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**Characterization of antimicrobial activity present in the cuticle of American  
lobster, *Homarus americanus***

A Thesis Presented

by

**Margaret Anne Mars**

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

**Master of Science**

in

**Marine and Atmospheric Science**

Stony Brook University

**December 2010**

**Stony Brook University**

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Abstract of the Thesis  
**Characterization of antimicrobial activity present in the cuticle of American  
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**Margaret Anne Mars**  
**Master of Science**

in

**Marine and Atmospheric Science**

Stony Brook University

**2010**

American lobster is an ecologically and socioeconomically important species. In recent years the species has been affected by disease and the catch in Southern New England has fallen dramatically. In order to fully understand how and why diseases affect lobster populations, it is imperative to fully understand lobster defense mechanisms. The cuticle, previously believed to act only as a physical barrier, has recently been shown to contain antimicrobial activity. This thesis focused on characterizing this activity and attempted to identify the molecules responsible. A sterile shell extract prepared from a pool of lobster shells was active against *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Escherichia coli*, and *Staphylococcus aureus*. The activity in the extract was found to be heat stable, as activity was not decreased after boiling (100°C, 5 min.). Activity was diminished when an extract was prepared from muffled lobster shells, indicating an organic component responsible for the observed activity. Size fractionation of the extract with centrifugal filter units decreased activity in extract unable to pass through a 10kDa filter, while extract that passed through a 10kDa and 3kDa filter retained antimicrobial activity, despite having much lower protein concentrations. Fast protein liquid chromatography (FPLC) of the shell extract revealed several protein peaks. Samples corresponding to FPLC peaks had varying antimicrobial activities. In contrast, the void volume, containing inorganic and anionic material, increased bacterial growth. Tris/tricine SDS-PAGE with the FPLC fractions revealed small peptides (<7kDa) in the fractions that displayed the highest and least variable antimicrobial activity. MALDI mass spectrometry revealed peptide peaks at 1.6, 2.8, 4.6, and 5.6 kDa. A partial sequence of the 5.6kDa peptide was determined. Manipulation of the unknown amino acids in the sequence in a search with BLAST led to a non-definitive, partial match with the wasp antimicrobial peptide mastoparan. Antimicrobial peptides have been described as being cationic peptides less than 10kDa with broad-spectrum antimicrobial activity. The small size and structure of the peptides make them very stable and able to withstand high heat. AMPs have been isolated from a wide variety of plants and animals and are an integral part of the invertebrate defense response. This thesis demonstrated that the broad-spectrum antimicrobial activity observed in the lobster shell is due to a component that is organic, cationic, heat stable, and less than 10kDa. Based on shared characteristics with antimicrobial peptides, it is likely that the activity observed in the shell is due to an antimicrobial peptide.

In addition, antimicrobial activity in the shells of a lobster population with a high incidence of Epizootic shell disease (Eastern Long Island Sound, ELIS) was compared with the activity in shells of lobsters from a population with a low incidence of the disease (Western Long Island Sound, WLIS). Extracts from the shells of WLIS lobsters had significantly higher antimicrobial activity when compared to extracts from ELIS lobsters. The antimicrobial activity in the shell may be a factor affecting susceptibility to the disease.

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## Acknowledgments

I would like to acknowledge the financial support provided for this project by NOAA through Rhode Island Sea Grant and the New England Lobster Research Initiative.

I would like to thank Margaret Homerding for her preliminary work that inspired this project.

I would like to thank Don Landers of the Millstone Environmental Laboratory and Rachel Britt of the New York State Department of Environmental Conservation for providing the lobsters used in this study.

I would like to thank Sue Pawagi of The Marine Animal Disease Lab at Stony Brook University for her assistance in processing lobsters and in overseeing and maintaining the laboratory facilities.

I am grateful to Toni Koller of the Proteomics Center of Stony Brook University for performing all mass spectrometry and peptide sequencing presented in this thesis.

I am especially grateful to the members of my thesis committee, Anne McElroy, Bassem Allam, and Emmanuelle Pales Espinosa, for all of their assistance, guidance, patience, and support.

## **Introduction:**

Lobsters, crabs, and other large crustaceans are ecologically important as they act as predators, scavengers, prey, and habitat modifiers. These animals can shape their prey's population size and distribution as well as prey animal morphology (Castilla & Paine 1987). Lobsters consume crabs, mussels, starfish, clams, sea urchins, shrimp, and small fish. As scavengers, lobsters, crabs, and other crustaceans are important in nutrient cycling as they consume a large amount of detritus (Castilla & Paine 1987). Large crustaceans create burrows in soft bottom sediments that provide habitat structure for other organisms once they are abandoned. Lobsters can move large rocks and boulders to create caves that also contribute to habitat structure (McKown et al. 2009). Additionally, large crustacean adults and juveniles are included in the diets of many demersal fish such as Atlantic cod, white hake, cunner, and shorthorn sculper (Davis et al. 2004). Lobsters are also prey for crabs, eels, other groundfish, and seals (McKown et al. 2009).

Large crustaceans, especially American lobster (*Homarus americanus*), are socioeconomically as well as ecologically important. American lobster is harvested on the continental shelf and continental margin of North Eastern North America. This fishery has historically been one of the most valuable fisheries in coastal New England and the Canadian Maritime Provinces. From 1986-88, the U.S. landed 46 million pounds of lobster with a net value of \$129 million. During the same period, Canada exported about 31 million pounds of lobster that was valued at approximately \$102 million (Cheng & Townsend 1993). In 2008, the U.S. lobster landing was 82 million pounds, which was worth \$306 million (McKown et al. 2009).

In the last decade, the lobster landing in Southern New England has sharply declined and has not shown signs of recovery (McKown et al. 2009). There have been several notable events in the decline of the Southern New England lobster fishery beginning with the 1999 Lobster mortality event in Western Long Island Sound. In August 1999, reports of lethargic, moribund, and dead animals proliferated and although most reports centered on lobsters, reports also included blue crabs, rock crabs, spider crabs, sea urchins, and starfish. By the end of the lobster season in late fall 1999, the

Long Island and Connecticut lobster catch had fallen 99% from previous years (Pearce & Balcom 2005). It is estimated that 11 million lobsters died during this event (Mullen et al. 2004).

The most recent threat facing lobsters is Epizootic Shell Disease (ESD). Epizootic shell disease causes lesions that make lobsters unmarketable and causes economic losses in the commercial lobster fishery (Smolowitz et al. 2005). These lesions do not usually lead to mortality, but if they become severe they can penetrate the shell, reaching the membrane and soft tissue below. Severe lesions can allow opportunistic infection and can prevent the lobster from molting properly, which can lead to death. ESD disproportionately affects female lobsters because they molt less frequently. In addition, the stress of ESD can cause a female lobster to molt when it is ovigerous and therefore lose its clutch. The larger effect on female lobsters could have population consequences in the future (Cobb & Castro 2006). ESD is much more prevalent in the Eastern Long Island Sound (ELIS) than the Western (WLIS) and Central Sound (CLIS). In 2004, 30% of lobsters in commercial trawls in ELIS were affected by ESD while only about 1% percent of lobsters in WLIS and CLIS trawls showed signs of the disease (Howell et al. 2005). Shell diseases can also affect a variety of other crustaceans including blue crab (*Callinectes sapidus*), king crab (*Paralithodes camtschatica* and *Paralithodes platypus*), white shrimp (*Penaeus setiferus*), pink shrimp (*Panaeus duoraum*), brown shrimp (*Penaeus aztecus*) (Cook & Lofton 1973), the edible crab *Cancer pagurus* (Vogan et al. 2001), and European lobster (Getchell 1989).

In order to understand diseases affecting lobsters and other crustaceans, it is important to have a comprehensive understanding of their immune defenses. Crustaceans do not have adaptive immune capabilities and therefore rely on their innate defense mechanisms (Soderhall et al. 1996). The crustacean cuticle is a physical barrier separating the external environment and the inside of the animal, thus providing a first line of defense against invading pathogens. Crustacean cuticle consists of chitin fibrils and inorganic salts (mostly calcium carbonate) embedded in a protein matrix (Nousiainen et al. 1998). It is likely that the exoskeleton plays a larger role in immune

defense than a simple physical barrier as recent investigation has demonstrated active participation by the cuticle in defense reactions and in storing proteins (Destoumieux et al. 2000, Ashida & Brey 1995, Asano & Ashida 2001, Ferrer et al. 1989, and Homerding 2009). Internal defense mechanisms include cell mediated and humoral responses. Cell mediated responses involve phagocytosis and encapsulation and humoral responses involve the phenoloxidase activating system and antimicrobial peptides (Hancock et al. 2006). The prophenoloxidase activating system recognizes small quantities of non-self material and initiates an immune response through an enzymatic cascade that leads to the production of melanin, which is toxic to microorganisms. The prophenoloxidase activating system is also believed to act in the initiation of antimicrobial peptide production (Sritunyalucksana & Soderhall 2000). Antimicrobial peptides are small cationic peptides found in the hemolymph of crustaceans. AMPs have broad-spectrum antibacterial activity at concentrations as low as 0.25-4µg/ml (Powers & Hancock 2003). Their small size allows AMPs to quickly diffuse to a site of infection and provide an immediate and rapid response to invading pathogens (Tincu & Taylor 2004).

Homerding (2009) investigated spatial differences in defense parameters of American lobster, as well as differences between healthy and diseased lobsters from Eastern Long Island Sound. The internal defense parameters analyzed included phagocytic activity, phenoloxidase activity, baseline production of reactive oxygen species (Native ROS), induced production of reactive oxygen species (oxidative burst), and antibacterial activity of plasma. The number of colony forming units (CFUs) present in plasma was also evaluated. Diseased lobsters from ELIS showed elevated plasma CFU levels and increased phagocytotic, phenoloxidase, and antibacterial activity when compared to healthy ELIS lobsters, although only phenoloxidase activity was significantly different. When compared to Western Long Island Sound lobsters, Eastern Long Island Sound lobsters showed a decrease in phagocytic, phenoloxidase, antibacterial activity, native ROS, oxidative burst, and plasma CFU levels. Of these differences, only the phenoloxidase activity and plasma CFU level was not significant. Homerding also conducted a preliminary evaluation of external defense parameters, including protein concentration, phenoloxidase-like activity, and antimicrobial activity of

shell extracts, and shell mass to surface area ratio. The antimicrobial assay of the shell extract was not optimized and no differences in activity were found between healthy and diseased ELIS lobsters or lobsters from Eastern and western Long Island Sound. The only parameter in which significant differences were found was the shell mass to surface area ratio; WLIS lobster shell was found to be significantly denser (larger shell mass to surface area) than shell from ELIS lobsters and shell from asymptomatic ELIS was denser than symptomatic ELIS lobster shells. These results indicate that internal immune capabilities vary regionally and may affect susceptibility and determine disease prevalence (Homerding 2009). Based on Homerding's preliminary results, further study and optimization of assays was warranted.

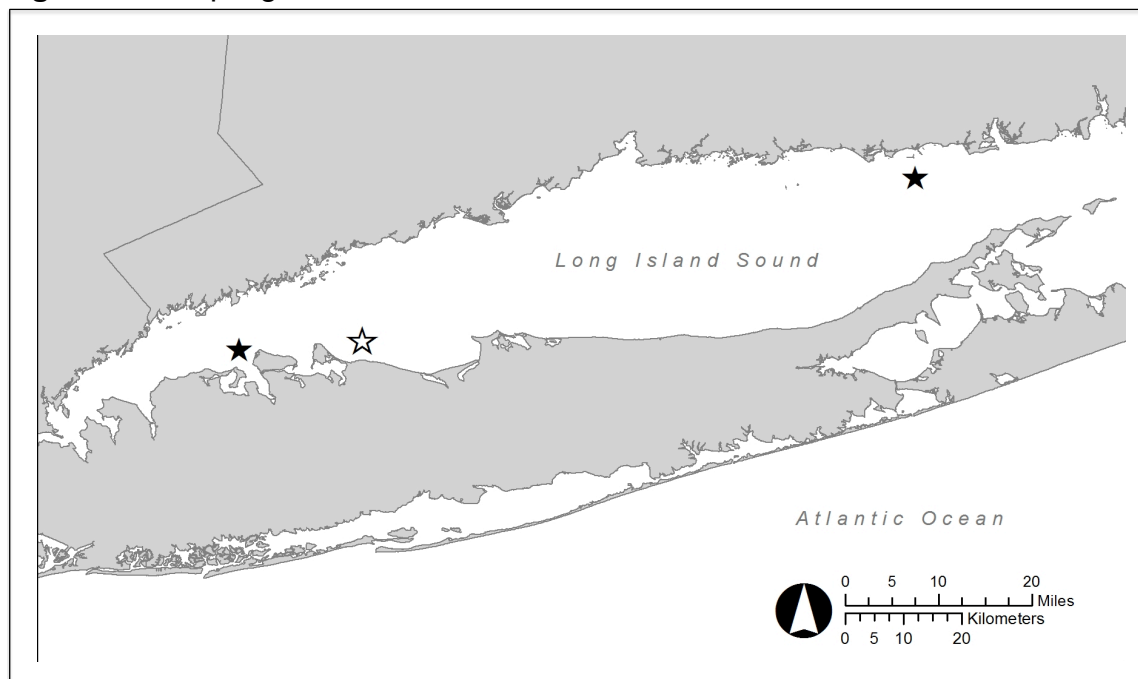
Further investigation into the role of the cuticle will be important when considering the full scope of the crustacean defense response to infections, especially in shell related diseases. The goals of this study were to further document and characterize antimicrobial activity in the shell of the American lobster. Additionally, antimicrobial activity in the shells of lobsters from regions that differed significantly in ESD incidence was evaluated to determine if antimicrobial activity in the shell is related to disease status or region.

## **Methods:**

### Lobster sampling

Lobsters from Western Long Island Sound (Figure 1) were supplied by the New York DEC. A set of 8 lobsters was collected in July, and a second set of 8 lobsters was collected in November 2009. All lobsters were kept on ice overnight, and were processed the following morning.

**Figure 1. Sampling locations**



The open star represents the sampling site for the lobsters sampled by the DEC in July and November 2009, which were used in the pooled shell extract. The solid stars represent Eastern and Western Long Island Sound sampling locations sampled by Homerding in 2008.

Lobsters were killed by cutting through the cephalothorax from the lower edge through the head. The carapace was removed from each lobster and all soft tissue was thoroughly scraped away. The carapaces were rinsed with DI water, dried, and placed in zip-lock bags prior to freezing at  $-80^{\circ}\text{C}$ . The lobsters in the second sample set were all in a premolt stage (D1 or D2), while the lobsters in the first sample set were in the intermolt stage of the molting cycle.

### Sterile shell extract

A sterile, pooled shell extract was prepared based on the modified procedures of Chen et al. (1991). Equal sized pieces of carapace from 8 lobsters (4 from the July sample and 4 from the November sample) were frozen in liquid nitrogen and then ground to a fine powder using a coffee grinder. Two grams of shell powder were added to 8ml of 0.05M potassium phosphate buffer [0.05M  $\text{K}_2\text{HPO}_4$ , 0.05M  $\text{KH}_2\text{PO}_4$  (pH 7.2)]. The mixture was stirred over ice on an orbital shaker for 1hr before being centrifuged at

2500g, 4°C, for 1hr. The supernatant was collected and sterilized with a 0.22µm syringe driven filter cartridge. Aliquots of sterile shell extract were stored at -80°C.

### Protein concentration

Protein concentrations of sterile shell extracts were determined using the microplate adapted protocol of the Thermo Scientific BCA Protein Assay (Rockford, IL) using bovine serum albumin as a standard.

### Antimicrobial activity assay

Bacterial suspensions were prepared by inoculating 50ml of sterile marine broth in a 125ml Erlenmeyer flask with one loop full of a single colony of a particular bacterial species (grown on marine agar) and incubating overnight at 37°C on an orbital shaker (200rpm). Bacterial cells were rinsed three times by spinning the suspensions at 300g, 28°C, for 5min, discarding the supernatant, and resuspending the pellet with sterile PBS. After rinsing, the suspensions were diluted with sterile PBS to an O.D of 0.1 at 570nm.

Antimicrobial activity was assessed using a photometric assay based on the methods of Noga et al. (1994) and adapted by Homerding (2009). In sterile 1.5ml microcentrifuge tubes, 10µl of sterile shell extract was combined with 10µl of bacterial suspension, and 30µl of sterile PBS. Negative controls included: 50µl of PBS; 40µl of PBS added with 10µl of potassium phosphate buffer (pH 7.2); 10µl of shell homogenate and 40µl of PBS. The growth control contained 10µl of bacterial suspension, 10µl of 0.05M potassium phosphate buffer (pH 7.2), and 30µl of PBS. The controls and treatments were each prepared in triplicate. All treatments were incubated at 28°C for 30 minutes prior to the addition of 450µl of cold marine broth to each tube. One hundred µl were then transferred from each treatment to a well in a sterile, clear, flat-bottomed, 96-well microtitre plate (Falcon, Franklin Lakes NJ). An initial absorbance reading was taken using a Wallac micro-plate reader (Wallac 1420 Multilabel Counter: Perkin Elmer, Wellesley, Massachusetts). The microcentrifuge tubes containing the controls and treatments were incubated at room temperature for 48 hours. At 24 and 48 hours, 100µl



of each treatment were transferred to a well in a new 96-well microtitre plate and the absorbencies were read.

Antimicrobial activity was evaluated by calculating the percent decrease in bacterial growth from the control for each treatment. This was done by subtracting the final absorbance at 48h from the initial absorbance at T<sub>0</sub> for each treatment and the control to provide a measure of bacterial growth over 48 hours. The control growth was an average of three replicates of the control treatment; there was very little variation in the amount of bacterial growth in the controls. The growth in each treatment was then subtracted from the averaged control growth, divided by the averaged control growth, and multiplied by 100 to give a percent. The percent decrease in bacterial growth in treatments was averaged from three replicates of each treatment and the standard deviation of the three replicates was calculated.

$$\% \text{ decrease} = \frac{(\text{averaged control growth} - \text{treatment growth})}{(\text{averaged control growth})} \times 100$$

## **Detection and characterization of antimicrobial activity in the shell extract**

### Evaluation of antimicrobial activity in the shell against a broad spectrum of bacteria

Bacterial suspensions of *Vibrio parahaemolyticus* (gram negative, halophilic), *Vibrio anguillarum* (gram negative, halophilic), *Escherichia coli* (gram negative), and *Staphylococcus aureus* (gram positive) were prepared. The activity of the pooled sterile shell extract against each species was measured using the antimicrobial assay described above. All treatments and controls were prepared in quintuplet in this assay.

### Heat sensitivity of antimicrobial activity in American lobster shell

To determine the effect of heat on the antimicrobial activity of the shell extract, the sterile pooled shell extract was boiled at 100°C for 5 minutes in a heating block. After boiling for 5 minutes, the extract was used in the antimicrobial assay with *V. parahaemolyticus*. Untreated shell extracts were used as controls in the assay in addition to the negative and positive growth controls.

### Effect of muffling on antimicrobial activity in American lobster shell

Lobster shell material was muffled in a high heat furnace to remove all organic matter. Four equal sized pieces of carapace from four lobsters from the July sample and four lobsters from the November sample were frozen in liquid nitrogen and ground to a fine powder using a coffee grinder. Two grams of the powder was placed in a crucible and heated in a furnace at 450°C for 4.5hr. The remaining shell material was allowed to cool overnight before being placed in 8ml of 0.05M potassium phosphate buffer (pH 7.2). The mixture was shaken over ice for 1hr on an orbital shaker before being spun at 2500g, 4°C, for 1hr. The supernatant was sterilized with a 0.22µm syringe driven filter cartridge. Aliquots of the furnace treated sterile extract were used in the antimicrobial assay with *V. parahaemolyticus*. Untreated shell extract was used as a control in the assay in addition to the negative and positive growth controls.

### Determination of antimicrobial activity in size fractionated shell extract (<3kDa, <10kDa, >10kDa)

To establish the size range of the component of the extract with antimicrobial activity, 3 and 10kDa microcon centrifugal size exclusion filter units (Millipore, Jaffrey, RI) were used to filter the extract. Five hundred µl of sterile shell extract were applied to the top of two 10kDa centrifugal filter units and the units were spun for 10min at 12000rpm and 4°C. After initial spinning, 250µl of filtered extract were taken from the bottom of each 10kDa filter unit and were added together to the top of a 3kDa filter unit. To the top of each 10kDa unit, an additional 500µl of sterile shell extract was added. The units were then spun for 10min at 12000rpm and 4°C. The extract that passed through the 10kDa filters was collected and combined. The two 10kDa filters were turned upside down over two new microcentrifuge tubes, and along with the 3kDa unit, were spun for 15 min at 12000rpm and 4°C. The extract from the top of the two 10kDa filters were combined and diluted with 100µl of sterile 0.05M potassium phosphate buffer (pH 7.2). The three filtered extracts, from the top of the 10kDa filter (10T), the bottom of the 10kDa filter (10B), and the bottom of the 3kDa filter (3B) were sterilized with 0.22µm syringe driven filter cartridges. Aliquots of the filtered extracts were used in the antimicrobial assay with *V. parahaemolyticus*.

### Fast protein liquid chromatography (FPLC) of shell extract

Fast protein liquid chromatography can separate proteins and other biomolecules out of a mixture based on different parameters depending on the type of column used in the stationary phase. A cation exchange column was used to separate cationic proteins and biomolecules from the sterile shell extract based on charge distribution. Sterile shell extract was applied to FPLC (BioLogic LP system, Bio-Rad Laboratories, Inc. Philadelphia, PA) using a HiTrap Q-sepharose FF column (1.6 x 2.5cm, 5ml, GE Healthcare, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl buffer (pH 8.5). After washing with the equilibration buffer, bound proteins were eluted with a linear gradient of 0–1M NaCl in 20mM Tris-HCl buffer (pH 8.5) at a flow rate of 1ml/min. Elutriate was collected for the first 4min (void volume), elutriate was discarded during minutes 5-9, and 1ml samples were collected every minute from minutes 10 to 25. In order to provide a control for the antimicrobial assay, 0.05M potassium phosphate buffer (pH 7.2) was also loaded into the instrument and samples were collected as described above.

### Evaluation of antimicrobial activity in FPLC fractions

The samples collected at time points corresponding to protein peaks in the chromatograph (fractions 3, 4, 5, 6, & 10) were sterilized using 0.22µm syringe driven filter cartridges and used in the antimicrobial assay with *V. parahaemolyticus*. Potassium phosphate buffer collected at the same time point in the blank run was used in positive controls for the corresponding FPLC fractions. Negative controls were not changed from the assay protocol described above. Untreated shell extract were used as a control in the assay in addition to the negative and positive growth controls.

### Tris/tricine SDS-PAGE with FPLC fractions

To determine the molecular sizes of proteins eluted from the FPLC exchange column (fractions 3, 4, 5, 6, &10), a 15% Tris/Tricine SDS PAGE polyacrylamide gel electrophoresis adapted from Schägger & von Jagow (1987) was conducted under reducing conditions. The molecular masses of the proteins were determined using an unstained protein ladder (Bio-Rad, Hercules, CA) and protein bands were visualized through silver staining.

### Evaluation of antimicrobial activity in size fractions (<10kDa, <3kDa, >10kDa) of FPLC fractions (3, 4)

FPLC fractions that exhibited the highest and least variable antimicrobial activity (3 and 4) were filtered using 3 and 10kDa centrifugal size exclusion filter units as described above. The filtered fractions of the FPLC samples (3-10T, 3-10B, 3-3B, 4-10T, 4-10B, & 4-3B) were then used in the antimicrobial assay with *V. parahaemolyticus*.

### Determination of molecular weights of small peptides in sterile shell extract

Ten µl of the sterile shell extract that had passed through the 10kDa centrifugal filter was cleaned up on a Zip Tip (Millipore, Philadelphia, PA) and the peptides eluted onto a MALDI target with alpha cyano-4-hydroxycinnamic acid (10mg/ml in 50% Methanol, 0.1% trifluoroacetic acid). MALDI spectra were acquired with an Applied Biosystems Voyager DE-STR mass spectrometer (Carlsbad, CA) from  $m/z$  1000 – 10,000. Four hundred µl of the peptide mixture was concentrated and buffer exchanged into Buffer A (2% Acetonitrile, 0.1% formic acid) with a Microcon YM-3 3kDa NMWL spin column (Millipore, Philadelphia, PA). Ten µl of the resulting 20µl concentrate was pressure-loaded onto a 10cm 100 µm inner diameter (i.d.) fused-silica capillary packed with 3µm C18 reverse phase (RP) particles (Magic, Michrom bioresources, Auburn, CA), which had been pulled to a 5µm i.d. tip using a P-2000 CO<sub>2</sub> laser puller (Sutter Instruments, Novato, CA). This column was then installed in-line with a Eksigent Nano2D HPLC pump running at 300nL/min. Peptides were eluted from the column by applying a 30min gradient from 5% buffer B to 40% buffer B (98% acetonitrile, 0.1% formic acid). The gradient was switched from 40% to 80% buffer B over 5min and held constant for 3min. Finally, the gradient was changed from 80% buffer B to 100% buffer A over 0.1min, and then held constant at 100% buffer A for 15 more minutes. The application of a 1.8kV distal voltage electrospayed the eluting peptides directly into an LTQ Orbitrap XL ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (Thermo Finnigan, San Jose, CA). Full MS spectra were recorded on the peptides over a 400 to 2000 $m/z$  range at 15,000 resolution, followed by one tandem

mass (MS/MS) event pair on the most intense ion, each pair containing of an HCD (higher Energy collision Dissociation) scan and a CID (collision induced dissociation) scan in the FT at 7,500 resolution of the same ion. Charge state dependent screening was turned on, and peptides with a charge state of 4<sup>+</sup> or higher charge state were taken. Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system. A second run with the remaining 10µl of concentrate was performed where only the peptides at m/z 1123.6 (for the 5+ ion), m/z 936.5 (for the 6+ ion) and m/z 1404.2 (for the 4+ ion) were fragmented either as a HCD scan or a CID scan in the FT at 7,500 resolution.

#### Determination of the amino acid sequence of a small peptide in the shell extract

As the peptide sequence is not in a protein database, manual *de novo* sequencing was performed on the 5.6kDa peptide (Toni Koller, per. com.).

#### **Evaluation of antimicrobial activity in the shells of lobsters from two populations with differing disease prevalence**

##### Comparison of antimicrobial activity in individual shell extracts from Eastern and Western Long Island Sound lobsters

In order to evaluate antimicrobial activity in the shells of lobsters from regions with differing disease prevalence, the antimicrobial activity of lobster shells from Eastern Long Island Sound (ELIS) and Western Long Island Sound (WLIS) was tested using the antimicrobial assay described above. Eastern Long Island Sound lobsters were collected in Waterford, Connecticut, an area with high disease prevalence and WLIS lobsters were collected of the shores of Oyster Bay, New York, an area with a disease prevalence of less than 5% (Homerding 2009). Sampling and processing of lobsters was performed by M. Homerding (2009) in 2008 and specimens were stored at -80°C until further analysis. Before use in this assay, the shell material from each lobster was thawed over ice, scraped thoroughly, rinsed with DI water, and blotted dry with a paper towel. The shell material from each lobster was then submerged in liquid nitrogen and ground to fine powder in a coffee grinder. The shell powder produced from each lobster was weighed and 0.05M potassium phosphate buffer (pH 7.2) was added in a 2:1 (v:w)

ratio. The mixtures were shaken over ice on an orbital shaker for 1hr and then spun at 2400g, 4°C, for 1hr. The supernatant was removed from each sample and sterilized using a 0.22µm syringe driven filter cartridge. The shells of 5 diseased lobsters from ELIS, 6 asymptomatic, apparently “healthy” lobsters from ELIS, and 11 healthy lobsters from WLIS were used to make 22 individual extracts. The extracts were used in the antimicrobial assay with *V. parahaemolyticus*.

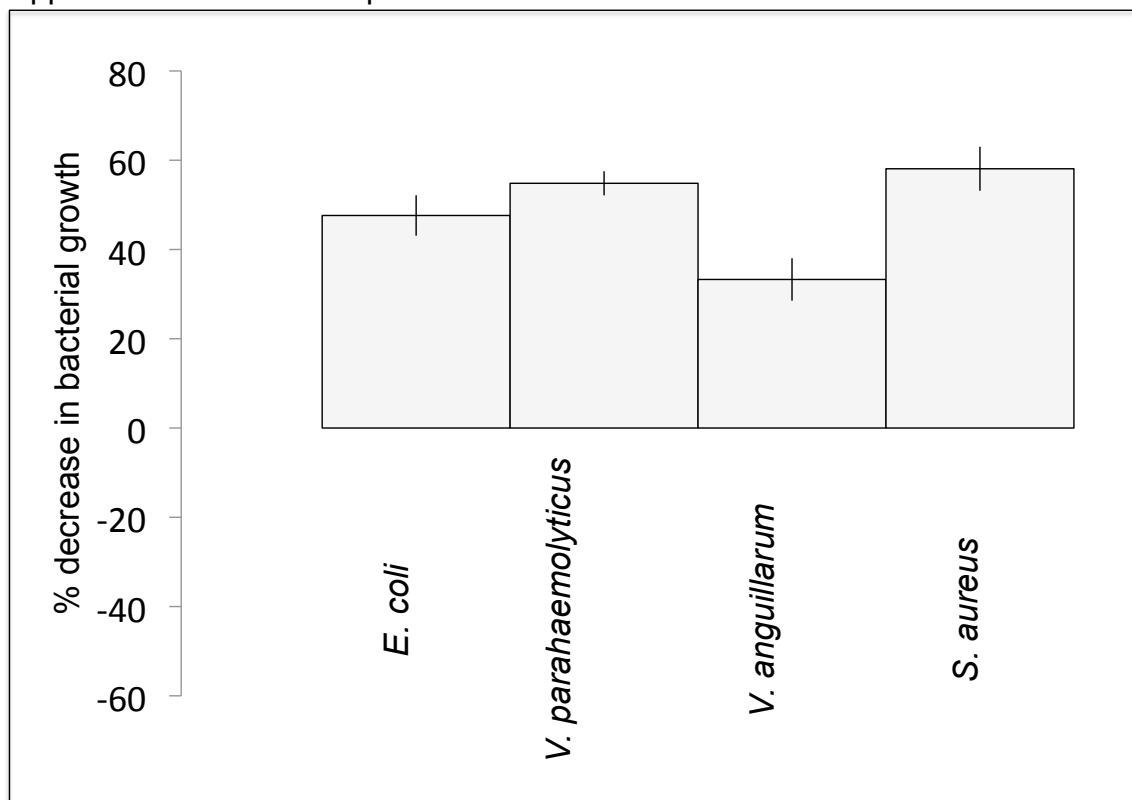
## **Results**

### **Detection and Characterization of Antimicrobial Activity in the Shell Extract**

#### **Evaluation of antimicrobial activity in the shell against a broad spectrum of bacteria**

The pooled shell extract was found to possess antimicrobial activity against *V. parahaemolyticus* (gram-negative, halophilic), *V. anguillarum* (gram-negative, halophilic), *E. coli* (gram-negative), and *S. aureus* (gram-positive) (Figure 2). The percent decrease in growth is highest in *S. aureus* (58±5%) and lowest in *V. anguillarum* (33±5%). The overall growth of *V. parahaemolyticus* in positive controls was highest and the shell extract caused a 55±3% decrease in the growth of this species, which was the least variable percent decrease in growth. *V. parahaemolyticus* was therefore used in all proceeding antimicrobial assays characterizing the antimicrobial activity in the American lobster shell.

**Figure 2.** Percent decrease in the growth of 4 species of bacteria in cultures supplemented with sterile pooled shell extract.

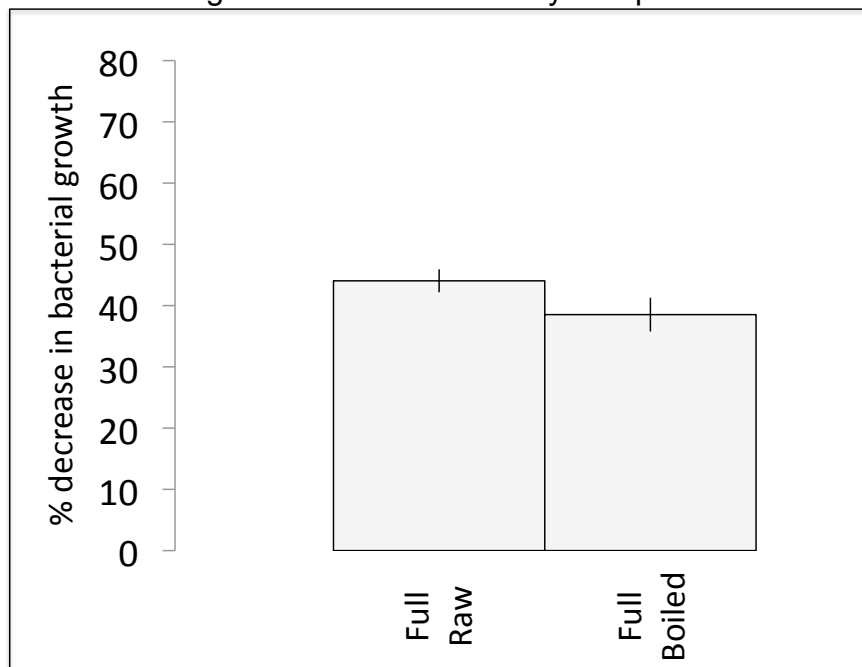


The final growth measurement was made 48h after  $T_0$ . Error bars illustrate one standard deviation. All assays were done in quintuplet with a shell extract prepared with lobster shell material from 8 individual lobsters.

#### Heat sensitivity of antimicrobial activity in American lobster shell

Boiling (5 minutes at 100°C) did not significantly affect the antimicrobial activity of the shell extract (Figure 3), indicating that the component of the shell extract responsible for the detected antimicrobial activity is heat stable.

**Figure 3.** Influence of boiling on antimicrobial activity of a pooled shell extract



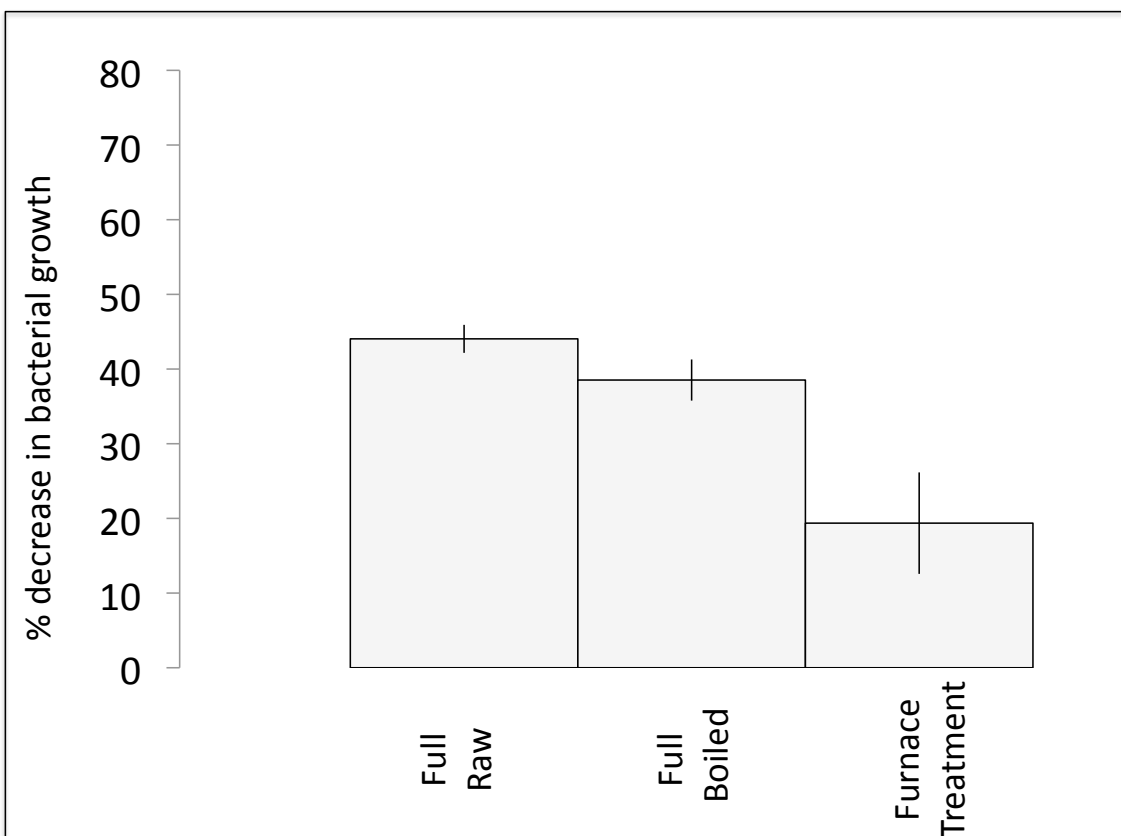
Percent decrease in *V. parahaemolyticus* growth in cultures supplemented with sterile pooled shell extract (Full Raw) and sterile pooled extract that had been boiled (Full Boiled). The final growth measurement was made 48h after  $T_0$ . Error bars illustrate one standard deviation. All assays were done in triplicate with a shell extract prepared with lobster shell material from 8 individual lobsters.

#### Effect of muffling on antimicrobial activity in American lobster shell

Heating the pooled shell material to 450°C for 4.5hr before preparing shell extract significantly decreased activity, but did not eliminate it (Figure 4). The furnace treated extract caused a 19±7% decrease in bacterial growth, which was a 56% smaller decrease in growth than that seen with the full raw extract (44±2%). The protein concentration of the furnace treated extract was 0.001% of the protein concentration of raw extract, verifying that the muffling treatment destroyed virtually all organic material in the extract. The reduced activity in the furnace treated extract indicates that the antimicrobial activity observed in the shell extract is likely due to an organic component, but observing residual activity in the furnace treated extract suggests that an inorganic component may be contributing to the overall activity.



**Figure 4.** Influence of muffling lobster shells on the antimicrobial activity of a pooled shell extract



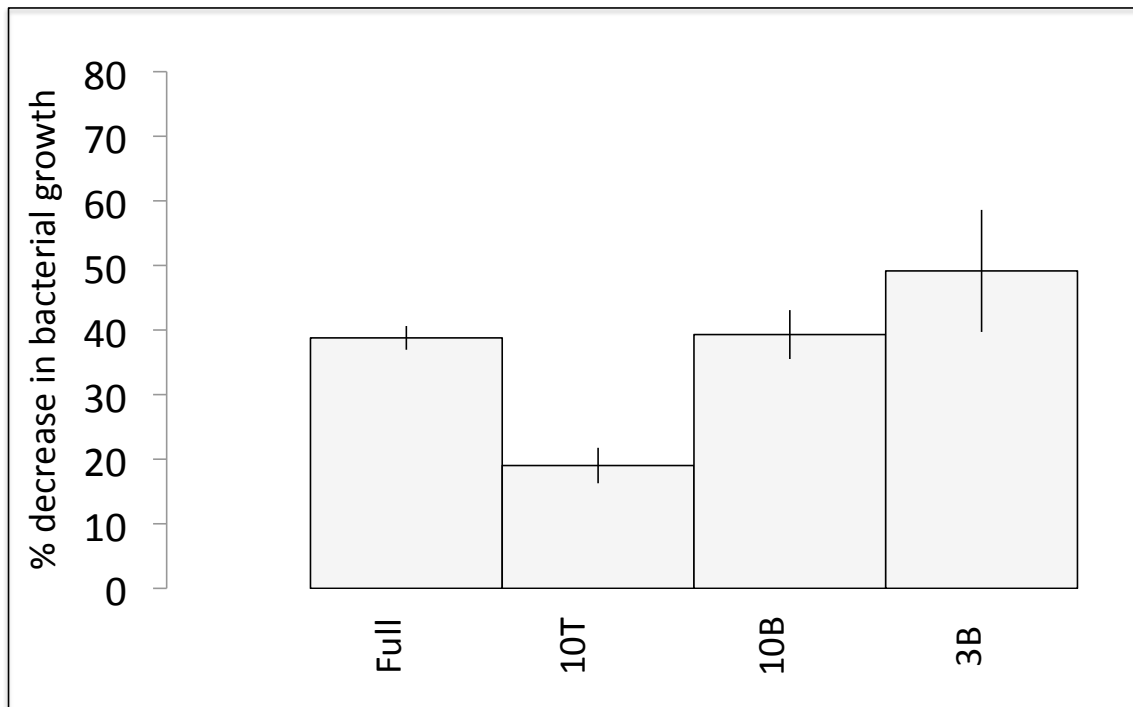
Percent decrease in *V. parahaemolyticus* growth in cultures supplemented with sterile pooled shell extract (Full Raw), boiled extract (Full Boiled), and a sterile pooled extract prepared with muffled lobster shells (Furnace Treatment). The final growth measurement was made 48h after  $T_0$ . Error bars illustrate one standard deviation. All assays were done in triplicate with a shell extract prepared with lobster shell material from 8 individual lobsters.

Determination of antimicrobial activity in size fractionated shell extract (<3kDa, <10kDa, >10kDa)

Size exclusion cartridges were used to produce three subsamples of the extract: extract that could not pass through a 10kDa filter (10T), extract that did pass through a 10kDa filter (10B), extract that passed through a 3kDa filter (3B). Antimicrobial activity in the extracts that passed through the 10kDa and the 3kDa filters was not significantly different from the activity observed in the full extract (Figure 5). The antimicrobial activity of the extract that was unable to pass through a 10kD filter was significantly lower than

that of the full extract (Figure 5). Additionally, the protein concentration of 10T (4800 $\mu$ g/ml) was much higher than that of the full extract (1360 $\mu$ g/ml), 10B (76.8 $\mu$ g/ml), or 3B (9.5 $\mu$ g/ml) treatments. These data indicate that the component of the extract responsible for antimicrobial activity is less than 10kDa and is likely less than 3kDa.

**Figure 5.** Evaluation of antimicrobial activity in size fractionated shell extract

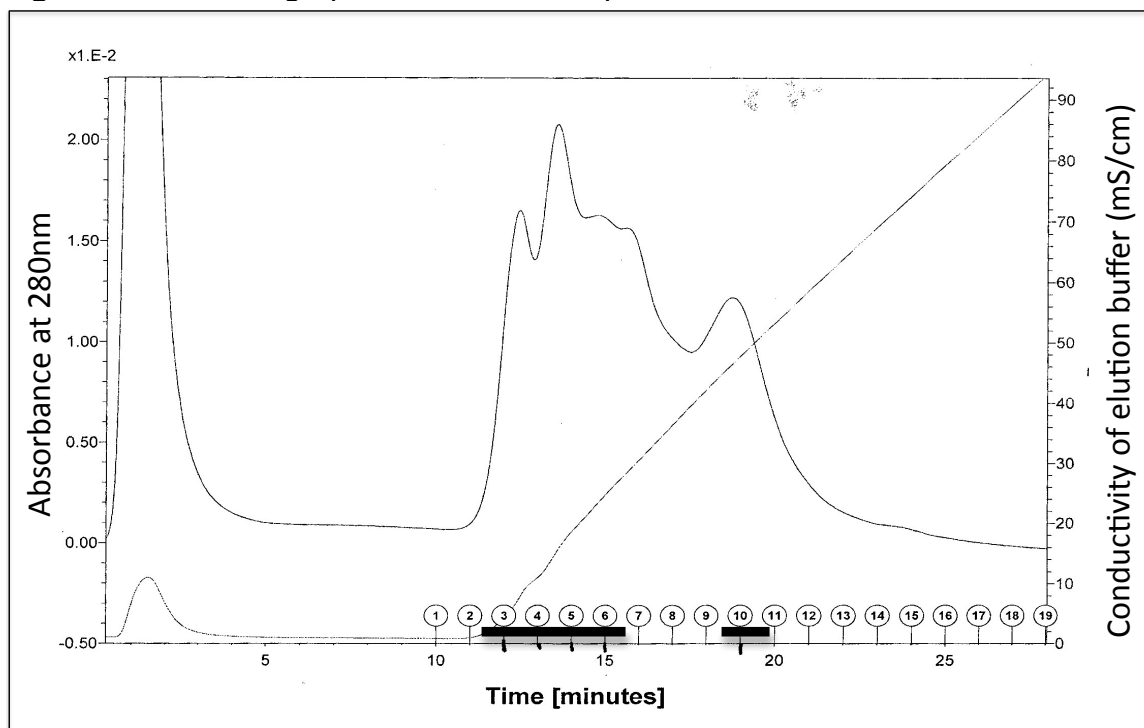


Percent decrease in *V. parahaemolyticus* growth in cultures supplemented with sterile pooled shell extract (Full), extract that did not pass through a 10kDa centrifugal filter unit (10T), extract that passed through a 10kD filter (10B), and extract that passed through a 3kD filter (3B). The final growth measurement was made 48h after  $T_0$ . Error bars illustrate one standard deviation. All assays were done in triplicate with a shell extract prepared with lobster shell material from 8 individual lobsters.

#### Fast protein liquid chromatography (FPLC) of shell extract

Fast protein liquid chromatography with a cation exchange column showed 5 protein peaks in the cationic fraction of the sterile shell extract (Figure 6). Samples collected at time points 3, 4, 5, 6, and 10 correspond to protein peaks in the chromatograph. The void volume peak (0-5min) corresponds to anionic organic compounds in the shell fraction.

**Figure 6.** Chromatogram from FPLC with pooled sterile shell extract



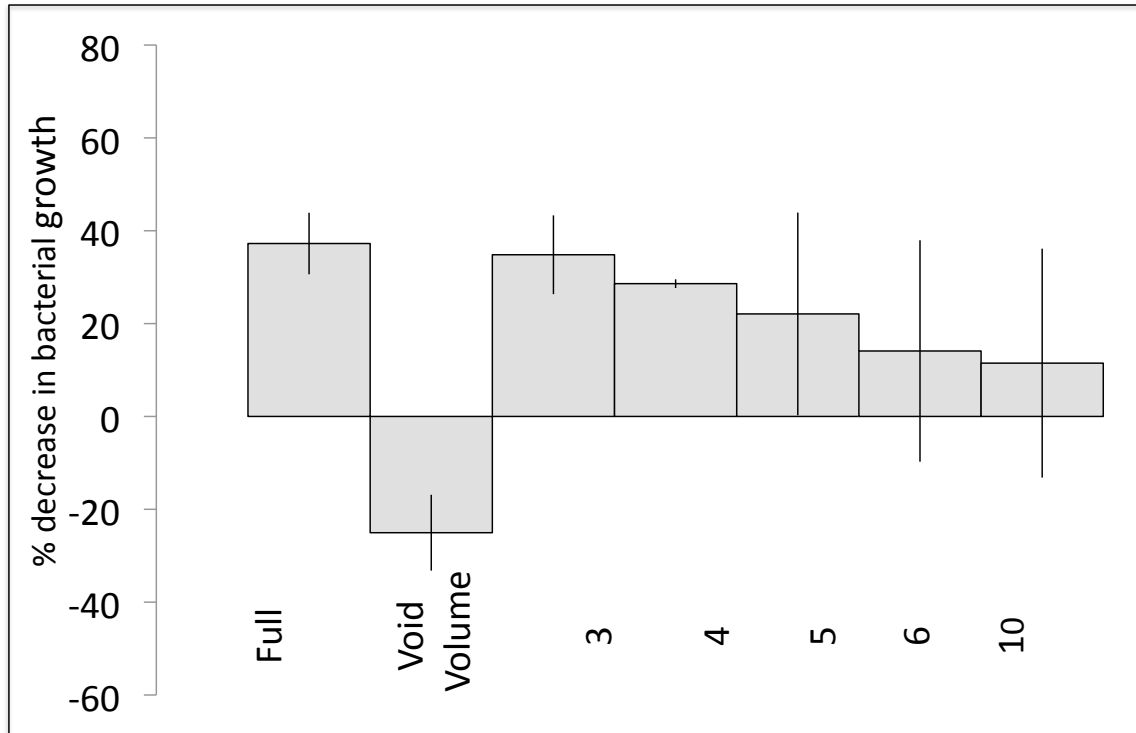
Full sterile shell extract was applied to an FPLC instrument with a cation exchange column and eluted with a linear gradient of 0–1M NaCl in 20mM Tris–HCl buffer (pH 8.5) at a flow rate of 1ml/min. The void volume was collected from minutes 1-4. One ml samples were collected from minutes 10-30 and labeled fractions 1-19 in the order they were collected. Fractions 3, 4, 5, 6, and 10 (underlined) correspond to protein peaks and were used in the antimicrobial assay.

### Evaluation of antimicrobial activity of FPLC fractions

The FPLC fractions 3, 4, 5, 6, and 10 were chosen to be analyzed for antimicrobial activity due to their correspondence to the protein peaks seen in the chromatogram (Figure 6). Antimicrobial activity observed in FPLC fractions 3 and 4 was not significantly different from that observed in the full extract (Figure 7). Fractions 5, 6, and 10 exhibited lower antimicrobial activity and this activity was highly variable (Figure 7). The void volume, which consists of all anionic and inorganic components of the extract, actually increased bacterial growth by 25% (Figure 7). These results indicate

that the component of the extract with antimicrobial activity is both organic and cationic in nature and is present in FPLC fractions 3 and 4.

**Figure 7.** Evaluation of antimicrobial activity in sterile shell extract fractionated with FPLC

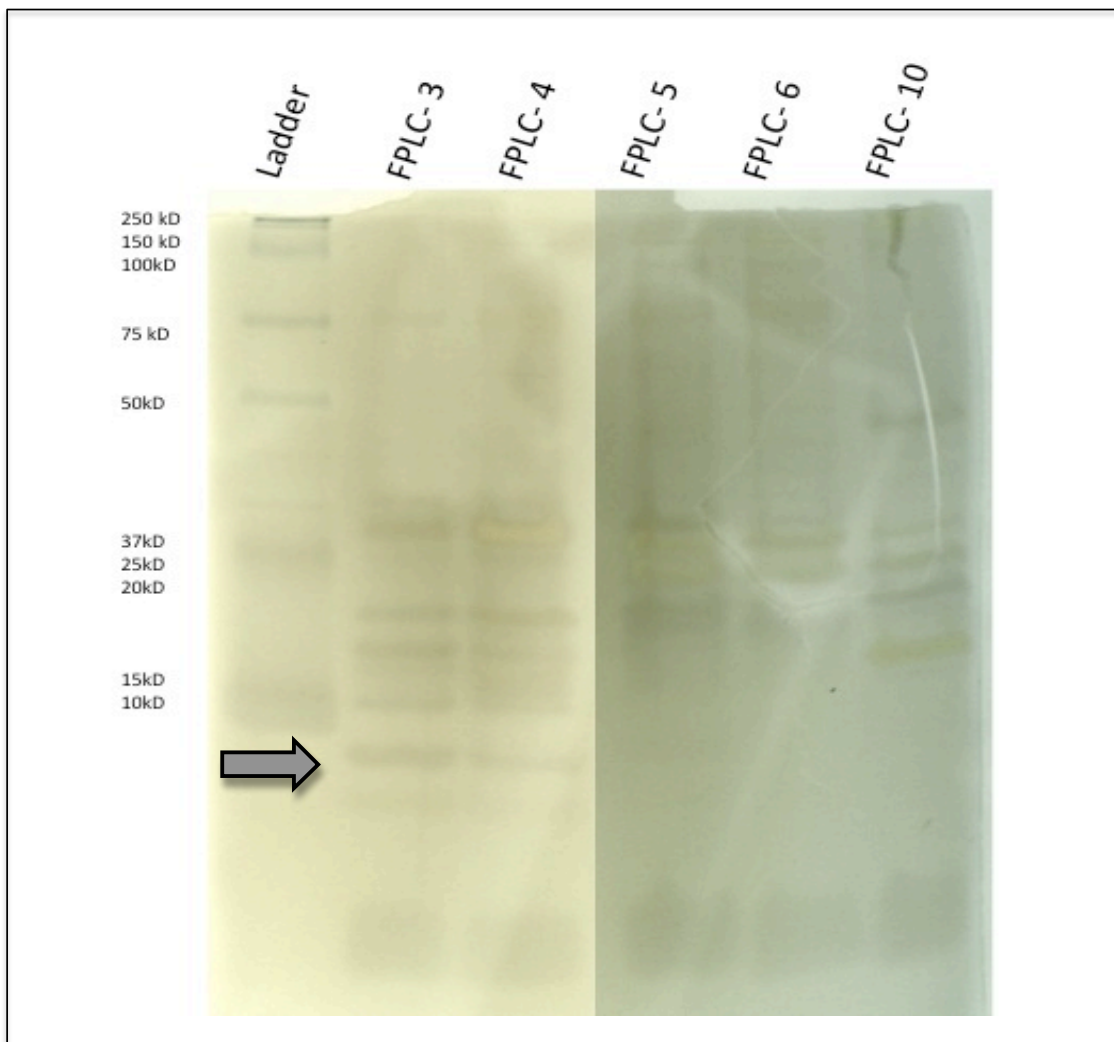


Percent decrease in *V. parahaemolyticus* growth in cultures supplemented with sterile pooled shell extract (Full), the void volume from the FPLC, and FPLC fractions taken at time points 3, 4, 5, 6, and 10. The final growth measurement was made 48h after  $T_0$ . Error bars illustrate one standard deviation. All assays were done in triplicate with a shell extract prepared with lobster shell material from 8 individual lobsters.

#### Tris/tricine SDS-PAGE with FPLC Fractions

Gel electrophoresis of the FPLC fractions revealed two faint bands in the region below the ladder's 10kD marker in fraction 3 and 4 that were not seen in the other fractions (Figure 8). This indicates that there are very small peptides (<10kD) present in FPLC fractions 3 and 4, but not in FPLC fractions 5, 6, 7, and 10.

**Figure 8.** Digital photograph of tris/tricine SDS-PAGE with FPLC fractions



The arrow in the image points to small protein bands (<7kDa) present in FPLC fractions 3 and 4.

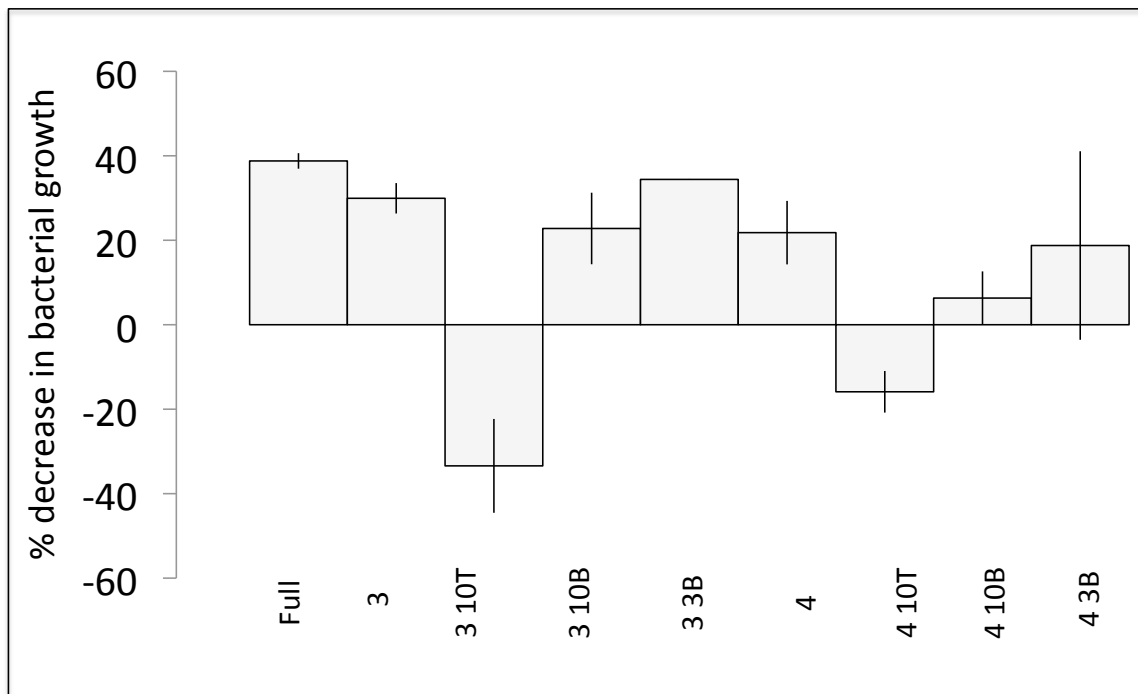
Note: The middle lanes of the gel did not contain samples and were removed from the photo. The tear seen in the right hand side occurred after the gel had finished running, during the staining process. The tear does not affect the ability to see small bands in the lanes it crosses.

#### Evaluation of antimicrobial activity in size fractioned FPLC fractions (3, 4)

FPLC fractions 3 and 4 were filtered using 10kDa and 3kDa centrifugal filter units. The extract that did not pass through the 10kD filter (3-10T, 4-10T), extract that

passed through the 10kD filter (3-10B, 4-10B), and extract that passed through a 3kD filter (3-3B, 4-3B) were evaluated for antimicrobial activity. The antimicrobial activity of the FPLC fraction 3 that passed through the 3kDa and 10kDa filters was not significantly different from that of the full FPLC fraction 3 or the full raw sterile shell extract (Figure 9). The FPLC fraction 3 that was unable to pass through a 10kDa filter increased bacterial growth (Figure 9). FPLC fraction 4 and its subsamples followed the same pattern as FPLC fraction 3, but it was less pronounced and much more variable (Figure 9). These results confirm that the component of the extract responsible for antimicrobial activity is less than 10kDa and is likely less than 3kDa.

**Figure 9.** Evaluation of antimicrobial activity in size fractionated FPLC fraction

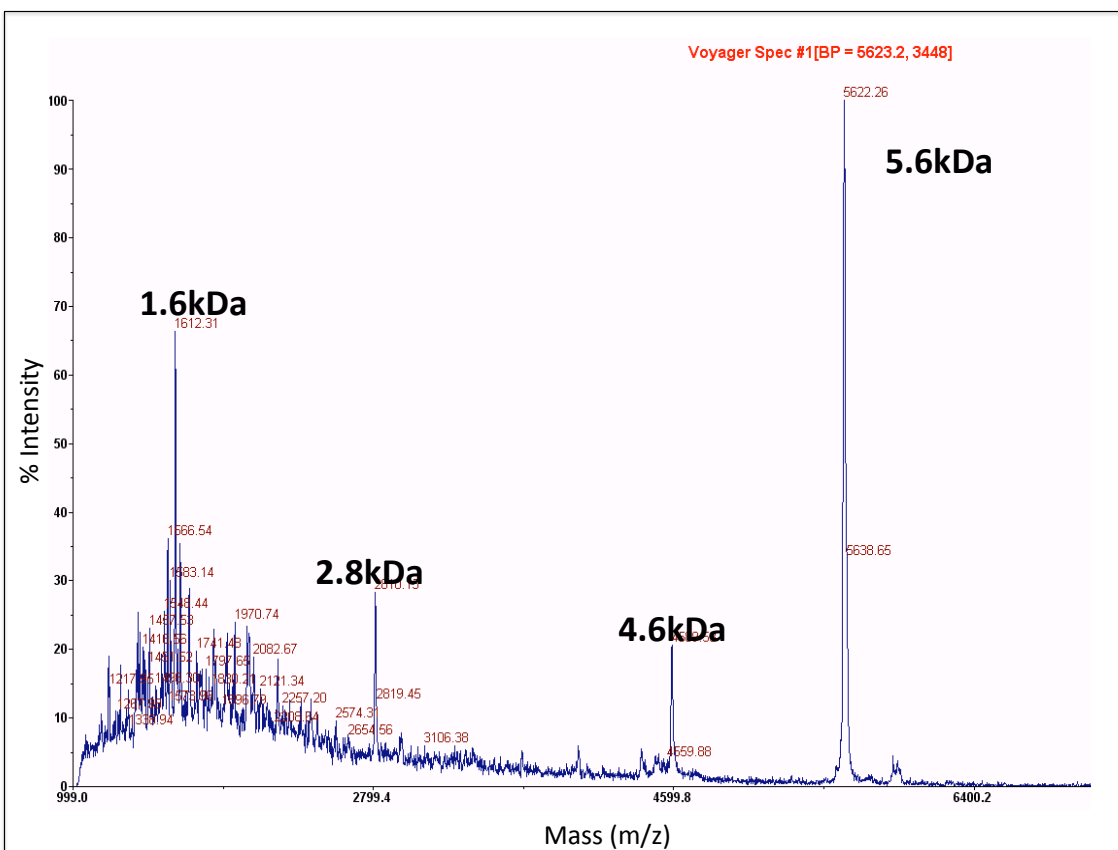


Percent decrease in *V. parahaemolyticus* growth in cultures supplemented with sterile pooled shell extract (Full) and both FPLC fractions 3 & 4 that did not pass through a 10kD filter (3-10T, 4-10T), passed through a 10kD filter (3-10B, 4-10B), and passed through a 3kD filter (3-3B, 4-10B). The final growth measurement was made 48h after  $T_0$ . Error bars illustrate one standard deviation. All assays were done in triplicate. The shell extract was prepared from lobster shell material from 8 individual lobsters.

## Determination of Molecular Weight of Small Peptides in Sterile Shell Extract

MALDI Mass Spectrometry shows 4 protein peaks at 1.6kDa, 2.8kDa, 4.6kDa, and 5.6kDa, with the most intense of these peaks at 5.6kDa (Figure 10). There is a large amount of noise in the lower mass range precluding further analysis of the smaller peptides (1.6, 2.8, and 4.6kDa), which, based on the size filtration studies, are likely to be involved in antimicrobial activity.

**Figure 10.** MALDI mass spectrometry of pooled sterile shell extract



MALDI MS spectrum obtained with sterile shell extract that passed through a 10kDa centrifugal filter unit. The most intense peak corresponding to a 5.6kDa peptide was further analyzed for amino acid sequence.

## Determination of the amino acid sequence of a small peptide in the shell extract

The 5.6kDa peak was ion fractionated and a partial amino acid sequence was determined (I/L V Y x N I/L x W N I/L V I/L x C R x G I/L I/L V N I/L W). I/L indicates

amino acids that are either leucine or isoleucine and x represents an unknown amino acid.

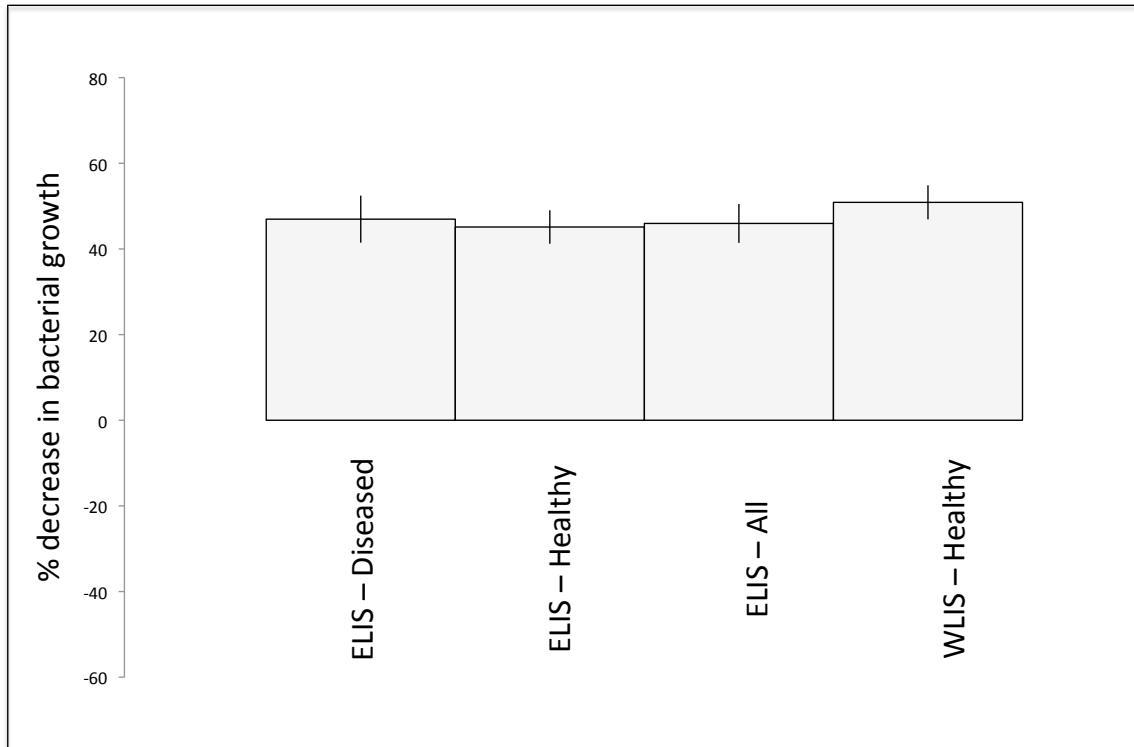
## **Evaluation of antimicrobial activity in the shells of lobsters from two populations with differing disease prevalence**

### Antimicrobial assay with shell extracts of lobsters from the Eastern and Western Long Island Sound

Sterile shell extracts were made from the shells of 22 individual lobsters, which were either apparently “healthy” from ELIS (n=5), diseased from ELIS (n=6), or healthy from WLIS (n=11). Antimicrobial activity against *V. parahaemolyticus* was tested for each individual extract and each individual extract was found to exhibit antimicrobial activity. The average % decrease in bacterial growth for the individuals in each group (ELIS Healthy, ELIS Diseased, ELIS All, and WLIS Healthy) was calculated. The difference in the mean % decrease in bacterial growth with extracts from ELIS and WLIS lobsters is significant at the 95% confidence level (Figure 11, Table 1). However, there was no difference in antimicrobial activity between shell extracts from healthy and diseased lobsters from ELIS (Figure 11).



**Figure 11.** Evaluation of antimicrobial activity in shell extracts from symptomatic and asymptomatic lobsters from Eastern and Western Long Island Sound



Averaged percent decrease in bacterial growth for ELIS diseased, ELIS healthy, All ELIS, and WLIS healthy lobster shell extracts. “ELIS Diseased” indicates Eastern Long Island Sound lobsters that exhibited ESD lesions on their carapace (n=5). “ELIS Healthy” (n=6) and “WLIS Healthy” (n=11) describes lobsters from Eastern and Western Long Island Sound with no visible ESD lesions. “ELIS ALL” (n=11) is an average of “ELIS Diseased” and “ELIS Healthy.” Final bacterial growth measurements were made 48h after T<sub>0</sub>. Error bars illustrate one standard deviation. All individual extracts were assessed for antimicrobial activity in triplicate.

**Table 1.** Analysis of Variance table of ELIS-All and WLIS-Healthy averaged % decrease in bacterial growth with shell extracts from individual lobsters from the two regions

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
% decrease	1	127.04	127.039	6.9371	0.01636 *
Residuals	19	347.95	18.313		

Signif. codes: 0 ‘\*\*\*\*’ 0.001 ‘\*\*\*’ 0.01 ‘\*\*’ 0.05

## **Discussion**

### **Detection and characterization of antimicrobial activity in American lobster shell**

The sterile pooled shell extract consistently exhibited antimicrobial activity against a broad spectrum of bacteria. Individual shell extracts from 22 lobsters each showed a comparable activity against *V. parahaemolyticus*. These data indicate that American lobster cuticle possesses an intrinsic antimicrobial activity and therefore likely plays a larger role in the defense against invading pathogens than a simple physical barrier. Haug et al. (2002) discovered broad-spectrum antimicrobial activity in the exoskeletons of four marine decapods: Northern Shrimp (*Pandalus borealis*), Hermit Crab (*Pagurus bernhardus*), Spider Crab (*Hyas araneus*), and King Crab (*Paralithodes camtschatica*). This activity was not fully characterized, but it was found to be heat resistant and sensitive to enzyme proteinase k (Haug et al. 2002). The antimicrobial activity discovered here, in the shell of American lobster, is also heat resistant. Furthermore, the antimicrobial activity in the shell of the American lobster is due to an organic, cationic component less than 10kDa and likely less than 3kDa.

Based on the data presented, it is likely that an antimicrobial peptide (AMP) in the shell of American lobster is responsible for the antimicrobial activity observed. AMPs are small (<10kDa), cationic peptides that exhibit broad-spectrum antimicrobial activity. Structural characteristics of AMPs and their small size make them stable and heat resistant. Although AMPs have not previously been reported to exist in crustacean exoskeletons, they are wide spread internal defense factors in crustaceans (Table 2). Three distinct families of crustacean AMPs have been characterized based on AMPs isolated from shrimp: the penaeidins, antilipoplysaccharide factors (ALFs), and crustins (Pisuttarachai et al. 2009). Because these defense molecules are ubiquitous internally, it is possible that AMPs may be present in the crustacean cuticle as well. The chitin-binding capabilities of shrimp penaeidins support this idea as they have been found bound to chitin in gill cuticle surfaces of shrimp (Destoumieux et al. 2000).

Of the three families of crustacean AMPs, the penaeidins (5.48-6.62kDa) found in shrimp are the most prominent (Bachere et al. 2000). These AMPs show antifungal activity and specific antibacterial activity against gram-positive bacteria, but do not show

strong activity against *Vibrio spp.* (Destoumieux et al. 1999). This group of AMPs is produced in hemocytes and they appear to be released when hemocytes lyse in response to infection (Destoumieux et al. 2000). The penaeidins are very stable and are resistant to proteolysis (Destoumieux et al. 1999). However, penaeidins have not been isolated from lobsters.

AFL's were first discovered in horseshoe crab plasma. Bacterial endotoxins trigger a cascade in horseshoe crab hemocytes leading to rapid degranulation and intracellular coagulation; horseshoe crab AFLs are capable of binding and neutralizing LPS, thus inhibiting this cascade (Iwanaga 1993). AFL's have also been isolated from the hemolymph of many shrimp species (Somboonwiwat et al. 2005) and the American lobster, *Homarus americanus* (Beale et al. 2008). AFL's have direct antimicrobial capabilities and have been shown to be active against gram-negative and positive bacteria and filamentous fungi (Somboonwiwat et al. 2005).

Crustins (7-14kD) have been isolated from the hemolymph of a variety of crustaceans and are categorized based on the crustaceans they are found in: type I crustins are found in crabs, lobsters, and crayfish; type II crustins are mostly found in shrimp; types III crustins are found in decapods. All crustins, however, have a cationic region at the carboxyl terminus with the first of twelve cysteine residues making up the whey acidic protein (WAP) domain, which is associated with antimicrobial activity (Pisuttharachai et al. 2009).

Specific examples of AMP's isolated from the hemolymph of large crustaceans (Table 2) include a proline-rich, 6.5 kD AMP (Shnapp et al. 1996) and a cysteine-rich, 11.5kD, gram-positive specific (Relf et al. 1999) AMP from the shore crab *Carcinus maenas*. The 11.5kD peptide has been shown to share similarities with crustins expressed in shrimp (Tincu & Taylor 2004). A smaller cationic antimicrobial peptide with molecular weight of 3.7kD has been found in blue crab (*Callinectes sapidus*) hemocytes (Khoo et al. 1999). Additionally, four isoforms of a type two crustin have been characterized from the Japanese spiny lobster, *Panulirus japonicus* (Pisuttharachai et al. 2009). In American lobster, cDNA encoding a 96 amino acid crustin-like peptide has been identified and sequenced (ACN: CN853187.1). This

crustin is referred to as Hoa-crustin and is most similar to a crustin isoform found in the European lobster, *Homarus gammarus* (Christie et al. 2007). In 2008, Battison et al. isolated and characterized two antimicrobial peptides from the hemocytes of American lobster. The first is approximately 12kD, expresses specific antibacterial activity against gram-positive bacteria, and contains amino acid sequences that were predicted by the cDNA sequenced by Christie et al. (2007). The second peptide, homarin, is composed of four 6kD subunits and is similar to amphibian temporins, but unlike any other marine invertebrate AMP. Homarin has been shown to exhibit antimicrobial activity against gram-negative bacteria as well as two cultured scuticociliate parasites (Battison et al. 2008).

**Table 2.** Selected examples of AMPs isolated from the of hemolymph large crustaceans

<b>Name</b>	<b>Species</b>	<b>Size</b>	<b>Description</b>	<b>Reference</b>
Penaeidins	shrimp <i>Panaeus vannamei</i>	2.7-8.32kD	Proline and cysteine rich, 3 disulfide bridges	Destoumieux et al. (2000)
ALF $Pm^3$	<i>Penaeus monodon</i>	10kD	Anti-lipopolysaccharide Factor	Somboonwiwat et al. (2006)
broad spectrum antibacterial protein	<i>Carcinus Maenus</i>	6.5kD	Proline rich	Schnapp et al. (1996)
Carcinin	<i>Carcinus Maenus</i>	11.5kD	crustin, cysteine rich, disulfide bridge	Relf et al.
Callinectin	<i>Callinectes sapidus</i>	3.7kD	proline is most abundant AA, but is not arranged in way typical to proline rich AMPs	Khoo et al. (1999)
Hoa-crustin	<i>Homarus americanus</i>	12kD	crustin	Battison et al. (2008)
<i>Homarin</i>	<i>Homarus americanus</i>	4-6kD	dissimilar to other crustacean AMPs, similar to amphibian temporins	Battison et al. (2008)
PJC1, 2, 3, 4	<i>Panulirus japonicus</i>	13.6-15.7kD	4 individual crustins more similar to shrimp than lobster crustins	Pisuttharachai et al. (2009)
ALFHa-1 & 2	<i>Homarus americanus</i>	13.7kD, 13.9kD	Anti-lipopolysaccharide factors	Beale et al. (2008)

Antimicrobial peptides (AMPs) are an integral component of the invertebrate internal defense system and have been found in every invertebrate species in which their presence has been investigated (Hancock et al. 2006). Over 2,000 AMP's have been identified in a large variety of organisms (Park et al. 1998), including plants, insects, marine invertebrates, marine vertebrates, amphibians, and mammals, including humans (Powers & Hancock 2003). A high degree of diversity exists in AMP sequences and the same peptide sequence has not been found in two species, but segments and motifs of amino acid sequence can be conserved within AMP classes across species (Zaslhoff 2002). AMPs are typically made up of 12-50 amino acids, 2-9 of which are positively charged lysine or arginine residues and up to 50% of the amino acids in AMPs are usually hydrophobic (Tincu & Taylor 2004). AMPs are usually less than 10kDa and many AMPs are less than 3kDa (table 3).

**Table 3.** Selected examples of small AMPs (<5kDa) that have been isolated from a variety of animal species

Name	Species	Size	Description	Reference
Styelin	<i>Styela clava</i> , solitary tunicate	3.7kDa	$\alpha$ -helical, phenyl-alanine rich GWFGKAFRSVSNFYKHKHTYIHAGLSAAT LLG effective against gram + & - bacteria, including marine bacteria, at a min conc. of 1.5 $\mu$ g/ml	Lee et al. (1997)
Clavanin	<i>Styela clava</i>	2.7kDa	$\alpha$ -helical, histidine rich VFQFL GKIIH HVGNF VHGFS HVF similar in structure and function to Magainins	Lee et al. (1997 B)
Clavaspirin	<i>Styela clava</i>	2.7kDa	$\alpha$ -helical, histidine rich amidated 23 residue peptide FLRFIGSVIHGIGHLVHHIGVAL broad spectrum, able to permeabilize inner and outer membranes	Lee et al. (2001)
Cynthaurin	<i>Halocynthia aurantium</i> , solitary tunicate	3.25kDa	$\alpha$ -helical, ILQKAVLDCLKAAGSSLSKAAITAIYNKIT often exists as dimer, Dicynthaurin, linked covalently with a cystine disulfide bond. Active against gram + and gram - bacteria.	Lee et al. (2001 B)
Tachypesin I	<i>Tachyplesus tridentatus</i> , Japanese horse shoe crab	2.26kDa	$\beta$ -hairpin structure with 2 cystine disulfide bonds, KWCFRVCYRGICYRRC, active against gram - bacteria at 0.8-12.5 $\mu$ g/ml and gram + bacteria at 3.1-6.3 $\mu$ g/ml depending on species	Nakamura et al. (1988)

**Table 3.** Continued from previous page

Tachyplesin II	<i>Limulus polyphemus</i>	2.5kDa	$\beta$ -hairpin structure w/ two cystine disulfide bonds RWCFRVCYRGICYRKCR active against gram + & - bacteria and the fungus <i>candida albicans</i> M9	Miyata et al. (1989)
Magainin 2	<i>Xenopus laevis</i> (African clawed frog)	2.5kDa	$\alpha$ -helical, GIGKFLHSAKKFGKAFVGEIMNS, forms pores in membranes and translocates the membrane	Matsuzaki et al. (1995)
Melittin	<i>Apis mellifera</i> , European Honey Bee	2.8kDa	Interrupted $\alpha$ -helical conformation, two helices separated by bend, GIGAVLKVLTTGLPALISWIKRKRQQ, increases membrane permeability	Bechinger (1996)
Buforin II	<i>Bufo bufo garagiozans</i> , Asian toad	2.0kDa	$\alpha$ -helical, TRSSRAGLQFPVGRVHRLLRK , strong activity against gram + & - bacteria, forms pores and inhibits cell function by binding with DNA and RNA	Park et al. (1998)
Pyrrhocoricin	<i>Pyrrhocoris apterus</i> , firebug	3.0kDa	linear, proline rich, VDKGSYLPRPTPPRPIYNRN, Broad spectrum, DNA binding	Kragol et al. (2002)
Thanatin	<i>Podisus maculiventris</i> , Spined Soldier Bug	2.4kDa	$\beta$ -hairpin structure with one disulfide bridge, GSKKPVPIIYCNRRTGKCQRM, active against gram + & - bacteria and fungi	Pascale et al. (1996)
Apidaecin	<i>Apis mellifera</i> , Honey Bee	2.0kD	linear, proline and arginine rich GNNRPVYIPQPRPPHPRI active against gram - bacteria only, stereospecific, but not pore forming, translocates and depolarizes inner membrane	Castle et al. (1999)
Indolicidin	<i>Bos taurus</i> , cow	1.5kD	linear, tryptophan and proline rich, smallest known AMP, ILPWKWPWPWRR, active against gram + & - bacteria, increases membrane permeability and inhibits DNA synthesis	Hsu et al. (2005)

Antimicrobial peptides are classified based on their structural characteristics. They can generally be assigned to one of three structural groups: (1) linear peptides that form  $\alpha$ -helical structures, (2) linear peptides rich in a specific amino acid, and (3) cysteine-rich open-ended peptides containing one or more disulfide bridges (Viziolo & Salzet 2002). AMPs with disulphide bridges independently maintain their secondary structure (Hancock et al 2006), typically a  $\beta$ -hairpin configuration. The hairpin configuration lends stability to the peptide and allows it to maintain structural integrity at

high temperatures and low pH's (Tincu & Taylor 2004). Linear peptides that form  $\alpha$ -helices and linear peptides that are rich in a particular amino acid exist in solution with little or no secondary structure and fold into an amphipathic structure when they contact a target membrane. Because these AMPs lack secondary and tertiary structure, they are also very stable and heat resistant (Hancock et al. 2006).

The mechanism of action has not been elucidated for all AMP's that have been isolated, but general structure-function relationships have been described for the major structural groups (Viziolo & Salzet 2002). Most AMPs interact with the outer membrane of gram-negative bacteria in a similar manner. AMPs associate with the anionic lipopolysaccharides (LPS) of the outer membrane through electrostatic interactions that are stronger than those between LPS and native divalent cations such as  $Mg^{2+}$  and  $Ca^{2+}$ . As a result, native divalent cations are displaced, which leads to a local disturbance in the outer membrane allowing the AMP to cross the outer membrane and reach the cytoplasmic membrane. Once an AMP reaches the cytoplasmic membrane, its mode of action becomes dependent on its structure (Powers & Hancock 2003). Linear amphipathic peptides increase bacterial membrane permeability through the interaction of their cationic charge with anionic membrane lipids or through membrane lipid displacement. Cysteine-rich peptides with disulfide bridges and  $\alpha$ -helical peptides may form ion-permeable channels in the lipid bilayer. The peptides may or may not cross the membrane into the cell, but it is likely that this is the case for most small AMPs. Microbial death follows either from the disturbance of membrane functioning or damage incurred to crucial intracellular targets after an AMP enters the cell (Viziolo & Salzet 2002).

In this study, MALDI mass spec of the shell extract revealed four small peptides (1.6, 2.8, 4.6, and 5.6 kDa) in the shell extract. These sizes are typical of antimicrobial peptides. Unfortunately only a partial sequence of the 5.6kDa peptide could be determined. The sequence, I/L V Y x N I/L x W N I/L V I/L x C R x G I/L I/L V N I/L W, has several unknowns and whether or not it is an AMP can not be determined conclusively. The sequence includes one (known) positively charged arginine, while most AMPs contain 2-9 arginine residues. Thirteen of the identified amino acids in the

22 amino acid sequence are hydrophobic; this is more than 50%, but it is close to the percent range of hydrophobic amino acids in AMPs. The peptide is not rich in proline or glycine as are many linear AMPs rich in a particular amino acid isolated from invertebrates (Viziolo & Salzet 2002). Additionally, when different combinations of unknowns in the unknown peptide were searched using the Basic Local Alignment Tool (BLAST), a partial match was made with the  $\alpha$ -helical peptide mastaparan, which is a broad-spectrum AMP isolated from wasps (Figure 12) (Hisada et al. 2000). This match is not conclusive, but the data suggest that the 5.6kDa peptide is also an  $\alpha$ -helical AMP.

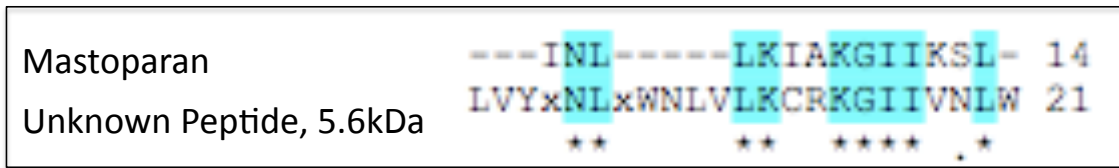
**Table 4.** Characteristics of the unknown 5.6kDa peptide

Abbreviation	Amino Acid	Charge	Hydrophobic or philic at pH 7	% of total amino acids
I/L	Isoleucine or Leucine	neutral	very hydrophobic	27.3%
V	Valine	neutral	very hydrophobic	13.6%
Y	Tyrosine	neutral *	hydrophobic	4.5%
x	-	-	-	18%
N	Asparagine	neutral*	hydrophilic	13.6%
x				
W	Tryptophan	neutral	very hydrophobic	9.1%
N	Asparagine	neutral*	hydrophilic	
I/L	Isoleucine or Leucine	neutral	very hydrophobic	
V	Valine	neutral	very hydrophobic	
I/L	Isoleucine or Leucine	neutral	very hydrophobic	
x	-	-	-	
C	Cysteine	neutral	hydrophobic	4.5%
R	Arginine	(+)*	hydrophilic	4.5%
x	-	-	-	
G	Glycine	neutral	aliphatic	4.5%
I/L	Isoleucine or Leucine	neutral	very hydrophobic	
I/L	Isoleucine or Leucine	neutral	very hydrophobic	
V	Valine	neutral	very hydrophobic	
N	Asparagine	neutral*	hydrophilic	
I/L	Isoleucine or Leucine	neutral	very hydrophobic	
W	Tryptophan	neutral	very hydrophobic	

\* Polar



**Figure 12.** BLAST sequence alignment of the unknown 5.6kDa peptide with the wasp antimicrobial peptide mastoparan



**Evaluation of antimicrobial activity in the shells of lobsters from two populations with differing disease prevalence**

When the antimicrobial activity in shells of lobsters from ELIS and WLIS were evaluated, extracts from WLIS lobsters showed significantly higher antimicrobial activity than those from ELIS lobsters. There was no difference in antimicrobial activity in shell extracts from healthy and diseased ELIS lobsters. Homerding (2009) found that hemocytes and plasma from WLIS lobsters have significantly increased phagocytotic rates, antimicrobial activity, native concentrations of reactive oxygen species, and induced concentrations of reactive oxygen species when compare to ELIS lobsters. It was also found that WLIS lobster carapaces are significantly thicker than ELIS lobster carapaces. While lobsters are known to migrate in and off-shore, tagged lobsters from Eastern and Western Long Island Sound are generally recaptured where they were initially tagged, suggesting limited migration between the two populations (Landers et al. 2007). Indeed, it has been shown that WLIS lobsters are genetically isolated from ELIS lobsters, but ELIS lobsters are not genetically isolated from off-shore lobsters (Crivello et al 2005). It is possible that WLIS lobsters having gone through a severe die-off during the 1999 mortality event, experienced a genetic bottleneck, and the population now has a more robust defense system. If this is the case, then the defense capabilities of the populations may be affecting their susceptibilities to Epizootic Shell diseased and decreased antimicrobial activity in the shell may play a role in ELIS lobsters' increased susceptibility.

## **Conclusions**

This study has demonstrated broad-spectrum antimicrobial activity in the shell of American lobster for the first time. Characterization of the activity has determined that it is caused by an organic, cationic component, less than 10kDa, and likely less than 3kDa. Based on these results, it is likely that an antimicrobial peptide is responsible for the activity observed. The presence of AMPs in crustacean cuticle has not been previously studied. This study has therefore contributed to building a more comprehensive understanding of crustacean defense mechanisms. Additionally, the difference in antimicrobial activity in the shells of ELIS and WLIS lobster shells suggests that the activity detected and characterized may play a role in susceptibility to shell disease.

## **Future Directions**

Continuation of this research requires the purification and full sequencing of all the small peptides observed in the MALDI mass spec of the sterile shell extract. Evaluation of the antimicrobial activity of purified peptides would allow for the determination of which peptide(s) is responsible for the activity observed in the shell extract. If the causative agent of Epizootic Shell Disease is determined, the activity of the antimicrobial peptide(s) from the lobster shell against this pathogen should be investigated. The activity of the peptide(s) should also be evaluated against other pathogens affecting lobster populations. Differences in concentrations of AMPs in lobster shells from different populations with varying disease prevalence can also be investigated.

A public health interest exists in identifying and characterizing new AMPs. Antimicrobial peptides often act by interrupting microbial cell membranes, an action that is believed to be unlikely to incite microbial resistance. This property, along with their small size, which facilitates their synthesis, and generally low toxicity to eukaryotic cells, makes AMPs an attractive prospect for biomedical development (Tincu & Taylor 2004).

This is especially the case since some AMP's are able to inhibit the replication of enveloped viruses, such as influenza A virus and human immunodeficiency virus (HIV-1). Some AMPs have also demonstrated anticancer capabilities and promote wound healing (Powers & Hancock 2003). Therefore, a better characterization of AMPs in lobster shells may also have significant biomedical applications.

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