INFORMATION TO USERS

ROLE OF IRON IN ATLANTIC SALMON (*SALMO SALAR*) NUTRITION: REQUIREMENT, BIOAVAILABILITY, DISEASE RESISTANCE AND IMMUNE RESPONSE

by

M. Niamul Naser

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
September 2000

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of

Graduate Studies for acceptance a thesis entitled "Role of Iron in Atlantic Salmon (*Salmo*

salar) Nutrition: Requirement, Bioavailability, Disease Resistance and Immune Response"

_____

by   Niamul Naser

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: _____ September 6, 2000 _____

External Examiner

Research Supervisor

Examining Committee
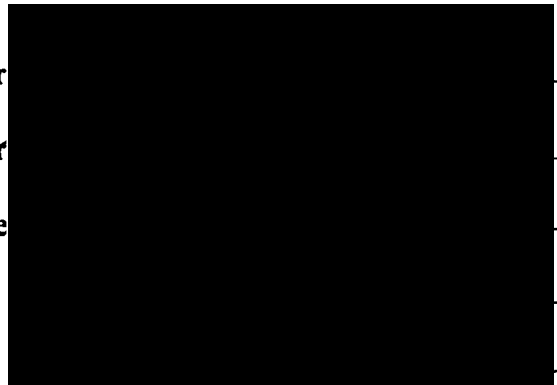
DALHOUSIE UNIVERSITY

DATE: September 6, 2000

AUTHOR: M. Niamul Naser

TITLE: Role of iron in Atlantic salmon (*Salmo salar*) nutrition: requirement,

bioavailability, disease resistance and immune response.

DEPARTMENT OR SCHOOL: Biology

DEGREE: Ph.D.   CONVOCATION: Fall   YEAR: 2000

"And we brought forth iron wherein is mighty power, as well as many benefits for mankind...." (Al Qur'an, 57:25)

DEDICATION

To my wife, Jinat Shumi,
And our children, Raheel Naser and Eruban Naser;
Who shared all of the good times during my graduate
studies.

To my parents, Mr. Nasaruddin and Mrs. Marjina Jahan,
And my sisters, Dr. Sirat Nasir and Mrs. Seephat Naser;
Who have always encouraged me!!

To my father-in-law, late M. Mozammel Hoque Ph.D.,
Who dreamed of a higher education for me.

To the people of my country Bangladesh,
Villagers of 'Panchgachi (Kurigram)', from where my
forefathers came, who inspired me to return to my birthplace.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abstract

The role of iron in the nutrition of Atlantic salmon (*Salmo salar*) was investigated in this study because of recent information on its clinical role in aquatic and terrestrial animals, detrimental effects of iron on nutrient stability in feeds, discharge of nutrients in aquaculture effluents and the increase in the prevalence of infectious diseases. Six experiments were designed to investigate the quantitative iron requirement of Atlantic salmon, its biological availability from potential inorganic and organic feed supplements and to examine the role of this element in immune function and disease resistance. The estimated quantitative dietary iron requirement of salmon based on broken line analysis of tissue iron concentration data was 60 mg iron/ kg of diet. Atlantic salmon fed a low iron semi-purified test diet developed hypochromic microcytic anemia. Fish growth and survival during the feeding trial were similar among six dietary treatments (0,15, 30, 60 and 120 mg iron / kg) and no other overt gross deficiency signs were observed. Results show that serum ferritin levels could be used as an indicator of the physiological iron status of Atlantic salmon. The biological availability of iron from various inorganic feed supplements (ferric chloride, ferric oxide) and animal protein sources (fish meal, blood meal) was measured by the hemoglobin regeneration assay. The relative bioavailability of iron from ferric chloride, ferric oxide, blood meal and herring meal was 98.9, 17.8, 52.3 and 47.1 %, respectively. Vitamin C (L-ascorbyl-2-polyphosphate) supplementation (300 mg/kg) of a fish meal based diet had no significant effect on iron absorption and deposition in groups receiving either no inorganic iron supplement or diet containing iron from an organic source (blood meal).

Furunculosis and vibriosis diseases experimentally induced in Atlantic salmon by *Aeromonas salmonida* and *Vibrio anguillarum* infections caused mobilization of iron from liver and spleen into plasma. Plasma iron concentrations were significantly higher in fish infected with *V. anguillarum* than *A. salmonicida*. Feeding fish during the *V. anguillarum* infection caused higher mortality than those fish receiving no food. An extensive experiment was conducted to determine the effects of heme (blood meal) and non heme ($FeSO_4.7H_2O$) iron on immune response, resistance to bacterial diseases (*Aeromonas salmonicida* and *Vibrio anguillarum*) and tissue iron accumulation. The following immune responses were investigated: bactericidal activity of serum, differential blood cell counts, serum lysozyme activity, phagocytosis by macrophages, myloperoxidase assays and respiratory burst activity of phagocytes and lymphocyte proliferation. No significant disease resistance against virulent *V. anguillarum* and *A. salmonicida* was observed among Atlantic salmon fed diets containing different levels of non-heme (0,100, 300, 600 or 1200 mg /kg) and heme (0,100 or 300 mg/kg) iron. Although, variability in several immune responses was observed in fish fed diet with varying concentrations and forms of dietary iron, the responses were not consistent. Excessive amounts of iron in salmonid feeds should be avoided because of its potential detrimental effects on nutrient stability and its influence on plasma iron concentration which may promote growth of bacterial pathogens.

LIST OF ABBREVIATIONS

AA- Ascorbic acid (Vitamin C)
ANOVA- Analysis of varience
AAS- Atomic Absorption Spectrophotometer
BHI- Brain heart infusion
BKD -*Renibacterium salmoninarum*
BM- Blood meal
C – Control
Cfu – Colony formation units
Con A- Conchanavalin A
CMI- Cell mediated immunity
CRP- C- reactive protein
DFO- Department of Fisheries and Oceans
ELISA- Enzyme linked immunosorbent assay
FBS- Foetal bovine serum
Fe – Iron
FE- Feed efficiency
g - Gram
H – Heme (organic)iron diet
Hb- Haemoglobin
Hct -Haematocrit
HBSS – Hank's balanced salt solution
HEPES-
IgM- Immunoglobulin$_M$
IFN - Interferon
IMB – Institute of Marine Biosciences
IP – Intraperitoneal
IU – International units
Kg - Kilograms
L - Litre
LD_{50}- Lethal dose at 50%
LPS – Lipopolysaccharide
MCH- Mean cell hemoglobin
MCHC- Mean cell hemoglobin concentration
MCV- Mean cell volume
MEM- Minimal essential media
Mg – Milligram
Mo - Macrophage
MPO- Myeloperoxidase
MS222- Tricane methanesulfonate
N- Natural killer cell
NaCl- Sodium chloride salt
NB- New Brunswick

NBT- Nitro blue tetrazolium
NCC- Non-specific cytotoxic cells
NCCLS- National committee for clinical laboratory standards
NH – Non heme (inorganic) iron diet
NK- Natural killer cells
No Fe – Non- iron
NRC – National Research Council of Canada
NS- Nova Scotia
OD- Optical density
PBS- Phosphate buffered saline
PEC- Peritoneal exuded cell
PHA – Phytohemagglutinin
PS- Peptone saline
RBC- Red blood cell
SI- Stimulation index
TBS- Tris buffered saline
TIBC- Total iron binding capacity
TSA- Tryptone soy agar
TSB- Tryptone soy broth
W- Weight
WK - Week

ACKNOWLEDGEMENTS

General Introduction

The world population is increasing at an alarming rate and is expected to reach seven billion by the year 2010. Aquaculture is becoming an important source of protein for feeding this increasing population. In 1995, according to statistics of the Food and Agricultural Organization (FAO), worldwide production from aquaculture alone was about 21.3 million tons, and this was 19% of total fish production (FAO, 1997). At the same time, the FAO suggested that fish production in the "capture" fisheries has reached its maximum level viz., about one hundred million tons annually. In 1995, the per capita global consumption of fish products was about 14 kg (FAO, 1997). It is assumed that world population growth will continue at the present rate, and productivity in the capture fisheries cannot increase, then aquaculture production needs to be doubled to 42 million tons per annum to maintain the current per capita consumption. Various attempts have been made by FAO in the last two decades to increase aquaculture production and popularise aquatic species of finfish and shellfish for human consumption worldwide. The practice of aquaculture is gaining acceptance as an effective means of production of high quality food for human consumption. At present, aquaculture products are in great demand and they are cultured throughout the world. Atlantic salmon has been an attractive candidate for aquaculture in Western Europe and North America for many years. Norway, which began salmon aquaculture in the 1970s, is now the major Atlantic salmon producer in the world. Canada, Chile, Scotland and Ireland also supply

1

Atlantic salmon from their expanding aquaculture industries. In 1978, marine

Atlantic salmon production began in the southwest Bay of Fundy in New

Brunswick and by 1998 sales of the salmon aquaculture industry of New

Brunswick reached C$107 million.

One of the major cost items in aquaculture is feed. The nutrients required

by fish for growth, reproduction, and other normal physiological functions are

similar to those required by land animals (Halver, 1996). Fish nutritionists are

attempting to establish the requirements for approximately 40 specific nutrients,

and they have found the higher requirements for certain nutrients at various

stages of development (NRC, 1993). The major nutrients necessary for growth

and development of fish include protein and amino acids, lipids and essential

fatty acids, carbohydrates, vitamins and minerals. These nutrients can come

either from natural aquatic organisms or from prepared diets, but when fish are

cultured in confinement (in cages) where accessibility to natural foods is

restricted, their diet must be nutritionally complete. Besides nutrition, fish health

is also a major concern in aquaculture. Most of the diseases of poultry and

livestock are transmitted by air, so it is possible to maintain a strict quarantine.

However, infection is a greater problem with aquaculture because fish are

susceptible to water-borne pathogens. The types and severities of infection are

also affected by water quality, fish handling stress, poor feed quality, uneaten food

and faecal material. Some biological constraints in aquaculture include bacterial

disease outbreaks, parasites, and predators like birds, seals etc. It is a common practice to vaccinate fish against bacterial diseases, but vaccination of fish is time consuming for fish farmers. Chemicals can be used to prevent disease and parasites threatening farmed salmon, but concerns are increasing about the safety of the environment and non-target species during the chemical treatment (Saunders, 1995). The significance of a proper diet in preserving the health of fish is widely recognized (Lall and Olivier, 1993). Nutritional status is considered one of the important components that determine the ability of the animal to resist diseases in nature or under cultured conditions.

All aquatic organisms require inorganic elements for their normal life processes. Of the 90 naturally occurring elements, 29 are known to be essential for animal life. Most living matter contains approximately 95% water. The dry weight consists mostly of carbon, hydrogen, nitrogen and oxygen, distributed in various organic compounds (protein, lipid and carbohydrate) (Vinogradov, 1953). The remaining fraction constitutes the ash or total mineral component of the body. The inorganic elements distributed in the body are broadly classified into two groups namely, macro and trace elements. The macro-elements, which include the structural elements (calcium (Ca), phosphorus (P) and magnesium (Mg)) and electrolytes (sodium (Na), potassium (K) and chloride (Cl) and sulfur (S)), occur in concentrations ranging from 0.1% to 2% of fish weight and their dietary requirement is generally higher than 100 mg/kg dry diet. The trace elements,

include iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), cobalt (Co),

molybdenum (Mo), chromium (Cr), selenium (Se), fluorine (F), iodine (I), nickel

(Ni), lithium (Li), silicon (Si), vanadium (V) and silver (Ag), they occur at much

lower concentrations (mg or μg per kg) and their dietary requirements are below

100 mg/kg of diet. Fish can absorb some trace minerals not only from their diets

but also from their external aquatic environment (Lall, 1989). The main functions

of inorganic elements include the formation of skeletal structure, electron transfer,

regulation of acid-base equilibrium, and osmoregulation. Inorganic elements are

also important components of hormones and enzymes, and they activate

enzymes. Several complex biochemical mechanisms control and regulate the

uptake, storage, and excretion of various inorganic elements. The electrolytes

Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, and HCO_3^- are essential in the osmotic and ionic

regulation of extra- and intracellular fluids in fish.

Iron has long been acknowledged as an essential trace element, but its

metabolic uses have only been understood recently mainly from studies on

humans and higher animals. It is now known that iron is involved in regulatory

mechanisms controlling blood formation and respiration. Also, iron has other

important regulatory effects associated with hormone synthesis and fatty acid

metabolism (Brody, 1994). Some iron enzymes, such as myeloperoxidase and

rinbonucleotide reductase are used in the immune system. Dietary iron is

important for expanding blood volume, avoiding anemia and providing a reserve

of iron in fish. An in depth understanding of how an essential nutrient like iron acts in both normal and disease states is of importance. Such information should offer an opportunity whereby specific nutritional manipulation of a dietary component may be used in a rational way to prevent and/or treat disease states. Iron exists as part of a number of distinct molecules within the blood and tissues; each molecule has a particular structure and function. Myoglobin, found in muscle is structurally similar to hemoglobin, but the protein is monomeric. Functionally, myoglobin plays an intermediary role between hemoglobin and tissue hemins (Bernat, 1983). Iron occurs in the blood as hemoglobin in the erythrocytes, but in small quantities in transferrin and ferritin in serum. Transferrin serves as the principal carrier of iron in blood serum and therefore plays an important role in iron metabolism. Ferritin and hemosiderin, the iron storage proteins, occur widely in liver and spleen. Although they differ chemically, they are intimately related by their similar functions. There is relatively little information available on the absorption and metabolism of iron in fish and other aquatic organisms. The mechanisms by which iron is absorbed from the digestive tract, stored and excreted, appear to be similar to those in other vertebrates (Lall, 1989). The intestinal mucosa is the major site of iron absorption. Thus, food is a major source of iron. Some absorption takes place across gill membranes (Walker and Fromm, 1976). Hence, the addition of ferrous sulphate to water was trend to thus enhance growth and the hemoglobin level in *Xiphophorus helleri* , a warm water fish

(Roeder and Roeder, 1966). Aisen et al. (1972) found a protein in hagfish that closely resembles human transferrin. In rainbow trout, iron is absorbed from the peritoneal cavity and stored in greatest concentrations in liver, spleen, and head kidney (Walker and Fromm, 1976).

Iron is an essential nutrient for microorganisms, and their ability to infect a host depends on its availability. A low concentration of free iron in mucus membranes and in other tissues is one of the first lines of host defence against bacterial infection. Microorganisms have several systems for acquiring iron from the host. The most efficient of these involves the synthesis of low molecular weight, high affinity chelators, called siderophores. Siderophores are secreted in response to iron deprivation, and once complexed with iron they are transported back into the cell. Certain iron-binding proteins (e.g. transferrin) withhold iron from microbes, while at the same time keeping it available to host cells. Iron is also important in maintaining the structural integrity of epithelium and thus helps to prevent access by pathogens to the host. Iron deficiency causes microcytic anemia in several fish species (Lall, 1989), but relatively little is known about its effect on immune response. Information from studies on terrestrial species supports two different views. One suggests that iron deficiency predisposes animals to infection (Humbert and Moore, 1983); thus iron supplementation might be expected to increase disease resistance. The second view implies that iron deficiency protects against infection by limiting the amount of iron available

to bacteria for multiplication. Consequently iron supplementation in this case

would increase susceptibility of the host infection (Weinberg, 1984). Hence iron

deficiency can reduce both host resistance and replication of an infectious agent,

increasing the dose of iron could exacerbate the infection before the host has an

opportunity to recover. However, iron deficiency is not a desirable means of

controlling infection because the deficiency makes the host more susceptible to

other infectious agents (Scrimshaw, 1990). Further, Paterson et al. (1985)

observed that if the supply of iron to the host is high, invading bacteria can

obtain iron for growth, and thus in turn can produce an active infection in Atlantic

salmon. Commercially available salmonid diets contain 150-800 mg iron/kg of

diet (Olivier, 1997). Whether higher levels of iron originating from blood meal or

fishmeal in commercial diets predispose salmonids to common bacterial

pathogens remains to be determined. Olivier (1997) suggested that the role of

dietary iron and its relationship to the immune system of fish should be explored

further. Therefore, this is one of the main goals of this thesis.

Objectives

The main objectives of the present study were as follows:

1. Determine the dietary iron requirement of Atlantic salmon for growth and development.

2. Determine the biological availability of iron from heme and non-heme iron sources in feed.

3. Determine the effect of deficiency and excess of dietary iron on haematology, tissue and serum iron levels and growth.

4. Determine the effect of vitamin C on the availability of iron and other minerals from practical diets.

5. Determine the effect of deficiency and excess of dietary iron on the immune response(s) and disease resistance of Atlantic salmon.

6. Determine the effect of bacterial infection on tissue iron concentrations in Atlantic salmon.

Accordingly, the following five experiments were conducted to achieve the foregoing objectives:

Experiment 1: To determine the quantitative iron requirement of Atlantic salmon fingerlings were fed semipurified diets containing graded levels of iron i.e., 0, 15, 30, 60, 120 or 240 mg/kg of diet in the form of $FeSO_4.7\ H_2O$ for a period of 20 weeks. Iron deficiency was characterised using hematological responses and tissue iron concentration results. The dietary iron requirement was determined on the basis of the following criteria: growth, feed efficiency, mortality, haematology and tissue iron concentrations.

Experiment 2: The bioavailability of iron from inorganic and organic sources to Atlantic salmon was assessed using the haemoglobin regeneration assay that was developed previously by Fritz et al. (1970, 1975) to test the bioavailability of iron in feed supplements for experimental animals. In this regards a purified test diet low in iron (14.6 mg Fe/kg diet) was fed to experimental fish for a period of 22 weeks. The biological availability of iron from ferrous sulfate, ferric chloride, ferric oxide, blood meal and herring meal was measured.

Experiment 3: This experiment was designed to determine the effects of an excess and a deficiency of iron on growth, tissue iron concentrations, immunity, and disease resistance in Atlantic salmon. A semipurified basal diet was supplemented with 0, 100, 300, 600 or 1200 mg of iron/kg in the form of ferrous sulfate and 0, 100 or 300 mg of iron/kg as hemoglobin iron (originated from spray-

dried haemoglobin). The fish were fed their prescribed diets for 20 to 32 weeks, then they were sampled to determine the effect of iron supplementation on tissue iron concentrations. The fish were further challenged with bacterial pathogens, namely, *Vibrio anguillarum* and *Aeromonas salmonicida* to determine if there were any differences in fish mortality due to dissimiler iron status. The LD_{50} challenge was performed to calculate the virulence of the bacteria when the fish were fed the diets with different levels and forms of iron. The tissue and serum iron contents of healthy, infected, and morbid fish were further investigated to determine any variations among the samples of challenged fish. The effect of dietary iron on the following immune parameters was investigated: serum bactericidal activity, lymphocyte proliferation, macrophage phagocytosis, lysozyme assay, respiratory burst, and differential leukocyte counts. These assays were undertaken to measure the non-specific immune responses of the fish to vibriosis (*Vibrio anguillarum*) and furunculosis (*Aeromonas salmonicida*).

Experiment 4: This experiment was conducted to follow changes in tissue mineral concentrations in fed and sterved fish after infection. Fish were fed a practical diet for 18 weeks and then they were infected with either *A. salmonicida* or *V. anguillarum*. One group of infected fish in each case was fed whereas the other was left unfed during the experimental period. Fish tissues were sampled at day 0, 2 and 4 and mineral compositions were determined. The data were

compiled to determine whether nutritional status influenced the tissue mineral compositions at the different sample times in Atlantic salmon.

Experiment 5: This experiment was conducted to determine whether there are any interactions between heme and nonheme iron with vitamin C in practical diets for Atlantic salmon. In this regard four diets containing 100 mg of supplemented iron from either blood meal (spray dried haemoglobin; heme source) or ferrous sulfate (nonheme source) with or without supplemental ascorbic acid (Stay-C) were tested. The experimental fish were reared in sea water for 18 weeks and then they were sampled to determine the deposition of vitamin C and iron as well as copper, zinc and manganese in the hepatic tissues in relation to diet treatment. Several blood parameters, including hemoglobin content, hematocrit, red cell abundance, mean cell volume and mean cell hematocrit content were measured along with concentrations of serum iron, transferrin saturation, and ferritin.

CHAPTER 1. Literature Review

Iron is the fourth most abundant element in the earth's crust and, after aluminum, the second most abundant metal. Since most living systems require iron for their growth and development, organisms have had to devise strategies to solublize and acquire this essential trace metal. Mineral regulation in animals involves many interrelated factors, including the systematic relationships leading to absorption of iron through the gills, skin, and gastrointestinal tract, with metabolism by liver, spleen, and kidney in aquatic animals. These relationships change with the homeostasis of the particular elements. The need for an optimum level of iron in preserving the health of fish is widely recognized. The nutritional status of iron is considered to be one of the most important factors determining the ability of animals to resist diseases. Egyptians recognized the role of iron in human health and disease 3500 years ago (Moore and Dubach 1962). Romans, East Indians, and Greeks used it for therapeutic purposes; Hippocrates, for example, used iron for treatment of dermatitis, hemorrhoids, wounds, gout, diarrhea, vomiting, weakness, edema, fever and cystitis (Loosli, 1978; Conard et al., 1980). However, Monarde in the sixteenth century was the first to relate Fe and blood.

1.1 Iron in animals and fish

Iron is present in all cells of the body and plays a key role in nutrition and metabolism. It is not available in the animal body in a free form, rather as a complex with protein molecules, and its biochemical functions depend on the

protein to which its it is attached. With respect to this, iron is found either as heme protein compounds (e.g. hemoglobin, myoglobin, cytochromes) or as a nonheme protein compounds (e.g. transferrin, ferritin, and hemosiderin), where the iron is usually bound tightly to sulfur atoms of cysteine residues.

A healthy adult 70-kg man can contain 4 to 5 g of iron (Bothwell and Charlton, 1979). Of this, approximately two-thirds is utilized as the functional iron of hemoglobin (60%), myoglobin (5%), and various heme and non-heme enzymes (5%) (reviewed by Carpenter and Mahoney, 1992). The remaining iron is stored in the body as ferritin (20%) and hemosiderin (10%). Only minor amounts of iron (<0.1%) are found as a transit chelate with transferrin. There is not much information available on the distribution of iron in fish. Cross et al. (1973) reported that the iron concentration in the white muscle of a small fish, the Bluefish (Pomatomus saltatrix) was about 8.23 µg iron /g wet wt., but in a Bathyl-Demersal fish (Antomora rostrata), it was 4.3 µg iron /g wet wt. They noted that the tissue iron concentrations of large fish were only half those found in small fish. Bjornevik and Maage (1993) observed that the iron concentration of Atlantic salmon (Salmo salar) parr (mean wt 31.3 g) was 16.3 mg/kg wet wt.

Hemoglobin (Hb) is the major protein of the red blood cells. It is a conjugated form of the pigment heme and the protein globin (Frausto da Silva and Williams, 1991). The heme complex consists of iron, four pyrrole groups

and hydrocarbon chains. Four heme molecules combine with one globin each to form Hb, which has a molecular weight of approximtely 64,500 (Brody, 1994). Two of these Hb subunits are designated as α and β-globulin.

Myoglobin, with a molecular weight of 16,900 , is a monomeric protein containing one iron atom per molecule. It is present in muscle and has higher affinity for O_2 at low oxygen tensions than does Hb. Consequently, the formation of oxymyoglobin is higher, and its formation occurs 2.5 to 5 times faster than that of oxyhemoglobin. This makes it useful for short-term storage of oxygen (Brody, 1994) and also facilitates the transfer of O_2 from oxyhemoglobin to the sites of oxidation in muscle cells (Fruton and Simmonds, 1958). Since myglobin tends to release O_2 at low oxygen tensions, it is useful in diving mammals, such as dolphins and seals, where it makes up 3 to 8% of the muscle protein. Physiologically, myoglobin obtains the O_2 from hemoglobin, but activation of the O_2 takes place in the cell by an oxyreduction process (Bernat, 1983).

The cytochromes occur in the mitochondria as well as in the endoplasmic reticulum. The former cytochromes (e.g. cytochrome c) are used in respiratory chains. The latter cytochromes include cytochrome P450 and cytochrome b5. Cytochrome c oxidase, one of the most important enzymes of the body, consists of a dozen proteins that include two cytochromes (a and a_3) and two copper metalloproteins. Cytochromes, along with some nonheme iron enzymes and

coenzymes participate in the respiratory cycle. NADH dehydrogenase, a

nonheme protein bound to the mitochondrial membrane, mediates the transfer of

an electron to coenzyme Q reductase a nonheme protein. Coenzyme Q

reductase receives electrons indirectly through succinate dehydrogenase, a

nonheme ezyme of the Kreb cycle. These electrons are transferred to

cytochrome c via cytochrome b. Finally, cytchrome c oxidase catalyzes the

transfer of electrons from cytochrome c to oxygen, forming water (Brody, 1994).

Cytochromes are also physiologically active in conversion of cholesterol to

steroid hormones. They also play an important role in lipid metabolism inside the

endoplasmic reticulum by desaturation of unsaturated fatty acids.

The heme proteins also include two important enzymes, catalase and

peroxidase. These two enzymes are used to remove toxic oxygen products like

hydrogen peroxide (HOOH) and organic peroxidases (R-OOH). Heme enzymes

like endoperoxide synthase and myeloperoxidase are important components in

the peroxidation of the fatty acids and in the immune system, respectively.

The nonheme-iron containing proteins ferritin and hemosiderin mainly act

in iron storage. The ferritin concentration in tissues, together with that of

hemosiderin, reflects the iron status of an animal (McDowell, 1992). Ferritin

without iron is known as apoferritin. In the presence of a positive iron balance, it

produces ferritin. Walters et al.(1973) showed a positive correlation between

human serum ferritin concentration and body iron stores. Ferritin is available

throughout the body and has a high concentration in the liver. Hemosiderin is relatively amorphous and in most animals is dominant in tissues. Ferritin is present at lower levels (McDowell, 1992), and contains ferric iron. At the onset of a biological reaction, iron enters apoferritin in the ferrous form and then it is oxidized within the interior of the protein to the ferric form, ferritin. When there is a shortage of iron in the body, the stored iron in ferritin is again reduced to the ferrous form. Hemosiderin, on the other hand, stores iron when there is an iron overload in the body. This protein is present in lysosomes and is believed to replace the partially degraded form of ferritin (Broody 1993). Miguel et al. (1991) isolated ferritin from rainbow trout (*Salmo gairdneri*). The molecular masses of ferritin and apoferritin are around 430 kDa. When saturated with iron the molecular mass becomes 900 kDa (Munro and Linder 1978). Miguel et al. (1991) also observed that the rate of release of iron increased with decreasing pH.

The nonheme transferrin also known as β-globulin is an iron transport protein, that is able to bind with two atoms of iron. Ferrous iron, after absorption, enters the blood serum and is oxidized to the ferric state. Ferric iron complexes with transferrin and is then transported to various sites in the body (McDowell, 1992). Transferrin also releases iron to the sites of storage like hepatic tissue, and also to the spleen or bone marrow (in mammals) for hemoglobin synthesis.

Utilization of transferrin depends upon a transferrin receptor. Thus the levels of transferrin receptor and ferritin are organized in a coordinated fashion. When a cell requires iron, an increase in transferrin receptor in the membranes of cells in the serum promotes iron uptake. Coinciding with this event, ferritin synthesis decreases, making iron available to the transferrin. The molecular weight of hagfish transferrin is 75,000 to 80,000 (Aisen et al.,1972); in carps (*Cyprinus carpio*) it is 70,000 (Valenta et al., 1977). Most proliferating cells utilize transferrin-bound iron. The transferrin level in blood has been considered in the evaluation of both iron and protein nutrition (McFarlane et al., 1969; Fletcher et al., 1987). Transferrin also acts as an inhibitor of bacterial growth by reducing free iron levels in the serum of vertebrate hosts (Weinberg, 1974).

1.2. Biochemistry of iron

Iron is important in cellular respiration both in oxidation-reduction and electron transport activity. Each of the Hb subunits, with the participation of its heme molecule, can bind one molecule of O_2. The iron atom in each heme pigment must be in its ferrous form to accept an O_2 molecule, but a small amount of (less than 1%) of the ferrous Hb spontaneously oxidizes to ferric Hb, called met-hemoglobin. The binding of O_2 depends on the O_2 concentration and O_2 affinity of Hb of the blood. The high concentration of O_2 in the lungs and gills, causes Hb to bind to O_2, but at the lower O_2 tensions in tissues the affinity of Hb for O_2 decreases, so O_2 is released to the tissues. At lower pH, Hb has a lower

affinity for oxygen. In higher animals, this is useful in promoting the release of O_2

by exercising tissues. The CO_2 produced in these tissues is converted to

carbonic acid by the action of carbonic anhydrase, and this induces Hb to

release O_2 into the tissues.

The nonheme enzyme ribonucleotide reductase is essential in the

cytoplasm of all growing cells. It converts ribonucleotides to the corresponding

deoxyribonucleotides, the unit of DNA. Ribonucleotide reductase uses the

diphosphate form of ribonucleotides instead of the commonly available

triphosphate form to synthesize deoxyribonucleotide diphosphates.

ADP $\longrightarrow$ dADP

Ribonucleotide reductase

In higher animals, iron deficiency has been associated with impairment of

the immune system. Some concepts developed from this association suggest

probable impairment of iron-containing enzymes of cells. Phagocytes engulf

bacteria and kill them by surrounding them with vesicles that expose them to

toxic forms of oxygen (O_2^-), hydrogen peroxide (HOOH) and hydroxyl radicals

(HO$^-$) through "Fenton-like" reactions.

$Fe^2 + HOOH \longrightarrow Fe^3 + HO^o + HO^-$

$HO^o + HOOH \longrightarrow O_2^- + H^+ + H_2O$

In addition, bacteria are exposed to highly toxic hypoclorous acid (HOCl), which is synthesized from HOOH and chloride ions with the help of the heme-metalloenzyme myeloperoxidase:

$$HOOH + Cl- \longrightarrow H_2O + HOCl$$

Myeloperoxidase

Some of the lymphocytes synthesize antibodies. Antibodies recognize a particular foreign organism or protein when they encounter it in the host. Lymphocytes need to proliferate to produce a very large number of antibodies. The iron enzyme most important for cell multiplication is ribonucleotide reductase.

1.3. Iron metabolism

1.3.1. Absorption

The oxidation state of the iron is an important factor in iron absorption as reduction of ferric to ferrous iron is preferred. Both divalent and trivalent iron ions are soluble and stable under highly acidic conditions (pH 2 or less) in the stomach. For absorption, depending on the nature of the food, 75-95% of Fe^{+3}, will be reduced to Fe^{+2}. Animals have limited capability to excrete iron from the body. Thus the regulation of total body iron is controlled at the site of absorption (Bothwell et al., 1979; Forth and Rummel, 1973). Iron absorption occurs in the duodenum and jejunum (Figure 1.1) and, to lesser extent, in the ileum and the

Dietary iron

Gut lumen   Fe^{2+} exfoliation

Mucosa   X ⟷ ferritin

Blood   transferrin

Tissues

Iron receptors
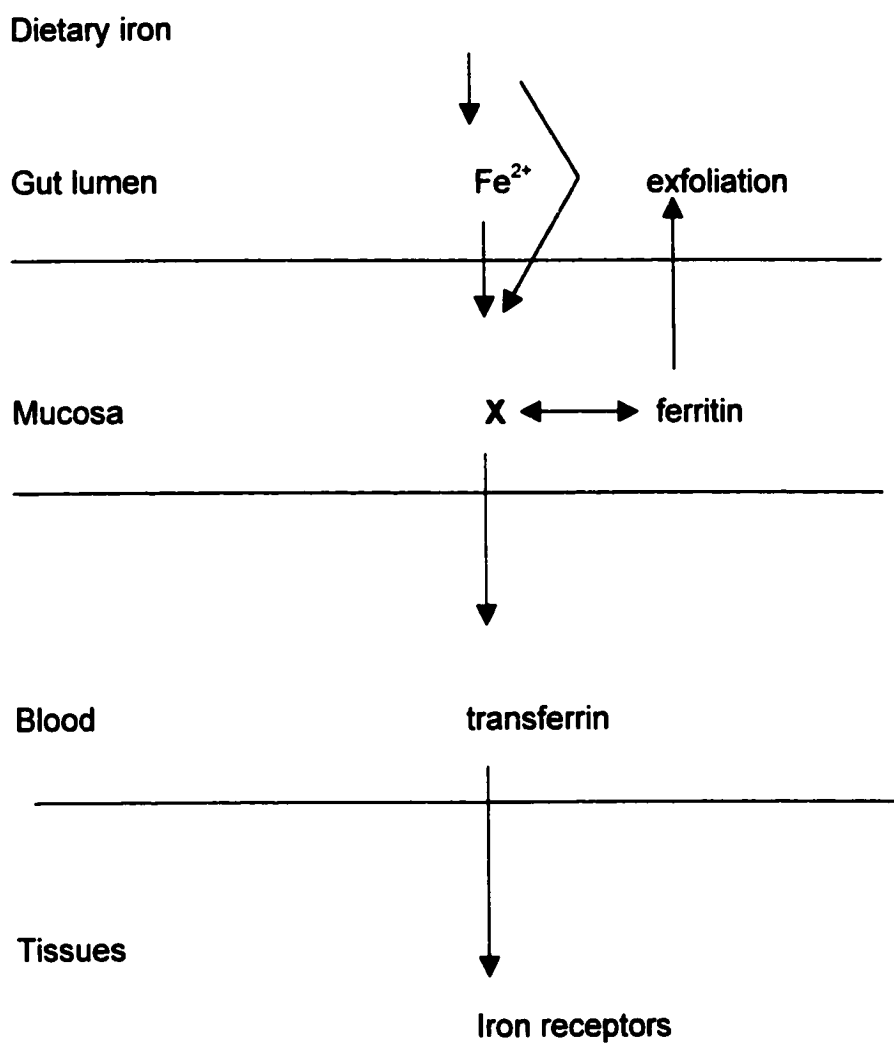
Figure 1.1. Iron absorption in an animal model (Bernat,1983)

stomach (Forth and Rummel, 1973). This is related to the fact that iron acceptor

sites that exist on the brush border of intestinal cells (Grasbeck et al.,1979;

1982) have greater iron-binding activity in the duodenal membranes than in

those from the ileum and other tissues of the gastrointestinal tract (Cox and

O'Donnel, 1980). However, if the influence of transit time is taken into

consideration, it is possible that the smaller absorption activity of the ileum may

be compensate for the longer transit time and exposure to iron (Schade et al.,

1969).

Heme and nonheme-iron are taken up by the mucosal cells via two

different pathways. Both absorption pathways are complex processes involving

three steps, namely, uptake from the intestinal lumen into mucosal cells, transit

through the cell, and release from the cells into the body. The heme complex

enters the mucosal cell intact. Once inside the cell, it is dissociated from the

porphyrin ring by heme oxygenase (Raffin et al.,1974).

Mucosal iron uptake: The uptake of iron in the lumen of the intestine into

the mucosal cells represents the first step in the process of iron absorption

(Manis and Schachter, 1962). Iron is taken up by enterocytes. Conard et al.

(1994) suggested that mucin plays a role in presenting the iron to the

enterocytes in an available form for absorption. They also suggested that mucin

was able to bind iron at acid pH and maintain the iron in solution at neutral pH.

In summary, Conard and co-workers postulated that acid-solubilized ferric iron forms complexes with gastric mucins that in turn keep iron soluble and available for mucosal uptake in the small intestine.

In general, there are two distinct microvillus carriers for nonheme iron, one for Fe^{+2} and other for Fe^{+3}, due to the different biochemical properties of the two froms of iron (Simpson et al.,1986). Some studies indicated that an electrogenic pathway requires cleavage of iron complexes at the mucosal surface prior to entry of iron (Raja et al.,1989). Presumably, most of the Fe^{+3} is converted to Fe^{+2} before absorption (Raja et al.,1991), and the Fe^{+2} entering the cell would be oxidized to Fe^{+3} after entry.

Transit through mucosal cell: Little information is available about how the mucosal transport of iron from brush borders occurs (Benito and Miller, 1998). It seems unlikely that iron is transported to the brush border of the mucosa in a free soluble form, which is toxic and may cause free radical mediated membrane damage (Halliwell and Gutteridge, 1984). Thus an intercellular protein is probably involved in temporary storage of iron. Recent studies from Conard et al. (1994) have reported that there are at least two iron- binding proteins in the intestinal mucosa and these differ biochemically and immunologically from the iron-binding proteins, ferritin and tansferrin. The mucosal proteins are mobilferrin and integrin. Mobilferrin is a water-soluble cytosol protein, while integrin is a water-insolubleTriton-soluble membrane protein. Conard et al.

(1994) hypothesized that the absorptive process is driven by various differences in the binding constants of proteins. According to this hypothesis, mucins bind iron at the acidic pH of the stomach-making it soluble and available for absorption in the more alkaline environment of the intestine. Integrin present in the duodenal mucosa or microvilli, seems to facilitate the transport of iron into the cell. Mobilferrin acquires iron from integrin and hence acts as a carrier protein for iron in the cellular cytosol. According to the hypothesis, the transfer of mucosal iron to serum takes place via integrins on the basal surface of the cell. In states of iron overload, ferritin would be synthesized as the top of the cell sequesters intracellular iron and prevents cellular damage from oxidation catalized by free iron.

Release of iron from the intestine to circulation and delivery tissues: The pathway by which iron in the cytosol of enterocytes is carried to the blood is unknown. However, most of the iron released to the circulation after absorption from the intestine passes directly into the blood (Morgan, 1980). Usually iron is bound to transferrin and release to the tissue takes place through the so-called "transferrin cycle" (Morgan, 1980). Here, iron-transferrin enters cells by receptor-mediated endocytosis, The transferrin receptors are disulfate-linked homodimers in the serum membrane of cells. These receptors bind with transferrin which itself possesses two binding sites for ferric iron. Transferrin receptors along with the transferrin are capsulated via endocytosis forming

endosomes. These endosomes help to create an acidic environment that is essential for release of iron from transferrin. The released iron may be used for incorporation into hemoglobin or other complexes, or stored in ferritin. The receptors along with the transferrin are recycled back into the membranes of cells in serum to continue the cycle (Benito and Miller, 1998).

1.3.2. Distribution and storage

From serum, iron bound to transferrin is transported to all tissues in the body for storage. However, the majority of iron-transferrin is transported to liver and bone marrow and, to a lesser extent, the spleen.

Ferritin and hemosiderin are two nonheme compounds utilized for storage of iron in the body. However, ferritin iron is a more readily available iron pool than hemosiderin iron (Shoden and Sturgeon,1961). Although similar in function, ferritin and hemosiderin have different chemical compositions. Hemosiderin and ferritin can contain up to 35 and 20 % iron respectively (Underwood, 1977). The release of iron from storage ferritin to serum requires the reduction of ferric to ferrous iron (Martin et al., 1981). The iron must subsequently be reoxidized to the trivalent form to enable transferrin to bind it. Ceruplasmin, a copper protein which acts as a ferroxidase, is thought to be involved in this process (Emery, 1978).

Total iron stores in adult humans have been estimated to be in the range of 600-1500 mg (Moore and Dubach, 1962), the highest quantities being in the

liver, spleen, bone marrow and muscles. During iron overload, iron concentrations in muscle, bone marrow and spleen do not respond as extensively as the concentration of iron in the liver. The latter appears to be the major site of iron storage in all animals (Cook et al., 1974; Linder and Munro, 1973). There appear to be two cellular compartments for iron storage in the liver: (1) Kupffer cells that contain ferritin and hemosiderin derived from the catabolism of red cells, and (2) parenchymal cells that contain iron taken up from serum transferrin, hemoglobin and ferritin (Cook et al., 1974). There are several metabolic cycles of iron and the quantitatively most important pathways are shown in Figure 1.2.

Serum transferrin is the link between the cycles and may regulate the distribution of iron in the body (Underwood, 1977). Normally, 70-90 % of the iron that leaves the serum goes to the bone marrow where an estimated two thirds is used for hemoglobin synthesis (Pollycove and Mortime, 1961).

All animals have a limited ability to excrete iron (McCance and Widdowson, 1937; Conrad et al., 1981). Most of the iron excreted is unabsorbed food iron and the amount will vary with iron intake. Most of the excreted iron is mostly from desquamated intestinal epithelial cells and from bile (Ingalls and Johnston, 1954). Most of the bile-iron is reabsorbed in the intestine (Underwood, 1977; Moore and Dubach, 1962). Other routes of iron excretion include dermal loss of cells from hairs, scales, skin, and also via mucus.
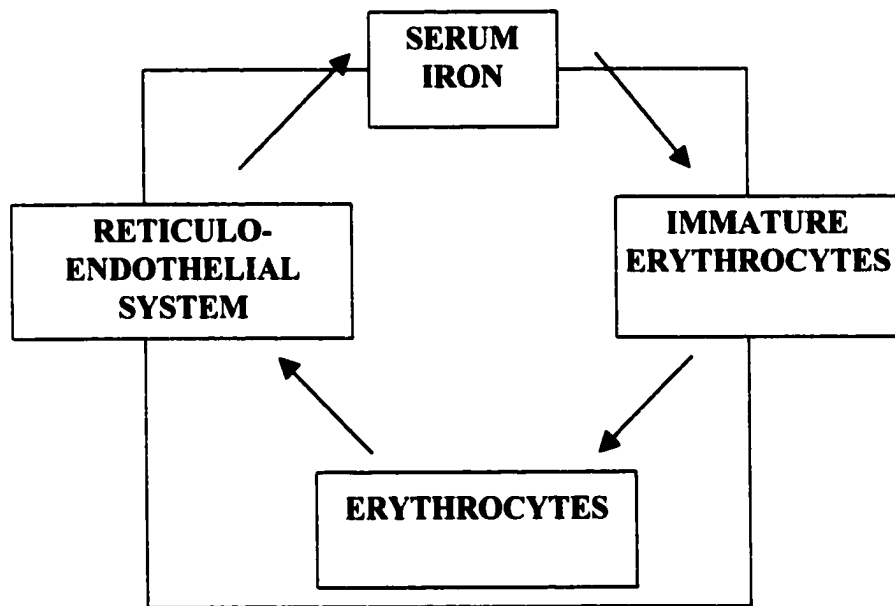
Figure. 1.2. Major metabolic cycles of iron utilization in vertebrate body.

1.4. Dietary iron requirement

The dietary requirements of iron in fishes have been established empirically by feeding graded levels of this element in semi-purified diets and then observing a variety of performance indicators such as growth, feed efficiency, tissue and vertebrae iron concentration and hemoglobin synthesis. The iron requirements of various species are listed in Table 1.1. The iron requirements of certain finfish have been quantitatively established. The dietary iron requirement of eel (Nose and Arai, 1979), catfish (Gatlin and Wilson, 1986) and red sea bream (Sakamoto and Yone, 1978) ranges from 30 to 170 mg /kg. Recently Andersen et al.(1996) estimated that dietary iron requirement of Atlantic salmon varies between 60 and 100 mg /kg. The iron supplied from purified diets about (39 mg /kg) may not be sufficient to avert iron deficiency in rainbow trout (Desjardins, 1987), and thus there may be a need for a dietary inorganic iron supplement.

Iron deficiency is often difficult to detect under commercial farming conditions, however, it may be readily produced experimentally in certain fish fed low-iron diets. Iron deficiency causes characteristic microcytic anaemia in brook trout (Kawatsu, 1972), red sea bream (Sakamoto and Yone, 1976), yellowtail (Ikeda et al., 1973), eels (Nose and Arai, 1979), and carp (Sakamoto and Yone, 1978). In most cases, fish growth was not influenced by iron deficiency. In catfish,

Table 1.1. Iron requirement of various species

Species	Iron requirement (mg/kg diet)	Reference
Chicken	50 - 80	NRC, 1984
Japanese quail	60 -100	NRC, 1984
Cow	50	NRC, 1989a
Sheep	30 - 50	NRC, 1985
Horse	40 –50	NRC, 1989b
Channel catfish	30	NRC, 1993
Rainbow trout	60	NRC, 1993
Cat	80	NRC,1986
Rats	35	NRC, 1978
Mice	25	NRC, 1978

iron deficiency was observed to suppress hematocrit, hemoglobin, plasma iron

levels as well as transferrin saturation (Gatlin and Wilson, 1986). Dietary iron

toxicity signs develop in rainbow trout that ingest greater than 1380 mg of iron/kg

(Desjardins, 1985). The major adverse effects of iron toxicity include reduced

growth, poor feed utilization, feed refusal, increased mortality, diarrhoea, and

histopathological damage to liver cells.

1.5. Iron bioavailability

For fish, the nutritional value of an iron supplement or a feed ingredient is

based not solely on its chemical composition, but also on the amount of nutrients

that fish can absorb and utilize. Nutrient bioavailability information can be used

in conjunction with dietary requirement values for cost effective feed formulation

and development of practical diets for fish. Biological availability is defined as

the empirical measure of the absorption efficiency of a nutrient from a diet.

There is no universally accepted definition of bioavailability as applied to dietary

minerals. However, bioavailability may be characterized as the proportion of a

given nutrient in a given food or diet which the body can actually utilize. In this

context, the term bioavailability includes the absorption and transport of nutrients

to the relevant body tissue as well as the in conversion into physiologically

active compounds (Godber, 1990; Fairweather-Tait , 1992; cited by Benito and

Miller, 1998). Iron bioavailability is influenced by the chemical form of the

element, the presence of chelating agents, the chemical and physical

characteristics of a particular feedstuff, the effects of antagonistic and other

nutrients, and the iron status of the animals.

1.5.1. Bioavailability studies

Four different approaches have been employed when bioavailability studies

of iron have been undertaken in domestic animals and fish.

a. Hemoglobin repletion assay: This is the oldest approach and dates back

to the early 20th century (Mitchell and Schmidt, 1926; Waddell et al., 1928;

Elvehjem et al., 1929). The bioavailability of iron sources was studied by

assessing hemoglobin regeneration in the subject. Ferrous sulfate heptahydrate

($FeSO_4.7H_2O$) was used as the standard. The subject was fed an iron-deficient

diet for 2 weeks. Subsequently, supplements with 0, 5, 10, 15 or 20 ppm iron as

$FeSO_4.7H_2O$ were fed for a week to create a standard curve from the hemoglobin

value (Pla and Fritz, 1970). Several iron sources each included as 20 ppm iron

supplements in test diets can then be compared` with this standard curve by using

the hemoglobin value in the formula (Fritz et al., 1970; Pla and Fritz, 1970). The

formula is as follows;

Relative bioavailability value or relative availability = 100 x (mg/kg iron as

$FeSO_4.7H_2O$ for measured Hb response)/ (m/kg iron as test iron source for equal

Hb response).

Hemoglobin regeneration measured after 2 weeks of feeding test diets to

rats (Pla and Fritz, 1970) showed that the availabilities of ferrous chloride and

ferric chloride were 106% and 78%, respectively compare with 100% for ferrous

sulfate. Ferrous carbonates had low relative availabilities (2% and 7%).

b. Slope ratio method: Amine et al.(1972) used a similar approach to that

described above but their procedure involved comparison of the slope ratios of

hemoglobin values to standard and test sources. In this regard, they depleted iron

in chicks for 3 weeks and then supplemented the diet with iron for 2 weeks.

Setting the response for $FeSO_4.7H_2O$ at 100%, the bioavailability values for ferric

orthophosphate, sodium iron pyrophosphate and ferrous carbonate were

estimated to be 27%, 4% and 2%, respectively. Andersen et al. (1997) used

similar methods to study iron bioavailability in Atlantic salmon. Instead of

hemoglobin, the liver iron content was used for determination of bioavailability

values. Details of the results are discussed in the iron bioavailability experiment

(chapter 3).

c. Radioisotopes: This method is used extensively in higher mammals and

humans. Both extrinsic and intrinsic radiolabels with ^{59}Fe have been used to study

nonheme iron bioavailability. [54]Fe, a stable iron isotope has been used in humans

but not in animals (Hansen et al., 1967). By using ^{59}Fe labelled ferrous sulfate,

ferric chloride, ferrous carbonate and ferric oxide in calf and lamb feed

(Ammerman et al., 1967), it was noted that ferrous sulfate and ferrric chloride had

equivalent bioavailability, while ferric oxide was unavailable. Absorptions of [59]Fe

forms of ferrous carbonate, ferrous sulfate and ferrous chloride were found to be

almost equal. The radioisotope method is not well established due to considerable

amounts of background interference that result in high values of bioavailability from

isotope readings. Another limitation of approach includes the reduced stability of

iron isotopes (Fairweather-Tait, 1992).

d. Injectable forms: Iron forms of iron dextrin, iron dextrin and gleptoferron

have been used as effective sources of iron when administered as single intra-

muscular injections to piglets in the first three days of life (NRC, 1988). The rate of

repletion has been compared with the anaemic subject (Fairweather-Tait, 1992).

1.5.2. Factors influencing bioavailability

Johnson et al. (1994) and Benito and Miller (1998) have reviewed the

factors influencing bioavailability of iron in animals.

a. Species: The bioavailability of iron in some feedstuffs can vary from

species to species. For instance, iron in corn gluten meal was found to be 84%

available for chicks but only 20% available for cats compared to 100% for ferrous

sulfate in both species.

b. Iron status: Individually within species, iron status is the most important

physiological factor. Iron absorption will increase in response to body iron

depletion (Bothwell et al., 1979; Cook et al., 1974; Cook, 1990).

c. Stomach physiology: Acid secretions in the duodenum (Cook, 1964)

and mucus secretion in digesta (Quarterman, 1987) are important factors that

influence iron absorption. The mechanism is already described in the section of this review dealing with iron absorption.

d. Ascorbic acid: Ascorbic acid effectively reduces ferric iron to the ferrous state. Moreover, this vitamin can chelate nonheme iron when they are ingested concurrently (Greenberg et al., 1957; Monsen, 1988). Thus iron uptake can be increased by these mechanisms.

e. Phytate: Phytate is a common constituent of plant seeds. It has a tendency to reduce metal absorption. However, the bioavailability (relative to ferrous sulfate) of iron from monoferric phytate of wheat bran was found to be 99% in rats (Morris and Ellis,1982). In the same experiment, other phytate forms, (di- or tetraferric phytate) showed low bioavailability. In calves, Bremner and Dalgarno (1973) reported that the formation of insoluble iron –phytate complexes resulted in low bioavailability of iron. Thus the bioavailability of iron the in presence of phytate appears to depend upon the chemical state of the latter component.

f. Protein: Iron from animal sources is generally more available than iron from plant sources (Morris,1987). This is due to the heme iron content in animal sources. Certain amino acids, such as cysteine, histidine, and lysine can form chelates that promote absorption of iron (van Campen, 1972, 1973). Low-protein diets appear to reduce iron uptake (Coons, 1964; Layrisse et al.,1968). Alternately higher protein diets have been observed to increase haematocrit and

hemoglobin values in rats. Caster and Resurreccion (1982), however, found that

low protein diets improved iron absorption. Plant protein, like soy protein also

influences iron absorption. Soy protein can deplete body iron storage in rats

(Thompson and Erdman, 1984).

g. Pectin: Pectin is a component of the dietary fibre from plants. Free

carboxyl groups of pectin can complex with polyvalent cations, including iron.

Consequently, they increase iron absorption and the efficiency of hemoglobin

regeneration in anaemic rats (Kim and Atallah, 1992,1993).

h. Minerals: Mineral interactions in feed may also have important

influences on iron bioavailability. For example, increasing dietary calcium and /or

phosphorus can reduce iron absorption in chicks (Waddell and Sell, 1964; Sell,

1965) and rats (Amine and Hegsted, 1971; Prathet and Miller, 1992). Further,

dietary cobalt (Forth et al.,1973), copper (Bradley et al., 1983), and zinc

(Settlemire and Matrone,1967a,b) can reduce iron availability in domestic animals.

Excess manganese in diets can also result in decreased hemoglobin in young

lambs (Hartman et al., 1955) and in chicks (Baker and Halpin,1991). Iron

absorption has also been observed to be lower in nickel-deficient rats (Morris,

1987). More information on mineral interaction is available in the section entitled

'Interactions with other nutrients'.

i. Feed or food processing: Heat and pressure processing of ferrous

sulfate, sodium ferric pyrophosphate, ferric orthophosphate, or ferric

pyrophosphate can increase the availability of iron in chicks (Wood et al.,1978).

Prolonged warming of meals for humans can reduce iron bioavailability from the

diet (Hallberg et al., 1982).

j. Other factors: Cellulose and oxalate may increase the bioavailability of

ferrous iron (Gordon and Chao,1984). However, Reinhold et al.(1986) showed

that fiber from corn or wheat decreased iron retention. Carbohydrate also may

influence iron metabolism (Amine and Hegsted 1971). For instance, sucrose was

observed to decrease hemoglobin and iron absorption in rats (Johnson and

Gratzek,1986) but starch had no effect.

1.5.3. Bioavailability in fish

Little is known about the forms of iron present in common feedstuffs and

their bioavailability to fish. Much of the research on the bioavailability of iron has

been conducted with domestic animals and humans. This information was recently

reviewed by Henry and Miller (1995). Andersen (1997) reported the bioavailability

of some iron sources for Atlantic salmon and the details are reviewed in Chapter 3.

Iron is present in food both in inorganic forms as ferrous and ferric compounds,

and in organic forms, with the most important of these being heme iron. In animal

protein, iron may be present as iron-porphyrin, myoglobin and haemoglobin. Heme

iron compounds are absorbed more efficiently from food sources than inorganic

iron. Unlike inorganic iron, heme is soluble in alkaline solutions and is precipitated

in acid milieus, making chelation less important in facilitating solubility. Many

substances that either enhance or diminish absorption of inorganic iron have no

effect upon absorption of heme iron because they do not chelate this form of iron.

Inorganic iron compounds are commonly used to increase the iron

concentration of experimental and commercial diets for fish. Iron in its two

oxidation states, ferrous and ferric, have comparable biological availability.

However, not all iron salts are bioavailable and capable of preventing anaemia.

Compounds considered to be good iron sources include ferric ammonium citrate,

ferric chloride, ferric sulphate, ferrous ammonium sulphate and ferrous fumarate

(Fritz et al., 1970). Iron forms classified as poor sources include ferric oxide, ferric

orthophosphate, ferrous carbonate and sodium iron pyrophosphate (Fritz et al.,

1970).

1.5.4. Interactions with other nutrients

Certain minerals can also affect the boavailability of dietary iron. Excess

copper has an antagonistic effect on iron utilization (Underwood, 1977). Cobalt

has a similar electronic configuration to iron. Consequently, iron absorption is

competitively inhibited by equimolar amounts of cobalt in the diet (Thomson et al.,

1971). Similarly, high levels of manganese in feed or water have been shown to

inhibit iron absorption in ruminants (Hartman et al., 1965). Sugars, particularly

fructose, can enhance iron absorption (Pollock et al., 1964). Ascorbic acid has a

dual role in iron absorption. It can form a stable complex with iron and/or reduce

iron to the more soluble ferrous form (Forth and Rummell, 1973). Other reducing

agents such as glutathione, cysteine, succinate, pyruvic and lactic acids also

appear to stimulate absorption by a similar mechanism (Forth and Rummel, 1973).

Iron absorption can be decreased by the following dietary components: oxalates,

phosphates, orthophosphates, pyrophosphate and phytate (Forth and Rummel,

1973). These phosphate compounds bind tightly to iron forming insoluble

compounds, which then decrease iron absorption.

1.6. Iron toxicity

A substance is considered to be a toxicant, when it harms living

organisms by its detrimental effect on tissues, organs, or biological processes.

Whether a substance is poisonous or not depends upon the type of organism the

amount of the substance, and the route of exposure (Manahan 1991).

Rivers that carry coal mine effluents may contain a high load of ferrous

iron (Gale et al., 1976). Depending upon the temperature, oxidation of ferrous to

ferric iron may affect the river ecosystem by reducing the phytoplankton and

zooplankton populations.

Coho salmon (Oncorhynchus kishutch) eggs were fertilized and fry were

incubated in 3 mg of suspended iron hydroxide /L (Brenner and Cooper, 1977).

No apparent effects on development, hatching rate, and survivability were found.

Iron toxicity has been observed in some studies on livestock (reviewed in NAS, 1980). The toxic effects have been found when the diets contained both deficient and excess iron supplementation. High levels of oral iron supplementation have caused acute toxicosis, which included, anorexia, oliguria, diarrhea, hypothermia, diphasic shock, metabolic acidosis, and finally death (Boyd and Shanas, 1963). Elevated serum iron levels accompanied the iron toxicosis. Histological evidence of cellular deformities was found in tissues of the gastrointestinal tract, liver, kidneys, heart, lungs, brain, spleen, adrenals, and thymus. When ferrous sulfate was given to dogs at levels of 150 to 600 mg of iron per kg body weight, various disorders ranging from vomiting and diarrhea to irritation of the gastrointestinal tract occurred (Reissman and Coleman, 1955; D'Arcy and Howard, 1962). In rabbits, supplementation with 750 mg of ferrous sulfate per kilogram of body weight caused hepatic congestion within 24 to 48 hours (Luongo and Bjornson, 1954). A dose of 2 g of ferrous sulfate per kg body weight killed all rabbits within a few hours of administration.

Iron overload diseases can cause disorders of iron metabolism such as hemosiderosis, hemochromatosis, and hemolytic jaundice, and they have resulted in accumulation of large numbers of hemosiderin granules in parenchymal hepatocytes. In these disease states, a nonheme form of soluble iron like ferritin is stored in lysosomes and this is subsequently degraded to insoluble hemosiderin (Bradford et al., 1969; Crichton, 1971; Trump et al., 1973).

Hemolytic jaundice can also result in increased erythrocyte lysis in subjects.

Low dietary levels of iron cause signs of chronic iron toxicosis in livestock. For

most species this results in reduced feed intake, lower growth rate and

decreased efficiency of feed conversion (Standish and Ammerman, 1971). In

fish, rainbow trout fed a diet with 5964 mg of iron/kg showed high levels of fecal

iron excretion (Desjardins, 1985). Serum protein increased with the increase in

dietary iron. Serum and carcass concentrations of iron, copper, and zinc also

increased with dietary iron levels in these fish. Liver, kidney, and spleen iron

concentrations increased only two folds and were as 536 μg/g and 493 μg/g at

5964 mg iron/kg (Desjardins, 1985).

Excessive dietary iron results in excess iron in the serum and reduces the

capacity of iron binding proteins. The loosely bound iron is rapidly removed from

the serum, and then this in turn, causes toxicity by stimulating the oxidative

Fenton reaction in the cells where it is stored. Because animal has limited

capacity for iron excretion, the toxicity of iron is largely controlled by its

absorption.

Toxic elements like cadmium, lead and aluminum can interact

metabolically with iron (Goyer 1997). Iron deficiency increases the absorption of

these elements. Cadmium and aluminum interact with calcium in the skeletal

system to produce osteodystrophies. Lead interacts with calcium in the nervous

system and this leads to impairment of cognitive development. Calcium deficiency along with low dietary magnesium intake may contribute to aluminum-induced degenerative nervous disease in fish.

Iron toxicity signs develop in rainbow trout fed diets with greater than 1380 mg of iron/kg (Desjardins, 1985). The major effects of iron toxicity include reduced growth, poor feed utilisation, feed refusal, increased mortality, diarrhoea, and histopathological damage to liver cells. The maximum tolerable levels will depend on the biological availability of the dietary iron. Iron toxicity tolerance can vary with species. Pigs are more tolerant of excess iron than cattle, sheep, or poultry. All animals probably tolerate much higher dietary levels when the iron is supplied from sources with low bioavailability.

1.7. Iron, immunity and disease resistance

1.7.1. Teleost immune system

Knowledge of the fish immune system has been gathered from two interdependent lines of research: (a) comparative immunology and (b) investigations of how fish respond to pathogenic infections. As ancestors of vertebrate evolution, fish have an immune system that is in between that of invertebrates and higher animals. Like invertebrates, fish possess the phagocytic mechanisms associated with granulocytes and mononuclear

phagocytic cells. Like higher vertebrates, they exhibit both humoral and cell-mediated immunity.

The immune system of fish consists of non-specific and specific systems. The non specific immune system acts as a first line of defence and, as in mammals, it is comprised of many humoral (non-cellular) and cellular responses. The specific immune system requires antigen-antibody complexes for its activation, thus forming a second line of defence. This review gives the mechanisms of defence against invading organisms, rather than a description of each factor.

1.7.1.1. Non-specific defence mechanisms

In terms of the chronology of events during the course of infection, the non-specific defence system provides the first obstacle by creating surface barriers. These are followed by non-specific humoral factors and non-specific cellular factors. For instance in fish, the mucosal secretion can be considered as the first line of defence, whereas the mucosal lining cells function as the second barrier against invading pathogens. Blood cells, especially granulocytes and monocytes, act as the third line of defence by destroying microbes in the blood (Dalmo et al., 1997).

a. **Surface barriers:** The mucus, skin, scales, gills, and gastrointestinal tract provide a very effective surface barrier against invading organisms. A layer of mucus covers the integument of fish (skin, gills, and gut), and a continuous

shedding of mucus either entraps microorganisms for sloughing or prevents their attachment for colonization. Mucus is a multifunctional material. Diverse functions of mucous have been reviewed recently by Shephard (1994). The rate of mucus production in fish increases in response to infection or by physical stress or chemical irritants. This effect may be related to the fact that various non-specific immune components have been found to play a role in the surface defence system. Peleteiro and Richards (1990) have shown that phagocytic macrophage cells are present in the epidermis of rainbow trout and they appear to be efficient in migrating to the basal lamina of skin. Fish mucus also provides lysozyme, lectins, proteinases, complement, C-reactive proteins, and trypsin-like enzymes (Fletcher and Grant, 1968; Harrell et al., 1976; Braun et al., 1990; Alexander and Ingram, 1992). Marginos et al. (1995) recently reported that the gel-forming fish-mucus glycoproteins account for the antibacterial action of mucus. The epidermal healing response in fish is remarkably fast (Bullock et al., 1978) and helps to minimize the chances of pathogenic invasion through the surface area.

b. Non-specific humoral factors:. The body fluids of fish, including serum, and mucus contain a variety of substances that non-specifically inhibit the growth of pathogens. The non-specific humoral defence system includes proteases, lysins, and agglutinins that are either proteins or glycoproteins in nature. They may be further classified according to their functions as follows:

Growth inhibitors: Growth inhibitors act by depriving microorganisms of essential nutrients or interfering with their metabolism. Some of the best examples of these inhibitors are as follows.

Transferrin: Transferrin is one of the iron binding proteins present in fish serum (reviewed by Yano 1996). By removing iron, an essential growth element for microorganisms, from serum or infected areas, it exerts a bacteriostatic and fungistatic effect (Putnam, 1975). To counteract it, many microorganisms produce their own iron-binding siderophores to compete with transferrin for iron. Substances related to transferrin are also found in higher vertebrates. They are lactoferrin in mucus secretions and milk of mammals and conalbumin in chicken egg white.

Interferon: Interferon (IFN) is a protein or glycoprotein that acts as an antiviral agent by inhibiting viral replication. IFN production has been observed in rainbow trout and carp but not in cyclostomes or cartilaginous fishes. Yano (1996) reviewed its biochemistry and physiology in detail. In higher vertebrates, three types of IFN have been identified (Stewart, 1980). They are IFN-α, IFN-β and IFN-γ. The first two are secreted by leukocytes and fibroblasts cells and IFN-γ is secreted by T helper-1, T cytotoxic, and natural killer cells. IFN-α and IFN-β act to inhibit viral replication in infected cells. IFN-γ has the same function as the others. In addition, it enhances the activity of macrophages, and

increases the expression of class I and class II MHC molecules (Kuby 1994). It also inhibits proliferation of T-helper-2 cells. Fish IFNs have been classified into two groups, 'αβ' and 'γ' (Gravell and Malsberger, 1965; Graham and Secombes, 1988).

Enzyme inhibitors: Pathogens produce enzymes to invade and obtain nutrients from the host. Host body fluids contain various pathogenic enzyme inhibitors like, α-2-macroglobulin (α-2M). In rainbow trout, α-2M has been shown to inhibit the proteolytic activity of furunculosis (*A. salmonicida*) protease (reviewed by Ellis 1987).

Lectin: Lectins are proteins that are capable of agglutinating and precipitating carbohydrate moieties that are freely associated with or attached to cell surfaces (Alexander and Ingram 1992). There are two types of lectins, the S-type and the C-type (Drickamer 1988). Lectins have been isolated from the eggs, serum, and mucous of a variety of fish species as reviewed by Alexander and Ingram (1992); and Yano (1996). However, it is not clear whether fish lectins are C- or S-type. Alexander and Ingram (1992) in their review suggested that the S-type of lectins might be dominant in the eggs and mucus of fish.

Complement: This is probably the most important component of the non-specific defence system because of its multiple roles in the elimination of foreign substances by mediating inflammatory vasodilation, chemotactically

attracting leucocytes and promoting ingestion by phagocytes. The complement

system is composed of 12 or more chemically and immunologically distinct

serum glycoproteins that are capable of interacting with each other, as well as

certain bacterial products, and cell membranes with or without antibody. Each

protein of the complement system is normally present in the circulation as a

functionally inactive molecule. In humans, they make up altogether

approximately 15 per cent (wt/wt) of the serum globulin fraction. The ability of

C1 (the first protein of the complement system) to bind to a specific site on

certain immunoglobulin molecules (which are themselves bound to antigen) can

activate a sequence of reactions. This leads to the production of a unit capable

of lysing a target cell membrane. This established the fact that the complement

system acts as the primary mediator of the antigen-antibody reaction (Mayer,

1973). After specific inactive complement proteins become activated, they serve

as enzymes that catalyze the activation of the next complement protein in the

cascade. The sequential activation of individual complement proteins from

inactive to functionally active substances is a dynamic event called the

complement cascade (Kuby, 1994).

In fish, there are two parallel but independent pathways that lead to the

activation of biologically important proteins that comprise the membrane attack

complex (MAC). These pathways are termed the classic complement pathway

(CCP) and the alternative complement pathway (ACP) (reviewed by Secombes

and Olivier, 1997). Biologically important substances that are involved in inflammatory responses can trigger activation of CCP or ACP. Both pathways converge at the midpoint of the complement cascade and thus share a common terminal activation sequence involving components C5 through C9 (Kuby, 1994). The MAC is a complex of complement molecules that polymerize to form a lytic hydrophobic plug that enters the bacterial cell membrane to form pores. Lysozyme (discussed later) is also thought to play an important role in complement lysis by dissolving the outer peptidoglycan layer, and exposing the membrane so the MAC can attach and create pores.

The classic complement pathway is considered to be a part of the specific immune system because it needs the presence of an antibody-antigen complex to be activated. The alternative complement pathway is considered to be a part of the non-specific immune system because it is activated by the presence of a variety of compounds, like the lipopolysaccharide (LPS) of Gram-negative bacterial cell wall, zymosan, red blood cells of rabbit etc. Activation of complement by the classical pathway requires immunoglobulin (IgM) from closely related species (reviewed by Yano 1996).

Lysozyme: Lysozyme is found in a wide range of vertebrates (Osserman et al., 1974), and is one of the defense factors against invading organisms. In fish, lysozyme is found in phagocytic cells, serum, and mucus. Some bacteria can be directly lysed by lysozyme, but gram-negative bacteria, are not directly

damaged by it. The enzyme becomes effective after complement and other

enzymes have disrupted the outer cell wall, thereby unmasking the thin

peptidoglycan layer of these bacteria (Glynn, 1969, Hjelmeland et al., 1983).

Lysozyme has been reported in a variety of freshwater and marine fishes

(Lie et al., 1989). Monocytes, macrophages and polymorphonuclear

granulocytes are known to synthesize and secrete lysozyme in fish (Murray and

Fletcher, 1976). Lysozyme has also been isolated from fish liver, spleen, kidney,

muscles, skin, mucus, gills, ovary and eggs (Takahashi et al.,1986; Lie et

al.,1989; Yousif et al.,1991; Takemura and Takano,1995). However, kidney

tissue appears to have the greatest concentration of lysozyme activity (Maack

and Sigulem, 1974).

Precipitins and agglutinins

C-reactive protein: C-reactive protein (CRP) is a regular serum

constituent, but it acts as an acute phase protein because its concentration is

greatly enhanced after tissue damage due to infection (Murai et al. 1990). C-

reactive protein binds to phosphoryl-ester groups that are present in many

microbial cell walls. By recognizing phosphorylcholine in the cell walls of

invading organisms, CRP plays a vital role in the host defence mechanism as a

substitute for immunoglobulin (IgM). C-reactive protein is also capable of

activating the complement system. It needs Ca^{2+} ions for the precipitation

reaction. CRP has been detected from different types of fish (reviewed by Yano

1996) and from different tissues of fish bodies including the serum, eggs, and sperm (Fletcher and Baldo, 1976; Fletcher et al., 1977; White et al., 1978). Season, sex, and stress can greatly influence the levels of CRP in serum. The CRP level in rainbow trout was elevated 3 to 20 fold over that in normal serum following injection of inflammatory agents like turpentine (Winkelhake and Chang, 1982) or Freund's complete adjuvant (Kodama et al., 1989).

c. **Non-specific cellular factors:** A variety of leukocyte types are involved in nonspecific cellular defences of fish. These include monocytes/ macrophages, neutrophils, and non-specific cytotoxic cells (NCC).

Phagocytes: Macrophages and neutrophils are mobile phagocytic cells in fish. In fish, phagocytic activity can be activated in two different ways. There are 'opsonins', which increase the phagocytic uptake of particles, and 'lymphokines', which are produced in response to specific antigen stimulation. The descriptions of some of the phagocytic cells are as follows:

Macrophages: Macrophages are monocytes and in teleosts they are distributed widely in the blood and tissues, including gills and the peritoneum. They are mainly found in reticuloendothelial cells in the head kidney and spleen but not in the liver as in the Kuppfer cells of mammals (Varichak,1938).

Monocytes are found in the kidney and a small number are present in blood. They are capable of migrating in the blood to inflammatory sites, giving rise to macrophages, and are thus considered to be precursors of tissue

macrophages. Macrophages and monocytes are avidly phagocytic for a wide range of particles, like carbon, bacteria, and yeasts. Macrophage containing melanosomes within lysosomes are known as melanomacrophages.

Neutrophils: Fish neutrophils are one of the granulocytes and are morphologically and histochemicially similar to mammalian neutrophils. Like macrophages, they are mobile and phagocytic in nature, and are found in blood, reticuloendothelial cells and sites of inflammatory lesions. Although isolated neutrophils exhibit highly mobile, phagocytic activity and produce reactive oxygen species their bactericidal activity is weaker than that of macrophages (Secombes, 1996). Griffin (1983) suggested that a certain stage of maturation is required before they can acquire active phagocytic competence.

Nonspecific Cytotoxic Cells: Nonspecific cytotoxic cells (NCC) of fish are considered to be equivalent functionally to mammalian natural killer (NK) cells. They are present in most fishes, and are morphologically similar to monocytes (Evans et al., 1984). These cells of several fish species are capable of cytotoxic reactions against most fish and mammalian cell lines, virus-infected cells, and fish protozoan parasites (Evans and Jaso-Friedmann,1992).

Other granulocytes: Other granulocytes in fish are represented by eosinophils, basophils, and mast cells. Eosinophils are abundant whereas basophils are absent from most of the fish (Secombes 1996). Eosinophilic cells found in the stratum granulosum of the gut, gills, skin, and surrounding blood

vessels are considered to be mast cells (Vallejo andEllis,1989). In mammals,

these cells play a role in inflammation and other defence mechanisms, but their

role in fish is not completely understood.

1.7.1.2. Specific defence mechanisms

Immune responses that are mediated by various lymphocytes and

antibodies are collectively termed the specific immune system.

Lymphocytes: Lymphocytes are responsible for initiating and mediating

three aspects of specific immunity; humoral immunity, cell-mediated immunity

(CMI) and memory. Humoral immunity results in the production of soluble

antibody i.e. immunoglobulin while CMI results in a response mediated by a

variety of cells including lymphocytes and macrophages. Memory has always

been considered to be an important aspect of specific immunity. It constitutes an

adaptive change in the lymphocyte's response to the same antigen. A secondary

response occurs and this is characterized by a shorter latent period and an

increased magnitude of response.

Lymphocytes are available in the circulation. Moreover, they originate in

the lymphoid organs (thymus, kidney, and spleen) and other tissues, especially

during the inflammatory stage. In higher animals, there are two types of

populations, namely, T (thymus-derived) lymphocytes and B (bone marrow-

derived) lymphocytes. The T lymphocytes provide the CMI and help the B

lymphocytes (through T helper cells) to produce antibodies. Sub-populations of T

and B cells are responsible for memory (or memory cells). In fish, it is now well established that lymphocyte sub-populations analogous in many respects to the T cells and B cells of mammals exit (Clem et al., 1991).

In vitro studies on lymphocyte populations in fish show that a number of substances, principally plant lectins such as phytohemagglutinin (PHA) and concavalin A (Con A), are specific for T cells (reviewed by Rowley et al., 1988). When present in appropriate doses these T-cell mitogens induce proliferation of T cells but not B cells. Other mitogens such as bacterial lipopolysaccharides (LPS) activate only B cells, but not T cells, in fish. The mitogen responses of fish lymphocytes when the fish are held under different environmental or health conditions should receive greater attention by fish immunologists (Warr and Simon, 1983; Albergoni and Viola, 1995; Daly et al., 1995).

1.7.2. Iron and immunity in humans and experimental animals

It has been widely recognized that iron is required by the host to mount an effective immune response and destroy infections and antigenic agents. However, the exact mechanism by which iron functions in disease resistance and immunity is not clear. In humans, iron deficiency is considered to be the most prevalent nutritional disorder in the world. It is associated with population groups when infection varies with factors like dissimilar geographical regions, very young and old, health and hygiene, etc. A considerable amount of research

has been conducted on human populations in countries where iron deficiency is widespread, and where increased infection and defective immune responses have also been reported (Sherman, 1990).

Many many confounding variables were present in most of the field studies published in the 1970's. For example, many of the groups studied suffered from multiple nutrient deficiencies, parasitic infections, and unhygienic conditions. It was difficult to conclude from these types of studies that iron was the single parameter causing the diminished immune response. It was also not possible to establish cause and effect relationships.

There is no doubt that microorganisms require iron for growth and survival (Bag and Neilands, 1987). It has been established that a rapid drop in the iron content of serum and an attendant decrease in transferrin saturation is common during infection and inflammation due to the action of cytokines such as interleukin-1(IL-1) (Keuch et al., 1983). Some investigators have suggested that this dramatic response of the host constitutes an important nonspecific antimicrobial defence tactic, and this is also referred to as "nutritional immunity" (Kochan, 1973). However, there is sufficient evidence from both animal and human infections that bacteria grow in vivo under iron-limiting conditions (Brown et al., 1984; Shand et al., 1985: Griffith et al., 1983: Kadurugamuwa, 1988), and this brings into question the impact of clinical iron deficiency as an added stress.

Iron serves many functions in microorganisms. It has a regulatory role for some microbial products, e.g. diphtheria toxins. Under *in vitro* conditions of iron deprivation, toxin production reaches a peak level, and is rapidly shut off when iron is added to the medium (Matsuda and Barksdale, 1967). If everything operates *in vivo* as it does *in vitro*, then iron deficiency states would be expected to turn on both siderophore and toxin production, with an increase in virulence of the organism. Conversely, iron administration might inhibit both responses of the bacterium. Many clinical studies suggest that iron deficiency promotes infection (Keuch, 1990). Several community studies have shown reductions in respiratory or enteric infections during the administration of iron either orally or parentally or that iron deficiency anemia was associated with higher infection rates (Arbeter et al., 1971). The conclusions from these studies cannot be considered definitive, however, due to poor experimental design, limited number of samples and relatively short-term observations. Moreover, there are conflicting results in which iron administration resulted in an increase in infection rates. For example, the use of prenatal iron dextran in a program of prophylaxis for iron deficiency was found to be associated with an apparent striking increase in the incidence of neonatal sepsis, primarily *Escherichia coli* meningitis, which decreased when routine iron dextran administration was discontinued (Becroft et al., 1977). However, the findings of these studies cannot be confirmed due to the lack of control subjects to determine the effect of the iron dextran administration.

Inferences regarding the adverse effects of iron administration have been made from observations on experimental animals and patients with various states of iron overload. One such state is hemochromatosis; a situation in which transferrin saturation is high and low-molecular weight iron is loosely complexed with albumin that is present in the circulation. This form of iron is readily available to microorganisms. Although lethal complications of the disease may be largely due to organ dysfunction caused by iron deposition, e.g. hepatic or cardiac failure or diabetes, the individuals may die from other infections such as pneumonia (Finch and Finch, 1955). Reports on iron deficiency in humans are not entirely clear despite some evidence that iron administration increases the prevalence of certain infections, notably malaria, in humans. It remains to be proven whether oral iron supplements for iron deficiency states have any major or life threatening effects. Recently, the approach has been to use clearly defined and controlled animal models to identify where and how iron functions in the immune response of animals and humans. The possible ways in which micronutrients, particularly iron, may function in immunity include the following: a) anatomical development of the lymphoid tissues, b) mucous production, c) synthesis of immunologically active substances, d) cellular proliferation, e) cellular activation and movement, f) intracellular killing, and g) modulation and regulation of immune processes.

In order to investigate the above mechanisms, animal and human nutritionists have used the rat model of iron deficiency. In this regard, the diets contain adequate levels of nutrients with the exception of iron. The effects of iron deficiency at two developmental stages have been investigated, i.e., in early development during the prenatal and neonatal period, and in a rapid growth phase after weaning. In these models, severe (< 5 mg Fe/kg of diet) and moderate iron deficiency (< 15 mg Fe/kg of diet) as well as iron overload have been studied. Cellular development of key lymphoid tissues was observed to be reduced in iron deficiency (Kochanowski and Sherman, 1982; 1985). Histopathological studies have confirmed that iron deficiency damages lymphoid cell development. Also, splenic and thymic tissue from young rats have shown decreased lymphopoiesis. A decrease in immune functions may develop due to the reduced cellular activity of immunocompetent tissue from iron deficient rats.

Studies conducted to determine the effects of iron deficiency on humoral components of laboratory animals and humans show that circulating immunoglobin concentration is not affected. Sherman (1985) injected sheep red blood cells (SRBCs) into rats fed diets containing 5, 15, 35 (optimum level) or 1250 mg of iron per kg for 8.5 weeks. Severe and moderate anemia were prevalent in rats fed with 5 and 15 mg of Fe, respectively. The production of both IgG and IgM by splenocytes was measured and found to be significantly lower in the severely iron deficient groups. In contrast, the circulating IgG level

in serum was not significantly influenced by either severe or moderate iron

deficiency. Serum IgM level was lower in severe but not moderate iron

deficiency. The data showed that the level of circulating immunoglobin did not

reflect actual antibody production in response to a known antigen challenge.

The level of serum immunoglobin alone failed to identify effects on antibody

production. Thus the conclusion that humoral immunity is protected during iron

deficiency in animals is false since it is based on measurements of serum

immunoglobin alone. It is important to note that these results clearly show that

during the marginal iron deficiency in humans where anemia was not present,

IgG and IgM production were both impaired in spleen but serum levels of IgM

appeared to be unaffected (Macdougall et al., 1975; Bagchi et al., 1980). The

implication of this in humans is significant. In contrast to the marked effects of

iron deficiency observed, no effects of iron overload on antibody production in

the spleen or on circulating immunoglobulins were found.

Iron deficiency in neonates has been found to cause severe impairement

of antibody production (Kochannowski and Sherman, 1985). To determine

whether the decrease in antibody production was due to impaired protein

synthesis, the incorporation of ^{14}C tyrosine was measured in vitro in the spleens

of rats from the same litter (Rosch et al. 1987). Protein synthesis was noted to

be significantly lower in severely and moderately iron deficient rat pups relative

to control pups. In severe iron-deficiency, a sharp decrease in RNA

concentration indicated a reduced capacity for protein synthesis. A reduction in RNA synthesis was also found in moderately iron deficient groups and this further suggested that protein synthesis in this group was less efficient.

Several components of cell-mediated immunity have been studied with this animal model of iron deficiency (Weiss et al, 1995). Lymphocyte blastogenesis was observed to be impaired in splenocytes of Fe-deficient weanling mice (Kuvibidila et al., 1983). Iron deficient children also have lower lymphocyte blastogenesis (Macdougall et al., 1975; Chandra, 1976a,b)

Natural killer (NK) cells identify and destroy virus cancerous cells without previous exposure. Sherman and Lockwood (1987) found that in severe and modest iron deficient rat pups, NK activity was significantly impaired. Peritoneal macrophages, another cytotoxic cell, is also less cytotoxic in iron deficient neonatal rats. When iron deficiency was initiated after weaning, macrophage cytotoxicity was not affected. This indicates that stage of development influences the susceptibility of cell mediated immunity to insult by iron deficiency. Macrophages appear to have a shorter period of sensitivity to iron deficiency insult than NK cells, that is limited to the prenatal and early neonatal period of development.

In order to determine whether defective endogenous interferon production is involved in iron deficiency, Hallquest and Sherman (1989) stimulated interferon production in vitro by incubating macrophages with polyinosinic and

polycytidylic acid. In iron deficient rats, NK activity after stimulation with interferon produced by the animals' own macrophages was lower than noted in control rats. This indicates that interferon plays a role in the decreased NK cell activity during iron deficiency, but it is likely the only reason for the response.

Interleukin 1 (IL 1) is another soluble factor that is produced by macrophages, and has widespread systematic effects on metabolism and immunity. Iron deficiency has been shown to impair production of IL I (Helyar and Sherman, 1987). Impairments in cell mediated immunity, humoral immunity, nonspecific immune functions, and other defenses against infection could all be affected by reduced IL I action. IL I plays an important role in T cell dependent processes, and these indirectly influences B cell functions. Bacterial killing by neutrophils is under partial control of IL I. Since all of these functions have been found to be impaired in iron deficiency, IL 1 mediation may play an important and central controlling role.

Prostaglandins are a group of phospholipid-derived mediators that are secreted by macrophages, and they have immunosuppressive effects. The following three observations summarize the potential effects of iron deficiency on prostaglandin and the immune response of animals:

1) Fe deficient animals generally show specific changes in n-6 and n-3 polyunsaturated fatty acid patterns, and these are particularly low concentrations of arachidonic acid in serum, liver (Sherman et al., 1982) and the membranes of

cells in serum (Hamm et al., 1989), indicating an impairment of prostaglandin synthesis,

2) lipoxygenase and prostaglandin endoperoxide synthetase involved in prostaglandin synthesis are both iron requiring enzymes,

3) PGE_2 (prostaglandin derived from arachidonic acid) is an important regulator of IL1 and IL1, in turn, regulates PGE production. Thus, changes in the types and levels of prostaglandins may provide a key to our understanding of the defects in immunity observed during iron deficiency.

Recent studies on the role of prostaglandins in iron deficient rats are not conclusive and require further investigation. The radioimmunoassay of prostaglandins from macrophages of iron deficient rats has shown that neither iron deficiency nor food restriction affected PGE_2 production. It appears that differences in PGE_2 production due to other nutrients are not necessarily responsible for defects in immunity observed in iron deficiency.

It is evident that iron deficiency results in impairment in the anatomical development of immune tissues. Also, it leads to a reduction in synthesis of immunologically active proteins such as antibody and IL 1, as well protein synthesis. Further, iron deficiency may lead to impairment in cellular activity and cell-mediated killing such as NK cytotoxicity, granulocyte phagocytosis, and tumor killing. The exact role of iron in immunity remains to be established, but possible mechanisms include a general role in cellular growth and development,

a general role in protein synthesis, or a possibly unrecognized specific function in immune cells.

The liver is the primary source of complement, components that include factor B of the alternative pathway (AP), but extra-hepatic synthesis of AP proteins has been recorded in cells of the monocyte/macrophage lineage. The main techniques adopted to demonstrate APC synthesis have involved the incorporation of radiolabelled amino acids into an antigenetically detectable protein of appropriate molecular weight, together with the reversible inhibition of its production by cycloheximide, an inhibitor of protein synthesis.

For many years, the paradoxical relationship between iron deficiency anaemia and infectious diseases has been recognized in the health of humans and animals. Certain human population groups with a high incidence of anaemia have been observed to have a high rate of infectious diseases. On the other hand, iron overload produced by administering large doses of iron parenterally or orally, has also been associated with increased infection. This apparent paradox results from a delicate balance between the iron requirements of the host and invading micro organisms, and the role of iron in mounting an effective immune response in the host.

1.7.3. Dietary iron, infection and the immune response in fish

The subject of iron nutrition, infection and immunological interactions in fish is clearly in its infancy. Relatively little is known about the absorption, metabolism

and deficiency of this element. In various fish species, the exchange of iron across gills and skin of fish complicates investigations on the relationship between iron deficiency and the immune response. A high concentration of iron in fish diets is of more concern than iron deficiency. The methodological approaches used to define the relationships between iron deficiency and overload and the immune response in warm-blooded animals are a useful guide to determine the optimum level of iron necessary for normal health and disease prevention in fish.

The need for nutritionally adequate diet to preserve the health of human and domestic animals has been recognized throughout history. Fish are no exception. Nutritional status is considered to be one of the important factors that determine the ability of the animal to resist diseases. The relationship between nutrition and immune response or disease resistance has been reviewed in homeotherms (Chandra 1988; Bendich and Chandra 1990) as well as in fish (Landolt 1989, Blazer 1992; Lall and Olivier 1991; Waagbo 1994).

Iron deficiency causes microcytic anemia in several fish species (Lall et al, 1989), but relatively little is known about its effect on immune responses. The relationship between iron deficiency and immune response in fish is difficult to study because deficiency signs develop slowly. Also, iron is required in such small amounts that it is difficult to formulate test diets that contain low concentrations of this element. In addition it is hard to maintain low levels of iron in water. Although iron deficiency signs are not commonly observed in

aquaculture operations, a combination of factors including marginal iron intake, environmental stress, altered gastrointestinal activity, disease state, drug-induced anorexia, metabolic defects and food contaminants may cause iron deficiency. Under these conditions, malnourished fish may harbor latent infections and they are likely to be susceptible to bacterial and viral infections. Acute or chronic infections generally deplete the body of important nutrients including iron (Lall and Olivier, 1993) and the resultant nutritional deficits then render fish more susceptible to secondary infections.

Because of the paucity of clinical information supporting the significance of iron deficiency and overload in determining the severity of infectious diseases in fish, the nutritional immunity hypothesis outlined for humans and experimental animals cannot be tested. A similar approach to the controlled studies conducted with animal models will define the mechanisms involved in iron metabolism and the immune response of fish and other aquatic organisms. Iron deficiency in fish that are maintained in water containing low amounts of this element will explain how iron functions in immunity especially with respect to the anatomical development of lymphoid tissues, mucous production, synthesis of immunologically active proteins such as antibody and IL 1 and cell-mediated killings.

The published information on fish provides some evidence that excess iron makes fish more susceptible to common bacterial pathogens. Commercial

salmonid feeds contain 150-800 mg iron/kg feed to the use of iron rich

ingredients particularly fish and blood meals (Olivier, 1997). Further

investigations are needed to determine whether high levels of iron in fish diets

will predispose fish to bacterial pathogens and hence this is one of the major

goals of this thesis.

In one of the few studies conducted on fish, Nakai et al. (1987) reported

that increasing the availability of free iron by intramuscular injection of ferric

ammonium citrate caused significant increases in the virulence of *V. anguillarum*

infections in eels (*Anguilla japonica*) and ayu fish, however, the infection was

more severe in eels than in ayu. Eels infected with *A. salmonicida* showed lower

concentrations of red blood cells, hemoglobin, hematocrit, magnesium, copper and

iron in serum than non-infected fish (Kakuta and Nakai, 1992; Kakuta and Murachi,

1993). Nakai et al. (1987) reported that a higher availability of intramuscular

injected ferric ammonium citrate caused a significant increase in the virulence of *V.*

anguilarum. Ravndal et al. (1994) examined iron concentrations in different

genetic strains of Atlantic salmon. Their extensive study revealed that fish stocks

with higher serum iron were more susceptible to *Vibrio* infections. Rorvik (1992)

found a direct relationship between concentration of iron in Atlantic salmon feeds

and fish mortality due to natural outbreaks of furunculosis.

When the supply of the iron in the host is high, invading bacteria can

obtain iron for growth, and flourish, producing active infection in Atlantic salmon

(Paterson et al., 1985). It is possible that higher levels of inorganic and heme iron in commercial diets of salmonids could predispose salmonids to common bacterial pathogens. Controlled laboratory experiments rather than field observations (Rorvik, 1992) are needed to determine the effects of excessive dietary iron on immune response and the susceptibility of fish to infectious diseases.

Iron is an essential nutrient for microorganisms and their ability to infect a host depends on its availability. Microorganisms attempt to obtain iron directly from host iron binding proteins. In most cases siderophores are employed to remove the iron. Toxic substances secreted by the pathogen can also release iron from hemoglobin. Thus the first line of defence in the fish involves nonspecific immunity like mucous. Mucus secretions that cover body orifices and other membranes are important. The low concentration of free iron in mucus membranes and in other tissues is one of the first lines of host defense against bacterial infection. Iron is also important in maintaining the structural integrity of epithelium and thus it helps to prevent access to the host by pathogens. Iron deficiency causes microcytic anemia in several fish species (Lall et al., 1989) but relatively little is known about its effects on the immune response. Information on other terrestrial species offers two different views. The first suggests that iron deficiency predisposes animals to infection (Humbert and Moore, 1983); thus dietary iron supplementation might be expected to increase

disease resistance. The second view implies that iron deficiency protects against infection by limiting the amount of this element that is available to bacteria for multiplication (Weinberg, 1984). In this situation dietary iron supplementation would increase susceptibility of the fish to infection.

Since iron deficiency reduces both host resistance and replication of an infectious agent, increasing the dose of iron could exacerbate the infection before the host could recover. However; iron deficiency is not a desirable means of controlling infection because the deficiency makes the host more susceptible to other infectious agents (Scrimshaw, 1990).

CHAPTER 2. Dietary iron requirement of Atlantic salmon (*Salmo salar* L.) parr

2.1. Introduction

Iron is essential in fish, as in other vertebrates, for oxygen transport, cellular response and mitosis (Robbins et al., 1979). Even though fish can absorb soluble iron from the water through gill membranes and intestinal mucosa (Roedar and Roedar 1968), food is vital as the major source of iron (NRC 1993). Iron deficiency has been found to cause microcytic anaemia in brook trout, *Salvelinus fontinalis* (Kawatsu, 1972), common carp, *Cyprinus carpio* (Sakamoto and Yone, 1978b), red sea bream, *Pagnus major* (Sakamoto and Yone, 1978a) and channel catfish, *Ictalurus punctatus* (Gatlin and Wilson, 1986). Iron deficiency in channel catfish has been characterized by suppressed growth and feed efficiency, as well as reduced hemoglobin concentrations, hematocrits, serum iron, transferrin saturation and red blood cell (RBC) count values (Gatlin and Wilson, 1986). The estimations of iron requirements in fish have been based on haematological parameters (Kawatsu 1972; Sakamoto and Yone, 1978 a,b; Gatlin and Wilson, 1986), tissue iron concentrations (Bjornevik and Maage, 1993), or both (Andersen et al., 1996). Andersen et al. (1996) observed in Atlantic salmon parr, that haematological parameters and tissue iron concentrations were significantly modified by dietary iron supplements.

66

Dietary iron requirements have been reported for several species of fish, including, red sea bream, *Chrysophrys major* (Sakamoto and Yone, 1976; 1978b), yellow tail, *Seriola quinqueradiata* (Ikeda et al., 1973), common carp, *Cyprinus carpio* (Sakamoto and Yone, 1978b), eel, *Anguilla japonica* (Nose and Arai, 1979), channel catfish, *Ictalurus punctatus* (Gatlin and Wilson, 1986) and recently Atlantic salmon, *Salmo salar* (Andersen et al., 1996). Bjornevik and Maage 1993) indicated that the iron requirement of Atlantic salmon is more than 33 mg/kg dry diet. When the present study was in progress, Andersen et al. (1996) estimated that the total dietary iron requirement for Atlantic salmon ranges between 60 and 100 mg of iron/kg. However, estimates of the dietary iron requirement can differ between species or even within the same species, depending on population and a number of environmental and dietary factors that change with conditions. Growth rate, life history stage, food source and particular environmental conditions such as the amount of dissolved minerals in the water during an experiment under different environmental conditions can result in variations in estimates of the dietary requirement. In addition, it is difficult to obtain a sufficiently low concentration of iron in purified diets. One of the major concerns about test diets is that the experimental fish do not grow well on purified diets (Rumsey and Ketola, 1975). To date, the iron requirement for Atlantic salmon parr has been studied by using a cod-muscle meal diet (Bjornevik and Maage, 1993) or a casein-gelatin diet (Andersen et al.,1996).

The present study used a modified casein-gelatin based basal diet. It was conducted to characterize iron deficiency in juvenile Atlantic salmon reared in freshwater and to determine the minimum dietary iron requirement.

2.2 Materials and Methods

2.2.1. Experimental design, fish, rearing system and diets

Atlantic salmon (*Salmo salar*) fingerlings were obtained from the Cobequid hatchery, Department of Fisheries and Oceans, Collingwood, Nova Scotia. They were acclimated for 2 weeks in 100 L circular fibreglass tanks and fed with a low iron purified diet twice daily. The experiment was conducted according to a randomised block design. Three sets of tanks were randomly selected and each set was considered as a block in the experimental design. Within each block the six diets were randomly assigned. Seventy five fish of uniform size (initial mean weight between 5 and 5.2 g) were randomly distributed to each tank. Freshwater was continuously aerated in a header tank, then circulated to the experimental tanks at approximately 2 L/min. The temperature was controlled at 14°C, and the photoperiod was set at a 12 h day and a 12 h night cycle.

Atlantic salmon fingerlings were fed a semi-purified diet (Table 2.1) that was supplemented with reagent grade ferrous sulfate ($FeSO_4.7H_2O$) at each of the following concentrations: 0, 15, 30, 60, 120 or 240 mg/kg. Feed ingredients were thoroughly mixed in a Hobart mixer and then steam pelleted in a laboratory pellet

mill (California Pellet Mill Co. Crawfordsville, Indiana, USA). The pellets were quickly air dried at room temperature in a forced air dryer. Diets were prepared every 8 weeks and stored in a freezer at –30 °C. The fish were fed three times daily to satiety for approximately 20 weeks. Dead fish were removed daily and gross anatomical changes were recorded.

2.2.2. Sampling

The average weight of the fish in each tank was measured every 4 weeks and food consumption was determined every week; the amount of diet fed was adjusted accordingly. The condition factor was calculated as $k = (W \times 100) / L^3$, where L and W were the standard length and weight of the fish.

Twenty fish were randomly sampled from each treatment, killed, and used for haematological measurements and tissue mineral analyse. The kidney, liver, spleen and heart were collected from each animal. Residual blood was removed by blotting with dry tissue paper. The samples were weighed and immediately frozen on dry ice at -45°C. Thereafter were stored at -80°C until analysis. The rest of the fish body was preserved similarly for bone preparation. With respect to this, the fish were thawed and cooked in a microwave oven for 3 to 4 minutes to collect the bones. The bones were then washed in deionized water and oven dried (110°C) for 18h. Bone ash was obtained after combustion at 550°C for 18h.

Table 2.1. Composition of the basal diet.

Ingredients	% of mix (as-fed basis)
Casein, vitamin free[1]	42.0
Gelatin[1]	3.0
Corn gluten meal[2]	6.0
Corn starch[3]	8.0
Dextrin[1]	11.3
Celufil[1]	5.0
Amino acid mixture[5]	1.5
Vitamin mixture[6]	1.0
Mineral mixture[7a,7b]	6.0
Choline chloride[1]	0.2
Herring oil[4]	16.0

[1]US Biochemical Corp., Cleveland, Ohio. [2]Corey Feed Mills Ltd., Fredericton, NB [3]National Starch and Chemical Company, Bridgewater, NJ. [4]Commeau Seafood Ltd., Saulnierville, NS., stabilized with ethoxyquin.

[5]Amino Acid mixture (g/kg diet) : L-Arginine.HCl, 4; DL-methionine, 6.

[6]Vitamin mix (mg or IU/kg diet) : vitamin A, 6000 IU; vitamin D_3, 4000 IU; vitamin E, 250 IU; vitamin K , 30; thiamin.HCl, 40; riboflavin, 50; d-calcium pantothenate, 150; biotin, 0.8; folic acid, 15; niacin, 200; vitamin B_{12}, 0.05; pyridoxine.HCl, 30; ascorbic acid (ascorbyl polyphosphate), 200; inositol, 400; butylated hydroxytoluene, 22.

[7a]Trace elements (mg/kg diet): Mn ($MnSO_4.H_2O$, 32.5% Mn),40; Cu ($CuSO_4.5H_2O$,25.4% Cu),10; Zn ($ZnSO_4.7H_2O$, 22.7%Zn),50; I (KI, 75.4%I),5; Se (Na_2SeO_3,45.6%Se),1; Co ($CoCl_2.6H_2O$, 24.9%Co),5; F (NaF, 45.2% F),4.5.

[7b]Macro-elements (%): P ($Ca(H_2PO_4)_3.H_2O$, 24.6%P), 0.54%; P, (NaH_2PO_4, 22.4% P), 0.08; P (KH_2PO_4, 22.8% P), 0.08; Mg ($MgSO_4$. $7H_2O$, 9.9 % Mg), 0.04; Salt (NaCl, 39.4% Na), 0.25.

Ten additional fish from each diet were randomly sampled for whole body

mineral analysis. Only the gut was removed to avoid any accidental feed residue

in the intestine. Each fish was minced in a grinder before digestion. Blood

samples were collected from the caudal vein of the fish into heparinized vials

using 22-gauge needles. For collection of serum, the vials were stored at 4°C for

an hour, then centrifuged at 1200g for 15 min to separate the serum from the

supernatant. Hematocrit (Hct), hemoglobin (Hb) concentrations and red blood

cell (RBC) count were determined after Sandnes et al. (1988). Hematocrit was

determined by a micromethod and Hb was determined by the Drabkin method

using Hb test kits (Sigma, USA). RBC were measured in a Neubauer-

haematocytometer.

2.2.3. Analytical procedures

The efficiency of feed utilization was determined from the ratio of feed

consumed to weight gained by the fish. Tissue percentages were calculated by

dividing tissue wt by body wt and multiplying each ratio by 100. The indices of

mean cell volume (MCV) and mean cell hemoglobin (MCH) were calculated by

using the following formulae: MCV= (Hct/RBC) x 10 and MCH=(Hb/RBC) x10.

The amounts of iron and other minerals in the fish feed, and in tissue

(liver, spleen, kidney, heart, whole body, serum, and bone) of fish were

measured with an atomic absorption spectrophotometer (Perkin Elmer 5000). A

known quantity of each diet and bone was ashed prior to acid digestion (AOAC., 1990). All reagents and acids used in the digestion were trace-metal analytical grades. The ashes were accurately weighed before being put into a 30-ml Kjeldahl flask. Fifteen milliliters of 6 M HCL, two glass beads and three drops of concentrated HNO_3 were added to each flask. Each sample was digested for one to two hours in a micro-digester (LabConco, Mo, USA., Model 6300). Subsequently, the digested samples were allowed to cool and then each was diluted with 100 ml of deionized water before the Atomic absorption spectrophotometer (AAS) reading was taken. Serum minerals were read directly after dilution with deionized water. Fish tissues were digested on a digester (LabConco, Mo, USA., Model 6300) in a 30-ml Kjeldahl flask with 15 ml of a 3:1 mixture of trace-metal analytical $HCl-HNO_3$ solution. The mixture was diluted before the AAS reading was taken. All digested samples were read by AAS to determine the concentration of various minerals. Each sample was read twice and after three samples a blank was taken to recalibrate the spectrophotometer. The accuracy and precision of the analysis of each element was controlled by comparison with a standard reference material (shark liver from the National Institute of Standards and Technology -NIST, Washington, USA).

2.2.4. Calculation of iron requirement

Nutrient-response curves characteristically ascend to a point and then level off. The iron requirement was determined by assuming a linear response between dietary iron and tissue iron retention. The broken line model of Robbins et al. (1979) was used (Figure 2.1). This model takes the form of;

$Y = L + U(R-X_{LR})$ where,

Y= measured tissue iron value

L= ordinate

U= slope

R= abscissa of the breakpoint

X_{LR}= Dietary iron contents (when X < R).

Spleen, liver, and body iron contents were analyzed for determination of iron requirement of the fish.

Other approaches involving the use of nonlinear models like quadratic regression analysis were avoided because they do not reflect the practically insignificant differences in tissue iron retention below and beyond the maximum values. Also, they do not consider that fish can adapt to a range of dietary iron concentrations between a deficiency on one side or toxicity on the other, i.e., there are minimum and maximum levels of iron intake within which fish can adapt to the nutrient supplied without substantial changes in metabolic processes.
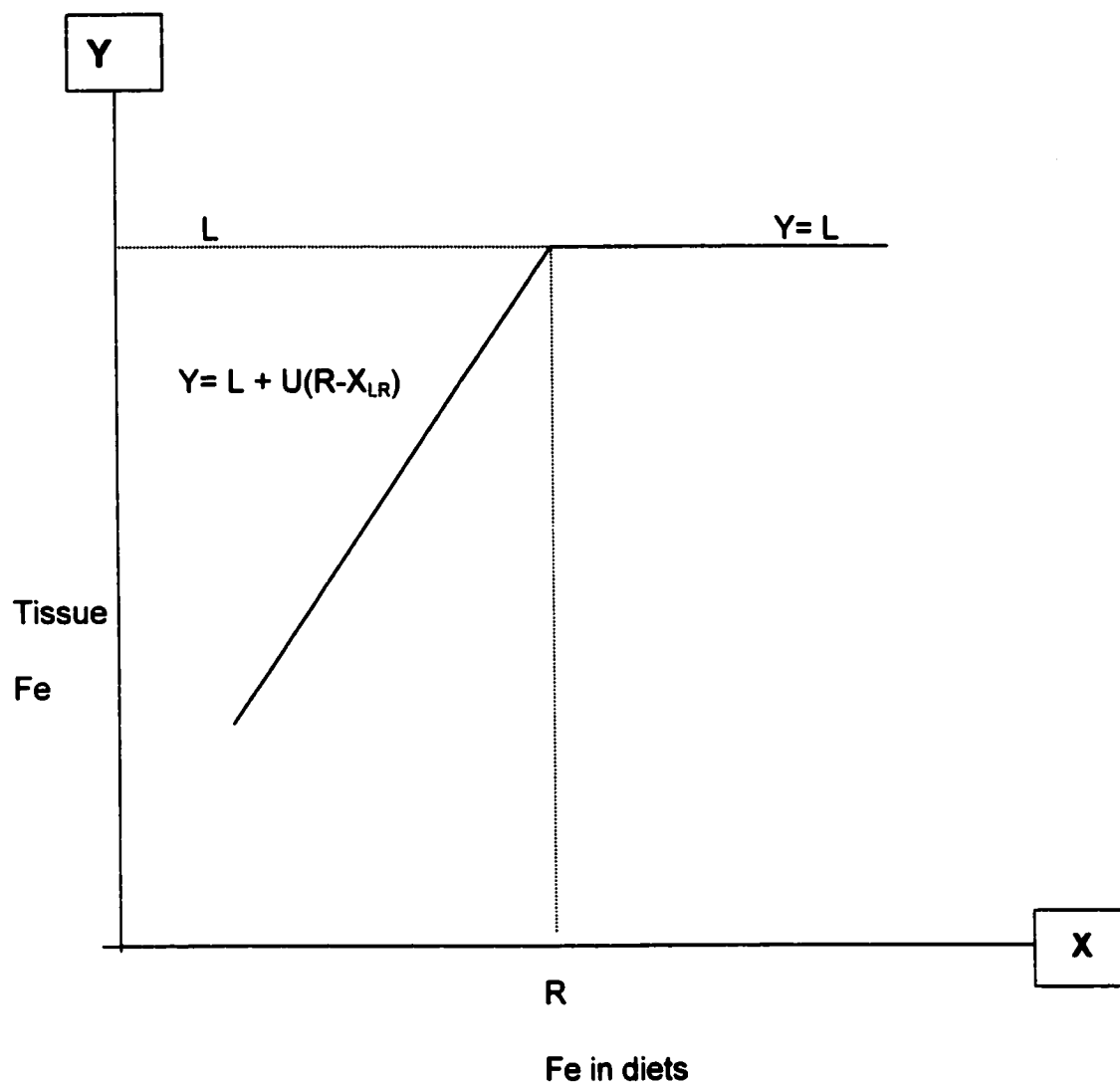
Figure. 2.1. Broken-line regression model used for the determination of the dietary iron (Fe) requirement using liver, spleen, and body iron levels against dietary iron concentration (based on Robbins et al. 1979)

2.2.5. Statistical analysis

The data were statistically evaluated by one-way ANOVA (Zar, 1984)
either for independent groups, or as randomized blocks for correlated
observations. Values of $p \le 0.05$ were considered to be significant. Tissue
percentage values were arc-sin transformed before analysis to avoid non-
homogeneity of error variance. In most analyses, a common error variance was
used. This ensured that effects of the same size had the same "significance"
and were not over or under evaluated because of an incidental small or large
error-variance value. Since the relevant sample sizes were equal, the findings
should be quite robust against heteroscedasticity. Where appropriate,
differences between treatment means were determined at the 5% probability
level using Tukey's test, as described by Zar (1984). All tests were performed
with Systat™ version 8.0 statistical software for Windows™ (SPSS Inc., USA
1998).

2.3. Results

2.3.1. Iron and other mineral concentrations in the experimental diet

The mineral composition of the basal diet was determined on an as-fed

basis and is provided in Table 2.2. The macro mineral concentrations were 0.93,

0.74, 0.06, 0.16 and 0.13% for calcium, phosphorus, magnesium, sodium and

potassium, respectively. The concentrations of trace elements including iron,

zinc, copper, manganese, cobalt, selenium and iodine were 16.4, 121.3, 22.4,

35.4, 10.6, 1.8, and 5.6 mg/kg, respectively. These quantities of macro and

micronutrients are lower than the values found in commercial salmonid feeds

used in Europe and North America (Tacon and De Silva, 1983; Crawford and

Law,1972). However, the values are within the limits of what is considered to be

acceptable for standard salmonid diets (NRC, 1993).

The iron contents in the test diets are provided in Table 2.3. The

determined mean iron contents for the diets supplemented with either 15, 30, 60,

120 or 240 mg of iron/kg were 32.4, 49.6, 78.1, 140.8 and 251.8 mg iron/kg

respectively. Thus, the test diets showed graded increments of iron and as

expected more iron was found in each of the diets than supplied by the

supplement alone. It is impossible in practice, to produce an iron-free diet, or to

provide the exact levels expected in feed because the ingredients used in diet

preparation contain iron, which in turn, cause higher values in the feed.

Table 2.2. Measured mineral concentrations in the basal diet[1].

Macro-minerals	(%)	Micro-minerals	mg/kg
Calcium	0.93± 0.08	Iron	16.4± 1.6
Phosphorus	0.74± 0.04	Zinc	121.3± 4.7
Magnesium	0.06± 0.01	Copper	22.4± 2.8
Sodium	0.16± 0.02	Manganese	35.4± 1.7
Potassium	0.13± 0.02	Cobalt	10.6± 2.4
		Selenium	1.8± 0.2
		Iodine	5.6± 0.9

[1]Mean±SE

Table 2.3. Iron contents of the experimental diets

(mg/kg)	Diet number					
	1	2	3	4	5	6
Iron supplemental[1]	0	15	30	60	120	240
Total iron content[2]	16.4± 1.6	32.4± 3.1	49.6± 3.2	78.1± 4.3	140.8± 3.8	251.8± 4.6

[1]Amount added to various experimental diets

[2]Iron concentrations (mean±standard error) as measured by atomic absorption spectrophotometer

2.3.2. Growth and feed utilization

The average weight gains, feed efficiency values, and percent mortalities, of Atlantic salmon parr during this experiment are given in Table 2.4. The initial mean weights of the groups varied from 5.0 to 5.2 g. After 20 weeks of feeding the various experimental diets, the average fish growth (weight gain) varied from 19.12 to 21.41 g, which is approximately a 4 fold increase over the initial weights. The feed efficiency values ranged from 0.83 to 0.93 and were considered to be acceptable for Atlantic salmon at this stage of life history. Fish mortality varied from 3.2 to 5.1%. Mortality was lowest in fish fed the diet with 60-mg of supplemental iron/kg. However, post mortem examinations showed that none of the deaths was related to dietary treatment. Moreover, there were no significant differences in weight gain, feed utilization,or mortality among dietary groups.

2.3.3. Body condition factors, bone ash and tissue and body weight percentage

Body condition factor (weight/length relationship), bone ash percentages, and tissue weight expressed as of the body weight of the fish from the different dietary groups are presented in Table 2.5.

Dietary treatment did not significantly influence any of the preceding parameters. The average condition factors for fish varied from 1.30 to 1.35. Percentages of bone ash varied in fish ingesting the different diets from 49.9 to

Table 2.4. Average weight gains, condition factors, feed utilization and percent mortalities of Atlantic salmon (Salmo salar) fed diets varying in iron content over a period of 20 weeks[1]

Supplemental iron (mg/kg)	Initial weight (g)	Weight gain (g)	Feed/ gain ratio	Mortality (%)
0	5.2[a]	20.80[a]	0.89[a]	5.1[a]
15	5.1[a]	21.41[a]	0.89[a]	4.3[a]
30	5.0[a]	19.49[a]	0.93[a]	4.4[a]
60	5.0[a]	20.20[a]	0.84[a]	3.2[a]
120	5.1[a]	19.32[a]	0.83[a]	3.4[a]
240	5.0[a]	19.12[a]	0.83[a]	4.6[a]

[1]Means in each column sharing common superscripts are not significantly different ($p \geq 0.05$).

Table 2.5. Effects of dietary iron levels on percentages of bone ash and liver, heart, spleen, and kidney weights (% of body weight)[1]

Supplemental dietary iron (mg/kg diet)	Mean condition factor	Liver (%)	Heart (%)	Spleen (%)	Kidney (%)
0	1.35[a]	1.26[a]	0.153[a]	0.080[a]	1.01[a]
15	1.32[a]	1.23[a]	0.158[a]	0.086[a]	0.92[a]
30	1.33[a]	1.22[a]	0.152[a]	0.095[a]	0.94[a]
60	1.35[a]	1.15[a]	0.137[a]	0.080[a]	0.95[a]
120	1.33[a]	1.24[a]	0.138[a]	0.088[a]	0.94[a]
240	1.30[a]	1.15[a]	0.150[a]	0.110[a]	0.94[a]

[1]Means in each column sharing common superscripts are not significantly different (p≥0.05).

51.8%. Tissue percentages in terms of total body weight also varied little

between groups despite the wide range in dietary iron concentration. In this

regard, liver (%) varied from 1.15 to 1.26, heart (%) from 0.137 to 0.158, spleen

(%) from 0.080 to 0.110 and kidney (%) from 0.92 to 1.01.

Hence the findings support the conclusion that the dietary iron

concentration used in this study (16.4- 252 mg iron/kg) did not have any effect

on the general performance of the fish. Also, there was no visible evidence that

the treatments damaged any of the tissues or affected their growth relative to the

overall growth of the fish.

2.3.4. Haematology

The haematological parameters for fish fed the diets with different

supplemental levels of iron (0, 15, 30, 60, 120 or 240 mg/kg) are provided in

Table 2.6. The levels of iron in the diets affected all parameters. Hematocrit

(Hct) values were significantly lower in fish given no dietary iron supplement than

in those given 15 mg of supplemental iron/kg diet. These values increased

again as the supplemental level of iron in the diet was increased from 15 to 30

mg iron/kg. However, increasing the supplemental dietary iron level above 30

mg of iron/ kg did not significantly increase the Hct level further.

Table 2.6. Haematology of Atlantic salmon (*Salmo salar*) fed diets with different supplemental levels of iron for 20 weeks[1].

Supplemental iron (mg/kg diet)	Haematocrit (%)	Haemoglobin (g/100ml)	RBC counts (10^6)	Mean cell volume (MCV) (nm^3)	Mean cell haemoglobin (MCH) ($\mu g\ cell^{-1}$)
0	30.6^a	6.1^a	1.68^a	182.1^a	3.63^a
15	36.7^b	6.6^{ab}	1.91^b	191.3^a	3.45^a
30	40.3^c	7.3^{bc}	1.81^b	224.5^b	4.03^b
60	40.5^c	7.7^{bc}	1.85^b	218.9^b	4.16^{bc}
120	40.4^c	8.1^c	1.83^b	220.8^b	4.43^c
240	41.5^c	8.0^c	1.86^b	223.6^b	4.30^{bc}

[1]Means in each column sharing common superscripts are not significantly different ($p \geq 0.05$).

Hemoglobin (Hb) concentrations increased as the amount of supplemented iron in the diet was raised to 120 mg of iron/kg and then leveled off. There was, however, no difference ($p \leq 0.05$) in Hb values between fish on the diet deficient in iron (0) and those fed the diet with 15 mg iron/kg. Further, fish ingesting diets with 15,30 or 60 mg of supplemental iron/kg and those consuming the diets with 30, 60, 120 or 240 mg of supplemented iron/kg did not show differences in Hb values. These results indicate that a minimum of 30 mg of supplemental iron/kg in the diet of Atlantic salmon or a total iron concentration of 50 mg/kg is essential for normal Hb levels. Red blood cell (RBC) counts increased significantly when the lowest iron supplement was added to the diet and then there was no additional increase. The maximum RBC value (1.86×10^6 cells/ml) was observed in fish fed diet with the 240 mg of supplemented iron /kg.

Mean cell volumes (MCV) and mean cell hemoglobin (MCH) levels of fish consuming the diets with 15 mg of supplemented iron or no iron supplement were significantly lower than noted for those fed the diets with 30 mg of supplemented iron /kg or more iron supplement. The maximum value of MCV was observed for the fish fed the diet with 30 mg of supplemental iron/kg. No significant differences in MCV values were observed amongst the fish fed the diets with more than 30 mg of supplemental iron/kg. The maximum MCH value was observed in the fish fed the diet with 120 mg of supplemental iron/kg. However there were no differences in mean MCH values in fish receiving 60 mg

or more of supplemental iron/kg diet. In addition, there were no differences in

mean MCH values for fish fed diets containing 30, 60 or 240 mg of supplemental

iron/kg. Therefore, normal MCH values in Atlantic salmon parr were obtained

when a minimum of 30 mg of supplemental iron/kg diet were provided (total of 50

mg/kg diet). Hence, it is evident that an increase in the iron content in the diet

resulted in a corresponding increase in Hb, Hct, RBC MCV and MCH values of

Atlantic salmon, but the increase reached a plateau when the supplemental iron

in the diet ranged from 30 to 60 mg/kg.

2.3.5. Iron content of tissues and serum

Iron concentrations in the liver, kidney, spleen, heart, and bone of salmon

fed the diets with different supplemental levels of iron are shown in Table 2.7.

The iron content of all of these tissues was generally directly related to the

amount of iron in the diet. The contents of iron in the liver, kidney, spleen, heart,

bone were lowest in animals fed the diet with no iron supplemented highest in

those the diet fed with 240 mg of supplemented iron/kg.

Liver values of iron in fish ingesting the diet with 30 mg of supplemented

iron /kg were significantly higher than those of in fish given no supplement of iron

or 15 mg of supplemented iron/kg, and they were significantly lower than in fish

given 60 mg of supplemented iron or more /kg. There were no differences in

liver values in fish fed diets with more than 60 mg of supplemented iron/kg.

Table 2.7. Vertebrate ash and tissue iron concentrations (µg /g) of Atlantic salmon parr fed diet containing different levels of iron for 20 weeks[1,2].

Supplemental iron (mg/kg diet)	Bone ash (%)	Liver (µg /g)	Kidney (µg /g)	Spleen (µg /g)	Heart (µg /g)	Bone (µg /g)
0	51.0 [a]	18.30 [a]	47.47 [a]	142.98 [a]	45.44 [a]	25.2 [a]
15	51.8 [a]	31.07 [a]	53.80 [a]	159.65 [a]	47.03 [a]	25.6 [ab]
30	51.6 [a]	56.69 [b]	54.09 [a]	175.90 [a]	46.56 [a]	28.9 [ab]
60	49.9 [a]	90.47 [c]	56.96 [a]	247.22 [b]	47.16 [a]	32.1 [c]
120	51.2 [a]	106.73 [c]	63.22 [a]	273.05 [b]	46.40 [a]	33.1 [c]
240	49.9 [a]	119.64 [c]	64.23 [a]	283.90 [b]	48.15 [a]	34.8 [c]

[1]Mean± SEM
[2]Means in each column sharing common superscripts are not significantly different ($p \geq 0.05$).

Supplementing a diet with 60 mg of iron/kg may be enough to exceed the response to dietary iron in fish tissue.

Kidney iron concentrations showed an increasing trend from fish fed the deficient diet (47.47 μg/g) towards those receiving the diets with higher supplementation levels. A maximum mean value of 64.23 μg/g was observed for fish fed the diet with 240 mg of supplemented iron /kg. However no significant differences were observed among fish receiving the various treatments. Thus, dietary iron supplementation up to 240 mg /kg did not have any significant effect on kidney iron concentration in this study.

The iron concentrations in the spleens of fish ingesting the diets with 60 mg of supplemented iron or more/kg were significantly higher than those noted in fish fed the diets with 30 mg of supplemental iron/kg or less. The highest concentration of spleen iron was found in the fish fed the diet with the highest level of iron supplementation (283.9 μg/g). The iron-deficient fish (group 1) showed the lowest value at 142.98 μg/g. Spleen iron concentration among fish fed diets 1-3 or 4-6 were not significantly different. This was probably due to saturation of the response to dietary iron.

The spleen always accumulated more iron than the other tissues examined. Considering its higher iron values, the spleen can be considered as being a sensitive tissue to iron. Iron concentrations in the heart were found to

vary from 45.44 to 48.15 µg/g irrespective of dietary treatment. No significant effects of dietary iron supplementation on heart iron concentrations were evident. Vertebrae iron contents (Table 2.7) showed a gradual increase with the level of iron supplementation of the diets. Saturation of the bone with iron was achieved when the supplemental dietary iron levels ranged between 30 to 60 mg/kg.

The amount of iron in the diet also had a significant effect on serum iron concentration, as shown in Figure 2.2. A trend was noted toward increasing serum iron concentration s the supplemental dietary iron concentration was increased. Serum iron levels showed no significant differences between the fish fed diets 1-3 (0, 15 and 30 mg of supplemented iron/kg) and between those ingesting 30 mg of supplemented iron/kg or more. This suggests that the serum is less helpful for estimating iron sufficiency than some of the other tissue examined. However, fish given no iron supplement did show a significantly lower level of iron in their serum than those given 15 mg of supplemented iron /kg or more. Thus, deficient fish exhibited significantly low serum values.

The mean iron concentration of the total body tissues with no supplement was 34.2 µg/g (Table 2.8). Gradual increases in the amounts of supplemental dietary iron resulted in higher values for body iron. The groups that received 60 mg or more of supplemental iron /kg diet had significantly higher whole body iron concentrations than the groups receiving diets with lower iron supplements. The
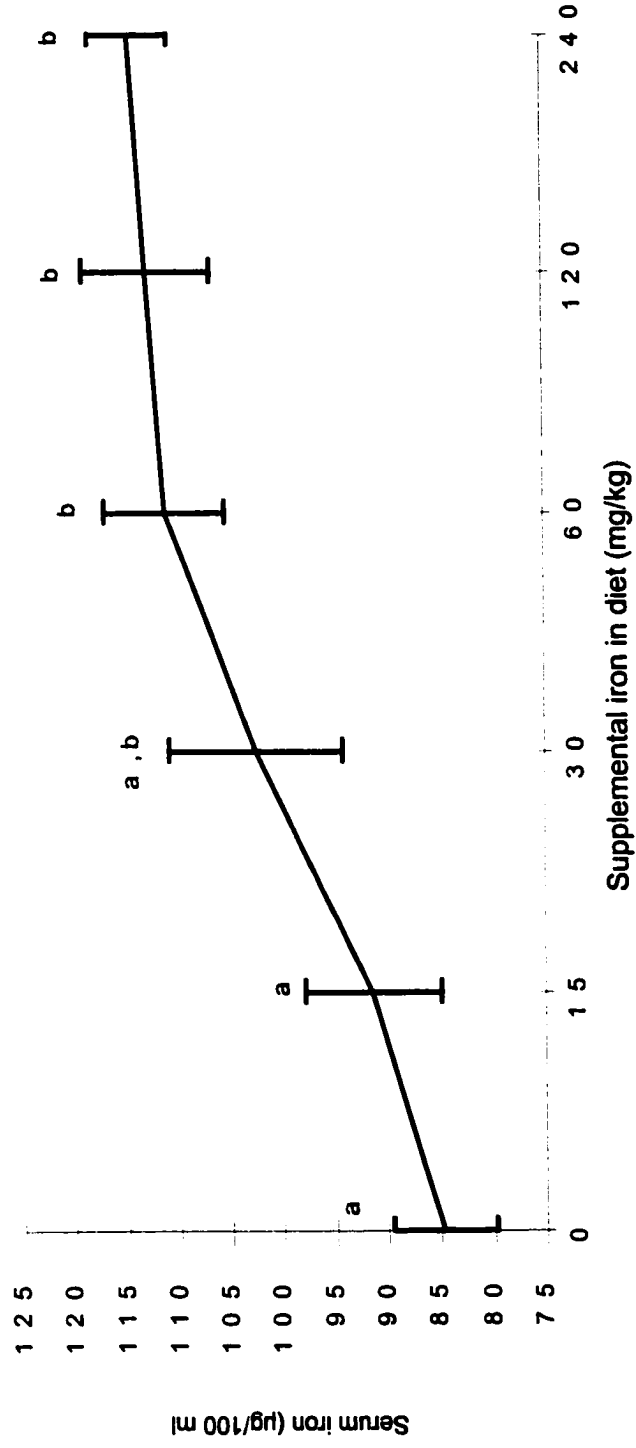
Figure 2.2. Serum iron concentrations in Atlantic salmon parr fed diets containing different levels of supplemental iron for 20 weeks. Values are expressed as mean±sd.

Table 2.8. Whole-body mineral concentrations of Atlantic salmon parr fed diets with different levels of supplemented iron for 20 weeks[1,2].

Supplemental iron (mg/kg diet)	Ca (%)	Na (%)	K (%)	Mg (%)	Mn (µg/g)	Zn (µg/g)	Fe (µg/g)
0	2.1[a]	0.48[a]	0.80[a]	0.09[a]	9.1[a]	125.2[a]	34.2[a]
15	2.2[a]	0.47[a]	0.75[a]	0.10[a]	5.0[a]	121.6[a]	43.5[b]
30	2.2[a]	0.24[a]	0.76[a]	0.09[a]	7.3[a]	124.3[a]	45.2[b]
60	2.2[a]	0.19[a]	0.75[a]	0.08[a]	5.4[a]	125.3[a]	56.4[c]
120	2.1[a]	0.23[a]	0.79[a]	0.09[a]	9.6[a]	140.9[a]	60.9[c]
240	2.2[a]	0.32[a]	0.85[a]	0.10[a]	5.8[a]	144.9[a]	67.0[c]

[1] Mean± SEM
[2] Means in each column sharing common superscripts are not significantly different ($p \geq 0.05$).

minimum dietary iron supplement that led to a saturation of whole body iron was found to be 60 mg/kg.

It is clearly evident from the data presented that the saturation of iron concentrations in liver, spleen and the body occurred when the fish were fed a diet with 60 mg of supplemental iron/kg (total iron concentration in diet was 78 mg/kg).

2.3.6. Concentration of minerals in whole body

Table 2.8. shows the concentrations of selected minerals in the whole bodies of the fish in relation to diet treatment. Dietary iron concentrations had no significant effects on whole body concentrations of all of the minerals examined viz., Ca, Na, K, Mg and Zn except iron itself. Therefore, it is concluded after considering the above data, that body mineral levels are not appreciably affected by dietary iron supplementation.

2. 4. Discussion

This study was designed, not only to determine the iron requirement of Atlantic salmon parr on the basis of biological and biochemical parameters, but also to establish the clinical parameters that are suitable to detect iron deficiency. Growth and feed efficiency were not significantly affected by diets with varying iron levels. The diet containing no iron supplement caused iron deficiency in Atlantic salmon, and this was characterized by reduced Hb concentration , Hct, and RBC counts as well as serum iron concentration (Table 2.6, Figure 2.2). Fish ingesting diets with 15 mg or more of supplemented iron/kg had normal RBC counts. A minimum of 30 mg of supplemented iron/ kg diet was required to maintain normal Hct, Hb and MCV values. MCV values were significantly reduced in fish fed diets with less than 30 mg of supplemented iron/kg, indicating that the availability of iron for erythropoietic tissue was reduced in fish fed iron deficient diets. Microcytic hypochromic anaemia has been widely recognized as a sign of iron deficiency in fish as well as in higher animals (Lall, 1989). Iron deficiency anemia has been reported for several fish species fed diets with low iron contents (< 20 mg Fe/kg of diet); however growth and feed efficiency have not always been adversely affected (Kawatsu, 1972; Sakamoto and Yone, 1976, 1978a).

In this study, Hb values of fish fed diets containing various levels of iron were lower than the values reported by Bjornevik and Maage (1993) and Andersen et al. (1996) for Atlantic salmon. Similarly, Hct values were also lower than those of Bjornevik and Maage (1993) but were similar to those of Andersen et al. (1996). Hematocrit values have been used in higher vertebrates to detect their iron status (Conard et al., 1983). However, fish stress may cause significant variations in the Hc values (Schiffman and Fromm, 1959). Recently, Iversen et al. (1998) reported a significant increase in Hct values in stressed Atlantic salmon. The Hc values can be used to demonstrate when fish are suffering from a severe iron deficiency, however, they are less useful as a clinical indicator of the nutritional adequacy of iron in salmon, this is because the values do not vary significantly over a wide range of dietary iron concentration. All three hematological parameters, Hct, Hb and RBC should therefore be used to detect anemia in fish caused by iron deficiency.

One of the challenges in this study was to formulate an experimental diet that was not only low in iron concentration but was also readily accepted by the salmon. A major problem in the earlier studies designed to investigate the nutrient requirements of fish was the poor acceptability of semi-purified diets (Rumsey and Ketola,1975; Gatlin and Wilson, 1986). Indeed, Atlantic salmon showed low feed intake and marked growth depression (Rumsey and

Ketola,1975) on these diets. Channel catfish fed a purified diet based on egg-white protein with no iron supplement also showed growth below that normally observed when practical diets were fed (Gatlin and Wilson, 1986). It was not clear whether the iron deficiency itself or the use of egg white as the protein source caused the lower growth rate. Several researchers have incorporated either fish meal, cod muscle or artemia into their experimental diets to overcome low diet palatability (Roeder and Roeder,1966; Sakamoto and Yone, 1978a; Bjornevik and Maage, 1993). In this investigation, a casein-gelatin based semi-purified diet was readily accepted by Atlantic salmon. This diet has been successfully used in several other micronutrient studies on this species (Lall and Hines, 1987).

The liver is a major iron storage organ (Walker and Fromm, 1976) and responds directly to changes in dietary iron concentration (Bjornevik and Maage, 1993). However, other organs can store significant amounts of iron so they must also be considered as possible indicators of dietary iron status. In this study, the range of iron concentrations determined in the salmon liver was similar to that observed by Andersen et al. (1996) but lower than that reported by Bjornevik and Maage (1993). The whole-body iron contents of fish fed the various experimental diets in this study were higher than those in the other two studies on iron utilization by salmonids (Bjornevik and Maage,1993; Andersen et

al.,1996) but lower than the values reported by Shearer et al. (1994) for Atlantic salmon. These differences may have arisen due to variations in the different genetic populations or because of the dissimilar life stages of fish that were examined (Shearer et al. 1994). However, the total body mineral contents of fish observed here showed similar trends to those reported by Shearer et al. (1994), Bjornevik and Maage (1993), and Andersen et al. (1996). In this study, the iron concentrations in the liver, kidney, spleen, heart, total body and serum varied significantly (p>0.05) with the level of dietary iron intake. In all cases, the iron content increased as the dietary iron concentration was increased, but in three cases, namely, the spleen, liver, and whole body a plateau in iron concentration was reached when the fish received 60 mg or more iron/kg diet. A similar plateau was reached in the serum iron level but at a lower level of dietary iron (30 mg of supplemented iron/kg).

There were no significant differences in heart and kidney tissue iron contents between fish fed the diets regardless of iron concentration, perhaps because these fish absorbed sufficient iron from water to maintain adequate levels in these vital organs during dietary deficiency.

It is noteworthly, that the spleen had a tendency to accumulate more iron than the liver or kidney. Indeed, it can retained about seven-fold more iron than the liver when no dietary iron supplement was given and about three-fold more

when 120 mg of supplemented iron/kg or more given. Thus, the spleen may be considered to be a sensitive organ for determining the dietary iron status of Atlantic salmon.

It appears from the haematological results in this study that a supplement of 30 mg of iron/kg diet is required to maintain the minimum acceptable iron status in Atlantic salmon. This conclusion is also partially supported by the results of measuring the serum iron concentrations in relation to diet treatment. Bjornevik and Maage (1993) used iron concentrations in the serum, liver, and whole body as indicators of iron requirement and found that the minimum dietary iron requirement for Atlantic salmon was met with a supplement of 60 mg of iron/kg. Above this level, 'large differences' were not seen in hepatic tissue iron levels. Similar results were obtained when Wekell et al.(1986) used the level of body iron. In the present study, the levels of iron in the spleen, liver, and whole body reach a plateau at 60 mg iron/kg diet, implying that 60 mg of supplemental or 78 of total iron/kg is the best estimate of dietary iron requirement for Atlantic salmon parr. Andersen et al. (1996) using the broken-line model (Robbins et al., 1979) related Hb and liver iron concentrations to dietary iron levels and they reported that the iron requirement for Atlantic salmon lies between 60 and 100 mg of iron/kg diet. However, when the same model was used to analyze the data for spleen, liver and whole body iron contents, in this study (Figures 2.3 to

2.5) the estimates of iron requirement varied between 50 and 70 mg of iron/kg

diet. In this regard, the iron requirement of Atlantic salmon was found to be 50

mg of supplemental iron /kg diet when the data from liver were plotted in relation

to dietary level (R^2=0.97). In the case of the spleen the iron requirement was

calculated to be 61.4 mg iron/kg (R^2=0.95). Lastly when whole body iron

contents were considered, an estimate of 70.4 mg of supplemental iron/kg diet

(R^2=0.96) was found.

The response of the spleen to dietary iron supplementation was highest,

followed by the liver and total body values. The continuous bioaccumulation of

iron from graded levels of dietary iron by the various tissues resulted in different

requirement values when fitting the model. Once again the lowest iron

requirement was obtained when the liver was used in the present study whereas

Andersen et al. (1996) obtained the highest requirement when the organ was

employed. Body iron values gave the highest requirement value. One of the

reasons for the differences in findings between in these studies may be the

shorter duration of the experiment by Andersen et al. (1996). It is possible that

during the shorter feeding trial, the fish were unable to metabolize sufficient

tissue iron to reach physiological homeostasis. Nevertheless, it is evident from

Figures 2.3, 2.4, and 2.5 for liver, spleen and whole body iron concentrations in
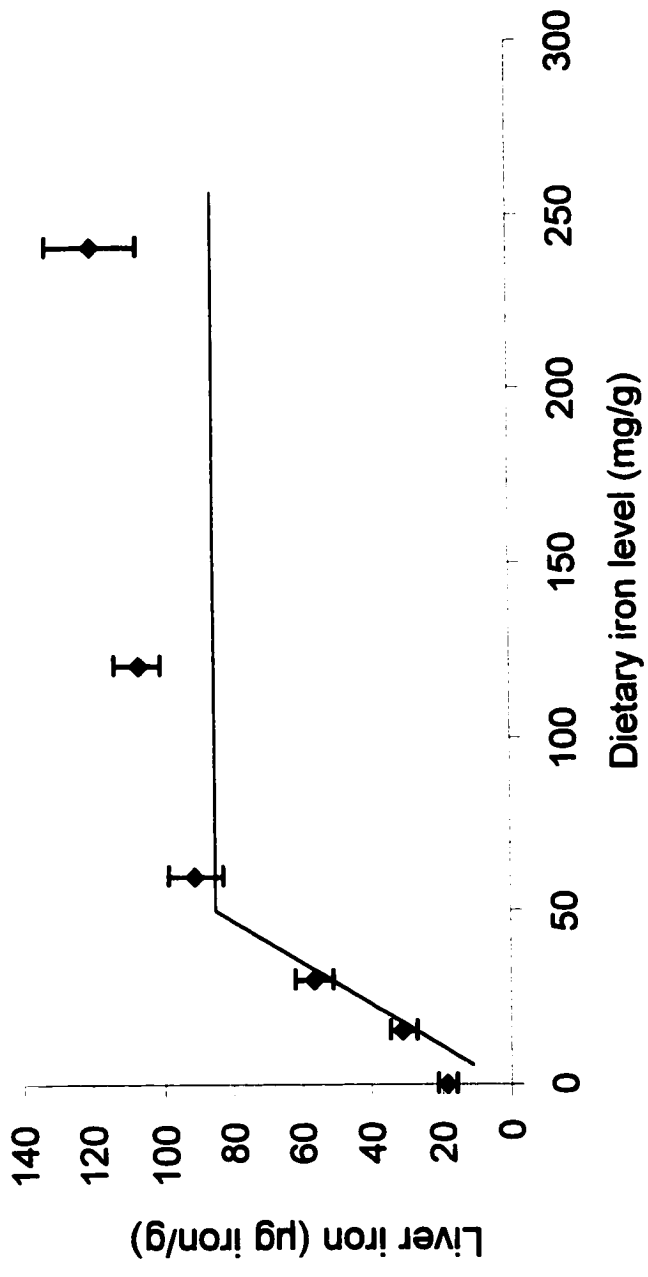
Figure. 2. 3. Relationship between liver iron concentration and dietary iron levels after fitted by broken-line model. (R^2=0.97).
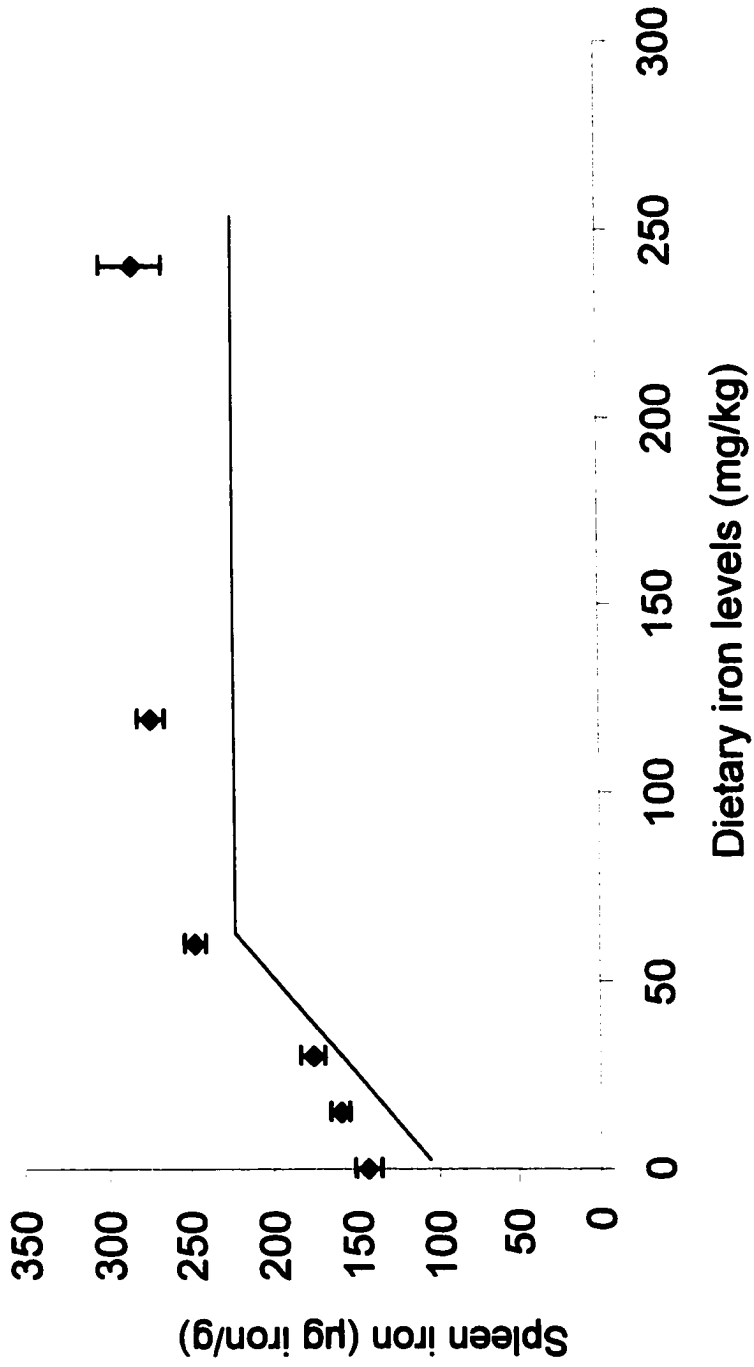
Figure. 2. 4. Relationship between spleen iron concentration and dietary iron levels after fitted by broken-line model. (R^2=0.95).
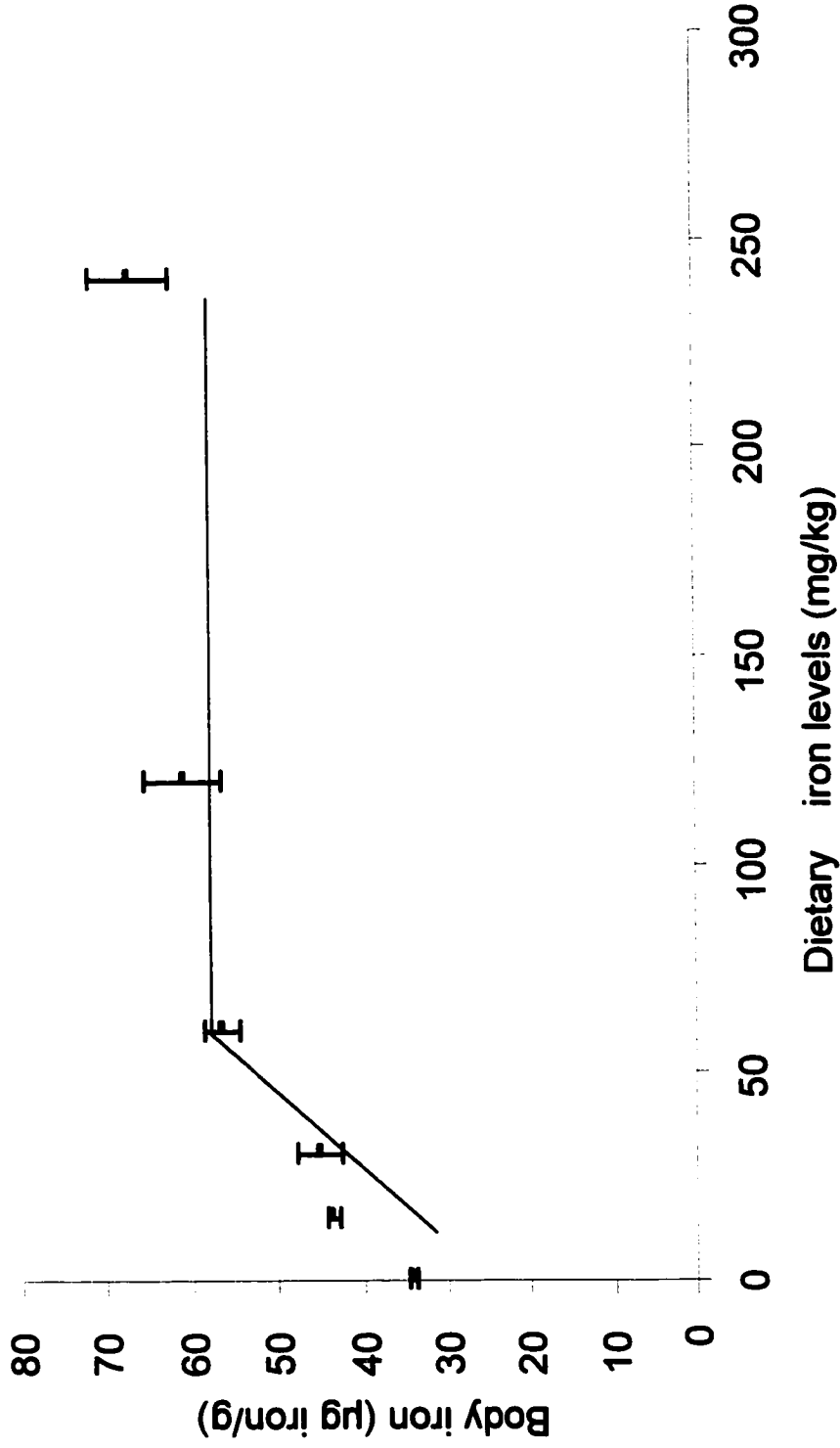
Figure. 2. 5. Relationship between body iron concentration and dietary iron levels after fitted by broken-line model. $(R^2=0.97)$.

response to dietary treatment that saturation was reached when the fish were fed a diet with 60 mg of supplemented iron/kg.

Several factors may have contributed to the differences in findings between studies for the iron requirement of Atlantic salmon. The major variables likely include genetic factors, growth rate, gut physiology, concentration and extent of absorption of iron from the water, bioavailability of iron from the diet, analytical methods used to measure iron in the feed and tissues and other dietary factors such as fiber, protein, copper and zinc concentrations. The metabolic demand for iron may vary significantly among populations. Often in nutritional studies all nutrients, except the one under study, are supplied far in excess, (Kaushik et al., 1998) or vice versa (Henry and Miller, 1995). This will result in requirement values that are either too low or too high. Andersen et al. (1996) used a much higher range for dietary iron supplements (0-400 mg iron/kg) in their experiment. In the current study, certain tissues, especially the liver and total body tissues, continued to accumulate iron in response to the increase in dietary iron concentration, even when the dietary concentrations were clearly above normal requirements. By adopting liver iron concentrations fitted to a 'broken-line' curve, Andersen et al. (1996) found that the iron requirement lies close to 100 mg of iron/kg diet. This value could be estimated more accurately if diets containing more than 200 mg of iron/kg could be excluded to avoid the

background error. The content of iron in the liver levels off at higher dietary iron levels. This could be a toxic effect rather than an indication of dietary requirement. In addition, the same 'broken-line' method using the iron content in body tissues gave an iron requirement of around 150 mg of iron/kg (Andersen, 1997).

A common pattern emerges from the hematology indicators and measurements of iron concentration in organs, body tissues and serum. Iron values increase as dietary iron increases from 15 to 60 mg of iron/kg diet, but most parameters level off as the supplements become higher. It can be concluded that the requirement of juvenile Atlantic salmon is 60 mg of supplemented iron/ kg diet and any level lower than this will reduce the amount of hemoglobin and the level of iron in the tissues and serum. This value agrees with the suggestion by Bjornevik and Maage (1993) that the iron requirement of Atlantic salmon will be more than 33 mg of iron/kg dry feed and it agrees with the lower limit estimated by Andersen (1996).

Since the iron content in feed ingredients such as fishmeal, soybean meal, corn gluten meal, wheat middlings, etc (NRC, 1993), can vary, depending upon the composition of the diet formulation, the level of iron in the feed can also vary. Most commercial diets for Atlantic salmon in Europe (Tacon and DeSilva, 1983) and in Canada (Lall and Hines, 1987) contain adequate iron (158 mg of

iron/kg to 800 mg of iron/kg). The results of the present study on Atlantic salmon parr suggest that it is not necessary to supplement commercial salmon feeds with iron. However it is necessary to measure bioavailability of iron in feed ingredients to ensure that the diet supplies iron in a form which can be used by the salmon. Accordingly, the next experiment in this investigation determined the bioavailability of iron from different sources for Atlantic salmon.

CHAPTER 3. Bioavailability of iron from inorganic and organic feed supplements in Atlantic salmon (*Salmo salar*)

3.1. Introduction

Iron is by far the most abundant trace element in the fish body and its nutritional value as a dietary constituent is widely recognized. Most plant and animal by-products used in fish feeds contain large, though variable, concentrations of iron (NRC, 1993). The utilization of feed and feed supplements as a source of iron depends not only on total iron concentration but also how much can be absorbed from the gut and used by the animal's cells and tissues (Henry and Miller, 1995). This, in turn, may depend on the age and species, the intake of the iron relative to the need, the chemical form in which it is ingested by animal, the amount and proportions of other dietary components with which it interacts metabolically (Hallberg, 1981; Forbes and Erdman, 1983; Carpenter and Mahoney, 1992), and environmental factors such as water quality and temperature (Hilton, 1989; Lall, 1989). The assessment of the physiological availability of minerals in feed supplements presents difficulties that do not exist with organic nutrients such as amino acids and sugars. Ordinary feed digestibility experiments are of limited value because feces constitute an important and variable pathway of excretion for many minerals and therefore contain minerals that have previously been absorbed. In addition, some iron may not be absorbed from the diet while in the gastrointestinal tract. Balance studies,

104

which measure the difference between the amount of mineral appearing in the urine and feces over a specified period and the amount ingested, can therefore be misleading indicators of availability. Furthermore, the influence of body stores may change as the study progresses and some losses (skin, scale and gill excretions) may go undetected (Lall, 1989).

Bioavailability is a quantitative measure of the utilization of a nutrient under specified conditions to support the organism's normal structure and physiological processes (Fox et al., 1981). The concentration of iron in common feed ingredients is highly variable and greatly influenced by the degree of contamination from ferrous metal during processing. In animal protein supplements, iron may be present as iron-porphyrin, myoglobin, and hemoglobin which is often referred to as heme iron. In cereal grains, a small proportion of iron occurs as an iron-phytin complex. Non-heme or inorganic forms of iron occur in the ferrous (Fe^{+2}) and ferric (Fe^{+3}) oxidative states. In terrestrial animals, solubility of the heme iron is not significantly influenced by gastrointestinal conditions because the heme molecule is absorbed as such and the porphyrin ring is split off within the mucosal cells of the gut (Wienk et al, 1999). The biological availability of inorganic compounds varies considerably. In monogastric animals, biological availability from ferric ammonium citrate, ferric chloride, ferrous sulphate, ferrous ammonium sulfate and ferrous fumarate is relatively high (Ammerman and

Miller, 1972). Iron forms classified as poor sources include ferric oxide, ferric

orthophosphate, ferrous carbonate and sodium iron pyrophosphate.

Information on the bioavailability of iron and other trace elements by fish is

limited. Paripatananont and Lovell (1995) reported that zinc methionine had a

lower bioavailability than zinc sulphate when used as a dietary source of zinc in

both purified and practical diets of channel catfish. In similar studies on channel

catfish, Lim et al. (1996) found that iron methionine and iron sulphate were equally

effective as iron supplements. Recently, Standal et al. (1999) using isotopic iron

reported that the bioavailability of heme and non-heme iron in Atlantic salmon may

be similar to the values reported for other vertebrates. The absorption of non-

heme iron is also affected by other dietary factors. For instances, it may be

enhanced by ascorbic acid (Hilton et al., 1978), or inhibited by substances like

phytic acid and phosphorus (Brody, 1994).

Although several methods are available to measure the bioavailability of

iron in terrestrial animals, the hemoglobin regeneration assay (Fritz et al., 1970)

or the extrinsic radioiron tag technique using single meals (Benito and Miller,

1998) have produced the most consistent results. Hemoglobin (Hb) regeneration

in anemic animals is considered to be a sensitive method to determine iron

bioavailability in monogastric animals (Fritz et al., 1970; Pla and Fritz, 1970), but

this method has not been used in fish studies. In this assay, test animals are

depleted on a low iron diet until hemoglobin and/or hematocrit deteminations

indicate that animals have developed anemia. The animals are then placed on test diets for a 2 week period. At the end of this period, individual blood samples are drawn and the responses of hemoglobin are compared between test animals that received known graded quantities of iron from ferrous sulfate and animals that received similar quantities of iron from test samples. Results are interpreted by comparing the quantity of iron that was supplied by the sample with the quantity of iron supplied by the reference ferrous sulfate that gave the same response.

The present experiment was conducted to measure the bioavailability of iron from two inorganic compounds (ferric chloride, ferric oxide) and two common organic feed supplements (herring meal, spray-dried blood meal), using a modification of the Hb regeneration assay (Fritz et al.,1970). Like most animal studies, reagent grade ferrous sulfate was used as the reference material in the test diet.

3.2. Materials and Methods

3.2.1. Experimental

Test ingredients

The following inorganic and organic feed ingredients were selected for the bioavailability assay: ferrous sulfate ($FeSO_4.7H_2O$), ferric chloride ($FeCl_3.6H_2O$) ferric oxide (Fe_2O_3), fish meal (Commeau Seafood Ltd., Saulnierville, NS.,

National Sea Products, Lulenberg, NS., Corey Feed Mills Ltd., Fredericton, NB)

spray-dried blood meal (Corey Feed Mills Ltd., Fredericton, NB). All inorganic

iron supplements were reagent grade (Fisher Scientific, Ontario). Several

samples of steam-dried fish meal and spray-dried blood meal were analyzed for

iron concentration before selecting them for bioassay. Norse-LT94® was selected

as the source of fish meal because of its high protein quality as determined in our

laboratory by Anderson et al (1993). Two spray dried bovine blood meal samples

were analyzed and their acceptability was determined in a separate protein

digestibility experiment (Lall, unpublished). A high quality spray dried blood (Corey

Feed Mills Ltd., Fredericton, NB)was selected for the hemoglobin regeneration

assay.

Test diets

Semi-purified test diets were prepared according to the formula described in

Table 2.1. In each case, the iron source was incorporated into the basal diet to

supply a total of 30 mg of iron kg^{-1} of food. In the low iron diet that contained no

iron supplement and the other test diets,10% deionized water was added and then

the diets were cold pelleted in a California pellet mill without any steam. After

processing, the diets were divided into 500 g batches, and subsequently these

were placed in sealed plastic bags and stored at $-30^{\circ}C$ to avoid lipid peroxidation.

Diets were prepared every 8 weeks and stored until used. The average iron,

copper and zinc levels in the basal diet were 13.1±1.8, 26.5±4.2 and 145 ±6.9

mg/kg, respectively.

Fish

Atlantic salmon fingerlings obtained from Cobequid hatchery, Department

of Fisheries and Oceans, Collingwood, Nova Scotia, were acclimated for a 2-

week period in 100L circular fiberglass tanks. During that time they were fed a

commercial diet (Corey Feed Mills Ltd., Fredericton, NB) twice daily. In order to

produce an iron deficiency, eight hundred Atlantic salmon juveniles were

transferred to four tanks (20 m^3) and then they were gradually acclimated to a

semipurified diet (Table 2.1) that contained no iron supplement for a period of one

week. Fish were maintained for 32 weeks on this low iron diet which contained

13.1±1.8 mg iron/kg that originated mainly from the dietary components such as

casein. At the same time to maintain a control group, 60 fish were fed a diet

containing reagent grade ferrous sulphate (64.1±2.8 mg iron/kg). The fish were

reared in filtered freshwater that had an average iron concentration of 26 µg/L.

Each tank received water at approximately 6 L.min^{-1}. Dissolved oxygen content of

the water during the experiment ranged from 9.3 to 10.4 mg/L. Water was

continiously aerated and the temperature controlled to 15±0.9 °C. The

photoperiod was 12h light and 12h dark. During the experimental period, fish were

hand fed three times per day during the week and daily on weekends. Records

were kept of weekly feed consumption, daily mortalities and behaviour. After 32

weeks when the fish were anemic, they were divided into groups of 75 fish and

transferred to 500 L circular tanks and fed, for 16 days, either a "standard" diet

(Table 2.1) supplemented with six different concentrations of ferrous sulphate

($FeSO_4.7H_2O$) or a "test" diet, supplemented with one of four different sources of

organic (fish meal and blood meal) or inorganic (ferric chloride and ferric oxide)

iron. Average weight of Atlantic salmon was 68± 4.6 g.

3.2.2. Hemoglobin regeneration assay

The "standard" diet was used to calculate a standard curve for Hb

concentration, and the "test" diet was used to calculate bioavailability by

comparing results with the standard diet. Six of the groups of fish were fed the

"standard" diet, which was the basal diet supplemented with 0, 10, 20, 30, 40 or

50 mg of iron as ferrous sulfate. After 16 days on this diet, 25 fish were

randomly sacrificed and blood was sampled to calculate hemoglobin levels and

produce a standard curve. Four other groups of fish were fed "test "diets

consisting of the basal diet supplemented with organic and inorganic sources of

iron. Again, they were fed their prescribed diets for 16 days. Then, 25 fish per

group were sacrificed and blood was drawn for the hemoglobin and hematocrit

assays according to the procedure described in Chapter 2. Blood haematocrit

(Hct) and hemoglobin (Hb) levels were measured as described in Chapter 2. A

standard curve of Hb concentration was developed by plotting blood hemoglobin

levels of fish against the amount of ferrous sulfate in the diet of animals fed the

"standard" diet. The relative bioavailability of iron was calculated from the standard

curve of FS using the method of Fritz et al. (1970).

Relative bioavailability value (RBV) = 100 x

$$\underline{\text{mg/kg Iron as } FeSO_4.7H_2O \text{ for measured Hb response}}$$

mg/kg Iron as test iron source for equal Hb response

3.2.3. Mineral analysis of feed

Samples of the fish meals, blood meals, test diets and experimental diets

were analyzed for Ca, P, Mg, K, Na, Cu, Fe, Mn and Zn using an atomic

absorption spectrophotometer (Perkin Elmer Model 503, Norwalk, CT, USA).

3.2.4. Statistical analysis

Hemoglobin regeneration data were subjected to analysis of variance for

completely randomized design. Differences among treatment means were

determined at the 5% probability level using Duncan's new multiple- range test,

as described by Steel and Torrie (1960). Where appropriate a t test was also

used to measure the significance of differences between treatment means

$(p>0.05)$.

3.3. Results

3.3.1. Proximate and mineral components of test materials

There were significant differences in the proximate and mineral

compositions of fish and blood meals (Table 3.1). The high ash contents of

some herring meals (HM2 and HM4) suggests that these meals were made from

filleted fish racks as the amounts of Ca, P and Mg were higher than in the other

meals. The iron concentrations of the various fish meals ranged from 176-300

mg kg^{-1}. These differences were mainly due to the processing conditions that

were used during the production of the fish meals. The iron levels in the two

blood meals were 2975 and 3180 mg kg^{-1}. The iron contents of the three

inorganic supplements were very close to the manufacturers specifications:

ferrous sulfate, 209 mg g^{-1}; ferric chloride 217 mg g^{-1}; ferric oxide, 702 mg g^{-1}.

3.3.2. Relative bioavailability of iron

A relatively long period of time was required to reach a low concentration

of hemoglobin in the blood of Atlantic salmon. Figure 3.1 shows a standard

curve, created by plotting concentration of hemoglobin in the blood of Atlantic

salmon against the concentration of iron in the diet, when ferrous sulfate was the

only source of iron in the diet. As the concentration of ferrous sulfate in the diet

increased from 0 to 50 mg kg^{-1} of diet, the concentration of hemoglobin in the

blood increased from 5.5 g 100ml^{-1} to 8.7 g 100 ml^{-1}.

Table 3.1. Proximate and mineral analyses of various fish meals and blood meals

	Herring meal (HM)				Menhaden meal	Anchovy meal	Norse-LT94®	Blood meal (BM)	
	HM1	HM2	HM3	HM4				BM1	BM2
Proximate analysis (%)									
Dry matter[a]	97.3	93.9	93.4	96.3	96.2	89.6	91.6	93.1	93.7
Protein[b]	83.7	78.5	78.6	78.4	67.7	70.4	80.6	86.4	88.1
Lipid[b]	9.4	11.5	14.3	9.6	10.7	11.4	12.0	1.1	1.2
Ash[b]	11.0	14.6	12.8	14.0	21.5	17.5	13.1	4.3	4.1
Macro elements (%)[b]									
Ca	2.79	2.81	3.01	3.94	6.89	4.13	2.39	0.45	0.39
P	2.06	2.17	2.19	2.44	3.65	2.60	2.07	0.33	0.30
Mg	0.23	0.26	0.20	0.30	0.27	0.28	0.19	0.15	0.15
K	0.76	1.05	0.96	0.43	0.54	0.79	1.64	0.13	0.14
Na	0.46	1.57	0.71	0.69	0.67	1.07	0.83	0.40	0.38
Micro elements (mg/kg)[b]									
Cu	5.7	5.8	6.5	4.9	7.6	4.4	7.5	8.2	10.2
Mn	9.0	7.0	15.0	16.0	27.0	16.0	9.0	7.2	6.7
Zn	120	79	86	107	122	69	108	210	249
Fe	176	168	181	220	249	300	263	2975	3180

[a] Expressed as a percentage of the fish meal (as-received).
[b] Expressed as a percentage of dry matter.
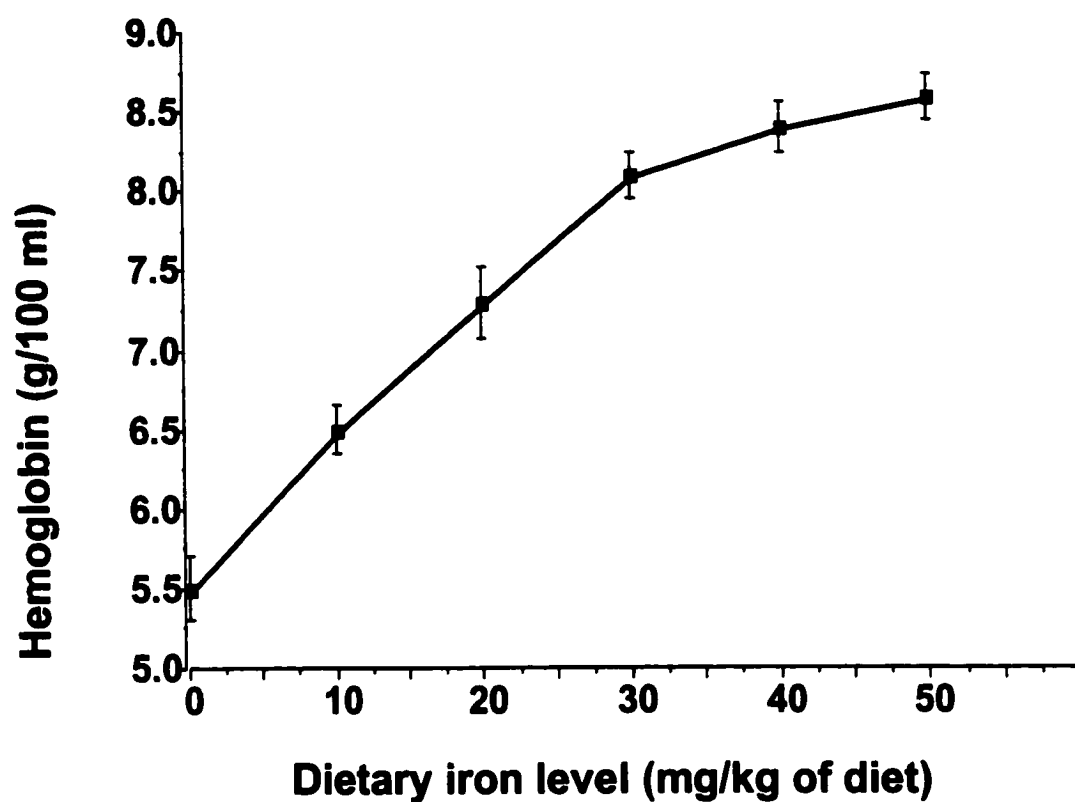
Figure 3.1. Effect of the iron concentration in the diet on the

concentration of hemoglobin in the blood of Atlantic salmon.

The relative bioavailability values (RBV) of organic and inorganic sources of iron are indicated in Table 3.2. The RBV of iron for ferric chloride, ferric oxide, blood meal and herring meal were 98.9, 17.8, 52.3 and 47.1, % respectively.

Iron from heme sources such as blood meal was readily available, but there was no significant difference ($p \geq 0.05$) between blood meal and herring meal in the bioavailability of their iron content. Herring meal had a significantly lower bioavailability of iron than noted for ferrous sulfate.

Table 3.2. Bioavailability of iron from different sources. The relative bioavailability of iron was measured using ferrous sulfate supplemented at 30 mg iron/kg of food as the standard.

Iron supplement / Test materials	mg/kg	Hb (g/100 ml)	Hct (%)	Relative bioavailability (mean ±SE)
None[a]	0	5.5±1.1	30.6±2.1	-
Ferrous sulfate	10	6.5±0.7	36.7±0.9	-
	20	7.3±0.8	42.2±1.8	-
	30	8.0±0.5	44.8±2.3	-
	40	8.4±0.9	44.7±1.9	-
	50	8.7±0.8	45.3±2.1	-
Ferric chloride	30	7.9±0.6	44.9±0.8	98.8±1.9
Ferric oxide	30	5.9±0.4	35.3±1.3	17.8±2.3
Blood meal	30	7.2±0.7	43.0±2.0	52.3±3.1
Herring meal	30	6.8±0.7	41.5±1.1	47.1±2.1

[a]13.1 mg iron/kg of diet.

3.4. Discussion

The elementary iron from different feed sources or within the same ingredient varied considerably. This, in turn, will affect the trace element status in the diet. As a result the iron status will vary from diet to diet. This finding agrees with the fact that the iron content of commercial fish feed varies from 200 to 800 mg kg^{-1} feed. The finding that the iron content varies between batches of feed or even within the same ingredient, suggests that the bioavailable iron content of feed is not constant.

The bioavailability of iron from any source is considered to be that portion of the total iron that is metabolizable. When assaying iron bioavailability, it is therefore necessary to use an organism whose need will not exceed the amount provided. In animal assays of iron bioavailability, iron needs and assessed by a growth phase and /or creation of iron deficiency through feeding iron deficient diets (Mahoney and Hendricks 1982). In Atlantic salmon, dietary iron content over a wide range did not have any effect on growth (Chapter 2).

The hemoglobin regeneration assay gave a reasonable estimate of iron bioavailability from both the organic (heme) and inorganic (non-heme) sources of iron. In addition, error was avoided by supplementing the basal diet with 30 mg of iron kg^{-1} since this amount was within the level of iron required by the fish.

The relatively low bioavailability of iron not only from ferric oxide, but also blood meal and fishmeal is in accordance with the findings of Pla et al. (1974) for

rats and chicks. However, the present findings contradict the results of Andersen (1997), perhaps because different methods were used to assess iron bioavailability. Anderson (1997) used higher levels of iron in the diets so that the fish may have been able to excrete the excess iron. This may have negated the effect on the fish of higher dietary iron concentrations and led to overestimation of the normal iron requirement.

Cells take up heme iron intact and then the iron is released within the cells. By contrast, non-heme iron needs to shed its ligands before entering the cell (Hallberg and Bjorn-Rassmussen, 1972; Turnbull et al., 1962). Thus, the two mechanisms of iron uptake likely have an effect on iron bioavailability. One of the most important indicators of adequate iron availability is normal hemoglobin or hematocrit levels in the subject, but Andersen (1997) used liver and kidney iron deposition as indicators of iron status. Heme iron, which can be taken up by the cell intact, could have greater availability for regeneration of Hb, and thus higher bioavailability values may be obtained relative to the analysis of liver or spleen tissues.

The bioavailability of iron from nonheme sources is closely related to the solubility of iron (Spiro 1977; Spiro and Saltman, 1974). Both ferric and ferrous iron are soluble at stomach pH, but only ferrous iron remains soluble at the higher pH of the intestine. Hence, ferric iron is almost unavailable unless it complexes with a soluble ligand. Consequently, it was evident from the results

of this study that ferric chloride had similar bioavailability to FS, whereas ferric oxide had lower bioavailability than FS. Since phosphorus and calcium are major components of diets based on herring meal (Vielma, 1998), the higher dietary levels of these elements may have depressed iron absorption (Mahoney and Hendricks, 1978) and consequently lowered the iron bioavailability estimated from herring meal in the present study. An excess of a chelating agent like ascorbate was not used in the diets of this experiment. Hence, this may be another explanation for the reduced bioavailability of iron from blood meal or fish meal in this study relative to the values reported by Andersen (1997).

Fish can absorb iron through their gills and skin (Lall 1989), and thus a period of at least 22 weeks is needed to create an iron deficiency in Atlantic salmon by feeding a low iron diet. The present observations support the view that some inorganic iron compounds are better utilized than organic sources. However, limited comparisons were made between salts of di- and trivalent iron to confirm the frequently held view in monogastric animals that the former absorbed better than the latter (Fritz et al., 1972). Certainly the results suggest that iron from both ferrous sulfate and ferric chloride was more available than from ferric oxide.

CHAPTER 4. Effect of dietary heme and non-heme iron on nutrition, disease resistance and the immune system of Atlantic salmon (*Salmo salar*)

4.1. Introduction

Optimal nutrient balance in fish diets is an important consideration in feed formulation. Imbalances could result in a nutritional deficiency or a toxic effect due to a nutrient overdose. The effects of a dietary nutrient imbalance are difficult to observe without measuring parameters associated with deficiency or toxicity. An imbalance in the diet is likely to predispose animals to disease.

Maintenance of homeostasis of cellular iron is a prerequisite for many essential biological processes such as growth and regulation of the immune system. Both iron deficiency and iron overload can exert subtle effects on the immune status of animals. This is partly due to the central role that iron plays as a cofactor for critical enzymes, such as mitochondrial aconitase or ribonucleotide reductase (Hoffbrand et al., 1976).

The ability of an invading pathogenic microorganism to multiply successfully within the host environment is a commonly shared essential factor in all infections (Grayson et al., 1995). Iron plays a key role in the pathology of bacterial infections by enhancing the multiplication and virulence of the invading micro-organism (Weinberg, 1984). The ability to obtain iron *in vivo* is an

120

important virulence determinant for many microbes, such as the human pathogens *Listeria monocytogenes* (Sword, 1966; Alford et al., 1991) and *Legionella pneumophila* (Byrd and Horwitz, 1989), and the fish pathogens *Vibrio anguillarum* (Lemos et al., 1991) and *Aeromonas salmonicida* (Hirst and Ellis, 1994). Accordingly, a concept has been developed that depriving the pathogen of iron may serve as a means of non-specific protection against infection (Bullen et al., 1978; Weinberg, 1984).

In serum, iron is usually bound to iron-binding proteins (serum transferrin, ferritin, etc.) which in turn restrict availability of the iron to bacteria. Bacteria have several mechanisms to overcome the ability of the host to use this defense strategy. The best-studied bacterial mechanism is to produce an iron chelator, known as a siderophore, which competes with the iron binding ability of transferrin (Griffiths, 1987; Otto et al., 1992). However, non-siderophore bacteria use other mechanisms to acquire iron from the host. These include a ferrous uptake system, as well as cytolysins and proteases that release iron from iron-binding proteins and hemoglobin (Byers and Arceneaux, 1998). *Aeromonas salmonicida* and *V. anguillarum*, the causative agents of furunculosis and vibriosis, respectively, in fish have both siderophore and non-siderophore strains (Hirst et al.,1994; Lemos et al., 1991). The non-siderophore strains of these fish pathogens overcome iron-restricted conditions encountered *in vivo* by independent mechanisms that include haemolytic, haemagglutinating and

cytotoxic activities (Toranzo et al., 1983; Massad et al., 1991), but the exact mechanism has not been established.

Differences in disease susceptibility of salmonids possessing different transferrin genotypes have been shown during infections with common bacterial diseases, such as bacterial kidney disease (BKD; *Renibacterium salmoninarum*, in rainbow trout (Suzumoto et al., 1977), and vibriosis and furunculosis in Atlantic salmon (Pratschner, 1978). Genetic variation in disease susceptibility among different Atlantic salmon families was identified by Ravandal et al., (1994), and was observed to be associated with total serum iron concentration. Families with higher levels of serum iron were more susceptible to vibriosis infection.

The influence of dietary factors on disease outbreaks in aquaculture has been recognized for many years and extensively reviewed (see, Lall, 1989; Landolt, 1989; Blazer, 1992; Lall and Olivier, 1993 and Waagbo, 1994). The iron content of most commercial fish feeds varies between 150 and 450 mg/kg. The iron requirement of Atlantic salmon has been estimated to range from 50 to 70 mg/kg diet (Chapter 2), but the bioavailability of iron varies with the source (Chapter 3). The excess iron in diets may have an adverse effect on fish and their immune system, as well as on the environment, if excreted (Hutchins et al., 1999). The effects of diets containing different iron sources i.e., non-heme (iron sulfate) and heme (blood meal), on the immune function in Atlantic salmon has not been examined before.

Studies of the effects of nutrients on the immune function of fish are complicated, because there may be confounding variables such as additional nutritional deficiencies or pre-existing infection that make it difficult to conclude that the test nutrient of the study is the only causative factor in the immune alternations. Lall et al., (1989) proposed that the relationship between nutrition and disease in fish can be studied by feeding the fish with diets, that are not only deficient in the nutrient of interest but also contain graded levels of the test nutrient, for a minimum of 12 weeks. Then the effects of the diets on the immunocompetence of the fish can be tested by several *in vivo* or *in vitro* techniques. In this regard, fish are either challenged with a specific disease agent under different challenge protocols, or they are subjected to a series of immunological assays to examine the effect of diet on various immune parameters.

Complex interactions with the host may elicit physiological changes in the pathogen growing *in vivo* that may not be reflected *in vitro*. Several studies have shown that the responses obtained with the *in vivo* approach do not necessarily correlate with enhancement or suppression of immune parameters using *in vitro* tests (Sheldon and Blazer,1991; Obach *et al.*, 1993; Fracalossi and Lovell, 1994). Therefore the studies described herein were done using a combination of the two approaches to examine the effects of dietary iron levels on the susceptibility of Atlantic salmon to outbreaks of vibriosis or furunculosis.

The objective of this study was to determine the effects of a deficiency or an excess of iron on growth, tissue iron concentrations, immune response, and disease resistance in Atlantic salmon. In this regard, Atlantic salmon from the same genetic population without prior exposure to disease were fed test diets with different sources and levels of iron. A semipurified diet was supplemented with either 0, 100, 300, 600 or 1200 mg of iron/kg from ferrous sulphate and either 0, 100 or 300 mg of iron/kg from spray-dried hemoglobin. Two experiments were conducted on Atlantic salmon held in fresh water for either 20 or 32 weeks. The effects of the diets on the following immune responses were investigated: serum bactericidal activity (complement system), lymphocyte proliferation, macrophage phagocytosis, total serum lysozyme, respiratory burst within phagocytic cells, and differential blood cell counts. LD_{50} challenges were performed to calculate the virulence of vibriosis and furunculosis in relation to diet treatment.

4.2. Materials and methods

4.2.1. Fish

Atlantic salmon parr (32 to 35 g) from the Mersey hatchery of the Department of Fisheries and Oceans (DFO) were acclimated in their experimental tanks for 3 weeks prior to each experiment. They were maintained in a flow-through system of 100 L freshwater circular tanks. Water was supplied

to each tank at a flow rate of 1.5 L per minute and temperature was maintained

at $14\pm1°C$. Photoperiod was controlled automatically on a 12-hour light and 12-

hour dark schedule. The fish were fed a commercial grower diet (Corey Feed

Mills Ltd., Fredericton, NB) that contained 96 ± 2.5 mg iron/kg before they were

switched to their prescribed experimental diets.

4.2.2. Mineral loads in experimental tank water

The concentrations of Ca, Mg, Zn, Fe, Cu and Mn in the water that was

supplied to the experimental tanks were determined at the outset of each

experiment. The average elemental compositions of the water for each

experiment are provided in Table 4.1. The levels of the selected elements in the

water were noted to be within the ranges of these elements reported by

Freedman et al., (1989) and in other fish nutrition studies.

4.2.3. Nutritional study

Experimental diets and feeding of fish

The composition of the basal diet used in the investigation is shown in

Table 4.2. Using this basal diet, seven experimental diets with different levels of

non heme and heme iron were prepared. The supplemental dietary iron levels

were 0, 100, 300, 600 or 1200 mg of non-heme iron/kg and 0, 100 or 300 mg

heme iron/kg. The basal diet was fed as a control. Ferrous sulfate heptahydrate

Table 4.1. Selected mineral concentrations in the freshwater at the start of experiment 1 and 2.

Elements	Concentrations (mg/L)[1]	
	Experiment 1 (20 wk)	Experiment 2 (30 wk)
Ca	16.6 ±1.20	17.9 ±1.80
Mg	0.5 ± 0.02	0.5 ± 0.03
Zn	0.005±0.001	0.004±0.003
Cu	0.041±0.061	0.045±0.034
Fe	<0.1±0.001	<0.1±0.000

[1]Means±SE

Table 4.2. Ingredient and chemical composition (g kg⁻¹) of the basal diet.

Ingredient	g kg⁻¹
Casein, vitamin free[a]	320
Corn gluten meal[b]	190
Corn starch pregelatinized[c]	80
Dextrin[a]	113
Celufil[a]	50
Choline chloride[a]	2
Amino acid mixture[e]	15
Vitamin mixture[f]	10
Mineral mixture[g]	60
Herring Oil[d,i]	160

[a] US Biochemical Corp., Cleveland, Ohio

[b] Corey Feed Mills Ltd., Fredericton, NB

[c] National Starch and Chemical Company, Bridgewater, NJ

[d] Comeau Seafood Ltd., Saulnierville, NS

[e] Amino acids were supplied at the following levels (%); DL-methionine, 0.5; L-arginine.HCl, 1.0.

[f] Vitamins were supplied at the following levels (I.U. or mg.kg⁻¹ diet): vitamin A, 8000 I.U.; vitamin D_3, 2400 I.U.; vitamin E, 300 I.U.; vitamin K (menadione sodium bisulfite), 40; thiamin 50; riboflavin, 60; d-calcium pantothenate, 200; biotin, 1; folic acid, 20; vitamin B_{12}, 0.1; niacin, 250; pyridoxine, 40; ascorbic acid 300; inositol, 400; butylated hydroxy toluene, 15.

[g] Mineral were supplied at the following levels (mg.kg⁻¹ diet): $MnSO_4.H_2O$, 153.9; $CuSO_4.H_2O$, 59.1; $ZnSO_4.7H_2O$, 440.5; $MgSO_4.7H_2O$, 2525.3; KI, 6.5; $Na_2Se_2O_3$, 2.2; $CoCl_2.6H_2O$, 60.5; NaCl, 2500; NaF,10; $CaH_4(PO_4)$. H_2O, 12195.1.

[i] Stabilized with 0.06% ethoxyquin.

and bovine hemoglobin were used in the preparation of the non-heme and heme iron diets, respectively. Experimental diets were fed to triplicate groups of 50 Atlantic salmon parr. The two experiments were conducted using a randomized complete block design with 3 blocks of 6-7 tanks in each. Each diet was represented in 1 tank within each block. The fish were fed to satiety 3 times daily, 5 days a week with two feedings on the 6th and 7th day. Mortality was noted daily and the feed consumption were recorded weekly, with the resultant feed-gain ratio determined at the 4th week and the end of each feeding trial. Growth of the fish was monitored every week. Dead fish were examined for gross abnormalities.

Two different feeding trials were conducted. In the first, six diets containing non-heme iron (0, 100, 300, 600 mg iron/kg diet) and heme iron (0, 100 and 300 mg iron/kg diet) were fed for 20 weeks. In the second trial, seven diets including the six above and a diet with 1200 mg of non-heme iron/kg were fed for 32 weeks.

Analytical methods:

Fish were randomly selected from each dietary group at the end of each experiment. Fish were killed by a hard blow to the head and liver, kidney and spleen were removed and the residual blood was blotted out by fresh tissue paper. Each of the organs was weighed and immediately frozen in dry ice. Samples were then stored at -80°C until analysis.

Minerals in the feed-ingredients, experimental diets and fish tissue samples were determined by dry-ash methods (Middleton and Stuckey, 1953). Duplicate samples of test materials were subjected to chemical analysis. Experimental diets were ashed or samples of liver, kidney and spleen were subjected to digestion as it was using 6M HCl and three drops of ultra-pure HNO_3. Concentrations of iron, copper, zinc and other minerals were determined with an atomic absorption spectrophotometer (Perkin Elmer, Model 503).

Dry matter contents of the diets and tissues were determined by weight loss after drying samples at $105^{\circ}C$ for 24 h (Association of Official Analytical Chemists, AOAC 1984). Ash was determined by the AOAC (1984) procedure. Crude protein (%N x 6.25) of diets was determined by the Dumas method (Ebeling, 1968) using a Leco Nitrogen estimator (Leco Corporation, St. Joseph, MI). Lipid content of the test diets was determined by the method of Bligh and Dyer (1959).

Growth and feed utilization

The average weight and feed utilization of each of the groups were monitored during the experiments. The value of feed/gain ratio was based on a four-week feeding period. In this study, the fish were found to be very sensitive to stress. Although precautions were taken to minimize stress during the handling of the fish, fish died in several tanks after weighing at the 4th week of experiment-1. After examining random samples of the fish from the groups in

which there was mortality, the DFO disease laboratory concluded that the

mortality was not due to bacterial infection. As a precaution, the fish in the

feeding experiments were weighed once after four weeks and then at the end of

the feeding experiment.

The following indices and ratios were calculated:

Feed /gain ratio = (dry feed fed in g/ fish weight gain in g)

Hepatosomatic Index (%) = (liver weight in g / fish weight in g) x 100.

Spleen-body wt ratio (%) = (spleen weight in g / fish weight in g) x 100.

The condition factor was calculated as k = (W x 100) / L^3, where L and W

were the standard length and weight of the fish, respectively.

Hematology

Sampled fish were anesthetized with tricane methanesulfonate (MS-222,

Argent Chemical Co., Redmond, Washington) using dose between 15 to 25 mg

/L. The blood was collected from the caudal vein of each fish using a syringe

fitted with a 22-gauge needle. To minimize rupture of erythrocytes, the needle

was removed from the syringe and the blood was gently placed into sterile tubes.

Hematocrit (Hct), hemoglobin (Hb) concentration and total red blood cell counts

(RBC) were measured as described in chapter 2 and 3.

The hematological parameters, mean cell volume (MCV), mean cell

hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were

measured. They were calculated as follows: MCV= (Hct/RBC) x 10; MCH= (Hb/RBC) x 10, MCHC = (Hb/Hct) x 100.

Serum iron, total Iron-binding capacity, transferrin saturation and ferritin analysis:

Serum collection: Blood was collected from anesthetized Atlantic salmon by caudal venipuncture into non-heparinized tubes and allowed to clot. The blood was then centrifuged at 800g for 15 minutes. Serum was obtained by drawing the fluid part after the centrifugation. All serum samples were stored at -80° C until used.

Serum iron: Serum iron was directly measured by AAS after dilution where necessary.

Total iron-binding capacity (TIBC): TIBC was analyzed using the method described by the National Committee for Clinical Laboratory Standards (NCCLS, 1990). Briefly, 100 μL serum were mixed with 200 μL of iron saturating solution (2 mg iron/L) by vortexing, and then this mixture was incubated at room temperature for 15 min. After incubation, 0.03 g of basic $MgCO_3$ (Fisher Analytic) were added and the aliquot was incubated at room temperature for 30 min, then centrifuged at 1000g for 15 min. The supernatants were collected for determination of TIBC. The samples were analyzed in an Atomic Absorption Spectrophotometer to determine their levels of iron. The levels were used for

TIBC calculation.

Serum transferrin percentage: Transferrin, a major serum protein, is

usually about a one-third saturated with iron (NCCLS 1990). The percent

transferrin saturation was calculated from the serum iron concentration and the

serum iron-binding capacity, by the following formula,

Transferrin saturation (%) = (serum iron μg/L / TIBCμg/L) x 100

Serum ferritin: Quantitative determination of ferritin was conducted using

in vitro ELISA methods (Enzymun-Test® kit, Boehringer Mannheim). Briefly, 30

μL of serum were placed in an incubation tube and left at room temperature for

30 min. Each tube was washed with washing solution (Enzymun test kit) and

1.0. mL of substrate (Chromogen solutiion) was added. After 30 min of

additional incubation, the samples were read at an OD of 405 nm. The value

was determined against a curve drawn from 0, 2.25, 4.5, 9 and 18 ng of ferritin

/ml standards.

4.2.4. The Immunological study

Experimental conditions for challenges

After the 20 to 32-week feeding trials, the fish were transferred into the

quarantine unit of the Halifax Research Laboratory where all disease challenges

were performed. Depending upon the size of the fish and the type of challenge

to be undertaken, fish were kept in either 30 L or 200 L tanks under the same

environmental conditions as those described for the feeding regime.

Bacteria

The fish pathogens, A. salmonicida and V. anguillarum, were isolated

from salmonid fish during outbreaks of furunculosis and vibriosis that occurred in

Atlantic Canada. Virulent isolates of A. salmonicida (80204, Restigouche River,

NB., 1980) and V. anguillarum (serotype02, Bay of Fundy, NB.,1989) from the

Halifax Laboratory, N.S., Department of Fisheries and Oceans were used for the

experiments.

Aeromonas salmonicida was routinely grown at 22°C in a brain/heart

infusion (BHI) broth (Difco, Detroit, MI.) while V. anguillarum was grown at 22°C

in a trypticase soy broth (TSB, Difco, Detroit, MI) supplemented with 2% sodium

chloride (NaCl).

Fish condition

The fish used for immunological experiments were reared strictly in

freshwater. They were not vaccinated and had no known previous exposure to

A. salmonicida and V. anguillarum pathogens. The fish were, therefore,

assumed to be immunologically naive to these bacterial pathogens.

In vivo disease challenge procedures

Two similar experiments were conducted with the bacteria. In the first

experiment, six diets that had been supplemented with different sources and levels of iron were fed to triplicate groups of fish for 20 weeks before the challenge. In the second experiment, fish were fed the seven test diets for 32 weeks before being challenged. Details of the diets were mentioned earlier under the section 'experimental diets and feeding of fish'.

Depending upon the bacterial species and goal of the experiment, three kinds of challenges were done with the fish namely, cohabitation, interperitoneal (ip) injection LD_{50} challenge, and bath challenges.

Cohabitation challenge

This challenge was conducted with A. salmonicida to determine the capacity of disease-free fish fed the diets with selected levels of supplemental iron from two sources to contract or resist disease when confined with experimentally-injected fish.

Groups of 15 fish from each dietary treatment were finclipped for identification and randomly distributed into three 250L aquaria. Immediately after transfer, four additional fish given an ip-injection of 0.1 ml of bacterial suspension of A. salmonicida (adjusted to 1.0×10^7 colony forming units (cfu)/ml) were added to each tank. Previous experiments conducted in our lab had shown that this concentration of bacteria resulted in 100% mortality within 3-4 days following injection. The cohabited fish were infected by the disease within a couple of

days after the death of injected fish. Further mortalities attributed to *V.*

anguillarum were confirmed by isolation from kidney smears on TSA with 2%

NaCl plates. Fish were monitored for 14 to 16 days and cumulative mortality

expressed as a percentage was calculated for fish in each diet treatment.

LD_{50}

LD_{50} trials were performed with two virulent strains of *V. anguillarum*

(serotype-02, Bay of Fundy,N.B., 1989). *Vibrio anguillarum* cells were grown at

22°C in TSB (2% salt) for 18 hrs and then they were harvested and transferred

to sterile peptone-saline (PS; 0.85% NaCl containing 0.1% peptone), and

homogenized with a sterile syringe. The concentration of the suspension was

adjusted to an optical density (OD) of 0.1 at 540 nm in PS. Various dilutions

were made and were kept in ice until the experiment was performed. Ten-fold

serial dilutions were made in PS and the cell concentrations were determined by

using the drop inoculation method (20 µl aliquots) onto TSA supplemented with

2% NaCl. Three groups (one group for each dilution) of four Atlantic salmon

parrs /treatment were injected IP with 0.1 ml dilutions of bacteria. Fish were

monitored for 21 consecutive days. Mortalities attributed to *V. anguillarum* were

confirmed by bacterial isolation from kidney smears on TSA with 2% NaCl plates.

The LD_{50} was calculated by the method of Reed and Muench (1938). The

cumulative mortalities of fish exposed to *V. anguillarum* were estimated from the

LD$_{50}$ experiment.

Bath challenge

Only *V. anguillarum* was used as the pathogen for this experiment. The bacteria were grown in overnight culture at 22 °C in 5 ml of TSB-2 % NaCl. Bacteria were washed in PS, resuspended in PS and adjusted to an O.D. of 0.1 at 540 nm. Ten-fold serial dilutions were prepared in PS and the number of cfu/ml was determined by plate count on TSA with 2 % NaCl.

Fish that had been fed their prescribed diets for 32 weeks in the second trial were used with three replicate groups of 7 fish each from each diet. Fish were exposed for 15 min in a 2% NaCl bath with 2 x 10^7 cfu/ml of *V. anguillarum* in a dip tank and then they were released into their assigned tanks. Fish mortality was recorded daily for two weeks. Kidney samples from dead fish were cultured on TSA-2%NaCl to confirm the cause of death. The average percentage mortality value of the three replicate groups in each case was used for further analysis.

Heat inactivated serum:

Heat-inactivated serum was obtained by incubating serum at 45°C for 30 minutes. The fetal bovine serum (FBS) used in all assays was heat-inactivated prior to use at 56°C for 30 minutes. All sera were stored at -80° C prior to use.

Assay for phagocytic activity:

Phagocytosis assays were performed with isolated peritoneal phagocytes. The procedure for the assay was adapted from Secombes (1990). Four Atlantic salmon were randomly chosen from the groups receiving the 0 and 100 mg/kg non-heme iron diets and then they were given ip injections of 2.0 ml of 2.5% w/v glycogen in physiological saline (0.89% NaCl) to elicit migration of phagocytes to fish peritoneal cavities. Peritoneal exuded cells (PECs) were collected by injecting 10 ml of Hank's balanced salt solution without magnesium or calcium, HBSS I, but containing 10 I.U. heparin, 100 I.U./ml penicillin with 100 µg streptomycin/ml and buffered to pH 7.2 with 10 mM HEPES, into the peritoneum followed by gentle massaging the body cavity. The body cavity was cut, opened, and rinsed with more HBSS I as PECs were removed by gentle pipetting. Peritoneal exuded cells (PECs) were pelleted by centrifugation at 800g for 10 minutes at room temperature and then they were resuspended in minimum essential medium (MEM) that contained 1 mM glutamine, 0.2% heat-inactivated foetal bovine serum (FBS), 100 I.U./ml penicillin, 100 µg/ml streptomycin, 25µg /ml amphotericin B, and pH was adjusted to 7.4 with 10 mM HEPES. Cell viability was checked by counting cells in 0.05% trypan blue. The concentration of the cell suspension was adjusted to 5.0 x 10^6 cells/ml and 0. 6 ml aliquots were seeded in each well of an 8-well tissue culture slide (Lab-Tek, Mile Lab. Inc. IL). After 2 hours of incubation at 15°C, non-adherent cells were removed

by washing with Hank's balanced salt solution with calcium and magnesium

(HBSS-II). The adherent phagocytes were cultured for 48 h at 15 °C in 0.5 ml of

MEM with 10% heat-inactivated FBS, with a media change at 24 h.

The assay was conducted by infecting phagocytes with 0.2 ml of

Escherichia coli bacterial suspension with heat-inactivated normal fish serum

(10% by vol) as described by Olivier et al., (1986). Phagocytosis was allowed to

proceed for an hour. Slides were rinsed in HBSS containing calcium,

magnesium and 0.01% gelatin (HBSS-G), air-dried, and stained with Diff-Quick®

(Dada diagnostics, PR., USA). Two hundred phagocytes per well were

examined. The results were expressed as the percentage of phagocytes

exhibiting phagocytosis and the mean number of bacteria associated per

phagocyte.

Serum bactericidal assay

The ability of normal or immunostimulated sera to kill *V. anguillarum*

bacterial strains *in vivo* was determined by the method of Secombes (1990) as

modified by Hajji et al., (1990). Serum was collected separately from regular and

glycogen-stimulated fish (n=5/diet; methods and doses similar in the

phagocytosis assay). A volume of 50 μl of each serum sample was placed

separately into two tubes for further treatment as non-heat-inactivated (NHI) and

heat inactivated (HI, 45° C, 30 min) serum. Fresh *V. anguillarum* suspensions

(grown as described earlier) were adjusted to a concentration of 10^{-7} (for NHI)

and 10^{-5} (for HI) cfu/ml. An equal volume (50 µl) of *V. anguillarum* suspension

was added to each tube. The aliquots were allowed to incubate for 0, 3 and 5

hours at 18 °C. At each time interval, 25 µl were taken out of each sample and

serially diluted to 10^{-4}. Three drops, 20 µl, of each dilution was plated on TSA-

1.5% NaCl plates for viable counts of cfu. The plates were incubated for 4-5

days at 18 °C and the cfu were recorded. Bactericidal activity was calculated by

subtracting the $\log_{10}$ cfu of untreated serum from $\log_{10}$ cfu of heat-inactivated

serum (control) at 0, 3 and 5 hours.

Serum lysozyme activity:

The purpose of the assay was to determine a quantitative value for the

amount of lysozyme in the serum of Atlantic salmon fed (n=5/diet) diets with

selected levels and sources of iron. The lysozyme assay of Ellis (1990) was

adapted for use in microplates. The assay is based upon the degree of lysis of

lysozyme-sensitive gram-positive bacteria *Micrococcus luteus* (or *M.*

lysodeikticus) by lysozyme present in a sample. Lysis was measured by

turbidimetry in a microplate assay reader (SPECTRA Max 300;Fisher).

Quadruplicate 25 µl serum samples were incubated with 25 µl of lysozyme buffer

(40mM NaH_2PO_4, pH6.2) at 30° C. In addition, 25 µL lysozyme (2500 units/ml)

and 25 µL of buffer were used as positive and negative controls, respectively.

Using a multi-tip dispenser, 50 μl of substrate (0.6 mg/ml suspension of *M. luteus*) were added into each well. Plate ODs were read at 450 nm for 60 min by using a microplate reader (Fisher) and SoftMax™ software (Fisher). Later the V_{max} values were determined by using the same software and these were used for further analysis.

Respiratory burst activity of phagocytes:

Respiratory burst activity of phagocytes results in the production of superoxide anion (O_2^-), which can be measured by reduction of the dye nitroblue tetrazolium (NBT) in a glass-adhered assay (Brown et al., 1996) and NBT-microplate assay (Secombes, 1990). Both the glass-adhered and microplate assays were used in this experiment.

Five fish from each diet were stimulated by ip injection of glycogen (described earlier in phagocytic assay solution) and kidney tissues were collected after day 5. The kidney tissues were placed in HBSS and then they were macerated through a coarse stainless still mesh. The resulting cell suspension was centrifuged at 1000g for 5 mins at 4°C. The supernatant was removed from the cell pellet and the cells were washed twice in fresh HBSS. In order to measure viable cell concentration, 50 μl of the cell suspension were mixed with an equal volume of 0.4% (w/v) trypan blue. The number of live cells were immediately determined using a haemocytometer viewed at 400x

magnification. Further aliquots were diluted in HBSS to a concentration

equivalent to 10^6 cells / ml. For the microplate assay, 50 µl were added to 4

wells each of a 96-well tissue culture plate. Aliquots of each sample (50 µl) were

dropped, in duplicate, on a 2-well glass slide for the glass-adhered assay. They

were incubated for 30 min in a moist chamber at 18°C. The slides and plated

cells were then rinsed with PBS. An amount of 25 µl NBT (0.2% w/v) was added

to each well.

The slides were examined under a compound microscope at 1000 x

magnification to determine the proportion of activated (blue) cells. Two hundred

cells per sample were counted and the proportion of activated cells was

determined. For the microplate method, plates were read at 590 and 650 nm.

The results were compared with the values obtained from the control wells with

cells and PBS only.

Differential cell counts

Fresh blood (approx. 20µL, n=8/diet) was smeared onto a clean glass

slide (Fisher) and then the blood smears were stained with a modified Wright-

Giemsa stain (Difquick®) according to the instructions of the supplier. Ten fields

per slide were examined at 1000 x magnification and the blood cells were

identified after Jordan and Speidel (1923-24) and Conroy (1972), and counted.

Differential cell ratios (%) were determined by relating the average cell number in

each field (approximately 200 per field), to the total blood cells.

Myeloperoxidase (MPO) disorder estimation

Blood smears (described above) from the various fish fed (n=5/diet) the various experimental diets were fixed with alcohol-formaldehyde fixative for 1 min at room temperature. They were washed gently for 15- 30 seconds in deionized H_2O. The smears were stained with peroxidase stain (0.3 g of benzidine dihydrochloride, Sigma; 1 ml of 0.132 M $ZnSO_4.7H_2O$, ICN; 1 g of sodium acetate, Sigma; 0.7 ml of 3% H_2O_2, Fisher; and 1.5 ml 1N NaOH, dissolved in 100 ml 30% ethanol) for 5 minutes at room temperature. To observe the nucleus, the cells were counter-stained with safranine (ICN) for 1 min, washed for 5 to 10 seconds in deionized H_2O, air dried, and examined at 1000 x magnification. Peroxidase-positive cells exhibit yellowish green to bluish and brownish green granules. Results were reported as either (+) positive or (-) negative for MPO granules in the cytoplasm.

Lymphocyte proliferation assay

Lymphocytes were isolated from the anterior kidney of three deficient (no supplemental iron diet) and three fish that had ingested the diet with 100 mg of iron /kg (NH100) following the methods of Daly et al., (1995). The head kidney was carefully removed and sieved through a sterile steel mesh into a mixture of equal volumes of Alsever's solution (GIBCO) and Hank's Balanced Salt Solution without calcium and magnesium (HBSS-I, GIBCO). Heparin was not used as the

solution acts itself as an anticoagulant suspension fluid to separate lymphocytes and macrophages from connective tissue. The fluid was transferred to a plastic centrifuge tube and the cells were pelleted at 800g for 5 min at room temperature for subsequent washing in HBSS-I/Alsver's solution. The cell pellet was then resuspended in HBSS-I/Alsver's solution and then the suspension was centrifuged at 800g for 5 min. The diluent and fat layers were removed and the pellet was resuspended in 6 ml of HBSS-I. Using a 5 ml pipette, 3 ml of the suspension were layered over a discontinuous Percoll gradient of 34% and 51% (Percoll in HBSS-I) and then it was centrifuged (Airfuge clinical centrifuge) at 200g for 30 min at room temperature to isolate the leukocyte population. The leukocyte fraction was removed from the 34% / 51% interface and placed into another centrifuge tube and then washed once with fresh HBSS-I solution.

Serum from fish blood was collected from five fish by using the same method mentioned earlier. Serum samples were pooled together and half of the sample was separated and heated at 45°C for 30 min to inactivate the complement. All samples were stored at -80° C until used as a blood serum source later in this assay.

Cells were resuspended in 1 or 2 ml of proliferation medium consisting of MEM supplemented with 1.0 mM non-essential amino acids, 50 μM 2-mercaptoethanol, 10 mM sodium pyruvate, 2mM L-glutamine, 100 IU/ml penicillin G, 100 μg/ml streptomycin, and 10% heat-inactivated foetal bovine

serum (all from GIBCO) and then 2% heat-inactivated fish serum was added.

The viability of cells was determined by the Trypan blue exclusion method, and

the cell number was adjusted to a concentration of 2.5 x 10^5 leukocytes in 180 µl

proliferation medium. Each well of a 96 well tissue culture plate (ICN) was

seeded with 200µL of adjusted cell suspension. Tissue culture plates were then

sealed with plastic wrap and incubated as described below. Minimum essential

medium without cells was used as a control.

The following assay was a modification of the colorimetric assay of

Mossmann (1983) and Daly et al., (1995). Mitogens in appropriate

concentrations were added to the wells in 20 µl volumes of proliferation medium

and incubated at 18°C for 9 days. The mitogens used were concanavalin A (25

µg/ml, ConA, ICN, Costa Mesa,CA.), lipopolysaccharide (LPS, from *Escherichia*

coli 026:B6 (200 or 500 µg/ml, Difco) and phytohemagglutinin (PHA, ICN, Costa

Mesa,CA). After 0, 6 and 9 days of incubation, 20 µl of Alamar blue™ (Bio-

Source, Camarillo,CA.) were added to each well. Reduction of the tetrazolium

dye, Alamer blue™ is accompanied by development of a dark blue colour.

Optical densities were read daily over a period of 6-9 days at 540 to 595 nm

using a multiscan plate reader (Titertek Multiscan) integrated with an Apple

Macintosh computer. Results were compared at 0, 6 and 9 days after calculating

the stimulation index (S.I) using the following formula:

S.I. = (mean OD of stimulated cells with test mitogen at day x)- (mean OD

of control cultured cells at day x)

4.2.5. Statistical analysis

The statistical package SYSTAT® 8.0 for Windows® (Systat, Inc., 1998) was used for all statistical analyses.

Normality of data was tested by using the statistical program. The percentage data were transformed by using arc-sin transformation before any statistical analyses were conducted. Differences between diets were analyzed by one-way analysis of variance (ANOVA). When differences were evident, multiple range testing was performed using Tukey's test with $p=0.05$ to determine whether there were any effects of the graded levels of dietary iron on the various growth and hematological parameters as well as the serum and tissue mineral concentrations. Non- parametric tests, including the G-test and paired t-test were performed for interpretation of the disease challenge data, when the data could not be normalized (Zar, 1984).

4.3. Results

4.3.1. Nutritional studies

<u>Analysis of iron in feed materials</u>

The contents of iron in the feed ingredients are presented in Table 4.3. Spray-dried bovine hemoglobin proved to have the highest (2265 mg/kg) iron content, followed by regular blood meal (1835 mg/kg), fish meal (312 mg/kg) and krill meal (289 mg/kg). Iron was lowest (0.4 mg/kg) in corn starch. Gelatin and α-starch (pre-gelatinized starch) had similar iron (14 mg/kg) contents. Casein-based diets were chosen for use in the present experiment to achieve the lowest iron content in the basal diet.

<u>Proximate and mineral compositions of feeds</u>

The proximate and mineral compositions of the experimental diets are presented in Table 4.4 and 4.5, respectively. No differences were found between diets for total lipid (%) and crude protein (%) in the diets and the values for crude protein were within 10% of these reported by Tacon and De'Silva (1983) for commercial diets. Mineral contents, including Cu, Zn, Mg and Mn and showed little variations in each case between diets. All minerals were within the levels referred to by NRC (1993). The determined levels of iron from the different diets were close to required expected values.

<u>Growth and feed efficiency</u>

Table 4.3. Moisture, ash and iron concentrations in the feed ingredients used in the experimental diets.

Ingredients	Moisture %	Ash %	Iron (mg/kg) (as is basis)
Blood meal[a]	10.5	3.8	1835.0
Bovine haemoglobin[b]	6.9	4.2	2265.0
Casein, vitamin free[b]	2.6	1.2	20.0
Cellulose[b] (Cellufil)	3.8	0.4	2.0
Corn gluten[a]	6.3	3.0	109.0
Corn starch[c]	10.1	0.04	0.4
Fish meal[d]	8.6	15.0	312.0
Gelatin[b]	11.1	0.4	14.0
Krill meal[a]	8.2	18.7	289.0
α-starch[c]	7.3	0.1	14.0
Soy concentrate[b]	7.0	5.8	82.0

[a]Corey Feed Mills Ltd., Fredericton, NB.
[b]US Biochemical Crop., Cleveland, OH.
[c]National Starch and Chemical Co., Bridgewater, NJ.
[d]Comeau Seafood Ltd., Saulnierville, NS.

Table 4.4. Levels of proximate constitutent (as is basis) in the test diets. The diets contained different supplemental levels of iron from non-heme (FeSO$_4$.7H$_2$O) and heme (Bovine Hemoglobin, US Biochemical Corp., Cleveland, Ohio) sources.

Diet no.	Iron supplementation (mg/kg)		Proximate composition (%)			
	Inorganic (NH)	Hemoglobin (H)	Moisture	Ash	Crude protein	Total lipid
1	0	0	3.8	8.1	49.0	18.0
2	100	0	4.6	8.6	49.4	17.8
3	300	0	3.4	8.6	48.3	18.2
4	600	0	4.2	8.2	48.4	17.5
5	1200	0	3.8	8.5	48.1	17.9
6	0	100	3.5	12.3	48.7	18.3
7	0	300	4.1	13.6	48.6	18.6

Table 4.5. Levels of selected minerals in the test diets. The diets contained different supplemental levels of iron from non-heme ($FeSO_4.7H_2O$) and heme (Bovine Hemoglobin, US Biochemical Corp., Cleveland, Ohio) sources.

Diet no.	Iron supplementation (mg/kg)		Mineral					
	Inorganic (NH)	Hemoglobin (H)	Mg (%)	Zn (mg/kg)	Mn (mg/kg)	Cu (mg/kg)	Fe (mg/kg)	
1	0	0	0.05	41.6	14.8	2.5	15.4	
2	100	0	0.06	39.0	17.0	3.6	103.4	
3	300	0	0.06	37.6	18.8	2.2	316.4	
4	600	0	0.06	42.9	20.7	2.6	629.5	
5	1200	0	0.05	35.0	18.2	2.9	1243.3	
6	0	100	0.05	37.0	19.8	3.1	101.8	
7	0	300	0.05	39.0	17.6	3.1	299.9	

The average final weight gains and feed efficiency values (first 4 weeks

of study) for the groups in each experiment are given in Tables 4.6 and 4.7.

After 4 weeks of feeding, in the 20-week feeding trial, there were no significant

differences in weight gain ($F_{20wk}=1.97$, $p\geq0.05$) and feed ($F_{20wk}=1.92$, $p\geq0.05$).

Average growth after 20 weeks did not significantly differ due to dietary treatment

($F_{20wk}=4.05$, $p\geq0.05$). The average weights of the fish in the 32-week feeding

trial were not significantly different at the beginning ($F_{32wk}=0.03$, $p\geq0.05$), of the

study and thus was also true at 4 weeks ($F_{32wk}=0.94$, $p\geq0.05$) and 32 weeks (F_{32wk}

$=0.68$, $p\geq0.05$). No significant differences were found in feed efficiency after 4

weeks. The sources and levels of supplemented iron did not appear to have any

effect on fish growth and feed efficiency in either experiment.

Hepatosomatic Index, spleen-body ratio and condition factors

Hepatosomatic indices (HSI) and spleen-body ratios (SBR) and condition

factors of the groups in each experiment are provided in Tables 4.8 and 4.9.

There were no significant differences in the hepatosomatic indices of the fish fed

the various iron supplemented diets for 20 ($F_{20wk}=1.03$, $p\geq0.05$) or 32 (F_{32wk}

$=2.09$, $p\geq0.05$) weeks. Spleen-body ratios of the 20-week ($F_{20wk}=2.92$, $p\leq0.05$)

fish groups did significantly vary, however, in the 32 week feeding experiment,

the SBR did not vary significantly among fish fed the different diets ($F_{32wk}=0.23$,

$p\geq0.05$). After pair-wise diet comparisons, it was evident that the SBR of the

Table 4.6. Effect of supplemental dietary level of inorganic and organic iron on weight gain and feed efficiency in Atlantic salmon (*Salmo salar*) (20-week feeding trial).

Diet No.	Iron supplement (mg kg⁻¹)		Initial weight (g)	Weight gain (g)		Feed/Gain
	Inorganic (NH)	Hemoglobin (H)		4 weeks	20 weeks	4 weeks
1	0	0	34.3±1.9[a]	47.9±1.6[a]	113.0±2.0[a]	0.80±0.23[a]
2	100	0	33.4±0.6[a]	48.9±2.2[a]	120.5±1.5[a]	1.05±0.28[a]
3	300	0	33.6±0.9[a]	49.3±1.8[a]	121.0±4.0[a]	1.14±0.15[a]
4	600	0	34.1±0.8[a]	47.0±0.6[a]	116.0±2.0[a]	0.90±0.07[a]
5	0	100	33.9±1.2[a]	52.6±2.3[a]	110.5±2.5[a]	1.33±0.19[a]
6	0	300	32.8±0.7[a]	51.8±2.3[a]	122.5±1.5[a]	1.10±0.70[a]

[a] Mean (± SE) in the same column are not significantly different ($p \geq 0.05$).

Mortality of fish due to secondary bacterial infections took place after weighing at the 4th week. Total mortalities ranged from 0-1% in tanks on 4th week of sampling.

Table 4.7. Effect of supplemental dietary level of inorganic (NH) and organic (H) iron on weight gain and diet utilization in Atlantic salmon (*Salmo salar*) parr (32-week feeding trial)[a].

Diet No.	Iron supplement (mg kg⁻¹)		Initial weight (g)	Weight gain (g)		Feed /Gain
	Inorganic (NH)	Haemoglobin (H)		4 weeks	32 weeks	4 weeks
1	0	0	34.4±1.1 [a]	46.2±1.2 [a]	306.0±5.3 [a]	1.0±0.32 [a]
2	100	0	34.4±0.6 [a]	47.6±1.3 [a]	304.3±3.7 [a]	1.1±0.22 [a]
3	300	0	34.0±1.5 [a]	43.3±3.2 [a]	312.3±5.4 [a]	1.1±0.45 [a]
4	600	0	34.5±1.1 [a]	46.7±0.4 [a]	300.7±5.2 [a]	0.9±0.71 [a]
5	1200	0	34.2±1.4 [a]	42.5±3.0 [a]	304.3±4.3 [a]	1.0±0.51 [a]
6	0	100	34.0±1.1 [a]	46.7±2.0 [a]	307.7±5.2 [a]	1.1±0.11 [a]
7	0	300	34.0±1.4 [a]	45.4±1.5 [a]	311.5±5.8 [a]	1.1±0.17 [a]

[a] No mortality was observed on or before 4th week sampling.

[b] Mean (± SE) in the same column are not significantly different ($p \geq 0.05$).

Table. 4.8. Hepatosomatic indices (HSI %), spleen-body ratios (%), and condition factors of Atlantic salmon (*Salmo salar*) fed six diets containing different levels of inorganic and organic (hemoglobin) iron for 20 weeks (experiment 1)[1].

Diet No.	Iron supplement (mg kg^{-1})		Body weight (g)	HSI (%)	Spleen (%)	Condition factor
	Inorganic (NH)	Hemoglobin (H)				
1	0	0	88.9±3.5[a]	1.28±0.14[a]	0.07±0.01[a]	0.96±0.02[abc]
2	100	0	116.8±8.6[a]	1.34±0.09[a]	0.08±0.01[ab]	1.04±0.02[ab]
3	300	0	118.1±27.3[a]	1.14±0.26[a]	0.13±0.04[ab]	1.03±0.07[abc]
4	600	0	112.1±6.5[a]	1.20±0.07[a]	0.13±0.02[ab]	1.06±0.03[a]
5	0	100	92.4±5.7[a]	1.06±0.12[a]	0.15±0.03[b]	0.89±0.02[cd]
6	0	300	118.6±7.7[a]	1.11±0.06[a]	0.13±0.02[ab]	0.95±0.02[bd]

[1]Values are means±SE. Means in the same column sharing a common superscript letter are not significantly different (p≥0.05).

Table. 4.9. Hepatosomatic indices (HSI %), spleen-body ratios (%), and condition factors of Atlantic salmon (*Salmo salar*) fed seven diets containing different levels of inorganic and organic (hemoglobin) iron for 32 weeks (experiment 2)[1].

Diet No.	Iron supplement (mg kg^{-1})		Body weight (g)	HSI (%)	Spleen (%)	Condition factor
	Inorganic (NH)	Hemoglobin (H)				
1	0	0	278.9±19.7[a]	1.05±0.05[a]	0.29±0.03[a]	0.97±0.03[a]
2	100	0	290.9±15.3[a]	0.96±0.07[a]	0.27±0.03[a]	0.99±0.02[ab]
3	300	0	314.8±28.4[a]	1.32±0.11[a]	0.23±0.02[a]	0.99±0.03[ab]
4	600	0	310.4±19.5[a]	1.06±0.10[a]	0.26±0.05[a]	1.06±0.03[ab]
5	1200	0	304.8±19.2[a]	1.16±0.07[a]	0.27±0.04[a]	1.03±0.05[ab]
6	0	100	313.8±19.2[a]	1.02±0.13[a]	0.25±0.06[a]	1.24±0.13[b]
7	0	300	262.2±13.3[a]	1.28±0.10[a]	0.26±0.03[a]	1.04±0.04[ab]

[1]Values are means±SE. Means in the same column sharing a common superscript letter are not significantly different (p≥0.05).

H100 fish group (in the 20 week feeding trial) was significantly higher than that of the fish fed the iron deficient diet ($p \leq 0.05$). The condition factors of the fish groups after 20 and 32 weeks of feeding showed significant differences among diet treatments ($F_{20wk} = 6.25$, $p \leq 0.05$; $F_{32wk} = 2.29$, $p \leq 0.05$ respectively). At the end of the 20-week trial, the condition factor of the H100 group of fish was significantly lower than those for all non-heme diet fed fish groups. However, there were no differences among heme iron fed fish or deficient and non-heme iron fed fish. The H100 group of fish in the 32-week trial showed a significantly higher ($p \leq 0.05$) condition factor value than the group ingesting the diet with no iron (0-iron) supplement. There were no significant differences in condition factor between any of the other groups in the 32-week trial.

Hematology

The mean hematological values of the groups fed diets containing no supplemental iron and selected levels of non-heme (NH) and heme (H) iron following the 20 and 32-week feeding period are shown in Tables 4.10 and 4.11.

Numbers of red blood cells (RBC), mean cell volumes (MCV) and mean cell hemoglobin contents (MCH) of the fish were affected significantly by diet treatment in the 20-week trial. Increased RBC were observed in fish ingesting the diets with 300 mg of iron supplementation from either heme or non-heme sources. A decrease in the RBC value was noted for fish fed the diet with 600

Table 4.10. Hematological parameters of Atlantic salmon (*Salmo salar*) fed six diets with different levels of inorganic and organic (hemoglobin) iron for 20 weeks (experiment 1)[1].

Diet no.	Iron supplementation (mg kg⁻¹)		Hct	Hb	RBC	MCV	MCH	MCHC
	Inorganic (NH)	Hemo-globin (H)	%	g100 ml⁻¹	10⁷ml⁻¹	nm³	µg cell⁻¹	g100ml⁻¹
1	0	0	34.6 ± 1.3^a	9.7 ± 0.5^a	5.2 ± 0.1^a	6.6 ± 0.2^b	18.4 ± 0.9^a	2.8 ± 0.2^a
2	100	0	35.7 ± 1.8^a	10.4 ± 0.3^a	5.6 ± 0.2^a	6.2 ± 0.2^{bc}	18.4 ± 1.0^a	3.0 ± 0.3^a
3	300	0	33.0 ± 3.8^a	9.5 ± 1.1^a	6.3 ± 0.7^{ab}	5.3 ± 0.3^{ac}	15.3 ± 0.6^{ab}	2.9 ± 0.1^a
4	600	0	33.1 ± 2.6^a	9.2 ± 0.5^a	5.1 ± 0.3^a	6.4 ± 0.2^b	18.0 ± 0.9^a	2.8 ± 0.2^a
5	0	100	36.8 ± 2.4^a	10.0 ± 0.6^a	5.9 ± 0.3^a	6.3 ± 0.2^b	17.0 ± 0.6^{ab}	2.8 ± 0.2^a
6	0	300	38.1 ± 1.3^a	10.3 ± 0.6^a	7.0 ± 0.3^b	5.4 ± 0.1^a	14.6 ± 0.6^b	2.7 ± 0.1^a

[1]Values are means±SE. Means in the same column sharing a common superscript letter are not significantly different ($p \geq 0.05$).

Table 4.11. Hematological parameters of Atlantic salmon (*Salmo salar*) fed seven diets with different levels of inorganic and organic (hemoglobin) iron for 32 weeks (experiment 2)[1].

Diet no.	Iron supplementation (mg kg^{-1})		Hct	Hb	RBC	MCV	MCH	MCHC
	Inorganic (NH)	Hemoglobin (H)	%	g100 ml^{-1}	10^{7}ml^{-1}	nm^3	µg cell^{-1}	g100ml^{-1}
1	0	0	24.7±1.5^a	7.2±0.3^{ab}	5.9±0.2^a	4.2±0.2^a	12.2±0.6^a	2.9±0.2^a
2	100	0	33.3±2.5^a	7.9±0.4^{ab}	6.6±0.5^a	5.3±0.7^a	12.1±0.4^a	2.5±0.3^a
3	300	0	28.0±2.7^a	7.3±0.7^{ab}	5.3±0.9^a	6.0±0.9^a	15.6±2.6^a	2.6±0.2^a
4	600	0	34.4±5.1^a	8.7±0.9^a	5.6±0.6^a	6.3±1.0^a	15.6±0.9^a	2.7±0.3^a
5	1200	0	27.5±3.4^a	6.1±0.4^b	5.2±0.8^a	5.4±0.8^a	12.5±1.2^a	2.4±0.3^a
6	0	100	26.0±2.5^a	8.0±0.7^{ab}	6.1±0.3^a	4.3±0.2^a	13.2±0.9^a	3.1±0.2^a
7	0	300	31.8±1.5^a	8.3±0.5^{ab}	6.8±0.6^a	4.8±0.5^a	12.3±0.4^a	2.7±0.2^a

[1]Values are means±SE. Means in the same column sharing a common superscript letter are not significantly different (p≥0.05).

mg of iron/kg (NH600). Values of MCV and MCH were observed to decrease as

the supplemental dietary level of iron was raised except for fish receiving diet 4

(NH600). MCV values for the groups fed the diets with 300 mg of supplemental

iron from heme and non-heme sources were found to be significantly below

those observed for fish receiving diet 1 (0-iron), diet 4 (NH600) and diet 5

(H100). Similar trends were also observed for MCH values among the groups.

There were no significant differences in hematocrit (Hct; $F=0.90$, $p\geq0.05$),

hemoglobin (Hb; $F=0.77$, $p\geq0.05$) and mean cell hemoglobin concentration

(MCHC; $F= 0.41$, $p\geq 0.05$) irrespective of diet treatment.

In the 32-week trial there were no significant effects of diet treatment on

Hct ($F=2.21$, $p\geq0.05$), RBC ($F=1.03$,$p\geq0.05$), MCV ($F=1.56$, $p\geq0.05$), MCH

($F=1.83$, $p\geq0.05$) and MCHC ($F=1.34$, $p\geq 0.05$). The Hb levels, however, did

vary significantly ($F=2.70$,p 0.05) and they differed among fish fed diet 4 (NH600)

and diet 5 (NH1200) in which the lower value was noted. All other differences

between groups were not significant.

Serum iron, total iron binding capacity, transferrin saturation and ferritin

Values for serum iron, total iron binding capacity (TIBC), transferrin

saturation and ferritin are shown in relation to diet treatment during the 20-week

feeding trial in Table 4.12.

Average serum iron levels (µg/ml) differed significantly among the fish

Table 4.12. Serum iron, total iron binding capicity (TIBC), transferrin saturation, and ferritin of Atlantic salmon (Salmo salar) fed six diets with different levels of inorganic and organic (hemoglobin) iron for 20 weeks[1].

Diet no.	Iron supplementation (mg kg^{-1})		Serum Iron	TIBC	Transferrin	Ferritin
	Inorganic (NH)	Hemoglobin (H)	µg/ml	µg/ml	(%)	ng/ml
1	0	0	3.0±0.07^a	14.2±0.07^a	21.2±0.4^a	7.4±0.06±^{a}
2	100	0	7.2±0.49^b	20.0±0.29^b	35.8±2.4^b	9.3±0.07^b
3	300	0	11.9±0.14^d	31.7±0.67^d	37.7±1.1^b	6.9±0.15^a
4	600	0	10.7±0.13^c	31.2±0.44^d	34.4±0.2^b	8.8±0.15^b
5	0	100	6.5±0.11^b	27.4±0.51^c	23.8±0.3^a	10.5±0.07^d
6	0	300	7.2±0.08^b	30.0±0.50^d	23.8±0.2^a	11.6±0.12^e

[1]Values are means±SE. Means in the same column sharing a common superscript letter are not significantly different (p≥0.05).

groups (F=207.29, p≤0.05). The mean serum iron concentration of fish fed the 0-iron diet (diet 1) was significantly lower than found in any of the other dietary groups. By contrast fish ingesting the NH300 diet had a mean serum iron content that was significantly higher than observed in the other groups. Fish fed the NH600 diet had the second highest serum iron level. There were no significant differences in serum iron levels between groups fed the NH100, H100 and H300 diets.

Average TIBC (μg/ml) values ranged from 14.2±0.07 (diet 1) to 31.7±0.67 (diet NH300) and they differed significantly among fish fed the test diets (F=244.05, p≤0.05). The serum of fish fed the diet with no iron supplement showed significantly lower values than those fed the iron-supplemented diets. The iron binding capacity of fish fed the NH100 diet was significantly lower than noted for all other groups receiving the diets with iron supplementation. Groups ingesting the NH300, NH600 and H300 diets showed no difference in TIBC values. These groups also showed significantly higher TIBC values than those fed diet 5 (H100).

Transferrin saturation (%) values of serum from fish fed diet 1 (0-iron) were significantly lower (21.2±0.4%) than found for all groups receiving the inorganic iron supplemented diets. However, no differences were noted between fish fed diet 1 and those fed the heme iron supplemented diets. The saturation value was highest in fish fed diet NH300 (37.7±1.1%), but this value was not

significantly different from those receiving the diets NH100 and NH600. Thus, transferrin saturation was significantly elevated when non-heme iron was included in the diets instead of heme iron.

Ferritin (ng/ml) values differed significantly (F=276.20, p≤0.00) among the fish groups. The value was significantly lower in fish fed the diets without supplemental iron (7.4±0.06 ng/ml) and 300 mg of inorganic iron/ kg (6.9±0.15 ng/ml) relative to the values found for the other fish groups. Heme-iron fed fish (H100 and H300) showed significantly higher values than the iron-deficient and non-heme iron supplemented fish. However, the values found for fish ingesting the NH100, NH600, H100 and H300 diets all differed from one another.

Therefore, the iron deficient diet resulted in significant reductions of serum iron and TIBC levels in the fish. Also, ferritin levels were significantly higher in the fish consuming the heme-supplemented diets than in those ingesting the non-heme iron supplemented diets.

Tissue iron deposition

The levels of iron in liver, spleen and kidney tissue after the fish had been fed diets with selected levels of heme (0, 100 and 300 mg /kg diet) and non-heme (0, 100, 300, 600 and 1200 mg/kg diet) iron for 32 weeks are shown in Tables 4.13, 4.14 and 4.15.

Liver iron deposition differed significantly (F=7.83, p≤0.00) with

Table. 4.13. Levels of iron (Fe), copper (Cu), magnesium (Mg) and zinc (Zn) in the liver of Atlantic salmon (*Salmo salar*) fed seven diets with different levels of inorganic and organic (hemoglobin) iron for 32 weeks[1].

Diet No.	Iron supplement (mg kg⁻¹)		Fe	Cu	Mg	Zn
	Inorganic (NH)	Hemoglobin (H)	(µg/g)	(µg/g)	(µg/g)	(µg/g)
1	0	0	36.9 ± 5.6^a	13.5 ± 1.5^b	131.7 ± 8.9^{ab}	17.6 ± 1.4^a
2	100	0	84.5 ± 8.3^{ab}	3.8 ± 0.8^a	113.1 ± 3.9^{ab}	11.6 ± 1.7^a
3	300	0	113.6 ± 10.3^b	11.5 ± 1.7^b	112.8 ± 5.2^{ab}	14.9 ± 1.2^a
4	600	0	133.4 ± 22.7^{bc}	9.9 ± 1.9^{ab}	128.9 ± 6.7^{ab}	14.8 ± 1.0^a
5	1200	0	155.0 ± 16.3^c	9.3 ± 1.4^{ab}	115.8 ± 14.0^{ab}	13.8 ± 2.2^a
6	0	100	144.7 ± 15.3^{bc}	11.3 ± 1.8^b	152.4 ± 7.9^b	14.4 ± 1.4^a
7	0	300	117.1 ± 6.2^{bc}	6.9 ± 1.0^{ab}	121.9 ± 4.0^a	14.1 ± 1.1^a

[1]Values are means±SE. Means in the same column sharing a common superscript letter are not significantly different (p≥0.05).

differences in the dietary level of iron (Table 4.13). It was lowest in fish fed the diet without supplemental iron (36.9±5.6 µg/g) and highest in fish fed NH1200 (155±16.3 µg/g). There was a clear trend towards an increase in liver iron content with increasing levels of dietary iron from the non-heme source. In comparison to fish fed the diet deficient in iron (0-iron), there was a significantly higher deposition of iron in the liver of fish fed diets with more than 300 mg of supplemental inorganic iron/kg (i.e. NH300, NH600 and NH1200) as well as heme-iron (H100 and H300) irrespective of the supplemented level. Hepatic iron content did not differ between fish fed the deficient diet and NH100 fed fish. Similarly, there were no significant differences between fish receiving the NH100, NH300, NH600, H100 and H300 treatments and between those given the NH600, NH1200 and heme iron-supplemented diets.

Spleen iron content (F=6.46, p≤0.05) was influenced significantly by the different sources and levels of dietary iron (Table 4.14). The spleen iron content of fish receiving NH100 was significantly higher than noted in the iron deficient fish (diet 1) and also higher than the levels observed in fish given the NH300 and NH600 treatments. Fish given the later two treatments had equivalent values and these were significantly below those of all other groups. Salmon ingesting the NH100, NH1200, and heme-supplemented diets had equivalent values that were generally higher than found in the other groups.

Kidney iron levels (F=3.24, p≤0.05) were significantly different among the

Table. 4.14. Levels of iron (Fe), magnesium (Mg) and zinc (Zn) in the spleen of Atlantic salmon (*Salmo salar*) fed diets with seven different supplemental levels of inorganic and organic (hemoglobin) iron for 32 weeks[1].

Diet No.	Iron supplement (mg kg⁻¹)		Fe (µg/g)	Mg (µg/g)	Zn (µg/g)
	Inorganic (NH)	Hemoglobin (H)			
1	0	0	300.8±22.3[b]	167.0±8.4[a]	36.2±3.6[a]
2	100	0	421.0±45.3[c]	191.6±8.1[a]	42.6±9.4[a]
3	300	0	207.4±38.9[a]	268.1±34.5[b]	51.0±20.5[a]
4	600	0	217.6±31.1[a]	269.4±13.2[b]	36.2±8.6[a]
5	1200	0	361.6±60.6[bc]	306.6±66.6[bc]	34.2±4.7[a]
6	0	100	461.1±36.1[c]	478.1±54.4[c]	55.3±6.0[a]
7	0	300	390.8±22.5[c]	384.9±41.1[c]	54.4±14.6[a]

[1]Values are means±SE. Means in the same column sharing a common superscript letter are not significantly different (p≥0.05).

Table. 4.15. Levels of iron (Fe), magnesium (Mg) and zinc (Zn) in the kidney of Atlantic salmon (*Salmo salar*) fed diets with seven different supplemental levels of inorganic and organic (hemoglobin) iron for 32 weeks[1].

Diet No.	Iron supplement (mg kg^{-1})		Fe (µg/g)	Mg (µg/g)	Zn (µg/g)
	Inorganic (NH)	Hemoglobin (H)			
1	0	0	81.8±10.8[a]	158.0±10.1[ab]	38.5±3.5[a]
2	100	0	104.0±15.7[ab]	178.1±4.0[b]	42.5±4.6[a]
3	300	0	88.0±6.3[ab]	161.6±3.4[ab]	42.2±5.5[a]
4	600	0	132.5±11.7[b]	164.2±4.4[b]	51.7±9.6[a]
5	1200	0	129.8±11.8[b]	137.4±8.5[a]	36.3±3.9[a]
6	0	100	107.5±5.3[ab]	158.1±3.0[ab]	60.8±9.7[a]
7	0	300	109.9±8.8[ab]	153.0±4.7[ab]	45.8±7.7[a]

[1]Values are means±SE. Means in the same column sharing a common superscript letter are not significantly different ($p \geq 0.05$).

dietary groups (Table 4.15). The highest iron deposition was found in the kidney of fish fed diet NH600 (132.5±11.7 µg/g). The fish fed diet 1 (no supplemental iron) deposited the lowest amount of iron, and which the mean value for these fish was significantly lower than noted for fish ingesting diets NH600 and NH1200. All other differences between groups were not significant.

Dietary iron interactions with other minerals

The average (± SE) liver Cu, Mg and Zn, and spleen and kidney Mg and Zn levels are shown in Tables 4.13, 4.14 and 4.15. Copper analyses are only reported for the liver tissues since the levels of this element in the spleen and kidney were below the detection limit of the AA method.

Liver Mg levels did not differ between groups except fish ingesting diet 7(H300) had a significantly lower level relative to fish fed diet 6 (H100). Similarly, there were no differences between groups for lower Cu content except fish fed diet 2 had a significantly lower value than those fed diets 1, 3 and 6. Liver Zn levels were unaffected by dietary treatment.

The spleen Mg content was significantly higher in the fish consuming diet 6 (H100) relative to the values observed in all other groups except those fed diet 7 (H300). The values for fish fed diets 1-5 were not found to be different and the dietary inclusion of ≥ 300 mg of supplemental inorganic iron fed to the same level

of Mg as noted for fish fed diet 7 (H300). The zinc contents of the spleen were unaffected by dietary treatment.

Kidney Mg levels were equivalent in all groups except those fed diets (NH1200) had a significantly lower mean value than those observed for fish fed diets 2 (NH100) and diet 5 (NH600). Kidney Zn levels were uninfluenced by diet treatment.

4.3.2. Imunological studies

In vivo disease challenges

Challenges with *A. salmonicida* after the 20-week feeding trial

Total cumulative mortalities from the *A. salmonicida* cohabitation challenge did not differ with diet (Figure 4.1). There was 100 percent mortality in all groups after 14 days of challenge. However, the rate of mortality in fish fed the diet with 100 mg of non-heme iron/kg (NH100) was quicker than observed in the other dietary groups. The mortality of NH100-fed fish started at day 5 (7%) and rose sharply to 67% by the next day and then to 100% by day 8 of post-challenge. Fish fed with the NH300 diet experienced 80% mortality by day 6. Fish fed with the diet without supplemental iron showed the longest delay in reaching 100% mortality. Among the groups receiving blood meal (heme) iron sources, fish fed the diet with 100 mg/kg (mortality 20% by day 6, 67% by day 8, 100% by day 10) showed a more delayed mortality after challenge than fish fed
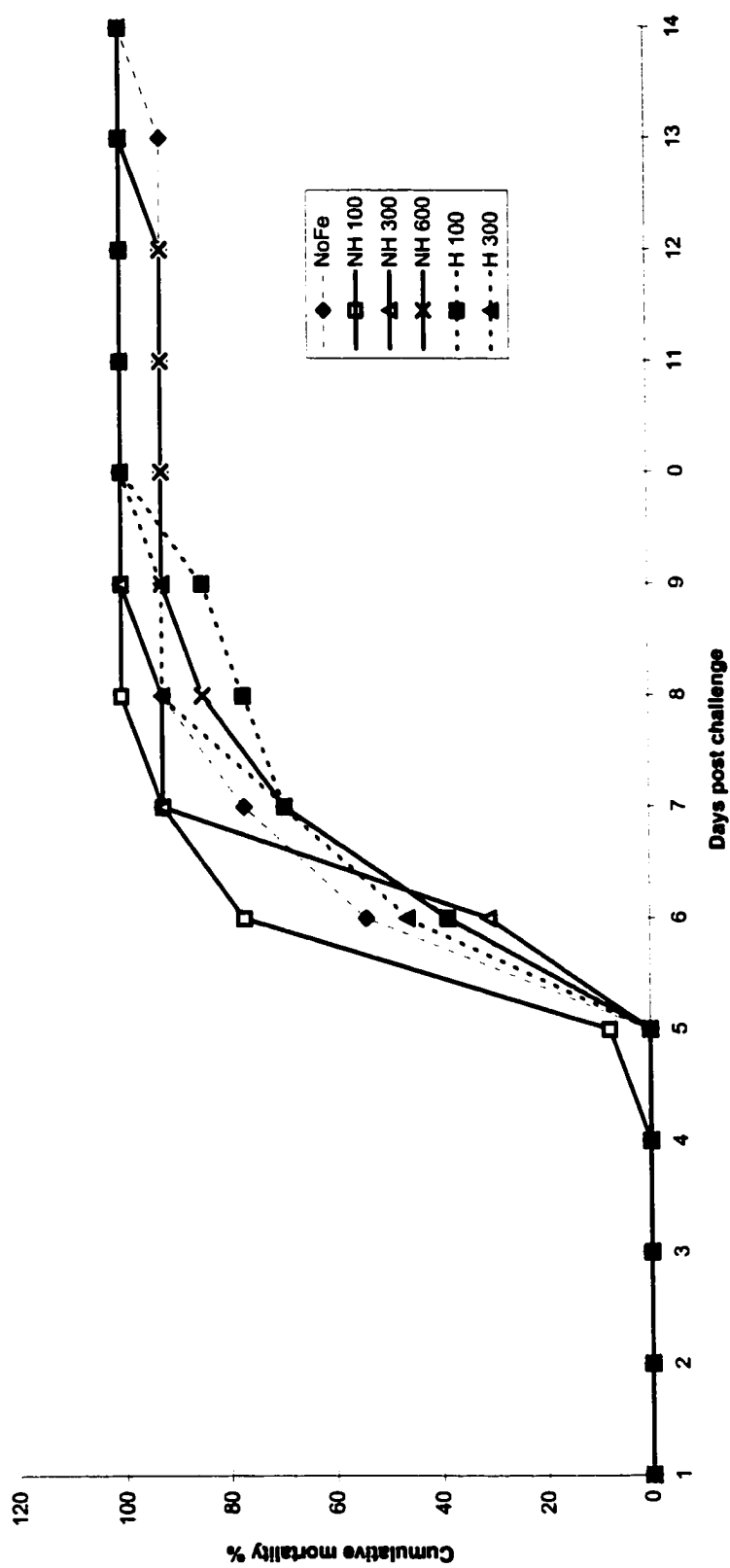
Figure. 4.1. Percent cumulative mortality of Atlantic salmon (*Salmo salar*) fed diets with selected levels of iron (0,100,300 and 600 mg non-heme iron/kg and 100 and 300 mg heme iron/kg) for 20 weeks before a cohabitation challenge with *Aeromonas salmonicida*. NH= non-heme, H= heme (hemoglobin) iron sources.

the diet with 300 mg of iron /kg (mortality of 40% by day 6, 80% by day 8, 100%

by day10). There were no significant differences in mortalities among groups fed

the diets at days 6, 9 and 12 post-challenge. Fish fed the NH100 and H100 diets

showed some differences in mortality (76.9% and 38.5%, respectively) among

groups at day 6 post-challenge, but these differences were not statistically

significant (X^2 =1.89, p≥0.05).

Challenges with A. salmonicida after the 32-week feeding trial

Total cumulative mortalities from the A. salmonicida cohabitation challenge after

the 32-week feeding trial are provided in Figure 4.2. Mortality of the NH300 fish

started at day 9 (50%), then rose sharply to 87.5% by day 10 and finally to 100%

by day 16, post-challenge. Mortality of the H100 fed fish started at day 8

(12.5%) and reached the maximum percentage (37.5%) by day 16, post-

challenge. There was a significant difference in percent mortality between these

groups. The only group, other than NH300, to reach 100% mortality at 16 days,

post-challenge was the one receiving no supplemental dietary iron. The overall

cumulative mortalities (%) of fish receiving NH100, NH600, NH1200 and H100

were 62.5%, 75%, 50% and 62.5%, respectively.

There were significant differences in total cumulative mortalities among

dietary groups (X^2= 12.84; p≤ 0.05) by 16 days, post-challenge (Figure 4.2). At

day 8, post-challenge, the cumulative mortalities were not significantly different
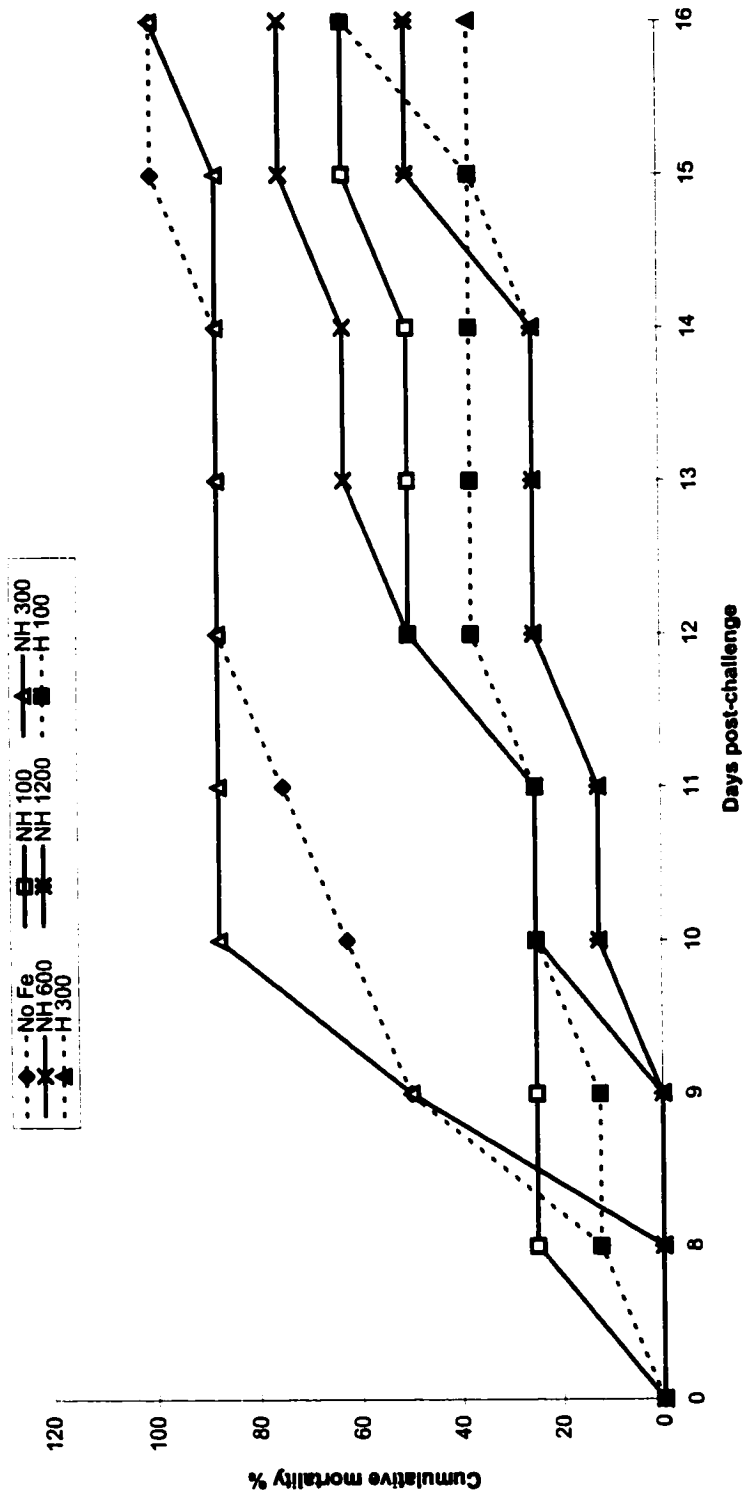
Figure 4.2. Percent cumulative mortality in Atlantic salmon (*Salmo salar*) fed diets with different levels of iron (0, 100, 300, 600 and 1200 mg non-heme iron /kg and 100 and 300 mg heme iron/kg) for 32 weeks and subsequently and challenged by cohabitation with *Aeromonas salmonicida*. NH= non heme, H= heme (hemoglobin) iron diets.

(X^2= 7.0; p≥ 0.05) but at day 12, post-challenge cumulative mortalities were

significantly different (X^2= 13.45; p≤0.05).

Challenge with ip-injected V. anguillarum

The percent mortalities of fish when challenged with strain 1 and strain 2 of V.

anguillarum are given in Table 4.16. The cumulative mortality was lowest

(43.8%) in the NH100 fish group for strain 1, and in the H300 group (37.5%) for

strain 2. The highest mortality was recorded in the NH300 group (87.5%) for

Table 4.16. Percent mortality of Atlantic salmon parr fed selected levels of heme

(H) and non-heme (NH) iron for 20 weeks, and then subsequently challenged

with Vibrio. Anguillarum strain 1, and in the iron-deficient group (no

supplemental iron,81.3%) for strain 2. Fish fed the non-heme 600 mg of iron/kg

diet (NH600) showed the same mortality rate (68.8%) for both strains. Heme

100 (H100) fed fish showed similar levels of mortality after challenge with both

strains.

V. anguillarum by bath challenge (experiment 2)

Stress mortality occurred in the 32-week feeding trial after the fish were

challenged with V. anguillarum on the first day due to a fall in the dissolved

oxygen levels of the water in the tanks. The results of mortality due to stress and

mortalities due to vibriosis are shown in Table 4.17. Stress-related mortality was

Table 4.16. Percent cumulative mortalities of Atlantic salmon (*Salmo salar*) fed diets with different levels of heme and non-heme iron for 20 weeks, and challenged with *Vibrio anguillarum*.

Diet No.	Dietary Iron in feed (mg/kg)		Mortality due to Strain 1[a] (%)	Mortality due to Strain 2[a] (%)
	Inorganic (NH)	Hemoglobin (H)		
1	0	0	75.0 (2)	81.3 (1)
2	100	0	43.8 (5)	68.8 (3)
3	300	0	87.5 (1)	75.0 (2)
4	600	0	68.8 (3)	68.8 (3)
5	0	100	62.5 (4)	56.3 (4)
6	0	300	68.8 (3)	37.5 (5)

[a] The ranking levels of mortality within the same strain from maximum (1) to minimum (5) are given in brackets.

Table 4.17. Percent cumulative mortalities of Atlantic salmon (*Salmo salar*) fed diets with different levels of heme and non-heme iron for 32 weeks, and challenged with *Vibrio anguillarum*.

Diet No.	Dietary Iron in feed (mg/kg)		Mortality due to stress[1,2] (%)	Mortality due to vibriosis[2] (%)
	Inorganic (NH)	Hemoglobin (H)		
1	0	0	19.1±4.8 (3)	94.5±5.6 (1)
2	100	0	33.3±17.2 (1)	63.9±21.7 (6)
3	300	0	4.8±4.8 (6)	73.8±14.5 (4)
4	600	0	4.8±4.8 (6)	66.7±9.6 (5)
5	1200	0	9.5±9.5 (5)	58.9±14.5 (7)
6	0	100	14.3±0.0 (4)	77.8±14.7 (3)
7	0	300	23.8±4.8 (2)	83.3±9.6 (2)

[1] Excludes mortality due to *Vibrio* challenge [2] The ranking of mortality within the same strain from maximum (!) to minimum (5) are given in brackets.

highest in the fish receiving NH100 (33.3% mortality), whereas the lowest stress-

Table 4.17. Percent mortality of Atlantic salmon parr fed selected levels of heme

(H) and non-heme (NH) iron for 20 weeks, followed by a *V. anguillarum* bath

challenge. Values given are the mean of 3 replicate tanks at 14 days, post-

challenge related mortality occurred in fish given the NH300 and NH600 (4.8%)

diets. The percent mortality of fish due to vibriosis was highest (94.5%) in the

iron-deficient (no supplemental iron) group and lowest (58.9%) in fish given

NH1200.

There was, however, no significant difference in mortality due to stress

$(X^2=10.62, p \leq 0.05)$ and due to the challenge with *V. anguillarum* $(X^2= 6.27,$

$p \leq 0.05)$.

The LD$_{50}$ vibriosis infection

The LD$_{50}$ values for vibriosis infection (Table 4.18) showed that fish fed a diet

without supplemental iron for 20 weeks were killed by fewer *V. anguillarum*

bacterial cells $(4.1 \times 10^2 \text{cfu}$ and $1.13 \times 10^2 \text{cfu}$ for strains 1and 2, respectively)

than fish given diets supplemented with iron except in the case of fish given

NH300 (strain 1). Fish in groups NH300 and NH600 for strain 1 and all non

heme supplemented iron fed fish for strain 2 showed lower LD$_{50}$ values.

However, fish in groups NH100 and both heme supplemented iron fed fish

showed higher LD$_{50}$ values for both strains. The LD$_{50}$ values generally followed a

trend corresponding to the mortality rates given in Table 4.16.

Table 4.18. LD$_{50}$ values of *Vibrio anguillarum* colony formation units (cfu) required to kill Atlantic salmon (*Salmo salar*) parr after feeding the fish diets with selected sources and levels of iron for 20 weeks (NH= non heme, H = heme)

Diet No.	Dietary Iron in feed (mg/kg)		LD$_{50}$ of *V. anguillarum*	
	Inorganic (NH)	Hemoglobin (H)	Strain 1 (cfu)	Strain 1 (cfu)
1	0	0	4.1×10^2	1.1×10^2
2	100	0	8.9×10^3	5.8×10^2
3	300	0	1.0×10^2	3.2×10^2
4	600	0	6.9×10^2	4.2×10^2
5	0	100	2.2×10^3	1.7×10^3
6	0	300	2.1×10^3	6.7×10^3

<u>Serum bactericidal activity</u>

Bactericidal activity (expressed as reduction in bacterial growth) in non-immunostimulated normal (Untrt) fish serum was relatively higher in iron-deficient fish (Figure 4.3). There was no evidence of differences between dietary groups with heat inactivation (Heat) of serum. Preliminary observations based on the survival of *V. anguillarum* in non-immunostimulated fish serum showed no significant differences ($p \geq 0.05$) in the bactericidal activity against *V. anguillarum* with normal fish serum. However, a trend for an increase in cfu values with non-heme iron supplementation was evident after 5 hour of incubation of serum from these fish.

The bactericidal activity of immuno-stimulated normal (Untrt) fish serum against *V. anguillarum* showed a significant difference at 3 and 5 hours compared to 0 hours of incubation (Figure 4.4). However, serum bactericidal activity was impaired in fish fed an iron deficient diet (0), and in groups: NH600, H100 and H300 ($\log_{10}$ cfu, 0.74, 0.40, 0.34 and 0.60, respectively). The bactericidal activity could be abolished after heating the sera (Heat) for 30 min at 45°C (Figure 4.4). Non-stimulated fish serum from the same feeding groups did not exhibit any enhanced bacterial growth (Figure 4.3) and showed no significant difference in bactericidal activity between normal (non-heat inactivated) and heat-inactivated fish serum.
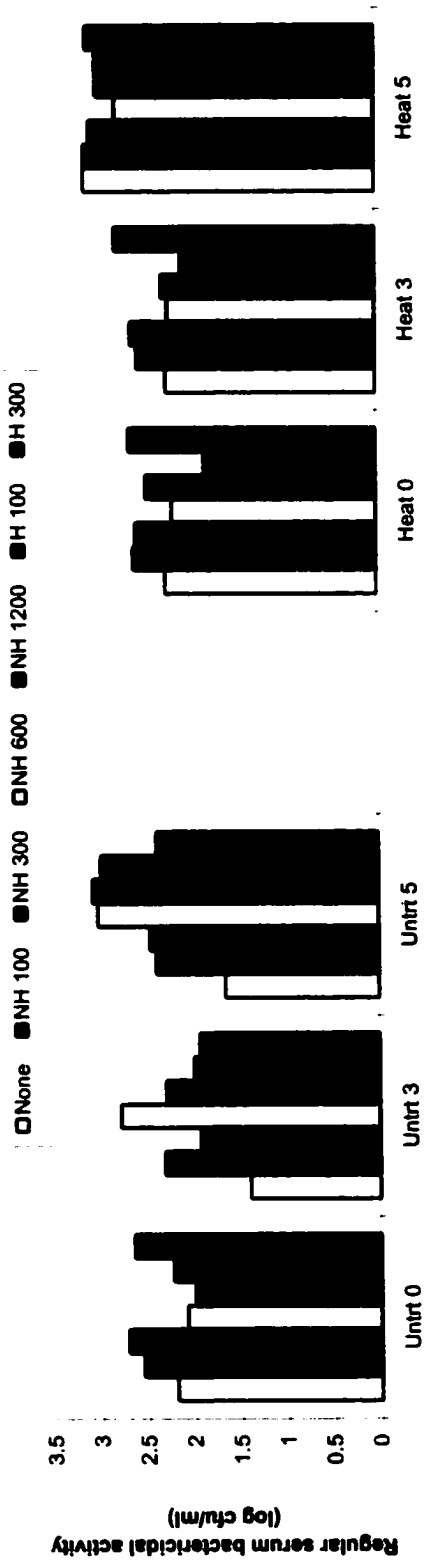
Figure 4.3. Bactericidal activity of serum from non-stimulated Atlantic salmon, fed diets containing 0, 100, 300, 600, and 1200 mg non-heme (NH) iron/kg from iron sulfate (FeSO₄·7H₂O) and 100 and 300-mg heme (H) iron/kg from hemoglobin for 32 weeks. Untreated (Untrt) and heat inactivated (Heat) serum were incubated with *Vibrio anguillarum* for 0, 3 and 5 hours.
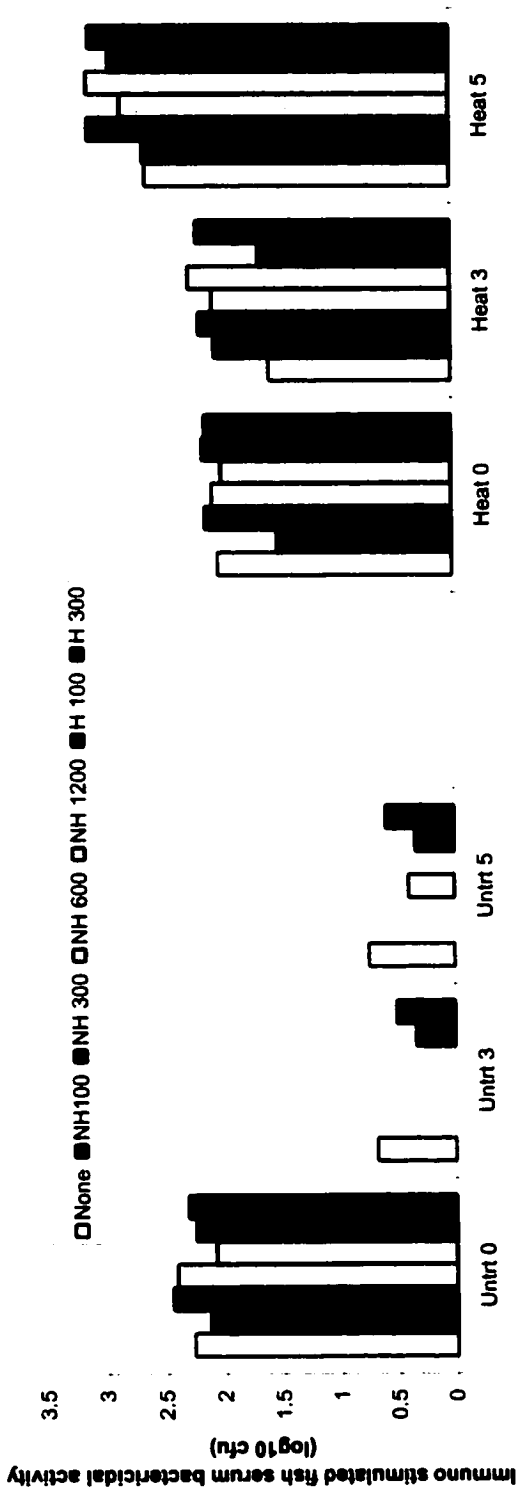
Figure 4.4. Bactericidal activity of immuno-stimulated serum from Atlantic salmon, fed diets containing 0, 100, 300, 600, and 1200 mg non-heme (NH) iron/kg from iron sulfate ($FeSO_4.7H_2O$) and 100 and 300-mg heme (H) iron/kg from hemoglobin for 32 weeks. Untreated (Untrt) and heat inactivated (Heat) serum were incubated with *Vibrio anguillarum* for 0, 3 and 5 hours.

Serum lysozyme activity

Lysozyme activities of regular and stimulated fish serum are represented in Figure 4.5. There were no significant differences in lysozyme activities in the serum of unstimulated fish irrespective of diet treatment (F=0.48, p≥0.05).

However, after immunostimulation, the lysozyme enzyme activities showed significant differences between the dietary groups (F=2.57, p≤0.05) with serum from fish receiving H100 having higher values than those noted for serum from fish given NH1200.

In addition to this, the stimulated fish showed significant differences in the serum lysozyme activity compared to the non-stimulated fish groups (t= 2.56, p≤0.05). After glycogen-stimulation, fish fed the heme 100 iron diet (H100) exhibited more lysozyme activity than fish fed the diet without iron supplementation and the NH600. In general, after stimulation lysozyme activity became higher relative to the corresponding non-stimulated groups except in the case of serum from fish given NH100 and NH1200. The lysozyme activities of serum from unstimulated fish fed NH100 and NH1200 were greater than those found for their respective stimulated counterparts but the differences were not significant.

T-tests carried out between and within stimulated and non-stimulated fish serum showed that lysozyme values significantly varied between stimulated NH1200 with non-stimulated 0 supplemental iron (t=3.37, p ≤0.05), or stimulated 0 iron (t=3.42, p≤0.05), or non-stimulated NH100 (t= 3.34, p≤0.05) or stimulated H100
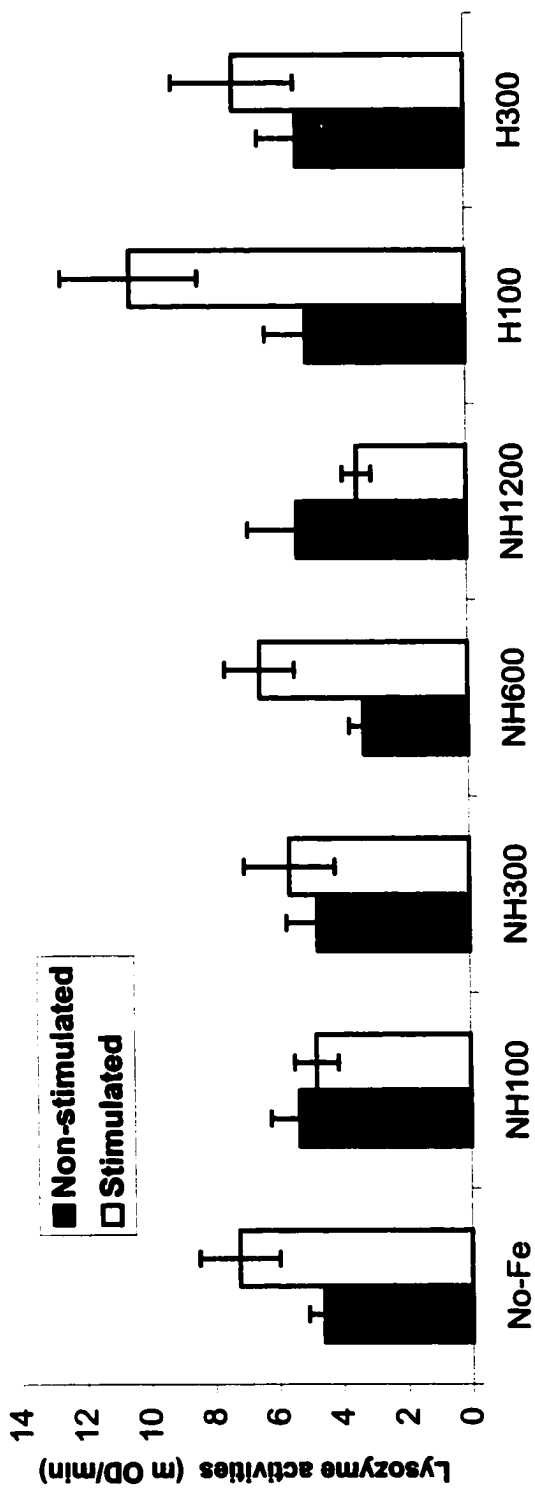
Figure 4.5. Serum lysozyme activity of non-stimulated and stimulated Atlantic salmon fed diets for 32 weeks with selected levels of iron (0, 100,300,600 or 1200 mg non-heme iron and 100 or 300 mg heme iron/kg). Immunostimulated fish received an i.p. glycogen injection 5 days before the sampling. Serum lysozyme activity was measured by the micro plate method. NH= non–heme iron level, H= heme iron level.

(t=-3.11, p≤0.05) groups. The lysozyme activities of stimulated H100 with non-stimulated H100 (t=2.92, p≤0.05), non-stimulated 0 iron (t=-2.80, p≤0.05) and non-stimulated NH600 (t=-3.65, p≤0.05) also varied significantly.

Respiratory burst activity of phagocytes

Respiratory burst (rb) activity of phagocytes measured by the microplate and plate count methods are represented in Figures 4.6 and 4.7, respectively. In general, it was evident from the microplate assay results that the rb activity was lowest in NH100 (0.273±0.05 at 590 nm; 0.349±0.08 at 650 nm) but increased as the non-heme dietary iron levels increased up to NH1200 (0.36±0.07 at 590nm; 0.48±0.09 at 650 nm). Phagocytes from fish fed the heme iron diets (H100 and H300) exhibited a similar trend like those from the fish fed diets with similar levels of non-heme iron (NH100 and NH300). However, the microplate methods indicated that the iron deficient fish kidney phagocytes showed highest rb activity (0.432±0.09 at 590nm; 0.568±0.11 at 650nm) but the activity was not significantly different than that noted for the iron supplemented diets (F_{590nm}=0.01 and F_{650nm}=0.03; p≥0.05). No such trend was observed in phagocyte cells by the plate counting methods. However, the mean values of rb activities of phagocytes by the plate count methods showed no differences among diets (F=0.02; p≥0.05).
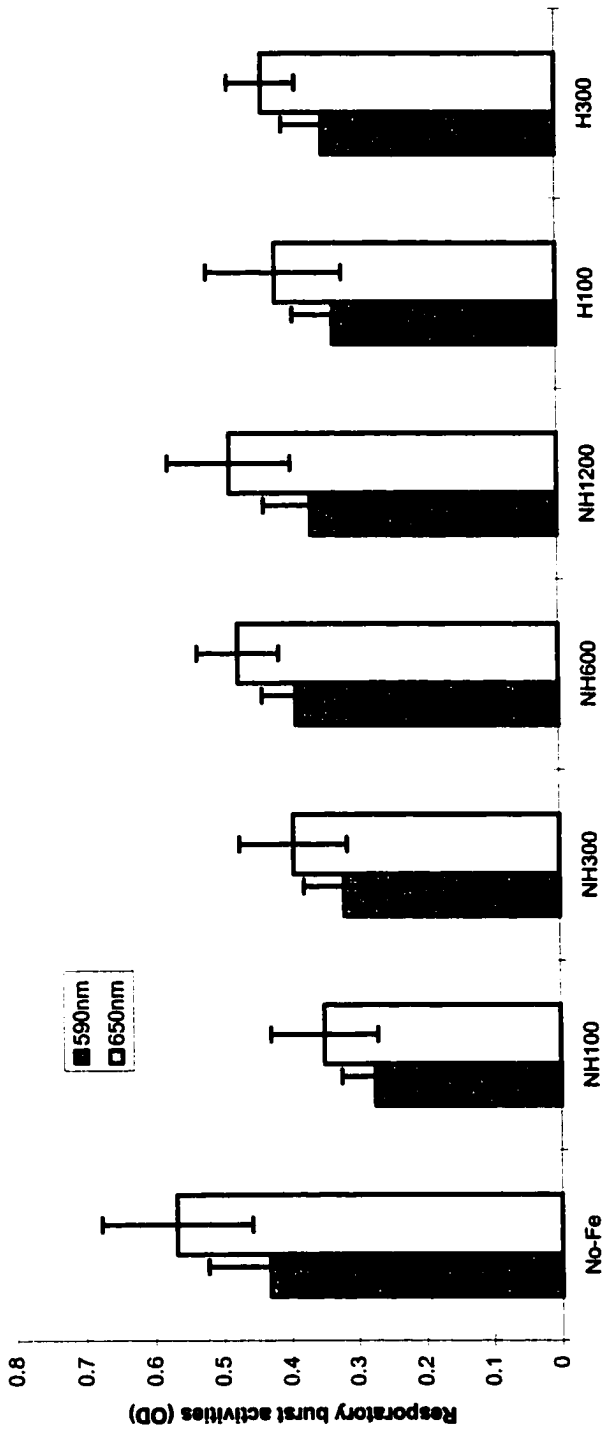
Figure 4.6. The respiratory burst activity of isolated phagocytic cells from fish fed diets with selected levels of iron (0,100,300,600 or 1200 mg non-heme iron and 100 or 300 mg heme iron/kg) for 32 weeks. The respiratory burst activity was measured by NBT assay and the results are expressed as OD at 590 and 650 nm wavelength. Non-heme iron levels (NH) and heme (H) iron levels.
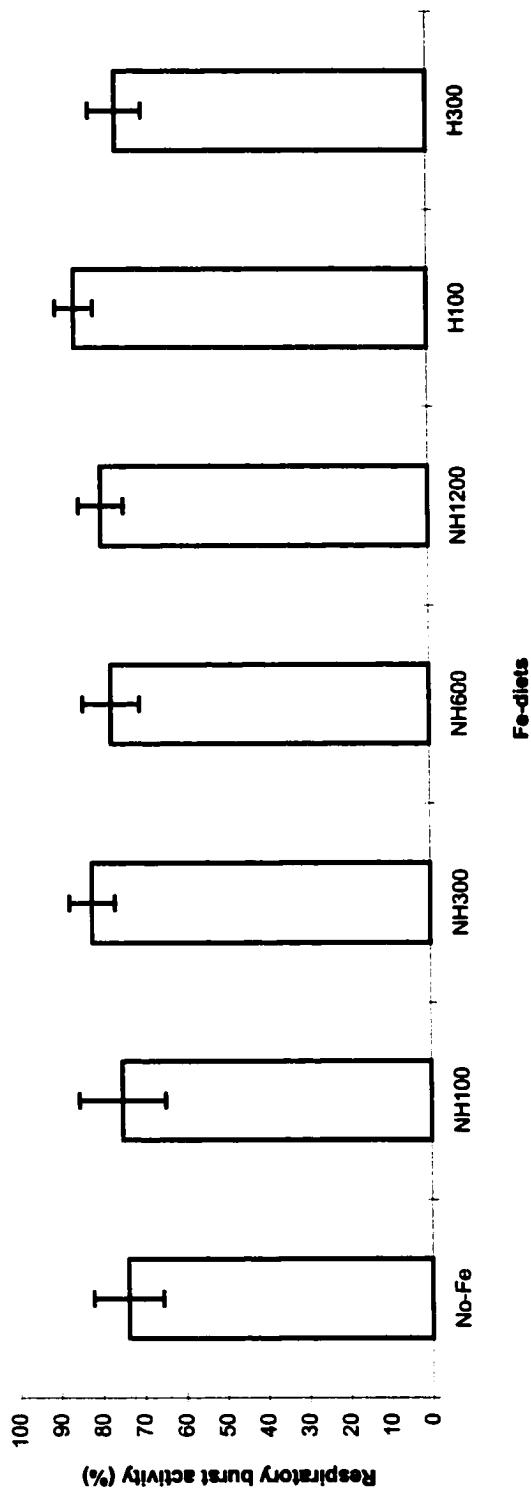
Figure 4.7. The respiratory burst activity of isolated phagocytic cells from fish fed diets with selected levels of iron (0, 100, 300, 600 or 1200 mg non-heme iron and 100 or 300 mg heme iron/kg) for 32 weeks. The respiratory burst activity was measured by NBT assay and the results are expressed as percent cell activation. Non-heme iron levels (NH) and heme (H) iron levels.

Lymphocyte proliferation activity

Three mitogens: Con A, LPS and PHA were used to determine the differences in proliferative responses of lymphocytes isolated from Atlantic salmon fed the NH100 and iron deficient (0 mg of supplemental iron /kg) diets for 32 weeks. The results of *in vitro* stimulation after 0, 6, and 9 days in culture are presented in Table 4.19. Proliferation of control lymphocytes, isolated from the anterior kidney of the 0 and NH100 groups, increased with time but were not significantly different among experimental groups. Proliferation of mitogen-induced lymphocytes, isolated from deficient and NH100 fish, also showed a trend of increase with time. However, they were no significant differences between the mitogenic activities of lymphocytes from the 0 and NH100 fish groups (F=1.02, 0.04, 0.06 ; p≥ 0.05).

The stimulation index (SI) of lymphocyte cells from the 0 supplemental iron fed fish was highest for all mitogens on day 6. The SI of LPS and PHA-induced lymphocyte cells increased significantly (p≤0.05) from day 0 to day 6 and 9. However, no significant differences between 6 and 9 days of incubation were evident for this diet treatment due to these mitogens. A similar trend was observed for mitogen ConA in the 0-iron fed fish only. The fish fed the diet with no supplemental iron exhibited in general a higher proliferation activity than the iron-supplemented group with all mitogens.

Table 4.19. In vitro stimulation of Atlantic salmon (*Salmo salar*) kidney lymphocytes by ConA , LPS and PHA after different days of culture. Fish were taken after 32 weeks of feeding diets without supplemental iron or 100 mg of inorganic iron/kg.

Dietary Iron supplement (mg/kg)	No of days in culture	Non-stimulated controls[1]	Stimulation index[1,2]		
			ConA	LPS	PHA
0	0	-0.076±0.018	0.030±0.006	0.021±0.008	0.020±0.004
	6	0.043±0.075	0.099±0.059	0.175±0.047	0.131±0.049
	9	0.111±0.050	0.089±0.062	0.126±0.036	0.076±0.049
	Pooled SEM	0.038	0.027	0.028	0.026
100	0	0.039±0.041	0.024±0.002	0.032±0.002	0.022±0.003
	6	0.087±0.029	0.049±0.019	0.128±0.017	0.079±0.028
	9	0.101±0.031	0.075±0.031	0.112±0.005	0.085±0.020
	Pooled SEM	0.028	0.012	0.016	0.014

[1]Optical density (OD)±SEM. [2]Stimulation index= mean OD of stimulated cells with test mitogen at day x- mean OD of control cultured cell at day x. (n=3).

Differential cell counts

The percentages of different cells in Atlantic salmon blood smears from fish fed diets with selected sources and levels of iron are presented in Figure 4.8. Only erythrocytes (RBC), monocytes (Mono) and eosinophils (Eos) were found in the smears. The blood smears consisted of large numbers of RBC (77.8 ± 1.5 to $89.3\pm1.1\%$), followed by Mono (8.1 ± 0.9 to $19.4\pm1.3\%$) and Eos (1.6 ± 1.2 to $6.3\pm1.9\%$).

The highest population of RBC was observed in the blood of the NH600 (89.3%) group and the lowest in the NH100 (77.8%) group. No significant differences in RBC and Eos abundance with respect to dietary iron were observed. Mono populations were significantly higher in the 0-iron and NH100 groups of fish than in the other non-heme or heme groups. Mono populations showed an inverse relationship with the dietary level of non-heme and heme iron. The group, H100 ($14.2\pm1.2\%$) exhibited a higher mono population than that noted in the H300 group ($12.8\pm0.6\%$), but the difference was not statistically significant.

Phagocytic activity of peritoneal phagocytic cells

The percentage of phagocytic cells and the mean number of associated *Escherichia coli* per phagocytic cells are summarized in Table 4.20. There is no significant difference in either of these parameters between deficient and 100 mg iron fed fish.
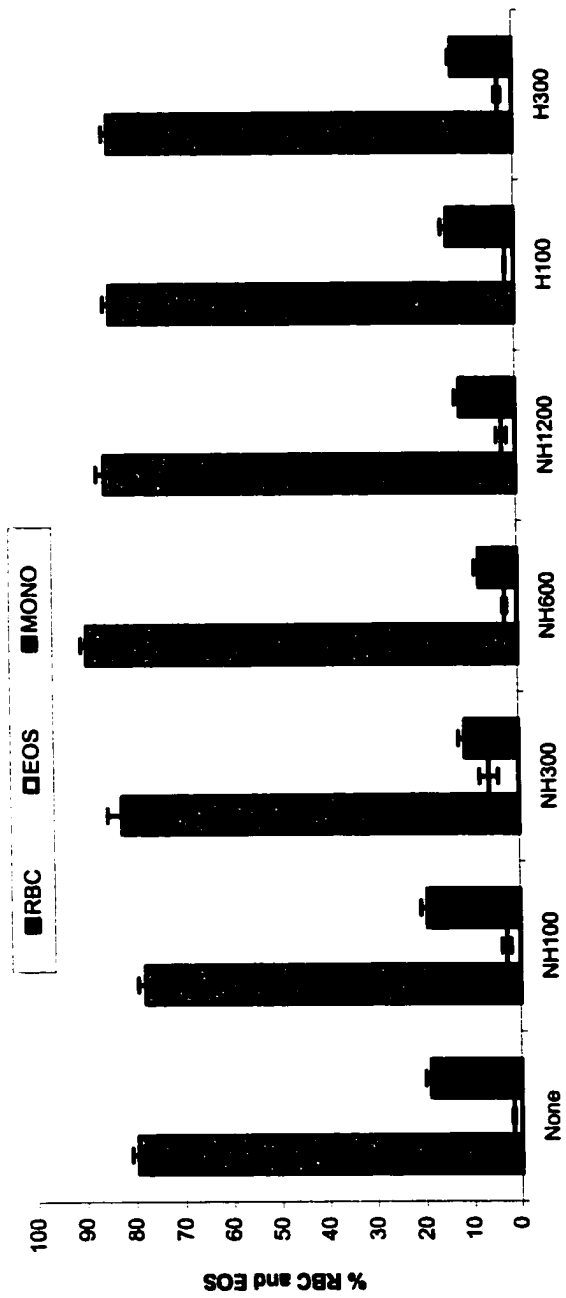
Figure 4.8. Differential cell counts (%) of Atlantic salmon parr fed with selected levels of iron in diets.

Table 4.20. *In vitro* phagocytic activity of Atlantic salmon peritoneal phagocytes against *E. coli* after fed iron-deficient and 100 mg inorganic iron/kg diet for 32 weeks[1]

Diet	Phagocytosis (%)	Number of bacteria /phagocytes	Number of Well
Deficient	73.5±6.3[a]	4.8 ± 1.3[a]	6
100 mg Fe/kg diet	74.2±7.5[a]	4.6 ± 1.6[a]	6

[1]Values are means±SE. Means in same column sharing a common superscript are not significantly different (p≥0.05).

Myloperoxidase (MPO) assay

After staining, colour developed in iron deficient and supplemented fish groups. Thus no differences were observed among fish fed deficient and iron supplemented diets, that is, they all exhibited a +ve stain for MPO.

4.4. Discussion

In this experiment, fish growth, feed efficiency and hepatosomatic index (HSI) were not affected by the level of dietary iron. In Chapter 2, I also reported no effect of iron supplementation on feed efficiency or HSI. Similar observations on growth have been reported earlier by Bjornevik and Maage (1993), Andersen et al.,(1996) and Lorentzen and Maage (1999) for Atlantic salmon. Further dietary iron content has no effect on the growth of other fishes, including brook trout (Kawatsu, 1972), carp (Sakamoto and Yone, 1978), red sea bream (Sakamoto and Yone, 1976) and yellowtail (Ikeda et al., 1973). However, iron deficiency has been reported to be related to retarded growth in *Xiphophorus spp.* (Roeder and Roeder, 1966) and channel catfish (Gatlin and Wilson, 1986; Lim et al., 1996 and Lim and Klesius, 1997). There is no published report on the effects of dietary iron supplementation on feed efficiency and HSI of Atlantic salmon. Iron deficiency can result in suppressed feed utilization in channel catfish (Gatlin and Wilson, 1986). However, catfish fed diets supplemented with 10 mg or more of iron were noted to have normal feed efficiency. Iron deficiency

has not been found to affect the feed efficiency of other fishes (Kawatsu, 1972; Ikeda et al., 1973; Sakamoto and Yone, 1976, 1978). Andersen et al., (1998) reported that the HSI of Atlantic salmon could be significantly increased by the combined effects of supplemental dietary iron and ethyl cellulose-coated ascorbic acid after 20 weeks of feeding the supplemented diets. Interestingly, this effect on HSI was not evident on or before 16 weeks of the same feeding trial. In addition, dietary iron and/or ascorbic acid polyphosphate reduced the HSI values after 20 weeks in the experiment conducted by these authors. Lim et al. (1996) did not observe any effect of iron deficiency on feed conversion in channel catfish.

In this study, spleen-body ratios varied significantly in the 20-week feeding trial. Fish fed the diet with 100 mg of heme iron/kg had a significantly higher spleen-body ratio than found for the iron deficient fish; however no other significant differences were evident from the data. In Chapter 2, no significant differences in spleen-body ratios were evident after 20 weeks of feeding the iron-supplemented diets. In this study, there were no significant differences in the spleen-body ratios of the fish fed the test diets for 32 weeks. The spleen-body ratios in the 32-week feeding trial were more than double those seen for fish fed all of the diets in the 20-week feeding trial. The reason for this difference between the trials is unknown. Agius (1979) observed that the spleen of starved and diseased fish increased due to the deposition of ferric iron. To our

knowledge, fish in the 32-week trial had never been exposed to any disease and no disease was evident during this trial. There were significant differences in the condition factors of the fish in both trials. However there were no consistent trends in condition factors between fish receiving similar diets in these trials. Anderson et al. (1996, 1998) did not observe any differences in condition factors of Atlantic salmon in response to dietary iron variation. However, Bjornevik and Maage (1993) reported a decline in fish condition factors when they were fed a diet with 160 mg of iron/kg. Differences in condition factor can be caused by water temperature variations (Russell et al., 1996) and 'fatness' or performance of fish (Bagenal and Tesch, 1978). Although the studies of Bjornevik and Maage (1993) and Andersen et al. (1996) were conducted in the same research station, different feeding trial periods were used. The relevant period of the experiment of Bjornevik and Maage (1993) was 8 weeks whereas that for Andersen et al. (1996) was 12 weeks. Moreover, both experiments were conducted using a river water supply, which resulted in seasonal fluctuations in water temperature. In contrast, the experiment of Andersen et al. (1996) was conducted at a water temperature between 10° to 12°C, which was close to the water temperature (12°C) maintained in the present study.

Iron deficient diets have been reported to cause reduced values for hematocrit (Hct), hemoglobin (Hb), mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) in fish, but

no effect on red blood cell (RBC) counts (Andersen et al.,1996). In this study,

fish did not exhibit any true anemic conditions due to iron deprivation. There

were no significant differences in Hct and MCHC due to diet treatment in the 20-

and 32-week trials. Hemoglobin varied significantly between groups in the 32-

week feeding trial, but there was no trend across the diets and no consistency of

the results in the 20-week feeding trial. The RBC counts varied significantly

among groups in the 20-week trial and this was also true for the MCV and MCH

values. The significant differences in MCV and MCH were expected, as these

values were derivatives of RBC with Hct or Hb. Thus when any of the preceding

parameters varied significantly, they resulted in variations in MCV and MCH

values. Bjornevik and Maage (1993) in their experiments did not find any

significant differences in Hb and Hct values in fish due to dietary iron differences.

A depletion of hematological parameters has been reported as a clinical sign of

iron deficiency in brook trout (*Salvelinus fontinalis*) (Kawatsu, 1972). In Chapter

2, as well as in other reports (Andersen et al.,1996; Rasmussen,1994; Bjornevik

and Maage,1993 in Atlantic salmon, Roeder and Roeder,1966; Walker and

Fromm.,1976; Gatlin and Wilson., 1986; Desjardins et al.,1987 and Lim et al.,

1996; in other fishes), the hemotology of fish has been observed to vary

remarkably with dietary iron content. In channel catfish, values for Hb, Hct, RBC

and MCH were found to be significantly lower in iron deficient fish (Gatlin and

Wilson, 1986). There were no significant differences in these blood parameters

in catfish when the supplemental dietary iron varied between 10 and 30 mg/kg. The insignificant differences of all hemotological parameters except Hb in the 32-week feeding trial may indicate that excess dietary iron does not have any strong adverse effects on fish hemotology. It is likely that fish have mechanisms by which they can compensate for the dietary iron variations like other domestic animals and humans (Morris, 1987; Sayers et al., 1994). Fish can absorb iron from water (Lall, 1989) and perhaps they have a mechanism for excreting excess iron. The values of Hb, Hct and RBC were always lower in this study than those of other studies on Atlantic salmon (Andersen et al., 1996; Sandnes et al., 1988). This difference in findings may have occurred because of dissimilar fish stocks, rearing periods, and life history stages of the fish that were used. Sampling techniques can also influence Hct and Hb values. For instance, increased values have been observed under different conditions like fish stress, and they may be affected by sampling interval and the way the blood samples are handled (Lowe-Jinde and Niimi, 1983). In this study, extreme care was taken to avoid fish stress and sampling error. Therefore the values, likely accurately represent the hemotological conditions of the experimental fish.

Not only did the level of iron in the diet, but also the form of iron (heme or non-heme iron) affect the concentrations of serum iron and iron-binding proteins in fish serum after 20 weeks. This probably occurred because heme iron and non-heme iron have different absorption mechanisms (Condrad and Umbreit,

1993). The highest levels of serum iron were noted in fish fed the diets with supplemental non-heme iron at 300 mg or 600 mg/kg. The higher iron levels in the serum may have resulted from greater absorption of inorganic iron in the intestinal lumen when the dietary concentrations were high. Significantly lower levels of serum iron, total iron binding capacity (TIBC), transferrin saturation and ferritin were observed in fish fed the iron deficient diet. In addition, transferrin saturations were also significantly lower when the heme iron source was employed. On the other hand, ferritin was significantly higher in fish fed the diets with heme iron and thus was also true for fish fed the NH100 diet. In this regard, the iron needs of the body are controlled in an unusual manner, which mainly involves changing the content of iron in the storage protein, ferritin. Standal et al., (1998) reported that expression of the iron regulatory element of the ferritin gene is controlled by levels of iron in the diet. In addition, the levels of transferrin receptor and ferritin are regulated in a coordinated manner. Details of these mechanisms in fish, however, are unknown. In higher animals, when a cell needs more iron, the transferrin receptor increases in number in the plasma membranes of the cells, thus promoting the uptake of iron. At the same time, ferritin synthesis decreases, to promote the uptake of iron (Klausner et al., 1993). The abundance of ferritin in these dietary groups may be related to the low level of transferrin saturation with iron in these fish. In support of this view, fish fed diets with an excess of iron, where transferrin saturation was evident, generally

showed low ferritin production. Two possible explanations can be given.

Transferrin may be used to carry excess iron for disposal. Secondly, transferrin

may be dedicated to stopping peroxidative damage of tissues by capturing the

excess iron absorbed from the diets. Assessment of iron intoxication in humans

includes evaluation of serum iron, transferrin and total iron binding capacity

(TIBC) (Arena, 1986). Standal et al., (1998) stated that the TIBC could not be

calculated using fish due to its low availability. In this study, TIBC values from

Atlantic salmon were analysed by atomic absorption spectrophotometry and they

definitely showed a positive correlation with the dietary iron supplements.

Trends similar to the serum iron values with transferrin saturation were reported

by Standal et al., (1998). From this experiment, it is evident that serum iron,

TIBC, transferrin saturation and ferritin levels in fish are useful for monitoring iron

in fish and they can be used to evaluate the iron status of the fish.

After the 32-week feeding trial, liver iron concentrations showed a gradual

increase with the iron level of the diet. Similar positive correlations were

observed by Desjardins et al. (1987), Bjornevik and Maage (1993), Andersen et

al.(1996) and Kvingedal et al. (1996), for salmonids following dietary iron

supplementation. In the present study, the actual concentrations of iron in the

liver of all groups were found to be lower than those reported in the other

studies. Moreover, the addition of iron to the diets in exceess of the requirement

did not increase concentration of hepatic iron in the same manner as reported by

Andersen et al. (1996). The reason for this difference may be due to differences in genetic stocks, experimental conditions, and protocols between this studies. Another possible reason is illustrated in Figure 4.9 and this will be constdered later. Diets containing heme iron generally led to a higher deposition of iron in the liver compared to those containing similar levels of non-heme iron.

The highest level of iron deposition in the spleen was found in the fish receiving the H100 diet followed by those ingesting the NH100 and H300 diets. In the kidney, iron deposition was higher in all groups fed the iron-supplemented diets relative to those consuming the low iron diet, except the fish consuming NH300. In general, spleen tissue absorbed 2 to 10 times more iron/g than kidney or liver tissue when all of the dietary treatments were considered. These differences in tissue iron contents are likely due to different metabolic pathways in these organs. In fish, blood flows from the spleen to the liver and from the liver to the gills (Smith and Bell, 1976). Kidney and liver blood flows directly to the gills. Thus, it is most likely that the liver and kidney have a mechanism to reduce excess iron by releasing it to the blood flows towards the gills. The gills, in turn, release this iron to the environment (Figure 4.9). There is no information available on iron metabolism in the fish spleen. In higher vertebrates, including humans, the RBC have a fixed life span. Indeed, the is an apparent mechanism to remove senescent RBC from blood molecules by special phagocytic cells called macrophages (Mo). Most of the senescent RBC cells are consumed by
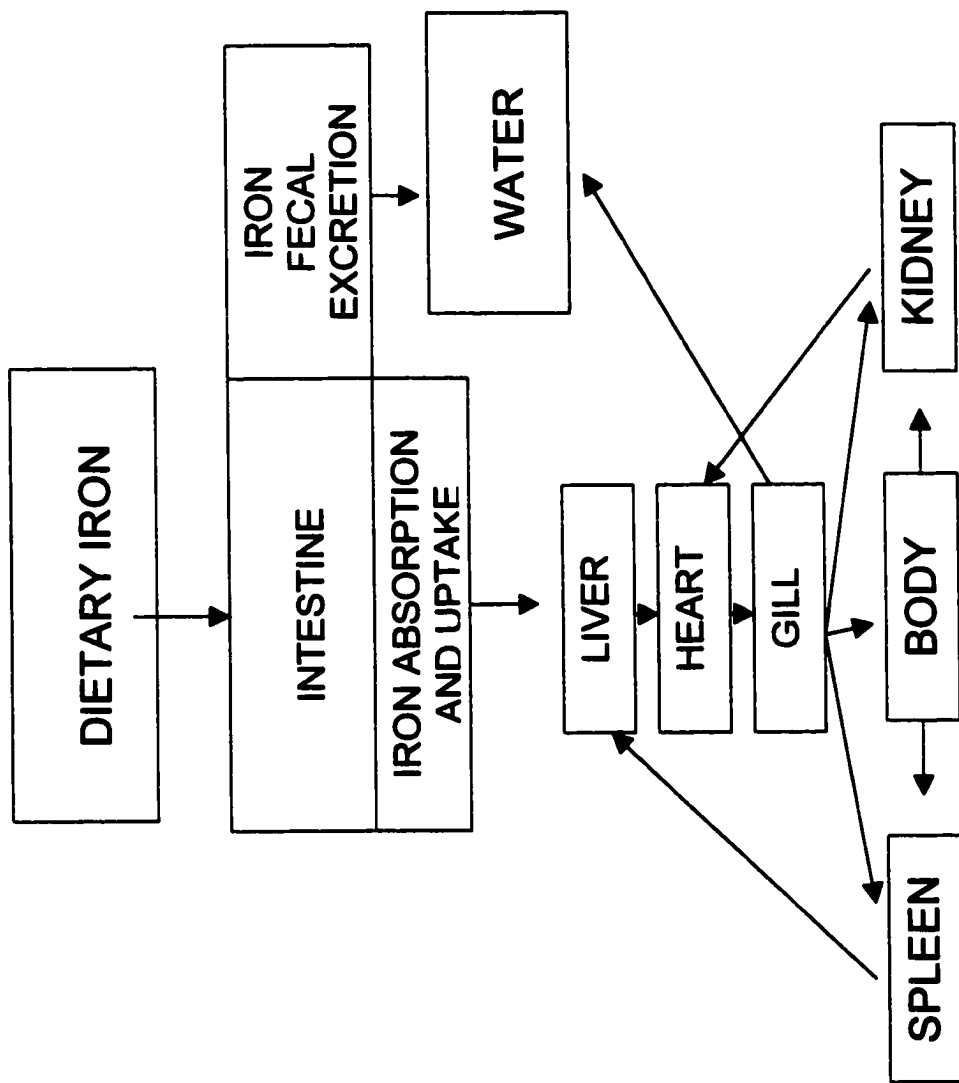
Figure 4.9. Possible excretion pathways of iron from fish body.

Mo in the spleen. The liver and kidney also contain Mo. Those in the liver are called Kupffer cells. Kupffer cells, however, do not consume lipoprotein particles. The relationship between RBC and spleen iron content has been discussed in Chapter 2. Such relationships were observed in the 32-week trial. The non-heme iron supplemented groups generally showed parallel trends between RBC values, and the levels of iron in spleen, except for fish receiving NH1200. The lower RBC in the NH1200 group may have been due to a toxic effect of dietary iron. The higher RBC values of the NH100 and heme-supplemented groups are consistent with the higher spleen iron values.

A possible pathway that illustrates how Atlantic salmon can overcome the effects of excess dietary iron I shown in Figure 4.9. In this scheme, dietary iron is first absorbed from the gut wall and then is transferred to the liver and to other reticuloendothelial tissues. Most of the functions of iron regulation in fish as well as other vertebrates are not clear. However, transferrin and ferritin are involved in tissue distribution and storage of iron. Fish fed higher dietary levels of iron can also directly excrete the iron in the feces without absorbing it through the gut wall. Knowledge of the renal or fecal excretion of iron in fish is very limited, due to difficulties in obtaining non-contaminated samples of urine or fecal material from fish. Dependable methods still need to be developed for mineral studies of fish. Excess iron could be transferred to the gills through the blood by transferrin to be released in to the environment. Fish can also obtain iron by mobilizing and

reabsorption of iron from the liver as well as from the gut wall depending upon the iron status of the body (Standal et al., 1999). Iron can be directed back to the liver through the blood. This model could explain why the hepatic iron contents were lower than reported in other studies (Chapter 2; Bjornevik and Maage.,1993; Andersen et al., 1996).

Zinc levels in the liver, spleen and kidney were unaffected by diet treatment. This finding agrees with those of Andersen et al., (1996) who reported no variation in Zn contents in body tissue in relation to the dietary level iron supplementation.

Copper levels could only be determined for the liver since the copper values found in this study for the spleen and kidney were below detectable levels using AAS. In Cu values for the liver samples showed that there was an interaction with the dietary level of iron supplementation period. Cu values were highest in fish ingesting the iron deficient diet followed by those consuming the NH300 and H100 diets. However, NH100 liver samples had the lowest copper content indicating there was no consistent relationship between liver copper content and dietary iron supplementation.

Liver magnesium (Mg) levels were highest in the iron deficient fish. In the spleen, Mg was highest in the heme iron-fed fish. By contrast, in the kidney, the Mg level was highest in NH100- fed fish and the iron-deficient and H100-fed fish exhibited the same levels. Thus, Mg did not show any consistent relationships

with dietary iron in the reticuloendothelial tissue.

An excess of iron in the diet is not desirable because feed quality can be lowered by the lipid peroxidation process (Desgerdins et al., 1986). However, an excess of iron (Standal et al., 1998) or a deficiency of iron (Andersen et al., 1996, Gatlin and Wilson, 1986) in the diet has been found to have an adverse effect on fish physiology. Shearer et al. (1994) showed only small variations in whole body iron concentrations in Atlantic salmon during their life cycle. This suggests that Atlantic salmon fed diets containing required levels of iron can maintain a stable body iron concentration during growth and development. It can be concluded that Atlantic salmon exhibit some clinical signs of iron deficiency. Also, there are mechanisms that help the fish to eliminate the excess iron from the body. However, any excess dietary iron if excreted into the environment, may cause environmental problems by facilitating the growth of diatoms (Muggli and Harrison, 1997), algae (Johnson et al., 1999) or bacteria (Reid et al., 1993; Hutchins et al., 1999). Such growth in the case of marine phytoplankton may possibly lead to the production of algal toxins or red tide or facilitate bacterial infection(s) in the vicinity of the fish cages.

Total cumulative mortalities of the fish fed the diets with different levels of iron for 20 weeks did not differ after a cohabitation challenge with A. salmonicida. A similar challenge conducted on fish from the 32-week feeding trial initially showed in some differences in percent mortalities among fish given different

diets (data shown for 16 days) but all groups had 100% mortality after 28 days. However, in both challenges iron deficient fish showed a similar trend for mortality trend until 13 days post challenge. There was no consistency in findings between the 20- and 32- week feeding trials with respect to the mortality patterns in relation to diet treatments. Nordmo and Ramstad (1999) reported that the mortality of fish in an *A. salmonicida* cohabitation challenge depended on the water temperature and the number of cohabitants added. The present experiments were conducted at constant water temperature and the same number of fish cohabitants was used.

Percent cumulative mortalities of fish challenged by two strains of *Vibrio anguillarum* were higher for fish fed iron-deficient diets in the 20 and 32-week trials relative to most other treatment groups. However, fish ingesting the iron-supplemented diets did not show any consistent trend in mortality for the same diet treatments with the two strains of pathogen used. These results were generally supported by the LD_{50} studies since iron-deficient fish were found to be infected by a small number of colony formation units (cfu). Dietary levels of iron had no effect on cumulative mortality at 4 days when channel catfish were exposed to the *Edwardsiella ictaluri* (ESC) bacterium (Lim et al., 2000). However, it was evident from earlier experiments that the iron deficient fish were more susceptible to infection than the fish supplied with sufficient iron in their diets (Lim et al., 2000; Lim and Klesius. 1997, Sealey et al., 1997). Considering

the source of dietary iron, fish fed organic heme iron showed prolonged survival compared with fish fed the inorganic non-heme iron diet in the 32-week trial. In general, a similar trend was observed in the 20-week fish until day 9.

Interestingly, Sealey et al. (1997) also reported that channel catfish could survive longer after an ESC challenge when they were given organic iron in their diet compared to those fed a diet with inorganic iron. The lack of clearcut differences in mortalities between diet treatment and the bacterial challenges may have been due to stress in the fish during the experiments. There are no standard for conducting nutrition/ health experiments in fish. Fish may be challenged with higher cfu, which, in turn, may result in intense infections and fish mortality. The cohabitation challenge results showed some differences due to treatment in the 32-week experiment as the bacteria were shed into the water from the cohabitated fish. This is further supported by the experiment of Nordmo and Ramstad (1999) where a bath challenge with Vibrio salmonicida infection was found to be related to the dose of cfu injected in to the fish. A relationship between dietary iron content and ESC challenge has been reported with some studies on channel catfish (Lim et al., 2000; Lim and Klesius, 1997; Sealey et. al., 1997). With respect to this, the iron-deficient fish were found to be more susceptible to infection. Sherman (1992) suggested that a delicate balance exists between the need for iron for host defense mechanisms and the need for iron to sustain microbial growth. However it should be kept in mind that the host

defense mechanism varies with the species of the host as well as the bacterial types (Secombes and Olivier, 1997).

With respect to the non-cellular immune systemof the salmon in this study, the fish challenged with *V. anguillarum* showed no differences in serum bactericidal activity. However, clear differences were observed after immunostimulation. Bactericidal activity was slightly suppressed in both iron-deficient fish and those fed diets with heme iron. Also, some bacterial growth was evident in one NH600 plate after 5 hours. As no bacterial growth was evident in the 3 hour slides, this may have been due to contamination in the plate. Trust et al. (1981) observed that pathogenic *V. anguillarum* are resistant to coho salmon serum bactericidal killing and perhaps this is due to species differences (Secombes and Olivier, 1997). However, in general Atlantic salmon serum is capable of efficient killing of the *V. anguillarum* bacterium. The impairment of bactericidal activity in the iron-deficient fish followed the trend observed earlier, but the reason for bacterial growth in fish fed diets with heme iron, even after immunostimulation was not clear.

In general, serum lysozyme activity was found to be highest in the NH100 and NH1200 unstimulated fish, but after immunostimulation serum lysozyme activity was reduced in fish given these dietary treatments. After immunostimulation lysozyme activities of serum increased in most dietary groups. However, there was no significant difference of serum lysozyme activity

due to diet treatment. Moyner et al. (1993) reported significant increases in lysozyme activities in Atlantic salmon after experimental infection with *A. salmonicida*, indicating that the activity was a normal defense measure in the fish. In the present experiment, Atlantic salmon exhibited lysozyme activity when unstimulated or imunostimulated but, once again there was no evidence for effects of dietary iron supplementation on serum lysozyme activit

CHAPTER 5. The effects of dietary iron intake on changes in tissue iron

content of Atlantic salmon (*Salmo salar*) infected with furunculosis

(*Aeromonas salmonicida*) and vibriosis (*Vibrio anguillarum*)

5.1. Introduction

One of the characteristic clinical signs of bacterial infection in animals and

fish is a significant decrease in plasma iron levels (Weinberg, 1989; Ravndal,

1994). Iron plays a key role in the pathology of bacterial infections by enhancing

the multiplication and virulence of the invading microorganisms. Therefore, tissue

iron concentration may determine the availability of iron to microorganisms.

Depriving pathogens of iron may serve as a non-specific protective mechanism

against infection (Weinberg, 1989).

Iron is a constituent of hemoglobin, and myoglobin and several enzymes.

In serum, iron is normally bound to and transported by serum transferrin.

Bacteria have several mechanisms to overcome the ability of the host to restrict

iron bioavailability. The best studied bacterial mechanism is that of iron

chelators, siderophores, which compete with the iron binding ability of transferrin

(Otto et al, 1992). Free iron plays a vital role in the proliferation of bacteria in

mammalian and fish species (Bullen et al., 1978; Ravndal, 1994). Injection of

fish with Fe enhances the virulence of some bacteria such as *V. vulnificus*

(Wright et al, 1981), *V. parahaemolyticus* (Karunsagar et al, 1984) *and V.*

anguillarum (Nakai et al, 1987). Copper is another important element in many

metal binding proteins and enzymes, some of which are essential for the proper

utilization of iron (Hemphill, 1992).

During an infection in animals, the availability of iron for acquisition by

microorganisms is sharply limited by two major mechanisms. The first, involves

rapid sequestering of iron in tissue storage forms (Beisel, 1979) and the second,

sequestering of iron by the presence of excess quantities of transferrin and

lactoferrin in body fluids. These iron-binding proteins have very high association

constants for iron (in the range of 10^{36}). This means that the amount of free iron in

equilibrium with these proteins is only about 10^{-18} M, which is too low for normal

bacterial growth. The theoretical aspects of this type of nutritional restriction have

been discussed in detail by Bullen et al. (1978). When excess iron is added to

saturate the iron-binding capacity of either transferrin or lactoferrin, their ability to

function as an antibacterial system may be lost (Bullen et al.,1978). Infection

induces abrupt redistribution of iron from serum into cellular storage sites in the

liver, spleen, and bone marrow of animals (Bullen et al., 1978).

Although iron is an essential nutrient for bacterial pathogens in fish (Crosa

and Hodges, 1981), little is known about its role in infection of aquatic organisms.

However, changes in tissue iron caused by bacterial or parasitic infections have

recently been reported (Nakai et al., 1987; Hjeltnes and Julshamn 1992; Kakuta

and Murachi, 1993; Grayson et al, 1995; Bakopoulos et al., 1997; Simko et al.,

1999). Nakai et al. (1987) reported that increasing the availability of free iron by

intramuscular injection of ferric ammonium citrate caused a significant increase

in the virulence of *V. anguillarum* in eels (*Anguilla japonica*) and ayu

(*Plecoglossus altivelis*). The infection was more persistent in eels than in ayu,

perhaps due to genetic differences between the two species. Izuru and Murachi

(1993), using the same species reported that serum and tissue iron levels

decreased as the infection progressed. Ravndal et al., (1994) studied different

families of Atlantic salmon (*Salmo salar*) and reported that families with higher

serum iron levels seem to be more susceptible to *V. anguillarum* infection but not

A. salmonicida infection. Earlier, Rorvik (1992) reported that a natural outbreak

of *A. salmonicida* correlated with the iron concentration in the diet of the fish but

not with the iron concentration in the serum.

Furunculosis and vibriosis caused by *A. salmonicida* and *V. anguillarum* ,

respectively are the two most widely distributed bacterial diseases in salmonid

fishes in Canada. It was evident from an earlier experiment that dietary iron

deficiency causes mortality in fish (Chapter 4), but there was no clear effect of

dietary iron supplementation on the immune system and disease resistance to

vibriosis and furunculosis in salmon. The main objective of this experiment was

to investigate the depletion of tissue iron after *A. salmonicida* and *V. anguillarum*

infections in Atlantic salmon fed a practical diet based on fish meal as a major

source of protein. It was anticipated that this information could be used to gain a

better understanding of the host's response to bacterial infections as well as to

tailor iron levels in the diet during the growout period when these pathogens are

highly prevalent.

5.2. Material and methods

Fish and feeding protocols

One hundred and fifty Atlantic salmon fingerlings were obtained from the

Department of Fisheries and Oceans (DFO), Collingwood, Nova Scotia. The fish

underwent a 3-week acclimation period during which they readily adjusted to the

aquaria and standardized environmental conditions. They were maintained in a

flow through system in three 100 L circular tanks and the general rearing

conditions were essentially the same as those described in chapter 2. Water

was supplied to each tank at a flow rate of 2L per minute and temperature was

maintained at $15\pm1^{\circ}C$. Fish were fed a practical diet based on herring meal

(Table 5.1) for four weeks. The experimental protocol for feeding, rearing

conditions and the care of experimental of fish was essentially the same as

described in Chapter 2. Average weight of Atlantic salmon was $61.4\pm 3.5g$.

Disease challenges

Prior to the disease challenge, 15 fish were checked for bacterial and

fungal pathogens as described in chapter 4 and all fish were found to be free of

infection. One hundred fish were equally divided into four groups and then they

were transferred to a quarantine laboratory and held in four tanks. Fifty fish in

two tanks were infected with V. anguillarum by bath immersion. Fish in the

remaining two tanks were used for a cohabitation challenge with A. salmonicida

to infect them with furunculosis. The protocols for these challenges, strains of

Table 5.1. Composition of the experimental diets

Ingredient	Diet 1 (%)
Herring meal (72% protein)[1]	41.0
Casein vitamin free[5]	7.6
Krill meal (54% protein)[2]	4.0
Corn gluten meal (60% protein)[2]	3.5
Wheat middlings (17% protein)[3]	16.5
Whey (13% protein)[4]	7.0
Celufil[5]	1.4
Mineral mix[6]	1.0
Vitamin mix[7]	1.0
Choline chloride	0.2
Herring oil [1,8]	16.8

[1] Comeau Seafood, Saulnierville, NS.
[2] Corey Feed Mills, Fredericton, NB.
[3] Dover Flour Mills, Halifax, NS.
[4] Farmers Dairy, Halifax, NS.
[5] US Biochemical, Cleveland, OH.
[6] Minerals added to supply the following (mg/kg diet): Mn,40 mg; Zn, 75 mg; I,5 mg; Co,5 mg; F,4.5 mg; Se 1 mg.
[7] Vitamin added to supply the following (IU or mg/ kg diet): vitamin A, 6000 IU; vitamin D_3, 3000 IU; vitamin E, 200 IU; vitamin K, 30 mg; thiamin, 40 mg; riboflavin, 50 mg; d-calcium pantothenate, 150 mg; biotin, 0.08 mg; folic acid, 15 mg; vitamin B_{12}, 0.10 mg; ascorbic acid, 300 mg; niacin, 200 mg; pyridoxine, 20 mg; inositol, 100 mg; butylated hydroxy toluene, 10 mg.
[8] Stabilized with 0.06% ethoxyquin.
[9] Rovimix Stay C25 (polyphosphate form)
[10] Added as premix after mixing in wheat middlings

infectious agents and inoculation and test procedures have already been described in Chapter 4. In each infected group, one tank of fish was starved whereas the second was fed the experimental diets.

Experimental

Plasma and tissue (spleen and kidney) samples were collected from five Atlantic salmon before subjecting them to *A. salmonicida* and *V. anguillarum* infections. Fish were held in the tanks for seven days and mortalities were monitored for bacterial pathogens. After 48 and 96 hours post-infection, six fish from each tank were sacrificed to remove tissues and blood for mineral analyses. These fish were checked for bacterial pathogens to make sure that the disease challenges for *V. anguillarum* and *A. salmonicida* were effective. Morbidity was characterized by dark coloration, lethargy and death within a few hours. Fish infected with *A. salmonicida* became morbid much earlier than those challenged with *V. anguillarum*; an average period of approximately 3.5 days and 6 days respectively with these challenges. All fish sampled for blood and tissue analysis showed positive signs of clinical infections, which was confirmed by tests already described in Chapter 4. Liver and spleen were quickly removed after the blood collection and stored in a -80°C freezer until analyzed. All tissue samples were thawed prior to mineral analysis. The serum mineral concentrations were analyzed directly by atomic absorption spectrophotometer (Perkin Elmer 5000).

The tissues were acid-digested prior to analysis. The analytical methods have been already described in Chapter 4.

5.3. Results

Plasma iron concentrations from fish sampled at various stages of the infections are provided in Table 5.2. The plasma iron levels were significantly $(p<0.05)$ higher in fish after $V.$ anguillarum infection than in fish sampled before the disease challenge in both groups, fed or not offered experimental diet. This response was not observed for plasma copper or zinc levels. After four days of infection, plasma iron and zinc levels decreased significantly regardless of the feeding level. Atlantic salmon infected with $A.$ salmonicida did not accept experimental diets soon after the disease challenge. They showed a gradual decrease in plasma iron, copper and zinc concentrations when tested at two and four days after the infection.

Tissue trace element concentrations of the fish fed the experimental diet were not monitored because fish infected with $A.$ salmonicida did not accept the experimental diet during the test period and extensive sampling was required to examine the fish for bacterial pathogens during the short duration of this study. Moreover, it was obvious from the results of the previous experiments and the data obtained in our laboratory, that dietary iron intake has little influence on tissue iron concentrations during a short experimental period.

Table 5.2. Iron, copper and zinc concentrations in the plasma of Atlantic salmon after the fish were infected with *Aeromonas salmonicida* and *Vibrio anguillarum*[1].

Bacterium challenged	Stages of infection (days)	Iron (µg/mL)		Copper (µg/mL)		Zinc (µg/mL)	
		Unfed	Fed	Unfed	Fed	Unfed	Fed
A. salmonicida	0	$5.30+0.85^c$	$5.30+0.85^c$	$0.63+0.05^a$	$0.63+0.05^a$	$11.62+0.70^a$	$11.62+0.70^a$
	2	$4.78+0.56^{cd}$	$5.02+0.53^c$	$0.65+0.03^a$	$0.67+0.03^a$	$10.94+0.65^a$	$10.88+0.65^d$
	4	$3.10+0.37^d$	$3.36+0.25^d$	$0.42+0.07^b$	$0.41+0.04^b$	$7.28+0.62^c$	$6.92+0.44^d$
V. anguillarum	0	$5.30+0.85^c$	$5.30+0.85^c$	$0.63+0.05^a$	$0.63+0.05^a$	$11.62+0.70^a$	$11.62+0.70^a$
	2	$12.94 +1.12^a$	$15.26+0.75^a$	$0.36+0.02^b$	$0.37+0.02^b$	$8.90+0.58^b$	$9.36+0.76^b$
	4	$7.08+0.70^b$	$10.38+0.58^b$	$0.41+0.03^b$	$0.39+0.03^b$	$5.72+0.48^d$	$8.80+0.63^c$

[1]Values are means±SE. Means in the same column sharing a common superscript letter are not significantly different (p≥0.05).

A significant (p<0.05) increase in liver and spleen iron levels was observed in starved fish at two days after challenge with *A. salmonicida* but not *V. anguillarum* (Table 5.3). After four days, iron levels in the spleen declined rapidly in fish infected with both pathogens. Liver zinc concentrations also increased 2 days after *A. salmonicida* infection and decreased after 4 days. Spleen zinc levels showed a gradual decrease after both of the disease challenges. Liver copper concentrations showed a decrease 4 days after the infections, however, a gradual reduction was observed in the spleen from 0- 4 days after infection. This response was consistent with *A. salmonicida* and *V. anguillarum* infections.

The cumulative percent mortalities of the fish infected with the two pathogens are shown in Table 5.4. Fish infected with *A. salmonicida* started to die after 2 days and 80 % of the fish were dead after 4 days. Most of the fish were dead by day 7. Since the fish did not consume the experimental diet, the mortality data between fed and starved groups was not compared. The magnitude of the response was essentially the same between the two groups. Feeding the experimental diet to the *V. anguillarum* infected fish caused high mortality. After 7 days of infection, 96 % of the fish died in the fed group but mortality was 60 % in the starved group. Furthermore, the mortality rate was higher in fish infected *with A. salmonicida* than *V. anguillarum*. All dead fish were examined for respective pathogens and they were found to be positive.

Table 5.3. Iron, copper and zinc concentrations in the liver and spleen of Atlantic salmon after the fish had been infected with *Aeromonas salmonicida* and *Vibrio anguillarum*[1].

Bacterium challenged	Stages of infection (days)	Liver			Spleen		
		Iron (µg/g)	Copper (µg/g)	Zinc (µg/g)	Iron (µg/g)	Copper (µg/g)	Zinc (µg/g)
A. salmonicida	0	103.8±3.31[b]	11.4±1.07[a]	13.8±0.87[b]	304.0±72.99[bc]	3.52±0.74[a]	35.4±12.53[a]
	2	134.8±10.65[a]	11.0±0.97[a]	17.6±1.58[a]	412.0±58.20[a]	1.66±0.32[b]	20.6±6.42[b]
	4	82.6±7.74[c]	8.1±0.95[b]	14.4±0.69[b]	247.2±20.43[cd]	1.38±0.23[b]	18.0±2.29[b]
V. anguillarum	0	103.8±3.31[b]	11.4±1.07[a]	13.8±0.81[b]	304.0±72.99[bc]	3.52±0.74[a]	35.4±12.53[a]
	2	96.0±12.81[bc]	10.7±0.96[a]	13.6±0.89[b]	365.9±74.49[ab]	1.89±0.37[b]	27.7±5.17[ab]
	4	106.6±11.82[b]	9.0±0.76[b]	14.6±0.79[b]	191.7±28.13[cd]	1.43±0.03[b]	15.6±2.95[c]

[1]Values are means±SE. Means in the same column sharing a common superscript letter are not significantly different (p≥0.05).

Table 5.4. Cumulative mortality (%) of Atlantic salmon after challenge with *Aeromonas salmonicida* and *Vibrio anguillarum*.

Stages of infection (days)	Mortality (%)			
	Aeromonas salmonicida		*Vibrio anguillarum*	
	Unfed	Fed	Unfed	Fed
0-2	20	0	0	0
0-4	80	80	20	45
0-7	100	96	60	96

5.4. Discussion:

A significant increase in the plasma iron concentration of salmon 2 days after infection with V. anguillarum suggests that iron must have been mobilized from tissues to supply an essential nutrient for the bacterial pathogens. However, with the increase in the severity of infection after four days, fish were likely unable to mobilize sufficient iron for bacterial growth at this stage (Table 5.2). This response was consistent whether fish were fed the experimental diet or starved. Plasma iron concentrations showed a gradual decrease in fish infected with A. salmonicida, which further supports the view that iron is utilized for bacterial growth. Nutrients are supplied to the pathogen either from the tissue reserves of the host or absorption from the diet. It is clear from the feed consumption records that the fish infected with A. salmonicida did not accept any food but there was some nutrient intake and utilization during the infective stages of V. anguillarum in the fish. The decrease in plasma iron levels was greater than noted for other elements. Iron is known to be essential for the proliferation of pathogenic bacteria in mammalian and fish tissues (Sherman, 1984).

The increase in plasma iron level was probably due to mobilization of iron from tissue reserves. In this regard, the liver and spleen generally showed a gradual decrease in tissue iron concentration (Table 5.1 and 5.2). Depletion of trace elements in the liver and spleen of salmon with an increase in the severity of infection was apparent, however, the fish infected with A. salmonicida showed an initial increase in iron and zinc concentration in the liver and spleen in the

case of iron. However, after 4 days zinc level decreased. These responses may have been due to the initial reaction of the fish to mobilize these elements from the liver and spleen, which are actively involved in trace element metabolism of aquatic animals (Lall, 1989). Both liver and spleen may later release iron and zinc into the blood for the growth of *A. salmonicida* and *V. anguillarum* during infections. Agius (1979) observed that *V. anguillarum*-infected rainbow trout deposited iron to a large extent in the spleen compared to the liver or kidney. The dynamics of trace element metabolism may vary with the virulence, species and strain of the pathogen differences between the host, nutritional status of the fish and the trace element concentrations in the aquatic environment.

Aeromonas salmonicida mobilized iron more efficiently than *V. anguillarum*. Perhaps *A. salmonicida* has different mechanisms to acquire iron from the host instead of depending upon body iron storage. One suitable substrate could be heme iron or hemoglobin (Hb) of fish. Kakuta and Murachi (1993) reported that in the Japanese eel, blood components, especially Hb, are reduced at the onset of *A. salmonicida* infection. Shieh and MacLean (1975) reported that in brook trout infected with *A. salmonicida*, Hb and total serum protein decreased. This may again indicate the possibility that *A. salmonicida* uses Hb as a source of iron. Efficient utilization of heme compounds by *A. salmonicida* was reported by Hirst and Ellis (1994). The distribution of hemoglobin and free inorganic iron in fish blood as well as its utilization by bacterial infections may be an interesting area for future investigation.

In eels infected with *A. salmonicida* (strain ET-83205), decreases in plasma osmotic pressure, iron, protein and electrolytes such as sodium, calcium and magnesium an increase in potassium have been observed (Kakuta and Murachi, 1992). The changes in the clinical chemistry of the blood of infected fish is generally associated with: increased osmotic water inflow and decreased iron uptake, extensive acidosis due to acceleration in anaerobic metabolism, depression in excretion of metabolic end products, and shifts of electrolytes between plasma and various tissues (Kakuta and Murachi, 1993).

Copper concentrations gradually decreased in the liver and spleen with the progression of both infections. Acute and chronic bacterial infections have been observed to deplete the tissue iron, copper and zinc levels as well as other nutrients, and the resultant nutritional deficits then render the fish more susceptible to secondary infections (Lall, 1988). Anorexia caused by infections, nutrient imbalance or other dietary factors depending on their severity will reduce intake of dietary nutrients to varying degrees, Losses of key intracellular elements such as potassium and magnesium, phosphate, sulfate and zinc from the body also occur during bacterial kidney disease (*Renibacterium salmoninarum*) infection (Lall and Olivier, 1993). Certain pathogens cause disturbances in gut motility, and inflammatory lesions within the mucosa and intestinal wall, which may interfere with absorptive functions and loss of blood.

The results suggest that the feeding of fish during the infection is not desirable since feeding provides the necessary nutrients for the growth of the

bacterial pathogen. This response was evident from the serum iron concentrations and mortality rates of the fish infected with *V. anguillarum*. In a tank system, uneaten food may also affect the hygiene of the system and release soluble nutrients, which will supply additional nutrients to fish. Fish have the ability to absorb trace elements from the water through their gills (Lall, 1989). The feeding response of the fish may vary with the pathogen. Damsgard et al. (1998) reported that fish infected with pancreatic necrosis virus must have relative by high virus titers before any changes in appetite or growth could be detected. However, anorexia is a common occurrence in fish infected with bacterial pathogens (Neji and de la Noue, 1998; Diggles et al, 2000). The results presented here clearly indicate that the extent of decrease in food intake may vary in fish challenged with different pathogens. Anorexia caused by infection appeared within a few hours after the *A. salmonicida* infection but the decrease in food intake was relatively slow in fish infected with *V. anguillarum*. The research in this area should be extended to develop proper feeding strategies for fish culture when the potential for infection with bacterial pathogens is high.

CHAPTER 6. Effects of ascorbic acid supplementation of diets containing blood meal on iron utilization by Atlantic salmon (*Salmo salar*)

6.1. Introduction

Atlantic salmon (*Salmo salar*), like most other fish, are unable to synthesize ascorbic acid (AA) (Halver et al.,1975) and they require a dietary supply to maintain an adequate level of this vitamin within their body. As long as this level is above a minimum critical level, fish are able to maintain normal growth and health. When the body stores of AA in fish reach a critically low level, deficiency signs occur (Hilton et al., 1978). By contrast, high tissue concentrations of AA have been associated with an increase in fish tolerance to environmental pollution (Eichbaum et al.,1977; Mayer et al., 1978) and increased resistance to bacterial infections (Navarre and Halver, 1989; Blazer, 1992; Verlhac and Gabaudan, 1994; Waagbo, 1994). Induction of mixed function oxidase activity, which is involved in hydroxylation of lysine and proline in collagen formation, is directly related to ascorbate concentration in the tissues (Zannoni et al., 1982).

Ascorbic acid is known to increase the absorption of iron from food in terrestrial animals as well as fish (Harper, 1975; Hilton, 1989; NRC, 1993). It affects the absorption and metabolism of both non-heme and heme-iron. The effect of AA on non-heme iron involves reducing the ferric form (Fe^{3+}) in the

acidic environment of the stomach, to the ferrous form (Fe^{2+}). Iron then forms a

soluble stable chelate that stays in solution in the alkaline environment of the

small intestine and then it is well absorbed by the animal (Forth and Rummel,

1973; Harper, 1975). To increase the absorption of iron, AA and iron must be in

the gut simultaneously. Ascorbic acid is also involved with adenosine

triphosphate (ATP) in the release and reduction of ferric (Fe^{3+}) iron from ferritin

and its subsequent incorporation with iron-binding proteins, apoferritin and

transferrin, into tissue ferritin (Mazur et al., 1960; Harper, 1975).

Ascorbic acid may also promote the utilization of heme-iron; however, the

mechanism of this effect is less understood. In blood meal, fish meal and other

animal by-products, the major proportion of iron is bound organically as iron-

porphyrin, myoglobin and hemoglobin. Cells take up heme-iron intact and then

iron is released within the cells, whereas non-heme iron needs to shed its ligand

before entering the cell (Hallberg and Bjorn-Rassmussen, 1972; Turnbull et al.,

1962). Ascorbic acid probably enhances absorption of heme-iron into its

intracellular storage form ferritin. Studies with cultured cells have shown that

ascorbic acid enhances the stability of ferritin by blocking its degradation through

reduced lysosomal autophagy of the protein . Thus the decline in ferritin and

accumulation of hemosiderin in scorbutic animals are reversed by ascorbic acid

treatment.

Studies on the effects of ascorbic acid on iron absorption in fish are

limited. Lanno et al. (1985), for example did not find any effect of high dietary

levels of AA on the absorption of iron in rainbow trout fed diets that were

concurrently supplemented with ferrous sulfate. Hilton et al. (1978) found that a

deficiency of AA cause a reduction in serum iron levels and a redistribution of

tissue iron stores in rainbow trout. A deficiency of AA has also been observed to

cause reductions of hemoglobin and hematocrit values in catfish, trout and

snakehead fish (Andrew and Murai, 1975; Hilton et al., 1978; Lim and Lovell,

1978; Agrawal and Mahajan,1980).

It is obvious from the discussions in the previous chapters that the dietary

iron level affects the iron concentration of several organs, particularly the spleen

and kidney, and several blood parameters are also affected by low dietary iron

intake. The major storage tissues for AA in fish are the liver, kidney, and skin

(Halver, 1972; Sandnes, 1991). Tissue concentrations are influenced by levels

of AA in the diet (Hilton et al. 1978). Using liver content as an indication of the

AA body pool, Lim and Lovell (1978) found that a liver concentration below 30

$\mu g/g$ was associated with scurvy in channel catfish Hilton et al. (1978) reported

20 $\mu g/g$ as the critical low level for hepatic AA in rainbow trout.

The present study was conducted to determine the effects of ascorbic acid

supplementation on the utilization of iron from a practical diet based on blood

meal in Atlantic salmon reared in seawater. The main parameters measured were growth, feed utilization, survival, serum and tissue iron concentrations and several hematological parameters. Although it had been planned to study the effects of the dietary treatments on the immune response of Atlantic salmon, the sudden decision of the Department of Fisheries and Oceans to close the aquarium and quarantine facilities of the Halifax Fisheries Research Laboratory, led to the premature termination of the experiment. Unfortunately, this work could not be continued elsewhere in the Halifax-Dartmouth area.

6.2 Material and methods

Fish

Three hundred Atlantic salmon smolts obtained from the Department of Fisheries and Oceans (DFO), Cobequid hatchery, Collingwood, Nova Scotia were reared in two 300 L circular tanks at the Halifax Laboratory of the DFO. Prior to the start of the experiment, the fish underwent a one-week acclimation period during which they readily adjusted to a commercial feed, the aquaria and standardized environmental conditions. They were gradually acclimated to sea water (32 ‰) within a 2- week period. Sea water, filtered through sand and gravel, was supplied to each tank at a flow rate of 4 liters per minute. These fish were maintained for approximately two weeks and during this period fish reached an average body weight of 88.1±1.74 g. Fish mortality during the acclimation and rearing period was 0.5 %. The Atlantic salmon smolts were also acclimated

from a water temperature of 8 °C to a heated water temperature of 15 ± 1 °C

prior to the commencement of the experiment. Two hundred and forty fish were

equally distributed into twelve, 0.91 m diameter fiberglass Tanks. Each tank

received separate inflow of continuously flowing water that was degassed by

spray bars and air stones. Oxygen saturation of 90% or better was maintained in

each tank. Photoperiod was controlled automatically on a 12 hour light and 12

hour dark schedule.

The experimental design consisted of a completely randomized block

design arranged in 3 blocks of 4 tanks each. Each diet was represented in one

tank within each block. The fish were fed to satiety twice daily during the week

and once daily on weekends. Salmon were bulk-weighed and counted after a 24

hour fast, at the beginning of the experiment and every 4 weeks thereafter for 16

weeks. Mortalities were monitored daily and dead fish were examined for gross

abnormalities. Kidney samples were streaked on tryptose soy agar (TSA-1.5 %

NaCl, Difco) to determine any signs of bacterial infection. Final weight gain and

feed intake were recorded to calculate feed-gain ratio (as is basis).

Experimental diets

The compositions of the experimental diets are shown in Table 6.1. The

basal diet was similar to the practical diet reported by Lall et al. (1990) with fish

Table 6.1. Composition of the experimental diets

Ingredient	Diet 1 (%)	Diet 2 (%)	Diet 3 (%)	Diet 4 (%)
Herring meal (72% protein)[1]	41.0	41.0	41.0	41.0
Casein, vitamin free[5]	7.6	7.6	-	-
Blood meal (86% protein)[2]	-	-	8.0	8.0
Krill meal (54% protein)[2]	4.0	4.0	4.0	4.0
Corn gluten meal (60% protein)[2]	3.5	3.5	3.5	3.5
Wheat middlings (17% protein)[3]	16.5	16.5	16.5	16.5
Whey (13% protein)[4]	7.0	7.0	7.0	7.0
Celufil[5]	1.4	1.4	1.0	1.0
Mineral mix[6]	1.0	1.0	1.0	1.0
Vitamin mix[7]	1.0	1.0	1.0	1.0
Choline chloride	0.2	0.2	0.2	0.2
Ascorbic acid (mg/kg)[9,10]	0	300	0	300
Herring oil [1,8]	16.8	16.8	16.8	16.8

[1] Comeau Seafood, Saulnierville, NS.
[2] Corey Feed Mills, Fredericton, NB.
[3] Dover Flour Mills, Halifax, NS.
[4] Farmers Dairy, Halifax, NS.
[5] US Biochemical, Cleveland, OH.
[6] Minerals added to supply the following (mg/kg diet): Mn,40; Zn, 75; I,5; Co,5; F,4.5; Se 1.
[7] Vitamins added to supply the following (IU or mg/ kg diet): vitamin A, 6000 IU; vitamin D_3, 3000 IU; vitamin E, 200 IU; vitamin K, 30 mg; thiamin, 40 mg; riboflavin, 50 mg; d-calcium pantothenate, 150 mg; biotin, 0.08 mg; folic acid, 15 mg; vitamin B_{12}, 0.10 mg; niacin, 200 mg; pyridoxine, 20 mg; inositol, 100 mg; butylated hydroxy toluene, 10 mg.
[8] Stabilized with 0.06% ethoxyquin.
[9] Rovimix Stay C25 (polyphosphate form)
[10] Added as premix after mixing in wheat middlings

meal being the major source of protein. Practical diets were formulated so that the experimental findings would be applicable to the salmon aquaculture industry. Four diets, each containing one of two levels of blood meal (0 and 8 %) and either 300 mg of supplemental AA in the form of ascorbic acid-2-polyphosphate/ kg (15 % activity, Hoffman La-Roche Ltd., Toronto, Ontario) or no AA were formulated from the basal diet. In diets that contained no blood meal, casein supplied the equivalent amount of protein. The AA premix was made by blending it with wheat middlings in a twin-shell dry blender (Patterson-Kelly Co., East Stroudsbury, Penn.) and then it was stored in a double-lined polyethylene bag at $-30\ ^{\circ}C$ until used. After thoroughly mixing the feed ingredients in a Hobart mixer (Rapids Machinery Co., Iowa, USA), each diet was steam pelleted (Laboratory California Pellet Mill Co., San Francisco, CA). Feed drying and storage conditions for the experimental diets were essentially the same as previous experiments (Chapter 5).

Sampling

At the end of the 16 week feeding trial, fifteen Atlantic salmon were randomly selected from the three dietary tanks of each dietary group and sacrificed for the measurement of tissue ascorbic acid concentration and various hematological analyses. Fish were killed by a cranial blow and blood was withdrawn as described in Chapter 3. The liver was quickly excised and mucus was isolated after gentle scraping of the body surface. All tissue and serum

samples were immediately frozen on dry ice and stored separately at -80°C until analysis.

Liver ascorbic acid analysis

Total ascorbate was determined colorimetrically after the methods of Carr et al. (1983). Livers were weighed and homogenized in 5 mL of deproteinizing buffer (5% trichloroacetic acid (TCA) in 250 mM $HClO_4$) using a polytron homogenizer for 3 to 5 s. The homogenates were centrifuged for 15 min at 15000g at 4°C and the supernatant was taken for further analysis. Blanks were prepared by hydrolyzing ascorbate by heating 1 ml of the supernatant at 90°C for 2 h. Another set of 1 ml supernatant samples was incubated for 1 h at room temperature with 200 µl of 2,6-dichlorophenol-indophenol (DCIP, Sigma, 0.2% in water). After hydrolysis, 200 µl of DCIP were added to each blank. Samples and blanks were again incubated with 1 ml of thiourea reagent (Sigma, 2% in 5% metaphosphoric acid) and 1 ml of 2,4-dinitrophenylhydrazine (Sigma, 2% in 12M H_2SO_4). All samples were incubated for 3 h in a water bath at 60 °C. Two ml of ice cold concentrated H_2SO_4 (18 M) were added to each sample and gently swirled. Fresh standards of L-AA (Sigma) were prepared daily and a standard curve was made using 0, 1, 2, 3, 4 and 5 µg L-ascorbic acid. The absorbance was measured in duplicate using a UV spectrophotometer (Perkin Elmer 1000), at 530nm. Finally, the AA concentration of each sample was determined from the standard curve after subtraction of the blank values.

Mineral analysis

Fish feed, serum, liver and mucus were collected for determination of iron

and other minerals. The tissues were digested in a mixture containing 6M HCL

with a few drops of HNO_3 and analyzed by an atomic absorption

spectrophotometer (AAS, Perkin Elmer 5000) using the same methods described

in Chapter 2.

Statistical analysis

The data were analyzed by means of two-way ANOVA with a 5%

acceptance level. Where appropriate, Tukey's test with $p=0.05$ was used to

detect significant differences among treatment means (Zar, 1984). Systat® 8.0

software was used to conduct all statistical analyses.

6.3. Results

Mineral content of seawater

Seawater collected at the beginning of the experiment from the reservoir

tanks was analyzed for various minerals by atomic absorption

spectrophotometry. The concentrations of trace elements (mg/L) were as

follows: Fe, 0.51±0.03; Cu, 0.17±0.02; Zn, 0.07±0.01; Mn, 0.06±0.01. The macro

element contents (g/L) were: Na, 10.8 ± 0.78; Mg, 0.97 ± 0.29; K, 0.39 ±0.02.

The iron content of the seawater was higher than noted for freshwater. The

levels of other elements in the seawater were with in the ranges observed for these elements in unpublished sea water analysis reports from the Halifax Fisheries Research Laboratory aquarium.

Mineral composition of experimental diets

The average iron concentration in diets 1 and 2 was 196 mg/kg and that for diets 3 and 4 was 412.3 mg /kg. This increase was mainly associated with the incorporation of blood meal in to the experimental diets 3 and 4 (Table 6.2). There was no significant ($p<0.05$) difference in iron concentration between diets 1 and 2 or between diets 3 and 4. The feed ingredients, particularly fish meal, supplied the major portion of iron in diets 1 and 2. No inorganic iron supplement was added to these diets. The concentrations of Cu, Zn and Mn in the four experimental diets were essentially the same and not affected by the inclusion of similar levels of casein or blood meal in the diets (Table 6.2).

Growth, feed efficiency, and survival

Growth and feed efficiency of Atlantic salmon were not significantly affected by the dietary level of ascorbic acid (Table 6.3). Feed/gain ratio varied from 1.1 ± 0.5 to 1.4 ± 0.6 and did not vary significantly among fish fed the various experimental diets. All fish ate actively and there were no gross signs of vitamin C deficiency in any of the fish examined at the end of the experiment. Percent survival of all groups of fish was $\geq 95\%$.

Table 6.2. Determined levels of certain trace elements in the diets to Atlantic salmon

Diet No.	Blood meal	Ascorbic Acid	Mineral composition (mg /kg of diet)			
	%	(mg/kg)	Fe	Cu	Zn	Mn
1	0	0	183.8[a]	10.6[a]	82.7[a]	44.0[a]
2	0	300	208.9[a]	10.1[a]	78.4[a]	44.0[a]
3	8	0	425.8[b]	9.6[a]	75.4[a]	41.5[a]
4	8	300	398.8[b]	10.2[a]	78.8[a]	44.2[a]

[1] Means sharing same alphabets in the same column with common superscript were not significantly different (p>0.05).

Table 6.3. Effects of dietary ascorbic acid and organic iron (from blood meal) supplementation on growth, feed utilization, and percentage survival of Atlantic salmon[1]

Diet No.	Blood meal %	Ascorbic Acid (mg/kg)	Initial weight (g)	Final weight (g)	Weight gain (g)	Feed/ gain	Survival (%)
1	0	0	88.6 ± 4.5[a]	287.9±8.6[a]	199.3 ± 6.9[a]	0.96±0.02[a]	96.7
2	0	300	87.5 ± 5.5[a]	298.9±6.2[a]	211.4 ± 5.1[a]	0.95±0.01[a]	98.4
3	8	0	89.4 ± 3.2[a]	307.3±10.8[a]	217.9 ± 8.0[a]	0.98±0.03[a]	95.0
4	8	300	86.5 ± 4.1[a]	279.0±9.8[a]	192.5 ± 8.1[a]	0.94±0.03[a]	98.4

[1] Mean (± SE) in the same column with a common superscript are not significantly different (p>0.05).

Liver ascorbic acid concentration

Liver ascorbic acid concentrations (µg/g wet weight) of fish fed the various experimental diets are presented in Figure 6.1. Fish fed diets containing 300 mg of AA /kg (diets 2 and 4) showed significantly ($p > 0.05$) higher hepatic levels of ascorbic acid than fish fed diets without any AA supplement (diets 1 and 3). The incorporation of 8 % blood meal into the control diet increased the heme-iron concentration of the diet considerably but had no significant effect on total ascorbic acid content of the fish liver. It therefore appears that the liver ascorbic acid concentrations were not influenced by the higher iron intake of the fish fed the practical diets containing a high concentration of heme-iron.

Serum and liver iron concentrations

Although serum and liver iron concentrations of the fish fed the various experimental diets showed wide differences in response to dietary treatment (Figure 6.2 and 6.3), these differences were not statistically significant ($p > 0.05$). Fish fed the diet containing blood meal without AA showed the highest levels of serum iron. Although there were no significant differences in the levels of iron in the serum and liver due to diet treatment, the lowest iron levels in the serum were found in fish fed the diet that contained BM with AA and in the case of liver iron, the control (C) diet. When ascorbic acid was added to the control diet (C+AA), iron levels increased in both the serum and liver. However, when AA
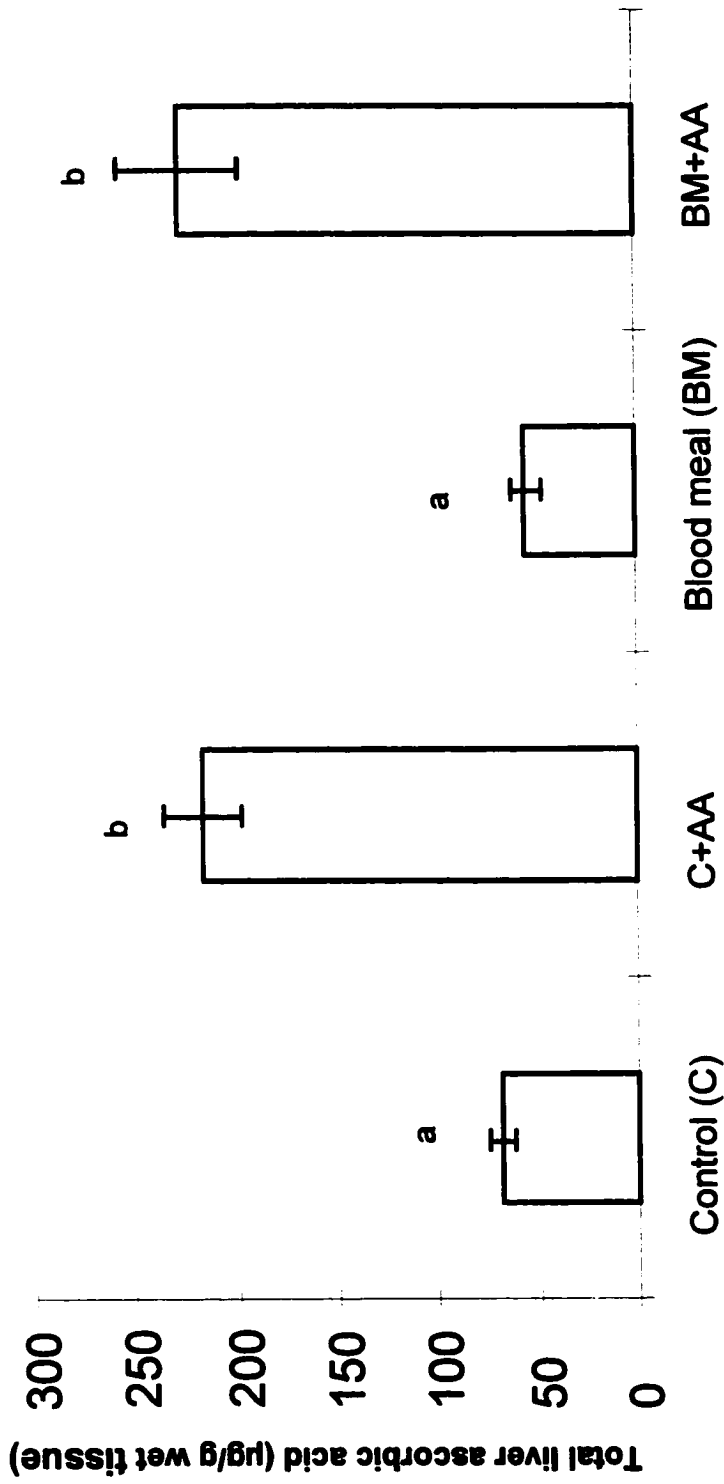
Figure 6.1. Total ascorbic acid (AA) concentrations in the liver of Atlantic salmon after feeding the control and blood meal diets with 0 or 300 mg AA /kg or without AA supplement for 16 weeks. C, control diet; C+AA, control diet with ascorbic acid; BM, Blood meal; BM+AA, BM with AA. Bars with same letters were not significantly different ($p \geq 0.05$)

Figure 6.2. Serum iron concentrations of Atlantic salmon fed the control and blood meal diet supplemented with 0 and 300 mg of ascorbic acid (AA) /kg or no AA for 16 weeks. C, control diet; C+AA, control diet supplemented with 300 mg AA /kg; BM, blood meal diet; BM+AA, BM diet supplemented with AA. Bars with same letters were not significantly different (p≥0.05).

Figure 6.3. Liver iron concentrations of Atlantic salmon fed the control and blood meal supplemented diet with 300 mg of ascorbic acid (AA) /kg or no AA for 16 weeks. Please see figure 6.2 for additional information. Bars with same letters were not significantly different ($p \geq 0.05$).

was added to the blood meal diet (BM+AA), iron absorption appeared to be reduced in the serum of the fish.

Mucous iron

The concentration of iron in the mucous of salmon is presented in Figure 6.4. There were no significant differences among the groups ingesting the four diets with respect to mucous iron levels. However, the fish fed the control diet that was supplemented with AA (C+AA) exhibited an increase in mucous iron. Fish fed the control diet (C) and blood meal diet that was supplemented with ascorbic acid (BM + AA) had the lowest levels of mucous iron. Thus, the addition of AA to the diet with blood meal appeared to reduce iron absorption.

Hematology

Hematological values are given in Figures 6.5 and 6.6. Although the level of hemoglobin (Hb) did not differ significantly with the addition of blood meal or AA, to the diets the hemoglobin concentration was highest in fish fed diets containing blood meal (BM) without AA. Similarly, there were no significant differences in RBC values, but the hematocrit (Hct) values were significantly higher in fish fed the control diet with added AA, relative to the values obtained for fish fed the control diet. Since there were no significant differences in Hb concentration and RBC values, there were no effects of diet treatment on MCV,
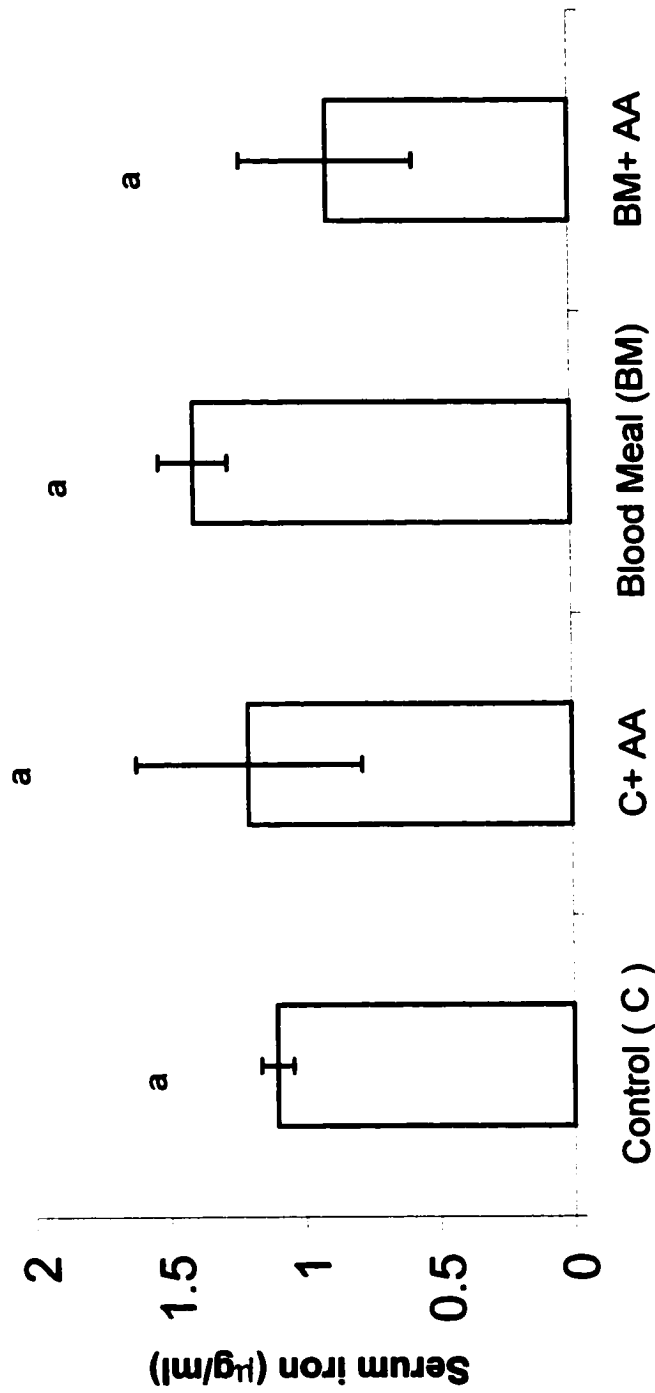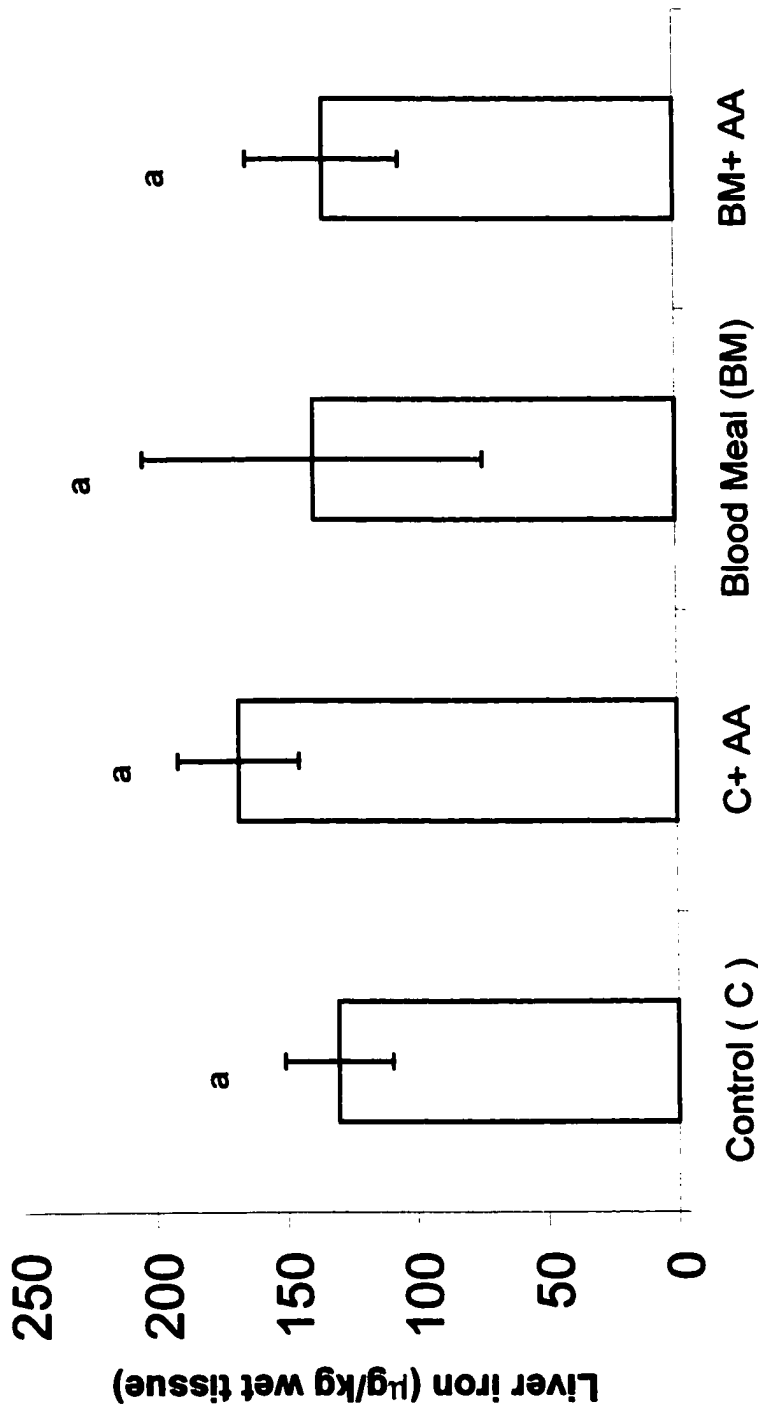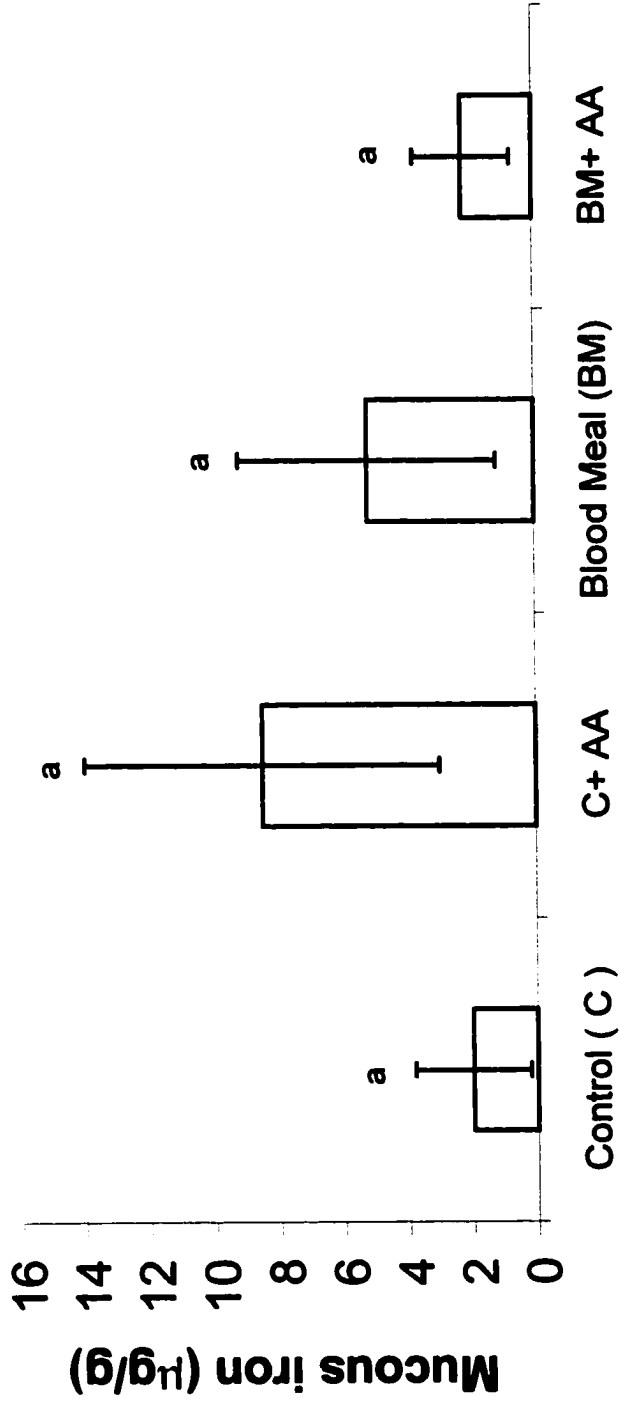
Figure 6.4. Mucous iron concentrations of Atlantic salmon fed control and blood meal diet supplemented with 0 or 300 mg of ascorbic acid (AA) /kg or no AA for 16 weeks. Please see figure 6.2 for additional information. Bars with same letters were not significantly different ($p \geq 0.05$).

Hb (g/dL)

12
10
8
6
4
2
0

a   a   a   a

C   C+AA   BM   BM+AA

Hct (%)

60
50
40
30
20
10
0

a   b   ab   ab

C   C+AA   BM   BM+AA

RBC (x 10^{6}cells /ml)

8
7
6
5
4
3
2
1
0

a   a   a   a

C   C+AA   BM   BM+AA
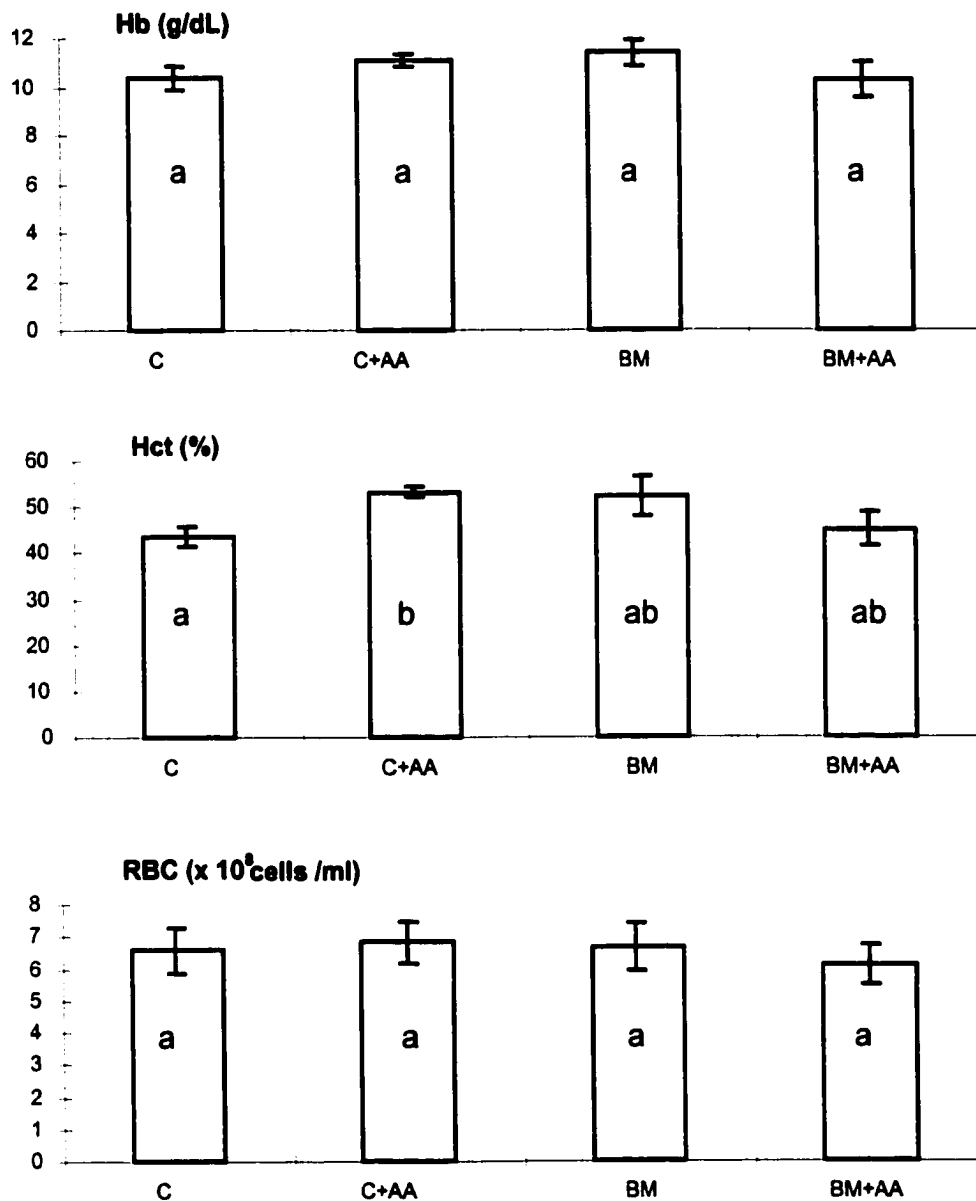
Figure 6.5. Hemoglobin concentrations, hematocrit and red blood cell counts in Atlantic salmon fed control and blood meal diet supplemented with 300 mg of ascorbic acid (AA)/kg or no AA for 16 weeks. Bars with same letters were not significantly different ($p \geq 0.05$).

MCV (x 10^8/ml)

90
80
70
60
50
40
30
20
10
0

a | a | a | a

C | C+AA | BM | BM+AA

MCH (10^8 cell/ml)

20

15

10

5

0

a | a | a | a

C | C +AA | BM | BM+AA

MCHC (g/dl)

25
20
15
10
5
0

a | a | a | a

C | C+AA | BM | BM+AA

Figure 6.6. Mean cell volume (MCV), mean cell hemoglobin (MCH) and

mean cell hemoglobin concentration (MCHC) in Atlantic salmon fed the control

and blood meal diets with 300 mg of supplemented ascorbic acid (AA)/kg or no

AA for 16 weeks. Bars with same letters were not significantly different (p≥0.05).

MCH and MCHC (Figure 6.6). Thus, it can be concluded that neither the dietary form of iron nor the addition of AA had an effect on the hematological parameters except in the case of Hct as noted above.

Total iron binding capacity (TIBC), transferrin saturation, ferritin

Total iron binding capacity (TIBC) values were not significantly influenced by diet treatment. However, as a general trend, TIBC values were higher in fish fed diets supplemented with BM or AA and they were lowest in fish fed the control (C) diet (Figure 6.7). Transferrin saturation was also influenced by diet treatment, but as a trend they decreased when the diet was supplemented with AA (Figure 6.7). Two-way ANOVA showed that AA had a significant effect on serum ferritin level of Atlantic salmon. The values of ferritin in serum (Figure 6.8) were significantly lower in fish fed the BM diet that was supplemented with AA. However, in comparison to the values found for the other dietary groups supplementation of the control diet with AA (C+AA) did result in an increase in the ferritin value. Other differences between groups were not significant. Therefore, dietary AA supplementation did have an effect on ferritin values.

Liver copper (Cu), zinc (Zn) and manganese (Mn) concentrations

The concentrations of Cu, Zn and Mn in the livers are given in Figure 6.9. The concentrations of Cu in the liver followed a trend that was similar to the concentrations of iron described previously, and Cu was absorbed most readily

TIBC (µg/ml)

5
4
3
2
1
0

a   a   a   a

C   C+AA   BM   BM+AA

Transferrin Saturation (%)

40
35
30
25
20
15
10
5
0

a   a   a   a

C   C+AA   BM   BM+AA
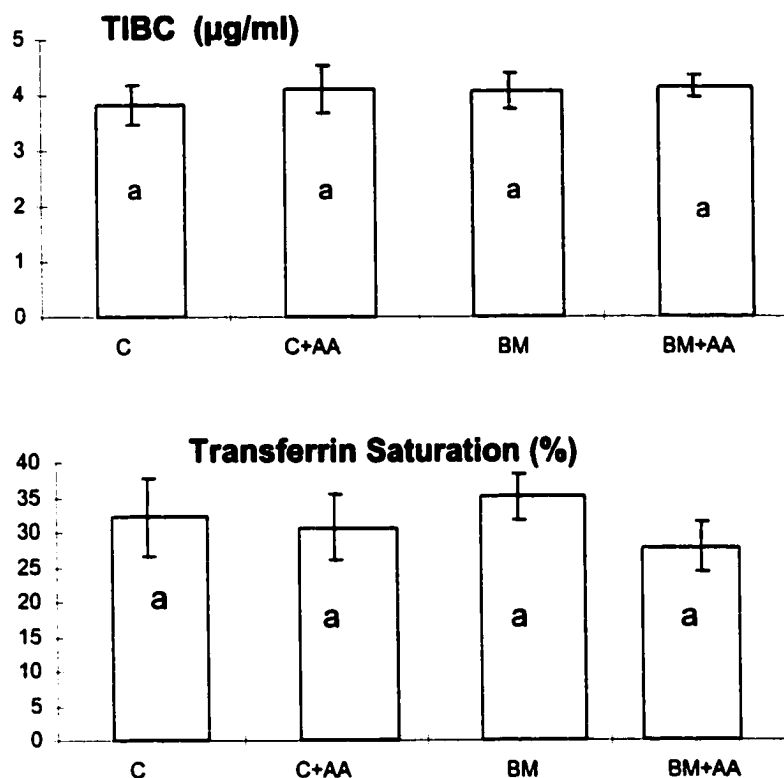
Figure 6.7. Total iron binding capacity (TIBC) and transferrin saturation (%) in Atlantic salmon fed the control and blood meal diets of 300 mg of supplemented ascorbic acid (AA)/kg or no AA for 16 weeks. Bars with same letters were not significantly different ($p < 0.05$).
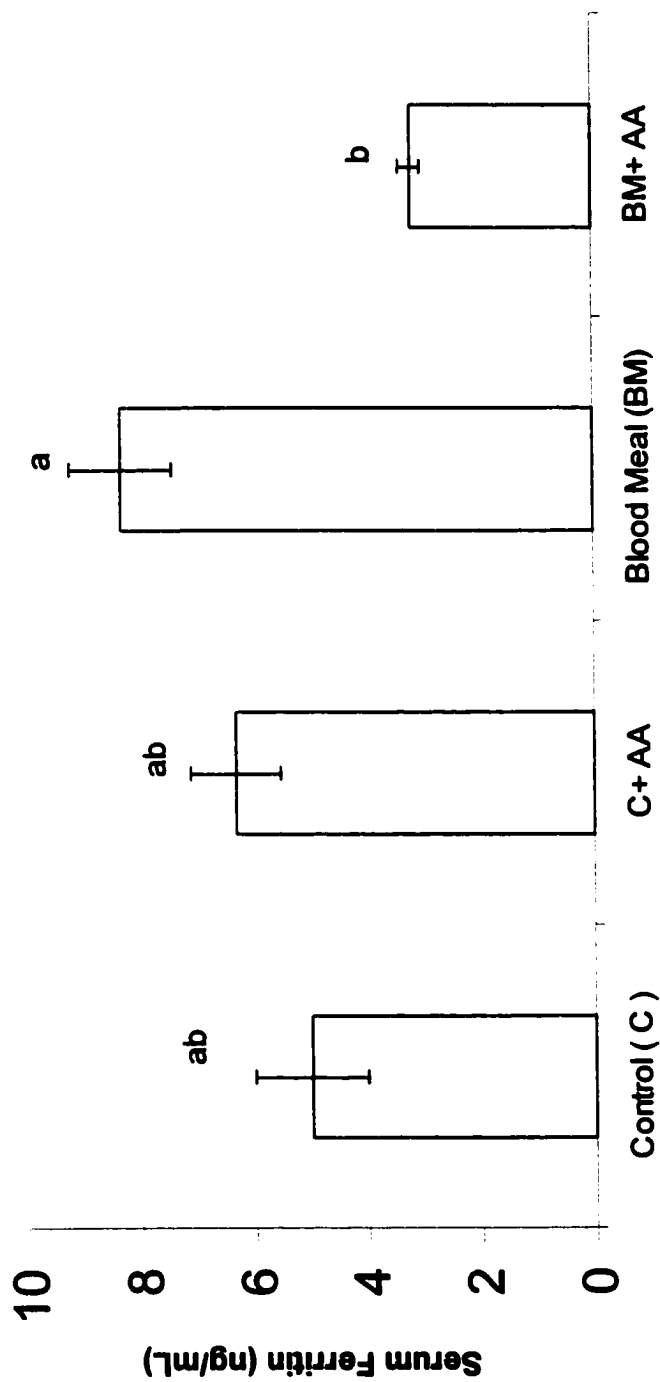
Figure 6.8. Serum ferritin concentrations in Atlantic salmon fed the meal being the major source of protein. Practical diets were formulated so that the experimental findings would be applicable to the salmon aquaculture control and blood meal diets supplemented with 300 mg of ascorbic acid (AA)/kg or no AA for 16 weeks. Bars with same letters were not significantly different (p ≥0.05).
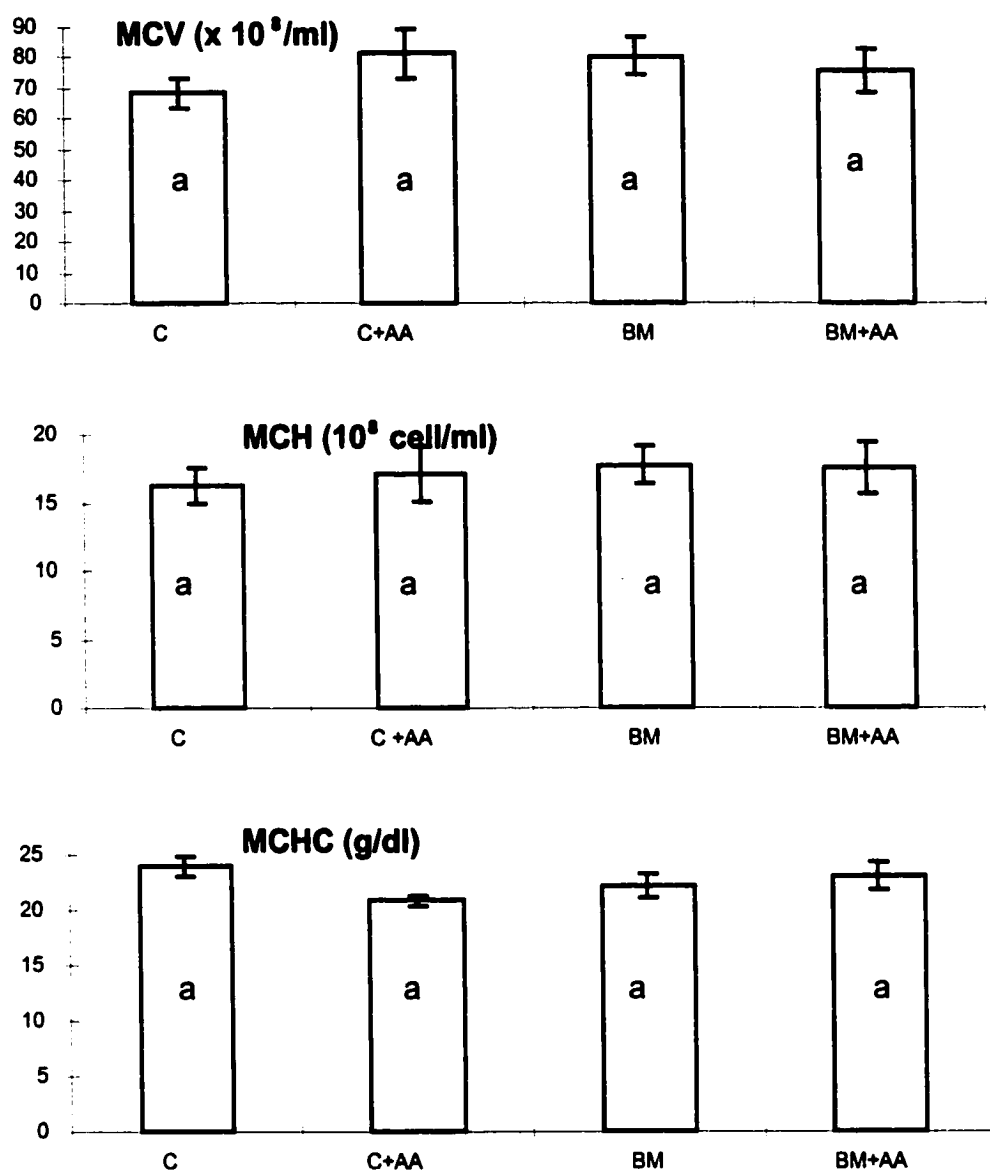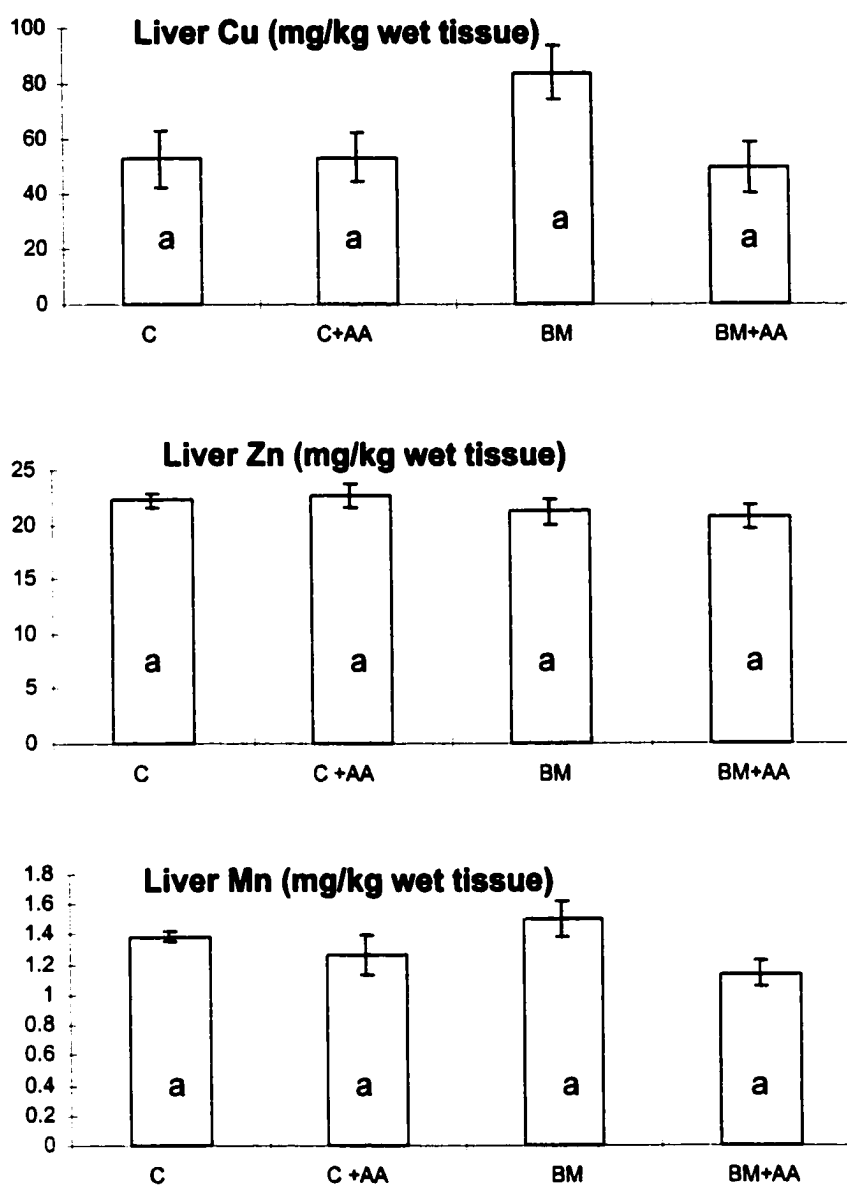
Figure 6.9. Copper (Cu), zinc (Zn) and manganese (Mn) (μg/kg wet tissue) levels in the liver of Atlantic salmon fed the control and blood meal diets supplemented 300 mg of ascorbic acid (AA) or no AA for 16 weeks. Bars with same letters were not significantly different (p≥0.05).

from the diet containing blood meal (BM). However, the differences between groups were not significant.

Liver Zn and Mn levels also did not differ significanty with diet treatment. Liver Mn levels were highest in fish fed the blood meal diet (BM). The addition of AA to the control and blood meal diets resulted in non-significant depressions in Mn levels.

6. 4. Discussion:

Growth and feed efficiency were not affected by either dietary AA supplementation or by incorporation of blood meal as a source of heme-iron in salmon diets. Similar response have also been observed in Atlantic salmon fed diets supplemented with AA (Chin, 1994) and heme-iron (Andersen et al., 1998). However, channel catfish fed an iron-deficient diet supplemented with ascorbic acid exhibited reduced growth (Lim et al., 2000), but feed efficiency was not affected. Sandnes et al. (1990,1992) observed reduced growth and severe AA deficiency signs in Atlantic salmon fed a purified diet without any AA supplement. It is possible that the duration of the present experiment was too short and that the fish were able to mobilize AA from other tissues. The ascorbic acid concentrations that were observed in the liver in this study were also higher than those reported by Sandnes (1990, 1992), which is a further indication that the fish in the present study did not deplete their stores of ascorbic acid sufficiently to produce clinical deficiency signs or suppress their growth.

A stable form of AA (L-ascorbyl-2-polyphosphate) was incorporated into the experimental diets and it is known that AA is readily available to rainbow trout (Grant et al., 1989) and Atlantic salmon (Waagbo et al., 1991; Sandnes and Waagbo, 1991) from this source. The high amount of AA found in the liver of fish fed the AA supplemented diets was within the same range of AA values observed in the liver of Atlantic salmon by Chin (1994). The liver AA concentrations were not affected by increase intake of heme-iron. Furthermore, the liver AA concentration was essentially the same in fish fed either the control or blood meal based diet containing no AA supplement. The experimental diets had a sufficient amount of vitamin E and other antioxidants to minimize the oxidation of AA in feed, both during absorption in the digestive tract and at the tissue level. The amount of AA in the liver was not sufficiently low to produce clinical signs of deficiency of this vitamin in salmon. Sandnes et al. (1992) found that Atlantic salmon develop scoliosis and lordosis, and the typical signs of vitamin C deficiency occurred when the liver AA level reached between10 to 20 $\mu g/g$ of liver. Atlantic salmon used in this study were relatively large compared to those used by Sandnes (1992). Recently, Andersen et al. (1998) also did not find clinical signs of vitamin C deficiency in Atlantic salmon smolts (body weight >200 g) fed a diet containing no AA supplement.

Dietary ascorbic acid supplementation had no significant ($p>0.05$) effect on serum, liver and mucus iron contents. It appears that the control diet supplied relatively high amounts of iron from the diet and a further increase in dietary

heme-iron concentration or AA supplementation did not enhance iron absorption or tissue iron deposition. A clear effect of dietary AA supplementation on heme-iron utilization by salmonid fishes fed purified diets has not been observed by other investigators (Hilton, 1989; Maage et al., 1990; Andersen et al., 1998). Fish reared in seawater absorb a significant amount of iron by drinking sea water (Lall, 1989). Hence, iron supplied from seawater and the control diet may have furnished sufficient iron to meet the requirement of Atlantic salmon this element in this study.

In the previous experiment (Chapter 4), heme-iron supplementation (300 mg /kg) of a purified diet also had no significant effect on tissue iron concentration. Fish fed the blood meal diet did show slightly higher mucus iron concentrations than those fed the control diet. It is well recognized that certain bacteria and parasites flourish on the body surface of fish. An increase in dietary iron concentration is likely to provide additional iron in fish mucus for the growth of pathogens. Although, the effect of dietary iron level on mucus iron concentration was relatively small, it is likely desirable not to add additional iron to fish feeds to prevent the prevalence of infectious diseases.

There was no significant effect of dietary heme-iron and AA supplementation on the various hematological parameters of Atlantic salmon (Figures 6.5 to 6.6) that were examined except for the hematocrit (Hct) level. The hematocrit level was significantly ($p<0.05$) increased in fish fed the control

diet with the AA supplement relative to fish fed the control diet. Reduced Hct

levels have been reported in fish ingesting iron (Kawatsu, 1972; Sakamoto and

Yone, 1978; Gatlin and Wilson, 1986; Andersen et al.,1996; Lim et al., 2000)

and/or AA deficient diets (Maage et al., 1990; Sandnes et al., 1990). None of the

experimental diets used in this study were iron deficient. It is possible that fish

the fed the diet containing no AA supplement were marginally deficient and their

supplementation of this vitamin may have been necessary to improve blood

formation in these fish. Unfortunately, the study was not continued for a sufficient

duration to produce a deficiency of either AA or iron.

The total iron binding capacity (TIBC) of serum was not significantly

affected by dietary heme-iron and AA intakes. However, the transferrin

saturation of serum was constantly lower relative to control values in fish fed

diets containing AA supplements. Serum ferritin level was significantly

depressed in fish fed the blood meal diet containing supplemental AA compare to

those fed the blood meal diet without AA. In terrestrial animals, AA is required for

the release of iron from transferrin and ferritin and its subsequent incorporation

into hemoglobin (Wegger and Palludan, 1990). Although, the effect of AA

supplementation on the serum ferritin level was relatively small, further studies

should be pursued to determine the effect of graded levels of this vitamin on iron

metabolism of Atlantic salmon.

The use of blood meal in a practical diet based on fish meal had no

significant effect on growth performance and iron metabolism of fish. Further,

there was no detrimental effect of heme-iron in salmonid diets. Ascorbic acid is considered to be an essential vitamin for all salmonid fishes, but the present finding suggest that there is no need to use higher dietary levels of this vitamin above the requirements level (NRC, 1993) to improve heme-iron utilization by Atlantic salmon. In higher animals, AA is known to increase inorganic iron absorption by reducing ferric to ferrous iron, which is essential for iron uptake into the mucosal cells (Hallberg et al., 1987). The existing practical salmonid diets, which are based on fish meal, to a large extent, supply sufficient iron for the metabolic needs of Atlantic salmon. However, there is a growing demand to replace fish meal with alternate plant protein sources in commercial salmonid feeds. Therefore, future studies should be directed to determine whether AA has a potential role in improving the utilization of iron in diets based on plant protein products regardless of the form of iron present in the feed compo

Summary and Conclusions

It is widely recognized that iron is a key nutrient for maintaining the host defence mechanisms and normal health of aquatic animals. However, the research in this area has proceeded very slowly during the past two decades. The present research was designed to determine the quantitative iron requirement of Atlantic salmon, its biological availability from potential inorganic and organic feed supplements and to examine the role of this element in immune function and disease resistance of salmon. Experiments were designed to gain preliminary information in this area as well as to provide some guidelines regarding the use of iron supplements in salmon feeds which would be useful for salmon feed producers, aquaculturists and environmentalists. On the basis of the experimental results obtained in this study as well as those reported in the literature, the following points summarize the major findings and conclusions of this study:

1. Iron is an essential nutrient for Atlantic salmon and is required for normal blood formation, growth and development. The estimated quantitative dietary iron requirement of Atlantic salmon based on broken line analysis of tissue iron concentration data is 60 mg iron/ kg of diet.

2. Dietary iron deficiency was characterized on the basis of several hematological parameters including hypochromic microcytic anemia. Iron deficiency signs can develop in salmon reared on semi-purified test diets

249

containing 13.1 $\pm$ 1.8 mg of iron/kg diet. A supplement of 30 mg of iron/kg diet appears to be adequate to prevent deficiency.

3. Biological availability of iron from inorganic or non-heme ($FeSO_4.7H_2O$; Fe_2O_3 etc) and organic or heme (e.g. fish meal, blood meal etc) sources was estimated by the hemoglobin regeneration assay. The highest bioavailability of iron was from ferric chloride (98.9%). The low bioavailability of iron in the form of ferric oxide (17.8%) indicates that salmon utilized the iron in this source poorly. Iron from heme sources such as blood meal (52.3%) and herring meal (47.1%) was about 50% as available compared to the iron in ferrous sulfate and ferric chloride. These are the first conclusive results reported for the bioavailability of iron in fish.

4. All existing commercial salmonid feeds contain a high amount of fish meal (~25 -50 % of the diet) that supplies approximately 150- 400 mg iron/kg diet. These diets supply 70.6- 188.4 mg of available iron (iron bioavailability ~47.1%) based on my study. Commercial Atlantic salmon feeds based on high percentages of fish meals do not require inorganic iron supplementation because the iron requirement of salmon is about 60 mg/kg of diet. It is also clear that fish excrete a major portion of dietary iron from commercial diets and attempts should be made to minimize the amount of iron excretion in aquaculture effluents by incorporating the minimal acceptable level of this element in fish feeds.

5. Investigations on iron metabolism in Atlantic salmon during V. anguillarum and A. salmonicida infection showed that mobilization of iron in plasma was

significantly higher in fish infected with V. anguillarum than A. salmonicida.

Feeding fish during the V. anguillarum infection caused higher mortality than

those fish receiving no food. Fish infected with A. salmonicida did not accept

any food during the infection. The results also indicated that levels of iron,

copper and zinc were gradually depleted from the liver and spleen of salmon

as the severity of bacterial infection increased.

6. Vitamin C (L-ascorbyl-2-polyphosphate) supplementation (300 mg of ascorbic

acid (AA)/kg) in a fish meal-based diet containing heme (organic)

supplemental iron had no significant affect on iron absorption and deposition

in Atlantic salmon. There was no significant effect of AA supplementation in

fish diets containing blood meal or heme iron.

7. This study clearly indicates that serum ferritin levels could be used as an

indicator of the physiological iron status of Atlantic salmon. This non-invasive

assay requiring a small amount of blood could be used either in laboratory or

field conditions.

8. A major study was conducted to determine the effects of heme (blood meal)

and non heme ($FeSO_4.7H_2O$) iron on the immune response, resistance to

bacterial diseases (Aeromonas salmonicida and Vibrio anguillarum) and

tissue iron accumulation in Atlantic salmon. The following immune responses

were investigated: bactericidal activity of serum, differential blood cell counts,

serum lysozyme activity, phagocytosis by macrophage, myloperoxidase assays

and respiratory burst activity of phagocytes and lymphocyte proliferation. The

main findings of this experiment were as follows :

- Iron deficient fish generally showed higher susceptibility to disease. However, no significant disease resistance against virulent *Vibrio anguillarum* and *Aeromonas salmonicida* was observed among Atlantic salmon fed diets containing different supplemental levels of non-heme (100, 300, 600 and 1200 mg /kg) and heme (100 and 300 mg/kg) iron.

- Cellular (phagocytic killing activity, respiratory burst, myloperoxidase assay, differential cell counts, lymphocyte proliferation assay) and humoral (serum bactericidal activity, lysozyme activity) immune responses were not affected in fish either by dietary iron concentration or the inorganic and organic forms of the iron supplements. Phagocytic cells including macrophages also had no effect on the oxygen dependent killing capacity in fish fed different dietary concentrations of heme or nonheme iron.

- Bactericidal activity of peritoneal macrophages (phagocytosis) from fish fed the iron deficient diet and the diet containing 100 mg of non-heme iron/kg were not significantly influenced by the dietary treatments.

- Blood smears from iron deficient and iron supplemented fish after 32 weeks developed peroxidase positive stains. Iron deficiency developed a negative melanoperoxidase (MPO) stain, which suggests that the MPO staining method may not be a useful indicator for iron deficiency in Atlantic salmon.

- An experiment was conducted to isolate and identify eosinophilis, monocytes and red blood cells. The monocyte population was significantly lower in fish fed diets containing higher amounts of non-heme (300, 600 or

1200 mg/kg) and heme (100 or 300 mg/kg) iron than in fish receiving an iron deficient diet or low dietary level of inorganic iron (100 mg/kg). No significant differences were observed in the red blood cells and eosinophil population of fish fed diets with either the heme or non heme iron supplements at various concentrations.

• The viability of Atlantic salmon lymphocytes was tested using fish fed an iron deficient diet or diet containing 100 mg of non-heme iron/kg for 20 weeks. There were no differences in viability of cells among deficient and 100 mg iron supplemented fed fish without mitogen stimulation. After mitogen stimulation, the stimulation index in general showed higher values after 6 days of incubation, compared to day 0. However, no significant differences among deficient and 100 mg/kg iron supplemented fish after mitogenic stimulation for T and B cell proliferation were evident at the 6th or 9th day of incubation.

• Bactericidal activity (complement system) of serum from Atlantic salmon fed deficient and varying dietary levels of iron for 32 weeks was evaluated. There were no differences in bactericidal activity in fish fed different dietary iron levels compared to iron deficient fed fish. However, after-inter peritoneal glycogen injection, fish fed the iron deficient diet and the heme-iron (100 or 300 mg iron/kg diet) supplemented groups failed to prevent *in vivo* growth of *Vibrio anguillarum*.

• No significant differences were observed in serum lysozyme activity of Atlantic salmon fed diets with 0, 100, 300, 600 or 1200 mg of non-heme iron/kg or 100 or 300 mg of heme iron/kg for 32 weeks.

- The supplementation of diets for Atlantic salmon with either heme or non-heme iron had no effect on the non-specific resistance of the fish to vibriosis and furunculosis.

9. Supplementation of diets for Atlantic salmon with either inorganic or organic iron (0, 100, 300, 600 or 1200 mg of non-heme iron/kg and 0, 100 or 300 mg of heme iron/kg) did not affect growth, feed efficiency and hepatosomatic index of the fish. The hematology of the fish did not vary consistently with the level of iron in the diet when the fish were fed for 20 and 32 weeks.

It is apparent that dietary iron supplementation with either inorganic or organic forms had no significant effect on the immune responses and disease resistance of the fish. The experiments for this thesis were conducted under controlled environmental conditions and all the necessary precautions were taken to minimize lipid peroxidation in the diets. In salmon hatcheries or cage operations where feed storage conditions, environmental conditions and physiological stress may vary considerably, it is possible that higher amounts of dietary iron may affect the nutrient stability of feeds and influence plasma iron concentrations. These, in turn, may ultimately promote growth of bacterial pathogens. Therefore, excessive amounts of iron in salmonid feeds should be avoided.

References

Agius,C., 1979. The role of melano-macrophage centres in iron storage in normal and diseased fish. J. Fish Dis., 2, 337-343.

Agrawal, N.K., Mahajan,C.L., 1980. Hematological changes due to vitamin C deficiency in *Channa punctatus* Block. J. Nutr., 110, 2172-2181.

Aisen,P., Leibmen,A., Sia,C. 1972. Molecular weight and subunit structure of hagfish transferrin. Biochemistry 11, 3461- 3464.

Ammerman, C.B., 1995. Methods for estimation of mineral bioavailability. In: Ammerman.B.B., Baker,D.H., Lewis,A.J., (Edts.), Bioavailability of nutrients for animals: amino acids, minerals and vitamins. Academic Press,Inc., San Diego, Calif., pp. 83-94.

Ammerman,C.B., Miller,S.M., 1972. Biological availability of minor mineral ions: a review. J. Anim. Sci., 35, 681-694.

Andersen, F., 1997. Studies on iron nutrition in Atlantic salmon (*Salmo salar*) with respect to requirement, bioavailability, interactions and immunity. Ph.D. Thesis, Department of Fisheries and Marine Biology, University of Bergen, Bergen, Norway (unpubl.), 58 pp.

Andersen, F., Maage,A., Julshamn,K., 1996. An estimation of dietary iron requirement of Atlantic salmon, *Salmo salar* L., parr. Aquaculture Nutrition 2, 41-47.

Andersen, F., Lygren, B., Maage, A., Waagbo,R., 1998. Interaction between two dietary levels of iron and two forms of ascorbic acid and the effect on growth, antioxidant status and some non-specific immune parameters in Atlantic salmon (*Salmo salar*) smolts. Aquaculture,161, 437-451.

Andersen, A., Standal, D.H., Giskegjerde, T.A., Karstensen, R., Rorvik, K.A., 1995. Two ferritin subunits of Atlantic salmon (*Salmo salar*); cloning of the liver cDNAs and antibody preperation. Mol. Marine Biol. Biotechnol., 4, 164-170.

Andrews, J.W., Murai, T., 1975. Studies on the vitamin C requirements of chennel catfish (*Ictalurus punctatus*). J. Nutr., 105, 557-561.

Arena, J.M., Poisoning:Toxicology, symptoms, treatments. 5th edn. Charles C. Tomas, Springfiled, pp. 579- 585.

Association of Official Analytical Chemists (AOAC). 1990. Official Methods of Analysis, 15th edition., Association of Official Analytical Chemists, Virginia, USA.

Bagchi,K., Mohanram, M., Reddy,V., 1980. Humoral immune response in children with iron-deficiency anaemia. Br. Med. J. 280, 1249- 1251.

Baker, D.H., 1986. Problems and pitfalls in animal experiments designed to establish dietary requirements for essential nutrients. J. Nutr., 116, 2239-2249.

Bakopoulos, V., Volpatti, D., Adams, A., Galeotti, M., Richards, R., 1997. Qualitative differences in the immune response of rabbit, mouse and sea bass, *Dicentrarchus labrax*, L. to *Photobacterium damsela* subsp. *piscicida*, the causative agent of fish pasteurellosis. Fish Shellfish Immunol., 7, 161-174.

Begenal, T.B., Tesch, F.W., 1978. Age and growth. In: Bagenal, T., (edt.). Methhods for assessment of fish production in freshwaters. (3rd edition). Blackwell Scientific Publications, Oxford, UK, pp.101-136.

Beisel, W.R., 1979. Metabolic balance studies--their continuing usefulness in nutritional research [editorial]. The American journal of clinical nutrition., 32, 271-274.

Beisel, W.R., 1982. Single nutrients and immunity. Am. J. Clin. Nutr., 35, 417-468.

Bendich, A., Chandra., R.K., 1990. Micronutrients and Immune functions. New York Academy of Sciences, New York.

Benito,P., Miller,D., 1998. Iron absorption and bioavailability: An updated review. Nutrition Research.,18, 581-603.

Bernát, I., 1983. Iron metabolism. Plenum Press, NY., 415 pp.

Bjørnevik,M., Maage,A., 1993. Effects of dietary iron supplementation on tissue iron concentration and haematology in Atlantic salmon (*Salmo salar*). Fisk. Dir. Skr., Ser. Ernæring., 6, 35-45.

Bjorn-Rasmussen, E., Hallberg,L., Isaksson,B., Arvidsson,B., 1974. Applications of the two-pool extrinsic tag method to measure heme and nonheme iron absorption from whole diet. J. Clin.Invest., 53, 247-255.

Bjorsten,B., Kaijser,B., 1978. Interaction of human serum and neutrophils with *Escheria coli* strains: differences between strains isolated from urine of patients with pyelonephritis or asymptomatic bacteria. Infect. Immun., 55, 1239- 1242.

Blake,I., Clark,J.C., 1931. Observations on experimental infection of trout by *B. salmonicida*: with particular reference to "carriers" of furunculosis, and to certain factors influencing susceptibility. Fishery Board of Scotland, Salmon Fisheries 7, Edinburgh: HMSO. pp.13.

Blazer,V.S., 1992. Nutrition and disease resistance in fish. Annu. Rev. Fish Dis., 2, 309-323.

Blazer, V.S., 1991. Piscine macrophage function and nutritional influences: a review. Journal of Aquatic Animal Health 3, 77- 86.

Bligh, E.H., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can J. Biochem. Physiol., 37, 911-917.

Bothwell, T.H., Charlton, R.W., Cook, j.D., Finch, C.A.,1979. Iron metabolism in man, Blackwell, Oxford, UK., 576 pp.

Bothwell,T.H., Finch,C.A., 1962. Iron metabolism. Little, Brown and Co., Boston.

Boussingault, M., 1872. *Du fer contenu dans le sang et dans les alimants.* Compt. Rend. Acad. Sci. Parts, 74, 1353-1359.

Brody,T., 1994. Nutritional Biochemistry. Academic Press, San Diego, Calif., 658pp.

Brown, E.B., Smith, E.B., Dubach, R., Moore, C.V., 1995. Lethal iron overload in dogs. J. Lab. Clin. Med., 53, 591-606.

Brown, L.L., Iwama, G.K., Evelyn, T.P.T., 1996. The effect of early exposure of coho salmon (*Oncorhynchus kisutch*) eggs to the p57 protein of *Renibacterium salmoninarum* on the development of immunity to the pathogen. Fish and Shellfish Immunology, 6, 149-165.

Bullen, J.J., Rogers, H.J., Griffiths, E., 1978. Role of iron in bacterial infection. Current Topics in Microbiology and Immunity,80, 1-35.

Bullock,G.L., Snieszko, S.F., 1969. Bacteria in blood and kidney of apparently healthy hatchery trout. Transaction of the American Fisheries Society 2, 268- 271.

Byers, B.R., Arceneaux, J.E.L., 1998. Microbial iron transport: Iron acquisition by pathogenic microorganisms. In: Sigel,A., Sigel,H.,(Eds.), Metal Ions In Biological Systems, Vol.35, Iron transport and storage in microorganisms, plants and animals. Marcel Dekker Inc, New York, USA, pp. 37-66.

Carpenter, C.E., Mahoney, A.W., 1992. Contributions of heme and nonheme iron to human nutrition. Crit. Rev. Food Sci. Nutr., 31,333-67.

Carr , R.S., Bally, M.B., Thomas, P., Neff, J.M., 1983. Comparison of methods for determination of ascorbic acid in animal tissues. Anal. Chem., 55, 1229-1232.

Carrol, M.E.W., Lackett, P.S., Arber, V.R., Lowrie, D.B., 1979. Phagolysosome formation, cyclic adenosine 3',5'-monophosphate and the fate of *Salmonella typhimurium* within mouse peritoneal macrophages. J. Gen. Microbiol. 110, 421- 429.

Chandra, R.K., 1976 a. Immune response in iron-deficient children. J. Pediatr. 88, 698-699.

Chandra, R.K., 1976 b. Iron-deficiency anaemia and immunological responses. Lancet II, 1200-1201.

Chandra, R.K.,1988. Nutrition and Immunology. Alan R. Liss, Inc. New York.

Chin, E.A., 1994. The effects of L-ascorbyl-2-polyphosphate on the nutritional status, immune response and disease resistance of Atlantic salmon (*Salmo salar*) reared in seawater. M.Sc. Thesis, Dalhousie University, Halifax, Canada. (unpubl.),120 pp.

Cho, C.Y., Cowey,C.B., 1991. Rainbow trout, *Oncorhynchus mukiss*. In: Wilson,R.P. (Ed.), Handbook of Nutrient Requirements of Finfish., CRC Press, Boca Raton, FL, pp. 131- 143.

Cipriano, R.C., Ford, L.A., Teska, J.D., Hale, L.E., 1992. Detection of *Aeromonas salmonicida* in the mucus of salmonid fishes. Journal of Aquatic Animal Health, 4, 114-118.

Cipriano, R.C., Ford, L.A.,Schachte, J.H, Petrie,C., 1994. Evaluation of mucus as a valid site to isolate *Aeromonas salmonicida* among asymptomatic populations of lake trout (*Salvelinus namaycush*). Biomedical letters, 49, 229-233.

Cipriano, R.C., Ford, L.A.,Starliper, C.E., Teska, J.D., 1996a. Control of external *Aeromonas salmonicida*: Tropical disinfection of salmonids with Chloramine-T. Journal of Aquatic Animal Health, 8, 52-57.

Cipriano, R.C., Ford, L.A., Nelson, J.T., Jenson, B.G., 1996b. Monitoring for early detection of *Aeromonas salmonicida* to enhance antibiotic therapy and control furunculosis in Atlantic salmon. Prog. Fish Cult., 58, 204-209.

Conard, J.H., Sousa, J.C., Mendes, M.O., Blue, W.G., McDowell, L.R., 1980. Iron, manganese, sodium and zinc interrelationships in a tropical soil, plant and animal system. In: Verde, L .S., Fernandez, A. (Eds.), Proceedings of fourth world conference on animal production IV. Buenos Aires, Argentina, pp. 458.

Conard, M.E., Schade, S.G., 1968. Ascorbic acid chelates in iron absorption: a role for hydrochloric acid and bile. Gastroenterology, 55, 35-45.

Conard, M.E., Umbreit, J.N., Moore, E.G., 1991. A role for mucin in the
absorption of inorganic iron and other metal cations. Gastroenterology,
100, 129 -136.

Conard, M.E., Umbreit, J.N., Moore, E.G., 1994. Iron absorption and cellular
uptake of iron. Adv. Exp. Med. & Biology, 356, 69-79.

Conard, M.E., Umbreit, J.N., Moore, E.G., Peterson, R.D.A., Jones, M.B., 1990.
A newly identified iron binding protein in duodenal mucosa of rats. J. Biol.
Chem., 265, 5273- 5279.

Conard, H.R., Zimmerman, D.R., Combs, G.F.,Jr., 1980. NFIA, Literature Review
on Iron in Animal and Poultry Nutrition. National Feed Ingredients
Association, West Des Moines, Iowa.

Cook, J.D., 1977. Absorption of food iron., Federation Proceedings, 36, 2028-
2032.

Cook, J.D., Layrisse, M., Martinez-Torres, C., Monsen, E., Finch, C.A., 1972.
Food iron absorption measured by an extrinsic tag., J. Clin. Invest., 51,
805-815.

Cook, J.D., Baynes, R.D., Skikne,B.S., 1992. Iron deficiency and the
measurement of ironstatus. Nutr. Res. Rev., 5, 189-202.

Cowey, C.B., 1992. Nutrition: Estimating requirements of rainbow trout.
Aquaculture, 100 (1-3): 177-189.

Crawford, D.L., Law, D.K., 1972. Mineral composition of Oregon pellet
production formulations. Prog. Fish Cult., 34, 126-130.

Crosa, J.H., Hodges, L.L., 1981. Outer membrane protein induced under
conditions of iron limitation in the marine fish pathogen Vibrio anguillarum.
Infection and Immunity, 31, 223-227.

Crosa, J.H., Walter, M.A., Potter,S.A., 1983. The genetics of plasmid-mediated
virulence in the marine fish pathogen Vibrio anguillarum. In: Crosa, J.H.,
(Ed.), Bacterial and viral diseases of fish, molecular studies., University of
Washington., Seattle, USA., pp. 21-30.

Cross, A.S., Kim, K.S., Wright, O.C., Sadoff, J.C., Gemski, P., 1986. Role of lipopolysaccharide and capsule in the serum resistance of bacteremic strains *Escherichia coli*. J. Infect. Dis. 154, 497- 503.

Daly, J.G., Moore, A. R., Olivier, G., 1994. Bactericidal activity of brook trout (*Salvelinus fontinalis*) peritoneal macrophages against virulent straints of *Aeromonas salmonicida*. Fish & Shellfish Immunology 4, 273-283.

Daly, J.G., Moore, A. R., Olivier, G., 1995. A colorimetric assay for the quantification of brook trout (*Salvelinus fontinalis*) lymphocyte mitogenesis. Fish & Shellfish Immunology 5, 265-273.

Daly, J.G., Stevenson, R.M.W., 1985. Importance of culturing several organs to detect *Aeromonas salmonicida* in salmonid fish. Transactions of the American Fisheries Society 114, 909- 910.

Damsgard, B., Mortensen, A., Sommer, A.I., 1998. Effects of infectious pancreatic necrosis virus (IPNV) on appetite and growth in Atlantic salmon, *Salmo salar* L. Aquaculture, 163, 185-193.

Desjardins, L.M., 1985. The effect of iron supplementation on diet rancidity and on the growth and physiological response of rainbow trout. M.Sc. Thesis. University of Guelph, Ontario, Canada. (unpubl.), 168 pp.

Desjardins, L.M., Hicks,B.D., Hilton, J.W., 1987. Iron catalysed oxidation of trout diets and its effect on the growth and physiological response of rainbow trout. Fish Physiol. Biochem., 3, 173- 182.

Diggles, B.K., Carson, J., Hine, P.M., Hickman, R.W., Tait, M.J., 2000. *Vibrio* species associated with mortalities in hatchery-reared turbot (*Colistium nudipinnis*) and brill (*C. guntheri*) in New Zealand. Aquaculture, 183,1-12.

Eichbaum, F.W., Guedes, A.O., Neto, J.P., Carvalho, F.V., 1977. Protecting effect of ascorbic acid in strychnine poisoning and tetnus(fish, mice, rats). International Journal for Vitamin and Nutrition Research,16, 31-39.

Ellis, A. E., 1982. Differences between the immune mechanisms of fish and higher vertebrates.In: Roberts,R.J. (Ed.), Microbial Diseases of fish. Academic Press.

Ellis, A.E., 1988. The immunology of teleosts. In: Roberts, R.J. (Ed.), Fish pathology. Bailliere Tindall, London, pp. 135- 152.

Ellis, A.E. 1997. The extracellular toxins of Aeromonas salmonicida subsp. salmonicida. In: Bernoth, E,M., Ellis, A.E., Midtlyng, P.J., Olivier, G., Smith, P. (Eds.) Furunculosis, Academic Press, SanDeigo, USA, pp. 366-381.

FAO. 1997. Review of the state of world aquaculture. FAO Fisheries Circular No. 886, Rev.1. Rome, Food and Agriculture Organization of the United Nations, 163pp.

Fillet,G.,Cook,J.D.,Finch,C.A., 1974. Storage iron kinetics. VII. A biologic model for reticuloendothelial iron transport. J. Clin. Invest., 53, 1527-1533

Fillet,G., Cook,J.D., Finch,C.A., Fletcher, T.C., 1982. Non-specific defence mechanisms of fish. Developmental and Comparative Immunology, Supl.2, 123-132.

Fletcher, T.C., White,A., Baldo, B.A., 1977. C-reactive protein-like precipitin in the lumpsucker Cyclopterus lumpus L. during the breeding season. Com. Biochem. Physiol., 57B, 353.

Finlay, B.B., 1999. Bacterial disease in diverse hosts. Cell, 96, 315-318.

Forbes, R.M., Erdman, J.W. Jr., 1983. Bioavailability of trace elements. Ann. Rev. Nutr., 3, 213-231.

Forth, W., Rummel, W., 1973. Iron absorption. Physiol. Rev., 53, 724-792.

Fox, M.R.S., Jacobs,R.M., Jones,O.L., Fry, B.E. Jr., Rakowska, M., Hamilton,R.P., Harland, B.F., Stone, C.L., Tao, S.H., 1981. Animal models for assessing bioavailability of essential and toxic elements. Cereal Chem., 58, 6-11.

Frausto da Silva, J.J.R., Williams, R.J. P., 1991. The biological chemistry of the elements. Clarendon Press, Oxford, pp. 323-367.

Fritz, J.C., Pla, G.W., 1972. Application to the animal hemoglobin repletion test to measurement of iron availability in foods. J. Assoc. Off.Anal. Chem., 55, 1128-1132.

Fritz,J. C., Pla, G. W., Robert, T., Boehme, J. W.,Hove, E. L. 1970. Biological availability in animals of iron from common dietary sources. J. Agr. Food Chem., 18, 647-651.

Fritz, J.C., Pla, G.W., Harrison, B.N., Clark, G.A., 1975. Estimation of the bioavailability of iron. J. AOAC., 58, 902-905.

Gatlin III, D.M., Wilson, R.P., 1986. Characterisation of iron deficiency and the dietary iron requirement of fingerling channel catfish. Aquaculture, 52, 191-198.

Gierer, W., 1990. Siderophore - ein Virulenzfaktor bei fischpathogenen Bakterien. Fischerei-Forschung., 29, 80-82.

Gierer, W., Rabsch, W., Reissbrodt, R., 1992. Siderophore pattern of fish-pathogenic Vibrio anguillarum, Aeromonas spp. and Pseudomonas spp. from the German Baltic coast. J. Fish. Dis., 15, 417-423.

Glynn, A.A. 1972. Bacterial factors inhibiting host defense mechanisms. In: Smith, H., Pearce, J.H. (Ed.), Microbial Pathogenicity in Man and Animals. Society for General Microbilogy Symposium no. 22. Society for General Microbiology, Reading, UK, pp. 75- 112.

Grace, M.F., Botham, J.W., Manning, M.J., 1981. Ontology of lymphoid organ function in fish. In: Solomon, J.B. (Ed.), Aspects of Developmental and Comparative Immunology I. Pergamon Press, Oxford and New York, pp. 467.

Grant, B.F., Seib, P.A., Liao, M.C., 1989. Polyphosphorylated L-ascorbic acid: A stable form of vitamin C for aquaculture feeds. Journal of the World Aquaculture Society, 20, 143-157.

Grasbeck, R., Majuri,R., Kuovonen, I., Tenhunen, R., 1982. Spectral and other studies on the intestinal haem receptor of the pig. Biochem. Biophys. Acta., 700, 137-291.

Grayson, T.H., Bruno, D.W., Evenden, A.J., Gilpin, M.L.,Munn, C.B., 1995. Iron acquisition by *Renibacterium salmoninarum*: Contribution of iron reductase. Dis. Aquat. Org., 22, 157-162.

Griffiths,E., 1987. The iron uptake systems of pathogenic bacteria. In: Bullen, J.J., Griffith, E., (Edt.) Iron and infection: molecular, physiological and clinical aspects. John Wiley and Sons, Chichester, pp. 69-137.

Griffiths, E., Chart, H., Stevenson, P., 1988. High-affinity iron uptake system and bacterial virulence. In: Roth, J.A. (Ed.), Virulence mechanisms of bacteiral pathogens. American Soc. of Microbiology, Washington, D.C. pp.121- 137.

Hallberg, L., 1974. The pool concept of iron absorption and some of its implications.Proc. Nutr. Soc., 33, 285- 291.

Hallberg, L., 1981. Bioavailability of dietary iron in man. Ann. Rev. Nutr., 1: 123-147.

Hallberg,L., Solvell,L., 1967. Absorption of hemoglobin in man. Acta. Med. Scand., 181, 335- 354.

Hallberg, L., Bjorn-Rassmussen,E., 1972. Determination of iron absorption from whole diet. A new two pool model using two radioiron isotopes given as haem and non-haem iron. Scand. J. Haematol., 9, 193-197.

Hallberg, L., Bjorn-Rassmussen,E., Howard, L., Rossander,L., 1977. Dietary heme iron absorption. A discussion of possible mechanisms for the absorption promoting effect of meat and for the regulation of iron absorption. Scand. J. Gastroenterol., 14, 769- 779.

Hallquist, N.A., Sherman, A.R., 1989. Effect of iron deficiency on the stimulation of natural killer cells by macrophage produced interferon. Nutr. Res., 9, 282- 292.

Halver, J.E., 1972. The role of ascorbic acid in fish disease and tissue repair. Bull. Japanese Soc. Scien. Fish.,38, 79-92.

Halver,J.E., 1996. Nutrition of salmonids. In: Pennell, W., Barton, B.A. (Eds). Principles of Salmonid Culture, Developments in Aquaculture and Fisheries Science , vol. 29., Elsevier, pp. 613-654.

Halver, J.E., Smith, R.R., Tolbert, B.M., Baker, E.M., 1975. Utilization of ascorbic acid in fish. Ann. N.Y. Acad, Sci., 258, 81-102.

Hamm, M.W., Helyar,L., Hallquist,N.A., Sherman, A.R. 1989. Effect of iron deficiency and excess on liver plasma membrane fatty acid composition. FASEB J., 3, A1075.

Hamre, K., Waagbo, R., Berge, R.K., Lie, O., 1997. Vitamins C and E interact in juvenile Atlantic salmon (*Salmo salar*, L.). Free Radical Biol. Med., 22, 137-149.

Harper, H.A., 1975. Review of physiological chemistry. 15 edn. Lange Medical Publ., Los Altos, CA.

Hartman, R. H., Matrone,G.,Wise, G. H., 1965. Effect of high dietary manganese on hemoglobin formation. J. Nutr., 57, 429-439.

Helyar,L., Sherman, A.R., 1987. The effect of iron deficiency on interleukin 1 production by rat leukocytes. Am. J. Clin. Nutr., 46, 346- 452.

Henry, P. R. , Miller, E. R., 1995. Iron bioavailability. In: Ammerman, C.B., Baker,D.H., Lewis,A.J. (Ed.), Bioavailability of nutrients for animals: amino acids, minerals and vitamins. Academic Press, San Diego, pp.169-199.

Hjeltnes, B., Julshamn, K., 1992. Concentrations of iron, copper, zinc and selenium in liver of Atlantic salmon *Salmo salar* infected with *Vibrio salmonicida* . Diseases of Aquatic Organisms, 12, 147-149.

Hilton, J.W., 1989. The interactions of vitamins, minerals and diet composition in the diet of fish. Aquaculture, 79, 223-244.

Hilton, J.W., Cho,C.Y., Slinger,S.J., 1978. Effect of graded levels of supplemental ascorbic acid in practical trout diets fed to rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can., 35, 431-436.

Hiney, M.P., Kilmartin, J.J., Smith, P.R., 1994. Detection of *Aeromonas salmonicida* in Atlantic salmon with asymptomatic furunculosis infections. Diseases of aquatic Organisms, 19, 161-167.

Hirst, I.D., Ellis, A.E., 1994. Iron-regulated outer membrane proteins of *Aeromonas salmonicida* are important protective antigens in Atlantic salmon against furunculosis. Fish Shellfish Immunol., 4, 29-45.

Hirst, I.D., Hastings, T.S., Ellis, A.E., 1994. Utilization of haem compounds by *Aeromonas salmonicida*. Journal of fish diseases,17, 365-373.

Hirst, I.D., Ellis, A.E., 1996. Utilization of transferrin and salmon serum s sources of iron by typical and atypical strains of *Aeromonas salmonicida*. Microbiology, 142, 1543-1550.

Hirst, I.D., Hastings, T.S., Ellis, A.E., 1991. Siderophore production by *Aeromonas salmonicida*. Journal of General Microbiology,137, 1185-1192.

Hoffbrand, A.V., Ganeshaguru, k., Hooton, J.W.L., Tattersall, M.H.N., 1976. Effect of iron deficiency and desferrioxamine ondna synthesis in human cells. British Journal of Haematology, 33, 517- 526.

Horne, J.H., 1928. Furunculosis in trout and the importance of carriers in the spread of the disease. Journal of Hygiene, 28, 67- 78.

Huang, Y., Liu,Y., 1989. Studies on the mineral requirement in juvenile grass carp (*Ctenopharyngodon idella* C. et V.). Acta Hydrobiol. Sinica., 13, 134

Hulsow 1913. Contribution to the study of furunculosis, especially with regard to the salmonidae. Allgemeine Fischerei Zeitung,38.

Humbert,J.R, Moore-L. L., 1983. Iron deficiency and infection: a dilemma. J-Pediatr-Gastroenterol-Nutr., 2(3), 403-406.

Hurst, J.K., Barrette, W.C., 1989. Leukocytic oxygen and microbial oxidative toxins. Critical Reviews in Biochemistry and Molecular Biology, 24, 271- 328.

Ikeda,Y., Ozaki,H.,Uematasu,K. 1973. Effect of enriched diet with iron in culture of yellowtail. J. Tokoyo Univ. Fish., 59, 91- 99.

Iversen,M., Finstad,B., Nilssen,K.J., 1998. Recovery from loading and transport stress in Atlantic salmon (*Salmo salar* L.) smolts . Aquaculture, 168, 387- 394.

Kawatsu, H., 1972. Studies on the anemia of fish-V. Dietray iron deficient anemia in Brook Trout, *Salvelinus fontinalis*. Bull. Freshwater Fish. Res. Lab., 22, 59 - 67.

Kakuta, I., Murachi, S., 1992. Blood changes in Japanese eels, *Anguilla japonica*, experimentally infected with typical or atypical *Aeromonas salmonicida*. Comparative Biochemistry and Physiology. 103A, 151-155.

Kakuta, I., Murachi, S., 1993. Changes of iron content in eels (*Anguilla japonica*) infected with atypical *Aeromonas salmonicida*. Comparative Biochemistry and Physiology 105C, 275- 278.

Kaushik,S.J., GouillouCoustans,M.F., Cho,C.Y., 1998. Application of the recommendations on vitamin requirements of finfish by NRC (1993) to salmonids and sea bass using practical and purified diets. Aquaculture, 161, 463-474.

Karunasagar,I., Joseph, S.W., Twedt, R.M., Hada, H., Colwell, R.R., 1984. Enhancement of Vibrio parahaemolyticus virulence by lysed erythrocyte factor and iron. Infection and Immunity, 46, 141-144.

Klontz, G.W., Yasutake, W.T., Roas, A.J., 1966. Bacterial diseases of the salmonidae in the western United States: pathogenesis of furunculosis in rainbow trout. American Journal of Veterinary Research, 27, 1455-1460.

Klonz, G.W., Wood,J.W., 1972. Observation on the epidemiology of Furunculosis disease in juvenile Coho salmon (*Oncorhynchus kisutch*). Food and

Agriculture Organization of the United Nations: European Inland Fisheries advisory Commission.

Kochan, I., 1973. The role of iron in bacterial infections with special considerations of host-tubercle bacillus interaction. Curr. Top. Microbiol. Immunol., 60, 1-30.

Kochanowski, B.A., Sherman, A.R., 1982 a. Cellular growth in iron-deficient rat pups. Growth., 46, 126-134.

Kochanowski, B.A., Sherman, A. R., 1984. Phagocytosis and lysozyme activity in granulocytes from iron-deficient rat and pups. Nutr. Res.,4, 511-520.

Kochanowski, B.A., Sherman, A. R., 1985 a. Cellular growth in iron-deficient rats: effect of pre- and postweaning iron repletion. J. Nutr., 115, 279- 287.

Kuvibidila,S., Nauss,K.M., Baliga,B.S., Suskind,R.M. 1983. Impairment of blastogenic response of splenic lymphocytes from iron-deficient mice: in vivo repletion. American journal of Clinical Nutrition, 37, 15-25.

Kvingedal, A.M., Dehli, A., Rorvik, K.A., 1996. Transferrin mRNA in relation to liver iron storage in farmed Atlantic salmon (Salmo salar). Fish Physiology and Biochemistry.,15,317-322.

Lall, S.P., 1988. Disease control through nutrition. In: Proceedings of the Aquaculture International Congress and Exposition,B.C., Pavilion Corp., Vancouver, Canada, pp. 607-612.

Lall, S.P., 1989. The Minerals. In: Halver, J.E. (Ed.), Fish Nutrition. 2nd Ed., Academic Press Inc., New York, pp. 219- 257.

Lall, S.P., 1991a. Nutritional value of fish silage in salmonid diets. Bull. Aquacult. Assoc. Can., 91,63-74.

Lall, S.P., 1991b. Salmonid nutrition and feed production. Special Session On Salmonid Aquaculture, World Aquaculture Society., 1991, Pp. 107-123, Can. Tech. Rep. Fish. Aquat. Sci., No. 1831.

Lall, S.P., 1995. Macro and trace elements in fish and shellfish. In: Ruiter,A., (Ed.), Fish and fishery products. CAB International., Oxon, UK.

Lall, S.P., Hines, J.A., 1987. Iron and copper requirement of Atlantic salmon (*Salmo salar*) II. Int. Symp. Feeding Nutr. Fish. Bergen, Norway, Aug 23-27.

Lall, S.P., Olivier, G., Weerakoon, D.E.M., Hines, J.A., 1990. The effect of vitamin C deficiency and excess on immune response in Atlantic salmon (*Salmo salar* L.). The Current Status Of Fish Nutrition In Aquaculture. The Proceedings Of The Third International Symposium On Feeding And Nutrition In Fish. August 28-September 1, 1989, Toba, Japan., 1990, pp. 427-441

Lall, S. P., Olivier, G., 1993. Role of micronutrients in immune response and disease resistance in fish. In: Kaushik, S.J., Luquet., P. (Eds.) Fish Nutrition in Practice, INRA , Paris, pp. 101- 118.

Lall, S.P., Paterson, W.D., Hines, J.A., Adams, N.J., 1985. Control of bacterial kidney disease in Atlantic salmon, *Salmo salar* L., by dietary modification. Journal of fish diseases. 8,113-124.

Landolt, M.L., 1989. The relationship between diet and the immune response of fish. Aquacuture 79, 193-206.

Lanno, R.P., Slinger,S.J., Hilton, J.W., 1985. Effect of ascorbic acid on dietary copper toxicity in rainbow trout (*Salmo gairdneri* Richardson). Aquaculture, 49, 257-268.

Laryrisse, M., Marinez-Torres, C., Roche, M., 1968. Effects of interaction of various foods on iron. Am. J. Clin. Nutr. 111, 2195-2202.

Lim, C., Lovell, R.T., 1978. Pathology of vitamin C deficiency syndrome in channel catfish. J. Nutr., 108, 137-114.

Lim,C., Sealey,W.M., Klesius,P.H., 1996. Iron methionine and iron sulfate as sources of dietary iron for channel catfish *Ictalurus punctatus*. J. World Aquacult. Soc., 27, 290-296.

Lim, C., Klesius, P.H., 1997. Responses of channel catfish (*Ictalurus punctatus*) fed iron-deficient and replete diets to *Edwardsiella ictaluri* challenge . Aquaculture, 157,83-93.

Lim, C., Klesius, P.H., Li, M.H., Robinson, E.H., 2000., Interaction between dietary levels of iron and vitamin C on growth, hematology, immune response and resistance of channel catfish (*Ictalurus punctatus*) to *Edwardsiella ictaluri* challenge., Aquaculture, 185, 313-327.

Littell,R.C., Lewis,A.J., Henry,P.R., 1995. Statistical evaluation of bioavailability assays. Bioavailability Of Nutrients For Animals. 1995, 5-33.

Lockwood, J., Sherman, A.R., 1988. Spleen natural killer cells from iron-deficient rat pups manifest an altered ability to be stimulated by interferon. J. Nutr.,118, 1158-1563.

Loosli, J.K., Harris, L.E., 1945. Methionine increases the value of urea for lambs. J. Animal Sci., 4, 435- 437.

Loosli, J.K., 1978. Mineral problems as related to tropical climates. In: Conard, J.H., McDowell, L.R., (Eds.). Proceedings of the Latin American symposium on mineral nutrition research with grazing ruminants., University of Florida.   Gainesville, Fla., USA, pp.5-9 and 54.

Lorentzen, M., Maage,A., 1999. Trace element status of juvenile Atlantic salmon *Salmo salar* L. fed a fish-meal based diet with or without supplementation of zinc, iron, manganese and copper from first feeding. Aquaculture Nutrition, 5, 163-171.

Maage, A., 1994. Trace elements in Atlantic salmon *Salmo salar* nutrition. Ph.D. Thesis., University of Bergen, Bergen, Norway.

Maage, A., Waagboe, R., Olsson, P.E., Julshamm, K., Sandnes, K. 1990. Ascorbate-2-sulfate as a dietary vitamin C source for Atlantic salmon (*Salmo salar*): 2. Effects of dietary levels and immunization on the metabolism of trace elements. Fish Physiology and Biochemistry, 8, 429-436.

Maage, A., Julshamn, K., Ulgenes,Y., 1991. A comparison of tissue levels of four essential trace elements in wild and farmed Atlantic salmon (*Salmo salar*). Fisk. Dir. Skr. Ser. Ernæring., 4, 111-116.

Macdougall, L.G., Anderson, R., McNab, G.M., Katz,J. 1975. The immune response in iron-deficient children: Impaired cellular defense mechanisms with altered humoral components. J. Pediatr., 86, 833-843.

Mackie, T.J., Menzies, W.J.M., 1938. Investigations in Great Britain of furunculosis of the salmonidae. Journal of Comparative Pathology and Therapeutics, 51, 225-234.

Mackie, T.J., Arkwright, J.A., Pyrce-Tannatt, T.E., Mottram, J.C., Johnson, W.D., Menzies, W.J., 1930. Interim report of the furunculosis committee. Edinburgh, HMSO.

Mahoney,A.W., Hendricks, D.G., 1978. Some effects of different phosphate compounds on iron and calcium absorption. J. Food. Sci., 43, 1473-1476.

Masawe, A.E., Muindi, J. M., Swai, G.B., 1974. Infections in iron deficiency and other types of anaemia in the tropics. Lancet., II, 314-317.

Mahoney,A.W., Hendricks, D.G., 1982. Efficiency of hemoglobin regeneration as a method of assessing iron bioavailability in food products. In: Kies, C. (Ed.) Nutrititional bioavailability of iron. ACS Symp. Series, 203, American Chemical Soc., Washington DC, pp.1-10.

Mayer, F.L., Mehrle, P.M., Crutcher, P.L., 1978. Interactions of toxaphene and vitamin C in channel catfish. Trans. Amer. Fish. Soc., 107, 326-333.

Mazoy, R., Conchas, R.F., Fouz, B., Lemos, M.L., 1992. Growth of *Vibrio anguillarum* in different fish sera. Aquaculture, 107, 277-281.

Mazur, A., Green,S., Carleton, A., 1960. Mechanism of plasma iron incorporation into hepatic ferritin. J.iol. Chem., 235, 595-603.

McCarthy,D.H., 1977. Some ecological aspects of the bacterial fish pathogen *Aeromonas salmonicida*. In: Skinner, F.A., Shewan,J.M. (Eds.) Aquatic

Microbiology. The Society for Applied Bacteriology Symposium Series No.6., Academic Press, London., pp. 299-324.

McDowell, L. R., 1992. Minerals in animal and human nutrition. Academic Press,Inc., pp.524.

Miguel, J.L., Pablos, M.I., 1993. An electrophoretical study of ferritin from rainbow trout (*Salmo gairdneri*, R) liver. Comparative Biochemistry and Physiology,106B,937-942.

Moore, C.V., Dubach,R., 1962. Mineral Metabolism., Vol.2., The Elements., Academic Press,Inc., NY, pp. 288-348.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival application to proliferation and cytotoxicity assays. Journal of Immunological methods, 65, 55-63.

Munro, H., 1993. The ferritin genes: their response to iron status. Nutr. Rev., 51, 65-73.

Murray, J., Stein, N. 1970. Iron. In: Mills, C.F. (Ed.). Trace Element Metabolism in Animals., Vol.1., Livingstone, Edinburgh, Scotland.

Murray,M.J., Murray,A.B., Murray, N.J., Murray, M.B., 1975. Refeeding-malaria and hyperferraemia. Lancet., I, 653- 654.

Murray, M.J., Murray, A.B., Murray,M.B., Murray, C.J. 1978. The adverse effect of iron repletion on the course of certain infections. Br. Med, J., 2, 1113-1115.

Nakai, T., Kanno, T., Cruz, E.R., Muroga, K., 1987. The effect of iron compounds on the virulence of *Vibrio anguillarum* in Japanese eels and ayu. Fish Pathology 22, 185-189.

Navarre,O., Halver, J.E., 1989. Disease resistance and humoral antibody production in rainbow trout fed high levels of vitamin C. Aquaculture, 79, 207-221.

Neilands, J.B., 1957. Some aspects of microbial iron metabolism. Bacteriol. Rev., 21, 101-111.

Neilands, J.B., 1995. Siderophores: Structure and function of microbial iron transport compounds. J. Biol. Chem., 270, 26723-26726.

Neji, H., Noue, J. De La., 1998. Effect of animal and vegetal protein diets on feed intake and apparent digestibility of nutrients in rainbow trout (*Oncorhynchus mykiss*) infected by *Aeromonas salmonicida*, with and without chronic hypoxia stress. Can. J. Fish. Aquat. Sci.,55, 2019-2027.

Nomura, T., Yoshimizu, M., Kimura, T., 1991 a. Prevalence of *Aeromonas salmonicida* in the kidney of chum salmon (*Oncorhynchus keta*) and masu salmon (*O. masou*) at various life stages. Gyobyo Kenkyu, 26, 149-153.

Nomura, T., Yoshimizu, M., Kimura, T., 1991 b. Prevalence of *Aeromonas salmonicida* in the chum salmon (*Oncorhynchus keta*) and pink salmon (*O. gorbuscha*) and masu salmon (*O. masou*) returning to rivers in Hokkaido. Gyobyo Kenkyu, 26, 139- 147.

Nomura, T., Yoshimizu, M., Kimura, T., 1992a. Detection of *Aeromonas salmonicida* in the coelomic fluid and kideny of mature of chum salmon (*Oncorhynchus keta*), pink salmon (*O. gorbuscha*) and masu salmon (*O. masou*). Gyobyo Kenkyu, 27, 69-72..

Nose, T., Arai,S., 1979. Recent advances in studies on mineral nutrition of fish in Japan. In: Pillay, T.V.R., Dill, W.A. (Eds.), Advances in Aquaculture, Farnham, Surrey, pp. 584-590.

NRC (National Research Council). 1978. Nutrient requirements of domestic animals, Nutrient requirements of laboratory animals., National Academy of Sciences, National Academy Press, Washington, DC, USA.

NRC (National Research Council). 1984. Nutrient requirements of domestic animals, Nutrient requirements of poultry., 8th Ed., National Academy of Sciences, National Academy Press, Washington, DC, USA.

NRC (National Research Council). 1985. Nutrient requirements of domestic animals, Nutrient requirements of sheep., 5th Ed., National Academy of Sciences, National Academy Press, Washington, DC, USA.

NRC (National Research Council). 1989a. Nutrient requirements of domestic animals, Nutrient requirements of dairy cattle., 6th Ed., National Academy of Sciences, National Academy Press, Washington, DC, USA.

NRC (National Research Council). 1989b. Nutrient requirements of domestic animals, Nutrient requirements of horses., 5th Ed., National Academy of Sciences, National Academy Press, Washington, DC, USA.

NRC (National Research Council). 1993. Nutrient requirements of fish. National Academy Press, Washington, DC, USA.

Olivier, G.,1997. Effect of nutrition on furunculosis. In: Bernoth, E.M., Ellis, A.E., Midtlyng,P.J., Olivier,G., Smith,P., (Eds.). Furunculosis, Academic Press, San Diego, pp. 327-344.

Olivier, G; Eaton, CA; Campbell, N., 1986. Interaction between Aeromonas salmonicida and peritoneal macrophages of brook trout (Salvelinus fontinalis). Veterinary Immunology and Immunopathology 12, 223-234.

Opal, S., Cross, A., Gemski, P., 1982. K antigen and serum sensitivity of rough Escherichia coli. Infect. Immun. 37, 956- 960.

Otto, B.R., Verweij-van Vaught, A.M.J.J., MacLaren, D.M., 1992. Transferrin and heme compounds as iron sources for pathogenic bacteria. Critical reviews in microbiology, 18, 217-233.

ParipatanAnont,T., Lovell,R.T., 1995. Responses of channel catfish fed organic and inorganic sources of zinc to Edwardsiella ictaluri challenge. J. Aquat. Anim. Health., 7, 147-154.

Paripatananont,T., Lovell,R.T., 1997. Comparative net absorption of chelated and inorganic trace minerals in channel catfish Ictalurus punctatus diets J. World Aquacult. Soc., 28, 62-67.

Paterson, W.D., Lall, S.P., Airdrie, D., Greer,P., Greenham, G.J., Poy, M., 1985. Prevention of disease in salmonids by vaccination and dietary modification. Fish pathology, 20, 427-434.

Pla, G.W., Harrison, B.N., Fritz, J.C., 1973. Comparison of chicks and rats as test animals for studying bioavailability or iron, with special reference to use of reduced iron in enriched bread. J. Assoc. Off. Anal. Chem.,56, 1369- 1373.

Pla, G.W., Fritz, J.C., 1970. Availability of iron. J. Assoc. Off. Anal. Chem.,53 , 791-800.

Pla, G.W., Fritz, J.C., 1971. Collaborative study of the hemoglobin repletion test in chicks and rats for measuring availability of iron. J. Assoc. Off. Anal. Chem., 54, 13-17.

Plehn,M. 1911. Die Furunkulose der Salmoniden. Centralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, I, Abteilung, Originale, 60, 609-624.

Pollock , S., Kaufman, R. M.,Crosby, W. H. 1964. Iron absorption effects of sugars and reducing agents. Blood, 24, 577-581.

Raffin, S.B., Woo,C.H., Roost,K.T., Price, D.C., Schmid, R., 1974. Intestinal absorption of hemoglobin iron heme cleavage by mucosal heme oxygenase. J. Clin. Invest., 54, 1344-1352.

Rainger, G.E., Rowley, A.F., 1993. Antibacterial activity in the serum and mucus of rainbow trout, Oncorhynchus mykiss, following immunisation with Aeromonas salmonicida. Fish and Shellfish Immunology, 3, 475-482.

Raja, K.B., Pountney, D.J., Simpson, R.J., Peters, T.J., 1999. Importance of anemia and transferrin levels in the regulation of intestinal iron absorption in hypotransferrinemic mice. Blood.,94, 3185-3192.

Ravandal, J., Løvold, T., Bentsen,H.B., Røed,K.H., Gjedrem,T., Rørvik,K.A., 1994. Serum iron levels in farmed Atlantic salmon: family variation and association with disease resistance. Aquaculture,125, 37-45.

Rassmussen, K.J., 1994. Spray-dired blood in diets to Atlantic salmon (Salmo salar). Fisk. Dir. Skr. Ser. Ern.,6, 151-161.

.

RDA. 1989. Recommended dietary allowances, 10th ed., Food and Nutrition Board, National Academy of Sciences, Washington, D.C. USA.

Reddy, M.B., Cook, J.D., 1991. Assessment of determinants of nonhaem-iron absorption in humans and arats. Am. J. Clin. Nutr., 54, 723- 728.

Robbins, K.R., Norton, H.W., Baker, D.H., 979. Estimation of nutrient requirements from growth data. J. Nutr., 109, 710-714.

Roed, KH; Dehli, AK; Flengsrud, R; Midthjell, L; Rorvik, KA., 1995. Immunoassay and partial characterisation of serum transferrin from Atlantic salmon (Salmo salar L.). Fish & Shellfish Immunology, 5,71-80.

Roed, K.H., Fjalestad, K.T., Stromsheim, A., 1993. Genetic variation in lysozyme activity and sponteneous haemolytic activity in Atlantic salmon (Salmo salar L.). Aquaculture,114, 19-33.

Roeder,M., Roeder,R.H. 1966. Effect of iron on the growth rate of fishes. J.Nutr. 90, 86-90.

Roitt, I., 1997. Roitt's Essential Immunology., 9th edt. Blackwell Science Ltd., Oxford, UK., pp. 3-39.

Rørvik, K.A., Salte, R., Bentsen,H., Thomassen,M., 1991. Effects of dietary iron and n-3 unsaturated fatty acids (omega-3) on health and immunological parameters in farmed salmon. Proc. Eur. Assoc. Fish. Pathol., Budapest, Hungary. p.71.

Rorvik, LM; Caugant, DA; Yndestad, M., 1995. Contamination pattern of Listeria monocytogenes and other Listeria spp. in a salmon slaughterhouse and smoked salmon processing plant. International Journal of Food Microbiology, 25,19-27.

Rose, A.S., Ellis, A.E., Munro, A.L.S., 1989b. The infectivity by different routes of exposure and shedding of Aeromonas salmonicida subsp. salmonicida in Atlantic salmon, Salmo salar L., held in sea water. Journal of fish diseases, 12, 573-578.

Rose, A.S., Ellis, A.E., Munro, A.L.S., 1990a. Evidence against dormancy in the bacterial fish pathogen *Aeromonas salmonicida* subsp. *salmonicida*. FEMS Microbiology Letters, 68, 105-108.

Rothenbacher,H., Sherman, A. R., 1980. Target organ pathology in iron-deficient suckling rats. J. Nutr. 110, 1648- 1654.

Rumsey,G.L., Ketola,H.G., 1975. Amino acid supplementation of casein in diets of Atlantic salmon (*Salmo salar*) and of soybean meal fopr rainbow trout (*Salmo gairdneri*) fingerlings. J. Fish. Res. Bd. Can., 32, 422-426.

Sakai, D.K., 1983. Lytic and bactericidal properties of salmonid sera. Journal of Fish Biology, 23, 457-466.

Sakamoto,S.,Yone, Y. 1976. Requirement of red sea bream for Fe. 1. Rep. Fish. Re. Lab. Kyushu Univ., 3, 59-64.

Sakamoto, S., Yone,Y., 1978 a., Requirement of red sea bream for dietary iron-II. Bull. Japan. Soc. Sci. Fish., 44, 223-225.

Sakamoto, S., Yone,Y., 1978 b., Iron deficiency symptoms of carp. Bull. Japan. Soc. Sci. Fish., 44, 1157-1160.

Sakamoto, S.,Yone, Y. 1979. Availabilities of three iron compounds as dietary iron sources for red sea bream. Bull. Jap. Soc. Scien. Fisher., 45, 231 - 235.

Salte,R., Rorvik, K.A., Reed,E., Nordberg,K., 1994. Winter ulcers of the skin in Atlantic salmon, Salmo salar L.: pathogenesis and possible aetiology. J. Fish. Diseases, 17, 661-665.

Sandnes, K., 1991. Vitamin C in fish nutrition- a review. Fisk. Dir. Ser. Ernaering, 4, 3-32.

Sandnes, K., Lie, Ø., Waagbø., 1988. Normal ranges of some blood chemistry parameters in adult farmed Atlantic salmon (*Salmo salar*). J. Fish. Biol., 32,129-136.

Saunders, R.L., 1995. Salmon aquaculture: Present status and prospects for the future. In: Boghen, A.D.(Edt.). Cold-Water Aquaculture In Atlantic Canada, 2nd Edition., Cirrd, Univ. Moncton, Nb, (Canada), pp. 35-81.

Schricker, B.R., Miller, D.D., van Campen, D., 1983. Effects of iron status and soy protein on iron absorption by rats. J. Nutr., 113, 996- 1001.

Scombes, C.J., Olivier, G., 1997. Host-Pathogen interactions in Salmonids. In: Bernoth, E., Ellis, A.E., Midtlyng, P.J., Olivier, G., Smith, P., (Eds.). Furunculosis. Academic Press, New York, pp. 269-296.

Scrimshaw, N.S.,1990. Minerals and immunity: Introduction. In: Watson, R.R. (Ed.). Malnutrition, Disease Resistance, and Immune Function. Marcel Dekker, New York. pp. 110-112.

Sealey, W.M., Lim, C., Klesius, P.H., 1997. Influence of dietary level of iron from Iron Methionine and Iron Sulfate on immune response and resistance of Channel Catfish to Edwardsiella ictaluri. Journal of World Aquaculture Society, 28, 142- 149.

Secombes, C.J., 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: Stolen, J.S., Fletcher, T.C., Anderson, D.P., Robertson, B.S., van Muiswinkel, W.B., (Eds.). Techniques in fish immunology., SOS Publications, Fair Haven, pp. 137- 154.

Secombes, C.J., 1994. Enhancement of fish phagocyte activity. Fish & Shellfish Immunology ,4, 421-436.

Secombes, C.J., Cross, A.R., Sharp, G.J.E., Garcia, R., 1992. NADPH oxidase-like activity in rainbow trout Oncorhynchus mykiss (Walbaum) macrophages. Developmental & Comparative Immunology. 16,405-413.

Shearer, K.D., 1984. Changes in elemental composition of hatchery reared rainbow trout (Salmo gairdneri) associated with growth and reproduction. Can. J. Fish. Aquat. Sci., 41, 1492-1600.

Shearer, K.D., 1991. Determination of the dietary requirement for the essential elements in fish., Ph.D. Thesis, University of Bergen, Bergen, Norway.

Shearer, K.D., 1994. Factors effecting the proximate composition of cultured fishes with emphasis on salmonids. Aquaculture, 119, 63-88.

Shearer, K.D., 1995. The use of factorial modeling to determine the dietary requirements for essential elements in fishes. Aquaculture, 133, 57-72.

Shearer,K.D., Åsgård,T., 1991. The effect of water-borne magnesium on the dietary magnesium requirement of rainbow trout (Oncorhynchus myiss). Fish. Phtsiol. Biochem., 9, 387-392.

Shearer, K.D., Christiansen, R., Daae,B., Torrissen,O.J., 1993. Evaluation of four semipurified diets for juvenile Atlantic salmon (Salmo salar). In: S.J. Kaushik., P. Luquet., eds. Fish nutrition in practice. INRA Editions, Paris.

Shearer,K.D., Åsgård,T., Andorsdöttir,G., Aas,G.H., 1994. Whole body elemental and proximate composition of Atlantic salmon (Salmo salar) during the life cycle. J. Fish. Biol., 44, 785-797.

Sherman, A.R., 1984. Iron, infection, and immunity. In: Watson, R.R. (Ed.). Malnutrition, Disease Resistance, and Immune Function. Marcel Dekker, New York. pp. 251-283.

Sherman, A. R. 1990. Influence of iron on immunity and disease resistance. In: Bendich, A., Chandra, R.K. (Ed.) Micronutrients and Immune Functions. Annals NY Acad. Scien. 587. New York, pp.140-146.

Sherman, A.R., Bartholmey, S.J., Perkins,E.G. 1982. Fatty acid patterns in iron deficient maternal and neonatal rats. Lipids.17, 639- 643.

Sherman, A.R., Lockwood,J. 1987. Impaired natural killer cell activity in iron-deficient rat pups. J. Nurt. 117, 567- 571.

Sherman, A.R., Wolinsky,I., 1978. Lysozyme levels in tissues of iron-deficient rats. Experientia, 34, 367-368.

Shieh, H.S., MacLean, J.R., 1975. Purification and properties of an extra-cellular portease of Aeromonas salmonicida, the causative agent of furunculosis. International Journal of Biochemistry, 6, 653-656.

Shoden, A., Sturgeon, P., 1961. Formation of haemosiderin and its relation to ferritin. Nature, 189, 846-847.

Smith, P.R., Davey, S., 1993. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. Journal of Fish Diseases,16, 521-524.

Spiro, T.G., 1977. Chemistry and biochemistry of iron. In: Brown, E.B., Aisen,P., Fielding,J., Crichton,R.R. (Eds.) Proteins of iron metabolism, Grune & Stratton, NY., pp. xxii-xxxii.

Spiro, T.G., Saltman,P., 1974. Inorganic chemistry. In: Jacobs,A., Worwood,M. (Eds.) Iron in biochemistry and medicine, Academic press, NY.

Standal, H., Rorvik, K.A., Lien, H., Andersen, O., 1997. Effects of acute iron overload on Atlantic salmon(*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Biol. Trace. Element Res., 59, 13-22.

Standal, H., Dehli, A., Rorvik, K.A., Andersen, O., 1999. Iron status and dietary levels of iron affect the bioavailability of haem and nonhaem iron in Atlantic salmon *Salmo salar*. Aquaculture Nutrition. 5,193-198.

Steel, R.G.D., Torrie, J.H., 1960. Principles and procedures of statistics. McGraw Hill Book Co., Inc., NY., pp.481.

Suzumoto, B.K., Schreck, C.B., McIntyre, J.D., 1977. Relative resistances of three transferrin genotypes of Coho salmon (*Oncorhynchus kisutch*) and their hematological responses to bacterial kidney disease. Journal of the fisheries research board of Canada, 34, 1-8.

Swartz, A.S. 1982. The uptake an distribution of erythromycin phosphate in a drug-surfactant bath with several species of salmonids. MSc Thesis, University of Idaho.

Tacon, A.G.J., De Silva, S.S., 1983. Mineral composition of some commercial fish feeds available in Europe. Aquaculture, 31, 11-20.

Thomson , A. B. R. , Shaver, C., Lee, D. J., Jones, B. L., Valberg, L. S. 1971. Effect of varying iron stores on site of intestinal absorption in the rat: Effect of cobalt. Am. J. Physiol., 220, 1080-1085.

Turnbull, A., Cleton,F., Finch,C.A., 1962. Iron absorption. IV. The absorption of hemoglobin iron. J. Clin. Invest. 41, 1897- 1906.

Underwood, E.J. 1977. Trace elements in human and animal nutrition. Academic Press. NY.

Underwood, E.J. 1981. The Mineral Nutrition of Livestock. Commonwealth Agricultural Bureau, London, UK.

Valenta, M., Stratil, A., Kalal, L., 1977. Polymorphism and heterogeneity of transferrins in some species of the fish family Cyprinidae. Anim. Blood Groups Biochem. Genet., 8, 93-109.

Van Campen, D., Gross, E. 1969. Effect of histidine and certain other amino acids on the absorption of iron-59 by rats. J. Nutr., 99, 68-74.

Verlhac, V., Gabaudan, J., 1994. Influence of vitamin C on the immune system of salmonids. Aquaculture and Fish. Manag., 25, 21-36.

Vielma, J., 1998. Utilization of dietary phosphorus in rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (Salmo salar). Ph.D. Thesis, Department of Applied Zoology and Veterinary Medicine, University of Kuopio, Kuopio, Finland.

Vinogradov, A.P. 1953. The elementary chemical composition of marine organisms. (translation from Russain) Sears Foundation for Marine Research, Yale Univ. 647 pp.

Waagbo,R. 1994. The impact of nutritional factors in the immune system in Atlantic salmon, Salmo salar L.: A review. Aquaculture and Fisheries Management, 25, 175- 197.

Waagbo, R., Glette, J., Raa-Nilsen, E., Sandnes, K., 1993. Dietary vitamin C, immunity and disease resistance in Atlantic salmon (Salmo salar)., Fish Physiology and Biochemistry, 12, 61-73.

Waagbo, R.,Sandnes, K. S.O., 1996, Title Effects of dietary vitamin C on growth and parr-smolt transformation in Atlantic salmon, *Salmo salar* L. Aquaculture Nutrition, 2, 65-69.

Walker,R.L., Fromn,P.O. 1976. Metabolism of iron by normal and iron deficient rainbow trout. Comp. Biochem. Physiol. 55A, 311- 318.

Walters,G.O., Miller, F.M., Worwood, M. 1973. Serum ferritin concentration and iron stores in normal subjects. J-Clin-Pathol., 26, 770-2 .

Weinberg, E.D., 1984., Iron and infection. Physiol Rev., 64, 65-102.

Weiss, G., Wacher, H., Fuchs, D., 1995. Linkage of cell-mediated immunity to iron metabolism. Immunology Today, 16, 495-500.

Wekell, J.C., Shearer, K.D., Gauglitz, E.J., 1986. Zinc supplementation of trout diets: tissue indicators of body zinc status. Prog. Fish. Cult., 48, 205-212.

Wienk,K.J., Marx,J.J., Beynen, A.C., 1999. The concept of iron bioavailability and its assessment. Eur. J. Nutr., 38: 51-75.

Wolinsky,I., Sherman, A.R., 1979. Maternal and neo-natal tissue lysozyme levels: effects of iron nutriture in rats. Proc. Soc. Exp. Biol. Med., 162, 369-373.

Wood, R.J., Han, O., 1998. Recently identified molecular aspects of intestinal iron absorption. J. Nutr., 128, 1841-1844.

Zannoni, V.G., Holsztynska,E.J., Lau, S.S., 1982. Biochemical functions of ascorbic acid: chemistry, metabolism, and uses. American Chemical Society, Washington, D.C., USA.

Zar, J.H., 1984. Biostatistical Analysis, 2nd Edn., Prentice-Hall., Englewood Cliffs., NJ.

Zettner, A., Sylvia, L.C., Capacho-Delgado, L., 1966. The determination of serum iron and iron-binding capacity by atomic absorption spectroscopy. The American Journal of Clinical Physiology, 45, 533-540.