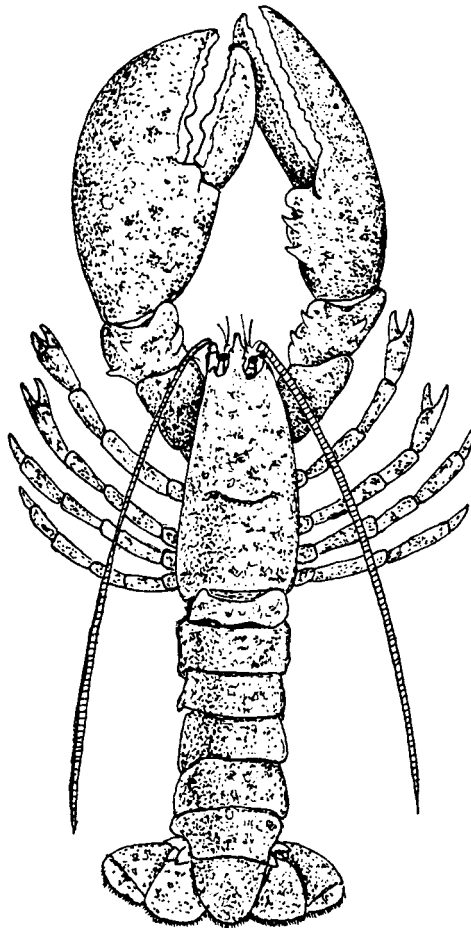# *Parasites and Disease*

Progress in *Paramoeba* Research

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

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

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

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

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

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

7. It has so far not been possible to cultivate the lobster pathogen. We have very recently had success, however, in cultivating *Neoparamoeba* species from moribund sea urchins (*Strongylocentrotus droebachiensis*)
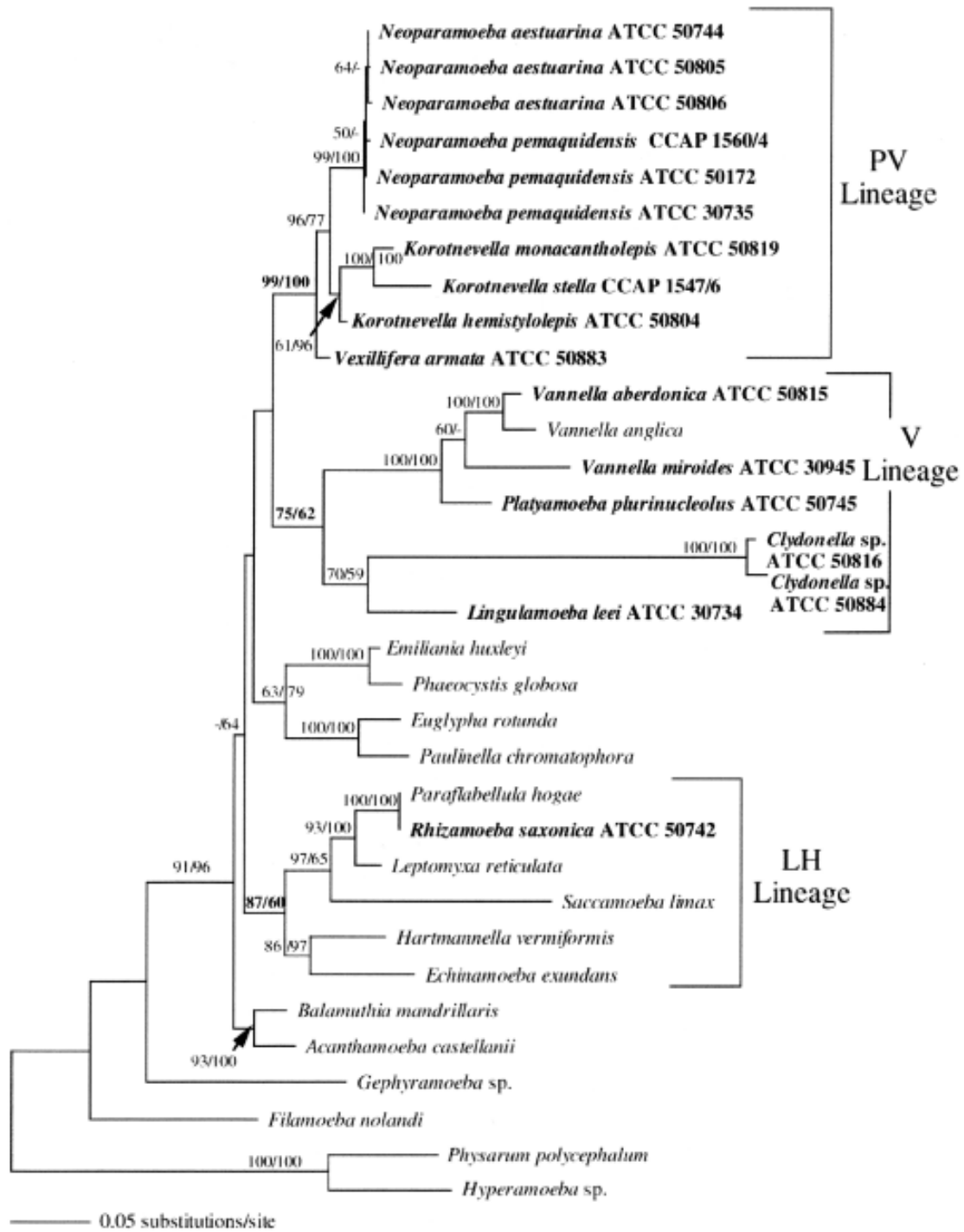
Figure 1. Phylogenetic tree showing the position of the Paramoeba assemblage (PV lineage) with respect to other groups of amoebae and selected other protists. From a manuscript submitted to the Journal of Eukaryotic Microbiology. Additional species not shown on the tree have been found to belong to the PV lineage in preliminary analyses, including the lobster pathogen (species of Neo-paramoeba), Paramoeba eilhardi, Pseudoparamoeba pagei, and species of Mayorella.
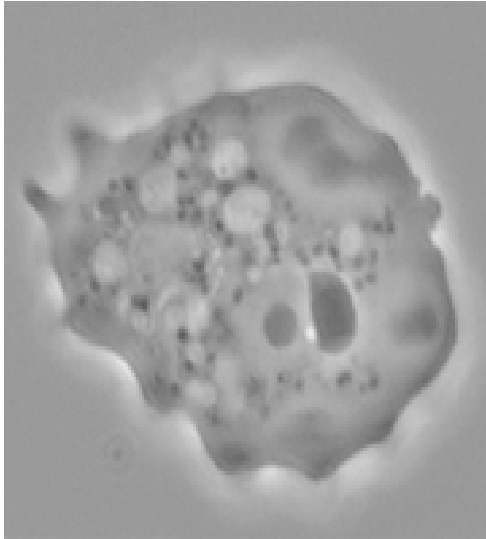
Figure 2. Light micrograph (phase contrast optics) of strain 1 of the Neoparamoeba species isolated (tentatively into axenic culture) from green sea urchins in the Gulf of Maine. The cell is ca. 10 μm in length. Unpublished. Cells of strain 2 are half again as large (ca. 15 μm length) and may have up to three parasomes.

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

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

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Introduction

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

Hypothesis and Objectives

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

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

Methods

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

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

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

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

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

Results

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

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

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

Acknowledgements

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Oligonucleotide-based Detection of Pathogenic *Neoparamoeba* Species

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Introduction

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

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

Objectives

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

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

Progress to Date

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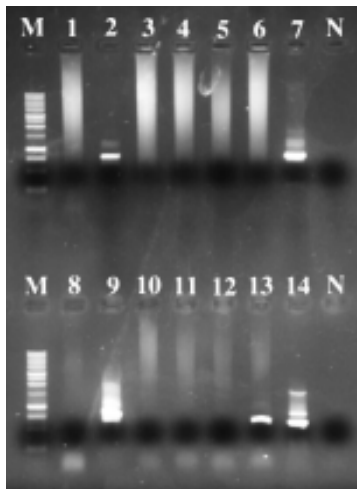

Figure 1. PCR amplification and reamplification of samples using Neoparamoeba-specific primers P1, P2 and P3R. Lane 1, 8 – uninfected lobster DNA; lane 2, 9 – LIS Neoparamoeba isolate A4S; lane 3, 10 – lobster DNA 1268; lane 4, 11 – lobster DNA 1280; lane 5, 12 – lobster DNA 1352; lane 6, 13 – lobster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

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

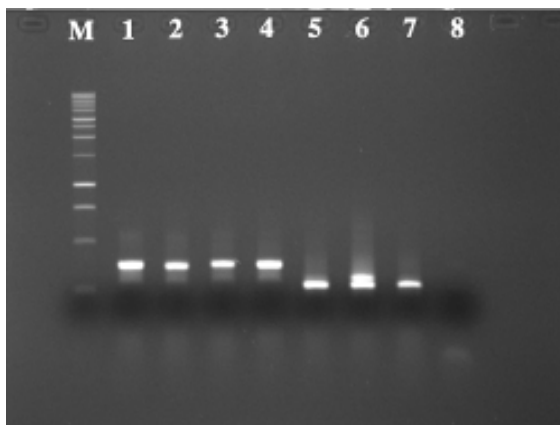*Figure 2. PCR amplification of bands picked from DGGE gel. Lanes 1 - 4 are products that are the correct size for the fragment of the Neoparamoeba small subunit ribosomal gene. Lanes 5 – 7 are products that are the correct size for the products from the lobster gene. Sequencing will be used to confirm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

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

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

Project Significance

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

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

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

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

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

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

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

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

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

Table 1. Bacteria in hemolymph of lobsters:

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

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

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

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

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

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

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

* NT, not tested.

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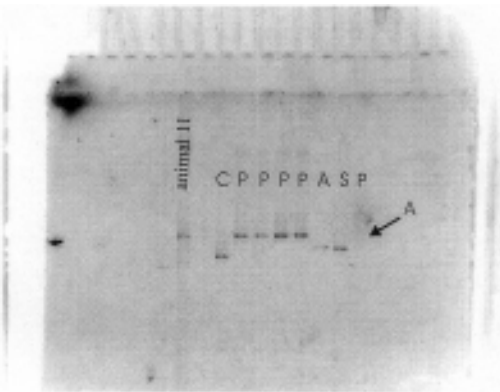

Figure 1. A DGGE gel of the whole bacterial community from lobster #1, along with individual isolates from the lesions of this animal.

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

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

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

Inflammation in epizootic shell disease is composed of increased numbers of mixed populations of hemocyte types in the underlying connective tissues and accumulations of usually necrotic hemocytes between layers of carapace. Inflammation, and cuticular proliferation, as well as melanization, of the affected cuticle are also seen in impoundment shell disease and are general responses to erosion of the cuticle for any reason.
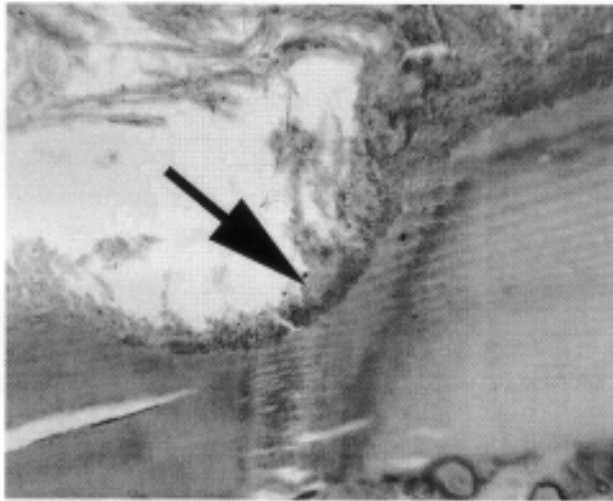
Figure 2. Early erosions into the epicuticle and exocuticle show invasion primarily around the carapace pores and vertical extension deep into the exocuticle. These erosive areas are melanized. As the lesions deepen, and the exocuticle is eroded, they leave behind cuticular matrix that forms skeletonal pillars in the holes.

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

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

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

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

Calcinosis in LIS Lobsters During Summer 2002

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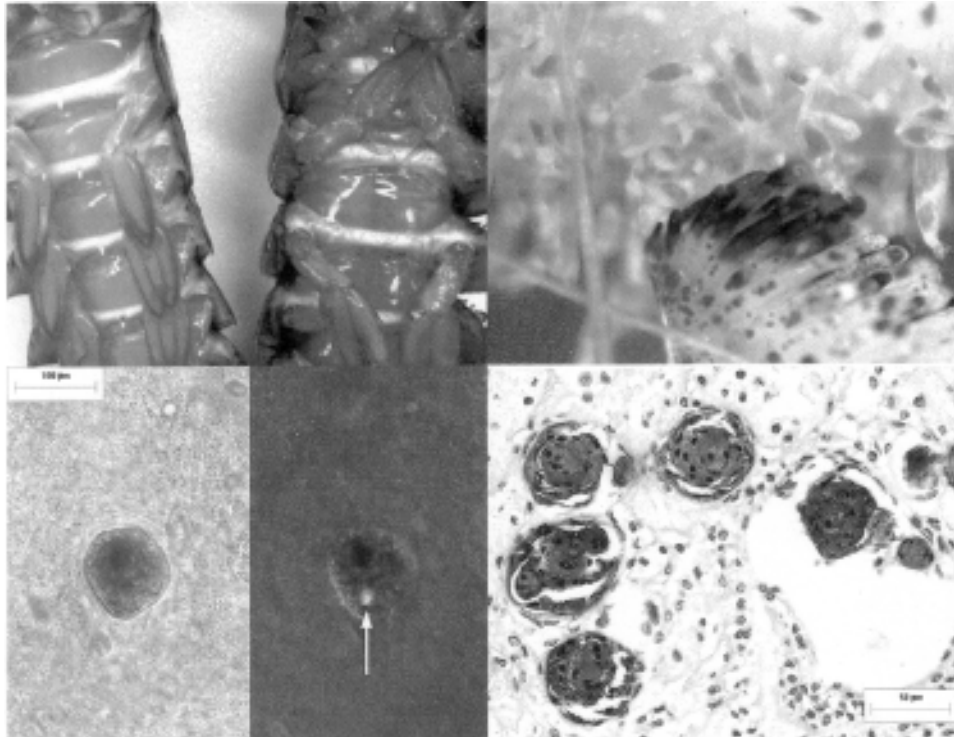[2]NY Department of Environmental Conservation, East Setauket, NY
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A significant number of moribund and dead lobsters were reported to state authorities by lobster fishers in Long Island Sound, New York, during the summer of 2002. The first of these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

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

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

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

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

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