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**Characterization of cuticular and hemolymph associated defense
parameters in shell diseased lobsters**

A Thesis Presented

by

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to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science in

Marine and Atmospheric Science

Stony Brook University

May 2009

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Abstract of the Thesis

Characterization of cuticular and hemolymph associated defense parameters in shell diseased lobsters

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Master of Science

in

Marine and Atmospheric Science

Stony Brook University

2009

Epizootic shell disease syndrome (ESD) is known to affect lobsters (*Homarus americanus*) in eastern Long Island Sound (ELIS) and the near shore waters of southern New England. Marring the shell of individuals, ESD decreases the economic value of infected lobsters, but is known to lead to mortality in only the most diseased individuals. While prevalence of ESD varies spatially and temporally, it tends to be the most common in areas around Buzzard's Bay Rhode Island and ELIS, and least prevalent in offshore canyons, off the coast of Maine, and in western Long Island Sound (WLIS).

To investigate the potential role of the immune system in determining an individual's or population's susceptibility to ESD, the immunocompetence of both ESD-affected and healthy lobsters from ELIS, WLIS, and Boothbay Harbor Maine was assessed in the late spring (June 2007) using several assays, including the measurement of phenoloxidase activity, phagocytosis rates and respiratory burst in hemocytes, and antimicrobial activity in plasma. In light of recent findings concerning the presence of defense-related factors in crustacean shells, we also evaluated healthy and diseased shell, measuring the shell mass to surface area ratio, and quantifying any phenoloxidase-like activity and antimicrobial-activity of a shell homogenate extract (SHE). To assess how the immune system of lobsters responds to ESD in relation to seasonal changes, we measured internal and external defense parameters of ELIS lobsters during mid-summer (August 2007), early fall (October 2007) and the following spring (June 2008) in addition to the initial late-spring sampling.

Despite high inter-individual variability in defense-related factors, results showed significantly higher protein concentrations and phenoloxidase activity of the hemocyte lysate in diseased lobsters as compared to healthy individuals. Mean values of phagocytic activity of circulating hemocytes and antimicrobial activity appeared to be higher in diseased lobsters, but these differences were not significant. Interestingly, ELIS lobsters had lower rates of phagocytosis, lower oxidative burst and antimicrobial activity in addition to higher initial concentrations of ROS, relative to WLIS lobsters, suggesting that the disproportionately high prevalence of ESD in ELIS may be related, at least in part, to the reduced immunocompetency of ELIS lobsters. Similarly, carapace thickness was higher in WLIS lobsters as compared to ELIS individuals as indicated by higher mass to surface area ratios. Results also revealed that lobster shell had measurable antimicrobial and phenoloxidase-like activities, but preliminary findings suggested that these did not appear to play a major role in determining the susceptibility of an individual to ESD.

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Preface

This work is part of a collaborative study designed to provide an integrative approach aiming to characterize the microbial community of healthy and epizootic shell disease (ESD) affected lobsters from Eastern and Western Long Island Sound as well as identify any immunological parameters that may make any one individual or population more susceptible to ESD, and characterize immunological response to ESD.

Results of this study will be reported to Rhode Island Sea Grant as part of a larger consortium of projects designed to address the issue of shell disease in New England waters.

Acknowledgments

I would like to acknowledge the financial support for this project provided by Rhode Island Sea Grant. I am also grateful to New York Sea Grant for channeling my scholarship funds through the Sea Grant Scholar program.

I am particularly grateful to Don Landers (Millstone Environmental Laboratory), and to the New York State Department of Environmental Conservation and Maine's Department of Marine Resources for providing lobsters and environmental information.

I am also grateful to Professor Gordon Taylor and his team (Sheryl Bell and Jezreel Otto) for generating and sharing bacterial data presented in this thesis, and members of the Marine Animal Disease Lab (Sue Pawagi and Sarah Winnicki) and all of our volunteers who helped with sample processing.

Finally, I would like to thank the members of my thesis committee (Anne McElroy, Bassem Allam and Mark Fast) for endless hours spent advising and re-advising me.

Introduction:

The American lobster (*Homarus americanus*: H. Milne Edwards 1837) is found in the cold near-shore waters of the Atlantic coast. Although lobsters are found as far south as North Carolina, the commercial lobster industry only extends as far south as New York and is dominated by the colder-water fisheries of Maine and maritime Canada, with the waters off of Massachusetts and Maine producing 90% of all American-caught lobsters (ASMFS 2005). Lobsters have long been one of the most economically important fisheries in the New England area, with 2005 Maine lobster landings being valued at \$318 million, representing 80% of the value for all commercial fish and shellfish species of that state (NOAA 2006). Within the past ten years, however, several diseases affecting lobsters have emerged or become more prevalent, raising concern about the future of the lobster fishery.

Shell disease in crustaceans is not uncommon and has been documented for over 100 years. Classified as the presence of spots and/or pits on the carapace, shell disease is generally bacterial in origin and typically affects only a small number of individuals in wild populations. There are three recognized forms of shell disease in *H. americanus*: black spot disease, impoundment disease (Hess 1937) and, most recently, epizootic shell disease syndrome (ESD: Smolowitz et al. 2005).

First documented in 1997, ESD is identified by the severe erosion of the non-chitinous components of the dorsal carapace, leaving semi-bilaterally focal and multi-focal lesions on the exoskeleton (Smolowitz et al. 2005). The progression of the disease has been broken down into four stages, with individuals displaying small infrequent pits and/or minor lesions in stage 1, individuals displaying moderate to deep pits and lesions over a considerable portion of the carapace in stages 2 and 3, and individuals with lesions

covering the majority of the carapace and severe lesions and ulcers exposing connective tissue in stage 4. The severity of ESD can range greatly from individual to individual, and can increase rapidly in a short period of time (Castro et al. 2005). The prevalence and severity of ESD, however, tends to increase with the amount of time since the last molt, with 37 % of individuals appearing disease-free after molting (Landers et al. 2005). This leads to the highest prevalence just prior to the summer and fall molts (Castro et al. 2005, and Landers 2005), with mature female –who go longer between molts in order to rear eggs- being the most severely affected (Glen & Pugh 2005).

While the percentage of individuals displaying signs of ESD varies greatly from region to region, ESD is most prevalent in the shallow near-shore waters of Eastern Long Island Sound (ELIS, ~35 % at peak) and Rhode Island (~32 % at peak), and has not had an incidence greater than 5 % in the waters of Western Long Island Sound (WLIS), the Gulf of Maine or off-shore canyons (Castro & Cobb 2006). Recent reports, however, have indicated that ESD is moving northward into the more productive waters of Massachusetts and Maine (Tlusty et al. 2007), potentially posing a large economic threat.

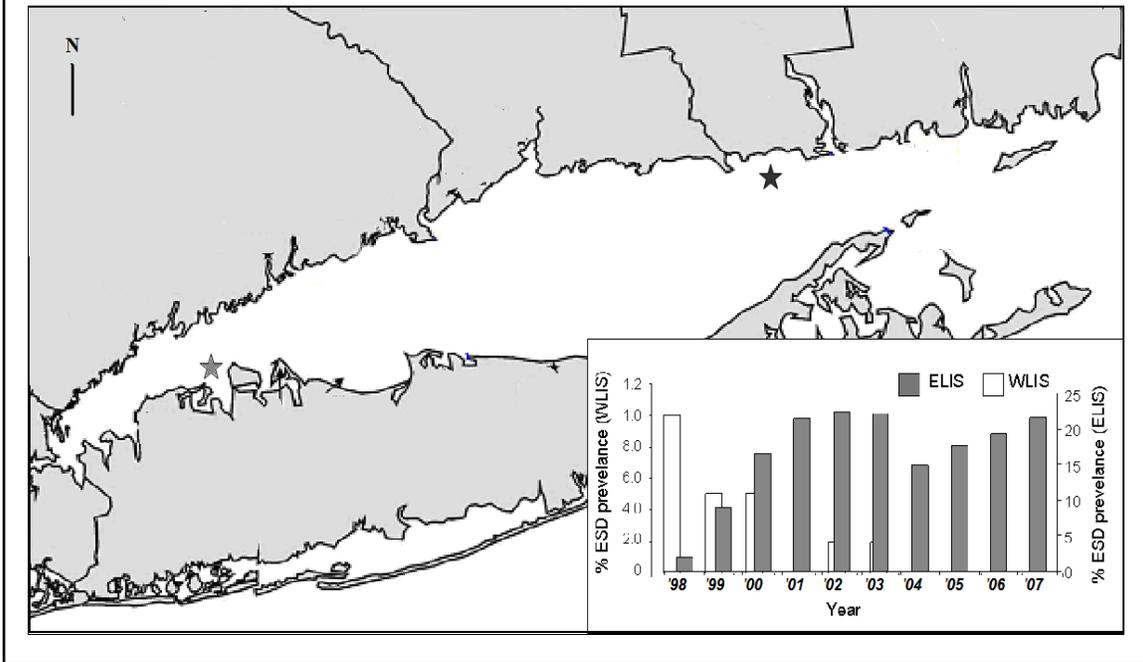
Immunocompetence is the most important factor in determining an individual's ability to resist infection and disease. Lacking an adaptive immune system, invertebrates, such as lobsters, depend on circulating hemocytes and innate antimicrobial substances in their plasma to ward off pathogens within the hemolymph. This can be done through the isolation of the pathogen by phagocytosis or the formation of nodules, or, the killing of the pathogen by the formation of melanin and other antimicrobial and lytic compounds. While the shell of the lobster has traditionally been viewed as solely a physical barrier to infection, recent findings in other crustaceans have provided evidence for the existence of antimicrobial proteins within the shell, as well as the presence of phenoloxidase (an

enzyme that regulates the formation of melanin) (Brey et al. 1995, and Asano & Ashida 2001).

Several studies have shown that the immune system of crustaceans can be weakened or suppressed under the effect of environmental stressors such as increased temperature (Sindermann 1971, Dove et al. 2005, and Zulkosky et al. 2005), the presence of contaminants (Smith et al. 1995, De Guise et al. 2004, and Draxler et al. 2005) or pathogens (Keith et al. 1992, and Hauton et al. 1997), and hypoxia (Le Moullac & Haffner 2000, Burnett & Stickle 2001, and Draxler et al. 2005). Additionally, the immunocompetence of an organism can vary between different genetically isolated populations (Bakke et al. 1990, Chevassus & Dorson 1990, and Ragone-Calvo & Burreson 2002).

Long Island Sound, with two lobster populations displaying very different ESD prevalence within a relatively small geographic range (see Figure 1), provided us with an interesting setting to examine factors related to differential susceptibility to ESD. By measuring a collection of internal and external defense parameters in healthy and ESD-affected individuals from ELIS and WLIS, we hoped not only to gain further insight into the lobsters' defense systems (this is especially true in regards to the external antimicrobial defenses), but to identify any differences in the immunocompetence of ELIS and WLIS lobsters- allowing us to better understand the difference in ESD prevalence between these two populations.

Figure 1: ESD prevalence within Long Island Sound.



Notes: Stars on map indicate sampling locations.

Materials and Methods

Study Design

To investigate potential differences in the immune capacity of lobsters from locations with different ESD prevalence within Long Island Sound, healthy and diseased lobsters were collected from 2 locations: Milford, Connecticut in Eastern Long Island Sound (ELIS, an area with a high ESD prevalence) and Oyster Bay, New York in Western Long Island Sound (WLIS, a more urbanized area with a maximum prevalence below 5 %). Healthy and diseased lobsters from Boothbay Maine (a remote area with a maximum ESD prevalence below 5 %) were used as a reference population.

To investigate temporal changes in lobster immunity relative to seasonal changes, healthy and diseased individuals were collected from ELIS in late spring (June 2007), mid-summer (August 2007), early-fall (October 2007), and the following spring (June 2008).

Lobsters

Long Island Sound lobsters were captured using double-entry vinyl-coated wire pots (76 × 51 × 30 cm; 2.5 cm mesh), identified as healthy (asymptomatic) or diseased (showing signs of ESD infection), banded and transported on ice to the lab and held in coolers overnight before processing. To minimize bacterial contamination, healthy and diseased lobsters were transported and kept in separate containers throughout processing. Lobsters from Maine were processed in a similar manner, being shipped overnight to the laboratory.

Before collecting hemolymph and tissue samples, swabs of each lobster's carapace were collected for bacterial culture analysis. Each lobster's weight, carapace length, disease status, and sex was also recorded, and the molt stage of each lobster was assigned based on the extent of setal development of the telson (Waddy et al. 1995).

Hemocyte Collection and Preparation

Using a 21-gauge needle, 1 ml of hemolymph was withdrawn from the base of the fifth walking leg into a 10 ml syringe containing 9 ml of ice-cold sterile crustacean anticoagulant (CAC, Soderhall & Smith 1983: 0.45 M NaCl, 0.1 M glucose, 0.3 M trisodium citrate, 0.026 M citric acid, and 0.01 M EDTA, pH 4.6). The diluted hemolymph was then transferred into two 5ml tubes and kept on ice until centrifugation (~10 minutes). Half of the hemolymph/CAC suspension was centrifuged (300 × g for 15 minutes at 4° C); the supernatant was discarded and the pellet was then resuspended in 1 ml filtered artificial seawater at 30 ppt, and used immediately in the phagocytosis and respiratory burst assays. The remaining half of the hemolymph/CAC suspension was centrifuged (800 × g, 10 minutes and 4° C), the supernatant was discarded and the pellet

was rinsed twice without suspension in 0.01 M sodium citrate cacodylate buffer (SCCB, Hauton et al. 1997: 0.45 M NaCl, 0.1 M Na₃ Citrate, and 0.01 M Sodium cacodylate, pH 7.0) air dried for 30 seconds and flash frozen using liquid nitrogen before being stored at -80° C until processing for the phenoloxidase assay.

Frozen hemocyte pellets were thawed on ice before adding 1 ml of ice-cold SCCB and disrupting the cells (10 seconds, pulsing at 50 % duty cycle) using an ultrasonic homogenizer (4710 series Cole-Palmer instrument Co., Vernon Hills, Illinois). The disrupted cell suspension was then centrifuged (1200 × g for 25 minutes at 4° C) in order to remove any cellular debris. The hemocyte lysate supernatant (HLS) was then decanted and placed on ice for immediate use in the phenoloxidase and protein assays.

An additional 1 ml of blood was drawn directly into a clean syringe and 200 µl were used to determine bacterial counts in hemolymph. The remaining volume was centrifuged (800 × g for 10 minutes at 4° C). The plasma was then decanted, flash frozen in liquid nitrogen, and stored at -80° C for the antimicrobial assay.

Shell Collection and Preparation

The exoskeleton of individual lobsters was removed and immediately placed in plastic bags and stored at -80° C for later processing. Samples were then thawed on ice and any excess tissue was removed from the exoskeleton using a weighing spatula. Samples were rinsed with sterile artificial seawater (30 psu) and the cellulose-like inner layer was removed before the shell was divided into healthy and diseased portions. Healthy and diseased portions of the shell were then weighed and amended (1:10, weight:volume) with sterile SCCB before homogenizing for 10 seconds on medium speed using a tissue homogenizer (Kinematica AG, Switzerland). The shell homogenate

was then centrifuged ($1000 \times g$ for 5 minutes at $4^{\circ} C$), before decanting and sterilizing the supernatant using a 22 μm syringe driven filter, to obtain sterile shell homogenate extract (SHE).

Measurement of Internal Defense Factors

Phagocytosis Assay

Phagocytic activity was measured using a plate reader method adapted from Dove et al. (2005). This assay measures the fluorescence of phagocytized fluorescently labeled *Vibrio* bacteria. Briefly, 100 μl of prepared hemocyte suspension was added to a 96 black-well plate and allowed to incubate in the dark at room temperature for 1 hour. Controls consisted of 100 μl of hemocyte suspension and 5 μl of 37 % formalin. After incubation, the supernatant was aspirated, and 100 μl of fluorescein isothiocyanate (FITC)-labeled *Vibrio* suspension was added to each well before incubating in the dark at room temperature for 1 hour. After incubation, the supernatant was aspirated and 100 μl of trypan blue solution (250 $\mu g/ml$ citrate buffer, pH 4.4) was added to each well in order to quench any extra-cellular fluorescence. After 1 minute, the dye was discarded and the fluorescence intensity (relative fluorescence units), was immediately determined at 485 nm excitation and 530 nm emission on a micro-plate reader (Wallac 1420 Multilabel Counter: Perkin Elmer, Wellesley, Massachusetts). Readings were averaged between 3 replicate test wells before being corrected for controls and standardized to protein concentration of the HLS.

Oxidative Burst Assay

Production of reactive oxygen species (ROS) by hemocytes was measured fluorometrically using micro-plate reader methods adapted from Moss and Allam (2006). In summary, 100 μl of prepared hemocyte suspension was added to each of four wells in a 96 black-well plate, before adding 20 μl of a working solution of dichlorofluorescein-diacetate (10 $\mu\text{g}/\text{ml}$ filtered artificial seawater (FASW) at 30 psu) (Rosenkranz et al. 1992) to each well. After an initial reading (485 nm excitation and 530 nm emission), 15 μl of a zymosan suspension (20 $\mu\text{g}/\text{ml}$ FASW at 30 psu) was added to each of 3 replicate-wells, with the exception of the control and then re-read on the plate reader. The plate was then incubated in the dark at room temperature for 30 minutes before being read a third and final time. Values were averaged between replicates and corrected for controls before being standardized to protein concentration of the HLS. The corrected value for the second reading was subtracted from the final reading to obtain the difference between initial and up-regulated concentrations of ROS in the hemocytes (level of oxidative burst).

Phenoloxidase Activity assay

Phenoloxidase activity of the hemolymph was measured using methods previously described (Zulkosky et al. 2005), which were adapted from an earlier enzymatic study (Soderhall & Smith 1983). A micro-plate version of the assay was performed by adding 75 μl of the prepared HLS to each of 3 wells on a 96 clear-well plate before adding 75 μl of trypsin solution (1 μg trypsin (from porcine pancreas)/ 1 ml SCCB), and incubating for 1 hour in the dark at 28° C. Control wells were incubated with 75 μl of buffer rather than trypsin solution. After incubation, 75 μl of saturated L-

Dopa solution (~1 mg L-Dopamine per 1 ml milli-q water) was added to all wells and absorbance at 490 nm was read immediately on a microplate reader, repeating every 30 seconds for 10 minutes. PO activity was measured as the rate of formation of dopachrome per minute. After subtraction for controls, the rate of change in the linear portion of the curve was averaged and normalized to protein content of the sample.

Protein Concentration of hemocyte lysate supernatant

Protein concentration of the hemocyte lysate supernatant (HLS) was determined using the Pierce Bicinchoninic acid protein assay micro-well plate protocol, using bovine serum albumin as the standard. To ensure consistency, each plate was incubated in the dark at room temperature for 30 minutes before reading at 590 nm on a micro-plate reader. The protein concentration of each sample was then determined by comparing the average absorbance of the replicates relative to that of a standard curve.

Antimicrobial Assay

The antimicrobial activity of the plasma was measured using a turbidimetric assay adapted from Noga et al. (1994), where *Escherichia coli* D31 (*E.coli* D31: Monner 1971) is treated with plasma and microbial growth is compared between treatments after 36 hrs of incubation. *E.coli* D31 was obtained from the culture collection at Yale University. Briefly, 5 ml of culture media (5 ml Tryptic Soy Broth with 1 % NaCl and 0.1 mg/ml streptomycin) was inoculated with *E. coli* D31 isolated from a Tryptic Soy Agar plate and incubated overnight at 35° C. The culture was then centrifuged at 300 × g for 5 minutes and rinsed in PBS solution twice. The resulting bacterial suspension was then

diluted to an optical density of 0.100 at 570 nm using sterile PBS solution, and then diluted an additional 200 times to obtain a prepared bacterial suspension.

Frozen plasma was thawed on ice before being sterilized using a 0.22 μm centrifuge driven filter cartridge. Ten μl of the sterile plasma was then mixed with 10 μl of the prepared bacterial suspension and 30 μl of PBS solution in a 1.5 ml tube and allowed to incubate for 30 minutes at 28° C. Negative controls included 10 μl of plasma and 40 μl of PBS solution, while the growth control was made of 10 μl of the prepared bacterial suspension and 40 μl of the PBS solution. After incubating, 450 μl of ice cold culture media was added to each tube and allowed to incubate at room temperature for an additional 36 hours. After incubation, 100 μl of each sample was pipetted into 3 separate wells (2 test and 1 control) on a 96-well clear micro plate and measured for absorbance at 570 nm. Inhibition was calculated using the average percent of growth in test wells relative to the growth control, once the negative control was subtracted from each sample.

Measurement of bacterial counts

Assessment of bacterial load in lobster hemolymph was carried out by Gordon Taylor's lab (Stony Brook University, New York). Briefly 100 μl of hemolymph was pipetted onto prepared marine agar and tryptic soy agar plates before being incubated at room temperature for 48 hours. The number of colony forming units (CFUs) on each plate was counted, and the highest number of CFUs between the two plates for each individual recorded.

Measurement of External Defense-Related Factors

Characterization of Phenoloxidase-like activity of the shell

Effect of trypsin

Preliminary trials indicated that the addition of trypsin did not enhance the phenoloxidase activity of SHE. To confirm this, the phenoloxidase-like activity of several randomly selected samples was run as described below, with the exception that 75 µl of prepared trypsin solution was added to the first two wells of each sample in place of 75 µl SCCB and the entire plate was incubated at 28° C for 30 minutes before the addition of L-Dopa solution.

Effect of size fractionation

Size exclusion cartridges were used in an attempt to characterize what component of the SHE contributed to the phenoloxidase-like activity measured. In summary, prepared SHE was left unfiltered or filtered at 10kDa, or 3kDa using centrifugal filter units (Millipore, microcon) to form 3 separate samples. Each sample was then measured for phenoloxidase-like activity as described below.

Effect of temperature

To investigate the role of enzymatic activity in the phenoloxidase-like activity of the SHE, prepared SHE was either boiled for 5 minutes at 100° C, or kept at 4° C. The phenoloxidase-like activity of each treatment was then measured as described below.

Phenoloxidase-like activity assay

The phenoloxidase-like activity of the shell homogenate extract (SHE) was measured using methods adapted from the hemolymph procedure above. The only change in the protocol was that the trypsin activation step was omitted since preliminary trials showed that the addition of this enzyme did not enhance the phenoloxidase-like activity of the SHE (see Results). Briefly, 75 μ l of sterile shell homogenate was pipetted into each of five wells (four test and 1 control), the volume of each well was brought up to 150 μ l using SCCB, and 75 μ l of saturated L-Dopa solution was subsequently added to each of the wells (with the exception of the control) just prior to reading on a micro-plate reader (490 nm). After subtraction of the control, the rate of change in absorbance of the linear portion of the curve was averaged between replicate wells and standardized to the weight of shell used to make the extract.

Protein concentration determination

Protein concentration of the SHE was determined using the Bradford method (Bradford 1976), using bovine serum albumin as a standard. Five μ l of prepared SHE was pipetted into each of two wells on a clear 96 clear-well microplate, 250 μ l of prepared Coomassie assay reagent (Bradford™: Pierce Protein Research Products) was then added to each well before being mixed on a plate shaker for 30 seconds. To ensure consistency, the plate was then incubated in the dark at room temperature for 10 minutes before measuring the absorbance (590 nm) of each sample. The protein concentration of each sample was then determined by comparing the average absorbance of the two replicates relative to that of a standard curve.

Antimicrobial activity

The antimicrobial activity of the shell homogenate was measured using the methods described for plasma above, with the exception that SCCB was used in place of PBS. Briefly, 10 µl of sterile SHE and 10µl of prepared bacterial suspension were combined in a sterile 1.5 ml Eppendorf tube with 30 µl of SCCB.

Negative controls consisted of 10 µl of sterile SHE and 40 µl of SCCB, while growth controls consisted of 10 µl of prepared bacterial suspension and 40 µl of SCCB. After incubation (28° C for 30 minutes), 450 µl of ice cold growth media was added to each tube and allowed to incubate at room temperature for an additional 36 hours. The absorbance of the resulting cultures and percent growth inhibitions were determined as described above.

Shell mass to surface area ratio

Shell mass to surface area was calculated by dividing the mass of a shell piece (mg) by its surface area (mm²).

Data analysis

Statistics

Data were analyzed using SigmaStat statistical software (Ver. 3.11. Systat Software, Inc., San Jose, CA). One-way analysis of variances (1-way ANOVA) and two-way analysis of variances (2-way ANOVA) were used to analyze differences in the internal and external defense parameters between differing health/disease status, locations, and sampling dates. ANOVA treatments that generated probability values below 0.05 were systematically followed by a Holm–Sidak post-hoc test comparing

different data points (treatments or time intervals). When necessary, data were transformed using the \log_{10} , reciprocal or square root transform function within the SigmaStat software prior to ANOVA to generate a normal distribution. When transformation of the data did not meet the criteria for parametric testing, raw data were analyzed using the Kruskal-Wallis ANOVA on ranks test to determine differences between groups. Kruskal-Wallis treatments that generated probability values below 0.05 were systematically followed by a Dunn's post-hoc test comparing different groups (health/disease status, location, or sampling date). Differences between groups were considered statistically significant when the p-value was at or below five percent.

Indexes

A large number of different parameters were measured in this study. To summarize observed trends (and to allow for easy visual comparisons among groups) indices of relative difference were calculated from the percent difference of the mean value of test parameter relative to the mean value of its control as determined by the formula:

$$\left(\frac{\overline{GroupA} - \overline{GroupB}}{\overline{highest}} \right) \times 100 = \%difference,$$

where Group A is the group in which the comparison is being made (e.g. ELIS or diseased individuals), Group B is the control counterpart (e.g. WLIS or Maine, or healthy individuals) and highest refers to the group with the highest mean value of the investigated parameter. Although these indices are useful to present general trends, statistical comparisons were made on individual measurements as described above.

Results

Molt status between samplings:

The great majority of the lobsters sampled were in molt stage 1 (92%). The molt status of the lobsters sampled, did not vary between sampling dates ($p= 0.385$) or between sampling sites ($p= 0.389$) (data not shown).

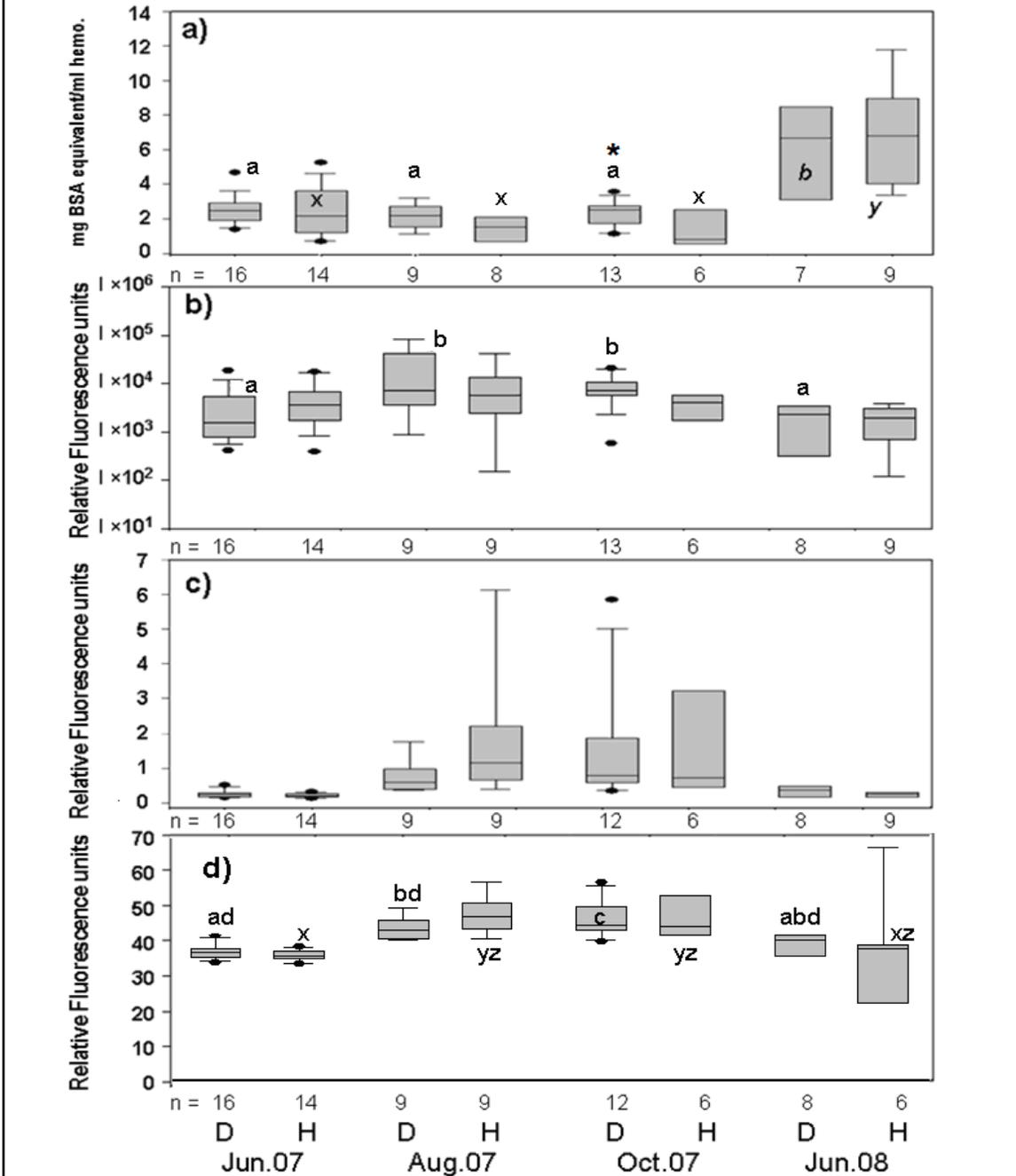
Internal immune defense parameters

Comparisons over time in ELIS lobsters

<u>Parameter</u>	<u>Test</u>	<u>Transformation</u>		<u>p-value</u>	<u>Significant?</u>	<u>Post-hoc test</u>	<u>Result</u>
Protein	2-way ANOVA	log 10	Date	<0.001	yes	Holm-Sidak	4>1,2,3 and 1>3
			Health status	0.010	yes	Holm-Sidak	Diseased > Healthy
			x	0.073	no	-	-
Phagocytosis	2-way ANOVA	log 10	Date	<0.001	yes	Holm-Sidak	4<2,3
			Health status	0.530	no	-	-
			x	0.244	no	-	-
Native ROS	2-way ANOVA	reciprocal	Date	0.013	yes	Holm-Sidak	4<1
			Health status	0.731	no	-	-
			x	0.462	no	-	-
Oxidative Burst	Kruskal Wallis	none	Date	<0.001	yes	Dunn's Method	2,3>1,4
	Kruskal Wallis	none	Health status	0.432	no	-	-
			x	-	-	-	-
Phenoloxidase	2-way ANOVA	none	Date	0.006	yes	Holm-Sidak	3>1
			Health status	0.018	yes	Holm-Sidak	Diseased > Healthy
			x	0.177	no	-	-
Antimicrobial	Kruskal Wallis	none	Date	<0.001	yes	Dunn's Method	2<1,3,4
	1-way ANOVA	none	Health status	0.309	no	-	-
			x	-	-	-	-
CFUs	Kruskal Wallis	none	Date	<0.001	yes	Dunn's Method	4>1,2,3
	Kruskal Wallis	none	Health status	0.538	no	-	-
			x	-	-	-	-

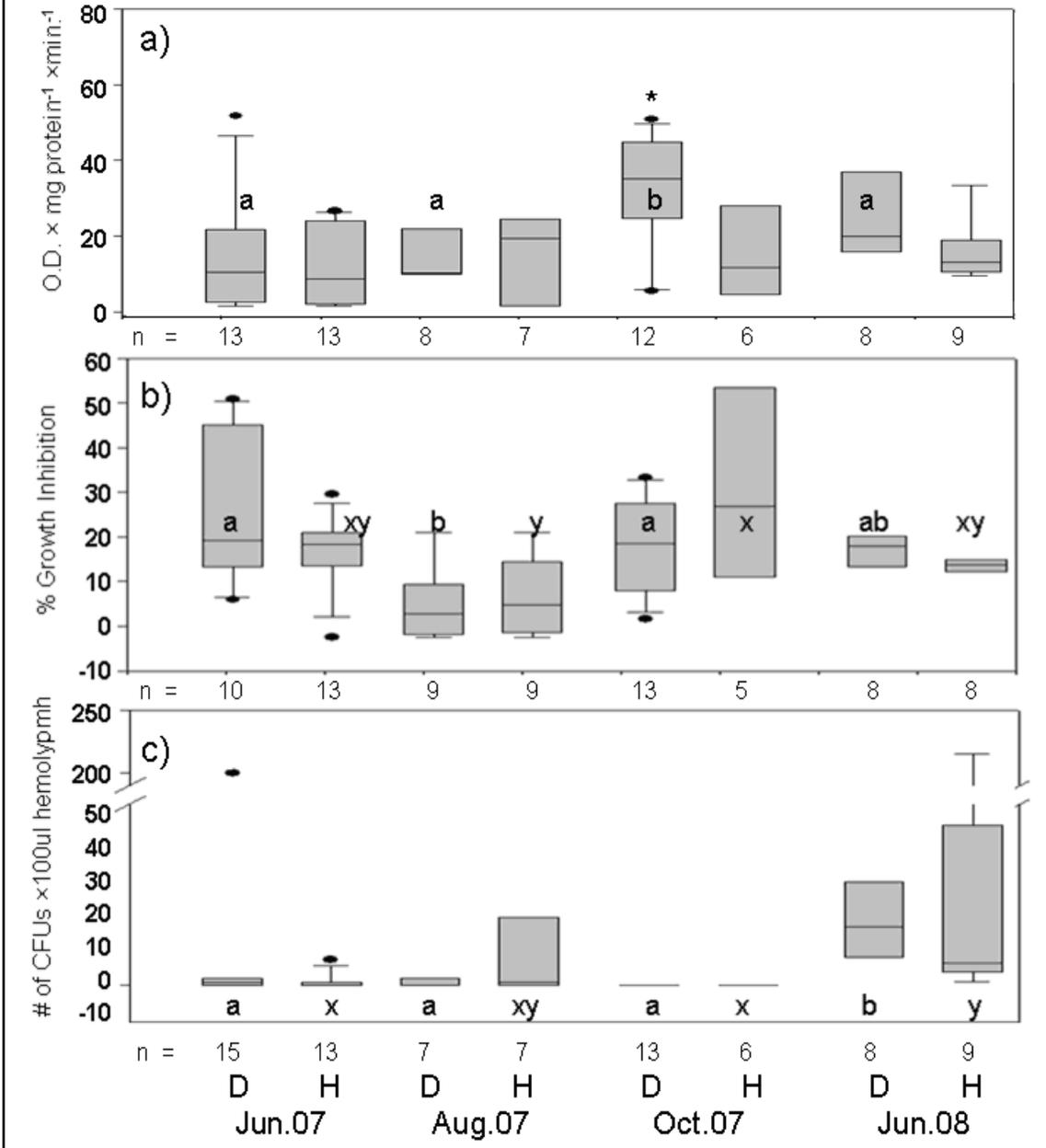
CFUs refers to colony forming unit counts in hemolymph. ANOVA's that were significant were followed by a Holm-Sidak post-hoc test and results were reported. When data did not fulfill requirements for parametric tests, a non-parametric Kruskal-Wallis test was performed followed by Dunn's Post-hoc test when applicable. In the Result column: 1, 2, 3 and 4 refer to lobsters sampled in June 2007, August 2007, October 2007 and June 2008, respectively. If data was not normally distributed, transformations were made as indicated. Differences were considered to be significant when $p \leq 0.05$.

Figure 2: Temporal variation of hemolymph parameters in healthy and diseased ELIS lobsters (a) protein contents in hemocyte lysates, (b) hemocyte phagocytic activity, (c) native and (d) induced ROS production in hemocytes.



The central line through each box indicates the median value of the represented sampling group, while the lower boundary and upper boundary of each box indicates the 25th percentile and the 75th percentile respectively; whiskers indicate standard error around the median and dots indicate outliers within the sample. Labels on the x-axis indicate the number of lobsters sampled, sampling dates and group health status, where D = diseased and H = healthy. Where significant differences were found, letters indicate which samples shared statistically equivalent (same letters) or different (different letters) means among symptomatic (a, b, c and d) or asymptomatic (x, y and z) individuals (1-way ANOVA). (*) indicate significant differences between healthy and diseased individuals within each sampling date (2-sample comparison). Significant differences in pooled samples (D and H) from each sampling date are expressed in Table 1.

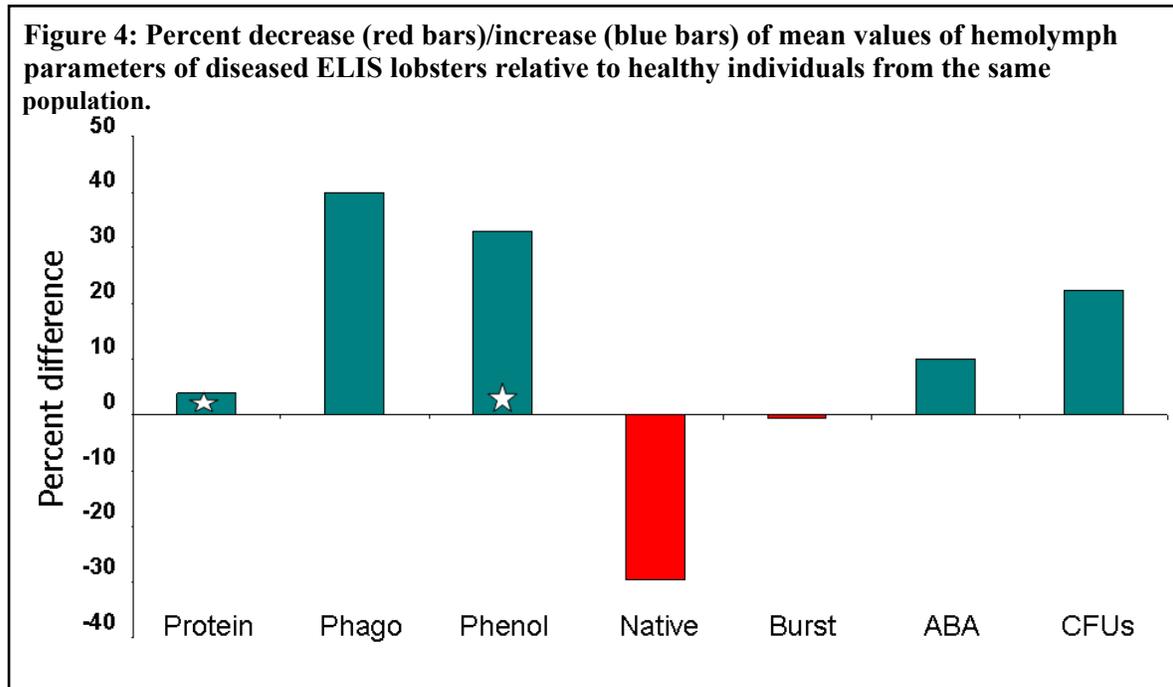
Figure 3: Temporal variation of hemolymph parameters in healthy and diseased ELIS lobsters: (a) phenoloxidase activity of hemocyte lysate, (b) percent bacterial growth inhibition by plasma, and (c) maximum # of bacterial colony forming units per 100µl hemolymph.



The central line through each box indicates the median value of the represented sampling group, while the lower boundary and upper boundary of each box indicates the 25th percentile and the 75th percentile respectively; whiskers indicate standard error around the median and dots indicate outliers within the sample. Labels on the x-axis indicate sampling dates and group health status where D = diseased and H = healthy. Where significant differences were found, letters indicate which samples shared statistically equivalent (same letters) or different (different letters) means among symptomatic (a and b) or asymptomatic (x and y) individuals (1-way ANOVA). (*)'s indicate significant differences between healthy and diseased individuals within each sampling date (t-test). Significant differences in pooled samples (D and H) from each sampling date are expressed in Table 1.

The internal immune parameters of lobsters (ESD-affected and healthy combined or separately analyzed) from ELIS varied significantly between sampling dates (Table 1, Figures 2 and 3). The protein concentration of the hemocyte lysate, which represents a proxy for circulating hemocyte counts, was significantly higher in June of 2008 than all other sampling dates (Table 1 and Figure 2). Additionally, the protein concentration of the hemocyte lysate from June 2007 was significantly higher than that measured in lobsters sampled in October of 2007. Hemocytes collected from lobsters sampled in June 2008 had significantly lower phagocytic activity than those withdrawn from lobsters sampled in August or October of the previous year (Table 1). Initial (native) production of reactive oxygen species (ROS) by hemocytes from lobsters sampled in June of 2008 was significantly lower than that measured in lobsters sampled in June of the previous year, while the increase in ROS production (oxidative burst) in hemocytes of lobsters sampled in June of both years was lower than that of lobsters sampled in August and October of 2007. The hemocyte lysate collected from lobsters sampled in June of 2007 had significantly lower phenoloxidase activity than that of hemocyte lysate obtained from lobsters sampled in October of 2007 (Table 1 and Figure 3). The antibacterial activity of the plasma withdrawn from lobsters sampled in August of 2007 was significantly lower than that of the plasma isolated from lobsters at all other sampling dates (Table 1), and the number of bacterial colony forming units (CFUs) found in the hemolymph of lobsters sampled in June 2008 was significantly higher than that of the hemolymph from lobsters sampled at any time point in 2007 (Table 1).

Overall comparison of internal immune status in healthy and diseased ELIS lobsters



Phago: phagocytic activity, Pheno: phenoloxidase activity, Native: baseline production of ROS, Burst: induced ROS production, ABA: antibacterial activity of plasma, CFUs: number of colony forming units in hemolymph. Stars indicate significantly higher or lower values (see Table 1, Appendix Table 1).

To allow for easy visual comparisons among groups, indexes of relative difference were calculated using the percent difference of the mean value of the test parameter to the mean value of its control counterpart. Significant trends of individual parameters were calculated using raw data and are reported above (see Table 1).

Diseased individuals had significantly higher protein concentrations (4 %) and phenoloxidase activity (33 %) in the hemocyte lysate when compared to healthy individuals (Table 1, Figure 4). While not significant, diseased individuals had 39 % higher phagocytic activity, and 22 % higher CFU counts in their hemolymph when compared to healthy individuals (Figure 4). Comparisons between healthy and diseased lobsters collected from Maine generated similar trends but differences were not significant because of the relatively small sample size (Appendix Table 1).

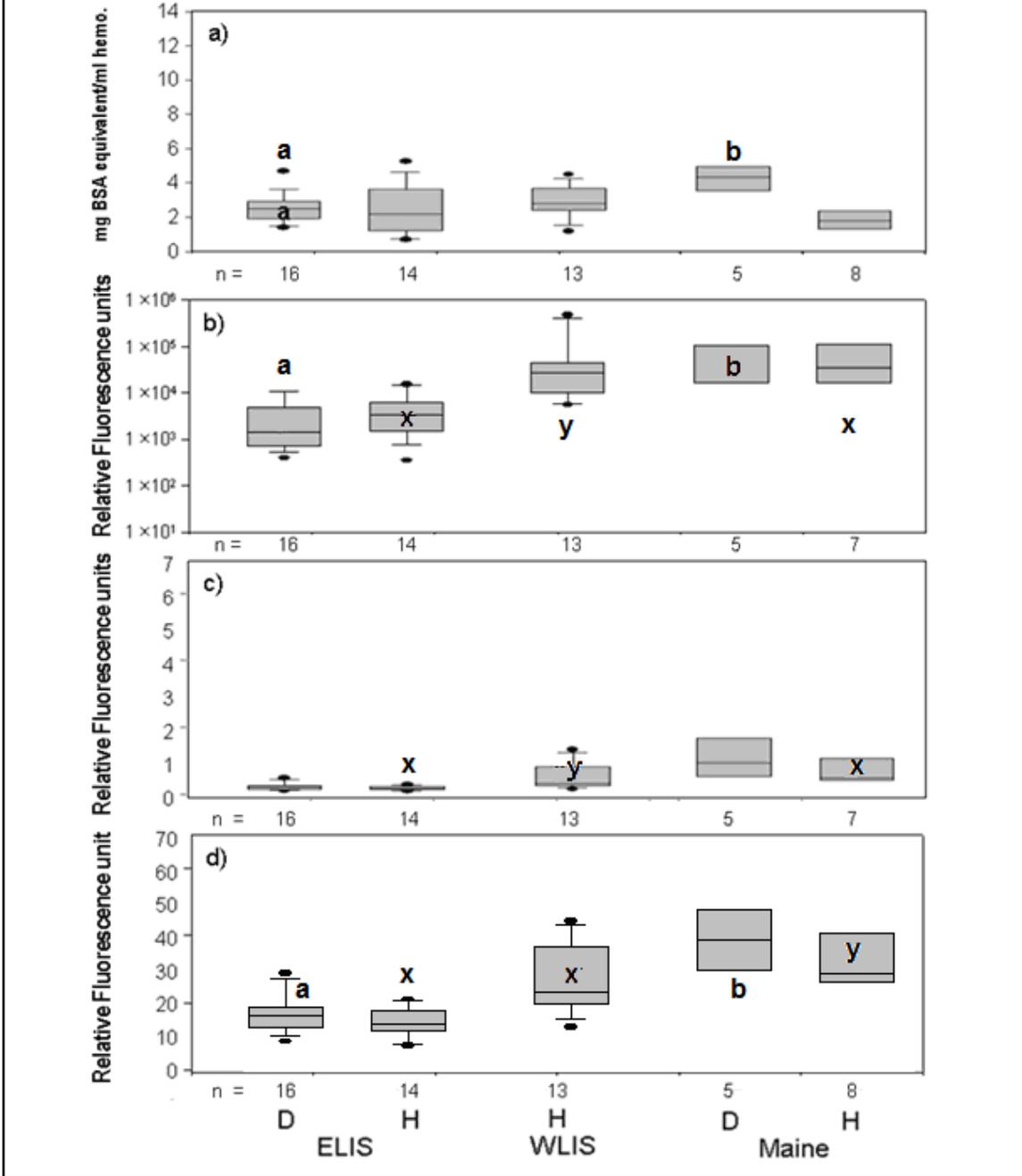
Spatial comparison of immune parameters

Table 2: Spatial analysis of hemolymph parameters between ELIS, WLIS and Maine in June 2007.

Parameter	Test	Transformation		p-value	Significant?	Post-hoc test	Result
Protein	1-way ANOVA	none	Healthy	0.191	no	-	-
Phagocytosis	1-way ANOVA	log10	Pooled	<0.001	yes	Holm-Sidak	E<W,M
Native ROS	Kruskal Wallis	none	Pooled	<0.001	yes	Dunn's Method	E<W
Oxidative Burst	1-way ANOVA	reciprocal	Pooled	<0.001	yes	Holm-Sidak	M>W>E
Phenoloxidase	1-way ANOVA	none	Healthy	0.083	no	-	-
Antimicrobial	Kruskal Wallis	none	Pooled	0.004	yes	Dunn's Method	E<W,M
CFUs	Kruskal Wallis	none	Pooled	0.019	yes	Dunn's Method	E<M

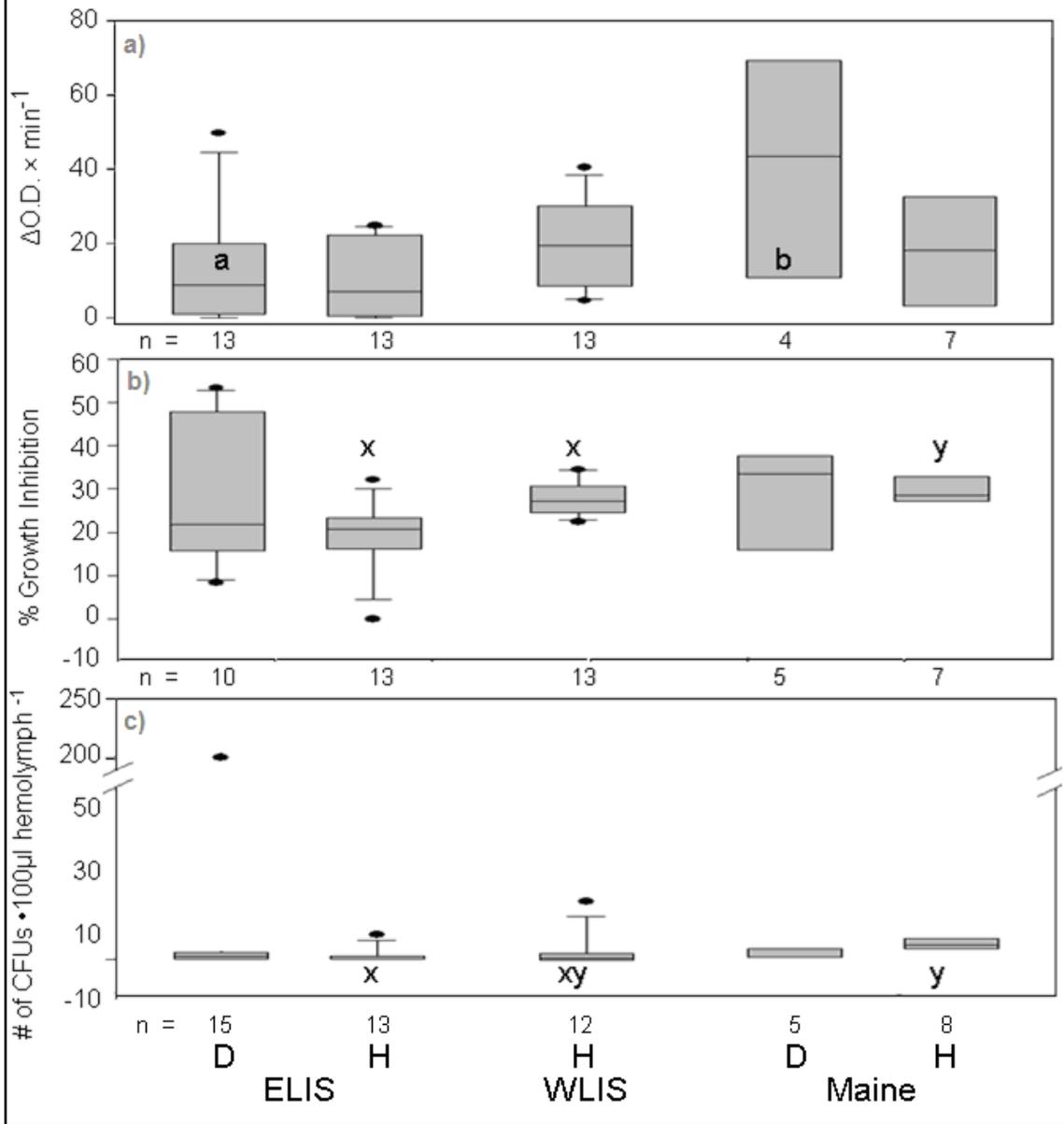
E, W and M refer to lobsters taken from Eastern Long Island Sound, Western Long Island Sound and Maine, respectively. Diseased and healthy individuals were pooled together, unless statistically different (refer to Table 1) in which case only samples taken from healthy individuals were considered. If data were not normally distributed, transformations were made as indicated. Tests that were significant were followed by Holm-Sidak post-hoc test and results reported. When 1-way ANOVAs were not possible, the non-parametric Kruskal Wallis tests were performed followed by Dunn's Post-hoc test when applicable. Differences were considered to be significant when $p \leq 0.05$, (-) indicates test was not applied.

Figure 5: Spatial variation of hemolymph parameters in healthy and diseased lobsters: (a) protein contents in hemocyte lysate, (b) hemocyte phagocytic activity, (c) native concentration of ROS in hemocytes, (d) increase in ROS species in hemocytes (respiratory burst).



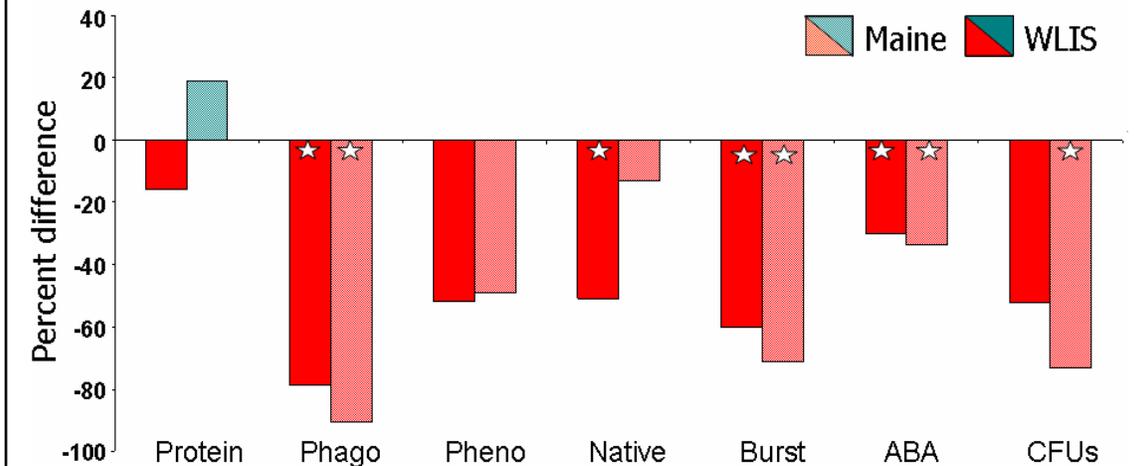
The central line through each box indicates the median value of the represented sampling group, while the lower boundary and upper boundary of each box indicates the 25th percentile and the 75th percentile respectively; whiskers indicate standard error around the median and dots indicate outliers within the sample. Labels on the x-axis indicate sampling dates and group health status where D = diseased and H = healthy. Where significant differences were found, letters indicate which samples shared statistically equivalent (same letters) or different (different letters) means among symptomatic (a and b, 2-sample comparisons) or asymptomatic (x and y, 1-way ANOVA) individuals. Significant differences in pooled samples (D and H) from each sampling location are expressed in Table 2.

Figure 6: Spatial variation of hemolymph parameters in healthy and diseased lobsters: (a) phenoloxidase activity of hemocyte lysate, (b) percent bacterial growth inhibition by plasma, and (c) maximum # of bacterial colony forming units per 100 μ l hemolymph.



The central line through each box indicates the median value of the represented sampling group, while the lower boundary and upper boundary of each box indicates the 25th percentile and the 75th percentile respectively; whiskers indicate standard error around the median and dots indicate outliers within the sample. Labels on the x-axis indicate sampling dates and group health status where D = diseased and H = healthy. Where significant differences were found, letters indicate which samples shared statistically equivalent (same letters) or different (different letters) means among symptomatic (a and b, 2-sample comparisons) or asymptomatic (x and y, 1-way ANOVA) individuals. Significant differences in pooled samples (D and H) from each sampling location are expressed in Table 2.

Figure 7: Percent decrease (red bars) / increase (blue bars) of mean values of hemolymph parameters of healthy ELIS lobsters relative to healthy individuals from WLIS and Maine.



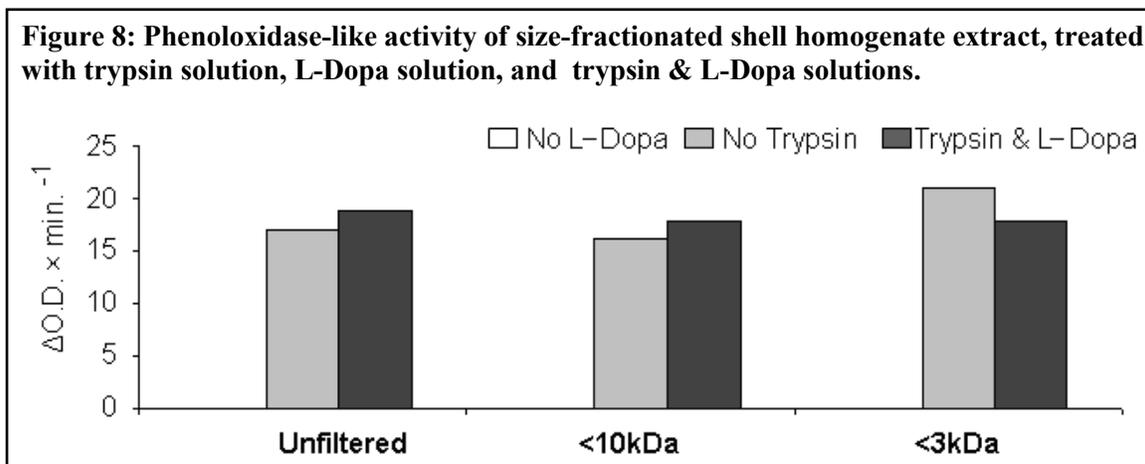
Phago: phagocytic activity, Pheno: phenoloxidase activity, Native: baseline production of ROS, Burst: induced ROS production, ABA: antibacterial activity of plasma, CFUs: number of colony forming units in hemolymph. Stars indicate significantly higher or lower values (see Table 2, Appendix Table 1).

While there was no significant difference in the protein concentration or phenoloxidase activity of the hemocyte lysate collected from lobsters sampled from ELIS, WLIS and Maine, ELIS lobsters displayed significantly lower immune responses relative to WLIS and Maine lobsters including: 78-90 % lower phagocytic activity of hemocytes, 29-33% lower plasma associated antibacterial activity, 49-51% lower oxidative burst in hemocytes, and 52-73% fewer CFUs in their hemolymph (Table 2, Figure 5, 6 & 7).

External defense related parameters

Characterization of phenoloxidase-like activity of the shell:

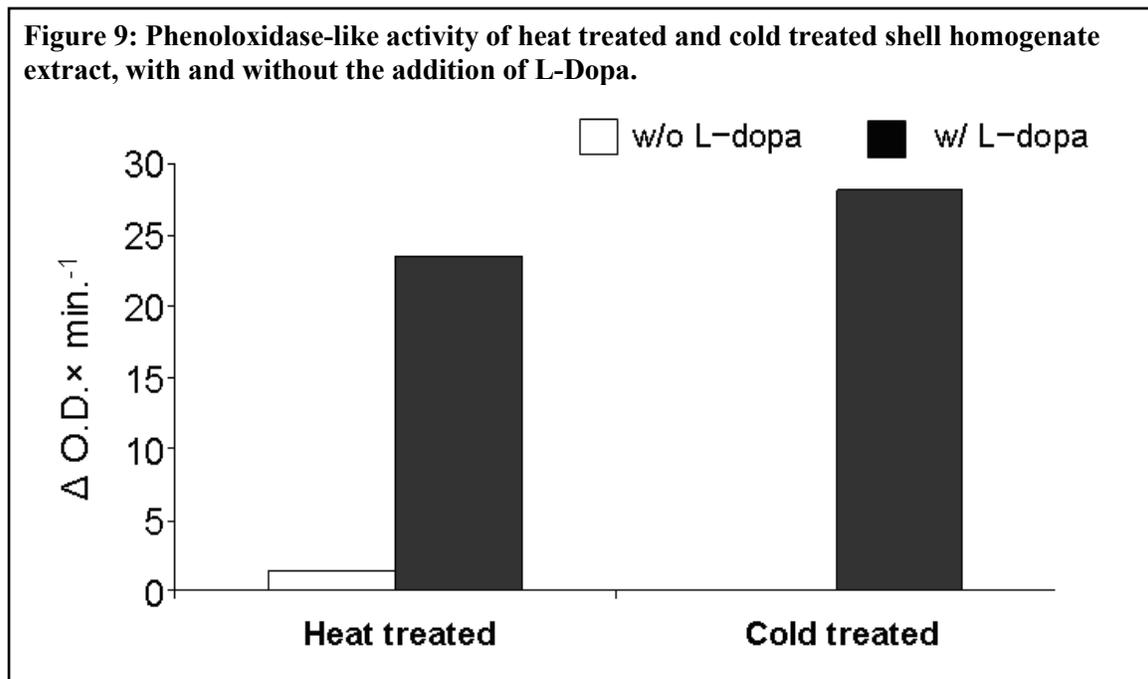
Effect of size fractionation and the addition of trypsin



Samples were obtained by pooling the shell homogenate extract from three separate individuals prior to size fractioning. Samples treated with trypsin solution (no L-DOPA), displayed no phenoloxidase activity.

A preliminary attempt was made to characterize the phenoloxidase-like activity of the shell homogenate. Phenoloxidase-like activity of the shell homogenate does not require the addition of trypsin to the sample. No dopachrome formation was observed without the addition of L-Dopa, however, ruling out the possibility that our results are due to the presence of an endogenous pigment absorbing at this wavelength (Figure 8). Size fraction had no affect on the rate of dopachrome formation, indicating that the molecule responsible for the phenoloxidase-like activity of the (SHE) is smaller than 3 kDa -the smallest size exclusion filter tested (Figure 8).

Effect of temperature



Boiling the sample for 5 minutes diminished the rate of dopachrome formation, but did not prevent the formation of dopachrome altogether (Figure 9). In addition, boiling the sample also appeared to trigger the formation of dopachrome, even prior to adding L-DOPA (Figure 9).

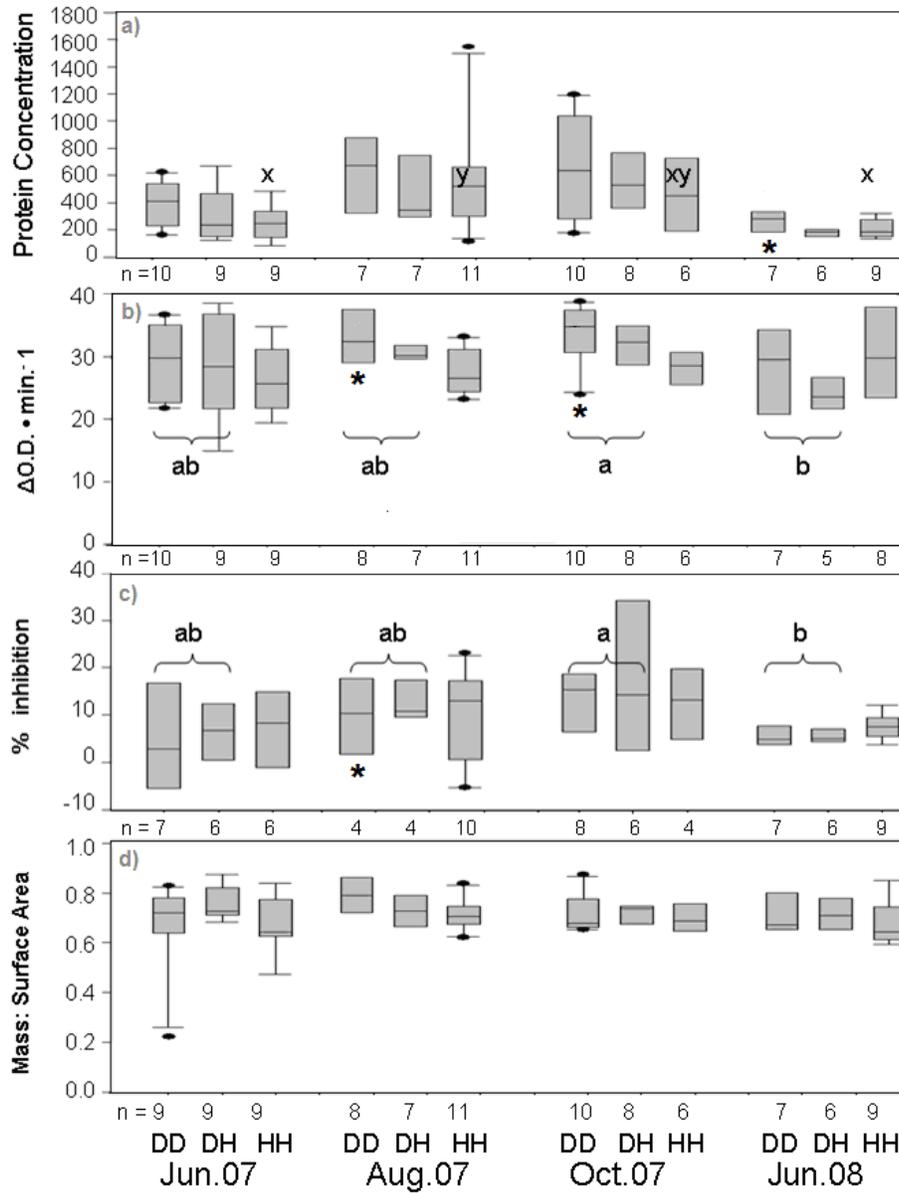
Comparison over time of shell associated defense parameters in ELIS lobsters:

Table 3: Statistical results for shell associated defense parameters of healthy and diseased shell from healthy and diseased ELIS lobsters sampled over time.

<u>Parameter</u>	<u>Test</u>	<u>p- Value</u>	<u>Post-hoc test</u>	<u>Result</u>
Protein	Kruskal Wallis	0.048	Dunn's	None
Phenoloxidase	Kruskal Wallis	<0.001	Dunn's	2,3>1,4
Antimicrobial	Kruskal Wallis	0.023	Dunn's	3>4
Mass : S.A.	Kruskal Wallis	0.141	-	-

In the result column, 1, 2, 3 and 4 refer to lobsters sampled in June 2007, August 2007, October 2007 and June 2008, respectively. When data did not fulfill requirements for parametric tests, a non-parametric Kruskal-Wallis test was performed followed by Dunn's Post-hoc test when applicable. Differences were considered to be significant when $p \leq 0.05$.

Figure 10: Temporal variation of shell defense parameters from ELIS lobsters: (a) mean protein concentration of shell homogenate, (b) Rate of phenoloxidase-like activity of shell homogenate as determined by relative fluorescence, (c) antimicrobial activity of shell homogenate, and (d) shell mass (mg) to surface area (mm²) ratio.



The central line through each box indicates the median value of the represented sampling group, while the lower boundary and upper boundary of each box indicates the 25th percentile and the 75th percentile respectively; whiskers indicate standard error around the median and dots indicate outliers within the sample. Labels on the x-axis indicate sampling dates and group health status. DD designates diseased shell from diseased individuals, DH designates healthy shell from diseased individuals, and HH indicates healthy shell from healthy individuals. Where significant differences were found, letters indicate which samples shared statistically equivalent (same letters) or different (different letters) means among symptomatic (a and b) or asymptomatic (x and y) individuals (1-way ANOVA). (*)'s indicate significant differences between healthy (HH) and diseased (DD and DH) individuals within each sampling date (2-sample comparison). Significant differences in pooled samples (DD, DH, and HH) from each sampling date are expressed in Table 3.

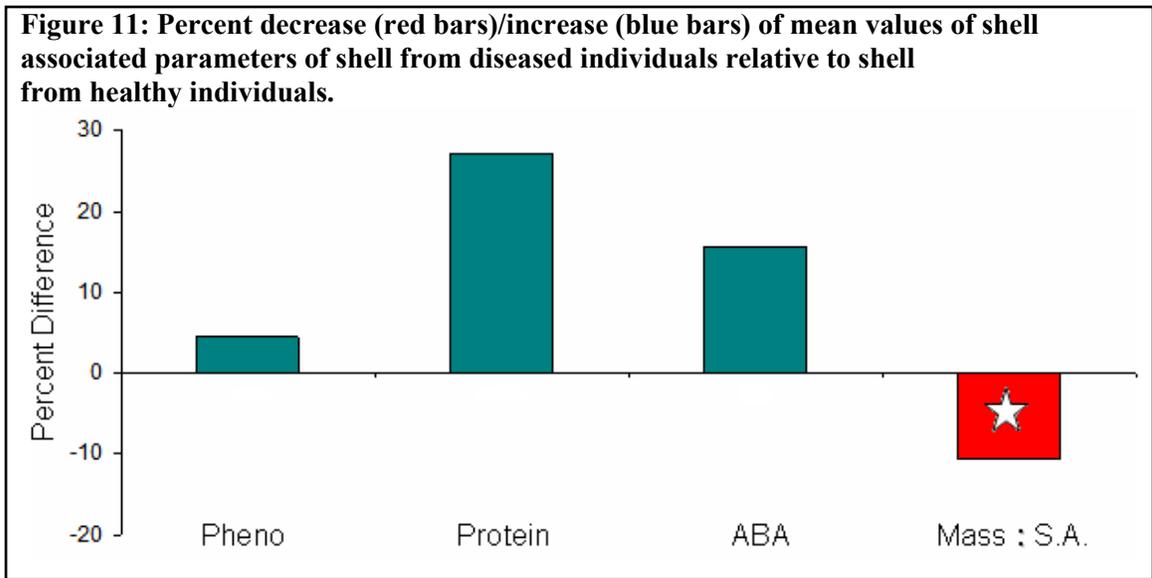
Results demonstrated the presence of antimicrobial activity and phenoloxidase-like activity in shell homogenates of healthy and diseased individuals at all sampling dates. While there was no significant difference in the shell mass to surface area ratio or protein concentration of the shell homogenate from lobsters collected at different sampling dates, the phenoloxidase-like activity of the shell homogenate collected from lobsters sampled in August and October of 2007 was significantly higher than that of the shell homogenate prepared from lobsters sampled in June of 2007 and 2008 (Table 3, Figure 10). Additionally, the antimicrobial activity of the shell homogenate from lobsters sampled in October of 2007 was higher than that measured in lobsters sampled in June of 2008 (Table 3, Figure 10).

Overall comparison of external parameters in healthy and diseased ELIS lobsters

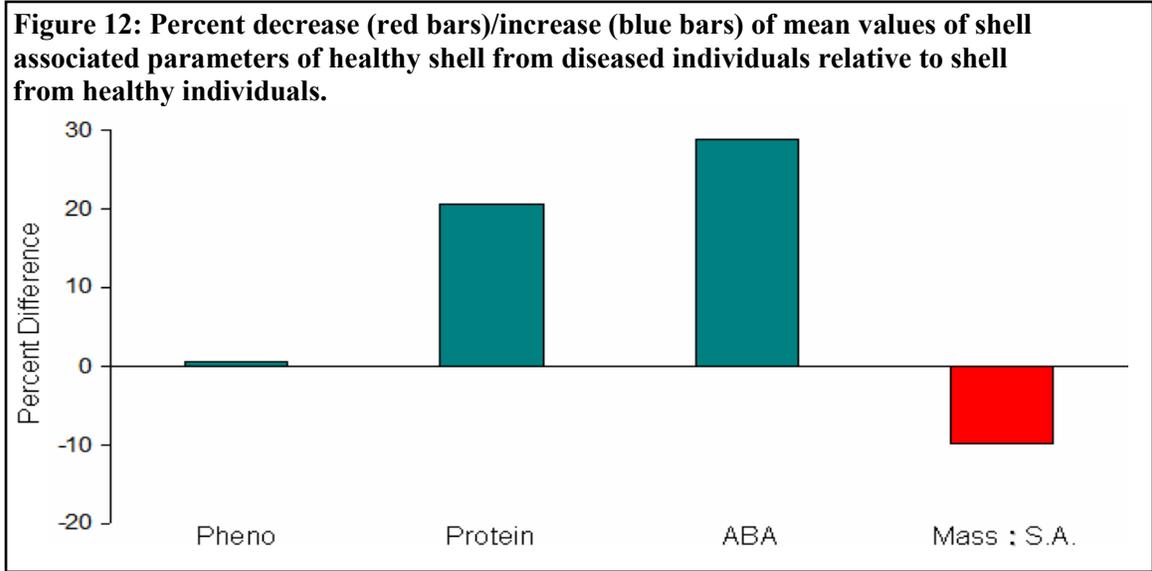
Table 4: Statistical results for shell associated parameters of healthy and diseased shell from ELIS lobsters

<u>Parameter</u>	<u>Test</u>	<u>Transformation</u>		<u>p-value</u>	<u>Significant?</u>	<u>Post-hoc test</u>	<u>Result</u>
<i>Protein</i>	1-way ANOVA	<i>Log₁₀</i>	<i>disease status</i>	0.564	no	-	-
	1-way ANOVA	<i>Log₁₀</i>	<i>shell status</i>	0.114	no	-	-
<i>Phenoloxidase</i>	1-way ANOVA	<i>none</i>	<i>disease status</i>	0.407	no	-	-
	1-way ANOVA	<i>none</i>	<i>shell status</i>	0.125	no	-	-
<i>Antimicrobial</i>	1-way ANOVA	<i>none</i>	<i>disease status</i>	0.734	no	-	-
	1-way ANOVA	<i>none</i>	<i>shell status</i>	0.739	no	-	-
<i>Mass to Surface Area Ratio</i>	1-way ANOVA	<i>none</i>	<i>disease status</i>	0.034	yes	<i>Holm-Sidak</i>	Diseased < Healthy
	1-way ANOVA	<i>none</i>	<i>shell status</i>	0.837	no	-	-

Disease status refers to the health status of the individual, while shell status refers to the status of the shell (taken from diseased individuals only). If data was not normally distributed, transformations were made as indicated. ANOVA's that were significant were followed by a Holm-Sidak post-hoc test and results were reported. When data did not fulfill requirements for parametric tests, a non-parametric Kruskal-Wallis test was performed followed by Dunn's Post-hoc test when applicable. Differences were considered to be significant when $p \leq 0.05$.



Pheno: phenoloxidase activity, Protein: protein concentration of shell homogenate, ABA: antibacterial activity of plasma, Mass: S.A.: Mass to surface area ratio of the shell. Stars indicate significant difference relative to healthy individuals.



Pheno: phenoloxidase activity, Protein: protein concentration of shell homogenate, ABA: antibacterial activity of plasma, Mass: S.A.: Mass to surface area ratio of the shell.

Although the shell had measurable antimicrobial activity, protein concentrations, and phenoloxidase-like activity, these did not differ between healthy and ESD-affected individuals (Table 4, Figure 11). The shell mass to surface area ratio, however, was significantly lower in ESD-affected lobsters (10%) when compared to healthy individuals

(Table 4, Figure 11). This 10% reduction was also seen when comparing only healthy shell from diseased individuals to shell from healthy individuals (Figure 12).

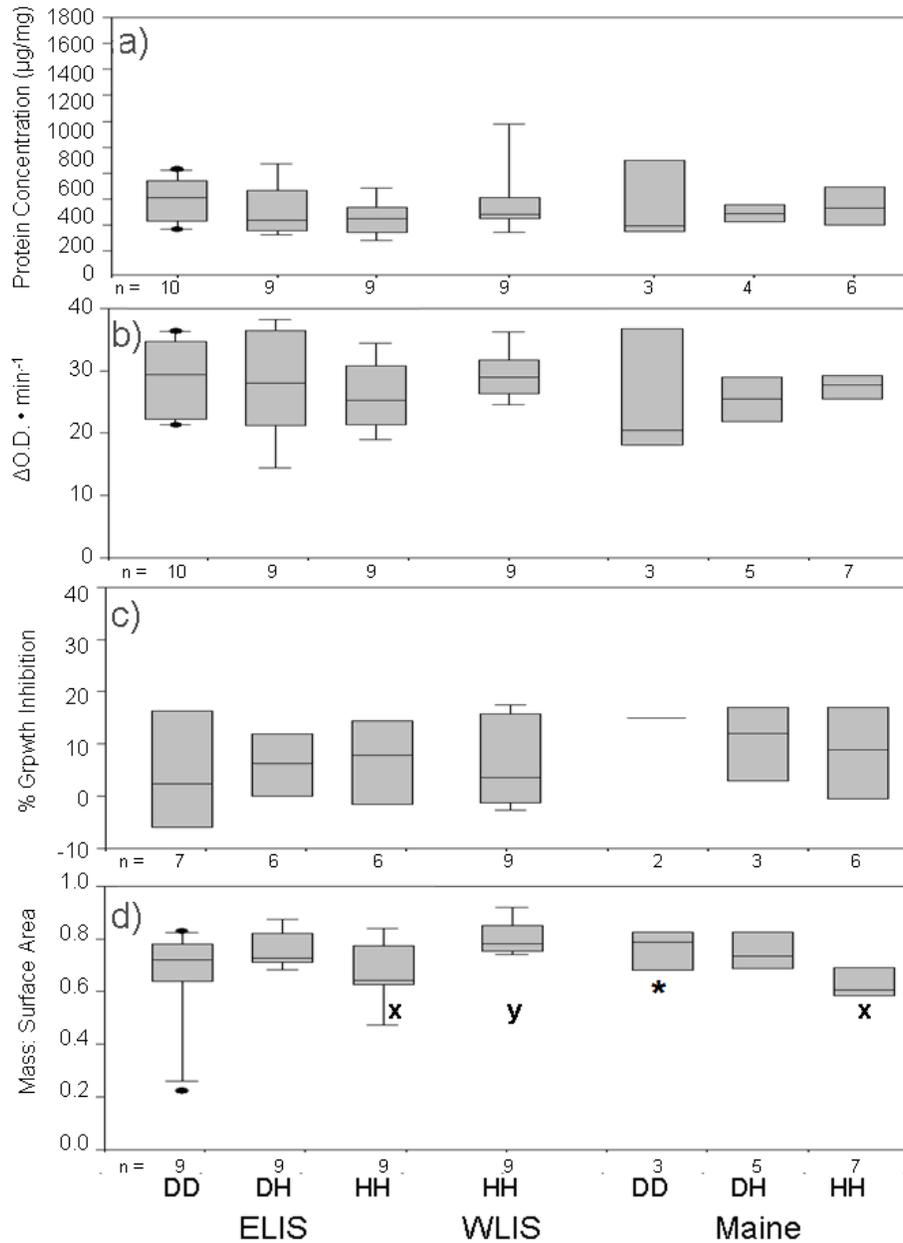
Spatial comparison of external parameters

Table 5: Spatial analysis of shell associated parameters.

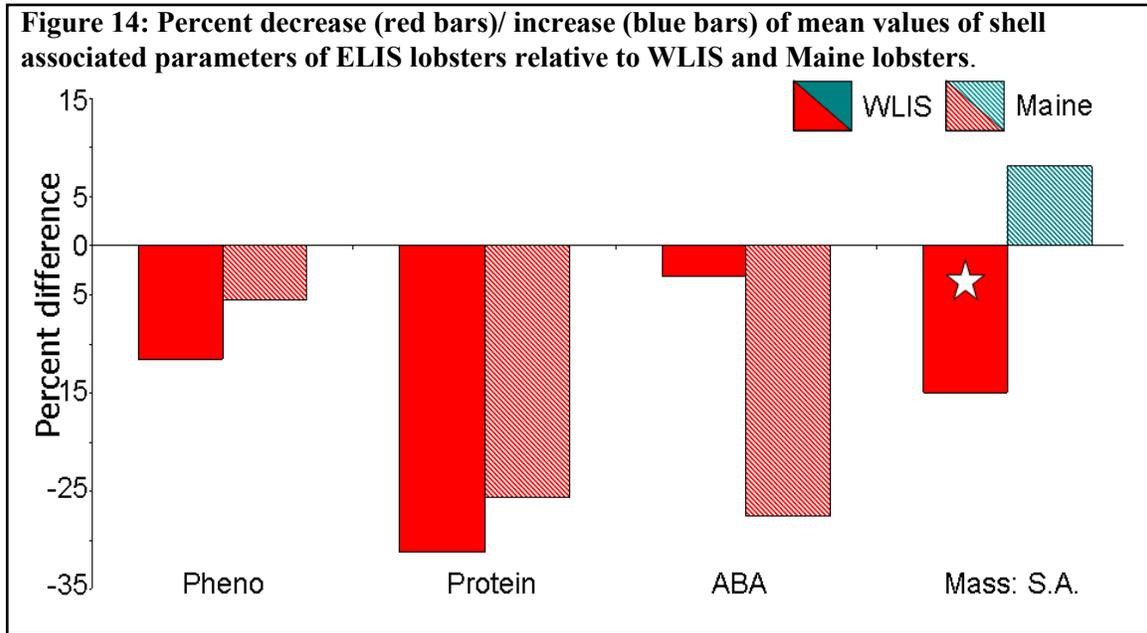
Parameter	Test	Transformation		p-value	Significant?	Post-hoc test	Result
Protein	Kruskal-Wallis	none	origin	0.923	no	-	-
	Kruskal-Wallis	none	disease status	0.309	no	-	-
	Kruskal-Wallis	none	shell status	0.216	no	-	-
Phenoloxidase	1-way ANOVA	none	origin	0.442	no	-	-
	Kruskal Wallis	none	disease status	0.963	no	-	-
	Kruskal Wallis	none	shell status	0.712	no	-	-
Antimicrobial	Kruskal Wallis	none	origin	0.593	no	-	-
	Kruskal Wallis	none	disease status	0.692	no	-	-
	Kruskal Wallis	none	shell status	0.974	no	-	-
Mass to Surface Area Ratio	Kruskal Wallis	none	origin	0.008	yes	<i>Dunn's Method</i>	W > M,E
	Kruskal Wallis	none	disease status	0.509	no	-	-
	Kruskal Wallis	none	shell status	0.833	no	-	-

All samples were taken in June 2007. E, W and M refer to lobsters taken from Eastern Long Island Sound, Western Long Island Sound and Maine, respectively. Disease status refers to the health status of the individual, while shell status refers to the status of the shell. Diseased and healthy individuals were pooled together, unless statistically different (refer to Table 4) in which case only samples taken from healthy individuals were considered. If data was not normal, transformations were made as indicated. Tests that were significant were followed by Holm-Sidak post-hoc test and results were reported. When 1-way ANOVAs were not possible, non-parametric Kruskal Wallis test were performed followed by Dunn's Method Post-hoc test when applicable. Differences were considered to be significant when $p \leq 0.05$, (-) indicates test was not applied.

Figure 13: Spatial comparison of shell associated parameters from healthy and diseased lobsters: (a) mean protein concentration of shell homogenate, (b) rate of phenoloxidase-like activity of shell homogenate as determined by relative fluorescence, (c) antimicrobial activity of shell homogenate, and (d) shell mass (mg) to surface area (mm²) ratio.



The central line through each box indicates the median value of the represented sampling group, while the lower boundary and upper boundary of each box indicates the 25th percentile and the 75th percentile respectively; whiskers indicate standard error around the median and dots indicate outliers within the sample. Labels on the x-axis indicate sampling dates and group health status where DD = diseased shell from diseased individuals, and DH = healthy shell from diseased individuals, and HH = healthy shell from healthy individuals. Where significant differences were found, letters indicate which samples shared statistically equivalent (same letters) or different (different letters) means between sampling groups (1-way ANOVA). (*)s indicate significant differences between healthy (HH) and diseased individuals (DD and DH) within each sampling date (2-sample comparison). Significant differences in pooled samples (DD, DH, and HH) from each sampling location are expressed in Table 5.



Pheno: phenoxidase activity, Protein: protein concentration of shell homogenate, ABA: antibacterial activity of plasma, Mass: S.A.: Mass to surface area ratio of the shell. Stars indicate significantly higher or lower values (Table 5).

Lobsters from WLIS had significantly higher shell mass to surface area ratios (12%) than those from ELIS and Maine (Table 5, and Figures 13 & 14).

Discussion:

Internal immune parameters:

Influence of season on immune activity

The antimicrobial activity of the plasma from ELIS lobsters sampled in August was significantly lower than that of lobsters sampled in October of 2007 or in June of either year. This corresponds to when bottom temperatures were highest at the sites from which the lobsters were sampled (Donald Landers, personal communication), suggesting that the decrease in antimicrobial activity of the plasma could be a result of temperature stress. Chisholm and Smith (1994) saw similar results in the crab *Carcinus maenus* in which the antimicrobial activity of hemocytes was lowest in February and August, when average water temperatures were at their lowest and highest for the year, respectively.

In contrast, the hemocytes of healthy ELIS lobsters sampled in August or October had significantly higher rates of phagocytosis and larger oxidative burst than those of hemocytes from ELIS lobsters sampled in June of either year (Table 1, Figure 2). Additionally, the phenoloxidase activity of the hemocyte lysate from ELIS lobsters was highest in October (Figure 3). Similar patterns have been seen in the prawn *Litopenaeus vannamei*, in relation to its molt cycle, with the lowest phagocytosis rates and oxidative burst levels seen just prior to and after molting in the early summer and the highest phenoloxidase activity occurring during the late inter-molt phase in late summer/early fall (Liu et al. 2003, and Cheng et al. 2003), suggesting that the immune health of lobsters within ELIS might be changing in relation to molt cycle. The molt status of the lobsters sampled, however, did not vary between sampling dates, therefore changes observed in immune parameters are more likely due to other factors such as temperature or bacterial abundance. Previous studies have indicated that the phenoloxidase activity of lobsters

increases in relation to temperature (Chisholm & Smith 1994, Zulkosky et al. 2005, and Bartlett et al. 2008) and bacterial abundance in the water column (Hauton et al. 1997, and Dove et al. 2005).

Influence of disease status on immune activity

With significantly higher protein concentrations and phenoloxidase activity, the immune system parameters of diseased individuals appear to be heightened in comparison to that of healthy individuals. This is further supported by the elevated, but not significantly, higher rates of phagocytosis and lower concentrations of latent ROS. The higher concentration of bacterial colony forming units in the hemolymph of diseased individuals suggests that this increase in immune activity might be in response to the presence of bacteria in the circulatory system.

Similar studies found infection led to lower hemocyte counts in the crayfish *Pacifastus leniusculus* (Persson et al. 1987) and the shrimp *Panaeus stylirostris* (Le Moullac et al. 1998), and the hemolymph-associated phenoloxidase activity of the amphipod *Gammarus pulex* (Plaistow 2003). Similar increases in phenoloxidase activity of the hemocytes have been seen in the shrimp *Pannaeus stylirostris* in response to *Vibrio* exposure (Le Moullac et al. 1998), and the shore crab *Carcinus maenus* in response to increased bacterial counts in the water column (Hauton et al. 1997). Increased rates of phagocytosis were seen in the clam *Ruditapes decussatus* displaying signs of brown ring disease, but decreased in the less resistant *Ruditapes philippinarum* (Allam et al. 2001); indicating that the immune response to bacterial infection can vary greatly between species and potentially between populations.

Influence of sampling location on immune activity

Lobsters from the ELIS population were found to have depressed immunocompetency compared to individuals from either WLIS or Maine. ELIS lobsters had 80-90% lower rates of phagocytosis, 50-60% lower phenoloxidase activity, 60-70% smaller oxidative burst, and ~30% lower antimicrobial activity relative to WLIS and Maine (Figure 7). The reasons for the reduced immune capacity of individuals within ELIS are not evident.

While decreased immunocompetence has been correlated with increased incidences of disease (Noga et al. 1994) and bacteremia (Persson et al. 1987, Hauton et al. 1997, Le Moullac et al. 1998, LaCoste et al. 2001, and Mucklow et al. 2004), the immune health of an organism has also been shown to be reduced by environmental stress such as reduced oxygen availability (Direkusarakom & Danayadol 1998, Le Moullac et al. 1998, Boyd and Burnett 1999, and Holman et al. 2004) thermal stress (Chisholm & Smith 1994, Vargas-Alboras et al. 1998, and Dove et al. 2005) and contaminants (Young & Pearce 1975, Smith et al. 1995, Cheng & Wang 2001, Hernroth et al. 2004, and DeGuise et al. 2004 & 2005). It is therefore surprising that the immunocompetence of lobsters from ELIS, an area that is relatively pristine, is lower than that of lobsters from WLIS, an area known to have low oxygen concentrations (Mecray & Buchholtz ten Brink 2000, and Lee & Lwiza, 2007) and to be more highly polluted with industrial contaminants and sewage (Mecray & Buchholtz ten Brink 2000). This incongruity between environmental health and immunological health suggests that there are additional factors influencing the immunocompetence of lobsters within Long Island Sound. While it is possible that the pathogen responsible for ESD syndrome is not

present or is less virulent in WLIS, the relatively close range of ELIS and WLIS makes this unlikely.

The movement of lobsters within Long Island Sound is limited, with the majority of lobsters being recaptured in the same area in which they were initially tagged (Stewart 1972, Howell et al. 2005, and Landers et al. 2007). While this would indicate that there would be a decreased rate in transmission of any potential pathogen between ELIS and WLIS lobsters it also suggests that there is limited genetic mixing between LIS populations. Crivelo (2005) found that while there is little difference in the genetic structure of ELIS lobster populations and offshore populations, WLIS lobster populations were genetically isolated from ELIS lobsters. It has been postulated that these differences arose as a result of the high selective pressure on WLIS lobsters due to the high concentrations of contaminants and fishing, coupled with the massive lobster die off in 1999, leaving only those individuals with the strongest immune systems to reproduce. Similar events have been seen in salmon populations in response to *gyrodactylus* infestations (Chevassus & Dorson 1990), and in the Quahog clam in response to QPX outbreaks (Dahl et al. 2008). It is possible that the lobsters from WLIS have developed stronger immune systems in response to the large amount of environmental stresses and therefore have become more readily able to defend themselves against certain disease agents including the microbes causing ESD.

External microbial defenses

Traditionally thought of as solely a physical barrier, research into the potential role of the carapace in determining the susceptibility of an organism to disease has been limited. Recent studies, however, have indicated that the carapace contains important biochemical defense mechanisms including the presence of antimicrobial peptides,

prophenoloxidase, and hemocyanins (Ashida & Brey 1995, Asano & Ashida 2001, Nagai et al. 2001, and Adachi et al. 2005). Responsible for regulating the melanization reaction and shell hardening, phenoloxidase plays an important role in the internal defense system and most likely plays an important role in the shell. Recent studies have found that hemocyanins- which are present in much higher concentrations in the shell than phenoloxidase- have similar enzymatic properties to phenoloxidase (Adachi et al. 2005) in addition to antifungal and antibacterial activities (Destroumieux-Garzon et al. 2001).

Influence of season on shell associated parameters

The phenoloxidase-like activity of the SHE isolated from lobsters sampled in August and October of 2007 was significantly higher than that of lobsters sampled in June of either year (Table 3). In addition, the antimicrobial activity of the SHE isolated from lobsters sampled in October 2007 is higher than that isolated from lobsters sampled in June 2008. These patterns are very similar to those found in the hemolymph. This similarity suggests that defense factors in the shell and hemolymph are tightly coupled, whether this is due to similar responses to physical and environmental cues or that they are controlled by the same mechanism is unknown. Previous studies have indicated the potential for hemocyanin and other proteins to be actively transported from the hemolymph to the shell across an epithelial layer (Adachi et al. 2005), allowing for modification of the shell in response to environmental and physical cues.

Influence of disease state on shell associated parameters

The shell from ESD-affected lobsters had significantly lower mass to surface area ratios than the shell from healthy lobsters. This is not surprising given that the shell of diseased lobsters is often highly corroded as a result of the disease. However, this was also seen

when comparing only the healthy shell from diseased lobsters to the shell from healthy lobsters indicating that the shell of diseased lobsters may have been more susceptible to disease even prior to infection.

While not significant, diseased shell had higher protein concentrations and phenoloxidase-like activity than healthy shell from both diseased and healthy lobsters, suggesting a localized response in the shell of ESD affected lobsters. However, this suggestion remains highly speculative without further studies comparing defense related factors in shell extracts from healthy and diseased lobsters.

Influence of lobster origin on shell associated parameters

Lobsters from WLIS had significantly higher shell mass to surface area ratios than those from ELIS and Maine. Since ESD appears to start on the outside of the shell and work its way inward, this “bulking” of the shell could play an important role in the defense of the lobster against disease.

Characterization of the phenoloxidase-like activity of the shell

Preliminary investigations into shell-associated phenoloxidase activity revealed that while there is phenoloxidase-like activity in the shell of lobsters, the molecule responsible for triggering the oxidation of dopachrome is most likely not phenoloxidase. While pro-phenoloxidase is 142 kDa, phenoloxidase is 71 kDa (Asano & Ashida, 2001), and the similarly acting hemocyanin is 67-77 kDa (Adachi et al. 2005), size fractionation of the SHE indicated that the molecule responsible for the oxidation of dopachrome in the samples is less than 3 kDa (Figure 8). Additionally, while phenoloxidase is a particularly heat sensitive protein, the molecule responsible for the phenoloxidase-like activity of the shell does not appear to be heat sensitive or a protein since boiling the samples for 5

minutes only reduced its overall activity level and did not quench it entirely (Figure 9). In recognition of this information, the oxidation of dopachrome by the SHE has been referred to as phenoloxidase-like activity throughout his paper.

To our knowledge, this is the first time shell related defense parameters have been identified in the lobster. Although, we did not see significant differences in phenoloxidase-like activity between healthy and diseased individuals, it does not mean that this system is not an integral component of lobsters' defense. Further investigation to characterize and identify the molecule responsible for this activity and the factors influencing this activity is needed.

Conclusions:

ELIS lobsters have a reduced immune capacity relative to WLIS and Maine lobsters. In the absence of known environmental stressors in ELIS, this finding suggests that differences in the immunocompetence of LIS lobster populations may be due in part to genetic differences between ELIS and WLIS populations and/or the potential differences in the bacterial communities of ELIS and WLIS. While previous studies have indicated that ELIS and WLIS lobsters are genetically isolated and have suggested that WLIS lobsters have undergone intense selective pressure (Crivello et al. 2005), it is impossible to determine the cause of the high ESD-prevalence rate in ELIS without further investigation into whether WLIS lobsters are truly less susceptible to ESD, and whether the pathogen responsible for ESD syndrome is as equally virulent in WLIS as ELIS. As part of the same project, Gordon Taylor's laboratory is investigating the bacterial communities of the shell and hemolymph in the same lobsters studied here. Their data should provide information about the physiology of bacteria associated with

healthy and diseased shell as well as healthy and diseased individuals. It would be advantageous to directly measure the disease susceptibility of the ELIS and WLIS lobsters, and the virulence of the microbial community at these two locations. A reciprocal transplant study of ELIS and WLIS lobsters could address these questions, but environmental and ecological concerns would likely preclude approval of such a study. Alternatively, a common garden experiment in the laboratory could address the relative susceptibility of these two populations to a model pathogen. Until the pathogen responsible for ESD is isolated and transmission successfully carried out in the laboratory, however, direct measurement of differential susceptibility to ESD cannot be accomplished.

This study has confirmed that the shell possesses biochemical defense mechanisms in addition to providing a physical barrier. Shell parameters appear to change in response to environmental and physical cues including infection, and are tightly coupled with those of the hemolymph -suggesting that the biochemical defense properties of the shell are regulated by the transport of molecules across the epithelium. Further characterization of antimicrobial defense parameters of the shell, including the identification of the responsible enzymes and their origins, is needed.

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Table 1: Descriptive statistics of hemolymph associated immune parameters.

	Protein concentration mg protein / ml hemolymph	Rate of Phagocytosis (Relative fluorescence / µg protein) × 10 ⁴					Initial ROS Concentration Relative fluorescence units / µg protein					Oxidative Burst (increase in fluorescence/ µg protein) × 10 ⁴									
		mean	min	max	sterror	n	mean	min	max	sterror	n	mean	min	max	sterror	n					
Maine	Diseased	4.24	3.44	5.30	0.34	5	4.83	1.68	1.11	3.13	3	537	292	1310	195	5	1.08	0.53	1.76	0.26	5
	Healthy	1.99	0.97	3.90	0.91	8	5.55	1.71	1.14	5.14	3	947*	336	2540	322*	7*	0.75	0.40	1.85	0.19	8
W LIS	Diseased	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Healthy	29.23	1.15	4.48	0.24	13	2.42	0.57	2.67	0.49	10	406	289	665	30	13	0.54	0.19	1.35	0.11	13
ELIS 1	Diseased	2.49	1.39	4.68	0.20	16	0.35	0.04	1.73	0.11	16	916	362	2290	136	16	0.26	0.15	0.51	0.02	16
	Healthy	2.46	0.68	5.24	0.38	14	0.52	0.04	1.59	0.13	14	823	371	1610	105	14	0.21	0.14	0.32	0.02	14
ELIS 2	Diseased	2.12	1.13	3.17	0.24	9	2.10	0.08	7.61	0.92	9	824	412	1690	165	9	0.73	0.38	1.77	0.15	9
	Healthy	1.50	0.60	2.82	0.28	8	0.10	0.01	3.93	0.40	9	785	399	1850	184	9	1.78	0.40	6.12	0.59	9
ELIS 3	Diseased	2.29	1.12	3.56	0.20	13	0.81	0.05	1.97	0.14	13	637	429	1010	51	12	1.46	0.35	5.84	0.46	12
	Healthy	1.31	0.53	2.63	0.40	6	0.39	0.15	0.74	0.09	6	589	419	1040	99	6	1.73	0.46	0.55	0.81	6
ELIS 4	Diseased	5.60	2.28	8.53	1.03	7	0.21	0.02	0.44	0.06	8	506	162	1080	98	8	3.55	0.06	0.56	0.06	8
	Healthy	6.75	3.38	11.78	0.98	9	0.19	0.01	0.37	0.04	9	702	277	2280	206	9	0.24	0.10	0.32	0.05	68
Maine	Diseased	4.11	0.68	7.06	1.58	4	28.4	8.6	41.9	5.2	5	2	0	3	6	5					
	Healthy	1.92	0	4.54	0.62	7	29.7	25.9	33.9	1.1	7	4	0	6	1	8					
W LIS	Diseased	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Healthy	2.02	0.44	4.04	0.33	13	28.2	22.7	34.7	1.1	13	2	0	17	1	12					
ELIS 1	Diseased	1.37	0	4.99	0.43	13	28.0	8.45	53.3	5.1	10	15	0	200	13	15					
	Healthy	0.96	0	2.47	0.28	13	19.1	0.0	32.1	2.7	13	1	0	7	1	13					
ELIS 2	Diseased	1.38	0.20	2.54	0.28	8	7.09	0.0	23.4	3.2	9	1	0	6	1	7					
	Healthy	1.55	0.04	2.59	0.39	7	8.44	0.0	23.4	3.8	9	5	0	22	3	7					
ELIS 3	Diseased	3.39	0.59	5.11	0.43	12	20.5	4.1	35.8	2.9	13	0	0	0	0	13					
	Healthy	1.67	0.06	4.74	0.69	6	24.9	7.0	42.7	12.9	5	0	0	0	0	6					
ELIS 4	Diseased	2.51	1.19	4.39	0.43	8	19.5	12.5	24.9	1.5	8	39	4	206	24	8					
	Healthy	1.59	0.95	3.34	0.25	9	14.5	0.0	18.4	2.8	8	37	1	215	24	9					

Notes: Maine refers to lobsters that were sampled from Boothbay Maine, W LIS refers to lobsters taken from Western Long Island Sound, and ELIS refers to lobsters taken from Eastern Long Island Sound.

ELIS 1 refers to lobsters sampled in June 07, ELIS 2 refers to lobsters sampled in August 07, ELIS 3 refers to lobsters sampled in October 07, and ELIS 4 refers to lobsters sampled in June 08.

Diseased refers to individuals displaying visual signs of ESD infection, while healthy refers to asymptomatic individuals.

*n values are not converted by category

Table 2: Descriptive statistics of shell associated microbial defense parameters

	<i>Health status</i>	<i>Shell status</i>	Phenoloxidase-like activity dopachrome formation × minute ⁻¹					Protein µg protein × mg shell ⁻¹					Antimicrobial % growth inhibition					Mass to S.A. Ratio (mg shell × mm ⁻²) × 10				
			mean	min	max	sterror	n	mean	min	max	sterror	n	mean	min	max	sterror	n	mean	min	max	sterror	n
Maine	<i>Diseased</i>	<i>Diseased</i>	25.0	18.0	36.7	5.89	3	348	154	698	175	3	15.5	15.0	16.0	0.5	2	7.60	6.75	8.21	4.36	3
	<i>Diseased</i>	<i>Healthy</i>	25.4	20.8	30.2	1.68	5	290	222	356	37	4	11.1	3.4	17.5	4.1	3	7.47	6.40	8.73	3.78	5
	<i>Healthy</i>	<i>Healthy</i>	27.2	23.5	31.1	0.95	7	340	145	535	62	6	9.9	0.0	25.9	4.0	6	6.23	4.94	7.58	3.21	7
WLIS	<i>Diseased</i>	<i>Diseased</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	<i>Diseased</i>	<i>Healthy</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	<i>Healthy</i>	<i>Healthy</i>	29.1	24.6	36.2	1.23	9	368	144	978	82	9	7.8	0.0	18.0	2.8	9	7.97	7.36	9.15	2.16	9
ELIS 1	<i>Diseased</i>	<i>Diseased</i>	29.3	21.3	36.4	1.84	10	396	168	631	54	10	6.7	0.0	18.3	3.8	7	6.73	2.21	8.38	2.58	9
	<i>Diseased</i>	<i>Healthy</i>	28.2	14.5	38.2	2.78	9	318	128	678	63	9	6.6	0.0	13.7	2.6	6	7.63	6.84	8.73	2.18	9
	<i>Healthy</i>	<i>Healthy</i>	25.7	19.0	34.5	1.77	9	253	86	486	43	9	7.9	0.0	16.9	3.5	6	6.78	4.73	8.39	3.75	9
ELIS 2	<i>Diseased</i>	<i>Diseased</i>	31.8	22.2	38.6	1.89	8	671	317	1290	32	7	9.6	0.8	17.6	4.4	4	8.01	7.14	9.40	2.61	8
	<i>Diseased</i>	<i>Healthy</i>	28.7	20.7	32.8	1.44	7	480	259	742	84	7	12.3	8.9	19.2	2.4	4	7.32	6.52	8.36	2.92	7
	<i>Healthy</i>	<i>Healthy</i>	26.6	22.2	32.3	1.06	11	610	111	1540	133	11	10.4	0.0	22.8	3.1	10	7.20	6.28	8.36	1.98	11
ELIS 3	<i>Diseased</i>	<i>Diseased</i>	33.2	23.5	38.5	1.49	10	668	177	1200	117	10	15.5	0.0	40.3	14.9	8	7.09	6.42	8.63	2.35	10
	<i>Diseased</i>	<i>Healthy</i>	31.6	26.9	35.8	1.17	8	569	260	906	79	8	18.3	0.0	40.1	8.4	6	7.10	6.31	8.31	2.22	8
	<i>Healthy</i>	<i>Healthy</i>	27.9	23.9	31.8	1.17	6	468	70	930	125	6	12.6	3.3	20.6	3.9	4	6.81	5.68	7.68	2.85	6
ELIS 4	<i>Diseased</i>	<i>Diseased</i>	27.2	17.4	34.5	2.52	7	282	158	444	36	7	5.3	3.4	8.07	0.7	7	7.08	6.34	7.99	2.98	7
	<i>Diseased</i>	<i>Healthy</i>	23.1	18.9	26.6	1.29	5	172	75	224	21	6	5.6	3.5	9.0	0.8	6	7.01	6.26	7.69	2.40	6
	<i>Healthy</i>	<i>Healthy</i>	29.3	21.3	39.2	2.48	8	205	133	317	23	9	7.6	3.7	12.0	0.9	9	6.77	5.82	8.39	2.81	9

Notes: Maine refers to lobsters that were sampled from Boothbay Maine, WLIS refers to lobsters taken from Western Long Island Sound, and ELIS refers to lobsters taken from Eastern Long Island Sound.

ELIS 1 refers to lobsters sampled in June 07, ELIS 2 refers to lobsters sampled in August 07, ELIS 3 refers to lobsters sampled in October 07, and ELIS refers to lobsters sampled in June 08.

Diseased refers to individuals and shell portions displaying visual signs of ESD infection, while healthy refers to asymptomatic individuals and disease-free portions of shell.

'n' values are not converted by category