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Control of Disease in Aquaculture

Proceedings of the Nineteenth U.S.–Japan Meeting on Aquaculture Ise, Mie Prefecture, Japan 29–30 October 1990

Ralph S. Svrjcek (editor)

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Ise, Mie Prefecture, Japan
29–30 October 1990
Satellite Symposium: 2 November

Ralph S. Svrjcek
Publications Unit
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PREFACE

The United States and Japanese counterpart panels on aquaculture were formed in 1969 under the United States–Japan Cooperative Program in Natural Resources (UJNR). The panels currently include specialists drawn from the federal departments most concerned with aquaculture. Charged with exploring and developing bilateral cooperation, the panels have focused their efforts on exchanging information related to aquaculture which could be of benefit to both countries.

The UJNR was begun during the Third Cabinet-Level Meeting of the Joint United States-Japan Committee on Trade and Economic Affairs in January 1964. In addition to aquaculture, current subjects in the program include desalination of seawater, toxic microorganisms, air pollution, energy, forage crops, national park management, mycoplasmosis, wind and seismic effects, protein resources, forestry, and several joint panels and committees in marine resources research, development, and utilization.

Accomplishments include increased communication and cooperation among technical specialists; exchanges of information, data, and research findings; annual meetings of the panels, a policy-coordinative body; administrative staff meetings; exchanges of equipment, materials, and samples; several major technical conferences; and beneficial effects on international relations.

Conrad Mahnken–United States
Seiji Sakaguchi–Japan

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Studies on Diseases of Cultured Juvenile Atlantic Halibut

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ABSTRACT

Bacterial infections by *Flexibacter* and *Vibrio* species are major causes of mortalities in Atlantic halibut (*Hippoglossus hippoglossus* L.) larviculture. Egg surface disinfection is a possible prophylactic treatment. This article summarizes and reviews several experiments concerning causes of mortality of Atlantic halibut eggs and larvae.

Introduction

Cultivation of Atlantic halibut (*Hippoglossus hippoglossus* L.) is presently at the verge of a commercial breakthrough in Norway. However, as is the case with all cultivated species, there are problems emerging concerning diseases related to opportunistic microorganisms (Bergh et al. 1992; Bergh and Jelmert 1990; Opstad and Bergh 1990; Pittman et al. 1990). The purpose of this work is to summarize several experiments by studying the effects of microorganisms on mortalities of halibut eggs and yolk sac larvae, possible prophylactic treatment procedures, and effects of some physical stressors.

Methods and Materials

Eggs from one female were artificially stripped and fertilized with sperm from two males and reared in 250-L upstream incubators between 6 and 7° C until further processing.

The eggs were transferred to polystyrene multiwell dishes (NUNC, Roskilde, Denmark) for the disinfection trials and the infection experiment. Each well contained one egg and 11 mL of sterilized seawater. The dishes were incubated in darkness between 5 and 6° C for the duration of the experiment.

For the infection experiment, eggs were divided into 6 groups, each containing 60 eggs. Four days before hatching, each well of each of the 5 treatment groups was infected with 200 μL of a suspension of an accenic culture of one of the following bacteria: *Flexibacter* sp. strains NCIMB 13128 and NCIMB 13127^T, (National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland) which were isolated from two different groups of halibut eggs and otherwise seemingly identical (Hansen and Bergh et al. 1992), *Vibrio* strain HI-10448 (Institute of Marine Research, Bergen, Norway) and *Vibrio anguillarum* NCMB 6 (National Collection of Marine Bacteria, Aberdeen, Scotland); and *Vibrio fischeri* strain ATCC 7744 (American Type Culture Collection, Rockville, MD). Final total counts of bacteria in the wells were measured by staining with DAPI (Porter and Feig 1980) and counting in a Nikon epiflourescence microscope at 600× to be in the order of 2–3 × 10^6 bacteria x mL^{-1}. One group of 60 eggs was not infected, serving as the control. Within 24 hours after hatching, visible remnants of the eggshell were removed along with 10 mL of the water, and 10 mL of sterile seawater were immediately added. Mortality was recorded until Day 37 after hatching. For a further description of the infection experiment, see Bergh et al. (1992).

For the disinfection experiment another egg group was disinfected one day before hatching. The following procedure was followed. Eggs were divided into four groups, which were exposed to different concentrations of the iodophor disinfectant Buffodine (Evans Vanodine, Preston, England): 0.5, 0.05, and 0.005%, plus one untreated control group. Applica-

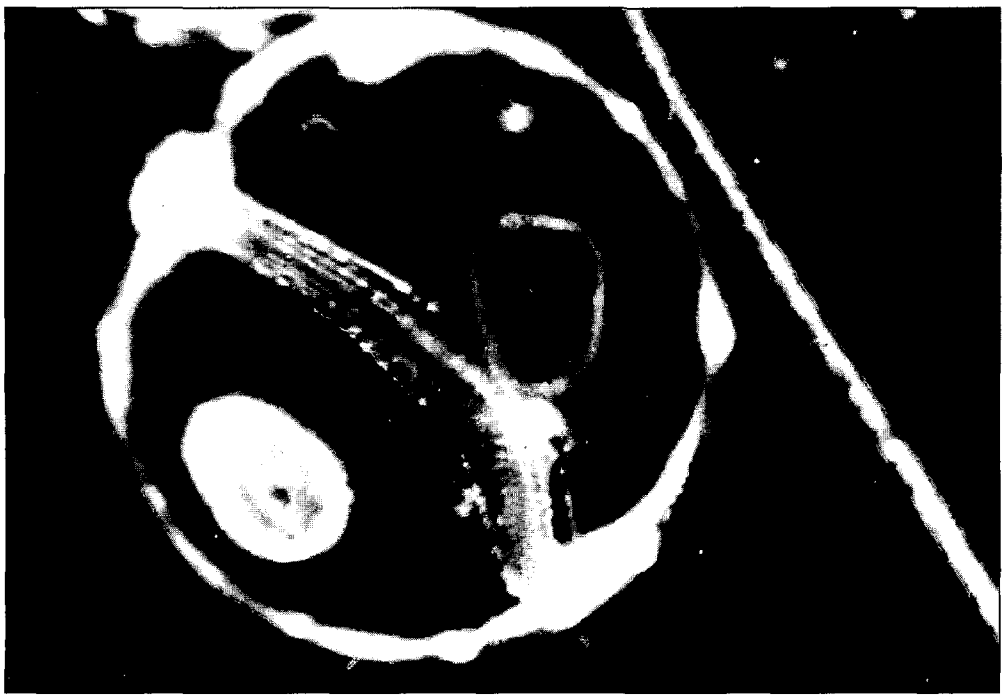

Figure 1
Halibut egg showing large surface wounds. This appearance is typical of eggs infected with *Flexibacter* sp. Egg diameter is approximately 3 mm. The photograph was taken from a Wild zoom binocular microscope operated in the dark field mode. Photo by Guri Grung and Vibeke Valkner.

tion time was 10 minutes. Immediately following the disinfection, the solution was carefully pipetted off the eggs, and more sterile seawater was added. This procedure was repeated three times. The control group was washed the same way as the other groups. Thereafter, 60 randomly chosen eggs from each group were incubated in polystyrene multiwell dishes as previously described. Within 24 hours after hatching, visible remnants of the eggshell were removed along with 10 mL of water, and 10 mL of sterile seawater was added. Mortality was recorded until Day 37 after hatching. The remaining living larvae were examined microscopically under a dissection microscope for developmental disorders. A further description of this experiment is given by Bergh and Jelmert (1990).

Results

Figure 1 shows a halibut egg with severe surface damage, an appearance typical for eggs infected with *Flexibacter* sp. Figure 2 demonstrates a normal egg, without visible damage.

Scanning electron microscopy of infected eggs revealed the chorion to be completely dissolved over large areas (up to 200 μm in diameter), whereas the zona radiata was severely damaged. Isolation of epibiotic bacteria from this egg group revealed an epiflora totally dominated (99% of colony-forming units) by *Flexibacter* sp. (Bergh et al. 1992)

The infection experiment revealed three different types of mortality patterns:

1 The uninfected control group showed very low mortality throughout the experiment, as only 5 out of 60 larvae died.

2 The two groups infected with *Flexibacter* sp. showed high mortalities at hatching; out of 60 larvae per group, 40 and 49 had died in the NCIMB 13127^T and NCIMB 13128 groups, respectively. At Day 18, these groups were terminated in order to gain material for re-isolation of bacteria. Eighty and 93% of the larvae were dead in Groups NCIMB 13127^T and NCIMB 13128, respectively.

3 The groups infected with *V. anguillarum* strains or with *V. fischeri* showed an intermediate mortality pattern. Only 1–4 larvae died per group at hatching, but high mortalities occurred throughout the rest of the experiment. When the experiment was terminated at Day 37, mortalities had risen to 95% in the *V. anguillarum* NCMB 6 group, 78% in the *V. fischeri* ATCC 7744 group, and 67% in the group infected with *V. anguillarum* 651.

In the disinfection experiment, 7 out of 56 remaining larvae in the group treated with 0.5% Buffodine were dead when the experiment was terminated, 9 out of 59 were dead in the 0.05% Buffodine group, 24 out of 59 were dead in the 0.005% Buffodine group, and 19 out of 58 in the untreated group.

Figure 2
A normal halibut egg. Diameter is approximately 3 mm. The photograph is taken from a Wild zoom binocular microscope operated in the dark field mode. Photo by Guri Grung and Vibeke Valkner.

The groups that were disinfected with 0.5% and 0.05% Buffodine could not be significantly distinguished for cumulative mortality at the end of the experiment ($P>0.05$, *t*-test with arcus sinus transformation of proportions). The two remaining groups were not statistically separable, but they both had significantly higher mortalities than the two groups that were treated with the highest Buffodine concentrations ($P<0.05\%$, *t*-test with arcus sinus transformation of proportions).

With respect to developmental disorders, 5 out of 49 living larvae treated with 0.5% Buffodine were found to posess at least one kind of disorder when the experiment was terminated in the group. In the other groups, scores were 16 out of 50 (0.05% Buffodine), 22 out of 36 (0.005% Buffodine) and 23 out of 39 (control). The most common disorder was the presence of black, probably necrotic, tissue in the gill, heart, or frontal yolk-sac region. Work is in progress to characterize the ultrastructural changes associated with these kinds of disorders.

Discussion

We have presented initial results showing that bacterial infections may be closely involved in the mortality of cultured halibut eggs and yolk-sac larvae. The infection experiment proves that *Flexibacter* sp. is a causative agent of mortality at the egg stage, at hatching, and at the early yolk-sac stage. It has earlier been shown that bacteria are able to cause at least minor destruction to the chorion (Hansen and Olafsen 1989), but we are not aware of other reports showing that bacteria are able to dissolve the chorion completely. It could not be deduced from the scanning electron micrographs that the zona radiata was completely destroyed; however, evidence of severe destruction was clear. This biotype is commonly isolated from Atlantic halibut eggs and could be considered a major problem in halibut larviculture.

With the two *Vibrio* species that were used in the infection experiment, the situation is different. These bacteria seemed to be harmless to the egg stage, causing no significant mortality. However, the profound mortality of the infected groups throughout the yolk-sac stage indicates that *Vibrio*-infections during the yolk-sac stage may help to explain the high mortality rates experienced so far.

Recent observations indicate that infectious pancreatic necrosis virus (IPNV), serotype N1, is prevalent in juvenile stages of the Atlantic halibut (Mortensen et al. 1990). The IPNV is associated with high mortalities, but it is not verified whether the virus is the principal lethal agent. The time of infection has so far not been shown. Also, nematodes could be present in relatively large amounts (G. Bristow, Zoological Laboratory, University of Bergen, pers. commun. 1991); these are probably introduced by feeding the larvae with collected zooplankton.

The disinfection experiment demonstrated that the bacterial epiflora on halibut eggs is at least partly

responsible for many of the developmental disorders commonly occuring in halibut yolk-sac larvae. Surface disinfection of eggs with iodophors should be an adequate prophylactic treatment, as they have good pathogen/host differential of toxicity (Amend and Pietsch 1972; Ross and Smith 1972; Amend 1974). However, more work is needed to establish reliable disinfection procedures.

Mortality rates could be augmented by several sublethal factors (Rosenthal and Alderdice 1976). Sublethal physical stressors might increase egg and larvae sensitivity to infectious microorganisms, rather than *per se* be the causative agent of death. The effects of sublethal stressors to early life stages of halibut have been investigated in several studies.

Jelmert and Naas (1990) reported that lowered O_2 concentrations, exposure to H_2S and exposure to high light levels led to a higher prevalence of deformed yolk-sac larvae.

Sensitivity of halibut eggs to physical shocks was investigated by Holmefjord and Bolla (1988), who found that eggs were most sensitive before the closure of the blastopore. In a more extensive study, eggs of halibut were compared with several other marine fishes, Opstad (I. Opstad, Austevoll Aquaculture Research Station, pers. commun. 1991) found similar results, with the addition that eggs during the hatching period were highly sensitive to physical stress. Effects of water flow on yolk-sac larvae were studied by Opstad and Bergh (1990), who concluded that high rates of water exchange in upstream incubators significantly increased mortality. Yolk-sac utilization was inversely related to rate of flow.

Absence of flow, however, caused rapid increase in the amount of bacteria in the incubators (Opstad and Bergh 1990; Skiftesvik et al. 1990), and subsequent larval mortality. Thus, these two effects must be carefully weighed against each other. Although normally not regarded as a sublethal stressor or causative agent of diseases, extreme light regimes have been shown to induce reduced yolk-sac utilization and increased mortality of halibut yolk-sac larvae (Skiftesvik et al. 1990).

Studying development and mortality of Atlantic halibut eggs and larvae at different temperatures, Pittman et al. (1990) concluded that 3° C is near the lower limit for development of halibut eggs and larvae. At this temperature, the larvae often showed incomplete caudal development and suffered higher mortality than those reared at 6° C. The groups reared at 9° C had high egg mortality and quickly developed abnormalities, such as small hearts and livers, and large peritoneal and pericardial spaces, indicating that this temperature was sublethal. No primary cause of larval death could be identified, although four critical periods of high mortality were identified for aquaculture systems at temperatures between 3 and 9° C: hatching, 10–14, 25–35, and 45–60 days after hatching. The latter was probably due to starvation (I. Opstad and A.B. Skiftesvik, Austevoll Aquaculture Research Station, pers. commun. 1991). For the three other critical periods of the yolk-sac stage, evidence presented here indicates that effects of bacteria could not be ruled out.

There are not yet any data available ranking the quantitative importance of the different causes of death of Atlantic halibut eggs and larvae, although early-life stage mortalities are still a major factor limiting the commercial success of halibut aquaculture. The data presented here, however, give evidence that pathogenic or opportunistic microorganisms are closely involved in some typical mortality and developmental disorder patterns of eggs and yolk-sac larvae.

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Scoliosis of Fishes Caused by Tryptophan Deficiency

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ABSTRACT

Scoliosis, caused by dietary tryptophan (Trp) deficiency, has been reported mainly in salmonids. Neither abnormality in the vertebra *per se* nor microscopically visible damage in the surrounding tissues was detected in the scoliotic fish, most of which returned to normal shape within a short period of time after restoration of Trp to the diet. Therefore, serotonin (5-HT), which is one of the Trp metabolites and a known neurotransmitter, was suspected as a key substance responsible for the symptom. This paper reviews several feeding studies where purified diets containing various combinations of L-Trp, 5-hydroxy-L- tryptophan (5-HTP, direct precursor of 5-HT), MK486 (local inhibitor of 5-HT synthesis only in periphery) and DL-*p*-chlorophenylalanine (PCPA, general inhibitor of 5-HT synthesis) were fed to chum salmon fry (*Oncorhynchus keta*). The findings indicate that occurrence of the spinal deformity is related to depletion of 5-HT in the central nervous system. In addition, the relationship between water temperature during rearing period and incidence of the scoliosis is also discussed.

Introduction

Although it is known that 10 amino acids are essential for normal fish growth, all of the quantitative requirements for essential amino acids have been determined only for chinook salmon, *Oncorhynchus tschawytscha,* coho salmon, *O. kisutsch,* carp, *Cyprinus carpio,* channel catfish, *Ictalurus punctatus,* Japanese eel, *Anguilla japonica,* and Nile tilapia, *Oreochromis niloticus.* The author conducted a series of dietary studies to determine the amino acid requirements for the fry of chum salmon, *O. keta,* which is one of the most important species in the salmon enhancement project in Japan. In these experiments, spinal deformity was observed in many of the fish fed a tryptophan(Trp)-deficient diet (Fig.1; Akiyama et al. 1985). Since the abnormality was first attributed to Trp deficiency in sockeye salmon by Halver and Shanks (1960), the same deficiency symptom has been reported in rainbow trout (Shanks et al. 1962; Kitamura 1969; Kloppel and Post 1975; Poston and Rumsey 1983; Walton et al. 1984) and coho salmon (Ogata and Arai 1981). So far, the biochemical pathway resulting in the occurrence of spinal deformity due to Trp deficiency has not been elucidated.

Characteristics of Spinal Deformity

The spinal deformity caused by Trp deficiency is mainly scoliotic or slightly lordoscoliotic, and neither lordosis or kyphosis has been noted. Scoliosis occurs after 1–2 weeks of feeding a Trp-deficient diet in rainbow trout (Kitamura 1969; Kloppel and Post 1975) and chum salmon (Akiyama et al. 1986a). Most scoliotic fish return to normal shape after restoration of Trp to their diet; therefore, this symptom is reversible. However, most spinal deformities caused by nutrient deficiency are not reversed even by restoring nutrients to their optimum level in the diet. For example, ascorbic acid-deficient fish form thermally labile underhydroxylated collagen which is denatured and digested at higher temperatures; this results in connective tissues with a low collagen content and in the development of a fragile bone structure, which finally results in irreversible symptoms of scurvy, such as lordosis and scoliosis (Sato et al. 1983; Ikeda et al. 1983). Although Kloppel and Post (1975) observed some minor abnormalities such as protrusions of the fibrous matrix sheath investing the notochord of scoliotic rainbow trout that were caused by Trp deficiency, serious lesions of the vertebrae and microscopically visible damage in the

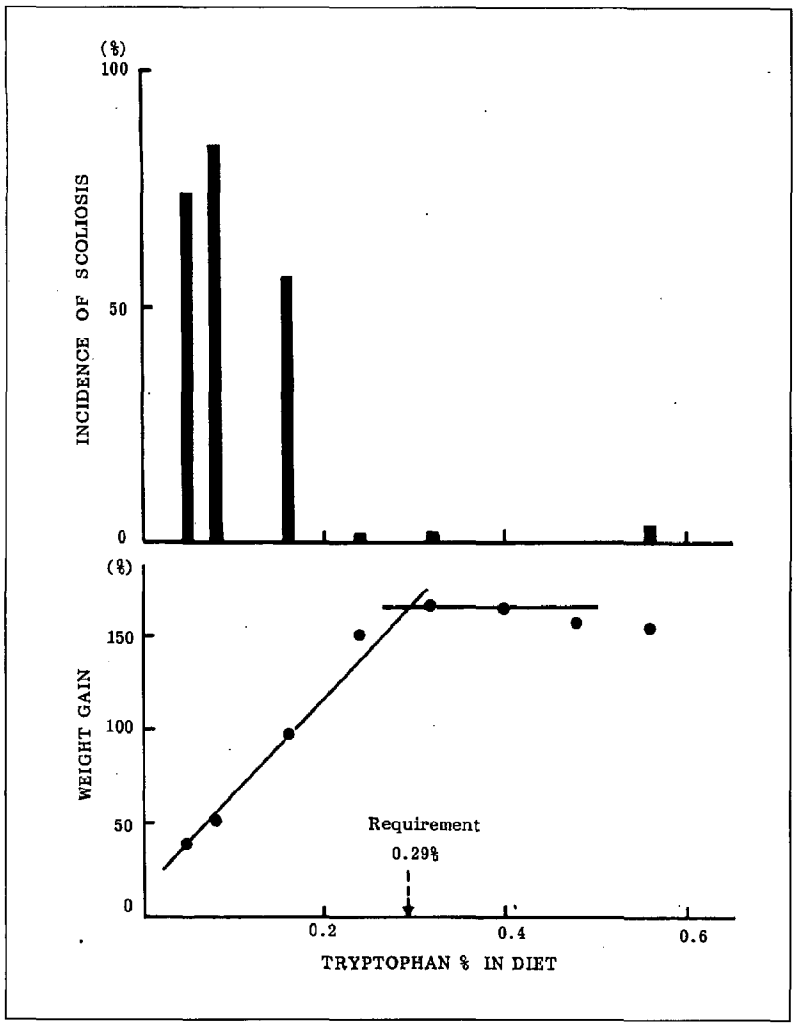

Figure 1
Relationships between tryptophan level in diet and weight gain or incidence of scoliosis; average value of duplicate tanks of 35 fish, each group fed for 4 weeks at 16.0° C (Akiyama et al. 1985).

surrounding tissues have not been recognized in rainbow trout (Kitamura 1969) and chum salmon (Akiyama et al. 1986b).

Construction of Hypothesis

Trptophan is not only an essential structural element of protein but also the precursor of nicotinamide adenine dinucleotide (NAD) and niacin in higher vertebrates (Fig.2). Therefore, attention was focused on the role of dietary niacin in the early studies of Trp metabolism. Poston and Combs (1980), however, reported that dietary Trp is not an efficient precursor of niacin in salmonids. Moreover, Poston and Rumsey (1983) showed that the deletion of dietary niacin did not significantly increase the incidence of scoliosis in rainbow trout fed a diet containing a low level of Trp.

It is possible that the symptom of scoliosis may be induced by an abnormal and involuntary contraction or relaxation of muscle due to a defect in the nervous system. Many reports on spinal deformity caused by a metabolic disfunction or lesion in the nervous system are available. In mammals, scoliosis develops in bipedal rats with brain-stem lesions (Tamura 1974) and in rabbits whose dorsal root in the spinal cord was removed (MacEwen 1973). In fish, yellowtail parasitized by cysts of *Myxobolus* in the 4th ventricle of the brain (Egusa 1985; Sakaguchi et al. 1987) showed scoliosis. Vertebral deformity was reported to occur in yellowtail whose brain was infected by beta-hemolytic streptococcal bacterium (Shiomitsu 1982; Kaige et al. 1984). Spinal deformities caused by an abnormality in the peripheral nervous system are well known in fishes exposed to pesticides such as diazinon, which develop severe spinal curvature together with fracturing (Hirose and Kitsukawa 1976; Hirose et al. 1979). It is speculated that these symptoms are induced by excess accumulation of acetylcholine in the neuromuscular junction. Thus, abnormality in the nervous system is one of the most important factors for occurrence of spinal deformity.

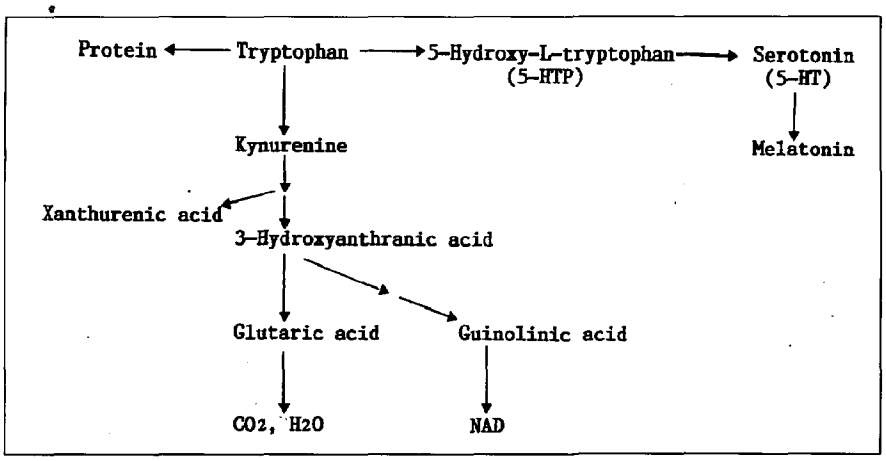

Figure 2
Map of tryptophan metabolism.

Among the various Trp metabolites, serotonin (5-HT) is known to function as a brain neurotransmitter or modulator and is involved in the regulation of sleep, body heat, sexual behavior, appetite, pain recognition, secretion of growth hormone and prolactin, besides classical functions such as the contraction of smooth muscle of blood vessels, the uterus, and the digestive tract. In addition, it is known that torticollis and abnormal posture can be induced by the destruction of rat midbrain in which both serotoninergic and dopaminergic neurons are located (Tanaka and Kimura 1984), and that 5-HT modulates the central pattern generator for locomotion in the spinal cords of the lampreys *Ichthyomyzon unicuspis* and *Petromyzon marinus* (Harris-Warrich et al. 1985). These reports suggest that muscular function can be partly controlled by a serotoninergic neuron. From these facts, I hypothesized that scoliosis caused by Trp deficiency would be induced by an imbalance of muscular tension due to a decreased 5-HT level in the nervous system.

5-HT Involvement in the Occurrence of Scoliosis

In the first experiment which tested the involvement of 5-HT in the occurrence of scoliosis an oral administration of 5-HT to Trp-deficient chum salmon fry decreased the incidence of scoliosis, but did not completely inhibit its occurrence (Akiyama et al. 1986a). Therefore, we fed fry a Trp-deficient diet supplemented with 5-hydroxy-tryptophan (5-HTP, 100–130 mg/100 g diet), which is a direct precursor of 5-HT

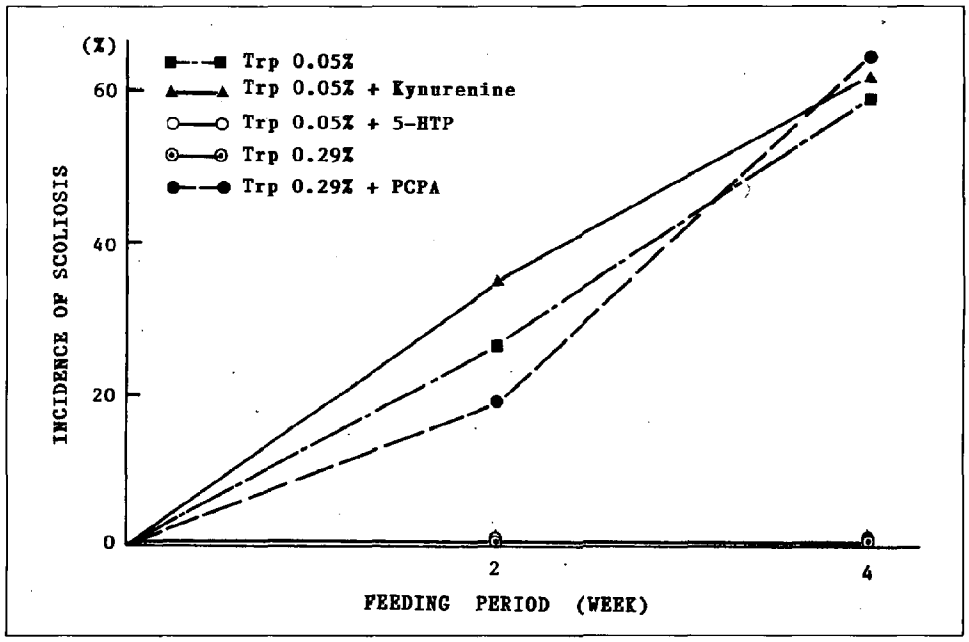

Figure 3
Effects of oral administration of tryptophan (Trp) metabolites to Trp-deficient or sufficient chum salmon fry. Administration of 5-hydroxy-L-tryptophan (5-HTP) to Trp-deficient fish completely prevented scoliosis, whereas kynurenine failed to inhibit the occurrence. DL-*p*-Chlorophenylalanine (PCPA) developed scoliosis even in the fish fed Trp-sufficient diet (Akiyama et al. 1986b).

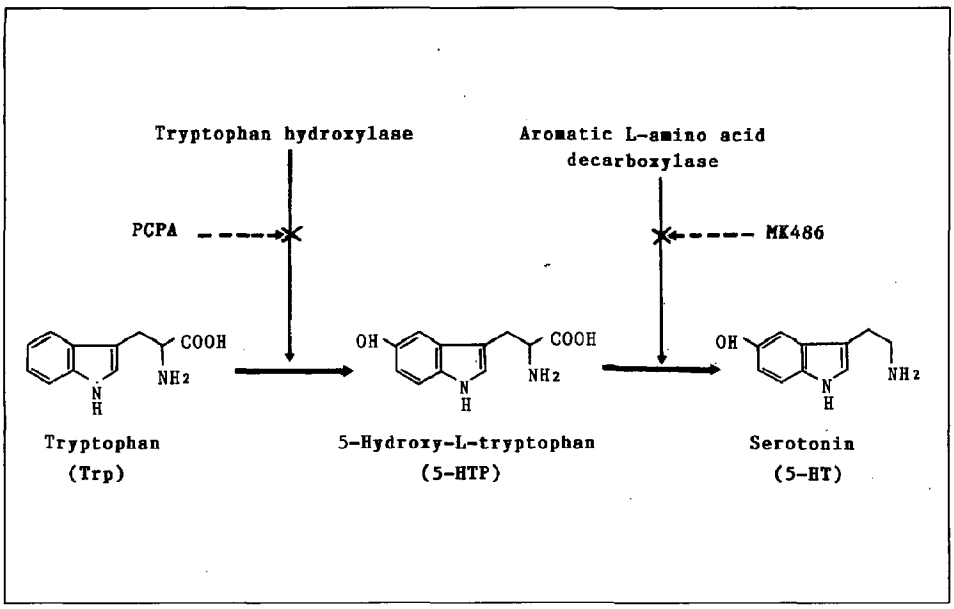

Figure 4
Functions of administrated drugs on serotonin (5-HT) pathway. DL-*p*-Chlorophenylalanine (PCPA) is an inhibitor of tryptophan-hydroxylase (TrpOHase) and inhibits a biosynthesis of 5-hydroxy-L-tryptophan (5-HTP) from Trp. L-2-hydrazino-alfa-methyl-beta-(3,4-dihydroxyphenylpropionic acid); (MK486) is an inhibitor of L-amino acid decarboxylase only in periphery and consequently induces a conversion of exogenous 5-HTP to 5-HT in central nervous system.

and can easily pass through a blood-brain barrier in contrast to 5-HT (Akiyama et al. 1986b, 1989). The treatment completely prevented the occurrence of scoliosis and increased the brain 5-HT level in Trp-deficient fish (Fig.3). Kynurenine, a precursor of niacin, NAD, and xanthurenic acid was fed at 120 mg (as L-kynurenine)/100 g diet to Trp-deficient fish, but failed to prevent scoliosis. Moreover, both scoliosis and decreased brain 5-HT levels were observed in fish fed a Trp-sufficient diet supplemented with DL-*p*-chlorophenylalanine (PCPA) (Akiyama et al. 1986a; 1986b), which is an inhibitor of tryptophan-hydroxylase (TrpOHase) and a potent depletor of both brain and peripheral stores of 5-HT (Fig. 4). TrpOHase is a rate-limiting enzyme on the 5-HT pathway. These findings indicated a relationship between the occurrence of scoliosis and 5-HT levels. The reduced ability of orally administrated 5-HT to inhibit the development of scoliosis compared with complete prevention with the use of 5-HTP suggested involvement of the serotoninergic neuron in the central nervous system. Therefore, the author prepared Trp-deficient diets (0.05%) containing various combinations of 5-HTP (10 or 50 mg/100 g diet) with or without L-2-hydrazino-alpha-methyl-beta-(3,4-dihydroxyphenylpropionic acid) (MK486, 1 or 5 mg/100 g diet). MK486 functions as an inhibitor of aromatic L-amino acid decarboxylase only in periphery and inhibits 5-HT biosynthesis from 5-HTP. Thus, it consequently functions to promote the conversion of exogenous 5-HTP to 5-HT in the central nervous system. Feeding a Trp-deficient diet supplemented with both 5-HTP and MK486 resulted in a significantly lower incidence of scoliosis and higher levels of brain 5-HT and 5-hydroxyindoleacetic acid than did adding 5-HTP alone (Akiyama and Murai, unpubl. data). The experiment suggests that the deficiency of 5-HT in the central nervous system was related to the occurrence of scoliosis.

Effect of Temperature on the Occurrence of Scoliosis

Scoliosis caused by Trp deficiency has been reported only in salmonids such as sockeye salmon, rainbow trout, coho salmon and chum salmon, although it has also been studied in chinook salmon (Halver et al. 1957), channel catfish (Dupree and Halver 1970), eel (Arai et al. 1972), carp (Nose et al. 1974; Nose 1979), red sea bream, *Pagrus major* (Yone 1976) and tilapia, *Tilapia zillii* (Mazid et al. 1978). At first I considered scoliosis to be a characteristic Trp deficiency symptom of all salmonids except chinook salmon. Arai et al. (1986), however, reported development of scoliosis in Trp-deficient Ayu fish (Plecoglossidae) reared at 16° C, although the incidence was low. I also found one scoliotic fish when a Trp-deficient diet was fed to yellowtail at 20° C (Akiyama, unpubl. data), even though the brain was not infected by *Streptococcus* and not parasitized by cysts of *Myxobolus*. These facts suggest that scoliosis due to Trp deficiency is unlikely to be peculiar to salmonids. Because salmonids are coldwater fish, and because most of the fishes in which scoliosis was not observed as a symptom of Trp deficiency are warmwater fish (except chinook salmon), I focused my attention on the influence of environmental temperature. It is

likely that among the fishes developing scoliosis, incidences of scoliosis decrease as the optimum temperature for each species rises. Moreover, spinal deformity has not been observed to be an external symptom of dietary Trp deficiency in mammals and birds, which are warm-blooded animals. In fact, the author presumed that the occurrence and incidence of scoliosis might be influenced by rearing temperatures, and therefore fed the Trp-deficient diet to chum salmon fry at three different temperatures: 10, 16, and 20° C. The experiment showed that as the rearing temperature was lowered, the incidence of scoliotic fish increased and brain 5-HT levels decreased (Akiyama and Murai, unpubl. data). It is still unknown why the brain 5-HT level in Trp-deficient fish varied under different temperature conditions.

Conclusion

All of these findings described above indicate that scoliosis caused by Trp deficiency is related to the level of 5-HT in the central nervous system. In fishes, hereafter, the central nervous system, especially the 5-HT neuron, should be considered as one of the important factors in an occurrence of idiopathic spinal deformity.

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Control of IHN Virus in Alaskan Sockeye Salmon Culture

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A recent review of trends in the prevalence and risk management of Infectious Hematopoietic Necrosis Virus (IHNV) in Alaskan sockeye salmon *Oncorhynchus nerka* culture has been reported by Meyers et al. (1990). The reader is referred to this report for further details, discussion, and references.

Prior to 1980, IHNV prevented successful culture of sockeye salmon in Alaska. This led the Fisheries Rehabilitation, Enhancement, and Development Division (FRED) of the Alaska Department of Fish and Game to develop a policy to control the negative effects of the virus in sockeye salmon culture. This policy included procedures for the collection and incubation of eggs and for the rearing of fry that were based upon the known and suspected biological characteristics of the virus-host relationship. Many of these criteria are common sense approaches such as: use of a virus-free water supply; disinfection of utensils, facilities, and external surfaces of broodfish; separate fertilization of eggs from each female using 1 or 2 males; separate water hardening of each family of eggs in a 100 ppm iodine disinfectant for 1 hour; compartmentalization of families into Kitoi Box incubators or into stacks of Nopad trays at egg densities of 250,000-300,000 (80–100 females), or into modified Bams Boxes used at one facility that are each loaded with 500,000 eggs; physical isolation of each sockeye stock and isolation of all sockeye stocks from any nonsockeye species; and release of fry unfed or after short-term rearing (4–6 weeks) with pooling of fry in raceways or start tanks according to the date of eggtake. These criteria nearly eliminate opportunities for horizontal virus transmission from the parents to offspring or from the water supply. They also further reduce the rare occurrence of vertical transmission of the virus within the egg and allow for compartmentalization of eggs and fry so that the occasional incubators or raceways of fish developing IHN can be destroyed and the virus contained to protect the remaining fish inventory. This "sockeye culture policy" has allowed Alaskan hatcheries a great measure of success in controlling IHNV at several different facilities around the state for nearly 10 years. Based on these guidelines, an average of 2–3 million sockeye salmon eggs can be spawned in a day and totals of 20–36 million eggs may be taken at certain facilities. Although vertical transmission of the virus generally occurs in fry almost every year at certain facilities, total losses are minimized to between 1 and 3% of the statewide fry production. In 1990 only 1% of the sockeye fry were destroyed owing to IHN of 68 million healthy fish that were released. Production data from various Alaskan sockeye salmon hatcheries suggest that vertical transmission of IHNV is more dependent upon the proportion of high virus-titered female fish rather than total virus prevalence. Also, as one would expect, the risk of vertical virus transmission increases with increasing numbers of eggs taken from females of a high-titered stock. Hence, IHN outbreaks are more common at the larger eggtake facilities that have greater prevalences of high-titered broodfish.

During the past 14 years, yearly monitoring of sockeye salmon stocks by FRED has resulted in a data base summarizing IHNV occurrence in over 96 wild and hatchery sockeye salmon stocks in Alaska. Yearly prevalence of IHNV ranges from 0 to 100% in both ripe and postspawned females with as many as 92% within a stock having high titers ($\geq 10^4$). Repeated yearly sampling has shown that all anadromous sockeye salmon tested in Alaska are positive for IHNV. The data base has been useful for examining general trends and has shown some differences from previously reported IHNV-sockeye salmon relationships. For example, no significant differences in mean IHNV prevalence were

found in ovarian fluids from postspawned female sockeye salmon vs. those from ripe females. Furthermore there were no significant differences between geometric mean virus titers of postspawned vs. ripe female fish, but postspawners did have a significantly greater mean proportion of high-titered fish. The log value of 10^4 was selected as the breakpoint for high virus titers owing to the tendency for bimodality of IHNV titers to occur at this level in most stocks of Alaskan sockeye salmon. The significance of this phenomenon needs further investigation. As found by other investigators, the mean virus prevalence in male fish from 27 stocks of sockeye salmon was significantly less (9%) than in female fish (40.1%).

This data base is a useful tool for examining general trends for IHNV within a geographic area or statewide. However, these trends may not always be true for certain individual sockeye salmon stocks that may be unique due to genetic reasons, the strain of the indigenous virus, or environmental factors affecting natural virus exposure and transmission.

Citation

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Identification of a Conserved Antigenic Domain in the Major Capsid Protein of Infectious Pancreatic Necrosis Virus

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ABSTRACT

The gene for the major capsid protein, VP2, of the Sp serotype of infectious pancreatic necrosis virus (IPNV) was cloned and expressed in *Escherichia coli*. Nonoverlapping fragments of the VP2 gene were recloned in *trpE* fusion vectors of the pATH series and the expressed fusion proteins were characterized for reactivity with antisera to three different serotypes of IPNV. One clone, pB10, which contained an insert encoding amino acids 99 to 206 of the VP2 protein, produced a fusion protein recognized by antisera for all three serotypes. In contrast, the pA43 clone, which contained an adjacent region on the VP2 gene encoding for amino acids 207 to 315, produced a fusion protein that was only recognized by homologous antisera in Western immunoblots. A comparison of the derived amino acid sequence for each clone with that reported for two other IPNV clones indicates that the pB10 region is conserved and the pA43 region is very heterogeneous.

Introduction

Infectious pancreatic necrosis virus (IPNV) is a birnavirus that causes one of the most serious diseases in trout and salmon farms in North America, Europe, and Asia. It can also kill a number of nonsalmonid fish species including striped bass (*Morone saxatilis*), turbot, menhaden, and eels (Wolf 1988), and it has been isolated from a variety of marine fish and molluscs. The ubiquitous nature of this birnavirus and its ability to infect such a wide variety of hosts make this virus important for scientific study. Most IPNV isolates are closely related antigenically and yet, they exhibit marked differences in host-range in vivo and in vitro, pathogenicity, and temperature of replication.

The most extensive study of the antigenic relationships of the aquatic birnaviruses compared 175 virus isolates from 44 fish and shellfish species from 11 countries by reciprocal plaque reduction tests using polyclonal sera (Hill and Way 1983). From these results, it was proposed that there are 2 major serogroups: 1 containing 9 serotypes which includes 171 isolates from fish, and the other containing 1 serotype which includes those viruses isolated from molluscs. These virus isolates also contain common immunoreactive determinant(s). Other studies have recognized only three major serotypes characterized by the following virus isolates: VR299, a North American strain; Sp, a European strain which is pathogenic for trout; and AB, a European strain which is nonpathogenic for trout (Wolf 1988). In our paper we review recent efforts to unravel the mechanisms that biologically distinguish the different IPNV isolates by characterizing the immunoreactive regions of the major capsid protein of the Sp serotype of IPNV.

Methods

The viral genome is composed of two double-stranded RNA segments, A and B. The B segment encodes the viral RNA polymerase, VP1. The A segment encodes the virion proteins, VP2 and VP3. The major capsid protein, VP2, is responsible for the induction of neutralizing antibodies (Lipipun et al. 1989). In addition, there is a nonstructural protein, NS, which is an autocatalytic protease responsible for cleavage of the polyprotein, VP2-NS-VP3, encoded by the viral genome A segment (Duncan et al. 1987; Manning et al. 1990).

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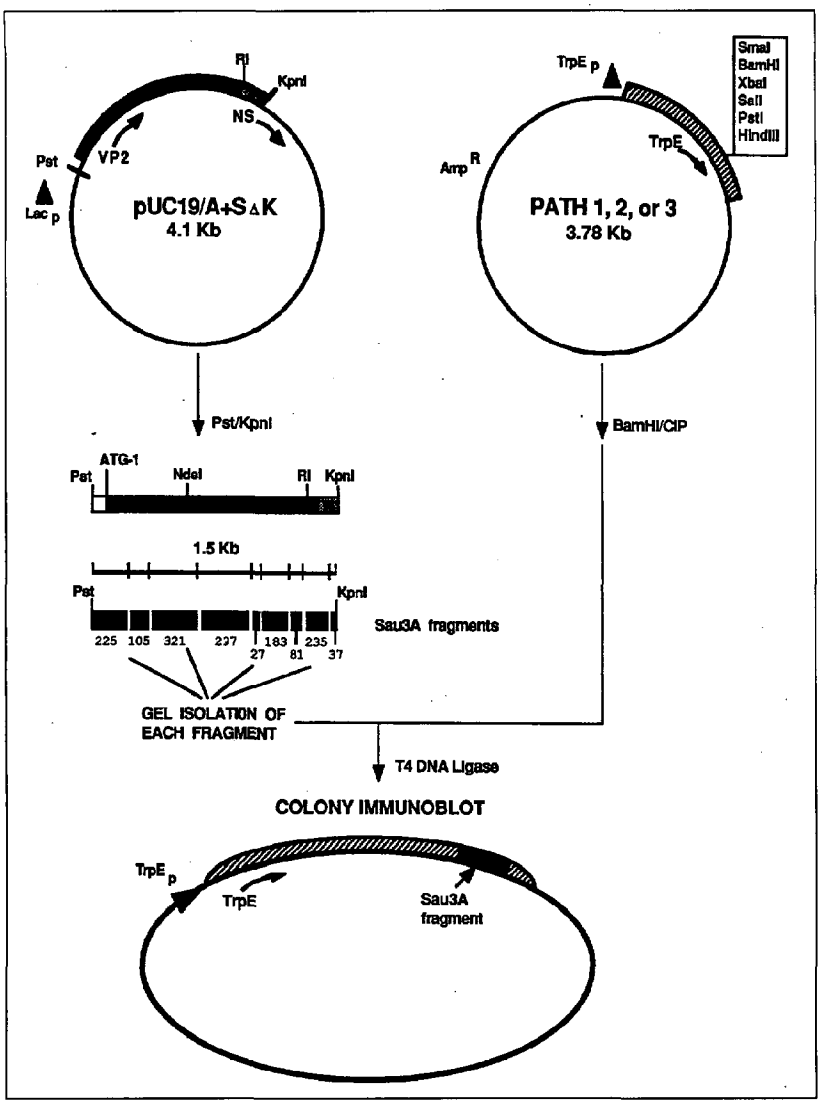

Figure 1
Construction of *trpE*-VP2 gene fusions. The 1.5 Kb cloned insert containing the IPNV-Sp VP2 gene was restricted with Sau3A1; the fragments were purified and subcloned into the *trpE* fusion expression vectors, pATH 1, 2, or 3. Recombinants containing fragments in the correct orientation and in the appropriate reading frame were selected by colony immunoblot as previously described (Gilmore et al. 1988).

Since the entire VP2 gene has been expressed in *Escherichia coli* as part of a *trpE* fusion protein (Manning and Leong 1990), it was possible to examine different regions of the VP2 gene for immunoreactivity with a panel of rabbit antisera and monoclonal antibodies to different serotypes of IPNV. The VP2 gene was excised from the plasmid pUC19/A+SΔK and cut with the restriction enzyme Sau3A1 which generated seven DNA fragments (Fig. 1). These fragments were inserted in-frame with the *trpE* gene in one of the three pATH vectors, pATH 1, pATH2, or pATH3, which put the resulting *trpE*-VP2 fusion gene under the control of the tryptophan operator and promoter (Dieckmann and Tzagaloff 1985). Recombinants expressing a portion of the VP2 gene were detected by direct colony immunoblot with anti-IPNV-Sp sera (Gilmore et al. 1988).

Results and Discussion

Two recombinant plasmids were identified from the clones derived from the pATH1 vector/insert ligation mixture, and the *trpE*-VP2 fusion proteins expressed by these plasmids were characterized by Western immunoblot analysis. The antisera used to detect the VP2-specific protein in the bacteria had been prepared against purified virions of IPNV-Sp. The recombinant plasmid, pB10, was found to produce a 47 kDa fusion protein and the recombinant plasmid, pA43, was found to produce a 52 kDa fusion protein (Fig. 2). The VP2 derivation of the IPNV sequence in the fusion proteins was verified by Western immunoblot using anti-VP2-specific antisera prepared against purified VP2 virion protein from the IPNV-Sp strain (Huang et al. 1986). The cell lysate prepared

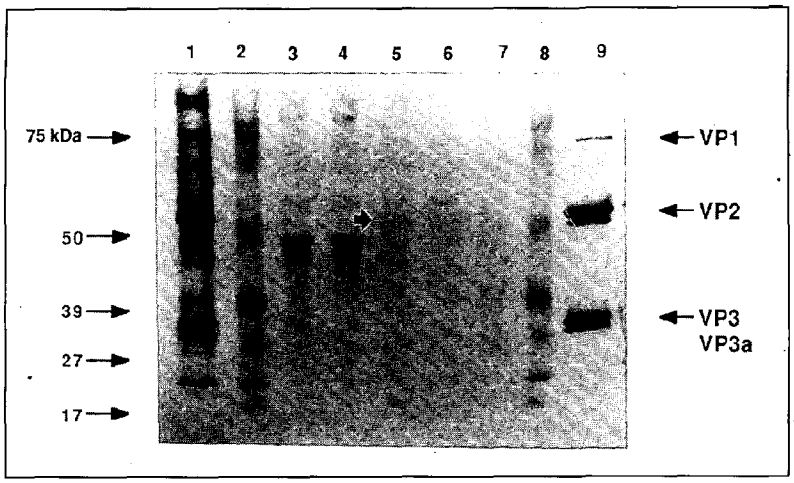

Figure 2
Immunoblot showing reactivity of *trpE*-VP2 fusion proteins with anti-IPNV-Sp sera. *E. coli* cells containing the recombinant plasmids (pB10 or pA43) were grown to mid-log phase before induction with 15 μg/ml indoleacrylic acid. The cultures were then grown to stationary phase before the cells were harvested by centrifugation for protein analysis. The cells were lysed and the proteins separated on an SDS-polyacrylamide gel as previously described (Gilmore et al. 1988). The proteins were transferred to nitrocellulose and then exposed to anti-IPNV-Sp antisera. Lane 1 contains IPNV-Buhl infected fish tissue culture cell lysate; lane 2, prestained low molecular weight markers from BioRad at 75 kDa, 50 kDa, 39 kDa, 27 kDa, and 17 kDa; lanes 3 and 4, pB10 induced bacterial cell lysate; lane 5, pA43 induced bacterial cell lysate; lane 6, bacterial cells containing the pATH vector with no insert; lane 7, bacterial cells without a plasmid; lane 8, low molecular weight markers; and lane 9, purified IPNV-Buhl. The arrow in lane 5 indicates the position of the *trpE*-VP2 fusion protein encoded by the recombinant plasmid, pA43. The computed molecular weight of the *trpE*-VP2 fusion protein was 56.5 kDa.

from induced cells containing pB10 or pA43 was found to contain protein bands that were strongly reactive with the anti-IPNV-Sp/VP2 sera (Fig. 3).

The DNA sequence of the viral insert in pB10 and in pA43 was determined after subcloning of the insert into the M13 sequencing vectors mp18 and mp19. Sequence analysis was performed by the modified Sanger dideoxy chain termination method (Davis et al. 1986). The pB10 insert comprised 323 nucleotides encoding 108 amino acids and mapped to amino acid number 99 to 206 of the VP2 protein of IPNV-Sp (Mason and Leong, unpubl. data). The pA43 insert comprised two neighboring Sau3A fragments of 297 and 27 nucleotides. This insert probably originated from a partial cleavage product (Fig. 1) and it was mapped to the adjacent region of the VP2 protein at amino acid number 207 to 315 of the VP2 protein. Although both pB10 and pA43 contained inserts encoding 108 amino acids, striking differences in the observed migration of the fusion proteins produced by each plasmid were noted. Careful analysis of the 3' terminal sequence of both plasmids by direct sequence analysis from the recombinant pATH fusion plasmids themselves (Wang et al. 1988) indicated that the translational termination codon, TAG, was present immediately after the end of both VP2 cDNA inserts. The calculated isoelectric points for both fusion proteins was 6.6, and there was

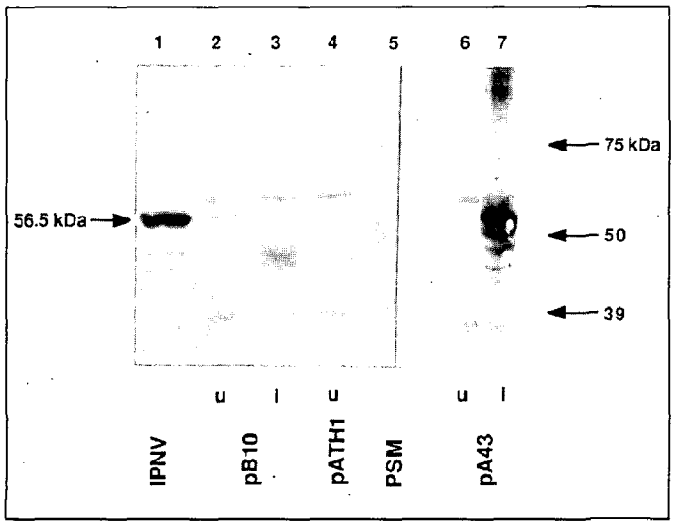

Figure 3
Immunoblot of *trpE*-VP2 fusion proteins with antisera to the VP2 protein of IPNV-Sp. *E. coli* cells containing the recombinant plasmid, pB10 or pA43, were grown and prepared as described in Figure 2. Lane 1 contains purified IPNV-Sp; lanes 2 and 3, lysates from uninduced and induced cells containing pB10; lane 4, lysate from uninduced cells containing the pATH1 vector without any VP2 insert; lane 5, prestained low molecular weight markers from BioRad at 75 kDa, 50 kDa, and 39 kDa; lanes 6 and 7, lysates from uninduced and induced cells containing pA43. The photograph taken for lanes 6 and 7 was a lighter exposure of the immunoblot and these lanes contained five times as much bacterial lysate as that used in lanes 2, 3, and 4.

```
pB10 insert

                                                                  *
SP  ISRKYDIQSSTLPAGLYALNGTLNAATFEGSLSEVESLTYNSLMSLTTNPQDKA
JA  ISRKYDIQSSTLPAGLYALNGTLNAATFEGSLSEVESLTYNSLMSLTTNPQDKV
N1  ISRKYDIQSSTLPAGLYALNGTLNAATFEGSLSEVESLTYNSLMSLTTNPQDKV

            *                        *     *
SP  NNQLVTKGVTVLNLPTGFDKPYVRLEDETPQGLQSMNGARMRCTAAIAPRRYEI
JA  NNQLVTKGITVLNLPTGFDKPYVRLEDETPQGPQSMNGARMRCTAAIAPRRYEI
N1  NNQLVTKGVTVLNLPTGFDKPYVRLEDETPQGLQSMNGAKMRCTAAIAPRRYEI

pA43 insert

        **  * *    *  *          *     * * *** *   *   *
SP  DLPSQSLPPVPATGTLTTLYEGNADIVNSTTVTGDINFSLAEQPANETRFDFQL
JA  DLPSERLPTVAATGTPTTIYEGNADIVNSTAVTGDITFQLEAEPVNETRFDFIL
N1  DLPSQRLPPVPATGTLTTLYEGNADIVNSTTVTGDINFSLAEQPANETKFDFQL

    ***   *       *  * * *     *   *       *          * **
SP  DLMGLDNDVPVVTVVSSVLATNDNYRGVSAKMTQSIPTENITKPITRVKLSYKI
JA  QFLGLDNDVPVVTVTSSTLVTADNYRGASAKFTQSIPTEMITKPITRVKLAYQL
N1  DFMGLDLDVPVVTVVSSVLATNDNYRGASAKMTQSIPTENITKPITRVKLSYKI
```

Figure 4
A comparison of the derived amino acid sequence of IPNV-Sp, IPNV- Jasper, and IPNV-N1 cDNA inserts present in pB10 and in pA43. The asterisks indicate differences in amino acids. The IPNV-Jasper (Ja) sequence was taken from Duncan and Dobos (1986). The IPNV-N1 sequence was taken from Havarstein et al. (1990).

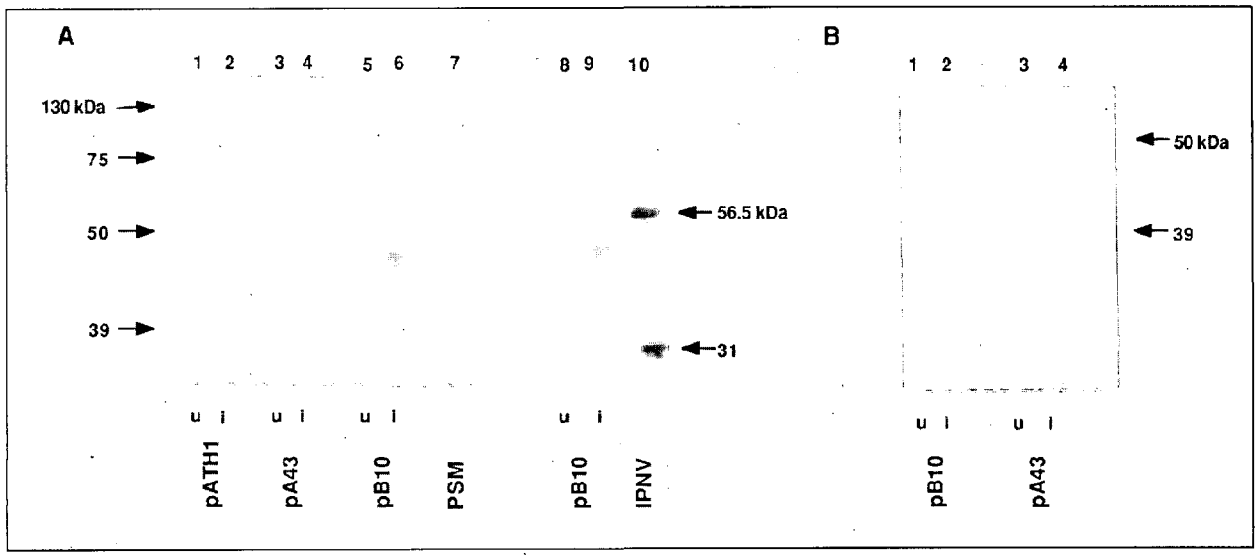

Figure 5
Immunoblots of *trpE*-VP2 fusion proteins with antisera to the heterologous IPNV strains, IPNV-Buhl and IPNV-EVE. *E. coli* cells containing *trpE*-VP2 fusion proteins encoded by the recombinant plasmids, pB10 or pA43, were analyzed for reactivity with antisera prepared to purified virus of the two heterologous IPNV strains. (**A**) Reactivity with antisera to IPNV-Buhl. Lanes 1 and 2 contain lysates from uninduced and induced cells containing the pATH1 expression vector with no viral insert; lanes 3 and 4, lysates from uninduced and induced cells containing pA43; lanes 5 and 6, lysates from uninduced and induced cells containing pB10; lane 7, prestained low molecular weight markers from BioRad; lanes 8 and 9, lysates from uninduced and induced cells containing pB10; lane 10, purified IPNV. (**B**) Reactivity with antisera to IPNV-EVE. Lanes 1 and 2 contain lysates from uninduced and induced cells containing pB10; lanes 3 and 4, lysates from uninduced and induced cells containing pA43.

no dramatic difference in the amino acid composition. Thus far, the only possible explanation for the slower migration of the pA43 fusion protein might be the series of four prolines found towards the amino terminus of this insert (Fig. 4).

Polyclonal rabbit antisera prepared to the different serotypes of IPNV will crossreact with the heterologous VP2 proteins in Western immunoblots (R. Barrie and J. Leong, unpubl. data). Thus, there are conserved linear epitopes among the IPNV strains. When the expressed *trpE*-VP2 fusion proteins were examined for reactivity with polyclonal anti-IPNV sera prepared to three different IPNV serotypes, only pB10 reacted with the heterologous antisera in West-

ern immunoblots (Fig. 5, A and B). The anti-IPNV-Buhl sera was prepared against purified virions of the Buhl virus isolate which had been previously characterized as a member of the IPNV-VR299 serotype found in rainbow trout (*Oncorhynchus mykiss*) in North America (Hill and Way 1983). The IPNV-EVE isolate was obtained from Japanese eels (*Anguilla japonica*) suffering from branchionephritis in Japan (Sano et al. 1981); it has been antigenically grouped with the AB serotype of IPNV, which is nonpathogenic for rainbow trout. The fusion protein encoded by pA43 was completely nonreactive with the heterologous antisera. Thus, it appears that the VP2 gene region encoded by pB10 contains an antigenic determinant(s) which is conserved among the IPNV strains examined and the insert in pA43 encodes a region which is highly variable.

A comparison of the nucleotide sequence and its derived amino acid sequence of each insert with that of other published sequences of the VP2 gene showed that the pB10 region was highly conserved at the nucleotide and amino acid level (Fig. 4; Christie et al. 1988; Havarstein et al. 1990). There were three amino acid differences between the Sp and the Jasper isolate (a member of the VR299 serotype), and there were only two amino acid differences between Sp and N1 isolates, the latter of which is another IPNV isolate from the Sp serotype (Havarstein et al. 1990). In contrast, 27 amino acid changes between the Sp and Jasper isolates were observed for the pA43 gene fragment. Only five amino acid changes were found between isolates Sp and N1. The similarity between the Sp and N1 genomes indicates that these two isolates are highly related, a finding that is consistent with the findings of Christie et al. (1988).

Conclusion

In summary, two immunoreactive regions of the viral major capsid protein, VP2, have been identified. One region from amino acids 99 to 206 contains a very conserved epitope(s) which was recognized by neutralizing antisera to three different IPNV serotypes. Another region from amino acids 207 to 315 contains a highly divergent epitope(s) that may encode the serotype-specific epitope(s) of an IPNV strain. A suggestion that the amino acid region from 206 to 350 encoded the serotype-specific epitope(s) of IPNV was made by Havarstein et al. (1990) when a comparison of the deduced amino acid sequence of IPNV-N1 and IPNV-Jasper capsid proteins revealed that this region was very heterogeneous.

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Cloning of Hemolysin Genes of Aeromonads

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ABSTRACT

The role of extracellular products is critical in the pathogenic mechanisms of bacterial infections. In *Aeromonas* spp., hemolysins may be the most important of these products in establishing and maintaining infections. This report reviews our knowledge of the structure and expression of hemolysin genes in *Aeromonas* and discusses preliminary results on gene homology and ancestry among various *Aeromonas* spp.

The related species *Aeromonas hydrophila* (Ljungh and Wadström 1982) and *A. salmonicida* (Titball and Munn 1985a) produce several extracellular proteins that are virulence factors. In the study of pathogenic mechanisms of these bacteria, there has been interest in the role of these extracellular substances as toxins. *A. hydrophila* produces a variety of extracellular products, including a protease, glycerophospholipid cholesterol acyltransferase (GCAT), cytotoxin, an enterotoxin, acetylcholinesterase (Nieto et al. 1991), and hemolysins (Ljungh and Wadström 1982). Extracellular products of *A. salmonicida* include hemolytic, leukocytolytic, proteolytic, and GCAT activities (Ellis et al. 1981, 1988). The virulence of *A. hydrophila* and *A. salmonicida* is significantly enhanced by their ability to secrete hemolysin. Hemolysin may be the most important of these products in causing tissue damage and in establishing and maintaining infections with *Aeromonas*.

There are many reports describing the number and nature of hemolysins found in *A. hydrophila* (Ljungh et al. 1981; Thune and Johnson 1986; Asao et al. 1986) and *A. salmonicida* (Titball and Munn 1985a; Rockey et al. 1988). Asao et al. (1986) purified and characterized two hemolysins from *A. hydrophila* which were biologically similar but immunologically distinct. Both hemolysins caused fluid accumulation in infant mouse intestines and rabbit ileal loops and elicited a cytotoxic effect on Vero cells. Two distinct hemolysins have also been found in *A. salmonicida*. One is a broad-spectrum hemolysin with maximum activity against horse erythrocytes (H-lysin) (Titball et al. 1985b), and the other is active against trout erythrocytes (T-lysin) (Rockey et al. 1988). H-lysin contains GCAT. Nomura et al. (1988) purified salmolysin, an extracellular hemo lytic toxin from *A. salmonicida*. Salmolysin was lethal to rainbow trout *Oncorhynchus mykiss* when it was injected intramuscularly.

Almost all isolates of *A. hydrophila* and *A. salmonicida* produce aerolysin, a substance with hemolytic activity. The level of aerolysin production is known to vary under different growth conditions, and individual isolates can alternate between high and low level phases of production. When this hemolysin gene is cloned into *E. coli*, the gene's characteristics can be more easily studied.

For this reason, we cloned the two hemolysin genes from *A. hydrophila* and the one hemolysin gene from *A. salmonicida* to study their structure and expression (Aoki and Hirono 1991; Hirono and Aoki 1992, a and b).

We have previously reported the cloning of two hemolysin genes (for aerolysins AHH-1 and AHH-2) from *A. hydrophila* ATCC7966 into a plasmid vector in *E. coli* K-12 (Aoki and Hirono 1991; Hirono and Aoki 1991, Table 1). Open reading frames (ORF) of the AHH-1 and AHH-2 genes were 1,734 and 981 base pairs (bp), respectively. The sequences included the –10 region and the –35 region of a promoter and a ribosome binding site (Shine-Dalgarno sequences) upstream from the ORF. Two palindromic sequences were found immediately following the termination site. Analysis of the deduced amino acid sequences indicated a highly hydrophobic N-terminal region in the AHH-1 gene with the characteristics of a leader

```
        10        20        30        40        50        60        70        80        90       100
GCATGCCGAATCATCCACCTTAGATCGTATCGGGATCGTTGTTCTGTCAACAATAAATGCATTCGGCGCCACACTGTTAATTCACGGCGAGAATAATGAATGTCA

       110       120       130       140       150       160       170       180       190       200       210
TGACAGGCAAGCAGAATAACGCCCCGAAATATAACCGTGAATAATATGGTTCTGCCGTATTCGTTCATTGTTTAAATAGCTTGGCTTGATTCGACAAGGAGATAA
                                                                                  -35
       220       230       240       250       260       270       280       290       300       310
CAATGAAAAACAAAAAACCACGCAAATTCATCACGCAAGCCCCCACTCTCAGTCTCGTGCGCTGGCCTTGTTGGCAGGCAGCGTGCAAGGCGAAGATATTGGCGA
-10
       320       330       340       350       360       370       380       390       400       410       420
ACGTACCGACCAGGGCACCGCCATGCTGGCCAGCCTGCAATCCGAACAGGGTCTGATTTACCTCAATGCCGACGTCTGGCTGAAGGGGCAGGGGCGGACGCCGCT
            SD        MetLeuAlaSerLeuGlnSerGluGlnGlyLeuIleTyrLeuAsnAlaAspValTrpLeuLysGlyGlnGlyAlaThrProLe

       430       440       450       460       470       480       490       500       510       520
CATGACCCGGGATCAGCTGCGCGAGCGGGTGCTGGCACGGGGTGAGCGTCTGTTCATCGATTTCAGCGCCGTCACCGACCAGATCGAGCGACAACAGGCCAGAAA
uMetThrArgAspGlnLeuArgGluArgValLeuAlaArgGlyGluArgLeuPheIleAspPheSerAlaValThrAspGlnIleGluArgGlnGlnAlaArgLy

       530       540       550       560       570       580       590       600       610       620       630
GGCCATGGAGCAGCTGGCCGGGATCTCTTTTGATGCGGACTGGGTGCTGGTGTCCGGCTACAAGGGGGAGCTGCTGTTCACCCCGCTGGGAGGCGTCCATGACCC
sAlaMetGluGlnLeuAlaGlyIleSerPheAspAlaAspTrpValLeuValSerGlyTyrLysGlyGluLeuLeuPheThrProLeuGlyGlyValAspAspPr

       640       650       660       670       680       690       700       710       720       730
GGCCTTCTATCAGCTGATGGAGCCGGGTCGAGAGCCTGGAAGGGCAGGGCAACCGCCACAAGCGCTCGCTGACCCAGCCGCCTGCCGCCGAGGCCCGTCTGCCCCA
oAlaPheTyrGlnLeuMetGluArgValGluSerLeuGluGlyGlnGlyAsnGlyHisLysArgSerLeuThrGlnProProAlaAlaGluAlaGlyLeuProHi

       740       750       760       770       780       790       800       810       820       830       840
TGTGGCCTTTTACCTCAACGTCAACCGCAAGATCAGCGATGCCGAGTGCACCTTCCCGGGCTCGCGTACCTGGAGCCGGGCCGACCGGCTGTTCTGCGATTCGCC
sValAlaPheTyrLeuAsnValAsnArgLysIleSerAspAlaGluCysThrPheProArgSerArgThrTrpSerArgGlyAspArgLeuPheCysAspSerPr

       850       860       870       880       890       900       910       920       930       940
GAACATCTCGCTGGTCTACCGAGTCAACCTGGAGCGCTCCCTGCAATTTGGCAACACGGCGTCCGCCACGCCGGATGCCAAGATAGTGCGGATCTCGCCTGGACGA
oAsnIleSerLeuValTyrArgValAsnLeuGluArgSerLeuGlnPheGlyAsnThrAlaSerAlaThrProAspAlaLysIleValArgIleSerLeuAspGl

       950       960       970       980       990      1000      1010      1020      1030      1040      1050
AGAGTCGGCCGGTGCCCGTATCCAGCTCAACGAGGATCTGAGCTGGAGCGAAAACATCGCGACTATCTGCTGCTGGACGGCTGGGCCCCCGCACTATGCCACCGA
uGluSerAlaGlyAlaGlyIleGlnLeuAsnGluAspLeuSerTrpSerGluAsnIleAlaAspTyrLeuLeuLeuAspGlyTrpAlaArgAspTyrAlaThrAs

      1060      1070      1080      1090      1100      1110      1120      1130      1140      1150
TGCCATCGCCCAGGATTATCGCTTCACGACTGAGGCGCTCCAACACCAAGGCGGCGGTGCTCAAGAGCCTGCCGACCAACCTCAACAGCAAGTACGAGCATCGCGA
pAlaIleAlaGlnAspTyrArgPheThrThrGluAlaSerAsnThrLysAlaAlaValLeuLysSerLeuProThrAsnLeuAsnSerLysTyrGluHisArgGl

      1160      1170      1180      1190      1200      1210      1220      1230      1240      1250      1260
GATCTCGGGTTTCGAGGTGGGGGTCACCGGCGGGGTCGAGGTGAACAAGGATGGCCCAAGGCCAAGCTGGAGGCGTCGGCCAAGTTCAGCCAGCAGCGCCAGCTC
uIleSerGlyPheGluValGlyValThrGlyGlyValGluValAsnLysAspGlyProArgProSerTrpArgArgArgProSerSerAlaSerSerAlaSerSe

      1270      1280      1290      1300      1310      1320      1330      1340      1350      1360
GCCTACAACACCCAGGATTACCGGGTTGAACGCTCCGGCCGAGCGCCCAGAAGCTGAGTTTCAGCTGGGTGCGCGATCAATACGCGACCGCAGAGTCCCTGCTCT
rProThrThrProArgIleThrGlyLeuAsnAlaProAlaGluArgProGluGlyGluPheGlnLeuGlyAlaArgSerIleArgAspArgArgValProAlaLe

      1370      1380      1390      1400      1410      1420      1430      1440      1450      1460      1470
CCTCCAAGACGGCCACGTCTGGGCATGGCTACGACGTGGATCACAACCGGCATCCAGCCGCTCAGCTACAAGGGGTTTGTGCCGAATCTGATGTCATCTACAAGGC
uLeuGlnAspGlyHisValTrpAlaTrpLeuArgArgGlySerGlnProHisProAlaAlaGlnLeuGlnGlyValCysAlaGluSerAspValIleTyrLysAl

      1480      1490      1500      1510      1520      1530      1540      1550      1560      1570
GGCGCCGGACGAGACGGGCAGCACCGAGTTCAAGATCGACTCCTGGTCAACATCCGCCCCATCTACACCGGCATCTACAAGCACTACTACGTGGTGGGGGCCCA
aAlaProAspGluThrGlySerThrGluPheLysIleAspSerSerValAsnIleArgProIleTyrThrGlyIleTyrLysHisTyrTyrValValGlyAlaHi

      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
TGTCTCCTTCCAGGGCTTCGAAGATACCGACAAGCGCGAGACGCGGTGGCGGCGTCCACCAGCTTCAAGGTGGACTGGAACCACCCGGTGTTTACCGGCGGTCGCCC
sValSerPheGlnGlyPheGluAspThrAspLysArgArgArgValAlaAlaSerThrSerPheLysValAspTrpAsnHisProValPheThrGlyGlyArgPr

      1690      1700      1710      1720      1730      1740      1750      1760      1770      1780
GGTCAACCTGCAGCTGGGGGGCTTTGACAACCGTTGCCTGAGCGCGGATGCCAACCATGGTCTGAGCGCGGTGACCTTTGACGAGACCTCGGCCGCCCAGTCCTC
oValAsnLeuGlnLeuGlyGlyPheAspAsnArgCysLeuSerAlaAspAlaAsnHisGlyLeuSerAlaValThrPheAspGluThrSerAlaAlaGlnSerSe

      1790      1800      1810      1820      1830      1840      1850      1860      1870      1880      1890
CATCTATGACGAGTACGGCCGCTATGTCAGCGCGCAGGATACCCTCCGCTGCCTGGATGGCAACAACCTTGGCCAGCTGCAGAGCTGCAGCCTGAGCCTGGGTCA
rIleTyrAspGluTyrGlyArgTyrValSerAlaGlnAspThrLeuArgCysLeuAspGlyAsnAsnLeuGlyGlnLeuGlnSerCysSerLeuSerLeuGlyGl

      1900      1910      1920      1930      1940      1950      1960      1970      1980      1990
GCGCTGGGAGTGGAAAGCGGACAGCGATGCGCTCAGCAACCTGAGTGCCCACCAGCTGCTGGTGCATGACAAGCAGAGCGGGGCCTGGGGCTCTACGACGAGAA
nArgTrpGluTrpLysAlaAspSerAspAlaLeuSerAsnLeuSerAlaHisGlnLeuLeuValHisAspLysGlnSerGlyAlaLeuGlyLeuTyrAspGluAs

      2000      2010      2020      2030      2040      2050      2060      2070      2080      2090      2100
CGGCAATCCGCAGAATGTGAGCGTACGGACCCTGACCTCCTACACCCGCATCTTCGGGCCACCTGCCAGTCACTGACAGAGCAGGGCCTTGCGCTCAAGACAACC
nGlyAsnProGlnAsnValSerValArgThrLeuThrSerTyrThrArgIlePheGlyProProAlaSerHis

      2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
ACGACGCCAAGTGCCGGGCGTTTTTATGAGGGGCGGATCAGGCCTTGTTTCTATGAGCGGGCGAGCACGTCCGCCAGCTTGTAGAAGGTCTGCACCCGGGTGG
palindrome structure
      2210      2220      2230      2240      2250      2260      2270      2280      2290      2300      2310
TGCTGGCGCCACACCAGCCCTATCTCGCGGTAGTGATGTTCACCGGCAGCGGGATGGCCACCAGATCCGTGTTGTCGACGATGCCGCCATCGATGGCCATCTGC

      2320      2330      2340      2350      2360      2370      2380      2390      2400      2410
GGCAGGAAGGTGGCTGAGCCCGGCGCCGACCATCTGCACCAGGGTGTGCAGGCTGGTGGCGGCAAACGGGTTGATCTTGCTCTTGTCACCCGCAGCCGGCAGGGC

      2420      2430      2440      2450      2460      2470      2480      2490      2500      2510      2520
TCACCGCGTGTTCGGTGAGACAGTCGTCCCGCTCCAGCAGGAAGATACTCTGGTTGGGCAGCTCCTTGTAGTCGAACGGGGTGTGCAGGCTGGCCATGCTGGCG
```

Figure 1

Nucleotide sequence and deduced amino acid sequence of hemolysin gene AHH-1 from *Aeromonas hydrophila* ATCC7966. The deduced amino acid sequence is given under the nucleotide sequence. A putative promoter is indicated by the areas marked –35 and –10, and ribosome binding site is indicated by SD. The palindromic structure sequence of a terminator-like region is also indicated downstream from the AHH-1 coding region.

Table 1
Maximum matching comparison of amino acid sequences of cloned hemolysin genes of *Aeromonas hydrophila* and *A. salmonicida*.

	AHH-1	AHH-2	ASH-1	*aer*
AHH-1				
AHH-2	17.4%			
ASH-1	17.0	17.0		
*aer**	17.1	16.9	18.5	

* Howard and Buckley 1986.

peptide (Figure 1). However, the N-terminal region of the AHH-2 gene was not hydrophobic.

Two aerolysin genes were also cloned from *A. hydrophila*, Ah65 (Howard and Buckley 1986; Howard et al. 1987) and AH2 (Chakraborty et al. 1986). The nucleotide sequence of the Ah65 aerolysin gene was 1,458 bp. There was very low homology between the aerolysin gene from Ah65 and each of the ATCC7966 genes, and there were no indications of similarity in the predicted amino acid sequences (Table 1).

We also cloned one hemolysin gene (ASH-1) from *A. salmonicida* ATCC14174 (Hirono and Aoki 1992, a and b) (Table 2) which had an ORF of 1,716 bp. It had the -10 region and the -35 region of a putative promoter and a ribosome-binding site up stream from the ORF, and the termination codon and palindromic sequences downstream from the ORF. The N-terminal region of the ASH-1 gene was highly hydrophobic. Comparative analysis of the fundamental molecular structures of our cloned AHH-1, AHH-2, and ASH-1 genes, and the previously reported aerolysin gene from Ah65 (Howard et al. 1987) suggests that they have not descended from a common ancestor.

The recombinant plasmids pAHH-1, pAHH-2, and pASH-1 were introduced into a maxicell strain CSR603 of *E. coli* and the plasmid-encoded proteins examined by polyacrylamide gel electrophoresis (Sancar et al. 1979). Strains containing pAHH-1 and pASH-1 produced proteins of 62 kDa and 56 kDa, respectively (Hirono and Aoki 1991, 1992a). These results are in agreement with the size of the proteins predicted by the DNA-sequenced ORF. However, the molecular size of novel protein synthesized by cells harboring the pAHH-2 plasmid was 26 kDa. The molecular size of the protein was clearly different from the size predicted by the hemolysin AHH-2 gene sequence which inferred a protein with a molecular weight of 37.7 kDa. The products of transcription or translation in AHH-2 may be either greatly truncated or reduced in quantity. As mentioned above, analysis of the deduced amino acid sequences indicated that the N-terminal region of the AHH-2 gene was not hydrophobic. As yet, it is difficult to explain the difference in the molecular size of the final protein.

Hemolysin was released by the *E. coli* cells containing the AHH-1 gene and those containing the ASH-1 gene. The hemolytic activity in a supernatant was inactivated by heating to 70° C for 10 minutes. However, the hemolysin was expressed but was not secreted from *E. coli* cells carrying the recombinant plasmid containing the AHH-2 gene. The aerolysin cloned by Howard and Buckley (1986) similarly was not released from the *E. coli* cells in which they were cultured. The *E. coli* hemolysin requires four cistrons encoded with the hemolysin structured protein for full expression of active protein to be achieved (Felmlee et al. 1985). These proteins are associated with the cell envelope and are involved in transporting the hemolysins out of the cells. This complex system is unlike the one producing the extracellular hemolysins in *A. hydrophila* and *A. salmonicida*. It is very interesting that the hemolysin release mechanism produced by *E. coli* cells containing the hemolysin gene is different from *Aeromonas* spp.

Using colony hybridization analysis, the cloned hemolysin genes were tested for presence of homologous regions in isolates *A. hydrophila* and *A. salmonicida* from humans and fish in Japan (Hirono

Table 2
Cloned hemolysin genes from *Aeromonas hydrophila* and *A. salmonicida*.

Hemolysin genes	Sources	Open reading frame (bp)	Predicted molecular size from DNA sequences (Da)	Molecular size from Maxicell (kDa)
AHH-1	*A. hydrophila*	1,734	63,658	62 (Hirono and Aoki 1991)
AHH-2	*A. hydrophila*	981	37,797	26 (Aoki and Hirono 1991)
ASH-1	*A. salmonicida*	1,716	64,780	56 (Hirono and Aoki 1992a)

and Aoki 1992, a and b). Ten of 15 strains of *A. hydrophila* isolated from humans and 14 of 33 strains from fish possessed sequences homologous to the hemolysin gene of AHH-1 (Table 3). The AHH-1 gene was detected in all 38 strains of *A. salmonicida*. The AHH-2 gene was detected only in the original strain ATCC7966 and not in the other strains of *A. hydrophila* and *A. salmonicida* tested. The ASH-1 gene was detected in two strains of *A. hydrophila* from fish and the original ATCC14174 strain. We used strains of *A. hydrophila* and *A. salmonicida* isolated in the United States for cloning the hemolysin gene and tested for homology with strains isolated in Japan. Homology of the tested strains with the cloned hemolysin was low. We shall attempt to survey the distribution of our cloned hemolysin genes in more isolates of aeromonads. The presented data, however, indicate that the hemolysin genes having different structures occur in *A. hydrophila* and *A. salmonicida* isolates from different geographical locations.

Table 3

Detection of DNA sequences homologous to the hemolysin genes from Aeromonas hydrophila and *A. salmonicida* by colony hybridization. (Hirono and Aoki 1991, 1992b.)

Strains	Sources	AHH-1	AHH-2	ASH-1
A. hydrophila	Human	10/15*	0/4	0/15
	Fish	14/33	0/5	2/33
	ATCC7966	1/1	1/1	0/1
A. salmonicida	Fish	38/38	0/19	0/38
	ATCC14174	1/1	0/1	1/1

* Number of strains containing hemolysin genes/Number of tested strains.
Hemolysin genes of the AHH-1 and AHH-2 of *A. hydrophila* ATCC7966 and the ASH-1 gene of *A. salmonicida* ATCC14174 were cloned into plasmid vectors in *E. coli* K-12.

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Harmful Red Tides of *Heterosigma akashiwo*

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ABSTRACT

The raphidophyte *Heterosigma akashiwo* is one of several species of flagellates that cause harmful red tides. This paper reviews the distribution of *H. akashiwo,* the relationship between eutrophication and the occurrence of red tides in the Seto Inland Sea, and environmental and biological features of red tide development (life cycle, growth dynamics, and allelopathy).

Introduction

The raphidophyte *Heterosigma akashiwo* is one of several species of flagellates causing prodigious red tides. The total damage by *H. akashiwo* red tides has amounted to about 2 billion yen over a period of 16 years (1972–87) in the Seto Inland Sea of Japan. Most of this damage affected fish culture operations. This paper seeks to advance research on red tides by reviewing the known ecological features of *Heterosigma* red tides.

Morphology

The cells of *Heterosigma akashiwo* (HADA) Hara et Chihara are yellow-brown and ovoid, and slightly compressed dorso-ventrally. The cell size is 8–25 × 6–15 μm. This organism is thought to lack a cell wall and be limited only by a single membrane. The delicate structure of the cell has hindered the study of its surface morphology, although the existence of an external structure, the glycocalyx, has been demonstrated (Yokote et al. 1985). In Japan, *H. akashiwo* has been referred to as *Entomosigma* sp. and *H. inlandica;* in many other countries it has been confused with the chrysophyte *Olisthodiscus luteus* CARTER. Recently, Hara and Chihara (1987) reported that *Entomosigma* sp. and *H. inlandica* are synonymous with *H. akashiwo,* and that most red tides ascribed to *O. luteus* actually involve *H. akashiwo.*

Distribution

Heterosigma akashiwo occurs in the temperate and subtropical embayments in Japan (Hara and Chihara 1987), Singapore (Taylor 1990), New Zealand (Taylor 1990), England (Lackey and Lackey 1963), and Belgium (Conrad and Kufferath 1954), and in the eastern (Tomas 1982) and western areas (Lackey and Clendenning 1965; Taylor 1990) of North America, Bermuda (Tomas 1982), and Chile (Taylor 1990) (Fig. 1). Damages to fish have been recorded for the Seto Inland Sea (yellowtail, *Seriola quinqueradiata* and red sea bream, *Pagrus major*), New Zealand (salmon), British Columbia (salmon), and Chile (salmon) (Taylor 1990).

Eutrophication and Red Tides

In the Seto Inland Sea, nitrate, inorganic nitrogen (ammonia + nitrite + nitrate), and phosphorous concentrations have increased rapidly since the mid-1960s (Fig. 2). Nutrient concentrations reached a maximum in the mid-1970s then gradually decreased thereafter. The total number of red tide occurrences in the Seto Inland Sea before 1965 was less than 50 cases per year. Beginning in 1968, the number increased year by year, reaching a peak of 299 cases in 1976. After 1976, the number decreased to 160–170 cases per year. The increase in the 1970s coincided with a rapid development in the Japanese economy, with a time lag of 2–3 years. The decrease in frequency after the first oil shock (1973–1974) cor-

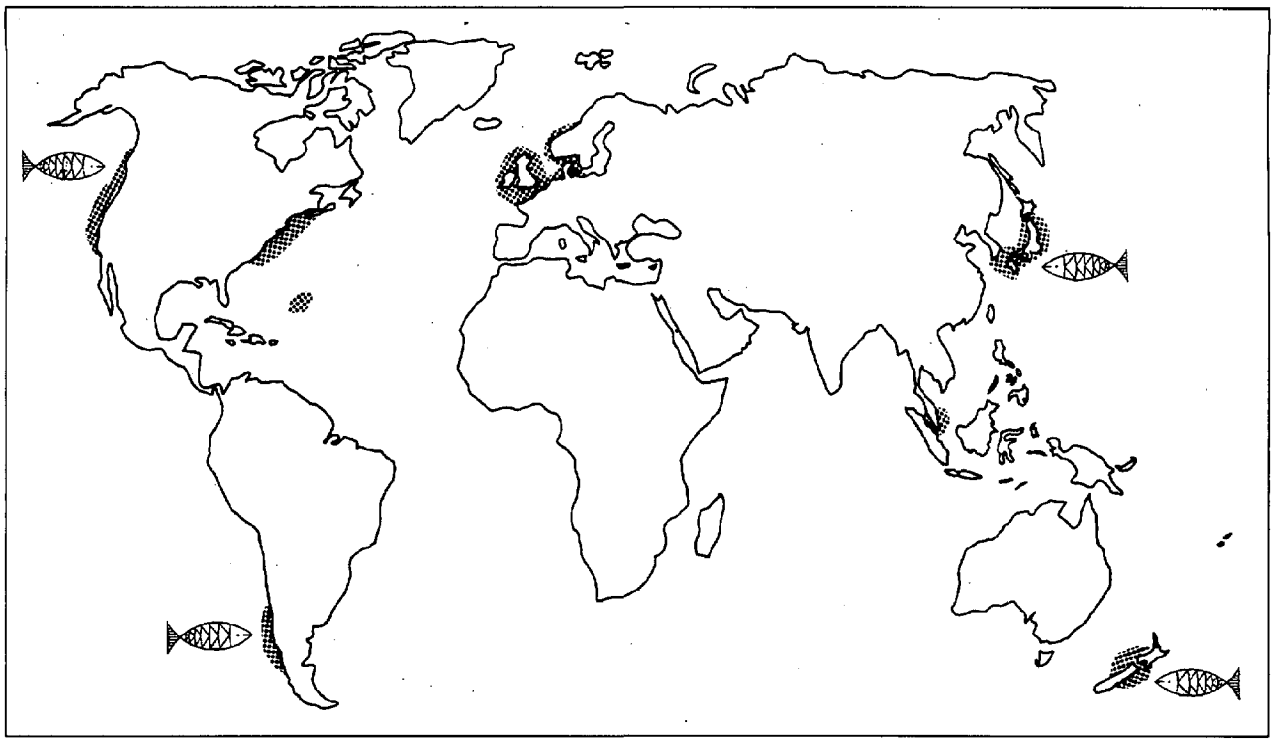

Figure 1
Distribution of *Heterosigma akashiwo* red tide (dotted areas). Fish indicate places where cultured fish have sustained damage.

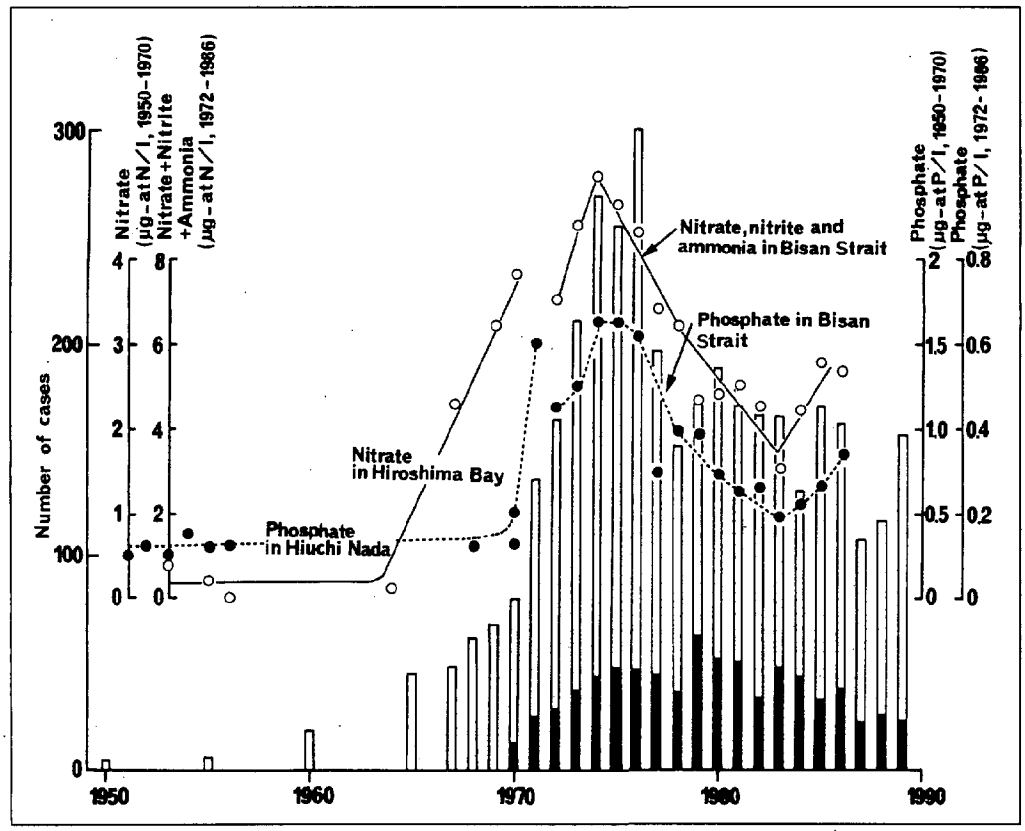

Figure 2
Total number of red tide cases (open bars) and of *Heterosigma akashiwo* red tides (black bars) and concentrations of nitrogen (open circles and solid lines) and phosphorous (black circles and dashed lines) in the Seto Inland Sea (1950-89).

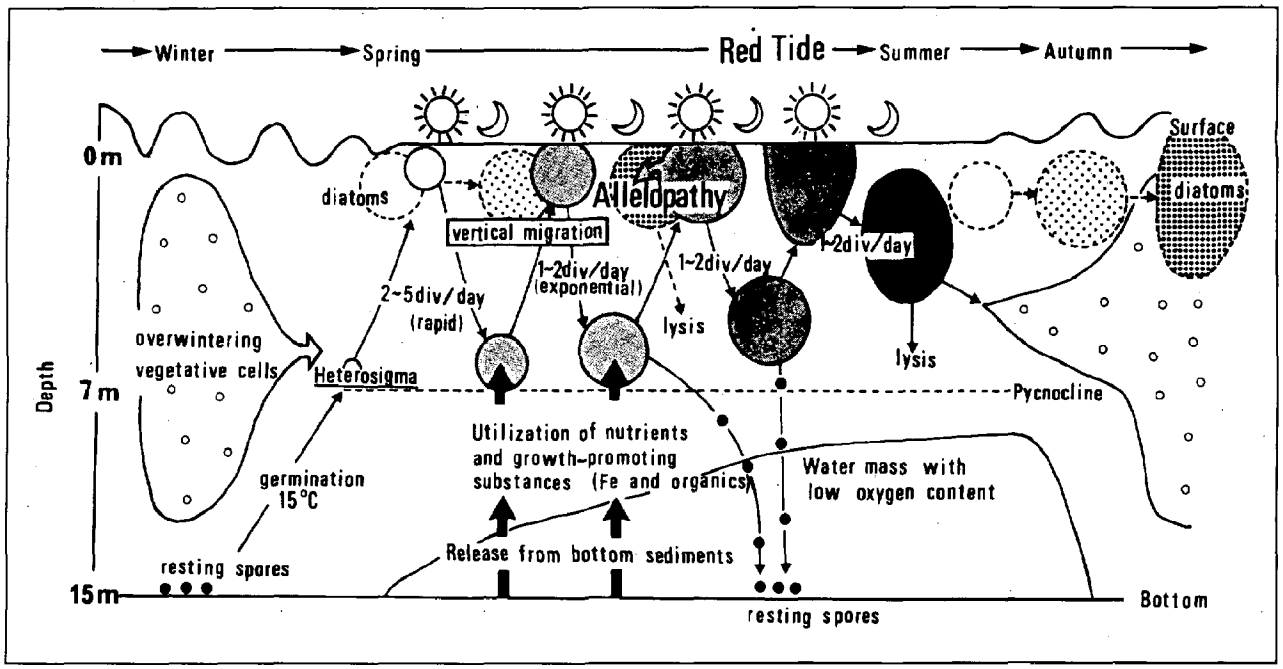

Figure 3
Schematic diagram of ecological features during the period of red tide development.

responds with the switchover of the Japanese economy to a lower rate of development. The patterns of change in the total number of tides and the number of *Heterosigma* red tides were similar to that of nutrient concentrations. Thus, the frequency of red tide occurrences is closely related to eutrophication.

Environmental Features During the Period of Red Tide Development

Temperatures suitable for the growth of *H. akashiwo* have been reported from culture experiments to be in the range of 15–30° C (Tomas 1978a; Mori et al. 1982; Yamochi 1989). This helps to explain why *Heterosigma* red tides tend to occur in coastal waters from May through late June. However, the range of suitable salinity differs among culture strains: 30‰ for a Fukuyama strain (Iwasaki et al. 1968); 10‰ for a Gokasho strain (Iwasaki and Sasada 1969); 27–28‰ for a Hakata strain (Honjo and Hanaoka 1973); 12–40‰ for a Narragansett strain (Tomas 1978a); and 12–28‰ for a Tanigawa strain (Mori et al. 1982). This suggests that these strains have developed a physiological acclimation to the range of salinity in each habitat.

Growth of *H. akashiwo* is usually initiated in early summer when a pycnocline is formed in the middle layer (Fig. 3). Oxygen concentrations in bottom water decrease rapidly after the formation of the pycnocline. The pH of the anoxic bottom water decreases to about 7. As a result, nutritive substances (inorganic nutrients, metals, and organic constituents including growth-promoting substances) are released from the bottom sediments and these substances accumulate to high levels in anoxic bottom waters (Honjo 1974). The *Heterosigma* population has access to these substances in the bottom water through diurnal vertical migration at night.

The Development of *Heterosigma* Red Tides

Growth originates from cell stocks that overwintered as motile forms (Yamochi 1989) and/or that germinated from resting cells (Imai et al. Nansei National Fisheries Research Institute pers. commun. 15 October, 1990). Tomas (1978b) and Yamochi (1989) found that motile cells aggregate and change to nonmotile cells. Population growth can be divided into two phases: rapid and exponential (Fig. 3; Honjo and Tabata 1985). The rapid phase is defined as the period when cells increase more than four-fold each

day and the exponential phase as that period when the correlation coefficient between log cell number and time is greater than or equal to 0.95 and there is an obvious increase in the number of cells for more than 4 days. The duration of *Heterosigma* red tides is shorter than for other flagellates. The abrupt disappearance of *Heterosigma* cells requires further study.

Growth Rate and Division Periodicity

Blooms of this organism develop so rapidly and dramatically that many workers have studied the growth of *H. akashiwo* in vitro and in situ as an approach to understanding the enigma of red tide formation. Their results, however, have conflicted (Honjo and Hanaoka 1973; Tomas 1978a).

Honjo and Tabata (1985) studied *Heterosigma* growth dynamics and division periodicity in outdoor tanks. They found that *H. akashiwo* has a potential for high growth rates (2–5 divisions per day). There was a strong tendency for large cells to dominate at the beginning of this fast growth phase and to be replaced by small cells toward the end of the phase. During culture experiments, a portion of a *H. akashiwo* patch in a tank was cultured in a 1-liter volumetric flask hung in the tank. The growth rate in this experiment was 2.3 divisions per day. Other *H. akashiwo* cells were collected from the tank and individually cultured in small tissue chambers. In these chambers the highest growth rate was 3.3 divisions per day; the growth rates of large cells inoculated into these chambers were much higher than those of small cells.

In the tank environment, *H. akashiwo* prefers dark-associated cell division. Cell division first occurred a little before sunset and continued through the night until the next morning. Similarly, in the small chambers cell division began just before the onset of the dark period and continued for 4–5 hours into the next light period. Seven of 24 *H. akashiwo* cells divided three times during the night in the small chambers, with a cell division interval of about 6 hours. Puiseux-Dao (1981) comprehensively reviewed the events of the cell cycle and Chisholm (1981) described the chronobiology of cell division in unicellular algae. In their reviews, the cell division cycle was discussed in terms of the length of the circadian period (1 division per day), but no consideration was given to the rapid division cycles of *H. akashiwo*.

Allelopathy

The study of allelopathy is important for elucidating the mechanisms of temporal succession of phytoplankton and of monospecific bloom events. Allelopathic interactions in which organic metabolites of one plant or microorganism suppress or enhance the growth of other plants or microorganisms are different from competitive interactions which involve the removal or reduction of certain factors such as water, minerals, foods, and light. The interactions among marine and freshwater phytoplankton have been well studied and have been reviewed by Smayda (1980), Maestrini and Bonin (1981), and Rice (1984). Some of the most dramatic changes of species composition in marine phytoplankton have been observed during *Heterosigma* red tide blooms, and allelopathic interactions have been observed between *H. akashiwo* and the centric diatom *Skeletonema costatum* during in vitro experiments (Pratt 1966; Honjo et al. 1978). Their studies imply that an allelopathic relationship between these organisms occurs during high cell densities of *H. akashiwo*. Stuart (1972) and Sakshaug (1977) examined physical features of the allelopathic substances but experienced difficulty in determining their molecular weight.

Recently Honjo et al. (unpubl. data) investigated the biological and chemical features of allelopathic substances from *H. akashiwo* (Fig. 4). During a bloom of *H. akashiwo* in an outdoor tank, an abrupt decrease in cell numbers of centric diatoms (dominant species, *S. costatum*) and an increase in the dinophyte *Prorocentrum triestinum* occurred when high concentrations of dissolved carbohydrates were detected in the water. Crude polysaccharide extracts from *Heterosigma* cells and from filtrates of the bloom seawater were both separated into two main fractions by gel chromatography. In bioassay experiments, a macromolecular fraction from *Heterosigma* cells greatly suppressed the growth of *S. costatum* at concentrations above 1.0 μg glucose per mL, whereas this same fraction enhanced the growth of *P. triestinum* and *H. akashiwo* and had no effect on the growth of *Phaeodactylum* sp. The other fraction caused moderate suppression of *S. costatum* growth. Results of bioassays using *S. costatum* and the two fractions from *H. akashiwo* bloom water were similar to those using the fractions from *Heterosigma* cells. Histochemical analysis of the crude polysaccharide extracts from *Heterosigma* cells indicated a polysaccharide-protein complex with features analogous to the glycocalyx on the cell surface of the organism. Results suggest that a polysaccharide-protein complex exfoliated from

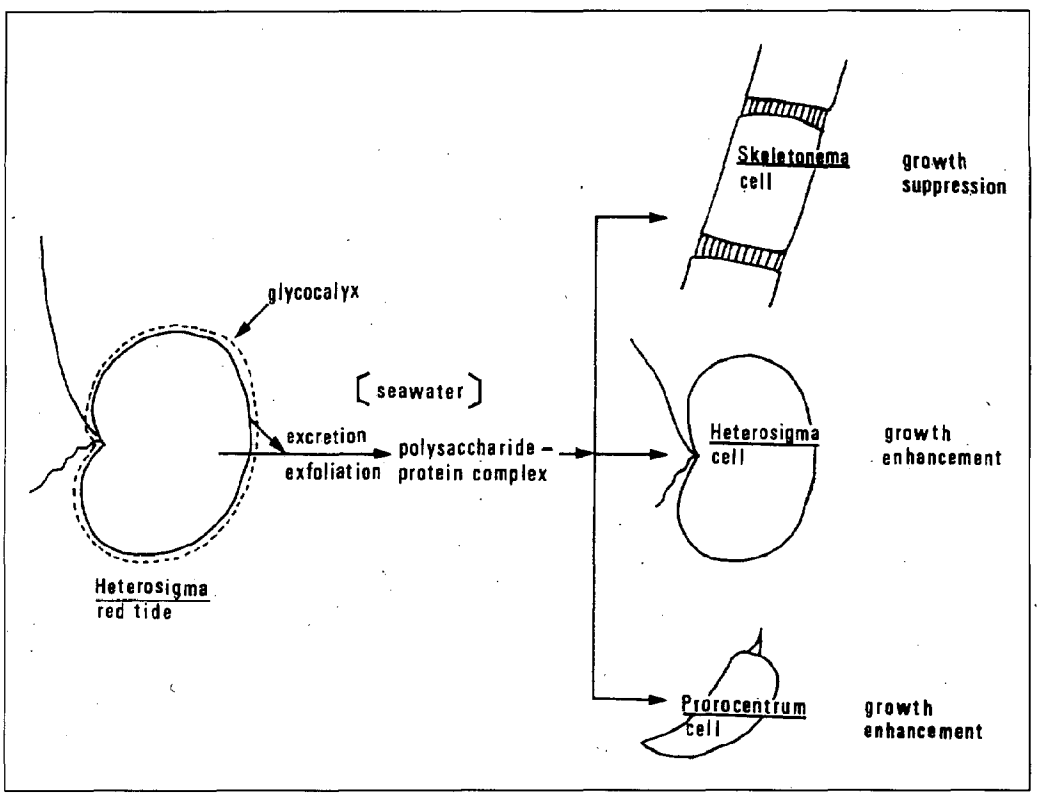

Figure 4
General scheme of allelopathic relationships between *Heterosigma akashiwo* and other phytoplankton.

and/or excreted by *H. akashiwo* is a species-specific allelopathic substance and plays an important role in causing dramatic changes in cell numbers of other phytoplankton during *Heterosigma* blooms.

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Impact of the Myxosporean Parasite *Ceratomyxa shasta* on Survival of Migrating Columbia River Basin Salmonids

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ABSTRACT

Columbia River Basin salmonids are exposed to the parasite *Ceratomyxa shasta* during both their seaward and return migrations. The impact of ceratomyxosis on the survival of migrating fish is difficult to assess because there are few data on causes of fish mortality once they are released from the hatchery. This study examines the impact of this disease on juvenile salmonids by 1) sampling the downstream migrants to determine what percent of fish leaving the basin are infected with the parasite and by 2) determining the effects of entering salt water on the progress of the infection. Results of comparisons of methods for diagnosing a *C. shasta* infection indicate that serological techniques using monoclonal antibodies are more sensitive than techniques for examination of spores.

Introduction

The Columbia River Basin has long supported an important Pacific salmon fishery for Oregon, Washington, and Idaho. However, the resource has steadily declined even with supplementation of fish from hatcheries. As research efforts are directed toward determining the causes behind the depletion of Columbia River Basin salmonid stocks, the impact of diseases must be considered. One disease that is enzootic to the basin is caused by the myxosporean parasite *Ceratomyxa shasta*. Although this parasite has devastated certain hatchery productions, it is not normally a disease of hatchery fish; therefore, its impact on survival of fish in the wild is difficult to evaluate. The parasite first infects and multiplies in the intestinal tract of the fish and from that site spreads to other tissues. The infection results in tissue necrosis accompanied by a severe host inflammatory reaction. Signs of infection may include lethargy, darkening of the body surface, abdominal distension, and hemorrhaging in the area of the vent (Bartholomew et al. 1989a). In addition, the disease has a prolonged incubation period and current diagnostic methods (Amos 1985) detect the parasite only in heavily infected fish.

Assessing the impact of ceratomyxosis requires examining a variety of factors. Studies of geographic distribution (Johnson et al. 1979; Hoffmaster et al. 1988) indicate that the parasite has spread within the Columbia River Basin, but the extent and cause of expansion is not yet known. The degree of resistance to infection among salmonid fish within the basin has also been examined and compared with resistance among fish populations from watersheds where the parasite is not found (Zinn et al. 1977; Buchanan et al. 1983). All groups of fish tested from the Columbia River Basin were relatively resistant. However, the ability of many wild and upriver stocks to resist infection has not been examined.

Further investigations of the impact of ceratomyxosis on migrating Columbia River salmonids were designed to answer 1) how many Columbia River salmonids become infected during their downstream migration, 2) how the disease progresses after the fish enter salt water, and 3) how effective are the methods currently used to diagnose ceratomyxosis.

Materials and Methods

Examination of Downstream Migrants for Infection

To estimate how many salmonids become infected during their downstream migration, outmigrants

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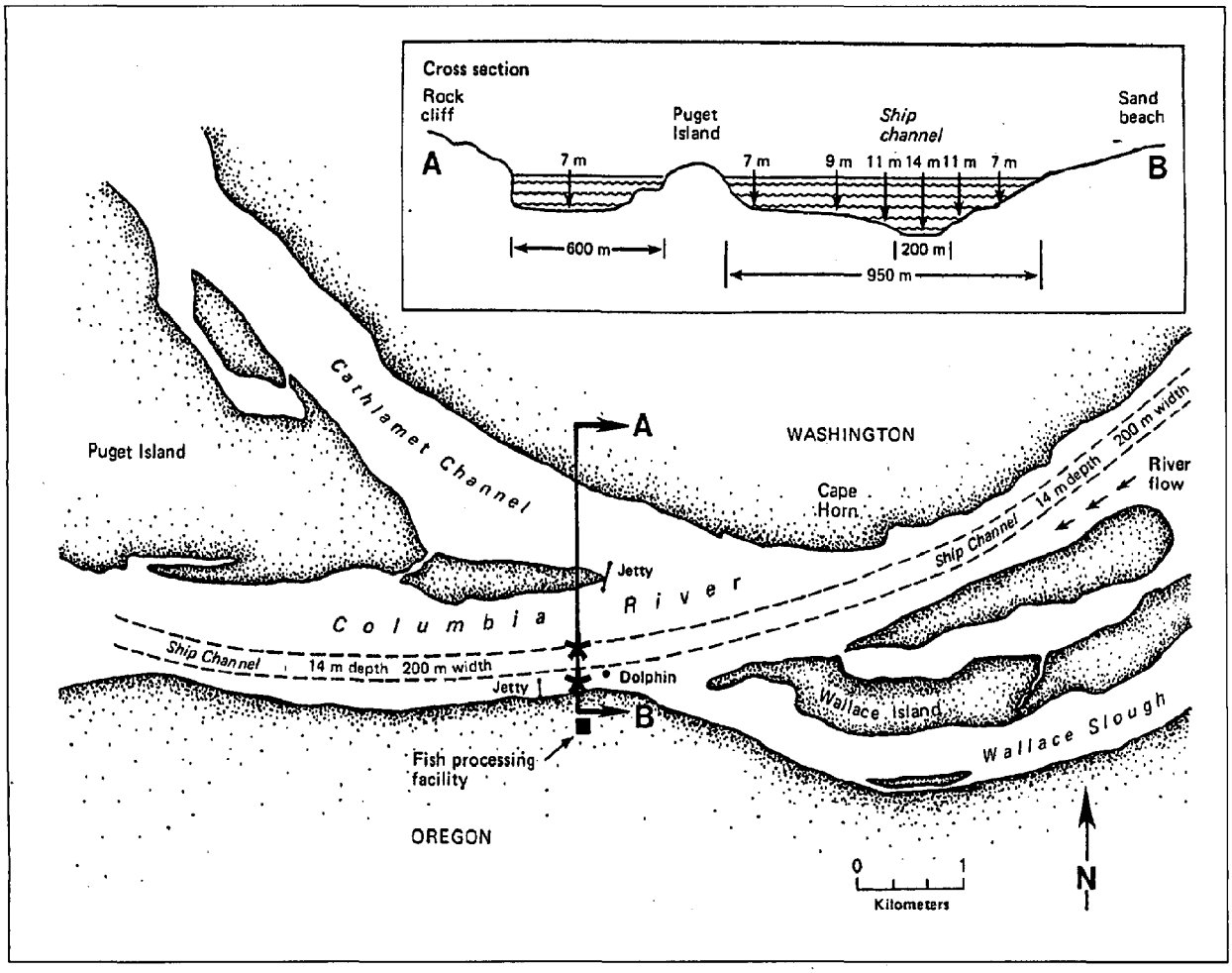

Figure 1

Jones Beach sampling site. The beach and purse seining areas are indicated by the two asterisks on the main map. From Dawley et al. (1984a).

were collected just prior to entering the estuary and were maintained in fresh water to monitor disease development. Outmigrating juvenile salmonids of different species and year classes were obtained by beach and purse seine from a collection facility operated by the National Marine Fisheries Service at Jones Beach (75 river km, measured upstream from the mouth of the Columbia River) on the Oregon side of the Columbia River (Fig. 1). Seining procedures were those described by Dawley et al. (1984a). A purse seine 206 m long and 11 m deep with a mesh of 1–2 cm was used in water about 9 m deep. The seine was set drifting with the current, then towed upstream for 5 minutes before closing and pursing. Beach seining with a net 95 m long, 5 m deep, with a mesh of 1–2 cm, was done in water about 6 m deep at the outer end of the net sweep. Fish were collected on 12 occasions between 20 May and 8 September in 1983 and on six occasions between 5 July and 20 September in 1984. In 1984, fish were collected by beach seine only. All fish were transported to the Round Butte Hatchery Isolation Facility on the Deschutes River, a facility operated by the Oregon Department of Fish and Wildlife. Holding tanks were 1-m circulars with a 375-L capacity. The water supply was free of pathogens and the water temperature was 10° C. All groups of fish were fed an Oregon Moist Pellet diet containing 3% terramycin in the form of TM_{50} (Pfizer) as a prophylactic measure against bacterial fish pathogens (Udey et al. 1975). Groups were held for at least 150 days and observed for development of ceratomyxosis. Fish deaths occurring within 10 days after transportation were attributed to handling mortality and were not included in the results.

In all experiments, dead fish were collected daily, and either immediately necropsied or frozen for later examination. All fish held in tanks that remained at the end of the observation period were killed and

examined for *C. shasta*. Wet mounts of intestinal tract scrapings were examined microscopically (as detailed later) for up to 5 minutes, and samples containing spores of *C. shasta* were considered infected.

Determining Saltwater Effects

Three strains of salmonid smolts were used to determine the effects of salt water on the progress of ceratomyxosis. Big Creek coho salmon (*Oncorhynchus kisutch*) were from Big Creek Hatchery located on the lower Columbia River and Round Butte chinook salmon (*O. tshawytscha*) were from the Round Butte Hatchery on the Deschutes River, a Columbia River tributary. Both strains migrate through waters enzootic for *C. shasta* and are relatively resistant to the parasite (Zinn et al. 1977; Johnson et al. 1979). Alsea steelhead trout (*O. mykiss*) were taken from the Alsea Hatchery. The Alsea River empties directly into the Pacific Ocean and does not harbor the infectious stage of *C. shasta*. Salmonids from this system have not developed resistance. All fish were held at the Oregon State University-Fish Disease Laboratory (OSU-FDL) in 1-m diameter tanks of 375 L capacity and were supplied with 12° C pathogen-free water until they reached smolt stage. Fish were exposed to the infectious stage of *C. shasta* in the Willamette River at Corvallis, Oregon, on three occasions. On the first occasion, two groups of 50 Alsea steelhead trout were exposed: one group for 3 days and the other for 5 days. On the second occasion, groups of 50 Alsea steelhead trout and 50 Big Creek coho salmon were held for 5 days. On the third occasion, 100 Alsea steelhead trout and 100 Round Butte chinook salmon were exposed for 3 days. Control groups of an equal number of each species were not exposed in the river. After exposure, the groups were divided and half of the fish were transported to freshwater holding facilities at the OSU-FDL and the other half to ultraviolet-treated saltwater facilities at the Mark O. Hatfield Marine Science Center in Newport, Oregon. The fish were fed Oregon Moist Pellets containing 3% terramycin in the form of TM_{50} (Pfizer). All groups were held for at least 100 days.

Comparison of Detection Methods

The sensitivity of the standard detection method of examining wet mounts of material from the lower

Table 1

Prevalence of *Ceratomyxa shasta* in chinook salmon smolts beach seined from the Columbia River at Jones Beach, Oregon.

Date collected (1983)	Number collected[a]	Holding mortalities	Mortalities infected with *C. shasta*	Percent of fish collected infected with *C. shasta*
May 20	91	3	3	3
27	81	6	1	1
June 3	75	8	2	3
10	53	1	1	2
17	65	36	3	5
24	130	21	3	2
July 1	113	24	17	15
15	141	46	17	12
29	68	33	2	3
Aug. 12	109	36	13	12
26	34	20	8	24
Sept. 8	112	39	25	22
Total	1072	273	95	9

[a] Number collected minus holding mortality during the first 10 days.

Table 2
Prevalence of *Ceratomyxa shasta* in chinook salmon smolts purse seined from the Columbia River at Jones Beach, Oregon.

Date collected (1983)	Number collected[a]	Holding mortalities	Mortalities infected with *C. shasta*	Percent of fish collected infected with *C. shasta*
May 20	9	5	3	33
27	37	6	2	5
June 10	58	17	0	0
17	47	18	4	9
24	98	25	8	8
July 1	128	46	21	16
15	43	38	5	12
29	47	33	11	23
Aug. 12	47	45	4	9
26	21	18	1	5
Sept. 8	14	12	2	14
Total	549	263	61	11

[a] Number collected minus holding mortality during the first 10 days.

Table 3
Prevalence of *Ceratomyxa shasta* in coho salmon smolts purse seined from the Columbia River at Jones Beach, Oregon.

Date collected (1983)	Number collected[a]	Holding mortalities	Mortalities infected with *C. shasta*	Percent of fish collected infected with *C. shasta*
May 20	82	13	4	5
27	38	4	3	8
June 3	23	1	0	0
10	5	4	1	20
17	1	2	0	0
24	12	2	1	8
July 1	1	0	0	0
15	1	0	0	0
Aug. 26	2	0	0	0
Total	179	26	9	5

[a] Number collected minus holding mortality during the first 10 days.

intestinal wall (Amos 1985) was compared with detection of *C. shasta* by serological methods (Bartholomew et al. 1989b). The lower portion of the intestine was excised from 121 adult salmon that had died prior to spawning. Two smears were made from each fish. The first smear was examined for spores as a wet mount at 400× magnification for a maximum of 5 minutes. The second smear was air-dried, fixed in 1:1 acetone:xylene solution, and incubated for 15 minutes with a monoclonal antibody specific for

Table 4
Prevalence of *Ceratomyxa shasta* in steelhead trout smolts purse seined from the Columbia River at Jones Beach, Oregon.

Date collected (1983)	Number collected[a]	Holding mortalities	Mortalities infected with *C. shasta*	Percent of fish collected infected with *C. shasta*
May 20	11	5	2	18
27	13	4	0	0
June 3	45	13	6	13
10	4	2	0	0
24	1	1	1	100
July 1	1	1	0	0
Total	75	26	9	12

[a] Number collected minus holding mortality during the first 10 days.

Table 5
Prevalence of *Ceratomyxa shasta* in chinook salmon smolts beach seined from the Columbia River at Jones Beach, Oregon.

Date collected (1984)	Number collected[a]	Holding mortalities	Mortalities infected with *C. shasta*	Percent of fish collected infected with *C. shasta*
July 5	88	76	6	7
26	75	22	12	16
31	84	31	14	17
Aug. 16	82	34	11	13
23	87	74	13	15
Sept. 20	47	29	9	19
Total	463	266	65	14

[a] Number collected minus holding mortality during the first 10 days.

prespore stages of *C. shasta*. Specific antibodies were detected using biotinylated horse anti-mouse IgG and fluorescein-conjugated avidin D (Vector Laboratories, Burlingame, CA). Methyl green dye (1% in distilled water) was used as a counterstain. Smears were examined with a Zeiss standard microscope containing an IV epifluorescence condensor at 250× magnification until prespore or spore stages of *C. shasta* were detected or for three minutes. All microscopic examinations were made by the same individual.

Results

Infections in Downstream Migrants

Chinook salmon were the largest group collected in 1983; 1072 subyearling smolts were captured in beach seines and 549 yearling chinook salmon were taken in purse seines. Smaller numbers of coho salmon (179) and steelhead trout (75) were also captured in purse seines. *Ceratomyxa shasta* was present in

Table 6

Effects of salt water on steelhead trout and coho and chinook salmon exposed to the infectious stage of *Ceratomyxa shasta*.

Salmonid	Exposure length (days)	Fresh water: No. of fish recovered[a]	Fresh water: No. of fish infected	Fresh water: Percent infected	Salt water: No. of fish recovered[a]	Salt water: No. of fish infected	Salt water: Percent infected
Alsea steelhead trout	3[b]	21	21	100	6	3	50
	3[c]	24	24	100	37	37	100
	control[d]	25	0	0	11	0	0
	5[b]	23	23	100	13	7	54
	5[b]	18	18	100	9	8	89
	control[d]	25	0	0	16	0	0
Big Creek coho salmon	5[b]	25	1	4	25	0	0
	control[d]	25	0	0	25	0	0
Round Butte chinook salmon	3[c]	27	0	0	30	0	0
	control[d]	25	0	0	27	0	0

[a] Number of fish exposed minus number of fish that died before spores were detected.
[b] Fifty fish exposed; 25 were transferred to fresh water and 25 to salt water.
[c] One hundred fish exposed; 50 were transferred to fresh water and 50 to salt water.
[d] Control fish were not exposed to the infectious stage of *C. shasta*.

1–24% of the individuals of the chinook salmon groups caught by beach seine (Table 1). The prevalence of infection was between 1–3% in groups collected from May through June but increased to 12–24% from July to the final sample period in September. The prevalence of infection among yearling, purse-seined chinook salmon also showed a tendency to increase during the later collection periods (Table 2). Among the yearling chinook salmon, the prevalence of infection in groups collected from May through June was generally less than 10%, except for a 20 May collection where three of nine fish developed ceratomyxosis. Infection incidence after July averaged 15%. The total incidence of *C. shasta* infection among chinook salmon was 9% for beach-seined groups and 11% for purse-seined groups. Smaller numbers of coho salmon and steelhead trout were collected in May and early June, during the peak of their migration. The prevalence of infection for these species was 5 and 12%, respectively (Tables 3 and 4).

In 1984, 463 chinook salmon smolts were caught in beach seines between 5 July and 20 September. Infection incidence among these subyearling salmon averaged 14% (Table 5).

Effects of Salt Water

All Alsea steelhead trout held in fresh water after exposure to *C. shasta* died from ceratomyxosis (Table 6). In groups of Alsea trout transferred to salt water, between 26 and 76% of the fish died prior to development of the disease; losses of 36 to 56% of unexposed, control fish held in salt water indicated that these deaths were caused by inability of the fish to adjust to saltwater conditions. The prevalence of infection among fish surviving the prepatent losses in salt water was lower in two of the four groups than among fish transferred to fresh water. Big Creek coho and Round Butte chinook salmon were resistant to infection when held in either fresh or salt water after exposure to *C. shasta*.

Sensitivity of Detection Methods

Detection of *C. shasta* infections by standard wet mount examination of intestinal tract scrapings was less sensitive than detection by indirect fluorescent antibody techniques (IFATs) in which a monoclonal

Table 7

Comparison of detection sensitivity between wet mount examination for spores and indirect fluorescent antibody techniques (IFAT) using monoclonal antibodies against *Ceratomyxa shasta* prespore stages.

		Wet mount		IFAT	
Sample origin[a]	No. samples	No. positive	% positive	No. positive	% positive
Spring chinook salmon					
Willamette Hatchery	20	8	40	19	95
Clackamas Hatchery	27	16	59	25	93
Fall chinook salmon					
Bonneville Hatchery	40	7	17	14	35
Coho salmon					
Big Creek Hatchery	14	14	100	14	100
Cascade Hatchery	20	17	85	20	100
Total	121	62	51	92	76

[a] Adult prespawning mortalities.

antibody was used. Of 121 intestinal samples examined where both methods were used, 51% of the samples were diagnosed as positive for the presence of spores by the wet mount technique and 76% were diagnosed positive for the presence of prespore and spore stages using IFATs (Table 7).

Discussion

Evaluating the impact of ceratomyxosis on salmonid populations requires consideration of a variety of factors. Two of these, geographic distribution of the parasite and resistance to infection of resident salmonids, have been examined in previous studies (Zinn et al. 1977; Johnson et al. 1979; Buchanan et al. 1983; Hoffmaster et al. 1988). Past studies have demonstrated the presence of the infectious stage of *C. shasta* in the mainstem of the Columbia and Snake Rivers and have shown that parasite presence and concentration vary between locations. There is also evidence that its distribution has increased within the basin. Because all salmonids migrating in the Columbia River Basin are exposed to *C. shasta,* native strains have developed some resistance to infection. However, resistance is not complete in the fish populations studied and little is known about the susceptibility of feral and upper Columbia and Snake River salmonids to infection by *C. shasta.*

The prevalence of infection among outmigrating fish was higher than the incidence predicted from susceptibility studies. In studies by Zinn et al. (1977) and Buchanan et al. (1983), the infection incidence among Columbia River strains of chinook salmon and steelhead trout was generally less than 5%, even when fish were exposed to the infectious stage for 120 days. However, of 2084 chinook salmon smolts collected at Jones Beach during 1983–84, 221 fish or 10.6% died from ceratomyxosis. The number of fish infected did not vary significantly between year classes, as the prevalence in yearling purse-seined chinook salmon paralleled that of the subyearling chinook salmon collected in beach seines. The prevalence also did not vary significantly between the 2 years of this study, although the number and type of samples collected were limited during 1984, but there was a trend toward increasing numbers of infected fish from the later collection dates.

The dates of peak migration at Jones Beach were determined by Dawley et al. (1984b) for each species. Coho salmon and yearling chinook salmon and steelhead trout migrated through the lower Columbia River during May and early June. Migration of subyearling chinook salmon past Jones Beach began to increase during May but reached its peak from early June to mid-July. This means that the majority of subyearling chinook salmon were migrating during periods of increasing water temperature when the impact from *C. shasta* is greater (Udey et al. 1975). Dawley et al. (1984b) also determined that migration rates were 22, 18, 17, and 35 km/day for subyearling chinook, yearling chinook and coho salmon, and steelhead trout, respectively. Therefore, the duration of exposure to *C. shasta* for subyearling and yearling salmon and steelhead trout migrating from Oxbow Dam on the Snake River, a distance of

over 1000 river miles from the Columbia Estuary, could be as long as 43, 53, and 27 days, respectively. Coho salmon originate from the lower Columbia River and therefore have a shorter time of exposure to the parasite. Long periods of exposure to *C. shasta* combined with the stress of migration and smoltification may explain why the numbers of chinook salmon and steelhead trout infected in our study are higher than numbers predicted from susceptibility studies. Also, it was not possible in this study to determine the strain origin of the infected fish so their resistance status cannot be assumed. Studies by Ratliff (1981) and Ching and Munday (1984) demonstrate that fish originating from rivers enzootic for the parasite can still be highly susceptible to infection. Ching and Munday exposed chinook salmon representing six stocks from the Fraser River, British Columbia, to the infectious stage of *C. shasta* in the lower Fraser River. Between 87 and 100% of the fish in each group died from ceratomyxosis. Ratliff (1981) further demonstrated that prevalence of infection may increase with increasing length of exposure to *C. shasta* and he calculated that 50–70% of all chinook salmon released into the Deschutes River may become infected. Prior to changes in the Columbia River Basin that resulted from the building of dams, upriver salmonids may also have avoided ceratomyxosis by migrating through infectious areas before parasite concentrations were high. However, the presence of dams and reservoirs has impeded outmigration, raised water temperatures (Raymond 1979) and may have created conditions favorable for proliferation of *C. shasta* (Ratliff 1981). These changes may have caused the range and numbers of the parasite to increase more rapidly in the upper portions of the basin than fish could adapt by developing resistance or avoidance strategies. Therefore, strains of salmonids from the upper Columbia and Snake Rivers may have lower or more variable levels of resistance than the strains that have been examined.

Because ceratomyxosis has a long incubation period, most salmonids that become infected during their downstream migration enter the ocean before the disease results in death. Acute ceratomyxosis has been reported in juvenile chum salmon (*O. keta*) captured off the coast of British Columbia (Margolis and Evelyn 1975). This finding indicates that the disease is not attenuated when fish enter salt water. To demonstrate this under laboratory conditions, Ching and Munday (1984) exposed chinook salmon to the infectious stage of *C. shasta* for 10 days, then held the fish in either fresh or salt water. They found that mortality was 100% in both groups. Similar experiments were conducted in this study using shorter exposure lengths and both susceptible and resistant strains of salmonids. Although survival was poor, in *C. shasta*-susceptible steelhead trout transferred to salt water after exposure, two of four groups had a lower incidence of infection than groups transferred to fresh water. It appears that migration to salt water may reduce the progress of ceratomyxosis if the fish are not overwhelmed by a large infectious dose. Entry into salt water did not impair the ability of resistant fish to block the infection.

While all of the studies described here show the impact of ceratomyxosis on captured fish held under laboratory conditions, many field studies rely on sampling fish prior to overt signs of ceratomyxosis. Reports by Yasutake et al. (1986) and Bartholomew et al. (1989a) indicate that spores do not form until late in the infection and that, in cases of acute ceratomyxosis, fish may die before spore development. Therefore, diagnosis made on the basis of identifying spores is likely to underestimate the prevalence of the parasite. To demonstrate this, an IFAT utilizing monoclonal antibodies specific for prespore stages of *C. shasta* was compared with examination by standard wet mount procedures. When both methods were used to examine samples collected from prespawning adult salmon mortalities, there was a 25% increase in the number of infections detected by using the serological method. An even greater increase in sensitivity could be expected when techniques are developed which can detect the earlier stages of *C. shasta* infection. Monoclonal antibodies and DNA probes that specifically recognize all life stages of the parasite are necessary for an accurate evaluation of the numbers of fish infected with *C. shasta*.

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Viral Infections of Cultured Fish in Japan

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ABSTRACT

Since infectious pancreatic necrosis virus and infectious hematopoietic necrosis virus were first isolated in the 1970s, more than 20 fish viruses have been isolated and at least 5 viruses have been observed by electron microscopy. Viral diseases are major problems and cause economic losses among cultured fishes in Japan and other countries. This paper reports our current understanding of the extent of viral infection in the cultured fishes of Japan.

Introduction

A virological study of cultured fishes in Japan was initiated when an unknown disease occurred among rainbow trout *Oncorhynchus mykiss* in the 1960s. The causative agent was identified as infectious pancreatic necrosis virus (IPNV) (Sano 1971). Subsequently, infectious hematopoietic necrosis virus (IHNV) was isolated from kokanee salmon *O. nerka* (Kimura and Awakura 1977). Since then, various viral infections of fish have been reported. At present, more than 20 fish viruses have been isolated and at least 5 viruses have been observed by electron microscopy studies (Table 1).

Viral Diseases of Salmonid Fishes

Infectious Pancreatic Necrosis

Infectious pancreatic necrosis is an acute systemic disease affecting the fry and fingerlings of rainbow trout. Its occurrence is widespread in Japan. Susceptibility of fish to IPNV depends on body weight; smaller fry are more susceptible. The signs of this disease are darkening of body coloring, moderate exophthalmia, and abdominal distention. Internally, the spleen, heart, liver, and kidneys are pale and the digestive tract is almost always devoid of food. Rainbow trout is the fish most affected by IPNV, but the virus has also been isolated from amago (*O. rhodurus*) and masu salmon (*O. masou*). Recently, the fry of rainbow trout were observed to be less susceptible to IPNV and, consequently, damage attributed to IPN has decreased (Okamoto et al. 1987).

Infectious Hematopoietic Necrosis

Infectious hematopoietic necrosis is an acute systemic disease which mainly affects the fry of rainbow trout and masu and kokanee salmon but which has also been isolated from moribund ayu *Plecoglossus altivelis* (Yoshimizu et al. 1987c). The characteristic sign of IHNV infection is V-shaped hemorrhages located in muscle tissue. Recently, a large rainbow trout, with a body weight of 50–80 g was found to be infected with IHNV and subsequently died (Mori et al. 1987). In this case, petechiae were observed in the fatty tissues and on the wall of the body cavity. This virus is widespread and especially prevalent in the central part of Honshu, the Japanese mainland (Sano et al. 1977). In several districts, river waters have been contaminated with IHNV and are now unsuitable for rainbow trout culture. Although the vertical transmission of IHNV is doubtful (Yoshimizu et al. 1988, a and b), it can be controlled by disinfecting eggs with iodine during the early eyed stage. Fish at the fry stage are very susceptible to IHNV. They should be reared in either well water or ultra-violet irradiated river water. When fish are past this sensitive stage, they can be transferred to the usual rearing ponds.

Table 1
Viral infection in cultured fishes in Japan.

Isolated virus	Host
DNA virus	
Nerkavirus in Towada Lake, Aomori and Akita Prefecture (NeVTA)	Kokanee salmon
Oncorhynchus masou virus (OMV)	Masu salmon
Yamame tumor virus (YTV)	Yamame (masu salmon)
Icosahedral cytoplasmic deoxyribovirus (ICDV)	Japanese eel
Herpesvirus cyprini	Fancy carp
Herpesvirus	Japanese eel
Unidentified small virus	Tiger puffer
RNA virus	
Infectious pancreatic necrosis virus (IPNV)	Salmonid fish
Infectious hematopoietic necrosis virus (IHNV)	Salmonid fish
Chum salmon virus (CSV)	Masu salmon
Yellowtail ascitic virus (YAV)	Yellowtail
Rhabdovirus olivaceus (HRV)	Various marine fish
Eel virus from European eel (EVE)	European eel
Eel virus of America (EVA)	American eel
Eel virus of Europe X (EVEX)	European eel
Papovavirus	Japanese eel
Birnavirus	Yellowtail
Birnavirus	Japanese flounder
Birnavirus	Red sea bream
Coronavirus cyprini virus (CACV)	Common carp
Picornavirus	Japanese eel
Reovirus	Common carp
Reovirus	Japanese eel
Observed by electron microscopy	
Viral erythrocytic necrosis virus	Various marine fish
Lymphocystis virus	Various marine fish
Paramyxovirus	Black rockfish
Herpesvirus	Japanese flounder
Picornavirus	Ishidai

Herpesvirus Infection

A herpesvirus, nerkavirus in Towada Lake Akita and Aomori Prefecture (NeVTA), was first isolated from diseased kokanee salmon in Towada Lake (Sano 1976). In 1978, another herpesvirus was isolated from the ovarian fluid of apparently normal mature masu salmon (Kimura et al. 1980). This virus was named *Oncorhynchus masou* virus (OMV) from the scientific name of the host fish. *Oncorhynchus masou* virus was found to be pathogenic and significantly more oncogenic in young masu salmon and several other salmonid fish (Kimura et al. 1981, a and b; Yoshimizu et al. 1987a). In 1983, a similar herpesvirus were isolated from tumor tissue of yamame (landlocked *O. masou*) and was named yamame tumor virus (Sano et al. 1983). Subsequent study showed that OMV is enzootic in the northern part of Japan (Yoshimizu et al. 1988b) and that the characteristics of these three herpesviruses are similar except that NeVTA lacks oncogenicity (Hedrick et al. 1987; Sano et al. 1988). In 1983, we recommended the disinfection of fish eggs with iodine at the early eyed stage in Hokkaido. Now OMV cannot be detected in most of the hatcheries in this area (Yoshimizu et al. 1988b). Although the host species of this virus is primarily masu salmon, OMV has also been isolated from the tumor tissues of pen-cultured coho salmon, *O. kisutch.*

Chum Salmon Virus Infection

In 1978, a reovirus was isolated from an apparently normal adult chum salmon, *O. keta* returning to its hatchery in Hokkaido (Winton et al. 1981). After initial isolation and characterization, it was named chum salmon virus (CSV). This virus was not observed again until 1986, during an episode of mass mortalities of masu salmon fry for which it was responsible. Since then, the virus has been detected in stocks of adult masu salmon at new locations in Hokkaido (Yoshimizu 1988). Artificial infection studies of this virus showed no significant mortality in several species of salmonid fishes (Winton et al. 1989).

Viral Erythrocytic Necrosis

Inclusion bodies stained with Giemsa were observed in the erythrocytes of chum and pink salmon, *O. gorbuscha,* collected in Okhotsuku and along the north Pacific coast of Hokkaido. The causative agent of viral erythrocytic necrosis (VEN), an iridovirus, was subsequently observed by electron microscopy (Yoshimizu et al. 1988b).

Viral Infections of Eels

Many viruses have been isolated from cultured eels (*Anguilla anguilla, A. japonicus,* and *A. rostrata*) by Sano (1976) and Sano and Fukuda (1987). They include a birnavirus, eel virus from the European eel (EVE); the rhabdoviruses, eel virus of America (EVA) and eel virus of Europe X (EVEX); papovavirus; herpesvirus; picornavirus; and a reovirus. These viruses are not recognized as pathogenic against eel except for EVE (Nishimura et al. 1981). Sorimachi (1982, 1984) reported having isolated icosahedral cytoplasmic deoxyribovirus (ICDV) from a diseased eel. This

virus was shown to be pathogenic against Japanese eels following artificial infection. Mortality was 40–75% at water temperatures of 14.5–18.5° C, 15% at 22.8° C, and 0% at 24.1° C. Infected fish showed signs of decoloration; congestion of the anal, pectoral, and dorsal fins; and an increase of mucus on the body surface.

Viral Infections of Carp

Herpesvirus cyprini was isolated from papilloma tissue of cultured fancy carp (*Cyprinus carpio,* also called common or asagi carp) and confirmed as the agent of infection by induction of epithelial tumors by artificial infection (Sano et al. 1985). *Coronavirus cyprini,* carp coronavirus (CACV), was isolated from diseased common carp raised in the laboratory. Fish infected with CACV showed acute mortality showing no external signs except erythematous skin on the abdomen. Experimentally, CACV was virulent for carp fry at 20° C. Cumulative mortality for 3-week-old fry was 72.5%. The affected fish manifested swollen and hemorrhagic abdomens filled with ascites and eventually died. Reovirus was also isolated from common carp (Sano and Fukuda 1987).

Viral Infection of Other Marine Fishes

Viral Pancreatic-Hepatic Necrosis of Yellowtail

A yellowtail ascites virus (a birnavirus) was isolated from the fry of yellowtail *Seriola quinqueradiata* (Sorimachi and Hara 1985). This epizootic is an acute viral infection of both naturally grown and hatchery-raised fry. The epizootic period occurs from May to June at water temperatures of 18 to 22° C. The moribund fry typically show anemic gills, hemorrhaging in the liver, and ascites and petechiae in the pyloric caea. The disease name, viral pancreatic-hepatic necrosis, was proposed by Egusa and Sorimachi (1986).

Rhabdovirus Infection of Japanese Flounder

The rhabdovirus, *Rhabdovirus olivaceus*—also referred to as hirame rhabdovirus (HRV)—was isolated from diseased hirame (Japanese flounder), *Paralichthys olivaceus,* and black sea bream, *Milio macrocephalus* (Gorie et al. 1985; Kimura et al. 1986). This virus is pathogenic for marine fish such as hirame, black sea bream, red sea bream *Pagrus major* and black rockfish *Sebastes inermis,* and also for salmonid species, especially rainbow trout and masu salmon (Yoshimizu et al. 1987b). Signs of HRV infection are gonadal congestion, focal hemorrhage of skeletal muscle and fins, and accumulation of ascitic fluid. Hirame rhabdovirus is distributed widely from Hokkaido to Honshu in Japan.

Kuchishiroshou of Tiger Puffer

From cultured tiger puffer, *Fugu rubripes,* an unidentified, small virus was isolated (Inoue et al. 1986). The epizootic period is from May to June during water temperatures of 18–22° C. Moribund fish had necrosis around the mouth and had been observed to be fighting with each other. From the signs of this infection, the disease was named "Kuchishiroshou", from the Japanese words "kuchi", meaning mouth, "shiro", meaning white, and "shou", meaning disease. Viral particles were observed in the brain by electron microscopy. Kuchichiroshou occurs in southwest Japan where tiger puffer are cultured.

Epidermal Hyperplasia of Japanese Flounder.

Outbreaks of a disease resulting in mass mortalities of larval and juvenile Japanese flounder was reported by Iida et al. (1989). Once the disease occurs in a pond, the resident fish populations usually become extinct within one month. Affected fish are characterized by opaque fins. Histopathologically, hyperplasia is observed in the epidermal layer of the fins and skin. In the epidermal tissues of infected fish, hexagonal virus particles were observed by electron microscopy. Japanese flounder larvae experimentally exposed to the filtrate of infected tissue homogenate suffered 18–50% mortalities with 93-100% of the survivors exhibiting epidermal hyperplasia. This virus has not been isolated in any of the 33 fish cell lines in which culture has been attempted, including that of the host species.

Birnaviruses were also isolated from Japanese flounder and red sea bream (Yoshimizu and Kimura, unpubl. data). These viruses were neutralized with antibody against IPNV; the pathogenicities of these birnaviruses have not been clarified.

Lymphocystis Disease

In several species of marine fishes, suzuki, *Lateolabrax japonicus,* yellowtail, red sea bream, Japanese flounder, and others, lymphocystis disease was re-

ported and iridovirus was observed by electron microscopy (Matsusato 1975; Miyazaki and Egusa 1972; Tanaka et al. 1984). Seasonal variation in the prevalence of lymphocystis was noted with increased prevalence in summer. Lymphocystis cells were observed mainly on the fins or body surface. The virus particles were polyhedral, presenting hexagonal or pentagonal profiles in tissue sections. They may be seen in a crystalline array and are always located in the cytoplasm.

Other Diseases

Yoshikoshi and Inoue (1988) reported picornavirus in moribund fry of ishidai, *Oplegnathus fasciatus*, and Miyazaki et al. (1989) reported herpesvirus in the epidermal necrosis of Japanese flounder and paramyxovirus in the epithelial necrosis of black sea bream. To date, these viruses have not been isolated.

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Some Important Infectious Diseases of Kuruma Shrimp, *Penaeus japonicus*, in Japan

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ABSTRACT

The kuruma shrimp, *Penaeus japonicus*, is the most widely cultured crustacean in Japan. Shrimp farming operations for this species have been greatly affected by several infectious diseases including baculoviral midgut gland necrosis (BMN), vibriosis, and black gill disease. This paper discusses our knowledge of the BMN virus in detail, and brieflly reviews the latter two diseases with respect to the culture of crustaceans.

Introduction

It may safely be said that kuruma shrimp, *Penaeus japonicus*, is the only crustacean species cultured on an industrial scale in Japan. About six to seven hundred million post-larval kuruma shrimp are produced at public and private hatcheries every year for stocking and farming (Japan Sea Farming Assoc. 1990). Annual production of cultured kuruma shrimp reached 3,020 metric tons in 1988 (DSI 1990).

The infectious diseases responsible for high mortalities to cultured kuruma shrimp in Japan are baculoviral midgut gland necrosis (BMN), vibriosis, and black gill disease. Baculoviral midgut gland necrosis is a very severe viral disease that occurs during the seed production process which usually causes 90% or higher mortalities in the population of the rearing tank. Recently, the frequency of BMN outbreaks has decreased greatly, probably because of the preventive measures executed at hatcheries. Vibriosis is a bacterial disease affecting the lymphoid organ that has been responsible for a great deal of damage to the shrimp farming industry over the past several years. Black gill disease is caused by *Fusarium solani*, a ubiquitous pathogen of shrimp and lobster, invading the gills, appendages, and various parts of the exoskeleton.

In the present paper BMN is discussed in detail, and vibriosis and black gill disease are touched briefly.

Baculoviral Midgut Gland Necrosis

To date, four baculoviruses have been recorded in penaeid shrimp worldwide (Lightner 1985; Lester et al. 1987; Johnson and Lightner 1988). They exhibit higher pathogenicity to larval or postlarval shrimp than to adults. Baculoviral midgut gland necrosis was first noticed in 1971, and since then it has often caused mortalities of over 90% during the mass production of kuruma shrimp larvae in Japan (Momoyama 1981; Sano et al. 1981). Heavy losses due to baculovirus infection during the larval production of *P. aztecus* (Couch 1978) and *P. monodon* (Lightner et al. 1983) have also been reported in other countries.

The results of our histological and epizootiological studies on BMN in kuruma shrimp are summarized as follows.

Histopathology

Midgut glands of diseased shrimp become soft and develop a white turbid appearance at the advanced stage of infection, whereas those of healthy postlarvae appear brown or colorless during the early developmental stages (Momoyama 1981). From histological examinations, it was confirmed that only the midgut gland and the intestine are affected by the disease. Disarrangement and exfoliation of epithelial cells are remarkable in the midgut gland of the diseased shrimp. Nuclear hypertrophy and chromatolysis of infected epithelial cells are the most characteristic cytopathological changes in BMN (Momoyama 1981). In contrast with other penaeid shrimp baculovirus infections, no inclusion bodies are present in the hypertrophied nuclei (Lightner 1985; Lester et al. 1987; Johnson and Lightner 1988).

Electron microscopy observations of the hypertrophied nuclei and the midgut gland lumen have revealed many rod-shaped particles having outer and inner envelopes, which represent virions of the baculovirus group. The average length and diameter of the virions are 310 nm and 72 nm, respectively (Sano et al. 1981).

Baculoviral midgut gland necrosis can be diagnosed by detecting the hypertrophied nuclei of affected midgut gland epithelial cells in both fresh and stained squash preparations. In stained squash preparations homogeneous hypertrophied nuclei about 20 to 30 μm in diameter appear among the smaller normal ones which are about 10 μm in diameter. The Feulgen reaction makes the difference clearer between hypertrophied and normal nuclei. The diagnostic technique of using a dark field microscope with a wet-type condenser has the advantages of precision and rapidity. Hypertrophied nuclei are clearly seen as white bodies of 10 to 30 μm diameter in fresh squash preparations from diseased samples under the dark field microscope. The reason why the infected nuclei appear white is thought to result from an increased number of reflected or diffracted rays due to the numerous virus particles in the nucleus (Momoyama 1983).

Source of Infection

Epizootiological investigations indicate that mature female kuruma shrimp spawners with latent BMN-virus infections and cultured young kuruma shrimp that have recovered from infection with the virus are the main source of infection in hatchery epizootics (Momoyama 1988). Histological examinations reveal nuclear hypertrophy of the midgut gland epithelial cells in both spawners and young cultured survivors of the disease. Fluorescent antibody techniques have been used to reveal the presence of BMN-specific virus antigen in the hypertrophied nuclei of spawners (Momoyama 1988).

The Effect of Developmental Stage of the Host on the Susceptibility to BMN Virus

The susceptibility of kuruma shrimp to BMN virus by waterborne infection (Momoyama and Sano 1988) was determined for fertilized eggs, nauplii, zoeae, mysis larvae, and postlarvae (2, 4, 6, 8, and 10 day old) (Momoyama and Sano 1989). When the fertilized eggs and nauplii were exposed to the virus, they showed no evidence of infection. But when the zoeae, mysis larvae, and postlarvae were exposed, some or all of the shrimp were diagnosed to be infected on the final day of the test period (6 to 16 days). Susceptibility to infection tended to decrease with the advance in developmental stages from zoea to P-10 (10-day-old postlarva). Cumulative mortalities decreased from 100% in zoeal stage larvae to 0% in P-6 larvae. The growth rates of test shrimp inoculated at stages P-2 through P-6 were lower than those of controls, but there were no differences in mortality or growth rates between test shrimp and controls inoculated at stages P-8 and P-10. These results indicate that kuruma shrimp from the zoeal to the P-6 stage are highly susceptible to BMN virus, but stage P-8 or older postlarvae become refractory to this disease.

The route of baculovirus infection in shrimp has generally been considered to be by oral ingestion of virus contaminated sediments or by cannibalism of diseased shrimp (Lightner et al. 1983; Couch 1974). In the previously mentioned study on waterborne susceptibility, kuruma shrimp were not administered any food during the inoculation period. However, peristaltic movements were frequently observed in the oesophageal part of the shrimp, suggesting that test shrimp ingest the seawater containing the virus particles through the mouth. This hypothesis was supported by an observation that azocarmine G accumulated in the stomach and midgut gland lumen of shrimp when they were dipped in seawater containing this dye (Momoyama and Sano 1989).

Inactivation and Survival of BMN Virus

The effects of disinfectants, heating, and ultraviolet irradiation on BMN virus, and the survival time of the virus in seawater at different temperatures were examined by waterborne infectivity experiments using larval and postlarval kuruma shrimp. The virus was inactivated by 10-minute exposure at 25° C to any of the following chemicals: 5 ppm chlorine, 25 ppm iodine, 100 ppm benzalkonium chloride and benzethonium chloride, 30% ethyl alcohol, and 0.5% formalin (Momoyama 1989a). The virus was still active after 2 hours of heat treatment at 35 and 40° C, but was inactivated within 2 hours at 45° C, 30 minutes at 50 and 55° C, and 5 minutes at 60° C (Momoyama 1989b). The virus was also inactivated within 20 minutes by ultraviolet irradiation with a 15-watt ultraviolet lamp at a distance of 30 cm (Momoyama 1989b). In seawater, the virus could not survive longer than 4 days at 30° C, 7 days at 25° C, 12 days at 20° C, and 20 days at 15° C (Momoyama 1989c).

Prevention

The following two preventive measures are now used against this epizootic in some hatcheries. One prevents vertical infection from spawners by rinsing the fertilized eggs with virus-free seawater then transferring them to a disinfected rearing tank. The other prevents horizontal infection by adding chlorine in the rearing tank to kill infected populations. Since 1985, the measure to prevent vertical infection has been carried out on an industrial scale, and BMN has never occurred in the hatcheries where this treatment has been practiced.

Vibriosis

Vibriosis has caused a great deal of damage to the shrimp farming industry over the last several years.

Although some dead shrimp infected with vibriosis develop white turbid muscle at the 6th abdominal segment, shrimp suffering from this disease do not usually show any specific external clinical signs.

The lymphoid organ (Oka 1969) is intensively invaded by the causative bacterium resulting in extensive necrosis and nodule formation (Egusa et al. 1988). The nodules are seen by the unassisted eye as very small black spots and are composed of a bacterial colony in the center, a melanized zone around the bacterial colony, and multiple layers of hemocytes encapsulating the melanized zone. Small nodules are also frequently observed in other organs such as the gills, heart, midgut gland, and abdominal musculature, but extensive necrotic lesions can not be found in these organs.

The *Vibrio* sp. isolated from kuruma shrimp has been identified as a new species (Takahashi et al. 1985a), and was tentatively named *Vibrio* sp. PJ (PJ is the abbreviation of the scientific name of kuruma shrimp *Penaeus japonicus*). *Vibrio* sp. PJ has very high pathogenicity to kuruma shrimp. LD_{50} values obtained by intramascular injection were about 20 to 100 cells/g body weight of shrimp.

Now, two antibiotics, oxytetracycline (Takahashi et al. 1985b) and oxolinic acid, are on the market with the government's approval. Although mortalities are decreased significantly by administering these antibiotics, disease often returns shortly after treatment.

Black Gill Disease

Black gill disease often occurs in the intensive culture systems in the Okinawa and Kagoshima districts, but rarely in those of the Chugoku district.

Black gill disease is caused by *Fusarium solani*, which is a member of the imperfect fungi. This fungus is a ubiquitous pathogen and infects penaeid shrimp (Cook 1971; Lightner 1975) as well as lobster (Lightner and Fontaine 1975; Alderman 1981) and freshwater shrimp (Burns et al. 1979). The fungus usually invades the gills, appendages, and various parts of the exoskeleton.

In kuruma shrimp, gills are most susceptible to the fungus and infected gills always become black (Ishikawa 1968; Bian and Egusa 1981). In the degenerating gill filaments, hemal channels are found extremely congested with hemocytes, encapsulated hyphae, and tissue debris. The fungus also often penetrates into the thoracic central nerve and sometimes into the ventral thoracic artery (Momoyama 1987). Tissue destruction, cellular inflammation, and hyphae encapsulated by multiple layers of hemocytes are always observed in the lesion. Failure to exchange gas in the gills and damage to the central nerve and ventral thoracic artery are thought to be responsible for death.

As blackening of the gills in shrimp is often induced by various causes, detection of macro- and micro-conidiospores is necessary for diagnosis of this disease (Egusa and Ueda 1972).

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The Application of Molecular Biology to the Detection of Infectious Hematopoietic Necrosis Virus

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ABSTRACT

Traditionally, the detection of most fish viruses, including infectious hematopoietic necrosis virus (IHNV), has relied upon isolation of the virus in cell culture and identification by serum neutralization or fluorescent antibody assays using polyclonal rabbit antisera. In recent years, the techniques of molecular biology have provided new strategies for rapid and sensitive detection of antigens, antibodies, and nucleic acids. This paper reviews the application of these powerful new methods for the detection of IHNV. Included, are discussions on the creation of monoclonal antibodies specific for viral proteins, development of enzyme-linked immunoassays for detection of viral antigens and antibodies, electrophoretic identification of radiolabeled viral proteins, a nonradioactive DNA probe specific for the nucleoprotein (N) gene messenger RNA of IHNV, and a polymerase chain reaction (PCR) based method for amplification of genomic or messenger RNA of the virus. As these methods become more widely accepted, they will result in significant improvements in the speed, precision, and sensitivity of IHNV detection.

Introduction

Infectious hematopoietic necrosis (IHN) is the most important viral disease of trout and salmon in western North America. Depending upon the species, stock, and size of the fish, strain of the virus, and environmental conditions such as temperature, an outbreak of IHN may result in losses approaching 100% when fish are infected at a small size. Among groups of larger fish, mortality is reduced, often becoming chronic, with a typical loss of 25% or less (Wolf 1988). Presently, no control measure for IHN is available other than avoidance of exposure to the causative rhabdovirus, infectious hematopoietic necrosis virus (IHNV). This has not proven practical at many large hatcheries with open water supplies or at commercial trout operations where production of fish occurs on a continuous basis. The chemical, physical, and serological characteristics of IHNV, essential features of the biology of the virus, and a description of the host and geographic range, have been extensively reviewed (McAllister 1979; Pilcher and Fryer 1980; Nicholson 1982; Wolf 1988).

Virological examinations are regularly conducted on stocks of trout and salmon. These include routine health checks, diagnostic examinations to identify causes of mortality, and pathogen-free certification examinations, which are required prior to moving fish from one location to another. Traditional methods for isolation and identification of IHNV rely upon cell cultures for recovering the virus and serum neutralization or fluorescent antibody assays to identify the agent, a time-consuming and expensive process (Amos 1985). Recently, new techniques from molecular biology utilizing monoclonal antibodies, enzyme-linked immunosorbent assays, nonradioactive DNA probes, and the polymerase chain reaction, promise to provide new tools for detection and identification of IHNV that are more rapid, sensitive, specific, and cost-effective.

Electrophoresis of Structural Proteins

Leong et al. (1981) described a method for identification of different strains of IHNV using

polyacrylamide gel electrophoresis (PAGE) and autoradiography of the virion structural proteins that had been radioactively labeled with ^{35}S-methionine. The method required approximately 48 hours and provided a type of fingerprint that would not only confirm the presence of IHNV, but yielded data about the strain of the virus, giving researchers additional epizootiological information. Hsu and Leong (1985) compared this electrophoretic method with two immunoblotting techniques using either ^{125}I-labeled Protein A or peroxidase to detect the rabbit anti-IHNV serum bound to structural proteins separated by PAGE and transferred to nitrocellulose membranes. When used to detect and confirm the presence of IHNV, the direct electrophoretic method was the most sensitive and rapid of the three. Although the need to use radioactive materials has limited the field application of this method, it has proven useful in analyzing the structural proteins of IHNV (Hsu et al. 1985) and in identifying strains of the virus (Hsu et al. 1986). This electropherotypic analysis (based upon variation in the molecular weights of structural proteins) has become widely used in various epizootiological studies of IHNV.

Monoclonal Antibodies

Monoclonal antibodies (MAbs) are useful for both research and diagnostic applications as they are highly reactive, very consistent, and do not cross-react with other antigens to any significant extent (Harlow and Lane 1988). The first monoclonal antibody against IHNV was developed by Schultz et al. (1985). While the antibody lacked neutralizing activity, it could be biotinylated and used to develop an immunoblot assay for IHNV (Schultz et al. 1989). For maximum sensitivity, the assay required initial amplification of the virus in cell culture where as few as 100 plaque forming units of IHNV could provide a positive diagnosis of IHNV as early as 36 hours after infection.

A monoclonal antibody was compared with polyclonal rabbit serum in a rapid fluorescent antibody test (FAT) for IHNV by LaPatra et al. (1989). The FAT used fish cell cultures grown on coverslips and infected with IHNV for approximately 48 hours at which time the test approached the plaque assay in sensitivity and offered a significant improvement in speed. One advantage of the MAb over the polyclonal serum was that it did not require extensive adsorption before use. Arnzen et al. (1991) used a monoclonal antibody that was conjugated with fluorescein isothiocyanate to produce a direct FAT that detected IHNV antigens in cell cultures within 6–8 hours after infection.

Another application of monoclonal antibodies for detection of IHNV includes an immunohistochemical staining method developed by Yamamoto et al. (1989). The method proved useful for in situ detection of fish cells initially infected with IHNV following waterborne exposure to the virus (Yamamoto et al. 1990). Monoclonal antibodies have also been use to detect neutralization variants among strains of IHNV (Winton et al. 1988) and in determining important epizootiological information about the distribution of IHNV strains (Ristow and Arnzen 1989).

Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) is an important tool for the rapid and sensitive detection of antigens. An ELISA was used to detect IHNV antigens present in infected cell cultures and fish tissues (Dixon and Hill 1984; Way and Dixon 1988). Because the assays used polyclonal antisera, low level cross-reactions were observed with other fish rhabdoviruses and with uninfected fish cell cultures or fish tissue extracts. While differences between antisera affected the results, the IHNV ELISA appeared to be quite specific and could detect infections in cell culture as early as 48 hours after infection at the time that cytopathic effect was first noted. The assay was also used to detect IHNV in acutely infected fry.

A modification of the ELISA, the dot blot, where IHNV antigens were spotted onto nitrocellulose paper and detected by labeled antisera, was reported by McAllister and Schill (1986). This assay was rapid and required no special instrumentation, but the polyclonal IHNV antiserum required extensive adsorption with both uninfected cells and fetal bovine serum to remove cross-reacting antibodies before use. While the assay was not suitable for use with tissues or fluids because the filter matrix became clogged, it was able to detect IHNV in cell culture supernatant fluids at concentrations of 10^5 to 10^6 plaque forming units (PFU)/mL when initial cytopathic effect was observed.

Although fish lack the complex immune system of the higher vertebrates, it is possible to confirm past infection with IHNV using methods that detect the presence of antibodies in serum. Amend and Smith (1974) reported that rainbow trout *Oncorhynchus mykiss* developed good titers of neutralizing antibodies that could be detected at least 90 days after immunization with live virus. Techniques that have been used to assay for antibodies to IHNV in fish serum include neutralization, FAT, and ELISA (Amend and Smith 1974; Hattenberger-Baudouy et al. 1989; Jorgensen et al. 1991). Of these, the ELISA

appeared to have the best combination of sensitivity and ease of processing the numerous samples needed for serological screening of fish populations.

Nonradioactive DNA Probe

A biotinylated DNA probe for rapid detection of IHNV was developed by Deering et al. (1991). This probe was designed to detect the nucleoprotein (N) gene messenger RNA (mRNA) of IHNV because this molecule is synthesized early and in high abundance during viral replication. A 30 nucleotide target site for the probe was chosen by computer search of the published sequence of the N gene of IHNV (Gilmore and Leong 1988). A synthetic oligonucleotide, complementary to this sequence, was made by automated chemical synthesis, coupled with biotin, and detected with a streptavidin-peroxidase conjugate. The probe was shown to be specific for IHNV mRNA and detected as little as one picogram of target sequence. It did not react with mRNA of viral hemorrhagic septicemia virus or hirame rhabdovirus, but it did recognize strains of IHNV representing each of the electropherotypes described by Hsu et al. (1986). The method required initial amplification of the virus mRNA in fish cell cultures, but at high multiplicities of infection, detectable levels of mRNA could be extracted from these cells after less than 24 hours yielding a positive diagnosis in less than 48 hours.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) uses two DNA primers and repeated cycles of DNA synthesis performed by a thermostable polymerase to amplify low copy numbers of a specific nucleic acid sequence to levels that can be easily detected (Erlich 1989; Innis et al. 1990). Arakawa et al. (1990) used PCR to amplify a 252 nucleotide portion of the N gene of IHNV that included the site for the DNA probe used by Deering et al. (1991). The primers directed the synthesis of large amounts of DNA from all strains of IHNV tested. The DNA produced was confirmed to be of the appropriate sequence by Southern and dot blot assays. Sufficient messenger RNA for cDNA synthesis using reverse transcriptase and subsequent PCR amplification could be extracted from cell cultures infected for as little as 24 hours. The PCR was able to amplify target sequences from IHNV-infected rainbow trout to levels that were easily detected with a biotinylated probe. Recent tests using clinical material from IHNV-infected fish revealed that the method approached cell culture in sensitivity and was able to detect IHNV infections in rainbow trout for up to 32 weeks post-infection.

Discussion

Control of IHNV continues to rest upon avoidance of the virus through use of virus-free fish reared in virus-free water supplies (Wolf 1988). Currently, assurance of the virus-free status of salmonid fish requires a series of time-consuming, expensive, and labor-intensive procedures (Amos 1985). Many of the methods reviewed in this paper will be important improvements in the speed, sensitivity, and precision of these assays. These novel assays and procedures will find increasing use in examination of fish and fish eggs to be moved to new locations and in providing early diagnosis of IHNV outbreaks allowing prompt management actions.

One area of concern has been the ability to transfer these new methods to field stations where much of the fish diagnostic work is being performed. In this regard, the use of radioactive material, expensive equipment, or technically demanding procedures have not proven attractive. While it seems likely that improvements in speed and sensitivity of diagnostic methods are still possible, they must be developed with these limitations in mind.

In the future, additional methods will become available for control of IHNV. These include the use of antiviral drugs (Hasobe and Saneyoshi 1985; Kimura et al. 1990), chemicals (Batts et al. 1991), or modern vaccines based upon recombinant DNA technology (Leong et al. 1988). The combination of newer detection methods and improved control strategies promises to provide substantial reduction in the losses caused by this important virus.

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Bacterial and Viral Diseases of Marine Fish During Seed Production

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ABSTRACT

In Japan, seed production techniques have been developed for about 40 species of marine fishes; however, mass mortalities due to infectious and noninfectious diseases have often occurred. Among these problems are the bacterial and viral diseases reviewed in the present paper. Vibriosis and other bacterial diseases have occurred in various marine fishes during their juvenile stage. These diseases have essentially the same pathology in both juvenile and adult fish in their tendency to terminate in systemic infection, except for *Flexibacter* infections. On the other hand, larvae most frequently develop intestinal infections, such as bacterial enteritis with *Vibrio* sp. INFL in Japanese flounder. It has been suspected that live diets contaminated with pathogenic bacteria serve as an important source of these intestinal infections. Recently, some new viral infections, such as viral epidermal hyperplasia and viral nervous necrosis, have been reported in larval and juvenile marine fishes. Although isolation of the causative viruses in cultured cells has not been successful, except for yellowtail ascites virus (YAV), these diseases were confirmed to occur only in the larval, or larval and juvenile, stages by infection experiments and epidemiological surveys.

Introduction

In Japan, demand for coastal fisheries and aquaculture products has been increasing drastically over the past few decades, especially since the establishment of the 200-mile economic zone. Consequently, governmental programs have concentrated on the improvement and development of coastal fisheries stocks, including mass release programs for important species. At present, more than 100 million juveniles of about 40 marine finfish species are produced annually at national, prefectural, and private hatcheries. Red seabream (*Pagrus major*), Japanese flounder (*Paralichthys olivaceus*), and black seabream (*Acanthopagrus schlegeli*) are the representative species produced in southern Japan. Fish production techniques, particularly for the three species mentioned above, are apparently well established (Fukuhara 1987). However, during fish production, difficulties are often encountered in controlling diseases of known and unknown etiology. The present paper will review bacterial and viral diseases of marine fishes in the course of seed production in Japan.

Bacterial Diseases

A wide range of pathogenic bacteria have been isolated from cultured fishes during their juvenile stage (Table 1). *Vibrio anguillarum* infection has been reported in red seabream, tiger puffer (*Takifugu rubripes*), and Japanese flounder (Muroga and Tatani 1982; Muroga et al. 1987b ; and Yamanoi et al. 1988; respectively) and *Vibrio ordalii* infection has been observed in juvenile rockfish (*Sebastes schlegeli*) (Muroga et al. 1986). *Pasteurella piscicida* was isolated from juveniles of black seabream and red grouper (*Epinephelus akaara*) as a disease agent (Muroga et al. 1977; Ueki et al. 1990; respectively). *Flexibacter maritimus* infection has been reported in red seabream, black seabream, and flounder (Wakabayashi et al. 1984, 1986; Baxa et al. 1986). Except for *Flexibacter* infections, these bacterial infections terminate in septicemia, or systemic infection in juvenile fishes as they do in adult fishes. On the other hand, these systemic infections have rarely been reported during the larval stage. Instead, high mortalities during the larval stage are usually associ-

Table 1
Bacterial diseases in larval and juvenile marine fishes.

Stage	Name of disease	Pathogen	Host fish
Larval stage	Abdominal swelling	*Vibrio alginolyticus*	Red seabream
		Other pathogenic organisms	Black seabream
	Bacterial enteritis	*Vibrio* sp. INFL	Japanese flounder
Juvenile stage	Vibriosis	*Vibrio anguillarum*	Red seabream
			Japanese flounder
			Tiger puffer
		Vibrio ordalii	Rockfish[a]
	Pasteurellosis	*Pasteurella piscicida*	Black seabream
			Red grouper
	Flexibacter infection	*Flexibacter maritimus*	Red seabream
			Black seabream
			Japanese flounder

[a]*Sebastes schlegeli.*

ated with intestinal infections. These intestinal infections occur in the larvae of various marine fishes.

Abdominal swelling ("Fukubu-boman-sho" in Japanese) occurs in red and black seabreams (Iwata et al. 1978; Kusuda et al. 1986; respectively). *Vibrio alginolyticus* has been reported to be the causative agent or to be associated with the disease in both fish species. In an investigation of this disease in red seabream reared in extensive nursery ponds, various species of the genus *Vibrio*, including *V. alginolyticus* and *V. vulnificus*, were the predominant isolates from different batches of diseased larvae. The role of these vibrios was not made clear, however, because significant mortality or abdominal swelling could not be produced by oral challenge in red seabream larvae with these isolates (Yasunobu et al. 1988). A marine turbellarian (*Allostoma* sp.) was also reported to be associated with abdominal swelling of black seabream (Yamaguti 1987). At present, abdominal swelling of seabreams can only be interpreted as being a syndrome caused by ingesting some pathogenic or toxicogenic organisms.

A disease called "Chokan-hakudaku-sho" in Japanese, which means a disease condition characterized by opaque intestine, occurs in larval Japanese flounder. The causative agent is thought to be a *Vibrio* species isolated from an affected intestine (Murata 1987). According to Murata (1987), this disease occurs in 14 to 30- or 40-day-old larval flounder; symptoms include darkening of body color and opaqueness and shrinkage of the intestine. Mortalities sometimes reach 90% or higher, especially when it occurs in younger fish. The causative bacterium is a gram-negative, short rod, which is motile by its single polar flagellum. It grows well on ZoBell's 2216e agar and heart infusion or brain heart infusion agar with 3% NaCl, forming small, circular, gray-colored colonies. Based on biochemical characteristics and GC (guanine and cytosine) value (44.1 mol%) of DNA, the organism was placed in the genus *Vibrio*. However, the characteristics of this pathogen differ from those of previously described fish-pathogenic *Vibrio* species. The pathogen was tentatively named *Vibrio* sp. INFL after the disease it causes: intestinal necrosis of flounder larvae (Masumura et al. 1989b).

Infection experiments using *Vibrio* sp. INFL were carried out by oral administration via rotifers (*Brachionus plicatilis*) and brine shrimp (*Artemia salina*) nauplii, by addition to rearing tanks, and by intraperitoneal injection (Masumura et al. 1989b). The disease was reproduced in flounder larvae only by oral challenge. On the contrary, *V. anguillarum*, which was used for comparison, killed flounder juveniles by intraperitoneal injection, but did not kill flounder larvae by oral challenge. The pathogenicity of *Vibrio* sp. INFL therefore seems to be quite different from that of *V. anguillarum*.

Six different age groups of flounder (11, 16, 17, 27, 41, and 60 day olds) were submitted to an oral challenge test with *Vibrio* sp. INFL (Muroga et al. 1990). The bacteria were incorporated into brine shrimp nauplii or rotifers and were given for three days. As a result, mortality of the test groups was significantly higher than that of the respective controls in the groups of 16, 17, and 27-day-old fish. The characteristic clinical sign of the disease, an opaque

intestine, occurred and subsequently *Vibrio* sp. INFL was isolated from the intestine in these test groups. In the older fish groups (41- and 60-day olds), no apparent changes were observed in either the test groups or the controls. Based on these results and the before-mentioned epidemiological data on natural outbreaks of the disease, it was concluded that this bacterial enteritis is confined to the larval stage of flounder. Histopathological and electron microscopic examinations revealed that pathogen multiplication and resultant pathological changes occurred only in the intestine (Miyazaki et al. 1990; Muroga et al. 1990). Although pili-like structures were not observed on the cells of the pathogen, an adhesive property was demonstrated on a chinook salmon embryo (CHSE-214) cell line. These findings led us to the conclusion that the necrotic enteritis caused by *Vibrio* sp. INFL in flounder larvae is similar to enteric colibacillosis of young mammals (Muroga et al. 1990).

Investigations of the intestinal bacterial flora of larval and juvenile marine fishes seem to be essential to elucidate the pathogenesis of the above-mentioned intestinal infections in larval fishes. A method for isolating and enumerating the aerobic intestinal bacteria of larval and juvenile fish was devised by Muroga et al. (1987a); the intestinal bacterial flora of seabreams, flounder, and other fishes was subsequently investigated (Tanasomwang and Muroga 1988, 1989a). The compositions of the intestinal flora of these marine fishes were characterized by two predominating groups, *Vibrio* and *Pseudomonas*, which were thought to be derived from the diets of live rotifers and brine shrimp nauplii. The same genera, *Vibrio* and *Pseudomonas*, were most frequently isolated from these live diets (Tanasomwang and Muroga 1990). Sodium nifurstyrenate bath proved effective in reducing bacterial contamination of rotifers (Hayashi et al. 1976; Yamanoi and Sugiyama 1987; Tanasomwang and Muroga 1989b), and recently, it was also reported that the bacterial contamination of rotifers and brine shrimp was significantly reduced by freezing at –15° C for 1 month (Yamanoi and Katayama 1989).

Viral Diseases

Recently, several viral diseases have been reported in the larvae and juveniles of marine fishes (Table 2). These include viral ascites of yellowtail (*Seriola quinqueradiata*), viral epidermal hyperplasia of Japanese flounder, viral epithelial necrosis of black seabream, and viral nervous necrosis of Japanese parrotfish (*Oplegnathus fasciatus*), red grouper and striped jack (*Pseudocaranx dentex*). Except for the first virus mentioned, the causative agents have not been isolated in cultured cells.

In 1983, an acute disease characterized by ascites occurred among yellowtail juveniles cultured in a hatchery, and an IPNV (infectious pancreatic necrosis virus) -like virus, which was named YAV (yellowtail ascites virus), was isolated in several cell lines including RTG-2 and CHSE-214. It was confirmed by an immersion challenge that the disease could be reproduced in yellowtail juveniles and that the mortality was higher in smaller fish (Sorimachi and Hara 1985; Sorimachi and Egusa 1986). The same disease occurred among wild juveniles of yellowtail which were caught as seedlings for net cage culture (Isshiki et al. 1989). Histopathological observations of naturally and experimentally infected fish suggest that acinous tissues of the pancreas and parenchymal tissue of the liver are the primary tissues involved in YAV infection (Egusa and Sorimachi 1986).

Since 1985, outbreaks of a disease resulting in mass mortalities have occurred in larvae and juveniles of the Japanese flounder cultured at several hatcheries.

Table 2
Viral diseases in larval and juvenile marine fishes.

Stage	Name of disease	Pathogen	Host fish
Larval stage	Viral epidermal hyperplasia (Viral epidermal necrosis)	Herpesvirus	Japanese flounder
	Viral epithelial necrosis	Paramyxovirus-like virus	Black seabream
	Viral nervous necrosis	Picornavirus-like virus	Japanese parrotfish Red grouper Striped jack Seabass[a]
Juvenile stage	Viral ascites	YAV (Yellowtail ascites virus=IPNV-like)	Yellowtail

[a]*Lates calcarifer.*

The disease occurred in 10- to 30-day-old fish reared at 18–20° C, and mortality usually reached 80–90% in a few weeks. The affected fish had opaque fins and a hyperplastic epidermis on the fins and skin. Electron microscopy revealed hexagonal virus particles in the nucleus and cytoplasm (diameter in enveloped state: 200 nm) of the affected epidermal cells. Although isolation of the causative agent by the use of several fish-cell cultures was not successful, the disease was transmitted to healthy larval flounder by exposing them to a 0.45 μm filtrate of diseased fish homogenate. Morphological and physiological characteristics of the virus indicate that the agent is a herpesvirus (Iida et al. 1989). When immersion challenge tests of flounder larvae were made at three different temperatures (15, 20, and 25° C), the disease progress was apparently delayed at the lowest temperature (15° C), though the cumulative mortality was the same for all three temperatures. Larvae younger than 20 days old (smaller than 9.5 mm in total length) were highly susceptible, but the susceptibility was significantly decreased in fish 23 days old or older (larger than 11.0 mm). Therefore, this disease proved specific to the larval stage of flounder (Masumura et al. 1989a). A similar disease was reported in Japanese flounder larvae and juveniles under a name of viral epidermal necrosis (Miyazaki et al. 1989); however, a precise comparison of these herpesvirus infections has not been made owing to the unsuccessful in-vitro culture of the agents. Another epithelial necrosis due to a paramyxovirus-like virus was reported from larval black seabream (Miyazaki et al. 1989).

Mass mortalities of hatchery-reared Japanese parrotfish larvae and juveniles have occurred in Nagasaki Prefecture, the westernmost part of Japan. Light and electron microscopic examinations revealed an extensive necrosis of the nervous system in the spinal cord, spinal ganglia, and brain. Numerous nonenveloped virus particles, icosahedral in morphology and measuring 34 nm in diameter, were found in the cytoplasm of the affected neurones and glial cells. The disease was named viral nervous necrosis (Yoshikoshi and Inoue 1990). A similar disease was found in red grouper larvae in 1989 and striped jack larvae in 1990 (Yoshikoshi 1990). Similar picorna-like viral particles were observed in degenerative areas of the brain and retina in seabass (*Lates calcarifer*) larvae cultured in Australia (Glazebrook et al. 1990). A comparative study on these new disease agents is a matter of great interest because traffic of live fishes has been increasing among south and southeast Asian countries. The frequent occurrences of these new viral diseases remind us of the necessity of rapid improvement in the current seed production system for marine fishes from the standpoints of quarantine and hygiene, both of which have almost been established for salmon hatcheries.

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An Ecological Study of the Parasitic Nematode *Hysterothylacium haze* in the Japanese Common Goby *Acanthogobius flavimanus*, in a Brackish Inlet

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ABSTRACT

The unique life cycle of the parasitic nematode *Hysterothylacium haze* in the Japanese common goby *Acanthogobius flavimanus* is presented. It is a direct cycle in which the fish become infected by ingesting fully developed eggs or hatched larvae of the nematode or by ingesting invertebrates that can act as transport hosts. Infections by the nematode were investigated monthly in a wild population of common goby in a brackish inlet over a period of five years. Seasonally, the rate of infection in the body cavity peaked in November in fish age 0+ and in May in fish age 1+. Annually, the infection rate declined during the investigation period. The intensity of infection in individual fish tended to fluctuate with the population's overall infection rate for both age 0+ and age 1+ fish. The influence of the infection on the host population is briefly discussed.

Introduction

Mass mortalities of the Japanese common goby *Acanthogobius flavimanus* (yellowfin goby) occurred in the inner part of Tokyo Bay every summer from 1973 to 1975. The body cavities of the dead fish were heavily infected with the eggs, larvae, and adults of a nematode; visceral adhesions were often noted (Takahashi et al. 1976; Takahashi et al. 1977). Takahashi et al. (1977) demonstrated that the heavily infected fish were intolerant of low dissolved oxygen concentrations and concluded that the mass mortalities of the goby were caused by the nematode infections in combination with the scarcity of dissolved oxygen. The nematode was identified as a new anisakid, *Thynnascaris haze,* by Machida et al. (1978) and later assigned to the genus *Hysterothylacium* as *H. haze,* a new combination by Deardorff and Overstreet (1981).

This nematode possesses unique features among the anisakid nematodes. Adult worms and eggs of *H. haze* were found in the body cavity of the host fish, whereas the adults of other anisakid nematodes harbor and deposit their eggs in the digestive tract of their definitive hosts. It also appears that *H. haze* has a unique life cycle and that infection is apparently harmful to the host goby, heavy infections causing the deaths of some hosts. There is no other known case in which an anisakid nematode infection is so harmful as to kill the host fish. From the extensive damage observed in heavily infected fish, parasitism by the nematode may even reduce the host population size.

A previous study of the biology of *H. haze* has already elucidated its peculiar life cycle (Yoshinaga et al. 1989). An ecological investigation was made for five years. In this paper, the seasonal and annual changes in *H. haze* infections in goby inhabiting a brackish inlet and the life cycle of this parasite are described. In addition, the influence of its infection on the population of the host is discussed briefly.

Life Cycle

In general, nematodes molt four times prior to becoming adults. The first-stage larvae are worms before the first molting. Following the first, second, and third moltings, the worms are called the second, third, and fourth-stage larvae, respectively, and after the fourth molting (the fifth stage), they are adults. It was reported by Yoshinaga et al. (1988) that all

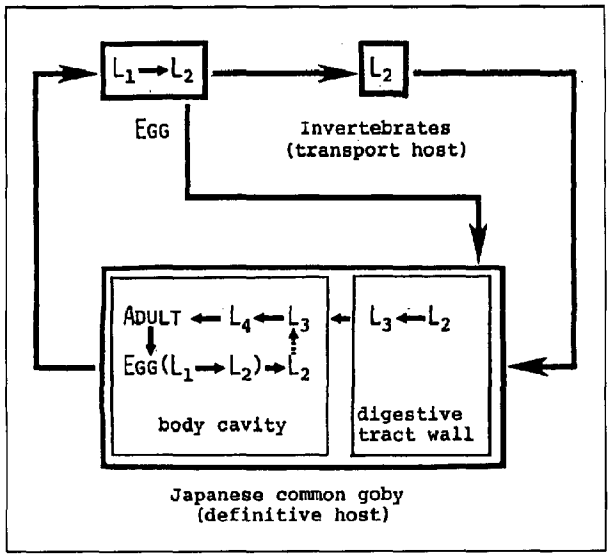

Figure 1
Life cycle of *Hysterothylacium haze*. L_1, L_2, L_3, and L_4 represent the first-, second-, third-, and fourth-stage larvae, respectively. The development from L_2 to L_3 in the body cavity of the Japanese common goby (dotted arrow) is uncertain. (From Yoshinaga et al. 1989.)

stages of *H. haze* are harbored in the body cavity of heavily infected fish; this is an exceptional feature among anisakid nematodes. Detailed observations of *H. haze* infections in goby and experiments with infected goby and invertebrates resulted in the elucidation of the *H. haze* life cycle (Yoshinaga et al. 1989). It is summarized as follows (Fig. 1): when a goby eats fully developed worm eggs, second-stage larvae in the eggs hatch and penetrate the digestive tract wall of the fish. The fish may also become infected by eating invertebrate transport hosts containing second-stage larvae in their body cavities; various invertebrates (e.g., polychaetes, amphipods, and mysids) can act as transport hosts. Second-stage larvae develop to the third stage in the tissue of the goby's digestive tract wall, then migrate into the body cavity. In the body cavity, third-stage larvae develop through the fourth stage to become adults, that mature and deposit eggs in the body cavity of the host. These eggs are released into the water after the death of heavily infected goby. It should be noted that the deaths of heavily infected goby are essential for egg dispersion and subsequent invasion of new hosts. The eggs hatch in the body cavity of the host. Although it is uncertain whether the second-stage larvae can develop to third-stage larvae in the body cavity, it seems that a large number of larvae are harmful to the host and contribute to its death even when the host is infected with only a small number of adults.

Ecological Study

An ecological study of *H. haze* was carried out in Lake Shonai, a brackish inlet of Lake Hamana, an estuary located on the Pacific coast of central Japan (Fig. 2). The Japanese common goby has a life span of one year and spawns in the spring (Miyazaki 1940); however, some fish do not become fully mature during the spawning season, probably because of delayed growth. These fish survive to become age 1+ in some areas. Thus, the goby population in Lake Shonai consists of two year classes from late spring to autumn.

Hysterothylacium haze infections in the Japanese common goby were investigated monthly from June 1981 to November 1982 and from December 1983 to September 1986. Fish captured commercially by trap net were sampled. They were examined for *H. haze* infection in the body cavity or in the digestive tract wall, or both, after age determination by scale readings. Infections in the body cavity were examined

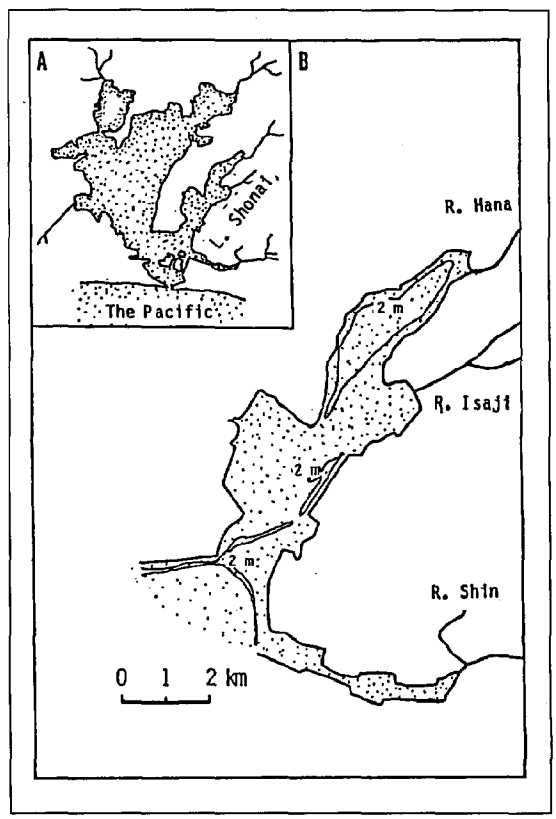

Figure 2
Map of Lake Hamana (A) and Lake Shonai (B). Lake Shonai is a brackish water inlet of Lake Hamana, an estuary located on the Pacific Coast in Shizuoka Prefecture, Honshu.

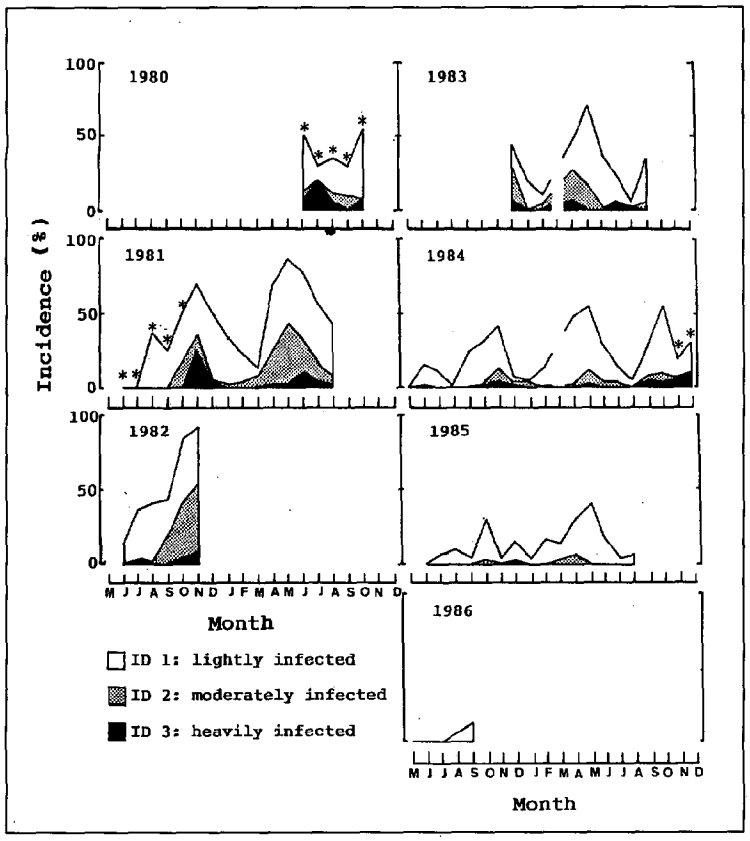

Figure 3
Monthly changes in the level of *Hysterothylacium haze* infection in the body cavities of the Japanese common goby by year class from 1980 to 1986. Asterisks indicate months when sample sizes were less than 30. ID = intensity index determined by macroscopical observation; ID 1 = lightly infected with 1–9 worms; ID 2 = moderately infected with 10–99 worms; ID 3 = heavily infected with more than 100 worms.

with a stereomicroscope during the entire investigation period. Infections in the digestive tract wall were examined microscopically by compressing the tract between glass slides.

Seasonal Changes

Infections in the body cavities occurred in a clear seasonal pattern, although there were slight differences in the pattern between years (Fig. 3). Infections in age 0+ fish were first noticed in early summer. The incidence of infected fish peaked in November and subsequently declined to a minimum level between January and March. A second peak occurred in May in age 1+ fish that had survived after their spawning season, and then the peak declined. When the prevalence of the nematode was high, heavily infected fish containing worm eggs in the body cavity were frequently found.

Although infection of the digestive tract walls was investigated for only two years, it was observed that the year when the infection level in the body cavity was lower and showed a less clear seasonal pattern, a seasonal pattern was still discernible in the incidence in the two sites of infection (Fig. 4). Characteristically, infection in the digestive tract wall preceded

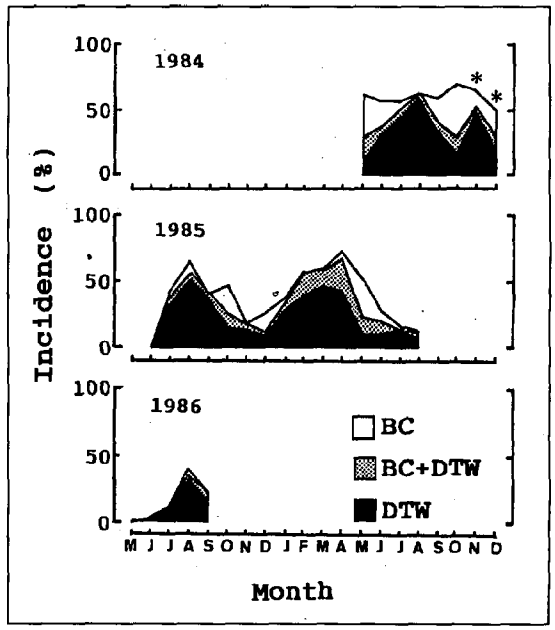

Figure 4
Monthly changes in the incidence of infection with *Hysterothylacium haze* in the two infection sites in the 1984–86 year classes of the Japanese common goby. Blank, semidark, and dark areas represent fish infected only in the body cavity (BC), both in the body cavity and digestive tract wall (BC+DTW), and only in the digestive tract wall (DTW), respectively.

that in the body cavity. Infection in the digestive tract wall began in June or July prior to or in the same month as the infection in the body cavity. The incidence of nematode infection in the digestive tract walls peaked in August and declined to its lowest levels in November and December (1985 year class) when the incidence of infection in the body cavities began to peak. The incidence of nematodes in the digestive tract walls continued to rise until April and declined in May, when it reached a second peak in the body cavity.

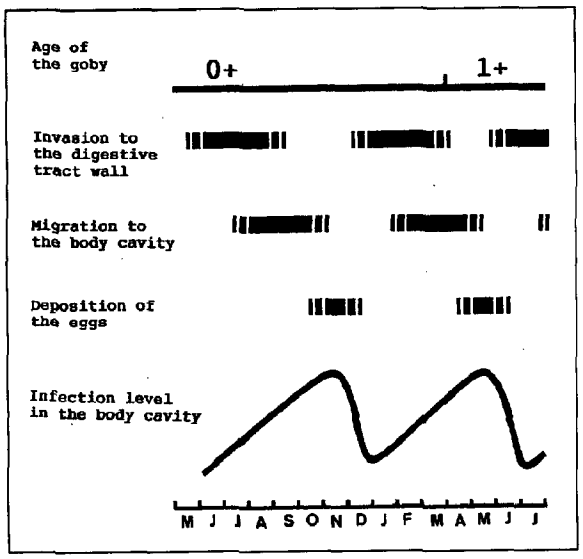

Figure 5
A diagram of the seasonal pattern of *Hysterothylacium haze* infection in the Japanese common goby in Lake Shonai.

The seasonal pattern of *H. haze* infection is summarized from these results as follows (Fig. 5): second-stage larvae invade the digestive tract wall of age 0+ goby in summer and develop to the third stage there. They migrate to the body cavity from summer to autumn, develop to the adult stage, mature, and then deposit their eggs there in late autumn through early winter when the infection level in the body cavity in the body cavity first peaks. Between December and March, new invasions into the digestive tract wall occur again and the infection level in the body cavity reaches a second peak in May in age 1+ fish.

Hysterothylacium haze has never been reported to occur in any fish other than the Japanese common goby; therefore it appears that the biology of the goby is closely related to the biology of the nematode. It is also apparent, from the seasonal pattern above, that the transmission of the nematode from age 1+ to age 0+ fish occurred in summer, suggesting that the survival of part of the goby population after the spawning season is indispensable to the nematode life cycle.

Annual Changes

During this 5-year investigation, the prevalence and intensities of *H. haze* infection in goby body cavities showed sharp annual declines when infection peaks were observed (Fig. 6). The declines were observed over the whole life span of the goby (Fig. 3). Also the beginning of the invasion of the body cavity was delayed to July in 1985 and to August in 1986, whereas it first occurred in June in the 1982 and 1984 year classes.

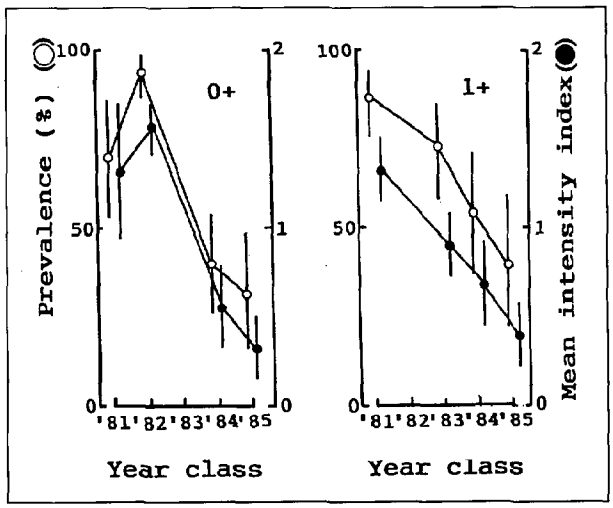

Figure 6
Annual changes in the level of *Hysterothylacium haze* in the body cavities of the Japanese common goby by year class from 1981 to 1985. The incidence and mean intensity index (total intensity indexes/number of fish examined) are shown for the first and second peaks in age 0+ and age 1+ goby, respectively. See Figure 3 caption for definition of intensity index. Vertical bars are 95% confidence limits.

If *H. haze* infection had regulated the host population size, some relationship would have been found between the levels of the host population and the nematode population. Figure 7 shows that the goby catch in Lake Shonai from 1982 to 1986 (data from the Shirasu Branch, Hamana Fisheries Cooperation). Though the total catch of goby showed considerable annual fluctuation, there was no apparent relationship between the infection levels and catch. It seems likely that the *H. haze* infection has little influence upon the population's size, despite the lethal damage

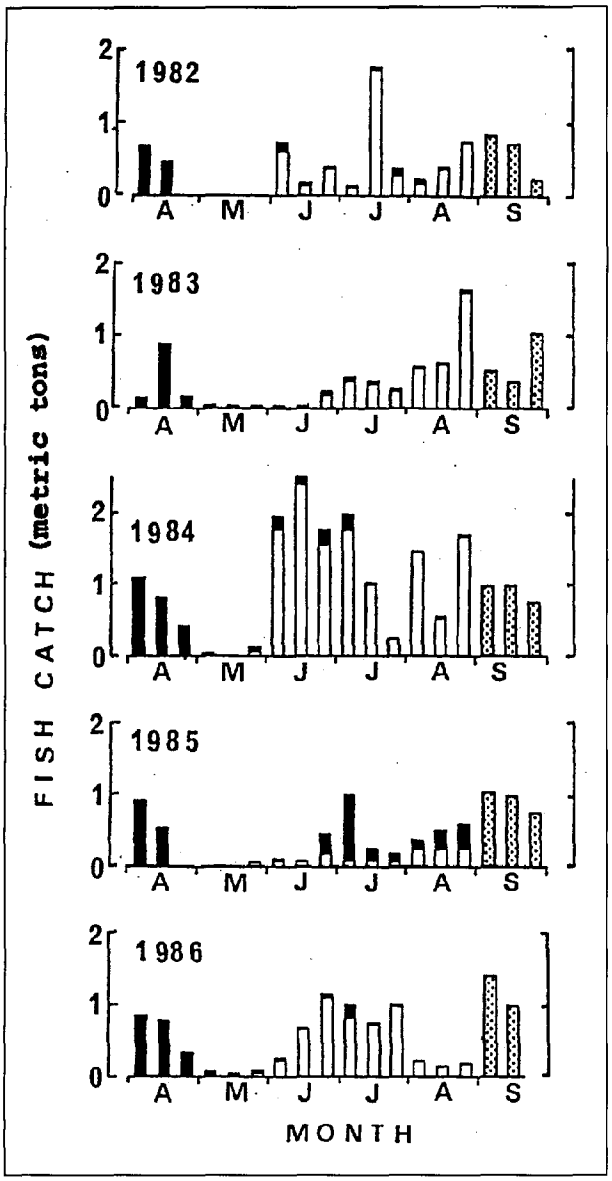

Figure 7
Changes in the catch (metric tons) of the Japanese common goby in Lake Shonai from 1982 to 1986 (based on data from Shirasu Branch, Hamana Fisheries Cooperation). Blank and dark areas represent age 0+ and age 1+ fish respectively. In the semidark area, age was not determined.

to heavily infected hosts. This is probably due to the fact that the Japanese common goby has a life span of only 1 year and the influence of the infection does not accumulate year by year, and because the goby population is regulated more by environmental factors than by the population size of the previous generation (Tokyo Metropolitan Fisheries Experimental Station 1981).

Another question arose from the annual decline of the *H. haze* infection level. Why did the nematode population decrease during the investigation? Although there were no data available to interpret it, two possible reasons may be assumed from the biology of the nematode: one is that the populations of the invertebrate transport hosts may have decreased, and another is that the biological conditions of the Japanese common goby in Lake Shonai (e.g., spawning season, survival after the spawning season, and growth) may have changed. Nevertheless, some ecological changes should have occurred in Lake Shonai, based on Price's (1980) contention that parasites generally exist in nonequilibrium states, where the departure of any one element in the patch may render the population inviable.

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Epidemiology of Marine Fish Diseases in the Warm Waters Along the Kuroshio Current

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ABSTRACT

In Japan, epidemiological studies have been carried out on the diseases of marine fish for many years. From 1981 to 1989, daily clinical record cards were used in every prefecture to obtain information on the outbreak of diseases in cultured marine fish. Observations were recorded on a standard form, translated into codes, and stored on a data base. During the analysis of this data, fish diseases were classified into six groups according to the causal agent: bacterial, viral, fungal, parasitic, nutritional, or unidentified disease. The most frequently reported group was bacterial disease, while the least common one was fungal disease. This paper describes the species-specific features and seasonal variations in outbreaks of these disease groups and discusses the relationship between fish size and diseases. Also, the relationship between locality and diseases are analyzed for yellowtail, *Seriola quinqueradiata*. From this epidemiological examination, it is clear that disease outbreaks by bacteria that are normally nonvirulent, such as streptococci and gliding bacteria, suggest that fish cultivation itself disturbs the natural relationship between fish and the environment. In other words, deteriorated environmental quality and poor fish health led to a beneficial situation for the growth of pathogens.

Introduction

Japan is surrounded by two major oceanic currents: the Kuroshio (Japan) Current and the Oyashio (Okhotsk) Current. The Kuroshio Current is warmer and its surface temperature varies from about 10° C in winter to about 30° C in summer (Fig. 1). Most marine fish cultures in Japan are carried out in the coastal waters along this current. The marine environment in these coastal waters is suitable for marine fish cultivation because of its warmer temperature, milder weather, abundant sunlight, and lower chlorinity. Nitrogenous and phosphorous nutrients supplied by many rivers make this area highly fertile. These factors support the high production of phytoplankton and zooplankton, which in turn assure food for fish and shellfish larvae. Also, the rias coastline is well suited for the culturing of marine fish because culture equipment can remain set up throughout the year and the daily work routine of fish culturing can be easily performed in the small gulfs and bays.

Fish species chosen for cultivation must be 1) adaptable to the culture environment, 2) easily cultured, 3) produce readily available seedlings, and 4) must be commercially valuable as sashimi (raw fish).

Among the 30 species now being cultured in Japan are *Seriola quinqueradiata* (yellowtail), *Pagrus major* (red sea bream), *Paralichthys olivaceus* (Japanese flounder), and *Takifugu rubripes* (tiger puffer). Yellowtail is the most popular and familiar species among these and accounts for about 70% of the total production of cultured marine fish.

The production of marine fish by artificial cultures and traditional methods is shown for the period 1976–86 in Figure 2 (SID 1981–90). Recently, the annual catch by marine fisheries (excluding shellfish and seaweeds) has amounted to about 10 million metric tons (t) yielding a gross income of about 210 billion yen. The harvest from marine cultivation has totaled about 0.2 million t valued at 20 billion yen. Although the net production of fish both by traditional marine fisheries (A) and marine cultures (B) has increased slightly since the early 1980s, the ratio between them (B/A) has remained fairly constant at about the 2% level. In contrast, the ratio of gross income (b/a, Fig. 2) has steadily increased and

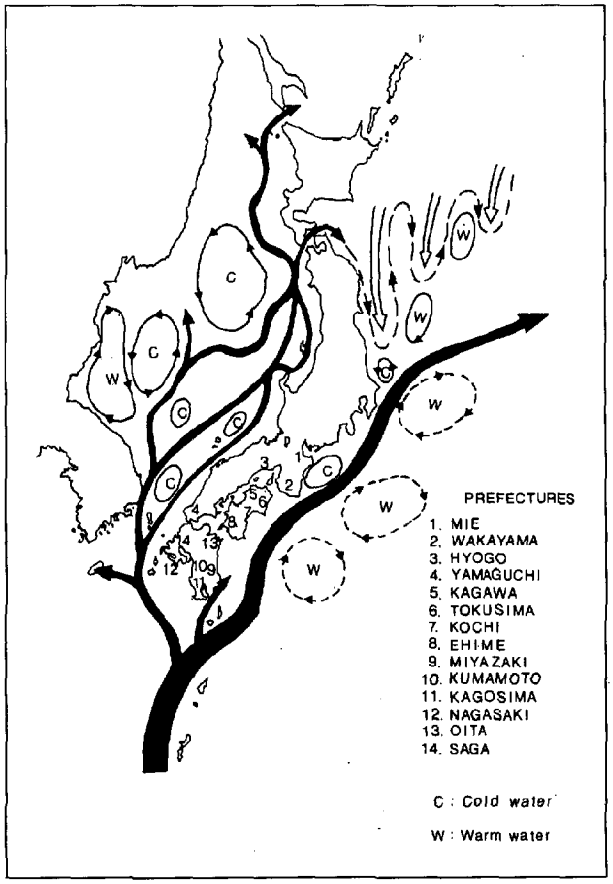

Figure 1
Currents around Japan.

amounted to about 10% in 1986. This suggests that the market not only demands cultured fish as a protein source but also holds their species-specific quality and freshness in high regard.

Because of the importance of cultured marine fishes to the Japanese economy, an epidemiological study of the fish diseases affecting the industry was undertaken. Data compiled by the various prefectural fisheries institutes from 1981 to 1989 were examined to establish trends in disease outbreaks and relationships between fish, their culture environment, and disease organisms.

Marine Fish Cultivation: Technical Features

The techniques used to culture marine fishes in Japan vary little between species and localities. In the popular case of yellowtail, fry or seed fish migrating with drifting algae in the surface of the Kuroshio Current are captured by a small purse seine. The fish are transferred to floating net pens located in a bay and are fed nonliving raw food, such as minced sand eels.

In the case of fish species such as red sea breams, where seedling production is done mainly in land-based ponds, fertilized eggs are collected by net and then transferred into a rearing tank. After hatching, fish are fed with live feed suitable for their size, such as oyster eggs, rotifers, brine shrimp, and zooplankton, which are collected from the sea. As they develop, they are eventually fed nonliving feed such as minced raw fish, chopped shrimp, or shellfish until development to the young fish stage is almost completed.

Intensive rearing in the marine net pen is then carried out for about 1 or 2 years until the fish grow to a marketable size. During this rearing period, fish are fed principally with sliced or cut, raw or frozen fish meat.

Intensive culture techniques have an adverse effect not only on fish health, but also on the conditions of the surrounding environment. Food containing high levels of protein and fat, such as raw fish meat, is supplied in large amounts in expectation of more rapid growth. The leftover food and fish excrement deposited on the sea bottom cause a deterioration of water quality, which is believed to be a cause of red tides and diseases. In addition, excessive feeding and restricted activity in the limited confines of the rearing pens also make fish fatty.

Investigating Disease Outbreak

At the request of an aquaculturist fish diseases are diagnosed at the prefectural experimental stations, where daily clinical records are maintained. Diagnostic data are recorded on a standard form (Table 1), translated into codes, and stored in data bases of marine fish diseases at the Nansei National Fisheries Research Institute. These data can then be entered into a personal computer (NEC 9801) and be statistically sorted and calculated using DATA BASE IV software (Nihon Integrated Software Co., Ltd.). The program for compiling, sorting, calculating, and printing disease case data was previously reported (Umezawa and Ishioka 1988). The data base of the present study represents diagnoses conducted by 20 prefectures during the period from 1981 to 1989.

The standard form consists of five parts, as shown in Table 1. The first part includes information essential for registering one diagnostic case: fish species name, region where the disease occurred, name of the fish farm or fish culturist, diagnosis date, and the disease name. Diagnosis relies upon physical observations, parasitology, and bacteriology of the diseased

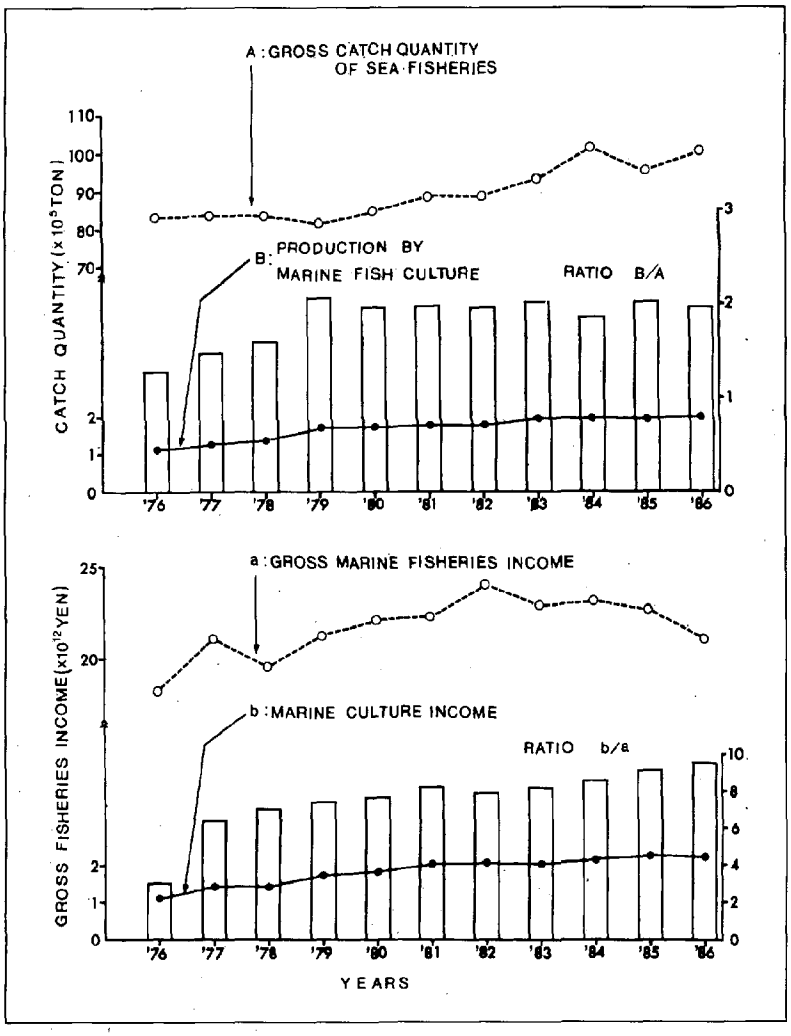

Figure 2
Catch quantity and gross income by marine fisheries.

fish. Because fish are cultured in a group, one disease is defined as one diagnostic case of one species in one fish culture establishment in a region.

The second part of the form contains specific information on the diseased fish; date of observation of unusual behavior or other abnormal symptoms, such as body color change or appetite loss; date of appearance of mortality; number of dead fish per pen; whether the progress towards death was sudden or prolonged; fish size (body length, and weight); and any treatment administrated to the diseased fish.

The third part details cultivation conditions such as food type and species, region of food production, size of healthy fish in the pen, the basic information necessary for calculating fish density, pen type, and seed information.

The fourth part contains environmental information on the cultivation area, that is, water depth, weather, degree of rain, temperature conditions, number of years the area was used as a fish farm, and years of fish cultivation experience accumulated by the aquaculturist.

The fifth part is a memorandum for recording other information, such as the results of bacterial checks, symptoms of unidentified diseases, or information on the occurrence of red tide.

Results

Number of diseases reported

The rate of fish farms requesting disease diagnosis in each prefecture was calculated by summing the total number of establishments requesting diagnosis by using the standard forms for that prefecture and dividing it by the total number of marine fish culture establishments quoted from governmental statistics (SID 1981–90). While both the number of disease cases reported and the diagnostic rate differed from one

Table 1
Standard data form used to compile disease diagnosis information.

Category	Content
Essential Information	Species name
	Region
	Name of fish farm
	Date of investigation
	Name of disease
Information on the diseased fish	Date abnormality was detected
	Date dead fish was observed
	Number of dead fish per pen
	Was death sudden or prolonged?
	Size of dead fish (cm)
	Weight of dead fish (g)
	Treatment used
Information on cultivation conditions	Food type
	Region of food production
	Age of the fish
	Size of healthy fish (cm)
	Weight of healthy fish (g)
	Number of the fish per pen
	Square measure of the pen (m^2)
	Length of the pen (m)
	Pen type
	Days after pen renewing
	Place of seed production
	Age of seed introduced
	Date of the seed supplied
Information on the cultivation environment	Water depth (m)
	Weather
	Degree of rain
	Temperature conditions
	Period the area was applied for fish cultivation (years)
	Length of experience of aquaculturist
Memorandum	Other information such as bacterial checks, symptoms of unidentified diseases, occurrence of red tide, etc . . .

prefecture to another, a considerably high correlation was calculated between the numbers of disease cases and numbers of marine fish culture establishments in each prefecture. Spearman's rank correlation coefficient between number of establishments requesting diagnoses by their prefectural experimental station and the total number of establishments in each prefecture was about 0.9 for yellowtail, and 0.7 for red sea bream in 1985. These results may suggest that collected data represent the trend of disease outbreaks in the fish of the warm current on a smaller scale. Mean diagnostic rates were about 20% for yellowtail, about 8% for red sea bream, and about 15% for total marine fishes cultured. Based on these values red sea breams appear to be less sensitive to disease than yellowtail.

During the 9 years from 1981 to 1989, 56 species fish were reported to be diseased and over 20,000 disease cases were registered in 20 prefectures. About three quarters of these cases involved yellowtail. Governmental statistics (SID 1981–90) indicate an increased number of fish species in marine cultivation. This increase in cultivation may contribute to the increased number of fish with disease; an especially high incidence of disease originated from the recent increase in the culture of Japanese flounder.

Disease Diversity

Fish disease was classified into six groups according to the causal agent: bacteria, parasite, virus, nutrition, fungus, and unidentified disease. When the diseases occurred simultaneously, the causal agents were classified into both partitions, so the total amount of relative frequencies were over 100%. Bacterial diseases were most frequently reported, accounting for approximately 80% of all disease cases (Fig. 3). Parasitic (8.3%), viral (3.9%), nutritional (6.5%), and unidentified (8.4%) diseases were less frequent. The partitioning of these values seems to be closely related to the fact that diagnoses were made during the advanced levels of disease progression. The number of dead fish recorded in diagnostic cards suggests that the diagnoses had been performed after a considerable number of dead fish were noticed.

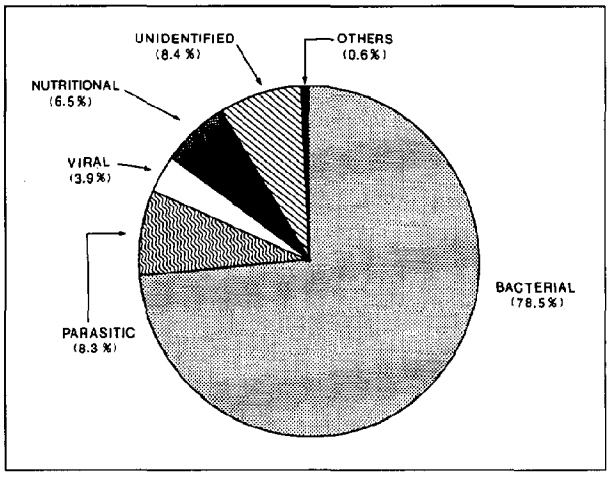

Figure 3
Disease groups observed in marine fish cultures located along the Kuroshio Current.

Species-specificity of diseases

The relative frequencies of the six disease groups var-

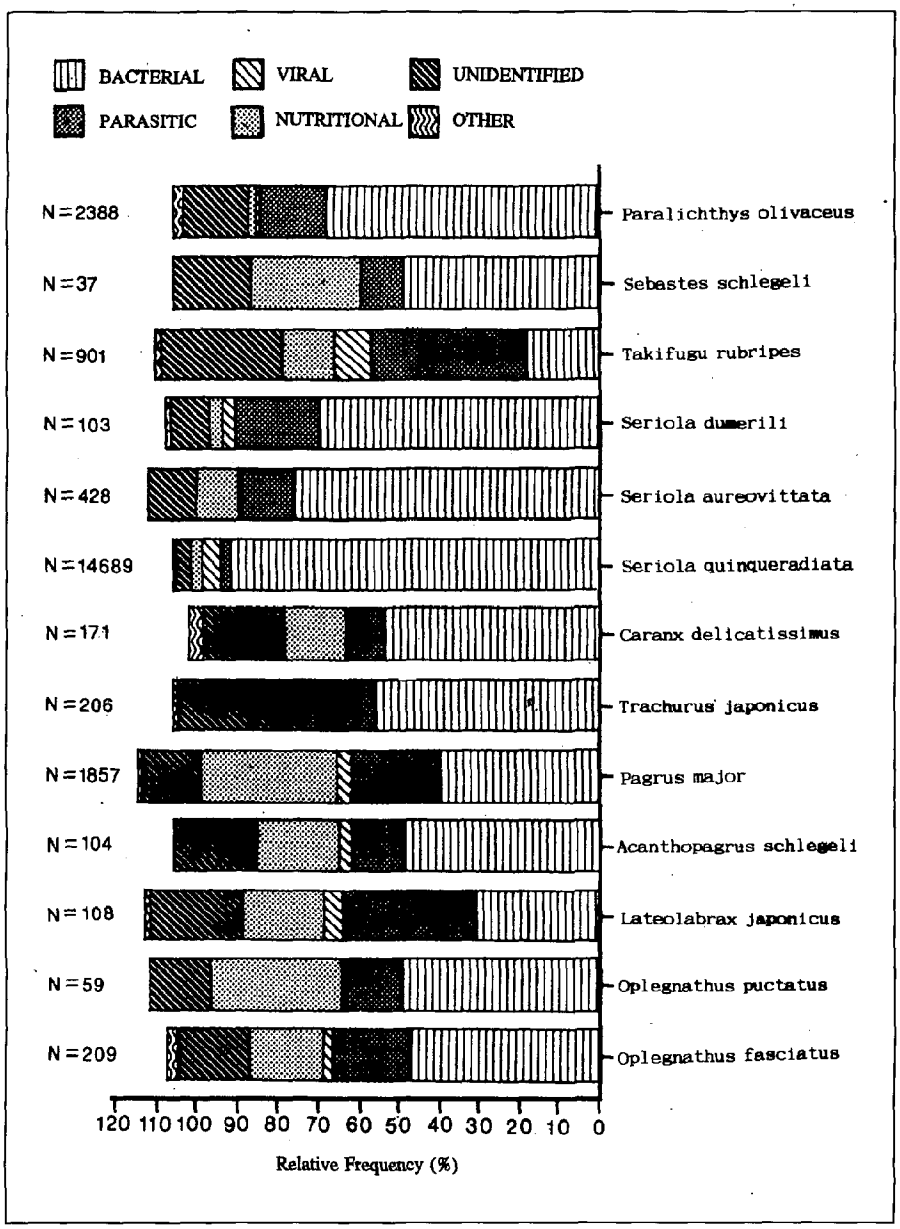

Figure 4
Composition of disease varieties in each species.

ied with fish species (Fig. 4). Over 90% of the diseased yellowtail experienced bacterial disease. In red sea bream, a high rate (22.1%) of parasitic disease resulted from parasitism by *Bivagina tai* on the gill, by *Longicollum pagrosom* in the rectum, and by other parasites. The high rate (33.4%) of nutritional disease involves yellow fat disease and peroxidative lipid intoxication caused by lipid metabolism disorders.

In tiger puffer, several serious parasitic diseases (*Heterobothrium tetradonis, Trichodina* sp., and *Cryptocaryon irritans*) break out frequently (39.2%). The specific viral disease (9.0%), named kuchi-jiro sho (white mouth symptom) is often reported; its pathogen has not yet identified officially. The rate of unidentified disease was highest among the 13 fish species listed in Figure 4.

In Japanese flounder, bacterial disease accounted for about 67% of all the cases registered, while the rate of parasitic disease, mainly due to a *Trichodina* sp. and *Cryptocaryon irritans*, was 17%.

The relative frequency of the six disease groups was not related to any taxonomical order nor to the ecological habitat of the cultured fish species.

The relative frequency of diseases caused by different pathogenic bacteria, are shown in Figure 5 for 13 fish species. Yellowtail, purple yellowtail (*Seriola aureovittata*), and gold striped yellowtail (*Seriola*

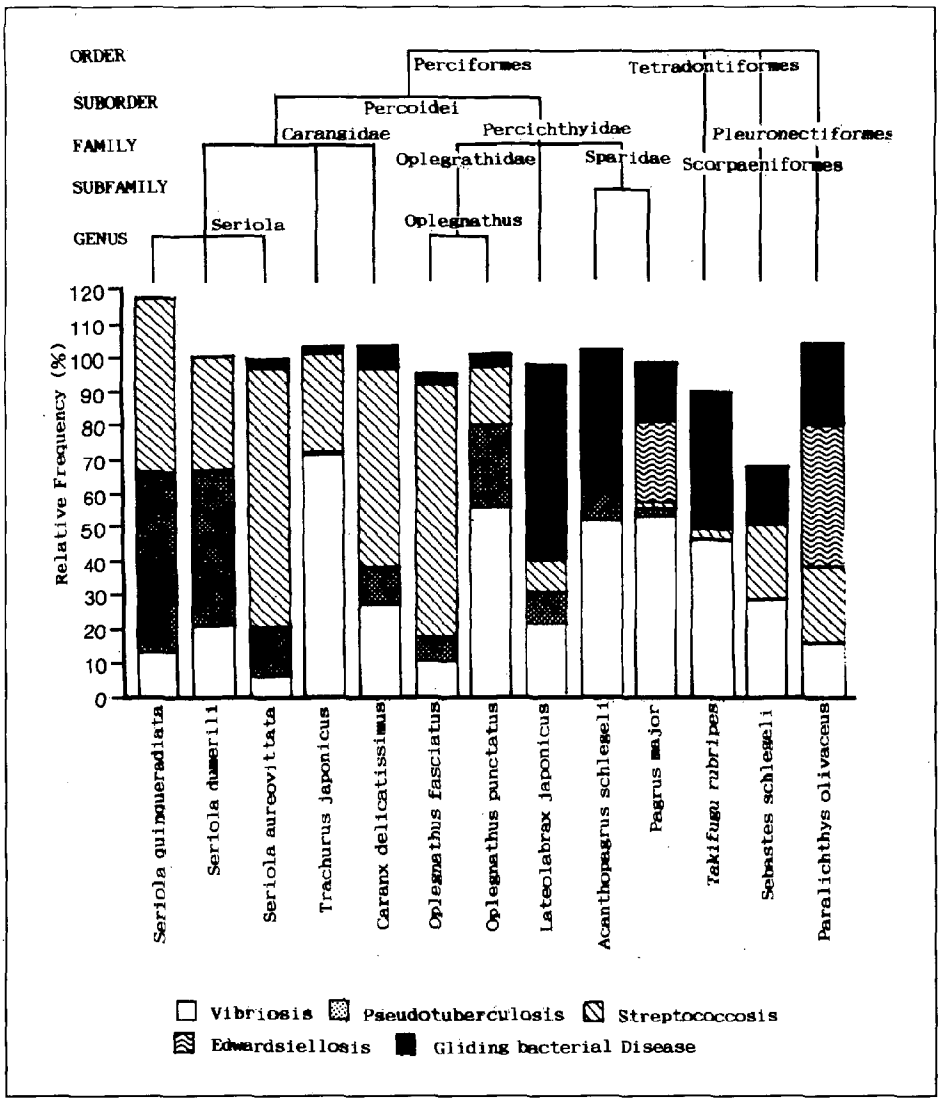

Figure 5
Composition of bacterial diseases in each species.

dumerili), showed higher rates of pseudotuberculosis and streptococcosis diagnoses, whereas jack mackerel (*Trachurus japonicus*), taxonomically closely related to yellowtail, had high rates of vibriosis and streptococcosis.

Red sea bream were frequently infected with *Vibrio* spp., *Edwardsiella tarda,* and gliding bacteria (mainly *Flexibacter* sp.), but less so with *Streptococcus* spp. and *Pasteurella piscicida.* No edwardsiellosis was reported from black sea bream (*Acanthopagrus schlegeli*), which are related to red sea bream.

Japanese flounder were seriously infected with *Edwardsiella tarda* and suffered heavy damage; the relative frequency of edwardsiellosis in Japanese flounder amounted to 41.8% of all bacterial disease cases. They were affected to a lesser extent by gliding bacteria and *Streptococcus* spp.

Tiger puffer suffered mainly from vibriosis and gliding bacteria infections.

With some exceptions, the relative frequency of bacterial disease seems to be related to the host by the host's ecological niche and related physiological characteristics. As shown in Figure 5, pelagic or migratory fish species such as yellowtail were more susceptible to *Pasteurella piscicida,* while bottom fishes like tiger puffer, Japanese flounder, and Japanese rockfish (*Sebastes schlegeli*), were more susceptible to gliding bacteria. Other species in Figure 5 fell between these two extremes.

A species' ecological habits are closely related to its anatomical, physiological, and biochemical characteristics. Pelagic and migratory fishes have a typical

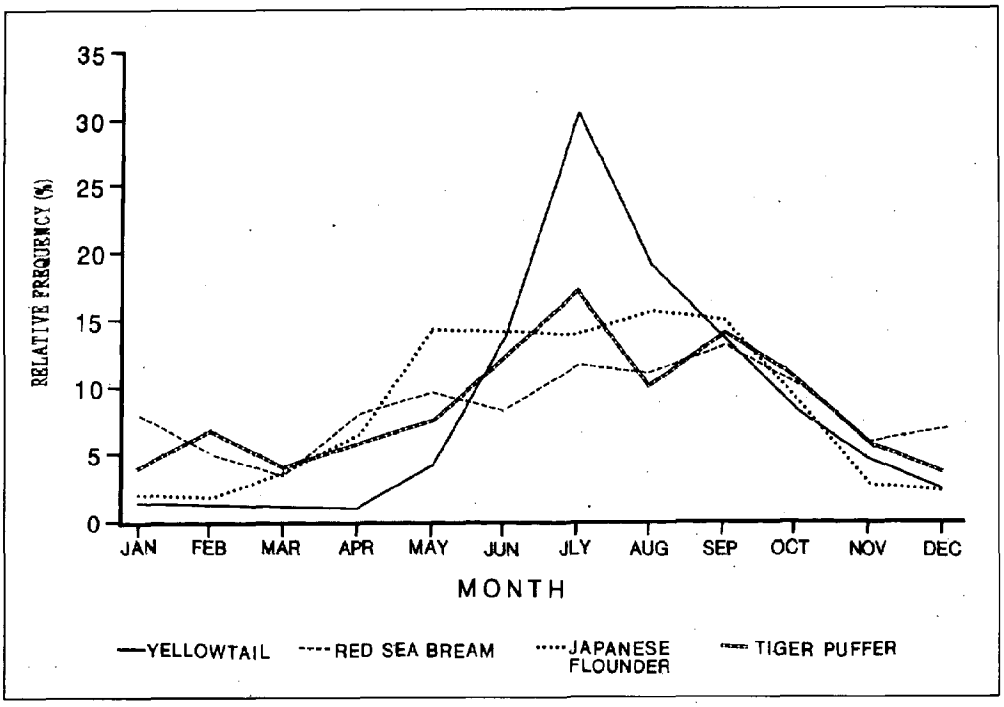

Figure 6
Seasonal changes in number of diseases registered between 1981 and 1989.

spindle shape suitable for swimming, developed cerebellum and vascular system, abundant superficial dark muscle, and an abundance of myoglobin in their ordinary muscle. On the other hand, the bottom fishes of coastal areas, such as Japanese flounder, rockfish, puffers, and sea breams have flat or rounded shapes, less-developed cerebellums, and characteristics that indicate that these fishes are generally less active. Their white ordinary muscle has less myoglobin and hemoglobin, and their superficial dark muscle is not as developed.

Because *Pasteurella piscicida,* a causative bacterium for pseudotuberculosis, migrates in the bloodstream of infected fish, active fish, with well-developed vascular systems, may be more likely to reach an exhausted state.

Infections by gliding bacteria tended to break out in benthic fish or fish living near the beach. Because these fish are less active than the pelagic species and may have a susceptible body surface, gliding bacteria, which move by means of surface mucus, may have an advantage in expanding their living area. Also, because these fish are more likely to come into contact with other individuals via their body surface, new infections and propagation of gliding bacteria are prevalent.

It is believed that the accumulation and integration of information on fish species and pathogens (such as cell or tissue susceptibility to pathogens; anatomical, histological, and physiological aspects of fish; environmental conditions; and characteristics of the pathogen) are very important to understand the phenomenon or mechanism of bacterial disease outbreak.

Seasonal Variations of Disease Outbreaks

Monthly variation in the relative frequency of disease cases reported is shown in Figure 6, for four fish species: over 900 cases for each species were reported during the 9-year period 1981–89.

The highest evidence of disease occurred during July for yellowtail (30%) and tiger puffer (17%), during August for Japanese flounder (15.5%), and during September for red sea bream (13%). Reports of disease for the latter three species peaked in the summer from June through September when the water temperature is higher than 20° C. These peaks were not as steep as that of yellowtail, yet the summer peaks suggest that fish under cultivation are exposed to physiological and environmental conditions favorable to infection by pathogens, in spite of possible efforts to promote the defensive activities of fish, such as immunological resistance.

In yellowtail, viral and bacterial diseases, especially pseudotuberculosis, show significantly sharp seasonal changes (Fig. 7). Since the official identification (Sorimachi and Hara 1985) of the viral disease viral ascites (VA), the rate of this viral disease has increased. VA disease breaks out in late May, reaches a

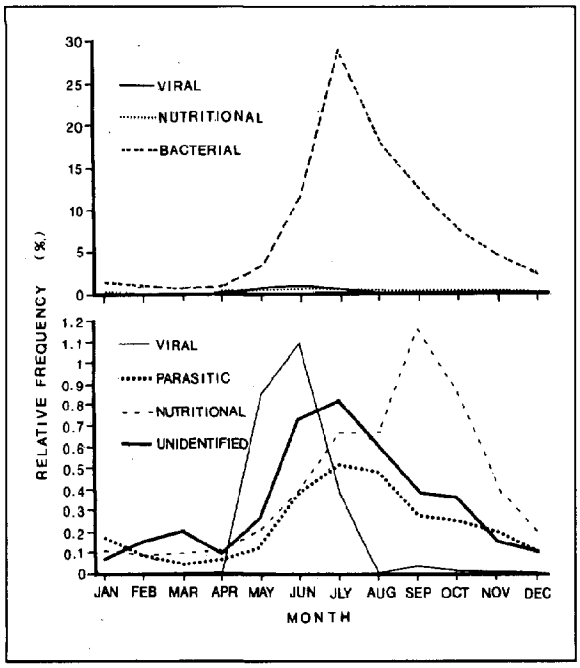

Figure 7
Seasonal changes in yellowtail diseases.

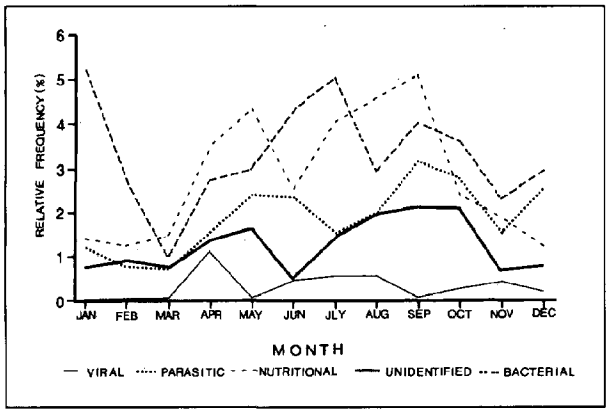

Figure 8
Seasonal changes in diseases of red sea bream.

peak in June, and ends in mid-July. The rate of VA disease diagnoses was very low and account for only 1% of yellowtail disease reported in June; its occurrence fluctuates yearly. The rate at which the major bacterial diseases were reported showed similar seasonal variations. Vibriosis accounted for about 10% of all the bacterial diseases reported and the number of cases peaked in June. Cases of pseudotuberculosis peaked sharply in July (*see* Fig. 11), and streptococcosis epidemics seem to occur between July and

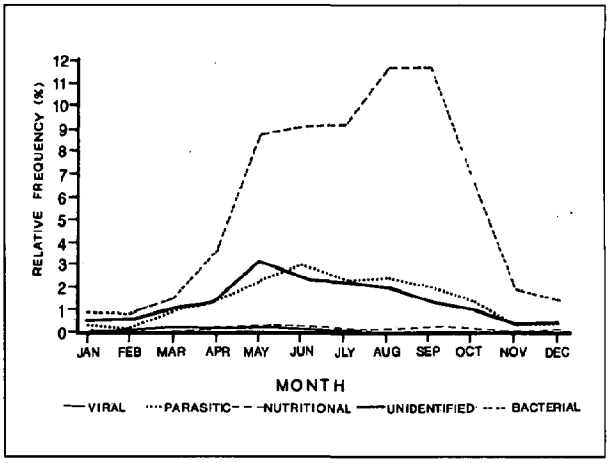

Figure 9
Seasonal changes in diseases of Japanese flounder.

October. Parasitic, nutritional, and unidentified diseases showed gentle seasonal changes in their occurrence. Parasitic and unidentified diseases broke out during summer, and the level of nutritional diseases peaked in autumn.

In red sea bream, viral diseases represented only 3.6% of the total disease cases reported (Fig. 8), and for the most part were observed as lymphocystis disease every month of the year. Parasitic diseases were reported least in February and March and most frequently between May and December. Nutritional disease outbreaks appeared between May and September, indicating that they are chronic in nature. Half of the bacterial disease cases reported were vibriosis (53.2%), which occurs all year round with a peak at winter (*see* Fig. 14). Gliding bacterial disease also occurred all year round but the higher rates were found in January and from May through July (*see* Fig. 15). Edwardsiellosis accounted for about 10% of all disease cases in red sea bream and was prevalent between July and November (*see* Fig. 13).

Since the successful development of seedling production methods, production of the Japanese flounder has gradually increased. The main viral disease observed in cultural flounder was epidermal necrosis, which occurred in spring during the fry stage and accounted for only 0.8% of all disease cases (Fig. 9). Japanese flounder suffered from parasitism by protozoa such as *Cryptocaryon* spp., *Trichodina* spp., and other Ciliata. Because there are no effective treatments for these parasites, infected fish are left untreated, resulting in a great deal of infection and death. A trial is now being conducted to see if conditions leading to infection can be effectively removed

by increasing the water supply to rearing areas. It is unknown why nutritional diseases are rare in Japanese flounder. Bacterial diseases are serious for this fish accounting for about 67% of all disease cases reported. The most virulent, edwardsiellosis, amounts to about 28% of all disease cases. Edwardsiellosis is most prevalent during the summer, from June through October, with the number of reports peaking in August. Streptococcosis is most prevalent from July to October and vibriosis and gliding bacterial disease are both most prevalent between April and August. Unidentified disease accounts for 16% of all disease cases reported.

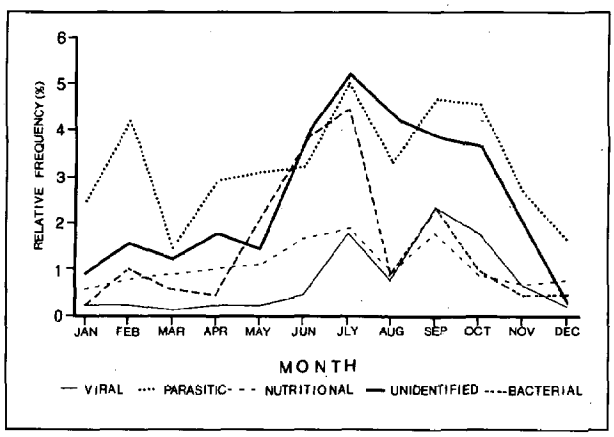

Figure 10
Seasonal changes in diseases of tiger puffer.

In tiger puffer (Fig. 10), kuchi-jiro sho was registered often throughout the year. Parasitic disease amounted to about 39% of all disease cases (Fig. 10). Fatal parasites included a *Trichodina* spp., a protozoa which was prevalent between April and October, and *Heterobothrium tetrodonis,* a monogenea prevalent mainly from April through August. Nutritional disease was also prevalent from spring through autumn. Higher levels of bacterial diseases were diagnosed from June to September: most were cases of vibriosis and gliding bacterial disease.

Pasteurella piscicida was a virulent pathogen for pseudotuberculosis in yellowtail and to a less extent in other cultured fish species (Fig. 11). This disease broke out seasonally, appearing during warmer months in all fish species.

Streptococcus sp. was most common in yellowtail and Japanese flounder and occurred less frequently in red sea bream and tiger puffer, showing similar seasonal variation patterns that peaked in September (Fig. 12). *Edwardsiella tarda* was virulent for Japanese flounder and red sea bream and most prevalent from

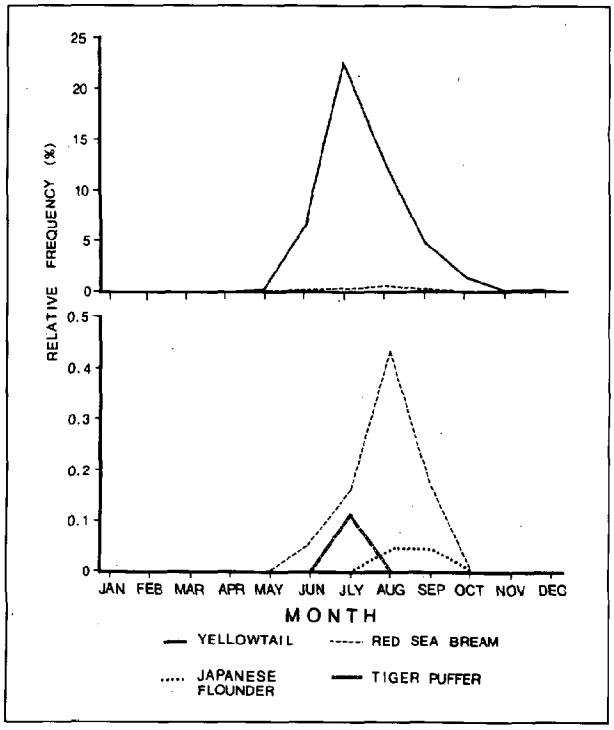

Figure 11
Seasonal changes in pseudotuberculosis for each species.

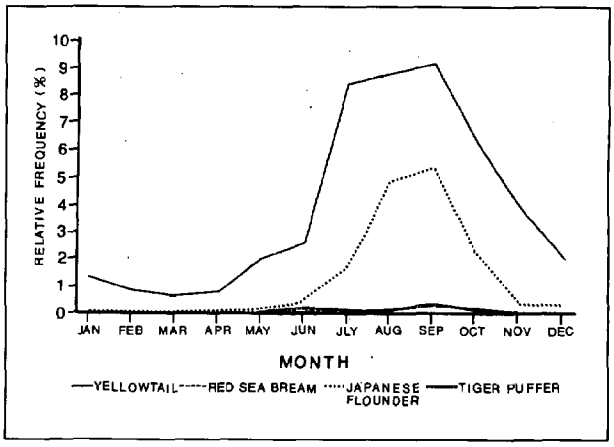

Figure 12
Seasonal changes in streptococco-sis for each species.

June through October; the level of reported cases peaked during August in Japanese flounder and showed a plateau from July to November in red sea bream (Fig. 13). The optimal water temperature ranges from 15 to 24° C for Japanese flounder and is slightly broader from 13 to 28° C for red sea bream. Cultured Japanese flounder suffer from exposure to

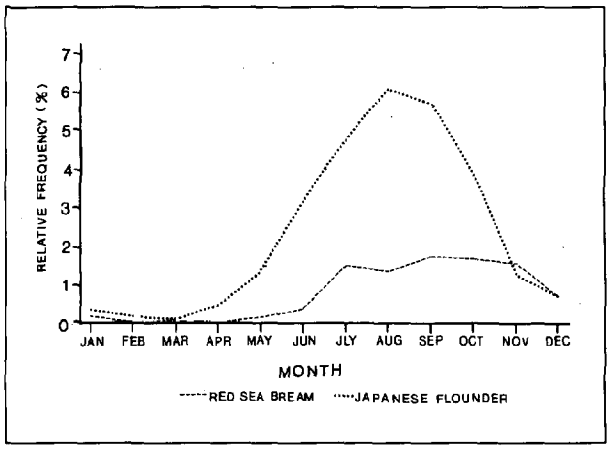

Figure 13
Seasonal changes in edwardsiello-sis for each species.

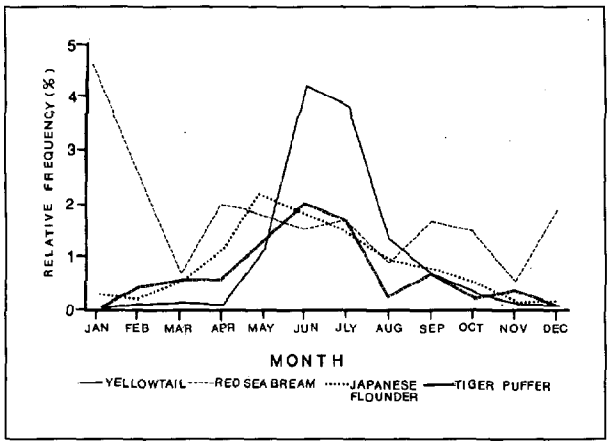

Figure 14
Seasonal changes in vibriosis for each species.

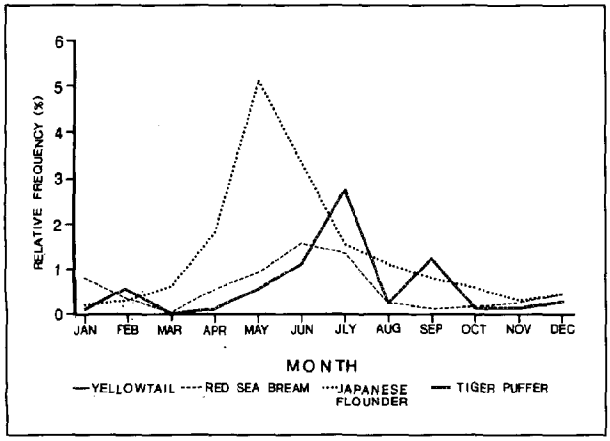

Figure 15
Seasonal changes in gliding bacterial diseases for each species.

high temperatures above 25° C in summer and become more susceptible to bacterial infection. On the other hand, red sea bream grow well during summer and suffer from nutritional disorders in autumn. Because *Edwardsiella tarda* can multiply more actively during the summer season, Japanese flounder, which are less tolerant of higher temperatures, are more susceptible to this pathogen. Conversely, red sea bream, which are more tolerant of higher temperatures, are less susceptible to this pathogen than Japanese flounder.

Seasonal variations in the relative frequency of vibriosis are complicated and differ according to fish species (Fig. 14). This complexity may result not only from the difference in susceptibility of each fish species but also from the presence of different varieties of *Vibrio*. *Vibrio anguillarum* is known to have varied serotypes. Susceptibility of fish to this pathogen would vary with the serotype and fish species involved.

The term "gliding bacteria" implies several bacterial species. The gliding bacteria resulting in marine fish diseases are mostly *Flexibacter, Cytophaga,* and *Sporocytophaga* spp. The unique characteristics of each gliding bacterium may contribute to the complicated patterns of seasonal variations in reported infection among the different fish species (Fig.15).

Fish size and disease

The variation in relative frequency of disease cases reported in yellowtail is illustrated according to fish size (body weight) in Figure 16. For this analysis, relative frequency was calculated by dividing the total number of reports for each disease in the defined size class by the total number of disease cases with body weight (bw) data for each species. Viral disease was more frequent in younger fish (<50g bw), while nutritional diseases were more prevalent in older fish (>250g bw) and season also an influencing factor. Occurrence of bacterial disease appears closely related to body weight. Yellowtail seemed to be successively attacked by VA virus first, then *Vibrio* and *Pasteurella piscicida,* and finally *Streptococcus.* This sequence of infection suggests that susceptibility of yellowtail to different pathogens varies with body weight or growth. This may imply not only the development of defense mechanisms to the pathogens but may also represent the changes in the overall physiology of the fish under cultivation.

In red sea bream, parasitic diseases occurred more frequently in fish between 50 and 250 g bw while reports of nutritional disease increased in fish over 250 g bw (Fig. 17). The rate of gliding bacteria diagnoses was highest in young fish below 10 g bw and that of vibriosis was highest in fish between 50 and 100 g bw. The diagnostic rate of edwardsiellosis was

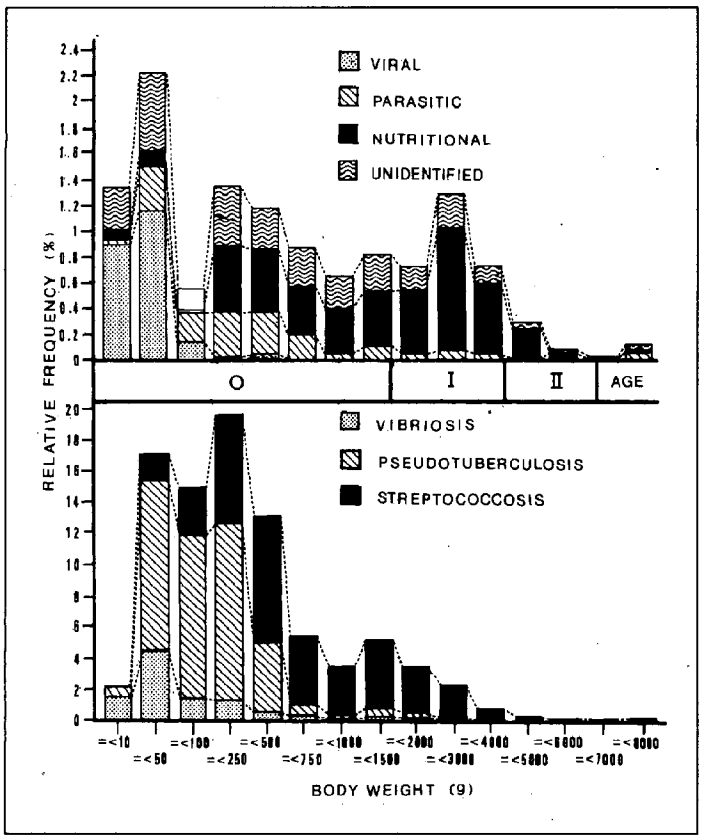

Figure 16
Relationship between fish size and disease outbreaks in yellowtail.

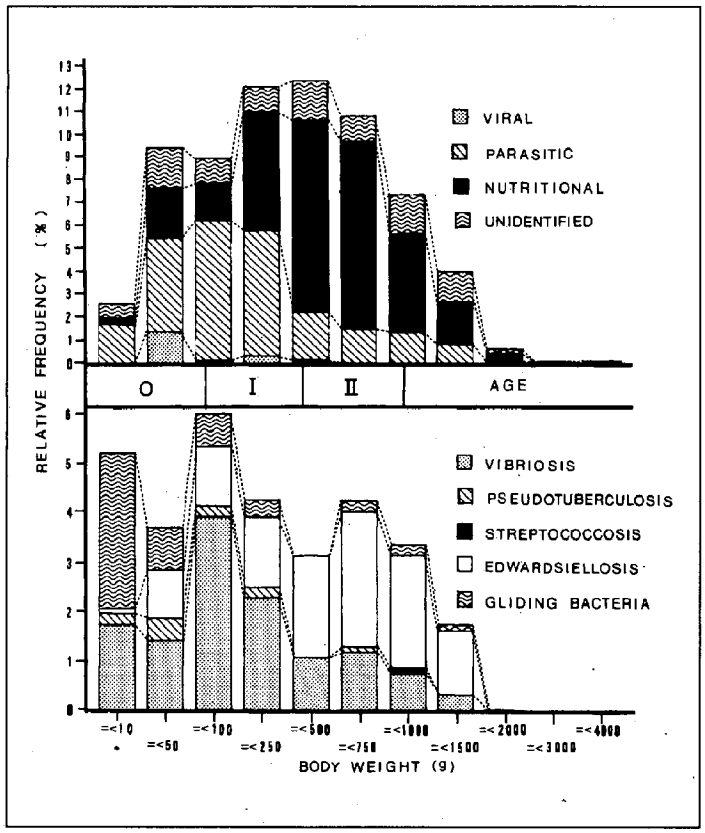

Figure 17
Relationship between fish size and disease outbreaks in red sea bream.

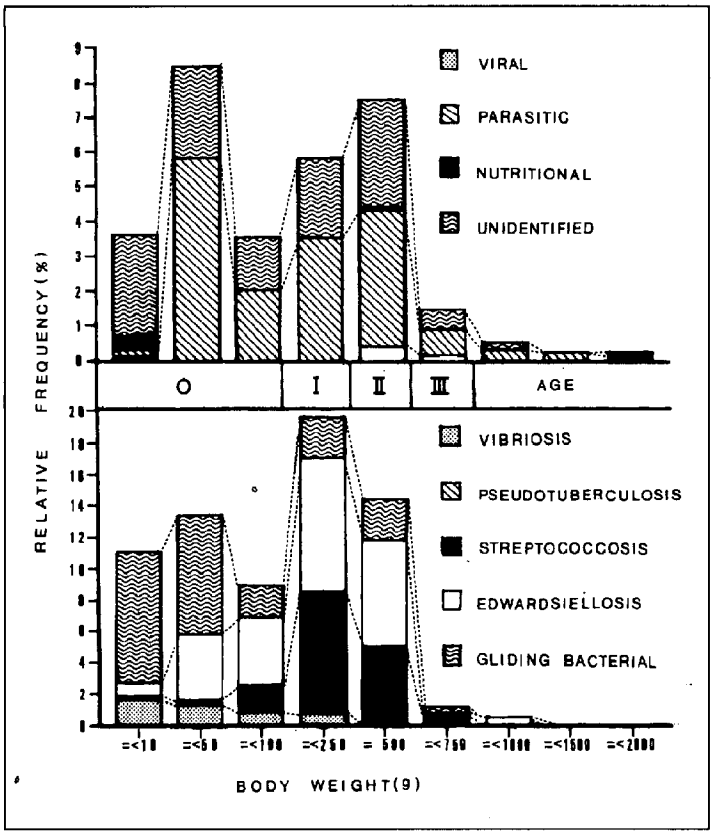

Figure 18
Relationship between fish size and disease outbreaks in Japanese flounder.

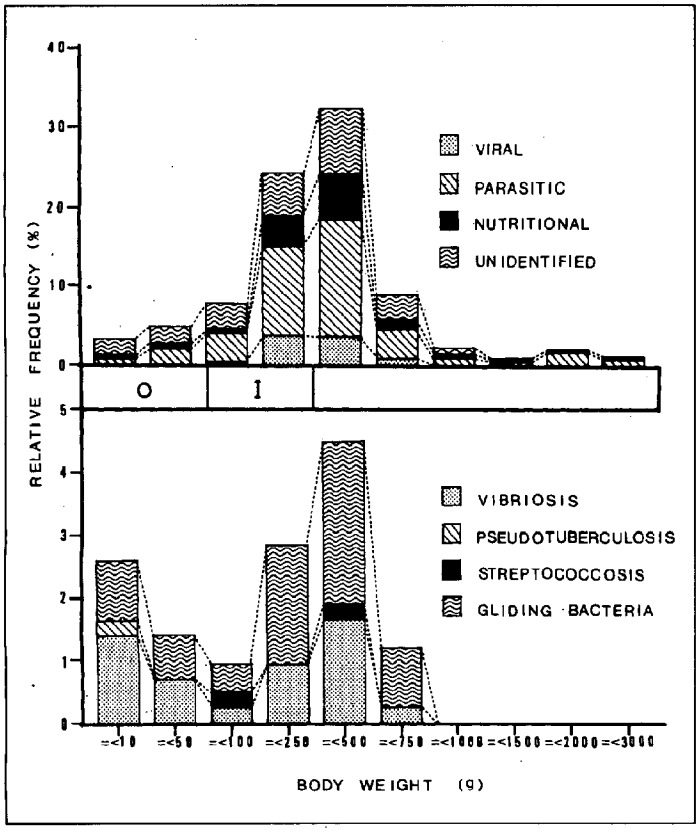

Figure 19
Relationship between fish size and disease outbreaks in tiger puffer.

higher in larger fish, especially those over 250 g bw.

All ages of Japanese flounder experienced outbreaks of parasitic diseases, while viral diseases were found only in fish below 10 g bw (usually herpes virus) (Fig. 18). Vibriosis and gliding bacterial disease outbreaks were more common in younger fish (<50g). Although streptococcosis and edwardsiellosis were both more prevalent in larger fish (>250 g), edwardsiellosis was more common than streptococcosis in the fish of 50–100 g bw.

In tiger puffer, a unique feature was found: fish 250 to 500 g bw appear to be most susceptible to pathogens (Fig. 19). This may be dependent on the method used to culture this species. Though this species is in great demand by the market, its cultivation from the egg through the spawning stage is very difficult. The eggs for seedling production are generally raised by the public sector and are in most cases supplied from the natural resource by fisheries, which also provides some large sized seedlings. Because the aquaculturist cultures mainly large-size seedlings, diagnoses may concentrate on the larger sized fish.

In all species, nutritional disease tended to occur in larger fish, probably as a result of prolonged nutritional treatment. Outbreaks of edwardsiellosis and streptococcosis were synchronized with nutritional disorders.

Locality and Disease

As shown in Figure 20, for yellowtail in 1986, some characteristics are common to all the prefectures listed. Although the relative frequency of bacterial disease was about 80% in all prefectures, the compositions of different bacterial diseases were to some extent different by locality. Mie Prefecture is the most northern of all the prefectures listed in Figure 20, while Kagoshima Prefecture is located in most southern part of Japan. Interprefectural analysis suggests that the composition of different bacterial diseases seems not to be related to geographical locality. A possible similarity exists between the composition of bacterial infections observed and the type of cultivation management used in some prefectures. There are two types of management: cultivation of large seedlings and production of fish of marketable size. Streptococcosis tended to be reported at a higher rate in the prefectures managing the larger fish (2-year-olds) of marketable size.

No characteristic difference in seasonal outbreak

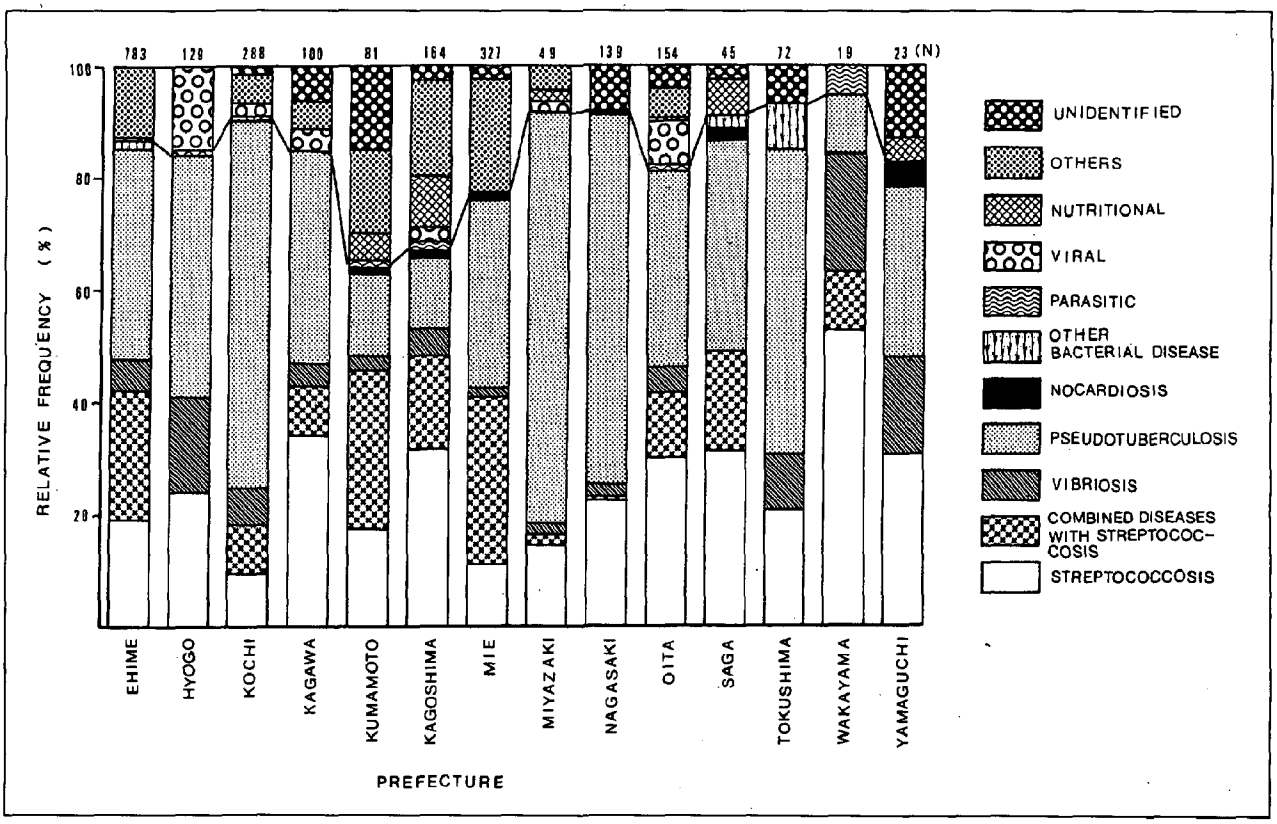

Figure 20
Composition of diseases of yellowtail by prefecture during 1986.

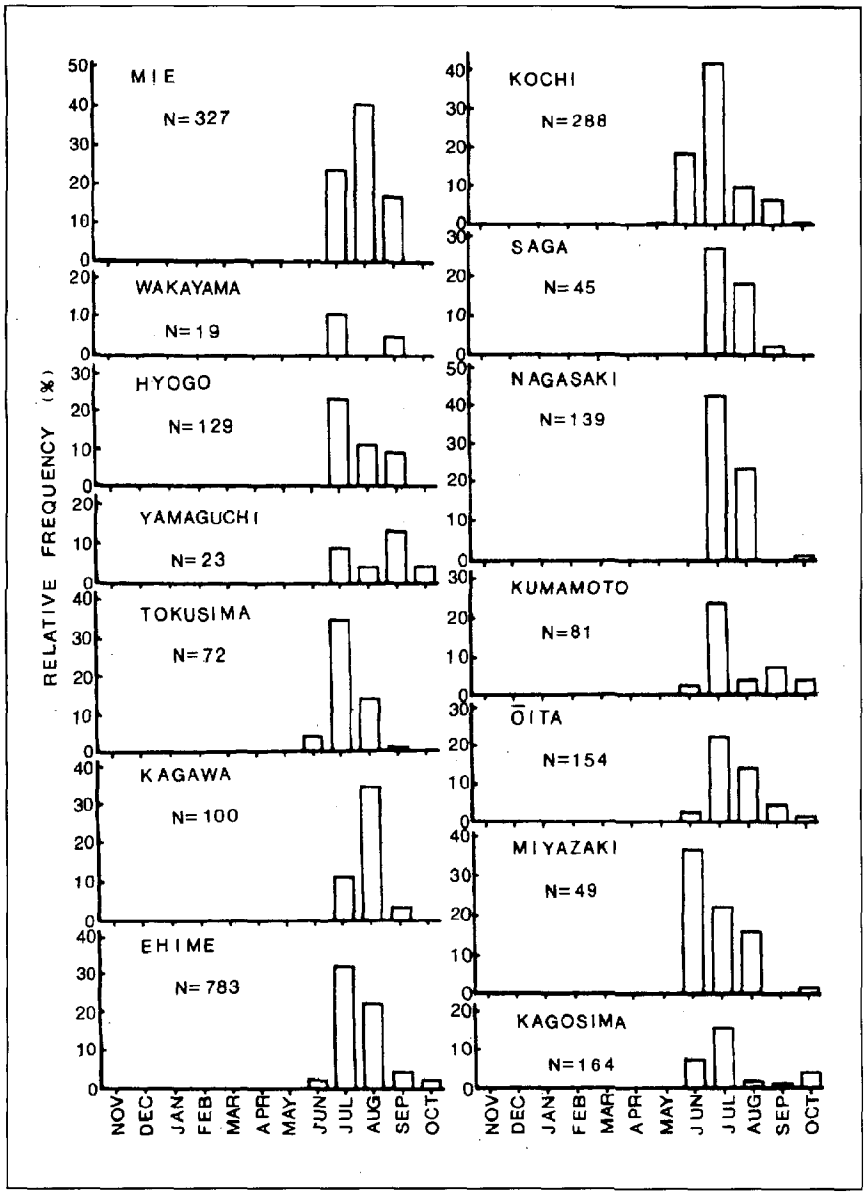

Figure 21
Patterns of the outbreak of pseudotuberculosis in yellow-tail in each prefecture in 1986.

of virulent disease was found according to geographical location. In the example of 1986 shown in Figure 21, pseudotuberculosis broke out earlier in warmer areas. This disease was reported to occur at a higher rate in June and July in Kagoshima, Miyazaki, and Kochi Prefectures, while the rates were higher in July and August in other prefectures. Because the optimal range (20 to 25° C) for multiplication of *Pasteurella piscicida* is strict, the location of outbreaks of this disease could be determined by calendar and locality or latitude.

As a whole, no characteristic local feature was observed that could explain particular disease outbreaks in the area surrounded by warm oceanic current, except perhaps slight differences. This suggests that all fish culture fields along the warm current are in an area favorable to the multiplication and propagation of pathogens.

Discussions

From this epidemiological examination, it is clear that the natural and delicate relationship between fish and their environment is disturbed on a local basis by fish cultivation in coastal areas and induces the propagation of pathogens and the outbreak of disease in the cultured animals. As shown in Figure 22, the means and methods of fish culture affect not only fish health but also water quality and pathogen

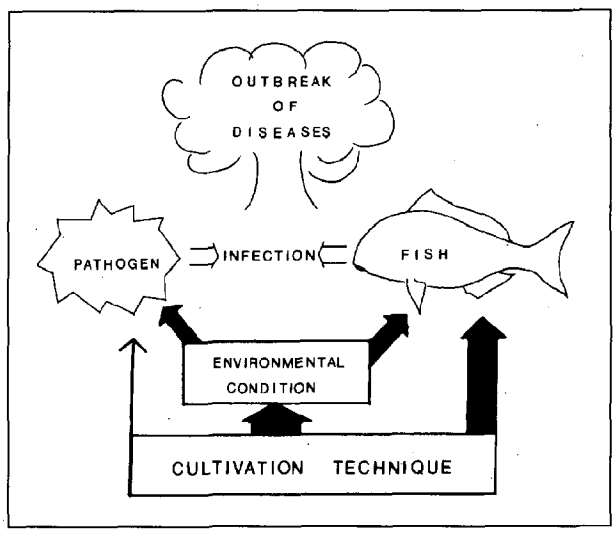

Figure 22
Disease outbreak flowchart for marine fish cultured in the Kuroshio Current.

distribution; major factors include fish density in a pen, location of the pen, pen density, handling, excess food supply, food quality, sanitary treatment of nets, type of food and other materials, and treatment of dead fish. Supplying unsuitable or unsanitary food induces the degeneration of fat in fish, whereas supplying excessive food makes them generate fat. Unsuitable handling techniques injure the body surface, especially in younger fish, and allows pathogens to invade. Fish are also susceptible to pathogens under some nutritional conditions and during certain developmental stages. Culturing fish under high density adds additional stress.

A high density of pens interrupts the water current and causes water quality to deteriorate. As excess food and fish excrement are deposited on the bottom and dissolve into seawater, the quality of water in the cultivation area degrades. These changes in environmental conditions are beneficial for pathogen multiplication, which, in turn, lead to infection of the fish and to outbreak of disease.

As previously described, the seasonal changes of disease cases are very remarkable, and it is thought that temperature is the most influential environmental factor. Higher temperature is beneficial in activating various physiological functions, including immunological processes in healthy fish. But this factor can also be a harmful to unhealthy fish under poor environmental conditions.

The results of this study help us to direct future study and research in this field. Unidentified diseases must be clarified as soon as possible, especially in new fish species introduced for cultivation. In this type of research, it is most important to have a thorough knowledge of the anatomy, physiology, ecology, pathology, and immunology of the fish and pathogens. This may lead to the development of improved cultivation techniques.

Effective methods to diagnose fish health should be developed, not only to prevent mass mortality by pathogens that are generally nonvirulent, but also to regulate cultivation techniques. A large amount of research is needed in this area, for example: physiological studies on poikilothermal fish, pathological studies that clarify the mode of action of pathogen within their hosts during infection, and immunological studies to explain fish defense mechanisms against pathogens.

Epidemiological research in a small area such as one bay unit or a fisheries cooperative association unit should be carried out in detail to clarify the direct relationships among disease outbreaks, environmental factors, and cultivation techniques. Such research could provide knowledge for a more successful cultivation of healthy fish.

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Characterization of Hematic Neoplasia in the Softshell Clam *Mya arenaria*

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ABSTRACT

A leukemia-like disease, variously termed hemic, hematopoietic, or hematic neoplasia, (HN) has been detected in bivalves from diverse geographic locales. The wide geographic range of the disease, coupled with mortalities due putatively to HN, makes it important to determine the nature of the abnormal cells characteristic of the disease. The studies reported here determined certain biochemical and genomic characteristics of HN in the softshell clam *Mya arenaria*. No significant difference in the total amount of lipid of normal and abnormal hemocytes was noted. Fatty acids of the omega 3 and omega 6 families were elevated in clams with the highest HN intensities. Similarly, in vitro incorporation of acetate into phospholipids and neutral lipids by normal lymphocytes was significantly different from HN hemocytes but unlike the pattern seen in mammalian leukemias. Examination of the DNA content of normal and HN cells by flow cytometry indicated a high degree of aneuploidy in the HN cells. This consisted of clams with hyperdiploid DNA content of 1.6 to 2.0 times normal, and a hypodiploid population with DNA 0.85 times normal. Hyperdiploid cells had significantly higher DNA synthesis than diploid cells. In this respect, HN cells are similar to true leukemias of mammals, but the combination of hypodiploid and hyperdiploid cells in one individual is uncharacteristic of mammalian neoplasia. In summary, hemocytes of *M. arenaria* affected with HN have some features coincident with mammalian leukemias but also several unique characteristics.

Introduction

The harvest of bivalves from commercial and cultured sources is of considerable economic importance on both worldwide and national scales. Disease affects natural as well as artificially propagated populations. Unfortunately, our knowledge of diseases in bivalves is quite restricted in comparison to the information base available for finfish. It is important that we establish a more detailed database for diseases of cultured and feral bivalves in order to predict sustainable harvests of this significant marine resource accurately.

Among the most widespread diseases of bivalves are the putative neoplastic diseases which have been detected in a wide variety of commercially important species and from a geographically disparate range

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(Couch 1969; Farley 1969; Brown et al. 1977). The primary tissue distribution of these diseases appears to be gonadal ("germinomas") (Yevich and Barszcz 1976, 1977) and hematic (variously termed hematopoietic neoplasms, hematic neoplasms, or hemic neoplasms). Hematic neoplasia (HN) has been associated with elevated levels of mortality in field and laboratory studies with the softshell clam *Mya arenaria* (Appeldoorn and Oprandy 1980; Cooper et al. 1982) and the edible oyster *Ostrea edulis* (Alderman et al. 1977). The prevalence rates for HN in *M. arenaria* range from 0.02 to 84% , and intensities of HN in individuals span the range from less than 0.01% to greater than 99% of hemocytes affected (Couch 1969; Mix 1986; Reno, unpubl. observations). The pervasive nature of this disease and its potential for killing feral and cultured bivalves makes it essential to understand the nature of the disease and its etiology. The results reported here reflect an initial attempt to define more clearly the nature of the disease in *M. arenaria*.

Hematic neoplasia is characterized by its proliferative nature and the resulting cytological morphological abnormalities assessed by microscopic examination of either unstained, or fixed and stained cells (Couch 1969; Farley 1969; Cooper et al. 1982). These criteria, however, are not adequate for accurately defining HN in bivalves as neoplastic because these invertebrates are evolutionarily distant from the mammals, the phylum on which virtually all definitive work has been done on the characteristics of neoplasia. This lack of definition is noted in two prominent reviews of presumed neoplasms of bivalves: "Some of the neoplasms may be of questionable validity . . . and little is known about the exact nature of the cells involved but, in general, they are considered neoplastic" (Mix 1986); and "In spite of intensive study, the true nature of these neoplastic cells is not yet quite clear." (Lauckner 1983). There are scores of structural, biochemical, immunological, and genomic alterations in mammalian cells associated with the transformed or neoplastic state (reviewed in Wood 1972; Marchesi 1976; Vasiliev and Gelfand 1981; Heim and Mitelman 1987; Iversen 1988). Among the most well characterized biochemical alterations that occur in transformed cells are those occurring in two fundamental classes of molecules: the nucleic acids and the lipids. The present work was designed to monitor potential differences between normal hemocytes and HN cells from *M. arenaria* compared by flow cytometric analysis and in vivo and in vitro lipid constitution.

Materials and Methods

Sample Collection, Evaluation of HN, and Animal Maintenence

Softshelled clams ranging in size from 45 to 120-mm valve length were collected from Long Cove, Searsport, Maine, during the summer, placed on ice, and returned to the laboratory for evaluation of HN levels. When necessary, animals were held in the wet laboratory at the University of Maine in a recirculating artificial seawater system at 14° C until needed. Animals were generally evaluated within 1 week of collection. Evaluation of HN was performed by a modification of the method of Cooper (1982). Approximately 100 μL of hemolymph was removed from the cardiac or anterior adductor sinus, 50 μL was placed in one well of a 96-well, flat-bottomed microtitration plate, and hemocytes were allowed to adhere for 20 to 30 minutes. Samples were then evaluated for the levels of HN by microscopic examination under phase optics at 200× with an inverted cell culture microscope. The number of nonadherant or loosely adherant, rounded cells in the hemolymph (HN cells) was compared to the number of fully adherent spreading normal hemocytes by counting 500 to 1000 cells and converting the proportion to percentages.

Lipid Analysis

The major lipids that are altered in neoplasia are the fatty acids, the neutral lipids, and the phospholipids. Several methods were used to evaluate lipid changes in HN cells, including gravimetric total lipid content, fatty acid methyl ester (FAME) profiles, and in vitro incorporation of radiolabelled acetate into neutral lipids and phospholipids.

Gravimetric analysis was carried out on washed hemocytes from normal and abnormal clams using the method of Sasaki and Capuzzo (1984). This involved a modified Folch procedure employing several extraction cycles with chloroform:methanol. Total lipid by weight was obtained using an ultramicrobalance (Cahn model 25 automatic electrobalance). Fatty acids were esterified to fatty acid methyl esters (FAMEs) to stabilize them prior to analysis, which was carried out on hemocytes from clams with various levels of HN and clams with no evidence of HN. Fatty acid separation was carried out using the modified Bligh and Dyer (1959) method of Jerkofsky and DeSiervo (1986). Washed, pelleted hemocytes were extracted with chloroform:methanol followed by a saline phase

separation, and final suspension in chloroform. A known quantity of a 19-carbon fatty acid was added to the cell pellet prior to extraction to serve as an internal standard for the extraction process. Samples were transesterified with a methanolic base reagent followed by methylene chloride and final suspension in hexane. Esterified samples were then applied to a gas chromatograph (Hewlett Packard model HP5890A) equipped with a 30 m DB carbowax capillary column. Peak areas were calculated with a disc integrator, and retention times were compared with known standard FAMEs.

In vitro assimilation of radiolabelled acetate into neutral and polar lipids was carried out using hemocytes that were removed from the clams within 8 hours of capture. To each of four replicate tubes containing the hemocytes, an equal volume of 0.45 μm-filtered sterile seawater containing 10 μCi of ^{14}C-acetate was added. The cell-acetate mixture was incubated at 15° C for 24 hours and extracted as described for FAMEs, except that, in order to improve the precipitation of labelled lipid, non-labelled carrier lipid was added. The carrier lipid was prepared from two shucked clams by removing the digestive diverticula and skin of the siphons, blending them in a blender, and extracting as previously described. The lipid extract was weighed and resuspended in chloroform to a concentration of 300 mg/mL; 30 mg of carrier lipid was added to each sample. A final suspension in solvent was divided into 2 aliquots which were then evaluated for either neutral lipids by one-dimensional silicic acid paper chromatography (Wuthier 1966) or for polar lipids by two-dimensional silicic acid paper chromatography, followed by autoradiography (Jerkofsky and DeSiervo 1986).

Flow Cytometric Analysis

In order to determine the configuration of the DNA in the genome of HN cells versus normal hemocytes, flow cytometric analysis of the total DNA content of the cells as well as the cell cycle status of the cells was carried out. Preliminary experiments were performed to determine whether live or fixed hemocytes were optimal for flow cytometric analysis. Fixation of HN hemocytes in either 2% formalin-seawater or 2% glutaraldehyde-seawater led to reduced resolution of DNA peaks compared to unfixed materials suspended in the anticoagulant 0.01 M cysteine in seawater. Consequently, fresh, unfixed, washed hemocytes were used for the flow cytometric analysis in all further studies. The processing of hemocytes for flow cytometric analysis of DNA followed standard methods used in human clinical oncology (Raber 1988). Cells were washed in buffered saline with 0.1% bovine serum albumin, counted, and suspended in a solution of 1 μg/mL RNAse and 0.01% NP-40 detergent to increase membrane permeability. This was followed by the addition of propidium iodide fluorochrome at the ratio of 10μg/10^6 cells in a solution of NP-40 and 0.1% polyethylene glycol 8000 at pH 5.0. Examination of the cells under a ultraviolet light microscope confirmed that nuclear morphology was normal and that no artifacts were present. The cells were examined in a flow cytometer (Becton-Dickinson, EPICS model CS) and the relative fluorescence (DNA content) and cell cycle analysis was performed using a Modfit analysis program (Verity Software Corp.), which integrated the area under the various peaks and estimated the percentage of total area for each peak, as well as their coefficients of variation.

Results

Lipid Analysis

Comparison of hemocytes from HN-affected and normal clams by gravimetric analysis indicated that there was no significant difference between the total extractable lipid in the cells (normal cells = 15.97±8.51 pg lipid/cell; HN cells = 23.21±11.88 pg lipid/cell, $P = 0.223$). In order to more fully define any differences that might exist between the two cell types, both the basic fatty acid components, as well as the complex lipid moieties derived from them were analyzed.

Gas chromatographic analysis of FAMEs from hemocytes taken from HN-affected clams was compared with that of normal clams as well as with the pattern found in normal and SV40 virus-transformed WI-38 cells (human diploid fibroblasts) (Table 1). When compared to normal clams, only clams with rates of HN in excess of 90% abnormal cells exhibited significant alterations in FAME composition. The saturated class of FAMEs remained stable in affected clams, with the exception of a decrease in palmitic acid and in stearic acid. Abnormal cells had significantly lower levels of monounsaturated fatty acids, as well as significantly higher levels of the longer chain fatty acids (20 and 22 carbon polyunsaturated groups). The changes found in the FAME profiles of HN cells were opposite in direction from those found in SV40-transformed human cells.

Evaluation of the in vitro incorporation of lipid precursors (acetate) into the more complex lipids also indicated significant changes in hemocytes from

Table 1
A summary comparison of lipid composition of normal and SV40-virus transformed mammalian cells and normal and HN cells from *Mya arenaria*. Levels of HN ranged from 5 to >95%.

Lipid class	WI-38[a]	SV-40 infected WI-38[a]	Normal hemocytes	HN hemocytes
Total lipid/cell (pg)	65.0	59.0	16.0	23.2 (P=0.223)
Neutral lipids (% of total cpm)	29.0	41.0*	5.0	8.0 (P<0.05)
Triacylglycerides (% of neutral)	27.0	25.0*	40.0	62.0 (P<0.005)
Free fatty acids (% of neutral)	21.0	26.0*	36.0	22.0 (P<0.01)
Cholesterol (% of neutral)	35.0	35.0	6.2	4.2 (P<0.05)
Cholesterol esters (% of neutral)	5.0	4.0	18.0	8.0 (P<0.05)
Phospholipids (% of total cpm)	71.0	59.0*	95.0	92.0 (P<0.001)
Cardiolipin (% of phospholipids)	6.1	2.7*	6.0	2.0 (P<0.001)
Phosphotidylethanolamine (% of phospholipids)	12.0	13.0	75.0	71.0 (P<0.001)
Phosphotidylinositol (% of phospholipids)	13.0	10.0*	1.0	2.0 (P<0.001)
Phosphotidylcholine (% of phospholipids)	57.0	57.0	2.0	8.0 (P<0.005)
Fatty acid methyl esters				
Palmitic acid (16:0) (% of FAME)	44.0	50.0*	70.0	59.0 (P<0.01)
Stearic acid (18:0) (% of FAME)	37.0	30.0*	20.0	25.0 (P<0.05)
18:1 Fatty acids (% of FAME)	63.0	78.0*	25.6	22.5 (P<0.05)
20:4 Fatty acids (% of FAME)	37.0	17.5*	1.2	4.0 (P<0.01)

[a] Data from Howard et al. 1977.
*Significant difference between WI-38 and SV40-transformed WI-38.

diseased clams relative to normal ones. The percentage of labelled acetate incorporated into the total neutral lipid fraction of the HN cells was significantly higher, especially the triacylglycerols (P<0.005), the primary storage form of lipids (Table 1). In fact, while the level of the other classes of neutral lipids evaluated, including free fatty acids, cholesterol, and cholesterol esters, decreased significantly, the elevation in triacylglycerides masked the depressed synthesis of these compounds in the total neutral lipid fraction. Oil red O staining, which preferentially stains triacylglycerols (Luna 1986), specifically stained the vacuoles or "droplets" seen in HN cells by phase contrast microscopy; these droplets are not apparent in normal hemocytes.

As with the neutral lipids, there were also significant changes in the incorporation of acetate into the polar lipids, which consist almost entirely of the membrane-associated phospholipids. The overall level of incorporation into the total phospholipid class decreased significantly (P<0.001). Likewise, decreases occurred in the amount of labelled phosphatidylglycerol and cardiolipin, which is derived from phosphatidylglycerol (P<0.001) (Table 1). By contrast, there were significant increases in the levels of incorporation into the phosphotidylinositols and the phosphotidylcholines(P<0.001 and P<0.05 respectively). Many of the changes occurring in the lipids of HN hemocytes differ with those documented in mammalian neoplastic cells (Howard et al. 1973).

Flow Cytometric Analysis

Preliminary experiments indicated that using formalin or glutaraldehyde fixatives for flow cytometric analysis of hemocytes altered the chromosomal material and resulted in poor separation of the normal diploid and aneuploid peaks in HN-positive clams. Therefore, for all of the experiments reported here, unfixed hemocytes were rapidly (within 5 minutes of removal from the clam) processed for flow cytometric analysis. This resulted in a clear separation of DNA peaks in the cells.

Flow cytometric analysis indicated that the DNA content of the abnormal hemocytes was distinctly aneupoid. As shown in Figure 1, there was a strong positive linear correlation (r = 0.95) between the abnormal microscopic appearance of the nonadherent cells and abnormal DNA content as detected by flow cytometry. Two forms of aneuploid cells were detected: those with a hypodiploid DNA content and

those with a hyperdiploid DNA content. Examples of the DNA contents of normal and abnormal hemocytes are shown in Figure 2. The amount of DNA in diploid clam hemocytes was approximately 3.2 pg, compared with 6.8 pg in a human lymphocyte standard. In contrast to diploid clam hemocytes, DNA profiles of cells from abnormal clams invariably had a population of cells containing hyperdiploid DNA comparable in number to the percentage of abnormal cells as assessed by microscopic observation. The DNA index (DI)—the quantity of aneuploid DNA relative to the DNA content of the normal diploid hemocyte—of clams showing hyperdiploidy varied from 1.6 to 2.0 in 12 HN-positive individuals. In most, but not all clams that were HN-positive, a hypodiploid cell population also occurred which comprised between 0.5 and 10% of the cells (Fig. 2). The DI of the hypodiploid cell population, ranging from 0.82 to 0.86, was more constant than the DI of the hyperdiploid population. This hypodiploid peak was not noted in the hemocytes of normal clams.

One of the hallmarks of neoplastic transformation is the proliferative nature of the cells. Cell cycle analysis of hemocytes from HN-positive clams indicated that this was indeed the case for this particular malady. Normal cells showed some indication of proliferative activity; 2 to 5% (mean = 4.4%) were in the S (DNA synthesis) phase and an approximately equal percentage were in the G_2/M (premitotic and mitotic) phases of the cell cycle. Hemocytes from clams that were aneuploid, however, had a significantly higher (mean = 16.4%, P = 0.0001) proportion of cells which were either undergoing DNA synthesis, in preparation for mitosis, or actually in the process of mitosis (Fig. 3). The proportion of HN cells in some phase of replicative activity ranged from 10 to 25%; most of this activity was in the hyperdiploid population, but the hypodiploid and even the diploid populations also were undergoing more extensive multiplication than normal.

Discussion

The present study was designed to help characterize the abnormal hemocytes in *M. arenaria* with hematic neoplasia. This disease, and similar diseases in a variety of other bivalves, have attracted much attention in the scientific community for two reasons. In the marine community, they can cause significant mortalities in the affected animals (Cooper et al. 1982; Brousseau and Baglivo 1991; Reno, Leavitt, and Capuzzo, unpubl. observations). In addition, these diseases have become the focus of epidemiological investigations assessing the potential causal interaction between anthropogenic pollutants in marine environments and the generation of neoplasia, and the potential for using HN disease in bivalves as sentinals in appraising the potential carcinogenic activity of these pollutants in humans. While the rationale of the first reason still holds true irrespective of the true nature of the disease, the assumption

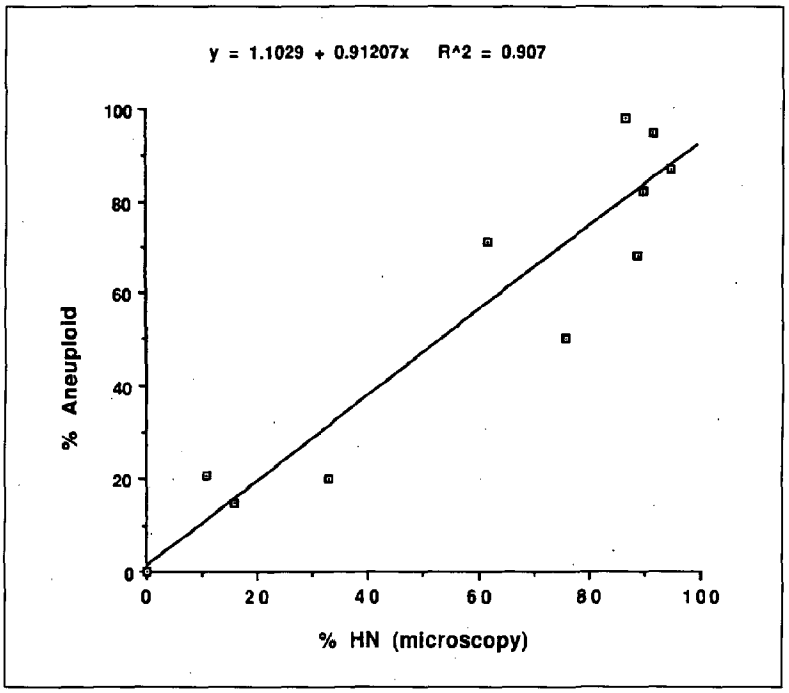

Figure 1
Relationship between the percentage of HN by microscopic evaluation and the percentage of DNA aneuploidy by flow cytometry.

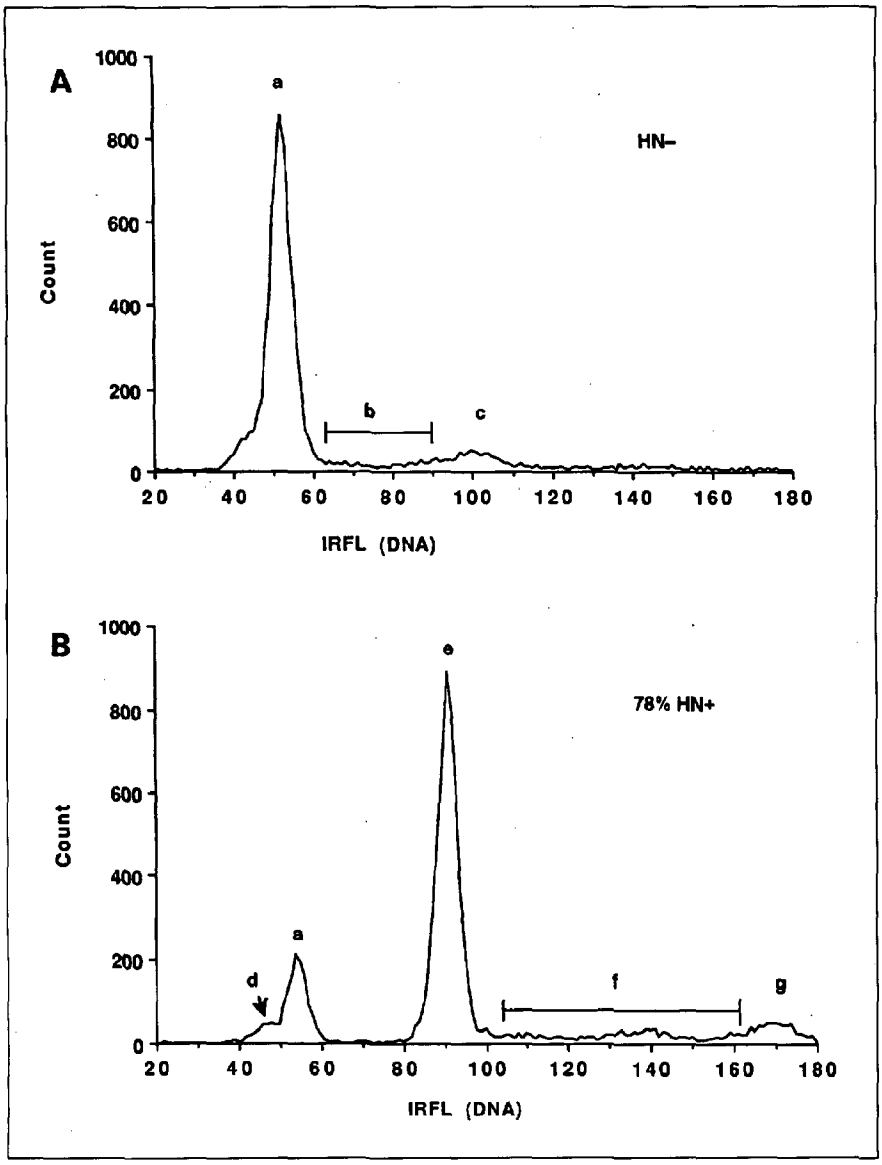

Figure 2
Examples of DNA distrib ution and cell-cycle analysis of normal and abnormal hemocytes. (**A**) Normal clam; (**B**) clam with approximately 78% abnormal cells. IRFL = integrated red fluorescence (DNA content).
Key to letters on graphs:
a: resting stage of cell cycle (G_0 and G_1 phases) for diploid cells
b: DNA synthesis phase of cell cycle (S phase)
c: Premitotic and mitotic phases of cell cycle (G_2 and M phases) for diploid cells.
d: Hypodiploid DNA peak
e: Hyperdiploid DNA peak
f: S phase for hyperdiploid cells
g: G_2/M phases for hyperdiploid cells

on which the second is based is subject to invalidation if the disease is not a true neoplasm, but is rather a hyperplastic or anaplastic disease—or even due to an exogenous source such as a protozooan parasite.

Information is readily available in the literature with respect to the characteristics of neoplasia and transformed cells in mammals and birds. Relatively little is present that deals stringently with the disease and nature of the altered cell in poikilothermic vertebrates; virtually none is available with respect to the "diseased cell" in invertebrates. Mix's comprehensive review (1986) of the nature of the interaction between anthropogenic pollutants and "neoplastic" diseases in aquatic vertebrates and invertebrates represents the most critical analysis of the extant data. The author, as well as others (Harshbarger et al. 1979; Lauckner 1983), prudently note that the criteria presently used to determine the nature of the disease are inadequate when compared with the manifold criteria that are demonstrable in mammals, and that it may be premature or unwarranted to describe the leukemia-like disease in bivalves as a true neoplasm without further investigations into the nature of the disease.

Mammalian and avian neoplastic cells derived from tumor tissue or transformed by biological, physical, or chemical agents have several dozen characteristics in common that differ significantly from normal cells. These characteristics are most readily evaluated when the cells are separated from their normal residence in vivo and are cultured in vitro, if only for short periods. This process removes the major confounding factor of the large number and wide variety

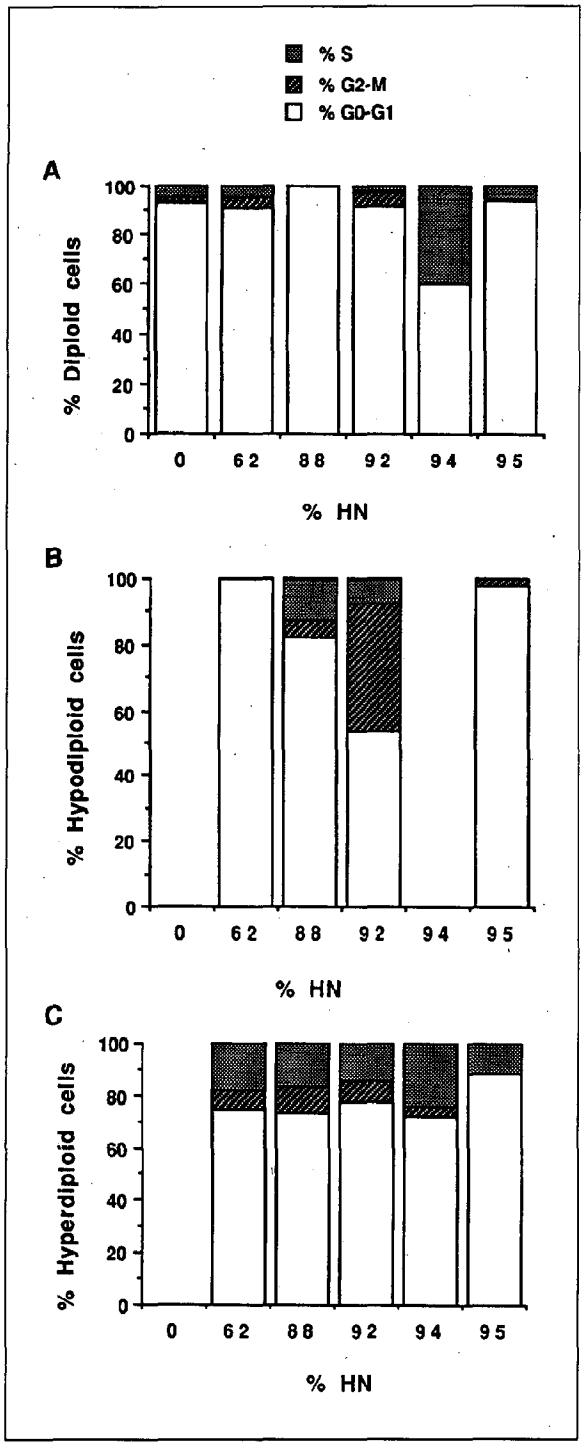

Figure 3
Percent of diploid and aneuploid cells in various phases of the cell cycle. (**A**) diploid cells; (**B**) hypodiploid cells; (**C**) hyperdiploid cells. % S = cells in DNA synthesis phase of cell cycle; G_2/M = cells in premitotic and mitotic phases of the cell cycle; G_0/G_1 = cells in the resting stages of the cell cycle.

of normal cells that are present in tumor tissue in vivo. Affected cells of the circulatory system are most readily divested of these contaminants by simply drawing off hematic fluids which contain the abnormal cells and placing them in culture for further analysis. This is the approach taken in the current investigations.

Lipids are the major constituents of cellular membranes, as well as a source of stored energy, and the specific lipid components that are present have a marked effect on the properties of the membrane (Wood 1972; Shinitzky 1984). Because a wide variety of the alterations occurring in neoplastically transformed cells are associated with the plasma membrane surface, it is not surprising that many alterations in the lipid composition of neoplastic cells have been documented in mammalian systems. Wood (1972) and others have published a variety of papers dealing with the alterations in lipid metabolism and biochemistry in mammalian neoplasia (e.g., Awad and Spector 1976; Yau et al. 1976; Montaudon et al. 1981; Schroeder and Gardiner 1984; Kier et al. 1988; Calorini et al. 1989). The SV40-induced transformation of human WI-38 lung fibroblast cells (Howard et al. 1973; Perkins and Scott 1978) is an in vitro system resembling HN of *M. arenaria* because transformation by viruses produces a homogeneous population of altered cells, as is found with HN hemocytes, and because it has been indicated that HN in *M. arenaria* is also viral-induced (Oprandy et al. 1981).

There are significant alterations in more than one half of the lipid moieties examined in the SV40-WI-38 cells in culture. With the essential building blocks of lipids, the fatty acids, there are increases in the levels of the saturated fatty acid palmitic acid (16:0), the monounsaturated fatty acid oleic acid (18:1Δ9), and the diunsaturated fatty acid linoleic acid (18:2Δ6). In contrast, in *M. arenaria* HN cells show a decrease in palmitic acid but a similar increase in the two unsaturated fatty acids. In the virally transformed WI-38 cells, a significant decrease occurs in the levels of stearic (18:0), palmitoleic (16:1Δ7), linolenic (18:3Δ3), and arachidonic (20:4Δ6) acids. In *M. arenaria* HN, however, there is a marked increase in the levels of stearic acid in conjunction with decreases in the levels of 16:1Δ7, 18:3Δ3, and 20:4Δ6 fatty acids. The changes seen in mammalian and bivalve cells are at variance in their levels of two major essential fatty acids derived from *de novo* sources: palmitic and stearic acids. These variations may reflect a difference in the process of synthesis of the fatty acids in invertebrates compared with mammals or may reflect a basic difference in the nature of the abnormal HN cell and the transformed WI-38 cell. With respect to the more complex lipids, a significant

increase is seen in the levels of cholesterol in both virally transformed WI-38 and in murine hepatoma cells (Steele and Jenkin 1977). Cholesterol is one of the most important lipids in the cellular membrane and contributes much to the attributes of the cell surface. In studies with transformed mammalian cells there is a significant increase in the levels of cholesterol in the cytoplasmic membrane (Howard et al. 1973). In our study, the incorporation of labelled acetate into cholesterol, and its derivatives, the cholesterol esters, of HN cells was significantly lower than the levels of incorporation seen in normal hemocytes. This is in contrast to uptake observed in mammalian neoplastic cells. Likewise, the changes in another class of neutral lipids, the free fatty acids, remained unchanged while in mammalian cells there is an increase in free fatty acids. Taken together, these major differences between the lipid biochemistry of HN cells and that of neoplastically transformed mammalian cells indicate that it would be presumptious to declare HN cells neoplastic on the basis of their lipid biochemistry.

A fundamental change that occurs in neoplastically transformed cells is the alteration of the genome of the cell, which is heritable and leads to the initiation and progression of tumors. These alterations may be considerable, such as heteroploidy, which can be easily detected by flow cytometric analysis, or more subtle, such as chromosomal rearrangements or alterations in specific gene sequences which must be detected by karyotyping or molecular DNA analysis. In this study, flow cytometric analysis was used to determine the potential lesions in the DNA content of HN cells and also to assess the proliferative potential of the disease. It was found that HN cells were distinctly aneuploid in nature and that the most consistent change in the DNA content in HN cells was to a hyperdiploid state. The level of this hyperdiploid population correlated well with the proportion of cells which appeared abnormal by microscopic examination. There was significant variation, however, in the amount of aberrant DNA in the cells, which ranged from 1.6 to 2.0 times normal. This would be consistent with the clonal development of the transformed cell (Iversen 1988). However, it was also found that most of the clams that were HN positive had a second, markedly stable hypodiploid population of cells, a finding that is inconsistent with the clonal development of transformed cells. This finding is problematic if one is making the assumption that HN in *M. arenaria* is a true neoplastic disease because it is unlikely that a stable hypodiploid population would coexist with a more variable hyperdiploid population in the same animal if this were true.

The available data on the etiology of HN in bivalves is scant. There is evidence that the abnormal hemocytes of HN clams possess unique surface antigens as detected with both polyclonal and monoclonal antibodies (Reinish et al. 1983; Miosky et al. 1989). In Smolowitz et al. (1989), this same group has also produced a monoclonal antibody that reacts both with a subpopulation of cells in the gut of normal clams and with HN cells, indicating an internal source for HN cells. However, with the well-known possibility of epitopic crossreactivity, or even with active mimicry of epitopes by parasites, the reaction of a single monoclonal antibody with a subpopulation of *M. arenaria* hemocytes is insufficient to confirm the endogenous source of the HN cells. As mentioned above, several authors have noted that the available information on the origin of the HN cell in clams does not preclude the possibility that the cells are parasitic rather than neoplastic in nature (Lauckner 1983; Mix 1986). Unfortunately, the data presented here does not alleviate this problem because, for example, the heteroploid cells may be parasitic protozooans or even algae, which would have a considerably different chromosomal makeup than clam hemocytes. Further work on genetic analysis of these cells using cytogenetic techniques, as well as molecular biological techniques, must be carried out before the resolution of this problem can be achieved.

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Kinetics of Bovine Serum Albumin Administered by the Immersion Method in Fishes Acclimatized to Seawater and to Fresh Water

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ABSTRACT

To clarify plasma BSA kinetics in marine fish after hyperosmotic infiltration (HI) treatment, chum salmon (*Oncorhynchus keta*) and blue tilapia (*Tilapia aurea*) were acclimatized to seawater or to fresh water, and were administered bovine serum albumin (BSA) by HI treatment or intravenous injection. First, by using a least squares program, regression curves were computed from the plasma BSA levels of fish injected with BSA to obtain the clearance rate of BSA from plasma. Then, by using a deconvolution method, the BSA release rate from the organ of BSA entry to plasma after HI treatment was calculated from plasma BSA levels after HI treatment and the clearance rate. The results showed that the clearance rates of both fishes acclimatized to seawater were higher than those of the fish acclimatized to fresh water. On the other hand, the release rates of both fishes acclimatized to seawater were much lower than those of the respective fish acclimatized to fresh water. Thus, it is quite plausible that the high clearance rates and low release rates of fish in seawater work synergistically, resulting in lower plasma BSA levels of fish in seawater compared with fish in fresh water after HI treatment.

Introduction

The immersion method, first reported by Amend and Fender (1976), is a useful method for mass immunization in aquaculture. Many papers have shown the effectiveness of this method on freshwater fish (Ellis 1988). On the other hand, there is a little knowledge as to the immersion method's effectiveness on marine fish. In a preliminary study (unpubl. data), the authors used hyperosmotic infiltration (HI) treatment to administer bovine serum albumin (BSA) to yellowtail *Seriola quinqueradiata*, which is the most important aquaculture species in Japan, and rainbow trout *Oncorhynchus mykiss*, and then measured the BSA levels in their plasma. We found that plasma BSA levels were much lower in yellowtail than in rainbow trout after HI treatment. This difference may have been caused by two factors: species specificity and the type of environmental water—fresh water or seawater. To analyze this difference it is necessary to compare the kinetics of BSA in the plasma of several species of fish that can reside in both seawater and fresh water. Thus, in the present study, chum salmon (*O. keta*) and blue tilapia (*Tilapia aurea*) were acclimatized to seawater or to fresh water, and then administered BSA by HI treatment or injection. By using the plasma BSA levels of these animals, the rate of BSA release into plasma and BSA clearance from the plasma were calculated.

Materials and Methods

Experimental Animals

Six-month-old chum salmon, 16 ± 1 g (mean ± standard deviation) in body weight, and 1-year-old blue tilapia, 157 ± 20 g in body weight, were used in this experiment. Tilapia were fed commercial floating dry pellets in a 1-ton tank supplied with running fresh water at 25° C, whereas chum salmon were kept in a 1-ton tank supplied with running fresh water at 15° C, and fed commercial dry pellets for rainbow trout. These tanks were kept indoors.

Acclimatization to Seawater

Before the administration of BSA, one half of the tilapia and chum salmon were acclimatized to seawater, and the other half were kept in running fresh

water throughout the experiment. Fish were acclimatized to seawater (salinity: 34 ppt) by increasing the proportion of seawater day by day to 50% on the first day, 75% on the second day, 87.5% on the third day, and 100% on the fourth day. Afterwards the fish were kept in the tank supplied with running seawater for 3 weeks before the experiment. During the acclimatizing period, no fish died or showed abnormal behavior. Experiments were carried out with four groups of fishes: chum salmon in fresh water (freshwater salmon); chum salmon acclimatized to seawater (seawater salmon); tilapia in fresh water (freshwater tilapia); and tilapia acclimatized to seawater (seawater tilapia). Water temperature was maintained at 15° C for freshwater or seawater salmon and at 25° C for freshwater or seawater tilapia.

Administration of BSA by HI Treatment

Fifty fish of each group were administered BSA (fraction V, Sigma Co.) by the two-step hyperosmotic infiltration technique according to the method of Amend and Fender (1976) with slight modification. Distilled water was used to prepare the two bath solutions; 5.3% NaCl solution and 2% BSA solution. Fish were immersed in 5.3% NaCl solution for 3 minutes and then placed directly into 2% BSA solution for 3 minutes. After the bath treatment, the treated fish were placed in tanks supplied with either running seawater or fresh water for 3 minutes. Afterwards they were returned to their original tanks. Blood samples were taken 0.5, 1, 2, 4, and 8 hours after the bath treatment. At each sampling time six fish were sacrificed and the blood was withdrawn from the caudal blood vessels using heparinized syringes.

Administration of BSA by Injection

Fifty fish of each group were administered BSA by intravenous injection. Bovine serum albumin was dissolved with sterilized 0.85% NaCl solution to 2 mg/mL and then injected into fish via the caudal blood vessels at the dose of 1 mg/kg body weight. Blood samples were taken 0.5, 1, 2, 4, 8, and 24 hours after the administration by the same method as mentioned above.

Immunoelectrophoresis

Bovine serum albumin content in the plasma was quantitatively assayed by rocket immunoelectrophoresis (Laurell 1966; Wallenborg and Andersson 1978). The gel plate was run at 1 V/mm and 15° C for 3 hours. The exact amounts of BSA in normal fish plasma were run in parallel with all tests for standard reference.

Calculations

Plasma BSA Clearance—By means of an unweighted least squares program, the plasma BSA levels $Cp^{inj}(t)$ at each time t after injection were fitted to following exponential equations:

$$Cp^{inj}(t) = a_1 \cdot \exp(-b_1 \cdot t); \text{ or}$$

$$Cp^{inj}(t) = a_1 \cdot \exp(-b_1 \cdot t) + a_2 \cdot \exp(-b_2 \cdot t).$$

For the index of BSA clearance from plasma, the mean residence time (MRT) of each experimental group was given by

$$\text{MRT} = 1/b_1; \text{ or}$$

$$\text{MRT} = 1/b_1 + 1/b_2 - (a_1 + a_3)/(a_1 \cdot b_2 + a_2 \cdot b_1).$$

The BSA distribution volume V of each experimental group was calculated by

$$V = D/Cp^{inj}(0),$$

where D is the amount of BSA administered by injection (1 mg/kg body weight).

BSA Release into Plasma—The BSA release rate into the plasma $U(t)$ at each time t was given by

$$Cp^{HI}(t) = \int_0^t U(t) \cdot Cp^{inj}(t-\tau) \cdot d\tau,$$

where $Cp^{HI}(t)$ is the plasma BSA level after the HI treatment and τ is the time constant. The gross BSA release into the plasma after the HI treatment was calculated from $U(t)$ by integration.

Statistics

Data were analyzed using the F test, and then by the Student's t test to detect significant differences.

Results

Plasma BSA Levels After the HI Treatment

Concentration of plasma BSA in the freshwater salmon increased until 4 hours after the HI treatment

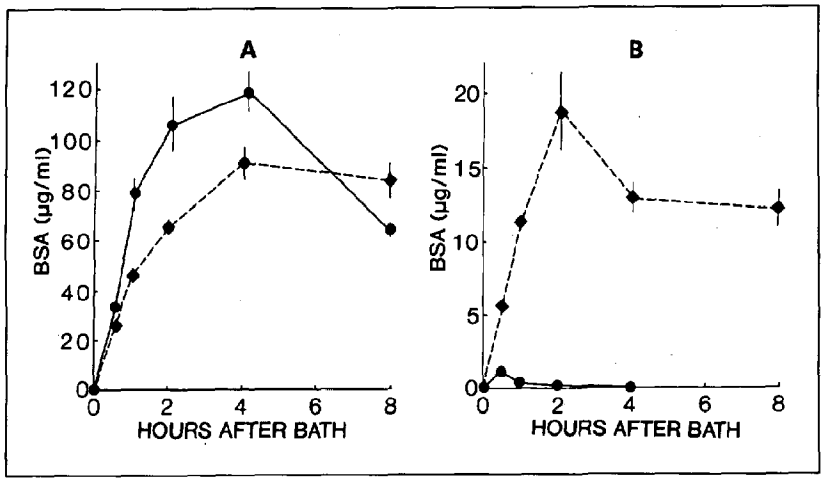

Figure 1
Changes in plasma concentrations of bovine serum albumin (BSA) after hyperosmotic infiltration treatment in freshwater-acclimatized fish (**A**) and seawater-acclimatized fish (**B**). Key: (•) = tilapia; (◆) = chum salmon. Data are presented as means ± standard errors.

and reached a maximum of 90.6 ± 6.5 µg/mL (mean ± standard error), and then decreased gradually (Fig. 1A). On the other hand, plasma BSA levels of the seawater salmon (Fig. 1B) were always significantly lower ($P<0.01$) than those of freshwater salmon. A maximum plasma BSA level of 18.8 ± 2.6 µg/mL was observed in the seawater salmon 2 hours after the bath; from then on, plasma BSA stayed at a rather constant level of 11–19 µg/mL. The plasma BSA level of freshwater tilapia increased until 4 hours following the bath when it reached a maximum of 119.0 ± 8.1 µg/mL and subsequently decreased to 64.1 ± 2.8 µg/mL 8 hours after the treatment (Fig. 1A). In contrast, plasma BSA levels of seawater tilapia (Fig. 1B) were always significantly lower ($P<0.001$) than those of freshwater tilapia. A low level of BSA (0.09–1.1 µg/mL) was detected in the plasma of the seawater tilapia for the initial 2 hours, but no BSA was detected in the plasma after 4 hours (minimum detectable level, 0.01 µg/mL).

Plasma BSA Levels After the Injection

Following BSA injection, the plasma BSA levels were always lower in seawater salmon than in freshwater salmon, and the difference was significant ($P<0.01$) 0.5, 1, 2, 8, and 24 hours after the injection (Fig. 2A). The difference between seawater and freshwater acclimatized fish was more distinct in tilapia than in salmon, and the plasma BSA levels of seawater tilapia were always significantly lower ($P<0.001$) than those of freshwater tilapia (Fig. 2B).

Plasma BSA Clearance

The regression equation

$$Cp^{inj}(t) = a_1 \cdot \exp(-b_1 \cdot t) + a_2 \cdot \exp(-b_2 \cdot t)$$

was adopted for express plasma BSA concentration for freshwater salmon after injection. On the other hand,

$$Cp^{inj}(t) = a_1 \cdot \exp(-b_1 \cdot t)$$

was adopted as the regression equation for seawater salmon, freshwater tilapia, and seawater tilapia (Fig. 2). The MRT and the distribution volume of BSA after the injection are shown in Table 1. Both in

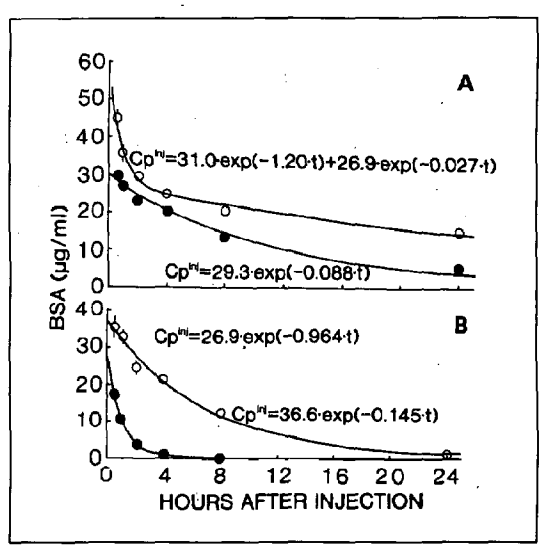

Figure 2
Changes in plasma concentrations of bovine serum albumin (BSA) after injection of BSA into chum salmon (**A**) and tilapia (**B**). Open circles indicate the fish in fresh water and closed circles indicate the fish acclimatized to seawater. Data are presented as means ± standard errors. Regression equations were calculated with a least squares program.

Table 1
Kinetics of plasma BSA administered by hyperosmoticinfiltration treatment.

Experimental group	Maximum BSA level in plasma (μg/mL)	Mean residence time (MRT) (hours)	Gross release into plasma (μg/mL)	Distribution volume (mL/100g b.w.)[a]
Freshwater salmon	84	36	167	1.7
		(37)[b]	(95)[b]	(3.7)[b]
Seawater salmon	19	11	24	3.4
Freshwater tilapia	119	6	146	2.7
Seawater tilapia	1	1	14	3.7

[a]b.w. = body weight.
[b]Calculated by the modified regression equation.

chum salmon and in tilapia, the plasma BSA clearance, as represented by the MRT, was more rapid in seawater acclimatized fish than in respective freshwater acclimatized fish. The MRT of seawater salmon and seawater tilapia were 1/3 and 1/6 of that of respective freshwater acclimatized fish. The distribution volume of freshwater salmon was smaller than those of other experimental groups.

BSA Release into the Plasma

The BSA release rate into the plasma, calculated from the clearance rate and the plasma BSA levels, is shown in Figure 3. The BSA release rate was always lower in seawater acclimatized fish than in freshwater acclimatized fish. In all experimental groups, the BSA release rate was high during the early period, and the peak of the rate was observed within one hour after the bath treatment. The rate rapidly decreased after one hour and became low six hours after the bath. The gross BSA release into plasma, which is given by the area under the curve in Figure 3, is shown in Table 1. Both in chum salmon and tilapia, the gross BSA release of freshwater acclimatized fish was larger than that of seawater acclimatized fish.

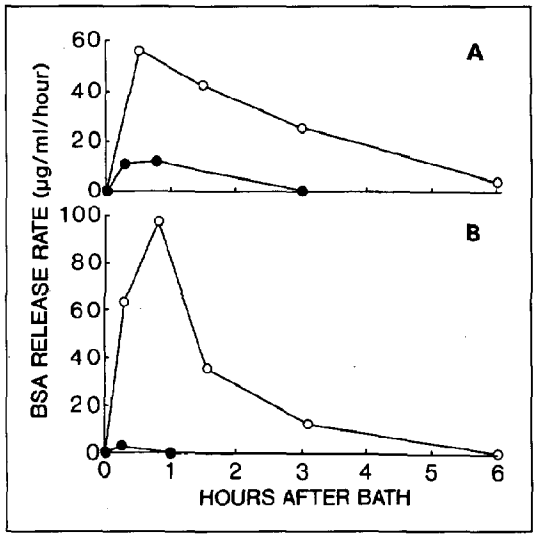

Figure 3
Changes in the BSA release rate into plasma after hyperosmotic infiltration treatment in chum salmon (**A**) and tilapia (**B**). Open circles indicate the fish in fresh water and closed circles the fish acclimatized to seawater. Data are presented as mean ± standard errors.

Discussion

In the present study, BSA clearance from the plasma after the intravenous injection differed greatly within the same species; in each species, the BSA clearance was much more rapid in seawater-acclimatized fish than in freshwater-acclimatized fish. These results strongly suggest that the environmental water affects fish physiology and consequently, by some mechanism(s), changes the plasma BSA clearance. Smith (1982) reported that plasma BSA administered by the immersion method was trapped in the spleen and the kidney of rainbow trout. Similarly, the trapping of plasma BSA by the spleen and the kidney has been observed in plaice administered BSA by intravenous injection (Ellis 1980). The kidney is one of the most important organs for osmoregulation in fish, and its function changes following seawater acclimatization (Hickman and Trump 1969). Thus it is possible that the seawater acclimatization mechanism influenced the BSA trapping in the kidney and affected the plasma BSA clearance. The plasma BSA clearance was

also different between species. Tilapia showed a rapid BSA clearance, while the chum salmon showed a slow BSA clearance. This difference may not only be attributed to species specificity, but also to the temperature of the rearing water.

The estimated gross BSA release into the plasma of seawater salmon and seawater tilapia was much smaller than the amount released into respective freshwater acclimatized fish. This indicates that the physiological changes that resulted from seawater residence reduced the gross BSA release after the HI treatment. Amend and Fender (1976) suggested that hyperosmotic pretreatment had a hydrating effect on membranes of fish tissues, and thus made BSA infiltrate into the fish more easily. However, fish residing in seawater are adapted to a hyperosmotic environment, and their membranes are more resistant to hydration than those of fish in fresh water. Therefore, it is reasonable to consider that hyperosmotic pretreatment stimulates BSA uptake more strongly in fish in fresh water than in fish in seawater. In any case, it is quite plausible that some factors in seawater influenced the fish physiology and induced the low BSA release rate and high BSA clearance rate. These two rates of seawater-acclimatized fish worked synergistically to make their plasma BSA levels after the HI treatment much lower than those of freshwater-acclimatized fish.

The BSA distribution volume after the intravenous injection is expected to agree with the whole plasma volume estimated by tracer-dilution methods using Evans blue dye, serum albumin, or red blood cells, all of which have been widely used to determine whole plasma (or blood) volumes. Using these methods, whole plasma volumes of fish have been determined to be 2.5–4.7 mL/100 g body weight in salmonids (Conte et al. 1963; Smith 1966; Huggel et al. 1969, Nikinmaa et al. 1981; Gingerich et al. 1987) and 3.5 mL/100 g body weight in yellowtail (Itazawa et al. 1983). The distribution volumes of the present study for seawater salmon, freshwater tilapia, and seawater tilapia agree well with those reported values. In freshwater salmon, however, the distribution volumes in the present study were smaller than the whole plasma volumes reported so far in the literature. Smith (1966) suggested that blood sampling periods of less than two hours after tracer injection tended to produce low plasma volume and high clearance rate estimates in salmonids because these periods did not allow for adequate circulation of tracers in the vascular system. Therefore, in the case of freshwater salmon in the present study, there is a possibility that the plasma BSA clearance rate was overestimated. In such a case, the gross BSA release is likely to be overestimated. Therefore, the BSA release and clearance rates in freshwater salmon were examined by modifying the regression equation to ignore the first term, which is the main component for the two hours after the injection. The distribution volume, the MRT, and the gross BSA release were recalculated by using this modified regression equation and the deconvolution method. Recalculation with the modified regression equation also showed more rapid BSA clearance and less gross BSA release in seawater salmon than in freshwater salmon (Table 1).

It has been reported that the plasma kinetics of several ions, such as Na^+, Ca^{2+}, Mg^{2+}, and Cl^-, are different in seawater- and freshwater-acclimatized fish (Hirano and Uchida 1972; Bentley 1971). There have also been reports comparing the kinetics of medicines in plasma of seawater- and freshwater-acclimatized fish, but the results are contradictory among the chemicals. Endo and Onozawa (1987) and Ishida (1990) reported that when oxolinic acid was administered to ayu, *Plecoglossus altivelis,* and coho salmon, *O. kisutch,* by bath and oral methods the seawater-acclimatized fish of both species showed lower oxolinic acid concentration and shorter retention time than freshwater-acclimatized fish. On the other hand, Bergsjo and Bergsjo (1978) reported that when sulfanilamide or sulfadimidine was administered by bath treatment to rainbow trout, the plasma concentration of seawater-acclimatized fish was higher than that of freshwater-acclimatized fish. It still remains unknown why there are such differences between medicines. In any case, from the view of vaccine application to marine fishes, it is quite important to clarify whether the phenomenon observed in the present study for BSA (seawater-acclimatized fish have a lower release rate and higher clearance rate) is common to protective antigens such as lipopolysaccharides and proteases.

Acknowledgments

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The Epidemiological Study of Furunculosis in Salmon Propagation

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ABSTRACT

The authors attempted to determine the distribution and prevalence of *Aeromonas salmonicida* in mature chum (*Oncorhynchus keta*), pink (*O. gorbuscha*), and masu salmon (*O. masou*) in Hokkaido that showed no apparent clinical signs of furunculosis. From September 1979 to November 1989, a total of 12,891 chum, pink, and masu salmon were collected from 30 rivers. The changing pattern of the annual prevalence of *A. salmonicida* in salmon was closely related to changes in fish density in the holding ponds: the prevalence of *A. salmonicida* increased in proportion to the increase in the number of fish in the ponds. We concluded from the results of histological and bacteriological examinations that fish with *A. salmonicida* in the kidney were not diseased but were carriers of *A. salmonicida*. The agent could not be isolated from the immature fish examined. *A. salmonicida* was also isolated from the ovarian fluid of fish showing no apparent clinical sign of furunculosis. Few *A. salmonicida* were found on the surface of the eggs one hour after fertilization. A survey of agglutination titers against *A. salmonicida* in sera of chum, pink, and masu salmon showed great variability within the species. The isolated strains were identified as *A. salmonicida* subsp. *salmonicida* and were pathogenic to salmonids. We concluded that the *A. salmonicida* carrier state in fish poses a serious problem in the prevention of furunculosis and its reduction plays a key role in salmon propagation. Both maturation of fish under conditions of low density in ponds, and disinfection of their eggs, are necessary to prevent fish furunculosis during artificial propagation of salmon.

Introduction

Furunculosis of salmonid fishes, caused by *Aeromonas salmonicida,* was first reported in 1890s by Emmerich and Weibel (1890, a and b). Since these first reports, furunculosis has been reported in virtually all parts of the world where wild or cultured salmonids occur (Smith 1960; Herman 1968; Snieszko 1972; Austin and Austin 1987).

Furunculosis is not a serious problem in rainbow trout (*Oncorhynchus mykiss*) culture in Japan because this species is resistant to the causative agent of the disease. However, serious mortality has been documented in juvenile amago salmon (*O. rhodurus*) and masu salmon (*O. masou*) in accordance with increased production of these fish. In Hokkaido, outbreaks of furunculosis have been reported to occur in chum salmon (*O. keta*) by Nishino (1967), and in masu and pink salmon (*O. gorbuscha*) by Kimura (1970) during the maturation of these species in holding ponds. Nomura and Kimura (1981), Nomura (1983), and Nomura et al. (1983, 1991, a and b) reported isolating *A. salmonicida* from the kidneys of mature chum, pink, and masu salmon that showed no apparent clinical signs of furunculosis.

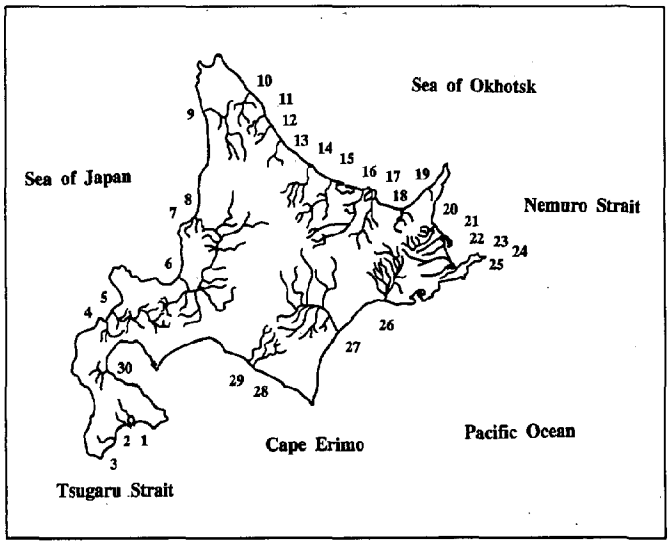

Figure 1
Rivers where chum, pink, and masu salmon were collected (after Nomura et al. 1991a).

1 Moheji	2 Hekirichi	3 Shiriuchi	4 Shubuto
5 Shiribetsu	6 Ishikari	7 Shokanbetsu	8 Nobusha
9 Teshio	10 Tonbetsu	11 Hrobetsu	12 Tokushibetsu
13 Horonai	14 Shokotsu	15 Yuubetsu	16 Tokoro
17 Abashiri	18 Shari	19 Iwaobetsu	20 Ichani
21 Shibetsu	22 Tohoro	23 Nishibetsu	24 Furen
25 Bettouga	26 Kushiro	27 Tokachi	28 Shizunai
29 Niikappu	30 Yurappu		

Recently, no systematic epidemiological studies have been done to establish control measures for furunculosis in salmonids from which quarantine and disease control policies could be based.

In this paper, we report the recent epidemiological study of *A. salmonicida* which was carried out for the purpose of establishing control measures for the disease.

Distribution of *A. salmonicida* in Salmonids in Hokkaido

We attempted to determine the distribution and prevalence of *A. salmonicida* in mature chum, pink, and masu salmon populations in Hokkaido that showed no apparent clinical signs of furunculosis (Nomura et al. 1991a).

From September 1979 to November 1989, a total of 12,891 chum, pink, and masu salmon were collected from 30 rivers (Fig. 1). At each sampling, a total of 60 fish of each species were randomly selected from the rivers' salmonid populations in accordance with Amos (1985). The fish were separated by species and river and held in individual ponds at each river for about 1 month until maturity. After spawning, they were processed for examination. Kidney materials were streaked onto nutrient agar plates (Eiken Co., Tokyo, Japan) and cultured at 20°C for 7 days. No clinical signs of furunculosis were observed in the examined fish. Bacterial colonies that produced a soluble brown pigment and showed the following characteristics were classified as *A. salmonicida:* Gram-negative staining, lack of motility, failure to grow at 37°C, tested positive for cytochrome oxidase, and had the ability to ferment on oxidative fermentative basal medium.

We isolated *A. salmonicida* from chum salmon in 11 of the 22 rivers examined; the percent occurrence of the bacterium in this species of fish ranged from 0.6 to 49.2%. Populations of pink salmon, from 13 rivers were tested and *A. salmonicida* was isolated from 6 of these rivers with percent occurrence ranging from 0.2 to 13.3%. In masu salmon the bacterium was isolated from 5 of 10 rivers examined and the percent occurrence ranged from 1.0 to 5.6%. Hence, A. salmonicida was determined to be distributed widely in the salmonid populations of Hokkaido, except those of rivers located between Tsugaru Strait and Cape Erimo (Fig. 2).

In the Ishikari, Shari, Iwaobetsu, Shibetsu, and Tokachi rivers, the prevalence of *A. salmonicida* was found to vary yearly. In the Ishikari river, the prevalence of *A. salmonicida* in chum salmon was high from 1979 to 1984 but has gradually been decreasing since 1985 (Fig. 3). In the chum salmon of the Tokachi River and in all three species in the Shibetsu River, the prevalence of the bacterium remained high throughout the examination period. In the Shari river from 1979 to 1988, *A. salmonicida* was not isolated from any of the fish examined; however, it was isolated from 4 of 60 fish examined in 1989.

From 1979 to 1984, changes in the monthly prevalence of the agent could be observed in fishes in the Ishikari river. The incidence of *A. salmonicida* increased until the middle of October and then decreased thereafter. The pattern of change was closely related with changes in fish density in the holding pond; the prevalence of the bacterium appeared to increase proportionately as density of fish in the pond increased (Fig. 4).

The number of *A. salmonicida* bacteria found in kidney tissues ranged from 10^1 to 10^5 colony forming units per gram (cfu/g) (Nomura et al. 1991a).

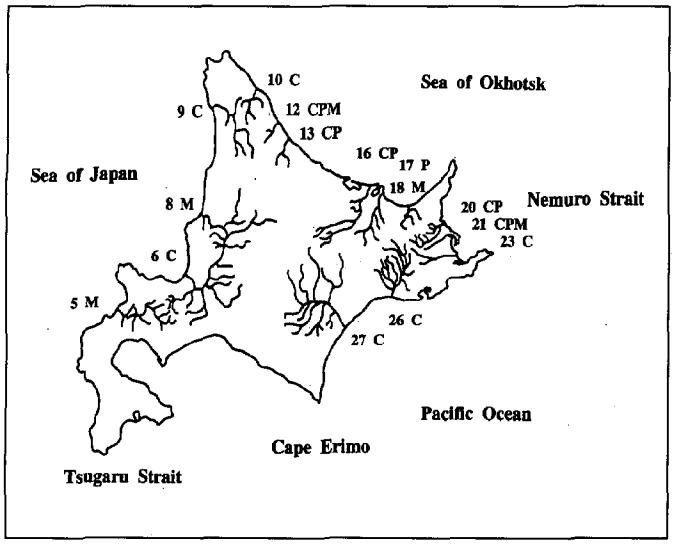

Figure 2

Rivers where *Aeromonas salmonicida* was isolated from chum, pink, and masu salmon from 1979 to 1989. C: isolated from chum salmon; P: isolated from pink salmon; M: isolated from masu salmon (after Nomura et al. 1991a).

5 Shiribetsu	6 Ishikari	8 Nobusha
9 Teshio	10 Tonbetsu	12 Tokushibetsu
13 Horonai	16 Tokoro	17 Abashiri
18 Shari	20 Ichani	21 Shibetsu
23 Nishibetsu	26 Kushiro	27 Tokachi

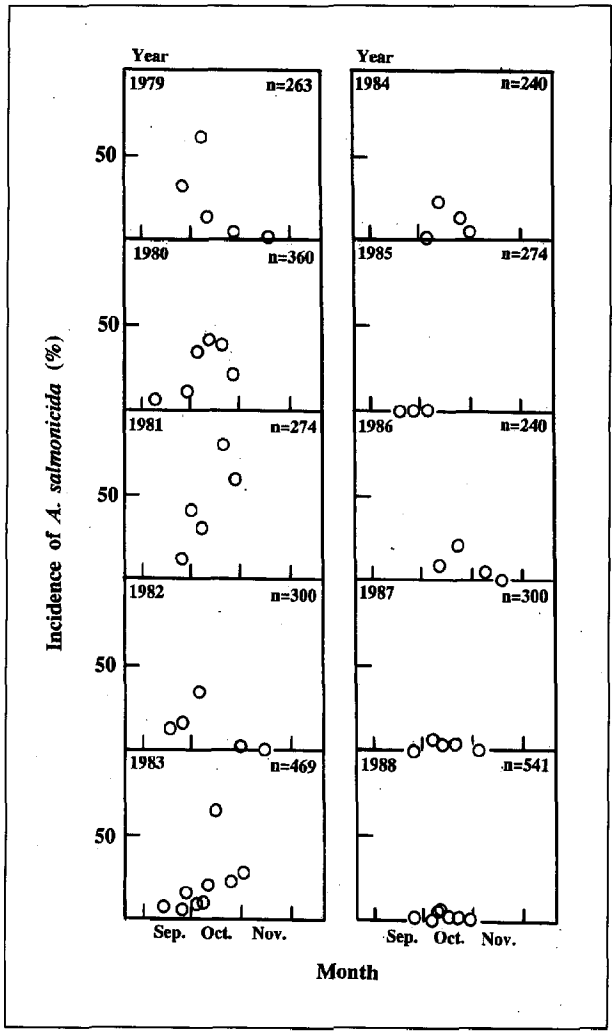

Figure 3

Changes in the monthly incidence of *Aeromonas salmonicida* in chum salmon collected from the Ishikari river, and held for maturation periods during the period September to November, 1979–88 (Modified from Nomura et al. 1991a).

The kidney materials of chum salmon in which *A. salmonicida* was isolated were fixed with Bouin's solution for histopathological examination. The kidney organs were dehydrated and embedded in Paraplast, and sections of the samples were made and stained with HE and Gimsa stain.

Histopathological examination of the infected fish did not, however, reveal colonies of *A. salmonicida* typically observed in fishes with furunculosis. Also, no outbreaks of furunculosis were recorded in the examined populations during the research period (Nomura et al. 1991a).

There are few reports examining the prevalence of *A. salmonicida* in the organs of apparently normal mature fish. In fact, as far as we know, there is only one report by Daly and Stevenson (1985). They reported that *A. salmonicida* was detected in 31 of 286 brown trout (*Salmo trutta*) sampled from spawning runs in the Ganaraska River, Ontario, Canada, over a period of two years. Our results showed that the incidence of this agent in apparently normal chum salmon was higher compared to that of Daly and Stevenson's (1985) estimated for brown trout, and that *A. salmonicida* is distributed widely in the river populations of salmonids in Hokkaido.

Morikawa et al. (1981) reported that the number of *A. salmonicida* in the kidneys of moribund amago salmon was 10^8 to 10^9 cfu/g. The reason why diseased fish were not found in the population we examined, even though they had *A. salmonicida* in their kidneys, was that the degree of infection was not high enough. From the results of histological and bacteriological examinations, we conclude that fish with *A. salmonicida* in the kidney are not diseased fish but carriers of *A. salmonicida*.

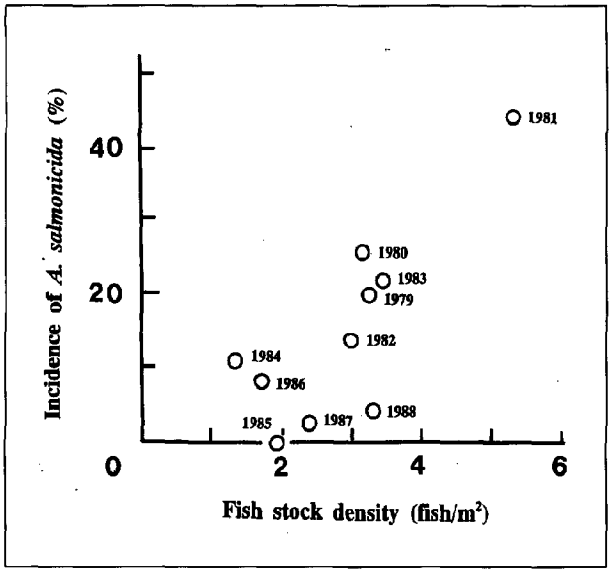

Figure 4
The relationship between average density of the fish in holding ponds and the incidence (% occurrence) of *Aeromonas salmonicida* in fish taken from the Ishikari River. Numbers in the figure indicate the year of examination (unpubl. data).

A. salmonicida in Immature Fish

We attempted to isolate *A. salmonicida* from immature chum and masu salmon (Nomura et al. 1991b). A total of 680 fish were collected in four coastal set nets off Hokkaido, and a total of 1,200 juvenile masu salmon and chum salmon fry were collected from 11 hatcheries of the Hokkaido Salmon Hatchery system.

The bacterium was not isolated from any of the examined fish.

A. salmonicida in Ovarian Fluid

In 1989 in Hokkaido, we attempted to isolate *A. salmonicida* from the ovarian fluids of mature chum, pink, and masu salmon.

Ovarian fluids were collected according to the method of Yoshimizu et al. (1985). The ovarian fluids were streaked onto nutrient agar plates (Eiken Co., Tokyo, Japan) and the plates were cultured at 20°C for 7 days. Number of viable counts of *A. salmonicida* in ovarian fluid and kidney were measured in accordance with the method of Nomura et al. (1991a).

A. salmonicida was isolated from the ovarian fluid of fish showing no apparent clinical signs of furunculosis. For example, *A. salmonicida* was isolated from the ovarian fluid of 22 of 120 fish examined from the Tokachi River. The number of *A. salmonicida* bacteria in ovarian fluids ranged from 10^1 to 10^7 cfu/mL in the populations from the Shibetsu and Teshio rivers.

Ovarian fluid containing *A. salmonicida* flows out of the fish at the time eggs are stripped or during the process of maturation in the pond. Consequently, infected water and infected ovarian fluid are expelled into the river because the sewage from the egg stripping areas and the holding ponds is not disinfected in Hokkaido. We suspect from these results that the agent drained from the fish may be a source of infection for other anadromous salmon that ascend the river for spawning.

Horne and Maj (1928), McCraw (1952), and Hastein and Lindstad (1991) stated that the most important source of *A. salmonicida* in the spread of furunculosis is the existence of fish carrying this agent. Fish carrying the bacterium pose a serious problem to the prevention of furunculosis, and their reduction in fish plays a key role in salmon propagation.

A. salmonicida on Egg Surfaces

The existence of the bacterium in the ovarian fluid suggests that the surface of eggs taken from the fish will also be contaminated. Contaminated eggs may spread the agent to areas where the eggs will be transplanted. We studied the existence of *A. salmonicida* on the surface of eggs by artificially contaminating chum salmon eggs with *A. salmonicida*.

The *A. salmonicida* 20-1, strain, which was isolated from the kidneys of chum salmon in the Tokachi River, was used as innoculum. The strain was cultured and harvested, then was suspended in phosphate buffer saline (PBS). The chum salmon eggs were bathed in PBS containing the agent for an hour. The eggs were incubated in well water at 8°C in the laboratory. At one hour and at 24 hours after fertilization, we took 20 eggs from the incubator and put them into sterilized water. The flask was shaken strongly for 5 minutes, we then measured the viable number of *A. salmonicida* in the water according to the method of Nomura et al. (1991b).

Egg surfaces were initially bathed with 1.1×10^4 to 4.3×10^6 cfu/egg of *A. salmonicida*. The number of *A. salmonicida* present on the egg surfaces decreased from 68 to 4.6 cfu/egg an hour after fertilization and *A. salmonicida* could not be isolated from the eggs cultured on plates 24 hours after the initial bath treatment.

We also attempted to isolate *A. salmonicida* from eggs in the incubation boxes at the Satsunai, Nakagawa, Nemuro, and Tokachi hatcheries. These eggs were taken from brood fish in which the preva-

lence of *A. salmonicida* was high (Nomura et al. 1991a). Fortunately, *A. salmonicida* was not isolated from any of the 15 hatcheries' eggs (Nomura et al. 1991b).

In Hokkaido, fertilized eggs are transported to a hatchery from the egg collection location 1 hour after fertilization. From the results of our experiment, it appears that *A. salmonicida* is able to exist on the surface of an egg. This makes us concerned that we may be transporting the bacteria to the hatchery with the fertilized egg. We believe that it is necessary to prevent the transfer of *A. salmonicida* via eggs in order to control furunculosis.

Survival of *A. salmonicida* in Water

By definition, *A. salmonicida* is considered to be an obligate pathogen (Popoff 1984) and is never found in surface water. Its ability to survive and remain infectious in the external environment may be a major determinant in the spread of furunculosis. We studied the viability of *A. salmonicida* in nonsterile, sterile filtered, and autoclaved fresh water and in salt water.

A. salmonicida strain 20–1 isolated from chum salmon in the Tokchi River was used in this experiment. The strain was cultured at 20° C and harvested, then suspended in fresh water or in sea water. The suspended cells were inoculated into 200 mL of nonsterile, sterile filtered, and autoclaved fresh water and saltwater and were incubated at 10° C.

In sterilized fresh water, *A. salmonicida* survived for 60 days and in nonsterile water, only 4 days. The survival of *A. salmonicida* in sterile salt water was 8 days; this was a shorter survival period than that in sterile fresh water.

It is believed that *A. salmonicida* is not able to exist for long time in water without fish, but McCarthy (1980) studied the survival of the agent in water using an antibiotic-resistant strain of *A. salmonicida* and found the agent could survive for 8 days in water.

The results of McCarthy (1980) and this study indicate that *A. salmonicida* survives long enough to infect other fish in the water.

Variation of Agglutination Titer Against *A. salmonicida* in the Serum

A serological survey of adult salmon was made from blood samples collected in 1988 in Hokkaido, from mature chum, pink, and masu salmon. Blood was aseptically extracted from the donsal artery with 10 mL of Vacteinor (Terumo Co., Tokyo, Japan). The resulting serum was separated from the blood-cell clot by centrifugation and was stored at –90°C until assayed. The serum was tested for agglutinating antibody titers individually, by test-tube methods with *A. salmonicida* ATCC14174.

Agglutinin titers against *A. salmonicida* in the serum of mature chum, pink, and masu salmon in Hokkaido in 1988 are shown in Table 1.

Of a total of 75 serum samples taken from mature chum salmon, 73.3% did not have the agglutinin, and the range of titers was 8 to 32. In pink salmon, 10% of the sample did not have the agglutinin, and the range was 4 to 32. In masu salmon, 16.6% of the examined serum did not have the agglutinin, and the range was 4 to 128.

The diversity in the incidence of agglutination titer within each of the three species indicates a continuous, widespread interaction between individuals of

Table 1
Prevalence and ranges of agglutinin titers against *A. salmonicida* in the serum of mature chum, pink, and masu salmon ascending various rivers in Hokkaido.

Species	River	No. of fish examined	Agglutinin titers		
			Negative (%)	Modes	Range
Chum salmon	Ichani	15	80.0	32	16– 32
	Tokachi	30	76.6	8	8– 32
	Shizunai	30	66.6	16	8– 32
Pink salmon	Shokotsu	30	16.6	16	4– 32
	Iwaobetsu	30	6.7	16	4– 32
	Nishibetsu	30	6.7	16	4– 32
Masu salmon	Shiribetsu	39	15.4	8	4–128
	Shari	18	0	16	4–128
	Nishibetsu	27	29.6	8	4– 32

the host populations and *A. salmonicida.* The difference in the amount of agglutination titer is proportionate to the period of *A. salmonicida* infection.

In general, the percentage of serologically reactive salmon increased as their length of freshwater residency increased. Weber and Zwicker (1979) reported that of a total of 43 serum sampled from Atlantic salmon (*Salmo salar*) in the Miramichi or Margareen rivers in Canada, none had A. salmonicida agglutinin, but of 27 Restigouche River salmon, four had a titer of 10, five had a titer of 20, and one had a titer of 640. They confirmed that Atlantic salmon have previously contacted *A. salmonicida* in the Restigouche River.

In our study, agglutinate titers in the serums were low. It was suggested that the fish were infected with *A. salmonicida* shortly before their eggs were stripped. Kimura (1970) reported that the immunological method of preventing furunculosis was useful in adult masu salmon during the holding period because these salmon stay in fresh water for a long enough period to allow them to produce antibodies after antigen inoculation. In chum salmon, however, the freshwater residency period is short, so this method of prevention would not be practical.

Isolation of the Bacteriophage of *A. salmonicida* from Water

There is no sensitive medium for selecting *A. salmonicida.* This means that when the number of *A. salmonicida* in water is low, the isolation of *A. salmonicida* from the water will be difficult. This is because *A. salmonicida* cannot grow on the culture medium under competitive conditions with other natural bacteria populations. We attempted to isolate the bacteriophage of *A. salmonicida* to ascertain the existence of *A. salmonicida* in the water.

Water samples, from 11 hatcheries and 4 rivers were examined. Nutrient agar (Eiken Co., Tokyo, Japan) was employed for the routine culture, dilution, and enumeration of *A. salmonicida* and its phage strain. One hundred mL of sample was added to 500 mL of cultured *A. salmonicida* Ar-32, Ar-43, Ar-71, and H-70 strains in the logarithmic phase. Detection and enumeration of phage were achieved using the medium and double agar layer technique (Paterson et al. 1969). The results are shown in Table 2.

McCraw (1952) stated that when the bacteriophage of *A. salmonicida* exists, its presence may indicate the existence of the bacterium. The bacteriophage was isolated from two samples of river water and five samples from hatchery water. From this result, it was suspected that *A. salmonicida* can survive for a long time in the river water in Hokkaido after leaving the fish and that its existence may be a source of infection to salmonid fish. The results suggest that such bacteriophage could be very useful for studying the existence of the agent in water.

Table 2
Isolation of *Aeromonas salmonicida* bacteriophage from the samples of river water and hatchery water.

Sample	Samples	
	Numbers examined	Isolated containing phage
River water	4	2
Hatchery water	9	2
Sewage of hatchery	13	3

Pathogenicity of the Isolated *A. salmonicida*

The isolated strains were identified as *A. salmonicida,* subspecies *salmonicida,* by their biological, biochemical, and immunological characteristics. All of the isolated *A. salmonicida* strains showed auto-agglutination and produced protease in the medium, so we also expected them to be pathogenic.

In order to examine the pathogenicity of the isolated strain, we injected it into chum and masu salmon fry and adult chum salmon.

A. salmonicida 20–1 was cultured for 48 hours at 20°C. The cells were washed three time in PBS and were suspended in PBS. The strain was injected into chum salmon fry, yearling masu salmon, and chum salmon brood fish at concentrations of 1.7×10^2, 1.8×10^3 and 6.0×10^5 cfu/fish, respectively.

All of the examined fish showed typical signs of furunculosis 3 to 4 days after injection. The number of *A. salmonicida* in the kidneys of moribund fish was around 10^8 cfu/g kidney tissue, the same number reported by Morikawa et al. (1981) in the kidneys of moribund amago salmon. On the basis of these results, we suspect the isolate is a pathogenic strain.

Control of *A. salmonicida* on the Surface of Egg

To establish a method of controlling *A. salmonicida* on the eggs, the bactericidal effect of popidon-iodine (Isodine), and the toxicity of this agent to the chum salmon egg were studied.

The bactericidal effects of popidon-iodine to *A. salmonicida* were determined in accordance with the method of Amend and Fryer (1972).

A. salmonicida was completely killed by treatment with 25 ppm isodine for five minutes and this solution was not toxic to the chum salmon eggs for treatments lasting up to one hour. Thus, the authors confirmed that isodine solution has a sanitizing effect on the agent, and that it does not have adverse effects on chum salmon eggs.

Method for Decreasing the Prevalence of *A. salmonicida* in Chum Salmon

From the results of our epidemiological study, we suspected that the incidence of *A. salmonicida* was affected by the density of fish during their maturation period in the holding ponds; as the average density of brood fish stocked in ponds decreased, the incidence of *A. salmonicida* in examined fish also decreased (Fig. 4). Therefore, we examined the relationship between the stocking density of fish in the pond and the prevalence of *A. salmonicida* in the fish.

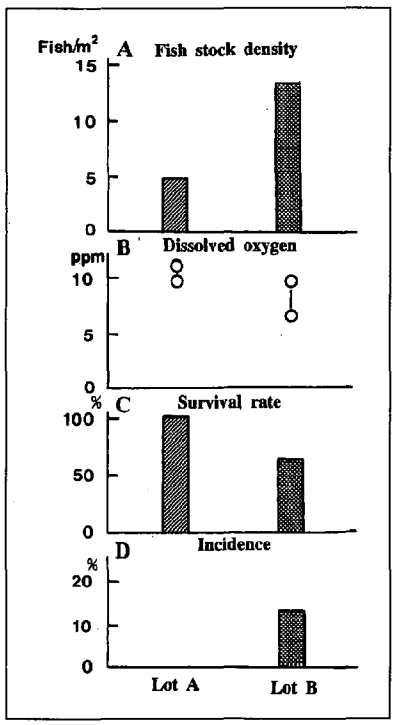

Figure 5
The relationship between the density of fish stock in holding ponds, the concentration of dissolved oxygen in the water, survival rate, and the incidence (% occurrence) of *Aeromonas salmonicida* (unpubl. data).

Chum salmon in the Ishikari River were randomly assigned to experimental holding ponds and held under low (4.9 fish/m^2) and high density (14.7 fish/m^2) conditions until maturation. The kidney tissues of all the fish used in experiment were cultured on nutrient agar in accordance with the method of Nomura et al. (1991).

As we expected, we found that 12.4% of the fish examined harbored *A. salmonicida* when they were stocked at a high density, but no examined fish contained the agent when they were stocked at a low density (Fig. 5A). The incidence of the bacterium in fish that were held under low dissolved oxygen conditions was higher than that of fish held under high dissolved oxygen levels (Fig. 5D). These results clearly indicate that high stocking densities and low dissolved oxygen levels in holding ponds have a marked effect on the prevalence of the agent in the fish. We concluded that fish maturation in the pond under low density conditions and disinfection of the eggs, are necessary to prevent fish furunculosis in the artificial propagation of salmon.

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Functions of Hemocytes During the Wound Healing Process in the Pearl Oyster

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ABSTRACT

Morphological evidence from previous studies suggests that bivalve hemocytes function in hemostasis and extracellular matrix production during the wound healing process. The present paper describes the use of an in vitro cell culture system to show that the agranular hemocytes of the pearl oyster *Pinctada fucata* have the ability to perform these healing functions. A possible wound healing system is also presented.

Introduction

Defense reactions against infection and the removal of tissue debris are the responses of the immune system that occur after wounding. On the other hand, tissue regeneration at the wound site is an organogenetic event in which cell-cell and extracellular matrix (ECM)-cell interactions occur. Thus, the wound healing process can be a useful model to study the functions of proteins and other cell components in both the immune and organogenesis systems. Because bivalves have no humoral clotting factors, platelets, or capillary vessels, it is assumed that their wound healing system differs from that of vertebrates and is probably less complex. The wound healing process has been described for several bivalves at the morphological level. It is known that the hemocytes prevent blood loss at the wound site by forming a cellular sheath and producing an ECM (DesVoigne and Sparks 1968; Pauley and Heaton 1969; Ruddell 1971). The healing process in the pearl oyster *Pinctada fucata* and the associated functions of hemocytes have been demonstrated (Suzuki et al. 1991). In this paper, the author describes a study using cultured hemocytes from the pearl oyster in which agranular hemocytes show the ability to form a cellular sheath and to produce an ECM. A possible wound healing system is also presented.

Methods

Hemocyte Culture

While the valves of *P. fucata* were held slightly open with a wedge, blood was drawn from the adductor muscle with a plastic syringe attached to a 24 gauge needle (Fig. 1). Approximately 2 mL of blood was collected from each animal. Blood pooled from five animals was centrifuged at 100 × g for 8 minutes and a pellet of hemocytes was obtained. The pellet was washed twice with a balanced salt solution for marine molluscs (MMBSS) prepared according to Machii and Wada (1989). The pellet was suspended on 2 mL of culture medium *Pf35* (Machii and Wada 1989), developed specifically for pearl oyster tissue. A 0.5-mL sample of the suspension was pipetted into a plastic culture flask (50 mL: Nunc). After an hour, to allow the hemocytes to adhere to the plastic surface, 2.0 mL of Pf35 was added to the flask. The cells were then incubated at 25°C. The culture medium was renewed on the fourth day of culture.

Microscopy

The cultured cells were fixed in Karnovsky's fixative containing 8% sucrose at 4°C for 1 hour. After washing in 0.1 M phosphate buffer (PB; pH 7.2), they were post-fixed in 1% osmic acid in PB for 1 hour, washed twice in PB, dehydrated through graded alcohols, and embedded in Taab 812 resin. Ultrathin sections were stained with uranyl acetate and lead acetate. All transmission electron microscopy was performed with a JEOL JEM- 1200EX electron microscope.

Results and Discussion

Aggregate Formation

The hemocytes formed cellular aggregates during blood collection and rinsing. Three hours after the

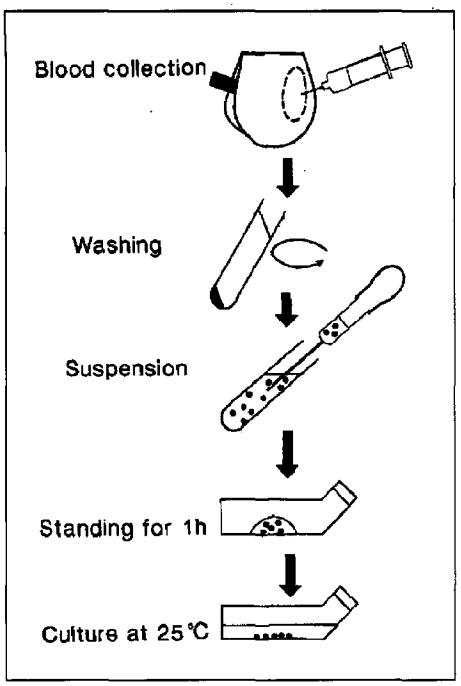

Figure 1
Procedure used for hemocyte culture.

beginning of culture, the aggregates were 80–200 µm in diameter and adhered well to the plastic surface of the culture flask (Fig. 2). In bivalves, two categories of hemocytes occur—agranular and granular hemocytes (Feng et al. 1971). It has been demonstrated that the former are macrophage-like cells with active phagocytic capabilities (Reade and Reade 1972; Moore and Lowe 1977). One hemocyte of the pearl oyster is able to take in 1–9 vertebrate erythrocytes (Suzuki and Mori 1990). As shown by transmission electron microscopy, the aggregate is formed only by agranular hemocytes (Fig. 3). They adhere to one another at an adhesion plaque from which microfilaments can be seen running into the cytoplasm (Fig. 4). Thus, agranular hemocytes can form a cellular aggregate in a relatively short time (perhaps several minutes if stimulation is provided), although it is not understood what factors stimulate the reaction. It is possible that this aggregate formation is a homologous reaction to the cellular sheath formation at the wound site.

ECM Production

During the first 2–3 days of culture, deposition of an ECM started in the agranular hemocyte aggregate. The matrix was observed as a transparent gel when viewed under phase-contrast microscopy (Fig. 5). The matrix continued to develop until about the seventh day of culture, after which time the number of living hemocytes rapidly decreased. Agranular hemocytes of 1–3 layers thickness were attached to the matrix, which was composed of fine fibrils and flocculent substances (Figs. 6 and 7). The fibrils were 20 nm in diameter and showed a faint banding pattern indicating that they are collagenous fibrils. The flocculent substances are probably proteoglycans. It has also been ascertained at the biochemical level that the matrix includes collagen (Suzuki et al. 1991).

Wound Healing System

In the pearl oyster, four noticeable cellular reactions occur during the healing process (Suzuki et al. 1991): 1) removal of tissue debris, 2) cellular sheath formation, 3) ECM deposition, and 4) epithelial regeneration. The first reaction is attributed to the phagocytic ability of agranular hemocytes. In addition, these cells also perform the second and third of these healing reactions, cellular sheath formation and ECM deposition, as suggested by the results of this paper.

Based on these results, a possible wound healing system in the pearl oyster is presented in Figure 8. When the pearl oyster is wounded, agranular hemocytes infiltrate the injured area to remove tissue debris (and possibly foreign particles) and then form a cellular sheath to prevent blood loss. After sheath formation, they begin to produce an ECM, which functions as a template for the regenerating epithelium. It is possible that the wound site is repaired through this sequence of processes. Assuming this explanation is accurate, the wound healing system of this bivalve is simple compared with that of vertebrates.

It is well known that in vertebrates growth factors secreted from the platelets, macrophages, and fibroblasts are the humoral factors that control cell growth and ECM production at the wound site. In bivalves, however, neither a growth factor nor its production cell has been identified. The agranular hemocyte is the most likely candidate to be a growth factor production cell, because, like macrophages and fibroblasts in vertebrates, it is the primary cell type in bivalves that appears at the wound site. In addition, platelets are absent from bivalve hemocytes. Future studies with in vitro cultures will improve our understanding of both the wound healing system and organogenesis in bivalves.

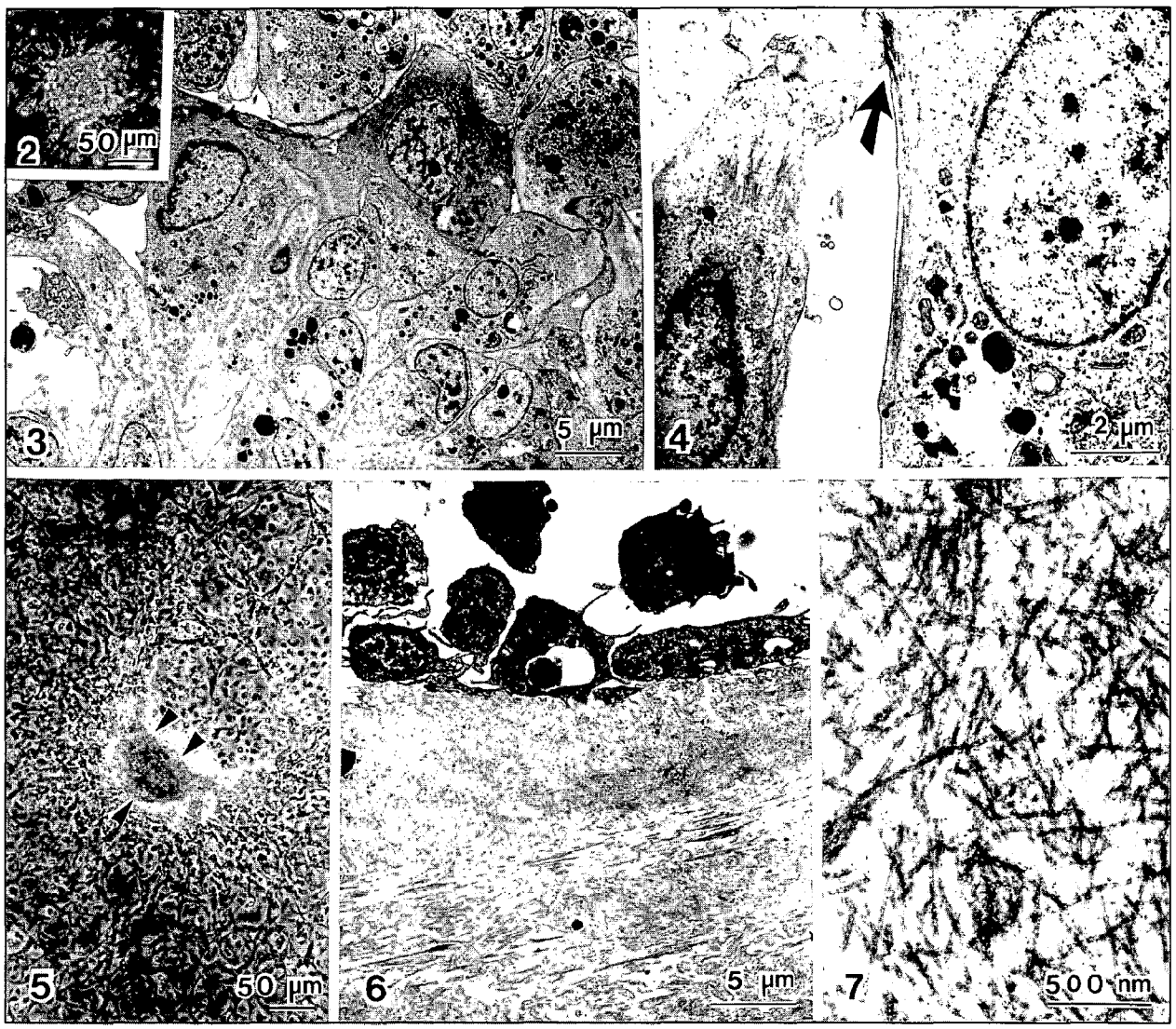

Figure 2 Hemocyte aggregate after 3 hours of culture. × 135.
Figure 3 Electron microscopy of aggregate after 1 day of culture. × 2,000.
Figure 4 Adhesion plaque formed at contacting site of agranular hemocytes. × 6,700.
Figure 5 Matrix (arrowhead) formed in a hemocyte aggregate after 7 days of culture. × 135.
Figure 6 Electron microscopy of matrix and agranular hemocyte layer. × 2500.
Figure 7 Fibrils in the matrix at high magnification. × 31,000.

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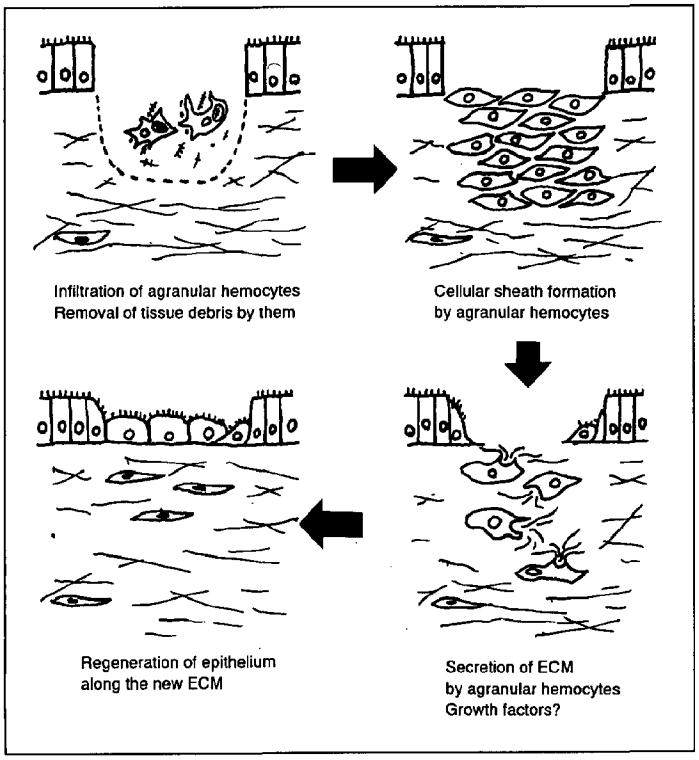

Figure 8
A possible wound healing system in the pearl oyster: ECM=extracellular matrix.

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Skeletal Abnormalities of Fishes Caused by Parasitism of Myxosporea

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ABSTRACT

The relationship between skeletal abnormalities and parasitic infection was studied for deformed cultured yellowtail, *Seriola quinqueradiata*, Japanese bluefish, *Scombrops boops*, and mullet, *Mugil cephalus*. Soft radiographic observations indicated that the deformities were due to skeletal abnormalities: deformed yellowtail, Japanese bluefish, and mullet were characterized by scoliosis, lordosis, and lordo-scoliosis, respectively. Myxosporean cysts were found in various parts of the brain of deformed fish such as the fourth ventricle, the cavity of the optic tectum, the surface of the olfactory lobe and bulb, and the optic lobe. The cysts were observed in the fourth ventricle in all deformed fish, but not in normal fish. From morphological characteristics, myxosporeans from the deformed yellowtail and Japanese bluefish were identified as *Myxobolus buri*, and that from deformed mullet was identified as *M. spinacurvatura*. These results suggest that *Myxobolus* species are responsible for the skeletal abnormalities observed in these deformed fish.

Introduction

Skeletal abnormalities of fish have been reported to be caused for various reasons, such as dietary deficiencies (Halver and Shanks 1960), pesticide exposure (Couch et al. 1977), heavy metal exposure (Holcombe et al. 1976), and bacterial (Kaige et al. 1984) and parasitic infections (Halliday 1976). Many studies report sporulation of myxosporeans in fish brains. However, only a few studies have described a possible correlation between skeletal abnormalities and myxosporean parasitism in the brain. While studying skeletal abnormalities in three species of fish, cultured yellowtail (*Seriola quinqueradiata*), Japanese bluefish (*Scombrops boops*), and mullet (*Mugil cephalus*), we found spores of a myxosporean in the brains of these fish. The present paper elaborates on the possible relationship between skeletal abnormalities and parasitism by myxosporeans with special reference to parasitized regions of the brain.

Materials and Methods

Seventeen yellowtail (age 1+), including 7 deformed ones, were collected from a fish farm in Mie Prefecture from 1987 to 1989.Four deformed Japanese bluefish and four deformed mullet were captured by a set net in Mie Prefecture in 1989.

After external observation, radiography of all fish was performed with Fuji Lx film at 40 milliamps and 50kV for 20 seconds and subsequently examined for parasitism in viscera by necropsy. From these animals, three deformed and three normal yellowtail, three Japanese bluefish, and three mullet were used for subsequent histological examination.

The brain, liver, kidney, and spleen were excised from each fish and fixed in 10% formalin, embedded in paraffin, cut serially at 4 μm, stained with Giemsa or hematoxylin and eosin, and then observed for the presence and location of cysts.

For the study of spore morphology, some myxosporean cysts were removed from the brain and crushed in phosphate buffer saline to get a suspension of fresh spores. Light microscopic observations were made both on fresh spores and those stained with Giemsa.

For the scanning electron microscopy, spore-suspensions were fixed in Karnovsky's fixative for 2 hours at room temperature. They were then dehydrated in a graded ethanol series, treated with isoamyl acetate three times, each for 15 minutes, processed in a critical point dryer, sputter-coated with

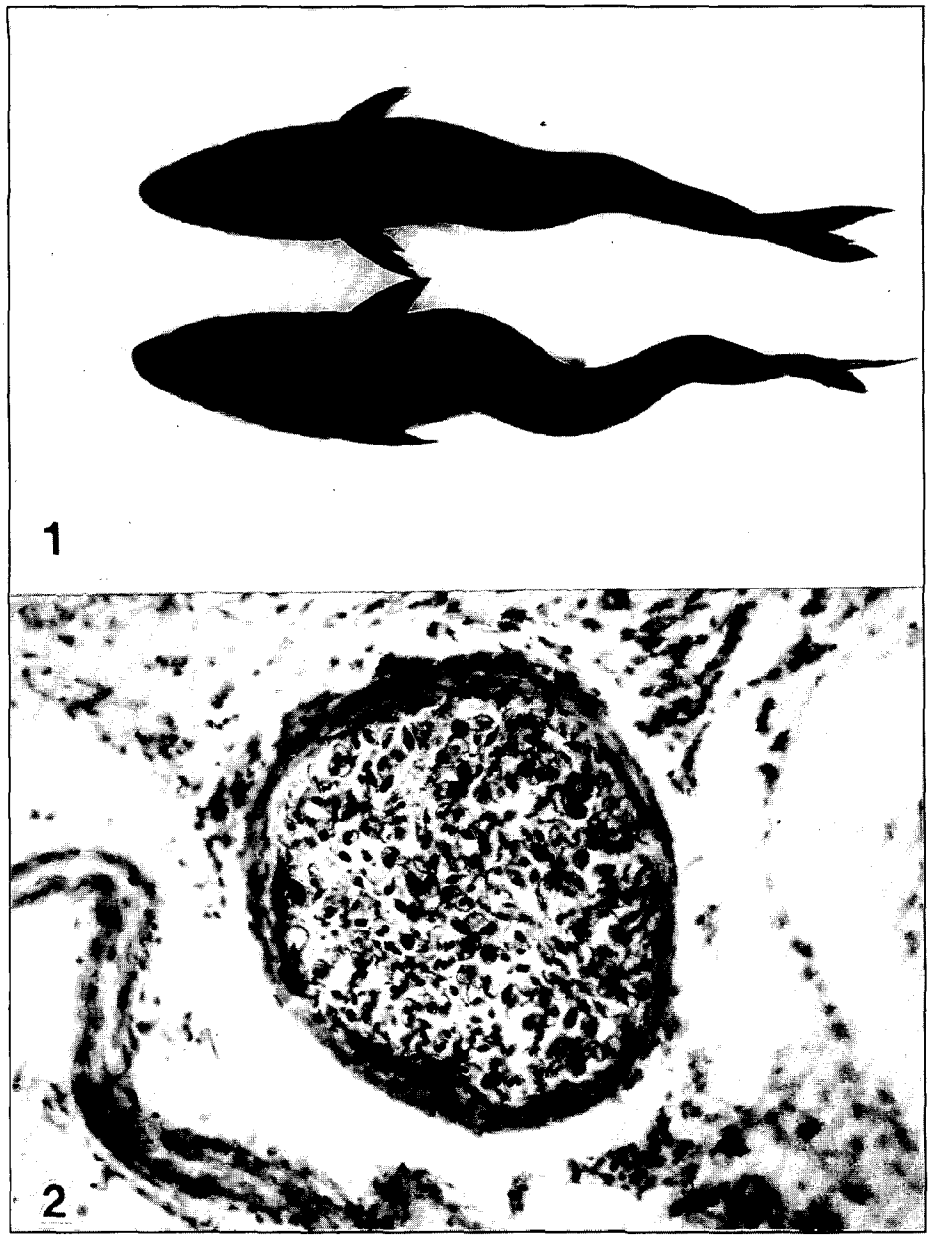

Figure 1
Scoliosis found in yellowtail *Seriola quinqueradiata* (dorsal view).

Figure 2
A cyst of *Myxobolus buri* found in the brain of yellowtail (*Seriola quinqueradiata*) Giemsa stain (X300).

gold, and examined with the JEOL (Japan Electronics Optical Limited) T220A scanning electron microscope.

Results

Cultured Yellowtail

The deformities were externally apparent as curvatures from the trunk to caudal region. Radiography of the deformed specimens revealed extreme skeletal curvatures consisting of a smooth S-like bend in the lateral plane, scoliosis (Fig. 1).

In the seven deformed yellowtail, myxosporean cysts were found in one or more of the following regions: beneath the meninges of the olfactory bulb and lobe, surface of optic lobe, cerebellum, medulla oblongata, inferior lobe, the cavity of optic tectum, and the fourth venticle. In particular, the cysts were found in the fourth ventricle of the brain in all deformed fish examined (Table 1). In the normal fish, on the other hand, no cysts were found in the fourth ventricle of three fish, even though seven fish had

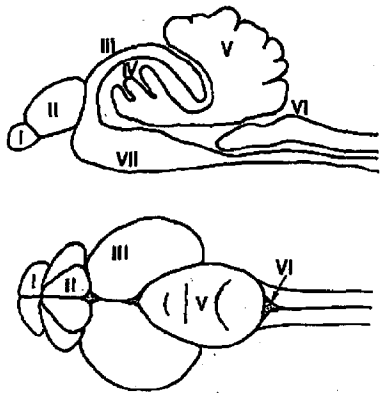

Table 1

Distribution of cysts in the brains of deformed (n=7) and apparently normal (n=10) yellowtail *Seriola quinqueradiata*. (I=olfactory bulb; II=olfactory lobe; III=optic lobe; IV=the cavity of optic tectum; V=cerebellum; VI=fourth ventricle; VII=inferior lobe.)

	I	II	III	IV	V	VI	VII
Deformed fish	5/7	5/7	2/7	3/7	1/7	7/7	2/7
Normal fish							
Infected	2/5	3/5	2/5	1/5	0/5	0/5	1/5
Non-infected	0/5	0/5	0/5	0/5	0/5	0/5	0/5

cysts in some other region of the brain. No cysts were observed in regions other than the brain.

The cysts range from 0.5 to 3 mm in diameter and were enveloped by a collagenous capsule with fibroblasts (Fig. 2). Most cysts were filled with nearly mature or mature myxosporean spores. Morphological observations showed spores to be broadly ellipsoidal and symmetrical in shape and to possess a prominent sutural ridge in the front view. In the side view they were lenticular and symmetrical and consisted of two shell valves. The polar capsules were pyriform and nearly equal in size, and a small intercapsular appendix was clearly seen. The length, width, and thickness of spores were 10.5, 9.0, and 6.3 μm, respectively. From these morphological characteristics they were identified as *Myxobolus buri* Egusa, 1985.

Japanese Bluefish

Radiographic observations demonstrated that the abnormality in the four Japanese bluefish was dorso-ventral deformity of the vertebral column and flexure of the neural spine and hemal spine (Fig. 3).

In deformed Japanese bluefish, cysts were always found in the fourth ventricle and less frequently found in the cavity of the optic tectum. In some cases cysts were found attached to the surface of the olfactory bulb and olfactory lobe and in the cavity of the optic tectum. No cysts were found in tissue other than the brain. Both light and scanning electron microscopy revealed that the spores from these fish were morphologically similar to those obtained from deformed yellowtail. The spores were broadly ellipsoidal in frontal view and broadly lenticular in side view; the shell valve of the spore had a sutural ridge with fold; each spore had two polar capsules nearly equal in shape and size; and an intercapsular appendix was present (Figs. 4 and 5). The average length of fresh spores was 10.4 μm, width 9.1 μm, and their thickness 6.2 μm.

Mullet

The four deformed mullet showed spinal curvature and lordo-scoliosis in radiography (Figs. 6 and 7). Histological examination revealed many myxosporean cysts on the surface of the gut and in various visceral organs such as the liver, kidney,

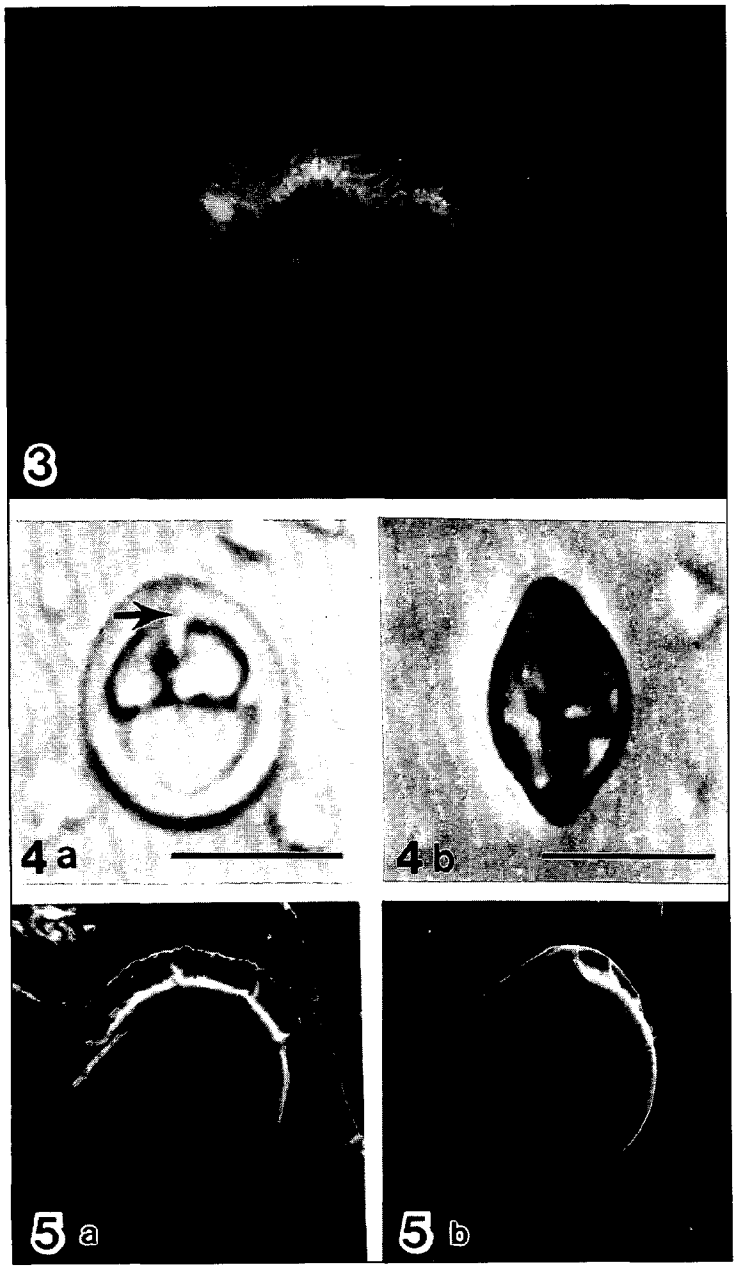

Figure 3
Soft radiograph of lordosis in a Japanese bluefish (*Scombrops boops*) (side view).

Figure 4
Fresh spore of *Myxobolus* sp. obtained from the brain of Japanese bluefish. (**a**) frontal view (arrow indicates intercapsular appendix); (**b**) side view (bar=10 µm). Phase contrast microscopy.

Figure 5
Scanning electron micrographs of spores of *Myxobolus* sp.obtained from the brain of Japanese bluefish. (**a**) frontal view; (**b**) side view.

spleen, pancreas, and brain of the deformed mullet. As in yellowtail and Japanese bluefish, cysts were found in various regions of the brain, such as the fourth ventricle, the cavity of the optic tectum, the surface of the olfactory lobe and bulb, and the optic lobe. Cysts were observed in the fourth ventricle of all four fish examined (Fig. 8).The average length of fresh spores was 11.5 µm, their width was 9.8 µm, and thickness 6.7 µm. From the morphological characteristics of the spores, such as their rounded frontal view, lenticular side view, two smooth shell valves, two teardrop-shaped polar capsules situated on the sutural plane, and lack of an intercapsular appendix (Fig. 9), these myxosporean spores were considered to belong to the genus *Myxobolus* (Lom and Noble 1984).

Discussion

The morphological characteristics of the spores clearly indicate that the myxosporean found in deformed yellowtail of the present study is *M. buri*. The light and scanning electron microscopic examina-

Figure 6
Soft radiograph of lordosis in a mullet (*Mugil cephalus*) (side view).

Figure 7
Soft radiograph of scoliosis in a mullet (*Mugil cephalus*) (dorsal view).

Figure 8
Diagrammatic view of the distributions of the cysts in the brain of mullet *Mugil cephalus* (★=loci of the cyst).

Figure 9
Fresh spore of *Myxobolus spinacurvatura* obtained from the brain of mullet. (**a**) frontal view; (**b**) side view (bar=10 μm).

tions also demonstrated a complete similarity in the morphology between the spores from the deformed Japanese bluefish and those from deformed yellowtail. Thus, the spores parasitic in the brain of the deformed Japanese bluefish are considered to be those of *M. buri*.

To date, more than 400 species of *Myxobolus* have been described. The morphological characteristics of *Myxobolus* from the deformed mullet differ from those of most species described. Only three *Myxobolus* species, *M. achmerovi* Shulman, 1966, *M. buri*, and *M. spinacurvatura* Maeno et al., 1990, have morphological similarities to those found in deformed mullet. However, *M. achmerovi*, which has been found from the gill, fin, and mesentery of common carp (*Cyprinus carpio*) and mullet (*Mugil cephalus*) (Shulman 1966) is different from the present *Myxobolus* in that the former spore has an ellipsoidal frontal view, and a distinct intercapsular appendix. *M. buri* also differs from the present *Myxobolus* in mullet because the spore of *M. buri* has a broadly ellipsoidal frontal view, distinct folds around the edge, an intercapsular appendix, and a distinct polar filament inside the polar capsule. On the other hand, the spores from the deformed mullet were quite similar to those of *M. spinacurvatura* in both morphological characteristics and in spore dimensions. Thus, the *Myxobolus* found in deformed mullet in the present study is considered to be *M. spinacurvatura*.

In the present study the myxosporean cysts were invariably observed in the fourth ventricle of the brain in all deformed fishes examined. Myxosporean parasitism in the central nervous system of fish have been studied in the bullhead *Cottus gobio* (Lom et al. 1989), the redfin perch *Perca fluviatilis* (Langdon 1987), the yellowtail *Seriola quinqueradiata* (Sakaguchi et al. 1987), the fathead minnow *Pimephales promelas* (Mitchell et al. 1985), and the Japanese river goby *Acanthogobius flavimanus* (Hoshina 1952). However, even in a heavily myxosporean-infected bullhead, motor or sensory disturbances were not observed. Also, in myxosporean-infected fathead minnow and Japanese river goby, no clear external signs of deformity were found. On the other hand, skeletal abnormalities were observed in the myxosporean-infected redfin perch and yellowtail. In the redfin perch, cysts were located in the regions of the mesencephalon, diencephalon, third ventricle, and medulla oblongata, while in the yellowtail, cysts were found in the cavity of the optic tectum, the surface of the optic and olfactory lobes, and in the fourth ventricle. These studies, as well as the present study, indicate some correlation between skeletal abnormalities and myxosporean parasitism in the brains of fish. In addition, the present study strongly suggests that skeletal abnormalities occur when cysts infect particular regions of the brain such as the fourth ventricle. Thus, it is quite possible that myxosporean infection in this region of the brain mechanically affects central nervous system function to produce skeletal abnormalities in fish.

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Presence of Oncogenes in Fish Tissues and in Fish Cell Lines

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ABSTRACT

Work during the past decade has shown that avian and mammalian proto-oncogenes are centrally involved in cell transformation in vitro and in the formation of tumors in vivo. The fish systems in which oncogenes have been described are reviewed as are the general methodologies used to detect oncogenes and their gene products. Also discussed is preliminary work on the development of a test system that measures oncogene activation in fish cell lines in order to evaluate carcinogenic chemicals in the environment. Sequences related to the *ras*^{k}, *ras*^{h}, v-*raf*, v-*erb*-B, c-*src*, c-*myc*, c-*abl*, and c-*fos* proto-oncogenes, and to the p53 suppressor gene, were detected by Southern, Northern, and Western blots. Taken together, the above findings indicate that proto-oncogenes are well conserved evolutionarily in vertebrates and may be responsible for development of the transformed phenotype in fish.

Introduction

Evidence has been accumulating in recent years that in mammals, particularly humans, tumor development and progression are correlated with changes in the structure or expression of cellular genes. Yet in a number of higher vertebrates, oncogenic retroviruses have long been known to be causally involved in the initial appearance and subsequent growth of a variety of naturally arising tumors (Bishop 1985; Klein and Klein 1985). These two statements are not contradictory. The retroviral oncogenes (v-*oncs*) responsible for the transformation event are now recognized to have arisen from the capture and processing of whole or portions of essential cellular genes termed proto-oncogenes or c-*oncs* (Bishop 1983; Stehelin et al. 1976). It is now considered important to understand the extent of evolutionary conservation of these cellular genes in order to fully characterize their normal function and delineate their involvement in the tumorigenic conversion of normal cells either directly or through retrovirus-induced transformation.

The family of *ras* proto-oncogenes, *ras*^{h} (Harvey), *ras*^{k} (Kirsten), and N-*ras* are highly conserved from mammals to yeast. The 21 kilo dalton *ras* protein is expressed in a wide variety of nontransformed cells, suggesting a normal cellular function. *Xenopus laevis* oocytes espress the *ras* protein throughout oogenesis and embryonic development (Baum and Bebernitz 1990). However, wild type *ras* protein can be transformed in a variety of carcinogen-induced animal tumor model systems. For example, in rats almost 90% of mammary carcinomas induced by a single dose of nitrosomethylurea possess a c-H-*ras*-1 oncogene, which is the activated form of the c-H-*ras* gene having a G to A transition at codon 12 (Barbacid 1986, 1987). In humans, highly amplified N-*ras* DNA sequences were found in small cell lung cancer, and this amplification seems to correlate with tumor progression and prognosis (Johnson et al. 1987). Changes in the structure (mutation) of one or more oncogenes, their amplification, and the level of expression of their mRNA, are all potential indicators of tumorigenic initiation and progression.

In view of the widespread occurrence of neoplasia in freshwater and saltwater fish species, and because fish are becoming recognized as convenient model systems for the study of natural and environmentally induced diseases (Powers 1989), the search for proto-oncogene-like sequences in teleosts is warranted.

Experimental evidence indicates that some fish species, when compared with rodents, are less sensitive to the toxic effects of chemical agents and more susceptible to their carcinogenic effects. Because of their aquatic habitat, fish are fully exposed to chemicals in the water at the gill, eye, skin, and gut levels,

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and therefore may be good indicators of carcinogens in the environment. Thus, it is worth investigating oncogenes and their expression in tumorigenesis in fish, especially when they are induced by chemicals. Such studies have now begun. Here we describe the general methodology used in detecting oncogenes and their expression and review those systems where oncogenes have been found in fish tissues and in continuously cultivated fish cell lines.

Methodology

Oncogene Detection

For the detection of oncogenes at the DNA level, it is important to isolate high molecular weight genomic DNA with a minimal size of 100–200 kb. Standardized procedures are available for the rapid isolation of good quality DNA from either cell cultures or directly from tissues excised from organisms. One procedure (Smith et al. 1988) employs a gentle sodium dodecyl sulfate (SDS) and proteinase-K lysis step followed by phenol extraction, ethanol precipitation, and spooling of DNA (contaminating RNA can be removed by RNAse treatment). Tissue samples are processed by this method by including liquid nitrogen freezing and tissue crushing steps prior to the lysis. Methods for isolating genomic DNA from fish cell lines are described in Smith et al. (1988) and Read-Connole et al. (1990).

Samples of DNA from the various sources are then digested to completion with one or more various restriction endonucleases (e.g., *Eco*RI, *Hind* III and *Bam* HI), and the resulting DNA fragments are resolved by size by agarose gel electrophoresis. After transfer of the DNA fragments to nitrocellulose or nylon membrane supports, the DNA samples can then be screened by Southern blotting for the presence of sequences homologous to nick-translated labelled DNA probes specific for mammalian and viral oncogenes. In addition, serial dilutions of samples of restriction-endonuclease-treated DNA can be directly spotted onto nitrocellulose or nylon membranes. These dot blots can then be probed in parallel with the Southern blots to aid in the quantification of hybridizable DNA in each sample, and to estimate any gene amplification that may occur in the tumor tissues.

Comparisons between normal and tumor tissues can be made from the banding patterns obtained in Southern blots probed by the different oncogenes. In this way one can obtain some idea on the similarity of the gross structure of the various fish oncogenes that are detected. Comparisons of the number and size of oncogene homologous fragments from the Southern blots with the more quantifiable data from the dot blot experiments should reveal whether any of the fish genes detected have been rearranged or amplified in the tumor tissues under study.

Detection of Oncogene Expression

For any gene to exert an effect on cells and tissues, mRNA must be transcribed and eventually translated into protein. One can therefore screen RNA isolated from the various sources for the presence of transcription products homologous to those of mammalian oncogenes. Enhanced expression of various oncogenes following the amplification of DNA may contribute to malignant progression as is suggested by the human c-N-*myc* gene involvement in the progression of human neuroblastomas (Schwab et al. 1984), or it may involve the loss of a gene and its expression as has been observed in the case of the human retinoblastoma susceptibility gene (Friend et al. 1986; Lee et al. 1987).

Described briefly, cells and tissues being examined for oncogene transcription products are first processed by the guanidinium thiocyanate/hot phenol procedure to extract the total cellular RNA. The total cellular RNA obtained is subjected to oligo-dT cellulose affinity chromatography to purify or enrich the poly A+ RNA fraction (putative mRNA). The poly A+ RNA species are then separated by size by denaturing (formaldehyde) agarose gel electrophoresis. After transfer of the RNA to nitrocellulose or nylon membranes, mature mRNA species with homology to oncogene probes can be detected by northern blotting procedures. In addition, RNA dot blot experiments can also be performed to quantify the levels of mature mRNA hybridizing with the oncogene probes (Smith et al. 1987; Louis et al. 1988). The mRNA studies give valuable information on whether any of the specific oncogenes detected in the various tumor tissue preparations are of different sizes (suggesting either truncation of the gene or rearrangements) and whether they are overexpressed or aberrantly expressed when compared with the normal counterparts.

Detection of Oncogene Products

The third approach to monitor oncogenes in cells is to assay for their gene products (e.g., tyrosine kinase for c-*src* expression, epidermal growth factor receptor for c-*erb*-B expression). Monoclonal antibodies

(Mabs) have been raised against synthetic peptides spanning the active site regions of the *src* and *ras* gene families and are available commercially. Nonidet P-40 (non-ionic detergent) lysates of cells are cleared by high speed centrifugation and the proteins immunoprecitated with Mab mixtures. Following electrophoresis on 10% SDS-polyacrylamide gels, the bands are transferred to nitrocellulose membranes, and immunoblotted with an appropriate antibody. These western hybridizations, in which Mabs are used against oncogene products, can reveal the size and level of expression of these proteins.

Oncogenes Detected in Fish Tissues

A certain population of platyfish (*Xiphophorus maculatus*) carry special genes (Tu or tumor genes) for macromelanophores. When they are interspecifically hybridized with swordtails (*X. helleri*), the F_1 offspring carrying these genes develop a preneoplastic state. When the F_1 offspring are backcrossed with swordtails, a certain percentage of the backcrossed offspring develop a heritable form of melanoma with a characteristic inheritance pattern (Ozato and Wakamatsu 1983; Anders et al. 1984). Expansion of these classic and elegant genetic studies involves the use of modern biotechnology techniques to define the molecular basis of the melanoma formation. For example, elevated levels of cellular *src* mRNA and phosphoprotein (pp 60) kinase activity are detected in melanomas or tumors induced by carcinogens in *Xiphophorus* (Schartl et al. 1985) and more recent research indicates that the signal to start the events leading to melanoma development may come from the platyfish *erb*B region (Zechel et al. 1988). They have named this gene *Xmrk*. An additional copy of the *Xmrk* gene is found linked to either sex chromosome of the platyfish when hybridized to a probe specific for this region (Schartl 1990). The cellular myc (c-*myc*) gene has been cloned from rainbow trout *Oncorhynchus mykiss* and sequenced (van Beneden et al. 1986). The results of this work were used for evolutionary comparison with other vertebrate c-*myc* genes. Using the rainbow trout c-*myc* clone as a probe, DNA was examined from various fish tumors, including hepatocellular carcinomas induced in the medaka *Oryzias latipes* by dimethylnitrosamine. Compared with DNA isolated from normal tissues, there was no apparent increase in the intensity of hybridizing bands or differences in restriction patterns noted in Southern blots (van Beneden et al. 1988). However such experiments would not detect mutations, which could be detected only by cloning and sequencing the entire c-*myc* gene. It was also reported (van Beneden et al. 1988) that the blots, when rehybridized to other viral oncogenes, gave no indication of gross amplification or rearrangement. Clearly, cloning and sequencing of the entire gene is necessary to show putative differences.

Similarly c-*ras*-related sequences were cloned from the genomic libraries of the goldfish (*Cerassius auratus*) by Nemoto et al. (1986). Comparison of the nucleotide sequences of one of these clones with those in mammalian c-*ras* genes showed extensive homology to the gene coding for the mammalian p21 protein (96% homology to the c-ras^k protein). Subsequent work (Nemoto et al. 1987) involved sequencing and comparison of the first exon and its flanking regions in the c-*ras*-related genes from normal goldfish liver tissues with those from goldfish erythrophoroma cells cultured in vitro. No differences were apparent in the first 245 nucleotides which covered the first exonic region and whose length was identical to the first exonic region in mammalian c-*ras* genes. Enhanced expression of c-*ras* gene was seen in the erythrophoroma cells.

The high incidence of hepatomas present in winter flounder *Pseudopleuronectes americanus* in Boston harbor has been associated with the high levels of polycyclic aromatic hydrocarbons (PAHs) found in the harbor's sediment. Genomic DNA from the liver tumors produced foci in the NIH-3T3 transfection assay and a c-ras^k oncogene was identified in a transformant derived from one of the tumors (McMahon et al. 1988). Sequencing of c-ras^k sequences from the tumor and from the transformants indicated the oncogene to be of flounder origin. Sequencing of the tumor cell DNA amplified by polymerase chain reaction (McMahon et al. 1987) showed GC to AT or GC to TA conversions in the 12th codon of this gene (McMahon et al. 1990). Such mutations in the 12th codon can activate c-*ras* protooncogenes to oncogenic forms (Barbacid 1987). DNA samples from livers taken from fish at a less polluted site did not transform NIH-3T3 cells, and only wild-type sequences (GGT) were seen at the 12 codon of c-ras^k.

A cellular 53 kilo dalton nuclear phosphoprotein, denoted p53, was discovered more than a decade ago in SV40 virus transformed mouse cell lines (Chang et al. 1979). Because p53 was found complexed to the transformation antigen (T antigen) of SV-40 virus, and because the p53 gene was thought by many investigators to function as a dominantly acting oncogene, it has been intensively investigated during the past decade. Current work on mutations in the p53 gene indicates that the wild-type gene product actually functions like a tumor suppressor gene (Finlay et al.

Table 1
Oncogenes Detected in Fish Tissues.

Fish Species	Tissue Tested	Oncogene	Transforms NIH-3T3 Cells	Reference
Platyfish - swordtail hybrid (*Xiphophorus* sp.)	Hereditary melanoma	X*mrk* (tu)		Schartl et al. 1985 Wittbrodt et al. 1989
		erb-B		Zechel et al. 1988
Rainbow Trout (*Oncorhyncus mykiss*)	Normal liver	c *myc*		van Beneden et al. 1986
Goldfish (*Carassius auratus*)	Normal liver	*ras*		Nemoto et al. 1986
Winter flounder (*Pseudopleuronectes americanus*)	Liver tumor	ras^k	+	McMahon et al. 1988, 1990
Tomcod (*Microgadus tomcod*)	Liver tumor	ras^k	+	Wirgin et al. 1989
Northern pike (*Esox lucius*)	External lymphomas	?	+	van Beneden et al. 1990

1989). Mutations in the p53 gene are frequently detected in diverse human tumor types (Nigro et al. 1989), and currently it is believed that p53 gene mutations play an important role in the development of many common human cell malignancies. The hypothesis is that the mutated p53, which develops during the process of tumorigenesis, binds to the wild-type p53 gene product, creating an inactive complex. Further loss of growth control can occur when the wild-type allele is deleted. Thus, p53, like the retinoblastoma gene, is under intensive study by many investigators as a potential tumor suppressor gene (Sager 1989). The tumor suppressor genes are wild-type alleles of genes that play regulatory roles in cell proliferation and differentiation, and it is their loss or inactivation that is oncogenic. The p53 gene is highly conserved in vertebrates including fish (Smith et al. 1988).

Oncogenes Detected in Fish Cell Lines

A number of oncogenes have been described in some of the more commonly used fish cell lines (Read-Connole et al. 1990). These have been detected by Southern, Northern, and Western blots (Table 2).

The presence of many bands in Southern blots suggests that, as in mammals, some of the fish proto-oncogenes may exist as families of homologous sequences that share common functions. Probing Southern blots with v-*erb-b*, v-*src*, and v-ras^h revealed a striking similarity in the banding patterns of homologous sequences between the rainbow trout *O. mykiss* and the chinook salmon *O. tshawytscha* cell line. This suggests that, as expected, these genes in the rainbow trout and chinook salmon are more closely linked in an evolutionary sense than they are to the homologous genes in the other fish species studied. A very high degree of homology has been shown in the protamine genes of chum salmon (*O. keta*) and rainbow trout (Moir and Dixon 1988).

Many of the monoclonal antibodies raised against mammalian viral or cellular oncogene protein products recognized fish proteins in Western blots. Immunoprecipitation followed by Western blotting is considered a highly sensitive and discriminatory procedure, especially when monoclonal antibodies are used that recognize discrete and specific epitopes on the proteins of interest. The apparent recognition of fish proteins by this technique shows that these regions of the oncoproteins may be well conserved from viruses to mammals and fish.

Both the c-ras^h and c-ras^k gene products were detected in all the fish cells, showing that these proteins are conserved in vertebrates and may be essential. It is possible, however, that the c-ras^k and c-ras^h antibodies recognized the same fish proteins, but the monoclonal antibodies used were raised against different peptides representing regions of the two viral proteins and did not cross-react.

Table 2
Oncogenes Detected in Fish Cell Lines.

Detection Method	Oncogene(s) Detected	Cell line(s)*
Southern blots	*ras*^{h}, *raf*,	BB, RTG-2,
Using mammalian	*erb*-B, *src*	EPC, CHSE-214
viral *onc* probes	p53	EPC
Western blots		BB, EPC,
Using Mabs against	c-*myc*, c-*abl*, c-*fos*,	CHSE-214
human and viral	v-*ras*^{h}, p53 (murine), v-*ras*^{k},	
oncogene products	v-*raf*, v-*src*	
Northern blots	*ras*^{h}	High expression in BB and EPC
Using mammalian		Moderate expression in RTG
viral *onc* probes		Low in CHSE-214
	abl	High expression in CHSE-214
		Moderate in RTG
	src, abl, myc, ras^k	BB, EPC,
		RTG-2, CHSE-214
	p53	EPC, CHSE-214

*BB = Brown bullhead catfish line (Wolf and Quimby 1969); RTG-2 = rainbow trout gonad (Wolf and Quimby 1962); EPC = epithelioma papulosum cyprini (Fijan et al. 1983); and CHSE-214 = chinook salmon embryo (Fryer et al. 1965).

Significance

The mechanisms by which normal cells acquire the malignant phenotype form the central focus of cancer research. Transforming genes have been detected in a variety of tumors, including human tumors, and in cell lines derived from tumors by their ability to tranform NIH-3T3 mouse cells in culture. Genetic alternations also accompany tumorigenesis in feral populations of aquatic organisms exposed to environmental carcinogens. Any unambigious signal regarding oncogene or tumor-repressor-gene expression that correlates with tumor formation could serve as the basis for a test system for evaluating carcinogenic chemicals in the environment. Changes in the structure (mutation) of one or more oncogenes, their amplification, or the level of expression of their mRNA are all potential indicators of tumorigenic initiation and progression.

The number of genes homologous to either the viral oncogenes or the mammalian cell counterparts found in fish genomes suggests that these genes are highly conserved evolutionarily, and therefore their role in both normal cells and those cells in various stages of tumorigenic transformation may be common to all vertebrates. The brown bullhead *Ictalurus nebulosus* is of particular interest to us because of the high prevalence of tumors that occur in them during the late spring and summer in certain areas of the Chesapeake Bay (unpubl. data). We are now attempting to establish tissue culture cell lines from these tumors with the intent of comparing the genomic DNA of the tumor cells to that of the normal skin tissue and the brown bullhead (BB) cell line to determine if any of the potential fish oncogenes are altered or expressed differently in the tumor cells. If mutations identified in specific fish oncogenes can be shown to result from chemically-induced DNA damage, then such mutations could serve as indicators for environmental disease.

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Streptococcal Infection in Cultured Yellowtail

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ABSTRACT

Streptococcal infection is widespread in yellowtail (*Seriola quinqueradiata*) cultured in southwestern Japan and results in extensive losses. It is urgent that control measures be established against this disease. This paper presents the etiology, prophylaxis, and treatment of streptococcal infections in cultured yellowtail.

Introduction

Yellowtail (*Seriola quinqueradiata*) culture developed rapidly in the 1960s, mainly in southwestern Japan. By 1988, the farming of yellowtail was being practiced in 27 prefectures (Fig. 1), the total production was about 166,000 metric tons, or 68% of the total production of marine fish cultured in Japan (Fig. 2). The incidence of diseases in cultured yellowtail is gradually increasing as the culture of this fish becomes more popular. About 30 diseases have been identified so far. Figure 3 shows the loss of cultured yellowtail due to disease in 1988; the cost to fish farmers was about 11,300 million yen. Bacterial diseases are responsible for the majority of the losses, whereas those due to viruses and parasites are minor. Among the bacterial diseases, streptococcosis was responsible for the highest losses (64%), pasteurellosis ranking second (21%). Thus, with yellowtail culture, it is very important to control the incidence of streptococcal infection.

The author describes here some aspects of streptococcal infection in cultured yellowtail with special emphasis on the etiology of the causative bacteria, prophylaxis, and methods used to treat the disease.

Causative Bacteria

Streptococcal infection in cultured yellowtail was first identified by Kusuda et al. in 1974 (Kusuda et al. 1976). Since then, three species of fish pathogenic *Streptococcus* have been reported. These bacteria are clearly distinguishable by their biochemical and physiological characteristics (Table 1; Sako, unpubl. data). The species reported by Kusuda et al. is generally called alpha-hemolytic *Streptococcus* sp. Almost all of the streptococci isolated from yellowtail were confirmed to be the alpha-hemolytic *Streptococcus* sp. (Kitao 1982). Recently, this species was transferred to genus *Enterococcus* (Riichi Kusuda, Kochi Univ., pers. commun., April 1990). Another species isolated from a yellowtail juvenile in 1976 by Minami et al. (1979) displayed beta-hemolysis on sheep blood agar, so it has been called beta-hemolytic *Streptococcus* sp. This species is classified with pyogenic streptococci based on its biochemical characteristics. Its biochemical characteristics also agreed well with those of *S. iniae* ATCC 29178 isolated from the Amazon freshwater dolphfin, *Inia geoffrensis* (Pier and Madin 1976). The last species is a nonhemolytic *Streptococcus* reported by Iida et al. (1986), which has biochemical characteristics similar to those of *S.agalactiae* and has the Lancefield grouping antigen B, type Ib.

A species that shows the same characteristics as alpha-hemolytic *Streptococcus* sp. was also isolated from cultured yellowtail in the Republic of Korea (Park et al. 1987). The nonhemolytic, group B, type Ib *Streptococcus* was also isolated from several marine fishes in the United States (Plumb et al. 1974).

Host Range

Large numbers of *Enterococcus* sp. and *S. iniae* have been isolated from wild and cultured marine fishes as

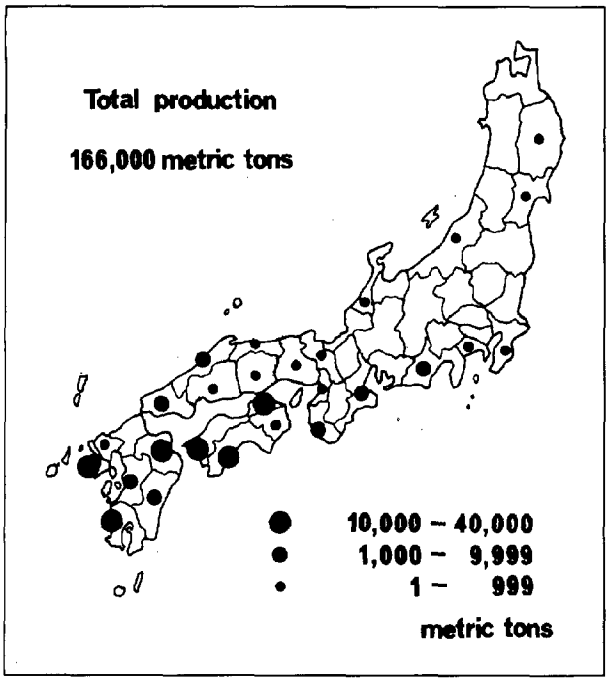

Figure 1
Yellowtail production in Japan by prefecture, 1988. Source: Statistics and Information Department, Ministry of Agriculture, Forestry, and Fisheries, Japan.

well as in small quantities from cultured freshwater fishes in Japan (Table 2; Kusuda et al. 1976; Kusuda et al. 1978; Minami 1979; Minami et al. 1979; Kitao et al. 1981; Ohnishi and Jo 1981; Kusuda and Kawai 1982; Kusuda et al. 1982; Ogawa et al. 1982; Yasunaga 1982; Hatai et al. 1983; Nakatsugawa 1983; Kaige et al. 1984; Iida et al. 1986; Sakai et al. 1986; Atsuta et al. 1990). Because sardines and anchovies, as well as their minced meats, are a major feed for yellowtail, it is possible that they are the origin of streptococcal infection in cultured fish.

Pathogenicity

Each of the *Streptococcus* spp. and their strains exhibit different degrees of virulence. *Enterococcus* sp. produces both intra- and extracellular toxins (Kimura and Kusuda 1979). In artificially induced infections, even small amounts of *Enterococcus* sp. cells killed yellowtail (Taniguchi 1982a). However, *S. iniae* killed yellowtail only occasionally although it frequently caused vertebral deformity (Sako, unpubl. data).

Streptococci is an indigenous bacteria in warm-blooded animals and grows rapidly at 30–35° C. *S. iniae* isolated from yellowtail have been confirmed to be pathogenic to mice by intraperitoneal injection. However, *Enterococcus* sp. and nonhemolytic group B, type Ib *Streptococcus* isolated from yellowtail were nonpathogenic to mice (Sako, unpubl. data).

Diagnosis

Major clinical signs caused by *Enterococcus* sp. are exophthalmos, petechiae, and ulceration on the inside of the opercula, and congestion of the pectoral and caudal fins. Signs such as congestion and haemorrhagia are also found in the intestine, liver, spleen, and kidney (Kusuda et al. 1976). Epicarditis is also seen often. Pathogenic *Streptococcus* is frequently isolated from the brains of diseased yellowtail

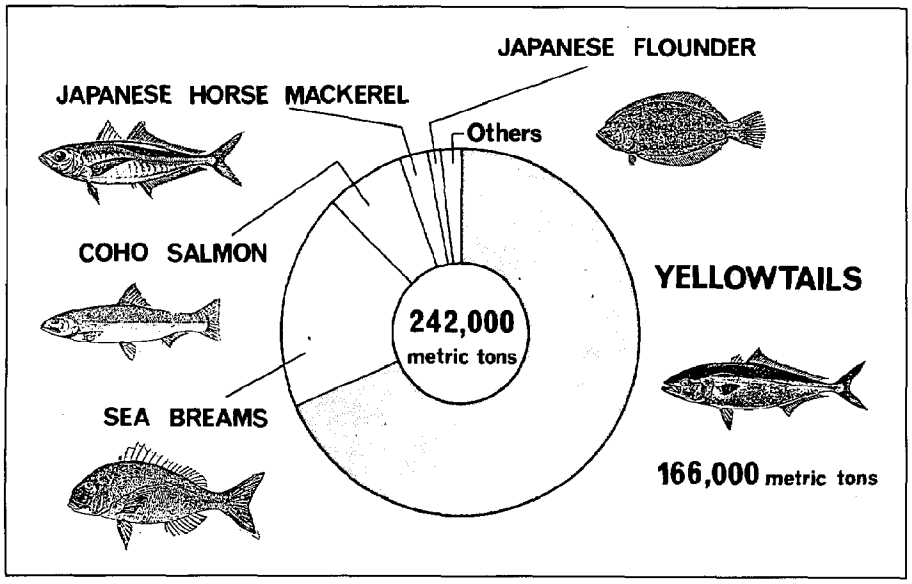

Figure 2
Marine fish culture production for 1988. Source: Statistics and Information Department, Ministry of Agriculture, Forestry, and Fisheries, Japan.

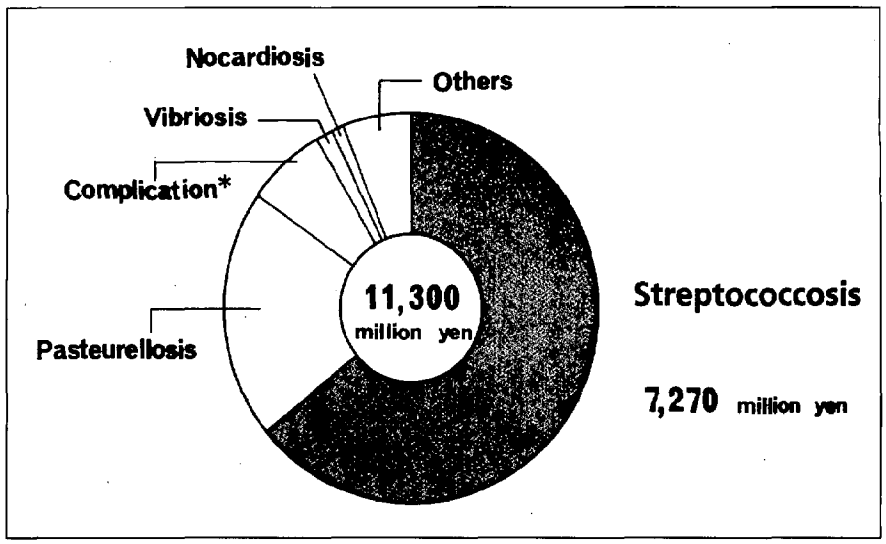

Figure 3
Loss of cultured yellowtail to diseases in 1988. *This classification contains cases that the cause of a disease are two or more: e.g. mixed infection. Source: Research Division, Fisheries Agency of Japan.

(Shiomitsu 1982; Kaige et al. 1984; Iida et al. 1986).

Immunological identification using fluorescent antibody techniques is also useful for the rapid identification of yellowtail pathogenic streptococci (Kusuda and Kawahara 1987).

A rapid identification system is also commercially available for the identification of fish pathogenic streptococci. Three species of streptococci from yellowtail can be easily distinguished using API 20 Strep System (BIO MERIEUX S.A.) within 4 hours (Table 3; Sako, unpubl. data).

Prevention

It was reported that fish meats used as feed for yellowtail have fish pathogenic *Streptococcus* (Minami 1979; Yasunaga 1982). The organisms grow quickly in the minced meat of fish within several hours under optimal temperature (Fig. 4; Sako, unpubl. data). In laboratory experiments, it was demonstrated that yellowtail can be infected orally by *Streptococcus* through feed such as minced meat (Taniguchi 1982a). In order to prevent contamination by the pathogen and to

Table 1
Biochemical characteristics of α, β and nonhemolytic *Streptococcus* sp. isolated from yellowtail.

Characteristics	α-hemolytic *Streptococcus* sp. (*Enterococcus* sp.)	β-hemolytic *Streptococcus* sp. (*S. iniae*)	nonhemolytic *Streptococcus* sp. (*S. agalactiae*)
Hemolysis	α, –	β	–
Voges-Proskauer reaction	+	–	+
Esculin hydrolysis	+	+	–
Starch hydrolysis	–	+	–
Hippurate hydrolysis	–	–	+
Pyrrolidonylarylamidase	+	+	–
β-galactosidase	–	+	–
Alkaline phosphatase	–	+	+
Sensitivity to bacitracin	–	+	+
cAMP test	–	+	–
Lancefield group antigen	not A, B, C, D, F, G[a]	not A, B, C, D, F, G[a]	BIb[b]

[a]No reaction at least against the antisera of A~G.
[b]BIb denotes type of group antigen.

Table 2

Fish species in Japan from which a pathogenic streptococci was isolated (Kusuda et al. 1976; Kusuda et al. 1978; Minami 1979; Minami et al. 1979; Ohnishi and Jo 1981; Kitao et al. 1981; Yasunaga 1982; Kusuda and Kawai 1982; Kusuda et al. 1982; Ogawa et al. 1982; Nakatsugawa 1983; Hatai et al. 1983; Kaige et al. 1984; Iida et al. 1986; Sakai et al. 1986; Atsuta et al. 1990).

Isolates	Marine fish		Freshwater cultured fish
	Cultured	Wild	
Enterococcus sp.	Yellowtail (*Seriola quinqueradiata*) Japanese horse mackerel (*Trachurus japonicus*) Purplish amber jack (*Seriola dumerili*) Goldstriped amber jack (*Seriola lalandi*) Largescale blackfish (*Girella punctata*) Black scraper (*Thamnaconus modestus*)	Sardine (*Sardinops melanosticta*) Anchovy (*Engraulis japonica*) Round herring (*Etrumeus microps*) Sand lance (*Ammodytes personatus*) Chub mackerel (*Scomber japonicus*) Moon dragonets (*Repomucenus lunatus*) Black scraper (*Thamnaconus modestus*) Multicolorfin (*Halichoeres poecilopterus*) Silver jewfish (*Pennahia argentata*) Red sea bream (*Pagrus major*)	Eel (*Anguilla japonica*)
Streptococcus iniae	Yellowtail (*Seriola quinqueradiata*) Japanese horse mackerel (*Trachurus japonicus*) Japanese flounder (*Paralichthys olivaceus*) Knifejaw (*Oplegnathus fasciatus*) Rabbit fish (*Siganus fuscescens*) Largescale blackfish (*Girella punctata*) Triggerfish (*Aluteres monoceros*) Jacopever (*Sebastes schlegeli*) Coho salmon (*Oncorhynchus kisutch*)	Yellowtail (*Seriola quinqueradiata*) Japanese horse mackerel (*Trachurus japonicus*) Chub mackerel (*Scomber japonicus*) Sardine (*Sardinops melanosticta*) Red sea bream (*Pagrus major*)	Ayu (*Plecoglossus altivelis*) Rainbow trout (*Oncorhynchus mykiss*) Amago salmon (*Oncorhynchus rhodurus*) Tilapia (*Tilapia nilotica*)
Streptococcus agalactiae	Yellowtail (*Seriola quinqueradiata*)		

slow its growth, the following techniques are recommended: 1) washing feed fish in uninfected water, 2) feeding fish without defrosting, and 3) feeding processed pellets (Taniguchi 1982b).

Yellowtail can acquire immunity after streptococcal infection (Kusuda and Takagi 1983). Indeed, many attempts have been made to prevent streptococcal infection using bacterin. Iida et al. (1982) reported on the efficacy of vaccination for control of streptococcal infection.

Therapy

It is not easy to cure yellowtail that are in the advanced stages of streptococcal infection. Minimal inhibitory concentrations (MIC) of antimicrobial agents against *Streptococcus* from yellowtail are shown in Figure 5 (Sako, unpubl. data). Sensitivities to several drugs are different between *Enterococcus* sp. and *S. iniae*, but both species have high sensitivities to penicillins, macrolides, tetracyclines, and lincomycin.

Table 3

Differential characteristics among *Enterococcus* sp., *Streptococcus iniae*, and *Streptococcus agalactiae* isolated from yellowtail by API 20 Strep System incubated at 35° C for four hours (Sako, unpubl. data). VP = Voges-Proskauer reaction; HIP = Hippurate hydrolysis; ESC = Esculin hydrolysis; PYRA = Pyrrolidonylaryl amidase; PAL = Alkaline phosphatase.

Species	Test (characteristics)				
	VP	HIP	ESC	PYRA	PAL
Enterococcus sp.	+	–	+	+	–
Streptococcus iniae	–	–	+	+	+
Streptococcus agalactiae	+	+	–	–	+

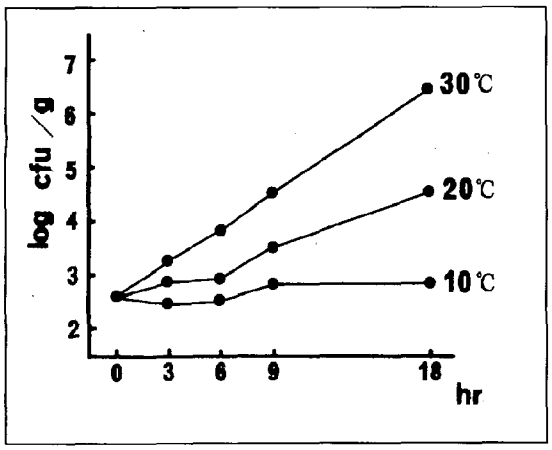

Figure 4
Growth of *Streptococcus iniae* in minced meat of sand lance (Sako, unpubl. data).

Table 4
Drugs used to cure yellowtail streptococcal infection in Japan. Source: Fisheries Agency of Japan.

Drugs	Daily dose (mg/kg body weight)
Alkyl trimethyl ammonium calcium oxytetracycline	50
Doxycycline hydrochloride	20–50
Spiramycin embonate	25–40
Kitasamycin	80
Erythromycin	25-50
Josamycin	30–50
Oleandomycin polystyrene sulfonic acid	25
Lincomycin hydrochloride	20–40
Florfenicol	40

At present, two kinds of tetracyclines, five kinds of macrolides, lincomycin, and florfenicol are permitted as the chemotherapy of streptococcal infection in yellowtail in Japan (Table 4). Recently, streptococci that have aquired resistance to macrolides and tetracyclines were found in many yellowtail farms (M. Fukudome, Kagoshima Pref. Fish. Exp. Sta., pers. commun., July 1990). Therefore, chemotherapy treatments for yellowtail infected with drug-resistant streptococci are likely to be ineffective.

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Drugs	MIC (μg/ml)															
	0.00625	0.0125	0.025	0.05	0.10	0.20	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	≥200
Penicillin G	○							●								
Ampicillin		○	○					●								
Erythromycin				●○												
Oleandomycin							●○									
Kitasamycin						○	●	●								
Spiramycin							○	○	●							
Josamycin						○		●								
Chloramphenicol									○	●						
Oxytetracycline							○	●								
Doxycycline					○●											
Streptomycin														●○	●	
Lincomycin				●○												
Colistin																●○
Sulfadimethoxine																●○
Sodium nifur-styrenate			○						●							
Trimethoprim						○	○							●	●	
Oxolinic acid																●○

Figure 5
Sensitivities of *Enterococcus* sp. and *Streptococcus iniae* isolated from yellowtail against various drugs: • *Enterococcus* sp.; ○ *Streptococcus iniae* (Sako, unpubl.data).

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Stress Induced Pathologies in Fish: The Cost of Stress

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ABSTRACT

Pathology is the consequence of prolonged stress. Maintaining fish under aquaculture conditions intensifies the problems of stress by adding the impact of stressors that are unique to culture conditions. Unlike terrestrial domestic animals, fish have not benefitted from the genetic pressures of generations of domestication that favor those individuals most suitable for culture. The physiological mechanisms by which stress induces the development of pathology remain unknown, although it is recognized that one of the major factors affecting health during stress is the response of the neuroendocrine system, a stress responsive system that directly regulated growth, reproduction, and the immune system. During stress, the neuroendocrine system shifts biological resources from pre-stress activities to new functions at a biological cost to the fish. As the biological cost of shifting these resources rises during stress, the fish is placed into a prepathological state, rendering it vulnerable to the development of pathology. It is by focusing on this biological cost of stress that it is possible to develop strategies to reduce stress in aquaculture and to understand the biological basis for the development of disease.

Introduction

Pathology is the consequence of prolonged stress. While we can not identify the exact mechanisms responsible for the development of pathologies during stress, there is indisputable evidence that stress can result in the development of a diverse number of pathologies that include not only disease, but the loss of reproduction, the failure to grow normally, and even the development of abnormal, deleterious behaviors (Moberg 1985).

Fish in culture are especially at risk to the adverse effects of stress. Unlike domesticated mammals, fish have not benefitted from generations of genetic selection for traits that would assist them in adapting to the restraints of confinement. In essence, fish are still wild animals, and when culture conditions do not duplicate their natural habitat, they are forced to make biological adjustments to survive. Exacerbating this problem is the failure of aquaculturists to make any serious effort to systematically define those culture conditions that the fish would find least stressful. Practical considerations have dictated most culture conditions with emphasis on such factors as the ease of cleaning, maximizing the number of fish, or using any holding facility that is readily available. Little concern is given to the fish, at least until stress becomes so severe that pathology occurs. Certainly this is an area of aquaculture that needs to be addressed, especially as increasingly intensified culture conditions are adopted.

Problem of Defining Stress

Although there is considerable evidence demonstrating the disruptive effects of stress on the well-being of fish (Schreck 1982; Pickering and Pottinger 1987; Maule et al. 1989; Vijayan and Leatherland 1990), it is surprising that there is not a better understanding of how stress actually leads to the development of pathologies. One reason for the difficulty in discovering the mechanisms involved is a lack of consistent responses to stress. Most fish do not develop a pathol-

ogy during stress. In fact, it is not possible to predict which fish will become vulnerable to a pathology due to stress. This is because stress, per se, is not necessarily harmful to an individual. Animals have developed defenses for coping with stress. Stress is a part of life, and for a species to be successful, it must have evolved biological defenses to stress. Therefore, stress responses are not bad. In fact, they are desirable and necessary. Pathology occurs only when the animal is confronted with stress of such a magnitude that the very biological responses that evolved for defense result in a biological cost to the individual and renders it vulnerable to pathology. To understand how the biological cost of stress leads to the development of pathology, it is necessary to first examine how fish respond to stress.

Model of the Stress Response

Numerous studies have identified the various biological responses that fish use in responding to and coping with stress (for monographs summarizing this work see Pickering 1981 and Adams 1990). Because these responses are so varied and complex, I will use a model of animal stress (Fig. 1) to organize current concepts of stress biology and examine how the biological cost of stress results in the development of pathology. The development of this model has been discussed elsewhere (Moberg 1985, 1987), but, in brief, the model divides the biological response to stress into three major components: recognition of a threat to homeostasis, the stress response, and the consequences of stress. It is the central nervous system that perceives a threat to homeostasis and organizes the fish's biological defense. If a threat (also referred to as a stressor) is perceived, three general types of biological responses are available to the fish: behavioral, autonomic nervous system, and neuroendocrine system responses. In the wild, behavior can be the most biologically efficient way for a fish to cope by simply allowing the animal to remove itself from the stressor. However, under the confinement of culture conditions, this option is severely limited. If the fish is unable to avoid the stressor, then the roles of the autonomic nervous and neuroendocrine systems become critical. While activation of the autonomic nervous system represents an important way for a fish to avoid or cope with a stressor, its effects are rapid in onset, of short duration, and relatively specific. For this reason, the importance of the autonomic nervous system in inducing stress-related pathologies is questionable. In contrast to the autonomic nervous system, the hypophyseal hormones of the neuroendocrine system undoubtedly play an important role in stress-induced pathology (Moberg 1985). These hormones have a widespread action on the animal and long-lasting effects on such diverse biological functions as reproduction, growth, metabolism, resistance to diseases, and behavior. Each of these functions are vital to the fish's well-being.

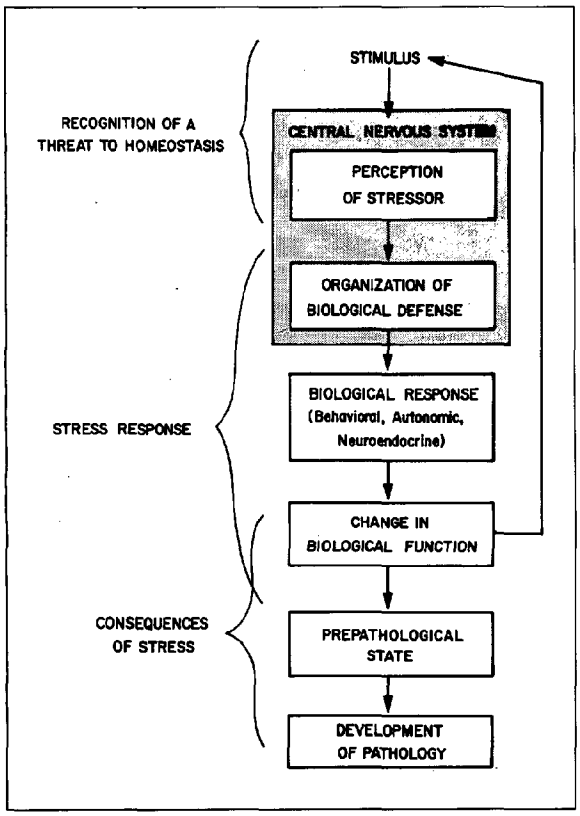

Figure 1
Model for the response of animals to a stressful event (from Moberg 1985).

When the central nervous system organizes the biological defense to a stressor, it is a combination of these three general biological systems that alters function. In mammals, we have found that a number of factors influence the pattern of this biological response, and we have found it impossible to predict how the individual systems will respond (Moberg 1985). While the data for fish is limited, there is evidence indicating that in fish there are also a number of factors that can alter the stress response. For example, water quality and temperature (Pickering and Pottinger 1987, 1989), genetics (Refstie 1982), and social interactions (Peters et al. 1988) have all been found to influence the stress response. Thus, measurement of the three general biological systems have not proved to be reliable as an indicator of stress

(Moberg 1987). However, what is important to the fish is not the pattern of the biological response but the resulting change in biological function—the ultimate consequence of stress.

Regardless of which of the general biological systems the fish chooses in response to the stressor, the result is a change in biological function. Depending upon the appropriateness of the response, this change in biological function may alleviate, or even eliminate the stressor. But the change in biological function can also lead to the development of a prepathological state which can eventually result in the development of a pathology (Fig. 1). For example, if the change in biological function during stress results in the suppression of the immune system (a prepathological state), then the animal is vulnerable to pathogens, and the opportunity for disease (a pathological state) exists. The longer the fish is stressed, the longer the immune system is suppressed and the greater the opportunity for disease to occur. However, disease is only one possible pathological state. Other examples would be the inability to reproduce, abnormal behavior, or the failure to grow normally.

Biological Cost of Stress

The change in biological function that occurs as part of the stress response results in a biological cost to the fish. The fish's resources are diverted from such pre-stress activities as growth to new activities. For example, the glucocorticosteroid hormone cortisol secreted during stress will induce gluconeogenesis, diverting metabolic resources supporting such functions as growth to the production of glucose. Barton and Schreck (1987) found in juvenile steelhead (*Oncorhynchus mykiss*) that acute stress reduced by about 25% the amount of energy available for other activities. Likewise, the biological cost of responding to a stressor may suppress the ability of the immune system to respond to pathogens, rendering the fish vulnerable to disease (Maule et al. 1989). It does not matter which pattern of responses the animal chooses; a change in biological function occurs that imposes a cost, whether or not the change in function is effective in helping the animal to cope with the stressor.

Fortunately, most stressors last for only a brief time and the biological cost of coping with them is relatively small. However, if a stressor is severe, if it persists, or if the fish experiences a series of stressors, the resulting biological cost may be sufficient to induce a prepathological state which in turn can lead to the development of pathology.

The concept that there is a biological cost associated with coping with stress also explains how the effects of subclinical stressors can accumulate, resulting in a significant stress that results in pathology (Moberg 1985, 1992). Separately, none of the subclinical stressors would result in a significant expenditure of the fish's resources, but combined, the subclinical stressors could cost the animal sufficient resources to induce a prepathological state and lead to the development of a pathology. For example, Jarvi (1989) found a greater mortality rate in Atlantic salmon smolts (*Salmo salar*) that were exposed simultaneously to both osmotic stress and the presence of predators than if the smolts experienced only one of the stressors. Because of the accumulation of biological costs, a series of apparently innocuous events can lead to disease, loss in reproduction, diminished growth, or mortality.

Conclusion

The biological cost of stress is the key to understanding why fish exposed to stress develop pathologies. For a fish to cope with a stressor, it must expend biological resources. The more prolonged the stressor or the greater the effort needed to cope with a severe stressor, the greater the diversion of biological resources, and thus the greater the biological cost of the stress. This diversion of resources occurs at the expense of other biological functions, leading to a prepathological state where the fish is at risk to the development of pathology. For some fish, the biological cost of coping with a stressor is greater than for other fish and these are the first to succumb to the stressor. As we manage fish in culture, every effort must be made to reduce stress and, as a result, lower the biological cost for fish living in culture.

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Control of Fish Disease in Japan

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ABSTRACT

The damage to Japanese aquaculture by fish disease in 1988 was estimated at 20,000 metric tons, worth 144 million dollars, and amounted to 6% of the total aquaculture production. The Fisheries Agency of the Government of Japan financially supports fish disease control projects carried out by prefectural governments and fisheries cooperative associations. It also entrusts the Japan Fisheries Resource Conservation Association (JFRCA) with the following tasks; 1) voluntary pathogen inspection of imported larval fish and fish eggs; 2) training of advisory personnel on fish disease control; 3) improvement of diagnostic methods; and 4) improvement of disease prevention methods and the publication of their findings. The main body of the Agency is augmented by the Shimonoseki University of Fisheries and nine national research institutes. One of these is the National Research Institute of Aquaculture, which has a Fish Pathology Division totalling 11 researchers. Each of the 47 prefectural governments in Japan has at least one Fishery Experimental Station. There are a total of 96 stations. Prefectural governments, with their institutions, undertake disease diagnosis and drug residue testing and provide guidance on fish disease control and drug use. Fisheries co-operative associations play important roles in fish disease prevention by giving guidance to fishermen and controlling local fishery practices. There are 17 universities that have a fishery faculty and 16 universities with a veterinary faculty. All these universities have curriculums for fish disease and conduct research on it.

Aquaculture and Fish Disease

Fishery Products and Aquaculture Production

In 1988, fishery production in Japan totaled 12,785,000 metric tons (t) valued at $19.4 billion (1 dollar = 140 yen) (MAFF 1988). Changes in amount and value of aquaculture products over the last 23 years are shown in Figure 1. Aquaculture production in 1988 reached 1,426,000 t or 11% of the total fishery output. These products were valued at $4.9 billion or 25% of the total fishery value.

Aquaculture has been rapidly developing and has become more important because 1) consumers are demanding higher quality and more variety in fishery products, 2) aquaculture techniques have improved, and 3) the total amount of aquacultural grounds has increased owing to the development of new aquacultural facilities, and the expansion of older ones.

There are two types of aquaculture; feeding aquaculture (i.e., fish culture) and aquaculture without feeding (i.e., shellfish culture). Production by feeding aquaculture has grown markedly in recent years and has reached 343,000 t or $2.5 billion in 1988—about 7 and 19 times the same values for those in 1965, respectively.

The production of marine fish by aquaculture in 1988 was valued at $1,811 million and amounted to 245,000 t. Yellowtail *Seriola quinqueradiata* production was greatest at $949 million (165,900 t), followed by red sea bream, *Pagrus major*, and coho salmon, *Oncorhynchus kisutch*. (Table 1). Coho salmon, which is cultured in fresh water until the smolt stage and then transferred to seawater, is one of the species the production of which has increased rapidly during recent years. Likewise the production of horse mackerel, *Trachurus japonicus*, striped jack, *Caranx delicatissimus*, and tiger puffer, *Takifugu rubripes*, have increased markedly. Increased variety

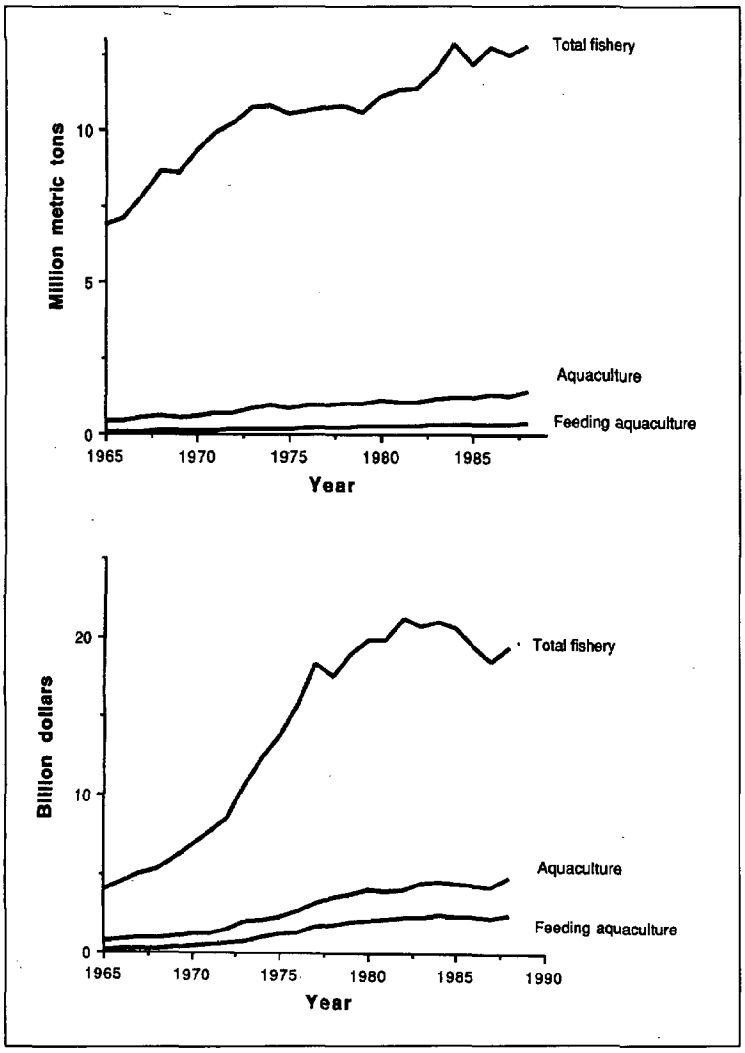

Figure 1
Cultured fish production (feeding aquaculture and aquaculture without feeding) and the total fishery production since 1965 (MAFF 1988) in metric tons and dollars. (1$= 140 yen.)

of cultured species is a current trend in Japanese aquaculture.

Production of freshwater fish by aquaculture was $732 million (97,800 t) in 1988. Japanese eel, *Anguilla japonica,* production was largest at 39,600 t ($417 million), followed by Ayu, *Plecoglossus altivelis,* salmonids, and common carp, *Cyprinus carpio.* A diversity of species is also seen in freshwater aquaculture. Among these are Japanese native salmonids such as landlocked salmon, *O. masou,* dwarf rill trout, *O. rhodurus,* and mountain trout, *Salvelinus pluvius.*

Fish Disease and Its Damage

The rapid development of aquaculture has also brought about a problem, namely with the occurrence of diseases. These are caused partly by the decline in the health of the cultured fish owing to environmental deterioration and overpopulation, and partly by the recent entry of foreign pathogens.

For example, infectious pancreatic necrosis (IPN), infectious hematopoietic necrosis (IHN), and bacterial kidney disease (BKD) are diseases considered to have been introduced from abroad. Both IHN and BKD are thought to have entered through eggs imported for propagation, while IPN probably entered through eggs imported for culture. These diseases have become prevalent in salmonid farms and are causing severe damage to salmonid production.

Based on a questionnaire sent to all aquaculture farmers through the prefectural governments every year, the Fisheries Agency has estimated the annual damage by fish diseases to feeding aquaculture. The results are shown in Figure 2.

The damage to aquaculture by fish disease in 1988 was estimated at 20,000 t or $144 million which is

Table 1
Cultured fish production by species and damage due to disease in 1988 (MAFF 1988).

Fish	Production		Damage		Ratio (Damage/Production)	
	Amount[a] (1,000 t)	Value[b] (million dollars)	Amount (1,000 t)	Value (million dollars)	Amount (%)	Value (%)
Marine fish	245.0	1811	17.3	119	7	7
yellowtail	165.9	949	14.4	81	9	9
red sea bream	45.5	431	0.5	5	1	1
coho salmon	16.5	109	1.0	7	6	6
flounder	3.1	64	0.4	6	13	10
horse mackerel	6.5	37	0.4	2	6	6
tiger puffer	1.2	37	0.3	7	27	19
kuruma prawn	3.0	140	0.2	10	7	7
others	3.3	44	0.1	1	3	4
Freshwater fish	97.8	732	2.8	25	3	3
eel	39.6	417	1.1	14	3	3
ayu	13.6	120	0.3	3	2	3
salmonids	19.1	73	1.0	5	5	7
common carp	18.1	57	0.2	1	1	1
others	7.4	65	0.2	2	3	4
Total	342.8	2,543	20.1	144	6	6

[a] in metric tons, t.
[b] 1$ = 140 yen.

about 6% of the total value of all aquaculture products. In 1979 estimated damage was $137 million and increased year by year to reach a peak of $190 million in 1982. It then decreased and has been maintained at around $140 million since 1985. During the 1980s, the total value of aquaculture products increased rapidly while the rate of damage by diseases either decreased or remained nearly constant. It is thought that two factors have worked effectively in reducing damage by diseases in recent years. One is the practice of control measures, such as disinfection, disposal of dead fish, and suitable layout of ponds. The other is the improvement of husbandry techniques, such as improvement of food and the control of population levels and pen location with regard to the environmental capacity.

The rate of disease damage (estimated damage value/total product value) for each species is shown in Table 1. Disease reduced yellowtail production by 9% in 1988. The damage rates of tiger puffer, Japanese flounder (*Paralichthys olivaceus*) and kuruma prawn (*Penaeus japonicus*) were 19, 10, and 7%, respectively, while only 1% of red sea bream production was affected. As for freshwater finfish, rainbow trout (*O. mykiss*) showed a high damage rate of 7%, while that of common carp was only 1%. In marine aquaculture, species with a long history of culture generally showed low damage rates, while species with a short history of culture, such as tiger puffer and japanese flounder, showed high damage rates. This difference may be caused by the lack of experience and less-developed management techniques for culturing new species.

In recent years, fish disease problems have become more diversified and complicated. In yellowtail culture, streptococcosis and pseudotuberculosis are the two major diseases. When an episode of mass mortality occurs, both of these agents are often found in a single fish. Nocardiosis, viral ascite disease (diseases for which treatments are not yet established), and a disease causing peduncle curvature have recently become an additional problem.

Fish Disease Control

System—Because of the complexity and variety of diseases occurring in Japanese aquaculture, the Fisheries Agency is carrying out systematic fish disease control in cooperation with prefectural governments, fisheries cooperative associations, and some universities in order both to decrease disease damage and to ensure the safety of cultured fish for human consumption. It is important for aquaculture

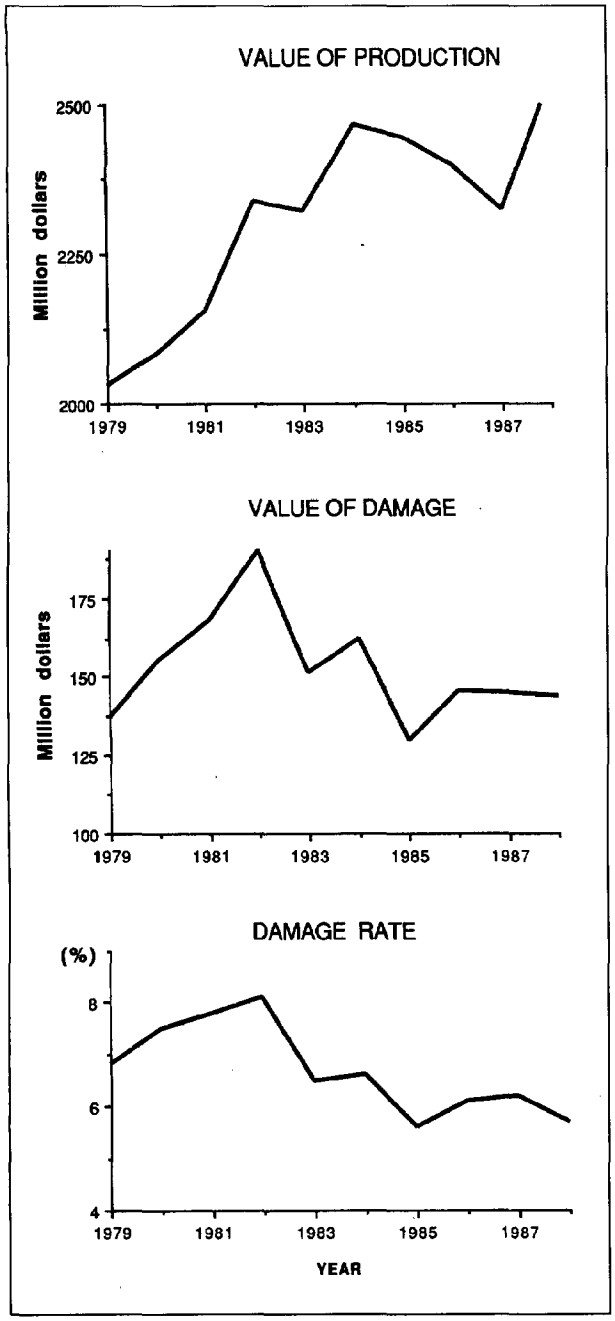

Figure 2
Changes in cultured fish production (feeding aquaculture) and damage by fish disease. (1$=140 yen.)

farmers to practice proper feeding and fish disease control. Thus, prefectural governments and fisheries cooperative associations have been requested to establish a system to assist the aquaculture farmer's practice of disease control (Fig. 3).

Fisheries Agency—This agency financially supports fish disease control projects carried out by prefectural governments and the fisheries cooperative associations. It also entrusts the Japan Fisheries Resource Conservation Association (JFRCA) with the following responsibilities: 1) voluntary pathogen inspection of imported live fish and eggs; 2) training of advisory personnel on fish disease control; 3) improvement of diagnosis methods; and 4) improvement of disease prevention methods and the publication of their findings (Fig. 4).

The Fisheries Agency oversees nine national research institutes, the Shimonoseki University of Fisheries, and the Hokkaido Salmon Hatchery. In these facilities either research or education in fisheries is carried out, but among these national research facilities, only the National Research Institute of Aquaculture, which was established in 1979 to carry out basic and leading research for aquaculture, has a fish pathology division. This division has 11 researchers and consists of 4 research sections; Pathogens, Pathophysiology, Pharmacology, and Immunology (Fig. 5).

Prefectural Government—Control measures on the aquaculture grounds are very important for disease prevention. Prefectural governments play important roles in guiding aquaculture farmers either directly or indirectly through the fisheries cooperative associations.

Prefectural governments and their research institutions undertake disease diagnosis and drug residue testing, and provide guidance on fish disease control and drug use (Fig. 6).

There are 96 prefectural fisheries experimental stations in Japan: at least one station in each prefecture. Investigation and research for aquaculture and fish disease is carried out in their propagation and aquaculture divisions. In most of these prefectures, fish disease control centers (20 in total) have been established, either as a part of the fishery experimental station or as an independent establishment. Prefectural governments have also established fisheries extension offices to give technical guidance to aquaculture farmers. These offices and fishery experimental stations undertake fish disease prevention measures in a cooperative manner.

The number of staff engaged in disease control measures in prefectural governments was 470 in 1986. In addition, 30 staff members from various cities, towns, and villages were also engaged in these measures.

Fisheries Cooperative Association—Fisheries cooperative associations play a very important role in fish disease control by giving guidance to aquaculture farmers and controlling local fishery grounds. How-

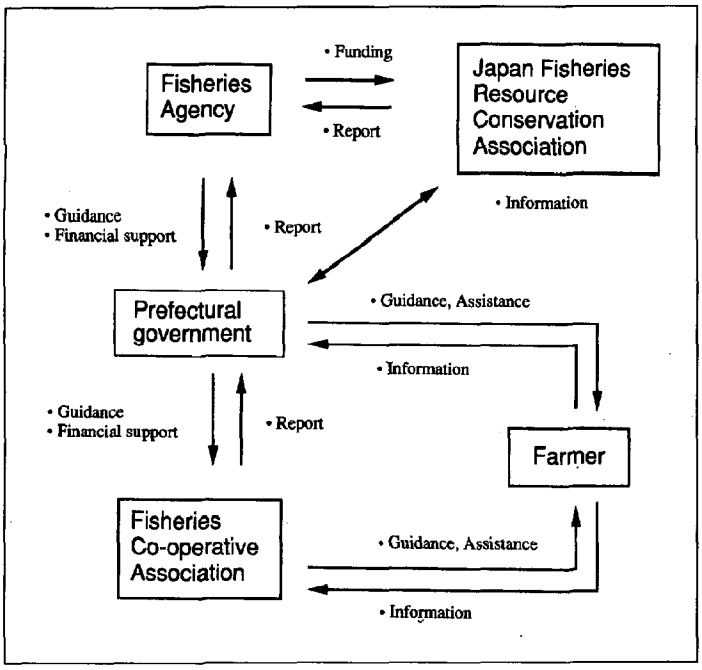

Figure 3
Fish disease control system in Japan.

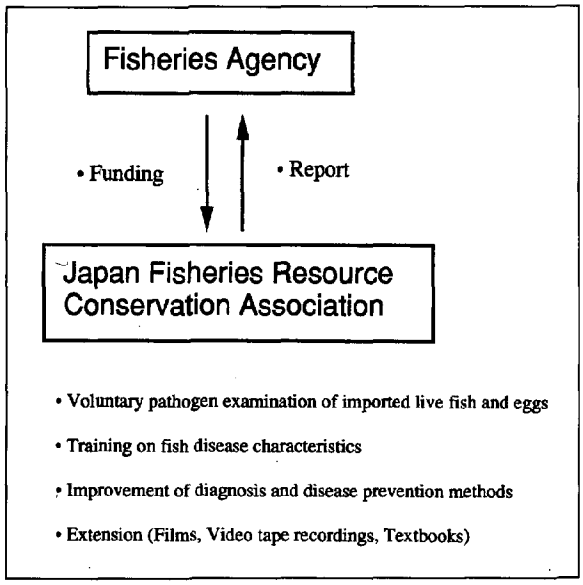

Figure 4
Role of the Japan Fisheries Resource Conservation Association in the control of disease in aquaculture.

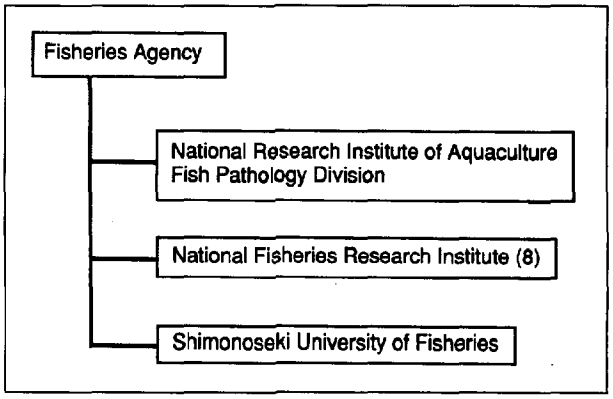

Figure 5
Fish disease research system of the Fisheries Agency.

ever, many of these associations are small in scale, and the level of guidance varies widely. Only a few of them carry out drug residue testing, diagnosis of fish disease, and observation of environmental conditions in their aquaculture grounds (Fig. 7). The number of staff engaging in disease control from those associations was only 80 in 1986.

Universities—Fish disease education is carried out by 17 fishery faculties in universities (including the Shimonoseki University of Fisheries, Fisheries Agency) and 16 veterinary faculties. All these faculties also have fish disease research facilities.

Prevention of the Introduction and Outbreak of Pathogens—In Japan, neither laws nor regulations have been established concerning fish quarantine. However, in order to avoid the introduction of fish pathogens from foreign countries, the Fisheries Agency requests importers (through prefectural governors) to take the following steps. Importers are requested 1) to import living eggs or fish in conjunc-

tion with health certificates issued by the government authority of the exporting country, 2) to receive an examination for pathogens in the fish or eggs at the time of import, and 3) to disinfect eggs immediately after importation.

Examination for pathogens at the time of import is carried out by the JFRCA as entrusted by the Fisheries Agency. Examination is done on viral diseases such as VHS (viral hemorrhagic septicemia), BKD and whirling disease of salmonid fish (*Myxosoma cerebralis*), and on viruses, bacteria, and parasites for eel. Examinations are also conducted for other species.

To prevent the spread of fish diseases, prefectural fish disease control centers and fisheries experimental stations examine live fish and eggs for pathogens upon the request of the aquaculture farmer. The Fisheries Agency encourages farmers to transport live fish and eggs with records of species, place of production, name and address of producer, dates of fertilization or hatching, history of fish disease, and medication history.

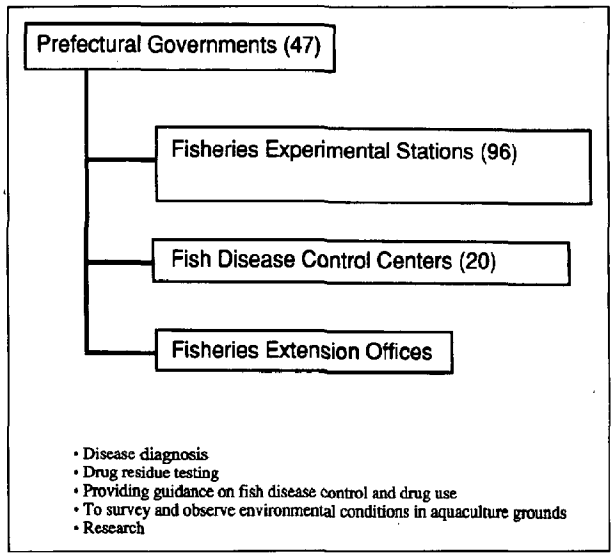

Figure 6
Role of the prefectural governments in the fish disease control system.

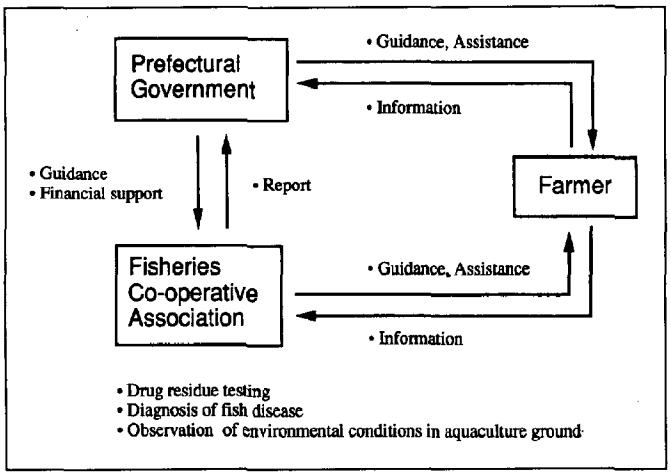

Figure 7
Role of fisheries cooperative associations in the control of fish disease.

Training—Fish disease specialists are indispensable for carrying out adequate control measures such as providing advice to the aquaculture farmers and diagnosing and disinfecting facilities and equipment. Since 1973, the Fisheries Agency has been training the staffs of prefectural governments—in 1984 staffs of fisheries cooperative associations were included in the training program. This training was aimed at educating new fish disease specialists and at improving the knowledge of the current specialists. In recent years, the Fisheries Agency has entrusted this training to JFRCA.

There are various training courses offered by the JFRCA for fish disease technical workers. A basic class consists of 20 day-lectures and practical exercises each year for 3 years. In 1989, there were 30 to 40 trainees for each year course; altogether, 100 people have participated in the training. Other training on fish disease is also carried out. Since 1974, the Japan Veterinary Medical Association has been providing a fish disease course for veterinarians every year as postgraduate education. In Japan, training for fish disease technical workers is carried out separately in the fishery and veterinary fields. On the other hand,

aquacultural guidance and leadership are mainly carried out in the fishery field.

Investigation and Extension—Under the present circumstances where fish disease can cause large-scale damage and is becoming more complicated to prevent, diagnose, and treat, it is becoming more important to promote research on fish disease and to give aquaculture farmers information and techniques to control this threat.

Studies on fish disease by the Fisheries Agency are mainly conducted at the National Research Institute of Aquaculture in cooperation with six of the nine regional national fisheries research institutes and the Hokkaido Salmon Hatchery. The Agency entrusts some universities with fundamental research, such as exploring the mechanisms of outbreak and infection of various fish diseases. It also entrusts prefectural fisheries experimental stations with applied research for fish disease control techniques, such as disinfection and vaccines (Fig. 8).

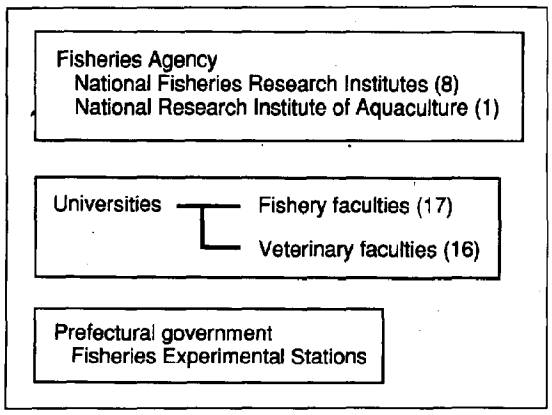

Figure 8
Major organizations involved in fish disease research in Japan.

Through its extension program, the Agency distributes textbooks about disease diagnosis and techniques of disease prevention and lends films and video tape recordings through the JFRCA to the prefectural staffs who are in charge of guiding aquaculture farmers. Prefectural governments also offer guidance courses and distribute textbooks or pamphlets on fish disease to the aquaculture farmer. These measures are used to impart knowledge on fish disease characteristics, disease prevention, aquaculture management, and use of drugs, and how to put all of this knowledge into practice appropriately (Fig. 9).

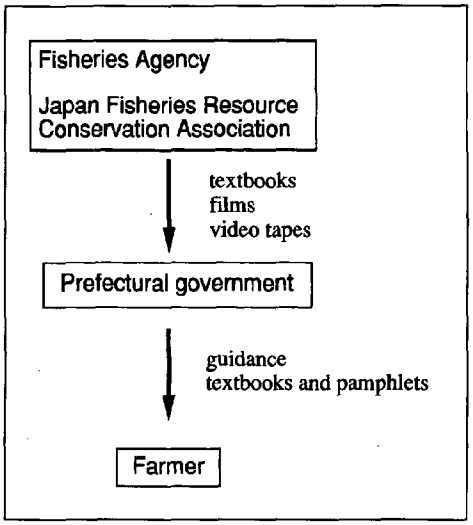

Figure 9
The Fisheries Agency's extension system for providing fish disease information in Japan.

Drugs—Appropriate aquaculture management is important to prevent or to reduce damage by fish disease. Drugs are also important to reduce damage when disease breaks out.

At present, there are 26 antibacterial medicines available for fish in Japan. In addition, there are vaccines for vibriosis, insecticides, anesthetics, and nutritive drugs. In 1988, all the fishery drugs sold in Japan amounted to 1,540 t, which was worth $55 million.

All drugs are strictly evaluated for safety, efficacy, residue accumulation in fish tissues, and other properties by the Central Pharmaceutical Affairs Council prior to receiving manufacture approval from the Ministry of Agriculture, Forestry, and Fisheries, based on the Pharmaceutical Affairs Law. Use of antibacterial drugs for fish as well as livestock is regulated by "The Standard to be Observed by User," which is based on the same law (Table 2).

Staffs of prefectural governments and fisheries cooperative association assist all aquaculture farmers to use drugs properly by visiting, distributing pamphlets, or carrying out guidance courses at regular intervals. The public health divisions of prefectural governments carry out drug residue testing by sampling cultured fish from shops. The fisheries divisions of prefectural governments and fisheries cooperative associations also examine aquaculture products submitted on a voluntarily basis for drug residues before harvest, in order to ensure that foods are safe for human consumption.

Table 2
The standard to be observed by user of drug (MAFF 1980).

Drug	Subject animal	Administration and dosage	Withdrawal period[a]
Feed additive containing alkyltrimethyl-ammoniumcalcium-oxytetracycline	Yellowtail	Administer orally, mixing not more than 50 g (potency) in 1 t of feed[b]	20 days
Feed additive containing ampicillin	Yellowtail	Administer orally, mixing not more than 20 g (potency) in 1 t of feed	5 days
Feed additive containing chlortetracycline hydrochloride	Yellowtail	Administer orally, mixing not more than 50 g (potency) in 1 t of feed	10 days
	Eel	Administer orally, mixing not more than 50 g (potency) in 1 t of feed	15 days
Feed additive containing erythromycin	Yellowtail	Administer orally, mixing not more than 50 g (potency) in 1 t of feed	30 days
Feed additive containing florphenicol	Yellowtail	Administer orally, mixing not more than 10 g in 1 t of feed	5 days
Feed additive containing kitasamaycin	Yellowtail	Administer orally, mixing not more than 80 g (potency) in 1 t of feed	20 days
Feed additive containing oxytetraccycline hydrochloride	Yellowtail	Administer orally, mixing not more than 50 g (potency) in 1 t of feed	20 days
	Red sea bream	Administer orally, mixing not more than 50 g (potency) in 1 t of feed	30 days
	Coho salmon	Administer orally, mixing not more than 50 g (potency) in 1 t of feed	30 days
	Eel	Administer orally, mixing not more than 50 g (potency) in 1 t of feed	30 days
	Rainbow trout	Administer orally, mixing not more than 50 g (potency) in 1 t of feed	30 days
Feed additive containing oxolinic acid (except liquid)	Yellowtail	Administer orally, mixing not more than 30 g in 1 t of feed	16 days
	Coho salmon	Administer orally, mixing not more than 20 g in 1 t of feed	21 days
	Eel	Administer orally, mixing not more than 20 g in 1 t of feed	20 days
	Rainbow trout	Administer orally, mixing not more than 20 g in 1 t of feed	21 days
	Ayu	Administer orally, mixing not more than 20 g in 1 t of feed	14 days
	Carp	Administer orally, mixing not more than 10 g in 1 t of feed	28 days
Feed additive containing oxolinic acid (liquid)	Yellowtail	Administer orally, mixing not more than 20 g in 1 t of feed	16 days

Table 2 (Continued)

Drug	Subject animal	Administration and dosage	Withdrawal period[a]
Bath agent containing oxolinic acid	Eel	Give bath, dissolving not more than 5 g in 1 t of water	25 days
	Ayu	Give bath, dissolving not more than 10 g in 1 t of water	14 days
Feed additive containing spiramaycin embonate	Yellowtail	Administer orally, mixing not more than 40 g (potency) in 1 t of feed	30 days
Feed additive containing sulfadimethoxine or its sodium salt	Rainbow trout	Administer orally, mixing not more than 100 g in 1 t of feed	30 days
Feed additive containing sulfamonomethoxine or its sodium salt	Yellowtail	Administer orally, mixing not more than 200 g in 1 t of feed	15 days
	Eel	Administer orally, mixing not more than 200 g in 1 t of feed	30 days
	Coho salmon	Administer orally, mixing not more than 100 g in 1 t of feed	30 days
	Ayu	Administer orally, mixing not more than 100 g in 1 t of feed	15 days
	Rainbow trout	Administer orally, mixing not more than 150 g in 1 t of feed	30 days
Bath agent containing sulfamonomethoxine or its sodium salt	Rainbow trout	Give bath, dissolving not more than 10 g in 1 liter of salt water (less than 1% concentration)	15 days
Feed additive containing sulfamonomethoxine and ormetoprim (compound drug)	Ayu	Administer orally, mixing not more than 15 g of sulfamonomethoxine and 5 g of ormetoprim in 1 t of feed	15 days
Feed additive containing thiamphenicol	Yellowtail	Administer orally, mixing not more than 50 g in 1 t of feed	15 days

[a] Withdrawal period defined as the required waiting period following drug administration prior to use of fish for human consumption.
[b] t = metric ton.

Citations

MAFF (Ministry of Agriculture, Forestry, and Fisheries)
1980. Ministerial ordinance regarding the control and use of drugs for animals. MAFF Ordinance No. 42, Sept. 30, 1980, MAFF, Government of Japan, Tokyo, Japan. (In English.)
1988. Annual statistics of fisheries and aquaculture production, MAFF, Government of Japan. (In English.)

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