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Changes in bacterial densities and hemocyte parameters in eastern oysters, *Crassostrea virginica*, affected by juvenile oyster disease

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Abstract

Juvenile oyster disease (JOD) in the eastern oyster, *Crassostrea virginica*, is characterized by a conchiolin deposit on the inner surface of the valves. Similarities to pathological syndromes affecting other oysters (pearl oyster mortality) and clams (brown ring disease), has suggested an infectious origin and a possible bacterial etiology. Bacteriological analysis (Total Heterotrophic Bacteria, THB and Total *Vibrio* sp., TVS) of oyster soft tissues, shell fluid and inner shell surfaces were monitored during the course of JOD onset and development. A significant increase in THB (but not TVS) occurred in shell fluid and at the surface of the inner shell of JOD affected oysters. At the same time, changes in cytometric parameters (Total Hemocyte Counts, THC; Differential Hemocyte Counts, DHC) in hemolymph and shell fluid were documented in symptomatic oysters. THC in the shell fluid showed a decrease in late-stage JOD oysters. The disease was also correlated with altered cell ratios in both hemolymph and shell fluid, resulting in an increase in the percentage of the granulocytes in both locations. Our results have showed that main pathological changes associated with JOD occurred in extrapallial region rather than in the soft tissues. The disease was transmitted in the laboratory by extracts from diseased oysters: anomalous conchiolin developed within 4 weeks and the prevalence was higher when two inoculations were performed rather than one. A number of bacterial strains were found at high levels in diseased oysters and isolated. However, none produced the JOD deposit when injected into asymptomatic oysters. The bacterial etiology hypothesis for JOD should be explored further, expanded beyond the family Vibrionaceae, and should include consideration of a multiple etiology.

Keywords: Shell disease, Bivalve, *Crassostrea virginica*, bacteria, hemocyte, cellular response, shell fluid.

Modifications de la charge bactérienne et des paramètres hémocytaires chez l'huître américaine, Crassostrea virginica, *affectée par la maladie des huîtres juvéniles.*

Résumé

La maladie des huîtres juvéniles chez l'huître américaine, *Crassostrea virginica* est caractérisée par un dépôt de conchyoline sur la face interne des valves. Du fait de similitudes avec les symptômes des maladies affectant les huîtres (mortalités d'huîtres perlières) et les palourdes (maladie de l'anneau brun), une origine infectieuse et une éventuelle étiologie bactérienne ont été suggérées. Ainsi, lors du développement de la maladie, des analyses bactériologiques (microflore bactérienne hétérotrophique totale, MBHT et microflore Vibrionnacées) ont été réalisées dans les tissus, les fluides extrapalléaux et à la surface interne des coquilles d'huîtres. Une augmentation significative de MBHT (mais non de *Vibrio* sp.) a été décelée au niveau des fluides extrapalléaux et à la surface des coquilles d'huîtres malades. Parallèlement, des modifications de paramètres cytométriques (comptage des hémocytes totaux, CHT; comptage différentiel des hémocytes, CDH) ont été mesurées au sein des fluides hémolymphatiques et extrapalléaux. Dans les stades évolués de la maladie, une diminution de CHT a été mise en évidence au sein des fluides extrapalléaux. Des

modifications de la formule hémocytaire ont été de plus enregistrées au sein des fluides hémolymphatiques et extrapalléaux, révélant une augmentation du pourcentage de granulocytes dans ces deux liquides. Les résultats de cette étude montre que les perturbations associées à la maladie des juvéniles d'huîtres affectent plutôt les compartiments extrapalléaux que ceux internes et tissulaires. Cette maladie a été transmise expérimentalement par inoculation de broyats d'huîtres malades à des huîtres asymptomatiques : le dépôt anormal de conchyoline est nettement visible à partir de 4 semaines et la prévalence de la maladie est plus forte après deux inoculations plutôt qu'une. Des souches bactériennes prédominantes ont été isolées chez des huîtres malades. Aucune de ces souches, inoculées à des huîtres asymptomatiques, a induit un dépôt de conchyoline caractéristique de la maladie des huîtres juvéniles. L'hypothèse d'une étiologie bactérienne doit cependant être poursuivie, y incluant des recherches au delà de la famille des Vibrionnacées mais aussi en lien avec l'effet synergique de facteurs physiologiques et environnementaux.

Mots-clés : Maladie coquillière, bivalve, *Crassostrea virginica*, bactéries, hémocytes, réponse cellulaire, fluides extrapalléaux.

INTRODUCTION

Juvenile Oyster Disease (JOD) affects cultured eastern oysters, *Crassostrea virginica*, in the northeastern United States (Bricelj *et al.*, 1992; Davis and Barber, 1994). Mortalities, which may reach 90% within 5-6 weeks, affect densely grown juveniles between 10 and 25 mm shell height, although larger oysters may show symptoms without high mortality. The most characteristic symptom of JOD is a conchiolin layer, raised into a ridge around the mantle edge, on the inner surface of one or both valves. Mantle retraction and lesions of the mantle epithelium, especially at the distal edge, are also common. The symptoms of JOD suggest that the mantle is reacting to a severe irritant, but an etiological agent has yet to be identified.

Several etiologies have been proposed for JOD, including poor nutrition; exotoxin-producing bacteria or phytoplankton; and protozoans (Bricelj *et al.*, 1992; Farley and Lewis, 1993; Farley and Lewis, 1994). Nutrional deficiencies and toxic phytoplankton have been eliminated as direct causes and most investigators report no consistent evidence of protozoans in tissues (Bricelj *et al.*, 1992; Lee, 1995). The disease can be transmitted in the laboratory, however (Lewis, 1993). Bacteria in the family Vibrionaceae have been implicated in two other diseases of marine bivalves that also include anomalous organic deposits on the inner shell. Brown Ring Disease of Manila clams, *Ruditapes philippinarum* in western Europe is caused by a species of *Vibrio* named *Vibrio* P1 by Paillard and Maes (1990), who isolated it from diseased clams and demonstrated that disease symptoms were induced when clams were challenged by the isolate. Another *Vibrio* is thought to be responsible for a disease of the pearl oyster *Pinctada maxima* in western Australia (Pass *et al.*, 1987). A characteristic of this disease is a layer of conchiolin on the inner shell, which interferes with pearl formation. A bacterium similar to *V. harveyi* was isolated from diseased oysters and caused the same symptom in experimentally challenged oysters, although another bacterium, *Pseudomonas putrefasciens*, also caused "morbidity" (Dybdahl and Pass, 1985).

The bacterial associations with these two diseases as well as the observation of bacteria adhering to the anomalous conchiolin layers in JOD (Bricelj *et al.*, 1992), led to intensive bacterial sampling of three cohorts of hatchery-produced oysters before, during, and after JOD disease development at the Frank M. Flower Oyster Company, New York, USA. Three cohorts, produced at different dates in 1993, each showed an exponential increase in total Vibrios one to two weeks before the start of mortality (Lee, 1995). Several bacterial isolates obtained during the immediate pre-mortality period were used in challenge experiments. Two, phenotypically similar to *V. anguillarum* and *V. algynolyticus,* caused high mortality, but failed to elicit the typical anomalous organic deposit (Lee, 1995). Thus, the role of bacteria in JOD remains unclear.

Defense mechanisms against disease agents, especially protozoans, that infect soft tissues of marine molluscs are under intense study in many laboratories. Investigation of protective mechanisms involved in shell and exoskeletal diseases are just beginning. They include studies of hemocytes in both hemolymph and extrapallial compartments and consideration of the organic deposit as a defense mechanism that embeds bacteria in a melanized substrate and prevents progression of the microbes (Oubella *et al.*, 1993; Oubella *et al.*, 1994; Paillard *et al.*, 1994; Allam, comm. pers.).

The objectives of this study were to follow changes in the density of bacteria in oyster soft tissues, shell fluid, and inner shell surfaces during the course of JOD, and to perform transmission experiments to help determine the etiological agent of the disease. Also, hemocyte numbers and types in hemolymph and shell fluid of the same oysters were documented to investigate potential cellular responses to this disease.

MATERIALS AND METHODS

Oysters

Juvenile oysters, 1-3 months old and 13-38 mm shell height, were obtained at two-week intervals during 1994 from the Frank M. Flower Co. and at one-month intervals from the Blue Points Co., both on Long Island, New York. One-time samples of JOD-affected juvenile and older oysters (13-74 mm) were also obtained from a nursery and a growout site near Southold, New York, at the eastern end of Long Island. All oysters were shipped on ice via overnight mail to the Haskin Shellfish Research Laboratory and processed immediately. Surplus oysters were placed in aerated, standing-water isolation tanks. They were fed cultured algae daily and water was changed every two weeks.

JOD detection and disease prevalence determination

Upon arrival at Haskin Laboratory, 30 oysters were opened and examined for evidence of the conchiolin deposits associated with JOD (Bricelj *et al.*, 1992). The presence of a deposit was considered evidence of the disease. Three stages of JOD deposit were established. Stage 1 corresponds to a deposit with an light brown intensity, located in a confined area; stage 2, to a deposit with a dark brown intensity, located in a confined area, and stage 3, to a deposit with dark brown intensity forming the typical brown ring. Surplus oysters placed in isolation tanks were examined periodically in the same way.

Shell fluid and hemolymph collection

From June to August 1994, the bacterial and hemocyte composition of nearly 250 oysters with and without JOD symptoms was assayed. Samples were collected from each oyster. Soft tissues, shell fluid and shells were analyzed separately for bacterial composition, and shell fluid and hemolymph analyzed, for hemocyte composition. At each sampling, the external shell surface of 10 oysters was washed by scrubbing with tap water to remove mud and attached organisms. The oysters were next swabbed with 70% ethanol and each oyster was positioned vertically, hinge end down, for about ten minutes so that shell fluid (primarily the extrapallial fluid between the shell and the mantle) would accumulate in the subarticular area. This fluid (3-1 300 μl depending on oyster size) was removed with a sterile syringe attached to a 26-gauge needle. The needle was inserted through a small notch on the ventral edge of the shell and pushed between the mantle and shell to the subarticular area.

When oysters were large enough (>23 mm shell height), hemolymph (35-200 μl depending on oyster size) was removed from the adductor muscle through the same notch using a 26-gauge needle and syringe. All volumes were recorded and amounts less than 200 μl were diluted with sterile seawater (SSW) to a final volume of 200 μl. Calculations were adjusted to reflect original shell fluid or hemolymph volume. All samples were kept on ice until processed. Bacterial cultures and hemocyte analyses of the shell fluid were made from the same oysters and completed within 3 hours.

Soft tissue and shell processing

After hemolymph and shell fluid had been collected, each oyster was opened by cutting the adductor muscle with a sterile knife and the soft tissue was transferred to a sterile plastic tube and weighed. The shell was also placed in a separate sterile tube and weighed. Samples from individuals were classified according to whether the oysters were symptomatic or asymptomatic for JOD and pooled in the following manner: two samples of 5 oysters each if the prevalence of JOD was 0%, one pool of 5 symptomatic oysters and one of 5 asymptomatic oysters if JOD was present but <20%; and two pools of 5 symptomatic oysters if the prevalence was >40%. Pooled samples of tissue and of shell were established as described above, using the same oysters and homogenized for 30 s in SSW (dilution w/v = 1/10) using a Virtis blender. Serial dilutions of the homogenates were prepared in SSW.

Bacterial analysis

A volume of 0.1 ml of each of the diluted homogenates and shell fluid was plated on marine agar (1 l distilled water, 15 g agar, 24.7 g sea salts [Sigma, USA], 4 g peptone, 0.1 g FE $(PO_4)_2$) and TCBS medium (thiosulfate citrate bile sucrose) and incubated for four days at 20°C. Total heterotrophic bacteria (THB) and Total *Vibrio* sp. (TVS) were counted and are presented as colony forming units.ml^{-1} (*e.g.* CFU.ml^{-1} of shell fluid) or g wet weight^{-1} (*e.g.* CFU.g^{-1} wet weight soft tissues). At each sampling, predominant bacterial colonies were isolated and purified. The biochemical characteristics of these colonies were analyzed using the API 20 E diagnostic system (Biomerieux Vitek, Inc., St. Louis, MO) to determine affinities with known species. Additional tests were performed on strains selected for the challenge experiment: morphology, mobility, Gram staining, oxydase test, API 50 CH diagnostic system (Biomerieux Vitek, Inc., St. Louis, MO), marine agar with blue Bromothymol as pH indicator was used as culture medium, oxydation and fermentation pathways were tested). Species affinities of bacterial isolates identified in the genera *Vibrio* or *Pseudomonas* by the API 20E diagnostic system were also determinated using manual of systematic bacteriology (Baumann and Baumann, 1981; Baumann *et al.*, 1984).

Hemocyte analysis

Two differential hemocytometer counts were performed on each sample to estimate granular, agranular, and total numbers of cells.ml ^{1}. The percentage of dead cells was determined by staining with the nucleic acid stain, ethidium homodimer^{-1} (EHD) (Molecular Probes, Eugene, OR, USA- Catalog number E-1169). A stock solution of EHD was prepared according to manufacturer's directions (dilute 12 mM EHD in DMSO to 150 μl with sterile Dulbecco's phosphate buffered saline (DPBS), and freeze in aliquots) and used at 3 μM, a concentration previously determined to be adequate for visualization with fluorescent microscopy. Shell fluid or hemolymph (100 μl) was placed on a microscope slide and 4 μl of EHD, diluted in 100 μl of 0.22 μl filtered seawater was added. Slides were incubated at room temperature for at least 30 minutes and then examined by fluorescent microscopy (Zeiss ICM 405 inverted scope at 320x magnification) using a long-pass 520 nm filter. Non-viable cells fluoresce red at >600 nm wavelength (Kaneshiro *et al.*, 1993). Each microscope field was first examined under phase contrast and all cells were counted. The filter was then inserted and the number of red-fluorescing cells was counted in the same field. At least 200, but more often 300 to 1 000 total cells, were counted in each sample and the percent fluorescing cells was calculated as a measure of non viability. Certain algae species, which can be present in shell fluid, autofluoresce red. Therefore, each sample was checked under the microscope for fluorescence before staining and these cells were subsequently eliminated from the count.

Transmission experiments

Two transmission experiments were performed according to the procedure already described by Paillard *et al.* (1989) and Paillard and Maes (1990, 1994) for Brown Ring Disease (BRD) in Manila clams, *Ruditapes philippinarum*. The first involved using extracts from JOD-affected oysters; the second used bacterial strains isolated from diseased oysters.

Inoculation procedure

Oysters (40-60 mm shell height) used as recipients in both experiments were obtained from stocks held at the Haskin Shellfish Laboratory Cape Shore Station in lower Delaware Bay where JOD has never been found. Oysters in this size range can have JOD, as indicated by shell deposits, but they have much lower mortality rates than smaller individuals. The shell of each oyster was notched at the ventral edge and shaken to remove about 100 to 200 μl of the pallial and shell fluid. Then, extracts or bacterial strains were inoculated directly into the pallial cavity. After inoculation, the oysters were kept out of the water for six hours to minimize leakage of the inoculate by forcing the oysters to remain closed. They were subsequently returned to aquaria where they were held in aerated water at 22°C and 25 ppt, and fed cultured algae daily.

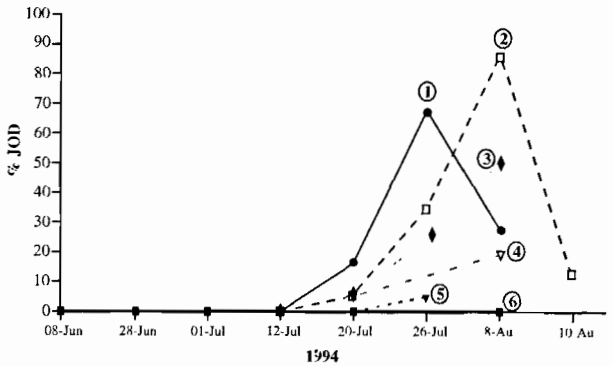

Figure 1. – Evolution of JOD prevalence in juvenile oysters originating directly from different nurseries (**2**: Flowers in nursery; **4**: Blue Points in nursery) or surplus placed in isolation tanks (received from Flowers Co. **6**: 28 June; **5**: 12 July; **3**: 20 July; and from Blue Points Co. **1**: 1st July).

Extract inoculation experiment

Extract inoculation experiments were done after obtaining the first diseased oysters. Supernatant from symptomatic oysters was obtained from oysters with 20% prevalence of JOD (BP in tanks, July 20; *fig.* 1). A sample of oysters showing 0% JOD was used for asymptomatic supernatant (FH in tanks, June 28; *fig.* 1). Oysters were washed and opened as described above for the bacteriological assays. Each was classified as symptomatic or asymptomatic for JOD. Tissues from 14 to 20 oysters of similar disease category (total = 0.8 g) were pooled and homogenized in SSW by means of a sterile potter grinder. The suspensions were then centrifuged (10 min, 200 g) and the supernatants immediately inoculated into experimental oysters.

Three batches of eighty oysters each were inoculated via the shell cavity with 100 μl of either: (1) SSW, (2) supernatant from asymptomatic oysters, or (3) supernatant from symptomatic oysters. Each batch was split into two replicates of 40 oysters and each replicate was held in a covered 15-l tank. A fourth batch of 22 oysters was held without inoculation in a single tank. Oysters were checked daily. Dead oysters were removed as they occured, recorded, and examined for evidence of conchiolin deposition on the inner valve surfaces. After 10 days, 5 live oysters from each tank were removed, opened, and examined. At this time, one half of the remaining oysters received a second inoculation; the other half received no further inoculate. Each of the new treatments was held in a separate tank. The experiment was terminated at day 28 when all remaining oysters were opened and examined.

Bacterial inoculation experiment

Bacterial suspensions of 10 predominant strains isolated from oysters with JOD symptoms were obtained from 72-hour pure cultures grown on marine agar. Twelve groups of 60 oysters were established and split into replicates of 30 oysters each. Each oyster received an inoculation into the shell cavity of 100 μl (10^7 to 10^9 cells.ml^{-1}) from one of the 10 isolates or one of two controls (SSW or *Vibrio* P1 – pathogenic agent of Brown Ring Disease). Dead oysters were removed, recorded, and examined as they occurred. Half of the live oysters were sampled after 10 days; the other half after 25 days.

Establishment of a classification system for JOD-like and other conchiolin deposits

A number of different types of conchiolin deposits were observed during the experimental transmission experiments. Therefore, a classification system permitting differentiation of the typical JOD deposit from other types was developed (*table* 1). Each deposit was rated as to type, intensity, and location. It has been clearly demonstrated by examination of naturally affected oysters that the JOD symptom is characterized by a brown ridge-type deposit, running from the subarticular area (location 2.5) along the ventral (location 2) and posterior areas (location 3), to finally form a typical brown ring (location 4). The shells of experimentally challenged oysters were scored according to this system. Only those with deposits classified as type II, locations 2 to 4 were judged to be JOD-like deposits (*fig.* 2). These were further categorized according to degree of development.

Table 1. – Establishment of a classification system for conchiolin deposit in eastern oysters, *Crassostrea virginica*. Only the type II, location 2 to 4 were judjed to be JOD-like deposits. H: heavy; L: light.

Type	I. Brown organic deposit, "C" calcified II. Ridge of organic deposit "Brown Ring" III. Calcified repair of broken shell IV. Organic sheet blister. Loose attachment
Intensity	L. Light gold H. Dark brown
Location	1. Site of notch 1.5. From notch into ventral area 2. Ventral 2.5. Sub articular 3. Dorsal 3.5. Adductor muscle 4. Entire shell 5. Extreme shell margin

The other combinations represent different host responses against various agents irritating the mantle or damaging the inner surface of the shell, like notching which stimulates a deposit associated with shell repair (type I, location 1, *fig.* 3), or the inoculation process itself, which can provoke blister formation (type IV, location 1 to 2, *fig.* 4).

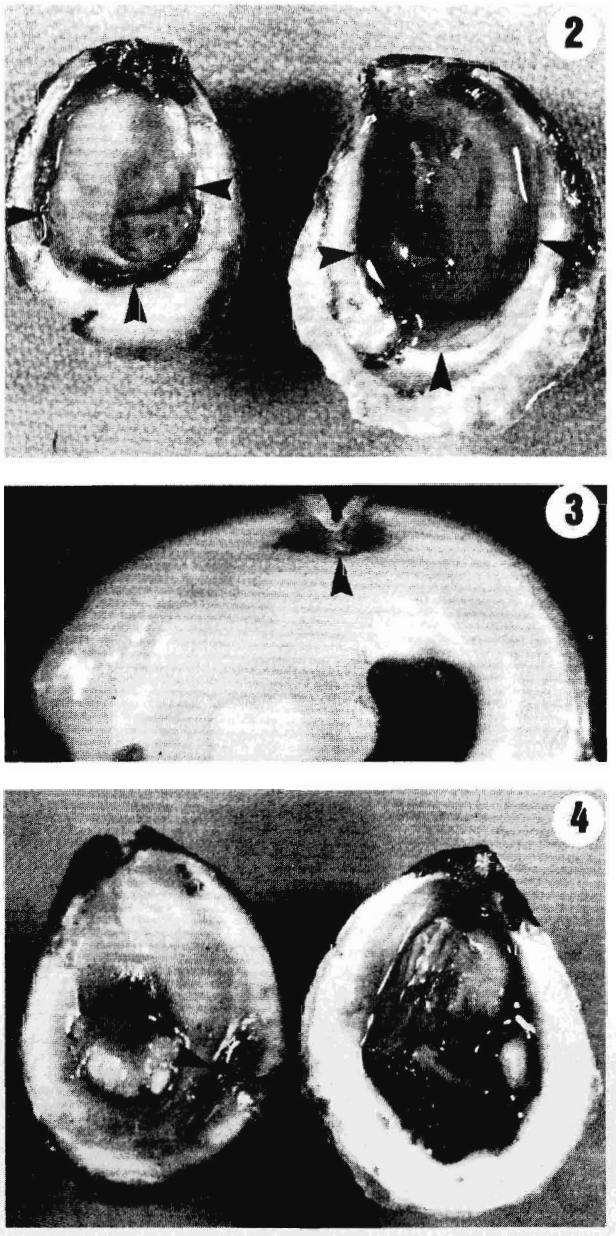

Figure 2. – Naturally diseased eastern oyster, *Crassostrea virginica*, presenting the characteristic symptom of Juvenile Oyster Disease (JOD), conchiolin layers adhering to the inner surface of the two valves and raised into a ridge around the mantle edge (arrows). Note the apparent greater growth of the cupped valve (right) in comparison with the flat valve (left).

Figure 3. – Experimental oyster that has been notched at the ventral edge. A brown deposit located at the site of notch (arrow) is associated with shell repair (Type 1, Intensity H, Location 1).

Figure 4. – Experimental oyster that has been notched at the ventral edge. Blister formation (arrow) is associated with the inoculation process (Type IV, Intensity H, Location 1-2).

Statistical analysis

Total heterotrophic bacteria (THB), total *Vibrio* sp. (TVS), and total and differential hemocyte (THC and DHC) counts and were $\log_{10}$-transformed before analysis and their geometric means (GM) calculated ($\log_{10}$ GM = 1/n [$\log_{10}$ x_1 + $\log_{10}$ x_2 .....+ $\log_{10}$ x_n]). Bacteriological and hemocyte means were compared using the Student *t*-test. Differences were considered significant at $\alpha = 0.05$.

RESULTS

Prevalence of JOD

The first signs of JOD in 1994 were observed on 20 July both in the field and in isolation tanks where excess oysters from earlier field collections had been placed (*fig.* 1). Oysters from the Blue Points Co. obtained on 1 July and held in isolation tanks developed a maximum prevalence of 70% on 26 July. Samples obtained directly from the Flower Co. reached maximum prevalence on 8 August. Samples obtained from the Flower Co. on 20 July and placed in isolation tanks developed JOD symptoms in parallel to those subsequently obtained from the field, whereas those obtained on 28 June showed almost no evidence of JOD in isolation tanks (*fig.* 1).

Bacteriological analysis

Meat

Total heterotrophic bacteria (THB) concentrations in the meat of both symptomatic and asymptomatic oysters varied between 2 × 10^5 and 1 × 10^7 CFU.g^{-1} (asymptomatic, GM = 1.66 × 10^6 CFU.g^{-1}; symptomatic, GM = 1.00 × 10^6 CFU.g^{-1}) (*fig.* 5*a*). Total *Vibrio* species (TVS) densities in meats of the same oysters ranged from 2 × 10^4 to 4 × 10^5 CFU.g^{-1} (asymptomatic, GM = 1.26 × 10^5 CFU.g^{-1}; symptomatic, GM = 1.58 × 10^5 CFU.g^{-1}) (*fig.* 5*b*). There were no significant differences in mean for either THB or TVS between symptomatic and asymptomatic oysters.

Shell fluid

The THB concentrations in the shell fluid of asymptomatic oysters were highly variable, ranging from 6 × 10^3 to 9.8 × 10^6 CFU.ml^{-1} (GM = 4.7 × 10^5 CFU.ml^{-1}) (*fig.* 5*a*). In symptomatic oysters, THB concentrations were also variable, with a range of 2 × 10^4 to 4 × 10^7 CFU.ml^{-1} (GM = 1.7 × 10^6 CFU.ml^{-1}) (*fig.* 5*a*). Difference between asymptomatic and symptomatic were statistically significant for THB. The higher concentrations in symptomatic oysters were especially noticeable in Stage 3 of the disease (GM = 2.2 × 10^6 CFU.ml^{-1}) (*fig.* 6*a*). Concentrations of TVS ranged from 5 10^2 to 2.8 × 10^6 CFU.ml^{-1} in asymptomatic oysters

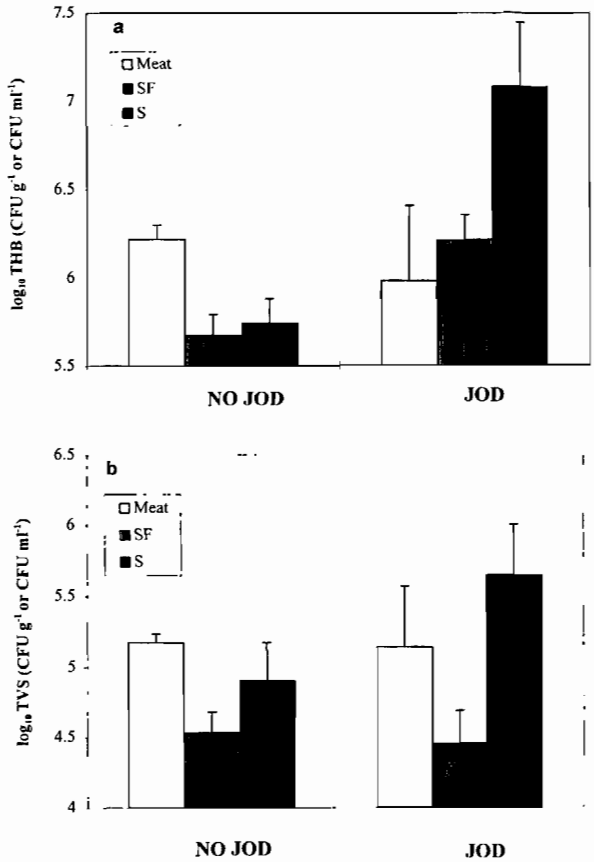

Figure 5. – Histograms showing geometric means for total heterotrophic bacteria (*a*: THB) and total *Vibrio* sp. (*b*: TVS) in asymptomatic oysters (JOD conchiolin deposits absent) and symptomatic oysters (JOD conchiolin deposits present) in different compartments: meat, shell fluid (SF) and shell (S). Means are expressed as $\log_{10}$ CFU.g^{-1} for meat and shell and CFU.ml^{-1} for shell fluid. Vertical bars are standart errors of the mean.

(GM = 3.4 × 10^4 CFU.ml^{-1}) and from 2 10^2 to 2.2 × 10^7 CFU.ml^{-1} in symptomatic oysters (GM = 2.8 × 10^4 CFU.ml^{-1}) (*fig.* 5*b*). There were no significant differences in mean of TVS between oysters with and without JOD symptoms (*fig.* 6*b*).

Shell

The THB and TVS concentrations on the shells of both symptomatic and asymptomatic oysters were also very variable, but were higher on average in the former. The THB densities ranged from 8 × 10^4 to 6.7 × 10^6 CFU.g^{-1} (GM = 5.5 × 10^5 CFU.g^{-1}) in asymptomatic oysters and from 3.8 × 10^5 to 2.6 × 10^8 CFU.g^{-1} (GM = 1.2 × 10^7 CFU.g^{-1}) in symptomatic individuals (*fig.* 5*a*). The TVS concentrations ranged from 4 × 10^3 to 5 × 10^5 CFU.g^{-1} (GM = 7.9 × 10^4 CFU.g^{-1}) in asymptomatic oysters and from 1 × 10^4 and 3 × 10^7 CFU.g^{-1} (GM = 4.4 × 10^5 CFU.g^{-1}) in symptomatic oysters (*fig.* 5*b*). Differences betwen asymptomatic and symptomatic oysters were statistically significant for THB.

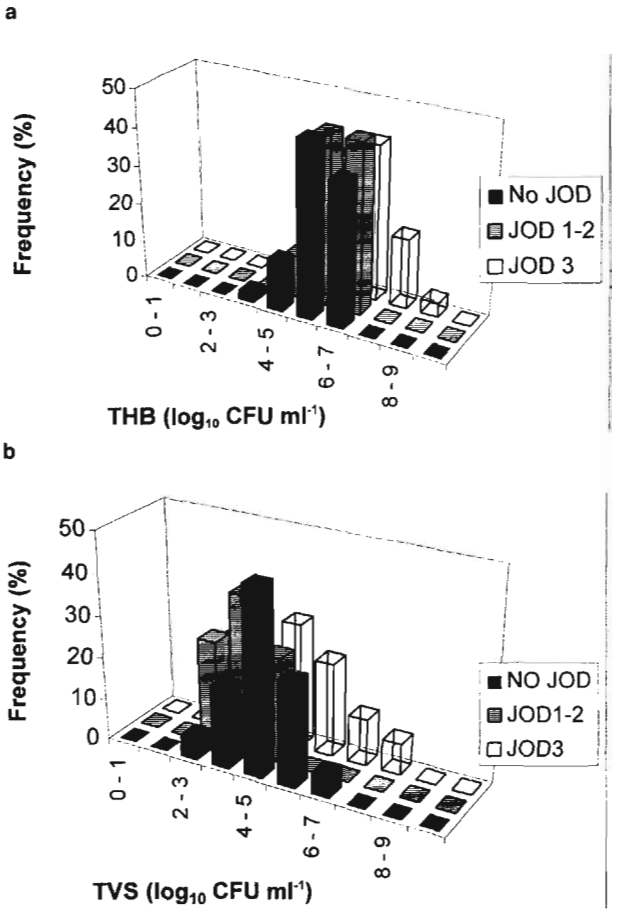

Figure 6. – Frequency distributions of total heterotrophic bacteria (*a*: THB) and total *Vibrio* sp. (*b*: TVS) between oysters with and without JOD symptoms in shell fluid. Diseased oysters were divided in two groups according degree of development, JOD 1-2 corresponding stage 1 and 2 and JOD 3 to stage 3. THB and TVS are expressed as $\log_{10}$ CFU.ml^{-1}.

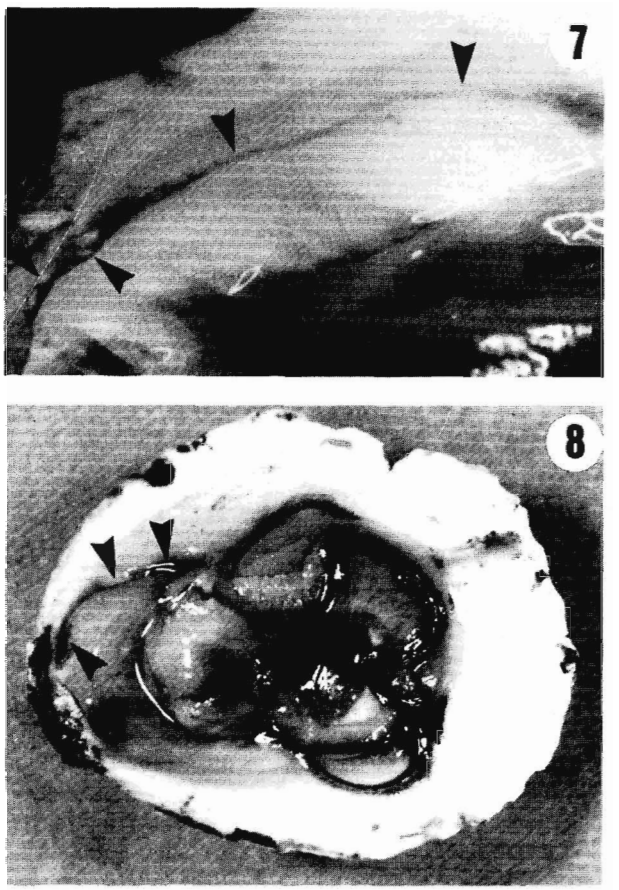

Figure 7. – Ten days after challenge with extract from symptomatic oysters, this oyster shows an early stage of JOD deposit. On the inner face of the anterior valve, a golden-brown ridge located from the subarticular to the ventral area (arrows) has formed.

Figure 8. – Four weeks after challenge with extract from symptomatic oysters, this oyster shows a stage 3 JOD deposit. On the inner face of the valves, a dark brown ridge is located from the subarticular to the ventral area (arrows).

Transmission experiments

Extract inoculation experiment (table 2)

Ten days after inoculation, the 20% oysters challenged with supernatant from symptomatic oysters had developed signs of JOD (*table* 2). At this time, they showed only early stages of the disease with a localized, very thin, golden-brown ridge from the subarticular to the ventral or dorsal area (*fig.* 7). Control (no inoculation, inoculation of SSW, or inoculation of asymptomatic supernatant) oysters did not exhibit typical signs of JOD. After 28 days, prevalence and intensity of JOD-like shell deposits in the groups inoculated with symptomatic supernatant had increased (*fig.* 8). Two successive inoculations resulted in a higher prevalence of JOD (40-45%) than did one (11-15% JOD) (*table* 2). There were no signs of JOD observed in the control groups except for a single oyster in asymptomatic inoculation tank which developed a JOD-like shell deposit.

At the end of the experiment, cumulative mortality was 0% in unnotched controls, 4% in SSW-injected controls, 2% in oysters inoculated with asymptomatic supernatant, and 9% in those given symptomatic supernatant.

Bacterial inoculation experiment (table 3)

Two hundred bacterial strains were isolated from oysters during the sampling phase of the study. Ten of these were systematically and predominantly detected only in individuals exhibiting signs of JOD. These ten strains along with *Vibrio* P1, pathogenic agent of Brown Ring Disease in *Ruditapes philippinarum* were inoculated in bacterial challenge experiments as described above. Analysis with the API 20 E system indicated that strains 113 and 130 belonged to the genus *Vibrio*; strains 69 and 111, to the genus *Aeromonas*; and strains 54, 122, and 128 to the genus *Pseudomonas*, probably *Pseudomonas putrefaciens*, because they produced H_2S.

Table 2. – Prevalence of JOD-like deposits in oysters 10 and 28 days after first and second extract inoculation (n, number of sampled oysters; SSW, sterile sea water; supernatant from asymptomatic or symptomatic oysters.

Days after experiment	n	No inoculation	SSW 1	SSW 2	Supernatant			
					Asymtomatic		Symptomatic	
					1	2	1	2
10	5	0	0	0	0	0	20	20
28 (1 inoculation)	17-20	0	0	0	0	6	15	11
28 (2 inoculations)	15-20	0	0	0	0	0	40	45

Table 3. – Prevalence of JOD-like deposit in oysters 14 and 25 days after bacterial inoculation. The 11 bacterial strains used for challenge were *Vibrio* P1 (VP1), pathogenic agent of Brown Ring Disease, S 54, S 69, S 90, S 111, S 113, S118, S122, S128, S130, S 131 and sterile sea water, SSW.

Days after inoculation	SSW 1	SSW 2	VP1	S 54	S 69	S 90	S 111	S 113	S 118	S 122	S 128	S 130	S 131
14	0	0	0	0	0	0	0	0	0	13	0	0	0
25	3	3	3	0	6	0	0	0	5	11	0	0	3

Fourteen days post-inoculation, several oysters (13%) injected with strain 122 showed early stages of JOD-like shell deposits. None of the other groups showed similar deposits at this time. After 25 days, the total prevalence of JOD-like deposits in strain 122-injected oysters was 11%. Similar deposits, however, had developed in a small proportion (3-6%) of oysters in some of the other groups inoculated with other bacterial strains, as well as controls.

Mortality was variable and occurred mostly in the first week after inoculation, at which time it ranged from 8 to 33%. Mean mortality in strain 122-injected oysters was 12%. No mortality occurred in SSW-injected control oysters and 4% was recorded in those challenged with the *Vibrio* P1 isolate.

Hemocyte analysis

Total hemocyte counts

Hemolymph – Hemocyte concentrations varied between 1.3×10^5 and 1.2×10^6 cells.ml^{-1} in both asymptomatic (GM = 6.8×10^5 cells.ml^{-1}) and symptomatic (GM = 6.9×10^5 cells.ml^{-1}) oysters, with no significant differences among means associated with disease (*table* 4).

Table 4. – Geometric mean total and differential hemocyte concentrations (cells.ml^{-1}) in hemolymph and shell fluid of oysters with and without JOD symptoms (n: number of oysters examined; SE: standard error of the mean; No JOD: asymptomatic oysters; JOD: all symptomatic oysters combined; JOD 1-2: only oysters with stage1 and 2 of JOD; JOD 3: only oysters with stage 3 of JOD; AGR: agranulocyte; GR: granulocyte). Mean and standard error are expressed as $\log_{10}$ GM and $\log_{10}$ SE; GM: geometric mean.

		Total hemocyte counts		Differential hemocyte counts			
		Shell fluid	Hemolymph	Shell fluid		Hemolymph	
				AGR	GR	AGR	GR
No JOD	n	45	18	41	41	18	18
	mean	5.79	5.83	5.49	5.37	5.63	5.33
	SE	0.05	0.09	0.04	0.07	0.11	0.09
JOD	n	50	13	50	50	13	13
	mean	5.54	5.84	5.25	5.17	5.60	5.38
	SE	0.05	0.08	0.05	0.06	0.08	0.12
JOD 1-2	n	20		20	20		
	mean	5.74		5.45	5.31		
	SE	0.08		0.07	0.13		
JOD 3	n	30		30	30		
	mean	5.4		5.1	5.1		
	SE	0.05		0.05	0.05		

Shell fluid – Cells found in the shell fluid were morphologically indistinguishable from those found in the hemolymph, and appeared to be typical hemocytes. Significantly fewer hemocytes (GM = 2.8 × 10^5 cells.ml^{-1}) were present in the shell fluid of oysters in stage 3 of the disease compared to those in stage 1-2 of the disease (GM = 5.5 × 10^5 cells.ml^{-1}) and in asymptomatic oysters (GM = 6.2 × 10^5 cells.ml^{-1}) (*fig.* 9, *table* 4).

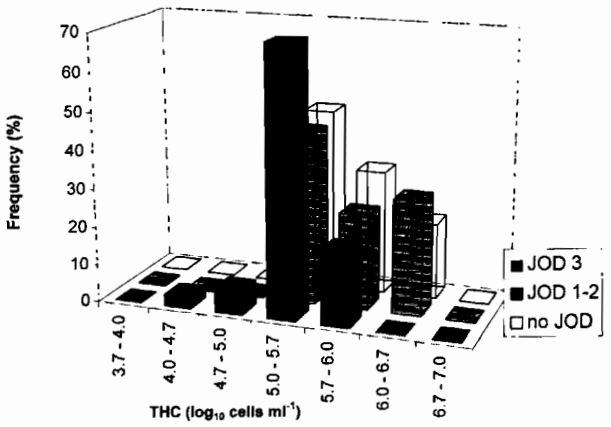

Figure 9. – Frequency distributions comparing total hemocyte concentrations (THC) in shell fluid of oysters with and without JOD symptoms. Diseased oysters were divided in two groups according to degree of development: JOD 1-2 corresponding to stages 1 and 2, and JOD 3 to stage 3. THC is expressed as $\log_{10}$ cells.ml^{-1}.

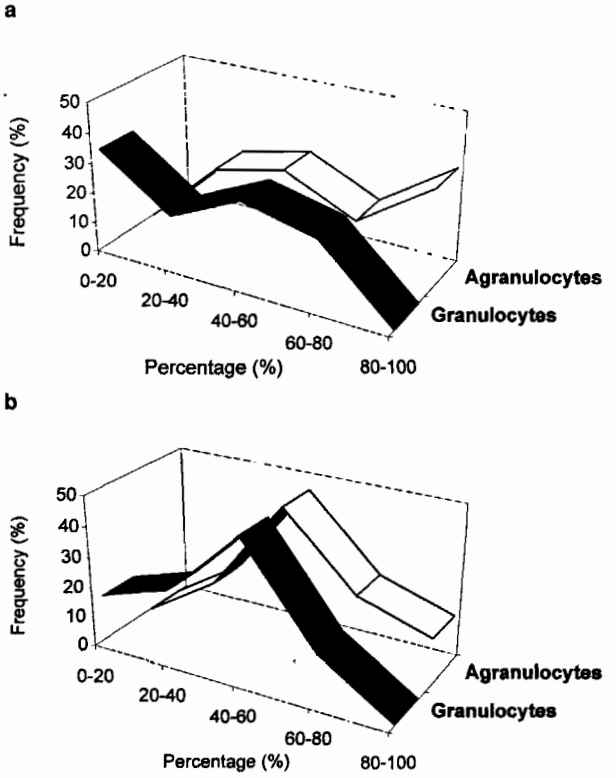

Figure 10. – Frequency distributions of agranulocytes (AGR) and granulocytes (GR) percentage in hemolymph of asymptomatic oysters (*a*: JOD conchiolin deposits absent) and symptomatic oysters (*b*: JOD conchiolin deposits present).

Differential hemocyte counts

Hemolymph – Symptomatic oysters had somewhat higher granulocyte (GR) counts and somewhat lower agranulocyte (AGR) counts than asymptomatic oysters but their means were not significantly different (*table* 4). The change in absolute counts resulted in a more striking change in relative proportions of the two cell types. The percentages of AGR and GR were highly variable in asymptomatic oysters, but with AGR predominating (*fig.* 10*a*). In contrast, hemocytes in the majority of symptomatic oysters were about evenly split between AGR and GR (*fig.* 10*b*).

Shell fluid – Oysters with JOD symptoms had fewer AGR and GR in the shell fluid than did oysters without symptoms (*fig.* 11*a* and *b*) and the means were significantly lower in stage 3 oysters (*table* 4). Further, JOD affected hemocyte proportions in the shell fluid in much the same manner as in the hemolymph. Most asymptomatic oysters tended

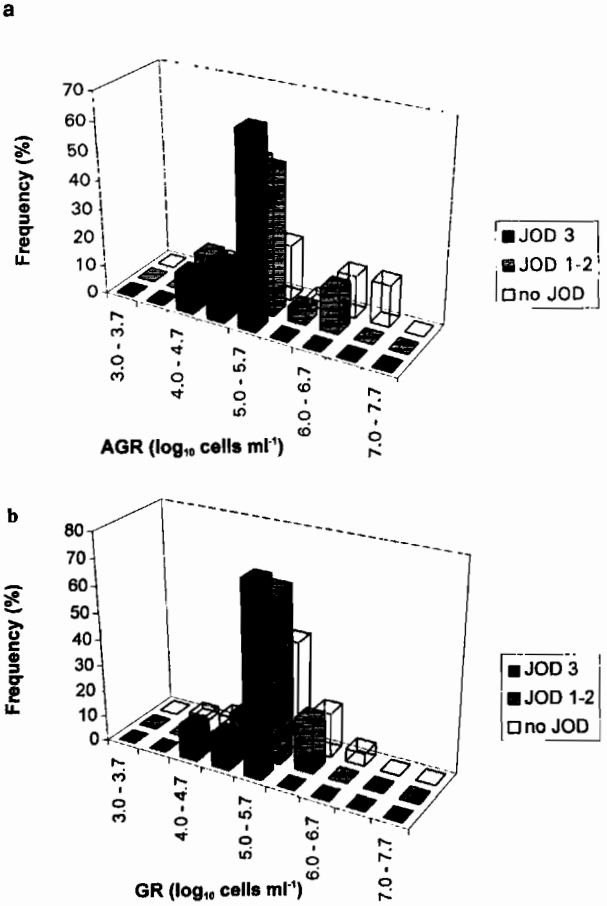

Figure 11. – Frequency distributions of differential hemocyte concentrations (DHC) in shell fluid of oysters with and without JOD symptoms. Total agranulocytes (*a*: AGR) and granulocytes (b: GR) concentrations are expressed as $\log_{10}$ cells.ml^{-1}.

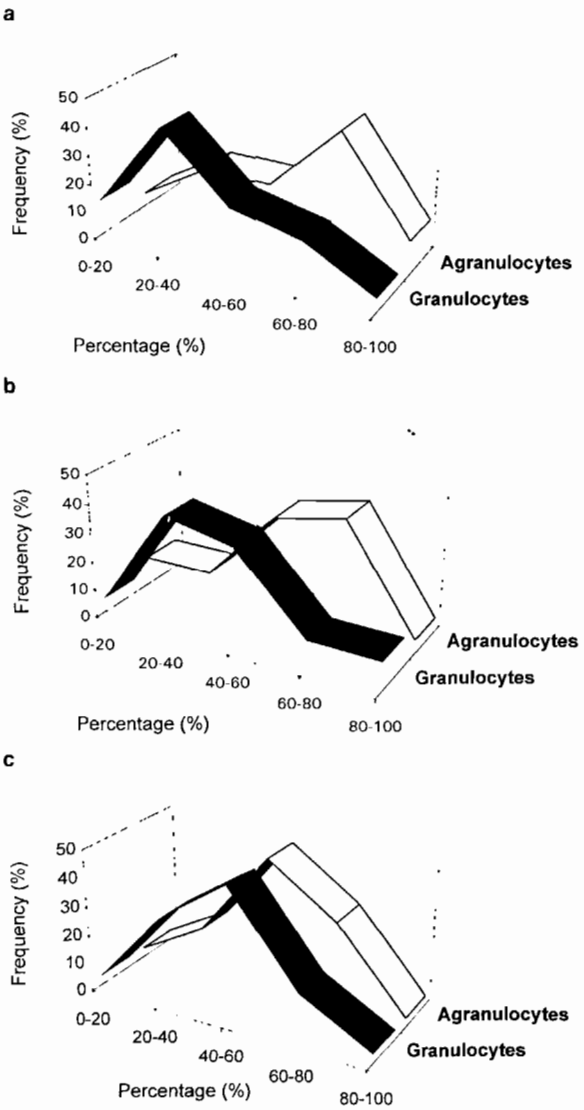

Figure 12. – Frequency distributions of agranulocytes and granulocytes percentage in asymptomatic oysters (*a*), in JOD 1-2 oysters (*b*), and in JOD 3 oysters (*c*).

to have a predominance of agranulocytes (60-80%) (*fig.* 12*a*). With the development of JOD, proportions became more nearly equal, reaching a ratio of 50:50 in most stage-3 oysters (*fig.* 12*b* and *c*).

Hemocyte viability

In asymptomatic oysters, the hemolymph and the shell fluid contained an average 6.1% and 7.3%, respectively, of nonviable hemocytes. These figures were not significantly different from each the 7.9% non viable cells found in hemolymph of symptomatic oysters. However, in shell fluid, symptomatic oysters had a significantly higher proportion (11.9%) of nonviable cells compared to asymptomatic ones.

DISCUSSION

In this study, we applied procedures developed for the identification of *Vibrio* P1, the bacterial agent of Brown Ring Disease (BRD) in Manila clams (Paillard *et al.*, 1994), to test the hypothesis of a bacterial cause for Juvenile Oyster Disease (JOD) (Bricelj *et al.*, 1992; Lee, 1995). We also investigated changes in hemocyte concentrations and ratios in the hemolymph and shell fluid of diseased oysters. Results showed that JOD was associated with statistically significant changes in both bacterial and hemocyte parameters in the shell fluid and at the inner shell surface, where the conchiolin deposits characteristic of the disease are found (*table* 5).

Bacteria and JOD

A bacteria etiology for JOD was first suggested by Bricelj *et al.* (1992), who described pathological signs of an agent that irritated the mantle, but could find no evidence of parasites in or on the tissue. Lee (1995) tracked total *Vibrio* species in specific cohorts before and during JOD onset and found an exponential increase in *Vibrio* densities just before mortalities began. This indicates that these species were not just secondary invaders of moribund animals.

Unlike Lee's (1995) whole oyster or shucked meat samples, our samples were divided into compartments (soft tissue, shell fluid, and inner shell) and separated oysters into those with and without JOD symptoms. We determined total heterotrophic bacteria (THB) densities and concentrations of *Vibrio* sp. (TVS), which are a subset of THB. We found statistically significant increases of THB in the shell fluid and on the surface of the inner shell from oysters with JOD symptoms, however, no change in bacterial densities occurred in the soft tissues. Lee (1995) also indicated that TVS densities were higher in the shell fluid and on inner shell scrapings of JOD-affected oysters compared to the soft tissues. These results agree closely with the findings of Maes (1992) who reported a 100-fold increase of THB in shell fluid from Manila clams with BRD but only a 4-fold increase in the meat. In our study, TVS densities paralleled THB concentrations but only THB were statistically correlated with JOD status (*table* 5). It is possible that if bacteria cause JOD, species other than, or in addition to, Vibrios are involved. Likewise, both *Vibrio* and *Pseudomonas* species have been implicated in the disease of pearl oysters, Pinctada maxima, which results in anomalous conchiolin shell deposits (Pass *et al.*, 1987; Dybdahl and Pass, 1985).

An interesting and relevant finding of our study was that oysters from some samples not showing JOD symptoms at the time of collection developed typical symptoms after a period in isolation tanks at the Haskin Shellfish Research Laboratory (*fig.* 1).

Table 5. – Summary of changes in bacterial flora and hemocytes parameters in JOD-affected oysters. AGR: Agranulocyte; DHC: Differential Hemocyte Count; GR: Granulocyte; THB: Total Heterotrophic Bacteria; THC: Total Hemocyte Count; TVS: Total *Vibrio* sp.

Bacteriological analysis	Meat		No changes in THB and TVS densities
	Surface of the inner shell		Significant increase in THB (not TVS) concentrations
	Shell fluid		Significant increase in THB (not TVS) concentrations
Hemocyte Counts	Hemolymph	*DHC*	Decrease AGR counts, but not statistically significant Increase GR counts, but not statistically significant Increase in percent GR.
	Shell fluid	*THC*	Significant decrease in hemocytes densities in stage 3 oysters.
		DHC	Significant decrease in both AGR and GR densities in stage 3 oysters. Increase in percent GR.
		Hemocyte viability	Significant increase in the percent nonviable hemocytes.

In the case of oysters from the Flower Co., JOD development in the isolation tanks was a function of when the samples were collected: an early sample never showed JOD symptoms, whereas 60% of a later sample developed JOD. A similar phenomenon was found for BRD: clams remaining in a nursery and those from the same site placed in isolation tanks at high densities both developed high BRD levels within three weeks from an initial prevalence of 0 to 0.1% (Paillard, 1992). Oysters from the Blue Points Co. where JOD had never been reported (Ford, unpubl. data), developed a 70% prevalence of JOD during 3 weeks, although no JOD episode was reported at the Blue Points Co. (Stan Czyzyk, pers. comm.). The Blue Points Co. nursery apparently did not provide a favorable environment for JOD development which may be a result of the increased water flow that they use in their upwellers (Rivara and Czyzyk, 1995). These data indicate that JOD outbreaks depend both on the presence of the causative agent and a proper environment for disease development. The disease agent must have already been present in the Blue Points Co. oysters and the 22 July shipment from the Flower Co., but absent or very low in earlier collections at the Flower Co.

Comparison of JOD, BRD, and other shell deposits

The JOD syndrome in eastern oysters is characterized by a conchiolin deposit on the inner shell that resembles the BRD shell lesion (Paillard *et al.*, 1989, Paillard and Maes, 1994). In both diseases, the deposits are considered a defense mechanism to isolate some agent that irritates the mantle and stimulates the response (Bricelj *et al.*, 1992; Paillard *et al.*, 1994). In BRD, a laminated deposit is located in the peripheral compartment of the extrapallial space, external to the pallial line and usually do not spread in the central and subarticular compartments of the extrapallial space. The JOD deposit is formed at the mantle edge well inside the shell edge since the mantle is typically retracted at the time. JOD deposits usually include sheet-like layers of conchiolin, secreted by the general mantle surface in the central and subarticular compartments (Bricelj *et al.*, 1992). Morphological differences between the two species probably explain the difference in deposit location. In clams the mantle is attached to the shell at the pallial line, separating the central from the peripheral compartments. In oysters, the mantle is not attached, leaving a relatively open central compartment and allowing the mantle more freedom to retract into it. Thus, agents invading the extrapallial cavity of the clam are likely to remain in the peripheral compartment and provoke a response in that compartment. In the oyster, such agents are apt to be less restricted and to stimulate a more generalized response.

The ridge-like brown deposits of JOD and BRD can easily be distinguished from other types of conchiolin deposits found in our transmission studies. Inoculation often elicited a blister-type deposit near the site of injection and even the injection of something as innocuous as SSW occasionally induced the secretion of brown conchiolin. This phenomenon has never been observed in *R. philippinarum* or *R. decussatus* inoculated with SSW or bacterial strains other than *Vibrio* P1 (Paillard, unpubl. data).

Transmission experiments

A series of experiments by Lewis (1993) demonstrated that JOD could be transmitted by proximity to diseased oysters. Our extract inoculation experiment showed that the transmissible agent is present in soft tissues, shell fluid, or both. BRD can also be transmitted by proximity to, and inoculation of extracts from, diseased clams (Paillard *et al.*, 1989). Two inoculations of extract from JOD-affected oysters provoked a higher prevalence of JOD than one indicating a dose-effect response typical of many pathogens.

None of the ten bacterial isolates injected into asymptomatic oysters produced strong or consistent JOD-like conchiolin deposits although the isolates were obviously pathogenic because they caused mortality. The mortality may have had nothing to

do with JOD, however, since all isolates caused mortality greater than control. Bacterial species not normally harmful may become facultative pathogens when inoculated at high doses to stressed oysters. For instance, Grischowsky and Liston (1974) demonstrated that *Vibrio anguillarum* and *V. alginolyticus* induced mortality in adult Pacific oysters, *Crassostrea gigas*, held at high temperature. Lewis (1993) found that if he improved oysters' metabolic condition by placing them in natural waters for four weeks before transmission experiments, he induced typical JOD deposits and mortality in the presence of JOD-affected oysters. If he did not condition the oysters, he induced only mortality without the shell deposits. For oysters to attempt defense against JOD by secreting conchiolin, it is apparently necessary for them to be in good metabolic condition. In this respect, it is relevant that JOD usually affects young oysters during a period of rapid growth and high metabolic activity (Bricelj *et al.*, 1992; Davis and Barber, 1994). Our experimental oysters were relatively large and had not been growing rapidly at the time of inoculation. They may not have been able to respond to bacterial challenge by fast synthesis and secretion of conchiolin.

The study of bacterial diseases of juvenile and adult bivalves is a new field of investigation in which experimental methods have yet to be perfected. To date, only three have been clearly demonstrated: Brown Ring Disease (Paillard and Maes, 1990; Paillard *et al.*, 1994), Pacific Oyster Nocardiosis (Elston *et al.*, 1987; Friedman and Hedrik, 1991; Friedman *et al.*, 1991), and Hinge Ligament Disease (Elston *et al.*, 1982; Dungan and Elston, 1988; Dungan *et al.*, 1989). Other bacterial diseases have been reported, but are either nonspecific bacterial infections as the Pearl oyster mortality affecting *Pinctada maxima* (Dybdahl and Pass, 1985, Pass *et al.*, 1987) or suspected bacterial conditions such as cardiac edema in *Crassostrea virginica* (Tubiash *et al.*, 1973). With the exception of cardiac edema, all affect oysters that are experiencing crowding in high density cultures, elevated temperature, or some other stressor. As intensive culture of bivalves increases, especially when the species is not native to the region, there will undoubtedly be more outbreaks of microbial disease as normally noninjurious species become facultative pathogens of stressed molluscs. To effectively investigate these outbreaks and demonstrate an etiology, appropriate methods for collecting samples, culturing organisms, and conducting transmission studies must be refined.

Hemocytes in hemolymph and shell fluid

Total hemocyte concentrations in hemolymph of juvenile oysters in our study ranged from 1.3×10^5 to 1.2×10^6 cells.ml^{-1}, somewhat lower on average than in reports for adult oysters and clams, where circulating blood cell densities were typically in the 10^6 to 10^7 range (Feng, 1965; Foley and Cheng, 1974; Suresh and Mohandas, 1990; Ford *et al.*, 1993; Oubella *et al.*, 1993). At the time we sampled oysters, they had been out of water with their valves closed for nearly 24 h. Feng (1965) demonstrated that the number of blood cells in circulation was correlated directly with heart pumping rate. During periods of shell closure, oyster heart rate decreases (Stauber, 1940) and the number of cells in suspension would decline correspondingly.

Only a few previous studies have described cells in bivalve shell fluid (Mulholland and Friedl, 1994; *J. Shellfish Res.*, 13, 299-300). Recent studies in our laboratory (CNRS, Brest) have shown that the cells in the shell fluids of *R. philippinarum* are morphologically and biochemically similar to hemocytes and probably arise through diapedesis from the mantle and gill. In the central extrapallial fluid of Manila clam, we have measured that cell concentrations were more variable and generally higher (4×10^5 to 9×10^6 cells.ml^{-1}) than we found for juvenile oysters in total shell fluid, but about the same densities as in hemolymph of the same species (Oubella *et al.*, 1993). Viability of hemocytes in the central extrapallial fluid of Manila clam (Allam, pers. comm.), however, was about the same (91%) as we found for oysters (93%). The function of these cells in the shell fluid, especially as it relates to defense against pathogens, is unknown.

JOD associated hemocyte changes

The principal soft-tissue lesions associated with JOD occur at the mantle surface and include hemocyte aggregations in the subepithelial tissues, diapedesis of hemocytes through mantle epithelium, and leakage of hemocytes through eroded epithelial areas (Bricelj *et al.*, 1992). This histological picture suggests that the concentration of hemocytes in the shell fluid of JOD-affected oysters should increase. Contrary to this, we found that hemocyte concentrations in the shell fluid remain comparable in non-diseased and early-stage JOD oysters but that they decrease by about one-half in late-stage JOD oysters. This apparent anomaly may result because many hemocytes become sequestered within layers of conchiolin as the conchiolin deposit is formed (Bricelj *et al.*, 1992). Histological sections also show moribund oyster cells being shed into the extrapallial fluid (Bricelj *et al.*, 1992) and we found a statistically significant increase in the percentage of nonviable cells in the shell fluid of symptomatic (12%) as compared to asymptomatic (7%) oysters.

Our finding that JOD symptoms were not associated with an increase in circulating hemocyte concentrations concurs with histological evidence that shows no general hemocytosis in tissue sections of JOD-affected oysters (Bricelj *et al.*, 1992). Similarly, circulating hemocyte concentrations were elevated in Manila clams for two weeks post-challenge with *Vibrio*

P1, but then returned to pre-challenge levels even as the prevalence of brown ring was increasing (Oubella *et al.*, 1993; Paillard *et al.*, 1994).

Juvenile Oyster Disease was correlated with altered cell ratios in both hemolymph and shell fluid resulting in an increase in the percentage of granulocytes in both locations. The same result was demonstrated in the hemolymph of Manila clams three days after inoculation with *Vibrio* P1, the BRD agent (Oubella, 1996).

The results of our study strengthen previous observations that the most of the pathological changes associated with JOD occur in the extrapallial region. The mantle is the earliest and primary site of soft-tissue lesions, the shell is the site of the characteristic conchiolin deposit, and the shell fluid experiences a decrease in hemocyte concentrations that does not appear to occur in the general circulation. Finally, the bacterial concentrations in JOD-affected oysters are elevated only in the shell fluid and on the inner shell, not in the meat. These characteristics of JOD are very similar to those of BRD, which is a bacterial disease. For these reasons the bacterial etiology hypothesis for JOD should be explored further, expanded beyond the family Vibrionaceae, and should include consideration of a multiple etiology.

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