University of Alberta

Antimicrobial Activity in the Egg Wax of the African Bont Tick *Amblyomma hebraeum*

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

Physiology and Cell Biology
Department of Biological Sciences

Edmonton, Alberta

Spring, 2005

Abstract

The female of the tick *Amblyomma hebraeum* coats her eggs with a waxy secretion that prevents desiccation and microbial attack. When incubated with bacteria, the eggs inhibited the growth of Gram-negative bacteria. The extract of the egg wax preserved antibacterial activity. The antibacterial activity of the extract disrupted the membranes of bacterial cells within 30 min. However, lysis was detected only after 1.5 h. The most evident cytological change observed by TEM was the formation of a precipitate within the cytoplasm. The egg wax is secreted by Géné's organ, a reproductive accessory gland active only during oviposition. Géné's organ grows during the first 7-10 days post-engorgement, although it lacks antimicrobial activity until about day 10, when oviposition starts. This suggests that growth and/or secretion of Géné's organ may be under hormonal control. However, injections of 20-hydroxyecdysone 20E did not stimulate growth of Géné's organ or secretion of antimicrobial activity.

Acknowledgements

I would like to thank my supervisor and co-supervisor, Dr. W. Reuben Kaufman and Dr. Brenda K. Leskiw, for their continuous support throughout my degree. Their efforts to make this project possible and their input on my research has been priceless. I would also like to thank Dr. Ronald Mc Elhaney for always being available to offer me scientific advice. In addition, I thank Dr. Warren Gallin, Donna Atherton, Dr. Greg Goss, Rakesh Bhatnagar, Jack Scott and Randy Mandryk, for the countless technical advice they offered. I would also like to thank my teaching advisors Kim Christopher, Maggie Haag, Anna Szenthe and Tom Hantos, for providing me with the confidence I will need to pursue this career in the future.

My family has provided me with moral and financial support during these years. Thank you very much for being there for me. Papi, muchas gracias por enseñarme a ser alguien responsable y por aceptar mis decisiones aunque no las compartamos. Mamá, infinitas gracias por darme, en 18 años, el ejemplo de mujer fuerte y de tener siempre una meta que cumplir. A ambos, gracias por su esfuerzo. Uds. son el verdadero motivo por el cual estoy aquí. A mi hermano y mis hermanas, muchas gracias por decidirse a seguir el buen ejemplo que nos enseñaron y por hacerme sentir orgullosa de cada uno de Uds.

My stay in Edmonton would have lacked of fun and unbelievable stories if I had not met my dear friends: Tara Klassen, Tanya Hope, Amanda Roe, Steve Kembel, Agnes Wong, Martín Tresguerres, Moira Igor, Elizabeth Orr, Kirsten Norris, Jonners Pinnell, Paul Venturelli, Todd Cole, Brian Weiss and Stephanie

Cheng. Thank you all for all the time and energy you spent with me. I developed a special bond to this part of the world thanks to you. Special thanks to my accomplice in life Lula Niehaus, for sharing and cherishing this Albertan experience with me (hasta el fin del mundo Lulis!!).

Finally, I would like to thank my soulmate Esteban Acuña for being my companion in Edmonton and in life. Without your understanding and encouragement this work may have not been possible. Sos lo mas valioso que tengo.

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List of Abbreviations

20E	20-Hydroxyecdysone
AMP	Antimicrobial peptide
CFU	Colony forming unit
LPS	Lipopolysaccharide
MH	Mueller Hinton
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TEM	Transmission electron microscopy

Chapter 1

General Introduction

1.1 Ticks: General Background

Ticks are widely distributed blood sucking arthropods; their phylogeny is described in Figure 1.1. Approximately 850 species have been described, subdivided in two major families: Ixodidae (hard ticks) and Argasidae, (soft ticks; Sonenshine, 1991). Argasid ticks have one larval stage and up to four or five nymphal stages, depending upon species, feeding success and other variables. The adult females can feed numerous times, and they lay a small batch of eggs following each meal (Aeschliman and Grandjean, 1973). Argasid ticks use a multihost feeding strategy, in which a new host is found for each developmental stage. Ixodid ticks have three active life stages: larva, nymph, and adult. Each stage feeds once only on a host, and uses the nutrients gained either for moulting (larva, nymph) or producing eggs and sperm (adult). The adult ixodid female lays a very large batch of eggs and then dies (Sonenshine, 1991). Most ixodid ticks use a multihost feeding strategy, although some species feed on the same host for two or more life stages (e.g. *Rhiphicephalus (Boophilus) microplus* and *Dermacentor albipictus*).

1.1.1 Amblyomma hebraeum

The tick used primarily in our laboratory is the African bont tick, *Amblyomma hebraeum* (Koch). This ixodid tick feeds on many mammalian

species in Zimbabwe, Botswana, South Africa, and Mozambique (Food and Agriculture Organization, 1998). The adult female feeds for 7 to 14 days, increasing its body weight up to 100 times. An engorged female produces up to 20,000 eggs, and then dies. The female oviposits on the soil or vegetation (Bryson *et al*, 1991), where hatching occurs 4 to 13 weeks later (F.A.O., 1998). Thus, during embryogenesis, the eggs can be exposed to many factors for an extended period of time, including the very abundant and diverse soil microflora. However, the adult female protects her egg batch by covering it with a waxy secretion that prevents desiccation (Kaufman *et al*, 1986) and, as shown in this thesis, microbial infestation.

1.2 Soil microbiology: General background

Due to a high concentration of organic matter, the soil represents a favourable habitat for the proliferation of microorganisms including viruses, bacteria, fungi, algae, and protozoans (Madigan *et al*, 2002). Generally, 10^6 - 10^9 bacteria per gram are found in soils.

Bacteria are divided into two major groups based on differences in cell wall structure: Gram-positive and Gram-negative (Figure 1.2). Briefly, the Gram-positive cell wall consists mainly of a single type of molecule, peptidoglycan, and it is much thicker than the Gram-negative cell wall. Gram-negative bacteria possess a more complex cell wall structure. Besides peptidoglycan, Gram-negatives have an outer membrane made of phospholipids (inner leaflet) and

lipopolysaccharide (LPS; outer leaflet). This layer acts as a second lipid bilayer (Madigan *et al*, 2002).

Some of the most abundant bacterial genera found in soils are the Gram-negatives: *Acinetobacter, Agrobacterium, Alcaligenes,* and *Pseudomonas,* the Gram-positives *Bacillus, Clostridium, Micrococcus, Staphylococcus* and *Streptococcus*, and the actinomycetes (filamentous Gram-positive bacteria) *Corynebacterium, Streptomyces, Mycobacterium, and Nocardia* (Alexander, 1997). Filamentous fungi and yeasts also constitute a high proportion of the soil microflora. The most common genera found in the soil include *Aspergillus, Penicillium, Trichoderma, Candida, Rhodotorula, and Cryptococcus* (Atlas and Bartha, 1993).

1.3 Insect immunity: General background

Insects possess a remarkable ability to resist microbial infections. Their innate immune system appeared very early in the evolution of animals and it is still active in animals that have an adaptive immune system. It differs significantly from the vertebrate adaptive immune system, which first evolved in cartilaginous fishes, and is dependent upon prior exposure to the foreign material (pathogens, parasites, etc.).

The immune system in insects lacks the components that provide molecular specificity and immunological memory in vertebrates, such as lymphocytes and immunoglobulins (Tzou *et al*, 2002). Insect immunity is an non-specific response that depends on receptors encoded by genes that are

already in final form in the genome, and do not undergo gene rearrangement after infection (Gillespie *et al*, 1997).

In order for pathogens or parasites to enter the hemocoel (the insect equivalent to the bloodstream), they first must breach the physical barrier of the integument. Pathogens also encounter the epithelia of the midgut, trachea, and Malpighian tubules, which all produce peptides that inhibit microbial growth (antimicrobial peptides, AMPs; Tzou *et al, 2002*). Organisms that penetrate these external barriers encounter two types of immune response: cellular and humoral. The cellular immune response involves haemocytes (plasmatocytes and granulocytes), which interact to produce three types of cellular response: *phagocytosis, nodulation* and *encapsulation* (Tzou *et al,* 2002). Small groups of bacteria and fungi are attacked by phagocytosis. Within minutes, they are surrounded by extensions of the haemocytes, which surround and enclose the pathogens such that they are brought into the cells where they are killed and digested. Larger numbers of bacteria or fungal spores are attacked via the formation of nodules. In this process, haemocytes move to where the microorganisms aggregate and release their cell contents, forming a matrix that traps the organisms. This matrix then melanizes (become infused with melanin). Larger nodules and parasites, such as protozoans or nematodes, are normally encapsulated. Haemocytes discharge granules onto the surface of the parasite, and then plasmatocytes arrive and form layers of flattened cells around the parasite, forming a tight capsule. The inner layer may then melanize. (Brey and Hultmark, 1998).

The humoral response in insects involves an enzymatic cascade leading to melanization and the secretion of antimicrobial compounds, primarily peptides and proteins (Hetru *et al,* 1999). Immune peptides are expressed *de novo*, usually in the fat body and haemocytes (Dimarcq *et al,* 1994), and are delivered to the affected site to defend the host from microbial infection (Boman, 1998).

1.3.1 Antimicrobial compounds in insects

Lysozyme was the first AMP isolated from insects (Powning and Davidson, 1973). This enzyme cleaves β-(1,4)-glycosidic bonds in the peptidoglycan layer of the bacterial cell wall and is bactericidal against Gram-positive bacteria (Boman *et al*, 1991). In contrast to all other insect AMPs, lysozyme is not produced in the fat body or in the haemocytes, but is mainly expressed in the digestive tract (Daffre *et al*, 1994).

Cecropins were originally isolated from the haemolymph of *Hyalophora cecropia* pupae (Lepidoptera) following infection with bacteria (Steinner *et al*, 1983). These compounds are strongly cationic linear peptides of ~ 4 kDa and contain amphipathic α-helices. They cause lysis of bacterial cells through disintegration of the cytoplasmic membranes (Figure 1.3). Cecropins are effective against Gram-positive and Gram-negative bacteria (Hetru *et al*, 1998).

Defensins were first isolated from the flesh fly *Sarcophaga peregrina* (Matsuyama and Natori, 1989). These are cationic peptides of ~4 kDa and they all contain a motif of six cysteines engaged in three disulfide bridges (Hetru *et al*, 1998). These peptides are mainly active against Gram-positive bacteria,

although in some cases they also kill Gram-negative bacteria or fungi (Götz and Trenczek, 1991). Although the mode of action has not yet been fully described for defensins, it has been suggested that they bind to the acidic components of the target cell membrane, inducing permeability changes (Shai, 1997).

Originally isolated from *Apis mellifera*, apidaecin is the prototypic member of the *proline-rich* peptide family (Casteels *et al*, 1989). These peptides are 16-20 residues long (2-3 kDa) and contain the conserved sequence PRPPHPRI/L. Their mechanism of action is not known but is assumed not to be the result of a membrane lytic mechanism because there is a complete lack of membrane permeabilization, as detected by the efflux of β-galactosidase and potassium ions (Hetru *et al*, 1998).

The family of *glycine-rich* AMPs includes several 9 – 30 kDa polypeptides, all with a 10-22% glycine content. These polypeptides are predominantly active on Gram-negative bacteria, inhibiting cell wall synthesis (Hoffman *et al*, 1996). The grouping of these peptides may be artificial because the sequences of the peptides included in this group are not as closely related to each other as in other families. This family includes the *attacins, sarcotoxins II*, and the *diptericins*. (Hetru *et al*, 1998).

Antifungal peptides have been isolated from *Drosophila*. One peptide, dromomycin, has potent antifungal activity against a broad spectrum of filamentous fungi, but shows no activity against bacteria (Fehlbaum *et al*, 1994). Dromomycin has a high degree of homology to other defensins; however, its mode of action has not been determined. (Hetru *et al*, 1998).

1.3.2 Antimicrobial compounds in ticks

Antimicrobial substances have also been identified in ticks. There is a brief report of a lysozyme in the haemocytes of *Ixodes ricinus*. This enzyme was detected during endocytosis after the injection of particulate material into the haemocoel (Kühn and Haug, 1994). A defensin has been isolated from the haemolymph of *Dermacentor variabilis* (Johns *et al*, 2001). This cationic peptide was identified in the haemolymph one hour post inoculation with *Borrelia burgdorferi* (the spirochaete causing Lyme disease) and *Bacillus subtilis* (Johns *et al*, 2001). This defensin also controls infections of *Escherichia coli* and *Staphylococcus aureus* (Johns *et al*, 1998). A 4 kDa peptide, with high homology to a scorpion defensin, was purified from the haemolymph of *Ornithodoros moubata*. This defensin is heat resistant and is present in the haemolymph even without challenging them with bacteria (van der Goes van Naters-Yasui *et al*, 2000; Nakajima *et al*, 2001).

In addition to the hemolymph – localized defensins, Kopacek *et al.* (1999) isolated a lysozyme from the gut of *O. moubata*. The presence of a host haemoglobin fragment in the gut of *Boophilus microplus* was reported by Fogaca *et al.* (1999). Nakajima *et al.* (2003) proposed that this fragment is used as an antibacterial agent in the midgut of the tick, because it proved to inhibit the growth of both Gram-negative and Gram-positive bacteria *in vitro*.

Potterat *et al.* (1997) observed that while dead females of the *B. microplus* may be covered with fungi, the eggs next to them remained clean. A

sterol amide (boophiline; Figure 1.4) was extracted from whole *B. microplus* females. This substance inhibited the growth of the fungi *Claudosporium cucumericum* and *Candida albicans,* the Gram-positive bacteria *B. subtilis* and the Gram-negative bacteria *E. coli.* Preliminary assays by the latter authors also detected the presence of an antifungal compound in an egg extract of *B. microplus*, possibly also boophiline.

Unpublished observations from our laboratory demonstrated that the eggs of *A. hebraeum* contain an antimicrobial substance. When one or more eggs were plated on agar plates inoculated with *E. coli* or *Serratia marcescens* (Gram-negative), a clear inhibition zone of bacterial growth was formed. However, the eggs failed to inhibit the growth of *S. epidermidis* and only a very small inhibition halo was observed for *B. subtilis* (Gram-positive).

To date, very little has been published on antimicrobial substances associated with the eggs of arthropods. I have found only one published report of an antibacterial component in an insect egg. This material, isolated from the Mediterranean fruit fly *Ceratitis capitata*, inhibits the growth of Gram-negative and Gram-positive microorganisms and is deposited onto the eggs with the secretion of the reproductive accessory glands of *C. capitata* (Marchini *et al*, 1997). The authors suggested that his represents a preventive measure against possible microbial infection. Unlike most antimicrobial proteins isolated from insects, this peptide, ceratotoxin, is not induced by bacterial infection. Moreover, it is expressed only in sexually mature females. (Marchini *et al,* 1995). The results of the latter study suggest that the antimicrobial substance

from tick eggs also may not be specifically induced by microbial infection and may also originate from the female reproductive organs.

1.4 The ixodid female reproductive system

1.4.1 Anatomy

The anatomy of the ixodid female reproductive system consists of an ovary, two oviducts, a vagina and a seminal receptacle (Figure 1.5). Two paired accessory glands (tubular and lobular) are present at the junction with the vagina. Géné's organ (the egg-waxing organ) is anatomically separate from the rest of the reproductive tract (see section 1.4.3).

1.4.2 Physiology of egg development

Briefly, the undeveloped oogonia and the primary oocytes project from the wall of the ovary, covered by a basement membrane (Figure 1.6). The mature oocyte is ovulated into the central lumen of the ovary and passes down to the oviduct (Lees and Beament, 1948). There is some controversy regarding the site of fertilization. However, both the oviducts and the ovarian lumen are filled with spermatozoa during ovulation, so it has been suggested that fertilization occurs somewhere in the ovarian lumen (Sonenshine, 1991). After the egg passes to the oviduct, secretions from the lobular accessory glands are deposited onto the shell, and this provides some degree of waterproofing (Lees and Beament, 1948). When the egg emerges from the genital pore, it is received by the everted, glandular part of Géné's organ (see next section).

1.4.3 Géné's organ

Géné's organ is an unusual organ found only in ticks (Figure 1.7a). It is located in the body cavity between the anterior end of the scutum and the mouth parts. This organ everts from the body cavity only during oviposition, and it coats each egg with a wax-like material. In combined action with the mouth parts, the eggs are pushed to the dorsal part of the tick's body (Sonenshine, 1991; Figure 1.7b).

The wax material that this organ produces provides vital protection against desiccation (Lees and Beament, 1948; Booth *et al*, 1984). Kaufman *et al* (1986) demonstrated that unwaxed eggs desiccate rapidly, even when held at 95% relative humidity. The egg wax contains long chain hydrocarbons, fatty acids, wax esters and steroids (McCamish *et al,* 1977). The wax produced in the horns of Géné's organ stains with Sudan black and dissolves in chloroform, suggesting that this secretion is primarily a lipid. Relatively crude histochemical tests suggested the absence of protein (Lees and Beament 1948), although this thesis suggests otherwise.

To date, most physiological studies of Géné's organ have focused on its structure and ultrastructure (Booth *et al*, 1985), the pharmacological control of its secretion (Booth *et al*, 1986), and the waterproofing effect of the wax secretion (Lees and Beament, 1948), but nothing has yet been reported about a potential role in coating the eggs with an antimicrobial substance.

Géné's organ is very inconspicuous in unfed ticks. It begins to enlarge during the feeding period, but only becomes prominent during the post-engorgement period, when it begins to function (Schöl *et al,* 2001), suggesting that its development may be under hormonal control.

1.5 Hormonal control of egg development in A. hebraeum

Following engorgement, oocytes mature rapidly (Sonenshine, 1991). Yolk (vitellin) accumulates within the oocytes and, by day 10 post-engorgement, the ovary reaches its maximum weight (Friesen and Kaufman, 2002; Figure 1.8). The ecdysteroid hormone, 20-hydroxyecdysone (20E), controls egg development in *A. hebraeum* by stimulating egg yolk synthesis in the fat body (Friesen and Kaufman, 2002). This hormone also triggers salivary gland degeneration four days after engorgement (Harris and Kaufman, 1985).

The ecdysteroid titer in hemolymph rises 10-100 times during the first week after engorgement in *A. hebraeum.* On the day of engorgement (day 0), 20E titers in the hemolymph are ≥ 10 ng/ml. By day 10 the amount rises to ~ 1000 ng/ml, and by day 18, ~ 1700 ng/ml of 20E were detected in the haemolymph (Friesen and Kaufman, 2002). During the post-engorgement period, Géné's organ constantly increases in size until days 12-14 post-engorgement. Thus, 20E may also play a role in the organ's growth and may also stimulate the secretion of the antimicrobial compound in the egg wax.

1.6 Thesis Objectives

My thesis comprises six major objectives:

- To confirm earlier unpublished observations from this lab that the surface of the tick eggs contains antimicrobial activity,
- To isolate an antimicrobial compound from the eggs,
- To evaluate the general mechanism that underlies the action of this antimicrobial substance.
- To determine whether Géné's organ and/or other section of the reproductive system produces the substance responsible for this antimicrobial activity,
- To determine whether 20E stimulates the development of Géné's organ and/or the secretion of the antimicrobial substance,
- To partially characterize the antimicrobial substance associated with the eggs.

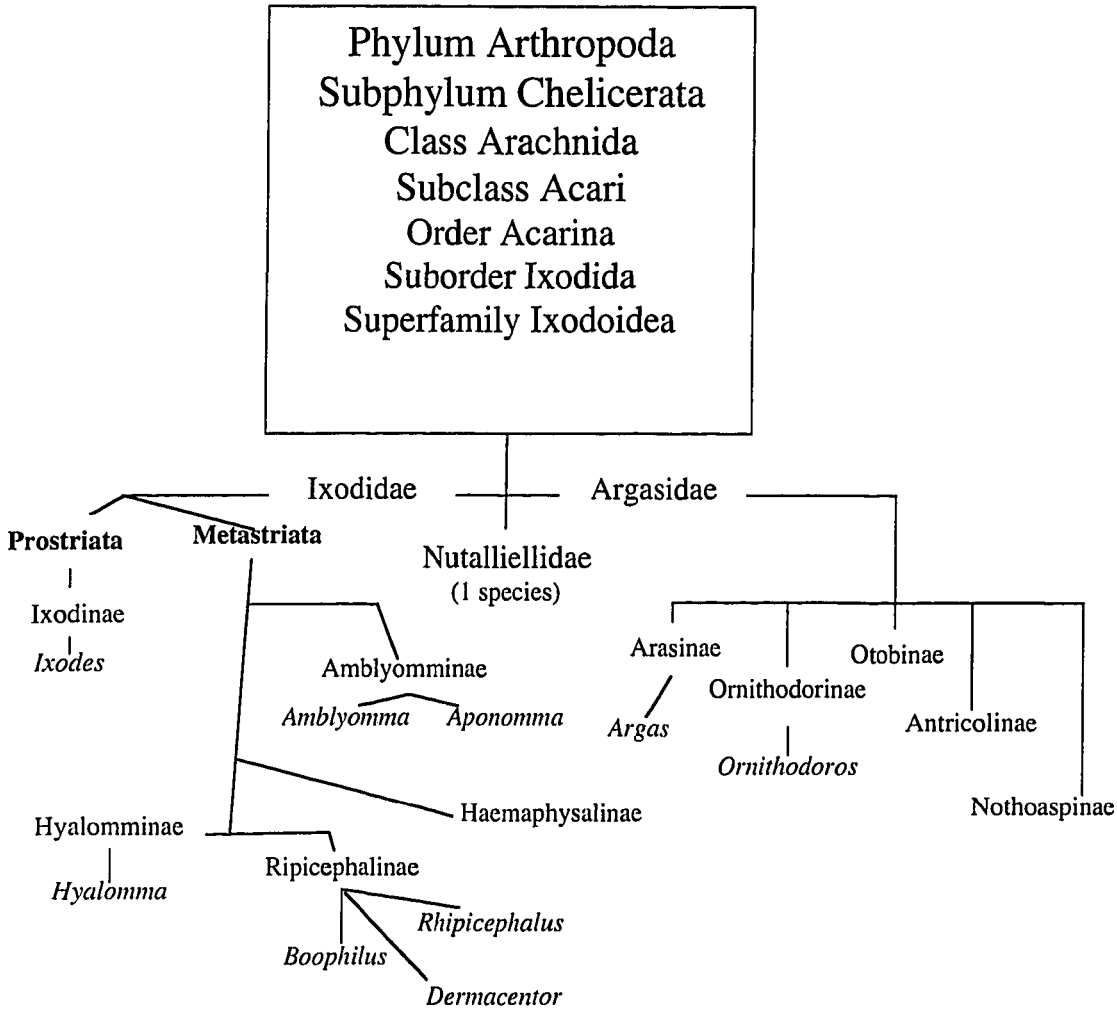


Figure 1.1 Phylogeny of ticks. Very recently the genus *Boophilus* has been collapsed into the genus *Rhipicephalus*. Modified from Sonenshine (1991).

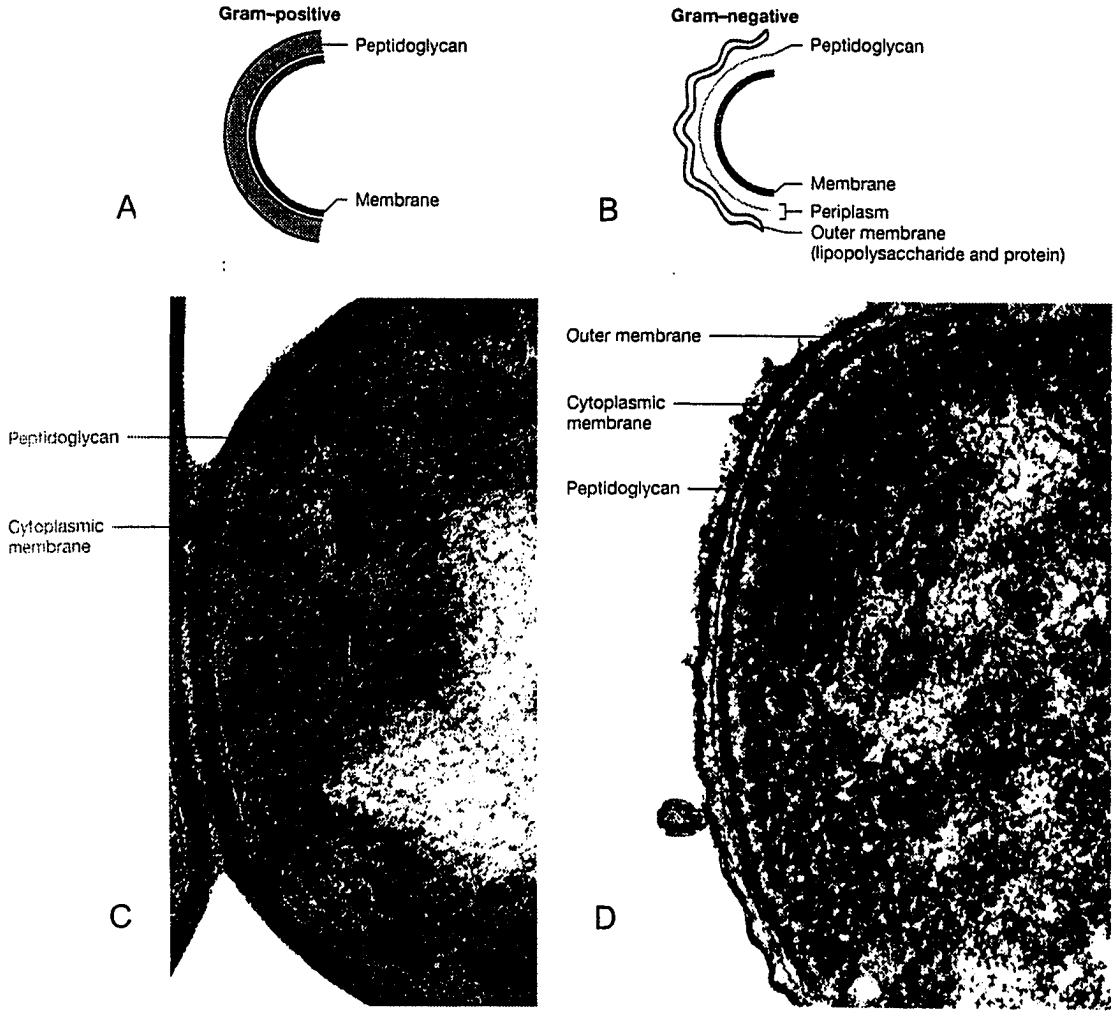
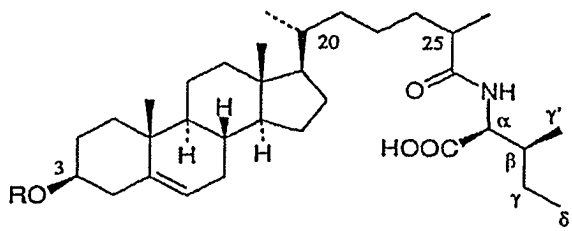


Figure 1.2 Schematic diagram of Gram-positive (A) and Gram-negative (B) cell walls. Electron micrographs showing the cell wall of the Gram-positive *Bacillus subtilis* (C) and the Gram-negative *Leucotrix mucor* (D; modified from Madigan, 2002).

Figure 1.3 Bacterial lysis caused by cecropin. Transmission electron micrographs of *E. coli* cells treated with the antibiotic cecropin B. (from Chen *et al*, 2003)



1 R = SO_3H

2 R = H

Figure 1.4 Boophiline. N-[3-sulfooxy)-25ξ-cholest-5-en-26-oyl]-L-isoleucine. A sterol amide isolated from homogenates of the tick *Boophilus microplus.* (from Potterat *et al.,* 1997).

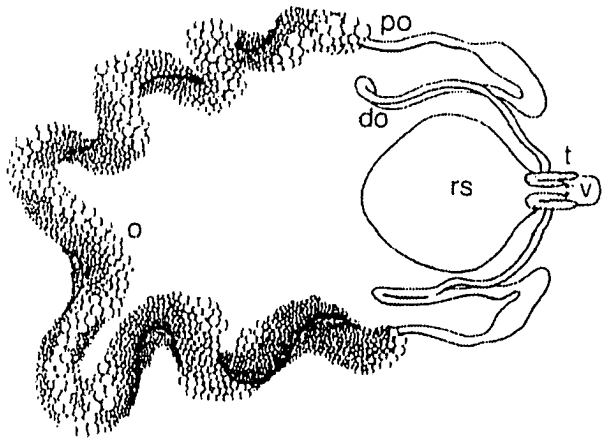


Figure 1.5 Anatomy of the female genital system of *A. hebraeum*. (O) ovary; (po) proximal oviduct; (do) distal oviduct; (rs) seminal receptacle; (v) vestibular vagina; (t) tubular accessory glands. Two of the accessory glands (lobular and Géné's organ) are not shown here. (modified From Diehl *et al.*, 1982).

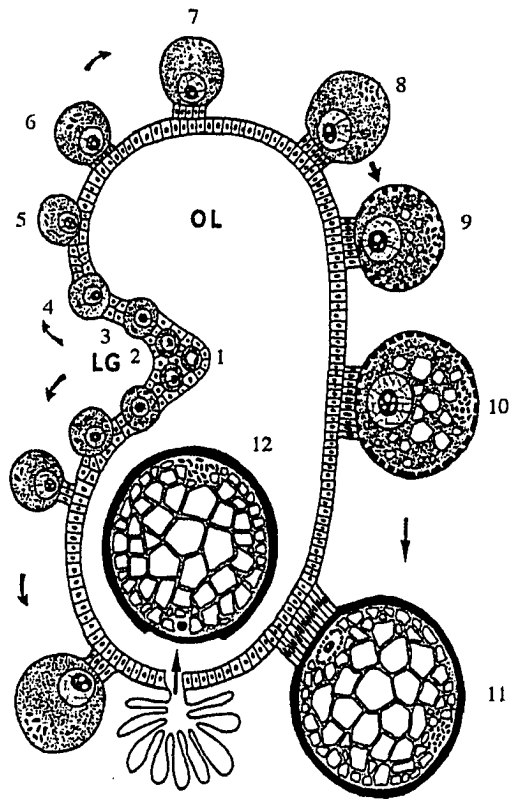


Figure 1.6. Oogenesis in a tick ovarian cross-section. Immature oocytes (1-3) develop within the longitudinal groove (LG). As oocytes continue their maturation they move away from the LG (4-7). As the oocytes enlarge, they protude significantly from the ovarian wall (6-10). At the end of this maturation process, the nucleolus and the nuclear membrane disappear (8-10) and ovulation occurs (12). OL, ovarian lumen. (modified from Diehl *et al.*, 1982)

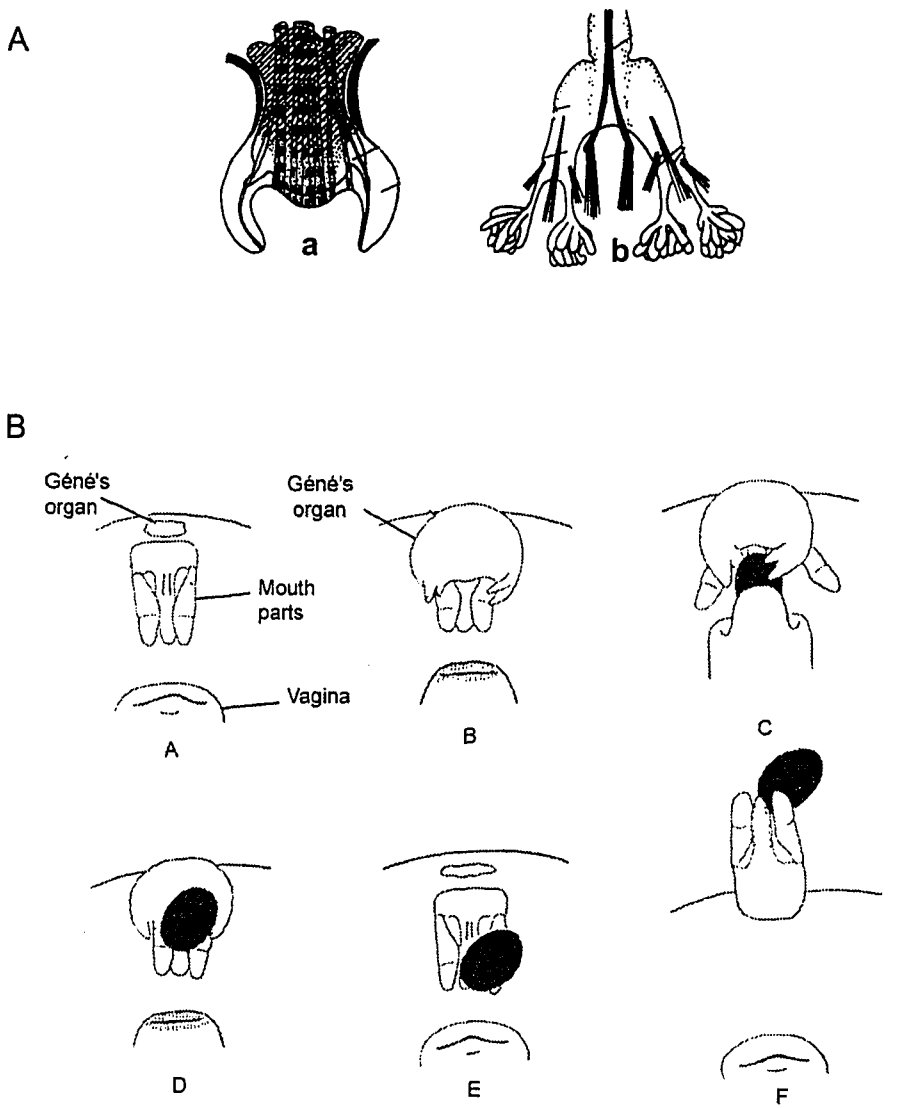


Figure 1.7 A. Anatomy of the everted Géné's organ in (a) *Ornithodoros moubata* (argasid) and (b) *Hyalomma asiaticum* (ixodid). (Modified from Sonenshine, 1991). **B.** The ovipositional process in female ticks. During oviposition, Géné's organ everts from the body cavity to cover each egg with a waxy deposit. Then, the mouth parts hold each egg and push it dorsally and away from the female's body. (Modified from Lees and Beament, 1948).

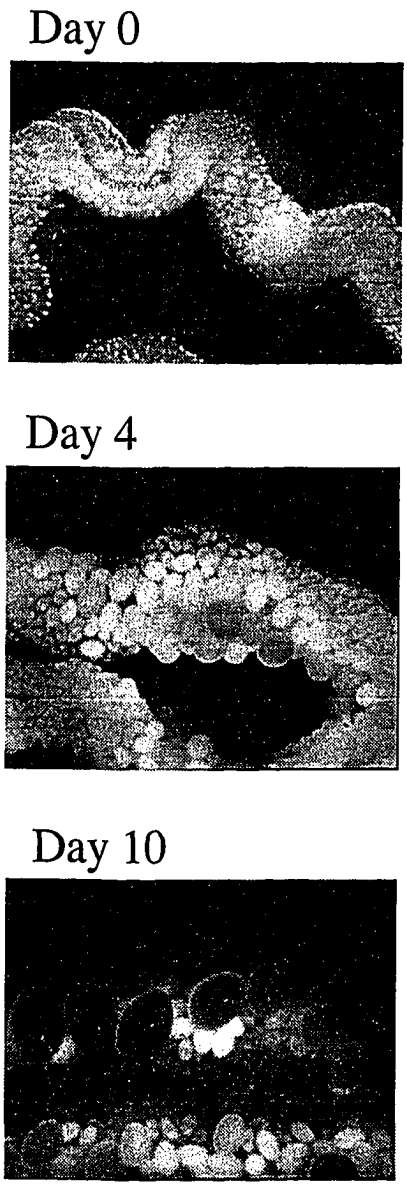


Figure 1.8 Ovarian development in *A. hebraeum* after engorgement. Note how the mature oocytes project from the wall of the ovary. By day 10, the ovary represents 7% of the total body weight (modified from Friesen, 2003)

Chapter 2

General Methods

2.1 Tick feeding and collection of eggs

Females and males were fed together in a cloth-covered foam pack on the backs of rabbits, as described originally by Kaufman and Phillips (1973). Engorged ticks detach spontaneously usually within 10 days. Engorged females were washed with distilled water, weighed and stored in the colony incubator (darkness at 26°C and 95% humidity). Egg laying begins about 10-12 days later, and continues for approximately 3 weeks. Only ticks over 1000 mg were used in this study.

The eggs were collected with a spatula from the females and stored in cloth-covered plastic vials. Because oviposition occurs over 3-4 weeks, the eggs were carefully mixed 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 the experiment, eggs used in this study were frozen at -20°C.

2.2 Collection of tissue

Just prior to dissection, engorged ticks were rinsed in water, immobilized on Petri dishes with cyanoacrylate glue and cooled in a refrigerator for 30 min. to reduce the risk of puncturing the gut during dissection. Ticks were then dissected from the dorsal surface and the following tissues collected: ovary,

oviducts, Géné's organ, salivary glands, trachea, and gut. All tissues were washed in modified Tissue Culture medium 199 (TC-199, no antibiotic added; Sigma) three times, to remove haemolymph, which might contain AMPs. Tissue samples were stored at -20°C in microfuge tubes containing 35 μl of TC -199. Just prior to use, frozen tissues were rapidly thawed in a 37°C water bath.

2.3 Solid culture diffusion assay of the antimicrobial activity of the tick eggs and egg wax extracts

The following bacterial species and fungal species were used: four Gram negative species (*E. coli* B5, *S. marcescens, Proteus vulgaris* and *Pseudomonas aeruginosa*); three Gram positive species (*S. epidermidis, B. subtilis* and *M. luteus)*; and the yeasts *C. albicans* and *Saccharomyces cerevisiae*. One colony of each strain was suspended in 10 ml of Mueller Hinton (MH) broth (Difco) and incubated overnight at optimal growth temperature: 37°C for all species, except for *B. subtilis* and *M. luteus* (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, 100 μl of this dilution was mixed with soft agar to overlay the agar plates (see below). The cell concentration used for the growth inhibition assays was 1-5 X 10^7 colony forming units (CFU)/ml (about $OD_{600\ nm}$ = 0.1).

MH agar (Difco) was prepared by dissolving the medium (containing 1% agar) in distilled water and sterilizing in an autoclave for 25 min. MH soft agar

(Difco) was prepared by adding agar to MH broth to a final concentration of 0.66% and sterilizing in an autoclave for 25 min.

Eighteen to 23 ml of MH agar were poured into sterile Petri dishes (100 mm diameter). One plate was incubated for 24 h at 37°C as a sterile control. Sterile test tubes were filled with 4 ml hot MH soft agar and kept at 45°C. Under aseptic conditions, 0.1 ml of each bacterial suspension (see above) was added to one soft agar aliquot, the tube was vortexed for 2-3 seconds, and the contents quickly poured and spread onto a MH agar plate.

Whole tick eggs, eggs denuded of wax, and paper discs (5 mm) containing 50 μl of egg wax extracts, were plated aseptically on MH agar plates overlaid with bacteria and incubated for 48 h at 37°C or 30°C. Unsoaked paper discs and paper discs soaked with solvent were plated as controls.

The clear zone around the eggs was measured with callipers or at 9X magnification, with an ocular micrometer fitted to a dissection microscope. When paper discs were used, the measurements included the disk diameter.

2.4 Bacterial growth inhibition assay in liquid culture

Two bacterial species were used: *E. coli and S. epidermidis.* Ten ml of MH broth were aseptically pipetted into 25 ml test tubes. One tube was incubated at 37°C for 24 h as an aseptic control. One colony of each bacterial species was inoculated into each tube and incubated overnight. Aliquots of the overnight cultures were diluted 1/100 in 10 ml tubes with 5 ml fresh MH broth.

Sterile paper strips soaked with 100 μl of egg wax extracts (100 mg/ml) and solvent controls were introduced to 10 ml tubes with MH broth. Bacterial suspensions were then added to each tube, and ODs (600 nm) were recorded at time 0, then each hour for 3 h and then at 20 h.

To study the basic mechanism of action of the aqueous extract of the egg wax, liquid cultures of *S. epidermidis* were started as described above, and $OD_{600\,nm}$ was recorded every hour until the culture reached the logarithmic growth phase (Abs= 0.2 - 0.3). At this point, ~10 mg of the aqueous extract dissolved in 200 μl of MH broth was added to the culture. 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 sample was then diluted $1:10^4$ –1: 10^{13} in MH broth. One hundred microliters of each dilution were plated onto MH agar plates in duplicate. After an incubation of 48 h at 37 °C, only those plates with 30-300 colonies were used to calculate CFU at each time point.

2.5 Wax extraction of the tick eggs

Organic solvents were mostly used to extract the wax material around the tick eggs. Two biphasic extraction methods: Folch, (1957) and Bligh & Dyer, (1959) were assayed as well. These two methods extract both polar and non-polar compounds, which are separated by an interphase. Only glassware was used for the following procedures. Several batches of eggs (100 mg each) were

extracted using various polar and non-polar solvent systems in order to choose the best extraction method. The methods used were as follows:

- 1 ml chloroform was added to the eggs and vortexed for 30 s. The solvent was collected in a clean test tube with a glass pipette, after allowing the particulate matter to settle.
- 1 ml methanol was added to the eggs and vortexed for 30 s. The solvent was collected in a clean test tube with a glass pipette, after allowing the particulate matter to settle.
- Folch method (1957): 1 ml of a mixture of chloroform and methanol (2:1) was added to the eggs and vortexed for 1 min. The solvents were collected in a clean test tube as with a glass pipette and 0.5 ml of distilled water was added to the eggs and vortexed for 1 min. The water was also collected and vortexed for 3 sec. with the first solvent mixture. The solvents were centrifuged for 1 min at 1000 rpm to separate the aqueous phase from the organic phase.
- Bligh and Dyer method (1959): 1.0 ml of a mixture of chloroform and methanol (1:2) was added to the eggs and vortexed for 1 min. The solvents were removed and 0.5 ml of water was added, vortexed and removed as described above for the Folch method.
- 1 ml acetonitrile was added to the eggs and the mixture was placed on a shaker for 20 min at 1200 rpm. The solvent was collected as described above.

- 1 ml phosphate buffered saline (PBS) 1X was added to the eggs and the mixture was placed on a shaker for 1 h at 1200 rpm.

All non-polar extracts were transferred into 2 ml pre-weighed ampoules (Wheaton) and all polar extracts to microfuge tubes. The solvents were evaporated under a nitrogen stream (non-polar) or lyophilized overnight (aqueous). The weight of the extract was calculated as the difference between the extract plus container and the container alone. The non-polar extracts were resuspended in 0.1 ml of the same extraction solvent to minimize lipid hydrolysis from air exposure. The samples were stored at -80°C until analysis for antimicrobial activity. These extracts were absorbed onto paper discs, air dried for 15 min, and the discs incubated for 48 h on the surface of agar plates that had been previously overlaid with soft agar containing the organisms mentioned in section 2.3.

2.6 Amino acid analysis of the aqueous phase of the egg wax extract

Analysis of the amino acid content of the Folch aqueous extract was performed by the Alberta Peptide Insitute using an ion-exchange-based Beckham 6300 analyzer. Prior to analysis, the sample was hydrolysed with HCl and dried under vacuum. The amount (nmol) of amino acid detected was used to estimate the amount (ng) of amino acid in the aqueous extract. The total nmoles of amino acid was multiplied by 110 ng/nmol (the average molecular weight of the individual amino acids in a "typical" protein).

2.7 Protein electrophoresis of the aqueous phase of the egg wax extract

Protein separation was performed using four one-dimensional electrophoretic systems:

1) Nondenaturing polyacrylamide gel electrophoresis (PAGE; Schagger, (1994): pH range 7.1-8.9, 15% acrylamide, 0.1% Bis acrylamide and 0.5M Tris buffer in the separating gel. The stacking gel was composed of 5% acrylamide and 0.2M Tris buffer. Samples were mixed with 0.5 M Tris, glycerol (13% v/v) and bromophenol blue (10 μg/ml) as a tracking dye. Electrophoresis was carried out at 100 V until the tracking dye reached the bottom of the gel (about 1.5 h).

2) Acetic acid nondenaturing PAGE (Hultmark, 1980): pH range 3.7-5.6. Briefly, polyacrylamide gel (15% acrylamide, 0.1% Bis) was prepared using 200 mM acetic acid (pH 4.3), but the stacking gel was omitted. Samples were mixed with 5 mM acetic acid, glycerol (13% v/v) and methyl green (1mg/ml) as a tracking dye. Electrophoresis was performed towards the cathode at 100 V for 1h and 150 V for 3 h.

3) Tris/glycine SDS-PAGE (Laemmli, 1970): to investigate the molecular weights of denatured proteins and peptides in the sample, a separating gel (15% acrylamide, 0.1% Bis) was prepared using 0.8 M Tris gel buffer and 0.1% sodium dodecyl sulfate (SDS). The stacking gel consisted of 5% acrylamide and 0.15 M Tris buffer. The samples were denatured with SDS and 2-mercaptoethanol, and boiled for 5 min prior to loading. The running buffer consisted of 0.02 M Tris base and 0.2 M glycine. Electrophoresis was

performed at 100 V until the tracking dye (bromophenol blue) reached the bottom of the gel (about 1.5 h).

4) Tris/tricine PAGE (Schagger and von Jagow, 1987): to improve resolution for small proteins and peptides, the sample was separated in a resolving gel containing 10% acrylamide, 0.1% Bis, 0.8 M Tris buffer (pH 8.45), SDS 0.1% and 7.5% glycerol. The stacking gel contained 5% acrylamide, 0.05% Bis and 0.5 M Tris buffer (pH 8.4) and 0.1% SDS. The sample was solubilized in tricine sample buffer and heated to 45°C for 60 min. prior to loading. The running buffer contained 0.1 M Tris, 0.1 M tricine and 0.1% SDS. Coomassie blue G-250 was used as a tracking dye because in this system it moves ahead of the smallest peptides. The gel was run at 30 V for 1h and then at 150 V until the dye reached the bottom of the gel (about 2 h). A heat exchanger was used to keep the electrophoresis chamber at room temperature.

All gels were rinsed with a fixative solution (40% (v/v) methanol, 10% (v/v) acetic acid, 2.5% (w/v) glycerol) for 15 min. The fixative solution was washed out with distilled water for 10 min. The gels were then stained with colloidal Coomassie brilliant blue G-250 (Sigma) for 30-60 min. The stained gels were rinsed with distilled water for 5 min., placed in a destaining solution (30% (v/v) methanol, 3% (w/v) glycerol) and gently shaken for 30 min.

2.8 Assay of antimicrobial activity of protein bands of the aqueous phase in non-denaturing conditions

The aqueous phase of the egg wax extract was subjected to non-denaturing electrophoresis using neutral and acidic buffer systems (pHs 7.1 - 8.9 and 3.5 - 5.6, respectively; see previous section). In both cases, 20 μl of the aqueous phase (10 μg amino acid/ ul) were loaded into wells as duplicate sets on each half of the gel. After electrophoresis, half of the gel was stained with Coomassie blue and the other half was washed with PBS 0.1 X in MH broth for 60 min. Then, a mixture of 3.0 ml soft MH agar and 0.1 ml of *S. epidermidis* culture (OD=1.0 at 600 nm, ~ 10^7 CFU/ml) were poured over the gel slab. The plate was incubated at 37°C for 24 - 48 h (Hultmark *et al.*, 1980). The antimicrobial peptides defensin and cecropin (Sigma-Aldrich; 6 μg each) were used as a positive control. The overlay was incubated for 48 h @ 37°C.

2.9 Protease and heat treatments

The antimicrobial activity of the aqueous residue of the egg wax extract was tested in liquid culture against *S. epidermidis* (see section 2.4) after exposure to Proteinase K (15 min @ 55°C), or Pronase (30 min @ 37°C), or after boiling for 10 min. Lysozyme, a bacteriolytic protein that is sensitive to protease and heat treatments, served as a positive control. To inactivate the proteases before the addition of the aqueous phase to the cultures, the samples were boiled for 5 min. Following protease treatments, samples were run in a SDS-PAGE system.

2.10 Transmission electron microscopy (TEM)

Samples (1 ml) of untreated bacterial cells and cells 2 h after the addition of the aqueous extract were prepared for TEM. Samples were pelleted by centrifugation (3000 rpm) and fixed in glutaraldehyde (2%) for 18 h. Samples were washed with 1X PBS three times for 10 min, and treated with osmic acid for 2 h, followed by three 15-min washes with 1X 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 resin (1:1) was added to the samples for 24 h. Finally, the samples were embedded in Spurr 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 1% uranyl acetate and Reynold lead citrate. Sections were examined under a Morgani 268 Philips Electron Microscope.

2.11 Live/Dead cell staining

S. epidermidis cells were treated with the aqueous phase of the egg wax extract as in Section 2.4, and stained with the LIVE/DEAD BacLight kit (Molecular Probes). The kit contains 2 nucleic acid stains, SYTOX-9 and propidium iodide, which have different spectral characteristics and abilities to penetrate membranes. Propidium iodide (red) is impermeant to live cells with undamaged membranes, while SYTOX-9 (green) is able to penetrate cell

membrane in normal conditions (information from manufacturer). Cells with an intact cytoplasmic membrane show green fluorescence and cells with a damaged cytoplasmic membrane show red fluorescence.

A mixture of SYTO-9 and propidium iodide was prepared (1:1, v/v) and diluted in PBS 1X (2:1000, v/v). One ml of the treated and untreated bacterial suspensions was centrifuged for 10 min at 10,000 rpm and was incubated for 15 min in the dark at room temperature (RT) with 0.2 ml of stain mix. Preparations were mounted with immersion oil on glass microscope slides and green or red cells were observed by confocal flourescence microscopy. Observations were done 30 min and 2 h after addition of the aqueous extract.

2.12 Antimicrobial activity of various female tick tissues

Various tissues of *A. hebraeum* (Géné's organ, oviducts, ovary, gut, salivary glands, and trachea) were dissected (see Section 2.2), weighed and plated on agar plates as mentioned in section 2.3. Two sets of tissues were assayed: tissues dissected before day 4 post- engorgement and those dissected after day 10. Géné's organ was also tested for antimicrobial activity on days 0, 2, 4, 6, 8, 10, 12, 16 and 20 post-engorgement.

Because the samples that were tested were not constant in size or form, the size of the egg batches or the tissue samples were not measured, only the size of the clear zones. Four measurements at different points of the clear zone were averaged. Antimicrobial activity was determined as the width (in mm) of

inhibition per mg of sample. Paper discs containing 50 μl of TC-199 served as control.

2.13 Injections of 20E

20E was dissolved in 70% ethanol and stored at -20°C freezer as a 4 mg/ml stock solution (Friesen and Kaufman, 2002). On the day of injection, 20E was diluted to working concentration in modified TC-199.

Engorged ticks were injected on the day of engorgement (day 0) with 4 μg 20E/g body weight with a 50 μl Hamilton syringe through the coxa of the 4th leg. Twenty microliters were injected per 1000 mg body weight. 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 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, Géné's organ was removed from each tick, weighed, and tested for antimicrobial activity against *S. epidermidis* in solid culture (see Section 2.11). Uninjected ticks and ticks injected with TC-199 and ethanol served as controls.

2.14 Statistical analysis

Unless otherwise stated, all data are reported as mean ± SEM (Standard error of the mean). Means were compared using a Student's t-test. Statistical difference is indicated at follows: $0.05 > \rho > 0.01$ (*); $0.01 > \rho > 0.001$ (**); $\rho < 0.001$ (***).

Chapter 3

Results

3.1 Antibacterial activity of the eggs

To confirm that the surface of the tick egg contains antimicrobial activity, conventional bacterial growth inhibition assays were performed in solid agar plates. Because experimental design would be easier if eggs could be collected and stored prior to experimentation, I tested whether antibacterial activity was affected by freezing for up to 4 weeks. Table 3.1 demonstrates that the clear zones produced by fresh and frozen eggs were similar. Consequently, frozen eggs were used for all subsequent experiments and extractions.

Tick eggs inhibited the growth of the Gram-negative bacteria *E. coli* (Figure 3.1, Table 3.1) and *S. marcescens* (Table 3.1) in solid agar culture. In contrast, when tested against Gram-positive bacteria, either no clear zones were visible (*S. epidermidis*; Figure 3.1, Table 3.1) or only a very small partial inhibition was observed (*B. subtilis*; Table 3.1). Eggs plated following wax removal (see Folch method; section 2.5) tested against *E. coli* did not inhibit bacterial growth (Figure 3.1).

When the eggs were plated over *E. coli* cultures in batches of 1, 5, 10 or 20 eggs, an increase in the size of the clear zone was observed as the number of eggs increased (Figure 3.2).

3.2 Wax extraction

Because the nature of the antimicrobial substance was suspected to be a lipid, mostly organic solvents were used for extraction of the egg wax. However, because ticks are known to produce AMPs, it was considered possible that the antimicrobial activity of the eggs is due to a peptide. Therefore, two extraction methods that involve both polar and non-polar solvents were tested as well (Bligh and Dyer (1959) and Folch (1957).

In total, six extraction methods were evaluated: Bligh and Dyer, Folch, chloroform, methanol, PBS (1X) and acetonitrile. The last two solvents did not extract a significant amount of residue nor antibacterial activity. The extraction with chloroform yielded 1.9 mg of egg wax residue per 100 mg of eggs and this extract produced a growth inhibition halo of 11.2 mm (Figures 3.3 and 3.4). The residue obtained after extraction with methanol weighed 0.7 mg and did not inhibit bacterial growth (Figures 3.3 and 3.4). The Bligh and Dyer and Folch methods each yielded an organic phase and an aqueous phase. The Bligh and Dyer method yielded an average of 1.9 mg of residue per 100 mg of eggs (organic plus aqueous) and produced a clear halo of 13.3 mm in agar plates with *S. epidermidis*. Only the aqueous phase caused growth inhibition (Figures 3.3 and 3.4). The Folch method yielded 3.1 mg/100 mg of eggs (organic plus aqueous) and produced an inhibition halo of 26.9 mm (organic plus aqueous; Figures 3.3 and 3.4). Consequently, I used the Folch method for all subsequent extractions. Table 3.2 shows that 1 g of eggs yielded 23.4 ± 6.9 mg residue (n=14) when extracted by the Folch method.

3.3 Antibacterial activity of the Folch extracts

3.3.1 Diffusion assays in solid culture

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*, *P. vulgaris* and *P. aeruginosa),* even after combining the organic and the aqueous residues (Table 3.3). The wax extracts produced a halo only when plated with Gram-positive bacteria (*S. epidermidis, B. subtilis* and *M. luteus).* The extracts failed to kill the yeasts *Candida albicans* and *Saccharomyces cerevisiae* (Table 3.3).

3.3.2 Growth inhibition assays in liquid culture

In liquid culture, only the aqueous extract (10 mg) was able to inhibit the Gram-positive *S. epidermidis.* Inhibition was noticeable within 1 h of incubation, and by 20 h, growth was inhibited by ~ 94% (Figure 3.5).

3.4 Basic mechanism of action of the aqueous phase

Antibacterial agents usually express one or more phenomenological effects when added to an exponentially growing bacterial culture. *Bacteriolytic* agents induce cell lysis. *Bactericidal* agents kill cells, but lysis does not occur. *Bacteriostatic* agents inhibit bacterial growth but do not kill or lyse bacterial cells (Madigan *et al,* 2002).

To evaluate the effect of the antimicrobial component of the aqueous extract of the egg wax, I monitored the growth of exponentially-growing bacteria after adding 10 mg of the aqueous phase. The aqueous phase was added to the *S. epidermidis* cultures 2.5 h after the cultures were started, and cell growth was monitored by optical density (OD) measurements and viable cell counts (see Chapter 2). Following addition of the aqueous phase, ODs started to decrease by 1.5 h, whereas viable cell counts began to decrease within 1 h. Viable cell counts dropped from 8.3×10^8 to 6.6×10^6 CFU/ml by 2 h. Control and solvent control cultures grew normally. (Figure 3.6)

To determine whether or not the bacterial cytoplasmic membrane and/or the cell wall is injured by the antimicrobial substance (classic mechanism of AMPs), treated and control *S. epidermidis* cells were stained using the LIVE/DEAD BacLight Kit (Molecular Probes) 30 min and 2 h after adding the aqueous phase of the tick egg extract to the culture. Control cultures reflected green light throughout the experiment. By 30 min, bacterial cells were losing membrane or cell wall integrity, and the normally impermeant dye, propidium iodide, was able to stain some cells red. By 2 h, all the cells were stained red (Figure 3.7). However, when bacterial cells were observed under TEM, their cell walls seemed thickened, but not disrupted. The most evident cytological change observed was the formation of circular inclusions within the cytoplasm (Figure 3.8).

3.5 Protein analysis of the egg wax aqueous extract

Because only the aqueous extract showed antimicrobial activity in liquid culture (Figure 3.5), I attempted to analyze the protein content of the aqueous phase. Colourimetric assays were not suitable due to the turbidity and yellow colour of the aqueous extract. Instead, I arranged for an amino acid analysis of the extract (see Chapter 2). The aqueous residue contained ~776.4 nmol amino acid/mg of residue (85.4μg amino acid/ mg od residue), representing ~6-10% of the total sample. Because this was a significant amount of protein, extracts were separated by 4 different PAGE systems (see Section 2.7). The extract resolved by Tris SDS-PAGE showed a clear band of ~14 kDa, a group of bands in the range of 20-36 kDa and a heavier band of ~64 kDa. The extract resolved by tricine SDS-PAGE showed a clear band of 14 kDa and a series of blurry bands between 25 and 36 kDa (Figure 3.9).

The protein bands of the aqueous phase did not show antibacterial activity when overlayed with soft agar seeded with *S. epidermidis* or *B. subtilis* under acidic or neutral buffer systems (data not shown). However, the AMP cecropin, which has been reported to show antibacterial activity in acidic protein gels (Hultmark, 1980), did not produce an inhibition halo either. The cecropin used was tested for biological activity in liquid culture against *S. epidermidis* and it showed a very potent antimicrobial effect (~6 μg caused 100% inhibition of bacterial growth within 10 min; data not shown).

The aqueous phase did not lose its antibacterial activity after being boiled for 10 min. nor following treatment with proteinase K or pronase (Figure 3.10 a). Lysozyme, a bacteriolytic protein, was inactivated with proteinase K and served as a control. Figure 3.10b shows that proteinase K had more of an effect on the lower bands of the aqueous extract. Enzymatic digestion of lyzozyme by proteinase K is shown in Figure 3.10c, although the reduction in molecular weight is not as evident as with the aqueous phase.

3.6 Antimicrobial activity of tick tissues

A number of tissues from *A. hebraeum* were assayed on agar plates with *S. epidermidis*: ovary, oviducts, Géné's organ, salivary glands, trachea, and gut. Some of the tissue samples were collected between days 0 – 4 post-engorgement, and some were collected between days 11-18 post engorgement; these are labeled in Table 3.4 as "before day 4" and "after day 10", respectively. All tissues had at least some antimicrobial activity against *S. epidermidis*, with the exception of Géné's organ before day 4, and the closed gut samples before day 4 and after day 10. When the gut contents were exposed (opened gut), bacterial growth was inhibited. But, when the gut contents were not exposed (closed gut), bacterial growth was not inhibited (Table 3.4).

Because Géné's organ secretes the egg wax, it is the likely source of the antimicrobial activity observed in the eggs. Therefore, Géné's organ was dissected out at various days post-engorgement and assayed against *S.*

epidermidis to determine when antimicrobial activity appears in this organ in relation to egg-laying. Figure 3.11 shows the weight of Géné's organ as a function of time post-engorgement. The organ was relatively small on days 0-2, but grew steadily thereafter, appearing to plateau at about 50 mg on day 16 (Figure 3.11).

Figure 3.12 shows antimicrobial activity in Géné's organ as a function of time post-engorgement. Antimicrobial activity appeared by day 10, just prior to the onset of oviposition (days 10-12). Both measures increased thereafter in parallel. Prior to egg laying, no Géné's organ showed any antimicrobial activity. With one exception, all ticks which had begun oviposition also had antimicrobial activity in Géné's organ.

3.7 Effect of 20E on the growth of Géné's organ and the development of antimicrobial activity

The ecdysteroid hormone 20E triggers vitellogenesis in *A. hebraeum* (Friesen and Kaufman, 2002) and triggers salivary gland degeneration during the post-engorgement period (Harris and Kaufman, 1985). Because the antimicrobial activity appears in Géné's organ during the post-engorgement period (Figure 3.12), it was considered possible that the secretion of antimicrobial activity is under hormonal control. To investigate whether 20E plays a role in the development of Géné's organ and its antimicrobial activity, several ticks were injected with 20E (4 µg/g body weight) in order to approximate normal hemolymph 20E titers 10 days post-engorgement, the time

at which antimicrobial activity in Géné's organ appears. Ticks injected with ethanol and uninjected ticks served as controls. Ticks injected with 20E on the day of engorgement were dissected after 2, 4 or 6 days, and Géné's organ was weighed and assayed for antimicrobial activity. Those ticks incubated for more than 2 days received a booster injection every 2 days (see Chapter 2). No differences in tissue weight were observed in any of the experimental groups, compared to their respective control groups (Figure 3.13).

Immediately after weighing, each Géné's organ was assayed against *S. epidermidis* on agar plates, and none of the Géné's organs showed antimicrobial activity (data not shown).

Table 3.1 Comparison of the antimicrobial activity of fresh and frozen tick eggs. Clear zones of inhibition were measured after 48 hours of incubation at 37°C. Batches of five eggs (n=4) of *A. hebraeum*, fresh or frozen, were plated onto cultures of four bacterial species.

Bacteria	Clear zone (mm) Fresh eggs	Clear zone (mm) Frozen eggs	p
Gram-negative			
E. coli	0.28 ± 0.02	0.31 ± 0.02	0.06
S. marcescens	0.11 ± 0.01	0.09 ± 0.02	0.7
Gram-positive			
B. subtilis	<0.09 ± 0.02*	<0.10 ± 0.02*	0.8
S. epidermidis	0	0	N/A

* Only partial clearing was observed.

Table 3.2. Weights of the organic and aqueous extracts of the egg wax extracted by the Folch method. Batches of 1g of eggs were extracted. Egg batches were collected and frozen over the course of the study.

Sample	Organic phase (mg)	Aqueous phase (mg)	Total (mg)
1	16.2	12.3	28.5
2	6.1	16.7	22.8
3	6.3	10.8	17.1
4	14.1	13.1	27.2
5	8.5	8.6	17.0
6	15.2	12.8	28.1
7	7.1	16.0	23.1
8	18.4	14.8	33.2
9	12.0	14.4	26.4
10	6.7	12.9	19.6
11	13.1	9.6	22.7
12	11.4	9.3	20.6
13	5.9	16.4	22.3
14	6.9	12.0	18.9
Mean	**10.6**	**12.8**	**23.4**
SEM	**4.3**	**2.6**	**6.9**

Table 3.3 Spectrum of antimicrobial activity of the egg wax extracts. Paper discs soaked with ~10 mg of the organic extract and ~ 13 mg of the aqueous extract were assayed against six bacterial and two fungal species in solid culture. A combination of both organic and aqueous phases was assayed against two Gram-positive and two Gram-negative species (not: not tested). All plates were incubated for 48 h @ 37°C, except for *B. subtilis*, *M. luteus* and the yeasts, which were incubated at 30°C, and the inhibition halos measured (n=7 for all groups).

Microorganism	Inhibition halo aqueous phase (mm)	Inhibition halo organic phase (mm)	Inhibition halo aqueous + organic phases (mm)
Gram-negatives			
E. coli	0	0	0
S. marcescens	0	0	0
P. vulgaris	0	0	Not
Gram-positives			
S. epidermidis	16.2 ± 3.0	23.9 ± 2.9	30.3 ± 4.1
M. luteus	9.2 ± 3.4	12.0 ± 1.1	21.0 ± 3.3
B. subtilis	10.2 ± 2.0	13.5 ± 4.6	Not
Yeasts			
C. albicans	0	0	Not
S. cerevisiae	0	0	Not

Table 3.4 Antimicrobial activity of various tissues of *A. hebraeum*. Clear zones formed around tissues of *A. hebraeum* after 48 hours of 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. (n=4)

Sample	tissue weight (mg)	clear zone (mm)	Ratio of clear zone to tissue weight (mm/mg)
Tissues collected before day 4 post-engorgement *			
Géné's organ	2.1 ± 0.6	0	0
Oviduct	7.1 ± 2.2	1.8 ± 0.8	0.2
Ovary	11.1 ± 4.6	0.8 ± 0.3	0.1
Gut (intact)†	16.3 ± 5.5	0	0
Gut (opened) ††	9.7 ± 4.8	2.3 ± 1.9	0.3
Salivary gland	13.7 ± 4.2	1.3 ± 0.3	0.1
Trachea	4.1 ± 1.9	0.7 ± 0.1	0.2
Tissues collected after day 10 post-engorgement *			
Géné's organ	37.1 ± 4.1	3.2 ± 1.5	0.1
Oviduct	8.4 ± 3.3	1.5 ± 0.9	0.2
Ovary	21.9 ± 6.0	1.6 ± 1.0	0.1
Gut (intact) †	15.0 ± 1.9	0	0
Gut (opened) ††	16.2 ± 4.9	3.1 ± 0.6	0.2
Salivary gland	6.4 ± 2.2	0.8 ± 0.1	0.1
Trachea	4.8 ± 1.0	0.5 ± 0.1	0.1

* "before day 4": tissues collected from ticks 0-4 days post-engorgement.
** "after day 10": tissues collected from ticks 11-18 days post-engorgement.

† Gut contents not exposed to the agar plate
†† Gut contents exposed to the agar plate

Figure 3.1 Antimicrobial activity of the eggs of *A. hebraeum*. Eggs were plated on agar plates with *S. epidermidis* (A) and *E. coli* (B). A clear halo of growth inhibition was observed only against *E. coli.* (C) Eggs treated with chloroform and methanol to remove the wax layer did not inhibit the growth of *E. coli.* Plates were incubated @ 37°C for 48 h. Images A and B are magnified 9X and image C is magnified 15 X.

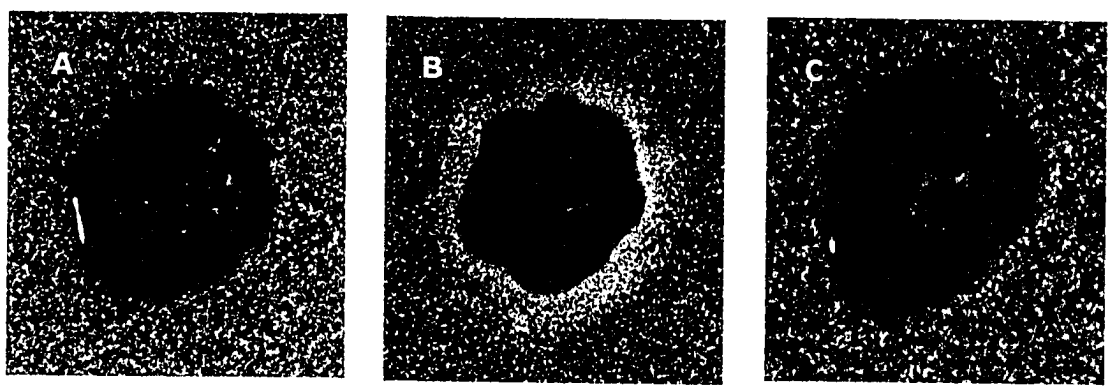


Figure 3.2 Measurements of the inhibition zones zones produced by batches of frozen eggs of *A. hebraeum* after 48 hours of incubation in agar plates with *E. coli*. Mean ± standard error of the mean (SEM) are shown. N is indicated above each bar.

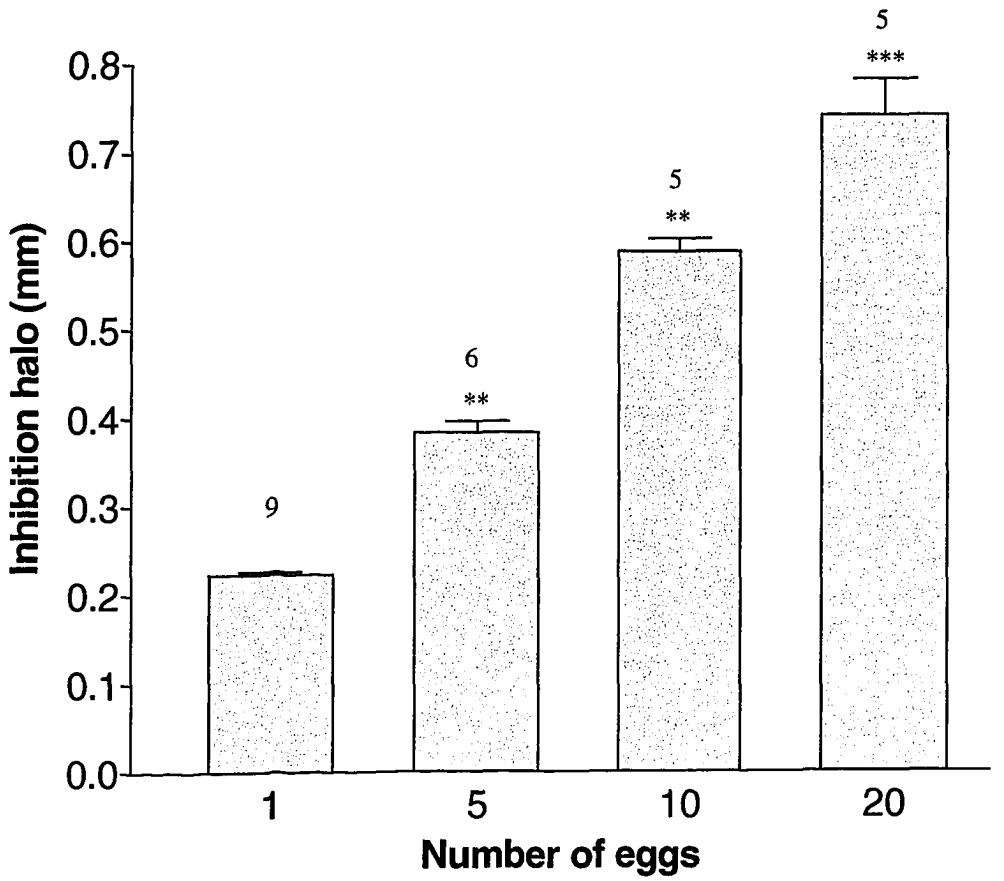


Figure 3.3 Growth inhibition zones (*S. epidermidis)* and extract yield produced after extracting batches of 100 mg eggs by four methods: Bligh and Dyer, Folch, chloroform and methanol (see Chapter 2). Inhibition zones were measured after 48 h in solid agar culture. Note that the methanol extract did not inhibit bacterial growth. Mean ± SEM and n are shown.

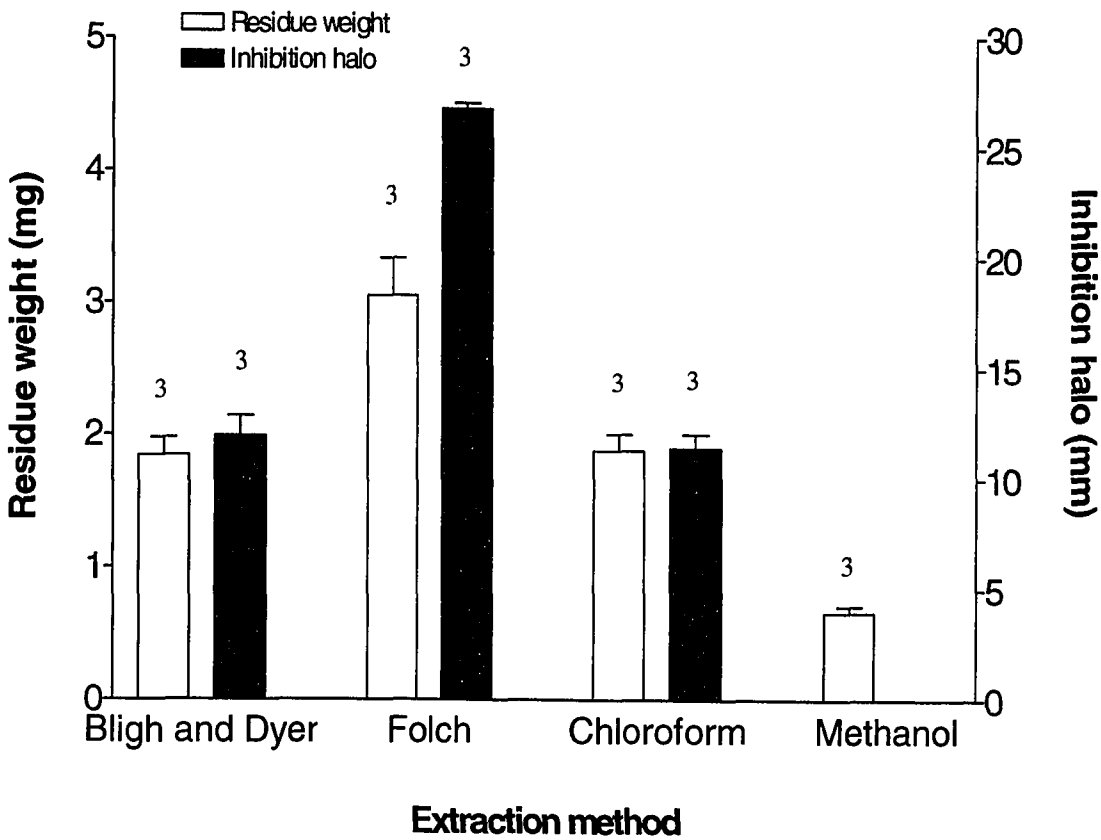


Figure 3.4 Antimicrobial activity of egg wax extracts. The extracts were applied to paper discs, air-dried (see Chapter 2) and tested against *S. epidermidis*. Plates were incubated @ 37°C for 48 h. **(A)** Organic and **(B)** aqueous Folch extracts, **(C)** methanol extract, **(D)** chloroform extract, **(E)** organic and **(F)** aqueous Bligh and Dyer extracts. Solvent controls of chloroform **(G)** and methanol **(H)** are also shown.

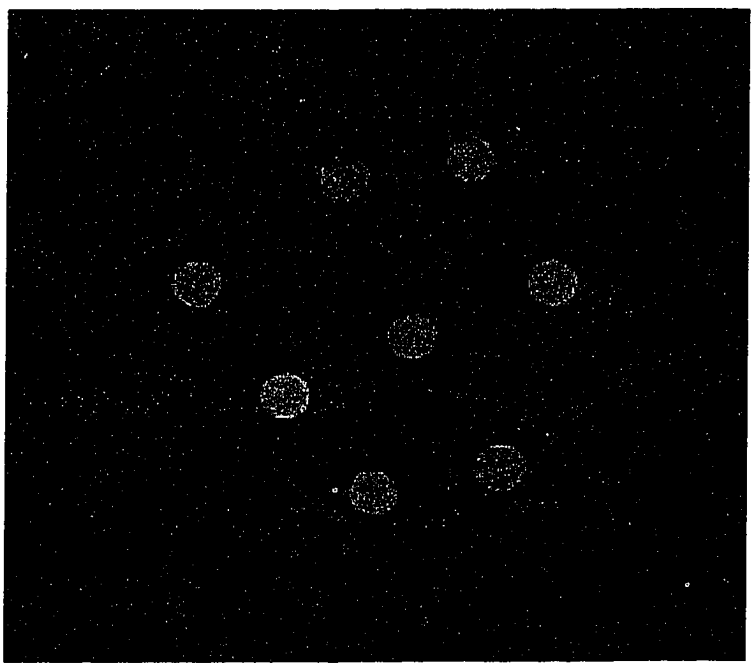


Figure 3.5 Bacterial inhibition assay in liquid culture. The aqueous and organic phases of the egg extracts (Folch method) were added to 5 ml tubes with sterile MH broth. These tubes were subsequently inoculated with *S. epidermidis* (time 0) and bacterial growth was measured for 1, 2 and 3 h, and then at 20 h. Solvent controls showed similar bacterial growth as the MH broth alone (Control). Notice the break in the axis between hours 3 and 20. Mean ± SEM are shown; n=4 for all treatments.

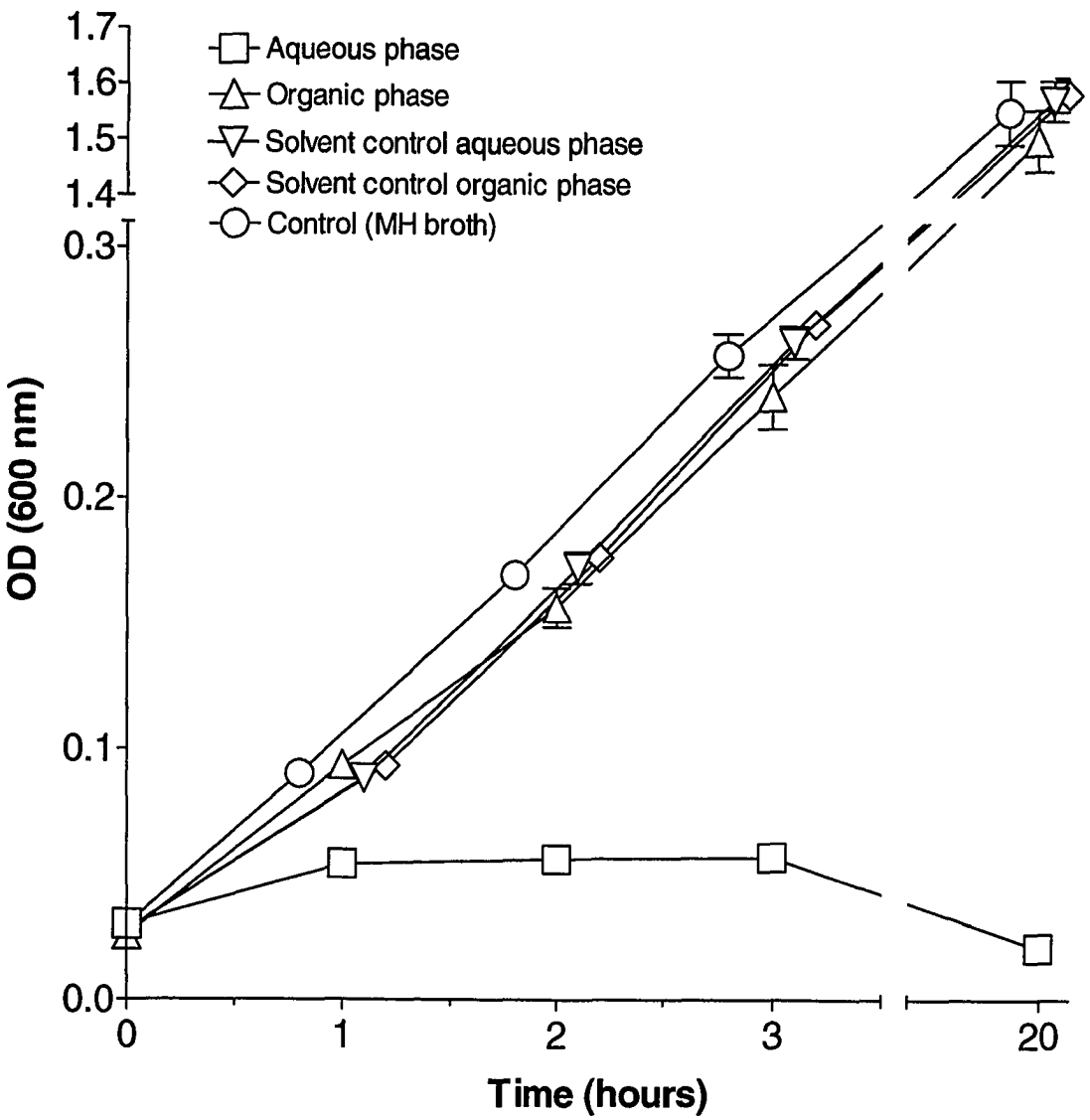


Figure 3.6 Bacteriolytic activity of the antimicrobial component(s) of the aqueous egg wax extract. The aqueous extract was added (10 mg) to a log phase culture of *S. epidermidis* at 2.5 h (arrow) and bacterial growth was measured by two methods every 30 min for 2 more hours. Solid lines represent optical densities (600 nm), dashed lines represent viable cell counts (CFU/ml). Mean ± SEM are shown; n=4 for all treatments.

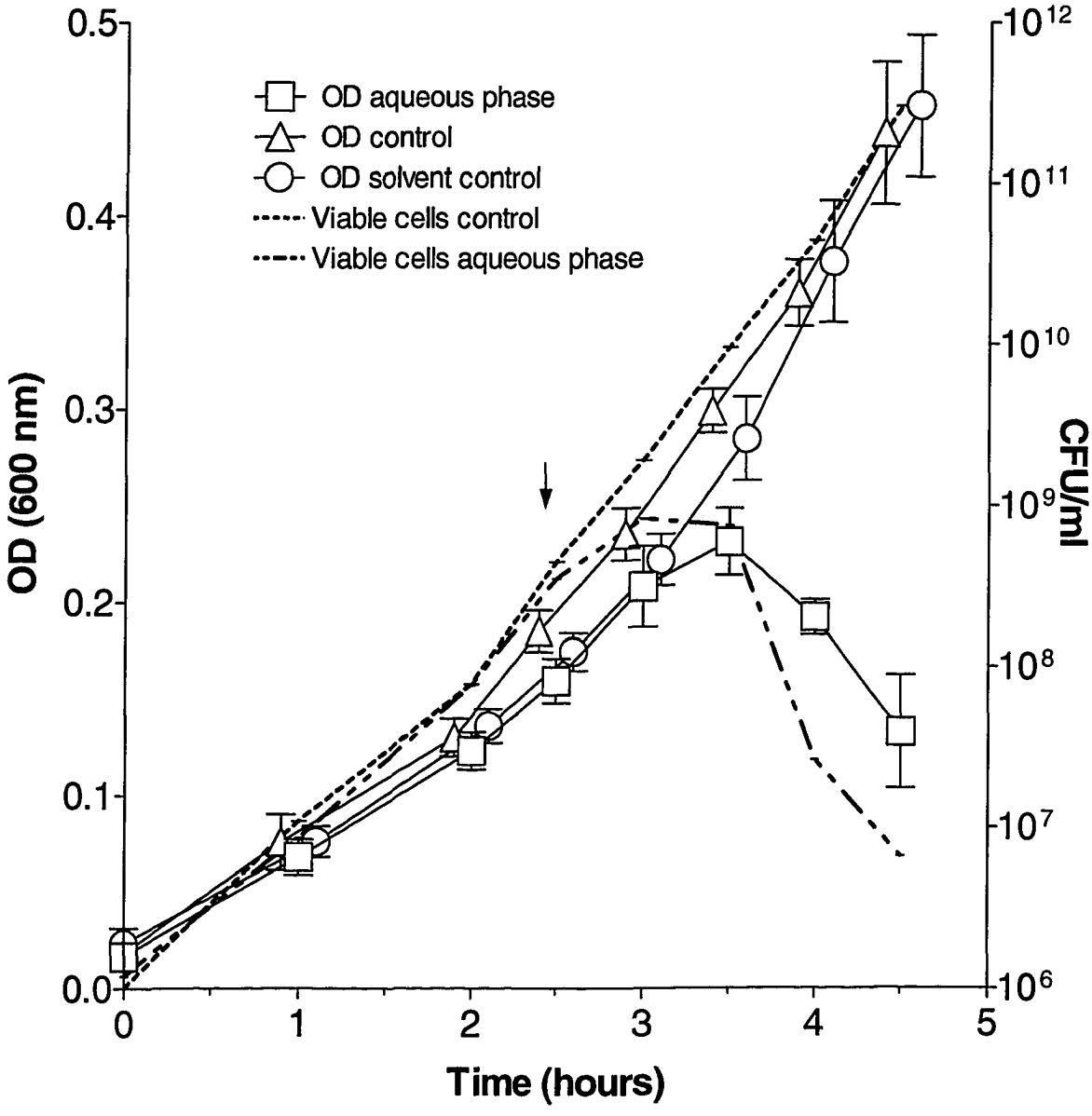


Figure 3.7 Loss of membrane integrity. *S. epidermidis* cells were stained using the LIVE/DEAD BacLight Kit (Molecular Probes), which contains 2 nucleic acid stains: SYTOX-9 (green fluorescence) and propidium iodide (red fluorescence). Cells with intact cell walls/membranes are green fluorescent and cells with damaged cell walls/membranes are red fluorescent. Orange indicates transition from green to red (see Chaper 4) Controls (A and B) and treated cells (C and D) were stained 30 min (A and C) and 2 h (B and D) after addition of the aqueous tick egg extract.

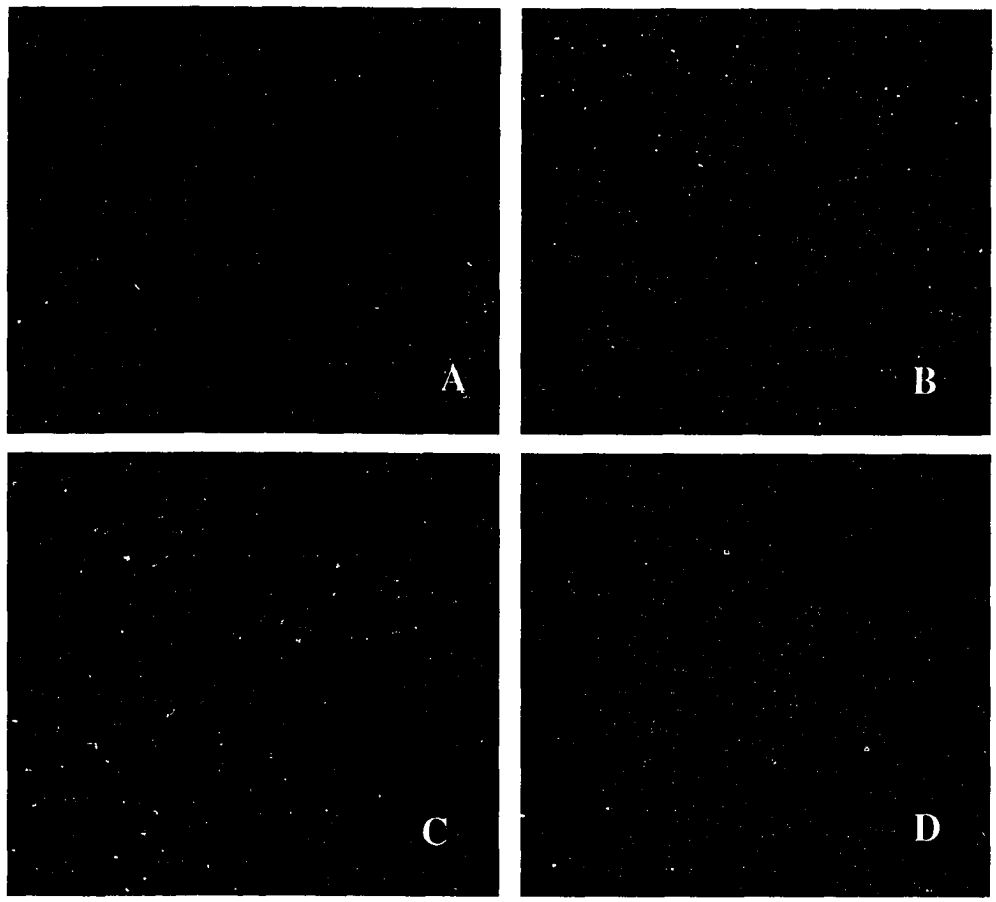


Figure 3.8 Ultrastructure of *S. epidermidis* treated with the 10 mg of the aqueous egg wax extract (A) for 30 min. and (B) for 2 h. (C) Control cells; 30 min, and (D) Control cells 2 h. Cytoplasmic inclusions (solid arrow) and thickened cell walls (dashed arrow) were observed in treated bacteria. Magnifications are: (A and C) 44K; (B and D) 56K.

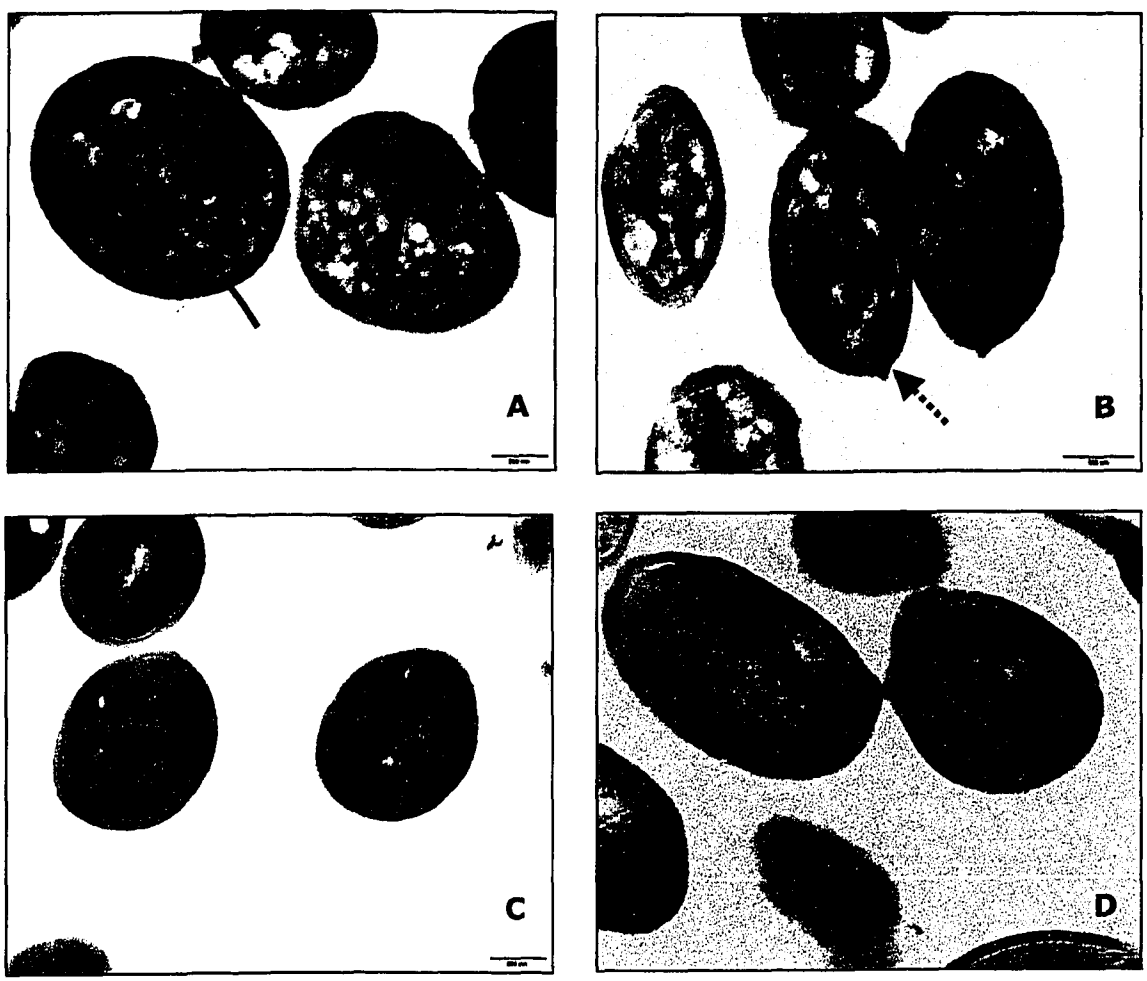


Figure 3.9 Coomassie blue stains of proteins of the aqueous phase: **A.**Tris-SDS PAGE, 15% polyacrylamide, run @ 100 V for ~ 1.5 h, molecular weight standards are shown in lane 1. ~10 µg and ~20 µg of the aqueous residue were loaded in lanes 2 and 3, respectively **B.** Tricine-SDS PAGE, 10% polyacrylamide, run @ 30 V for 1h and @ 150 V for ~1.5 h. Molecular weight standards are shown in lane 1. ~8 µg and ~16 µg were loaded in lanes 2 and 3, respectively.

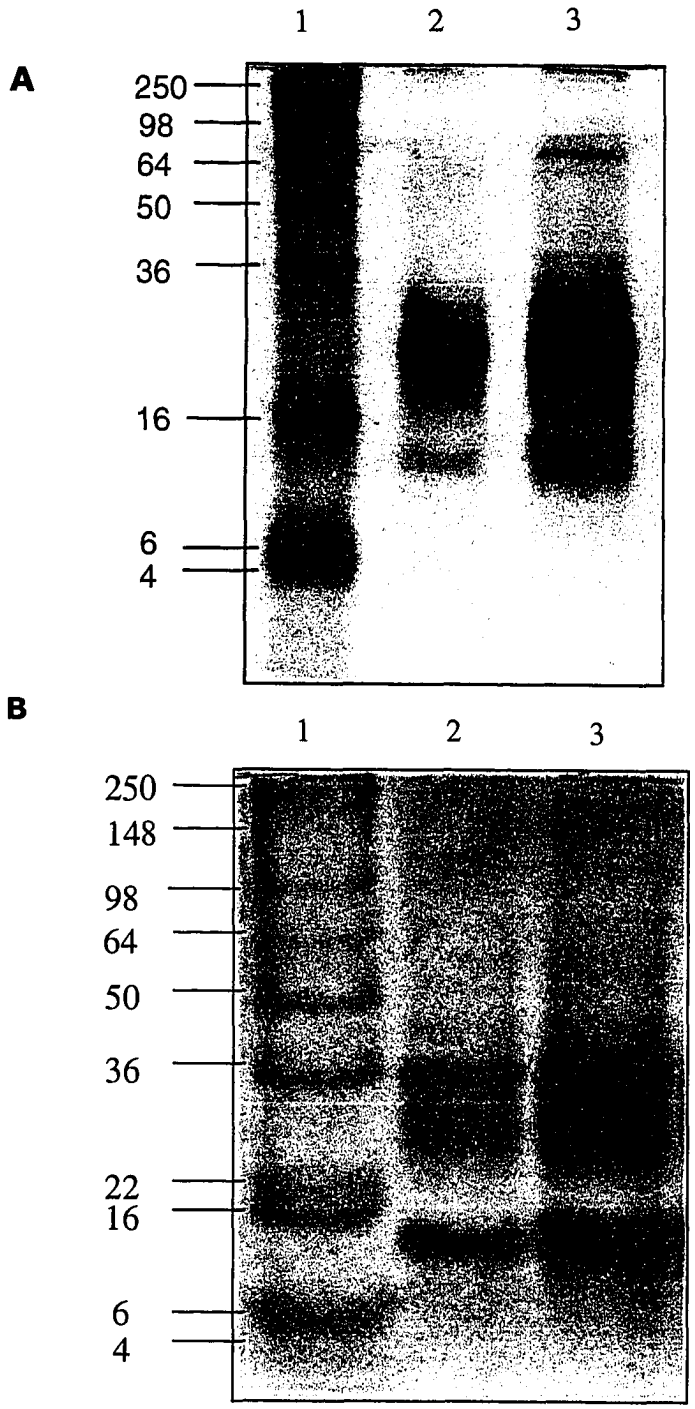


Figure 3.10 Effect of heat and protease treatments on the antibacterial activity of the egg wax aqueous extract. **A.** Aqueous phase (10 mg) incubated with proteinase K (15 min @ 55°C; triangles), pronase (15 min @ 56°C; inverted triangles) or heated (10 min @ 100°C) (squares) were tested in liquid culture against *S. epidermidis*. Lyzozyme (10 mg/ml) was used as a control (circles) of growth inhibition. Lyzozyme treated with proteinase K (stars) did not inhibit bacterial growth. Measurements were done over 20 h. **B.** Digestion of the aqueous phase by Proteinase K in a tricine-SDS PAGE gel. **C.** Digestion of lyzozyme by Proteinase K in a Tris/glycine SDS PAGE. In both gels, the lanes correspond to pre- and post- digested samples, respectively. Means ± SEM are shown. n= 4 or 5.

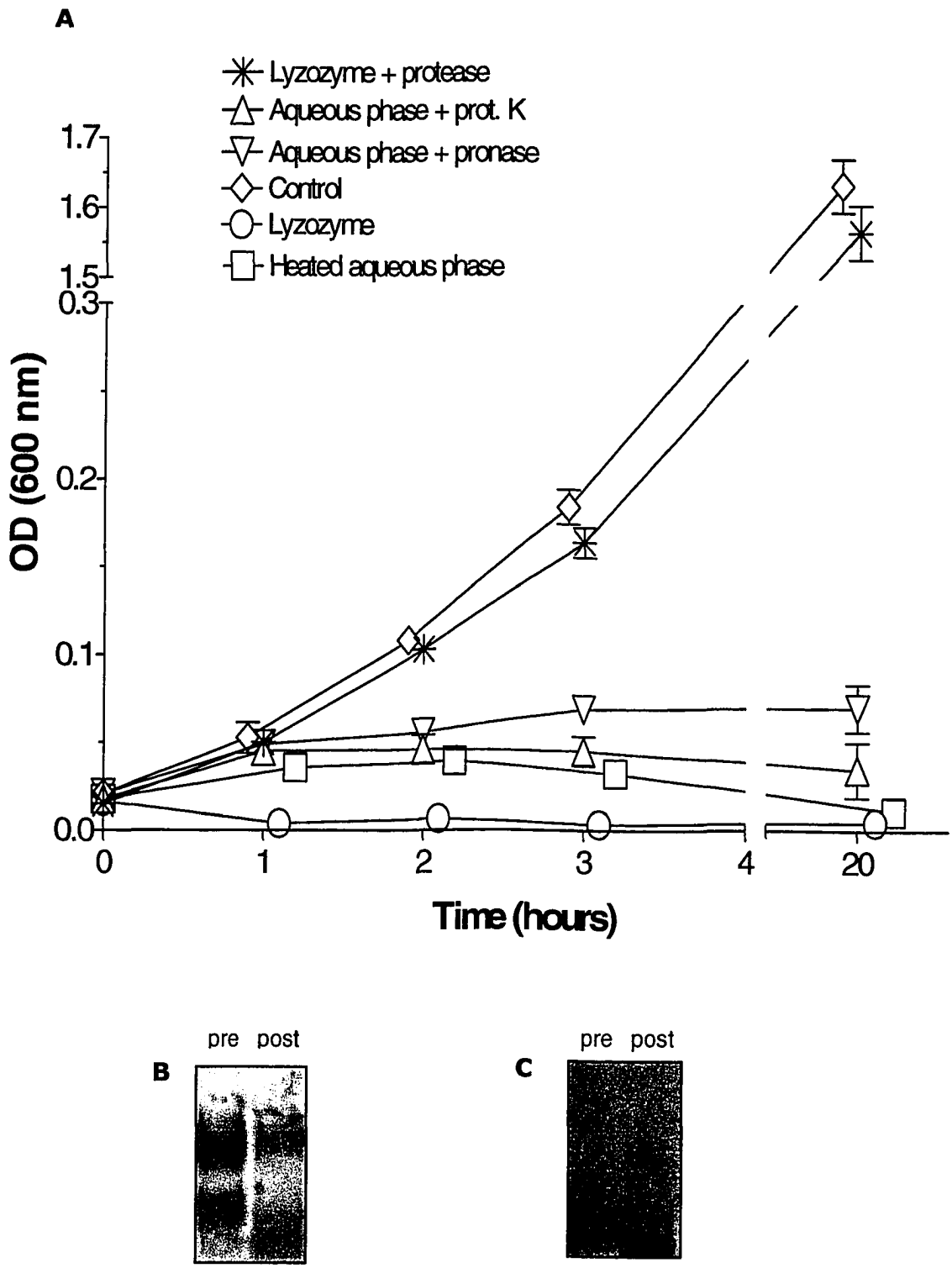


Figure 3.11 Géné's organ development after engorgement. Géné's organ was dissected and weighed between days 0 -20 post-engorgement. Only ticks above 1000 mg were used. Means ± SEM and n are shown.

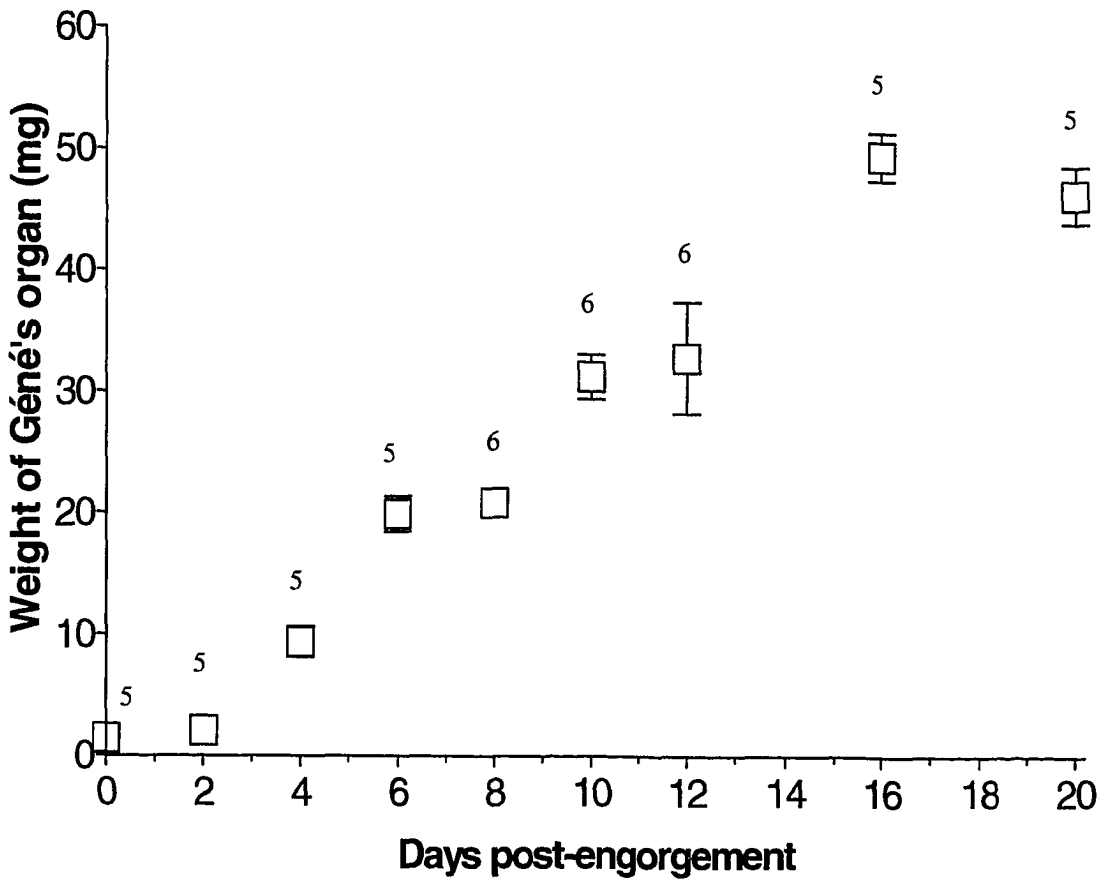


Figure 3.12 Appearance of antimicrobial activity in Géné's organ during the post-engorgement period. Géné's organs from ticks over 1000 mg were dissected and assayed for antimicrobial activity on agar plates with *S. epidermidis*. Antimicrobial activity is shown as mm inhibition halo normalized to the weight of the tissue. Egg laying was monitored daily for each of the ticks and eggs were weighed (when present) on the day of dissection. Arrow indicates when oviposition begins. Mean ± SEM and *n* are shown.

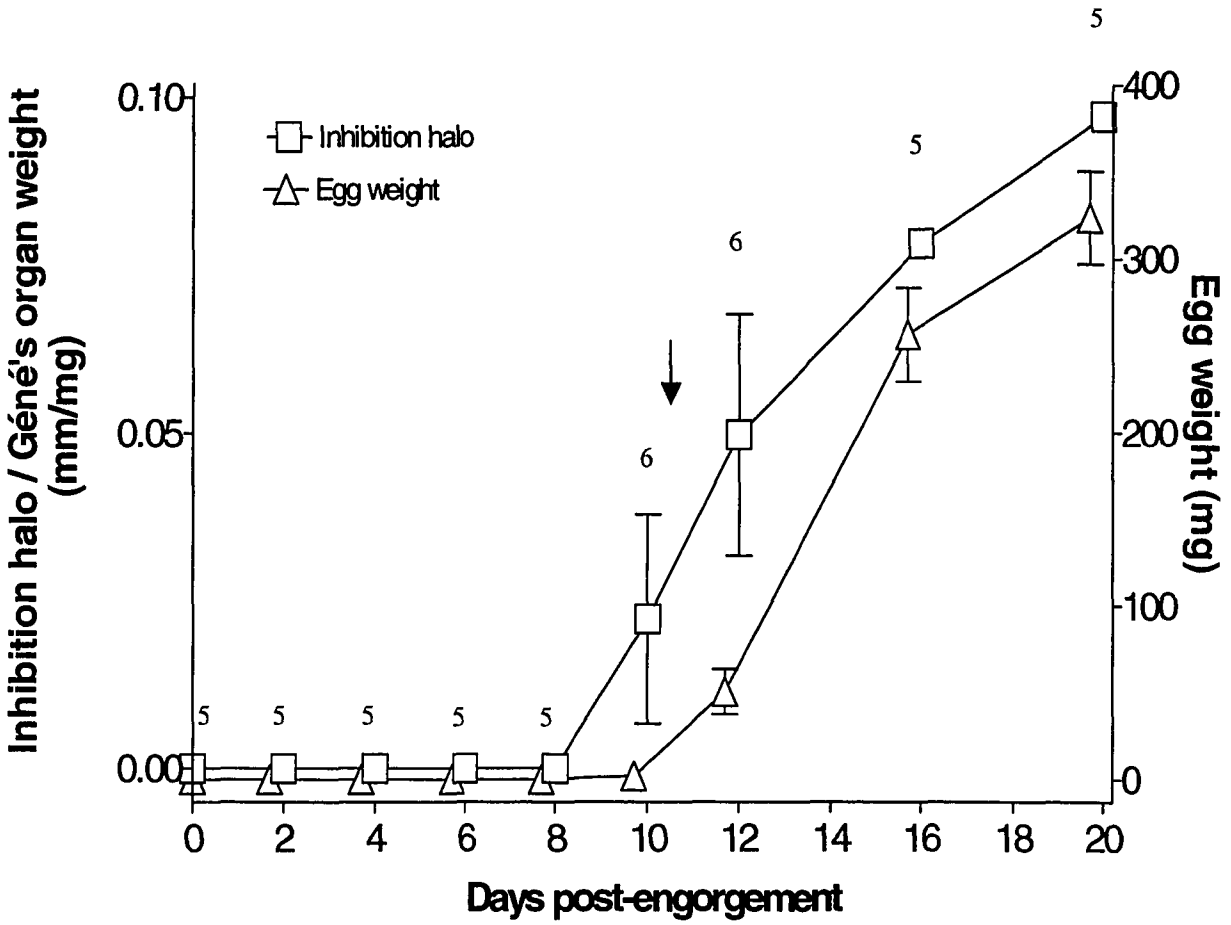
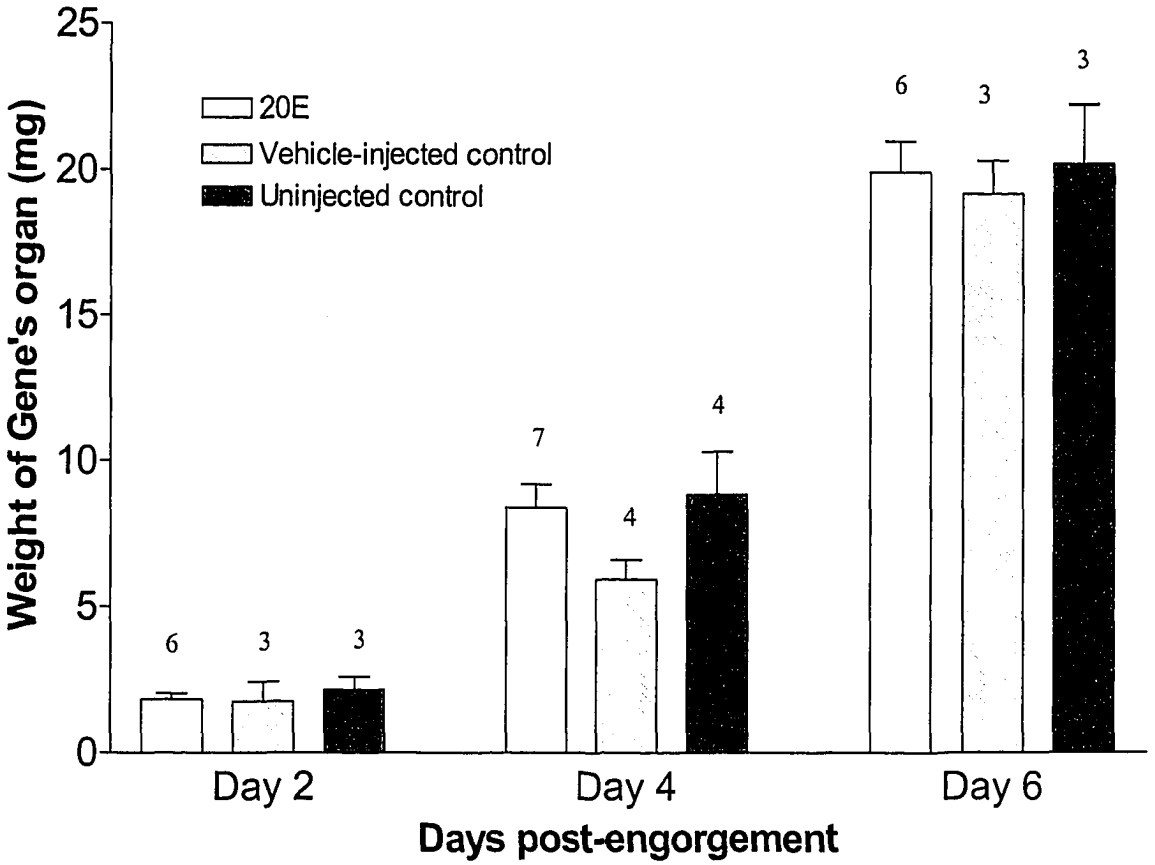


Figure 3.13 Effect of 20E on Géné's organ development. Several ticks were injected with 4 µg/g body weight of 20E on the day of engorgement. These ticks were incubated for 2, 4 or 6 days, and their Géné's organ was dissected and weighed. Ticks that were kept for 4 and 6 days after engorgement received 2 and 3 boost injections respectively. Controls injected with ethanol (<1% v/v) and uninjected controls showed similar values as the experimental groups. Mean ± SEM and n are shown.



Chapter 4

General Discussion and Conclusion

Because ticks lay their egg mass in the soil, the eggs are exposed to the rich microflora for at least a few weeks (until they hatch). I have found only a single published observation in the literature suggesting that insects eggs are protected by an antimicrobial substance (Marchini *et al*, 1997), but it seems reasonable to propose that other organisms may have evolved a similar strategy to protect their eggs against microbial flora in the environment. In this thesis, I have established the following major points:

1) When eggs of *A. hebraeum* were incubated on solid agar culture, they inhibited the growth of selected Gram-negative bacteria and Gram-positive bacteria.
2) When the egg wax was extracted with a non-polar solvent, and the latter was further partitioned with a polar solvent, both phases possessed antimicrobial activity. However, the polar and non-polar extracts inhibited only Gram-positive species. Following extraction, no antimicrobial activity remained on the surface of the eggs, indicating that only the egg wax provides protection from microbial attack.
3) The antimicrobial substance found in the aqueous phase of the egg wax extract possibly has a cytoplasmic target.
4) The antimicrobial component(s) of the aqueous phase was resistant to heat and protease digestion (proteinase K and pronase).

5) The major source of this antimicrobial substance (or substances) was Géné's organ, although it cannot be ruled out that the other accessory glands (tubular and lobular accessory glands) may also incorporate an antimicrobial substance in their secretions.
6) Géné's organ grew enormously during the first 7-10 days post-engorgement, although it lacked antimicrobial activity until about day 10. Even though this suggested that the growth and/or secretion of Géné's organ may be under hormonal control, I was not able to demonstrate this *in vivo*.

4.1 Extraction and antimicrobial activity of the egg wax

Because the antimicrobial substance is embedded in a wax, it was initially suspected to be a lipid. However, many AMPs are amphiphilic molecules with a high proportion of hydrophobic residues (~50%; Hancock, 2001), and so presumably could also be incorporated into a wax. The Folch extraction method, which consists of both polar and non-polar components, extracted the largest amount of egg wax residue and resulted in the largest growth inhibition halo, compared to the other five methods attempted. The Folch method produced two phases: an upper, aqueous phase, in which the proportions of chloroform:methanol:water are reported to be 3:47:48, and a lower, organic phase, with a proportion of 86:14:1 (Hamilton and Hamilton, 1992). Recall from Figure 3.4 that the biggest inhibition halo was produced by the component(s) of the organic phase. The size of a zone of inhibition is, in principle, proportional to

the amount and potency of the antimicrobial agent, as well as on its solubility and diffusibility (Madigan *et al,* 2002). Considering that the organic extract weighed less than the aqueous extract (Table 3.2), and that diffusion of a hydrophilic material should be favoured over that of a hydrophobic one in an agar plate, I suggest that the organic phase probably contains most of the antimicrobial substance. I consider it unlikely that the antimicrobial substance in the organic phase is different from that of the aqueous phase, primarily because both residues showed the same spectrum of activity against all the microorganisms tested (Table 3.3). For the moment, I consider it more likely that the antimicrobial activity observed in the agar plate corresponds to the same substance(s), but which partitioned differentially in the two phases. Obviously, a complete characterization of the two extracts would be required to confirm this.

Perhaps the most surprising result in this thesis was the change in antibacterial activity before and after wax extraction. The unextracted eggs inhibited the growth of two Gram-negative species, but only produced a partial inhibition halo against one of the Gram-positive species (*B. subtilis*). After the extraction, the material from both the organic and aqueous extracts inhibited the growth of the Gram-positives only. Another research group reported a similar result when isolating and characterizing antimicrobial peptides from the haemolymph of the soft tick *O. moubata* (Van der Goes Van Naters-Yasui *et al,* 2000). The hemolymph was initially purified on a Sephadex column from which three fractions showed antibacterial activity: two against *S. aureus* and one against *E. coli.* Further purification with gel filtration chromatography resulted in

the loss of activity against the Gram-negative bacteria, though the authors did not offer an explanation. Until the chemical nature of the antimicrobial substance of the egg wax is known, one can only speculate as to what caused the loss of antimicrobial activity against Gram-negative bacteria and the gain in activity against Gram-positives. At least two possibilities could explain the loss of activity against Gram-negatives in the egg wax:

(1) The antimicrobial activity against Gram-negatives might have been inactivated during the extraction in several ways. Strong organic solvents may have denatured protein components of the egg wax. A change in pH – which unfortunately was not controlled during the experiment – could cause loss of activity. 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. Or, 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-negatives 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. Thus, intact eggs show very low anti-Gram-positive activity, but this material becomes concentrated during the extraction, and the concentration accounts for the apparent increased antimicrobial activity. Both organic and aqueous phases were combined in a paper disc to determine if the activity against Gram-negatives could be recovered, but this was not the case (Table 3.3). This ruled out the possibility that

a component of the antimicrobial substance that conferred activity against Gram-negatives could have partitioned into the other phase.

(2) A single antimicrobial substance could have been modified during the extraction in such a way that it became active only against Gram-positive bacteria. There are some examples in the literature of changes in antibiotic selectivity by chemical alteration. The antibacterial action of polymixins and octapeptins, both cationic polypeptide antibiotics, is directed particularly against Gram-negative bacteria. This selectivity can be dramatically altered by chemical modification. Thus, the penta-*N*-benzyl derivative of these antibiotics is highly active against Gram-positive bacteria (Franklin and Snow, 1989). Whether the organic solvents used here could cause such a change is not known.

The interaction between cationic antimicrobial compounds and bacterial cell walls and plasma membranes depends on a specific conformation, the disposition of charges, and the presence of hydrophobic groups in the peptide secondary structure (Vaara, 1992). In general, AMPs bind electrostatically to the anionic bacterial cell wall before penetrating and disrupting the cytoplasmic membrane (Franklin and Snow, 1989). Defensins, mostly active against Gram-positive species, bind to the acidic peptidoglycan molecules of the Gram-positive cell wall (Bulet, 1999). Other AMPs capable of killing Gram-negative bacteria bind to the polyanionic LPS of the outer membrane (refer to Figure 1.2). LPS confers a higher anionic state to the Gram-negative cell envelope compared to the Gram-positive cell wall. LPS molecules are linked electrostatically by Ca^{2+} or Mg^{2+}, and polycationic AMPs displace these divalent cations, disorganizing the

outer membrane (Hancock, 1984; Nikaido and Vaara, 1985). Vaara (1992) examined the net positive charge/mass ratio of the various AMP families and determined that defensins have the smallest ratio. This could explain why defensins are not capable of disrupting the Gram-negative cell wall. It is possible that a loss of net positive charge occurred to the antimicrobial substance of the tick eggs during the extraction procedure. Consequently, the compound would have lost the ability to bind to Gram-negative bacteria, and gained the ability to bind to Gram-positive bacteria after it was concentrated during the extraction. This should be tested by extracting the egg wax at various pHs.

4.2 Mechanism of action of the antimicrobial component(s) of the aqueous phase of the egg wax extract

The organic extract was not able to inhibit bacterial growth in liquid culture, perhaps because it was too hydrophobic to dissolve in the aqueous medium. The aqueous phase did not seem to kill bacteria as fast as most AMPs isolated from insects. Only when all the extract obtained from 1 g of eggs was applied to the culture (~ 10 mg) was a noticeable growth inhibition observed after 1.5 h. Most defensins have an almost immediate lytic effect on bacteria. A 1-min exposure of recombinant defensin (2.2 μg) to exponentially growing *M. luteus* was enough to lyse all cells in the culture (Cociancich *et al,* 1994). However, the proline-rich AMP drosocin, which is active only against Gram-negatives, needed 6-12 h to kill exponentially growing bacteria in vitro (Bulet *et al*, 1996; Cudic *et al*, 1999), and the defensin isolated from *O. moubata* took 2.5 h to begin inhibiting

bacterial growth (Dr. DeMar Taylor, University of Tsukuba, Japan; personal communication).

One can get clues about the mode of action of an AMP from the speed of its effect. For example, membrane-active AMPs (most cecropins and defensins), kill bacteria very quickly (Otvos, 2000). This is because they disrupt the cell membrane rapidly, resulting in a rapid loss of cytoplasmic potassium, a decrease in cytoplasmic ATP, and an inhibition of respiration (Bulet *et al,* 1992; Cociancich *et al,* 1994). The results shown in Figure 3.6 indicate that *S. epidermidis* cells were not lysed rapidly. On the contrary, during the first hour of exposure to the aqueous extract, the bacteria continued to grow and divide. The change in fluorescence from green to orange (LIVE/DEAD BacLight kit) indicated that cell wall disruption was detected as soon as 30 min post-treatment (Figure 3.7). This suggests that the egg wax extract damaged the cell membrane. However, it is unlikely that this is the only disruptive effect. The appearance of the cell wall in Figure 3.8 is not similar to the classical appearance of bacterial lysis (see Figure 1.3). Even though the cell membrane may have partially lost its permeability barrier function in the first hour of exposure, the cells were still able to divide and were recovered in agar plates (Figure 3.6). The most noticeable morphological effect was the formation of what appear to be flocculent precipitates within the cytoplasm. Such an effect, also seen with the AMP, bactenecin, was described as condensed DNA and cytoplasmic proteins (Wu and Hancock, 1999). Hancock (1992) suggested that the increased membrane permeability in *S. epidermidis* cells treated with bactenecin allows the peptide to cross the membrane and

reach the cytoplasm, where it causes precipitation of the nucleic acids and proteins, resulting in cell death. Under these circumstances, leakage of the cell contents is possibly hindered by simple mechanical blockage by the precipitate. This may also be the case for the antimicrobial substance studied here. Nevertheless, lysis eventually occurs, as indicated by the loss of turbidity in the culture (Figure 3.6). Perhaps if samples for TEM were taken after 2 h of exposure or if they were taken from the bottom of the culture tube instead of the supernatant, visibly damaged membranes might have been observed. The most important conclusion from these experiments is that this antimicrobial substance, like others isolated from insects, interacts not only with the bacterial cell envelope, but probably also has a cytoplasmic target.

4.3 Protein analysis and stability of the antimicrobial component(s) of aqueous phase of the egg wax extract

Most of the information regarding mechanisms of action of antimicrobial substances from insects that I have encountered corresponds to AMPs. Even though it is not yet proven that the antimicrobial substance associated with tick eggs is a protein or a peptide, it seems unlikely that a significant amount of a strong hydrophobic substance could be present in the aqueous extract. It was interesting to observe that the aqueous phase contained proteins mainly within the range of 14 – 40 kDa because, with only a few exceptions, the size of the AMPs isolated from insects is under 15 kDa (Boman, 1995). Nondenaturing continuous electrophoresis was done to test the antimicrobial activity of specific

protein bands. The traditional method to test biological activity of cationic AMPs is done under low pH conditions (Cytrynska *et al*, 2001), so that all amino groups are protonated and the compound becomes more soluble in water (Dr. John Vederas, Dept. of Chemistry, U. of Alberta; personal communication). After assaying the antimicrobial activity of the proteins of the aqueous extract under neutral and acidic conditions (see section 2.8), no activity was shown for either the aqueous phase or the positive control in several trials. It was not possible to obtain a useful conclusion from this technique. Perhaps it was due to the fact that there was not enough sample loaded in the gel (only one fifth of the amount that had previously shown antimicrobial activity in liquid culture), or, that the proteins present in the gel diffused out after washing the gel with PBS and MH broth for 1h. Another method that could be used to address this question would be to fractionate the aqueous phase through a gel filtration chromatography system, and to assay the different fractions in a liquid culture inhibition assay.

Certain AMPs, like the antifungal drosomycin, possess a remarkable resistance to proteases. It has a highly compact structure due to an extra disulfide bond between the first cysteine residue and the C-terminal cysteine residue (Fehlbaum *et al*, 1994; Michaut *et al*, 1996). On the other hand, some compounds that have shown excellent antimicrobial activity *in vitro*, like the AMP from horseshoe crabs polyphemusin I, loses its activity in the presence of proteases (Zhang *et al,* 2000). The antimicrobial substance in the aqueous phase of the tick egg wax extract did prove to be very stable to heat and protease treatment. Proteinase K and pronase are both non-specific serine proteinases

that randomly cleave peptide bonds (Lehninger, 2000). Although these enzymes hydrolyze most proteins, some short peptides or cyclic polypeptides may be resistant. Degradation of short peptides can be accomplished by peptidases, which remove amino or carboxy-terminal residues. It would be instructive to treat the aqueous extract with amino and carboxypeptidases and test for its effect on antimicrobial activity.

4.4 Antimicrobial activity of various tissues of the engorged tick

Antimicrobial activity was found in several tissues (Table 3.4). Of all the tissues, the gut was interesting in that it showed antimicrobial activity only when the wall was broken and the contents leaked out. This indicates that the gut contents, but not the serosal surface, contain antimicrobial activity. Recall from Chapter 1 that the argasid tick, *O. moubata,* uses a fragment of mammalian hemoglobin as an antimicrobial peptide and that this fragment was isolated from the gut (Nakajima *et al,* 2002). Perhaps *A. hebraeum* uses a similar defense strategy.

Géné's organ dramatically increases in size until day 16 post-engorgement (Figure 3.11), but it does not inhibit bacterial growth until after day 10, when egg laying begins (Figure 3.12). The other tissues tested (ovary, oviduct, gut, salivary gland and trachea), however, showed a similar level of antimicrobial activity throughout the post-engorgement period. Together, these results suggest that antimicrobial activity in Géné's organ is under physiological

control, and timed specifically to correspond with the onset of oviposition. This is further discussed immediately below.

4.5 Effect of 20E on development of Géné's organ and its antimicrobial activity

As mentioned in Chapter 1, most AMPs isolated from insects are induced only following bacterial challenge. Ceratotoxin, the AMP isolated from the eggs of the Mediterranean fly, is one of the few examples of non-induced AMPs in insects. It is constitutively present in the eggs and in the reproductive accessory glands of sexually mature females (Marchini *et al,* 1997). The antimicrobial substance studied here, although also expressed independently of bacterial challenge, seems to be under physiological control. Manetti *et al.* (1997) suggested that gene expression of ceratotoxin is controlled by juvenile hormone (JH). This hormone controls vitellogenesis in many insect orders (Wyatt *et al*, 1994). Manetti *et al* (1997) showed that gene expression of ceratotoxin was blocked by precocene II in a dose-dependent manner. Precocene II is a "antiallotropin"; it inhibits JH biosynthesis in some insects (Staal, 1986). JH is apparently not produced by ixodid ticks (Neese *et al*, 2000). Instead, the 20E is the vitellogenic hormone in ixodid ticks (Friesen and Kaufman, 2002, 2004), and also the hormone that triggers salivary gland degeneration in the engorged female (Harris and Kaufman, 1985). Although I tested the effect of 20E on Géné's organ *in vivo*, it neither stimulated growth of Géné's organ nor secretion

of the antimicrobial substance (Figure 3.13). However, 20E is rapidly metabolized when bolus injections are made, 99% being lost within 24 hours (Weiss and Kaufman, 2001). It is possible that 20E does not regulate development of Géné's organ or its secretory activity, or that it was inactivated before eliciting an effect. Future work should include: (1) testing a series of 20E concentrations *in vivo*; (2) infusing 20E continuously via a microliter syringe pump (see, for example, Harris and Kaufman, 1985); and (3) testing 20E in organ culture, where 20E would not be metabolized rapidly.

4.6 Conclusion

The knowledge of arthropod immunity in the egg stage is still very limited. As demonstrated in this thesis, female *A. hebraeum* protects her egg batch against microbial infection with a secretion of its reproductive accessory gland, Géné's organ. Future research in this topic should be directed at (1) purifying the antimicrobial substance from the organic and aqueous phases and from Géné's organ, (2) chemically characterizing the antimicrobial compound(s), (3) elucidating the mechanism of action of this substance(s) and determining if there is a cytoplasmic target, and (4) establishing what triggers the development of Géné's organ and its production of this antimicrobial compound.

Chapter 5

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