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Alterations in Hemolymph and Extrapallial Fluid Parameters in the Manila Clam, *Ruditapes philippinarum*, Challenged with the Pathogen *Vibrio tapetis*

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In a recent study, we demonstrated the presence of defense factors, competent hemocytes and high enzymatic activities (peptidases, hydrolases, lytic, etc.), in the extrapallial fluid, located between the mantle and the shell, of the Manila clam, *Ruditapes philippinarum.* In Europe, this species is affected by brown ring disease, an epizootic disease caused by the bacterium *Vibrio tapetis.* The present work focused on the effect of the development of the disease on cellular and humoral defense parameters in the hemolymph and the extrapallial fluid of experimentally infected clams. Results indicate significant changes in total and dead hemocyte counts, as well as modifications in lysozyme activity and protein content, in the hemolymph and extrapallial fluid of challenged animals. Hemocyte counts and lysozyme activity increased significantly in the hemolymph, but particularly in the extrapallial fluid, where the highest values were observed. A healing (recalcification) process was observed 7 weeks following challenge, suggesting defense system efficiency at neutralizing the pathogen. These results are discussed with emphasis on the role of extrapallial fluids in the defense process against invading microorganisms. © 2000 Academic Press

Key Words: **shell disease; bivalve; immune defense; lysozyme; hemocyte; bacteria.**

INTRODUCTION

In common with many invertebrates, host defense in bivalves is largely nonspecific, based on activities of circulating hemocytes in the soft tissues (Bayne, 1983; Millar and Ratcliffe, 1994; Cheng, 1996) and in the extrapallial fluid (EF) between the mantle and the inner face of the shell (Allam and Paillard, 1998). Hemocyte defense functions include phagocytosis (Lopez *et al.,* 1997), encapsulation and wound repair (Sparks and Morado, 1988), and synthesis of humoral defense factors (Feng, 1988; Cheng, 1996). Diverse types of serum defense factors also occur, such as agglutinin/opsonins, bactericidins, and antiparasitic and lytic factors (Chu, 1988; Hubert *et al.,* 1996). The lytic activity of bivalve lysozyme has also been demonstrated against many species of Gram-positive and -negative bacteria (Chu, 1988; Cheng, 1983).

Both the cellular and the biochemical composition of bivalve hemolymph (HE) have been studied during natural or experimental contact with pathogens. Changes in the number and type of circulating hemocytes have been described in parasitized bivalves (Ford *et al.,* 1993) or following experimental challenge with pathogens (Suresh and Mohandas, 1990; Oubella *et al.,* 1996). Modifications in molluscan serum components, such as protein and lysozyme, have also been associated with parasitism and experimental challenge with pathogens (Feng and Canzonier, 1970; Ford, 1986; Chu and La Peyre, 1993). Moreover, it has been demonstrated that *in vitro* challenge of bivalve hemocytes with bacteria induced hypersynthesis and release of hydrolytic enzymes into the serum (Cheng, 1992).

Since the late 1980s, European culture of the Manila clam, *Ruditapes philippinarum,* has been seriously decimated by a new shell disease, brown ring disease (BRD). This disease is characterized by an abnormal conchiolin deposit on the inner face of the shell, within the extrapallial space. Laboratory experiments have shown that challenge with the causative bacterium, *Vibrio tapetis* (Borrego *et al.,* 1996a), induces cellular and humoral responses, reflected by changes in total and differential hemocyte counts and leucine aminopeptidase activity in the hemolymph (Oubella *et al.,* 1994, 1996). Recently, we developed a new research strategy, taking into account the study of defense fac-

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tors in clam extrapallial fluid, the fluid in contact with the site where the pathogen is mainly present (mantle edge and periostracal lamina) (Allam, 1998; Allam and Paillard, 1998). In these studies, we demonstrated the presence of numerous active hemocytes and high enzymatic activities (peptidases, hydrolases, lysozyme-like, etc.) in the extrapallial fluid, at levels comparable to those found in the hemolymph. Additionally, an increase in hemocyte counts and lysozyme activity was observed in the hemolymph, and particularly in the extrapallial fluid, of naturally diseased clams, raising questions about a possible role of this fluid in defense processes (Allam, 1998).

Experimental challenge of clams with the pathogen is a way to clarify and understand the defense strategy used to fight infection and repair damage. Moreover, in contrast to observations made on diseased animals collected in the field, the kinetics of the development of the disease also can be followed. In the present study, both the hemolymph and the extrapallial fluid were investigated in clams challenged with *V. tapetis,* to better understand the defense mechanisms of the host against the pathogen. We examined alterations in hematological parameters (total cell counts) and lysozyme concentrations in response to pathological stress. The percentage of dead hemocytes and protein contents in the plasma were also measured to evaluate physiological alterations in clams during infection.

MATERIALS AND METHODS

Animals

Adult (35–40 mm) Manila clams showing no signs of brown ring disease were collected from wild populations living in the Bay of Brest (France). The animals were maintained in aerated marine aquaria at 15°C and fed daily on cultured algae (*Dunaliella euchlora, Pavlova lutheri,* and *Isochrysis galbana*) throughout the experiments.

Infection Experiment

The *V. tapetis* strain P 21 used in the infection experiment was isolated from naturally diseased clams from the Bay of Brest in 1993 (C. Palliard and P. Maes, unpublished data) and used according to Allam *et al.* (1996). The clams were left out of water for 12 h before inoculation to incite them to open their valves during the injection procedure. A *V. tapetis* suspension of 10^8 cells ml^{-1} in sterile seawater (SSW) was obtained from a 72-h-old culture grown on marine agar. A volume of 0.5 ml (5×10^7 bacteria) from this suspension was inoculated into the pallial cavity of each experimental clam. Care was taken not to damage the mantle edge and the shell. Challenged and control (SSW-inoculated) clams were left out of water for 6 additional h and then placed in separate aquaria. Twenty-five clams were kept apart before the beginning of the experiment (time zero clams) and sampled before the overnight emersion. After the challenge, test and control clams were sampled at each time interval (6 h corresponding to clams sampled at the end of the emersion period, 1 day, 3, 7, 14, 28, and 49 days, $n = 20$ clams/sample, except for h 6 where $n = 15$ clams/sample).

Fluid Sampling

Hemolymph samples were withdrawn from the posterior adductor muscle. Extrapallial fluid was collected by drilling a hole through the external face of the shell with a round dental burr without cutting the mantle (Allam and Paillard, 1998). For each clam, the extrapallial fluid obtained from both valves (400 μl/valve) was pooled, since previous studies have shown significant positive correlation in cell number in the left and the right valve (Allam and Paillard, 1998).

Hemolymph and Extrapallial Fluid Parameters

Cell counts and viability. Total and viable cells were simultaneously counted with a hemocytometer on mixtures (v/v) of sample with 0.2% ice-cold SSW–trypan blue solution. Results of total and viable cell counts are presented as 10^6 cells ml^{-1} and percentage of dead cells (PDC), respectively.

Lysozyme activity measurement. HE and EF samples were centrifuged (10 min, 400*g,* 4°C) and supernatant (SN) was withdrawn and stored below −20°C until use. Sterile seawater was added to cell pellets to obtain a final concentration of 10^6 cells ml^{-1}, which then sonicated (40 min in ice-cold water). Cell lysate (CL) was frozen (−20°C) until use. Lysozyme concentration was determined as previously described (Allam and Paillard, 1998). Chicken egg white lysozyme (CEW; Sigma) served as the standard. Supernatant protein was measured using bovine serum albumin as the standard. Lysozyme activity is given in CEW lysozyme equivalents expressed in micrograms per mg protein for the SN and per 10^6 cells for the CL.

Bacterial analysis. EF samples from clams collected at day 49 were examined for the presence of bacteria according to the method previously described (Allam and Paillard, 1998). Subsamples of EF were serially diluted and plated on standard marine agar or inoculated in standard marine culture broth. Enumeration was made after 2 weeks incubation at 20°C.

Characterization and Classification of BRD

Disease progression was monitored according to the scale established by Paillard and Maes (1994). In this classification, the degrees of symptoms ranged from microscopic brown spots on the inner face of the shell in

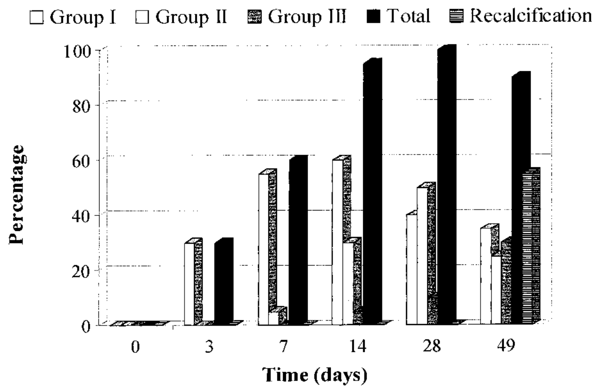

FIG. 1. Development of brown ring disease and recalcification in clams following challenge with *V. tapetis.* Total = sum of diseased animals of Groups I, II, and III; n = 20 clams for each time interval.

early stage (stage 1) to a complete thick brown ring in advanced stage (stage 7). In accordance with this classification, three main groups are considered: Group I: stages 1 and 2; Group II: stage 3; Group III: stages 4 to 7. The shell repair process, which consists of recovering the brown deposit with new calcified layers (Paillard and Maes, 1994), was also monitored.

Statistical Analysis

For all parameters, differences between test and control clams were assessed using Student's t test. Results are expressed as mean ± standard error (SE) and differences were considered statistically significant at $P < 0.05$.

RESULTS

Development of the BRD

Microscopic BRD symptoms appeared 3 days post-challenge (Fig. 1). Thereafter, the percentage of clams with the symptoms and the degree of development of the disease increased. Group I and Group II clams attained their maximum percentage at day 14 and day 28, respectively. Heavily diseased clams (Group III) appeared at day 14 (5%) and increased in number until the end of the experiment (30% at day 49). The shell repair process was observed only at day 49 (55% of collected clams). BRD signs were never observed in controls.

Cellular Parameters

In hemolymph samples, an early increase in total hemocyte counts (THC) was observed in both test and control clams (Fig. 2a). Following reimmersion at h 6, THC decreased only in control animals. Differences between test and control clams became significant from day 1. The value was maximal at day 7 and then steadily decreased, but still remained significantly higher than in controls until day 28. At day 49, the difference between test and control animals was not significant ($P = 0.37$).

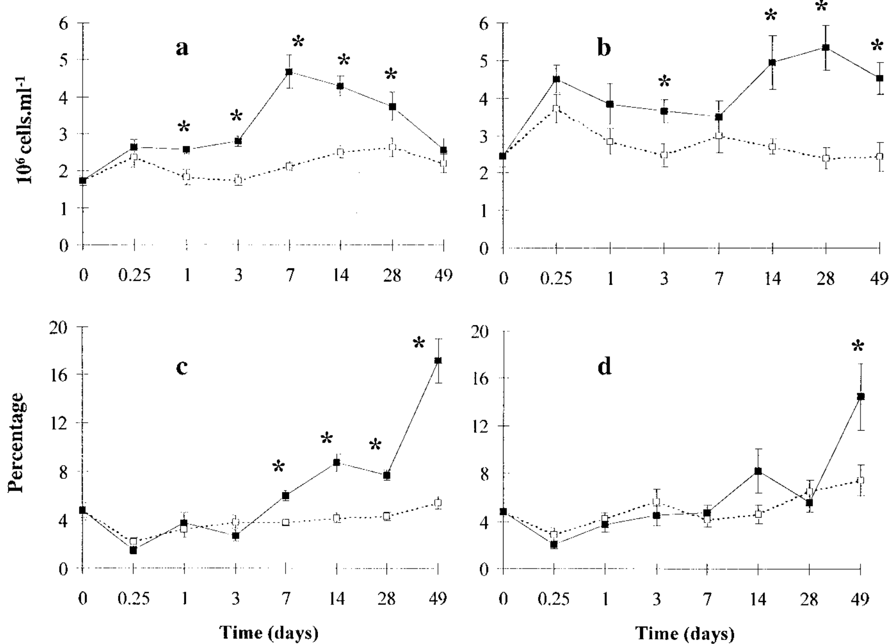

FIG. 2. Effect of the challenge of clams with *V. tapetis* on total hemocyte counts (a and b) and percentage of dead cells (c and d) in the hemolymph (a and c) and the extrapallial fluid (b and d). * Significant differences ($P < 0.05$, Student's t test) between challenged (solid lines) and control (SSW-injected, dotted lines) animals; n = 20 clams, except for h 6, for which n = 15; mean ± SE.

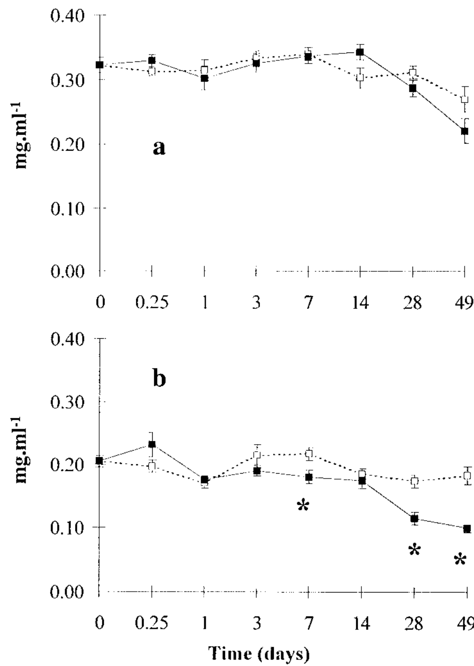

FIG. 3. Protein contents in the hemolymph (a) and the extrapallial fluid (b) of *V. tapetis*-challenged (solid lines) and SSW-injected (dotted lines) animals. * Significant differences ($P < 0.05$, Student's t test); $n = 20$ clams, except for h 6, for which $n = 15$; mean ± SE.

In extrapallial fluid samples, THC were always higher in test than in control clams (Fig. 2b). An early increase was observed in both control and test clams until reimmersion. Thereafter, THC decreased in both control and challenged animals. The first statistically significant differences between test and control clams appeared at day 3 (Fig. 2b). A second phase of increase appeared in challenged clams between day 7 and day 14, with values being significantly higher in test clams than in controls from day 14 until the end of the experiment. In challenged clams, the maximal value was reached at day 28.

The percentage of dead cells increased significantly in the HE of challenged clams from day 7 to day 49 (Fig. 2c). In the EF, the only significantly higher value occurred on day 49 (Fig. 2d). In both fluid compartments, maximum percentages of dead cells were observed at day 49 (about 17 and 14% for HE and EF, respectively).

Supernatant Protein

A general decrease in hemolymph protein concentration occurred after day 14, but no correlation to *V. tapetis* inoculation was observed (Fig. 3a). In the EF, the protein concentration was significantly lower in challenged clams than in controls at days 7, 28, and 49 (Fig. 3b). Control clams showed fairly stable protein levels, whereas challenged animals showed an apparent decrease at the end of the experiment.

Lysozyme

The lysozyme activity in hemolymph cell lysate increased in challenged clams after day 1 (Fig. 4a). How-

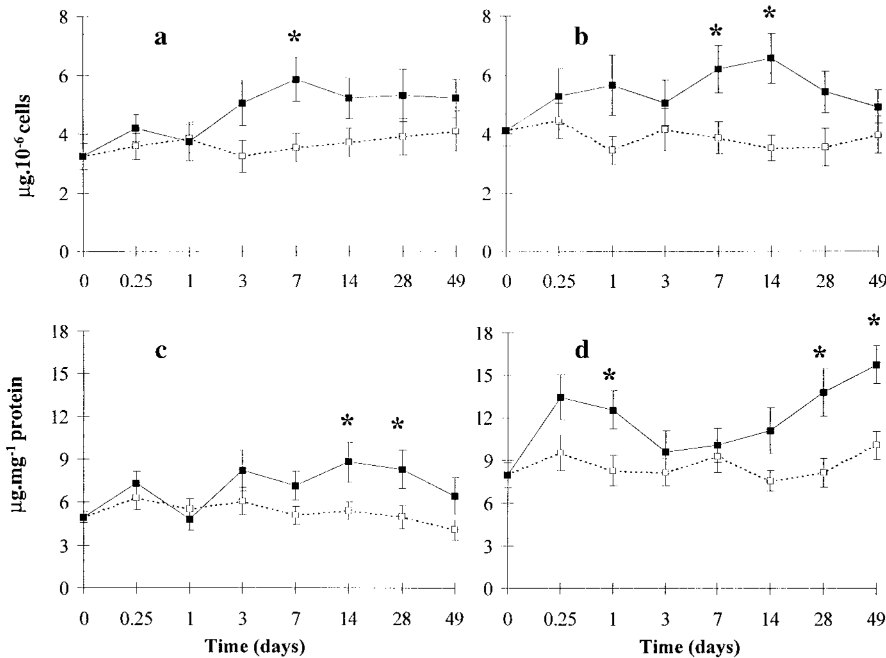

FIG. 4. Effect of the challenge of clams with *V. tapetis* on lysozyme activity in cell lysates (a and b) and supernatants (c and d) of the hemolymph (a and c) and the extrapallial fluid (b and d). * Significant differences ($P < 0.05$, Student's t test) between challenged (solid lines) and control (SSW-injected, dotted lines) animals; $n = 20$ clams, except for h 6, for which $n = 15$; mean ± SE.

ever, the difference from lysozyme activity in the control clams was significant only at day 7. In the EF of challenged clams, the lysozyme activity was higher than in the controls from h 6 until day 49 (Fig. 4b), but the differences were significant only on days 7 and 14.

The lysozyme activity in hemolymph supernatant increased in challenged clams after day 1 (Fig. 4c). Significant differences were obtained at day 14 (9 μg CEW lysozyme mg^{-1} protein, maximum value) and day 28, after which levels started to decrease. In the EF supernatant, lysozyme activity increased from the first hours in challenged animals ($P = 0.06$ and 0.018 at h 6 and day 1, respectively) compared to SSW-injected clams (Fig. 4d). After a decrease at day 3, EF supernatant lysozyme increased again in test clams and reached maximum values by day 49.

Bacteriology

Forty-nine days following inoculation, there were no culturable bacteria in the EF samples from either control ($n = 8$) or challenged ($n = 14$) clams, despite the development of the BRD in all contaminated animals. Consequently, they were considered to be bacteriologically sterile.

DISCUSSION

In the present study, cellular and humoral responses were observed in the hemolymph and the extrapallial fluid of *R. philippinarum,* following challenge with *V. tapetis,* the etiologic agent of BRD. In molluscan hemolymph, hemocytosis is one of the cellular responses of the defense system (Feng, 1988; Cheng, 1996), and previous work has demonstrated increased THC in bivalves challenged with bacteria (Suresh and Mohandas, 1990; Oubella *et al.,* 1996). The kinetics of the rise observed in the HE of challenged clams, i.e., a response within 24 h postchallenge and maximum values at 7–14 days, is in accordance with the observations by Oubella *et al.* (1994, 1996). These authors interpreted changes in THC by two processes: (1) the short-term increase of circulating cells corresponded to hemocyte mobilization from tissues into circulation and (2) the decrease observed after day 14 could result from "a mobilization of circulating cells into the sites of infection." The major site of infection in diseased clams is the periostracal lamina–mantle area, which is quickly colonized by the pathogen (Paillard and Maes, 1995; Allam *et al.,* 1996). This site is in contact with the extrapallial fluid. Since the increase in THC in EF at day 14 was accompanied by an increasing prevalence of BRD in challenged clams, it could result from an effective mobilization of hemocyte toward the site of infection. Simultaneously, THC decreased in HE and increased in EF between day 7 and day 28, the period of maximal BRD development. Infiltration of hemocytes toward infected tissues has been observed in several bivalve species affected with pathogens (Ford *et al.,* 1993). Intensive infiltration of hemocytes to the damaged mantle and the extrapallial compartment has been also reported by Bricelj *et al.* (1992) in young oysters, *Crassostrea virginica,* affected with juvenile oyster disease (a shell disease with clinical signs similar to those of BRD). They described hemocytes on and within the organic deposit characterizing this disease. Similar observations of hemocyte infiltration were associated with the presence of bacterial bodies in the extrapallial space of juvenile *C. virginica* with extrapallial abscesses (Elston *et al.,* 1999). We have already reported significantly higher THC values in the EF of naturally infected *R. philippinarum* and the presence of hemocytes on the periostracal lamina (Allam, 1998). Data presented here show that experimental induction of BRD causes a mobilization of hemocytes to the EF, probably caused by the perception of the pathogen and damage associated with the infection in this peripheral compartment.

High percentages of dead cells (PDC) have already been described in HE and EF of naturally diseased clams (Allam, 1998) and in EF of oysters affected by the juvenile oyster disease (Paillard *et al.,* 1996). Since it is a ratio, changes in PDC may have two different origins: (1) a toxic effect of *V. tapetis* directly on the hemocytes, or indirectly, causing a physiological disturbance or failure to remove dead cells (autophagocytosis) or (2) a decrease in viable cell counts leading to an increase in PDC. In the HE of test clams sampled at day 7, high PDC and high THC suggested a true increase of dead cell numbers. The increase in PDC after day 7 was accompanied by a decrease in THC and was possibly due to the concomitant effect of migration of active ("viable") cells from the HE toward other sites (for example, mantle and EF), leading to an increase in the proportion of dead cells accumulated in the HE. The maximum PDC values corresponded to the lowest THC in HE (day 49). In the EF, high PDC in heavily diseased clams (day 49) imply a real increase in dead cell counts and may be the result of interaction between the hemocytes and the pathogen. Even if the EF did not contain viable bacteria, the presence of bacterial products in this compartment cannot be excluded. *V. tapetis* has hemolytic, cytotoxic, and exotoxic properties (Borrego *et al.,* 1996b), and preliminary results of *in vitro* experiments show a toxic effect of *V. tapetis* cells and/or extracts against *R. philippinarum* hemocytes (unpublished data and Lane, 1997).

The protein contents of the serum were not altered by BRD development. This is in accordance with the observations of Oubella *et al.* (1994) and Plana *et al.* (1996). The EF supernatant from diseased clams had significantly lower protein contents than the controls at day 28 and day 49. This may be related to modifications of metabolic activities reflected by low shell

growth rate, as reported in diseased individuals (Goulletquer *et al.,* 1989).

The level of lysozyme activity measured here could be biased by the kinetics of synthesis and secretion from hemocytes into the extracellular medium (Cheng *et al.,* 1975). Indeed, the increase of lysozyme activity in EF supernatant noted at days 28 and 49 was associated with a decrease of the activity in cell lysate during the same period and could be related to release of lysozyme from the hemocytes into the surrounding medium. Feng and Canzonier (1970) demonstrated that lysozyme activity increased in the hemolymph of *C. virginica* infected by a trematode. However, it was not altered in oysters infected by the protistans *Haplosporidium nelsoni* and *Perkinsus marinus* (Feng and Canzonier, 1970; Chu and La Peyre, 1989, 1993). Chu and La Peyre (1993) speculated that a "specific stimulus," possibly a polysaccharide (e.g., bacterial cell wall component), may be responsible for rapid increase in lysozyme levels in oysters. Even if the lytic activity of *R. philippinarum* lysozyme on *V. tapetis* has not been tested, this enzyme probably plays an important role during the pathologic process in clams. In addition to the direct lytic effect on bacteria, lysozyme enhances the bactericidal effect of antibacterial proteins on Gram-negative bacteria, even if the bacteria are not lysed (Smith *et al.,* 1995). The increased lysozyme activity observed here in HE and EF of diseased clams probably plays a defensive role against *V. tapetis* and/or possible secondary infections of the mantle.

Clams experimentally infected with *V. tapetis* showed evidence of shell repair 7 weeks postchallenge. Shell repair, considered as recovery (or healing) by Paillard and Maes (1994), is associated with a decrease in *V. tapetis* (and total bacterial microflora) burden (Allam *et al.,* 1996). The repair process is probably related to the efficiency of the defense response in the EF (increase in THC and lysozyme activity, etc.). Hemocytes from the HE and EF of *R. philippinarum* phagocytose and degrade *V. tapetis* both *in vivo* and *in vitro* (Allam, 1998; Allam and Paillard, 1998; Lopez-Cortez *et al.,* 1999). The growth of *V. tapetis* is also reduced by hemocyte extracellular factors (unpublished data). The EF of experimentally infected clams was bacteriologically sterile 49 days following challenge. This result does not exclude the possible presence of noncultivable bacteria or bacterial products in the EF but underlines the efficiency of defense factors in the EF in neutralizing invading microorganisms. Hemocyte counts and lysozyme activity in the EF at day 49 were approximately two times higher than values measured at day 0. Culturable bacteria, particularly *V. tapetis,* were present in the EF of diseased animals collected in the field and exhibiting extremely advanced stages of BRD that are never reached under experimental conditions (Allam, 1998). Similarly, the death due to BRD is observed only in the field and is rare following experimental infection (unless under starvation conditions; Allam *et al.,* 1996). A relationship between invasion of the extrapallial compartment, and subsequently tissues, and clam death was suspected (Allam, 1998).

In conclusion, we demonstrate for the first time increases in hemocyte counts and lysozyme activity in the extrapallial fluid of experimentally challenged clams. This response was followed by a healing process. These findings confirm that the extrapallial fluids of bivalve molluscs may play a crucial role in defense processes during infection with shell diseases. A better consideration of this pseudo-internal fluid should be made in future work on bivalve immunity.

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