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Fish diseases and various parameters associated with disease caused mortality of fish were monitored at the Oregon State University Marine Science Center and at a private mariculture facility on Yaquina Bay during a period of five years. Nearly all disease problems observed were caused by Vibrio anguillarum and Vibrio spp. Infection by Vibrio spp. resulted in substantial mortality (as high as 50% of a population) among fish which were immunized against two serotypes of V. anguillarum and among non-immunized fish. Naturally occurring levels of V. anguillarum in Yaquina Bay were determined to be ten or less viable cells per ml water. Effluent water from groups of salmonids with naturally acquired vibriosis contained 1.0 to 4.3 x 10^3 viable cells of V. anguillarum per ml.

The histopathology associated with naturally acquired and experimentally induced infections of vibriosis in chum salmon fingerlings was described for the two serotypes of V. anguillarum which

commonly cause epizootic levels of mortality among salmonids reared at mariculture facilities in the Pacific Northwest United States. Results of these studies indicate that different histopathologic changes are produced by the two serotypes of V. anguillarum. One serotype (referred to as V. anguillarum serotype I) produced a bacteremia in early stages of disease with the following organs and tissues being the main targets: blood, loose connective tissue, kidney, spleen, posterior gastrointestinal tract, and gills. The second serotype (referred to as V. anguillarum serotype II) produced a bacteremia in late stages of disease with the following organs and tissues being main targets: skeletal muscle, cardiac muscle, anterior gastrointestinal tract, posterior gastrointestinal tract, and gills. Vibrio anguillarum serotype I cells were evenly dispersed throughout infected fish tissues while V. anguillarum serotype II formed distinct colonies in tissues of fish.

Experimentally induced infections of chum, coho, and chinook salmon were studied to compare the histopathologic changes associated with infections of V. anguillarum serotypes I and II and to obtain quantitative data concerning some specific effects produced in fish infected with these organisms. Differences in histopathology noted above were observed in all three species of fish when infections of the two serotypes of V. anguillarum were compared. Cellular responses were rarely observed during early or late stages of vibriosis.

The data suggest that both serotypes of V. anguillarum used in these studies produce a leukocidin in fish because infected fish had 80% to 95% less leukocytes than non-infected control fish. Extremely high levels of V. anguillarum were shown to be present in fish tissues. Pathology observed in the mucosa of the gastrointenstinal tract of infected fish was apparently related to pH. The anterior gastrointestinal tract was strongly acidic and contained no necrosis of the mucosa while the posterior gastrointestinal tract was not acidic and contained massive necrosis and sloughing of epithelial cells in the mucosa.

Experimentally induced infections of vibriosis with water born exposure of fish to live bacteria were used to study the progress of disease. Both serotypes of V. anguillarum used in these studies were shown to enter fish by penetrating the descending intestine and rectum. Penetration of the skin is a second means by which V. anguillarum serotype II enters fish. Moribund fish in all studies suffered from hypoxia, possible accumulation of toxins (although not highly potent), loss of fluids in the posterior gastrointestinal tract, and dysfunction of various organs. Death of fish was apparently due to a combination of these ill effects.

Bacteriologic, Immunologic and Pathologic Studies of *Vibrio spp.* Pathogenic to Salmonids

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INTRODUCTION

Vibriosis is considered to be one of the most serious diseases affecting salmonids in the Pacific Northwest as well as in other areas of the world. Vibrio anguillarum, the causative agent of vibriosis, can also infect many other species of fish (Wood, 1968; Anderson and Conroy, 1970). Antibiotics, antimicrobial agents, and active immunity through immunization are used to keep mortality at a minimum among cultured fish exposed to V. anguillarum. Protection from V. anguillarum through use of vaccine, which is commercially available in the United States, can be greatly reduced by stressful rearing conditions or exposure to pathogenic organisms which are not homologous to the organisms used in producing the vaccine. Mortality from vibriosis has been reported to reach 90% among non-vaccinated salmonids during three months of brackish water rearing (Cisar and Fryer, 1972) while losses of 50% have been reported during a period of four days (Evelyn, 1971). Protection of fish from vibriosis through immunization has resulted in significant decreases in mortality when fish were not exposed to heterologous strains of pathogenic vibrios or stressful rearing conditions (Nelson, 1972; Rohovec, 1974; Gould, 1977; Johnson, personal communication; Tebbit, personal

communication).

Various strains of V. anguillarum have been studied in respect to serology, DNA homology, or biochemical characteristics (Nybelin, 1935; Smith, 1961; Kiehn and Pacha, 1969; Pacha and Kiehn, 1969; Evelyn, 1971; Egidius and Anderson, 1976; Johnson, 1976; Schiewe, 1976; Gould, 1977; and others). However, definitive data concerning the best means of typing V. anguillarum isolates has not been reported. Serology has been most frequently used for typing V. anguillarum isolates in this laboratory because accurate results can be quickly obtained and large numbers of isolates can be tested if ample quantities of antisera are available. Two distinct serotypes of V. anguillarum commonly cause epizootic levels of mortality among non-immunized salmonids reared at mariculture facilities in the Pacific Northwest. Vibrio anguillarum isolates LS 1-74, MSC 1-73, 78 SKID, and 775 are examples of one serotype that some investigators refer to as the "fast grower" and will be referred to as V. anguillarum serotype I in this thesis. Vibrio anguillarum isolates MSC 2-75 and MAN 1669 are examples of the second serotype that some investigators refer to as the "slow grower" and will be referred to as V. anguillarum serotype II in this thesis.

The present investigations were undertaken with the following objectives: (1) to monitor disease problems among fish held in salt water at the OSU Marine Science Center and at Oregon Aqua-Foods,

Inc.; (2) to determine quantitative estimations of V. anguillarum in untreated Yaquina Bay water; (3) to test the use of booster immunization using V. anguillarum bacterin administered orally to coho salmon in untreated Yaquina Bay water; (4) to compare results of biochemical and immunodiffusion tests with V. anguillarum isolates and an unknown species of vibrio which is pathogenic to salmonids; (5) to compare the histopathologic changes in salmonids with naturally acquired and experimentally induced infections of vibriosis produced by the two serotypes of V. anguillarum commonly recovered from diseased fish in the Pacific Northwest United States; and (6) to determine where V. anguillarum enters fish at the onset of infection.

LITERATURE REVIEW

Pacha and Kiehn (1969) studied cultural and serological relationships among marine vibrios pathogenic to fish and compared them with three known isolates of Vibrio anguillarum. The strains of vibrio had been isolated from Pacific herring and salmonids in the Pacific Northwest and were compared to strains of V. anguillarum which had been isolated from cod and finnock in Europe. These investigators found that the majority of the vibrios were similar morphologically and physiologically to the strains of V. anguillarum but that they differed antigenically. The vibrios from the Pacific Northwest and the isolates of V. anguillarum were placed into three serologically distinct groups. Vibrios isolated from salmonids in the Pacific Northwest were referred to as serotype 1. Vibrios isolated from fish in Europe were referred to as serotype 2. Vibrios isolated from Pacific herring were referred to as serotype 3. The three serologically distinct groups were differentiated on the basis of heat stable antigens using slide agglutination procedures with specific rabbit antisera.

Kiehn and Pacha (1969) studied the relationship among strains of pathogenic marine vibrios according to DNA homology and base composition. The strains of vibrio used in their previous study (Pacha andKiehn, 1969) were compared. These investigators

reported that the three groups of vibrio which were differentiated according to heat stable antigens could also be differentiated through DNA homology and base composition analyses. They felt that strong correlations existed between phenotypic and genotypic analysis of the vibrios and that serological, cultural, physiological and DNA nucleotide sequence comparisons were useful in differentiating the organisms.

Evelyn (1969) described an archetype of V. anguillarum based on physiological characteristics and the criteria required for identification of the genus Vibrio. Results from other investigations with 20 isolates of V. anguillarum were reviewed and compared for a description of the emerging archetype which hypothetically possesses features characteristic of a majority of the isolates. Evelyn indicated that the system of typing V. anguillarum into types A, B, and C according to biochemical reactions (Nybelin, 1935; Smith, 1961) should be discontinued and that a large number of features should be used to type isolates of V. anguillarum. Evelyn felt that many isolates of V. anguillarum should be considered variants of this species and that pathogenicity might not be an important criterion in determining species.

Funabashi, Miyazaki, Odera, and Kubota (1973) conducted histopathological studies of moribund and dead ayu (Plecoglossus altivelis) which had suffered naturally acquired or experimentally induced

infections of V. anguillarum. Epidermal application and intramuscular injection of bacteria were used as means of experimentally inducing vibriosis in the ayu. Infections acquired naturally and by epidermal application were essentially the same. Gross signs of disease included skin discoloration, external lesions, dark swollen spleens, and kidneys described as fragile. Stomachs and intestines of infected fish were filled with fluid but neither petechiae nor large hemorrhagic areas were observed internally. Histopathologic changes associated with external lesions included the following: proliferation and swelling of slime cells, vacuolar degeneration of epithelial cells, hemorrhaging and edema of the corium, breakdown of collegen fibers and edema in areolar tissue, and hyaloid degeneration of underlying muscle. Colonies of bacteria were observed in the corium while many individual bacteria were present around dilated vascular walls in the surrounding stroma, and into the sarcolemma. No marked cases of phagocytosis or cellular envolvement were observed in or around lesions of the skin. Internal histopathologic changes in ayu with advanced infections of vibriosis were most severe in the kidney and spleen but were also present in the liver, heart, connective tissue of the gastrointenstinal tract, and around blood vessels of gill lamellae. The only cellular response observed internally was occasional phagocytosis in the kidney. Fish with mild infections had external lesions with no reported internal histopathologic changes.

These authors concluded the V. anguillarum enters ayu via an injured area of the skin and causes death after a bacteremia is produced.

Harbell (1976) examined moribund coho salmon (Oncorhynchus kisutch) for histological changes associated with an experimentally induced infection of V. anguillarum. A water-born exposure of approximately 0.25-2.2 x 10^8 bacteria per ml was used to infect fish (average of 85 g each) held in 10‰ saltwater (freshwater plus NaCl) for 30 minutes. These fish were then held in freshwater (15 C) until moribund. Samples of selected organs were fixed in Bouins solution and stained with May-Grunwald Giemsa stain. Gross symptoms of the disease included the following: enlarged spleen, excess fluid in the gut, and hemorrhages in the heart, liver, skin, gut, and striated muscle. Focal necrosis was reported in the spleen, kidney, and liver. Hyperplasia and degenerative cytoplasmic alterations were observed in the gills. The only histopathologic change noted in the gut was sloughing of the mucous layer.

Levin, Wolke, and Cabelli (1972) conducted histopathological examinations of winter flounder (Pseudopleuronectes americanus) infected with V. anguillarum. Major microscopic changes noted in fish used in this study were in the kidney and areas surrounding dermal lesions. Necrosis of renal tubules was indicated by pyknosis, increased cytoplasmic eosinophilia and sloughing of renal epithelium, and the presence of amorphous acidophilic debris in the lumen of

many tubules. Kidneys of fish contained excess amounts of Prussian Blue positive pigment which was identified as hemosiderin. Infected fish contained a greater number of stem hematopoietic cells than controls. Focal areas of coagulation necrosis were present in renal interstitial hematopoietic tissue. Necrosis of the dorsal fin was frequently observed with adjacent dermal ulceration. Zenkers degeneration (acute myolosis) was observed in striated muscle beneath areas of dermal necrosis. These investigators noted that winter flounder infected with V. anguillarum were anemic as indicated by increased renal hematopoiesis and low hematocrit values. Inflammatory cells were reported to be few in number and solely lymphocytic.

Miyazaki and Kubota (1977) conducted histopathological studies on rainbow trout (Salmo gairdneri) and amago salmon (O. roduras) infected with Vibrio sp. The most common sign of disease in these fish was the presence of external lesions described as boils or erosive lesions. Bacterial colonies were reported in skin, muscle, liver, kidney, heart, and spleen. Hemorrhages were described as slight but infiltration of inflammatory cells was observed in muscle. Dermal necrosis with epithelial cell sloughing and edema was observed in the skin. These authors did not mention pathology in the gastrointestinal tract or observation of bacteria in the blood of fish in their study.

Miyazaki et al. (1977) studied the histopathology associated with naturally induced infections of vibriosis in the Japanese eel (Anguilla japonica). The main lesion observed in the eel was in the skin and surrounding musculature. Edema, hemorrhage, and epithelial cell sloughing was present in the skin. Only advanced cases were described as systemic diseases. These authors believed that an external route of entry through the skin allowed onset of vibriosis in eels. Necrosis was noted in the spleen, liver, heart, gills, kidney, and intestine of fish with systemic infections. Bacteria were evenly dispersed throughout infected tissues and not aggregated in colonies.

Murchelano and Ziskowski (1977) reported the results of a histopathologic study of acute fin lesions from two summer flounder (Paralichthys dentatus). Loss of epidermal, dermal, and fin ray tissue was characteristic of early lesions while these tissues were totally absent in advanced lesions of the caudal fin. Aggregates of gram negative rod-shaped bacteria were present on the surface of lesions, between muscle fibers, in the heart, and in sinusoids of the liver. Germ negative coccobacilli were present within the dermis. The genus or species of bacteria infecting the fish in this study was not reported. Inflammatory responses observed in these fish included increased numbers of macrophages and lymphocytes in lesions. No histopathologic changes were observed in the kidney, intestine, spleen, heart, or gills.

MATERIALS AND METHODS

Examination of Diseased Fish in Yaquina Bay

Sources of Diseased Fish

Fish from two locations in Yaquina Bay were examined for pathogens from January 1, 1973 through December 31, 1977. These two locations were the Oregon State University Marine Science Center and the Oregon Aqua-Foods Inc. saltwater fish rearing facility. Both locations are approximately 2.5 km from the mouth of Yaquina Bay which is an estuary approximately eight km long.

Diseased fish from a variety of experiments at the OSU Marine Science Center were examined for fish pathogens. These fish were from many different studies conducted by members of the departments of Zoology, Microbiology, and Fisheries and Wildlife. Diseased specimens of the following species were examined: chum salmon (Oncorhynchus keta), pink salmon (O. gorbuscha), English sole (Parophrys vetulus), dover sole (Microstomas pacificus), and starry flounder (Platichthys stellatus). All fish in these studies were juvenile.

The most extensive disease study with fish at the Marine Science Center was conducted with coho salmon (O. kisutch) from oral immunization experiments conducted by the author. These experiments

were designed to test efficacy of bacterins administered to coho salmon which were subsequently exposed to natural levels of V. anguillarum in Yaquina Bay. The oral immunization experiments were carried out to 1) test the concept of oral immunization for protection of salmonids from vibriosis in an area suitable for a mariculture facility, and 2) test the value of oral booster immunization in salt water.

Vibrio anguillarum serotype I (isolate MSC 1-73) antigen used in the oral immunization experiments was prepared, incorporated into Oregon Moist Pellet (OMP) (Hublou, 1963) diet, and administered to fish according to the methods of Rohovec (1974). Vibrio anguillarum serotype II (isolate MSC 2-75) antigen was prepared according to the methods of Rohovec with the exceptions that BHI broth was used in place of tryptone sucrose broth and the cultures were incubated for 19 h instead of 24 h. Incubation was terminated at 19 h because the pH of the medium had declined to 6.85 and previous work with V. anguillarum serotype II indicated this organism formed spheroplasts at pH 6.6. Vibrio anguillarum serotype II antigen was incorporated into OMP diet and administered to fish according to the methods of Rohovec (1974). The immunization procedure consisted of feeding vaccine diet containing five mg (wet weight) formalin killed V. anguillarum cells per g OMP to fish on a daily basis for two weeks. Oral immunization studies conducted during the summers

conducted during the summers of 1974 and 1975 were designed to test the value of oral booster immunization of coho salmon smolts in Yaquina Bay using V. anguillarum serotype I antigen. Schedules for administering oral immunization boosters in salt water are presented in Table 1. Diseased fish were removed from fish tanks every 24 h and kidney tissue streaked onto BHI agar for the isolation of V. anguillarum. An oral immunization study conducted in 1976 was designed to test the value of oral booster immunization of coho salmon smolts in Yaquina Bay using V. anguillarum serotypes I and II antigens. The experiment in 1976 was also designed to test for cross protection by the two serotypes using oral immunization. Groups of fish were given V. anguillarum serotype I antigen according to Table 1. Other fish were given serotype II antigen according to Table 1. Groups of fish were given a diet containing both antigens using the same schedule. The leading edge of external lesions in many fish from the 1976 study in addition to kidney tissues was streaked onto BHI agar for the isolation of pathogenic vibriosis.

Diseased fish from the Oregon Aqua-Foods Inc. saltwater rearing facility in Yaquina Bay were examined for pathogens whenever significant mortality occurred (greater than 0.1% per week). Although Oregon Aqua-Foods Inc. has converted entirely to ocean ranching, both intensive and extensive rearing of fish occurred during this study. Coho salmon, chinook salmon (O. tshawytscha) and rainbow trout (S. gairdneri) were raised in cement raceways

TABLE 1. Immunization Schedules used for Testing Orally Administered Vibrio anguillarum Vaccine Boosters with Coho Salmon (O. kisutch) in 1974, 1975, and 1976.

Group	Freshwater Immunization	Saltwater Immunization
1	Vaccine diet fed daily for Two Weeks	None
2	Vaccine diet fed daily for Two Weeks	Vaccine diet fed daily One Day/Week
3	Vaccine diet fed daily for Two Weeks	Vaccine diet fed daily Two Days/Week
4	Vaccine diet fed daily for Two Weeks	Vaccine diet fed daily One Day/Two Weeks
5	Vaccine diet fed daily for Two Weeks	Vaccine diet fed daily Two Days/Two Weeks
6	Vaccine diet fed daily for Three Weeks	Vaccine diet fed daily Seven Days/Week
7	No Vaccine diet fed (controls)	No Vaccine diet fed

(approximately 20 m by 100 m) supplied with untreated Yaquina Bay water. The intake for this facility is approximately six m from the bottom of the main channel of the estuary. Many fish were immunized against V. anguillarum using one of the following methods: 1) intraperitoneal injection of bacteria suspended in Freund's complete adjuvent, 2) oral administration of bacteria in food, and 3) hyperosmotic dip using VAB-2 Vibrio vaccine commercially produced by Wildlife Vaccines Inc. All fish were fed moist pellets made by Oregon Aqua-Foods Inc.

Examination of Animals

Diseased fish were examined using standard methods for the detection of bacterial fish pathogens. These methods are described in the Canadian Fish Health Protection Regulations Manual of Compliance and in the American Fisheries Society Fish Health Section Suggested Procedures for the Detection and Identification of Certain Infectious Diseases of Fishes. No signs of mortality caused by viral agents were observed and all salmonids were from stocks certified to be free of infectious pancreatic necrosis virus and infectious hematopoietic necrosis virus; therefore, methods for virus isolation were not used. Kidney tissue and tissue beneath the skin near leading edges of external lesions on fish were streaked onto BHI agar and incubated at 18 C or room temperature (20-22 C). Bacteria isolated from

diseased fish in salt water were determined to be the genus Vibrio if they were motile, Gram negative rods which were inhibited by novobiocin and the vibriostat 0/129 and produced cytochrome oxidase. Identification of V. anguillarum was based on rapid slide agglutination tests using rabbit antiserum specific for V. anguillarum serotype I (isolate LS 1-74) and rabbit antiserum specific for V. anguillarum serotype II (isolate MSC 2-75). Four to five mg (wet weight) of live V. anguillarum cells suspended in 0.5 ml Freund's complete adjuvant was administered into the foot pads of New Zealand white rabbits for production of antisera. Antiserum was harvested three weeks after injection.

Biochemical and Immunodiffusion Tests

Four vibrio isolates were compared using biochemical and immunodiffusion tests. An isolate of V. anguillarum serotype I designated MSC 1-73 was recovered from the kidney of a diseased, juvenile chum salmon at the Marine Science Center in 1973. This isolate was positive when tested by the rapid slide agglutination test using rabbit antiserum specific for V. anguillarum serotype I (isolate LS 1-74). An isolate of V. anguillarum serotype II designated MSC 2-75 was also used. This isolate was recovered from the kidney of a diseased coho salmon smolt that had been orally immunized against V. anguillarum serotype I and was held at the Marine Science Center in 1975.

The MSC 2-75 isolate agglutinated rapidly when tested by the rapid slide agglutination test using rabbit antiserum specific for V. anguillarum serotype II (isolate MAN 1669). An unknown species of vibrio, designated Vibrio sp. MSC 1-76 had been recovered from the kidney of a diseased coho salmon smolt at the Marine Science Center in 1976. An organism thought to be a third serotype of V. anguillarum was also employed in these studies. This organism was designated V. anguillarum 507 and was isolated from a diseased coho salmon by Sawyer of the University of Maine. Biochemical tests were performed using API-20E[1] test systems and recommended procedures designed for analytical profiles of the Entero-bacteriaceae and other gram negative bacteria. All tests were repeated three times.

Antigen preparations for immunodiffusion tests were made by sonicating washed whole bacteria which had been resuspended in phosphate buffered saline (pH = 7.2) and adjusted to an optical density of 1.8 at 525 nm. Cell debris were removed from sonicated preparations by centrifugation for ten minutes at 3090 x G. Antigen and specific rabbit antisera were placed in wells of double-diffusion plates containing 20 ml 1% agarose (pH = 7.2-7.4) and allowed to diffuse for 24-48 h at room temperature. Immunodiffusion reactions were read using a light box equipped with a fluorescent light.

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[1] Analytab Products Inc., Plainview, N. Y.

Quantitative Estimation of Vibrio Anguillarum in Yaquina Bay Water

Throughout these studies, attempts were made to determine the number of V. anguillarum cells per ml of salt water under natural conditions. Five media were tested using the standard plate count technique. These included: BHI agar (DIFCO), BHI agar with sodium chloride added to a final concentration of 2.5%, Bacto TCBS Agar (DIFCO), Vibrio Agar (NISSUI)[2] and TCBS Agar (EIKEN).[3] The three latter media are differential and selective for V. cholera and V. parahaemolyticus but other species of vibrio are able to grow on them. Known broth cultures of V. anguillarum were tested on each medium before attempts were made to quantify levels of V. anguillarum in Yaquina Bay water. Bacterial counts of intake and effluent water from tanks of experimental fish and from Oregon Aqua-Foods Inc. were estimated during vibriosis outbreaks in order to determine the effect that moribund fish and dead fish have on the number of V. anguillarum present in salt water. Water intakes for both facilities are located approximately six m from the bottom of the main channel of Yaquina Bay. Suspect vibrio colonies were removed from the media with a sterile loop and tested serologically using rapid slide agglutination with specific antisera.

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[2]Eiken Chemical Co., Ltd., Tokyo, Japan.

[3]Tanabe Seiyaku Co., Ltd., Osaka, Japan.

Histopathology Study of Naturally Acquired Vibriosis in Chum Salmon Fingerlings

Specimens for this study were selected from 2800 non-immunized chum salmon held at the OSU Marine Science Center from May 15, 1976 to June 29, 1976. These fish were exposed to untreated salt water pumped from Yaquina Bay and experienced 100% mortality between May 27 and June 29. Although all fish were not examined for bacterial pathogens, it was assumed that vibriosis was responsible for all the mortality. This assumption was based on positive identification of V. anguillarum serotypes I and II using rapid slide agglutination tests with bacterial isolates recovered from peritoneal fluid and kidney tissue of 1420 fish. Non-infected control fish used in this study were held in disease-free water at the OSU Fish Disease Laboratory in Corvallis, Oregon.

Peritoneal fluid and kidney tissue from moribund fish weighing one and a half to two g each were streaked onto BHI agar using micro-dissecting instruments and aseptic technique. These fish and non-infected control fish were fixed in Bouins solution for approximately one week. Non-infected control fish and moribund fish from which a pure culture of V. anguillarum serotype I or serotype II was recovered were decalcified for 48 h in 70% ethanol with 3% hydrochloric acid, dehydrated and cleared in an ethanol-toluene series, and embedded in paraffin.

Fish embedded in paraffin were sectioned with a rotary microtome. Transverse sections 5 μm thick were made from the entire bodies of three fish infected with V. anguillarum serotype I, three fish infected with V. anguillarum serotype II, and three non-infected control fish. Every tenth section was mounted on slides, stained with either Delafield's hematoxylin and eosin Y or Giemsa's stain and examined microscopically. Photographs were made with a Carl Zeiss C35 camera and model 14 microscope using Kodak Ektachrome and Plus-X Pan films.

Histopathology Study of Experimentally Induced Vibriosis in Chum Salmon Fingerlings

The pathology observed in chum salmon with naturally acquired infections of V. anguillarum serotype I was very different from the pathology observed in chum salmon with naturally acquired infections of V. anguillarum serotype II. Differences in pathology were not expected; therefore a study was carried out to confirm these differences and to determine if the same differences would occur with experimentally induced infections of vibriosis in chum salmon fingerlings.

Chum salmon used in this study were the same size (approximately one and a half to two g) and stock (Netarts Bay) as the fish examined for histopathogic changes associated with naturally acquired

infections of vibriosis. Fish were held in 144 l fiberglass tanks supplied with air and Yaquina Bay water that had been filtered through number eight mesh sand and sterilized with an Aquanomics[4] ultraviolet light sterilizer.

Experimentally induced infections were achieved through waterborne exposure of fish to live bacteria. The water temperature was raised from 10 C to 14.5 C by stopping the water supply for 24 h and increasing the laboratory air temperature to 30 C. Optical density of BHI broth cultures was measured at 525 nm to estimate the number of bacteria in the cultures. These estimations were based on data collected by Gould (1977). Five hundred fifty ml of 0.85 optical density culture of V. anguillarum serotype I was added to a tank containing 40 fish. Thirty-seven hundred ml of 0.40 optical density culture of V. anguillarum serotype II was added to a second tank containing 40 fish. Cultures of both serotypes had been incubated at 14 C for 24 h. The contents of each tank were mixed and samples removed for quantitative estimates of bacteria by the standard plate count method (Rand, Greenberg and Taras, 1976) using BHI agar. Bacterial counts were made every 24 h to determine the number of bacteria shed into the water from fish. Fish in one tank were initially exposed to an estimated 7.0×10^6 cells for V. anguillarum serotype I

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[4]Aquanomics, Inc., Santa Fe Springs, Cal. 90670.

per ml for 1.5 h. Fish in the second tank were initially exposed to an estimated 2.6×10^7 cells from V. anguillarum serotype II per ml for 1.5 h.

Fish were removed from tanks, examined for bacteria by streaking kidney tissue on BHI agar and identifying cultures, and fixed in Bouins solution before and after signs of disease were apparent. This was done to determine the initial and final sites of infection. Three fish were sampled from each tank 4 h, 48 h, and 72 h after exposure to bacteria. Ten moribund fish, three dead fish, and three fish with no signs of disease were also sampled from each tank at the onset of mortality. All animals sampled were prepared for sectioning in the same manner that was described for chum salmon with naturally acquired infections of vibriosis. Selected areas of embedded fish were sectioned rather than serially sectioning the entire bodies of fish. These selected areas included the major tissues and organs where pathology was noted in chum salmon with naturally induced infections of vibriosis. Stained sections were examined and photographed as previously described. Five non-infected control fish were removed from salt water before bacteria were added and processed in the same manner as infected fish.

Studies with Experimentally Induced Vibriosis in Coho Salmon

A study was carried out with coho salmon with experimentally induced infections of vibriosis using the same methods described for experimentally induced infections of vibriosis in chum salmon. The two objectives of this experiment were: 1) to examine coho salmon salmon for histopathology associated with vibriosis, and 2) to determine the level of bacteria in blood of fish infected with V. anguillarum serotype I and compare it with the level of bacteria in blood of fish infected with V. anguillarum serotype II.

Three fish exposed to V. anguillarum serotype I and three fish exposed to V. anguillarum serotype II were removed from tanks before and every eight h after initial exposure to bacteria. Fish were anesthetized with ethyl-m-aminobenzoate, their gills rinsed with sterile distilled water, and 0.2 to 0.3 ml blood removed from the duct of Cuvier with a sterile, heparinized one ml tuberculin syringe and sterile, heaprinized 26G needle. Blood was then diluted 1:100 in sterile, phosphate buffered saline (0.85% NaCl: pH = 7.2) and serial ten fold dilutions made for quantitative estimation of bacteria using the standard plate count technique. Three moribund fish from each tank were examined for bacteria, by streaking kidney tissue on BHI agar and identifying cultures, placed in Bouins solution, and processed for microscopic examination. Since the coho salmon used in this study weighed approximately 25 g each, only selected

areas of each fish were sectioned for examination.

Studies with Experimentally Induced Vibriosis in Chinook Salmon

A study similar to the previously described study with experimentally induced vibriosis in coho salmon was performed using chinook salmon (O. tshawytscha). In this experiment, the white blood cell counts of infected and non-infected fish were determined in addition to bacterial levels in blood, pH of the lumen of the gastrointestinal tract of fish, and histopathologic changes associated with vibriosis.

The sample used in enumeration of white blood cells was a stained aliquot of blood diluted in phosphate buffered saline which was used for bacterial enumeration as described in the experiment with coho salmon. A 0.5 ml aliquot of 1:100 diluted blood was added to 0.5 ml crystal violet staining solution. The staining solution was made with 5% crystal violet in 95% ethanol and then diluted 1:20 with phosphate buffered saline. A drop of stained, diluted blood was placed in a hemacytometer and the white blood cells counted.

The gastrointestinal tracts of fish were excised and a micro-pH electrode was inserted through the lumen beginning at the esophagus and ending at the posterior rectum. Values for pH of the esophagus, stomach, ascending and descending intestines and the rectum were

recorded. This was done to gather more data in support of the hypothesis that the histopathologic changes observed in the gastrointestinal tract of salmonids infected with vibriosis is related to pH. Tests were also run to determine if V. anguillarum serotypes I and II would grow on BHI agar adjusted to the following values of pH: 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5.

The sampling schedule for this experiment differed from the schedule followed in the experiment with coho salmon. Moribund fish and fish with no signs of disease were sampled on the first day that external signs of infection were evident. This was done because gradual changes in the blood and pH of the gastrointestinal tract were not noted in the previous experiment with coho salmon. Therefore, it was possible to sample moribund and non-moribund fish in the same day.

RESULTS

Examination of Diseased Fish

Nearly all bacteria isolated from diseased fish in these studies were members of the genus Vibrio when tested using criteria described in the materials and methods. The following isolates occurred in decreasing order of frequency: V. anguillarum serotype I, V. anguillarum serotype II, Vibrio spp. from fish with external lesions, and Vibrio spp. from fish with no external lesions or hemorrhages.

Chum and pink salmon were more susceptible to vibriosis than any other specis of fish in these studies. Total mortality reached 95% to 100% in many groups of chum and pink salmon. In June 1976, 1568 moribund or dead chum salmon fingerlings from a vibriosis epizootic were examined to determine the relative mortality caused by serotypes I and II of V. anguillarum. The following data were collected:

	Number of Fish	Mortality (%)
Pure culture of V. anguillarum serotype I	864	55
Pure culture of V. anguillarum serotype II	421	27
Mixed cultures of V. anguillarum serotypes I and II	135	9
No growth on medium	148	9
Total	1568	100

This example is presented because it indicates typical results observed during severe vibriosis epizootics of chum and pink salmon.

While V. anguillarum serotypes I and II were most frequently isolated from chum and pink salmon, mortality from Vibrio spp. associated with severe necrosis of skin and the caudal fin (Figures 1 and 2) occurred every year. Mortalities as high as 33% occurred in groups of fish as large as 3,000 fish per group. Mortality from the disease usually followed a normal bell shaped curve during a two to three week period. Disease problems did not appear to be chronic nor associated with a specific type of fish tank. Pure cultures of Vibrio spp. were isolated from kidneys and from muscle below skin near leading edges of external lesions. Pink salmon suffering from this disease in 1977 were treated successfully with sodium sulfamethazine added to the water. Isolations of V. anguillarum were not common during these epizootics. Myxobacteria were not observed in nor isolated from external lestions of these or other fish studied.

Bacterial isolations from kidney tissue of English sole and starry flounder included V. anguillarum serotype I and Vibrio spp. The main disease problem of these two species of fish was infection with Vibrio spp. which caused severe skin, fin, and muscle necrosis (Figures 3 and 4). Prophylactic and therapeutic treatment with sulfamethazine and furanace resulted in only limited success.

No bacteria isolated from diseased dover sole agglutinated with

Figure 1. Moribund pink salmon (O. gorbuscha) suffering from an infection by Vibrio sp. which caused complete necrosis of the caudal fin.

Figure 2. Dead chum salmon (O. keta) infected with Vibrio sp. which caused complete necrosis of the caudal fin.

Figure 3. Juvenile English sole (Parophrys vetulus) with necrosis (N) of fins during early stages of infection by Vibrio sp.

Figure 4. Necrotic (N) tissue adjacent to dorsal fin of juvenile English sole (Parophrys vetulus) infected with Vibrio sp.

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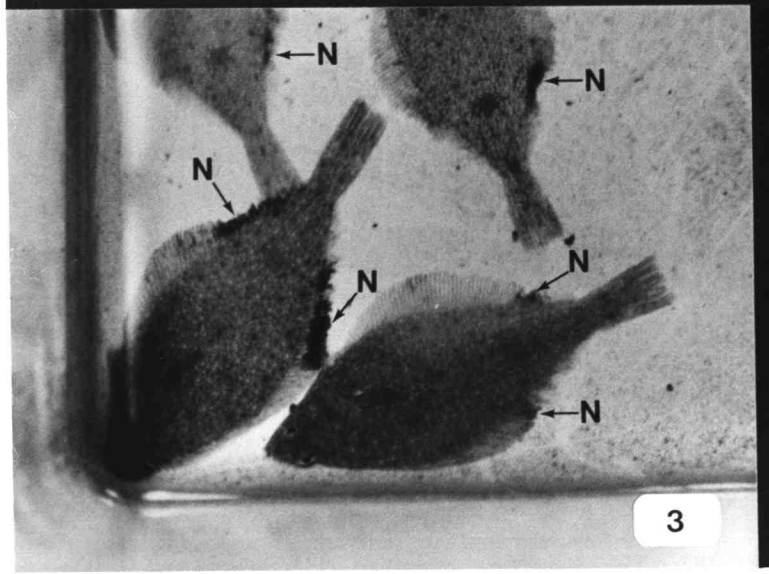

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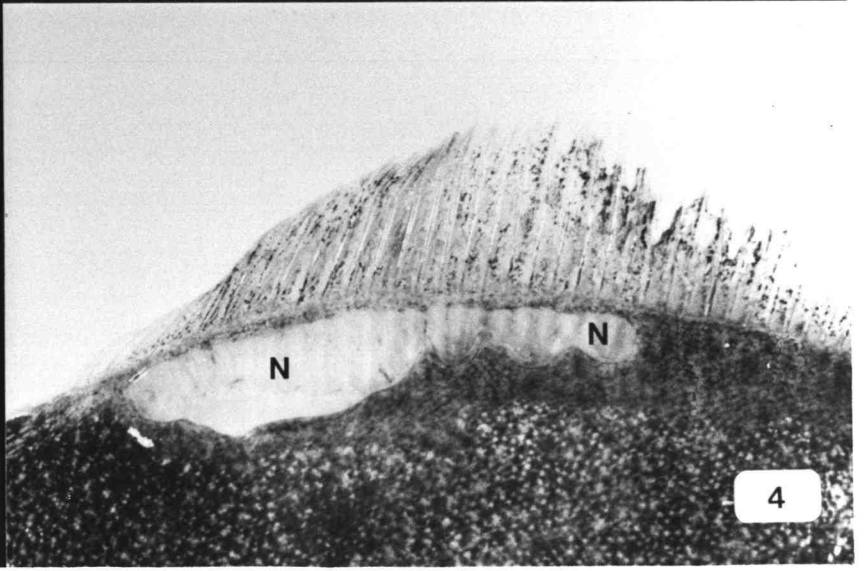

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rabbit antisera specific for V. anguillarum. However a Vibrio sp. was isolated from three dover sole with severe skin and muscle necrosis near the dorsal fin. It was not possible to assess the significance of these isolations because very few dover sole were held at the Marine Science Center.

It is possible that English sole, dover sole, and starry flounder held at the Marine Science Center suffered a high rate of infection by Vibrio spp. because rough surfaces of fiberglass tanks were more abrasive than natural habitats. However, external lesions did not occur on the ventral surface of these fish where abrasions should have been most numerous as they are flat fish.

No bacteria isolated from immunized or non-immunized coho salmon in 1974 agglutinated with rabbit antiserum specific for V. anguillarum serotype I or with rabbit antiserum specific for V. anguillarum serotype II. Rapid slide agglutination tests were performed on bacteria isolated from 556 diseased coho salmon. Known cultures of V. anguillarum serotypes I and II were positive controls for rapid slide agglutination tests. Fourteen of the 556 isolates were not inhibited by novobiocin and 0/129 while 542 isolates were determined to belong to the genus Vibrio. No bacteria were recovered from kidney tissue of 677 diseased coho salmon in this study. Daily mortality never exceeded 5% in any group of fish but total mortality reached 40% to 75% in every group of fish.

Vibrio anguillarum serotypes I and II were recovered from diseased coho salmon in the 1975 immunization study. Hemorrhages in skeletal muscle typical of V. anguillarum serotype I and areas of raised scales typical of infections of V. anguillarum serotype II were observed in these fish (Figures 5 and 6). Both organisms were responsible for epizootic levels of mortality (as high as 82%). Fish given daily booster immunizations against V. anguillarum serotype I were not protected against V. anguillarum serotype II but did possess increased protection against the homologous serotype when compared to fish with no booster immunizations. Isolation of Vibrio spp. ranged from three to ten percent among groups of fish. No bacteria were recovered from kidney tissue of 15% to 40% of the fish in different groups.

In contrast to the studies with immunized coho salmon in 1974 and 1975, immunized coho salmon were taken to salt water in late summer and held into winter in 1976 rather than being held in salt water during June, July and August. As a result, these fish were subjected to salinity fluctuations caused by runoff from winter storms but were not stressed by temperature changes which commonly occur in Yaquina Bay during summer months. Vibrio anguillarum serotypes I and II were isolated from coho salmon less frequently during 1976 than during 1975. Mortality resulting from infection of V. anguillarum serotypes I and II was less than 25% for most groups of coho

salmon in the 1976 study. The major disease problems among fish in this study were caused by Vibrio spp. Vibrio spp. were isolated from 46% of the diseased coho salmon from 17 groups of fish. The number of different Vibrio spp. isolated from coho salmon in 1976 was not determined, but it was concluded that one of the isolates was responsible for a majority of the mortality. This was determined by selecting six Vibrio sp. isolates, based on colony morphology on BHI agar, preparing rabbit antisera specific for each isolate, and performing rapid slide agglutination tests with cultures which had been maintained on sea water cytophaga agar deeps. In every case when this particular isolate (designated Vibrio sp. MSC 1-76) was recovered from a fish, severe skin necrosis had occurred in the fish (Figures 7 and 8). Most isolations of Vibrio sp. MSC 1-76 from kidney tissue and tissues beneath the skin near leading edges of external lesions were pure cultures. Occasionally pure cultures of Vibrio sp. MSC 1-76 were recovered directly from the surface of lesions. Signs of disease produced by Vibrio sp. MSC 1-76 progressed rapidly with death usually occurring two to three days after the appearance of external lesions. Transmission of the disease among fish within a group was suggested by normal bell shaped mortality curves.

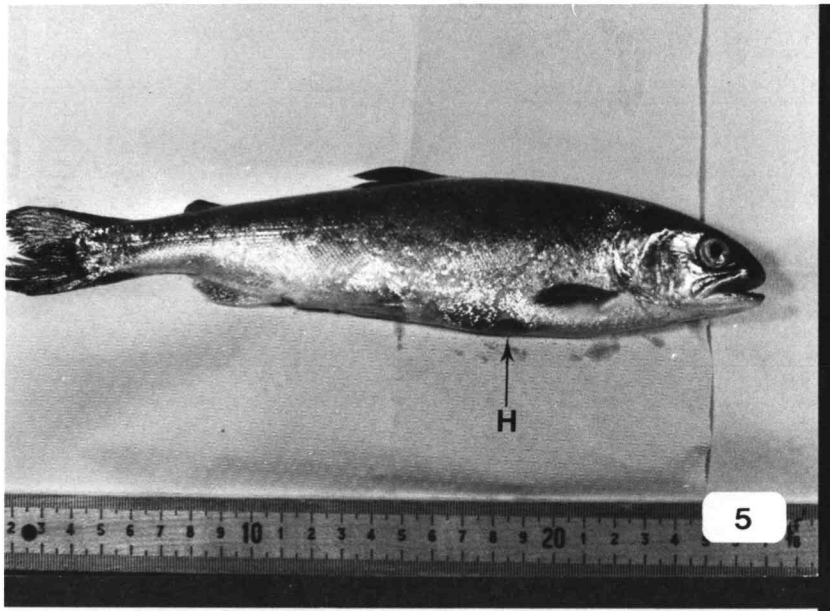
Vibrio anguillarum serotypes I and II were isolated from diseased coho and chinook salmon, and rainbow trout at the Oregon Aqua-Foods Inc. saltwater fish rearing facility. Chinook salmon

Figure 5. Sacrificed juvenile coho salmon (O. kisutch) with hemorrhagic (H) abdominal muscle produced by infection with V. anguillarum serotype I.

Figure 6. Moribund juvenile coho salmon with raised scales produced by infection with V. anguillarum serotype II.

Figure 7. Sacrificed juvenile coho salmon with external lesions associated with infection by Vibrio sp. MSC 1-76. Note caudal fin is absent because of necrosis.

Figure 8. Moribund juvenile coho salmon with external lesions associated with infection by Vibrio sp. MSC 1-76. Extensive necrosis of caudal fin was absent while lesions occurred between the dorsal and caudal fins.



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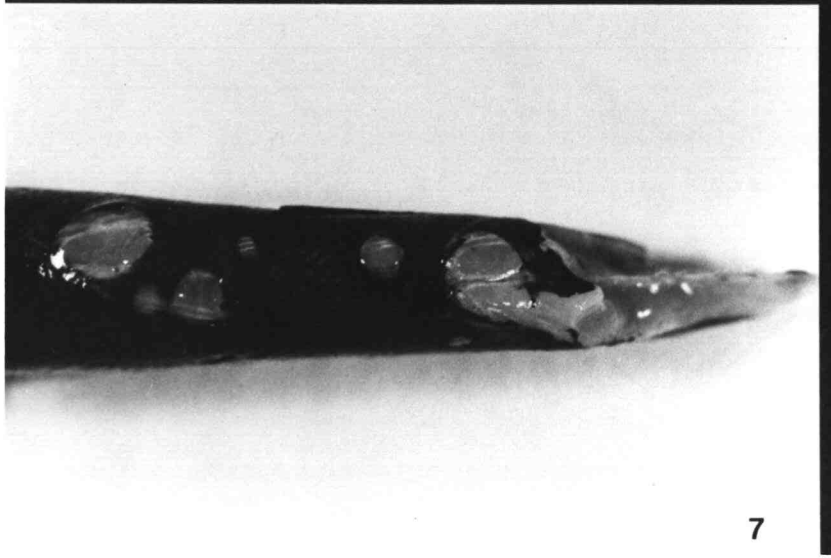

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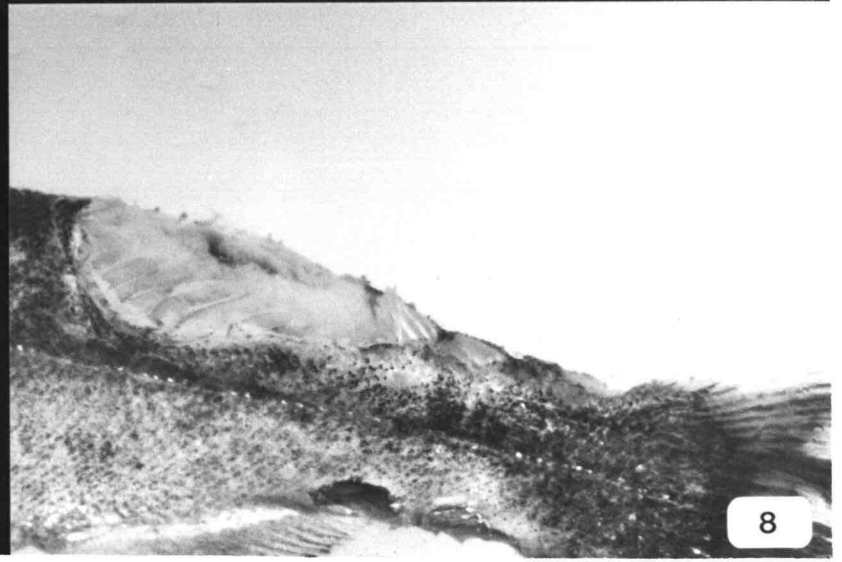

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were the most susceptible to vibriosis. Fish infected with kidney disease in fresh water were taken to salt water with no apparent increased susceptibility to vibriosis until the kidney disease reached advanced stages of infection one year later. At that time, an epizootic occurred when the fish had grown to approximately 225 g. However, it was not possible to determine if vibriosis, kidney disease, or both of these diseases was responsible for the mortality.

Various isolates of Vibrio spp. that did not agglutinate with rabbit antiserum specific for V. anguillarum were also recovered from diseased fish at the Oregon Aqua-Foods Inc. saltwater fish rearing facility. Twenty percent of 20,420 rainbow trout immunized against V. anguillarum serotypes I and II died with external lesions during a two week period in October 1977. Terramycin was administered orally and may have kept the mortality from increasing beyond 20%. A Vibrio sp. which was positive when tested by rapid slide agglutination with rabbit antiserum specific for Vibrio sp. MSC 1-76 was recovered from most dead or moribund fish. Approximately 75 fish were examined. Vibrio anguillarum serotype I or II was not recovered from any fish during this epizootic.

Biochemical and Immunodiffusion Tests

Results of biochemical tests with V. anguillarum serotype I, V. anguillarum serotype II, V. anguillarum isolate 507, and Vibrio sp.

MSC 1-76 are presented in Table 2. Biochemical reactions by V. anguillarum serotype I and V. anguillarum isolated by Sawyer were identical with the exception of melibiose utilization. Vibrio anguillarum serotype II and Vibrio sp. MSC 1-76 were closely related according to biochemical reactions and were similar to V. anguillarum serotype I and V. anguillarum isolate 507.

Results of immunodiffusion tests indicated that the four vibrios were less closely related antigenically than biochemically. A single cross reacting precipitin band existed between V. anguillarum serotypes I and II and V. anguillarum isolate 507. No cross reacting bands existed between these three isolates and Vibrio sp. MSC 1-76.

Quantitative Estimation of Vibrio anguillarum in Yaquina Bay Water

Comparison of Media

Known cultures of V. anguillarum grew well at room temperature (20 to 22 C) on all five media (BHI agar, BHI agar containing 2 1/2% NaCl, Difco TCBS agar, Eiken TCBS agar, and Nissui Vibrio agar) used. A color change characteristic of the pH indicator in the media accompanied sucrose utilization by V. anguillarum when grown on the three Vibrio agars. This color change allows differentiation of V. anguillarum from V. cholera, V. parahaemolyticus, and other bacteria which do not utilize sucrose aerobically.

TABLE 2. Biochemical Reactions of Four Vibrio Isolates.

	Vibrio Anguillarum serotype I (isolate MSC 1-73)	Vibrio Anguillarum serotype II (isolate MSC 2-75)	Vibrio Anguillarum (isolate 507)[a]	Vibrio sp. MSC 1-76
Beta-Galactosidase Production	+	-	+	-
Arginine Dihydrolase Production	d+[b]	-	+	-
Lysine Decarboxylase Production	-	-	-	-
Ornithine Decarboxylase Production	-	-	-	-
Citrate Utilization	-	-	-	-
Hydrogen Sulfide Production	-	-	-	-
Urease Production	-	-	-	-
Tryptophane Deaminase Production	-	-	-	-
Indole Formation	+	-	+	+
Voges-Poskauer Reaction	-	-	-	-
Gelatinase Production	+	-	+	-
Acid From:				
Glucose	+	+	+	+
Manitol	+	+	+	+
Inositol	d+	d+	d+	d+
Sorbitol	+	d+	+	+
Rhamnose	d+	d+	d+	-
Sucrose	+	+	+	d+
Melibiose	-	d+	d+	+
Amygdalin	+	d+	d+	d+
Arabinose	+	d+	+	-

TABLE 2. (Continued)

	Vibrio Anguillarum serotype I (isolate MSC 1-73)	Vibrio Anguillarum serotype II (isolate MSC 2-75)	Vibrio Anguillarum (isolate 507)[a]	Vibrio sp. MSC 1-76
Cytochrome Oxidase	+	+	+	+
Nitrate Reduction	+	+	+	+
Catalase Production	+	-	+	+
Fermentative	+	+	+	+
Growth @ 12 C	+	+	+	+
Growth @22 C	+	+	+	+
Growth @ 36 C	+	-	+	-
Inhibited by 0/129	+	+	+	+
Inhibited by Novobiocin	+	+	+	+

[a] Vibrio anguillarum isolate 507 was recovered from a diseased Coho salmon by Evelyn Sawyer of the University of Maine.

[b] d+ = delayed positive reaction. These reactions were negative at 24 h incubation and positive at 48 h incubation.

In comparing the five media tested, BHI agar was best for enumerating V. anguillarum in salt water. This medium contains 0.5% NaCl which allows V. anguillarum to grow well while the bacteria which require high levels of salt cannot grow. This partial selectivity exhibited by normal BHI agar could be observed when comparing total counts with those on BHI agar containing 2 1/2% NaCl. Total counts were always higher (up to 100% more bacteria per ml) on BHI agar containing 2 1/2% NaCl than on normal BHI agar. The three Vibrio agars were not useful in enumerating V. anguillarum in salt water. The number of sucrose utilizing organisms could be determined using these media but no rapid slide agglutination tests using the sucrose utilizing colonies were ever positive. Further investigation indicated that while V. anguillarum grew well on the Vibrio media, cell surface antigens were apparently altered in some way such that specific rabbit antibodies could not react with the bacteria to produce agglutination.

Determination of Presumptive V. anguillarum Colonies

Colony characteristics on BHI agar were useful in choosing presumptive V. anguillarum colonies for use in rapid slide agglutination tests. Colonies of V. anguillarum serotype II were typically very small and slightly white while colonies of V. anguillarum serotype I were larger and cream colored. Colonies of each serotype of

V. anguillarum were measured to determine if colony size was a parameter which could be used as a valid means of differentiating the two serotypes. A dissecting microscope with an ocular micrometer was used to measure 50 colonies of each serotype at 120X magnification. The following are the results of these measurements:

DIAMETER OF COLONIES (mm)[A]

Time	V. anguillarum serotype I	V. anguillarum serotype II
24 h	$\overline{X}$ = 0.79 (R=0.40-1.17)	$\overline{X}$ = 0.23 (R=0.06-0.38)
48 h	$\overline{X}$ = 2.33 (R=1.53-2.92)	$\overline{X}$ = 0.98 (R=0.24-1.29)

[A] $\overline{X}$ = Mean, R = range.

Vibrio anguillarum serotype I colonies appeared to have a blue colored edge when a BHI agar plate containing them was placed on a fluorescent light box normally used to read gel diffusion plates. When examining Yaquina Bay water samples, all colonies with this characteristic were positive V. anguillarum serotype I when tested by rapid slide agglutination while no colonies without the characteristic were positive. In one case this was true for three plates containing 143, 144, and 145 presumptive V. anguillarum serotype I colonies and 103, 153, and 113 colonies respectively without a blue edge when observed on the light box.

Enumeration of Bacteria

Enumeration of V. anguillarum cells in salt water appeared to be unsuccessful in 1974 and 1975 because the organism was not frequently recovered from water. Repeated sampling at various locations at the Marine Science Center and Oregon Aqua-Foods Inc. revealed no V. anguillarum colonies among sucrose utilizers on the Vibrio differentiating media and usually zero or one V. anguillarum colony when 0.1 ml undiluted salt water was inoculated onto BHI agar. Although many of the colonies grown on both BHI media did not have to be tested serologically because their morphology was not typical of V. anguillarum, slide agglutination tests with many suspect colonies were tedious and required large quantities of antiserum.

There were two opportunities in 1976 to sample water from fish tanks during a vibriosis epizootic. In both cases, naturally occurring levels of V. anguillarum were low (ten or less per ml) in water entering the tanks while effluent water contained many V. anguillarum bacteria (1.0 to 4.7 x 10^3 per ml). Repeated sampling of untreated salt water supplied to the Marine Science Center and effluent water from fish tanks containing healthy fish confirmed that naturally occurring levels of V. anguillarum present in Yaquina Bay were consistently ten or less per ml. These findings confirmed the results of plate counts run in 1974 and 1975 which had appeared to be unrealistically low compared to counts of bacteria in salt water in Lint Slough

(Rohovec, 1974).

Histopathology Study of Naturally Acquired Vibriosis in Chum Salmon Fingerlings

A brief comparison of the histopathology associated with V. anguillarum serotypes I and II is presented in Table 3. This table does not cover details associated with the histopathology of vibriosis and only presents a comparison of some basic changes associated with infections of fish by these two organisms. Data summarized in this table were collected by examination of three moribund fish with naturally acquired infections of V. anguillarum serotype I, three moribund fish with naturally acquired infections of V. anguillarum serotype II, ten moribund fish with experimentally induced infections of V. anguillarum serotype I, and ten moribund fish with experimentally induced infections of V. anguillarum serotype II.

Phagocytosis of bacteria was the only cellular response observed in fish infected with either serotype of V. anguillarum. Few white blood cells were present in fish infected with V. anguillarum serotype II while a definite decrease in white blood cells was observed in fish infected with serotype I.

Control Fish

No abnormalities were observed in histological sections of non-infected control fish. Photomicrographs of some of the tissues

and organs are presented in Figures 9 to 16. These photomicrographs can be used in the most important comparisons of diseased and non-diseased fish. More photomicrographs of non-infected control fish tissue were excluded for economic reasons.

Blood

Vibrio anguillarum Serotype I

Blood appeared to be the main target for V. anguillarum serotype I. Bacteria were numerous throughout the vascular system as well as in all hemorrhagic areas of infected fish (Figures 17, 38, 44, and 49). Bacteria in most hemorrhages appeared to be present as a result of infiltration via the blood rather than from infection of surrounding tissues. Blood in the liver, thymus, gills, skeletal muscle, and eyes contained many bacteria, but the organs themselves were either only slightly infected or not infected at all. It was evident that V. anguillarum serotype I produced a classic bacteremia in chum salmon fingerlings.

Vibrio anguillarum Serotype II

Although V. anguillarum serotype II produces a bacteremia in salmonids (shown in experiments discussed later) bacteria were never

Figure 9. Section through kidney and esophagus of non-infected chum salmon fingerling. Note void areas beneath mucosal epithelium resulted during fixation and do not represent areas of edema. X100.

Figure 10. Section through skin, skeletal muscle, pyloric caecae, and pancreas of non-infected chum salmon fingerling. Void areas in muscle resulting from fixation were inconsistently observed throughout muscle of control fish. X100.

Figure 11. Section through ascending intestine of non-infected chum salmon fingerling. X100.

Figure 12. Section through descending intestine of non-infected chum salmon fingerling. X100.

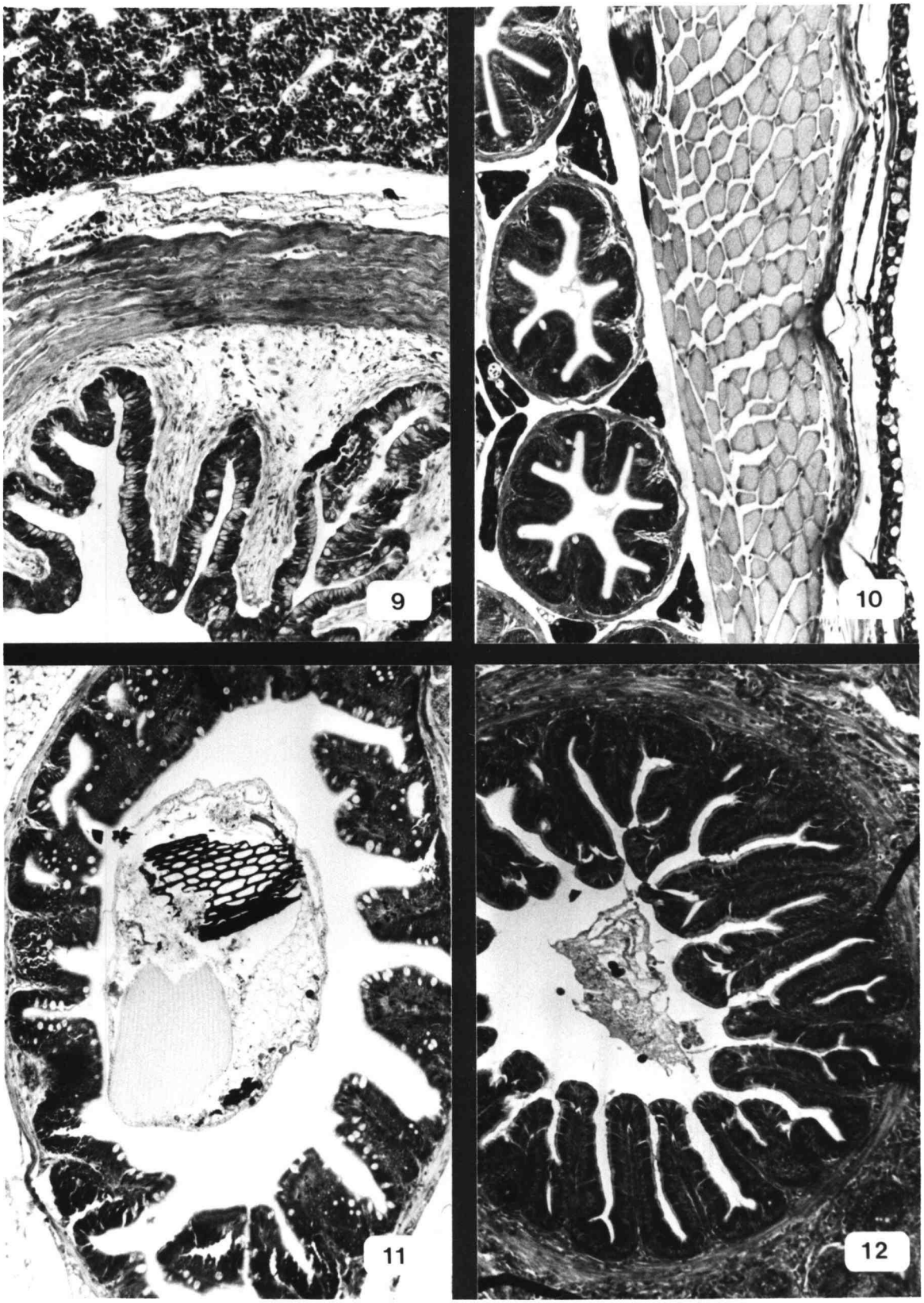
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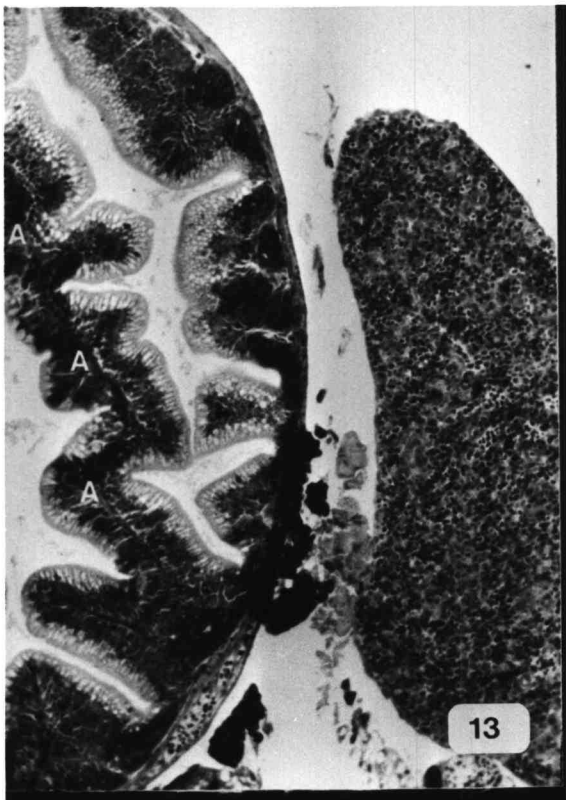
Figure 13. Section through rectum and spleen of non-infected chum salmon fingerling. Note annulo-spiral septum (A) in rectum. X100.

Figure 14. Section through trunk kidney of non-infected chum salmon fingerling. Note gray area within renal tubules is artifact and not edematous fluid. X400.

Figure 15. Section through pancreas of non-infected chum salmon fingerling. Note exocrine tissue (E). X400.

Figure 16. Section through gill arch of non-infected chum salmon fingerling. Note lamellar epithelium (LE) is adjacent to the capillary bed in lamellae of healthy fish. X400.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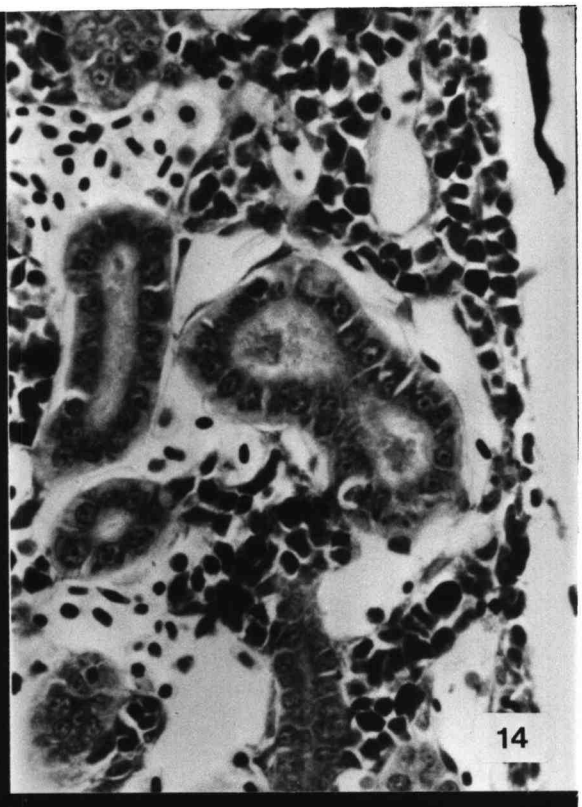
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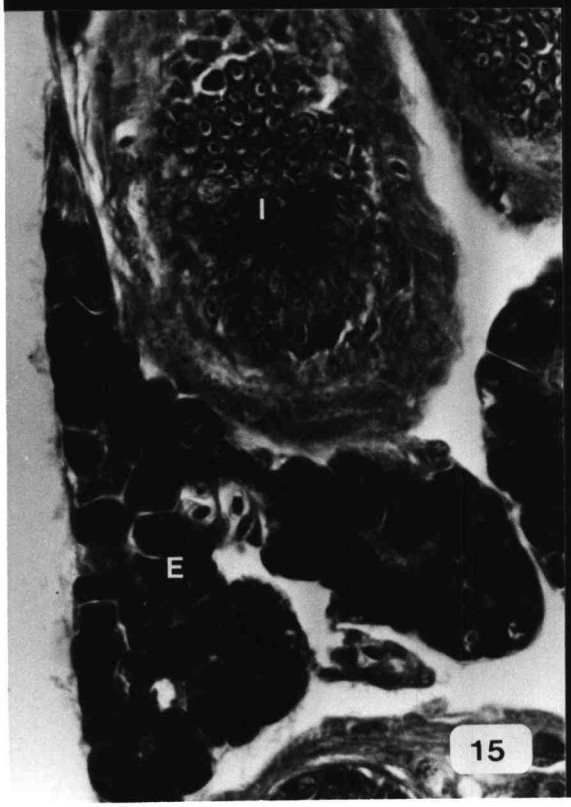
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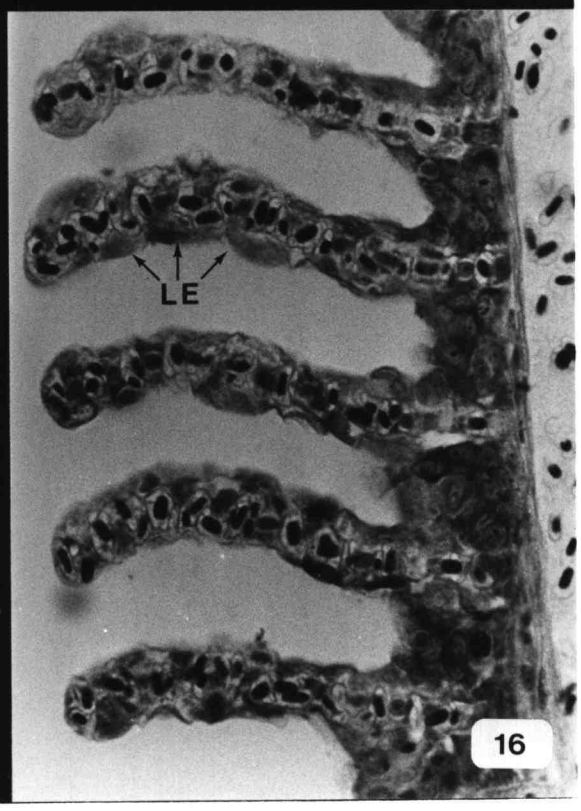
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positively identified in blood of chum salmon fingerlings with naturally induced infections of this agent (Figures 18, 45, and 46). No bacterial cells of serotype II were observed in blood vessels that were comparable in size to blood vessels in which 200-300 bacterial cells could be observed in fish infected with V. anguillarum serotype I. In contrast to infections with V. anguillarum serotype I, serotype II cells in hemorrhaged areas were associated with tissues of the hemorrhaged organ rather than with blood cells.

Gastrointestinal Tract

Vibrio anguillarum Serotype I

Blood was the most heavily infected tissue in the gastrointestinal tract of chum salmon infected with V. anguillarum serotype I. Bacteria were numerous and evenly distributed throughout all blood and in prevalent hemorrhages. No bacteria or pathological changes were observed in the mucosal epithelium of the buccal cavity, pharynx, esophagus, cardiac stomach, or pyloric stomach. However, in these areas, low numbers of bacteria were observed in connective tissues (Figure 19). The peritoneum was heavily infected throughout, but usually did not contain as many bacteria as blood (Figure 20). Bacteria occurred in the lumen of the cardiac stomach and the pyloric stomach but had not invaded the mucosal epithelium.

No pathology was observed in most pyloric caecae (Figure 21). However, necrosis and sloughing of mucosal epithelial cells were noted in some pyloric caecae (Figure 22) and in the ascending intestine. Necrosis and epithelial cell sloughing were severe in the descending intestine (Figure 23) and massive in the rectum (Figures 24 and 25). Connective tissue in the mucosa was also necrotic in the descending intestine and rectum. In some areas of the rectum, necrosis extended through the mucosa and into the muscularis resulting in destruction of the annulospiral septa (Figures 24 and 25). Pathology observed in the rectum appeared to be the result of extensive infection of the supportive connective tissue in the mucosa. This layer of the mucosa contained most of the bacteria present in the rectum.

Vibrio anguillarum Serotype II

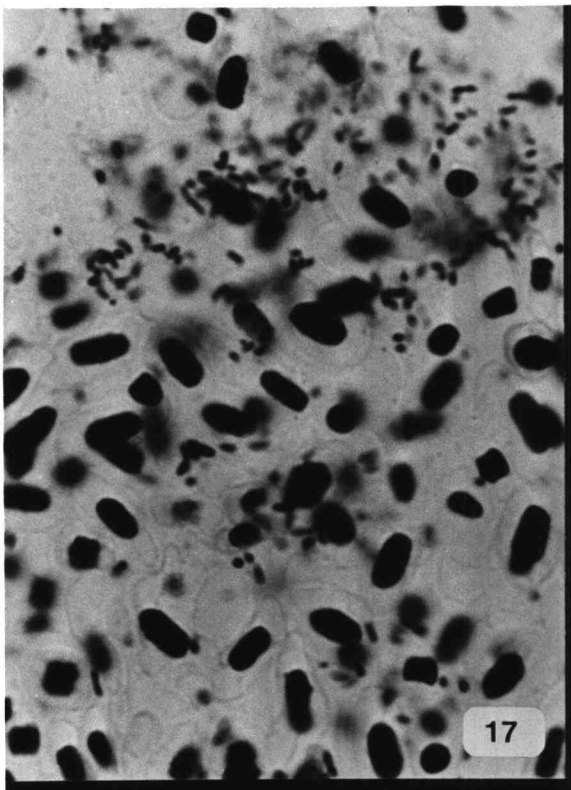
Bacterial colonies containing thousands of organisms were observed along the entire gastrointestinal tract of chum salmon fingerlings infected with V. anguillarum serotype II. These colonies were observed in all layers of tissue between the intact peritoneum and the mucosal epithelium (Figures 26 through 29). In the areas of the buccal cavity, pharynx, esophagus, cardiac stomach, and pyloric stomach, the epithelium was intact and necrosis absent. Epithelial cells and bacteria were observed in the lumen of the ascending intestine and pyloric caecae. The number of bacterial and epithelial cells

Figure 17. Blood of chum salmon fingerling infected with V. anguillarum serotype I. Note large number of bacteria. X1,000.

Figure 18. Blood of chum salmon fingerling infected with V. anguillarum serotype II. Two dark objects (B?) were the only suspect bacteria observed in blood of chum salmon with naturally induced infections of V. anguillarum serotype II. X1,000.

Figure 19. Section through cardiac stomach of chum salmon fingerling infected with V. anguillarum serotype I. Necrosis and bacteria were absent in the mucosa. Bacteria (B) were occasionally observed in the muscularis. X400.

Figure 20. Photomicrograph of heavily infected peritoneum adjacent to muscularis of the esophagus in chum salmon fingerling with naturally acquired infection by V. anguillarum serotype I. X1,000.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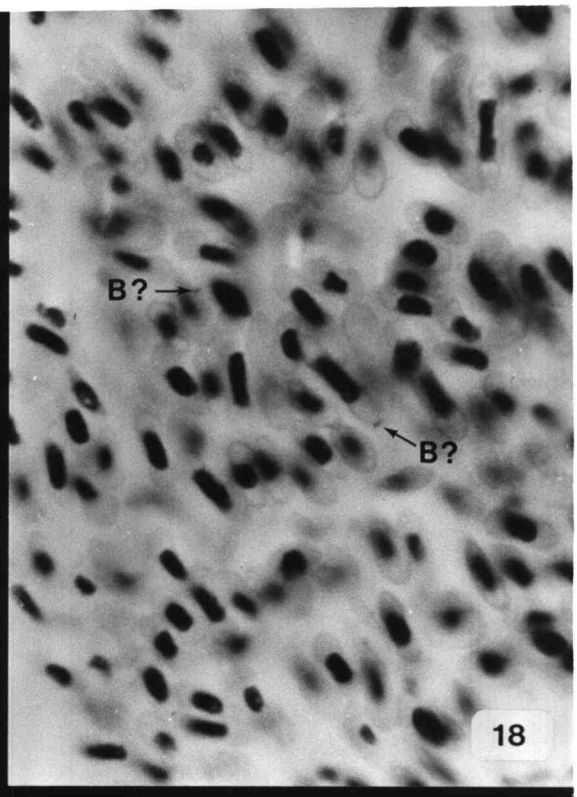
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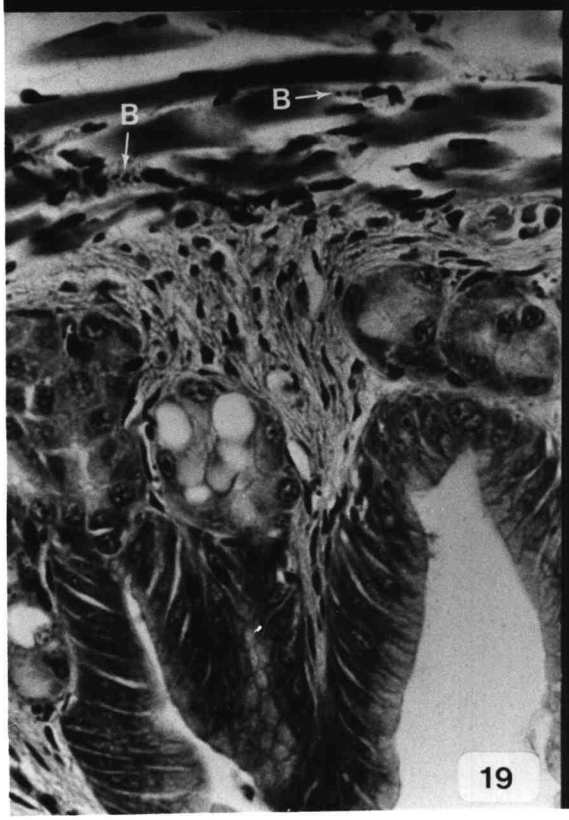
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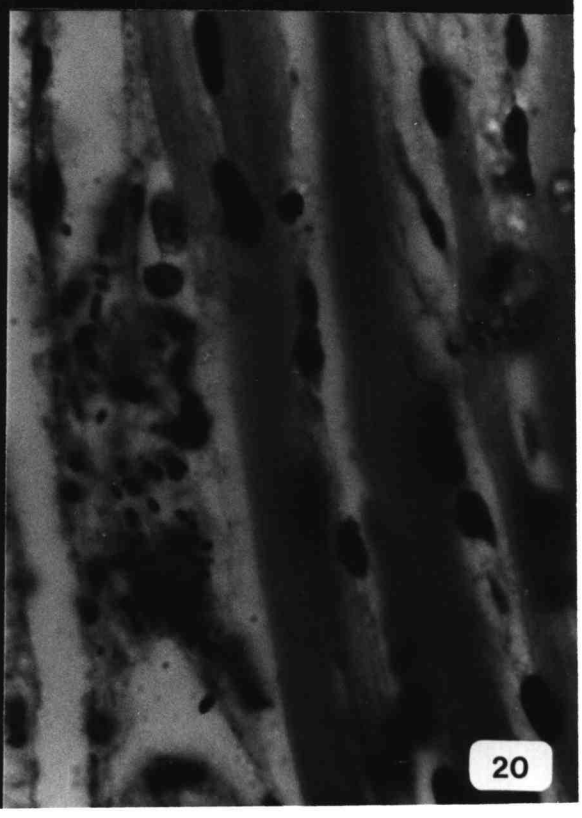
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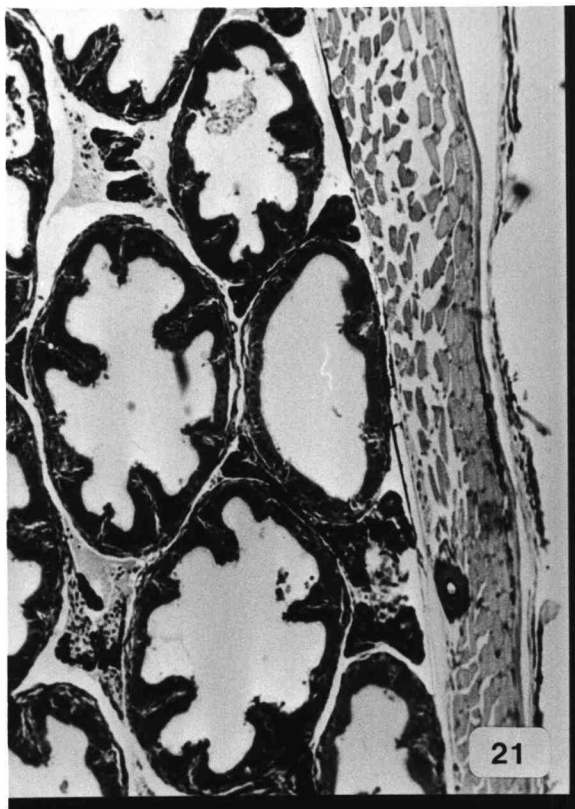
Figure 21. Section through pyloric caecae of chum salmon fingerling infected with V. anguillarum serotype I. Note intact mucosal epithelium. X100.

Figure 22. Section through an intact pyloric caecum and a pyloric caecum with necrosis and sloughing of mucosal epithelium in chum salmon fingerling infected with V. anguillarum serotype I. Loose connective tissue between pyloric caecae was infected with bacteria (B). X400.

Figure 23. Extensive sloughing and necrosis of the mucosa in the descending intestine of chum salmon fingerling infected with V. anguillarum serotype I. Folds of the mucosa are absent due to necrosis. X100. Compare with Figure 12.

Figure 24. Extensive necrosis in rectum of chum salmon fingerling infected with V. anguillarum serotype I. Note absence of annulo-spiral septa due to necrosis. X100. Compare with Figure 13.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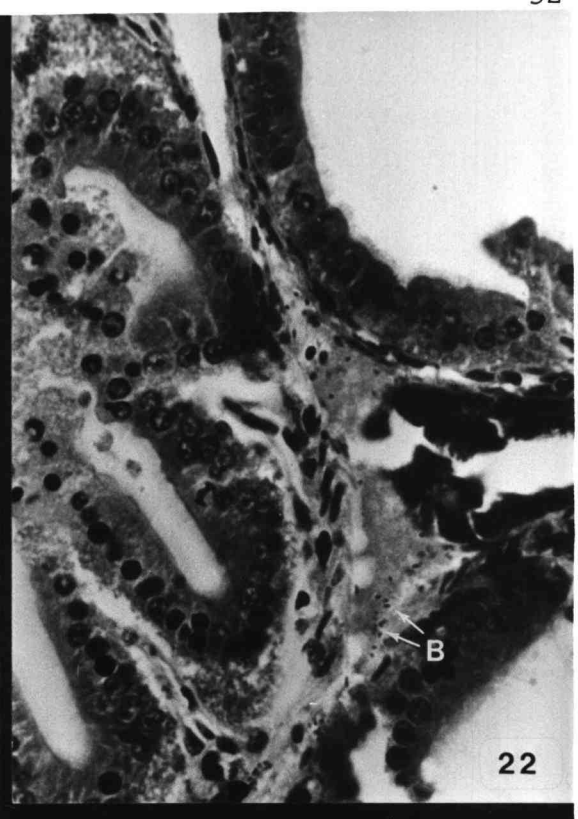
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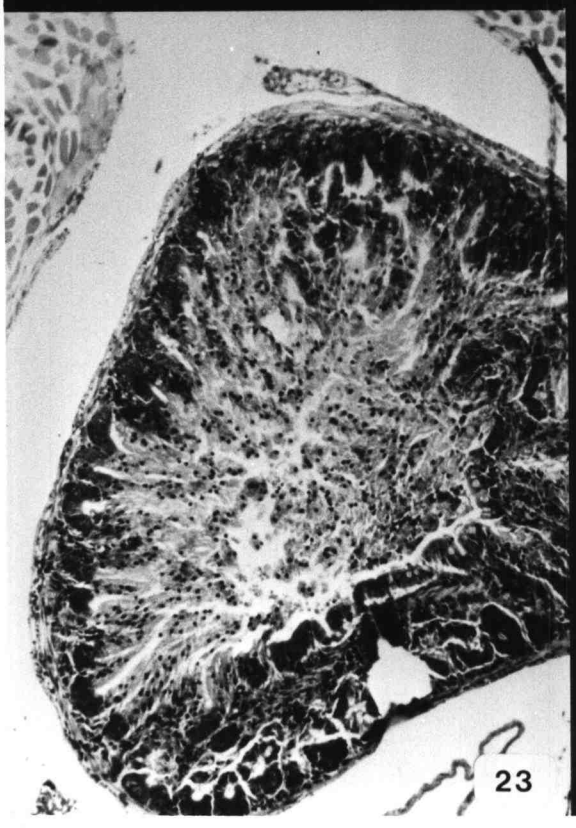
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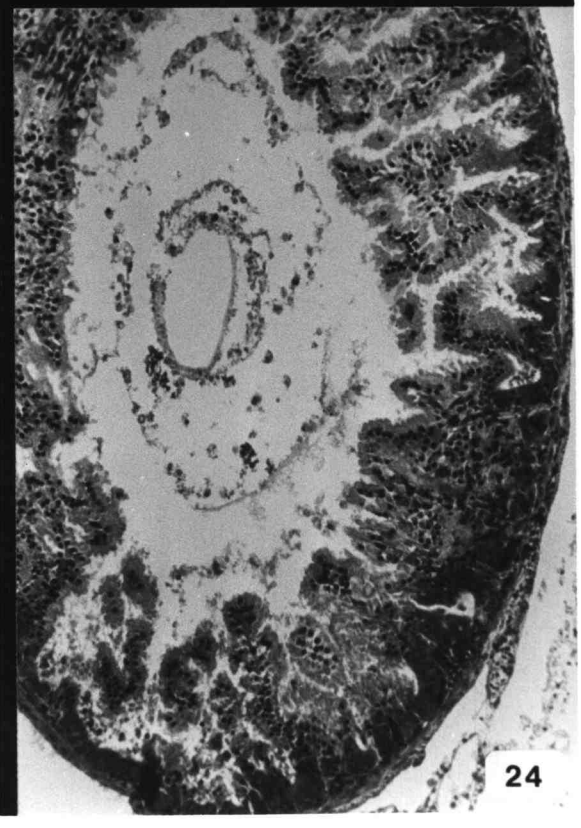
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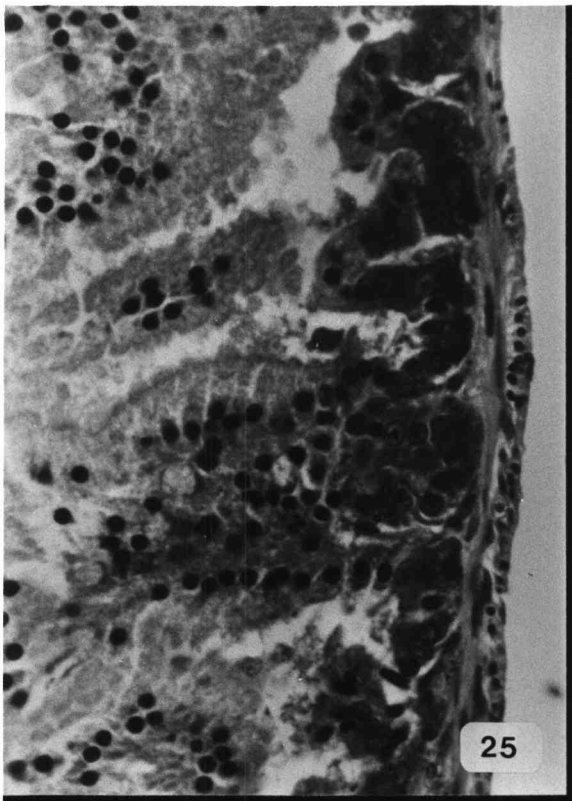
Figure 25. Magnification of Figure 24 showing necrotic and intact tissues. A few normal nuclei are present in the mucosal epithelium while most nuclei are pyknotic. X400.

Figure 26. Section through liver and esophagus near the cardiac stomach of chum salmon fingerling infected with V. anguillarum serotype II. Note colonies (C) of bacteria located between the intact peritoneum and the mucosal epithelium but not extending into the liver. X100.

Figure 27. Section through esopahgus with colonies (C) of bacteria located between the intact peritoneum and the mucosal epithelium in a chum salmon fingerling infected with V. anguillarum serotype II. X100.

Figure 28. Magnification of Figure 27 showing lack of necrosis in the mucosal epithelium and supporting connective tissue. Note void area below mucosal epithelium is also present in sections of non-infected chum salmon and is due to fixation rather than edema. C = bacterial colony. X400.



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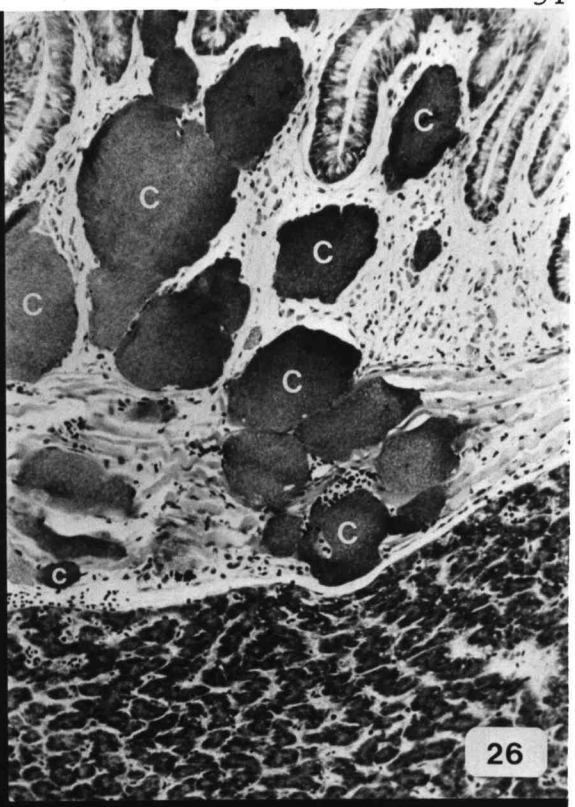

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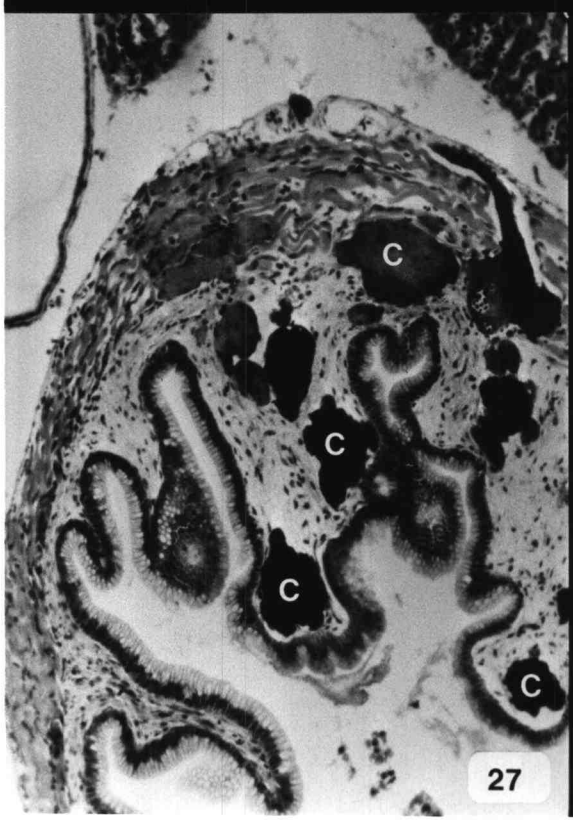

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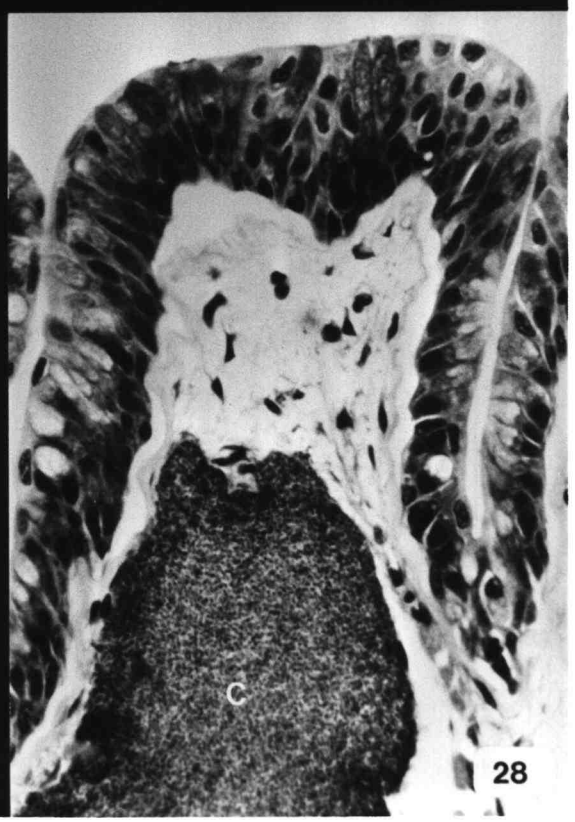

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in the lumen of the gut as well as the degree of necrosis in the mucosa was greatest in areas most posterior to the stomach. This suggested a possible correlation of pathology with pH which was tested during subsequent experiments. Colonies in the pyloric caecae were common and were usually located just beneath the intact mucosal epithelium. Occasionally colonies extended through the epithelium releasing bacteria into the lumen of a caecum (Figures 29 through 31). Necrosis and sloughing of the mucosa were present in the ascending intestine, most pyloric caecae, the descending intestine, and the rectum (Figures 32 through 36). Necrosis was most severe in the rectum where many of the annulo-spiral septa were completely destroyed (Figure 36).

Muscle

Vibrio anguillarum Serotype I

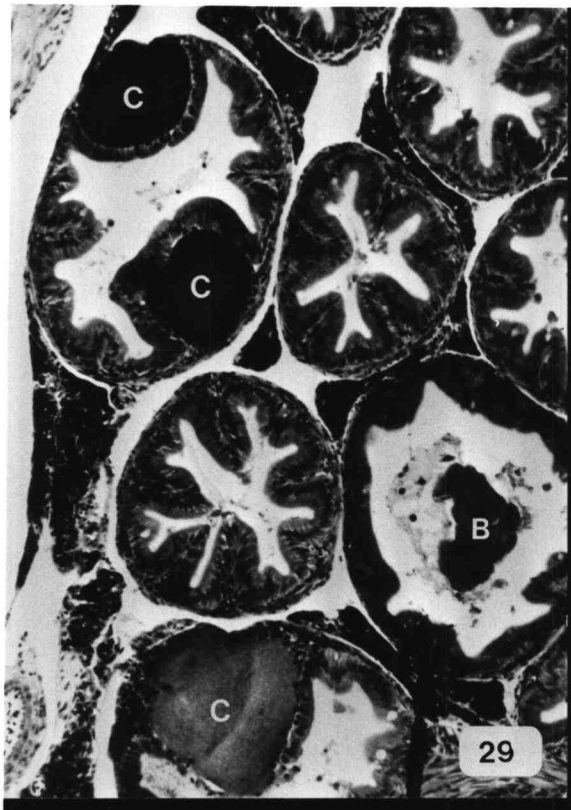
High concentrations of bacteria infecting skeletal muscle were not observed in chum salmon fingerlings (Figure 37). Blood associated with hemorrhages was the most common location of serotype I cells in muscle (Figure 38). Bacteria appeared to be present in hemorrhages due to infection of blood rather than infection of muscle. The perimysium and loose connective tissue adjacent to muscle containing no apparent bacteria were consistently infected (Figure 39).

Figure 29. Section through pyloric caecae of chum salmon fingerling infected with V. anguillarum serotype II. Two colonies (C) of bacteria were bound by intact mucosal epithelium and the tunica serosa. The colony at the bottom of the figure had broken through the mucosal epithelium. Free bacteria (B) were located in the lumen of one pyloric caecum. X100.

Figure 30. Magnification of Figure 29 showing lack of necrosis in tissues adjacent to in vivo colony (C) of V. anguillarum serotype II in pyloric caecum of chum salmon fingerling. X400.

Figure 31. Magnification of Figure 29 showing a colony of bacteria breaking through the mucosal epithelium of a pyloric caecum. Note many free bacteria in the lumen of the caecum at the bottom of the figure. X400.

Figure 32. Section through a pyloric caecum of chum salmon infected with V. anguillarum serotype II. Note bacteria (B) free in lumen and held from penetrating mucosa by microvilli. Epithelial cell (E1) was in process of being sloughed while epithelial cell (E2) had just been sloughed from the mucosa. X400.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C
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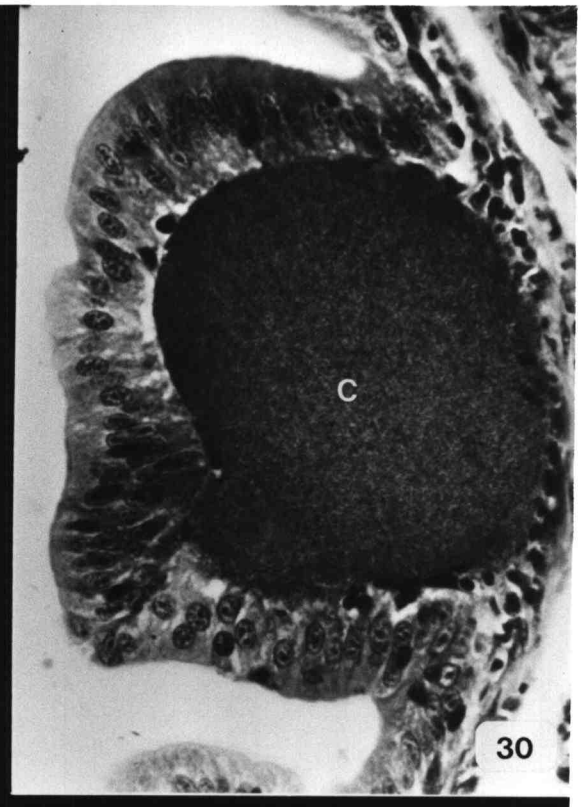
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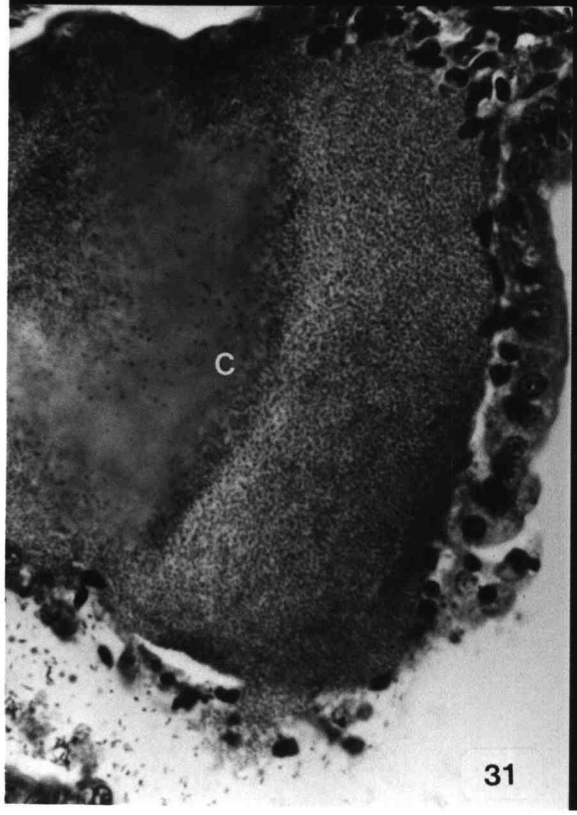
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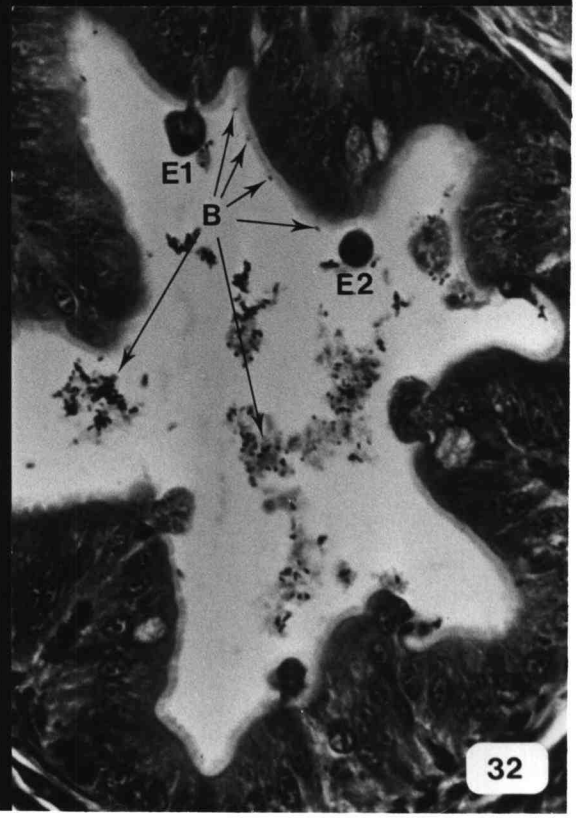
E1
B
E2
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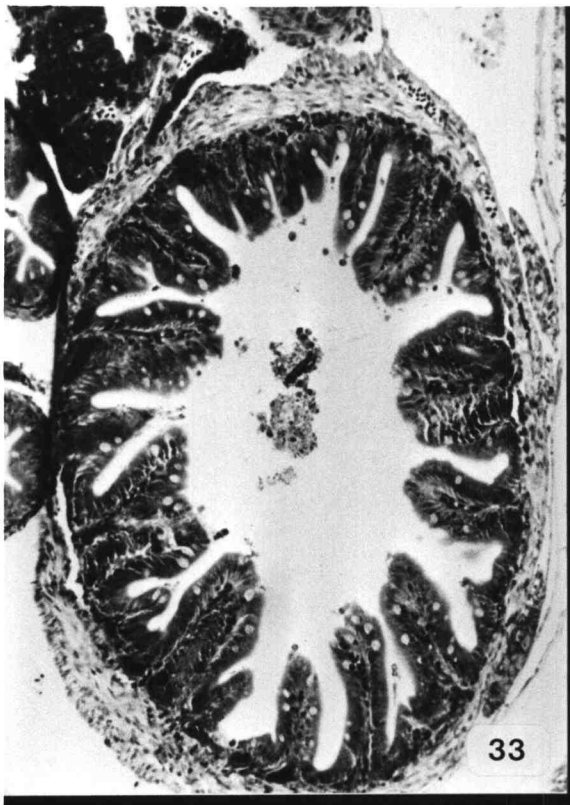
Figure 33. Minor sloughing of epithelial cells in ascending intestine of chum salmon fingerling infected with V. anguillarum serotype II. X100.

Figure 34. Magnification of Figure 33 showing bacteria and epithelial cells in lumen of ascending intestine. D = diatom. X1,000.

Figure 35. Necrosis and extensive sloughing of epithelial cells in descending intestine of chum salmon fingerling infected with V. anguillarum serotype II. X400.

Figure 36. Complete necrosis of nearly all mucosa and muscularis in the rectum of chum salmon fingerling infected with V. anguillarum serotype II. Note absence of annulospiral septa due to necrosis. Debris in lumen is fecal material and cell breakdown. X100. Compare with Figure 13.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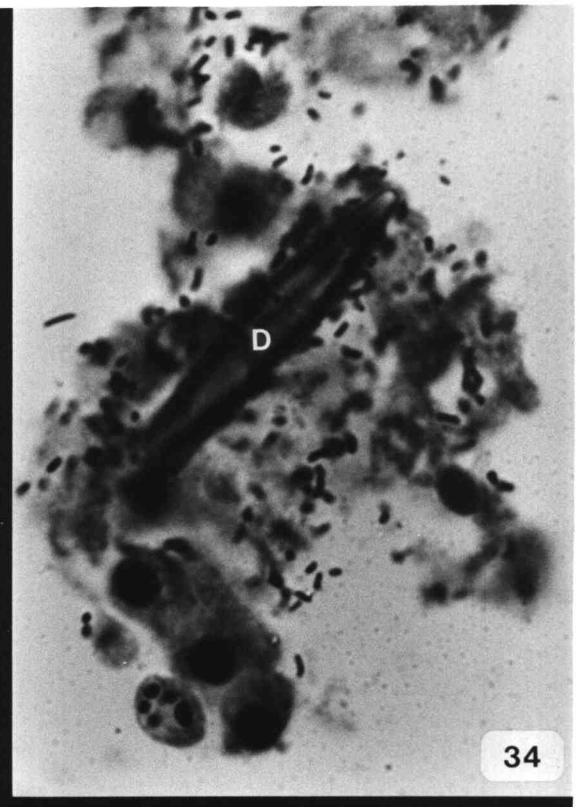

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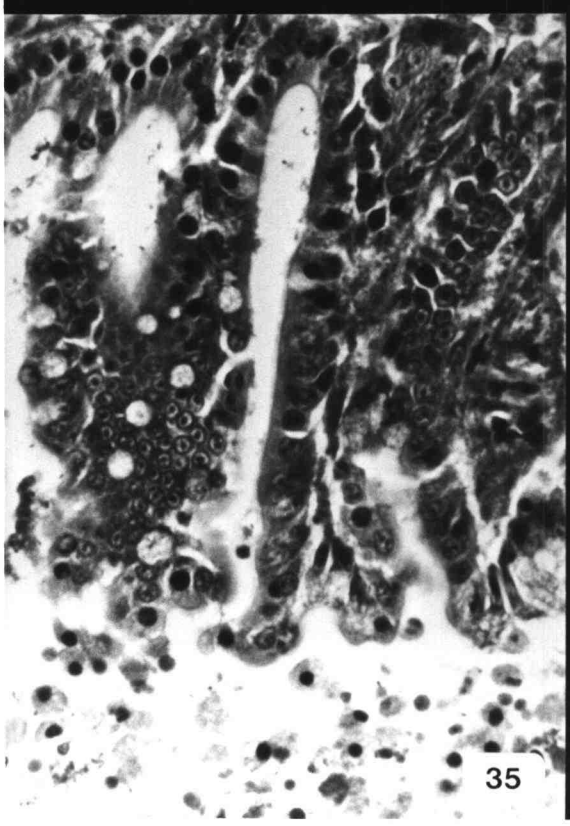
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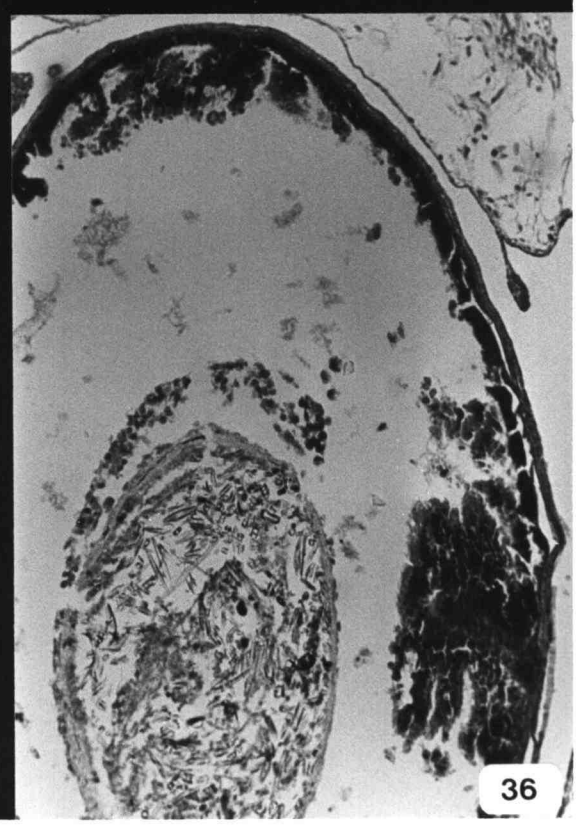
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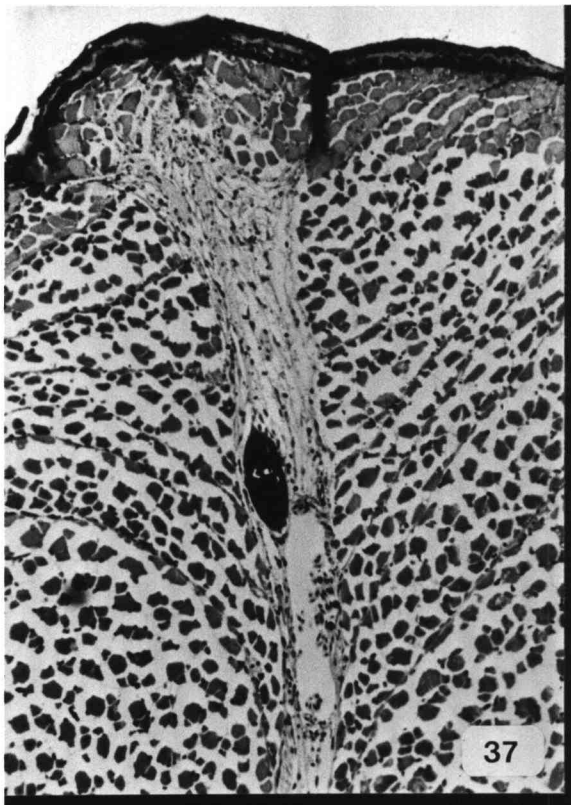
Figure 37. Skeletal muscle of chum salmon fingerling infected with V. anguillarum serotype I. This was a typical section without infection of muscle fibers. X100.

Figure 38. Hemorrhage in skeletal muscle of chum salmon fingerling infected with V. anguillarum serotype I. Bacteria are evenly dispersed throughout blood. X400.

Figure 39. Magnification of Figure 37 showing extensive infection of connective tissue between muscles. N = muscle cell nuclei, B = bacteria. X400.

Figure 40. Extensive infection of skeletal muscle with typical in vivo bacterial colonies (C) in chum salmon fingerling infected with V. anguillarum serotype II. An external route of entry and progress of infection was suggested by extensive infection in outer layers of muscle (top of figure) while inner layers of muscle (bottom of figure) contained few bacteria. A definite line of demarcation between infected and non-infected areas existed, which indicated that muscle fascia was as a physical barrier to this organism. H = hemorrhage, C = bacterial colony. X100.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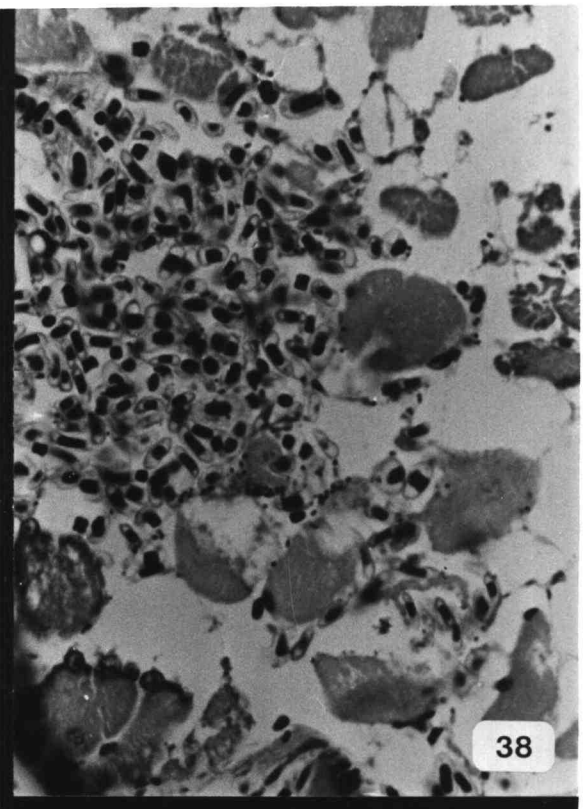
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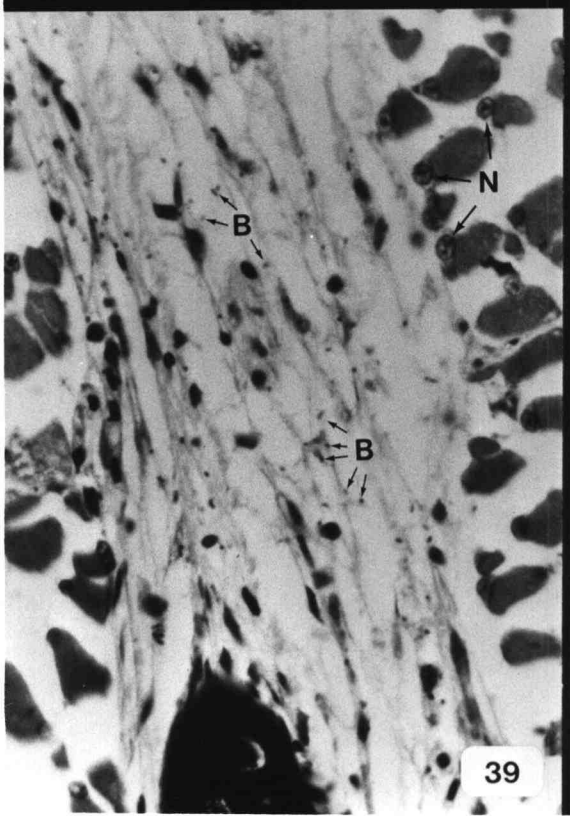
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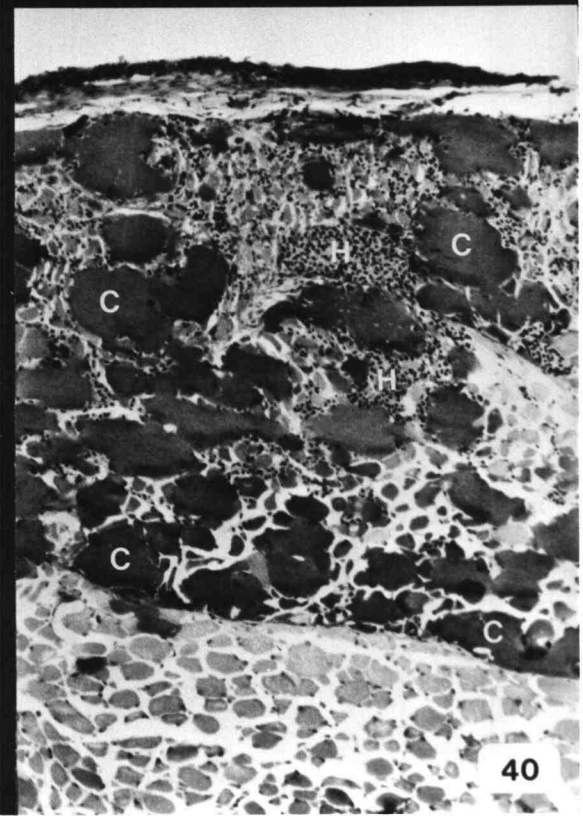
B
39


C
C
H
H
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C
40

It was concluded that muscle was not a preferred target of V. anguillarum serotype I because infections were contained within hemorrhages in muscles and within loose connective tissue surrounding muscles. Atrophy of skeletal muscle was indicated by void areas between most muscle bundles (Figure 37) but the degree of atrophy could not be assessed because void areas were occasionally observed in non-infected control fish.

Vibrio anguillarum Serotype II

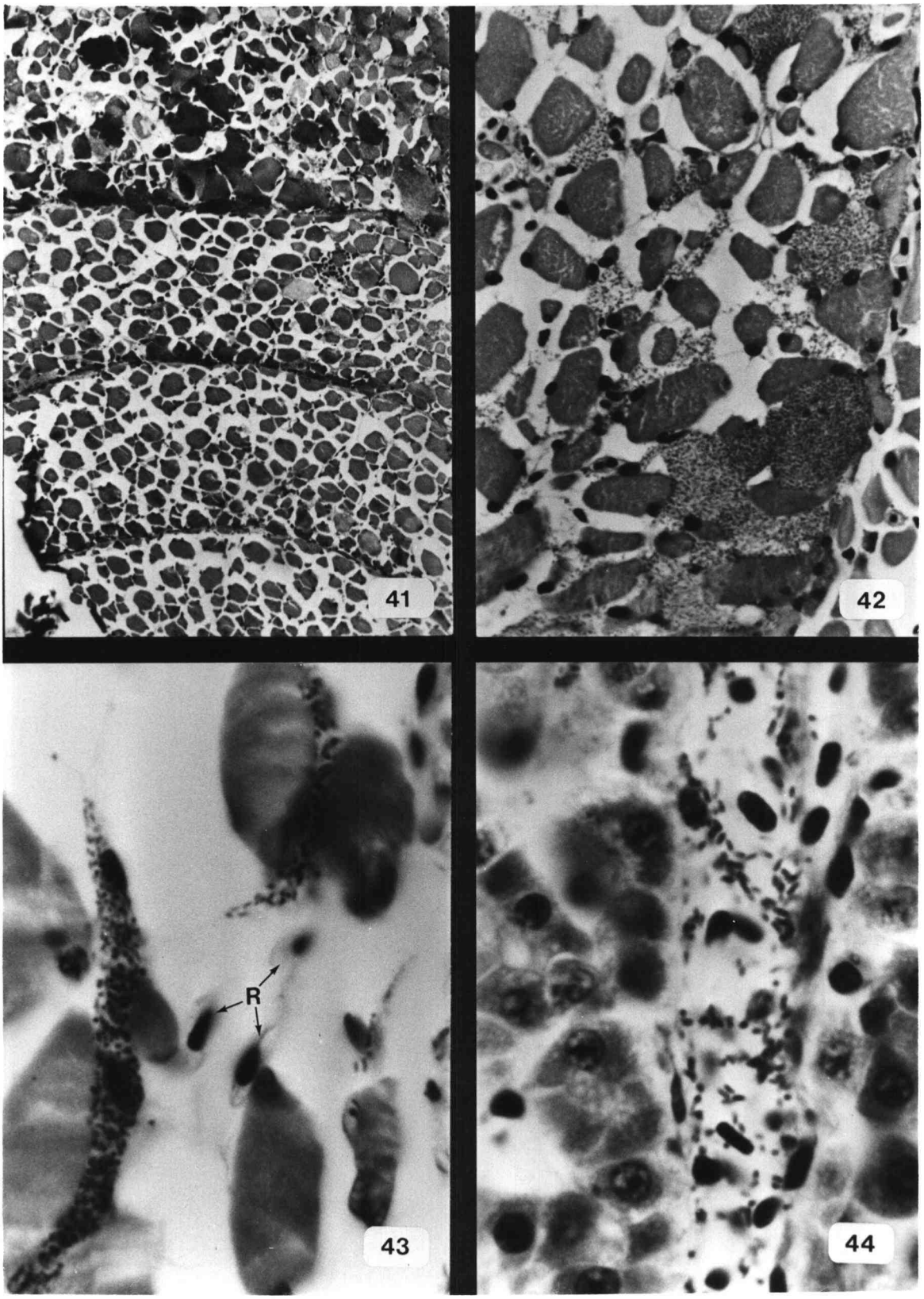
The pathology observed in skeletal muscle of chum salmon fingerlings was more severe with infections of serotype II than with infections of serotype I. More colonies of bacteria were present in muscle than in any other organ (Figure 40). Infections were not uniform; some areas were relatively free of bacteria while others appeared to contain more bacterial mass than tissue mass (Figure 40). Outer layers of muscle were most highly infected, suggesting that organisms may have spread inward from the skin (Figure 40). The degree of infection followed definite lines of demarcation between muscle layers suggesting that muscle fascia might serve, to a limited extent, as a physical barrier to serotype II (Figure 40). Bacteria were concentrated against the muscle fascia with infection spreading laterally between muscle layers (Figure 41). This was evident where heavy infection extended along the fascia from areas

Figure 41. Section through skeletal muscle of chum salmon fingerling infected with V. anguillarum serotype II. Note dark areas of infection along fascia between muscle layers where bacteria were concentrated and appeared to be spreading laterally through muscle. White area in lower left of figure is a defect in the section. X100.

Figure 42. Infection of skeletal muscle of chum salmon fingerling infected with V. anguillarum serotype II. Most muscle fibers were free of bacteria but contained pyknotic nuclei. Note high number of bacteria and absence of hemorrhage. X400.

Figure 43. Magnification of Figure 42 showing V. anguillarum serotype II in the areolar endomysium of skeletal muscle. R = red blood cell. X1,000.

Figure 44. Section through liver of chum salmon fingerling infected with V. anguillarum serotype I. Blood in capillary was infected with many bacteria but hepatic tissue immediately adjacent to the capillary contained few bacteria. X1,000.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R
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with large numbers of bacteria into areas where few bacteria were located. Acute necrosis was present in muscle but in many areas bacterial colonies were limited to the areolar endomysium surrounding muscle fibers (Figures 42 and 43).

Liver

Vibrio anguillarum Serotype I

Livers of chum salmon fingerlings with naturally induced infections of V. anguillarum serotype I contained many bacteria which were located mainly in the blood supply of this organ (Figure 44). Focal infection and necrosis were observed in hepatic cells immediately adjacent to sinusoids and the hepatic vein. The hepatic vein in one fish was surrounded by a band of infected hepatic tissue three to four cells deep. These cells were necrotic and contained pyknotic nuclei while pyknosis was not observed in any intact liver cells. The lack of necrosis in cells adjacent to highly infected blood or focal infection of hepatic cells indicated a potent toxin was not present. Bacteria were not observed in bile ducts surrounded by infected connective tissue.

Vibrio anguillarum Serotype II

No bacteria, necrosis, or edema were observed in livers of

chum salmon fingerlings infected with V. anguillarum serotype II (Figures 45 and 46).

Kidney

Vibrio anguillarum Serotype I

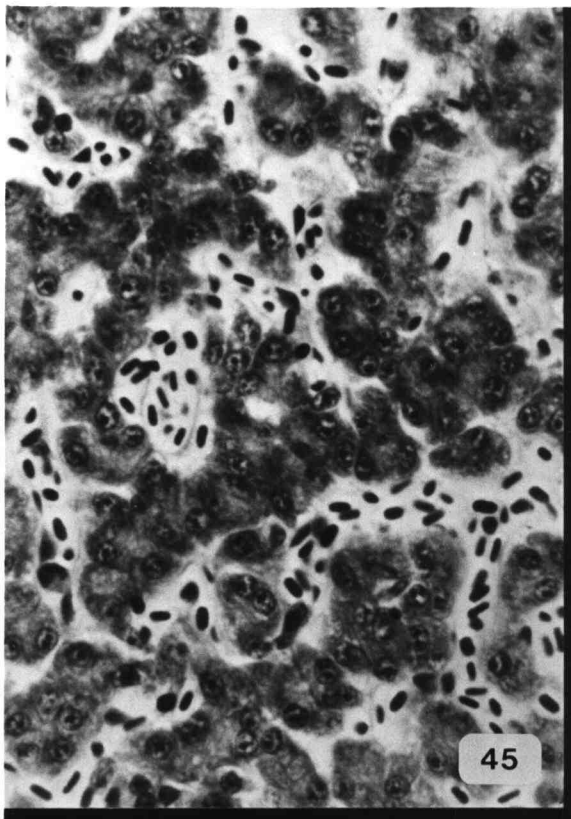
Kidneys of fish infected with serotype I contained many bacteria. Nearly all bacteria were distributed evenly throughout the blood, interrenal tissue, and hematopoietic tissues, although areas where bacteria were concentrated and might be interpreted as focal infections were present. However, bacteria in these focal areas were never as highly concentrated as bacteria in the in vivo colonies produced by serotype II. In many areas of hematopoietic tissue, there appeared to be an abundance of stem cells (compared to hematopoietic tissue in kidneys of non-infected control fish) suggesting that fish infected with serotype I suffered from anemia. Phagocytosis of bacteria was also noted in these areas. An overall swelling of the kidney indicated edema was present, but cell-free areas of edematous fluid were masked by congestion (Figure 47). Various stages of cellular breakdown and the presence of a large amount of cell debris were manifestations of necrosis of interstitial tissues between renal tubules (Figure 48). Nearly all renal tubules in moribund fish were indistinguishable from renal tubules in

Figure 45. Section of liver in chum salmon fingerling infected with V. anguillarum serotype II. No bacteria, necrosis, or edema were observed. X400.

Figure 46. Magnification of Figure 45. No pathology was observed. Note lack of bacteria in blood. X1,000.

Figure 47. Section through head kidney of chum salmon fingerling infected with V. anguillarum serotype I. Note congestion as indicated by abnormally high number of blood cells throughout this organ. X100.

Figure 48. Magnification of Figure 47 showing necrosis and infection of hematopoietic tissue of the head kidney. Additional cell debris not evident in this photomicrograph was observed in other planes of focus. X1,000.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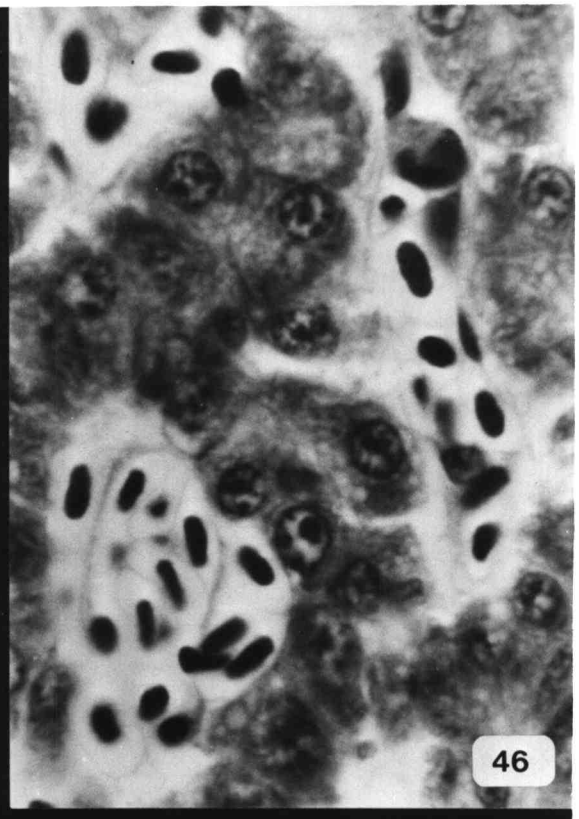
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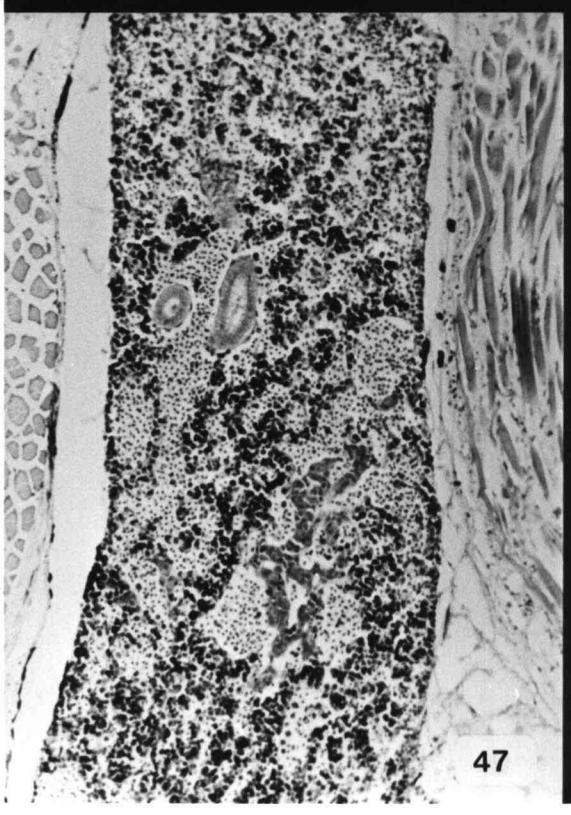
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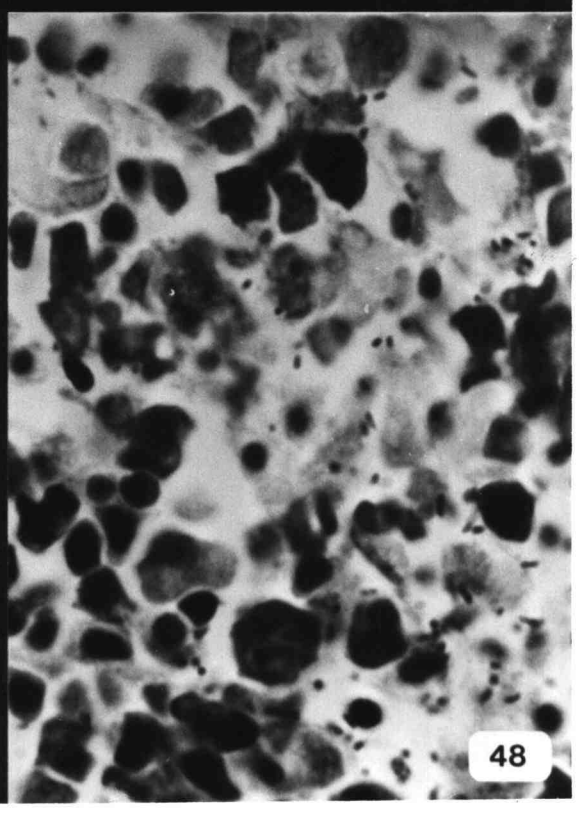
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non-infected control fish (Figure 49). Therefore, it was evident that renal tubules of fish might be fully functional during early stages of this disease. Bacteria and necrosis were not observed in the mesonephric duct or the collecting tubules.

Vibrio anguillarum Serotype II

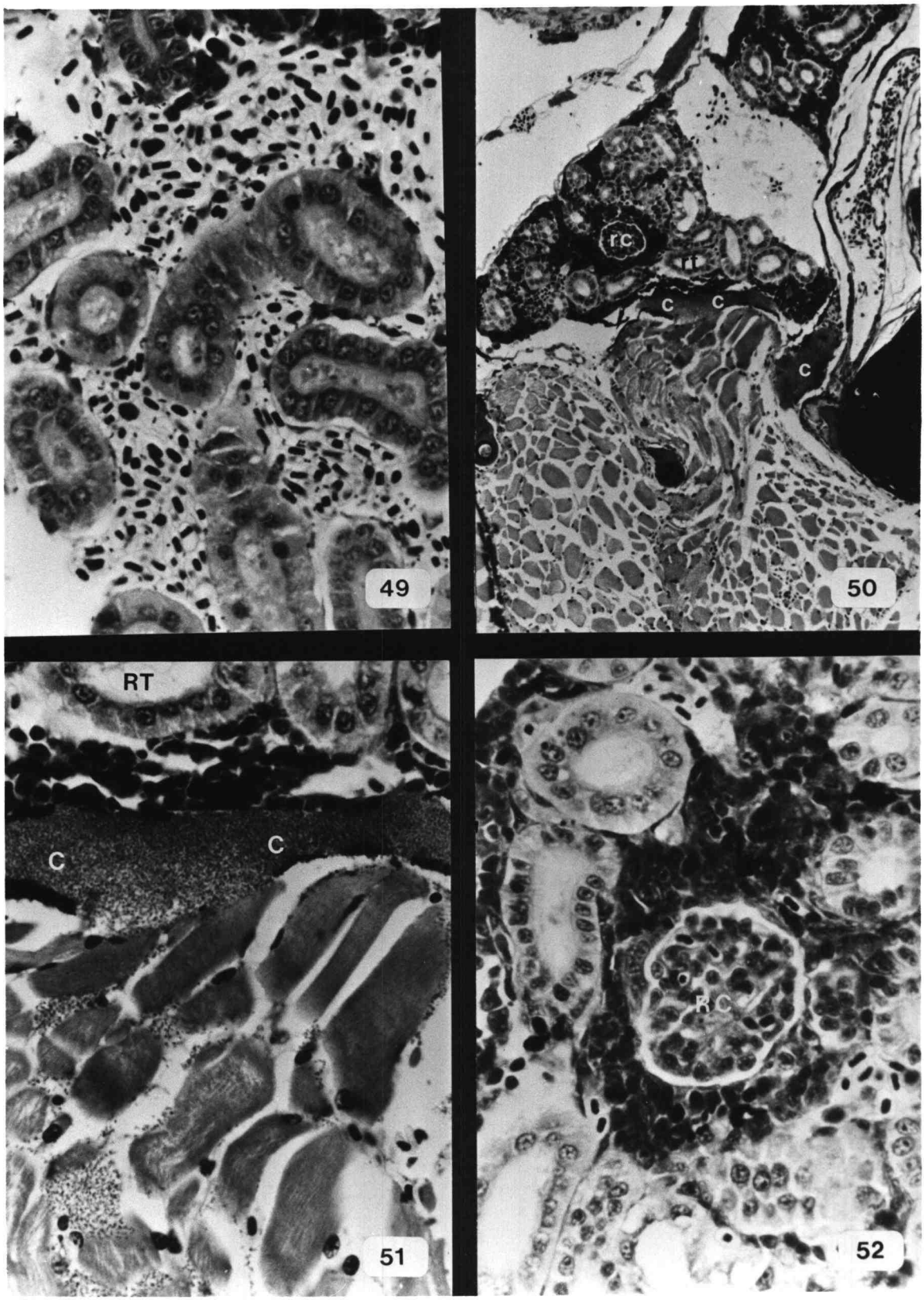
No bacteria were observed in kidneys of two chum salmon infected with V. anguillarum serotype II. However, small numbers of bacteria must have been present because pure cultures of V. anguillarum serotype II were recovered from the kidney and peritoneal fluid of fish in this study. Bacteria were occasionally observed in the kidney of the third chum salmon infected with serotype II. Colonies of bacteria were located immediately adjacent to the kidney of this third fish without spreading into the kidney tissue (Figures 50 through 52). The lact of bacteria in renal tissue adjacent to colonies of bacteria in muscle indicated that kidney tissue was not a preferred target of V. anguillarum serotype II. Kidneys of fish infected with serotype II were not edematous or congested and did not appear to contain increased numbers of stem cells.

Figure 49. Section through trunk kidney of chum salmon fingerling infected with V. anguillarum serotype I. Many bacteria were present in the blood while infection and necrosis in renal tubules was absent. X400.

Figure 50. Section through kidney and skeletal muscle of chum salmon fingerling infected with V. anguillarum serotype II. Muscle tissue was heavily infected while kidney tissue immediately adjacent to bacterial colonies (C) was not infected. Note renal corpuscle (RC) and renal tubule (RT) indicated as reference points for Figures 51 and 52. X100.

Figure 51. Magnification of Figure 50 showing highly infected muscle and non-infected kidney. X400.

Figure 52. Magnification of Figure 50 with no apparent bacteria in the kidney. Figures 50 through 52 indicate that kidney tissues are not preferred targets for V. anguillarum serotype II. X400.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rc
rt
c
c
c
50
RT
C
C
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RC
52

Integument

Vibrio anguillarum Serotype I

Small areas of focal infection were not common but could be observed in fins and under scales of chum salmon fingerlings (Figure 53). However, there was no necrosis observed in any layers of the skin. Because the epidermis over many infected areas was intact and not infected, it is presumed that V. anguillarum serotype I cells reached the skin via the blood rather than by an external route. Loose connective tissue between the dermis and underlying muscle was consistently infected with bacteria which were usually limited to the connective tissue without spreading to the dermis or musculature.

Vibrio anguillarum Serotype II

The skin of chum salmon naturally infected with V. anguillarum serotype II contained varying amounts of bacteria. Many areas were heavily infected with bacteria (Figure 54) while others were indistinguishable from skin of control fish. There was no pattern indicating that one area of skin was more susceptible to infection than another area. In many cases, colonies of bacteria extended through the epidermis, dermis, and underlying connective tissue. When this had occurred, loss of scales, hemorrhage, and sloughing of the skin were usually present. Frequently, breakdown of collagen fibers and

disorganization of the dermis was observed in these areas. Necrosis occurred only in areas where colonies of bacteria were present. Bacteria were commonly observed in the epidermis and dermis without accompanying necrosis. In many areas, the stratum compactum of the dermis was not penetrated by bacteria. In these areas, the epidermis and the dermal loose connective tissue surrounding scales were infected with colonies of bacteria while the stratum compactum contained few or no bacteria. This indicated that the stratum compactum acted as a barrier to infection. However, the barrier provided by the stratum compactum was not a significant host defense because once bacteria had penetrated the skin at any location, bacteria could spread along the underlying loose connective tissue and into areolar connective tissue in muscle.

Pancreas

Vibrio anguillarum Serotype I

Although blood within the pancreas contained many bacteria, lesions of the pancreas of chum salmon were uncommon. In areas where infection was present, bacteria were usually located in the islets of endocrine tissue rather than in exocrine tissue. While exocrine tissue was usually not infected, bacteria were commonly observed in edematous fluid and hemorrhages immediately adjacent

to the pancreas (Figure 55).

Vibrio anguillarum Serotype II

Bacteria were uncommon in the pancreas and were present in the islands of endocrine tissue rather than in exocrine tissue (Figure 56). Frequently, non-infected pancreatic tissue was adjacent to colonies of bacteria in a pyloric caecum or abdominal muscle.

Loose Connective Tissue

Vibrio anguillarum Serotype I

Chum salmon fingerlings infected with V. anguillarum serotype I contained many bacteria evenly dispersed throughout most loose connective tissues (Figures 39, 57, and 58). Loose connective tissues supporting the gastrointestinal tract were the most heavily infected, with some tissues containing as many bacteria as were present in blood.

Vibrio anguillarum Serotype II

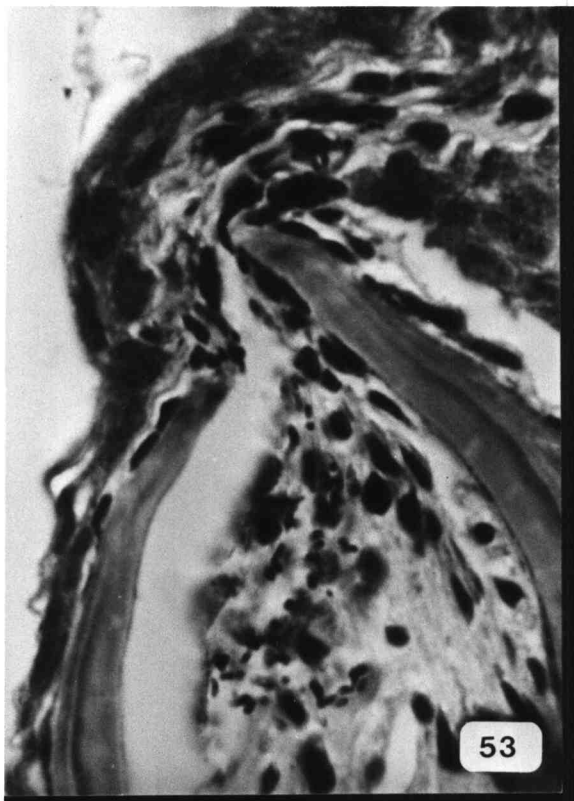
Bacteria were frequently observed in loose connective tissues of chum salmon fingerlings. However, the number of bacteria in loose connective tissues of these fish was consistently less than the number of bacteria in loose connective tissues of chum salmon

Figure 53. Section through base of pectoral fin of chum salmon fingerling infected with V. anguillarum serotype I. Focal infection of bacteria was located between two scales. X1,000.

Figure 54. Section of skin with bacterial colonies (C) and muscle without bacterial colonies in chum salmon fingerling infected with V. anguillarum serotype II. X100.

Figure 55. Bacteria in edematous fluid surrounding non-infected exocrine tissue in chum salmon fingerling infected with V. anguillarum serotype I. X1,000.

Figure 56. Section through pyloric caecum and pancreas of chum salmon fingerling infected with V. anguillarum serotype II. The islet of endocrine tissue in this photomicrograph is masked by a colony (C) of bacteria while the exocrine tissue contained almost no bacteria. X400.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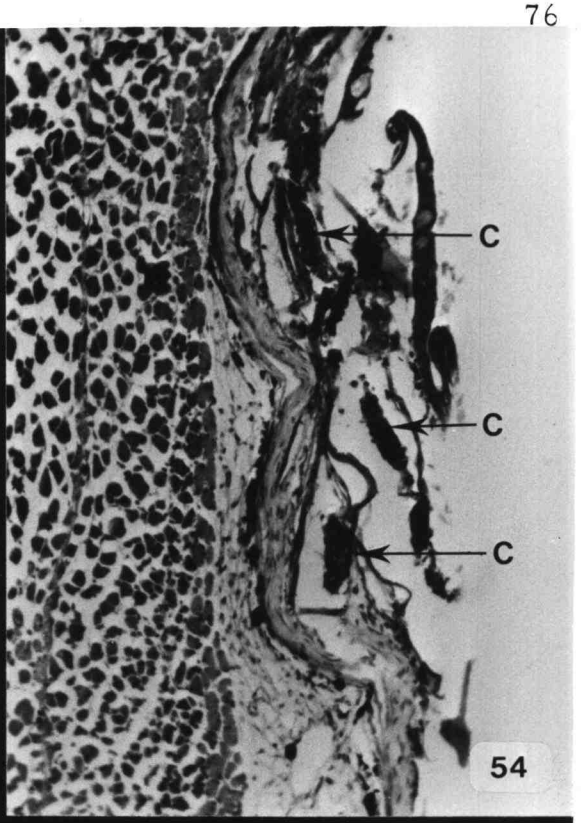
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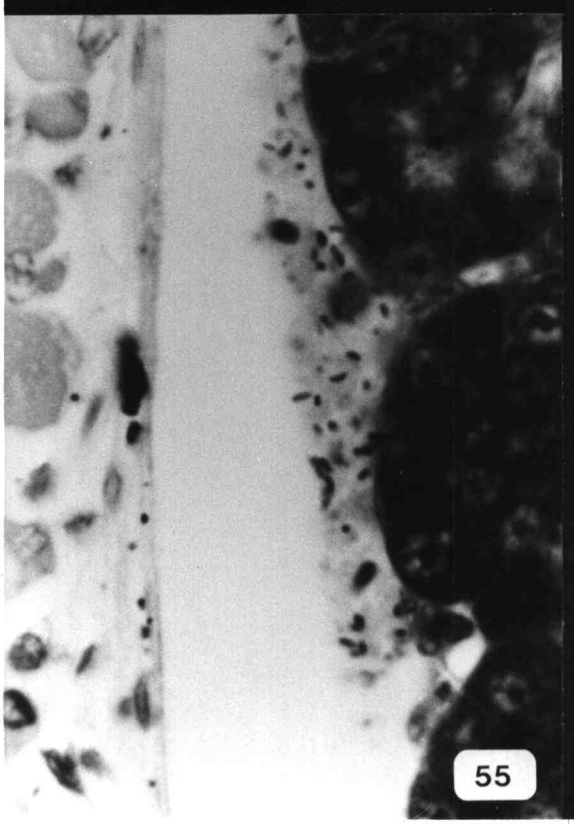
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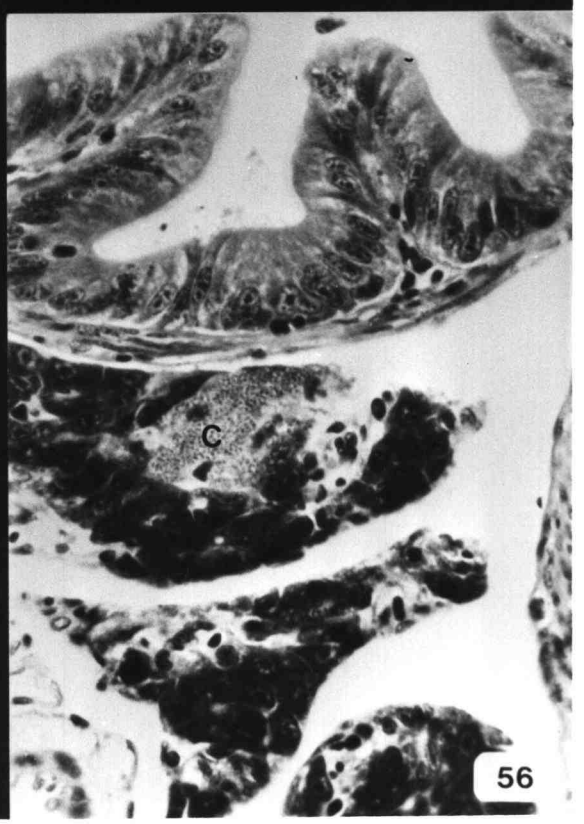
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infected with serotype I. Typical serotype II colonies were observed.

Gills

Vibrio anguillarum Serotype I

Vibrio anguillarum serotype I produced severe edema in gills of chum salmon. The lamellar epithelium was completely separated from the capillary bed in many lamellae (Figure 59). However, there was no noticeable infection of gill tissues even though many bacteria were present in the blood. Bacteria appeared to coat some red blood cells (5-10 per cell), but it is probable that this was due to the high concentration of bacteria rather than an attraction of bacteria to red blood cells. Some hemorrhaging was evident with blood cells located between filaments and between lamellae. Hyperplasia was not observed in gills of these fish.

Vibrio anguillarum Serotype II

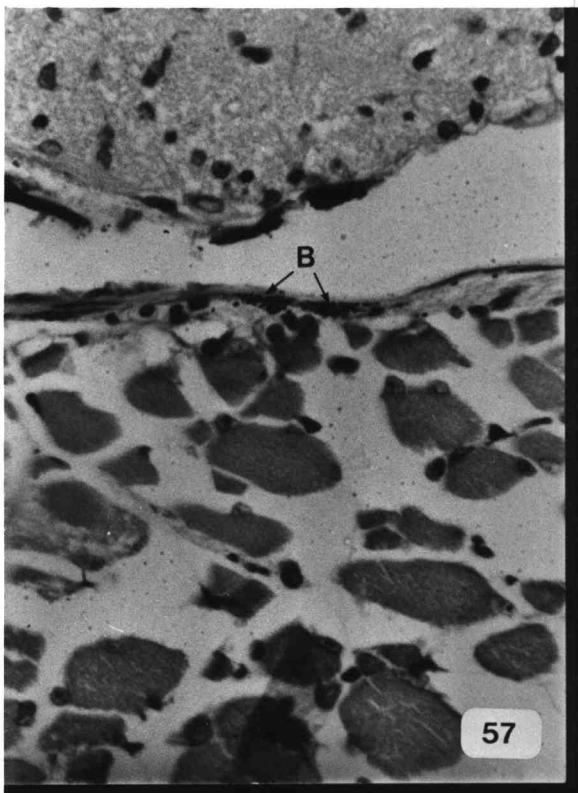
Severe edema was also present in gills of chum salmon naturally infected with V. anguillarum serotype II. The lamellar epithelium was completely separated from the capillary bed in many areas of the gills but no bacteria were observed in most lamellae (Figure 60). Bacteria were not observed in the blood but typical serotype II

Figure 57. Section through muscle and spinal cord of chum salmon fingerling infected with V. anguillarum serotype I. The perimysium was infected with bacteria (B) while skeletal muscle was not infected. Note spinal cord at top of figure is not infected. X400.

Figure 58. Infection of loose connective tissue between skeletal muscle and skin of chum salmon fingerling infected with V. anguillarum serotype I. It is difficult to distinguish bacteria (B) from melanin (M) in a black and white photomicrograph. X1,000.

Figure 59. Gill filament of chum salmon fingerling infected with V. anguillarum serotype I. Edema caused complete separation of the lamellar epithelium (LE) from the capillary bed. X400.

Figure 60. Gill filament of chum salmon fingerling infected with V. anguillarum serotype II. Edema caused complete separation of the lamellar epithelium (LE) from the capillary bed. X400.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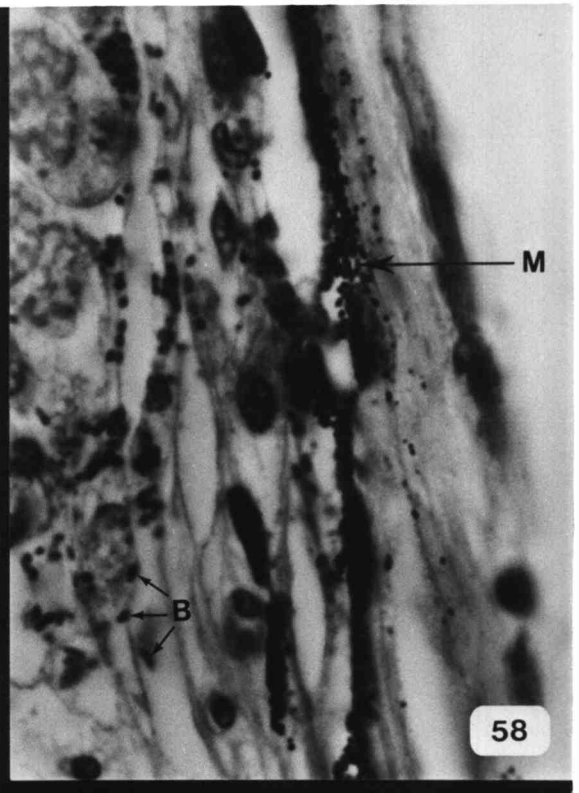
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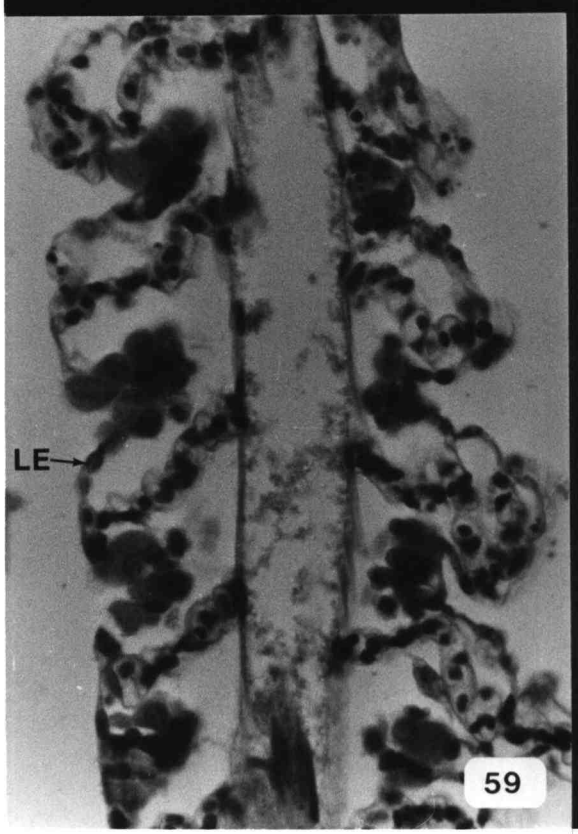
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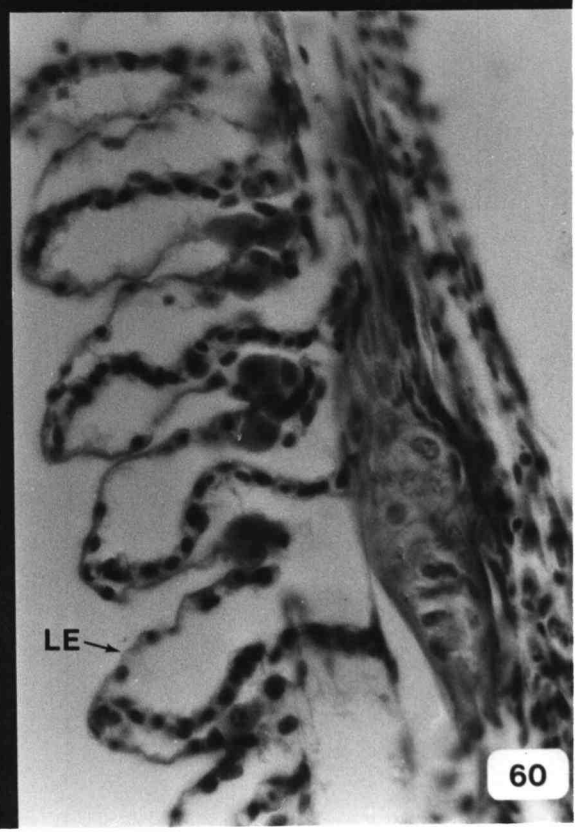
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colonies were occasionally observed under gill epithelium. Hyperplasia and hemorrhaging were not present in gills of these fish.

Spleen

Vibrio anguillarum Serotype I

The spleen of fish naturally infected with serotype I contained many bacteria. Pyknotic nuclei, edema, and necrosis were present throughout this organ (Figures 61 and 62). The spleen of moribund chums in this study appeared to be too heavily infected and necrotic to be functional. This pathology was consistent with the degree of infection of blood in these fish.

Vibrio anguillarum Serotype II

Few bacteria were present in the spleen of chum salmon infected with serotype II, but small colonies of bacteria with associated necrosis were occasionally observed (Figures 63 and 64). Most tissues in the spleen appeared to be normal when compared to spleen sections of control fish.

Other Organs

Gas Bladder

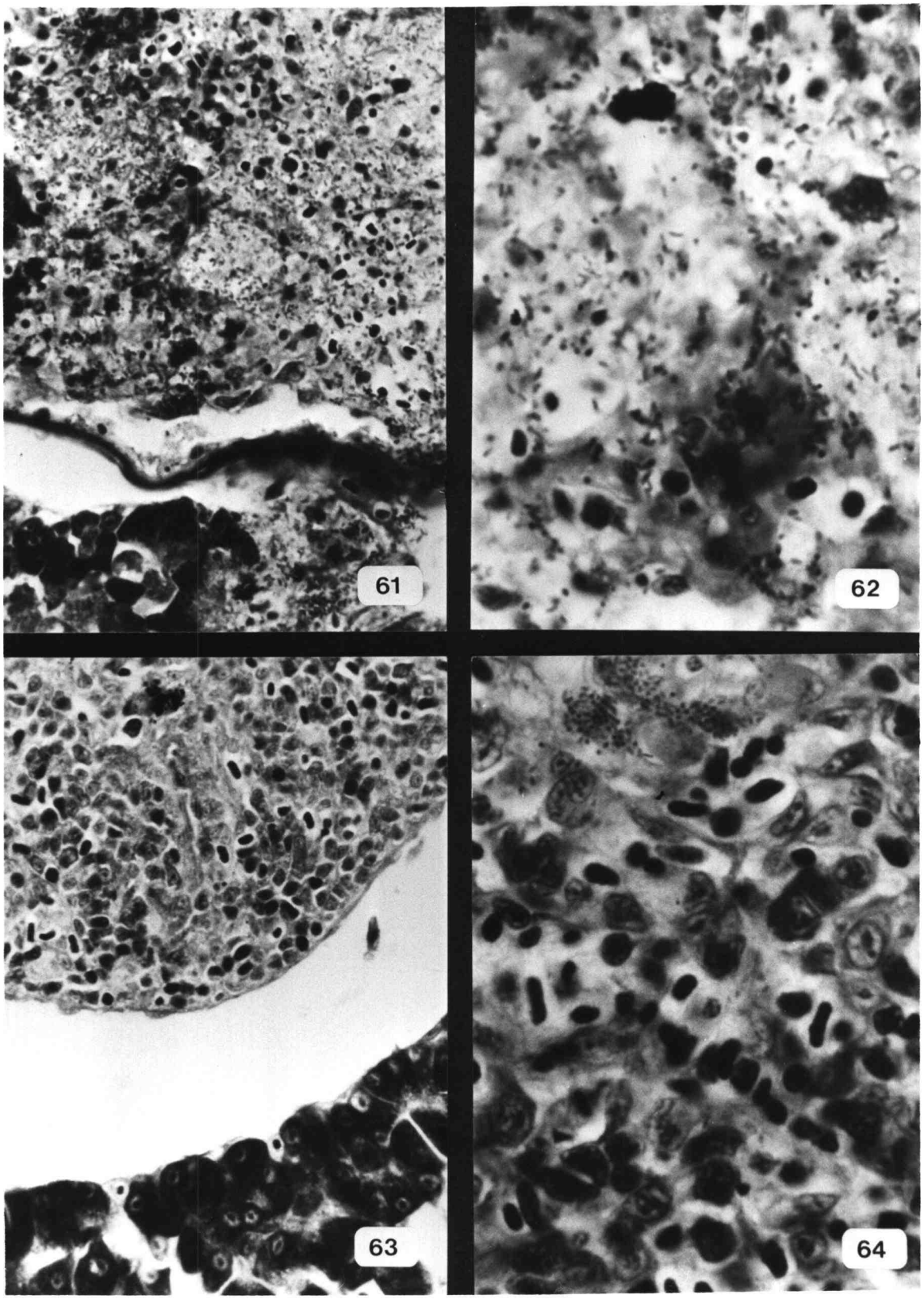
Small numbers of bacteria were observed in the tunica

Figure 61. Section through spleen of chum salmon fingerling infected with V. anguillarum serotype I. Many bacteria and extensive necrosis are present. X400.

Figure 62. Magnification of Figure 61 showing a focal area of necrosis of many bacteria present in the spleent. The pathology observed in the spleen was consistent with the number of bacteria in blood of chum salmon infected with V. anguillarum serotype I. X1,000.

Figure 63. Section through spleen and pancreas of chum salmon fingerling infected with V. anguillarum serotype II. The spleen was indistinguishable from spleens of non-infected control fish at this magnification. Lack of extensive pathology in spleens from fish infected with V. anguillarum serotype II was consistent with the lack of bacteria observed in blood. X400.

Figure 64. Focal area of infection in magnification of Figure 63. X1,000.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muscularis of the gas bladder in fish infected with V. anguillarum serotype I while surrounding connective tissues contained many evenly distributed bacteria. Vibrio anguillarum serotype II colonies were located adjacent to the intact lamina epithelialis of the gas bladder in fish infected with this agent.

Eye

No pathology was observed in eyes of chum salmon with naturally acquired infections of either serotype of V. anguillarum. This was not consistent with commonly occurring exophthalmos and pyoid lesions in eyes of salmonids with vibriosis. Observations in this laboratory indicate that lesions of the eye are more common in fish infected with serotype II than in fish infected with serotype I. These observations have been made during naturally and experimentally induced infections of chum, coho, and chinook salmon, and rainbow trout.

Eggs

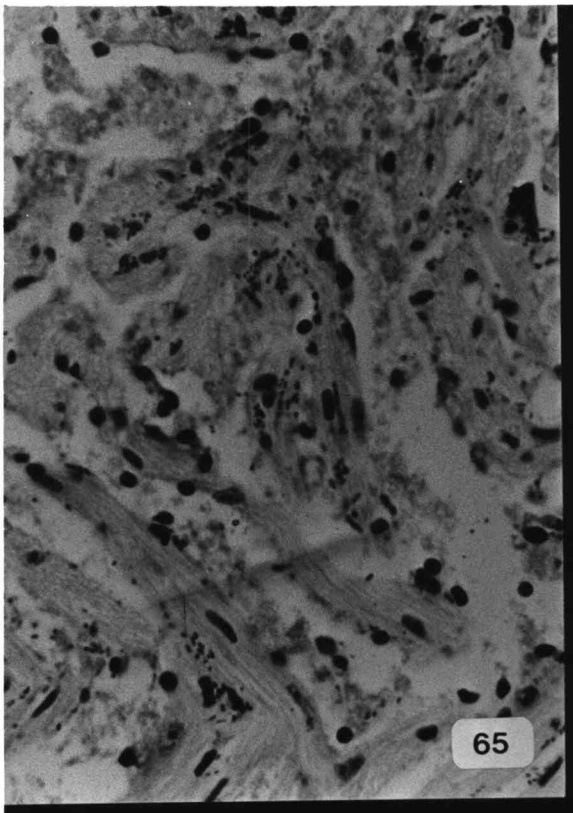
Immature eggs were present in chum salmon fingerlings weighing approximately one and one half grams. Bacteria were observed in peritoneal fluid immediately adjacent to non-infected eggs in fish from which either serotype of V. anguillarum had been recovered. Bacteria were never observed in eggs.

Figure 65. Section through heart of chum salmon fingerling infected with V. anguillarum serotype I. X400.

Figure 66. Section through heart of chum salmon fingerling infected with V. anguillarum serotype II. Colonies (C) of bacteria were located in cardiac muscle (exterior to cells) but few bacteria were present in blood of the heart. X400.

Figure 67. Extensive necrosis in rectum of chum salmon fingerling experimentally infected with V. anguillarum serotype I and sampled 48 h after water born exposure to live bacteria. X100. Compare with Figure 13.

Figure 68. Extensive necrosis in rectum of chum salmon fingerling experimentally infected with V. anguillarum serotype II and sampled 72 h after water born exposure to live bacteria. X100. Compare with Figure 13.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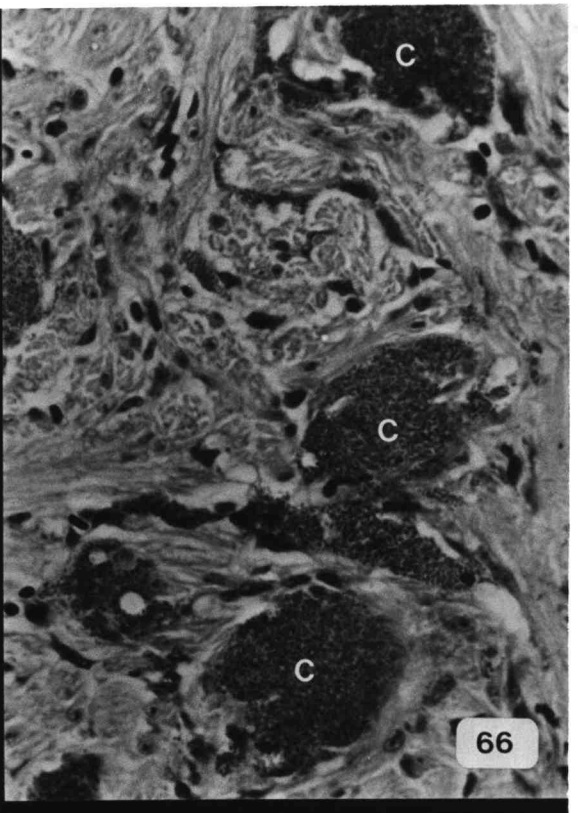
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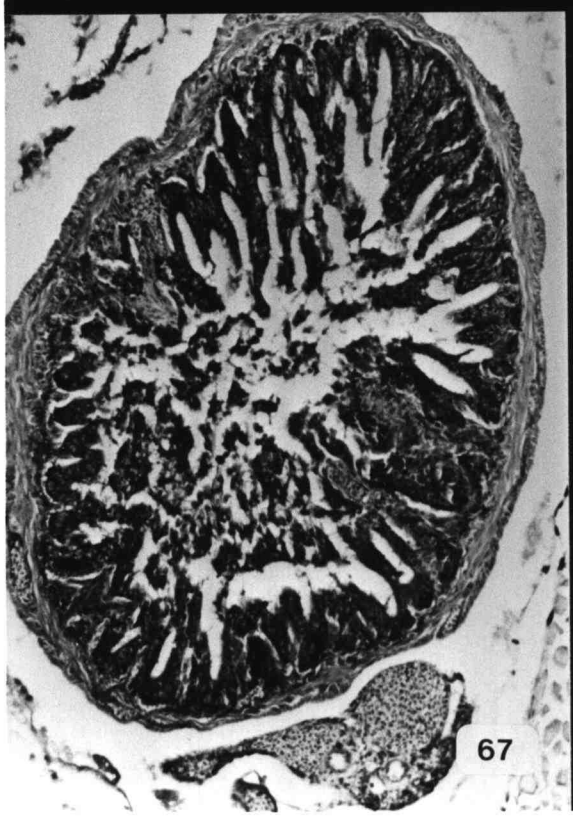
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TABLE 3. A Brief Comparison of the Sites of Infection for Vibrio anguillarum serotype I and Vibrio anguillarum serotype II in chum salmon (O. keta) fingerlings.

Organ or Tissue Examined	Vibrio anguillarum serotype I	Vibrio anguillarum serotype II
Blood	+++[a]	-
Loose Connective Tissue	+++	+
Kidney	+++	±
Spleen	+++	+
Skeletal Muscle	+	+++
Heart	++	+++
Anterior Gastrointestinal Tract	+	+++
Posterior Gastrointestinal Tract	+++	+++
Integument	±	++
Liver	+	+
Pancreas	+	+
Gills	+++	+++
Bacteria in Colonies	No	Yes
Bacteria Evenly Dispersed	Yes	No

[a](-) bacteria not observed, (+) bacteria observed, (++) pathology or many bacteria observed, (+++) main site of infection.

Spinal Cord and Brain

Bacteria were not observed in tissues of the spinal cord or brain in any fish in this study.

Thymus

Chum salmon fingerlings infected with V. anguillarum serotype I contained many bacteria in blood of the thymus but no bacteria were observed in other tissues of this organ. No bacteria were observed in any area of the thymus of fish infected with serotype II.

Heart

The heart of chum salmon infected with V. anguillarum serotype I contained many evenly dispersed bacteria which were usually associated with blood (Figure 65). Cardiac muscle in fish infected with serotype II contained many colonies of bacteria (Figure 66). Necrosis associated with both serotypes did not appear to be severe.

Histopathology Study of Experimentally Induced Vibriosis in Chum Salmon Fingerlings

Major differences observed in the pathology associated with naturally acquired infections of V. anguillarum serotypes I and II in chum salmon were also observed in chum salmon fingerlings with experimentally induced infections of vibriosis. Each serotype of

V. anguillarum infected similar tissues and produced the same types of histological changes that were present in naturally acquired infections. Experimental infections of V. anguillarum serotype II appeared to be more severe than natural infections, with a greater number of bacteria in fish and infection of more organs.

Histological sections from ten moribund chum salmon fingerlings which had been experimentally infected with V. anguillarum serotype I were indistinguishable from sections of chum salmon fingerlings which had been naturally infected with this organism. Blood, loose connective tissues, kidney, and spleen were heavily infected with bacteria which were evenly distributed throughout the tissues. Mucosal epithelium of the anterior gastrointestinal tract was intact but the ascending and descending intestines contained massive sloughing of mucosal epithelial cells and most annulo-spiral septa were absent in the rectum because of necrosis. Infection was not observed in the skin or underlying skeletal muscle as might have been expected in fish exposed to approximately 7.0×10^6 cells of V. anguillarum serotype I per ml water. Some areas of gills were edematous with complete separation of lamellar epithelium from the capillary bed.

Although the same tissues were infected and in vivo colonies of bacteria were formed in naturally and experimentally induced V. anguillarum serotype II infections of chum salmon, histological sections of naturally and experimentally infected fish were different.

The sections differed in the following four ways: 1) colonies between the intact peritoneum and the mucosal epithelium of the gastrointestinal tract were less frequent in fish with experimentally induced infections than in fish with naturally acquired infections; 2) epithelial cell sloughing in the ascending intestine and pyloric caecae was more extensive in fish with experimentally induced infections than in fish with naturally acquired infections; 3) bacteria were observed in more organs of fish with experimentally induced vibriosis than in fish with naturally acquired vibriosis caused by serotype II. The liver and spleen of infected fish contained colonies of bacteria. One of ten fish examined contained many colonies of bacteria in the kidney while colonies were occasionally observed in kidneys of eight of the remaining nine fish. Colonies of bacteria were occasionally observed along the inside wall of blood vessels in the one fish that contained many bacteria in the kidney; 4) the skin and underlying muscle were uniformly infected in fish with experimentally induced infections and did not contain bacteria in localized areas which occurred in fish with naturally acquired infections of V. anguillarum serotype II. Bacteria were present in high numbers in outer layers of skeletal muscle of fish with naturally acquired infections while very few bacteria were present in inner layers of skeletal muscle of fish with experimentally induced infections of V. anguillarum serotype II. These differences may have been due to rapid onset of disease and the high concentration

of bacteria used in experimentally induced infections with this organism.

White blood cells were not frequently observed in fish exposed to V. anguillarum serotype I and II. The only cellular response observed in fish infected with V. anguillarum serotypes I and II was occasional phagocytosis of bacteria.

Evidence indicating that both serotypes of V. anguillarum enter hosts by penetrating the wall of the descending intestine and rectum was obtained through histological examination of fish sampled before gross signs of disease were observed. Bacteria were not recovered from nor pathology observed in any of three fish exposed to serotype I or in any of three fish exposed to serotype II which were sampled four hours post-exposure. Three fish which were exposed to V. anguillarum serotype I and sampled 48 h post-exposure contained necrosis in the mucosa and muscularis of the descending intestine and rectum (Figure 67). Histopathology was not observed in any other tissues in these fish. Vibrio anguillarum serotype I was recovered from kidney tissue of the three fish sampled 48 h post-exposure but bacteria were not observed in stained sections of kidney. Similar data was collected with fish exposed to V. anguillarum serotype II which were sampled 72 h post-exposure. Extensive necrosis was present in the mucosa and muscularis of the descending intestine and rectum (Figure 68). Bacteria were not recovered from kidney tissue nor

pathology observed in other internal tissues of fish exposed to V. anguillarum serotype II and sampled 72 h post-exposure. External infection was indicated as a second route of entry for V. anguillarum serotype II. Small focal areas of V. anguillarum serotype II infection were observed in epithelial cells covering scales at 48 h post-exposure (Figure 69). Fish sampled 72 h post-exposure contained colonies of serotype II cells under scales (Figure 70). Infection by serotype II cells had spread into muscle immediately beneath the skin of fish sampled at 140 h post-exposure (Figure 71) but not into the deep layers of muscle.

Few bacteria were shed from fish before gross external signs of disease were apparent. Concentrations of V. anguillarum serotype I could not be accurately estimated in water at 24 h and 48 h post-exposure because few colonies were present on BHI agar plates. Concentrations of V. anguillarum serotype I rose to approximately 2.3×10^2 cells/ml and 2.1×10^2 cells/ml at 72 h and 96 h post-exposure. The increase in bacterial concentration coincided with signs of disease and mortality among these fish. Vibrio anguillarum serotype II concentrations in water followed a similar pattern with the exception that shedding of bacteria, signs of disease, and mortality of fish occurred at 196 h post-exposure. At this time, V. anguillarum serotype II concentrations rose to approximately 4.1×10^3 cells/ml.

Moribund chum salmon which had been exposed to V. anguillarum

serotype I, placed in sterile PBS, and ground in a blender for quantitative estimation of the extent of infection were found to contain many bacteria. The number of bacteria per gram of fish as estimated by standard plate count technique was 1.3×10^8, 1.2×10^8, and 1.1×10^8 for three different moribund fish. Three moribund fish exposed to V. anguillarum serotype II contained 2.1×10^9 bacteria/g fish, 7.4×10^8 bacteria/g fish, and 2.7×10^9 bacteria/g fish. Selected colonies from BHI agar plates were all identified as the appropriate serotype by rapid slide agglutination tests.

Studies with Experimentally Induced Vibriosis in Coho Salmon

The histopathological changes associated with experimentally induced infections of vibriosis in chum salmon fingerlings were also observed in coho salmon smolts exposed to high concentrations of either V. anguillarum serotype I or V. anguillarum serotype II. The pathology unique to each serotype which was observed in chum salmon was also observed in coho salmon. Bacterial colonies of V. anguillarum serotype II were not as prevalent in coho salmon as they were in chum salmon. Early formation of serotype II colonies in pyloric caecae of one fish, sampled before overt signs of disease were present, indicated that spread of infection throughout the gastrointestinal tract might occur via peritoneal fluid. These colonies were small and located between the peritoneum and the muscularis of the pyloric

caecum (Figure 72). Other pathology observed in coho salmon will not be discussed because it was essentially the same as the pathology observed in chum salmon which was previously described in detail.

Levels of bacteria in blood were higher in fish infected with serotype I than in fish infected with serotype II. Blood from moribund fish infected with serotyype I contained 4.0×10^6 to 2.1×10^8 bacteria per ml. Blood from moribund fish infected with serotype II contained 5.0×10^3 to 2.9×10^4 bacteria per ml. These levels of V. anguillarum serotype II in blood did not appear to be consistent with results of histopathology studies because bacteria were rarely observed in stained blood. The discrepancy was dependent upon the number of bacteria required for microscopic detection and is explained in the results of the experiment with experimentally induced vibriosis in chinook salmon.

Studies with Experimentally Induced Vibriosis in Chinook Salmon

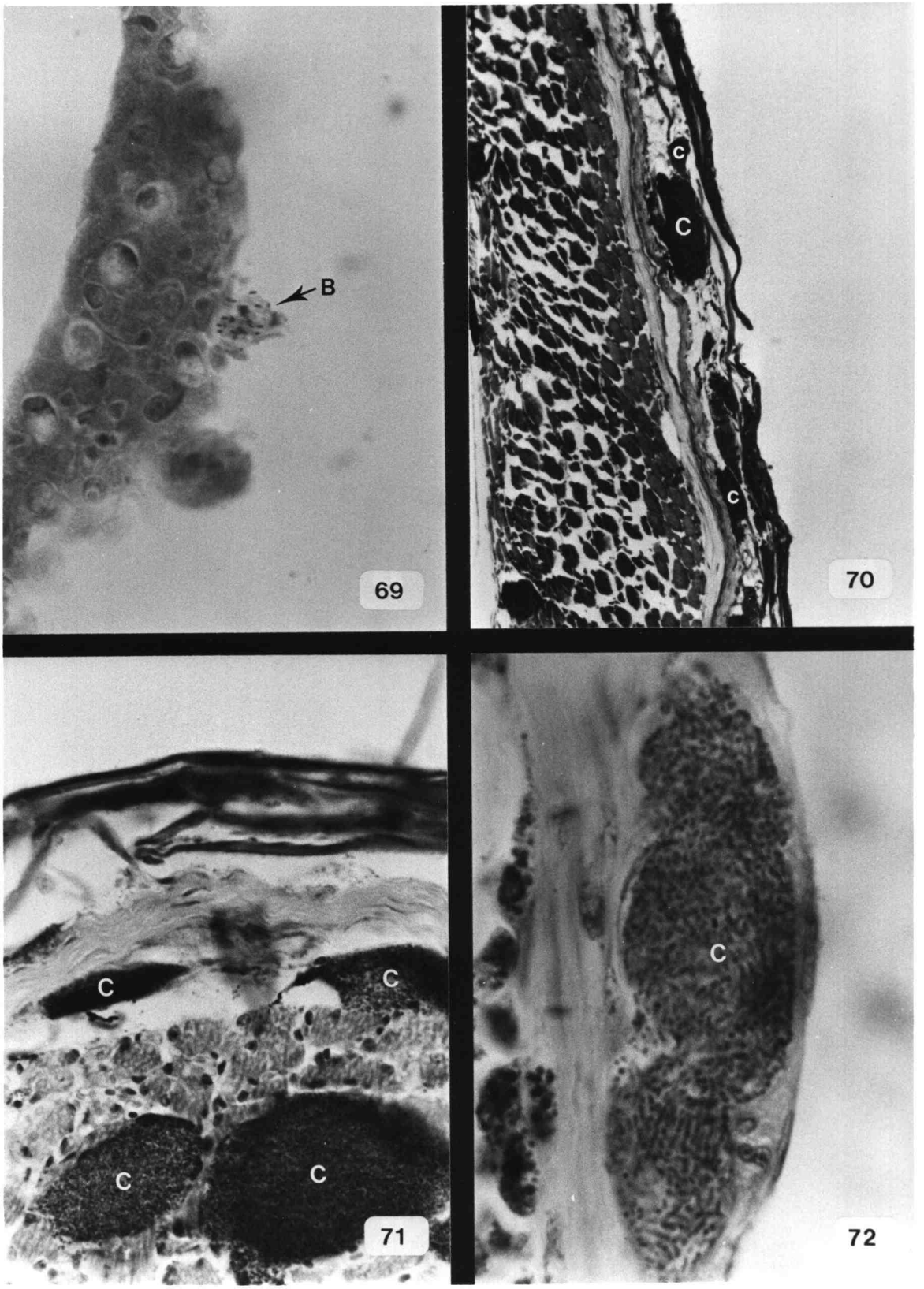
Histopathology of experimentally induced vibriosis in chinook salmon was similar to the histopathology observed in experimentally induced infections of chum and coho salmon. The main targets and pathology unique to each organism in the previous experiments were no different in this study. Colonies of V. anguillarum serotype II were less common in tissue of the gastrointestinal tract of chinook

Figure 69. Focal infection of V. anguillarum serotype II in epithelial cells covering a scale in fish sampled 48 h after water born exposure to live bacteria. Bacteria were not recovered from kidney tissue or peritoneal fluid of this fish. B = bacteria. X1,000.

Figure 70. Colonies (C) of V. anguillarum serotype II under scales in chum salmon fingerling sampled 72 h after water born exposure to live bacteria. X100.

Figure 71. Colonies (C) of V. anguillarum serotype II in skeletal muscle adjacent to skin of chum salmon fingerling sampled 140 h after water born exposure to live bacteria. X400.

Figure 72. Colony (C) of bacteria between the peritoneum and muscularis of a pyloric caecum in a coho salmon experimentally infected with V. anguillarum serotype II. Bacterial colonies in this fish, which was sampled during early stages of disease, were not located near the mucosa. The number of eosinophilic granule cells adjacent to muscularis is not abnormally high. X1,000.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salmon than were observed in chum salmon.

Vibrio anguillarum serotype I occurred in blood of diseased fish at levels ranging from 3.0×10^3 cells/ml to 2.4×10^8 cells/ml. Blood from a fish which had expired just prior to sampling contained 7.0×10^8 cells/ml. Fish from which bacteria were not recovered from kidney tissue did not have bacteria in their blood. Examination of Giemsa-stained blood smears revealed that 3.0×10^6 cells/ml were required for microscopic observation of bacteria in blood. Four bacteria were observed in 20 fields of view at 400X in a stained blood smear from blood which contained 3.0×10^6 cells/ml. Bacteria were not observed in stained smears of blood containing less than 3.0×10^6 cells/ml.

Vibrio anguillarum serotype II was present in blood of diseased fish at levels ranging from 1.0×10^3 cells/ml to 1.5×10^6 cells/ml. Bacteria were not observed in Giemsa-stained blood smears of any fish. This data indicated that fish previously examined for histopathology associated with V. anguillarum serotype II probably suffered from a bacteremia which was not detectable using microscopic methods. However, levels of serotype I in blood were usually 10^2 to 10^3 times greater than levels of serotype II in blood: this confirmed a difference in pathology associated with infections of the two organisms.

The pH of the gastrointestinal tract of non-infected control fish

varied greatly within each fish. The following values of pH were recorded for five healthy chinook salmon in salt water: stomach 2.1 to 3.8, ascending intestine 6.6 to 6.8, descending intestine 6.7 to 7.0, and rectum 7.5 to 8.0.

Growth studies with V. anguillarum serotype I indicated that this organism cannot grow below pH = 6.0 on BHI agar. Vibrio anguillarum serotype II did not grow below pH = 6.5 on BHI agar.

Changes in pH of the gastrointestinal tract occurred when fish became diseased. This was determined by measuring the pH of the stomach and rectum of fish that were sampled (the pH of the ascending and descending intestines was not measured). The pH of the stomach of fish infected with serotype I was less acidic than non-infected control fish; pH of stomachs ranged from 4.4 to 6.6 in these fish. Changes in pH of the stomach accompanied increased levels of bacteria in blood (Table 4). The pH of the stomach of the fish that was sampled just after it had expired was 7.1. Two fish from which V. anguillarum serotype I was not recovered had stomachs with a pH of 2.1 and 2.8 respectively. Similar but less drastic changes occurred with fish infected with serotype II (Table 4). The pH of the stomach of these fish ranged from 2.8 to 5.0. The pH of the rectum of fish infected with serotype I or II ranged from 7.0 to 8.0 but was consistently lower than the pH of control fish rectums. The pH of gastrointestinal tracts of fish that survived exposure to V. anguillarum

serotypes I or II was similar to the pH observed in non-infected control fish.

Severe decreases in numbers of leukocytes accompanied high levels of bacteria in the blood of chinook salmon infected with vibriosis (Table 4). Fish with 5×10^7 to 2×10^8 cells of serotype I per ml blood suffered a 90% to 95% decrease in leukocytes. Although V. anguillarum serotype II never exceeded 1.5×10^6 bacteria/ml blood in fish of this experiment, decreases in leukocyte counts of 80% and 90% occurred in two fish with 1.0×10^6 bacteria/ml blood and 1.5×10^6 bacteria/ml blood respectively. Data presented in Table 4 indicates correlations between bacteria in blood, decrease in leukocytes, and changes in pH of the gastrointestinal tract of fish infected with vibriosis. This experiment was undertaken twice because fish did not become infected with serotype II during the first trial. However, similar data concerning bacteria in blood, destruction of leukocytes, and changes in pH of the gastrointestinal tract was collected from fish infected with serotype I in the first trial.

TABLE 4. Changes in Blood and the Gastrointestinal Tract of Chinook Salmon (*O. tshawytscha*) with Experimentally Induced Vibriosis.

Number of Fish	Infecting Agent	Bacteria in Blood (per ml)	White Blood Cell Count (per ml)	pH of stomach	pH of rectum
5	None (controls)	0	3.4×10^4 to 7.0×10^4	2.1 to 3.8	7.5 to 8.0
7	*V. anguillarum* serotype I	2.0×10^3 to 3.0×10^6	1.9×10^4 to 6.2×10^4	2.1 to 6.5	7.0 to 7.9
7	*V. anguillarum* serotype I	4.0×10^6 to 2.4×10^8	1.6×10^3 to 6.0×10^3	5.4 to 7.7	7.0 to 8.0
6	*V. anguillarum* serotype II	0 to 9.0×10^5	1.1×10^4 to 4.8×10^4	2.8 to 5.0	7.3 to 7.8
2	*V. anguillarum* serotype II	1.0×10^6 to 1.5×10^6	5.7×10^3 to 7.2×10^3	3.1 to 3.2	7.6

DISCUSSION

Many disease problems from Vibrio spp. associated with severe external lesions were observed among six species of fish in these studies. While good evidence of pathogenicity was gathered for only one isolate, many Vibrio spp. were recovered in pure culture from kidney tissues and external lesions and are believed to be pathogenic to fish. These bacteria may or may not be new isolates of V. anguillarum.

Vibrio sp. isolate MSC-1-76 was repeatedly recovered from coho salmon at the OSU Marine Science Center and caused an epizootic among rainbow trout reared in salt water by Oregon Aqua-Foods Inc. This bacterium produced severe external lesions posterior to the dorsal fin and was usually not systemic in early stages of infection. The disease progressed rapidly with death usually occurring two to three days after the appearance of lesions and was transmitted among fish. Infections of Vibrio sp. MSC 1-76 were not preceded by mechanical damage of skin or fins and occurred among fish in cement raceways as well as a variety of types of fish tanks. Although myxobacteria are most frequently associated with skin and fin erosion in saltwater fishes, Hoskins and Hulstein (1977) reported isolating a Vibrio sp. from coho salmon with external lesions. These salmon had been immunized against V. anguillarum.

Chum and pink salmon were very susceptible to vibriosis and frequently suffered 95% to 100% mortality. It is likely that high mortality from vibriosis does not occur among these species of salmon

in natural habitats because they migrate directly to the ocean without long term exposure to stress and disease in estuaries. However, if mortality from V. anguillarum does occur in the open ocean, chum and pink salmon would be good choices as indicator organisms for detection of vibriosis. Neither of these species should be incorporated into a mariculture program designed to rear fish in an estuary where V. anguillarum is enzootic. Chum and pink salmon held at the OSU Marine Science Center usually experienced epizootics after being exposed to untreated Yaquina Bay water for approximately one month.

Coho salmon which were given boosters of oral vaccine in salt water possessed a slight increase in protection from infection by the homologous serotype of V. anguillarum. Statistical analyses could not be made with data from these experiments due to disease problems caused by Vibrio spp. However, it was determined that administering V. anguillarum bacterin orally while fish were exposed to natural levels of V. anguillarum in salt water did not increase susceptibility to vibriosis. Some groups of fish received vaccine diet exclusively while in salt water when vibriosis epizootics occurred among other fish. Prior to this study members of this and other laboratories suspected that administration of vaccine to fish in the presence of disease might result in increased susceptibility to the disease because antibodies and phagocytic cells would be blocked by antigens.

There was little or no protection from infection by the

heterologous serotype when V. anguillarum serotype I or serotype II bacterin was administered orally to coho salmon. Similar data has been collected in other studies using different means of administering V. anguillarum bacterin (Johnson, personal communication; Tebbit, personal communication; Gould, 1977). These data indicate that common antigens identified by bands of identity in double immunodiffusion tests with V. anguillarum serotypes I and II are not antigens which elicit immune responses for protection in fish.

Mortality from vibriosis among fish which had been vaccinated against V. anguillarum serotypes I and II did not indicate that protective immune responses failed to occur among fish held at the OSU Marine Science Center and Oregon Aqua-Foods Inc. Because protection by active immunity is not absolute, factors such as stress must be considered when evaluating the efficacy of a vaccine. It is likely that immunized salmonids at the OSU Marine Science Center and Oregon Aqua-Foods Inc. possessed protective immunity against vibriosis but that the degree of protection was reduced by stress from fluctuation in salinity and/or water temperature. In addition, the health of these fish was affected by pathogenic Vibrio spp.

During the time that these studies were conducted, Oregon Aqua-Foods Inc. converted their mariculture program from one emphasizing intensive saltwater rearing of fish to one which is exclusively extensive in the saltwater phase except for a brief holding period for

imprinting fish prior to ocean release. One of the reasons that Oregon Aqua-Foods Inc. changed their mariculture program was that mortalities of approximately 13% to 30% occurred among fish reared in Yaquina Bay water. Fish released to the ocean will not be subjected to stress in Yaquina Bay and should not experience high mortality from disease.

Infections of V. anguillarum serotypes I and II were common in fish exposed to untreated Yaquina Bay water above 12 C and uncommon in fish exposed to untreated Yaquina Bay water below 11 C. However, a direct correlation between disease incidence and water temperature could not be made because temperature fluctuation was most extreme at times when the bay water was the warmest. Incidence of vibriosis at Lint Slough in Waldport, Oregon has been highest in summer when water temperatures exceeded 12 C (Rohovec, personal communication). Unpublished reports of vibriosis epizootics among intensively reared salmonids at mariculture facilities in the Pacific Northwest have been most common during summer months when fish are held in warm water. Didier (1974) reported that mortality of chinook salmon infected with vibriosis correlated with water temperature when fish were stressed by handling. Data presented by Fryer and Pilcher (1974) and Fryer et al. (1976) indicate that many other diseases cause increased mortality among salmonids held at temperatures above 12 C.

Vibrio anguillarum was isolated from diseased fish and untreated

Yaquina Bay water. Limited attempts to isolate this organism from Yaquina Bay mud and healthy wild fish were unsuccessful. Didier (1974) isolated V. anguillarum from water and diseased fish but was unable to recover this organism from plankton or fowling material on saltwater net pens. Vibrio anguillarum was isolated more frequently from fish and water at the OSU Marine Science Center after introduction of large numbers of salmonids at Oregon Aqua-Foods Inc. than before introduction of these fish. During 1973 and 1974 non-vaccinated coho held at the Marine Science Center were never known to be infected with vibriosis. Vibriosis has caused mortality among all groups of non-vaccinated coho from the spring of 1975 to the present. This data is similar to data concerning mortality from vibriosis among non-vaccinated fish at other saltwater or brackish water aquaculture projects. Non-vaccinated salmon were initially reared without mortality from vibriosis at the Oregon Department of Fish and Wildlife Lint Slough rearing station in Oregon and at the National Marine Fisheries Service Little Port Walter experiment station in Alaska. However, vibriosis epizootics occurred in later years and continue to cause problems among non-vaccinated fish reared at these two locations.

Data concerning quantitative estimation of V. anguillarum in salt water indicate that while normal levels of V. anguillarum are low in Yaquina Bay, large numbers of organisms are shed from

salmonids infected with vibriosis. Brain Heart Infusion Agar (DIFCO) was the best of five media tested for enumerating V. anguillarum in salt water. Normal levels of V. anguillarum in Yaquina Bay water were ten or less cells/ml. Effluent water from tanks of fish suffering from vibriosis contained 1.0 to 4.7 x 10^3 cells/ml. High concentrations of V. anguillarum from small groups of fish were diluted with no noticeable effect on levels in bay water. However, future projects at Oregon Aqua-Foods Inc. and the OSU Marine Science Center include very large numbers of salmonids which may shed enough bacteria to increase levels of V. anguillarum in Yaquina Bay. Didier (1974) enumerated "Vibrio anguillarum-like" bacteria in water from Henderson Inlet, Washington and determined that levels were consistently about 10^3 cells/ml and independent of vibriosis epizootics.

Data collected from experiments with artificially induced vibriosis in chum salmon substantiates that large numbers of bacteria can be shed from infected fish. Moribund chum salmon infected with V. anguillarum serotype I contained approximately 1.2 x 10^8 bacteria/g fish tissue. Moribund chum salmon infected with V. anguillarum serotype II contained approximately 1.0 x 10^9 bacteria/g fish tissue. Bacteria shed into water from live fish in this experiment reached approximately 2.3 x 10^2 cells/ml with V. anguillarum serotype I and approximately 4.1 x 10^3 cells/ml with V. anguillarum serotype II. Fish were not allowed to die and degrade in tanks in

this experiment. Fish that die from vibriosis and go unnoticed at aquaculture facilities would shed many times the number of bacteria that were shed from live fish in this study. It is hoped that these data will encourage fish culturists to remove dead fish from saltwater holding facilities as frequently as possible.

Three isolates of V. anguillarum which are assumed to be different serotypes and a Vibrio sp. (isolate MSC 1-76) were similar according to biochemical reactions but dissimilar when compared using double immunodiffusion tests. In addition, at least three of these organisms produced unique pathology in salmonids (data concerning pathology produced by V. anguillarum isolate 507 were not collected). Therefore, biochemical reactions were not very useful for comparing these pathogenic vibrios. Evelyn (1971) suggested that the system of dividing isolates of V. anguillarum into three biochemical types (Nybelin, 1935; Smith, 1961) should be abandoned and that pathogenicity should not be considered an important criterion for determining species. Evelyn indicated that the 20 strains of V. anguillarum, with which he compared data from the literature, should be considered as variants of this species. Many different Vibrio spp. were isolated from diseased fish in the present study. It is the opinion of this author that many variants of V. anguillarum may be present in Yaquina Bay and that the virulence of these variants could differ greatly. Although Bergy's Manual of Determinative Bacteriology

might recognize more than five species of vibrio at some time in the future, it seems apparent that most isolates of Vibrio sp. pathogenic to fish will never be accepted as separate species and should be considered variants of V. anguillarum.

Differences in pathology associated with infections of V. anguillarum serotypes I and II were consistent among a small number of chum salmon with naturally induced infections of vibriosis. Histopathologic examination of chum salmon with experimentally induced infections of vibriosis substantiated these differences and indicated that experimental infection by water born exposure to live V. anguillarum results in pathology which is similar to the pathology observed in fish with naturally acquired infections of vibriosis. Studies with experimentally induced infections of vibriosis in coho and chinook salmon indicated that the differences in pathology produced by serotype I and serotype II were not unique to infection of chum salmon. It is apparent from these data that water born challenges of V. anguillarum should be used when testing the efficacy of vaccines made to protect fish from vibriosis. Experimental infections of V. anguillarum serotype II were more severe than natural infections which indicates that the pathology may vary between chronically and acutely infected fish.

Differences in pathology associated with different serotypes or biotypes of V. anguillarum have not been reported by other

investigators. However, it is probable that differences would have been observed by others if fish infected with different isolates of V. anguillarum had been included in their studies. The pathology of a Vibrio sp. infection in rainbow trout and amago salmon (O. roduras) described by Miyazaki and Kubota (1977) was similar to the pathology produced in salmonids infected with V. anguillarum serotype II (isolate MSC 2-75) in this study. Harbell (1976) described some histopathologic changes similar to changes observed in fish of the present study which were infected with V. anguillarum serotype I (isolate MSC 1-73) and did not report observing colonies of bacteria in fish. It is probable that the organism in Miyazaki's and Kubota's study was similar to V. anguillarum serotype II (isolate MSC 2-75) and that the organism in Harbell's study was similar to V. anguillarum serotype I (isolate MSC 1-73).

One difference associated with the pathology produced by V. anguillarum serotypes I and II was the form of bacterial growth within fish tissues. Serotype I cells were evenly dispersed throughout tissues while serotype II cells grew in colonies. A possible explanation for this difference is that surface components of serotype I cells could cause bacteria to repel one another and surface components of serotype II cells could cause bacteria to attract one another.

Although isolation of bacteria from kidney and blood indicated that V. anguillarum serotype I and II produced bacteremias in fish,

bacteria were not observed in blood of fish infected with serotype II. Pacha and Ordal (1967) collected similar data with columnaris disease in juvenile salmon. These investigators were unable to observe cells of Flexibactor columnaris in stained sections of tissue from kidneys that low numbers of organisms were recovered from. Bullock (personal communication) found that 10^6 cells/slide were required before the kidney disease bacterium could be observed in Gram stained smears of kidney tissue. Data in the present study indicated that approximately 3.0×10^6 cells/ml were required for microscopic observation of V. anguillarum in Giemsa stained blood smears.

Quantitative estimation of bacteria in blood and results of histopathology studies indicated that blood and blood producing organs were main targets for V. anguillarum serotype I but not for V. anguillarum serotype II. Bacteria were present in blood of fish with advanced infections of V. anguillarum serotype II but the concentrations were 10^2 to 10^3 times less than the number of V. anguillarum serotype I cells in blood of diseased fish. The number of bacterial colonies grown on BHI agar was consistently less from kidney tissue of moribund fish infected with V. anguillarum serotype II than from moribund fish infected with V. anguillarum serotype I. Miyazaki et al. (1977) reported that only advanced cases of vibriosis in their study were systemic in the Japanese eel (Anguilla japonica). Data from the present study indicate that onset of disease with mortality

occurs more rapidly among fish infected with V. anguillarum serotype I than among fish infected with V. anguillarum serotype II and that this may be due to differences in pathology of the circulatory system.

Few cellular responses to infections of V. anguillarum serotype I or II were observed in fish in these studies. This was true in early and late stages of infection and in areas where bacteria were concentrated. Enumeration of leukocytes in healthy and diseased fish indicated that a leukocidin is produced by V. anguillarum serotypes I and II and that 80% to 95% of the leukocytes are usually destroyed in moribund fish infected with these agents. This leukocidin(s) might also act on other inflammatory cells though no data were collected to indicate this.

Reports by other investigators indicate that lack of cellular response is not uncommon in diseased fish. Parisot and Wood (1960) reported "almost complete absence of cellular response to bacterial invasion" in their histopathologic study of fish mycobacteriosis. Parisot and Wood did observe an increase in eosinophilic granule cells in the muscle wall of the intestinal tract of fish in their study. Levin, Wolke and Cabelli (1972) and Funabashi et al. (1973) reported depressed cellular responses in fish infected with V. anguillarum.

Klontz, Yasutaki, and Ross (1966) observed a leukopenia in rainbow trout infected with Aeromonas salmonicida. Fuller, Pilcher and Fryer (1977) described the leukocytolytic factor produced by A. salmonicida as a glycoprotein which was released into supernatant fluid of broth cultures and was thought to be a virulence factor.

Bacteria were never observed in eggs within moribund fish infected with vibriosis. Peritoneal fluid adjacent to non-infected eggs in moribund fish frequently contained many bacteria; this might indicate that the bacteriocidal lectin described by Banowetz (1978) is present in immature eggs of chum salmon as small as two grams. Parisot and Wood (1960) reported that immature eggs in moribund juvenile chinook salmon infected with fish mycobacteriosis were never observed to be infected in their study. The possibility therefore exists that bacteriocidal activity of lectin in fish eggs could be detected through histopathologic examination.

Pathology observed in the mucosa of the gastrointestinal tract of fish infected with V. anguillarum serotype I or II was related to pH. Data from histopathology studies indicating the possibility of this relation were substantiated by pH measurements in different areas of gastrointestinal tracts of healthy and diseased fish and by the lack of growth of serotypes I and II on an acidic medium.

Infection of V. cholera in humans is also related to pH of the gastrointestinal tract where gastric acidity is an important defense

(Davis et al., 1973). In contrast to human cholera, bacteria invade the mucosa of the descending intestine and rectum resulting in sloughing of epithelial cells in fish infected with vibriosis.

Vibrio anguillarum serotypes I and II enter fish by penetrating the mucosa of the descending intestine and rectum. This was determined through histological examination of fish which had been exposed to these organisms and sampled with time until signs of disease were apparent. Initial signs of pathology produced by both organisms occurred in the rectum which is the most alkaline area of the gastrointestinal tract.

A second route of entry into fish was determined for V. anguillarum serotype II using the same methods described above. This organism can gain entry across the skin. Infection began in the epidermis covering scales, spread into the deep layers of the skin, and eventually resulted in infection of underlying skeletal muscle. It was apparent that external injury was not required for V. anguillarum serotype II to enter the skin of fish. Miyazaki et al. (1977) and Funabashi et al. (1973) reported that infection in an injured area of the skin was the probable route of entry of V. anguillarum among fish in their studies.

Death of fish infected with vibriosis probably results from a combination of causes. It is apparent that hypoxia occurs in moribund fish because gas exchange at the gills is impaired by edema and high

levels of bacteria in fish tissues increase the oxygen demand. Edema observed in many gills of fish infected with vibriosis was as severe as the gill edema observed by Pacha and Ordal (1967) in salmon experimentally infected with columnaris disease. It is likely that accumulation of toxins in tissues also contributes to the death of fish. Concentrations of toxins in moribund fish in these studies had to be high because levels of viable bacteria were determined to be as high as 2.7×10^9 cells per gram of fish tissue. Necrosis and cell sloughing in the posterior gastrointestinal tract of fish infected with vibriosis would cause loss of fluids which would also be a contributing factor in the death of fish. Dysfunction of various organs was apparent in fish infected with V. anguillarum serotype I but may not be as important in fish infected in V. anguillarum serotype II.

Extremely high levels of viable bacteria in tissues of fish indicated that toxins produced by V. anguillarum serotype I or II were not highly potent. Other investigators (Bullock et al., 1971; Snieszko and Axelrod, 1971; Umbreit and Ordal, 1972; Umbreit and Tripp, 1975) have speculated or presented data indicating that toxic factors are important in the pathogenesis of vibriosis. Data from the present study indicate that toxic factors may be important in the pathogenesis of vibriosis, but that high concentrations of toxins and action from other aggresins are probably required to bring about death of the host.

SUMMARY AND CONCLUSIONS

1. Nearly all disease problems observed from January 1, 1973 to December 31, 1977 among fish held at the OSU Marine Science Center and Oregon Aqua-Foods, Inc. were caused by V. anguillarum or Vibrio spp.
2. Infection by Vibrio spp. resulted in substantial mortality among fish were immunized against V. anguillarum serotypes I and II and among non-immunized fish. Many Vibrio spp. were recovered in pure culture from diseased fish. Vibrio sp. isolate MSC 1-76 is a virulent organism which produces severe external lesions in salmonids. This organism produced high mortality among many groups of coho salmon at the OSU Marine Science Center and was recovered in pure culture from moribund rainbow trout during an epizootic in which 20% of 20,420 fish at Oregon Aqua-Foods, Inc. died during a two week period.
3. Naturally occurring levels of V. anguillarum in Yaquina Bay were determined to be ten or less bacteria per ml water. Effluent from tanks of fish suffering from naturally acquired infections of vibriosis contained 1.0 to 4.7 x 10^3 cells of V. anguillarum per ml water.
4. In comparing five media, BHI agar (DIFCO) was best for enumerating V. anguillarum in salt water. This medium contains

0.5% NaCl which allows growth of V. anguillarum and is partially selective because organisms with higher NaCl requirements do not grow. Surface antigens of V. anguillarum were apparently altered by each of three vibrio differentiating media resulting in false negative rapid slide agglutination tests with specific rabbit antisera.

6. Biochemical and immunodiffusion tests using three serotypes of V. anguillarum and Vibrio sp. isolate MSC 1-76 indicated that these organisms are similar biochemically but dissimilar antigenically.

7. Histopathology studies of chum salmon fingerlings with naturally acquired infections of vibriosis indicated that diseases produced by two serotypes of V. angullarum were different. Vibrio anguillarum serotype I produced a bacteremia in early stages of disease with the following organs and tissues being the main targets: blood, loose connective tissue, kidney, spleen, posterior gastrointestinal tract, and gills. Vibrio anguillarum serotype II produced a bacteremia in late stages of disease with the following organs and tissues being main targets: skeletal muscle, cardiac muscle, anterior gastrointestinal tract, posterior gastrointestinal tract, and gills. Vibrio anguillarum serotype I cells were evenly dispersed throughout infected fish tissues while V. anguillarum serotype II formed distinct

colonies in tissues of fish.

8. Phagocytosis of bacteria was the only cellular response observed in salmonids infected with vibriosis. These observations were made with early and late stages of infection with V. anguillarum serotypes I and II.

9. Experimentally induced infections of vibriosis using water born exposure of fish to live bacteria resulted in pathology which was nearly identical to the pathology observed in naturally acquired infections of chum salmon fingerlings. These data substantiated that V. anguillarum serotypes I and II produce different diseases. This was true with experimentally induced infections of chum, coho, and chinook salmon. Naturally acquired and experimentally induced infections of V. anguillarum serotype I could not be differentiated in chum salmon fingerlings. Experimentally induced infections of V. anguillarum serotype II resulted in more extensive pathology in chum salmon fingerlings than naturally acquired infections of these fish. Experimental infections of serotype II may have been more severe because the disease progressed differently due to exposure of fish to large numbers of bacteria.

10. Enumeration of bacteria in tissues of moribund chum salmon

fingerlings indicated that extremely high levels of bacteria are present in fish prior to death caused by vibriosis. Standard plate count methods using ground whole fish indicated that approximately 1.2×10^8 cells of V. anguillarum serotype I and approximately 1.0×10^9 cells of V. anguillarum serotype II can be present per gram of tissue in moribund fish.

11. Enumeration of bacteria in blood of moribund coho and chinook salmon with experimental infections of vibriosis indicated that extremely high levels of bacteria are present in blood of fish prior to death. Vibrio anguillarum serotype I was present in concentrations as high as 2.4×10^8 cells per ml blood. Vibrio anguillarum serotype II was present in concentrations as high as 1.5×10^6 cells per ml blood. Levels of serotype I in blood were usually 10^2 to 10^3 times greater than levels of serotype II in blood. It was determined that at least 3.0×10^6 cells/ml were required for microscopic detection of V. anguillarum in Giemsa stained blood smears.

12. Large decreases in numbers of leukocytes accompanied high levels of bacteria in the blood of chinook salmon infected with vibriosis. Decreases of 90% to 95% were observed in blood of fish infected with serotype I. Moribund fish infected with serotype II had 80% to 90% less leukocytes than non-infected control fish. These data suggest that both serotypes of V. anguillarum

used in these studies produce a leukocidin in fish.

13. Vibrio anguillarum serotypes I and II enter fish by penetrating the mucosa of the descending intestine and rectum. Penetration of the skin is a second means by which V. anguillarum serotype II enters fish. Data indicating these routes of entry were collected by histopathologic examination of fish sampled at various times after water born exposure to live V. anguillarum.

14. Pathology observed in the mucosa of the gastrointestinal tracts of fish infected with V. anguillarum serotype I and II was apparently related to pH. Experiments concerning the progress of vibriosis indicated that necrosis of the mucosa in the gastrointestinal tract began and was most severe in the descending intestine and rectum where the pH was 6.7 to 7.0 and 7.5 to 8.0 respectively (pH measurements were made with infected and non-infected fish). The stomach was the most acidic (pH = 2.1 to 3.8) area of the gastrointestinal tract and contained no necrosis of the mucosa even in moribund fish. Gastrointestinal tracts of fish infected with vibriosis were less acidic than gastrointestinal tracts of non-infected control fish. Tests indicated that neither serotype of V. anguillarum used in these studies can grow on an acidic medium which would explain why bacteria and necrosis were not observed in acidic areas of the gastrointestinal tracts of infected fish.

15. Death of fish infected with vibriosis probably results from a combination of causes. It was apparent that moribund fish in these studies were suffering from hypoxia, possible accumulation of toxins (although not highly potent), loss of fluids in the posterior gastrointestinal tract, and dysfunction of various organs.

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