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PATHOPHYSIOLOGY OF IHN VIRUS DISEASE IN RAINBOW TROUT.

University of Washington, Ph.D., 1973
Health Sciences, general

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PATHOPHYSIOLOGY OF IHN VIRUS

DISEASE IN RAINBOW TROUT

by

Donald F. Amend

A dissertation submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

UNIVERSITY OF WASHINGTON

1973

Approved by Lynwood S. Smith
(Chairman of Supervisory Committee)

Department College of Fisheries
(Departmental Faculty sponsoring candidate)

Date 4 December 1973

UNIVERSITY OF WASHINGTON

Date: November 14, 1973

We have carefully read the dissertation entitled Pathophysiology of IHN Virus Disease in Rainbow Trout submitted by Donald F. Amend in partial fulfillment of the requirements of the degree of Doctor of Philosophy and recommend its acceptance. In support of this recommendation we present the following joint statement of evaluation to be filed with the dissertation.

The thesis reading committee for Donald Amend has read and evaluated his thesis. We believe that it is noteworthy in several respects.

First, the thesis is directed to a problem of great concern to trout growers -- a virus disease of trout for which the only cure at present is to destroy the infected fish and sterilize the water system in which they were grown. The objectives were to understand the course of the disease, what organs and functions were most infected, and to look for clinical methods which could be of diagnostic value early in the course of the disease. These objectives were realized. A cure for the disease was not expected as a result of these studies and none was found.

The methods used in the research were comparable in most respects to those used in biomedical research, but were adapted as necessary for trout. This had the double advantage of making the data from the fish reasonably comparable to that for people and mammals, plus it saved much of the time that would have been needed to develop new methods uniquely for fish.

In moribund fish infected with infectious hematopoietic necrosis virus (IHN) there were many changes. It was concluded that the fish died from severe electrolyte and fluid imbalance caused by renal failure. Before external symptoms were apparent it was possible to detect changes in the plasma lactic dehydrogenase and in acid-base imbalance. These two changes in combination were unique to IHN virus in comparison to several other viral and bacterial diseases of trout.

Preliminary experiments were carried out which show that trout can develop immunity against IHN virus and that vaccination of trout is a possible preventative for the disease.

These data contribute significantly to the understanding of diseases of cultured trout and we, therefore, recommend the acceptance of this thesis in partial fulfillment of his Ph.D. Degree.

DISSERTATION READING COMMITTEE: Lynwood S. Smith

C. A. Evans

Harold O. Hodgins

Doctoral Dissertation

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I especially thank my major professor, Dr. Lynwood Smith, for his assistance and continued support throughout this study and for his many helpful suggestions in preparing this dissertation. I also sincerely appreciate the guidance and support of my graduate committee, Drs. Charles Evans, Harold Hodgins, Kenneth Chew, and Jack Matches.

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The technical assistance and help in securing samples by the following individuals also deserve a special acknowledgement: Mr. John Pietsch, Mr. Norman Buroker, Mr. Dennis Crouch, Ms. Kathy Smith, and Ms. Nancy Nelson.

Above all I thank my wife Jan, for her willing assistance in typing this dissertation and her loving encouragement. Also the understanding and patience of my sons Dale, Brian, and Steven is appreciated.

PREFACE

The information obtained from this study might be of interest to both biomedical and fishery scientists; therefore, this dissertation was prepared in three sections. Section I and II were prepared as manuscripts to be submitted for publication. Section I was prepared for a biomedical journal describing the physiological status in clinically ill fish. Section II was prepared for a fisheries journal and describes the changes found during the course of disease. Section III contains appendices which review the relevant literature and contains statistical analyses of the collected data. The reader is referred to the appendices for a description of the virus and a current synopsis of the epizootiology of the disease.

General Background

Infectious Hematopoietic Necrosis (IHN) virus can cause serious mortality in juvenile sockeye salmon (*Oncorhynchus nerka*), chinook salmon (*O. tshawytscha*), and rainbow trout (*Salmo gairdnerii*) and is prevelent in these species along the Pacific Coast. Although a disease of sockeye salmon believed to be of viral origin was first recognized in 1951, the etiological agent was not isolated until the mid-1960's when cell culture systems became available. Now much national and international concern has arisen because of recent IHN epizootics across North America from the transportation of infected fish. Consequently, there is a great need for information concerning identification of carrier fish and control of the disease. To obtain this information necessitates an understanding of the virus and epizootiology of the disease.

There have been several studies where the IHN virus was studied and characterized (see appendix A). Gross clinical signs of the disease and the histological pathogenesis have been described. Some information is available on the epizootiology of the virus, but the data are not complete. There have been no reports to my knowledge of the pathophysiology in fish due to IHN disease. There have been several reports which associate a low water temperature with a high disease potential. Because fish are poikilothermic animals, various physiological functions which are temperature dependent must operate to combat disease and influence viral persistance.

Experimental Objectives

In modern clinical laboratories, various tests of physiological functions are followed to determine the disease status of individuals, and some of these tests are of diagnostic value. Because similar tests could be of value in fisheries medicine, the objective of the experiments was to determine which physiological tests could be used to give a better understanding of the disease process due to IHN virus, and to determine if certain physiological changes could be diagnostically useful.

The general approach was to select various physiological and biochemical tests for which microprocedures were available and these tests should give information regarding functional changes in blood, kidney, liver, pancrease, gill, and other vital functions of rainbow trout infected with IHN virus. Comparisons were made at water temperatures which are conducive to disease and at temperatures where disease does not occur.

Comparisons were also made with some other fish diseases. Lastly, the immunological response of rainbow trout following infection with IHN virus was determined.

SECTION I

PATHOPHYSIOLOGY OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS DISEASE IN RAINBOW TROUT: HEMATOLOGICAL AND BLOOD CHEMICAL CHANGES IN MORIBUND FISH[1]

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(This manuscript is to be submitted to Proceeding of Experimental Biology and Medicine.)

INTRODUCTION

Infectious Hematopoietic Necrosis (IHN) virus infects sockeye salmon (*Oncorhynchus nerka*), chinook salmon (*O. tshawytscha*), and rainbow trout (*Salmo gairdneri*) and is prevalent along the Pacific Coast. The virus has the properties of Rhabdoviruses (1, 2). The primary pathological lesion in diseased fish is extensive necrosis of the hematopoietic tissues, but in terminal cases necrosis of the liver, pancreas, and granular cells of the lamnia propria of the gut is also found (3,4). The epizootic potential is highest at 10° C and disease does not occur naturally above 15° C (5, 6). However, in cell culture the virus replicates and causes cytopathic effects at temperatures up to 18° C (3, 7).

Although some information is available regarding the properties of the virus and epizootiology of the disease (8), only limited data exists regarding the physiological pathogenesis of the disease and the mechanisms by which fish resist

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1 This paper reports a portion of the work encompassed by a thesis submitted to the University of Washington School of Fisheries in partial fulfillment of the requirements for a Phd degree.

infection. Various differential hematological, physio-chemical, enzyme, and organ function tests are used in mamalian medicine to aid in disease diagnosis and to follow the pathogenesis and recovery from disease. In this study various hematological and blood chemical tests were performed on clinically ill fish to determine if some tests may be of diagnostic value, and to gain a better understanding of the response of fish to this viral infection.

MATERIALS AND METHODS

Rainbow trout (Kamloop variety) averaging about 25 g each were placed in 700 L aquaria supplied with flowing 9° C dechlorinated municipal water. There were 200 experimental and 200 control fish in separate aquaria. All fish were acclimated to the water for 2 weeks prior to the experiment and were maintained on a commercial dry pellet diet. The water had a total hardness of about 20 ppm (as $CaCO_3$) and a pH of about 6.6. Further characteristics of the water supply were described by Amend (9).

The fish were fasted for 24 hrs. prior to injection. A 50 ppm Benzocaine solution was used to anesthetize the fish, then 0.1 ml of IHN virus ($10^{7.5}TCID_{50}$/ml) was injected ip into each fish. The controls were handled similarily except that just cell culture media was injected. The virus was originally isolated from rainbow trout and was neutralized by IHN specific antisera.

As the fish became lethargic and showed signs of the disease (3), the fish were again anesthetized. After excess water was removed from the surface of the fish, the caudle peduncle was severed and blood collected into heparinized capillary tubes from the caudal artery. Also, hemoglobin and red blood cell counts were determined and blood smears and anterior kidney imprints prepared (10). The capillary tubes were sealed and centrifuged at 1000 xg for 10 min. The packed cell volume was calculated, then the plasma pH, bicarbonate, and chloride was frozen (-10° C) and the other biochemical tests were performed within 1 month. Control fish were randomly selected and handled

similarily except all fish were processed on one day about the time peak mortality was occurring in the infected group (7 day post injection). All analyses were performed on individual fish.

Blood smears and tissue imprints were air dried, fixed in methanol, then stained with Lieshman-Giemsa. The cells were classified according to Klontz (12). Ascorbate analyses were performed as described by Wedemeyer (13) using homogenized anterior kidney tissue, glucose was determined by method of Duboloski (14), and bilirubin by the modified Nosslin method (15). Bicarbonate and calcium were determined by titration and the plasma pH with a pH meter at 37° C, then calculated to 10° C. Osmolality was measured by the freezing point depression method and phosphorus by the Molybdate method. The pCO_2 was determined mathematically using the Henderson-Hasselbalch equation for blood carbonate.

Horizontal starch gel electrophoresis was used for detecting alterations in plasma lactic dehydrogenase (LDH), esterase, peptidase, and glutamic oxalacetic transaminase (GOT) isozymes concentrations (16). Using a photometric analyser, the optical density from polaroid negatives of the stained gels was used to semi-quantitate alterations in the LDH concentrations. A more quantitative measurement was attempted using a densitometer but the difference between peaks was too great to standardize on the machine. Perhaps standardized serum dilutions would resolve the problem. Disc acrylamide gel electrophoresis was used to separate plasma proteins and the concentration of each peak was quantitated by direct scanning of the gel with a densitometer equipped with an internal integrator.

Fluid balance was determined by weighing individual fish to the nearest 5 mg. In this test 15 rainbow trout averaging about one gram each were injected intraperitoneally (ip) with 0.01 ml IHN virus ($10^{6.0}TCID_{50}$/ml) and 15 fish with the same amount of cell culture media. Each fish was held in a separate container and weighed daily for 19 days. The fish were anesthetized and excess water removed by blotting on absorbant paper before each weighing. The fish were not fed during the test and a virological examination was performed on all fish that died.

During these tests IHN virus was grown on established epitheliod embryonic rainbow trout cells. The cells were grown in Eagle's minimal essential media (Earl's Salts) containing 10 percent agamma fetal calf serum, 100 iu penicillin G, and 100 mg streptomycin per ml. Virus infected cells were grown at 15° C and virus was harvested in about 6 days when all cells showed cytopathic effects. The virus preparation was centrifuged at 1000 xg for 10 min. to sediment cellular debris. Viral titrations were quantitated by the Reed-Muench method.

RESULTS

Hematology

The hemoglobin, hematocrit, and red blood cell (RBC) levels were significantly depressed in all moribund fish (Table 1). However, the mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were normal. This suggests that there was a normal reduction in the cellular concentration. The % immature RBC's was significantly increased ($P<.05$) in the infected fish but the difference of means between infected and control fish was only 2.2%. The % leukocytes in the total blood smear remained unchanged, but there were some significant changes in the differential abundance of leukocytes (Table 1). There was no difference in the % monocytes but the % lymphocytes increased and the % neutrophils greatly diminished. The relative % increase in lymphocytes was probably due to the absolute reduction in neutrophils. It appears that the neutrophils are the predominant type of circulatory cell being affected by this disease. This is perhaps most apparent by comparing the control:moribund ratio in Table 1.

The data above indicates a normocytic anemia with a selective reduction in the abundance of neutrophils. There were no abnormal lymphocytes observed in the circulatory smears, but there appeared to be excess debris in the smears from infected fish. There were areas where basophilic granules were common and occasionally unidentifiable necrobiotic disintegrating particles were seen (Figure 1). These necrobiotic particles were found only on slides from infected fish.

The anterior kidney is the major site of hemopoiesis in

Table 1: Hematological comparison of uninfected controls with moribund rainbow trout infected with IHN virus. (mean values with standard error and sample size in parenthesis)

Hematological Test	Units	Controls	Moribund	Statistic[1]	C/M[2]
Whole Blood					
Hemoglobin	g/100 ml	9.4 ± 0.2(20)	7.1 ± 0.4(20)	**	1.32
Red Blood Cells (RBC)	X10^6/ml	1.33 ± 0.03(20)	0.97 ± 0.07(20)	**	1.37
Packed Cell Volume	%	47 ± 1(19)	35 ± 3(20)	**	1.34
Mean Corpuscular Volume	cμ	359 ± 11(20)	347 ± 19(20)	NS	1.04
Mean Corpuscular Hemoglobin	μμg	72 ± 2(20)	74 ± 2(16)	NS	0.97
Mean Corpuscular Hemoglobin Concentration	%	20.1 ± 0.4(19)	19.6 ± 1.7(16)	NS	1.03
Blood Smears[3]					
Mature RBC	%	91.1 ± 0.7(20)	87.8 ± 2.4(18)	NS	1.04
Immature RBC	%	7.0 ± 0.6(20)	9.2 ± 1.1(18)	*	0.76
Total Leukocytes	%	2.0 ± 0.2(20)	2.5 ± 1.3(18)	NS	0.80
Differential Leukocyte Count[4]					
Monocytes	%	5.3 ± 1.0(20)	3.0 ± 1.0(18)	NS	1.7
Lymphocytes	%	90.7 ± 1.7(20)	96.0 ± 1.0(18)	**	0.94
Neutrophils	%	4.0 ± 1.0(20)	0.7 ± 0.3(18)	**	5.7

1. Statistical Significance*P=<.05 **P=<.01, NS=not significant

2. C/M = control:moribund ratio

3. Immature RBC and Leukocytes are expressed as % of a total of about 500 cells counted, including mature RBC.

4. Monocytes, Lymphocytes, and Neutrophils are differential percentages of about 100 Leukocytes counted.

SECTION I

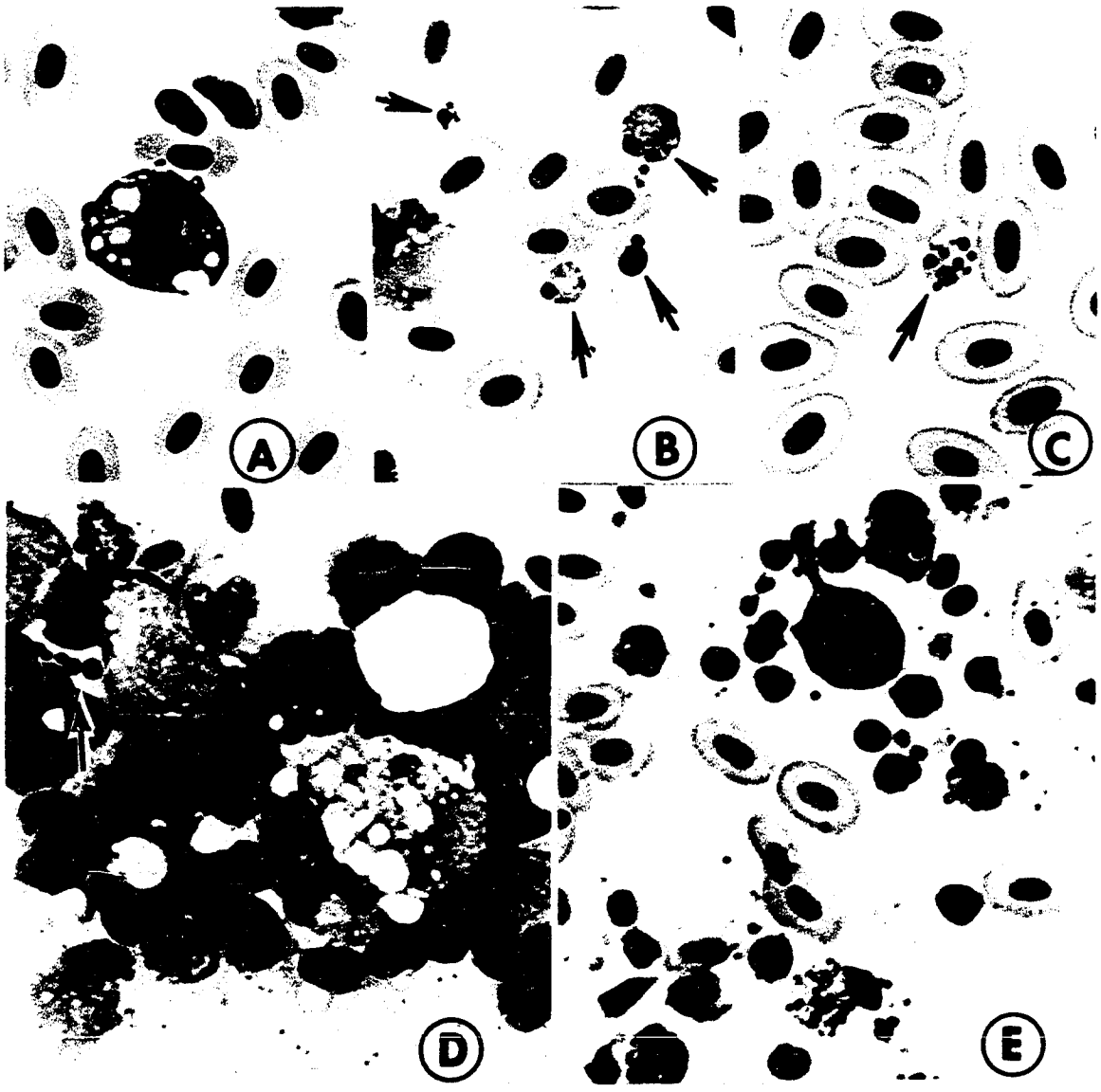

Figure 1: Cellular changes in blood of rainbow trout infected with IHN virus: (A) monocyte in blood smear showing vacuolated cytoplasms; (B) and (C) arrows indicate necrobiotic disintegrating particles in blood smear; (D) macrophage with vacuolated cytoplasm and early necrotic debris in anterior kidney imprint; and (E) extensive necrotic debris in anterior kidney imprint.

fish and the imprints prepared from this area indicated areas of severe necrosis (Figure 1). Although no attempt was made to quantitate the number of necrotic particles, there appeared to be considerably more debris and necrotic particles in the kidney imprint than in the circulatory smears. Also, many of the early blast cells were affected. A "vacuolated" cytoplasm condition was seen in some macrophages and this appeared to be more prominent in fish where necrosis was less evident. Again there appeared to be a lack of neutrophils. There was no evidence of an inflamatory response.

Biochemical Changes

The most severe changes resulted in a reduction of the plasma electrolytes (Table 2). Calcium, phosphorus, osmolality, and bicarbonate levels were greatly reduced and there was a significant decrease in the plasma chlorides. The bilirubin level was also reduced, but there was no significant change in plasma glucose or in anterior kidney ascorbate levels. There is considerable difference in the mean glucose values between Test 1 and the replicate Test 2. The values in Test 1 are normal values. The values in Test 2 are high and may be due to excessive handling stress (13), but the trend of both tests is the same.

Plasma protein and enzyme analyses were determined on the same fish. Tests for total protein indicated no significant difference between moribund and control fish. Plasma was then subjected to disc electrophoresis to determine if there were alterations in any of the various proteins fractions. Table 3 shows that there was a highly significant increase in the

Table 2: Biochemical comparison of plasma from uninfected controls with plasma from moribund rainbow trout infected with IHN virus. (mean values with standard error and sample size in parenthesis)

Clinical Test	Control	Moribund	Statistic[1]
Bicarbonate (mEq/L)			
Test 1	9.8 ± 0.5 (20)	5.8 ± 0.8 (6)	**
Test 2	10.5 ± 0.5 (20)	6.1 ± 0.4 (15)	**
Chloride (mEq/L)			
Test 1	115 ± 1 (10)	107 ± 4 (12)	*
Test 2	119 ± 2 (20)	113 ± 2 (16)	*
Glucose (mg/100ml)			
Test 1	81.1 ± 6.3 (15)	136.0 ± 43.4 (11)	NS
Test 2	429 ± 85 (10)	381 ± 81 (10)	NS
Osmolality (mOsm)	315 ± 2 (7)	277 ± 6 (9)	**
Phosphorus (mg/100ml)	15.38 ± 0.57 (10)	12.11 ± 0.49 (10)	**
Calcium (mg/100ml)	10.8 ± 0.39 (10)	8.07 ± 0.58 (10)	**
Bilirubin (mg/100ml)	2.1 ± 0.2 (16)	1.2 ± 0.4 (4)	*
Interrenal Ascorbate (µg/g)	51.5 ± 3.4 (4)	45.8 ± 2.1 (10)	NS

1. See Table 1 for explanation

Table 3: Comparison of plasma protein fractions following disc electrophoresis from 6 uninfected controls and 6 moribund rainbow trout infected with IHN virus. (mean values with standard error)

Test	Fraction (% of total)					
	Albumin	α 1	α2	α 3	β 1	β 2
Control	29.4 ± 1.3	3.1 ± 0.3	11.7 ± 1.0	27.8 ± 1.7	11.6 ± 1.1	16.5 ± 1.1
Moribund	32.0 ± 1.7	3.9 ± 0.6	18.9 ± 2.3	17.6 ± 2.1	13.0 ± 0.5	14.7 ± 1.9
Statistical Significance[1]	NS	NS	**	**	NS	NS

1. see Table 1

alpha-2 fraction and a decrease in the alpha-3 fraction of moribund fish. Figure 2 shows a typical densitometric scan of plasma from a moribund and a control fish. In the same serum samples, no difference could be detected in the plasma concentration of esterase, GOT, or peptidase isozymes, but there was a significant increase in the $B^{2'}_{4}$ LDH isozyme (Table 4). Two major regions of LDH activity were evident in both control and moribund fish (Figure 3), but the change was limited to the $B^{2'}_{4}$ isozyme. Utter and Hodgins (16) showed that there was genetic variation with LDH isozymes, but the $B^{2'}_{4}$ gene frequency is most prevalent in serum. LDH isozyme patterns were also compared in muscle, anterior, kidney, posterior kidney, liver, spleen, and gill, but no differences were detected.

Acid-Base and Fluid Balance

The reduced bicarbonate levels shown in Table 2 indicated that acid-base regulations were being affected, perhaps associated with alteration of the body water balance. As can be seen in Table 5, the acid-base balance was severly affected. The total buffering capacity was greatly reduced and the pH was significantly increased. This means that the pCO_2 (H_2CO_3) was reduced more than the HCO_3^- level thus increasing normal base acid ratio. The condition suggests a trend towards an uncompensated alkalosis (higher pH) which may be complicated by a metabolic acidosis (lower alkali reserve).

The body fluid balance was determined by daily weighing individual fish. The weight of each fish was combined into 3-day composites, and then the 3-day mean of all fish in each group was determined. The data were then subjected to linear

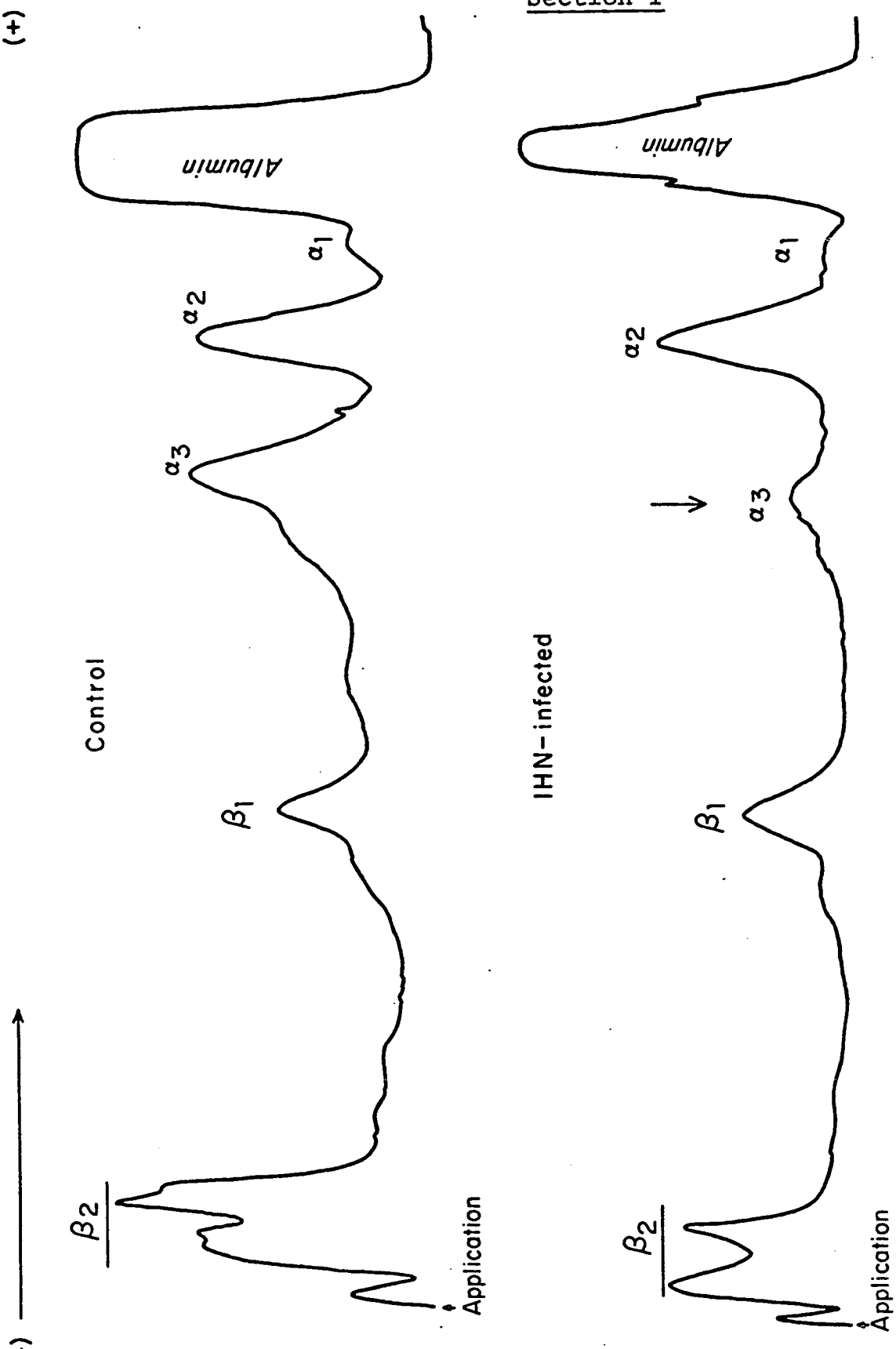

Figure 2: Scan of a disc electropherogram of plasma proteins from control and IHN infected rainbow trout. Arrow at α 3 indicates major reduction in α 3 fraction.

Table 4: Comparison of $B^{2'}_{4}$ LDH isozyme concentration in plasma from control and moribund rainbow trout infected with IHN virus. Values are photometric measurements from photograph negatives of stained starch-gel electrophoretic determination. (mean values with S.E.)

Test	Sample Size		Optical Density		Statistic[1]
	Control	Infected	Control	Moribund	
Test 1[2]	4	4	2.51 ± .07	1.94 ± .10	**
Test 2[3]	9	9	0.851 ± .016	0.677 ± .021	**

(1) see Table 1

(2) cumulative values of 3 readings taken at center and ± 1 mm of center of enzyme activity for each fish

(3) reading taken at center of enzyme activity

Figure 3: Lactic dehydrogenase isozymes from starch-gel electrophoresis. (C) control serum from 4 rainbow trout; (T) serum from 4 moribund rainbow trout infected with Infectious Hematopoietic Necrosis virus.

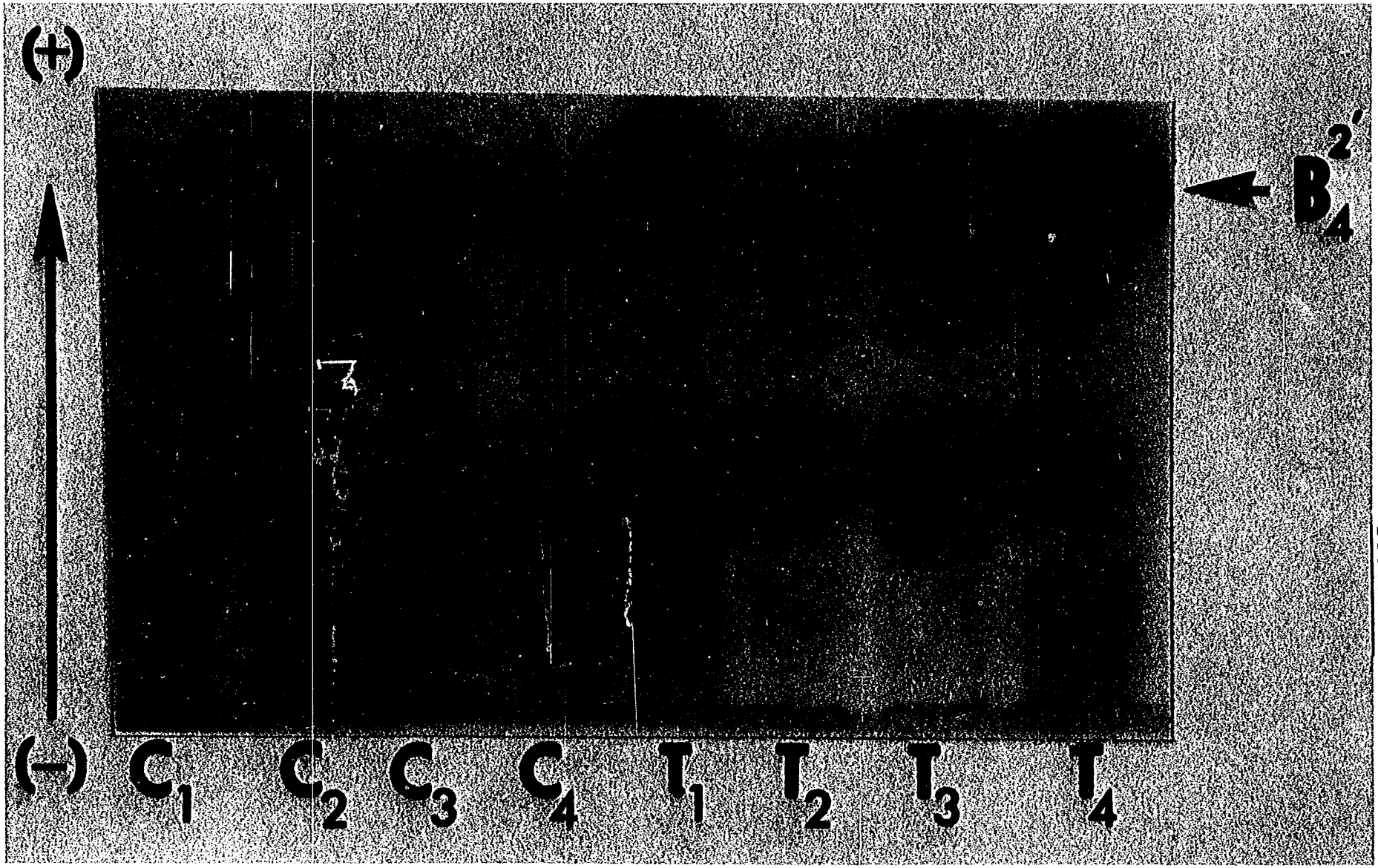

Table 5: Comparison of acid-base balance between 15 uninfected controls and 15 moribund rainbow trout infected with IHN virus. (mean values with standard error)

Clinical Test	Control	Moribund	Statistical Significance (1)
HCO_3^- (mEq/L)	10.54 $\pm$ 0.48	6.14 $\pm$ 9.41	**
pH (2)	7.454 $\pm$ 0.019	7.594 $\pm$ 0.022	**
pCO_2 (mmHg)	15.56 $\pm$ 0.68	6.45 $\pm$ 0.33	**
H_2CO_3 (mEq/L)	0.47 $\pm$ 0.02	0.20 $\pm$ 0.01	**
HCO_3^-/H_2CO_3	22.9 $\pm$ 1.0	31.5 $\pm$ 1.6	**
Total CO_2 (mEq/L)	11.01 $\pm$ 0.49	6.33 $\pm$ 0.42	**

(1) see Table 1

(2) mean hydrogen ion concentration (H^+) expressed as pH

regression analysis. Because all fish were fasted over the 19 day test, the control fish lost weight at a rate of about 5 mg per day or a total 6.5% weight loss (Figure 4). This weight loss for fasting fish is comparable to Brett's data (17) for starved sockeye salmon. In contrast, the infected fish continued to gain weight until they died. The average weight gain was about 15 mg per day or about a 20% total weight gain. This difference was highly significant and demonstrates that the infected fish had lost their ability to regulate their fluid balance.

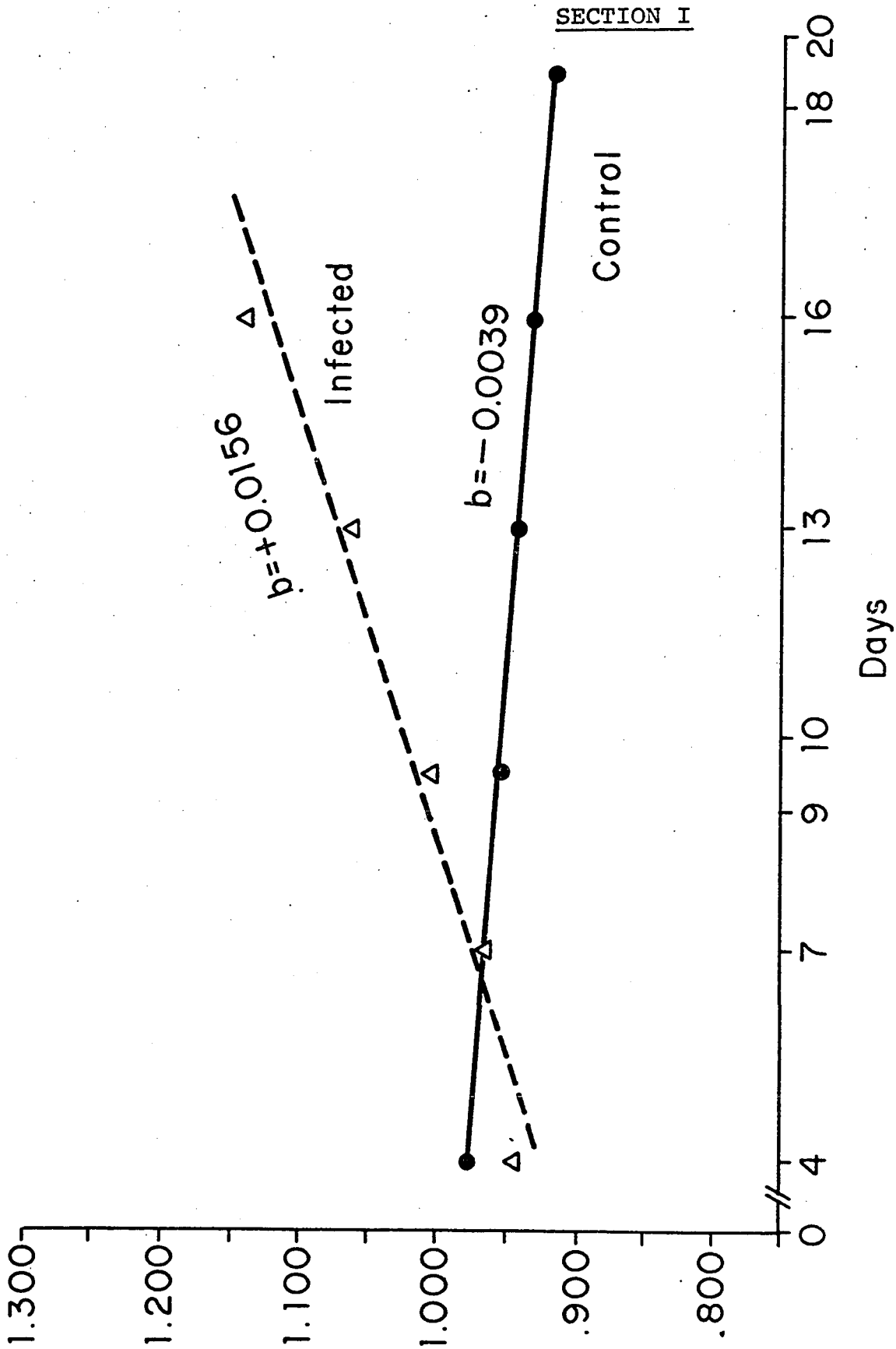

Figure 4: Comparison of mean weight of fasting rainbow trout uninfected and infected with IHN virus at 9° C.

DISCUSSION

The first pathological lesions in IHN disease occur in the hematopoietic tissues of the anterior kidney and in the spleen (4), and anemia is a typical sign of disease (3). Watson (18) described some of the cellular changes, but this report is the first to characterize and quantitate these changes.

It appeared that the fish underwent a normocytic aplastic anemia. Although hematopoietic tissue appeared to be responding by an increase in % of immature cells, the degree of response was less then expected. Perhaps there was some paralysis of the hematopoietic tissue as evidenced by the necrosis seen in the kidney imprints. This was apparent even in the most anemic fish. Several fish had hemoglobins as low as 4.0 g/100 ml and a hematocrit of 7% and still there was no evidence of increased hemopoiesis. However, the mean value for hemoglobin, hematocrit, and RBC, though significantly lower than control values, were still in the normal range (19, 20).

Watson (18) and Amend and Chambers (21) reported the appearance of necrotic particles in diseased sockeye salmon and suggested that the degenerate cells were probably lymphocytes. Parisot (22) reviewed Watson's work and surmised that these degenerate cells were immature erythrocytic nuclear fragments leaking from the kidney. Leukopenia was also said to be characteristic of the disease (3); however, no quantitative data was presented in the above papers. The data presented here does not resolve the identity of the necrotic particles, but does dispute the idea of a generalized leukopenia. On the contrary, there was no evidence of a generalized leukopenia,

but there was definitely a neutropenia associated with the disease. Neutrophils and macrophages are common targets of systemic viral infections and often serve as the source for spread of the agent throughout the host (23). Perhaps the necrobiotic particles, in part, are debris from disintegrating neutrophils. Fluorescent antibody studies should help resolve this question.

Although the hematopoietic tissues of the anterior kidney are the major site of necrosis, histological examination of the renal tubules and glomeruli show little change (3, 4). This, however, does not necessarily mean that there is no physiological alteration of the renal functions, and the data presented here suggests that kidney dysfunction may be the primary cause of death.

The teleost kidney functions primarily as a regulator of body fluid and secondarily as an excretory organ and electrolyte regulator (24). The freshwater fish is hypertonic to its environment and water continually diffuses across the gills into the blood. The kidney eliminates this excess water and re-absorbs most of the body electrolytes. The gill function not only as a respiratory organ, but also as an excretory and ion regulating organ (25).

These experiments indicated that the normal physiology of the kidney was altered. The weight increase most clearly demonstrated this, and much of the loss of electrolytes and perhaps some of the hematological changes can be explained by hemodilution. The condition was also probably heightened

by loss of other renal regulatory functions. The alteration in acid-base balance could have been due to physiological dysfunction of the renal elements of the kidney.

It should be noted that some of the values I reported for acid-base balance are derived values and do not necessarily represent normal values. Garey (26) pointed out that different methods for determining blood pH result in different values. Blood pH is considerably higher if measured by chronic catherization as compared to cardic puncture. Because catherization is impractical in a 25 g fish, the pH values I reported are more comparable to those of Garey's cardic puncture and Wedemeyer's (personal communication) caudal artery values. Furthermore, the values I reported were calculated by the standard formula used for humans:

$$pH = 6.1 + \text{Log} \frac{(HCO_3^-)}{.03pCO_3}$$

at 38° C. This, of course, is not temperature corrected for pK and solubility constant (27, 28). However, for just comparing moribund fish to control fish the predictions derived from the data are still valid because all fish and samples were handled the same.

As Wedemeyer and Chatterton noted (29), the terms alkalosis and acidosis are relative to temperature and because of this, it cannot be said that the IHN infected fish were suffering from alkalosis. In fact the pH was still within the normal range at 10° C, even though there was significant alteration of the acid-base ratio and severe depletion of the alkali reserve. Randall and Cameron (30) stated that changes in blood ph with

temperature were regulated by varying the (HCO_3^-) and the pCO_2 remained constant. However, at a constant temperature any raise in the blood pH when the (HCO_3^-) is below normal must be due to a greater reduction in the pCO_2 (H_2CO_3). The alkali deficit could be due to metabolic acidosis, but the increased pH indicates an over compensation, most likely by respiratory mechanisms. The anemic condition also must contribute because the PO_2 would be reduced, which in turn must affect the pCO_2 levels (28). Furthermore, the regulatory mechanisms of the kidney may be reduced which would contribute to uncompensated regulation of acid-base balance. The mechanisms of acid-base regulations by each organ is just beginning to be understood; therefore, a more realistic evaluation of the changes noted in this paper must await further information regarding the relative importance of gill and kidney in regulating acid-base balance.

Pathological changes are also observed in the liver and pancreas of fish terminally infected with IHN disease (3, 4). However, none of the physiological tests indicated a change in function of these organs. Glucose regulation was unchanged and bilirubin levels were lower in infected fish. Enzymes can sometimes indicate pathological changes due to viral infections (31), and Bell (32) showed that certain enzymes could be of diagnostic value with some fish diseases. However, no enzyme differences were noted here except for an increased plasma $B^{2'}_4$ LDH. Various pathological conditions can result in an increase in LDH (33). The LDH virus of mice inhibits the reticular endothelial system from clearing LDH, thus LDH accumulates in the serum (34). In other viral infections, the reasons for LDH

increase are not clear, but tissue damage is usually suspected. The reasons for the specific $B^{2'}_{4}$ LDH increase in this test were not clearly evident. It is also not known what change would occur in the few fish which have been shown not to express this genetically variable enzyme (Utter and Hodgins, 1972).

Diminished levels of serum proteins have been reported for several diseases of fish (35, 36). However, with IHN disease there was no apparent overall reduction in total plasma protein, but there were specific alterations in some of the serum proteins. Klontz, et al (37) using cellulose acetate strips showed an increase in the beta-2 serum fraction of chinook salmon surviving an IHN infection and suggested it may be of immunological significance. The antibody activity of fish serum resides in the slowest migrating macroglobulin (33, 39). In these tests alterations were observed in the alpha-2 and alpha-3 fractions. Although the reasons for this are not clear, tissue damage or some other host response may be responsible for this change. The separation of control plasma was similar to that previously reported with disc electrophoresis (4).

In conclusion, the cause of death seems most closely correlated with the reduction in serum electrolytes. The anemic condition, raised blood pH, and other changes found were not incompatable with survival. However, the very low blood osmolality and plasma bicarbonate were considerably below normal values (20). The very low osmolality value indicated a very poor prognosis (41) and also suggested a considerable loss of sodium. All of these data implicated acute kidney failure as the most probable cause of death. Urine pH, HCO_3^-, water

clearance, and osmolality would have given much additional information, but these tests were not possible on a 25 g fish. Future studies should include analysis of serum sodium and potassium.

SUMMARY

Rainbow trout were infected with Infectious Hematopoietic Necrosis (IHN) virus, and various hematological and biochemical measurements of moribund fish were compared to uninfected controls. Infected fish had reduced corpuscular counts, hemoglobin, and packed cell volume, but normal MCV, MCH, and MCHC. The % immature erythrocytes was increased but the % leukocytes were unchanged. Differential leukocyte counts showed a significant decrease in neutrophils, increase in lymphocytes, but no change in monocytes. Unidentifiable necrobiotic disintigrating particles were prevalent in blood smears and hematopoietic tissue imprints.

Plasma bicarbonate, chloride, calcium, phosphorus, bilirubin,and osmolality were significantly reduced, but plasma glucose and anterior kidney ascorbate were unchanged. Plasma pH increased and there were alterations in the alpha fractions of the serum proteins. No change was found in plasma enzymes, except that the $B^{2'}_{4}$ LDH isozyme was significantly increased. The alkali reserve was diminished and there were alterations in acid-base and fluid balance.

Death probably resulted from a severe electrolyte and fluid imbalance caused by renal failure.

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SECTION II

PATHOPHYSIOLOGY OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS DISEASE IN RAINBOW TROUT: EARLY CHANGES IN BLOOD AND ASPECTS OF THE IMMUNE RESPONSE AFTER INJECTION OF IHN VIRUS.[1]

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ABSTRACT

Juvenile rainbow trout (*Salmo gairdneri*) were injected with Infectious Hematopoietic Necrosis (IHN) virus and various hematological and blood chemical changes were followed over a nine day period of taking daily random samples. The packed cell volume, hemoglobin, red blood cell count, and plasma bicarbonate were significantly depressed by day 4. No change occurred in plasma chloride, calcium, phosphorus, total protein, or in any of the blood cell types during the 9 day period. Furthermore, plasma $B^{2'}_{4}$ LDH isozyme was significantly increased by the fourth day, and fish infected with Infectious Pancreatic Necrosis virus, *Vibrio anguillarum*, *Aeromonas salmonicida*, and redmouth bacterium did not show specific LDH isozyme alterations. Acid-base alterations occurred at 10° C but not at 18° C. The acid-base imbalance and elevation of the $B^{2'}_{4}$ LDH isozyme were consistantly associated with the early development of the disease.

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1. This paper reports a portion of the work encompassed by a thesis submitted to the University of Washington School of Fisheries in partial fulfillment of the requirements for a Phd degree.

The immune response after injection of IHN virus was determined and protection from disease was tested by passive immunization. Actively immunized fish developed IHN neutralizing antibodies within 54 days after injection of virus and the antibodies were protective when juvenile fish were passively immunized and experimentally challenged with IHN virus.

INTRODUCTION

The physiological changes associated with disease are used extensively in mamalian medicine for diagnostic purposes and to determine the prognosis of disease. Several attempts have been made to determine the physiological status of fish infected with various pathogens (Hunn, 1964; Bell, 1969; Wedemeyer and Ross, 1973; Mulcahy, 1969; Field, et al, 1944; Foda, 1973; Cardwell and Smith, 1971) and with some toxic chemicals (McKim, et al, 1970; Wedemeyer, 1971). However most physiological tests are not routinely used in fisheries medicine because an insufficient number of diseases have been studied for comparative purposes and because normal values have not been widely established or the tests standardized. The recent works of McCarthy, et al (1973) and Wedemeyer and Nelson (1973) have aided much to establishing the normal ranges of many physiological parameters of rainbow trout (*Salmo gairdneri*).

Amend (1973) described the physiological status of moribund rainbow trout infected with Infectious Hematopoietic Necrosis (IHN) virus. The disease was characterized by a severe depletion of the alkali reserve and an imbalance of blood electrolytes resulting in decreased blood osmolality. The reason for this was believed to have been due to a loss of renal function as a result of the viral infection. The objective of this paper is to describe some of the early changes following experimental injection of IHN virus. Objectives also include a comparison of infected fish at two water temperatures, a comparison of plasma lactic dehydrogenase (LDH) isozymes of IHN infected fish with the LDH isozymes of some bacterial diseases, and a pre-

liminary description of the immune response of rainbow trout to IHN virus.

MATERIALS AND METHODS

Experimental Procedures

Four series of tests were conducted. In the first test about 600 rainbow trout 10 to 13 cm long were placed in each of two 700 L circular tanks containing 9° C flowing municipal water. Chemical characteristics of the water are described elsewhere (Amend, et al, 1960). The fish were acclimated for two weeks, then all fish in one tank were anesthetized with 50 ppm Benzocain and injected intraperitoneally (ip) with 0.1 ml of IHN virus preparation containing $10^{6.4}TCID_{50}$/ml grown in fathead minnow cells. The fish in the other tank were handled similarily except they received only 0.1 ml ip of cell culture medium. Fish to be sampled were randomly selected 24 hours after inoculation and sampling continued at 24 hour intervals for nine days. The fish were not fed during the experiment. At each sampling period, 40 fish were anesthetized, the caudal peduncle severed, and blood collected in heparinized capillary tubes. Precautions were taken to prevent hemolysis. From 10 fish, a red blood cell count (RBC), packed cell volume (PCV), hemoglobin (Hb), and blood smear were taken. A capillary tube of blood from each of these 10 fish was plugged for plasma bicarbonate analysis. All capillary tubes were centrifuged at 1000 xg for 10 min., then the plasma from the 30 unplugged tubes were pooled into 3 10-fish groups and frozen at -20° C for other biochemical analyses.

In the second test, 14 rainbow trout (20-28 cm) were placed in each of four 75 L troughs. Two of the four troughs received 10° C flowing water and the other two troughs received

18° C water. The fish in one trough at each temperature were injected with 0.1 ml ip of IHN virus preparation ($10^{6.6}TCID_{50}$/ml) and the other fish received 0.1 ml ip of cell culture media. This gave an infected and control group at each temperature. After six days all fish were anesthetized and the blood from each fish was collected in heparinized capillary tubes and sealed. Plasma pH, bicarbonate, chloride, and LDH were determined on each fish.

In the third test, the plasma LDH isozyme patterns were compared from rainbow trout (8-9 cm) infected with *Vibrio anguillarum, Aeromonas salmonicida,* and redmouth (RM) bacterium (Hagerman strain). Ten fish were injected ip with 0.1 ml of a standardized bacterial suspension for each pathogen and held at 15° C. The bacteria were grown on trypticase soy agar (plus 1% NaCl for Vibrio) 24 hr. at 20° C, then harvested and washed (1X) with saline, and adjusted to 40% transmittance at 625 mμ. Ten control fish were injected with saline. Blood was collected as described above when signs of disease were evident within 24-72 hours post injection. The 10 control fish were bled 72 hours after injection. Plasma was also obtained from moribund 6-7 cm brook trout (*Salvelinus fontinalis*) naturally infected with Infectious Pancreatic Necrosis (IPN) virus. Plasma from IHN infected rainbow trout from a previous test was used for comparison.

The fourth test was to determine if rainbow trout formed antibodies to IHN virus and to determine if these antibodies would confer protection from the disease by passive immunization. Five adult rainbow trout (about 500 g each) were actively imm-

unized by injecting intravenously 1 ml of cell culture grown (rainbow trout embryonic) IHN virus ($10^{6.5}TICD_{50}$/ml). Five control fish received 1 ml of cell culture media from non-infected cells. All fish were held in flowing 8° C water for 5 days, then the temperature raised to 17° C for 21 days. The fish were given a second injection the same as before at 8° C, and the temperature was again raised to 17° C after 5 days. The temperature remained at 17° C for the remainder of the test. Each fish was fin-clipped for identification. Serum samples were taken 0, 26, 54, and 90 days after the first injection, next complement inactivated at 45° C for 30 min., and then tested for neutralization against IHN virus.

The rationale for the above procedure is that animals respond to live viruses better than to killed viruses (Fenner and White, 1970). Rainbow trout are readily infected at 8° C, but the disease does not develop at 17° C (Amend, 1970). By injecting the fish at a low temperature, a viremia is established, but the infection stops before death occurs. Furthermore, the immune response is faster at 17° C. This procedure optimizes the antigen dose and the antibody response.

To test if the virus neutralizing ability of the serum would confer protection against the disease, the serum from 3 immunized fish was pooled. Fifteen rainbow trout fingerlings (5-6 cm) were then passively immunized by injecting 0.1 ml ip of undiluted immune serum, and the same number of control fish were injected with non-immune serum. Additional controls consisted of nonchallenged and IHN-challenged fish which received no serum. All fish, except the non-challenged controls, were

injected subcutaneously anterior to the mid-dorsal line with 0.01 ml of IHN virus preparation ($10^{6.6}TCID_{50}$/ml). All tests were duplicated and mortalities counted for 20 days.

Biochemical Test

The Hb, RBC, PCV, plasma bicarbonate and pH were determined immediately on fresh blood. Blood smears were prepared, fixed in methanol, and stained by the Lieshman-Giemsa method as described by Klontz and Smith (1971) and the cells classified as described by Klontz (1972). Plasma chlorides, calcium, phosphorus, protein, and LDH were determined on frozen samples. Methods of analyses were as follows: chloride and protein (Wedemeyer and Chatterton, 1970); Hb, PVC, bicarbonate, and pH (Wedemeyer and Chatterton, 1971). Calcium was determined by titration, phosphorus by the Molybdate Method, and RBC with a coulter counter. Horizontal starch gel electrophoresis was used for LDH analysis and the isozymes identified according to Utter and Hodgins (1972). The pCO_2 and H_2CO_3 concentrations were determined mathematically using the Henderson-Hasselbalch equation for blood carbonate.

Virological Tests

A permanent cell line of fathead minnow (FHM) and an established cell line of rainbow trout embryonic (RTE) cells were used. In all cases Eagle's minimal essential medium (Earl's salts) containing 10% agamma newborn calf serum, 100 iu per ml penicillin G, and 100 µg per ml of streptomycin was used. IHN virus was from a laboratory stock (1274) originally isolated from rainbow trout and was injected into fish to test for virulence. The reisolated virus was specifically neutralized by

anti-IHN serum. Viral preparations were grown at 15° C until all cells showed cytopathic effects, 4 to 6 days after inoculation. All preparations were centrifuged at 1000 xg for 10 min. to sediment cellular debris. The method of Reed and Meunch was used to determine viral titers and all titrations were performed in microtiter plates. Serum for neutralization tests was pre-absorbed with cells and media prior to conducting the test. Two-fold dilutions of serum against a constant 50-100 $TCID_{50}$ of IHN virus was used in the neutralization tests.

RESULTS

Hematological and Biochemical Changes

In a preliminary test to determine the susceptibility of the test fish to the disease, only 42% of the fish died, with maximal mortality occuring 9 days after injection. Consequently, during subsequent tests I only expected between 40 and 60% mortality. In the first experiment, signs of the disease were noted (fecal casts) 4 days after injection and the first mortality occurred on the sixth day. Mortalities increased considerably on the eighth day. Because I was interested only in the early changes associated with the disease, the experiment was terminated on the ninth day.

Samples of control and infected fish were taken daily. Consequently, at the termination of the experiment, there were about 90 individual analyses on fresh blood, and 27 pooled samples representing 270 fish on the frozen samples from both test groups. The data were then tested by analysis of variance. Because no significant differences ($P<.05$) were found within the control values for each analytical test, the composite 9 day mean and standard deviation were determined and used as representative of the population. These values were: RBC($X10^6$/ml)= 1.300 ± 0.158, PCV (%) = 46 ± 5, Hb (g/100ml) = 9.2 ± 0.8, calcium (mg/100 ml) = 10.1 ± 0.4, phosphorus (mg/100 ml) = 13.3 ± 0.6, protein 5.6 ± 0.3, bicarbonate (mEq/L) = 10.2 ± 2.5, and chloride (mEq/L) = 120 ± 7. The t-test was then used to analyze daily means of the infected fish. To illustrate these changes more consicely, the % change from control values was calculated and are shown in Figures 1 and 2.

Significant reduction in PCV, Hb, RBC, and bicarbonate

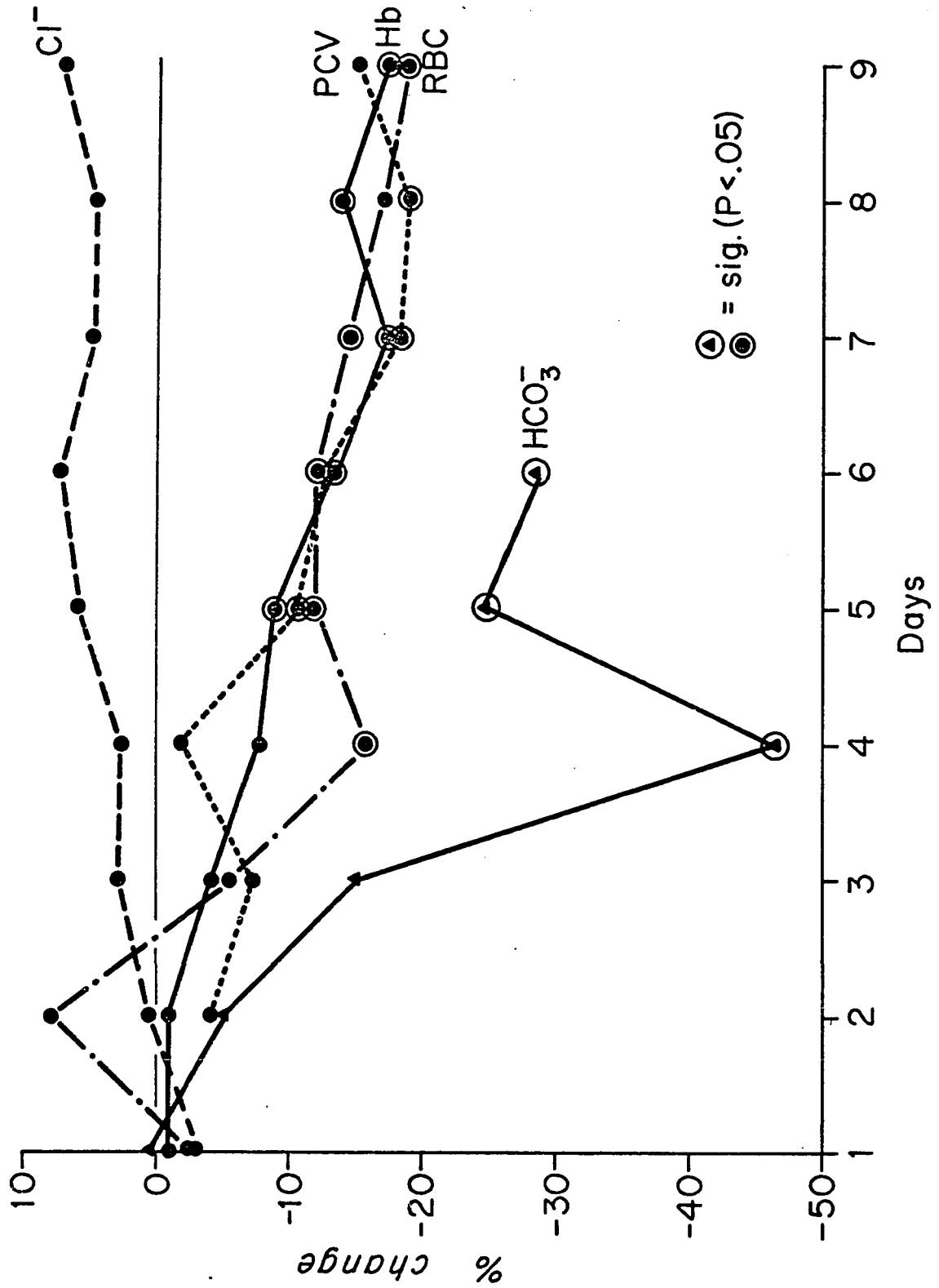
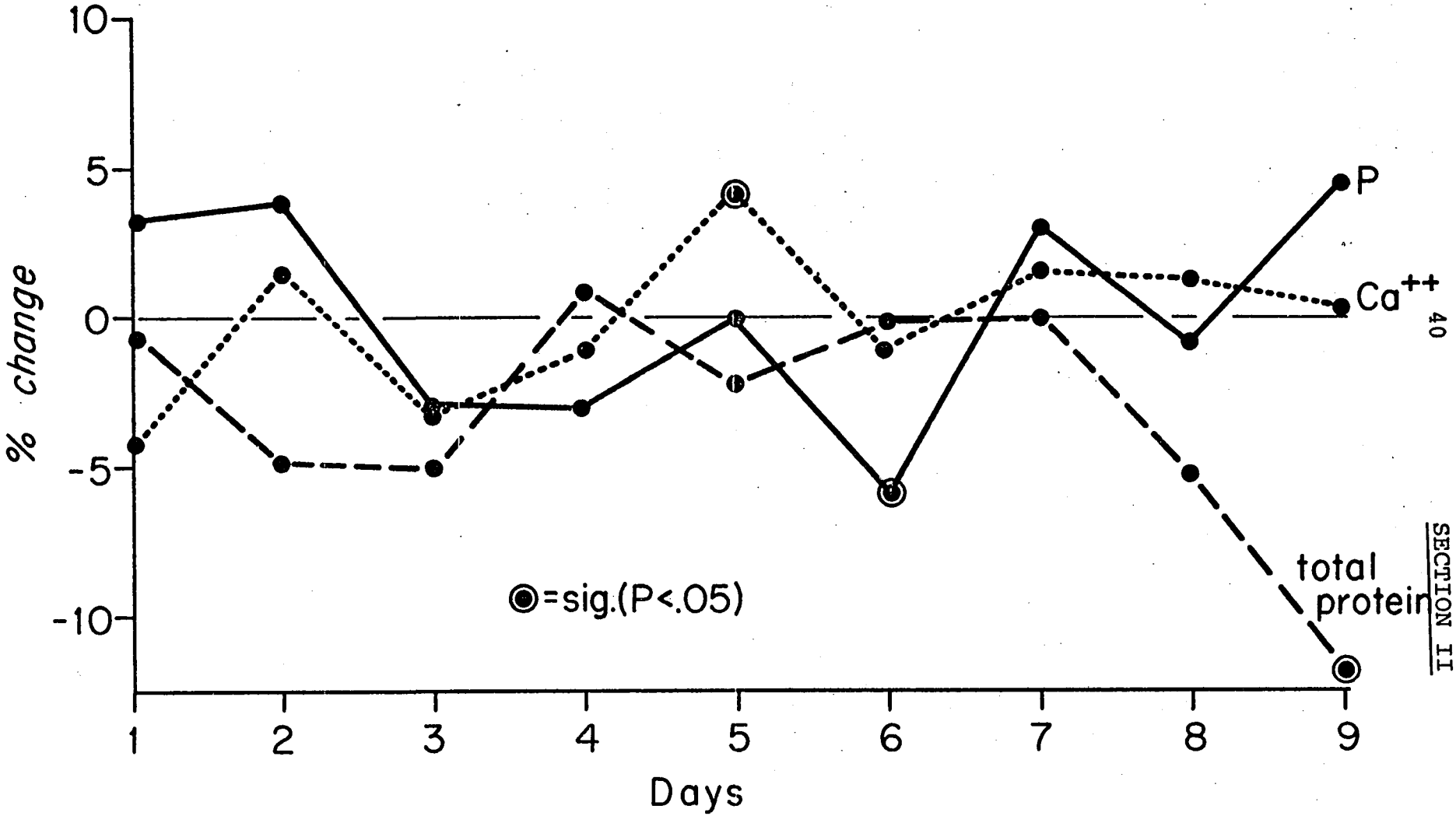


Figure 1: Daily chloride, packed cell volume, hemoglobin, red blood cell count, and bicarbonate values from IHN infected rainbow trout expressed as the percent change from mean values of the uninfected population.

Figure 2: Daily phosphorus, calcium, and total protein values from IHN infected rainbow trout expressed as the percent change from mean values of the uninfected population.



occurred on the fourth or fifth day which was at least 24 hours prior to the first mortality (Figure 1). The changes were most evident by the severe depletion of the serum bicarbonate. However, there was no significant differences in the daily change in plasma chloride, phosphorus, calcium, or total protein (Figure 2). Furthermore, there was no significant change in the % immature RBC, % total leukocytes, % monocytes, % lymphocytes, or % neutrophils (Table 1). The $B^{2'}_{4}$ plasma LDH isozyme was increased on the fourth day and remained elevated (Figure 3).

It was apparent that some physiological changes could be detected prior to the onset of mortalities, and serum bicarbonate and LDH levels were most obviously affected. Reduction in serum bicarbonate suggests alteration in the acid-base regulation. A test was then conducted to determine if acid-base and LDH changes occur at both 10° C and 18° C. At 10° C the plasma bicarbonate was not significantly changed, but significant reduction ($P<.05$) of pCO_2 and an increase in plasma pH and HCO_3^-/H_2CO_3 ratio had occurred (Table 2). At 18° C there was no significant change between control and infected fish. The acid-base alteration was again confirmed, but occurred only at the lower temperature.

The results of the LDH tests were similar; increase in the $B^{2'}_{4}$ LDH isozyme was present in 10 of 13 infected fish at 10° C, but no change occurred in an equal number of fish at 18° C or in any of the control fish. Plasma LDH isozyme patterns were then compared on moribund fish infected with *A. salmonicida, V. anguillarum,* RM bacterium, and IPN virus to determine if the $B^{2'}_{4}$ LDH change was specific for IHN.

SECTION II

Test 1: Hematological comparison of blood smears from uninfected controls and from rainbow trout infected with IHN virus.[1]

Cell Type	Control		IHN infected		Statistic[2]
	Number	Mean ± SE	Number	Mean ± SE	
Immature RBC					
Day 1	10	9.2 ± 0.9	10	8.2 ± 1.0	NS
Day 5	10	7.0 ± 1.0	10	8.0 ± 0.7	NS
Day 9	10	6.9 ± 0.9	10	10.3 ± 1.9	NS
Leukocytes (Total)					
Day 1	10	0.9 ± 0.2	10	1.5 ± 0.3	NS
Day 5	10	2.0 ± 0.3	10	1.9 ± 0.6	NS
Day 9	10	1.9 ± 0.3	10	3.4 ± 2.3	NS
Monocytes					
Day 1	10	3.3 ± 0.8	10	2.3 ± 1.0	NS
Day 5	10	4.8 ± 1.0	10	4.0 ± 1.3	NS
Day 9	10	5.8 ± 1.8	10	6.2 ± 1.6	NS
Lymphocytes					
Day 1	10	91.2 ± 1.6	10	89.8 ± 3.0	NS
Day 5	10	93.0 ± 1.0	10	92.8 ± 2.2	NS
Day 9	10	88.4 ± 3.2	10	91.8 ± 2.9	NS
Neutrophils					
Day 1	10	5.6 ± 1.6	10	7.9 ± 2.6	NS
Day 5	10	2.2 ± 0.8	10	3.2 ± 1.1	NS
Day 9	10	5.8 ± 1.7	10	2.0 ± 1.6	NS

1. Immature RBC and leukocytes are percentages of about 500 cells counted, including mature RBC. Monocytes, lymphocytes, and neutrophils are differential percentages of about 100 leukocytes counted.

2. NS = not significant ($P<.05$)

Figure 3: Starch-gel electrophoresis of plasma LDH isozymes from IHN infected (T) rainbow trout and uninfected controls (C). Subscript indicates days following injection. Arrow indicates location of B^2_4 LDH isozyme.

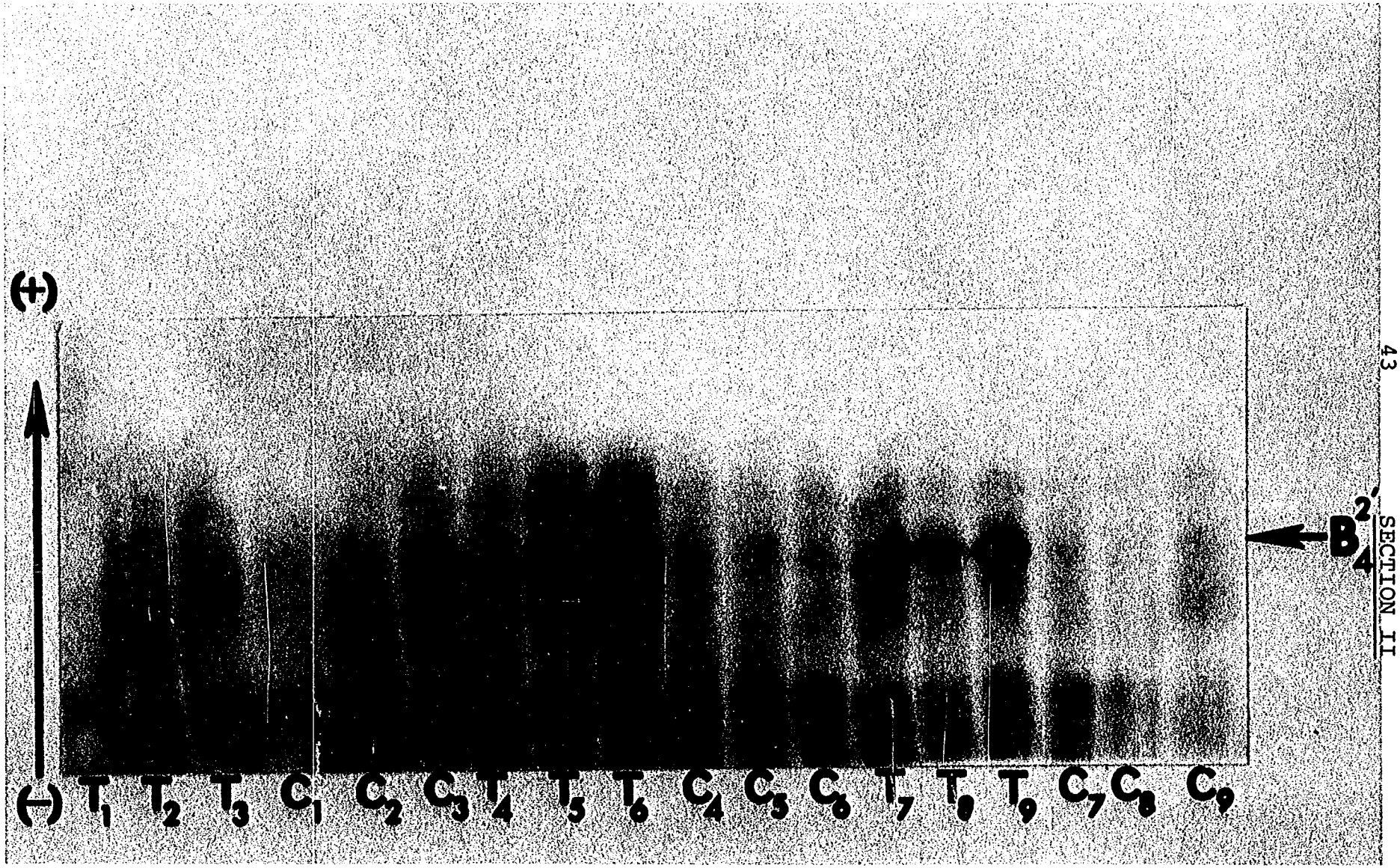


Table 2: Comparison of acid-base balance between uninfected control and IHN infected rainbow trout at two water temperatures.

Clinical Test[1]	Control Number	Mean ± SE	Infected Number	Mean ± SE	Statistical Significance[2]
at 10° C					
pH[3]	11	7.508 ± 0.030	13	7.599 ± 0.038	*
HCO_3^- (mEq/L)	13	11.4 ± 0.3	14	11.0 ± 0.5	NS
pCO_2 (mmHg)	10	15.87 ± 0.81	12	12.69 ± 1.38	*
H_2CO_3 (mEq/L)	10	0.476 ± 0.024	12	0.381 ± 0.041	*
HCO_3^-/H_2CO_3	10	25.31 ± 1.8	12	30.9 ± 2.0	*
Total CO_2 (mEq/L)	10	12.18 ± 0.33	12	11.40 ± 0.58	NS
at 18° C					
pH	6	7.454 ± 0.013	14	7.485 ± 0.023	NS
HCO_3^- (mEq/L)	6	11.7 ± 0.5	14	11.2 ± 0.5	NS
pCO_2 (mmHg)	6	17.30 ± 0.94	14	15.58 ± 0.84	NS
H_2CO_3 (mEq/L)	6	0.519 ± 0.028	14	0.468 ± 0.025	NS
HCO_3^-/H_2CO_3	6	22.7 ± 0.7	14	24.7 ± 1.3	NS
Total CO_2	6	12.22 ± 0.53	14	11.71 ± 0.56	NS

1. pCO_2, H_2CO_3, HCO_3^-/H_2CO_3, and total CO_2 are calculated values and were derived by the formula[3] $pH = 6.1 \text{ Log } \frac{HCO_3^-}{.03pCO_2}$ which is valid only at 38° C.

   These data, except for pH, are not temperature corrected and are not to be taken as normal values, but are included for comparative purposes because all data and fish were handled similarily.

2. *=$P<0.05$, NS=not significant

3. the mean hydrogen ion concentration (H^+) expressed as pH

In all cases the disease was confirmed by reisolation of the agent, and with IPN, histological examination, and serum neutralization confirmation were also performed. The number of samples showing increased LDH on the $B^{2'}_{4}$ isozyme was as follows: IPN - 0/10, RM - 0/5, *A. salmonicida* - 1/7, *V. anguillarum* - 1/7. The one fish with elevated $B^{2'}_{4}$ LDH in *A. salmonicida* and *V. anguillarum* also showed an increase in the slower migrating isozymes. With IHN, only the $B^{2'}_{4}$ isozyme was elevated. Therefore, of the 5 diseases compared, only IHN could be distinguished by the LDH isozyme pattern.

Aspects of Immunology

Adult rainbow trout were injected with a live virus preparation and the temperature manipulated to prevent disease. Sera were then tested for viral neutralization. All sera, including those from control fish were toxic to cell cultures at a dilution of 1:20 or less; therefore, the lowest dilution recorded was 1:40. Prebleeding prior to injection of virus showed no detectable anti-IHN activity (Table 3). No antiviral activity was detected 26 days after injection, but 4 of 5 fish had significant IHN neutralizing antibody by day 54. The results were only slightly changed on day 90. These sera neutralized the IHN virus, but showed no activity against IPN virus. Furthermore, absorption of the sera with $10^{5}TCID_{50}$ IHN virus completely removed the antiviral activity.

The above data show that rainbow trout can be immunized against IHN virus, and in other tests, trout which survived a natural epizootic showed anti-IHN neutralization (unpublished data). To demonstrate whether the antibody was protective, the

SECTION II

Table 3: Serum neutralization titer of rainbow trout immunized against IHN virus. Sera were tested against 50 to 100 $TCID_{50}$ IHN virus.

Fish	Reciprocal of 100% neutralization titer			
	Days			
	0	26	54	90
1	<40	<40	>320	>160
2	<40	<40	160	<80
3	<40	<40	< 40	<40
4	<40	<40	not tested	1280
5	<40	<40	160	320
Controls (5 fish)	<40	---	---	<40

sera of 3 fish were pooled (average 50% neutralizing titer<1:320), injected into fingerling rainbow trout, and then the fish were challenged with virulent virus. None of the passively immunized fish died, but an average 55% of the fish receiving control serum died (Table 4). These data show that the antibody formed by rainbow trout against IHN disease are protective antibodies.

SECTION II

Table 4: Passive immunization of fingerling rainbow trout by ip injection of undiluted sera from adult rainbow trout actively immunized with IHN virus. Fingerlings were challenged by subcutaneous injection of $10^{6.6}TCID_{50}$/ml of IHN virus at 10^{o} C and mortalities counted for 20 days.

	Control (no virus)		Control (virus)		Antiserum		Serum Control	
	A	B	A	B	A	B	A	B
No. dead	0	0	13	6	0	0	9	7
No. alive	15	15	1	9	12	12	5	8
Total	15	15	14	15	12	12	14	15
% dead	0	0	93	40	0	0	64	47
Combined	0/30		19/29		0/24		16/29	
% Ave.	0		66		0		55	

DISCUSSION

Anemia is a characteristic sign of IHN disease during natural epizootics (Amend, et al, 1969), but severe anemia may or may not occur during experimental infections or during acute natural epizootics. Anemia, though helpful as a diagnostic aid, is not a specific sign of any disease. There are numerous diseases and conditions that can cause anemia in fish. Many anemic conditions in fish have not been characterized, but Amend (1973) described IHN disease as a normocytic aplastic anemia. In this paper I showed that the changes in blood occurred early in the infection. Although there were significant reductions in mean blood values, they were still in the normal range and compatible with survival (Wedemeyer and Nelson, 1973; McCarthy, et al, 1973).

Plasma calcium, phosphorus, chloride, and total protein remained unchanged, and there was no detectable change in the abundance of immature RBC, total leukocytes, macrophages, lymphocyte, or neutrophils. Amend (1973) showed that fish terminally infected with IHN disease showed a significant depression of plasma electrolytes and a reduction of neutrophils. In this test random samples were selected from a population which only 50% of the fish were estimated to have contracted a fatal infection. Consequently, the data would not reflect the severe changes one would expect to find in moribund fish. More severe changes may have been present in these tests, but were inapparent either because of the method of selecting fish or because the clinical changes had not developed to the extent previously observed. This would also explain the greater variation in the data.

Alteration in plasma $B^{2'}_{4}$ LDH isozyme concentrations and in acid-base balance are two changes which appear early in the infection and are consistantly associated with the disease. The degree of base deficit or pH change would depend largely on the ability of the fish to compensate for acid-base changes. In terminally infected fish there were severe depletions in alkali reserve and an increase in blood pH (Amend, 1973). In this paper, alkali depletion was suggested in the first test, but an uncompensated alkalosis was indicated in the second test. Although bicarbonate was not significantly changed in the second test, elevated pH and elevation of the bicarbonate/carbonate ratio indicates a loss of acid (pCO_2). The specificity of the acid-base imbalance due to IHN disease is not known because I am unaware of any similar analyses with other fish diseases.

The specificity of the $B^{2'}_{4}$ LDH isozyme concentration changes is also not known, but it appears that similar changes do not occur with IPN, *A. salmonicida*, *V. anguillarum*, or RM disease. However, Wroblewski (1957) points out that several conditions are capable of causing an increase in serum LDH in mammals, and may not be specific. Furthermore, Utter and Hodgins (1972) showed that genetic variability of the $B^{2'}_{4}$ isozyme occurs in the serum of rainbow trout. It is not known what change might occur in fish without this isozyme.

Amend (1970) showed that elevating the water temperature on IHN infected sockeye salmon from 10° C to 18° C prevented the disease. Preliminary tests also showed that the disease would also not develop in rainbow trout at 18° C (unpublished data). However, it was not known in either case if the fish

were not infected or they only experienced a subclinical infection which did not result in death. The data presented here indicates that the infection was terminated at the higher temperatures. There was no change in acid-base balance or increase in $B^{2'}_{4}$ LDH levels which was characteristic early in the infection at lower temperatures. DeKinklin and Dorson (1973) demonstrated the presence of an interferon at 18° C in rainbow trout infected with viral hemorhagic septicema (VHS) but only weakly at lower temperatures. Whether this or some other physiological explanation is responsible for the temperature effect is not known for IHN disease.

The immune response of fish to viral infections is just beginning to be understood. Klontz (1965) suggested that certain protein changes in the serum macroglobins may be of immunological significance in sockeye salmon following infection with IHN virus. Wolf (1969) showed that rainbow trout which were injected with IPN virus developed IPN neutralizing antibodies. Dorson (1972) also showed that rainbow trout were capable of responding immunologically to bacteriophages. However, Jørgensen (1971) was not able to demonstrate antibody in rainbow trout naturally infected with VHS virus, but was able to demonstrate neutralizing antibodies in artifically infected fish only after 18 mos. post injection. IHN resembles VHS in many ways, but in this test rainbow trout responded very well to IHN virus. Perhaps it was the virulent virus and temperature minipulation that optimized the immunological response.

Passive immunization is one way of demonstrating the relative importance of cellular immunity and humoral antibody in conferring

protection from disease. The cellular immune response in mammals is sometimes important in combating initial viral infections and may even be responsible for some of the signs of the disease (Fenner and White, 1970). However, humoral antibody is most important for preventing second attacks from the same disease. The results presented here demonstrate that humoral anti-IHN antibody in rainbow trout can prevent fatal infection of IHN disease.

The ability to control viral infections in fish must depend on a thorough understanding of the disease. Knowing what physiological changes occur can help us to choose what environmental, diet, or gas regulation we should try to help alleviate the disease. Chemotherapeutics are useless to combat viral infections; therefore, we must rely on environmental control, immunization, or eradication of the agent. Eradication is not always practical. Environmental control is being practiced and as diseases become better understood, may become more widely practiced. Results presented here suggest that salt and acid-base regulations may influence the course of IHN disease. Immunization is the most widely utilized method of protection from viral diseases in mammalian medicine. In fact, almost all major breakthroughs in controlling viral diseases has been through the development of effective vaccines. This goal may be attainable with the fish viruses when we obtain a better understanding of the immune mechanisms of fish, the antigenic makeup of the viruses, and the diseases they cause.

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APPENDIX A

A REVIEW OF LITERATRUE

History of IHN Virus

A suspected viral disease of juvenile sockeye salmon (*Oncorhynchus nerka*) was first reported by Rucker, et al (1953) in several Columbia River salmon hatcheries in the state of Washington. A filterable agent was demonstrated, but the virus was not isolated (Watson, et al, 1954). A similar disease of sockeye salmon occurred in Oregon in the late 1950's, but in this case, infected fish were preserved (ultrafrozen). The virus was subsequently isolated when cell culture techniques for fish tissue became available (Wingfield, et al, 1969). Because the virus from the Washington hatcheries was not available for comparison, the two outbreaks were sometimes referred to as two diseases. Parisot, et al (1965) referred to the Washington cases as the Columbia River Sockeye Disease and the Oregon case as the Oregon Sockeye Disease. A virus which causes disease in sockeye salmon similar to that originally reported (Rucker, et al, 1953; Guenther, et al, 1958) is still prevalent in sockeye salmon in Washington (Amend and Wood, 1972).

A virus-like disease of chinook salmon (*O. thsawytscha*) in the Sacramento River, California was described by Ross, et al, (1959) and Parisot (1962). Although this disease resembled the sockeye disease (Yasutake, et al, 1965), differences in species specificity distinguished the sockeye virus from the chinook virus (Parisot, et al, 1965). The chinook disease became known as the Sacramento River Chinook Disease. Later studies showed that there was also no difference in physical

and biochemical characteristics between the sockeye and chinook virus isolates (Wingfield, et al, 1969; Wingfield and Chan, 1970; Amend and Chambers, 1970).

In 1967 a viral disease of rainbow trout (*Salmo gairdneri*) was described (Amend, et al, 1969). The trout were dying in a locality where severe losses of sockeye salmon were also occurring. In subsequent studies, the rainbow trout virus isolate was compared to the sockeye virus (Oregon strain) and to the chinook virus (California strain) and no difference was found between the 3 isolates in morphological, biochemical, or physical characteristics (Amend and Chambers, 1970; Darlington, et al, 1972). A serological comparison by McCain, et al, (1971) showed that all 3 isolates were closely related, if not identical.

It is now generally agreed that all 3 diseases are probably caused by the same virus or a group of closely related viruses (Amend, et al, 1972; Wolf, 1972). There still is some degree of species specificity which can be demonstrated, but cross transmission has been experimentally shown (Parisot, et al, 1965; Amend, et al, 1969). In all 3 species, the hematopoietic tissues of the kidney and spleen are the first and most severly affected tissues in both natural and experimental infections (Yasutake, et al, 1965; Yasutake and Amend, 1972). Therefore, the descriptive name Infectious Hematopoietic Necrosis (IHN) was introduced (Amend, et al, 1969) and is now referred to all 3 diseases.

The epizootiology of the disease has been recently reviewed (Amend, et al, 1972). The virus has the properties and morphology of rhabdoviruses (Amend and Chambers, 1970; Darlington, et al, 1972). Virions are about 90 mμ wide by 160 mμ long and contain

an axial pore about 20 mμ in diameter. Substructural units have been described. The virus contains a RNA nucliocapsid and is sensitive to chloroform or ether. It is readily inactivated by heat (50° C for 30 min) and low pH.

The disease is characterized by severe loss of fish at water temperature between 8 and 12° C. Signs of the disease typically include exophthalmos, anemia, fecal casts, lethargy, and petechial hemorrhaging about fins, adipose tissue, and visceral mesentaries (Amend, et al, 1969; Rucker, et al, 1953; Ross, et al, 1959). Histopathologic lesions first occur in the hematopoietic tissues of the anterior kidney and spleen, and extensive necrosis occurs in these tissues and in the lymphoid tissues of the gut at the time of death (Yasutake and Amend, 1972). In terminal cases, focal necrosis is also seen in the liver and pancreatic tissues.

IHN is a disease of the young and the severity of disease decreases with age. Survivors of epizootics became asymptomatic carriers and shed virus in sex products during spawning. The disease can be transmitted both vertically, via contaminated ova, and horizontally. Vertical transmission can possibly be prevented by disinfecting eggs prior to hatching and horizontal transmission can be prevented by rearing fish at water temperatures above 14° C (Amend, et al, 1972; Amend, 1970).

The virus replicates and causes a distinctive cytopathic effect (CPE) on several fish cell lines (Nims, et al, 1970; Amend, et al, 1969; and Wingfield, et al, 1969). The optimum temperature for replication in cell culture is between 12 and 15° C, but the virus replicates and causes CPE between 4 and

18° C. CPE is characterized by margination of nuclear chromatin giving the appearance of a thickened nuclear membrane. The cells eventually round-up into grape-like clusters and form distinctive plaques which are of diagnostic value (Wolf and Quinby, 1973).

IHN is prevelent along the Pacific Coast and is a concern to all conservation agencies. Because Pacific Coast salmon and trout eggs and fry are being distributed around the world, the threat of spreading the agent is increasing. In fact, epizootics have now been recorded throughout the United States and in Japan from the shipment of fish from the Pacific Coast (Amend, et al, 1972; Kimura, personal communication). There is a world wide need for a better understanding of this disease.

Biochemical Changes in Animals due to Viral Infections

Viruses in general are not susceptible to chemotherapeutics; therefore, little can be done to treat viral diseases except to help alleviate the signs and symptoms of the host, and provide optimal physiological conditions for the host to combat the infection. This requires an understanding of what the physiologic effect the virus has on the host and what can be done to counteract these changes. In general, the approach in mamalian medicine has been to determine which tissues or organs are affected, then to prescribe a treatment regimen to replace or help alleviate the complications of the damaged tissue or organ. For example, in respiratory infections respirometers and decongestants are used to keep the respiratory tract open and functioning properly. Fluids and electrolytes are administered with acute intestinal infections when excessive diarrhea occurs. Bicarbonate or gas mixtures are used when acid-base imbalances

occur, drugs and ice baths are used to reduce high body temperatures, salt intake and diets are regulated with kidney failure, and many other examples (Sodeman and Sodeman, 1967; Ruch and Fulton, 1960).

It is beyond the scope of this review to examine the physiologic effect of all viral infections, but some generalities can be made. The type of physiologic alteration depends largely upon the invasive properties of the virus. The more invasive the infection, the more pronounced is the physiologic effect. Also, the viral infections of the alimentary tract and respiratory tract generally cause greater changes than skin infections (Fenner and White, 1971). The spread of virus throughout the host can occur via the blood, lymphatics, or the central nervous system. Once the target organ is infected and dysfunction occurs, the physiologic effect can be detected; however, changes may also occur while the virus is multiplying in various tissue before reaching the target organ. Sometimes, diagnosis of the infection can be accomplished, and the course of disease can be followed by determining the physiological status of the affected tissues. Cytocidal viruses, which cause necrosis of the affected tissue, can also cause elevation of centain enzymes (Mahy, et al, 1964). The type of enzyme appearing in serum depends on the tissue being affected.

In the late 1950's and early 1960's attempts were made to demonstrate specific enzyme changes in animals infected with oncogenic viruses (Notkins, 1965). From these investigations the lactic dehydrogenase (LDH) virus of mice was discovered. Though once believed to have been associated with

neoplastic tissue, it was found that the virus was a contaminant of one of the oncogenic viruses (Notkins, 1965). Tests eventually showed that the virus was inhibiting the normal clearance of LDH via the lymphatics and thus the enzyme accumulated. The LDH increase was not due to any pathologic lesion. In many previous studies, elevated LDH was believed to be due to tissue damage, causing release of abnormal levels of enzyme into the intercellular space. Although elevated LDH can be caused by tissue damage, it can be caused by many factors including toxic chemicals as well as infectious agents (Wroblewski, 1957).

At present, the physiological status of animals infected with virus can be determined by following the biochemical and physiological changes which occur. Blood cellular and chemical changes are used both as a diagnostic tool, and to determine the prognosis for recovery.

Normal Blood Chemistry of Fish

The value of using changes in blood chemistry to aid in diagnosing fish diseases has been recognized by many investigators. However, realization of this value has been slow in developing because many variables, such as different diets, water temperatures, water conditions, stresses, strains of fish, etc., can effect "normal" values. In recent years, the approach has been to establish normal ranges based on a statistical approach which would include the influences from these variables (Wedemeyer and Chatterton, 1970; McCarthy, et al, 1973). Consequently, the normal ranges are wide in comparison to mammalian normal values. Therefore, biological significance of any change must be standardized to each situation. Normal ranges, however,

do have a value for comparative purposes, and may even have significant clinical value when abnormal situations are more thoroughly understood. Table 1 compares the normal ranges for rainbow trout of Wedemeyer and Chatterton (1970) and McCarthy, et al (1973) to the values obtained during the experimentation of this dissertation. The values for the control fish compare favorably to those already reported for rainbow trout and the values for fish infected with IHN are included for comparison.

APPENDIX A

Table A1: Comparison of ranges of normal rainbow trout blood chemistry to mean values reported in this dissertation.[1]

Parameter	Wedemeyer[2]	McCarthy[3]	Amend[4]	
			Control	IHN
Protein (g/100 ml)	2.0-6.0	4.0-6.6	5.6	5.5
Chloride (mEq/L)	84-132	-------	119	107
Bicarbonate (mEq/L)	8.9-15.9	-------	10.5	5.8
pH	7.50-7.83	-------	7.51	7.60
Calcium (mg/100 ml)	6.7-10.6	-------	10.8	8.1
Glucose (mg/100 ml)	41-151	71-207	81	136
Bilirubin (mg/100 ml)	0.4-1.7	-------	2.1	1.2
Phosphorus (mg/100 ml)	8.4-12.7	-------	15.4	12.1
Osmolality (mOsm)	288-339	-------	315	277
RBC (10^6/ml)	-------	0.77-1.58	1.33	.97
Hb (g/100 ml)	5.4-9.3	5.6-10.3	9.4	7.1
PCV (%)	23.9-42.7	32-48	47	35
MCV (cμ)	-------	274-513	359	347
MCH (μμg)	-------	54-98	72	74
MCHC (%)	-------	12.7-25.3	20.1	19.6
Lymphocytes (%)	-------	89-98	91	96
Granulocytes (%)	-------	1.0-9.0	4.0	0.7

1 Ranges are reported at the 95% confidence interval.
2 Wedemeyer and Chatterton, 1970; Wedemeyer and Nelson, 1973
3 McCarthy, et al, 1973
4 mean values reported in this dissertation

Biochemical Changes in Fish Due to Infectious Diseases

Anemia is a common sign of many viral infections of fish but the anemia has not been characterized for any of them. IPN, Viral Hemorrhagic Septicemia (VHS), channel catfish virus (CCV), and spring viremia of carp (*Rhabdovirus carpio*) all multiply in and can cause necrosis of the hematopoietic tissues (Wolf, 1972; Fijan, 1972). Depressed Hb and PCV are reported, but there have been no differential comparisons of the cellular components of the blood or hemopoietic tissues. To my knowledge, there have been no pathophysiological tests reported for any of the fish viral diseases.

There have been several reports on the physiological comparisons of diseased and non-diseased fish infected with bacterial pathogens. *Aeromonas salmonicida* is perhaps the most thoroughly studied fish pathogen, but only a limited amount of data exists regarding the pathophysiology of the disease (furunculosis). Field, et al (1944) reported the first study describing the biochemical changes with carp (*Cyprinis carpio*) infected with the furunculosis. They concluded that no significant changes occurred in the RBC, Hb, albumin, or total plasma protein, but a large reduction in blood glucose and increase in non-protein nitrogen was found. In contrast to these results, Amlacher (1970) reported a significant decrease in serum proteins, especially albumin. Klontz, et al, (1966) working with rainbow trout infected with furunculosis, also reported no changes in PCV, RBC, or Hb levels, but described a severe leukopenia. The hematopoietic tissues consistantly showed degenerative changes. Foda (1973), however, described greatly depressed PCV and Hb levels in

Atlantic salmon (*Salmo salar*) infected with furunculosis, but these fish showed signs of extensive internal and external hemorrhaging which would account for these unusual symptoms.

Corynebacterial kidney disease is prevalent in many species of salmonids. Although many organs can be infected, the kidney is the primary target organ. Anemia is characteristic of this disease, and lesions are commonly found in the hematopoietic tissues (Bullock, 1971; Hunn, 1964; Wedemeyer and Ross, 1973). Wedemeyer and Ross (1973) compared the pathophysiology of coho salmon (*Oncorhynchus kisutch*) with kidney disease, but maintained on different diets. Not only did they find differences between fish on different diets, but showed that plasma chloride, interrenal ascorbate, liver glycogen, and blood glucose were depleted in infected fish. Furthermore, blood pH and blood urea nitrogen was significantly elevated. Hunn (1964) further showed that the blood albumin levels were greatly depressed. Bell (1968) also showed that the plasma glutamic-oxalacetic transaminase (GOT) were elevated in diseased sockeye salmon, and concluded that measurement of GOT levels might be of diagnostic value.

The physiological changes occurring as a result of other fish diseases are just beginning to appear in the literature. The hematological changes in chinook salmon (*O. tshawytscha*) infected with *Vibrio anguillarum* have been characterized as a microcytic, normochromic anemia (Cardwell and Smith, 1971). Specific serum protein changes occurred in Atlantic salmon infected with ulcerative dermal necrosis (UDN) that may be of diagnostic value (Mulcahy, 1969). Recent reviews by Bullock (1971) and Amlacher (1970),

and a bibliography of fish hematology (Hawkins and Mawdesley-Thomas, 1972) described the current knowledge regarding the physiological changes associated with fish pathogens.

Immunization Against Viral Diseases in Mammals and Fish

Immunization is the most widely practiced method of preventing viral diseases. Pasteur prepared one of the first viral vaccine by using a killed rabies vaccine from the spinal cord of infected rabbits. Live, low virulent or avirulent vaccines have also been used for many years. For example, the vaccinia virus confers protection from small pox. Small pox was at one time the most dreaded virus disease in the world, but through vaccination it is now eradicated from most developed countries. However, it was not until routine methods of cultivating viruses were available that most viral vaccines were developed. The polio, mumps, rubella, and other viral vaccines were developed only after cell culture technique became available (Fenner and White, 1970).

The effectiveness of viral vaccines depends largely on the antigenic stability of the virus and the site(s) of entry and replication. Influenza, for example, confers a stable immunity, but antigenic variants continue to appear which are not controlled by vaccines prepared from earlier isolates. Rhinoviruses infect cells only in the upper respiratory tract, and the rubella virus enters via the respiratory tract, but multiplies in various transitory cells (leukocytes) only to produce disease symptoms at some other site. Only secretory antibody (IgA) would be effective in the former example, but hummoral antibody confers protection in the latter example

(Fenner, 1968).

Vaccines with live, avirulent virus usually produce a more permanent immunity than killed-virus vaccines because they can be given by the natural route and produce local as well as humoral antibody. Killed virus vaccines primarily produce humoral antibody and require frequent booster injections. The Sabin live virus polio vaccine, for example, confers intestinal secretory and humoral antibody response which protects from the intestinal infection as well as from poliomyelitis. The Salk, killed-virus polio vaccine, however, only protects against poliomyelitis and vaccinated people can still experience an intestinal infection which could be a risk to unvaccinated individuals. The disadvantage of live virus vaccines is that the avirulent virus may mutate back to a virulent form or that contaminating virus may be inadvertently included in the vaccine (Fenner and White, 1970).

Elasmobranchs and marine teleosts respond immunologically to mammalian viruses (Sigel, 1967), but Wolf (1963) was the first to demonstrate that trout responded immunologically to a viral agent from fish. He showed that rainbow trout naturally infected with IPN virus would develop neutralizing antibodies following infection. Wolf and Quimby (1969) further showed that rainbow trout would develop high IPN neutralizing titers when injected with virulent IPN virus. Dorson (1972) while studying the immune response in rainbow trout used a bacteriophage (FH_5) as an antigen. Neutralizing antibodies were formed and the antibody was purified and described. Jørgensen (1971) also showed that rainbow trout formed neutralizing antibodies to injected

VHS virus, but was not able to demonstrate antibody following natural infection with the virus. The response was slow, for it took 18 mos. to detect neutralizing antibodies, and the titers were at a very low level. Low water temperature was used to explain the poor response in the fish immunized against VHS.

These reviews clearly show that rainbow trout are capable of responding immunologically to viruses. Therefore, it would be reasonable to suspect that IHN virus would stimulate an antibody response. However, the only reported results of fish tested for antibody following infection with IHN virus were negative (Klontz, et al, 1965). There was, however, an alteration in the macroglobulins that was suggested to be of immunological importance. IHN is more closely related to VHS than any of the other viruses. It would appear from the above reports that these viruses produce a poor immunological response. The results presented in this dissertation show that if the right combination of dosage and temperature regulation is maintained, IHN is as strong of stimulus immunologically as other viral antigens tested on fish. The next obvious step is to develop vaccines for the protection of fish against viral diseases.

APPENDIX B

STATISTICAL ANALYSIS

The statistical procedures for variance ratio, t-test, and two way analysis of variance were followed as described by Remington and Schork (1970), and for linear regression analysis as described by Finney (1971). For all tests of control vs infected fish the sample mean, range, variance (s^2), standard deviation (s), and standard error (se) was calculated. The ratio of variance

$$F = \frac{s_1^2}{s_2^2}$$

was used to test the assumption that the sample variances were not different ($P<.05$). If the sample variance was not different, the difference between sample means was tested by

$$t = \frac{\bar{x}_1 - \bar{x}_2}{sp \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where $\bar{x}$ is the sample means, *sp* the average variance of the two samples, and n the number in each sample. However, if the sample variances were not the same, the difference between sample means was tested by

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

In the case of comparing samples between control and infected fish over a period of time, the data was first subjected to a two-way analysis of variance. If control samples were essentially the same over the test period, the control values were averaged to obtain a population mean and variance. The

daily changes of the infected fish were then tested as described above using the control population means and variance in the test statistic.

In the experiment where fish were weighed daily for 19 days the data, of course, was not a random sample of the weight change in each population, but was a record of the weight change of each individual fish. Consequently, the population means did not change much, but there were individual weight fluctuations from day to day. If the weight of each fish was averaged into a 3-day composit, this eliminated the individual daily fluctuations. Therefore, when the 3-day mean of control and infected fish was plotted, a straight line relationship was found. The two groups were then tested by linear regression analysis.

In all blood chemistry tests, it was assumed that the mean values were from a random selection of non-infected or virus infected population of fish. Wedemeyer (1973) has argued that a non-parametric analysis would be more appropriate, but for the purpose of these experiments, parametric analyses were conducted, especially because a normal range estimation was not intended.

APPENDIX B

Table B1: Hematological comparison of uninfected controls with moribund rainbow trout infected with IHN virus.[1]

Clinical Test	Infected					Control					Statistic[4]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	F	t
Whole Blood												
Hemoglobin (g/100 ml)	20	7.1	4.0-9.8	1.89	0.4	20	9.4	7.6-11.0	0.924	0.2	4.192**	4.820**
Corpuscular Count ($X10^6$)	20	0.97	0.40-1.44	0.290	0.07	20	1.33	1.03-1.51	0.152	0.03	3.652**	5.000**
Packed Cell Volume (%)	20	35	7-53	14.0	3	19	47	34-57	6.49	1	4.661**	3.578**
Mean Corpuscular Volume(cu)	20	347	117-417	83.3	19	19	59	279-466	49.8	11	2.798*	0.527
Mean Corpuscular Hemoglobin (uug)	16	74	64-93	8.12	2	20	72	59-99	10.0	2	1.523	0.794
Mean Corpuscular Hemoglobin Concentration (%)	16	19.6	16.1-22.7	2.75	1.7	19	20.1	15.6-23.8	1.85	0.4	2.201	0.627

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APPENDIX B

Table B1: Hematological comparison of uninfected controls with moribund rainbow trout infected with IHN virus.[1] --- Continued

Clinical Test	Infected					Control					Statistic[4]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	F	t
Blood Smear												
% Mature RBC	18	87.8	53.0-94.7	10.16	2.4	20	91.1	86.0-96.4	2.92	0.7	12.15**	1.329
% Immature RBC	18	9.2	4.2-23.0	4.50	1.1	20	7.0	2.8-13.4	2.88	0.6	2.481*	1.787*
% Total Leukocytes	18	2.5	0.2-24.0	5.53	1.3	20	2.0	0.4-3.6	0.981	0.2	31.73**	0.409
% Monocytes	18	3	0-12	3.89	1	20	5.3	0-16	4.46	1.0	1.315	1.616
% Lymphocytes	18	96	86-100	4.39	1	20	90.7	64-100	7.60	1.7	3.004*	2.764
% Neutrophils	18	0.7	0-4	1.19	0.3	20	4.0	0-20	4.40	1.0	13.73**	3.226**

1. see Section I, Table 1, page 7
2. standard deviation
3. standard error
4. *=P<.05 **=P<.01

APPENDIX B

Table B2: Biochemical comparison of plasma from uninfected controls with moribund rainbow trout infected with IHN virus.[1]

Clinical Test (Plasma)	Infected					Control					Statistic[4]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	F	t
Bicarbonate(mg/L)												
EX (1)	6	5.8	4.0-9.0	1.86	0.8	20	9.8	5.8-14.2	2.24	0.50	1.451	4.204**
EX (2)	15	6.1	2.9-8.7	1.58	0.4	20	10.5	5.2-13.1	2.13	0.50	1.808	6.854**
Chloride (mEq/L)												
EX (1)	12	107	88-128	12.4	4	10	115	108-120	4.22	1	8.634**	2.120*
EX (2)	16	113	92-124	9.85	2	20	119	106-134	8.35	2	1.482	1.956*
Glucose (mg/100 ml)												
EX (1)	11	136	40-427	144	43	15	81	44-132	24.4	6	34.83**	1.339
EX (2)	10	321	122-852	257	81	10	429	77-786	270	85	1.104	0.917
OsM (mOsm)	9	277	248-297	18	6	7	315	325-305	6.07	2	9.168**	5.811**
Phosphorus (mg/100 ml)	10	12.11	9.45-14.64	1.56	0.49	10	15.38	13.09-17.82	1.81	0.57	1.353	4.336**
Calcium (mg/100 ml)	10	8.07	5.83-11.57	1.82	0.58	10	10.80	8.74-12.38	1.24	0.39	2.149	3.917**
Bilirubin (mg/100 ml)	4	1.2	0.2-2.1	.802	0.4	16	2.1	1.5-4.1	.781	0.2	1.054	2.034*
Ascorbate[5] (µg/g)	10	45.8	35.2-53.4	6.55	2.1	4	51.5	43.2-59.9	6.86	3.4	1.097	1.437

Continued next page

APPENDIX B

Table B2: Biochemical comparison of plasma from uninfected controls with moribund rainbow trout infected with IHN virus.[1] ------Continued

1. see Section I, Table 2, page 10
2. sd = standard deviation
3. se = standard error
4. *=P<.05. **=P<.01
5. tissue homogenate of anterior kidney

APPENDIX B

Table B3: Comparison of electrophoretic separations of plasma protein fractions from uninfected controls with moribund rainbow trout infected with IHN virus.[1]

Fraction (% of total)	Infected					Control					Statistic[4]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	F	t
Albumin	6	32.0	25.2-38.0	4.20	1.7	6	29.4	23.9-32.5	3.14	1.3	1.789	1.21
α1	6	3.9	2.2-5.4	1.44	0.6	6	3.1	2.4-4.0	0.672	0.3	4.592	1.23
α2	6	18.9	12.8-29.5	5.72	2.3	6	11.7	9.7-16.5	2.50	1.0	5.235*	2.83**
α3	6	17.6	9.8-17.6	5.01	2.1	6	27.8	22.5-31.3	4.17	1.7	1.444	3.83**
β1	6	13.0	11.6-14.6	1.20	0.5	6	11.6	8.1-14.7	2.72	1.1	5.138*	1.15
β2	6	14.7	6.3-19.6	4.75	1.9	6	16.5	12.4-19.5	2.58	1.1	3.380	0.82

1. see Section I, Table 3, page 11
2. standard deviation
3. standard error
4. *=P<.05; **=P<.01

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Table B4: Comparison of $B^{2'}_{4}$ LDH isozyme concentration in plasma from uninfected controls with moribund rainbow trout infected with IHN virus. Values are photometric optical density measurements from photograph negatives of stained starch-gel electrophoretic determinations.[1]

	Infected					Control					Statistic[4]	
Test	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	F	t
1	4	1.94	1.76-2.11	.197	0.10	4	2.51	2.31-2.66	.146	0.07	1.80	4.69**
2	9	.68	.57-.76	.063	.02	9	.85	.75-.91	.048	.02	1.74	6.52**

1. see Section I, Table 4, page 14
2. standard deviation
3. standard error
4. **=P<.01

APPENDIX B

Table B5: Acid-base balance in the plasma of uninfected controls and moribund rainbow trout infected with IHN virus.[1]

Clinical Test	Infected					Control					Statistic[5]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	F	t
HCO_3^-	15	6.14	2.93-8.65	1.58	0.41	20	10.54	5.20-13.07	2.13	0.48	1.808	6.854**
pH	15	7.594	7.433-7.754	0.0866	0.022	20	7.454	7.269-7.593	0.0837	0.019	1.071	4.828**
pCO_2 (mmHg)[4]	15	6.45	5.51-8.63	1.29	0.33	20	15.56	10.00-20.65	3.03	0.68	5.559**	11.43**
H_2CO_3 (mEq/L)[4]	15	0.20	0.14-0.26	0.0368	0.01	20	0.47	0.30-0.62	0.0916	0.02	6.000**	12.14**
HCO_3^-/H_2CO_3[4]	15	31.51	20.93-44.76	6.25	1.61	20	22.87	15.10-31.81	4.47	1.00	1.956	4.654**
Total CO_2[4]	15	6.33	4.27-8.88	1.61	0.42	20	11.01	5.50-13.60	2.18	0.49	1.837	7.134**

1. see Section I, Table 5, page 16
2. standard deviation
3. standard error
4. calculated values using the formula on page 21
5. ** = $P<.01$

APPENDIX B

Table B6: Linear regression analysis of weight change of uninfected controls and moribund rainbow trout infected with IHN virus.[1]

Nature of Variation	Control Fish			
	Degrees of Freedom	Sum of Squares	Mean Squares	F^2
Regression	1	.065450	.065450	112**
Deviations from linearity	4	.000499	.000125	0.213
Between days	5	.065949	.013190	22.5**
Between fish	29	6.6852	.23052	393**
Error	125	.0732	.000586	
Total	159	6.8243		

	Infected Fish			
Regression	1	.326082	.326082	31.7**
Deviations from linearity	3	.021998	.007333	.71
Between days	4	.348080	.087020	6.8**
Between fish	17	8.837259	.519839	51**
Error	53	.545233	.010287	
Total	74	9.730572		

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APPENDIX B

Table B6: Linear regression analysis of weight change of uninfected controls and moribund rainbow trout infected with IHN virus.[1] ----- Continued

Nature of Variation	Combined Control Fish and Infected Fish			
	Degrees of Freedom	Sum of Square	Mean Square	F^2
Between groups	1	.214428	.214428	223**
Regression	1	.003053	.003053	3.17
Parallelism	1	.388479	.388479	403**
Linearity	7	.041598	.005943	6.2**
Between days	10	.647558	.064756	67**
Between fish	47	15.73886	.334827	348**
Error	177	.170401	.000963	
Total	234	16.554846		

1. see Section I, Figure 4, page 18

2. ** = P<.01

APPENDIX B

Table B7: Comparison of successive samples of hemoglobin, red blood cell count, and packed cell volume from rainbow trout infected and not infected with IHN virus.[1]

	Control					Infected					Statistic[4]	
Parameter	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	t	F
Hemoglobin (g/100ml)												
Day 1	10	9.2	8.0-11.8	1.026	0.3	10	9.1	7.5-11.1	0.998	0.3	0.240	1.530
2	9	9.2	8.6-10.4	0.581	0.2	10	9.1	7.8-11.1	1.010	0.3	0.238	1.567
3	10	9.0	8.0-10.0	0.680	0.2	10	8.8	7.3-10.3	0.987	0.3	0.966	1.496
4	10	9.2	8.1-10.6	0.822	0.3	10	8.5	6.8-10.1	1.219	0.4	1.483	2.275
5	10	9.8	8.4-11.0	0.809	0.3	10	8.4	7.4-9.5	0.717	0.2	2.279*	1.267
6	10	9.4	8.0-10.7	0.918	0.3	10	8.0	6.8-9.6	0.859	0.3	3.133**	1.134
7	10	9.0	8.2-10.0	0.651	0.2	10	7.6	4.0-9.3	1.472	0.5	2.936**	3.329
8	10	8.6	7.4-9.5	0.775	0.3	10	7.9	4.0-9.8	2.057	0.7	1.813*	6.499*
9	10	9.0	7.6-10.4	0.899	0.3	10	7.6	4.0-10.8	2.163	0.7	2.136*	7.187*
9-day mean		9.2		0.807								

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APPENDIX B

Table B7: Comparison of successive samples of hemoglobin, red blood cell count, and packed cell volume from rainbow trout infected and not infected with IHN virus.[1]--Continued

	Control					Infected					Statistic[4]	
Parameter	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	t	F
Red blood cell count ($X10^6$)												
Day 1	10	1.252	1.17-1.42	0.089	0.03	10	1.271	0.92-1.43	0.173	0.06	0.377	1.200
2	8	1.465	1.27-1.64	0.138	0.05	10	1.403	1.23-1.70	0.158	0.05	1.411	1.000
3	10	1.204	0.92-1.54	0.195	0.06	10	1.230	1.03-1.56	0.164	0.05	0.946	1.080
4	10	1.282	0.70-1.51	0.247	0.08	10	1.094	0.81-1.39	0.214	0.07	2.368*	1.840
5	10	1.397	1.29-1.50	0.063	0.02	10	1.151	0.92-1.27	0.105	0.03	2.403*	2.273
6	10	1.384	1.12-1.60	0.164	0.05	10	1.143	0.92-1.31	0.114	0.04	2.492*	1.923
7	10	1.278	1.00-1.52	0.161	0.05	10	1.114	0.60-1.43	0.226	0.07	2.067*	2.040
8	10	1.190	1.00-1.34	0.114	0.04	10	1.083	0.40-1.49	0.362	0.11	1.695	5.240*
9	10	1.252	1.03-1.51	0.182	0.06	10	1.058	0.40-1.46	0.324	0.10	2.068*	4.200
9-day mean		1.300		0.158								

Continued next page

APPENDIX B

Table B7: Comparison of successive samples of hemoglobin, red blood cell count, and packed cell volume from rainbow trout infected and not infected with IHN virus.[1]----------Continued

Parameter	Control					Infected					Statistic[4]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	t	F
Packed Cell Volume (%)												
Day 1	--	----	-----	-----	---	--	----	-----	-----	---	-----	-----
2	10	51.0	45-61	5.011	1.6	10	43.9	38-50	4.654	1.5	0.932	1.288
3	10	46.2	41-52	3.853	1.2	10	42.7	37-49	3.889	1.2	1.547	1.845
4	10	48.4	37-58	6.381	2.0	10	45.2	37-55	6.286	2.0	0.327	1.416
5	10	51.0	46-57	4.028	1.3	10	41.2	31-48	5.432	1.7	1.929*	1.058
6	10	45.7	37-58	6.865	2.2	10	40.5	34-50	5.318	1.7	2.229*	1.014
7	10	44.6	37-51	4.326	1.4	9	38.0	7-46	11.948	4.0	1.841*	5.117*
8	10	39.9	30-46	5.021	1.6	10	37.4	12-53	13.100	4.1	1.915*	6.151*
9	9	42.7	35-50	5.937	2.0	10	39.1	11-50	14.640	4.6	1.402	7.682*
9-day Mean		46.1		5.282								

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APPENDIX B

Table B7: Comparison of successive samples of hemoglobin, red blood cell count, and packed cell volume from rainbow trout infected and not infected with IHN virus.[1]----------Continued

1. see Section II, Figure 1, page 39
2. standard deviation
3. standard error
4. *=P<.05; **=P<.01

APPENDIX B

Table B8: Differential blood comparison of rainbow trout infected and not infected with IHN virus.[1]

Parameter	Control					Infected					Statistic[4]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	t	F
Blood smear												
% Imm. RBC												
Day 1	10	9.2	3.4-11.7	2.692	0.9	10	8.2	2.6-13.2	3.064	1.0	0.240	1.136
Day 5	10	7.0	2.8-13.4	3.144	1.0	10	8.0	4.6-12.0	2.226	0.7	0.154	1.668
Day 9	10	6.9	3.0-11.2	2.767	0.9	10	10.3	4.0-23.0	5.847	1.9	0.848	4.136
3-day mean		7.7		2.875								
Leukocytes												
% Total												
Day 1	10	0.9	0-1.7	0.495	0.2	10	1.5	0-2.8	0.860	0.3	0.228	1.020
Day 5	10	2.0	0.4-3.6	0.942	0.3	10	1.9	0-5.5	2.015	0.6	0.284	5.377
Day 9	10	1.9	0.4-3.3	1.066	0.3	10	3.4	0.4-24.0	7.258	2.3	0.754	69.774*
3-day mean		1.6		0.869								

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APPENDIX B

Table B8: Differential blood comparison of rainbow trout infected and not infected with IHN virus.[1]------Continued

Parameter	Control					Infected					Statistic[4]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	t	F
Differential Leukocyte Count												
% Monocytes												
Day 1	10	3.3	0-8.0	2.544	0.8	10	2.3	0-10.0	3.057	1.0	0.995	1.710
Day 5	10	4.8	0-8.0	3.011	1.0	10	4.0	0-10.0	4.110	1.3	0.236	1.057
Day 9	10	5.8	0-16.0	5.692	1.8	10	6.2	0-14.0	5.029	1.6	0.525	1.583
3-day mean		4.6		3.997								
% Lymphocytes												
Day 1	10	91.2	84-98	4.934	1.6	10	89.8	70-100	9.601	3.0	0.196	2.054
Day 5	10	93.0	90-100	3.300	1.0	10	92.8	82-100	6.877	2.2	0.432	1.054
Day 9	10	88.4	64-96	9.969	3.2	10	91.8	70-100	9.114	2.9	0.177	1.851
3-day mean		90.9		6.699								

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APPENDIX B

Table B8: Differential blood comparison of rainbow trout infected and not infected with IHN virus.[1]-----Continued

Parameter	Control					Infected					Statistic[4]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	t	F
% Neutrophils												
Day 1	10	5.6	0-14.5	5.040	1.6	10	7.9	0-28.0	8.333	2.6	0.765	3.526
Day 5	10	2.2	0-6.0	2.394	0.8	10	3.2	0-10.0	3.553	1.1	0.507	1.560
Day 9	10	5.8	2.0-20.0	5.287	1.7	10	2.0	0-16.0	4.989	1.6	0.818	1.264
3-day mean		4.5		4.438								

1. see Section II, Table 1, page 42
2. standard deviation
3. standard error
4. *-P<.05; **=P<.01

APPENDIX B

Table B9: Comparisons of plasma bicarbonate (mEq/L) of rainbow trout infected and not infected with IHN virus.[1]

Days after Injection of IHN virus.	Control					Infected					Statistic[4]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	t	F
1	--	----	--------	----	---	10	10.2	8.3-15.1	2.14	0.7	0.142	1.335
2	10	9.7	5.1-11.4	1.79	0.6	10	9.7	6.6-2.5	1.6	0.5	0.279	2.307
3	10	10.4	3.4-14.6	3.41	1.1	10	8.7	5.5-10.7	1.89	0.6	1.095	1.717
4	10	10.3	7.0-13.0	2.24	0.7	10	5.5	2.9-10.3	2.24	0.7	3.496**	1.216
5	10	10.0	5.8-14.2	2.60	0.8	10	7.7	4.9-11.2	1.76	0.6	1.973*	1.965
6	10	9.7	8.0-13.4	1.94	0.6	10	7.3	5.3-11.2	2.12	0.7	2.159*	1.365
5-day mean		10.2		2.47								

1. see Section II, Figure 1, page 40
2. standard deviation
3. standard error
4. *=P<.05; **=P<.01

APPENDIX B

Table B10: Comparison of plasma chloride (mEq/L) of rainbow trout infected and not infected with IHN virus.[1]

Days after injection of IHN virus	Control					Infected					Statistic	
	n[2]	mean	range	sd[3]	se[4]	n	mean	range	sd	se	t	F
1	3	118.7	116-120	5.333	2.309	3	116	112-120	16	4.0	0.437	5.064
2	3	118.7	116-120	5.333	2.309	3	120	0	0	0	.119	-----
3	3	120.0	0	0	0	3	122.7	120-124	5.333	2.309	.741	1.778
4	3	118.7	116-120	5.333	2.309	3	122.7	120-124	5.333	2.309	.741	1.778
5	3	118.7	116-120	5.333	2.309	3	126.7	124-128	5.333	2.309	1.696	1.778
6	3	121.3	120-124	5.333	2.309	3	128.0	124-132	16	4	1.019	5.064
7	3	121.3	120-124	5.333	2.309	3	125.3	124-128	5.333	2.309	1.362	1.778
8	3	122.7	120-124	5.333	2.309	3	125.3	124-128	5.333	2.309	1.362	1.778
9	3	116	112-120	16	4.0	3	128	124-132	16	4	1.019	5.064
9-day mean		119.6		7.11								

1. see Section II, Figure 1, page 39
2. represents 3 10-fish pools
3. standard deviation
4. standard error

APPENDIX B

Table B11: Comparison of plasma inorganic phosphorus (mg/100ml) of rainbow trout infected and not infected with IHN virus.[1]

Days after injection of IHN virus	Control					Infected					Statistic[5]	
	n[2]	mean	range	sd[3]	se[4]	n	mean	range	sd	se	t	F
1	3	13.5	13.1-14.0	0.451	0.3	3	13.7	13.4-14.1	0.361	0.2	1.194	2.885
2	3	13.4	12.5-14.3	0.902	0.5	3	13.8	13.2-14.3	0.586	0.3	1.253	1.093
3	3	12.0	11.5-12.6	0.550	0.3	3	12.9	12.3-13.2	0.520	0.3	1.058	1.389
4	3	13.7	13.2-14.8	0.924	0.5	3	12.9	12.3-13.6	0.650	0.4	0.950	1.128
5	3	13.4	13.2-13.6	0.200	0.1	3	13.3	18.0-13.6	0.305	0.2	0.000	4.032
6	3	14.3	13.9-14.6	0.378	0.2	3	12.5	12.3-12.8	0.265	0.2	2.548*	5.357
7	3	13.3	12.9-13.8	0.458	0.3	3	13.7	13.4-13.9	0.288	0.2	1.254	4.518
8	3	13.1	12.5-13.6	0.557	0.3	3	13.2	12.9-13.6	0.378	0.2	0.295	2.622
9	3	13.1	12.3-13.6	0.700	0.4	3	13.9	12.9-15.0	1.050	0.6	1.047	2.941
9-day mean		13.3		0.612								

1. see Section II, Figure 2, page 40
2. represents 3 10-fish pools
3. standard deviation
4. standard error
5. *=P<.05

APPENDIX B

Table B12: Comparison of plasma calcium (mg/100ml) of rainbow trout infected and not infected with IHN virus.[1]

Days after injection of IHN virus	Control					Infected					Statistic[5]	
	n[2]	mean	range	sd[3]	se[4]	n	mean	range	sd	se	t	F
1	3	9.56	9.38-9.69	0.161	0.09	3	9.70	9.19-10.32	0.574	0.33	1.285	2.570
2	3	11.14	10.72-11.83	0.602	0.35	3	10.26	9.84-10.50	0.367	0.21	0.620	1.055
3	3	9.74	9.62-9.91	0.152	0.09	3	9.79	9.62-9.91	0.152	0.09	1.739	5.565
4	3	9.78	9.34-10.15	0.410	0.24	3	9.99	9.80-10.08	0.161	0.09	0.652	4.923
5	3	10.08	9.56-10.54	0.493	0.29	3	10.53	10.45-10.67	0.118	0.07	2.373*	9.143
6	3	10.07	9.86-10.32	0.232	0.13	3	10.00	9.93-10.13	0.110	0.06	0.625	10.667
7	3	10.06	9.62-10.56	0.473	0.27	3	10.26	9.97-10.61	0.322	0.19	0.661	1.231
8	3	9.81	9.71-9.89	0.095	0.06	3	10.24	10.13-10.32	0.095	0.06	0.743	14.222
9	3	10.74	10.54-10.94	0.200	0.12	3	10.13	9.84-10.54	0.363	0.21	0.083	1.031
9-day mean		10.11		0.358								

1. see Section II, Figure 2, page 40
2. represents 3 10-fish pools
3. standard deviation
4. standard error
5. *=P<.05

APPENDIX B

Table B13: Comparison of plasma total protein (g/100ml) of rainbow trout infected and not infected with IHN virus.[1]

Days after injection of IHN virus	Control					Infected					Statistic[5]	
	n[2]	mean	range	sd[3]	se[4]	n	mean	range	sd	se	t	F
1	3	5.81	5.45-6.21	0.382	0.22	3	5.51	5.15-5.64	0.313	0.18	.207	1.400
2	3	6.40	6.18-6.67	.249	.14	3	5.28	5.12-5.44	0.161	0.09	1.849*	2.692
3	3	5.51	5.32-5.61	.167	.10	3	5.27	4.93-5.56	0.318	0.18	1.436	1.443
4	3	5.41	5.29-5.64	.202	.12	3	5.59	5.56-5.64	.045	.03	0.315	35.000
5	3	5.35	5.15-5.44	.179	.10	3	5.43	5.32-5.53	0.105	0.06	0.889	6.364
6	3	5.39	4.99-5.73	.373	.22	3	5.54	4.99-6.06	0.536	0.31	.035	4.100
7	3	5.18	5.05-5.25	.118	.07	3	5.55	5.53-5.56	.032	.02	.000	70.000*
8	3	5.20	4.93-544	.257	.15	3	5.26	4.90-5.53	.326	.19	1.465	1.514
9	3	5.66	5.29-5.88	.324	.19	3	4.89	4.70-5.06	.182	.11	4.342**	2.121
9-day mean		5.55		0.265								

1. see Section II, Figure 2, page 40
2. represents 3 10-fish pools
3. standard deviation
4. standard error
5. *=P<.05; **=P<.01

Table B14: Comparison of plasma acid-base balance of rainbow trout infected and not infected with IHN virus at 10 and 18° C.[1]

Clinical Test	IHN Infected					Control					Statistic[4]	
	n	mean	range	sd[2]	se[3]	n	mean	range	sd	se	F	t
at 10° C												
pH	13	7.599	7.342-7.862	0.135	0.038	11	7.508	7.342-7.692	0.100	0.030	1.300	1.872*
HCO_3^-	14	11.0	8.2-14.0	1.81	0.5	13	11.4	10.1-13.8	1.10	0.3	2.717*	0.699
pCO_2	12	12.69	8.12-26.05	4.78	1.38	10	15.87	10.92-20.05	2.55	0.81	3.523*	1.989*
H_2CO_3	12	0.381	.244-.782	0.144	0.041	10	0.476	.328-.602	0.0762	0.024	3.552*	1.983*
HCO_3^-/ H_2CO_3	12	30.87	17.45-40.44	6.95	2.01	10	25.33	17.46-39.07	5.80	1.83	1.435	2.022*
Total CO_2	12	11.40	8.67-14.58	2.02	0.58	10	12.18	11.10-14.30	1.06	0.33	3.642*	1.162
at 18° C												
pH	14	7.485	7.234-7.658	0.0860	0.023	6	7.454	7.403-7.496	0.0316	0.013	7.400**	1.170
HCO_3^- (mEq/L)	14	11.2	9.4-17.9	2.06	0.5	6	11.7	9.5-13.0	1.24	0.5	2.767	0.590
pCO_2 (mmHg)	14	15.58	8.67-20.95	3.14	0.84	6	17.30	13.51-19.58	2.29	0.94	1.877	1.282
H_2CO_3 (mEq/L)	14	0.468	.260-.629	.094	.025	6	.519	.405-.587	.0686	.028	1.894	1.275
HCO_3^-/ H_2CO_3	14	24.69	15.26-36.15	4.81	1.28	6	22.66	21.85-24.90	1.61	0.66	8.926**	1.407
Total CO_2 (mEq/L)	14	11.71	9.66-18.52	2.10	0.56	6	12.22	9.91-13.57	1.29	.53	2.655	0.599

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APPENDIX B

Table B14: Comparison of plasma acid-base balance of rainbow trout infected and not infected with IHN virus at 10 and 18° C.[1] ----- Continued

1. see Section II, Table 2, page 44
2. standard deviation
3. standard error
4. *=P<.05, **=P<.01

APPENDIX B

Table B15: Two-way analysis of variance for blood parameters from rainbow trout uninfected and infected with IHN virus. (see Section II)

Parameter	Source	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio	Significance[1]
Hemoglobin	between days	3,517	8	440	(8,8)0.853	NS
	between groups	4,045	1	4,045	(1,162)17.9	**
	interaction	4,129	8	516	(8,162)2.28	**
	within groups	36,571	162	226		
Packed cell Volume	between days	359,000	8	44,875	(8,8)3.75	*
	between groups	190,096	1	190,096	(1,162)18.2	**
	interaction	95,780	8	11,972	(8,162)1.14	NS
	within groups	1,699,344	162	10,489		
Corpuscular Count	between days	860,144	8	107,518	(8,8)0.474	NS
	between groups	746,496	1	746,496	(1,162)7.148	**
	interaction	1,812,384	8	226,548	(8,162)2.17	*
	within groups	16,918,812	162	104,437		

Continued next page

APPENDIX B

Table B15: Two-way analysis of variance for blood parameters from rainbow trout uninfected and infected with IHN virus. (see Section II)-----Continued

Parameter	Source	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio	Significance[1]
Immature RBC	between days	1,034	2	517	(2,2)0.36	NS
	between groups	1,082	1	1,082	(1,54)0.013	NS
	interaction	2,864	2	1,432	(2,54)0.017	NS
	within groups	4,506,528	54	83,454		
% Total Leukocytes	between days	1,246	2	623	(2,2)3.18	NS
	between groups	376	1	376	(1,54)0.633	NS
	interaction	391	2	196	(2,54)0.329	NS
	within groups	32,086	54	594		
Monocytes	between days	6,144	2	3,072	(2,2)17.9	NS
	between groups	196	1	196	(1,54)0.196	NS
	interaction	344	2	172	(2,54)0.172	NS
	within groups	53,880	54	988		

Continued next page

APPENDIX B

Table B15: Two-way analysis of variance for blood parameters from rainbow trout uninfected and infected with IHN virus. (see Section II) ---------- Continued

Parameter	Source	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio	Significance[1]
Lymphocytes	between days	5,504	2	2,752	(2,2)1.47	NS
	between groups	324	1	324	(1,54)0.089	NS
	interaction	3,744	2	1,872	(2,54)0.513	NS
	within groups	197,208	54	3,652		
Neutrophils	between days	10,386	2	5,193	(2,2)1.68	NS
	between groups	25	1	25	(1,54)0.015	NS
	interaction	6,194	2	3,097	(2,54)1.86	NS
	within groups	89,706	54	1,661		
Bicarbonate	between days	4,395	4	1,099	(4,4)0.74	NS
	between groups	12,254	1	12,254	(1,90)24.9	**
	interaction	5,885	4	1,471	(4,90)2.98	*
	within groups	44,410	90	493		

Continued next page

APPENDIX B

Table B15: Two-way analysis of variance for blood parameters from rainbow trout uninfected and infected with IHN virus. (see Section II)------Continued

Parameter	Source	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio	Significance[1]
Chloride	between days	14,720	8	1,840	(8,8)1.28	NS
	between groups	14,400	1	14,400	(1,36)37.5	**
	interaction	11,520	8	1,440	(8,36)3.75	**
	within groups	13,824	36	384		
Calcium	between days	299	8	37.4	(8,8)2.34	NS
	between groups	.001	1	0.001	(1,36)0.00	NS
	interaction	128	8	16.0	(8,36)2.11	NS
	within groups	275	36	7.6		
Phosphorus	between days	339	8	42	(8,8)0.71	NS
	between groups	.001	1	0.001	(1,36)0.00	NS
	interaction	476	8	59	(8,36)3.28	**
	within groups	651	36	18		

Continued next page

APPENDIX B

Table B15: Two-way analysis of variance for blood parameters from rainbow trout uninfected and infected with IHN virus. (see Section II)----Continued

Parameter	Source	Sum of Squares	Degrees of Freedom	Mean Square	F Ratio	Significance[1]
Total Protein	between days	94.3	8	11.8	(8,8)0.605	NS
	between groups	22,8	1	22.8	(1,36)5.89	**
	interaction	155.7	8	19.5	(8,36)5.04	**
	within groups	139.4	36	3.87		

1. NS = not significant

* = significant at 5% level

** = significant at 1% level

APPENDIX C

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