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ARTICLE *in* MARINE ENVIRONMENTAL RESEARCH · SEPTEMBER 2001

Impact Factor: 2.76 · DOI: 10.1016/S0141-1136(01)00098-8 · Source: PubMed

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Effects of molting and environmental factors on trace metal body-burdens and hemocyanin concentrations in the American lobster, *Homarus americanus*

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Received 20 September 2000; received in revised form 15 October 2000; accepted 4 January 2001

Abstract

Hemocyanin concentrations in the hemolymph of marine crustacea are dependent on the molt cycle and on environmental conditions. Studies in our laboratories have found that hemocyanin levels in blue crabs are reduced after ecdysis and under conditions of environmental stress (Engel, Brouwer, & McKenna, 1993. Hemocyanin concentrations in marine crustaceans as a function of environmental conditions. *Marine Ecology Progress Series*, *93*, 233–244). We have extended those studies to include the American lobster, *Homarus americanus*. Hemolymph and digestive gland tissues from Long Island Sound lobsters were analyzed for hemocyanin, copper, and zinc during different stages of the molt cycle. Hemocyanin, copper and zinc in the hemolymph were highest in premolt stages (D_1–D_4), and lowest in the postecdysal papershell stages (B_1–B_2). Concomitantly, copper in digestive glands decreased significantly following ecdysis, but no significant changes in the metals bound to metallothionein (MT) were observed. Copper-MT was the predominant form throughout the molt cycle, presumably because lobsters were obtained from copper-contaminated areas. To examine the effects of environmental factors, intermolt lobsters were collected from locations

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0141-1136/01/$ - see front matter Published by Elsevier Science Ltd.
PII: S0141-1136(01)00098-8

of different environmental quality along the Atlantic coast, and were analyzed for hemocyanin and trace metals. In general, animals from areas with a history of contamination showed the highest hemocyanin concentrations. Published by Elsevier Science Ltd.

Keywords: Homarus americanus; Lobster; Hemocyanin; Copper; Zinc; Pollution; Molt cycle

1. Introduction

The American lobster, *Homarus americanus*, is found along the east coast of the USA and Canada from Cape Hatteras, North Carolina to the Arctic Ocean and inhabits remote areas as well as those in close proximity to population centers in both countries. Such a distribution means that because of the migratory behavior of lobsters, there is a high probability that they will be exposed to anthropogenic contaminants released into the coastal waters. Of particular interest are trace metals (i.e. copper, cadmium, zinc) normally found in their tissues and the physiological and biochemical processes that regulate them.

The American lobster, *Homarus americanus,* along with other marine crustaceans, use hemocyanin as the oxygen carrying protein in the hemolymph. The structure and function of crustacean hemocyanins have been studied extensively (Brouwer, 1992; Ellerton, Ellerton, & Robinson, 1983; Van Holde & Miller, 1982). They are large copper containing proteins composed of a minimum of six subunits, each of 75,000 dalton molecular weight. The number of hexamers that make up the hemocyanin molecule varies between one and four among various groups of crustaceans.

During the molt cycle, trace metal concentrations in the digestive gland and hemocyanin concentrations in the hemolymph of blue crabs change significantly. It was shown that the ratios of copper to zinc bound to metallothionein changed from predominantly copper during intermolt to zinc during premolt, and then back to copper after ecdysis (Engel, 1987; Engel & Brouwer, 1987, 1989, 1991). These studies suggested that both copper and zinc were lost from the digestive gland at ecdysis, and that the loss of copper correlated with decreases in hemolymph hemocyanin concentrations (Engel & Brouwer, 1991). Increased concentrations of copper and zinc in the stomachs of softshell crabs suggest that the metal is voided from the digestive gland into the gut and excreted. In other experiments, we examined the effects of accumulated cadmium in the hepatopancreas using MRI imaging techniques (Brouwer, Engel, Bonaventura, & Johnson, 1992), and found significant differences in cadmium, copper and zinc concentrations in the hepatopancreas of exposed crabs before and after molting.

Our research has shown that in addition to the physiological and biochemical processes related to the molt cycle, environmental factors could affect the synthesis and turnover of hemocyanin, and the metabolism of copper and zinc (Engel & Brouwer, 1987, 1991; Brouwer, Winge, & Gray, 1989; Engel et al., 1993). Extrinsic factors, such as elevated temperature and hypoxia in the environment, can influence the concentration of hemocyanin in the hemolymph of crabs. DeFur, Mangum, and

Reese (1990) demonstrated in the laboratory that decreased salinity and low dissolved oxygen concentrations caused an increase in hemocyanin in blue crabs. Engel et al. (1993) showed an inverse relationship between hemocyanin concentrations in blue crabs and temperature and hypoxia. Studies with the Dungeness crab, *Cancer magister*, from the waters around Vancouver, British Columbia, have shown that decreased dissolved oxygen concentrations in the vicinity of sewage outfalls may be associated with increased hemocyanin concentrations (J. Thompson, unpublished data, Institute for Ocean Science, Sidney, British Columbia).

In the studies described here, we examined how the molting process affected hemocyanin and metal concentrations in the hemolymph and metal concentrations in the digestive glands of lobsters collected from Long Island Sound in the vicinity of Milford Harbor. We also examined the effects of environmental factors on the concentrations of copper, cadmium, and zinc in the digestive glands and hemocyanin concentrations of lobsters collected from different locations in the northeast.

2. Methods and materials

Lobsters used for these measurements were collected in Long Island Sound by the staff of the NMFS, Milford Laboratory and along the north Atlantic coast by the NOAA, National Status and Trends, Benthic Surveillance Program. The lobsters used in the molting portion of the investigation were staged at the Milford Laboratory, and identified as being premolt (D_1–D_4), soft shell ($A_{1\ \&\ 2}$), paper shell ($B_{1\ \&\ 2}$), and intermolt (C_4). All of the lobsters collected by NOAA were adults, intermolt, and not segregated by sex. Hemolymph samples were collected and then the animals were euthanized and hepatopancreas samples taken.

Hemolymph samples were collected by severing an appendage between the joints. The cut was made with a sharp pair of scissors between the joints near the base of the appendage. The samples were collected in plastic vials, placed on ice, allowed to clot and frozen at $-70^{\circ}C$, and shipped to Beaufort on dry ice. The clotted hemolymph was homogenized with a Polytron homogenizer, and then centrifuged at 20,000 x ***g*** for 30 min. The resulting supernate or serum was then decanted and kept at $0^{\circ}C$.

The hemocyanin measurements were made spectrophotometrically. The hemolymph serum samples were diluted with buffer, 50 mM Tris/10 mM $CaCl_2$ pH 8.0, and readings taken at 280 and 334 nm. The concentration of hemocyanin was calculated with $E_{280nm} = 13.5$ and $E_{334nm} = 2.30$ as determined for intact undissociated hemocyanin (Johnson, Bonaventura, & Bonaventura, 1984).

For trace metal analysis, hemolymph and hepatopancreas samples were dried at $100^{o}C$, wet ashed in concentrated HNO_3, and measured for trace metal concentrations (i.e. cadmium, copper, and zinc) using flame atomic absorption spectrophotometry (Engel & Brouwer, 1987). The TORT-I certified reference material (lobster hepatopancreas) was used to verify our preparative and analytical measurement techniques for metals.

3. Results

3.1. Effects of molting

The concentrations of hemocyanin, copper and zinc in the hemolymph of lobsters varied with molt stage (Fig. 1). The highest concentrations of all three elements of the hemolymph were seen in the premolt stages (D_1–D_4) and the lowest in the postecdysal papershell stages (B_1, B_2). As would be expected, there was a close correlation between the concentrations of hemocyanin and copper in the hemolymph, since hemocyanin is a copper containing protein. Zinc also covaried positively with both copper and hemocyanin, which suggests that hemocyanin may be acting as a zinc transporter in the hemolymph.

In the digestive glands, copper, cadmium, and zinc all covaried with the stage of the molt cycle (Fig. 2). Copper showed a significant decrease ($P<0.05$) of about 2.5× from intermolt through the postecdysal softshell into the papershell stage. The amounts of copper in the digestive gland were high 12 mM/kg wet weight (762 ppm wet weight) among the intermolt animals, and decreased after ecdysis to 5 mM/kg wet weight (318 ppm wet weight) which is still elevated relative to other crustaceans we have measured (D. Engel, unpublished data). Tissue concentrations of cadmium and zinc were orders of magnitude lower than copper. While the concentrations of zinc decreased about two-fold, cadmium concentrations showed only minor fluctuations.

The cytosolic distribution of metals also was examined in digestive glands from lobsters at different stages of the molt cycle, but no significant changes were observed in the metals bound to the metallothionein (MT) peak. A typical gel-filtration elution profile (Fig. 3) shows that the vast majority of the cytosolic copper is bound to the MT peak along with some zinc and cadmium. It is hypothesized that the lack of change in metals bound to MT during the different stages of the molt cycle may be due to the high concentrations of copper in the digestive glands.

3.2. Effects of the environmental factors

Lobsters were collected over a period of 2 years, 1989 and 1990, at locations ranging from the coast of Maine to Raritan Bay in New Jersey as a part of the NOAA, National Status and Trends Benthic Surveillance Program sampling effort. The hemolymph and digestive gland samples were collected from the animals and the concentrations of hemocyanin in the hemolymph and cadmium, copper, and zinc in the digestive glands were measured (Table 1). The concentrations of hemocyanin ranged from a high of 90.4 to a low of 32 mg/ml in hemolymph. During both years, the lowest concentrations were observed at locations along the Maine coast; Casco Bay in 1989 and Johns Bay and Cape Elizabeth in 1990. The highest hemocyanin concentrations were in animals from Boston Harbor and Salem Harbor and ranged from 72 to 90.5 mg/ml during the 2 years of collections. Trace metal concentrations in the digestive glands of lobsters also varied with location. Copper was generally higher than zinc at all locations with the exception of Casco Bay and Duxbury, and

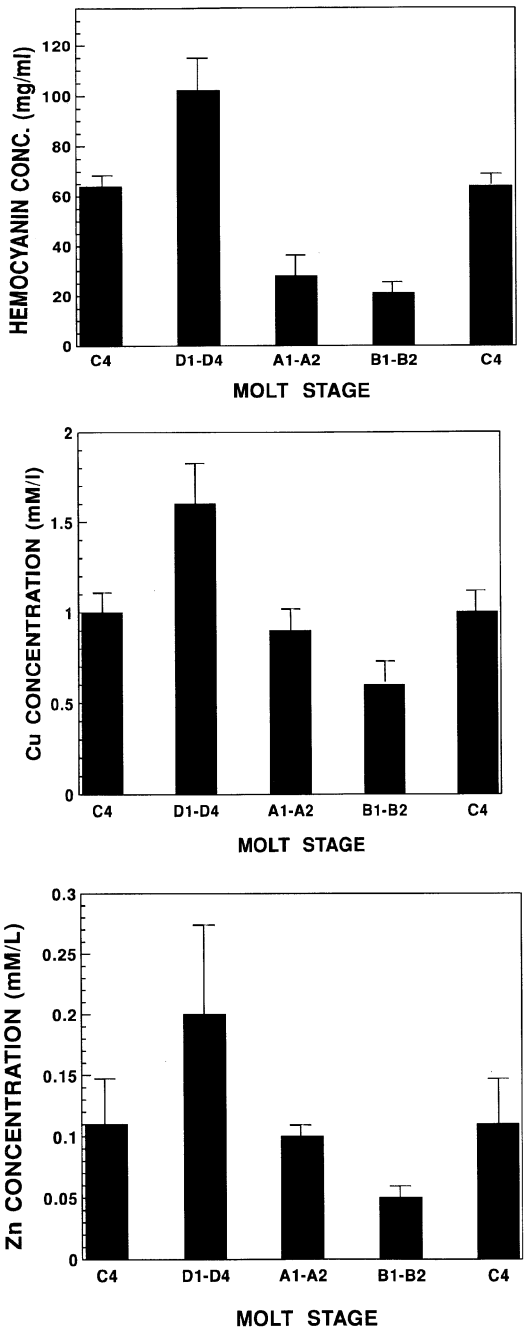

Fig. 1. Hemolymph concentrations of hemocyanin, copper, and zinc from American lobsters during different stages of the molt cycle. Molt stages were intermolt: (C_4); premolt (D_1–D_4); soft shell (A_1–A_2); and paper shell (B_1–B_2). Each mean represents three to six individual hemolymph samples ± 1 S.E.

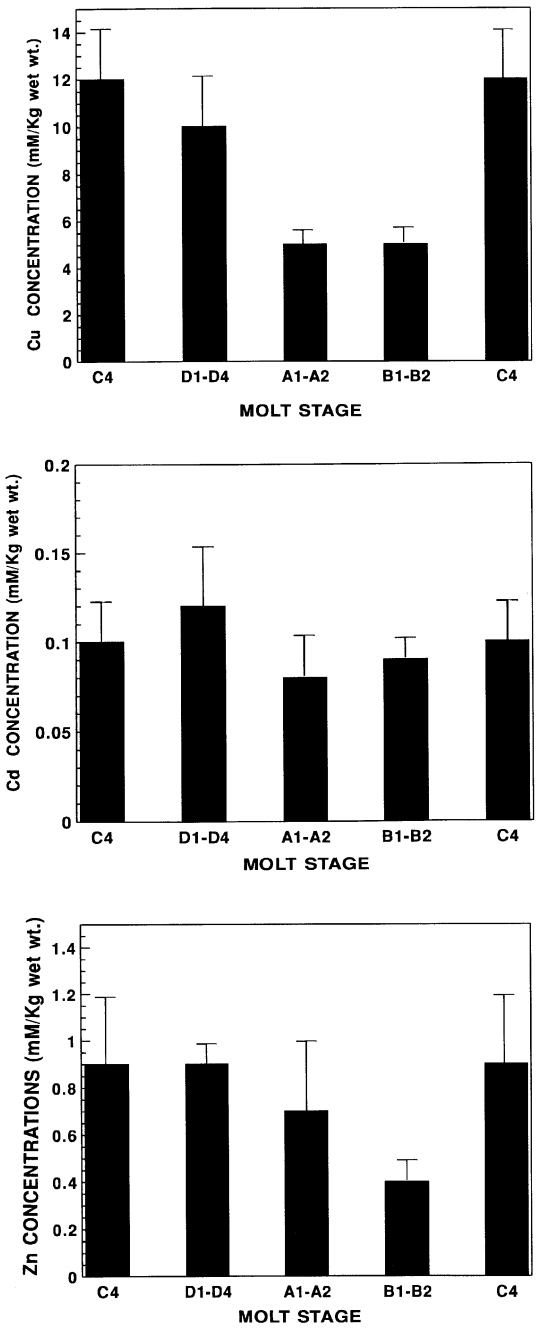

Fig. 2. Digestive gland concentrations of copper and zinc from American lobster during different stages of the molt cycle (Fig. 1). All mean values represent three to six individual samples of digestive gland ±1 S.E.

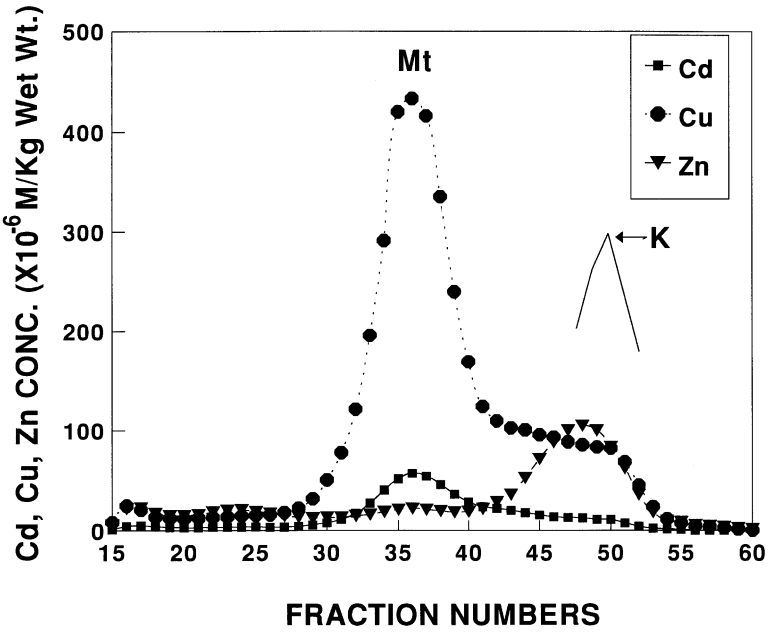

Fig. 3. Elution profiles of the cytosolic fraction of the digestive gland of an intermolt American lobster showing the partitioning of metals. Chromatographic conditions were: Sephadex G-75 gel filtration media; 2.6×65 cm column; elution buffer 60 mM Tris-HCl pH 7.9 with 2 mM 2-mercaptoethanol. The peak denoted (K) represents the position of the potassium peak (very low molecular weight).

an order of magnitude or more than cadmium at all locations in the 1990 sampling. Copper and hemocyanin concentrations were highest at urbanized locations, Boston Harbor and western Long Island Sound, but neither zinc or cadmium concentrations showed any correlations between site of collection and level of human activity.

Another series of collections of lobster hemolymph were made by the Division of Marine Fisheries of the state of Massachusetts during the spring and summer of 1989 and 1990, and the hemocyanin concentrations were measured at our Laboratory. Those locations included: Cape Ann, Boston Harbor (inside and outside), Plymouth/Cape Cod Bay, Nomans, Buzzards Bay, and New Bedford Harbor (Fig. 4). Among the lobsters that were sampled there were only two locations, Buzzards Bay (1989) and Cape Ann (1990), where the hemocyanin concentrations were below 60 mg/ml. Unlike the NBSP sampling strategy, most of these sites were in close proximity to urbanized areas with the exception of Cape Ann.

4. Discussion

The relationships between trace metal concentrations in the hemolymph and digestive glands of lobsters during the molt cycle were similar to the observed changes among blue crabs, *Callinectes sapidus*, in earlier studies (Engel, 1987; Engel

& Brouwer, 1987, 1991). Unlike the blue crabs, the lowest hemocyanin, copper, and zinc concentrations (Fig. 1) occurred in the papershell stages (B_1, B_2) rather than the softshell stages (A_1, A_2; Engel, 1987; Engel & Brouwer, 1991). The range of observed hemocyanin concentration changes in the hemolymph also were similar to those observed with the blue crab. However, unlike the blue crabs, there was a measurable increase in hemolymph hemocyanin concentration prior to ecdysis (D_1–D_4), which was mirrored in the concentrations of copper and zinc. The observed elevated metal and hemocyanin concentrations are probably associated with increased hemolymph osmotic pressure and covarying total protein concentrations prior to molt (Mercaldo-Allen, 1991). Increased hemolymph osmotic pressure would facilitate the uptake of water that is a component of the process of ecdysis.

The concentrations of copper, cadmium and zinc in the digestive glands of lobsters were related to the molt cycle, but not necessarily in the same manner (Fig. 2). Copper and zinc concentrations decreased by about a factor of two at ecdysis and remained relatively constant through the papershell stages which is similar to that observed in post-molt blue crabs (Engel, 1987; Engel & Brouwer, 1991). As with the blue crab and other crustaceans, dilution of the hemolymph after ecdysis and the reduction hemocyanin and metal concentrations is caused by the imbibition of water. The concentrations of copper in the digestive glands of lobsters prior to molt, however, were about 100-fold higher than in the blue crab digestive glands. These lobsters were collected in Long Island Sound near the Housatonic River which has been shown to have high concentrations of available copper (Sunda & Huntsman, 1991). Historically, this area of the Connecticut coast had a large brass and copper

Table 1
Cadmium, copper and zinc concentrations in the hepatopancreas and hemocyanin concentrations in the hemolymph of adult lobsters collected from coastal waters along the northeast coast of the USA in 1989 and 1990 in collaboration with the NOAA Status and Trends, Benthic Surveillance Program[a]

Location	N	Cadmium	Copper	Zinc	Hemocyanin
Quincy Bay, Boston, Harbor	10	NA	3.4±0.5	0.5±0.08	74.0±6
	10*	0.02±003	2.9±0.1	0.7±0.2	90.4±5
Deer Island, Boston Harbor	5	NA	1.6±0.3	0.4±0.08	80.0±4
	10*	0.01±0.002	0.7±0.1	0.4±0.03	79.5±5
Hull Harbour, Boston Harbor	3	NA	1.5±0.3	0.4±0.003	72.0±5
	10*	0.01±0.001	1.2±0.2	0.3±0.01	84.1±5
Duxbury	5	NA	0.6±0.2	0.6±0.003	60.0±6
Salem Harbour	6	NA	1.9±0.4	0.5±0.006	85.0±9
Raritan Bay	6*	0.05±0.01	1.4±0.4	0.6±0.03	62.8±8
W. Long Island Sound	10*	0.04±0.004	3.6±0.5	0.8±0.1	64.4±16
Narragansett Bay	5*	0.02±0.01	1.8±0.6	0.5±0.1	59.2±3
Mystic River	10*	0.01±0.002	1.7±0.5	0.8±0.1	68.6±5
Cape Elizabet	10*	0.05±0.006	2.4±0.3	0.3±0.01	41.6±4
Penobscot Bay	7*	0.05±0.005	0.6±0.1	0.3±0.03	57.1±9
Johns Bay	10*	0.06±0.006	0.8±0.1	0.3±0.03	39.5±8
Casco Bay	7	NA	0.4±0.6	0.5±0.006	32.0±5

[a] Metal concentrations are mM/kg wet of tissue and hemocyanin concentrations are mg/ml of hemolymph (* denotes samples collected in 1990; NA = not measured).

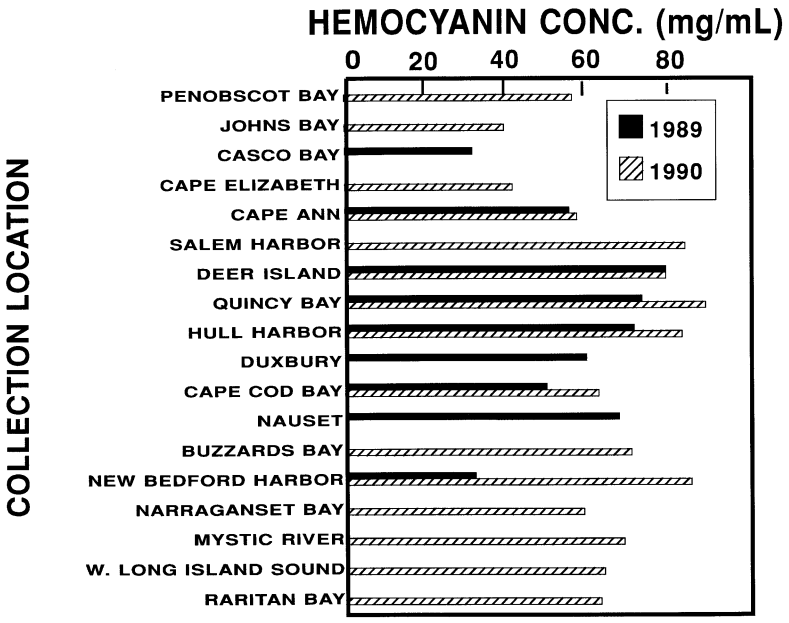

Fig. 4. American lobster hemolymph hemocyanin concentrations collected form animals captured at different locations in the coastal waters of the state of Massachusetts by the Massachusetts Division of Marine Fisheries in 1989 and 1990.

industry. Cadmium, which was not detected in blue crabs, did not change significantly throughout the molt cycle of lobsters. In experiments with blue crabs, where the animals were fed cadmium enriched food, a large proportion of the accumulated cadmium was lost at molt, ~60% (Brouwer et al., 1992). The lobsters may not eliminate metals from their digestive glands at molt in the same way as the blue crabs, as indicated by the lack of change in cadmium in the lobster. Due to the high concentrations of copper in lobster digestive glands small changes in total copper that may have occurred at molt could not be detected.

The concentrations of zinc in lobster digestive glands, and changes during the molt cycle, about a factor of two, were similar to those of blue crabs (Fig. 2). Zinc metabolism in lobsters and blue crabs is similar and appears to be well regulated, but if lobsters are fed a high zinc diet, oysters, they accumulate significant amounts of zinc (Engel & Brouwer, 1986) and will retain it over time. Blue crabs, however, will neither accumulate nor retain zinc, whether it is injected or forcefed (Engel & Brouwer, unpublished data).

Lobsters did not show any differences in the cytosolic partitioning of digestive gland metals during the different stages of the molt cycle. Using gel permeation chromatography, we were unable to detect meaningful changes in the Cu/Zn ratios associated with metallothionein, MT, at any point in the cycle. The lack of change in the metal ratios associated with MT may be attributed to the elevated concentrations of copper in the digestive glands of lobsters that ranged from 12 to 5 mM/kg.

Thus, small changes in cytosolic copper would not be detectable due to the large pool of exchangable copper. In blue crabs the ranges in copper concentrations were lower (i.e. 0.8–0.1 mM/kg) allowing short-term subtle changes in copper partitioning to be detected (Engel & Brouwer, 1991).

Digestive gland concentrations of copper, cadmium, and zinc also were measured in lobsters collected from different locations in the northeast and compared to sediment concentrations of those same metals (Harmon, Gottholm, & Robertson, 1998). Since copper is most abundant in the digestive glands of the lobsters, mean digestive gland copper concentrations are plotted against mean sediment copper concentrations at locations where both data sets were available. A poor correlation between tissue concentration and sediment concentration resulted ($R^2 = 0.258$; Fig. 5A). Weakness of the correlation suggests that other factors such as seasonal migrations, food web relationships, and environmental conditions may play major roles in trace metal (copper) bioaccumulation in marine crustaceans.

Zinc is also abundant in the sediments of all the sites where lobsters were collected in the northeast (Harmon et al., 1998). The measured concentrations of zinc in the digestive glands of the lobsters from all locations, however, are very similar (Table 1), which reflects the degree of regulation. While zinc concentrations in lobsters can be elevated in the laboratory, under normal environmental conditions lobsters closely control their body-burden of zinc.

Hemocyanin concentrations also were measured in lobster hemolymph collected from different locations in the northeast, and these data suggested that environmental conditions might have an influence on hemocyanin concentrations (Table 1, Fig. 4). It appears that lobsters collected from locations with heavy industrial and urban inputs (i.e. Boston Harbor, New Bedford Harbor, and Salem Harbor) had higher hemocyanin concentrations than those from clean areas. Sediment copper concentrations, used as surrogate indicators of human and industrial inputs, were plotted against hemocyanin concentrations from lobsters collected from those same locations (Fig. 5B). Lobsters collected at locations with higher sediment copper concentrations tended to have the highest hemolymph hemocyanin concentrations. Such a correlation suggests that anthropogenic inputs may modify environmental conditions, i.e. low dissolved oxygen or increased food availability, which in turn may be influencing the hemocyanin concentrations. A similar response pattern was observed among Dungeness crabs, *Cancer magister*, collected at sites in the vicinity of sewage outfalls, potentially hypoxic areas, around Vancouver, British Columbia harbor. Those crabs also had hemocyanin concentrations that were higher than those collected from uncontaminated, well flushed sites in the Strait of Georgia (i.e. water temperature rarely $> 13^{\circ}C$; J. Thompson, Sidney, British Columbia, unpublished data). Thus the both lobsters and Dungeness crabs could be responding to reduced dissolved oxygen in the presence of adequate food by increasing their hemocyanin concentration. Hagerman and Baden (1988) showed that under chronic severe hypoxia, Norway lobsters, *Nephrops norvegicus*, had reduced feeding and lowered hemocyanin concentrations. Baden, Phil, and Rosenberg (1990) showed, however, that under moderate hypoxia, hemocyanin concentrations increased, lobsters could still feed, but with severe hypoxia the concentrations decreased. In North

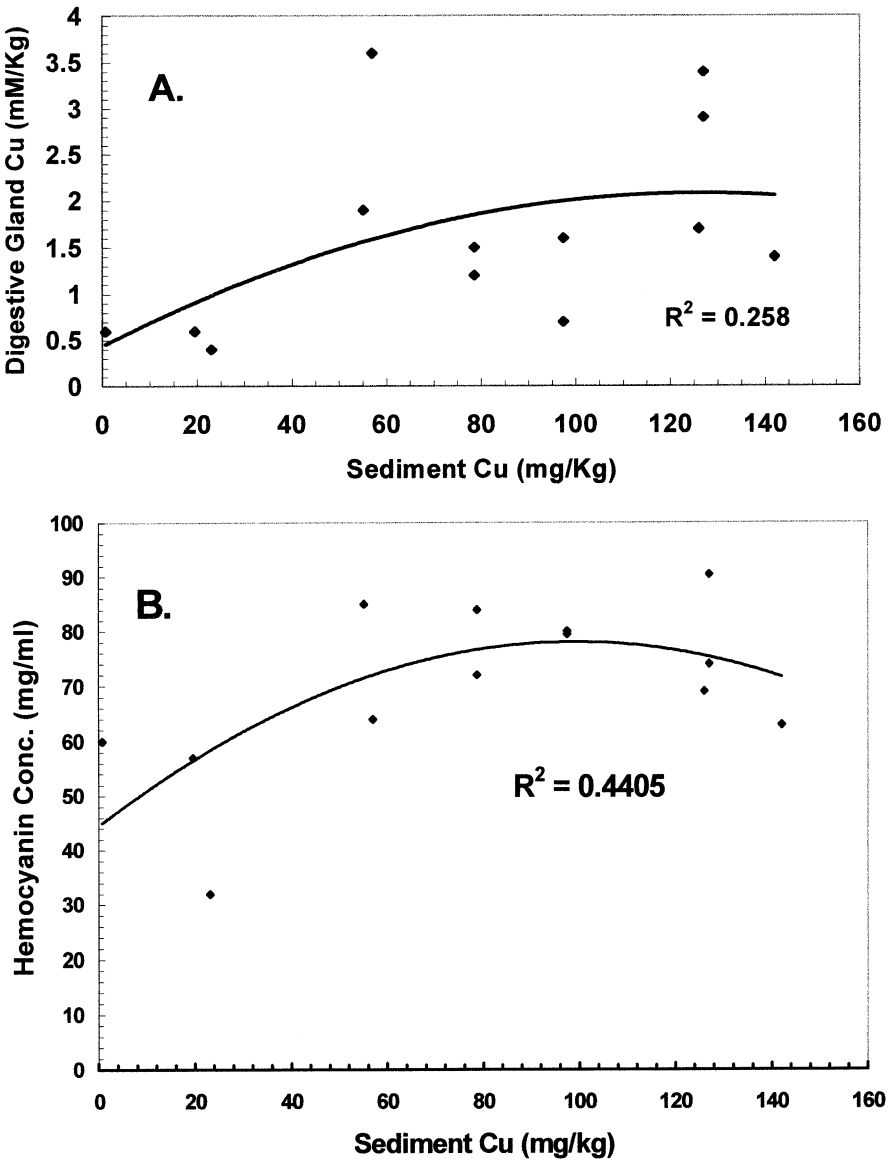

Fig. 5. Lobster digestive gland copper (A) and hemolymph hemocyanin concentrations (B) regressed against sediment copper concentrations from the sites of collection along the northeast coast. Trend lines are quadratic regressions. Digestive gland copper and hemolymph hemocyanin concentrations are from this study and the sediment metal data are taken from Harmon et al. (1998). All data points are mean values for those specific locations.

Carolina coastal rivers during the midsummer months, Engel et al. (1993) demonstrated a correlation between low dissolved oxygen concentrations elevated water temperatures and reduced concentrations of hemocyanin in the hemolymph of blue crabs. It was suggested that low dissolved oxygen and elevated temperatures led to reduced feeding, either through reduced mobility or the lack of prey organisms, which resulted in hemocyanin concentrations 20% of normal. DeFur et al. (1990), however, induced hemocyanin synthesis in blue crabs in the laboratory by subjecting them to reduced dissolved oxygen and moderate temperatures, but supplied adequate food throughout the period of stress. Thus, hypoxia, temperature, and food availability may interact to modify hemocyanin concentrations in marine crustaceans both in the presence and absence of contaminants.

Crustacean hemolymph hemocyanin concentration may be a useful field measure of health status. Such a measurement is attractive due to the ease of hemolymph collection and the measurement of hemocyanin. It is necessary, however, to evaluate and acknowledge the various environmental factors that may affect the measurement in order to interpret the data.

Acknowledgements

The authors thank Dr. Bruce Estrella and the personnel of the Massachusetts Division of Marine Fisheries and Mr. Peter Crumley of the National Marine Fisheries Service, Beaufort Laboratory for collecting the lobster hemolymph and tissue samples that were used in this investigation, and the use of the facilities of the Duke University Marine Laboratory, Beaufort, North Carolina. This investigation was supported by the US Department of Commerce, NOAA/National Marine Fisheries Service.

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