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THE REDUCTION OF HATCHERY AND AQUACULTURE DISEASES BY THE USE OF MOLECULAR BASED THERAPEUTIC NUTRITION

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FINAL REPORT

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ABSTRACT

This project addressed the problem of mortalities among hatchery-reared salmonids. The hypothesis was that health and disease resistance could be improved- not through costly drugs and chemicals, but rather, through dietary supplementation with micronutrients and antioxidants. This approach was based upon previously published evidence relating to differences that exist between wild and hatchery-reared salmonids. Specifically, hatchery-reared salmonids have been shown to be seriously lacking in some key natural micronutrients that govern the efficient functioning of host immune response systems. The present study was the first phase of a continuing effort to find ways to replenish these insufficiencies by introducing molecular based therapeutic micronutrition with interactive antioxidant vitamins. The long term goal is to utilize natural nutrients to produce healthy fish without the use of costly drugs and chemicals. Success of this approach will be measured in enhanced hatchery survival and improved adult returns of salmonids--including threatened and endangered stocks.

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EXECUTIVE SUMMARY

OVERALL GOAL OF STUDY

The overall goal of this study was to pursue a line of investigation originating from a previous observation, namely that hatchery-bred fish differed significantly from their wild counterparts in body concentrations of the micronutrient selenium. The goal was to study physiological changes and health effects that might occur when selenium levels in hatchery-reared fish were made equal to those of wild fish. The project included long-term feeding studies using elevated levels of dietary selenium, as well as selenium coupled with antioxidants, such as vitamins C and E. Also included were stress tests (such as thermal, seawater and disease) using fish fed different levels of selenium. The results of the feeding trials and stress tests were validated by biochemical measurements.

YEAR 1

Hatchery-bred coho salmon (*Oncorhynchus kisutch*) were fed elevated levels of selenium (as Na_2SO_3) in order to raise body burdens to the level measured in wild counterparts. The goal was to find a dietary concentration that would achieve the desired effect without causing damage to growth and normal development. Whole body selenium concentration and changes in enzyme activity (hepatic glutathione peroxidase and superoxide dismutase) were measured at the end of the feeding trial in order to evaluate the effects of the various supplements (1.1, 8.6, 11.1, 13.6 $\mu g\ g^{-1}$ Se). Enzyme activity was also measured following exposure of the fish to transportation stress and to seawater adaptation.

The results indicated that a dietary supplement of 8.6 $\mu g\ g^{-1}$ selenium was capable of inducing body burdens similar to those found in wild fish. The elevated selenium levels persisted throughout the freshwater rearing phase, but declined following seawater adaptation. Superoxide dismutase (SOD) levels did not increase above control levels, but glutathione peroxidase (GSH-Px) levels increased in fish fed the supplemented diets. The elevated GSH-Px activity persisted following exposure to transportation stress and seawater adaptation. Following seawater entry, the 11.1 $\mu g\ g^{-1}$ and 13.6 $\mu g\ g^{-1}$ diet groups had cumulative mortality rates of 45% and 60%, respectively. These results indicate that the higher concentrations of selenium may have been detrimental to seawater adaptation. The survival rate of fish fed 8.6 $\mu g\ g^{-1}$ Se was the same as that of the control group (80%).

YEAR 2

The goal was to use the optimum dietary selenium concentration found in Year 1 (8.6 $\mu g\ g^{-1}$) and combine it with varying concentrations of the antioxidant vitamins E and C. The primary end points measured were changes in GSH-Px and SOD activity, concentration of selenium and vitamin C, mortality rates, and response to stress. The Year 2 studies were plagued by a series of disease outbreaks and water system failures that frustrated our attempts to collect high quality data.

GSH-Px activity generally increased in the supplemented diets. The highest GSH-Px level was obtained with the diet that contained elevated concentrations of selenium and vitamin C. Analysis of eviscerated carcass samples indicated that high levels of ascorbic acid were retained for up to 18 wk in fish that were fed diets supplemented with vitamin C. It was interesting to note that SOD levels decreased, a subject that will be discussed in section V, Findings.

YEAR 3

Utilizing the information gained during the first two years, a long term feeding study was conducted in Year 3. The antioxidant additions (vitamins C and E) were further refined and were combined with the optimum selenium concentration (8.6 $\mu g\ g^{-1}$) as well as with a reduced selenium concentration (4.3 $\mu g\ g^{-1}$). Additional micronutrients (copper and zinc) were also examined. During this period of study, key parameters measured included growth, changes in enzyme activity, concentrations of selenium, zinc and copper, and survival following pathogen challenge.

The best growth was obtained in fish that were fed a diet containing 8.6 $\mu g\ g^{-1}$ selenium, vitamin E (at 25X the recommended daily allowance) and 20 IU g^{-1} vitamin A. GSH-Px activity was increased in all of the supplemented diet groups relative to the level in control fish. SOD activity decreased in most of the diet groups, but was modestly elevated when fish were fed diets that contained high concentrations of vitamin E and selenium. Concentrations of zinc were similar for all diet groupss (including the control) and were similar to those measured in hatchery-reared fish. Copper was somewhat conserved in fish that were fed diets containing selenium plus an antioxidant; however, this finding was not uniform. The pathogen challenge test used *Vibrio anguillarum* . The results indicated that the best protection was afforded by the diet containing high concentrations of vitamin E, vitamin C and selenium, as well as the diet containing high concentrations of vitamin E, vitamin A and selenium. Mortalities in those groups were only 20-21% versus 53% in control fish.

This study revealed the extreme complexity of applying micronutrient therapy to the maintenance of salmonid health. Single nutrient and/or single vitamin supplementation is not sufficient. Rather, it is necessary to identify the optimal concentrations of vitamins, minerals and metals that will enhance the health of fish at different stages in the life cycle.

PURPOSE OF RESEARCH

PROBLEM ADDRESSED

This research addressed the adverse impact of diseases on salmonid production and adult return rates. Disease problems cost private and public hatcheries millions of dollars annually. Salmon hatcheries may experience mortality rates ranging from 20% to 90%; typical mortality rates in trout hatcheries are 20 to 30% annually. Returns to public hatcheries on the Columbia River average about 0.1 to 0.15 %. The ultimate priorities of this study are reduction of these mortalities and improvement of returns. The production of robust fish is crucial if significant numbers are to reach the marketplace, to enter the sport and commercial fisheries, or to meet escapement goals.

Recent evidence indicates that hatchery-reared coho salmon (*Oncorhynchus kisutch* Walbaum) adults and smolts differ significantly in whole body selenium concentration when compared with their wild counterparts (Felton et al. 1990). Similar findings have been shown for adult Atlantic salmon (*Salmo salar* L.) (Poppe et al. 1985, Julshamn et al. 1990, and Maage et al. 1991). For example, Felton et al. (1990) found that selenium levels in hatchery-reared coho salmon smolts measured 1.97 $\mu g\ g^{-1}$ dry wt. whereas wild fish of the same age and from the same river system had concentrations of 3.6 $\mu g\ g^{-1}$ dry wt. This disparity could place hatchery-reared fish at a competitive disadvantage and might contribute to poor adult return rates.

Selenium is an essential component of three mammalian enzymes: glutathione peroxidase (GSH-Px), phospholipid hydroperoxide glutathione peroxidase (PHG-Px) and Type I tetra iodothyronine 5' deiodinase (Diplock 1992). PHG-Px and GSH-Px are detoxifying enzymes that catalyze the reduction of hydroperoxides and peroxides, respectively, to less reactive alcohols and water. Berry et al. (1991) recently purified the selenocysteine-containing enzyme, a type I iodothyronine deiodinase, which is involved in the conversion of T4 to T3 in vertebrates. Related to this finding, Arthur et al. (1992) found that a concurrent deficiency of selenium and iodine affected the severity of iodine deficiency in rats. Selenium deficiency can lead to peroxidative damage to cell membranes and organelles (Koller and Exon 1986). Selenium deficiency can also affect host defense mechanisms by reducing the ability of phagocytes to kill ingested bacteria (Gyang et al. 1984), depressing chemotactic and phagocytic functions of neutrophils (Aziz et al. 1984), and suppressing stimulation of IgM antibody-forming cells (Sheffey and Schultz 1979). Selenium may also play a critical role in metabolic regulation. Selenium may play a key role in the speed at which a virus attacks an organism (Taylor et al. 1994).

The selenium-requiring enzyme GSH-Px is a part of the complex protective system that is dependent upon the often referred-to antioxidant micronutrients, which include selenium, manganese, copper, and zinc, as well as vitamins A, C, E, and beta carotene. Deficiency of any of these micronutrients may cause impairments in immune systems, leading to micropathological lesions. The persistence of such impairments will precipitate disease (Diplock 1992).

PROJECT OBJECTIVES

Year 1

The primary objective was to increase whole body selenium levels in hatchery-reared coho salmon (1.97 $\mu g\ g^{-1}$ dry wt) to a level equaling that found in wild fish (3.6 $\mu g\ g^{-1}$ dry weight). A basic commercial feed (Oregon Moist Pellet) was used as a basal (control) diet. Selenium supplements were added to the basal diet as specified in the accompanying tables.

Year 2

The primary objective was to utilize the dietary selenium level established in Year 1, and to add therapeutic levels of vitamins E and C. Fish were maintained on the supplemented diets for a period of 6 mo and monitored pathologically and biochemically for health effects.

Year 3

The primary objective was completion of a long-term (9-mo) feeding study using test diets containing therapeutic levels of vitamins C, E, and A plus selenium. The study culminated in a disease resistance test in which fish from all diet groups were injected with *Vibrio anguillarum*, a bacterial pathogen of salmonid fish.

APPROACH

DESCRIPTION OF WORK PERFORMED

Year 1

The experiments were conducted at the U.S. Fish and Wildlife Service research station on Marrowstone Island, Washington. The facility was utilized because (1) it had the required number of 200 gallon circular tanks for fish containment, (2) both fresh and saltwater systems were available, (3) flow rates were adequate to sustain optimum fish health, (4) cold and freezer storage areas for fish foods were available, (5) the personnel at the facility had extensive prior experience with the husbandry of coho salmon.

The experimental system consisted of four duplicate sets of tanks with 250 fish per tank and four dietary regimes. Two thousand juvenile coho salmon (4–5 g) were obtained from the Washington Department of Fish and Wildlife (WDFW) hatchery at Issaquah, Washington. Fish were distributed equally among the eight tanks.

An acclimation period of 3 wk was completed before experimental feeding began. Fish were fed twice a day for 9 mo. Enzyme and selenium data were obtained at weeks 5, 12 and 25. Visual observations of fish health and well being were made daily.

It should be noted that an extremely successful first year's endeavor made possible early completion of the objective. Therefore, the remaining time was utilized to perform two unproposed experiments that were valuable adjuncts to the first year's study. A transportation stress test was done at week 21, and a saltwater (SW) adaptation test was conducted at end of the feeding study, week 25.

At the beginning of the tenth month, the fish were transferred from freshwater to saltwater (SW) and fish in all dietary groups were switched to the basal diet without supplementation. This change reflected the fact that selenium concentrations could no longer be controlled due to the presence of selenium in sea water. The saltwater exposure continued for 6 mo during which time blood samples were periodically taken to evaluate the degree of seawater adaptation. The purpose of this SW experiment was to mimic the natural life history of salmon and to observe any changes related to the selenium supplementation.

The test diets were made using the basic ingredients of the Oregon Moist Pellet diet (OMP; Moore Clark Co., La Conner, Washington), including dry and moist components in the unpelletized forms (Table 1). The ration contained all of the ingredients necessary for a normal fish diet with the exception of vitamin C. Each diet was made as outlined in Table 1 and contained a standard amount of vitamin C (0.15%). Selenium, as Na_2SO_3, was added to Diets #2, 3, and 4 at levels of 8.6, 11.6, and 13.6 $\mu g\ g^{-1}$, respectively. The control diet (#1) contained an average concentration of 0.9 to 1.1 $\mu g\ g^{-1}$ selenium. Diets were stored at - 20°C until needed.

Year 2

The established Year 1 dietary concentration of selenium (8.6 $\mu g\ g^{-1}$) was combined with two antioxidants, vitamins C and E. Seven diets (in duplicate) were formulated as shown in Table 2.

Due to facility renovation and construction, the Year 2 study could not be conducted at the Marrowstone Island research station. The alternate site chosen was the University of Washington (UW) Big Beef Creek research station at Seabeck, Washington.

Table 1. Diet composition for Year 1 feeding study. A basic commercial diet (OMP) was supplemented with additions of selenium as Na_2SO_3. The amounts listed are for 7 kilograms of pelletized diet.

Diet number	Dry comp. (kg)	Wet comp. (kg)	Vitamin C (g)	Selenium as Na_2SO_3 (mg)
#1 Control(1.1)	4.410	2.590	10.5	0
#2 8.6 $\mu g\ g^{-1}$ Se	4.410	2.590	10.5	115
#3 11.6 $\mu g\ g^{-1}$ Se	4.410	2.590	10.5	153.33
#4 13.6 $\mu g\ g^{-1}$ Se	4.410	2.590	10.5	191.66

Table 2. Dietary composition for Year 2 feeding study.

Diet #1 control	Diet #2	Diet #3	Diet #4	Diet #5	Diet #6	Diet #7
No added vitamins or Se	Vitamin E at 25 X[a]	Vit E 25X Se at 8.6 μg g^{-1}	Selenium at 8.6μg g^{-1}	Vitamin C at 10 X & Se 8.6	Vitamin C at 10 X & E at 25 X	Vitamin C at 10 X E at 25 X Se at 8.6
Duplicate	Duplicate	Duplicate	Duplicate	Duplicate	Duplicate	Duplicate

X[a] = concentration normally found in commercial diets (Vit. C = 0.15%; Vit. E = 40 IU/kg).

In preparation for the Year 2 study, 3,500 young-of-the-year coho salmon were transported for holding from the Issaquah Hatchery (WDFW) to the University of Washington Seward Park Hatchery. During the holding period, the fish developed a serious helminth infestation that resulted from being held in open tanks that were exposed to sea gulls and other birds. The fish could not be used for research and were sacrificed.

A new group of salmon was obtained from the George Adams Hatchery (WDFW). Five thousand juveniles (11-13 g) were transported to the Big Beef Creek research station and distributed equally among 14 (250 gal) tanks. The fish were allowed to acclimate for 2 wk before the feeding study was initiated.

Using the diet formulations shown in Table 2, feeding was begun in October 1991 and continued without incident until January 28, 1992. On that date there was a rupture in the main underground water line supplying the test tanks, causing a total loss of the feeding study. An extension of the project was granted so that this phase of the study could be repeated.

For the repeat study fish had to be held in two facilities in Seattle, Washington, because there were limited facilities available at both sites. Half of the fish were taken to the University of Washington School of Fisheries and half were taken to the Northwest Fisheries Science Center (NMFS). Both labs receive dechlorinated city water. Water temperatures differed by two degrees at the start of study (UW = 12°C; NMFS = 14°C) and by 5°C at the end of May 1992. Although this emergency situation of holding fish in two locations was not desirable it did allow continuation of the study.

On March 23, 1992 , coho salmon (3,200) averaging 35 g each were transferred from the Issaquah Hatchery (WDFW) and distributed as follows: (1) 300 per tank were placed in the NMFS tanks (712-liter capacity), and (2) 150 per tank were placed in the UW tanks (350 liter capacity). Three weeks were allowed for acclimation, and feeding began on April 15, 1992. Fish were fed to satiation once daily. Biochemical analyses were done at the beginning of the study and at 6-wk intervals for a period of 6 mo.

In mid May, mortalities began to occur in the NMFS facility and increased into the first week of June 1992. During this same period, no mortalities occurred at the UW labs.

Dr. Lee Harrell, a fish pathologist employed by NMFS, was asked to determine the cause of mortalities. He concluded that the fish suffered from a bacterial disease known as furunculosis (caused by *Aeromonas salmonicida*). At his request, the NMFS portion of the study was terminated and the fish were destroyed on June 9, 1992. Prior to euthanasia, five fish were sampled from each dietary group and were compared with five fish taken from the same dietary group at the UW laboratory. GSH-Px, SOD enzyme activities and whole body vitamin C concentrations were compared in the two separated groups. On June 10, 1992, mortalities began to occur at the UW laboratory, but the prevalence was lower than that experienced at the NMFS laboratory.

The UW experiment was continued for 5 mo. In the sixth (final) month, a stress test was conducted. The fish were transferred from a cool simulated stream water habitat to that of a warmed river water habitat.

Year 3

The final phase of the research encompassed a long term feeding study. The study started the first week of December 1992 and continued until September 1993. The experiment consisted of seven diets (in duplicate) with 150 fish per tank. Table 3 shows the supplementation made to the basic commercial diet. The average starting weight of the fish was 10 g. The fish were housed at the UW Big Beef Creek research station.

There was a change in the diet during the last phase of the study because the producers of Oregon Moist Pellet went out of business. A replacement diet was obtained from Bioproducts, Inc. (Warrenton, OR). The choice was made after conferring with Dr. Ronald Hardy, a National Marine Fisheries Service nutritionist, and with other fish culturists. The product closely resembled OMP in terms of proximate analysis as well as selenium and mineral content. The seven diets were made as shown in Table 3. The term "basic," as used in Table 3, refers to the basal (control) diet which contained all of the nutrient requirements for cold water fish (National Research Council 1981).

Table 3. Diet composition for Year 3 feeding study.

Diet No.	Comp.	Vit. C	Vit. E	Vit. A	Se
#1 Control	Basic	1 X[a]	no add.	no add.	no add.
#2	Basic	1 X	25 X	no add.	8.6 ppm
#3	Basic	1 X	12.5 X	no add.	4.3 ppm
#4	Basic	1 X	25 X	20 IU g^{-1}	8.6 ppm
#5	Basic	10 X	no add.	no add.	8.6 ppm
#6	Basic	10 X	50 X	no add.	8.6 ppm
#7	Basic	10 X	12.5 X	no add.	4.3 ppm

X[a] = Normal constituents as defined in Table 2.

Every 6 wk during the Year 3 feeding study, enzyme analyses were conducted. Selenium concentrations were checked periodically using eviscerated, whole body samples. Fish were fed daily to satiation and weights were measured at 6-wk intervals (see Table 17). Water temperatures were checked daily at the time of feeding and the temperatures remained at 12°± 0.5°C. Mortalities were limited to fish injured during tank maintenance. Disease challenges were performed during the last 4 wk of the 9-mo study.

A pathogen challenge test was performed in cooperation with personnel from Biomed, Inc., Bellevue, Washington. *Vibrio anguillarum* (Biomed Inc., strain 1575), frozen in glycerol, was thawed and cultured in 100 ml trypticase soy broth (pH 7.4) for 18 hr. Serial dilutions (10^{-4} M, 10^{-5} M, and 10^{-6}M) of the broth culture were made in sterile PBS (pH 7.4). Fish were anesthetized with benzocaine/alcohol (10 g 100 ml^{-1}), using 1 ml 6 L^{-1} of water. Pathogen-containing media (0.1 ml) was injected intraperitoneally into each fish using a 1-ml syringe fitted with a 26 gauge x 3/8" needle. A preliminary challenge was conducted using 15 fish from the control diet for each of the three pathogen dose levels. The results indicated that the 10^{-6} M dose was optimum, producing 50% mortality in 10 d.

The day before the pathogen challenge test was to be performed, there was a disturbance in the water system. The nature of the disturbance was not determined; however, it was assumed to be a transient dechlorination problem. The disturbance resulted in the loss of control fish in both duplicates and also fish from diets #4, 6, and 7 in one duplicate set. Only duplicate sets and singles that experienced no losses were used for the challenge. Loss of the control fish made it necessary to substitute control fish from the preliminary pathogen challenge. All of the fish were allowed to acclimate before further experiments were begun. The disease challenge was conducted using the procedures described above, with the exception that methane tricaine sulfonate (MS 222; Argent Laboratories) was used as the anesthetic.

The Washington Department of Fish and Wildlife operates a fish trap in association with the Simson Hatchery on Bingham Creek, a tributary of the Satsop River (Grays Harbor County, Washington, USA). When adult coho salmon migrate upstream in the fall, a portion of the fish are trapped and used to supply eggs for the Bingham Creek Hatchery. The remaining fish are allowed to pass above the trap to spawn naturally. Approximately 15 mo later, the hatchery-reared smolts are released and allowed to migrate to the sea, along with naturally spawned smolts. The practices employed at this hatchery allow for comparison of wild and hatchery-reared smolts derived from the same parent stock. Twenty wild and twenty hatchery-reared coho smolts were obtained from this facility and analyzed for zinc and copper concentration.

GENERAL PROCEDURES FOR YEARS 1, 2, AND 3

Fish Transfers

Whenever transfer of fish was necessary, the fish were placed in an insulated fiberglass tank containing water cooled by ice. The dissolved oxygen level was maintained at saturation using bottled oxygen. Care was taken to prevent any physical damage to the fish.

Chemical Analyses

Fish were anesthetized with MS-222 in order to facilitate weighing and sacrificing. Weights were measured to the nearest g for fish, and to the nearest milligram for tissue samples.

Superoxide Dismutase Assays

Superoxide dismutase assays were performed on liver samples utilizing the method as described by this investigator (Roberts et al. 1987). The technique involves the generation of luminol-enhanced chemiluminescence by the superoxide anion produced in the xanthine-xanthine oxidase system. The basis of the measurement is the ability of SOD to sequester the superoxide ion and reduce chemiluminescence by 50%. Activity is related to concentration of protein required for this inhibition; therefore the smaller the amount of protein required for the inhibition, the greater the activity of the enzyme. The luminescence was monitored on a model 300 SATechnology Co. luminometer at 25°C. The enzyme source in the preparation is described below. In a previous study of fish liver tissue, this modified method was successfully employed (Roberts et al. 1987).

Hepatic glutathione peroxidase activity was measured following a modification of the method described by Flohé and Gunzler (1984). Oxidized glutathione (GSSG) is continuously reduced by an excess of glutathione reductase, actively providing for a constant level of reduced glutathione (GSH). The activity is determined by the concomitant oxidation of NADPH monitored photometrically at 340 nm.

Eviscerated carcass selenium was initially assayed by the indirect fluorescence procedure as specified by the American Society for Testing and Materials (Koh and Benson 1983). Later, a more sensitive method was tried (cathodic stripping voltometry at a dropping mercury electrode in a Princeton Applied Research Model 384B Polarograph). The benefit of this improved method lies in the fact that it is a direct, rather than an indirect, assay which measures the element itself instead of relying on the indirect measurement of a chromaphoric substance-to-element bonding. To conduct the procedure, the eviscerated carcass of the fish and an equal volume of distilled water were homogenized for 2-5 min to produce a smooth slurry. This slurry was then freeze dried to produce a powder. The dried tissue and blood samples were digested in a mixture of nitric and sulfuric acid in a ratio of 5:2. The digestion and sample preparation followed the method described by Adeloju and Bond (1983).

Blood selenium was measured in some fish. Blood was collected by severing the caudal peduncle. The blood was collected in heparinized tubes and used as whole blood for selenium assays. Digestion was made as described in Adeljou and Bond (1983). Assays were conducted using the polarographic procedure outlined in the paragraph above.

Eviscerated carcass vitamin C concentrations were measured to confirm that the fish were retaining the vitamin supplements. Ascorbic acid was analyzed using a method developed in this laboratory (Felton et al. 1994a). Briefly, tissue was deproteinized by TCA and assayed by HPLC.

Liver homogenates were prepared for superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and protein assays as follows: Livers were homogenized in 9 volumes of 0.1 M

K_2PO_4 buffer (pH 7.4) for 1 min with polytron homogenizing at top speed. Homogenates were centrifuged at 27,000 x g for 30 min; the precipitate was discarded and the supernatant was decanted and divided into equal aliquots. To 0.65 ml of one aliquot was added 0.034 ml of 0.1 M reduced glutathione. The pH was adjusted to 5.2 with 3 N HCl and the supernatant was allowed to incubate in the cold (5°C) for 1 h. The pH 5.2 solution was then centrifuged in the cold (5°C) at top speed in an Eppendorf Minifuge for 20 min. Supernatant solution was again collected and the pH was adjusted to 7.0 with 1 N NaOH. The GSH-Px assay and protein assay were performed on this final solution. The second aliquot of the ultra centrifuged supernatant solution was used for the SOD assay and commensurate protein determination. Protein concentrations were determined using the Lowry et al. (1951) procedure.

PROJECT MANAGEMENT

The study was managed and directed by Samuel P. Felton, principal investigator. The chemical analyses were performed primarily by Richard Grace with assistance and guidance from the principal investigator. Chemical analyses were performed in the biochemistry laboratories of the P.I. A number of the earlier selenium assays were performed in Dr. P.D. Whanger's laboratory (Department of Agricultural Chemistry, Oregon State University) and later confirmed by polarography in the laboratory of the principal investigator. Dr. Marsha Landolt, co-principal investigator, assisted in matters of pathology and report editing. Dr. Ronald Hardy (NMFS) was consulted on matters of diet and logistics. Dr. Lee Harrell (NMFS pathologist) was consulted on mortalities and disease problems. Dr. Robert Stickney was consulted on matters of nutrition. Mr. Evan Greger (Biomed, Inc., Bellevue, WA) provided invaluable technical assistance in the preparation and conduct of the pathogen challenge experiments.

FINDINGS

Accomplishments and findings will be discussed with respect to each year's objectives.

YEAR 1

The primary objective was to produce hatchery-reared fish with a body burden of selenium comparable to that found in wild counterparts. The diet used was a commercial feed supplemented with selenium. An additional saltwater adaptation experiment was possible with the cooperation of staff of the U.S. Fish and Wildlife Service's Marrowstone Island field station.

Table 4 presents the results of the Year 1 study. Diet #2 produced the dietary concentration of selenium needed to achieve the level found in wild fish. Midway through the feeding experiment the body burden of selenium appeared to decrease; however, by the 25th week it had returned almost to the level found at week 5. This decrease might be attributable to some type of metabolic change. Although Diet #2 level did not produce exactly the same selenium concentration as that found in the wild fish (2.93 vs. 3.6 $\mu g\ g^{-1}$ dry wt), it produced the desired growth rate. The only mortalities noted resulted from injuries incurred during tank maintenance and from fish that escaped from the tanks.

Table 4. Eviscerated carcass selenium concentration (μg g^{-1} dry weight). Data based on pooled samples (three fish each). The two sets of numbers represent data from duplicate tanks.

Sampling period	Fish on Diet 1 1.1 μg g^{-1} Se	Fish on Diet 2 8.6 μg g^{-1} Se	Fish on Diet 3 11.6 μg g^{-1} Se	Fish on Diet 4 13.6 μg g^{-1} Se
End of Week 5	1.10	3.23	3.89	4.77
	1.00	3.35	3.90	4.70
End of Week 12	0.65	2.35	3.03	3.70
	0.88	1.73	2.70	3.90
Avg. wt. in g	12.2	11.4	12.7	11.2
End of Week 25	1.25	2.56	4.00	4.35
	1.18	3.29	4.67	5.49
Avg. Se Dup.	1.22	2.93	4.34	4.92
Avg. wt. in g	22.7 ±2.62	23.5 ±7.09	22.7 ±7.51	28.4 ±4.37

Table 5 contains enzyme data from fish subjected to transportation stress. The test was conducted in Week 21 of the feeding trial. Fifteen fish from each diet group were transported from the Marrowstone Island field station to the University of Washington (2 hr travel time). During transportation the fish were held in 20-gallon containers that were bubbled with oxygen and cooled by ice. The fish were sampled before trucking and after arrival at UW. SOD decreased slightly in all groups except Diet #4. GSH-Px, the selenium sensitive enzyme, was virtually unchanged in Diet #2 but decreased by about 50 % in Diets #1, 3, and 4 even though there were no significant changes in blood selenium levels. One would expect that a 50% loss in activity by the GSH-Px enzyme would be accompanied by a loss of whole body selenium (Felton et al. 1989, Felton 1993). Such a loss would normally be reflected by an increase in blood selenium. The findings in this study contradicted that expectation.

To test the validity of this observation a second transportation experiment was performed analyzing individual fish instead of pooled samples. Table 6 shows GSH-Px and SOD activity in the dietary groups at week 21 before the stress test. Relative to fish fed diet #1, GSH-Px activity was increased by 32.4%, 52.5% and 23.5% in fish fed diets #2,3, and 4, respectively. Following exposure to transportation stress, hepatic GSH-Px activities within diets measured as follows: an increase in diet # 1; a decrease in diet # 3; no significant change in diets # 2 and # 4 (Table 2). At this time there is no clear explanation for the reversal of effects shown in diets # 1 and # 3.

When comparing the significance of the GSH-Px activities in fish fed the control diet 1 with fish fed diets 2, 3 and 4 before transfer, the following p values can be calculated: Diet 1 vs. Diet

Table 5. Changes in enzyme activities prior to and after transportation stress. Data collected at week 21. (Note: The smaller the protein concentration, the greater the SOD activity.) SOD data based upon pooled samples (three fish each) from duplicate tanks. GSH-Px data based on pooled samples (3 fish each); data include the mean and standard deviation of five assays. Year 1 study.

	Diet 1 1.1 μg g^{-1}	Diet 2 8.6 μg g^{-1}	Diet 3 11.6 μg g^{-1}	Diet 4 13.6 μg g^{-1}
SOD				
Before transfer (mg of protein needed for 50% inhibition)	4.51 5.17	6.90 6.42	6.16 6.58	10.17 9.27
After transfer	8.62 4.90	7.16 6.92	7.36 6.51	7.10 6.80
GSH-Px (liver) (nM min^{-1} mg $prot^{-1}$)				
Before transfer	12.20 ± 2.08	6.7 ± 1.03	10.02 ± 4.09	10.56 ± 2.41
After transfer	6.30 ± 3.12	7.08 ± 4.27	5.01 ± 1.70	6.32 ± 2.61
Selenium (whole blood) ppm				
Before transfer	0.224	0.690	1.050	1.194
After transfer	0.244	0.668	0.858	1.235

2 = 0.009; Diet 1 vs. Diet 3 = 0.003 and Diet 1 vs. Diet 4 = 0.010. However, when making the same comparison after transfer, the only diets showing any significance were Diet 1 vs. Diet 3 with a p value of 0.044.

Before the stress test, SOD activity was essentially the same in dietary groups # 1,2, and 3, and was depressed in the group fed diet # 4. The SOD activities following transfer were significantly depressed in diet # 4 but remained unaffected in diets # 1,2 and 3.

Tables 7, 8, and 9 show the effect of saltwater transition on enzyme levels and on selenium retention. Twenty fish from each diet group (including duplicates) were placed in tanks supplied with seawater at the Marrowstone Island field station. Mortalities were recorded weekly. Blood chemistries, enzyme activities and selenium concentrations were measured periodically. As explained earlier, SW contains unknown amounts of Se. Therefore, although the dietary groups were maintained separately, all fish were fed the basal (control) diet.

Table 7 illustrates the decline of eviscerated carcass selenium levels during the 6-mo period when all fish were fed the control diet. These data indicate that higher levels of dietary selenium may need to be fed during the freshwater phase of the life cycle if body burdens are to remain the

Table 6. Hepatic GSH-Px and SOD (mean ± SD) measurements before and after exposure to transportation stress at week 21. Dietary selenium = $\mu g\ g^{-1}$; GSH-Px = nM min^{-1} mg $protein^{-1}$; SOD = mg of protein needed for 50% inhibition. (n = 8) p value shows significance within diets before and after transfer stress. * denotes the diets with significant changes.

Sample I D	Diet #1 (1.1 $\mu g\ g^{-1}$ Se) (control diet)	Diet #2 (8.6 $\mu g\ g^{-1}$ Se)	Diet #3 (11.1 $\mu g\ g^{-1}$ Se)	Diet #4 (13.6 $\mu g\ g^{-1}$ Se)
GSH-Px				
Before transfer	7.36 ± 1.29 *	9.76 ± 1.77	11.24 ± 2.53 *	9.10 ± 1.02
After transfer	10.10 ± 1.12 *	10.61 ± 1.24	9.07 ± 0.53 *	9.04 ± 0.89
p =	0.0006	0.28	0.05	0.19
SOD				
Before transfer	5.20 ± 0.4	5.26 ± 0.6	5.28 ± 0.3	6.25 ± 0.7 *
After transfer	5.46 ± 0.5	5.01 ± 0.4	5.31 ± 0.7	7.54 ± 0.6 *
p =	0.23	0.32	0.89	0.0005

Before transfer:

Diet # 2 represents an increase of 32.4% over control diet # 1.
Diet # 3 represents an increase of 52.5% over control diet # 1.
Diet # 4 represents an increase of 23.5% over control diet # 1.

same as those of wild fish. Alternatively, it is possible that when fish are feeding under natural conditions, they could sustain selenium levels through their marine forage base.

After 21 wk, fish fed the elevated selenium diets (#2, 3, 4) had increased GSH-Px activity relative to that of control fish (Table 8). The same was generally true following seawater transfer. Fish fed the control diet maintained GSH-Px activity following seawater transfer, but fish in all of the supplemented diets lost GSH-Px activity. The conservation of GSH-Px by the control fish may reflect an attempt to conserve selenium.

Table 9 records the SOD enzyme data before and after 6 mo residence in saltwater. All groups had an increase in activity (the smaller the quantity of protein needed for 50 % inhibition, the greater the activity of the enzyme). SOD is an enzyme that is not dependent upon selenium for activity. This increase might have resulted from exposure to marine pollutants which were not present in freshwater (Roberts et al. 1987).

Table 7. Eviscerated carcass selenium concentrations before and after seawater adaptation. All fish fed control diet at point of entry to SW. Concentration based dry weight. Year 1 study.

Diet treatment	Before SW	After 2 mo	After 4 mo	After 5+ mo
#1 1.1 μg g^{-1}(control)	1.22	1.55	0.791	1.106
#2 8.6 μg g^{-1}	2.93	2.38	1.42	1.450
#3 11.6 μg g^{-1}	4.35	2.12	1.61	1.870
#4 13.6 μg g^{-1}	4.92	3.08	1.96	1.820

SW = Seawater

Table 8. Hepatic glutathione peroxidase levels (nM min^{-1} mg $prot^{-1}$) before and after seawater adaptation. The "before SW" data were taken at week 21 of the feeding trial. Year 1 study. All fish fed control diet at point of entry to SW.

Diet treatment	GSH-Px before SW	GSH-Px after 2 mo	GSH-Px after 4 mo	GSH-Px after 5+ mo
#1. 1.1 μg g^{-1} control	7.37	8.37 ± 0.80	8.75 ± 0.92	7.68 ± 1.33
#2. 8.6 μg g^{-1}	9.76	7.16 ± 0.33	8.90 ± 0.74	7.03 ± 1.30
#3. 11.6 μg g^{-1}	11.24	4.20 ± 1.12	11.64 ± 0.52	9.88 ± 1.61
#4. 13.6 μg g^{-1}	9.10	5.43 ± 1.14	8.36 ± 0.47	8.41 ± 0.47

Table 9. Hepatic SOD levels before and after seawater adaptation. n = 10. Year 1 study. All fish fed control diet at point of entry to SW.

Diet conc. SE	SOD before SW	SOD after 5+ mo in SW
#1 1.1 μg g^{-1} (Control)	5.20 ± 0.4	3.87
#2 8.6 μg g^{-1}	5.26 ± 0.6	4.10
#3 11.6 μg g^{-1}	5.28 ± 0.3	4.14
#4 13.6 μg g^{-1}	6.25 ± 0.7	3.97

SOD conc. = μg of protein needed to produce 50% inhibition. The lower the protein concentration the greater the activity.

Figure 1 illustrates cumulative weekly mortality during the 6 mo saltwater residence period. Note that there was no difference in cumulative mortality between the control (Diet #1) and the optimum selenium diet (Diet #2). Both groups lost 20%, indicating that the 8.6 $\mu g\ g^{-1}$ selenium concentration was not toxic and did not affect seawater adaptation. By contrast, mortality was higher in Diets #3 and 4. In Diet #4, especially, mortality began soon after seawater entry and reached almost 65% by the end of the study.

YEAR 2

The Year 2 study was fraught with difficulties. As discussed earlier, an extension was granted in order to repeat the 6-mo feeding study which was terminated following interruption of water flow. The objective for Year 2 was to utilize the optimum dietary selenium concentration found in Year 1 and to examine the effects of added antioxidants (vitamins C and E). The dietary compositions are shown in Table 2.

The data shown in Table 10 were accumulated before the water problem occurred at the Big Beef Creek field station. After 6 wk of feeding, there was an increase in the GSH-Px enzyme activity, commensurate with the enhanced dietary Se supplement. SOD activity decreased in all of the diets to which antioxidants were added. This type of decrease has been reported by other investigators studying similar antioxidant additions to diets. Dietary antioxidants have the ability to neutralize free radicals, reducing the need for a free radical scavenger such as the inducible SOD enzyme (Gaziano et al. 1992).

Table 11 shows differences in whole body concentrations of vitamin C in fish fed low and high vitamin C diets. The data indicate that the dietary vitamin C was accumulated in the body and was not lost via the urine. Vitamin C was, therefore, available for antioxidant needs in the fish (as indicated in the SOD activity decrease above).

A new stock of coho salmon was obtained from the George Adams Hatchery (WDFW) to restart the aborted 6-mo study. Table 12 shows baseline data on GSH-Px and eviscerated carcass selenium content before the feeding study began. It also demonstrates the consistency of the two measurements, Se and GSH-Px.

As mentioned previously, the Se duplicates had to be separated, one at UW and the other at NMFS. Table 13 compares the enzyme data for the two duplicate sets after the first 6 wk of the new feeding study. Basically, the enzyme data seem consistent with the first assay periods of the Big Beef Creek study. However, GSH-Px data from control fish at the UW were much higher than the control diets tested before and were higher than the NMFS control levels. The explanation for this discrepancy is unknown. The GSH-Px data from the other two groups were reasonable. The SOD activity of the NMFS group was slightly higher than that of the UW group (possibly indicative of pathogen presence among the NMFS group).

At the height of smoltification, there was an outbreak of furunculosis in the NMFS fish. Table 14 compares percent mortality in UW vs. NMFS fish at the time the NMFS fish were destroyed. There are several important observations to be noted in this comparison. First, the high Se diet (#4) in the NMFS set appears to have afforded some protection irrespective of the

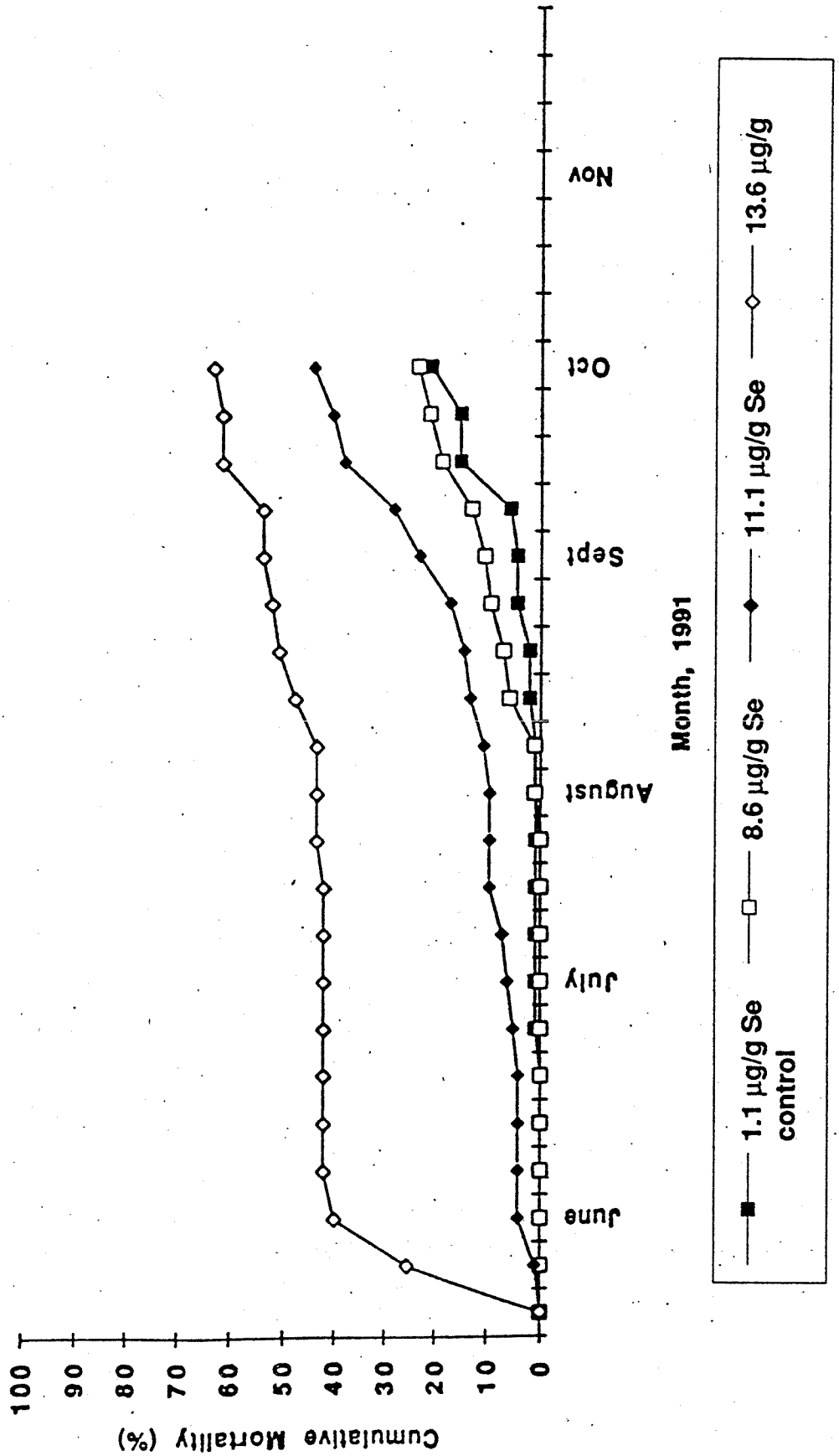

Figure 1. Cumulative weekly mortality during the 6-month saltwater residence period.

Table 10. Enzyme levels in first 6 mo feeding study at weeks 6, 12 and 18 (before water system broke). Units for GSH-Px are nM min^{-1} mg $protein^{-1}$; units for SOD are µg of protein needed to produce 50% inhibition. The lower the protein concentration the greater the activity. Set 1 and 2 are duplicates. n = 4 for enzymes and n = 2 for selenium concentrations. Year 2 study.

(A) Results after 6 wk

Diet	SOD at 6 wk	Selenium at 6 wk	GSH-Px at 6 wk. Set 1	GSH-Px at Set 2
Baseline	3.1 ±1.4	0.96 ± 0.31	6.41 ± 0.62	6.41 ± 0.62
#1 Control	3.1 ± 1.4	1.05	7.20 ± 0.53	6.40 ± 1.03
#2 E	4.2 ± 1.5	0.89	6.67 ± 0.51	7.34 ± 0.42
#3 Se + E	4.4 ± 0.51	2.80	7.05 ± 0.61	9.81 ± 1.82
#4. Se	5.2 ± 0.76	2.90	7.90 ± 1.02	7.69 ± 0.89
#5 Se + C	6.4 ± 0.65	3.10	8.31 ± 1.39	8.59 ± 0.91
#6 E + C	6.9 ± 1.20	0.99	8.17 ± 0.74	8.95 ± 0.58
#7 Se + E + C	7.4 ± 0.73	4.44	7.23 ± 1.70	7.39 ± 0.43

(B) Results after 12 and 18 wk

Diet	GSH-Px at 12 wk Set 1	GSH-Px at 12 wk Set 2	GSH-Px at 18 wk Set 1	Set 2 Destroyed
#1 Control	7.33 ± 0.23	7.54 ± 0.11	7.50 ± 0.41	nd
#2 E	7.38 ± 1.26	13.42 ± 3.62	7.95 ± 0.71	nd
#3 Se + E	9.09 ± 0.11	7.97 ± 1.08	8.79 ± 2.11	nd
#4 Se	6.31 ± 0.52	8.12 ± 0.59	8.34 ± 0.27	nd
#5 Se + C	7.56 ± 0.25	7.93 ± 0.83	9.57 ± 0.68	nd
#6 E + C	8.43 ± 0.39	8.46 ± 1.37	6.98 ± 1.24	nd
#7 Se + E + C	8.35 ± 0.85	7.27 ± 0.34	6.63 ± 0.21	nd

Table 11. Eviscerated carcass concentration of vitamin C in fish fed diets with recommended level of vitamin C vs. elevated vitamin C. Conc. μg g^{-1} wet weight. Data collected at week 18 on fish selected from diet #1 (regular vit. C) and diet # 5 (10X vit. C). Year 2 study.

Sample number	Reg. Vit C Diet	Supplemented Vit C Diet
1	21.48 μg	83.20 μg
2	30.23 μg	108.50 μg
3	31.30 μg	79.10 μg
4	21.21 μg	54.69 μg
Mean	24.96 μg	75.55 μg
S D	±5.32 μg	±23.09 μg

Table 12. Baseline eviscerated carcass selenium and hepatic GSH-Px data in new stock of George Adams Hatchery coho smolts before 6-mo feeding study. Year 2 study.

Sample	Se conc. in diet (mg kg^{-1})	GSH-Px (nM min^{-1} mg $protein^{-1}$)	Whole body Se (mg kg^{-1})
1	0.894	6.98	1.006
2	"	5.73	1.072
3	"	6.22	1.021
4	"	6.18	0.775
5	"	6.50	1.023
6	"	7.51	0.782
7	"	6.99	1.002
8	"	5.48	1.012
9	"	6.80	1.103
10	"	6.22	1.029
Mean	0.894	6.46	0.983
S D		±0.62	±0.112

Table 13. Enzyme activity comparisons of the two duplicates located at UW and at NMFS before the loss of the NMFS fish. n = 5. GSH-Px = nmol min^{-1} mg $protein^{-1}$; SOD = amt. of protein needed to produce 50% inhibition; vitamin C = µg g^{-1} wet wt. Year 2 study.

Diets	6 wk GSH-Px UW	6 wk GSH-Px NMFS	Whole body Vit C UW	Whole body Vit C NMFS	6 wk SOD UW	6 wk SOD NMFS
#1 Control	9.68 ± 1.87	7.22 ± 0.62	45.61	41.25	4.99 ± 0.78	4.87 ± 0.83
#2 E	9.95 ± 0.46	7.15 ± 0.38	30.66	nd	6.82 ± 0.31	5.33 ± 0.86
#3 Se + E	8.62 ± 1.58	8.88 ± 1.39	40.83	31.15	7.1 ± 0.92	4.82 ± 0.37
#4 Se	8.18 ± 0.64	6.52 ± 0.62	nd	nd	6.47 ± 1.03	4.95 ± 0.67
#5 Se + C	8.48 ± 1.23	8.52 ± 0.71	35.45	nd	7.17 ± 0.61	5.8 ± 1.37
#6 C + E	7.63 ± 0.72	8.02 ± 0.92	nd	nd	6.63 ± 0.56	4.64 ± 0.78
#7 Se + E + C	8.08 ± 0.56	6.94 ± 0.98	40.30	59.06	6.78 ± 1.32	5.91 ± 0.74

nd = not determined

Table 14. Percent mortality comparison between NMFS and UW fish (6/1/92 to 6/9/92). Vitamin E is at 25 X and C is at 10 X. High Se is at 8.6 µg g^{-1} as compared to the control at 0.9-1.1 µg g^{-1}. NMFS temperature = 19-20°C and UW temperature = 12-14°C. Year 2 study.

Diet Number	Added Components	NMFS Laboratory	U of W Laboratory
1	Control	75.2	5.4
2	E	69.5	0
3	Se + E	63.2	24.5
4	Se	45.2	11.4
5	Se + C	93.0	0
6	C + E	73.0	2.0
7	Se + E + C	83.0	44.0

high temperature and pathogen exposure. The presence of selenium in Diets # 3, 4, and 7 did not, however, offer any protection. Second, UW fish fed three of the diets (#2, 5, 6) were virtually unaffected by the pathogen exposure that was occurring at NMFS; however, NMFS fish fed the same diets did not have reduced mortalities. In the UW fish, highest mortalities occurred in Diet #7 which had high levels of vitamin C, vitamin E and selenium. This may be a reflection of immune suppression found by Blazer (1991) under similar conditions.

Table 15 documents enzyme activities in the UW fish prior to the stress test. The data are representative of healthy fish. It is noteworthy to point out the interaction of low SOD activity and high GSH-Px activity in Diets #4 and 5 at week 18. This phenomenon is not well understood since the role of SOD is to produce substrate for the GSH-Px enzyme. Selenium may be serving some antioxidant role other than as a cofactor for the GSH-Px system.

The UW study continued for the full 6 mo of feeding. During this time, normal smoltification occurred. Table 16 is a comprehensive table comparing three things: percent mortalities during the smolting period and the post smolting period; the results of the thermal stress test; the final 24-wk enzyme data. The highest mortalities among the smolts occurred in Diet #7. The post smolt period lasted more than 2.5 mo. There were no mortalities except for tank-cleaning injuries.

The thermal stress test was performed at the end of the post smolting period, after the final enzyme activities were measured. The test involved the transfer of 60 fish per diet to tanks supplied with Lake Washington water (unregulated temperature). The temperature change was from 14 to 19°C. Also, the purity of the water was unknown. An attempt was made to simulate the transfer of fish from a more or less pristine hatchery water environment to that of a less pure receiving water environment. It is important to note that the lowest number of stress test mortalities occurred in Diets #3 and 5. The commonalities in these diets were high concentrations of vitamin C or vitamin E combined with selenium. It is also important to note that selenium alone (Diet #4) did not offer the same protection. The next best diet was Diet #7 with all antioxidants at

Table 15. Enzyme assays on UW fish at weeks 12 and 18 after loss of NMFS fish. GSH-Px=nmoles/min/mg protein; SOD µg of protein needed for 50% inhibition. The means of 3 fish are shown. Year 2 study.

Diets	12 week GSH-Px	18 week GSH-Px	12 week SOD	18 week SOD
#1 Control	6.31 ± 0.90	8.86 ± 1.39	7.25 ± 0.70	7.4 ± 0.57
#2 E	6.83 ± 1.07	7.16 ± 0.90	8.66 ± 1.43	8.11 ± 0.60
#3 Se + E	7.87 ± 1.83	8.83 ± 1.54	7.85 ± 0.74	8.02 ± 0.64
#4 Se	9.8 ± 1.45	7.86 ± 0.66	10.29 ± 1.05	7.21 ± 0.85
#5 Se + C	9.1 ± 0.60	9.81 ± 0.93	10.62 ± 2.27	6.69 ± 0.41
#6 C + E	7.59 ± 1.34	7.2 ± 1.10	8.99 ± 0.80	6.32 ± 0.47
#7 Se + E + C	8.33 ± 1.34	8.88 ± 0.42	8.62 ± 1.00	5.9 ± 0.44

Table 16. Percent mortalities in the UW fish during smolting period, post smolting period, and the thermal stress test. Comparative enzyme activities at week 24 are from pooled samples (3 fish each). Year 2 study. LW = lake water.

Diets	Smolting % morts 6/10-6/30	Post-smolt % morts 7/10-9/28	Stress test in 19° LW % morts	GSH-Px in nmol min^{-1} mg $protein^{-1}$	SOD in µg of prot. for 50% inhib.
#1 Control	5.4	0.6	24.7	6.79 ± 0.76	7.41 ± 0.86
#2 E	0	0	28.0	6.49 ± 0.65	7.41 ± 0.60
#3 Se + E	24.6	0	9.1	7.45 ± 0.78	7.24 ± 0.96
#4 Se	11.4	0	76.0	8.26 ± 0.40	7.19 ± 0.78
#5 Se + C	0	0	1.4	8.37 ± 1.29	6.40 ± 0.39
#6 C + E	2.0	0.6	53.3	7.90 ± 1.27	6.03 ± 0.47
#7 Se + E + C	44.0	0	18.9	8.70 ± 0.94	5.55 ± 1.58

the elevated level. This finding contrasts with the results of the natural mortality data presented earlier.

GSH-Px activity was increased in all diets containing added selenium (Table 16). SOD activity was enhanced in diets # 5, 6, and 7. This was somewhat unexpected in view of the fact that vitamins C and E are considered free radical scavengers as is SOD.

YEAR 3

In the final year of the study the diets were further refined in terms of antioxidant additions. Concentrations were shown previously in Table 3. The objective was to conduct a long term feeding study measuring enzyme activities and whole body copper and zinc concentrations. The culmination was a pathogen challenge.

Figure 2 illustrates weight gain among all dietary groups. The average weight of the coho smolts at the time they were obtained from the WDFW George Adams hatchery was 8.73 grams. At 18 wk, all of the test diet groups had a higher percent gain when compared to the control diet, with Diet #4 giving the best average weight gain of 22.5 g. Diet #4 was the same as Diet #2 with the addition of 20 IU g^{-1} of vitamin A. The Diet #4 fish were visually brighter in skin color and the muscle tissue had a rich pink coloration.

Tables 17 and 18 contain enzyme data collected at the designated 6-wk periods. In each of the supplemented diets, the GSH-Px activities were elevated. It is interesting to note that in diets # 6 and 7, GSH-Px activities were more consistently elevated as compared to the control diet, suggesting that the high concentration of vitamin C, vitamin E was in some way maintaining selenium in a more active valence state, a state that favors the inducibility of the enzyme GSH-Px. A similar observation was made by Poovaiah et al. (1987).

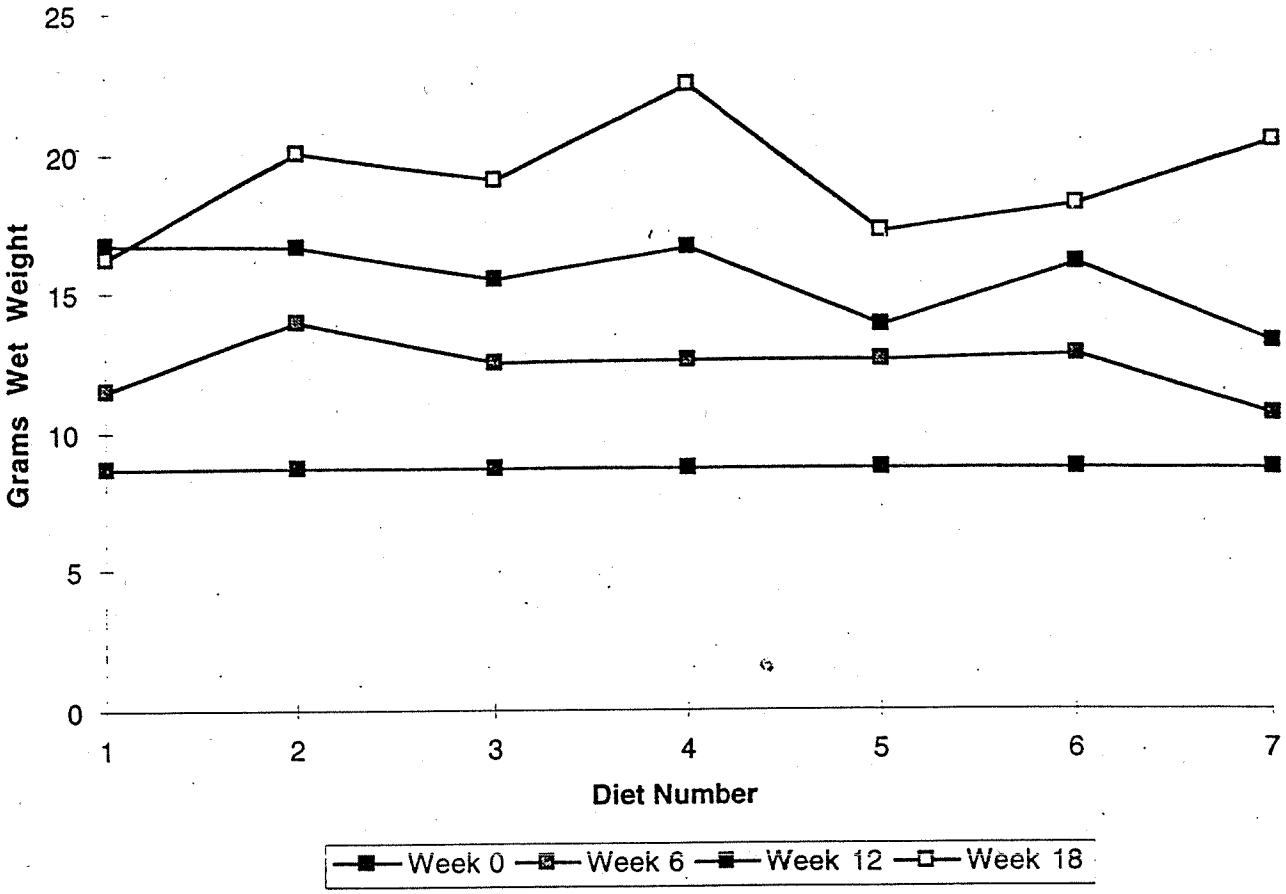

Figure 2. Cumulative weight gains in grams at weeks 6, 12, and 18. Starting weight = 8.73 g. Year 3 study.

Figure 3 shows the 24 week plot of the means from the GSH-Px activities at 6 wk. Diets 2 through 7 show an overall improvement trend in the GSH-Px activity when compared to diet 1 (Control). A similar average is shown in Figure 4 for SOD activities. The erratic SOD activity is clearly displayed in this figure. Table 18 illustrates the erratic behavior of the SOD enzyme (which is now believed to be due to zinc and copper deficiencies). These apparent deficiencies will be discussed later.

In Figure 4, one can see the same erratic behavior as shown in Table 18. The decrease in activity of the SOD enzyme may have been due to the fact that other added antioxidants in diets 2 through 7 decreased the free radicals, thereby making less substrate for the SOD enzyme (See reaction 1 below). Vitamin C has the capability of reducing a free radical, thereby helping to neutralize its destructive power.

In Year 3, the objective was to measure copper and zinc in the dietary groups. This measurement was planned in order to evaluate any changes in two other important micronutrients which are involved in the immune response system. In order to have a base of comparison for

Table 17. Glutathione peroxidase (GSH-Px) activities at the 6-wk periodic intervals of the 9-mo feeding study, expressed as nmol min^{-1} mg $protein^{-1}$ of protein. A and B are data from duplicate sets. Year 3 study.

(A) Set 1

Diet	6 wk	12 wk	18 wk	24 wk
#1 Control	5.99 ± 1.05	7.15 ± 1.22	5.76 ± 0.38	6.82 ± 0.26
#2 Hi Se + Hi E	6.30 ± 0.26	6.99 ± 0.36	7.85 ± 0.90	7.55 ± 0.68
#3 Lo Se + Lo E	5.90 ± 0.78	7.20 ± 0.61	7.38 ± 0.79	8.47± 0.47
#4. Hi Se + Hi E + A	7.47 ± 0.38	7.08 ± 1.00	7.48 ± 0.80	7.74± 1.20
#5 Hi Se + Hi C	8.37 ± 1.44	6.71 ± 0.99	6.79 ± 0.12	7.85 ± 0.79
#6 Hi Se + Ex Hi E + Hi C	7.35 ± 1.19	7.73 ± 0.80	7.31 ± 0.19	7.88 ± 0.71
#7 Lo Se + Lo E + Hi C	8.11 ± 2.13	6.95 ± 0.23	7.41 ± 0.94	7.72 ± 0.84

(B) Set 2

Diet	6 wk	12 wk	18 wk	24 wk
#1 Control	7.16 ± 1.53	6.31± 0.64	6.79 ± 0.65	6.45 ± 0.99
#2 Hi Se + Hi E	6.37 ± 0.82	7.05 ± 0.75	6.66 ± 0.16	7.36 ± 0.44
#3 Lo Se + Lo E	7.58 ± 1.13	7.06 ± 0.98	7.87 ± 1.81	7.61 ± 0.80
#4 Hi Se + Hi E + A	6.96 ± 0.67	7.97 ± 0.86	7.86 ± 0.90	7.21 ± 0.61
#5 Hi Se + Hi C	6.85 ± 0.50	7.47 ± 1.24	7.70 ± 0.69	8.78 ± 0.47
#6 Hi Se + Ex Hi E + Hi C	9.16 ± 1.21	7.20 ± 1.96	8.64 ± 0.86	7.67 ± 0.77
#7 Lo Se + Lo E + Hi C	8.48 ± 0.25	7.58 ± 1.71	8.34 ± 1.24	7.89 ± 0.43

these metals, it was necessary to return to the same stock of coho (Bingham Creek) in which the existence of a selenium deficiency was originally found in 1990.

Table 19 illustrates an important finding regarding whole body concentration of copper and zinc. The measurements were made on eviscerated carcasses and were related to wet weight, dry weight and milligram of protein. The data obtained produced a comparative basis for evaluating the two groups; but in addition they suggested why the SOD enzyme activity was so erratic, and possibly why the disease challenge was not more definitive. SOD is a key enzyme in the immune system which requires both copper and zinc in order to function. The key reactions may be visualized in two steps: In step 1 the free radical of oxygen is dismutated in the presence of the SOD enzyme to hydrogen peroxide. In step 2 the hydrogen peroxide is detoxified further to water with the aid of reduced-glutathione and the enzyme GSH-Px.

$$O_2^- + 2H\text{--(SOD)->} H_2O_2 \quad (1)$$

$$H_2O_2 + 2GSH\text{--(GSH-Px)-->}GSSG + 2H_2O \quad (2)$$

Table 18. Superoxide dismutase(SOD) activities at six week intervals of the final long term feeding study. Expressed as µg of protein required to produce 50 % inhibition. A and B are results from duplicate sets. Year 3 study.

Table 18 (A) Set 1

Diet	6 wk	12 wk	18 wk	24 wk
#1 Control	8.98±	7.36 ± 0.85	7.44 ± 1.27	11.07 ± 0.88
#2 Hi Se + Hi E	9.55 ±	7.21 ± 0.64	7.98 ± 1.05	9.82 ± 0.30
#3 Lo Se + Lo E	9.27 ±	7.63 ± 0.67	8.19 ± 0.21	10.25 ± 0.28
#4 Hi Se + Hi E + A	9.86 ±	8.02 ± 0.69	8.45 ± 0.32	10.36 ± 1.47
#5 Hi Se + Hi C	9.27 ±	7.69 ± 1.17	8.78 ± 0.17	10.58 ± 1.69
#6 Hi Se + Ex Hi E + Hi C	9.91 ±	6.59 ± 0.16	8.10 ± 0.51	10.55 ± 0.25
#7 Lo Se + Lo E + Hi C	9.27 ±	7.55 ± 0.57	7.46 ± 0.95	9.54 ± 0.07

Table 18 (B) Set 2

Diet	6 wk	12 wk	18 wk	24 wk
#1 Control	8.77± 0.20	7.33 ± 0.40	7.31 ± 0.43	9.34 ± 0.42
#2 Hi Se + Hi E	9.86 ± 0.15	7.93 ± 0.42	7.10 ± 0.31	9.10 ± 0.78
#3 Lo Se + Lo E	9.52 ± 0.30	8.31 ± 0.62	7.61 ± 0.92	9.86 ± 0.67
#4 Hi Se + Hi E + A	9.32 ± 1.04	8.77 ± 0.61	6.51 ± 0.96	8.69 ± 0.47
#5 Hi Se + Hi C	8.55 ± 1.51	8.32 ± 0.96	7.19 ± 0.53	9.09 ± 0.59
#6 Hi Se + Ex Hi E + Hi C	8.62 ± 1.22	9.39 ± 0.82	7.26 ± 0.61	8.56 ± 1.21
#7 Lo Se + Lo E + Hi C	7.85 ± 0.90	8.89 ± 0.66	6.84 ± 0.47	10.45 ± 1.02

Reaction (1) requires SOD, a copper/zinc requiring enzyme, to form substrate(H_2O_2) which then becomes the substrate for reaction (2). Therefore if SOD activity is limited by copper and zinc then the GSH-Px activity is limited as well, due to the lack of substrate, H_2O_2. Without deficiencies of selenium, copper and zinc it is expected that the reaction will be driven to the right and that SOD will show a concomitant increase with GSH-Px enzyme.

Tables 20 and 21 show the concentrations of zinc and copper in eviscerated carcasses of pooled fish (n=3) from each of the dietary groups at weeks 0, 12, 18, and 24. The concentrations of zinc and copper found in all of the test diets resembled those that were found in the hatchery-reared fish. This finding is to be expected since these diets were basically a commercial diet similar to that used in hatcheries. In Diets #2, 6 and 7, however, there seemed to be a conservation of copper similar to that seen in wild fish. This fact is interesting in that it may reflect the demands being placed on the SOD system by the enhanced GSH-Px enzyme.

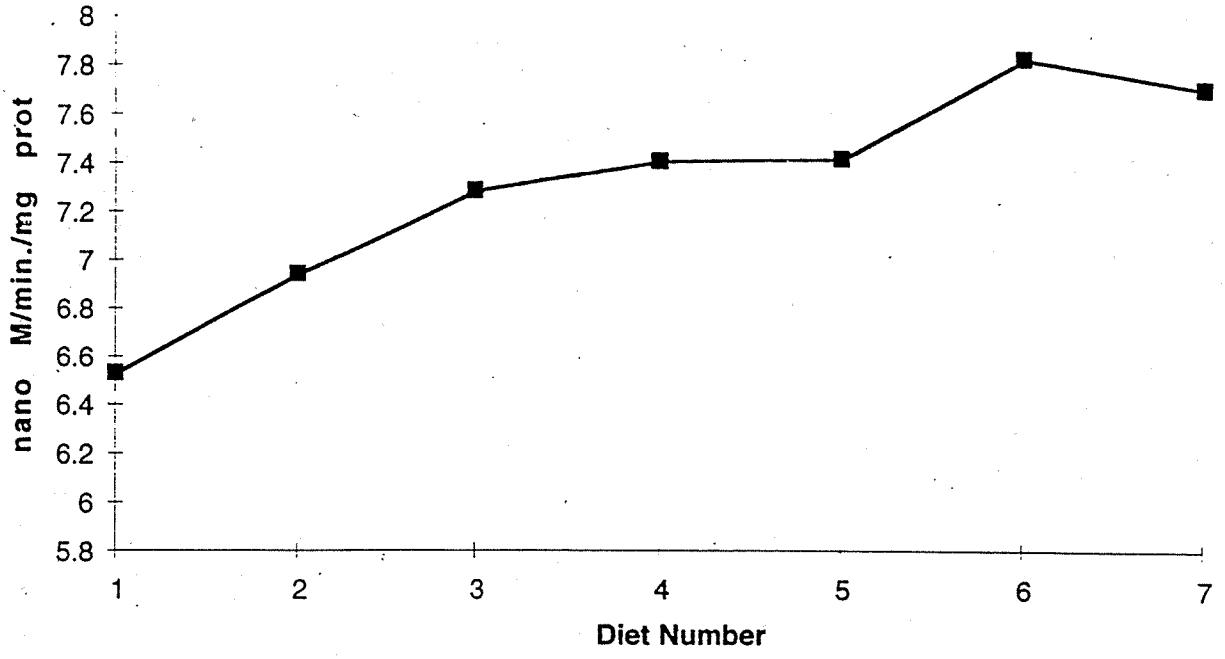

Figure 3. Average of GSH-Px 6-wk mean at end of 24-wk period.

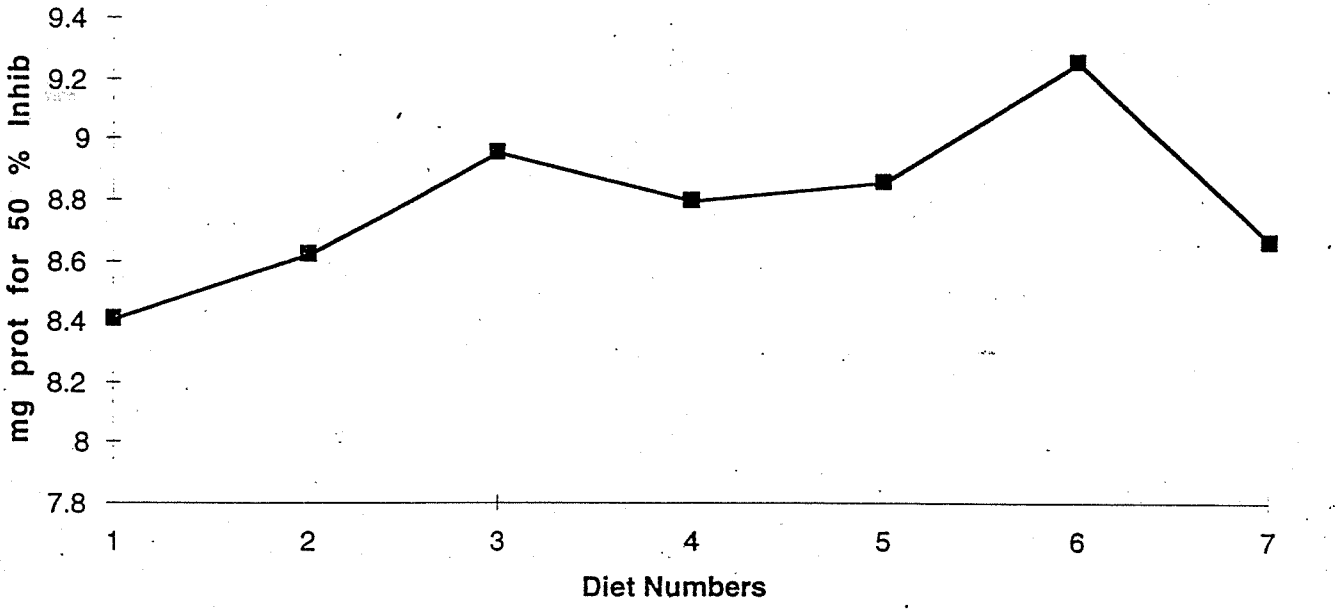

Figure 4. Average of SOD 6-wk means at end of 24-wk period.

Table 19. Zinc and copper concentrations (mean and standard deviation) in eviscerated carcass samples of hatchery-reared and naturally reared coho salmon smolts. (Felton et al. 1994b). Year 3 study.

Sample ID	Zinc concentration			Copper concentration		
	$\mu g\ g^{-1}$ wet wt	$\mu g\ g^{-1}$ dry wt	$\mu g\ mg^{-1}$ Prot.	$\mu g\ g^{-1}$ wet wt	$\mu g\ g^{-1}$ dry wt	$\mu g\ mg^{-1}$ Prot.
Hatchery	11.50	51.01	0.1110	1.705	7.54	0.0165
Std Dev	1.12	7.39	0.0163	0.103	0.67	0.0023
SEM	0.25	1.7	0.0036	0.023	0.15	0.0005
n=20						
Wild	16.79	81.3	0.1602	1.908	9.15	0.0185
Std Dev	4.35	25.0	0.0412	0.186	1.00	0.0039
SEM	0.97	5.6	0.0092	0.042	0.22	0.0008
n=20						

Table 20. Eviscerated carcass concentration of zinc in $\mu g\ g^{-1}$ wet weight tissue based on pooled samples (n=3). Year 3 study.

Diet	0 wk	12 wk	18 wk	24 wk	Mean of 24 wk
#1 Control	14.90	12.05	10,70	10.66	12.08
#2 Hi Se + Hi E	9.27	9.21	11.63	10.42	10.13
#3 Lo Se + Lo E	10.15	10.35	12.06	13.55	11.53
#4 Hi Se + Hi E + A	12.40	11.11	11.03	14.11	12.16
#5 Hi Se + Hi C	10.60	11.80	10.66	16.90	12.49
#6 Hi Se + Ex Hi E + Hi C	14.94	10.82	10.26	16.82	13.21
#7 Lo Se + Lo E + Hi C	NA	9.28	11.33	13.77	11.46

Table 21. Eviscerated carcass concentration of copper in $\mu g\ g^{-1}$ wet weight tissue based on pooled samples (n = 3). Year 3 study.

Diet	0 wk	12 wk	18 wk	24 wk	Mean of 24 wk
#1 Control	1.82	1.71	1.80	1.35	1.67
#2 Hi Se + Hi E	1.90	1.97	2.04	1.72	1.91
#3 Lo Se + Lo E	1.85	1.84	1.57	1.81	1.77
#4 Hi Se + Hi E + A	1.85	1.73	1.63	1.92	1.78
#5 Hi Se + Hi C	1.86	1.55	1.44	1.80	1.66
#6 Hi Se + Ex Hi E + Hi C	1.62	1.96	1.93	2.04	1.89
#7 Lo Se + Lo E + Hi C	NA	1.83	1.95	2.00	1.92

Table 22. Eviscerated whole body selenium concentrations at 12 and 24 wk compared to the baseline level of coho smolts at week 0. Concentration = $\mu g\ g^{-1}$. Year 3 study.

Diet	Week 0	Week 12	Week 24
#1 Control	1.003	1.123	1.228
#2 Hi Se + Hi E		1.924	1.850
#3 Lo Se + Lo E		1.450	1.510
#4 Hi Se + Hi E + A		1.810	1.950
#5 Hi Se + Hi C		1.315	1.835
#6 Hi Se + Ex Hi E + Hi C		1.641	1.750
#7 Lo Se + Lo E + Hi C		1.350	1.380

In the third year, diets #2, 4, 5, and 6 contained the optimum Se level established in the first year ($8.6\ \mu g\ g^{-1}$). Diet #1 (control) contained no additional selenium. Diets #3 and 7 contained half of the optimum level of $4.3\ \mu g\ g^{-1}$. The whole body selenium content held constant over the course of the feeding study, and was consistently higher than that in controls (Table 22).

The final experiment planned at the end of the long term feeding study was the direct inoculation of the test fish with a live pathogen. Inoculation is not a normal manner in which fish are exposed to pathogens; however, it is a sure method of exposure. There were a number of problems associated with this experiment (as described above). Periodic checks were made of ammonia, pH, dissolved oxygen and temperature in the test tanks. Ammonia levels were maintained at 0.1 to 0.4 ppm, dissolved O_2 at 90 to 100 %, pH at 6.8 to 7.1 and temperature at 15.0 to 15.2°C. The results of the challenge are shown in Tables 23 and 24. Table 23 illustrates daily mortality.

Table 23. Daily mortality and cumulative percent mortality resulting from *Vibrio anguillarum* challenge. Media refers to the media control (fish injected with media that did not contain pathogen). Some diets are not duplicated. (") = duplicate diet. n = 15 fish per tank. Year 3 study.

Diet	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Morts %
#1 n=15	0	0	2	5	1	0	0	0	0	0	53
#2 n=30	0	0	1	4	2	3	4	0	1	0	50
" n=30	0	0	2	3	3	2	1	0	0	0	37
#3 n=31	0	0	1	9	1	2	1	0	0	0	45
" n=31	0	0	0	3	0	1	0	0	0	0	13
#4 n=29	0	0	0	5	1	0	0	0	0	0	21
#5 n=28	0	0	0	4	3	1	1	0	0	0	32
" n=29	0	0	1	5	3	2	1	0	0	0	42
#6 n=31	0	0	1	2	2	1	0	0	0	0	20
#7 n=28	0	0	1	5	3	3	0	0	0	0	42
Media n=30	0	0	0	0	0	0	0	0	0	0	0
" n=30	0	0	0	0	0	0	0	0	0	0	0

Table 24. Summary of disease challenge, comparing means of duplicates to single test percentages. (*) indicates mean of duplicate sets. Year 3 study.

Diet	% morts
#1 Control	53
#2 Hi Se + Hi E	43*
#3 Lo Se + Lo E	29*
#4 Hi Se + Hi E + A	21
#5 Hi Se + Hi C	37*
#6 Hi Se + Ex Hi E + Hi C	20
#7 Lo Se + Lo E + Hi C	42

Tables 23 and 24 indicate that Diets #4 and 6 had the best survival. The better survival in diets # 4 and 6 seem to indicate that the combination of three antioxidants warrants further investigation. It may also indicate that there was increased cellular protection from free radical destruction. Diet 3 had the next best survival even though one duplicate was high.

CONCLUSIONS

Selenium alone was not expected to solve the problem of hatchery disease, since it is only one of a number of vital micronutrients. The study did show, however, that the dietary level of selenium could be elevated to a point where hatchery-bred fish would equal their wild counterparts and still remain healthy.

It was interesting that SOD and GSH-Px enzyme measurements did not reveal consistent or expected activities. GSH-Px, a selenium-dependent enzyme, changed in activity but reached a plateau that did not correlate well with selenium supplementation. Changes in SOD activity were erratic and demonstrated an inverse relationship to GSH-Px activities. While the survival data were not definitive, they did indicate other existing weaknesses in the immune system. The inconsistent activities of SOD and GSH-Px are more easily understood in light of the findings concerning copper and zinc deficiencies in hatchery-reared fish.

Phase I results bode well for the next phases of the on-going attempt to utilize natural nutrients in order to produce healthy fish without the use of costly drugs and chemicals.

NEED FOR ADDITIONAL WORK

In order to further this nutritional approach of improving disease resistance in fish, there is a need to develop a more inclusive database. In addition to the micronutrients studied above, iron needs to be evaluated. In addition to the enzymes studied above, it is necessary to analyze the activities of cytochrome c oxidase, alkaline phosphatase and ribonuclease. The data can be

acquired from the following salmonid runs where wild and hatchery-reared smolts of the same parentage are available:

Idaho: The Snake River Falls, Lions Ferry Hatchery and NMFS fish ladder study site (Deschutes Pelton Ladder).

Washington: Yakima River, Leavenworth Hatchery (Wenatchee River) and Bingham Creek Hatchery.

Others may be selected. This more-inclusive database will provide the information needed to design a dietary study that compensates for discovered deficiencies, therefore making it possible to raise healthy and viable salmon .

EVALUATION

GOALS ACHIEVED

1. Coho salmon were reared on elevated levels of dietary selenium for a period of 9-11 mo. They suffered no mortalities when fed concentrations as high as 10 times that found in commercial diets. GSH-Px, a key enzyme in the host defense system, was enhanced in activity by 30 to 50 %. SOD, a copper and zinc requiring enzyme, was not significantly affected.
2. When hatchery-reared coho salmon were fed selenium at a level of 8.6 $\mu g\ g^{-1}$ (as compared with 1.1 $\mu g\ g^{-1}$ in the commercial diet), they attained a body burden of selenium equal to that found in wild coho.
3. The fish fed the optimal dietary selenium concentration (8.6 $\mu g\ g^{-1}$) adapted to saltwater as well as did fish fed a normal diet(1.1 $\mu g\ g^{-1}$).
4. Stress data indicated that when coho salmon were transported by truck from one laboratory to another there was a loss of activity in glutathione peroxidase (a selenium requiring enzyme), thus placing a burden on host defense mechanisms.
5. When coho salmon were transferred from a cold water hatchery environment (12°C) to a warmer (19°C) and more contaminated environment, a significant resistance to mortality was demonstrated by fish reared on enhanced diets.
6. In the stress test, there was an apparent interaction between vitamin enhancement and micronutrient enhancement . Vitamin E enhancement alone was not sufficient; however, with both vitamin E and Se enhancement there was substantially improved survival. A similar combined effect has been noted in human studies (Willett 1986). With Se enhancement alone mortality was exceptionally high. However when Se was coupled with vitamin C, the lowest mortality was observed. Interaction between vitamin C and Se has been noted in guinea pigs as well (Poovaiah et al. 1987). Without increased Se enhancement, vitamin C and vitamin E yielded very poor survival. When all three

antioxidants were enhanced in the diet at the same time, there was an apparent decrease in protection (as compared with the same combination using normal vitamin C supplement). A similar observation was made by Blazer (1991).

7. There was an apparent inconsistency in the SOD data. In all probability this inconsistency reflects the low copper and zinc concentrations found in hatchery-reared fish (Felton et al. 1994b). GSH-Px activity was related to Se enhancement in the diets. The SOD and GSH-Px determinations represent the mean of three measurements taken on fish from each dietary group.
8. A long-term feeding study was completed, using an optimum selenium concentration of 8.6 $\mu g\ g^{-1}$ with varying antioxidant concentrations, producing the following results:
   (a) No significant mortalities occurred.
   (b) Some test diets appear to afford protection against transportation trauma and stress.
   (c) The enzyme GSH-Px was consistently more active in fish fed the test diets than in fish fed the control diet.
   (d) A pathogen challenge test was performed to evaluate the assistance of the selenium and antioxidant-rich diets in host immune systems.
   (e) Copper and zinc were measured in the eviscerated carcasses of fish from the seven dietary treatments (all of which contained the same concentrations of metals as those found in commercial diets). In all dietary groups the concentration of zinc was less than that found in wild fish. This observation is consistent with measurements that were made in other hatchery-reared fish.

All goals were realized with the exception of the disease challenge. However a more reliable method is now available for the evaluation of disease resistance and can be employed in the continuation of this study. The overall information supports the hypothesis that hatchery-reared fish are treatable with supplemented diets which improve their health and disease resistance.

DISSEMINATION OF PROJECT RESULTS

The results are being circulated to the fishing industry and fish feed industry through the media of journal and trade publications. A paper on the zinc and copper deficiency already has been published (Significantly higher levels of zinc and copper found in wild compared to hatchery-reared coho salmon smolts *Oncorhynchus kisutch* . Diseases of Aquatic Organisms 1994 Vol 18: 233-236). A second paper has been accepted for publication by *Aquaculture and Fisheries Management* (Effects of selenium dietary enhancement on hatchery-reared coho when compared with wild coho: Hepatic enzymes and seawater adaptation evaluated). In June 1994, personal conferences with colleagues in Norway brought to their attention the impact of this approach.

A definitive paper is in preparation to cover the essential findings of years 2 and 3. Other areas of exposure will be: a University of Washington publication, *Research in Fisheries;* invitations and

inquiries from industry representatives such as Dr. E. English of Battelle Northwest; Dr. Subramanyam, vice president of research for Zeigler Brothers Feed Company; and Dr. David Erickson, director of technical services for Clear Springs Foods, Inc., of Buhl, Idaho.

As a result of the *Diseases of Aquatic Organisms* paper (cited above), requests have been submitted by Dr. James Congleton (Cooperative Fish and Wildlife Research Unit, University of Idaho) and Dr. Steven Schroder (State of Washington Department of Fish and Wildlife). Their proposals are for surveys of deficiencies in other species and in other locations. In addition, there has been a very large demand for reprints of this paper (worldwide). An even larger response is anticipated for the selenium paper now in press.

Armed with the information from this first phase, the focus for Phase II is clear—to follow-up the supplementations of the micronutrients copper and zinc, and consider adding iron. When these findings have been tallied, there is a realistic promise for a stronger immune response system in salmon, leading to a greater return of threatened and endangered stocks.

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