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ANTIMICROBIAL ACTIVITY IN THE PALLIAL CAVITY FLUIDS OF THE OYSTER *CRASSOSTREA VIRGINICA* (GMELIN) FROM A HIGHLY IMPACTED HARBOR IN WESTERN LONG ISLAND SOUND

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ABSTRACT Fluid and its associated mucus from the pallial (mantle) cavity of eastern oysters *Crassostrea virginica* (Gmelin) from Black Rock Harbor, Bridgeport, Connecticut, inhibited growth of both Gram-positive (*Bacillus subtilis, Staphylococcus aureus*) and Gram-negative (*Escherichia coli, Serratia marcescens*, and *Vibrio parahaemolyticus*) bacteria in antimicrobial assays. In the presence of oyster fluid, *E. coli* resulted in significant reduction in growth after 26 h. Soluble lysozyme activity in pallial cavity fluid of oysters collected in the fall was 3 times greater than that measured in combined winter–spring–summer samples ($P = 0.0008$). During the course of the study, copper concentrations in pallial cavity fluid ranged from 0.60–2.49 ppm and zinc concentrations ranged from 9.7–61.0 ppm. Copper concentrations remained relatively constant throughout the study; the highest zinc concentrations were recorded in the fall. Fall antimicrobial assays showed heightened antimicrobial activity compared with the spring, which may be the result of increased lysozyme activity and higher zinc concentrations present in the pallial cavity fluid at that time of year. Results of this study suggest that pallial cavity fluid and its associated mucus likely serve an important role in defense-related functions as the first line of defense against infections from environmental pathogens in *Crassostrea virginica*.

KEY WORDS: antimicrobial activity, pallial cavity fluid, lysozyme, copper, zinc, *Crassostrea virginica*

INTRODUCTION

Eastern oysters (*Crassostrea virginica* Gmelin) are often found inhabiting relatively polluted bays and estuaries along the Atlantic and Gulf coasts of North America. As molluscan filter feeders, they ingest microalgae, bacteria, and detritus readily from the surrounding environment. Cavallo et al. (2009) showed that high bacterial densities in bivalve tissues can be lethal if the accumulated bacteria are pathogenic. For protection against microorganisms, bivalves rely primarily on cellular defense functions such as phagocytosis and encapsulation, as well as wound repair, and humoral defense factors including lysosomal enzymes, agglutinin/opsonins, and antimicrobial peptides (Cheng 1996, Chu 2000, Canesi et al. 2002, Cheng-Hua et al. 2009).

Most studies investigating cellular and humoral defense mechanisms in oysters have focused on the hemolymph (Chu 1988, Cheng 1996). Relatively little is known about the effectiveness of other body fluids in killing microorganisms. In bivalves, the first line of defense against infections by pathogenic microorganisms and parasites is the fluid in the cavities, which surrounds the mantle (pallial and extrapallial cavity fluid) and the soft body of the mollusc. Previous studies have confirmed the presence of lysozyme- (McDade & Tripp, 1967a) and mucus-derived agglutinins (Fisher 1992) in oyster pallial fluid, suggesting an important role for this fluid in the overall microbial defense system. Recently, oyster pallial mucus has been implicated in the regulation of the acquisition, growth, and virulence of the oyster parasite *Perkinsus marinus* (Allam et al. 2013, Espinosa et al. 2013). However, the extent of the defense-related properties of bivalve pallial fluid and its associated mucus have not yet been investigated thoroughly.

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The literature contains many accounts of heavy-metal toxicity effects in marine organisms, yet the eastern oyster thrives in polluted environments (Abbe & Sanders 1986). Many tissue burden studies have found extraordinarily high concentrations of copper and zinc in the oyster (Bodansky 1920, Wolfe 1970, O'Connor 1996), and several investigators have shown that these metals accumulate against a concentration gradient (Korringa 1952, Boyden & Romeril 1974, Boyden 1975, Simkiss et al. 1982 Roesijadt 1996). Brown (1975) and Ruddell and Rains (1975) presented evidence that much of the copper and zinc is sequestered in oyster amoebocytes, particularly the basophilic granulocytes. In addition, the ability of oysters from copper- and zinc-contaminated sites to inhibit microbial growth is enhanced (Fisher et al. 2003, Oliver et al. 2003). Based on this evidence, Fisher (2004) proposed a role for copper and zinc as antimicrobial agents in oyster hemocyte function.

It is widely accepted that pallial fluid and its associated mucus has an important function in lubrication and particle capture in filter-feeding bivalves (see Jorgensen [1996] for review); however, its role in defense is less well studied. The purpose of this research was (1) to investigate bactericidal activity of oyster pallial fluid using challenge experiments, (2) to determine the amount of lysozyme present in that fluid, and (3) to measure pallial fluid copper and zinc concentrations. The information gained from this study suggests an important role for pallial fluid/mucus in the defense system of *Crassostrea virginica*.

MATERIALS AND METHODS

Cultivation of Organism

Cultures of *Escherichia coli* (ATCC 29425), *Bacillus subtilis, Serratia marcescens, Salmonella typhimurium, Vibrio*

parahemolytica, and *Staphylococcus aureus* were maintained at room temperature on Tryptic Soy Agar (TSA) slants. The bacteria necessary for experiments were obtained in the following manner: The applicable organism was grown at 37°C overnight on TSA slants. Cells were washed and calibrated in sterile 0.05 M phosphate-buffered saline (pH 7.2), and calibrated to a 0.20 absorbance using a Spectronic 20 set at a 550-nm wavelength.

Experimental Animals and Sample Collection

Adult (shell length, >70 mm) *Crassostrea virginica* were collected from Black Rock Harbor, Bridgeport, Connecticut, from September 2010 to November 2011 and again in 2013. The shellfish growing classification for this area is "prohibited" (Connecticut Department of Agriculture, Bureau of Aquaculture). Black Rock Harbor receives effluent (secondary treatment) from Bridgeport's West End sewage treatment plant. Only wild oyster populations are present at this site. Animals were kept overnight in a cold room at 5°C to prevent possible changes in lysozyme activity (Wang et al. 2008) and mucus quantity/quality (Rosa et al. 2012) resulting from heat stress before being processed the following day. Oyster shells were V-notched at the posterior–ventral edge and the pallial cavity fluid was drained according to the method described by Fisher (1992). Mucus was aspirated from the surface of the mantle, gills, and visceral mass of opened oysters using a sterile syringe. Gaping oysters or those with an excessive amount of mucus production (presence of stringy material in the pallial fluid) were discarded. Combined pallial cavity fluid and mucus samples from each animal were centrifuged (5 min, 400*g*, 20°C) to remove debris. The supernatant was withdrawn and a fresh subsample was used in the antimicrobial challenge experiments. The remainder was stored at –80°C until use in the *Escherichia coli* growth experiment and the heavy-metal studies. Pallial fluid from 10–15 animals was pooled and antimicrobial analysis was done within 2 h for each sampling date.

Plate Antimicrobial Assay

To determine the range of bacterial inhibition by *Crassostrea virginica*, pallial fluid—both Gram-positive and Gram-negative strains—was tested in inhibition assays using a modification of the method described by Ritchie (2006). Oyster fluid-treated media were used to test the growth inhibition of bacteria by plating out 400 µL undiluted oyster fluid onto TSA plates and allowing it to dry for 10 min. Oyster fluid-treated plates were sterilized using UV radiation by placing uncovered Petri plates face up in a Baker SterilGARD III laminar flow hood for 10 min at a 253-nm wavelength. Any hemocytes not removed during centrifugation were killed by the UV treatment. Experimental bacterial strains were diluted serially and plated on oyster fluid-treated and untreated control TSA plates and incubated at 37°C for 24 h. At the conclusion of incubation, the colonies on the treated and control plates were counted visually. Each experiment was done in triplicate, and dilutions containing between 50 colonies and 200 colonies were used for comparison of treated and control plates. To derive measures of inhibition, the experimental mean was divided into the control mean for each experiment. These plating experiments were done within 2 h of oyster fluid extraction.

Growth Curve of Escherichia coli

Growth of *Escherichia coli* in the presence and absence of oyster pallial cavity fluid extract (sampled October 18, 2011) was analyzed using sterile Corning 96-well flat-bottom microplates. The control wells contained 250 µL Tryptic Soy Broth. The treated wells contained 10, 25, or 50 µL oyster fluid extract and the appropriate amount of Tryptic Soy Broth to bring the volume to 250 µL. Both control and treated wells were inoculated with 10 µL of an *E. coli* suspension in phosphate-buffered saline (optical density, 0.14 at 550 nm). This experiment was repeated 4 times. Cell growth was monitored for 26 h at a 595-nm wavelength using a BioRad Benchmark Microplate Reader. The average absorbance values of 8 replicates of each concentration of pallial fluid were graphed for each hour assayed. Growth rate was determined using a computer program (Growth Curves, Zappy Laboratory). The growth curve results of *E. coli* for the oyster fluid treatments (0, 10, 25, and 50 µL) were compared statistically.

Lysozyme Determination

Lysozyme activity was determined using an EnzChek Lysozyme Assay Kit E-22013 (Molecular Probes). This is a fluorescence-based assay that measures lysozyme activity on *Micrococcus lysodeikticus* cell walls. The lysozyme substrate was *M. lysodeikticus* labeled with fluorescein that was quenched. Lysozyme action relieved this quenching, yielding an increase in fluorescence that was proportional to the lysozyme activity in the pallial cavity fluid tested. Relative fluorescence units (RFU) was measured on a Tecan fluorescence microplate reader using excitation/emission of 485/535 nm. A lysozyme standard curve for the measured fluorescence was established using known concentrations of chicken egg white lysozyme (measured in RFU per milliliter), and the amount of lysozyme in the experimental mucus extract was determined. The Bradford Assay determined protein concentration of the oyster fluid extracts, and lysozyme activity is reported as the number of RFU per microgram of extract protein.

Copper and Zinc Analysis

Oyster pallial cavity fluid samples were stored at –80°C in plastic tubes, separated by date of collection, until the day of analysis. On the day of analysis they were defrosted to room temperature by soaking the tubes in tap water. After defrosting, the samples were removed from the tubes, and collections from different days of the same month were combined to increase total sample available per month. Combined samples were then vacuum-filtered using Whatman glass microfiber filters (GF/A) prior to analysis of each month of oyster extract. Copper and zinc standards were purchased from Perkin Elmer (1,000 ppm in aqueous 2% nitric acid). Each standard was diluted to 100 ppm with water (the water used was 18MΩcm; Millipore Direct-Q), and these diluted standards were used to produce the standard solutions for analysis of varying concentrations. The Perkin Elmer Atomic Absorption Spectrophotometer 5100 PC was used for analysis with Perkin Elmer single-element zinc and copper lamps.

The method of standard additions was used for zinc analysis of the samples collected from 2010 through 2013. Concentrations of added standard ranged from 0.1–1.0 ppm in 10-mL

aqueous samples that included 0.2 mL filtered oyster fluid extract.

An external standard calibration method of analysis was used for copper analysis of the samples collected from 2010 through 2011. Copper calibration curve standards of 0.5, 1.0, 1.5, 2.0, and 2.5 ppm were made in 10-mL total volumes, and each solution was analyzed twice and averaged to obtain an external standard calibration curve. Filtered oyster fluid extract samples separated by month of collection were analyzed in 3-mL aliquots for copper. Fresh calibration standards were made and analyzed for each day that copper analysis was completed.

To ascertain any possible matrix effects on both copper and zinc analysis in these oyster samples, additional samples were collected in 2013 and analyzed using both external standard calibration and the method of standard additions for both metals (Campbell 1977, Weisel et al. 1979). The results of these analyses were compared. The samples were stored, prepared, and filtered as described earlier. Copper external standard calibration and zinc standard addition analyses were performed as described previously for the samples collected in 2010 and 2011. For the method of copper standard addition, concentrations of added standard were 0.5, 1.0, 1.5, 2.0, and 2.5 ppm in 3-mL total volume samples that also included 2 mL filtered oyster fluid. Zinc external calibration curve standards ranged from 2–10 ppm. The oyster fluid extract was diluted by a factor of 2 or 3 to ensure its measurement would be within the limits of the calibration curve for the zinc analysis.

Replicate measurements of any analyte sample or standard addition solution were not made because of the small sample volumes available. Standard deviations obtained from performing replicate measurements were, therefore, not obtained. The error associated with the reported zinc and copper concentrations is the propagation of uncertainty related to the least squares fit of the external standard calibration curves (Salter 2000) and standard addition curves (Bruce & Gill 1999) used to determine the concentrations to straight lines.

Statistical Analyses

Nonparametric and permutation tests were used to analyze the data.

RESULTS

Antimicrobial Activity of Oyster Pallial Cavity Fluid

Pallial cavity fluid of *Crassostrea virginica* collected at 2 different times of the year (May 2010 and October to November 2010) inhibited the growth of both Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) tester strains (Fig. 1). Results suggest the inhibitory effect of pallial fluid on both isolates was greater in the fall than in the spring (Table 1), but because of the small sample size no statistical analysis was done. In addition, *C. virginica* fluid samples collected in October to November 2010 inhibited the growth of both the Gram-positive bacteria *Staphylococcus aureus* (4-fold) and the Gram-negative bacteria *Serratia marcescens* (3-fold) and *Vibrio parahemolyticus* (3-fold). These strains were not tested against May 2010 samples of oyster fluid.

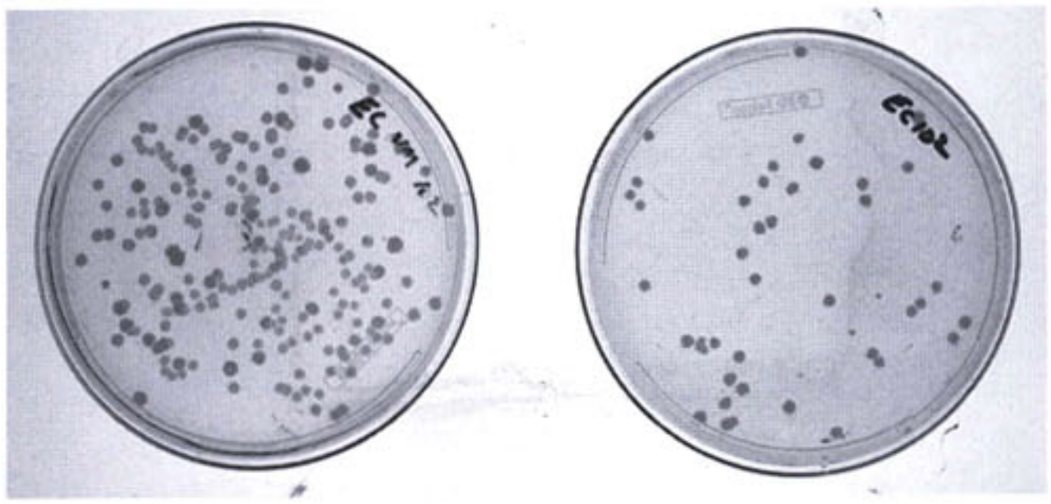

Figure 1. Plate treated with pallial fluid of *Crassostrea virginica* collected in September 2010 showing inhibition of *Escherichia coli* growth on Tryptic Soy Agar medium (right) and the untreated control (left).

Lysozyme Activity

Soluble lysozyme values averaged 0.175 ± 0.06 RFU/µg protein throughout the sampling period (September 2010 to November 2011). A statistically significant difference in seasonal (meteorological) lysozyme activity was observed (2-sided Wilcoxon's rank-sum test, $P = 0.0008$). Lysozyme activity was low throughout the winter–spring–summer months (mean, 0.08 RFU/µg protein) but increased 3-fold in the fall (mean, 0.25 RFU/µg protein). During the study, protein concentrations ranged from 1,192–2,363 µg/mL.

Growth Curve of Escherichia coli

During the screening experiments, a reduction in colony number as well as colony size in pallial fluid-treated plates versus controls was observed. A 26-h growth study using 0, 10, 25, and 50 µL oyster pallial fluid showed a significant end point decrease in *Escherichia coli* growth (Kruskal-Wallis test, $P = 0.0001$; Fig. 2). For the dilutions chosen, no clear-cut dose effect was observed. However, the 26-h end point in the 50-µL group was significantly less than all other groups, and the 25-µL group was significantly less than the control (Table 2). Estimated reduction in *E. coli* growth rate was 66% in the 50-µL group and 10% in the 25-µL group when compared with controls.

Zinc and Copper Analysis

Oyster pallial cavity fluid concentrations of zinc (standard addition analysis) and copper (external standard calibration analysis) in the 2010 to 2011 samples are shown in Table 3. In all months sampled, zinc concentrations were at least an order of magnitude greater than copper concentrations. Zinc concentrations in October and November 2010 remained relatively constant. However, on average, there was a 50% decrease in zinc concentrations in the following 7 mo studied. In contrast, copper concentrations remained relatively constant throughout the course of the study.

Zinc analysis of the 2013 samples using the method of standard additions resulted in a higher measured concentration of zinc in 6 of the 7 mo compared with the external standard calibration method, which may be caused by a complex oyster matrix affecting the zinc measurement. The zinc concentrations measured by the standard additions in the 2013 samples were all generally less than those measured in 2010 to 2011 (Table 3). They ranged from 8.9 ± 0.5–24 ±2 ppm. The average concentration of

TABLE 1.

Inhibition of *Crassostrea virginica* pallial fluid collected at 2 different times of the year on bacterial tester strains.

Inoculum	May 2010			October–November 2010		
	No. of colonies			No. of colonies		
	Ctrl	Exptal	Fold inhibition	Ctrl	Exptal	Fold inhibition
Escherichia coli	271 ± 19	115 ± 32	2.4	265 ± 52	28 ± 11	9.5
Bacillus subtilis	70 ± 11	34 ± 3	2.1	53 ± 7	21 ± 4	2.5
Serratia marcescens	—	—	—	270 ± 4	87 ± 5	3.1
Staphylococcus aureus	—	—	—	49 ± 5	13 ± 2	3.9
Vibrio parahaemolyticus	—	—	—	154 ± 27	47 ± 15	3.3

Control plates (Tryptic Soy Agar [TSA] + UV radiation) were compared with corresponding tester plates (TSA + PF + UV). Numbers are based on mean ± SD colony forming units of 3 plates for each sample pool tested. Fold inhibition was estimated by dividing the experimental mean into the control mean. —, no data available; Ctrl, control; Exptal, experimental; PF, pallial fluid.

zinc in April, May, and June 2013 was approximately 50% less than the average value in September and November 2013. Copper analysis of the 2013 samples using 2 different analytical methods does not indicate a clear matrix effect on the measured copper concentrations in the pallial fluid because the copper values obtained are similar for both methods. In addition, there is not a clear trend of 1 method always showing higher results than the other. The copper concentrations (measured by the external standard calibration method) were slightly less to similar, on average, in the 2013 samples compared with the 2010 to 2011 samples and ranged from 0.58 ± 0.06–2.3 ± 0.3 ppm.

The correlation between monthly mean levels of lysozyme and monthly mean levels of zinc was found to be 0.2446. Although the observed value is positive, there is insufficient evidence to conclude that average lysozyme and zinc levels are associated (exact permutation test, $P = 0.56$).

DISCUSSION

Although screening studies of antibacterial activity in marine invertebrate phyla (Porifera, Cnidaria, Echinodermata, Mollusca) have shown that Gram-positive bacteria are more sensitive than Gram-negative strains to extracts or tissues from marine organisms (Haug 2004), several studies have demonstrated that bivalve hemolymph lyses both Gram-positive and Gram-negative bacteria (Chu 1988, Cheng 1983). McDade and Tripp (1967b) found oyster hemolymph inhibits the growth of Gram-positive *Bacillus subtilis* and *Bacillus megaterium*. Later, it was shown effective against *Escherichia coli, Gaffkya tetragena, Salmonella pullorum*, and *Shigella sonnei* (Cheng 1983). In the current study, the inhibitory effect of oyster pallial cavity fluid on Gram-negative *Serratia marcescens* and *Serratia marcescens* is reported. Often found in warm coastal waters *V. parahaemolyticus* has been linked to recent outbreaks of illness from eating contaminated shellfish harvested in western Long Island Sound (Cuda 2013).

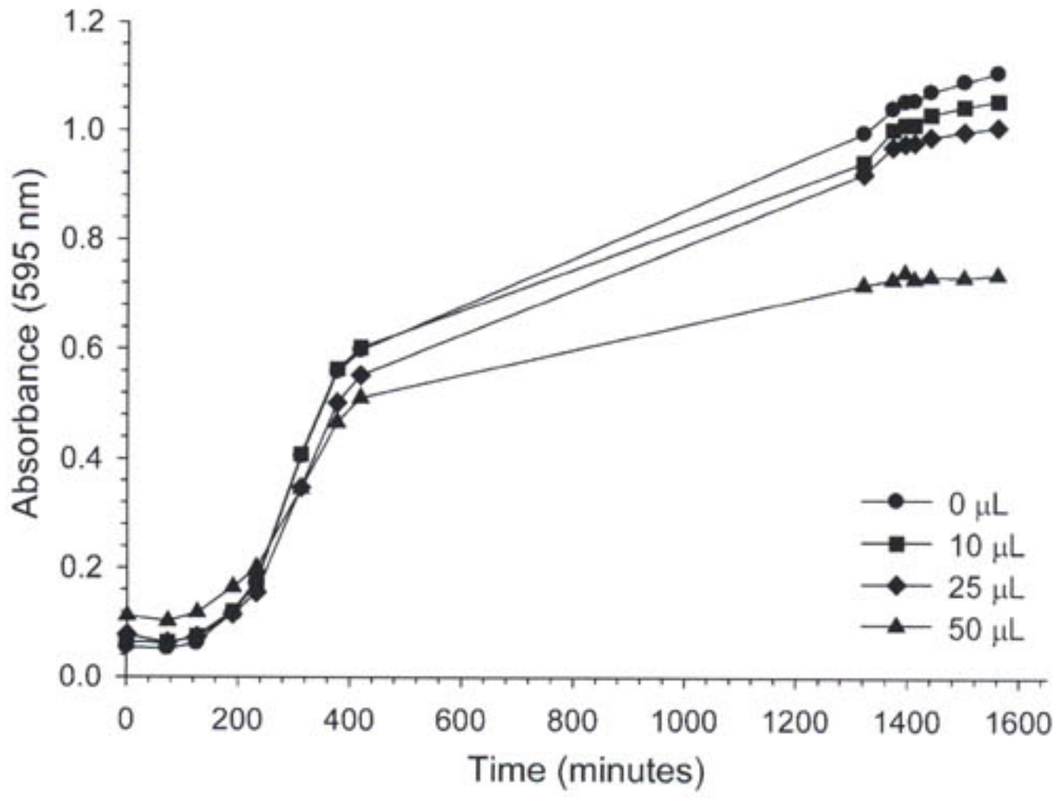

Figure 2. Growth curves from a single experiment of *Escherichia coli* growth in the presence of 0, 10, 25, and 50 µL pallial fluid from *Crassostrea virginica* collected in October 2011.

Antimicrobial factors (antimicrobial peptides, lysosomal enzymes, lectins) are synthesized in the hemocytes (Mohandas et al. 1985, Tunkijjanukij & Olafsen 1998, Mitta et al. 1999, Mitta et al. 2000), where they destroy bacteria, and when released into plasma contribute to a systemic microbial response (Cheng et al. 1975, Cheng 1986, Mitta et al. 1999). Lysozyme is found in oyster hemocytes and serum (Cheng & Rodrick 1974) as well as in mantle mucus (McDade & Tripp 1967a). Several investigators have shown the presence of hemocytes in shell fluids of oysters (Mulholland & Friedl 1994, Paillard et al. 1996), and Allam and Paillard (1998) documented the presence of lysozyme activity in the extrapallial fluids of the Manila clam *Ruditapes philippinarum*. Therefore, the high concentration of lysozyme found in the oyster mantle fluid in the current study is not surprising. The presence of this enzyme in pallial fluid of both clams and oysters is likely a result of diapedesis of hemocytes into the mantle cavity and/or mantle epithelial cell secretions.

Studies of serum lysozyme levels in oysters from the mid Atlantic found low levels of lysozyme activity during the summer months (Feng & Canzonier 1970, Chu & LaPeyre 1989, Chu & LaPeyre 1993). Similarly, the result of the current study show low summer lysozyme levels in oysters from Black Rock Harbor, but increased mantle fluid lysozyme levels during the fall. This result is also similar to those reported for the mussel *Mytilus galloprovincialis* from the Adriatic Sea, in which increasing serum lysozyme activity was recorded in the fall (Ciacci et al. 2009). In addition, the finding in the current study of increased lysozyme levels in shell liquors during periods of

TABLE 2.

Calculated *P* values for 2-sided Wilcoxon's rank-sum tests for all possible pairs of groups (0, 10, 25, and 50 µL pallial fluid) in the *Escherichia coli* growth experiment.

	10-µL group	25-µL group	50-µL group
0-µL group	0.0938617	0.00932401	0.0003108
10-µL group		0.160528	0.0001554
25-µL group			0.0001554

TABLE 3.

Concentrations of zinc and copper in oyster pallial fluid collected in 2010 to 2011 and 2013.

Month and year	Zinc concentration* (ppm)	Copper concentration† (ppm)
October 2010	59 ± 3	2.49 ± 0.06
November 2010	61 ± 3	1.31 ± 0.07
December 2010	28 ± 4	0.78 ± 0.08
February 2011	38 ± 2	—
March 2011	28 ± 3	2.14 ± 0.03
April 2011	28 ± 2	1.18 ± 0.06
May 2011	30 ± 2	2.14 ± 0.08
July 2011	21 ± 4	1.59 ± 0.03
October 2011	37 ± 2	1.61 ± 0.08
January 2013	17 ± 2	1.3 ± 0.3
February 2013	—	2.3 ± 0.3
March 2013	24 ± 2	1.6 ± 0.5
April 2013	8.9 ± 0.5	0.8 ± 0.2
May 2013	10 ± 1	0.6 ± 0.2
June 2013	9.7 ± 0.5	1.1 ± 0.8
July 2013	20.3 ± 0.7	1.05 ± 0.06
September 2013	19 ± 3	0.58 ± 0.06
November 2013	23.3 ± 0.5	0.91 ± 0.02

* The error associated with the zinc concentrations in this table is the propagation of uncertainty related to the least squares fit of the standard curves to straight lines (Bruce & Gill 1999), rather than an SD obtained from performing replicate measurements.

† The error associated with the copper concentrations is the propagation of uncertainty associated with the least squares fit of the external standard calibration curves to straight lines (Salter 2000), rather than a standard deviation obtained from performing replicate measurement.

elevated water temperatures (Brousseau unpubl.), and corresponding high bacterial loads at the study site are in line with experimental evidence indicating increased lysozyme activity in the extrapallial fluid of several bivalves species after bacterial challenge (Allam et al. 2000a, Allam et al. 2000b, Paillard et al. 2004, Allam et al. 2006).

The antimicrobial role of the metals copper and zinc is well documented in the literature (for review, see Stafford et al. [2012]). It has been reported that both copper and zinc can be absorbed and retained in the tissues of oysters in larger quantities than is needed to sustain normal metabolic processes (Marks 1938). Brown (1975) found that copper and zinc were ubiquitous in granular amoebocytes of oysters, and estimated cell-free hemolymph contained 0.159 µg/mL copper and 8.37 µg/mL zinc. *In situ* experiments designed to study the dynamics of several metals in the shell and soft tissues of *Crassostrea virginica* demonstrated that copper and zinc accumulated in soft tissues at levels of 450 ppm copper and 4,100 ppm zinc, compared with 60 ppm copper and 1,700 ppm zinc for controls (Frazier 1976). Mantle tissue of oysters from Jamaica Bay, New York, contained 125–200 ppm copper and 1,100–1,600 ppm zinc (Rodney et al. 2007). As expected, metal concentrations in Black Rock Harbor oyster pallial cavity fluid fell between hemolymph and soft tissue values, averaging 1.4 ppm copper and 24 ppm zinc. Metal concentrations in seawater samples taken at the study site in November 2013 were nearly undetectable (copper, 0.003 ± 0.02 ppm; zinc, 1.9 ± 0.6 ppm). Zinc uptake appeared to be dependent seasonally, with the highest concentrations measured during the fall, whereas copper concentrations remaining relatively steady throughout the year. In addition, Frazier (1976) reported rapid uptake of metals in the summer and fall by oyster soft tissues. Although the data from the current study suggest a possible association between seasonal levels of lysozyme activity and zinc concentrations in the oyster pallial fluid analyzed, there is insufficient evidence to conclude that correlation between the variables is statistically significant. Additional study with larger sample sizes is needed.

Copper and zinc accumulation in oyster amoebocytes and soft tissues may account, in part, for the defensive properties of mantle cavity fluid demonstrated in the current study. Oyster pallial cavity fluid contains hemocytes as well as mucus and agglutinins derived from soft tissues such as the mantle, which have been shown to sequester these heavy metals. Lysozyme is known to be most effective against Gram-positive bacteria as a result of its ability to degrade the exposed peptidoglycan layer in cell walls. However, pallial cavity fluid tested in the current study was equally effective against Gram-negative strains. This effectiveness may be the result of the presence of high copper and zinc concentrations. Copper and its alloys (brasses, bronzes, copper–nickel–zinc) attack membrane integrity in microbes and have been shown to kill 99.9% of *Escherichia coli* within 2 h of exposure (Yamada 2010). Furthermore, heightened antimicrobial activity was measured in the fall, the season when not only soluble lysozyme levels, but also zinc concentrations during the periods 2010 to 2011 and 2013 were at their highest. Although further testing of the importance of these metals is needed, the inhibitory action of pallial cavity fluid demonstrated in the current study, adds to the already strong circumstantial evidence of antimicrobial activity by accumulated copper and zinc in oysters.

Black Rock Harbor is arguably one of the most highly affected sites in western Long Island Sound. It receives large volumes of sewage effluent daily, and the bottom sediments are contaminated with high amounts of the heavy metals copper, chromium, and zinc, as well as PCBs, PAHs, and chlorinated pesticides (Gardner et al. 1991, Brandon et al. 1996). The ability of oysters to thrive in this location is yet another example of the apparent contradiction that, despite its known toxicity to many marine organisms (Neff 2002), oyster survival may be enhanced by the presence of copper and zinc. The presence of detectable amounts of lysozyme, as well as copper and zinc, in the pallial fluid links all 3 to the antimicrobial activity demonstrated in the current study. Furthermore, these results support previous work demonstrating the possible

involvement of mucosal immunity at the pallial interface (Jing et al. 2011). The suggestion of a seasonal pattern of pallial fluid lysosomal activity and sequestration of the heavy-metal zinc found in the current study, underscore the need for further investigation regarding possible seasonality in defense-related functions for the pallial cavity fluids in the eastern oyster *Crassostrea virginica*.

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