ANEMIC BLUE CRABS FROM THE ALBEMARLE-PAMLICO ESTUARY: HEMOCYANIN CONCENTRATIONS AS A MEASURE OF ENVIRONMENTAL QUALITY

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ALBEMARLE-PAMLICO ESTUARINE STUDY

From

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Anemic Blue Crabs, *Callinectes sapidus*, From the Albemarle-Pamlico Estuary: Hemocyanin Concentrations as a Measure of Environmental Quality.

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Introduction

The hemolymph of blue crabs has a distinct, intense blue color, which is caused by the presence of its respiratory pigment called hemocyanin. Hemocyanin is a large, multisubunit, copper-containing protein, which is functionally equivalent to hemoglobin, i.e. hemocyanin takes up oxygen from the environment and releases oxygen in the respiring tissues (Miler and Van Holde, 1982). In blue crabs hemocyanin is responsible for the uptake and transport of approximately 95% of the oxygen consumed by the animals. During a study on the etiology of blue crab shell disease, initiated by Dr. Engel from the National Marine Fisheries Service in Beaufort and Dr. Noga, from NCSU, it was noted that hemolymph samples from blue crabs obtained from the Pamlico River were significantly less blue than hemolymph samples from crabs obtained from the Beaufort area. Subsequent measurements showed that the lack of blue color was associated with diminished concentrations of hemocyanin in the hemolymph. Whereas "normal" hemocyanin concentrations in blue crabs range from 40-60 mg/ml, Pamlico River crabs generally contained only 10-30 mg hemocyanin/ml. Such crabs seemed therefore to be moderately to severely anemic. Similar measurements carried out on blue crabs obtained from clean and contaminated environments (Houston Ship Channel) strongly suggest that low hemocyanin concentrations and deteriorating water quality are linked phenomena. These observations suggest that hemocyanin may have the potential of being used as a biomarker of environmental contamination. We define a biomarker as a biochemical or physiological response to anthropogenic contaminants, which can provide a sensitive index of exposure or sublethal stress at the organismal level. Understanding the molecular reasons for the change in biomarker concentration or activity may lead to identification of the active contaminants and their source, which in turn may lead to regulatory action.

Objectives.

The objective of the research was to measure hemocyanin concentrations in blue crabs throughout coastal North Carolina on a spatial and temporal basis. These measurements were designed to:

(1) determine if the abnormal hemocyanin levels, which we have observed in September, 1988 in Pamlico River crabs, are associated with a particular season, or persist throughout the year

(2) to delineate the area(s) in which the abnormalities occur

(3) to obtain necessary background information on seasonal variability in hemocyanin concentrations in "unstressed" populations of blue crabs.

The proposed research will demonstrate the feasibility of using hemocyanin as an indicator of environmental stress, and lay the groundwork for more elaborate studies aimed at elucidating the factors responsible for the decrease in hemocyanin levels.

Experimental Design.

Blue crabs were obtained from the Alligator River, eastern Pamlico Sound, Long Shoal River, Pamlico River (South Creek and North Creek), Pungo River, Bay River, Neuse River (Broad Creek and Oriental). Reference areas outside of the Albemarle/Pamlico area were Beaufort/Bogue and Core Sound. Blue crabs were selected for molt stage. Only C4 (intermolt, hard crabs) or early premolt D1-D3 male crabs and terminal molt female blue crabs without eggs were used. Samples were collected by biologists in the Washington, N.C. office of the North Carolina Division of Marine Fisheries as part of their juvenile sampling program.

Hemolymph was collected by either severing the paddle appendage or a walking leg between the joints with a sharp pair of scissors. The samples were collected in plastic vials, placed on ice and allowed to clot. The clotted material was homogenized with a tissue homogenizer and then centrifuged at 20,000 x g for 20 minutes. The supernatant was diluted approximately 100 times with 50 mM Tris buffer, containing 10 mM $CaCl_2$ at pH 8.0. Absorbance spectra, of the diluted samples were measured with a Hewlett Packard Photodiode Array spectrophotometer over a wavelength interval from 220 to 400 nm. Hemocyanin concentrations were calculated from the absorbance values at 280 and 334 nm : E_{280nm} = 13.5 and E_{334nm} = 2.30 for 10 mg hemocyanin/ml.

Results

Initial collections of blue crab hemolymph and measurements of hemocyanin concentrations were made in southwestern Pamlico Sound during September of 1988. The data showed that hemocyanin concentrations in blue crabs from the southwestern portion of the Sound were low relative to the reference area, Beaufort (Figure 1). 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.

In 1989 blue crabs in the Pamlico Sound estuarine complex were sampled at seven test sites and two reference sites, from May through October. Spatial and temporal differences occurred within and among the different sampling locations: Northwestern Pamlico Sound, Pamlico River, and Neuse River (Figure 2). The reference areas that were used are Core Sound and Bogue Sound which were separated from the areas of concern (Figures 1 and 3). The monthly average 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 one in the Pungo River, a tributary of the Pamlico. 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 are interesting, because the lowest values are from the two most isolated areas, while the Oriental site remained relatively constant. This is particularly puzzling since that area has the heaviest pleasure boat traffic and large marinas, which would suggest poorer water quality than the other two isolated areas. While there were fluctuations in the hemocyanin levels observed in the reference areas, all measurements were higher than those seen in the Pamlico and Neuse Rivers. The Pungo River was particularly interesting because after a very low mean hemocyanin concentration in July the value rebounded in August, indicating that some change had occurred in the population.

To explore the reason for the observed temporal changes in hemolymph hemocyanin concentrations in crabs from the Pungo River, frequency distributions were plotted for each of the months sampled (Figure 4). The mode of the distribution shifts toward lower concentrations for the first two months and then becomes highly skewed in the low concentration range in July. The large number of crabs with hemocyanin concentrations in the 0-10 mg/ml range is surprising since such values suggest that the crabs were extremely anemic. The shift to a bimodal character of the distribution pattern in August may be accounted for by the immigration of adults crabs into the Pungo River from Pamlico Sound during the late summer (N.C. Division of Marine Fisheries, personal communication).

Six month average blue crab hemolymph concentrations for crabs collected from the reference and Pamlico Sound locations show differences between areas (Figure 5). The pooled data underscores the differences between the southwestern Sound and the Beaufort/Bogue and Core Sound sites. An analysis of variance of all the data showed that there were significant differences ($P<0.05$) between locations and between months, but no significant interactions. At this time we do not know whether the observed temporal and spatial differences are natural, or are the result of some stressing agent that is affecting hemocyanin metabolism of blue crabs from southwestern Pamlico Sound.

Discussion

The data that has been collected in the southwestern Pamlico Sound and its tributaries shows that there are both spatial and temporal differences in the hemocyanin concentrations among the blue crab population in this area. Low concentrations of hemocyanin were first measured in September 1988, in crabs from different locations in Pamlico Sound in a single set of collections. Another set of observations were made during the summer of 1989 at similar locations that showed similar trends. In the latter data set significant differences ($P<0.05$) in hemocyanin concentrations were demonstrated both between and within sampling locations. The lowest concentrations occurred during July and August and then increased in September and October. Also, the lowest concentrations of hemocyanin were measured in crabs from some of the more isolated locations, the Pungo River, Bay River, and Broad Creek on the Neuse River. In many cases crabs from these locations have lower values than those measured in crabs from South and Durham Creeks on the Pamlico River, that are adjacent to the Texas Gulf Sulfur phosphate mining and processing facility.

The factors responsible for causing lowered hemocyanin concentrations have not been demonstrated, but some information is available that relates environmental conditions (salinity, hypoxia and disease) to hemocyanin concentrations in 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 (M. Brouwer and D. Engel, unpublished data, and C. Mangum, personal communication). Therefore, crabs from low salinity water of the Pamlico system should have higher concentrations of hemocyanin than those collected near Beaufort where salinities are higher, but the opposite is true. deFur and coworkers (1990) have shown that hypoxia causes an increase in hemocyanin concentration in blue crabs from Lake Pontchartrain where hypoxic events occur routinely. In contrast, Hagerman and Baden (1988) demonstrated that low oxygen caused reduced feeding and lowered hemocyanin concentrations in *Nephrops norvegicus*. Hagerman (1986) also demonstrated that chronic hypoxia caused increases in hemocyanin concentrations in *Crangon crangon*, and that starvation alone will decrease hemocyanin. Hypoxic conditions also occur in the tributaries entering Pamlico Sound in eastern North Carolina during the summer (data from the N.C. Division of Environmental Management). Therefore, higher hemocyanin concentrations would be expected among crabs from those locations if low oxygen was the only variable. From the information at our disposal, we cannot determine whether hypoxia alone, or combination of hypoxia and reduced feeding caused the observed reduced hemocyanin concentrations.

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 perniciosa* which causes "gray crab disease". A documented symptom of this disease is a significant decrease in the hemocyanin concentration and a loss of the clotting mechanism in the hemolymph (Newman and Ward, 1973; Pauley et al, 1975). Examinations of smears from Pamlico River crabs have not shown any marked *Paramoeba* infections that could account for the lowered hemocyanin concentration.

In addition to environmental 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 (Meister and Anderson, 1983), also is involved in copper metabolism (Freedman et al., 1989). In our laboratory we have demonstrated that that a complex of copper and glutathione can reactivate copper-free hemocyanin (Brouwer, unpublished results). 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 (Lee et al., 1988). This information suggests a possible pathway by which exposure to polycyclic hydrocarbons and pesticides may result in diminished hemocyanin synthesis due to the lack of copper-glutathione complex. The measured hemocyanin concentrations in the hemolymph of blue crabs collected at different sites in the Houston Ship Channel and in Tampa Bay support this hypothesis. Those data showed a negative correlation between water quality and hemocyanin concentration in crabs (Engel and Brouwer, unpublished results).

A negative correlation between hemocyanin and water quality has also been observed in lobsters (Engel and Brouwer, unpublished results). Hemolymph samples from lobsters collected in the northeast in the vicinity of polluted estuaries, such as Boston Harbor, showed lower hemocyanin concentrations than lobsters collected from cleaner areas, such as Cape Cod Bay.

At this time we cannot document a cause and effect relationship that explains the observed reductions in blue crab hemocyanin concentrations. Our data does provide evidence for a positive correlation between environmental quality and lowered hemocyanin concentrations. The intriguing nature of this field data set suggests that further investigations should be done to determine physiological/toxicological processes that affect the turnover and synthesis of hemocyanin, and to determine the value of hemocyanin concentrations as a surrogate measure of environmental quality.

Summary

A variety of surrogate physiological and biochemical measures have been proposed as bioindicators of environmental quality. Here we report studies which were designed to determine if hemolymph hemocyanin concentrations in blue crabs, *Callinectes sapidus*, could be correlated with water quality. To this end hemocyanin concentrations were measured in hemolymph samples collected from Eastern North Carolina (and Florida and Texas). Blue crabs from isolated estuarine systems in eastern N.C. (i.e. Albemarle/Pamlico Sound area) had significantly lower concentrations of hemocyanin in their hemolymph than crabs from reference areas (i.e. Core and Bogue Sounds), but these lowered concentrations could not be correlated with any known sources of contamination.. Among crabs from Tampa Bay and Houston Ship Channel, however, there was a negative correlation between the degree of industrialization and hemocyanin concentration. The lower concentrations of hemocyanin were not correlated with either the apparent health or sex of the crabs, or with salinity or oxygen tension. These data suggest that the lowered hemocyanin concentrations in blue crabs may be related to water quality. However, factors other than toxic contaminants, such as natural phenomena and epizootics, also may be contributing to the observed differences.

(This Abstract is from a manuscript (in preparation) titled: "Hemocyanin Concentrations in Marine Crustaceans as a Measure of Environmental Quality" by David. W. Engel, Marius Brouwer and Sean McKenna)

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Figure legends

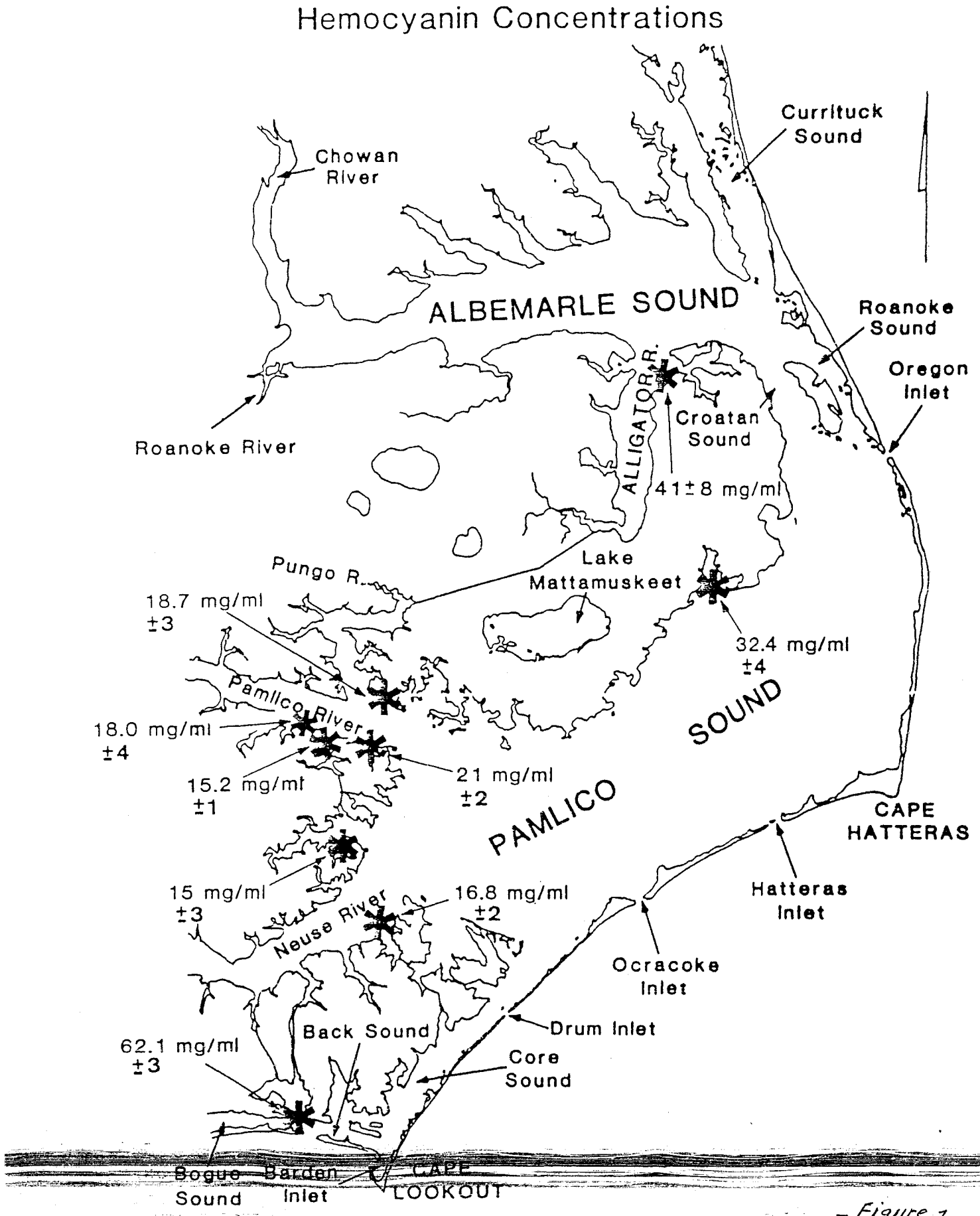
Figure 1. A map of Albemarle and Pamlico Sounds showing the mean hemocyanin concentrations of blue crabs collected in September 1988.

Figure 2. Spatial and temporal differences in hemocyanin concentrations in blue crabs collected in 1989 from Northwestern Pamlico Sound, the Pamlico River area, and Neuse River area. Each monthly point is the mean of five to ten blue crabs.

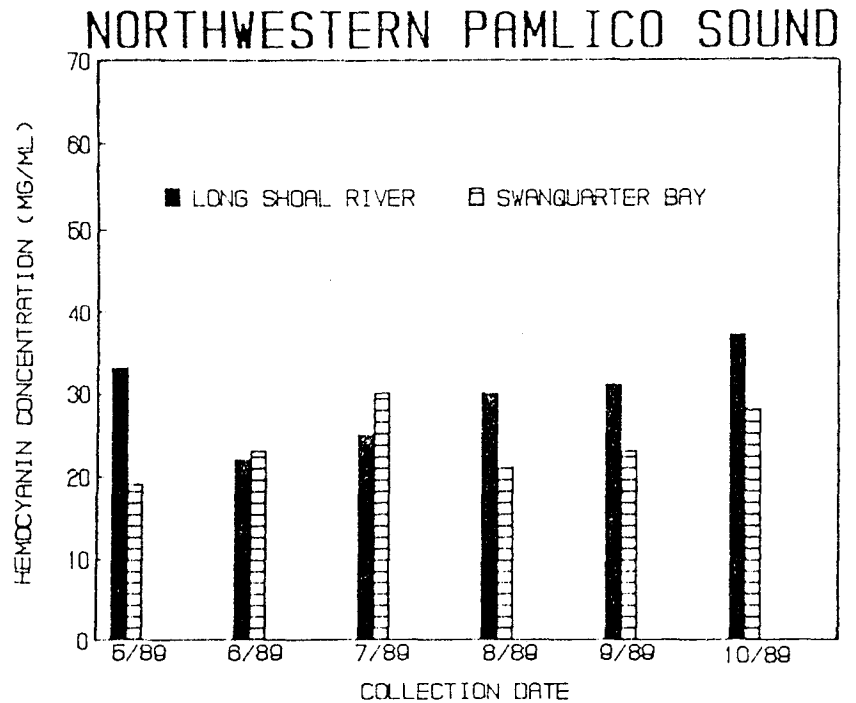
Figure 3. Mean concentrations of hemocyanin in blue crabs collected from reference areas, Core and Bogue Sounds, in 1989. Each mean represents five to ten blue crabs plus or minus one standard error.

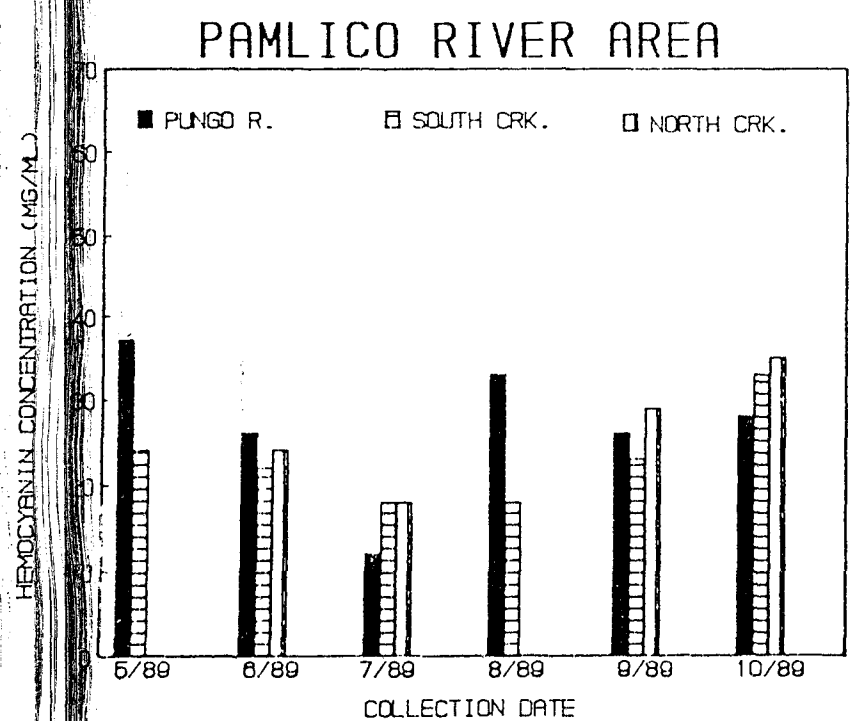
Figure 4. Frequency distribution of monthly hemocyanin concentrations in blue crabs collected from the Pungo River in 1989.

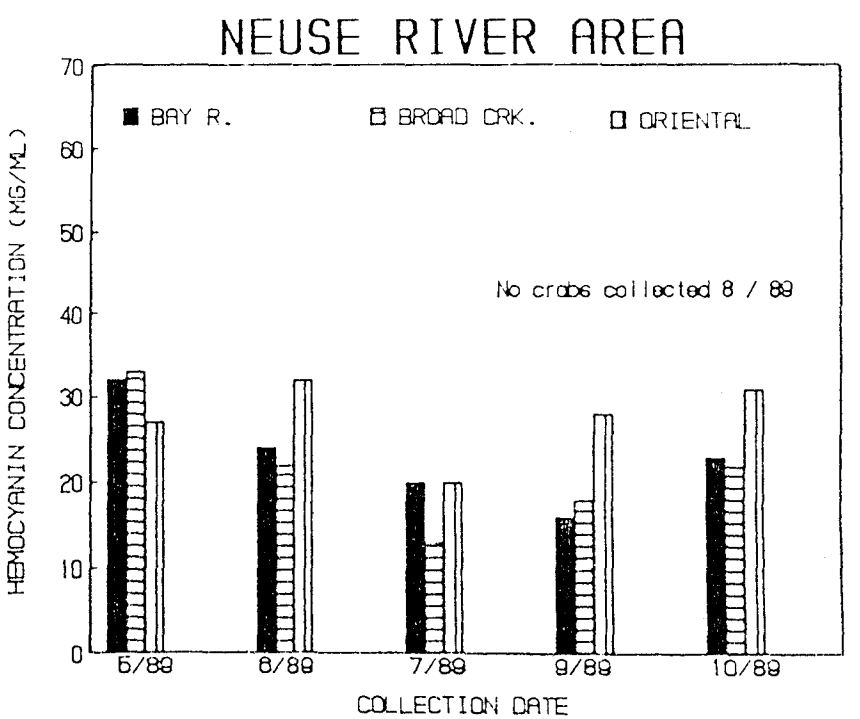
Figure 5. Average hemocyanin concentrations measured in blue crabs from reference and Pamlico Sound locations during the six month sampling period. The vertical bar indicates one standard error. The locations from left to right are: Cntl: control/reference; Ls. R.: Long Shoal River; Swq. B: Swanquarter Bay; Dur. C: Durham Creek; Sth.C: South Creek; Nth.C: North Creek; Pgo.R: Pungo River; Bay.R: Bay River; Brd.C: Broad Creek; and Ortl: Oriental.


Hemocyanin Concentrations
Currituck Sound
Chowan River
ALBEMARLE SOUND
Roanoke Sound
Oregon Inlet
ALLIGATOR R.
Croatan Sound
Roanoke River
41±8 mg/ml
Pungo R.
Lake Mattamuskeet
18.7 mg/ml ±3
32.4 mg/ml ±4
Pamlico River
SOUND
18.0 mg/ml ±4
15.2 mg/ml ±1
21 mg/ml ±2
PAMLICO
CAPE HATTERAS
15 mg/ml ±3
16.8 mg/ml ±2
Neuse River
Hatteras Inlet
Ocracoke Inlet
Drum Inlet
Back Sound
62.1 mg/ml ±3
Core Sound
Bogue Sound
Barden Inlet
CAPE LOOKOUT

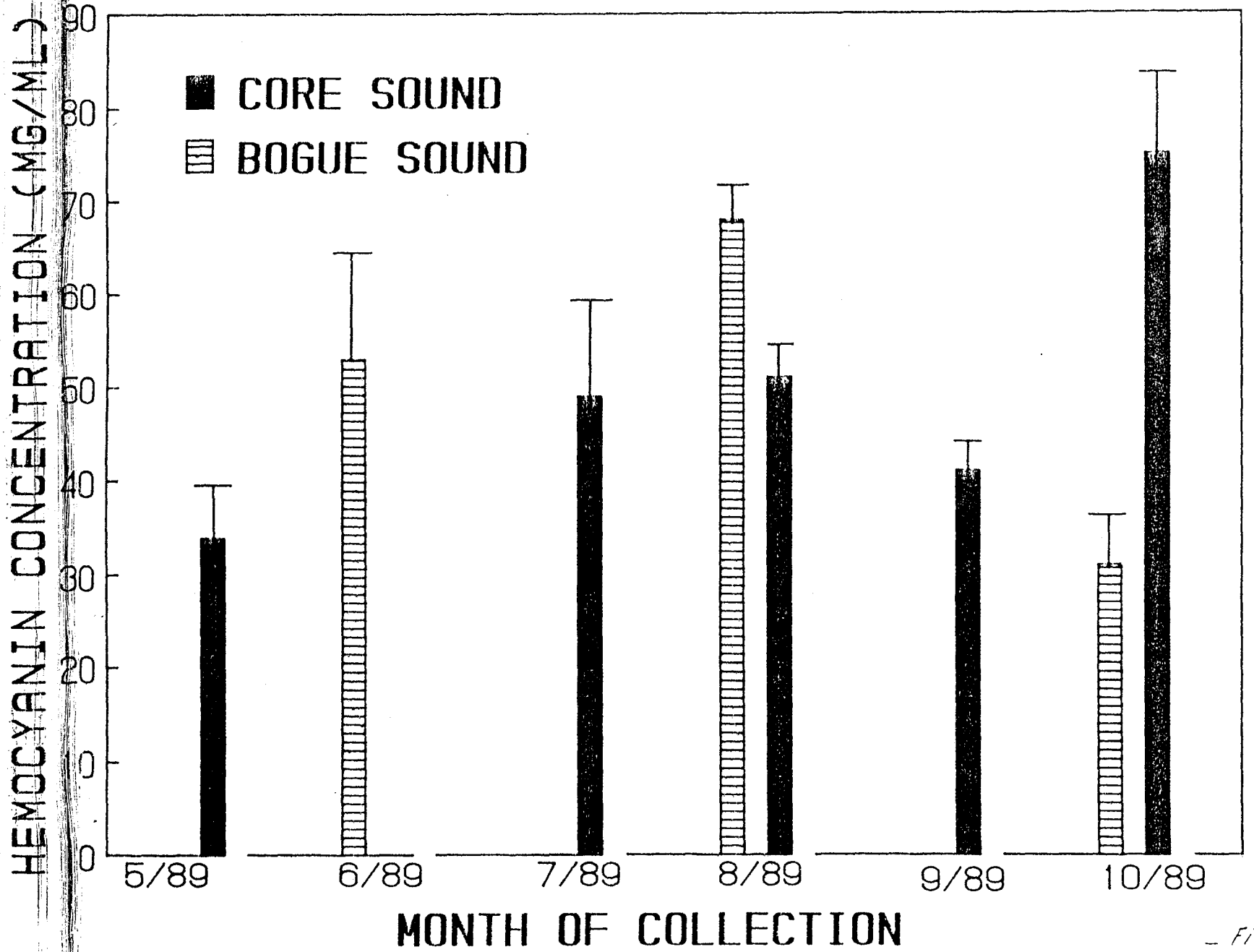
- Figure 1 -


NORTHWESTERN PAMLICO SOUND
HEMOCYANIN CONCENTRATION (MG/ML)
70
60
50
40
30
20
10
0
LONG SHOAL RIVER
SWANQUARTER BAY
5/89
6/89
7/89
8/89
9/89
10/89
COLLECTION DATE


PAMLICO RIVER AREA
HEMOCYANIN CONCENTRATION (MG/ML)
PUNGO R.
SOUTH CRK.
NORTH CRK.
5/89
6/89
7/89
8/89
9/89
10/89
COLLECTION DATE

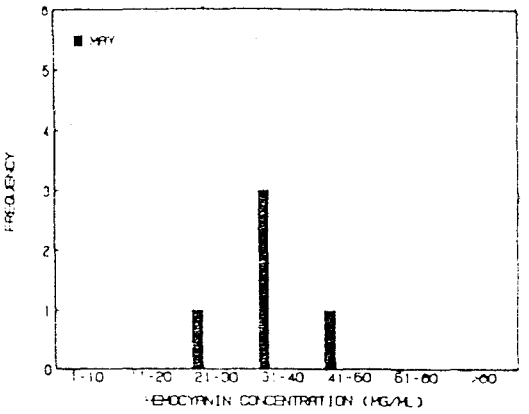

NEUSE RIVER AREA
HEMOCYANIN CONCENTRATION (MG/ML)
70
60
50
40
30
20
10
0
BAY R.
BROAD CRK.
ORIENTAL
No crabs collected 8 / 89
5/89
6/89
7/89
9/89
10/89
COLLECTION DATE

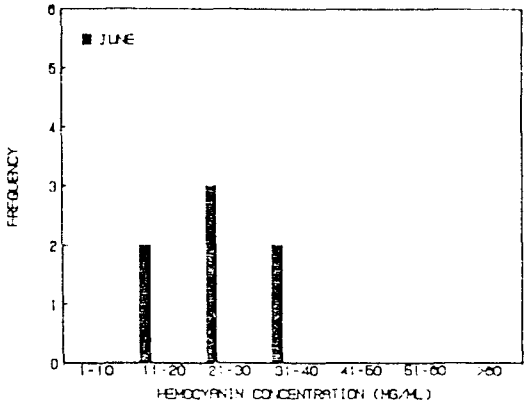
– Figure 2 –

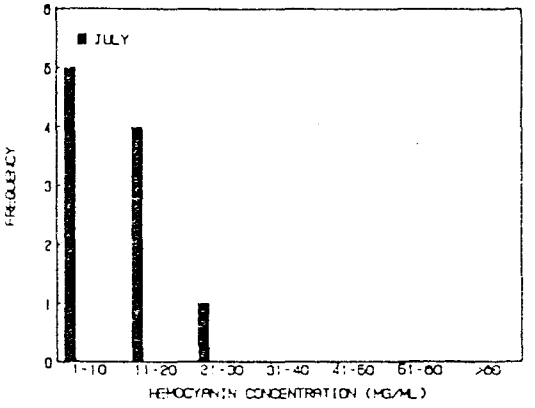

HEMOCYANIN CONCENTRATION (MG/ML)
0
10
20
30
40
50
60
70
80
90
CORE SOUND
BOGUE SOUND
5/89
6/89
7/89
8/89
9/89
10/89
MONTH OF COLLECTION

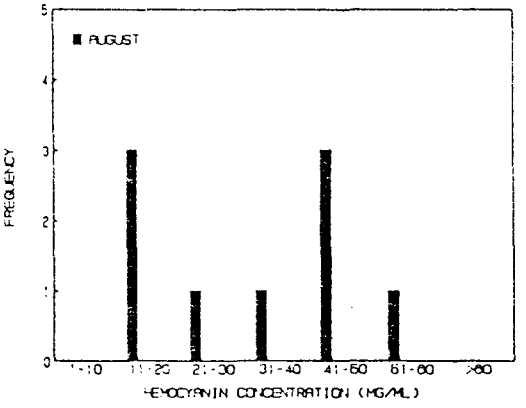
- Figure 3 -

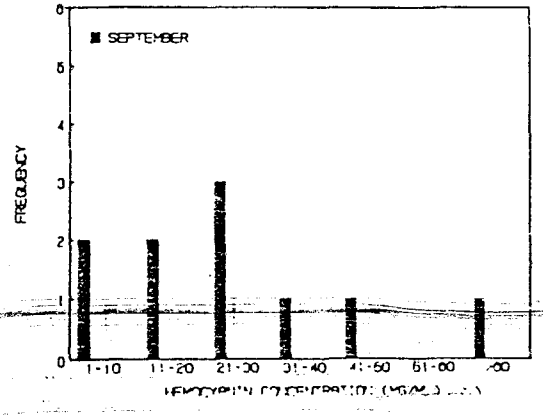
PUNGO RIVER 1989

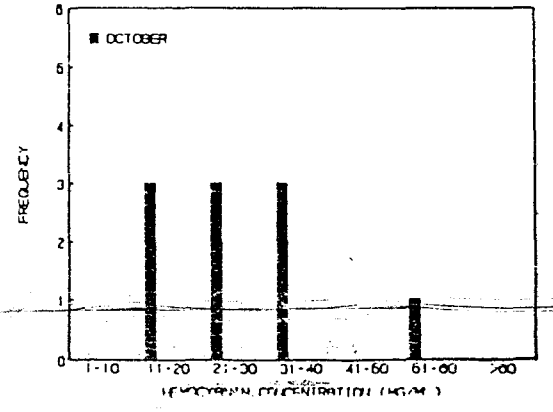




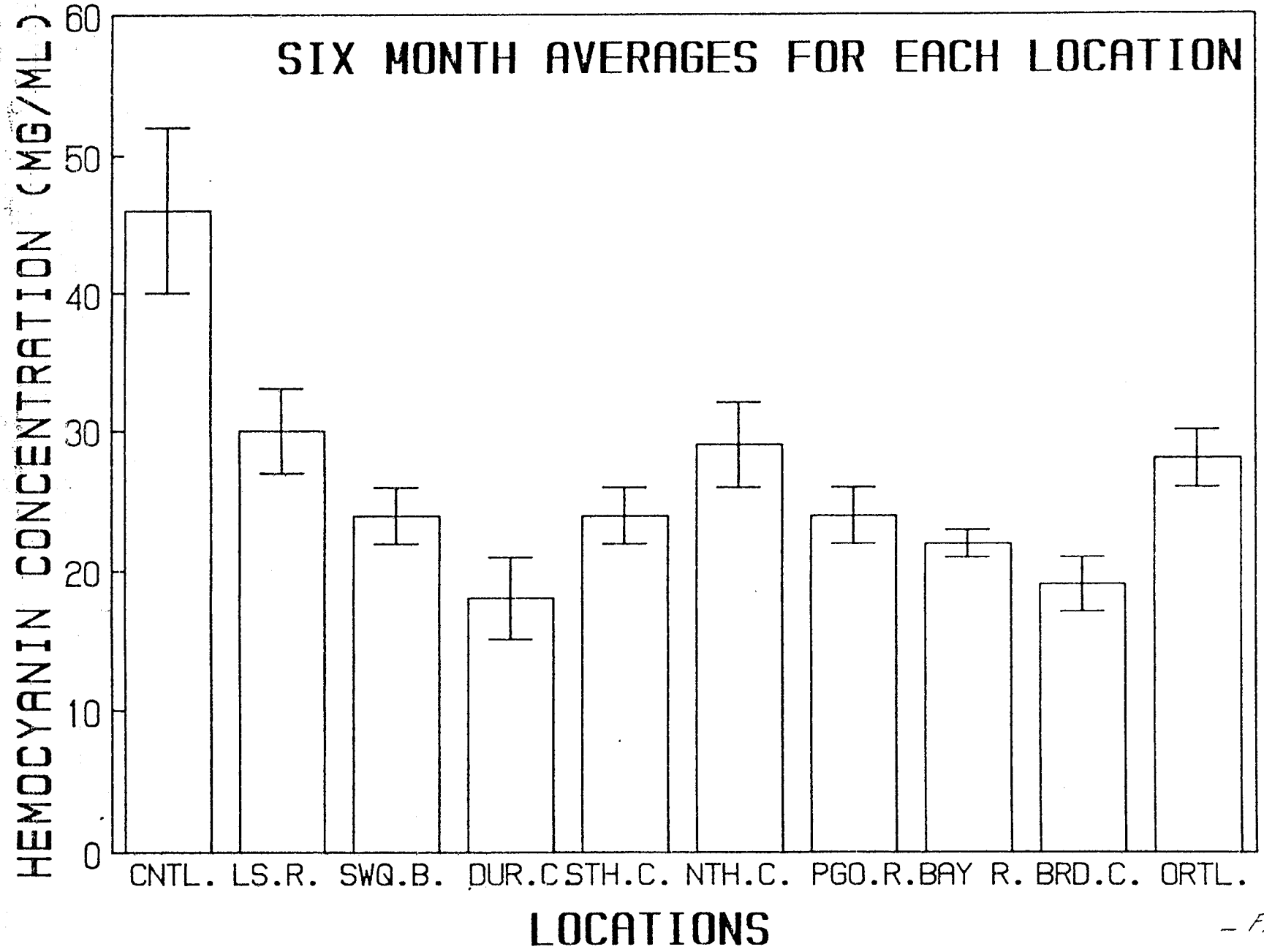








- Figure 4 -


SIX MONTH AVERAGES FOR EACH LOCATION
HEMOCYANIN CONCENTRATION (MG/ML)
0
10
20
30
40
50
60
CNTL.
LS.R.
SWQ.B.
DUR.C
STH.C.
NTH.C.
PGO.R.
BAY R.
BRD.C.
ORTL.
LOCATIONS

– Figure 5 –