AN ABSTRACT OF THE THESIS OF

Nitimulyo Kamiso Handoyo for the degree of Doctor of Philosophy in Fisheries presented on August 12, 1985

Title: DIFFERENCES IN PATHOGENICITY AND PATHOLOGY OF VIBRIO ANGUILLARUM AND VIBRIO ORDALII IN CHUM SALMON (ONCORHYNCHUS KETA) AND ENGLISH SOLE (PAROPHRYS VETULUS) UNDER LABORATORY CONDITIONS

Redacted for privacy

Abstract approved: _____
Dr. Robert E. Olson

Both Vibrio anguillarum and Vibrio ordalii caused the same pathological changes in chum salmon (Oncorhynchus keta) or English sole (Parophrys vetulus). Hemorrhaging in gills, within the mouth, body musculature, intestine and other internal organs was very common in diseased fish of both fish species after water-borne exposure. Red necrotic lesions in the body musculature and exophthalmia were observed in most diseased chum salmon and were not observed in diseased English sole. In chum salmon, hemorrhagic ulceration around the injection site was observed after intraperitoneal injection with bacteria. This pathological sign was not observed in injected English sole. In histological preparations, bacteria were observed in the blood, loose connective tissue, kidney, spleen, skeletal muscle,

gastrointestinal tract, integument, liver, pancreas, and gills. However, no clear necrotic lesions were observed except in kidney and gills. Bacteria dispersed in infected tissues except in muscle tissue of chum salmon infected with V. ordalii where the bacteria formed small and loose colonies.

Effects of water temperature on infection were examined at 10, 14, and 18°C. The mortality rates increased and mean times to death decreased at increased water temperatures. Size of fish also had a significant effect on mortality rates of English sole infected by either Vibrio species. The mortality rates were significantly higher in small sized than in large sized English sole, however there was no significant difference in mean time to death between the two size groups. In both fish species the LD_{50} of water-borne exposure of both V. anguillarum and V. ordalii was significantly higher than the LD_{50} of intraperitoneal injection.

Immune response and duration of antibody titers were determined in chum salmon and English sole after immunization with either V. anguillarum or V. ordalii bacterin by intraperitoneal injection. Both fish species developed good immunity against vibriosis. However, chum salmon had a higher relative percent survival (RPS) than did English sole. Antibody titers in both fish species were detected 1 week post immunization. Peaks were reached in about 4 weeks after immunization, and about 5 months after immunization

both fish species still had positive antibody titers. The results of passive protection experiments indicated that the internal protection to vibriosis in English sole could not be transferred to chum salmon by intraperitoneal injection of unimmunized English sole serum.

Changes in the virulence of V. anguillarum or V. ordalii due to selection after three passages through either chum salmon or English sole were not observed. There was also no significant difference in mean time to death of chum salmon or English sole exposed to these bacterial isolates. However, the LD_{50} of both V. anguillarum and V. ordalii after passage was lower for the homologous species of fish.

In general, the results of this study showed that English sole is more resistant than chum salmon to both Vibrio species. Cross infection of V. anguillarum and V. ordalii between chum salmon and English sole is possible, at least under laboratory conditions. However, it is unlikely that cross infection occurs very often under natural conditions, unless potential hosts are stressed.

DIFFERENCES IN PATHOGENICITY AND PATHOLOGY OF *VIBRIO ANGUILLARUM* AND *VIBRIO ORDALII* IN CHUM SALMON (*ONCORHYNCHUS KETA*) AND ENGLISH SOLE (*PAROPHRYS VETULUS*) UNDER LABORATORY CONDITIONS

by

Nitimulyo Kamiso Handoyo

A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed August 12, 1985

Commencement June 1986

APPROVED:

Redacted for privacy

Associate Professor of Fisheries in charge of major

Redacted for privacy

Head of Department of Fisheries and Wildlife

Redacted for privacy

Dean of Graduate School

Date thesis is presented August 12, 1985

Typed by Harvey McCloud for Nitimulyo Kamiso Handoyo

ACKNOWLEDGEMENTS

I would like to thank:

Dr. Robert E. Olson, my major professor, for excellent guidance and encouragement in my study and in finishing this thesis.

Special thanks to Dr. James R. Winton, for his guidance and for reviewing this thesis.

Drs. James D. Hall, Lawrence R. Curtis, and Dr. Paul W. Adams, for their advice and for reviewing this thesis.

Ms. Cathlene Lannan for teaching me many of the technical methods concerning this research.

The Rockefeller Foundation for the financial support which they provided and for giving me the opportunity to study in the United States.

And finally, I wish to thank also my wife, Endah, for help and encouragement, and my daughters, Wiwid and Ratih, for a cheerful atmosphere which helped me complete my study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DIFFERENCES IN PATHOGENICITY AND PATHOLOGY OF *VIBRIO ANGUILLARUM* AND *VIBRIO ORDALII* IN CHUM SALMON (*ONCORHYNCHUS KETA*) AND ENGLISH SOLE (*PAROPHRYS VETULUS*) UNDER LABORATORY CONDITIONS

CHAPTER I

INTRODUCTION

Vibriosis is one of the diseases of greatest concern in salt-water fish culture because it can be the cause of high mortality levels (Fryer *et al.*, 1972). Cisar and Fryer (1969) found that vibriosis caused losses in juvenile chinook salmon (*Oncorhynchus tshawytscha*) in a saltwater impoundment on the Oregon coast. In Maine and New Hampshire coastal waters, vibriosis was considered to be a major problem, causing losses up to 80% of pen culture of coho salmon (*O. kisutch*) (Sawyer, 1978). Further study has shown that all species of salmon reared in saltwater are subject to vibriosis and according to Wood (1974), pink salmon (*O. gorbuscha*) and chum salmon (*O. keta*) are the most susceptible.

Cases of vibriosis have been described from a wide variety of marine and migratory fishes, and outbreaks have been recorded from the Americas, Asia and Europe (Andersen and Conroy, 1970). The marine culture of salmonids has increased substantially in Norway since the late sixties, and vibriosis has been economically the most important disease. In the Pacific Northwest vibriosis is considered

to be one of 10 bacterial diseases of major concern because it can cause significant losses in commercially important fish (Winton, 1983).

Vibriosis can also cause disease in wild fish (Strout, et al., 1978), and strains of V. anguillarum have been reported to become adapted to particular species of hosts (Egidius and Andersen, 1978). However, studies of the pathogenicity and pathology in wild fish are few. One of the studies was done by Levin et al. (1972), and they found that pathogenicity differed between strains of V. anguillarum that were isolated from wild fish (Pseudopleuronectes americanus) and V. anguillarum that were isolated from cultured fish (Oncorhynchus kisutch). The majority of research papers concerning vibriosis describe the disease in salmonids.

In a study of V. anguillarum and V. ordalii, Ransom et al. (1984) found that under laboratory conditions, both bacteria caused different histopathology in salmonid species. Vibrio anguillarum produced a bacteremia that was distributed throughout the tissues of fish. Vibrio ordalii also produced a bacteremia, but the bacteria often formed distinct colonies in fish tissue.

The English sole is found along the Pacific coast from San Cristobal Bay in Baja California to Unimak Island in Western Alaska and is an important component of the commercial fishery (Hart, 1973). Olson and Pratt (1973)

found that many juvenile English sole use the estuaries as a nursery ground during at least a part of their first year of life. It is possible that during this period, the young fish are exposed to high levels of Vibrio species. In a study of rearing conditions and their effects on growth, food conversion, and survival English sole, Williams (1974) found that the fish occasionally suffered from systemic bacterial infections. The dead juvenile English sole were usually reddened around the bases of the fins and the gill cover and occasionally had extensive hemorrhaging in various parts of the body. Further study of the disease was not done, however he suggested that Vibrio species was probably one of the causative agents that infected the weakened fish.

The goal of this study was to gain a better understanding of two causative agents of vibriosis, Vibrio anguillarum and Vibrio ordalii, with special emphasis on the comparative effects of these bacteria on salmonid and non-salmonid fishes. Specific objectives were to compare the pathogenicity and pathology of V. anguillarum and V. ordalii for English sole (Parophrys vetulus) and chum salmon (O. keta); to determine the effect of water temperature on the pathogenicity and pathology in English sole and chum salmon under laboratory conditions; and to measure changes that may occur in the virulence of the bacteria after several passages through either English

sole or chum salmon. Other objectives were to determine: the effects of different methods of exposure to the bacteria (water-borne exposure or intraperitoneal injection); the susceptibility of different sizes of English sole; and the immune capability of English sole and chum salmon when exposed to V. anguillarum and V. ordalii.

CHAPTER II

LITERATURE REVIEW

Causative Agents of Vibriosis

Vibriosis is an acute, systemic disease of fish caused by the bacteria Vibrio anguillarum and V. ordalii. Before the causative agents were identified, the disease was often called saltwater furunculosis since the lesions resembled those caused by Aeromonas salmonicida and occured in saltwater (Wood, 1974). The bacteria are world-wide in distribution and are known to be infectious to a large number of fish species, primarily of marine fishes (Rucker, 1959; Cisar and Fryer, 1969; Anderson and Conroy, 1970; Evelyn, 1971; Hastein and Holt, 1972; Wolke, 1975).

Early isolations and descriptions of V. anguillarum were by Canestrini in 1893 and Bergman in 1909 (Rucker, 1959; Groberg, 1981). There has been confusion about the nomenclature of this bacterium since its first isolation and it has been called Bacterium anguillarum, Vibrio anguillarum, Vibrio piscium, Achromobacter ichthyodermis, and Pseudomonas ichthyodermis (Post, 1983). A proposal was made by Hendrie et al. (1971) to combine all strains as a single species. The name Vibrio anguillarum was officially recognized in 1974 (Cowan et al., 1975).

Serotypes

Vibrio anguillarum has proven to be a highly variable species. The variation can be serological (Pacha and Kiehn, 1969; Johnsen, 1977; Lewis, 1979; Ezura et al., 1980; Crosa, 1981; Kitao et al., 1983; Muroga et al., 1984), biochemical (Smith, 1961; Muroga et al., 1976; Ohnishi and Muroga, 1976; Hastein and Smith, 1977; Kusuda et al., 1979; Ezura et al., 1980; Schiewe and Crosa, 1981; Tajima et al., 1981; Muroga and Tatani, 1982; Ruiz, 1982; Muroga et al., 1984), or in terms of pathogenicity and host specificity (Harrell et al., 1976; Egidius and Andersen, 1978).

In Japan during the period from 1965 to 1980, 267 strains of V. anguillarum were isolated from vibriosis outbreaks in various parts of the country. Two hundred sixty three of the V. anguillarum strains were from ayu (Plecoglossus altivelis), two from rainbow trout (S. gairdneri) and two from eel (Anguilla japonica). On the basis of cross-agglutination and cross-absorpsion tests with thermostable (O) antigens, they were divided into six serotypes (A, B, C, D, E, and F) (Aoki et al., 1981; Kitao et al., 1983). From these six serotypes, serotype A, B and C were considered to be the most important serotypes causing vibriosis in cultured ayu. There are 163 isolates of V. anguillarum that have been cultured from diseased fish in Norwegian waters. Based on biochemical

tests they were divided into two groups; nearly all those isolated from farmed salmonids were in group I, arabinose positive, and those from wild fish were in group II, arabinose negative (Hastein and Smith, 1977).

There is no exact number of strains of V. anguillarum known in the United States, however Harrel et al. (1976) indicated that heterogeneity of the bacterium was observed in isolations from diseased salmonids in Puget Sound. Based on serological analysis of thermostable antigens, Pacha and Kiehn (1969) suggested that there were two serotypes of V. anguillarum found in the United States, serotype I and serotype II. According to this arrangement, serotype I was most common and considered to be the most important pathogen in marine fishes, growing rapidly in culture and causing rapid mortality in fish. Serotype II characteristically grew more slowly in culture and the onset of the disease was delayed (Gould, 1977; Gould et al., 1979; Shiewe, 1983). According to Ransom (1978), serotype I produced a bacteremia, with bacteria distributed throughout tissues of fish, with the major sites being blood, loose connective tissue, kidney, spleen, posterior gastrointestinal tract and gills. Serotype II also produced a bacteremia, but the bacteria often formed distinct colonies in fish tissues, and the major sites were skeletal muscle, heart, anterior and posterior gastrointestinal tract and gills.

Characteristics of Vibrio anguillarum and Vibrio ordalii

Since serotype I and serotype II not only differ antigenically but also in biochemical, genetic composition and in the pathology they cause, Schiewe et al. (1981) proposed the creation of two species. Serotype I was considered to represent typical V. anguillarum and serotype II was designated a new species V. ordalii (Schiewe et al., 1982). The characteristics that V. anguillarum and V. ordalii have in common are: motile, gram-negative rod or slightly curved, anaerogenic, asporogenic, cytochrome oxidase positive, sensitive to vibriostat 0/129 and novobiocin, and need NaCl for growth (Smith, 1961; Cisar and Fryer, 1969; Handrie, 1971; Levin et al., 1972; Cowan et al., 1975; Harrell et al., 1976; Bullock, 1977; Nishibuchi and Muroga, 1977; Kusuda et al., 1979; Schiewe, 1983).

Geographic Distribution

Vibrio anguillarum and Vibrio ordalii are ubiquitous in nature, worldwide in distribution, and are infectious to a large number of fish species (Anderson and Conroy, 1970; Evelyn, 1971; Fryer et al., 1972; Hastein and Holt, 1972; Wolke, 1975). In addition to the well know disease problems caused in saltwater aquaculture, Ohnishi and Muroga (1976) isolated Vibrio from rainbow trout (S. gairdneri) in freshwater ponds. The causative agent was identified as V. piscium, a species that now is considered

to be V. anguillarum. Muroga et al. (1984) isolated V. anguillarum from ayu (P. altivelis) that were caught in seawater, brackish water and freshwater environments. Hacking and Budd (1971) also identified V. anguillarum as a causative agent of an epizootic in tropical freshwater fishes.

Currently vibrosis is found in many countries and causes serious problems wherever mariculture is practiced, including Japan (Ezura et al., 1980; Kitao, 1983), Norway (Egidius and Andersen, 1978), Great Britain (McCarthy, 1976), United States (Cisar and Fryer, 1969; Novotny, 1978; and Sawyer, 1978), and Canada (Evelyn, 1971). Most problems are caused by V. anguillarum rather than V. ordalii.

Pathogenicity

Our knowledge of the method by which V. anguillarum and V. ordalii penetrate into the host is still poorly understood. It is suspected that the pathogenic bacteria gain entrance into the body through mouth or gastrointestinal tract, gills, skin and lateral line (Horne, 1982). In a study utilizing water-borne exposure of fish to bacteria, Ransom et al. (1984) suggested that both V. anguillarum and V. ordalii enter the fish by penetrating the descending intestine and rectum, and that penetration of the skin is a second means by which V. ordalii enters the fish. Using immersion of tissues in V. anguillarum

solution, Horne and Baxendale (1983) found that the number of bacteria adhering to the gut tissue was affected by the concentration of the bacteria in the medium and the time of immersion. The immune state of the fish also played an important role in determining the number of bacteria adherence. By the same method, they compare the adherence of V. anguillarum to the gut tissue of vaccinated and unvaccinated fish. The result indicated that bacterial adherence to the gut tissue of vaccinated fish was substantially less than to the gut tissue of unvaccinated fish. After comparing the adhesion of the bacteria to different tissue organs Horne and Baxendale (1983) suggested that the gut may be an important site of bacterial multiplication in slowly developing infections.

In general, bacterial virulence factors promote growth in the antagonistic environment established by the host defense mechanisms. One line of defense is provided by proteins transferrin and lactoferrin, which bind iron, rendering it unavailable to pathogens (Bullen et al., 1978). According to Crosa et al. (1977), most of the high-virulence strains of V. anguillarum have a plasmid that carries genetic determinants. This plasmid can increase the capability of the bacteria to grow in the host and establish infection (Crosa, 1979; Trust et al., 1981). In a recent study, Crosa (1980) proved that the V. anguillarum virulence plasmid specifies a very efficient iron sequestering system

enabling the bacteria strains to survive and grow *in vitro* under limited iron availability in a culture medium containing high concentration of transferrin. Under the same conditions, low virulence cured strains (without plasmid) are completely inhibited. In an experimental infection of fish, Crosa *et al.* (1980) found that the LD_{50} of virulent *V. anguillarum* (with PJM1 plasmid) in juvenile coho salmon was lower (1.1 to 4.0 x 10^3 cells/ml) than non virulent *V. anguillarum* (plasmidless) (between 1.5 x 10^6 and 7.6 x 10^7 cells/ml). Concomitant with an efficient iron uptake by plasmid-carrying strains of *V. anguillarum*, new outer membrane proteins are induced, one of them is OM2 (Crosa, 1981; Crosa and Hodges, 1981). This outer membrane protein may play a role as a receptor the ironsiderophore complex (Crosa *et al.*, 1983).

Vibrio anguillarum produces both exotoxin and endotoxin, however these toxins are not the primary cause of the disease (Harbel *et al.*, 1979). Innoculation with 38 mg of *V. anguillarum* endotoxin could kill 50% of mice tested, but little effect was observed when this amount was injected into fish (Harbel *et al.*, 1979). There was no mortality or significant change in hematocrit or total plasma protein concentration in coho salmon injected with cell free supernatant, 1 mouse LD_{50} of endotoxin, bacterial cell lysate, or combination of endotoxin, supernatant and lysate (Umbreit and Tripp, 1975). However, it was found that

non-toxic supernatant fluid became toxic to fish after heating for 15 minutes at 100°C. The source and the nature of the toxin is not completely understood and it was suggested that the active substance may be released from cells after heating. Munn (1980) and Trust et al. (1981) studied various toxic factors including hemolysin, cytotoxin and hemagglutinin in relation to the virulence of V. anguillarum. However, they could not determine what role the toxins play in the pathogenicity. According to Inamura et al. (1984), the release of active substance is affected by nutrients in the medium, culture conditions (temperature), time and strains of V. anguillarum. In their experiment, some strains of V. anguillarum produced exotoxin on nutrient agar of BHI agar at 20°C or 25°C for 18-36 hours that were toxic to goldfish (Carassius auratus), but did not produce exotoxin in 24 hours BHI broth culture at 25°C.

Effects of Environment on Vibriosis

Stress is an important factor affecting the severity of a vibriosis outbreak (Wood, 1974; Roberts, 1975; McCarthy, 1976; Egidius and Andersen, 1977; Winter et al., 1981; Piper et al., 1982; Baker et al., 1982; Refstie, 1982). Temperature is one of the most important factors governing the growth rate of the bacterium and the severity of the disease (Egidius and Andersen, 1977; Gould, 1977;

Muroga et al., 1984). Harrell et al. (1976) reported that high mortality of pen-reared coho salmon occurred when temperature exceeds 9°C. Wood (1974) suggested that any attempt to rear fish in saltwater impoundments subject to considerable warming should consider vibrio disease as a factor that will possibly limit the success of the project. He also indicated that the disease can cause losses at temperatures less than 10°C. However, nearly all outbreaks have occurred at temperatures above 10°C, and the most severe ones have occurred at temperatures over 15°C.

The effect of water temperature upon mortality of turbot (Schophthalmus maximus) was studied by Horne et al. (1977). Under laboratory conditions, high mortality of young turbot due to an acute outbreak of vibriosis occurred at a temperature above 10°C. Therapy by using antibiotics was only marginally successful. The most effective method of treatment reducing losses was by decreasing the temperature of water to below 10°C. Groberg (1981) also studied the effect of temperature on the growth rate of V. anguillarum and on the mortality of coho salmon to vibriosis. He found that the doubling time of V. anguillarum in brain heart infusion (BHI) broth in the logarithmic growth phase was 442 minutes at 6°C, 151 minutes at 12°C, and 80 minutes at 18°C. The mortality of coho salmon after injection by V. anguillarum increased from 0% at 3°C and 6°C to 60% at 21°C, and the mean time to death decreased with increasing temperature from 23 days at 6°C to 2.7 days at 21°C.

Host Specificity

During the past few years the frequency of outbreaks of vibriosis has increased considerably among wild marine fish and migratory fish, especially along the Norwegian coast (Hastein and Holt, 1972). From their observations, Hastein and Smith (1977) believed that a major reservoir for vibriosis in farmed salmonids was wild fish, especially saithe (Gadus virens) feeding around the holding facilities. Wood (1974) suggests that if salmonids in pens and non-salmonid fishes live in close proximity, these fish may represent a reservoir for vibriosis. However, Smith (1961), Egidius and Andersen (1978), and Strout et al. (1978) suggest that there is a possibility of host specificity of strains of V. anguillarum. From their study, Egidius and Andersen (1978) found that V. anguillarum isolated from rainbow trout were pathogenic to salmonids but of only very low pathogenicity for saithe, while strains isolated from saithe were pathogenic to saithe and non-pathogenic to salmonids.

Pathology of Vibriosis

In general, the external and internal gross pathology of vibriosis is very similar to that of all gram negative septicemias seen in fish. In winter flounder (Pseudopleuronectes americanus) it can cause dermal lesions, fin necrosis and petichiae in the acute phase, and ulceration

in the more chronic phase of the disease (Levin et al., 1972). The affected fish become anemic and the kidney contains a greater proportion of haematopoietic cells. The anemia may be due to the destruction of red blood cells by bacterial hemolysins or possibly to chronic blood loss associated with necrosis of the fins. In salmonids, Ransom (1978) found that vibriosis can cause severe skin, fin, and muscle necrosis, a large decrease in the number of leukocytes and high levels of bacteria in the blood. The diseased fish probably died due to a combination of hypoxia, accumulation of toxins, loss of fluids in the posterior gastrointestinal tract, and dysfunction of various organs. In infected, cultured turbot (Scopthalmus maximus) and Dover sole (Solea solea), gross lesions and clinical signs varied with species of fish, age and temperature, but could be classified into peracute, acute and chronic diseases (Richards, 1980). In peracute, death occurred without visible gross symptoms except for a darkening of coloration. In young fish, abdominal distention and white circlets of tissue surrounding the eye were observed and skin lesions were absent. In the acute stage, fish were anorexic, dark in color and lethargic. Erosion in the area of the jaw was frequently found in turbot of all ages. Further lesions developed in skin and superficial musculature, or internal organs. Hemorrhages occurred in some areas, especially the fins, sloughing of the skin associated with hemorrhaging

was often seen and the gills were usually pale in color. In the chronic condition, skin lesions were deep ulcers with hemorrhaging and fibrin deposition. In some cases ulceration penetrated the abdominal wall leading to the viscera. In Dover sole, dark colored necrotic skin areas developed very rapidly, and the gills were pale.

Control and Immunization

The potential importance of marine fish farming and the serious problem caused by vibriosis has stimulated efforts toward the prevention and control of the disease (Fryer et al., 1972; Antipa, 1976; Munn, 1980). Under conditions of captivity, vibriosis may be controlled by careful husbandry practices that reduce stress, and rational use of antibiotics and immunization (Anderson and Conroy, 1970; Bullock, 1977; Snieszko, 1978; Sako and Kusuda, 1979; Richards, 1980; Tabata et al., 1982). The antibiotic oxytetracycline is commonly incorporated into the feed (Snieszko, 1957; Herman, 1970; Fryer et al., 1972; Sawyer and Strout, 1977; Post, 1983; Winton, 1983). However, it would not be recommended to use subtherapeutic levels of dietary antibiotics for a long period of time because resistant strains of pathogenic bacteria could develop (Snieszko, 1958; Herman, 1970; Aoki and Kitao, 1978; Sako and Kusuda, 1978; Hahnel and Gould, 1982). In addition, antibiotics could have side effects on the fish

including suppresive effects on the immune response (Rijkers et al., 1980; Rijkers et al., 1981; Grondel and Boesten, 1982; Lewis et al., 1985). Grondel and Boesten (1982) found that oxytetracycline administered either by mixing with food or by intraperitoneal injection could delay or inhibit cellular and humoral immune response of fish. Pearse et al. (1974) tried to control vibriosis in plaice (Pleuronectes platessa), Dover sole (Solea solea), and brill (Scophthalmus rhombus) using furanace by an immersion (bath) method. They found that minimum inhibition concentrations depend on species of fish, strain of Vibrio and time exposure.

There are different methods of vaccination to prevent the outbreak of vibriosis in farmed salmonids. These include: bath or direct immersion (Egidius and Andersen, 1979; Gould et al., 1979; Kusuda et al., 1980; Laurencin and Tangtrongpiros, 1980; Groberg, 1981; Davina et al., 1982; Egidius and Andersen, 1982; Johnson et al., 1982a; Johnson et al., 1982b; Kawano et al., 1983; Tatner and Horne, 1983; Aoki et al., 1984; Amend and Johnson, 1984; Egidius and Andersen, 1984; Kawano et al., 1984; Sakai et al., 1984; Tatner and Horne, 1984), oral bacterin incorporated with feed (Fryer et al., 1972; Nelson, 1972; Fletcher and White, 1973; Rohovec, 1975; Bratein and Hodgins, 1976; Fryer et al., 1976; Gunnel et al., 1976; Prescott, 1977; Fryer et al., 1978; Inebi and Horne, 1979;

Evelyn and Ketcheson, 1980; Groberg, 1981; Agius et al., 1983; John'son and Amend, 1983; Kawamoto et al., 1984; Kawano et al., 1984), spray (Gould, 1977; Gould et al., 1978; Itami and Kusuda, 1980), intraperitoneal or intramuscular injection (Fletcher and White, 1973; Rohovec, 1975; Bratein and Hodgins, 1976; Sawyer and Strout, 1977; Schiewe and Hodgins, 1977; Harrell, 1978; Sawyer, 1978; Hastein et al., 1980; Groberg, 1981; Rosenkvist-Jensen, 1982; Agius et al., 1983; Amend and Johnson, 1984; Aoki et al., 1984; Horne et al., 1984; Sakai et al., 1984; Tatner and Horne, 1984), hyperosmotic infiltration (Antipa and Amend, 1977; Croy and Amend, 1977; Aoki and Kitao, 1978; Lannan, 1978; Antipa et al., 1980; Rosenvist-Jensen, 1982; Aoki et al., 1984), and passive immunization (Harrell et al., 1975; Gould, 1977; Viele et al., 1980; Groberg, 1981; Aoki et al., 1984).

The degree and duration of protection varies with the route of vaccination (Rohovec, 1975; Antipa and Amend, 1977; Fryer et al., 1978; Evelyn and Ketcheson, 1980; Agius et al., 1983; Nelson et al., 1985), type of V. anguillarum bacterins (Harrell et al., 1975, Antipa, 1976), size and species of fish (Johnson et al., 1982a; Tatner and Horne, 1983; Tatner and Horne, 1984), and temperature of water (Snieszko, 1958; Fryer et al., 1976; Groberg, 1981; Johnson et al., 1982b). In general intraperitoneal injection of bacterin gives much longer and higher protection than oral

or immersion methods of immunization (Rohovec, 1975; Evelyn and Ketcheson, 1980; Agius et al., 1983). However, hyperosmotic immunization is more practical for large numbers of fish, and the result is comparable to intraperitoneal injection (Antipa and Amend, 1977). Antipa (1976) found that heat-killed V. anguillarum antigen injected intraperitoneally gave more protection than formalin-killed, or combination of both heat-killed and formalin-killed antigens. The addition of alum adjuvant enhanced the response of both oral and intraperitoneal immunizations (Agius et al., 1983; Horne et al., 1984). Freund's complete adjuvant was used in an immunization experiment by Harrell et al. (1975), and results showed that the immune response was higher in the fish that were immunized with bacterin and adjuvant than that were immunized with bacterin without adjuvant.

After direct immersion into V. anguillarum bacterin suspension, Johnson et al. (1982a) found that immunity lasted longer in large fish than in small sized fish. They also found that coho salmon (O. kisutch) and sockeye salmon (O. nerka) had the longest immunity, pink salmon (O. gorbuscha) the shortest, and chinook salmon (O. tshawytscha) and rainbow trout (S. gairdneri) were intermediate. The onset of protective immunity was also affected by water temperature. In general, the higher the water temperature the earlier the immunity was detected and the longer the protection lasted (Groberg, 1981; Johnson et al., 1982b).

Passive immunization against V. anguillarum was studied by Harrell et al. (1975), Viele et al. (1980), and Aoki et al. (1984), and results varied among the experiments. Harrell et al. (1975) injected rainbow trout with trout anti-V. anguillarum serum from immunized fish and then challenged with living bacteria by parenteral injection. The trout were protected against vibriosis, but the protection began to decrease after eleven days. The antibody was detected within ten minutes after injection, and reached a peak titer of 512 after 96 hours. Although the titer decreased sharply after 96 hours, some antibody could be detected in the fish serum 64 days after passive immunization.

Viele et al. (1980) tried to inject different substances from different organs of immunized fish to control vibriosis under laboratory conditions. The results showed that plasma was most effective in transferring vibriosis protection, followed by pronephros cells. Thymus tissue gave negative results and splenocytes gave variable results. Contrasting results were found by Aoki et al. (1984) in the study of passive immunization. They injected immune serum from fish immunized by immersion in lyophylized formalin-killed bacterin one month previously. Four hours later all fish were challenged by intraperitoneal injection with V. anguillarum and held at 20°C for ten days. Controls were injected with serum of non-vaccinated fish. No protection from this attempt at passive immunization was observed.

CHAPTER III

MATERIALS AND METHODS

Bacteria

Cultures of Vibrio anguillarum (isolate LS 173) and Vibrio ordalii (isolate MSC 275) were provided by the Fish Disease Laboratory at the Hatfield Marine Science Center, Oregon State University. These bacteria were inoculated onto tryptic soy agar (TSA) and incubated at 18°C. Colonies obtained from resulting pure cultures were identified by colony morphology, cell motility, shape and gram stain, sensitivity to novobiocin and vibriostat 0/129, and rapid slide agglutination with specific rabbit antiserum. Before the bacteria were used in experiments they were passed through chum salmon (Oncorhynchus keta) or English sole (Parophrys vetulus) three times by water-borne exposure. After each passage bacteria were isolated from kidney tissue of the fish that died by streaking onto TSA. Pure cultures of the bacteria from each passage were identified by colony morphology, sensitivity to novobiocin and vibriostate 0/129, and rapid slide agglutination.

Fish

Juvenile English sole (P. vetulus) were collected with a 5-m otter trawl in Yaquina Bay, Oregon and transported to the Fish Disease Laboratory. To remove ectoparasites, especially Gyrodactylus sp. that are very commonly found on

their fins, the juvenile English sole were treated with a 1:4,000 formalin solution for one hour (Putz and Hoffman, 1963). Salmon used in experiments were fingerling chum salmon (O. keta) hatched from eggs obtained at the Oregon State University Experimental Fish Hatchery on Netarts Bay. Prior to use in experiments, the fish were held in circular tanks (87 cm in diameter and containing 125 l of seawater) supplied with pathogen free seawater (3-4 per minute) and fed with a commercial moist salmon diet. The juvenile English sole that had been treated with formalin were held in the laboratory for 1 to 2 weeks before used in experiments to allow feeding to begin and to adapt to laboratory conditions. Both English sole and chum salmon were tested for vibrio antibody titers to determine if they had previous infections with V. anguillarum or V. ordalii. Fish used in experiments were selected for uniform size and absence of abnormalities. Measurements were made in weight (g ± SD) and total length (Cm ± SD).

Challenge Method

Water-borne exposure to bacteria was used in most experiments. The procedures followed the method described by Gould (1977) for water-borne exposure of fish to V. anguillarum. Pure cultures were grown in 10 ml trypticase soy broth (TSB) and incubated at 18°C. After 24 hours the cultures were transferred into 250 ml TSB in 500 ml

Erlenmeyer flasks and incubated at 18°C with slow agitation. Twenty four hours later these 250 ml cultures were transferred into 1 or 2 l Erlenmeyer flasks containing 500 ml or 1,000 ml TSB (depending upon the amount of broth culture needed). The final broth cultures were incubated at 18°C on an agitator for about 20 hours. The bacterial suspensions were made in circular tanks containing 50 liters of seawater. The concentration of the bacterial suspension varied with the species of the bacteria, fish species and particular experiment. Approximate bacterial cell numbers were estimated by optical density measurements of the cultures at 525 nm using a spectrophotometer. More accurate bacterial cell counts were determined by triplicate plate counts.

The fish were exposed to the bacterial suspension for 30 minutes with aeration. After 30 minutes water flow was restored to normal levels with flow rate between 2 and 3 liters per minute. All experiments using water-borne exposures were terminated in 14 days and fish were not fed. Kidney tissue smears from all moribund or dead fish were innoculated on TSA plates. The bacteria were identified by colony morphology, sensitivity to novobiocin and vibriostat 0/129. To confirm the results, about 25 to 40% of the isolates were tested with specific rabbit antiserum in a rapid slide agglutination test.

General Serological Methods

1. Vibrio anguillarum and Vibrio ordalii bacterin preparation

A 10-ml broth culture of each bacterium was prepared in TSB and incubated at 18°C for 24 hours. The purity of the cultures was determined by gram stain, motility, colony morphology on TSA, sensitivity to novobiocin and vibriostat 0/129, and rapid slide agglutination with specific antiserum. Each of these cultures was then transferred into 250 ml TSB and incubated at 18°C on an agitator. After 24 hours each was transferred again into 1 liter TSB and incubated at 18°C on an agitator for about 20 hours. After incubation the purity was tested and the cells were harvested. Harvesting the bacterial cells from these broth cultures was accomplished with the aid of a Beckman Model J2-21 centrifuge. The cells were washed three times in sterile phosphate-buffered saline (PBS, pH 7.0) with centrifugation at 3,000 rpm for 10 minutes at 0°C. The harvested cells were resuspended in 40 ml sterile PBS containing 0.3% formaldehyde and kept in refrigerator for 24 hours. After 24 hours the cells were again washed and centrifuged three times (10 minutes, 3,000 rpm, at 0°C) in sterile PBS. Vaccines were prepared by resuspension of the wet-packed cells in sterile PBS about 0.85 optical density at 525 nm. Three 0.1 ml aliquots of the suspensions were inoculated into TSA to test sterility. The absence of growth indicated

non-viability of the bacterial cells. The vaccines were frozen until they were used (Rohovec et al., 1981).

2. Rabbit Anti-Vibrio Sera

Each bacterium (V. ordalii or V. anguillarum) was innoculated on an agar slant in an eight ounce bottle and incubated at 18°C. Two bottles were prepared for each bacterium. When the cultures were confluent on agar (about 48 hours for V. anguillarum and 96 hours for V. ordalii), the cells were removed from the agar surface with 10 ml PBS and washed three times with sterile PBS (pH 7.0) by centrifugation (3,000 rpm, for 10 minutes, at 0°C). The pellet of each bacterium from the last washing was suspended in 10 ml PBS. Two New Zealand white rabbits were used for antisera production. One rabbit for anti V. ordalii and the other for anti V. anguillarum serum. Each rabbit was injected (by intradermal injection) at 15 sites with 0.1 ml suspension of antigen and Freund's complete adjuvant. A booster injection (by intramuscular injection) was given one week after the first injection with a 1.0 ml suspension of antigen and Freund's incomplete adjuvant. The first, second and the third bleedings were done at 2, 3 and 4 weeks after the booster injection. A second booster was given by injecting each of the hind feet with 1.0 ml antigen and Freund's incomplete adjuvant. The first hind foot was injected about one week after the third bleeding. The second hind foot was

injected one week after the injection of the first foot. The fourth, fifth and the sixth bleedings were done at 1, 2 and 3 weeks after the second booster injections. For the first through the fifth bleeding, blood was collected from the marginal ear vein (about 25 ml per bleeding). The sixth bleeding was done by cardiac puncture and about 50 ml of blood was collected from each rabbit. The blood was allowed to clot at room temperature for two hours and then placed at 4°C overnight for clot retraction. The serum was collected by centrifugation (3,000 rpm, for 10 minutes, at 0°C) and frozen in sterile tubes. The titer of the harvested serum was determined after each bleeding by microtiter technique.

3. Harvesting Fish Sera

Fish blood was collected by cutting the caudal peduncle. Individual or pooled blood samples were allowed to clot at room temperature for one hour and overnight at 4°C. The serum fraction was harvested by centrifugation (3,000 rpm, for 10 minutes, at 0°C). The serum was used immediately or frozen in a sterile tube until use.

4. Antigen Preparations for Microtiter

Antigens for microtiter tests were prepared by the same procedures as in vaccine preparation except for formalin treatment. The antigens were made by resuspending the harvested cells that had been washed in PBS without formalin

treatment. The concentration of the suspensions were adjusted to an optical density of 0.85 at 525 nm using a spectrophotometer and stored at 4°C until they were used.

5. Microtiter Technique

Fifty µl of serum was used in the microtiter technique for determination of antibody titer. Serial doubling dilutions of the serum sample using PBS were made in a 96 well microtiter plate. After addition of antigen (50 µl per well), the plate was shaken gently for two minutes before incubating two hours at room temperature and overnight at 4°C. The antibody titer was determined after incubation by observing the microtiter plates under a dissecting microscope.

Comparative Pathology

Thirty juvenile English sole (8.2 ± 0.6 cm, 5.0 ± 1.1 g) and thirty fingerling chum salmon (11.4 ± 1.2 cm, 14.5 ± 4.4 g) were used in this experiment. Each species of fish was divided into two groups, each group consisted of 15 fish that was challenged by water-borne exposure to a bacterial suspension for 30 minutes at 12°C in a rectangular tank containing 40 l saltwater. The first group of English sole was exposed to *V. ordalii* (10^7 cells/ml) and the second group to *V. anguillarum* (10^7 cells/ml). The first group of chum salmon was exposed to *V. ordalii* (10^6 cells/ml) and the second group to *V. anguillarum* (10^6 cells/ml). The identity of the bacterial pathogen was confirmed by testing

bacteria isolated from kidney tissue of moribund or dead fish. Pathology caused by the bacteria was documented by observing the external, internal and histopathological changes in the diseased fish. Histological preparations were made after fixation of moribund fish in bouin's solution for about two weeks. After fixation one of the gills and a transverse section of the body (5 mm in thickness) behind the operculum was taken and embedded in paraffin after standard dehydration procedures. The embedded tissues were sectioned at 7 µm thickness with a rotary microtome and mounted on 25 x 75 mm slides. The mounted tissues on slides were stained with either hematoxylin and eosin or Giemsa's stain and examined by compound microscope. Photographs were taken using Kodak Plus-X Pan film.

Comparative Pathogenicity of Vibrio ordalii and Vibrio anguillarum on Juvenile English sole and Chum salmon at Different Temperatures of Water

To compare the pathogenicity of the two species of Vibrio in salmonid and non-salmonid fishes, juvenile English sole (8.2 ± 0.6 cm, 5.0 ± 1.1 g) and chum salmon (11.4 ± 1.2 cm, 14.5 ± 4.4 g) were experimentally infected either with V. ordalii or V. anguillarum at 10, 14 or 18°C by water-borne exposure. Twelve degrees centigrade approximated ambient temperature (Frolander et al., 1973) and fish to be maintained at 10 or 18°C were acclimated prior to the experiment. The acclimation was done by increasing or decreasing

temperature 1°C per day until 18 or 10°C was reached. The experiment was started about one week after the fish had reached the acclimated temperature. Because results of a preliminary study indicated that each fish species had a different susceptibility to V. ordalii and V. anguillarum, different concentrations of bacteria were used in each combination between species of fish and species of bacteria. Juvenile English sole were exposed to 4.2×10^7 cells/ml of V. ordalii or 6.8×10^7 cells/ml of V. anguillarum. Fingerling chum salmon were exposed to 2.0×10^5 cells/ml of V. ordalii or 2.3×10^5 cells/ml of V. anguillarum. All fish were exposed for 30 minutes at 10, 14 or 18°C in 50 l of bacterial suspension. The experiment consisted of two replicates with 16 fish per replicate. The exposed fish were held at 10, 14 or 18°C in pathogen free flowing seawater for 14 days. Mortality of the fish was checked daily. Relative pathogenicity was determined by the percent of mortality of each treatment. Causative agents were identified from kidney tissue smears on TSA from all fish that died.

Comparative Pathogenicity of Vibrio ordalii and Vibrio anguillarum in different sizes of English sole

To determine if the size of fish affects susceptibility to vibrosis, two different sizes of English sole were experimentally infected with either V. ordalii (7.6×10^7 cells/ml) by water-borne exposure for 30 minutes at

14°C. Each treatment had six replicates with 20 fish per replicate. The small size group of English sole measured 7.7 ± 0.7 cm and 4.4 ± 0.7 g, and the large size group measured 12.8 ± 0.6 cm and 16.2 ± 1.4 g per fish. The mortality was checked daily and the experiment was terminated after 14 days. The comparison of the pathogenicity was based on the percent mortality in each treatment. Bacteria were isolated and identified as described earlier.

Comparative Pathogenicity of Vibrio anguillarum in English sole and Chum salmon by Water-borne Exposure and Intraperitoneal Injection

An experiment was designed to determine whether external or internal protective factors were operating in the protection of English sole and chum salmon from vibriosis. Three doses of bacteria were used in both water-borne exposure and intraperitoneal injection of each fish species. There were 12 treatment combinations with three replicates per treatment, and 10 fish per replicate. Vibrio anguillarum was the only bacterium used in the experiment. The bacterium was passed three times through English sole or chum salmon before being used in the experiment. The size of English sole was 8.5 ± 0.7 cm, and 5.6 ± 1.2 g, and for chum salmon was 14.9 ± 0.9 cm, and 28.3 ± 6.8 g per fish. The concentrations of V. anguillarum used in water-borne exposure were 3.8×10^6, 3.8×10^7, and 3.8×10^8 cells/ml for English sole, and 1.8×10^4, 1.8×10^5, and 1.8×10^6 cells/ml for

chum salmon and fish were exposed for 30 minutes at 14°C.

For intraperitoneal injections, the bacteria from broth culture were harvested and washed three times in PBS (3,000 rpm, 10 minutes, at 0°C), and resuspended in PBS before use. Each individual fish was injected with 0.1 ml washed V. anguillarum cells suspension using one mililiter tuberculin syringe and 26 G x ½ inch needle. The concentrations of the bacterial suspensions were 6.1×10^6, 6.1×10^7, and 6.1×10^8 cells/ml for English sole, and 1.8×10^4, 1.8×10^5, and 1.8×10^6 cells/ml for chum salmon. The fish were anesthetized with tricaine methane sulfonate (MS 222), 50 mg/l, before injection. The infected fish were held in 125 liters of seawater supplied with flowing ultraviolet filtered seawater at about 2 to 3 liters per minute and 14°C.

Fish mortality was checked daily until the experiment was terminated. The comparative pathogenicity of V. anguillarum in water-borne exposure and intraperitoneal injection was based on the LD_{50} of each exposure method. The LD_{50}s were determined based on the method used by Reed and Muench (1938). During the experiment the fish were not fed and bacteria were isolated and identified as before.

Immune Response Experiments

Experiments were designed to determine the immune response and duration of antibody titers in the sera of

English sole and chum salmon after immunization either with V. ordalii or V. anguillarum bacterin. Two experiments were conducted to achieve these purposes. There were 160 juvenile English sole (11.2 ± 0.9 cm, 7.4 ± 1.7 g) and 160 fingerling chum salmon (15.8 ± 1.5 cm, 36.0 ± 7.5 g) used in the first experiment. Each fish species was divided into two groups consisting of 80 fish and maintained separately in circular tanks containing 125 liters seawater supplied with pathogen free seawater (2 to 3 l/min) at 14°C for 15 days. The fish were fed daily with Oregon Moist Pellets, about 5% of body weight for English sole, and about 3% for chum salmon. After 15 days the first group of each species was immunized by intraperitoneal injection with 0.1 ml (of 10^9 cells/ml) of formalin killed V. ordalii bacteria using 1.0 ml tuberculin syringe and 26 G x 1/2 inch needle. The second group of each species was injected with 0.1 ml sterile PBS (pH 7.0) and used as a control. Tricaine methane sulfonate (MS 222), 50 mg/l was used as an anesthetic while the fish were being injected.

The second experiment was identical to the first except that V. anguillarum was source of the bacterin. The second group, also consisting of 80 English sole and 80 chum salmon, was injected with 0.1 ml sterile PBS and used as a control.

Each week 2 or 3 fish were sampled from each tank to check the immune response. The microtiter method was used

to determine the antibody titers of the pooled serum of fish from each tank. When the antibody titers of immunized fish had reached a peak, about 4 weeks post immunization, the fish were challenged with live V. ordalii or V. anguillarum. Forty fish were taken randomly from each tank and divided into four groups (10 fish per group). The first two groups were challenged with the bacteria that had been passed through English sole, and the other two groups were challenged with the bacteria that had been passed through chum salmon. Water-borne exposure was used for this purpose, and conducted under conditions previously described. The bacterial concentrations used for water-borne exposures are shown in Table 1. Mortality was checked daily and bacterial pathogens were isolated and identified as described earlier. The relative percent survival (RPS) was calculated using the formula adapted by Johnson et al. (1982).

$$RPS = \left(1 - \frac{\% \text{ mortality of vaccinated fish}}{\% \text{ mortality of unvaccinated fish}} \right) \times 100\%$$

The remainder of the fish in each tank was sampled weekly until the eighth week, and monthly after eight weeks, to determine the duration of immune response.

Passive Protection Experiments

Two experiments were designed to determine if the relative resistance to vibriosis in English sole can be

Table 1. Concentrations of bacteria in water-borne exposure in immune response experiments (cells/ml)

Fish species (bacterin)	Challenged with	
	V. ordalii(E)*)	V. ordalii(C)**)
Experiment I		
English sole (V. ordalii, E)*)	5.5×10^{7}***)	5.2×10^{7}
English sole (PBS)	5.5×10^{7}	5.2×10^{7}
Chum salmon (V. ordalii, C)**)	5.0×10^{6}	5.0×10^{6}
Chum salmon (PBS)	5.0×10^{6}	5.0×10^{6}
Experiment II		
	V. anguillarum(E)*)	V. anguillarum(C)**)
English sole (V. anguillarum, E)*)	4.8×10^{7}***)	4.3×10^{7}
English sole (PBS)	4.8×10^{7}	4.3×10^{7}
Chum salmon (V. anguillarum, C)**)	2.9×10^{6}	2.6×10^{6}
Chum salmon (PBS)	2.9×10^{6}	2.6×10^{6}

*) (E) or E) : the bacterium was passed through English sole.

**) (C) or C) : the bacterium was passed through chum salmon.

***) There were two replicates in every dose (with 10 fish/replicate).

****) Water-borne: the fish were exposed for 30 minutes at 14°C.

transferred to chum salmon by intraperitoneal injection of English sole serum. In the first experiment, 96 fingerling chum salmon (17.9 ± 1.2 cm, 43.0 ± 18.5 g) were divided into two groups. In the first group, each fish was given an intraperitoneal injection with 0.1 ml serum of chum salmon using a 1.0 ml tuberculin syringe 26 G x 1/2 inch needle. The second group was injected with 0.1 ml English sole serum. The chum salmon serum used was the pooled serum of 20 fingerling chum salmon averaging 17.6 ± 1.2 cm, and 47.5 ± 7.6 g. The English sole serum was the pooled serum of 10 adult English sole averaging 22.5 ± 1.3 cm, and 126.3 ± 4.8 g. The adult English sole were reared from juvenile English sole captured in Yaquina Bay that had been maintained in pathogen free seawater under laboratory conditions for 3 to 4 years. Both chum salmon serum and English sole serum had slightly positive reactions to V. ordalii and V. anguillarum antigens (in 1:2 titer) when tested by the microtiter technique. When the fish were to be injected they were anesthetized with MS 222, 50 mg/l. Four days after the injection each group was divided into four subgroups consisting of 12 fish each. The first and the second subgroups were challenged by water-borne exposure to V. ordalii (8.6×10^6 cells/ml) for 30 minutes at 14°C. The third and the fourth subgroups were challenged with V. anguillarum (6.2×10^6 cells/ml) in the same manner. The mortality was checked daily, and

the bacteria isolated were identified from TSA cultures as in the other experiments.

A second experiment was conducted to determine if the amount of serum and the time between injection and challenge affect the degree of protection. Both fingerling chum salmon and adult English sole were taken from the same stock as they were in the first experiment. Thirty chum salmon (18.8 ± 4.3 cm, 55.5 ± 11.6 g) were injected intraperitoneally with 0.25 to 0.30 ml chum salmon serum, and another group of 30 was injected with 0.25 to 0.30 ml English sole serum. The chum salmon serum was pooled serum collected from 12 fingerling chum salmon, and English sole serum was pooled from 8 adult English sole. Both sera had a slightly positive reaction to V. anguillarum antigen tested by microtiter technique (in 1:2 titer). As previously, MS 222 (50 mg/l) was used as an anesthetic when the fish were to be injected. After injection each group was divided into three subgroups and kept separately in 125 liters of flowing seawater (2-3 l/min) at 14°C. Five hours after injection the fish were challenged with V. anguillarum (5.1×10^6 cells/ml) by water-borne exposure for 30 minutes at 14°C. Each subgroup consisting of 10 fish was used for a replicate. The mortality was checked daily, and the isolated bacteria identified as described above.

Virulence Selection Experiment

An experiment was designed to measure changes in the virulence of the two species of Vibrio after several passages through either chum salmon or English sole. Vibrio ordalii isolate MSC 275 and V. anguillarum isolate LS 173 were inoculated onto TSA and incubated at 18°C for 2-4 days. Identification was confirmed by gram stain, colony morphology, sensitivity to novobiocin and vibriostat 0/129, and rapid slide agglutination test. Each species of the bacterium was then passed three times either through chum salmon or English sole by the water-borne exposure method. Kidney tissue from dead fish was inoculated onto TSA after each passage and identified as above. Any changes in virulence for the homologous fish species were determined after the three passages on the basis of LD_{50} of water-borne exposure method. Based on a preliminary study, three doses of V. ordalii and V. anguillarum were used in the water-borne exposure. There were 24 treatment combinations in this experiment (two species of fish, four "isolates" of bacteria, and three doses of each isolate). Each treatment consisted of two replicates with 16 fish per replicate. The English sole that were used in the experiment measured 9.5 ± 1.2 cm and 8.4 ± 2.6 g, and the chum salmon measured 10.5 ± 1.1 cm and 8.6 ± 2.8 g. The fish were experimentally infected by water-borne exposure in a suspension of the bacteria at 12°C. The concentrations

of the bacteria that were used in the water-borne exposures are shown in Table 2. Dead fish were sampled each day until the experiment was terminated. Kidney tissue from each of the dead fish was innoculated onto TSA for identification of the causative agent. The method for calculation of the LD_{50} was adopted from that of Reed and Muench (1938).

Statistical Methods

The treatments in all experiments that were used to determine the difference in percent mortality and mean death time under laboratory conditions were arranged in a completely random design (Cochran and Cox, 1957). To determine the difference between treatments within an experiment, factorial analysis of variance was used (Steel and Torrie, 1980), except in the second passive immunization experiment where the difference in percent mortality and mean death time between groups was analyzed by a t test. The difference between treatments in an experiment both in factorial and t-test analysis was based on F or t-table with $P < 0.05$.

Table 2. Concentrations of the bacteria in water-borne exposure in virulence selection experiment (cells/ml)

Bacteria species		Fish species	
		English sole	Chum salmon
Vibrio ordalii (E)*)	1.	3.6×10^6 ***)	2.3×10^5
	2.	3.6×10^7	2.3×10^6
	3.	3.6×10^8	2.3×10^7
Vibrio ordalii (C)**)	1.	3.1×10^6	2.3×10^5
	2.	3.1×10^7	2.3×10^6
	3.	3.1×10^8	2.3×10^7
Vibrio anguillarum (E)*)	1.	2.3×10^6	1.9×10^4
	2.	2.3×10^7	1.9×10^5
	3.	2.3×10^8	1.9×10^6
Vibrio anguillarum (C)**)	1.	2.5×10^6	1.4×10^4
	2.	2.5×10^7	1.4×10^5
	3.	2.5×10^8	1.4×10^6

*) V. ordalii and V. anguillarum that have been passed through English sole.

**) V. ordalii and V. anguillarum that have been passed through chum salmon.

***) There were two replicates (16 fish/replicate) for each dose.

CHAPTER IV

RESULTS

Comparative Pathology

Both Vibrio species caused similar pathological changes in both fish species, but the onset of pathological signs in V. anguillarum infected fish was faster than in those infected with V. ordalii. Chum salmon infected with bacteria by the water-borne route commonly had a darkening of the dorsal part of the body, erythema at the base of the fins and erected scales (Fig. 3 and 4). The fish became sluggish, lost balance, and often developed exophthalmia and a distended abdomen with excessive fluid in the body cavity. Hemorrhaging was common in gills, within the mouth, body musculature, intestine and other internal organs. If the fish did not die until this stage, sloughing skin and red necrotic lesions in the musculature were often found shortly before fish died.

Most of the chum salmon that were injected intraperitoneally with bacteria developed a lesion around the injection site. This lesion became a hemorrhagic ulceration at the center with necrosis at the periphery (Fig. 4). Causative agents were easily isolated from kidney tissue of moribund or dead fish infected by water-borne or intraperitoneal injection routes.

Similar gross pathological changes were found in English sole infected with either V. ordalii or V. anguillarum. Most of the pathological signs were observed on the blind side of the fish. Hemorrhages occurred in fins, base of the fins, operculum, body musculature (normally along the lateral line), and in internal organs such as intestine, kidney, spleen and liver. Necrotic fins and very severe hemorrhaging were observed in the late stages of the disease (Fig. 1). A few fish also developed a grey-colored lesion on the skin of the eyed side. A distended abdomen was observed in some diseased English sole but no fish with exophthalmia was observed. In slowly progressing infections, hemorrhages developed in the body musculature posterior to the abdomen (Fig. 2).

There were no major differences in pathological changes in English sole infected by the water-borne or intraperitoneal injection routes. In some cases hemorrhaging was present around an injection site, but ulceration was not normally observed. Causative agents were easily isolated from kidney tissue of the moribund or the dead fish. In both fish species, there were some fish that died without any clear signs of the disease, especially fish which died about 1 to 2 days after exposure to bacteria.

Histological preparations from moribund individuals (about 3 to 9 days after exposure) showed that necrotic

Figure 1. Dead English sole (Parophrys vetulus) after water-borne exposure to Vibrio anguillarum. (H = hemorrhages; N = necrotic fin).

Figure 2. Dead English sole (Parophrys vetulus) after water-borne exposure to Vibrio ordalii. (H = hemorrhage extensive in posterior portion of body).

Figure 3. Dead chum salmon (Oncorhynchus keta) with hemorrhages (H) at the base of fins and vent after water-borne exposure to Vibrio ordalii.

Figure 4. Dead chum salmon (Oncorhynchus keta) infected with Vibrio anguillarum.

above: with epidermal necrosis (N) after water-borne exposure to the bacteria, and

bottom: after intraperitoneal injection with bacteria which caused hemorrhagic ulceration (HU) around injection site.

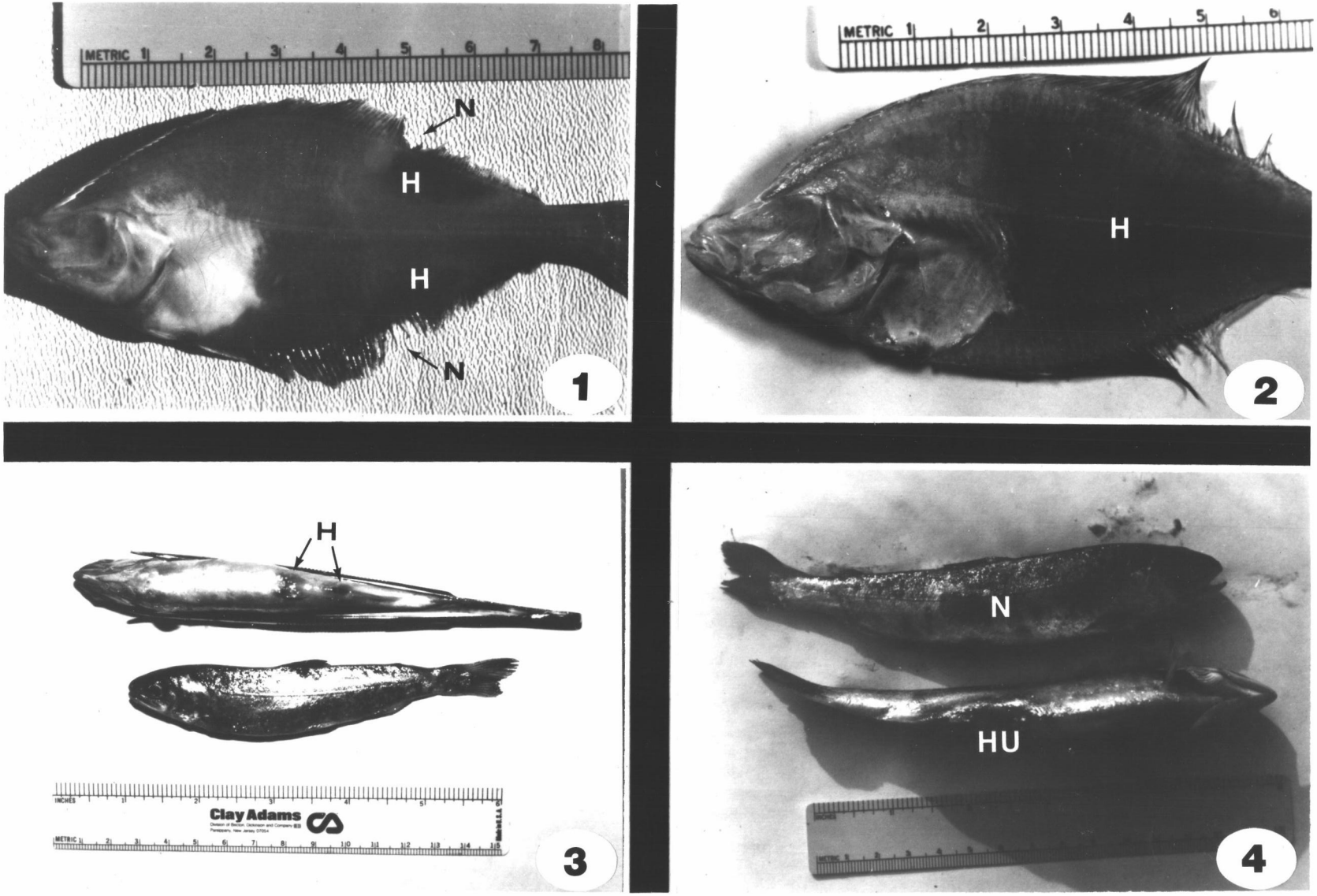
METRIC
N
H
H
N
1
H
2
H
3
Clay Adams
N
HU
4

Figure 5. Section of gill of uninfected English sole (Parophrys vetulus). (LE = lamellar epithelium) (400 x)

Figure 6. Section of gill of moribund English sole (Parophrys vetulus) after water-borne exposure to Vibrio anguillarum. (Most of lamellar epithelium has been lost) (400 x)

Figure 7. Section of kidney of uninfected English sole (Parophrys vetulus). (400 x)

Figure 8. Section of kidney of moribund English sole (Parophrys vetulus) after water-borne exposure to Vibrio anguillarum. (N = necrosis of hematopoietic tissue; B = bacteria) (400 x)

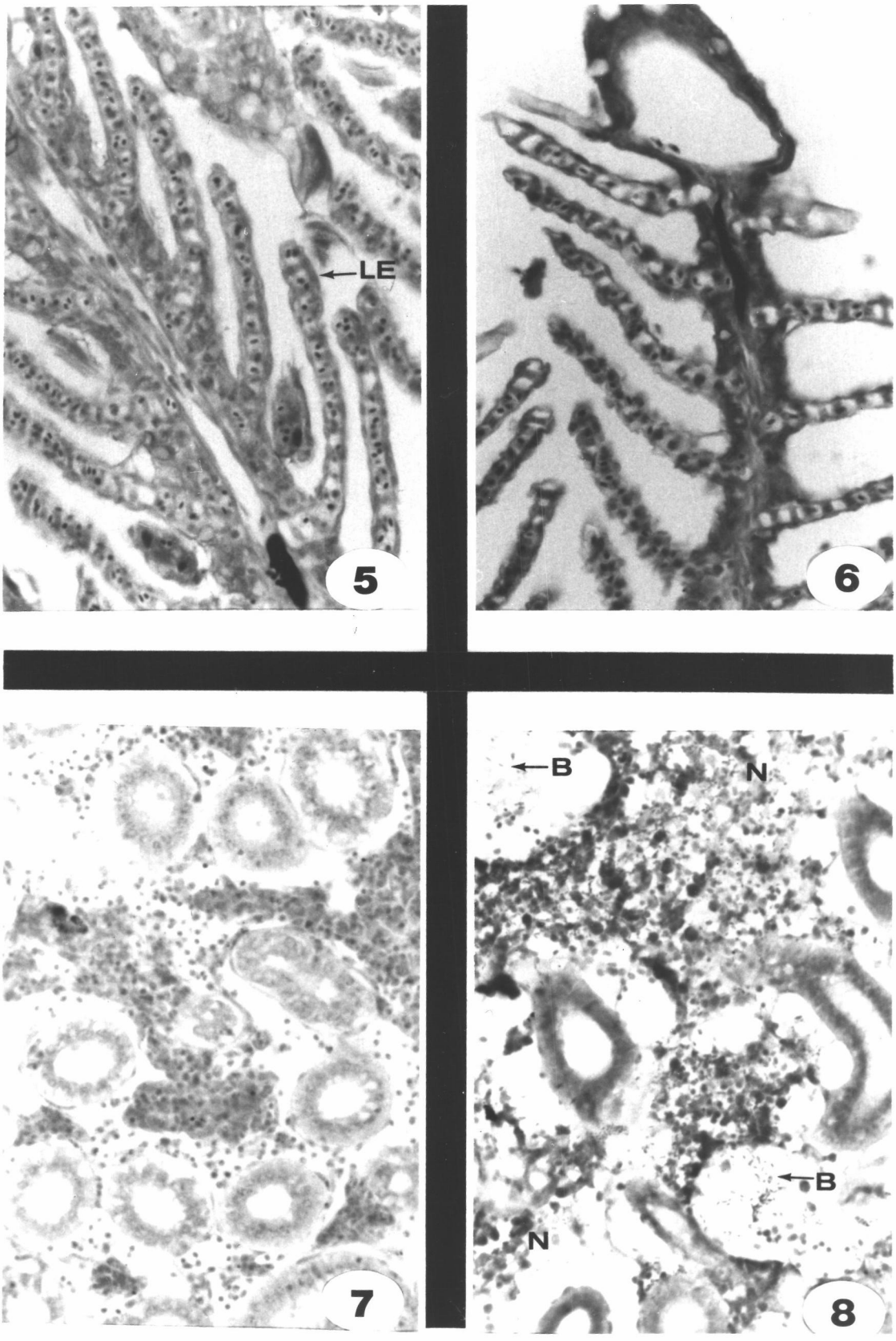

FIGURE 5 - 8

Figure 9. Section of muscle of uninfected English sole (Parophrys vetulus). (400 x)

Figure 10. Section of muscle tissue of moribund chum salmon (Oncorhynchus keta) after water-borne exposure to Vibrio anguillarum (B = bacteria). (400 x)

Figure 11. Section of muscle tissue of moribund English sole (Parophrys vetulus) after water-borne exposure to Vibrio ordalii (B = bacteria) (400 x)

Figure 12. Section of muscle tissue of moribund chum salmon (Oncorhynchus keta) after water-borne exposure to Vibrio ordalii (C = small colonies of bacteria). (1,000 x)

Figure 13. Bacteria in connective tissue of moribund English sole (Parophrys vetulus) after water-borne exposure to Vibrio anguillarum. (B = bacteria) (400 x)

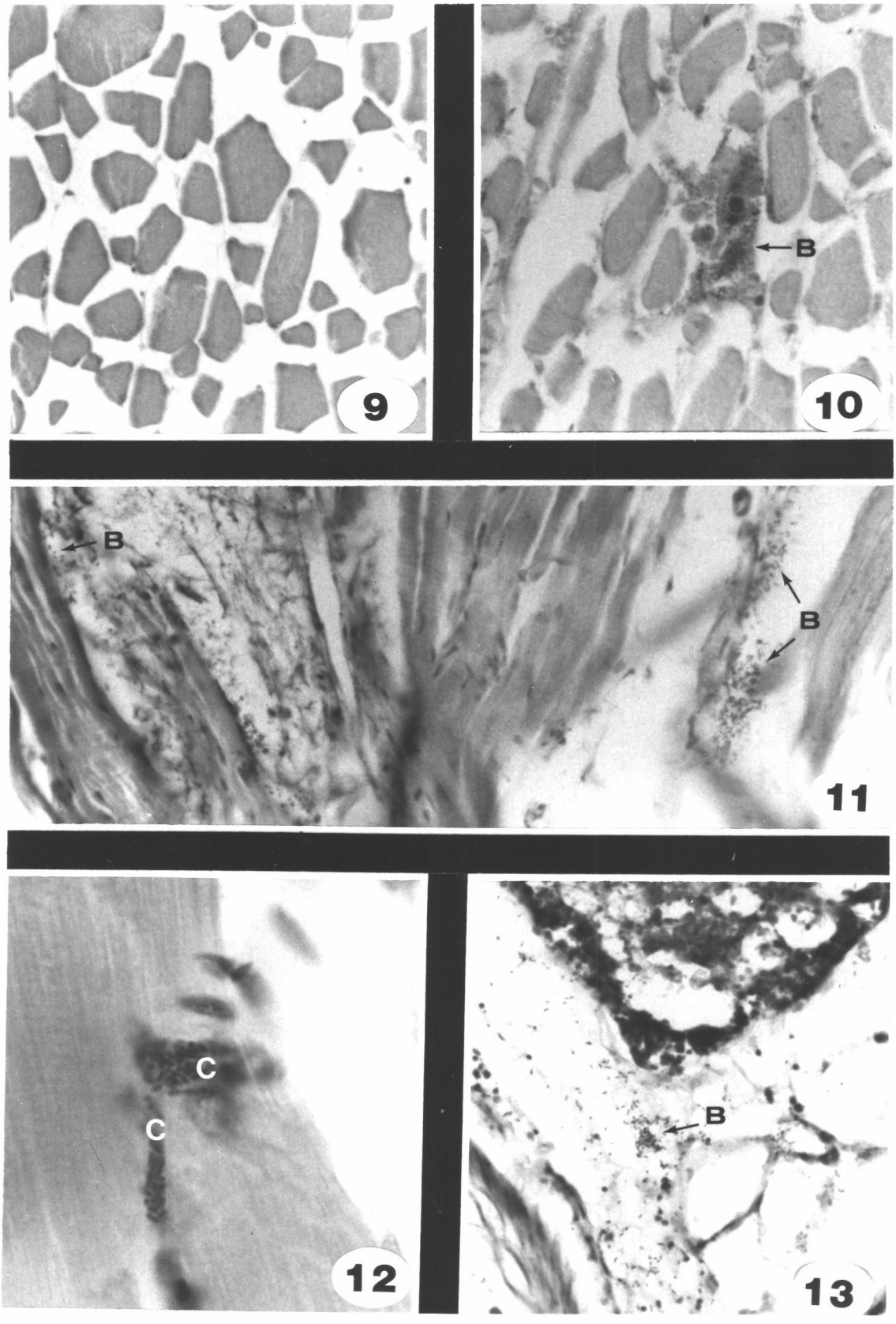

FIGURE 9 - 13

kidney tissue and damaged gill filaments with sloughing epidermis were normally observed in both chum salmon and English sole infected with either V. ordalii or V. anguillarum. Although the bacteria were present in liver, spleen, pancreas and intestine there was no obvious necrotic tissue observed. Anatomical sites where both V. ordalii and V. anguillarum were found in chum salmon and in English sole are presented in Table 3. In general, both Vibrio species had similar sites of infections in both chum salmon and English sole. Variations in the number of bacteria within or between sites of infections were observed in both bacterial species and both fish species. In most cases both bacterial species dispersed in infected tissue except V. ordalii which sometimes formed small and loose colonies within the muscle tissue of infected chum salmon. No colonies of V. ordalii were observed in muscle tissue of infected English sole.

Comparative Pathogenicity of Vibrios at Different Water Temperatures

Variation between replicates in percent mortality and mean time to death was very common in both English sole and in chum salmon (Table 4). The higher the water temperature the greater the variation in percent mortality. However, mortality generally increased with increasing water temperature, and statistically significant differences in percent mortality were found between 10 and 14°C,

Table 3. Sites of infections for Vibrio anguillarum or Vibrio ordalii in chum salmon (Oncorhynchus keta) or English sole (Parophrys vetulus)

Organ or tissue examined	Chum salmon		English sole	
	V. anguillarum	V. ordalii	V. anguillarum	V. ordalii
Blood	+ +	+	+ +	+
Loose connective tissue	+ +	+	+ +	+ +
Kidney	+	+	+ +	+
Spleen	+	+	+	+
Skeletal muscle	+	+	+	+
Gastrointestinal tract	+ +	+ +	+ +	+ +
Integument	+	+ +	+ +	+
Liver	+	+	+	+
Pancreas	+ +	+ +	+ +	+
Gills	+ +	+ +	+ +	+
Bacteria in colonies	No	few & small	No	No
Bacteria dispersed	Yes	Yes	Yes	Yes

+ = Bacteria observed, but were in low numbers and difficult to find at 400 x.

+ + = Bacteria were observed in a higher number than (+) and were easily found at 400 x.

Table 4. Mortality rates of English sole (Parophrys vetulus) and chum salmon (Oncorhynchus keta) infected with Vibrio ordalii or Vibrio anguillarum at 10, 14 and 18°C

Fish species/ Bacteria species	Rep. *)	Mortality rates (%) at 10°C	14°C	18°C**)
English sole				
V. ordalii (4.2×10^7 cells/ml)	1.	18.8	43.8	81.3
	2.	25.0	37.5	68.8
Average		21.9	40.6	75.0
V. anguillarum (6.8×10^7 cells/ml)	1.	37.5	62.5	81.3
	2.	31.3	56.3	93.8
Average		34.4	59.4	87.5
Chum salmon				
V. ordalii (2.0×10^5 cells/ml)	1.	43.8	75.0	93.8
	2.	50.0	68.8	81.3
Average		46.9	71.9	87.5
V. anguillarum (2.3×10^5 cells/ml)	1.	68.8	81.3	100.0
	2.	62.5	75.0	100.0
Average		65.6	78.1	100.0

*) 16 fish per replicate.

**) There were significant differences in mortality rates between 10 and 14°C, 10 and 18°C, and 14 and 18°C ($P < 0.05$).

10 and 18°C, and between 14 and 18°C, in all treatment combinations. The results also showed that V. anguillarum caused a significantly higher percent mortality than did V. ordalii in both English sole and chum salmon at each water temperature.

Water temperature not only affected mortality rate, but also significantly affected mean time to death (Table 5). In general, the higher the water temperature the shorter the mean time to death. Statistically, the mean time to death was significantly different between 10 and 14°C, 10 and 18°C, and between 14 and 18°C in all treatment combinations. Significant differences in mean time to death were found between English sole and chum salmon at all water temperatures. In general, English sole had a higher mean time to death than did chum salmon infected with either bacterial species. If two bacterial species are compared, there were significant differences in mean time to death between V. ordalii and V. anguillarum at all water temperatures. Vibrio ordalii infection resulted in a longer mean time to death than did V. anguillarum in both fish species. Statistical analysis also showed that there were interactions between water temperature and fish species, and between water temperature and bacterial species in the mean time to death.

In addition, water temperature also affected the period between exposure to the bacteria and the beginning

Table 5. Mean time to death of English sole (Parophrys vetulus) and chum salmon (Oncorhynchus keta) infected with Vibrio ordalii or Vibrio anguillarum at 10, 14 and 18°C

Fish species/ Bacteria species	Rep. *)	Mean time to death (days) at 10°C	14°C	18°C**)
English sole				
V. ordalii (4.2×10^7 cells/ml)	1.	9.0	7.9	6.9
	2.	8.8	7.3	6.9
Average		8.9	7.6	6.9
V. anguillarum (6.8×10^7 cells/ml)	1.	8.7	4.8	4.1
	2.	7.8	5.3	4.5
Average		8.3	5.1	4.3
Chum salmon				
V. ordalii (2.0×10^5 cells/ml)	1.	6.6	5.1	2.5
	2.	7.0	5.6	3.1
Average		6.8	5.4	2.8
V. anguillarum (2.3×10^5 cells/ml)	1.	6.3	4.6	2.3
	2.	6.1	4.7	2.5
Average		6.2	4.7	2.4

*) 16 fish per replicate.

**) There were significant differences in mean time to death between 10 and 14°C, 10 and 18°C, and 14 and 18°C ($P < 0.05$).

of fish mortality. At 18°C, in both English sole and chum salmon that were infected by either V. ordalii or V. anguillarum, mortality began in 2 to 3 days, at 14°C in 2 to 4 days, at 10°C in 3 to 5 days after exposure. In general, at high water temperatures the mortality was high and occurred in a relatively short period of time, while at low water temperatures, the mortality was low and tended to extend over a relatively long period of time.

Comparative Pathogenicity on Different Sizes of English sole

When the susceptibility of different sized English sole to Vibrio species was compared, there were variations between the replicates in percent mortality and mean time to death (Tables 6 and 7). The variations were greater in the small sized than in the large sized English sole. However, mortality started at almost the same time in both groups, between 3 and 4 days after exposure for V. ordalii and between 2 and 3 days for V. anguillarium. In V. anguillarum exposed fish, heavy mortality in both small and large size groups occurred between 3 and 5 days after exposure. In V. ordalii a similar heavy mortality did not occur, the mortality rate was low and continued until 11 days after exposure.

Mortality was statistically significantly higher in small sized than in large sized English sole (Table 6). In

Table 6. Mortality rates in different sized English sole (Parophrys vetulus) infected with Vibrio ordalii or Vibrio anguillarum after water-borne exposure at 14°C (small size: 7.7 ± 0.7 cm, 4.4 ± 0.7 g; large size: 12.8 ± 0.6 cm, 16.2 ± 1.4 g)

Bacteria species	Replicates *)	Mortality of English sole	
		small size %	large size % **)
V. ordalii (7.8×10^7 cells/ml)	1.	55.0	20.0
	2.	40.0	30.0
	3.	45.0	20.0
	4.	35.0	25.0
	5.	40.0	20.0
	6.	30.0	20.0
	Average	40.8	22.5
V. anguillarum (7.6×10^7 cells/ml)	1.	85.0	35.0
	2.	70.0	30.0
	3.	70.0	25.0
	4.	55.0	30.0
	5.	65.0	30.0
	6.	45.0	30.0
	Average	65.0	30.0

*) 20 fish per replicate.

**) There was significant difference in mortality rates between small and large groups ($P < 0.05$).

Table 7. Mean time to death of different sized English sole (*Parophrys vetulus*) infected with *Vibrio ordalii* or *Vibrio anguillarum* after water-borne exposure at 14°C (small size: 7.7 ± 0.7 cm, 4.4 ± 0.7 g; large size: 12.8 ± 0.6 cm, 16.2 ± 1.4 g)

Replicates *)	Mean time to death (days)			
	Vibrio ordalii (7.8×10^7 cells/ml)		*Vibrio anguillarum* (7.6×10^7 cells/ml)	
	small size	large size	small size	large size**
1.	7.9	7.5	4.2	6.0
2.	7.4	7.8	3.9	5.5
3.	6.3	6.0	4.6	4.0
4.	7.6	6.6	3.6	4.0
5.	6.4	7.5	5.3	4.5
6.	6.0	8.3	4.9	4.5
Average	6.9	7.3	4.4	4.8

*) 20 fish per replicate.

**) There was no significant difference in mean time to death between small and large groups ($P < 0.05$).

English sole infected with V. ordalii, the mortality in the small sized group ranged between 30 to 55% (average 40.8%), and in the large sized group ranged between 30 and 30% (average 22.5%). The mortality of English sole caused by V. anguillarum in the small group was between 45 and 85% (average 65%), and in the large group between 25 and 35% (average 30.0%).

Mean time to death of fish that were challenged with V. ordalii ranged from 6.0 to 7.9 days (average 6.9 days) in the small size group, and from 6.0 to 8.3 days (average 7.3 days) in the large size group (Table 7). In the fish that were challenged with V. anguillarum, the mean time to death was 3.6 to 5.3 days (average 4.4 days) in the small size group, and from 4.0 to 6.0 days (average 4.8 days) in the large size group. The mean time to death of fish in the two size groups of English sole was not significantly different whether infected with V. ordalii or V. anguillarum. When the comparison was made between species of Vibrio, mean time to death of English sole caused by V. anguillarum was significantly shorter than that caused by V. ordalii in both size groups.

Comparative Pathogenicity of Vibrio anguillarum in English sole and Chum salmon Exposed by Water-borne and Intraperitoneal Injection

In English sole the LD_{50} for water-borne exposure was higher than the LD_{50} for intraperitoneal injection of

V. anguillarum (Table 8). In English sole the LD_{50} of water-borne exposure varied between 4.1 and 35.7 times the LD_{50} of intraperitoneal injection. In chum salmon the LD_{50} of water-borne exposure varied between 28.4 and 130.8 times the LD_{50} of intraperitoneal injection. Statistically the LD_{50} of water-borne exposure was significantly higher than the LD_{50} of intraperitoneal injection in both English sole and chum salmon. Further statistical analysis also showed that the LD_{50} in English sole was significantly higher than in chum salmon after water-borne exposure and intraperitoneal injection. After water-borne exposure the LD_{50} for English sole was between 33.1 and 363.1 times (average 151.4 times) the LD_{50} of chum salmon, and after intraperitoneal injection, was between 259.1 and 1,096.5 times (average 575.4 times). In general, the ratio of LD_{50} after intraperitoneal injection of English sole to chum salmon was higher than after water-borne exposure.

In addition to greater LD_{50} values, water-borne exposure also resulted in a greater mean time to death than did intraperitoneal injection in all bacterial concentrations (Table 9). Regardless of bacterial concentration, English sole had a mean time to death between 2.0 and 4.5 days after water-borne exposure, and between 2.3 and 4.3 days after intraperitoneal injection. Chum salmon had mean time to death between 4.2 and 7.7 days in water-borne exposure and between 3.3 and 4.6 days in intraperitoneal

Table 8. The LD_{50} of English sole (Parophrys vetulus) and chum salmon (Oncorhynchus keta) after water-borne exposure and intraperitoneal injection with Vibrio anguillarum at 14°C.

Fish species	Rep.	LD_{50} (cells/ml)	
		Water-borne	i.p. injection*)
English sole	1.	1.7×10^7	1.4×10^6
	2.	5.8×10^6	5.6×10^5
	3.	2.0×10^7	8.1×10^5
	Average	1.4×10^7	9.2×10^5
Chum salmon	1.	6.2×10^4	1.9×10^3
	2.	5.4×10^4	1.3×10^3
	3.	1.7×10^5	1.4×10^3
	Average	9.5×10^4	1.5×10^3

*) There was significant difference in LD_{50} between water-borne and i.p. injection ($P < 0.05$).

Table 9. Average of mean time to death of English sole (Parophrys vetulus) and chum salmon (Oncorhynchus keta) after water-borne exposure and intraperitoneal injection with Vibrio anguillarum at 14°C

Fish species	Bacteria (cells/ml) **)	Average mean time to death (days)	
		Water-borne	i.p. injection***)
English sole	10^6	3.5	3.3
	10^7	3.3	3.3
	10^8	3.6	2.3
Chum salmon	10^4	6.7	4.1
	10^5	4.9	3.5
	10^6	4.4	4.5

*) i.p. injection: 0.1 ml of bacterial concentration per fish.

**) There were 3 replicates per bacterial concentration.

***) There was significant difference in mean time to death between water-borne and i.p. injection ($P < 0.05$).

injection. Statistically, the mean time to death after water-borne exposure was significantly higher than mean time to death after intraperitoneal injection. When the comparison was made between fish species, the results showed that the mean time to death of chum salmon was significantly longer than that of English sole after water-borne exposure and intraperitoneal injection (Table 9).

Immune Response Experiments

The mortality rate of English sole challenged with V. ordalii ranged from 10 to 20% in vaccinated fish, and from 30 to 70% in unvaccinated fish. In English sole that were challenged with V. anguillarum the mortality rate ranged from 10 to 20% in vaccinated fish, and from 40 to 60% in unvaccinated fish. There was no mortality in vaccinated chum salmon challenged with V. ordalii, and only one out of four replicates had a 10% mortality when the fish were challenged with V. anguillarum. In unvaccinated chum salmon challenged with V. ordalii the mortality rate was between 20 and 70%, and in those which were challenged with V. anguillarum, between 70 and 90%. Statistical analysis showed that the mortality rate was significantly higher in unvaccinated fish than in vaccinated fish in all treatment combinations. Within bacterial species, there was no significant difference in mortality rate caused by bacteria that were passed through English sole or chum

salmon in both vaccinated and unvaccinated fish. The average mortality rates of both vaccinated and unvaccinated fish are given in Table 10.

In most treatments, mortality in unvaccinated fish began sooner (about two days after challenged) than in vaccinated fish (about three days after challenged). However, statistical analysis did not show that there was a significant difference in mean time to death between vaccinated and unvaccinated groups, or between V. ordalii and V. anguillarum within fish species. In English sole, mean time to death of the vaccinated group was between 3.0 and 5.0 days, and in the unvaccinated group was between 2.3 and 7.3 days. In unvaccinated chum salmon the mean time to death was between 5.4 and 8.5 days. The average mean time to death in each treatment is presented in Table 11.

Relative percent survival (RPS) of English sole challenged with V. ordalii ranged between 50 and 71.4% (average 65.2%), and with V. anguillarum was between 50 and 80% (average 69.7%). In chum salmon challenged with V. ordalii the RPS was 100%, and with V. anguillarum was between 85.7 and 100% (average 96.9%). Chum salmon had a higher RPS than did English sole whether challenged with V. ordalii or V. anguillarum. When the comparison of RPS was made between fish exposed to bacteria that passed through the heterologous fish species, slightly different RPS were

Table 10. Average mortality and relative percent survival (RPS) of vaccinated and unvaccinated English sole (Parophrys vetulus) and chum salmon (Oncorhynchus keta) challenged with Vibrio ordalii and Vibrio anguillarum at 14°C

Fish species/ Bacteria species	Average mortality rates (%) Vaccinated	Unvaccinated	RPS (%)
English sole			
V. ordalii (E) (5.5×10^7)	15.0 *)	45.0	66.7 **)
(C) (5.2×10^7)	20.0	55.0	63.6
Average	17.5	50.0	65.2
V. anguillarum (E) (4.8×10^7)	15.0	55.0	72.7
(C) (4.3×10^7)	15.0	45.0	66.7
Average	15.0	50.0	69.7
Chum salmon			
V. ordalii (E) (5.0×10^6)	0.0	30.0	100.0
(C) (5.0×10^6)	0.0	60.0	100.0
Average	0.0	45.0	100.0
V. anguillarum (E) (2.0×10^6)	0.0	80.0	100.0
(C) (2.6×10^6)	5.0	80.0	93.8
Average	2.5	80.0	96.9

(E): The bacteria were passed three times through English sole.

(C): The bacteria were passed three times through chum salmon.

*): There was significant difference in mortality rates between vaccinated and unvaccinated fish ($P < 0.05$).

**): There were two replicates per treatment, with 10 fish per replicate.

Table 11. Average mean time to death of vaccinated and unvaccinated English sole (Parophrys vetulus) and chum salmon (Oncorhynchus keta) challenged with Vibrio ordalii and Vibrio anguillarum at 14°C

Fish species/ Bacteria species		Average mean time to death (days) Vaccinated	Unvaccinated*)
English sole			
V. ordalii	(E) (5.5×10^7)	3.5	2.8**)
	(C) (5.2×10^7)	4.0	3.1
	Average	3.75	2.95
V. anguillarum	(E) (4.8×10^7)	3.5	4.1
	(C) (4.3×10^7)	3.5	5.8
	Average	3.50	4.95
Chum salmon			
V. ordalii	(E) (5.0×10^6)	-***)	7.3
	(C) (5.0×10^6)	-	5.4
	Average	-	6.35
V. anguillarum	(E) (2.9×10^6)	-	5.7
	(C) (2.6×10^6)	3.0****)	6.5
	Average	3.0	6.1

(E): The bacteria were passed three times through English sole.

(C): The bacteria were passed three times through chum salmon.

*): There was no significant difference in mean time to death between vaccinated and unvaccinated fish ($P < 0.05$).

**): There were two replicates per treatment, with 10 fish per replicate.

***): There was no fish mortality.

****): Only one out of two replicates.

observed. Exposure to bacteria that had been passed through the heterologous fish species resulted in a lower RPS in English sole, and the same or higher RPS in chum salmon than did exposure to the bacteria that had been passed through homologous fish species (Table 10).

Both juvenile English sole and fingerling chum salmon produced antibody titers against V. ordalii and V. anguillarum bacterins, and the titer level and duration was similar in both fish species (Figure 14 and 15). These fish species had higher antibody titers (about twofold) when they were immunized with V. anguillarum bacterin than when V. ordalii bacterin was used. Antibody titers were first detected 1 week after immunization, and reached a maximum in 3 or 4 weeks. After reaching peaks (1:32 for V. ordalii bacterin, and 1:64 for V. anguillarum bacterin), the antibody titers decreased slowly until the end of the experiment. However, when the experiment was terminated about 5 months after immunization both English sole and chum salmon still had positive antibody titers (between 1:2 and 1:4).

Some juvenile English sole (about 5-10%) from both vaccinated and unvaccinated groups died about one month after immunization. Kidney tissue was innoculated onto TSA and no bacterial infections were detected. The dead fish were usually thin and the digestive tract contained no food suggesting that they were not feeding and starved to death.

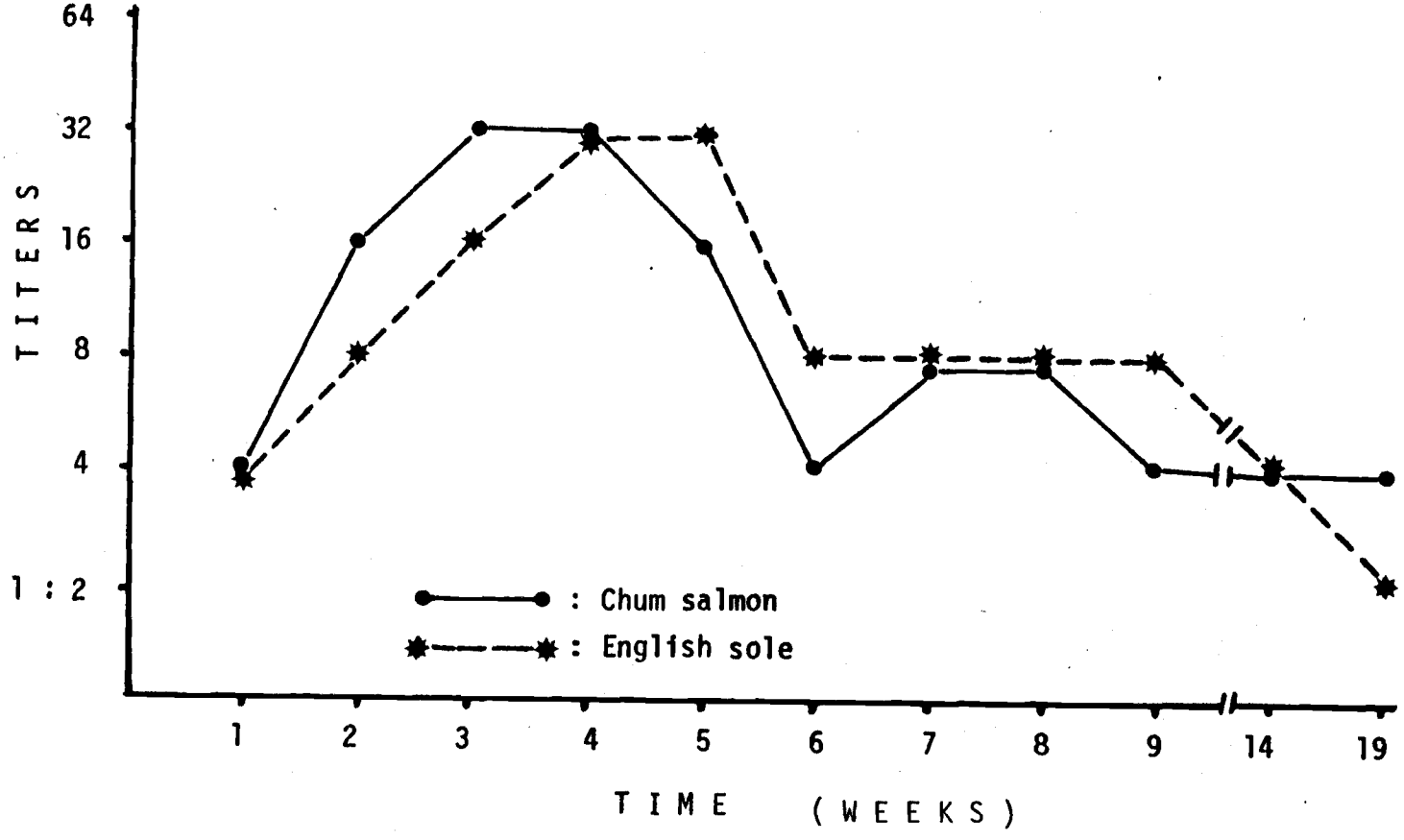

FIGURE 14. Antibody titers of chum salmon (Oncorhynchus keta) and English sole (Parophrys vetulus) serum after immunization with Vibrio ordalii bacterin

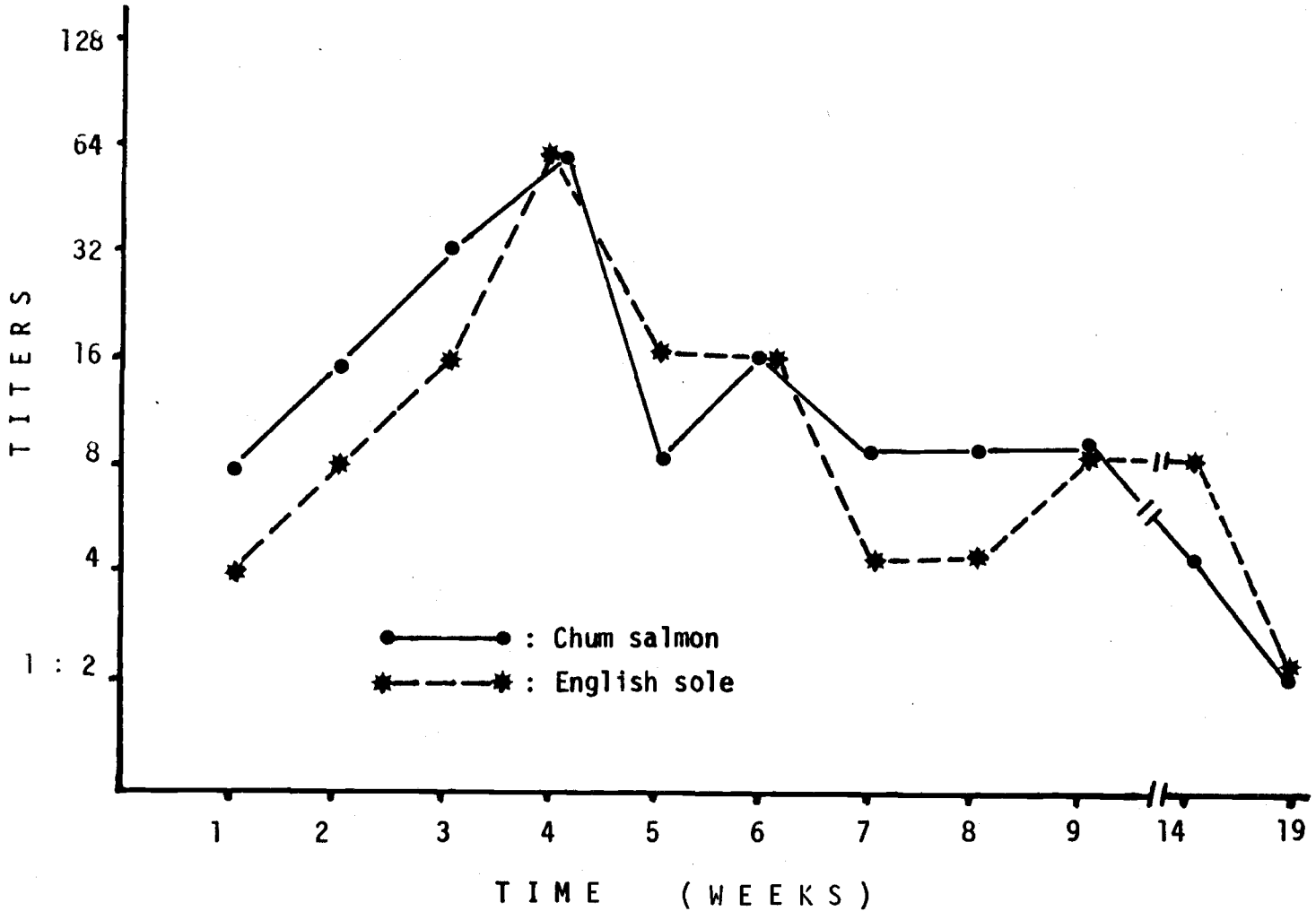

FIGURE 15. Antibody titers of chum salmon (Oncorhynchus keta) and English sole (Parophrys vetulus) serum after immunization with Vibrio anguillarum bacterin

Passive Protection Experiments

The average mortality rate of chum salmon that were injected with chum salmon serum and challenged with V. ordalii was 62.5% and with V. anguillarum was 58.4% (Table 12). In chum salmon that were injected with English sole serum and challenged with V. ordalii the average mortality was 45.9% and with V. anguillarum was 41.7%. The mortality rates in fish that were injected with English sole serum were generally lower than those that were injected with chum salmon serum. However, there was no statistically significant difference in percent mortality between the two groups.

The mean time to death for chum salmon that were injected with English sole serum and exposed to V. ordalii was higher than that for fish injected with chum salmon serum (Table 13). However, if the chum salmon were challenged with V. anguillarum, those that were injected with chum salmon serum had a higher mean time to death than those injected with English sole serum. There was no statistically significant difference in mean time to death between the two chum salmon groups. A significant difference in mean time to death was found only in chum salmon that were injected with English sole serum and then challenged with V. ordalii and with V. anguillarum. The mean time to death for chum salmon challenged with V. ordalii was higher (between 6.0 and 8.8 days) than the

Table 12. Mortality rates of chum salmon (Oncorhynchus keta) that were injected with chum salmon serum or English sole (Parophrys vetulus) serum and challenged with Vibrio ordalii or Vibrio anguillarum at 14°C

Challenged with	Rep. *)	Mortality rates (%) of chum salmon injected with	
		Chum salmon serum	English sole serum**)
V. ordalii (8.6×10^6 cells/ml)	1.	75.0	50.0
	2.	50.0	41.7
	Average	62.5	45.9
V. anguillarum (6.2×10^6 cells/ml)	1.	66.7	50.0
	2.	50.0	33.3
	Average	58.4	41.7

*) There were 12 fish per replicate.

**) There was no significant difference in mortality rates between chum salmon injected with chum salmon serum or English sole serum ($P < 0.05$).

Table 13. Mean time to death of chum salmon (Oncorhynchus keta) injected with chum salmon serum or English sole (Parophrys vetulus) serum and challenged with Vibrio ordalii or Vibrio anguillarum at 14°C

Challenged with	Rep. *)	Mean time to death (days) of chum salmon injected with	
		Chum salmon serum	English sole serum**)
V. ordalii (8.6×10^6 cells/ml)	1.	7.0	8.3
	2.	6.0	8.8
	Average	6.5	8.6
V. anguillarum (6.2×10^6 cells/ml)	1.	6.5	5.3
	2.	6.0	4.8
	Average	6.3	5.1

*) There were 12 fish per replicate.

**) There was no significant difference in mean time to death between chum salmon injected with chum salmon serum or English sole serum ($P < 0.05$).

mean time to death of chum salmon that were challenged with V. anguillarum (between 4.8 and 6.5 days). In addition, regardless of serum injection, the fish that were challenged with V. ordalii began to die later (5 days after challenge) than did those challenged with V. anguillarum (4 days). Increasing the dose of serum and earlier challenge did not cause significant effects on fish mortality rates and mean time to death. Chum salmon that were either injected with chum salmon serum or with English sole serum had relatively the same mortality rates and the same mean time to death (Table 14).

Virulence Selection Experiment

The LD_{50} of bacteria that were passed through different fish species was higher than that of bacteria that were passed through the same fish species (Table 15). The only exception occurred in replicate number two of the treatment combination between English sole and V. ordalii (C) where the homologous passage resulted in higher LD_{50}. However, statistical analysis showed that, within either bacterial species, there was no significant difference between LD_{50}'s of the bacteria that were passed through English sole or through chum salmon. In English sole, the ratio of LD_{50} V. ordalii (C) to V. ordalii (E) was between 0.74 and 2.03 (average 1.23), and V. anguillarum (C) to V. anguillarum (E) was between 2.08 and 2.43 (average 2.13). In chum salmon,

Table 14. Mortality and mean time to death of chum salmon (Oncorhynchus keta) injected with chum salmon serum or English sole (Parophrys vetulus) serum, and challenged with Vibrio anguillarum (5.1×10^6 cells/ml) at 14°C

Injected serum	Rep. *)	Mortality %**)	Mean time to death days***)
Chum salmon serum	1.	90.0	4.4
	2.	80.0	4.9
	3.	100.0	4.9
	Average	90.0	4.7
English sole serum	1.	70.0	6.1
	2.	100.0	5.2
	3.	80.0	6.0
	Average	83.3	5.8

*) 10 fish per replicate.

**) There was no significant difference in mortality rate between chum salmon injected with chum salmon serum or English sole serum ($P < 0.05$).

***) There was no significant difference in mean time to death between chum salmon injected with chum salmon serum or English sole serum ($P < 0.05$).

Table 15. LD_{50} of *Vibrio ordalii* or *Vibrio anguillarum* after three passages through either English sole *Parophrys vetulus*) or chum salmon (*Oncorhynchus keta*) at 12°C

Fish species/ Bacteria species		LD_{50} (cells/ml) Rep. 1	Rep. 2	Average*)
English sole				
V. ordalii	(E)	3.6×10^7	5.3×10^7	4.4×10^7
	(C)	7.3×10^7	3.9×10^7	5.4×10^7
V. anguillarum	(E)	1.2×10^7	7.0×10^6	9.4×10^6
	(C)	2.5×10^7	1.7×10^7	2.0×10^7
Chum salmon				
V. ordalii	(E)	8.9×10^5	7.3×10^5	8.0×10^5
	(C)	2.8×10^5	5.2×10^5	3.8×10^5
V. anguillarum	(E)	4.1×10^5	3.3×10^5	3.6×10^5
	(C)	1.4×10^5	6.1×10^4	9.0×10^4

(E): The bacteria were passed through English sole.

(C): The bacteria were passed through chum salmon.

*): There was no significant difference in LD_{50} between bacteria that passed through either chum salmon or English sole ($P < 0.05$).

the ratio of LD_{50} V. ordalii (E) to V. ordalii (C) was between 1.40 and 3.18 (average 2.11), and V. anguillarum (E) to V. anguillarum (C) was between 2.93 and 5.41 (average 4.00).

The results of this experiment also allowed observations on the differences in virulence between the two bacterial species. Statistical analysis indicated that there was a significant difference between the LD_{50} of V. ordalii and V. anguillarum in both fish species regardless of whether the bacteria were passed through English sole or chum salmon. The LD_{50} of V. ordalii was higher than the LD_{50} of V. anguillarum in both English sole and chum salmon. Significant differences were also found in the LD_{50} measurements between fish species. The LD_{50}'s of both species of Vibrio for English sole were higher than the LD_{50}'s for chum salmon.

Results when the mean time to death was calculated were similar to those based on LD_{50} (Table 16). There was no significant difference between mean time to death in either fish species when exposed to bacteria that were passed through either English sole or chum salmon. When the results at all bacterial exposure concentrations were combined, the mean time to death for English sole infected with V. ordalii was between 6.8 and 10.3 days, and between 3.7 and 6.5 days for those infected with V. anguillarum. In chum salmon infected with V. ordalii the mean time to

Table 16. Average of mean time to death of English sole (Parophrys vetulus) and chum salmon (Oncorhynchus keta) infected with Vibrio ordalii or Vibrio anguillarum after waterborne exposure at 12°C

Fish species/ Bacteria species	Average mean time to death (days)		
English sole	10^6 cells/ml	10^7 cells/ml	10^8 cells/ml*)
V. ordalii (E)	8.5	7.6	9.3
(C)	7.2	9.2	7.4
V. anguillarum (E)	5.3	5.1	6.1
(C)	5.7	5.5	4.3
Chum salmon	10^4 cells/ml	10^5 cells/ml	10^6 cells/ml
V. ordalii (E)	8.3	6.1	5.3
(C)	11.0	11.0	9.3
V. anguillarum (E)	7.3	7.8	6.5
(C)	9.4	6.3	6.1

(E): The bacteria were passed through English sole.

(C): The bacteria were passed through chum salmon.

*): There was no significant difference in mean time to death between bacteria that passed through chum salmon or English sole ($P < 0.05$).

death was between 4.8 and 12 days, and when infected with V. anguillarum between 5.6 and 9.4 days. In English sole, the results also showed that V. anguillarum could kill the fish significantly faster (average mean time to death between 4.3 and 6.1 days) than V. ordalii (average between 7.2 and 9.2 days). In chum salmon, the mean time to death of V. ordalii (averaged between 5.3 and 11.0 days) was not significantly different from V. anguillarum (averaged between 6.1 and 9.4 days). When the comparison was made between fish species there was no signficant difference in mean time to death between English sole and chum salmon when exposed to either bacterial species.

The mean time to death was affected by the concentration of the bacteria in the suspension at exposure. The higher the concentration of the bacteria the shorter the mean time to death. This was more consistently observed in chum salmon (Table 16), but there was variation in the onset of mortality. In English sole, both V. ordalii and V. anguillarum killed fish within two days after a waterborne exposure of 10^8 cells/ml. In this fish species, the greatest mortality caused by V. ordalii occurred between 5 and 10 days, and by V. anguillarum between 3 and 8 days after exposure. In chum salmon, the mortality started in three days after exposure to 10^6 cells/ml of V. ordalii, and four days after exposure to 10^6 cells/ml of V. anguillarum. Greatest mortality in chum salmon occurred between

5 and 10 days after exposure to *V. ordalii*, and between 5 and 8 days after exposure to *V. anguillarum*.

CHAPTER V

DISCUSSION

The major pathological changes cuased by Vibrio ordalii and Vibrio anguillarum in chum salmon and English sole were hemorrhages externally at the base of the fins and in body musculature and internally in the kidney, liver, spleen and intestine. Although variation was observed, both Vibrio spp. caused similar pathological signs in both chum salmon and English sole. However, the onset of the disease in chum salmon was faster than in English sole. In addition, diseased chum salmon tended to develop more extensive pathological changes including loss of scales and necrotic lesions not commonly observed in diseased English sole. The most severe pathological changes in English sole were hemorrhages in musculature of the posterior half of the body and necrosis of fins. In general, V. anguillarum caused an earlier onset of clinical signs than did V. ordalii in both chum salmon and English sole. The most likely explanation is the faster reproductive rate of V. anguillarum when compared to V. ordalii (Gould, 1977; Gould et al., 1979; Schiewe, 1983).

Different methods of experimentally infecting fish caused different pathological changes, especially in chum salmon. Water-borne exposure to bacteria resulted in pathological signs similar to pathological signs observed

in naturally infected fish and in fish exposed in this manner by other investigators (Cisar and Fryer, 1969; Fryer et al., 1972; Hastein and Holt, 1972; Levin et al., 1972; Hastein and Smith, 1977; Horne et al., 1977; Richards, 1980; Tajima et al., 1981; Ransom et al., 1984). When exposed by intraperitoneal injection, most of the infected chum salmon developed a hemorrhagic ulceration around the injection site and had fewer pathological signs in the other parts of the body than did fish exposed by the water-borne route. A possible explanation could be that in acute infections resulting from intraperitoneal injections the bacteria did not spread throughout the body as rapidly as did those in fish exposed by way of water where the bacteria could penetrate the body more generally from the gills (Tatner and Horne, 1983), the descending intestine, rectum and skin (Ransom et al., 1984). Similar studies have been done by Smith (1961), Chart and Munn (1980) and Kawano et al. (1983), who have reported similar pathological signs around the injection sites.

The examination of histological preparations made from moribund fish that were infected by water-borne exposure indicated that both V. ordalii and V. anguillarum had similar sites of infection in both chum salmon and English sole (Table 3). Although in some individuals certain organs were infected with relatively high numbers of bacteria, it was not possible to conclude that these particular

organs were primary sites of infection because high variations were observed in both fish species infected with both species of bacteria. These variations were probably due to the different time periods between exposure to the bacteria and the time fish became moribund (between 3 and 9 days).

No pathological changes were observed in histological preparations made 24 hours after fish were exposed to bacteria, and no bacteria were found in internal organs or muscle tissue. A few bacteria were observed in skin of a moribund chum salmon 24 hours after it was exposed to V. anguillarum. It is possible that skin is a route of entry of V. anguillarum. However, Ransom (1978) reported that V. ordalii and V. anguillarum entered the fish by penetrating the mucosa of the descending intestine and rectum, and only V. ordalii used the skin as a second route of entry.

A detailed study of histopathological changes in three species of Pacific salmon infected with V. ordalii or V. anguillarum has been reported by Ransom et al. (1984). In chum salmon that were infected with V. anguillarum, they found that kidney and spleen were heavily infected, and heavy concentration of bacteria were observed in liver, visceral peritoneum, cardiac muscle and connective tissue, and the bacteria were uniformly distributed in the infected areas. In contrast, in chum salmon that were infected with

V. ordalii they found that the bacteria were most frequently observed in muscle and skin, and that the bacteria formed colonies.

Although the same isolates of bacteria used by Ransom et al. (1984) were used in this study (V. ordalii MSC 2-75, and V. anguillarum LS 1-73), different results were obtained, especially in the fish that were infected with V. ordalii. In this study, only a few small, loose colonies were found in the muscle tissue of chum salmon infected with V. ordalii and no colonies were observed in infected English sole. In most cases both V. ordalii and V. anguillarum were dispersed in the infected tissue of the diseased fish. In addition, the number of bacteria observed was less than reported by Ransom et al. (1984) and in some fish bacteria were hard to find. These differences may be due to the different sizes or species of fish that were used in the study. Ransom et al. (1984) used chum salmon with a mean weight 1.75 g per fish, while in this study the fingerling chum salmon measured 14.5 g, and the juvenile English sole weighed 5.0 g per fish. These differences might affect the pathogenicity of the bacteria, and cause a different manifestation of infection because of differences in host defense mechanisms. According to Johnson et al. (1982a) and Johnson et al. (1982b), larger fish tend to have a stronger immune response than do smaller fish and Tatner and Horne (1983) indicated that larger fish have a more

mature immune system. Ransom et al. (1984) found only a few colonies of V. ordalii in 80 g chinook salmon and 25 g coho salmon although otherwise these species exhibited similar histopathological changes to those in chum salmon.

Another aspect of this study was an experiment conducted to compare the pathogenicity of the two species of Vibrio in salmonid and non-salmonid fishes at different water temperatures. The results indicated that in both English sole and chum salmon, increasing water temperature from 10 to 18°C increased mortality rates and decreased the mean time to death caused by both V. ordalii and V. anguillarum. These results were probably due to a combination of an increasing reproductive rate of the bacteria and increasing stress on the host.

Under laboratory conditions, the optimum temperature for English sole is about 10°C (Alderdice and Forrester, 1968; Williams, 1975; Williams and Caldwell, 1978), and for salmon, including chum salmon, is about 10-12°C (McNeil and Bailey, 1975; Sedgwick, 1982). In contrast, V. anguillarum can grow between 6.5 and 37°C (Muroga et al., 1976; Schiewe, 1981; Schiewe et al., 1981), with optimum growth at about 20°C (Horne et al., 1977; Ohnishi and Muroga, 1977; Tabata et al., 1982; Inamura et al., 1984; Muroga et al., 1984). The doubling time of the bacteria also decreases as the temperature increases (Groberg, 1981; Hahnel et al., 1982; Muroga et al., 1984). There is less information about the

range of temperatures at which V. ordalii grows. According to Schiewe (1981) and Schiewe et al. (1981), V. ordalii grew very well at 15 and 25°C but did not grow at 37°C.

In all experiments most fish died in a relatively short period of time (between 2.3 and 9.0 days after exposure to bacteria) and apparently did not produce enough antibody to give protection at any of the water temperatures employed. A few samples of serum from survivors, both English sole and chum salmon, about 15 days after they were exposed to the bacteria indicated that the fish did not have measurable antibody titers against either Vibrio spp. antigen. Antibody production in fish usually occurs at temperatures within the normal environmental range of the species (Fletcher, 1982), and the peak response occurs at the higher end of the range (Avtalion, 1969; Muroga and Egusa, 1969; Snieszko, 1970; Umminger, 1970; Avtalion et al., 1973; Corbel, 1975; Bell, 1977; Rijkers et al., 1980; Avtalion, 1981; Rijkers, 1982; Stolen et al., 1982; Wishkovsky and Avtalion, 1982). Above the normal temperature range, increasing temperature could cause thermal stress and decrease the immune response due to alterations in the physiology and biochemistry of the fish. Under such conditions non-lymphoid defenses may become more important (Snieszko, 1974; Fletcher, 1982; Rijkers, 1982).

If infected chum salmon and English sole produced antibody at high temperatures, levels would be low and most

likely would not be able to compensate for the increased reproductive rate of the bacteria. In addition, increasing water temperature could decrease the susceptibility of the bacteria to bacteriostatic or bactericidal factors that were produced by the fish. According to Hahnel and Gould (1982), the susceptibility of bacteria to some antibiotics decreased when the incubation temperature increased. From their observations, Horne et al. (1977) indicated that in an outbreak of acute vibriosis in young turbot (Scophthalmus maximus), antibiotic therapy was only marginally successful compared to temperature reduction.

Similar results were found by Groberg (1981), in the study of the effect of water temperature on the mortality rate of juvenile coho salmon infected with V. anguillarum. In his study, the mortality rates of fish increased from 4% at 6°C to 60% at 21°C and the mean time to death decreased from 23 days at 6°C to 2.7 days at 21°C. Using Aeromonas salmonicida Fryer and Pilcher (1974) also found that the mortality rates of coho salmon increased from 14% at 4°C to 100% at 21°C, while the mean time to death decreased from 18.4 days at 4°C to 2.9 days at 21°C. In juvenile chinook salmon, A. salmonicida killed 26% at 7°C and 98% at 22°C, and mean time to death were 12.2 days at 7°C, and 2.2 days at 22°C (Fryer et al., 1976).

During a 14 day experiment, results showed that size had a significant effect on the mortality rate of English

sole infected with both Vibrio species, with small fish having higher mortality rates than large fish. It is most likely that the difference in mortality rates between small and large sized English sole was due to differences in stress resistance and immune response mechanisms. Large English sole probably had a lower mortality rate because they were less susceptible to stress and had more mature immune system than did the small sized English sole. There is some evidence that small fish are more susceptible to stress than are larger fish of the same species. Braum (1978) found that young fish were very sensitive to abnormal conditions and mortality increased considerably if conditions were not optimum. Under laboratory conditions, Holeton (1979) found that large sized fish were more resistant than small sized fish to low dissolved oxygen. Reasons for this could include the lower weight-specific oxygen consumption (Holeton, 1979) and lower metabolic rate (Brett and Groves, 1979). Anderson and Conroy (1970), Horne et al. (1977), Egidius and Andersen (1977), and Horne et al. (1984) have made similar observations. Although they used different fish species in their studies, results showed that small fish were more susceptible to vibriosis than were large fish.

There is some evidence that in the first few months of life the immune system of fish is not fully developed (Ellis, 1977; Rijkers and van Muiswinkel, 1977; Manning

et al., 1982; Tatner and Manning, 1983). During this life stage effective production of antibody does not occur (van Loon et al., 1981), and the fish rely on macrophages as a first line of defense (Grace et al., 1977). Epidermis and its associated mucus are also important defense mechanisms (Fletcher and Grant, 1969; Di Conza, 1970; Bradshaw et al., 1971; Di Conza et al., 1971; Roberts, 1971; Harrel et al., 1976; Ingram, 1980; Uskova et al., 1980). According to Roberts et al. (1973), and Blackstock and Pickering (1982) the epidermis and its associated layers of mucus may undergo marked structural and functional changes based on environmental conditions and life cycle stage of the fish, particularly during the early life stages.

The majority of the experiements conducted in this study showed that the English sole is more resistant to both Vibrio spp. than is chum salmon. One possible explanation is that the bacteria were originally isolated from salmonids and may therefore be more specific to these hosts than to English sole. Another explanation could involve the host defense mechanisms. It is possible that the English sole has a stronger defense against infections by the two species of Vibrio.

Other workers have observed host specific differences in susceptibility to V. anguillarum. Hastein and Smith (1977) isolated two strains of V. anguillarum from fish in Norwegian waters. Vibrio anguillarum group I was arabinose

positive and more likely to cause vibriosis in farmed fish while group II was arabinose negative and most often caused disease in wild fish. For example, they observed a mass mortality due to vibriosis in saithe (Pollachius virens) that were gathered around salmonid holding pens while the captive salmonids remained uninfected. Further studies were done by Egidius and Andersen (1978) who tested the pathogenicity of the two groups of V. anguillarum that were isolated by Hastein and Smith (1977). The results indicated that under laboratory conditions, strains isolated from salmonids were pathogenic to salmonids but only slightly to saithe. Strains isolated from saithe were pathogenic to saithe but non-pathogenic to salmonids. Strout et al. (1978) also compared the pathogenicity of V. anguillarum isolated from cultured and feral fishes to salmonids under laboratory conditions. They found that most isolates from farmed fishes were pathogenic to salmonids and that most isolated from feral fishes (mostly winter flounder, Pseudopleuronectes americanus) were non- or less pathogenic to salmonids.

Hacking and Budd (1971), Kawano et al. (1983), and Inamura et al. (1984) also showed that there is a possibility of host specificity to V. anguillarum. Inamura et al. (1984) suggested that this host specificity was probably due to different capability of the strains to produce toxins. In an experiment they tested six different

strains of V. anguillarum. The results showed that under the same culture conditions each strain produced different amount of exotoxins (protease, hemolysin and hemaglutinin) and in an intraperitoneal injection experiment they found that most of the strains caused different mortality rates in fish.

Host specificity has also been found for other species of bacterial fish pathogens. De Figuieredo and Plumb (1977) compared the pathogenicity of nine Aeromonas hydrophila strains isolated from diseased fish, diseased Macrobrachium sp. and from pond water. The results showed that two of the isolates of A. hydrophila from Macrobrachium sp. failed to kill channel catfish (Ictalurus punctatus) fingerlings at injection levels of 1.0×10^7 cells. Based on LD_{50}, bacteria that were isolated from water were less virulent than those from diseased fish even if they were isolated from the same pond and had similar biochemical reactions.

In addition to differences in the capability of bacteria to invade fish, differences in fish susceptibility also plays an important role in determining host specificity to V. anguillarum. According to Snieszko (1958) and Ellis (1982) the difference in susceptibility of fish to certain diseases is caused by natural resistance that can be differentiated from the resistance between host species (interspecific) and the resistance within host species

(intraspecific). All these differences are due to genetic variations within or between species (Wolf, 1953; Gjedrem and Aulstad, 1974; Hines et al., 1974; Robohm and Sparrow, 1981; Refstie, 1982). The genetic factors involved in diseases and abnormalities are generally complex and difficult to detect, but the evidence for their existence is undeniable (Gordon, 1953).

In this study, experimentally induced infections using water-borne and intraperitoneal exposures were conducted to determine if external or internal resistance factors are more important when English sole and chum salmon are exposed to V. anguillarum. In a 14 day experiment the results showed that for both fish species both the LD_{50} and the mean time to death of fish exposed by the water-borne route were significantly higher than the same values for fish exposed by intraperitoneal injection. The difference may be explained by the fact that both external and internal defense mechanisms are involved in protecting fish exposed to bacteria in the water while only internal defense mechanisms (cellular and humoral) are available to protect fish injected with bacteria intraperitoneally.

The ratio between the LD_{50} of water-borne exposure and that of intraperitoneal injection between the two fish species indicated that the internal protection of English sole was stronger than that of chum salmon. However, it was not possible to transfer this protection by the

intraperitoneal injection of English sole serum into chum salmon. In a passive protection experiment, the mortality rate of chum salmon that were injected with unimmunized English sole serum was not statistically different from chum salmon that were injected with unimmunized chum salmon serum when these two groups were challenged with V. anguillarum by water-borne exposure four days after injection. Increasing the doses of the sera and shortening the period between serum injection and challenge did not result in a significant difference in the mortality rate or mean time to death of either group. This indicated that humoral defense mechanisms in serum may not be the primary internal protection factor to vibriosis in non-immune English sole. Rather the internal cellular defense mechanism is probably the most important defense system that results in the English sole being more resistant than chum salmon to both bacterial species.

Harrell et al. (1975) injected trout anti-V. anguillarum serum into juvenile rainbow trout and found that the anti-serum could be detected within 10 minutes, but that titers in recipient fish were drastically reduced after 96 hours. In other passive immunization experiments, Harrell et al. (1975), and Viele et al. (1980) found that the mortality rates of fish that were injected with anti-V. anguillarum serum was significantly lower than that of control fish. In addition, Viele et al. (1980) found that plasma

was the most effective in transferring vibriosis protection when compared to pronephros cells, splenocytes and thymus cells. Passive immunization against A. salmonicida has been studied by Spence et al. (1965) who found that protection against furunculosis was produced in coho salmon by administration of serum from rainbow trout containing antibody produced by intraperitoneal vaccination. In contrast, Aoki et al. (1984) found no difference in the protection against vibriosis between ayu that received serum from immersion vaccinated fish and those which received serum from unvaccinated fish.

Different fish species may develop different levels of protection even though they were immunized with the same bacterin and have the same optimum temperature ranges. In oral immunization studies using four species of marine tropical fish, Prescott (1977) found that the protection against V. anguillarum for each of the four species tested varied to a considerable degree. Other investigators have done similar experiments and found the results to vary from one experiment to another. The results were affected by environmental conditions especially water temperature, fish species, size of fish, type of V. anguillarum bacterins and immunization methods (Harrell et al., 1975; Rohovec, 1975; Antipa, 1976; Antipa and Amend, 1977; Fryer et al., 1978; Evelyn and Ketcheson, 1980; Groberg, 1981; Johnson et al.,

1982a; Agius et al., 1983; Harrell et al., 1983; Tatner and Horne, 1983).

In the experiment conducted to determine the duration of antibody titers and the immune response of English sole and chum salmon after immunization by intraperitoneal injection, differences in protection were observed. In general, immunization gave protection to both English sole and chum salmon and mortality rates of immunized fish were significantly lower than controls. However, when a comparison was made between the two fish species, English sole had a lower relative percent survival (RPS) than chum salmon. This indicates that English sole had a lower level of protective immunity than chum salmon even though they had similar antibody titers. This could also be interpreted to indicate that the antibody titers had a different meaning in different fish species, and do not necessarily serve as a direct indication of protection level.

Similar results were found by Spence et al. (1965) in studies of rainbow trout immunized against Aeromonas salmonicida. They suggested that although agglutinins may be present in sera at high levels, this does not necessarily indicate the existence of protecting antibodies. In contrast, Nelson (1972), Sawyer (1978), Laurencin and Tangtrongpiros (1980), Aoki et al. (1984), Kawano et al. (1984), and Sakai et al. (1984) could not detect agglutinating antibodies in fish serum which were immunized by oral or

direct immersion with V. anguillarum bacterins. However, they did find significant differences in mortality rates between immunized fish and control after being challenged by water-borne exposure or intraperitoneal injection with V. anguillarum with mortality rates of immunized fish generally lower than controls.

Although the immunized English sole and chum salmon had significantly lower mortality rates than did the controls, they did not have a significantly longer mean time to death. It is possible that in this case the immune response only helps to prevent infection and if the fish become infected, the development of the disease is the same in both immunized and control fish. In a similar study with A. salmonicida, Spence et al. (1965) found that immunization not only decreased mortality rates, but also delayed the mean time to death as compared to controls.

The peaks of antibody titers observed in this experiment were substantially lower than the peaks of antibody titers found by most other investigators. One of the reasons may be the size of fish used in the experiments which was much smaller than the average sizes of fish used by other investigators and could influence the immune response (Johnson et al., 1982a; Tatner and Horne, 1983; Tatner and Horne, 1984). When the experiment was terminated 19 weeks after immunization, both English sole and chum salmon still had positive antibody titers (about 1:2 to 1:4). However,

the fish were not challenged and the level of protection against vibriosis is not known.

Changes in the virulence of V. ordalii or V. anguillarum due to selection after three passages through either English sole or chum salmon were not observed and there was no significant difference in mean time to death of English sole or chum salmon exposed to these bacterial isolates. However, in general, the LD_{50} of V. ordalii or V. anguillarum after passage was lower for the homologous species of fish. This suggests that the virulence of the bacteria was modified slightly after passage through fish, but the change was small. It is possible that further passages would have increased the difference.

Another observation was that the ratio of LD_{50} values between heterologous and homologous fish species was always larger than one (Table 15). For example, 4.4×10^7 cells/ml of V. ordalii (E) were required to kill 50% of the English sole, and 1.2 times that amount of V. ordalii (C) were required to reach an LD_{50} in sole. Similarly the LD_{50} of V. ordalii (C) for chum salmon was 8.0×10^5 cells/ml and was 2.1 times that with V. ordalii (E). Similar results were observed with V. anguillarum providing an indication that the bacteria lost virulence for the original host after passage through a different fish species. Virulence to heterologous fish species appeared to decrease at a higher rate if the bacteria were passed through English sole

than through chum salmon. This may be related to the fact that both Vibrio species were originally isolated from salmonid fishes, and may have been better adapted to chum salmon than to English sole.

It was also observed that V. anguillarum changed virulence to a greater extent than did V. ordalii. An explanation for this could be related to reproductive rates. Vibrio anguillarum has a shorter doubling time than V. ordalii (Gould, 1977; Gould et al., 1979; Schiewe, 1983), so in the same period of time V. anguillarum would have more generations than V. ordalii. If the virulence changed at the same rate per generation, V. anguillarum would change more than V. ordalii during the same time period.

Homologous or heterologous passage of V. anguillarum does not always change the virulence to the hosts. Kawano et al. (1983) compared the virulence of V. anguillarum strain SG 7754 after five passages through ayu (Plecoglossus altivelis) with the original freshly isolated from naturally infected ayu and found that the LD_{50} for homologous fish species did not change. Contrasting results were found by Aoki et al. (1984) who compared the LD_{50} of an original isolate of V. anguillarum strain AG 7011 from diseased ayu with an isolate after the third passage in ayu and with an isolate after thirteen passages in a heart infusion broth and found that pathogenicity increased after passage through the host fish.

In this study, variation in pathology, onset of mortality, mortality rates and mean time to death between replicates within an experiment and between experiments were found. Variations were usually greater in English sole than in chum salmon. Part of the reason could be genetic heterogeneity in the English sole since they were obtained from a wild population. Further, the stress of capture and laboratory rearing conditions may depress the defense mechanisms of English sole. According to Miller and Tripp (1982), newly collected fish had a stronger immune response than did fish that had been in the laboratory for an extended period. A partial explanation of this result was that these fish were stressed by environmental changes.

In general, the results of this study indicate that cross infection of V. ordalii and V. anguillarum between English sole and chum salmon is possible, at least under laboratory conditions. However, it is unlikely that cross infections occur very often under natural conditions, unless high water temperatures and crowded conditions result in massive epizootics. The number of bacteria that occur in natural waters is usually low (Rohovec, 1975; Ransom, 1978) so epizootics are more often the result of stress on potential hosts than of high bacteria levels.

The kinds of factors that could influence the greater resistance of English sole to both Vibrio spp. other than host specificity of the bacteria may include: non-specific

defense (external and internal), previous exposure to naturally occurring antigens, behavioral patterns and physiological state of the fish. External and internal humoral and cellular non-specific defenses could involve phagocytic cells, lysozyme, enzymes and other substances that lyse the bacteria, natural agglutinins and natural immunoglobulins, and unfavorable pH. In addition, blood may also contain C-reactive protein and complement that are involved in lysis of bacteria, and transferrin and lactoferrin that bind iron and make it unavailable for bacterial growth (Wolf, 1953; Anderson, 1974; Corbel, 1975; Bell, 1977; Fletcher, 1978; Ingram, 1980; Ellis, 1981; Fletcher, 1982). Previous exposure to a broad spectrum of naturally occurring antigens may cause fish to produce antibodies that cross react with a particular bacterial pathogen (Ingram, 1980). Although the production of natural agglutinating antibody in English sole is low and could not be detected by microtiter technique, it could be a factor that increases the resistance of the fish to both bacterial species.

Behavioral patterns and the physiological state of fish also result in differences in susceptibility to certain diseases (Corbel, 1975; Ellis, 1981). Chum salmon is a more active fish than is the English sole. According to Bone and Marshal (1982), active fish have a higher weight-specific oxygen consumption than do non-active fish. To

fulfill this high oxygen consumption the fish has to pass a greater volume of water through the gills and increase area of the gills. In seawater, a larger gill area may result in greater losses of water than would a smaller gill area. To compensate for the water that is lost through the gills, the fish have to drink seawater. Since active fish lose more water than do non-active fish, the active fish has to drink more seawater to maintain osmotic balance. If gills (Tatner and Horne, 1983) and gastrointestinal tract (Ransom, 1978, Tatner and Horne, 1983) are a main entry site for both V. ordalii and V. anguillarum, it is likely that the bacteria have greater access to chum salmon than to English sole in water-borne exposures, because of the greater volume of water that passes through the gills and that enters the gastrointestinal tract. In intraperitoneal injection, bacteria avoided external defense systems and were delivered directly into the body. As was discussed earlier, the English sole has a stronger cellular internal defense mechanism than does chum salmon. This defense mechanism may be the factor that allowed English sole to be more resistant to both Vibrio species than chum salmon when infected by intraperitoneal injection.

Differences in susceptibility of chum salmon and English sole to both Vibrio spp. may also be caused by differences in stress resistance of both fish species. Stress can reduce the realized capacity of fish (Schreck, 1982),

and in relation to defense mechanisms, stress results in many changes in the physiological activities of fish and decreases the immune response (Wedemeyer, 1970; Ellis, 1981). Refstie (1981) reported a correlation between stress resistance and the susceptibility of fish to vibriosis.

However, the interrelationship between host, pathogen and environment is complex, and further study is needed to determine which of these possibilities are operating to make English sole the more resistant species. Future studies probably should emphasize cellular non-specific defense mechanisms, especially those that are generated by the spleen (splenocytes), kidney (pronephros cells), and thymus (thymus cells). Stress resistance should also be studied to determine if this factor is important in allowing English sole to become more resistant to both Vibrio species than are chum salmon. Studies on host specificity and mechanisms of pathogenicity of both Vibrio species are also necessary to determine how the bacteria infect the fish, route of entry, primary site of infection and the mechanism by which bacteria cause disease in susceptible hosts.

LITERATURE CITED

Agius, C.; M. T. Horne, and P. D. Ward, 1983. Immunization of rainbow trout, Salmo gairdneri Richardson, against vibrosis: Comparison of an extract antigen with whole cell bacterins by oral and intraperitoneal routes. J. Fish Dis., 6 : 129-134.

Alderdice, D. F., and C. R. Forrester, 1968. Some effects of salinity and temperature on early development and survival of the English sole (Parophrys vetulus). J. Fish. Res. Bd. Can., 25 : 494-521.

Amend, D. F., and K. A. Johnson, 1984. Evidence for lack antigenic competition among various combinations of Vibrio anguillarum, Yersinia ruckeri, Aeromonas salmonicida and Renibacterium salmoninarum bacterins when administered to salmonid fishes. J. Fish. Dis., 7 : 293-299.

Anderson, D. P., 1974. Disease of fish. Book 4 : Fish immunology, ed. by S. F. Snieszko, and H. R. Axelrod, TFH Pub., Neptune City, 108 p.

Anderson, J. I. W., and D. A. Conroy, 1970. Vibrio disease in marine fishes. 266-272, in : A symposium on diseases of fishes and shellfishes, S. F. Snieszko (ed)., Am. Fish. Soc. Spec. Pub. no. 5., Washington, D. C.

Antipa, R., 1976. Field testing of injected Vibrio anguillarum bacterins in pen-reared Pacific salmon. J. Fish. Res. Bd. Can., 33 : 1291-1296.

Antipa, R., and D. F. Amend, 1977. Immunization of Pacific salmon: Comparison of intraperitoneal injection and hyperosmotic infiltration of Vibrio anguillarum and Aeromonas salmonicida bacterins. J. Fish. Res. Bd. Can., 34 : 203-208.

Antipa, R.; R. Gould, and D. F. Amend, 1980. Vibrio anguillarum vaccination of sockeye salmon Oncorhynchus nerka (Walbaum) by direct and hyperosmotic immersion. J. Fish Dis., 3 : 161-165.

Aoki, T., and T. Kitao, 1978. Vibriosis in ayu. Fish Pathol., 13 : 19-24.

Aoki, T.; T. Kitao, M. Fukuda, S. Takahashi, and S. Egusa, 1984. Modification of the hyperosmotic infiltration method of vaccination against vibriosis in cultured ayu, Plecoglossus altivelis Temmic and Schlegel. J. Fish Dis., 7 : 149-156.

Aoki, T.; T. Kitao, T. Habashi, Y. Wada, and M. Sakai, 1981. Proteins and lipopolysaccharides in the membrane of Vibrio anguillarum. Dev. Biol. Standard. 49. Serodiagnostics and vaccines, 225-232.

Aoki, T.; M. Sakai, and S. Takahashi, 1984. Protective immunity in ayu, Plecoglossus altivelis, vaccinated by immersion with Vibrio anguillarum. Fish Pathol., 19 (3) : 181-185.

Avtalion, R. R., 1969. Temperature effect on antibody production and immunological memory, in carp (Cyprinus carpio) immunized against bovine serum albumin (BSA). Immunol., 17 : 927-931.

_____, 1973. Influence of environmental temperature on the immune response in fish. Cur. Top. Micr. and Immunol., 61 : 1-35.

_____, 1981. Environmental control of the immune response in fish. Crit. Rev. Env. Cont., 11 : 163-188.

Baker, R. J.; M. D. Knittel, and J. L. Fryer, 1983. Susceptibility of chinook salmon, Oncorhynchus tshawytscha, and rainbow trout, Salmo gairdneri, to infection with Vibrio anguillarum following sublethal copper exposure. J. Fish Dis., 6 : 267-275.

Bell, G. R., 1977. Aspects of defense mechanisms in salmonids. Proc. Int. Symp. Dieseases of cultured salmonids, Seattle, 56-70.

Blackstock, N., and A. D. Pickering, 1982. Changes in the concentration and histochemistry of epidermal mucous cells during the elevin and fry stages of the brown trout Salmo trutta. J. Zool. Soc. London., 197 : 463-471.

Bone, Q., and N. B. Marshall, 1982. Biology of Fishes. Blackie, London, 253 p.

Braatein, B. A., and H. O. Hodgins, 1976. Protection of steelhead trout Salmo gairdneri against vibriosis with a living low-virulence strain of Vibrio anguillarum. J. Fish. Res. Bd. Can., 33 : 845-847.

Bradshaw, C. M.; A. L. Richard, and M. M. Sigel, 1971. IgM antibodies in fish mucus. Proc. Soc. Exp. Biol. and Med., 136 : 1122-1124.

Braum, E., 1978. Ecological aspects of the survival of fish eggs, embryos and larvae. 102-131, in : Ecology of freshwater fish production, S. D. Gerking (ed.), John Wiley and Sons, New York.

Brett, J. R., and T. D. D. Groves, 1979. Physiological energetics. 279-352, in : Fish physiology, vol. VIII, W. S. Hoar, D. J. Randall, and J. R. Brett (eds.), Academic Press, New York.

Bullen, J. J.; H. J. Rogers, and E. Griffiths, 1978. Role of iron in bacterial infection. Cur. Top. Mic. and Immunol., 80 : 1-35.

Bullock, G. L., 1977. Vibriosis in fish. Fish Disease Leaflet 50, U. S. Dept. Inter., Fish and Wildlife Service, Washington, D. C., 11 p.

Chart, H., and C. B. Munn, 1980. Experimental vibriosis in the eel (Anguilla anguilla). in : Fish diseases, W. Ahne (ed.), third COPRAQ-Session, Springer-Verlag, Berlin, 39-43.

Cisar, J. O., and J. L. Fryer, 1969. An epizootic of vibriosis in chinook salmon. Bull. Wildl. Dis. Assoc., 5 : 73-76.

Cochran, W. G., and G. M. Cox, 1957. Experimental designs. Second ed., John Wiley and Sons, Inc., New York, 611 p.

Corbel, M. J., 1975. The immune response in fish : Review. J. Fish. Biol., 7 : 539-563.

Cowan, S. T.; J. G. Holt, J. Liston, R. G. E. Murray, C. F. Niven, A. W. Ravin, and R. W Stanier, 1975. in : Buchanen, R. E., and N. E. Gibbons (eds.), Bergey's manual of determinative bacteriology, 8th ed., Williams and Wilkins Co., Baltimore, 1,268 p.

Crosa, J. H., 1979. Vibrio anguillarum : An iron uptake mechanism as a factor of virulence. Fish Health News, 8 (4) : vii-viii.

_____ , 1980. A plasmid associated with virulence in the marine fish pathogen Vibrio anguillarum specifies an iron-sequestering system. Nature, 284 : 566-568.

_____, 1981. Cell envelop proteins induced under conditions of iron limitation in different serotypes of Vibrio anguillarum. Dev. Biol. Standard. Serodiagnostics and Vaccines., 49 : 181-188.

Crosa, J. H., and L. L. Hodges, 1981. Outer membrane proteins induced under conditions of iron limitation in the marine fish pathogen Vibrio anguillarum. Infect. Immunol., 31 (1) : 223-227.

Crosa, J. H.; L. L. Hodges, and M. H. Schiewe, 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen Vibrio anguillarum. Infect. Immunol., 27 (3) : 897-902.

Crosa, J. H.; M. H. Schiewe, and S. Falkow, 1977. Evidence for plasmid contribution to the virulence of the fish pathogen Vibrio anguillarum. Infect. Immunol., 18 (2) : 509-513.

Crosa, J. H.; M. A. Walter, and S. A. Potter, 1983. The genetics of plasmid-mediated virulence in the marine fish pathogen Vibrio anguillarum. 21-30, in : Bacterial and viral diseases of fish, J. H. Crosa (ed.), Univ. of Washington.

Croy, T. R., and D. F. Amend, 1977. Immunization of sockeye salmon (Oncorhynchus nerka) against vibriosis using the hyperosmotic infiltration technique. Aquaculture, 12 : 317-325.

Davina, J. H. M.; H. K. Parmentier, and L. P. M. Timmermans, 1982. Effect of oral administration of Vibrio bacterin on the intestine of cyprinid fish. Dev. Comp. Immunol., Sup. 2 : 157-166.

De Figueiredo, J., and J. A. Plumb, 1977. Virulence of different isolates of Aeromonas hydrophila in channel catfish. Aquaculture, 11 : 349-354.

Di Consa, J. J., 1970. Some characteristics of natural haemagglutinins found in serum and mucus of the catfish, Tachysurus australis. Aust. J. Exp. Biol. Med. Sci., 48 : 515-523.

Di Consa, J. J., and W. J. Halliday, 1971. Relationship of catfish serum antibodies to immunoglobulin in mucus secretions. Aust. J. Exp. Biol. Med. Sci., 49 : 517-519.

Egidius, E. C., and K. Andersen, 1978. Host specific pathogenicity of strains of Vibrio anguillarum isolated from rainbow trout Salmo gairdneri and saithe Pollachius virens. J. Fish Dis., 1 : 45-50.

Egidius, E. C., and K. Andersen, 1979. Bath-immunization a practical and non stressing method of vaccinating sea farmed rainbow trout Salmo gairdneri against vibriosis. J. Fish Dis., 2 : 405-410.

Egidius, E., and K. Andersen, 1977. Norwegian reference strains of Vibrio anguillarum. Aquaculture, 10 : 215-219.

Egidius, E., and K. Andersen, 1982. Bath-vaccination against vibriosis. Dev. Comp. Immunol. Sup. 2, 193-196.

Egidius, E., and K. Andersen, 1984. Disease problem in cod rearing. Inst. Mar. Res., ed. by E. Dahl, D. S. Danielssen, E. Moksness, and P. Solemdal, Flodevigen Biol. Sta., Arendal, 761-769.

Ellis, A. E., 1977. Ontogeny of the immune response in Salmo salar. 225-231, in : Dev. Immunobiol., J. B. Solomon, and J. D. Horton (eds.).

_____ , 1981. Stress and the modulation of defence mechanisms in fish. 147-183, in : Stress and fish, A. D. Pickering (ed.), Academic Press.

_____ , 1982. Non-specific defense mechanisms in fish and their role in disease processes. Dev. Biol. Standard., 49., Serodiagnostics and vaccines, 337-352.

Evelyn, T. P. T., 1971. First records of vibriosis in Pacific salmon cultured in Canada, and taxonomic status of the responsible bacterium, Vibrio anguillarum. J. Fish. Res. Bd. Can., 28 : 517-525.

Evelyn, T. P. T., and J. E. Ketcheson, 1980. Laboratory and field observations on antivibriosis vaccines. 45-52, in : Fish diseases, W. Ahne (ed.), third COPRAQ-Session, Springer-Verlag, Berlin.

Ezura, Y.; K. Tajima, M. Yoshimizu, and T. Kimura, 1980. Studies on the taxonomy and serology of causative organisms of fish vibriosis. Fish Pathol., 14 (4) : 167-179.

Fletcher, T. C., 1978. Defence mechanisms in fish. 189-222, in : Biochemical and biophysical perspectives in marine biology, D. C. Mallins, and J. R. Sargent (eds.), Academic Press, London, Vol. 4.

_____ , 1982. Non-specific defence mechanisms of fish. Dev. Comp. Immunol., Sup. 2, 123-132.

Fletcher, T. C., and P. T. Grant, 1969. Immunoglobulins in the serum and mucus of the plaice (Pleuronectes platessa). Biochem. J., 115 : 65.

Fletcher, T. C., and A. White, 1973. Antibody production in the plaice (Pleuronectes platessa) after oral and parenteral immunization with Vibrio anguillarum antigens. Aquaculture, 1 : 417-428.

Frolander, H. F.; C. B. Miller, M. J. Flinn, S. C. Myers, and S. T. Zimmerman, 1973. Seasonal cycles of abundance in zooplankton populations of Yaquina Bay, Oregon. Mar. Biol., 21 : 277-288.

Fryer, J. L.; J. S. Nelson, and R. L. Garrison, Vibriosis in fish. 129-133, in : Progress in Fishery and Food Science, Univ. Washington, Pub., Fish New Series, 5.

Fryer, J. L.; J. S. Rohovec, and R. L. Garrison, 1978. Immunization of salmonids for control of vibriosis. Mar. Fish Rev., 1290 : 20-23.

Fryer, J. L.; J. S. Rohovec, G. L. Tebbit, J. S. McMichael, and K. S. Pilcher, 1976. Vaccination for control of infectious diseases in Pacific salmon. Fish Pathol., 10 : 155-164.

Fryer, J. L.; K. S. Pilcher, J. E. Sander, J. S. Rohovec, J. L. Zinn, W. J. Groberg, and R. H. McCoy, 1976. Temperature, infectious diseases, and the immune response in salmonid fish. U.S. EPA, Environmental Research Laboratory, EPA-600/3-76-021, Duluth, 48 p.

Gordon, M., 1953. The genetics of fish diseases. Am. Fish. Soc., 83 : 229-237.

Gould, R. W., 1977. Development of a new vaccine delivery system for immunizing fish and investigation of the protective antigens in Vibrio anguillarum. Ph.D. thesis, Oregon State University, Corvallis, 145 p.

Gould, R. W.; R. Antipa, and D. F. Amend, 1979. Immersion vaccination of sockeye salmon (Oncorhynchus nerka) with two pathogenic strains of Vibrio anguillarum. J. Fish. Res. Bd. Can., 36 : 222-225.

Gould, R. W.; P. J. O'Leary, R. L. Garrison, J. S. Rohovec, and J. L. Fryer, 1978. Spray vaccination: A method for the immunization of fish. Fish Pathol., 13 (1) : 63-68.

Grace, M. F., J. W. Botham, and M. J. Manning, 1981. Ontogeny of lymphoid organ function in fish. 467-468, in : Aspect of Dev. Comp. Immunol., J. B. Solomon (ed.), Pergamon Press, Oxford : Vol. I.

Groberg, W. J., Jr., 1981. Infection and the immune response induced by Vibrio anguillarum in juvenile coho salmon (Oncorhynchus kisutch). Ph D. thesis, Oregon State University, Corvallis, 107 p.

Grondel, J. L., and H. J. A. M. Boesten, 1982. The influence of antibiotics on the immune system, I : Inhibition of the mitogenic leukocyte response in vitro by oxytetracycline. Dev. Comp. Immunol., Sup. 2., 211-216.

Gunnels, R. D.; H. O. Hodgins, M. H. Schiewe, 1976. Failure of vaccines to protect salmon from vibriosis enzootic in Puget Sound, Washington. Am. J. Vet. Res., 37 (6) : 737-740.

Haastein, T., and G. Holt, 1972. The occurrence of Vibrio disease in wild Norwegian fish. J. Fish. Biol., 4 : 33-37.

Hacking, M. A., and J. Budd, 1977. Vibrio infection in tropical fish in a freshwater aquarium. J. Wildl. Dis., 7 : 273-280.

Hahnel, G. B., and R. W. Gould, 1982. Effects of temperature on biochemical reactions and drug resistance of virulent and avirulent Aeromonas salmonicida. J. Fish Dis., 5 : 329-337.

Harbel, S. C.; H. O. Hodgins, and M. H. Schiewe, 1979. Studies on the pathogenesis of vibriosis in coho salmon Oncorhynchus kisutch. J. Fish Dis., 2 : 391-404.

Harrell, L. W., 1978. Vibriosis and current salmon vaccination procedures in Puget Sound, Washington. Mar. Fish. Rev., 1291 : 24-25.

Harrell, L. W.; A. J. Novotny, M. H. Schiewe, and H. O. Hodgins, 1976. Isolation and description of two Vibrios pathogenic to Pacific salmon in Puget Sound, Washington. U. S. Fish and Wildlife Service, Fish Bull., 74 : 447-449.

Harrell, L. W., H. M. Etlinger, and H. O. Hodgins, 1975. Humoral factors important in resistance of salmonid fish to bacterial disease. I : Serum antibody protection of rainbow trout (Salmo gairdneri) against vibriosis. Aquaculture, 6 : 211-219.

Harrell, L. W.; H. M. Etlinger, and H. O. Hodgins, 1976. Humoral factors important in resistance of salmonid fish to bacterial disease. II : Anti-Vibrio anguillarum activity in mucus and observations on complement. Aquaculture, 7 : 363-370.

Hart, J. L., 1973. Pacific fishes of Canada. Bull. 180, Fish. Res. Bd. Can., 740 p.

Hastein, T., and J. E. Smith, 1977. A study of Vibrio anguillarum from farmed and wild fish using principal components analysis. J. Fish Biol., 11 : 69-75.

Hastein, T.; F. Hallingstad, T. Refstie, and S. O. Roald, 1980. Recent experience of field vaccination trials against vibriosis in rainbow trout (Salmo gairdneri). 53-59, in : Fish diseases, W. Ahne (ed.), third COPRAQ-session, Springer-Verlag, Berlin.

Hendrie, M. S.; W. Hodgkiss, and J. M. Shewan, 1971. Proposal that the species Vibrio anguillarum Bergman 1909, Vibrio piscium David 1927, and Vibrio ichthyodermis (Wells and Zo Bell) Shewan, Hobbs, and Hodgkiss 1960 be combined as a single species Vibrio anguillarum. Int. J. Syst. Bact., 21 (1) : 64-68.

Herman. R. L., 1970. Chemotherapy of fish diseases : A review. J. Wild. Dis., 6 : 31-34.

Hines, R. S.; G. W. Wohlfarth, R. Moav, and G. Hulata, 1974. Genetic differences in susceptibility to two diseases among strains of common carp. Aquaculture, 3 : 187-197.

Holeton, G. F., 1979. Oxygen as an environmental factor of fishes. 7-32, in : Environmental physiology of fishes, M. A. Ali (ed.), Plenum Press, New York.

Horne, M. T., 1982. The pathogenicity of Vibrio anguillarum. 171-182, in : Microbial diseases of fish, R. J. Roberts (ed.), Academic Press, London.

Horne, M. T., and A. Baxendale, 1983. The adhesion of Vibrio anguillarum to host tissues and its role in pathogenesis. J. Fish Dis., 6 : 461-471.

Horne, M. T.; R. J. Roberts, and M. Tatner, 1984. The effects of the use of potassium alum adjuvant in vaccines against vibriosis in rainbow trout, Salmo gairdneri. J. Fish Dis., 7 : 91-99.

Horne, M. T.; R. H. Richards, R. J. Roberts, and P. C. Smith, 1977. Peracute vibriosis in juvenile turbot Scophthalmus maximus. J. Fish. Biol., 11 : 355-361.

Inamura, H.; K. Muroga, and T. Nakai, 1984. Toxicity of extracellular products of Vibrio anguillarum. Fish Pathol., 19 (2) : 89-96.

Inebi, W., and M. T. Horne, 1979. A complement-fixation method for the detection of Vibrio - specific antibodies in rainbow trout, Salmo gairdneri. J. Fish Dis., 2 : 159-160.

Ingram, G. A., 1980. Substances involved in the natural resistance of fish to infection. A review, J. Fish. Biol., 16 : 23-60.

Itami, T., and R. Kusuda, 1980. Studies on spray vaccination against vibriosis in cultured ayu. II : Duration of vaccination efficacy and effect of different vaccine preparations. Bull. Jap. Soc. Sci. Fish., 46 (6) : 699-703.

Johnsen, G. S., 1977. Immunological studies on Vibrio anguillarum. Aquaculture, 10 : 221-230.

Johnson, K. A., and D. F. Amend, 1983. Efficacy of Vibrio anguillarum and Yersinia ruckeri bacterins applied by oral and anal intubation of salmonids. J. Fish Dis., 6 : 473-476.

Johnson, K. A.; J. K. Flynn, and D. F. Amend, 1982a. Onset of immunity in salmonid fry vaccinated by direct immersion in Vibrio anguillarum and Yersinia ruckeri bacterins. J. Fish Dis., 5 : 197-205.

Johnson, K. A.; J. K. Flynn, and D. F. Amend, 1982b. Duration of immunity in salmonids vaccinated by direct immersion with Yersinia ruckeri and Vibrio anguillarum bacterins. J. Fish Dis., 5 : 207-213.

Kawano, K.; T. Aoki, and T. Kitao, 1983. Immersion vaccination and water-borne challenge of ayu Plecoglossus altivelis against vibriosis. Fish Pathol., 18 (3) : 143-149.

Kawano, K.; T. Aoki, and T. Kitao, 1984. Duration of protection against vibriosis in ayu Plecoglossus altivelis vaccinated by immersion and oral administration with Vibrio anguillarum. Bull. Jap. Soc. Sci. Fish., 50 (5) : 771-774.

Kitao, T.; T. Aoki, M. Fukudome, K. Kawano, Y. Wada, and Y. Mizuno, 1983. Serotyping of Vibrio anguillarum isolated from diseased freshwater fish in Japan. J. Fish Dis., 6 : 175-181.

Kusuda, R.; H. Sako, and K. Kawai, 1979. Classification of vibriosis isolated from diseased fish. I : On the morphological, biological and biochemical properties. J. Fish Pathol., 13 (3) : 123-137.

Kusuda, R.; K. Kawai, and T. Itami, 1980. Efficacy of bath-immunization against vibriosis in cultured ayu. Bull. Jap. Soc. Sci. Fish., 46 (8) : 1053.

Lannan, J. E., 1978. Vibriosis vaccination of chum salmon by hyperosmotic infiltration. Prog. Fish Cult., 40 (2) : 43-45.

Laurencin, F. B., and J. Tangtrongpiros, 1980. Some results of vaccination against vibriosis in Brittany. 60-68, in : Fish diseases, W. Ahne (ed.), third COPRAQ-session, Springer-Verlag, Berlin.

Levin, M. A.; R. E. Wolke, and V. G. Cabelli, 1972. Vibrio anguillarum as a cause of disease in winter flounder (Pseudopleuronectes americanus). Can. J. Microbiol., 18 : 1585-1592.

Lewis, E. H.; R. J. Tarpley, J. T. Marks, and R. F. Sis, 1985. Drug induced structural changes in olfactory organs of channel catfish Ictalurus punctatus. J. Fish. Biol., 26 : 355-358.

Manning, M. J.; M. F. Grace, and C. J. Secombes, 1982. Ontogenetic aspects of tolerance and immunity in carp and rainbow trout : Studies on the role of the thymus. In : Dev. Comp. Immunol., Sup. 2., 75-82.

McCarthy, D. H., 1976. Vibrio disease in eels. J. Fish. Biol., 8 : 317-320.

McNeil, W. J., and J. E. Bailey, 1975. Salmon rancher's manual. U.S. Nat. Mar. Fish. Service, Seattle, Wa., 95 p.

Miller, N. W., and M. R. Tripp, 1982. The effect of captivity on the immune response of the kilifish, Fundulus heteroclitus. J. Fish. Biol., 20 : 301-308.

Miyasaki, T., and S. S. Kubota, 1977. Histopathological study on vibriosis of the salmonids. Fish Pathol., 12 (2) : 93-98.

Munn, C. B., 1980. Production and properties of a haemolytic toxin by Vibrio anguillarum. In : Fish diseases, ed. by W. Ahne, third COPRAQ-session, Springer-Verlag, Berlin, 69-74.

Muroga, K.; H. Yamanoi, Y. Hironaka, S. Yamamoto, M. Tatani, Y. Jo, S. Takahashi, and H. Hanada, 1984. Detection of Vibrio anguillarum from wild fingerlings of ayu Plecoglossus altivelis. Bull. Jap. Soc. Sci. Fish., 50 (4) : 591-596.

Muroga, K.; K. Satoh, and T. Wakai, 1984. Growth temperature of five bacterial pathogens for eel. Fish Pathol., 19 (3) : 193-196.

Muroga, K., and M. Tatani, 1981. Isolation of Vibrio anguillarum from juvenile red sea-bream (Pagrus major). Fish Pathol., 16 (4) : 211-214.

Muroga, K., and S. Egusa, 1969. Immune response of the Japanese ell to Vibrio anguillarum. I : Effect of temperature on agglutinating antibody production in starved eels. Bull. Jap. Soc. Sci. Fish., 35 (9) : 868-874.

Muroga, K.; Y. Jo, and M. Nishibuchi, 1976. Pathogenic Vibrio isolated from cultured eels. I : Characteristics and taxonomic status. Fish Pathol., 11 (3) : 141-143.

Nelson, J. S., 1972. Oral immunization for the control of vibriosis in Pacific salmon. MS thesis, Oregon State University, Corvallis, 48 p.

Nelson, J. S., J. S. Rohovec, and J. L. Fryer, 1985. Tissue location of Vibrio bacterin delivered by intraperitoneal injection, immersion and oral routes to Salmo gairdneri. Fish Pathol., 19 (4) : 263-269.

Nishibuchi, M., and K. Muroga, 1977. Pathogenic Vibrio isolated from cultured eels. III : NaCl tolerance and flagellation. Fish Pathol., 12 (2) : 87-92.

Novotny, A. J., 1978. Vibriosis and furunculosis in marine cultured salmon in Puget Sound, Washington. Mar. Fish. Rev., 1297 : 52-55.

Ohnishi, K., and K. Muroga, 1976. Vibrio sp. as a cause of of disease in rainbow trout cultured in Japan. I : Biochemical characteristics. Fish Pathol., 11 (3) : 159-164.

Ohnishi, K., and K. Muroga, 1977. Vibrio sp. as a cause of disease in rainbow trout cultured in Japan. II : Physiological characteristics and pathogenicity. Fish Pathol., 12 (1) : 51-55.

Olson, R. E., and I. Pratt, 1973. Parasites as indicators of English sole (Parophrys vetulus) nursery grounds. Trans. Am. Fish. Soc., 102 (2) : 405-411.

Pacha, R. E., and E. D. Kiehn, 1969. Characterization and relatedness of marine Vibrios pathogenic to fish : Physiology, serology and epidemiology. J. Bact., 100 (3) : 1242-1247.

Pearse, L.; R. S. V. Pullin, D. A. Conroy, and D. McGregor, 1974. Obervations on the use of furanace for the control of Vibrio disease in marine flatfish. Aquaculture, 3 : 295-302.

Piper, R. G.; I. B. McElwain, L. E. Orme, J. P. McCraren, L. G. Fowler, and J. R. Leonard, 1982. Fish hatchery management. U. S. Dept. Int., Fish and Wild. Service, Washington, D. C., 517 p.

Post, G., 1983. Text book of fish health. TFH Pub., Neptune City, 256 p.

Prescott, S. R., 1977. A technical report on oral immunization of marine tropical fish against Vibrio anguillarum. Aquaculture, 11 : 281-283.

Ransom, D. P., 1978. Bacteriologic, immunologic and pathologic studies of Vibrio spp. pathogenic to salmonids. Ph D. thesis, Oregon State University, Corvallis, 123 p.

Ransom, D. P.; C. N. Lannan, J. S. Rohovec, and J. L. Fryer, 1984. Comparison of histopathology caused by Vibrio anguillarum and Vibrio ordalii in three species of Pacific salmon. J. Fish Dis., 7 : 107-115.

Reed, L. J., and H. Muench, 1938. A simple method of estimating fifty percent endpoints. Am. J. Hygiene, 27 (3) : 493-497.

Refstie, T., 1982. Preliminary results : Differences between rainbow trout families in resistance against vibriosis and stress. Dev. Comp. Immunol., Sup., 2., 205-209.

Richards, R. H., 1980. Observations on vibriosis in cultured flatfish. 75-81, in : Fish diseases, W. Ahne (ed.), third COPRAQ-Session, Springer-Verlag, Berlin.

Rijkers, G. T., 1982. Kinetic of humoral and cellular immune reactions in fish. In : Dev. Comp. Immunol., Sup., 2., 93-100.

Rijkers, G. T.; A. G. Teunissen, R. van Oosteromm, and W. B. van Muiswinkel, 1980. The immune system of cyprinid. The immunosuppresive effect of the antibiotic oxytetracycline in carp (Cyprinus carpio) Aquaculture, 19 : 177-189.

Rijkers, G. T.; E. M. H. Frederix-Wolter, and W. B. van Muiswinkel, 1980. The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (Cyprinus carpio). Immunol., 41 : 91-97.

Rijkers, G. T.; R. van Oosterom, and W. B. van Muiswinkel, 1981. The immune system of cyprinid fish. Oxytetracycline and the regulation of humoral immunity in carp. Vet. Immunol. Immunopathol., 2 : 281-290.

Rijkers, G. T., and W. B. van Muiswinkel, 1977. The immune system of cyprinid fish. The development of cellular and responsiveness in the rosy barb (Barbus conchonius). 233-240, in : Dev. Immunobiol., J. B. Solomon, and J. D. Horton (eds.), Elsevier/North-Holland.

Roberts, R. J., 1971. Studies on the skin of plaice (Pleuronectes platessa). I : The structure and ultrastructure of normal plaice skin. J. Fish. Biol., 4 : 87-98.

_____, 1975. The effects of temperature on diseases and their histopathological manifestations in fish. In : The pathology of fishes, ed., by W. E. Ribelin, and G. Migaki, Univ. of Wisconsin Press, 477-496.

Roberts, R. J.; M. Bell, and H. Young, 1973. Studies on the skin of plaice (Pleuronectes platessa). II : The development of larval plaice skin. J. Fish. Biol., 5 : 103-108.

Robohm, R. A., and D. S. Sparrow, 1981. Evidence for genetic selection of high antibody responders in summer flounder (Paralichthys dentatus) from polluted areas. Dev. Biol. Standard., Serodiagnostic and vaccines, 49 : 273-278.

Rohovec, J. S., 1975. Oral and parenteral immunization for the control of Vibrio anguillarum, the etiological agent of vibriosis in salmonid fish. Ph D. thesis, Oregon State University, 82 p.

Rohovec, J. S.; J. R. Winton, and J. L. Fryer, 1981. Bacterins and vaccines for control of infectious diseases in fish. In : Proc. Rep. of China - United States Cooperative Science Seminar on Fish Diseases. G. H. Kou, J. L. Fryer, and M. L. Landolt (eds.), Nat. Sci. Council, Taipei, 115-121.

Rosenkvist-Jensen, L., 1982. Results from vaccination of Danish rainbow trout against vibriosis. Dev. Comp. Immunol., Sup., 2., 187-191.

Rucker, R. R., 1959. Vibrio infections among marine and freshwater fish. Prog. Fish Cult., 21 : 22-25.

Ruiz, M. S., 1982. Studies of naturally occurring disease during the production of ayu in seawater (Plecoglossus altivelis). Fish Pathol., 17 (3) : 205-212.

Sakai, M.; T. Aoki, T. Kitao, J. S. Rohovec, and J. L. Fryer, 1984. Comparisons of the cellular immune response of fish vaccinated by immersion and injection of Vibrio anguillarum. Bull. Jap. Soc. Sci. Fish., 50 (7) : 1187-1192.

Sako, H., and R. Kusuda, 1978. Chemotherapeutical studies on trimethoprim against vibriosis of pond-cultured ayu. I : Microbiological evaluation of trimethoprim and sulfonamides on the causative agent Vibrio anguillarum. Fish Pathol., 13 (2) : 91-96.

Sawyer, E. S., 1978. Vibriosis in Maine and New Hampshire salmonids. Mar. Fish. Rev., 1332 : 9-10.

Sawyer, E. S., and R. G. Strout, 1977. Survival and growth of vaccinated, medicated and untreated coho salmon (Oncorhynchus kisutch) exposed to Vibrio anguillarum. Aquaculture, 10 : 311-315.

Schiewe, M. H., 1981. Taxonomic status of marine Vibrios pathogenic for salmonid fish. Dev. Biol. Standard., Serodiagnositcs and vaccines, 49 : 149-158.

_____ , 1983. Vibrio ordalii as a cause of vibriosis in salmonid fish. 31-40, in : Bacterial and viral diseases of fish, J. H. Crosa (ed.), Univ. of Washingto, Seattle, Wa.

Schiewe, M. H., and H. O. Hodgins, 1977. Specificity of protection induced in coho salmon (Oncorhynchus kisutch) by heat-treated components of two pathogenic vibrios. J. Fish. Res. Bd. Can., 34 : 1026-1028.

Schiewe, M. H., and J. H. Crosa, 1981. Molecular characterization of Vibrio anguillarum biotype 2. Can. J. Microbiol., 27 : 1011-1018.

Shiewe, M. H.; T. J. Trust, and J. H. Crosa, 1981. Vibrio ordalii sp. nov. : A causative agent of vibriosis in fish. Cur. Microbiol., 6 : 343-348.

Schiewe, M. H.; T. J. Trust, and J. H. Crosa, 1982. Vibrio ordalii sp. nov. In : List no. 9 Validation of the publication of new names and new combinations previously effectively published outside the IJSB. It. J. Syst. Bacteriol., 32 : 384-384.

Schreck, C. B., 1981. Stress and compensation in teleostean fishes : Response to social and physical factors. 295-321, in : Stress and fish, A. D. Pickering (ed.), Academic Press, New York.

Schreck, C. B., 1982. Stress and rearing of salmonids. Aquaculture, 28 : 241-249.

Sedgwick, S. D., 1982. The salmon handbook. Andre Deutsch London, 247 p.

Smith, I. W., 1961. A disease of finnock due to Vibrio anguillarum. J. Gen. Microbiol., 24 : 247-252.

Snieszko, S. F., 1957. Use of antibiotics in the diet of salmonid fishes. Prog. Fish Cult., 19 (2) : 81-84.

_____ , 1958. Natural resistance and susceptibility to infections. Prog. Fish Cult., 20 : 133-136.

_____ , 1970. Immunization of fishes. A Review. J. Wild. Dis., 6 : 24-29.

_____, 1974. The effects of environmental stress on outbreaks of infectious diseases of fishes. J. Fish. Biol., 6 : 197-208.

_____, 1978. Control of fish disease. Mar. Fish Rev., 1301 : 65-68.

Spence, K. D.; J. L. Fryer, and K. S. Pilcher, 1965. Passive immunization of certain salmonid fishes against Aeromonas salmonicida. Can. J. Microbiol., 11 (3) : 397-405.

Steel, R. G. D., and J. H. Torrie, 1980. Principles and procedures of statistics. A biometrical appl., second ed., McGraw Hill Book Co., New York, 633 p.

Stolen, J. S.; T. Gahn, and J. Nagle, 1982. The humoral antibody formation to erythrocyte antigens in three species of flatfish. In : Dev. Comp. Immunol., Sup., 2., 101-106.

Strout, R. G.; E. S. Sawyer, and B. A. Coutermarsh, 1978. Pathogenic Vibrios in confinement-reared and feral fishes of the Maine-New Hampshire coast. J. Fish. Res. Bd. Can., 35 : 403-408.

Tabata, K. S.; S. Karata, and M. S. Ruiz, 1982. Studies on naturally occurring disease during the production of ayu (Plecoglossus altivelis) in seawater. II : Dynamics of Vibrio anguillarum. Fish Pathol., 17 (3) : 205-212.

Tajima, K.; M. Yoshimizu, Y. Ezura, and T. Kimura, 1981. Studies on the causative organisms of vibriosis among the pen-cultured coho salmon Oncorhynchus kisutch in Japan. Bull. Jap. Soc. Sci. Fish., 47 (1) : 35-42.

Tatner, M. F., and M. J. Manning, 1983. Growth of the lymphiod organs in rainbow trout, Salmo gairdneri, from one to fifteen months of age. J. Zool. London, 199 : 503-520.

Tatner, M. F., and M. T. Horne, 1983. Factors influencing the uptake of ^{14}C-labelled Vibrio anguillarum vaccine in direct immersion experiments with rainbow trout, Salmo gairdneri. J. Fish. Biol., 22 : 585-591.

Tatner, M. F., and M. T. Horne, 1984. The effects of early exposure to Vibrio anguillarum vaccine on the immune response of the fry of the rainbow trout, Salmo gairdneri. Aquaculture, 41 : 193-202.

Trust, T. J.; I. D. Courtice, A. G. Khouri, H. H. Crosa, and M. H. Schiewe, 1981. Serum resistance and hemagglutination ability of marine Vibrios pathogenic for fish. Infect. Immun. 34 (1) : 702-707.

Umbreit, T. H., and M. R. Tripp, 1975. Characterization of the factors responsible for death of fish infected with Vibrio anguillarum. Can. J. Microbiol., 21 : 1272-1274.

Umminger, B. L., 1970. Effects of temperature on serum protein components in the killifish, Fundulus heteroclitus. J. Fish. Res. Bd. Can., 27 (2) : 404-409.

Uskova, Y. T.; A. V. Chaykovskaya, and S. I. Davidenko, 1980. On the chemical nature of the skin mucosa in the mullet, Mugil auratus. J. Ichthyol., 20 (2) : 112-118.

van Loon, J. J. A.; R. van Oosterom, and W. B. van Muiswinkel, 1981. Development of the immune system in carp (Cyprinus carpio). 469-470, in : Aspects of Dev. Comp. Immunol., I., J. B. Solomon (ed.), Pergamon Press, Oxford.

Viele, D.; T. H. Kerstetter, and J. Sullivan, 1980. Adaptive transfer of immunity against Vibrio anguillarum in rainbow trout Salmo gairdneri vaccinated by the immersion method. J. Fish. Biol., 17 : 379-386.

Wedemeyer, G., 1970. The role of stress in the disease resistance of fish. 30-35, in : A symposium on diseases of fish and shellfishes. S. F. Snieszko (ed.), Spec. pub., 5, Am. Fish. Soc., Washington, D. C.

Williams, S. F., 1974. Rearing conditions and their effects on growth, food conversion, and survival of English sole (Parophrys vetulus). Ph D thesis, Oregon State University, Corvallis, 95 p.

Williams, S. F., and R. S. Caldwell, 1978. Growth, food conversion and surival of O-group English sole (Parophrys vetulus) at five temperatures and five rations. Aquaculture, 15 (2) : 129-139.

Winter, G. W.; C. B. Schreck, and J. D. McIntyre, 1981. Resistance of different stocks and transferrin genotypes of coho salmon, Oncorhynchus kisutch, and steelhead trout, Salmo gairdneri, to bacterial kidney disease and vibriosis. Fish. Bull., 77 (4) : 795-802.

Winton, J.R.; J. S. Rohovec, and J. L. Fryer, 1983. Bacterial and viral diseases of cultured salmonids in the Pacific Northwest. 1-20, in : Bacterial and viral diseases of fish, J. H. Crosa (ed), Univ. of Washington, Seattle, Wa.

Wishkovsky, A., and R. R. Avtalion, 1982. Induction of helper and suppressor functions in carp (Cyprinus carpio) and their possible implication in seasonal disease in fish. Dev. Comp. Immunol., Sup., 2., 83-91.

Wolf, L. E., 1953. Development of disease-resistant strains of fish. Trans. Am. Fish. Soc., 83 : 342-349.

Wolke, R. E., 1975. Pathology of bacterial and fungal diseases affecting fish. 33-116, in : Pathology of fishes, W. E. Ribelin and G. Migaki (eds.), Univ. of Wisconsin Press, Madison, Wisconsin.

Wood, J. W., 1974. Diseases of Pacific salmon their prevention and treatment. Second ed., State of Washington, Dept. of Fish., Olympia, 81 p.