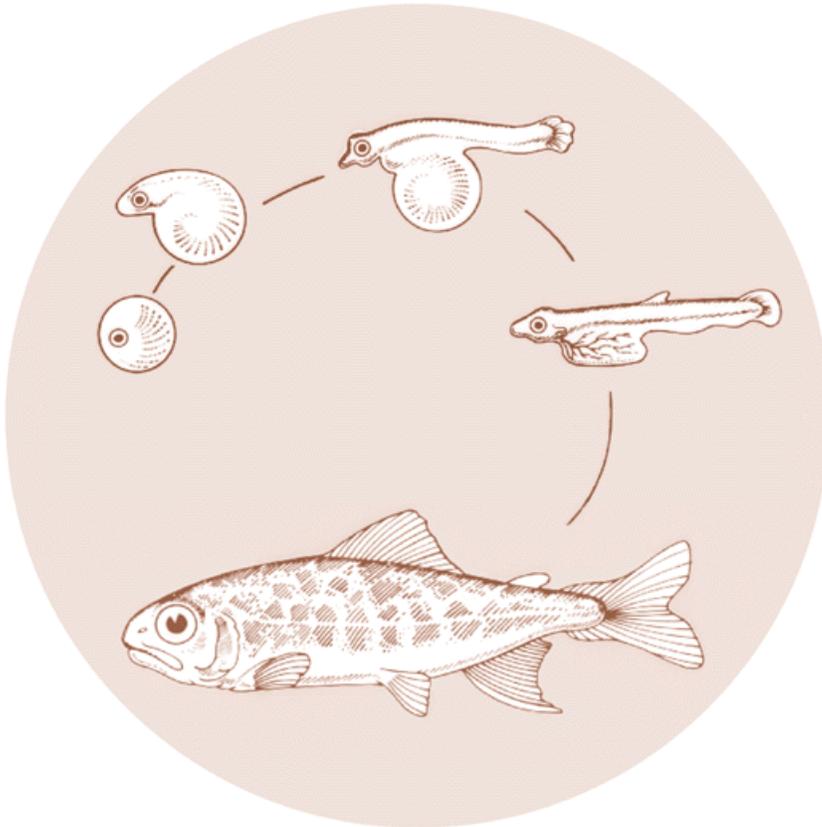


July 1989

# EFFECTS OF VITAMIN NUTRITION ON THE IMMUNE RESPONSE OF HATCHERY-REARED SALMONIDS

Final Report



DOE/BP-18010-2



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# EFFECTS OF VITAMIN NUTRITION ON THE IMMUNE RESPONSE OF HATCHERY-REARED SALMONIDS

Final Report

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July 1989

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## ABSTRACT

The objectives of this study were to define the dietary concentrations of folic acid, pyridoxine, riboflavin, pantothenic acid, ascorbic acid, and alpha-tocopherol required to assure maximum immune responses and resistance to Aeromonas salmonicida and Renibacterium salmoninarum in juvenile spring chinook salmon (Oncorhynchus tshawytscha).

In the first year of the study a number of *in vitro* assays were evaluated as tools for measuring the immunological status of juvenile chinook salmon. Those assays included: response of lymphocytes to mitogens, mixed lymphocyte reactions, migration inhibition factor, measures of phagocytosis, plaque-forming cell counts, and polyclonal activation. The plaque-forming cell and mitogenic stimulation assays were selected as the most appropriate tests to **use** in our scheduled studies to evaluate the effects of vitamin nutrition. The plaque-forming cell assay measures the capacity of lymphocytes to produce a specific immune response to an antigen, while the mitogen assay measures the general immunocompetency of an entire population of lymphocytes. Also, the technical requirements for both of these measurements made it practical to perform the tests on the large number of fish involved.

During the final three years of the project, graded levels of the test vitamins were incorporated in both the Oregon Test Diet and the Abernathy Diet and fed to small groups of spring chinook salmon juveniles. The fish were held in constant **12°** well water under laboratory conditions for up to six months. During that period, the fish's immunocompetency was measured using plaque-forming cell and mitogenic assays. In addition, their resistance to laboratory challenges with Aeromonas salmonicida and Renibacterium salmoninarum was determined.

Results indicated that the dietary levels of the test vitamins recommended for maximum growth by the National Research Council (NRC) were ample for growth and immunological function under our laboratory conditions. In **fact**, evidence indicated that, in some cases, concentrations of individual vitamins as low as one third of the NRC recommendation were adequate. The findings suggest that the NRC recommendations should be re-examined and refined.

In the majority of cases, the values of the immunological parameters significantly increased with fish age or size. Further experiments will be required to determine the degree to which each factor is important. The results also suggest that attempts to immunize fish may be most successful if they are performed when the fish are older than six months.

It should be noted that there was considerable variation in immunological measurements among fish within replicate lots fed individual diets. This has important implications for the immunological competency of significant segments of fish populations in hatcheries.

## INTRODUCTION

The presence of infectious diseases is one of the most important problems affecting the efficiency and economics of salmonid culture. Total mortality experienced during the egg, fry, and fingerling stages of Pacific salmon (*Oncorhynchus* spp.) culture, for example, normally ranges from **15-20%** (Hublou and Jones, 1970). About 50% of that mortality occurs during the fry and fingerling phases, and most of those deaths are probably attributable to infectious disease. This results in the loss of many millions of fish each year. Additionally, mortality can significantly exceed normal ranges in the event of epizootics resulting in even larger losses during artificial propagation.

Diseases can also produce debilitated fish or survivors which harbor latent infections. Low-grade or latent infections have the potential for significantly reducing the post-release survival of smolts from hatcheries. For example, Sanders (1979) and Banner et al. (1983) have found that coho salmon (*O. kisutch*) fingerlings, suffering a low-grade infection of bacterial kidney **disease (*Renibacterium salmoninarum*)**, died at high rates after transfer to seawater. Also, Wedemeyer et al. (1976) reported similar effects when coho **smolts** had subclinical cases of furunculosis (*Aeromonas salmonicida*).

Considerable research has been done to better understand fish diseases and develop means for their prevention and treatment. That work has concentrated primarily upon identification of agents causing disorders, improving diagnostic methods, exploring chemotherapeutic treatments, and developing vaccines. Very little work, however, has been done to determine the relationships among nutritional state, immunocompetence, and disease resistance in fish (Bell et al., 1984; Blazer, 1982; Blazer and Wolke, 1984; Bowser et al., 1988; Durve and Lovell, 1982; Hardy et al., 1979; Lall et al., 1985; Li and Lovell, 1985; Navarre, 1985; Paterson et al., 1981; Salte et al., 1988).

Research with livestock, laboratory animals, and humans has provided a large body of information which demonstrates the importance of host nutrition in combating disease. Observations made by pathologists at hatcheries indicate this is also true for fish. For example, Wood (1974) reports case histories in which juvenile coho salmon fed either a dry diet or a moist pellet containing corn gluten meal suffered higher mortality from bacterial kidney disease than did fish receiving the standard moist pellet formula. Wedemeyer and Ross (1973) could not confirm that corn gluten meal increased kidney disease mortality, but fish fed the corn product exhibited a more severe, nonspecific, stress-response to the infection.

Many vitamins have significant roles in the functioning of immune systems in man and other animals. Several researchers and reviewers (Axelrod and Traketellis, 1964; Beisel, 1982; Blalock et al., 1984; Cunningham-Rundles, 1982; Debes and Kirskey, 1979; Scott et al., 1976; Wilgus, 1977) have reported that a **deficiency** of pyridoxine suppresses the immune response in a number of animals. The requirement is dependent, in part, on the amount of dietary protein (Scott et al., 1976). Hardy et al. (1979) found that increased pyridoxine in a high-protein diet rendered chinook salmon fingerlings more resistant to *Vibrio anguillarum*, but additional pyridoxine in a low-protein feed did not have the same effect. Cell mediated responses have been

demonstrated to be dramatically affected by reduced pyridoxine, as evidenced by depressed mixed lymphocyte reactivity, prolonged allograft survival, and reduced skin hypersensitivity (Axelrod and Traketellis, 1964). Numbers of blood lymphocytes are dramatically reduced, as well as the weight and size of the spleen and **thymus** during pyridoxine deficiency (Debes and Kirskey, 1979). Antibody formation is decreased as determined by bacterial agglutination and plaque forming cell responses in rats, swine, chickens and humans fed diets deficient in pantothenic acid (Beisel, 1982; Nelson, 1978; Panda and Combs, 1963; Scott et al., 1976), however, that vitamin apparently has little effect on cell mediated immunity. Insufficient amounts of dietary riboflavin have also caused decreased antibody responses to Streptococcus pullorum in chicks (Beisel, 1982). Deficiencies of **folic** acid lead to reduced host resistance to salmonella and impaired **humoral** and cellular immune function in both man and experimental animals (Beisel, 1982; Cunningham-Rundles, 1982; Scott et al., 1976; Siddons, 1978). Impaired functioning of phagocytes and reduced response to phytohemagglutinin have also been observed in vitamin B-12 deficiency states. Inadequate biotin intake in rats causes a reduced passive hemagglutinating antibody response to diphtheria toxoid, and reduced development of splenic plaque-forming cells after inoculation with sheep red blood cells (Beisel, 1982). The amount of ascorbic acid in the feed has reportedly influenced disease susceptibility in several animals (Chatterjee, 1978). Durve and Lovell (1982) found that channel catfish (Ictalurus punctatus) were more resistant to the bacterium, Edwardsiella tarda when fed elevated doses of ascorbic acid and the effect was more pronounced at lower temperatures. Li and Lovell (1985) reported that channel catfish fed increased amounts of dietary ascorbic acid experienced decreased mortality due to E. ictaluri. Blazer (1982) found that ascorbic acid deficiency depressed non-specific resistance, decreased serum iron binding capacity, reduced phagocytosis, and depressed cellular responses to Yersinia ruckeri in rainbow trout (Oncorhynchus mykiss). Navarre (1985) stated that the defenses of rainbow trout against Vibrio anguillarum were directly related to dietary supplementation with ascorbic acid. On the other hand, Bell et al. (1984) had equivocal results when studying the effects of ascorbate on the development of bacterial kidney disease in sockeye salmon (O. nerka) roles performed by this vitamin are still unclear and controversial; however, it may play an important part in the normal functioning of phagocytic cells (Beisel, 1982; **Cunningham-Rundles**, 1982). Two fat soluble vitamins, A and E, have recognized effects on immune systems of other animals. Vitamin **A** probably influences resistance to infections through its role in maintaining the integrity of epithelial and mucosal membranes as well as affecting **humoral** and cellular immune responses (Beisel, 1982; Pan & Combs, 1963). Vitamin E has been shown to improve the **humoral** immune response of mice, chicks, turkeys, swine, sheep, and guinea pigs when they are challenged with either non-living antigens, living bacteria, or live viruses (Beisel, 1982; Colnago et al., 1984; Ellis and Vorhies, 1976; Heinseling et al., 1974; **Nockels**, 1980). Blazer (1982) and Blazer and Wolke (1984) found that rainbow trout showed a significantly reduced **humoral** immune response and reduction of several non-specific resistance factors when fed diets deficient in vitamin E. On the other hand, Salte et al. (1988) could show no beneficial effect of dietary vitamin E supplementation alone or in combination with selenium as a prophylaxis for Hitra Disease in Norway. Since vitamin E is an antioxidant which prevents autooxidation of lipids and/or protects animals from toxic effects of oxidation products (Lee and Sinnhuber, 1972), the amount of lipid in the diet and its degree of rancidity could be important factors in the

relationship between this vitamin and immunity. Past research to define the quantitative vitamin needs of juvenile salmonids has concentrated primarily on determining requirements for maximum growth and **most** efficient feed utilization (Halver, 1972; National Academy of Sciences, 1973) without attempts to assess the immunocompetency of test animals. Work is needed to determine the **quantities** of key vitamins required to insure optimal **functioning** of immune systems and high resistance to common diseases.

CHAPTER I

DEVELOPMENT OF **IMMUNOLOGICAL** ASSAYS

## OBJECTIVES

The primary objective of the first year of this project was to develop and assess the efficacy of a variety of assays that could be used to evaluate, at regular intervals, the immunological status of juvenile chinook salmon (*Oncorhynchus tshawytscha*) held on various diet treatments. In choosing the most appropriate assays for this project three criteria had to be met: 1) the assays must be performed on a large number of individuals over a relatively short period of time (12-24 individuals from 10 duplicated treatments within a period of one day), 2) the assays must be quantitative and 3) optimally the assays should monitor different aspects of immunological function.

The assays which were evaluated were mitogen, mixed lymphocyte reaction, migration inhibition factor, phagocytosis, plaque-forming cell, and polyclonal activation.

## MATERIALS AND METHODS

**Animals:** Spring chinook salmon were used as test animals in all experiments. They were obtained as eyed eggs resulting from adult fish spawned at Carson National Fish Hatchery located about 15 miles north of Carson, WA on the Wind River. Eggs were transferred to the Abernathy Salmon Culture Technology Center (The Center). Upon arrival at The Center, the eggs were surface disinfected with an iodophor (Argentine, Argent Chemical Laboratories, Redmond, WA) according to the protocol recommended by Wood (1974). At a size of 20-100 g the juvenile salmon were transferred to the Oregon State University Fish Disease Laboratory (OSUFDL). All fish were maintained in 460-liter circular fiberglass tanks supplied with 12° well water.

**Culture Media:** Media components were purchased from Whittaker H.A. Bioproducts, Walkersville, MD, unless otherwise noted. Media were selected after testing two media [Roswell Park Memorial Institute (RPMI) 1640 and Leibowitz-15 (L-15)] and two serum sources (fetal calf and chinook salmon). Mishell-Dutton holding medium consisted of 100  $\mu\text{g/ml}$  gentamicin and 10% fetal calf serum in RPMI 1640 (GIBCO, Grand Island, NY). Mishell-Dutton modified (MDM) RPMI was used for tissue culture and consisted of RPMI 1640 supplemented with: non-essential amino acids, sodium pyruvate, L-glutamine, 10% fetal calf serum (hybridoma screened), 100  $\mu\text{g/ml}$  gentamicin, 50  $\mu\text{M}$  2-mercaptoethanol (Cincinnati, OH), and the nucleosides adenosine, uracil, cytosine, and guanine (10  $\mu\text{g/ml}$ , Sigma, St. Louis, MO) as described by Yui and Kaattari, 1987 (See Appendix). A nutritional supplement was also prepared as described by Tittle and Rittenberg (1978) and fed daily to the cultures as described below.

**Cell Cultures:** A single cell suspension of each organ was obtained by aspiration through a 1 ml syringe after which holding medium was added. Organs from several fish were pooled when necessary to obtain the required number of cells for culture. The resulting cell suspension was incubated on ice to allow organ fragments to settle. The supernatant medium, containing single cells in suspension, was then washed two times in holding medium by centrifugation at 500 x g for 10 minutes at 4°C. The final washed cell pellet was resuspended in MDM. Lymphocytes were enumerated by the use of a hemocytometer or Coulter counter (Coulter Electronics, Hialeah, FL) adjusted

for counting **salmonid** leukocytes. The **cell concentration** was then adjusted with **MDM** to yield a concentration of  $1 \times 10^7$  cells/ml and held on ice until cultured. Fifty  $\mu\text{l}$  aliquots of the final cell suspension were added to the wells of a 96-well, flat-bottomed, tissue culture plate (Corning, Corning, NY) containing antigen or mitogen. Tissue culture plates were then incubated in plastic culture boxes (C.B.S. Scientific, Del Mar, CA) in an atmosphere of 10%  $\text{CO}_2$  at  $16^\circ\text{C}$ . The cultures used for the PFC (plaque-forming cell) assays were maintained by adding 10 ml of nutritional supplement daily.

**Mitogens and Antigens:** A stock solution of lipopolysaccharide B (LPS) from E. coli 055:B5 (Difco, Detroit, MI) was pasteurized for 30 minutes at  $70^\circ\text{C}$  in distilled water. Stock solutions of phytohemagglutinin (PHA, Sigma), **pokeweed** mitogen (Sigma), and concanavalin A type III (Sigma) were made up in MDM and sterilized by filtration through a  $0.45 \mu\text{m}$  filter. Trinitrophenylated-LPS (TNP-LPS) was prepared by the method of Jacobs and Morrison (1975). All mitogens and antigens were diluted into tissue culture medium at two times the final concentration, so that the addition of an equal volume of cells would yield the desired concentration of all components.

**Vibrio anguillarum Extract:** The *Vibrio* extract was prepared from V. anguillarum strain LS-174 which had been **formalin** killed and stored frozen. Fifty ml thawed packed cells were suspended in 10 volumes of 2% saline and placed in a boiling water bath for 2 hours. Cells were washed 3 times in 2% saline, centrifuged at  $10,000 \times g$  for 10 min at  $4^\circ\text{C}$ , resuspended in 95% ethanol, and incubated 48 hours at  $37^\circ\text{C}$ . The cells were then washed 2 times in acetone, centrifuged at  $3,000 \times g$  for 10 min and dried to paste overnight at  $37^\circ\text{C}$ . The paste was ground to a fine powder with mortar and pestle and stored at  $4^\circ\text{C}$ . The soluble *Vibrio* extract used for these studies was prepared by boiling the powder in phosphate buffered saline (PBS) at  $10 \text{ mg/ml}$  in a boiling water bath for 1 hour with frequent agitation. This suspension was then centrifuged at  $1,000 \times g$  to remove **particulates** and filter sterilized. Protein concentration were determined by the method of Lowry et al., (1951).

**Mitogen Assays:** The ability of lymphocytes to undergo proliferation upon stimulation by various mitogens, **was** assessed by the uptake of tritiated thymidine. The radioactive counts/minute (cpm) increase as the amount of proliferation increases. This ability to proliferate then **becomes** a measure of lymphocytic activation. Briefly, fifty  $\mu\text{l}$  containing  $5 \times 10^5$  cells were placed in individual wells of a 96-well flat bottom tissue culture plate with  $50 \mu\text{l}$  of mitogen or culture medium. The plates were then incubated in gas boxes under 10%  $\text{CO}_2$  at  $17^\circ\text{C}$ . Twenty four hours **b**efore harvest each well was pulsed with  $1 \mu\text{Ci}$  of tritiated thymidine (methyl- $^3\text{H}$ , ICN **Biomedicals**, Irvine, CA) in  $50 \mu\text{l}$  of MDM. Cells were harvested with distilled water onto glass fiber filters with a Skatron cell harvester (Sterling, VA). The filters were then dried, placed in scintillation vials with a solubilizing agent [**6g 2,5-diphenyloxazole (PPO), Sigma, 5 mg p-bis(2-(5-phenyloxazolyl))benzene (POPOP), Amersham, Arlington Heights, IL, in 1 liter toluene, after Etlinger et al., 1976**], and counted on a Beckman liquid scintillation counter (LS 8000). Data are reported as mean or as stimulation indices (SI) defined as  $(\text{experimental cpm})/(\text{control cpm})$ .

**Mixed Lymphocyte Reaction (MLR).** The ability of lymphocytes to recognize and respond to dissimilar antigens expressed on lymphocytes from other

heterologous individuals is measured by a mixed lymphocyte assay. As in the mitogen assays, incorporation of tritiated thymidine is a direct measure of the proliferation caused (in this case) by the stimulation by heterologous lymphocytes. These mixed lymphocyte reactions were performed utilizing the same media and culture conditions described for the mitogen assays. Briefly, 50  $\mu\text{l}$  of a lymphocyte suspension ( $1 \times 10^7$  cells/ml) from individual fish were coincubated with fifty  $\mu\text{l}$  of lymphocytes from a heterologous source. Each combination of the mixed lymphocytes was cultured in triplicate, pulsed with tritiated thymidine 24 hours prior to harvesting, which was performed as described above. Cell proliferation was measured by the uptake of the tritiated thymidine.

**Migration Inhibition Factor (MIF):** The ability of the lymphocytes to express normal levels of important regulatory factors was determined by assaying for the production of migration inhibition factor. Antigen specific stimulation of lymphocytes causes the release of migration inhibition factor, which in turn, inhibits the migration of macrophages away from the antigen. The MIF assays used were a modification of the agarose microdroplet assay described by McCoy (1976). Fish anterior kidney cells were prepared as single cell suspensions in L-15 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum and 100  $\mu\text{g/ml}$  gentamicin. Cells were washed twice and resuspended to a concentration of  $5 \times 10^5/12 \mu\text{l}$  in a preparation of 0.2% Sea Prep agarose (FMC Corp., Marine Colloids, Rockland, ME) in L-15. These preparations were made with or without the test antigen. Two  $\mu\text{l}$  droplets of cells were placed in the center of sterile flat-bottom 96-well tissue culture plates held on ice. The droplets were then allowed to solidify for 15-20 minutes. This incubation was then followed by an addition of 100  $\mu\text{l}$  of 0.1% Sea Prep in L-15, containing the same concentration of test antigen. This second layer of agarose was added gently so as not to dislodge the droplet. The plate was then placed in a sealed humidified box at room temperature for 48 hours. Migration was scored by inspection with an inverted microscope. Positive (+), or normal migration, occurred when the white blood cells appeared in a continuous exterior halo to the non-migrating red blood cells and pigmented kidney cells, which were held in the agarose droplet. Moderate migration (+/-) was indicated when only scattered white blood cells were found to be exterior to the central red blood cells and kidney cells. No migration (-) was determined when no or few white blood cells were found outside the droplet.

**Radiolabelling of Renibacterium salmoninarum:** One ml of a suspension of formalin-killed *R. salmoninarum* (one unit of O.D. at 520 nm) was washed twice in PBS, pH 7 and resuspended in 250  $\mu\text{l}$  of PBS. This solution was placed in an ice bath and 500  $\mu\text{Ci}$  of  $\text{I}^{125}$  (Amersham, Irvine, CA) in 5  $\mu\text{l}$  was added while constantly stirring. Immediately following the  $\text{I}^{125}$ , 0.2 ml of 1% chloramine T was added. After five minutes, 0.2 ml of a 1% solution of sodium metabisulphite was added to stop the reaction. The labelled bacteria were then washed with PBS at 6,500 x g until the supernatant contained less than 1% of the total cpm incorporated.

**Phagocytosis Assay:** Levels of phagocytic activity against *R. salmoninarum* were quantitatively measured by determining the uptake of radiolabeled bacterial cells by salmon leukocytes. Briefly, 0.5 ml of a cellular suspension ( $3 \times 10^6$  cells/ml) in 5% FCS (fetal calf serum)-L-15 was mixed with 0.05 ml of radioiodinated bacteria and diluted to a concentration of  $2 \times 10^6$  cpm/ml. Phagocytosis was measured by removing non-phagocytized bacteria by

two washes at 500 x g. The final pellet was counted and the per cent phagocytosis calculated as:

$$\frac{\text{cpm in leukocyte pellet} - \text{cpm w/o leucocytes}}{\text{total cpm} - \text{cpm w/o leukocytes}} \times 100$$

**Plaque-forming cell (PFC) Assay:** Antibody production by lymphocytes *in vitro* was measured by the enumeration of lymphocytes producing plaques (cleared areas) in lawns of antigen coated red blood cells. Briefly, single cell suspensions of  $1 \times 10^7$  cells/ml were prepared in MDM, as described above. Aliquots of 0.05 ml of the suspension were cultured with 0.05 ml of the appropriate dilution of antigen in MDM or in MDM alone. Cultures were fed 10  $\mu$ l of nutritional supplement daily until harvested. The contents of each microculture were assayed and three such microcultures were tested per data point. Cells secreting anti-trinitrophenyl (TNP) antibodies were detected by a modification of the Cunningham plaque assay (Cunningham and Szenberg, 1968). One hundred  $\mu$ l of the lymphocyte suspension, 25  $\mu$ l of a 10% suspension of sheep red blood cells (SRBC) or TNP-SRBC (Rittenberg and Pratt, 1969) in modified barbital buffer (MBB), and 25  $\mu$ l of steelhead serum were mixed, diluted in MBB, and deposited into individual wells of a 96-well microtiter plate (Lindbro, McLean, VA). The contents of each well was pipetted into a slide chamber thus producing a thin lawn of cells. The chamber was sealed by dipping the edge in melted paraffin and incubated for 1-3 hours at 16°C. Plaques were then enumerated under low power with the aid of a dissecting microscope.

**Polyclonal Activation:** Polyclonal activation of lymphocytes was done by the method of Yui and Kaattari (1987) - see appendix.

## RESULTS AND DISCUSSION

**Mitogen Studies:** Mitogen-induced lymphocyte proliferation in response to four distinctly different mitogens was studied. These four mitogens were *Escherichia coli* lipopolysaccharide (LPS), phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM). In addition, we discovered a new mitogen, an extract of *Vibrio anguillarum* (VAE), which is very effective for the mitogenic stimulation of salmonid lymphocytes and directly applicable to studies with salmon (See appendix). Dose responses of all five mitogens for both anterior kidney and spleen are depicted in Table 1. and the kinetics of the response to LPS, PHA, and VAE are given in Table 2. The data presented are the results from single experiments which are representative of the typical responses seen. The responses do tend to vary with each preparation of mitogen with respect to the optimal dose. For this reason, dose responses were conducted on fish prior to the use of a new preparation of mitogen.

Lipopolysaccharide, a typical mammalian B cell mitogen, was consistently found to induce high stimulation indices in lymphocytes isolated from the spleen or anterior kidney. Both lymphoid populations demonstrated stimulation indices of 20-30. The peak stimulatory dose was found to be approximately 0.25-1.0 mg/ml.

Table 1. Dose responses of spring chinook salmon (Oncorhynchus tshawytscha) lymphocytes to various mitogens.

<b>Mitogen<sup>1</sup></b>	Cell Source	DOSE (mg/ml)				
		<u>0.25</u>	0.5	1.0	2.0	
<b>LPS</b>	A. Kidney <sup>2</sup>	<b>23.5<sup>3,4</sup></b>	30.8	31.9	21.9	
	Spleen	20.8	27.7	35.4	30.4	
-----						
		2	5	<u>10</u>	<u>50</u>	<u>100</u>
PHA	A. Kidney	5.0	13.4	17.4	9.4	1.8
	Spleen	1.2	1.1	1.6	1.0	0.6
-----						
		<u>10</u>	<u>50</u>			
<b>PWM</b>	A. Kidney	6.2	6.6			
	Spleen	6.0	2.0			
-----						
		<u>2</u>	5	10	<u>50</u>	<u>250</u>
<b>ConA</b>	A. Kidney	1.0	1.2	4.0	2.4	0.6
	Spleen	0.6	0.4	1.4	0.8	0.6
-----						
		<u>0.05</u>	<u>0.26</u>	1.3	<u>6.5</u>	<u>32.5</u>
VAE	A. Kidney	5.6	16.0	28.8	28	32.8
	Spleen	4.4	8.8	18.8	35.6	30.0

<sup>1</sup>Mitogens used in this assay were E. coli lipopolysaccharide (**LPS**), phytohemagglutinin (PHA), **pokeweed** mitogen (**PWM**), concanavalin A (**ConA**), and V. anguillarum extract (VAE).

<sup>2</sup>Anterior kidney.

<sup>3</sup>Stimulation index = (cpm of cultures with **mitogen**)/cpm of cultures without mitogen).

<sup>4</sup>**Standard** errors of the mean were <5% for all values.

Table 2. Kinetics of the mitogenic response of spring chinook salmon (Oncorhynchus tshawytscha) lymphocytes to selected mitogens.

<b>Mitogen<sup>2</sup></b>	ANTERIOR KIDNEY				
	Day of Harvest <sup>1</sup>				
	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
VAE	27.2 <sup>3,4</sup>	76.0	44.0	16.8	27.2
LPS	5.6	16.8	20.0	16.8	20.0
PHA	2.4	5.6	6.4	1.6	5.6

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Mitogen	SPLEEN				
	Day of Harvest <sup>1</sup>				
	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
<b>VAE</b>	41.6	52.0	25.6	32.8	24.8
LPS	6.4	12.1	12.8	24.8	20.0
PHA	1.6	1.6	1.0	0.9	1.1

<sup>1</sup>Cells were harvested 24 hours after pulsing with titrated thymidine on day 4 of culture.

<sup>2</sup>Mitogens used in this assay were V. anguillarum extract (VAE), E. coli lipopolysaccharide (LPS), phytohemagglutinin (PHA).

<sup>3</sup>Standard errors of the mean were <5% for all values.

<sup>4</sup>Values expressed as stimulation indices.

In contrast, PHA, a T cell mitogen. was found to be capable of inducing stimulation indices of 15-20 in the anterior kidney only. No stimulation was consistently found in the spleen. Occasionally, PHA stimulation can be seen in the spleen, particularly if fish serum supplementation is used (data not shown). This form of serum supplementation, however, would be impractical for these studies since the procurement of large quantities of chinook serum with consistent culture-supportive activity could not be assured. These data may suggest that there is a distinct PHA responsive subpopulation of lymphocytes in the anterior kidney which does not exist in the spleen. This inconsistency of responsiveness in the spleen cautioned us against the possibility of using this mitogen. It was our desire to have the potential capability of using either immune organ or both if the need arose. We were also unsure of responsiveness in the anterior kidney if the spleen did not possess consistent responsiveness. In the end we utilized solely anterior kidney samples, since at our first sampling date (the fish were **10-15 g**), only this organ was capable of supplying a sufficient number of lymphocytes for the assays.

**Pokeweed** mitogen. a mammalian B and T cell mitogen was found to produce stimulation indices of 5 to 10 in both anterior kidney and spleen. This value was consistently below that found with either LPS or VAE. Thus, we felt that either LPS or VAE would be superior to **PWM**.

Concanavalin A, another T cell mitogen, produce the lowest stimulation index which was approximately 3.0 in the anterior kidney, while no stimulation was seen in the spleen. It is of considerable interest that both of the T cell mitogens possess the ability to elicit responses from the anterior kidney **only**, while lipopolysaccharide and **pokeweed** mitogen stimulates responses in both anterior kidney and spleen. This may suggest that either there are few, if any, T cell-like lymphocytes in the spleen, or that T cell responses in the spleen are heavily regulated or suppressed in response to mitogens.

The mitogenesis induced by VAE is of particular interest because it is derived from a **salmonid** pathogen. This material has been found to produce similar or much higher mitogenic responses than the other  itogens. It also stimulated a non-specific anti-TNP plaque-forming responses (see polyclonal activation section). This high degree of stimulation was found in both anterior kidney and spleen. This material was eventually deemed to be the preferred mitogen due to these factors. It was not, however, incorporated in the first year's studies with pyridoxine and **folic** acid. since it was a fortuitous discovery that was made near the end of the optimization study. It, therefore, required some additional time before it was ready for use and deemed equivalent to **LPS** in activity (Appendix).

The kinetics of the mitogenic responses of the three most stimulatory agents (**VAE, LPS, PHA**) was determined at their respective optimal doses. VAE appears to stimulate an early peak response in both anterior kidney and spleen, whereas lipopolysaccharide demonstrates a rather protracted peak response over the seven days of culture for anterior kidney lymphocytes, and distinct peak response at 6 days of culture for the splenic lymphocytes. Thus, it appears that VAE is similar to lipopolysaccharide in its ability to stimulate both anterior kidney and splenic lymphocytes, the difference in kinetics may indicate a different mechanism of stimulating lymphocytes.

Phytohemagglutinin responses were inconclusive as to the resolution of a peak response day, however, the absence of any mitogenic responses with splenic lymphocytes was consistently observed at all times.

**mixed Lymphocyte Reactions (MLR):** It has been found that chinook salmon can exhibit a strong MLR when cells from two or more fish are mixed (Table 3).

**Table 3. Mixed lymphocyte reaction of spring chinook salmon (Oncorhynchus tshawytscha) anterior kidney lymphocytes.**

	Cell Mixtures <sup>1</sup>						
	A	B	C	AB <sup>2</sup>	AC <sup>2</sup>	BC <sup>2</sup>	ABC <sup>2</sup>
Day 5 <sup>3</sup>	333(27) <sup>4</sup>	500(47)	667(65)	2167(1000)	1500(310)	4167(1100)	1135(312)
Day 7	1000(99)	834(79)	2167(845)	1010(153)	4167(995)	2313(653)	2431(1113)
Day 9	1515(153)	1254(121)	1267(11)	3843(2015)	6893(675)	4120(1153)	3000(1175)

<sup>1</sup>Total number of lymphocytes per culture -  $1 \times 10^6$

<sup>2</sup>Equal numbers of cells from each individual (A, B, or C) were added to result in the final concentration of  $1 \times 10^6$ /cultures.

<sup>3</sup>Cultures were pulsed with titrated thymidine 24 hours prior to harvest.

<sup>4</sup>Values represent mean cpm of triplicate cultures (SEM).

The occasional weak response or lack of a response, is most probably due to the partial histocompatibility of certain individuals. As can be seen in the table, there appears to be a general increase in the cpm over time, the greater stimulation occurring at day nine. To counter the variability of this MLR, we feel that it would be necessary to standardize this assay by incorporating stimulatory lymphocytes from a single, uniform xenogeneic source (**i.e. trout**), rather than to use an allogeneic source (other chinook cells). This should provide a constant stimulus to the chinook cells, independent of the individual tested. To prevent incorporation of tritiated thymidine by xenogeneic cells, trout lymphocytes should be irradiated with 1000 rads from a Co<sup>60</sup> irradiator prior to coculture. Preliminary evidence demonstrates that this amount of irradiation is sufficient to prevent tritiated thymidine uptake.

**Migration Inhibition Factor (MIF) Assay:** Previous studies (McKinney et. al, 1976) have demonstrated that cell-mediated immunity in fish can be assessed by the analysis of the inhibition of macrophage migration. This assay measures the ability of "T"-like cells to elaborate a factor in response to antigen specific stimulation. This factor then induces the inhibition of macrophage migration.

Initial studies employing the defined antigen TNP-bovine serum albumin (TNP-BSA), revealed that TNP-BSA immunized fish were sensitive to TNP-BSA at concentrations of 1 to 100  $\mu\text{g/ml}$  in culture (Table 4). The lowest concentration

Table 4. Migration inhibition factor responses of immune and non-immune spring chinook salmon (Oncorhynchus tshawytscha) anterior kidney lymphocytes<sup>a</sup>.

	TNP-Bovine Serum Ablumin $\mu\text{g/ml}$											
	0			0.01			1		100			
	1	2	3	1	2	3	12	3	1	2	3	
Injected fish <sup>b</sup>	+ <sup>c</sup>	+	+	+	+	+/-	/	.	-	+/-	-	-
Naive or Native Fish	+	+	+	+	+	+	/	+	++	+	+	+

<sup>a</sup>Anterior kidney cells ( $5 \times 10^5/12 \mu\text{l}$ ) were cultured in 0.2% agarose in L-15. Cultures were maintained in 96 well tissue culture plates, held in a humidified chamber at room temperature for 48 hours.

<sup>b</sup>Cells were obtained from fish which had been injected one week previously with  $100\mu\text{g}$  TNP-bovine serum albumin in complete adjuvant.

Migration scored as follows:

- + extensive migration
- +/- moderate migration
- no migration (inhibition)
- / not **scorable**

of TNP-BSA (0.01  $\mu\text{ml}$ ) was unable to stimulate sufficient elaboration of MIF activity to inhibit this response. Cells from naive or non-immune animals demonstrated no **macrophage** inhibition at any of the tested concentrations of antigen. Thus, within the chinook system, we should be able to evaluate the effects of vitamin nutrition on the ability of lymphocytes to produce an antigen specific cellular immune function.

Also, since macrophages play an important role in the progress of bacterial kidney disease (Fryer and Sanders, 1981; Young and Chapman, 1978), this assay may provide informative *in vitro* correlates to the *in vivo* studies relating resistance to **BKD** to vitamin nutrition.

**Phagocytosis Assay :** The data in Table 5 demonstrate that the kidney and spleen possess varying degrees of phagocytic activity to R. salmoninarum. Leukocytes from either the anterior or posterior kidney **produced** the greatest amount of activity (approximately 23% within one hour), while the spleen produced considerably less (approximately 6%). Also, it should be noted that the spleen demonstrated no increased uptake of **labelled R. salmoninarum** during the time period examined.

Table 5. Phagocytosis (%)<sup>1</sup> of I<sup>125</sup> labelled R. salmoninarum by spring chinook salmon (Oncorhynchus tshawytscha) anterior kidney, posterior kidney, and splenic lymphocytes.

<u>Cell Source</u>	<u>Duration of Incubation (Minutes)'</u>		
	<u>20</u>	<u>60</u>	<u>180</u>
Posterior kidney	<b>18.5<sup>3</sup></b>	22.7	21.5
Anterior kidney	14.2	<b>20.8</b>	<b>20.8</b>
Spleen	6.1	6.5	<b>8.8</b>

<sup>1</sup>Percent phagocytosis =  $\frac{\text{cpm w/leukocytes} - \text{cpm w/o leukocytes}}{\text{total cpm} - \text{cpm w/o leukocytes}} \times 100$

<sup>2</sup>**Total** time that leukocytes were coincubated with radiolabelled R. salmoninarum.

<sup>3</sup>Mean cpm of triplicated samples, SEM was less than 5% of the mean value for all samples.

It is felt that the close examination of the kinetics of phagocytosis may allow for the differentiation between non-specific and specific uptake of bacteria within the spleen.

The use of MIF and phagocytosis assays allow for the examination of two distinct phases of the cell mediated immune response. The MIF assay examines the initial antigen specific lymphocytic response to antigen and, thus, represents the inductive phase of the macrophage's response.

**Plaque-forming: Cell (PFC) Assay:** The antigen TNP-LPS has been found capable of inducing an in vitro antibody response as ascertained by the PFC assay. The spleen (Table 6) demonstrated significant PFC responses to TNP-coated sheep red blood cells. At no time were PFCs to uncoated sheep red blood cells detected (data not shown). Approximately 20 µg/ml produced an optimal antibody response of 30 PFCs/10<sup>6</sup> total lymphocytes harvested. Retitering of antigen preparations throughout this study revealed different optimal antigen concentrations for each antigen lot. Thus, the optimal dose utilized changes in the vitamin study reported in chapter 2. The optimal PFC response occurred in seven to nine days of culture.

This assay represents an extremely powerful tool for the analysis of the immune response. This is due to the fact that we are now capable of examining

Table 6. In vitro plaque-forming cell (PFC) response of spring chinook salmon (*Oncorhynchus tshawytscha*) lymphocytes to trinitrophenylated-lipopolysaccharide' (TNP-LPS).

	TNP-LPS Concentration ( $\mu\text{g/ml}$ )			
	0	2	20	200
Day 7	0.8(0.1) <sup>2</sup>	22.4(8.0)	30.8(10)	20(12)
Day 9	0	-		14.8 (2.4)

<sup>1</sup>Lipopolysaccharide was derived from *Escherichia coli* strain 055:B5.

<sup>2</sup>Mean number of PFC/10<sup>6</sup> lymphocytes harvested for triplicate cultures (SEM).

quantitatively, the capability of lymphocytes to produce a specific antibody without the immunization of whole animals. Thus, this and future investigations will not be required to use immunized groups of fish. Also, in utilizing this system, fish from various sources may be screened for their ability to produce a specific immune response without having to be subjected to the stress of transport to other holding facilities.

**Polyclonal Activation:** Mitogenic materials were examined to determine if they might also be capable of stimulating spring chinook lymphocytes non-specifically to secrete antibodies. Such non-specific stimulation of antibody secretion and proliferation was demonstrated by incubation with VAE (Appendix). The data presented in the appendix demonstrate that when naive chinook lymphocytes are cultured with optimal concentrations of VAE (6.5  $\mu\text{g/ml}$ ), a significant response is elicited. This stimulation results also in the non-specific generation of anti-TNP antibodies (figure 4 and Table I in the appendix) for coho salmon (*Oncorhynchus kisutch*). Although of interest from an immunological perspective, it was felt that specific antibody (PFC) induction in vitro would be of greater value in assessing the effects of dietary vitamin manipulations, than would polyclonal activation (See below)

#### SUMMARY

**Plaque-forming cell and mitogen assays:** The two in vitro assays that were chosen to be used throughout the following study with vitamins (Chapter 2) were the plaque-forming cell (PFC) assay and the mitogen assay. These choices were made for the following reasons: 1) the PFC assay measures the capacity of lymphocytes to produce a specific immune response to an antigen, while the mitogen assay is a measure of the general immunocompetency of an entire population of lymphocytes 2) technically these assays were capable of being performed on the large number of animals that had to be assayed during the testing periods. Taken together with the disease challenges, which would indirectly focus on innate immune defenses (especially in the case of *Aeromonas salmonicida*), we felt that a very thorough examination of the immune

status of the salmon could be undertaken with this collection of assays. The **assays** not chosen and the specific reasons for their not being selected are listed below.

**Mixed lymphocyte reaction:** Although this reaction demonstrated significant responses, it was not chosen for logistical reasons. The measurement of non-self recognition requires the recognition of an allogeneic stimulator cells. Usually these are simply the cells from another individual of the same species. Some degree of variability in the response of a population of individuals can occur simply due to a non-sharing of histocompatibility antigens. Thus, it is possible that within a large population of individuals some amount of non-responsiveness may be due to genetic differences in histocompatibility rather than differences in immunocompetency. Thus, for the sake of consistency, it would be best to have a constant cell source which can be used as a stimulator cell population for all individuals tested. Although it is felt that this condition may present an insurmountable problem, it may be possible to use a xenogeneic source of stimulator cells (i.e. trout) to alleviate these difficulties. Thus the salmon, in this case, would recognize the major differences found in trout antigens rather than the minor ones found in individual chinook. Mixed lymphocyte reaction testing would be even more efficient with a source of homogeneous stimulators such as with an isogenic strain of trout or other salmonid, which are currently being developed by other research groups. However, given the time and material constraints of this project, it was not feasible to use this assay.

**Phagocytosis assay:** This assay appears to have many advantages such as its simplicity, ease of preparation, quantitative nature, and its ability to assess a distinctly different function of the immune system. The assay requires further optimization to work efficiently with transported cells. Such was not a concern with the PFC assay, the mixed lymphocyte assay, and the mitogen assays. This assay only worked well with cells freshly taken from the animal. Since this assay could not be conducted entirely at The Center, due to restrictions on the use of radioactive materials. Other forms of phagocytosis evaluation required the microscopic examination of the ingestion of bacterial cells or particles by the phagocytic cell, this would have been too time consuming to have been feasible for this project.

**Migration inhibition assay:** The MIF assay, in its present form, requires the specific immunization of the fish before testing. We, however, did not possess enough space to effectively immunize the test animals.

**Polyclonal activation:** Since this assay would simply examine the non-specific induction of antibody-producing cells, and since the mitogen assays would monitor the induction of proliferation (the initial phase of polyclonal activation) it **was** felt that the use of this assay would be somewhat redundant. The PFC and mitogen assays would cover these functions well without the need to resort also to polyclonal activation.

CHAPTER II

EFFECTS OF VITAMIN NUTRITION

## **EXPERIMENTAL DESIGN**

The vitamins chosen for study were pyridoxine, pantothenic acid, riboflavin, **foli**c acid, alpha-tocopherol, and ascorbic acid. A practical fish feed formulation and a semi-purified test diet were compounded to contain incremental amounts of the test vitamins. **Each** vitamin was studied singly while dietary levels of all other vitamins were held constant, using the supplementation rates specified in the vitamin packages for each diet. The test diets were fed for up to 24 weeks to duplicate lots of spring chinook juveniles held indoors in cylindrical tanks supplied with well water. During rearing, each lot of fish was weighed at 2-week or **4-week** intervals to: **(1)** determine growth rates, (2) calculate food conversion efficiencies, and (3) provide population weights on which to base feeding rates. Dead fish were removed and recorded daily to determine survival rates. Samples of test feeds were analyzed regularly to verify vitamin **dietary** concentrations, and fish organs were analyzed for vitamin levels at the termination of each study. Assays were then applied to fish from each diet group at regular intervals to assess their immune competence. The assays examined in vivo and in vitro parameters of **humoral** immunity, cellular immunity, and disease resistance. Data concerning growth, food conversion efficiencies, rearing mortality, times to death in disease challenges, and in vitro **assays** of immune responsiveness were tested by analyses of variance **and t tests** to determine significance of differences (**5%** level of **significance**) and, where **applicable**, regression techniques were used.

## MATERIALS AND METHODS

Facilities: The feeding and rearing phases of the experiments were performed at the Abernathy Salmon Culture Technology Center (**SCTC**), Longview, Washington. Immunological assays and disease challenges were performed on chinook salmon obtained from Abernathy SCTC and housed at the Oregon State University Department of **Microbiology** and the Fish Disease Laboratory (**OSUFDL**), Corvallis, Oregon.

During rearing, the fish were held in 700-liter, cylindrical, fiberglass tanks furnished with constant temperature (**12°C**) well water. During disease challenges, fish were housed in 30-liter fiberglass tanks supplied with **12°C** well water. The compositions of the two water sources are given in Table 1.

The amount of water flowing into each 700-liter rearing tank was maintained at 12 liters per minute throughout the studies. The 30 liter tanks used to hold fish during disease challenges received flows of 0.40 l ters per minute. Fish population densities (Kg/liter **inflow/min** and Kg/m<sup>4</sup>) never exceeded the guidelines of Banks et al. (**1979**).

Illumination of the experimental tanks at the Abernathy **SCTC** was provided by fluorescent lights controlled by a photocell system to simulate the natural photoperiod at the latitude of the Abernathy SCTC.

Test Fish: Spring chinook salmon (**O. tshawytscha**) were used as test animals in all experiments. Except **for two** experiments (riboflavin and pantothenic acid) the fish were obtained as eyed eggs from fish spawned at Carson National Fish Hatchery (**NFH**) located about 15 miles north of Carson, **WA** on the Wind River. Eggs were transferred to the Abernathy SCTC, cushioned in moist burlap bags, supported in 40 cm x 40 cm x 25 cm egg baskets in order to prevent physical trauma. Upon arrival at the Abernathy SCTC, the eggs were surface-disinfected with an iodophore (Argentine) according to the protocol recommended by Wood (1974). The eggs were then placed in incubator trays (Heath Techna Corp.) supplied with well water. During the riboflavin and pantothenic acid studies, eggs and milt were collected from adult fish at Carson **NFH** and transported separately to Abernathy **SCTC** in chilled containers. The eggs were fertilized upon arrival at Abernathy SCTC and placed immediately into the Heath incubators.

After hatching and yolk absorption, the resulting fry were stocked in a **1200-liter**, steel, circular tank furnished with well water at 40 liters/min. They were fed either **BioDiet** (Bloproducts, Inc., Warrenton, OR) or BioDiet followed by Abernathy Diet until they reached an average size of 2.0 grams or larger. At that time they were randomly distributed into 700-liter, cylindrical, fiberglass tanks by the following procedure. Groups of ten fish were hand counted sequentially into separate baskets until a total of 300 fish per basket was reached. Each diet used in a given study, and its replicate, were assigned a number which was written on a separate piece of paper. All of the pieces of paper were then placed into a container, mixed, and removed one at a **time** and assigned to a basket of fish. Each basket was then assigned to

Table 1. Composition of water supplies. Immunological study. Abernathy Salmon Culture Technology Center.

Measurement	Unit	Water Supply	
		Fish Rearing <sup>1</sup>	Immunological Testing <sup>2</sup>
Total gas saturation	% <sup>3</sup>	100	100
N <sub>2</sub> saturation	%	99	101-103
O <sub>2</sub> saturation	%	89	91
pH		7.7	7.4
Conductivity (@ 25°C)	umhos/cm	244	--4
Alkalinity, total (as CaCO <sub>3</sub> )	mg/l	76	--
Hardness, total	mg/l	90	--
Phosphate, ortho (as PO <sub>4</sub> )	mg/l	0.023	--
Solids, dissolved (@ 105°C)	mg/l	180	154
Solids, suspended (@ 105°C)	mg/l	1.2	0.1
Ammonia, total (as NH <sub>3</sub> -N)	mg/l	<0.02	<0.002
Nitrate (as NO <sub>3</sub> -N)	mg/l	0.33	0.97
Nitrite (as NO <sub>2</sub> -N)	mg/l	<0.002	<0.001
Carbon dioxide	mg/l	3.8	--
Chloride	mg/l	14	22
Cadmium	µg/l <sup>4</sup>	<0.0003	--
Calcium	mg/l	18	17
Cobalt	mg/l	<0.13	<1.0
Copper	mg/l	<0.001	0.0025
Iron	mg/l	0.17	0.016
Lead	mg/l	0.005	0.016
Magnesium	mg/l	11	8
Manganese	mg/l	0.10	0.01
Molybdenum	mg/l	<0.16	<0.025
Potassium	mg/l	2.8	1.5
Sodium	mg/l	15	8.8
Zinc	mg/l	19	--
Sulfate	mg/l	19	--
Turbidity	JTU	11	--

<sup>1</sup>Abernathy SCTC well

<sup>2</sup>Oregon State University well

<sup>3</sup>Percent of Barometric pressure

<sup>4</sup>N.A.

a rearing tank using the same procedure. In some cases, a random numbers table was used, instead of the numbered papers in a container, to assign baskets and diets to tanks.

**Diets and Feeding:** In each experiment, the vitamin under study was incorporated, at five graded levels, into two types of diets: (1) a practical, dry pellet formulation (Abernathy diet, Table 2) and (2) a semi-purified feed (Oregon test diet, Table 3).

Table 2. Composition of Abernathy Diet used as a vitamin test ration for spring chinook salmon (Oncorhynchus tshawytscha) fingerlings. Immunological study, Abernathy Salmon Culture Technology Center.

Component	Percent
Herring meal, minimum protein 65%	40.57
Dried whey, minimum protein 12%	5.00
Wheat germ meal, minimum protein 23%	5.00
Wheat standard middlings, minimum protein 15%	26.65
Blood meal, spray dried, minimum protein 80%	10.00
Vitamin <b>premix</b> <sup>1</sup>	1.50
Ascorbic acid	0.10
Choline chloride, 60% product	0.58
Trace mineral premix'	0.10
Herring oil, stabilized with 0.04% BHA-BHT <b>(1:1)</b>	<u>10.50</u>
	100.00

<sup>1</sup>**Mg/kg** of diet unless otherwise indicated: riboflavin, 53; niacin, 220; **folic acid**, 12.7; thiamine, 43; biotin, 0.60; B-12, 0.06; vitamin K, 9; inositol, 132; d-pantothenic acid, 106; pyridoxlne, 31; vitamin E 503 IU; vitamin D3, 441 IU; and vitamin A, 6614 **IU**.

<sup>2</sup>**Mg/kg** of diet: zinc, 75.0; manganese, 20.1; copper, 1.54; and iodine, 10.0. Mineral sources were zinc sulfate, manganese sulfate, copper sulfate, and potassium iodate.

The total vitamin levels provided represented that contributed from both natural sources in ingredients and the crystalline supplements. Generally, the vitamin supplementation in the Abernathy diet was aimed at obtaining dietary vitamin levels starting with the amount recommended by the National Research Council (National Academy of Sciences, 1973. Table 4) and multiples of this base requirement in the remaining four graded levels. The lowest vitamin level in the Oregon test diet (**OTD**) was usually aimed at 25% or 50% below the National Research Council (NRC) recommendation. The remaining four graded vitamin level values being paired with the vitamin levels incorporated in the Abernathy diet (AD). As a result of this supplementation scheme, we achieved four vitamin concentrations which were common to both test feeds.

Table 3. Composition of the Oregon Test Diet formula (National Academy of Sciences, 1973). Immunological study, Abernathy Salmon Culture Technology Center.

Components	Percentage of Dry Components
Vitamin-free <b>casein</b>	45.9
Gelatin	8.1
Dextrin	15.6
<b>Carboxy</b> methyl cellulose	1.3
alpha-cellulose	12.129
alpha-tocopherol (250 <b>IU/g</b> )	0.264
Choline chloride (99%)	0.707
Mineral mix <sup>1</sup>	4.0
Vitamin <b>premix</b> <sup>2</sup>	2.0
Herring oil, stabilized with 0.04% BHA-BHT (1:1)	<u>10.0</u>
	100.0

<sup>1,2</sup>are identical in composition to those described by the National Academy of Sciences (1973).

Table 4. Dietary vitamin levels recommended for salmonids by the National Research Council, 1973.

Vitamin	Recommended level' (ng/Kg feed)
<b>Folic</b> acid	5
Pyridoxine	10
Riboflavin	20
Pantothenic acid	40
Ascorbic acid	100
Alpha-tocopherol	30

<sup>1</sup>Total vitamin contribution from all sources. Other amounts may be more appropriate to offset losses resulting from the effects of formulation and storage, or when feeding other than small fish at SET.

The Abernathy diet was prepared as follows: Meal ingredients were either purchased in a finely ground state or were ground through a hammer mill until they passed entirely through a 20 mesh/inch screen. Components were blended in a paddle mixer and pelletized (2.5 mm diameter x 2-6 mm length) through a small, compaction-type pellet mill (California Pellet Mill Co.) without steam conditioning. Sufficient feed for 90 days was prepared at one time and stored at room temperature (**22°C**) until fed.

The Oregon test diet was prepared in the following manner: During experiments with pyridoxine and folic acid, 35 parts of dry semi-purified ingredients sufficient for 20-35 days of feeding were mixed with 65 parts water in a dough mixer, then frozen (-60°C) in air tight containers until used. Daily allotments of the Oregon test diets were thawed and extruded through a ricer (3-mm diameter holes) from which pellets of desired length (3-10 mm) were cut with a laboratory spatula. For all remaining experiments, 50 parts dry ingredients were mixed with an equal weight of water. When cool, the diet was passed through a custom-made, laboratory extruder which compacted the dough and cut it into pellets ranging from 2.4-3.2 mm in diameter and 2-7 mm in length. The diet was then stored at -60°C. All Oregon test diets were fed within five weeks of preparation.

Daily feed allotments for all types of diets were based on a uniform weight of dry food per weight of live fish computed by the methods of Buterbaugh and Willoughby (1967). Feeding was done by hand according to frequencies recommended by Fowler (1989).

**Growth and Feed Efficiency:** During the rearing phases of experiments with pyridoxine and folic acid each lot of fish was weighed biweekly to the nearest gram in a water-filled container. During all other experiments, weigh-days were held at 4-week intervals. Dead fish were removed from the tanks daily and information about their weight and numbers recorded.

Computation of specific growth rates (Mahnken et al., 1980) and gross feed efficiencies (Brett et al., 1969) were done as follows:

**Specific growth rate, SGR:**

$$\text{SGR} = \frac{\text{Log}_e \text{ of weight at end} - \text{Log}_e \text{ of weight at start}}{\text{days fed}} \times 100$$

**Gross feed efficiency, GFE:**

$$\text{GFE} = \frac{\text{Total fish weight gain in grams}}{\text{food offered in grams (dry weight)}} \times 100$$

**Vitamin Analysis:** To document actual vitamin levels achieved in the test diets, samples were collected from each batch of feed immediately after manufacture and when the batch was nearly exhausted (90 days for Abernathy diet and three weeks for the Oregon test diet) and sent to commercial laboratories for analysis.

At the end of each experiment, samples of liver tissue were collected from fish in each replicate tank and assayed to determine vitamin storage. Sufficient numbers of fish were sampled to provide a minimum of five grams of liver tissue. This usually required pooling livers from 30-40 fish per tank. Assays were performed by commercial laboratories and the National Marine Fisheries Service, Seattle, WA. We were unable to obtain reliable information on liver stores of folic acid and pyridoxine because the commercial laboratory (Coffee Laboratories, Portland, OR) could not resolve technical difficulties in their analytical methods.

**Fish Sampling for Assays:** Following 11-15 weeks of feeding (when the fish had reached an approximate size of 10 grams) the first set of fish samples were collected for *in vitro* immunological assays. A second set was collected 16-21 weeks later and a final sampling was done at the end of 22-25 weeks of feeding. During the tests with pyridoxine and **folic** acid, 12 fish were randomly sampled from each tank (total of 24 per diet). In all other experiments, 24 fish were sampled per tank (48 per diet) on each sampling date. In all cases, response to mitogenic stimulation and plaque-forming cell assays were both determined on the same fish along with a weight measurement. To obtain the samples, the fish were rapidly crowded into two large dip nets and the requisite number of fish were randomly selected and placed immediately in a 200 **mg/l** solution of tricaine methane sulphonate (**MS-222**). The fish died within 20 seconds and were then promptly dissected to collect tissues for immunological analyses.

To obtain fish for the disease challenges, the fish were **crowded** and netted in the same manner as for the immunological tests, except that they were not placed in anesthetic. Fifty fish from each tank were randomly selected from the population and weighed in a water-filled container before being placed in **20-gallon** plastic cans filled with 15 gallons of well water for transport to Oregon State University by truck. During transit, the water was aerated with bottled oxygen and cooled with block ice. The fish were allowed to acclimate to their new tanks at Oregon State University for two days before any challenge with disease organisms was administered.

**Disease challenges (*Aeromonas salmonicida*):** Twelve 2-liter flasks with one liter of Brain Heart Infusion broth (BHI; **Difco**, Detroit, MI) in each were inoculated with ten milliliters of a 48 hour *A. salmonicida* culture grown from a single plate colony. The cultures were **gently** agitated at 17°C for 24 hrs. Contents of all twelve flasks were pooled **and** plate counts made on BHI agar plates. Cultures contained approximately 10<sup>9</sup> bacteria/ml as determined by colony counts.

For the immersion challenge, the water level in each tank was drained to 20 liters and the water flow was terminated. Five hundred milliliters of the *A. salmonicida* broth culture was added to each tank. Water flow was resumed **20** minutes after the bacteria were added. Daily mortalities were counted and agar plate checks were performed to verify the presence of the disease organism.

**Disease challenges (*Renibacterium salmoninarum*):** Bacteria were grown in KDM-II (Evelyn, 1977) for 7-10 **days** at 17°C with agitation to 1 O.D. unit. Each tank of fish was anaesthetized in a benzocaine bath consisting of 3 ml of a stock solution (10% **w/v** ethyl p-aminobenzoate in 95% ethanol) per 10 liter of water. Each fish was injected *i.p.* with 0.05 ml of the unwashed bacterial suspension (10<sup>9</sup> bacteria/ml) using a 26 g **1/2** inch needle and 1-cc syringe. Daily mortalities were counted and kidney smear checks were performed to verify the presence of the disease organism.

**statistical Analysis:** The data are presented as means with standard errors of the mean (**SEM**, Snedecor and **Cochran**, 1971). Data on SGR, GFE, liver vitamin concentrations, and mean times to death in the disease challenges were used directly in one-way analyses of variance and Student-Newman-Keuls Multiple Range Testing (Sokal and Rohlf, 1969). Mortality rates (**%**) during

rearing and disease challenges were transformed using an **arcsine** function before analysis.

Data on the responses of fish lymphocytes to mitogens were expressed as stimulation indices (ratios of stimulated counts per minute to control counts per minute) and transformed to log, values before analysis. Plaque-forming cell counts were transformed cubed root values. Analyses of both sets of transformed data were done using an Unweighted Analysis of Cell Means (Snedecor and **Cochran**, 1971). If the analyses of variance indicated significant interactions, the treatment means were compared using the Student's t test. Where appropriate, regression techniques were used to classify relationships.

## RESULTS

**FOLIC ACID:** The average folic acid levels achieved in the test feeds were very close to the nominal concentrations we had planned (Table 5).

Table 5. Vitamin **analysis**<sup>1</sup> of folic acid diets. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Nominal vitamin level <sup>2</sup>	Actual vitamin level <sup>2</sup>		
		Mean	(SEM)	n
Oregon Test	2	2.6	(0.1)	7
	6	6.8	(0.1)	7
	10	11.0	(0.3)	7
	14	14.3	(1.3)	7
	18	19.7	(0.6)	7
Abernathy	6	6.1	(0.2)	5
	10	9.9	(0.2)	5
	14	13.1	(0.4)	5
	18	17.3	(0.7)	5
	22	20.6	(0.5)	5

<sup>1</sup>All analyses were performed by **Hazelton** Laboratories America, Inc., Madison, Wisconsin.

<sup>2</sup>Values are mg vitamin/Kg dry diet.

Total rearing mortalities were uniformly low and averaged less than 0.5% for the entire experiment. Specific growth rates and feed efficiencies obtained are summarized in Table 6. The fish fed AD grew at significantly greater rates and had better feed utilization than fish fed OTD.

The response of lymphocytes to **E. coli** lipopolysaccharide is shown in Table 7 and the statistical analyses of those data appear in Table 8. Dietary levels of folic acid had no significant influence on this measure of immunocompetency.

The results of the plaque-forming cell (**PFC**) assays are shown in Table 9. Statistical analyses of these data (Table 10) showed that vitamin level and diet type were significant sources of variation. However, there also were significant interaction effects among those variables making it impossible to interpret their biological significance. Furthermore, time, which was found to be a highly significant factor, **had a confounding effect on these variables**. Inspection of the data showed no consistent relationship of PFC to vitamin level in either diet.

Table 6. Specific growth rate and gross feed efficiency of spring chinook (*Oncorhynchus tshawytscha*) fed two diets containing graded dietary concentrations of folic acid for 167 days. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Specific Growth <sup>1,3</sup> Rate		Gross Feed <sup>2,3</sup> Efficiency	
		Mean	(SEM)	Mean	(SEM)
Oregon test	2	1.518	(0.000) <sup>a</sup>	83.45	(0.47) <sup>c</sup>
	7	1.517	(0.005) <sup>a</sup>	82.35	(1.08) <sup>c</sup>
	11	1.502	(0.004) <sup>a</sup>	81.39	(0.17) <sup>c</sup>
	14	1.499	(0.002) <sup>a</sup>	81.18	(0.17) <sup>c</sup>
	20	1.497	(0.001) <sup>a</sup>	78.02	(1.29) <sup>c</sup>
Abernathy	6	1.606	(0.010) <sup>b</sup>	90.44	(1.17) <sup>d</sup>
	10	1.630	(0.017) <sup>b</sup>	92.31	(2.20) <sup>d</sup>
	13	1.610	(0.005) <sup>b</sup>	90.94	(0.63) <sup>d</sup>
	17	1.605	(0.003) <sup>b</sup>	90.31	(0.46) <sup>d</sup>
	20	1.601	(0.004) <sup>b</sup>	89.65	(0.85) <sup>d</sup>

$$^1\text{Specific growth rate SGR} = \frac{\log_e \text{ of weight at end} - \log_e \text{ of weight at start}}{\text{days fed}} \times 100$$

$$^2\text{Gross feed efficiency GFE} = \frac{\text{total fish gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

<sup>3</sup>Values are means of duplicate lots of fish, values with different superscripts are significantly different, Student-Newman-Keuls test ( $p \leq 0.05$ ).

Table 7. Mitogenic response (stimulation index) to *E. coli* lipopolysaccharide by anterior kidney lymphocytes from chinook salmon (*Oncorhynchus tshawytscha*) fed graded concentrations of folic acid in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by diet Mean (SEM)
		97 Mean (SEM)	125 Mean (SEM)	153 Mean (SEM)	
Oregon Test	2	3.8 (1.0)	11.2 (0.2)	38.0 (1.3)	17.7 (10.4)
	7	2.9 (0.3)	11.9 (0.2)	33.7 (5.2)	16.2 (9.1)
	11	2.4 (0.3)	8.5 (0.3)	36.5 (14.6)	15.8 (10.5)
	14	9.5 (1.0)	16.7 (0.2)	19.9 (1.2)	15.4 (8.1)
	20	7.0 (0.8)	13.4 (0.0)	42.4 (2.8)	20.9 (10.9)
<b>Time means</b>		5.1 (1.3)	12.3 (1.3)	34.1 (3.8)	-
Abernathy	6	3.0 (0.4)	16.1 (1.3)	49.4 (0.2)	22.8 (13.8)
	10	2.6 (0.1)	12.0 (1.2)	44.0 (10.8)	19.5 (12.5)
	13	2.1 (0.8)	15.1 (0.4)	50.1 (17.1)	22.4 (14.3)
	17	1.8 (0.1)	15.6 (0.0)	46.6 (16.2)	21.3 (13.2)
	20	1.6 (0.3)	13.8 (1.3)	37.6 (5.0)	17.6 (10.6)
<b>Time means</b>		2.2 (0.2)	14.5 (0.7)	45.5 (2.3)	-

Table 8. Analysis of variance. Mitogenic response (stimulation index) to *E. coli* lipopolysaccharide (100 µg/ml) by anterior kidney lymphocytes from spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of folic acid in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion <sup>2</sup>
			Calculated	Table <sup>1</sup>	
Groups	9	0.203	3.14	2.21	Sig.
Times	2	31.436	486.60	3.32	Sig.
Groups x time	18	0.332	5.14	1.95	Sig.
Diet	1	0.026	0.40	4.17	N.S.
Vitamin level <sup>3</sup>	3	0.185	2.86	2.92	N.S.
Diet x vitamin level <sup>3</sup>	3	0.326	5.05	2.92	Sig.
Diet x time <sup>3</sup>	2	1.303	20.20	3.32	Sig.
Vitamin level x time <sup>3</sup>	6	0.134	2.07	2.42	N.S.
Diet x time x vitamin level <sup>3</sup>	6	0.311	4.81	2.42	sig.
Error	30	0.064			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>Significant (Sig.),  $p \leq 0.05$ . Not significant (N.S.)  $p > 0.05$ .

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

Table 9. In vitro antibody response (plaque-forming cell) to TNP-lipopolysaccharide (0.5 µg/ml) by anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of folic acid in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by diet Mean (SEM)
		97 Mean (SEM)	125 Mean (SEM)	153 Mean (SEM)	
Oregon Test	2	205 (4)	284 (31)	374 (20)	288 (49)
	7	314 (27)	421 (51)	517 (2)	417 (58)
	11	300 (1)	240 (9)	401 (15)	314 (47)
	14	202 (28)	319 (10)	460 (4)	327 (74)
	20	178 (1)	418 (12)	470 (7)	355 (98)
<b>Time means</b>		<b>240 (28)</b>	<b>336 (36)</b>	<b>444 (25)</b>	
Abernathy	6	208 (945)	354 (54)	368 (66)	310 (51)
	10	278 (65)	258 (1)	457 (25)	331 (63)
	13	191 (57)	273 (22)	418 (20)	294 (66)
	17	256 (8)	406 (6)	429 (14)	369 (54)
	20	189 (4)	324 (42)	505 (25)	339 (91)
<b>Time means</b>		<b>224 (18)</b>	<b>323 (27)</b>	<b>435 (22)</b>	

Table 10. Analysis of variance. In vitro antibody response (plaque-forming cell) to DIP-lipopolysaccharide (0.5 µg/ml) of anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of folic acid in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion <sup>2</sup>
			Calculated	Table <sup>1</sup>	
Groups	9	5.88	6.00	2.21	Sig.
Times	2	115.90	118.40	3.32	Sig.
Groups x time	18	3.70	3.78	1.95	Sig.
Diet	1	6.18	6.32	4.17	Sig.
Vitamin level <sup>3</sup>	3	4.71	4.81	2.92	Sig.
Diet x vitamin level <sup>3</sup>	3	5.09	5.20		Sig.
Diet x time <sup>3</sup>	2	0.70	0.71	3.32	N.S.
Vitamin level x time <sup>3</sup>	6	6.20	6.33	2.42	Sig.
Diet x time x vitamin level <sup>3</sup>	6	0.88	9.90	2.42	Sig.
Error	30	0.98			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>Significant (Sig.) p ≤ 0.05. Not significant (N.S.) p > 0.05.

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

It is of particular interest to note that **OTDs** containing only 52% of the NRC recommended level produced growth and mitogen responses comparable not only to those feeds containing the NRC recommended level but also those with a 6-fold excess.

When the fish were challenged with either Aeromonas salmonicida (Table 11) or Renibacterium salmoninarum (Table 12) no significant differences were found in total mortality or mean times to death related to vitamin level or diet type.

**Table 11. Total mortality (%) and mean time to death (days) of spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of folic acid in Oregon test diet and Abernathy diet and challenged with Aeromonas salmonicida. Immunological study. Abernathy Salmon Culture Technology Center. 1986.**

Diet	Mean vitamin concentration (mg/Kg dry diet)	Total mortality <sup>1</sup>		Mean time <sup>1</sup> to death (Days)	
		Mean	(SEM)	Mean	(SEM)
Oregon Test	2	89.0	(3.5) <sup>a</sup>	7.3	(0.2) <sup>b</sup>
	7	80.0	(9.9) <sup>a</sup>	7.0	(0.3) <sup>b</sup>
	11	82.0	(4.2) <sup>a</sup>	7.3	(0.5) <sup>b</sup>
	14	63.0	(0.7) <sup>a</sup>	7.5	(0.0) <sup>b</sup>
	20	72.0	(9.9) <sup>a</sup>	7.4	(0.5) <sup>b</sup>
Abernathy	6 <sup>2</sup>	48.0	(33.9) <sup>a</sup>	6.4	<sup>b</sup>
	10	70.0	(12.7) <sup>a</sup>	7.5	(0.4) <sup>b</sup>
	13	81.0	(0.7) <sup>a</sup>	7.5	(0.1) <sup>b</sup>
	17	71.0	(3.5) <sup>a</sup>	7.1	(0.1) <sup>b</sup>
	20	58.0	(7.1) <sup>a</sup>	7.8	(0.4) <sup>b</sup>

<sup>1</sup>Values are means of duplicate lots of 50 fish : post challenge rearing 12 days.  
Values with different letter superscripts are significantly different, Student-Newman-Keuls test (p ≤ 0.05).

<sup>2</sup>Mortality occurred in only one replicate.

Overall it appears that the passage of time had the most significant, direct effects on immune function (mitogen responses and **PFCs**). This was not simply due to an increase in absolute numbers of lymphocytes but rather to increased percentages of functional cells.

**PYRIDOXINE:** The average pyridoxine levels were within 18% of the planned nominal concentrations (Table 13). The average rearing mortalities were approximately 1.1% with no significant differences between diets. Specific growth rates and feed efficiencies are summarized in Table 14. Fish fed AD grew at a significantly greater rate than those fed OTD. There were significant differences in these growth values among vitamin levels; however no trends related to the

Table 12. Total mortality (%) and mean time to death (days) for spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of folic acid in Oregon test diet and Abernathy diet and challenged with *Renibacterium salmoninarum*. Immunological study Abernathy Salmon Culture Technology Center, 1986.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Total mortality <sup>1</sup> %		Mean time <sup>1</sup> to death (Days)	
		Mean	(SEM)	Mean	(SEM)
Oregon test	2	1.0	(0.7) <sup>a</sup>	3.0	(2.1) <sup>b</sup>
	7	5.0	(2.1) <sup>a</sup>	13.7	(3.0) <sup>b</sup>
	11	3.0	(2.1) <sup>a</sup>	6.5	(4.6) <sup>b</sup>
	14	21.0	(4.9) <sup>a</sup>	12.9	(0.4) <sup>b</sup>
	20	11.0	(2.1) <sup>a</sup>	8.1	(2.2) <sup>b</sup>
Abernathy	6	13.0	(2.1) <sup>a</sup>	14.9	(2.1) <sup>b</sup>
	10	3.0	(2.1) <sup>a</sup>	7.3	(5.2) <sup>b</sup>
	13	15.0	(4.9) <sup>a</sup>	12.0	(0.3) <sup>b</sup>
	17	9.0	(0.7) <sup>a</sup>	15.6	(0.6) <sup>b</sup>
	20	13.0	(2.1) <sup>a</sup>	15.0	(0.3) <sup>b</sup>

<sup>1</sup>Values are means of duplicate lots of 50 fish, post challenge rearing 21 days. Values with different letter superscripts are significantly different, Student-Newman-Keuls test ( $p \leq 0.05$ ).

vitamin levels were observed. The AD, on the whole, was more efficient than OTD, although no significant differences related to the vitamin levels were observed.

The mitogenic responses to *E. coli* lipopolysaccharide are shown in Table 15 and the statistical analyses of the data appears in Table 16. There were no significant differences in mitogenic responses related to vitamin levels. Significant differences related to the effect of time were observed. However the confounding effects of diet formulation do not allow for a determination of any particular trends.

The results of the PFC assays are shown in Table 17 and the statistical analyses of these data in Table 18. No differences related to the vitamin level were observed. A significant difference in the PFC response was found between diet formulations, with OTD achieving higher responses than those seen with AD. Also, there were statistical differences associated with time, but no trend was noted. The absence of any such trend is most likely due to the significant interaction between diet and time.

Disease challenge data for this study are only available for the *A. salmonicida* challenge (Table 19). No data are available for the *R. salmoninarum* challenge due to a loss attributable to technical failure.

Table 13. Vitamin **analysis**<sup>1</sup> of pyridoxine diets. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Nominal vitamin level <sup>2</sup>	Actual vitamin level <sup>2</sup>		
		Mean	(SEM)	n
Oregon Test	5	5.9	<b>(0.6)</b>	<b>7</b>
	15	17.3	(0-4)	<b>7</b>
	35	41.3	(1.4)	<b>7</b>
	55	64.1	(3-0)	<b>7</b>
	75	80.8	(4.1)	<b>7</b>
Abernathy	15	17.1	(1.4)	<b>5</b>
	35	37.1	<b>(2.6)</b>	<b>5</b>
	55	49.3	(3-0)	<b>5</b>
	95	88.4	<b>(2.2)</b>	<b>5</b>

<sup>1</sup>All analyses were performed by **Hazelton** laboratories America, Inc., Madison, Wisconsin.

<sup>2</sup>Values are mg vitamin/Kg dry diet.

Table 14. Specific growth rate and gross feed efficiency of spring chinook (*Oncorhynchus tshawytscha*) fed two diets containing graded dietary concentrations of pyridoxine for 167 days. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Specific Growth <sup>1,3</sup>		Gross Feed <sup>2,3</sup>	
		Rate		Efficiency	
		Mean	(SEM)	Mean	(SEM)
Oregon test	6	1.532	<b>(0.003)<sup>c</sup></b>	76.45	<b>(0.30)<sup>f,g</sup></b>
	17	1.479	<b>(0.003)<sup>a</sup></b>	<b>71.03</b>	<b>(0.23)<sup>f</sup></b>
	41	1.508	<b>(0.002)<sup>b,c</sup></b>	<b>73.58</b>	<b>(1.79)<sup>f</sup></b>
	64	1.492	<b>(0.012)<sup>a,b</sup></b>	72.97	<b>(1.07)<sup>f</sup></b>
	81	1.497	<b>(0.007)<sup>a</sup></b>	73.21	<b>(0.51)<sup>f</sup></b>
Abernathy	17	1.597	<b>(0.005)<sup>d,e</sup></b>	79.42	<b>(0.11)<sup>g</sup></b>
	37	1.584	<b>(0.006)<sup>d</sup></b>	79.30	<b>(0.62)<sup>g</sup></b>
	49	1.608	<b>(0.004)<sup>e</sup></b>	80.86	<b>(1.36)<sup>g</sup></b>
	69	1.609	<b>(0.008)<sup>e</sup></b>	80.07	<b>(1.17)<sup>g</sup></b>
	88	1.593	<b>(0.002)<sup>d,e</sup></b>	79.93	<b>(0.49)<sup>g</sup></b>

$$^1\text{Specific growth rate SGR} = \frac{\log_e \text{ of weight at end} - \log_e \text{ of weight at start}}{\text{days fed}} \times 100$$

$$^2\text{Gross feed efficiency GFE} = \frac{\text{total fish gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

<sup>3</sup>Values are means of **duplicate** lots of fish, values with different superscripts are significantly different, Student-Newman-Keuls test (p ≤ 0.05).

Table 15. Mitogenic response (stimulation index) to *E. coli* lipopolysaccharide by anterior kidney lymphocytes from spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of pyridoxine in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by diet Mean (SEM)
		110 Mean (SEM)	138 Mean (SEM)	166 Mean (SEM)	
Oregon Test	6	14.7 (1.2)	8.5 (0.2)	<b>12.2</b> (1.9)	<b>11.8</b> (1.8)
	17	15.8 (1.6)	7.1 (1.9)	17.4 (0.2)	13.4 (3.2)
	41	<b>15.4</b> (3.4)	8.6 (2.2)	8.4 (0.3)	<b>10.8</b> (2.3)
	64	16.4 (1.6)	9.4 (1.0)	7.2 (0.8)	<b>11.0</b> (2.8)
	81	23.0 (0.6)	9.1 (1.5)	<b>12.0</b> (0.2)	14.7 (4.2)
<b>Time means</b>		17.1 (1.5)	8.5 (0.4)	<b>11.4 (1.8)</b>	
Abernathy	17	7.8 (1.0)	9.6 (4.2)	<b>15.0</b> (1.2)	<b>11.5</b> (1.8)
	37	<b>11.2</b> <sup>1</sup>	10.9 (3.7)	16.0 (0.7)	<b>12.7</b> (1.7)
	49	<b>11.2</b> (1.8)	10.0 (0.4)	17.0 (1.4)	12.7 (2.2)
	69	12.2 (0.4)	8.8 (2.0)	15.0 (1.4)	12.0 (1.8)
	88	7.4 (0.2)	<b>12.4</b> (1.5)	13.6 (0.4)	<b>11.1 (1.9)</b>
<b>Time means</b>		<b>10.4</b> (0.8)	<b>10.3</b> (1.0)	15.3 (0.6)	-

<sup>1</sup>Only one replicate.

Table 16. Analysis of variance. Mitogenic response (stimulation index) to *E. coli* lipopolysaccharide (100 µg/ml) by anterior kidney lymphocytes from spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of pyridoxine in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion <sup>2</sup>
			Calculated	Table <sup>1</sup>	
Groups	9	0.051	0.94	2.22	N.S.
Times	2	0.848	15.70	3.33	Sig.
Groups x time	<b>18</b>	<b>0.187</b>	3.46	1.98	Sig.
Diet	1	0.004	0.07	4.18	N.S.
Vitamin level <sup>3</sup>	3	0.033	0.61	2.93	N.S.
Diet x vitamin level <sup>3</sup>	<b>3</b>	0.109	2.02	2.93	N.S.
Diet x time <sup>3</sup>	<b>2</b>	0.734	13.60	3.33	Sig.
Vitamin level x time <sup>3</sup>	6	0.097	1.80	2.43	N.S.
Diet x time x vitamin level <sup>3</sup>	6	0.069	1.28	2.43	N.S.
Error	29	0.054			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>Significant (Sig.)  $p \leq 0.05$ . Not significant (N.S.),  $p > 0.05$ .

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

Table 17. In vitro antibody response (plaque-forming cell) to TNP-lipopolysaccharide (0.5 µg/ml) by anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed pyridoxine in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by diet Mean (SEM)
		110 Mean (SEM)	I.38 Mean (SEM)	166 Mean (SEM)	
Oregon Test	6	3042 (52)	1666 (125)	2675 (431)	2461 (411)
	17	3010 (80)	1479 (23)	2536 (164)	2342 (453)
	41	3504 (539)	2904 (20)	3658 (742)	3355 (360)
	64	3286 (550)	2477 (85)	3702 (127)	3155 (360)
	81	2434 (668)	1401 (122)	2188 (72)	2008 (312)
<b>Time means</b>		<b>3055 (179)</b>	<b>1985 (299)</b>	<b>2952 (308)</b>	
Abernathy	17	1836 (58)	716 (400)	2906 (50)	1816 (632)
	37	1645 <sup>1</sup>	1052 (36)	3228 (483)	1675 (369)
	49	1054 (284)	1280 (38)	3752 (112)	2029 (864)
	69	1170 (102)	1347 (5%)	2401 (53)	1639 (384)
	88	6% (128)	942 (173)	2836 (352)	1491 (676)
<b>Time means</b>		<b>1278 (204)</b>	<b>1067 (115)</b>	<b>2845 (254)</b>	

<sup>1</sup>Only one replicate.

Table 18. Analysis of variance. In vitro antibody response (plaque-forming cell) to TNP-lipopolysaccharide (0.5 µg/ml) by anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of pyridoxine in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion <sup>2</sup>
			Calculated	Table <sup>1</sup>	
Groups	9	0.890	8.09	2.22	Sig.
Times					
Groups x time	18	0.036	27.60	2.69	Sig. Sig.
Diet	1	4.665	42.40	4.18	Sig.
Vitamin level <sup>3</sup>	3	0.225	2.32	2.93	N.S.
Diet x vitamin level <sup>3</sup>	3	<b>0.135</b>	1.23	2.93	N.S.
Diet x time <sup>3</sup>	2	0.894	<b>8.12</b>	3.33	Sig.
Vitamin level x time <sup>3</sup>	6	0.1%	1.78	2.43	N.S.
Diet time x vitamin level <sup>3</sup>	6	0.090	0.82	2.43	N.S.
Error	29	0.110			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>Significant (Sig.) p ≤ 0.05. Not significant (N.S.) p > 0.05.

<sup>3</sup>Analysis performed ally on vitamin levels approximately common to both diets.

Table 19. Total mortality (%) and mean time to death (days) of spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of pyridoxine in Oregon test diet and Abernathy diet and challenged with Aeromonas salmonicida. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Total mortality <sup>1</sup> %		Mean time <sup>1</sup> to death (Days)	
		Mean	(SEM)	Mean	(SEM)
Oregon Test	6	86.0	(1.4) <sup>a</sup>	7.8	(0.2) <sup>c,d</sup>
	17	84.0	(1.4) <sup>a</sup>	7.5	(0.0) <sup>c</sup>
	41	93.0	(3.5) <sup>a</sup>	6.8	(0.0) <sup>c</sup>
	64	81.0	(2.1) <sup>a</sup>	8.0	(0.1) <sup>c,d</sup>
	81	87.0	(4.9) <sup>a</sup>	7.9	(0.2) <sup>c,d</sup>
Abernathy	17	51.0	(0.7) <sup>b</sup>	9.5	(0.2) <sup>d,e</sup>
	37	55.0	(6.4) <sup>b</sup>	9.3	(0.5) <sup>d,e</sup>
	49	34.0	(4.2) <sup>b</sup>	9.8	(0.2) <sup>e</sup>
	69	35.0	(2.1) <sup>b</sup>	9.3	(0.3) <sup>d,e</sup>
	88	40.0	(1.4) <sup>b</sup>	9.3	(0.0) <sup>d,e</sup>

<sup>1</sup>Values are means of duplicate lots of 50 fish, post challenge rearing 17 days. Values with different letter superscripts are significantly different, Student-Newman-Keuls test (p ≤ 0.05).

The A. salmonicida challenge revealed a significantly higher mortality for OTD groups over AD groups. The AD-fed fish also demonstrated a longer mean time to death than found with OTD-fed groups. No relationship was found among vitamin concentrations and the mean time to death or average mortality. All statistically significant effects were strictly related to the diet formulations.

An OTD containing only 60% of the NRC recommendation produced growth and immunological responses comparable not only to those diets containing the NRC recommended level, but also those with an 8-fold excess of this recommended level.

Another interesting observation can be made from these studies. Although the OTD-fed fish experience higher mortalities than AD-fed fish, the OTD-fed produce a higher PFC response. It must, however, be remembered that unimmunized fish experiencing an Aeromonas challenge rely strictly on innate defense mechanisms (i.e. phagocytosis) for protection. The PFC assay, on the other hand, measures the generation of specific immunity. Therefore, two distinctly different aspects of the immune defense system are being monitored through these assays.

**RIBOFLAVIN:** Table 20 summarizes the actual amounts of riboflavin achieved in the experimental diets.

Table 20. Vitamin **analysis**<sup>1</sup> of riboflavin diets. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Nominal vitamin <b>level</b> <sup>2</sup>	Actual vitamin level <sup>2</sup>		
		Mean	(SEM)	n
Oregon Test	5	7.3	(1.5)	8
	20	20.9	(1.0)	8
	65	63.2	(2.3)	8
	110	104.7	(5.6)	8
	155	146.9	(4.7)	8
Abernathy	20	19.4	(0.9)	6
	65	61.0	(1.7)	6
	110	107.2	(4.0)	6
	155	153.1	(4.1)	6
	200	197.5	(11.7)	6

<sup>1</sup>Vitamin analyses were performed by Woodson-Tenet Laboratories, Inc., Memphis, Tennessee; or Hazelton Laboratories America, Inc., Madison, Wisconsin.

<sup>2</sup>Values are mg vitamin/Kg dry diet.

All values, except for one diet, were within 6% of the objectives. The low-level OTD averaged 46% higher than desired, but it still as within 2 **mg/Kg** of the planned levels.

Analyses of the riboflavin concentrations in fish livers (Table 21) revealed no differences due to diet type or vitamin level in the feeds.

Table 21. Liver riboflavin analysis' of spring chinook (Oncohynchus tshawytscha) fed graded dietary concentrations of riboflavin in Oregon test diet and Abernathy diet for 161 days. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean dietary concentration (mg/Kg dry diet)	Liver riboflavin <sup>2</sup> ( $\mu$ g/g dry liver)	
		Mean	(SEM)
Oregon Test	7	52.4	(7.5) <sup>a</sup>
	21	46.8	(1.9) <sup>a</sup>
	63	46.8	(1.9) <sup>a</sup>
	105	39.3	(1.9) <sup>a</sup>
	147	48.5	(11.4) <sup>a</sup>
Abernathy	19	54.3	(5.6) <sup>a</sup>
	61	61.7	(9.3) <sup>a</sup>
	107	52.4	(0.0) <sup>a</sup>
	153	52.4	(0.0) <sup>a</sup>
	197	63.6	(7.5) <sup>a</sup>

<sup>1</sup>Analysis performed by **Hazelton** Laboratories America, Inc., Madison, Wisconsin.

<sup>2</sup>**Values** are means of duplicate pooled liver sample of 30-40 fish. Values with different superscripts are significantly different. Student-Newman-Keuls test (p 10.05).

The results suggest that 35% of the level recommended by the NRC is adequate to maintain tissue stores under our experimental conditions. Adding increasing amounts of riboflavin to the feed up to 10-fold the NRC value did not increase storage.

The average rearing mortalities were less than 0.1% over all diet groups. Specific growth rates and gross feed efficiencies (Table **22**) did not differ among diets or vitamin levels within diets. Fish fed riboflavin in 35% of the **NRC** recommendation grew and utilized their feed as well as fish receiving feeds containing up to 10 times the **NRC** recommendation.

The responses of lymphocytes to Vibrio anguillarum lipopolysaccharide are given in Table 23, and results of the plaque-forming cell assays are shown in Table 25. Statistical analysis of those data appear in Tables 24 and 26, respectively.

Table 22. Specific growth rate and gross feed efficiency of spring chinook (*Oncorhynchus tshawytscha*) fed two diets containing graded dietary concentrations of riboflavin for 159 days. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean vitamin concentration (ng/Kg dry diet)	Specific Growth <sup>1,3</sup> Rate		Gross Feed <sup>2,3</sup> Efficiency	
		Mean	(SEM)	Mean	(SEM)
Oregon Test	7	1.201	(0.006) <sup>a</sup>	97.84	(0.96) <sup>b</sup>
	21	1.201	(0.001) <sup>a</sup>	98.66	(0.52) <sup>b</sup>
	63	1.209	(0.002) <sup>a</sup>	98.98	(0.85) <sup>b</sup>
	105	1.205	(0.005) <sup>a</sup>	97.96	(0.09) <sup>b</sup>
	147	1.960	(0.002) <sup>a</sup>	97.92	(0.73) <sup>b</sup>
Abernathy	19	1.199	(0.003) <sup>a</sup>	94.19	(0.42) <sup>b</sup>
	61	1.198	(0.001) <sup>a</sup>	94.96	(0.02) <sup>b</sup>
	107	1.170	(0.011) <sup>a</sup>	91.84	(2.80) <sup>b</sup>
	153	1.184	(0.000) <sup>a</sup>	93.42	(1.07) <sup>b</sup>
	197	1.170	(0.011) <sup>a</sup>	92.39	(1.72) <sup>b</sup>

$$^1\text{Specific growth rate SGR} = \frac{\log, \text{ of weight at end} - \log, \text{ of weight at start}}{\text{days fed}} \times 100$$

$$^2\text{Gross feed efficiency GFE} = \frac{\text{total fish gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

<sup>3</sup>Values are means of duplicate lots of fish, values with different superscripts are significantly different, Student-Newman-Keuls test (p ≤ 0.05).

Table 23. Mitogenic response (stimulation index) to Vibrio anguillarum extract by anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of riboflavin in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by Diet Mean (SEM)
		90 Mean (SEM)	125 Mean (SEM)	160 Mean (SEM)	
Oregon Test	7	8.1 (0.3)	8.3 (2.1)	6.9 (0.0)	7.8 (0.4)
	21	8.4 (3.3)	8.0 (2.3)	2.8 (1.0)	6.4 (1.8)
	63	7.6 (2.7)	3.9 (0.0)	7.5 (1.3)	6.3 (1.8)
	105	14 (0.0)	6.1 (3.6)	3.2 (0.9)	7.8 (3.2)
	147	6.7 (1.8)	10.6 (0.0)	3.9 (2.7)	7.1 (1.9)
T i n e -		8.9 (1.3)	10.6 (0.0)	4.8 (1.0)	-
Abernathy	19	7.4 (2.9)	20.9 (0.9)	1.7 (0.05)	10.0 (5.7)
	61	11.6 (2.2)	16.3 (0.5)	1.6 (0.2)	9.8 (4.3)
	107	6.1 (2.1)	19.7 (5.1)	1.5 (0.6)	9.1 (5.5)
	153	5.9 (1.9)	19.5 (1.1)	1.6 (0.8)	9.0 (5.4)
	197	7.5 (0.7)	17.1 (0.5)	2.0 (0.0)	8.9 (4.4)
<b>Time means</b>		7.7 (1.0)	18.7 (0.4)	1.7 (0.1)	-

Table 24. Analysis of variance. Mitogenic response (stimulation index) to Vibrio anguillarum extract by anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of riboflavin in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion <sup>2</sup>
			Calculated	Table <sup>1</sup>	
Groups	9	0.076	0.36	2.27	N.S.
Times	2	11.351	54.10	3.37	Sig.
Groups x time	18	0.798	3.80	2.01	Sig.
Diet	1	0.000	0.00	4.23	N.S.
Vitamin level <sup>3</sup>	3	0.025	0.12	2.98	N.S.
Diet x vit <sup>3</sup> in level <sup>3</sup>	3	0.066	0.31	2.98	N.S.
Diet x time	2	4.144	19.70	3.37	Sig.
Vitamin level x time <sup>3</sup>	6	0.318	1.51	2.47	N.S.
Diet x time x vitamin level <sup>3</sup>	6	0.303	1.44	2.47	N.S.
Error	26	0.210			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>Significant (Sig.) p < 0.05. Not significant (N.S.) p > 0.05.

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

Table 25. In vitro antibody response (plaque-forming cell) to **TNP-lipopolysaccharide** (0.5 &ml) by anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of riboflavin in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by Diet Mean (SEM)
		90	125	160	
Oregon Test	7	620 (263)	-	246 (40)	433 (187)
	21	636 (97)	-	236 (217)	436 (126)
	63	525 (256)	-	416 (394)	470 (34)
	105	389 (0)	-	539 (66)	424 (125)
	147	511 (137)	-	49 (33)	280 (231)
<b>Time means</b>		536 (44)	-	297 (84)	-
Abernathy	19	1231 (219)	-	52 (36)	641 (589)
	61	911 (182)	-	17 (8)	464 (447)
	107	944 (163)	-	20 (1)	482 (456)
	153	933 (249)	-	20 (2)	476 (456)
	197	1153 (424)	-	46 (0)	599 (553)
<b>Time Means</b>		1034 (66)	-	31 (8)	-

<sup>1</sup>Assay lost due to culture contamination.

Table 26. Analysis of variance. In vitro antibody response (plaque-forming cell) to TNP-lipopolysaccharide (0.5 &ml) by anterior kidney lymphocytes from spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of riboflavin in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion'
			Calculated	Table1	
Groups	9	1.51	0.57	2.39	N.S.
Times	1	212.10	79.40	4.35	sig.
Groups x time	9	8.33	3.12	2.39	sig.
Diet	1	1.04	0.39	4.35	N.S.
Vitamin level <sup>3</sup>	3	1.73	0.65	3.10	N.S.
Diet x vitamin level <sup>3</sup>	3	1.86	0.70	3.10	N.S.
Diet x time <sup>3</sup>	1	48.10	18.00	4.35	sig.
Vitamin level x time <sup>3</sup>	3	2.79	1.04	3.10	N.S.
Diet x time x vitamin level <sup>3</sup>	3	2.54	0.95	3.10	N.S.
Error	20	2.67			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>**Significant** (Sig.) p 10.05. Not significant (N.S.) p >0.05.

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

Vitamin levels and diet type had no significant impacts on either of the indices of **immunocompetency**. Although length of feeding was a significant source of variance, significant interaction terms with diet formulation confounded the interpretation. There were no consistent positive or negative trends with time for either type of test of immune response.

Results of the disease challenges with *A. salmonicida* and *R. salmoninarum* are presented in Tables 27 and 28, respectively. There were no **significant** differences in our measures of resistance to either of these two pathogens.

Overall, the lack of any differences in growth, immune responses, or disease resistance probably reflect the lack of any differences in the vitamin status of the fish.

Table 27. Total mortality (%) and mean time to death (days) for spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of riboflavin in Oregon test diet and Abernathy diet and challenged with Aeromonas salmonicida. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Total mortality <sup>1</sup> %		Mean time <sup>1</sup> to death (Days)	
		Mean	(SEM)	Mean	(SEM)
Oregon Test	7	14.0	(7.1) <sup>a</sup>	11.1	(1.5) <sup>b</sup>
	21	10.0	(4.2) <sup>a</sup>	10.01	(3.2) <sup>b</sup>
	63	8.0	(2.8) <sup>a</sup>	7.5	(2.5) <sup>b</sup>
	105	16.0	(1.4) <sup>a</sup>	9.0	(0.2) <sup>b</sup>
	147	13.0	(0.7) <sup>a</sup>	9.7	(1.2) <sup>b</sup>
Abernathy	19	13.0	(2.1) <sup>a</sup>	12.0	(0.7) <sup>b</sup>
	61	15.0	(2.1) <sup>a</sup>	13.1	(0.8) <sup>b</sup>
	107	12.0	(1.4) <sup>a</sup>	12.9	(0.3) <sup>b</sup>
	153	12.0	(5.7) <sup>a</sup>	13.5	(0.7) <sup>b</sup>
	197	10.0	(2.8) <sup>a</sup>	14.8	(1.3) <sup>b</sup>

<sup>1</sup>Values are means of duplicate lots of 50 fish; post challenge rearing 20 days. Values with different superscripts are significantly different, Student-Newman-Keuls test (p ≤ 0.05).

Table 28. Total mortality (%) and mean time to death (days) for spring chinook (Oncorhynchus tshawytscha) fed graded diet concentrations of riboflavin in Oregon test diet and Abernathy diet and challenged with Renibacterium salmoninarum. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Total mortality <sup>1</sup> %		Mean time <sup>1</sup> to death (Days)	
		Mean	(SEM)	Mean	(SEM)
Oregon Test	21 <sup>2</sup>	63.0	(0.7) <sup>a</sup>	51.3	(0.4) <sup>b</sup>
	63	80.0	(2.0) <sup>a</sup>	51.2	(0.3) <sup>b</sup>
	105	71.0	(3.5) <sup>a</sup>	50.3	(1.0) <sup>b</sup>
	147	56.0	(2.8) <sup>a</sup>	51.2	(0.7) <sup>b</sup>
Abernathy	19	87.0	(7.8) <sup>a</sup>	51.1	(0.2) <sup>b</sup>
	61	85.0	(10.6) <sup>a</sup>	52.6	(0.7) <sup>b</sup>
	107	86.0	(0.0) <sup>a</sup>	50.9	(0.6) <sup>b</sup>
	153	80.0	(1.4) <sup>a</sup>	51.5	(0.6) <sup>b</sup>
	197	85.0	(2.1) <sup>a</sup>	50.4	(0.7) <sup>b</sup>

<sup>1</sup>Values are means of duplicate lots of fish, post challenge rearing 59 days. Values with different superscripts are significantly different, Student-Newman-Keuls test (p 10.05).

<sup>2</sup>Replicate lot lost to water flow failure.

**PANTHOTHENIC ACID:** Table 29 presents the mean pantothenic acid concentrations obtained in our experimental diets compared to nominal-levels. Most values were within 10% of target amounts.

Table 29. Vitamin **analysis**<sup>1</sup> of **pantothenic** acid diets. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Nominal vitamin level <sup>2</sup>	Actual vitamin level <sup>2</sup>		
		Mean	(SEM)	n
Oregon Test	10	17.0	(0.9)	8
	40	48.5	(1.9)	7
	130	132.1	(4.0)	8
	220	213.9	(7.7)	8
	310	320.4	(6.9)	8
Abernathy	40	40.1	(3.5)	6
	130	128.9	(5.1)	6
	220	205.5	(5.5)	6
	310	278.5	(7.8)	6
	400	367.2	(10.3)	6

<sup>1</sup>Vitamin analyses were performed by Woodson-Tenet Laboratories, Inc., Memphis, Tennessee; or **Hazelton** Laboratories America, Inc., Madison, Wisconsin.

<sup>2</sup>Values are mg vitamin/Kg dry diet.

Pantothenic acid concentrations in the livers of experimental fish are presented in Table 30. There were significant differences in storage among vitamin levels. The data suggested a positive trend in liver stores as a function of dietary vitamin level in both types of feed. Regression analyses confirmed this. Correlation coefficients for OTD and AD were 0.65 and 0.87, respectively. These values are significant at the 0.05 level.

Mortality during the feeding phase of the trial was very low. Total death rates averaged less than 0.08% and there were no significant differences among treatments. Growth and feed deficiencies were similar in all treatments and did not differ **significantly** among feed types or vitamin levels (Table 31).

Table 30. Liver pantothenic acid analysis<sup>1</sup> of spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of **pantothenic** acid in Oregon test diet and Abernathy diet for 154 days. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Liver pantothenic acid <sup>2</sup> ( $\mu\text{g/g}$ dry liver)	
		Mean	(SEM)
Oregon Test	17	54.3	(5.6) <sup>a</sup>
	48	56.1	(3.7) <sup>a</sup>
	132	65.5	(1.9) <sup>a,b</sup>
	214	73.0	(1.9) <sup>b,c</sup>
	320	65.5	(5.6) <sup>a,b</sup>
Abernathy	40	54.3	(1.9) <sup>a</sup>
	129	76.7	(1.9) <sup>b,c</sup>
	205	78.6	(7.5) <sup>b,c</sup>
	278	82.3	(3.7) <sup>b,c</sup>
	367	<b>89.8</b>	(0.0) <sup>c</sup>

<sup>1</sup>Analysis performed by **Hazelton** Laboratories America, Inc., Madison, Wisconsin.

<sup>2</sup>**Values** are means of duplicate pooled liver sample of 40 fish. Values with different superscripts are significantly different, Student-Newman-Keuls test (p < 0.05).

Table 31. Specific growth rate and gross feed efficiency of spring chinook (*Oncorhynchus tshawytscha*) fed two diets containing graded dietary concentrations of pantothenic acid for 152 days. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Specific Growth <sup>1,3</sup>		Gross Feed <sup>2,3</sup>	
		Rate	(SEM)	Efficiency	(SEM)
		Mean		Mean	
Oregon Test	17	1.147	(0.005) <sup>a</sup>	99.30	(0.12) <sup>b</sup>
	48	1.136	(0.020) <sup>a</sup>	97.40	(1.56) <sup>b</sup>
	132	1.138	(0.002) <sup>a</sup>	97.57	(0.85) <sup>b</sup>
	214	1.117	(0.006) <sup>a</sup>	96.15	(0.20) <sup>b</sup>
	320	1.137	(0.015) <sup>a</sup>	97.90	(0.29) <sup>b</sup>
Abernathy	40	1.149	(0.017) <sup>a</sup>	97.32	(2.67) <sup>b</sup>
	129	1.162	(0.014) <sup>a</sup>	99.54	(0.87) <sup>b</sup>
	205	1.134	(0.009) <sup>a</sup>	93.93	(0.97) <sup>b</sup>
	278	1.151	(0.027) <sup>a</sup>	98.36	(3.25) <sup>b</sup>
	367	1.151	(0.001) <sup>a</sup>	97.24	(1.18) <sup>b</sup>

$$^1\text{Specific growth rate SGR} = \frac{\log_e \text{ of weight at end} - \log_e \text{ of weight at start}}{\text{days fed}} \times 100$$

$$^2\text{Gross feed efficiency GFE} = \frac{\text{total fish gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

<sup>3</sup>Values are means of duplicate lots of fish. Values with different superscripts are significantly different, Student-Newman-Keuls test ( $p \leq 0.05$ ).

Dietary pantothenic acid levels did not significantly influence our measurements of lymphocyte responses to mitogenic stimulation (Tables 32 and 33). The length of time that the fish were fed the test diets was associated with a significantly increased response. Also, fish which were fed the AD exhibited a significantly increased mitogenic response compared to OTD-fed fish.

The **PFC assays** (Table 34) appeared to show effects of time and diet type which were similar to those observed with mitogen testing. However, the results were much more variable and the differences were not statistically significant (Table 35). The PFC responses were also not affected by vitamin level in either diet.

Fish fed graded concentrations of pantothenic acid did not experience any significantly different percent mortalities or mean times to death when challenged with A. salmonicida or R. salmoninarum (Tables 36 and 37).

Table 32. Mitogenic response (stimulation index) to Vibrio anguillarum extract by anterior kidney lymphocytes from spring shinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of pantothenic acid in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by Diet Mean (SEM)
		83 Mean (SEM)	118 Mean (SEM)	1531 Mean (SEM)	
Oregon Test	17	2.5 (0.1)	3.4 (0.0)		3.0 (0.4)
	48	2.4 (0.0)	1.9 (0.1)		2.2 (0.2)
	132	2.2 (0.8)	6.8 (0.8)		4.5 (2.3)
	214	2.7 (0.4)	8.4 (3.4)		5.6 (2.8)
	320	2.2 (0.5)	4.7 (0.7)		3.4 (1.2)
<b>Time means</b>		2.4 (0.1)	5.0 (1.2)		
Abernathy	40		14.6 (8.2)		14.6 (8.2)
	129	6.8 (0.2)	<b>12.1</b> <sup>2</sup>		9.4 (2.6)
	205	6.2 (0.3)	13.0 (0.5)		9.6 (3.4)
	278	7.1 (2.2)	5.1 (4.2)		6.1 (1.0)
	367	5.0 (0.4)	13.6 (0.8)		9.3 (4.3)
<b>Time means</b>		6.3 (0.5)	11.7 (1.7)		

<sup>1</sup>Immunological assay lost to culture contamination.

<sup>2</sup>Only one replicate.

Table 33. Analysis of variance. Mitogenic response (stimulation index) to Vibrio anguillarum extract of anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of pantothenic acid in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion <sup>2</sup>
			Calculated	Table <sup>1</sup>	
Groups	9	0.958	2.48	2.39	sig.
Times	1	1.714	4.43	4.35	sig.
Groups x time	9	0.537	1.39	2.39	N.S.
Diet	1	3.588	9.35	4.35	sig.
Vitamin level <sup>3</sup>	3	0.447	1.16	3.10	N.S.
Diet x vitamin level <sup>3</sup>	3	0.500	1.29	3.10	N.S.
Diet x time <sup>3</sup>	1	1.021	2.64	4.35	N.S.
Vitamin level x time <sup>3</sup>	3	0.304	0.79	3.10	N.S.
Diet x time x vitamin level <sup>3</sup>	3	0.843	2.18	3.10	N.S.
Error	20	0.387			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>Significant (Sig.) p < 0.05. Not significant (N.S.) p > 0.05.

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

Table 34. In vitro antibody response (plaque-forming cell) to **TNP-Lipopolysaccharide** (0.5 µg/ml) of anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of pantothenic acid in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by Diet Mean (SEM)
		83	118	1531	
Oregon Test	17	368 (122)	461 (354)		414 (46)
	48	268 (6)	644 (82)		456 (188)
	132	218 (8)	816 (279)	-	517 (299)
	214	558 (122)	407 (13)		482 (76)
	320	396 (56)	803 (250)		600 (204)
<b>Time means</b>		362 (59)	626 (85)	-	-
Abernathy	40		206 <sup>2</sup> (350)		590 (350)
	129	440 (166)			323 (117)
	205	241 (31)	534 (193)	-	388 (146)
	278	412 (60)	263 (263)	-	338 (74)
	367	178 (48)	1246 (132)	-	712 (534)
<b>Time means</b>		318 (64)	568 (185)	-	-

<sup>1</sup>Immunological assay lost to culture contamination.

<sup>2</sup>Only one replicate.

Table 35. Analysis of variance. In vitro antibody response (plaque-forming cell) to TNP-lipopolysaccharide (0.5 &ml) of anterior kidney lymphocytes from spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of pantothenic acid in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion <sup>2</sup>
			Calculated	Table <sup>1</sup>	
Groups	9	2.16	0.64	2.49	N.S.
Times	1	7.87	2.33	4.45	N.S.
Groups x time	9	5.92	1.75	2.49	N.S.
Diet	1	10.80	3.18	4.45	N.S.
Vitamin level <sup>3</sup>	2	0.33	0.10	3.59	N.S.
Diet x vitamin level <sup>3</sup>	2	1.71	0.51	3.59	N.S.
Diet x time <sup>3</sup>	1	8.67	2.56	4.45	N.S.
Vitamin level x time <sup>3</sup>	2	1.16	0.34	3.59	N.S.
Diet x time x vitamin level <sup>3</sup>	2	9.25	2.74	3.59	N.S.
Error	17	3.38			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>Significant (Sig.) p < 0.05. Not significant (N.S.) p > 0.05.

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

Although fish in our studies apparently had not reached maximum liver storage of pantothenic acid when they were fed a level recommended by NRC, absorption of additional vitamin from fortified diets did not affect their growth, improve their immune response, or influence resistance to two common fish pathogens.

ASCORBIC ACID: The results of dietary vitamin analysis for ascorbic acid trials (Table 38) show considerable variation from planned levels in the AD. Due to the labile nature of ascorbic acid it is difficult to obtain precise dietary concentrations. Mean vitamin values found in AD represented only 50% of the planned vitamin levels. Table 39 presents storage loss of ascorbic acid in both diet formulations. Although supplemented ascorbic acid was in a stable form (soy oil encapsulated) storage loss in AD over 90 days at room temperature (22°C) ranged from 44 to 89%. Storage loss was minimal in the OTD stored 20 to 35 days at -60°C.

Variation in dietary ascorbic acid level had no significant effect on liver ascorbate levels between diet formulations or within a diet formulation (Table 40). Values were highly variable. These values may not fully reflect the vitamin status of the fish because liver tissue does not represent the primary storage site in salmonids (Halver, 1979).

Minimal rearing mortality (0.2%) was experienced in all diet trials except those having no supplementation of ascorbic acid and alpha-tocopherol. Fish fed nonsupplemented OTD had a mean mortality value of 10.7%. Groups fed nonsupplemented AD experienced a mean mortality value of 31.0%. In both diet formulations some fish had scoliosis or lordosis which are signs of ascorbic acid deficiency. Except for the nonsupplemented diet, specific growth rate (Table 41) was significantly greater

Table 36. Total mortality (%) and mean time to death (days) for spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of pantothenic acid in Oregon test diet and Abernathy diet and challenged with *Aeromonas salmonicida*. Immunological study. Abernathy Salmon Culture Technology Center, 1987

Diet	Mean vitamin concentration (mg/Kg dry diet)		Total mortality <sup>1</sup>		Mean time <sup>1</sup> to death (Days)	
			Mean	(SEM)	Mean	(SEM)
Oregon Test	17		7.0	(0.7) <sup>a</sup>	16.6	(1.0) <sup>b</sup>
	48 <sup>2</sup>		4.0	(2.8) <sup>a</sup>	17.2	<sup>b</sup>
	132		10.0	(2.8) <sup>a</sup>	14.3	(0.0) <sup>b</sup>
	2	1 4	15.0	(3.5) <sup>a</sup>	11.8	(0.8) <sup>b</sup>
	320		16.0	(1.4) <sup>a</sup>	12.3	(1.2) <sup>b</sup>
Abernathy	40		13.0	(2.1) <sup>a</sup>	13.7	(0.7) <sup>b</sup>
	129		23.0	(2.1) <sup>a</sup>	13.8	(1.5) <sup>b</sup>
	205		10.5	(1.1) <sup>a</sup>	15.3	(0.4) <sup>b</sup>
	278		21.0	(2.1) <sup>a</sup>	13.9	(2.5) <sup>b</sup>
	367		9.0	(3.5) <sup>a</sup>	15.8	(1.5) <sup>b</sup>

<sup>1</sup>Values are means of duplicate lots of 50 fish, post-challenge rearing 23 days. Values with different superscripts are significantly different, **Student-Newman-Keuls** test (**p ≤ 0.05**).

<sup>2</sup>Mortality occurred in only one replicate.

Table 37. Total mortality (%) and mean time to death (days) for spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of pantothenic acid in Oregon test diet and Abernathy diet and challenged with *Renibacterium salmoninarum*. Immunological study. Abernathy Salmon Culture Technology Center, 1987.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Total mortality <sup>1</sup>		Mean time <sup>1</sup> to death (Days)	
		Mean	(SEM)	Mean	(SEM)
Oregon Test	17 <sup>2</sup>	100.0	a	26.6	b
	48	71.0	(20.5) <sup>a</sup>	25.9	(3.6) <sup>b</sup>
	132	92.0	(5.6) <sup>a</sup>	20.8	(0.1) <sup>b</sup>
	214	92.0	(2.8) <sup>a</sup>	23.7	(2.1) <sup>b</sup>
	320	100.0	(0.0) <sup>a</sup>	26.5	(1.2) <sup>b</sup>
Abernathy	40	97.0	(0.7) <sup>a</sup>	20.3	(1.0) <sup>b</sup>
	129	93.0	(0.7) <sup>a</sup>	24.5	(4.4) <sup>b</sup>
	205	92.0	(2.8) <sup>a</sup>	23.1	(3.4) <sup>b</sup>
	278	98.0	(1.4) <sup>a</sup>	25.4	(0.3) <sup>b</sup>
	367	93.0	(2.1) <sup>a</sup>	27.0	(0.4) <sup>b</sup>

<sup>1</sup>Values are means of duplicate lots of 50 fish, post challenge rearing 40 days. Values with different superscripts are significantly different, Student-Newman Keuls test (p. 10.05).

<sup>2</sup>Replicate lot lost to water failure.

Table 38. Vitamin analysis<sup>1</sup> of ascorbic acid diets. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Nominal vitamin level <sup>2</sup>	Actual vitamin level <sup>2</sup>		
		Mean	(SEM)	n
Oregon Test	10	16.4	(2.5)	14
	20	29.9	(1.2)	14
	100	139.5	(5.3)	14
	500	661.1	(50.2)	14
	2500	2884.7	(39.5)	14
	0 <sup>3</sup>	11.2	(0.8)	14
Abernathy	20	10.1	(2.5)	3
	100	31.7	(9.1)	4
	500	272.5	(108.8)	4
	2500	1406.4	(565.4)	4
	0 <sup>3</sup>	23.2	(4.9)	4

<sup>1</sup>All analysis were performed by Hazelton Laboratories America, Inc., Madison, Wisconsin.

<sup>2</sup>Values are mg vitamin/Kg dry diet.

<sup>3</sup>Represents diets not supplemented with either ascorbic acid or a-tocopherol.

Table 39. Storage loss (%) of dietary ascorbic acid'. Immunological study.  
Abernathy Salmon Culture Technology Center, 1988.

Diet	Nominal <sup>2</sup> Level	Mean initial <sup>2</sup> Level	Mean Final <sup>2</sup> level	Storage Loss (%)
Oregon Test <sup>3</sup>	0	11.4	11.0	3.5
	10	16.1	16.7	
	20	28.7	31.1	
	100	139.6	139.3	0.2
	500	622.8	699.4	
	2500	2917.2	2852.1	
Abernathy <sup>4</sup>	0	29.7	16.7	43.8
	20	15.1	7.5	50.3
	100	49.9	13.4	73.1
	500	485.0	59.9	87.6
	2500	2532.1	280.7	88.9
	5000	5076.2	653.3	87.1

<sup>1</sup>Soy oil encapsulated ascorbic acid, Delchem Corp., Statehill, New York.

<sup>2</sup>Values are mg vitamin/Kg **dry** diet.

<sup>3</sup>Diet stored 20-35 days, -60°C.

<sup>4</sup>Diet stored 90 days, room temperature.

Table 40. Liver ascorbate analysis<sup>1</sup> of spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of ascorbic acid in Oregon test diet and Abernathy diet for 252 days. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean dietary concentration (mg/Kg dry diet)	Liver ascorbate <sup>2</sup> ( $\mu\text{g/g}$ dry liver)	
		Mean	(SEM)
Oregon Test	16	54.2	(17.6) <sup>a</sup>
	30	56.9	(3.2) <sup>a</sup>
	139	--	--
	661	171.0	(84.6) <sup>a</sup>
	2885	76.6	(6.7) <sup>a</sup>
Abernathy	10	67.3	(20.7) <sup>a</sup>
	32	42.2	(1.1) <sup>a</sup>
	272	69.9	(9.3) <sup>a</sup>
	1406	80.3	(3.0) <sup>a</sup>
	2864	63.3	(17.7) <sup>a</sup>

<sup>1</sup>Analysis performed by Northwest and Alaska Fisheries Center, NMFS, Seattle, Washington.

<sup>2</sup>Values are means of duplicate pooled liver sample of 12-14 fish. Values with different superscripts are significantly different. Student-Newman-Keuls test (p 10.05).

Table 41. Specific growth rate and gross feed efficiency of spring chinook (*Oncorhynchus tshawytscha*) fed two diets containing graded dietary concentrations of ascorbic acid for 252 days. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Specific Growth <sup>1,3</sup> Rate		Gross Feed <sup>2,3</sup> Efficiency	
		Mean	(SEM)	Mean	(SEM)
Oregon Test	16	1.025	(0.009) <sup>a</sup>	102.95	(1.20) <sup>e,f</sup>
	30	1.078	(0.004) <sup>b</sup>	107.45	(0.47) <sup>f,g</sup>
	139	1.079	(0.003) <sup>b</sup>	105.97	(0.25) <sup>f,g</sup>
	661	1.078	(0.004) <sup>b</sup>	105.72	(0.18) <sup>f,g</sup>
	2885	1.082	(0.000) <sup>b</sup>	107.57	(0.75) <sup>f,g</sup>
	<b>11<sup>4</sup></b>	0.987	(0.007) <sup>c</sup>	100.50	(0.73) <sup>e</sup>
Abernathy	10	1.118	(0.000) <sup>a</sup>	108.27	(0.92) <sup>g</sup>
	32	1.132	(0.000) <sup>a</sup>	110.38	(0.73) <sup>g</sup>
	272	1.106	(0.003) <sup>a</sup>	107.29	(0.63) <sup>f,g</sup>
	1406	1.115	(0.004) <sup>a</sup>	107.00	(0.59) <sup>f,g</sup>
	2865	1.106	(0.001) <sup>a</sup>	106.97	(1.16) <sup>f,g</sup>
	<b>23<sup>5</sup></b>	0.962	(0.007) <sup>d</sup>	107.20	(0.98) <sup>f,g</sup>

$$^1\text{Specific growth rate SGR} = \frac{\log_e \text{ of weight at end} - \log_e \text{ of weight at start}}{\text{days fed}} \times 100$$

$$^2\text{Gross feed efficiency GFE} = \frac{\text{total fish gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

<sup>3</sup>Values are means of duplicate lots of fish. Values with different superscripts are significantly different, Student-Newman-Keuls test (p ≤ 0.05).

<sup>4</sup>Represents diet not supplemented with either ascorbic acid or a-tocopherol (17.4mg/Kg dry diet).

<sup>5</sup>Represents diet not supplemented with either ascorbic acid or a-tocopherol (13.9mg/Kg dry diet).

with the AD formulation. Within the OTD formulations, the nonsupplemented diet had the lowest growth rate. The OTD containing 30% of the NRC recommended level did not produce growth rates significantly different from an OTD containing a megadose (i.e. 380 times the NRC recommended level) of ascorbic acid. Except for the OTD diet that was unsupplemented, there were few feed efficiency differences between and within diet formulations.

The mitogenic responses to Vibrio anguillarum lipopolysaccharide are found in Table 42 with statistical analysis found in Table 43. Results show that there was no significant difference due to vitamin level. Both diet type and passage of time were found to have **significant** effects. OTD had significantly higher mitogenic responses than AD. In both diet treatments the mitogenic response improved with increasing days of feeding.

Results of the PFC assay are shown in Table 44 with statistical analysis on Table 45. Although there is a significant difference between diet types there is evidence of an interaction between the diet type and time which prevents clear cut conclusions.

When the fish were challenged with A. salmonicida (Table 46) there appeared to be no consistent difference due to **vitamin** level. Generally, total mortality was higher in lots fed AD, however, the differences are not distinct. Mean time to death was not significantly different between or within diet types.

Results of challenges with R. salmoninarum are presented in Table 47. It should be noted that fish fed **the unsupplemented** OTD and AD were not challenged because the fish stocks were reduced to extremely low levels by immunological assays and an elevated mortality rate. There were no significant differences in total mortality due to diet type or vitamin level. Mean time to death data showed no consistent relationship to diet type or vitamin level.

**ALPHA-TOCOPHEROL:** The actual dietary levels of alpha-tocopherol were within 10% of the nominal concentration for the four OTD and AD formulations containing the highest concentrations of vitamin (Table 48). The actual concentrations for the two lowest levels of either formulation were at least 62% of the nominal level.

Analysis of the liver alpha-tocopherol content at the termination of this experiment revealed a direct correlation between the concentration of the vitamin in the feed and the amount stored within the tissues (Table 49). For both OTD and AD the correlation coefficient was found to be 0.99 and each was significant at a probability value of **.01**.

The average rearing mortality was never greater than 0.1%. The specific growth rates and gross feed efficiencies demonstrated no differences related to vitamin concentration or diet formulation (Table 50).

Assessment of mitogenic responses (Table 51) revealed statistical differences (Table 52) among the levels of vitamin; however, no distinct trend was observed. Overall OTD demonstrated significantly higher mitogenic responses than did AD. It is also quite apparent that the most significant influence on the mitogenic response was that of time. PFC data (Table 53) and statistical analysis (Table 54) reveal much the same effects, as were seen with mitogenesis. There was, however, a much

Table 42. **Mitogenic** response (stimulation index) to Vibrio anguillarum extract of anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of ascorbic acid in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by Diet Mean (SEM)
		90 Mean (SEM)	132 Mean (SEM)	174 Mean (SEM)	
Oregon Test	<b>11<sup>1</sup></b>	4.7 (2.4)	1.5 (0.3)	12.1 (0.3)	<b>6.1 (3.1)</b>
	16	2.9 (0.2)	1.1	15.5 (7.0)	6.5 (0.6)
	30	3.6 (1.0)	6.9 (5.6)	15.0 (4.2)	6.6 (3.2)
	139	4.9 (0.6)	3.3 (2.2)	13.0 (2.8)	7.1 (3.0)
	661	3.4 (2.1)	12.5 (9.4)	5.2 (1.5)	7.0 (2.8)
	2885	6.0 (2.8)	5.8 (4.6)	12.0 (2.8)	7.9 (2.0)
<b>Time means</b>		4.2 (0.4)	5.2 (1.7)	<b>12.1 (1.5)</b>	
Abernathy	<b>23<sup>2</sup></b>	1.3 (0.1)	1.7 (0.1)	5.1 (1.7)	2.7 (0.6)
	10	1.0 (0.1)	4.5 (2.7)	2.1 (1.0)	2.5 ( <b>1.0</b> )
	32	1.1 (0.1)	1.7 (0.3)	2.2 (0.1)	<b>1.7 (0.3)</b>
	272	1.1 (0.1)	1.2 (0.2)	6.6 (1.2)	3.0 (1.8)
	1406	1.2 (0.1)	8.9 (3.7)	12.8 (3.9)	7.6 (3.4)
	2864	1.1 (0.1)	1.5 (0.6)	1.9 (0.2)	1.5 (0.2)
<b>Time means</b>		<b>1.1 (0.4)</b>	3.2 (1.2)	<b>5.1 (1.7)</b>	-

<sup>1</sup>Represents diet not supplemented with either ascorbic acid or a-tocopherol (17.4 mg/Kg dry diet).

<sup>2</sup>Represents diet not supplemented with either ascorbic acid or a-tocopherol (13.9 mg/Kg dry diet).

Table 43. Analysis of variance. Mitogenic response (stimulation index) to Vibrio anguillarum extract by anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of ascorbic acid in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion <sup>2</sup>
			Calculated	Table <sup>1</sup>	
Groups	11	1.65	3.89	2.09	sig.
Times	2	9.29	21.90	3.28	sig.
Groups x time	22	0.69	1.63	1.87	N.S.
Diet	1	10.25	24.20	4.13	sig.
Vitamin level <sup>3</sup>	4	0.66	1.56	2.65	N.S.
Diet x vitamin level <sup>3</sup>	4	1.00	2.36	2.65	N.S.
Diet x time <sup>3</sup>	2	1.11	2.62	3.28	N.S.
Vitamin level x time <sup>3</sup>	8	0.65	1.53	2.23	N.S.
Diet x time x vitamin level <sup>3</sup>	8	0.71	1.68	2.23	N.S.
Error	35	0.42			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>Significant (Sig.) p < 0.05. Not significant (N.S.) p > 0.05.

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

Table 44. In vitro antibody response (plaque-forming cell) to TNP-lipopolysaccharide (0.5 µg/ml) of anterior kidney lymphocytes from spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of ascorbic acid in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by Diet Mean (SEM)
		90 Mean (SEM)	118 Mean (SEM)	153 Mean (SEM)	
Oregon Test	11 <sup>1</sup>	427 (147)	244 (27)	545 (372)	405 (87)
	16	492 (239)	6	253 (63)	250 (99)
	30	378 (178)	167 (161)	568 (259)	371 (116)
	139	640 (315)	341 (68)	682 (148)	480 (103)
	661	640 (315)	432 (62)	753 (116)	608 (94)
	2885	450 (4)	223 (73)	863 (505)	512 (187)
<b>Time means</b>		<b>467 (38)</b>	<b>235 (60)</b>	<b>611 (86)</b>	-
Abernathy	23 <sup>2</sup>	142 (42)	219 (58)	33 (29)	131 (54)
	10	45 (13)	226 (223)	55 (51)	109 (59)
	32	35 (220)	91 (29)	65 (36)	64 (16)
	272	53 (35)	145 (80)	253 (162)	150 (58)
	1406	68 (30)	345 (333)	289 (129)	234 (84)
	2864	122 (54)	236 (64)	151 (114)	170 (34)
<b>Time means</b>		<b>77 (18)</b>	<b>210 (35)</b>	<b>141 (44)</b>	-

<sup>1</sup>Represents diet not supplemented with either ascorbic acid or α-tocopherol (17.4 mg/Kg dry diet).

<sup>2</sup>Represents diet not supplemented with either ascorbic acid or α-tocopherol (13.9 mg/Kg dry diet).

Table 45. Analysis of variance. In vitro antibody response (plaque-forming cell) to TNP-lipopolysaccharide (0.5  $\mu\text{g/ml}$ ) by anterior kidney lymphocytes from spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of ascorbic acid in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1988.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion <sup>2</sup>
			Calculated	Table 1	
Groups	11	13.72	4.18	2.08	Sig.
Times	2	3.52	0.97	3.27	N.S.
Groups x time	22	3.03	0.84	1.86	N.S.
Diet	1	<b>99.50</b>	27.50	4.12	Sig.
Vitamin level <sup>3</sup>	4	7.88	2.18	2.64	N.S.
Diet x vitamin level <sup>3</sup>	4	0.47	0.13	2.64	N.S.
Diet x time <sup>3</sup>	2	14.00	3.87	3.27	Sig.
Vitamin level x time <sup>3</sup>	8	1.55	0.43	2.22	N.S.
Diet x time x vitamin level <sup>3</sup>	8	0.95	0.26	2.22	N.S.
Error	36	3.61			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>Significant (Sig.)  $p \leq 0.05$ . Not significant (N.S.)  $p > 0.05$ .

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

Table 46. Total mortality (%) and mean time to death (days) for spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of ascorbic acid in Oregon test diet and Abernathy diet and challenged with Aeromonas salmonicida. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Total mortality <sup>1</sup>		Mean time <sup>1</sup> to death (Days)	
		Mean	(SEM)	Mean	(SEM)
Oregon test	112	19.1	(0.6) <sup>a, b</sup>	17.6	(1.1) <sup>c</sup>
	16	10.0	(1.9) <sup>a</sup>	15.4	(0.5) <sup>c</sup>
	30	11.8	(3.2) <sup>a</sup>	16.1	(0.7) <sup>c</sup>
	139	43.6	(3.8) <sup>a, b</sup>	12.8	(0.4) <sup>c</sup>
	661	10.9	(1.3) <sup>a</sup>	13.8	(1.0) <sup>c</sup>
	2885	3.6	(0.0) <sup>a</sup>	14.7	(2.3) <sup>c</sup>
Abernathy	<b>23<sup>3</sup></b>	25.4	(5.1) <sup>a, b</sup>	14.8	(1.1) <sup>c</sup>
	10	32.7	(10.3) <sup>a, b</sup>	15.5	(0.7) <sup>c</sup>
	32	25.4	(7.7) <sup>a, b</sup>	17.3	(0.5) <sup>c</sup>
	272	52.7	(3.6) <sup>a, b</sup>	15.3	(0.9) <sup>c</sup>
	<b>1406<sup>4</sup></b>	60.0	<sup>a, b</sup>	12.1	<sup>c</sup>
	2864	62.7	(14.8) <sup>b</sup>	13.2	(2.0) <sup>c</sup>

<sup>1</sup>Values are means of duplicate lots of 55 fish, post-challenge rearing 23 days. Values with different superscripts are significantly different, **Student-Newman-Keuls** test ( $p < 0.05$ ).

<sup>2</sup>Represents diet not supplemented with either ascorbic acid and a-tocopherol (17.4 mg/Kg dry diet).

<sup>3</sup>Represents diet not supplemented with either ascorbic acid or a-tocopherol (13.9 mg/Kg dry diet).

<sup>4</sup> Replicate lot lost to water flow failure.

Table 47. Total mortality (%) and mean time to death (days) for spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of ascorbic acid in Oregon test diet and Abernathy diet and challenged with Renibacterium salmoninarum. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Total mortality <sup>1</sup> (%)		Mean time <sup>1</sup> to death (Days)	
		Mean	(SEM)	Mean	(SEM)
Oregon Test	16	98.2	(1.3) <sup>a</sup>	23.9	(1.2) <sup>bc</sup>
	30	98.2	(0.0) <sup>a</sup>	25.0	(0.6) <sup>bc</sup>
	139	97.2	(1.9) <sup>a</sup>	25.7	(0.4) <sup>bc</sup>
	661	65.4	(14.1) <sup>a</sup>	21.6	(2.0) <sup>b</sup>
	2885	86.3	(3.2) <sup>a</sup>	21.3	(3.9) <sup>b</sup>
Abernathy	10	88.1	(4.5) <sup>a</sup>	30.7	(1.1) <sup>c</sup>
	32	94.5	(3.8) <sup>a</sup>	27.3	(0.3) <sup>bc</sup>
	272	90.0	(0.6) <sup>a</sup>	24.8	(0.1) <sup>bc</sup>
	1406	94.5	(1.3) <sup>a</sup>	25.9	(1.6) <sup>bc</sup>
	2865 <sup>2</sup>	94.5	<sup>a</sup>	31.5	<sup>c</sup>

<sup>1</sup>Values are means of duplicate lots of 55 fish, post challenge rearing 43 days. Values with different superscripts are significantly different, **Student-Newman-Keuls** test ( $p \leq 0.05$ ).

<sup>2</sup>Replicate lot lost to water failure.

Table 48. Vitamin **analysis**<sup>1</sup> of a-tocopherol diets. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Nominal vitamin level <sup>2</sup>	Actual vitamin level <sup>3</sup>		
		Mean	(SEM)	n
Oregon Test	30	18.5	(1.2)	12
	60	45.5	(1.1)	12
	120	102.2	(1.5)	12
	240	213.2	(6.3)	12
	480	443.9	(8.3)	12
	<b>0</b> <sup>3</sup>	17.4	(1.1)	14
Abernathy	60	47.2	(3.4)	6
	120	97.1	(5.0)	6
	240	208.4	<b>(6.2)</b>	6
	480	429.4	(10.7)	6
	960	964.7	(29.2)	6
	<b>0</b> <sup>3</sup>	13.9	<b>(1.2)</b>	4

<sup>1</sup>All analysis were performed by **Hazelton** Laboratories America, Inc., Madison, Wisconsin.

<sup>2</sup>Values are mg vitamin/Kg dry diet.

<sup>3</sup>Represents diets not supplemented with either ascorbic acid or a-tocopherol.

Table 49. Liver total a-tocopherol analysis<sup>1</sup> of spring Chinook (**Oncorhynchus tshawytscha**) fed graded dietary concentrations of a-tocopherol in Oregon test diet and Abernathy diet for 238 days. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean dietary concentration (mg/Kg dry diet)	Liver <b>alpha-tocopherol</b> <sup>2</sup> ( $\mu$ g/g dry liver)	
		Mean	(SEM)
Oregon Test	18	152	(17) <sup>a</sup>
	45	427	(17) <sup>a</sup>
	102	1350	(86) <sup>a</sup>
	213	4767	(86) <sup>ab</sup>
	444	13551	(257) <sup>c</sup>
Abernathy	47	1778	(0) <sup>a</sup>
	91	3400	(51) <sup>ab</sup>
	208	6374	(1453) <sup>b</sup>
	429	21428	(1436) <sup>d</sup>
	865	46992	(2564) <sup>e</sup>

<sup>1</sup>**Analysis** performed by **Hazelton** Laboratories America, Inc., Madison, Wisconsin.

<sup>2</sup>**Values** are means of duplicate pooled liver sample of 21-37 fish. Values with different superscripts are significantly different, Student-Newman-Keuls test (p 10.05).

Table 50. Specific growth rate and gross feed efficiency of spring chinook (*Oncorhynchus tshawytscha*) fed two diets containing graded dietary concentrations of a-tocopherol for 245 days. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Specific Growth <sup>1,3</sup>		Gross Feed <sup>2,3</sup>	
		Rate	(SEM)	Efficiency	(SEM)
Oregon Test	18	1.147	(0.027) <sup>a</sup>	80.37	(0.00) <sup>b</sup>
	45	1.188	(0.005) <sup>a</sup>	83.99	(0.64) <sup>b</sup>
	102	1.155	(0.015) <sup>a</sup>	82.41	(0.42) <sup>b</sup>
	213	1.167	(0.017) <sup>a</sup>	82.41	(0.92) <sup>b</sup>
	444	1.127	(0.003) <sup>a</sup>	81.88	(0.68) <sup>b</sup>
Abernathy	47	1.154	(0.004) <sup>a</sup>	79.52	(2.08) <sup>b</sup>
	97	1.190	(0.009) <sup>a</sup>	81.01	(0.27) <sup>b</sup>
	208	1.174	(0.006) <sup>a</sup>	79.53	(0.33) <sup>b</sup>
	429	1.172	(0.020) <sup>a</sup>	80.36	(0.71) <sup>b</sup>
	865	1.192	(0.002) <sup>a</sup>	884.24	(0.36) <sup>b</sup>

$$^1\text{Specific growth rate SGR} = \frac{\log, \text{ of weight at end} - \log, \text{ of weight at start}}{\text{days fed}} \times 100$$

$$^2\text{Gross feed efficiency GFE} = \frac{\text{total fish gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

<sup>3</sup>Values are means of duplicate lots of fish. Values with different superscripts are significantly different, Student-Newman-Keuls test (p 10.05).

Table 51. Hitogenic response (stimulation index) to Vibrio anguillarum extract by anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of alpha-tocopherol in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by Diet Mean (SEM)
		81 Mean (SEM)	123 Mean (SEM)	165 Mean (SEM)	
Oregon Test	18	3.6 (0.3)	1.1 (0.1)	10.6 (5.7)	5.1 (2.3)
	45	3.8 (0.0)	1.4 (0.1)	10.2 (5.5)	5.1 (2.1)
	102	4.1 (0.8)	2.1 (0.3)	11.1 (3.1)	5.8 (2.2)
	213	4.0 (1.1)	7.2 (4.6)	9.7 (0.5)	7.0 (0.9)
	444	4.0 (0.4)	1.4 (0.0)	13.0 (0.3)	6.1 (2.9)
<b>Time means</b>		3.9 (0.1)	2.6 (1.2)	10.9 (0.6)	
Abernathy	47	0.9 (0.0)	3.4 (1.5)	8.4 (4.8)	4.2 (4.8)
	91	1.5 (0.0)	1.3 <sup>1</sup>	2.3 (0.5)	1.7 (0.2)
	208	1.2 (0.2)	1.0 <sup>1</sup>	1.7 (0.1)	2.3 (0.2)
	429	2.5 (0.3)	5.4 (1.0)	8.9 (4.2)	5.6 (1.8)
	865	2.0 (0.6)	5.7 (2.6)	8.7 (4.9)	5.5 (1.6)
<b>T h e -</b>		1.6 (0.3)	3.4 (1.0)	6.0 (1.6)	

<sup>1</sup>Only one replicate.

Table 52. Analysis of variance. Hitogenic response (stimulation index) to Vibrio anguillarum extract by anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of a-tocopherol in Oregon test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion
			Calculated	Table'	
Groups	11	1.25	2.52	2.09	Sig.
Times	2	8.31	16.80	3.28	Sig.
Groups x time	22	0.44	0.89	1.87	N.S.
Diet	1	6.06	12.20	4.13	Sig.
Vitamin level <sup>3</sup>	3	0.65	1.31	2.89	Sig.
Diet x vitamin level <sup>3</sup>	3	1.48	2.99	2.89	Sig.
Diet x time <sup>3</sup>	2	1.16	2.34	3.28	N.S.
Vitamin level x time <sup>3</sup>	6	0.13	0.26	3.38	N.S.
diet x time x vitamin level <sup>3</sup>	6	0.46	0.93	3.38	N.S.
Error	34	0.49			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>**Significant** (Sig.) p 10.05. Not significant (N.S.) p >0.05.

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

Table 53. In vitro antibody response (plaque-forming cell) to **TNP-** lipopolysaccharide (0.5 &ml) of anterior kidney lymphocytes from spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentrations of a-tocopherol in Oregon test diet and Abernathy diet. Immunological Study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean Vitamin Concentration mg/Kg Dry Diet	Time on Diet (Days)			Mean by Diet Mean (SEM)
		81	123	165	
		Mean (SEM)	Mean (SEM)	Mean (SEM)	
Oregon Test	18	1 (1)	107 (30)	729 (11)	279 (185)
	45	11 (4)	199 (52)	761 (238)	324 (184)
	102	24 (12)	58 (39)	1458 (12)	513 (336)
	213	33 (24)	298 (15)	960 (79)	430 (225)
	444	8 (0)	128 (42)	1573 (365)	570 (411)
<b>Time means</b>		<b>1s (6)</b>	158 (42)	<b>1096 (177)</b>	-
Abernathy	47	0 (0)	300 (70)	1031 (641)	444 (250)
	91	4 (3)	199 <sup>1</sup>	183 (33)	129 (51)
	208	0 (0)	89 <sup>1</sup>	266 (110)	118 (64)
	429	2 (2)	445 (116)	651 (106)	366 (156)
	865	2 (0)	211 (34)	295 (103)	169 (71)
<b>Time Means</b>		<b>2 (1)</b>	249 (59)	<b>485 (158)</b>	

<sup>1</sup>Only one replicate.

Table 54. Analysis of variance. In vitro antibody response (plaque-forming cell) to TNP-lipopolysaccharide (0.5  $\mu\text{g/ml}$ ) of anterior kidney lymphocytes from spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of a-tocopherol in Oregon Test diet and Abernathy diet. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Source of Variation	Degrees of Freedom	Mean Square	F value		Conclusion <sup>2</sup>
			Calculated	Table <sup>1</sup>	
Groups	11	5.39	2.38	2.08	Sig.
Times	2	197.40	87.10	3.27	Sig.
Groups x time	22	9.18	4.05	1.86	Sig.
Diet	1	15.74	6.94	4.12	Sig.
Vitamin level <sup>3</sup>	3	2.13	0.94	2.87	N.S.
Diet x vitamin level <sup>3</sup>	3	3.35	1.48	2.87	N.S.
Diet x time <sup>3</sup>	2	17.92	7.91	3.27	Sig.
Vitamin level x time <sup>3</sup>	6	1.55	0.68	2.37	N.S.
Diet x time x vitamin level <sup>3</sup>	6	4.17	1.84	2.37	N.S.
Error	36	2.27			

<sup>1</sup>Level of significance 0.05.

<sup>2</sup>**Significant** (Sig.) p  $\leq$  0.05. Not significant (N.S.) p  $>$  0.05.

<sup>3</sup>Analysis performed only on vitamin levels approximately common to both diets.

more dramatic association with time in this function. No effect of vitamin concentration or diet formulation was observed with respect to total mortality or mean time to death for either challenges with A. salmonicida (Table 55) or R. salmoninarum (Table 56).

As in previous studies, a concentration below the NRC recommended level in the OTD resulted in no diminution of growth, or noticeable change in immune status as opposed to AD. It is also quite apparent that the most significant influence on the mitogenic response was that of time. Much the same sort of effect was observed in the PFC responses (Table 53 and 54).

Table 55. Total mortality (%) and mean time to death (days) for spring chinook (Oncorhynchus tshawytscha) fed graded dietary concentration of  $\alpha$ -tocopherol in Oregon test diet and Abernathy diet and challenged with Aeromonas salmonicida. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Total mortality'		Mean time <sup>1</sup> to death (Days)	
		Mean	(SEM)	Mean	(SEM)
Oregon Test	18	43.6	(2.6) <sup>a</sup>	9.6	(0.3) <sup>b</sup>
	45	47.2	(11.6) <sup>a</sup>	10.0	(1.1) <sup>b</sup>
	102	58.1	(5.1) <sup>a</sup>	8.8	(0.2) <sup>b</sup>
	213	41.8	(1.3) <sup>a</sup>	10.4	(0.9) <sup>b</sup>
	444	32.7	(7.7) <sup>a</sup>	11.8	(1.3) <sup>b</sup>
Abernathy	47	39.1	(1.9) <sup>a</sup>	11.0	(0.1) <sup>b</sup>
	91	56.3	(3.8) <sup>a</sup>	10.1	(0.0) <sup>b</sup>
	208	29.0	(3.8) <sup>a</sup>	10.1	(0.2) <sup>b</sup>
	429	62.7	(1.9) <sup>a</sup>	9.1	(0.1) <sup>b</sup>
	965	42.7	(1.9) <sup>a</sup>	12.4	(0.2) <sup>b</sup>

'Values are means of duplicate lots of 55 fish; post challenge rearing 23 days. Values with different superscripts are significantly different, Student-Newman-Keuls test ( $p \leq 0.05$ ).

Table 56. Total mortality (%) and mean time to death (days) for spring chinook (*Oncorhynchus tshawytscha*) fed graded dietary concentrations of  $\alpha$ -tocopherol in Oregon test diet and Abernathy diet and challenged with *Renibacterium salmoninarum*. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Diet	Mean vitamin concentration (mg/Kg dry diet)	Total mortality <sup>1</sup> (%)		Mean time <sup>1</sup> to death (Days)	
		Mean	(SEM)	Mean	(SEM)
Oregon Test	18	90.9	(1.3) <sup>a</sup>	42.3	(0.0) <sup>b</sup>
	45	85.4	(1.3) <sup>a</sup>	41.5	(0.4) <sup>b</sup>
	102	75.4	(0.7) <sup>a</sup>	40.6	(0.3) <sup>b</sup>
	213	78.2	(6.4) <sup>a</sup>	38.9	(0.7) <sup>b</sup>
	444	89.9	(3.2) <sup>a</sup>	39.4	(1.5) <sup>b</sup>
Abernathy	47	96.3	(2.6) <sup>a</sup>	36.7	(0.7) <sup>b</sup>
	97	82.7	(5.8) <sup>a</sup>	41.3	(1.8) <sup>b</sup>
	208	90.9	(2.5) <sup>a</sup>	41.3	(0.1) <sup>b</sup>
	429	92.7	(2.6) <sup>a</sup>	29.5	(2.2) <sup>b</sup>
	865	69.0	(4.2) <sup>a</sup>	40.3	(0.5) <sup>b</sup>

<sup>1</sup>Values are means of duplicate lots of 55 fish; post challenge rearing 56 days. Values with different superscripts are significantly different, Student-Newman-Keuls test ( $p \leq 0.05$ ).

## DISCUSSION

The objectives of this study were to determine the concentrations of **selected** vitamins required for optimal growth, immune function, and disease resistance of juvenile chinook salmon. We have found that vitamin fortification of the fish's diets did not enhance these functions nor did they have any positive or deleterious effects. It was also found that the NRC recommended concentrations were not only ample for optimal function, but it appears that the recommended levels may be in considerable excess of what is actually required. For example, when ascorbic acid and riboflavin were provided at a third of the NRC recommendation there were no decreases in any of these functional parameters. We feel these observations warrant further examination to clarify the absolute vitamin requirements for optimal growth and immune function. If the actual requirements for these essential functions are confirmed to be at these reduced, or even lower levels, a revision of the NRC recommendations may be in order.

We recognize that these studies were conducted under laboratory conditions, thus other important factors such as the stress of field conditions **may** differentially affect these results if conducted under practical rearing conditions. We, therefore, feel that future studies should also be conducted under field conditions.

From our study there is a strong indication that fish fed diet formulations that differ in major ways have differences in their immune responses and disease resistance. With all vitamins tested, except for riboflavin, differences in immunological responses were found between diet types. Disease challenges with A. salmonicida in experiments involving pyridoxine showed significantly **higher** total mortality in fish fed OTD versus those fed AD. Our study and others (Blazer and **Wolke, 1984**), indicate that immunological status is influenced by diet formulation. This points to the need to examine the performance of commercially manufactured feeds. Having a ranking of commercial diets based on the quality of resulting immunological health would enable decisions on feed choice to include information on immunological quality.

Our experiments with ascorbic acid and alpha-tocopherol produced some intriguing results which suggest important nutrient interactions. When fish were fed diets containing suboptimal amounts of ascorbic acid (10 **mg/Kg** diet for OTD or 16 **mg/Kg** diet for AD) growth was only moderately reduced and mortality was normal compared to fish fed ascorbic acid at or above the NRC recommendation (>100 **mg/Kg** of diet). Similarly, when an OTD which was deficient in alpha-tocopherol (18 **mg/Kg** of diet) was fed, growth and mortality were not significantly different from lots receiving feeds fortified with alpha-tocopherol (>30 **mg/Kg** diet). On the other hand, when both ascorbic acid and alpha-tocopherol were simultaneously reduced to suboptimal levels (OTD - 16 mg ascorbic acid and alpha-tocopherol = 17 mg of diet; or AD with 10 mg of ascorbic acid and 14 mg of alpha-tocopherol per Kg of diet) growth rates were reduced, death rates increased dramatically, and symptoms reported for ascorbic acid deficiency were noted (lordosis and scoliosis). These results suggest to us a synergistic effect of combining suboptimal levels of these two important biological antioxidants. This possibility needs to be further explored. Similar investigations should also be performed with other vitamins to determine the synergistic effects of multiple deficiencies.

Another important finding was that a majority (13 out of 19) of the experiments in which a highly significant time-dependent effect was observed, demonstrated increased immune function with age or size (Table 57). We feel that this trend is highly significant; however, we also strongly recommended that future studies be designed to directly address this question. Our studies only incorporated three distinct time points simply as a window for the possible observation of vitamin concentration-dependent differences in immune responsiveness, not time-dependent effects. Experiments designed to address possible time/age/weight effects should evaluate more time points than were covered in this study. Furthermore, it is possible that the tremendous variability in immune function (addressed below) may have clouded our ability to discern these effects in experiments where no statistically significant time-dependent differences were observed. Therefore, experimental designs for future experiments should be modified as suggested below.

Since the time-related increase in immune function was observed within a set number of lymphocytes per sample (i.e.  $10^6$ ), the changes over time were due to an increase in the **functionality** of the lymphocyte population as a whole. Such age-dependent increases in immune function are not unique and have been observed in other species (Klingman and Press, 1975; Teale, 1985). This incidental, yet striking, observation deserves additional study because of its important implications for fish culture and experimentation. For example, this time-dependent relationship suggests that it may be fruitless to attempt vaccination during early life stages. Also, it may be a prudent practice to keep young fish under more environmentally controlled conditions that would not be necessary for fish of a slightly greater age. Such timed protection would limit the exposure of young fish to pathogens that they would be better able to control later in life.

Another important finding was that the results of our immunological assays varied widely among fish within populations receiving a given diet. At any selected sampling date, a number of the fish were seemingly incapable of developing appreciable reactions to certain antigens. At the same time, other individuals in the population demonstrated marked responses to the same antigen. Several investigators (Hastings and Ellis, 1988; Cossarini-Dunier, Desvaux, and Dorson, 1986; Wetzel and Charlemange, 1985) have reported that other fish species do not have a large repertoire of antibodies to a range of antigens; thus, they may be deficient in their responses to a number of pathogens. If this is true of juvenile salmon populations in hatcheries under normal conditions, a sizable proportion may not be competent to mount defenses against certain diseases. It may be useful to screen fish stocks to determine the prevalence of individuals capable of responding to insults from important pathogens.

Our initial observations of the tremendous variability in the mitogenic and PFC assays among responsive fish within a single replicate initially caused us considerable concern. Although this variability is considerable (see above), statistical comparison of such biological data without transformation of the raw data confounds this analysis and thus is inappropriate. Therefore, we performed such transformations of our data.

Before applying any form of statistical analysis to immunological data it is incumbent upon the investigators to understand the biological nature of the phenomena that lead to the measured response. Specifically, in this study,

Table 57. Analysis of variance. Effect of time (age/size) on immunological assay responses. Immunological study. Abernathy Salmon Culture Technology Center, 1988.

Vitamin	Immunological Assay	Diet	F-value	Probability	Relation <sup>1</sup>
Alpha-tocopherol	PFC	OTD	59.0	<.00005	positive
"	"	AD	40.8	<.00005	positive
Ascorbic acid	"	OTD	4.48	.02	positive
Folic acid	"	OTD	50.3	<.00005	positive
"	"	AD	35.3	<.00005	positive
<b>Panthenic acid</b>	"	OTD	1.81	n.s.	-
"	"	AD	0.85	n.s.	-
Riboflavin	"	OTD	8.58	.01	negative
"	"	AD	89.1	<.00005	negative
pyridoxine	"	OTD	4.14	.03	
"	"	AD	25.4	<.00005	positive
Alpha-tocopherol	Mitogenic	OTD	8.96	.001	positive
"	"	AD	2.86	n.s.	-
Ascorbic acid	"	OTD	11.2	.0002	positive
"	"	AD	8.40	.001	positive
Folic acid	"	OTD	112.0	<.00005	positive
"	"	AD	279.9	<.00005	positive
Panthenic acid	"	OTD	5.18	.04	positive
"	"	AD	0.00	n.s.	-
Riboflavin	"	OTD	6.33	.006	negative
"	"	AD	62.0	<.00005	-
Pyridoxine	"	OTD	20.7	<.00005	negative
"	"	AD	10.5	.0005	positive

<sup>1</sup>Positive = a significant increase with time, negative = a significant decrease with time and - = no consistent trend  
<sup>2</sup>n.s. = not significant, p >0.05.

both mitogenesis and PFC responses are the end result of an exponential biological function (i.e. the replication of lymphocytes). Therefore an arithmetic increase or decrease of the number of precursor lymphocytes (those giving rise to the proliferating cells) would result in an exponential variation of the final response. Thus, in order to accurately assess such responses it is necessary to mathematically transform the raw data prior to an analysis. In this study it was found that the most appropriate transformation of the mitogenic data was through natural logarithms, while the PFC data required cubed root transformation. It is of interest that although both functions, on the surface, seem dependent on the same exponential function, different types of transformation were required. It is possible that this difference may be reflective of the fact that, although both functions are the result of the same general class of functions (exponential), the responses themselves rely on populations of cells at different phases of differentiation.

Many immunological studies that analyze similar sorts of functions and are reported in the literature do not make use of transformations, and thus may be failing to detect important differences that may exist between experimental groups. However, in the case of the use of isogenic animals (extremely common in immunological studies), the amount of individual variation may be so low as to obviate the use of logarithmic transformation. This would be less likely with **outbred** species such as man and salmon.

In the future design of similar studies, investigators should consider the wide variability that can be found among individual juvenile spring chinook within a single replicate. In studies involving different species or possibly even different stocks, variability among individual fish should be assessed. Given the economic restriction of sampling a set number of fish within an experiment, statistical strength can be maximized and economic cost might be lowered by increasing the number of replicates. The use of more replicates allows the investigator to lower the number of fish sampled per replicate without a loss of statistical sensitivity.

## SUMMARY AND CONCLUSIONS

The Abernathy Salmon Culture Technology Center and the Department of Microbiology at Oregon State University, with funding from the Bonneville Power Administration, conducted studies on the effects of vitamin nutrition on immunity, disease resistance, and growth of juvenile spring chinook salmon (Oncorhynchus tshawytscha). The goal was to define the dietary levels of **folic acid**, pyridoxine, riboflavin, pantothenic acid, ascorbic acid, and alpha-tocopherol required to assure maximum immune responsiveness and resistance to Aeromonas salmonicida and Renibacterium salmoninarum **infections**. Each vitamin was studied individually and incorporated at five graded levels into a semi-purified diet (Oregon Test Diet) and a practical feed (Abernathy Diet) with all other vitamins held at or above the National Research Council (NRC) recommended levels. The test feeds were fed at a uniform, dry weight of feed per wet weight of fish to duplicate lots of 300 fish each held in **12°C** well water for approximately 24 weeks. During the feeding phase, fish were randomly sampled periodically from each lot for immunological tests including (1) responses of lymphocytes to lipopolysaccharides from coli or Vibrio anguillarum, and (2) enumeration of plaque-forming cells. During each experiment, samples of fish from each lot were challenged with A. salmonicida and R. salmoninarum in a laboratory supplied with disease containment facilities at Oregon State University. Total mortality and mean times to death were used to **assess** impact of vitamin nutrition on disease resistance.

Results indicated that the dietary levels recommended for the test vitamins by the NRC were ample. In fact, evidence indicated that, in some cases, vitamin concentrations as low as one third of the NRC recommendations were adequate under our laboratory conditions. Results suggest that the NRC recommendations should be re-examined and refined.

In the majority of cases, immunological parameters significantly increased with fish age/size/or time. Further experiments will be needed to sort out the degree to which either age or size are important. The results suggest that attempts to immunize fish may be most fruitful if performed when the fish are older than six months.

There was considerable variation in immunological measurements among fish within replicates fed individual diets. This has implications for design of similar experiments in the future as well as the immunological competency of significant segments of fish populations in hatcheries.

Results indicated that studies on vitamin interactions could be fruitful endeavors, especially with suboptimal dietary levels of ascorbic acid and alpha-tocopherol.

There is some indication that feeds that differ in major aspects of their formulation may affect immunocompetency by mechanisms other than vitamin nutrition alone.

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**APPENDIX**

Vibrio anguillarum Antigen Stimulates  
**Mitogenesis** and Polyclonal Activation'  
of Salmonid Lymphocytes

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ABSTRACT. An antigen preparation of Vibrio anguillarum, a salmonid pathogen, acts as a potent **in vitro** mitogenic stimulator of splenic and pronephric (anterior kidney) lymphocytes from coho salmon (Oncorhynchus kisutch), chinook salmon (O. tshawytscha) and rainbow trout (Salmo gairdneri). This antigen (VA) is comparable in its mitogenic activity to **Concanavalin A** (Con A), Escherichia coli lipopolysaccharide (LPS), and **phytohemagglutinin** (PHA). VA gives peak mitogenic responses in coho fish days after initiation of cell culture. VA also appears to be a nonspecific **polyclonal** activator as determined by the generation of plaque forming cells to **trinitrophenyl** (TNP) and fluorescein (FI) haptenic determinants. Chemical characterization is limited, but it appears that Vibrio LPS could be responsible for these activities.

INTRODUCTION

**Mitogens**, substances which stimulate **in vitro** lymphocyte proliferation, have proved useful as probes of **immune responsiveness** (1,2). Mitogens have been found to induce differentiation of lymphocyte populations by the selective stimulation of B lymphocytes by lipopolysaccharide (3), purified protein derivative of tuberculin (4), and dextran sulfate (5), or of T lymphocytes by **Concanavalin A** (6), and **phytohemagglutinin** (6). or by stimulation of both B and T lymphocytes by **pokeweed mitogen** (7). Although initially used with mammalian lymphocytes, fish lymphocytes have also been found to respond to these **mitogens in vitro**. Mitogen responses have been studied in rainbow trout (8,9,10), carp (11), and channel catfish (12). Whether the same functional distinctions between B and T cells as is found with mammalian lymphocytes can be made with fish lymphocytes is not, as of yet, clear, although recent evidence suggests that this may be possible (12).

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Numerous substances **derived** from bacteria and **metazoans** have been found to be polyclonal activators of mammalian B cells in that they stimulate both host B cell proliferation and antibody secretion. Some best characterized B cell polyclonal **activators** include lipopolysaccharides from gram negative bacteria (3), the purified protein **derivative** of tuberculin (4), protein A from *Staphylococcus aureus* (13), and polymerized flagellin from *Nocardia brasiliensis* (14). **These substances** have **proven** to be useful **immunological** tools for delineating the ontogeny of B cell responsiveness and dissecting the mechanism of B cell **activation** (15).

Past work (16,17) has **shown** that **an** antigen. extract of *Vibrio anguillarum* (VA) stimulates a specific anti-*Vibrio anguillarum* antibody response **in vivo**. It is possible, however, that this response maybe due in part to non-specific mitogenic and/or polyclonal activating components, working **in concert** with specific *V. anguillarum* antigens. Thus, in order to determine the mitogenic and polyclonal activating properties of VA, we have utilized an **in vitro** culture system, developed for the mitogenic **stimulation** (18) and induction of antibody responses (19) of **salmonid** lymphocytes. Since *V. anguillarum* is a fish pathogen in a wide variety of marine fish including salmonids (20,21), the **in vitro** analysis of its effects on their lymphocytes may assist in **understanding mechanisms** of both the pathogenesis of this bacteria and the **immune** responsiveness of the host fish.

#### MATERIALS AND METHODS

##### Animals and Facilities

All fish **were** supplied by the Oregon Department of Fish and Wildlife and maintained in 460 liter circular fiberglass tanks supplied with 12°C well water at the Oregon State University Fish Disease Laboratory (OSUFDL), Corvallis, Oregon. The fish **were** fed Oregon Hoist Pellets daily. **Seventy** to one hundred gram fish **were** used for these studies.

##### Culture media

**Media** Components were purchased from Whittaker M.A. Bioproducts, Walkersville, MD, unless otherwise noted. Cell suspensions were made in holding medium which consisted of 100 ug/ml gentamicin and 10% fetal calf serum in RPMI 1640 with L-glutamine (Gibco, Grand Island, NY), supplemented with 0.2% (v/v) sodium bicarbonate. Mishell-Dutton modified tissue culture medium (TCM) was used for tissue culture and consisted of RPMI 1640 with L-glutamine and bicarbonate supplemented with: 10 mM L-glutamine, 10% fetal calf serum. (hybridoma screened), 50 ug/ml gentamicin, 50 uM 2-mercaptoethanol (MCB, Cincinnati, OH), and the nucleosides; adenosine, uracil, cytosine, and guanosine (10 ug/ml, Sigma, St. Louis, MO). A media supplement (nutritional cocktail) was also prepared as previously described (22) and fed on alternate days to the cultures as described below.

##### Mitogens

stock solutions of lipopolysaccharide B (30 mg/ml) from *E. coli* 055:B5 (Difco, Detroit, MI) were pasteurized for 30 minutes at 70°C in distilled water. Further dilutions for tissue culture were made in TCM. stock solutions of PEA (Mfco, Detroit, MI), and Con A (Sigma, St. Louis, MO) were made in TCM (1 mg/ml) and sterilized by filtration through a 0.45 um filter.

Vibrio anguillarum O-antigen extract and lipopolysaccharide

The Vibrio extract was prepared from V. anguillarum strain LS-174 as described in Sakai et al. (16) with minor modifications. Fifty ml of thawed packed cells which had been formalin killed and stored frozen were suspended in 10 volumes of 2% saline and placed in a boiling water bath for 2 hours. Cells were washed 3 times in 2% saline by centrifugation at 10,000 x g for 10 minutes at 4°C. The final pellet was resuspended in 95% ethanol, and incubated 48 hours at 37°C. The cells were then washed 2 times in acetone by centrifugation at 3,000 x g for 10 minutes and dried by an overnight incubation 37°C. The cells were then ground to a fine powder with mortar and pestle and stored at 4°C. The soluble Vibrio extract used for these studies was prepared by boiling the powder in 0.1 M phosphate-buffered saline, pH 7.2 (PBS) at 10 □ g/ml in a boiling water bath for one hour with frequent agitation. This suspension was then centrifuged at 1,000 x g to remove particulates and filter sterilized (0.45 μm). -- The protein concentrations of the filtered extract was determined by the method of Lowry et al. (24).

Vibrio anguillarum lipopolysaccharide was prepared as described by Westphal et al. (25) and resuspended to the appropriate dilution in TCM. Concentrations of V. anguillarum lipopolysaccharide were expressed as ug/ml carbohydrate as determined by the phenol/sulfuric acid method (26).

Lymphocyte preparation

Fish were euthanized by cerebral concussion. The pronephric and splenic tissues were then dissociated in tissue culture medium as described by Kaattari, et al. (19). Briefly, single cell suspensions of each organ were obtained by repeated aspiration and expulsion of the tissues through a 1 ml syringe. The resulting cell suspension was incubated on ice to allow organ fragments to settle. The supernatant, a single cell suspension, was then washed once in tissue culture medium (TCM) by centrifugation at 500 x g for 10 min at 4°C. The cell pellets were resuspended in TCM to a concentration of 2 x 10<sup>7</sup> viable lymphocytes per ml, as determined by trypan blue exclusion.

Mitogen assay

Fifty ul (10<sup>6</sup> cells) of the lymphocyte suspension were added to individual wells of a 96-well flat-bottom microculture plate (Corning Glass Works, Corning, NY). Fifty ul of the mitogen were then added to these suspensions. The cultures were incubated in an airtight gasbox, (Model 624, C.B.S. Scientific Co., Del Mar, CA) at 17°C, in a blood-gas environment containing 10% CO<sub>2</sub>. The cells were fed 10 ul of the media supplement every other day for the duration of the culture period. Twenty-four hours before harvest, cells were pulsed with 1.0 uCi of tritiated thymidine (NEN, Wilmington, DL) in 10 ul of TCM. Cells were harvested with distilled water onto glass fiber filters, with a Skatron cell harvester (Sterling, VA). The filters were then dried, placed in scintillation vials with cocktail (6s PPO, Sigma, 5 mg POPOP, Amersham, Arlington Heights, IL, in 1 L toluene. after Etlinger et al. (9)), and counted on a Beckman liquid scintillation counter, (Model EL 3800, Fullerton, CA).

### Plaque forming cell assay

Two hundred  $\mu\text{l}$  of the lymphocyte suspension were added to each well of a **24-well** flat-bottom microculture plates (Corning Glass Works, Corning, NY). To this suspension 200  $\mu\text{l}$  of the appropriate dilutions of antigen were added. The cultures were incubated in an airtight **gasbox** at 17 C, in a blood-gas environment containing **10% CO<sub>2</sub>**. The cells were fed 50  $\mu\text{l}$  of feeding cocktail (22) every other day **for** the duration of the culture period. After 9 d of incubation, cell cultures from individual wells were removed via gentle aspiration using a Pasteur pipette, and deposited into separate plastic radioimmunoassay tubes (VWR Scientific, Seattle, WA) and held on ice. The tubes were centrifuged at **500 x g** for 10 min at **4°C**. The supernatants were removed, and the cell pellets resuspended in RPMI 1640 to an appropriate lymphocyte concentration for plaque forming cell (PFC) enumeration. Cells secreting **anti-TNP** antibody were then detected by the Cunningham modification of the Jerne plaque assay (27). One hundred  $\mu\text{l}$  of the lymphocyte suspension, 25  $\mu\text{l}$  of TSRBC (28) or Fluoresceinated-SRBC (29), and 25  $\mu\text{l}$  of an appropriate dilution of steelhead serum in MBB, were mixed in individual wells of a **96-well** round-bottom microtiter plate (Falcon, Oxnard, CA). This mixture was deposited in a Cunningham slide chamber, sealed with paraffin and incubated for 2 h at **17°C**. Plaques were enumerated using a low power dissecting microscope. Lymphocyte numbers were determined for each lymphocyte suspension, using a Coulter Counter, (Model ZM, Coulter, Hialeah, FL) with 0.08 M PBS as a diluent. Plaque forming responses were expressed as **PFC/culture**.

## RESULTS

### Mitogenic studies

Coho salmon (*Oncorhynchus kisutch*) anterior kidney and splenic lymphocytes undergo **mitogenesis in** response to a range of VA concentrations (Fig. 1). The data are a summary of results from 7 to 15 individual fish, **there is considerable variability** in cpm between individuals, however, lymphocytes from all fish tested exhibited **mitogenesis to VA**. The optimal dose for **stimulation** of lymphocytes was variable, between species or individuals within a species, usually occurring between 5 and 500  $\mu\text{g/ml}$  for a particular **mitogen** preparation.

VA is also **mitogenic** for lymphocytes from the other two salmonid species tested, chinook (*O. tshawytscha*) and rainbow trout (*Salmo gairdneri*) (Fig. 2). The data presented are from **representative experiments** with cells from a pool of organs from 5 fish. As with coho, all the fish tested **responded mitogenically** to the extract.

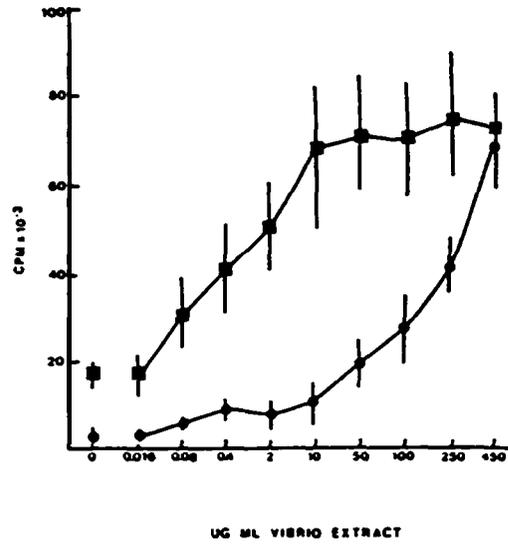
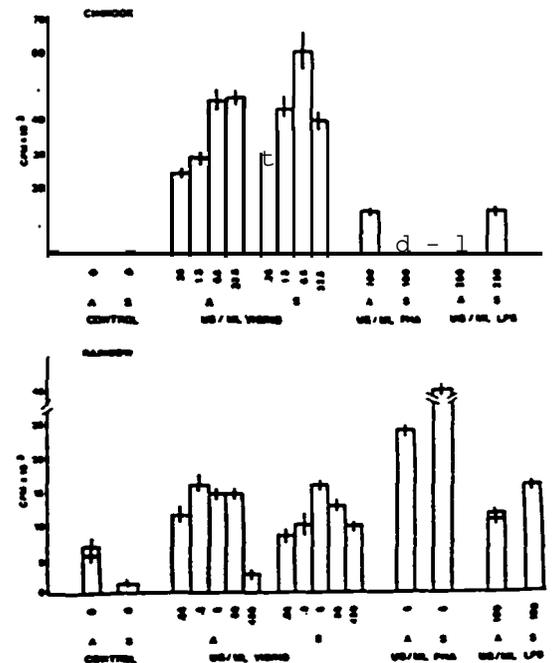


FIG. 1

Mitogenic responses of coho lymphocytes to Vibrio anguillarum extract. Splenic (●) and anterior kidney (■) lymphocytes were cultured with various doses of Vibrio extract and mitogenesis determined by the uptake of tritiated thymidine. Data given represent the mean CPM ± one standard error. Seven fish were used at each dose for the splenic responses, except for the 450 ug/ml dose which is represented by five replicates. Fifteen fish were used at each dose for the anterior kidney responses, except for the 100 ug/ml and 450 ug/ml doses which are represented by 14 and 10 replicates, respectively.

FIG. 2

Mitogenic responses of chinook salmon and rainbow trout lymphocytes to Vibrio anguillarum extract. Anterior kidney (A) and splenic (S) lymphocytes from chinook salmon and rainbow trout were cultured with various doses of Vibrio extract and optimal doses of PHA and LPS. Mitogenesis determined by the uptake of tritiated thymidine (<sup>3</sup>H). Data are presented as the mean CPM ± one standard error of triplicate cultures.



### Kinetics of VA mitogenesis with coho lymphocytes

VA demonstrates similar kinetics of tritiated **thymidine** uptake as is seen with other mitogens such as E. coli LPS and PEA (Fig. 3). **Maximal** proliferation appears to occur between day 4 and 5 for all **mitogens** in the anterior kidney, maximal proliferation for VA and PEA also appears to occur during **this time** period with splenic lymphocytes, whereas peak proliferation in response to **LPS** continues through day 6. There was no significant stimulation of **cpm** in the absence of antigen in the anterior kidney, however, a significant increase occurred between day 4 and 9 with the splenic lymphocytes.

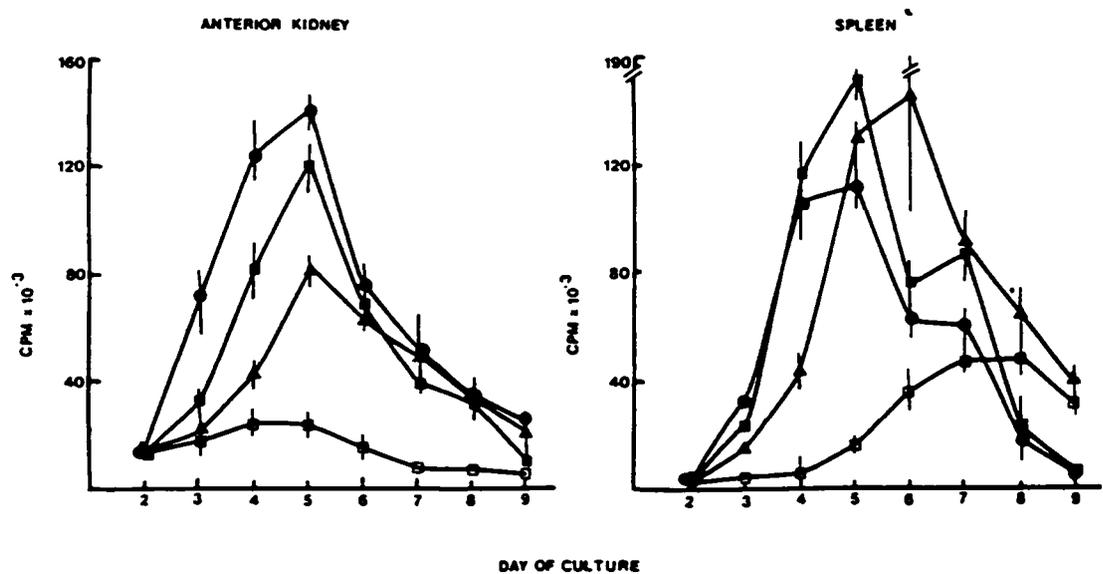


FIG. 3

**Kinetics** of mitogenesis. Anterior kidney and splenic **lymphocytes** were cultured with optimal concentrations of the Vibrio extract (■, 250 ug/ml), **LPS** (▲, 100 ug/ml), **PHA** (●, 5ug/ml), or without mitogen (□). Each day represents the **CPM** of cultures harvested after a 24 hour pulse of tritiated **thymidine**. Data are presented as the mean CPM  $\pm$  one standard error of triplicate cultures.

### Polyclonal activation of coho B cells by Vibrio extract as assessed by PFC production

Vibrio extract was able to stimulate PFC responses in anterior kidney and splenic lymphocytes to TNP and fluorescein haptenic determinants (Fig. 4). These responses were comparable to those elicited by **PHA** or **LPS**.

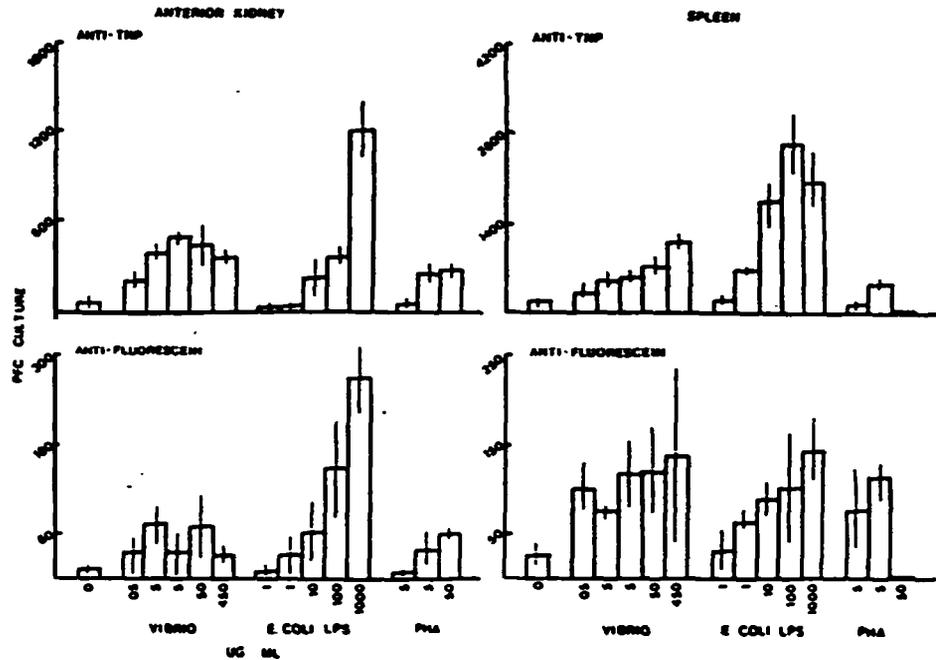


Table I. **Mitogenesis and Polyclonal Activation by V. anguillarum Lipopolysaccharide (Westphal)**

<u>V. anguillarum</u> <u>LPS (ug/ml)</u>	<u>PFC/culture</u> <sup>1</sup>	<u>CPM</u> <sup>2</sup>
40	247 <u>±</u> 17	26443 <u>±</u> 1822
4	209 <u>±</u> 28	16091 <u>±</u> 1791
.4	209 <u>±</u> 40	11502 <u>±</u> 779
0	90 <u>±</u> 10	8845 <u>±</u> 403

1. Data on day nine is presented as the mean number of **TNP plaque-forming** cells/culture ± one standard error of triplicate cultures. No **PFC** were detected against unhaptenated **SRBC**.
2. Data on day five is presented as mean **CPM** ± one standard error of triplicate cultures on day five.

The data demonstrate that V. anguillarum lipopolysaccharide is capable of inducing **TNP** plaque-forming cells as well as lymphocyte proliferation.

#### DISCUSSION

A commonly used somatic antigen extract of Vibrio anguillarum, a pathogen of marine fish including salmonids, induces a high level of specific immunity in vivo (16,17). A possible reason for the immunogenicity of this extract, as well as for killed cells (30-32) and other V. anguillarum extracts (33) may be the existence of substance(s) that are mitogenic or polyclonal activators for fish lymphocytes.

Utilizing a culture system for the expression of lymphocyte proliferation and antibody secretion (18,19) we demonstrate that Vibrio anguillarum somatic antigen extract (VA) contains a mitogenic substance(s) which stimulates anterior kidney and splenic lymphocytes (Fig. 1 and 2) of all three salmonid species tested (coho salmon, chinook salmon and rainbow trout). The mitogenic substance(s) appears to stimulate nonspecific lymphocyte proliferation and B cell differentiation in coho salmon lymphocytes, as determined by incorporation of tritiated thymidine and non-specific antibody production (Fig. 4). These responses suggest that the Vibrio extract acts as a polyclonal activator, in a similar manner to LPS and PHA (18).

The kinetics of mitogenic stimulation is also quite similar to other mitogens PEA and E. coli LPS tested suggesting a similar mode of stimulation. A likely candidate for such non-specific stimulation would be the lipopolysaccharide (LPS) of V. anguillarum. Previous work with mouse lymphocytes (34) has demonstrated the mitogenicity and adjuvant-like effects of V. anguillarum LPS. Using the same extraction procedures (25) we have isolated V. anguillarum lipopolysaccharide and cultured comparable concentrations (on the basis of carbohydrate content) to mitogenic concentrations of the somatic antigen. It can be seen from Table I that this LPS extract of V. anguillarum also possesses mitogenic and polyclonal activating properties for salmon lymphocytes.

The fact that Vibrio anguillarum is an important fish pathogen (20,21), and the ability of this extract to cause such potent in vitro immunological responses, suggests that this material may have some role in a immunity from this disease. Thus, it may have some practical application, in either crude or purified form, through conjugation to other non-immunogenic antigens to produce immunogenic vaccines.

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