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INVESTIGATION OF EPIZOOTIC SHELL DISEASE IN AMERICAN LOBSTERS (*HOMARUS AMERICANUS*) FROM LONG ISLAND SOUND: II. IMMUNE PARAMETERS IN LOBSTERS AND RELATIONSHIPS TO THE DISEASE

MARGARET HOMERDING,[1]† ANNE MCELROY,[1] GORDON TAYLOR,[1] ALISTAIR DOVE[2] AND BASSEM ALLAM[1]*

[1]*School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY 11794;* [2]*Georgia Aquarium, 225 Baker Street, NW Atlanta, GA 30313*

ABSTRACT Epizootic shell disease (ESD) affects 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 and can lead to mortality in severely affected individuals. ESD tends to be most common in areas around Buzzards Bay, RI, and ELIS, and least prevalent in offshore canyons, off Maine's coast, 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 and disease status of lobsters with and without signs of ESD from ELIS, WLIS, and Boothbay Harbor, ME, were assessed during late spring (June) 2007. We also measured internal defense parameters of ELIS lobsters during midsummer (August 2007), early fall (October 2007), and the following spring (June 2008) to assess how lobster immune systems respond temporarily. Despite high interindividual variability in defense-related factors, multivariate analyses showed that lobsters from ELIS presented significantly reduced immune responses relative to lobsters from either WLIS or Maine. Disease severity correlated negatively with several immune parameters, suggesting that the higher prevalence of ESD in ELIS may be related, at least in part, to reduced immunocompetency of ELIS lobsters.

KEY WORDS: epizootic shell disease, American lobster, *Homarus*, immunity, defense

INTRODUCTION

The American lobster (*Homarus americanus*: Milne Edwards 1837) is found in cold waters of the Atlantic coast of North America (Herrick 1911). Although lobsters are found as far south as North Carolina, the near-shore commercial lobster industry only extends as far south as New York and is dominated by the fisheries of Maine and Canada, with the waters off Massachusetts and Maine producing 90% of all American-caught lobsters. Although only a small portion of the total catch, the lobster fishery in Long Island Sound (LIS) was formerly the most economically important fishery in the New York–Connecticut area, with a value of approximately $42 million at its peak in 1998 (Atlantic States Marine Fisheries Commission 2005).

Shell disease in crustaceans is not uncommon, has been reported for more than 100 y, and is characterized by spots and/or pits on the carapace of affected individuals generally thought to be of bacterial origin. There are 3 recognized forms of shell disease in *H. americanus*: black spot disease, impoundment shell disease (ISD), and, most recently described, epizootic shell disease (ESD) (Hess 1937, Smolowitz et al. 2005). First documented in 1997, ESD is identified by severe erosion of nonchitinous components of the dorsal carapace, leaving semibilateral focal and multifocal lesions on the exoskeleton (Smolowitz et al. 2005). Disease progression after lesion onset can be broken down into 4 stages, with individuals displaying small infrequent pits and/or minor lesions representing stage 1, individuals displaying moderate to deep pits and lesions over a considerable portion of the carapace representing stages 2 and 3, and individuals with lesions covering the majority of the carapace, and severe lesions and ulcers exposing connective tissue representing stage 4 (Castro et al. 2005). ESD severity can range greatly from individual to individual and can increase rapidly in a short period of time (Castro et al. 2005). Prevalence and severity of ESD, however, tends to increase with the amount of time since the last molt, and Landers (2005) has reported that up to 37% of affected individuals do not show lesions after molting. Highest prevalences are observed just prior to summer and fall molts (Castro et al. 2005, Landers 2005), with mature females, which go longer between molts to rear eggs, being the most severely affected (Glenn & Pugh 2006). Initial investigations suggested an increase in ESD prevalence with carapace length, although Cobb and Castro (2006) reported a shift in disease frequency toward smaller lobsters, particularly among males. Although prior reports showed ESD is most prevalent in shallow near-shore waters of eastern LIS (ELIS, ~35% at peak) and Rhode Island (~32% at peak), and occurrence has not exceeded 5% in other areas, such as western LIS (WLIS), the Gulf of Maine, or off-shore canyons (Cobb & Castro 2006), recent reports, however, have indicated that ESD is moving northward into the more productive waters of Massachusetts and Maine (Glenn & Pugh 2006, Tlusty et al. 2007), potentially posing a serious economic threat.

Immunocompetence is a key factor in determining an individual's ability to resist infection and disease. Invertebrates, such as lobsters, which lack an adaptive immune system, rely primarily on circulating hemocytes and innate antimicrobial substances in their plasma to ward off pathogens (Smith & Chisholm 1992, Soderhall & Cerenius 1998, Destoumieux-Garzon et al. 2001). This can be done through the elimination or isolation of the pathogen by phagocytosis and intracellular digestion or by encapsulation, often combined with the action of a wide array of oxidative and other antimicrobial and lytic compounds. Several studies have shown that crustacean immune systems can be weakened or suppressed by environmental

*Corresponding author. E-mail: Bassem.Allam@stonybrook.edu.
†Current address: N. 541 Tribal Center Road, Shelton, WA 98584
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stressors, such as extreme (out of optimal ranges for the species) temperatures (Sindermann 1991, Dove et al. 2005a, Zulkosky et al. 2005), the presence of contaminants (Smith et al. 1995, De Guise et al. 2004, Draxler et al. 2005), or pathogens (Keith et al. 1992, Hauton et al. 1997b), and hypoxia (Le Moullac & Haffner 2000, Burnett & Stickle 2001, Draxler et al. 2005, Moss & Allam 2006). In addition, an organism's immunocompetence can vary between different genetically isolated populations (Bakke et al. 1990, Chevaussus & Dorson 1990, Ragone-Calvo & Burreson 2002). Differential ESD prevalence among different lobster populations may be related to prevailing environmental factors, genetic determinants, or both.

LIS provides an excellent natural setting to examine factors associated with differential susceptibility to ESD because it has 2 lobster populations that display very different ESD prevalence within a relatively small geographical range (Fig. 1). In this study, a suite of immune parameters was examined in lobsters with and without signs of ESD from WLIS, ELIS, and a remote population in Maine to investigate potential links between immune status and disease prevalence and susceptibility.

MATERIALS AND METHODS

Study Design

Lobsters were collected in early summer (late June 2007) from 2 locations: Waterford, CT, in ELIS (an area with a high ESD prevalence) and Oyster Bay, NY, in WLIS (a more urbanized area with a maximum prevalence less than 5%). Bottom water temperatures during these collection events were 16.5°C and 14.0°C for ELIS and WLIS, respectively. Throughout the rest of this article, lobsters are referred to as diseased or healthy according to whether they show signs of ESD or not, respectively. Lobsters were collected during the first week of July 2007 from Boothbay Harbor, ME (a remote area with a maximum ESD prevalence less than 5%; bottom water temperature, 10.9°C), and were used as a reference population. In addition to the initial sampling in June 2007, individuals were also collected from ELIS in midsummer (August 2007; bottom water temperature on collection day, 19.9°C), early fall (October 2007, 17.5°C), and the following spring (June 2008, 14.6°C) to investigate temporal changes in lobster immunity relative to the time of the year.

Lobster Collection and Initial Processing

LIS lobsters were captured using double-entry vinyl-coated wire pots (76 × 51 × 30 cm, 2.5-cm mesh), then classified as diseased or healthy based on the presence or absence of shell lesions, banded, and transported on ice to the laboratory and held in coolers overnight before processing. To minimize bacterial contamination, diseased and healthy lobsters were transported and kept in separate containers throughout processing. Healthy and diseased lobsters from Maine were shipped overnight to the laboratory and processed in a similar manner.

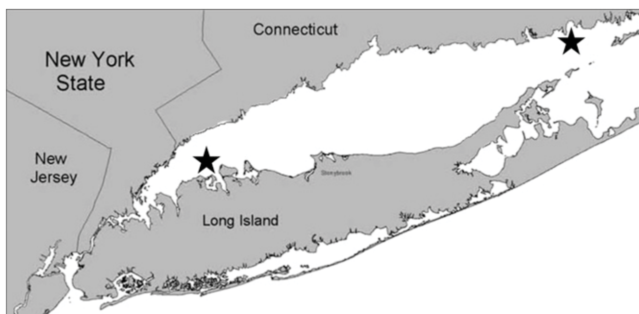

Figure 1. Map of sampling locations (stars) in eastern Long Island Sound (ELIS) and western Long Island Sound (WLIS).

Before collecting hemolymph and tissue samples, swabs of each lobster's carapace were made for bacterial culture analysis as described in Bell et al. (2012). Each lobster's weight, carapace length, disease status, and sex were 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). Disease severity was ranked using a modified version of the approach of Castro et al. (2005). Briefly, 11 disease stages were recorded based on the severity and the percent coverage of the lesions on the carapace, with healthy lobsters scored as stage 0 and those displaying ESD lesions ranging from stage 1 (1 small superficial lesion) to stage 10 (ulcers exposing connective tissue on the majority of the carapace). Lobster activity was also recorded on a scale ranging from 1 (lethargic) to 5 (very active and reactive to physical stimuli).

Hemolymph Collection and Processing

Using a 21-gauge needle, 1 mL hemolymph was withdrawn from the base of the fifth walking leg into a 10-mL syringe containing 9 mL 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 2 5-mL tubes and kept on ice until centrifugation (~10 min). Half the hemolymph/CAC suspension was centrifuged (300*g* for 15 min at 4°C); the supernatant was discarded and the pellet was then resuspended in 1 mL filtered artificial seawater (FASW) at 30 ppt and was used immediately in the phagocytosis and respiratory burst assays. The remaining half of the hemolymph/CAC suspension was centrifuged (800*g*, 10 min at 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. 1997a: 0.45 M NaCl, 0.1 M Na_3 citrate, and 0.01 M sodium cacodylate, pH 7.0)], air-dried for 30 sec and flash frozen using liquid nitrogen before being stored at –80°C until processing for the phenoloxidase (PO) and protein assays.

Frozen hemocyte pellets were thawed on ice before adding 1 mL ice-cold SCCB and disrupting the cells (10 sec, pulsing at 50% duty cycle) using an ultrasonic homogenizer (4710 series; Cole-Palmer Instrument Co., Vernon Hills, IL). The disrupted cell suspension was then centrifuged (1,200 *g* for 25 min at 4°C) to remove any cellular debris. The hemocyte lysate supernatant was then decanted and placed on ice for immediate use in the PO and protein assays.

An additional 1 mL hemolymph was drawn directly into a clean syringe, and 200 μL was used to determine bacterial counts. The remaining volume was centrifuged (800*g* for 10 min at 4°C). The cell-free hemolymph (referred to as plasma) was then decanted, flash frozen in liquid nitrogen, and stored at –80°C for the antimicrobial activity assay.

Measurement of Immune Parameters

Phagocytosis Assay

Phagocytic activity was measured using a plate reader method adapted from Dove et al. (2005a). This assay measures the

fluorescence of phagocytized fluorescently labeled *Vibrio* bacteria. Briefly, 100 μL prepared hemocyte suspension was added to a 96-black-well plate (Corning 3916) and allowed to incubate in the dark at room temperature for 1 h. Controls consisted of 100 μL hemocyte suspension and 5 μL 37% formalin. After incubation, the supernatant was removed by aspiration, and 100 μL fluorescein isothiocyanate (FITC)-labeled *Vibrio parahaemolyticus* suspension was added to each well before incubating in the dark at room temperature for 1 h. Labeling of *V. parahaemolyticus* cells was done as described by Allam et al. (2001). The hemocyte-to-bacteria ratio was on the order of 1:50. After incubation, the supernatant was again removed, and 100 μL Trypan blue solution (250 μg/mL citrate buffer, pH 4.4) was added to each well to quench any extracellular fluorescence (Dove et al. 2005a). After 1 min, the dye solution was removed and fluorescence was measured using 485 nm excitation and 530 nm emission on a microplate reader (Wallac 1420 Multilabel Counter; Perkin Elmer, Wellesley, MA). Readings were averaged among 3 replicate test wells before being corrected for controls by subtraction and standardized to protein concentration of the hemocyte lysate supernatant as determined later.

Reactive Oxygen Species Production

Production of reactive oxygen species (ROS) by hemocytes was measured fluorometrically using microplate reader methods adapted from Moss and Allam (2006). In summary, 100 μL prepared hemocyte suspension was added to each of 4 wells in a 96-black-well plate (Corning 3916), before adding 20 μL of a working solution of dichlorofluorescein–diacetate (10 μg/mL 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 μg/mL in FASW at 30 psu) was added to 3 of the 4 replicate wells. The plate was then incubated in the dark at room temperature for 30 min before being read a second time. Values were averaged among replicates and corrected for controls before being standardized to protein concentration of the hemocyte lysate supernatant. The corrected value for the initial reading was subtracted from the second reading to obtain the difference between native and upregulated (burst) concentrations of ROS in the hemocytes (level of oxidative burst).

Phenoloxidase Activity Assay

PO activity of the hemolymph was measured using methods described previously (Zulkosky et al. 2005), which were adapted from earlier work by Soderhall and Smith (1983). A microplate version of the assay was performed by adding 75 μL of the prepared hemocyte lysate supernatant to each of 3 wells on a 96-clear-well plate (Corning 3370) before adding 75 μL Trypsin solution (1 μg Trypsin (from porcine pancreas) in 1 mL SCCB), and incubating for 1 h in the dark at 28°C. Control wells were incubated with 75 μL buffer rather than Trypsin solution. After incubation, 75 μL saturated L-dopa solution (~1 mg/L dihyroxyphenylalanine/1 mL deionized water) was added to all wells, and absorbance at 490 nm was read immediately on a microplate reader, repeating every 30 sec for 10 min. 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 was determined using the Pierce bicinchoninic acid protein assay microwell plate protocol, using bovine serum albumin as the standard. To ensure consistency, each plate was incubated in the dark at room temperature for 30 min before reading at 590 nm.

Antimicrobial Assay

The antimicrobial activity of the plasma was measured using a turbidimetric assay adapted from Noga et al. (1994), where *Escherichia coli* D31 (Monner et al. 1971) is treated with plasma, and microbial growth is compared among treatments after 36 h of incubation. The bacterium *E. coli* D31 was obtained from the culture collection at Yale University. Briefly, 5 mL 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 min 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 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 microliters of the sterile plasma was 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 min at 28°C. Negative controls included 10 μL plasma and 40 μL PBS solution, whereas the growth control was made of 10 μL prepared bacterial suspension and 40 μL PBS solution. After incubation, 450 μL ice-cold culture media was added to each tube and allowed to incubate at room temperature for an additional 36 h. A 100-μL aliquot from each tube was pipetted into 3 separate wells (2 test and 1 control) on a 96-clear-well microplate (Corning 3590) and measured for absorbance at 570 nm. Bacterial growth inhibition was calculated using the average percent of growth in test wells relative to the growth control after the negative control was subtracted from each sample.

Measurement of Bacterial Counts

Assessment of bacterial load in lobster hemolymph was carried out using standard culture methods. Briefly 100 μL hemolymph was spread on prepared marine agar and Tryptic Soy Agar plates and incubated at room temperature for 48 h. The number of colony forming units (CFUs) on each plate was counted, and the number of CFUs in the plate with the highest count was recorded to assess the abundance of total microflora with disregard to the selectivity of each culture media.

Data Analysis

Data (relative fluorescent or absorbance units) were analyzed using SigmaStat statistical software (v.3.11; Systat Software, Inc., San Jose, CA). One-way analysis of variances (ANOVAs) and 2-way ANOVAs 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 less than 0.05 were followed systematically by a Holm-Sidak post hoc test comparing different data

points (treatments or time intervals). Data from diseased and healthy lobsters collected at the same time and from the same site were compared using Student's *t*-test. When necessary, data were transformed using the $\log_{10}$, reciprocal, or square root transform functions within the SigmaStat software prior to parametric testing 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 by ranks test or the 2-sample Mann-Whitney rank sum test to determine differences between groups. Kruskal-Wallis analyses that generated probability values less than 0.05 were followed systematically by a Dunn's post hoc test comparing different groups (health/disease status, location, or sampling date). Differences between groups were considered statistically significant at $P < 0.05$. Nontransformed data were displayed on an arbitrary scale ranging from 0–40 to facilitate data presentation.

Multivariate analyses were performed on all measured immune parameters combined using discriminant analysis (DA) to determine linear combinations of variables (hemolymph parameters) that maximize differences among *a priori* defined groups (lobsters from different origins), and principal component analysis (PCA) to analyze relationships between variables in diseased and healthy lobsters. DA and PCA were performed with Statgraphics plus software (Statistical Graphics Corporation, Warrenton, VA). Significance level (α) was set at 0.05.

Lastly, correlation analyses were used to evaluate the relationships between the various immune parameters (Pearson's product moment), and between immune parameters and categorical health data such as disease stages and lobster activity (Spearman rank order).

RESULTS

General Factors

The great majority of lobsters sampled (92%) were in the intermolt stages. Molt status of sampled lobsters did not vary among sampling dates ($P = 0.385$) or among sampling sites ($P = 0.389$; data not shown). Sex was also found not to be associated with any difference in response variables, so was not considered further in the analysis. None of the lobsters collected in June 2007 from WLIS ($n = 13$) showed signs of ESD, whereas diseased and healthy animals were retrieved from all sampling dates in ELIS ($n = 17$, 9, 13, and 9 diseased lobsters; and $n = 16$, 9, 6, and 9 healthy individuals for the 4 sampling dates) and from a single sampling from Maine ($n = 6$ diseased and 9 healthy lobsters).

Immune Parameters in Lobsters from Different Locations

Differences in immune parameters were not statistically significantly different between diseased and healthy lobsters collected in early summer (late June) 2007 from ELIS and Maine (early July 2007); therefore, data of lobsters with and without signs of ESD were pooled before comparing immune parameters in lobsters from the various sampling sites. Results showed clear differences in immune parameters among lobsters collected from ELIS, WLIS, and Maine (Fig. 2). For instance, lobsters from ELIS displayed significantly lower antimicrobial activity in their plasma ($P < 0.03$) and a lower hemocyte respiratory burst ($P < 0.01$) than lobsters from either WLIS or Maine. Mean hemocyte PO activity tended to be lower in the

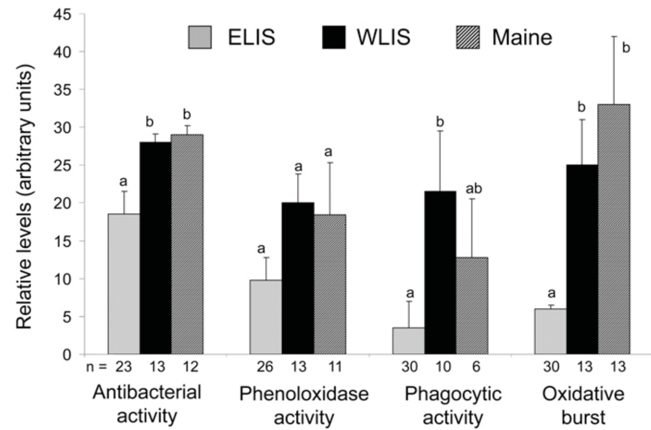

Figure 2. Immune parameters (arbitrary scale, mean ± SE) in lobsters collected from different locations. The letters a and b represent differences among different populations for the same parameter (1-way ANOVA followed by Holm-Sidak or Dunn post hoc test, $P < 0.05$).

ELIS population than in lobsters from either WLIS or Maine, but these differences were not statistically significant (ANOVA, $P = 0.083$).

Results showed differences in bacterial counts in hemolymph based on the geographical origin of lobsters (ANOVA on ranks in healthy lobsters, $P = 0.045$), and counts were lower in WLIS compared with ELIS and Maine lobsters (Table 1), although post hoc nonparametric tests did not provide sufficient statistical power to evaluate site-to-site comparisons ($P > 0.05$).

Influence of Sampling Date and Disease Status on Immune Parameters in ELIS Lobsters

Prevalence of lobsters with signs of ESD was highest in ELIS, so in this population we were able to evaluate changes in immune responses to infection over time and to follow immune parameters as a function of sampling date. Immune parameters in ELIS lobsters varied significantly between sampling dates (Table 2, Fig. 3). Data were analyzed using 2-way ANOVA to evaluate sampling time and disease status as factors (Table 2, Fig. 3). 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 2). This was driven mainly by significant temporal changes among diseased lobsters (Fig. 3). The oxidative burst in hemocytes of lobsters sampled in June of both years was lower than that of lobsters sampled in August and October 2007 (Table 2) as a result of differences among healthy lobsters (Fig. 3). The hemocyte lysate collected from lobsters sampled in June 2007 had significantly lower PO activity than that of hemocyte lysate obtained from lobsters sampled in October 2007. Interestingly, temporal changes in PO activity were particularly visible among diseased lobsters (Fig. 3). Antibacterial activity of the plasma withdrawn from lobsters sampled in August 2007 was significantly lower than that of plasma isolated from lobsters at all other sampling dates (Table 2), and this appears driven by trends in both diseased and healthy lobsters (Fig. 3). On the other hand, the number of bacterial CFUs was significantly higher in the hemolymph of diseased lobsters compared with healthy individuals when considering all data combined ($P = 0.005$,

TABLE 1.

Bacterial counts (median colony forming units per milliliter) in lobster hemolymph.

	WLIS June 2007	Maine July 2007	ELIS			
			June 2007	August 2007	October 2007	June 2008
Healthy	10 (20, $n = 13$)	100 (58, $n = 8$)	30 (5, $n = 14$)	10 (329, $n = 9$)	ND	70 (450, $n = 9$)
Diseased*	NA	100 (10, $n = 6$)	65 (206, $n = 16$)	60 (433, $n = 9$)	ND	160 (227, $n = 9$)

* Significant differences (Mann-Whitney rank sum test) between diseased and healthy lobsters when considering all data combined ($P = 0.005$) or data from ELIS alone ($P = 0.025$). The numbers in parentheses represent the SEM and sample size. NA, not applicable; ND, not determined.

Mann-Whitney rank-sum test, Table 1) or data from ELIS alone ($P = 0.025$). Two-sample comparisons of bacterial counts also revealed a general trend for higher levels in diseased lobsters compared with healthy individuals, although significant differences were detected in June 2007 only. No clear differences among collection dates were detected for bacterial counts in hemolymph.

PO activity was the only immune parameter measured that showed a significant difference in the 2-way ANOVA between diseased and healthy organisms; diseased lobsters showed higher levels, although 2-sample comparisons (Student's t-test) also showed significant differences in oxidative burst between diseased and healthy lobsters collected in August 2007 (Fig. 3D).

Multivariate and Discriminant Analysis

To characterize the pattern of immune response and disease status among all individuals sampled, we also used PCA and DA methods (Fig. 4). Biplots of the PCA on all lobsters combined clustered immune factors on component 1 in opposition to bacterial counts in the hemolymph, with diametrically opposing trends between bacterial counts and antibacterial activity of the plasma suggesting a negative correlation between these 2 parameters (Fig. 4A). Opposing trends were also detected on component 2 for oxidative burst (zymosan-stimulated) and native (unstimulated) ROS production. One-way ANOVA showed significant differences between ELIS lobsters and those collected from WLIS or Maine for extracted component 1 ($P < 0.0002$, Fig. 4B)

TABLE 2.

Summary of 2-way ANOVA and post hoc test results assessing the effect of sampling date and disease status on immune parameters in ELIS lobsters.

	Sampling Date	Disease Status
Antibacterial activity	$P < 0.001$ August 2007 < All other dates	$P = 0.3$
Phenoloxidase activity	$P = 0.006$ June 2007 < October 2007	$P = 0.018$ Diseased > healthy
Phagocytic activity	$P < 0.001$ June 2008 < August 2007 and October 2007	$P = 0.5$
Respiratory burst	$P < 0.001$ June 2007 = June 2008 < August 2007 = October 2007	$P = 0.4$

There were no significant interactions between factors.

and component 2 ($P = 0.036$, data not shown). Similar trends were observed when only healthy lobsters were analyzed (data not shown). Segregation of lobsters based on their geographical origin was clearly visible in DA on all lobsters combined (Fig. 4C, $P < 0.0001$ and $P = 0.022$ for factors 1 and 2, respectively) and among healthy lobsters alone (Fig. 4D, $P = 0.0003$). Results of the Spearman rank correlation showed an increase in bacterial counts in hemolymph with increased disease severity ($r = 0.29$, $P = 0.005$, $n = 90$; Table 3). PO activity also tended to increase in hemocytes from severely diseased lobsters ($r = 0.21$, $P = 0.04$, $n = 99$) whereas negative correlations were detected between disease severity and hemocyte phagocytic activity ($r = -0.19$, $P = 0.05$, $n = 103$), and between lobster activity and bacterial counts in hemolymph ($r = -0.21$, $P = 0.04$, $n = 90$). Interestingly, bacterial counts in hemolymph were correlated negatively with antibacterial activity of the plasma (Pearson correlation; $r = -0.21$, $P = 0.05$, $n = 82$), which matched well with the diagonally opposed trends observed in the PCA.

DISCUSSION

Like other invertebrates, lobsters fight off microbial pathogens through innate immune responses associated with hemocytes and antimicrobial molecules found in the hemolymph (Soderhall & Cerenius 1998). This study evaluated a suite of lobster immune parameters including both functional responses, such as plasma antimicrobial activity in plasma and hemocyte phagocytosis, as well as PO activity and oxidative burst in hemocytes. We also assessed more general measures of health, including severity of ESD lesions, activity level of the lobsters, and bacterial load in their hemolymph. The data obtained not only provide a picture of how immune status varies with location, sampling date, and presence or absence of disease signs, but also show how these parameters may relate to each other.

Influence of Sampling Location on Immune Performance

Lobsters from the ELIS population had depressed immunocompetency compared with individuals from either WLIS or Maine; ELIS lobsters had 70–80% lower rates of phagocytosis, 40–50% lower PO activity, 70–80% smaller oxidative burst, and 30–40% lower antimicrobial activity (Fig. 2). These findings are also supported by the PCAs and DAs shown in Figure 4. The reasons for reduced immune capacity of individuals within ELIS are not evident.

Although decreased immunocompetence has been correlated with increased incidences of disease (Noga et al. 1994) and

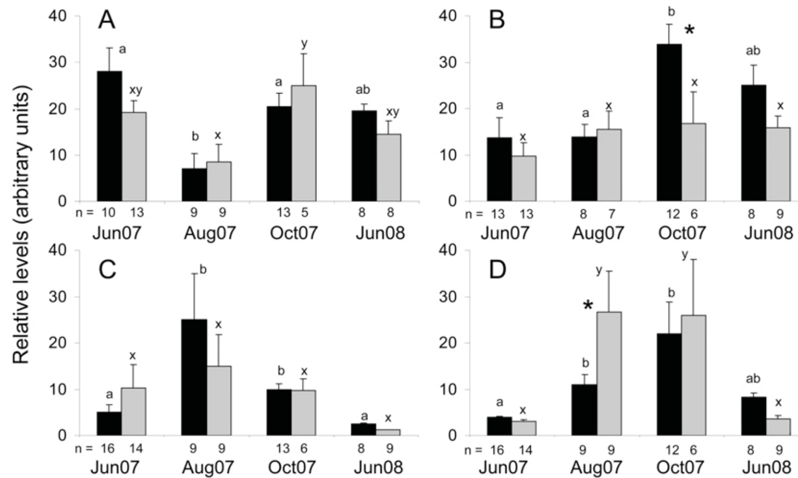

Figure 3. (A–D) Temporal variation in antibacterial activity in plasma (A), and phenoloxidase activity (B), phagocytic activity (C), and oxidative burst (D) in hemocytes from diseased (black bars) and healthy (gray bars) lobsters collected from ELIS. For each parameter, letters (a and b for diseased; x and y for healthy) denote significant differences (2-way ANOVA followed by Holm-Sidak or Dunn post hoc test, $P < 0.05$) between lobsters collected at different sampling times. *Significant differences between diseased and healthy lobsters collected at the same time. All values are represented as mean ± SE on an arbitrary scale. The number of lobsters (*n*) in each sample is given along the x-axis.

bacteremia (Persson et al. 1987, Hauton et al. 1997b, Le Moullac et al. 1998, Lacoste et al. 2001, Mucklaw et al. 2004), the immune heath of an organism can be reduced by environmental stress, such as reduced oxygen availability (Direkbursarakom & Danayadol 1998, Le Moullac et al. 1998, Boyd & Burnett 1999, Holman et al. 2004), thermal stress (Chisholm & Smith 1994, Vargas-Albores et al. 1998, Dove et al. 2005a), and contaminants (Young & Pearse 1975, Smith et al. 1995, Cheng & Wang 2001, Hernroth et al. 2004, DeGuise et al. 2004, DeGuise et al. 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, Lee & Lwiza 2008), 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 as-yet undiscovered factors influencing the immunocompetence of lobsters within LIS. Although it is possible that the pathogen(s) responsible for ESD is not present or is less abundant or less virulent in WLIS, the relatively close range of ELIS and WLIS–and water exchange between them–makes this unlikely. Results from the companion study evaluating the microbial communities also indicates no large differences between the microbial communities associated with the shells of these lobsters (Bell et al. 2012).

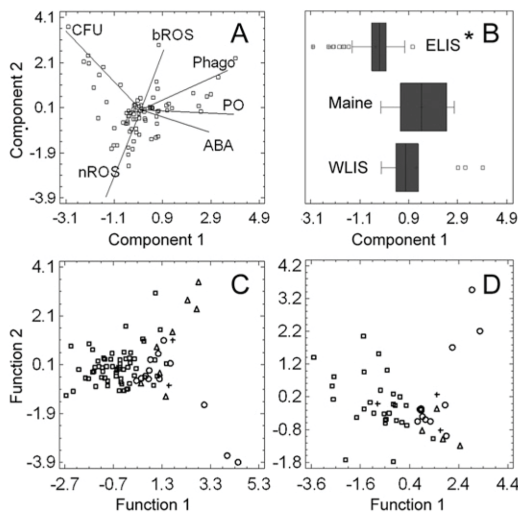

Figure 4. (A) Principal component analysis of immune parameters from all lobsters combined. ABA, antibacterial activity of plasma; bROS, oxidative burst in response to zymosan; CFU, bacterial counts in hemolymph; nROS, native (unstimulated) reactive oxygen species production; Phago, phagocytic activity of hemocytes; PO, phenoloxidase activity. (B) Plots of extracted component 1 showing significant differences (*; Holm-Sidak post hoc test, $P < 0.0001$) between ELIS lobsters and lobsters collected from WLIS and Maine. (C, D) Discriminant analyses using immune data from all lobsters combined (C) or from healthy lobsters alone (D) (□, ELIS; △, Maine, ○, WLIS).

The movement of lobsters within LIS is limited, with the majority of lobsters being recaptured in the same area in which they were initially tagged (Stewart & Zwicker 1972, Howell et al. 2005). Although this would indicate that there would be a decreased rate in transmission of any potential pathogen between

TABLE 3.

Spearman rank correlation coefficients between lobster disease stages or activity and different hemolymph parameters.

	Phagocytic Activity	Phenoloxidase Activity	Bacterial Counts
Disease stage	–0.19 (0.05, $n = 103$)	0.21 (0.04, $n = 99$)	0.29 (0.005, $n = 90$)
Lobster activity	NS	NS	–0.21 (0.04, $n = 90$)

The numbers between parentheses represent the probability value and sample size. NS, not significant.

ELIS and WLIS lobsters, it also suggests that there is limited genetic mixing between LIS populations. Crivello et al. (2005) found that, although 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 because of the strong selective pressure on WLIS lobsters resulting from high concentrations of contaminants and fishing, coupled with the massive lobster die-off in 1999, leaving only those well-adapted individuals to reproduce (Crivello et al. 2005). Similar events have been seen in salmon populations in response to *Gyrodactylus* infestations (Chevaussus & Dorson 1990), and in the Quahog clam in response to QPX outbreaks (Dahl et al. 2008). It is possible that lobsters from WLIS have developed stronger immune systems in response to a plethora of environmental stresses and therefore have become more readily able to defend themselves against certain disease agents, including the microbes causing ESD.

Influence of Sampling Date on Immune Activity

Antimicrobial activity of the plasma from ELIS lobsters sampled in August was significantly lower than that of lobsters sampled in October 2007 or in June of either year (Table 2). August is when water bottom temperatures were highest at the lobster sampling sites (Donald Landers, pers. comm.), suggesting that the decrease in antimicrobial activity of the plasma could be associated with 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. The reduction in the antibacterial activity of the plasma was associated with an increase in bacterial counts in hemolymph, suggesting that this humoral defense factor plays a central role in controlling the proliferation of invading microorganisms.

In contrast, hemocytes of ELIS lobsters sampled in August or October had significantly higher rates of phagocytosis and a larger oxidative burst than those of hemocytes from ELIS lobsters sampled in June (Table 2, Fig. 3). In addition, PO activity of the hemocyte lysate from ELIS lobsters was highest in October. 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 early summer, and highest PO activity occurring during the late intermolt phase in late summer/early fall (Cheng et al. 2003, Liu et al. 2003). These data suggest that the immune performance in ELIS lobsters might be influenced by molt cycle. Molt stage, however, did not vary among sampling dates; therefore, changes observed in immune parameters are more likely the result of other factors such as temperature or bacterial abundance in the environment. Previous studies have indicated that PO activity of lobsters increases at higher temperature (Chisholm & Smith 1994, Zulkosky et al. 2005, Bartlett et al. 2008) as a possible result of increasing bacterial abundance in the water column (Hauton et al. 1997a, Dove et al. 2005b).

Overall, temporal trends differed among measured immune parameters, suggesting possible tradeoffs and compensation mechanisms that help American lobsters maintain efficient protection against invaders during stressful summer months along the southern limit of their distribution range. Nevertheless, reduction in antibacterial activity of the plasma during summer was associated with an increase in bacterial loads in the blood, suggesting a limited ability of lobsters to cope with temperature stress. These findings are in agreement with our prior studies showing a significant reduction in immune competency among lobsters maintained at stressful, but ecologically relevant, temperatures (23°C) for 1 wk (Dove et al. 2005a).

Influence of Disease Status on Immune Activity

It is difficult to separate the influence of disease susceptibility from disease response on the immune factors evaluated in this study. Significant numbers of diseased animals were collected only from ELIS, whereas none were available from WLIS, and only a small number were collected from Maine. As discussed earlier, lobsters from ELIS showed a depressed immune system compared with lobsters from either WLIS or Maine. Results in ELIS lobsters showed temporal variations in the response to the disease for the various immune parameters investigated. In October, a higher PO activity was clearly displayed in diseased lobsters when compared with healthy individuals. This temporal elevation in PO activity could have been the result of activation of this system for wound healing in response to ESD lesions. The prophenoloxidase activating system in hemocytes responds to foreign materials by initiating an enzymatic cascade that leads to production of melanin, which is toxic to microorganisms and contributes to parasite encapsulation (Sritunyalucksana & Söderhäll 2000). In the cell, microbial cell wall components activate serine proteases, which convert prophenoloxidase to PO and then catalyze the oxidation of mono- and diphenols to quinones that eventually polymerize to form melanin. Both phenols and quinones as well as melanin have antimicrobial activity (Soderhall 1982, Soderhall & Cerenius 1998). Using RNA interference techniques to silence prophenoloxidase, Fagutao et al. (2009) recently reported significantly elevated levels of bacteria and viruses in shrimp hemolymph that were also associated with increased mortality, demonstrating the importance of this system in the innate immune response. Our data suggest an important role of the prophenoloxidase system in combating ESD. Further research should be aimed at elucidating this role.

Interplay between the immune parameters in response to short-term and more chronic stress is complicated and difficult to interpret based on single-time point sampling. The absence of a clear-cut response is evident in other investigations as well. For example, Plaistow et al. (2003) showed a decrease in the hemolymph-associated PO activity in the amphipod *Gammarus pulex* in response to infection with the acanthocephalan *Pomphorynchus laevis*. In contrast, increases in PO activity were reported in the shrimp *Pannaeus stylirostris* in response to *Vibrio* exposure (Le Moullac et al. 1998), and the shore crab *C. maenus* in response to increased bacterial counts in the water column (Hauton et al. 1997a). Similarly, increased rates of phagocytosis were seen in the clam *Ruditapes decussatus,* displaying signs of brown ring disease, but decreased rates were observed in the less resistant *Ruditapes philippinarum* (Allam et al. 2001). Collectively, the results indicate that immune responses to infections are highly variable and depend on the nature of the pathogen as well as a set of interrelated intrinsic (such as physiological conditions, reproductive and molting cycles) and extrinsic factors, such as season and/or temperature. Nevertheless, our findings showed

that ESD is associated with significant changes in lobster immunity in agreement with findings made by Tarrant et al. (2010, 2012), who reported significant modulation of genes involved in metabolic (arginine kinase) and immune (alpha-2 macroglobulin) activities in affected lobsters.

Perhaps not surprisingly, bacterial counts in the hemolymph increased with severity of the disease (Table 3). This may result from alteration of the physical barrier represented by the shell and/or reduction in the ability of diseased lobsters to clear bacterial agents from the circulatory system. As a matter of fact, the phagocytic activity of hemocytes correlated negatively with disease development, suggesting that disease may reduce lobster immunity. Most important, increasing bacterial counts in hemolymph were associated with a reduction in lobster activity. Therefore, lobsters with ESD were not only immune compromised but also were more lethargic than apparently healthy lobsters. Overall, these findings suggest that the morbidity of ESD-affected lobsters might result from uncontrolled bacterial proliferation in hemolymph leading to septicemia.

CONCLUSIONS

This research demonstrated that 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 a result, in part, of genetic differences between ELIS and WLIS populations, and/or the potential differences in the microbial communities of ELIS and WLIS. However, results from microbial community analysis of the shells of these lobsters (Bell et al. 2012) fail to demonstrate striking differences in shell-associated microbial communities between these sites, suggesting that disease incidence and susceptibility is the result of other factors supporting the host susceptibility hypothesis described by Tlusty et al. (2007). 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). However, determining the cause of the high ESD prevalence rate in ELIS is impossible without further investigation into whether WLIS lobsters are truly less susceptible to ESD, and whether the pathogen(s) responsible for ESD syndrome is equally abundant and virulent in WLIS as ELIS. Disease challenge studies could address this issue. Until the pathogen responsible for ESD is isolated and transmission is carried out unambiguously in the laboratory, direct measurement of differential susceptibility to ESD cannot be accomplished. This study is the first, to our knowledge, to address a comprehensive suite of immune response parameters in American lobster, particularly in response to a specific disease, such as ESD. Data indicate that the prophenoloxidase system may be adaptive in helping lobsters combat ESD, and that more general immune responses such as hemocyte phagocytic activity and oxidative burst are either affected negatively by ESD or are suppressed in individuals susceptible to the disease.

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