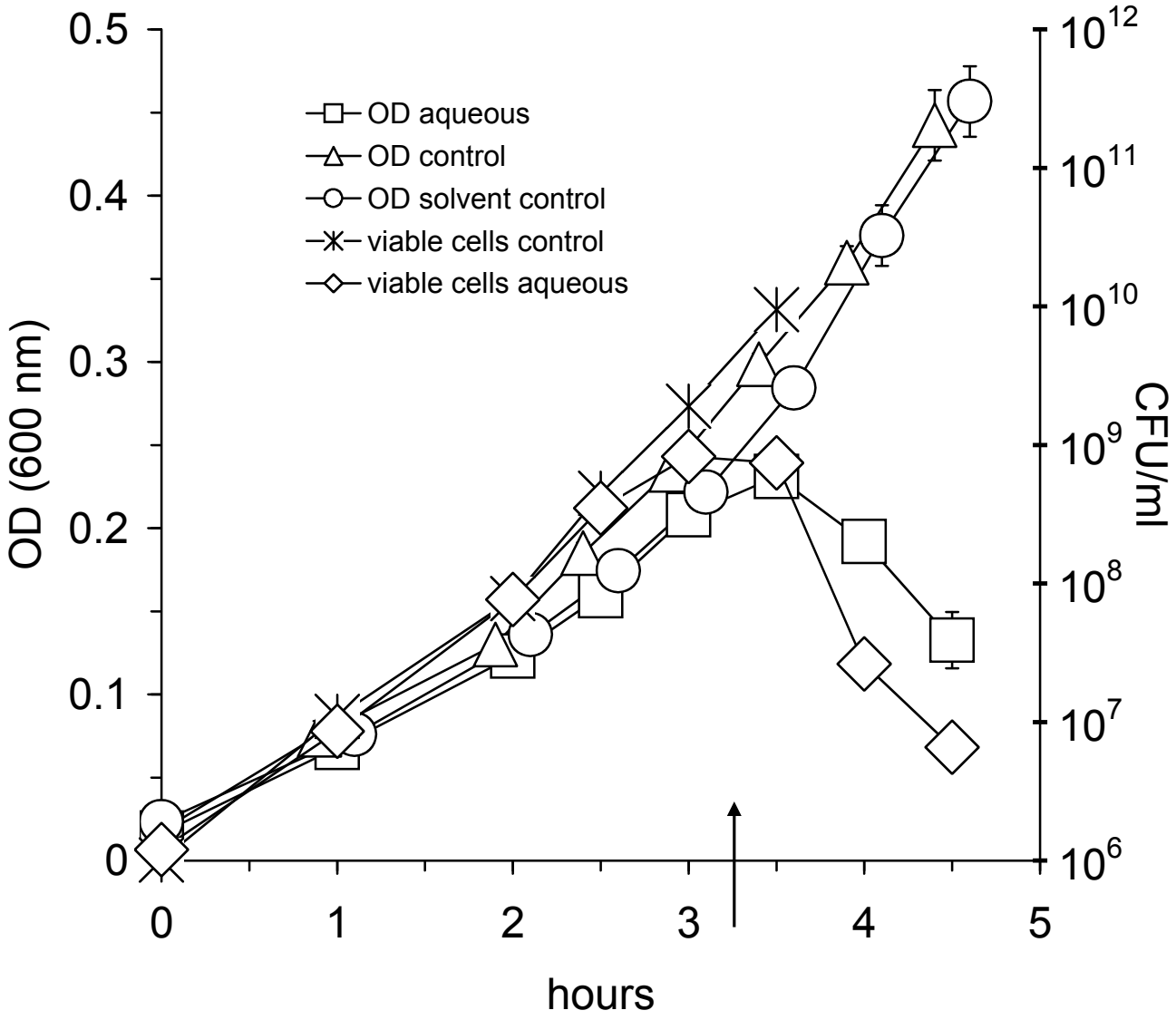

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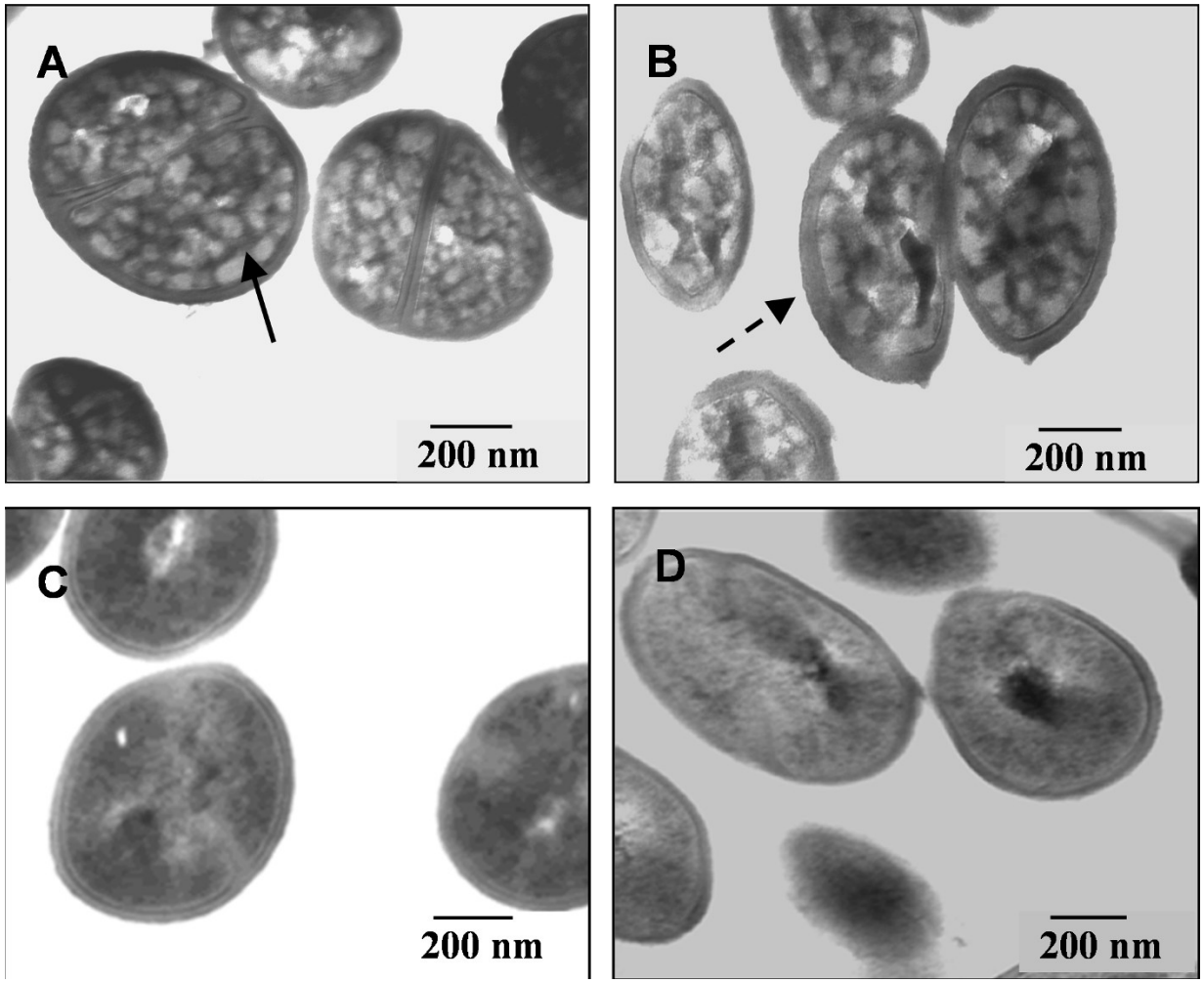

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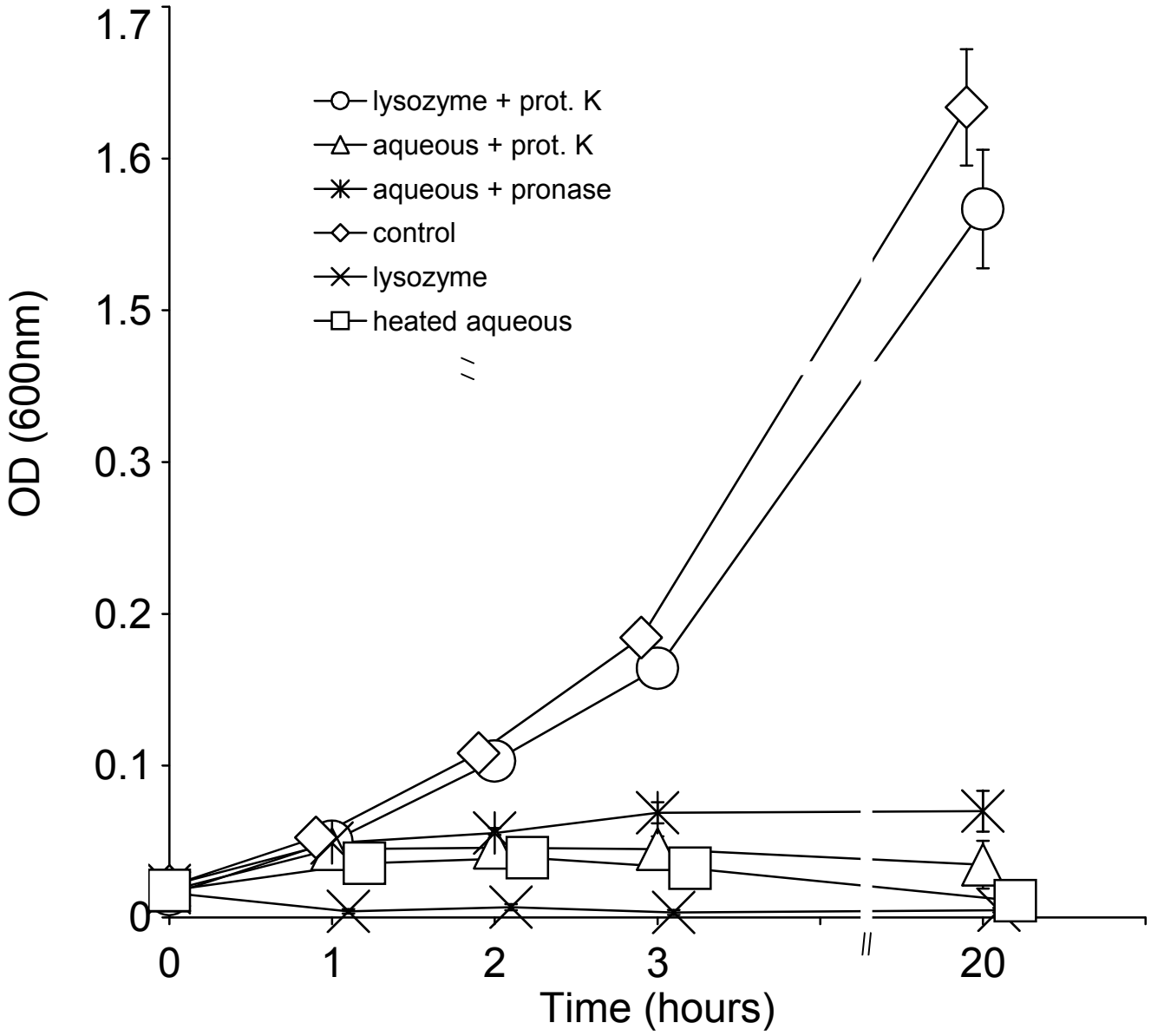

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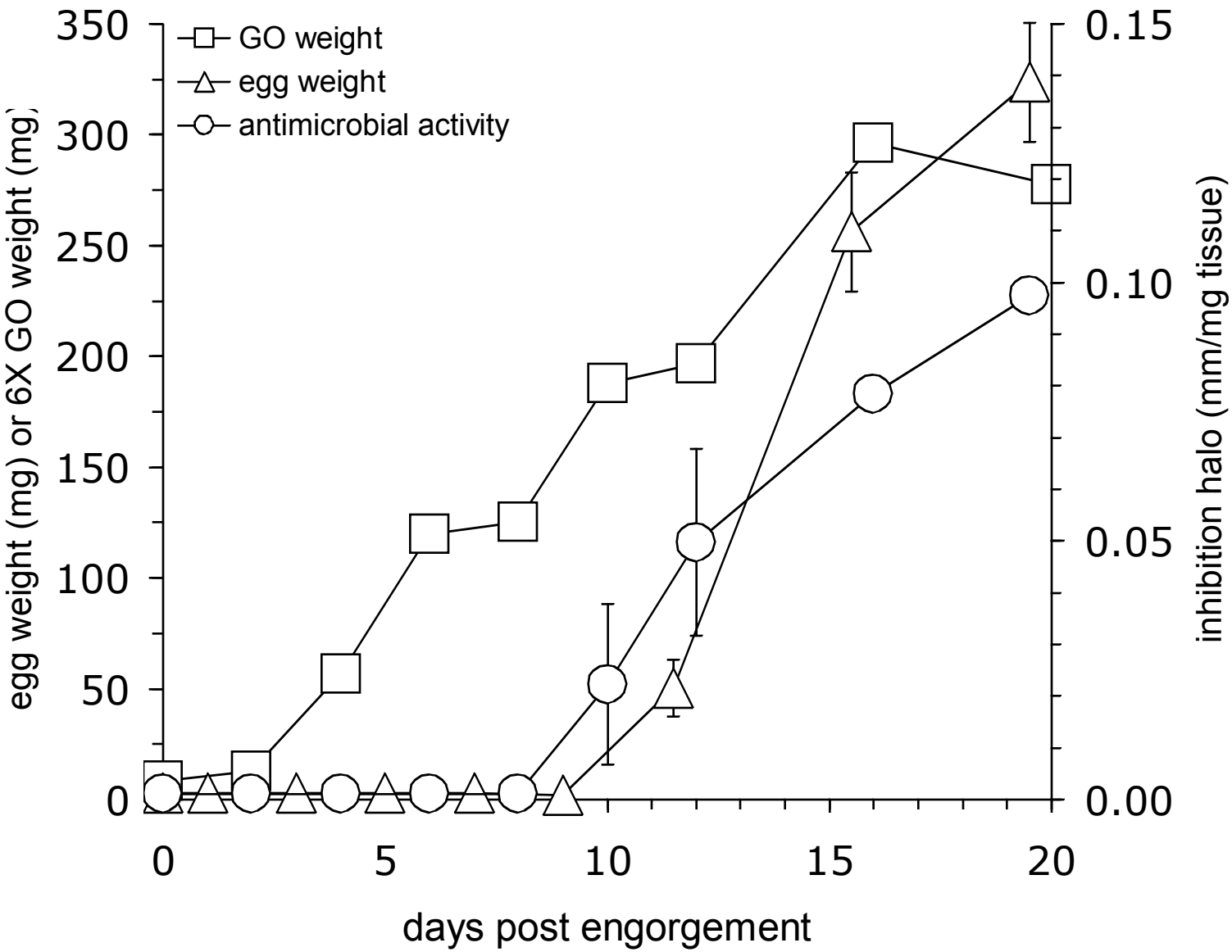

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Fig 6