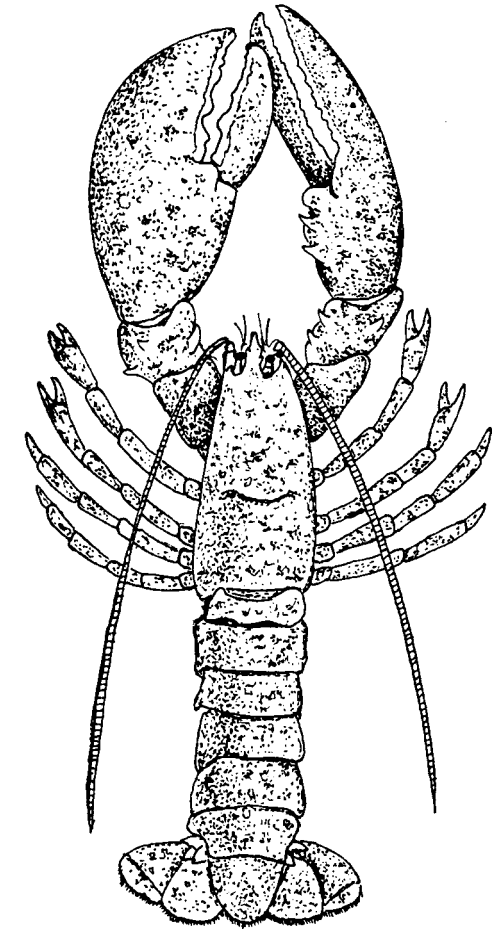
Physiological Responses to Stress



Immunological Health of Lobsters

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

Hemocyte-mediated immunity

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

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

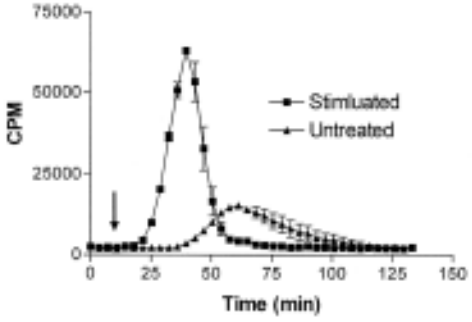

Figure 1. Superoxide production by lobster hemocytes.

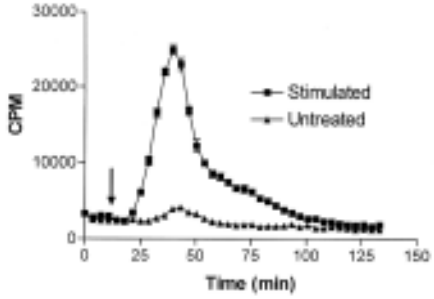

Figure 2. HOCl production by lobster hemocytes.

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

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

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

Plasma-mediated Immunity

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

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

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

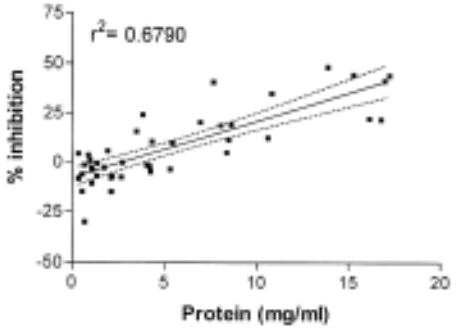

Figure 3. Percent inhibition of Listonella anguillarum by unseparated lobster plasma.

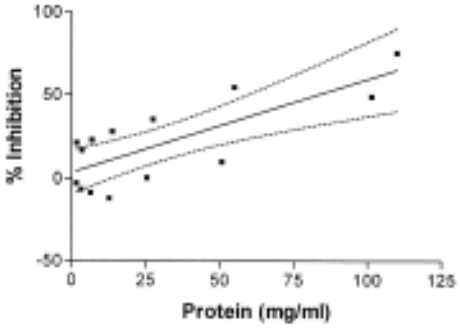

Figure 4. Percent inhibition of Listonella anguillarum by the >100kDa fraction of lobster plasma.

Plasma protein concentrations and immune status

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

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

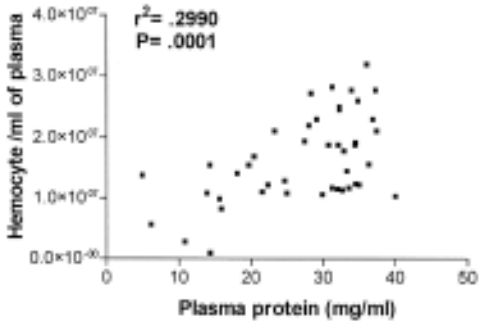

Figure 5. Plasma protein concentration positively correlated with hemocyte counts.

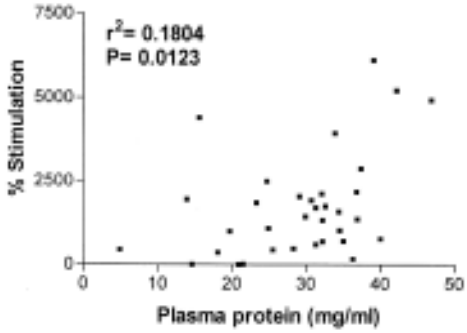

Figure 6. HOCl production increases with plasma protein concentration.

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

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

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Introduction

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

Materials and Methods

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

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

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

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

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

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

Results and Discussion

Effects of Stress on CHH Levels

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

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

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

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

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

Effects of Stress on HSP Levels

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

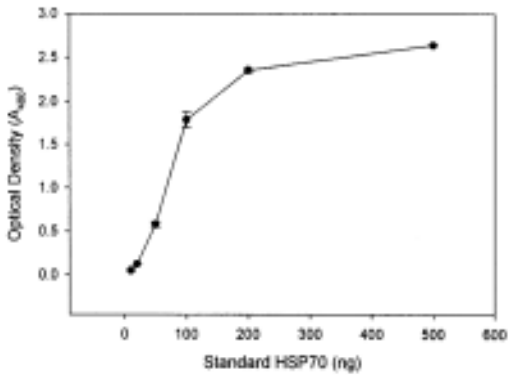

Figure 1. Standard curve for a direct ELISA. Wells were coated with human HSP70. The primary antibody was a mouse monoclonal made against chicken HSP70 (Stressgen). The secondary antibody was goat anti-mouse IgG conjugated to peroxidase. The reaction was developed with the addition of hydrogen peroxide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

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

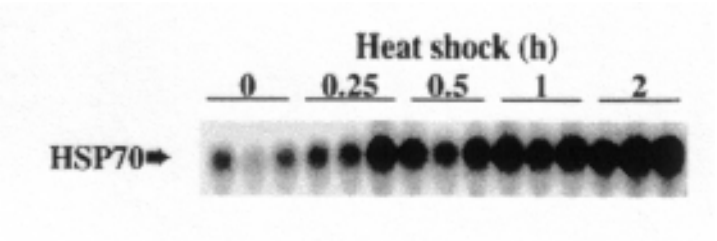

Figure 2. Northern blot of HSP70 mRNA isolated from abdominal muscle that was obtained from lobsters that were thermally shocked 13°C. Three individual lobsters were sampled at each time interval.

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

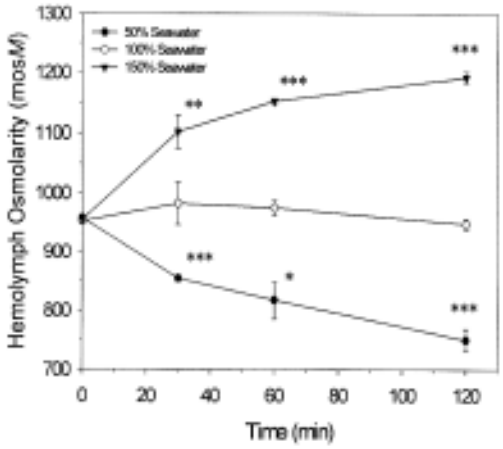

Figure 3. Hemolymph osmolarity (mosM) of individual lobsters repeatedly sampled at 0, 30, 60, and 120 min of exposure to control (100%), hypo-osmotic (50%), or hyper-osmotic (150%) seawater. N=4 for each time point. Error bars represent one S.D. of the mean. Asterisks indicate significant difference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

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

Effects of Molt Cycle on HSP Levels

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

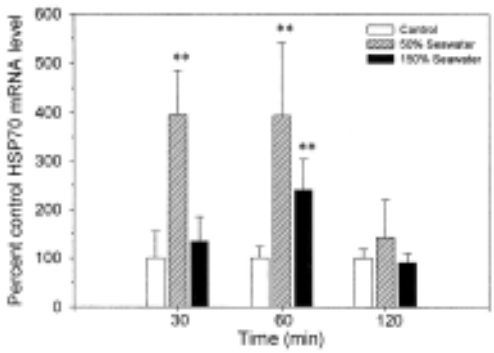

*Figure 4. Quantitative analysis of lobster HSP70 gene expression in abdominal muscle during hypo- and hyper-osmotic stress (50% and 150% seawater) for 30, 60, and 120 min. Data are normalized against the actin signal (indicator of equal loading) and expressed as percent control (100% seawater) mRNA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

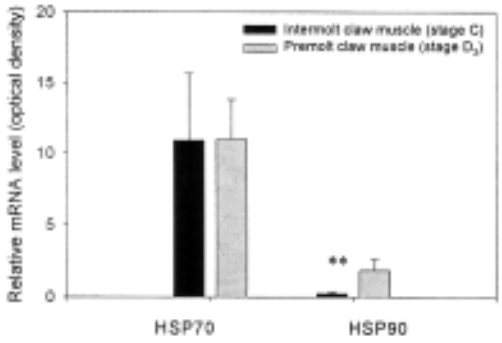

*Figure 5. Relative mRNA levels for molecular chaperones and polyubiquitin in intermolt versus premolt claw muscle. Data from (A) were normalized against the actin signal. N=6 for each bar. Absolute expression levels for one transcript should not be compared to any other because of potential differences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

Induction of Thermal Tolerance

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

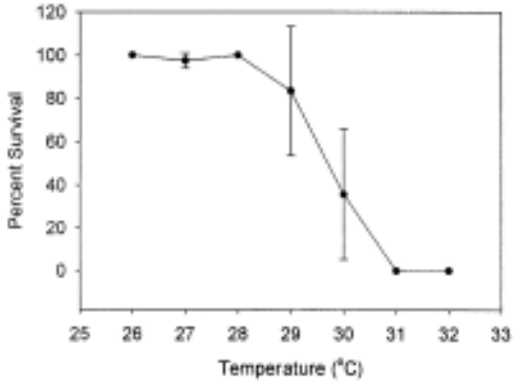

Figure 6. Percentage survival of stage I lobster larvae. Groups of 25 larvae were placed into a tea strainer and transferred to a jar placed in a water bath that had been heated to the indicated temperatures. Survival after 2 h was then determined.

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

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

Acknowledgments

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

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

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

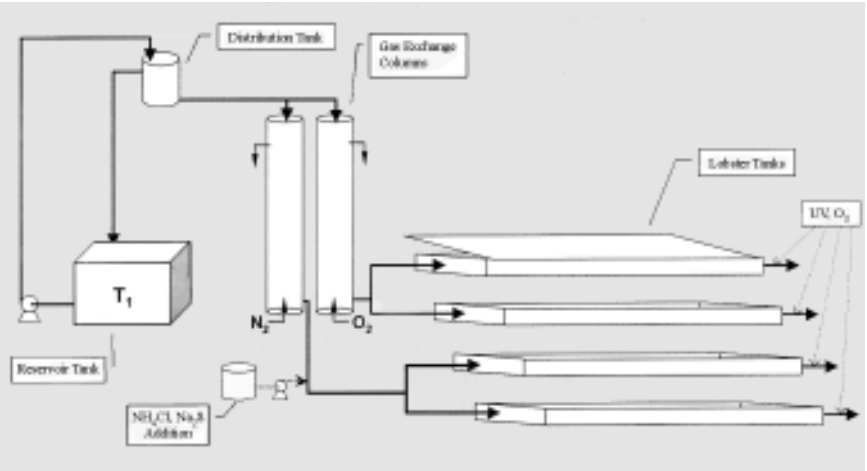

Figure 1. System for testing the effects of biogeochemicals.

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

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

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

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

* LIS: Lobsters taken from central Long Island Sound

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

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

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

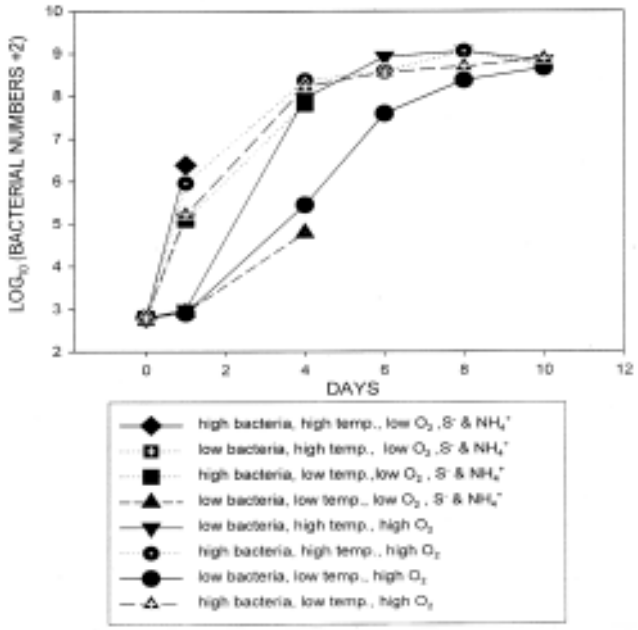

Figure 2. Aerococcus growth in hepatopancreas.

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

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

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

[b] RIS: Lobsters taken from Rhode Island Sound

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

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

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Introduction

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

Material and methods

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

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

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

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

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

Results

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

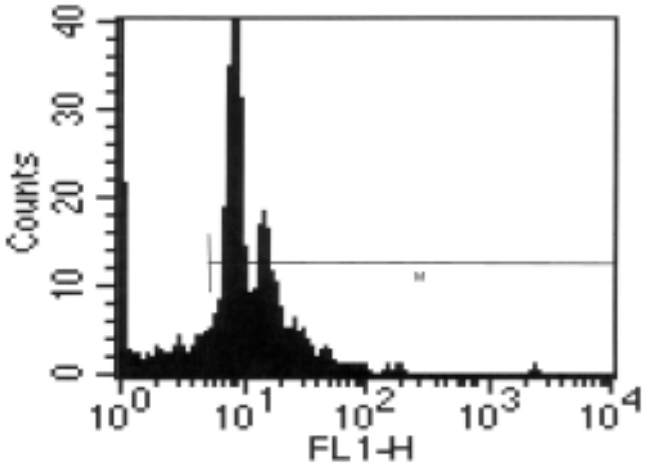

Figure 1: Histogram of fluorescence of lobster hemocytes phagocytizing fluorescent latex beads.

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

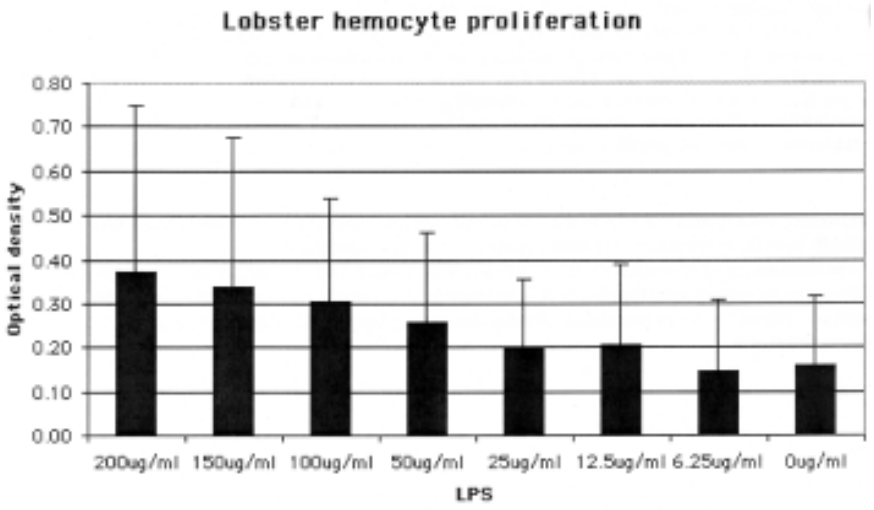

Figure 2. Proliferation of lobster hemocytes (mean of 4 animals) upon stimulation with LPS.

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

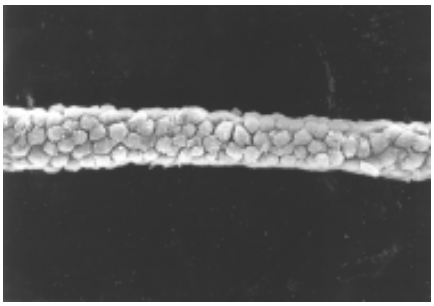

Figure 1. An isolated terminal hepatic arteriole, showing fixed phagocytes covering its outer surface; uninjected control; scanning electron micrograph; 410x in microscope.

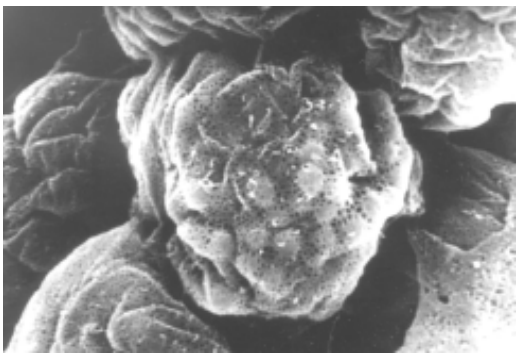

Figure 2. A fixed phagocyte from an isolated terminal hepatic arteriole, showing the outline of 1.0 µm fluorescent microspheres under the perforated membrane after removal from the blood; scanning electron micrograph; 6100x in microscope.

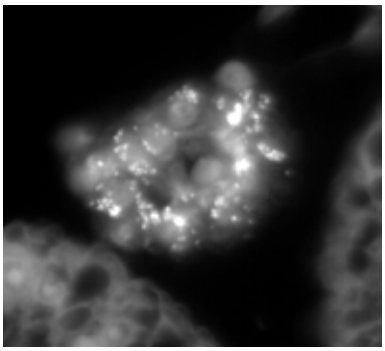

Figure 3. A histological section through a terminal hepatic arteriole that was harvested from a lobster 65 minutes after it was injected with 1.0 µm fluorescent microspheres (similar to the one in Figure 1); fluorescence microscopy shows uptake and distribution of fluorescent microspheres by the fixed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

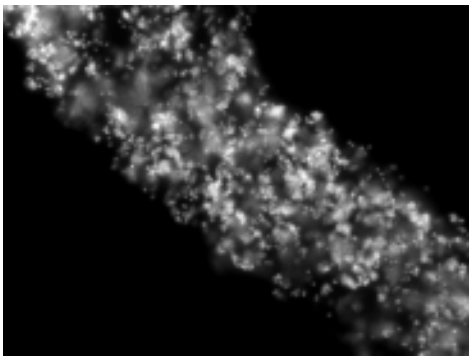

Figure 4. This light micrograph illustrates a terminal hepatic arteriole that was harvested from the digestive gland (=hepatopancreas) of a lobster 65 minutes after it was injected with 1.0 µm fluorescent microspheres. The same view of the terminal hepatic arteriole in Figure 2; fluorescence microscopy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

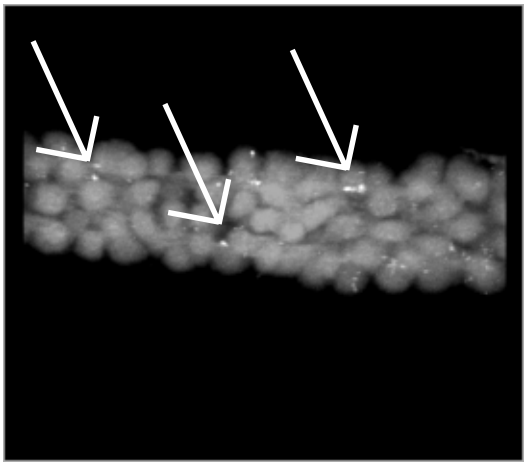

Figure 5. This light micrograph is made with a confocal fluorescence microscope and illustrates a single terminal hepatic arteriole that was harvested from the digestive gland (=hepatopancreas) of a lobster 30 minutes after it was injected with 1.0 µm fluorescent microspheres.

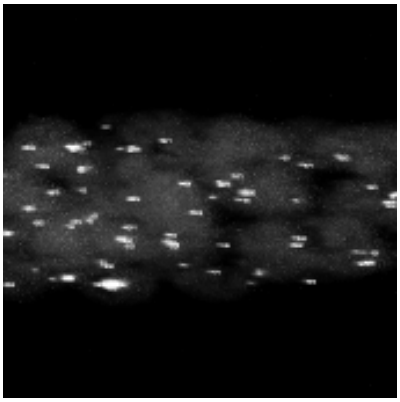

Figure 6. This micrograph demonstrates a method for quantifying fluorescent microspheres taken up by fixed phagocytes of a terminal hepatic arteriole that was harvested from the digestive gland (=hepatopancreas) of a lobster 1440 minutes after it was injected with 1.0 µm fluorescent microspheres. Terminal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

Hormonal Responses of Lobsters to Stresses, an Interim Report

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Introduction

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

1. Ecdysone levels in lobsters

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

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

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

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

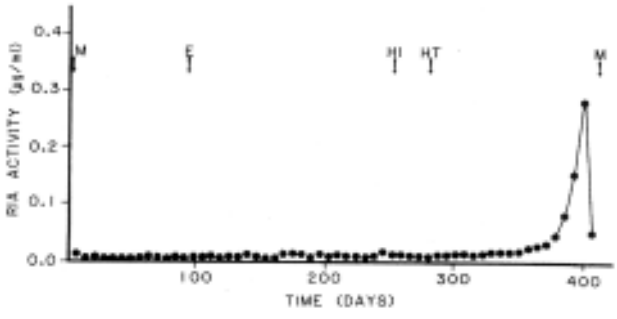

Figure 1A. Ecdysteroid titers of a female adult lobster that underwent extrusion and embryo incubation."RIA activity"represents hemolymph ecdysteroid concentrations. "M" represents the molts, "E" the day of extrusion, and "HI" and "HT" represent the initiation and termination of larval hatching, respectively.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

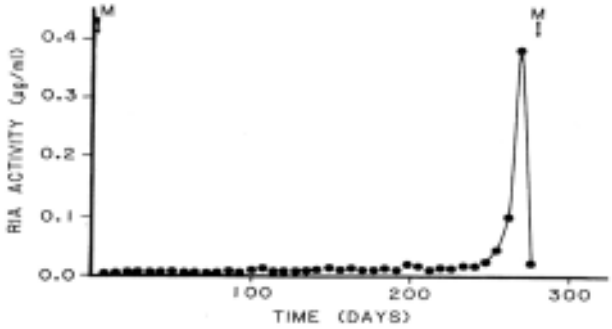

Figure 1B. Ecdysteroid titers of a female adult lobster that did not undergo egg extrusion. Legends, symbols, and source are the same as in Figure 1A.

Results

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

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

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

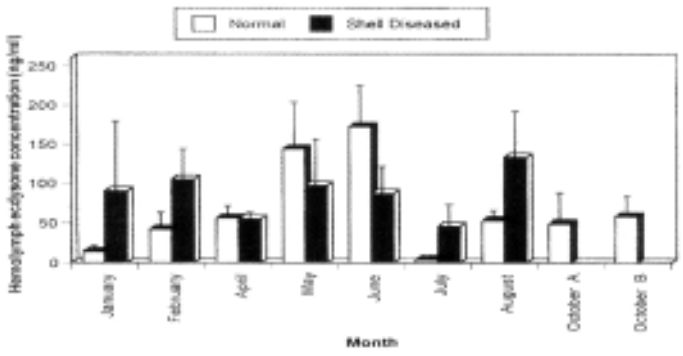

Figure 2. Hemolymph ecdysone concentrations in normal lobsters versus shell diseased lobsters collected during the year. Error bars show standard error of the mean. October A: Lobsters collected from Long Island Sound. October B: Lobsters collected from the Massachusetts Lobster Hatchery.

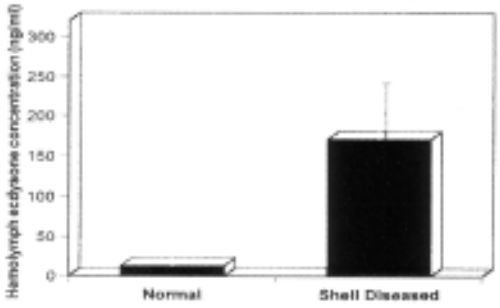

Figure 3. Comparison of hemolymph ecdysone concentrations between a normal lobster carrying eggs (n=1) and shell diseased lobsters carrying eggs (n=5). Ecdysone concentrations were found to be much higher in the shell diseased lobsters, which had recently released eggs (a few eggs still remained on pleopods),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

2. Identification of CHH functions

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

Results

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

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

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

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

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

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

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

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

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

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

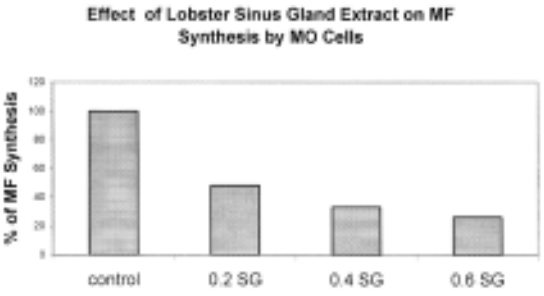

Figure 4. Inhibition of the MO by different quantities of sinus gland extract. Inhibition increases proportionally to increasing amounts of SG peptides.

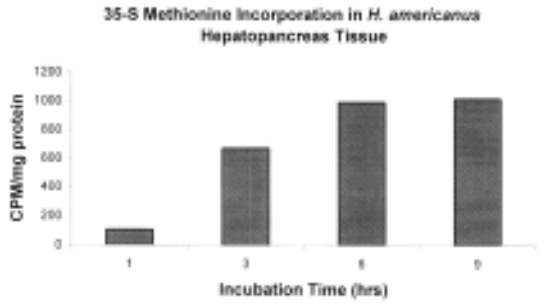

Figure 5. Amino acid incorporation into vitellogenin proteins over time. Proteins are produced linearly in vitro for up to 6 hours.

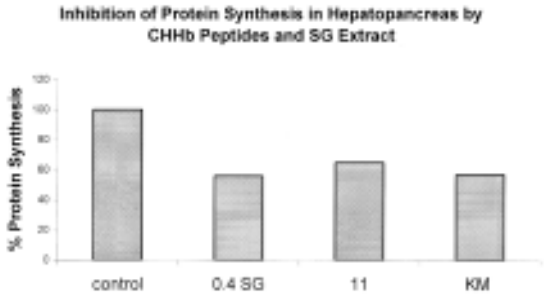

Figure 6. The effect of a CHHB cloned peptide on hepatopancreas protein synthesis.

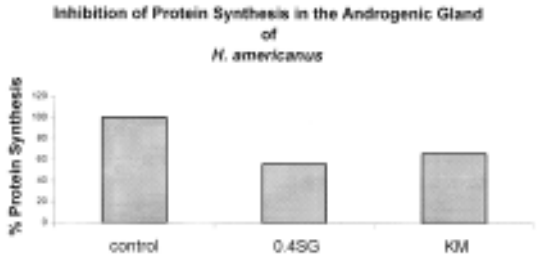

Figure 7. The effect of a CHHB cloned peptide on the AG. The KM CHHB peptide clone exhibits inhibition of the AG similar to sinus gland extracts.

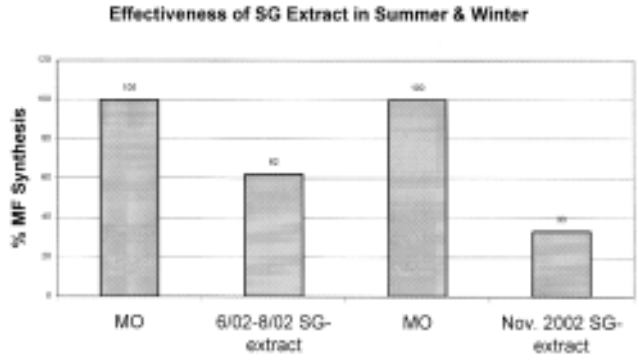

Figure 8. A comparison of MOIH activity found in the sinus gland of lobsters over the summer and winter months. SG Extracts from lobsters in the winter show 1/3 more MO inhibition.

3. Stresses on Larvae

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

Results

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

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

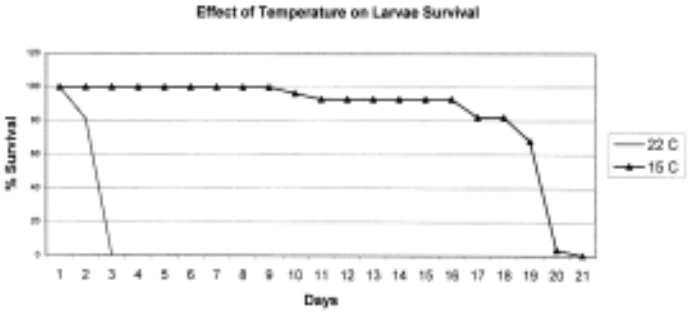

Figure 9. Larvae survival at different temperatures. Larvae kept at 15 ºC in the laboratory live 7 times as long as larvae kept at 22 ºC.

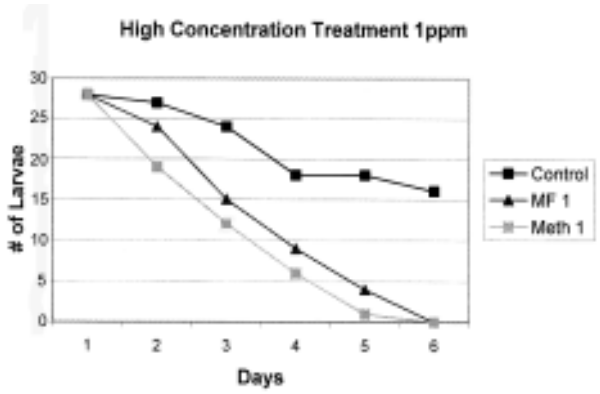

Figure 10. Effect of MF and Methoprene on larvae. A concentration of 1ppm of each compound kills 2nd stage larvae within 6 days at 15 ºC in the laboratory.

Conclusions

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

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

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