AN ABSTRACT OF THE THESIS OF

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Title: ORAL IMMUNIZATION FOR POSSIBLE CONTROL OF FURUNCULOSIS IN FISH

Abstract approved [redacted]
Dr. J. L. Fryer

This project was initiated in an attempt to control furunculosis (Aeromonas salmonicida) in a production hatchery rearing coho salmon. An oral vaccine was prepared and administered to 520,000 juvenile coho salmon (Oncorhynchus kisutch) at the Siletz River Salmon Hatchery prior to the onset of a predictable naturally occurring epizootic of furunculosis. A group of 65,000 salmon served as a nonimmunized control.

The Fermacell Fermentor was used to grow the Aeromonas salmonicida cells. The cell yield was more than doubled with the addition of 175 grams of dextrose to the culture media accompanied by automatic control of the pH. The cells were subjected to ultrasonic disruption and the supernatant fluid was precipitated with alum. The resulting material was lyophilized and incorporated into the Oregon Moist Pellet diet at a concentration of 201.73 milligrams $\pm$ 3

milligrams per kilogram of pellets.

Immunization with vaccine-containing food was begun on March 26, 1967 with 14 consecutive days of initial vaccination followed by eight weekly boosters. It was calculated that each fish could receive approximately 360.7 micrograms of vaccine during the immunization period.

During the period of May 1 through July 12, 1967, all hatchery mortalities were collected and examined for the presence of Aeromonas salmonicida. Although the nonimmunized control group showed a higher total loss and a higher furunculosis loss than the immunized groups, the results fail to indicate any distinctive difference between these two lots. The lower mortalities observed in the immunized group may indicate that slight immunity had been induced by the vaccine. However, any low-level immunity which may have been produced was not sufficient to provide protection against infection by Aeromonas salmonicida.

The number of mortalities appeared to be closely associated with the hatchery water temperature. An increase in water temperature was accompanied by a corresponding increase in losses. A decline in losses appeared to be associated with a decrease in water temperature.

Of the fish examined, 146 animals showed the presence of an acid-fast organism tentatively identified as Mycobacterium

fortuitum (Cruz).

Agglutinating antibody titers on serum samples from the immunized and nonimmunized groups of fish failed to indicate any meaningful difference between these groups.

Oral Immunization for Possible Control of Furunculosis in Fish

by

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INTRODUCTION

Of the bacterial diseases of fish, probably the most important, economically, is furunculosis of the Salmonidae. Furunculosis and its control have been reviewed in detail by McCraw (1952) and Snieszko (1954).

The etiologic agent of furunculosis is the bacterium Aeromonas salmonicida (Lehmann and Neumann), a short, Gram-negative, non-motile rod. The occurrence of this disease often causes large losses in hatchery-reared fish. Because of its importance to fish culture, control of furunculosis in production hatcheries is of particular interest.

Sulfonamides and antibiotics have been effectively employed in hatcheries to help control the disease, however drug resistant strains of A. salmonicida are becoming more prevalent. The problem of drug resistance along with the high cost of treating hatchery populations of fish with drugs have prompted research on other methods of controlling the disease. Since the demonstration of immunological responses in various poikilothermic vertebrates, emphasis has shifted from administration of drugs to the development of immunization procedures for use in furunculosis control.

Two immunization procedures are possible with fish. The first procedure involves parenteral injection of the antigen and the second consists of oral administration of an antigen preparation.

Parenteral injection, on a hatchery production basis, is not at all practical. The injection of each individual fish in a hatchery population would require a great deal of manpower and excessive handling of fish.

Because of the disadvantages of parenteral administration of the antigen, a study of the effectiveness of oral immunization in a complete hatchery production population totalling 585,000 juvenile coho salmon, Oncorhynchus kisutch (Walbaum), was undertaken. For this purpose, the Siletz River Salmon Hatchery located on Rock Creek near Siletz, Oregon was selected as the experimental site. This hatchery was considered ideal inasmuch as the fish experience a naturally occurring predictable epizootic of furunculosis each year thus eliminating the necessity of artificial challenge of the test animals with A. salmonicida.

This study follows a pilot experiment which clearly demonstrated definite protection against A. salmonicida using oral immunization (Overholser, Fryer and Klontz, 1966). The vaccines used in the pilot experiment and in the present study were prepared in accord with the method developed by G.W. Klontz at the Western Fish Disease Laboratory, U.S. Fish and Wildlife Service, Seattle, Washington.

LITERATURE REVIEW

Since the discovery of bacteria as disease agents, almost all of the research on such diseases has been concerned with human or warm-blooded animal pathogens. In recent years, research on bacterial diseases of fish has increased considerably. Many of the principles involved in the disease processes of poikilothermic vertebrates are similar to those of warm-blooded animals.

Oral immunization of fish against various bacterial diseases has been of particular interest in view of the increasing numbers of drug-resistant strains of disease-causing bacteria. Aeromonas salmonicida, the causative agent of furunculosis in fish, is no exception in this regard. A review of past work in the areas of drug resistance and immunity in A. salmonicida infections will aid in the understanding of the present research.

Drug Therapy and Drug Resistance in Aeromonas salmonicida Infections

The therapy of bacterial diseases of poikilothermic vertebrates is essentially the same as in any other warm-blooded animal. In drug therapy of fish, large populations are treated collectively. Higher animals, however, are often treated on an individual basis.

Sulfonamide therapy of furunculosis was introduced by Gutsell (1946, 1948). Initially, treatment of hatchery populations with

sulfonamides was very effective in decreasing losses due to furunculosis (Wolf, 1947; Gutsell, 1948; Snieszko, Gutsell and Friddle, 1950; Snieszko, Griffin and Friddle, 1952). More recently, however, the prolonged treatment and indiscriminate use of drugs have played a major role in the increasing appearance of highly resistant mutant strains of A. salmonicida (Snieszko and Bullock, 1957a, b).

In cases where sulfonamide resistance represented the major obstacle in treatment of furunculosis, antibiotics such as chloramphenicol (chloromycetin) and oxytetracycline (terramycin) were effectively used to control the disease (Snieszko and Bullock, 1957a). These authors recommended treatment with sulfamerazine, sulfamethazine or a mixture of sulfamerazine and sulfagaunidine followed by a three-day treatment with either chloramphenicol or oxytetracycline. The final three-day treatment cleared the population of drug-resistant pathogens which may have developed during the initial administration of sulfonamides.

The occurrence of oxytetracycline-resistant strains of A. salmonicida (Snieszko and Bullock, 1957b) have further complicated the use of sulfonamide and antibiotic therapy in furunculosis control. Because of the increasing drug resistance problem, recent research interests have been extended into the area of oral immunization against A. salmonicida.

Antibody Response in Poikilothermic Vertebrates

Today it is generally recognized that immunity or some immunological response occurs in almost all classes of animals. However, the immune response in lower vertebrates is not always the same as that observed in warm-blooded animals.

Bisset (1947a) cites that the early work of Metschnikoff (1884, 1887), Mesnil (1895) and others placed a great deal of emphasis upon the role of phagocytosis for protection against bacterial diseases in lower vertebrates. The ability of cold-blooded vertebrates to produce humoral antibodies was thought to be lacking. However, subsequent investigations provide evidence of antibody production in poikilothermic vertebrates which is similar to the antibody mechanism of higher animals (Bisset, 1947a).

Demonstrable antibodies have been produced in fish, reptiles, and amphibians by inoculation of these animals with bacterial antigens (Kulp and Borden, 1942; Smith, 1940; Bisset, 1946, 1947a, b, c, 1948; Evans and Cowles, 1959; Elek, Rees and Gowing, 1962; Evans, 1963a, b; Maung, 1963; Evans et al., 1965) and foreign proteins (Allen and McDaniel, 1937; Cushing, 1942; Dreyer and King, 1948; Clem and Sigel, 1965). Sigel and Clem (1965) used viral antigens in studying the immune response in marine fishes.

The Effect of Temperature on Antibody Production

A number of factors are involved in successful attempts to demonstrate an immune response. Probably the most important factor affecting the immune response in poikilothermic vertebrates is the environmental temperature. Evidence supports the assumption that a high degree of potential immunological competence exists in cold-blooded vertebrates, but antibody production seems to be directly related to the temperature.

Allen and McDaniel (1937) found that frogs developed hemolysins against human red blood cells at 22-27°C but not at refrigerator temperature (8-10°C). Smith (1940) showed that carp (Cyprinus carpio), rainbow trout (Salmo gairdnerii) and brown trout (Salmo trutta) formed antibodies against the bacterium A. salmonicida when injected intraperitoneally at 10°C. Additional studies by Cushing (1942) demonstrated that carp and gold fish produce agglutinins against sea urchin sperm more rapidly at 28°C than at 15°C. This work suggests that low temperatures affect the rate of antibody formation rather than completely inhibiting its production. Bisset (1946) noticed that fish were able to clear bacterial infections better as the water temperature was increased, while at low temperatures even non-pathogenic organisms were eliminated with difficulty. In later investigations, Bisset (1947b, c) found that production of circulating

antibodies in frogs (Rana temporaria) against Pseudomonas fluorescens was rapid at 20°C but completely inhibited at 8°C. Similarly, an animal immunized at a high temperature did not exhibit immunity if a low temperature was maintained. This suggested that two mechanisms are involved in the immunization process: (1) acquisition of the potential for antibody production and (2) actual production of circulating antibody (Bisset, 1948).

Using toads (Xanopus laevis), Elek, Rees and Gowing (1962) found antibody production evident at room temperature or above but not at 8°C. The toads were found to show demonstrable circulating antibodies within 60 hours after transfer to 27°C. This work indicates that antibodies were formed in the cold but were not released until the animals were placed at the higher temperature. These investigators also demonstrated that release of antibody could be brought about in the cold by injection of ACTH or cortisone. Similar results were obtained using the reptile Dipsosaurus dorsalis (Evans and Cowles, 1959; Evans, 1963b). It was shown by Bisset (1949) that the release of antibody in fish and frogs, which is inhibited at low temperatures, can be restored by injection of adrenal cortical extracts. The inhibition of antibody release at low temperatures is presumed to be due to inhibition of adrenal cortical hormone production at these temperatures.

Antibody Response in Poikilothermic Vertebrates to Aeromonas salmonicida

Smith (1940) demonstrated an increase in agglutinin production at 10°C for A. salmonicida when carp, rainbow trout and brown trout were inoculated intraperitoneally, over a period of seven weeks, with heat-killed cells at a concentration of 5×10^6 bacteria per ml. Each fish received one ml per week. Additional studies (Gee and Smith, 1941) included the use of painted turtles (Chrysemys eleganz) and a snapping turtle (Chelydra tricarinata). In all cases, agglutinins for A. salmonicida were demonstrated within six months, one painted turtle showing a titer of 1:2,560 during the seventh month.

Studies on the immune response of brown trout (Salmo trutta) and brook trout (Salvelinus fontinalis) to the injection of formalin-killed cells of A. salmonicida were performed by Krantz, Reddecliff and Heist (1964a). The results indicate that one injection of formalin-killed bacteria in adjuvant produced high lasting agglutinin titers and prophylactic immunity while the trout which received formalin-killed cells without adjuvant failed to develop protective immunity.

Passive immunity against A. salmonicida was demonstrated in juvenile coho salmon by injection of immune serum from adult rainbow trout (Spence, Fryer and Pilcher, 1965). The same studies failed to demonstrate agglutinating antibody or protective immunity by using an oral vaccine consisting of formalin-killed cells mixed in

the Oregon Moist Pellet diet at a concentration of 5×10^{10} cells per gram of food.

Oral Immunization Against *Aeromonas salmonicida*

Oral administration of vaccines has been successful in affording protection against disease. Using the same principles, investigators have attempted to develop an effective oral vaccine against furunculosis. Duff (1942) used chloroform-killed cells which were incorporated into the food of cutthroat trout (*Salmo clarki*). Forty daily doses of the vaccine failed to stimulate measurable immunity, but 64 or more feedings produced encouraging results. Some of the immunized trout failed to exhibit a detectable agglutinin titer, whereas some non-immunized trout showed the presence of agglutinins. Upon challenge with the bacterium, approximately 75% of the untreated fish and 25% of the immunized fish died. Oral administration of viable *A. salmonicida* cells also failed to elicit sufficient antibody titers to afford protection.

Snieszko and Friddle (1949) prepared an oral vaccine containing 0.5 ml of packed heat-killed *A. salmonicida* cells in one kilogram of food. It was found that this method did not provide protection to brook trout (*Salvelinus fontinalis*) when challenged by large doses of virulent bacteria administered both orally and by injection. Post (1963) used heat-killed cells of *Aeromonas hydrophila* in the diet of rainbow trout

for 272 days. The trout were challenged with intraperitoneal inoculations on four different occasions. There was 25-28% survival in the immunized groups and 12.5% survival in the nonimmunized control group. In contrast, Ross and Klontz (1965) used orally administered phenol-killed cells of the etiologic agent of "redmouth disease" and found that 90% of the immunized fish and 20% of the nonimmunized control fish survived a challenge dose of virulent organisms.

Krantz, Reddecliff and Heist (1964b) used the method of Duff and were not successful in demonstrating agglutinating antibody in trout with an oral vaccine of chloroform-killed A. salmonicida cells. Oral administration of whole viable cells also failed to produce agglutinating antibody. Formalin-killed cells incorporated into the diet of coho salmon also failed to produce agglutinating antibody (Spence, Fryer and Pilcher, 1965).

Overholser, Fryer and Klontz (1966) demonstrated definite protection against A. salmonicida in a group of 600 juvenile coho salmon at the Siletz River Salmon Hatchery. The vaccine used in this work was an alum-precipitated cell sonicate preparation which was incorporated into the regular fish diet of Oregon Moist Pellets. Mortalities attributable to furunculosis in the immunized lots of fish ranged from 0% to 0.7%, and the nonimmunized fish showed a 22% loss from furunculosis. These results provided the groundwork upon which the present production study was formulated and carried out.

EXPERIMENTAL MATERIALS AND METHODS

Culture Media

Trypticase Soy Broth (Baltimore Biological Laboratories, Inc): The Trypticase Soy Broth was rehydrated by suspending 30 g of the material in one liter of distilled water. Sterilization was done in the Fermacell Fermentor for 20 minutes at 120°C.

Furunculosis Agar (Difco Laboratories): The agar was prepared by reconstituing 33.5 g of the dry powder with one liter of distilled water. The preparation was autoclaved for 15 minutes at 15 pounds pressure at 121°C.

Solutions and Reagents

One percent para-phenylenediamine Solution (Eastman Organic Chemicals, Rochester, New York): A 1% solution was prepared by dissolving one g of the crystal in 100 ml of distilled water. The solution was stored in the dark under refrigeration and was made fresh each month.

Silicone Antifoam Y-4988 (Union Carbide Corporation, New York, New York): Two ml of the antifoam solution was added to each batch of Trypticase Soy Broth in the Fermacell Fermentor prior to the sterilization cycle. This solution was added to prevent excessive foaming of the media caused by agitation and aeration.

Dextrose Solutions: Concentrated dextrose solutions were prepared in distilled water. The solutions were filter-sterilized using a Millipore filter system prior to introduction into the Fermacell Fermentor.

Cultures Used

Aeromonas salmonicida Cultures: The culture of A. salmonicida used for preparation of the vaccine was the most recent isolate from the 1966 furunculosis epizootic at the Siletz River Salmon Hatchery and was maintained on Furunculosis Agar or in Trypticase Soy Broth.

Cultures used in preparation of the antigen solution for serum antibody titers were obtained from fresh isolates during the present epizootic (1967) at the Siletz River Salmon Hatchery.

Culture of Aeromonas salmonicida Cells

All A. salmonicida cells needed for the vaccine preparation were cultured in Trypticase Soy Broth (TSB) in the Fermacell Fermentor, Model CF-50 (New Brunswick Scientific Co., Inc.) in four lots of approximately 40 liters each. Two ml of silicone antifoam Y-4988, Lot No. 817033066 (Union Carbide Corp.) were added to each lot of TSB in the fermentor vessel and sterilized for 20 minutes at 120°C with continuous agitation. After cooling to 23°C, the broth was

inoculated with one liter of an 18 to 24 hour culture of A. salmonicida in TSB and incubated at 23°C ± 1°C. The vessel contents were aerated at a level of 1.75 cubic feet per minute and mixing was carried out at an impeller speed of 200 rpm. The automatic pH control was preset at pH 7.0 and pH adjustment of the culture was made using 1 N HCl and 1 N NaOH throughout most of the fermentation process.

During the production of the third lot, 525 ml of a sterile solution containing 175 g of dextrose was added to the 40 liter culture. The addition of dextrose replaced that utilized during the fermentation process.

At intervals during the growth cycle, samples were aseptically withdrawn from the bottom valve of the culture vessel. Sample purity was determined microscopically by phase contrast observation of a wet mount for cell motility and examination of a Gram-stained smear for cell morphology and typical Gram reaction. In addition, the samples were inoculated on Furunculosis Agar plates and allowed to incubate at 25°C for 48 hours. Resulting growth was examined for motility and the colonies were subjected to testing with a 1% solution of para-phenylenediamine. The plates were also examined for the appearance of the characteristic soluble brown pigment in the medium. The lack of motility, presence of the soluble brown pigment and a purple-black reaction when para-phenylenediamine is applied to the colony confirms the presence of A. salmonicida (Griffin, 1951a,

b; Griffin, Snieszko and Friddle, 1953). All samples proved to be A. salmonicida in pure culture.

After 40 to 48 hours, the contents of the fermentor vessel were collected through the bottom valve and the cells harvested using the Sharples Super Centrifuge, Type T 1P. A total of 152 liters of culture was grown in the manner described above.

Preparation of Aeromonas salmonicida Vaccine

The method used for production of the A. salmonicida vaccine was developed by G.W. Klontz at the Western Fish Disease Laboratory, U.S. Fish and Wildlife Service, Seattle, Washington. Two modifications of the method were instituted by this laboratory: (1) in order to produce the required weight of A. salmonicida cells, the Fermacell Fermentor was utilized for cell culture and (2) instead of precipitating the cell sonicate to a final pH of 7.0 with NaOH, all precipitation was carried out to a final pH of 5.5. It was found that maximum precipitation had taken place at pH 5.5; at pH 7.0 a portion of the precipitate had redissolved.

The packed A. salmonicida cells were resuspended in five volumes, by weight, of 0.86% NaCl solution using a magnetic stirring bar to insure complete suspension. Small portions (100 to 150 ml) of the resulting cell suspension were subjected to ultrasonic disruption for approximately 45 minutes using the "Sonifier", Model LS75

(Bronson Instruments, Inc.). This procedure was carried out in an ice bath to prevent excessive heating of the cell suspension. The sonicate was periodically examined to determine the degree of cell disruption. It was found that treatment of 100 to 150 ml of cell suspension for 40 to 45 minutes gave approximately 90% cell breakage. The sonicated material was pooled and centrifuged at 15,000 rpm for 60 minutes in the Servall Superspeed Centrifuge, Type SS3 (Ivan Sorvall, Inc.). The supernatant fluid was decanted and an equal volume of 10% aluminum potassium sulfate (alum) was added slowly with constant stirring. Using a Corning pH meter, Model 7, the solution was adjusted to pH 5.5 using 5 N NaOH. The suspension was then centrifuged at 2,500 to 3,000 rpm and the supernatant fluid examined for further precipitation by the addition of one drop of 5 N NaOH. The precipitate was washed three times with distilled water to remove all traces of NaOH. After the final washing, the material was lyophilized, weighed and stored at -10°C in sealed containers until incorporation into the fish diet.

Preparation of Oregon Moist Pellet Diet Containing Vaccine

The lyophilized vaccine was incorporated into Oregon Moist Pellets (OMP) at Bioproducts, Inc., Warrenton, Oregon on February 23, 1967.

The Oregon Moist Pellet is composed of a mixture of dry meals

and wet fish supplemented with oil, vitamins and an antioxidant (Tenox IV). The final product contains approximately 33% moisture. The composition of the OMP is given in Table 1.

Table 1. Composition of Oregon Moist Pellets.[1]

Ingredient	Percent
DRY MIX	
Cottonseed oil meal	22.00
Herring meal	22.00
Crab meal	4.00
Wheat germ meal	3.00
Distillers dried corn solubles	3.00
Kelp meal	2.00
Vitamin premix	1.50
Ascorbic acid	
Biotin	
B_{12}	
d alpha tocapheryl acetate	
d calcium pantothenate	
Folic acid	
Inositol	
Menadione sodium bisulfate	
Niacin	
p-aminobenzoic acid	
Pyridoxine hydrochloride	
Riboflavin	
Thiamin hydrochloride	
WET MIX	
Tuna viscera and salmon viscera	40.00
Corn oil	1.80
Choline chloride	0.65
Tenox IV	0.05
Total	100.000

[1] Figures obtained from Bioproducts, Inc., Warrenton, Oregon.

A total of 189.5 g of the dry A. salmonicida vaccine was mixed with approximately 15 lb of dry meal in a Patterson-Kelley Mixer for

five minutes. The mixer was emptied and an additional two lb of meal added and mixed to adsorb any residual vaccine left on the interior walls. The vaccine-meal mixture was then slowly added to the remaining 1,131 lb of meal and allowed to mix for 30 minutes in a hopper. The wet ingredients were added to the meal-vaccine preparation and allowed to mix well to provide even distribution of the vaccine. The combined material was then passed through an extruder and the resulting pellets were sacked in 50 lb bags. Each bag was labeled, quick frozen and stored at -30°F. Three pellet sizes were prepared:

3/64 inch pellets--1025 lb

1/16 inch pellets-- 375 lb

3/32 inch pellets-- 651 lb

The calculated concentration of vaccine in the OMP was found to be 201.73 mg$\pm$3 mg per kg of OMP.

Immunization of Fish

The experimental population consisted of 65,000 juvenile coho salmon in each of nine concrete ponds at the Siletz River Salmon Hatchery. The salmon in eight ponds (Pond No. 1 through Pond No. 8) received the Oregon Moist Pellet containing the alum-precipitated vaccine at a concentration of 201.73 mg$\pm$3 mg of vaccine per kg of food according to the following schedule:

1. Fourteen consecutive days of feeding beginning on March 26 and continuing through April 8, 1967.
2. Booster feedings of vaccine food on April 17, 24, May 1, 7, 11, 15, 22 and 29, 1967.

The feeding periods and amounts of vaccine food received by the fish in each of the eight ponds during the experimental period are presented in Table 2.

Table 2. Feeding schedule and amount of vaccine food received during immunization period in Pond No. 1 through Pond No. 8.[1]

Date of feeding	Pounds of food fed per day	Pounds of food per pond per day	Pellet size	mg. vaccine received per pond	µg. vaccine per fish[2]
March 26-April 1	64	8	3/64	5,128.5	78.9
April 2-April 8	72	9	3/64	5,769.5	88.8
April 17	88	11	3/64	1,007.4	15.5
April 24	104	13	1/16	1,190.5	18.3
May 1	120	15	1/16	1,373.7	21.1
May 7	136	17	1/16	1,556.9	23.9
May 11	160	20	3/32	1,831.6	28.2
May 15	144	18	3/32	1,648.4	25.4
May 22	160	20	3/32	1,831.6	28.2
May 29	184	23	3/32	2,106.3	32.4
TOTAL	2,048	256		23,444.4	360.7

[1] Pond #9 did not receive any vaccine food.

[2] Calculated on the basis of 65,000 fish per pond.

During periods when vaccine food was not being fed, all fish received the OMP containing 0.7 g of sulfamethazine per pound to

control "cold-water disease" caused by the bacterium Cytophaga psychrophila. This medicated food was fed only during the period of February 11, 1967 to May 5, 1967 after which non-medicated OMP were fed exclusively, except on days when vaccine boosters were administered. The day prior to the booster dose, all feeding was suspended in order to insure complete consumption of the booster on the following day.

The fish in Pond No. 9 served as a nonimmunized control group and did not receive vaccine-containing food. These fish did receive food containing sulfamethazine during the same period as indicated for fish in Pond No. 1 through Pond No. 8. As with the nonimmunized group, control group feeding was suspended on the days prior to booster administration.

On June 29, each of the eight experimental groups was divided, half of the fish being placed in a rearing lake adjoining the hatchery and half remaining in their respective ponds. The control group (Pond No. 9) was divided and half of the fish were placed in Pond No. 10 which had remained empty until this time. Those fish remaining in the first nine concrete ponds continued to function as experimental and control groups for the duration of the experiment. The population division was necessary to prevent crowding as a result of increased fish size.

Examination of Mortalities

Collection of Mortalities

Beginning on May 1, 1967 all mortalities in the nine concrete ponds were collected, enumerated and placed in plastic bags with proper identification. The dead fish were collected twice daily and immediately placed in the freezer. Specimens from each pond were kept separate on a daily basis. Prior to examination, the fish were allowed to thaw at room temperature.

Microbiological Examination of Mortalities

Each fish examined was dipped into a mild tincture of iodine solution and wiped clean with 95% ethyl alcohol. Using a sterile Bard-Parker blade, an incision was made through the body wall slightly dorsal and parallel to the lateral line. In this manner, the kidney was exposed for easy access. A separate sterile blade was used for each individual animal to prevent cross-contamination.

Using a heavy inoculum from the kidney tissue, cultures were made from each fish on Furunculosis Agar plates. Six inoculations were made on each agar plate and incubated at room temperature (approximately 25^{o}C).

After 48 to 72 hours of incubation, all cultures were examined for the presence of typical A. salmonicida colonies. Suspicious

colonies were tested with a 1% solution of para-phenylenediamine and observed for typical brown pigment production in the medium (Griffin, 1951a, b; Griffin, Snieszko and Friddle, 1953). Cell motility was examined using phase contrast microscopy.

All cultures were held for one week and re-examined for colonies of A. salmonicida before discarding.

Tube Agglutination Tests

Collection of Blood Samples

Ten blood samples were collected from experimental and control fish at the Siletz River Salmon Hatchery on July 20, 1967. Each sample contained a pooled specimen of blood obtained from ten fish from each of the nine concrete ponds. In addition, a pooled blood sample was collected from ten juvenile coho salmon at the Alsea River Salmon Hatchery. This served as a normal control specimen since fish at this station were reported to be free of furunculosis.

Using a Bard-Parker blade, the tail of each fish was severed at the caudal peduncle and blood allowed to drip freely into a centrifuge tube. Each specimen was kept cool in an ice bucket during the collection period and was stored overnight at 5°C for complete clot retraction. The serum was recovered and stored at 5°C until used.

Preparation of A. salmonicida antigen

A recent isolate (July 1967) was subcultured on Furunculosis Agar and the resulting growth washed off of the agar surface with cold 0.85% saline. The cell suspension was washed three times in saline to remove all traces of media. After the third washing, the cells were diluted with 0.85% saline to a turbidity approximating that of a number nine McFarland nephelometer tube.

Tube Agglutination Tests

Antibody titers were performed in duplicate on 11 serum samples: one sample from fish in each of the nine concrete ponds at the Siletz Hatchery, a sample from the fish at the Alsea Hatchery and a positive specimen of rabbit anti-A. salmonicida serum which was prepared in this laboratory.

Eleven groups of ten tubes each were arranged in metal racks with appropriate labelling to insure identification. Nine-tenths ml of 0.85% saline was added to the first tube in each group and 0.5 ml of saline to the remaining tubes. One-tenth ml of the serum sample to be tested was added to the first tube and serial dilutions made through the tenth tube by transferring 0.5 ml from tube no. 1 to tube no. 2, 0.5 ml from tube no. 2 to tube no. 3 and continuing through tube no. 10 from which 0.5 ml was discarded. The resulting dilutions in the

ten tubes were 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1,280, 1:2,560, 1:5,120 and 1:10,240 respectively.

Five-tenths ml of the A. salmonicida antigen suspension was added to all tubes and mixed thoroughly. A saline control tube was prepared by adding 0.5 ml of the antigen suspension to 0.5 ml of 0.85% saline.

All tubes were incubated at room temperature for four hours after which they were placed at 5°C for overnight incubation. Agglutination of the antigen was read and graded according to the degree of agglutination. The serum antibody titer was recorded as the highest serum dilution showing definite agglutination.

RESULTS

Culture of A. salmonicida Cells and Vaccine Preparation

Four lots of A. salmonicida cells were cultured in the Fermacell Fermentor as described. The cells from each lot were harvested, weighed and the cell yield per liter of media was determined (Table 3). It was noted that the addition of 175 g of dextrose to the third lot greatly increased the cell yield.

Table 3. Yields of Aeromonas salmonicida cells from Fermacell Fermentor.

Lot No.	Lot volume (liters)	Total cell yield (grams)	Cell yield (grams/liter)
1	31	227.1	7.57
2	40	313.9	7.84
3	41	702.6	17.56
4	40	444.0	11.10

A total of 152 liters of culture was harvested resulting in a total yield of 1,645 g of packed wet cells. The highest cell yield was obtained with the third lot which was characterized by a dark brown pigment throughout the culture medium. Pigment production in the other three lots did not develop to such an extent, if at all.

A total of 245.5 g of the alum-precipitated vaccine was produced from 1,645 g of packed wet cells. Of this amount, 189.5 g were incorporated into the Oregon Moist Pellet diet for

use at the Siletz River Slamon Hatchery.

The materials used in the production of 245.5 g of vaccine are listed in Table 4. The price of each item and total cost of the materials are also presented. The cost of materials to produce one gram of vaccine amounted to approximately $0.38.

In addition to the materials listed in Table 4, approximately 250 man-hours were required to prepare the quantity of vaccine needed.

Table 4. Cost of production of Aeromonas salmonicida vaccine.

Materials	Unit Cost	Total Cost
1.5 lb. aluminum potassium sulfate J.T. Baker Chemical Company	$2.35/lb.	$ 3.52
12 lb. Trypticase Soy Broth Baltimore Biological Laboratories, Inc.	7.25/lb.	87.00
175 g dextrose J.T. Baker Chemical Company	1.35/lb.	0.70
0.5 lb. sodium hydroxide pellets J.T. Baker Chemical Company	1.67/lb.	0.84
75 g sodium chloride J.T. Baker Chemical Company	1.25/lb.	0.20
8 ml silicone antifoam solution Union Carbide Corporation	----	0.00[1]
	Total	$92.26

[1] The silicone solution used was an experimental sample provided by the Union Carbide Corporation. Similar antifoam agents are available from other chemical companies.

Immunization of Fish

The total calculated amount of vaccine received by each fish in Pond No. 1 through Pond No. 8 during the first 14-day vaccination period was 167.6 μg. The amount of vaccine received by the test animals in these eight ponds during the eight booster feedings was 193 μg per fish. The total amount of vaccine received per animal during the entire experimental period was 360.7 μg. All of the above figures are based on the following three assumptions: (1) the vaccine concentration was constant, (2) all of the food was consumed and (3) each fish consumed an equal quantity of vaccine-containing food.

Examination of Mortalities

The monitoring of each pond began on May 1, 1967 and continued through July 12, 1967. The data, as presented, has been grouped into ten seven-day periods beginning on May 4. The final examination date reported here was July 12, however enumeration of mortalities continued through July 31, 1967.

Mortalities were divided into two groups: those animals from which A. salmonicida was isolated and those which were not infected with the organism. Fish in which A. salmonicida was not found were considered negative for furunculosis. Together, these two groups constitute the total loss from all causes. The cause of death in the

furunculosis-negative animals was not determined.

The initial experimental population in the hatchery on May 4, 1967 was calculated to be approximately 585,000 animals. Each of the nine concrete ponds contained approximately 65,000 fish. On June 29, all of the pond populations were divided, thus reducing the experimental number to half that existing on June 28. For example, on June 28 Pond No. 1 contained 63,685 animals and on June 29 this figure was reduced to 31,842.

Total and Percent Losses During Monitoring Period

In order to assess the effectiveness of the immunizing antigen and procedure, it was desirable to compare the total and percent losses for each of the nine ponds. The total losses, the losses in which furunculosis was diagnosed and percent furunculosis in each individual pond for the entire monitoring period are presented in Table 5. The population in Pond No. 2 experienced the highest loss from furunculosis (729) and the lowest figure (382) was obtained from Pond No. 7. In Pond No. 9 (nonimmunized control), the diagnosed cases of furunculosis made up 27.8% of the total pond loss. This value indicates that, of the nine test ponds, the nonimmunized group experienced the highest percent loss from furunculosis during the experimental period. The next highest value (25.4%) was obtained for Pond No. 5. Low values were calculated for Pond No. 3 and Pond No. 8

which were 19.6% and 19.3% respectively.

Table 5. Losses in individual ponds during the period of May 4-July 12, 1967.

Pond No.	Total loss	Loss in which furunculosis diagnosed	Percent furunculosis in total loss
1	1,644	409	24.9
2	2,951	729	24.7
3	2,678	524	19.6
4	2,557	529	20.7
5	2,347	597	25.4
6	2,389	503	21.0
7	1,734	382	22.0
8	2,657	512	19.3
9	2,430	676	27.8

Losses in the immunized groups (Pond No. 1 through Pond No. 8) during each of the ten seven-day periods are tabulated in Table 6. The values for the nonimmunized control group (Pond No. 9) appear in Table 7. Due to the daily pond loss, the initial population for each seven-day period was constantly being reduced as the experiment progressed.

It was noted that the highest total loss and highest loss in which furunculosis was diagnosed occurred during the period of June 15 through June 21. During this period, the immunized groups experienced a loss of 4,683 fish of which 41.2% were diagnosed as having furunculosis. In contrast, the nonimmunized control group lost a total of 705 animals during this period of which 43.1% were found to be infected with A. salmonicida.

Table 6. Summary of losses in immunized groups (Pond No. 1-Pond No. 8) for each seven-day period.

Period	Initial population	Total loss (all causes)	Loss in which furunculosis diagnosed	Percent furunculosis in total loss
May 4-May 10	520,000	673	0	0
May 11-May 17	519,289	359	0	0
May 18-May 24	518,872	670	165	24.6
May 25-May 31	518,009	510	86	16.9
June 1-June 7	517,424	845	109	12.9
June 8-June 14	516,565	660	90	13.6
June 15-June 21	515,825	4683	1931	41.2
June 22-June 28	510,437	4167	874	21.0
June 29-July 5[1]	252,809	5189	580	11.2
July 6-July 12	247,061	1201	349	29.1

[1] Pond populations divided in half on June 29.

Percent values from Table 6 and Table 7 are illustrated in Figure 1. This figure shows two definite peaks, one during the third week (May 18 through May 24) and one during the seventh week (June 12 through June 21) of the experimental period. Both the immunized and nonimmunized groups showed increases in the percent furunculosis during these periods, however there is very little difference

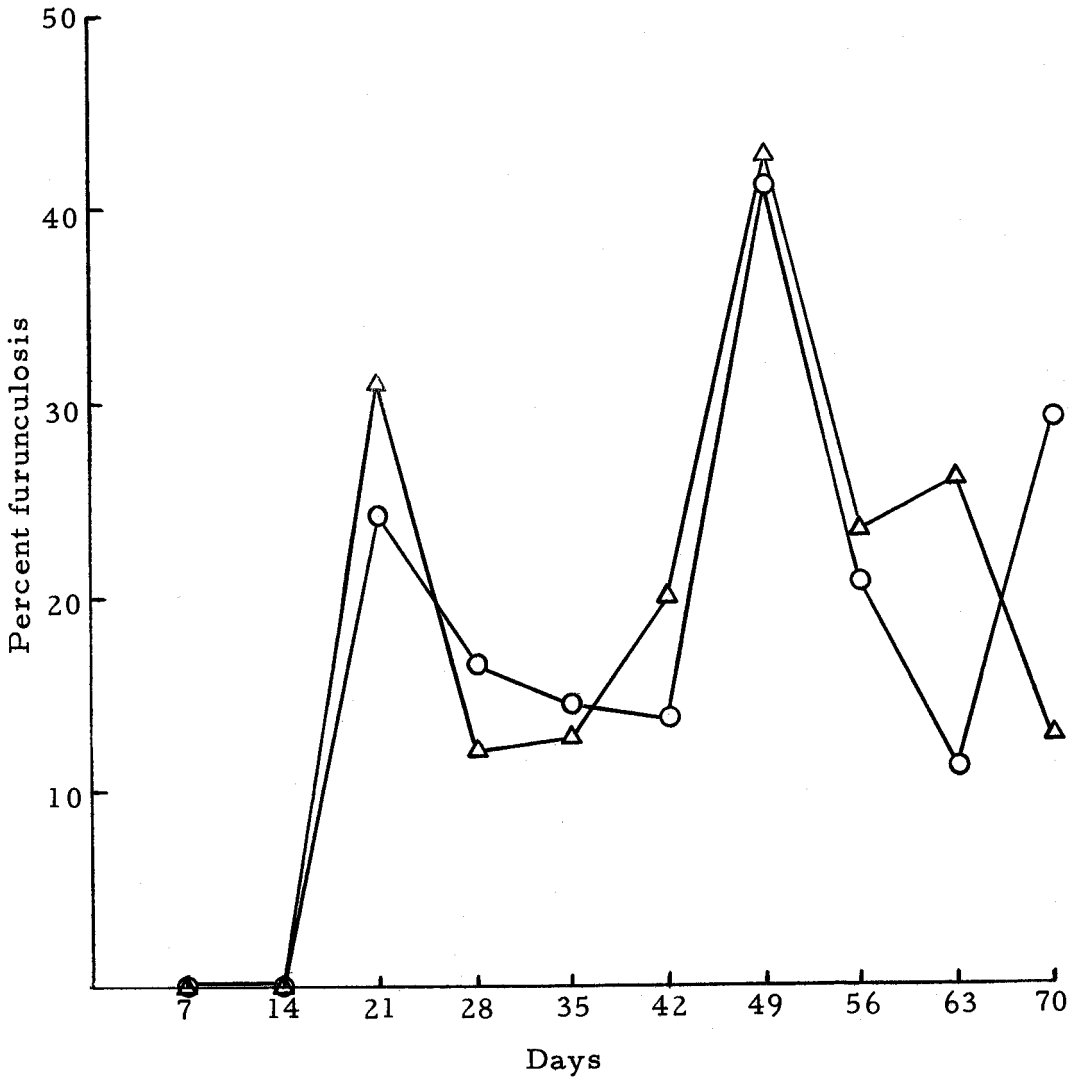

Figure 1. Percent furunculosis in the total loss of juvenile coho salmon in immunized groups (O) and nonimmunized group (Δ) during each seven-day period from May 4-July 12, 1967.

between these two groups.

Table 7. Summary of losses in nonimmunized control pond (Pond No. 9) for each seven-day period.

Period	Initial population	Total loss (all causes)	Loss in which furunculosis diagnosed	Percent furunculosis in total loss
May 4-May 10	65,000	38	0	0
May 11-May 17	64,962	58	0	0
May 18-May 24	64,904	93	29	31.2
May 25-May 31	64,811	75	9	12.0
June 1-June 7	64,736	114	15	13.2
June 8-June 14	64,622	80	16	20.0
June 15-June 21	64,542	705	304	43.1
June 22-June 28	63,837	653	150	23.0
June 29-July 5[1]	31,918	559	146	26.1
July 6-July 12	31,359	55	7	12.7

[1] Pond population divided in half on June 29.

The total number of mortalities in the hatchery during the period of May 4 through July 12 was 21,387 animals, of which 4,861 were diagnosed as being caused by A. salmonicida. In the nine ponds, 22.7% of the deaths resulted from A. salmonicida infections.

Although the culturing of individual mortalities in the nine ponds was terminated on July 12, a record was kept of the daily losses through July 31, 1967. These values are as follows:

July 13 through July 19 712

July 20 through July 26 767

July 27 through July 31 346

The mean hatchery water temperature for each of these periods was 64°F.

Comparison of Mean Losses in Immunized Groups and Total Losses in the Nonimmunized Group

For comparison of the total losses (all causes) and losses due to furunculosis in the experimental and control groups, mean values were calculated for the eight ponds containing immunized animals. These values are compared with total losses and losses from furunculosis in the nonimmunized control group (Table 8). The mean total loss in the immunized groups and the total loss in the nonimmunized group are graphically illustrated in Figure 2. The mean loss and total loss from furunculosis in these two groups are illustrated in Figure 3. The total losses from furunculosis are somewhat higher in the control group than in the immunized group except during the periods of May 25 through May 31 and July 6 through July 12, during which the control ponds values are lower.

Table 8. Mean losses in immunized groups (Pond No. 1-Pond No. 8) and total losses in nonimmunized control group (Pond No. 9) for each seven-day period.

Period	Immunized Group (Pond No. 1-Pond No. 8)		Nonimmunized Control Group (Pond No. 9)	
	Mean total loss (all causes)	Mean loss from furunculosis	Total loss (all causes)	Loss from furunculosis
May 4-May 10	84	0	38	0
May 11-May 17	45	0.1	58	0
May 18-May 24	84	21	93	29
May 25-May 31	64	11	75	9
June 1-June 7	106	14	114	15
June 8-June 14	83	11	80	16
June 15-June 21	585	241	705	304
June 22-June 28	521	109	653	150
June 29-July 5	649	73	559	146
July 6-July 12	150	44	55	7

Comparison of Accumulated Mean Loss Values in the Immunized Groups and Accumulated Total Losses in the Nonimmunized Group

In order to show what, if any, difference occurred in the total losses between the experimental and control groups, accumulated loss values were determined (Table 9). In Figure 4, a comparison is made

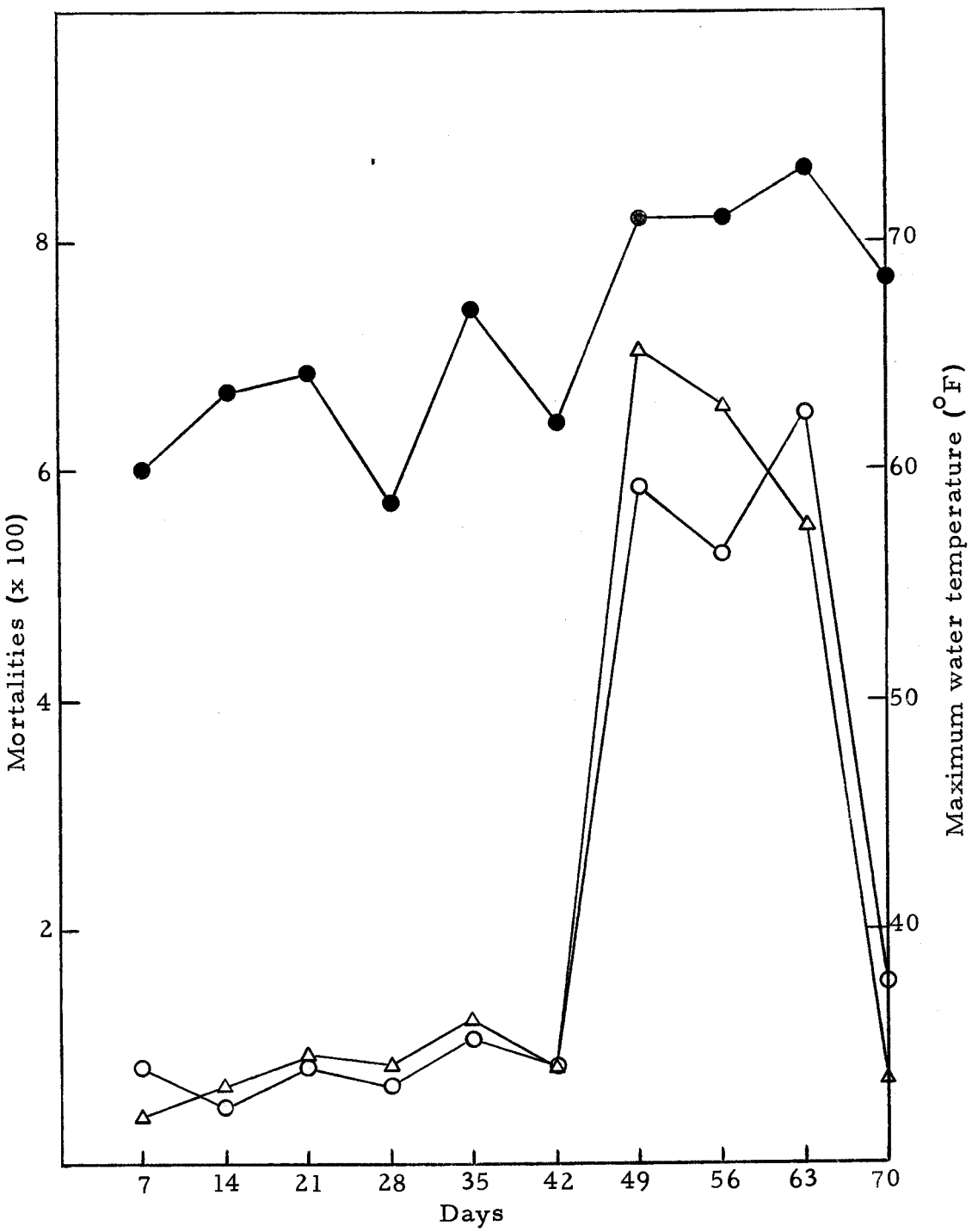

Figure 2. Mean weekly loss of juvenile coho salmon from all causes in immunized group (O) and weekly loss (all causes) in the nonimmunized group (△) compared with the maximum hatchery water temperature (●) during the period of May 4-July 12, 1967.

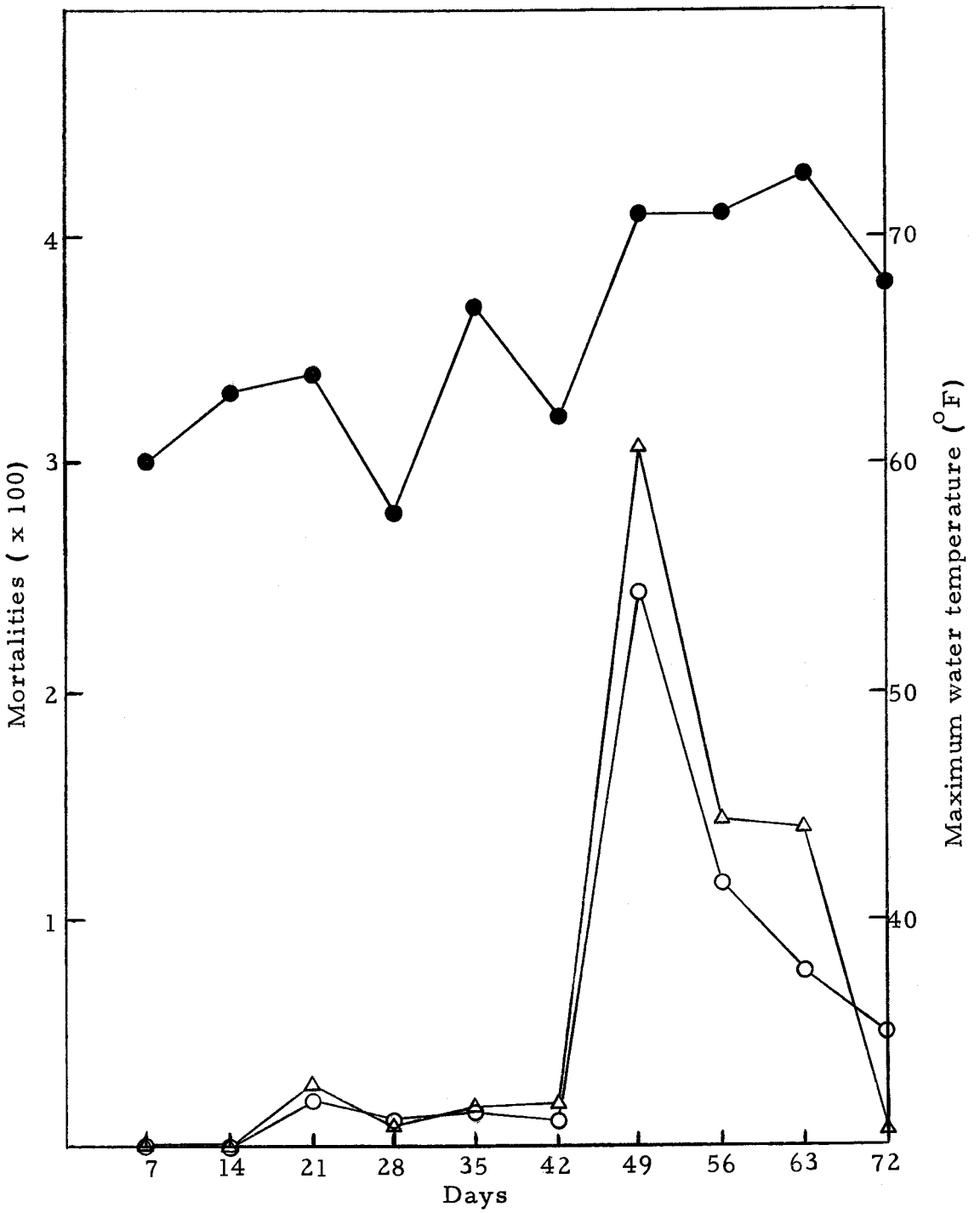

Figure 3. Mean weekly loss of juvenile coho salmon from furunculosis in immunized group (O) and weekly loss from furunculosis in the nonimmunized group (Δ) compared with maximum hatchery water temperature (●) during the period of May 4-July 12, 1967.

Table 9. Accumulated mean losses in immunized groups (Pond No. 1-Pond No. 8) and accumulated total losses in nonimmunized control group (Pond No. 9) for each seven-day period.

Period	Immunized Group (Pond No. 1-Pond No. 8)		Nonimmunized Control Group (Pond No. 9)	
	Mean total loss (all causes)	Mean loss from furunculosis	Total loss (all causes)	Loss from furunculosis
May 4-May 10	84	0	38	0
May 11-May 17	129	0	96	0
May 18-May 24	213	21	189	29
May 25-May 31	277	32	264	38
June 1-June 7	382	46	378	53
June 8-June 14	465	57	458	69
June 15-June 21	1050	298	1163	373
June 22-June 28	1571	407	1816	523
June 29-July 5	2220	480	2375	669
July 6-July 12	2370	524	2430	676

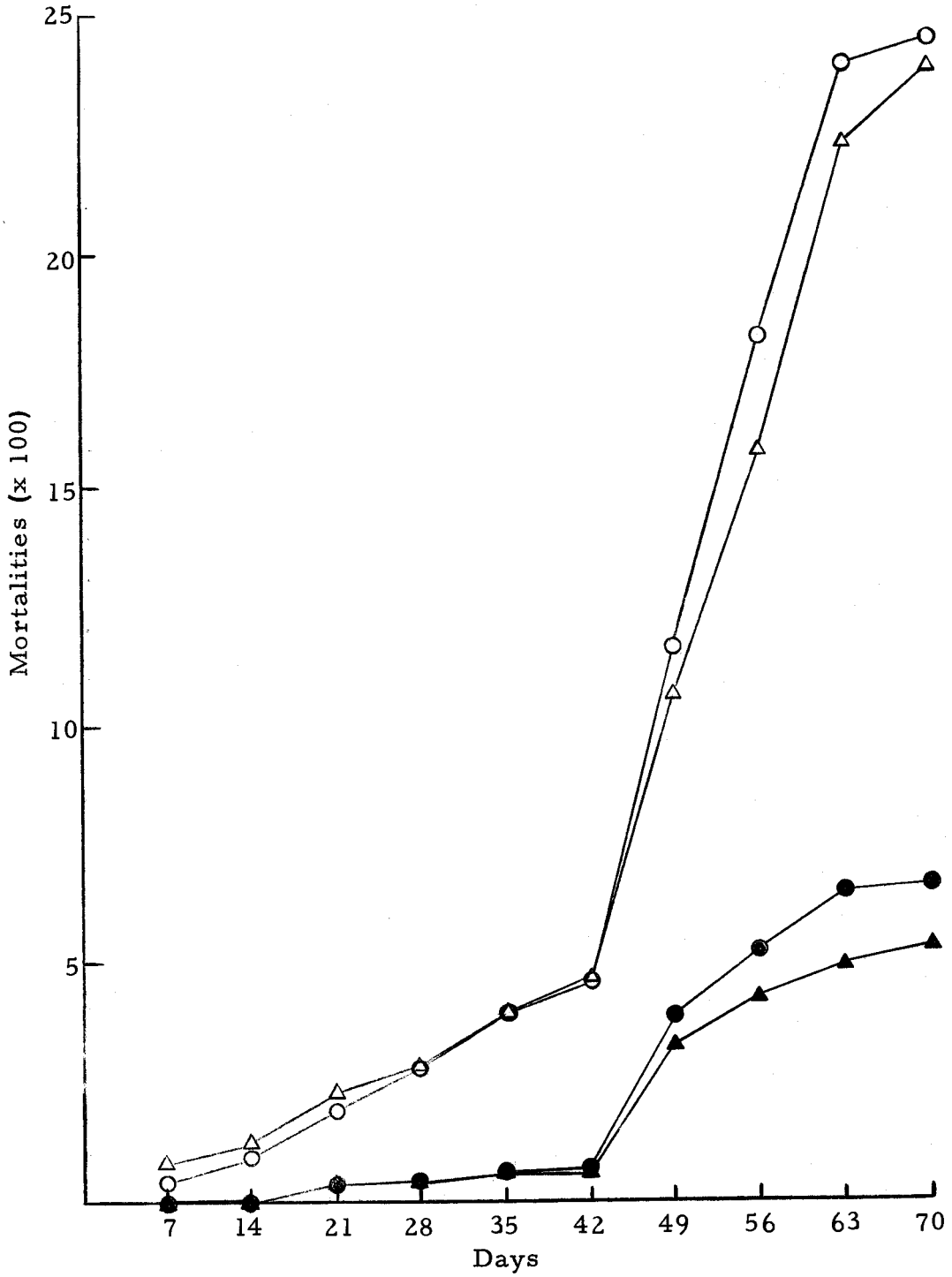

Figure 4. Accumulated total losses from all causes (O) and accumulated total losses from furunculosis (●) in the nonimmunized group (Pond No. 9) compared with accumulated mean loss from all causes (Δ) and accumulated mean loss from furunculosis (▲) in the immunized groups (Pond No. 1-Pond No. 8).

of the accumulated mean total loss (all causes) in the immunized group and the total loss (all causes) in the nonimmunized group. This figure also illustrates the relationship between the mean loss due to furunculosis in the immunized group and the loss from furunculosis in the nonimmunized group. The values for the accumulated mean total loss and the accumulated mean loss from furunculosis in the control group are higher than the corresponding values for the immunized animals. The differences between these two groups do not appear large enough to be meaningful.

Influence of Water Temperature on Hatchery Losses

During the monitoring period, the hatchery water temperature fluctuated considerably. Minimum, maximum and mean water temperatures for each of the ten seven-day periods are tabulated in Table 10.

Table 10. Hatchery water temperatures during experimental period.

Period	Water Temperatures (°F)		
	Minimum	Maximum	Mean
May 4-May 10	46	60	52.0
May 11-May 17	47	63	52.0
May 18-May 24	50	64	57.7
May 25-May 31	48	58	53.4
June 1-June 7	50	67	58.0
June 8-June 14	51	62	54.8
June 15-June 21	56	71	62.0
June 22-June 28	56	71	62.6
June 29-July 5	62	73	66.8
July 6-July 12	59	68	63.0

It was noted that increases in both the total loss and loss in which furunculosis was diagnosed corresponded closely with increases in the hatchery water temperature. A comparison of the water temperatures and losses during each seven-day period indicates that a decrease in water temperature was accompanied by a decrease in mortalities.

The periods of June 15 through June 21, June 22 through June 28 and June 29 through July 5 all show high losses (Table 6 and Table 7), and during these periods the highest water temperatures were attained (Table 10).

Figure 5 illustrates the relationship between the maximum water temperature and total losses in the hatchery for each seven-day period. Two peak mortality values are evident. The first peak occurred during the seventh week (June 15 through June 21) and was followed by a decrease during the eighth week and an even higher peak during the ninth week (June 29 through July 5). The first peak corresponds to an increase in the maximum temperature from 62°F to 71°F. A similar picture was seen in the comparison of the water temperature and mean total losses (Figure 2).

Figure 3 shows that the mean loss from furunculosis reached a maximum during the seventh week and decreased sharply thereafter. This decrease may be due, in part, to the sudden drop in maximum daily temperature from 71°F to 60°F during the last three

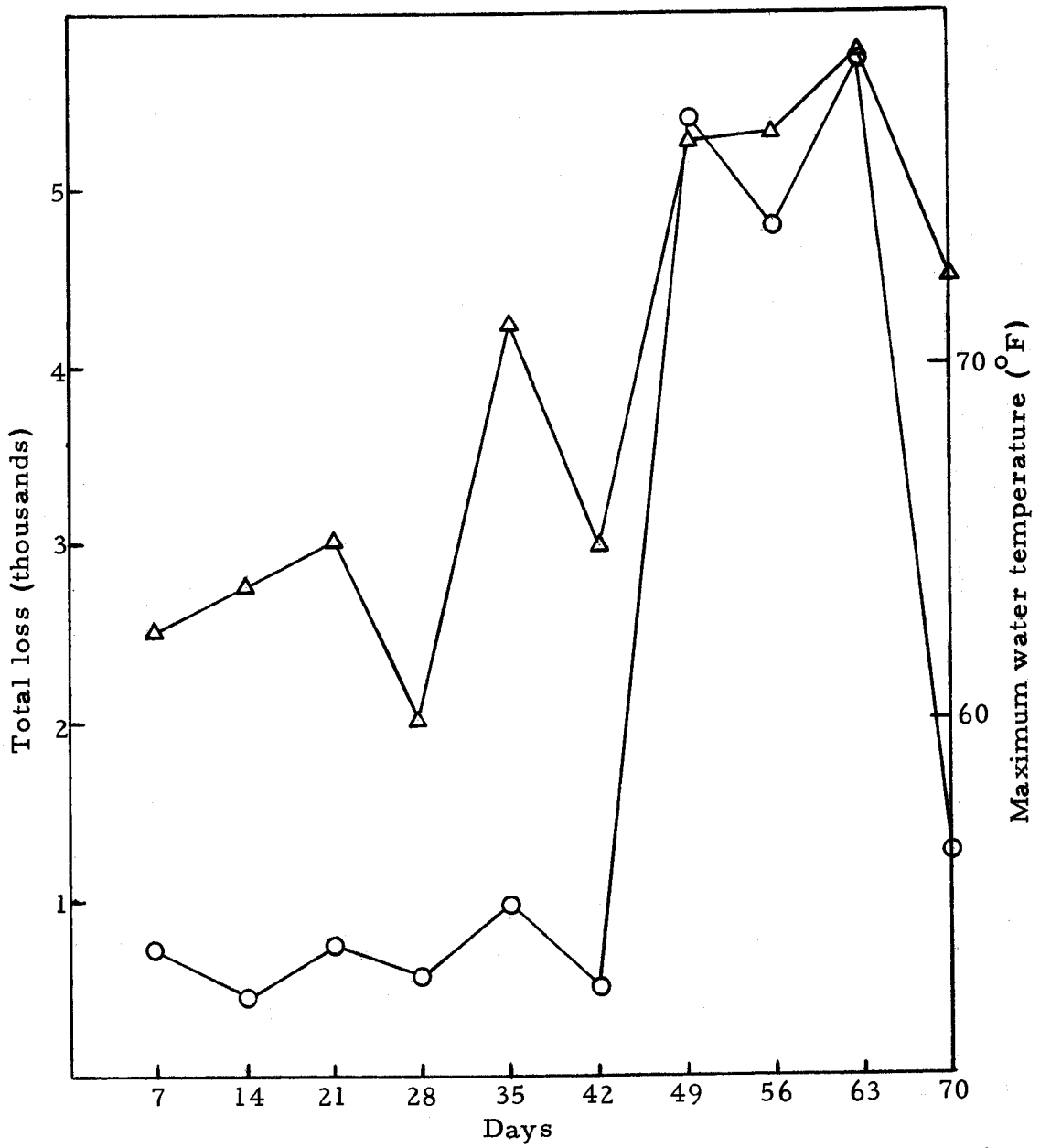

Figure 5. Comparison of total losses (O) of juvenile coho salmon in Pond No. 1 through Pond No. 9 with maximum hatchery water temperature (Δ) for each seven-day period from May 4-July 12, 1967.

days of the eighth week. This same temperature drop may account for the eighth week depression seen in Figure 5.

Occurrence of Acid-Fast Organism During Mortality Examination

Throughout the examination period, cultures from a number of animals showed the presence of rough, dry, cream-colored colonies in pure culture on Furunculosis Agar. Representative colonies were picked, stained and examined microscopically. The organism was tentatively placed in the genus Mycobacterium. Preliminary studies indicate that the bacillus probably is Mycobacterium fortuitum (Cruz). Systematic identification of this organism is now in progress.

The acid-fast organism was isolated from 146 fish and it appeared to be fairly evenly distributed throughout the nine groups of animals.

It is interesting to note that the acid-fast bacillus was not found in animals which were infected with A. salmonicida. The bacillus was obtained in pure culture from fish showing no apparent gross pathology. The organism was also observed in stained smears from kidney tissue.

Tube Agglutination Tests

The results of tube agglutination titers on 11 serum specimens are presented in Table 11. The serum samples from the Siletz Hatchery exhibited antibody titers ranging from 1:160 for Pond No. 5 and

Pond No. 9 to 1:640 for Pond No. 2, Pond No. 3 and Pond No. 4. The remaining ponds all showed titers of 1:320. A 1:80 titer was obtained with the Alsea Hatchery specimen and the rabbit serum had a titer of 1:5,120.

Table 11. Results of tube agglutinations on serum specimens from immunized and nonimmunized groups of juvenile coho salmon.

Serum Specimen	Tube Number 1	2	3	4	5	6	7	8	9	10	Titer
Pond No. 1	3+	2+	1+	1+	1+	0	0	0	0	0	1:320
Pond No. 2	3+	1+	1+	1+	1+	1+	0	0	0	0	1:640
Pond No. 3	3+	2+	2+	1+	1+	1+	0	0	0	0	1:640
Pond No. 4	3+	2+	2+	1+	1+	1+	0	0	0	0	1:640
Pond No. 5	3+	2+	1+	1+	0	0	0	0	0	0	1:160
Pond No. 6	3+	2+	1+	1+	1+	0	0	0	0	0	1:320
Pond No. 7	2+	1+	1+	1+	1+	0	0	0	0	0	1:320
Pond No. 8	3+	2+	1+	1+	1+	0	0	0	0	0	1:320
Pond No. 9	3+	1+	1+	1+	0	0	0	0	0	0	1:160
Alsea[2]	1+	1+	1+	0	0	0	0	0	0	0	1:80
Rabbit[3]	4+	3+	3+	2+	1+	1+	1+	1+	1+	0	1:5,120

[1]Nonimmunized control from Siletz River Salmon Hatchery.

[2]Negative control from Alsea River Salmon Hatchery.

[3]Positive control from rabbit immunized against A. salmonicida.

DISCUSSION

Culture of A. salmonicida Cells and Vaccine Preparation

The primary goal of the present investigation was to demonstrate definite protection against A. salmonicida in juvenile coho salmon on a production level after administration of an alum-precipitated oral vaccine.

Because of the excellent results obtained in the 1966 pilot study, oral immunization of an entire hatchery population was attempted. Except for the method of cell culture, the vaccine used in this study was prepared in the same manner as that used in the pilot experiment of 1966.

During the culture of Lot No. 1 and Lot No. 2, the pH of the culture medium dropped from an initial reading of pH 7.4 to approximately pH 5.8. This decrease in pH appeared to result from acid production during the utilization of dextrose in the Trypticase Soy Broth. It was felt that replacement of the utilized dextrose and pH control would increase the cell yield.

A total of 175 g of dextrose was added to the culture vessel at intervals throughout the fermentation of Lot No. 3. The results indicate that the yield of Lot No. 3 was more than double that of either Lot No. 1 or Lot No. 2 (Table 3). The effect of dextrose on the growth of A. salmonicida is currently being investigated.

The cost of producing the alum-precipitated A. salmonicida vaccine for this investigation is presented in Table 4. The cost of the ingredients, preparation and feeding of the Oregon Moist Pellet is not included since the OMP is normally prepared for and used in the rearing of coho salmon at the Siletz River Salmon Hatchery. The pellet manufacturing company made no additional charge for addition of the vaccine to the OMP.

Immunization of Fish

Based on figures obtained from preliminary studies, the amount of vaccine received by each animal during the immunization period (360.7 μg) was approximately three times that needed to afford protection.[1] The increased amount was administered in order to insure that each individual animal received a minimum protective dose of vaccine.

Examination of Mortalities

The experimental population consisted of approximately 520,000 animals which received the oral vaccine in their diet and approximately 65,000 animals which served as a nonimmunized control group.

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[1] Personal communication with G.W. Klontz, Western Fish Disease Laboratory, U.S. Fish and Wildlife Service, Seattle, Washington.

To assess the effectiveness of the immunization, all mortalities from the immunized and nonimmunized groups were collected and examined for the presence of A. salmonicida. If protection was afforded to the immunized group of animals, a definite difference between this group and the nonimmunized group should be apparent. Small differences between the immunized and nonimmunized groups are of little or no value in assessing the usefulness of the immunizing antigen or procedure.

The examination of each fish was carried out using heavy inoculation on Furunculosis Agar with material from the kidney of these animals. In fish, the kidney is generally considered an ideal site from which to take microbiological specimens. Furunculosis Agar was developed specifically for the isolation and detection of A. salmonicida and therefore was considered the medium of choice in this study. Following inoculation, the Furunculosis Agar plates were held for one week before discarding in order to detect any slow-growing colonies of A. salmonicida. By using a heavy inoculum from the kidney and prolonged incubation on Furunculosis Agar, it was felt that most, if not all, of the furunculosis-positive animals would be detected provided that the organism was present in blood or kidney tissue.

Both furunculosis-positive and furunculosis-negative animals were enumerated. The cause of death in furunculosis-negative

individuals was not determined due to the large variety of factors which may have contributed to the death of these animals.

From the results, it appears that insufficient antibody was produced in the immunized groups to protect these individuals from succumbing to A. salmonicida infection. The values in Tables 5, 6, 7, 8, 9 and 11 show no distinctive difference between the immunized and nonimmunized groups. Although the nonimmunized control group shows a higher total loss and a higher loss from furunculosis than the immunized group, the difference between these two groups fails to suggest any definite protection against A. salmonicida.

A review of the literature concerning the immune response of poikilothermic vertebrates reveals an important relationship between the defense mechanism of these animals and the environmental temperature. Antibody production in poikilotherms appears to take place slowly at low temperatures. However, immunity is developed rapidly in those animals which can be held at temperatures above 37°C (98.6°F). The complete absence of circulating antibody production at temperatures below 10°C (50°F) has been reported (Bisset, 1947b). There is also evidence that supports the hypothesis that, in poikilothermic vertebrates, antibody is formed at low temperatures but not released until the environmental temperature is raised (Elek, Rees and Gowing, 1962). In any case, the effect of temperature on the immune response cannot be overlooked.

Probably the most important observation made during the investigation was the relationship between the increase in mortalities and the increase in hatchery water temperature.

During the monitoring period (May 4 through July 12), increases in water temperature were accompanied by corresponding increases in mortalities. Decreases in water temperature were generally followed by a decrease in the number of deaths (Figure 5). Temperatures much above 70°F can be lethal to salmon, particularly if other conditions of stress are present, e.g. low dissolved oxygen, crowding, etc. The abnormally high water temperatures which occurred during the epizootic appear to have caused an increase in mortalities and, as a result, the data have been affected. It is possible that many A. salmonicida-positive cultures were obtained from low-grade infections in immunized fish which might have survived were it not for the additional stress of high water temperature. At low temperatures, the generation time of the bacterium would be increased. It is possible that had the temperatures remained low, these fish may have had enough antibody to fight the initial invasion of the organism. As the water temperature approached the optimum for the invading pathogen, the generation time would decrease and a generalized septicimia would result. Death would follow if sufficient protective antibody were not present to neutralize the overwhelming infection.

Water temperatures during the immunization period may also

be an important factor influencing the results of this study. The average daily temperatures during the first 14-day immunization period ranged from 44°F to 48°F (6.6°C to 8.9°C) and the average water temperatures on the days of booster feeding ranged from 44°F to 59°F (6.6°C to 15°C). Following the last booster on May 29, the daily water temperatures continued to increase slowly. The water temperatures during the first 14-day feedings could have been responsible for low protective antibody production in the immunized group of salmon.

In previous years, the epizootic of furunculosis appeared to correspond with the upstream migration of the adult lamprey, Lampetra tridentata. These lampreys, after spawning, were found on the intake screens at the point where water enters the hatchery. During the 1966 epizootic, 36 adult lampreys were collected and examined for the presence of A. salmonicida. Of these, 29 showed heavy infections of the organism. The presence of A. salmonicida in these adult lampreys at the time of a furunculosis epizootic suggested that this animal may serve as a carrier of the etiologic agent of the disease.

During the present investigation, the number of adult lampreys appearing in the incoming water supply seemed lower than in previous years. A few lampreys were collected from a point below the hatchery and, upon examination, were found to be free of A. salmonicida.

The appearance of acid-fast bacilli in pure culture on Furunculosis Agar is worth noting. Enumeration of the number of fish harboring the acid-fast organism was begun on June 1, 1967 when it was noticed that a number of smaller-than-average animals proved positive in this regard. Normal sized animals also carried the organism but positive cultures in these animals were observed less frequently. During the first half of the experimental period, a great many small fish were observed in the mortalities. It was suspected that these small animals were wild fish which had found their way into the hatchery. This year, as in previous years, adult salmon were passed above the hatchery for spawning purposes. These small fish may be the result of natural spawning.

The importance of this acid-fast organism as a fish pathogen is currently under investigation in this laboratory.

Tube Agglutination Tests

It was desirable to monitor the antibody titers throughout the course of the experiment beginning with specimens taken prior to the administration of the vaccine. This procedure proved impossible due to the small size of the animals during this time. In July, each animal was large enough to yield approximately 0.1 ml of whole blood for examination. The small volume of blood which could be collected from each fish necessitated the use of pooled blood specimens for the

tube agglutination procedure.

Tube agglutination titers were run to detect what, if any, difference in antibody levels was present in the nonimmunized control population as compared with the immunized population. Two control sera were utilized. The serum specimen from the Alsea Hatchery was used as a negative control since this population was reported to be free of furunculosis. Rabbit anti-A. salmonicida serum was used as the positive control.

The results show no significant difference in agglutinating antibody levels between the immunized groups and the nonimmunized group of fish. The nonimmunized control pond (Pond No. 9) does show a low titer (1:160) but Pond No. 5 also shows the same titer. These two ponds also show the highest percent loss from furunculosis (Table 5). Since the serum was obtained approximately two months after the first case of furunculosis was diagnosed, the titers obtained may be a result of natural immunity developed during the epizootic.

The use of pooled specimens has one disadvantage in that the titer measures the mean antibody level in the fish examined. The pooled specimen may contain blood from individual animals having titers above and below the figure obtained for the pooled sample.

It is important to note that high agglutinating antibody titers, in poikilothermic animals, does not necessarily indicate the presence of "protecting" antibody (Spence, Fryer and Pilcher, 1965).

In view of the studies carried out in 1966 at the Siletz Hatchery and investigations at the Western Fish Disease Laboratory in Seattle, Washington, mass immunization against furunculosis appears to be promising. The large scale production and administration of a vaccine involves problems not encountered during laboratory-scale investigations. Despite the fact that this study failed to demonstrate protection against furunculosis, it is felt that effective mass immunization with the alum-precipitated A. salmonicida vaccine can be carried out. It is realized that the vaccine used is a crude preparation. Purification techniques, methods for vaccine assay and quality control standards must be developed in future investigations utilizing this vaccine.

SUMMARY AND CONCLUSIONS

1. The addition of dextrose to the culture medium and pH control during the fermentation process increased the cell yield of A. salmonicida to more than twice that obtained when dextrose was not added.
2. The oral vaccine used in this study was an alum-precipitated cell sonicate of A. salmonicida which was lyophilized and incorporated into the fish diet of Oregon Moist Pellets.
3. The cost of materials necessary for preparation of 245.5 g of vaccine was $92.26 or $0.38 per g of vaccine. Approximately 250 man-hours were required for the vaccine preparation.
4. Immunization of approximately 520,000 juvenile coho salmon with the oral vaccine was started on March 26, 1967 with 14 consecutive days of initial vaccination followed by weekly boosters through May 29, 1967. Each fish received approximately 360.7 μg of vaccine during the immunization period. Approximately 65,000 fish served as a nonimmunized control group.
5. All mortalities were examined for the presence of A. salmonicida during the period of May 1 through July 12, 1967. Although the nonimmunized control group showed a higher total loss and a higher furunculosis loss than the immunized groups, the results fail to indicate any distinctive difference between these two

groups.

6. Losses appeared to be closely associated with the hatchery water temperature. An increase in water temperature was accompanied by a corresponding increase in losses. A decline in losses appeared to be associated with a decrease in water temperature.

7. Of the fish examined, 146 animals showed the presence of an acid-fast organism tentatively identified as Mycobacterium fortuitum (Cruz).

8. The presence of A. salmonicida in adult lampreys (Lamptera tridentata) at the time of a furunculosis epizootic suggests that this animal may serve as a carrier of the organism.

9. Tube agglutinations showed only slight differences between the agglutinating antibody levels in the immunized and nonimmunized groups of fish.

10. From the experimental data, it can be concluded that the immunized groups of animals failed to develop sufficient antibody to confer protection against furunculosis. The slightly lower values obtained for the immunized groups may indicate that some immunity had been induced by the vaccine. However, any low-level immunity which may have been produced was not adequate to provide protection against infection by A. salmonicida.

11. The failure to induce sufficient protective immunity may have

been caused by any one or a combination of the following factors: (a) administration of insufficient amounts of vaccine to afford protection, (b) administration of an excessively high immunizing dose which inhibited antibody formation, (c) administration of a vaccine which was weakly antigenic, (d) inadequate mixing of the vaccine during its incorporation into the OMP, (e) loss of vaccine antigenicity due to chemical or biological degradation after incorporation into the OMP, (f) low water temperature during immunization period which may have inhibited antibody formation, and (g) one or more factors which prevented the translation of this immunizing procedure from successful laboratory-scale experimentation to a production situation.

12. Future investigations using the alum-precipitated vaccine should include the development of quality control standards to be used throughout the vaccine preparation.

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