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A synopsis of juvenile oyster disease (JOD) experimental studies in *Crassostrea virginica*

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Abstract

In the late 1980's juvenile oysters, *Crassostrea virginica*, spawned and cultured in New England and New York, began experiencing up to nearly 100% mortalities in some batches of juveniles. The cause of these mortalities was not ascertained immediately, but examination of dead and dying oysters did not reveal a previously recognized disease syndrome. Early studies showed that it was not an environmental or genetic problem, thus we hypothesized that a new, transmissible disease agent caused the observed mortality. This was verified under laboratory conditions where the disease was readily transmissible. Transmission was enhanced by warm water temperatures, 22-26°C, and salinities of 18-30 ppt. Also, the infectious agent was filterable and sensitive to erythromycin, supporting the hypothesis that the causative agent may be a protistan parasite in the 2-6 μm size range. No evidence to support a viral or bacterial etiology was found.

Keywords: *Crassostrea virginica*, juvenile oyster disease, mortality, protist, conchiolin, bacteria.

Synopsis des études expérimentales de la maladie des jeunes huîtres (JOD) chez Crassostrea virginica.

Résumé

À la fin des années 80, les jeunes huîtres *Crassostrea virginica*, cultivées en Nouvelle-Angleterre commencèrent à atteindre 100% de mortalité chez certains lots. La cause de ces mortalités n'a pas été identifiée immédiatement mais l'examen d'huîtres mortes ou mourantes n'a pas révélé de syndrôme de maladie connue. Les premières études montrent qu'il n'y a pas de problèmes environnementaux ou génétiques, aussi nous faisons l'hypothèse qu'un nouvel agent d'une maladie transmissible provoque les mortalités observées. Ceci est vérifié en conditions de laboratoire où la maladie est facilement transmissible. La transmission est accrue avec des températures élevées, 22 à 26°C et des salinités de 18 à 30‰. L'agent infectieux est filtrable, sensible à l'érythromycine, supportant l'hypothèse que l'agent responsable peut être un parasite protiste de 2 à 6 μm. Aucune évidence concernant une étiologie virale ou bactérienne n'a été trouvée.

Mots-clés : *Crassostrea virginica*, pathologie, mortalité, protiste, conchioline, bactérie.

INTRODUCTION

Since 1988, cultured juvenile oysters, *Crassostrea virginica,* grown at salinities of 25 ppt and above have experienced heavy mortalities of unknown cause in the northeastern United States from New York north to Maine (Bricelj *et al.*, 1992; Davis and Barber, 1994; Lewis and Farley, 1994). These mortalities have come to be known as juvenile oyster disease (JOD). To date, the causative agent is unknown, but the syndrome is well documented.

This new disease consists of a number of specific characteristics that in combination comprise the JOD syndrome. The first indication of disease is the sudden onset of mortality in juvenile oysters, typically 7-30 mm in size, which may lead to a 90-100% cumulative mortality. Mortalities are closely associated with abnormal production of a conchiolinous deposit on the internal shell surface of one or both valves (*fig.* 1). These conchiolinous lesions often cover the majority of the inner shell of both valves and may completely envelop the oyster. Mantle recession is frequently observed as the disease progresses. Conspicuous "shell checks" (growth pauses) are often found in surviving oysters visible as distinct ridges, or growth bands, on the external shell (*fig.* 2). These checks occur where growth temporarily stops at disease onset. In severe cases of JOD, regrowth of shell may be vertical for several millimeters, or the oysters may completely change the direction of growth. Occasionally, abnormal deep cupping of shell, or separation of the adductor muscle from the shell occurs. The presence of small 2-6 μm singular intracellular inclusion bodies, with Feulgen-positive nuclei, found focally in healthy mantle epithelia of infected oysters, are routinely observed in histological examination of JOD-infected oysters. These inclusions progress in size and number to ultimately form ulcerations in the mantle epithelium (*figs.* 3-5). Hemocytic infiltration occurs into areas underlying the presence of the inclusion bodies (Bricelj *et al.*, 1992; Farley and Lewis, unpubl.).

Two hypotheses for the causative agent are being pursued. Some scientists consider the cause of JOD to be a toxin of bacterial or microalgal origin (Bricelj *et al.*, 1992). Alternatively, the authors of the present study feel a putative protistan agent may be the cause. One protist, similar to intracellular inclusion bodies seen in histological sections of JOD-infected oysters, has been isolated repeatedly from several JOD-infected water samples (Small, unpubl.). To date, however, the organism has not been isolated from water samples holding uninfected or control oysters.

Several different broodstocks were tested in our early field studies which showed that genetics was not the cause of JOD (Farley and Lewis, unpubl. data). All strains of oysters were equally susceptible to JOD and suffered heavy mortalities.

Our experimental studies reported herein demonstrate the yet unidentified cause of JOD to be a filterable, temperature and salinity sensitive biological agent that is readily transmissible in laboratory controlled experiments using JOD-infected oysters or material filtered from the water column at JOD-infected sites, and is susceptible to erythromycin. These results also serve to eliminate an environmental cause for JOD.

METHODS

Experimental studies summarized herein were conducted primarily at the National Marine Fisheries Service (NMFS) Cooperative Oxford Laboratory in conjunction with private growers in the northeastern United States. Most JOD scientific studies were made possible with the assistance of personnel from the Frank M. Flower and Sons Oyster Co. of Long Island, New York.

The first study determined the transmissibility of JOD in laboratory controlled experiments. This study also showed that a dinoflagellate was not a likely causative agent of JOD. Other studies evaluated the effect of salinity and temperature, saltwater aquarium medications, water filtration and protists associated with JOD-infected oysters.

All laboratory experiments were carried out in quarantine 38 l glass aquaria with synthetic seawater adjusted to the desired salinity. Each aquarium used a power filter, without the filter cartridge, to circulate water. Water temperatures were maintained at room temperature, or adjusted using submersible aquarium heaters. Oysters were monitored for indications of JOD and mortality once or twice a week, depending on the experiment. Uninfected, susceptible oysters were obtained from the University of Maryland Horn Point Environmental Laboratory hatchery (HP). The JOD-infected oysters were obtained from growers in New York and New England.

Confidence limits for percentages were used to suggest statistical differences in mortality between experimental exposures in the study (Sokal and Rohlf, 1973). However, confidence limits can be viewed only as an indication of differences in these treatments.

Transmission experiments

Transmission experiments began in 1991 and were completed in 1993. To assure that experimental oysters were in the best condition possible, uninfected HP oysters were suspended in pearl nets from the NMFS laboratory dock for approximately 3 weeks prior to beginning a transmission experiment. Studies were carried out at water temperatures of 18, 20, 22, and 24°C and at 26 ppt salinity (*table* 1). Depending on the experiment, groups of 175-500 uninfected juvenile oysters each, mean size 12-22 mm, were added to aquaria containing 30-50 recently infected

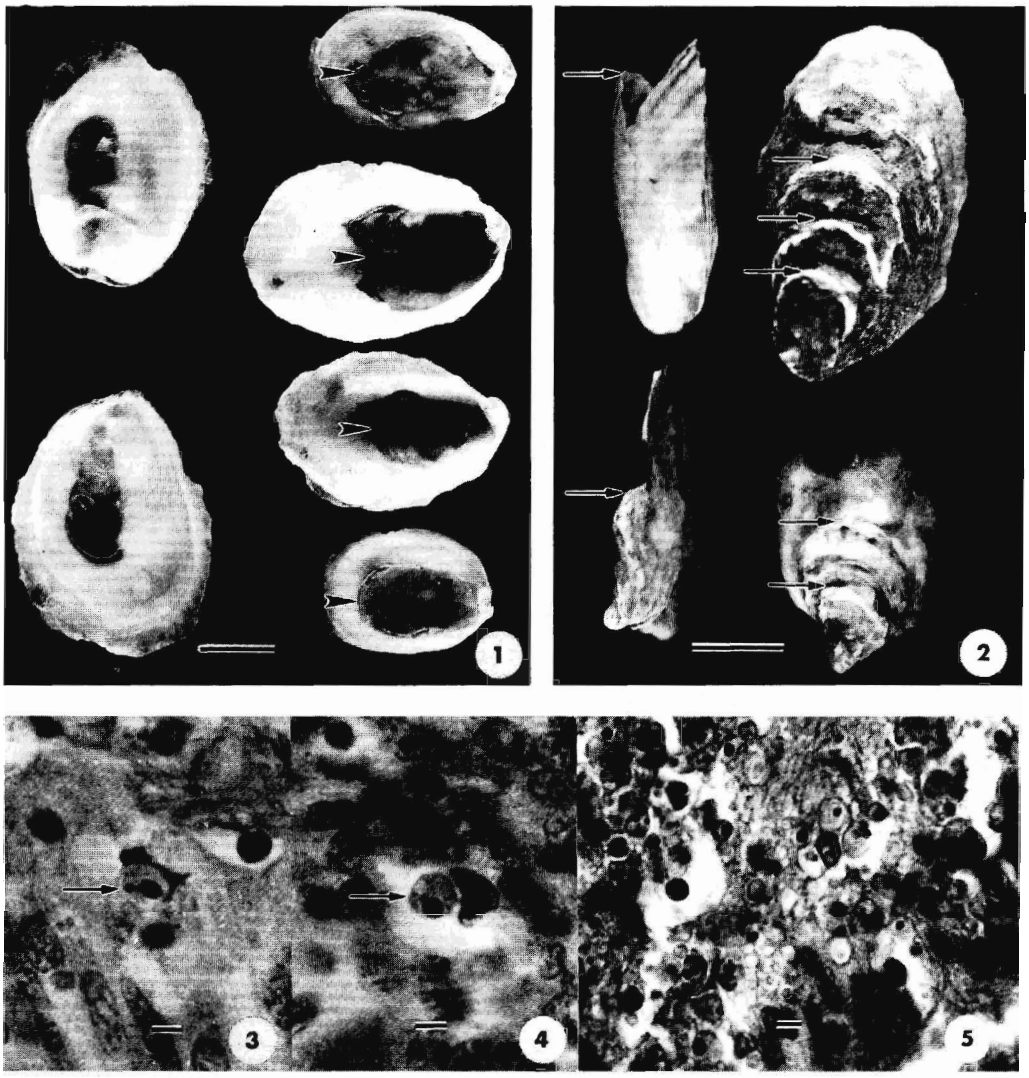

Figure 1. – Shells of uninfected oysters (left) and JOD-infected oysters with conchiolinous shell lesions on internal shell surface (right). Conchiolinous lesions indicated by arrow. Bar = 10 mm.

Figure 2. – Shell checks on exterior shell surface of oysters surviving JOD (arrows). Bar = 11 mm.

Figures 3-5. – Inclusion bodies in mantle epithelium. Feulgen picromethyl blue (FPM) stain. **3** – Inclusion body with 2 Feulgen positive bodies similar to macro- and micronucleus of a ciliate. Bar = 2 μm. **4** – Single intracellular inclusion body in healthy mantle epithelium. Bar = 2 μm. **5** – Advanced lesion in mantle epithelium. Bar = 3 μm.

or overwintered survivors of the previous year's JOD epizootic. A control aquarium was maintained at 26 ppt salinity without JOD-infected oysters. Oysters were fed a diet of algal paste resuspended in water. At the end of the first successful transmission experiment, top and bottom water samples from each aquarium were taken and examined for the presence of dinoflagellates, which had been proposed as a possible causative agent of JOD (Bricelj *et al.*, 1992). At the end of each trial, 30 oysters from each aquarium were processed for histology by NMFS standard methods (Howard and Smith, 1983).

In addition, 158 survivors of the 1990 and 1991 JOD epizootics were held in aquaria for 8 months simulating extended winter temperatures (4-9°C), then slowly warmed to 21-23°C and held for an additional 6 weeks to determine if gross signs of JOD would recur.

Effects of salinity on JOD development

Oysters naturally infected by JOD were exposed in aquaria at room temperature (20-27°C) and salinities of 10, 15, 20 and 26 ppt in experiment 1 and 14, 18, 22, 26 and 30 ppt in experiment 2 to determine the effect of salinity on disease progression (*table* 2). After completion of the second experiment, infected oysters were removed from aquaria; water flow and

Table 1. – Results on the effect of temperature on transmission of JOD. Approximately 175 susceptible oysters placed in each aquarium plus an additional 30 JOD-infected oysters in experimental transmission aquaria. Mean size of susceptible oysters = 21 mm, mean size of JOD-infected oysters = 26 mm, salinity = 26 ppt.

Exposure [1] (°C)	Mean temp. (°C)	Disease onset (weeks)	Total mortality (%)	Mortality confidence limits (%) ($p = 0.01$)	Prevalence of conchiolin (%)
Control – 18	17.6	–	2	1-12	0
Control – 22	21.6	–	19	12-27	0
Exp – 18	18.0	7	41	32-50	26
Exp – 22	21.4	5	63	54-72	25
Exp – 24	24.0	4	74	65-82	40
Exp – 26	25.9	3	70	61-78	31
Inf – 22	21.4	–	77	68-84	70

[1] Exposure denotes control, experimental transmission (Exp), or JOD-infected (Inf) oysters exposed at a specific temperature.

Table 2. – Prevalence of mortality and conchiolin in JOD-infected oysters after treatment at various salinities. Exposures made at room temperature for 6 weeks. Mean temperature = 21-22°C, N = 200 oysters in each aquarium treatment.

Experiment	Salinity (ppt)	Total mortality (%)	Mortality confidence limits (%) ($p = 0.01$)	Conchiolin (%)
1	10	51	42-60	70
1	15	44	36-54	87
1	20	75	66-82	80
1	26	85	77-91	79
2	14	40	31-49	76
2	18	60	51-69	57
2	22	65	56-73	80
2	26	65	56-73	79
2	30	68	59-76	76

Table 3. – Timing of disease onset and cumulative mortality after a 4-week exposure at various salinities. Approximately 200 uninfected oysters placed in each aquarium, plus an additional 30 JOD-infected oysters in experimental transmission aquaria. Mean size of uninfected oysters = 21 mm, mean size of JOD-infected oysters = 38 mm, experiment carried out at room temperature (22-26°C).

Salinity (ppt)	Time of disease onset (weeks)	Total mortality (%)	Mortality confidence limits (%) ($p = 0.01$)
Control – 10	–	12	8-17
Control – 15	–	5	2-9
Control – 26	–	3	1-6
Exp – 10	–	16	11-22
Exp – 14	– [1]	5	2-9
Exp – 18	3	41	34-48
Exp – 22	3	38	31-45
Exp – 26	3	43	36-50
Exp – 30	3	37	30-44

[1] Experimental exposure at 14 ppt did not result in mortality with conchiolinous shell lesions after 4 weeks. Previous exposure at 15 ppt resulted in an increase in mortality, but without conchiolinous shell lesions, beginning at week 5.

salinity were maintained; but aquaria were otherwise left dormant for 8 months over the winter at room temperature (10-22°C). The water was then used for transmission experiments, without the addition of JOD-infected oysters, to determine if the JOD agent was able to survive and reinfect susceptible oysters.

Room temperature (23-27°C) transmission experiments were attempted at salinities of 10, 14, 18, 22, 26 and 30 ppt to determine the effects of salinity on the disease process (*table* 3).

Additionally, a sample of 57 oysters was obtained from a grower after being imported from a JOD-infected site in New England and held in Maryland waters at a salinity <5 ppt for 7 months. The oysters were placed in an aquarium and maintained at 26 ppt to determine if gross indications of JOD would be expressed.

Effects of therapeutants on the JOD organism

Therapeutic treatments were investigated strictly to determine if a particular therapeutant might yield information on the identity of the causative agent of JOD. Experiments were not designed to identify a therapeutic treatment for JOD in aquaculture.

Naturally infected oysters that had begun to experience JOD mortality were also used to test the effect of medications on disease progression. Therapeutants tested were over-the-counter Mardell and Jungle Laboratories [1] products used by tropical fish hobbyists to control diseases of bacteria, fungi and ectoparasites in saltwater aquaria (*table* 4). Medications used were: chlorine oxides (Saltwater MarOxy), copper sulfate (Saltwater CopperSafe), erythromycin (Maracyn), minocycline (Maracyn-Two), and triethylene glycol, Victoria green, nitromersol, and acriflavine (Ick Guard). Two hundred oysters in each aquarium treatment were held at room temperature 21-26°C and dosages were administered according to manufacturer's recommendations. An aquarium with

Table 4. – Therapeutic treatments tested on JOD-infected oysters. Length of exposure – 6 weeks at room temperature, N = 200 oysters in each aquarium treatment.

Experiment	Active ingredient(s)	Target organisms	Total mortality (%)	Prevalence of conchiolin (%)
1	26 ppt control (JOD-infected)	–	85	79
1	Copper sulfate [1]	Ectoparasites	69	79
1	Chlorine oxides [1]	Fungi and bacteria	100	53
1	Erythromycin minocycline combined [1]	Gram positive and gram negative bacteria	43	90
2	26 ppt control (JOD-infected)	–	65	79
2	Minocycline [2]	Gram negative bacteria	68	63
2	Erythromycin [2]	Gram positive bacteria	9	69
2	Erythromycin minocycline combined [2]	Gram positive and Gram negative bacteria	9	76
2	Triethylene glycol [2, 3]	*Ichthyophthirius*	77	61

[1] Medication administered once at the beginning of the experiment.

[2] Medication administered every 3 weeks.

[3] Other active ingredients include – Victoria green, nitromersol, and acriflavine.

no therapeutant treatment of oysters was used as a control.

A second experiment to separately test the effects of erythromycin and minocycline and additional therapeutants was made following the same methods as in experiment 1. At the end of six weeks, 6 oysters each from the control, erythromycin and minocycline treatments in experiment 2 were cultured for bacteria, particularly *Vibrio* spp. Oysters were soaked 3-5 minutes in a bleach solution (minimally 20 ml bleach/100 ml water) to sterilize the outer shell, then rinsed well and opened using sterile implements. The mantle and inner shell were swabbed and sent off for culture. Swabs were inoculated into trypticase soy broth (TSB) with 2% sodium chloride, marine broth, and alkaline peptone water. Cultures were also originated by inoculation onto trypticase soy agar (TSA) with 2% sodium chloride, thiosulfate-citrate-bile-sucrose agar (TCBS) and marine agar plates by standard methods. Pure cultures were identified by traditional methods as well as API 20 E [2], Rapid NFT [2], and gas chromatography. All strains of bacterial isolates were tested for antibiotic sensitivity.

Filtration studies

In a field filtration study, 1-2 mm oysters from 2 different broodstocks were divided into 6 groups of 1 000 oysters for each of the broodstocks. Replicate groups of 1-2 mm oysters from each broodstock were exposed to ambient water pumped through separate upwellers in 15 000 l reservoir tanks at a commercial hatchery (*fig.* 6). Oysters were exposed to ambient unfiltered water, or water filtered through 50 or 25 μm mesh bag filters. No algal supplement was added since adequate food was available from the ambient water. Each tank was drained and refilled three times a week and otherwise maintained according to standard hatchery routine.

In laboratory experiments, material held in sequential bag filters was used to investigate transmission of JOD without JOD-infected oysters. Approximately 11 000-14 000 l of ambient water from a JOD-infected facility was sequentially filtered through a 50, 25, and 5 μm bag filter and backflushed in 4 l of filtered water. The resultant suspensions were then used as an inoculum for aquarium transmission experiments. Before backflushing, each bag filter was dipped repeatedly in 4 l of synthetic seawater to rinse smaller particles from the bag filter. In addition to the filtered solids from the 50, 25 and 5 μm bag filters, the 4 l of material rinsed from the 5 μm filter was also used to challenge uninfected oysters. Two control aquaria were set up at 26 ppt with only synthetic seawater and uninfected oysters from Horn Point, as used for the experimental transmission experiment.

During week 8 of the aquarium filtration experiment, while mortalities were occurring, 10 oysters from each aquarium were cultured for bacteria using the methodology described for the therapeutant experiments.

(1) The use of trade names does not suggest endorsement by the National Marine Fisheries Services.

(2) BioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042, USA.

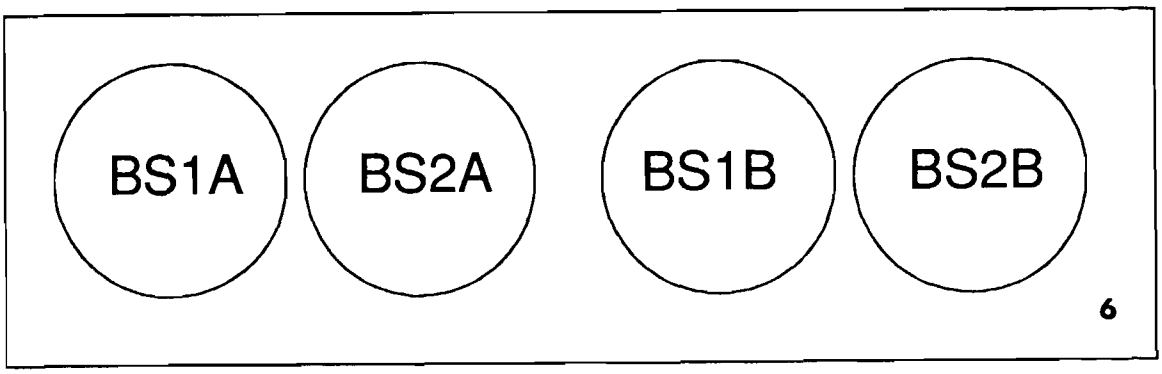

Figure 6. – Diagram of upwellers in 15 000 l reservoir tanks for field filtration study. Four upwellers, each containing 1 000 oysters, were placed in a 15 000 l tank. Replicates of two different broodstocks (BS1 and BS2) were exposed in all reservoir tanks. A separate tank was used for each of 3 levels of filtration.

RESULTS

Transmission experiments

Our first experiments showed that oysters need to be in good condition to produce the conchiolinous shell lesions characteristic of JOD. Prior to conditioning uninfected oysters overboard in pearl nets before beginning an experiment, fewer than 8% of the dead oysters produced conchiolinous shell lesions. Dead oysters that had been assessed visually to be in good condition at the beginning of the experiments had a 25-40% prevalence of conchiolinous shell lesions after various experimental exposures. Oysters in poor or watery condition at the beginning of an experiment failed to produce a strong conchiolinous response (1-8%). No indications of JOD, or comparable mortalities, were observed in unexposed control animals.

Uninfected, hatchery-reared oysters showed an acute onset of JOD mortalities 3 to 7 weeks after exposure to JOD-infected oysters in recirculating aquaria at a salinity of 26 ppt. Timing of disease onset varied according to water temperature. Cumulative mortality in experimentally challenged oysters, n = 175 oysters in each aquarium, ranged from 41% (18°C) to 74% (24°C) (*table* 1). Confidence limits of individual treatments suggest there was no significant difference between observed mortalities in oysters exposed at temperatures of 22, 24, and 26°C. There was a significant difference in mortalities from transmission at 18°C and the three higher temperatures ($p = 0.01$).

Consistent with our JOD field studies, histological examination of experimentally-infected oysters routinely revealed focal lesions of small round intracellular bodies in vacuoles inside otherwise healthy mantle tissue. Intracellular bodies ranged in size from 2-6 μm and had one or more densely staining Feulgen-positive nuclei. The presence of small, paired, dense staining, Feulgen positive nuclei in many intracellular bodies, one larger than the other, was suggestive of ciliate macro- and micronuclei (*fig.* 3). No control oysters revealed these mantle lesions.

No living phytoplankton were found in water from control aquaria, or JOD transmission aquaria at 21 or 24°C. Two transmission aquaria, 18 and 26°C, plus the aquarium with only JOD-infected oysters, had diatom populations of *Nitzschia* sp. or *Navicula* sp. The 26°C aquarium also contained heterotrophic microflagellates. No evidence of dinoflagellates was found in any of the aquaria.

Gross signs of JOD were observed to recur in overwintered survivors of previous JOD epizootics held in aquaria. At the beginning of the exposure, no mortality was evident in 158 survivors of the 1990 and 1991 JOD epizootics. A 30 oyster sample from each group showed that 3% (3/102) of oysters from the 1991 group expressed evidence of conchiolin. After approximately 10 months, oysters from both groups expressed mortality and internal conchiolinous shell lesions characteristic of JOD. Mean size of dead oysters was 31 and 54 mm, respectively. Survivors of the 1990 epizootic experienced a 27% (7/26) mortality with a 100% (7/7) expression of conchiolin in dead oysters compared to 75% (54/72) mortality and 35% (19/54) prevalence of conchiolin in the 1991 group.

Aquaria water that had contained JOD-infected oysters from a previous year's salinity study, and maintained through the winter at room temperature without the presence of JOD-infected oysters, did not transmit JOD.

Laboratory transmission of JOD consistently resulted in the expression of the typical JOD syndrome seen in natural infections. Bacterial lesions were not found in histological sections of diseased oysters from the present experimental studies.

Salinity experiments

Transmission of JOD to uninfected, hatchery-reared oysters readily occurred at salinities of 18 ppt and higher, with cumulative mortalities of up to

75% (n = 200 in each aquarium) after 4-6 weeks of exposure (*table* 3). Mortalities declined at salinities less than 18 ppt; however, transmission success of JOD at salinities of 10-15 ppt is uncertain. At the end of 4-6 weeks, cumulative mortalities at salinities of 10 and 14 ppt were 16% and 5%, respectively. No conchiolinous shell lesions were observed in oysters exposed at 10 or 14 ppt. Evaluation of confidence limits suggests no difference in mortalities from treatment of JOD-infected oysters, or in transmission of JOD, at salinities of 18-30 ppt. There was a significant difference in mortalities at lower salinities (10-15 ppt) compared to those above 18 ppt ($p = 0.01$). In addition, the infective agent was found to have apparently survived salinities of <5 ppt in oysters for 7 months after importation of juvenile oysters into Maryland from a New England area affected by JOD. Mortalities (79%, n = 57) and conchiolinous shell lesions (62%) characteristic of JOD were observed after placing these juveniles in aquaria at 26 ppt.

A low salinity laboratory exposure of oysters naturally infected by JOD demonstrated reduced mortalities over a period of 6 weeks. The naturally infected oysters originated from 2 different sources and revealed 1-16% (n > 100) JOD-related mortalities with conchiolinous shell lesions at the beginning of the experiments. In both salinity experiments (*table* 2), mortality decreased with a reduction in salinity, although the lowest cumulative mortalities were still high (40% at 14 ppt). Higher cumulative mortalities, ranging from 60-85%, were observed at salinities of 18-30 ppt after the room temperature exposure (21-22°C).

Effects of therapeutants on the organism responsible for JOD

Two hundred naturally infected oysters from populations showing JOD-related mortality and conchiolinous shell lesions were placed in each of 7 aquaria at 26 ppt and exposed to separate therapeutants (*table* 4). The most effective therapeutant for reducing mortalities (from 85% in unmedicated oysters to 43%) in the first experiment was a combination of erythromycin and minocycline. A repeat experiment which separated the two antibiotics showed erythromycin was the effective component in reducing JOD-related mortalities. Erythromycin reduced mortalities from 65% in unmedicated oysters, and 68% in minocycline-treated oysters, to 9%. Optimum mortality reductions were achieved with repeat treatments every 3 weeks. Mortalities increased if the antibiotic was not repeated. The copper sulfate mixture was less effective, but reduced cumulative mortalities from 85% in unmedicated oysters to 69% in the first exposure. Other medications were either toxic to the oysters or had no positive effects in reducing mortalities.

Table 5. – Bacterial isolations from oysters after second therapeutic experiment. NV = Non-*Vibrio*, N = 6 oysters from each treatment.

Treatment	Conch.[1]	Bacteria	
		Non-*Vibrio*	*Vibrio*
Erythromycin[2]			
6-2	VH	NV	–
Minocycline			
7-1	VL	–	–
7-2	M	NV	–
7-3	L	–	*V. alginolyticus*
7-4	–	–	–
7-5	M	–	*V. alginolyticus*, *V. logei*
7-6	L	–	*V. pelagicus*
Control 26 ppt[3]			
C-1	VL	–	*V. anguillarum*
C-2	–	–	*V. alginolyticus*
C-3	L	–	*V. logei*
C-4	M	–	–
C-5	–	–	*V. alginolyticus*
C-6	–	NV	–

[1] Intensity of conchiolin deposited on the inside of the shell is listed as VH = very heavy, M = moderate, L = light, VL = very light.

[2] All other oysters from the erythromycin-treated exposures were negative for bacteria and conchiolin.

[3] Positive control oysters that were naturally infected by the JOD organism and exhibiting the typical JOD syndrome.

Four species of vibrios were isolated from 7 of 18 (7/18) JOD-infected oysters tested after the second therapeutic exposure (*table* 5). The bacteria were not abundant and could be isolated only after enrichment in broth culture. In this study, no bacteria were isolated from oysters by direct plating on agar media. All vibrio isolates were from oysters held in the control aquarium (JOD infected), or those exposed to minocycline. Isolates were *V. alginolyticus* (3/18), *V. anguillarum* (1/18), *V. logei* (2/18), and *V. pelagicus* (1/18). None of the isolates were found to be sensitive to erythromycin which greatly reduced JOD mortalities, but, with the exception of 1 strain of *V. alginolyticus*, all were susceptible to minocycline. No *Vibrio* sp. were isolated from erythromycin-treated oysters, even though JOD remained at low levels in these oysters. One unidentified non-*Vibrio* sp. was isolated from 1 oyster in each of the 3 test groups. There also was no relationship between the occurrence of characteristic conchiolinous shell lesions and the isolation of bacteria in this study (*table* 5).

Filtration studies

Juveniles reared in 15 000 l tanks and 25 μm filtered water in 1992 showed reduced mortalities (less than 8%), compared to 66% from grow-out rafts in open water. A 10-week field experiment in 1994 reinforced the earlier observation that water filtration to 25 μm did not prevent JOD, but did reduce cumulative

mortalities (31%) and delay disease onset by 1-2 weeks compared to oysters in unfiltered (45% mortality), or 50 μm filtered water (82% mortality). A concurrent experiment in the same 15 000 l tanks with oysters selectively spawned for possible JOD resistance suffered only 6-12 % cumulative mortalities.

In laboratory experiments, material retained in sequential 50 μm, 25 μm, and 5 μm bag filters, plus what passed the 5-μm filter, was used to successfully transmit JOD in aquaria. Heaviest JOD-induced mortalities (64%, n = 200) occurred in the 5 μm exposure. Mortalities in oysters exposed to 25 μm and < 5 μm filtered particles were 16% and 28% (n = 200), respectively. A previously unknown species of suctorian ciliate was also found in the 5 μm filtrate. Confidence limits suggest the mortality observed in oysters exposed in aquaria inoculated with material from the 50 or 5 μm filters was different from all other experimental exposures. There was no significant difference in mortalities between oysters in the < 5 and 25 μm exposures.

Attempts to isolate bacteria revealed no specific bacterium associated with JOD-infected oysters in this transmission study. Vibrios isolated included *V. diazotrophicus* (9/40), *V. fluvialis* (1/40), *V. mimicus* (1/40), and *V. ordalii* (5/40). Both *V. diazotrophicus* (1/10) and *V. ordalii* (3/10) were also isolated from uninfected control oysters. Neither *V. alginolyticus*, *V. anguillarum*, *V. logei*, or *V. pelagicus*, isolated from the 3 therapeutant treatment groups tested earlier, were cultured from any of the 4 JOD-infected groups from the filtration study. The most common non-Vibrio isolates were *Alcaligenes faecalis*, *Micrococcus* sp., *Pseudomonas fluorescens*, and *Shewanella putrefaciens*. All were isolated from control and JOD-infected oysters.

Protistan isolations

Efforts to isolate potential disease agents from JOD-infected water and oysters since 1992 routinely yielded an unidentified spirochaete, numerous protists and other organisms (*table* 6).

The same parasitic suctorian protist, *Endosphaera* sp., was isolated from the 5 μm filter material, water samples from a JOD-infected oyster growing facility, and aquaria used for transmission experiments. This previously undescribed species of ciliate was not isolated from low salinity aquaria water used for transmission experiments, uninfected control aquaria, or oysters.

Endosphaera sp. was found free-living in the above samples and also as an intracellular parasite of a stichotrichan ciliate host, *Uroleptoides* sp. The adult free-living form of the suctorian measured approximately 9-11 μm in diameter. Intracellular embryos of the suctorian were 4-8 μm in diameter. The intracellular form of the parasite was morphologically similar to the intracellular bodies observed in mantle tissue of JOD-infected oysters by light microscopy.

Other parasitic protistan organisms, *Mesanophrys* sp., *Metanophrys* sp., and *Paranophrys* sp., were routinely cultured from tissues of JOD-infected oysters. To date we have not isolated these ciliates from cultures of uninfected oysters.

Table 6. – A partial listing and tentative identification of organisms from JOD-infected water and oysters to date.

Source of organism	Preparation	Tentative identification Group	Genus
Experimental aquaria	Live	Tintinnids	
	Live	Suctorian ciliate	*Acineta* sp.
	Live	Peritrich ciliates	*Vorticella* sp.
			Zoothamnium sp.
	Live	Scuticociliate	*Cohnilembus* sp.
	Live	Hypotrich	*Euplotes* sp.
	Stained	Suctorian ciliate	*Endosphaera* sp.
	Stained	Stichotrich ciliate	*Uroleptoides* sp.
	Live	Euglenoide flagellate	
	Live	Heliozoa	
JOD-infected oysters	Live	Hypotrich ciliates	*Aspidisca* sp.
	Live		*Euplotes* sp.
	Live	Scuticociliates	*Glauconema* sp.
	Live		*Uronema* sp.
	Live		*Cyclidium* sp.
	Live		*Mesanophrys* sp.
	Live		*Metanophrys* sp.
	Live		*Paranophrys* sp.
	Live	Rotifers	
	Live	Gastrotrichs	
JOD-infected oysters	Stained	Scuticociliates	*Mesanophrys* sp.
	Stained		*Metanophrys* sp.
	Stained		*Paranophrys* sp.
JOD-infected water [1]	Stained	Suctorian ciliate	*Endosphaera* sp.
	Stained	Stichotrich ciliate	*Uroleptoides* sp.
5 μm-filtered water-sample	Stained	Suctorian ciliate	*Endosphaera* sp.
	Stained	Stichotrich ciliate	*Uroleptoides* sp.

[1] Sample obtained at F. M. Flower and Son, Inc.

DISCUSSION

Combined results of these experiments, along with our histological data, continue to support the hypothesis that the JOD disease agent may be a protistan parasite in the 2-6 μm size range. A bacterial etiology for JOD appears unlikely from our results which show inconsistent isolation of specific species of bacteria from JOD-infected oysters, a lack of sensitivity of isolated bacteria to therapeutants that reduced JOD mortality, and the isolation of some of the same bacteria from uninfected control oysters.

No evidence of a virus was found in either of 2 pools of JOD-infected oysters tested in a

separate study (Lewis and Farley, unpubl. data). In an evaluation of naturally infected oysters, 2 pools of JOD-infected oysters, 10 oysters each, from 2 locations were tested for virus according to methods modified from Ganzhorn and LaPatra (1994). Since no molluscan cell lines currently exist for testing molluscan viral diseases, the following fish cell lines were used: *Epithelioma papillosum cyprini* (EPC), chinook salmon embryo (CHSE), fathead minnow (FHM), and brown bullhead (BB).

Results from transmission studies clearly indicate that JOD is transmissible under laboratory conditions. The incubation period for transmission was found to have an inverse correlation to water temperature: 3 weeks at 26°C to 7 weeks at 18°C. Four years of concurrent field studies (Farley and Lewis, unpubl. data) showed conchiolinous shell lesions present in 0-80% of oysters from numerous weekly and monthly samples of 100 live JOD-infected oysters each, depending on the stage of infection. The prevalence of conchiolin reached 100% in dead oysters from some samples. In comparison, conchiolinous lesions were found in 25-40% of dead oysters from experimental transmission. Transmission also occurred using survivors of JOD overwintered at 4-9°C, and material filtered from the water column at 25, 5, and < 5 μm. Transmission dynamics were similar to those observed with infected oysters as the source of infection. These results clearly demonstrate that oysters surviving JOD can still be carriers of the etiologic agent. JOD was not transmitted using infected aquarium water that had been held through the winter without JOD-infected oysters.

To date, JOD has only occurred naturally in oysters from high salinity growing waters (> 24 ppt). In salinity controlled transmission experiments, JOD was readily transmitted at salinities of 18-30 ppt; however, more studies are required to understand the effect of low salinity on the transmission process. Salinities below 18 ppt appear to either increase the incubation period for disease onset, or prevent transmission of JOD. However, the causative agent of JOD survived in oysters held at salinities < 5 ppt for an extended period of time. This conclusion is based on the observation of mortalities with the presence of typical JOD conchiolinous shell lesions in the oysters grown at < 5 ppt salinity after placing them in aquaria at 26 ppt salinity. Simply exposing control oysters to 26 ppt in aquaria has not caused mortalities or the production of conchiolin in our experiments and it is highly unlikely that they became infected by JOD after importation to Maryland. Furthermore, an east coast survey of oysters failed to reveal JOD-type conchiolinous shell lesions in oysters other than those infected by JOD (Lewis and Farley, unpubl.). It is clear that exposing JOD-infected oysters to low salinity has some degree of benefit in reducing mortalities; it also seems possible that low salinity may, at least partially, inhibit transmission of the disease.

Our histological examination of naturally infected oysters from field studies consistently revealed intracellular bodies in mantle tissue that we believe to be a protistan organism. The bodies were associated with mantle epithelia and conchiolinous shell lesions of JOD-infected oysters only. The combination of histological observations, therapeutant effects on JOD mortality, results of filtration studies, and the isolation of *Endosphaera* sp. from JOD-infected sources all support the hypothesis of a protist as the possible cause of JOD. The 5-25 μm and < 5μm size fractions of filtered material which were responsible for inducing the highest JOD mortalities in the filtration transmission experiments show a direct correlation in size with what we believe to be the causative agent observed in histological sections. Size and morphology of intracellular bodies observed in the histological samples are also compatible with that of *Endosphaera* sp. isolated from JOD-infected water samples. We feel that this tentative association warrants further investigation to determine if *Endosphaera* sp., or a similar organism, may be the causative agent of JOD.

Of the other protists isolated from JOD-infected material, *Mesanophrys* sp. is only known as a parasite of invertebrates; no free-living form is known. Species of *Metanophrys* and *Paranophrys* are also known parasites. However, none of these ciliates are believed to be the JOD causative agent. None of them are intracellular parasites and their size does not agree with histological data.

Antibiotics and other therapeutants were used to determine if they could provide insight into the possible identity of the causative organism. Sensitivity tests showed minocycline was effective against *Vibrio* sp. isolated from experimental oysters. This suggests that minocycline should have had a positive effect in reducing JOD-related mortalities if they were caused by a *Vibrio* sp. However, cumulative mortalities of minocycline-treated and unmedicated infected oysters were nearly identical. Furthermore, the failure to recover of vibrios from minocycline-treated and unmedicated oysters without enrichment indicates very few vibrios were present in the infected oysters from the second therapeutants experiment. Conversely, vibrios isolated were not sensitive to erythromycin, although this antibiotic greatly reduced the effects of JOD without fully curing the oysters of the disease. In preliminary tests, we found that some of the protists isolated from JOD-infected oysters were susceptible to erythromycin.

No association of either a vibrio or dinoflagellate with JOD was found in our studies. Transmission of JOD in the absence of dinoflagellates clearly indicates that a dinoflagellate is not the causative agent. Bacterial cultures from a total of 58 JOD experimentally-infected oysters showed no consistent association of a specific bacterium with JOD. Also, bacteria were not found in histologic sections of mantle lesions in field or experimental studies. In all, 7 *Vibrio* sp. were isolated and identified; 2 species

remain to be identified. *Vibrio* sp. isolated from the first 18 experimental oysters (3 experimental groups) were different from those isolated from 40 additional oysters from 4 experimental groups of the filtration transmission experiment. Two of the 7 species isolated, *V. diazotrophicus* and *V. ordalii*, were also isolated from uninfected control oysters, suggesting that they are not the cause of JOD. While *V. diazotrophicus* was the most common isolate from JOD experimentally-infected oysters (9/40), it was isolated from only 1 of 7 experimental groups of JOD-infected oysters cultured. The remaining vibrios were isolated too infrequently to suggest they are responsible for JOD.

CONCLUSION

While the identity of the JOD agent remains unknown, our experimental studies suggest that a protist continues to be a valid candidate. Our work does not favorably support any of the isolated bacteria as the JOD causative agent. The effects of low salinity on the transmission of JOD and the possible association of *Endosphaera* sp. and JOD need to be more thoroughly investigated. Studies are currently underway to pursue the possibility of *Endosphaera* sp. as the causative agent for JOD. Other ongoing experimental and field studies investigating a possible association of vibrios with JOD will soon be completed.

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REFERENCES

Bricelj V. M., S. E. Ford, F. J. Borrero, F. O. Perkins, G. Rivara, R. E. Hillman, R. A. Elston, J. Chang 1992. Unexplained mortalities of hatchery-reared, juvenile oysters, *Crassostrea virginica* (Gmelin). *J. Shellfish Res.* **11**, 331-347.

Davis C. V., B. J. Barber 1994. Size-dependent mortality in hatchery-reared populations of oysters, *Crassostrea virginica* Gmelin, 1791, affected by juvenile oyster disease. *J. Shellfish Res.* **13**, 137-142.

Ganzhorn J., S. E. LaPatra 1994. General procedures for virology. In: Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. 4th ed., Version 1, J. C. Thoesen ed. Fish Health Section, American Fisheries Society, Bethesda.

Howard D. W., C. S. Smith 1983. Histological techniques for marine bivalve mollusks. U.S. Dept. Commerce, NOAA Tech. Memo. NMFS-F/NEC-25, 97 p.

Lewis, E. J., C. A. Farley 1994. A proposal for the study of a protozoan disease agent(s) associated with mortalities of hatchery-reared juvenile oysters in the northeastern United States. Northeastern Regional Aquaculture Center final report, project # 555327, 33 p.

Sokal R. R., F. J. Rohlf 1973. Introduction to Biostatistics. W.H. Freeman & Co., San Francisco, 368 p.