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BLUE CRAB, *CALLINECTES SAPIDUS*, HEMOCYANIN CONCENTRATIONS AS AN INDICATOR OF ENVIRONMENTAL CONDITIONS IN THE ALBEMARLE-PAMLICO ESTUARY

ALBEMARLE-PAMLICO ESTUARINE STUDY

NC Department of
Environment, Health,
and Natural Resources

ALBEMARLE-PAMLICO

Environmental
Protection Agency
National Estuary Program

BLUE CRAB, CALLINECTES SAPIDUS, HEMOCYANIN CONCENTRATIONS AS AN INDICATOR OF ENVIRONMENTAL CONDITIONS IN THE ALBEMARLE/PAMLICO ESTUARY

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Dr. Marius Brouwer of the Duke University Marine Laboratory and Dr. Jerry B. Stevens and Dr. Edward J. Noga of the NCSU, College of Veterinary Medicine, contracted with the NMFS, Beaufort Laboratory to provide information used in the preparation of this report.

ABSTRACT

A survey of the hemolymph hemocyanin concentrations in blue crabs, Callinectes sapidus, from the Albemarle/Pamlico study area was initiated in 1989 to determine if environmental quality influenced the concentration of hemocyanin in crabs. The investigation, which was conducted during the summers of 1989 through 1991, was collaborative effort between the National Marine Fisheries Service, North Carolina Division of Marine Fisheries, and the Duke University Marine Laboratory. The project was funded by EPA/APES only during 1991.

Blue crabs were collected in 1991 May through October at three locations in the Pamlico River area (North Creek, South Creek, and Pungo River), at three locations in the Neuse River area (Bay River, Broad Creek, and Oriental), in the New River, and two reference sites (Currituck Sound and Core Sound). Hemolymph was collected from the crab by severing the paddle appendage between the joints with a sharp scissors, allowing it to clot, centrifuging out the clot, and measuring the hemocyanin concentration spectrophotometrically. Data also were collected on the sex and size of the crabs.

Analysis of the samples showed that there were both spatial and temporal differences in the hemocyanin concentrations. The concentrations of hemocyanin in the blue crabs was the lowest during July and August in both the Pamlico and Neuse Rivers and on average for the six summer months were about 50% lower than the concentrations measured for crabs collected from Currituck and Core Sounds. The 1991 data follows the same general trends that were established in 1990 and 1989. These data also correlated well with decreased concentrations of dissolved oxygen and increased water temperature.

Hemolymph from blue crabs collected from the Pamlico River and Core Sound was examined for differences in the number and types of hemocytes and the presence of parasites. Hemocyte numbers in the hemolymph of crabs from Core Sound were significantly higher ($P < 0.0001$) than those that were collected from the Pamlico River. The lower hemocyte numbers correlate with previous data that showed similar differences in antibacterial activity among the two groups of crabs (Noga et al. 1990). The antibacterial activity is thought to be associated with the hemocytes. Examination of numerous hemolymph smears revealed only one incidence of a parasitic Hematodynium infection, so apparently the lower hemocyanin concentrations among the crabs from the Pamlico River could not be attributed to that cause.

Experiments designed to demonstrate the effects of hypoxia on the concentrations of blue crab hemocyanin were equivocal. While the two week exposure to low dissolved oxygen concentrations caused a slight increase, the differences were not significant.

The data collected from blue crabs in the Albemarle/Pamlico systems on hemolymph

hemocyanin concentrations suggests that long-term exposure to reduced dissolved oxygen may indirectly cause the observed reductions in hemocyanin concentrations among the blue crabs in the Pamlico and Neuse River systems. Crabs with markedly reduced levels of hemocyanin would be expected to be at a physiological disadvantage which could result in increased susceptibility to parasitic infection, inability to molt successfully, and an inability to repair shell trauma which could serve as a focus for shell disease, but those types of responses were not observed. The earlier work of Noga et al. (1990), however, showed that blue crabs from the areas where the hemocyanin concentrations were the lowest also had the lowest levels of antibacterial activity in the hemolymph. These data suggest that levels of dissolved oxygen normally considered not to be stressful, 30-50% saturation, may not be sufficient for the long-term maintenance of physiological integrity, especially if the temperature of the water is also elevated. If such a hypothesis is correct, it could significantly affect the interpretation of water quality data in the Pamlico and Neuse River systems as well as other coastal estuaries with histories of hypoxic events. Currently low dissolved oxygen is not considered an environmental problem until the concentrations drop below 20% of saturation, and temperature is not considered to be an exacerbating factor.

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SUMMARY AND CONCLUSIONS

The investigation of the effects of environmental quality on blue crabs using hemolymph hemocyanin concentrations as an indicator of crab fitness was initiated because of two facts. The first fact was that during the investigation of the etiology of shell disease among blue crabs collected from the Pamlico River we observed that the hemocyanin concentrations in both healthy and diseased crabs appeared to be low relative to crabs collected at Beaufort. The second fact was that since hemocyanin in blue crabs was analogous to hemoglobin in vertebrates, the observed low concentrations indicated that the crabs in the Pamlico River may be under some type of stress, and possibly "anemic". In order to determine the extent of this phenomenon, we began a survey of hemocyanin concentrations in crabs from a number of different locations/estuaries in western Pamlico Sound and Core Sound and other southern estuarine systems.

Blue crab hemolymph samples were collected at predetermined sites in the Albemarle/Pamlico study area and in the southeastern part of the state. The collection sites in 1989, 1990, and 1991 were as follows: Currituck Sound, Long Shoal River, Swanquarter Bay, Pungo River, North Creek, South Creek, Bay River, Broad Creek, Oriental, Adams Creek, Core Sound, Bogue Sound, White Oak River, New River, and Cape Fear River. Reference areas were designated as Core Sound, a high salinity area, and Currituck Sound, a low salinity area. The sampling was conducted monthly by the staff of the N.C. Division of Marine Fisheries, Washington, Wilmington, Morehead City, and Elizabeth City offices as part of their juvenile fish sampling program. **[Only the data collected in 1991 was funded by APES/EPA. The data collected in 1988, 1989, and 1990 was supported through a collaborative agreement between NMFS, Beaufort Laboratory, N. C. Division of Marine Fisheries, and the Duke University Marine Laboratory.]**

Hemolymph was collected from the crab by severing the paddle appendage between the joints with a sharp scissors, allowing it to clot, centrifuging out the clot, and measuring the hemocyanin concentration spectrophotometrically. Data also were collected on the sex and size of the crabs.

A preliminary single data set collected in September, 1988 indicated that blue crabs collected from the Pamlico River and southwestern Pamlico Sound had lower hemocyanin concentrations than crabs from Beaufort. Further investigations during the summers of 1989, 1990, and 1991 showed that there were both spatial and temporal differences in blue crab hemolymph hemocyanin concentrations. The concentrations of hemocyanin in the blue crabs were the lowest during July and August in both the Pamlico and Neuse Rivers and were generally about 50% lower than the concentrations measured for crabs collected from Currituck and Core Sounds. The 1991 data follows the same general trends that were established in 1990 and 1989. These data also correlated well with decreased concentrations of dissolved oxygen and increased water temperature.

The primary finding of these investigations, which have covered three summers 1989 - 1991, is that the concentration of hemocyanin in the hemolymph of blue crabs from selected eastern North Carolina estuaries appears to be correlated strongly with the location of collection and time of year. The lowest hemocyanin concentrations tended to be associated with crabs collected during the warmest summer months and from specific localities in the Pamlico and Neuse River systems (Pungo River, South Creek, Bay River, and Broad Creek). The presence of poor water quality or a specific contaminant is attractive, because a cause and effect relationship could be shown between the contaminant and the observed depressed hemocyanin concentrations. This scenario does not appear to have much support, because in both the Pamlico and Neuse Rivers there is no evidence of high toxic contaminant concentrations. This line of reasoning also is supported by the results of organic contaminant measurements made on pooled samples of hepatopancreas collected from crabs in the Pamlico River and Core Sound in 1989 (analyses were done at the USEPA Laboratory in Duluth). The Pamlico River animals did not have higher contaminant bodyburdens or a significantly different array of contaminants than those measured in crabs from Core Sound. In earlier investigations in the Pamlico River concerned with shell disease in blue crabs (Noga et al. 1990), we measured the trace metal concentrations in the hepatopancreas of both diseased and healthy blue crabs and were unable to show any significant elevation of metals. It did appear that the concentrations of copper and zinc in the Pamlico River crabs were somewhat depressed relative to those collected from the reference areas, Core and Bogue Sounds. It appears, therefore, that there is no direct correlation between contaminant accumulation and either depressed hemocyanin concentrations or shell disease among the blue crabs from the Pamlico and Neuse Rivers.

Hemolymph from blue crabs collected from the Pamlico River and Core Sound was examined for differences in the number and types of hemocytes and the presence of parasites. Hemocyte numbers in the hemolymph of crabs from Core Sound were significantly higher ($P < 0.0001$) than those that were collected from the Pamlico River. The lower hemocyte numbers correlate with previous data that showed similar differences in antibacterial activity among the two groups of crabs (Noga et al. 1990). The antibacterial activity is thought to be associated with the hemocytes. Examination of numerous hemolymph smears revealed only one incidence of a parasitic Hematodynium infection, so apparently the lower hemocyanin concentrations among the crabs from the Pamlico River could not be attributed to that cause.

"Natural" stressors (temperature, salinity, and dissolved oxygen) also were examined to determine if correlations or cause and effect relationships could be demonstrated. Thus, a hypothesis was formulated that may implicate both dissolved oxygen concentration and elevated temperature as factors that together may negatively affect the concentrations of hemocyanin in blue crabs. This proposed hypothesis suggests that reduced dissolved oxygen in combination with elevated temperature entrains the crabs in the rivers and creeks, and because the hypoxic conditions are wide spread, the crabs can not escape to higher oxygen conditions. Also, the hypoxic conditions intensify at night

which exacerbates the situation. Once the crabs are entrained, the combination of elevated temperature, which increases the metabolic rates, and the lowered dissolved oxygen may cause lethargy in the crabs and decreased in feeding success. Decreased feeding and increased energy requirements would result in a nutritional deficit that could be expressed as a decrease in hemocyanin concentration in the affected crabs.

Laboratory experiments were conducted to determine if low dissolved oxygen concentrations alone could cause significant changes in the hemolymph hemocyanin concentrations of exposed crabs. The results of these experiments were not conclusive. While the hemocyanin concentrations in crabs exposed to low oxygen for two weeks were slightly higher the differences were not significant. It is important to note, however, that the concentrations did tend to go up in response to low dissolved oxygen which is the expected physiological response.

The three summers worth of data on hemocyanin concentrations in the hemolymph of blue crabs suggests that long-term exposure to reduced dissolved oxygen may indirectly cause the observed reductions in hemocyanin concentrations among the crabs in the Pamlico and Neuse River systems. Thus levels of dissolved oxygen normally considered not to be stressful, 30-50% saturation, may not be sufficient for the long-term maintenance of physiological integrity, especially if the temperature of the water is also elevated. Crabs with markedly reduced levels of hemocyanin would be expected to be at a physiological disadvantage which could result in increased susceptibility to parasitic infection, inability to molt successfully, and an inability to repair shell trauma which could serve as a focus for shell disease. This hypothesis is supported by the work of Noga et al. (1990) that shows that blue crabs from the areas where the hemocyanin concentrations were the lowest also had the lowest levels of antibacterial activity in the hemolymph. If the antibacterial activity is part of a nonspecific immune response, then crabs with lowered hemocyanin concentrations would be expected to more susceptible to various types of pathogens. Acceptance of the hypothesis that chronic low dissolved oxygen concentrations can affect resident blue crab populations could affect the interpretation of water quality data in the Pamlico and Neuse River systems as well as other coastal estuaries with histories of chronic low oxygen events.

RECOMMENDATIONS

The recommendation that has been developed from these investigations is that a monitoring program should be instituted to determine: (1.) if lowered hemocyanin concentrations among the blue crab population in the Pamlico and Neuse River systems indicate that these animals are stressed relative to the reference locations; (2.) whether crabs are being entrained by the reduced dissolved oxygen concentrations and elevated temperatures in the study area; and (3.) whether hemocyanin measurements can be used as a quick and inexpensive method of monitoring the relative health of crustacean populations, and as an integrated measure of dissolved oxygen. The following is an outline of a monitoring project that would help to achieve the above mentioned objectives.

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VALIDATION OF BLUE CRAB HEMOCYANIN CONCENTRATION AS A MEASURE OF ENVIRONMENTAL CONDITION IN THE PAMLICO AND NEUSE RIVERS

GOAL:

Determine if chronic low dissolved oxygen concentrations in the Pamlico and Neuse Rivers has a detrimental effect on blue crab survival and growth.

OBJECTIVE:

Conduct a monitoring program at selected locations on the Pamlico and Neuse Rivers to validate the use of blue crab hemocyanin concentration as an indicator of chronic low dissolved oxygen conditions.

PROTOCOLS:

Sampling Locations:

Six sites with variable salinities will be chosen.
Neuse River: Oriental, Broad Creek, and Bay River Pamlico River: South Creek, North Creek, and Pungo River

Two reference sites with relatively stable salinities will be chosen.
Core Sound (Oyster Creek)
Currituck Sound

Physical and Chemical Measurements:

* Nutrients - nitrates, nitrites, phosphorus
* Chlorophyll a
* Turbidity
* pH
* Salinity
* Temperature
* Dissolved oxygen
* Organic contaminants and trace metals (pooled blue crab hepatopancreas samples) at beginning and end of study
* Weather data - wind direction and speed and rain fall amounts (for the region)
* Blue crab hemocyanin concentrations
* Blue crab stomach contents (an indicator of feeding)

SAMPLE COLLECTION:

* The sample sites will be selected based upon the data that is already in place from previous studies.
* At each site a continuous salinity/temperature/oxygen monitor will be installed.
* Sites will be sampled once a month for six months, May - October, on the same calendar day, if practical.
* Other measurements will be made at that same location on a monthly basis.
* Blue crab samples will be taken by otter trawl at each site. Hemolymph samples will be collected at the same time.

CRAB COLLECTION AND MEASUREMENTS:

* Twenty (20) crabs >100 mm carapace width will be collected at each site, ten males and ten females. These numbers are the optimal numbers that will be collected. Obviously, there could be circumstances where the sex ratio would not work or the crabs would be scarce.
* Hemolymph samples will be taken in the field at the site of collection using the techniques already developed (See: Engel, APES Annual Report #92-09) and taken to the lab for hemocyanin measurements.
* Crabs will be numbered, placed on "ice" or refrigerated, and taken to the lab where hepatopancreas samples will be taken and quick frozen.
* Hemolymph will be processed in the laboratory and hemocyanin measured using established spectrophotometric techniques.

* Hepatopancreas samples will be measured for total trace metals using established trace metal analytical techniques.
* The pooled hepatopancreas samples will used for the determination of total organic contaminant body burdens (these measurements will be made if a certified laboratory can be contacted).

RATIONALE:

The primary focus of the proposed monitoring activity is to determine whether the observed reductions in blue crab hemocyanin concentrations can be linked in real time with low dissolved oxygen conditions at the site of collection. The hypothesis is that long-term exposure to chronic low dissolved oxygen concentrations (40-50%) may cause a reduction in the blue crabs ability to forage. If lethargy is induced, it could then result in reduced feeding and general malnutrition. In the literature, it has been shown that starvation will cause a significant decrease in hemocyanin concentration in other species of crustaceans. So the observed low hemocyanin would be a secondary effect of chronic hypoxia. The interesting thing is that the measured oxygen levels in the areas of the Pamlico River were about 50% of saturation which is generally considered to be adequate, but since the measurements were made during the day, the night-time oxygen levels will probably be much lower. The result is that during the night there could be acutely hypoxic conditions, but moderately low dissolved oxygen during the day.

COLLABORATIVE ORGANIZATIONS:

NMFS, Beaufort Laboratory; N.C. Division of Environmental Management; USGS; N.C. Division of Marine Fisheries.

INTRODUCTION

As part of a research project on the etiology of shell disease among the blue crab population in the Pamlico River, tissue samples from diseased and healthy crabs were collected. One of the tissues sampled was the hemolymph or blood of the crabs. During the collection of hemolymph from healthy and diseased crabs, it was observed that the samples of hemolymph, when fully oxygenated, did not have the characteristic dark blue color of hemolymph collected from crabs in the Beaufort area. To check on this apparent anomaly samples of hemolymph from blue crabs collected from the Pamlico River were measured for hemocyanin concentration. These measurements showed that the concentrations in the crabs collected from the Pamlico River were about 1/3 of the concentration measured in crabs from Beaufort. This observation was intriguing, because it indicated that the crabs were "anemic" and, possibly, under physiological stress. The N.C. Division of Marine Fisheries was informed of our observations. This interaction led to the collection of a single set of hemolymph samples from blue crabs in Pamlico Sound and its tributaries in September, 1988 by biologists in the Washington, N.C. office of the North Carolina Division of Marine Fisheries as part of their juvenile sampling program. Due to the intriguing nature of these observations monthly samples also were collected from similar locations during the summers of 1989, 1990, and 1991 (**SEE: Results and Discussion section for complete description of the data**).

The importance of hemocyanin to health, survival and normal physiological function of blue crabs, and all other crustaceans, can not be understated since it serves as the oxygen carrying protein in the hemolymph. Crustacean hemocyanins are large copper containing proteins composed of a minimum of six subunits, each of 75,000 dalton molecular weight. The number of hexamers and their arrangement is species specific. The structure and function of hemocyanins have been studied extensively (Van Holde & Miller, 1982; Ellerton et al., 1983; and Brouwer, 1991). Other researchers have examined the physiological and biochemical processes that control the synthesis and turnover of hemocyanin and metallothionein, and the metabolism of copper and zinc during the molt cycle in blue crabs (Engel & Brouwer 1987, 1991, and in press; Brouwer et al., 1989).

Certain extrinsic natural environmental factors have a significant effect on the turnover and synthesis of hemocyanin in marine crustaceans. Low salinity has been shown to act as a stimulus for the synthesis of hemocyanin in blue crabs exposed to hyposaline conditions under laboratory conditions (Marius Brouwer, unpublished data and Charlotte Mangum, personal communication). In 1988 we measured hemocyanin in blue crabs from a "clean" low salinity estuary on the Gulf of Mexico, Heron Bay, MS, and the hemocyanin concentrations were elevated relative to higher salinity locations. Therefore, crabs from low salinity water of the Pamlico system should have higher concentrations of hemocyanin than those collected near Beaufort where the salinities are higher, but apparently the

opposite situation is true. Oxygen concentration in the water also has been shown to have an effect on hemocyanin concentration in crustaceans. DeFur and colleagues (1990) have shown in laboratory experiments that hypoxia causes an increase in hemocyanin concentrations in blue crabs from Chesapeake Bay, where hypoxic events occur routinely. Since hypoxic conditions also are known to occur in the Pamlico River and many of the other coastal creeks and rivers in eastern North Carolina during the summer (data from the N.C. Division of Environmental Management), higher concentrations would be expected among those animals, but once again, that does not appear to be the case. Therefore the Pamlico River crabs do not appear to be able to cope with normal environmental stresses as effectively as blue crabs from other locations.

In addition to natural stressors, the presence of anthropogenic contaminants, particularly organics (hydrocarbons and pesticides), may interfere with normal copper metabolism and thus the synthesis and turnover of hemocyanin in blue crabs. For example, it has been shown that glutathione, a compound that has long been known to play a role in the detoxification of certain aromatic hydrocarbons (PAHs) (Meister and Anderson 1983), also is involved in copper metabolism (Freedman et al. 1989). Brouwer (unpublished data) demonstrated that a complex of Cu(I) and glutathione can reactivate copper-free hemocyanin. In addition, it has been reported that the hepatopancreatic levels of the enzyme glutathione S-transferase, which conjugates hydrocarbons with glutathione, increased when blue crabs were exposed to an organic contaminant (BHT) (Lee et al. 1988). This information suggests a possible pathway by which exposure to PAH and pesticides may result in diminished hemocyanin synthesis, due to the lack of Cu(I)-glutathione complex. To explore the possible link between water quality and hemocyanin concentrations, we measured hemocyanin in the hemolymph of blue crabs collected at three different locations in the Houston Ship Channel. The sites formed a gradient of increasing industrial and domestic sewage contamination up the Channel towards Houston. The data showed a negative correlation between the distance up the Houston Ship Channel and hemocyanin concentrations in the crabs (Engel and Brouwer in press) which may be interpreted as an indicator of water quality in the Ship Channel. Hemolymph samples also were collected from American lobsters from estuaries with historical contaminant loads, such as Boston Harbor. The hemocyanin concentrations measured in those samples were lower than those from cleaner areas, such as Cape Cod Bay. At this time the evidence for a positive correlation between degraded environmental quality (i.e. elevated levels of organic contaminants) and lowered hemocyanin concentration is circumstantial, but the intriguing nature of these data suggests that further investigations should be conducted.

Another possible cause for the lowered concentrations of hemocyanin in the hemolymph of blue crabs is the presence of disease organisms or agents, such as parasites, bacteria, and viruses. One of the internal parasites of blue crabs from North Carolina is Paramoeba pernicosa which causes "grey crab disease". One symptom of this disease is a significant decrease in the hemocyanin concentration and a loss of the clotting mechanism in the hemolymph (Sprague and Beckett 1966; Sawyer et al. 1970; Newman and Ward 1973; and Pauley et al. 1975). Another parasite shown to occur in blue crabs is the dinoflagellate,

Hematodinium sp., that has caused crab mortalities in N.C. waters (Newman and Johnson 1975). It would be desirable, therefore, to determine if disease processes among blue crabs from the Albemarle/Pamlico Sound area may be contributing to the aberrant concentrations of hemocyanin observed in that population.

The investigation of the hemocyanin concentrations among the blue crab populations of the Albemarle/Pamlico study area in 1991 was designed to work toward the following two objectives:

a. to determine if the blue crab populations in the Albemarle/Pamlico estuarine system continue to have low hemocyanin concentrations in the hemolymph

b. to determine if epizootic disease organisms occur in the hemolymph and tissues of blue crabs showing low hemocyanin concentrations

METHODS AND MATERIALS

Hemocyanin Collection and Measurement

Blue crab hemolymph samples were collected at predetermined sites in the Albemarle/Pamlico study area (Figure 1). The collection sites included some of the sites that were sampled in 1988 and 1989. The sites where samples were collected are: Currituck Sound, Core Sound, Pamlico River (South Creek and North Creek), Pungo River, Bay River, and Neuse River (Oriental and Broad Creek). Reference areas were designated as Core Sound, a high salinity area, and Currituck Sound, a low salinity area. Additional non-APES sites also were sampled in the summers of 1990 and 1991 (White Oak, New, and Cape Fear Rivers). The sampling was conducted monthly by the N.C. Division of Marine Fisheries, Washington, Wilmington, and Elizabeth City offices. [The individuals and organizations that did the sampling, analyzed the samples for hemocyanin, and generally assisted all were volunteer (1989 and 1990 and partially in 1991). The Principal Investigator on this study thanks the N.C. Division of Marine Fisheries for conducting the sampling of blue crabs for three years without obtaining any additional funding for the task. It is hoped that the data that has been generated and the conclusions that have been drawn will be of use to NCDMF in the management of both environmental and fishery resources.]

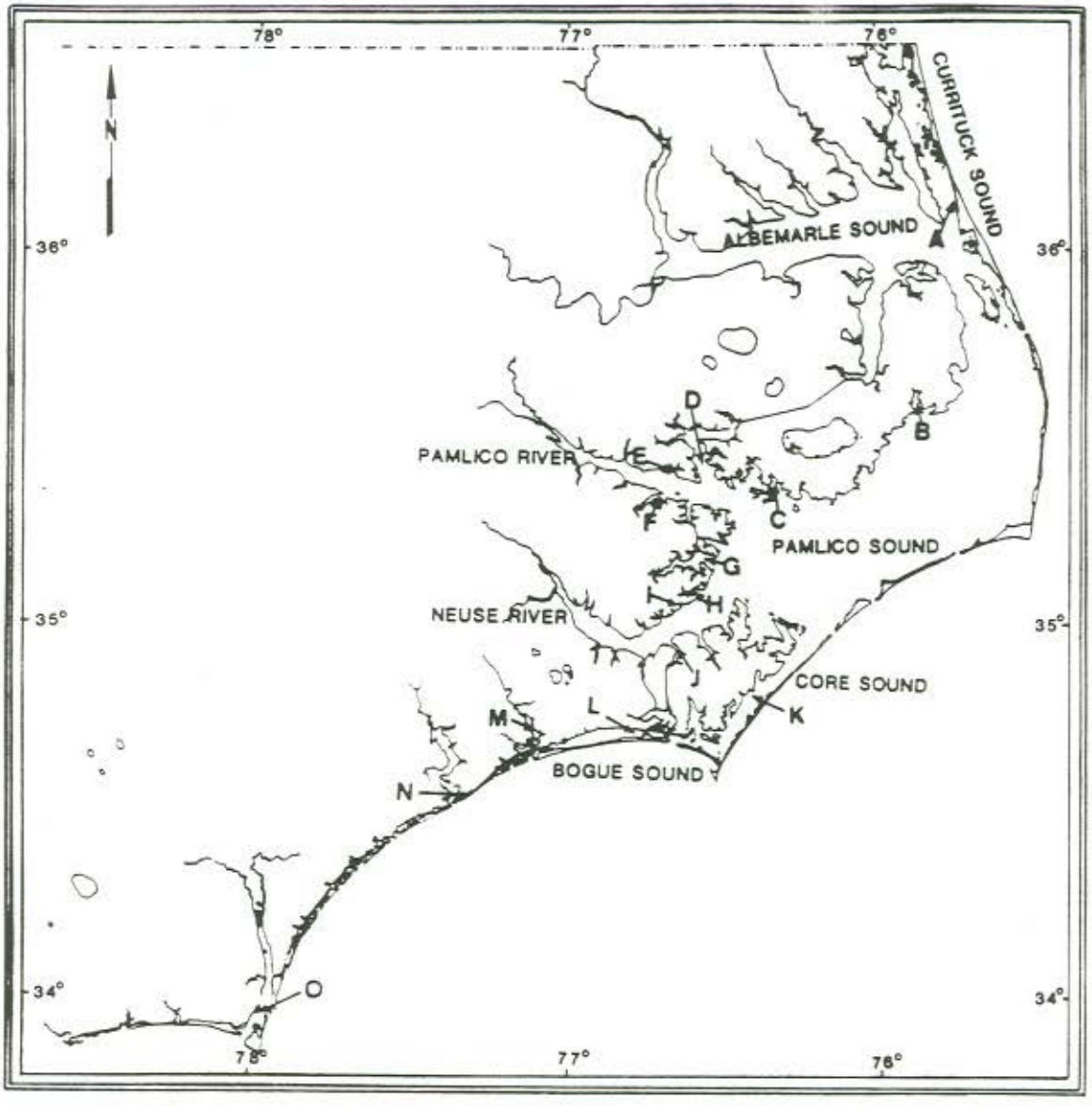

FIGURE 1. A map showing the locations of all sampling sites used during 1989, 1990, and 1991. The letters indicate the following locations: A. Currituck Sound; B. Long Shoal River; C. Swanquarter Bay; D. Pungo River; E. North Creek; F. South Creek; G. Bay River; H. Broad Creek; I. Oriental; J. Adams Creek; K. Core Sound; L. Bogue Sound; M. White Oak River; N. New River; and O. Cape Fear River. The sites M., N., and O. are not in the APES study area.

Blue crabs were selected for molt stage and sex. Only C_4 or early premolt D_1 - D_3 male crabs and terminal molt females without eggs were collected so that the effects of molt cycle on hemocyanin concentration will be negated as much as possible (Engel, 1987). Previous investigations have indicated that there is no significant sex difference in hemocyanin concentration among blue crabs (unpublished data). A sample size of ten individual crabs, five males and five females were collected at each site when available. Vital statistics were recorded on each individual crab: date, collection location, sex, carapace width [mm], water temperature, salinity, pO_2 and health rating of each crab.

Hemolymph samples were collected by severing an appendage between the joints. The appendage of choice was the paddle appendage but a walking leg was also acceptable. The cut was made with a sharp pair of scissors between the joints through the meropodite. When cut in this manner, the hemolymph flows freely from the wound. The samples were collected in plastic vials, placed on ice, and allowed to clot. The samples were shipped to the Beaufort Laboratory on ice usually within two days of collection. Tests conducted previously in our laboratory demonstrated that clotted hemolymph was stable for at least seven days after collection when held at $<4°C$.

To prepare the samples for analysis of hemocyanin concentration, the clotted hemolymph was homogenized with a Polytron homogenizer, and then centrifuged at 20,000 x G for 30 minutes. The resulting supernate or serum was decanted and kept at $<4°C$.

The hemocyanin measurements were made spectrophotometrically using either a Hewlett-Packard diode array spectrophotometer or a Beckman DU 7500 spectrophotometer. The spectrophotometers were intercalibrated and used interchangably during the study. To determine the hemocyanin concentration, hemolymph serum was diluted with 50 mM Tris/ 10 mM Ca buffer at pH 8.0 (i.e. 50 uL hemolymph into 2.95 mL of buffer in a quartz cuvette). Measurement were made at both 280 and 334 nm. These readings were compared to standards and the concentration of hemocyanin calculated using the extinction coefficients for blue crab hemocyanin (i.e. $E_{280nm}=13.5$ and $E_{334nm}=2.30$ [Johnson et al., 1989]).

<u>Hemolymph Hemocyte Determinations:</u>

To develop methods for the collection, preservation, and staining of crab hemolymph cells, clinically healthy male crabs were selected. Hemolymph samples were drawn with a tuberculin syringe with a 25 Gage needle. A 5/32 inch steel ball was placed in the barrel of the syringe along with 0.6mL of 10% formalin. The steel ball facilitated mixing of the sample with the formalin. About 0.5mL of hemolymph was drawn from the hemolymph sinus at the base of the paddle fin. This technique allowed for the collection of multiple samples from a single crab over time. The technique described earlier can be used only once due to the trauma and loss of hemolymph. These fixed samples are stable and do not require refrigeration.

For unpreserved and non-clotted hemolymph the same bleeding technique was used except both the crab and the syringe are cooled with ice to 5°C. The hemolymph was aspirated from the crab and mixed 1 : 1 with isotonic saline. The ionic strength of the saline should be adjusted to reflect the salinity of the water to which the crab was acclimated (i.e. NaCl concentration in hemolymph of crabs acclimated to 5 to 20 ppt, 350 mM and at 35 ppt, 500 mM [Engel 1971]). The rapid dilution in saline prevents clotting. These samples, however, should be refrigerated and used as soon as possible.

The preparation of the slides for differential counting and for observation of cell types, and the techniques for staining the preparations are outlined in detail in APPENDIX II.

Effects of Hypoxia Experiment:

To examine the effect of hypoxia on blue crab hemolymph hemocyanin concentration, crabs were maintained for 14 days in 30 ppt seawater with an oxygen concentration of about 30% saturation (50-55 mm Hg). The hypoxic conditions were produced by bubbling nitrogen gas into the water to reduce the Po_2 from 150 to approximately 55 mm Hg in 3 to 4 hours. Thereafter the nitrogen input was stopped and air was used. Additional nitrogen was added as required through the use of an oxygen electrode coupled with a nitrogen regulator. Hemocyanin was measured at the end of the experiment using the techniques described above (SEE: Hemocyanin Collection and Measurement).

RESULTS AND DISCUSSION

1988 STUDIES:

The first trial collections of blue crab hemolymph and measurements of hemocyanin concentrations were made in southwestern Pamlico Sound during September of 1988. This single collection showed that hemocyanin concentrations in blue crabs from the southwestern portion of the Sound were low relative to the reference area, Beaufort (Fig. 2). The concentration in Beaufort crabs was 62 mg/ml while the concentrations in crabs from the tributaries of Pamlico Sound ranged between 15-21 mg/ml . This information indicated that a possible problem existed in the area covered by this single set of measurements.

Measurements also were made of hemocyanin concentrations in healthy blue crabs and those with shell disease that were collected at Etlas Henries crab house on South Creek. These data showed that both groups of crabs had reduced hemocyanin concentrations, and

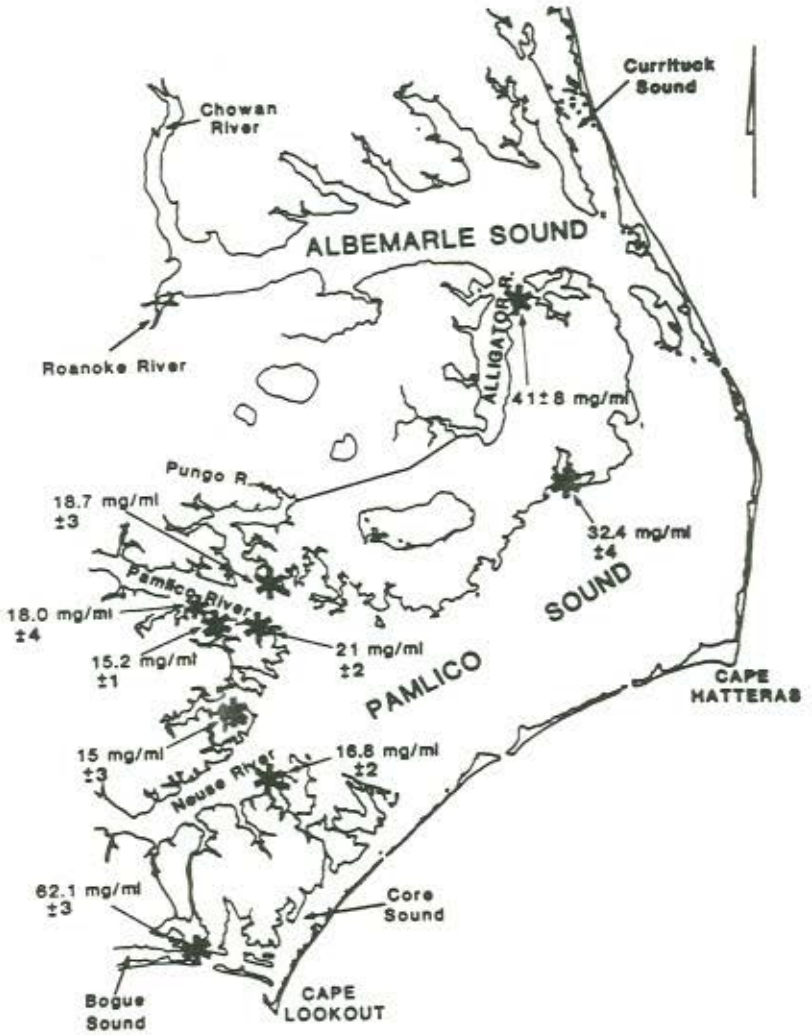

FIGURE 2. A map of Albemarle and Pamlico Sounds with stars indicating the location of blue crab sampling in September, 1988, and the numbers are the mean hemolymph hemocyanin concentrations. Each mean represents an average of four to ten crabs plus/minus one standard error. The crabs were collected by N.C. Division of Marine Fisheries, Washington Office.

that there was no significant difference between the two groups (i.e. Diseased 22±3 mg/ml and Healthy 24±4 mg/ml) ($P > 0.05$) (Noga et al. 1990). This observation was further evidence that some undefined factors were interacting in the environment to affect possibly both the health and physiology of the blue crab population in the Pamlico River.

1989 STUDIES:

To further examine distribution of hemocyanin concentrations in the blue crab population in the Pamlico Sound estuarine complex, seven test sites and a number of reference sites were sampled from May through October. Spatial and temporal differences occurred within and among the different sampling locations in the northwestern Pamlico Sound, Pamlico River, Neuse River, and reference locations (Fig. 3 and Table I). The reference locations were separated from the areas of concern but were in the same general locale. The average of monthly measurements of hemocyanin concentrations in blue crabs collected from the two locations in the northwestern Sound were similar. Major temporal differences were shown, however, at two locations in the Pamlico River and at one location in the Pungo River. In July the hemocyanin concentrations in crabs collected from the Pungo River averaged 13 mg/ml which is about 25% of the reference area crabs. Stations in the Neuse River area, Broad Creek and Bay River, also had large temporal changes. The changes observed between locations in the Neuse River were interesting, because the lowest values were from the two most sparsely populated areas, while crabs from the Oriental site had higher hemocyanin concentrations. This is particularly intriguing since the Oriental site has the heaviest pleasure boat traffic and large marinas, which would suggest poorer water quality than the other two less populated areas. While there were fluctuations in the hemocyanin levels among the crabs collected from reference areas, measured concentrations were generally higher than those seen in the Pamlico or Neuse Rivers. The Pungo River crabs had a mean hemolymph hemocyanin concentration in May of 33± mg/ml, but the concentrations decreased to 12±2 mg/ml by July. In August mean concentration rebounded to the same level as May and then remained relatively constant through October. The higher hemocyanin concentrations in August may have been the result of immigration of adult crabs into the Pungo River from Pamlico Sound during the late summer (N.C. Division of Marine Fisheries, Washington, N.C., personal communication).

1990 STUDIES:

In 1990, the geographical range of sampling sites was changed somewhat after consultation with the N.C. Division of Marine Fisheries. The primary change was to include sampling that was outside of the APES area of interest (White Oak River, New River, and Cape Fear River), drop two of the Pamlico Sound sites (Long Shoal River and Swanquarter Bay), and add Currituck Sound. The additions gave us sites that were low salinity and were therefore more comparable to the Pamlico and Neuse Rivers. One of the inherent weaknesses of the 1989 study was that we did not have any true low salinity reference areas.

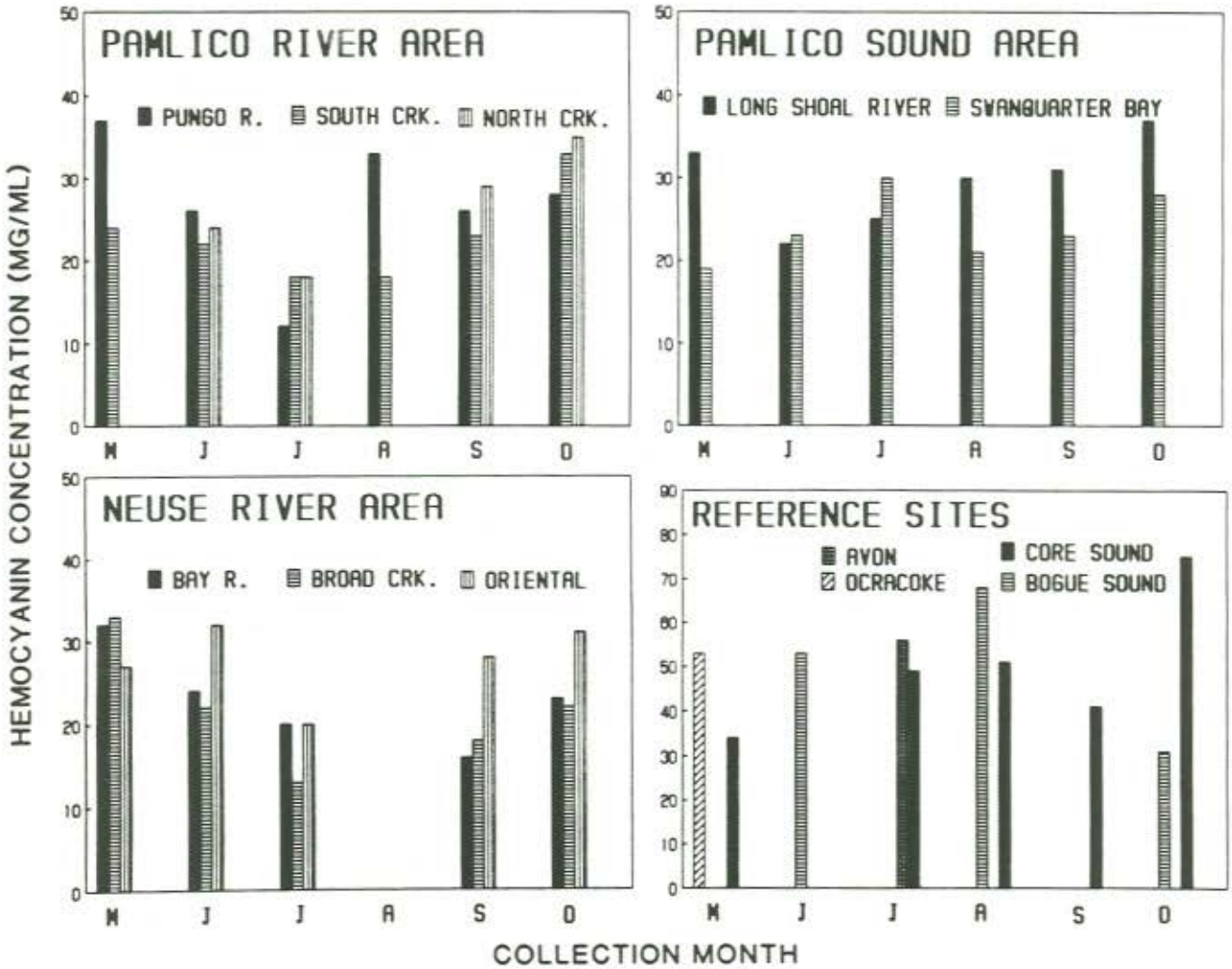

FIGURE 3. Mean hemolymph hemocyanin concentrations for blue crabs collected from the Pamlico and Neuse River areas, Pamlico Sound and the Reference sites in 1989. Each bar is a mean of five to ten crabs. Locations are shown in Figure 1. The crabs and hemolymph samples were collected by N.C. Division of Marine Fisheries personnel from the Washington Office.

TABLE I. Mean hemocyanin concentrations in the hemolymph of blue crabs collected in the Albemarle/Pamlico study area during the summers of 1989, 1990, and 1991. Data is presented as the mean ± SE Mg hemocyanin / Ml hemolymph.

1989 LOCATION	MAY	JUNE	JULY	AUGUST	SEPTEMBER	OCTOBER
SWANQUARTER BAY	19±1	23±2	30±4	21±2	23±4	28±3
LONG SHOAL RIVER	32±3	22±4	25±4	30±4	31±8	37±6
PUNGO RIVER	33±5	25±3	12±2	33±5	26±6	28±4
NORTH CREEK	NC	24±3	19±3	NC	29±3	35±7
SOUTH CREEK	23±3	22±6	18±4	18±3	23±3	33±4
BAY RIVER	32±4	22±4	20±3	NC	16±2	23±3
BROAD CREEK	33±4	22±3	13±2	NC	18±3	22±3
ORIENTAL	28±3	32±4	20±4	NC	28±4	31±3
AVON	NC	NC	56±13	NC	NC	
OCRACOKE	53±6	NC	NC	NC	NC	NC
CORE SOUND	34±6	NC	50±8	51±6	41±3	76±12
BOGUE SOUND	NC	53±10	NC	68±6	NC	31±5
1990						
PUNGO RIVER	30±3	30±3	29±4	20±2	22±3	26±2
NORTH CREEK	NC	33±5	27±4	22±2	14±1	16±2
SOUTH CREEK	23±2	37±4	23±3	22±2	14±2	26±3
BAY RIVER	NC	NC	23±1	28±3	24±3	17±1
BROAD CREEK	28±6	18±2	27±4	24±5	25±4	21±3
ORIENTAL	49±8	34±3	32±3	25±2	36±7	24±3
ADAMS CREEK	NC	32±4	29±8	38±3	25±4	29±4

(1990 CONT.)

LOCATION	MAY	JUNE	JULY	AUGUST	SEPTEMBER	OCTOBER
WHITE OAK RIVER	41±12	25±3	34±5	38±5	30±7	21±2
NEW RIVER	NC	16±3	18±1	17±1	23±4	25±4
CAPE FEAR RIVER	NC	54±11	49±10	53±6	33±4	44±6
CURRITUCK SOUND	48±3	36±6	48±12	36±8	16±7	32±4
CORE SOUND	36±3	37±4	47±3	52±6	43±5	41±3

1991

PUNGO RIVER	25±2	23±2	18±2	26±2	28±6	29±9
NORTH CREEK	27±4	18±3	17±2	23±4	38±4	20±5
SOUTH CREEK	26±3	16±3	19±2	19±3	26±2	25±5
BAY RIVER	17±2	20±4	25±4	19±3	25±3	26±5
BROAD CREEK	21±3	19±3	17±2	15±4	21±3	14±2
ORIENTAL	17±2	23±5	18±2	23±2	28±2	20±2
NEW RIVER	20±2	19±3	17±2	25±4	9±3	NC
CURRITUCK SOUND	18±2	30±7	49±9	34±8	21±8	46±8
CORE SOUND	35±5	41±3	40±6	48±3	42±2	41±2

The data showed that there were differences between the Pamlico and Neuse Rivers and the "Southern Rivers" and also the reference locations (Figure 4 and Table I). One of primary differences between 1989 and 1990 in the Pamlico and Neuse Rivers was that the July low in hemocyanin concentrations did not occur universally. The hemocyanin levels in crabs from the Neuse River fluctuated very little over the sampling interval and were somewhat higher than in 1989 at all locations. There was, however, a decrease in hemocyanin concentrations in September and October in both the Neuse and Pamlico areas. This relation could be correlated with the water temperatures in the two regions. Among the "Southern Rivers" the Cape Fear River crabs had the highest concentrations, the White Oak River was next and the New River crabs had the lowest concentrations of hemocyanin. The values for the Cape Fear and White Oak were as high or higher than the reference sites. These high concentrations may be correlated with low salinity, since both of these sites had lower salinities than the Core Sound area, but not as low as Currituck Sound.

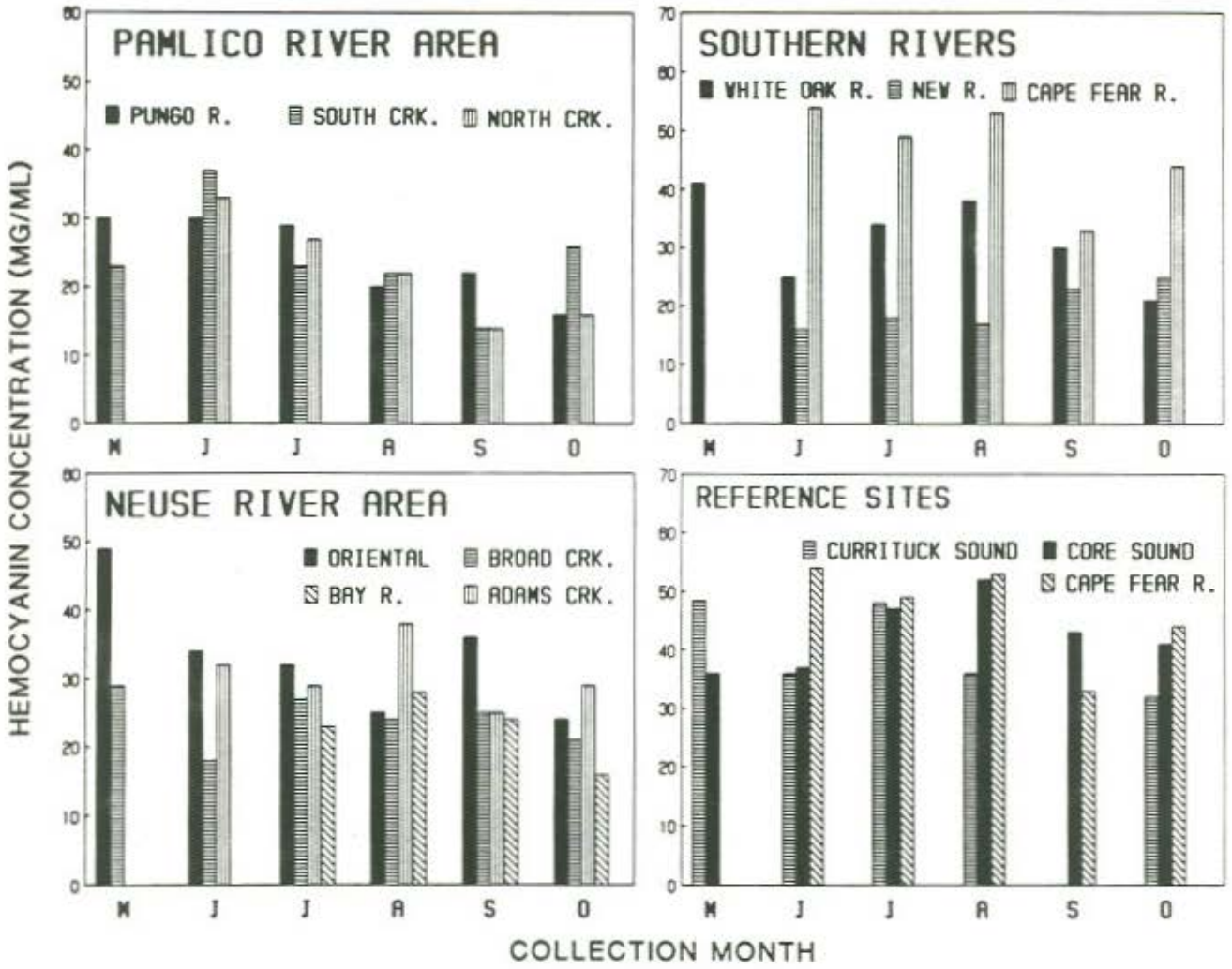

FIGURE 4. Mean hemolymph hemocyanin concentrations for blue crabs from the Pamlico and Neuse River areas, Southern Rivers and the Reference sites in 1990. Each bar of the graph is a mean of five to ten crabs. Locations are shown in Figure 1. The crabs and hemolymph samples were collected by N.C. Division of Marine Fisheries personnel from the Washington, Morehead City, Elizabeth City, and Wilmington Offices.

1991 STUDIES:

In 1991 the number of sites to be sampled was changed after consultation with the N.C. Division of Marine Fisheries, because of reduced funding from the State. The primary changes occurred outside of the APES area of interest: the White Oak and Cape Fear Rivers were dropped, and Adams Creek on the Neuse River was dropped. The New River and Currituck Sound were retained as sampling locations. In addition to collecting and sampling hemolymph in crabs, temperature, salinity, and dissolved oxygen was measured at each of the collection sites.

The hemolymph hemocyanin concentrations among the blue crabs that were sampled showed differences between the Pamlico, Neuse and New Rivers and the reference locations (Figure 5 and Table II). The crabs from the Pamlico River had the lowest hemocyanin values in July as they did in 1989, but they did not drop as low in the crabs from the Pungo River. The hemocyanin concentrations is crabs from the Neuse River area were uniformly lower than in either 1989 or 1990. The lower hemocyanin concentration was particularly noticeable in May where the average for the three sites (i.e. Oriental, Broad Creek, and Bay River) was less than 20 mg/ml, whereas in 1989 and 1990 the average was 30 mg/ml or greater. The hemocyanin values for crabs from the New River showed an overall decrease through September even with a transient increase in August. The average for September, 9.3 ± 3 mg/ml, was the lowest average that we have recorded in three years of collecting blue crab hemolymph samples. The New River also was the only site to have crabs obviously infected with Parameoba and Hematodinium that were examined at our laboratory. These samples were milky white, opaque, and low in hemocyanin (similar samples have been collected from the New River in 1992).

As a further indication that the blue crabs in the Pamlico River system were not behaving in a normal fashion was the lack of food in the stomach. Mr. Sean McKenna as a part of his hemolymph collecting activity also examined some of the stomachs of crabs that were sampled and found that very few food items were present. This activity was not part of the planned sampling program, so the data only can be treated as anecdotal. It does suggest that the crabs were not feeding normally and deserves to be followed up by a more complete study.

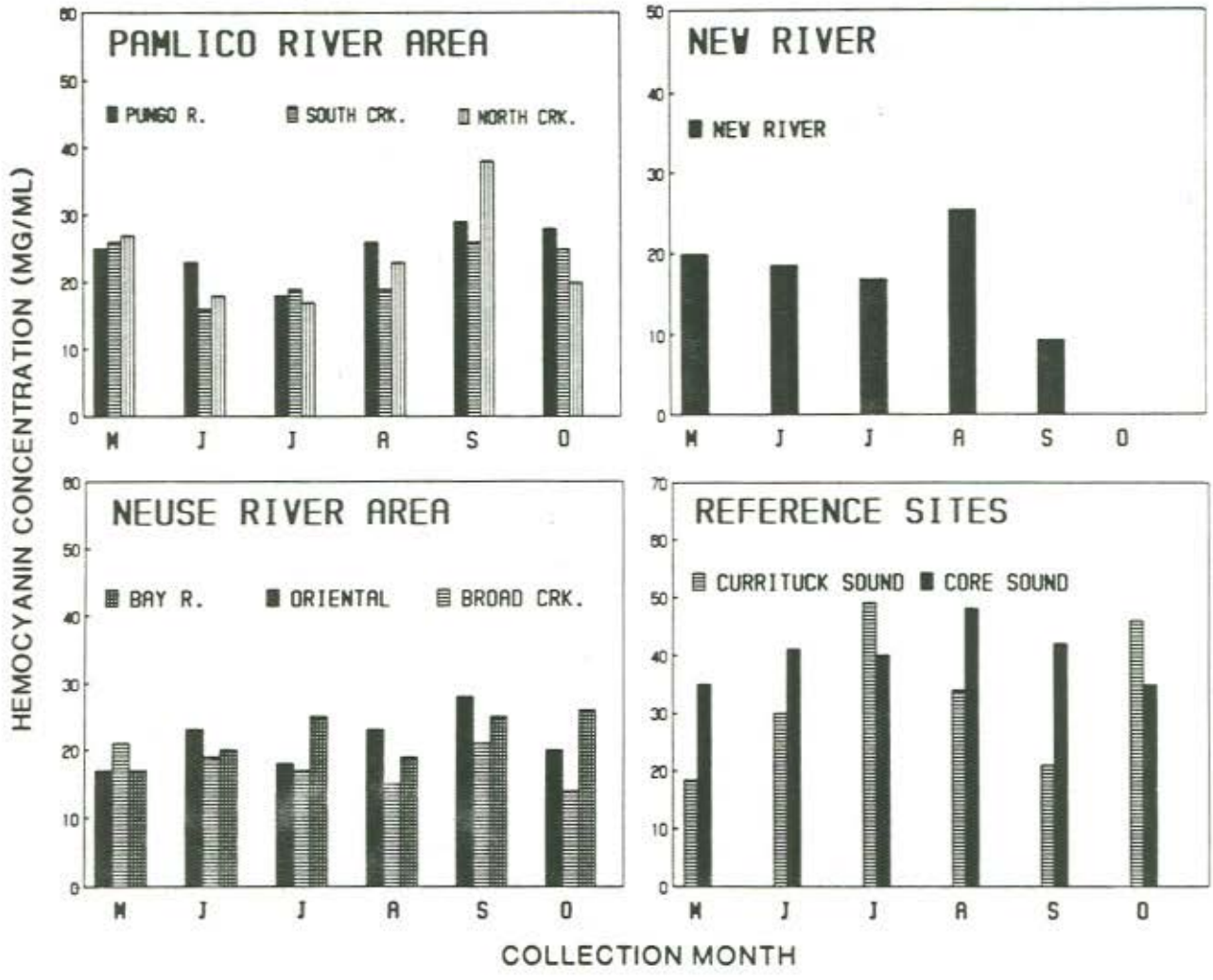

FIGURE 5. Mean hemolymph hemocyanin concentrations for blue crabs collected from the Pamlico and Neuse River areas, New River and the Reference sites in 1991. Each bar of the graph is a mean of five to ten crabs. Locations are shown in Figure 1. The crabs and hemolymph samples were collected by N.C. Division of Marine Fisheries personnel from the Washington, Morehead City, Elizabeth City, and Wilmington Offices.

Table II. Temperature, salinity and dissolved oxygen data for the different sampling locations in the Pamlico and Neuse Rivers and Core and Currituck Sounds by month during 1991. All values are measurements at the surface and bottom. Hemocyanin concentrations are a mean of 10 individual blue crab hemolymph measurements at 335 nm (Mean±SE).

LOCATION	MONTH	TEMPERATURE C°	SALINITY P.P.T.	DISSOLVED O_2 P.P.M.	HEMOCYANIN MG/ML
PAMLICO RIVER SYSTEM					
SOUTH CREEK	May	23.6/23.2	8.5/6.4	7.3/6.9	25.5±2
	June	27.3/27.0	10.5/10.5	7.8/7.5	16.4±3
	July	30.7/30.1	14.7/14.7	6.7/6.5	19.3±2
	August	27.5/27.1	12.8/13.0	6.4/5.4	19.0±3
	September	NM	NM	NM	26.2±2
	October	19.7/19.6	11.7/11.8	8.2/8.1	24.9±5
NORTH CREEK	May	26.3/25.7	7.9/8.4	8.5/6.5	27.4±4
	June	27.8/25.6	11.0/11.6	7.0/4.0	18.0±3
	July	31.0/30.8	14.0/18.8	5.9/5.4	17.3±2
	August	27.4/27.3	14.1/14.1	6.9/6.3	22.7±4
	September	NM	NM	NM	38.2±4
	October	20.5/19.9	12.7/13.3	8.3/8.0	20.1±5
PUNGO RIVER	May	23.7/23.7	10.7/10.7	7.2/6.7	25.3±2
	June	25.6/ -	14.0/ -	NM	22.7±2
	July	29.5/29.2	14.6/14.6	5.5/4.5	18.2±2
	August	27.5/27.5	11.2/11.2	8.2/8.2	25.7±2
	September	NM	NM	NM	28.5±6
	October	19.8/19.6	14.8/14.8	8.5/8.5	28.3±9
NEUSE RIVER SYSTEM					
ORIENTAL	May	27.7/ -	15.0/ -	NM	17.3±2
	June	28.4/27.5	14.5/15.7	7.3/7.2	23.0±5
	July	30.8/28.8	17.0/18.6	7.5/4.7	17.7±2
	August	27.6/27.5	15.0/15.0	NM	23.0±2
	September	30.0/28.9	15.4/15.9	7.6/6.8	28.3±2
	October	20.8/20.6	13.1/13.5	8.6/7.3	20.0±3
BROAD CREEK	May	29.7/28.2	15.6/16.1	NM	21.1±3
	June	29.1/27.6	15.0/16.0	7.3/3.6	19.1±3
	July	31.0/29.5	18.8/19.7	7.5/4.7	16.7±2
	August	27.4/27.1	17.0/18.0	NM	14.9±4
	September	29.9/28.7	18.4/19.7	7.0/4.3	21.0±3
	October	21.0/20.5	16.1/16.2	8.5/8.4	14.4±2

BAY RIVER	May	29.0/27.9	15.8/16.9	NM	17.2±2
	June	27.6/26.5	15.7/16.9	6.1/4.0	20.2±4
	July	30.8/29.5	19.1/19.2	7.5/4.5	25.4±4
	August	29.9/26/9	19.2/19.4	NM	19.0±3
	September	29.6/29.9	21.0/21.2	7.2/7.4	24.8±3
	October	18.4/18.4	18.0/18.0	8.6/8.6	26.1±5
REFERENCE SITES					
CORE SOUND					
	May	23.0/23.0	30.0/30.0	NM	34.6±5
	June	28.5/27.0	30.0/30.0	NM	40.6±3
	July	32.0/31.5	20.0/25.0	NM	39.8±6
	August	NM	NM	NM	48.3±3
	September	26.1/ -	30.0/ -	NM	42.3±2
	October	NM	NM	NM	41.1±2
CURRITUCK SOUND					
	May	NM	NM	NM	17.6±6
	June	23.6/23.6	1.3/1.9	NM	30.4±7
	July	27.6/27.5	3.4/3.3	NM	48.6±9
	August	27.3/ -	2.3/ -	NM	33.6±8
	September	NM	NM	NM	21.2±8
	October	19.2/ -	1.2/ -	NM	45.6±8

Data collected on the Pamlico River and Neuse River were collected by the N.C.D.M.F., Washington Office, and Currituck Sound by the Elizabeth City Office, and Core Sound by a volunteer crab fisherman.

In 1991, the NC Division of Marine Fisheries also collected dissolved oxygen, salinity, and temperature data at the sites where the blue crabs were collected, particularly in the Pamlico and Neuse Rivers (Table II). While these data represent a single day-time measurement of dissolved oxygen on the days when the collections occurred, they do indicate that there may be a correlation between the oxygen concentrations and the blue crab hemocyanin concentrations. This correlation, low dissolved oxygen and low hemocyanin concentrations, was most convincing among the crabs collected from North and Broad Creeks (Table II).

Since the emphasis of this investigation was on the blue crab populations in the Pamlico and Neuse River systems, the statistical analyses were directed toward testing for statistically significant interactions in those systems. If additional sampling sites in the southern portions of State were included, they could influence the interpretation of the data from the target sites. Analysis of the blue crab hemocyanin concentrations by ANOVA for 1989, 1990, and 1991 revealed highly significant interactions ($p < 0.001$) between months and locations (Table III A.). The data from collections for the years 1989 and 1990, indicated that the patterns of change in hemocyanin concentrations over

Table III. Analyses of variance of blue crab hemocyanin concentr-ations for 1989, 1990, and 1991 (A.) at all locations by month and (B.) in three regions by month and year. (** - P<0.05)

A.

1989

SOURCE	DF	MEAN SQUARE	F VALUE	
LOCATION	7	1509.61868	7.97	**
MONTH	1	56.39812	0.30	
MONTH X LOCATION	7	1273.56809	6.72	**

1990

SOURCE	DF	MEAN SQUARE	F VALUE	
LOCATION	7	656.123809	4.12	**
MONTH	1	1558.955035	28.63	**
MONTH X LOCATION	7	838.241664	5.26	**

1991

SOURCE	DF	MEAN SQUARE	F VALUE	
LOCATION	7	84.697913	0.51	
MONTH	1	1920.879951	11.53	**
MONTH X LOCATION	7	237.281143	1.12	

B.

SOURCE	DF	MEAN SQUARE	F VALUE	
REGION	2	12187.22412237	134.83	**
YEAR	2	1956.03185933	15.73	**
MONTH	5	1733.08371860	6.01	**
REGION X YEAR	1	1566.27973910	7.25	**
REGION X MONTH	10	12137.88189020	7.89	**
REGION X YEAR X MONTH	25	21750.32670610	5.52	**

time (months) varied among the locations sampled. In 1991, however, no interaction was detected, nor were differences among locations, although significant differences among months were, implying that in 1991 there was some consistency among the locations in the pattern of change in hemocyanin over time. For further analysis by ANOVA, the data were grouped by region (Pamlico, Neuse, and Reference) (Table III

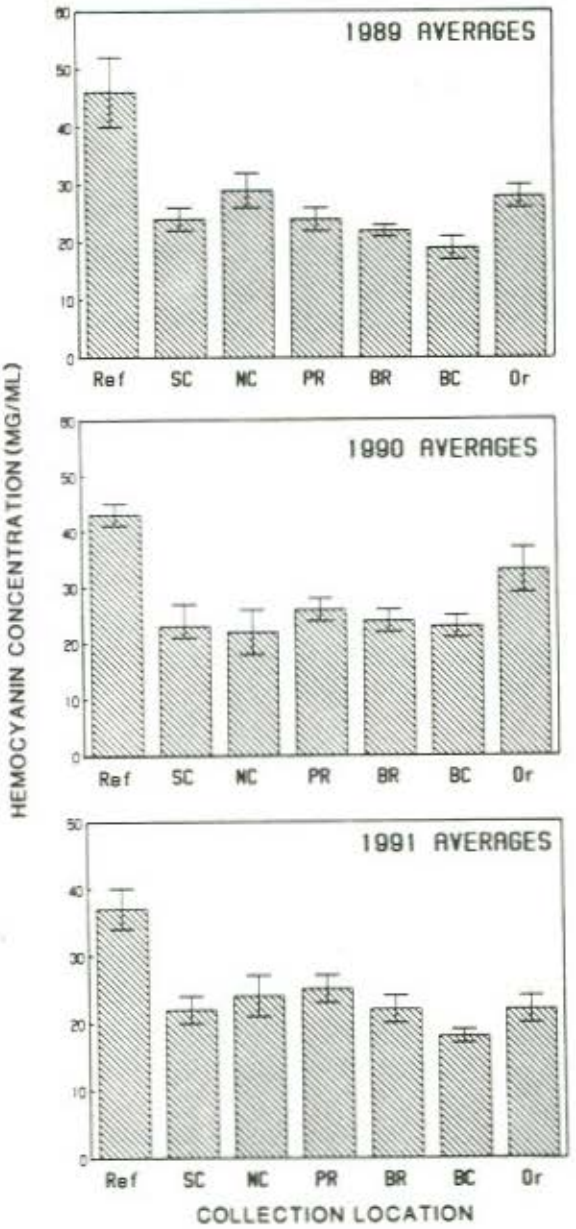

FIGURE 6. Six month average hemocyanin concentrations measured in blue crabs from reference and Pamlico/Neuse River locations during the 1989, 1990, and 1991 sampling periods. The vertical line indicates one standard error. The locations from left to right are: Ref., control/reference; S.C., South Creek; N.C., North Creek; P.R., Pungo River; BR., Bay River; B.C., Broad Creek; and Or., Oriental. Each bar of the graph is an average of 50 to 100 crab samples.

B.). The analysis detected significant region x year x month interaction, indicating a lack of consistency in the temporal patterns among regions and years. Examination of mean hemocyanin concentrations reveal that the Pamlico and Neuse regions exhibited similar seasonal patterns in 1989 and 1991. However, in 1990, the Pamlico region, after reaching a maximum in June showed a steady decline in average hemocyanin concentration through September. In contrast, the Neuse region declined from a maximum in May to a minimum in June and thereafter exhibited intermediate hemocyanin concentrations until October. With the exception of May, June and September of 1990, the concentrations of hemocyanin in crabs from the Reference region averaged well above those for the other two regions.

1989, 1990, & 1991 AVERAGES:

Six month averages in hemocyanin concentrations for crabs collected from the reference and Pamlico Sound locations were calculated for each of three years of the study (Figure 6). The pooled data underscores the differences between the southwestern Sound and the Beaufort/Bogue and Core Sound sites. At this time we do not know whether the observed temporal and spatial differences in hemocyanin concentrations are natural, or are the result of some stressing agent that is affecting hemocyanin metabolism of blue crabs from the Pamlico Sound estuarine systems.

HEMOLYMPH HEMOCYTE CONCENTRATION:

Intermolt male blue crabs of similar size were collected from either Core Sound or the Pamlico River. Crabs were bled using a 22 GA needle and 3 ml syringe having 2 ml of formalin. Cells were counted with an automated cell counter (Model ZM, Coulter Electronics) and blood smears were prepared with a cytocentrifuge (Shandon Instruments) and stained with eosin-phloxine (Appendix II). A total of 38 and 39 crabs from each site were sampled. As can be seen in Table IV, the hemocyte counts for the Pamlico River crabs were significantly lower than those from Core Sound animals ($P < 0.0001$ by t-test and ANOVA).

Table IV. Hemocyte counts of hemolymph collected from blue crabs from Core Sound and the Pamlico River in the Albemarle/ Pamlico Estuary. Cell counts are cells/ul.

	Core Sound	Pamlico River
Number of crabs	(n=38)	(n=39)
Mean	28,289	14,529
Std Dev	±11,431	±5,934
Range	13,648 - 47,600	5,168 - 27,740

These hemocyte counts are similar to those reported by Sawyer et al. (1970) for male blue crabs, with cell counts ranging from 6,000 to 56,000 cells/ul with a mean of 35,000 $\pm$ 23,700 cells/ul (N=25).

The lower cell counts in the Pamlico River crabs substantiates our previous studies (Noga et al. 1990) that showed that blue crabs from low salinity areas of the Albemarle/Pamlico Estuary had significantly lower levels of hemolymph antibacterial activity. Most, if not all, of the hemolymph antibacterial activity resides in the hemocytes (Noga and Arroll, unpublished data) and thus there should be a correlation between hemolymph hemocytes and antibacterial activity. The cause of the reduced hemocyte numbers/antibacterial activity is unknown, but of concern, since it may predispose Pamlico River crabs to opportunistic infections, such as shell disease, which is most prevalent in the low salinity tributaries of the River .

Numerous smears of blue crab hemolymph were examined microscopically during this study and there was only one documented case of a hemolymph parasite. The identified organism was Hematodynium which is a parasitic dinoflagellate. As a result it should be safe to say that parasitic diseases were not responsible for the observed low hemocyanin concentrations in the Pamlico River.

EFFECTS OF HYPOXIA ON HEMOLYMPH HEMOCYANIN CONCENTRATION:

The results of the hypoxic exposure did not show any significant effects on blue crab hemolymph hemocyanin concentration ($P > 0.05$) (Table V). This type of short-term exposure (14 days) did not elicit an immediate increase in hemocyanin synthesis in the crabs that were tested. In addition, the ratio of optical density at 280 and 334 nm also did not change significantly, which suggests that protein synthesis (i.e. measured at 280 nm) was not affected by the reduced oxygen.

Table V. Hemolymph hemocyanin concentrations and OD_{280}/OD_{334nm} ratios from blue crabs exposed to ambient and hypoxic dissolved oxygen concentrations for 14 days in the laboratory.

	CONTROL	HYPOXIA
Hemocyanin (mg/mL)	31.7 $\pm$ 11.3 (n=6)	34.7 $\pm$ 12.0 (n=8)
$OD_{280}/OD_{334\ nm}$	7.9 $\pm$ 1.5 (n=6)	7.0 $\pm$ 0.8 (n=8)

DISCUSSION:

The primary finding of these investigations, which have covered three summers 1989 - 1991, is that the concentration of hemocyanin in the hemolymph of blue crabs from selected eastern North Carolina estuaries appears to be correlated strongly with the location of collection and time of year. Since water quality has always been a major concern of the APES mission, it would be attractive, therefore, to hypothesize that some specific toxicant is causing the observed depressed concentrations. This scenario does not appear to have much support, because in both the Pamlico and Neuse Rivers there is no evidence of high contaminant concentrations (there are some "hot spots" in the sediments [Riggs et al. 1989], but it is questionable whether that is relevant). This line of reasoning also is supported by the results of organic contaminant measurements made on pooled samples of hepatopancreas collected from crabs in the Pamlico River and Core Sound in 1989 (analyses were done at the USEPA Laboratory in Duluth). The Pamlico River animals did not have higher contaminant bodyburdens or a significantly different array of contaminants than those measured in crabs from Core Sound (Copies of data are attached as APPENDIX II). In earlier investigations in the Pamlico River concerned with shell disease in blue crabs (Noga et al. 1990), we measured the trace metal concentrations in the hepatopancreas of both diseased and healthy blue crabs and were unable to show any significant elevation of metals. It did appear that the concentrations of copper and zinc in the Pamlico River crabs was somewhat depressed relative to those collected from the reference areas, Core and Bogue Sounds. It appears, therefore, that there is no direct correlation between contaminant accumulation and either depressed hemocyanin concentrations or shell disease among the blue crabs from the Pamlico or Neuse Rivers.

Hypoxia, which is known to occur in both the Pamlico and Neuse systems during the summer (data from the N.C. Division of Environmental Management) when the water temperatures are high, is a candidate for being a major contributor to the observed effects on hemocyanin concentrations in blue crab populations. DeFur and colleagues (1990), however, have shown experimentally that hypoxia causes increased hemocyanin concentrations in blue crabs from Chesapeake Bay where historically hypoxic events occur routinely. Brouwer (APES Report #92-09) was unable to demonstrate significant increases in hemocyanin in blue crabs held under laboratory controlled hypoxia. Pihl et al. (1991) in a field investigation measured hemocyanin in blue crabs in relation to hypoxic events in the lower York River, Chesapeake Bay. In their investigation they did not find any significant effect of these events on the hemocyanin concentrations in the hemolymph of the blue crabs tested. It is reasonable, however, to expect that exposure to chronic low dissolved oxygen should result in increased hemocyanin synthesis in blue crabs exposed to such conditions, but the data does not support such speculation. The crabs in the Pamlico and Neuse systems apparently can not cope with the stresses of reduced dissolved oxygen and elevated temperatures as effectively as blue crabs from other locations within and outside the state.

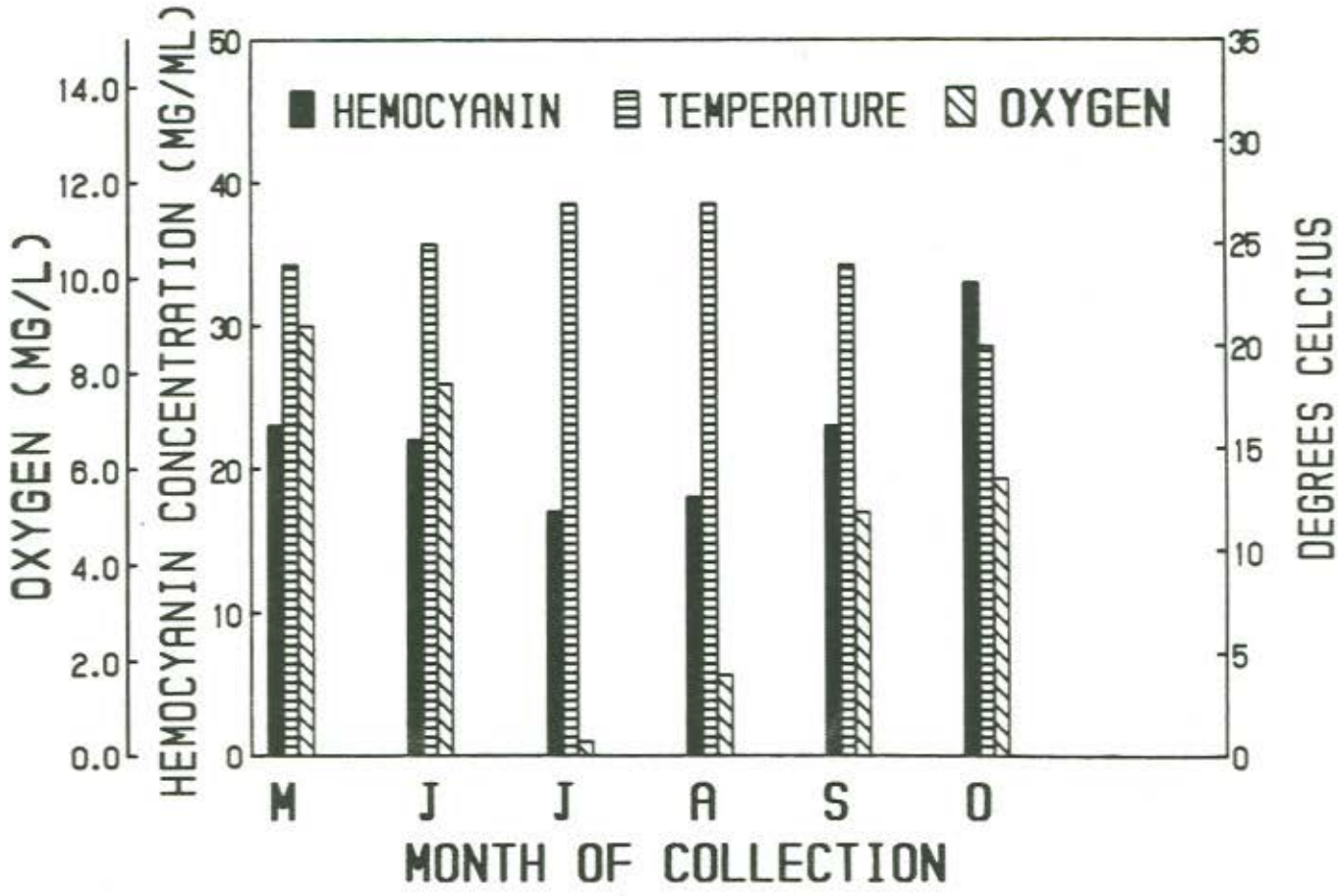

FIGURE 7. Relationship between blue crab hemolymph hemocyanin concentrations and water temperature and dissolved oxygen concentrations on South Creek in 1989. The hemocyanin measurements are the same as in Figure 3. The temperature and dissolved oxygen data was collected by the N.C. Division of Environmental Management at a location in the vicinity of where the blue crabs were collected by otter trawl. Both the oxygen and temperature data was collected at a depth of 4 meters.

In addition to dissolved oxygen reduced salinity has a significant positive effect on hemocyanin metabolism in marine crustaceans. It has been shown that low salinity stimulates the synthesis of hemocyanin in blue crabs under laboratory conditions (Marius Brouwer, unpublished data; Engel and Noga, unpublished data; and Charlotte Mangum, personal communication). In 1988 as part of a field monitoring program, we measured hemocyanin in blue crabs from a "clean" low salinity estuary on the Gulf of Mexico, Heron Bay, MS. Those animals had higher hemocyanin concentrations than animals collected from high salinity environments which supports the above mentioned observations. Also, in 1990 and 1991 blue crabs collected from the Cape Fear River and Currituck Sound, both of which are low salinity areas, tended to have elevated concentrations of hemocyanin relative to all other sites. Therefore, crabs from the Pamlico and Neuse River systems which have medium to low salinities, depending on rainfall, should have concentrations of hemocyanin equivalent to or higher than those collected in Core Sound where the salinities are routinely higher. This relationship has not been demonstrated by the data collected in this investigation.

South Creek on the Pamlico River is a sampling location where the blue crabs have been shown to have depressed concentrations of hemocyanin in their hemolymph in all three years of our investigations. Utilizing data supplied by the NCDEM on dissolve oxygen and water temperatures in South Creek during the summer of 1989, changes in water temperature and dissolved oxygen in the Creek could be correlated with changes in hemocyanin among the crabs collected from that locality (Figure 7). In July, when the water temperature was the highest, and dissolved oxygen the lowest, the hemocyanin concentrations were the lowest. From August through October the dissolved oxygen in the Creek increased and the hemocyanin concentrations also increased by almost a factor of two. This same type of correlation between oxygen and hemocyanin concentrations can be made in 1991 in North Creek and Broad Creek by comparing the oxygen measurements made at the time of collection by NCDMF and the hemocyanin concentrations that were measured (SEE: Table II and Figure 5). It is also interesting that the crabs from the South Creek area have significantly lower hemocyte concentrations than crabs from the Core Sound area (SEE: Results - Hemolymph Hemocyte Concentrations). This observation may be another example of a stress response.

It has been suggested that the consistent low hemocyanin concentrations measured in crabs from South Creek may be associated with the higher prevalence of shell disease among the crabs from that locality. As part of our investigation of the etiology of shell disease in blue crabs (study funded by APES in 1988, 1989, and 1990; see Noga, et al. 1990), we measured the concentrations of hemocyanin in the hemolymph of both "healthy" and diseased crabs that were captured in the same general area of the Pamlico River. The measurements show that there are no consistent differences in hemolymph

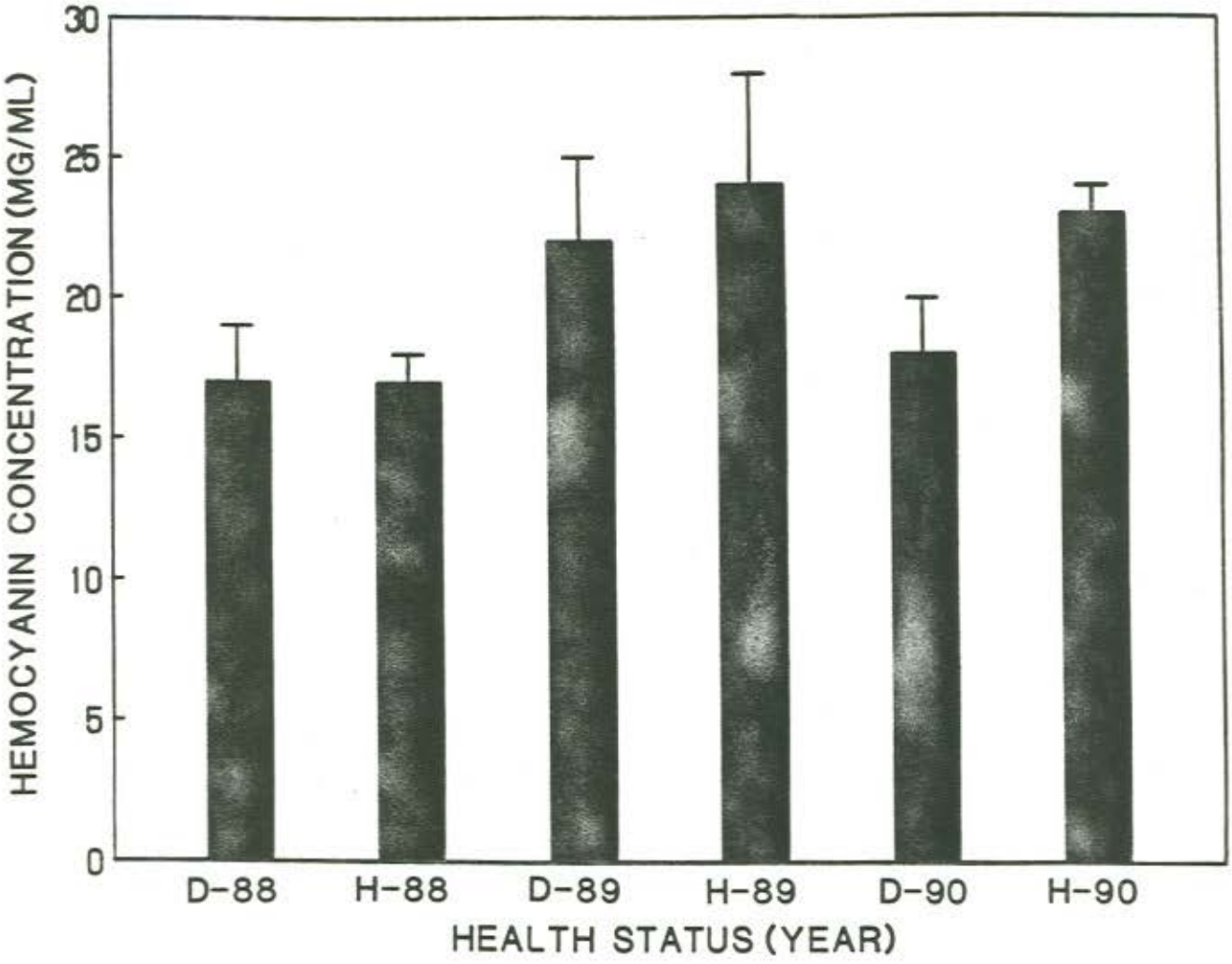

FIGURE 8. Hemolymph hemocyanin concentrations in blue crabs collected in the Pamlico River in 1988, 1989, and 1990 that have been shown to have shell disease (D) and those that appear to be clinically healthy (H). Each bar of the graph represents a mean plus and minus one standard error. All crabs were collected from commercial fishermen at Etlas Henries Crab House on the Pamlico River.

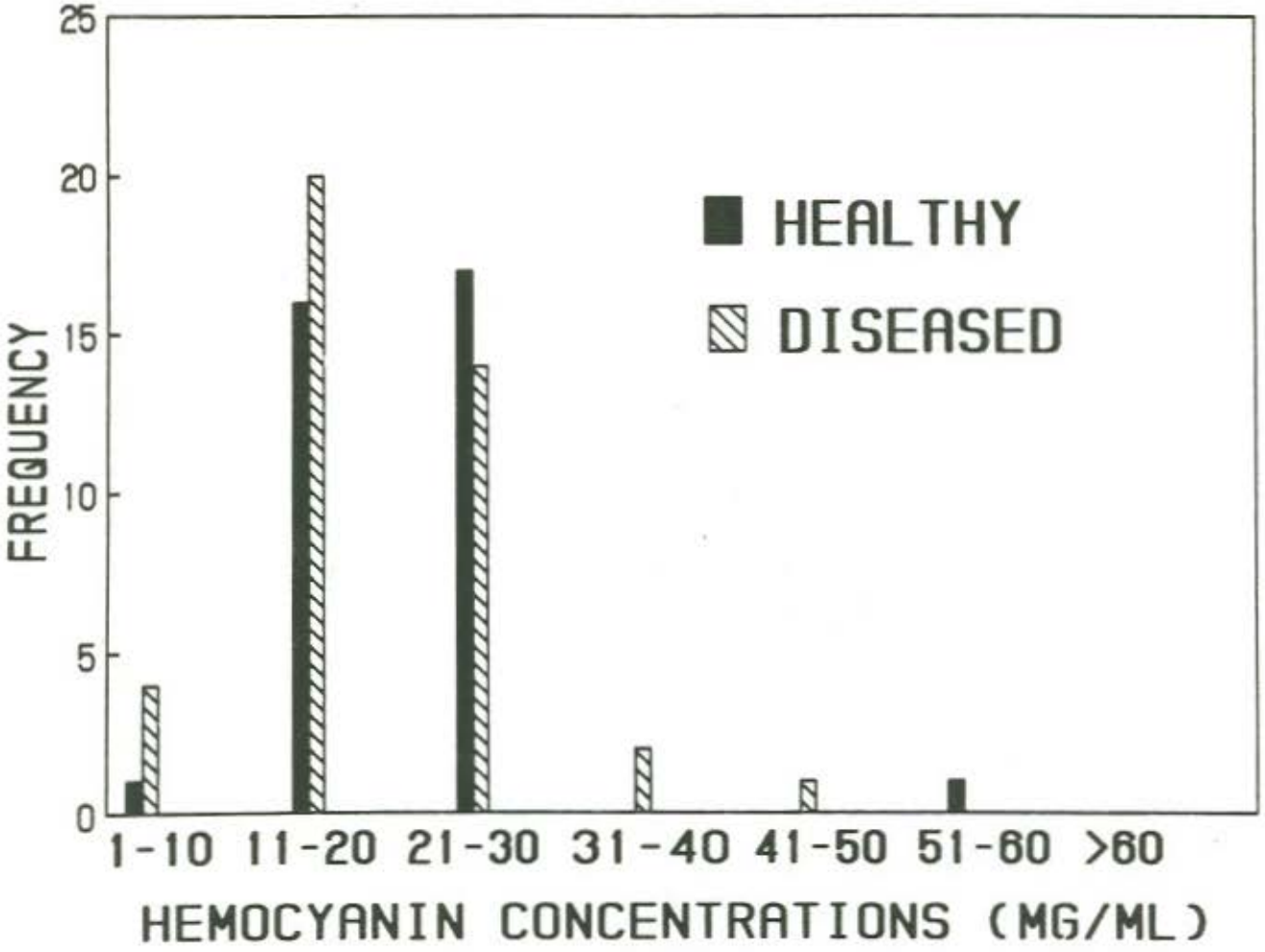

FIGURE 9. Frequency distribution of hemocyanin concentrations in the hemolymph of clinically healthy blue crabs and those that have shell disease (Noga et al. 1990) collected from the Pamlico River during 1988, 1989, and 1990.

hemocyanin concentrations when healthy or diseased crabs are compared (Figures 8). This observation is also supported by the lack of difference in the frequency distributions for healthy and diseased animals collected over the three years of the study (Figure 9). While the hemocyanin concentrations were low, they were not significantly different from those measured in crabs collected from the Pamlico River area during the same period. Even though there is no demonstrable correlation between hemocyanin concentration and shell disease, there is a direct correlation between low hemocyanin concentrations and strength of the crab's nonspecific immune response (Noga et al. 1990). Since the relationship between hemocyanin and the bacteriocidal protein concentration have not been determined, it is not possible to speculate about the relationship between the two proteins and crab health. Apparently, however, the shell disease process itself does not seem to be a controlling factor in the metabolism and turnover of hemocyanin in Pamlico River blue crabs.

The physiological processes that are involved in producing the observed variations in hemocyanin concentration in blue crabs are the result of complex interactions between dissolved oxygen concentration, temperature, salinity, and nutrition. The proposed hypothesis is that reduced oxygen tension in combination with elevated temperature entrains the crabs in the river and creeks, and, because the hypoxic conditions are wide spread, the crabs can not escape to higher oxygen conditions. The hypoxic conditions intensify at night which exacerbates the situation. Once the crabs are entrained, the combination of elevated temperature, which increases the metabolic rates, and the lowered dissolved oxygen may cause lethargy in the crabs and a decrease in feeding success. The decreased feeding and increased energy requirements could result in a nutritional deficit in the crabs that may be expressed as a decrease in hemocyanin concentration. This hypothesis is supported by the observation that crabs from the Pamlico River system have relatively empty stomachs relative to crabs collected in the Sound (Sean McKenna, NCDMF, Washington, NC, personal communication). The lowest concentrations tended to occur during July and August when the water temperatures tend to be the highest, and then increase in September and October as the water cools (data supplied by the N.C. Division of Environmental Management and the Division of Marine Fisheries). Temperature and dissolved oxygen measurements made in the Pamlico and Neuse Rivers (Garrett and Bales 1991) show that the lowest bottom and midwater dissolved oxygen and highest temperatures were measured in June and July in 1989 and in August and September in 1990. These data correlate well with the lowest hemocyanin concentrations measured in blue crabs collected from areas close to where oxygen-temperature data was obtained. Such a correlation strongly implicates dissolved oxygen and temperature as contributing factors to the reduced hemocyanin concentrations. An alternative explanation for the reduced feeding success could be that the organisms on which the crabs feed leave the area or are killed due to low dissolved oxygen concentrations. Both sets of events would produce the same effects on the crabs if they were unable to escape the affected area.

The scenario spelled out above is supported by laboratory studies of the effects of fasting with Carcinus maenas (Uglow 1969) and molting and feeding with Homarus gammarus (Hagerman 1983), and in field studies with lobsters, Nephrops norvegicus, from the North Sea (Hagerman and Baden 1988; Baden et al. 1990). In the laboratory experiments, it was demonstrated that fasting and lack of feeding during the premolt period caused a significant decrease in hemolymph protein concentration (hemocyanin comprises about 90% of the total protein in the hemolymph). In both cases, the hemolymph proteins were used as an alternative protein source. In the field investigation with lobsters, hypoxia caused the lobsters to decrease their feeding activity which resulted in lowered hemocyanin concentrations (Hagerman and Baden 1988). Baden et al. (1990) also indicated that when the oxygen was reduced below 15% there was a rapid catabolism of hemocyanin by lobsters. In our own experience (Engel and Brouwer 1987), we demonstrated that blue crabs that were dredged up from the mud in early spring had reduced levels of hemocyanin apparently caused by overwintering and not feeding during the hibernation period. Resumption of feeding caused an immediate increase in the hemocyanin concentration and an apparent stimulation of hemocyanin synthesis.

An alternative explanation is that the lowered hemocyanin concentrations in blue crabs from the Pamlico and Neuse Rivers was the result of a reduced food supply. This situation could have occurred if the low dissolved oxygen either killed or forced out the organisms that the blue crab feeds upon. The results would once again by poor nutrition and reduced hemocyanin. The difficulty is that we have no data on food availability, but this scenario would also fit the results that we have observed.

This data set, therefore, suggests that long-term exposure to reduced dissolved oxygen may indirectly cause the observed reductions in hemocyanin concentrations among the blue crabs in the Pamlico and Neuse River systems. Crabs with markedly reduced levels of hemocyanin would be expected to be at a physiological disadvantage which could result in increased susceptibility to parasitic infection, inability to molt successfully, and an inability to repair shell trauma which could serve as a focus for shell disease. This hypothesis is supported by the work of Noga et al. (1990) that shows that blue crabs from the areas where the hemocyanin concentrations were the lowest also had the lowest titers of antibacterial activity in the hemolymph. The data suggest that levels of dissolved oxygen normally considered not to be stressful, 30-50% saturation, may not be sufficient for the long-term maintenance of physiological integrity, especially if the temperature of the water is also elevated. If such a hypothesis is correct, it could have a significant effect on the interpretation of water quality data, because generally low dissolved oxygen concentrations are not considered a problem until they fall below 20-30% saturation.

INFORMATION ADDED WHILE REPORT WAS IN REVIEW:

As part of a cooperative research effort with investigators at the Canadian Fisheries and Oceans, Institute of Ocean Sciences, Victoria, B.C. hemolymph samples were taken from Dungeness crabs, Cancer magister, that were collected from areas near the outfalls of Kraft pulp mills. These areas are characterized by having low dissolved oxygen concentrations and high nutrients. Hemocyanin concentrations were measured in hemolymph samples from three pulp mills and a reference site in the Strait of Georgia, B.C. The hemocyanin concentrations of the hemolymph samples of the crabs from the pulp mills all were higher than the reference site by almost a factor of two. Since the temperature is never elevated and food is readily available, these crabs apparently respond to low dissolved oxygen concentrations by increasing the hemocyanin concentration. There is also evidence of elevated digestive gland metallothionein concentrations which would be expected if hemocyanin was being synthesized. While these data are preliminary, they do suggest that our approach is appropriate.

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APPENDIX I

1991 BLUE CRAB HEMOCYANIN DATA

ecord#	LOCATION	HEMOLYMPH	RATIO	SEX	DATE	MONTH
1081	SOUTH CREEK	27.97	8.3	M	05/10/91	5
1082	SOUTH CREEK	18.05	11.7	M	05/10/91	5
1083	SOUTH CREEK	19.85	6.6	M	05/10/91	5
1084	SOUTH CREEK	21.37	8.4	M	05/10/91	5
1085	SOUTH CREEK	50.32	10.0	M	05/10/91	5
1086	SOUTH CREEK	18.52	5.3	M	05/10/91	5
1087	SOUTH CREEK	18.42	9.1	M	05/10/91	5
1088	SOUTH CREEK	18.73	14.1	M	05/10/91	5
1089	SOUTH CREEK	34.12	9.8	M	05/10/91	5
1090	SOUTH CREEK	28.75	5.1	M	05/10/91	5
1091	PUNGO RIVER	24.86	10.7	M	05/13/91	5
1092	PUNGO RIVER	26.01	5.5	M	05/13/91	5
1093	PUNGO RIVER	17.14	10.1	F	05/13/91	5
1094	PUNGO RIVER	26.35	5.3	M	05/13/91	5
1095	PUNGO RIVER	40.02	6.6	M	05/13/91	5
1096	PUNGO RIVER	29.56	11.0	F	05/13/91	5
1097	PUNGO RIVER	22.43	6.3	M	05/13/91	5
1098	PUNGO RIVER	14.79	10.8	M	05/13/91	5
1099	PUNGO RIVER	20.48	5.4	M	05/13/91	5
1100	PUNGO RIVER	31.12	6.7	M	05/10/91	5
1101	NORTH CREEK	26.97	7.9	M	05/14/91	5
1102	NORTH CREEK	46.28	8.4	M	05/14/91	5
1103	NORTH CREEK	31.72	9.3	M	05/14/91	5
1104	NORTH CREEK	37.93	10.3	M	05/14/91	5
1105	NORTH CREEK	19.46	6.8	M	05/14/91	5
1106	NORTH CREEK	40.62	6.9	F	05/14/91	5
1107	NORTH CREEK	10.88	10.4	M	05/14/91	5
1108	NORTH CREEK	29.95	4.9	M	05/14/91	5
1109	NORTH CREEK	13.80	7.0	M	05/14/91	5
1110	NORTH CREEK	14.95	6.4	M	05/14/91	5
1111	CORE SOUND	41.06	5.1	M	05/15/91	5
1112	CORE SOUND	18.08	5.6	M	05/15/91	5
1113	CORE SOUND	27.68	5.1	M	05/15/91	5
1114	CORE SOUND	28.96	5.0	M	05/15/91	5
1115	CORE SOUND	42.42	5.1	M	05/15/91	5
1116	CORE SOUND	29.63	5.0	M	05/15/91	5
1117	CORE SOUND	10.38	5.4	M	05/15/91	5
1118	CORE SOUND	45.08	5.1	M	05/15/91	5
1119	CORE SOUND	40.41	5.1	M	05/15/91	5
1120	CORE SOUND	62.61	5.0	M	05/15/91	5
1121	NEW RIVER	15.37	18.0	F	05/16/91	5
1122	NEW RIVER	16.25	11.5	M	05/16/91	5
1123	NEW RIVER	36.68	10.0	F	05/16/91	5
1124	NEW RIVER	22.93	15.1	F	05/16/91	5
1125	NEW RIVER	15.91	6.4	M	05/16/91	5
1126	NEW RIVER	14.77	10.0	M	05/16/91	5
1127	NEW RIVER	26.84	13.7	F	05/16/91	5
1128	NEW RIVER	16.80	20.4	M	05/16/91	5
1129	NEW RIVER	19.49	7.6	F	05/16/91	5
1130	NEW RIVER	12.70	6.9	M	05/16/91	5
1131	ORIENTAL	21.05	4.8	M	05/24/91	5
1132	ORIENTAL	17.11	4.7	M	05/24/91	5
1133	ORIENTAL	16.36	6.8	F	05/24/91	5
1134	ORIENTAL	11.41	7.2	M	05/24/91	5
1135	ORIENTAL	5.51	7.4	F	05/24/91	5
1136	ORIENTAL	19.16	5.5	M	05/24/91	5
1137	ORIENTAL	27.56	7.1	M	05/24/91	5
1138	ORIENTAL	16.46	6.3	F	05/24/91	5
1139	ORIENTAL	18.51	5.1	F	05/24/91	5
1140	ORIENTAL	20.00	4.7	M	05/24/91	5
1141	BROAD CREEK	17.34	7.6	F	05/28/91	5
1142	BROAD CREEK	38.22	8.6	M	05/28/91	5

1143	BROAD CREEK	30.69	6.4	M	05/28/91	5
1144	BROAD CREEK	28.73	6.4	M	05/28/91	5
1145	BROAD CREEK	22.99	6.2	M	05/28/91	5
1146	BROAD CREEK	16.87	8.5	M	05/28/91	5
1147	BROAD CREEK	9.11	7.2	M	05/28/91	5
1148	BROAD CREEK	12.24	6.6	M	05/28/91	5
1149	BROAD CREEK	25.13	5.8	M	05/28/91	5
1150	BROAD CREEK	9.37	5.5	M	05/28/91	5
1151	BAY RIVER	17.95	12.9	M	05/30/91	5
1152	BAY RIVER	22.07	12.3	M	05/30/91	5
1153	BAY RIVER	19.44	9.1	M	05/30/91	5
1154	BAY RIVER	27.33	5.3	M	05/30/91	5
1155	BAY RIVER	11.97	6.1	F	05/30/91	5
1156	BAY RIVER	21.62	5.3	M	05/30/91	5
1157	BAY RIVER	13.36	8.6	F	05/30/91	5
1158	BAY RIVER	21.49	9.3	M	05/30/91	5
1159	BAY RIVER	9.00	8.5	F	05/30/91	5
1160	BAY RIVER	7.78	12.7	F	05/30/91	5
1161	CURRITUCK	18.27	5.5	M	05/30/91	5
1162	CURRITUCK	11.23	4.9	M	05/30/91	5
1163	CURRITUCK	7.19	6.4	M	05/30/91	5
1164	CURRITUCK	13.22	6.1	M	05/30/91	5
1165	CURRITUCK	27.77	4.9	M	05/30/91	5
1166	CURRITUCK	14.13	5.0	M	05/30/91	5
1167	CURRITUCK	12.71	6.0	M	05/30/91	5
1168	CURRITUCK	30.21	4.8	M	05/30/91	5
1169	CURRITUCK	17.57	5.3	M	05/30/91	5
1170	CURRITUCK	24.17	4.7	M	05/30/91	5
1171	CAPE FEAR	53.49	5.2	F	06/05/91	6
1172	CAPE FEAR	22.61	5.1	M	06/05/91	6
1173	CAPE FEAR	24.36	4.6	F	06/05/91	6
1174	CAPE FEAR	58.06	4.7	F	06/05/91	6
1175	CAPE FEAR	34.34	4.8	F	06/05/91	6
1176	CAPE FEAR	50.04	5.7	M	06/05/91	6
1177	CAPE FEAR	51.01	5.3	M	06/05/91	6
1178	CAPE FEAR	50.74	4.7	F	06/05/91	6
1179	CAPE FEAR	36.74	5.5	M	06/05/91	6
1180	CAPE FEAR	39.11	4.8	M	06/05/91	6
1181	CORE SOUND	38.59	6.8	M	06/11/91	6
1182	CORE SOUND	26.56	4.8	M	06/11/91	6
1183	CORE SOUND	50.91	6.1	M	06/11/91	6
1184	CORE SOUND	31.45	6.6	M	06/11/91	6
1185	CORE SOUND	33.48	5.4	M	06/11/91	6
1186	CORE SOUND	42.15	5.9	M	06/11/91	6
1187	CORE SOUND	54.11	4.9	M	06/11/91	6
1188	CORE SOUND	52.38	4.9	M	06/11/91	6
1189	CORE SOUND	40.60	6.3	M	06/11/91	6
1190	CORE SOUND	35.39	4.8	M	06/11/91	6
1191	PUNGO RIVER	34.60	6.1	M	06/13/91	6
1192	PUNGO RIVER	15.68	6.5	M	06/13/91	6
1193	PUNGO RIVER	23.47	6.3	F	06/13/91	6
1194	PUNGO RIVER	22.50	10.7	F	06/13/91	6
1195	PUNGO RIVER	16.65	6.9	F	06/13/91	6
1196	PUNGO RIVER	22.35	5.0	M	06/13/91	6
1197	PUNGO RIVER	20.73	6.8	M	06/13/91	6
1198	PUNGO RIVER	23.29	5.0	F	06/13/91	6
1199	PUNGO RIVER	23.31	5.0	F	06/13/91	6
1200	PUNGO RIVER	22.48	6.6	M	06/13/91	6
1201	NORTH CREEK	13.42	6.2	M	06/13/91	6
1202	NORTH CREEK	16.79	7.7	M	06/13/91	6
1203	NORTH CREEK	12.37	6.4	M	06/13/91	6
1204	NORTH CREEK	29.88	8.8	F	06/13/91	6
1205	NORTH CREEK	18.21	4.9	F	06/13/91	6
1206	NORTH CREEK	31.09	13.3	IF	06/13/91	6

1207	NORTH CREEK	11.79	7.7	M	06/13/91	6
1208	NORTH CREEK	24.82	6.1	M	06/13/91	6
1209	NORTH CREEK	10.59	7.2	M	06/13/91	6
1210	NORTH CREEK	9.04	6.4	M	06/13/91	6
1211	SOUTH CREEK	14.34	6.4	F	06/13/91	6
1212	SOUTH CREEK	28.99	7.6	M	06/13/91	6
1213	SOUTH CREEK	20.54	6.5	M	06/13/91	6
1214	SOUTH CREEK	9.24	5.7	M	06/13/91	6
1215	SOUTH CREEK	25.70	6.7	M	06/13/91	6
1216	SOUTH CREEK	8.02	6.4	M	06/13/91	6
1217	SOUTH CREEK	20.50	7.6	M	06/13/91	6
1218	SOUTH CREEK	11.61	6.3	F	06/13/91	6
1219	SOUTH CREEK	10.48	6.1	F	06/13/91	6
1220	SOUTH CREEK	8.20	5.9	M	06/13/91	6
1221	NEW RIVER	32.11	7.3	M	06/19/91	6
1222	NEW RIVER	16.91	15.5	F	06/19/91	6
1223	NEW RIVER	15.82	10.1	F	06/19/91	6
1224	NEW RIVER	29.13	8.2	F	06/19/91	6
1225	NEW RIVER	16.87	6.6	M	06/19/91	6
1226	NEW RIVER	8.40	8.5	F	06/19/91	6
1227	NEW RIVER	13.79	7.5	M	06/19/91	6
1228	NEW RIVER	16.71	16.9	F	06/19/91	6
1229	NEW RIVER	23.86	7.2	M	06/19/91	6
1230	NEW RIVER	11.96	7.6	F	06/19/91	6
1231	ORIENTAL	55.41	5.8	M	06/20/91	6
1232	ORIENTAL	17.97	7.4	M	06/20/91	6
1233	ORIENTAL	33.88	6.1	M	06/20/91	6
1234	ORIENTAL	15.66	5.6	M	06/20/91	6
1235	ORIENTAL	28.39	6.0	F	06/20/91	6
1236	ORIENTAL	9.11	8.0	F	06/20/91	6
1237	ORIENTAL	14.41	6.5	F	06/20/91	6
1238	ORIENTAL	9.54	7.7	F	06/20/91	6
1239	ORIENTAL	23.64	5.6	F	06/20/91	6
1240	ORIENTAL	22.93	5.5	F	06/20/91	6
1241	BROAD CREEK	20.34	5.2	F	06/20/91	6
1242	BROAD CREEK	8.61	5.4	M	06/20/91	6
1243	BROAD CREK	15.91	6.2	F	06/20/91	6
1244	BROAD CREEK	35.30	7.2	F	06/20/91	6
1245	BROAD CREEK	15.03	5.5	M	06/20/91	6
1246	BROAD CREEK	31.76	6.1	M	06/20/91	6
1247	BROAD CREEK	13.39	5.2	F	06/20/91	6
1248	BROAD CREEK	12.26	6.2	F	06/20/91	6
1249	BROAD CREEK	21.98	4.8	F	06/20/91	6
1250	BROAD CREEK	16.95	5.2	F	06/20/91	6
1251	BAY RIVER	26.96	8.3	M	06/21/91	6
1252	BAY RIVER	15.77	8.2	F	06/21/91	6
1253	BAY RIVER	6.18	7.8	F	06/21/91	6
1254	BAY RIVER	7.78	8.0	M	06/21/91	6
1255	BAY RIVER	22.14	7.0	M	06/21/91	6
1256	BAY RIVER	10.40	11.5	F	06/21/91	6
1257	BAY RIVER	27.46	12.7	M	06/21/91	6
1258	BAY RIVER	14.14	6.2	M	06/21/91	6
1259	BAY RIVER	49.28	9.1	F	06/21/91	6
1260	BAY RIVER	22.30	7.2	F	06/21/91	6
1261	CURRITUCK	19.35	5.4	M	06/25/91	6
1262	CURRITUCK	50.99	6.3	M	06/25/91	6
1263	CURRITUCK	21.47	5.0	M	06/25/91	6
1264	CURRITUCK	25.36	5.3	F	06/25/91	6
1265	CURRITUCK	50.94	5.1	M	06/25/91	6
1266	CURRITUCK	14.21	5.9	M	06/25/91	6
1267	CORE SOUND	79.55	5.2	M	07/10/91	7
1268	CORE SOUND	23.58	5.6	M	07/10/91	7
1269	CORE SOUND	53.83	5.5	M	07/10/91	7
1270	CORE SOUND	45.95	5.0	M	07/10/91	7

1271	CORE SOUND	29.53	5.5	M	07/10/91	7
1272	CORE SOUND	45.02	5.0	M	07/10/91	7
1273	CORE SOUND	27.90	5.2	M	07/10/91	7
1274	CORE SOUND	39.82	5.9	M	07/10/91	7
1275	CORE SOUND	37.70	5.1	M	07/10/91	7
1276	CORE SOUND	15.36	5.3	M	07/10/91	7
1277	NEW RIVER	18.65	6.9	M	07/17/91	7
1278	NEW RIVER	19.67	6.6	M	07/17/91	7
1279	NEW RIVER	15.00	7.9	M	07/17/91	7
1280	NEW RIVER	8.77	8.5	F	07/17/91	7
1281	NEW RIVER	15.12	9.5	F	07/17/91	7
1282	NEW RIVER	25.70	6.5	M	07/17/91	7
1283	NEW RIVER	20.87	14.4	F	07/17/91	7
1284	NEW RIVER	13.33	14.4	F	07/17/91	7
1285	NEW RIVER	11.47	8.7	M	07/17/91	7
1286	NEW RIVER	19.56	8.4	M	07/17/91	7
1287	ORIENTAL	13.98	7.5	F	07/23/91	7
1288	ORIENTAL	18.38	8.6	F	07/23/91	7
1289	ORIENTAL	21.09	4.9	F	07/23/91	7
1290	ORIENTAL	24.62	6.2	M	07/23/91	7
1291	ORIENTAL	18.36	8.4	M	07/23/91	7
1292	ORIENTAL	12.60	5.4	M	07/23/91	7
1293	ORIENTAL	12.76	5.4	M	07/23/91	7
1294	ORIENTAL	11.30	6.1	M	07/23/91	7
1295	ORIENTAL	30.29	8.3	M	07/23/91	7
1296	ORIENTAL	13.75	6.3	M	07/23/91	7
1297	BROAD CREEK	16.13	5.8	M	07/23/91	7
1298	BROAD CREEK	22.16	4.9	M	07/23/91	7
1299	BROAD CREEK	20.91	5.2	F	07/23/91	7
1300	BROAD CREEK	23.11	8.2	M	07/23/91	7
1301	BROAD CREEK	8.98	5.4	M	07/23/91	7
1302	BROAD CREEK	11.12	5.9	M	07/23/91	7
1303	BROAD CREEK	25.36	8.8	F	07/23/91	7
1304	BROAD CREEK	14.93	5.7	M	07/23/91	7
1305	BROAD CREEK	12.94	5.7	M	07/23/91	7
1306	BROAD CREEK	11.84	5.0	M	07/23/91	7
1307	ALLIGATOR	25.00	5.2	M	07/23/91	7
1308	ALLIGATOR	13.57	5.5	M	07/23/91	7
1309	ALLIGATOR	25.76	5.3	M	07/23/91	7
1310	ALLIGATOR	19.39	5.9	M	07/23/91	7
1311	ALLIGATOR	20.12	5.2	F	07/23/91	7
1312	ALLIGATOR	32.78	6.4	M	07/23/91	7
1313	ALLIGATOR	11.30	11.4	M	07/23/91	7
1314	ALLIGATOR	5.88	6.0	M	07/23/91	7
1315	ALLIGATOR	17.86	5.2	M	07/23/91	7
1316	ALLIGATOR	16.05	5.3	M	07/23/91	7
1317	BAY RIVER	23.70	5.2	M	07/24/91	7
1318	BAY RIVER	13.25	6.7	M	07/24/91	7
1319	BAY RIVER	32.21	6.2	M	07/24/91	7
1320	BAY RIVER	26.89	4.8	M	07/24/91	7
1321	BAY RIVER	48.20	9.0	M	07/24/91	7
1322	BAY RIVER	32.74	7.8	M	07/24/91	7
1323	BAY RIVER	22.45	7.2	F	07/24/91	7
1324	BAY RIVER	28.03	5.4	F	07/24/91	7
1325	BAY RIVER	8.83	10.8	F	07/24/91	7
1326	BAY RIVER	17.88	14.8	F	07/24/91	7
1327	PUNGO RIVER	20.85	9.1	M	07/25/91	7
1328	PUNGO RIVER	13.33	5.4	M	07/25/91	7
1329	PUNGO RIVER	19.35	5.3	M	07/25/91	7
1330	PUNGO RIVER	26.88	7.6	M	07/25/91	7
1331	PUNGO RIVER	23.07	7.4	M	07/25/91	7
1332	PUNGO RIVER	17.05	6.9	F	07/25/91	7
1333	PUNGO RIVER	11.93	9.8	F	07/25/91	7
1334	PUNGO RIVER	12.21	9.8	F	07/25/91	7

1335	PUNGO RIVER	15.12	8.2	F	07/25/91	7
1336	PUNGO RIVER	22.45	13.7	F	07/25/91	7
1337	NORTH CREEK	20.69	6.0	M	07/25/91	7
1338	NORTH CREEK	27.73	5.5	M	07/25/91	7
1339	NORTH CREEK	18.04	4.9	M	07/25/91	7
1340	NORTH CREEK	21.86	6.0	M	07/25/91	7
1341	NORTH CREEK	12.00	6.8	M	07/25/91	7
1342	NORTH CREEK	12.76	7.2	F	07/25/91	7
1343	NORTH CREEK	9.10	10.3	F	07/25/91	7
1344	NORTH CREEK	13.81	7.4	F	07/25/91	7
1345	NORTH CREEK	27.57	5.0	M	07/25/91	7
1346	NORTH CREEK	9.93	9.4	M	07/25/91	7
1347	SOUTH CREEK	28.98	6.6	F	07/25/91	7
1348	SOUTH CREEK	11.94	4.9	M	07/25/91	7
1349	SOUTH CREEK	17.68	5.9	M	07/25/91	7
1350	SOUTH CREEK	19.84	7.5	M	07/25/91	7
1351	SOUTH CREEK	28.50	9.8	M	07/25/91	7
1352	SOUTH CREEK	8.30	7.8	M	07/25/91	7
1353	SOUTH CREEK	18.66	4.7	M	07/25/91	7
1354	SOUTH CREEK	22.49	6.8	M	07/25/91	7
1355	SOUTH CREEK	15.60	6.5	M	07/25/91	7
1356	SOUTH CREEK	20.95	4.8	M	07/25/91	7
1357	CURRITUCK	64.84	5.4	M	07/31/91	7
1358	CURRITUCK	46.13	5.6	M	07/31/91	7
1359	CURRITUCK	24.97	5.0	M	07/31/91	7
1360	CURRITUCK	67.54	5.2	M	07/31/91	7
1361	CURRITUCK	74.90	6.1	M	07/31/91	7
1362	CURRITUCK	16.19	4.8	M	07/31/91	7
1363	CURRITUCK	60.66	5.9	M	07/31/91	7
1364	CURRITUCK	23.99	4.6	M	07/31/91	7
1365	CURRITUCK	14.36	4.8	M	07/31/91	7
1366	CURRITUCK	92.42	5.0	M	07/31/91	7
1367	NEW RIVER	30.62	11.4	M	08/14/91	8
1368	NEW RIVER	18.17	16.5	M	08/14/91	8
1369	NEW RIVER	22.65	13.7	M	08/14/91	8
1370	NEW RIVER	27.47	15.2	M	08/14/91	8
1371	NEW RIVER	20.59	9.7	M	08/14/91	8
1372	NEW RIVER	22.65	11.9	IF	08/14/91	8
1373	NEW RIVER	26.79	8.3	M	08/14/91	8
1374	NEW RIVER	18.27	6.9	M	08/14/91	8
1375	NEW RIVER	12.32	5.9	M	08/14/91	8
1376	NEW RIVER	53.28	6.7	M	08/14/91	8
1377	CURRITUCK	26.52	5.1	F	08/22/91	8
1378	CURRITUCK	10.59	8.8	F	08/22/91	8
1379	CURRITUCK	25.48	5.8	M	08/22/91	8
1380	CURRITUCK	69.78	5.1	M	08/22/91	8
1381	CURRITUCK	35.78	7.4	M	08/22/91	8
1382	CURRITUCK	49.30	5.7	M	08/22/91	8
1383	CURRITUCK	72.90	5.3	M	08/22/91	8
1384	CURRITUCK	13.59	6.9	M	08/22/91	8
1385	CURRITUCK	15.97	6.8	F	08/22/91	8
1386	CURRITUCK	16.27	5.9	F	08/22/91	8
1387	SOUTH CREEK	24.19	5.7	F	08/26/91	8
1388	SOUTH CREEK	17.90	4.9	M	08/26/91	8
1389	SOUTH CREEK	14.94	5.2	M	08/26/91	8
1390	SOUTH CREEK	32.72	5.4	M	08/26/91	8
1391	SOUTH CREEK	20.27	6.9	M	08/26/91	8
1392	SOUTH CREEK	15.07	4.9	F	08/26/91	8
1393	SOUTH CREEK	36.49	5.5	M	08/26/91	8
1394	SOUTH CREEK	10.61	5.0	F	08/26/91	8
1395	SOUTH CREEK	28.36	6.0	M	08/26/91	8
1396	SOUTH CREEK	20.04	5.5	F	08/26/91	8
1397	NORTH CREEK	14.65	6.7	F	08/26/91	8
1398	NORTH CREEK	17.61	8.2	F	08/26/91	8

1399	NORTH CREEK	10.59	6.4	F	08/26/91	8
1400	NORTH CREEK	17.50	7.2	F	08/26/91	8
1401	NORTH CREEK	19.18	5.9	F	08/26/91	8
1402	NORTH CREEK	36.35	6.1	M	08/26/91	8
1403	NORTH CREEK	16.01	5.7	M	08/26/91	8
1404	NORTH CREEK	29.04	7.3	M	08/26/91	8
1405	NORTH CREEK	21.73	6.5	F	08/26/91	8
1406	NORTH CREEK	44.72	5.2	M	08/26/91	8
1407	PUNGO RIVER	24.16	5.5	F	08/26/91	8
1408	PUNGO RIVER	30.81	10.1	F	08/26/91	8
1409	PUNGO RIVER	27.49	6.0	F	08/26/91	8
1410	PUNGO RIVER	22.84	5.2	F	08/26/91	8
1411	PUNGO RIVER	22.00	5.5	F	08/26/91	8
1412	PUNGO RIVER	25.16	4.1	M	08/26/91	8
1413	PUNGO RIVER	36.05	5.2	M	08/26/91	8
1414	PUNGO RIVER	16.09	6.1	M	08/26/91	8
1415	PUNGO RIVER	18.74	6.3	M	08/26/91	8
1416	PUNGO RIVER	34.18	6.3	M	08/26/91	8
1417	BROAD CREEK	13.40	10.1	F	08/26/91	8
1418	BROAD CREEK	12.54	6.0	F	08/26/91	8
1419	BROAD CREEK	6.09	17.8	F	08/26/91	8
1420	BROAD CREEK	17.92	8.6	F	08/26/91	8
1421	BROAD CREEK	2.29	32.3	F	08/26/91	8
1422	BROAD CREEK	6.48	14.2	M	08/26/91	8
1423	BROAD CREEK	7.60	11.6	M	08/26/91	8
1424	BROAD CREEK	32.14	8.2	M	08/26/91	8
1425	BROAD CREEK	10.81	10.3	M	08/26/91	8
1426	BROAD CREEK	40.29	8.4	M	08/26/91	8
1427	BAY RIVER	25.99	6.9	M	08/26/91	8
1428	BAY RIVER	11.41	5.7	M	08/26/91	8
1429	BAY RIVER	30.91	7.4	M	08/26/91	8
1430	BAY RIVER	15.52	7.6	M	08/26/91	8
1431	BAY RIVER	14.58	8.1	M	08/26/91	8
1432	BAY RIVER	13.85	11.2	M	08/26/91	8
1433	BAY RIVER	7.78	6.2	F	08/26/91	8
1434	BAY RIVER	32.15	3.0	M	08/26/91	8
1435	BAY RIVER	26.21	7.2	M	08/26/91	8
1436	BAY RIVER	11.58	2.4	M	08/26/91	8
1437	ORIENTAL	33.60	7.2	F	08/26/91	8
1438	ORIENTAL	17.26	5.2	F	08/26/91	8
1439	ORIENTAL	23.40	4.4	F	08/26/91	8
1440	ORIENTAL	19.29	5.9	F	08/26/91	8
1441	ORIENTAL	30.14	5.5	F	08/26/91	8
1442	ORIENTAL	21.57	4.3	M	08/26/91	8
1443	ORIENTAL	22.05	4.4	M	08/26/91	8
1444	ORIENTAL	17.57	4.2	M	08/26/91	8
1445	ORIENTAL	20.79	5.7	M	08/26/91	8
1446	ORIENTAL	24.35	5.9	M	08/26/91	8
1447	CORE SOUND	36.92	6.5	M	09/17/91	9
1448	CORE SOUND	42.28	5.4	M	09/17/91	9
1449	CORE SOUND	51.52	5.2	M	09/17/91	9
1450	CORE SOUND	43.49	6.0	M	09/17/91	9
1451	CORE SOUND	49.63	5.4	M	09/17/91	9
1452	CORE SOUND	35.48	6.2	M	09/17/91	9
1453	CORE SOUND	40.61	5.4	M	09/17/91	9
1454	CORE SOUND	49.51	5.5	M	09/17/91	9
1455	CORE SOUND	37.55	6.3	M	09/17/91	9
1456	CORE SOUND	35.69	5.2	M	09/17/91	9
1457	PUNGO RIVER	27.19		M	09/13/91	9
1458	PUNGO RIVER	35.28		M	09/13/91	9
1459	PUNGO RIVER	17.49		M	09/13/91	9
1460	PUNGO RIVER	33.36		M	09/13/91	9
1461	PUNGO RIVER	19.51		F	09/13/91	9
1462	PUNGO RIVER	25.65		F	09/13/91	9

1463	PUNGO RIVER	13.22		F	09/13/91	9
1464	PUNGO RIVER	16.67		F	09/13/91	9
1465	PUNGO RIVER	19.23		F	09/13/91	9
1466	NORTH CREEK	60.13		M	09/13/91	9
1467	NORTH CREEK	26.04		F	09/13/91	9
1468	NORTH CREEK	41.53		F	09/13/91	9
1469	NORTH CREEK	25.93		M	09/13/91	9
1470	NORTH CREEK	26.89		F	09/13/91	9
1471	NORTH CREEK	46.97		F	09/13/91	9
1472	NORTH CREEK	26.22		M	09/13/91	9
1473	NORTH CREEK	55.92		M	09/13/91	9
1474	NORTH CREEK	34.18		M	09/13/91	9
1475	SOUTH CREEK	19.22		M	09/13/91	9
1476	SOUTH CREEK	21.14		M	09/13/91	9
1477	SOUTH CREEK	23.22		M	09/13/91	9
1478	SOUTH CREEK	21.09		M	09/13/91	9
1479	SOUTH CREEK	23.31		M	09/13/91	9
1480	SOUTH CREEK	25.81		F	09/13/91	9
1481	SOUTH CREEK	30.54		F	09/13/91	9
1482	SOUTH CREEK	29.77		F	09/13/91	9
1483	SOUTH CREEK	42.23		F	09/13/91	9
1484	SOUTH CREEK	25.33		F	09/13/91	9
1485	NEW RIVER	1.24		F	09/18/91	9
1486	NEW RIVER	14.75		M	09/18/91	9
1487	NEW RIVER	15.67		M	09/18/91	9
1488	NEW RIVER	8.86		M	09/18/91	9
1489	NEW RIVER	9.31		F	09/18/91	9
1490	NEW RIVER	5.78		F	09/19/91	9
1491	NEW RIVER	1.59		F	09/18/91	9
1492	NEW RIVER	6.82		M	09/18/91	9
1493	NEW RIVER	28.14		F	09/18/91	9
1494	NEW RIVER	0.69		F	09/18/91	9
1495	BROAD CREEK	10.77	0.0	F	09/16/91	9
1496	BROAD CREEK	37.28		F	09/16/91	9
1497	BROAD CREEK	14.14		F	09/16/91	9
1498	BROAD CREEK	19.63		F	09/16/91	9
1499	BROAD CREEK	33.08		F	09/16/91	9
1500	BROAD CREEK	20.52		M	09/16/91	9
1501	BROAD CREEK	15.14		M	09/16/91	9
1502	BROAD CREEK	30.22		M	09/16/91	9
1503	BROAD CREEK	17.96		M	09/16/91	9
1504	BROAD CREEK	11.36		M	09/16/91	9
1505	ORIENTAL	20.75		M	09/16/91	9
1506	ORIENTAL	32.84		M	09/16/91	9
1507	ORIENTAL	21.33		M	09/16/91	9
1508	ORIENTAL	27.51		M	09/16/91	9
1509	ORIENTAL	35.14		M	09/16/91	9
1510	ORIENTAL	31.47		F	09/16/91	9
1511	ORIENTAL	22.21		F	09/16/91	9
1512	ORIENTAL	28.90		F	09/16/91	9
1513	ORIENTAL	38.02		F	09/16/91	9
1514	ORIENTAL	24.96		F	09/16/91	9
1515	BAY RIVER	43.19		M	09/16/91	9
1516	BAY RIVER	22.94		F	09/16/91	9
1517	BAY RIVER	16.34		M	09/16/91	9
1518	BAY RIVER	5.36		F	09/16/91	9
1519	BAY RIVER	25.59		M	09/16/91	9
1520	BAY RIVER	29.75	0.0	M	09/16/91	9
1521	BAY RIVER	25.40		M	09/16/91	9
1522	BAY RIVER	22.06		M	09/16/91	9
1523	BAY RIVER	25.36		M	09/16/91	9
1524	BAY RIVER	31.99		M	09/16/91	9
1525	NORTH CREEK	16.28	5.0	M	10/14/91	10
1526	NORTH CREEK	15.60	8.4	F	10/14/91	10

1527	NORTH CREEK	25.85	5.2	M	10/14/91	10
1528	NORTH CREEK	10.51	5.6	M	10/14/91	10
1529	NORTH CREEK	58.68	7.3	M	10/14/91	10
1530	NORTH CREEK	13.70	5.4	F	10/14/91	10
1531	NORTH CREEK	22.05	5.2	F	10/14/91	10
1532	NORTH CREEK	7.82	5.1	F	10/14/91	10
1533	NORTH CREEK	24.44	5.3	F	10/14/91	10
1534	NORTH CREEK	6.21	4.7	F	10/14/91	10
1535	BAY RIVER	26.73	5.9	M	10/14/91	10
1536	BAY RIVER	25.82	5.1	F	10/14/91	10
1537	BAY RIVER	17.55	5.2	M	10/14/91	10
1538	BAY RIVER	9.40	5.5	M	10/14/91	10
1539	BAY RIVER	16.12	5.1	M	10/14/91	10
1540	BAY RIVER	13.03	5.5	M	10/14/91	10
1541	BAY RIVER	21.78	5.4	M	10/14/91	10
1542	BAY RIVER	32.31	5.5	M	10/14/91	10
1543	BAY RIVER	32.26	7.6	M	10/14/91	10
1544	BAY RIVER	66.25	5.3	M	10/14/91	10
1545	BROAD CREEK	18.67	6.0	M	10/14/91	10
1546	BROAD CREEK	8.95	9.7	M	10/14/91	10
1547	BROAD CREEK	18.10	5.1	M	10/14/91	10
1548	BROAD CREEK	22.38	12.2	M	10/14/91	10
1549	BROAD CREEK	18.88	4.9	M	10/14/91	10
1550	BROAD CREEK	16.10	6.1	F	10/14/91	10
1551	BROAD CREEK	11.56	8.3	F	10/14/91	10
1552	BROAD CREEK	6.01	9.0	F	10/14/91	10
1553	BROAD CREEK	11.21	5.9	F	10/14/91	10
1554	BROAD CREEK	11.98	7.7	M	10/14/91	10
1555	PUNGO RIVER	25.10	4.9	F	10/14/91	10
1556	PUNGO RIVER	83.50	5.6	M	10/14/91	10
1557	PUNGO RIVER	62.39	8.7	M	10/14/91	10
1558	PUNGO RIVER	13.65	5.8	M	10/14/91	10
1559	PUNGO RIVER	26.41	8.4	M	10/14/91	10
1560	PUNGO RIVER	8.53	5.8	M	10/14/91	10
1561	PUNGO RIVER	12.70	5.6	F	10/14/91	10
1562	PUNGO RIVER	11.52	5.8	F	10/14/91	10
1563	PUNGO RIVER	4.84	6.2	F	10/14/91	10
1564	PUNGO RIVER	34.86	5.5	M	10/14/91	10
1565	CURRITUCK	51.67	5.1	M	10/14/91	10
1566	CURRITUCK	48.92	2.0	F	10/14/91	10
1567	CURRITUCK	50.15	5.2	M	10/14/91	10
1568	CURRITUCK	26.27	5.1	M	10/14/91	10
1569	CURRITUCK	35.42	5.2	F	10/14/91	10
1570	CURRITUCK	26.37	5.7	M	10/14/91	10
1571	CURRITUCK	83.01	5.2	M	10/14/91	10
1572	CURRITUCK	18.83	6.0	M	10/14/91	10
1573	CURRITUCK	69.60	5.6	M	10/14/91	10
1574	SOUTH CREEK	16.85	7.6	M	10/15/91	10
1575	SOUTH CREEK	18.42	9.7	M	10/15/91	10
1576	SOUTH CREEK	35.28	9.3	M	10/15/91	10
1577	SOUTH CREEK	21.38	7.3	M	10/15/91	10
1578	SOUTH CREEK	10.41	10.0	M	10/15/91	10
1579	SOUTH CREEK	18.58	4.5	M	10/15/91	10
1580	SOUTH CREEK	10.39	5.3	M	10/15/91	10
1581	SOUTH CREEK	29.07	4.9	M	10/15/91	10
1582	SOUTH CREEK	64.56	5.7	M	10/15/91	10
1583	SOUTH CREEK	24.42	4.9	M	10/15/91	10
1584	ORIENTAL	29.02	4.8	M	10/14/91	10
1585	ORIENTAL	24.28	10.8	M	10/14/91	10
1586	ORIENTAL	14.82	7.0	M	10/14/91	10
1587	ORIENTAL	22.69	6.5	M	10/14/91	10
1588	ORIENTAL	21.21	6.5	M	10/14/91	10
1589	ORIENTAL	10.33	6.9	F	10/14/91	10
1590	ORIENTAL	12.70	9.1	F	10/14/91	10

1591	ORIENTAL	14.38	5.7	F	10/14/91	10
1592	ORIENTAL	34.06	6.3	F	10/14/91	10
1593	ORIENTAL	16.87	5.2	F	10/14/91	10
1594	CORE SOUND	54.90	5.9	M	10/03/91	10
1595	CORE SOUND	55.45	7.1	M	10/03/91	10
1596	CORE SOUND	49.62	5.7	M	10/03/91	10
1597	CORE SOUND	23.04	8.6	M	10/03/91	10
1598	CORE SOUND	53.24	6.2	M	10/03/91	10
1599	CORE SOUND	59.00	7.6	M	10/03/91	10
1600	CORE SOUND	38.21	7.5	M	10/03/91	10
1601	CORE SOUND	49.54	5.3	M	10/03/91	10
1602	CORE SOUND	56.26	5.2	M	10/03/91	10
1603	CORE SOUND	53.05	5.9	M	10/03/91	10

APPENDIX II

HEMOLYMPH HEMOCYTE STAINING TECHNIQUES

PROCEDURE

COLLECTING, FIXING, AND STUDYING

CRAB HEMOLYMPH

CRAB HANDLING & COLLECTION SITES

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NCSU - COLLEGE OF VETERINARY MEDICINE

December 28, 1990

PROCEDURE

CRAB HANDLING & HEMOLYMPH COLLECTION

1. CRAB HANDLING

Crabs are usually handled at ambient temperature with heavy rubber gloves. However, under some circumstances, crabs are cooled to 5 C°. The crab is positioned with the pincher claws folded up tight to the body.

2. HEMOLYMPH COLLECTION TECHNIQUE

NEEDLE BLEEDING TECHNIQUE: One of the exposed joints of the pincher legs is prepared with alcohol to remove all the debris. An alternative site is at the ventral side of the swimmerette where it meets the body. The needle is inserted just under the epidermis followed by gentle aspiration. One volume hemolymph is aspirated into one volume of 10% formalin. The sample is mixed vigorously as soon after collection as is possible. This sample is used for hematology. If the sample is for bioassay it is collected without formalin.

NEEDLE BLEEDING - SMALL VOLUMES: For repeated bleeding from a crab, a tuberculin syringe (1 ml) is used with .6 ml of formalin. A 5/32 inch chrome ball is placed in the barrel of the syringe before the 10% formalin is introduced into the syringe. Care is taken to mix the hemolymph immediately after it enters the syringe.

BLEEDING TECHNIQUE FOR LARGER VOLUMES: A swimmerette is severed using sharp scissors. Free flowing hemolymph is collected rom the wound into a glass tube and kept refrigerated until processed. This is used for the bacteriocidal assay and hemocyanin concentration measurement.

BLEEDING TECHNIQUE FOR NOT PRESERVED NON-CLOTTED HEMOLYMPH: The crabs, syringes, needles are all cooled to 5 degrees centigrade with ice. Hemolymph is collected for one of the sites described above. Endotoxin contamination must be avoided to be successful.

PROCEDURE

HEMOLYMPH COLLECTION & PRESERVATION

EQUIPMENT:

1. GLASS SLIDES

Commercial slides have very light film of oil and may have endotoxin on the surface. Wash them with detergent to rid them of oil and then bake them at 190 C° for 2 hours to rid them of any endotoxin if a fixative is not used. Cleaned slides, when required, are usually wrapping groups of 10 or more slides in aluminum foil packages.

2. SYRINGES & NEEDLES

One ml plastic syringes and one inch 25 Gage needles are used. A 5/32 inch chrome steel ball is placed in the syringe. The syringe is then filled up to the 0.6 ml mark with 10% formalin before use. A new needle is used for sampling. The chrome ball allows immediate mixing when hemolymph enters the syringes.

3. ANTICOAGULANT / PRESERVATIVE

Buffered formaldehyde (25 ml 40% to 75 ml distilled water is used. The fluid is used at ambient temperature.

Buffered neutral formalin saline solution (1 Liter)

37% formaldehyde	100 ml
Distilled water	900 ml
Sodium phosphate monobasic	40.0 Gm
Sodium Phosphate dibasic (anhydrous)	6.5 Gm

4. HOLDING AND TRANSPORTING

Glass test tube with plastic caps are used for holding and transporting anticoagulated/preserved hemolymph samples although transportation in the syringe may be convenient for returning to the laboratory. Refrigeration is not required. Hemolymphocytes are stable for at least 2 months with this method of preservation.

PROCEDURE

SLIDE PREPARATION & STAINING

DIFFERENTIAL CELL SLIDE PREPARATION

A Shandon Cytospin Centrifuge (Shandon Southern Instruments, Inc. 515 Broad St. Drawer 43, Sweickley, PA, 15143) is employed to make slides from the preserved specimens. Washed slides and a reusable sample chambers are used. One drop of 22% Bovine Serum Albumin is added to 20 lambda of a 1:500 diluted hemolymph sample to increase cell adherence to the slides.

SEDIMENT SLIDE PREPARATION

A Monarch automated clinical chemistry instrument cup is filled with 1 ml of non-preserved hemolymph diluted with isotonic saline. A thin layer of Eukitt coverslip mounting media is applied to the rim of the cup. A precleaned and heat treated slide is applied to the cup and after the cup is fixed to the slide the cup and slide are inverted. Cells are allowed to settle to the surface of the slide for approximately 10 minutes. After the settling period the slide is reinverted and the cup is removed. The slide is air dried and stained as described elsewhere in these procedures. The excess Eukitt on the slide from the cup is removed with a scalpel blade before the slide is cover slipped.

SLIDE STAINING

Meyer's Hematoxylin
(If used with Harris Hematoxylin mix Meyer's (47 ml) and Harris (16 ml) and filter before use).

Mix Eosin-Phloxine working solution as following:

Eosin-Phloxine stock	35 ml
80% ETOH	35 ml
Acetic Acid	.15 ml (3 drops)

Filter before use.

Procedure for paraffin embedded tissue or go directly to tap water if hemolymph slides:

Xylene	5 min
Xylene	5 min
100% ETOH	2 min
100% ETOH	2 min
95% ETOH	1 min
95% ETOH	1 min
80% ETOH	1 min

Start here with hemolymph slides or continue with tissue

Tap Water	1 min
Meyers Hematoxylin	10 min (or Harris Meyer's Mixture 5 min)
Tap water (warm running)	10 min
80% ETOH	30 sec
Eosin-Phloxine	2 min
100% ETOH + 3 Drops Acetic Acid	30 sec
100% ETOH + 3 Drops Acetic Acid	30 sec
100% ETOH	2 min

Delete the next two steps for hemolymph samples but not for tissue.

Xylene	3 min
Xylene	3 min

Coverslip with standard #1 22 X 22 mm glass cover slips using Eukitt Mounting Reagent (Calibrated Instruments, Inc. 731 Saw Mill River Rd., ARdsley, NY 10502).

PROCEDURE

HEMOLYMPH CELL COUNTING

TOTAL CELL COUNTS

A Coulter Particle Counter Model ZM (Coulter Electronics LTD. Northwell Drive, Luton, Beds., LU3 3RH England) is used with a 50 micro orifice and threshold adjusted to accommodate crab hemolymph. A total of 50 micro liters of hemolymph is counted. The count is repeated. Raw counts are coincidence corrected. The sample is diluted 20:0.5 with isotonic saline for counting.

The count is calculated as follows:

Coulter Counter Count X (Syringe dilution)
X 50 (Counting dilution)
divided by 50 (microliters counted)
Yields cell count per microliter.

DIFFERENTIAL CELL COUNT

Granulocyte and hyaline cells are recognized by size and staining characteristics as described in the literature. A differential count of 100 cells is made from each slide.

Absolute granulocyte and hyalinocytes are calculated as per cent of absolute count as obtained from the Coulter counter.

PROBIT CELL COUNT

Threshold steps of 4 are used i.e. 25 data points. The cell count should be at least 10,000//microliter.

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