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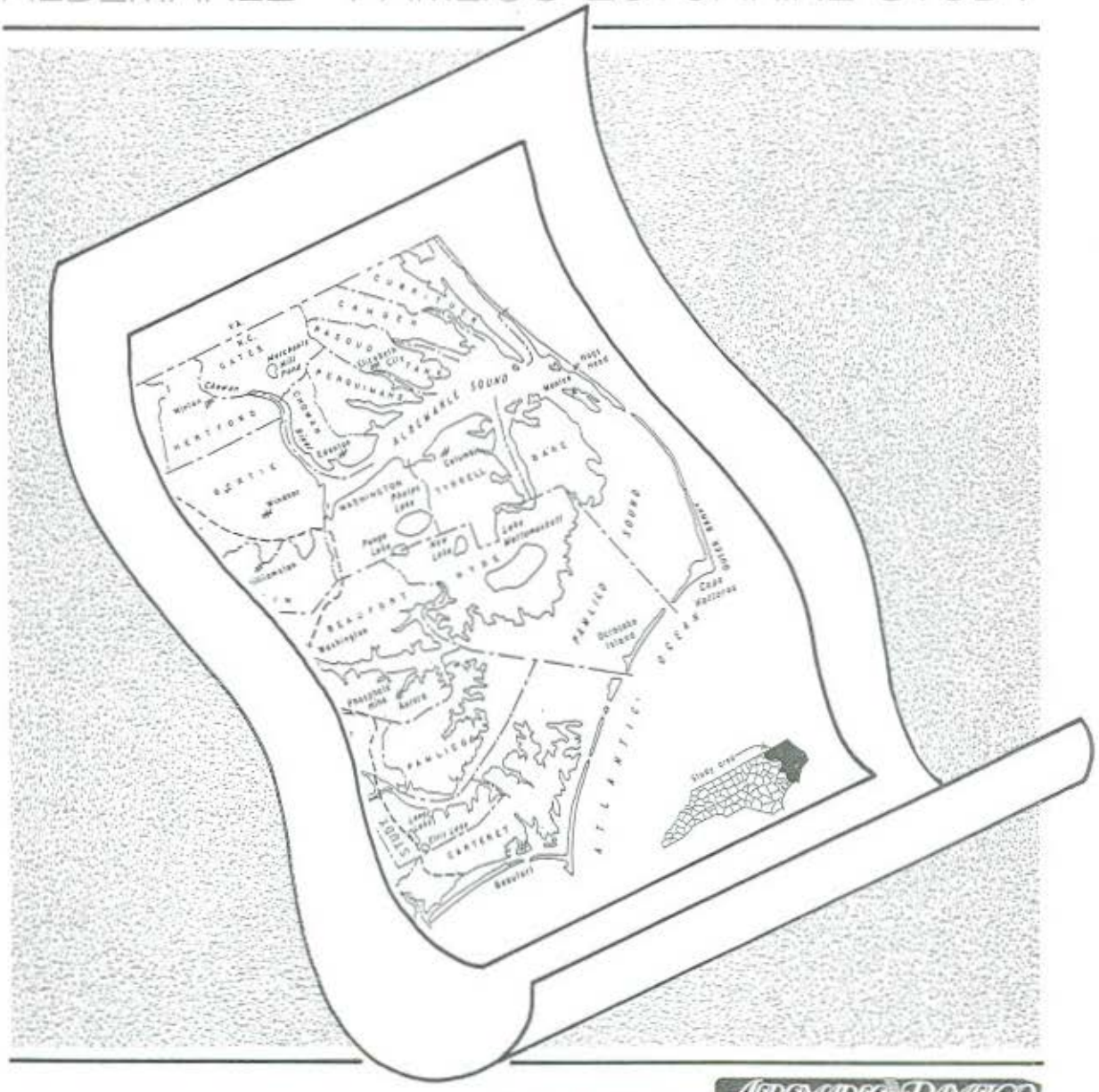
SHELL DISEASE IN BLUE CRABS, CALLINECTES SAPIDUS, FROM THE ALBEMARLE-PAMLICO ESTUARY

ALBEMARLE - PAMLICO ESTUARINE STUDY



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ALBEMARLE-PAMLICO

SHELL DISEASE IN BLUE CRABS, CALLINECTES SAPIDUS, FROM THE ALBEMARLE-PAMLICO ESTUARY

by

Edward J. Noga
Department of Companion Animal and Special Species Medicine
College of Veterinary Medicine
North Carolina State University
Raleigh, North Carolina 27606

David W. Engel
Beaufort Laboratory
National Marine Fisheries Service
Beaufort, North Carolina 28516-9722

and

Thomas W. Arroll
Department of Companion Animal and Special Species Medicine
College of Veterinary Medicine
North Carolina State University
Raleigh, North Carolina 27606

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ABSTRACT

Shell disease has recently been recognized as a common problem in blue crabs in certain areas of the Albemarle-Pamlico Estuary. This disease is a common problem in crustacean (crab, shrimp, lobster) populations and is most commonly associated with stressful conditions, such as polluted ecosystems or adverse aquaculture environments. While a number of pathogens, especially bacteria, have been isolated from shell disease lesions, the mechanisms leading to development of this disease are unclear. The difficulty in reproducing the disease by simply challenging healthy crustaceans with the putative causative bacteria suggests that host immunity may play a very important role in disease development.

Our studies have shown that shell disease lesions from blue crabs in the A/P Estuary have large numbers of bacteria; all of these bacteria have enzymes, such as chitinase and lipase, that are believed to be pathogenic markers that allow these bacteria to degrade the blue crab shell. However, the shell of clinically normal crabs that do not have shell disease also have large numbers of bacteria. These bacteria have the same enzymatic activities as those cultured from crabs with shell disease. This evidence supports the hypothesis that a change in host defenses may be pivotal to defending against these endogenous pathogens.

We have found that the blood (hemolymph) of blue crabs has a potent activity that kills many of the bacteria that are present on both clinically normal crabs and those having shell disease. Our demonstration of activity in shell extracts also suggests a primary defensive role in this activity in protecting against shell disease. This antibacterial activity is bactericidal (i.e., it kills these bacteria). Our studies have also demonstrated that this activity is sensitive to high temperatures and is inhibited by sodium chloride. The activity is also stable after multiple freeze-thaw cycles and is very stable during storage at -70^{o}C. The latter properties make this activity a potentially useful substance for field monitoring, as described below.

We developed a sensitive, reproducible and quantitative assay for measuring this antibacterial activity in individual blue crabs. This modified turbidometric assay has allowed us to test activity using very small samples. This test demonstrated that there were statistically significant differences in the antibacterial activity of different groups of crabs. First, there was a significantly lower antibacterial activity in crabs with shell disease compared to clinically normal crabs collected from the same geographic area. Second, there was a significantly lower activity in blue crabs collected from riverine areas of the A/P Estuary (e.g., Pamlico River, Pungo River) compared to those

collected from more oceanic sites (e.g., Core Sound, Bogue Sound). In some cases, the difference in antibacterial activity between these different geographic sites was greater than 500%.

We also found that the level of hemocyanin in the hemolymph was significantly lower in crabs in the Pamlico River compared to reference sites (i.e., Core and Bogue Sounds). Hemocyanin is the oxygen-carrying protein in crab hemolymph. There was no difference in hemocyanin levels between clinically normal crabs and those with shell disease.

The lower immunological competence of blue crabs in the riverine areas of the A/P Estuary may be a natural consequence of physiological changes that are associated with adaptation to these low salinity environments. However, this lower immunocompetence may also indicate that some type of pollutant(s) is(are) reducing the natural resistance of these crabs. In either case, it may explain why shell disease problems have been centered in these riverine areas. Evidence that crabs in these riverine areas have a higher risk of developing shell disease is also suggested by our preliminary studies where we held crabs in tanks exposed to either water from Bogue Sound or the Pamlico River. The former remained clinically normal, while crabs exposed to Pamlico River water developed shell disease lesions that have been seen in naturally affected crabs from that area.

The results of these studies suggest that there are significant physiological/immunological changes between crabs in different areas of the A/P Estuary. Further studies should be performed to determine whether these changes are due to natural environmental effects or are instead due to anthropogenic factors. The results of these studies may allow the development of a sensitive and ecologically relevant indicator for assessing the health of crustacean fishery populations and might be useful in a broader sense for assessing the general health of these aquatic ecosystems.

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SUMMARY AND CONCLUSIONS

The major conclusions from this study are as follows:

1) Shell disease lesions in blue crabs from the Pamlico River have many bacteria that possess enzymatic activities, including lipase and chitinase, that are potentially capable of degrading the shell.

2) The shell of clinically normal blue crabs from Core Sound also has large numbers of bacteria that have similar enzymatic properties.

3) Blue crabs possess a potent antibacterial activity in their hemolymph which can kill a wide range of the flora both from the shell of clinically healthy blue crabs as well as bacteria isolated from shell disease lesions.

4) The blue crab antibacterial activity is bactericidal, pH and temperature-sensitive, and inhibited by sodium chloride. It is also very stable after multiple freeze-thaw cycles and after prolonged storage at -70^{o}C, making it easy to collect and archive for assay.

5) There are marked differences in the immunocompetence of individual blue crabs in the A/P Estuary, as indicated by levels of antibacterial activity:

   a) Blue crabs with shell disease have significantly lower levels of antibacterial activity than clinically normal blue crabs.

   b) Blue crabs in the riverine parts of the A/P Estuary have significantly lower antibacterial activity than do blue crabs from the more oceanic parts of the system.

6) Blue crabs in the Pamlico River have significantly lower levels of hemocyanin than do crabs from the more oceanic reference site. The cause of the depressed hemocyanin levels is unknown.

7) There is no difference in hemocyanin levels between clinically normal blue crabs and those having shell disease. However, since Pamlico River crabs had lower hemocyanin levels that crabs from oceanic sites, the relationship of shell disease development to hemocyanin levels is uncertain.

8) Copper, cadmium or zinc levels are not important factors in the prevalence of shell disease in the blue crabs of the A/P Estuary.

9) The aggregate results of these studies suggest that there is a definite physiological/immunological difference between blue crabs in the more riverine parts of the A/P Estuary compared to more oceanic sites, which may account for the high prevalence of disease in the more riverine areas.

RECOMMENDATIONS

These studies have pointed to some potentially important and useful physiological changes in the blue crab populations of the A/P Estuary that may help to explain some of the deleterious changes that are believed to be occurring in this ecosystem. Monitoring studies should continue to collect information on the antibacterial activity and hemocyanin levels in blue crab populations in various parts of the A/P Estuary in order to substantiate the site-related immunological and physiological differences that have been identified in the present study.

In order to definitively determine the importance of man-made changes to these effects, controlled studies need to be performed to ascertain the mechanism(s) responsible for the reduced antibacterial activity and hemocyanin levels in crabs from the riverine areas of the Albemarle-Pamlico Estuary. This should include exposure of clinically healthy blue crabs from a single, control site (e.g., Core Sound) to water from riverine and oceanic areas, in order to experimentally confirm our findings that crabs exposed to riverine water are more likely to have lower immunocompetence and increased prevalence of shell disease. The importance of salinity per se as an influence on immunocompetence should also be examined and the identification of low salinity sites that are at low risk for shell disease should be included if possible.

In a complimentary study, blue crabs from the Pamlico River should be placed into tanks at different geographic sites to determine the reversibility of the changes associated with exposure to the high-risk-of-shell-disease environments.

Special emphasis should also be placed on nutritional status, since inadequate nutrition has been associated with both shell disease and depressed hemocyanin levels in other crustaceans. Conditions that may lead to poor nutrition (hypoxia or other adverse water quality factors, decreased prey abundance, etc.) should also be examined.

Once putative water quality factors have been identified that are associated with an increased risk of shell disease, controlled experimental studies should be performed to verify the importance of these factors to blue crab health. With such knowledge, management decisions about regulating the input of various substances into the Albemarle-Pamlico Estuary might then be made.

INTRODUCTION

The deterioration of water quality in coastal North Carolina has been documented with a number of physico-chemical and biological methods. One of the most obvious and publicly visible aspects of this degradation is the increasing prevalence of disease in aquatic organisms, especially those associated with dermatological (skin) lesions (i.e., ulcerative mycosis, lernaeosis, etc.). They affect many important fishery populations, such as menhaden, flounder, bass and sea trout (Noga 1986, Noga and Dykstra 1986) and there is increasing evidence that a number of these diseases have reached epidemic proportions in the Albemarle-Pamlico estuary.

While lesions on finfish have been most intensively studied, problems with shellfish have also been recently reported. In June 1987, fishermen in the Pamlico River began to report large numbers of blue crabs (Callinectes sapidus) having lesions on their carapace (McKenna et al 1988). Examination of these lesions by staff of the NCSU College of Veterinary Medicine revealed a diagnosis of shell disease. During the course of the outbreak, up to 90% of crabs in crab pots were affected (S. McKenna, personal communication). Since crabs with significant amounts of shell disease are unsalable, this has caused considerable concern among commercial fishermen (J. Hawkins, personal communication).

Shell disease (also known as rust disease, black spot, or brown spot) is a common syndrome in both freshwater and marine decapod crustaceans (Johnson 1983). It is considered an infectious disease and a number of pathogens have been reported from the lesions. The most commonly isolated pathogens are chitinoclastic bacteria, belonging to the genera Vibrio and Pseudomonas (Johnson 1983). Vibrio and Pseudomonas were among the pathogens isolated from shell disease lesions in A/P Estuary blue crabs (McKenna et al 1988). However, other agents including myxobacteria, psychrophilic luminescent bacteria, and fungi have also been associated with some cases, placing the true cause of this syndrome in question.

Shell disease has been reported in many natural populations of crustaceans (Sindermann 1977), but the prevalence has usually been very low. However, stressful environments increase the risk of disease. Thus, captive or cultured populations (Sindermann 1977) or those in polluted environments, such as sewage dump sites (Young and Pearce 1975), often exibit a high prevalence. Crustacean populations having shell disease can suffer considerable mortalities. Fisher et al (1976) found a mortality rate of 71% due to shell disease in cultured lobsters compared to only 6% in unaffected animals. Thus, shell disease might reduce the productivity of crustacean populations. Chronic presence of this disease has been a possible factor contributing to the

significant, progressive decline in the blue crab landings in the Pamlico River since 1984 (Table 1).

While the blue crab landings have declined in the Pamlico River since 1984, they have fluctuated in Pamlico Sound and have returned to the 1984 level. This suggests that conditions affecting blue crab survival have deteriorated in the Pamlico River since 1984. (This is assuming that no recruitment occurs between sites, which at present is uncertain). It is interesting to note that the recent major finfish epidemics have also occurred primarily in the Pamlico River, not Pamlico Sound (J. Hawkins, personal communication).

Shell disease of blue crabs fits a pattern which is similar to the dermatological syndromes which have been diagnosed in many finfish species of the A/P Estuary. The similarities are:

1) It is an infectious disease which is associated with a number of different pathogens which can cause a similar clinical syndrome.

2) The pathogens associated with the lesions are endemic to estuarine waters and thus are considered to be opportunistic agents.

3) A syndrome of similar appearance has been described in a number of other crustaceans, again suggesting the opportunistic nature of the pathogens involved.

4) The disease is typically associated with stressful environmental conditions.

While a number of infectious agents have been isolated from shell disease lesions, the underlying cause of the disease has been a mystery. It is generally felt that some form of damage to the surface of the carapace (i.e., the epicuticle) is needed for development of the disease. This is believed to allow the colonization of chitinoclastic bacteria which can feed on the deeper layers of the shell. However, this hypothesis does not explain several aspects of the pathogenesis of this disease:

First, most crabs do not develop severe shell disease from simply the minor mechanical cuticular damage associated with normal daily activity; second, the bacteria associated with shell disease are common and ubiquitous (Johnson 1983, Kaneko and Colwell 1975), making the apparent risk of infection very high; third, shell disease is difficult to reproduce experimentally.

Bacteria isolated from shell disease lesions often have lipase or chitinase enzymes (Cipriani et al 1980, Malloy 1978). Lipolytic bacteria have been suggested as the cause of initial damage to the lipid-rich epicuticle, subsequently exposing the

Table 1. Total number of pounds and value of blue crabs landed from the Pamlico River and Pamlico Sound, N.C. (1982-1989). Unpublished data from National Marine Fisheries Service, Division of Fishery Statistics. (Data in millions)

	YEAR					
	1982	1983	1984	1985	1986	1987
Pamlico River						
Pounds	3.4	3.8	5.1	3.5	2.2	1.7
Value	$0.63	$0.9	$1.1	$0.62	$0.65	$0.4
Pamlico Sound						
Pounds	18.3	15.0	14.4	11.6	7.8	14.5
Value	$3.4	$3.7	$2.8	$2.4	$1.8	$3.3

	YEAR	
	1988	1989
Pamlico River		
Pounds	2.5	1.8
Value	$0.60	$0.5
Pamlico Sound		
Pounds	17.1	15.9
Value	$5.2	$3.9

chitin-rich procuticle to chitinoclastic bacteria (Cipriani et al 1980). Yet again, the question remains as to why these bacteria rarely attack crabs in healthy environments. Bacteria are common epibionts of crustaceans. Yet in natural or unstressed environments, they cause little or no harm (Johnson 1983).

Shell disease is considered contagious (Johnson 1983) and normal animals placed in impoundments with diseased ones are likely to acquire the condition. Yet most attempts to transmit the disease, other than placing normal animals with diseased ones, have had equivocal results or have met with failure (Johnson 1983). The few successful experimental infections have usually required abrading the cuticle in combination with the

inoculation of the lesion with large numbers of bacteria (Cipriani et al 1980, Malloy 1978). At least 3 genera of bacteria can produce presumptive shell disease using this technique (Cipriani et al 1980). This challenge with large numbers of bacteria, which may overwhelm normal host protective mechanisms, makes the true meaning of such results open to question.

We felt that these observations strongly suggested that it is the environment and not the presence of bacteria per se, that is responsible for the induction and development of shell disease. We hypothesized that it is not simply the presence of bacteria but the presence of some immunosuppressive event that compromises the crab's ability to control its endemic microflora. Further, we hypothesized that potent antibacterial compounds must be present in normal crabs that keep the normal bacterial flora in check, and that disease induction is a function of reduced antibacterial activity in crabs exposed to stressful environments.

The blue crab exoskeleton (cuticle) consists of three major layers: the epicuticle, the procuticle, and the epidermis (Stevenson 1985). The thin outer layer, the epicuticle, is characterized by the absence of chitin. It is principally composed of protein, lipid, and calcium salts. Beneath it is the thick procuticle, which is composed primarily of chitin, protein, and calcium salts. These two layers are secreted by an underlying epidermis, usually one cell layer thick.

One feature of the crustacean cuticle which differentiates it from the body surface of vertebrates is the acellular nature of the epicuticle and procuticle. This has important ramifications for the defense of the cuticle since it implies that humoral immunity (i.e., antimicrobial chemicals), not cell-mediated immunity, is what protects the cuticle against microbial invasion. While intact immune cells are not found within the cuticle, fine cytoplasmic extensions of the epidermal cells extend vertically through the cuticle in pore canals (Halcrow 1978). In addition, ducts of the tegumental glands also extend to the surface of the cuticle. The tegumental glands are situated just beneath the epidermis. These canals and ducts may serve as portals for supplying antimicrobial agents. Stagner and Redmond (1975) found that tegumental gland secretion of the horseshoe crab (Limulus polyphemus) could agglutinate red blood cells and algae, suggesting that it might also recognize pathogens. Agglutination, cytolysis, and related activities have been identified from a number of marine invertebrates, including crustaceans, but their role in fighting infection has not been precisely defined (Kinne 1980).

Invertebrates do not have a specific immune response (e.g., antibody) as found in the vertebrates, and thus cannot recognize

specific pathogens (Manning 1984). Instead, their defensive responses are based upon broad-spectrum antimicrobial mechanisms. These include physical barriers, such as a chemically resistant shell, and cellular responses, such as phagocytosis and encapsulation (Sparks 1985). There is increasing evidence that natural antibacterial compounds also provide an important defense in lower animals. Potent antibiotics have been recently isolated from insects (Van Hofsten et al 1985). These agents, called cecropins, are believed to be the primary antibacterial defense in certain insects (Steiner et al 1981). Other agents having potent antimicrobial activity have recently been isolated from the skin of aquatic frogs (Zasloff 1987). Non-specific antimicrobial activity has also been reported in many different species of fishes (Ingram 1980).

Crustacean cuticle has high concentrations of phenol oxidase, which converts phenol and similar compounds into the polymer, melanin (Bang 1983). This metabolic pathway may be important in defending against microbes (e.g., through the production of free-radicals). However, this response is probably not the only mechanism which crustaceans use for protection, since shell disease lesions are often highly pigmented with melanin (e.g., "black spot"), even though the lesions continue to enlarge (Johnson 1983, McKenna et al 1988).

Protection from microbial invasion of the cuticle requires that the normal bacterial flora on the external surface of the crab be kept in check; otherwise, pathogenic changes will occur. These changes are the result of chemical degradation by these potentially pathogenic bacteria. Protection could be conferred via production of bacteriotoxic (antibiotic) substances that kill or slow the growth of bacteria that attempt to enter the cuticle.

PURPOSE AND OBJECTIVES

1) Determine if any antibacterial substances were present in selected crab tissues.

2) Partially characterize antibacterial activity that may be present.

3) Determine if there were any quantitative differences in the protective capabilities of individual crabs.

4) Determine if selected toxicants were elevated in A/P Estuary crabs.

5) Reproduce shell disease in blue crabs.

PROCEDURES

A. TISSUES SAMPLED FOR ANTIBACTERIAL ACTIVITY

For the great majority of studies, we used hemolymph. We primarily tested hemolymph because it was easily and reproducibly collected for quantitative studies and may act as a reservoir for antimicrobial agents, which may then be transported into the carapace by epithelial cells or tegumental glands.

Blue crabs were collected from various sites in the Albemarle-Pamlico Estuary by commercial fishermen and N.C. Division of Marine Fisheries personnel (Figures 1,2). Crabs were sampled immediately on site by severing the 5th pereiopod at the merepodite, collecting the hemolymph in a sterile tube and allowing it to clot on ice. Samples were transported to the laboratory, homogenized to break up the clot and centrifuged at 50,000 x g for 20 min. The resulting supernatant was frozen at -70^{o}C for later testing for antimicrobial activity as described in part D (p. 21), or stored at 4^{o}C for analysis of hemocyanin content as described in part H (p. 24).

The entire dorsal cuticle (epicuticle, procuticle, and epidermis) of 6 soft crabs was harvested and pooled as one group. Soft crabs had been purchased from a commercial shedding operation near Beaufort and maintained in flowing seawater at the National Marine Fisheries Service Laboratory, Beaufort, for several days prior to sampling. Biochemical extraction was performed as described below (part F.4).

Digestive gland was also collected from individual blue crabs and frozen at -70^{o}C for later examination for metal content.

B. BACTERIAL CULTURES

Cultures were taken from a) clinically normal crabs collected from Core Sound, b) shell disease lesions of crabs from the Pamlico River and c) clinically normal carapace of group b) crabs. Samples a) and c) were taken from the right anterior quadrant of the dorsal carapace. Sites were sampled by scraping the carapace with a sterile plastic loop and applying the loop to a small area of a culture plate having trypticase soy agar with 5% defibrinated sheep blood. The inoculum was then spread on the plate using a sterile swab. Plates were incubated at room temperature and predominant colonies were picked after 24 hr incubation. Isolates were purified by restreaking three times and then identified using standard procedures (Krieg 1986).

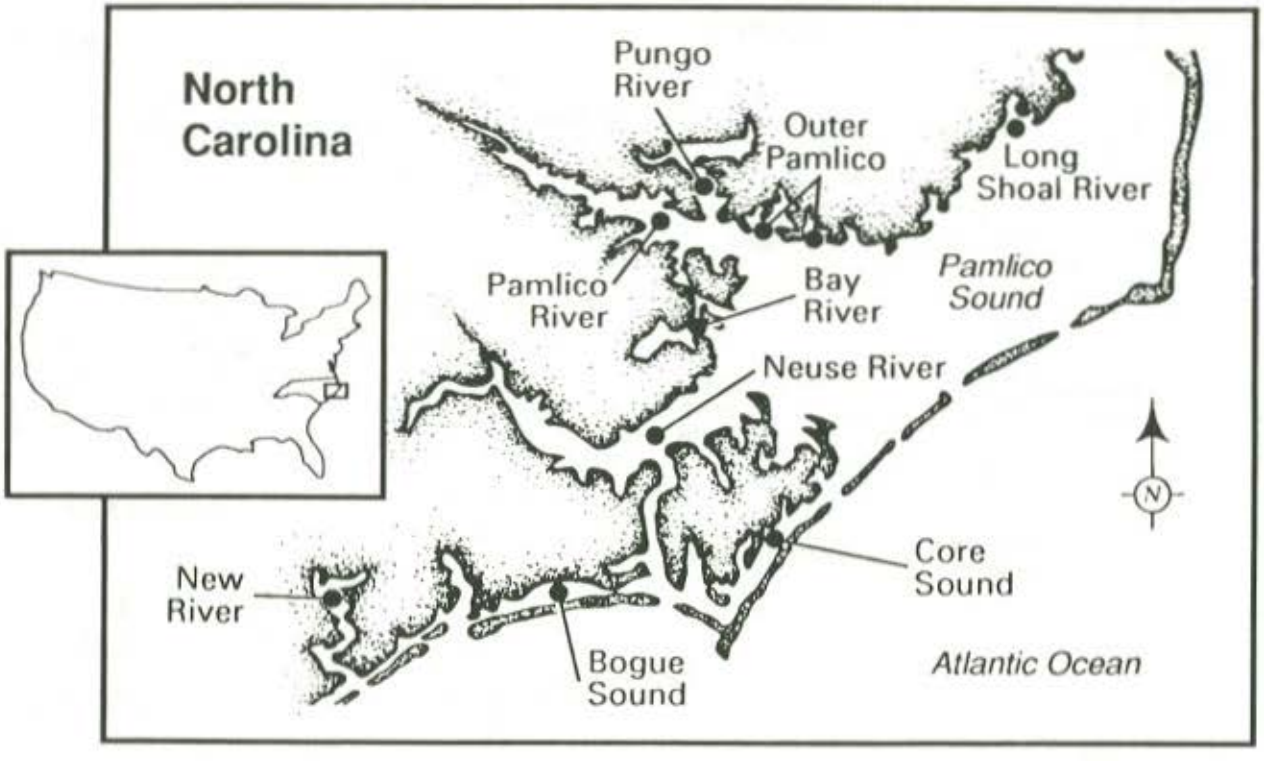

Figure 1. Sites in the Albemarle-Pamlico Estuary where blue crabs were collected in this study.

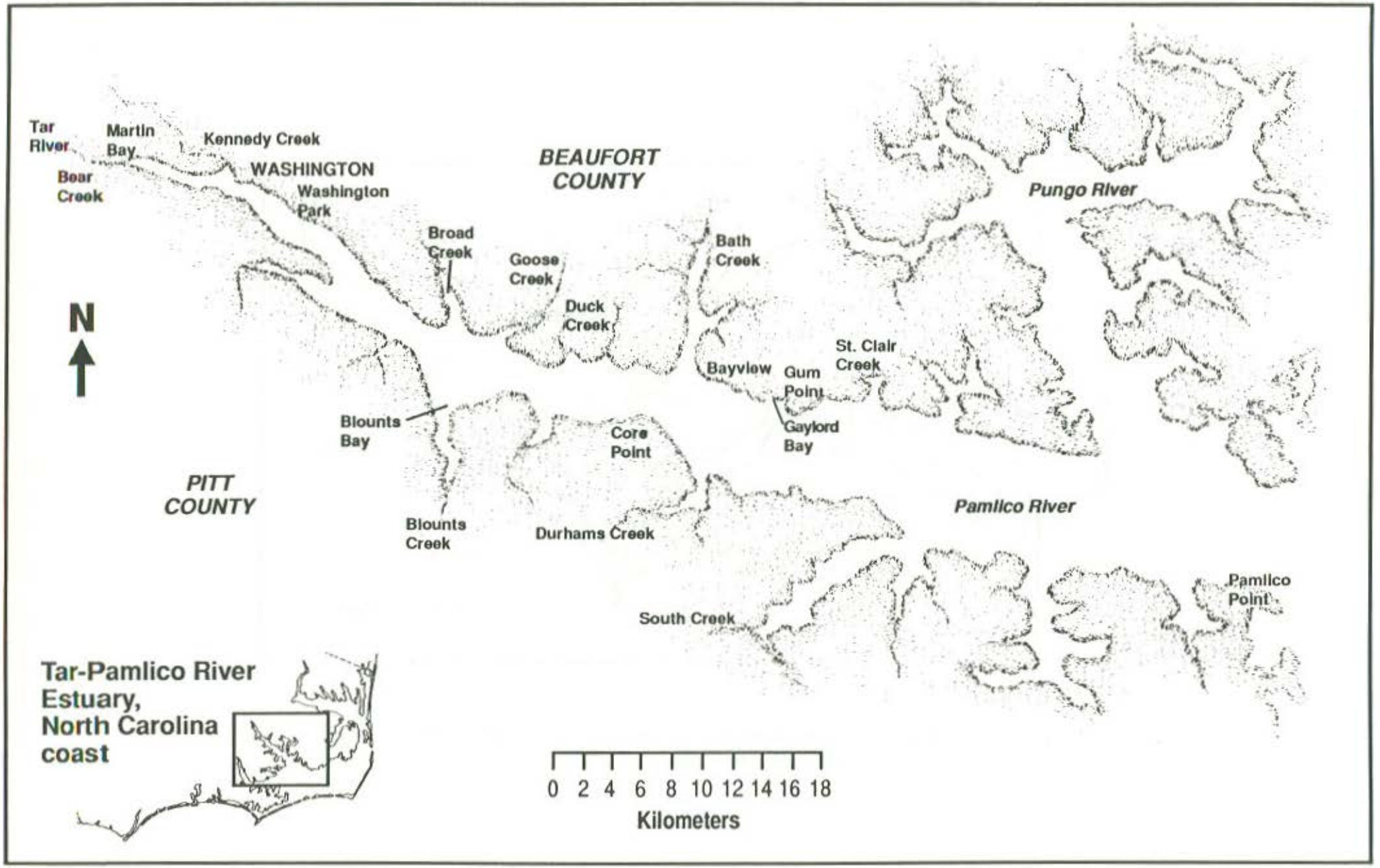

Figure 2. Sites in the Pamlico River where blue crabs were collected in this study.

Other bacteria used in this study were obtained from the American Type Culture Collection (Rockville, MD), including Staphylococcus aureus (ATCC # 12598), Pseudomonas aeruginosa (ATCC # 19154), Micrococcus luteus (ATCC # 381) and Serratia marcescens (ATCC # 990). Escherichia coli D31 was obtained from Dr. M. Postlewaite, University of Oregon.

Stock cultures were maintained at -70°C in brain heart infusion broth having 10% glycerol. Working cultures were maintained at room temperature on trypticase soy agar with 1% NaCl.

C. ASSAY FOR ENZYMATIC ACTIVITY IN CRAB BACTERIAL ISOLATES

We assayed crab bacteria for anti-lipase activity by streaking isolates onto lipase agar plates (Colwell and Wiebe 1970) having 1% NaCl. Lipolytic activity of the bacteria produced a cloudiness in the agar; a rough estimation of lipase activity could be determined by the size of the cloudy zone around the colonies. Activity of samples was compared to a control culture of Pseudomonas aeruginosa (ATCC # 19154), known to be a strong producer of lipase.

We assayed bacteria for anti-chitinase activity by streaking isolates onto chitinase agar plates (Colwell and Wiebe 1970) having 1% NaCl. Chitinolytic activity of the bacteria produced a clearing of the agar; a rough estimation of chitinase activity could be determined by the size of the clear zone around the colonies. Activity of samples was compared to a control culture of Serratia marcescens (ATCC # 990), known to be a strong producer of chitinase.

D. ASSAYS FOR ANTIBACTERIAL ACTIVITY

D. 1. Spot Assays

Two assays were used to rapidly screen for antibacterial activity.

D. 1. a. Modified Zasloff assay

In the modified Zasloff assay (Zasloff 1987), bacteria were grown in LB broth (Boman et al 1974) to a concentration of about 10^9 colony forming units/ml. About 3×10^7 bacteria were added to 100 ml of 1.5% agarose in LB broth with phosphate buffer (ph 7.0) and 100 ug/ml streptomycin sulfate. About 20 ml was poured into a 150 mm Petri dish. Test samples were spotted onto this plate, which was then incubated for 24 hr. Growth inhibition was

indicated by the presence of a clear zone (no bacterial growth) in the spotted area.

D. 1 a. Streak assay

In the streak assay, we streaked various bacterial isolates onto a nutrient agar; we then spotted either 10 ul of sample (hemolymph or shell extract) or saline onto the streak and incubated the plate for 24-48 hr. Growth inhibition was indicated by the absence of bacterial growth at the site of sample spotting, with no growth inhibition where saline was spotted.

D. 2. Turbidometric assay

Quantitative differences in antibacterial activity were measured using a procedure modified from that of Anderson and Chain (1982) and Muschel and Treffers (1956). Bacteria were inoculated into trypticase soy broth + 1% NaCl (TSBB) at either 25° or 37°C. After 18-24 hr the bacterial suspension was washed with 0.10 M phosphate buffer, pH 7.0, with 1% NaCl (PBS) and then diluted to an OD_{570} of about 0.100. This suspension was then diluted 1:100 with PBS. Two ul of each test sample was added to 38 ul of PBS. Ten ul of the diluted bacterial suspension were then added and the tubes were incubated for 30 min at 25°C. Controls included tubes where PBS or or a known strongly active hemolymph was substituted for the test samples, and samples that had no bacteria added. The reaction was simultaneously stopped by placing all tubes in a 4°C water bath; 450 ul of cold (4°C) TSBB was then added to all tubes.

One hundred ul aliquots of each sample were added to quadruplicate wells of a 96-well tissue culture plate. The optical density of each well was recorded using an automated spectrophotometer when the optical density of the negative control cultures (having bacteria but no tissue sample added) reached an optical density at 570 nm (OD_{570}) of 0.090 - 0.110.

D. 2. a. Comparison of individual crabs

Using the turbidometric assay, we determined if there was any relationship between antibacterial activity and presence/absence of shell disease and geographic location of the collected crabs.

E. IDENTIFICATION OF BACTERICIDAL ACTIVITY IN SAMPLES

To determine if the antibacterial activity could kill bacteria, we performed a plating assay as described by Anderson

and Chain (1982). Bacteria were inoculated into trypticase soy broth + 1% NaCl (TSBB) at either 25° or 37°C. After 18-24 hr the bacterial suspension was diluted to an OD_{570} of about 0.100. This suspension was then diluted 1:100 with TSBB. Various amounts of hemolymph were added to 100 ul of TSBB and incubated for 30 min at 25°C. Controls included samples that were inoculated with phosphate buffered saline (PBS) or seawater (IO) instead of hemolymph, and samples that had no bacteria added. The reaction was then simultaneously stopped by placing the tubes in a 4°C water bath and diluting them with 440 ul of cold (4°C) TSBB. One hundred ul of each sample was then spread onto triplicate plates of trypticase soy agar with 1% NaCl and incubated for 24 hr. Colonies were counted after 24 hr and the percentage inhibition determined.

F. BIOCHEMICAL CHARACTERIZATION OF ANTIBACTERIAL ACTIVITY

F. 1. Effect of pH on antibacterial activity

We tested the effect of pH on antibacterial activity by incubating a pooled hemolymph sample in 0.10 M phosphate buffer having a pH of 5.2, 5.5, 6.0, 6.5, 7.0, 7.5, or 8.0. Samples were incubated for 30 min and then diluted and cultured using the turbidometric assay as described previously.

F. 2. Effect of temperature on antibacterial activity

The effect of temperature on antibacterial activity was tested by heating a pooled hemolymph sample to 7.8, 25.8, 37.8, 40.8, 53, 56.6, 60, 65, or 70°C for 30 min and then immediately cooling the samples in an ice bath. Samples were then incubated with bacterial suspension for 30 min and then diluted and cultured using the turbidometric assay as described previously.

F. 3. Effect of salt concentration on antibacterial activity

The effect of salt concentration on antibacterial activity was tested by incubating various concentrations of a pooled hemolymph sample in 32 ppt artificial seawater (about 3.2% NaCl) or in phosphate buffer having 1% NaCl, 2% NaCl, 3% NaCl. Samples were incubated for 30 min and then diluted and cultured using the turbidometric assay as described previously.

F. 4. Extraction of agents from shell

Soft shell cuticle samples from six crabs were frozen individually and then cut into small pieces while frozen. Pieces were placed into 10 mM Tris buffer, pH 7.5, at 0°C and homogenized using a Brinkman Polytron tissue homogenizer. The homogenate was centrifuged at 105,000 x g for 60 min. The

supernatant was filtered through a glass fiber filter and individual aliquots were frozen at -70°C for later testing for antibacterial activity using both the modified Zasloff assay and the turbidometric assay.

F. 5. Preliminary determination of molecular weight of antibacterial activity

Fractionation of blue crab hemolymph was done using a low pressure gel permeation column using Sephadex G-75 (Pharmacia). The column was calibrated by using known molecular weight standards (blue dextran [> 10^6 daltons]; bovine serum albumin [67K daltons]; ovalbumin [43K daltons]; chymotrypsinogen [25K daltons]; and ribonuclase A [13.7K daltons]). Column eluants were monitored continuously at 280 nm, and antibacterial activity was determined for each fraction using a spot assay.

Hemolymph samples were also run on native polyacrylamide gels using the procedure of Gabriel (1971). Gels were tested for the location of antibacterial activity using "bug blots" (Robertson and Postlethwait 1986).

G. TRACE METAL MEASUREMENTS

Cadmium, copper and zinc concentrations were measured in the digestive glands of blue crabs captured in two reference sites (Core and Bogue Sounds) and in the Pamlico River. Metal analyses were done using standard wet ashing techniques and atomic absorption spectrophotometry (Engel and Brouwer 1984a). The National Bureau of Standards (NBS) Oyster Reference Material #1566 was used to verify our preparative procedures and analytical measurements of cadmium, copper, and zinc. Our measurements fell within the 95% confidence intervals given for the three elements by NBS.

H. DETERMINATION OF HEMOCYANIN CONCENTRATIONS

As a part of the procedure to collect hemolymph for the determination of antibacterial activity, it was noticed that the color (i.e., the characteristic blue color of oxygenated hemocyanin) was less distinct. Thus, we decided to also examine the hemocyanin levels of crabs from the Pamlico River. Hemolymph hemocyanin concentration was measured spectrophotometrically; after diluting samples with buffer, optical density readings were taken at 280 and 335 nm for each sample. These readings were compared to standards and the hemocyanin concentration was calculated. Hemocyanin measurements were made by Dr. Maurius Brouer of the Duke University Marine Laboratory at Beaufort, N.C.

I. EXPERIMENTAL REPRODUCTION OF SHELL DISEASE IN BLUE CRABS: PRELIMINARY STUDY

Clinically healthy blue crabs were collected from crab pots placed in Core Sound and acclimated from ambient salinity (~30 ppt) to 7 ppt using a mixture of well water and estuarine water. After about 2 weeks, representative samples of crabs were assigned to two groups: One group (30 crabs) was scraped on the dorsal carapace and the other group (30 crabs) was not scraped. Half of each group (scraped and unscraped) was transported to the Pamlico Aquaculture Center, Aurora, NC and placed into separate 500 gallon round fiberglass tanks (15 crabs/tank) having flow-through, 6-8 ppt salinity river water. The remaining 30 crabs were kept at the National Marine Fisheries Service Beaufort, N.C. Laboratory in 7 ppt salinity, consisting of a mixture of estuarine water (~30 ppt) and well water (0 ppt). Crabs were fed every other day on fish or squid and observed for the development of shell disease lesions.

RESULTS

A. CLINICAL FEATURES OF SHELL DISEASE

Our initial observations and collections of blue crabs were focused on the Pamlico River. Affected blue crabs from this area usually had a very severe form of shell disease that was much more aggressive than that previously described in outbreaks in other geographic areas (Rosen 1967, Rosen 1970, Sandifer and Eldridge 1974). As had been previously described by McKenna et al (1988), lesions commonly affected the dorsum and the lateral aspects of the carapace. Lesions often were so aggressive that they extended into the soft tissues. In this report, we refer to these lesions as "Pamlico River shell disease" to distinguish it from the more benign shell disease lesions described by Rosen (1967). However, it should be noted that "Pamlico River shell disease" is not confined to the Pamlico River; we have also seen such lesions in blue crabs from the Alligator River, NC, the Houston ship channel, TX and Biscayne Bay, FL.

B. PATHOGENS ASSOCIATED WITH SHELL DISEASE LESIONS

Representative colonies from carapace cultures were identified using standard techniques. Members of the genera Vibrio, Aeromonas, Plesiomonas, Pseudomonas and Achromobacter were identified. Most agents had either lipase or chitinase activity and several had both activities.

C. IDENTIFICATION OF ANTIMICROBIAL ACTIVITY IN BLUE CRABS: BACTERIAL SUSCEPTIBILITY AND ANATOMICAL SITES OF ACTIVITY

Using the streak assay, we identified potent antibacterial activity, which was evident as an area with reduced bacterial growth compared to surrounding areas of the plate. Activity was detected in both hemolymph and soft shell carapace extracts. Most bacteria cultured from both clinically healthy crabs and those with shell disease were inhibited by hemolymph. All of these were gram-negative organisms.

Selected isolates were also tested with the turbidometric assay. There was a wide range of sensitivity between various isolates. None of the gram-negative or gram-positive reference bacteria (i.e., those not cultured from blue crabs) were susceptible to hemolymph, except E. coli. We found that Escherichia coli strain D31, which has been used as a standard pathogen for antimicrobial assays (Boman et al 1974), appeared to be the most sensitive of all bacteria tested. We also achieved the most reproducible results in the turbidometric assay using this bacterium; thus it was chosen as our test bacterium for quantitative comparisons of individual samples (see part E).

D. BIOCHEMICAL CHARACTERISTICS OF THE ANTIBACTERIAL ACTIVITY

The blue crab antibacterial activity is bactericidal (Figure 3), heat-labile, somewhat pH-dependent (Table 2) and inhibited by sodium chloride (Figure 4). Activity was also retained after at least 3 freeze-thaw cycles and remained stable after at least 12 months at -70^{o}C. Attempts were made to isolate and identify the antibacterial factor in the hemolymph. The activity was in the high molecular weight (>50K daltons) fractions of a gel filtration chromatography column. Electrophoretic separation on native gels suggested the presence of multiple components.

E. GROUP-SPECIFIC DIFFERENCES IN ANTIBACTERIAL ACTIVITY

While the spot assays allowed us to detect antibacterial activity in samples, we could not use this assay to assess quantitative differences between samples. For quantitative comparisons, we developed a modified turbidometric assay which was very reproducible and quantitative.

Over 350 crab hemolymph samples were examined, representing samples collected from crabs between July and November of 1988 and 1989. Our initial statistical analyses suggested that sex might have an influence on levels of antibacterial activity. Due

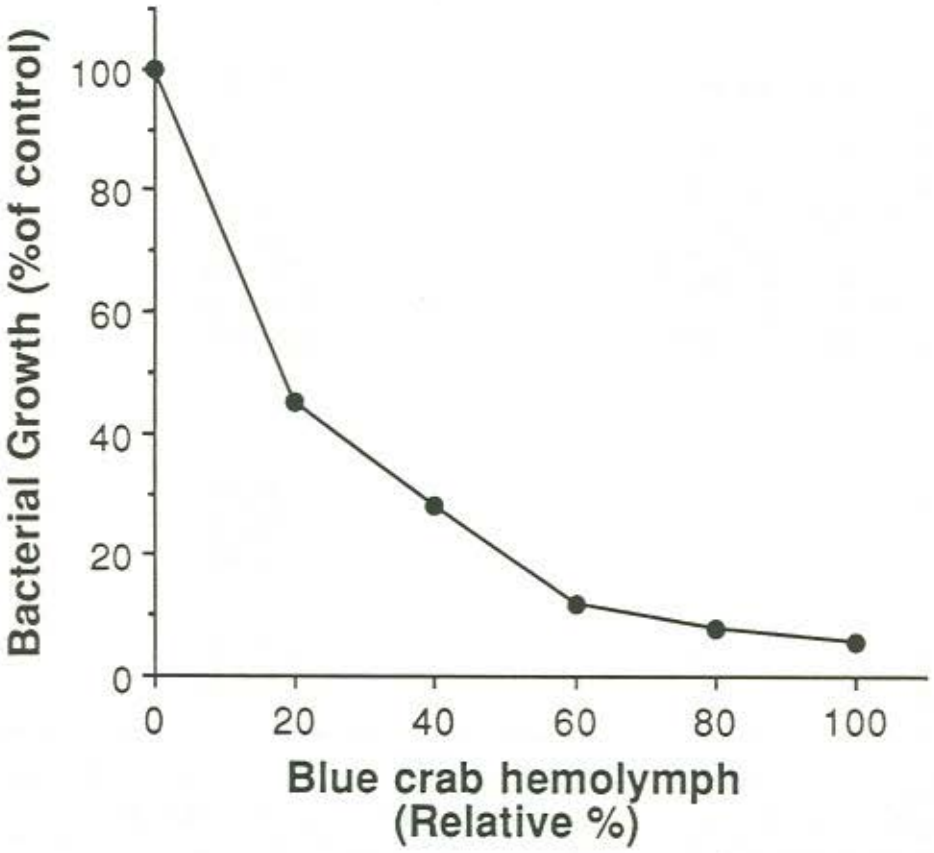

Figure 3. Effect of blue crab hemolymph on the survival of Escherichia coli D31 as measured by the plating assay. Two replicate experiment yielded similar results.

Table 2. Effect of heat and pH on antibacterial activity of blue crab hemolymph against Escherichia coli D31 as measured by the turbidometric assay.

Hemolymph Treatment	Activity (% of Negative Control)
Heat	
7.8°C for 30 min	1.0%
25.8°C for 30 min	0.2%
37.8°C for 30 min	2.9%
40.8°C for 30 min	1.9%
53.0°C for 30 min	35.0%
56.6°C for 30 min	58.0%
60.0°C for 30 min	79.0%
65.0°C for 30 min	105.0%
70.0°C for 30 min	106.0%
pH	
5.2	0.9%
5.5	0.9%
6.0	0.9%
6.5	2.7%
7.0	5.3%
7.5	12.9%
8.0	19.0%

to the relatively smaller number of females collected, all analyses were performed using intermolt (stages C1 to D1) males. For comparison of different geographic sites, we only used crabs without shell disease (see below).

Due to the nature of the sample collection, no specific sample run order was employed, which necessitated the use of controls to correct for run-to run variation. The positive and negative control variables were used as covariates in a two way ANOVA, which was run in a SAS linear models program (SAS Institute). The effects of geographical location, date of collection, presence versus absence of shell disease, as well as the variability of the positive and negative controls, was examined. When raw data was used in this analysis, there was an increase in the difference between the actual and predicted values as the relative optical density values increased, indicating that the model was a poor fit. This prompted us to perform a natural log transformation of all data points in order to correct for the observed multiplicative variation. In order

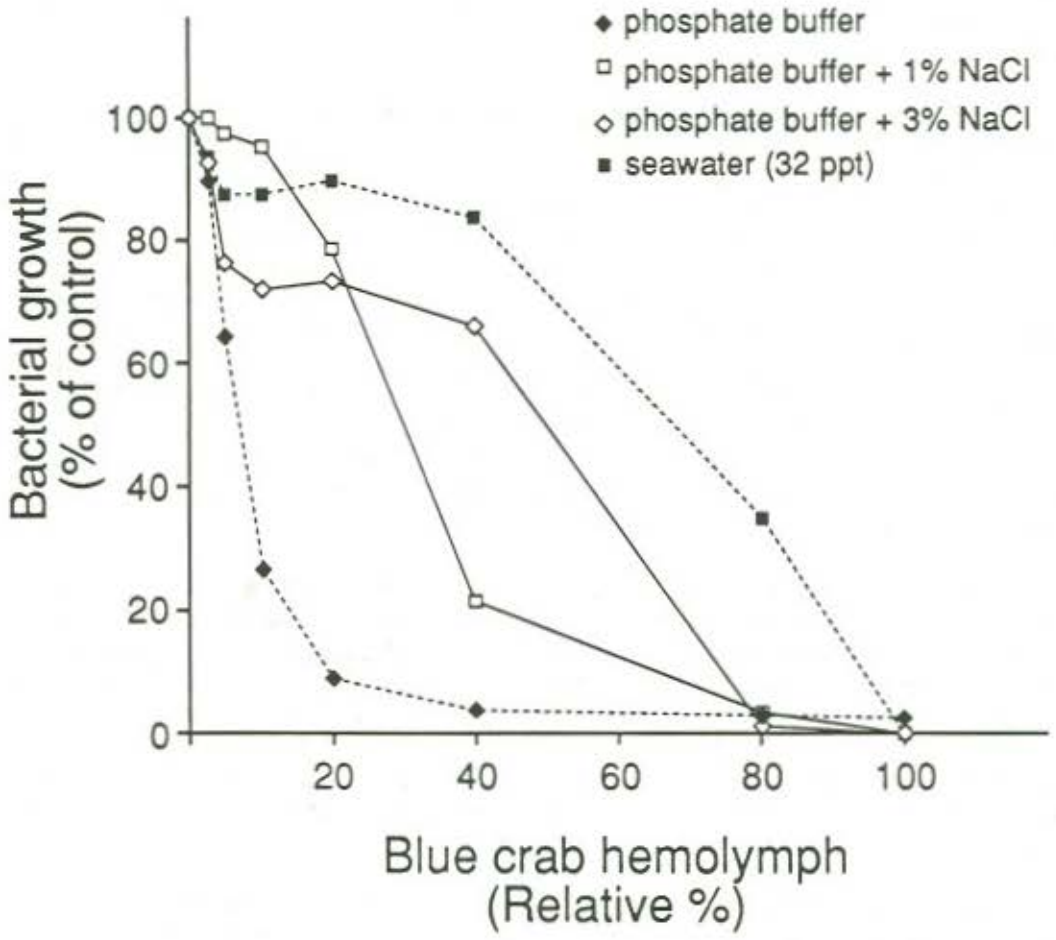

Figure 4. Effect of sodium chloride on the antibacterial activity of blue crab hemolymph against Escherichia coli D31 as measured by the turbidometric assay. Two replicate experiments yielded similar results.

to log-convert the data, all zero values had to be increased by a small factor. The model was run using a range of small correction factors added to the values, as well as to the zero values, in order to identify any significant skewing of the data. The result was that small values between 0.0001 and 0.001 did not significantly affect the data set; thus, a factor of 0.0005 was added to all values.

The transformed data was analyzed using the same model as previously mentioned. The results were that the control variables did not have any significant effect on the model, indicating that the assay and specifically the positive and negative controls were very consistent. There was also no significant effect of sample collection date, which prompted us to remove the control covariant and the sample collection date from the model. Least significant difference t-tests were calculated from all model outputs, as well as least square means and standard error. The data outputs were exponentiated in order to return the values back to the original scale. The exponentiated values were shifted lower due to the nature of the transformation, but they serve as an acceptable estimate of the predicted values on the original scale.

Our analysis indicated that there was a significantly lower level of antibacterial activity in blue crabs collected from the Pungo River than at any other site (Figure 5). There was also significantly lower activity in crabs collected from the Pamlico River, Neuse River and Bay River, compared to either Bogue Sound or Core Sound. Crabs collected from Bogue Sound and Core Sound had the highest activity, with a mean antibacterial activity that was from 250% to over 500% higher than crabs from the Pamlico or Pungo Rivers.

We also compared crabs having shell disease to those without shell disease. Due to the marked differences in antibacterial activity at different geographic locations, we only compared crabs collected in the Pamlico River. There was a significantly lower level of antibacterial activity in crabs having shell disease (Figure 6).

F. HEMOCYANIN CONCENTRATION IN BLUE CRABS FROM THE PAMLICO RIVER

The six month averages for hemolymph hemocyanin concentrations in blue crabs from a number of sites in the Pamlico River were significantly lower ($p < 0.05$) than the control/reference site (Figure 7), as indicated by two way ANOVA. Hemocyanin concentrations in crabs having shell disease were similar to levels in clinically normal blue crabs (Figures 8,9; t-test for significance, $p > 0.05$).

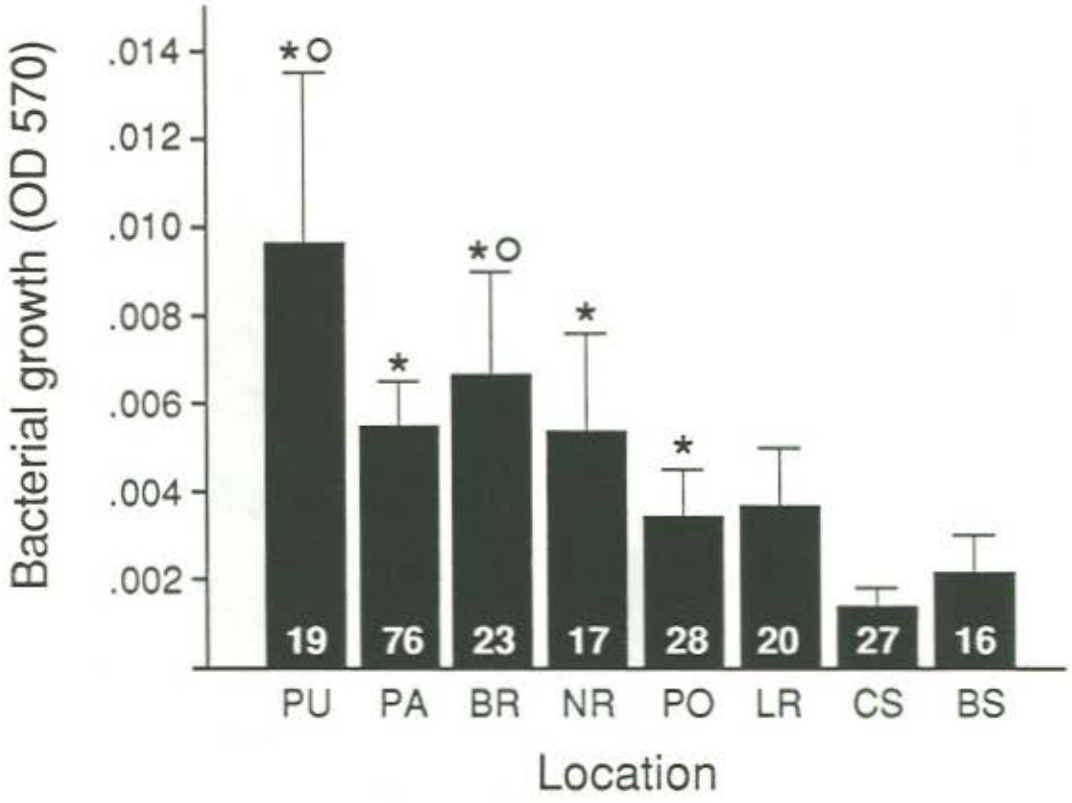

Figure 5. Antibacterial activity of intermolt, clinically normal, male blue crabs collected from different geographic locations in the Albemarle-Pamlico Estuary. Values are expressed as the concentration of bacteria present after exposure to hemolymph followed by a standardized incubation period in culture medium. Thus, the lowest bacterial growth indicates the highest antibacterial activity. BR = Bay River, BS = Bogue Sound, CS = Core Sound, LR = Long Shoal River, NR = Neuse River, PA = Pamlico River, PO = outer Pamlico River (includes Rose Bay and Swanquarter Bay), PU = Pungo River. Values are expressed as predicted least square means, as described in the text. Values with (*) are significantly different from mean activity of Core Sound crabs; values with (o) are significantly different from mean activity of Bogue Sound crabs ($t = 1.96927$, $p < 0.05$). No other values are significantly different from each other. Ranking of sites is from lowest (Pungo River) to highest (Bogue Sound) mean salinity based upon the hydrographic data of Wood (1967). Number inside the bar = # crabs sampled

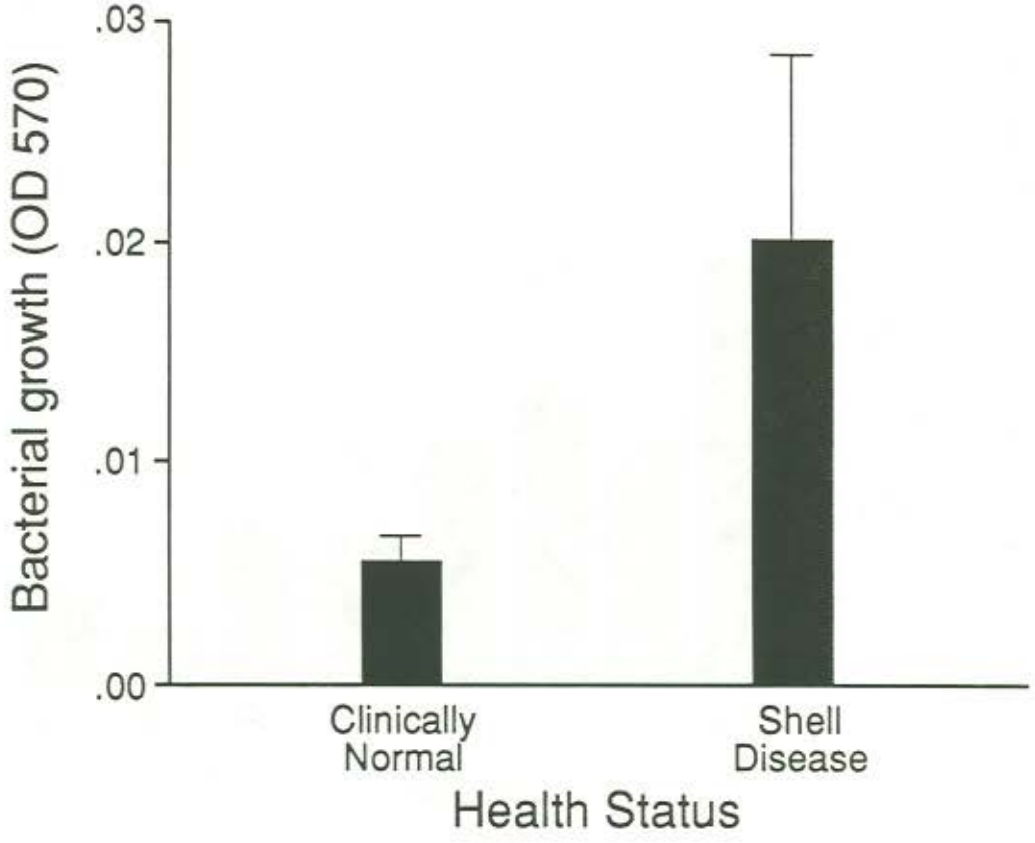

Figure 6. Antibacterial activity of intermolt male blue crabs that were either clinically normal or had clinical signs of "Pamlico River shell disease". Values are expressed as the concentration of bacteria present after exposure to hemolymph followed by a standardized incubation period in culture medium. Thus, the lowest bacterial growth indicates the highest antibacterial activity. Values are expressed as predicted least square means, as described in the text. Clinically normal crabs had significantly ($t = 1.96927$, $p < 0.05$) greater antibacterial activity.

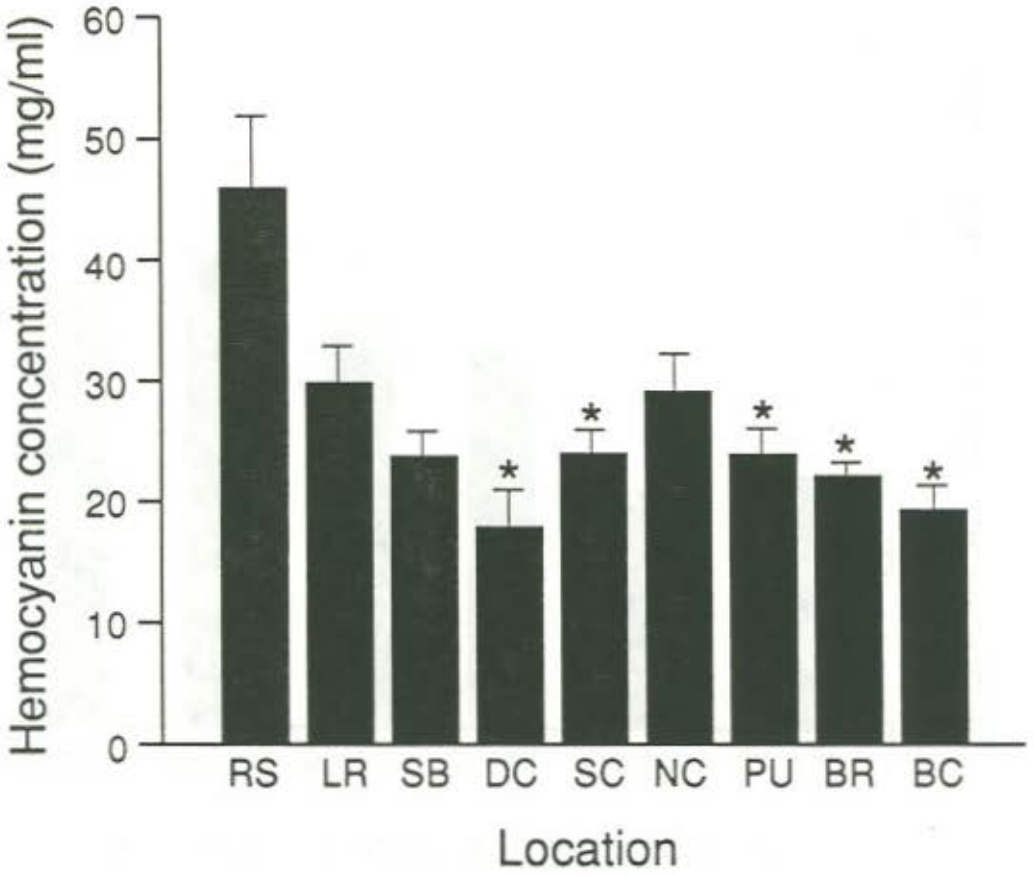

Figure 7. Average hemocyanin concentrations (± SE) measured in blue crabs from reference and various other Albemarle-Pamlico Estuary locations during the entire sampling period (June 1988- November 1989). Bars with an (*) indicate sites that are significantly different from the reference site. RS = Reference site (Core Sound), LR = Long Shoal River, SB = Swanquarter Bay, DC = Durham Creek, SC = South Creek, NC = North Creek, PU = Pungo River, BR = Bay River, BC = Broad Creek. (Samples were collected by the N.C. Division of Marine Fisheries and were not funded by the A/P Study).

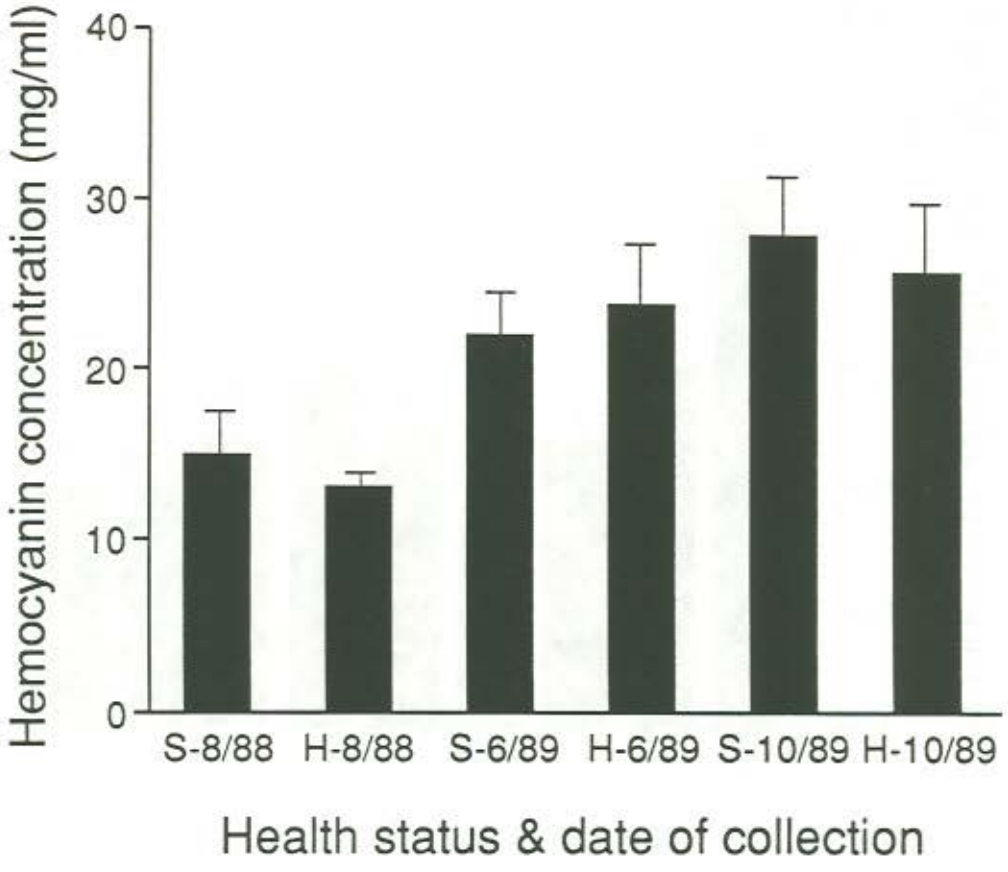

Figure 8. Average hemocyanin concentrations (± SE) measured in clinically normal blue crabs (H) and those with "Pamlico River shell disease" (S) collected from the Pamlico River during various sampling periods (June 1988- November 1989).

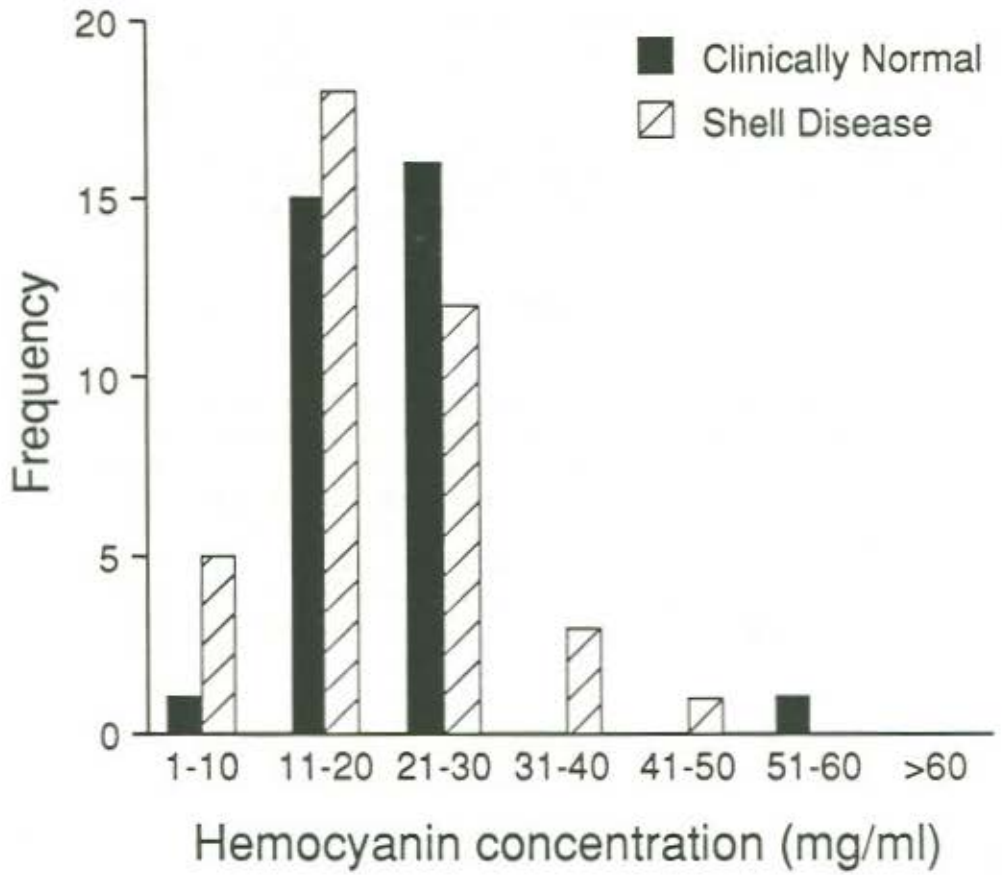

Figure 9. Frequency distribution of hemocyanin concentrations in the hemolymph of clinically normal blue crabs and those with "Pamlico River shell disease" collected from the Pamlico River during the entire sampling period of June 1988-November 1989.

G. TRACE METAL CONCENTRATIONS IN BLUE CRABS FROM THE PAMLICO RIVER

Cadmium concentrations of crabs collected from the Pamlico River were the same as crabs from the Bogue and Core Sound reference locations (Table 3). Copper and zinc were significantly lower ($p < 0.05$) in crabs collected from the Pamlico River than those from the reference sites. Metal measurements on Pamlico River crabs include both clinically normal and shell-diseased animals because there was no significant difference between these two groups.

H. DEVELOPMENT OF SHELL DISEASE IN NORMAL, HEALTHY BLUE CRABS

Blue crabs exposed to water from the Pamlico River developed shell disease lesions. These lesions included the deep, necrotic holes that characterize shell disease in blue crabs from the Pamlico River, but also included lesions that were more characteristic of classical shell disease as described by Rosen (1970). Blue crabs maintained in similar systems at Beaufort had no such lesions. There was no evidence that scraping the carapace increased the risk of shell disease in any animals.

DISCUSSION

A. CLINICAL FEATURES OF SHELL DISEASE IN THE A/P ESTUARY

Classical shell disease, as described by Rosen (1970), typically affects primarily the ventral aspect of the carapace, causing a darkening and/or shallow pitting of the shell. Deep lesions such as we have commonly seen in Pamlico River crabs, have not been commonly reported previously. However, during our investigations, we also observed crabs with similar aggressive lesions in the Alligator River, NC, the Houston Ship Channel, TX and Biscayne Bay, FL. The reason blue crabs in the Pamlico River develop this type of shell disease is unknown.

B. RELATIONSHIP OF ANTIBACTERIAL ACTIVITY TO PATHOGENIC POTENTIAL OF CRAB MICROFLORA

Bacteria which we isolated from both healthy and sick blue crabs include agents which have been commonly reported from shell disease lesions (Cipriani et al 1980, Cook and Lofton 1973,

Table 3. Cadmium, copper, and zinc concentrations in digestive glands of blue crabs collected from the Pamlico River, Beaufort area, and Tampa Bay. (All measurements reported as mg/kg wet weight of tissue ± SE)

LOCATION	N	CADMIUM UNITS (mg/kg)	COPPER UNITS (mg/kg)	ZINC UNITS (mg/kg)
Pamlico River, NC	35	2 ± 0.2	5 ± 0.3	30 ± 3
Beaufort, NC (Core and Bogue Sounds)	28	2 ± 0.3	20 ± 2	43 ± 4
Tampa Bay, FL				
N. Hillsborough Bay	22	7 ± 0.6	165 ± 28	46 ± 4
S. Hillsborough Bay	21	8 ± 0.6	90 ± 15	37 ± 3
Old Tampa Bay	10	6 ± 1.0	89 ± 19	43 ± 5
Gadsen Point	25	9 ± 0.8	50 ± 10	49 ± 3

N = number of crabs sampled

The Tampa Bay crabs were collected as part of the NOAA Status and Trends Biological Effects Survey in 1989. This data is presented for comparative purposes (see Discussion).

McKenna et al 1988). Many of these agents are also common in estuarine waters (Krieg 1986) and thus do not appear to be obligate pathogens. All of the bacteria which we isolated from shells of both normal blue crabs and those with shell disease had either chitinase or lipase enzymes and several had both activities, indicating that all isolates possessed biochemical markers that have been traditionally associated with shell disease pathogens (Cipriani et al 1980, Cook and Lofton 1973). In addition, most of these bacteria were susceptible to the antibacterial activity present in hemolymph.

The demonstration of antibacterial activity in the hemolymph suggests that this may be an important defensive mechanism in protecting crabs against their endogenous flora. Our demonstration of activity in shell extracts also suggests a

primary defensive role for this activity in protecting against shell disease. In summary, the importance of the antibacterial activity which we describe in this report may be substantial, since it appears to have a broad-spectrum of activity against many potential pathogens which colonize the surface of the crab shell.

C. CHARACTERISTICS OF THE ANTIBACTERIAL ACTIVITY

This is the first report of natural antimicrobial activity in the blue crab. Our data suggests that the biological activity present is bactericidal (i.e., it kills bacteria and does not simply stop their growth) and is directed at gram-negative organisms. Its high degree of activity against E. coli D31, also suggests that it may be a membrane-active agent, since this bacterium is especially sensitive to membrane active-antibiotics (Boman et al 1974). The resistance of Micrococcus luteus suggests that the blue crab antibacterial activity probably does not include lysozyme, since this organism is highly susceptible to such activity.

As demonstrated in our attempts to isolate the active substance(s) from the hemolymph, the molecular weight of the antibacterial activity is in the same range as the hemocyanin subunit, 70K daltons. Because of the separation techniques that were used, small differences in molecular weight (< 50K daltons) could not be resolved. The active component may either have a similar molecular weight to hemocyanin or may be bound to hemocyanin.

Our polyacrylamide gel electrophoresis studies suggest that there may be more than one substance present. Some insects (Boman et al 1974, Chadwick and Dunphy 1986) have several classes of antibacterial factors. The blue crab antibacterial factor shares some features with the blue crab agglutinin, including resistance to multiple freeze-thaw cycles and susceptibility to heating to 50^{o}C (Pauley 1974). Determination of whether these two activities are due to the same agent must await further studies.

While humoral antibacterial activity has been described in other invertebrates, the great majority of research has been performed in insects (Chadwick and Dunphy 1986). Relatively few studies have described such activity in marine invertebrates. Among those species where antibacterial activity has been described include the sipunculid worm Dendrostomun zostericolum (Johnson and Chapman 1970a), the tunicate Ciola intestinalis (Johnson and Chapman 1970b), the sea hare Aplysia californica (Johnson and Chapman 1970b); the American lobster Homarus americanus (Acton et al 1969), the horseshoe crab Limulus

polyphemus (Furman and Pistole 1976), the spiny lobster Panulirus argus (Evans et al 1968), and a polychaete worm Glycera dibranchiata (Anderson and Chain 1982). Most of the agents described have exhibited at least some activity against bacterial flora isolated either from the host organism or its natural environment. But in some cases, the greatest activity was seen against organisms not normally encountered by the invertebrate under study.

D. DIFFERENCES IN IMMUNOCOMPETENCE AMONG DIFFERENT GROUPS OF BLUE CRABS

For quantitative comparisons of antibacterial activity among various samples, we had initially intended to use a diffusion assay for measuring activity, as described by Boman et al (1974). However, we were unable to obtain reproducible results with this assay. We subsequently used an assay that had been developed by Muschel and Treffers (1956) and had been modified by Anderson and Chain (1982). Our primary modification of this assay was the development of a replicate plating method for rapidly measuring the optical density of small samples. The advantages of our modified assay were that first, it required a much smaller volume of sample for testing (as little as 2 ul, while the other assays mentioned often required at least 50 ul) and second, many samples could be run simultaneously because the reading of optical density is performed automatically using an automated spectrophotometer. Because the optical reader can measure and record 96 samples in less than 2 minutes, the error associated with time involved in reading the samples is significantly reduced. Since the bacteria are growing very rapidly, even relatively short differences between when readings are taken for different samples can have an important influence on results. This was probably a major reason why we had such a low variation in our control samples and thus had higher reproducibility between tests when using the D31 bacterium in our assay.

Our studies initially were designed to compare the antibacterial activity of samples from clinically healthy crabs versus those with shell disease. However, during the course of this investigation, we decided to expand our studies to include a much larger number of individuals from not only the Pamlico River but from a number of other geographic locations in North Carolina.

We compared the antibacterial activity of crabs of different sexes, collected from different geographic areas, collected during different times and clinically normal crabs versus those with shell disease.

We found that crabs collected from the riverine areas of the

Albemarle-Pamlico Estuary had much lower levels of antibacterial activity than blue crabs collected from the more oceanic parts of the system. Although not proven statistically, the evidence suggests that there is a gradient of decreasing antibacterial activity as one moves from the oceanic to the riverine areas of the estuary (Figure 5). Interestingly, it is these riverine areas that have also been plagued by the prevalence of shell disease (McKenna et al 1988), as well as fish disease epidemics (Noga, submitted).

There are many environmental and biological factors which vary along this riverine-oceanic gradient, the most obvious being salinity. However, salinity changes, as influenced by runoff, tidal flow and other factors, are also associated with many other ecological differences.

Differences in immunocompetence might be due to presently unidentified pollutants (e.g., pesticides, ammonia, or other toxins), which may impact the riverine ecosystems more severely, since they are closest to the initial discharge of these substances. This might explain why shell disease is more common in the riverine areas. Other diseases which are not as readily discernible as shell disease may be affecting the crabs in the riverine areas. Alternatively, blue crabs in the riverine areas may normally have lower antibacterial activity, due to presently unknown physiological changes that are associated with adaptation to this particular environment. For example, the natural stresses associated with the riverine ecosystem may cause a reduction in immunocompetence. Nutritional differences due to prey abundance or other factors (e.g., hypoxia; see Section E. below) may be important. Fisher et al (1976) found that inadequate nutrition caused shell disease in cultured lobsters (Homarus americanus) and Stewart et al (1967) found that inadequate nutrition caused a reduction in hemocyte numbers in lobsters. Most, if not all, of the blue crab antibacterial activity resides in the hemocytes, not the plasma (Noga and Arroll, unpublished data) and our preliminary data suggests that hemocyte numbers are also depressed in Pamlico River blue crabs (Stevens, Noga and Arroll, unpublished data).

Nonetheless, even if lower antibacterial activity in riverine crabs is a normal physiological change, it may still explain why blue crabs in the riverine parts of the ecosystem may be at higher risk of developing shell disease. It could also imply that these riverine ecosystems may be especially vulnerable to added stressors associated with pollution.

One somewhat puzzling aspect of this antibacterial activity is its inhibition by sodium chloride (Figure 4). The greater activity at low salt concentration would initially suggest that antibacterial defenses would be enhanced in low salinity environments. However, this would depend upon the

microanatomical site(s) where the antibacterial activity normally resides. We have recently determined that a major portion (and possibly all) of the antibacterial activity resides in the circulating hemocytes. Thus, the activity may not play a role in defense until the carapace is entirely breached, exposing potential pathogens to hemocyte attack. Shell disease lesions in Pamlico River crabs often appear to be very deep, even in the small, apparently early, stages. Thus, hemocyte activity may be important even at this early stage. Detailed studies of the pathology of these shell disease lesions, as well as labeling studies for identifying where the antibacterial activity resides in the carapace, will be needed to answer this question.

E. POSSIBLE CAUSES FOR LOWER HEMOCYANIN LEVELS IN PAMLICO RIVER BLUE CRABS

Hemolymph hemocyanin concentrations were lower in the riverine areas of the Albemarle-Pamlico Estuary than in the more oceanic reference sites, Core and Bogue Sounds (Figure 7). Investigations by deFur and co-workers (deFur et al 1990 and C. Mangum, College of William and Mary, personal communication) have shown that both low oxygen and low salinity stimulate an increase in hemocyanin concentration of blue crab hemolymph. Acclimation experiments conducted at our laboratory this year have confirmed this observation (i.e., crabs adapted to 5 ppt salinity from 30 ppt salinity had a 15% increase in hemocyanin concentration). Thus, this experimental data contradicts the gradient in hemocyanin levels that we have seen in the A/P Estuary, since both lower salinity and episodic hypoxia are typical of the riverine areas.

One possible cause for the low hemocyanin concentrations in the riverine areas is an infectious disease. Paramoeba pernicosa ("grey crab disease") causes a significant decrease in hemocyanin concentration (Sprague and Beckett 1966; Sawyer et al. 1970; Newman and Ward 1973; and Pauley et al. 1975). The dinoflagellate, Hematodinium sp., may also reduce hemocyanin levels (Newman and Johnson 1975).

Inadequate nutrition has also been associated with decreased hemocyanin levels in the American lobster (Stewart et al 1967) and the crab Carcinus maenas (Uglow 1969). Hagerman and Pihlbaden (1988) found that depressed hemocyanin levels in Norwegian lobster (Nephrops norvegicus) were due to inanition. The inanition resulted from anorexia that was caused by a hypoxic environment (i.e., the animals did not eat when environmental oxygen levels were low).

While hemocyanin concentrations of crabs with shell disease were low, they were not significantly different from those

measured in clinically normal crabs collected from the Pamlico River during the same period. This might suggest that shell disease is not related to hemocyanin level. However, this cannot yet be ruled out, since hemocyanin levels were highest in crabs collected in the sites at lowest risk for shell disease (i.e., high salinity areas).

F. POTENTIAL IMPORTANCE OF METAL CONTAMINANTS ON THE HEALTH OF BLUE CRABS IN THE A/P ESTUARY

Crabs collected from the Pamlico River had body burdens of copper, cadmium and zinc that were similar to crabs from areas considered to be relatively uncontaminated by these metals (i.e., Bogue and Core Sounds). In fact, copper and zinc concentrations in the Pamlico River crabs were significantly lower than those from the reference sites, suggesting that Pamlico River crabs may be deficient in copper or zinc. However, the zinc concentrations for Pamlico River and reference groups fall well within the range observed among crabs from the Atlantic and Gulf of Mexico coasts. The data on the crabs collected in Tampa Bay, Florida (Table 3) were included so that comparisons could be made with the North Carolina crabs. All the Tampa Bay crabs have 3-4 times the mean cadmium levels of the North Carolina crabs, as well as having higher copper concentrations. This comparison is relevant since the Tampa area is a major phosphate mining, processing and shipping location, as well as being heavily industrialized; high levels of both cadmium and copper are generally associated with industrialized areas.

It is also interesting that no Pamlico River shell disease has been reported in the Tampa Bay area, also supporting the contention that cadmium, copper or zinc contamination is probably not important in the development of Pamlico River shell disease. Also, most, if not all transition metals are complexed with naturally occurring chelators that are used in metabolism. From our measurements made on crabs in highly contaminated locations, most of the copper and 95% of the cadmium in in the digestive glands was associated with metallothioneins and thus, not available to produce effects at the intracellular level in exposed crabs (Engel and Brouwer 1984a, 1984b, and unpublished data). However, the potential importance of other toxicants, such as arsenic, mercury, nickel, lead, fluoride or selenium is uncertain.

G. POTENTIAL USEFULNESS OF THE ANTIBACTERIAL ASSAY TO FISHERIES HEALTH MONITORING

We have provided evidence that the immunocompetence of crabs with shell disease is lower than clinically normal crabs.

Furthermore, we have found that crabs collected from more riverine parts of the A/P Estuary have significantly lower antibacterial activity than crabs collected from more oceanic sites.

In order to further substantiate the geographic differences which we have seen in this study, it would be highly desirable to place healthy crabs from a reference, pristine site (e.g., Core Sound) at various sites in the A/P Estuary and closely monitor them for the development of changes in antibacterial activity and health status. This would allow a more precise assessment of the water quality factors that may be associated with this immunosuppression and, ultimately, how it relates to the health status of the fishery. The development of shell disease in Bogue Sound crabs that were held in water from the Pamlico River suggests that this may be a very useful tool.

If experimental studies support our empirical evidence that hemolymph antibacterial activity can be used as an indicator of health status, it may allow the rapid assessment of crab fishery populations. An example may be the development of a simple in vitro diagnostic test for assessing the health of a crab population based upon the antibacterial activity. The antibacterial activity is very stable, being resistant to multiple freeze-thaw cycles and maintaining activity after many months of storage, making it attractive for field sampling. The test which we developed for measuring antibacterial activity is also very reproducible and allows for the testing of at least 20 samples simultaneously. Test to test variation was also very low, but there was a wide variation in response among individual crabs. While significant differences were clearly evident even with this high degree of individual variation, it might be possible to improve the test by using another measurement of the antibacterial factor, such as by an enzyme-linked immunosorbent assay. Development of such a test will require the purification of the antibacterial factor(s).

This antibacterial assay may also facilitate the testing of water quality conditions that predispose to disease. This information may also aid in controlling the disease in crab-shedding operations and other crustacean aquaculture situations where the disease is often prevalent. This mechanistic approach might allow a direct cause and effect relationship to be established between disease and pollution in the A/P Estuary and other aquatic ecosystems.

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