Long Island Sound Lobster Health Symposium

Proceedings of the Long Island Sound Lobster Health Symposium
November 29 - 30 2001

NOAA
NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION
U.S. DEPARTMENT OF COMMERCE

Sea Grant

CONNECTICUT DEPARTMENT OF ENVIRONMENTAL PROTECTION

Second Annual

LONG ISLAND SOUND LOBSTER HEALTH SYMPOSIUM

RESEARCH SUMMARIES

HOLIDAY INN
Ronkonkoma, New York
November 2001

Hosted by the Sea Grant Programs in New York & Connecticut under the auspices of Atlantic States Marine Fisheries Commission (ASMFC) Steering Committee for Lobster Disease Research

For additional information contact:

New York Sea Grant Extension, 3059 Sound Avenue, Riverhead, NY 11901,
Telephone: 631.727.3910, Fax: 631.369.5944

Connecticut Sea Grant, 1080 Shennecossett Road, Groton, CT 06340,
Telephone: 860.405.9127, Fax: 860.405.9109

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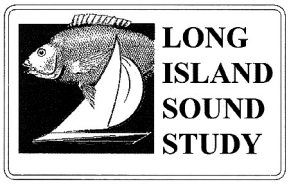
Acknowledgements

The chairman of the Lobster Steering Committee wishes to thank all researchers and staff in NOAA Sea Grant — Connecticut, New York and National Office, NOAA National Marine Fisheries Service, Connecticut Department of Environmental Protection, New York State Department of Environmental Conservation, U.S. EPA — Long Island Sound Office, Atlantic States Marine Fisheries Commission, and Messrs. Nick Crismale and Joseph Finke, who represent Long Island Sound lobster fishing industry. The hard work and dedication of these individuals made this meeting possible.

Long Island Lobster Research Initiative is a collaboration funded by National Oceanic and Atmospheric Administration (NOAA) National Marine Fisheries Service, Connecticut Department of Environmental Protection, NOAA Sea Grant — Connecticut, New York and National Office.



Design and Production by
New York Sea Grant Communications
121 Discovery Hall
SUNY Stony Brook
Stony Brook, NY 11794-5001
www.nyseagrant.org

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Assessment and monitoring of the American lobster resource and fishery in Long Island Sound

David Simpson
Marine Fisheries Division, Connecticut Department of Environmental Protection

Key Words: American lobster, habitat, genetic stock identification, tagging, abundance, distribution

Objectives

This project comprises six jobs that address the need for expanded sea-sampling and trawl survey monitoring, sample collections for researchers, stock movements and identification and habitat use. The goal and general approach for each job is summarized below.

Task 1

Expanded sea-sampling of the commercial lobster-pot fishery in Long Island Sound, to accurately characterize the commercial lobster fishery for stock assessment and management purposes by better estimating catch composition in terms of size, sex, egg-bearing status, incidence of shell disease or damage, and general physical condition; and to assist lobster researchers by providing lobsters for laboratory and field tagging studies. Connecticut Dept. of Environmental Protection (CT DEP) has conducted a sea-sampling program in the commercial lobster fishery since 1984. However, sampling was limited to peak periods in the fishery. The expanded sampling program will enable complete seasonal coverage and enhance spatial coverage as well (Table 1).

Season	**Week 1-16 (01/01-05/4)**	**Week 17-28 (05/04-07/27)**	**Week 29-34 (07/28-09/07)**	**Week 35-42 (09/08-11/02)**	**Week 43-52 (11/03-12/31)**
Samples/Area	2ELIS 2CLIS 2WLIS	4ELIS 4CLIS 4WLIS	6ELIS 6CLIS 6WLIS	2ELIS 2CLIS 2WLIS	4ELIS 4CLIS 4WLIS
Samples/Season	6	12	18	6	12

Table 1. Number of planned sea-sampling trips from Connecticut ports by area (eastern, central, western LIS) and season. A total of 54 trips are planned annually for two years.

Task 2

Expansion of CT DEP Long Island Sound Trawl Survey, an ongoing fishery-independent monitoring program for lobsters and other living resources, to include the waters between Norwalk, CT and Hempstead, NY (waters known as the Narrows). The Long Island Sound Trawl Survey has been ongoing since 1984, and it is conducted during the spring and fall from Groton to Norwalk, CT. From 1986-1990 limited sampling also was done west of Norwalk, generally in the area of Hempstead Harbor, NY to investigate the effects of hypoxia on finfish and crustaceans in that area. Due to the lobster die-offs, this has again become an important issue in this area. In addition to monitoring lobster abundance and condition, the general health of the area will be monitored in terms of abundance and diversity of finfish and crustaceans. Other areas of the Sound with similar depth and substrate characteristics will be used as reference sites.

Task 3

Coordinate activities to ensure that lobster samples can be collected, preserved, packaged, and delivered to state and Sea Grant researchers in an efficient manner. Researchers requiring specimens for laboratory research are encouraged to submit their requests to CT DEP staff. Samples will be provided from commercial sea-sampling trips or the trawl survey.

Task 4

Conduct a lobster tagging study to characterize movement and migration to better understand seasonal variation, population abundance, habitat preference, and response to environmental conditions. This activity also will determine population response to large-scale mortalities in terms of the repopulation of areas significantly affected by lobster mortalities. Between 5,000 to 7,500 lobsters, 60 mm and larger, will be tagged in each of the first two years of this three-year program (Table 2). Connecticut and New York fishermen received a package describing the program and they were asked to provide information on recaptured lobsters including size, sex, shell condition and egg-bearing status. Interval measuring gauges were supplied to all Connecticut commercial fishing license holders to collect growth information. Two tag colors are being used; white tags will be removed upon recapture and returned to CT DEP. One hundred white tags will be deployed each year, each carrying a $100 reward. Orange tags carry a $5 reward and fishermen must record the tag number and specified data, before releasing the lobster with the tag in place. To enhance the return rate, a lottery is used to randomly select 100 orange tags reported each year, and fishermen reporting such will receive a bonus. One fisherman will receive $1,000 and 99 fishermen will each receive a $100 bonus. Lobsters are being collected both from commercial fishing vessels and in the trawl survey, and are being released throughout the Sound, among five east-west zones. Tagging began in August 2001 and more than 3,000 lobsters have been tagged and released through October 2001.

Sex	Egg-Bearing Status	Size Group	Zone 1	Zone 2	Zone 3	Zone 4	Zone 5
Male	**N/A**	**Pre-recruit**	100-200	100-200	100-200	100-200	100-200
		Recruits	100-200	100-200	100-200	100-200	100-200
		Full recruits	100-200	100-200	100-200	100-200	100-200
Female	**No Eggs**	**Pre-recruit**	100-200	100-200	100-200	100-200	100-200
		Recruits	100-200	100-200	100-200	100-200	100-200
		Full recruits	100-200	100-200	100-200	100-200	100-200
	Egg-Bearing	**Pre-recruit**	100-200	100-200	100-200	100-200	100-200
		Recruits	100-200	100-200	100-200	100-200	100-200
		Full recruits	100-200	100-200	100-200	100-200	100-200

Table 2**.** Target range of lobsters to be tagged each year by geographic zone, sex, egg-bearing status and size group (pre-recruits 60 to <72 mm, recruits 72 to <82.6 mm, full recruits 82.6+ mm). A total of 5,000 lobsters will be tagged each year. Lobsters will be tagged on an opportunistic basis during the spring and fall trawl surveys and during commercial sea-sampling trips throughout the fishing season.

Task 5

Undertake stock identification and origin of lobster larvae in Long Island Sound using microsatellite markers to determine if there are functionally distinct lobster stocks in Long Island Sound; and determine the potential for interchange between lobsters of different regions within the Sound and from outside the Sound through larval drift. In year one, 100-150 berried females will be collected from each of four areas (western basin, central basin, eastern basin and offshore) to extract tissue samples (from walking legs) to be used to genetically characterize the parent stock in each area. In year two, larvae will be collected from these four areas for genetic comparison to the parent stock in order to determine whether genetically distinct breeding stocks exist within the Sound or between the Sound and offshore waters. Analysis and a final report will be completed in the third year of the project. Field collections will be made by CT DEP (central and western basins), Millstone Environmental Lab (eastern basin) and RI DEM (offshore). Dr. Joseph Crivello, University of Connecticut, is the principal investigator on this project and he will oversee laboratory work and analysis.

Task 6

Conduct spatial analysis of lobster population characteristics in Long Island Sound in relation to habitat structure and distribution to understand the variation in these spatial characteristics. Also, address basic questions arising from the recent die-off including the possible role of environmental stressors such as hypoxia, and sediment contamination; and assess how population spatial structure may affect the ability of this resource to recover from the die-off. The general approach includes the development of a GIS for the project, comprising relevant environmental and population data layers; these data will be used in a series of GIS-based statistical analyses. Dr. Roman Zajac of the University of New Haven will be contracted to develop the GIS layers and conduct these analyses in consultation with CT DEP Marine Fisheries staff. Dr. Zajac will acquire existing environmental datasets to characterize sea floor habitats (e.g., bottom temperature and dissolved oxygen, sediment type, depth) and assemble them as GIS data-layers. GIS data-layers also will be developed using LIS Trawl Survey data that depict lobster population characteristics at the survey locations (e.g. abundance, size, sex, egg-bearing status and shell condition). Using the analytical capabilities of GIS in conjunction with statistical analyses, we will either assess or characterize:

1. How lobster population characteristics differ among habitats in LIS
2. The extent to which spatial differences change over time
3. The existence of population "hot spots"
4. The extent to which there is spatial correlation in the distribution of potential stressors (e.g. low dissolved oxygen)
5. The spatial distributions and habitat responses of other macro-invertebrates (e.g. horseshoe crab, blue crab, rock crab) in the same fashion, and assess how they compare to those exhibited by lobsters.

Distribution, movement, and health of American lobster (*Homarus americanus*) in Long Island Sound, with emphasis on western Long Island Sound

Carl LoBue
Division of Fish, Wildlife, and Marine Resources, New York State Department of Environmental Conservation

Key Words: Lobster, Monitoring, Assessment, Long Island Sound, young-of-the-year, stock, population

Introduction

Over the last three decades, the New York State Department of Environmental Conservation (NYS DEC) has studied and described many aspects of its local lobster population and its commercial lobster fishery. The lobster fishery has been the most valuable commercial fishery in the region. Much of this work involved collecting data from commercial catch while at sea. The commercial lobster sea-sampling project was expanded significantly in 1999 compared to the previous four years. However this program was still not extensive enough to document precisely where, when, and how many lobsters were affected by the mass mortalities that occurred in the fall of 1999. Reliance on the commercial fleet proved problematic when catches could no longer sustain commercial fishing activities.

This study will combine fishery-independent and fishery-dependent sampling to describe the existing population of lobsters in western Long Island Sound (WLIS), document the commercial lobstering activities throughout the entire LIS, and develop methods to properly index the recruitment strength of young-of-the-year (YOY) lobsters each year.

Objectives

This study is comprised of three general tasks. Task 1 is to conduct an intensive three-year survey of WLIS using standard and modified commercial lobster trap gear. Task 2 is to expand (in terms of the number of sampling trips and the areas covered) the scope of NYS DEC at-sea observer program. Task 3 is to establish a YOY lobster-settlement index. The following is a list of the objectives for each task.

Task 1

1. Utilize a fishery-independent sampling design that will provide a map of where and when lobsters occur in the WLIS. Short-term changes in abundance can be tracked spatially and temporally and linked with environmental data.
2. Provide short-term indices of abundance for other species that are caught in commercial lobster pot gear. Seasonal changes in the abundance of these animals also may provide evidence of spatially seasonal unfavorable environmental conditions.
3. Estimate how often lobsters re-enter lobster trap gear by marking every lobster that is captured.
4. Document the movement of lobsters throughout the survey area by keeping track of multiple recaptures of marked animals.

Task 2

1. Increase the areal coverage and sampling frequency of New York's lobster sea-sampling program to improve the statistical precision of the catch and harvest data used to estimate western and central LIS landings, reported in pounds, to numbers of male and female lobsters. This is an essential component of many stock assessment models.
2. Maintain a presence in central and western LIS to document the incidence and severity of the shell disease syndrome that is affecting lobsters throughout many areas of southern New England. Track changes in these parameters within and among years.

Task 3

1. Establish a time series of the settlement strength of YOY lobsters in several locations in Long Island Sound.
2. Compare the rate of settlement of YOY lobsters in LIS by location and strata (depth and substrate type).
3. Design a cost-effective long-term YOY lobster settlement survey that can be continued after these project funds are exhausted.

Methods

Task 1: WLIS trap survey

A trap survey will be conducted throughout western Long Island Sound. The basic design of the survey will be to fish 25 five-trap strings (trawls) of lobster traps in three areas of WLIS (Figure 1). Each trawl will contain four standardized commercial style lobster traps and one small-mesh trap without escape vents. Traps will be fished from June through December each year (2002-2004). Stations (location of a string of lobster traps) will be arranged in seven transects that cross Long Island Sound (Figure 1).

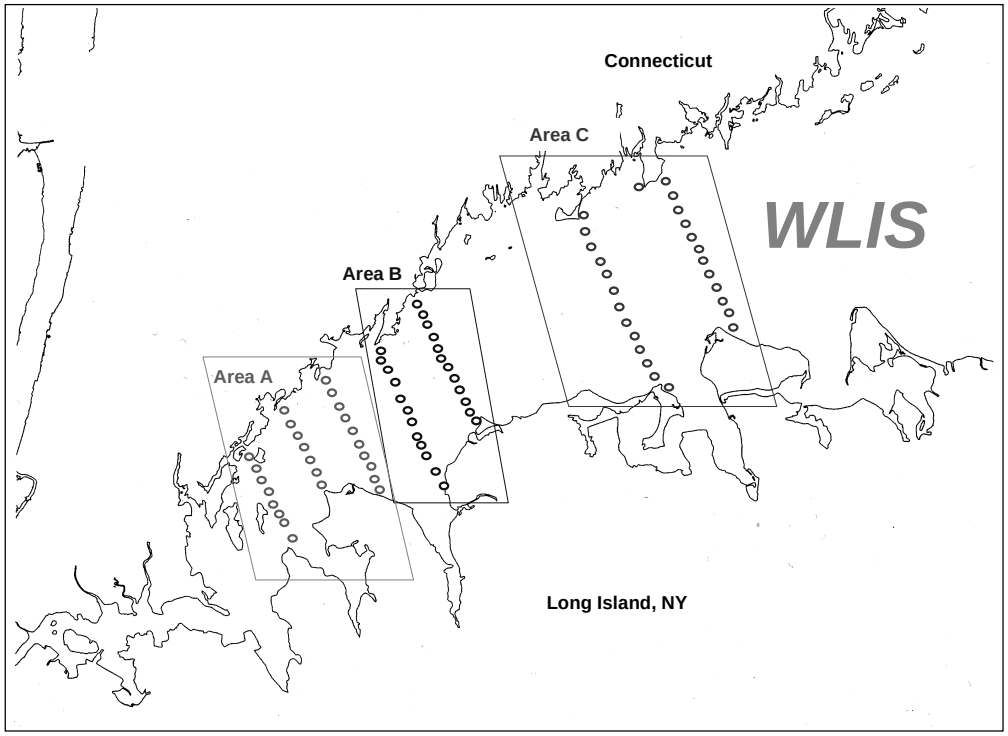

Figure 1: Approximate locations of gear for the WLIS lobster trap survey. Gear is arranged in three areas, each containing 25-five trap trawls,

One remote temperature sensor will be placed in each trawl. Temperature data will be downloaded from the sensors approximately every six to eight weeks. In addition, a temperature, salinity and dissolved oxygen reading also will be taken at the surface, and from approximately one meter above the bottom each week when hauling gear. All lobsters caught in each trap will be measured and sexed. All the released lobsters will be banded around the arm of their ripper claw (not around the claw itself) with a serially numbered plastic truck seal. Marking every lobster that is released will allow us to track short-term movements among many animals. It will also allow us to estimate re-capture frequency.

Task 2: Fishery-dependent sea sampling

The areal coverage and sampling frequency of the commercial lobster sea-sampling program in New York State will be increased. The methods used for this phase of monitoring will be identical to methods currently used by NYS DEC and CT DEP while aboard commercial lobster vessels (see LoBue *et al.* 2001).

Task 3: Establish a YOY lobster settlement index for LIS

This task will use two field techniques conducted simultaneously. They will consist of deploying YOY lobster-settlement collectors and utilizing the suction sampling techniques of Incze and Wahle (1991). Both methods have been used in Maine and Rhode Island. Collectors will be set in groups of 15-20 at each of eight locations in LIS. They will be deployed in late May or early June and retrieved in late September or October. Diver- and collector-generated indices will be compared by area and substrate type.

Expected Outputs

All the data collected will be disclosed to all interested parties in a timely fashion. Reports will be prepared according to the NMFS reporting requirements for federally-funded projects. It is expected that this body of work will result in peer-reviewed publications to be presented at professional meetings. When appropriate, data will be entered into GIS software to map lobster abundance in relation to the other environmental variables that are being collected as part of this study and variables that are available from other studies.

Task 1 will be used to describe the abundance and distribution of lobsters in western LIS. Recently, this area has been difficult to sample in the fishery-dependent work because much of the area no longer supports a viable commercial fishery. Additionally, the CT DEP trawl survey has been unable traditionally to fish in this area because of numerous obstructions to trawl gear. This phase of the project is expected to produce maps of the distribution of lobsters throughout this area. The use of small-mesh ventless traps should allow us to document the abundance of lobsters that are smaller than those typically caught in commercial trap gear. By marking each animal, and tagging some animals with sphyrion tags, we should be able to estimate the number of animals that are continually re-captured and track lobsters as they move through the study area. This information will be compared to dissolved oxygen, temperature, and salinity data collected at each site. Comparing the environmental data to the distribution of lobsters may help to identify areas with poor habitat quality for lobsters.

The expanded sea-sampling program hinges in part on the continued cooperation of commercial lobstermen. The success of the objectives of this task can be evaluated by comparing the number of sampling trips to the number of trips outlined in this proposal. Data collected by at-sea observers is the only detailed information available on the composition of the entire commercial catch. This information will be used to translate the LIS landing estimates into a format useable in stock assessment models.

Currently, the preferred areas for YOY lobster settlement in LIS are poorly understood. Several studies in other areas suggest that YOY lobsters prefer to settle in cobble habitat (Incze and Wahle, 1991; Wahle and Steneck, 1991; Cobb and Wahle, 1994), however, central and western LIS has relatively little cobble (Poppe *et al*., 2000). Understanding which habitats are most important to newly settling YOY lobsters in LIS is critical to properly evaluate the ecological impacts of underwater construction projects and alternative dredge material disposal locations. At the end of this three-year experimental study, there will be comparisons of the abundance of YOY lobsters in several locations and habitat types within LIS. Similarity in methodology will allow comparisons with other YOY lobster surveys in the Gulf of Maine and inshore Rhode Island.

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Exposure of lobsters to the varied chemical and biological environment of Long Island Sound

Andrew F.J. Draxler
Marine Chemistry Branch, Howard Laboratory, NOAA/NMFS Northeast Fisheries Science Center

Anthony Paulson[1] **and Ashok Deshpande**[1]
[1]Marine Chemistry Branch, Howard Laboratory, NOAA/NMFS Northeast Fisheries Science Center

Key Words: environmental stress, hypoxia, sulfide, lobster disease

Introduction

The scientific evidence suggests that the lobster die-off in western Long Island Sound was caused by biological infection, probably intensified by environmental stressors (extended periods of high temperature, low dissolved oxygen, and high concentrations of hydrogen sulfide and ammonia) and anthropogenic factors (sewage treatment discharges, dredge disposal, endocrine disrupting compounds, overall contamination of Long Island Sound sediments by a variety of metals and chlorinated hydrocarbons, and pesticides for mosquito control). This field experiment will attempt to relate lobster health to both ambient water and sediment quality conditions in Long Island Sound and determine the importance of each in influencing the health of these lobsters.

Objectives

1. To determine the relationships between lobster health and environmental factors (water and sediment quality) under ambient Long Island Sound conditions.
2. To determine levels of Mn accumulation in lobster gills that can be used as an indicator of exposure to natural biogeochemicals, such as hydrogen sulfide and ammonia.

Methodology

- Deploy lobsters in cages for up to 45 days at nine stations exhibiting varying water and sediment quality conditions during the summers of 2001 and 2002, while measuring sediment quality and temporal changes in water quality along the axis of LIS from Hewlett Point to Stratford Shoals between July 1 and Aug 15 of 2001 and 2002, the expected period of minimum dissolved oxygen.
- Determine the effects of anthropogenic chemicals by pairing near-shore, muddy-bottom stations with the sandy-bottom stations.
- Determine the health of lobsters as determined by survival, growth, lipid distribution, accumulation of metals in gills, accumulation of chlorinated hydrocarbons in hepatopancreas, and biological infections and examine the relationships with environmental exposure.

Expected Outputs

This study will determine whether bioaccumulation of natural biogeochemicals in LIS lobsters is related to their exposure from the interstitial waters of sediments. It will also help to determine whether uptake of environmental chemicals and microbiota are associated with measurable infection and pathology. The results of this work will contribute essential information, which, together with the work of others, may reveal the probable cause(s) of the mass mortality of lobsters. The results of the project will be reported at a Lobster Health Symposium, in a National Marine Fisheries Service Technical Memorandum (archived at the National Technological Information Service), and in peer-reviewed scientific journals.

Effects of temperature and body size on metabolic stress in Long Island Sound lobsters

Glenn Lopez
Marine Sciences Research Center, Stony Brook University, NY

Robert M. Cerrato
Marine Sciences Research Center, Stony Brook University, NY

Key Words: Lobster, temperature stress, body size

Introduction

The biological range of lobsters strongly suggests that LIS lobsters are susceptible to high temperature stress in the late summer and early fall. The primary goal of our study is, therefore, to test the hypothesis that high summer temperature in the bottom-waters of LIS causes a bioenergetic deficit in lobsters. In addition, we expect that the effect of high temperature will be size-specific. That is, because the allometric relationships between feeding rate and metabolism vary with body size, large lobsters will be more susceptible to temperature stress than small lobsters.

The proposed study will help scientists and resource managers understand both normal patterns of stress and mortality of lobsters in LIS, and shed light on the extraordinary mortality event of late summer/fall of 1999. The results of this study will also be useful in predicting the effects of long-term changes in summer temperatures on the health of LIS's lobster population. Slight changes in maximum bottom-water temperature, and duration of maximum temperature, will have major impacts on the population. These predictions will be useful for fishery resource managers and lobstermen in making management and investment decisions. Finally, understanding the response of lobsters to temperature, a natural environmental variable, is necessary in teasing apart the complex effects that anthropogenic influences may have. The 1999 mortality event may have been caused by the confluence of several factors, and no single study will unravel these factors. But timing of this event, together with the biogeographic distribution of lobsters, strongly implicates summer temperature maxima as an important factor to investigate.

Objectives

1. Determine effect of high temperature on bioenergetic deficit and production of stress proteins in the lobster, *Homarus americanus*.
2. Determine the role of body size in the effects of temperature in lobsters.

Methodology

1. Conduct laboratory experiments to quantify the relationship between energy balance, stress proteins, body size, and temperature for LIS lobsters.
2. Undertake a field study to search for evidence of temperature stress by examining the pattern of stress proteins in LIS lobsters.
3. Develop a model to predict the bioenergetics of LIS lobsters with respect to temperature and body size.

Progress

Most of our initial efforts have focused on acquiring equipment and supplies needed for the temperature stress experiments and for Western blot analysis. We changed the design of the thermal stress setup to avoid pseudoreplication. This improved setup is almost completed, and we will begin preliminary trials to determine thermal stability. We acquired all equipment and most of the reagents needed for conducting Western blot analysis of stress proteins. We will run initial gels to test our approach.

Environmental change in Long Island Sound in the recent past: eutrophication and climate change

J.C. Varekamp
Earth & Environmental Sciences, Wesleyan University

Ellen Thomas[1], Mark Altabet[2], Sherri Cooper[3], and Marilyn Buchholtz ten Brink[4]
[1]Dept. of Earth Environmental Sciences, Wesleyan University; [2]Dept. of Chemistry & Biochemistry, University of Massachusetts; [3]Biology Dept.,Byrn Athyn College; [4]U.S. Geological Survey, Coastal Marine Program.

Key words: eutrophication, paleoclimate, stable isotopes, paleontology, paleohydrology.

Introduction
The recent lobster die-off in Long Island Sound (LIS) probably has a variety of causes, which may include severe hypoxia, pollution by metals and organic substances, temperature change in the bottom waters as well as biological factors such as the introduction of new parasites and diseases. The research of our group will determine several environmental factors in modern LIS bottom environments and variations in these parameters over the recent past (last 50-100 years).

Objectives
Possible environmental causes that may have contributed to lobster die-off include eutrophication, metal pollution, insecticide spraying, and regional or global climate change. We will develop a detailed time-line of environmental changes in LIS, and its linkages to diatoms populations primary producers) and benthic foraminifera (bottom-dwelling organisms that are low in the food chain). Ultimately, we will address the question if the lobster die-off is more a result of global or regional climate change (temperature, precipitation with related water column stratification intensity) or can be tied in to environmental changes related to local causes such as the nutrient/eutrophication dynamics.

Methods
As part of this study, we have collected during the first week of November (2001), thirteen new gravity sediment cores in central and western LIS, up to a length of 220 cm. We have focused our attention to areas of high sediment accumulation rate, as indicated by our earlier studies of short cores, and the extreme western end of LIS, where most of the lobster die-off occurred. We will be measuring a set of parameters in sample slices of all these cores (Table 1).

Our current data deal with the calibration of our methods, such as maps of diatoms and benthic forams, pollutants and stable isotope values in surface samples from LIS. The combination of Ca/Mg and Sr/Ca thermometry with $\delta^{18}O$ has led to a method to reconstruct average water temperature and salinity from carbonate samples in surface samples, which is compared with observed water column data. Data from short cores in western LIS allowed us to compile a history that goes back in some cores more than 1000 years.

From 1200 AD until about 1700, apparent salinities (salinities calculated at constant temperature) increased, which we tentatively correlate with the transition from the Medieval Warm Period to the Little Ice Age. The apparent salinities decreased again somewhat over the last 300 years. The last 100 years was studied in more detail and shows substantial variations in salinity, which can be correlated with Connecticut's precipitation record (hurricane of 1938, wet periods of 1955, 1975 and early 1990's). The records of organic carbon and nitrogen in these cores show strong increases over the last 150 years, while the isotopic composition of nitrogen evolved towards heavier values. Mercury profiles correlate closely with *Clostridium perfringens* abundances, indicating that the Hg contamination is an anthropogenic signal. The $\delta^{13}C$ core records are complex, but show that the core

tops were deposited from waters that had seen a substantial amount of oxidation of organic matter. The latter suggests that the rate of carbon oxidation has increased over the last 40-50 years compared to the earlier centuries. The fine structure in $\delta^{13}C$ in the core tops has not yet been resolved in detail but it appears that the periods with low salinity coincide with very light carbon in the carbonate shells. After correction for the salinity effect on $\delta^{13}C$, these "dilute periods" still stand out as having light carbon, suggesting that intense stratification occurred in the western Sound during these high run-off periods.

PARAMETER	**INDICATOR**	**PI**[1]
Benthic foram assemblages in cores	Water oxygenation, food sources, temperature/salinity, turbidity	ET
Diatoms in cores	Water temperature/salinity, turbidity	SC
Metal Pollutants: Cu, Zn, Cr, Pb, Ni, As, Cd	Are toxic levels reached?	MB/TB
Clostridium perfringens	Indicator for sewage inputs into LIS	MB/TB
Sedimentology: grain size, major elements	Support-data for interpretation	MB/TB
Dating: ^{210}Pb, ^{137}Cs	Develop age models for cores	MB/TB
Metal Pollutants: Hg	Are toxic levels reached?	JCV
$\delta^{18}O$, Ca-Sr-Mg in carbonates	Reconstruct salinity/temperature history	JCV
$\delta^{13}C$ in carbonates	Reconstruct water oxygenation history	JCV
Biogenic silica in cores	Construct time-line of diatom productivity	JCV
Nitrogen concentrations in core samples	Construct time-line of nutrient inputs	MA
Nitrogen isotope ratios in core samples	Distinguish changes in nutrient sources over time	MA

Table 1. Parameters to be measured on core samples from LIS [1]Key: J.C. Varekamp (JCV); E. Thomas (ET); M. Altabet (MA); S. Cooper (SC); M. Buchholtz ten Brink (MB)

The foraminiferal studies have shown significant changes in the Sound over the last 50 years, with the disappearance of one species and the emergence over the last 10 years of a new species (*Ammonia beccari*). The latter is common in warmer waters elsewhere in the world and may indicate the influence of changes in temperature, but also may reflect changes in the local food chain with the inputs of large amounts of sewage (as indicated by the *C. perfringens* counts). The only other part of the LIS cores where *A. beccari* occurs in modest abundance are those deposited during the Medieval Warm Period (100-1200 AD).

The biogenic silica studies have just started and we have found biogenic silica levels on the order of 2-4 %. The methodology is still being developed because ~90% of LIS sediment consists of silicate material. We are optimizing existing techniques for the specific challenges of LIS sediments.

Relationship between American lobster mortality in Long Island Sound and prevailing water column conditions

Robert E. Wilson
Marine Sciences Research Center, Stony Brook University, SUNY

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

[1]Institute for Terrestrial and Planetary Atmospheres, Stony Brook University, SUNY; [2]Marine Sciences Research Center, Stony Brook University, SUNY

Key Words: temperature anomaly, dissolved oxygen, meteorological forcing

Background

The Long Island Sound lobster population has been dramatically affected by a mortality event whose cause remains undetermined. Although considered hardy, American lobsters are vulnerable to stress and sometimes mortality result when they are exposed to unfavorable environmental conditions. Environmental factors can act singly or in combination to cause sublethal stress that increases sensitivity to events that would normally be tolerated (Mercaldo-Allen and Kuropat, 1994). Particularly, the synergistic effect of water chemistry factors such as temperature, salinity and dissolved oxygen (as well as pollutants) could reduce water quality and detrimentally affect lobsters.

Laboratory studies by McLeese (1956) addressed tolerance limits for the American lobster under certain environmental conditions. McLeese examined how the interaction of temperature, salinity and dissolved oxygen affects survival of lobsters. He observed that the lethal limit for each parameter is related to the various acclimation levels of all 3 factors. When acclimation histories for lobsters are similar, lethal levels of temperature, salinity and oxygen are not influenced by size differences, variation in life-history or short-term starvation. Temperature affected lethal levels for salinity and oxygen. The McLeese paper synthesizes these data to create a diagram which can be used to assess the lethal effects of any one temperature, salinity or dissolved oxygen level with respect to the other two factors.

Lobsters may be especially susceptible to stressful environmental conditions during the molt cycle. Many metabolic demands are placed on molting crustaceans and these demands are often reflected by an elevation in oxygen consumption to support this increased metabolic activity (Penkoff and Thurberg, 1982). Physiological parameters such as heart rate and gill bailer activity increase with rising seawater temperature (Mercaldo-Allen and Thurberg, 1987). Penkoff and Thurberg (1982) report an increase in oxygen consumption rates as molting approaches, peaking just prior to the molt. Molting lobsters are less resistant to high temperatures, low salinity, and low oxygen conditions than hard shelled animals (McLeese, 1956). The timing of the LIS mortality event corresponds to seasonal molting cycles in the lobster.

Young (1973) reported an October kill of lobsters off the New Jersey coast that may have been due to an influx of water which resulted in reduced oxygen levels combined with high temperatures. Testimony of sport divers suggests the kill may have resulted from the synergistic effect of low dissolved oxygen and high temperature that approached the critical thermal point for the animal. McLeese (1964) observed that the rate of oxygen consumption by lobsters doubled over the temperature range of 12 to 25 ^{o}C and that oxygen consumption rates doubled after feeding. McLeese (1956) and McLeese and Wilder (1958) report that at optimal levels of 30 psu salinity and 6.4 mg/L dissolved oxygen, maximum lethal temperature for American lobsters is approximately 32 ^{o}C. Lower temperatures can be lethal when combined with unfavorable salinity or dissolved oxygen conditions.

For example lobsters acclimated at 15 ^{o}C, 30 psu salinity and 6.4 mg/L DO experienced mortality when exposed to temperature of 28.4 C, a salinity of 6 psu and a DO of 0.20 mg/L (McLeese, 1956).

Hypothesis

It is our hypothesis that the Long Island Sound lobster mortalities resulted from the convergence of several environmental factors. These conditions may have stressed the animals enough to result in mortality or may have weakened them to the extent that they were vulnerable to opportunistic pathogens. These observed anomalies in bottom temperature described above point to temperature. It is our contention a long-term decline in summer-time bottom water temperatures in the western Sound has contributed to a more suitable lobster habitat. However, while the general range in summer bottom temperatures is consistent with suitable lobster habitat, the Sound is subject to climatic temperature fluctuations that can be detrimental to lobsters.

Our specific hypothesis is that anomalies in water column properties including temperature, dissolved oxygen and salinity are correlated with anomalies in lobster mortality rates within the Sound. We hypothesize that covarying fluctuations in these water column properties may violate lobster habitat requirements and thereby contribute directly to an increase in lobster mortality rates. The apparently elevated salinities during the 1999 mortality events would lead us to focus primarily on covariations in temperature and dissolved oxygen.

Objectives

The overall goal of the proposed research is to test our hypothesis that anomalies in water column conditions are a causative factor for the anomalies in lobster mortality rate throughout the Sound. To achieve this goal we identified three specific objectives:

(1) Define the spatial patterns and time varying amplitudes which characterize the co-varying anomalies in water column properties T,S,DO) and we propose to identify the scenarios in meteorological/hydrological forcing responsible
(2) Interpret the temperature/dissolved oxygen/salinity 'profiles' obtained under objective (1) during mortality periods in light of the model developed by McLeese to determine the extent to which parameters approach lethal limits during the time frame
(3) Establish statistical relationships between the time variations in dominant modes characterizing the spatial patterns of water column anomalies and the time variations in patterns of lobster mortality rate anomalies.

Methods

Objective (1)

Under objective (1) we propose to use principal component analysis (PCA) to examine the spatial-temporal relationships between the variability of temperature (T), dissolved oxygen (DO), and salinity (S) in the Sound.

Objective (2)

Under objective (2) we propose to use available models of the type developed by McLeese (1956) to determine percent mortality in response to variations to T, DO and S. We propose to use this model to evaluate the possibility mortality response for PCA modes defined under objective (1). We propose to evaluate the response for an individual mode during periods when that mode dominates, and for combined modes during those periods when both must be considered.

Objective (3)

Under objective (3) we propose to develop statistical relationships between PCA time series and lobster abundance and catch per unit effort time series available from historical CTDEP trawl and catch data.

Significance

Lobsters, like other marine animals, respond to environmental conditions differently during the various stages of their life cycles. Adult lobsters are known to experience stress at elevated water temperatures and depressed DO concentrations. This is particularly true during molting. Over the long term, summer-time bottom water temperatures in western areas have declined. This is accompanied by a general decline in basin-wide summer bottom DO concentrations. The decline in bottom temperatures, in general, may extend the southern limit of the lobster's range. However, it is also true that inter-annual variability is increasing and the periodicity of the variability is decreasing. This research is intended to provide a description of the spatial patterns and time scales of variability associated with covarying anomalies in temperature and DO in the Sound.

The lobster population in the Sound may be quite unstable in the coming years and unpredictable from the point of view of fishery management. Sustainable yield estimates may need to be scaled downward. If our contention that if the lobster population in the Sound is being affected by physical environmental processes that are associated with regional climatic changes is correct, there may be little opportunity for environmental management to intervene to increase or even stabilize the population. Fishery managers may have to adopt innovative management practices to sustain the population under uncertain conditions. The results of this research should be of direct importance to agencies such as CTDEP, NYSDEC, NYCDEP and NMFS responsible for managing the resource.

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Determination of the toxicity and sublethal effects of selected pesticides on the American lobster (*Homarus americanus*)

Sylvain De Guise
Department of Pathobiology and Veterinary Science, University of Connecticut

Richard A. French[1,] **Salvatore Frasca Jr.**[1] **and Christopher Perkins**[2]
[1]Dept. of Pathobiology and Veterinary Science, University of Connecticut; [2]Environmental Research Institute, University of Connecticut

Keywords: Toxicology, immunology, malathion, resmethrin, methoprene, lobster

Introduction & Rationale

The immune system is central to health and resistance/susceptibility to pathogens in all species. Interestingly, it is also one of the most susceptible and sensitive systems for the effects of xenobiotics. It is therefore highly possible that environmental stressors (chemical or others) that would have affected the immune system of lobsters in LIS would have rendered them more susceptible to infections (for example with paramoeba) and have played a significant role in the 1999 LIS lobster die-off. It is for this reason that our group has developed methods to quantify the immune response of lobsters and incorporated this parameter in our evaluation of the health status of lobsters.

Since lobster is a highly-valued food item for human consumption, lobster tissues (especially muscle and hepatopancreas) have received particular attention. For example, chlorobiphenyls (CBs) were measured in tissues of lobsters and other marine "edible" tissue samples (seafood), to find that in some instances (such as in hepatopancreas from Boston Harbor lobsters), total and dioxin-like CBs exceeded the U.S. Food and Drug Administration (FDA) tolerance limits (2,000 ng/g for total CBs and 25 pg/g for toxic equivalents (TEQs) of dioxin-like CBs) (Ylitalo *et al*., 1999). This extensive database as well as the current literature does not include specific information on the toxicity or exposure of lobsters to malathion, resmethrin and methoprene. Although some toxicants have been measured in lobsters, their effects on the health of lobsters, including subtle modulation of immune functions, have practically not been measured.

The present proposal suggests the evaluation of the effects, upon controlled experimental exposure, of pesticides and other environmental contaminants on the immune functions and pathology of American lobsters (*Homarus americanus*) in order to determine their potential role in the 1999 die-off in LIS. Experimental exposure to individual toxicants is a direct method that will allow the evaluation of the direct toxicity (LC_{50}) of chemicals in lobsters compared to other species. Evaluation of effects on immune functions will help determine relevant sublethal effects that may have been directly involved in the die-off. This work will allow us to better understand the importance of pesticides and other contaminants in the recent lobster die-off, allowing more focus in actions to stop/solve the die-off and prevent similar events, further.

Hypothesis

Xenobiotics to which lobsters in Long Island Sound possibly have been exposed have sublethal toxic effects on lobster health that could have contributed to the recent die-off.

Objectives

1. To determine lobster LC_{50} for malathion, resmethrin and methoprene.
2. To determine the accumulation of selected xenobiotics in lobster tissues from known sublethal concentrations in water.

3. To determine the effects of sublethal exposure to xenobiotics in lobsters, including dose-response relationships, on immune functions and pathology.
4. To evaluate the risk of exposure to those xenobiotics for lobsters.

Methods

Experimental exposure of lobsters to known xenobiotics and testing of immune functions, determination of tissue concentrations of xenobiotics accumulated, and pathological examination.

Expected Outputs

The proposed study will address several issues relative to the potential contribution of toxic chemicals on the recent lobster die-off in LIS. First, we will examine the lobster's ability to absorb and accumulate different toxicants from the water column, and the distribution of such chemicals in different tissues. Second, this study will examine the sublethal acute, subacute and chronic effects of such chemicals on the health of lobsters. The immune system is particularly relevant for its central role in susceptibility to pathogens. The determination of dose-response relationships will help in the evaluation of the risk associated to exposure to those toxicants in the environment. This will be done in conjunction with an ongoing study to determine the concentrations of pesticides in the water column and resulting exposure of lobsters after spraying events. Taken together, those results will help determine the relative importance and possible contribution of chemical pollutants to the recent lobster die-off in LIS, and provide a framework for the evaluation of other possible environmental stressors suspected to affect the health of lobsters. Those tools will allow better identification of responsible factors, better focus for actions, and goals/references against which progress can be monitored.

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Acute effects of Methoprene on survival, cuticular morphogenesis and shell biosynthesis in the American lobster, *Homarus americanus*

Michael N. Horst
Division of Basic Medical Sciences, Mercer University School of Medicine

Anna N. Walker[1], and Thomas G. Wilson[2]
[1]Department of Pathology, Mercer University School of Medicine; [2]Department of Biology, Colorado State University.

Key words: lobster, cuticle, biosynthesis, methoprene, endocrine disruption

Introduction

The lobster mortality in western Long Island Sound (WLIS) coincided with the application of several pesticides, including methoprene. Lethal effects were not restricted to lobsters, as lobstermen noted similar massive death of several species of crabs, suggesting a more general mechanism was involved. Elevated water temperatures and decreased salinity of WLIS waters due to massive rain associated with Hurricane Floyd may be related to increased stress. Reports of gravid female lobsters that died while attempting to molt suggest endocrine disruption occurred, since molting is normally inhibited in gravid females. Two hormones, 20-hydroxyecdysone (20-HE) and methyl farnesoate (MF), regulate crustacean growth and metamorphosis.

Methoprene is a juvenile hormone analog (JHA) that closely resembles JH-I in structure. JHAs interfere with metamorphosis by maintaining abnormally high JH activity during the last larval stage or in the pupal stage when the JH titer normally decreases (Chamberlain, 1975). Several studies indicate methoprene acts at both nuclear and membrane sites (Braun and Wyatt, 1996). JH and JHAs inhibit ecdysterone action by preventing expression of specific adult genes while still allowing larval molts to take place (Borst, *et al*., 1987). Methoprene stimulates the Na^+/K^+ ATPase (Ahl and Brown, 1991); direct membrane effects of methoprene are observed within 2-4 h. Survival studies on crustaceans indicated a range of sensitivity to methoprene, from 1.5-125 ppb. Horst and Walker (1999) found that methoprene caused subcellular morphologic derangements and decreased cuticular synthesis in blue crabs. Methoprene exposure causes unique cytopathology in blue crabs. At the TEM level, methoprene causes disruption of the endoplasmic reticulum, distension of the perinuclear compartment/blebbing of the outer nuclear membrane and dispersed heterochromatin. We failed to detect the Golgi apparatus in methoprene treated tissues; lack of this organelle would block intracellular trafficking and cuticle secretion. Methoprene may disrupt the secretory pathway in cuticular epithelium by altering the pathway of secretion and/or extracellular deposition of products. In embryos of blue crab, we observed penetration of the cuticle by ^3H methoprene (Horst and Walker, 1999). Thus, methoprene may disrupt embryonic events, e.g., cuticle synthesis, that takes place inside the egg before emergence of the first larval stage. With the exception of the blue crab (Horst and Walker, 1999), we know little about the biochemical effects of methoprene on cuticle synthesis in crustaceans, and, more specifically, lobsters.

Hypothesis & Objectives

We hypothesize that three factors may have contributed to the WLIS mortality: acute dose of methoprene, lower than normal salinity, and higher than normal water temperatures. Our objectives are to: (1) determine both the 96-hour LC_{50} and sublethal doses for methoprene in all life stages of *H. americanus* at various salinities and temperatures; (2) investigate the cytopathology of methoprene-treated tissues, including epithelium, hepatopancreas, gills and nerve tissue, at the light and TEM levels; (3) conduct biochemical studies on the effects of methoprene on biosynthesis of cuticular protein and chitin components in lobsters; (4) evaluate stress protein induction in lobsters by an acute

methoprene dose and (5) examine deposition, metabolism and bioaccumulation of methoprene in various lobster tissues.

Methodology

Lobsters will be obtained from the Gulf of Maine, shipped overnight and held in 20-L tanks containing filtered, aerated seawater (27 ‰ ; 18-20°C; LD 12:12). After equilibration (5 d), lobsters (various life stages) will be exposed to S-methoprene (0.1-1 ppm) in seawater. Groups of animals will be exposed continually to methoprene at various concentrations for up to 96 h., to calculate 96-h LC_{50} values. Afterward, surviving animals will be sacrificed, fixed and embedded in paraffin or LR White resin. Additional groups of animals will be exposed to sub LC_{50} concentrations of methoprene (1- 4 h), transferred to fresh seawater and held for observation. Mortality will be plotted versus time of exposure to obtain a value for minimal time of exposure for accumulation of a lethal dose. We will determine if lobsters accumulate methoprene by ingestion of pesticide-laden food. Ingested methoprene may be absorbed in the hepatopancreas; effects on this tissue will be compared with epithelium, gill and muscle.

Explant cultures will be prepared from the dorsal carapace of intermolt and postmolt lobsters as previously described for blue crabs (Horst and Walker, 1999). After pretreatment of tissue with and without methoprene (10-50 ppb; 2-4 h), ^{3}H-N-acetyl-D-glucosamine (25 µCi/mL) or Tran-^{35}S-Label (100 µCi/mL) will be added and incubated for 4-8 h. Cells will be separated from cuticle and homogenized. After centrifugation (5500 x g/15 min) the supernatant will be removed and the pellet will be extracted with boiling SDS. The 5500 supernatant will be centrifuged (30,000 x g /45 min) to obtain a crude microsomal pellet and cytoplasmic supernatant. The supernatant will be dialyzed and the microsomal pellet will be extracted with SDS as above. Radioactivity in dialyzed fractions and SDS-insoluble residue will be measured. Samples will be analyzed by SDS-PAGE on 4-20% gradient polyacrylamide gels or by two-dimensional electrophoresis. For autoradiography, gels will be semi-dry electroblotted, placed on a BioMax LE screen and exposed to Kodak XAR-5 film. For Western blots**,** membranes will be probed with primary Ab to a specific stress protein/ secondary antibody-peroxidase and detected by chemiluminescence. Heat shocked HeLa cell lysate (HSP positive control) and anti-HSP antibodies will be obtained from StressGen. For TEM, samples of integument (0.5 x 0.5 cm) will be processed by standard methods and examined in a Hitachi H-600 electron microscope at 100 kV. For statistical analysis, we will use Logistic Regression to examine pesticide level, salinity and temperature, as well as interactions and synergistic effects. For LC_{50} calculations, a 3x3x5 design with 10 organisms per treatment combination will be utilized. A power analysis (computed using nQuery Advisor 3.0) will determine the number of juvenile and adult lobsters needed. Logistic regression statistical analysis will be performed using SAS V. 8.0 (Elashoff, 1996).

Relationship to LIS Lobster Mortality Priorities

The proposed research deals with lobster health and biology; pathology and toxicology related to acute methoprene exposure; anthropogenic inputs/water quality as the source of the methoprene in the lobster mortality; environmental factors and oceanographic conditions as related to Hurricane Floyd that may have altered normal water temperature and/or salinity and thereby caused an exacerbation of the methoprene effects.

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Hormonal responses of lobster stresses of western Long Island Sound

Hans Laufer
Department of Molecular and Cell Biology, University of Connecticut

Key Words: lobster, stress response, hormones, methyl farnesoate, hyperglycemic hormones

Introduction

Healthy lobsters require a properly functional endocrine system in order to survive. A compromised endocrine system is a sensitive indicator of impending problems affecting the survival, the development through critical stages such as metamorphosis, growth (to legal size), and ultimately through maturation to reproduction. It is essential for propagation and maintenance of the abundance of the species.

Adverse environmental factors (both of human origin and of nature), such as were present in western Long Island Sound (WLIS) in late 1999, are known to interfere with appropriate functioning of the major endocrine factors affecting the health and survival of lobsters. Of particular relevance are stresses, such as unusually high temperatures, and the use of the insect growth regulator (IGR) methoprene, a compound being used to control West Nile Virus by targeting larval mosquitoes; methoprene is known to interfere with the proper metamorphosis of mosquito larvae into pupae, preventing them from becoming adults because methoprene is an analog of their own juvenile hormone (JHIII).

The research proposed is an experimental investigation into the tolerance and resistance of lobsters to important physiological stresses. The effects of prolonged elevated temperature and the endocrine disrupter, methoprene, will be used to examine their effects on the ability of lobsters to sustain specific protein synthesis and on their ability to maintain a functionally integrated endocrine system, at critical developmental stages. Of major importance are the effects of stresses on methyl farnesoate (MF), a growth hormone, involved in differentiation and maturation as well as in reproduction. Another key hormone is ecdysone, the molting hormone. Hormones from the central nervous system — crustacean hyperglycemic hormone (CHHs), function mostly as inhibitory hormones, molt inhibiting hormones (MIH), differentiation, reproduction, and gonad inhibiting hormone (GIH). CHHs have been found to respond to short-term heat shocks. A major objective of the proposal is to assess the effects of long-term stresses (heat, methoprene, and infection) on MF, ecdysone, and their CHH regulators.

Laboratory experiments on lobsters will be compared to field-collected specimens. Particularly sick, dying, and amoebae-infected animals from WLIS will be compared to field collected healthy animals and experimentally dosed animals, to assess the extent and ability of lobsters to respond to significant stresses in the environment.

Rationale

Hormonal levels usually in delicate balance are required for survival, appropriate growth, development, metamorphosis, molting, maturation, and reproduction in lobsters. Hormones are sensitive indicators of normal health. Several stresses in WLIS have been suggested as being involved in the deaths of many lobsters in August and September of 1999. One stress of great interest is the insect growth regulator, methoprene, used on mosquito larvae to control the West Nile virus. Methoprene is an endocrine disrupter and a close and effective analog of a crustacean juvenile hormone, methyl farnesoate (MF). It is expected that temperature stress and infections from parameba seriously affect the lobster endocrine system. These factors will be investigated as indicators of endocrine disruption.

Objectives

1. Establish normal hormone levels for embryos, larvae (stages 1-3), metamorphosing juveniles in transition to stage four and in later stages, to maturation, for both molting hormone (ecdysteriods) and methyl farnesoate.
2. Examine the hormone levels for ecdysteroids and MF of lobsters, embryos, larvae, metamorphosing and later stages to maturation and reproduction from areas affected in 1999, during several sampling periods (at least once per month during June, July, August, September and October during 2001-2003) when stress may again be apparent. We will obtain lobsters diagnosed to be diseased and compare them to asymptomatic animals.
3. Treat lobsters to determine the effects of the IGR, methoprene, at a minimum of four levels of activity, low, medium and high sublethal, concentrations, as well as determine lethal concentrations on eggs, larvae, metamorphosing juvenile and later stages to maturation and reproduction to find concentration ranges for methoprene that may be safe for lobsters yet may be useful for the control of West Nile Virus for mosquitoes.
4. Examine the effects of prolonged methoprene exposure and temperature on stress proteins (HSPs) including CHHs with gonad inhibiting activity (GIH) and MOIH activity and MIH activity and on MF and ecdysteroid hormone levels.

Methodology

Adult lobsters will be matured, and embryos and larvae will be cultured with circulating seawater. Morphological stages of eggs, embryos, larval, post larval development, and later stages through to the legal size will be examined. Normal biochemistry will be assessed as well as morphology using microscopy. MF and ecydsteroid titers in the different developmental stages will be determined using normal phase high performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) for MF and a sensitive radioimmunoassay (RIA) for ecdysteroids. Sublethal and lethal effects of exogenous stresses such as paramoeba infection, methoprene, and elevated temperatures will be evaluated for their effects on hormone levels at critical stages. Protein synthesis will be measured by ^{35}S-methionine incorporation into HSPs and crustacean hyperglycemic hormone by polyacrylamide gel electrophoresis followed by radioautography and immunoassay; mRNA expression will be measured by Northern blot analysis.

Coordination

We will share lobsters and data to integrate our study with those of collaborating colleagues: Dr. R. French, Dr. Paul DeFur, Dr. Chris Perkins , Dr. Shili Liu, Dr. Lance Stewart (University of Connecticut), and Dr. Ernest Chang (Bodega Bay Marine Laboratory, CA). Normal larvae, post larvae, and juvenile lobsters will be reared and maintained at the University of Connecticut and at the Massachusetts State Lobster Hatchery and the New England Aquarium. Temperature experiments will be conducted at the Lobster Hatchery and at the Marine Biological Laboratory (MBL), Woods Hole, MA and at the University of Connecticut. Methoprene treatments and hormone analyses will be carried out at the University of Connecticut and at the MBL. Lobsters from WLIS will be collected with the assistance of the Connecticut Department of Environmental Protection, Dr. Lance Stewart, and his contacts from the area of the former and any new lobster devastation as it may occur. Dr. French will provide blood and tissue samples of infected specimens and data on their disease status. Dr. Charles Yarish is providing special temperature controlled environmental facilities to conduct treatment experiments under precisely controlled conditions. The members of the Environmental Research Institute (ERI) at UConn will share their analytical capabilities as well as their data concerning polluting components found in the bottom samples and lobster tissue samples. We will be sharing tissue and blood samples of diseased lobsters as well as data with the previously mentioned cooperating investigations and with Dr. E. Chang of the Bodega Bay Marine Lab. He, in turn, will supply us with his ecdysteroid antibodies.

Effects of pesticides on lobster health: trace level measurements and toxicological assessment at environmentally realistic concentrations

Anne McElroy and Bruce Brownawell
Marine Sciences Research Center, Stony Brook University, SUNY

Key Words: pesticides, Malathion, Methoprene, Pyrethroids, immune response

Introduction

The devastating mortalities recorded for adult lobster *(Homarus americanus)* in western Long Island Sound (WLIS) that coincided with the wide-spread application of pesticides during late summer and early fall of 1999, has led many to suspect that pesticides were in some way responsible. While it is unlikely that pesticide use alone caused these lobsters to die, it is possible that animals inhabiting areas receiving high levels of pesticide run-off could have been exposed to levels of pesticides (or their more persistent metabolites), that may have compromised their health in some way, which made the lobsters vulnerable to infectious disease agents. The very high densities of lobsters recorded in the Sound immediately preceding the die-off would have exacerbated the spread of disease once established.

Two key types of information are essential to assess the potential role of pesticides in lobster health in WLIS. We need a better assessment of pesticide exposure, and acute and sublethal toxicity data in lobsters exposed to environmentally realistic concentrations of the pesticides used. The proposed work addresses both issues.

Objectives

Specifically, our objectives are: 1) to measure acute mortality and chronic sublethal immune response in pre- and post-settlement lobster larvae and juveniles (stage II larvae and 9-12 month old juveniles) exposed to environmentally realistic levels of the Malathion, selected pyrethroids, and Methoprene at stressful (22-24^{o}C) and non-stressful (16-18^{o}C) temperatures; 2) to develop sensitive and selective methods to measure levels of these specific pesticides and their metabolites in seawater, sediment; and tissue samples; 3) to measure the levels of these compounds in LIS surface water, sediments and lobsters during periods when exposure is likely to be at a maximum exposure (after storm events following spraying).

Approach and Methodology

During the first year a combination of GC-MS and HPLC-MS methods will be developed to analyze pesticide and more persistent pesticide metabolites with pg to ηg/L detection levels not currently achievable with standard techniques, including solid-phase extraction (SPE) cartridges packed either with graphitized carbon (Carbograph 4, Crescenzi *et al.*, 1997) or LiChrolut polymer (Steen *et al.* 1999) to efficiently extract polar pesticides polar metabolites. Application of HPLC-MS techniques will also allow us to simultaneously monitor persistent metabolites without the need for derivitization. Acute toxicity tests with all three pesticides using both stage II planktonic lobster larvae and approximately year old juvenile lobsters will also be conducted. We have made arrangements to obtain test organisms from Dr. Michael Tlusty from the Lobster Rearing Facility (LRF) at the New England Aquarium.

Toxicity tests will be conducted at 16°C (a temperature not likely to be stressful to young lobsters) and 22°C (a temperature at the upper end of their tolerance range) to determine the combined effects of pesticide exposure and temperature stress. During year one, we also will get the phenol oxidase assay using the methods described by Smith *et al.* (1995) for immune response. In the second year of the project we will measure pesticide and metabolites levels in water, sediment and lobster samples from WLIS, and conduct sublethal toxicity tests with juvenile lobsters. Field sampling will be

accomplished through collaboration with NYC DEP for the extreme western Sound, and using MRSC vessels to collect samples in areas not presently included in routine monitoring. Concentrations tested in sublethal tests with juvenile lobsters will be based on results of acute toxicity experiments and pesticide measurements in WLIS surface waters.

Progress

In consultation with Michael Tlusty we have modified our experimental plan to provide sufficient numbers of high quality larval and juvenile lobsters for toxicity testing; and redesigned our exposure system plan to maximize survival and we are currently building the systems. We plan on conducting our acute toxicity dose response studies in December and January. We have been fortunate to attract a MS students, Ann Zulkosky to accept the position as the Sea Grant Scholar on this project. She is currently writing up her MS thesis on a related topic (sediment toxicity in benthic amphipods) and will begin working on this project full-time in January. Although currently only working part time on this project, with assistance from a student intern, Ann completed preliminary studies assessing acute toxicity of two pyrethroid pesticidies (Sumethrin and Permethrin) to benthic amphipods. LC_{50} values range from 0.4 µg/g for Permethrin to 1.0 µg/g for Sumethrin. Calculations using reasonable sediment/water partition coefficients for Sumethrin indicate these sediment concentrations with general dissolved concentrations of 10-100 ŋg/L have been found to be acutely toxic to other crustacean species. Calculations using actual pesticide application data from New York City for 1999 suggest that toxic concentrations could have been achieved in WLIS waters if appreciable pesticide run-off had occurred.

We have only recently concluded our search for the project assistant to conduct the chemical analyses for this project. Despite the lack of a dedicated technician, progress has also been made in enhancing our analytical capabilities. Two pre-owned mass spectrometers obtained by our laboratory were repaired, and they are ready to conduct analyses in support of this project. Another recently funded project provided money to purchase a new time-of-flight LC-MS from Micromass, to be delivered in January 2002. The sensitivity of this instrument will greatly expand our ability to detect ambient levels of pesticides and their metabolites in LIS samples.

Expected Outputs

The results of this project will be presented at national and international meetings and be published in the peer-reviewed literature. The results of this study will indicate whether or not pesticide use is likely to be in any way responsible for degraded lobster health in LIS. Such data should be beneficial for assessing the merits of public concerns and for present or proposed litigation seeking remedies for possible harm from pesticide use. This study will also provide needed information related to the effects of temperature on the immune response of early life stage lobsters.

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Immunological health of lobsters: assays and applications

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

Keywords: immunity, reactive oxygen intermediates, phagocytosis, defense mechanisms

Introduction
Homarus americanus is subject to numerous microbial diseases that may be exacerbated by environmental stressors. The internal defense mechanisms of lobsters include hemocyte-mediated and serum-mediated antimicrobial activities that control the progression of infectious diseases. Much of our knowledge of the immune system of the lobster was gained >30 years ago; therefore, it is important to re-examine this subject using state-of-the-art methodology. The recent mass mortality events have triggered renewed interest in developing techniques to quantify key aspects of immunity in normal and stressed lobsters. We plan to test the hypotheses that accurate measurements of immune status will not only provide needed insight regarding health and survival in the face of disease challenge, but also will serve as uniquely sensitive biomarkers of the effects of exposure.

Objectives
The main objective is to develop a suite of assays to define the immune health status of lobsters. This will include hemocyte parameters (total and differential counts, phagocytic activity, chemotaxis intracellular antimicrobial activity, and production of reactive oxygen intermediates) and serum parameters (bacterial agglutinins, prophenol oxidase, lysozyme, and other antimicrobial proteins and peptides.

Methods & Results
This is a report of preliminary data produced during the first several months of this project.

Phagocytosis assay
Yeast cells were labeled by reaction with fluorescin succinimidyl ester and incubated with hemocytes in primary cultures to initiate phagocytosis. After 30 min., ice-cold trypan blue solution was added to quench the fluorescence of unphagocytosed test particles, no quencher was added to controls. The assay places were read in an IDEXX fluorescence concentration analyzer; λ_{ex} / λ_{em} = 485/535. Since trypan blue quenching solution had not entered the hemocytes by the time of reading, the fluorescence of quenched preparations measured phagocytosed particles only. We are optimizing this assay, as well as fluorescence-based phagocytosis assays for pathogenic marine bacteria. Hemocyte anticoagulants and suspension media were examined for suitability in this assay, a cysteine based anticoagulant solution seems better than one containing EDTA and citrate, and the best medium is a phosphate buffered, balanced salt solution containing glucose, essential amino acids and vitamins. Preliminary results indicate that in 30 min. 10^5 hemocytes phagocytose ~$4.5x10^5$ yeast cells (~14K RFU, relative fluorescence units). Opsonization of the test particles with homologous serum was not required for phagocytosis.

Serum agglutinin assay
Lobster agglutinins have been reported to serve as immune recognition molecules that bind to foreign particles in the hemolymph making them more likely to be phagocytosed by the hemocytes. This process is termed opsonization. Since the phagocytosis assay described here employed yeast cells, it was of interest to look for anti-yeast agglutinins in lobster sera, and to determine if these agglutinins promoted phagocytosis. Serial dilutions of sera were prepared in microliter plates and titers were determined at 3 hrs. at 20EC and 18 hrs. at 6EC. The average titer was 32. Yeast pre-treated with the highest non-agglutinating hemolymph concentration were not opsonized; their phagocytosis was no greater than untreated controls.

Reactive oxygen intermediate production

Lobster hemocytes were treated with anticoagulants and resuspended in medium as per the phagocytosis assay. Hemocytes were incubated with particulate (yeast cells) or soluble (PMA) stimuli in the presence of a chemiluminescent probe (luminol). Production of cytotoxic reactive oxygen intermediates (ROI) by the cells produces luminol-dependent chemiluminescence (CL), which was continuously quantified in a liquid scintillation counter modified for single photon counting. Phagocytosis of yeast triggered immediate low level CL, which persisted for at least 2 hrs; treatment of the cells with 1-5Φg/ml phorbol myristate acetate (PMA) produced a much greater CL response than yeast, higher PMA doses produced hemocyte toxicity. The ROI assay using CL is highly quantitative and permits evaluation of overall magnitude of the response (peak height and total response, as measured by area under the curve), as well as response kinetics.

Expected Outputs

The phagocytosis assay is an automated, short-term method to measure accurately the capacity of lobster hemocytes to internalize foreign particles. The method can easily be adapted to study uptake of any test particle, microbe, pathogen, etc. of interest. Phagocytosis is a frequently required first step for hemocyte-mediated killing of microorganisms. One of the most important mechanisms for intrahemocytic killing depends on the production of superoxide anions and other ROI by the cells. The CL-based assay developed here can measure the capacity of hemocytes to generate ROI with great sensitivity, thus providing an indicator of the lobster's ability to destroy ingested microbes. These tests will be components of a battery of assays developed to measure the immunocompetency of lobsters.

Stress indicators in lobsters (*Homarus americanus*): hormones and heat shock proteins

Ernest S. Chang
Bodega Marine Laboratory, University of California at Davis

Key Words: heat shock proteins, hormones, stress

Introduction

Stress in animals is deleterious to survival, growth, development, and reproduction and it can arise from many sources. These sources include physical stressors from the environment (temperature, salinity, oxygen availability), anthropogenic stressors (pesticides, fisheries activities), and biological stressors (competition, predation, parasitism). Lobsters are particularly sensitive to the above stressors because of their particular developmental pattern.

We propose to determine the physiological responses of lobsters to various environmental stresses. The specific objectives are to determine the stress responses of lobsters: 1) at various stages of the molt cycle and during various developmental stages to thermal and hypoxic stress; 2) to various pathologies (parasitic protozoa and bacteria); and 3) to anthropogenic causes, such as pesticides. Indicators of stress will include measurements of the concentrations of the steroid molting hormones (ecdysteroids) and of the neuropeptide crustacean hyperglycemic hormone (CHH).

In addition, we will quantify alterations in the amounts of heat shock proteins (HSPs, also known as stress proteins). The primary function of HSPs is to act as molecular chaperones, promoting the initial folding of other proteins at the ribosome and the refolding of unfolded proteins when they are partially denatured. Environmental stresses such as changes in temperature, oxygen, and metal ion concentration, induce the synthesis of HSPs that act to prevent protein aggregation and to maintain functional conformations.

By comparing the endocrine and HSP responses in unstressed, laboratory-stressed, and field-stressed populations of lobsters, we will be able to evaluate the relative importance of these stresses, focus upon the most important of them and subsequently devise methods to prevent or diminish them.

Objectives

The overall objective of this proposal is to determine the physiological responses of lobsters to various environmental stresses. The specific objectives are to determine the following stress responses of lobsters:

1. At various stages of the molt cycle and during various developmental stages to thermal and hypoxic stress.
2. To various pathologies (parasitic protozoa and bacteria).
3. To anthropogenic causes, such as pesticides.

Methodology

Endocrinology**:**- Colleagues will obtain hemolymph samples from lobsters that are part of an on-going study and they will be characterized as to their degree of infection by parasites and/or bacteria. The possible prior exposure of these lobsters will be noted (e.g., were they from sites that have had exposure to high concentrations of pesticides, herbicides, or other toxicants). We will assay these samples for CHH concentrations with our enzyme-linked immunosorbent assay (ELISA). In addition to these samples from the field, we will conduct laboratory-based experiments. These experiments will involve stressing juvenile lobsters at different molt stages to elevated temperature, hypoxia, and addition of insecticides. Similar experiments will be conducted with the four larval stages. From the

same field and laboratory samples described above, we will measure ecdysteroids with our radioimmunoassay (RIA).

Heat Shock Proteins**:-** The same experimental strategies as described for lobster hormones will be employed for the examination of HSP responses. Western blots will be used to quantify the existing tissue concentrations of HSP70 and 90. Antisera are commercially available. Some samples will also be prepared for mRNA extraction. The mRNA analysis (Northern blots), will be indicative of HSP gene expression. The partial lobster HSP70 clone isolated by our laboratory will be labeled and used as the probe.

Expected Outputs

The primary products resulting from the proposed research are potential diagnostic reagents. These include antisera for lobster CHH, antisera for ecdysteroids, and DNA probes for lobster HSPs. Although these reagents have already been developed in our laboratory, the utility will be further validated through the proposed research.

Prior to the formulation of models attempting to explain any sudden changes in the population biology and ecology of a species, it is necessary to completely understand the basic physiology of the target species. After this baseline information has been obtained, then, and only then can laboratory- and field-based experiments be devised to determine the ultimate causes and effects of any short- or long-term environmental or anthropogenic disturbances on a population. The results from these experiments will provide basic and applied information concerning specific environmental stresses that may affect the endocrine systems of lobsters and also their heat shock responses. From these data, scientists and regulators will be able to pinpoint the most important stressors and subsequently make policy decisions based upon those factors that have the greatest influence upon lobster physiology.

Development of assays for the evaluation of immune functions of the American lobster (*Homarus americanus*) as a tool for health assessment

Sylvain De Guise
Department of Pathobiology and Veterinary Science, University of Connecticut

Richard A. French[1] **and Salvatore Frasca Jr.**[1]
[1]Dept. of Pathobiology and Veterinary Science, University of Connecticut

Keywords: immunology, lobster

Introduction & Rationale

Invertebrates lack adaptive or acquired immunity, and their defense mechanisms consists of an innate immune system, defined in mammalian immunology as the first-line host defense that serves to limit infection in early hours after exposure to micro-organisms. The last few years have seen a resurgence of literature on the almost forgotten innate immunity, with invertebrates often used as models for similar, evolutionary conserved systems and mechanisms in mammals (Ulevitch and Tobias 1999; Franc *et al*., 1999). Nevertheless, relatively little is known on the characteristics of the immune system of lobsters, and their ability to respond to insults/changes in environmental conditions.

The immune cells of lobsters are called hemocytes. They are classified according to their morphology into hyaline hemocytes or hyalinocytes, which are generally smaller and contain few granules, and granulocytes (small and large) which are larger and contain a larger number of granules (Martin and Hose, 1995). Hyalinocytes are thought to be the major cells involved in coagulation, while granulocytes are thought to be responsible for most of the phagocytosis (Martin and Hose, 1995). The functions of hemocytes have not been evaluated in detail.

The immune system is central in health. Evaluation of modern aspects of innate immunity in lobster will be interesting in the context of comparative immunology but also will have practical applications. New tools to evaluate and quantify the lobster immune system will be useful in the investigation of the subtle effects of particular stressors on lobsters, evaluation and management of health of lobsters, and therefore, fisheries.

Hypothesis

Assays can be developed to evaluate innate immune functions in lobsters.

Objectives

1. Develop and optimize an assay to quantify hemocyte respiratory burst in lobsters.
2. Develop and optimize an assay to quantify hemocyte natural killer cell-like activity in lobsters.
3. Develop and optimize an assay to quantify hemocyte proliferation in lobsters.
4. Develop and optimize an assay to quantify hemocyte apoptosis in lobsters.
5. Explore the expression of immunologically relevant surface molecules on lobster hemocytes.
6. Evaluate the relationship between the different immune functions in healthy lobsters.

Methods

Development/adaptation of *in vitro* assays on hemolymph of laboratory-kept lobsters, using modern methods (including flow cytometry) and reagents of current interest in mammalian and invertebrate models of innate immunology.

Expected Outputs

The proposed study will develop new tools to quantify immune functions in lobsters. Given the central role of the immune system in controlling pathogens and remaining healthy, these new tools will greatly improve our ability to assess the health of lobsters, and detect sub-clinical indications of health problems (before they are sick and/or dying). This will become part of our group's efforts to monitor the health of populations of lobsters in Long Island Sound. This and other efforts will be helpful to (1) investigations to further our understanding of the cause(s) of the recent die-off, (2) predict susceptibility of lobsters to subsequent epizootics/mortality events, and (3) make informed management decisions that will result in sustainable lobster fisheries in Long Island Sound.

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Development of an assay for phagocytic activity in the immune system of lobsters

Jan Robert Factor
Division of Natural Sciences, Purchase College, SUNY

Key Words: lobster, *Homarus americanus*, cellular defense, immune system, phagocytosis, Long Island Sound

Introduction
We face serious limitations in our ability to determine the physiological effects of various environmental stresses and toxic anthropogenic substances on the American lobster, *Homarus americanus*. Tests to determine lethality directly, such as the LD_{50}, are well known, however, the paucity of techniques to assess sublethal effects is a serious deficiency (McEnroe, 1991; Woodhead and McEnroe, 1992). Methods to assess the ability to the stressed lobster to protect itself against invading disease agents are needed and should become an important tool in determining the impact of environmental conditions on the lobster population of Long Island Sound (LIS). They have the potential to help explain the recent mortalities of Long Island Sound lobsters by determining sublethal impacts on the immune system that may lead to lethal infections.

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 LIS.

Objectives & Hypothesis
The overarching hypothesis of this work is that exposure to sublethal levels of environmental stresses, anthropogenic toxins, or combinations of these, depresses the immune system of lobster, thereby impairing their ability to defend against infective agents. 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).

Methods
Fluorescent microspheres will be injected into the blood, samples of the two populations of phagocytic cells (circulating hemocytes and fixed phagocytes) will be prepared and deposited on microslides, and the degree of phagocytic uptake will be assessed using fluorescence microscopy. An attempt to develop a more efficient technique will use results from the accurate histological/ cytological methods to calibrate a possible fluorometric method.

Progress
Equipment needed for this project has been ordered, received, and installed. Procedures have been developed and are being refined for: 1. microspheres, including type, size, manufacturer, fluorescent dye; 2. injection, including procedure, volume, concentration of microspheres; 3. cell separation, including isolation of hepatic arterioles, separation of fixed phagocytes, mounting methods; 4. visualization, including fluorescence light microscopy, scanning electron microscopy.

Effects of environmental stressors on disease susceptibility in lobsters: a controlled laboratory study

Richard A. Robohm
Biotechnology Branch, Milford Laboratory, NOAA/NMFS Northeast Fisheries Science Center

Andrew F.J. Draxler
Marine Chemistry Branch, Howard Laboratory, NOAA/NMFS Northeast Fisheries Science Center

Key Words: environmental stress, immunity, disease

Introduction

A proposed cause for the significant lobster mortalities in western Long Island Sound (WLIS) in 1998 and 1999 is that environmental stress weakened lobster immune systems and resulted in lethal microbial infections. A parasitic amoeba, found in moribund lobsters, may be associated with the mortalities; however, it is not clear that it is the primary cause since the parasite also is found in tissues of apparently healthy lobsters, and in some lobsters it is present without indication of inflammatory processes. Few studies have been done to link environmental stress with disease processes in lobsters. It is known that *in vitro* phagocytosis of the bacterium — *Aerococcus viridans* is reduced when lobsters are exposed to temperatures of 22ºC and higher and there is some indication that stress may be associated with shell disease. For example, it is associated with inadequate diets in juvenile lobsters. Also, shell disease in blue crabs coincides with low serum antibacterial activity (an indication of immune-system depression).

Two bacterial infections cause significant mortalities in the American lobster. The gram positive, coccoid bacterium, *A. viridans*, successfully competes with the lobster for its own energy reserves and causes a non-toxic, non-invasive, lethal bacteremia. Recently, *Vibrio fluvialis* was identified as the cause of limp lobster syndrome which caused an estimated $2.5 million economic loss since 1997 in Maine coastal waters This bacterium releases at least one toxin, so its pathogenesis is different from that of *A. viridans*. This project will use both of these bacterial species (as well as the parasitic amoeba if it becomes available in culture) as tools to test whether specific environmental stressors can reduce immunity in lobsters.

Objective

Determine whether exposure of lobsters to four putative environmental stressors will affect immunological indices and susceptibility to microbial infections.

Hypothesis

Exposure of lobsters to ammonia, sulfide, hypoxia, and increased temperature will not suppress immunological indices and will not increase susceptibility to microbial infections.

Methodology

- Market-size lobsters from central Long Island Sound will be exposed in sealed, flow-through tanks to normal conditions and to environmentally relevant concentrations of stressors (i.e., stressor exposures of 20° C temp, 20 mM and 75 mM dissolved O_2, 20 µM and 60 µM sulfide, and 100 µM and 200 µM ammonium). Seven days after initiation of exposure, lobsters will be injected with high and low doses of *A. viridans* or *V. fluvialis*.
- Initial experiments will employ reference conditions and sublethal, maximum stressful conditions in a 2x2x2 design with replication. Second level trials will employ one bacterium and one temperature in high oxygen with manipulation of the concentration of ammonia or sulfide in the absence of the other in a one-way, replicated design. In third level experiments, hypoxia will be manipulated in the presence of determined sublethal ammonia or sulfide.

- Treated lobsters will be sampled weekly, levels of bacteria in the hepatopancreas and the hemolymph counted in appropriate bacteriological media, and representative isolates confirmed in a bacterial identification system.
- The Gehen significance test will be applied to radian-transformed data to determine differences in mean hours till death for lobster treatments. Also, the relative numbers of bacterial counts in lobster hepatopancreas and serum will be compared statistically (T-tests, ANOVA, or non-parametric tests depending on the data distribution.
- Immune function tests will be applied to hemocytes and serum from test and control lobsters in the absence of bacterial exposure. Phagocytic response will be tested by the microplate assay of Wan *et al.*[1] (1993), in conjunction with the Vybrant Phagocytosis Assay Kit of Molecular Probes, Inc., Eugene OR. The method of Soderhall *et al.*[2] (1982) will be used to detect cytotoxic activity, but modified for use in 96-well plates and employing the LIVE/DEAD Eukolight Viability/Cytotoxicity kit from Molecular Probes. Antibacterial activity of lobster serum will be tested by inhibition zone assay[3]. Agglutinins against *A. viridans*, *V. fluvialis*, and *E. coli* will be tested in 96-well, v-bottom plates[4]. Data will be analyzed statistically using T-tests, ANOVA, or non-parametric tests depending on the data distribution.

Expected Outputs

This work will show whether exposure to four biogeochemical factors, known to occur in WLIS, can increase the susceptibility of lobsters to microbial infections. Also it will show effects (if any) on measurable immunological indices and may provide reference points for rapid, practical testing of lobsters for immunological stress. The results of this work will fill in essential pieces of the puzzle, which, together with the work of others under this Congressional funding, may reveal the probable cause(s) of the mass mortality of lobsters. The results of the project will be reported at a Lobster Health Symposium, in a National Marine Fisheries Service Technical Memorandum (archived at the National Technological Information Service), and in peer-reviewed scientific journals.

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Bacterial assemblages involved in the development and progression of shell disease in the American lobster, *Homarus americanus*

Andrei Y. Chistoserdov
Marine Sciences Research Center, University at Stony Brook, SUNY

Roxanna Smolowitz
Marine Biological Laboratory

Key Words: bacterium, pathogen, lobster, shell disease, stress, immune response

Introduction

Shell disease is a commonly recognized problem of lobsters; primarily seen in lobsters held in impoundments during the winter months[4]. However, in the last three years, this disease has been found at unusually high levels in free-living lobster populations from Eastern Long Island Sound to Buzzards Bay, Massachusetts. The disease is characterized by extensive cuticular erosions and melanization[4]. Although generally thought to be non-lethal, the disease affects the appearance and health of the animals. The disease has decreased substantially the commercial value of afflicted animals, and profitability of lobster fisheries, in Connecticut, Rhode Island and southern Massachusetts, as well as, Long Island Sound.

The causes of shell disease are not clear. However, mechanical or enzymatic damage of epicuticle under circumstances that prevents its ready repair, and possibly heavy metal and other water pollutants, may have triggering effects. It is widely accepted that shell disease etiology is bacterial[3] and that a consortium of bacteria rather than an individual pathogen is involved in shell disease progression. Indeed, chitinolytic representatives of *Vibrio*, *Pseudomonas* and *Aeromonas* spp. can be enriched easily and/or isolated from lobsters with shell disease (Prince and Bayer, presentation at Lobster Health Symposium, 2000, Stamford, CT). *Vibrio* and *Flavobacterium* spp. were implicated in shell disease in deep sea red crab[2] and an unidentified Gram-positive bacterium has been shown to be involved in shell disease in the snow crab[1]. It is also possible that additional pathogens weaken the immune system of Long Island Sound lobsters preventing effective defense against chitinolytic bacteria. If such additional pathogens existed they would be likely to cause a latent, otherwise unnoticeable infection.

Our proposal will be dealing with identification of the bacterial consortium involved in the development of shell disease in lobsters, the pathogenesis of shell disease and the crustacean inflammatory and immune response during the progression of the disease. We will also look for pathogens that can cause a concomitant latent infection. Since classical microbiological techniques alone may substantially under represent the complexity of the microbial consortia involved in shell disease, we propose to use a combination of culture-based and molecular techniques to study the consortium of bacteria causing shell disease and those present in hemolymph.

Objectives & Hypotheses

A modern paradigm of microbial ecology states that not more than 1% of bacteria present in the environment are culturable. We hypothesize that classical techniques alone substantially under represent the complexity of the microbial consortia involved in shell disease. Another hypothesis worth testing is that only one, or few, bacteria participate in initial invasion of the lesion and that the consortium is formed at later stages. This hypothesis would imply that a specialized group of pathogens is involved in shell disease initiation and progression rather than opportunistic microorganisms. To test our hypothesis we propose the following experimental objectives:

1. To characterize bacterial assemblages involved in development of shell disease, using classical culture techniques **in conjunction** with techniques of molecular characterization of bacterial assemblages. The final goal is identification of the pathogen(s). This will allow us to design a set of specific probes, which will be used to test for the pathogen(s) in various environmental samples.
2. To elucidate possible succession of pathogen communities during onslaught of the disease and to verify the identity of the pathogen(s) using Kochs postulates.
3. To compare shell disease pathogenesis in lobster from two different locations: Eastern Long Island Sound and Buzzards Bay.
4. To correlate lesion severity using gross and histological evaluations with the microbial findings.
5. To correlate general lobster health (stress and immune system response) with the progression of the disease.

Methods

Sampling:- Healthy and diseased lobsters will be collected from Eastern Long Island Sound (CT, NY) and Buzzard Bay (MA). Animals will be rated for shell disease severity using the methods of Bruce Estrella (MA Division of Marine Fisheries). These lobsters, primarily in substages C3 and C4 of cuticular development, will be used both for histology and for isolation of the bacterial consortium (BC) present in lesions.

Isolation of bacterial consortia:- This procedure will be carried out on diseased lobsters by scraping lesioned carapaces with a sterile razor blade and homogenizing this material in sterile seawater. The BC will be used to isolate DNA for molecular analysis, to enrich various groups of bacteria, to carry out infection experiments and for *in situ* hybridization probes. The hemolymph will be used in infection experiments along with BC isolated from lesions.

Characterization of BC assemblages and microbiological analysis of hemolymph using culture-dependent and molecular techniques:- BC from individual lesions and hemolymph will be used to isolate DNA. DGGE will be a method of choice for comparative molecular characterization of bacteria in lesions. The availability of 16S rDNA sequences for bacteria will allow us to design oligonucleotide probes for each individual bacterium identified in BC and hemolymph. The oligonucleotide probes will be used for *in situ* hybridization experiments in order to quantify bacteria present in histological lesion material. Simultaneously, with molecular analysis of BC and hemolymph, we will culture bacteria possessing chitinolytic activity as well as possible bacteria present in hemolymph.

Verification of the Kochs postulate and analysis of a possible succession of pathogen communities during onslaught of the disease:- This will be carried out with the BC and individual bacteria isolated from BC alone, or in combination with, hemolymph isolates. Bacteria present in the various stage of lesion formation will be monitored by *in situ* hybridization. Small amounts of lesion material will be collected and hybridized against the oligonucleotide probes described in the previous subsection.

Gross and Histological evaluations:- The lesions will be dissected from the animal making sure the underlying soft tissues remain attached to the shell. They will be fixed, decalcified, embedded in paraffin and sectioned using standard methods. Additionally, the internal organs of each sampled lobster will be examined grossly for abnormalities, sampled and examined histologically. Gross and microscopic lesion severity, based on the ratings of the lesions, will be then correlated with the BC identified from the same lesions with the goal of determining possible changes in the make up of the BC as the lesions advance in severity/depth through the carapace.

General lobster health (stress and immune system response) analysis:- This will include measurement of polyphenol oxidase and bactericidin activity and total counts of hemocytes. In order to estimate the

levels of stress in lobsters, we will measure concentrations of hyperglycemic hormone or morphine production in lobsters.

Expected Outputs

The immediate result of the project will be identification of bacteria responsible for shell disease in lobsters. Molecular probes will be designed to detect and enumerate these microorganisms in the environment. Since each individual animal may become infected several times during its life span, the causative agent of shell disease has a natural reservoir outside the lobster. First, using the molecular probes these reservoirs will be identified. Second, the molecular probes would allow environmental managers to determine whether activities and intrusion into the coastal environment (dredging, sewage dumping, coast area development, lobster fishing practices, etc.) increase numbers of lobster shell disease pathogens in their natural reservoirs and hence a risk for spreading the disease. Isolation of the pathogen in a pure culture will allow researchers to analyze environmental conditions and behavioral traits of lobsters, which facilitate spreading of the disease. This in turn may lead to modifications in environmental and lobster fisheries management, to decrease chances of shell disease spreading.

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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*

Salvatore Frasca Jr.
Dept. of Pathobiology and Veterinary Science, University of Connecticut

Key Words*: in situ* hybridization, lobster epizootic, molecular systematics, paramoeba, polymerase chain reaction

Introduction

Epizootic mortalities of American lobster (*Homarus americanus*) occurred in western Long Island Sound (WLIS) during the autumn of 1999. Lobstermen reported that dead or dying lobsters were "limp," i.e. flaccidly paretic or paralyzed. At the same time, there were reports from WLIS of increased numbers of dead or dying crabs and sea urchins. Pathologists from the Department of Pathobiology and Veterinary Science, University of Connecticut, performed necropsies on dead and dying "limp" lobsters. Bacterial cultures of hepatopancreas and hemolymph yielded several potential pathogens (e.g., *Vibrio* spp or *Aerococcus* spp), but not in any significant number or consistent pattern. Toxicologic testing of hepatopancreas and muscle did not demonstrate elevated levels of pesticides or their degradation products. 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, hemocytic infiltrates, the cytoplasm of neurons, and between nerve fibers. This protozoan possessed a paranuclear body, which stained positively for DNA using the Feulgen technique and was ultrastructurally consistent with a parasome, or "*Nebenkörper",* a feature common to protozoa of the genus *Paramoeba* (Schaudinn, 1896) (Page 1970).

Several potential sources of ecological contamination have been postulated to cause this mass mortality of WLIS lobsters, and 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). 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.

The principal goal of this research is to develop sensitive, specific, and rapid DNA-based tests for the detection of this parasitic paramoeba in host tissues. However, development of such DNA-based tests is predicated upon determination of the molecular systematics of the genus, since DNA sequence data, currently nonexistent, is essential for primer and probe design.

Hypothesis and Objectives

The principal objective of this research is to determine the molecular systematics of the genus *Paramoeba* through 18S SSU rRNA gene sequencing in order to exploit this nucleotide sequence data in the design of primers and probes for polymerase chain reaction (PCR)- and *in situ* hybridization (ISH)-based diagnostic tests to identify parasitic paramoeba in lobster tissue. The following specific aims will be addressed:

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.

Methodology

The following methods will be employed to achieve the specific aims:

- 18S SSU rDNA from amoeba will be amplified by PCR initially using universal primers to eukaryotic 18S SSU rDNA, then later using primers to consensus sequences of paramoeba 18S SSU rDNA.
- Amplicons will be cloned into TA vectors and sequenced by primer-walking of plasmid inserts and dideoxynucleotide chain termination reactions.
- Phylogenetics will be determined by nucleotide sequence comparisons using parsimony and distance matrix analysis.
- Nucleotide sequence data from the lobster paramoeba will be used to create primers and probes for PCR- and ISH-based diagnostic tests, which will be tested using host tissue from lobster, urchin and crabs.

Expected Outputs

The anticipated products of this proposed research are the following:

- 18S rDNA sequence data that allows for characterization of the molecular systematics of the genus *Paramoeba* Schaudinn, 1896.
- Determination of the 18S SSU rRNA gene sequence of the lobster paramoeba and subsequent phylogenetic characterization of the parasite.
- Development of PCR- and ISH-based tests for the detection of this paramoeba in host tissue.

Molecular characterization of the paramoeba associated with lobster neurologic disease is fundamental to determining the ecological source of this parasite. This has direct implications to management practices for natural populations of commercially important shellfish and will be contributory to an understanding of the interrelationships of other environmental factors, such as pollution, dredging, and pesticide run-off, on the health of the commercial lobster population. The results will deliver the essential nucleotide sequence data that is the basis for development of rapid and specific PCR- and ISH-based tests to detect the parasite in host tissues. The diagnostic tests proposed will improve stock enhancement initiatives of environmental protection agencies.

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Oligonucleotide-based detection of pathogenic *Paramoeba* species

Rebecca J. Gast
Woods Hole Oceanographic Institution

Keywords: molecular biology, paramoeba, DNA sequencing, oligonucleotide, srDNA

Introduction

Monitoring the environmental distribution of the *Paramoeba* species that infects lobsters is an essential component to understanding how infections occur and predicting their spread. For example, if *Paramoeba* 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 *Paramoeba* species is based primarily upon culture of the organism and/or analysis of morphologic characters by light or electron microscopy.

Serious drawbacks are associated with these approaches. Culture methods are biased for the recovery of particular species because they presuppose knowledge of growth requirements that may not be valid, especially for parasitic organisms. Even parasites that are able to exist as free-living species may not respond well to laboratory culture conditions, thereby reducing the likelihood of their recovery and generating the impression that they are absent from the sample. Without culture, studies of the natural distribution of paramoebae would require assessment of large volumes of fixed samples. Sorting through preserved samples is extremely time consuming and not amenable to screening large numbers of samples.

The inherent variability of morphologic characters can also render them insufficient for the discrimination between species of protozoan parasites. In fact, parasitic species seem to often be described as being morphologically very similar to free-living species, with the most distinguishing characteristic being that observed in an infection. The demonstration of host infection would be unambiguous for the presence of the parasite, but a lack of infection may indicate that the level of parasite present was sub-optimal for establishing an infection, or that the organism was dormant. Morphologic and infectivity methods, therefore, are not adequate for assessing the persistence or distribution of paramoebae in the environment.

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 *Paramoeba* species. Small subunit ribosomal gene (srDNA) sequences contain both invariant and variable regions that can be used as templates for the design of oligonucleotide probes (very short pieces of DNA) with specificities ranging from kingdom to individual isolate levels. The large number of srDNA sequences available in databases, such as GenBank, also makes this molecule useful because of the large volume of comparative sequence information available. In the past 12 years, the use of ribosomal probes 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 (see Amann *et al*., 1990; Manz *et al*., 1993; Lim *et al*., 1999). 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 lobster *Paramoeba* from other parasitic, and non-parasitic, paramoebae. Therefore, the first objective of this project is to develop a method for the detection of the lobster *Paramoeba* parasite that can be used to

analyze environmental samples for the presence of the parasite, as well as to analyze tissue samples. I will employ a PCR-based dot blot method that utilizes hybridization to oligonucleotides specific for the organism of interest. The suite of oligonucleotides that will be developed will include one specific for the *Paramoeba* genus, in addition to at least one specific for the lobster parasite (and eventually others that are specific for parasitic species from fish, crab, and urchin). PCR amplification of a portion of the small subunit ribosomal gene is employed to generate a probe from the sample of interest, and this probe is hybridized to a membrane containing an array of the different oligonucleotides. Colorimetric detection of hybridization to the oligonucleotide spots indicates the type(s) of organisms present in the sample. I have successfully developed and used this type of method for the detection and identification of another amoeba, *Acanthamoeba* (Gast, in press).

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

Progress to Date

This project was initiated in July 2001. An undergraduate summer student in my laboratory spent 2 months working on extracting DNA from lobster tissues, developing amplification primers that would be selective for paramoebae and establishing amplification parameters to recover paramoeba ribosomal gene amplification products. We have been successful in recovering amplifiable DNA from lobster tissues, but were not able to test the paramoeba primers due to difficulties obtaining cultured material to act as a positive control. We have acquired an ATCC culture of *Paramoeba pemaquidensis*, and will use it to optimize amplification with the several primers sets that we have designed. We already have tissue samples from a lobster that was confirmed as being infected with paramoebae, and we have made arrangements to acquire more potentially infected, and dead, lobsters from Connecticut Department of Environmental Protection. These samples will be analyzed using our *Paramoeba* primers, once optimal amplification conditions have been determined. The paramoebic srDNA sequences that are recovered will be used to design specific oligonucleotide probes for the reverse dot blot array. 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 several stations. DNA is extracted from water and sediment within two weeks of collection, and the samples are tested to confirm general amplification competence. The DNA is then archived at –20ºC until analysis by the reverse dot blot method.

Expected Outputs

Once developed, the *Paramoeba*-specific reverse dot blot 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 to study these organisms. We will use our method to survey LIS 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.

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Phenotypic and molecular identification of environmental specimens of the genus *Paramoeba* associated with lobster mortality events

Patrick M. Gillevet
Institute for Bioscience, Bioinformatics & Biotechnology, George Mason University

Charles J. O'Kelly[1], Thomas A. Nerad[2] and Thomas K. Sawyer[3]
[1]Bigelow Laboratory for Ocean Sciences; [2]American Type Culture Collection; [3]Rescon Associates

Key Words: lobster, *Paramoeba*, fingerprinting, systematics, environmental monitoring

Introduction
The protozoon consistently observed in dead and dying lobsters from western Long Island Sound during the die-off events of 1999-2000, has been identified as a species of *Paramoeba*. This is not the first record of *Paramoeba*-type amoebae in lobsters (Sawyer, T.K., 1976; Sawyer and MacLean, 1978), but it is the first to be associated with catastrophic mortality. The *Paramoeba* in lobsters has been most commonly assigned (Sawyer, T.K., 1976; Sawyer and MacLean, 1978) to *P. pernicoisa* [4], the agent of gray crab disease in the blue crab, *Callinectes sapidus*. However, reported differences in the histopathology of *Paramoeba* in blue crabs versus lobsters, raises the possibility that the lobster protozoon is a species other than *P. perniciosa*. It is also uncertain whether the paramoeboid organism is the causative agent in the Long Island Sound lobster die-off, or is symptomatic of some challenge to the environment, such as elevated summer temperatures in the Sound, or pesticide spraying in and around New York City. Ironically, much debate took place during significant gray crab disease, even though Koch's postulates have not been satisfied fully.

Objectives
1. To test the hypothesis that the lobster *Paramoeba* is a new species, and it is a genotypically unique population.
2. Determine whether other marine invertebrates serve as a reservoir for the lobster *Paramoeba*.

Methodology
We will use a combination of morphological, molecular, and phylogenetic methods to characterize the microbial consortia (bacteria and protists) associated with lobster deaths in an attempt to identify the causative agent. Specifically, we will characterize the *Paramoeba* that has been associated with lobster deaths and determine whether the reservoir for the lobster *Paramoeba* is other marine invertebrates or benthic sediments in western Long Island Sound. We will attempt to develop cultures of lobster neuronal cell lines, and determine whether endocytic bacteria are present in any cultures that are established. We will publish a database of the morphological and ultrastructural characters that may be used to identify amoebae now considered to belong to the genus *Paramoeba*. We will also publish a taxonomic treatment of species now considered to belong to the genus *Paramoeba*, based on consistent interpretations of morphological characters and phylogenetic analyses of SSU gene sequences. Using the latter molecular information, we propose to develop a sensitive DNA fingerprinting tool (ALH) for the unambiguous detection of the lobster *Paramoeba* in the environment, with special reference to finding reservoirs for the species and detecting infestations at low cell density. Ultimately, we hope to provide cultures that can be used for the verification of Koch's postulates.

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APPENDIX – List of Research Topics

PHYSICAL & CHEMICAL ENVIRONMENTAL PARAMETERS

Exposure of Lobsters to the Varied Chemical and Biological Environment of Long Island Sound. *Principal Investigators*: Andrew F.J. Draxler and Ashok Deshpande, NOAA/ NMFS/NEFSC Howard Laboratory, NJ.

Effects of Temperature and Body Size on Metabolic Stress in Long Island Sound Lobsters. *Principal Investigators***:** Glenn Lopez and Robert M. Cerrato, Marine Sciences Research Center, Stony Brook University, NY.

Environmental Change in Long Island Sound in the Recent Past. *Principal Investigator:* Johan C. Varekamp, Dept. of Earth & Environmental Sciences, Wesleyan University, Connecticut. *Co-investigators*: Ellen Thomas, Dept. of Earth & Environ. Sciences, Wesleyan University; Sherri R. Cooper, Biology Dept., Bryn Athyn College; Mark A. Altabet, Dept. of Chemistry and Biochemistry, University of Massachusetts; and Marilyn Buchholtz ten Brink, US Geological Survey, Coastal Program, MA.

Relationship Between American Lobster Mortality in Long Island Sound and Prevailing Water Column Conditions. *Principal Investigators:* Robert E. Wilson, R. Lawrence Swanson, and Duane E. Waliser, Marine Sciences Research Center, Stony Brook University, NY.

ANTHROPOGENIC INPUTS (PESTICIDES)

Determination of the Toxicity and Sublethal Effects of Selected Pesticides on the American Lobster (*Homarus americanus*). *Principal Investigator:* Sylvain De Guise, Dept. of Pathobiology, UConn, CT. *Co-investigators:* Richard A. French, Dept. of Pathobiology, UConn, CT; and Christopher Perkins, Environmental Research Institute, UConn, CT.

Acute Effects of Methoprene on Survival, Cuticular Morphogenesis and Shell Biosynthesis in the American Lobster, *Homarus americanus*. *Principal Investigator:* Michael N. Horst, Dept. of Pathobiology, Mercer University School of Medicine, Macon GA. *Co-investigators:* Anna N. Walker, Dept. of Pathobiology, Mercer University School of Medicine, and Thomas G. Wilson, Dept. of Biology, Colorado State University, CO.

Hormonal Responses of Lobsters to Stress in Western Long Island Sound. *Principal Investigator:* Hans Laufer, Dept. of Molecular & Cell Biology, UConn, CT. *Co-investigators:* S. Lin, C.R. Perkins, Environmental Research Institute, UConn; Richard French, Dept. of Pathobiology, UConn; Lance Stewart, UConn Extension Service; Michael Syslo, Massachusetts Lobster Hatchery, MA; Michael Tlusty, New England Aquarium, ME; and Charles Yarish, Dept., of Ecology & Evolutionary Biology, UConn, CT.

Effects of Pesticides on Lobster Health: Trace Level Measurements and Toxicological Assessment at Environmentally Realistic Concentrations. *Principal Investigator:* Anne E. McElroy, Marine Sciences Research Center, Stony Brook University, NY. *Co-investigator:* Bruce J. Brownawell, Marine Sciences Research Center, Stony Brook University, NY.

APPENDIX — List of Research Topics

IMMUNE & ENDOCRINE SYSTEMS RESPONSE FAILURE

Immunological Health of Lobsters: Assays and Applications. *Principal Investigator:* Robert S. Anderson, Chesapeake Biological Laboratory, Center for Environmental Sciences, University of Maryland, MD.

Stress Indicators in Lobsters: Hormones and Heat Shock Proteins. *Principal Investigator:* Ernest S. Chang, Bodega Marine Laboratory, University of California, CA.

Development of Assays for the Evaluation of Immune Functions of the American Lobster (Homarus americanus) as a Tool for Health Assessment. *Principal Investigator:* Sylvain De Guise, Dept. of Pathobiology, UConn, CT. *Co-investigators:* Richard A. French and Salvatore Frasca, Jr. Dept. of Pathobiology, UConn, CT.

Development of an Assay for Phagocytic Activity in the Immune System of Lobsters. *Principal Investigator:* Jan Factor, Division of Natural Sciences, SUNY Purchase, NY

Effects of Environmental Stressors on Disease Susceptibility in Lobsters: A Controlled Laboratory Story. *Principal Investigator:* Richard Robohm, NOAA NMFS Fisheries Laboratory, Milford, Connecticut, and Andrew F.J. Draxler, NOAA/ NMFS/NEFSC Howard Laboratory, Sandy Hook, NJ.

SHELL DISEASE SYNDROME & PARAMOEBA INFESTATION

Bacterial Assemblages Involved in the Development and Progression of Shell Disease in the American Lobster, *Homarus americanus*. *Principal Investigator:* Andrei Chistoserdov, Marine Sciences Research Center, Stony Brook University, NY. Co-investigator: Roxanna Smolowitz, Marine Biological Laboratory, MA.

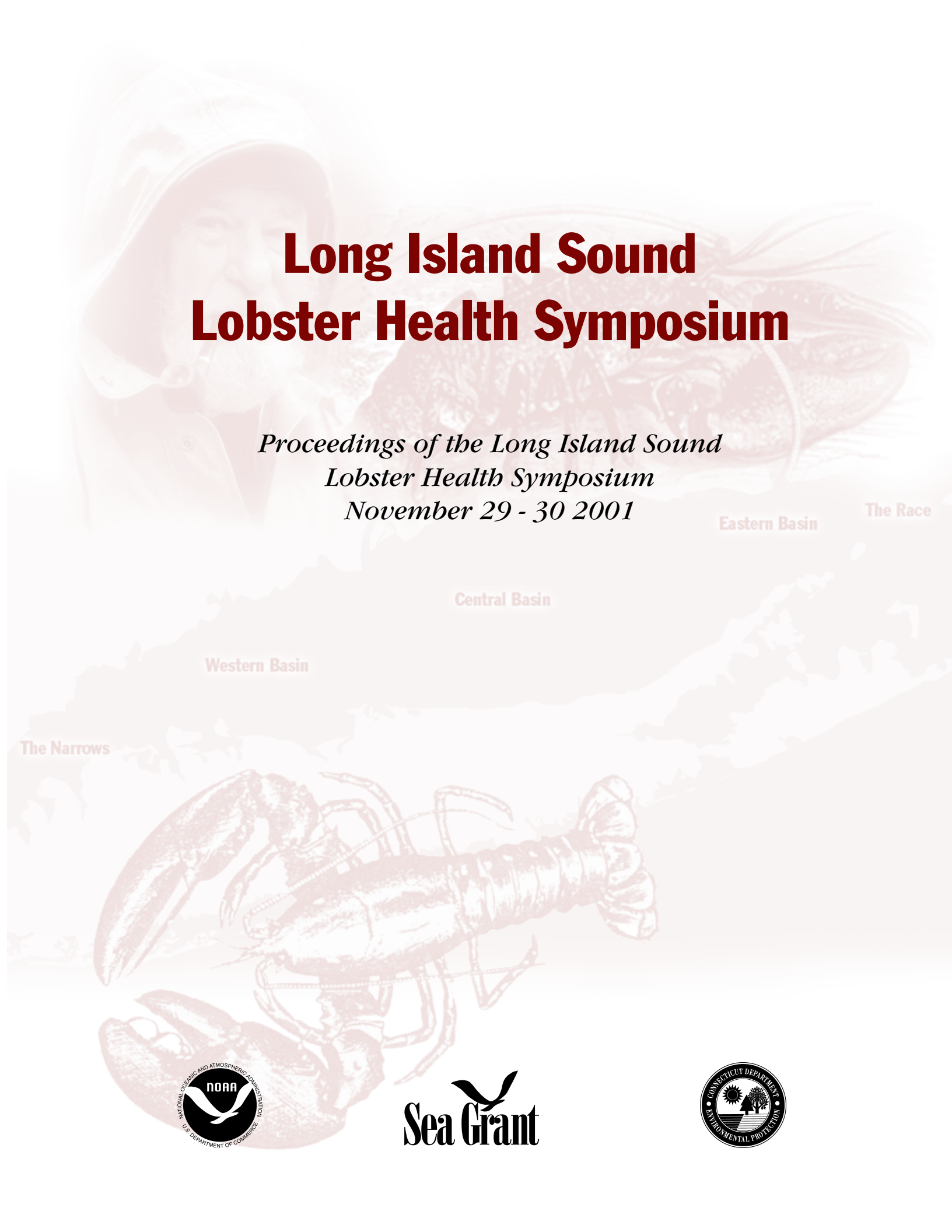
Development of Polymerase Chain reaction and *in situ* Hybridization-based Tests for the Specific Detection of the Paramoeba Associated with Epizootic Lobster Mortality by Determination of the Molecular Systematics of the Genus Paramoeba. *Principal Investigator:* Salvatore Frasca, Jr., Dept. of Pathobiology, UConn, CT. *Co-investigators:* Richard French and Sylvain De Guise, Dept. of Pathobiology, UConn, Storrs, CT.

Oligonucleotide-based Detection of Pathogenic Paramoeba Species. *Principal Investigator:* Rebecca J. Gast, Woods Hole Oceanographic Institution, MA.

Phenotypic and Molecular Identification of Environmental Specimens of the Genus Paramoeba Associated with Lobster Mortality Events. *Principal Investigator:* Patrick M. Gillevet, George Mason University, VA. *Co-investigator:* Charles J. O'Kelly, Bigelow Laboratory for Ocean Sciences, ME.

Long Island Sound Lobster Health Symposium

Proceedings of the Long Island Sound Lobster Health Symposium
November 29 - 30 2001



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