INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

"A MODEL OF GROWTH SUPPRESSION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) ASSOCIATED WITH A HIGH DOSE OF HYDROGEN PEROXIDE EXPOSURE WITH EVALUATION OF SELECTED HORMONAL AND BLOOD CHEMICAL CONSTITUENTS"

TITLE

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

VERONICA CARVAJAL DE LA FUENTE

Charlottetown, P.E.I.

October 23rd, 1998

CONDITION OF USE

The author has agreed that the Library, University of Prince Edward Island, may make this thesis freely available for inspection. Moreover, the author has agreed that permission for extensive copying of this thesis for scholarly purposes may be granted by the professor or professors who supervised the thesis work recorded herein, or, in their absence, by the Chairman of the Department or the Dean of the Faculty in which the thesis work was done. It is understood that due recognition will be given to the author of this thesis and to the University of Prince Edward Island in any use of the material in this thesis. Copying or publication or any other use of the thesis for financial gain without approval by the University of Prince Edward Island and the author's written permission is prohibited.

Requests for permission to copy or to make any other use of material in this thesis in whole or in part should be addressed to:

Chairman of the Department of Pathology and Microbiology
Faculty of Veterinary Medicine
University of Prince Edward Island
Charlottetown, P.E.I.
Canada C1A 4P3.

SIGNATURE PAGES

iii-iv

REMOVED

ABSTRACT

Hydrogen peroxide is an oxidizing compound which has been used as a therapeutic agent to control sea lice infestations of salmonids. However, it is known that concentrations needed to control these copepods may cause serious branchial injury. The effect of this treatment on fish growth performance is unknown. In this thesis, a treatment simulation model, in which rainbow trout (*Oncorhynchus mykiss*) juveniles reared in fresh water received a single exposure to hydrogen peroxide at 1250 ppm for 30 min, was used in order to develop an understanding of the interaction of this treatment with salmonid growth performance. The growth performance of fish before and after treatment was compared with that of untreated fish held in separate tanks and in a cohort design in which treated fish co-habitated with untreated fish. Those fish which were kept in separate tanks had a transient significantly reduced SGR (specific growth performance) after treatment through a significant interference with feed conversion ability, with no significant effect on appetite. However no significant effect on growth was noted in those fish reared with untreated cohorts. Other studies were designed to investigate whether growth alterations were related to disturbances in hormonal and blood chemical compounds. It was demonstrated that hydrogen peroxide activated the primary and secondary stress response characterized by increased serum cortisol concentrations, increased plasma glucose concentrations, and decreased leucocrits which were characterized by a decreased number of circulating lymphocytes. Thyroxine levels were also transiently increased in treated fish while levels of triiodothyronine were significantly decreased. Because the gills are a known target organ for hydrogen peroxide, changes in gill structure were investigated as well. Treatment-related histopathological changes in gills were lamellar necrosis, edema and epithelial hyperplasia. A significant increase of the interlamellar space (the thickness of the epithelial layer in the region between the bases of adjacent lamellae) was found when evaluated with a Bioquant system. Based on our results, we conclude that the activation of the primary and secondary stress response, and alteration of the gill morphology may be, in part, contributory factors in the transient decreased growth performance associated with a single high dose of hydrogen peroxide.

DEDICATION

A mis padres: Pedro y Guadalupe quienes lucharon para darme la oportunidad de ser quien soy.

A mis hermanos: Eva María, Beatríz Adriana, Pedro y Erika con mucho cariño y respeto.

A Oscar, porque siempre me brindo su apoyo, amor y cariño. Gracias por todo.

ACKNOWLEDGEMENTS

I wish to acknowledge my co-supervisors Dr. Barbara Horney and Dr. David Speare for offering me the opportunity to work on this project.

Thanks to the members of my supervisory committee, Dr. Fred Markham, and Dr. John Burka whose helpful suggestions and comments helped in the improvement of this thesis.

I would like to thank the financial support of the Canadian International Development Agency for my student stipend.

I am particularly grateful to Dr. Alfonso López for his encouragement, and support and for offering me the opportunity of being part of the CIDA corporation project between the University of Prince Edward Island and the Universidad Autónoma de Tamaulipas.

I would also acknowledge and thank people from the AVC of UPEI that helped me with technical assistance: Garth Arsenault, Joanne Daley, Allan Mackenzie, Kathy Mitton, and Linda Kerr.

I would also thank people from the Universidad Autónoma de Tamaulipas, especially to M.V.Z. Fernando Arizpe García, M.V.Z. Sergio Garza Quiroga and M.V.Z. Julio Martínez Burnes for their support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

TABLE OF ABBREVIATIONS

ACTH	: Adrenocorticotropic hormone
ANOVA	: Analysis of variance
DWBC	: Differential of white blood cells
DNA	: Deoxyribonucleic acid
e.g.	: Exampli gratia, for example
FCI	: Feed conversion index
FDA	: Food and Drug Administration
Fig.	: Figure
g	: Gram
h	: Hour
i.e.	: id est; that is
H&E	: Hematoxylin and Eosin
HPI	: Hypothalamic-pituitary-interrenal
kg	: Kilogram
L	: Liter
Lct	: Leucocrit
LD_{50}	: Lethal dose
m^3	: Cubic metre
mRNA	: Messenger ribonucleic acid
min	: Minute
mg	: Milligram
ml	: Millilitre
mm	: Millimetre
mmol	: Milimmoles
ppm	: Parts per million
p	: p value
PCV	: Packed cell volume
pH	: Hydrogen ion concentration (neg. Log)
RIA	: Radioimmunoassay
rpm	: Revolutions per minute
sec	: Second
SD	: Standard deviation
SGR	: Specific growth rate
T_3	: Triiodothyronine
T_4	: Thyroxine
™	: Trade mark
TSH	: Thyroid stimulating hormone
TRH	: Thyrotropin releasing hormone
°C	: Degree Celsius
%	: Percent
µl	: Microliter

1. GENERAL INTRODUCTION

1.1 Fish gills

The fish gill is a multifunctional tissue. It participates in the gas transfer of oxygen and carbon dioxide (Randall and Daxboeck 1984), and plays an important role in osmoregulation and active ion uptake (ie. Na^+, Cl^-, Ca^{2+}) (Evans 1993), acid-base regulation, elimination of ammonia and the excretion of many other metabolites (Heisler 1984).

Basically, gills consist of a network of capillaries, covered by a thin epithelium and arranged to allow surrounding water to pass through very fine channels. The blood is separated from the surrounding water by only one or two layers of epithelial cells, creating a system for carrying red blood cell hemoglobin in close contact with the water, so that oxygen can be absorbed and carbon dioxide released efficiently (Randall and Daxboeck 1984).

The constant exposure of this tissue to the external environment and the fragile structure of gills make them highly vulnerable to adverse environmental factors (Roberts 1978). Therefore, one might expect these organs to be frequently involved in disease problems.

1.1.1 Normal gill structure and function

The anatomical structure of the gills is very complex, each gill arch having a double row of filaments (also known as primary lamellae). Each filament has a series of lamellae (also known as secondary lamellae) located anteriorly and posteriorly, perpendicular to the

filament (Fig. 1).

1.1.2 The filaments and arch.

The filaments and arch supply structural support for the lamellae and blood vessels. The whole gill is covered with epithelium that consists of unspecialized protective and supportive cells (these cells cannot be differentiated with light microscopy), chloride cells and mucous cells which are particularly important for gill function (Morgan and Tovell 1973). The filaments are composed of an internal cartilaginous core which sometimes may be deformed during chronic disease processes. There are two arteries in the gill, the afferent filament artery which is in close apposition to the cartilage core and the efferent filament artery which is found at the other side of the filament. Between these two vessels there is a central venous sinusoid which is a venolymphatic sinusoid which receives lymphatic drainage from the gills and oxygenated blood from the efferent filament artery.

1.1.3 The lamella

The lamellae are the sites of exchange for oxygen, carbon dioxide, ammonia, and several cations and anions (Heisler 1984, Isaia 1984; Randall and Daxboeck 1984; de Renzis and Bornancin 1984). The typical morphology of the lamella is shown in Fig. 1. Basically, each lamella is composed of a network of interconnected spaces separated and supported by pillar cells which form pillar cell channels through which red blood cells percolate. The marginal channels are composed of true endothelial cells and these in turn are separated from the double cell layer of epithelial cells by the basement membrane (Lauren 1984). The thin

endothelial vascular channels between the pillar cells are the site of gas exchange, removal of nitrogenous waste and some electrolyte exchange (Lauren 1984).

A thick unstratified epithelium covers the filament between gill lamellae. In this interlamellar epithelium are scattered cells of two special types: mucous cells and chloride cells. In a healthy fish the lamellae have only low numbers of mucous cells which are granule-filled and vacuolated when observed with light microscopy (Yasutake and Wales 1983). Chloride cells are scattered along the entire lamellae epithelium, being more numerous at the proximal rather than the distal end of the lamella. These types of cells participate in the transport and secretion of ions required for controlling the osmoregulatory function of fish (Payan and Girard 1984).

1.1.4 Gill defence systems

The gills are the principal target site for many waterborne pathogens and toxicants, due to their intimate contact with the external environment (Wendelaar Bonga and Van der Meij 1989). The first line of defence of the gills is the production of mucus. This secretion helps to eliminate various particles such as metals and some water-borne pathogens which selectively bind to the mucus; the mucus then quickly sloughs the particles off the gill surface (Playle and Wood 1989). In addition to the mechanical action of the mucus, biochemical and cellular constituents such as lysozyme, haemagglutinins, antibodies, and leukocytes also play a defensive role (Playle and Wood 1989).

Strongly acidic or alkaline water may be a problem for fish. Modification of the pH of water near the gills is another mechanism of protection used by fish gills. Fresh water fish

are able to alter the composition of the water that they breathe by removing oxygen and ions and releasing carbon dioxide, ammonia and other metabolic end products across the gills (Playle and Wood 1989). This transfer of CO_2 and ammonia may acidify or alkalinize the water close to the gills, but is dependent on water pH. The local pH changes induced adjacent to gills may be sufficient to protect the fish from injury in very acidic or very basic water (Playle and Wood 1989).

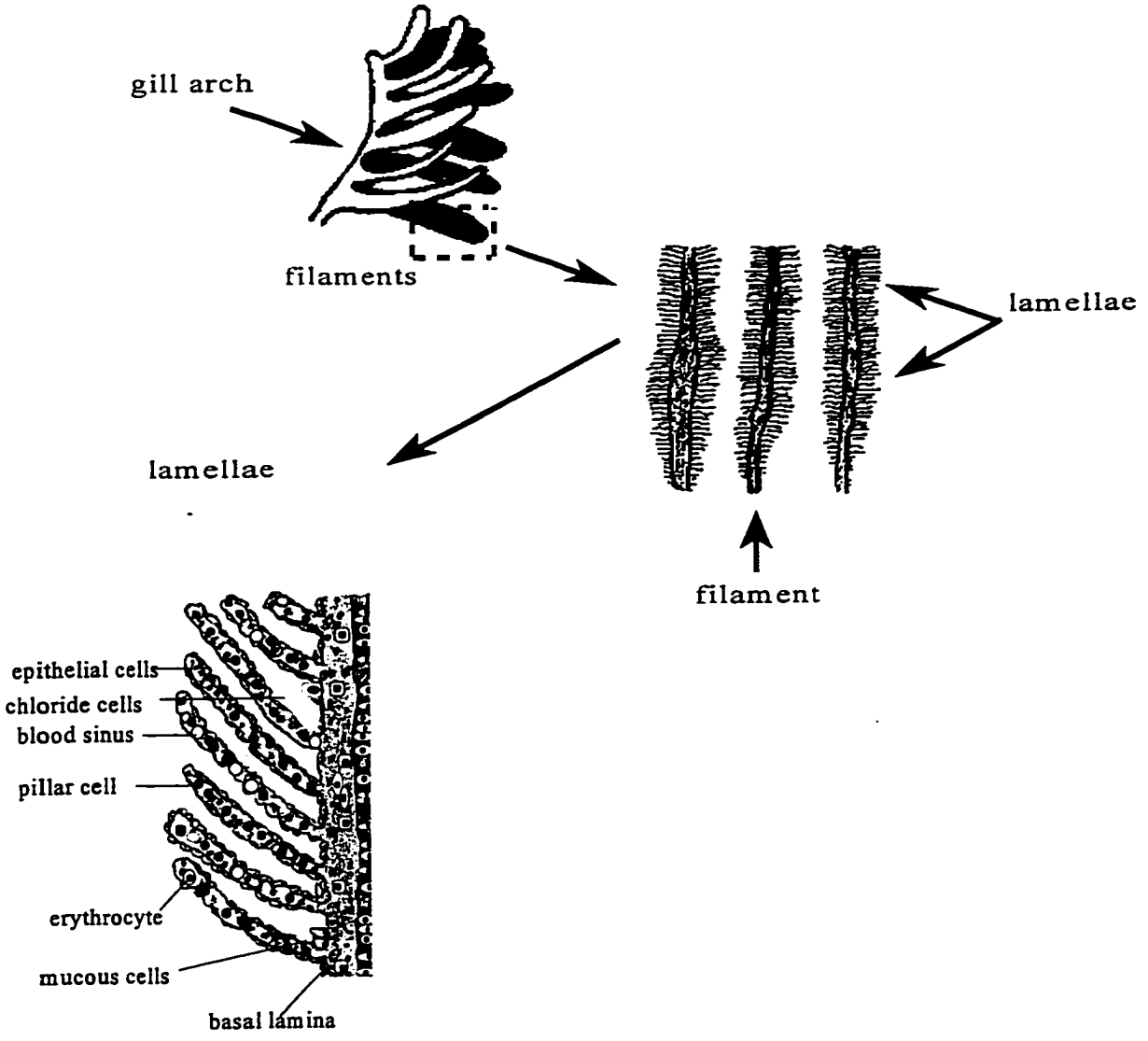


Fig. 1.1 Drawing of the gill architecture of the teleost fish.

1.1.5 Effects of toxicants and other stressors on gill structure and function

1.1.5.1 Alterations in gill structure

Gill structure may be affected by various toxic agents during acute or chronic exposure. An illustration of histological alterations caused by different kinds of irritants are shown in Fig. 1.2. Prolonged exposure to toxicants may stimulate cell division and lead to hyperplasia of the epithelial layer. Further hyperplasia may lead to clubbing of the lamellar channels and, finally, result in fusion of the adjacent lamella which can result in impairment of the gill functions.

Other frequent histopathological changes include: epithelial lifting, necrosis, and rupture of the gill epithelium, hypersecretion and proliferation of mucocytes, and changes in chloride cells and gill vasculature (Mallatt 1985; Satchell 1984).

Toxicants may or may not affect chloride cells. Some studies have reported that chloride cell numbers increase, decrease or do not change at all during chronic toxic stress exposure (Wendelaar and Lock 1992).

1.1.5.2 Alterations in gill function

Gill function may be altered by chemical stressor agents. In general, alterations frequently observed when fish are exposed to different kinds of toxins are: passive ion loss and water uptake in fresh water fish; an increase in passive ion influxes and water loss in seawater fish, and inhibited active ion exchange in both seawater and freshwater fish. These effects are associated with disturbed plasma homeostasis and acid-base balance (Evans 1987; Wendelaar Bonga and Lock 1992). Some toxic water pollutants and acidified water may

interfere with or impair oxygen uptake. This has been attributed to alteration of the branchial epithelium which may cause hemodynamic malfunction and clogging of the branchial cavity by accumulation of mucus (Evans 1987; McDonald 1983). Lamellar clubbing and thickening, or hyperplasia, of the lamella epithelium produced by some chemical irritants may also interfere with O_2 transport, since transfer of oxygen across the gill surface is directly proportional to its area and inversely proportional to its thickness (Hughes 1984).

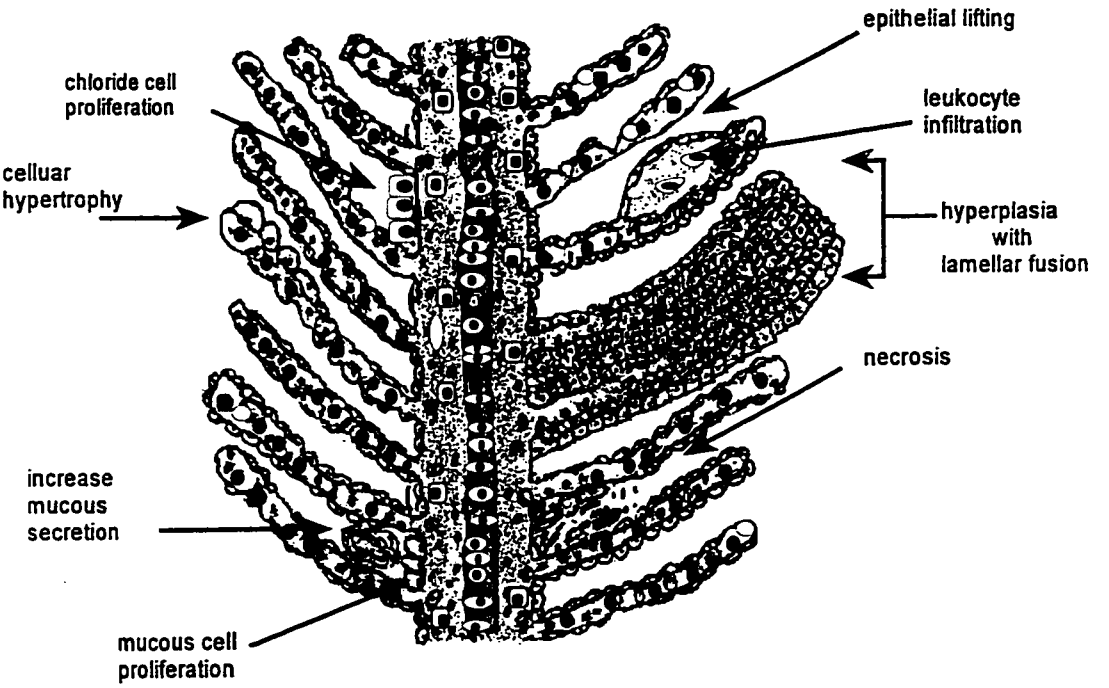


Fig. 1.2 Schematic depicting common irritant-induced gill lesions. Modified from Mallatt (1985).

1.2 Therapeutants in fish diseases

The use of therapeutic drugs is one of the most important current issues in aquaculture operations in North America. A major problem is the lack of approved drugs to diminish disease-related mortality and enhance production efficiency and product quality in both public and private aquaculture facilities. Public interest about human food safety, human health, and environmental effects have resulted in increasingly strict implementation of regulations by the U.S. Food and Drug Administration (FDA).

There are only a few therapeutic treatments that are currently approved and available for fish food in Canada (Armstrong 1994). As a result, fish farmers sometimes use unapproved drugs, general purpose chemicals that are not tagged for drug use or use approved drugs in a manner that differs from the labelled instructions. These practices sometimes result in therapeutant toxicities (FDA 1996). Aquaculture drugs approved in Canada and the United States are listed in Table 1. Unregulated drugs that are used in aquaculture fish may sometimes be a potential human health hazard. These substances can have negative effects on consumers as these drugs may be carcinogenic, allergenic, and/or may contribute to the increase in antibiotic resistant organisms.

Chemical treatments by bath are routinely prescribed for many fish diseases. Some of the more commonly used chemicals used in bath treatment protocols include formalin (37% formaldehyde), chloramine-T (sodium para-toluene-sulphonchloramide), concentrated quaternary ammonium compounds, salt, copper sulphate, organophosphates, malachite green and sodium chloride (Herwig 1979; From 1980; Salte et al. 1987; Speare and Ferguson 1989; Cusack and Johnson 1990; Grave et al. 1990; Thorburn and Moccia 1993).

Table 1. Approved Aquaculture Drugs in Canada and the USA

Canada	USA
- Oxytetracycline	- Formalin solution
- Sulfadimethoxine/ ormetoprim	-Tricaine methanesulfonate (MS-222)
- Sulfadiazine/trimethoprim	- Oxytetracycline
	- Sulfadiazine/trimethoprim

1.2.1 Adverse effects

Many side effects may result from the use of chemical treatments, such as physical damage to the gill and skin and loss of the protective mucus (Piper 1982).

The use of malachite green is no longer allowed in fish culture because it is a potential teratogen (Meyer and Jørgenson 1983; Alderman 1985). Use of sodium chloride continues; however there are disadvantages when using salt eg,. transporting and storing the large quantities that are needed for treatment (Rach et al. 1997). Formalin, which is used as a bath treatment to control parasites and fungal infections, has been reported to cause marked gill pathology in rainbow trout when used at therapeutic levels (Smith and Piper 1972). Because of its toxicity, there are concerns associated with its discharge into the environment after treatment. The use of quaternary ammonium compounds in salmonids at levels just above suggested therapeutic levels has been associated with gill pathology and ionic disturbances (Hoskins and Dalziel 1984; Byrne et al. 1989; Abel and Skidmore 1975; Mallatt 1985). Chloramine-T, a topical antimicrobial (Scott 1993) may produce serious gill damage when used as a high-dose dip. Weekly exposure of rainbow trout to chloramine-T has been shown to cause a dose-related reduction in growth rate and increased susceptibility to different types of dermatitis (Powell et al. 1994; Sánchez et al. 1996). Copper sulphate is used in fish farming as a treatment for ectoparasite infections. Dips and baths are used to administer this compound and have been shown to produce a serious astringent effect and remove mucus from gills (Scott 1993). Dichlorvos, an organophosphate used in controlling sea lice, has been shown by Høy et al. (1991) to significantly reduce acetylcholinesterase levels in the treated fish.

1.2.2 Hydrogen peroxide

Hydrogen peroxide is a chemical disinfectant agent which has currently used in salmonids in both fresh and salt water aquaculture. It is used as a treatment to remove sea lice (*Lepeophtheirus salmonis*) in Atlantic salmon (Thomassen et al. 1993; Bruno and Raynard 1994) and to treat bacterial gill diseases in fresh water-rearing facilities (Roberts 1995). Hydrogen peroxide has also been found to be effective in controlling fungal infections in fish eggs (Marking et al. 1994) and is considered an excellent candidate for controlling fungal dermatitis in chinook salmon (Fitzpatrick et al. 1995).

When comparing hydrogen peroxide with traditional therapeutants, this chemical offers numerous benefits when used in hatcheries. Hydrogen peroxide is environmentally safe because the compound degrades into water and oxygen, and it is relatively safe for users because no harmful fumes are released when it is applied (Rach et at. 1997). Because of its breakdown to water and oxygen, the FDA has classified hydrogen peroxide as a drug of "low regulatory priority" when used at low concentration levels (of up to 500 ppm) to control fungal infections on all species and life stages of fish, including eggs (Beaulieu et al. 1992).

Bath treatments are used to administer this chemical, providing an inexpensive method to treat a large number of fish in a short period of time. Fish do not need to be manipulated, avoiding handling stress. The withdrawal of the medication after the treatment is achieved by restoring the flow of the water and allowing it to flush the tank (Herwig 1979; Scott 1993).

It has been hypothesized that the mechanism of action of hydrogen peroxide is due to its reactivity and breakdown to release reactive oxygen species and molecular oxygen

which are acutely toxic to parasites (Bruno and Raynard 1994). The hydroxyl radicals, which can be generated from hydrogen peroxide, can cause peroxidation of lipids in cellular and organelle membranes, cross-linking of proteins leading to enzyme inactivation, as well as inactivation of DNA replication (Cotran et al. 1989). Others have suggested that hydrogen peroxide induces mechanical paralysis caused by the formation of gas bubbles in the gut and hemolymph of lice in salt water (Thomassen 1993; Bruno and Raynard 1994). As for the target-animal safety of this chemical in production settings, few studies have addressed the effective dosages, reporting that this chemical may be harmlessly used for fish in concentrations up to 500 ppm (Beaulieu et al. 1992). On the other hand, it is reported that when hydrogen peroxide is used at higher concentrations it may cause serious lesions to the fish gills, such as extensive epithelial lifting and necrosis causing respiratory distress or even death (Thomassen et al. 1993; Bruno and Raynard 1994; Arndt et al. 1997; Johnson et al. 1993). The toxicity of hydrogen peroxide varies among species and is dependent on the condition of the fish, life stage, and water temperature (Rach et al. 1997).

1.3 Stress in fish

The stress response is a mechanism which has resulted under natural selection pressures to enable any animal to cope with a potentially hostile environment (ie. physical disturbance, prophylactic treatments, overcrowding, water quality deterioration, intense social interaction etc.). The study of stress physiology in fish is well established and it is known that the stress response in teleost fish is very similar to that of the mammals (Barton et al. 1980; Pickering 1992).

Stress can cause multiple changes in hormone balance, which generally invoke the activation of two components of the neuro-endocrine system: 1) the sympathetic-chromaffin system, resulting in the secretion of the catecholamines, adrenaline, noradrenaline and dopamine; and 2) the hypothalamic-pituitary-interrenal (HPI) axis, resulting in the release of cortisol which is the principal corticosteroid in salmonids (Pickering 1992).

According to Selye (1936), all these changes are needed to return the body to homeostasis. Variations in the hormonal balance are accompanied by several secondary effects, including an increased susceptibility to diseases (Wedemeyer 1970; Snieszko 1974).

1.3.1 Acute stress

The natural stress response can been seen as an acute response which has developed to enable the fish to mobilize its energy reserves as it attempts to overcome the immediate stressor. Acute stress caused by capture, handling, transport, hypoxia, osmotic and temperature shocks, social stressors or exposure to water pollutants has often been reported to result in a rapid rise in muscle and plasma lactate and decreased blood pH and oxygen content. All these changes are associated with the release of catecholamines from the chromaffin cells and are followed by a rapid rise in ventilation, branchial blood flow, gas exchange and increase of blood glucose levels (Mazeaud et al. 1981; Pickering and Pottinger 1995; Witters et al. 1991).

1.3.2 Chronic stress

A chronic stress is one which is continuous over a prolonged period of time. In this

case, plasma cortisol may remain elevated producing damaging effects on the fish's state of health by increasing its susceptibility to diseases, suppressing growth rate, and interfering with reproductive processes (Espelid et al. 1996).

1.3.3 Types of stress response

The physiological responses to stress occur at three levels: primary, secondary, and tertiary stress responses (Adams 1990; Barton and Iwama 1991).

1.3.3.1 Primary stress response (the endocrine system)

The primary stress response is mediated by the activation of HPI axis and is characterized by an increase of plasma levels of catecholamines, ACTH and cortisol (Barton and Iwama 1991). Cortisol levels are generally used to monitor the primary stress response because this hormone is easily detectable and its rise and fall is a relatively direct measurement of the severity and duration of the primary stress response (Wedemeyer et al. 1990; Barton and Iwama 1991).

1.3.3.2 Secondary stress response

The secondary stress response results from the physiological effects of factors released during the primary stress response. Secondary changes are characterized by increased plasma glucose, alterations in hematocrit, leucocrit, red blood cell count, and hydromineral balance (Barton and Iwama 1991; Iwama et al. 1995).

As a general rule, the metabolic effects of the primary stress mediators remain longer

than do the elevated mediator levels (days vs hours). Hence, the hormone changes caused by stress may be of relatively short duration, but the physiological effects of these hormones will persist for longer periods (Pickering and Pottinger 1995).

Administration of exogenous glucocorticosteroids can cause a reduction in the number of circulating lymphocytes in the peripheral blood of fish (McLeay 1973). Fish exposed to various stressors such as infectious diseases or adverse environmental conditions can also have low peripheral lymphocyte counts.

1.3.3.3 Tertiary stress response

The tertiary or whole animal stress response is measured by parameters such as abnormal behaviour, increased metabolic rate, poor feed consumption, poor feed conversion efficiency and reduced growth rate, increased incidence of diseases and mortality rates, decreased resistance to infectious disease and disturbance in reproductive success (Espelid et al. 1996). All these changes indicate that unfavourable environmental conditions have exceeded the tolerance limits of fish (Wedemeyer et al. 1990). Increased cortisol release is the major factor that is responsible for the damaging effects of stress on survival, growth and reproduction (Pickering 1992). Cortisol has a catabolic action which is responsible for the mobilization of energy reserves by gluconeogenesis and lipolysis, resulting in a net catabolic effect and a reduction in the growth rate (Pickering 1990).

1.4 Growth in fish.

Growth has been defined as the sum of a series of biochemical, physiological and

behavioural processes which involve the assimilation of consumed food into the deposition of body material (Brett, 1979). This process can be positive or negative, temporary or long-lasting (Busacker et al. 1990).

1.4.1 Reporting fish growth

There are different methods of measurement that researchers use when reporting growth. Absolute growth rate, relative growth rate, and specific growth rate are the most common methods used (Hopkins 1992). Each of these measurements is a numerical representation of growth which can be used for various purposes including: 1) statistical evaluation of the effects of various treatments on growth; 2) presentation of growth data in a standard format which allows comparison of growth in different experiments; 3) providing the basis for management decisions (for example, when assessing how long it will take to grow fish under various conditions) (Hopkins 1992).

Measurements of growth expressed in terms of some intervals of time (day, month, year) constitute a growth rate. Relative growth and relative growth rate frequently are multiplied by 100 and expressed as a percentage. These calculations imply a linear growth rate over the time interval of interest.

Growth rates of fish are typically found to increase exponentially over short intervals of time, requiring that growth be expressed as an instantaneous rate (Busacker et al. 1990). This measurement has also been called the intrinsic, exponential, or logarithmic growth rate (Ricker 1979). The specific growth rate (SGR) is the difference between the natural logarithms of successive weights over a period of time. The SGR is particularly useful for

reporting growth of small fish at intervals for less than a year, since, in short intervals, it is considered that growth of young fish is exponential (Ricker 1979; Busacker et al. 1990; Hopkins 1992). The following formula is used to calculate the specific growth rate:

$$SGR = [(Ln\ wt - Ln\ wi) / t] \times 100$$

Where:

Ln wt = natural logarithm of the final body weight
Ln wi = natural logarithm of the initial body weight
t = length of the culture period
100 = express the result as SGR in %

This equation for SGR assumes that fish weight increases exponentially. This assumption is valid, as mentioned above, for most young fish cultured for short periods, but it is not valid for larger fish or longer culture periods.

1.4.2 Factors affecting growth

A variety of modifying factors, both biotic and abiotic, exist which alter feeding and growth patterns of fish during growth studies. It is essential that these factors be considered during the design of an experiment in which growth is evaluated to ensure that the resulting data be easily interpretable and comparable with values from other studies.

1.4.2.1 Abiotic Factors

Temperature: Temperature represents the major rate-controlling force regulating

growth (Jobling 1994). In most fish, metabolic activity is greatly stimulated as water temperature is increased (Elliott 1976), resulting in an exponential relationship of required maintenance ration with temperature.

Light/Photoperiod: It is believed that light stimulates the pituitary gland via endocrine and sympathetic systems inducing the production of growth hormone. Hogman (1968) was able to show that increased growth occurring during spring was best correlated with increasing day length and not with elevated water temperature. Photoperiod regime has also been shown to influence schooling behaviours, likely altering feeding patterns (Weber and Spieler 1994).

Salinity: Salinity is an abiotic factor that may have different effects on growth depending on the species of fish. Salmonids are able to tolerate increased salinity levels during smoltification, a period associated with high growth capacity. In comparison, Atlantic salmon parr (pre smolt) had an increased maintenance ration requirement and reduced conversion efficiencies during sea-water acclimation compared to freshwater (Shaw et al. 1975).

Oxygen: Increased food consumption has been shown to result in elevated oxygen consumption. Interestingly, exposure for only short periods of time to low-levels of oxygen resulted in decreased growth rates (Brett 1979).

1.4.2.2 Biotic factors

Feeding regime: Feeding regime can greatly contribute to the feeding and growth potential of the fish. Generally, feeding fish several times throughout the day results in

greater rates of food consumption than if fish are only fed one maximal ration daily (Cho 1992; Lanno 1989).

Fish density: Stocking density is another factor which may affect growth in some species. For example, high densities reduced feeding activities and growth rate in coho salmon, brook char and rainbow trout but have a positive effect on these parameters in Arctic charr (Alanärä and Brännäs 1996). Establishment of social hierarchies at low fish density may be developed. Dominant fish will typically feed more aggressively than subordinate fish resulting in large differences in the growth rate data. This problem may be further complicated if food ration is limited (McCarthy et al. 1993).

1.4.3 Growth and disease

As with all animal production systems, infectious and non-infectious diseases in cultured fish can cause negative effects in growth performance which is a clinical sign frequently observed during disease. A poor growth performance is a consequence of a variable number of factors during disease, including appetite, stress response, food absorption and metabolic rate.

1.4.3.1 Appetite

Subclinical (asymptomatic) diseases may reduce or completely inhibit appetite. Since lack of appetite is a sign common to many diseases, it is probably one of the most important factors initiating a poor growth performance. Reduced appetite and food intake are considered to be the principal causes of the growth suppression in brown trout under

crowded conditions (Pickering and Stewart 1984) and the main cause of reduced growth in subordinated eels (Peters and Hong 1985).

1.4.3.2 Stress

Stress is often associated with outbreaks of fish diseases. When fish are under conditions of stress, suppression of a series of hormones involved in the regulation of growth, such as growth hormone, insulin, thyroid hormones, and gonadal steroids, may be affected negatively by factors released during these conditions. Catecholamines and cortisol are the main hormones released during periods of stress and are associated with inhibition of growth (Pickering 1990). The growth suppression caused by cortisol is due to its catabolic action which is responsible for the mobilization of energy reserves by gluconeogenesis and lipolysis (Pickering 1990).

1.4.3.3 Food absorption:

The role of the digestive tract in the conversion of food into a living tissue is an important factor in fish nutrition. Diseases that directly and indirectly affect the digestive system may cause nutrient losses and consequently poor growth. Reichenbach-Klinke (1975) reported that malachite green blocked intestinal enzymes affecting feed conversion efficiency in fish treated with this chemical.

1.4.3.4 Metabolic rate:

Acute and chronic stress are typically associated with increased metabolic rate.

Increased metabolic rate is further reflected by stimulated respiratory movements and oxygen consumption. A 20% increase in respiratory rate was associated with a reduction of 8-18% in growth rate of coho salmon (Vaughan et al. 1982).

1.4.3.5 Feed conversion efficiency:

The efficiency of food conversion or food utilization, which is the ratio of food intake to the output of animal product, may be altered by several factors. For example, the amount of nutrients absorbed by an animal depend on the level of intake and efficiency of digestion. Different drugs such as malachite green may interfere with efficiency of digestion by blocking intestinal enzymes (trypsin and amylase) causing a poor conversion efficiency (Reichenbach-Klinke 1975; Gerundo et al. 1991). Many diseases may directly or indirectly affect the digestive system causing multi-functional disorders. Diseases such as nocardiosis or mycobacteriosis frequently result in the formation of multifocal granulomas in the lamina propria or sub-mucosa causing malabsorption and nutrient losses (Ferguson 1989).

1.4.4 Growth and thyroid hormones

The thyroid system of teleost fish is usually believed to conform to that of mammals in that the thyroid gland secretes primarily L-thyroxine (T_4) in response to thyroid stimulating hormone (TSH), whose pituitary release is regulated by hypothalamic hormones, including thyrotropin releasing hormone (TRH). Thyroid hormones are essential for the function of every cell in the body since they help to regulate their growth and the rate of cell metabolism.

In mammals, over 99% of T_4 binds to plasma proteins, but free T_4 enters cells and is deiodinated by a T_4 5'-monodeiodinase (5'D) to several iodothyronines including 3,5,3' triiodo-L-thyronine (T_3) in the liver and possibly in other peripheral tissues (Shields and Eales 1986). In comparison to mammals, a large proportion of plasma T_4 exists as free thyroxine in fish (Allen 1977).

The active hormone in teleost fish is believed to be T_3 since it binds strongly to nuclear receptors promoting mRNA and protein synthesis, with an anabolic effect on somatic growth and protein metabolism in fish (Higgs et al. 1982; Eales 1990).

1.4.4.1 Extrathyroidal factors altering thyroid hormone metabolism and circulating thyroid hormone concentrations

In humans, a wide range of clinical conditions such as chronic starvation or malnutrition, surgery, diabetes mellitus, hepatic and renal diseases, and other chronic systemic illnesses may result in decreased serum T_3 concentrations. This situation results from the inhibition of 5'-D. The reduction in the production of T_3 appears to be a beneficial adaptive mechanism by which the body limits the loss of protein and perhaps blunts the metabolic rate during disease (Utiger 1980).

When animals are faced with a situation of negative energy balance, a decrease in circulating thyroid hormones occurs as a protective mechanism by the body. In acute and severe illnesses, both T_4 and T_3 concentrations may fall precipitously (Wartofsky et al. 1982; Faber 1987; Kaptein 1982). Several pathophysiologic mechanisms may contribute to this low T_4 state. Impaired serum protein binding of T_4 caused by an endogenous inhibitor of

binding (such as fatty acids) or a reduction in binding protein concentrations results in reduced total serum T_4 concentrations (Leatherland 1994). Thyroxine concentrations are also related to testosterone, season, time of day and nutritional conditions (Hunt and Eales 1979; Milne et al. 1979).

1.4.4.2 Effect of stress on thyroid hormones in fish

Some studies have demonstrated that chronic environmental stress causes a suppression of thyroid activity in teleost fish (Brown et al. 1991). It is not known whether such effects of stress on the thyroid system are direct (cortisol effect), or if they are a reflection of the reduced food intake normally associated with chronic stress. Starvation is known to suppress thyroid functions in almost all teleosts studied since the pituitary-thyroid axis may be very sensitive to the nutritional status in these species. Reduced food intake also reduced the generation of T_3 from T_4 (Milne et al. 1979).

Assessing the correlations between growth rates and plasma T_3 and T_4 concentrations may give useful information about thyroid function and nutritional status of fish.

1.5 Rationale for research

Therapeutic use of different chemical bath treatments in fish have been associated with several negative effects on fish health. A reduced growth performance may be the result of chemical exposure (Powell et al. 1994; Sanchez et al.1996). Histological evidence of severe gill and skin damage and some evidence of biochemical alterations have been reported with the use of many chemotherapeutants (Herwig 1979; From 1980; Salte et al. 1987;

Speare and Ferguson 1989; Cusack and Johnson 1990; Grave et al. 1990; Thorburn and Moccia 1993).

Hydrogen peroxide is a disinfectant which is currently being used in aquaculture. It is environmentally compatible and, when used as a therapeutic bath, has successfully been used to treat different topical infections (low dose levels) (Beaulieu et al. 1992; Marking et al. 1994). Its effects on fish have been partially examined (Powell and Perry 1997). However, little work has been done to investigate the effects of higher doses of hydrogen peroxide which are used for sea lice treatment, on the growth performance and clinical pathology of fish. Although these treatments are commonly applied to marine cultured fish, we are using a fresh water model. This was proposed since the development of a treatment model that most closely simulates culture situation involves a large number of constraints. Additionally, conducting a study in salt water would be prohibitively expensive compared to that in fresh water.

1.6 Objectives

This study was designed to achieve the following objectives:

1. To assess growth performance in rainbow trout after exposure to a high dose of hydrogen peroxide (simulating a sea lice treatment episode) in fresh water.

2. To use this exposure model to assess clinical chemistry and hormonal parameters in treated rainbow trout.

3. To identify potential mechanisms through which hydrogen peroxide suppresses growth (assuming the hypothesis is confirmed).

4. To assess the degree of damage, if any, to the gills of rainbow trout after exposure to a high dose of hydrogen peroxide.

2 EFFECTS OF HYDROGEN PEROXIDE ON GROWTH PERFORMANCE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*).

2.1 ABSTRACT

Short duration hydrogen peroxide bath treatments, at concentrations ranging from 1250-1500 ppm, are used to treat net-pen reared salmonids infested with sea lice. The effect of this treatment on fish growth performance is not known. In order to develop an understanding of the interaction of this treatment with salmonid growth performance, a treatment simulation model was used, based on pilot study results, in which juvenile rainbow trout (*Oncorhynchus mykiss*) reared in fresh water received a single exposure to hydrogen peroxide at 1250 ppm for 30 min. The growth performance before and after treatment was compared with untreated fish held in separate tanks. In trial 1, treated fish after one week had a significantly ($p < 0.01$) depressed specific growth rate (SGR) through a significant ($p < 0.05$) interference with feed conversion ability, with no significant effect on appetite. In the subsequent two weeks, the SGR of treated fish was less than that of control fish, but the difference was not statistically significant. The effect on growth rate was less dramatic than that which had been observed in a pilot study which was based on a cohort design in which treated fish co-habitated with untreated fish. Therefore, a second growth trial was designed to determine whether the negative effect of hydrogen peroxide treatment on growth performance is more pronounced when treated fish co-habitate with untreated controls. Curiously, however, the opposite effect was found. Similar to trial 1, those fish which were kept in separate tanks had a significantly ($p < 0.001$) reduced SGR after treatment. However no significant effect

on growth was noted in those groups of fish reared with untreated cohorts. Based on the typical use-pattern of hydrogen peroxide treatment for sea lice in which entire netpens of salmon are treated, it was concluded that there is potential for a period of post-treatment growth suppression. The differences noted between the two study-design approaches are intriguing, and possible implications of these findings for future growth trial studies are discussed.

2.2 INTRODUCTION

Hydrogen peroxide is a strong oxidizing compound which has been widely used as a therapeutic agent in salmonids in both fresh and saltwater aquaculture in recent years. Hydrogen peroxide (50 to 250 ppm, for up to 60 min exposure) has recently become a popular treatment for fungal dermatitis and bacterial gill diseases in freshwater-rearing facilities (Rach et al. 1997; Roberts 1995). Higher dose levels (up to 1500 ppm for 20 min) are used to remove sea lice (*Lepeophtheirus salmonis*) from Atlantic salmon in saltwater (Thomassen et al. 1993; Bruno and Raynard 1994).

It is thought that the mechanism through which hydrogen peroxide works as a therapeutic agent is due to hydroxyl radicals which are released during the treatment. These oxygen species may cause acute toxicity to parasites (Bruno and Raynard 1994). Others have suggested that hydrogen peroxide induces mechanical paralysis caused by the formation of gas bubbles in the gut and hemolymph of lice (Thomassen 1993; Bruno and Raynard

1994).

Hydrogen peroxide is a low priority regulatory drug that is considered safe for fish and eggs when used for up to 500 ppm with no specified application time limit (Beaulieu et al. 1992). However, it is known that higher concentrations of H_2O_2 may cause serious branchial injury in salmonids (Thomassen et al. 1993; Bruno and Raynard 1994; Kiemer and Black 1997).

It has been reported that the LD_{50} of hydrogen peroxide for rainbow trout is about 500 ppm between 17 and 22°C for 60 min, but the toxicity depends on several factors which include condition of the fish, life stage, and water temperature (Rach et al. 1997). Although toxicity of a compound is generally assessed based on death losses during or following exposure, subclinical effects of commonly used chemotherapeutics can be of importance and are generally not fully recognized in fish farming.

Recently, with the increasing problem of sea lice infestations of marine net pen reared salmonids and the environmental concern regarding excessive use of organophosphates, hydrogen peroxide treatment has gained popularity as a means of lice control (Bruno and Raynard 1994; Johnson et al. 1993). However, our understanding of this compound's effect on salmonids is limited. The high concentrations used (up to 3000 ppm/20 min) are known to have detrimental effects on the gills of treated fish (Thomassen et al. 1993; Bruno and Raynard 1994; Kiemer and Black 1997). However, little is known about the effect of these treatments on subsequent growth performance of fish. A study by Speare and Arsenault (1997) provided evidence that exposure of juvenile rainbow trout to hydrogen peroxide twice weekly at low dose levels (200 ppm) for seven weeks leads to a reduction in growth rate

through a negative effect on feed conversion efficiency. This study, which simulated prophylactic use patterns in hatcheries, is of a considerably different design compared to one which would probe the effects of a single high dose treatment for sea lice.

The purpose of the present study is to characterize the effects of a single, high-dose hydrogen peroxide exposure (simulating that used in sea lice treatment) on the growth performance of *ad libitum*-fed juvenile freshwater-reared rainbow trout in a controlled laboratory setting which permits assessment of specific growth rates, appetite, and feed conversion index calculations before and after treatment. Two trials were conducted to determine whether a cohort design, in which treated fish co-habitate with untreated fish, yields different results from a trial design in which treated and control fish are held in separate tanks.

2.3 MATERIAL AND METHODS

2.3.1 Pilot study

Because the use patterns of hydrogen peroxide in sea lice control are largely anecdotally reported (ie. precise determination of both dose and exposure time are lacking), a pilot study was designed using doses within the range of anecdotal reports to gain preliminary data from which the major trials would be based. Eighteen tanks containing 35 rainbow trout (30.4 g average weight) each were utilized. The volume of each tank was 78 L, yielding a stocking density of 12.5 kg/m^3. Water temperature was 10°C. Five fish from each tank were exposed to one of three different concentrations of hydrogen peroxide (1500

ppm, 1250 ppm, 1000 ppm) and the remaining 30 fish in each tank were used as controls. Exposure was limited to one 30 minute bath and the specific growth rate (SGR) from each group was calculated every 21 days (a total of 3 measurements and compared to the cohort controls). Results from the pilot study showed that significant growth suppression persisted for 42 days post exposure when 3 different high dose levels were used. However, the dose with the most marked growth suppression was 1250 ppm (Speare et al. 1998) . The results from this pilot study were used to choose the dose of hydrogen peroxide used in the subsequent studies. Based on this pilot study, two growth trials were carried out to evaluate the growth performance of rainbow trout: one in which treated fish were held in separate tanks from control fish, as well as a trial in which treated fish were held in the same tank as control fish.

2.3.2 Growth trials

2.3.2.1 Experimental design

Rainbow trout (*Oncorhynchus mykiss*) were purchased from a commercial hatchery in Prince Edward Island, Canada.

Trial 1: Fish with an average weight of 30.3 ± 9.2 g ($\bar{x}$ ±SD), and length of 12.8 ± 1.4 cm were allocated to each of 10 circular fibreglass tanks. Each tank contained 30 animals. The total tank volume was 100 L. However, the tanks were filled only with a total volume of 78 L, creating an initial stocking density of 13.5 kg/m^3. Tanks received 12 h of light and 12 h of darkness. Fresh water temperature was maintained at 10°C. The water flow rate for each tank was maintained at 2.5 L/min by adjusting the flow valves. Flow was

assessed by measuring the time taken to fill a 2-L measuring device. The oxygen levels (measured with an oxygen meter) were above 7.6 mg/L during the whole growth trial.

Trial 2: Fish (average weight 34.7 ± 7.8 g), were allocated to one of 6 tanks so that each tank contained 20 animals. The system consisted of 6 circular fibreglass tanks containing each 78 L yielding a stocking density of 9.1 kg/m^3. Temperature, water flow rate, oxygen levels, hours of light and methods of monitoring were similar to those for trial 1. Prior to experimentation, all fish were anaesthetized with benzocaine (40 mg/L), weighed, and marked for identification by using individually numbered plastic labels implanted on the dorsal fin.

2.3.2.2 Allocation of treatment

Trial 1. The 10 tanks were allocated to 5 treatment and 5 control tanks. Each exposure tank was paired with an adjacent control tank to ensure that they would be subjected to the same ambient effects (Appendix A). This allocation was done because it has been reported that growth indices may be affected by the location of the tank (Speare et al. 1995). After allocation of tanks, fish were acclimated for three weeks to the trial conditions, including water temperature, photoperiod, feed and feeding regimens. The trial lasted 12 weeks.

Trial 2, consisted of 2 treatment tanks and 2 control tanks for the separate study and 2 tanks for the cohort. There were 20 fish in each tank (for the cohort study there were 10 control and 10 treated, see Appendix A). The trial lasted 8 weeks.

2.3.2.3 Feeding

The fish were carefully fed by hand to satiation once every morning using a commercial ration (High Pro Grower, pellet size # 3, Corey Feed Mills Ltd, Fredericton, NB). The proximate analysis (dry weight basis) of the feed is shown in Appendix B. Fish were fed every day and food was withheld the day before weighing. Feeding fish 6 days a week does not affect their growth performance (Cho 1992). The trial was 'single blind', since the person feeding the fish was unaware of treatment allocations. The order of tank feeding was different each time to avoid artifacts created by feeding sequence on appetite or anticipatory stress. Fish were observed for satiation; food was constantly offered to the fish until pellets were noted by the feeder to fall to the tank bottom. The amount of unconsumed feed was therefore negligible. The tanks were flushed after every feeding by lifting their standpipes and draining the tank for approximately 10 sec. The amount of food consumed every week was recorded.

2.3.2.4 Hydrogen peroxide assay

The final concentration of hydrogen peroxide was determined once the dilution was made by using a potassium permanganate ($KMnO_4$) titration (Douglas and Donald 1982). A solution of 0.333 ml of the water containing hydrogen peroxide was diluted in 100 ml of distilled water, and 5 ml of sulphuric acid (20%); this solution was thoroughly mixed and then titrated with 0.02 M $KMnO_4$. The concentration of hydrogen peroxide was determined and the results expressed in parts per million (ppm).

2.3.2.5 Exposure to hydrogen peroxide

After a 3 wk acclimation period, fish were exposed once to hydrogen peroxide in a static bath for 30 min for trial 1 and 40 min for trial 2. Exposure time in the second trial was prolonged to 40 min, based on the transient histopathological lesions found in trial 1. It was considered that a longer exposure time may result in more persistent lesions. The treatment was first pre-diluted in a bucket with water and it was added to the tank until a final concentration of hydrogen peroxide of 1250 ppm was obtained. Before the treatment was added to tanks, the inflow of the water was turned off. The inflow of the control tanks was also stopped to simulate conditions in the hydrogen peroxide treated tanks. Aeration was maintained throughout the bath period in both groups. After exposure the tanks were flushed with clean water and the normal water flow resumed. Mortality was monitored throughout the trial following the administration of the treatment. Since in trial 2, many fish died during the treatment, fish numbers were re-balanced to compensate for any post treatment losses (some control fish were treated within 24 h and then mixed with previously treated fish in a tank. Then, without knowing which fish was treated first or later, they were allocated to tanks that had sustained major losses). Appendix C shows the percentage of mortality which occurred in both trials.

2.3.2.6 Collection of growth indices

Fish were weighed during a single day at the end of each week. Whole body weight (g) was measuring by using an analytical balance (Mettler PJ 6000, Zurich, Switzerland).

For trial 1, all fish from each tank were netted and placed in a holding tank with a known quantity of water and weighed. In trial 2, fish were anaesthetized with benzocaine solution (40 mg/L) dried by wrapping them in a paper towel to absorb the excess of water and then individually weighed. Growth performance was evaluated weekly by calculating feed conversion index (FCI)[1], specific growth rate (SGR) [2], and appetite. Appetite refers to feed consumed by fish weekly, expressed as percent (%) of fish body weight.

2.3.3 Statistics

Trial 1: The null hypothesis tested was that treatment with H_2O_2 has no effect on the SGR, FCI, or appetite of fish. For growth trial 1, the unit of study was the tank of fish and not the individual animal. Statistical analysis for SGR, FCI and appetite was calculated for each tank in both groups at each week and compared by a one-way analysis of variance (ANOVA) using Minitab program software to test the null hypothesis. If the ANOVA was significant, Tukey's multiple comparison post test was used to determine significant differences between the two treatments. Values were considered significantly different at the $p \leq 0.05$ level of probability.

---

[1]FCI = (sum FI/N) / (MwT- Mwt)
Where :
sumFI = sum of food consumed during an interval of interest
MwT = mean final body weight for an interval of interest
Mwt = mean initial body weight for an interval of interest
N = number of fish

[2]SGR = [(Ln wt - Ln wi) / t] x 100
Where:
Ln wt = natural logarithm of the final body weight
Ln wi = natural logarithm of the initial body weight
t = length of the culture period
100 = express the result as SGR in %

Trial 2: the null hypotheses tested were: 1) that treatment with H_2O_2 has no effect on SGR when using a design in which controls and treated fish were in separate tanks and 2) that treatment with H_2O_2 has no effect on SGR when using a design in which controls and treated fish were in the same tank. In this second growth trial, the unit of study was the fish since each measurement of growth was made on individual fish. A two-way ANOVA (factors: treatment and time) was used. Differences were considered significantly different at a $p \leq 0.05$ level of probability.

2.4 RESULTS

Behaviour of fish during the treatment

It was observed that the fish exposed to H_2O_2 became agitated 5-10 min after treatment and then fish congregated near the water surface after 10-15 min. This was followed by short bursts of swimming in random directions after 15-20 min. Towards the end of treatment some jumping activity occurred and fish began to lose equilibrium, at which point tanks were rapidly flushed. At the end of the treatment, the treated fish were more lethargic than the controls. However, within 2-3 h they returned to normal activity. Fish were fed the day after a treatment was given. Treated fish initially appeared to consume less than the control ones. However, by 3 days post treatment this was no longer obvious.

Growth indices:

Trial 1.

No significant differences in SGR, FCI or appetite were detected during the acclimation period for fish destined to be controls compared to those destined to receive treatment (Fig. 2.1, 2.2, 2.3 ; Table 2.1). However, in the week which followed the bath exposure to hydrogen peroxide, there was a significant reduction ($p = 0.01$) (difference in means = 24.7%) in the SGR of peroxide treated fish compared to controls. At the same time, a significant increase ($p = 0.05$) in the FCI (difference in means = 26.1%) compared to controls was seen. Growth performance and FCI varied in treated and control fish from week to week. Due to this inherent variation, statistical comparison between weeks was not considered valuable.

The daily food intake dropped during the first and second day post-treatment. However, appetite, as addressed by the amount of feed consumed in the week following treatment, was not significantly affected by treatment (Table 2.2).

Although the SGR of treated fish remained less than that of control fish in the second and third week post treatment (average reductions of 11.1% and 4.6% respectively), the feed conversion index was greater in treated fish compared to controls. These differences were not statistically significant (Table 2.1; Figures 2.1 and 2.2). Beyond the third week after treatment, no obvious trends or effects were noticed.

Trial 2

A significant interaction between treatment and week was found ($p = 0.01$). This indicates that, indeed, the effect of the treatment is time dependent.

Similar to trial 1, when treated and control fish were kept in separate tanks, treated fish had significantly reduced SGR following treatment. In this trial the effect was significant for two weeks ($p < 0.001$) post treatment (31 % reduced during the first week after treatment; 16 % reduction during the second week after treatment) (Table 2.3; Fig. 2.4). When fish were kept in cohabitation with untreated fish, although the SGR of treated fish appeared suppressed, the differences were not statistically significant (Fig. 2.5) . However, when compared to the differences seen in the separate tank study, the treatment-induced changes were considerably less in magnitude (13% reduced during the first week after treatment; 6.8% reduction during the second week after treatment).

2.5 DISCUSSION

The trials completed in this study are the first to demonstrate the extent and the nature of growth performance reduction associated with exposure to a single high-dose hydrogen peroxide exposure. Although these treatments are typically applied to marine cultured salmon suffering from ectoparasitic diseases, our laboratory model utilized healthy, juvenile rainbow trout in fresh water. Therefore, applying our findings to a scenario such as predicting treatment-related growth suppression in sea lice infested Atlantic or Pacific salmon, or even marine culture steelhead rainbow trout, must be done with caution since the actual effect of treatment can be modified by species differences, fish age, interaction of salinity on dissociation rates of hydrogen peroxide, and interaction of disease stages (ie. both clinical and subclinical problems) (Rach et al.1997; Thomassen 1993). Despite this

cautionary note, the advantages of our model system are many and various. These include the ability to accurately control treatment dose and contact time of the treatment chemical with the fish. Secondly, weight-gain and growth rates can be determined at weekly intervals, thus permitting insight into the temporal effects of the treatments on growth. Thirdly, the satiation without waste feeding method can be accomplished in our trial setting, thus allowing accurate and meaningful determination of both appetite and feed conversion values. These latter indices are critical in order to establish further directions to pursue to completely understand the mechanisms through which hydrogen peroxide treatment affects growth.

Although high doses of hydrogen peroxide have been shown to effectively control ectoparasites in salmonids, some researchers have reported that this chemical may have severe pathological effects on the fish. Gill epithelial tissue seems particularly sensitive. Epithelial lifting and necrosis are among the histopathological lesions most frequently reported in fish dying following hydrogen peroxide treatment (Margolis 1993; Johnson et al. 1993). In the present study, mortalities were also observed following therapy. In contrast to previous studies, the focus of our study was to evaluate only effects manifested in surviving fish. It is known that hydroxyl radicals may cause peroxidation of lipids in cellular and organellar membranes, and cross-linking of proteins leading to enzyme inactivation (Cotran et al. 1989) which may help to explain the morphological lesions noted on gills of treated fish. However, neither the precise pathogenic events nor the mechanism(s) that lead to physiological changes, such as reduced growth, have been well characterized.

The data from this study indicated that a single, brief, high dose exposure of hydrogen peroxide (1250 ppm) produces a significant suppressive effect on growth in

rainbow trout when treated and control fish were kept in different tanks. The maximal effect of hydrogen peroxide, according to the SGR, occurred principally during the first two weeks post-treatment. After that period of growth suppression, the effect appeared to lessen over the rest of the trial, suggesting that the effects of the hydrogen peroxide were no longer altering the growth performance. However, a compensatory growth phase did not occur in treated fish during the study period. The development of compensatory growth is important when subclinical effects (growth performance) of chemical treatments are evaluated in a long-term growth trial. By definition, compensatory or, 'catch up' growth, is a phase of rapid growth, higher than control growth rates, associated with adequate feeding and an increased efficiency of food utilization following a period of weight loss (Sumpter 1992; Mortensen and Damsgard 1993). This phenomenon can be economically important with respect to feed conversion efficiency and 'time to market weight' in cultured fish (Dobson and Holmes 1984). The reduced specific growth rates and feed efficiency after a single high dose of H_2O_2 treatment are in agreement with preliminary findings where rainbow trout were exposed to repeated treatments of H_2O_2 at low dose levels (200 ppm) during seven weeks. However, compensatory growth occurred at the end of that growth trial (Speare and Arsenault 1997).

Typically, sea lice management would result in all fish in a netpen being treated. This approach, as shown in this experiment, may have negative effects on the growth performance in the entire population (ie. sick and healthy animals). The actual treatment outcome in an industrial setting cannot be predicted solely by this model since several variables must be considered. Numerous factors are known to affect hydrogen peroxide

treatment outcome. For example, water temperature, water chemistry, fish species, life stage, and time of exposure are components which may dramatically influence the toxic levels and effects of hydrogen peroxide (Thomassen 1993; Johnson et al. 1993; Rach et al. 1997).

In our study, growth suppression resulted when water temperature was at 10°C. If hydrogen peroxide treatment occurs during the summer when water temperature may reach 15°C or even higher, the effect on growth performance may be more dramatic since fish are more susceptible to the toxic effects of this chemical as water temperature increases (Thomassen 1993; Johnson et al. 1993; Rach et al. 1997). Sea lice problems are most noted during warm periods of the year, a time during which salmon growth would be maximal. Therefore, a treatment induced growth suppression during that period is of special concern economically. The effects of hydrogen peroxide treatment in a farm situation may be exacerbated as compared to the single treatment model, since farmers may treat the fish on a repeated basis to keep sea lice at low levels (McAndrew et al. 1998; Treasurer and Grant 1997; Bruno and Raynard 1997; Johnson et al. 1993; Thomassen 1993).

Fish species and health status could also affect treatment outcomes. Healthy rainbow trout were studied in this model. However, during disease or stress periods, fish may be less tolerant to the effects of treatments than are healthy fish. This difference may be accentuated if the species under study are aggressive, such as salmonids (Cutts et al. 1998), causing hierarchical, intraspecific competition. This condition has been associated with marked differences in the growth performance of fish where dominant fish tend to grow faster due to suppression of feeding of subordinated fish (Metcalfe 1994). There are some suggestions that through generations of domestication, rainbow trout may be more adapted to aquaculture

and laboratory rearing than other salmonid species. This tolerance to handling stress may result in differences in responses to stressful situations. Therefore, species differences may be important.

Our results support the hypothesis that hydrogen peroxide decreased growth rate through reductions in feed conversion efficiency rather than by decreased appetite. Several mechanisms through which feed conversion efficiency can be altered have been proposed by previous researchers: a) many diseases may directly or indirectly affect the digestive system causing multi-functional disorders such as malabsorption, nutrient losses or impairment of enzymes needed for digestion (Reichenbach-Klinke 1975; Vignier et al. 1992; Gerundo et al. 1991); b) alteration may occur in hormones that are essential to regulate growth and the rate of cell metabolism such as thyroid hormones (Milne et al. 1979; Brown et al. 1946); c) the stress response has been reported to stimulate the HPI axis resulting in the release of cortisol which has a catabolic action causing the mobilization of energy reserves directly affecting the FCI (Pickering 1990); d) changes may occur in the loss and replacement physiology of tissue and proteins (Powell et al. 1995).

The exact mechanism through which FCI was affected in this research was not determined. However, it is known that H_2O_2 may cause severe pathological effects on gill epithelial tissues including epithelial lifting (lamellar edema) and necrosis (Margolis 1993; Johnson et al. 1993). These alterations may be associated with functional disturbance in the gills which may interfere with both oxygen transport and acid-base regulation in the fish (Powell and Perry 1996). Therefore, rather than converting food into somatic growth, energy consumption is directed towards cellular kinetics trying to compensate for loss of gill

functions and repair damaged tissue. Alternatively, H_2O_2 may activate the stress response raising the levels of cortisol with its associated catabolic effect (Pickering 1990).

In contrast to the separate tank study, significant growth suppression did not occur at any time in the cohort tanks. Close examination of results in the cohort study demonstrated a trend toward decreasing SGR values; however, statistical significance was not present. The latter was an interesting finding because significant differences were expected in the cohort fish. This investigation was conducted based on culture situations where groups of diseased fish are sometimes treated uniformly or may be treated separately and returned to the general population. Social and competitive interaction, such as feeding behaviour and hierarchical standing (Brown 1946; Newman 1956; Noakes and Leatherland 1977), may result within these combined groups in which sub-dominant fish are characterized by a slower growth rate (Li and Brocksen 1977; Metcalfe 1994). There was no evidence of this hierarchical behaviour developing in our study, judging from the comparison of the SGR from the treated vs untreated controls. Also, there was no evidence to suggest the development of changes in fish behaviour or the formation of hierarchical groups based on color changes where dominant fish tend to be lighter than subordinated fish (Newman 1956).

One possible explanation for the lack of identification of significant differences in the cohort study might be the small sample size used in trial 2. In addition, the study design could have served to limit the effect of food competition through feeding to satiation. For example, if a restricted amount of food had been used it is possible that treated fish would have had less opportunity to eat as stronger fish would eat more aggressively than treated

fish resulting in decreased growth performance. It must also be considered that social behaviour may have benefited the treated group by stimulating feeding activity by association with their untreated cohorts. This stimulated feeding could serve to dampen any difference between the groups in growth rate. The effect of cohort design systems requires further study to determine the true effect of social interaction on treated fish.

In summary, results originating from this research demonstrated that a single high dose of hydrogen peroxide will suppress the growth of rainbow trout, specifically in the period immediately following treatment. The reduced growth is associated with an increased FCI rather than reduced appetite. The extent of the effect of this chemical may depend on design methodology of treatment. The separate tank study design showed significant growth suppression in the peroxide treated fish compared to a failure to demonstrate a significant suppression in treated fish kept in the same tank as untreated fish. Specific results may differ with other aquaculture conditions and species of fish being used.

Hydrogen peroxide is an effective chemical agent against many infectious diseases, but fish culturists must be aware of the potential subclinical effects that this treatment may have on the treated fish. Additional research is required to identify the exact mechanism (s) through which FCI may be affected and to determine whether repeated high doses of H_2O_2 further reduce SGR or if fish would develop tolerance to the effects of this chemical. Further studies are also needed to examine if treated fish from a cohort study respond differently than untreated fish maintained in a separate tank. The knowledge thus obtained is important to the aquaculture industry in determining the cost/benefit ratio of therapeutic intervention as well as the most effective method of treatment.

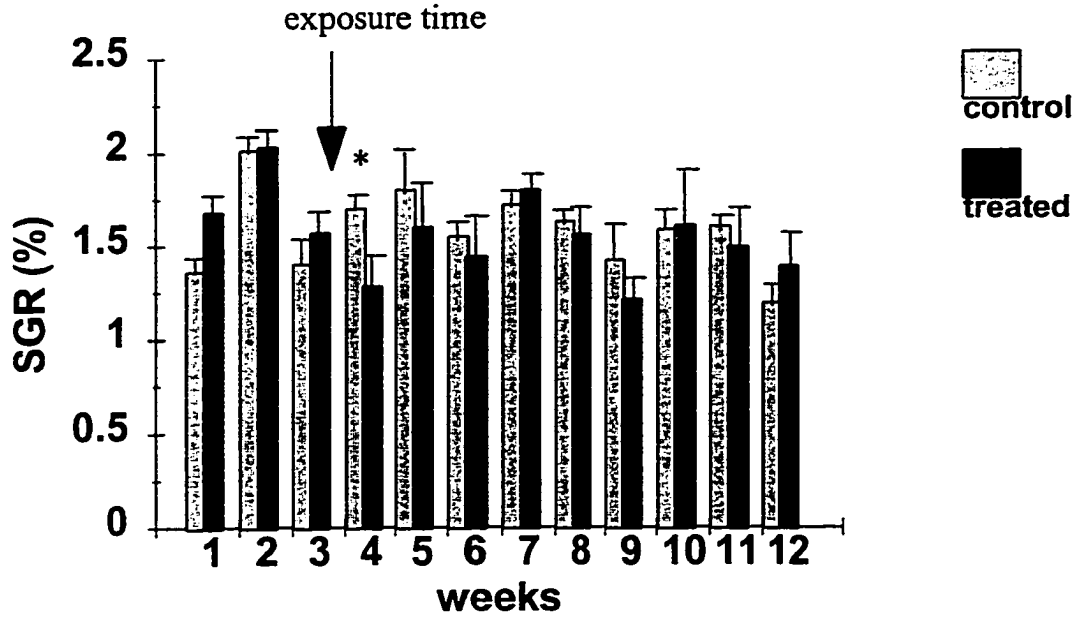


Fig. 2.1 Specific growth rate (SGR) for hydrogen peroxide treated and control fish for each week during the growth trial 1. (Mean + SD), * indicates significant differences between groups.

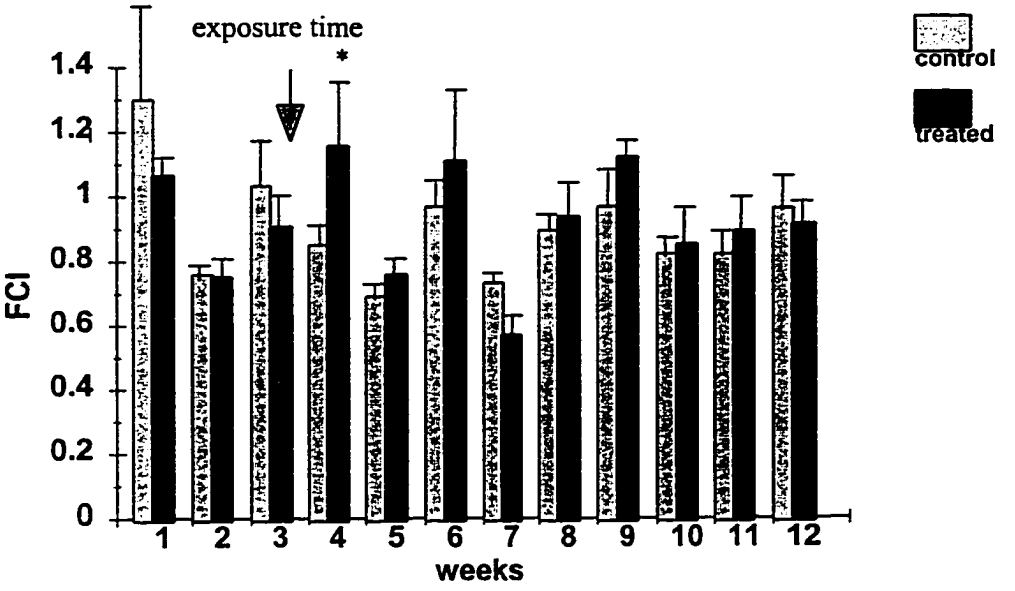


Fig. 2.2. Feed Conversion Index (FCI) for hydrogen peroxide treated and control fish for each week during the growth trial 1 (Mean + SD), * indicates significant differences between groups.

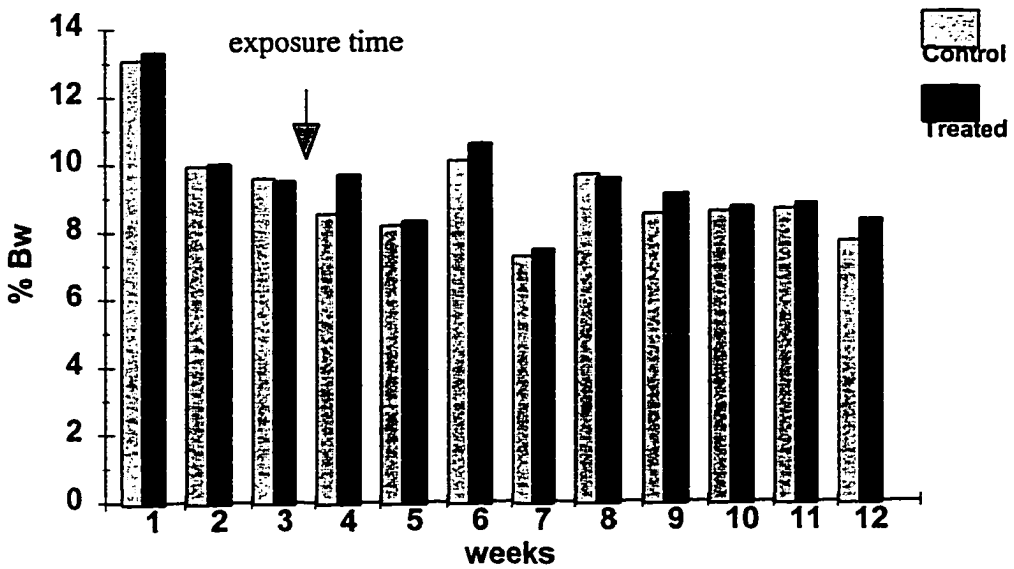


Fig. 2.3. Weekly feed intake for hydrogen peroxide treated and control fish, expressed as the percentage of body weight (%Bw) during the growth trial 1.

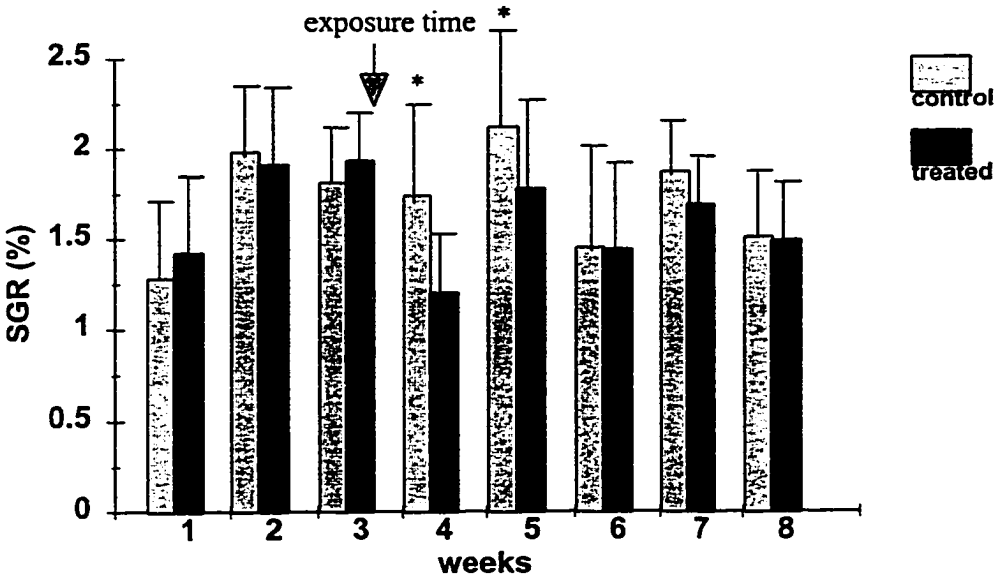


Fig. 2.4. Specific growth rate (SGR) for hydrogen peroxide treated and control fish for each week during the growth trial 2 (separate tanks), (Mean + SD), * indicates significant differences between groups.

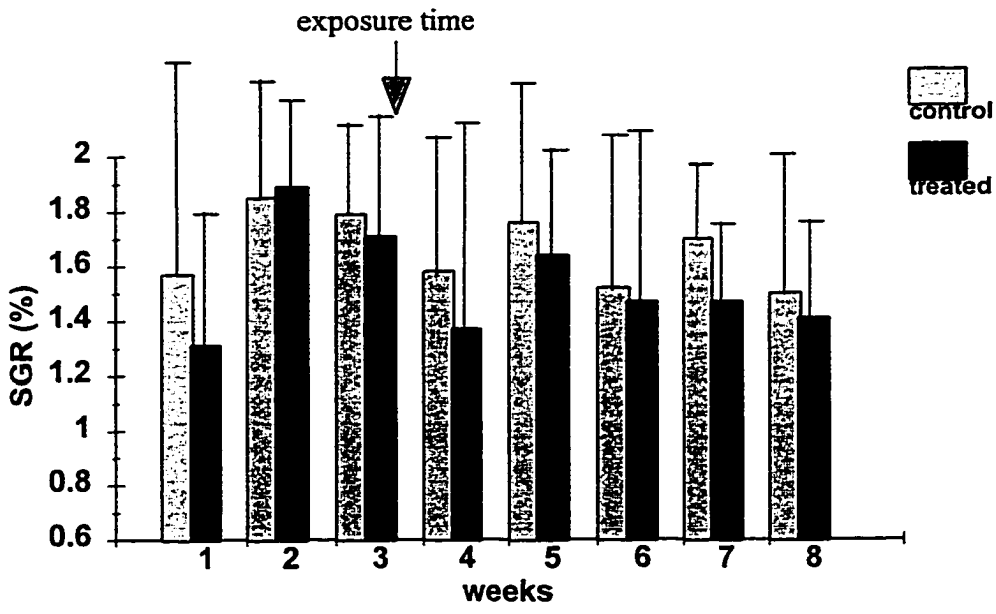


Fig. 2.5. Specific growth rate (SGR) for hydrogen peroxide treated and control fish for each week during the growth trial 2 (cohort study), (Mean + SD).

Table 2.1 Feed Conversion Index (FCI) and Specific Growth rate (SGR)(Mean ±SD) for control and hydrogen peroxide treated rainbow trout. N = 5. Trial 1.

	Feed Conversion Index (FCI)			Specific Growth Rate (SGR%)		
Week	**Treated**	**Control**	**P**	**Treated**	**Control**	**P**
1	1.06±0.06	1.29±0.34	0.21	1.68±0.12	1.36±0.14	0.00
2	0.75±0.07	0.75±0.05	0.86	2.03±0.16	2.01±0.11	0.80
3	0.90±0.10	1.03±0.14	0.15	1.56±0.20	1.39±0.19	0.2
[3]4	1.15±0.24	0.85±0.09	0.05*	1.28±0.22	1.70±0.10	0.01*
5	0.75±0.09	0.69±0.04	0.26	1.60±0.22	1.80±0.25	0.24
6	1.10±0.23	0.96±0.08	0.27	1.44±0.25	1.51±0.11	0.43
7	0.57±0.08	0.63±0.02	0.17	1.80±0.12	1.72±0.07	0.26
8	0.93±0.13	0.89±0.04	0.53	1.56±0.18	1.63±0.05	0.45
9	1.12±0.09	0.96±0.13	0.06	1.21±0.16	1.42±0.22	0.14
10	0.85±0.13	0.82±0.08	0.67	1.60± 0.31	1.58±0.11	0.90
11	0.89±0.13	0.81±0.07	0.32	1.49±0.28	1.60±0.13	0.46
12	0.91±0.11	0.96±0.13	0.56	1.39±0.23	1.19±0.15	0.17

* Significant differences between groups ($p \leq 0.05$)

[3] One week post-treatment

Table 2.2. Feed consumption (Mean ± SD)for control and hydrogen peroxide treated fish. Mean weekly values are expressed as percentage of the body weight of the fish. N= 5. Trial 1.

Appetite (% of body weight of the fish)			
week	Treated	Control	P
1	13.32±0.95	13.07±1.13	0.71
2	10.00±1.48	9.93±0.63	0.93
3	9.51±0.15	9.58±0.31	0.66
[4] 4	9.69±1.30	8.52±0.57	0.13
5	8.30±1.31	8.18±0.90	0.88
6	10.61±0.62	10.09±0.25	0.15
7	7.46±0.51	7.25±0.48	0.53
8	9.57±0.27	9.67±0.30	0.62
9	9.10±0.89	8.51±0.77	0.30
10	8.70± 0.44	8.57±0.29	0.62
11	8.81±0.42	8.65±0.39	0.54
12	8.32±0.34	7.69±0.89	0.20

[4] One week post-treatment

Table 2.3 Growth trial 2. Specific growth rate (Mean ±SD) of rainbow trout fish used in separate and cohort tanks. N = 40 (separate study) N = 20 (cohort study).

Specific Growth Rate (SGR%)						
	Separate tanks			Cohort tanks		
Week	Treated	Control	P	Treated	Control	P
1	1.35±0.48	1.28±0.48	0.53	1.31±0.48	1.57±0.79	0.21
2	2.29±0.48	2.31±0.44	0.88	2.16±0.37	2.23±0.46	0.61
3	1.93±0.33	1.81±0.33	0.12	1.71±0.54	1.79±0.38	0.59
[5]4	1.20±0.48	1.74±0.61	0.00*	1.37±0.79	1.58±0.51	0.32
5	1.78±0.55	2.12±0.53	0.00*	1.64±0.40	1.76±0.50	0.40
6	1.44±0.51	1.45±0.57	0.92	1.47±0.68	1.52±0.61	0.81
7	1.69±0.29	1.87±0.33	0.06	1.70±0.32	1.76±0.24	0.53
8	1.49±0.40	1.51±0.40	0.80	1.41±0.45	1.50±0.53	0.57

* significant differences between groups ($p \leq 0.05$)

[5] One week post-treatment

3. EVALUATION OF DIFFERENT PLASMA BIOCHEMICAL PARAMETERS, HORMONAL COMPOUNDS AND GILL STRUCTURAL CHANGES IN RAINBOW TROUT (ONCORHYNCHUS MYKISS) AFTER TREATMENT WITH A HIGH DOSE OF HYDROGEN PEROXIDE.

3.1 ABSTRACT

Hydrogen peroxide is a chemotherapeutic agent used in the aquaculture industry at high concentrations as a delousing agent. The effects of treatments at high concentrations on fish physiology has not previously been investigated. A single bath treatment at 1250 ppm for 30 min was used to evaluate the effects of this chemical on blood components and hormones of juvenile rainbow trout *(Oncorhynchus mykiss)*. Serum cortisol, thyroxine, triiodothyronine and plasma glucose concentrations as well as packed cell volume, leucocrit and differential white blood cell count were determined at intervals through 2 weeks after treatment. Serum cortisol and glucose concentrations in treated groups increased significantly ($p \leq 0.05$) 1, 24 and 96 h post-treatment, whereas the leucocrit and absolute number of lymphocytes decreased significantly ($p \leq 0.05$) at 24 h post-treatment. Thyroxine levels were also significantly ($p \leq 0.001$) increased in treated fish (1 h post-treatment) while levels of triiodothyronine were significantly ($p \leq 0.05$) decreased (24 h post-treatment). Hematocrit and absolute numbers of monocytes and granulocytes did not change significantly. Gills were examined at various intervals of time (24 h before treatment, 24 h, 96 h and 1 wk post-treatment) using light microscopy and a Bioquant morphometric system (BQ). Lamellar edema, necrosis, inflammation, epithelial hyperplasia, and fusion of the lamellae were indices evaluated with light microscopy and the thickness of the

blood-to-water diffusion barrier and the thickness of the epithelial layer between lamellae were evaluated with the BQ. Significant increases ($p \leq 0.05$)in the percent of lamellae with necrosis (24 h) and hyperplasia (1 wk) were observed in gills from treated fish. No significant differences were observed for the rest of the indices when compared with control groups. Gill necrosis found in this study was less dramatic than that found in previous studies where hydrogen peroxide exposure was used at similar concentration levels. Epithelial hyperplasia was only found in the treated group which was assessed one week post-treatment. This study shows that H_2O_2 activates the primary (↑ cortisol) and secondary stress (↑ glucose, ↓ leucocrit and ↓ number of circulating lymphocytes) response and causes gill morphological alterations when used at therapeutic dose levels (1250 ppm). Therefore, caution should be taken when using this chemical at high dose levels.

3.2 INTRODUCTION

To maintain the health of the fish in a culture unit, the use of prophylactic and therapeutic chemical treatments are often required. However, the use of pharmaceutical compounds in aquaculture is restricted and only a small number are approved for use (Armstrong 1994; Burka et al. 1997). The use of these agents is closely regulated because some therapeutic agents may have adverse effects on fish and human health in addition to possible negative environmental consequences. Therapeutants reported to cause several side effects in fish include: formalin, chloramine-T, concentrated quaternary ammonium compounds, salt, copper sulphate, and organophosphates (Herwig 1979; From 1980; Salte

et al. 1987; Speare and Ferguson 1989; Cusack and Johnson 1990; Grave et al. 1990; Thorburn and Moccia 1993). Alternative chemical treatments are being pursued to avoid these problems.

Hydrogen peroxide (H_2O_2), a chemical agent gaining acceptance in fish farms, is being used to control bacterial gill diseases, ectoparasites and fungal infections (Roberts 1995). Based on its low aquatic environmental impact and the minor toxic effects to fish when used at low dose levels (up to 500 ppm), this therapeutic agent has been classified as a low regulatory priority agent (Marking et al. 1994; Beaulieu et al. 1992; FDA 1996). Recently, H_2O_2 at high concentrations (1500 ppm) has become a popular treatment with salmon growers for controlling sea lice (*Lepeophtheirus salmonis*) (Thomassen et al. 1993; Bruno and Raynard 1994), an external copepodid which is causing high losses in the salmonid industry (MacKinnon 1997; Chang et al. 1997). Although H_2O_2 is considered to be non toxic to salmon at low doses, evidence of severe gill pathological changes have been reported when used at high concentrations (1500-2000 ppm) (Thomassen et al. 1993; Johnson 1993; Bruno and Raynard 1994; Kiemer and Black 1997). In acute cases, characteristic gill lesions include swelling of the gill epithelium with interlamellar edema or epithelial lifting and extensive epithelial necrosis (Ronney and Wagner 1997; Margolis 1993; Johnson et al. 1993). However, subacute or chronic gill changes have not yet been characterized.

It has been reported that relatively low-dose intermittently applied hydrogen peroxide (200 ppm) exposure of juvenile rainbow trout induces growth suppression which may be associated with induced gill lesions (Speare and Arsenault 1997). Constant physiological

effects on growth performance may result from systemic hormonal and blood chemical alterations or persistent functional alteration of the gills. For example, some chemical treatments may induce a stress response with concomitant release of cortisol (Barton 1980; Pickering 1992). If the stress is severe, hormonal abnormalities may be followed by several secondary effects, including biochemical and physiological changes, such as increased blood glucose and changes in the cellular composition of the blood (Barton and Iwama 1991; Pickering 1995; Wedemeyer 1970; Snieszko 1974). Parameters frequently used to indicate the secondary effects of the stress response are changes in the hematocrit or packed cell volume (PCV) (ie. percent of blood composed of erythrocytes) and the leucocrit (ie. percent of blood composed of leucocytes) (Wedemeyer et al. 1983).

In addition to changes in the blood parameters, it has been reported that morphological alterations in the gills may be seen when different chemotherapeutants are used. Hyperplasia of gill epithelium is a common morphological alteration induced by several chemical agents (Mallatt 1985; Satchell 1984). Functional alterations, such as respiratory impairment, may result as a consequence of this lesion (Ross et al. 1985). Since the transfer of oxygen across the gill surface is directly proportional to its area and inversely proportional to its thickness (Hughes 1984), measurement of the thickness of the barrier that separates the water from the blood can be used to assess the gas exchange function of the gill.

Little information is available about pathophysiological changes in fish caused by exposure to hydrogen peroxide. Identification of chemical/hormonal changes may help to

identify the mechanism by which fish can be negatively affected by treatment. Treatment doses and methods can be modified to minimize these changes and lessen the impact of therapy. Therefore, the objectives of the current study were 1) to characterize the clinical pathological changes on selected blood chemistry and hormonal constituents in rainbow trout treated with a high dose of hydrogen peroxide (1250 ppm), and 2) to assess the effects of this chemical on gill structure by performing a morphometric analysis using light microscopy and a Bioquant system.

3.3 MATERIALS AND METHODS

3.3.1 Trial 1. Evaluation of the primary stress response (serum cortisol), thyroxine and triiodothyronine

3.3.1.1 System design and allocation of treatment

One hundred and eighty juvenile rainbow trout with an average weight of 38 ± 7.1g ($\bar{x}$ ± SD) (60 fish were selected from the population and weighed) were used. Fish were purchased from a commercial hatchery in Prince Edward Island, Canada.

The fish were maintained in 6 circular fibreglass tanks. Groups of 30 fish were allocated to each tank as follows: 5 fish were netted from the large group and added sequentially until each tank had a total of 30 fish. This was done to avoid any bias caused by keeping the smallest or biggest fish in the same tank. The total tank volume of each was 78 L, yielding an initial stocking density of 13.8 kg/m^3. Tanks received 12 hours of

light and 12 hours of darkness. Water temperature was maintained at 10°C. The water flow rate for each tank was kept at 2.7 L/min by adjusting the flow valves. Flow was assessed by measuring the time taken to fill a 2-L measuring device. The oxygen levels (measured with an oxygen meter) were maintained above 7.6 mg/L. Fish were fed by hand to satiation once daily with a commercial ration (High Pro Grower, pellet size # 3, Core Feed Mills Ltd, Fredericton, NB). The proximate analysis (dry weight basis) of the feed is shown in Appendix C.

3.3.1.2 Exposure to hydrogen peroxide

After the allocation to tanks, fish were maintained for a 3 wk acclimation period and then the fish in 3 of the 6 tanks were exposed once to hydrogen peroxide in a static bath. The hydrogen peroxide (6%, Drug Trading Company Ltd., Scarborough, Ont., Canada) was pre-diluted in a bucket with water and then added to the tank until a concentration of 1250 ppm was obtained. The final concentration of hydrogen peroxide was determined once the dilution was made by using a potassium permanganate titration method as previously described in chapter 2. The flow of the water in both treated and control tanks was turned off prior to the treatment. After 30 min exposure, the tanks were flushed with clean water and the normal water flow resumed. Aeration was maintained in both groups during the experiment and oxygen levels did not fall below 7.6 mg/L.

3.3.1.3 Sampling procedure

Two ml of blood were collected from 5 fish from each group at each sampling period.

Time of sampling was as following: 24 h before treatment, 1, 24, and 96 h, 1, and 2 wks post-exposure. At each sampling time the fish were netted from their tanks, and anaesthetized in a solution of 40 mg/L benzocaine (Barton et al. 1985). No more than one or two fish per tank were sampled and the netting operation was restricted to less than 90 seconds, since stressful stimuli longer than this period cause a sharp rise in plasma cortisol (Barton et al. 1980). Blood was obtained from the caudal vessel (Houston 1990). Blood samples were collected in tubes without anti-coagulant. After blood collection, fish were euthanized with an overdose of benzocaine (80 mg/L). Blood was separated by centrifugation (7,000 rpm for 10 min) and the serum stored at -20 °C until assayed.

3.3.1.4 Determination of cortisol, thyroxine and triiodothyronine

Serum cortisol, thyroxine and triiodothyronine were analysed by using radioimmunoassay (RIA) kits (Coat-A-Count™ Cortisol, Canine T_4, and Canine T_3 kit Diagnostic Products Corporation, Los Angeles, CA). The details of the assays have been reported previously (Sumpter and Donaldson 1986; Burke and Leatherland 1983).

The measurement of serum cortisol involves ^{125}I-labelled cortisol which competes for a fixed time with cortisol in the sample for antibody sites. The antibody is immobilized to the walls of a propylene tube, so decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabeled cortisol.

Measurement of serum triiodothyronine (T_3) and thyroxine (T_4) are similarly based on competition assays with ^{125}I-labelled hormones. The reaction takes place in the presence of blocking agents which serve to liberate the bound T_3 and T_4 from carrier proteins; so the

assay measures total hormone, since both free and protein-bound hormone from the sample are able to compete with radiolabeled hormone for antibody sites.

All samples for cortisol, T_3 and T_4 were analyzed in duplicate and the final values reported were the mean of the two samples (Appendix D).

3.3.2 Trial 2. Evaluation of the secondary stress response

3.3.2.1 System design and allocation of treatment

Thirty rainbow trout (avg. weight of 40 ± 5.8 g; $\bar{x} \pm SD$) were allocated to each of 6 circular fibreglass tanks. The total tank volume of each tank was 78 L yielding an initial stocking density of 14.2 kg/m^3. After the allocation to tanks, fish were maintained for a 3 week acclimation period and then the fish in 3 of the 6 tanks were exposed once to hydrogen peroxide (1250 ppm) for 30 min in a static bath. In this study, 5 fish from each group were sampled at 24 h before treatment, 1, 24, and 96 h and 1 week post-treatment. After blood collection, fish were euthanized with an overdose of benzocaine (80 mg/L).

Either lithium heparin or sodium fluoride was used as anticoagulant. Plasma glucose concentration was determined from the sample in the fluoride tube by using an automated discrete analyzer (Hitachi 911) using Boerhringer Mannheim reagents. A manual packed cell volume (microhaematocrit method) was determined on the heparinized sample. The tubes were then spun for 5 min at 11,500 rpm. A graduated table was used to read the hematocrit value (Critocaps ™ St. Louis Mo. 63103 USA). Leucocrit values were determined from the same tubes. The width (mm) of the white cell layer was measured at a magnification of 40X using a microscope equipped with an ocular scale, and the leucocrit value was calculated as

the fraction or percentage of the total blood column height.

Differential white blood cell counts were performed on smears made from the heparinized sample. After Wright Giemsa staining, the blood smears were examined (magnification of 1000X) for relative numbers of lymphocytes, granulocytes and monocytes which were determined by counting 100 white blood cells per smear. After the differential was made, the fraction of each white blood cell (WBC) was multiplied by the leucocrit to obtain an absolute value for each cell type (fraction of that cell type in the total volume of blood) using the following formula.

Abs. fraction of each WBC (L/L) = [leucocrit fraction (L/L)] [% of each WBC cell /100]

3.3.3 Effect of hydrogen peroxide on gill structure (lamella)

Gills collected from fish in trial 2 were used for this study. After blood collection, the 5 fish from each group were euthanized with an overdose of anesthetic (80 mg/L benzocaine). Sampling time was: 24 h before treatment, 24 and 96 h and 1 wk post-treatment. All four gill arches were removed and fixed in neutral buffered formalin (10%) for at least 24 h. The second gill arches, after fixation, were placed in a histology cassette and then processed routinely for histopathology. Cassettes of tissues were washed, dehydrated in graded concentrated ethyl alcohol (70% up to 100%), cleared in xylene and embedded in paraffin wax; 4-6μm-thick sections were made. Samples were stained with haematoxylin and eosin (H&E) and examined by light microscopy.

3.3.3.1 Light microscopy

To determine the range of lesions of the gill filaments, the second gill arch was examined from 5 fish per group at each sampling time. Three well oriented filaments (ventral, central and dorsal filaments) of each arch were assessed. Well oriented filaments were defined as those with lamellae of the same length bilaterally present to near the tip of the filament and with well defined interlamellar zones (Speare and Ferguson 1989)

Each selected filament was evaluated for the following parameters: 1) Lamellar edema: as the percent of lamellae affected with either visible separation of two layers of epithelial cells or separation of epithelial cells from the pillar cells; 2) Epithelial hyperplasia: percent of lamellae with one or more points of thickened areas of epithelial tissue; 3) Lamellar necrosis: percent of lamellae with one or more necrotic epithelial cells as judged by changes in the nucleus (pyknosis, karyorrhexis and karyolysis); 4) Lamellar fusion: percent of lamellae that were fused to adjacent lamellae in the filament; and 5) Lamellar inflammation: percent of lamellae that had one or more clusters (>5 cells) of inflammatory cells. All parameters were evaluated under light microscope using the 40x objective.

3.3.3.2 Bioquant system (BQ) (measurement of the lamellar and interlamellar width)

A computerized Bioquant image analysis system (R&M Biometrics, Inc. Nashville, TN) was used to determine the thickness of both the lamella and the interlamellar area. The BQ is a system composed of computer hard and software and a digitizing pad (Hipad Digitizer Model; Dt 11, Houston Instruments, Austin TX.). The software is equipped with a television camera (JE3012/A, Javelin Electronics) that obtains information from a light

microscope (Carl Zeiss Canada Ltd., Don Mills, ON) and the image observed in the microscope is projected to a computer monitor screen. The lamellar and interlamellar thickness was measured in microns (μm).

In this morphometric analysis, the diffusion distances (barrier) between water and blood were measured (lamellar thickness) as well as the thickness of the base of the lamellar area (interlamellar thickness). Five fish from each group were evaluated similar to a procedure previously described (Speare and Ferguson 1989). Briefly, three well oriented filaments from each arch were evaluated as with light microscopy examination. Lamellar thickness was measured as follows: for every twentieth lamella a measurement was taken at the midpoint of the lamella (lamellar width) by measuring the transverse distance between both sides of the lamellae (Fig. 3.1). The other parameter evaluated was the interlamellar thickness (the thickness of the epithelial layer in the region between the bases of adjacent lamellae). This measurement was done by measuring at the middle part of the interlamellar space from the epithelial : water interface to the epithelial basement membrane (Fig. 3.1). Four to six measurements were done in each filament (depending on the length of the filament). A magnification of 400 diameters (40 x 10) at the light microscope level was chosen, but the actual magnification (2,591 x) of the image on the monitor screen was obtained by calibration using a stage micrometer.

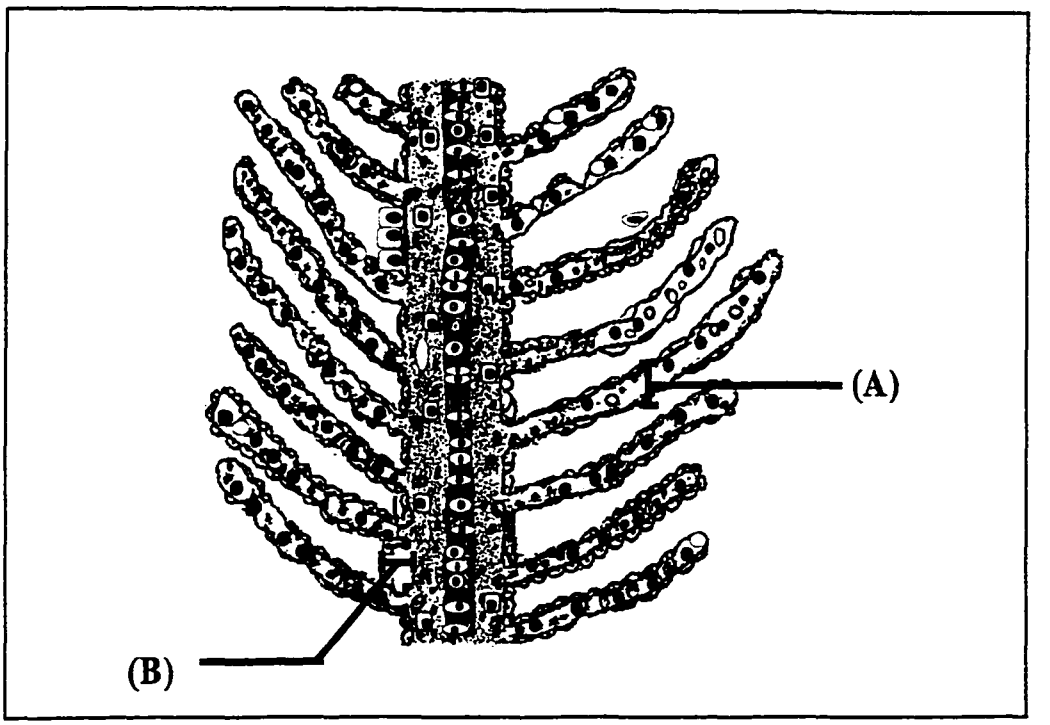


Fig. 3.1 This scheme shows the gill areas that were measured with the BQ. A: transverse measurement of the thickness of the middle part of the lamellar. B: transverse measurement of the thickness of the epithelium in the interlamellar space.

3.3.4 Statistical analysis

The null hypothesis tested in the chemistry trial was that hydrogen peroxide does not elicit significant changes in different blood components in rainbow trout. This hypothesis was tested by using one way analysis of variance (ANOVA) and, when significant, the treatment means were compared using the Tukey-Kramer multiple pairwise comparison procedure. The level of significance was 95% (α = 5%). Analyses were made by using MINITAB software for Windows (Minitab Inc. 1994; 3081 Enterprise Drive State College, PA).

Indices from the gill morphometric analysis were evaluated as follows: the values from each index from a given fish were expressed as a pooled mean from the three filaments evaluated. One way -ANOVA was used for each index to test the null hypothesis that there was no difference between the two groups. Parameters measured with the BQ were analyzed with two-way ANOVA (SAS software program). Values were considered significantly different at the $p \leq 0.05$ level of probability.

3.4 RESULTS

No death losses occurred during and following the treatment in both trials.

Trial 1

Exposure to hydrogen peroxide resulted in a marked significant increase ($p = 0.005$) in serum cortisol concentration 1 h after exposure ($\bar{x}$ 480 ± 243). Cortisol concentrations remained increased for 24 h, and 96 h post-treatment (Fig. 3.2; Table 3.1). The thyroxine

levels were also significantly increased ($p = 0.001$) in treated fish after 1 h. For the remaining times, concentrations of T_4 did not show any significant difference when compared with those of control fish (Fig. 3.3; Table 3.1). Serum T_3 levels were significantly decreased ($p = 0.002$) 24 h post-treatment. No significant differences were observed for the remaining sampling times (Fig. 3.4; Table 3.1).

Trial 2

Plasma glucose concentrations from treated fish was significantly higher ($p = 0.007$) after 1, 24 and 96 h. No significant changes were present after that time (Fig. 3.5; Table 3.2). Treated fish had a significant reduction in the leucocrit fraction ($p = 0.02$) which was characterized by a significant decrease ($p = 0.02$) in the circulating lymphocytes (24 h post-treatment) (Fig 3.6). No significant differences were found in the number of monocytes and granulocytes at any sampling time (Fig. 3.7 & 3.8; Table 3.2). Hematocrit did not show any significant differences at any sampling time (Fig. 3.9; Table 3.2). The blood cell profile in treated fish was similar to controls 96 h after treatment.

Light microscopy

Lamellar edema, epithelial necrosis, and epithelial hyperplasia were identified in the treated fish to some degree throughout the entire study (Fig. 3.10; 3.11; 3.12). Focal areas of lamellar edema were also observed in control fish and no significant differences were found between treated and control groups in this index percentage (Table 3.3). Multifocal epithelial necrosis was significantly higher in the treated group at 24 h post-exposure ($p =$

0.009). However, there was no significant evidence of infiltration of lamellae with inflammatory cells (Fig. 3.13). The percent of lamellae with epithelial hyperplasia was significantly increased ($p = 0.03$) a week after the treatment in the treated fish (Table 3.3). Lamellar fusion was not significantly different between groups at any time (Fig. 3.14).

BQ system analysis

A significant increase in the interlamellar epithelial thickness in treated fish was found a week after the treatment ($p = 0.014$) (Table 3.3). In contrast, lamellar thickness did not show differences between treated and control groups at any time (Table 3.3). For both the lamellar and interlamellar epithelial thickness in the treated groups, there was a linear relationship between the thickness and post-exposure time (for lamellar $r^2 = 0.14$ and for interlamellar $r^2 = 0.18$), which could be described by the equation $y = 15.5337 + 0.3282x$ (lamellar thickness) and $y = 28.8946 + 0.9684x$ (interlamellar thickness); where y is the thickness (μm) and x is the time (units) post-exposure. The slope of the regression was significantly different from zero ($p < 0.001$) in both parameters. Control fish did not show a significant slope for lamellar or interlamellar thickness.

3.5 DISCUSSION

This study has demonstrated the potential for hydrogen peroxide treatment, in a dosage similar to that used for sea lice control, to activate the stress response in juvenile rainbow trout. The prolonged increase in cortisol levels observed in this study (up to 96 h

post-treatment) indicated that treatment results in continued stimulation of the primary stress response in fish even after the chemical had been removed. Cortisol levels for the control fish (110 nmol/L or 40 ng/ml or less) were characteristic of unstressed fish (Barton and Iwama 1991) which indicated that the procedure used to handle fish during treatment and sampling did not induce an elevated serum cortisol concentration (Pickering et al. 1992). Assessment of serum catecholamines and serum cortisol concentrations are the main methods for monitoring the primary stress response in fish. In this study, cortisol was chosen as an indicator of stress because cortisol is more specific than catecholamines and it has better stability when stored frozen (Wedemeyer et al. 1990; Barton and Iwama 1991).

Thyroxine and triiodothyronine are hormones secreted by the thyroid gland which control the body's rate of metabolism. In mammals, a wide range of clinical conditions are associated with decreased serum concentration of thyroid hormones during acute and severe illnesses (Wartofsky et al. 1982; Faber et al. 1987; Kaptein et al. 1982). It has been suggested that this condition may also occur in fish since it has been demonstrated that during periods of stress these hormones are suppressed (Brown et al. 1991). In our study, the increase of T_4 (after 1h) and decrease of T_3 (after 24 h) were small and may not be physiologically significant. These alterations may have been the result of mild changes in the metabolism of thyroid hormones such as decreased stimulation of peripheral conversion of thyroxine to triiodothyronine (ie. reduced 5'-monodeiodinase) (Eales et al. 1989; Eales 1985) causing the accumulation of T_4 in serum. Cortisol concentration may affect the metabolism of these thyroid hormones, although the specific effect varies between species. For example, in the eel (*Anguilla anguilla*), both T_4 and T_3 levels were reduced by cortisol

injection (Redding et al.1986), whereas in salmonids chronic cortisol treatment appears to suppress plasma T_3 without influencing T_4 (Redding et al.1984; Brown et al. 1991). Several other factors are known to affect the concentration of these hormones, such as levels of testosterone, season, and the time of day (Hunt and Eales 1979; Milne et al. 1979).

The release of cortisol during the primary stress response causes several physiological changes, and these may also be used as an index of measuring the stress response (secondary stress response). Alterations in blood glucose levels (hyperglycemia) and white blood cell numbers are parameters frequently used to estimate the secondary stress response in fish (Houston 1990; Iwama et al. 1995). Characteristic blood changes associated with stress are leucopenia (decreased number of leukocytes), lymphopenia (decreased number of lymphocytes), and heterophilia (increased number of heterophils) (Ellis 1977; Bruno & Munro 1986; Pickering 1986; Blaxhall and Daisley 1973; Hoffmann and Lommel 1984). Our results clearly showed that an acute increase of the serum cortisol level in treated fish was followed by marked decrease of the leucocrit (which was characterized by a decreased numbers of circulating lymphocytes 24 h), and by hyperglycemia (1,24 and 96 h) which reflect the secondary stress response. Increased plasma glucose concentration is likely due to cortisol induced glucose mobilization from the fish liver (Pickering and Pottinger 1995) but the exact mechanism involved is unclear. It is believed that the rapid rise of blood glucose concentration following an acute stress response is not only caused by the cortisol action but may also be attributed to the glycogenolytic action of the catecholamines (Wendelaar Bonga 1997). Lymphopenia has been associated with cortisol release during periods of stress (Barton and Iwama 1991). In some mammals, such as mice and rabbits,

lymphopenia has been attributed to the lympholytic properties of corticosteroids (Dougherty and White 1945). The reported fraction values for the white blood cells are inexact due to the impreciseness of the methods used to measure them. However, leucocrit continues to be the most practical method in fish for estimating the number of white blood cells due to the presence of nucleated red blood cells interfering with manual and automated cell counting methods.

The hematocrit may be significantly affected by stress in rainbow trout (Casillas and Smith 1977; Railo et al. 1985). Powell and Perry (1997) found that, the hematocrit of rainbow trout was increased following exposure of hydrogen peroxide at 500 ppm for 20 min. However, the hematocrit returned to control levels 3 h after treatment. This acute change was mainly attributed to the effect of catecholamines. In our results, hematocrit was not altered at any sampling time with no indication of red cell destruction causing anemia or swelling of the erythrocytes caused by fluid shift (osmotic regulation problems) causing an increased hematocrit (Heath 1995).

It has been reported that hydrogen peroxide at high concentrations produces severe histological lesions in fish gills, such as extensive epithelial edema and necrosis causing respiratory distress or even death (Thomassen et al. 1993; Kiemer and Black 1997, Bruno and Raynard 1994; Johnson et al. 1993). Most of the lesions previously described were obtained from moribund or dead fish during exposure to hydrogen peroxide. No information was available about subsequent changes produced in gills after hydrogen peroxide was withdrawn. Therefore, in the present study, samples were collected from surviving fish at various times post-treatment; histological findings from moribund or dead fish were not

included.

The presence of dead epithelial cells along with the absence of inflammatory cells may indicate that cell death might have been induced by processes other than classic necrosis with cell lysis which usually induces inflammatory mediators. One process to consider may be apoptosis. Apoptosis is a different form of cell death (also known as programmed cell death) in which the cell plays an active role in its own death (Wyllie 1981; Uker 1991). In general, it is difficult to distinguish *in situ* cells undergoing apoptosis by light microscopy. Specific techniques, such as detection of DNA fragmentation which is a molecular change characteristic of apoptotic cell death, may be required to differentiate these changes (Yael et al. 1992; Sanders 1997). The morphological characteristics of cells dying by apoptosis differ from those of cells dying by necrosis. During apoptosis, cells become smaller and rounder. After that, the nuclear chromatin undergoes condensation and fragmentation. The apoptotic cells then split into many plasma membrane-bound vesicles (apoptotic bodies) which contain fragments of condensed chromatin and intact organelles such as mitochondria. In contrast, necrotic cells usually swell and then die, lysing to release cytoplasmic material causing injury and inflammation in surrounding tissue (Lieberthal and Levine 1996). Hydrogen peroxide is considered a cellular oxidant which causes its damage via the production of hydroxyl radicals (Cotran 1994). It has been reported to cause both necrotic and apoptotic cell death (Xu et al. 1997; Satoh et al. 1996; Gotoh and Cooper 1998; Sugiyama et al. 1996).

The increased percentage of lamellae with epithelial hyperplasia in the treated fish

from the present study was not described in the reports of previous researchers. One possible explanation of this difference may be the longer period of post-treatment sampling used in this study. The gill epithelium is the site of multiple functions. Increased proliferation may occur following exposure to chemicals such as copper sulfate, zinc chloride and inorganic mercury (Daoust 1984; Karan et al. 1998; Hemalatha and Banerjee 1997). Thickening of lamellar epithelium, and thus the blood-to-water diffusion barrier, may impair gas transfer (Hughes 1970). Additionally, this type of disruption may be associated with negative effects on blood pH and acid-base balance (Evans 1987; Wendelaar Bonga and Lock 1992). None of the latter parameters were measured in this study. However, Powell and Perry (1997) found that after exposing rainbow trout to hydrogen peroxide (500 ppm), an acute reduction in the amount of O_2 (i.e. hypoxemia) occurred along with respiratory acidosis. Further studies may help to determine whether other physiological changes, such as gas exchange, and blood ionic disturbances, as well as accumulation of metabolites (e.g. ammonia), are associated with hydrogen peroxide exposure.

The increased thickness of the epithelium of the interlamellar zone found in treated fish may simply reflect hyperplasia in response to loss of cells on the lamellae. Neither the type of lesion nor type of cells at the base of the lamella (ie. interlamellar space) from treated fish were characterized in this study. Common pathological changes produced by chemical-induced lesions at the base of the lamellae include hypertrophy and/or proliferation of chloride cells and proliferation of mucous cells (Mallatt 1985). Further studies are necessary to identify the cells that are involved in the increased thickness of the interlamellar space in order to better understand the pathophysiology of this type of lesion.

In summary, the present investigation has shown that H_2O_2 treatment at a high dose level results in activation of the primary and secondary stress response as indicated by the increased serum cortisol concentrations, hyperglycemia, and decreased numbers of circulating lymphocytes. Thyroid hormones showed mild alterations which require further study. Histological changes seen in treated fish include evidence of epithelial hyperplasia as well as areas of necrosis. These changes may affect fish production performance even when death losses are minimal. Fish culturists should apply hydrogen peroxide with the awareness that this chemical may induce a stress response and gill pathology.

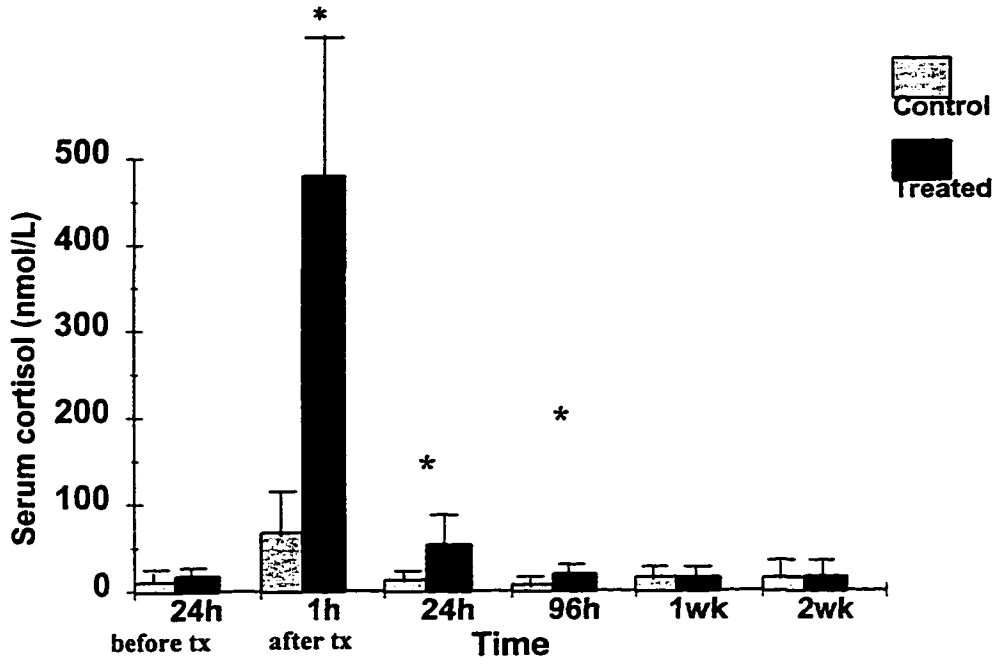


Fig. 3.2. Mean (+SD) of serum cortisol concentrations in hydrogen peroxide treated and control fish; * indicates significant difference between groups.

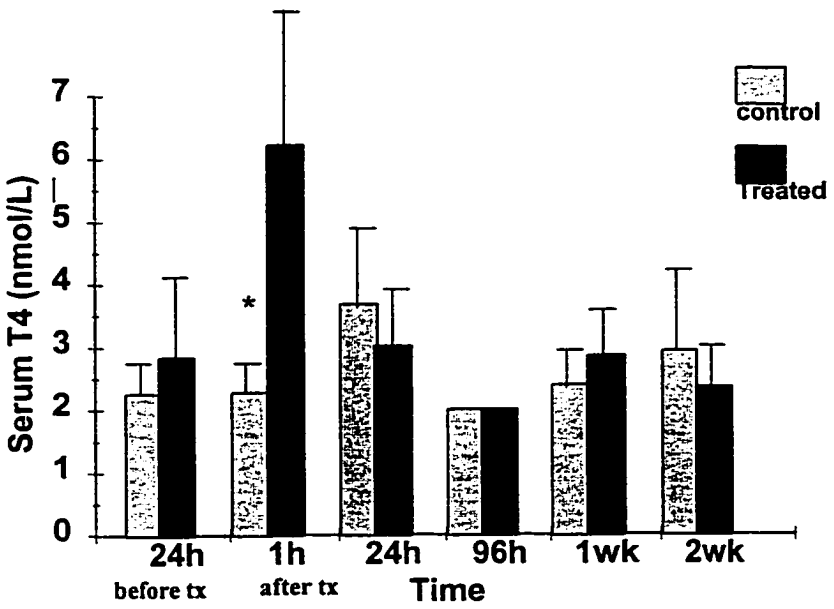


Fig. 3.3. Mean (+SD) of serum thyroxine concentrations in hydrogen peroxide treated and control fish; * indicates significant difference between groups.

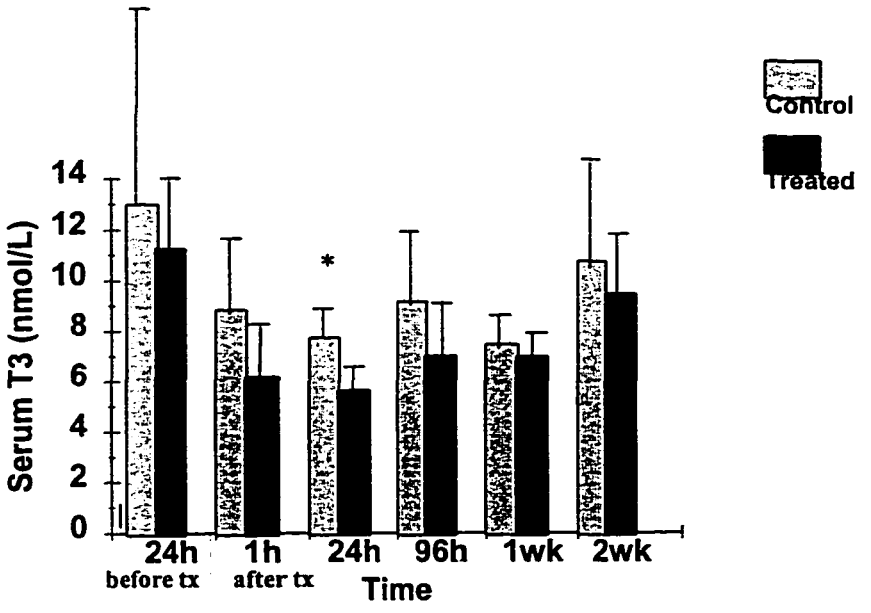


Fig. 3.4. Mean (+SD) of serum triiodothyronine concentrations in hydrogen peroxide treated and control fish; * indicates significant difference between groups.

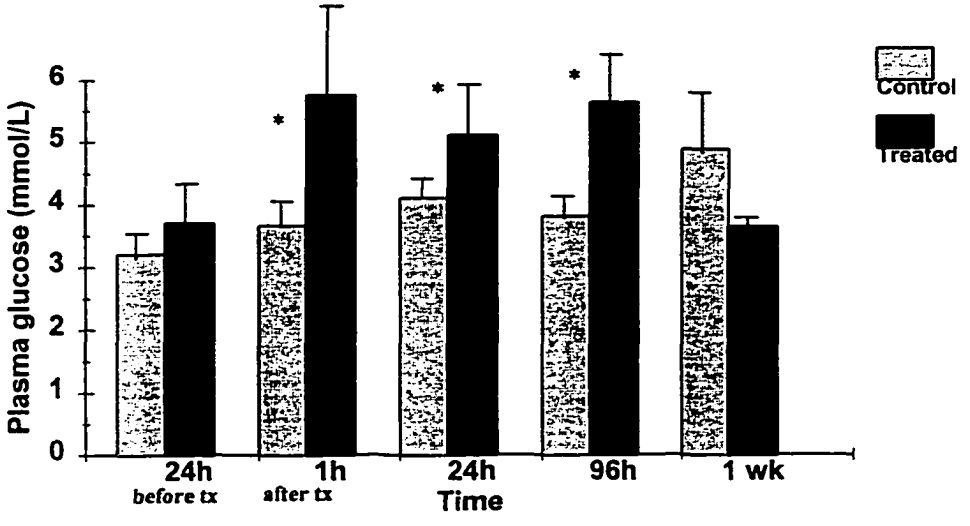


Fig. 3.5. Mean (+SD) of plasma glucose concentrations in hydrogen peroxide treated and control fish; * indicates significant difference between groups.

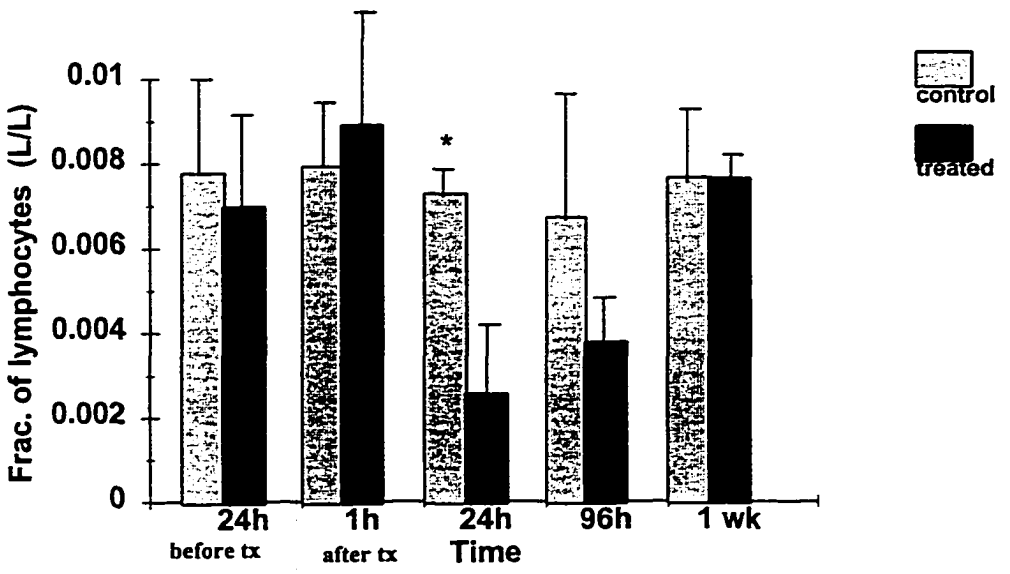


Fig. 3.6. Mean (+SD) of fraction of lymphocytes in blood of hydrogen peroxide treated and control fish; * indicates significant difference between groups.

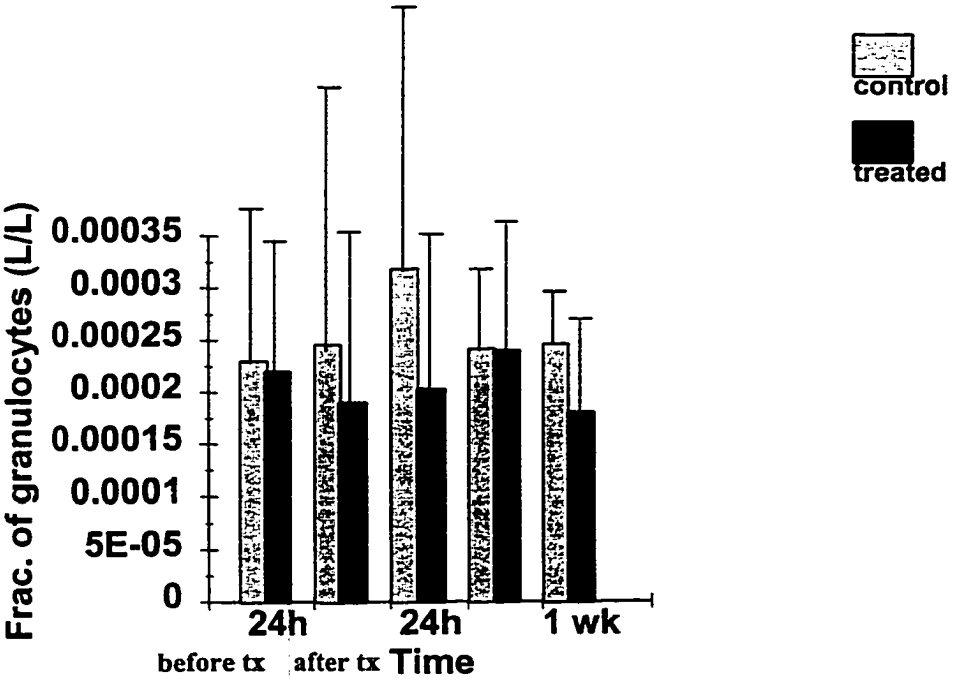


Fig. 3.7 Mean (+SD) of fraction of granulocytes in blood of hydrogen peroxide treated and control fish.

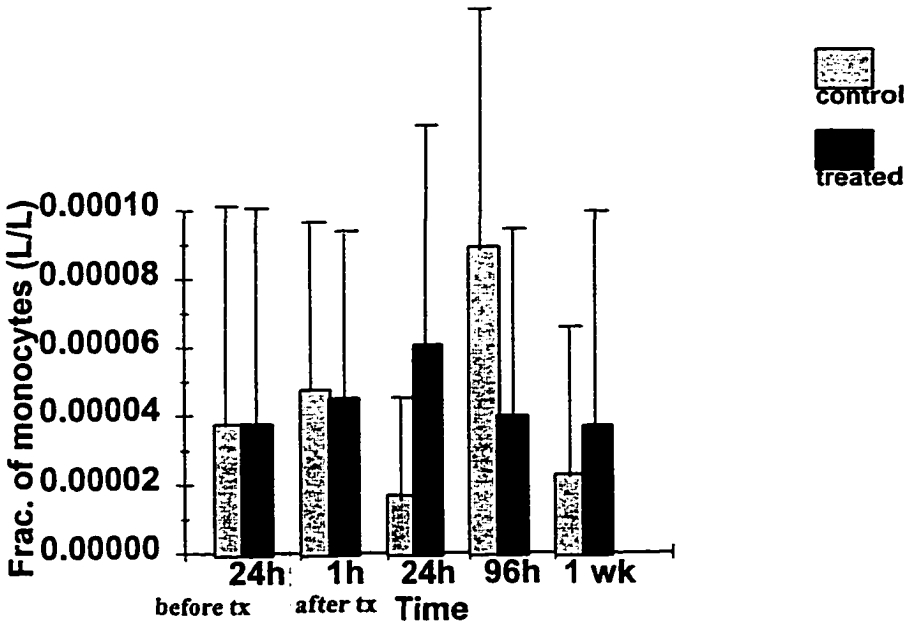


Fig. 3.8 Mean (+SD) of fraction of monocytes in blood of hydrogen peroxide treated and control fish.

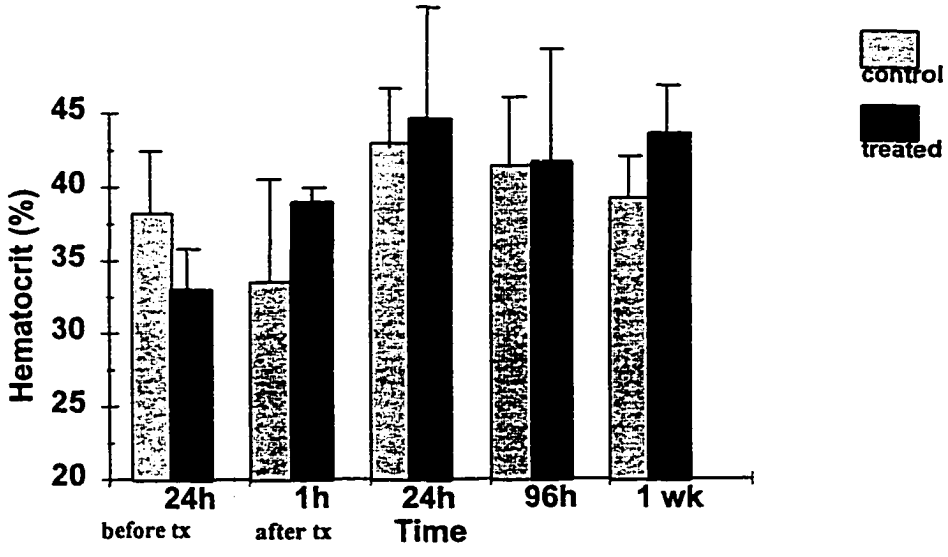


Fig. 3.9 Mean (+SD) of hematocrit in hydrogen peroxide treated and control fish.

Table 3.1 Mean (± SD) of serum cortisol, thyroxine and triiodothyronine levels (trial 1). N = 6

	24 hr	1 hr	24 hr	96 h	1week
cortisol (nmol/L)	C 10.4 ± 6.2 T 16.9 ± 7.6	C 67.2 ± 42 T 480 ±243*	C 12.1± 6.9 T 52.8± 30.8*	C 6.6±3.57 T 19.0±9.03	C 15.3 ±12.9 T 15.0 ±8.13
T_4 (nmol/L)	C 2.2 ± 0.37 T 2.8 ± 1.25	C 2.28 ±0.31 T 6.2 ± 2.05*	C 3.68±1.19 T 3.02±1.02	C 2.0 ± 0 T 2.0 ± 0	C 2.40 ±0.58 T 2.86 ±0.81
T_3 (nmol/L)	C 12.9 ± 6.6 T 11.25±2.7	C 8.84 ±3.0 T 6.2 ±2.1	C7.74 ±1.05 T 5.64±0.90*	C 9.18±2.85 T 7.02±1.90	C 7.48 ± 1.38 T 7.00 ± 1.26
	before tx	after tx →			

* Significant differences between control and treated fish ($p \leq 0.05$)

Table 3.2 Mean (± SD) of glucose, hematocrit (PCV), leucocrit, lymphocytes, granulocytes and monocytes (Trial 2). N = 5.

	24 hr	1 hr	24 hr	96 h	1week
glucose (mmol/L)	C 3.06 ± 0.47 T 3.78 ± 0.65	C 3.66 ± 0.48 T 5.76 ± 1.36	C 4.52 ±0.38 T 4.72 ±0.79*	C 3.82±0.37 T 5.66±0.98*	C 4.9 ± 1.01 T 3.66± 0.18
PCV (%)	C 38.2 ± 4.3 T 33.0 ± 2.7	C 33.5 ± 7.33 T 39.0 ± 0.81	C 43.0± 3.6 T 44.7± 7.2	C 41.5 ± 4.7 T 41.75±7.3	C 39.33±2.5 T 43.75±2.9
leucocrit (L/L)	C 0.79 $x10^{-2}$ ± 0.20 $x10^{-2}$ T 0.73 $x10^{-2}$ ± 0.19 $x10^{-2}$	C 0.82 $x10^{-2}$ ±0.2 $x10^{-2}$ T 0.91 $x10^{-2}$ ±0.2 $x10^{-2}$	C 0.76 $x10^{-2}$±0.06 $x10^{-2}$ T 0.20 $x10^{-2}$±0.2 $x10^{-2}$*	C 0.6 $x10^{-2}$±0.23 $x10^{-2}$ T 0.4 $x10^{-2}$±0.12 $x10^{-2}$	C 0.79$x10^{-2}$±0.71$x10^{-2}$ T 0.70$x10^{-2}$ ±0.04$x10^{-2}$
lymph. (L/L)	C 7.70 $x10^{-3}$ ± 19.7 $x10^{-4}$ T 6.90 $x10^{-3}$ ± 19.3 $x10^{-4}$	C 7.9 $x10^{-3}$ ± 16.8$x10^{-4}$ T 8.9 $x10^{-3}$ ± 25.9 $x10^{-4}$	C 7.20 $x10^{-3}$ ±5.49$x10^{-4}$ T 2.50 $x10^{-3}$ ±17.9$x10^{-4}$*	C 6.7 $x10^{-3}$±30.5$x10^{-4}$ T 3.7 $x10^{-3}$±12.3$x10^{-4}$	C 7.6 $x10^{-3}$ ± 17.6$x10^{-4}$ T 7.6 $x10^{-3}$ ± 4.29$x10^{-4}$
granul. (L/L)	C 2.30 $x10^{-4}$ ± 1.55 $x10^{-4}$ T 2.20 $x10^{-4}$ ± 1.32 $x10^{-4}$	C 2.4 $x10^{-4}$ ±3.19 $x10^{-4}$ T 1.9 $x10^{-4}$ ±1.82 $x10^{-4}$	C 3.1 $x10^{-4}$ ± 3.01 $x10^{-4}$ T 2.0 $x10^{-4}$ ± 1.55 $x10^{-4}$	C 2.4 $x10^{-4}$ ± 0.7 $x10^{-4}$ T 2.4 $x10^{-4}$ ± 1.3$x10^{-4}$	C 2.4 $x10^{-4}$ ± 0.42 $x10^{-4}$ T 1.8 $x10^{-4}$ ± 0.83 $x10^{-4}$
mono. (L/L)	C 3.70 $x10^{-5}$± 0.75 $x10^{-4}$ T 3.70 $x10^{-5}$± 0.75 $x10^{-4}$	C 4.7 $x10^{-5}$ ±0.56 $x10^{-4}$ T 4.5 $x10^{-}$ ± 0.55 $x10^{-4}$	C 1.6 $x10^{-5}$ ± 0.33 $x10^{-4}$ T 6.0 $x10^{-5}$ ± 0.82 $x10^{-4}$	C 8.9 $x10^{-5}$ ± 0.93$x10^{-4}$ T 3.9 $x10^{-5}$ ± 0.62$x10^{-4}$	C 2.3 $x10^{-5}$ ± 0.46 $x10^{-4}$ T 3.7 $x10^{-5}$ ± 0.75$x10^{-4}$
	before tx	after tx →			

* Significant differences between control and treated fish ($p \leq 0.05$)

Table. 3.3 Mean (± SD) percentage of gill lamellae that present structural changes. N = 4.

	24 h	24 h	96 h	1 week
Lamelllar edema	C 1.90 ± 2.00 T 1.30 ± 1.00	C 2.40 ± 1.1 T 5.20 ± 3.3	C 2.80 ± 1.60 T 5.20 ± 2.90	C 2.50 ± 0.8 T 4.00 ± 4.5
Lamellar hyperplasia	C 2.75 ± 1.80 T 2.91 ± 4.40	C 1.66 ± 1.2 T 5.00 ± 1.9	C 2.50 ± 2.00 T 3.33 ± 2.70	C 2.75 ± 1.7 T 4.52 ± 3.3 *
Lamellar fusion	C 0.16 ± 0.30 T 0.75 ± 1.50	C 1.30 ± 1.1 T 0.66 ± 0.6	C 0.40 ± 0.50 T 1.66 ± 0.90	C 0.70 ± 0.9 T 2.00 ± 0.8
Lamellar necrosis	C 0.80 ± 0.16 T 0.16 ± 0.30	C 0.33 ± 0.4 T 5.90 ± 4.2 *	C 0.33 ± 0.20 T 2.80 ± 2.06	C 1.33 ± 1.0 T 1.33 ± 0.2
Lamellar inflamm.	C 0.83 ± 0.60 T 1.50 ± 1.10	C 2.20 ± 1.2 T 2.60 ± 0.9	C 1.33 ± 0.50 T 2.00 ± 0.70	C 1.83 ±1.3 T 1.58 ± 0.8
Lamellar width (μm)	C 14.7 ± 1.90 T 15.1 ± 3.00	C 15.00 ±1.3 T 16.80 ±1.0	C 15.6 ± 1.10 T 17.4 ± 2.60	C 16.0 ± 2.2 T 17.3 ± 3.0
Interlamellar width (μm)	C 27.1 ± 5.70 T 29.8 ± 5.80	C 31.80 ± 2.6 T 32.60 ± 2.4	C 26.2 ± 5.10 T 33.9 ± 5.40	C 26.3 ± 4.0 T 34.5 ± 8.1*
	before tx	after tx →		

* Significant differences between control and treated fish ($p \leq 0.05$)

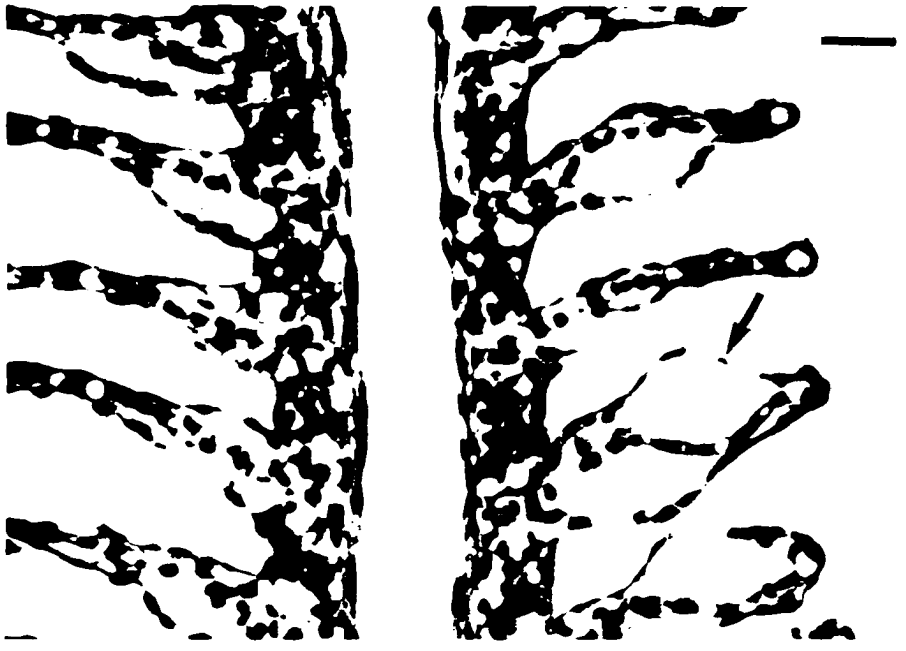


Fig. 3.10 Histological section of the gill of a hydrogen peroxide-treated rainbow trout showing edema (arrow). H&E. Scale bar = 25 μm.

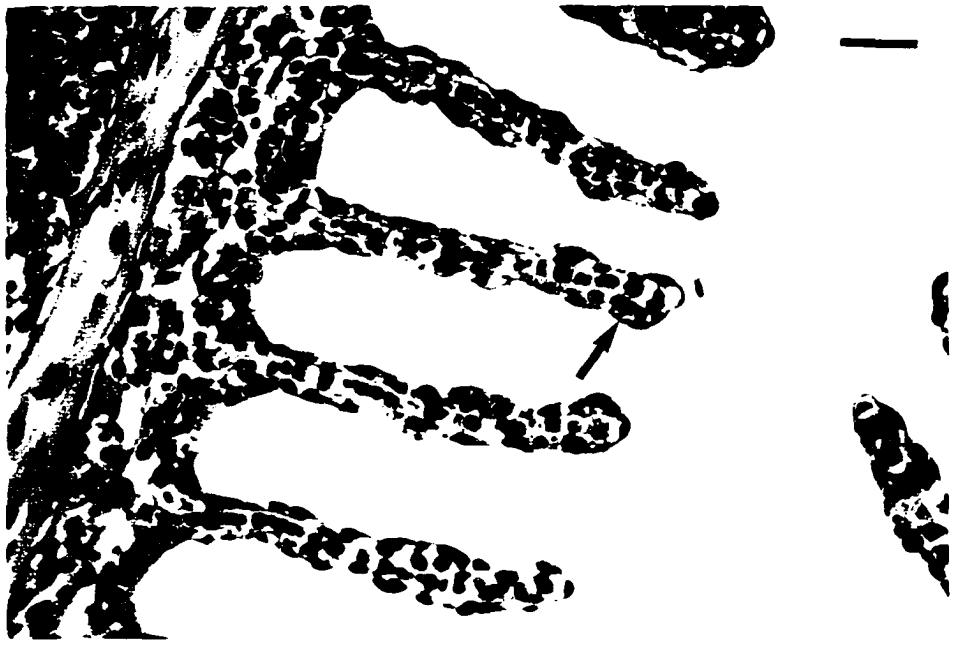


Fig. 3.11 Histological section of the gill of a hydrogen peroxide-treated rainbow trout showing necrosis. Note the lamellae with areas of necrosis (arrow) H&E. Scale bar = 25 μm.

Fig. 3.12 Histological section of the gill of a hydrogen peroxide-treated rainbow trout showing lamellar hyperplasia. H&E. Scale bar = 25 μm.

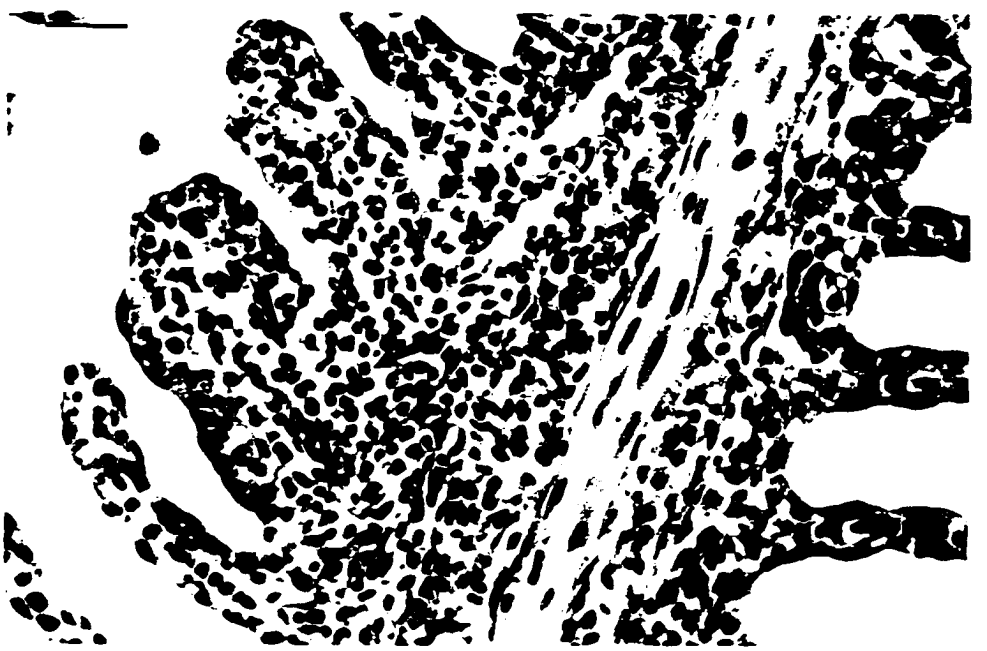


Fig. 3.13 Histological section of the gill of a hydrogen peroxide-treated rainbow trout showing lamellar inflammation. Note the infiltration of inflammatory cells (arrows). H&E. Scale bar = 25 μm.

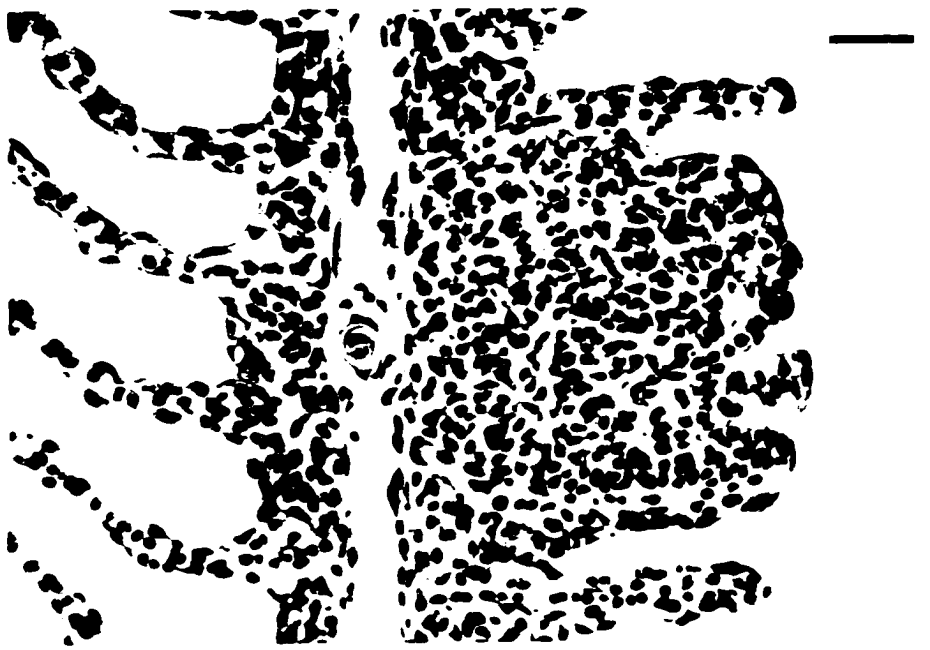


Fig. 3.14 Histological section of the gill of a hydrogen peroxide-treated rainbow trout showing lamellar fusion. H&E. Scale bar = 25 μm.

4. GENERAL DISCUSSION

The effects of toxic chemicals on fish physiology and anatomical structures (especially the gill) are frequently reported in the fish literature (Wendelaar Bonga and Lock 1992). Fish are often exposed to chemical toxicants through the large and sensitive respiratory surfaces of the gills. In addition to the toxic effects at the cellular and tissue levels, exposure to toxins can evoke a stress response in fish (Wendelaar Bonga 1997).

In these studies, a model of hydrogen peroxide exposure in rainbow trout which consisted of a single bath at 1250 ppm in fresh water was used to mimic the high dosage levels used to treat sea lice infestations. This model has been utilized to elucidate possible clinical and subclinical effects of this treatment and to describe possible mechanisms through which hydrogen peroxide negatively affects fish physiology. Data from this study has shown that H_2O_2 induces multiple pathological changes in fish, including transient suppressed growth performance, activation of the stress response and histopathological changes in the gills.

The growth reduction seen in this study was associated with an increased feed conversion index (decreased feed efficiency) rather than reduced appetite. Reduced growth has also been reported in rainbow trout exposed to repeated hydrogen peroxide treatments at 200 ppm (Speare and Arsenault 1997). Reduced growth performance following hydrogen peroxide therapy is not surprising, as many other chemicals have also been associated with growth suppression. These include chloramine T (Sánchez et al. 1996) and malachite green (Gerundo et al. 1991). Feed is considered to be the highest recurrent cost in a fish farm,

often ranging from 30 to 60% of production costs depending of the intensity of the operation (De Silva and Anderson 1995). Therefore, treatment-related growth suppression associated with a poor feed conversion efficiency is an important production concern. This situation may be aggravated when therapeutants, which are frequently administrated to the entire population, affect both healthy and sick animals negatively. In addition to the persistent effects of a disease outbreak, all treated fish might take a longer time and a greater amount of feed to reach market size, resulting in economic losses. The exact mechanisms through which chemical treatments interfere with growth performance are poorly understood. A number of possible implicated mechanisms in fish are suggested including: diseases that may affect the digestive system (malabsorption, nutrient losses or impairment of enzymes needed for digestion) (Reichenbach-Klinke 1975; Vignier et al. 1992; Gerundo et al. 1991); alteration in hormones that are essential to regulate growth and the rate of cell metabolism such as thyroid hormones (Milne et al. 1979; Brown et al. 1946); the stress response in which cortisol may cause mobilization of energy reserves, acceleration of the enzymatic breakdown of muscle proteins and conversion of their amino acids into glucose and mobilization of fat in adipose tissue for energy purposes (Pickering 1990); changes in the loss and replacement physiology of tissue and proteins (Powell et al. 1995).

Our study has demonstrated that when all fish are treated and kept in the same tank the growth performance is reduced in all individuals. However, we have to consider that our studies were conducted with healthy rainbow trout. The outcome may differ when therapies are applied to a mixture of healthy and diseased fish. Additional studies should be conducted to investigate whether diseased fish (e.g., sea lice infested) respond differently to hydrogen

peroxide therapy when compared to healthy controls.

We have demonstrated that pathological alterations are produced in the gills of fish exposed to a single high dose hydrogen peroxide treatment bath. This was not a surprising observation since a wide variety of pathological changes in the gills of fish have been attributed to exposure to different chemicals (Mallatt 1985; Johnson et al. 1993). These morphological gill alterations may be associated with functional disturbances such as respiratory impairment (Powell and Perry 1995). Therefore, rather than converting food into somatic growth, energy consumption would be directed towards cellular kinetics trying to compensate for loss of gill functions and repairing damaged tissue (Powell and Perry 1995).

It is also known that growth suppression can occur as a consequence of stress (Pickering et al. 1991). However, the exact mechanism linked to this indicator of the tertiary stress response is poorly understood. Increased serum cortisol concentrations identified in the treated fish in this treatment model indicates activation of the stress response. Increased cortisol levels are related to a shift towards catabolic metabolism and tissue breakdown (Pickering 1990) which is often associated with an increased plasma glucose concentration. This physiological response suggests that cortisol caused glucose mobilization from the fish liver (↑ gluconeogenesis) (Pickering and Pottinger 1995).

Increased serum cortisol concentrations have been associated with alterations in the metabolism of other growth-related hormones, such as thyroid hormones (Eales and MacLatchy 1989). It has been suggested that cortisol may influence the peripheral conversion of T_4 to T_3 (by deiodinase enzymes) in salmonids. Redding et al. (1984) demonstrated a reduction in plasma T_3 concentration without changes in plasma T_4

concentration associated with prolonged cortisol administration. Plasma concentrations of T_3 and T_4 were altered shortly following treatment in our model. It is possible that repeated treatment with hydrogen peroxide may have a similar or more pronounced effect.

The activation of the pituitary-thyroid axis is very sensitive to the nutritional status in fish. Hence, it has been suggested that changes in hormone metabolism may be related to poor food intake, which is normally associated with stress (Eales and MacLatchy 1989). Although the appetite of treated fish in our study was not significantly different from control fish, a reduced food intake was observed 1 and 2 days post-treatment. It is possible that this reduced food consumption caused disturbances in the thyroid hormone metabolism (Eales and MacLatchy 1989) . The significance of hormonal alterations and the mechanism through which fish thyroid hormones are altered during periods of stress caused by exposure to chemical treatments still warrants further investigation. Decreased growth rate following high dose H_2O_2 therapy documented in our model may therefore be a result of increased plasma cortisol concentrations and altered thyroid hormone metabolism.

Study design differences appear to have some influence on the growth suppression seen in this model of H_2O_2 exposure. A more dramatic suppression of growth was seen in the treated fish held in the same tank as controls in the pilot study. However, when compared to the separate tank study, growth suppression in treated fish was not significant when the cohort study was repeated. The small number of fish used in the cohort tanks may have affected the statistical significance of results. These studies need to be enlarged to identify differences resulting from treatment design.

Growth suppression and decreased feed conversion efficiency may reflect the tertiary

stress response (Pickering 1992; Pickering and Pottinger 1995; Wedemeyer et al.1990). It is possible that alterations associated with this level of stress response may also lead to a reduced disease resistance. Future studies could include evaluation of these parameters to fully investigate the effect of treatment.

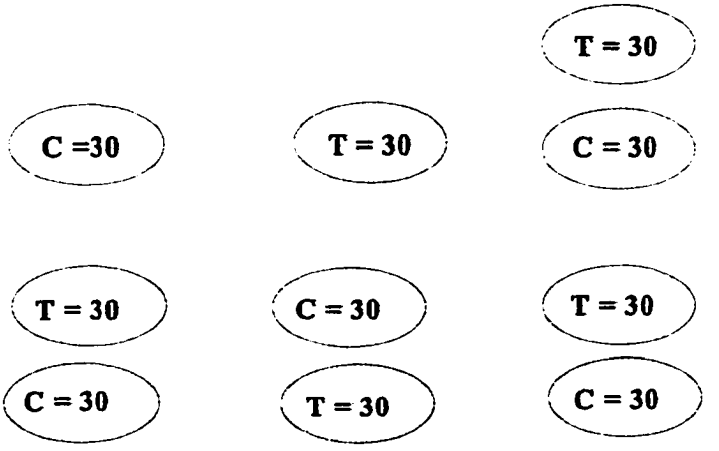
Application of the findings of studies using this treatment model to industrial situations involving disease/sea lice outbreaks in net pen reared Atlantic salmon must be done with caution. A large number of constraints impede the development of a treatment model that more closely mimics the culture situation. For example, environmental and management variables could not be controlled, as well as the presence of confounding diseases. Conducting a study in salt water would be prohibitively expensive compared to that in fresh water. Therefore, rainbow trout were used as an alternative species since it has been used successfully in many similar experimental situations (Speare et al. 1996; Speare et al. 1997; Sánchez et al. 1996). Some of the advantages in using these species of fish are availability and low cost of acquisition, ease of handling, well documented growth, and physiological characteristics and relatively less aggressive nature compared to salmon (Speare et al. 1998).

Other researchers have reported the development of histopathological lesions (epithelial necrosis and edema) in the gills of Atlantic salmon exposed to a high dose of hydrogen peroxide (Johnson et al. 1993; Kiemer and Black 1997). These findings suggest that the histopathological changes found in our laboratory model mimicked those that develop in aquaculture situations, thus validating the use of this model for investigative purposes. Nevertheless, care should be taken when applying our results to field cases such

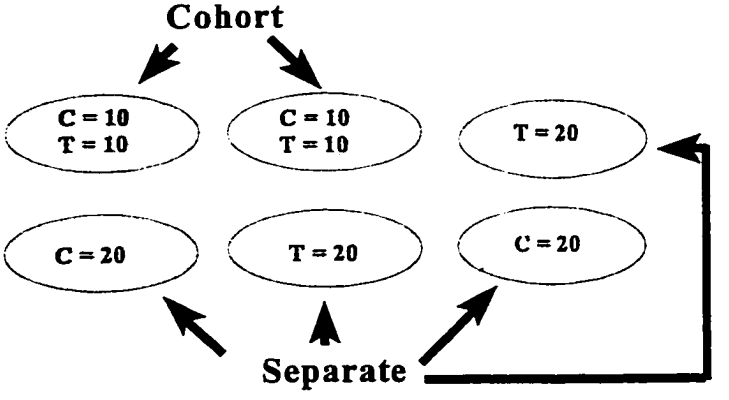
as predicting the effects of peroxide on growth suppression in sea lice infested Atlantic or Pacific salmon, or even marine culture steelhead rainbow trout, since the actual effect of treatment can be modified by several factors such as water temperature, condition of the fish, and life stage (Rach et al.1997; Thomassen 1993). Further studies should be conducted to investigate subclinical effects of hydrogen peroxide on various target species in salt water. The outcome of various chemical treatments may be altered by water quality such as hardness and salinity (Noga 1996). It is possible that the stress response, gill lesions and growth suppression seen in the present study may be ameliorated or exacerbated in saltwater. Furthermore, during a sea lice infestation, repeated hydrogen peroxide treatments are usually applied and subclinical effects resulting from repeated therapies are unknown. Thus, histopathological changes and physiological disturbances in fish caused by repeated exposure to hydrogen peroxide should be an area of future investigation.

The objectives of this study were to assess growth performance in rainbow trout after exposure to a high dose of hydrogen peroxide simulating a sea lice treatment episode and to establish possible mechanisms of growth disturbance such as chemical/hormone alterations and gill structural damage. Based on our results, we concluded that activation of primary and secondary stress responses and alteration of the gill morphology result from a single, high dose exposure to H_2O_2. These structural and physiological alterations may, in part, contribute to the demonstrated decreased growth performance in treated fish. The use of hydrogen peroxide as a chemotherapeutic agent at high doses should not be considered an innocuous procedure. Future research is required to investigate the effects of H_2O_2 in an industrial use pattern.

APPENDIX A



Allocation of tank treatments in growth trial 1.



Allocation of tank treatments in the growth trial 2.

APPENDIX B

Proximate Analysis of High Pro Grower fish feed[1]

Component	
Crude protein (min)	43.0%
Crude fat (min)	20.0%
Crude fibre(max)	3.0%
Sodium (actual)	0.6%
Calcium (actual)	0.8%
Phosphorus(actual)	1.0%
Vitamin A (min)	5,200 I.U./Kg
Vitamin D3 (min)	3,200 I.U./Kg
Vitamin E (min)	200 I.U./Kg

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

APPENDIX C

Percentage of mortality occurred during the two growth trials.

Trial No. 1					Trial No. 2					
Tank	# fish	dead fish	mort. (%)	Final # fish	Tank	# fish	dead fish	mort. (%)	survi-ving fish	Final # fish
Control 1	30	0	0.0	30	Control 1	30	0	0.0	30	20
Control 2	30	0	0.0	30	Control 2	30	0	0.0	30	20
Control 3	30	1	3.0	29	Treated 1	30	7	23.3	23	20
Control 4	30	1	3.0	29	Treated 2	30	20	66.6	10	20
Control 5	30	1	3.0	29	Cohort 1	30 (15 C; 15T)	11*	73.3	19	20 (10 C; 10 T)
Treated 1	30	1	3.0	29	Cohort 2	30 (15 C;15 T)	10*	66.6	20	20 (10 C; 10 T)
Treated 2	30	4	13.3	26						
Treated 3	30	11	36.6	19						
Treated 4	30	3	10.0	27						
Treated 5	30	7	23.3	23						

Note: Mortalities reported in trial 2 occurred in treated fish. Some control fish were treated within 24 h and then allocated to treated groups that had sustained major losses.

* 11 and 10 fish out of 15.

APPENDIX D

Radioimmunoassay procedure for the measurement of blood cortisol levels.

All samples were performed at room temperature (manufacture's recommended range 15-28°C)

1. **Plain tubes**: Four plain (uncoated) polypropylene tubes of 12 x 75 mm were labelled and NSB (nonspecific binding) in duplicated.

Coated tubes: Twelve Cortisol Ab-Coated tubes A (maximum binding) and B through F were labelled for the calibrators. Tubes with Cortisol Ab-Coated for control and samples were also labelled. All samples were analyzed in duplicate.

Calibrators	μg/dL	nmol/L
A(MB)	0	0
B	1	27.6
C	5	138
D	10	276
E	20	552
F	50	1,380

2. 25 μL of the zero calibrator A were pipetted into the NSB and A tubes. 25 μL of each remaining calibrator, control and fish samples were pipetted into the tubes prepared.

3. 1 ml of ^{125}I Cortisol was added to every tube and mixed in a vortex mixer.

4. The tubes were incubated for 45 min at 37°C.

5. Tubes were decanted thoroughly.

6. The tubes were placed in a gamma counter.

Radioimmunoassay procedure for the measurement of serum T_4 levels.

1. **Plain tubes**: Four plain (uncoated) polypropylene tubes of 12 x 75 mm were labelled and NSB (nonspecific binding) in duplicated.

Coated tubes: Twelve T_4 Ab-Coated tubes A (maximum binding) and B through F were labelled for the calibrators. Additional Antibody-coated tubes were labelled for controls and fish samples.

Calibrators	μg/dL	nmol/L
A(MB)	0	0
B	0.5	6.4
C	1.5	19.3
D	3	38.6
E	6	77.3
F	15	193

2. 25μL of the zero calibrator A were pipetted into the NSB and A tubes. 25μL of each remaining calibrator, control and fish samples were pipetted into the tubes prepared.

3. 1 ml of ^{125}I Canine T_4 was added to every tube and mixed in a vortex mixer briefly and gently.

4. The tubes were incubated for 2 hours at room temperature.

5. Tubes were decanted thoroughly.

6. The tubes were placed in a gamma counter.

Radioimmunoassay procedure for the measurement of serum T_3 levels.

1. **Plain tubes**: Four plain (uncoated) polypropylene tubes of 12 x 75 mm were labelled and NSB (nonspecific binding) in duplicated.

Coated tubes: Twelve T_3 Ab-Coated tubes A (maximum binding) and B through F were labelled for the calibrators. Additional Antibody-coated tubes were labelled for controls and fish samples.

Calibrators	μg/dL	nmol/L
A(MB)	0	0
B	20	0.31
C	50	0.77
D	100	1.54
E	200	3.07
F	600	9.22

2. 100 μL of the zero calibrator A were pipetted into the NSB and A tubes. 100 μL of each remaining calibrator, control and fish samples were pipetted into the tubes prepared.

3. 1 ml of ^{125}I Canine T_3 was added to every tube and mixed in a vortex mixer briefly and gently.

4. The tubes were incubated for 2 hours at 37°C.

5. Tubes were decanted thoroughly.

6. The tubes were placed in a gamma counter.

REFERENCES

ABEL PD, SKIDMORE JF. Toxic effects of an anionic detergent on the gills of rainbow trout. Water Res 1975; 9: 759-765.

ADAMS SM. Biological indicators of stress in fish. American Fisheries Society, 1990.

ALANÄRÄ L, BRÄNNÄS E. Dominance in demand-feeding behaviour in Arctic charr and rainbow trout: the effect of stocking density. J Fish Biol 1996; 48: 242-254.

ALDERMAN DJ. Malachite green: a review. J Fish Dis 1985; 8: 289-298.

ALLEN DM.. Measurement of serum thyroxine and the proportions of rhodopsin and porphyropsin in rainbow trout. Can J Zool 1977; 55: 836-842.

ANDERSON DP, MAYER FL. Salmonid pharmacology and toxicology. In: Stoskopf MK, eds. Fish Medicine. Philadelphia: WB Saunders, 1993: 432-439.

ARMSTRONG R. Therapeutant availability priorities of the Canadian salmon farming industry. Bull Aquacult Assoc Can 1994; 93-3: 8 -18.

ARNDT RE, WAGNER EJ. The toxicity of hydrogen peroxide to rainbow trout (*Oncorhynchus mykiss)* and Cutthroat trout (*Oncorhynchus clarki*) fry and fingerlings. J World Aquacult Soc 1997; 28: 150-157.

BARTON BA, IWAMA GK. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann Rev Fish Dis 1991; 1: 3-26.

BARTON BA, PETER RE, PAULENCU CR. Plasma cortisol levels of fingerling rainbow trout (*Salmo gairdneri*) at rest and subjected to handling, confinement, transport, and stocking. Can J Fish Aquat Sci 1980; 37: 805-811.

BARTON BA, SCHRECK CB, EWING RD, HEMMINGSEN AR, PATINO R. Changes in plasma cortisol during stress and smoltification in coho salmon, *Oncorhynchus kisutch.* Gen Comp Endocrinol 1985; 59: 468-471.

BEAULIEU A, GEENLESS K, HALEY C, KAHN L, OELLER M, BELL T, ORIANI JA. Aquaculture drug use: answers to commonly asked questions. FDA workshop-requirements for investigational new animal drugs- Eastern Fish Health Group and the American Fisheries Society Fish Health Section. Center for Veterinary Medicine, Rockville MD, US Food and Drug Administration, 1992.

BLAXHALL PC, DAISLEY KW. Routine haematological methods for used with fish blood. J Fish Biol 1973; 5: 771-781.

BOWSER PR. Clinical pathology of salmonid fishes. In: Stoskopf MK, eds. Fish Medicine. Philadelphia: WB Saunders, 1993: 327-332.

BRETT JR. Environmental factors and Growth. In: Hoar WS, DJ Randall and JR Brett, eds. Fish Physiology, Vol 8, London: Academic, 1979: 599-675.

BROWN ME. The growth of brown trout *(Salmo trutta Linn)*. I. Factors affecting the growth of trout fry. J Exp Biol 1946; 22:118-129.

BROWN SB, MACLATCHY DL, HARA TJ, EALES JG. Effects of cortisol on aspects of 3,5,3'-triiodo-L-thyronine metabolism in rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol 1991; 81: 207-216.

BRUNO DW, RAYNARD RS. Studies on the use of hydrogen peroxide as a method for the control of sea lice on Atlantic salmon. Aquacult Internat 1994; 2: 10-18.

BRUNO DW, MUNRO LS. Haematological assessment of rainbow trout *Salmo gairdneri* Richardson, and Atlantic salmon, *Salmo salar* infected with *Renibacterium salmoninarum.* J Fish Dis 1986; 9: 195-204.

BURKA JF, HAMMELL KL, HORSBERG TE, JOHNSON GR, RAINNIE DJ, SPEARE DJ. Drugs in salmonid aquaculture-a review. J Vet Pharmacol Ther 1997; 20:333-349.

BURKE MG, LEATHERLAND JF. Seasonal changes in the serum thyroid hormones levels of the feral brown bullhead, *Ictalurus nebulosus* Le sueur. J Fish Biol 1983; 23:585-593.

BUSACKER GP, ADELMAN IR, GOOLISH EM. Growth. In: Schreck CB, Moyle PB, eds. Methods for Fish Biology. Bethesda MD: Am Fish Soc 1990: 363-387.

BYRNE P, SPEARE D, FERGUSON HW. Effects of a cationic detergent on the gills and blood chemistry of rainbow trout *Salmo gairdneri.* Dis Aquat Org 1989; 6: 185-196.

CASILLAS E, SMITH LS. Effect of stress on blood coagulation and hematology in rainbow trout (*Salmo gairdneri*). J Fish Biol 1977; 10: 481-491.

CHANG B, PAGE G, MCCLELLAND G. Alternative treatment for sea lice in farmed salmon. Biological Station, St Andrews NB Fisheries Research Laboratory. DFO. Progress Report, 1997.

CHO CY. Feeding systems for rainbow trout and other salmonids with reference to current

estimates of energy and protein requirements. Aquacult 1992; 100: 107-123.

COTRAN RS, KUMAR R, ROBBINS SL. Cellular injury and cellular death. In: Cotran RS, Kumar R, Robbins SL, eds. Robbins Pathological Basis of Diseases, 4th ed. Toronto: WB Saunders, 1994:1-34.

COWEY CB. Nutrition: estimating requirements of rainbow trout. Aquacult 1992; 100: 177-189.

CUSACK R, JOHNSON G. A study of dichlorvos (Nuvan;2,2 dichloroethenyl dimethyl phosphate), a therapeutic agent for the treatment of salmonids infected with sea lice (*Lepeophtheirus salmonis*). Aquacult 1990; 90: 101-112.

CUTTS CJ, METCALFE NB, TAYLOR AC. Aggression and growth depression in juvenile Atlantic salmon: the consequences of individual variation in standard metabolic rate. J Fish Biol 1998; 52: 1026-1037.

DAOUST PY, WOBESER G, NEWSTEAD JD. Acute pathological effects of inorganic mercury and copper in gills of rainbow trout.Vet Pathol 1984; 21:93-101

DAOUST PY, FERGUSON HW. Nodular gill disease: a unique form of proliferative gill disease in rainbow trout *Salmo gairdneri* Richardson. J Fish Dis 1985; 8: 511-522.

DE RENZIS G, BORNANCIN M. Ion transport and gill ATPases. In: Hoar WS, Randall DJ, eds. Fish Physiology, Vol 10, Pt B. Orlando: Academic, 1984: 65-104.

DE SILVA SS, ANDERSON TA. Fish nutrition in Aquaculture. Chapman and Hall Aquaculture Series 1. Bury St Edmunds Suffolk Great Britain 1995: 1-14

DOBSON SH. HOLMES RM. Compensatory growth in the rainbow trout, *Salmo gairdneri* Richardson. J Fish Biol 1984; 25:649-656.

DOUGLAS AS, DONALD MW. Fundamentals of Analytical Chemistry. 4th ed. Philadelphia: WB Saunders, 1982: 366-371.

DOUGHERTY TF, WHITE A. Functional alterations in lymphoid tissue induced by adrenal cortical secretion. Amer J Anat 1945; 77:81-116.

EALES JG. The peripheral metabolism of thyroid hormones and regulation of thyroidal status in poikilotherms. Can J Zool 1985; 63: 1217-1231.

EALES JG, MACLATCHY DL. The relationship between T_3 production and energy balance in salmonids. Fish Physiol Biochem 1989; 7:289-293.

EALES JG. Thyroid function in poikilotherms. Prog Clin Biol Res. 1990;342:415-420.

ELLIOTT BJM. Body composition of brown trout (*Salmo trutta*) in relation to temperature and ration size. J Animal Ecology 1976; 45: 273-289.

ELLIS AE. The leukocyte of fish.: a review. J Fish Biol 1977; 11:453-491.

ESPELID S, LOKKEN GB, BOGWALD J. Effects of cortisol and stress on the immune system in Atlantic salmon (*Salmon salar L*). Fish Shellfish Immunol 1996; 6:95-110.

EVANS DH. The fish gill: site of action and model for toxic effects of environmental pollutants. Exp Health Perspect 1987; 71:47-58.

EVANS DH. Osmotic and Ionic regulation. In: Evans DH, eds. The Physiology of Fishes. Boca Raton: CRC, 1993: 315-341.

FABER J. Pituitary-thyroid axis in critical illness. J Clin Endocrinol Metabol 1987; 65: 325.

FERGUSON HW. Systemic Pathology of fish. A text and atlas of comparative tissue responses in diseases of teleosts. Iowa State University. 1989: 125-145.

FDA. Aquaculture Drugs. Ch. 11. In: Fish and Fishery Products Hazards and Controls Guide, Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, DC. 1996: 103-116.

FITZPATRICK MS, SCHRECK CB, CHITWOOD RL. Evaluation of three candidate fungicides for treatment of adult spring chinook salmon. Progr Fish Cult 1995; 57:153-155.

FROM J. Chloramine-T for control of bacterial gill disease. Progr Fish Cult 1980; 42: 85-86.

GERUNDO N, ALDERMAN DJ, CLIFTON-HADLEY RS, FEIST SW. Pathological effects of repeated doses of malachite green: a preliminary study. J Fish Dis 1991; 14: 521-532.

GOTOH Y, COOPER JA. Reactive oxygen species-and dimerization-induced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor-alpha signal transduction. J Biol Chem 1998; 28:17477-17482.

GRAVE K, ENGELSTAD M, SOLI NE, HÅSTEIN T. Utilization of antibacterial drugs in salmonid farming in Norway during 1980-1988. Aquacult 1990; 86: 347-358.

HEATH AG. Water Pollution and Fish Physiology 2nd ed. Boca Raton: Lewis Pub., 1995.

HEISLER N. Acid-base regulation in fishes. In: Hoar WS, Randall DJ, eds. Fish Physiology, Vol 10, Pt A. Orlando: Academic, 1984: 315-401.

HEMALATHA S, BANERJEE TK. Histopathological analysis of sublethal toxicity of zinc chloride to the respiratory organs of the air breathing catfish *Heteropneustes fossilis* (Bloch). Biol Res 1997; 30:11-21.

HERWIG N. Handbook of drugs and chemicals used in the treatment of fish diseases. Springfield IL: Charles C. Thomas Pub, 1979.

HESSER EF. Methods for routine fish hematology. Bureau of Sport Fisheries and Wildlife, Western Fish Nutrition Laboratory. Progr Fish Cult. 1960: 164-171.

HIGGS DA, FAGERLUND UHM, EALES JG, MCBRIDE JR. Application of thyroid and steroid hormones as anabolic agents in fish culture. Comp Biochem Physiol 1982; 73 B: 143-176.

HIGGS DA, FAGERLUND UHM, McBRIDE JR, EALES JG. Influence of orally administered L-thyroxine or 3.5.3'-triiodo-L-thyroxine on growth, food consumption and food conversion of under yearling coho salmon (*Oncorhynchus kisutch*). Can J Zool 1979; 57: 1974-1979.

HILLE S. A literature review of the blood chemistry of rainbow trout, *Salmo gairdneri* Richardson. J Fish Biol 1982; 20:535-569.

HOFFMANN R, LOMMEL R. Effect of repeated blood sampling of some blood parameters in fresh water fish. J Fish Biol 1984; 24: 245-251.

HOGMAN WJ. Annulus formation on scales of four species of coregonids reared under artificial conditions. J Fish Res Board Can 1968; 25: 2111-2112.

HOPKINS KD. Reporting fish growth: a review of the basics. J World Aquacult Soc 1992; 23: 173-179.

HOSKINS GE, DALZIEL FC. Survival of chinook fry (*Oncorhynchus tshawytscha*) following exposure to benzalkonium chloride in soft water. Progr Fish Cult 1984; 46: 98-101.

HOUSTON AH. Blood and circulation. In: Schreck CB, Moyle PB, eds. Methods for fish Biology. Bethesda: American Fisheries Society; 1990: 273- 334.

HØY T, HORSBERG TE, WICHSTRØM R. Inhibition of acetylcholinesterase in rainbow trout following dichlorvos treatment at different water oxygen levels. Aquacult 1991; 95: 33-

40.

HUGHES GM. Morphological measurements on the gills of fishes in relation to their respiratory function. Folia Morphol (Praha) 1970;18:78-95.

HUGHES GM. General anatomy of the gills. In: Hoar WS, Randall DJ, eds. Fish Physiology, Vol 10, Pt A. Orlando: Academic, 1984: 1-72.

HUNT DW, EALES JG. Iodine balance in rainbow trout (*Salmo gairdneri*) and effects of testosterone propionate. J Fish Res Bd Can 1979; 36: 282-285.

ISAIA J. Water and non-electrolyte permeation In: Hoar WS, Randall DJ, eds. Fish Physiology, vol 10, Pt B. Orlando: Academic, 1984: 1-38.

IWAMA GR, MORGAN JD, BARTON BA. Simple field methods for monitoring stress and general condition of fish. Aqua Res 1995; 25:272-282.

JOBLING M. Fish Bioenergetics. London: Chapman & Hall, 1994: 309.

JOHNSON SC, JM CONSTIBLE, J RICHARD. Laboratory investigations on the efficacy of hydrogen peroxide against salmon louse *Lepeophtheirus salmonis* and its toxicological and histopathological effects on Atlantic salmon *Salmo salar* and chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 1993; 17: 197-204.

KAPTEIN EM. Relationship of altered thyroid hormone indices to survival in nonthyroidal illnesses. Clin Endocrinol 1982; 16: 565.

KARAN V, VITOROVI&CACUTE S, TUTUNDZI&CACUTE V, POLEKSI&CACUTE V. Functional enzymes activity and gill histology of carp after copper sulfate exposure and recovery. Ecotoxicol Environ Saf 1998; 40:49-55.

KIEMER MCB, BLACK KD. The effect of hydrogen peroxide on the gill tissue of Atlantic salmon, *Salmo salar* L. Aquacult 1997; 153: 181-189.

LANNO RP. Nutrition and toxicity testing with juvenile and adult fish. Proceedings: Nutritional considerations in toxicity testing, 10th Annual SETAC Meeting, Toronto, Ontario, 1989.

LAUREN P. Gill internal morphology. In: Hoar WS, Randall DJ, eds. Fish Physiology, Vol 10, Pt A. Orlando: Academic, 1984: 73-183.

LEATHERLAND JF. Reflections on the thyroidology of fishes: from molecules to humankind. Guelph Ichthyol Rev 1994; 2:1-67.

LI HW, BROCKSEN RW. Approaches to the analysis of energetic costs of intraspecific competition for space by rainbow trout (*Salmo gairdneri*). J Fish Biol 1977; 11:329-334.

LIEBERTHAL W, LEVINE JS. Mechanisms of apoptosis and its potential role in renal tubular epithelial cell injury. Am J Physiol 1996 271:477-488.

MACKINNON BM. Sea lice: a review. World Aquacult 1997(a); 28: 5-10

MACKINNON BM. Control of sea lice infections in salmonids aquaculture: alternatives to drugs. Bull Aquacult Assoc Canada. AAC Special Publication 1997(b); 2:61-64.

MALLATT J. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Can J Fish Aquat Sci 1985; 42: 630-48.

MARGOLIS L. The efficacy of hydrogen peroxide against the salmon louse *Lepeophtheirus salmonis*. Its toxicological effects on Atlantic and Chinook salmon, its stability in sea water, and its toxic effects on some non-target marine species. Canada Department of Fisheries and Oceans, Pacific Biological Station, Aquaculture update 63, December 30. Nanaimo, 1993.

MARKING LL, RACH JJ, SCHREIER TM. Evaluation of antifungal agents for fish culture. Progr Fish Cult 1994; 56:225-231.

MAZEAUD MM, MAZEAUD F. Adrenergic response to stress in fish. In: Pickering AD, ed. Stress and Fish. London: Academic, 1981: 49-75.

McANDREW KJ, SOMMERVILLE C, WOOTTEN R, BRON JE. The effect of hydrogen peroxide treatment on different life-cycle stages of the salmon louse, *Lepeophtherius salmonis* (krøyer 1837). J Fish Dis 1998; 21:221-228.

McCARTHY ID, HOULIHAN DF, CARTER CG, MOUTOU K. Variation in individual food consumption rates of fish and its implications for the study of fish nutrition and physiology. Proc Nutr Soc 1993; 52: 427-436.

McDONALD DG. The effect of H^+ upon the gills of fresh water fish. Can J Zool 1983; 61: 691-703.

MCLEAY DJ. Effect of cortisol and dexamethasone on the pituitary-internal axis and abundance of white blood cell types in juvenile coho salmon, *Oncorhynchus kisutch*. Gen Comp Endocrinol 1973; 21: 441-450.

METCALFE NB. The role of behaviour in determining salmon growth and development. Aquacult Fish Mgt 1994; 25:67-76.

MEYER FP, JØRGENSON TA. Teratological and other effects of malachite green on development of rainbow trout and rabbits. Trans Amer Fish Soc 1983; 112: 818-824.

MEYER F P, ROBINSON J. Branchiomycosis: a new fungal disease of North American fishes. Prog Fish Cult 1973; 35: 74 -77.

MILNE RS, LEATHERLAND J, HOLUB B. Changes in plasma thyroxine, triiodothyronine and cortisol associated with starvation in rainbow trout (Salmo gairdneri). Envir Biol Fish 1979; 4: 185-190.

MORGAN M, TOVELL WA. The structure of the gills of the trout, *Salmo gairdneri*. Z. Zellforsh. Microsk Anat 1973; 142: 147-162.

MORTENSEN A, DAMSGARD B. Compensatory growth and weight segregation following light and temperature manipulation of juvenile Atlantic salmon (*Salmon salar* L.) and Arctic charr (*Salvenius alpinus* L.) Aquacult 1993; 114:261-272.

NEWMAN MA. Social behaviour and intraspecific competition in two trout species. Physiol Zool 1956; 29:64-81.

NOAKES DL, LEATHERLAND JF. Social dominance and interrenal cell activity in rainbow trout *(Salmo gairdneri)*. Envir Biol Fish 1977; 2:131-136.

NOGA EJ. Fish Disease: Diagnosis and Treatment. St. Louis: Mosby, 1996. 367 p

PAYAN P, GIRARD JP. Branchial ion movements in teleosts: The role of respiratory and chloride cells. In: Hoar WS, Randall DJ, eds. Fish Physiology, Vol 10, Pt B. Orlando: Academic, 1984: 39-63.

PETERS G. The effect of stress on the stomach of the European eel, *Anguilla anguilla* J Fish Biol 1982; 21: 497-512.

PETERS G, HONG LQ. Gill structure and blood electrolyte levels of European eels under stress. In: Ellis AD, eds. Fish and Shellfish Pathology. London, 1985: 183-196.

PICKERING AD, STEWART A. Acclimation of the interrenal tissue of brown trout *Salmo trutta* L. to chronic crowding stress. J Fish Biol 1984; 24:731-740.

PICKERING AD. Changes in cell blood composition of brown tout *Salmo trutta* L. During the spawning season. J Fish Biol 1986; 29: 335-347.

PICKERING AD. Stress and the suppression of somatic growth in teleost fish. In: Epple A, Canes CG, Stetson MH, eds. Progress in Comparative Endocrinology. New York: Wiley-

Liss, 1990: 473-479.

PICKERING AD, POTTINGER TG, SUMPTER JF, CARRAGHER JF, LE BAIL PY. Effects of acute and chronic stress on the levels of circulating growth hormones in the rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol 1991; 83: 86-93.

PICKERING AD. Rainbow trout husbandry: management of the stress response. Aquacult 1992; 100: 125-139.

PICKERING AD. Growth and stress in fish production. Aquacult 1993; 111:52-63.

PICKERING AD, POTTINGER TG. Biochemical effects of stress. In: Hochachka PW, Mommsen TP, eds. Environmental and Ecological Biochemistry. Amsterdam: Elsevier, 1995: 349-379.

PIPER RG. Fish hatchery management. US Fish and Wildlife Services. Washington DC. 1982.

PLAYLE RC, WOOD CM. Water chemistry changes in the gill microenvironment of rainbow trout: experimental observations and theory. J Comp Physiol 1989; 159: 527-537.

POWELL MD, SPEARE DJ, MACNAIR N. Effects of intermittent chloramine-T exposure on growth, serum biochemistry, and fin condition of juvenile rainbow trout (*Oncorhynchus mykiss*). Can J Fish Aquat Sci 1994; 51: 1728-1736.

POWELL MD, WRIGHT GM, SPEARE DJ. Morphological changes of rainbow trout (*Oncorhynchus mykiss*) gill epithelia following repeated intermittent exposure of chloramine-T. Can J Zool 1995; 73: 154-165.

POWELL MD, PERRY SF. Respiratory and acid-base pathophysiology of hydrogen peroxide in rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquat Toxicol 1997; 37:99-112.

RACH JJ, SCHREIER TM, HOWE GE. Toxicity of hydrogen peroxide to different life stages and species of fish. Amer Fish Soc Fish Hlth Newsletter 1995; 23: 12-14.

RACH JJ, SCHREIER TM, HOWE GE, REDMAN SD. Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. Progr Fish Cult 1997; 59: 41-46.

RAILO E, NIKINMAA M, SOIVIO A. Effect of sampling on blood parameters in the rainbow trout Salmo gairdneri Richardson. J Fish Biol 1985; 26: 725-732.

RANDALL D, DAXBOECK C. Oxygen and carbon dioxide transfer across fish gills. In:

Hoar WS, Randall DJ, eds. Fish Physiology, Vol 10, Pt A. Orlando: Academic, 1984: 263-314.

REDDING JM, BIRKS CB, EK, EWING RD. Cortisol and its effects on plasma thyroid hormone and electrolytes concentrations in fresh water and during seawater acclimation in yearling coho salmon, *Oncorhynchus kisutch*. Gen Comp Endocrinol 1984; 56: 146-155.

REDDING JM, DE LUZE A, LELOUP-HATEY J, LELOUP J. Suppression of plasma thyroid hormone concentrations by cortisol in the European eel *Anguilla anguilla*. Comp Biochem Physiol. 1986; 83 A: 409-413.

REICHENBACH-KLINKE H. Lesions due to drugs. In: Ribelin WE, Migaki G, eds. Pathology of Fishes. University of Wisconsin Press, 1975: 647-656.

RICKER WE. Growth rates and models In: Hoar WS, Randall DJ and Brett JR, eds. Fish Physiology, Vol 8, Bioenergetics and growth. New York: Academic, 1979: 677-743.

ROBERTS RJ. The pathophysiology and systemic pathology of teleosts. In: Roberts RJ, eds. Fish Pathology. Bailliere Tindall, 1978: 55-91.

ROBERTS S. Treatment of bacterial gill disease with hydrogen peroxide. Amer Fish Soc Fish Hlth Newsletter 1995; 23: 4-5.

RONNEY EA, WAGNER EJ. The toxicity of hydrogen peroxide to rainbow trout (*Oncorhynchus mykiss*) and Cutthroat trout (*Oncorhynchus clarki*) fry and fingerlings. J World Aquacult Soc 1997; 28:150-157.

ROSS LG, WARD KMH, ROSS B. The effect of formalin, malachite green, and suspended solids on the respiratory activity of rainbow trout (*Salmo gairdneri*, Richardson). Aquacult Fish Mgt 1985; 16:129-138.

ROWLEY AF, et al. Fish. In: Rowley AF, Ratcliffe HA eds. Vertebrate Blood Cells. New York: Cambridge University Press, 1988: 19-127.

SALTE, SYVERTSEN C, KJONNOY M, FONNUM F. Fatal acetylcholinesterase in salmonids subjected to a routine organophosphate treatment. Aquacult 1987; 61: 173-179.

SANCHEZ JG, SPEARE DJ, MACNAIR N, JOHNSON G. Effects of intermittent prophylactic chloramine-T treated on growth performance and condition indices of rainbow trout (*Oncorhynchus mykiss*). J Aquat Anim Hlth 1996; 8: 278-284.

SATCHELL GH. Respiratory toxicology of fish. In: Weber LJ, eds. Aquatic Toxicology, Vol 2. New York: Raven, 1984:1-50.

SATOH T, SAKAI N, ENOKIDO Y, UCHIYAMA Y, HATANA. Free radical-independent protection by nerve growth factor and Bcl-2 of PC12 cells from hydrogen peroxide-triggered apoptosis. J Biochem 1996; 120: 540-546.

SANDERS EJ. Methods for detecting apoptotic cells in tissues. Histol Histopathol 1997; 4: 1169-1177.

SELYE H. A syndrome produced by diverse noxious agents. Nature Lond 1936; 138: 32.

SCOTT P. Therapy in aquaculture. In: Brown LB, eds. Aquaculture for Veterinarians. Oxford: Pergamon, 1993: 131-152.

SHAW HM, SAUNDERS RL, HALL HC, HENDERSON EB. The effect of dietary sodium chloride on growth of Atlantic salmon (*Salmo salar*). J Fish Res Bd Can 1975; 32: 1821-1824.

SHIELDS CA, EALES JG. Thyroxine 5'-monodeiodinase activity in hepatocytes of rainbow trout *Salmo gairdneri*: distribution, effects of starvation and exogenous inhibitors. Gen Comp Endocrinol 1986; 63: 334-343.

SMITH CE, PIPER RB. Pathological effects in formalin-treated rainbow trout (*Salmo gairdneri*). J Fish Res Bd Can 1972; 29: 328-329.

SNIESZKO SF. The effect of environmental stress on outbreaks of infectious diseases of fishes. J Fish Biol 1974; 6:197-208.

SPEARE DJ, FERGUSON HW. Fixation artifacts in rainbow trout (*Salmo gairdneri*) gills: A morphometric evaluation. Can J Fish Aquat Sci 1989; 46: 780-785.

SPEARE DJ, FERGUSON HW. Clinical and pathological feature of common gill diseases of cultured salmonoids in Ontario. Can Vet J 1992; 30: 882-887.

SPEARE DJ, MACNAIR N, HAMMELL L. Demonstration of tank effect on growth indices of juvenile rainbow trout (*Oncorhynchus mykiss*) during an *ad libitum* feeding trial. Am J Vet Res 1995; 56: 1372-1379.

SPEARE DJ, MACNAIR N. Effects of intermittent exposure to therapeutic levels of formalin on growth characteristics and body condition of juvenile rainbow trout. J Aquat Anim Hlth 1996; 8: 58-63.

SPEARE DJ, ARSENAULT GJ. Effects of intermittent hydrogen peroxide exposure on growth and columnaris disease prevention of juvenile rainbow trout (*Oncorhynchus mykiss*). Can J Fish Aquat Sci 1997; 54: 2653-2658.

SPEARE DJ, CARVAJALV, HORNEY BS. Growth suppression and branchitis in trout from hydrogen peroxide exposure: a pilot study model to examine a current sea lice treatment strategy. J Comp Pathol in press - 1998.

STOSKOPF MK. Clinical pathology. In: Stoskopf MK, eds. Fish Medicine. Philadelphia: WB Saunders, 1993: 113-131.

SUGIYAMA H, KASHIHARA N, MAKINO H, YAMASAKI Y, OTA Z. Reactive oxygen species induce apoptosis in cultured human mesangial cells. Am Soc Nephrol 1996; 7:2357-2363.

SUMPTER JP, DONALDSON EM. The development and validation of radioimmunoassay to measure plasma ACTH levels in salmonids fishes. Gen Comp Endocrinol 1986; 62: 367-376.

THOMASSEN JM. Hydrogen peroxide as a delousing agent for Atlantic salmon. In: GA Boxhall and Defaye D, eds. Pathogens of wild and farmed fish: sea lice. Chichester: Ellis Horward, 1993: 290-295.

THORBURN MA, MOCCIA RD. Use of chemotherapeutics on trout farms in Ontario. J Aquat Anim Hlth 1993; 5: 85-91.

TREASURER JW, GRANT A. The efficacy of hydrogen peroxide for the treatment of farmed Atlantic salmon, *Salmo salar* L. infested with sea lice (Copepoda: Caligidae). Aquacult 1997; 148:265-275.

UCKER PS. Death by suicide: one way to go in mammalian cellular development? New Biol 1991; 2: 103-109.

UTIGER RD. Decreased extrathyroidal triiodothyronine production in nonthyroidal illness: Benefit or harm?. Am J Med 1980; 69: 807.

VAUGHAN DS, YOSHIYAMA RM, BRECK JE, ANGELIS DI. Review and analysis of existing modeling approaches for assessing population-level effects of multiple stresses on fish and shellfish. Oak Ridge Natl. Lab Publication 1982; 1-36

VIGNIER V, VANDERMEULEN JH, FRASER AJ. Growth and food conversion by Atlantic salmon parr during 40 days' exposure to crude oil. Trans Am Fish Soc 1992; 121: 322-332.

WARTOFSKY L, BURMAN KD. Alteration in the thyroid function in patients with systemic illness: The "euthyroid sick syndrome". Endocr Rev 1982; 3: 164-217.

WEDEMEYER GA.The role of stress in disease resistance of fish. In: Snieszko SF, eds. A Symposium of Diseases of Fishes and Shellfishes. Am Fish Soc Spec Publ 1970; 5: 30-35.

WEDEMEYER GA.The stress of formalin treatments in rainbow trout (*Salmo gairdneri*) and coho salmon (*Oncorhynchus kisutch*). J Fish Res Bd Can 1971; 28:1899-1905.

WEDEMEYER GA, GOULD RW, YASUTAKE WT. Some potentials and limits of the leucocrit test as a fish health assessment method. J Fish Biol 1983; 23: 711-716.

WEDEMEYER GA, BARTON BA, MCLEAY AJ. Stress and acclimatization. In: Schreck CB, Moyle PB, eds. Methods for Fish Biology. Bethesda: American Fisheries Society, 1990: 541-489.

WENDELAAR BONGA SE, VAN DER MEIJ CJM. Degeneration and death, by apoptosis and necrosis of the pavement and chloride cells in the gills of teleost *Oreochromis mossambicus*. Cell Tiss Res 1989; 255: 235-243.

WENDELAAR BONGA SE, LOCK RAC. Toxicants and osmoregulation in fish. Neth J Zool 1992; 42: 478-493.

WENDELAAR BONGA SE. The stress response in fish. Physiol Rev 1997; 77:591-625.

WITTERS HE, PUYMBROECK SV, VANDERBORHT. Adrenergic response to physiological disturbances in rainbow trout, (*Oncorhynchus mykiss*), exposed to aluminum at acid pH. Can J Fish Aquat Sci 1991; 48: 414-420.

WYLLIE AH. Cell death: A new classification separating apoptosis from necrosis. In: Bowen IDL, eds. Cell Death in Biology and Pathology. London, Chapman and Hall, 1981: 9-34.

YAEL G, SHERMAN Y, BEN-SASSON A. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol 1992; 119: 493-501.

XU Y, BRADHAM C, BRENNER DA, CZAJA MJ. Hydrogen peroxide-induced liver cell necrosis is dependent on AP-1 activation. Am J Physiol 1997; 273:795-803.

YASUTAKE WT, WALES JH. Microscopic Anatomy of Salmonids: An Atlas. Washington: Department of the Interior Fish and Wildlife Service, 1983.

IMAGE EVALUATION TEST TARGET (QA-3)

150mm

6″

APPLIED IMAGE . Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989