Third
Long Island Sound Lobster Health
Symposium

Friday, March 7, 2003
Bridgeport, Connecticut

Co-Sponsored by

The Long Island Sound
Lobster Research Initiative
and the
Connecticut Department
of Environmental Protection
Long Island Sound Research Fund

CT-SG-03-02

The Long Island Sound Lobster Research Initiative

A collaboration funded by the National Oceanic and Atmospheric Administration (NOAA), National Marine Fisheries Service, NOAA Sea Grant Programs of Connecticut and New York, the Connecticut Department of Environmental Protection Long Island Sound Research Fund, and the U.S. Environmental Protection Agency, Long Island Sound Study Office, in collaboration with the Atlantic States Marine Fisheries Commission.In July 2000, Congress made $6.9 million available to support research into the causes of the 1999 lobster mortality event and to support assessment and monitoring of the Long Island Sound lobster resource.

Lobster Mortality Research Steering Committee

- Atlantic States Marine Fisheries Commission
- Connecticut Department of Environmental Protection
- New York State Department of Environmental Conservation
- NOAA, National Marine Fisheries Service
- NOAA, National, Connecticut, New York Sea Grant Programs
- U.S. Environmental Protection Agency, Long Island Sound Study Office
- Connecticut and New York lobster industry representatives

Connecticut Department of Environmental Protection Long Island Sound Research Fund

The Long Island Sound Research Fund, administered by the Connecticut Department of Environmental Protection, was established in 1989 to promote scientific research through in-state academic institutions directed toward priority environmental protection and management of the Long Island Sound estuary. In July 2000, Governor Rowland made one million dollars available to the fund to help determine the causes of recent lobster mortality and identify methods to prevent future declines.

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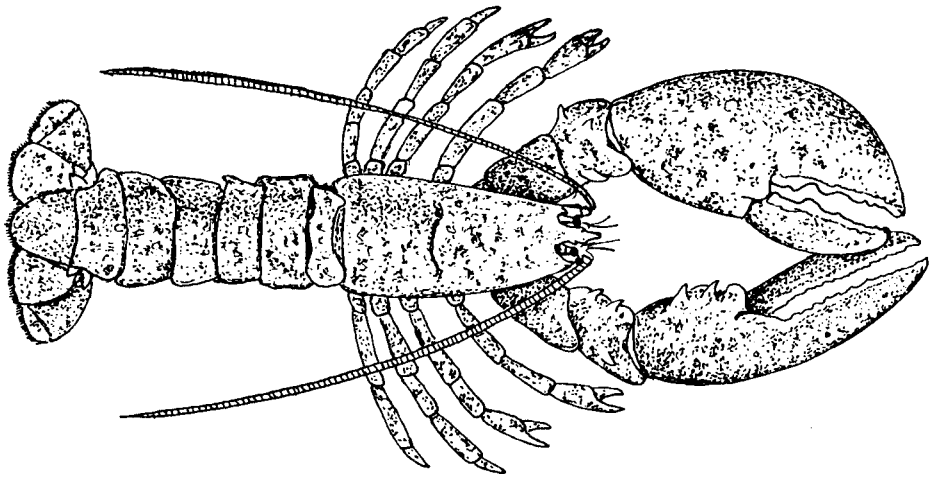

Hosted by the NOAA, Sea Grant Programs in Connecticut and New York under the auspices of the Atlantic States Marine Fisheries Commission (ASMFC) Lobster Mortality Research Steering Committee.

For additional information, contact:

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Visit our website at **www.seagrant.sunysb.edu/LILobsters**

Symposium Agenda

9:00 a.m. **Welcoming Remarks**

Anthony Calabrese, Chair, Lobster Mortality Research Steering Committee, NOAA, NMFS
Arthur J. Rocque, Jr., Commissioner, Connecticut Department of Environmental Protection
Gerald Barnhart, Director, NYS DEC, Division of Fish, Wildlife, and Marine Resources
(TBA), NOAA, National Marine Fisheries Service, Department of Commerce

9:15 a.m. **The Status of the Long Island Sound Lobster Resource**

Carl LoBue, New York State Department of Environmental Conservation
Penny Howell, Connecticut Department of Environmental Protection

This presentation combines the work of the following agencies and investigators:

- **Connecticut Department of Environmental Protection**
- **Jospeh Crivello,** University of Connecticut
- **New York State Department of Environmental Conservation**
- **Roman Zajac,** University of New Haven

10:00 a.m. **Discussion Period**

10:20 a.m. **Break**

10:40 a.m. **Environmental Stressors**

Carmela Cuomo, Yale University

This presentation combines the work of the following investigators:

- **Carmela Cuomo,** Yale University; **Raymond Valente,** SAIC
- **Andrew F.J. Draxler and Ashok Deshpande,** NOAA, NMFS, Howard Lab
- **Glenn Lopez and Robert Cerrato,** SUNY Stony Brook
- **Johan C. Varekamp, Ellen Thomas,** Wesleyan University; **Marilyn Buchholtz ten Brink,** USGS; **Mark Altabet,** UMASS, Dartmouth; **Sherri Cooper,** Bryn Althyn College
- **Robert E. Wilson, R. Lawrence Swanson, and Duane Waliser,** SUNY Stony Brook

11:20 a.m. **Discussion Period**

11:40 a.m. **Physiological Responses to Stress**

Richard A. Robohm, NOAA, National Marine Fisheries Service, Milford Laboratory

This presentation combines the work of the following investigators:

- **Robert S. Anderson,** University of Maryland
- **Ernest S. Chang,** University of California, Davis
- **Sylvain De Guise, Jennifer Maratea, Inga Sidor, James Atherton, Brenda Morsey,** University of Connecticut

- **Jan R. Factor,** SUNY Purchase
- **Hans Laufer, William Biggers, M. Johnson, N. Demir,** University of Connecticut; **J. Bagshaw,** Worcester Polytechnic Institute
- **Richard A. Robohm,** NOAA, NMFS, Milford Lab; **and Andrew F.J. Draxler,** NOAA, NMFS, Howard Lab

12:20 p.m. **Discussion Period**

12:40 p.m. **Lunch**

1:40 p.m. **Pesticides**

Sylvain De Guise, University of Connecticut

This presentation combines the work of the following investigators:

- **Sylvain De Guise, Jennifer Maratea, Christopher Perkins,** University of Connecticut
- **Michael N. Horst, Anna N. Walker,** Mercer University; **Thomas Wilson,** Colorado State University; **Parshall Bush,** University of Georgia; **Ernest Chang,** University of California, Davis; **Tim Miller,**University of Maine; **Robert Vogel,** Mercer University
- **Hans Laufer, William Biggers, M. Johnson, N. Demir,** University of Connecticut; **J. Bagshaw,** Worcester Polytechnic Institute
- **Anne McElroy, Bruce Brownawell,** SUNY Stony Brook

2:20 p.m. **Discussion Period**

2:40 p.m. **Parasites and Disease**

Salvatore Frasca, Jr., University of Connecticut

This presentation combines the work of the following investigators:

- **Andrei Chistoserdov,** University of Louisiana; **Roxanna Smolowitz, Andrea Hsu,** Marine Biological Laboratory
- **Alistair Dove, Paul Bowser,** Cornell College of Veterinary Medicine; **Carl LoBue,** NYS DEC
- **Salvatore Frasca, Jr., Kathleeen Nevis, Thomas E. Mullen,** University of Connecticut
- **Rebecca Gast,** Woods Hole Oceanographic Institution
- **Patrick M. Gillevet ,** George Mason University; **Charles O'Kelly,** Bigelow Laboratory for Ocean Sciences

3:20 p.m. **Discussion Period**

3:40 p.m. **Open Discussion**

4:00 p.m. **Summarizing Remarks**

4:30 p.m. **Adjourn**

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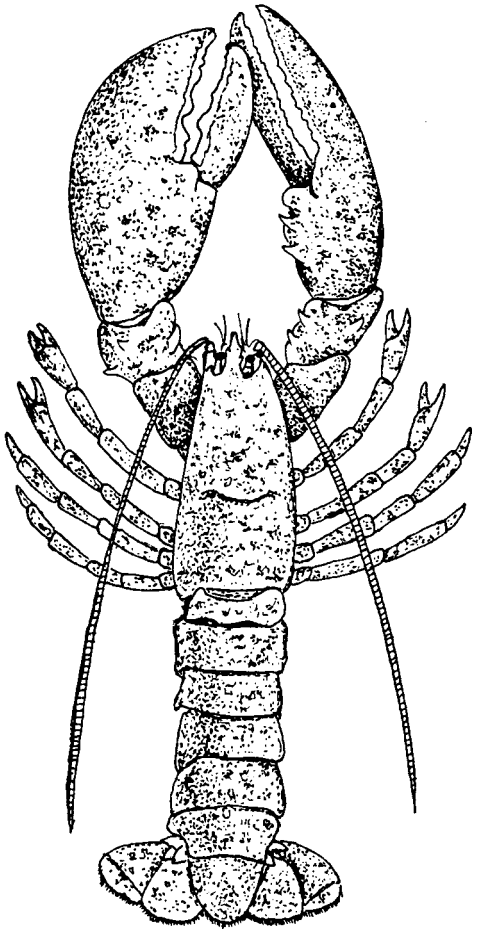


Fishery-Dependent Monitoring of the Long Island Sound Lobster Fishery

Carl LoBue[1] and Penny Howell[2]

[1]New York State Department of Environmental Conservation, East Setauket, NY
[2]Connecticut Department of Environmental Protection, Old Lyme, CT

The New York State Department of Environmental Conservation (NYS DEC) and the Connecticut Department of Environmental Protection (CT DEP) monitor the lobster harvest in each state through fishermen's logbooks, annual recall surveys, and at sea observer programs. This information shows that there had been an increase in the annual harvest of lobsters each year from the late 1980s though the late 1990s. This increase was correlated with an increase in lobster fishing effort as measured by the number of traps fished, as well as an increase in the abundance of lobsters as measured by the CT DEP Long Island Sound trawl survey. These trends were reversed around the time of the mortality event that affected Long Island Sound (LIS) lobsters and crabs in 1999. The recent harvest levels are approximately 30% of their pre-die-off peak.

By mid-winter of 2000, observations and reports of dead lobsters had decreased to background levels. The observations and reports of dead lobsters increased again in the late summer and fall of 2001 and 2002. The event in 2002 began earlier than in 1999 (mid-August), occurred Sound wide, and was restricted to lobsters (crabs seemed unaffected).

Despite a declining number of licensed commercial lobstermen and reductions in the number of permitted traps, the Long Island Sound lobster fishery remains over capitalized. Although exploitation rate is down, the combined natural and fishing mortality rate is up. The size distribution of lobsters in the Long Island Sound remains truncated at the first molt increment above legal size. Therefore, the number of lobsters available for harvest is directly dependent upon the number of lobsters molting into legal size each season.

Shell disease symptoms have remained fairly uncommon in the western LIS and are more common further east in the Sound. October and November of 2002 had the highest prevalence (15-22%) of shell disease in the eastern half of the Sound for the three-year time series. Shell disease symptoms continue to be prevalent in about 40% of the total catch east of the Connecticut River. Although symptoms in this area were less prevalent in 2002 than in 2001, the symptoms were more severe in 2002. Overall symptoms prevalence is lower during the molting seasons. Shell disease symptoms were also common in samples from the Atlantic Ocean about 8 miles off Jones Beach. The low incidence of symptoms in the WLIS suggests that there is little movement of infected lobsters from east to west. Minimal westward movement of lobsters is corroborated by results of more than two decades of tagging studies preformed by Don Landers at Millstone Environmental Lab.

The global economic effects on demand in concert with strong lobster landings from down east Maine and parts of Canada have kept lobster prices fairly stable. Without high wholesale prices, much of the LIS lobster fishery became commercially nonviable in the fall of 2002 and many fishermen stopped fishing or hauled their traps very infrequently. In New York this resulted in a rapid closeout of a federal and state funded effort reduction program as fishermen were forced to divest from the industry.

The future of the Long Island Sound lobster fishery over the next several years does not look optimistic. The combination of declining abundance and truncated size distribution has reduced the potential annual reproductive output of the LIS lobster population. Evidence of limited westward migration in addition to a reduced stock size in much of southern New England suggests there will not be rapid immigration to the central and western Sound. Cyclic mortality surges in late summer and early fall over the past several years suggest environmental stressors continue to contribute to population declines. Increased time intervals between checking lobster trap gear because of poor catches could exacerbate existing lobster health problems. The fishery's reliance on first molt group lobsters restricts annual harvest to the number of animals molting to legal

size each year. Global economics and alternative sources of lobsters are likely to keep dockside lobster prices from increasing significantly. Region-wide over-capitalization, the high cost of doing business in coastal NY/CT communities, and the limited opportunities available in other commercial fisheries suggests that the Long Island Sound lobster industry will continue to be in economic distress over the next several years. The long-term viability of this industry is uncertain.

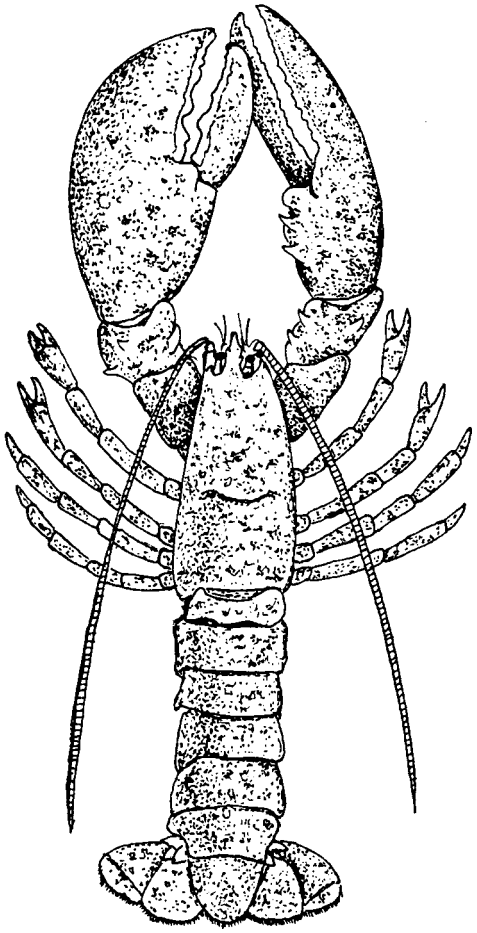
Fishery-Independent Monitoring of the Long Island Sound Lobster Fishery

Penny Howell, Connecticut Department of Environmental Protection, Old Lyme, CT

CT DEP monitors the status of important marine populations through a Long Island Sound Trawl Survey (LISTS) conducted in spring (April-June) and fall (September-October) every year since 1984. Indices of relative abundance for lobster catches rose dramatically from 1997 through 1999 and declined just as dramatically from 2000-2002. Although the 2002 spring index ranked 11th out of 19, the 2002 fall index is the lowest in the time series (19th).

CT DEP also initiated several studies in 2001 to investigate causes and consequences of a major die-off of lobsters in western Long Island Sound (LIS). Two studies meant to complement each other are a tagging program and a genetic analysis. As of January 1, 2003, a total of 7,888 lobsters have been tagged and released throughout the three basins of Long Island Sound (east, central, west). Preliminary movements have been calculated for recaptures during the first year at large. For the majority of recaptured lobsters, net movement was less than 1 kilometer. Few animals crossed from one basin to another or left the Sound. In order to examine long term differences among the Sound's lobsters, a genetic analysis was conducted by Dr. Joseph Crivello at UCONN, Storrs. Examination of DNA microsatellite markers of egg-bearing lobsters from the three LIS basins and offshore (Hudson Canyon) indicate that eastern and central LIS lobster populations are more genetically similar than offshore and western LIS populations. There were much larger levels of genetic difference between western LIS lobsters and other tested populations. These differences suggest that some unknown factors are limiting gene flow between the Sound and offshore, and between central and western LIS. If these preliminary findings remain consistent through the 3-year study, the implications are that (1) lobsters within Long Island Sound function as a distinct population; (2) lobsters within the Sound's western basin normally have little biological communication with the rest of the Sound; and (3) population rebuilding in the west following the die-off will have to occur primarily through enhanced local reproduction and survival rather than immigration from adjacent areas.

Environmental Stressors



Monitoring of Bottom Water and Sediment Conditions at Critical Stations in Western Long Island Sound

Carmela Cuomo[1], Raymond Valente[2], and Deren Dogru[3]

[1]Yale University and The University of New Haven, New Haven, CT
[2]SAIC, Newport, RI
[3]Yale University, New Haven, CT

The collapse of the lobster fishery in WLIS during September of 1999 served to raise concerns in the scientific and regulatory communities about the environmental processes that regularly occur at the bottom in WLIS. REMOTS® sediment-profile images taken immediately after the lobster die-off showed the presence of an extremely shallow (< 1 cm deep) apparent Redox Potential Discontinuity (aRPD) in the sediments underlying the majority of lobster fishing grounds in the western Sound. Such an aRPD is usually indicative of a recent hypoxic/anoxic event.

The summer of 1999 was not the first summer, however, during which WLIS experienced severe, widespread occurrences of hypoxic conditions and fisheries loss – it was simply the most widespread and the most economically-costly occurrence. One common environmental thread that runs through all of the lobster die-offs in LIS, both the massive ones and the more minor ones, is the presence of hypoxic/anoxic waters, as recorded by the CT DEP as part of their ongoing monitoring of water conditions in Western Long Island Sound. Although many other factors (e.g. paramoebas, high water temperatures, pesticide spraying, bait degradation products) may, undoubtedly, have been the ultimate cause of the death of the lobsters, bottom water hypoxia *was* recorded early during the summer of 1999 (CT DEP monitoring data), as well as during the massive die-off itself (as recorded by REMOTS®).

Hypoxia in Long Island Sound is a long-standing problem. Its main cause appears to be nitrogen enrichment from both point and non-point sources of pollution (CT DEP Bureau of Natural Resources, Marine Fisheries Office, 2000; Kaputa, N.P. & C.B. Olsen, 2000; Welsh, B.L. & F.C. Fuller,1991; Welsh, B.L, *et al.*,1994). Beginning in 1988, a comprehensive monitoring of western LIS waters for physical, chemical, and biological data has been undertaken by the CT DEP in conjunction with the USEPA in order to track, among other things, the development of hypoxic and anoxic waters. It should be noted that water samples are routinely taken as part of this analysis at approximately 1 meter off the bottom. While this provides important information, it does not necessarily reveal the environmental conditions at the sediment-water interface that benthic organisms are experiencing at any given point in time.

In the Spring of 2000, the USEPA and NEIWPCC authorized a four month study (August-November) of bottom water (< 5 cm above the bottom) and sediment chemistry at 36 sites in WLIS. The majority of these sites coincided with stations previously sampled immediately following the die-off in October of 1999. In addition to collecting REMOTS® sediment-profile images, the USEPA-NEIWPCC study also analyzed bottom waters for dissolved oxygen, hydrogen sulfide, and ammonia. Data from this study revealed that a number of stations, mainly concentrated in the western-most Sound (Western Narrows), had extremely black (i.e., anoxic/sulfidic) sediment and thin redox depths. Hypoxia (as defined by dissolved oxygen measurements) at the bottom of the water column in WLIS persisted during the summer of 2000 for a longer time period of time than in the waters present at 1 m or more above the bottom (CT DEP data). In fact, dissolved oxygen concentrations indicative of moderately severe hypoxia were observed at stations in the extreme western Sound during late August 2000. Bottom water dissolved oxygen levels increased steadily from August to November, indicative of the breakdown of thermal stratification and system wide re-aeration of the bottom waters.

In addition, measurable levels of hydrogen sulfide and ammonia were detected in bottom waters during August, September, and November indicating that the anaerobic decomposition processes occurring in the

sediment at that time dominated the bottom water benthic environment. Although much valuable data was collected in this study, the timing was such that the study missed most of the months when WLIS is, in fact, most susceptible to hypoxia (June-August), as well as some of the time during which the lobster mortality occurred (September-October). The work discussed herein sought to address this lack of information, particularly as it relates to seasonal changes in sediment dissolved oxygen levels, benthic infaunal communities, and releases of hydrogen sulfide and ammonia into WLIS bottom waters.

The chemical basis of hypoxia is described in a simple manner in the paragraphs that follow.

1) Oxygen is renewed in marine surface waters in two general ways. Phytoplankton productivity in the upper water column (photic zone) introduces oxygen into water via photosynthesis. Surface wind mixing of the waters facilitates oxygen exchange with the atmosphere, renewing the oxygen content of the surface waters. Downward advection of surface waters then brings oxygen to the bottom waters. Normal average oxygen saturation for LIS waters is approximately 7 mg/l.

2) Oxygen is consumed in marine waters in several ways. The organisms that live in the marine environment consume oxygen during respiration; plant respiration in the photic zone also consumes a certain amount of oxygen although significantly less than what is produced by the phytoplankton. Finally, the sediments, depending on their organic content, consume oxygen via bacterial degradation of organic matter.

3) This last point is extremely important for sediments that have high organic carbon, such as those of Western Long Island Sound. Such sediments, in contact with oxygen, will consume oxygen via aerobic bacterial decomposition of organic matter. This effects of sediment oxygen demand can become quite large if bottom water oxygen renewal is limited by either water temperature or the existence of a stratified water column which prevents vertical mixing, ultimately setting the stage for the onset of hypoxic and perhaps even anoxic bottom waters (within 2 cm of the sediment-water-interface).

The research undertaken specifically investigated bottom water dissolved oxygen, as well as hydrogen sulfide and ammonia (two end-products of organic matter degradation that are known to negatively affect many organisms), as structuring influences on the benthic habitat quality and associated benthic communities of western Long Island Sound. It is hypothesized that long-term exposures to low oxygen, ammonia, and hydrogen sulfide may have contributed to a chronic low-level, physiologically-stressed state in the lobsters and other organisms that died in 1999, weakening their immune system, and setting them up for disease.

The results of this study are as follows:

1) The REMOTS® images reveal that the sediment surface at stations in the Eastern Narrows appeared to be oxygenated, with aRPD depths on the order of 2-4 cm. Extremely black (ie. sulfidic) sediments were more routinely observed at stations in the Western Narrows in all three REMOTS® surveys. REMOTS® photos in May revealed that all of the stations in the Western Narrows had either well-developed aRPD depths of 2-4 cm or, at least, a thin band (< 1-2 cm) of oxygenated sediment at the sediment-water interface overlying black, sulfidic sediment. The September REMOTS® photos revealed black, sulfidic sediments exposed at the sediment surface at five stations in WLIS, including one that had obvious methane bubbles present. The December REMOTS® photos revealed a thin band (1-4 cm) of oxygenated sediment overlying black, sulfidic sediments at all stations.

2) There were linear trends in the data, corresponding to depth and substrate type. Measured bottom water dissolved oxygen levels were consistently lower than those recorded by the DEP during their water column sampling of WLIS for the same time period as this study.

3) Bottom waters (within 5 cm of the bottom) at two-thirds of the sites sampled contained their highest dissolved oxygen levels in May and their second highest oxygen levels in December. Near-bottom waters (approximately 1 meter above the bottom) most likely follow the same trend, however data for May were not collected due to sampling constraints at the time.

4) Hydrogen sulfide was present in bottom waters at almost all stations sampled throughout the duration of the study, with peak levels occurring in May and October. Near-bottom water levels of hydrogen sulfide peaked in September and October and were barely present in August at all stations.

5) Ammonia was present in bottom waters at all stations during all sampling times; however, peak ammonia values were obtained from stations in the western Sound during the month of October. Near-bottom water ammonia levels also followed this exact trend.

The REMOTS® photos were similar to those obtained in WLIS in past years, with sulfidic sediments and thin aRPD depths characterizing stations in the Western Narrows, and a significant amount of small-scale spatial variability present in the observed aRPD. These images record the chemical changes that occur in the presence of hydrogen sulfide and iron and are a record of overall seasonal changes but do not capture the actual chemical variability that occurs on a weekly to monthly time scale.

Chemical data collected from this study reveals that the chemistry of the bottom waters in WLIS is very dynamic and varies dramatically over the course of a year. The variation in the ammonia and sulfide data correlates not with oxygen levels in the upper water column but with temperature and organic matter availability in the sediments themselves. Thus, a disconnect appears between water column dissolved oxygen levels and conditions at the sediment-water interface. It appears that seasonal variations in water column conditions simply lead to variations in the magnitude of the disconnect between the water column and the sediment but do not directly control processes within bottom waters. In turn, sediment processes and their end-products exert effects upon the dissolved oxygen levels at the bottom. The existence of the disconnect between the chemical environment that is experienced by benthic organisms and that which is occurring 1 meter or more above the bottom means that, for systems like Long Island Sound, it is the sedimentary environment that strongly structures the bottom waters in which benthic organisms live. The presence of the disconnect is best detected with chemical methods and does not show up as distinctly with REMOTS®, which appears to record a seasonal time averaging of benthic phenomena rather than the weekly shifts that are, apparently, present and significant to organisms living in the environment.

One of the issues central to management of the lobster fishery in western Long Island Sound is the condition of the bottom waters with respect to hypoxia. This study has documented that the sediments play a significant role in structuring bottom water chemistry in WLIS although it must be recognized that the onset of hypoxia in western Long Island Sound results from the interplay of many different factors, including air and water temperature, rainfall, currents, amount and type of organic matter, initial bottom water oxygen levels, anthropogenic inputs, and degree of stratification. The individual contribution made by any of these factors can vary from year to year. This makes it all the more necessary to know the "standing" background conditions of the sediments, sediment geochemistry, and the benthic communities in WLIS in order to be able to effectively predict how well the Sound can support a lobster fishery in any given year, as well as provide important information to be used to gain further insights into the lobster mortality event of 1999.

Literature Cited:

Kaputa, N.P. & C.B.Olsen, 2000. Long Island Sound Ambient Water Quality Monitoring Program: Summer Hypoxia Monitoring Survey 1991-1998 Data Review. State of Connecticut, DEP.

Welsh, B.L. & F.C.Fuller, 1991. Mechanisms controlling summertime oxygen depletion in western Long Island Sound. *Estuaries* 14:265-278.

Welsh, B.L., R.I.Welsh, & M.L.DiGiacomo-Cohen, 1994. Quantifying hypoxia and anoxia in Long Island Sound in: K.R.Dyer & R.J.Orth,eds., *Changes in Fluxes in Estuaries: Implications from Science and Management*, Olsen & Olsen, pp.131- 137.

Prevailing Water Column Conditions in Long Island Sound and the Relationship to Lobster Mortality Events

Robert E. Wilson[1], R. Lawrence Swanson[1], and Duane Waliser[2]

[1]Marine Science Research Center, Stony Brook University, Stony Brook, NY
[2] ITPA, Stony Brook University, Stony Brook, NY

Our activities have included an analysis of long term records for water column temperature, salinity and dissolved oxygen. We have used available data from NY DEP and CT DEP within Long Island Sound, and additional data from Block Island Sound. Our objective has been to describe spatial and temporal patterns in anomalies (from climatology) in these parameters and covariation between parameters.

Results provide evidence for climate variability, especially for bottom temperature, with large winter anomalies which persist for more than one year. They also provide evidence for weather induced anomalies, some associated with anomalous vertical mixing events.

We provide here an example of results from an analysis of the covariation in bottom temperature and bottom dissolved oxygen over the last decade for six CT DEP stations distribute over the length of Long Island Sound. Figures 1 through 4 show results of principal component analysis applied to these twelve series. Each series consists of monthly averaged anomalies for the period 1991 through 2002. Figure 1 shows the eigenvector for Mode l; it represents the spatial structure associated with this mode of variability.

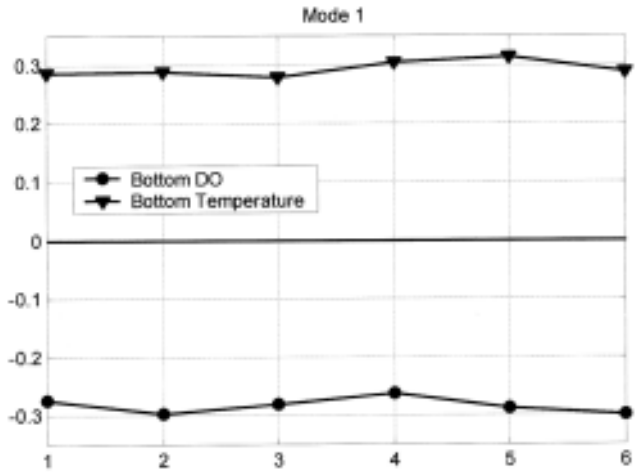


Figure 1. Eigenvector for Mode 1, representing the spatial structure associated with this mode of variability.

Mode 1 accounts for 52% of the total variance in these data. The horizontal axis in Figure 1 is series number so it can be considered distance down the axis of the Sound from west to east. Series 1 is from CT DEP station A4 in the western Sound. Series 6 is from CT DEP station M3 to the east of Mattituck sill in the far eastern Sound. Figure 1 shows that fluctuations in bottom temperature and near bottom dissolved oxygen have opposite signs with very little spatial structure over the length of the basin. It should be mentioned, however, that the series have each been normalized by their standard deviations and that there are significant spatial variations in these standard deviations.

Figure 2 shows the amplitude time series for Mode 1. It shows the strong positive winter anomalies which occurred in 1998, 1999 and 2002. It also shows a strong positive anomaly in the fall of 1999. A positive amplitude corresponds to a positive bottom temperature anomaly and a negative near bottom dissolved oxygen anomaly.

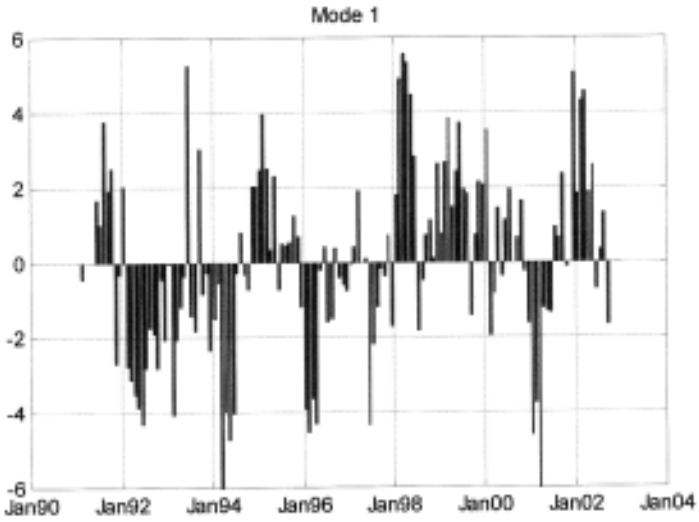


Figure 2. Amplitude time series for Mode 1. It shows the strong positive winter anomalies which occurred in 1998, 1999 and 2002. It also shows a strong positive anomaly in the fall of 1999. A positive amplitude corresponds to a positive bottom temperature anomaly and a negative near bottom dissolved oxygen anomaly.

Figure 3 shows the eigenvector for Mode 2 which accounts for 30% of the total variance in the 12 series. In contrast to Mode 1 it shows that the eigenvector components for bottom temperature and dissolved oxygen have the same sign throughout the basin. There is some longitudinal variation associated with the structure of this mode. The amplitude time series for Mode 2 (Figure 4) shows the strong positive anomalies which occurred in 1999 and 2002. For this mode a positive amplitude corresponds to positive bottom temperature bottom and dissolved oxygen anomalies. Both modes 1 and 2 are associated with positive bottom temperature anomalies in 1999 and 2002. Mode 2 exhibits very long term trends which is characteristic of the first mode results for bottom temperature alone.

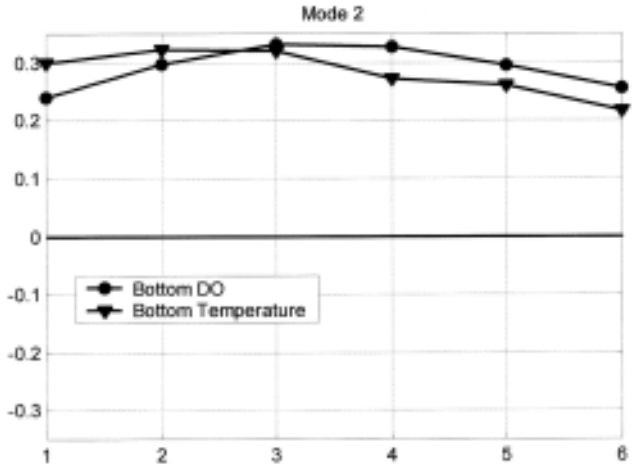
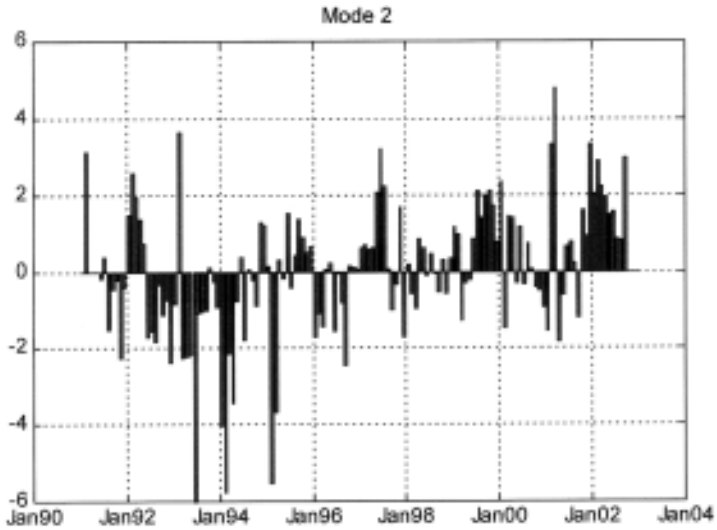


Figure 3. Eigenvector for Mode 2.

Figure 4. Amplitude time series for Mode 2.



Exposure of Lobsters to the Varied Chemical and Biological Environment of Long Island Sound

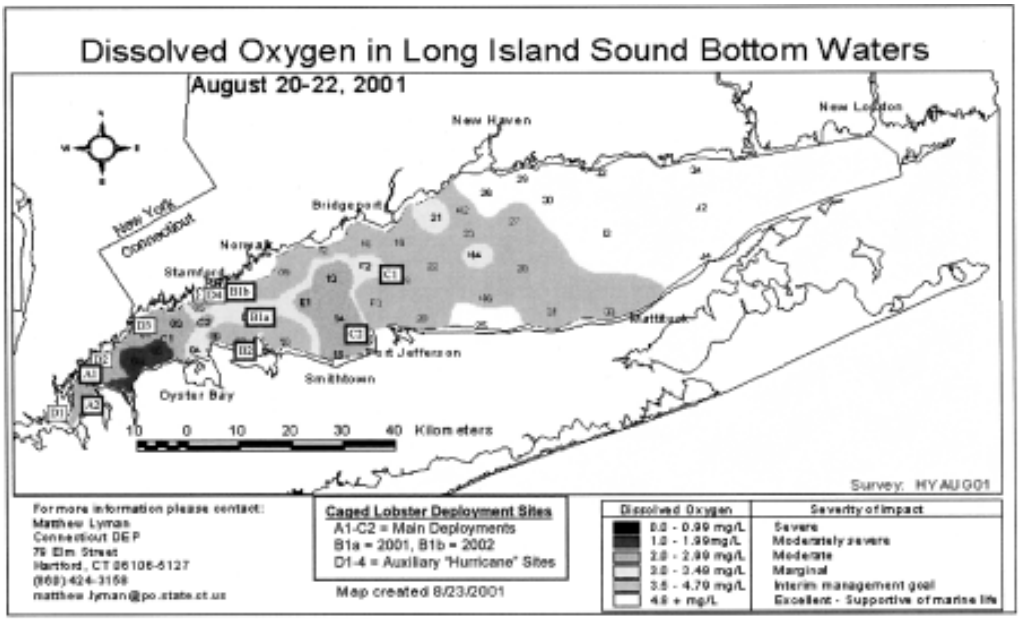
Andrew F.J. Draxler and Ashok Deshpande

NOAA, NMFS, NEFSC, James J. Howard Marine Sciences Laboratory, Highlands, NJ

This field experiment was designed to increase understanding of the relationship between lobster health and the ambient chemical and biological environment of Long Island Sound. During the first weeks of July 2001 and 2002, we deployed between 9 and 24 chemically naive lobsters (from Atlantis Canyon) in individual cages at each of six locations in western Long Island Sound (Figure 1). The sites were selected to expose the lobsters to the west to east cultural eutrophication gradient emanating from the New York metropolis, and to either sandy or muddy sediments. Each site was equipped with a temperature recorder and up to four sites had temperature-salinity-dissolved oxygen recording instruments. At two-week intervals, divers retrieved a subset of cages from each site and collected sediment and water column samples, exchanged hydrographic instruments and fed the remaining lobsters. Samples were aliquoted for the analysis of biogeochemicals in the water column and porewaters, and trace metals and chlorinated hydrocarbons in sediments. Retrieved lobsters were sampled to assess infection by bacteria and other pathogens, lipid distribution, accumulation of metals in gills, brain, and muscle tissue, and accumulation of chlorinated hydrocarbons in hepatopancreatic tissue. An auxiliary set of caged lobsters was deployed each year at four shallow water stations along the New York-Connecticut coast to examine the role that storm activity plays in exposing lobsters to biogeochemicals from resuspended sediments.

Raw mortality among lobsters caged in the main deployment reached 37% in 2001. However, this value may have been influenced by methodological problems which were subsequently addressed in the 2002 deployment when the maximum observed mortality was only 23%. Chemical analyses of habitat and tissue samples are currently in progress with about half of the variable classes completed.

Figure 1. Locations of deployed lobster cages in western Long Island Sound.



Environmental Change in Long Island Sound in the Recent Past: Eutrophication and Climate Change

Johan C. Varekamp[1], Ellen Thomas[1], Marilyn Buchholtz ten Brink[2], Mark A. Altabet[3] and Sherri Cooper[4]

[1]Earth & Environmental Sciences, Wesleyan University, Middletown CT
[2]U.S. Geological Survey, Coastal Geology, Woods Hole, MA
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[4]Biology Department, Bryn Althyn College, Bryn Althyn, PA

Goal of the research

The goal of our research is to document recent environmental changes in Long Island Sound (LIS) and their effects on the ecosystem as shown in microbiota which leave fossil evidence (photosynthesizing diatoms and dinoflagellates, and heterotrophic foraminifera and dinoflagellates), and put these into the historical perspective of the last 500-1000 years. We emphasize changes that occurred over the last 150 years, with the main anthropogenic imprint since the middle part of the 19th century, the changes that occurred over the last 30-40 years since anoxia/hypoxia in western LIS became common after the late 1960s-early 1970s, and changes in the late 1990s when the lobster die-off occurred. In order to document these environmental and biotic changes we have studied samples from sediment cores to record changes in dinoflagellate and diatom floras and benthic foraminiferal faunas, to obtain evidence for changes in water temperature and salinity (using $\delta^{18}O$ and Mg/Ca in carbonate foraminiferal shells), to document pollutant burdens, degree of bottom water oxygenation (using $\delta^{13}C$ in the calcite of foraminiferal shells), to provide evidence for changes in the magnitude of sewage input (using abundances of the bacterial spore *C. perfringens*), changes in diatom productivity (using analyses of sediment-stored biogenic silica), and changes in the storage and origin of C_{org}, and N_{org}, using C and N abundances and isotopic compositions ($\delta^{15}N$ and $\delta^{13}C$) and changes in sulfur abundances. We have also put effort into calibrating these indicators used in the core samples in the modern LIS environment through water sampling, surface sediment sampling and measurements of water column parameters (temperature, dissolved oxygen, salinity). We are working on providing age models for the sediment cores to obtain temporal records of the environmental and biotic changes.

Results

Nutrient fluxes have increased in the Sound with increased population density and changes in land use patterns. The Narrows and Western LIS have the largest inputs of effluents from wastewater treatment plants (WWTP) and there we find the most pronounced increased organic productivity as well as the resulting bottom water hypoxia/anoxia as a result of this eutrophication process.

Records of C_{org} concentrations in sediment cores show an increase from ~ 1850 on, in central LIS going from 1.2 to 2.5 % and in western LIS up to 4.5 %. A core in western LIS also shows an increase in N concentration from ~0.1 at the bottom to ~0.2 % at the top. Recalculation of C_{org} data as C_{org} accumulation rates shows that these rates have increased by a factor of 5 between 1850 and 2000. Accumulation rates of biogenic silica have increased as well by a factor of 4-5 over the last 150 years. This finding confirms and quantifies the long-held suspicion that the primary productivity and resulting flux of organic carbon to the LIS bottom waters has increased strongly as a result of eutrophication. We can, however, not conclude without further consideration that this enhanced carbon flux is the direct cause of the bottom water hypoxia/anoxia, because the carbon is covered more rapidly by sediment as the result of the higher sediment accretion rates of the last 150 years.

The records of paleo-salinity and -temperature (mean bottom water temperature over several years) show strong variations over the last millennium, with a positive correlation between temperature and salinity (warm and dry versus cool and wet). The highest temperatures occurred about 1,000 years ago (Medieval

Warm period) whereas the lowest water temperatures were reached about 200 years ago (end of Little Ice Age). The water temperatures have increased over the last 100 years. The paleo-salinity shows a narrow window between 26 and 31 ‰, with more extreme events during the 20th century. We assume that these short, low-salinity events are the result of wet periods, which, with changes in land use, resulted in more direct pulses of fresh water input into the Sound than before. The $\delta^{13}C$ values of carbonates in LIS sediment become substantially lighter over the last 200 years, the result of the oxidation of organic carbon in the bottom waters. The Nitrogen isotope signal becomes heavier by almost 2 per mille over that same period, evidence for the influx of anthropogenic nitrogen into the LIS system.

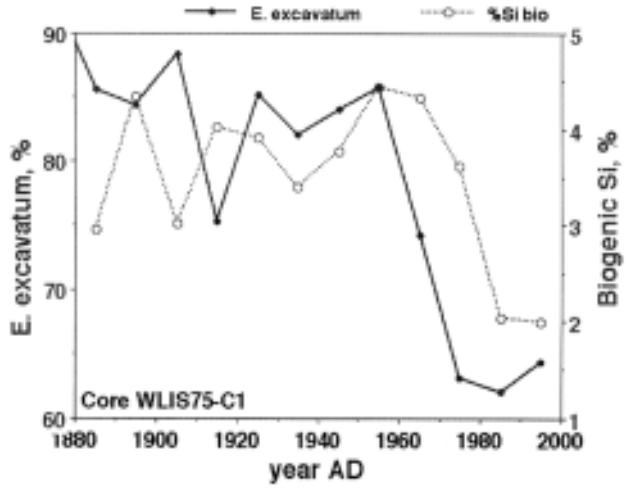
Diatom floras from a core in western LIS show a major decrease in diversity and species richness and an increase in the centric/pennate ratio (C:P; an indicator of eutrophication and increasing water turbidity) starting in the middle 19th century, when C_{org} data indicate increasing organic storage in the sediments. During the last few decades, the number of diatom valves declined as did diatom diversity, while the ratio of centric to pennate diatoms increased even more. Preliminary data on dinoflagellates show a strong east-west gradient in surface samples, with heterotrophic dinoflagellates (indicators of eutrophication) more abundant in western LIS. The heterotrophic forms increase in abundance from bottom to top in cores in western LIS.

Benthic foraminiferal faunas show the most severe changes with time in western LIS. In most cores, the total abundance of foraminifera (nr/gr bulk sediment) increased from the middle of the 19th century, but decreased again during the last ~30 years, and most prominently in the last few years. The mid 19th century increase in number of benthic foraminifera was caused by an increase in absolute and relative abundance of the diatom-consuming species, *Elphidium excavatum*. The decrease in total foraminiferal numbers in western LIS and the Narrows was caused by the decrease in numbers of *E. excavatum* during the last few decades. The decrease in relative abundance of this species was caused by an increase in abundance of the omnivorous *Ammonia beccarii*, a cosmopolitan omnivorous species which was very rare in LIS before the mid-1960s.

We speculate that the major changes in the benthic foraminiferal faunas were largely caused by the increased diatom productivity in the middle 19th century, followed by the decrease in diatom abundance in the last few decades. The increase in C:P in diatoms may be explained by increasing eutrophication and resulting increase in turbidity of the water column. The recent decrease in abundance of diatom valves and decrease in diversity could be explained by silica limitation during the spring phytoplankton bloom. The strong increase in relative abundance of the benthic foraminifer, *A. beccarii*, in western LIS at a time of decreasing abundance of the diatom-consuming species could have been caused by blooming of non-diatom phytoplankton. In the last few decades, the main primary producers in LIS may thus have changed from diatoms to organic-walled phytoplankton such as dinoflagellates, which has potentially a major impact on all LIS biota.

Conclusions

The paleo environmental records (examples shown below for core WLIS 75 –in the Narrows; cores A1C1 and A4C1 in West LIS) show clear evidence for eutrophication of the Sound since the mid-19th century, as evidenced by enhanced storage of organic carbon, biogenic silica, and nitrogen, heavier nitrogen isotopes, lighter carbon isotopes, and changes in benthic foraminiferal faunas and diatom floras. A decrease in biogenic silica and change in fauna in the last 20-30 years in the extreme western Sound may signal the onset of new changes in the LIS ecosystem. Salinity and water temperature have not moved dramatically outside their long-term range, but over the last century stronger variations in salinity seem to occur and waters have been warming. The warming with the occurrence of more extreme events in low-salinity together with the enhanced production of organic carbon is most likely the cause for the hypoxia/anoxia in western LIS. The documented changes in the LIS microfloral and faunal ecosystem may have propagated throughout the LIS ecosystem.


E. excavatum
%Si bio
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Core WLIS75-C1
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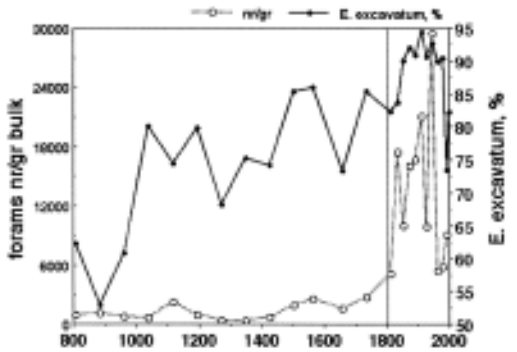
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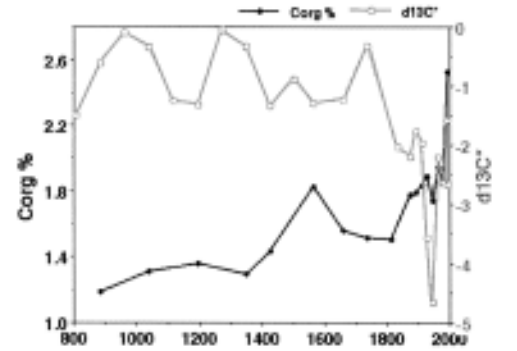
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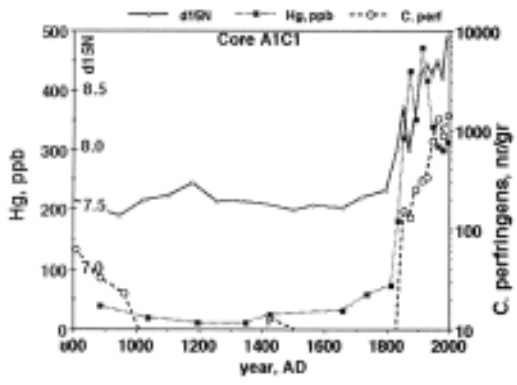
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Hg, ppb
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Core A1C1
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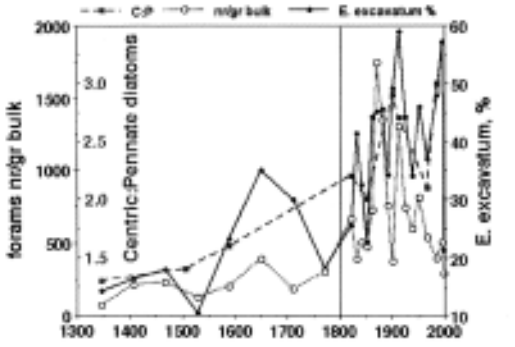
year, AD


C/P
nr/gr bulk
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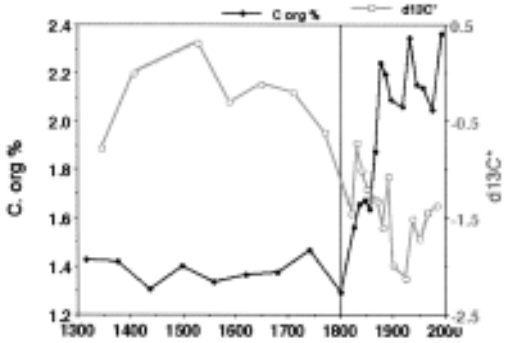
Centric:Pennate diatoms
E. excavatum, %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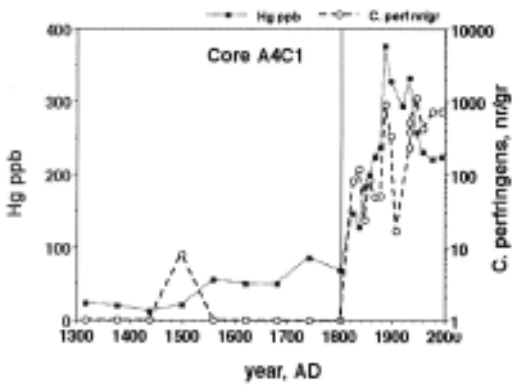
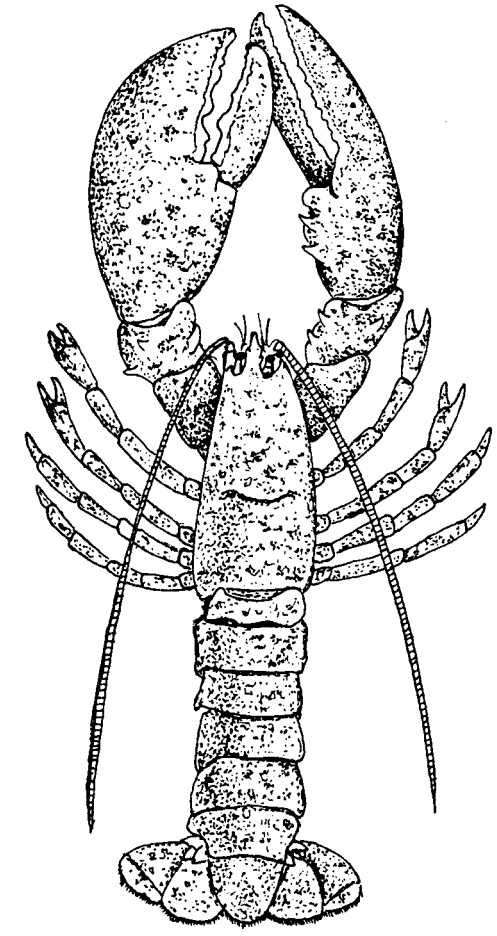
d13C'
C. org %
d13C'


Hg ppb
C. perf nr/gr
Core A4C1
Hg ppb
C. perfringens, nr/gr
year, AD

Physiological Responses to Stress



Immunological Health of Lobsters

Robert S. Anderson, University of Maryland Center for Environmental Science

In order to understand more fully the basis of resistance/susceptibility of lobsters to infectious diseases, an attempt was made to characterize its blood cell (hemocyte) mediated and humoral (plasma protein) mediated immune systems. As is the case for all invertebrates, lobsters lack antibody-dependent immunity but rely on phagocytic hemocytes and molecules secreted by these cells for host defense. The objective of this project is to characterize and provide methods to quantify the major immune mechanisms of the lobster; this information should be useful in studying responses to microbial pathogens and in predicting the immune status of lobsters collected in the field.

Hemocyte-mediated immunity

Circulating hemocytes represent the principal immune effector cells of the lobster. The total hemocyte count per ml hemolymph was found to be $1.7x10^7 \pm 7.0x10^6$, n=44. Phagocytosis, the ability of hemocytes to engulf microbes and other foreign particulates, was quantified using fluorescein-labeled particles. A fully-automated phagocytosis assay was developed in 96-well microtiter plates using a fluorescence concentration analyzer. Initial studies with labeled yeast cells showed extensive phagocytosis, with about 7.5 yeast cells taken up per hemocyte. Phagocytosis of the aquatic pathogen *Listonalla anguillarum* was also studied.

Using a colorimetric azo dye reduction method to measure intracellular killing of bacteria, we measured 10-20% killing of *L. anguillarum* at hemocyte:bacteria ratios of 1:10 – 1:50. These studies are continuing with *Aerococcus viridans* and *Hyphomicrobium indicum*. In order to better understand hemocyte-mediated antibacterial mechanisms, the ability of activated cells to produce antimicrobial reactive oxygen species (ROS) was determined using chemiluminescent (CL) probes. ROS are generated by activation of membrane-associated NADPH oxidase in response to membrane perturbations caused by phagocytosis and/or ligand-receptor interactions. Superoxide, the initial cytotoxic ROS produced after cell activation, was seen in lobster hemocytes by use of the CL probe lucigenin. Untreated cells produced peak superoxide response at ~60 minutes in culture; addition of the classical ROS stimulator phorbol myristate acetate (PMA) triggered a more rapid CL response that peaked at ~37 min. The physiological significance of this kinetic shift in superoxide response induced by PMA is not known (Figure 1).

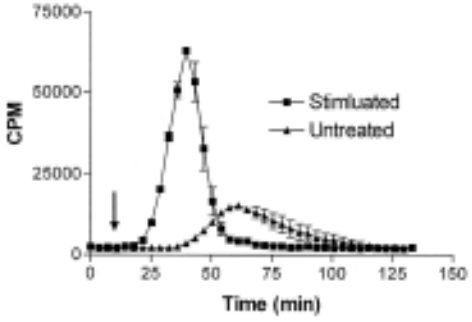


Figure 1. Superoxide production by lobster hemocytes.

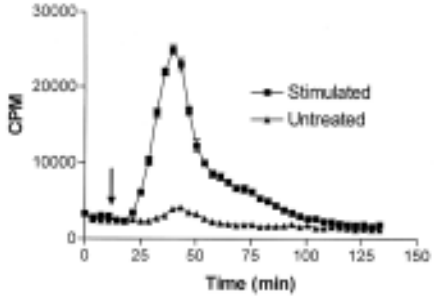


Figure 2. HOCl production by lobster hemocytes.

Total superoxide produced (obtained by integration of the area under the CL curve) was not significantly enhanced by PMA treatment. Superoxide is enzymatically converted to hydrogen peroxide, which is subsequently converted into hypochlorous acid (HOCl) by the hemocyte enzyme myeloperoxidase, in the presence of chloride ions. HOCl is an extremely cytotoxic antimicrobial agent used by blood cells; its presence in

lobster hemocytes was shown by use of the CL probe luminol. PMA treatment produced significant net HOCl induction (>10-fold greater than untreated cells), without shifting the peak release time of ~40 minutes (Figure 2).

This strongly suggests that luminol-dependent CL can be used to assess the ROS responsiveness (a parameter of immuno-competence) of lobster hemocytes. It appears that the stimulated ROS response of lobster cells is total, based on the lack of subsequent stimulation by additional PMA exposure. *In vitro*, phagocytic stimuli such as zymosan and latex beads showed little ability to activate the ROS pathway, compared to the protein kinase C (PKC) mimic PMA. PKC plays a role in the signal transduction pathway involved in assembly and activation of NADPH oxidase in mammals.

Plasma-mediated Immunity

In many invertebrate species, the hemocytes passively or actively secrete immuno-effector molecules into the cell-free plasma. For example, lysozyme is thought to play a role in molluscan defense by its ability to kill various bacteria. We found comparatively low lysozyme levels in lobster plasma (~0.1 µg/ml, vs ~20µg/ml in Eastern oyster plasma). Plasma agglutinins have sometimes been shown to act as opsonins, i.e. molecules that interact with foreign particles so as to make them more recognizable to phagocytic hemocytes. Although lobster plasma contained low and variable agglutinin titers against yeast and *L. anguillarum* cells, there was little evidence that they were recognition factors**.**

Lobster plasma was shown to effect the growth/viability of bacteria. In brief, bacteria were incubated in the presence of various concentrations of whole or fractionated plasma, allowed a short grow-out period, and their number determined by the MTS/PMS assay similar to that used to measure hemocyte-mediated killing. Whole plasma produced dose-dependent inhibition of *L. anguillarum* (Figure 3).

In an attempt to isolate and characterize the active antibacterial components of the plasma, anti-*L. anguillarum* activity was determined in fractions after ultrafiltration. The <10kDa fraction showed little activity; therefore peptides may play only minor roles in lobster immunity. However, both the >50kDa and the >100kDa fractions showed significant activity (Figure 4). We plan to use an AKTA prime chromatography system (Pharmacia) to purify and characterize the antibacterial molecule(s) present in lobster plasma.

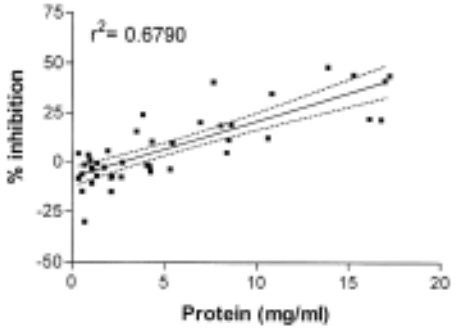


Figure 3. Percent inhibition of Listonella anguillarum by unseparated lobster plasma.

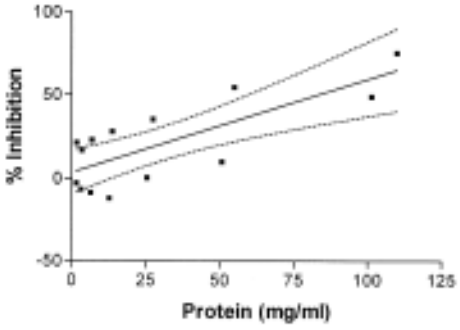


Figure 4. Percent inhibition of Listonella anguillarum by the >100kDa fraction of lobster plasma.

Plasma protein concentrations and immune status

For many years plasma protein levels have been associated with the general health of lobsters, without much speculation as to mechanism(s). Our data suggests that two indicators of immune status are positively correlated to plasma protein concentration. Plasma protein level was routinely determined in every hemolymph sample withdrawn for our studies; it was found to be 27.24 ±9.00 mg protein/ml (n=44). The number of

hemocytes in circulation at any given time is a measure of the number of immune effector cells available to the host organism; the hemocyte counts were correlated with plasma protein concentration (Figure 5).

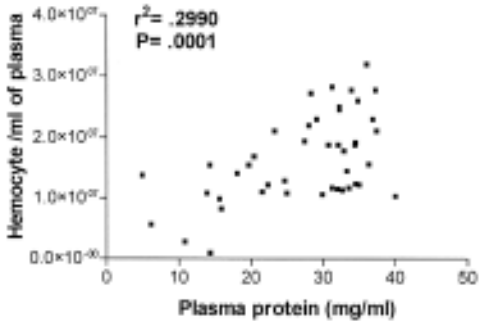


Figure 5. Plasma protein concentration positively correlated with hemocyte counts.

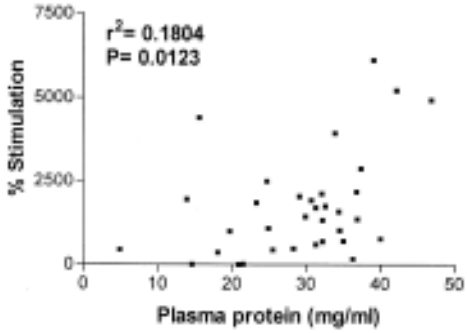


Figure 6. HOCl production increases with plasma protein concentration.

ROS responsiveness (PMA-induced HOCl release per hemocyte) can be taken as a measure of the total potential antimicrobial ability of hemocytes; per cell HOCl production was also correlated with plasma protein concentration (Figure 6). It will be interesting to follow these correlations as more data are gathered as the study progresses.

Environmental and Physiological Stresses in Lobsters: Effects on CrustaceanHyperglycemic Hormone and Heat-Shock Proteins

Ernest S. Chang, Bodega Marine Laboratory, University of California, Bodega Bay, CA

Introduction

The purpose of this project is to develop molecular and immunological assays for the characterization of biological responses in lobsters (*Homarus americanus*) to various environmental stresses (such as heat, osmotic stress, and hypoxia). In particular, we have focused on changes in the concentration of the stress hormone, crustacean hyperglycemic hormone (CHH), and the amount of heat-shock proteins (HSPs; also known as stress proteins) and their mRNAs.

Materials and Methods

CHH was quantified by an enzyme-linked immunosorbent assay (ELISA). The details of this assay have been published (Chang *et al*., 1998).

HSPs were initially quantified by Western blotting. Samples were homogenized and separated by denaturing polyacrylamide gel electrophoresis (Criterion, Bio-Rad). After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were first incubated with antisera specific to HSPs and then with a second antibody conjugated to horseradish peroxidase. The proteins were visualized following incubation with chemiluminescent reagents (Pierce), the images were electronically captured, and the bands quantified using imaging software (NIH). HSP mRNA was quantified with Northern blots. These procedures have been published previously (Spees *et al*., 2002a,b).

For the osmotic stress experiments, jars were filled with either 50, 100, or 150% seawater. We ran a parallel experiment to examine the effect of salinity on hemolymph osmolarity (Spees *et al*., 2002b).

Biological samples were obtained from natural populations in collaboration with Dr. Richard French (University of Connecticut). Lobsters were captured from five different locations within Long Island Sound, NY and were necropsied. Hemolymph and other tissues were obtained.

In the laboratory, lobster embryos and larvae were obtained from gravid females caught near Vineyard Haven, MA (Chang and Conklin, 1993) and subjected to thermal shocks of 13°C above ambient for 0.5 and 2 h. The embryos and larvae were then processed for CHH and HSP quantification. Juvenile lobsters were raised in the laboratory as previously described (Conklin and Chang, 1993).

Induced thermal tolerance was demonstrated by first determining the survival of animals subjected to various elevated temperatures for 2 h. This established the lethal temperature. Different lobsters were subjected to an induction temperature of 13°C above ambient for various times, placed back into ambient seawater for various times, and then subjected to the lethal temperature for 2 h. These results were compared to data obtained from lobsters that were not previously subjected to a non-lethal thermal shock.

Results and Discussion

Effects of Stress on CHH Levels

We have previously observed that elevated temperature significantly increases the amount of CHH in the hemolymph of juvenile lobsters (Chang *et al.*, 1998). We conducted analogous experiments on embryos and larvae at different developmental stages. These samples are currently being processed.

We assayed the hemolymph samples that were obtained from wild-caught adults. These adults were obtained from five different zones of Long Island Sound, NY. Zone 1 is at the western end of the Sound and

includes the waters west of a line between Eaton's Neck, NY and Norwalk, CT; it contains The Narrows. Zone 2 is the Western Basin (Norwalk to Stratford Shoal). Zone 3 is the western end of the Central Basin (Stratford Shoal to Herod Point Shoal). Zone 4 is the eastern end of the Central Basin and extends from Zone 3 to the Connecticut River. Zone 5 is at the east end of the Sound; it is east of Zone 4 and includes The Race. The hemolymph concentrations of CHH from Zone 4 lobsters were significantly higher than those from Zone 1 lobsters (Table 1). There were no other significant differences between the zones. We have no hypotheses to explain this difference at this time. We are currently examining the data for other correlations (such as diseases and trauma).

Table 1. Mean hemolymph CHH concentrations in lobsters collected from different locations in Long Island Sound, NY. Values with an asterisk (*) are significantly different from each other (P<0.05, ANOVA).

Zone	1	2	3	4	5
CHH (fmol/ml)	8.79*	10.91	19.82	29.23*	13.10
Standard Deviation	11.88	14.47	33.35	47.53	17.42
Sample Size	58	79	73	59	67

Effects of Stress on HSP Levels

We initially quantified thermal stresses by measuring changes in the amounts of the heat-shock proteins. However, we observed some variability in the responses and concluded that this variability was most likely due to the different batches of primary antisera that we used. These antisera were obtained from commercial sources (Stressgen) and were made against species other than lobsters (e.g. human, chicken, or mouse). We investigated the use of an enzyme-linked immunoassay (ELISA) for lobster HSP70 or HSP90. We tried several variations of the assay, including direct and sandwich assays. Typical results for a standard curve are shown in Figure 1. Unfortunately, the sensitivity was insufficient for lobster samples (HSP concentrations are in the pictogram range).

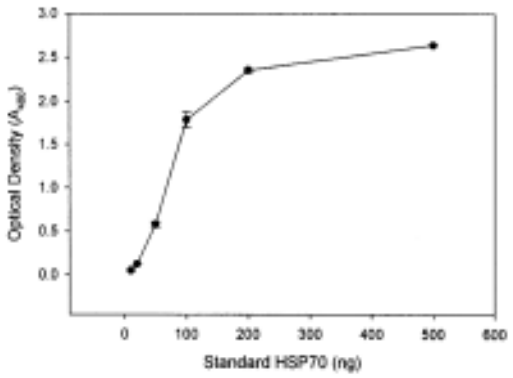


Figure 1. Standard curve for a direct ELISA. Wells were coated with human HSP70. The primary antibody was a mouse monoclonal made against chicken HSP70 (Stressgen). The secondary antibody was goat anti-mouse IgG conjugated to peroxidase. The reaction was developed with the addition of hydrogen peroxide and a color reagent (ABTS). The plate was read at 480 nm.

Subsequent analyses therefore were directed at measurement of HSP mRNAs. In some ways it is more precise to measure the mRNAs since they reflect recent stresses since their half-life is on the order of a few hours, as opposed to the HSP proteins that have half-lives on the order of a few days. Figure 2 shows typical Northern blot data following a 13°C heat shock of juvenile lobsters. Maximal response was observed after 2 h.

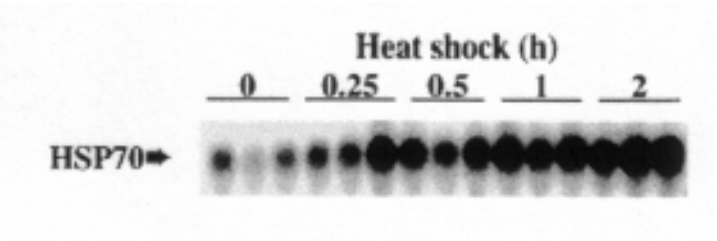


Figure 2. Northern blot of HSP70 mRNA isolated from abdominal muscle that was obtained from lobsters that were thermally shocked 13°C. Three individual lobsters were sampled at each time interval.

The induction of HSP mRNA was expected following thermal shock. The effects of osmotic shock are more novel. We observed that hemolymph osmolarity differed significantly between control animals and those incubated in either hypo- or hyper-osmotic conditions at all time points examined after the start of the experiments (Figure 3).

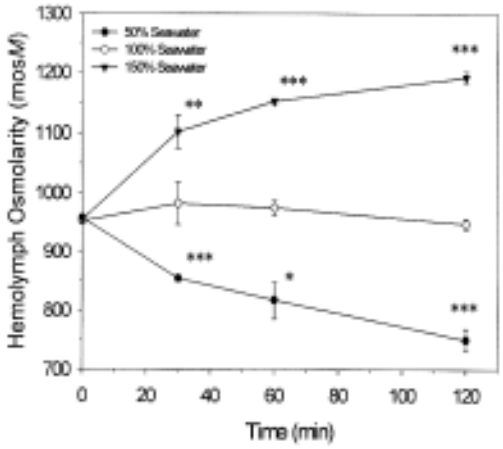


Figure 3. Hemolymph osmolarity (mosM) of individual lobsters repeatedly sampled at 0, 30, 60, and 120 min of exposure to control (100%), hypo-osmotic (50%), or hyper-osmotic (150%) seawater. N=4 for each time point. Error bars represent one S.D. of the mean. Asterisks indicate significant difference between treatment and respective control at a given exposure time (P≤0.05, ** P≤0.01, *** P≤0.001).*

Abdominal muscle HSP70 mRNA levels were significantly induced by both hypo- and hyper-osmotic stress (Figure 4). HSP70 mRNA levels were significantly higher than control levels by 30 min of incubation in 50% seawater and continued to be elevated at 60 min of incubation. Exposure to 150% seawater resulted in a significant induction of abdominal muscle HSP70 mRNA levels over control levels at 60 min. HSP70 mRNA levels returned to control levels in both salinity exposure groups by 120 min.

Effects of Molt Cycle on HSP Levels

We observed that different tissues have different degrees of HSP expression during the course of the molt cycle. HSP90 gene expression was significantly induced in premolt versus intermolt claw muscle (P=0.002; Figure 5). However, there was no significant difference between intermolt and premolt HSP90 mRNA levels in abdominal muscle. HSP70 gene expression did not differ during the molt cycle in either claw or abdominal muscle.

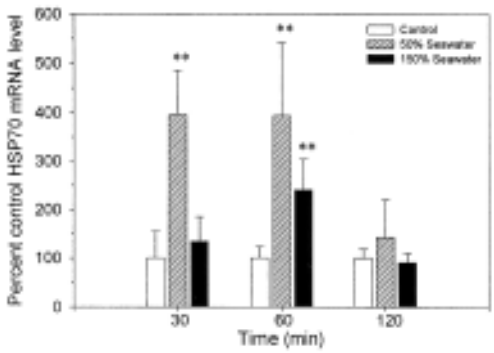


*Figure 4. Quantitative analysis of lobster HSP70 gene expression in abdominal muscle during hypo- and hyper-osmotic stress (50% and 150% seawater) for 30, 60, and 120 min. Data are normalized against the actin signal (indicator of equal loading) and expressed as percent control (100% seawater) mRNA level. N=4 for all time points. Error bars represent one S.D. of the mean. Significance between treatment and control HSP70 mRNA levels is indicated (** P≤0.01).*

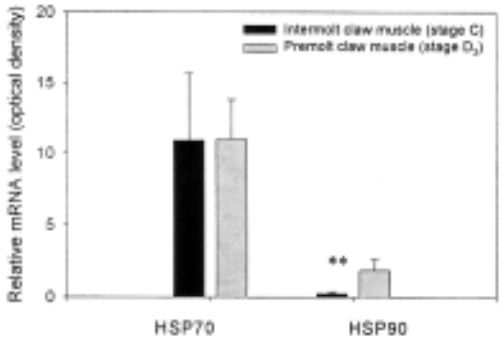


*Figure 5. Relative mRNA levels for molecular chaperones and polyubiquitin in intermolt versus premolt claw muscle. Data from (A) were normalized against the actin signal. N=6 for each bar. Absolute expression levels for one transcript should not be compared to any other because of potential differences in probe strength and film exposure. Significant difference between the intermolt and premolt stages is indicated; ** P≤0.01.*

Induction of Thermal Tolerance

Induction of thermal tolerance is characterized by initially exposing an organism to a sub-lethal thermal shock and subsequently subjecting it to a usually lethal thermal shock. Often the organism survives this shock, whereas control organisms that do not experience the prior sub-lethal shock succumb. This phenomenon has not previously been demonstrated in crustaceans. In our laboratory, larval lobsters display the thermal survival curve shown in Figure 6.

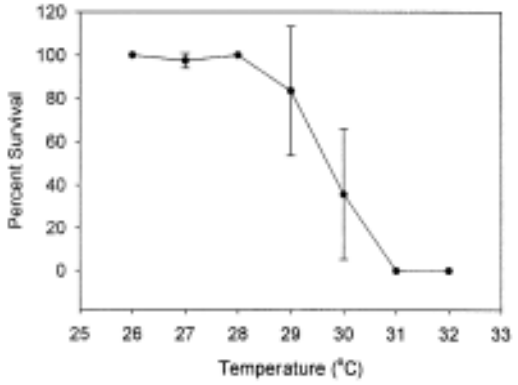


Figure 6. Percentage survival of stage I lobster larvae. Groups of 25 larvae were placed into a tea strainer and transferred to a jar placed in a water bath that had been heated to the indicated temperatures. Survival after 2 h was then determined.

In a typical experiment, if larvae were first exposed to an induction temperature of 13 °C above ambient (i.e., moved from 11.5 °C ambient water into a 24.5 °C water bath) for 2 h, returned to ambient (11.5 °C) for 2 h, and then subjected to 30 °C for 2 h, survival was 95.5% compared to 1.67% (P≤0.001) for the controls (larvae that were not previously exposed to the induction temperature). Significantly enhanced survival could be observed if the thermal induction period was as short as 15 min.

Parallel experiments are being conducted with juvenile lobsters (approx. 125 g wet weight). A survival curve for juveniles was obtained that is similar to that of larvae (Figure 6). Preliminary experiments indicate that juvenile lobsters do not display induced induced thermotolerance to the lethal temperature to the dramatic extent that was observed in larvae.

Acknowledgments

The following colleagues at the Bodega Marine Laboratory contributed to the work described in this abstract: Sharon A. Chang, W. Arthur Hertz, Mark J. Snyder, and Jeffrey L. Spees. This work was supported by the California (grant R/A-108, project NA66RG0477), and Connecticut (LR/LR-1) Sea Grant College Programs.

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Effects of Environmental Stressors on Disease Susceptibility in Lobsters: A Controlled Laboratory Study

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The environmental cause of the unprecedented lobster mortalities in western Long Island Sound (LIS) in 1998 and 1999 is not established. The research needed to understand the problem was discussed in the First LIS Lobster Health Symposium and developed into work plans (LIS Lobster work plans, 2000). This project addresses two of the seven objectives recommended in the Pathology/Toxicology section of the Work Plans. The objectives of this work are (1) determine whether increased (but environmentally realistic) conditions of temperature, hypoxia, sulfide, and ammonia, alone or in combination, can heighten susceptibility to bacterial infections and (2) after effective susceptibility levels have been confirmed, determine whether several indices of immunological health will be suppressed. Since the parasitic amoeba found in LIS lobsters cannot, at this point, be cultured, we have chosen two known bacterial pathogens of lobsters as surrogates to help reveal the effects of stressors on susceptibility to microbes. The Gram positive coccoid bacterium, *Aerococcus viridans*, causes a terminal infection, "gaffkemia", that is non-toxic and non-invasive, but competes with the lobster for its own energy reserves (Johnson *et al.*, 1981). The second organism, *Vibrio fluvialis*, has a different mechanism of action; it releases at least one toxin (Tall *et al.*, 2000) and causes a limp-lobster syndrome (Tall *et al.*, 1999) similar to that described in the reports of the western LIS mortalities. We report here on how selected combinations of stressors influence susceptibility to one of the bacteria, *A. viridans*.

Market-size lobsters procured from commercial harvesters in central and eastern LIS (and in one case, Rhode Island Sound) were acclimated to conditions of temperature and bottom light of western LIS in September, and to the salinity at the Howard Laboratory for 7 days. Each lobster was inoculated in the ventral sinus with 1 X 10^3 or 1 X 10^6 *Aerococcus* or with a sham (saline) solution. Experimental conditions were generated in a flow-through system of sealed tanks with concentrations of chemicals developed using existing technologies at the Howard Laboratory of counter-current gas exchange and metering-pump introduction of aqueous solutions. The tanks were provided with individual lobster shelters. Figure 1. shows one branch of the exposure system.

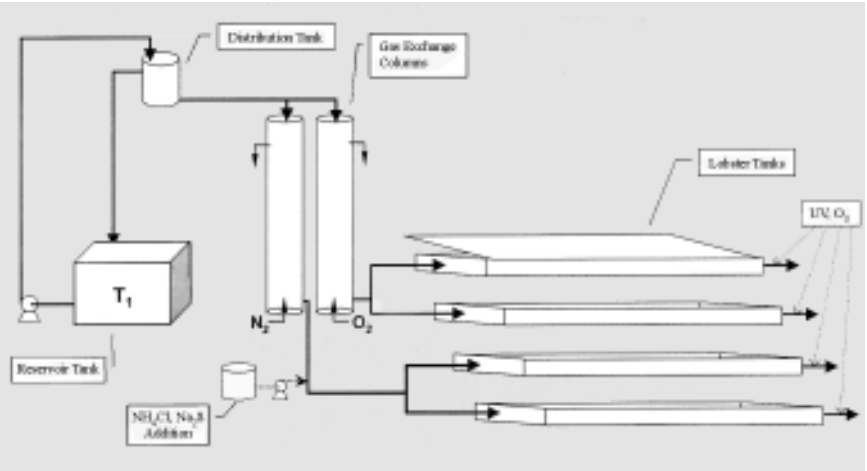

Figure 1. System for testing the effects of biogeochemicals.

Lobsters were monitored twice daily for viability, behavioral response, and death. At appropriate intervals, lobsters were removed, placed in coolers, and transported to the Milford Laboratory for enumeration of bacteria in the hepatopancreas and hemolymph. Bacterial counts were determined by plating aliquots of diluted, macerated hepatopancreas or dilutions of hemolymph on trypticase soy agar plates supplemented with 2% NaCl. The distinctive *Aerococcus* colonies on the plates showed typical tetrad grouping of cells when observed by phase-contrast microscopy. This was confirmed by re-injection of random colonies into lobsters.

The design of the first experiement is shown in the structure of Table 1. At either 14.5 °C or 19.5 °C, adequate oxygen, and no exposure to sulfide or ammonia, 50% survival of lobsters was about 9 days to greater than 10 days with minor variations depending on the temperature or bacterial load. When the oxygen level was dropped to 80 μM and sulfide plus ammonia were added, mortality was dramatically accelerated; again infection with the higher bacterial dose only slightly accelerated lobster deaths. Although the higher temperature and higher bacterial dose produced the most severe effect in the presence of hypoxia, sulfide, and ammonia; the design of this initial experiment did not distinguish between the effect of hypoxia or sulfide plus ammonia.

Table 1. Initial experiment to establish effects of stressors on lobsters.

Exposure Conditions				Approx. Days Till 50% Mortality
Temp. (C°)	Oxygen (μM)	Bacterial dose	Sulfide, NH_4^+ (μM)	LIS lobsters*
14.5	200	1 X 10^6	0, 0	>10
19.5	200	1 X 10^3	0, 0	>10
14.5	200	1 X 10^3	0, 0	8.9
19.5	200	1 X 10^6	0, 0	8.8
14.5	80	1 X 10^3	20, 70	4.3
14.5	80	1 X 10^6	20, 70	3.6
19.5	80	1 X 10^3	20, 70	3.4
19.5	80	1 X 10^6	20, 70	2.6

* LIS: Lobsters taken from central Long Island Sound

The bacterial counts in lobster hepatopancreas are shown in Figure 2. The counts on three of the four sets of lobsters exposed to low oxygen plus sulfide and ammonia could not be carried out to 10 days because all animals in those tanks died prematurely. Counts for the remaining lobsters reached about 1 x 10^9 gm^{-1} of tissue within 6 to 10 days. Bacterial growth patterns were similar in lobster hemolymph (data not shown).

A follow-up experiment, in which one temperature (19.5 °C) and three levels of sulfide and ammonia were selected (see Table 2), provided additional information on the stressor effects. In this experiment it was necessary to obtain lobsters from Rhode Island Sound as well as LIS because of insufficient numbers available from LIS. The two populations were separated in the experiment. Again with adequate oxygen and no sulfide or ammonia, 50% survival was 11-13 days in injected lobsters (longer in non-infected lobsters). When 6 and 24 μM sulfide and ammonia, respectively, were added, significant acceleration of death occurred in the presence of adequate oxygen. However, when oxygen was dropped to 80 μM, even greater acceleration of death occurred in the same amount of sulfide and ammonia. Paradoxically, when sulfide and ammonia were absent at 80 μM oxygen, equal deaths occurred. This was confirmed by adding a smaller amount of sulfide and ammonia (3 and 12 μM, respectively); there was no change in death acceleration.

In other words, it appeared that, at adequate oxygen levels, sulfide and ammonia strongly accelerated deaths in lobsters. However, low oxygen levels alone were sufficient to accelerate lobster deaths regardless of the presence of sulfide and ammonia. A suggestion of lobster strain differences was seen; compared with LIS lobsters, RIS lobsters had better survival at adequate oxygen levels, but accelerated deaths at low oxygen levels. Additional experiments are needed to separate the effects of sulfide and ammonia. Additional studies also will examine whether the same effects are seen in lobsters infected with *V. fluvialis*.

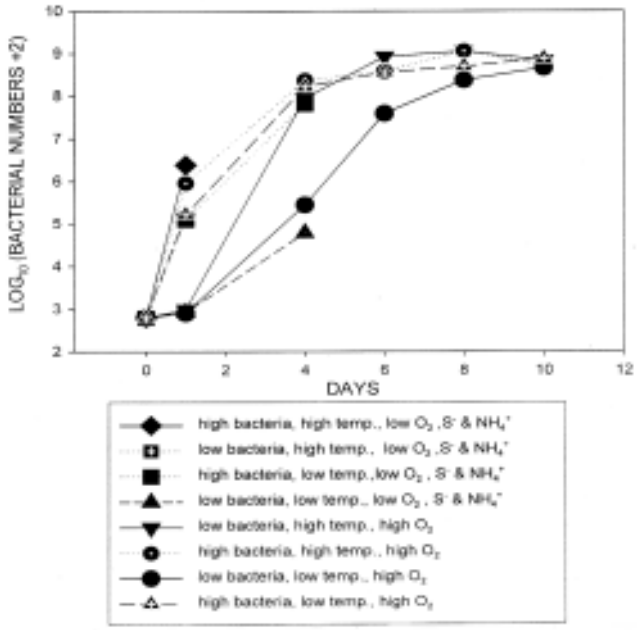

Figure 2. Aerococcus growth in hepatopancreas.

Table 2. Follow-up experiment to test effects of additional stressor levels.

Exposure Conditions				Approx. Days Till 50% Mortality	
Temp. (C°)	Oxygen (µM)	Bacterial dose	Sulfide, NH_4^+ (µM)	LIS[a] lobsters	RIS[b] lobsters
19.5	200	0	0, 0	>13	>13
19.5	200	1 X 10^6	0, 0	11.1	12.7
19.5	200	1 X 10^6	6, 24	4.6	8.6
19.5	80	1 X 10^6	6, 24	3.7	2.5
19.5	80	1 X 10^6	0, 0	2.9	2.5
19.5	80	1 X 10^6	3, 12	2.9	N. D.[c]

[a] LIS: Lobsters taken from eastern Long Island Sound

[b] RIS: Lobsters taken from Rhode Island Sound

[c] ND: Not done with Rhode Island Sound lobsters

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Development of Assays for the Evaluation of Immune Function of the American Lobster as a Tool for Health Assessment

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Introduction

A lobster die-off reduced the 1999 fall landings in western Long Island Sound by up to more than 99%. The die-off corresponded in time with the application of pesticides for the control of mosquitoes that carried West Nile virus, a new emerging disease in North America at the time. The lobsters examined suffered from a *Paramoeba* sp. infection that mainly affected the nervous system. Nevertheless, very few tools existed to assess the health of lobsters. We proposed the development of assays to quantify different parameters of the immune system of lobsters as tools to better assess the health of lobsters.

Material and methods

The endpoints tested include evaluation of different the immune functions using hemocytes. Briefly, hemolymph was collected and immediately transferred to Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing acid citrate dextrose (ACD) or 3.8% citrate. Cells were then counted using a hemocytometer and Trypan blue to determine viability.

Phagocytosis was evaluated as previously described (De Guise *et al.,* 1995) with some variations. Hemocytes were incubated in their hemolymph at room temperature (20-25 °C) and compared to samples incubated on wet ice (0 °C), which reduces metabolic activity and phagocytosis. One µm diameter fluorescent latex beads (Molecular probes, Eugene, OR) were diluted 1:10 in PBS and 5 µl of the bead mixture was added for every 200 µl of helolymph. After a 1 hour incubation in the dark, 200 µl of each cell suspension was analyzed by flow cytometry. The fluorescence of approximately 10,000 hemocytes was evaluated with a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer. Phagocytosis was evaluated as the proportion of hemocytes that had phagocytized 1 or more beads and the mean fluorescence of hemocytes. The results were reported as the phagocytic index, which represents the ratio of phagocytosis at room temperature to that on ice. A ratio higher than 1 represents active phagocytosis, and the higher the ratio is, the more effective phagocytosis.

Proliferation of immune cells is an important feature of the response to pathogens. The ability of lobster hemocytes to proliferate upon stimulation will be evaluated through the incorporation of bromodeoxyuridine (BrdU), a thymidine analogue, into the nucleus of proliferating cells, using an ELISA plate reader.

Respiratory burst consists of the ability of immune cells to produce oxygen free radicals in order to destroy foreign particles/organisms. We attempted to measure resporatory burst in lobster hemocytes using dichlorofluorescein diacetate (DCFDA), a “pro-fluorescent” dye that becomes fluorescent upon production of hydrogen peroxide, a common free radical in most species. The changes in fluorescence were measured at the single cell level using flow cytometry.

Natural killer cell (NK) activity is an important feature of the innate immune system. Natural killer cell-like activity has been observed in several species and consists of a non-major histocompatibility complex (MHC) restricted ability to kill foreign or infected cells. NK-like activity of lobster hemocytes will be measured against K-562 cells using two-color flow cytometry.

Results

Phagocytosis of fluorescent latex beads was evaluated using flow cytometry. Typical results, expressed as a fluorescence histogram, are shown in Figure 1.

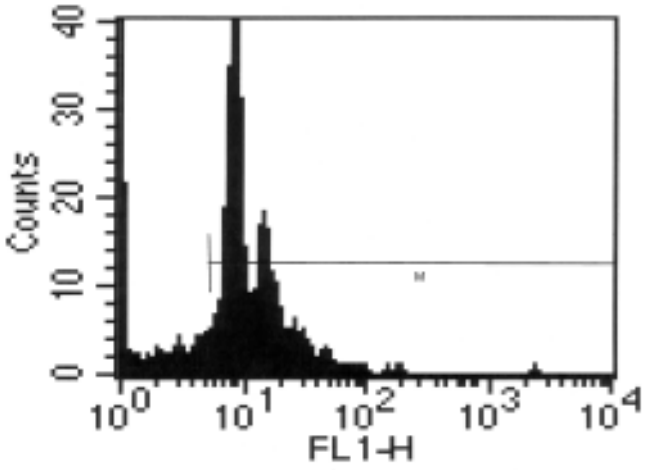

Figure 1: Histogram of fluorescence of lobster hemocytes phagocytizing fluorescent latex beads.

We also developed cell culture methods that allowed lobster cells to proliferate upon stimulation with LPS (Figure 2). The dose response curve represents preliminary data in the course of optimization studies.

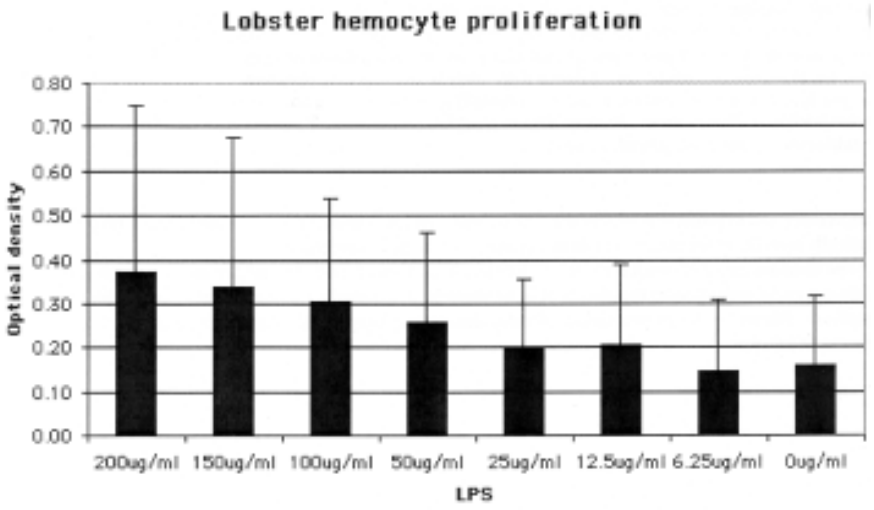

Figure 2. Proliferation of lobster hemocytes (mean of 4 animals) upon stimulation with LPS.

Attempts at measuring a respiratory burst in lobster hemocytes have so far been unsuccessful. The evaluation of cell surface molecules in lobster hemocytes appears interesting. It appears that CD14 is expressed constitutively on lobster hemocytes, while the expression of Toll-Like Receptor (TLR)-4 is inducible upon exposure to LPS. The results for TLR-2 are not yet conclusive. We are now in the process of evaluating the expression of those molecules in different sub-populations of hemocytes as defined by flow cytometry. Ongoing experiments are evaluating the Natural Killer cell (NK)-like activity in lobsters, after preliminary experiments to assess feasibility.

Discussion

We are in the process of optimizing several assays to measure immune functions in lobsters. Those assays will be useful in the assessment of subtle changes in the health status of lobsters.

Development of an Assay for Phagocytic Activity in the Immune System of Lobsters

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Objective: The objective of this study is to develop a method for assessing the ability of the immune system of the lobster, *Homarus americanus*, to remove foreign particles from the blood (phagocytic activity).

Rationale: By developing a method for determining phagocytic activity, this project will allow assessment of the state of cellular defenses of the lobster's immune system, and therefore a measure of their ability to protect against disease. This method may prove useful in comparing lobsters from different areas, or lobsters exposed to different environmental conditions in Long Island Sound.

Preliminary Results: Development of an assay for phagocytic activity has involved the development and adaptation of a variety of methods, techniques, and procedures, which can be described in the following areas.

1. Microspheres and injection experiments. Procedures for the use of fluorescent microspheres in uptake experiments have been developed. The type, size, manufacturer, and fluorescent dye appropriate for these experiments has been tested and determined. Procedures have been developed for carrying out experiments involving injections of microspheres, including volume, concentration, injection procedure, time needed for uptake, and harvesting and fixing tissues for microscopy.

2. Cell separation. Techniques have been developed for the isolation and separation of terminal hepatic arterioles from the surrounding tissues of the digestive gland. Procedures for handling isolated arterioles and preparing arterioles and fixed phagocytes for microscopy have been developed.

3. Histology. Tissues from injection experiments have been successfully prepared for microtomy, using methods that preserve the microspheres and their fluorescent dye. Digestive gland tissues embedded in plastic have been sectioned (0.5-1.5 µm thick sections) and observed and photographed to document the uptake of microspheres by fixed phagocytes (Figure 3).

4. Scanning electron microscopy. Tissues from injection experiments have been successfully prepared for scanning electron microscopy, using methods that preserve the microspheres (Figure 1). Fixed phagocytes have been observed and photographed to understand the mechanism and sequence of events during uptake of microspheres (Figure 2).

5. Fluorescence light microscopy. Microspheres taken up by fixed phagocytes have been visualized in isolated arterioles using epifluorescence light microscopy, and photographed using film and digital cameras (Figure 4).

6. Confocal microscopy. Because of the thickness of the cluster of fixed phagocytes attached to the arteriole, laser confocal microscopy offers the significant advantage of creating multiple, layered images that allow better resolution of microspheres. This technique also allows collecting images based on multiple fluorescent spectra, so that microspheres can be imaged separately from the fixed phagocytes, or can be combined into a single image (Figure 5).

7. Quantification. Using stacked images from a confocal microscope opens the opportunity to quantify microspheres in a sample using a counting program. This approach has been demonstrated to be feasible (Figure 6).

Work In Progress: Work in progress and planned for the immediate future involves completion of the quantification methods.

8. Quantification, continued. We are currently working on counter-staining cell nuclei for confocal microscopy, which should allow the counting software to count fixed phagocytes as well as microspheres. This will allow quantification of uptake by determining the average number of microspheres taken up per cell.

9. Fluorometric quantification method. Work is beginning on detection and quantification of microspheres from ground tissues of the digestive gland using fluorometry.

10. Transmission electron microscopy. Techniques are under development for preparing isolated arterioles for transmission electron microscopy, using methods that preserve the microspheres. These include methods for infiltrating arterioles and embedding in an embedding plastic, without the use of harsh solvents that would dissolve the microspheres, preparing ultrathin sections, staining, and observing in the transmission electron microscope. The goal of the work with transmission electron microscopy, as well as fluorescence light microscopy of histological sections and scanning electron microscopy, is to better understand the mechanism of uptake of foreign particles by the fixed phagocytes.

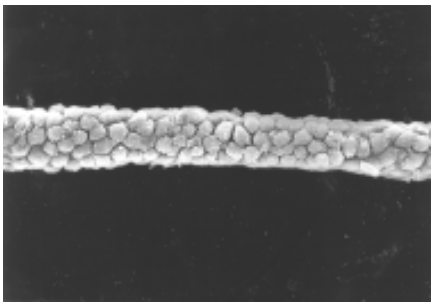

Figure 1. An isolated terminal hepatic arteriole, showing fixed phagocytes covering its outer surface; uninjected control; scanning electron micrograph; 410x in microscope.

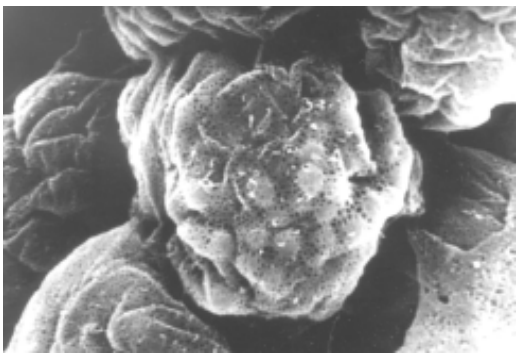

Figure 2. A fixed phagocyte from an isolated terminal hepatic arteriole, showing the outline of 1.0 µm fluorescent microspheres under the perforated membrane after removal from the blood; scanning electron micrograph; 6100x in microscope.

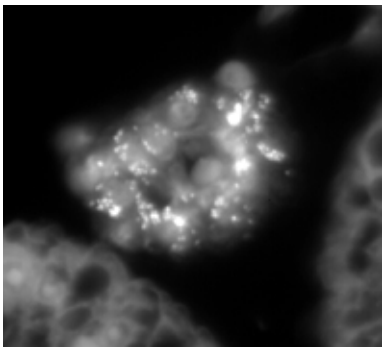

Figure 3. A histological section through a terminal hepatic arteriole that was harvested from a lobster 65 minutes after it was injected with 1.0 µm fluorescent microspheres (similar to the one in Figure 1); fluorescence microscopy shows uptake and distribution of fluorescent microspheres by the fixed phagocytes; fluorescence light micrograph; high mag. (180x in microscope).

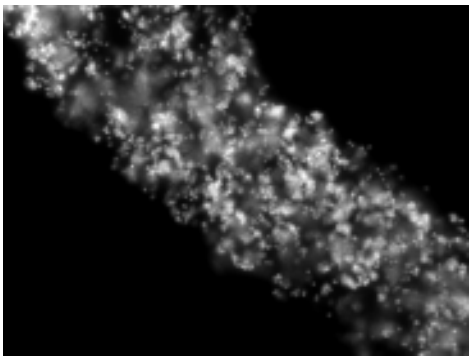

Figure 4. This light micrograph illustrates a terminal hepatic arteriole that was harvested from the digestive gland (=hepatopancreas) of a lobster 65 minutes after it was injected with 1.0 µm fluorescent microspheres. The same view of the terminal hepatic arteriole in Figure 2; fluorescence microscopy shows the uptake and distribution of the injected 1.0 µm fluorescent microspheres; fluorescence light micrograph; high mag. (180x in microscope).

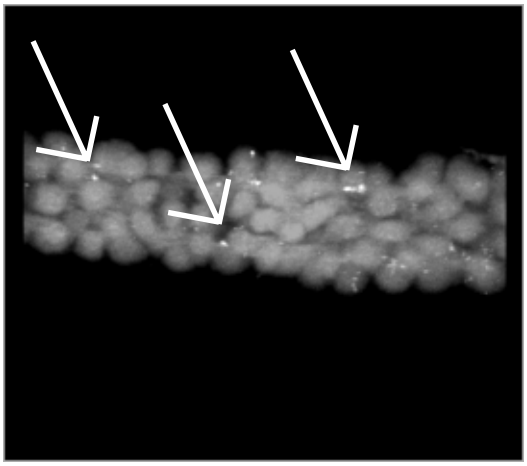

Figure 5. This light micrograph is made with a confocal fluorescence microscope and illustrates a single terminal hepatic arteriole that was harvested from the digestive gland (=hepatopancreas) of a lobster 30 minutes after it was injected with 1.0 µm fluorescent microspheres.

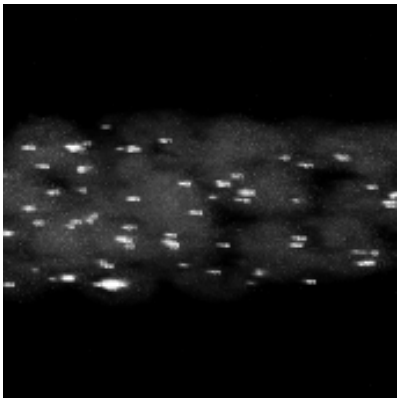

Figure 6. This micrograph demonstrates a method for quantifying fluorescent microspheres taken up by fixed phagocytes of a terminal hepatic arteriole that was harvested from the digestive gland (=hepatopancreas) of a lobster 1440 minutes after it was injected with 1.0 µm fluorescent microspheres. Terminal hepatic arteriole; after analysis by a counting program, which numbers each microsphere as it is counted.

Hormonal Responses of Lobsters to Stresses, an Interim Report

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Introduction

We are undertaking research in three areas related to the health of lobsters in Long Island Sound, including (1) determining ecdysone levels in healthy lobsters, heat stressed lobsters, and lobsters with shell disease, (2) identification of functions of lobster crustacean hyperglycemic hormones (CHHs), and (3) determining effects of stresses on larval lobsters.

1. Ecdysone levels in lobsters

We are defining normal seasonal baseline levels of ecdysones, a family of steroid hormones, which regulate molting, and observing how elevated temperature and methoprene affect ecdysone levels. Ovigerous lobsters from Long Island Sound during the late summer of 1999 have been found to be dying in the process of molting while carrying eggs; this suggests a hormonal imbalance in female lobsters. Normally, ecdysone levels in lobsters carrying eggs are low. High levels of ecdysone would induce a molt that would cause the animals to prematurely lose their eggs. This observation suggests that the ecdysone levels for unknown reasons may become elevated in stressed animals.

We are investigating whether hormonal imbalances in MF, ecdysone, and CHH neuropeptides exist in shell diseased lobsters. Shell disease is abundant in lobsters from eastern LIS (Castro and Angell, 2000). An assemblage of microorganisms destroys the cuticle, causing lesions on the shell. Five different levels of shell disease have been reported which vary in the degree of destruction of the inner and outer epidermis and cuticle (Smolowitz *et al.*, 1992).

As part of our studies in lobsters, we have been measuring baseline hemolymph ecdysone levels by radioimmunoassay as described by Chang (1984) using a polyclonal antibody against ecdysones. We have measured ecdysones in lobsters taken from Long Island Sound over the past year, including both normal lobsters, environmentally stressed lobsters, and lobsters with varying degrees of shell disease. So far, we have measured ecdysone in lobsters collected from 2/26 to 10/24, total of 154 lobsters, and are in the process of analyzing more.

In results published by Chang, Figure 1A shows the ecdysone concentrations in the blood of a female that molted, extruded eggs, hatched eggs between days 260 and 270 after the first molt and then molted again shortly after day 400. Ecdysone peaked only shortly before the molt. In Figure 1B the adult female did not lay eggs but molted a second time (M) after an ecdysone peak at about 275 days after the first molt. During the rest of the molt cycle ecdysone levels were exceedingly low.

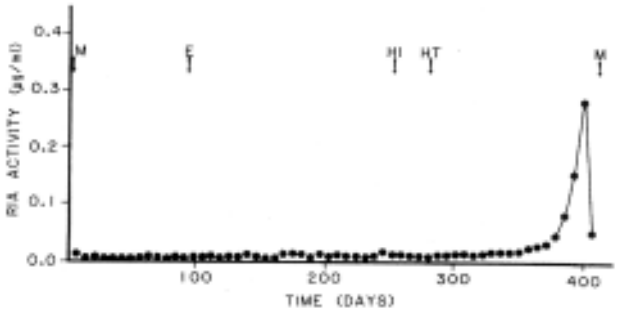

Figure 1A. Ecdysteroid titers of a female adult lobster that underwent extrusion and embryo incubation."RIA activity"represents hemolymph ecdysteroid concentrations. "M" represents the molts, "E" the day of extrusion, and "HI" and "HT" represent the initiation and termination of larval hatching, respectively. From: E. Chang (1984), "Ecdysteroids in Crustacea: Role in Reproduction, Molting, and Larval Development" In: Advances in Invertebrate Reproduction.

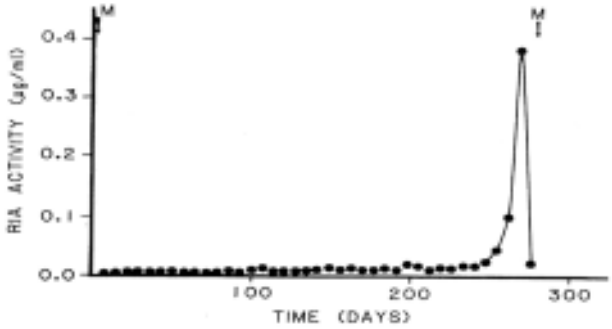

Figure 1B. Ecdysteroid titers of a female adult lobster that did not undergo egg extrusion. Legends, symbols, and source are the same as in Figure 1A.

Results

Ecdysone levels in healthy lobsters, heat stressed lobsters, and lobsters with shell disease

For all lobsters analyzed which did not have shell disesase (both males and females), the average hemolymph concentrations of ecdysones in January was 14 ng/ml (n=7), and increased gradually in February to 42 ng/ml (n=15) and April to 56 ng/ml (n=10), and then increased dramatically in May to 144 ng/ml (n=10) and peaked in June, where the average ecdysone concentration was 173 ng/ml (n= 14) (Figure 2). The average ecdysone concentrations then dropped to 4 ng/ml (n=3) in July and then rose again to 53ng/ml (n=16) in August. The animals sent in September died during shipping and could not be analyzed. Ecdysone levels found in October averaged 49ng/ml indicating that the levels went back to February and April levels may be heading down again for the winter.

Our preliminary data indicate that in five lobsters with shell disease that are ovigerous ("eggers") the average ecdysone concentration is much higher than in the one normal ovigerous lobster that we have examined (Figure 3).

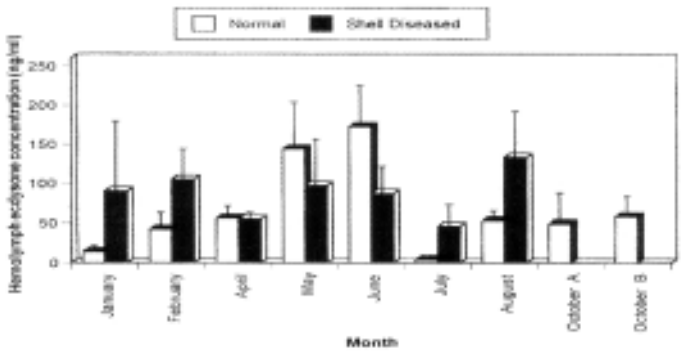

Figure 2. Hemolymph ecdysone concentrations in normal lobsters versus shell diseased lobsters collected during the year. Error bars show standard error of the mean. October A: Lobsters collected from Long Island Sound. October B: Lobsters collected from the Massachusetts Lobster Hatchery.

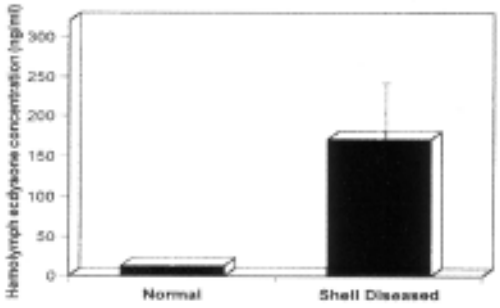

Figure 3. Comparison of hemolymph ecdysone concentrations between a normal lobster carrying eggs (n=1) and shell diseased lobsters carrying eggs (n=5). Ecdysone concentrations were found to be much higher in the shell diseased lobsters, which had recently released eggs (a few eggs still remained on pleopods), compared with a normal lobster that recently released eggs.

2. Identification of CHH functions

We are evaluating the function and ability of different crustacean hyperglycemic hormones (CHHs) to inhibit synthesis of methyl farnesoate in isolated mandibular organs. CHHs are a family of peptides that regulate glucose metabolism, but in most cases have multiple functions. In the lobster there are at least four CHH neuropeptides, which are produced by the sinus gland/x-organ complex located in the eyestalk. One such neuropeptide is molt-inhibiting hormone (MIH), which inhibits synthesis of ecdysone. There are also CHHa and CHHb, which are known for their function as CHHs. CHHa resembles MIH in structure and presumably functions as an MIH. Vitellogenin inhibiting hormone (VIH) inhibits ovarian maturation and also should affect vitellogenin synthesis by the hepatopancreas (HP). VIH also has CHH activity. In further defining the function of these CHH peptides, we are evaluating the effects of several of these neuropeptides on MF synthesis, as well as other functions, in order to identify important hormonal activities among the known CHHs. We have accomplished this by using isolated mandibular organs in *in vitro* bioassays. Also we have devised new bioassays using HP cells and androgenic gland cells to determine the range of function of CHHS. Thus, a CHH that affects vitellogenin synthesis by HP cells in culture can be considered to have VIH activity. Any CHH that regulates AG function would be a gonad inhibiting hormone or a GIH. Finally, we are initiating studies of specific lobster hyperglycemic hormones (CHHs) for their responses to different stressors such as increased temperatures, methoprene and shell disease. As part of these studies we have begun to examine the effects of particular CHHs on the androgenic gland (AG) and hepatopancreas (HP).

Results

MO cells in culture: Three proportions of extract from sinus glands: 0.2, 0.4, and 0.6 SG, inhibited the MO by 48%, 33%, and 26% respectively (Figure 4).

Hepatopancreas (HP) in culture: Lobster hepatopancreas tissue fragments were placed in culture and ^{35}S labeled amino acids were incorporated into proteins between one to nine hours. After 6 hours protein synthesis remained level (Figure 5).

SG extracts affect HP cultures: Sinus gland effects the ability of the hepatopancreas to produce proteins by 34%.

CHHB affects HP cultures: Figure 6 shows that CHHB peptides 11(78µg/ml) and KM (98µg/ml) inhibit protein synthesis by hepatopancreas cells compare to controls. CHHB peptide, KM (50µl), and 0.4 SG showed equal amounts of inhibition.

CHHB affects androgenic gland fragments: Protein synthesis of androgenic gland fragments treated with CHHB peptide (KM) shows reduced protein synthesis of 66% . Androgenic gland with peptide inhibition with 0.4SG showed 56 % of controls (Figure 7).

CHHB affects dissociated androgenic gland cell preparations: Dissociated androgenic gland cells inhibit more protein synthesis; 22 % synthesis was obtained when compared to controls. The inhibition of androgenic gland cells was greater than androgenic gland fragments.

CHH antibodies: Antibodies to CHHA did not remove a significant amount of inhibition from the SG extract. The antibodies to VIH appeared to remove some inhibition. In addition, experimental data collected (n=4) from the months of 6/02-8/02 showed less than 40% inhibition of the MO by the SG during the summer season suggesting a lower CHH production.

HPLC: All the peptides collected by HPLC have MO inhibiting activity. The third and fourth peaks exhibited the most inhibition, more than the total SG extract. Peptides 1 and 2 appear to be weaker MOIHs.

CHHB peptide effect on the MO: CHHB proteins inhibited MF production to some degree. Clone KM was the most potent, slightly less than 50% inhibition per 50µl (5µg) but more potent than the equivalent of SG extract, which inhibited about 30% of MF production.

Seasonal differences in CHH production: Sinus gland extract was more potent during winter months. Sinus gland peptides extracted over the summer only showed 28% inhibition, while those extracted in the late fall showed 66% inhibition of the MO (Figure 8).

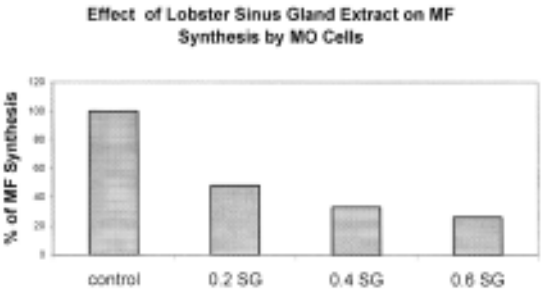

Figure 4. Inhibition of the MO by different quantities of sinus gland extract. Inhibition increases proportionally to increasing amounts of SG peptides.

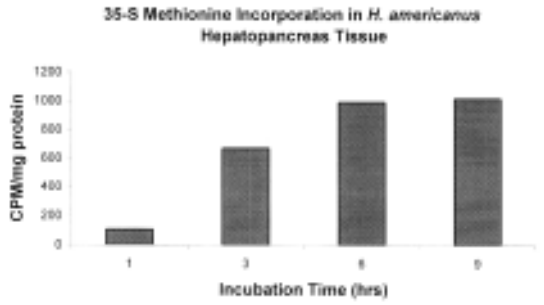

Figure 5. Amino acid incorporation into vitellogenin proteins over time. Proteins are produced linearly in vitro for up to 6 hours.

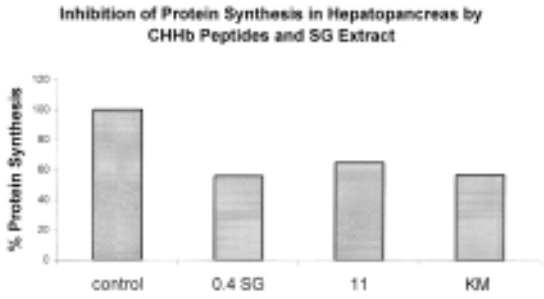

Figure 6. The effect of a CHHB cloned peptide on hepatopancreas protein synthesis.

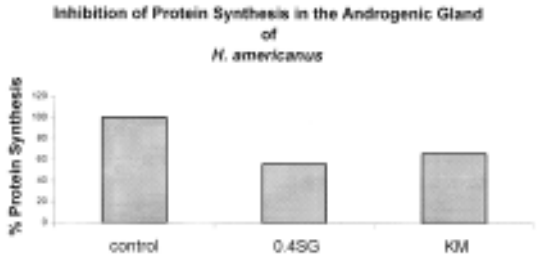

Figure 7. The effect of a CHHB cloned peptide on the AG. The KM CHHB peptide clone exhibits inhibition of the AG similar to sinus gland extracts.

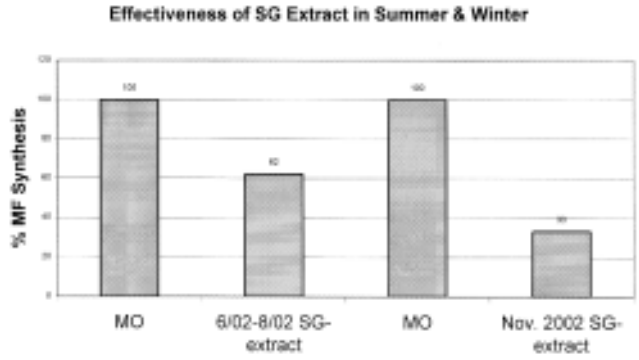

Figure 8. A comparison of MOIH activity found in the sinus gland of lobsters over the summer and winter months. SG Extracts from lobsters in the winter show 1/3 more MO inhibition.

3. Stresses on Larvae

We have carried out preliminary experiments looking at the effects of temperature, MF, and methoprene on survival of larvae.

Results

All 2nd stage larvae kept at 22 ºC died in 3 days (Figure 9). Treatment with MF or MP did not appear to have an effect on the death rate of these larvae. Larvae kept in control dishes at 15 ºC generally took 1-2 days before the first molt. Larvae with treatment of 0.1ppm MF or MP generally began the first molt slightly later, 2-3 days. By day 7, all larvae reached the second stage. For 2nd and 3rd stage transitions there is no significant difference in molting patterns. All larvae exhibited a prolonged 3rd stage and died approximately 18-21 days after their introduction into the dishes (Figure 9). Metamorphosis into juvenile larvae never occurred in experimental or control dishes kept at 15 ºC or 22 ºC.

High concentration trials at 15 ºC: 100ppm, 10ppm, and 1ppm, involving 2nd stage larvae generally allowed a 6-day life span. Over the course of a week, no larvae reached the 3rd stage. The control larvae demonstrated a capability of living well beyond the 6 days of the experiment (Figure10). Larvae treated with 100ppm of MF, MP, all show a rapid killing rate. 10ppm and 1ppm concentrations of the same chemicals show a less rapid killing rate. The killing rate of the control larvae remains somewhat uniform in all trials. At 3 ½ days about half of the treated larvae die.

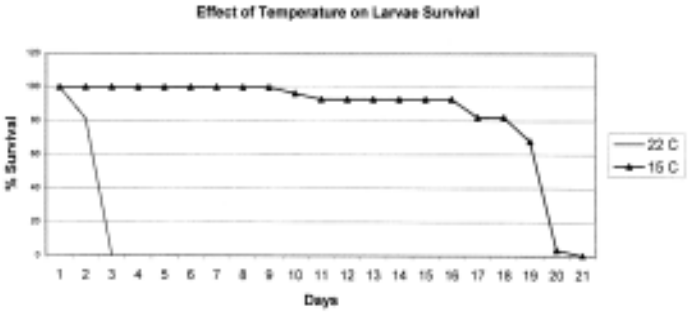

Figure 9. Larvae survival at different temperatures. Larvae kept at 15 ºC in the laboratory live 7 times as long as larvae kept at 22 ºC.

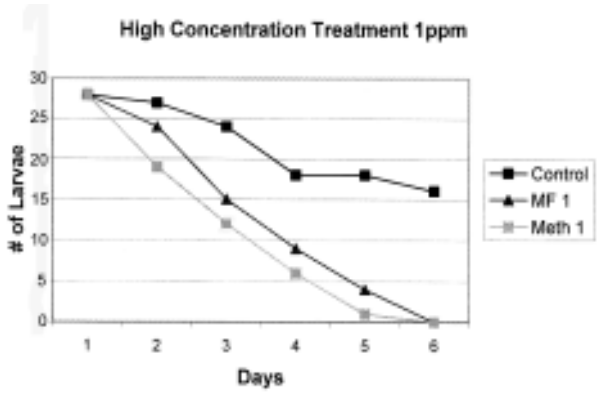

Figure 10. Effect of MF and Methoprene on larvae. A concentration of 1ppm of each compound kills 2nd stage larvae within 6 days at 15 ºC in the laboratory.

Conclusions

1. The concentration of the molting hormone ecdysone rises and falls significantly in populations of lobsters throughout the year and varies in important ways in shell diseased animals. It rises from January through June and then falls significantly in July after a molt. The June peak anticipates the molt, which occurs in early July in the population we observed. Ecdysone levels rise again from August to October. In contrast, in shell diseased animals the level of ecdysone remains relatively high when compared to normal lobsters. This may indicate that they undergo more frequent molts. The most unexpected result was the high level of ecdysone in ovigerous females with shell disease. Under normal conditions these levels are very low (10-20ng).

2. The ability of different crustacean hyperglycemic (CHH) hormones to exhibit mandibular organ inhibiting (MOIH) activity was carried out through the use of CHH specific antibodies, synthetic CHHB peptides, and HPLC separation of neuropeptides and testing of individual fractions. All MO experiments used the technique of MO cell dissociation in multiple assays. Immunoabosorption with antibodies to crustacean hyperglycemic hormone A (CHHA) did not remove a significant amount of MOIH activity. While VIH antibodies reversed some of the MOIH inhibition, data collected over the summer months, using eyestalk extract from lobsters in the molting season, showed less MO inhibition by the sinus gland (SG) peptides. Synthetic CHHB peptides tested *in vitro* during this period of time demonstrated more MO inhibition than the SG neuropeptides. Four peptides separated and collected with HPLC showed inhibition of the MO. CHHs inhibited androgenic gland protein synthesis and hepatopancreas protein synthesis.

3. The insecticide, methoprene (MP), which may be present in the waters of Long Island Sound (LIS) in harmful concentrations, is very similar in structure to Methyl Farnesoate (MF), a crustacean hormone that plays a role in the metamorphosis and reproduction of crustaceans. Its presence in the late 3rd larval stage should interfere with metamorphosis into the lobster juvenile stage, as does methoprene with insect larval metamorphosis. We attempt to determine an LD_{50} for methoprene by exposing 1st, 2nd, and 3rd stage larvae of the lobster *H. americanus* species to different concentrations of the insecticide. Concentrations were applied at .001, .01, 0.1, 1, 10, and 100ppm. MF and MP at lower concentrations, .001, .01, and 0.1ppm, did not appear to delay stage transitions, through the third larval stage. We surmise that due to some deficiencies in the laboratory dishes all experimental larvae died by 22 days and before metamorphosis into a juvenile stage. Larvae maintained in running seawater metamorphosed in about 10-12 days. Higher concentrations at 1, 10, and 100ppm were applied to 2nd instar larvae in filtered seawater with a 1.96% alcohol content. Control larvae survived 6 days while those treated with MF and MP died earlier. Experiments found temperatures of 22 ºC or higher to be a major factor contributing to the death of larvae maintained under laboratory conditions.

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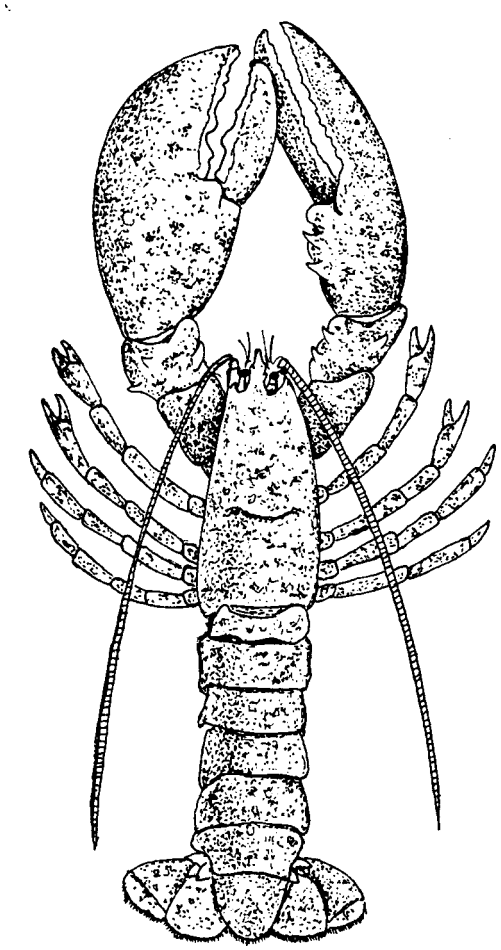
Pesticides



Effects of Pesticides on Lobster Health: Trace Level Measurements and Toxicological Assessment at Environmentally Realistic Concentrations

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Goal: Our goal is to better understand the fate and effects of pesticide use in the environment, specifically focusing on acute and chronic effects of pesticides on lobsters.

Approach:
1) Develop trace methods to analyze pesticides and their metabolites in water, sediment and possibly tissue samples;
2) measure levels occurring in the environment after applications and after significant rain events;
3) conduct toxicity studies on larval and juvenile lobsters to access acute toxicity and immune suppression after exposure to pesticides at stressful and non-stressful temperatures;
4) compare toxic levels to environmental concentrations to access risk.

Progress to date: An liquid chromatography-mass spectroscopy (LC-MS) system with electrospray ionization has been acquired and set-up, and methods developed that can measure 0.1-0.5 parts per trillion of pesticides in water samples of one liter. Using these methods, resmethrin, sumethrin, piperonyl butoxide, methoprene, and malathion can be analyzed together with comparable sensitivities. Analysis of the pesticides as sodium adducts in positive ionization mode is one of the keys to achieving high sensitivity. These methods are over 1,000 times more sensitive than those used previously to access pesticide levels in Long Island Sound, and at least ten times more sensitive than the best gas chromatography-MS based method developed to date. Sensitive methods are important both to trace inputs of highly reactive pesticides in receiving waters and to assess the risk of select pesticides that that are highly toxic. For example, there are many reports of lethal toxicity of pyrethroids to crustaceans at levels in the very low parts per trillion range.

Analytical methods have been applied to assist in the development of laboratory dosing systems and to monitor pesticide levels in surface waters following spraying events. Much of the field work was conducted collaboratively with the United States Geological Service (USGS) and the Suffolk County Department of Health. Our work to date has focused on making water measurements in shallow ponds, marshes, tidal inlets and coastal waters in the hours after spraying. In one case, following spraying on Staten Island, we followed concentrations in two water bodies before and after heavy rains that occurred the day after spraying. Most of the spraying events studied involved the pesticide formulation Scourge which consists of resmethrin and the synergist piperonyl butoxide (PPO). PPO was detected in 15 out of 19 samples at concentrations ranging from 0.7 to 15,000 parts per trillion; resmethrin was detected in only 5 of these samples at concentrations ranging from 1.7 to 980 parts per trillion. Based upon the low ratios of resmethrin to PPO in field samples relative to that of the formulations sprayed, it appears that resmethrin is being removed (probably by transformation) faster than PPO.

Toxicity assessment has been delayed by the significant time needed to develop an effective dosing system, facility and staffing issues at our marine laboratory, and limited availability of lobster larvae. We have worked on developing three different systems that have sufficient flow to maintain larval lobsters in suspension without use of aeration, that can deliver trace levels of pesticides reliably. Our third generation system has finally come on-line. It has the capability of delivering 6 different pesticides concentrations to five replicate 2 L chambers at a seawater flow rate of 0.5 L/min per chamber. With this system, measured levels of resmethrin are approximately 75-80 % of delivery calculations. This system can maintain constant temperatures

between 16 and 24 °C. A preliminary 24 hr acute test with Stage II lobster larvae indicates significant toxicity occurs near 300 parts per trillion. We anticipate that 96 hr LC50s will be significantly less.

During development of our dosing system we conducted preliminary experiments with other crustacean species to begin to assess toxicity of pyrethroids. Ten day LC50s for sediment exposures of the benthic amphipod *Leptocheirus plumulosus* were 1,000 ng/g for sumethrin and 400 ng/g for permethrin. Assuming exposure was predominately through pore waters, this roughly translates to water concentrations of 5-100 parts per trillion. We also conducted 96 hr static and flow-through acute tests with adult brine shrimp (*Artemia*). In static tests toxicity was only observed above nominal dosing concentrations of 2,000 parts per trillion. However, actually measured concentrations were much less and decayed significantly during the course of the exposure from 1,000 parts per trillion initially to 4 % of dosed levels at 24 hrs and 0.3% of dosed levels after 96 hrs highlighting the problems associated with static exposures. Measurements in flow-through systems showed toxicity at much lower levels (approximately 200 parts per trillion) after 96 hrs.

Work will continue focusing on additional field sampling for pesticides and determination of acute toxicity and immune suppression in larval and juvenile lobsters exposed to resmethrin with and without PBO, malathion, and methoprene at stressful (22-24 °C) and non-stressful (16-18 °C) temperatures.

The authors wish to thank Michael Tlusty and Denise Fiore of the Lobster Rearing Facility at the New England Aquarium, who provided organisms and technical assistance on lobster care and with the design and set-up of the dosing systems; Ken Gerold who conducted the sediment exposures with sumethrin and permethrin as part of his Intel Science Fair project; Steve Terracciano and Mike Thurman of USGS who assisted with sample collection and methods analytical methods development, and New York Sea Grant who provided financial support.

Acute Effects of Methoprene on Survival, Cuticular Morphogenesis and Shell Biosynthesis in the American Lobster, *Homarus americanus.*

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The acute effects of methoprene on various life stages of the lobster are being investigated in five areas: bioaccumulation, exposure/survival studies, cytopathology/TEM studies, metabolic incorporation studies and SDS-PAGE analysis. After exposure of adult intermolt lobsters to field level concentrations of methoprene (25 ppb/ 24 h), various tissues were dissected, extracted and the concentration of methoprene in each tissue was determined by GC-MS. The highest accumulation of methoprene was observed in epithelial tissue, gonads and hepatopancreas (6.17, 5.18, and 3.97 ppm), up to 250-fold concentration of the pesticide from the surrounding seawater. Conversely, concentrations of methoprene in the stomach, connective tissue and muscle were at nearly baseline levels (0.14, 0.71, 0.16 ppm). Similar results have been obtained with other intermolt lobsters. Standard trans-S-methoprene epoxide has been analyzed by GC-MS and exhibits its own unique GC-MS profile. Analysis of S-methoprenic acid has proved unsuccessful, thus we will utilize an ELISA procedure for this component. These results indicate that adult intermolt lobsters accumulate methoprene in specific tissues "against a gradient".

Our initial metabolic studies involved exposure of intermolt juvenile lobsters to 10 ppb methoprene (18 h), injection with ^{35}S Translabel, and incubation for 24 h. We observed 90% reduction in cytosolic, mitochondrial and microsomal protein synthesis in the hepatopancreas, 50% reduction in the gills, and no effect in muscle tissue. We conclude that *in vivo*, methoprene causes tissue specific decrease in protein synthesis in juvenile lobsters.

Acute toxicity studies have been initiated in all life stages of the lobster. Following exposure of stage I larvae to 25 ppb methoprene at 17 ^{o}C, we observed 100% survival of the larvae up to 48 hours. Stage II larvae were collected and individuals were exposed to methoprene (0.1, 0.5 and 10 ppb) at 18 ^{o}C for up to three days; these larvae were fed adult brine shrimp twice daily. Mortality after 24, 48 and 72 h was recorded. After 24 h exposure, no death was observed at any concentration of methoprene. After 48 and 72 h, we observed mortality in the methoprene-treated larvae, up to a maximum of 82% at 10 ppb. The estimated 72 h LC_{50} for methoprene was 2 ppb in stage II larvae. Postlarvae, juveniles and intermolt adults survive 24 h acute exposures up to 25 ppb methoprene. Following exposure of several postmolt adult lobsters to 25 ppb methoprene, we have observed death after 18 h. Since such animals were likely subjected to multiple stressors, additional studies are planned to further investigate this point.

In vitro metabolic studies with ^{35}S Translabel on postmolt adults utilized explant cultures in DMEM media supplemented with 10% fetal bovine serum and containing 1/10th the normal levels of methionine and cysteine. After 18 h incubation, we observed minimal effect of methoprene on total shell protein synthesis, but did detect a shift in the distribution of extractable proteins from the shell. The epithelial tissues from these cultures exhibited increased increased protein labeling but showed shifts of labeled precursors into buffer and urea soluble fractions. In order to study chitin synthesis, *in vitro* metabolic studies with ^{3}H D-glucosamine (GlcN) were performed as described above for ^{35}S Translabel. Methoprene reduces ^{3}H GlcN incorporation into epithelial cells and the associated microsomal fraction by 62%. Extraction of the shell fractions from this experiment indicate that methoprene decreases total ^{3}H GlcN incorporation by 17% and causes a shift in the

distribution of precursors from buffer to urea soluble. Taken together these metabolic studies indicate that even at very low levels, methoprene may cause a block in the exocytosis and/or crosslinking of ^{3}H GlcN labeled precursors into the nacent cuticle.

As part of our studies of the individual fractions solubilized from control and methoprene treated shell and epithelial cells, we have analyzed these fractions by SDS-PAGE. The gels have then been examined by total protein staining (colloidal Coommassie Blue G-250 or Sypro Ruby) and by Western blotting followed by probing with either the chitin specific lectin, ToL, or with monoclonal antibodies to specific stress proteins (HSPs). In intermolt adult lobsters, methoprene causes shifts in membrane-associated proteins of the hepatopancreas; no such changes were observed in muscle tissue. In postmolt adult epithelial cells, methoprene decreases most major cytosolic proteins and increases several, e.g., HSP-70. Decreased synthesis of tomato lectin-positive chitoproteins was observed in the cellular membrane fraction and in all fractions extracted from the shell of these samples. These results indicate that methoprene alters the synthesis, secretion and/or incorporation of chitoproteins in the postmolt lobster.

To date, our cytopathology studies have failed to show any consistent aberrations caused by low levels of methoprene (1-10 ppb); some preliminary indication of focal necrosis in the hepatopancreas has been noted but requires further investigation. Additional cytopathology and TEM studies are ongoing.

Malathion immunotoxicity in the American lobsters (*Homarus americanus*) upon experimental exposure

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Introduction

A lobster die-off reduced the 1999 fall landings in western Long Island Sound by up to more than 99%. The die-off corresponded in time with the application of pesticides for the control of mosquitoes that carried west Nile virus, a new emerging disease in North America at the time. The lobsters examined suffered from a *Paramoeba* sp. infection that mainly affected the nervous system. In order to determine the possible implication of pesticide application as a direct cause or contributing factor in the die-off, we studied the effects of experimental exposure to malathion on the health of lobsters.

Material and methods

Experimental exposures were performed in aerated 20 gallon tanks each containing 3 lobsters, with a total of 9 lobsters (in 3 tanks) per dose. Lobsters were kept at 10 °C in artificial sea water and exposed to malathion using different regimes. Standard LC50 experiments were performed in the course of 96 hours. Acute exposure lasted 5 days, with sampling on day 1, 3 and 5, and consisted of either a single dose of malathion or repeated doses through daily water changes. Subacute exposure was performed over the course of 4 weeks, with weekly sampling. At the end of each study, lobsters were sacrificed and tissues sampled for the presence of gross and histological lesions, and for determination of concentrations of the chemical used in pooled muscle, hepatopancreas and hemolymph, in comparison to water concentrations.

Water and tissue samples were analyzed at the Environmental Research Institute (ERI) based upon a modified form of EPA Method 616. This EPA method is not validated for sediment and tissue from the EPA Office of Pesticide Programs (personal communication). The primary changes from the EPA method is the use of capillary column techniques in lieu of the packed column specified in the methods, and the use of GC/MS instead of a flame ionization detector. EPA method 616 is based upon older techniques and the ERI improvements to the method allow for the identification and quantification at lower levels.

The endpoints tested include evaluation of the immune system using hemocyte counts and phagocytic index on hemolymph samples. Briefly, hemolymph was collected and immediately transferred to Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing acid citrate dextrose (ACD). This proved to be the best anticoagulant for use with lobster hemolymph cells in preliminary studies in our lab. Cells were then counted using a hemocytometer and Trypan blue to determine viability. Phagocytosis was evaluated as previously described (De Guise *et al.*, 1995) with some variations. Hemocytes were incubated in their hemolymph at room temperature (20-25°C). One µm diameter fluorescent latex beads (Molecular probes, Eugene, OR) were diluted 1:10 in PBS and 5 µl of the bead mixture was added for every 200 µl of hemolymph. After a 1 hour incubation in the dark, 200 µl of each cell suspension was analyzed by flow cytometry. The fluorescence of approximately 10,000 hemocytes was evaluated with a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer. Phagocytosis was evaluated as the proportion of hemocytes that had phagocytized 2 or more beads.

At the end of all studies, lobsters were sacrificed and a gross and histopathological examination performed to determine the presence/absence of pathological conditions. Tissues were fixed in Bouin's fixative for 48 hours, then in 70% ethanol for 24 hours, and further trimmed and processed for paraffin embedding. Tissues were sectioned at 4µm, routinely stained with hematoxylin and eosin, and examined by light microscopy for the presence/absence of lesions.

Results

The direct toxicity was determined through a standard 96-hour LC50, the calculated concentration that killed 50% of the animals. To do so, lobster mortality was recorded daily over a 4 day exposure. The cumulative mortality was then plotted against the concentrations of malathion used and a linear regression curve was determined using the Microsoft Excel software. The LC50 was calculated using the equation determined by the software for the regression curve. The 96 hour LC50 was 33.5 µg/L (or ppb) upon single exposure (Figure 1).

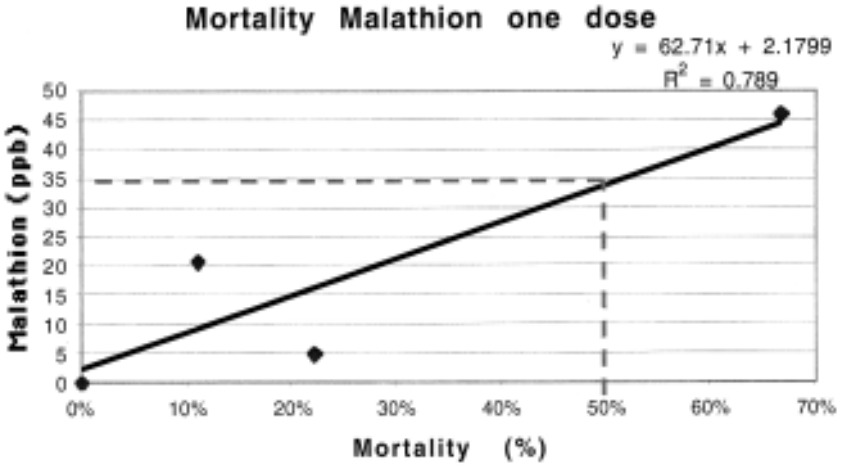

Figure 1. LC50 of malathion in lobsters.

Malathion degraded rapidly in our system, with 65-77% lost after one day and 83-96% after three days (Figure 2). No malathion was detectable in lobster tissues at the end of the 5 day exposure.

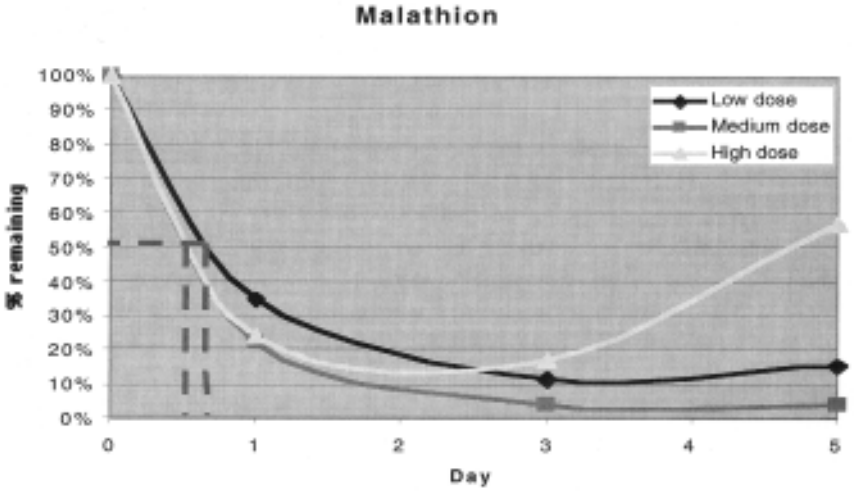

Figure 2. Concentrations of malathion in water decreased rapidly in our system.

Relatively high concentrations of malathion, upon repeated exposure, initially (day 1) increase phagocytosis, with no effects on day 3 and 5 (data not shown). Phagocytosis was significantly decreased 3 days (but not 1 or 5) after a single exposure to water concentration as low as 5 ppb (the lowest concentration tested), when water concentrations were as low as 0.55 ppb (Figure 3).

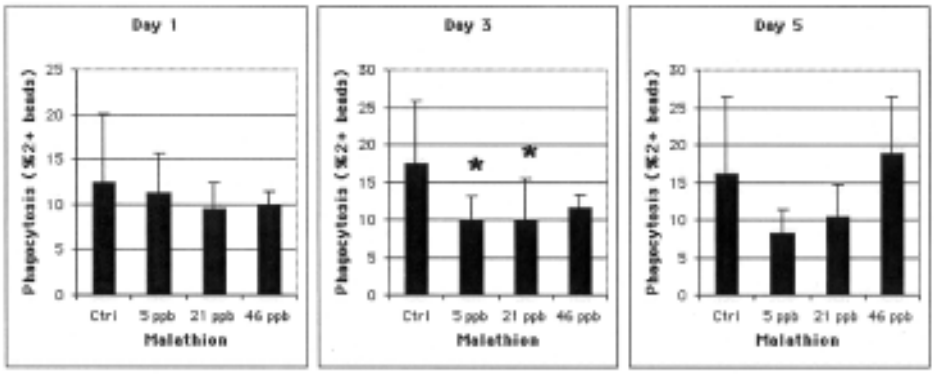

Figure 3: Phagocytosis of lobster cells after a single exposure to increasing concentrations of malathion.

Phagocytosis was also significantly affected in the course of the sub-acute (month long) exposure (Figure 4). There was a significant reduction of phagocytosis one week after the initial exposure to 21 ppb, and two weeks after the initial exposure to 5 ppb. There was a significant reduction of phagocytosis at all concentrations tested three weeks after the initial exposure.

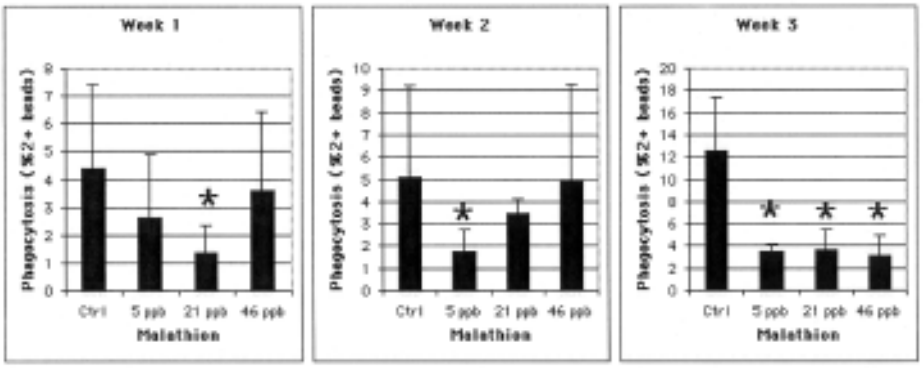

Figure 4: Phagocytosis of lobster cells after a weekly exposure to increasing concentrations of malathion.

Cell counts did not differ significantly upon exposure to malathion. Gross or histological lesions were not observed upon exposure to malathion.

Discussion

Malathion has a wide range of toxicities in fish, extending from very highly toxic in the walleye (96-hour LC50 of 0.06 mg/L) to highly toxic in brown trout (0.1 mg/L) and the cutthroat trout (0.28 mg/L), moderately toxic in fathead minnows (8.6 mg/L) and slightly toxic in goldfish (10.7 mg/L) and mosquitofish (12.68 mg/L) (Johnson and Finley 1980, Tietze *et al.*, 1991,Kidd and James, 1991, U.S. Public Health Service, 1995). Various aquatic invertebrates are extremely sensitive, with EC50 values from 1 ug/L to 1 mg/L (Menzie, 1980). Lobsters, with a LC50 of 33.5 ppb, appear to be very sensitive to the acute lethal effects of malathion compared to other aquatic species.

The very rapid breakdown of malathion in our system suggests that failure to measure malathion in water samples does not necessarily mean lack of exposure. At day 3 of our acute exposure study, the concentrations of malathion in the water were very low, yet effects on phagocytosis were demonstrated in lobsters.

Our data suggest that evaluation of phagocytosis using flow cytometry is a sensitive indicator of subtle sub-lethal effects of malathion, and that transient exposure to relatively small concentrations of malathion (6-7 times lower than the LC50) can affect lobsters' defense mechanisms, even with rapidly decreasing water concentrations. Those results are not surprising given that the immunotoxicity of malathion has been documented in several species of laboratory animals including effects on both humoral and cellular immune responses of mice, rats and rabbits (Banerjee *et al.,* 1998). Malathion was also documented to affect the natural and acquired immunity of fishes (Japanese medaka), in addition to decreasing resistance to a common pathogen (Beaman *et al.,*. 1999). Nevertheless, it is interesting to note that the initial water concentrations that resulted in immunotoxicity in lobsters (5 ppb or 5 µg/L) are 40 times lower that those which resulted in reduction in immune functions and 20 times lower than those which resulted in reduced resistance to a pathogen in the fish study (Beaman *et al.,* 1999).

In conclusion, our results suggest that lobsters are highly sensitive to both the lethal and sub-lethal toxicity of malathion in sea water. A reduction in immune functions could likely result in an increase susceptibility to infectious agents, and could have contributed to the mass mortality if exposure was sufficient.

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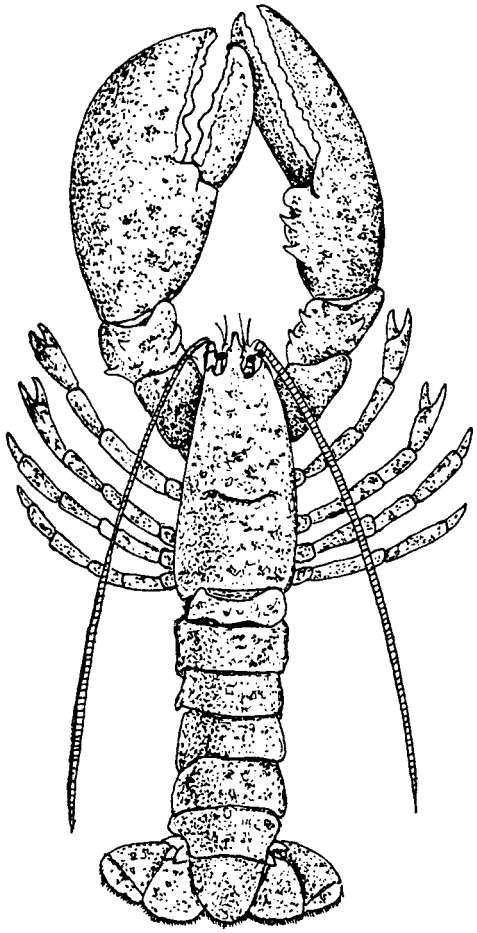
Parasites and Disease



Progress in *Paramoeba* Research

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1. *Paramoeba* and its relatives form a clade of eukaryotes that was previously unrecognized at the molecular level, though it had previously been proposed on the basis of the type of pseudopodia ("dactylopodia") produced by amoebae in this group. This lineage, designated the PV lineage (Figure 1), includes scale bearing species with (*Paramoeba*) and without (*Mayorella, Korotnevella*) parasomes, and species without scales both with ("*Paramoeba*"=*Neoparamoeba*) and without (*Vexillifera*, *Pseudoparamoeba*) parasomes.

2. The amoebae in the PV lineage had previously been placed in one or other of three different families; Mayorellidae, Paramoebidae, and Vexilliferidae. If the topology of Figure 1 continues to be supported, all of these amoebae will be placed in a single family, the Paramoebidae.

3. The type species of *Paramoeba, P. eilhardi*, differs from other parasome-containing amoebae in both ultrastructure and molecular sequence. *Paramoeba eilhardi* is a large amoeba that eats diatoms and is unlikely to be pathogenic to other organisms. The other parasome-containing amoebae for which sequence data are available belong together, and contain the type species of *Neoparamoeba*, *N. pemaquidensis*. These species, which include the lobster pathogen and other known pathogenic strains, belong in *Neoparamoeba*, not *Paramoeba.*

4. *Paramoeba eilhardi* is a parasome-containing amoeba. Another species has been described, under the name *Korotnevella nivo*, that is identical to *P. eilhardi* in gross morphology, habitat, food preference, and ultrastructure, but no parasome is present. Molecular sequence data indicate that the two entities are very similar, close enough so that both may be treated as species of *Paramoeba*, perhaps even as strains of *P. eilhardi*. In *P. eilhardi*, therefore, the parasome may not be a fixed character. So far, no parasome-free amoebae related to species of *Neoparamoeba* have been found, and amoebae (genus *Vexillifera*) formerly classified in the same family (Vexilliferidae) as *Neoparamoeba* are not even the closest relatives of *Neoparamoeba* species. Nevertheless, the possibility of parasome-free strains of *Neoparamoeba* cannot be ignored in attempts to assess presence or absence of pathogenic amoebae.

5. Among strains of *Neoparamoeba* so far examined, there is considerable variation in normally conservative domains of the nuclear-encoded small subunit ribosomal RNA (SSU rRNA) gene. The significance of this variation is not yet clear, but it is likely that there are many more genotypes of *Neoparamoeba* in the environment than are apparent from phenotype. If so, this complexity needs to be considered when attempting to define gene "markers" for identifying potentially pathogenic *Neoparamoeba* in the environment.

6. We are in the process of fingerprinting sediment and water samples collected by Mathew Lyman (CT Department of Environmental Protection, Bureau of Water Management) to try and identify potential pathogenic *Neoparamoeba* in the environment and determine their geographic distribution. As mentioned above, we are working to develop a series of specific PCR primers to amplify species from the *Neoparamoeba* clade, and need to take into account the atypical variations in the normally-conserved domains of the *Neoparamoeba* SSU rRNA.

7. It has so far not been possible to cultivate the lobster pathogen. We have very recently had success, however, in cultivating *Neoparamoeba* species from moribund sea urchins (*Strongylocentrotus droebachiensis*)

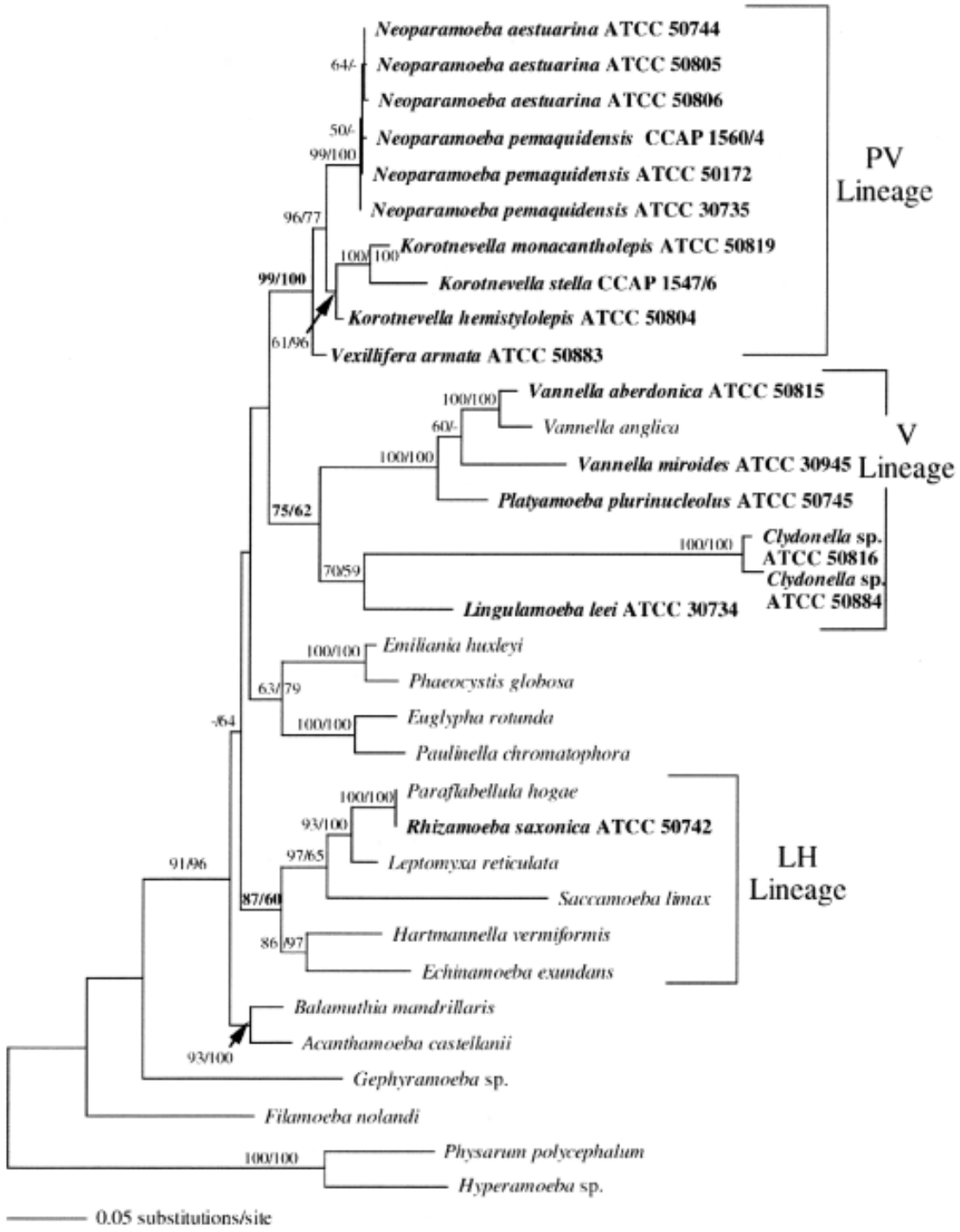

Figure 1. Phylogenetic tree showing the position of the Paramoeba assemblage (PV lineage) with respect to other groups of amoebae and selected other protists. From a manuscript submitted to the Journal of Eukaryotic Microbiology. Additional species not shown on the tree have been found to belong to the PV lineage in preliminary analyses, including the lobster pathogen (species of Neo-paramoeba), Paramoeba eilhardi, Pseudoparamoeba pagei, and species of Mayorella.

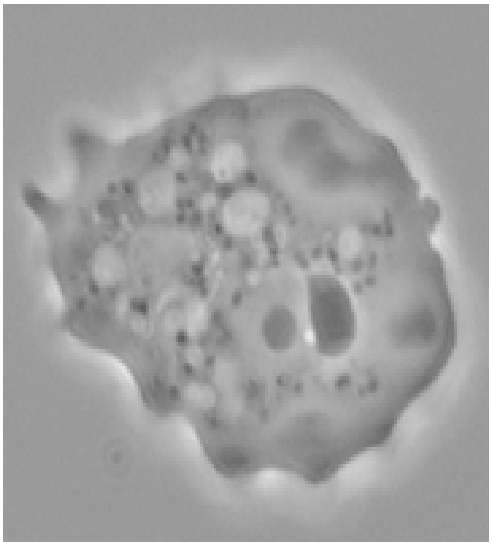

Figure 2. Light micrograph (phase contrast optics) of strain 1 of the Neoparamoeba species isolated (tentatively into axenic culture) from green sea urchins in the Gulf of Maine. The cell is ca. 10 µm in length. Unpublished. Cells of strain 2 are half again as large (ca. 15 µm length) and may have up to three parasomes.

in the Gulf of Maine (putative sea urchin pathogen *N. invadens*; Figure 2). Moreover, two strains of the urchin amoeba are presently in axenic culture; if these cultures continue to passage successfully, they will represent the first axenic cultivation of paramoebid amoebae known to us. The two strains isolated (both from a single urchin) differ in size and in parasome number; if both are pathogenic, then the possibility that multiple infective amoebal strains may be present must be considered for other disease conditions involving *Neoparamoeba* strains.

We gratefully acknowledge the contributions of:

Michael T. Peglar and **Thomas A. Nerad** (American Type Culture Collection)
Thomas F. Mullen and **Salvatore Frasca** (University of Connecticut)
Rebecca Gast (Woods Hole Oceanographic Institution)
Linda A. Amaral Zettler and **Mitchell L. Sogin** (Marine Biological Laboratory)
O. Roger Anderson (Columbia University)
Jeffrey D. Silberman (Dalhousie University and UCLA)

Development of Polymerase Chain Reaction- and *in situ* Hybridization-based Tests for the Specific Detection of the Paramoeba Associated with Epizootic Lobster Mortality by Determination of the Molecular Systematics of the Genus *Paramoeba*

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Introduction

Mass mortalities of American lobster (*Homarus americanus*) occurred in western Long Island Sound (LIS) during the autumn of 1999. Lobstermen reported dead or dying "limp" lobsters, and there were concurrent reports of increased numbers of dead or dying crabs and sea urchins from LIS. Histopathologic evaluation of viscera and nervous tissues from dead or dying lobsters revealed hemocytic neuritis and ganglioneuritis. A protozoan parasite was identified in foci of neuritis, and this protozoan possessed a paranuclear body that stained positively for DNA using the Feulgen technique. Transmission electron microscopic studies of this parasite conducted by Dr. Thomas Burrage (USDA, Plum Island Animal Disease Center, Greenport, L.I., NY) demonstrated ultrastructural features consistent with a parasome, or "*Nebenkörper,*" a feature common to protozoa of the genus *Paramoeba* Schaudinn, 1896 (Page 1970). The role of this neurotropic paramoeba as a primary or secondary agent in the lobster die-off has not yet been determined, nor have Koch's postulates been fulfilled. However, species of *Paramoeba* have been identified as the causative agents of mass mortalities of several commercially relevant marine invertebrates, e.g., sea urchins (*Strongylocentrotus droebachiensis*) (Jones *et al.*, 1985) and blue crabs (*Callinectes sapidus*) (Sprague *et al.*, 1969). A description of the paramoeba from LIS lobsters and the results of initial investigations into the occurrence of parameobiasis in association with the epizootic mass mortality of American lobsters in LIS have been submitted for journal publication (Mullen *et al.*, In submission). Very little is known about free-living and parasitic species of the genus *Paramoeba* Schaudinn, 1896. Molecular data derived from phylogenetically relevant genes (i.e. ribosomal RNA genes) are absent for this genus.

Hypothesis and Objectives

The principal goal of this research is to develop sensitive and specific DNA-based tests for the detection of this parasitic paramoeba in host tissues by determining the molecular systematics of the genus *Paramoeba* through 18S SSU rRNA gene sequence analysis. Development of such DNA-based tests is predicated upon determination of the molecular systematics of the genus, since DNA sequence data essential for primer and probe design are currently nonexistent.

The following are the specific aims of this research:

1. Determine the molecular systematics of known (i.e. previously identified) species of *Paramoeba*, or morphologically related free-living or parasitic amoeba.
2. Determine the phylogenetic relationship of the lobster paramoeba to other known paramoeba species.
3. Develop molecular tests based on 18S SSU rDNA sequence data to detect this parasitic paramoeba in host tissue.

Methods

Specific Aim 1. *In vitro* cultures of strains of three Paramoebid amoebae and five Vexilliferid amoebae were obtained for 18S SSU rRNA gene sequence analysis. DNA was isolated using silica-gel spin column extraction kits (DNeasy DNA extraction system, Qiagen, Inc., Chatsworth, CA). 18S SSU rDNA was amplified by

PCR from genomic DNA extracts using universal 18S SSU oligonucleotide primers adapted from Medlin *et al.*, 1988; Hillis and Dixon, 1991; and Weekers *et al.*, 1994. For each strain, PCR products from three separate reactions were treated independently of one other. PCR products were purified, ligated into TA cloning vectors (TOPO TA Cloning Vector for Sequencing, Invitrogen Corp., Carlsbad, CA), and cloned. Multiple clones from each of three independent cloning reactions per strain were screened, plasmids were isolated, and one representative clone from each cloning reaction was sequenced in the sense direction. Sequences were determined by oligonucleotide-directed dideoxynucleotide chain-termination sequencing reactions, sequence ABI files were assembled, and consensus sequences were generated from three separate sense sequences for each strain using Sequencher 4.1.1 for Macintosh. The 18S SSU rRNA gene sequences were then aligned against a set of pre-aligned eukaryotic rDNA sequences available through the Michigan State Ribosomal Database Project II (Maidak *et al.*, 2001) and Genbank (Benson *et al.*, 2002) using ClustalX v1.81 (Thompson *et al.* 1994). Phylogenetic trees were inferred by distance and parsimony optimality criteria using PAUP* (Swofford, 2002). Distance-based phylogenetic trees were created using the minimum evolution optimality criterion, and a maximum parsimony tree was obtained via random stepwise addition and tree-bisection-reconnection branch-swapping algorithm. The confidence of branching in each case was assessed by 1,000 bootstrap re-samplings of the data set, and 200 random sequence addition replicates were used to search for the most parsimonious tree.

Specific Aim 2. To amplify 18S SSU rDNA from the lobster parasite, paramoeba-infected lobster tissues were used to provide genomic DNA. Lobsters were either collected by trawl survey by the CT DEP or submitted independently by fisherman or biologists. Lobster tissues were processed for paraffin-embedding, evaluated microscopically, and frozen tissue samples from specimens diagnosed as infected by histopathological examination were subjected to nucleic acid extraction (DNeasy DNA extraction system, Qiagen, Inc., Chatsworth, CA). Order-based and genus-based primers were identified from multiple sequence alignments involving the 18S SSU rRNA gene sequences of Paramoebid and Vexilliferid amoebae from the first stage in this project, along with 18S SSU rDNA sequence data from American lobster available through GenBank. Sequences of oligonucleotide primers were selected so as to be conserved among Paramoebid and Vexilliferid genes and not homologous to lobster 18S SSU rDNA using comparative sequence alignments through ClustalX v1.81 and DNAMAN (Linnon Biosoft, Quebec, Canada). Three sets of inner and outer primer pairs for three separate nested PCR protocols were identified in order to amplify three overlapping regions of the 18S SSU rRNA gene of the parasitic paramoeba. Primers were tested for specificity by using purified plasmid SSU rDNA of Paramoebid and Vexilliferid amoebae as positive controls and genomic DNA of American lobster (*Homarus americanus*), blue crab (*Callinectes sapidus*) and green sea urchin (*Strongylocentrotus draebachiensis*) as negative controls. Multiple tissues from infected lobsters were tested in triplicate using one or more of the three, separate, nested PCR protocols. PCR products were treated independently of each other, and each was purified, ligated into a TA cloning vector, and cloned. One clone from each independent cloning reaction, i.e. representative of an individual PCR product, was sequenced in the sense and antisense directions, sequences were assembled using Sequencher 4.1.1 for Macintosh, and consensus sequences were constructed. Consensus sequences for each of the three overlapping regions of 18S SSU rDNA were in turn aligned to construct a final overall consensus representative of the nucleotide sequence of the 18S SSU rRNA gene of the parasite. To infer the relationships of the lobster parasite to other taxonomically and pathobiologically relevant species, phylogenetic trees were construct based on distance and parsimony optimality criteria using PAUP* (Swofford ,2002).

Specific Aim 3. Comparative sequence alignments of 18S SSU rDNA were constructed through ClustalX v1.81 and DNAMAN using sequence data from the lobster parasite, Paramoebid and Vexilliferid amoebae, and American lobster in order to identify target sites for nucleic acid-based diagnostic tests. Oligonucleotide primers were designed to variable regions of the 18S SSU rRNA gene of the parasite that were not homologous to lobster SSU rDNA. Primer pairs were evaluated theoretically using DNAMAN sequence analysis software

and in PCR experiments for thermodynamic compatibility, heterodimer and homodimer formation, spurious reactivity, and product length, and an inner and outer set of primer pairs was selected for a nested PCR protocol. PCR primers were tested for specificity using purified plasmid SSU rDNA of Paramoebid and Vexilliferid amoebae as positive controls and genomic DNA of American lobster, blue crab and green sea urchin as negative controls. Genomic DNA extractions from fresh lobster tissue samples and from formalin-fixed paraffin-embedded tissue sections, examined histologically for the presence of the parasite, are being prepared to test the application of this nested PCR protocol for the identification of parasite DNA in lobster tissue.

Results

Specific Aim 1. The 18S SSU rRNA genes of 3 Paramoebid and 5 Vexilliferid amoebae were amplified, cloned and sequenced, and the contig assemblies of independently derived clones for each particular strain of amoeba demonstrated less than 1.5% nucleotide ambiguity. Molecular systematic studies undertaken to describe the taxonomic relationships of these amoebae generated a rooted phylogenetic tree with bootstrap values from distance and maximum parsimony analyses. In both instances, the Paramoebid-Vexilliferid (P-V) clade arose as a distinct line of descent separate from genera belonging to the subclass Gymnamoebia. The P-V clade arose before the crown taxa of higher eukaryotes and after basal radiations by eukaryotes such as *Vannella anglica.* In addition within the P-V clade, there is a distinct branching that separates the Vexilliferid species, e.g. *Neoparamoeba* spp, from the Paramoebid species, e.g. *Paramoeba* sp and *Korotnevella* spp. The results of these SSU rDNA sequence data and phylogenetic interpretations have been contributed to a manuscript that is currently in review by the *Journal of Eukaryotic Microbiology* and represents a collaborative research effort with Drs. P. Gillevet, T. Nerad, and C. O'Kelly [Peglar M.T., L.A. Amaral Zettler, R. Anderson, T.A. Nerad, P.M. Gillevet, T.E. Mullen, S. Frasca, Jr., J.D. Silberman, C. O'Kelly, and M. Sogin. Two new small-subunit ribosomal RNA gene lineages within the morphologically defined subclass Gymnamoebia].

Specific Aim 2. Amplification of SSU rDNA of the parasite from genomic DNA extractions of paramoeba-infected lobster tissue was accomplished by using three separate nested PCR protocols targeted at overlapping regions of the 5'-end, internal 1.3-kilobase segment, and 3'-end. For each target region, PCR products amplified once or in duplicate from one or more tissue sources from a minimum of three different lobsters were visualized, purified and cloned. Consensus sequences for each target region were assembled from cloned PCR products from a minimum of three separate lobster hosts, representing a minimum of six double-stranded sequences. An overall consensus SSU rDNA sequence representing the nucleotide sequence of the 18S SSU rRNA gene of the parasite was generated by aligning the separate consensus sequence of each target region. Phylogenetic relatedness inferred by distance and parsimony analyses and assessed by bootstrap re-samplings of the data set revealed a very high relatedness of the 18S SSU rRNA gene sequence of the lobster parasite with those of the Vexilliferid amoebae, particularly *Neoparamoeba pemaquidensis*. Branching of the lobster parasite with species of *N. pemaquidensis* was supported by very high distance and parsimony bootstrap values.

Specific Aim 3. Comparative sequence analyses, along with theoretical and applied evaluations of primer pairings, generated a nested PCR protocol targeted at a variable region of Paramoebid and Vexilliferid SSU rDNA. In preliminary studies, this nested PCR protocol yielded a 144-base pair product from the 3 Paramoebid and 5 Vexilliferid amoebae tested in specificity experiments, as well as from paramoeba-infected lobster tissue, without amplifying genomic DNA from lobster, blue crab or green sea urchin. Sequence analysis of this 144-base pair product distinguishes the lobster parasite from other Vexilliferid or Paramoebid amoebae. DNA extractions and formalin-fixed paraffin-embedded tissue sections are being prepared to test the applicability of this nested PCR protocol for the detection of parasite DNA in lobster samples from a large sample group in comparison to other means of parasite detection, e.g. histopathological evaluation. In addition, probes are being prepared to begin *in situ* hybridization experiments to develop techniques to label the parasite in tissue sections.

Acknowledgements

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Oligonucleotide-based Detection of Pathogenic *Neoparamoeba* Species

Rebecca J. Gast

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Introduction

Monitoring the environmental distribution of the *Neoparamoeba* species that infects lobsters is an essential component to understanding how infections occur and predicting their spread. For example, if the amoeba is an opportunistic pathogen that can normally exist as a free-living organism in the same place as its host, the potential for repeated infections may be more likely than if the organism needed to be reintroduced from another area. Unfortunately, a good understanding of the etiology and distribution of paramoebiasis has been difficult to attain, due largely to the inability to reliably detect and identify the parasitic species of interest. The identification of *Neoparamoeba* species is based primarily upon culture of the organism and/or the analysis of morphologic characters by light or electron microscopy.

Issues of culture bias, morphologic variability, level of infectivity and the potential for dormancy have led us to propose the utilization of gene sequences for the detection and identification of the organism infecting lobsters. Small subunit ribosomal gene (srDNA) sequences contain both invariant and variable regions that can be used as templates for the design of oligonucleotide primers (very short pieces of DNA) with specificities ranging from kingdom to individual isolate levels. The very large number of srDNA sequences available in databases, such as GenBank, also make this molecule useful because of the large volume of comparative sequence information available. In the past 12 years, the use of ribosomal sequences in ecological studies has allowed researchers to detect an organism of interest, to determine natural abundances and to follow the organism's occurrence over time (for examples, see Amann *et al.*,1990; Lim *et al.*,1999; Manz *et al.*,1993). Coupled with polymerase chain reaction amplification (PCR), the results obtained are not only specific, but they can also be sensitive.

Objectives

Detection Currently there is no simple and reliable method for the discrimination of the *Neoparamoeba* that infects lobsters from other parasitic, and non-parasitic, paramoebae. Therefore, one of the objectives of my project is to develop a method for the detection of the lobster parasite that can be used to analyze environmental samples for the presence of the parasite, as well as to analyze tissue samples. I have been working to develop primers and protocols for denaturing gradient gel electrophoresis (DGGE) that will permit detection of the lobster parasite (and eventually for the parasitic species from fish, crab, and urchin). PCR amplification of a portion of the small subunit ribosomal gene is employed to generate fragments of DNA from the sample of interest. These fragments are separated on a gel with a gradient of denaturant, and the bands DGGE in my lab to study the genetic diversity of protists in environmental samples, but the method is also useful for detecting a particular organism. To increase the sensitivity of the method, I have designed primers that are specific for *Neoparamoeba* species and use them in amplifications prior to amplification with the DGGE primers.

Environmental monitoring The second objective in my project is to use the DGGE method to examine the natural occurrence of paramoebae in Long Island Sound. I will analyze sediment and water samples that have been collected, on a monthly basis, for 18 months. This will allow me to determine whether the lobster *Neoparamoeba* parasite is present year-round in the Sound, and will potentially help us to predict the likelihood for future outbreaks of infection.

Progress to Date

This project was initiated in July, 2001. With the assistance of an undergraduate summer student in my laboratory, we have established protocols for extracting DNA from lobster tissues, and developed five amplification primers that would be selective for paramoeba and established amplification parameters to recover neoparamoeba-like ribosomal gene amplification products. We have tissue samples from lobsters and crabs with confirmed and putative paramoebic infections, and have established an unofficial collaboration with the other research groups working on the molecular biology of paramoebae in order to make the exchange of samples and sequences easier. We have been successful in recovering amplifiable DNA from lobster and crab tissues, and have been able to recover a neoparamoeba product from the lobster tissues (Figure 1).

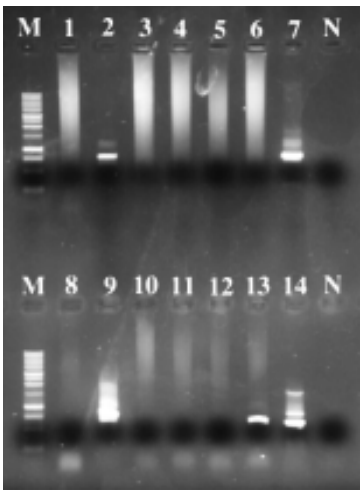

Figure 1. PCR amplification and reamplification of samples using Neoparamoeba-specific primers P1, P2 and P3R. Lane 1, 8 – uninfected lobster DNA; lane 2, 9 – LIS Neoparamoeba isolate A4S; lane 3, 10 – lobster DNA 1268; lane 4, 11 – lobster DNA 1280; lane 5, 12 – lobster DNA 1352; lane 6, 13 – lobster DNA 1697; lane 7, 14 – Neoparamoeba pemaquidensis DNA. Samples in lanes 1-7 were amplified with P1 and P3R. Samples in lanes 8-14 were P1/P3R reactions reamplified with P2 and P3R. M = size marker; N = negative control.

Crab samples have not yet yielded neoparamoeba-like products with any of our primers. The crab parasite sequences would be useful for determining whether the lobster amoeba is the same as the one infecting crabs, and for developing specific probes to be used in the detection method, but they are not necessary for further development of the DGGE method. DGGE gels have recently been run using products from infected lobster samples to identify the *Neoparamoeba* band (Figure 2), and we are currently working on determining the level of sensitivity that we can expect for this method.

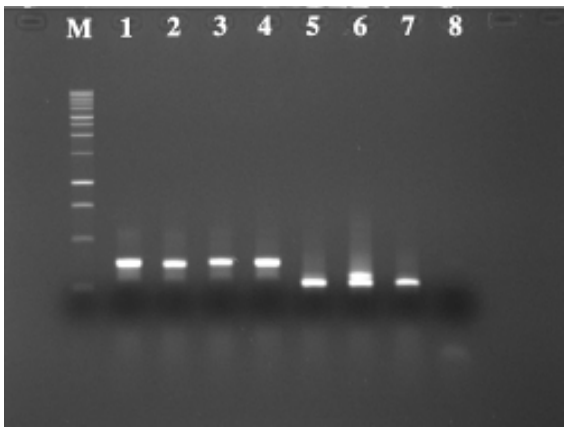

Figure 2. PCR amplification of bands picked from DGGE gel. Lanes 1 - 4 are products that are the correct size for the fragment of the Neoparamoeba small subunit ribosomal gene. Lanes 5 – 7 are products that are the correct size for the products from the lobster gene. Sequencing will be used to confirm the identity of the fragments. Lane 8 – negative control; M = size markers.

Sampling of Long Island Sound sediment and water began in August 2001 in conjunction with the CT DEP Water Quality Monitoring program. Samples are collected once a month at stations A4, B3, C2, D3, E1, H4, J2 and N3 (Figure 3). We completed our sampling this fall and are continuing the processing of these samples. DNA is extracted from water within two weeks of collection, and the samples are tested to confirm general amplification competence. Sediment samples are being stored at –80 °C until extraction this year. Although we have successfully recovered DNA from sediments in the past, we are currently testing several new protocols that may provide cleaner nucleic acids in less time. All isolated DNA is archived at –20 °C until analysis by the DGGE method.

In addition to collecting samples for DNA extraction from Long Island Sound, we have collected water and sediment samples for enrichment culture of amoebae to determine what paramoeba-like species are present. Many amoebae have been cultured, including several paramoeba-like isolates. Three of these have been confirmed as *Neoparamoeba* species based upon their small subunit ribosomal sequences. Two are very similar to *N. pemaquidensis*, and the other is most similar to *P. eilhardi*. Neither of the *N. pemaquidensis* isolates has a sequence that is the same as that of the lobster parasite, although they are very similar to each other. These confirmed sequences come from isolates recovered from sediment samples, but we have also obtained paramoeba-like isolates from the water column samples.

Project Significance

Once developed, the *Neoparamoeba*-specific DGGE will be a simple and relatively quick method for detecting the presence of the lobster parasite from environmental or tissue samples. Despite the impact of parasitic paramoebae on several different marine fisheries, we know very little about their natural distribution. The reliable identification of parasitic paramoebae from natural samples would represent a huge step forward in our ability study these organisms. We will use our method to survey Long Island Sound water and sediment samples to determine whether the lobster parasite is endemic to the region. Our results on the natural distribution of the organism may eventually help researchers to predict the potential for outbreaks of disease and the impact on host populations.

Figure 3. Map of Long Island Sound showing a subset of the 18 sampling stations monitored monthly throughout the year by the Connecticut DEP Long Island Sound Water Quality Monitoring Program. The sites indicated on this map are the ones where we collect water and sediment samples. (Image modified from the 1998 Summer Hypoxia Survey, online at <http://www.epa.gov/region01/eco/lisLis98tot.pdf>.)

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Bacterial Assemblages involved in the Development and Progression of Shell Disease in the American Lobster

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Sampling. 25 lobsters with lesions and 6 healthy lobsters were collected from the Eastern Long Island Sound (ELIS) by Connecticut DEP and were made available for this research. Five lobsters, provided by NY DEC, were collected from Long Island coastal waters (LICW). Ten lobsters with shell disease collected from the Buzzards Bay (BB) and one lobster with lesions from the Vineyard Sound (VS) was a gift from Bruce Estrella (MA Division of Marine Fisheries). All lobsters had various degrees of severity of cuticular lesions and primarily substages C3 and C4 of cuticular development. The lobsters were sacrificed and used for (1) collection of hemolymph for microbiological analysis, (2) collection of lesion material for microbiological analysis, (3) histological examination.

A half of the carapace lesion(s) was used to collect bacterial biomass and a half was preserved in 4% formaldehyde for further histopathological examination. The scraped material from healthy carapaces was also suspended in sterile seawater to optical densities similar to those of lesion material suspensions and was used for DNA isolation. Typically, we collected material only from carapace lesions. However, for nine lobsters (eight from ELIS and one from LICW) we collected material from both carapace and tail lesions. Approximately, 5 ml of hemolymph were drawn directly from hearts of each lobster into sterile Vacutainer® tubes and refrigerated.

Materials collected for gross and microscopic histopathological examination included: fragments of shell with lesions, hepatopancreas, nerve cord, portions of stomach, gonads, heart, green gland and antennae. This material was fixed in 10 % formalin in seawater. Fixed tissues were trimmed, decalcified, processed in paraffin and hematoxylin and eosin slides were prepared for examination using standard histological methods.

Culture-dependent microbiological analyses of shell lesion material. Our experiments have shown that Seawater Agar II (SAII; seawater with 1.7% of agar, 0.1% peptone, 0.01% Tween 80 and vitamin mix) and Marine Agar 2216 are the most adequate media for isolation of chitinolytic and non-chitinolytic bacteria associated with shell lesions. Chitinolytic bacteria were selectively cultured in media containing crude chitin powder from crab shells, which is an adequate imitation of lobster shell material in its biochemical composition, since it contains not only chitin but also proteins and lipids.

We could successfully isolate from each lobster three to eight unrelated bacterial strains. Bacterial strains isolated from different lobsters, colonies of which appeared very similar, turn out to be either identical or closely related (based on 16S rDNA analysis). Generally, shell lesion material from many but not all lobsters contained chitinolytic bacteria. However, their isolation in pure cultures was difficult, due to a gliding motility of non-chitinolytic bacteria and a long time response in the development of positive reactions (sometimes up to two weeks). During this time, gliding bacteria completely engulf colonies of chitinolytic bacteria. Through multistage re-streaking, however, we managed to isolated pure cultures of chitinolytic bacteria from five lobsters one of which was from ELIS and four from BB and one horseshoe crab.

Culture-dependent microbiological analyses of hemolymph. 5 µl of hemolymph from all lobsters and a horseshoe crab was streaked on rabbit or sheep blood agar plates. One set of the plates was incubated at room temperature and another at 37 °C. No growth was observed on plates incubated at 37 °C. Some bacterial growth was detected on plates incubated at room temperature with plated hemolymph from ELIS lobsters #2, 3, 6, 12 and 13 (Table 1). We concluded that there is no correlation between shell disease and

Table 1. Bacteria in hemolymph of lobsters:

Animal #	Location	Isolates	Density in hemolymph (cells ml^{-1})
1, 4, 7-11, 14,15	Fishers Island	sterile	<200
2	Fishers Island	*Pseudomonas fragi* H2	1200
3	Fishers Island	*Brochothrix thermosphacta* H3	$>2\times10^6$
5, 27-30	Long Island Coast	sterile	<200
12	Fishers Island	isolateH12.1*, H12.2, H12.3, H12.4**	2×10^4
13	Fishers Island	isolate H13.1*, H13.2, H13.3**	3200
16-25	Buzzards Bay	sterile	<200
26	horseshoe crab from MBL aquarium	sterile	<200 <200

* - isolates H12.1 and H13.1 are identical;
** - isolates H13..3 and H12.4 are identical.

hemolymph infection. Hemolymph of only four lobsters contained some bacterial contamination and only one lobster (#3) was heavily infected.

16S rRNA analysis of bacterial isolates. 16S rRNA genes from bacteria isolated from hemolymph and chitinolytic bacteria isolated shell lesions were amplified as described by Borneman *et al.* (*Appl. Environ. Microbiol.* 62:1935-1943.). On average a 700 bp portion of 16S rRNA gene was sequenced (approximately bases 600 through 1300, *E. coli* numbering). The sequence information was used to identify close relatives of our isolates in the GenBank and Ribosomal DNA project II databases. Based on the similarity with 16S rRNA sequences of closest relatives, the isolates were given either genus (less than 98% identity) or species (98% or more of identity) designation. For the hemolymph isolates H12.1-12.4 and H13.1-13.3 and the shell lesion isolate BA2, identification was inconclusive. The 16S rRNA analysis data are summarized in Tables 1 and 2.

No typical bacterial pathogens (*Aerococcus viridans* or *Vibrio fluvialis*) were found among hemolymph isolates. Chitinolytic bacteria forming yellow colonies, belong to one of the four closely related strains of *Cytophaga* sp., indicating that these bacteria are ubiquitous in shell lesions. Identical bacteria, i.e. *Cytophaga* sp. strain 23c1, was isolated from ELIS and BB lobsters as well as the horseshoe crab. All *Pseudoalteromonas gracilis* isolates were identical, indicating that this bacterium is also ubiquitous in shell lesions. It appears that microbial communities found in lesions of lobsters from ELIS and BB are similar to each other. However, the microbial community in lesions of the Vineyard Sound lobster was different.

Table 2. 16S rDNA analysis of isolates from lesions of lobsters with shell disease.

Animal #	Location	Number of independent isolates	Name of the isolate	Number of independent identical isolates from from all animals (≥99% identity)	Reaction with chitin
7	Fishers Island	1	*Psychroserpens* sp. AN7.1	2	NT*
		4	*Pseudoalteromonas gracilis* AN7.3	13	positive
11	Fishers Island	3	*Cytophaga* sp. 11a2	4	positive
		1	*Psychroserpens* sp. MA11.3	2	NT*
		5	*Pseudoalteromonas gracilis* 11b1white	10	positive
		1	*Shewanella frigidimarina* AN11.1		NT*
		1	*Alteromonas arctica* AN112.2		NT*
18	Buzzards Bay	4	*Cytophaga* sp. 23c1	12	positive
19		4	*Cytophaga* sp. 19b2	3	positive
		1	*Pseudoalteromonas gracilis* 19b1white	11	positive
23	Buzzards Bay	3	*Cytophaga* sp. 23c1	12	positive
24	Buzzards Bay	8	*Cytophaga* sp. 23c1	12	positive
		3	*Cytophaga* sp. 11a2	4	positive
26	MBL aquarium	1	*Cytophaga* sp. 23c1	1	positive
(horseshoe crab)		3	*Cytophaga* sp. 26a	2	positive
BA	Vineyard Sound	1	*Shewanella fidelis* BA1		negative
		1	*Vibrio* sp. BA2		negative
		1	*Vibrio lentus* BA3		negative

* NT, not tested.

Culture-independent microbiological analyses. Denaturing gradient gel electrophoresis (DGGE) was a method of choice to compare microbial communities in the lesions and individual isolates. PCR and DGGE were carried out as described by Ferris *et al.* (AEM, 1996, 62:340-346) under conditions, which we optimized earlier. The best results with PCR products of DNA from shell disease lesions were achieved using the following conditions: temperature of run 60 °C, 14 hours at 100 v, 40-55% gradient of the UF solution, and 9% acrylamide gel. The gel shown in Figure 1 depicts a DGGE gel of the whole community from the lobster #11 along with individual isolates from the lesions of this animal (C - *Cytophaga sp*., P - *P. gracilis*, A - *Alteromonas arctica*; S - *Shewanella frigidimarina*). Both *Cytophaga* sp and *P. gracilis* strains used in these experiments are chitinolytic and clearly present as members of the community. *P. gracilis* appears to be a dominant component of the community. Overall, the composition of bacterial communities in the lesions of BB and ELIS lobsters are very similar in that they have few individual bacterial strains and at least two or three of these strains are present in all analyzed animals. The composition of the microbial community in the lobster #5 (from LICW), however, was very different from those of ELIS and BB lobsters.

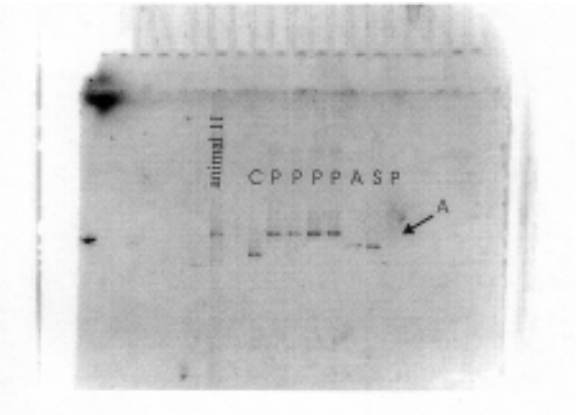

Figure 1. A DGGE gel of the whole bacterial community from lobster #1, along with individual isolates from the lesions of this animal.

Histopathological assessment. Gross examination of affected animals show moderate to deep erosions in the hard cuticle. Lesions are most common along the dorsum of the cephalothorax and abdomen, but in severe cases may extend to the claws and lateral and ventral hard carapace. Early lesions appear symmetrically, but further work needs to be done to verify this. Deeper lesions are often brown to black and result in softened carapace tissue overlying internal connective tissues of the lobster.

Histopathologically, carapace erosions are of variable depth; but deep, extensive erosions are common. Early erosions into the epicuticle and exocuticle show invasion primarily around the carapace pores and vertical extension deep into the exocuticle. These erosive areas are melanized. As the lesions deepen, and the exocuticle is eroded, they leave behind cuticular matrix that forms skeletonal pillars in the holes (Figure 2). This contrasts with impoundment shell disease, where erosions do not routinely occur as thin vertical erosions into the carapace, but rather appear as progressive areas of scooped out cuticle that leave no cuticular matrix behind.

More severe lesions show erosions that extend deeper into the calcified and uncalcified endocuticle. Melanization of the affected tissue is diffuse and in the deeper lesion the pillars of cuticle break off. Variable but often extensive layers of new uncalcified carapace (endocuticle/membranous layer) are produced by the intact hyperplastic epithelium underling the eroded site. This mechanism appears to prevent eventual ulceration into the underlying soft tissues of the animals body that could result from progressively deepening erosions.

Inflammation in epizootic shell disease is composed of increased numbers of mixed populations of hemocyte types in the underlying connective tissues and accumulations of usually necrotic hemocytes between layers of carapace. Inflammation, and cuticular proliferation, as well as melanization, of the affected cuticle are also seen in impoundment shell disease and are general responses to erosion of the cuticle for any reason.

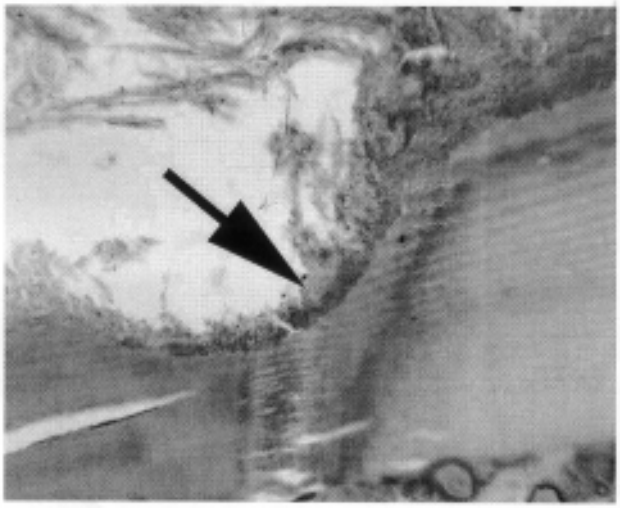

Figure 2. Early erosions into the epicuticle and exocuticle show invasion primarily around the carapace pores and vertical extension deep into the exocuticle. These erosive areas are melanized. As the lesions deepen, and the exocuticle is eroded, they leave behind cuticular matrix that forms skeletonal pillars in the holes.

Ulceration, focal loss of all carapace and invasion into underlying lobster connective tissues is very rare in tissues examined to date. Once ulceration occurs, intense inflammatory reaction produces a melanized pseudomembrane that covers the lesions. These foci can inhibit molting by causing attachments between old and new carapace.

No other disease has consistantly been identified to date in animals affected by epizootic shell disease (determined by examination of other body tissues), thus indicating epizootic shell disease is not secondary to some other primary disease (such as parameoba infections or gaffkemia).

Various organisms are identified in the shell erosions. Nematodes, sponge, algae and ciliates are occasionally seen. An as yet unidentified smaller protozoan is often seen in lesions and may be secondarily important in lesion development. But, by far the predominate organisms found at the interface of necrotic and live shell in both shallow and deep erosions into the cuticle are bacteria. Gram staining shows Gram negative bacteria in this position.

Infection experiments. A series of infection experiments has been commenced at the Flax Pond Marine Laboratory. Groups of healthy lobsters (5 individual each) were exposed to isolated *P. gracilis* and individual *Cytophaga* strains (10^6 cells of each per liter of seawater) for 24 hours. The carapace of two out of the five lobsters in each experiment was mechanically breached. To elucidate transmittance of shell disease, in a separate experiment, four healthy lobsters (epicuticle of two of them was mechanically damaged) are kept in the same tank with two lobsters with shell disease. No transmission of infection occurred after 6 month of incubations.

Calcinosis in LIS Lobsters During Summer 2002

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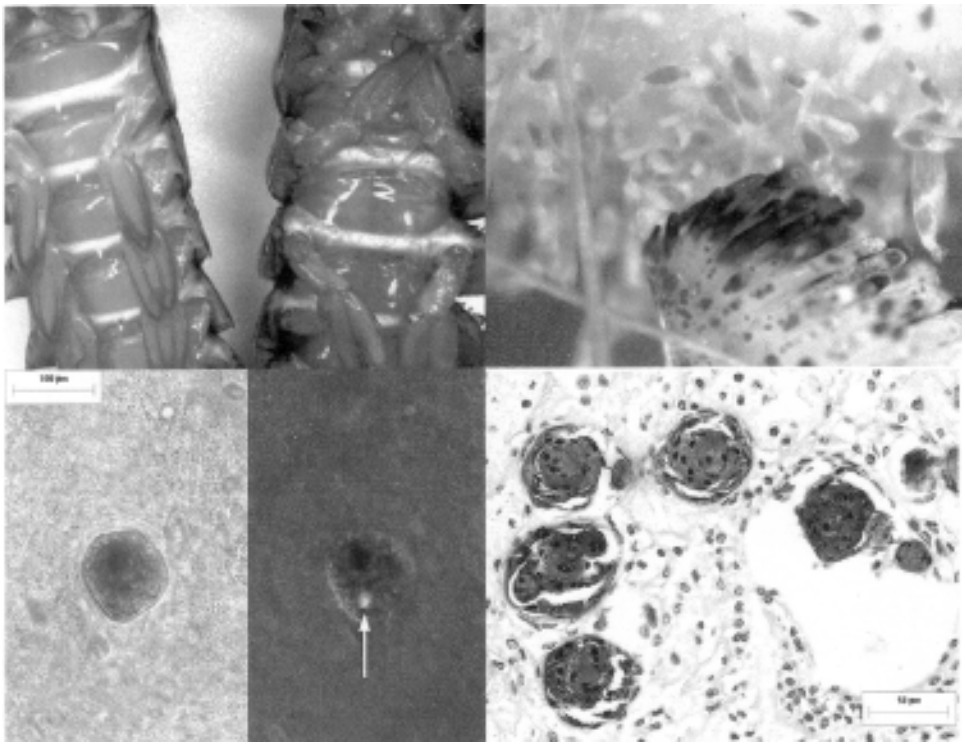
A significant number of moribund and dead lobsters were reported to state authorities by lobster fishers in Long Island Sound, New York, during the summer of 2002. The first of these were reported by a lobster fisher operating out of Mattituck who reported them to Cornell Cooperative Extension. Samples from this fisher were submitted to the new Marine Disease Laboratory at SUNY Stony Brook where they were necropsied and examined for infectious and other diseases. Morbid lobsters were characterized by an orange discoloration of the abdomen, lethargy, an excess of epibionts and poor post-capture survival. Most affected lobsters were in late intermolt or premolt stages. On necropsy, severe extensive multifocal or diffuse granulomatous inflammation of the gills and antennal glands was the most striking pathology. In the gills, granulomas were frequently seen to be embolised in filaments, resulting in congestion, ischemia (circulatory blockage) and coagulative necrosis of gill tissues. In the antennal glands, granulomas were concentrated along the border between the filtration and resorption zones of the organ. Affected lobsters lacked observable reserve inclusion cells (energy storage cells) and thus appeared to be either malnourished or metabolically exhausted (we suspect the latter). No significant pathogens were recovered from diseased individuals, suggesting that the disease is of metabolic origin. In prechronic individuals, however, it was evident that granulomas were focused around calcium carbonate (aragonite) crystals. Aragonite crystals were identified by their spheroid shape, radial striations, clear to golden brown coloration and strong birefringence. In early stage individuals, naked aragonite crystals were observed, whereas in later stage individuals, aragonite crystals were observed to be at the centre of granulomas. In most cases, the granulomas had continued to mineralize in an amorphous fashion.

As far as we are aware this is the first report of such a disease in lobsters. While microgranulomas of the type we observed are not unusual in lobsters, the number observed in our study, their focus around inorganic mineral crystals and their potential to cause what appear to be fatal pathologies in the gills and antennal glands has not been reported previously.

While it is not yet clear why this disease occurs, our best hypothesis of etiology is as follows. Calcinosis is probably caused by anomalously high sea bottom temperatures in Long Island Sound (~23 ºC) during the summer of 2002 and associated disruptions of the calcium chemistry of lobsters in favor of deposition of calcium minerals in soft tissues. We hypothesise that temperature-related respiratory stress results in hypercapnia (excess CO_2 in the hemolymph). Some of this CO_2 enters to carbonate/bicarbonate hemolymph buffering system of the lobster and becomes available as anionic conjugates for circulating calcium ions. We suspect that the excess anions combined with high hemolymph calcium concentrations during some phases of the molt cycle push the reaction in favor of deposition of calcium as crystalline $CaCO_3$ (aragonite). The concentration of the calculi in the two main filtration and excretion organs of the lobster to the exclusion of most other tissues suggests that the crystals form in the hemolymph and are subsequently filtered out in the antennal gland and (later) the gills, where they lodge and become the focus of granulomatous inflammation. Exactly why the calculi should elicit an inflammatory response is not clear, given that calcium carbonate is often exposed to lobster tissues in the form of the exoskeleton and gastroliths; this phenomenon may represent a tissue specific reaction since neither the gills nor the antennal glands would be exposed to crystalline aragonite in a healthy lobster. We suspect that under appropriate conditions the disease represents a fatal positive feedback loop in which continuing obstruction of the gills reduces the lobsters' ability to rid itself of CO_2, thus increasing the hypercapnia and favoring the disease progression. There may be thresholds of



***Top Left**: Healthy (L) and calcinotic (R) lobster abdomens showing distinctive orange colour in affected lobster.* ***Top Right**: gill tissue with multiple granulomatous lesions (brown areas) and characteristic abundance of epizootic colonial bryozoan Triticella sp.* ***Bottom Left**: brightfield (L) and darkfield (R) images of the same granuloma resolving crystalline aragonite nucleus (arrow) under darkfield illumination.* ***Bottom Right**: Histological section of mineralized granulomas in antennal gland tissues.*

temperature and hypoxia that trigger the disease state, and we suspect that even moderate hypoxia may be clinically significant for calcinotic lobsters at hypermetabolic temperatures.

The ultimate cause of death for affected lobsters is probably respiratory failure due to reduced effective surface area, exacerbated by the effects of hyperthermia on metabolic rate and an excess of epibionts. High amplitude climate cycles or permanent climatic change may play a role in the emergence of this new fatal disease of lobsters.

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