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FLUOROMETRIC MEASUREMENT OF OXIDATIVE BURST IN LOBSTER HEMOCYTES AND INHIBITING EFFECT OF PATHOGENIC BACTERIA AND HYPOXIA

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ABSTRACT The stimulation of hemocytes during phagocytosis leads to the generation of a series of oxygen radicals known as reactive oxygen species (ROS). Among these, hydrogen peroxide plays an important microbicidal role by directly killing microorganisms or by serving as an intermediate for other antimicrobial radicals. In this study, we adapted a technique using 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) to measure H_2O_2 production in lobster hemocytes. After oxidation by hydrogen peroxide, this molecule produces a fluorescent product that can be easily detected. The respiratory burst was successfully activated in lobster hemocytes by the addition of zymosan particles, but not with phorbol myristate acetate. After optimization, we used the technique to investigate the effect of different bacterial strains, including lobster pathogens, on the oxidative burst. Results demonstrate that *Aureococcus viridans*, a bacterial pathogen that is able to survive phagocytosis by lobster hemocytes, quenches ROS production. The comparison of ROS production in lobsters collected from field sites submitted to different levels of dissolved oxygen suggests that this technique provides a good indicator of lobster physiological status and immunocompetency.

KEY WORDS: lobster, *Homarus americanus*, oxygen, *in vitro*, dichlorofluorescein diacetate, ROS

INTRODUCTION

The American lobster, *Homarus americanus*, is one of the most commercially important species in the Northeastern United States and Canada. This species is subject to different infections, including those caused by bacterial agents such as the Gram-positive *Aerococcus viridans* (Stewart et al. 1969, Battison et al. 2003) and the Gram-negative *Vibrio fluvialis* (Tall et al. 2003), as well as those related to protozoan parasites such as Paramoeba (Mullen et al. 2004) and the ciliate Anophryoides hemophila (Cawthorn 1997, Athanassopoulou et al. 2004). Against these pathogens, lobsters have a set of cellular and humoral defense factors. In common with other crustaceans, host defense in lobsters is nonspecific, based on activities of circulating hemocytes (Bauchau 1981, Paterson & Stewart 1974, Paterson et al. 1976, Cornick & Stewart 1978, Battison et al. 2003). In these animals, hemocytes defense functions include coagulation, phagocytosis, encapsulation and wound repair (Bauchau 1981, Johansson & Söderhäll 1989, Bachère et al. 1995) and synthesis and secretion of humoral defense factors (Destoumieux et al. 2000, Bachère et al. 2004).

The stimulation of hemocytes during phagocytosis leads not only to release of lysosomal enzymes, but also to superoxide radical (O2-) generation catalyzed by NADPH oxidase associated with the cytoplasmic membrane. Superoxide radical is metabolized to hydrogen peroxide (H2O2) by superoxide dismutase (SOD), and the resulting peroxide is further converted to hypochlorite (OCl-) by myeloperoxidase (MPO). Hydrogen peroxide, superoxide radical and hypochlorous acid exhibit direct antimicrobial activity and are widely called reactive oxygen species (ROS) or intermediates (ROI). This phenomenon, known as respiratory burst, plays an important microbicidal role (Anderson 1996). Prior studies reported that ROS production is inhibited in shellfish experimentally submitted to environmental (pollutants, hypoxia) and pathogenic (bacterial) stresses (Anderson 1994, Anderson et al. 1997, Lambert & Nicolas 1998, Le Moullac et al. 1998, Cheng et al. 2002, Lambert et al. 2001).

Because of their importance in the defense process, various tools have been developed to measure ROS production in vertebrates, including microscopy, luminescence methods and flow cytometry techniques. In their recent paper, Anderson & Beaven (2005) described a chemoluminescence method measuring the oxidative burst in lobsters after activation with Phorbol myristate acetate (PMA). An appealing alternative to this technique is a method using 2′,7′-dichlorofluorescein-diacetate (DCFH-DA), initially developed in human phagocytic cells (Rosenkranz et al. 1992). After oxidation by ROS (particularly H_2O_2), this molecule produces a fluorescent product that can be easily detected using fluorescence microscopy, or measured using appropriate fluorometers (plate readers, flow cytometers).

There were several goals for this study. First, a simple fluorometric technique allowing detection and quantification of ROS production in lobster hemocytes was devised and optimized. This technique was then used to investigate the effect of pathogenic (bacterial) challenge on ROS production. Finally, as field evaluation of the optimized method, this study examined ROS production in lobsters collected from three different locations in Long Island Sound known to be submitted to different levels of dissolved oxygen.

MATERIALS AND METHODS

Lobsters

Lobsters (1.25–1.50 lb.) used for technique-setup experiments were obtained from a commercial source located in Port Jefferson, New York. Lobsters used to investigate the effect of bacteria on ROS production were collected from traps deployed north of Oak Neck, Long Island Sound (Fig. 1). Animals were maintained in 200-L tanks (3 lobsters per tank), filled with aerated recirculating seawater containing artificial shelters made of PVC pipes. The water, which was maintained at 32 ± 1 ppt and 15 ± 1°C, was continuously pumped through a canister filter system containing active biofilter media made from sintered glass (Eheim Ehfisubstrat, Aquatic Eco-Systems Inc., Apopka, Florida) and active carbon. Nitrate/nitrite and ammonia levels were monitored, and water changes were made when needed. Lobsters were fed twice a week, using clam meat for a minimum period of 7 days before bleeding and ROS measurement. To investigate ROS production in lobsters exposed to different levels of dissolved oxygen, additional lobsters

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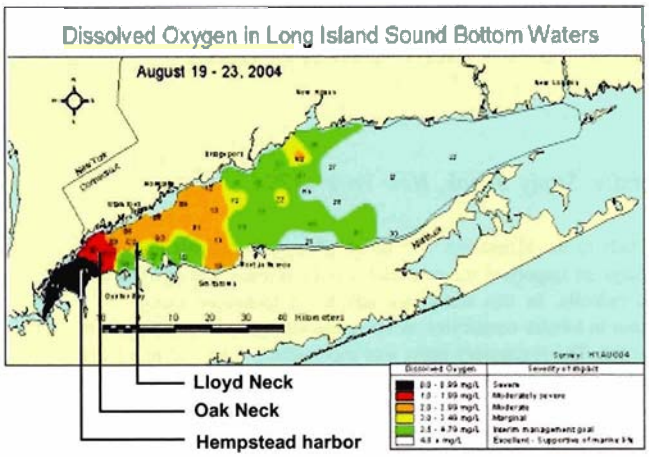

Figure 1. Map showing lobster sampling sites in Long Island Sound and dissolved oxygen in bottom waters during the sampling period (Map generated by CT DEP Long Island Sound Water Quality Monitoring Program, accessible online at: http://www.dep.state.ct.us/wtr/lis/monitoring/summer2004.htm)

were collected from different locations within Long Island Sound (Fig. 1): 12 lobsters were collected from traps located north of Hempstead Harbor, 15 from traps located north of Oak Neck, and 15 from traps located north of Lloyd Neck. These lobsters were maintained in seawater tanks onboard of the fishing vessel until transferred to the laboratory for immediate hemolymph sampling and processing.

Chemicals and Buffers

Crustacean anticoagulant (CAC, 0.45 M NaCl, 0.1 M glucose, 0.03 M trisodium citrate, 0.026 M citric acid, and 0.01 M EDTA, pH 4.6) and marine crustacean saline (MCS, 0.58 M NaCl, 0.013 M KCl, 0.013 M CaCl2, 0.026 M MgCl2, 0.00054 M N2HPO4, and 0.05 M Tris-HCL buffer, pH 7.6) solutions were prepared according to Smith and Söderhäll (1983). Dichlorofluorescin diacetate (DCFH-DA, Sigma) was dissolved in DMSO to obtain 100 mM stock solution (aliquoted and stored at −20°C). Work solutions of DCFH-DA were produced by diluting aliquots in filtered (0.22 μm) autoclaved seawater (FASW) at different concentrations as described later. Phorbol myristate acetate (PMA, Sigma) stock solution was made by dissolving 1 mg PMA in 1 mL dimethyl sulfoxide (DMSO). This solution was divided into 25-μL aliquots and stored at −20°C. Zymosan (Sigma) was directly suspended in FASW (20 mg 1 mL^{-1}), then heated in a boiling water bath for 30 min. The resulting suspension was then washed twice by centrifugation and resuspension in MCS. The particle count was checked microscopically and aliquots were frozen at −20°C.

ROS Measurement

General Design

Hemolymph (0.5 mL) was withdrawn from the ventral sinus by inserting a needle at the base of the last left walking leg. Hemolymph was directly collected in a 3-mL syringe containing 2.5 mL CAC to prevent blood clotting. Diluted hemolymph was then centrifuged (300*g*, 4°C, 10 min). The supernatant was discarded, and the cells were resuspended in 1 mL MCS. An aliquot (100 μL) of resuspended hemocytes was added with formalin (2% final concentration) and used for hemocyte count. Remaining hemocytes were added to a 96-well black microplate (100 μL per well, in 3–4 replicates depending on experiments) and were supplemented with DCFH-DA. An initial reading of the fluorescence (485 nm excitation and 535 nm emission) was then performed to measure the native (prior to activation) ROS production in hemocytes using a Wallac 1420 fluorometric plate reader. After the first reading, hemocytes were added with targeted activator (PMA or zymosan, see below), then additional fluorescence readings were recorded after 5 and 30 min of incubation in the dark.

Activation With PMA Versus Zymosan

Hemocytes resuspended in MCS were added to a 96-well microplate as follows: for each lobster (n = 3 lobsters), nine replicate wells containing 100 μL per well were performed: 3 wells received 100 μL MCS containing 2 μL PMA stock solution (10 μg mL^{-1} final concentration), another three received 100 μL Zymosan suspension made in MCS (1:50 hemocyte:zymosan ratio), and the last three received MCS as a control. Then 2 μL DCFH-DA stock solution were added to each well (final DCFH-DA concentration = 1 mM). Readings were taken using the Wallac fluorometric plate reader at 5, 20, 60, 90 and 120 min postactivation.

Determination of Optimal DCFH-DA Concentration

Hemocytes were processed in the same way as above. For each lobster (n = 3), four replicate wells were performed, and each of these wells received a different concentration of DCFH-DA. The different concentrations tested were: 0.1, 0.5, 1, and 2 mM DCFH-DA. The Wallac plate reader was set up to take automatic readings at 5-min intervals for a total of 90 min after activation with zymosan (1:50 hemocyte:zymosan ratio).

Effect of Bacterial Challenge on ROS Production

The significance of pathogenic stress was investigated by studying the effect of *in vitro* challenge with bacterial compounds on ROS production in lobster hemocytes. Four bacterial strains were investigated: *Aerococcus viridans* var. *homari* (Robohm et al. 2005) and *Vibrio fluvialis* (Tall et al. 2003), both known to be pathogenic to lobsters, *Listonella anguillarum* (strain 775, Crosa et al. 1977), a pathogen to several marine organisms including fish and shellfish, and *Escherichia coli*, which is not known to cause disease in marine organisms (here used as control). Bacteria were cultured in sterile marine broth (Difco 2216) at room temperature on a shaker table for about 24 h (exponential phase of growth). After spectrophotometrically estimating bacterial counts, bacterial cells were collected by centrifugation (3,000*g*, 15 min, 4°C), resuspended in MCS and used immediately for challenge experiments. Bacterial supernatants were filtered (0.22 μm) and saved (−20°C) for use in subsequent experiments.

The first experiment investigated the effect of challenge of hemocytes with bacterial cells on ROS production using additional lobsters collected from Lloyd Neck. For each lobster (9 total), 12 wells received 100 μL of hemocytes resuspended in MCS, supplemented with DCFH-DA at a final concentration of 0.5 mM. After an initial reading to measure native ROS activity, duplicate wells received 20 μL of one of the following suspensions (at a ratio of 1:50 hemocytes:test particles, e.g., cfu or zymosan): *A. viridans*, *V. fluvialis*, *L. anguillarum*, *E. coli*, zymosan, and two wells received 20 μL MCS as a control. After a 30-min incubation in the dark, a second reading was taken, then 10 μL zymosan were added to each well and two more readings were taken after 5 and 30 min addi-

tional incubation. Aliquots of hemolymph samples were diluted in CAC, fixed with formalin (1.9% final concentration) and used for hemocyte counts with a hemocytometer. ROS production was normalized to hemocyte counts.

The second experiment investigated the effect of bacterial supernatants on ROS production by hemocytes. For each lobster (9 total), 12 replicated wells received 100 μL of hemocytes resuspended in MCS. Duplicate wells were added with 100 μL of bacterial (*A. viridans*, *V. fluvialis*, *L. anguillarum*, *E. coli*) supernatants. The volume of supernatant added per hemocyte was estimated to be equivalent to the volume of culture medium containing 50 cfu (supernatant-cfu equivalent). Two other wells received 100 μL MCS as a control and two additional wells received 100 μL sterile culture media as a second control. After 1-h incubation, DCFH-DA was added to each well at a final concentration of 0.5 mM and baseline fluorescence reading was taken. Then 10 μL zymosan was added to each well and readings were taken 5 and 30 min after incubation in the dark. ROS production was normalized to hemocyte counts as described above.

Effect of Hypoxia on ROS Production

This experiment investigated ROS production in lobsters collected from areas on Long Island Sound known to be submitted to different levels of dissolved oxygen as determined by the Connecticut Department of Environmental Protection (CT DEP) Long Island Sound Water Quality Monitoring Program (Fig. 1). The biweekly summer survey generates hypoxia maps based on the monitoring of different stations across the sound using a CTD recorder. Lobsters were transferred to the laboratory and hemocytes were withdrawn as previously described. For each lobster, four wells received 100 μL of hemocytes resuspended in MCS, supplemented with DCFH-DA at a final concentration of 0.5 mM. After an initial fluorometric reading to measure native activity, 10-μL Zymosan suspension was added to each well to activate ROS production (about 1:50 hemocyte:zymosan ratio). Two additional readings were then made 5 and 30 min after incubation in the dark. ROS production was normalized to hemocyte counts as described for the bacterial challenge experiments.

Statistics

Average fluorescence signals were calculated for all replicated wells before being processed for statistical analyses. All data were analyzed using the Statgraphics statistical software. Statistical tests were a one-way general linear model analysis of variance (ANOVA) or repeated measure ANOVA followed by a Fisher's LSD posthoc test, as appropriate. Because raw data were not always normally-distributed, a Log_{10}-transformation was made on data before running statistical testing. Differences were considered significant at $\alpha = 0.05$.

RESULTS

Activation of Hemocytes for ROS Production

The addition of 1 mM dichlorofluorescein diacetate (DCFH-DA) to hemocytes induced the production of baseline fluorescence that increased with incubation time, up to 120 min after incubation (maximal incubation period assayed in this study, Fig. 2). Because the fluorescence signal is an indicator of hydrogen peroxide production, these results suggest that ROS are produced in untreated hemocytes. Hydrogen peroxide production was significantly

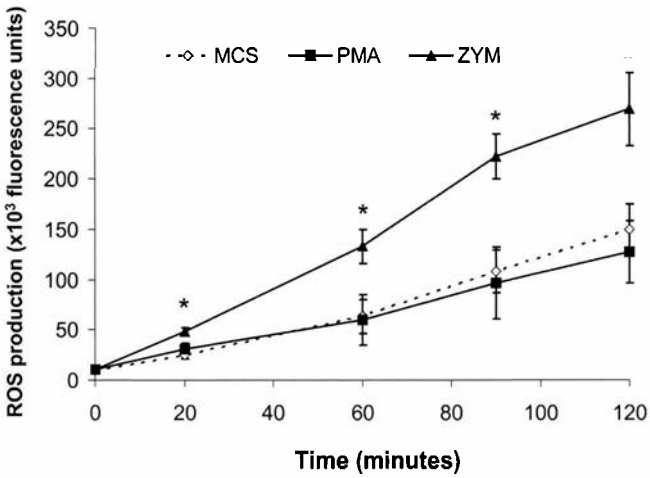

Figure 2. Oxidative burst in hemocytes activated with phorbol myristate acetate (PMA) or zymosan (ZYM). Control hemocytes were incubated with saline (MCS). Fluorescence signals were significantly higher (*) in hemocytes added with zymosan when compared with the 2 other treatments (Fisher's LSD posthoc test, $P < 0.05$, $n = 3$).

stimulated by the addition of zymosan A ($P < 0.001$, repeated measure ANOVA) within 20 min after activation, but was not modified by the addition of PMA, when compared with untreated hemocytes (added with saline). These trends were not modified over the 2-h duration of the experiment.

Determination of Optimal DCFH-DA Concentration

Fluorescence signals intensified with the increase of DCFH-DA concentration from 0.1–0.5 mM (Fig. 3). Further increase in concentration to 1 or 2 mM did not enhance the intensity or modify the kinetics of H_2O_2 production. Based on these results, a concentration of 0.5 mM was used in all subsequent experiments.

Effect of Bacterial Cells on the Oxidative Burst

Thirty minutes after the addition of bacterial cells to hemocytes, ROS production significantly increased in wells incu-

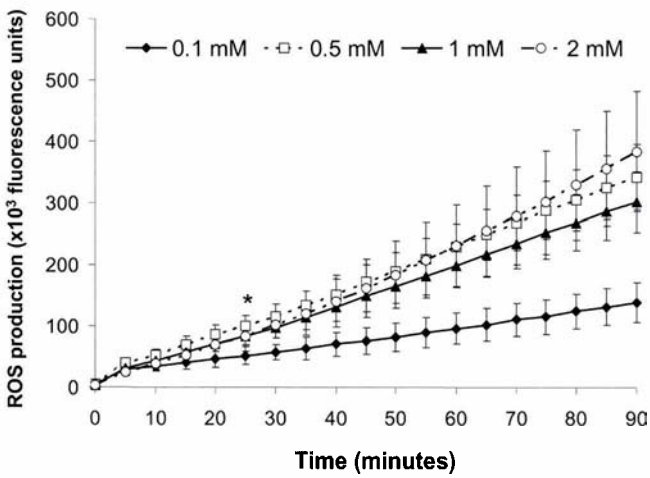

Figure 3. Oxidative burst in hemocytes added with different concentrations of dicholorofluorescein-diacetate (DCFH-DA) and activated with zymosan. *: significant differences between hemocytes added with 0.1 mM and all other treatments appeared 25 min after incubation and continued until the end of the experiment (Fisher's LSD posthoc test, $P < 0.05$, $n = 3$).

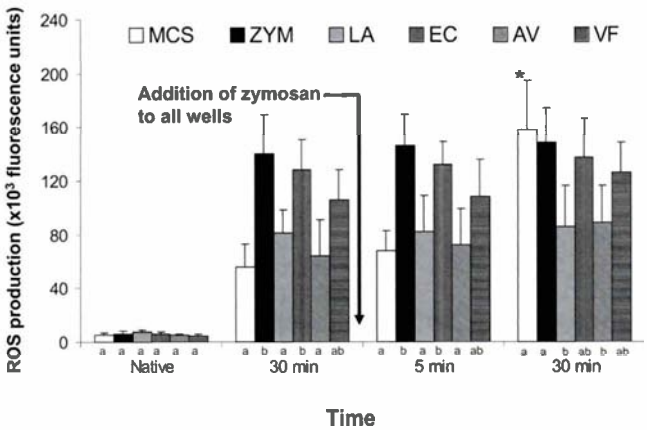

Figure 4. Effect of bacterial cells on reactive oxygen species production in lobster hemocytes. Zymosan was added to all wells after the second fluorescence reading. MCS: saline, ZYM, zymosan, LA: *Listonella anguillarum*, EC: *Escherichia coli*, AV: *Aerococcus viridans*, VF: *Vibrio fluvialis*. For each time interval, letters (a and b) represent significant differences among different treatments (Fisher's LSD post hoc test, $P < 0.05$, $n = 9$). *: significant increase when compared with the prior sampling point (Student t-test, $P < 0.05$).

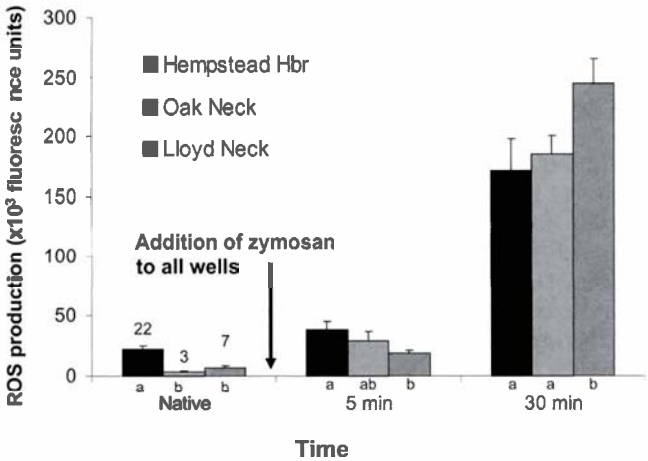

Figure 6. Reactive oxygen species production in lobsters collected from three different locations in Long Island Sound (see Figure 1 for more details). Each data point represents 12 (Hempstead Harbor) or 15 (Oak Neck Harbor and Lloyd Neck) lobsters. Letters (a and b) represent significant differences among samples (Fisher's LSD posthoc test, $P < 0.05$).

bated with *E. coli*, when compared with control hemocytes added with saline (Fig. 4). The other bacterial species caused only a slight increase in fluorescence signals that did not significantly differ from control hemocytes. Among this group, *V. fluvialis* caused the highest signals, followed by *L. anguillarum* and finally by *A. viridans*. The subsequent activation of hemocytes with zymosan did not significantly change the trends observed in hemocytes preincubated with bacteria. The major changes were observed in hemocytes initially used as controls (added with saline), in which the addition of zymosan produced the strongest signals (Fig. 4). A second addition of zymosan to hemocytes did not noticeably increase ROS production.

Effect of Bacterial Extracellular Products on the Oxidative Burst

Pre-incubation of hemocytes with bacterial extracellular products (ECP) did not affect native hydrogen peroxide production in inactivated hemocytes. However, hemocytes preincubated with ECP from *L. anguillarum* were unable to express oxidative bursts in response to zymosan activation (Fig. 5). Preincubation of hemocytes with ECP produced by the other bacterial species slightly increased ROS production when compared with saline control. However, ROS signals in these samples were not significantly different from those of control hemocytes incubated in sterile bacterial culture media.

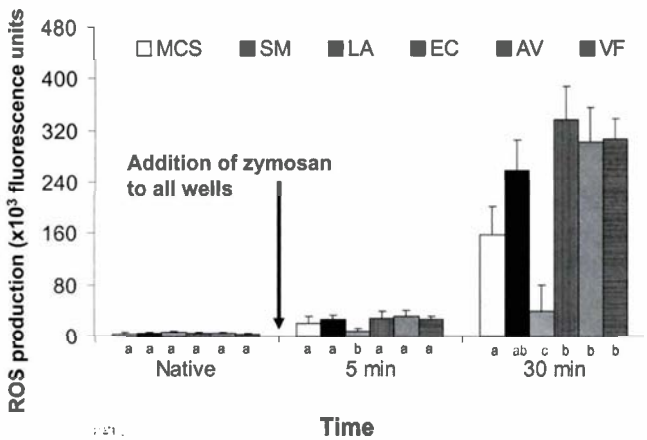

Figure 5. Effect of bacterial extracellular products (ECP) on reactive oxygen species production in lobster hemocytes. See legend of Figure 4 for abbreviations. For each time interval, letters (a, b and c) represent significant differences among different treatments (Fisher's LSD posthoc test, $P < 0.05$, $n = 9$).

Effect of Hypoxia on ROS Production

Native hydrogen peroxide production in inactivated hemocytes was significantly higher in lobsters collected from the most western field site (Hempstead Harbor), when compared with lobsters from Oak Neck and Lloyd Neck (Fig. 6). This trend is inverted after activation. For instance, thirty minutes after the addition of zymosan, oxidative burst was significantly higher in lobsters collected from the most eastern site when compared with the two other locations. Hemocyte counts were not significantly different among the different field sites and ranged from 1.9×10^6 to 4.8×10^6 hemocytes mL^{-1}.

DISCUSSION

Oxidative burst by blood cells is a major antimicrobial mechanism in vertebrates and invertebrates. This oxidative process starts when stimulation of macrophages leads to increased consumption of oxygen, the reduction of which, catalyzed by a membrane-bound NADPH oxidase, initiates the cascade and production of several reactive oxygen species. This study demonstrates that the optimized fluorometric technique represents a valuable method for the measurement of oxidative burst by lobster hemocytes, and provides a viable means of evaluating the effects of pathological and environmental stresses on lobster immunity. Our results show that dichlorofluorescein-diacetate (DCFH-DA) is cleaved into fluorescent dichlorofluorescein in hemocytes incubated in saline at a significantly lower rate than in hemocytes added with zymosan. Higher cleavage rate clearly indicates a higher production of hydrogen peroxide through activation of the ROS cascade (Rosen-

kranz et al. 1992). The fluorescent signal increased with incubation time, including that in control hemocytes, as a result of accumulation of dichlorofluorescein in cells. Fluorescent signals observed in control hemocytes correspond to spontaneous H_2O_2 production, which may reveal a normal physiological cell activity. Alternatively, such production can be related to an activation of hemocytes during the isolation procedure, or when the hemocytes adhere to the plastic as observed, for instance, for bivalve (Pipe 1992) or hypothesized for crustacean (Bachère et al. 1995) hemocytes. Incubation of hemocytes with phorbol myristate acetate (PMA, 10 $\mu g\ mL^{-1}$) did not induce any increase in hydrogen peroxide production. This is in agreement with several studies that found that PMA is not as efficient in inducing ROS production as zymosan in crustaceans (Song & Hsieh 1994, Bachère et al. 1995, Muñoz et al. 2000) and in mollusks (Ordas et al. 2000, Austin & Paynter 1995, Torreilles et al. 1997), but in disagreement with a prior report that showed higher ROS production in lobster (*H. americanus*) hemocytes activated with PMA, when compared with cells added with zymosan (Anderson & Beaven 2005). This apparent contradiction could be explained by the fact that Anderson & Beaven measured the production of a subsequent reactive intermediate in the cascade, namely HOCl, using a chemiluminescence detection method. The fact that the hemocyte:zymosan ratio used here (1:50) is significantly higher than ratios used by those authors (1:2–1:25) may provide another explanation of the differences observed between the two studies, because our preliminary results showed an increase in hydrogen peroxide production with the increase of hemocyte:zymosan ratio up to 1:50 (data not shown).

The possible role of lobster hemocyte ROS in the elimination of pathogenic microorganisms was investigated by studying the effect of selected bacteria on ROS production *in vitro*. Our results demonstrated that *E. coli* and *V. fluvialis* cells caused an increase in ROS production, but differences compared with the control were only significant in the case of *E. coli*. Although a prior report also demonstrated that *E. coli* cells induced the highest ROS response in shrimp hemocytes when compared with shrimp bacterial pathogens (Muñoz et al. 2000), the biological significance of these results is not clear, because *E. coli* is not significantly present in natural lobster habitat. It seems, however, that some bacterial pathogens have developed strategies to avoid the trigger of ROS production by hemocytes. For instance, Muñoz et al. (2000) demonstrated that a pathogenic strain of *Vibrio harveyi* did not induce ROS production when added to shrimp hemocytes, whereas the addition of a strain of *V. alginolyticus* used as probiotic in shrimp aquaculture did enhance ROS production. Similarly, our study revealed that *Aerococcus viridans*, a pathogen that is able to survive phagocytosis by lobster hemocytes (Stewart 1975), does not elicit ROS production. Similarly, the universal pathogen *L. anguillarum* did not induce the production of ROS. More importantly, ROS production in these two samples was not increased after the addition of zymosan, suggesting an active neutralization of the oxidative metabolism by *A. viridans* and *L. anguillarum*. Although bacterial cells from both strains have similar effects, the mechanisms underlying their ROS inhibition might be different. It has already been demonstrated that the survival in hosts' cells of pathogenic bacteria and fungi is often linked to the ability of these microorganisms to quench ROS through the production of catalases/peroxidases, which are thus considered virulence factors (Day et al. 2000, Lefebre & Valvano 2001, Pongpom et al. 2005). It is thus possible that *A. viridans* quenches ROS production in lobster hemocytes as a mean to colonize and survive within host's cells. The antioxidant enzyme catalase is not present in this bacterial species, however, suggesting that such quenching might be performed through other processes. The effect of *L. anguillarum* on ROS production is more pronounced when bacterial extracellular products (ECP) are used. *L. anguillarum*, which possesses catalase activity, may suppress fluorescence signals by altering H_2O_2 production as suggested by Bramble and Anderson (1997). Alternatively, the same strain of this universal pathogen has demonstrated wide cytotoxic effects on hemocytes from different bivalve species under similar experimental conditions (Allam & Ford 2006). The viability of hemocytes was not specifically monitored in the present work, but anecdotal observations suggested increased mortality only in hemocytes incubated with *L. anguillarum* (data not shown). The effect of *L. anguillarum* observed here might simply be a result of hemocyte killing by bacterial cells and ECP, because dead hemocytes could not participate in ROS production.

The comparison of ROS production in lobsters collected from field sites submitted to different environmental conditions suggests that this technique represents a good indicator of lobster physiological status and immunocompetency. The major environmental condition that varies among experimental sites was the level of dissolved oxygen, which ranged from levels below 1 mg L^{-1} in Hempstead harbor to about 3 mg L^{-1} in Lloyd Neck. Native hydrogen peroxide production was 3 times higher in hemocytes from lobsters collected in the former, when compared with those harvested from the latter. High native (base) ROS production may reveal stressful or injurious situations caused by *in situ* factors. These findings suggest that lobsters from Hempstead harbor had been submitted to oxidative stress, which may have been caused by low oxygen availability in the environment (Storey 1996, Pan et al. 2003). Animals typically respond to hypoxia by reducing metabolic rate (Perez-Rostro et al. 2004), but side effects of hypoxia include the formation of excess free oxygen radicals (Yu 1994, Pan et al. 2003), causing severe alterations in cellular activities. Although these microbiocidal agents are typically generated in the phagocytic vacuoles, an important quantity crosses into the extravacuolar environment and may cause damage to cells (Warner 1994, Thompson et al. 1995). ROS also damage cytoskeletal proteins, leading to a probable loss in defense function, because hemocyte adhesion, motility, endocytic ability and the capacity to phagocytose foreign particles all depend on the cytoskeleton (Bellomo & Mirabelli 1992). The prevention of such damages is normally made through different defense strategies, which involve the use of small antioxidant molecules (ascorbate, glutathione, carotenoid pigments) that can directly neutralize oxidative radicals, or rely on a variety of enzymes that metabolize ROS (superoxide dismutase, catalase, glutathione peroxidase) (Warner 1994). In any case, subsequent stimulation with a specific trigger of the respiratory burst could be impaired, ineffective or unreliable. For instance, hemocytes from hypoxic areas (Hempstead and Oak Neck) did not respond to zymosan activation as efficiently as those collected from Lloyd Neck. Similarly, Le Moullac et al. (1998) demonstrated a decrease in ROS production, measured by the nitroblue tetrazolium method, in shrimps submitted to hypoxia. In our study, the lowering of ROS production may hamper the lobster's ability to mount an effective defense against invading microorganisms in areas affected with hypoxia, thus amplifying stress to such animals and leading to mass mortality events such as those that have been observed in western Long Island Sound.

In conclusion, this paper describes a simple technique to measure oxidative burst in lobster hemocytes. The technique was used to demonstrate a deleterious effect of some bacterial pathogens on ROS production in lobster. Additionally, the technique seems promising for field monitoring of lobster immunocompetency. Our results suggest that pathogen and environmental stresses may combine and act together to create potentially disastrous conditions for lobsters.

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