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[Article]

An Investigation of the Pathology Associated with Mass Mortality Events in the Cultured Japanese Pearl Oyster *Pinctada fucata martensii* at Four Farms in Western Japan

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Abstract.—The epidemiological and histopathological characteristics of the mass mortality of cultured Japanese pearl oysters *Pinctada fucata martensii* were investigated. Rearing experiments with Japanese pearl oysters in farms revealed that the mass mortality occurs as a regular annual event in particular farms in western Japan. Diseased oysters had marked atrophy and red-brown discoloration of the soft parts of the body. Light microscopy revealed that the epithelia of the stomach, the ducts of the digestive diverticula (DD), and the DD themselves showed marked blebbing and necrosis to varying degrees during earlier stages of the disease. At advanced stages, muscle fibers of the adductor muscle, heart, mantle, and other parts of the body and the connective tissues of various organs involving the vascular system also exhibited considerable atrophy and necrosis. There were no remarkable changes in the branchial and pallial epithelia. No viral, bacterial, mycotic, or parasitic causative organisms were found in diseased oysters. The results of a case study of mass mortality at one farm suggested that there is some causal relationship between outbreaks of this disease and the existence of neighboring fish farms. These findings suggest that the mass mortality is not due to an infectious disease. We discuss pathological features and possible causes of this disease.

The production of pearls by the culture of Japanese pearl oysters *Pinctada fucata martensii* is one of the most important traditional marine industries in Japan and has a history of over 100 years. The first case of mass mortality in cultured Japanese pearl oysters was identified in the Kagoshima prefecture in 1993. In 1994, similar mortality events were identified in the Ehime and Oita prefectures. The mass mortality was prominent throughout western Japan by 1997. Although there were several crises in the industry's past, the mass mortality of cultured oysters caused unprecedented devastation.

Several studies have been carried out to elucidate the cause of the mass mortality in Japanese pearl oysters. Kurokawa et al. (1999) performed laboratory infection trials involving cohabitation and tissue transplantation into healthy oysters and reported that the mass mortality was reproducible. However, they did not identify the causative agent. Miyazaki et al. (1999) described a causative virus that was isolated from diseased oysters by use of two fish cell lines. However, Nakajima (1999) reported that attempts to isolate the causative virus with 14 fish cell lines, including the cell lines employed by Miyazaki et al. (1999), were unsuccessful. In addition, we reported that contact infection trials in the sea failed to reproduce the disease condition associated with the mass mortality (Hirano et al. 2002). Accordingly, there are

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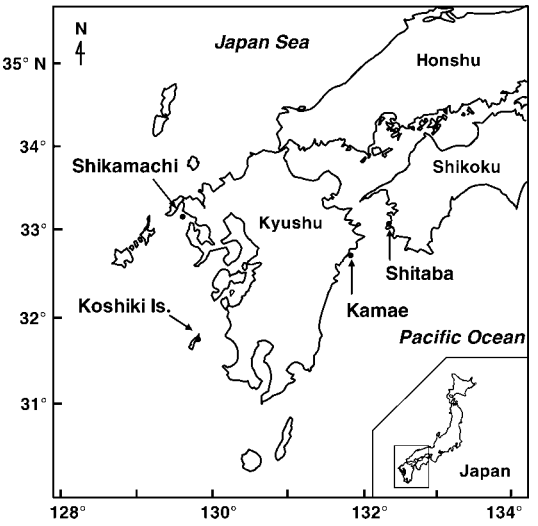

FIGURE 1.—Map of western and southern Japan, showing locations of Japanese pearl oyster farms where rearing experiments and a case study of oyster mass mortality were conducted.

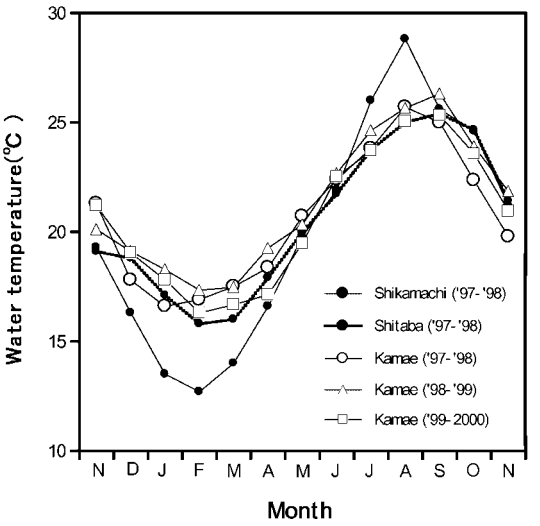

FIGURE 2.—Monthly mean water temperatures at Japanese pearl oyster farms located at Kamae, Oita prefecture; Shitaba, Ehime prefecture; and Shikamachi, Nagasaki prefecture, Japan.

discrepancies in the causal understanding of this disease and in the description of the pathological changes (Kurokawa et al. 1999; Miyazaki et al. 1999).

To elucidate the pathological profile of this disease, we first monitored the growth and mortality of experimentally cultured Japanese pearl oysters at several farms. Secondly, we performed a case study of mass mortality and examined the method of recovery. We also compared the histopathology of healthy and diseased oysters.

Methods

Test oysters and rearing experiments in farms.—Oysters of the same strain were produced and cultured by conventional methods at Nishimura Shinju Corp., Nagasaki prefecture, to an age of 2 years. Rearing experiments with these 2-year-old oysters were conducted at a farm in Kamae, Oita prefecture, during 1997–1998, 1998–1999, and 1999–2000 and at a farm in Shikamachi, Nagasaki prefecture, during 1997–1998. At the Kamae farm, we also conducted a rearing experiment with another hatchery-reared strain during 2000–2001. In a rearing experiment conducted during 1997–1998 at a farm in Shitaba, Ehime prefecture, we used hatchery-reared 2-year-old oysters that were produced and cultured by conventional methods at Tasaki Shinju Co., Ltd., Kagoshima prefecture (Figure 1). Among these farms, those in Kamae and Shitaba are known for high mortality of cultured Japanese pearl oysters. It is also known that mass mortality events have occurred at the Shikamachi farm in mother oysters (those not inserted with pearl nuclei) delivered in spring from high-mortality farms such as those at Kamae and Shitaba, but no mass mortality occurred in mother oysters delivered from high-mortality farms in autumn. Test oysters were cultured in these farms by use of lantern-type net-cages dangled on farming ropes at a depth of 1.0–2.5 m in the bay. Growth and mortality were monitored, sessile organisms growing on test oysters were removed, and rearing cages were changed every month. Figure 2 shows the monthly mean water temperatures at these farms during the experimental periods.

Growth and mortality data analyses.—To monitor the growth of test oysters in each farm, the shell heights of 50 oysters were measured by a digimatic caliper (Mitutoyo Corp., Kawasaki, Japan). Shell measurements were expressed as mean ± SE. Monthly mortality was calculated as the percentage of oysters that survived between consecutive sampling times. Cumulative mortality was calculated by accumulating consecutive monthly mortalities. Five-hundred test oysters were used in the first three rearing experiments conducted at the Kamae farm (1997–2000) and in the experiment at the Shitaba farm (1997–1998). We used 1,100 test oysters for the rearing experiment at the Shikamachi farm (1997–1998) and the

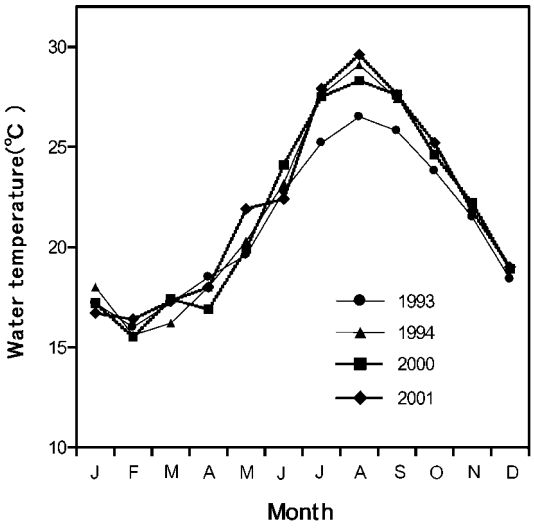

FIGURE 3.—Monthly mean water temperatures at a Japanese pearl oyster farm located at Koshiki Island, Kagoshima prefecture, Japan (1993–1994 and 2000–2001).

additional experiment at the Kamae farm (2000–2001).

Case study of mass mortality.—A case study was performed on the mass mortality that occurred in a Japanese pearl oyster farm operated by Kamimura Shinju Co., Ltd., at Koshiki Island, Kagoshima prefecture (Figure 1). The Koshiki farm was established in 1952. The farm's production records from 1993–1995, when the mass mortality occurred, were analyzed. Figure 3 illustrates the monthly mean water temperature at the Koshiki farm during 1993, 1994, 2000, and 2001. A 54-pen (each pen 10 × 10 m in size) fish farm neighboring the Koshiki farm was established in 1984. Originally, this fish farm cultured red seabream (also known as madai) *Pagrus major* and tiger puffer *Takifugu rubripes*, but since 1987 only tiger puffer have been cultured there.

Light microscopy and transmission electron microscopy.—We conducted sequential histopathological examinations of specimens that were usually sampled once per month when the growth and mortality of the test oysters were monitored. We collected 337 oysters for light microscopy (LM) and transmission electron microscopy (TEM) from the Kamae (1997–2001), Shitaba (1997–1998), and Shikamachi (1997–1998) farms. Table 1 lists locations, sampling times, and numbers of oysters used for LM and TEM. For LM, seven oysters were randomly sampled at each sampling time for histopathological examination. Sampled oysters were transported live to the laboratory of the respective farms and were sacrificed by removing valves. The soft parts of a total of 280 oysters were fixed in chilled 20% formalin in seawater after dissection. After fixation in the formalin solution for 1 week or more, various organs and tissues were trimmed, dehydrated in a graded ethanol series, and embedded in paraffin. Paraffin sections (3 μm thick) were cut and stained with hematoxylin and eosin. For TEM, a total of 57 oysters (40 randomly selected; 17 selected based on disease status [5 healthy and 12 diseased]) were fixed in a chilled solution of 4% paraformaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) after dissection. The specimens were then trimmed under a dissection microscope, postfixed in 2% osmium tetroxide

TABLE 1.—Location, time of sampling, and number of Japanese pearl oyster specimens used for light microscopy (LM) and transmission electron microscopy (TEM) in the study of mass mortality of cultured oysters at three farms in Japan.

	Kamae						Shitaba (1997–1998)		Shikamachi (1997–1998)	
	1997–1998		1998–1999		2000–2001					
Month	LM	TEM	LM	TEM	LM	TEM	LM	TEM	LM	TEM
Dec	7						7		7	
Jan	7						7		7	
Feb	7						7		7	
Mar	7						7		7	
Apr	7						7		7	
May	7		7	4			7		7	
Jun	7	3	7	4			7		7	3
Jul	7	3					7		7	3
Aug	7	3	7			6	7		7	3
Sep	7	3	7	4		5	7		7	3
Oct	7	3	7	4			7		7	3
Nov	7								7	
Total	84	15	35	16		11	77		84	15

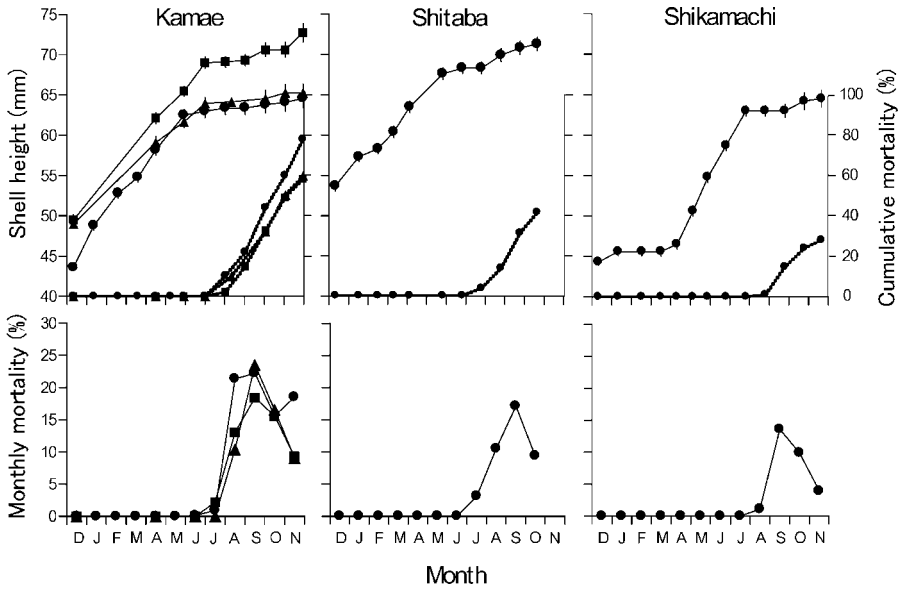

FIGURE 4.—Monthly changes in the growth (shell height) and mortality of Japanese pearl oysters cultured at farms in Kamae, Shitaba, and Shikamachi, Japan. For the Kamae farm, results from 1998 (circles), 1999 (triangles), and 2000 (rectangles) are presented. The strain cultured at Shitaba was different from that cultured at Kamae and Shikamachi. The broken lines (lower parts of top panels) indicate cumulative mortality (right scale).

in the cacodylate buffer for 1.5 h, dehydrated in a graded ethanol series, and embedded in Spurr's resin (Spurr 1969). The thin 0.5-μm sections were cut on a Porter-Blum MT-1 ultramicrotome and were stained with 0.5% toluidine blue in 1% Borax. The ultrathin sections were cut on a Leica Ultracut UCT microtome, stained with uranyl acetate and lead citrate (Reynolds 1963), and examined on a JEOL JEM-100S electron microscope.

The distinguishing characteristics for healthy oysters are the well-developed marginal projection of valves and well-ripened soft parts of the body. On the other hand, the distinguishing characteristics for oysters with advanced disease are distinct discoloration and moderate emaciation of the body. Moribund oysters are those that exhibit less conspicuous discoloration, strong emaciation of the body, and atrophy of various organs. We monitored the progression of pathological changes by means of two indicators: (1) the size of muscle fibers of the adductor and pallial muscles and (2) the number of necrotic foci in the adductor muscle on selected specimens. Cross sections of the adductor and subepithelial pallial muscles of these specimens were histologically prepared, and the diameters of 20 muscle fibers per oyster were measured microscopically by means of a micrometer. Numbers of necrotic foci per section were counted by carefully examining the total area of each section. Differences in the sizes of muscle fibers were analyzed by means of *t*-tests.

Results

Growth and Mortality of Cultured Oysters

Figure 4 shows that cultured oysters at the Kamae farm grew vigorously from December to May or June, after which growth was markedly stunted. The mortality of cultured oysters began in late July and continued until December or later. Mortality peaked in September, and cumulative mortality was as high as 56% (in 2000) to 79% (in 1998). A striking similarity existed in the growth and mortality trends recorded during three consecutive years. Growth and mortality trends at the Shitaba farm were very similar to those at the Kamae farm (Figure 4). The cumulative mortality at Shitaba reached 40.9% by mid-October of 1998. At the Shikamachi farm, low water temperatures inhibited the growth of cultured oysters from January to March, but vigorous growth was observed from April to July. Mortality at the farm began in late August and peaked in September. By late November, the cumulative mortality at Shikamachi was 28.5% (Figure 4).

Case Study of Mass Mortality in the Koshiki Farm

Table 2 presents a full account of field conditions during the mass mortality and the recovery

TABLE 2.—Mass mortality of Japanese pearl oysters cultured at a farm at Koshiki Island, Kagoshima prefecture, Japan (mother oysters are those not inserted with pearl nuclei; operated oysters are those inserted with pearl nuclei).

Year	Oyster type	Number dead	Mortality (%)	Note
1952–1991				No mass mortality occurred.
1992				Growth stagnation and increasing mortality after autumn were noted.
1993	Mother	1,169,000	74	
	Operated	208,000	57	
1994	Mother	1,072,000	72	
	Operated	197,000	62	
1995	Mother	1,357,000	84	Nearby fish farming was stopped in August.
	Operated	68,000	66	
1996–present				No mass mortality occurred.

that occurred in the Koshiki Japanese pearl oyster farm. In autumn 1992, growth stagnation of cultured oysters was noted and the mortality rate increased. Mass mortality occurred in 1993–1995. Out of 5,885,783 mother oysters and operated oysters (those inserted with pearl nuclei) at the Koshiki farm from 1993 to 1995, 4,071,000 died (69.2%). According to the Koshiki farm personnel (personal communication), cultured oysters showed vigorous growth during the winter and spring, whereas the mortality began in late July and peaked in September. Most diseased oysters showed considerable emaciation and red-brown discoloration of the soft body parts. Such growth and mortality trends, clinical signs, and gross pathology were identical with those observed at the Kamae and Shitaba farms. After a lengthy period of negotiation, fish farming was halted in August 1995. Since that time, no mass mortality has occurred at the Koshiki farm, and harvests of pearls and mother oysters have been consistently good.

Clinical Signs, Gross Pathology, and Histopathology

Figure 5 demonstrates the external appearance of the soft parts of healthy and diseased oysters. Diseased oysters had red-brown discoloration and emaciation of the soft parts to varying degrees. The discoloration of the body began in June and continued to January or later at the Kamae farm, according to Japanese pearl oyster culturists. At the Shikamachi farm, the discoloration began in September and continued until November or later. Most moribund oysters showed extreme emaciation of the body due to severe atrophy of various tissues. There were no differences in clinical signs and gross pathology among the diseased oysters collected at different farms, although the onset of disease and the degree of pathological changes varied among farms.

At the earlier stages of the disease, marked surface blebbing, loss of cytoplasm, and necrosis were found in the epithelial cells of the stomach

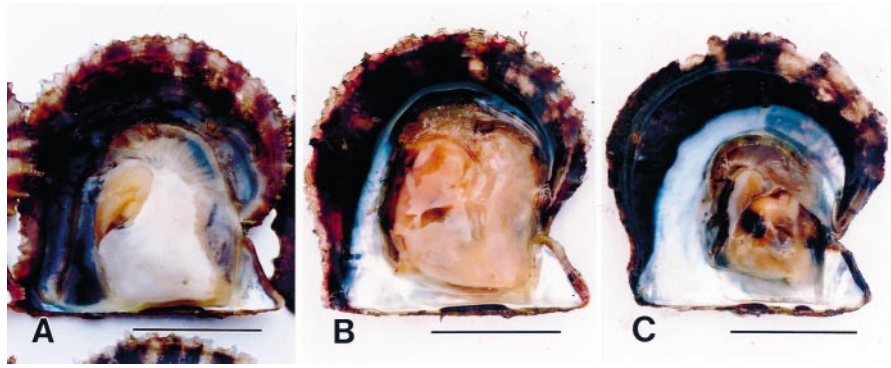

FIGURE 5.—External view of the soft body parts of **(A)** a healthy Japanese pearl oyster (scale bar = 36 mm); **(B)** an oyster at an advanced stage of disease, having distinct red-brown discoloration in the soft parts (scale bar = 30 mm); and **(C)** a moribund oyster showing strong emaciation of the body and less conspicuous red-brown discoloration (scale bar = 26 mm).

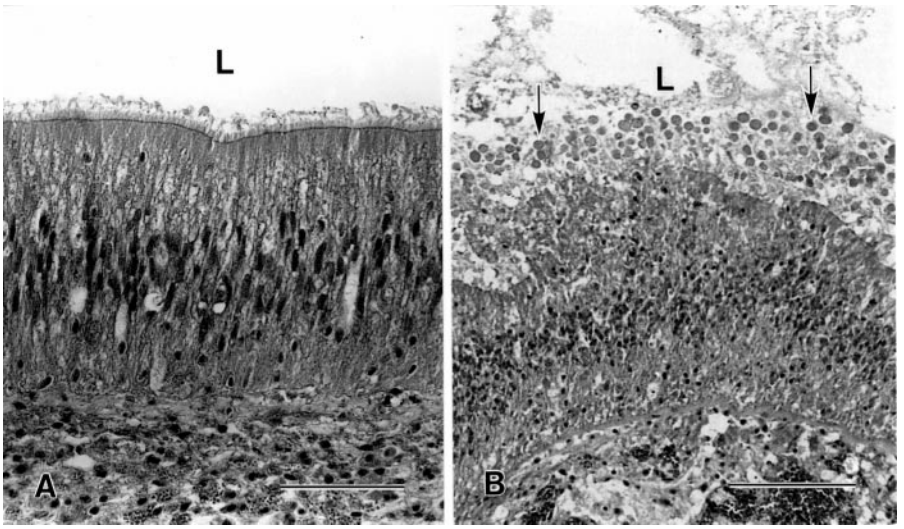

FIGURE 6.—Light micrographs (L = gut lumen; hematoxylin and eosin stain; scale bars = 50 μm) showing the stomach epithelia of Japanese pearl oysters. Panel **(A)** shows a healthy oyster whose epithelial cells have both cilia and microvilli in the apical surface; panel **(B)** shows an oyster at an early stage of disease exhibiting necrotic tissue and blebs. Arrows indicate blebs and cellular debris caused by the blebbing and necrosis of epithelial cells.

(Figure 6); the ducts of the digestive diverticula (DD) also showed remarkable clinical signs (Figure 7). These pathological features were also found in the DD themselves (Figure 8). Features like the filling of the gut lumen with necrotic cells and blebs occurred more frequently at earlier stages of the disease (May–July) in the Kamae farm specimens (Figure 7C). Transmission electron microscopy revealed that blebs formed in the apical portion of the epithelial cells and that these structures contained free ribosomes and cytoplasmic matrix. At advanced stages of the disease, oysters were moderately emaciated and had distinct red-brown discoloration. Oysters with advanced disease lost most of the glycogen deposition in the body; ducts of the DD were often found closed, and the DD were atrophied (Figures 7D, 8C). In the Kamae farm specimens, a marked decrease in the number of DD was noted in advanced and moribund stages, and diverticular epithelium showed marked atrophy. Such pathological features were not found in healthy oysters. We found no virus in the damaged epithelium of the digestive organ.

The adductor muscle of bivalves is composed of two parts, which are structurally and functionally different. In anatomical terms, they are called ''transparent and opaque parts.'' In the transparent part, myofibrils are thin and muscle fibers occupy the greater part of the adductor and contract quickly. In contrast, in the opaque part, myofibrils are thicker, and muscle fibers are white in color and are believed to function as ''catch muscle'' to sustain contraction for long periods.

In these experiments, the adductor muscle showed marked atrophy at advanced and moribund stages of the disease. Remarkable necrosis was observed in the connective tissue surrounding muscle bundles (epimysium and perimysium) and muscle fibers (Figure 9). Myofibrils of affected muscle fibers showed condensation or swelling. Infiltration of phagocytes was observed in necrotic foci, but no fibrosis was evident. Figure 10 shows that adductor muscle fibers decreased in size with the progression of disease in the same manner as did the pallial musculature. Necrosis of muscle fibers tended to advance with the progression of the disease (Figure 11). Such pathological changes were not found in healthy oysters. Analysis by TEM indicated that muscle fibers of healthy oysters contained abundant glycogen granules in the peripheral sarcoplasm and, to a lesser extent, between myofilaments. Thick and thin filaments were clearly observed; vacuoles that contained glycogen granules and that resembled glycogenosomes were frequently observed in the sarcoplasm (Figure 12). In diseased oysters, particularly at advanced and moribund stages, muscle fibers lost most of their glycogen granules and showed marked condensation of myofibrils accompanied by decomposition of myofilaments. In necrotic foci, liquefaction of affected muscle fibers was observed (Figure 13). Muscle fibers of moribund oys-

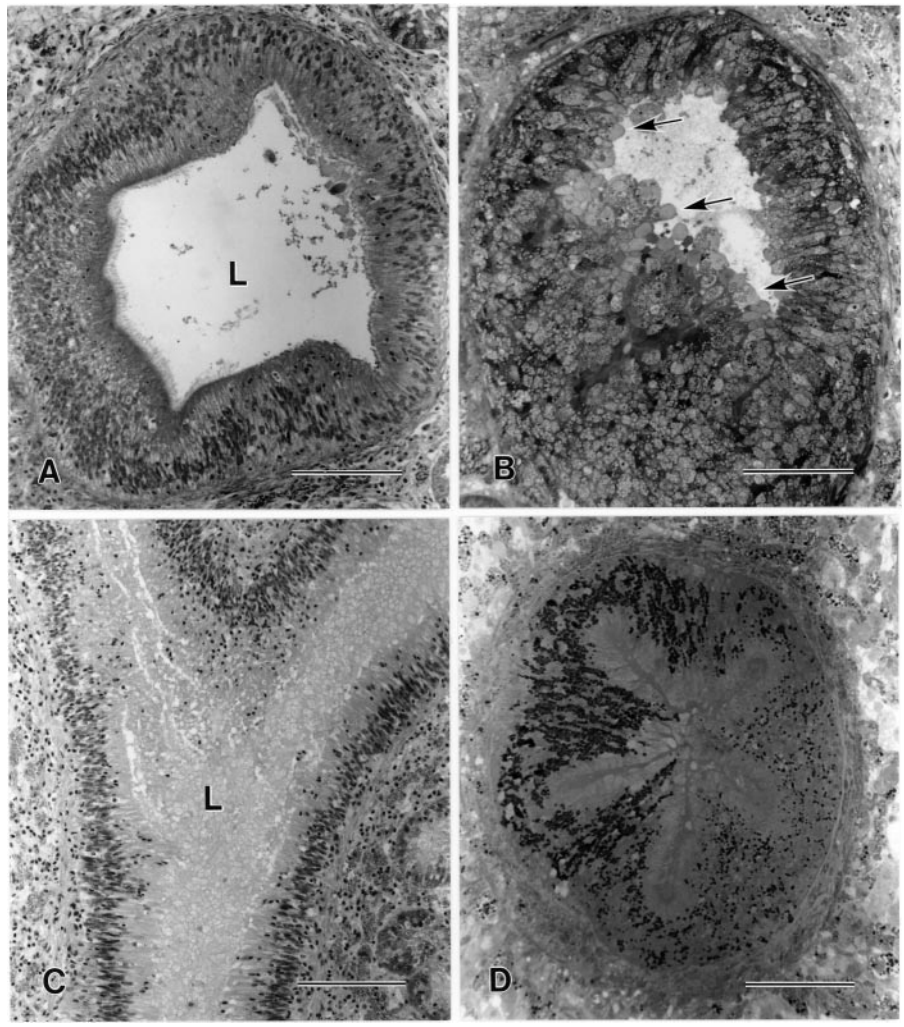

FIGURE 7.—Light micrographs (scale bars = 100 μm) showing the ducts of the digestive diverticula of Japanese pearl oysters. Panel **(A)** shows a healthy oyster with epithelial cells having both cilia and microvilli in the apical surface (L = gut lumen; hematoxylin and eosin stain [HE]); panel **(B)** shows an oyster at an early stage of disease, in which epithelial cells contain numerous, lightly stained, fine lipid droplets in the cytoplasm (arrows indicate prominent blebs in the apical portion of the epithelial cells; toluidine blue stain [TB]); panel **(C)** shows a diseased oyster at an early stage, in which the gut lumen is filled with blebs and cellular debris (HE); and panel **(D)** shows a diseased oyster at an advanced stage, in which the epithelial cells show atrophy and the gut lumen is closed (TB).

ters contained a few cytoplasmic organelles and reduced numbers of myofilaments, some of which were decomposed (Figure 14). No virus was found in the affected muscle fibers of oysters in either advanced or moribund stages.

Cardiac muscle fibers of diseased oysters showed marked atrophy and necrosis at the advanced stage of disease (Figure 15). Myofibrils were slender, markedly condensed, and lacked striations. Necrotic nuclei of muscle fibers were frequently observed. The endothelial tissue was necrotic and in some cases had disappeared. Similar pathological changes were not observed in healthy oysters.

No appreciable changes were observed in the branchial and pallial epithelia. The pallial muscle fibers and connective tissue showed marked atrophy at advanced stages (Figure 10). The pedal

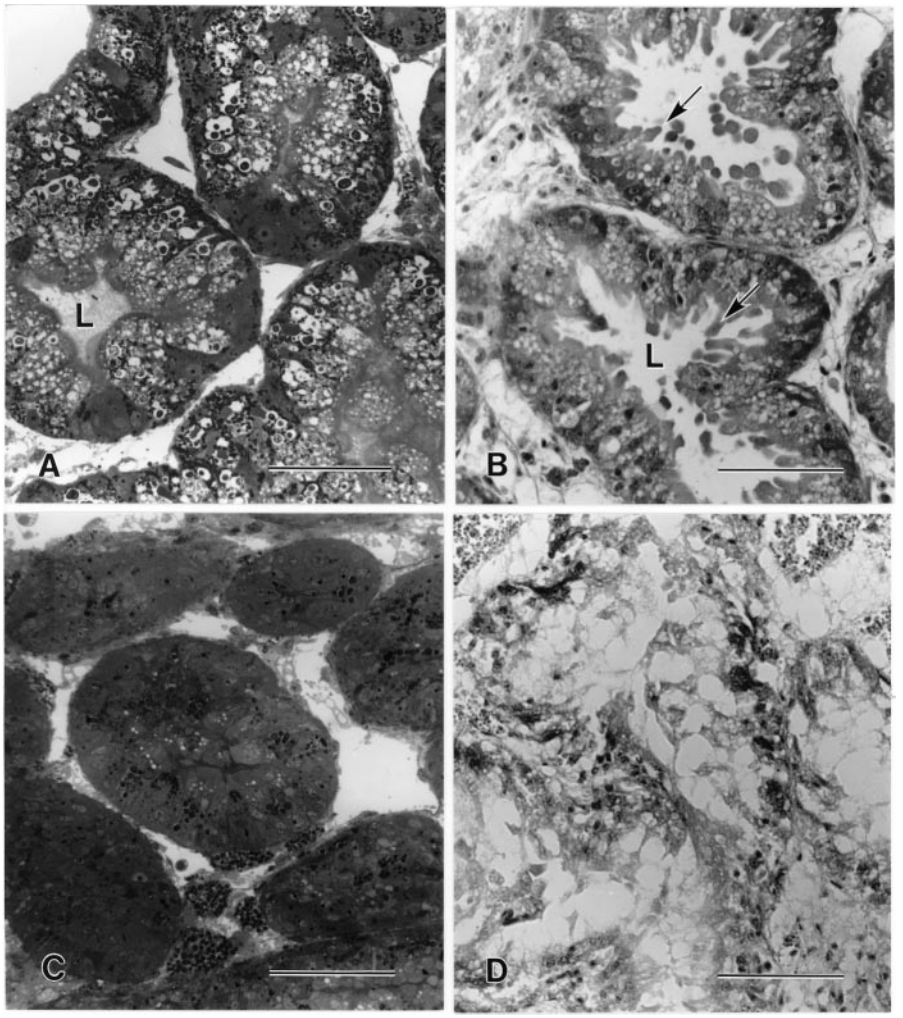

FIGURE 8.—Light micrographs (scale bars = 50 μm) showing the digestive diverticula of Japanese pearl oysters. Panel **(A)** shows a healthy oyster whose epithelial cells contain numerous vacuoles associated with digestion and absorption (L = gut lumen; toluidine blue stain [TB]); panel **(B)** shows an oyster at an early stage of disease, exhibiting prominent blebs (arrows), some of which contain lipid droplets, and visible cellular debris due to blebbing in the gut lumen (hematoxylin and eosin stain [HE]); panel **(C)** shows a diseased oyster at an advanced stage, in which the epithelial cells show atrophy and the gut lumina are closed (TB); and panel **(D)** shows a diseased oyster at an advanced stage, in which the epithelial cells are highly necrotic (HE).

musculature and connective tissue also showed notable atrophy. No bacterial, mycotic, or parasitic organisms were found in any tissue lesions.

The pathological changes described above were observed in specimens collected from all of the examined farms, but the disease clearly occurred earlier and was more severe at the Kamae and Shitaba farms than at the Shikamachi farm, and was clearly correlated with the growth and mortality trends. There was no clear difference in the progression of tissue lesions between the Kamae and Shitaba farms.

Discussion

It is well known that the mass mortality of cultured Japanese pearl oysters occurs in particular farms in the Ehime, Kochi, Oita, and Kagoshima prefectures of western Japan (Kurokawa et al. 1999; Miyazaki et al. 1999; Morizane et al. 2001; Hirano et al. 2002). It is also understood that the mass mortality occurs in summer to late autumn and peaks in September or October. In this study, we obtained sufficient materials to investigate the mass mortality sequentially by conducting rearing experiments on farms.

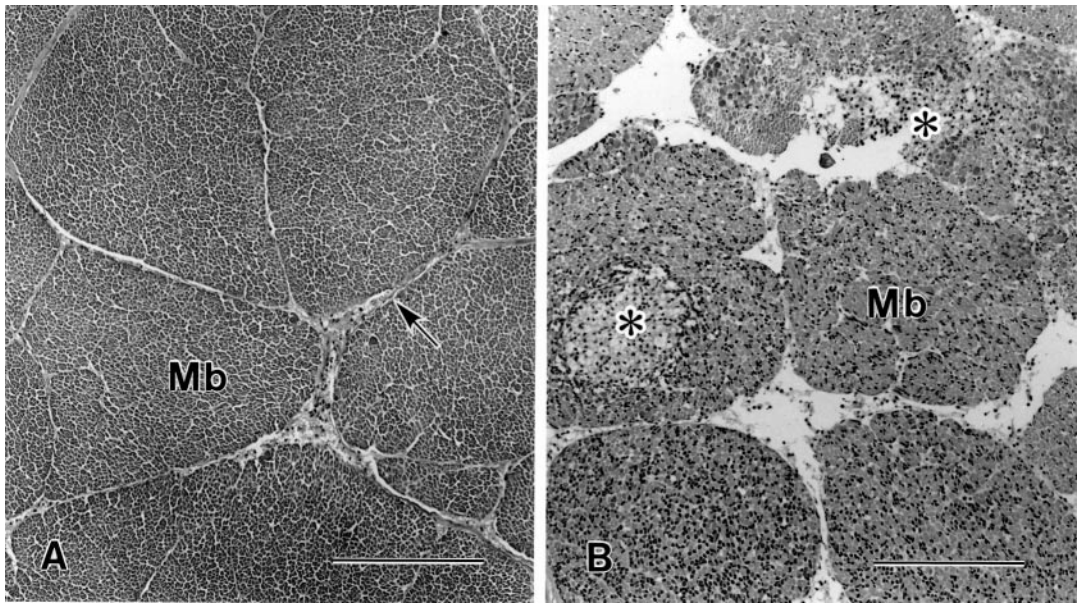

FIGURE 9.—Light micrographs (Mb = muscle fiber bundle; hematoxylin and eosin stain; scale bars = 200 μm) showing **(A)** the adductor muscle (transparent part) of a healthy Japanese pearl oyster (arrow indicates the connective tissue) and **(B)** the adductor muscle (opaque part) of a moribund oyster, in which numerous darkly stained muscle fibers are visible and the connective tissue is highly necrotic (asterisks indicate necrotic foci).

The pathological changes observed in diseased oysters in this study were characterized by blebbing and cell necrosis that spread from the digestive organ to various other organs; no viral, bacterial, mycotic, or parasitic organisms were detected. Dealing with diseased Japanese pearl oysters obtained from farms and infection trials, Kurokawa et al. (1999) reported that histopathological changes commonly appeared in the loose connective tissue of the mantle and less frequently

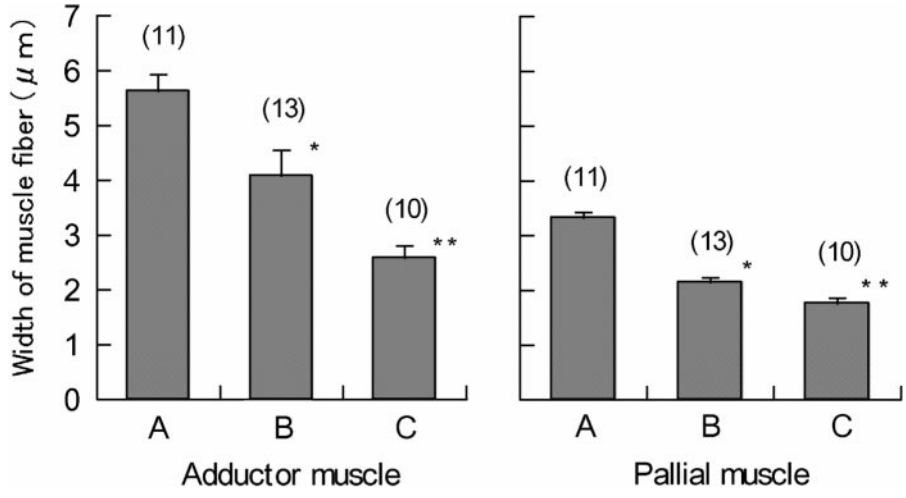

FIGURE 10.—Size changes in muscle fibers of the adductor muscle (opaque part; left panel) and pallial muscle (subepithelial muscle; right panel) occurring with the progression of disease in cultured Japanese pearl oysters. Bar labels indicate (A) healthy oysters, (B) diseased oysters at advanced stages, and (C) moribund oysters. Sample sizes are listed in parentheses above each bar. Asterisks indicate significant differences between diseased or moribund oysters and healthy oysters ($P < 0.05$*; $P < 0.01$**).

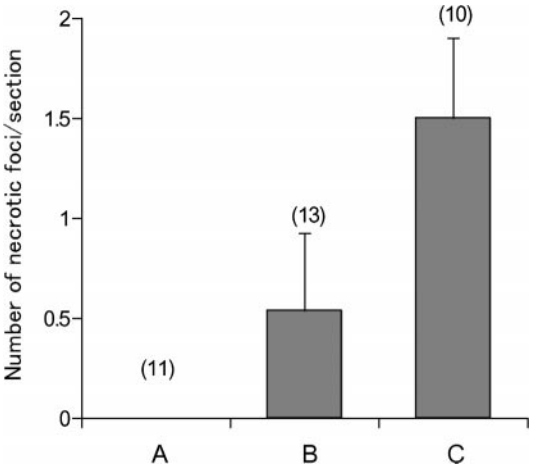

FIGURE 11.—Changes in the number of necrotic foci in the adductor muscle observed with the progression of disease in cultured Japanese pearl oysters. Bar labels indicate (A) healthy oysters, (B) diseased oysters at advanced stages, and (C) moribund oysters. Sample sizes are listed in parentheses above each bar.

in the adductor muscle, and that changes induced necrosis of muscle fibers and fibrosis. However, Kurokawa et al. (1999) also reported that no appreciable change was observed in the digestive organ. Miyazaki et al. (1999) also reported no appreciable change in the digestive organ of diseased oysters, except for atrophy of the DD. In contrast, we detected marked surface blebbing and necrosis in the epithelium of the stomach, ducts of the DD, and the DD themselves; these were characteristic changes that occurred at earlier stages of the disease. These changes were followed by necrosis of connective tissues involving the vascular system and by necrosis of parenchymal cells of other organs, such as the heart, adductor muscle, and mantle. This sequential pathology of diseased oysters suggests that some cellular injury factors were ingested with food, were absorbed, and caused damage to cells in various organs.

Miyazaki et al. (1999) reported that necrosis of the adductor muscle occurred because of viral infection, as they detected viral particles in the inclusion bodies. They treated thin sections prefixed in Karnovsky solution within 1% amylase solution at 36°C for 24 h. Differentiation between viral particles and glycogen granules is sometimes required in pathological studies of aquatic organisms. However, we seriously question the finding of viral particles by Miyazaki et al. (1999). First, we wonder why such differentiation is needed, because most glycogen granules tend to disappear in cells of affected tissues during the advanced stages of disease, and loss of glycogen granules is almost complete in moribund oysters. Second, it is uncertain whether glycogen granules in cytoplasmic (membrane-bounded) vacuoles could be digested by amylase based on their method, because membrane systems in aldehyde-fixed cells are often semi-permeable. If the differentiation between viral particles and glycogen granules is necessary, another method, such as specific staining for glycogen granules combined with amylase digestion, should be employed. Moreover, Miyazaki et al. (1999) did not describe how they differentiated viral particles from glycogen granules in infected eel kidney (EK-1) cells. It is impossible to determine whether the fine granules in the electron micrograph of an EK-1 cell are viral particles without appropriate differentiation. Finally, we could not discern any morphological difference between these viral inclusion bodies and the glycogenosomes that are found in mammalian cells under certain diseased conditions (e.g., Hug and Schubert 1967; Pfeifer 1970; Sato et al. 1978; Iwamasa et al. 1980) or the glycogenosome-like vacuoles that are found in the adductor muscles of healthy oysters.

We used the same hatchery-reared strain from year to year during our rearing experiments at the Kamae farm, and thus we were able to demonstrate that the mass mortality occurs as a regular annual event. We conducted a rearing experiment at the Kamae farm in 2001 with another hatchery-produced strain and also obtained a mortality pattern that was very similar to the pattern observed during the previous 3 years (data not shown). Morizane et al. (2001) also showed that there were similar mortality patterns at Japanese pearl oyster farms in the Ehime prefecture during 1998 and 1999 that were identical with the mortality pattern in our rearing experiment at the Shitaba farm. These results suggest that the mass mortality occurrences are strongly influenced by environmental factors common to high-mortality farms in which the mass mortality occurs as a regular annual event. Two possible environmental influences are water temperature and the presence of neighboring fish farms.

An average winter water temperature greater than 15°C, which enables the vigorous growth of cultured oysters, is a common condition in high-mortality farms. There is a striking difference in the average winter water temperature between the Shikamachi farm and the farms at Kamae, Shitaba, and Koshiki. At the Shikamachi farm, where win-

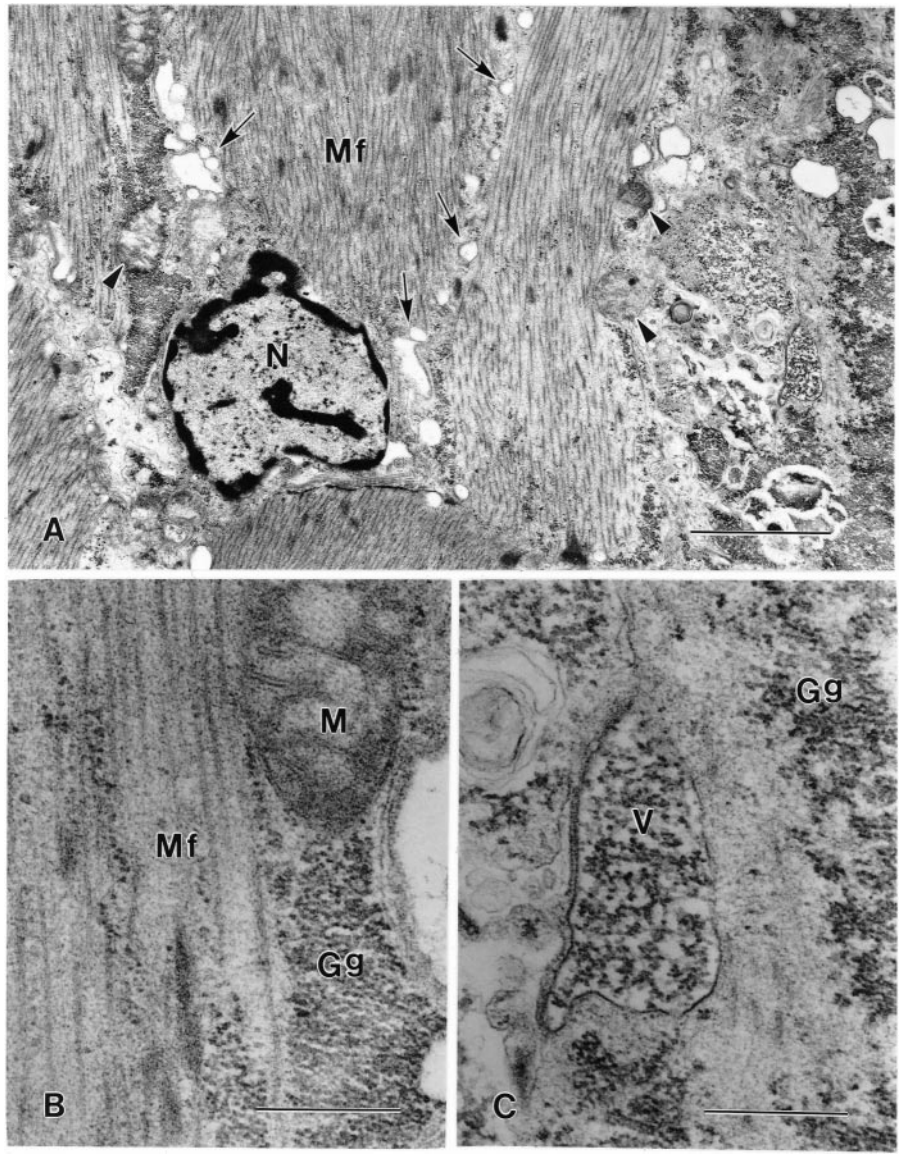

FIGURE 12.—**(A)** Electron micrograph showing muscle fibers of the adductor muscle (transparent part) of a healthy Japanese pearl oyster (arrow = sarcoplasmic reticulum, arrowheads = mitochondria, Mf = myofibril, and N = nucleus; scale bar = 2 μm). Panel **(B)** shows the upper left part of panel (A) at higher magnification (M = mitochondrion and Gg = glycogen granules; scale bar = 500 nm). Panel **(C)** shows the lower right part of panel (A) at higher magnification (V = vacuole containing glycogen granules; scale bar = 500 nm).

ter water temperature is usually low and summer water temperature is high, no mass mortality occurs; however, the cumulative mortality of experimental oysters there reached 28.5% because of repeated treatments involving the cutting of the byssus (for body measurements) and removal of sessile organisms on valves under unusually high water temperature in 1998 (Hirano et al. 2002). It is noteworthy that mass mortality events at the Shikamachi farm usually occurred in mother oysters that were cultured at high-mortality farms (e.g., Kamae and Shitaba) in winter and spring and delivered to the Shikamachi farm in spring. No mass mortality occurred in mother oysters delivered to the Shikamachi farm in autumn. The growth pattern of oysters reared at Shikamachi

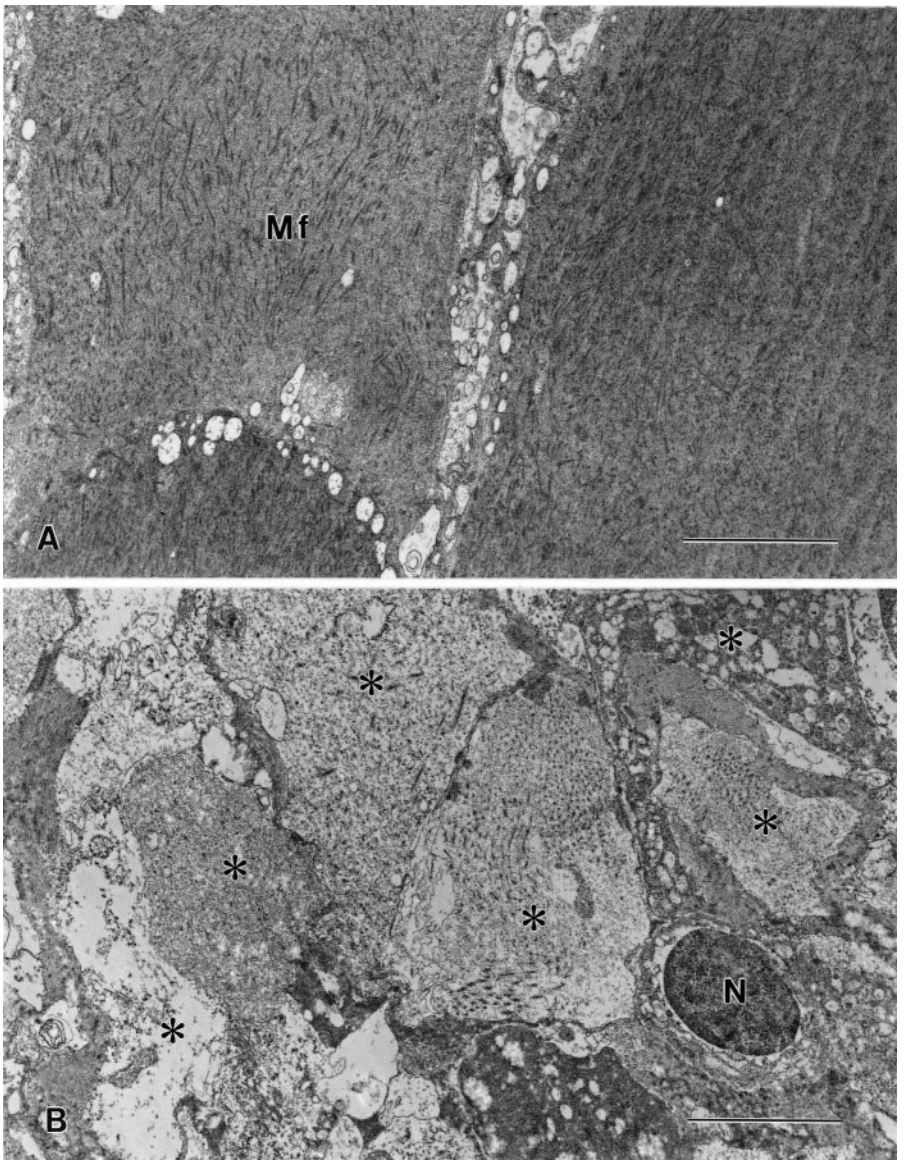

FIGURE 13.—Electron micrographs (scale bars = 2 μm) showing **(A)** condensed muscle fibers of the adductor muscle (transparent part) of a Japanese pearl oyster at an advanced stage of disease (Mf = myofibril) and **(B)** a necrotic focus in the adductor muscle (transparent part) of an oyster at an advanced stage of disease (asterisks indicate necrotic muscle fibers, some of which showed liquefactive change; N = nucleus).

suggests that low water temperature in winter shifts the growth period forward and shortens the critical period of this disease, resulting in comparatively low mortality. Thus, temperature has an indirect effect on the occurrence of mass mortality. On the other hand, cultured oysters grew vigorously in winter and spring at the Kamae and Shitaba farms but became debilitated about 2 or 3 months earlier than those at Shikamachi and subsequently experienced high mortality. The present study revealed remarkable lesions that spread from the digestive organ to various other organs in diseased oysters. These pathological changes occurred earlier and the degree of pathological changes was much higher at the Kamae and Shitaba farms than at the Shikamachi farm. It is therefore possible that the mass mortality may be associated with the food and feeding of cultured oys-

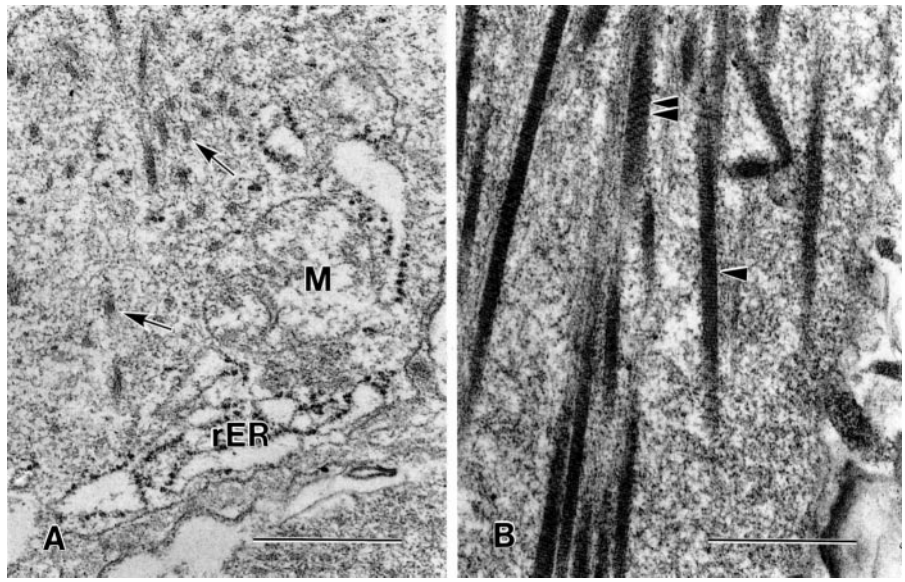

FIGURE 14.—Electron micrographs (scale bars = 500 nm) showing **(A)** a muscle fiber of the adductor muscle (transparent part) of a moribund Japanese pearl oyster (arrows indicate thick filaments, rER = rough endoplasmic reticulum, and M = mitochondrion) and **(B)** a muscle fiber of the adductor muscle (opaque part) of a moribund oyster (the arrowhead and double arrowhead indicate the transverse and diagonal banding of thick filaments, respectively).

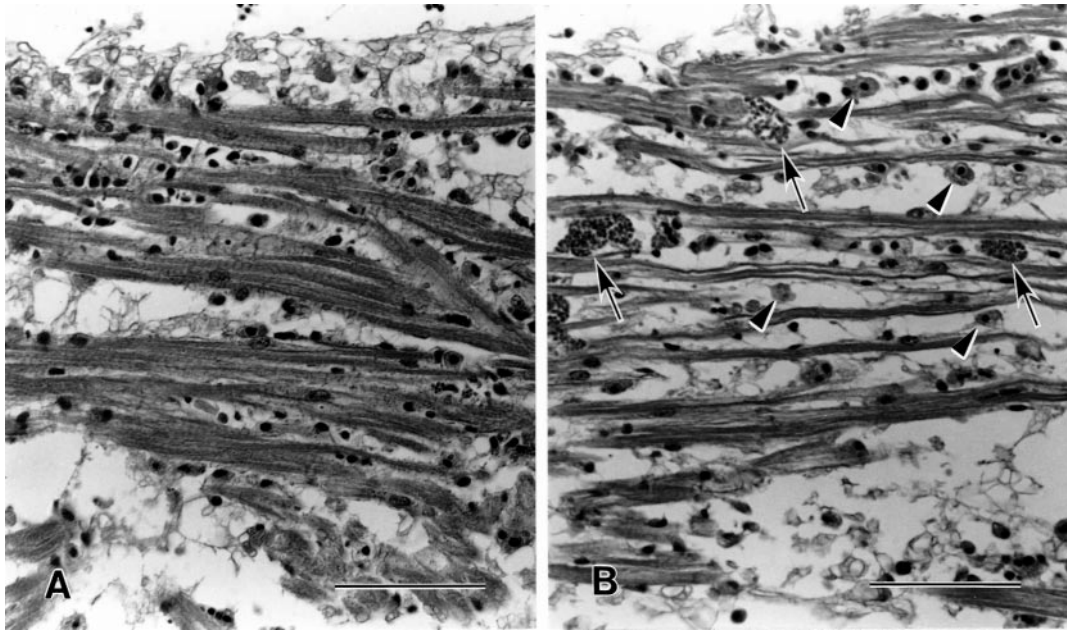

FIGURE 15.—Light micrographs (scale bars = 50 μm) showing the cardiac muscles of **(A)** a healthy Japanese pearl oyster in which myofibrils are thick and the striation is clearly visible and **(B)** an oyster at an advanced stage of disease (the arrows and arrowheads indicate infiltrated granular and agranular cells, respectively).

ters rather than with water temperature itself. Average winter and summer water temperatures did not have a direct effect on mass mortality because water temperature in affected farms was not appreciably different before versus after the occurrence of mass mortality, as clearly shown in the case study of the Koshiki farm.

Another suspected factor is the influence of neighboring fish farms. At Kamae, pearl oyster farms are surrounded by fish farms. At Koshiki Island, a fish farm is located at the mouth of the small bay where pearl oyster farming has been practiced (Figure 16). Water pollution around fish farms has been a serious problem in coastal areas of southern and western Japan. Several reports have shown that organic wastes discharged from fish farms cause the deoxygenation of the surrounding waters and considerable changes in the sediment chemistry and macrofauna (Hirata et al. 1994; Tsutsumi 1995; Yokoyama 2002). If the tidal flow, currents, and the scale and locations of fish farms are taken into consideration, it is likely that most high-mortality oyster farms are influenced by organic pollution discharged by neighboring fish farms. In this regard, the case study of the Koshiki farm is intriguing. Because of the great difficulty involved with eliminating fish farms that reside next to Japanese pearl oyster farms, this is the only case that we are aware of in which a high-mortality oyster farm experienced perfect recovery from mass mortality problems. However, according to personal communications with pearl oyster culturists, there have been some cases of successful pearl production in the Nagasaki prefecture after fish farming was withdrawn for various reasons.

Tomaru et al. (2001) reported that cultured Japanese pearl oysters in the Uwa Sea, Ehime prefecture, were weakened by starvation created by the dominance of inedible food, and these oysters then contracted an infectious disease that resulted in mortality. According to pearl oyster culturists (Z. Yamamoto, Yamamoto Shinju Corp., Oita prefecture, personal communication), starvation of cultured oysters has never occurred at the Kamae farm. Numaguchi (1995) reported that Japanese pearl oysters could survive without feed for more than 2 months under natural water temperatures (June–July). It is clear, therefore, that starvation is not a necessary condition for the occurrence of mass mortality.

Others have suggested that annual mass mortality events observed at Japanese pearl oyster farms since 1993 have an infectious etiology (e.g., Kurokawa et al. 1999; Muroga et al. 1999), but

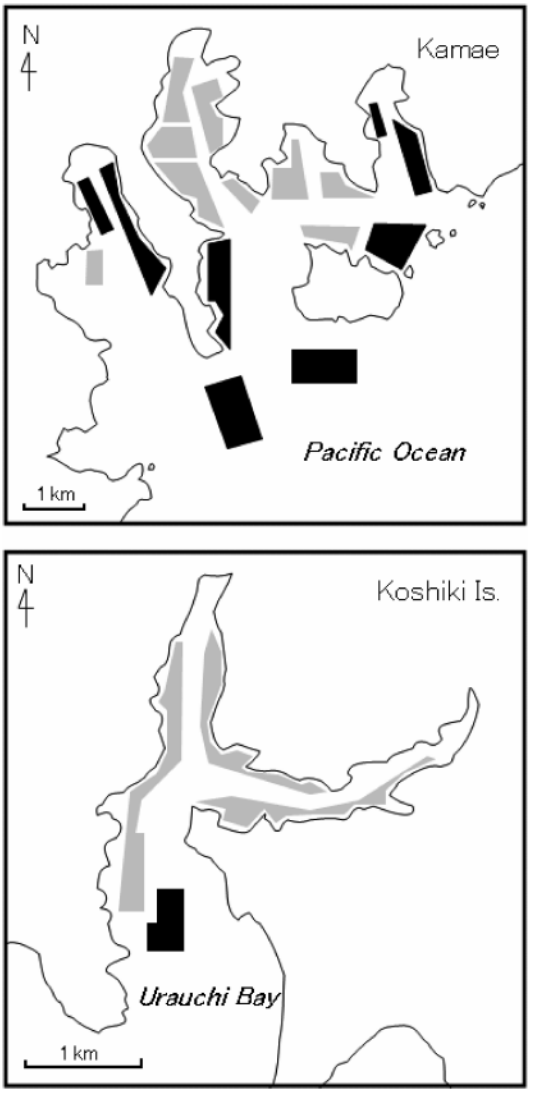

FIGURE 16.—Maps showing the locations of Japanese pearl oyster farms (gray areas) and fish farms (black areas) at Kamae and Koshiki Island, Japan.

our previous study showed that the mass mortality in pearl oyster farms is not consistent with an infectious disease etiology (Hirano et al. 2002). Our present sequential histopathological study has demonstrated the absence of viral, bacterial, parasitic, or mycotic agents in the various organs of diseased and healthy oysters. Our evaluations and analyses suggest that a more likely explanation is the environmental (ecological) impact of organic loading associated with neighboring fish farms. Studies to determine the cellular injury factors that

potentially exist in high-mortality farms and cause mass mortality are now in progress.

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