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An oral vaccine was developed for control of vibriosis in Pacific salmon. The vaccine consists of a lyophilized sonicate of cells of *Vibrio anguillarum*, the causative agent of this disease. The preparation was incorporated into a ration and fed to salmon in fresh water before the fish were transported and exposed to the disease at the Lint Slough marine rearing facility, Waldport, Oregon. Spring and fall chinook salmon were fed vaccine at a level of 300 μg per fish over a 14-day period. This resulted in 27% mortality in vaccinated salmon as compared to 97% in nonvaccinated fish. Vaccine was fed to spring chinook salmon at 100, 200, and 300 μg per fish over a 14-day interval. At a dosage of 100 μg, mortality sharply increased to 58% whereas 200 and 300 μg resulted in 33% mortality. Salmon receiving no vaccine had a 90% mortality. Spring chinook were fed vaccine at 200, 300, 500, 700, and 1,000 μg per fish over a 14-day period. Increasing the dosage

did not lower the mortality. The mortality was about 20% in the vaccinated groups of fish as compared to 90% in the nonvaccinated fish. Feeding booster vaccine in fresh or salt water did not further reduce mortality. Coho salmon were fed vaccine at 1,000 μg per fish over a 28-day period instead of a 14-day period. This resulted in less than three percent mortality for vaccinated animals as compared to 90% mortality in nonvaccinated fish.

The nature of the immunity was investigated. Salmon which demonstrated high levels of protection after oral immunization were examined for humoral antibody. None was found. This suggested a localized immunity. There is a suggestion of some specificity for vibriosis as a result of receiving vibriosis vaccine. A vaccine was prepared from *Aeromonas salmonicida*, another Gram negative fish pathogen. Mortality in *A. salmonicida* vaccinated salmon was as high as in nonvaccinated animals when exposed to vibriosis.

Oral Immunization for the Control of Vibriosis in Pacific Salmon

by

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APPROVED:

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Associate Professor of Microbiology
in charge of major

Redacted for Privacy

Chairman of the Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented 4/12/72

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ORAL IMMUNIZATION FOR THE CONTROL OF VIBRIOSIS IN PACIFIC SALMON

INTRODUCTION

At present there is an increased interest in utilizing marine aquaculture for production of food fishes. Various problems have to be solved before maritime areas can be developed. Perhaps the major obstacle is the fish disease vibriosis (causative agent: *Vibrio anguillarum*) which has long been recognized as a major fish disease in saltwater environments (Conroy and Anderson, 1970).

Antimicrobial agents fed in salmon rations have been the principal method of controlling bacterial infections in fishes (Wood, 1968). Salmon are often not fed a prepared ration in marine aquaculture but allowed to forage naturally. Therefore, feeding antimicrobials would defeat the purpose of these rich marine environments as a source of natural food. These investigations were performed to determine if oral immunization of salmon could be useful in controlling vibriosis prior to transfer to the marine area.

A lyophilized sonicate of *V. anguillarum* was used as the experimental oral vaccine. The parameters investigated were the dosage of vaccine required to elicit an immunity, the value of oral boosters in elevating this immunity, and the length of the feeding period required

for optimum immunization. Vaccinations were performed at the Oregon State University Fish Disease Laboratory, Corvallis, Oregon before vibriosis challenge at Lint Slough marine rearing facility, Waldport, Oregon.

LITERATURE REVIEW

Bacterial Oral Immunization

Ehrlich (1908) was the first to study peroral immunization. His research dealt with a means of preventing castor bean poisoning by orally immunizing against ricin (poisonous protein in castor beans).

Later, Besredka (1924) postulated a mechanism of immunity independent of circulating antibody. He found that a localized cellular immunity was attained by orally administering a vaccine against bacillary dysentery. Patients receiving this preparation failed to produce circulating antibody but did develop a resistance to dysentery.

Peroral immunization became unpopular because it was found to be less durable than intramuscular injections (Ross, 1934a,b,c,d; Hoffstad and Thompson, 1929). Interest in the use of oral vaccines did not revive until an oral viral immunity was demonstrated against poliomyelitis (Sabin, 1957, 1959, 1960; Cox, *et al*., 1959). This vaccine was composed of a live attenuated virus which, when fed orally, infects the intestinal mucosa eliciting local cellular and circulating antibody response against poliomyelitis.

Peroral immunization against typhoid (*Salmonella typhi*) has been demonstrated in mice (Raettig, 1961, 1962, 1964, 1965, 1967). Raettig (1962) found that oral doses

of vaccine could control epidemics in mice whereas subcutaneous vaccination often provoked more cases of typhoid. Raettig postulated that by perorally immunizing large populations during epidemics, the dangers of parenteral epidemic vaccination could be avoided. Levanon, Raettig, and Rossetini (1968), after orally immunizing mice against typhoid, found a positive immunological reaction of the intestinal mucosa by passive cutaneous anaphylaxis.

Fahey and Cooper (1970a,b) and Cooper and Fahey (1970) fed 10^8 to 10^9 living temperature sensitive mutants of *Salmonella enteritidis*. The oral vaccine conferred up to 80% protection with a 100 LD_{50} challenge in the absence of circulating antibody.

Desoxycolate extracts of *Dyspepsia coli* (enteropathogenic *Escherichia coli*) fed orally have produced up to 60% protection in mice with the absence of circulating antibody. The bacterin was orally administered in bread (Ocklitz *et al.*, 1967a,b, 1968; Mochman *et al.*, 1967, 1968).

A streptomycin-dependent *Vibrio cholerae* has been developed as an oral vaccine against cholera (Felsenfeld *et al.*, 1970). An oral cholera vaccine has been developed consisting of living apathogenic *Vibrio cholerae* El Tor strain. Caproantibody (intestinal antibody) levels rose in human patients after feeding this live preparation (Sanyal and Murkerjie, 1969). Freter (1970) found cholera oral immunization in the absence of circulating antibody,

but the immunization was found to be associated with antibody in the intestine that does not allow adsorption of the pathogen to the intestinal mucosa.

Bacterial Oral Immunization in Fish

Duff (1942) first considered oral immunization in fish. A chloroform killed preparation of *Aeromonas salmonicida*, the causative agent of furunculosis, was fed to cutthroat trout (*Salmo clarki*). The fish were challenged by exposure to live *A. salmonicida*. Twenty-five percent mortality was observed in the vaccinated group as compared to 75% mortality in a control group which did not receive vaccine.

Endo (1961) fed a multivalent vaccine consisting of four formalin killed fish pathogens: *Vibrio piscium*, *A. salmonicida*, *Chondrococcus columnaris* and *Haemophilus piscium*. During an epizootic of a disease of unknown etiology this multivalent vaccine was incorporated into a hatchery diet. A reduction of about 60% in mortalities was observed after administering this vaccine. A control group was not used. However, previous epizootics had killed most of the hatchery population.

Hayashi *et al*. (1964) fed formalin (1%) killed etiologic agent of vibriosis to rainbow trout. When challenged by intramuscular injection with 10^3 cells per ml of the agent, mortality was greater than 90% which was about the same as the mortality in nonvaccinated fish.

Post (1963) used a heat killed A. hydrophila oral vaccine. The vaccine was fed to rainbow trout (Salmo gairdneri). On challenge by injection, a 30% mortality was noted in the group which received the vaccine, compared to 90% mortality in the group which did not receive vaccine.

Ross and Klontz (1965) fed a phenol killed preparation of the etiological agent of red mouth disease to brook trout (Salvelinus frontinalis). On challenge by injection, fish which were fed vaccine had no mortality as compared to 100% mortality in unvaccinated controls.

Overholser (1968) fed an alum precipitate antigen of A. salmonicida to coho salmon (Ohcorhynchus kisutch). Using a natural challenge at Siletz River Salmon Hatchery, orally immunized fish had less than one percent mortality as compared to an unvaccinated control group which had 35% mortality.

Fujihara and Nakatani (1971) fed heat killed C. columnaris to coho salmon. On exposure to live C. columnaris, an eight percent mortality was observed in immunized fish as compared to 48% mortality in unimmunized controls.

Two review papers have been published on immunization of fish. Snieszko (1969) surveyed several methods of immunizing fish. In his opinion, "with the present state of knowledge, only a guarded optimism is justified towards the effectiveness of oral immunization of fishes." Klontz and Anderson (1970) reviewed oral immunization and also

concluded that more information is required concerning oral immunization of fishes.

Vibriosis

Vibriosis caused by *V. anguillarum* is characterized by a hemorrhagic septicemia resulting in discoloration of the skin, hemorrhaging of the fins and ulcer formation preceding death. Vibriosis among fish is known to be worldwide in distribution. Consequently, this disease is an important factor in the management of fisheries and in the operation of marine fish farms (reviews by Conroy and Anderson, 1970; Fryer, Nelson and Garrison, 1971; Evelyn, 1971).

The etiological agent of eel red pest was first described by Canestrini in 1893 and designated *Bacterium anguillarum* (as cited by Conroy and Anderson, 1970). Bergman (1912) later named the etiological agent of eel red pest as *V. anguillarum*.

Nyblin (1935) described two biotypes of *V. anguillarum*, Types A and B. Type A produced indole and fermented sucrose and mannitol producing acid and gas. Type B did not produce indole and did not produce acid and gas from sucrose or mannitol. Smith (1961) identified a third biotype, Type C, which did not produce indole but produced acid and gas from sucrose and mannitol.

The etiological agent of a disease in fall chinook

salmon (*O. tshawytscha*) at Lint Slough, Waldport, Oregon was identified as *V. anguillarum* Type A (Cisar and Fryer, 1969).

MATERIALS AND METHODS

Culture Media

Marine Broth (Morita, 1969): 5.0 g Neopeptone (Difco Laboratories), 1.0 yeast extract, 0.1 g soluble $FePO_4$, and 25.25 g Rela Salts per liter of distilled water. This medium was sterilized in the Fermacell Fermentor for one hour at 121°C.

Tryptic Soy Broth (Difco Laboratories): Tryptic Soy Broth was rehydrated using 30 g of dry powder with one liter of distilled water. Sterilization was accomplished in the Fermacell Fermentor for one hour at 121°C.

Furunculosis Agar (Difco Laboratories): Furunculosis Agar was rehydrated using 33.5 g of dry powder with one liter of distilled water. The agar was autoclaved for 15 minutes at 15 p.s.i. at 121°C.

Cytophaga Sea Water Agar (Pacha and Ordal, 1967): 0.2 g beef extract, 0.5 g tryptone, 0.5 g yeast extract, 0.2 g sodium acetate, 25.0 g Rela Salts and 4.0 g agar (Difco Laboratories) mixed into one liter of distilled water. This preparation was autoclaved for 15 minutes at 15 p.s.i. at 121°C.

Cultures

Vibrio anguillarum cultures were isolated from mortalities of salmon at Lint Slough. Culture LS-2-68 refers

to *V. anguillarum* Type A isolated by Cisar and Fryer (1969) from an epizootic at Lint Slough. Isolate LS-2-69 was obtained in June, 1969 from a fall chinook salmon mortality at Lint Slough. Stock cultures were maintained in Cytophaga Sea Water Agar. Working cultures were maintained on Furunculosis Agar.

Aeromonas salmonicida cultures were isolated in 1969 from an epizootic in coho salmon at the Siletz River Salmon Hatchery. Cultures were maintained on Furunculosis Agar.

Serological Methods

Antigen Preparation

Antigen preparations were formalin (0.3%) killed cell suspensions of either LS-2-68, LS-2-69 or other *V. anguillarum* cultures. To determine the proper concentration of antigen, the bacterium was suspended in physiological saline and the absorbance determined using a Bausch and Lomb Spectronic 20 set at 525 mμ.

Antiserum Preparation

Antiserum against *V. anguillarum* was prepared by injection of an antigen preparation of LS-2-68 at an absorbance of 0.3 into a young New Zealand rabbit. The injection schedule is given in Table I (Kolmer *et al.*, 1951).

TABLE I. INJECTION SCHEDULE FOR THE PREPARATION OF VIBRIO ANGUILLARUM IMMUNE SERUM.

Day	Amount Injected (ml)	Route of Injection
1	0.1	i.v.[a]
2	0.2	i.v.
3	0.5	i.v.
8	3.0	i.p.[b]
9	1.5	i.v.
10	2.0	i.v.

a. Intravenous
b. Intraperitoneal

Macroscopic Tube Agglutination

The macroscopic tube agglutination procedure was used for analysis of agglutinating antibody in sera. Two fold serum dilutions from 1:10 to 1:2560 were used (Kolmer *et al.*, 1951). The antigen consisted of a formalin (0.3%) killed saline suspension of *V. anguillarum* (LS-2-68) whole cells at an absorbance of 0.3.

Microtiter Agglutination

The microtiter method (Cooke Engineering Co.) was used to analyze serum for agglutinating antibody, and two fold serum dilutions from 1:2 to 1:128 were employed (Witlin, 1967; Benenson, Saad and Paul, 1968; Vedros and Hill, 1966). The antigen consisted of a formalin (0.3%) killed saline suspension of *V. anguillarum* (LS-2-68) whole cells at an absorbance of 0.9.

Rapid Slide Agglutination

The rapid slide agglutination method was performed to confirm the presence of *V. anguillarum* (Kolmer *et al.*, 1951). Immune serums with a titer of 1:320 against LS-2-68 were used. Cells from suspect *V. anguillarum* colonies were suspended in saline to an absorbance of 0.3 and employed as the antigen for this test.

Mass Culture Method

All bacterial cells used for vaccines were cultured in broth in a Fermacell Fermentor, Model CF-50 (New Brunswick Scientific Co., Inc.). Table II lists the isolates used, the culture media, and the growth temperature employed for each organism.

TABLE II. ISOLATES, MEDIA, AND GROWTH TEMPERATURE USED FOR VACCINE PREPARATION.

Isolate	Media	Growth Temperature
V. anguillarum		
LS-2-68	Marine Broth[a]	28°C
LS-2-69	Tryptic Soy Broth[b]	28°C
A. salmonicida		
1969 Siletz	Tryptic Soy Broth	23°C

a. Morita, 1969
b. Difco Laboratories

Five milliliters of Medical Antifoam C Emulsion (Dow Corning Corp.) was added to the broth before sterilization.

After sterilization at 121°C for one hour, the broth was adjusted to the proper growth temperature for each organism. The sterile broth was inoculated with a 24- to 36-hour-old two liter culture of the isolate used. The broth was aerated at a rate of 0.25 cubic feet per minute and mixed with an impeller at 200 rpm.

Before harvesting, each culture was examined for purity by means of phase microscopy, Gram stain and culture on Furunculosis Agar. The rapid slide agglutination method was used to confirm the presence of *V. anguillarum*. The formation of a characteristic soluble brown pigment on Furunculosis Agar was observed with *A. salmonicida* cultures. Rapid slide agglutination was also used to confirm the presence of *A. salmonicida*.

Each culture (approximately 30 l) was harvested after 24 to 36 hours incubation using a Sharples Super Centrifuge, Type T 1P. The flow rate through the centrifuge was set at 200 ml per minute. After harvesting, the cells were stored at 4°C for about two hours before continuing preparation of the vaccine.

Vaccine Preparation

Three vaccines were prepared: 1968 vaccine from LS-2-68, 1969 vaccine from LS-2-69, and *A. salmonicida* vaccine from the 1969 Siletz isolate.

In preparing the vaccines, 250 g of cells (wet weight)

were resuspended in one liter of 0.3% formalin saline solution and mixed for 24 hours. One milliliter of each formalin killed suspension was inoculated on Furunculosis Agar and incubated for 24 to 72 hours. These plates were examined for viability of the formalin killed cells.

The Banson W185 Sonifier Cell Disruptor (Heat Systems, Inc.) was used to sonically disrupt the preparation. The Sonifier's W horn was suspended in a 50 ml glass continuous flow disruptor vessel. The flow rate of the suspension was set at 25 ml per minute with a power setting of eight at 100 watts. The suspension was examined for disruption using phase contrast microscopy. About 90% disruption was observed using this method.

The sonified suspension was lyophilized using a VirTis model 10-145 Lyophilizer (VirTis Research Equipment, Inc.). The dried material was removed from the lyophilizer and powdered using a mortar and pestle.

Each vaccine preparation was stored at room temperature under vacuum. The vaccine preparations were used over a two-year period from July, 1969 to May, 1971.

Determination of the Amount of Vaccine and Diet Fed

The following methods were used to determine (1) the total μg vaccine required for a group of fish and (2) the total grams of diet required over a selected feeding period: (1) Total μg vaccine = number of fish X μg

vaccine/fish; (2) Total grams of diet required = (number of fish ÷ number of fish/lb) X 454 g/lb X percent body weight to be fed per day X total number of days (Amend, 1965).

Preparation of Test Diet Containing Vaccine

The ration used in the oral vaccination experiment was a formulation of the Oregon Test Diet[1] (Lee, Roehm and Sinnhuber, 1967). The premix (Table III) and vitamin mix (Table IV) were prepared separately. To compound the test diet, 308 g premix, 7 g vitamin mix, 35 g salmon oil (Bumble Bee Corp., Astoria, Oregon) and 650 ml unchlorinated hot water were mixed for about five minutes. The vaccine was mixed into the diet and the preparation poured into plastic freezer containers and allowed to gel at 4°C. After 24 hours, the diet was cut into 1/16- to 1/8-inch cubes. The ration was then stored at -15°C in plastic bags containing individual daily portions. One bag was thawed the day before the portion was fed.

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1. The formula was modified by Joseph H. Wales, Associate Professor, Dept. of Food Science and Technology, Oregon State University, Corvallis, Oregon.

TABLE III. OREGON TEST DIET PREMIX USED FOR PREPARATION OF RATION.

Component[1]	Percentage
Casein (vitamin free)	49.4
Gelatin	8.7
Dextrin	15.6
Bernhart-Tomerelli Salt Mixture	4.0
Calcium Carbonate	0.9
Carboxymethyl Cellulose	1.3
Alpha Cellulose	6.4
70% Choline Chloride Solution	1.0
Vitamin A	0.1
Vitamin E	0.6

1. Nutritional Biochemicals Corp.

TABLE IV. OREGON TEST DIET VITAMIN MIX USED FOR PREPARATION OF RATION

Component[1]	Percentage
Alpha Cellulose	73.58
Inositol	12.50
Ascorbic Acid	6.00
Niacin	2.56
Para Amino Benzoic Acid	2.00
Calcium Pantothenate	1.44
Riboflavin	0.72
Thiamin	0.32
Pyridoxine	0.24
Vitamin B_{12} (3,000 μg diluted in 1 g alpha cellulose)	0.267
Folic Acid	0.096
Menadione	0.08
B.H.A.	0.075
B.H.T.	0.075
Vitamin D	0.04
Biotin	0.008

1. Nutritional Biochemicals Corp.

Oral Vaccination Procedure

The experimental and control salmon were fed vaccine diet and control diet (Oregon Test Diet without vaccine) at a rate which allowed the salmon to consume the entire portion in one day (feeding one to four times per day). This procedure was followed from 14 to 28 days. After the vaccination period, the salmon were fed Oregon Moist Pellet (O.M.P.) (Hublou, 1963) for 14 to 21 days before natural challenge.

Experimental Animals

Chinook salmon were obtained from the Marion Forks Salmon Hatchery and coho salmon from the Trask River Salmon Hatchery. Both hatcheries are operated by the Fish Commission of Oregon.

Oral Vaccination Facilities

Salmon used in the oral vaccination experiments were administered the vaccine at the Oregon State University Fish Disease Laboratory. During the feeding period the experimental animals were held in fresh water obtained from a well with a temperature range of 13 to 15°C (approximately 54 to 56°F).

Natural Challenge Facilities

Lint Slough marine rearing facility, Waldport, Oregon

was used as the source of the natural challenge for vibriosis. This facility is operated by the Oregon State Game Commission. Lint Slough epizootics of vibriosis with mortalities of about 90% of the total population have been reported (Cisar and Fryer, 1969).

Controls (nonvaccinated fish) and orally vaccinated salmon were transferred from the Fish Disease Laboratory to Lint Slough after the vaccination procedure was completed and were exposed to the disease agent for 40 days. Water temperature at Lint Slough ranged from 8°C (45°F) in winter to 21°C (70°F) in summer.

Examination of Mortalities

Mortalities were collected twice a day during the period of heaviest loss; subsequently they were collected once a day. The fish were placed in plastic bags, properly identified, and frozen. Mortalities were examined within one month of death.

The mortalities were examined for gross pathology and the presence of *V. anguillarum* in the kidney. A sterile loop was aseptically inserted into the kidney and inoculated on Furunculosis Agar. The cultures were incubated at 28°C and examined daily for the presence of *V. anguillarum*. The criteria for identification of this bacterium were gross pathology of the fish, Gram stain of kidney and isolates, motility, and slide agglutination.

Oral Vaccination Experiments

In these experiments vaccine dosage, oral boosters, and the length of the vaccination period were examined. Each experiment used the same general procedure: a vaccination period and a holding period in fresh water followed by 40 days of natural challenge at Lint Slough in sea water.

Preliminary Experiment

In this experiment, 300 µg 1968 vaccine per fish was fed to 200 fall chinook salmon (mean weight 2.2 g/fish) and 100 spring chinook salmon (mean weight 2.2 g/fish) over a 15-day period, followed by a 20-day holding period. Two hundred spring chinook were fed diet without vaccine as controls. Each 100 fish received 12 grams of diet per day.

Vaccine Dosage

Paired groups of 100 spring chinook salmon (mean weight 2.7 g/fish) were administered 300 µg 1968 vaccine per fish and 100, 200 and 300 µg 1969 vaccine per fish over a 14-day period. This feeding schedule was followed by a 20-day holding period. Paired groups of 100 salmon were not fed vaccine as controls. Each 100 fish received ten grams of diet per day.

In another experiment, paired groups of 100 spring chinook salmon (mean weight 5.5 g/fish) were fed 200, 300,

500, 700 and 1,000 μg 1969 vaccine per fish over a 14-day period, followed by a 20-day holding period. Paired control groups of 100 salmon were not fed vaccine. Each 100 fish received 30 grams of diet per day.

Marine and Fresh Water Boosters

The size of fish and vaccine levels in this experiment were identical to those used in the first dosage experiment. Following vaccination and transfer to Lint Slough, oral boosters were administered. This schedule consisted of 15 oral boosters spaced over a 30-day period, feeding vaccine diet for three days followed by an alternate feeding of O.M.P. for three days. Feeding the oral boosters doubled the concentration of vaccine received by each fish.

The fresh water booster experiment was identical to the marine booster experiment except the oral booster was administered in fresh water before transfer to Lint Slough.

Length of Vaccination Period and Specificity of Vaccine

In this experiment two groups of 160 and 80 coho salmon (mean weight 1.5 g/fish) were fed 1,000 μg 1968 vaccine per fish over a 28-day period followed by a 14-day holding period. In addition, two groups of 160 and 80 coho salmon received 1,000 μg of *A. salmonicida* vaccine per fish over the same period. Two groups of 160 and 80 salmon were not fed vaccine as controls. Ten grams of diet were fed to 100

salmon per day.

Determination of Agglutinating Antibodies in Serum from Orally Vaccinated Salmon

At the beginning of each vaccination period, end of the fresh water holding period, and end of the exposure at Lint Slough, individual and pooled blood samples were taken. The tail of the fish at the peduncle region was excised and blood was collected in tubes. The blood was allowed to clot at room temperature and then retract at 4°C overnight. Serum from the fish in each experimental group was analyzed for agglutinating antibody using either microtiter or tube agglutination tests.

RESULTS

Oral Vaccination

Preliminary Experiment

In this experiment 300 μg per fish of 1968 vaccine was fed to 200 fall chinook and 100 spring chinook salmon over 14 days. The results of this experiment demonstrated that administration of this vaccine lowered mortality to about 28% in both vaccinated groups as compared to a 97% loss in the control animals (Figure 1). Table V lists the cumulative percent mortality for each experimental group.[2]

TABLE V. CUMULATIVE PERCENT MORTALITY OF IMMUNIZED FALL AND SPRING CHINOOK SALMON RECEIVING 300 μg VIBRIOSIS ORAL VACCINE (1968) PER FISH OVER A 14-DAY PERIOD AND NONVACCINATED SPRING CHINOOK SALMON.

	Cumulative Percent Mortality		
Day	Spring Chinook	Fall Chinook	Spring Chinook Control
2	0	0	0
4	6	4	84
6	13	11	91
8	17	15	96
10	19	16	96
20	19	17	96
30	19	17	96
40	27	28	97

2. This vaccine was prepared by Mr. Thomas Staley, and the experiment was conducted by Mr. R. L. Garrison.

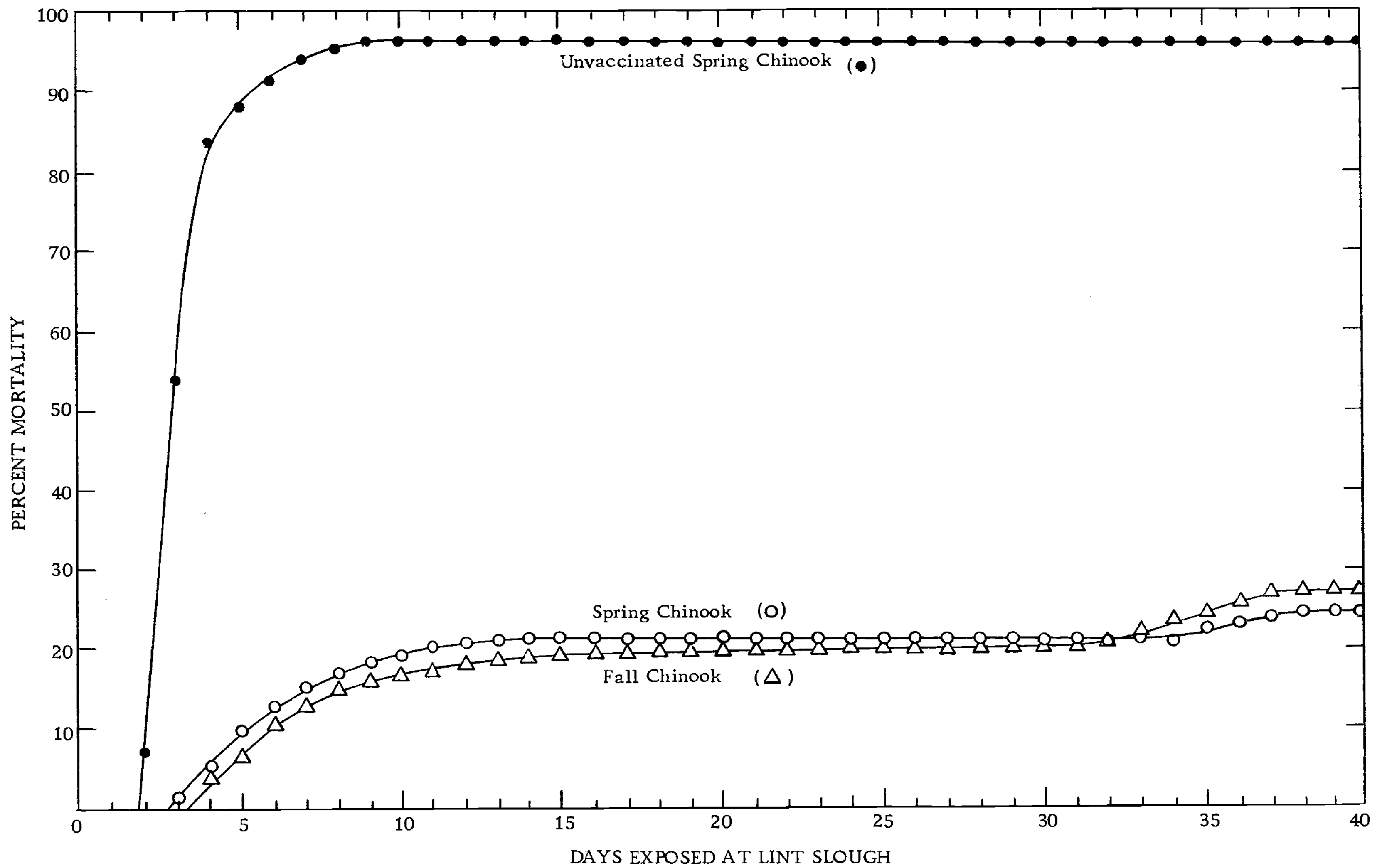

Figure 1. The effect of oral vaccination with 300 μg 1968 vaccine per fish for control of vibriosis in 200 spring chinook and 100 fall chinook as compared to 200 non-vaccinated spring chinook salmon fed for 14 days.

Vaccine Dosages

In this experiment dosages of 100, 200 and 300 μg 1969 vaccine were fed per fish over 14 days to paired groups of 100 spring chinook salmon to determine if dosages below 300 μg were effective in controlling vibriosis. To compare the effectiveness of the two experimental vaccines, 300 μg 1968 vaccine was fed per fish to two groups of 100 spring chinook. The results obtained from the paired groups were averaged. As shown in Figure 2, a greater percent loss was observed in the group fed 100 μg 1969 vaccine per fish, which indicated concentration was a factor in oral immunization against vibriosis. The results also demonstrated that 300 μg of 1968 vaccine was more effective than 300 μg 1969 vaccine in reducing mortality. Table VI presents the averaged cumulative percent mortality and the percent infected with *V. anguillarum*.

TABLE VI. CUMULATIVE PERCENT MORTALITY OF IMMUNIZED AND CONTROL GROUPS OF SALMON RECEIVING 100, 200 AND 300 µg 1969 VACCINE AND 300 µg 1968 VACCINE PER FISH OVER A 14-DAY PERIOD.

Day	Cumulative Percent Mortality				
	100 µg 1969	200 µg 1969	300 µg 1969	300 µg 1968	Control
2	0	0	0	0	0
4	3	1	3	1	14
6	18	7	5	2	64
8	48	19	17	4	69
10	56	30	25	4	82
20	58	32	30	8	87
30	58	33	33	10	87
40	58	33	33	12	87
% Infected[1]	95	90	93	88	96

1. Percent of cumulative mortality infected with *V. anguillarum* as determined by autopsy.
Example: of the 58% mortality in the group receiving 100 µg 1969 vaccine, 95% of these fish were infected with *V. anguillarum*.

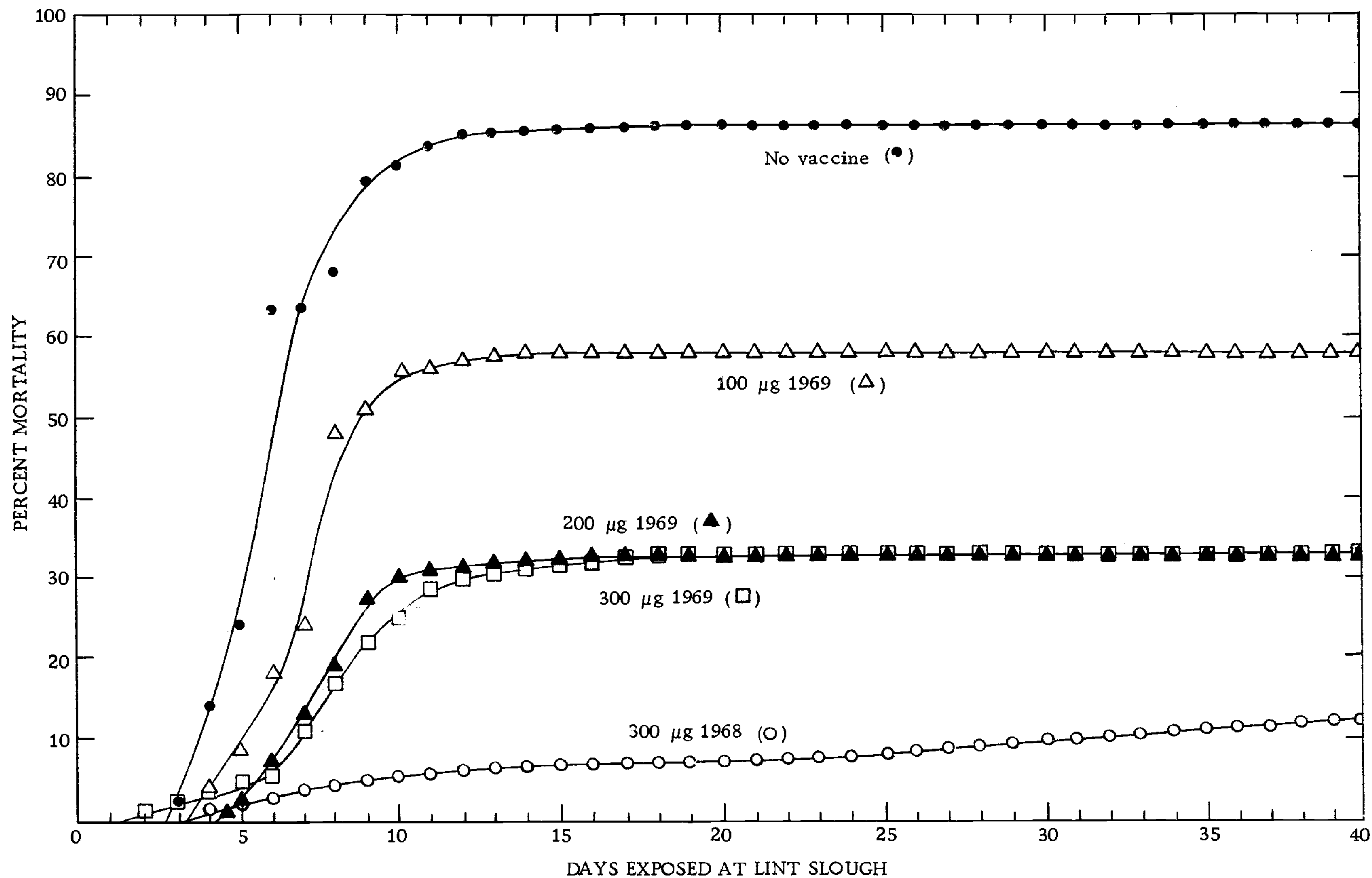

Figure 2. The effect of dosages of oral vaccines on percent mortality for the control of vibriosis in paired groups of 100 spring chinook salmon fed no vaccine, 100, 200 and 300 μg 1969 vaccine and 300 μg 1968 vaccine fed for 14 days.

Paired groups of 100 spring chinook salmon were fed 200, 300, 500, 700 and 1,000 μg 1969 vaccine per fish over 14 days. This experiment suggested that increasing the amount of vaccine received by each fish did not result in further reduction in the number of mortalities caused by *V. anguillarum*. The averaged results from the paired groups are shown in Figure 3. Table VII presents the average cumulative percent mortality and the percent of *V. anguillarum* infected fish in each group.

TABLE VII. CUMULATIVE PERCENT MORTALITY OF IMMUNIZED AND CONTROL GROUPS OF SALMON RECEIVING 200, 300, 500, 700 and 1,000 μg 1969 VACCINE PER FISH OVER A 14-DAY PERIOD.

	Cumulative Percent Mortality					
Day	200 μg	300 μg	500 μg	700 μg	1,000 μg	Control
10	0	0	0	0	0	0
12	0	0	0	0	1	2
14	4	4	1	2	1	19
16	6	7	4	3	2	43
18	13	12	7	6	4	60
20	15	14	11	9	5	70
30	24	18	18	14	14	76
40	24	18	18	14	14	76
% Infected[1]	90	95	97	96	73	92

1. Percent of cumulative mortality infected with *V. anguillarum* as determined by autopsy.

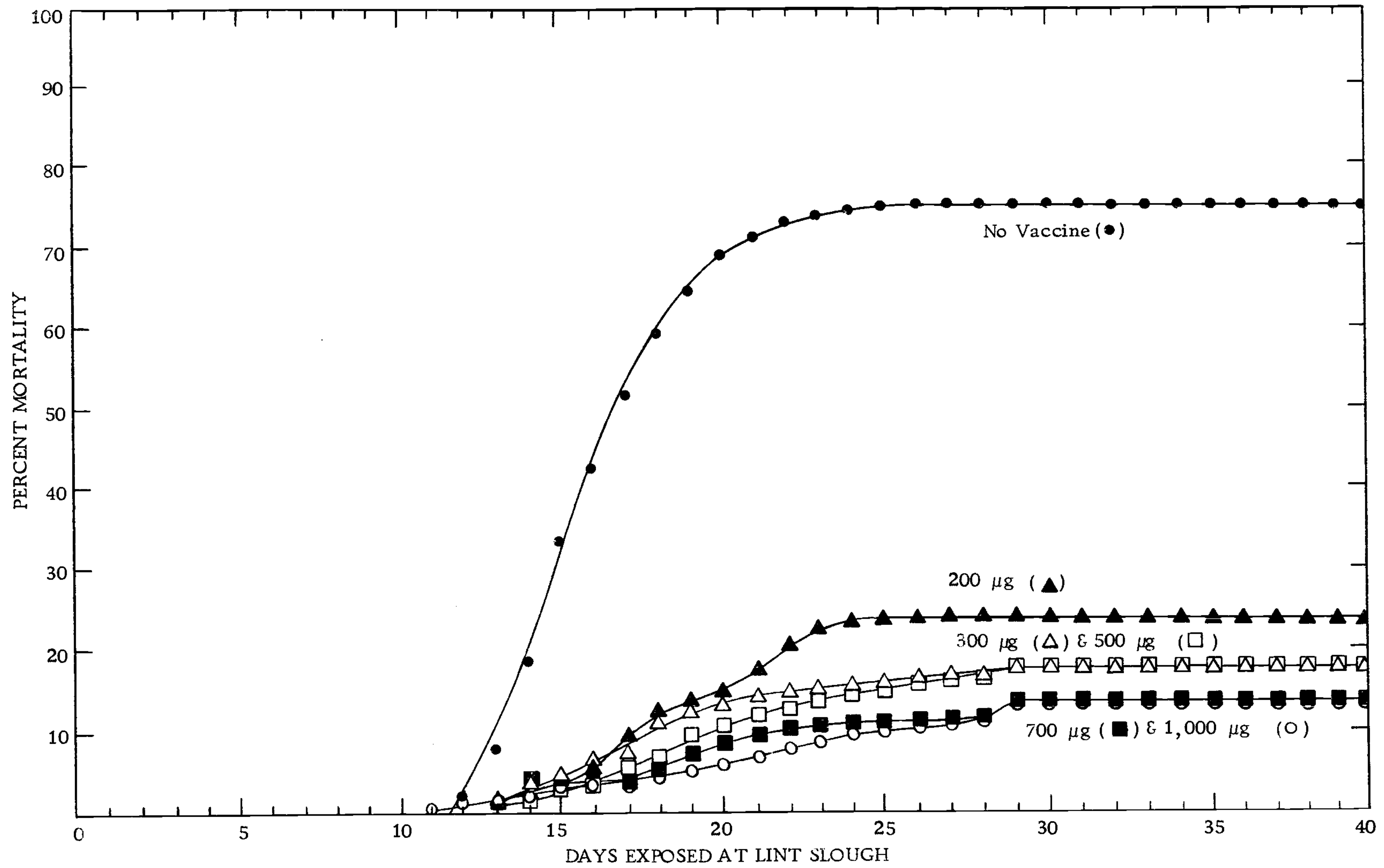

Figure 3. The effect of dosages of oral vaccines on percent mortality for the control of vibriosis in groups of 100 spring chinook salmon fed no vaccine, 200, 300, 500, 700 and 1,000 µg 1969 vaccine fed for 14 days.

Marine Booster

In this experiment, groups of spring chinook were fed 100, 200 and 300 μg 1969 vaccine and 300 μg 1968 vaccine before transfer to Lint Slough. After transfer, booster feedings equivalent to the amount received in fresh water were given to each group. The averaged results of two experiments as shown in Figure 4 demonstrated that oral boosters fed in salt water were not effective in reducing mortalities. Table VIII provides the averaged cumulative mortality and the percent *V. anguillarum* infected fish in each group.

TABLE VIII. CUMULATIVE PERCENT MORTALITY OF IMMUNIZED AND CONTROL SALMON RECEIVING 100, 200 AND 300 μg 1969 VACCINE AND 300 μg 1968 VACCINE PER FISH OVER 14 DAYS WITH AN EQUIVALENT BOOSTER FED AT LINT SLOUGH.

Day	Cumulative Percent Mortality				
	100 μg 1969 plus 100 μg Booster	200 μg 1969 plus 200 μg Booster	300 μg 1969 plus 300 μg Booster	300 μg 1968 plus 300 μg Booster	Control
2	0	0	0	0	0
4	4	2	1	1	11
6	12	5	5	3	52
8	34	13	13	5	87
10	48	29	23	8	92
20	54	31	32	12	94
30	55	32	32	14	95
40	55	34	33	15	95
% Infected[1]	96	91	91	83	95

1. Percent cumulative mortality infected with *V. anguillarum* as determined by autopsy.

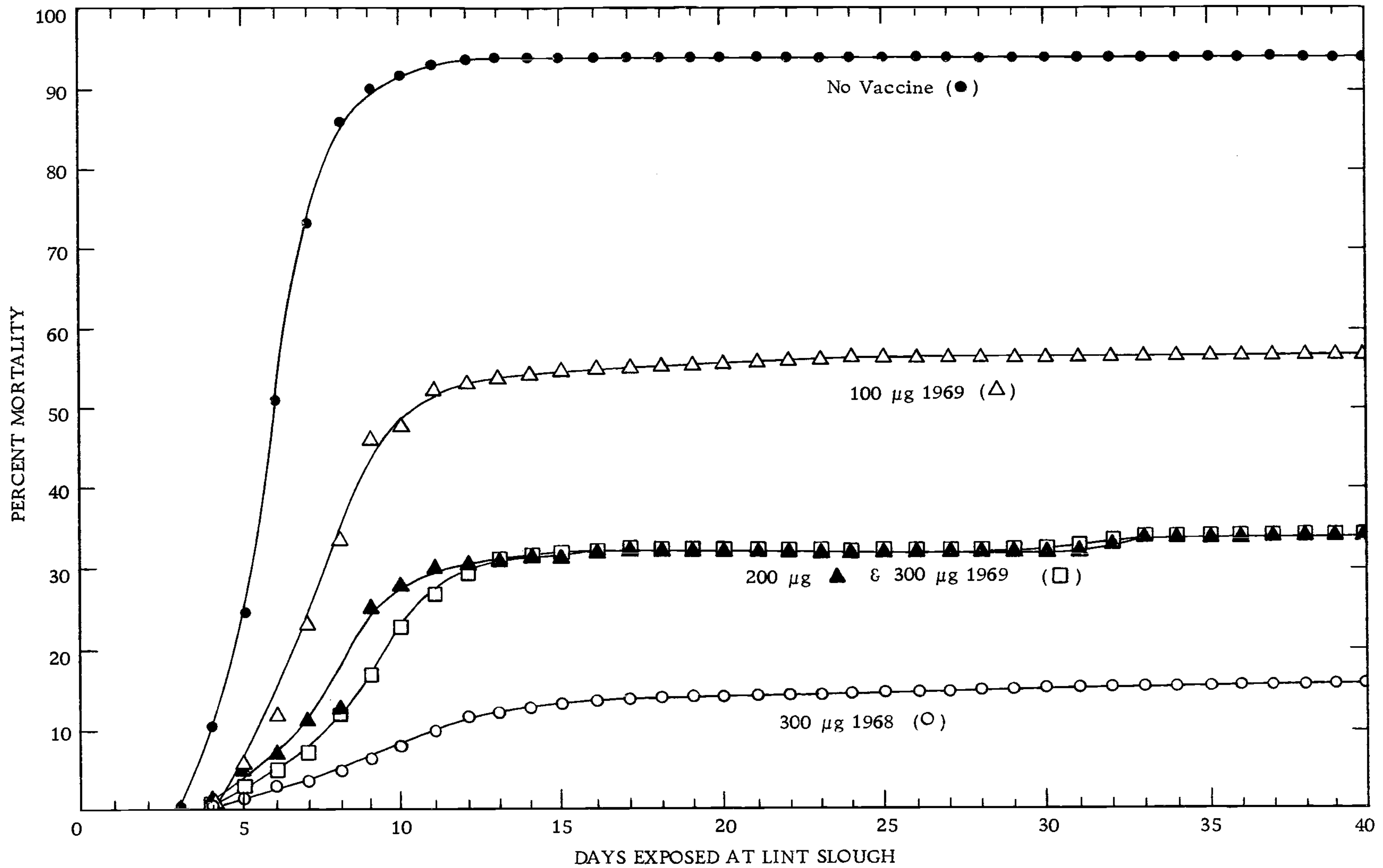

Figure 4. The effect of oral vaccine boosters in spring chinook salmon fed no vaccine, 100, 200, and 300 μg 1969 vaccine and 300 μg 1968 vaccine for 14 days before transfer to Lint Slough and an equivalent booster after transfer for 30 days.

Fresh Water Booster

In this experiment the oral booster was fed in fresh water before transfer to Lint Slough. The averaged results as shown in Figure 5 demonstrate that an oral booster fed in fresh water did not have an appreciable effect on the incidence of losses. Table IX presents the average cumulative mortality and the percent *V. anguillarum* infected fish in two paired experimental groups.

TABLE IX. CUMULATIVE PERCENT MORTALITY OF IMMUNIZED AND CONTROL SALMON RECEIVING 100, 200, AND 300 µg 1969 VACCINE PER FISH OVER 14 DAYS PLUS AN EQUIVALENT BOOSTER FED IN FRESH WATER FOR 30 DAYS.

Day	Cumulative Percent Mortality				
	100 µg 1969 plus 100 µg Booster	200 µg 1969 plus 200 µg Booster	300 µg 1969 plus 300 µg Booster	300 µg 1968 plus 300 µg Booster	Control
2	0	0	0	0	0
4	0	0	0	0	0
6	4	1	0	0	9
8	13	6	2	2	34
10	19	10	3	3	50
20	29	26	8	12	65
30	30	26	10	12	65
40	30	26	10	12	65
% Infected[1]	92	94	95	76	90

1. Percent of cumulative mortality infected with *V. anguillarum* as determined by autopsy.

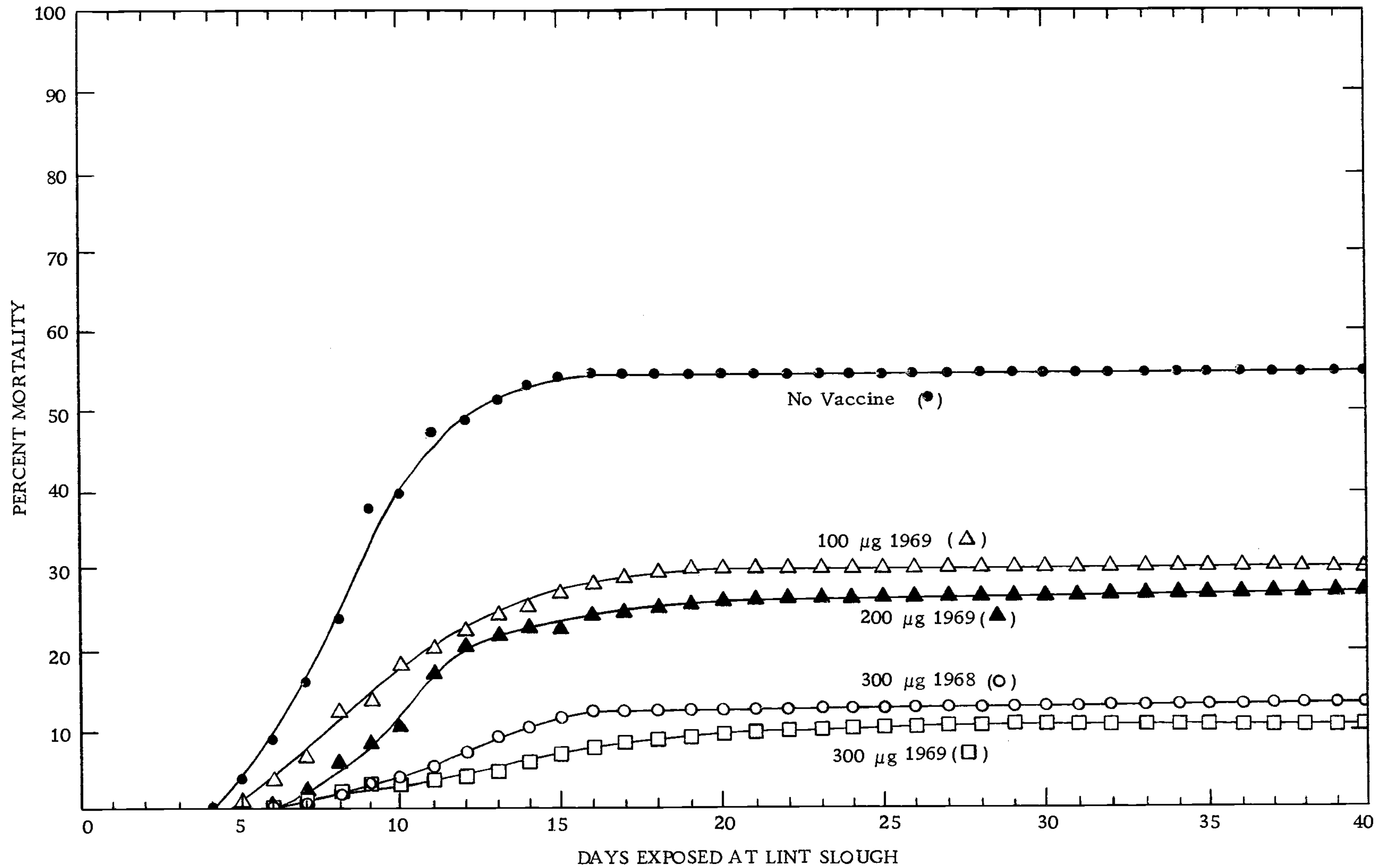

Figure 5. The effect of oral vaccine boosters in spring chinook salmon fed no vaccine, 100, 200, and 300 μg 1969 vaccine and 300 μg 1968 vaccine for 14 days and an additional equivalent booster fed in fresh water for 30 days.

Length of Vaccination and Specificity of Vaccine Experiments

The effect of feeding vaccine over a greater period was investigated in these experiments. The vaccine was administered over a 28-day period, or twice the interval used in the preceding studies. The results of two experiments were averaged, indicating approximately 85% of the control fish became infected with *V. anguillarum*; in contrast, less than three percent of the vaccinated group became infected (Figure 6). An additional group included in this experiment received 1,000 µg of *A. salmonicida* vaccine over the 28-day period. The averaged results from paired groups shown in Figure 6 imply that a nonspecific type of immunity against *V. anguillarum* was not elicited by feeding a vaccine composed of *A. salmonicida* cells. Table X provides the averaged cumulative mortality in each group and the percent of *V. anguillarum* infected fish.

TABLE X. CUMULATIVE PERCENT MORTALITY OF IMMUNIZED AND CONTROL SALMON RECEIVING 1,000 µg 1968 VACCINE AND 1,000 µg *A. SALMONICIDA* VACCINE FOR 28 DAYS.

Day	Cumulative Percent Mortality		
	1968 Vaccine	Control	*A. salmonicida* Vaccine
2	0	2	2
4	0	2	2
6	0	15	12
8	0	42	26
10	1	84	41
20	1	91	81
30	1	91	81
40	3	91	81
% Infected	40	90	86

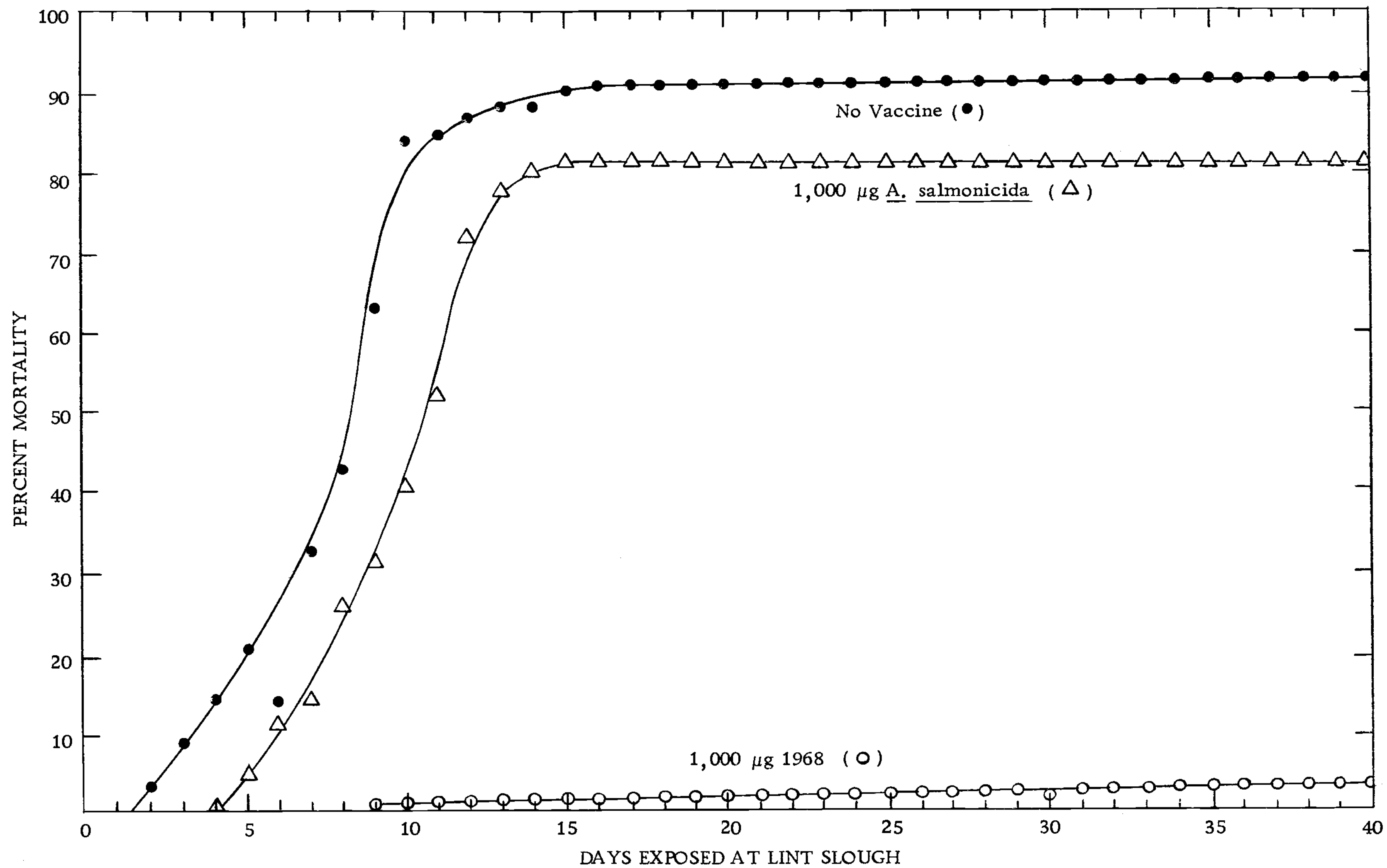

Figure 6. The effect of oral vaccination on percent mortality in groups of Coho salmon fed 1,000 μg 1968 vaccine and *A. salmonicida* vaccine for 28 days compared to Coho salmon fed no vaccine.

Determination of Agglutinating Antibody in Serum from Orally Vaccinated Salmon

In each experiment, serum samples were collected and analyzed for agglutinating antibody, using either the microtiter or tube agglutination technique. An additional serum sample was also collected 70 days after the fish in the preliminary experiment were exposed at Lint Slough. Using these methods, no agglutinating antibody was detected in fish from any of the experimental groups.

DISCUSSION

Vibriosis at Lint Slough is the major obstacle to overcome before the natural forage in this environment can be used for rearing salmon. The objective of this study was to develop a method of controlling this disease. An oral vaccine was developed which was easy to administer and could be fed before the salmon were transferred to Lint Slough. To determine if oral vaccination supplied an acceptable protective level, a series of experiments were performed.

A preliminary oral vaccination indicated that considerable reduction in mortality (70%) was attained when individual fish were fed 300 μg of 1968 vaccine.

Two experiments were performed to determine if oral vaccines administered at various dosages had an effect on the level of mortality. To find the lower limit of protection, 300, 200, and 100 μg of 1969 vaccine were fed per fish. The results of this experiment indicated that fish fed a dosage level below 200 μg demonstrated an increased mortality. Since low dosages elevated mortalities, an experiment was performed to determine if raising the dosage level of vaccine in the diet would reduce mortalities. No appreciable difference was observed in the number of mortalities between groups of fish fed vaccine levels ranging from 200 μg to 1,000 μg. These results suggested that the dosage of vaccine per fish may not account for the

protection attained, since varying the dosage per fish also varies the vaccine concentration per gram of ration. For example, 0.21 mg and 0.24 mg of vaccine, respectively, were incorporated per gram of ration and used at the 300 μg (first experiment) and the 1,000 μg (second experiment) level. Therefore, the total μgs of vaccine fed per fish differed considerably but the mortality and the mg/g of diet were similar in each experiment. This suggested that the amount of vaccine per gram of diet may be a better criterion for determining the level of vaccine required.

Oral boosters were examined to determine if this method would reduce the numbers of mortalities caused by vibriosis. The results of these experiments imply that boosters fed in either fresh or sea water did not enhance the original immunization. Boosters fed at Lint Slough may not have increased the immunity due to the rapid onset of the disease which would precede any practical protection the booster could offer. However, the boosters fed in fresh water were not effective. The boosters in these experiments were fed over a 30-day period in 15 feedings which were administered for three days and alternated with three days of O.M.P. diet without vaccine. Therefore the booster feedings did not allow exposure to the antigen as frequently as the daily exposure in the vaccination period. Perhaps the vaccine has to be fed daily in order for a greater immunity to be elicited.

An experiment was performed in which the vaccination period was extended to 28 days or double the number of vaccine administrations which were given in previous experiments. The fish in this experiment were fed 1,000 μg of 1968 vaccine over a 28-day period (0.5 mg/g of diet). This method resulted in the lowest mortality (less than three percent) when compared to previous vaccinated groups. Results of this experiment corroborate a study by Raettig (1967) in which mice orally immunized with an increased number of portions of typhoid vaccine experienced a greater reduction in mortality as compared to mice receiving fewer portions of vaccine.

Perhaps these experiments suggest that the oral vaccine should be fed to salmon over as long a period as possible at a concentration which exceeds the amount of vaccine necessary for protection. This concept would allow all the fish in a population to become completely vaccinated. This method would probably require the vaccine to be fed from the time the fish were able to feed until the time of transfer to Lint Slough.

A lyophilized sonicate was used in this investigation for two purposes: The preparation was sonicated to expose the maximum number of antigens to the vaccinated animal; the vaccine was lyophilized for ease of weighing and storage. Sonication and lyophilization are time consuming and expensive. Further studies should be instituted to

determine if either of these procedures is necessary. If neither is necessary, a cell paste of V. anguillarum may be all that would be required.

Two experimental results tend to demonstrate the nature of vibriosis vaccine. When the A. salmonicida preparation was fed to salmon, the resultant mortality from vibriosis was almost as high as in the controls. This experiment seems to indicate that a degree of specificity is associated with the immunity elicited by vibriosis vaccine. Secondly, all of the vaccinated groups of salmon had no demonstrable circulating agglutinating antibody at serum dilutions as low as 1:2 after vaccination. The absence of circulating agglutinating antibody has been reported many times after animals have been orally immunized (Besredka, 1924; Fahey and Cooper, 1970; Ocklitz et al., 1967; Mockman et al., 1967, 1968). Speculatively, the immunity elicited against V. anguillarum may resemble the cellular type immunity elicited after oral immunization reported by Freter (1970) and Levanon, Raettig and Rossetini (1968).

Vibriosis vaccine could be practical in vaccinating large hatchery populations. However, the transition from experimental vaccination to field vaccination has been difficult (Klontz, 1967, 1968). Feeding procedures, aquaria facilities and other general environmental conditions are considerably different in a hatchery than in an

experimental laboratory. Therefore, before the vibriosis vaccine can be applied to field use, this transition will have to be examined.

SUMMARY AND CONCLUSIONS

1. An oral vaccine was found to be effective in controlling the salmon disease, vibriosis.
2. The vaccine was a preparation of a lyophilized sonicate of _V_. _anguillarum_, the causative agent of vibriosis.
3. The vaccine was incorporated into a semi-purified ration and fed to salmon in fresh water. The fish were transferred to Lint Slough marine rearing facility on the Oregon coast, which has a natural epizootic of vibriosis.
4. A 70% reduction in mortality was found with 97% in nonvaccinated spring chinook salmon to 27% in spring and fall chinook salmon fed vaccine at 300 μg per fish over a 14-day period.
5. Reducing the dosage of vaccine from 300 μg to 100 μg per spring chinook salmon doubled the mortality from about 30% to 60%. The nonvaccinated salmon had an 87% mortality.
6. Elevating the dosage of vaccine from 300 μg to 1,000 μg per spring chinook salmon did not reduce the mortality.
7. The amount of vaccine per gram of diet was correlated more closely to protection than the dosage per fish.

8. Oral boosters fed after oral vaccination had little effect in lowering the mortality when fed either in fresh water or salt water at Lint Slough.

9. Doubling the number of feeding days from 14 to 28 reduced mortalities to less than three percent in coho salmon fed vaccine at 1,000 μg per fish.

10. A lyophilized sonicate of *A. salmonicida* was fed at 1,000 μg per coho salmon. When the fish were exposed to the disease this vaccine did not protect them, indicating that vibriosis vaccine has some specificity against vibriosis.

11. No circulating agglutinating antibody was detected in the sera of orally vaccinated animals, which indicated a localized immunity.

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