# EFFECTS OF A CHLORAMINE-T REGIME ON JUVENILE RAINBOW TROUT

## (*Oncorhynchus mykiss)*

A Thesis

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## ABSTRACT

Chloramine-T is a chlorine-based disinfectant agent, widely used in salmonid aquaculture to treat and prevent disease outbreaks, despite the paucity of studies which address its target animal safety. In this thesis, an experiment was devised to address the effects of this chemical on the production performance of juvenile rainbow trout during an 11-week growth trial conducted under conditions which simulated those of intensive aquaculture. Chloramine-T significantly suppressed the growth of treated fish ($\delta$=7.3%) through a significant suppressive effect on feed conversion efficiency. The suppressive effects of chloramine-T were most dramatic in the early phase of the growth trial, but persisted throughout its duration. Subsequent studies were designed to address whether growth disturbances in treated fish related to activation of a stress response and/or changes to gill and skin microanatomy. From these studies we found that exposure of trout to a chloramine-T treatment regime used on the growth trial did not cause a significant change in cortisol levels (the marker for activation of the primary stress response) or hematocrit, plasma glucose, sodium and chloride (the markers for the secondary stress response), leading to the conclusion that chloramine-T treatment was non-stressful to the experimental animals in this study. The hypothesis that chloramine-T was acting as gill toxicant was also largely rejected. Specifically, chloramine-T did not cause an increase in the frequency of a range of gill lesions which typically arise due to water-borne toxicants (such as free chlorine). However, fish treated with chloramine- T had an upward shift in the ratio of sialic-acid containing mucous cells compared to neutral mucous cells. Ultrastructural changes in the skin and gills of treated fish were assessed using a non-aqueous fixative regime adapted from methodologies used in mammalian airway studies. This method allows the simultaneous evaluation of the ultrastructure of gill or skin tissue along with the overlying mucous biofilm. Chloramine-T caused alterations in the epidermis of treated fish by decreasing its thickness, and increasing the number of electron-dense vesicles in epithelial pavement cells. Gill lamellae of treated fish had expanded interstitial spaces and increased numbers of chloride cells. However, treatment did not affect the morphology of the mucous covering of the gill or skin, nor did it change the number of mucous cells/mm$^2$, or the electron density of the mucosomes of the skin. These tissue changes are probably insufficient to be considered as significant mechanisms accounting for the effect of chloramine-T on growth rates, and further study is therefore warranted to derive the mechanism. It is concluded that treatment of rainbow trout with chloramine-T is likely to be relatively innocuous. Repeated treatment of healthy rainbow trout with a dose level currently recommended in the literature does not appear to evoke a stress response and evokes only minor changes in gill and morphology. The effect on growth is of concern. However, this regime helps to reduce the incidence (and economic effects) of infectious gill diseases in intensively reared populations of salmonids.

## DEDICATION

A mis padres, Genaro y Josefina, con amor y respeto. Gracias

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| AB2 | - | Alcian Blue pH 2.5 |
| ACTH | - | Adrenocorticotropic hormone |
| A.D. | - | Anno Domini |
| ANOVA | - | Analysis of variance |
| B.C. | - | Before Christ |
| Bw | - | Body weight |
| bl | - | Basal lamina |
| ca. | - | circa |
| CC | - | Chloride cell |
| cd | - | Cell debris |
| cm | - | Centimetre |
| CRF | - | Corticotropin release factor |
| D | - | Dermis |
| DNA | - | Deoxyribonucleic acid |
| δ | - | Difference |
| E | - | Epidermis |
| e.g. | - | Exempli gratia; for example |
| FAO | - | Food and Agriculture Organization |
| FCE | - | Feed conversion efficiency |
| FCI | - | Feed conversion index |
| Fig. | - | Figure |
| Fl | - | Fork length |
| g | - | Gram |
| GAS | - | General adaptation syndrome |
| h | - | Hour |
| ha | - | Hectare |
| H&E | - | Haematoxylin and Eosin |
| HPI | - | Hypothalamic-pituitary-interrenal |
| i.e. | - | id est; that is |
| Inflamm. | - | Inflammation |
| Interlam. | - | Interlamellar |
| K | - | Condition factor |
| kg | - | Kilogram |
| L | - | Litre |
| Lam. | - | Lamellar |
| m | - | microridge |
| M | - | Mucus; mucous layer |
| $m^3$ | - | Cubic metre |
| MC | - | Mucous cell |
| MCNv | - | Mucous cell number density |

| | | |
|---|---|---|
| MCVv | - | Mucous cell volume density |
| mEq | - | Milliequivalent |
| min | - | Minute |
| mg | - | Milligram |
| ml | - | Millilitre |
| $mm^2$ | - | Square millimetre |
| ms | - | Mucosome |
| Mw | - | Mean weight |
| ng | - | Nanogram |
| P | - | P value |
| PAS | - | Periodic-acid Schiff |
| PCV | - | Packed volume cell |
| ppm | - | Parts per million |
| RBC | - | Red blood cells |
| RIA | - | Radioimmunoassay |
| RNA | - | Ribonucleic acid |
| s | - | Second |
| SD | - | Standard deviation |
| sec | - | Second |
| SEM | - | Scanning electron microscopy |
| SGR | - | Specific growth rate |
| ™ | - | Trade mark. |
| TEM | - | Transmission electron microscopy |
| TMS | - | Tricaine methane sulphonate |
| vs | - | Versus |
| $X^2$ | - | Chi square |

# 1. GENERAL INTRODUCTION

## 1.1 Aquaculture.

Aquaculture is a broad term that describes the rearing and production of aquatic animals and plants by man, under controlled or semi-controlled conditions. Aquaculture has four main categories of production: algae, mollusks, crustaceans and fish (Barnabé 1994).

The objectives of aquaculture vary depending on the economic context in which they are found. In industrialized countries, the main objective is the production of high value products for human consumption. In the non-industrialized countries the objective is the production of animal protein to support the usually growing population. Aquaculture also plays an important role in the production of fish for commercial or recreational fishing, for restocking natural populations, for the introduction of new species, and for the supply of ornamental fish.

A working definition of aquaculture is the following:

> "Aquaculture is the farming of aquatic organisms, including fishes, mollusks, crustaceans, and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as stocking, fertilizing, feeding, habitat manipulation, and protection from predators. Farming also implies individual or corporate ownership of the stock being cultivated" (FAO 1991).

### 1.1.1 History of Aquaculture

Aquaculture has been practiced in various parts of the world for at least ten thousand years. As long as four thousand years ago, it played an important role in the Chinese culture, while the Egyptians cultivated tilapia (*Oreochromis niloticus)* in ponds at around 2500 B.C., and the Japanese cultured oysters (*Crassostrea* sp.) as long ago as 2000 B.C. (Boghen 1995). The first known text on aquaculture was written by Fan Li in 475 B.C. in China (Jolly and Clonts 1993, Ackefors et al. 1994, Barnabé 1994, Boghen 1995), and addressed the lucrative potential of spawning carp (*Cyprinus carpio*) in captivity. Europe saw the introduction of aquaculture about two thousand years ago, with mentions of carp culture by Aristotle. Carp were fattened in ponds by the Greeks and Romans after being caught in the wild (Ackefors et al. 1994). The rearing of fishes through their entire cycle did not develop until much later in Europe (ca. 1150 A.D.), a tradition that was continued and disseminated by monks so that by the eighteenth century, carp farming was practiced in most European countries, and by the 1850's this activity had become well-established (Ackefors et al. 1994).

Aquaculture has been a more recent phenomenon in America, associated with the restocking of rivers for the purpose of food production (Boghen 1995). During the nineteenth century rainbow trout *(Oncorhynchus mykiss*) was bred for sport fisheries and was transplanted from its indigenous to its actual culture range all over the world (Ackefors et al. 1994). In Canada, the earliest recorded attempts concerning aquaculture date from 1857 in Quebec, when the incubation and hatching of Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*) eggs were studied (Boghen 1995).

As the methodology for rearing fish developed with the advent of supplemental life support systems, like air pumps for oxygen and filters for toxin removal (Noga 1996), the possibilities of increasing the carrying capacity of a tank led to a sharp increase in the intensification of fish farming. However, with this increase in intensification of fish culture, the possibilities of a disease outbreak also increase, since the water quality conditions, created by unnatural crowding, can quickly deteriorate if they are not closely monitored (Saunders 1995). Several of these water quality problems frequently result in opportunistic infections (Noga 1996).

### 1.1.2 Aquaculture in the world.

The aquaculture industry is growing rapidly. In only 15 years, the aquaculture production has more than tripled, from 6 million tons in 1976 to over 18 million tons in 1992. By projecting these figures to the year 2000, fish production due to aquaculture should reach 22 million tons. Presently aquaculture represents 18.5% (Boghen 1995) of the total production from fisheries and it is estimated that this will reach 25% by the year 2000 (Barnabé 1994). Fisheries depend heavily on natural ecosystems. It is estimated that the biomass yield for the earth's oceans is less than 2 kg/ha per year (Ackefors et al. 1994). The low yield and the increasing demand for food from the world population are depleting the marine resources. As an alternative to this, aquaculture ecosystems are highly intensive, have a much higher yield per unit area, and are a good alternative to the traditional fishery production, since the demand for fish keeps increasing (Ratafia 1995).

Most of the aquaculture production in the world is done in Asia (84.1%), followed

by Europe (8.5%), North America (3.7%), South America (1.8%), former USSR (0.9%), Africa (0.5%) and Oceania (0.5%) (Boghen 1995). Finfish account for 52.3% of the total world aquaculture production, mollusks for 21.6% and algae for 21.4% (Ackefors et al. 1994). In 1994, there were 1111 companies and other organizations involved in aquaculture in 43 countries (Ratafia 1995). The most important species being cultured are carp, tilapia, catfish (*Ictalurus punctatus*), oysters, shrimp (*Penaeus* sp.), rainbow trout, Atlantic salmon, and coho salmon (*Oncorhynchus kisutch*). Most of the companies involved in aquaculture are rearing shrimp (28.2%), with the number of companies rearing salmon (16.77%) and trout (13.65%) following closely, and catfish (13.16%) in fourth place (Ratafia 1995). The remaining 28.22% of aquaculture companies cultivate, in decreasing order of importance, tilapia, eel, carp, clam, mussels, etc.

### 1.1.3 Aquaculture in Canada.

North America accounts for 3.7% of the global aquaculture production. Canadian production has increased considerably between 1984 and 1992, when it climbed from the fiftieth to the twenty-ninth position among the 154 nations where aquaculture is practiced (Boghen 1995). The trend in Canadian aquaculture is the farming of salmonids, which command the highest price in Canada.

The development of aquaculture in Canada has not been as fast nor as spectacular when compared to the development observed in other industrialized countries with a similar climate and suitable water resources. Several reasons have been attributed to this, one being the harsh climate in some parts of the country, but also the fact that Canada has

a rich tradition of fisheries. By having the longest fishing coastline in the world, it still has one of the most productive fisheries in the world. This has somewhat marginalized aquaculture related activities. Another reason for the slow development of aquaculture is that domestic rates of fish consumption are relatively low.

Despite all this, the aquaculture industry has developed in Canada because of a growing awareness that aquaculture was being successfully conducted in other parts of the world and that it represented the possibility of financial benefits and employment opportunities (Boghen 1995). Aquaculture producers seek to explore more efficient ways to produce larger and healthier fish, making the global aquaculture industry a good source of opportunities to develop and produce growth enhancing feed, feed additives, chemicals, pharmaceuticals, vaccines, and therapeutics (Ratafia 1995).

Most aquaculture in Canada occurs on the West Coast and the Atlantic Region. In the West Coast, the industry developed rapidly after its foundation in the late 1970's, with producers in British Columbia growing primarily indigenous species, such as coho and chinook salmon, but more recently also Atlantic salmon. Fish farming has developed more steadily in the Atlantic coast than in British Columbia, with a slow, orderly growth. This industry primarily produces Atlantic salmon, which sell for high prices (Price Waterhouse Management Consultants 1991).

### 1.1.4 Aquaculture in Atlantic Canada

Aquaculture has steadily evolved in Atlantic Canada. Several species are being cultured or are in a research stage to assess the potential for their culture. Among the

cultured organisms, the ones of greatest value in Atlantic Canada are: Atlantic salmon, blue mussel (*Mytilus edulis*), American oyster, rainbow trout and steel head trout. Atlantic salmon have the greatest value, followed by trout, blue mussel and the American oyster.

## 1.2 Salmonids and freshwater salmonid culture

### 1.2.1 Salmonid biology and culture

Salmonid farming is a fairly recent activity even though the farming of fish has been practiced for thousand of years (Roberts and Shepherd 1979). Fish from the family Salmonidae are commonly referred to as salmonids, with Atlantic salmon being the best known. Salmonids originated in the colder waters of the northern hemisphere, although they are now widely distributed throughout the world (Smith 1993). Rainbow trout originated in the eastern Pacific Ocean and the area west of the Rocky Mountains from northwestern Mexico to Alaska (Drummond 1995; Dubé and Mason 1995). Since then, they have been introduced all over the world (Laird and Needham 1988). There are two varieties of rainbow trout, a salt-water form known as steel head and a freshwater form, with a number of strain types (Drummond 1995).

The second half of the nineteenth century saw the introduction of fish culture in Atlantic Canada, the main objective being the stocking of lakes to maintain natural populations. However, the 1950s and 1960s saw the emergence of a new retail market (Dubé and Mason 1995), with rainbow trout being the major fish cultured. The main genera cultured in Atlantic Canada are: *Oncorhynchus* (rainbow trout, cutthroat trout), *Salvelinus* (brook trout, lake trout, arctic charr) and *Salmo* (Atlantic salmon).

Salmonid culture has experienced a substantial growth all over the world in the past decades, although some of the basic techniques have been practiced for as long as two hundred years. The life cycle of a typical salmonid involves spawning in shallow fresh water streams during the autumn (October - November). The development of the spawned eggs proceeds slowly due to the low water temperatures during winter, and hatching takes place in early spring after which they swim up to the surface in May or June when the water temperature rises and appropriate food becomes available. The fry (later called parr) remain in freshwater for approximately 2 years in the wild, after which they reach the smolt stage and migrate towards the sea in May or June (Roberts and Shepherd 1979).

Culture practice with salmonids follows this pattern, with the difference that the hatching time can be reduced by raising the water temperature, and the growth to the smolt stage can be compressed into one or two years (Saunders 1995). In aquaculture situations, salmonids, like Atlantic salmon, are reared in freshwater until the smolt stage, while others, like rainbow trout, are entirely cultured in freshwater. When these two species share the common freshwater conditions, the problems arising during this shared stage of their culture are similar; for example, the range of important infectious agents is the same.

### 1.2.2 Salmonid culture in Atlantic Canada

Freshwater culture of salmon has taken place since 1900 in Atlantic Canada. Most of these hatcheries were developed for production of juvenile salmon to restock wild populations or to establish new populations (Saunders 1995). Atlantic Canada is a major stronghold for the Atlantic salmon. However, the efforts to enhance stocks in this region

have been fragmented and relatively ineffective (Ritter and Carey 1980) until recently. In the past decade, the Maritime provinces have experienced a rapid expansion in farmed salmon production and the industry has diversified with hatcheries, equipment suppliers, feed manufacturers and processing facilities (Price Waterhouse Management Consultants 1991).

## 1.3 Disease in cultured fish

Disease control is particularly difficult in aquatic systems due to the role of environmental conditions. Some of the major diseases are caused by changes or deterioration in the aquatic environment.

### 1.3.1 Factors which predispose fish to disease

*1.3.1.1 Non-infectious*

Several environmental or water quality indices are known to directly or indirectly contribute to disease pathogenesis in farmed fish. These include: temperature, pH, supersaturation, suspended solids, exogenous toxins, sunburn, predation, physical damage due to handling, nutrition, starvation and dietary toxicity (Southgate 1993). Several of these are key promoters of infectious diseases. For example, hyperthermia increases susceptibility to opportunistic infections like columnaris or edwardsiellosis, while hypothermia can lead to fungal infections. As well, low oxygen, high ammonia, high turbidity and overcrowding lead to bacterial dermopathies; high temperatures, overcrowding, organic pollution, and hypoxia are predisposing factors for motile

aeromonad septicemia (Noga 1996).

*1.3.1.2 Infectious*

There are many infectious agents that cause disease in farmed fish. These include viruses, bacteria (*Aeromonas, Vibrio, Pseudomonas, Yersinia, Edwardsiella, Cytophaga*), fungi (*Saprolegnia, Branchyomices*), and parasites (ecto- and endo parasites: protozoan and metazoan) (Southgate 1993; Noga 1996).

**1.3.2 Endemic diseases.**

An endemic disease is one that is generally or constantly found among organisms that share similar conditions, usually in a particular area. In the case of salmonids, there are a range of diseases that are shared among different species when the rearing conditions are similar, e.g. when the fish are reared in freshwater. Among the non-infectious diseases endemic to salmonids are: environmental gill disease, nephrolithiasis, strawberry disease, fin nipping, soreback, sestonosis, sunburn, brown blood disease, hypoxia, anoxia, gas bubble disease, oxygen supersaturation, and therapeutant toxicities (Klontz 1993). Some of the infectious endemic diseases of salmonids can be caused by a)bacteria: furunculosis (*Aeromonas salmonicida*), enteric red mouth (*Yersinia ruckeri*), bacterial gill disease (*Flavobacterium* spp.), bacterial kidney disease (*Renibacterium salmoninarum*), lactobacillosis (*Lactobacillus piscicola*) (Shotts and Nemetz 1993); b) fungi and algae: saprolegniasis (*Saprolegnia* spp.), branchiomycosis (*Branchyomyces* spp.), candidiasis (*Candida sake*), cerebral mycetoma (*Exophiala salmonis*), ichthyophoniasis

(*Ichthyophonus hoferi*), phaeohyphomycosis (*Ochrocomis* spp.), paecilomycosis (*Paecilomyces farinosus*), phomamycosis (*Phoma herbarum*) (Chacko 1993); c) viruses: salmonid herpesvirus, Japanese salmonid herpesvirus, infectious pancreatic necrosis, landlocked salmon virus, focal necrotizing hepatitis, Atlantic salmon papilloma, infectious hematopoiesis necrosis virus, rhabdoviral hepatitis of salmonids, viral hemorrhagic septicemia (McAllister 1993); d) parasites: *Costia* spp., *Ichthyophtirius multifiliis, Myxobolus cerebralis, Henneguya salmonis, Loma salmonae,* among others (Heckmann 1993).

### 1.3.3 Topical diseases of salmonids.

Topical diseases of the skin and gills occur frequently in salmonid farming operations, and are, in some instances, the most significant disease problem. This group of diseases usually appear in the form of an "outbreak", with an accelerating pattern of the morbidity and mortality (Speare and Ferguson 1989b; Thorburn and Moccia 1993). Accordingly, the development of effective therapies to control these diseases is a top research priority cited by Canadian salmonid producers (Armstrong 1994).

An emerging practice in North American salmonid aquaculture involves the prophylactic treatment of these external diseases, which are considered secondary infections that follow gill alteration due to water anomalies such as poor water quality (Holliman 1993). The incidence of these diseases is high, to the point of limiting production of rainbow trout in Ontario (Speare and Ferguson 1989b). Prophylaxis involves the intermittent treatment of healthy fish with chemicals such as formalin,

hydrogen peroxide and chloramine-T (Speare and Ferguson 1989; Bullock et al.1991; Thorburn and Moccia 1993). Some of the specific diseases against which chemoprophyiactic regimes are used, include bacterial gill disease (BGD), columnaris disease, protozoal infections, and saprophytic fungi (Daoust and Ferguson 1983; Speare and Ferguson 1989; MacMillan 1991; Byrne et al. 1995; Noga 1996).

## 1.4 Therapeutants in fish diseases

Therapeutants are used to treat and prevent the incidence of disease. An obstacle commonly encountered is the low number of chemicals that are available for use in food fish, which reflects the minimal research that has been conducted on fish therapeutants. There are only three therapeutants that have been reviewed by Health Canada and are available for use in food fish, while in the United States, there are only four (Armstrong 1993).

The use of therapeutics in the prevention of disease is gaining popularity because they provide an effective method of controlling disease, since the negative effects on fish include reduced feed conversion efficiency and impaired growth which increases the cost of production. The treatments with these therapeutants need to be effective and economical to keep production costs to a minimum and maintain the producers' competitivity.

### 1.4.1 Types of therapeutants

*1.4.1.1 Antiseptics and Disinfectants*

Sepsis is the contamination of an organic system by the introduction of pathogenic organisms or their toxins to the blood or tissues. An antiseptic is a substance which prevents or inhibits growth of these organisms (Herwig 1979), while a disinfectant is just a stronger solution which sterilizes objects, places, or surfaces that may harbor pathogens. Generally antiseptics and disinfectants are applied topically, or in dips or baths (Herwig 1979). The effectiveness of these substances varies depending on the amount of organic matter present. While they are among the most dangerous chemicals to use on or in the presence of fish, some of the most frequently used therapeutants used in the treatment of fish diseases belong to this group. Among the chemicals used for fish are potassium permanganate, hydrogen peroxide, heavy metal salts, (e.g. copper, zinc or mercury); formalin, alcohol, chlorine, iodine, and sodium chloride. Dyes like acriflavine, methylene blue, and malachite green are also used as disinfectants. Disinfectants are unduly used in fish farms as 'fish treatments' since so many fish diseases are 'topical', and the agent causing the disease is therefore susceptible to the action of a waterborne disinfectant, which is really compared to most other farm animals.

*1.4.1.2 Chemotherapeutants*

A chemotherapeutic agent is one that, when used in the treatment of infectious diseases, will cause little or no injury to the host species while effectively killing the causative organism. Chemotherapeutants include antibiotics, antimicrobials, topical

parasiticides and fungicides. Some of the most effecacious antibacterial agents are the sulfonamides, synthetic drugs discovered in 1932 in Germany (Herwig 1979), which have been very efficient against gram-negative bacteria. Topical parasiticides provide an efficient way to treat ectoparasitic conditions, they include: malachite green (used in the control of fungi in fish eggs), formalin and quaternary ammonium compounds (used to control bacterial gill disease in fry). Other ectoparasiticides include copper sulphate, acriflavine and potassium permanganate (Alderman 1988; Scott 1993).

### 1.4.2 Therapy

The above mentioned therapeutants need to be administered in an adequate way to maximize the healing effect that they may have on the fish. The therapeutants can be used as a treatment for the disease once present, or prophylactically, to prevent the appearance of host diseases.

#### *1.4.2.1 Methods of administration*

The aquatic nature of the fish habitat poses special problems and considerations regarding the administration of drugs to fish. The following routes of administration are or have been used:

a) Topical applications: In this form of application the drug is applied directly over the surface of the fish. This is a good method for treating superficial wounds and ulcerations. However this method involves intense handling of the fish and is not economically sound when treating large numbers of fish (Herwig 1979).

b) Baths are the most commonly used form of treatment, since they can be applied to the fish in one aquarium or to the fish in a farm. A large number of pathogens can be treated using this technique. There are different types of baths depending on the time of the application and the concentration of the chemical used: Dips may vary from a few seconds to several minutes, with a maximum of three minutes. The chemical is used at a very high concentration and it is effective in the treatment of ectoparasites and general external conditions. Short baths range from 15 minutes to one or several hours with a maximum of 24 hours. In a fish farm, it is typical to treat the fish for 1 hour in a static bath by stopping the water flow. The removal of medication after treatment is accomplished by turning on the flow of water and allowing it to flow out of the tank or raceway. This method has the advantage that the fish do not need to be handled. Flushing is a method exclusive to flow through systems in which the drug is applied at the inlet and allowed to go through the system and out the effluent pipe. Long baths use very low concentrations of the therapeutant and this makes it one of the most inefficient methods to treat disease in fish (Herwig 1979; Scott 1993).

c) Oral administration is usually done through medicated feed, and is used extensively to combat systemic infections, both prophylactically and for treatment of disease.

d) Injections are useful if they can be administered economically and without harming the fish. There are several ways to introduce the therapeutant by injection: intramuscularly, intravenously or intraperitonealy. The most common method of injection is intraperitoneal. The drug administered must be highly absorbable and able to pass

through the peritoneal cavity and other membranes to be absorbed into the cardiovascular system (Herwig 1979; Anderson and Mayer 1993).

### 1.4.3 Adverse effects

Chemicals must be carefully monitored, since they may cause damage to the exposed epithelial surfaces (gill membranes and epidermis). This would then affect the physiological functions of the fish, most commonly by causing iono- and osmoregulation disturbances, which may, in turn, affect respiratory gas exchange, acid-balance and the excretion of waste products (Jobling 1994). Therapeutant intoxications are very common and usually involve an overdose of the chemical intended to treat a disease condition (Klontz 1993). However, even in the cases when the proper dose is being used, a chemical may still have adverse effects, since the chemical may have toxic properties depending in the characteristics of the water in the tank. The toxicities of a wide range of substances can change depending in the pH, total hardness, salinity, organic carbon content, temperature and dissolved oxygen (Jobling 1994).

## 1.5 Chloramine-T

Chloramine-T is one of the chemicals used to treat or prevent topical infections. Chloramine-T has been used to treat wounds, sterilize drinking water, and to sanitize dairies (From 1980), in addition to its use as a fish therapeutant. Chloramines are compounds that contain one or more chlorine atoms attached to nitrogen (Sheltmire 1962). When chloramines are dissolved in water, the N-Cl bond is partially hydrolyzed, causing

the release of hypochlorous acid or free chlorine, which is responsible for the bleaching and disinfecting properties of the chloramine solution (Sheltmire 1962, Scott 1993). The bactericidal action of the N-chloro sulfonamides (chloramine-T) depends on the activation of the chlorine by the sulfonyl group, which also prevents the migration of the chlorine to the ring structure (Sheltmire 1962). Other factors involved in the efficacy of the action of chloramine-T are stocking density and the presence of suspended solids in the tank which affect the bioavailability of chloramine-T (Schnick 1988; Ostland et al. 1995).

Chloramines have several advantages: 1) the percentages of hypochlorous acid present in a chloramine solution are very small when compared with those of a hypochlorite solution of comparable available chlorine strength; 2) chloramines can be marketed as dry powders which makes storage convenient and the loss of available chlorine minimal (Sheltmire 1962). Chloramine-T (sodium *p*-toluenesulfonchloramide) has been used to treat bacterial gill disease in salmonids (Anderson and Mayer 1993) and is among the eight therapeutants considered in the greatest need of registration (Schnick 1988). Chloramine-T is a N-chloro sulfonamide derivative whose formula is:

$$CH_3\text{-}C_6H_4\text{-}SO_2\text{-}NClNa \cdot 3H_2O$$

At 25°C it is 12% soluble in water and the available chlorine content is ca. 25% (Sheltmire 1962). Chloramine-T is used successfully in the fish farming industry to treat myxobacteria, *Costia, Trichodina*, and *Gyrodactylus* (Scott 1993). From (1980) reports that a dose of 6.5 or 8.5 mg/L control bacterial gill disease in rainbow trout, a dosage well

below the toxic levels of chloramine-T to rainbow trout. Chloramine-T at a dose of 8.5 mg/L is most effective in the treatment of bacterial gill disease, when it is applied in the early stages of the disease (Bullock et al.1991) and when bacteria are the sole etiological agents (Ostland et al. 1995). The results obtained with the treatment with chloramine-T are better than those obtained with other therapeutants that have been used to combat bacterial gill disease, such as copper sulphate, acetic acid, vinegar, salt, potassium permanganate, Roccal™, pyridylmercuric acetate, lignasan X, and hyamine. All this has made chloramine-T the primary treatment of choice on 66% of the rainbow trout farms in Ontario that are using therapeutic agents (Thorburn and Moccia 1993). This same study also reports that the chemical is used both as a therapeutic and a prophylactic agent. Smith et al. (1993) reported that a similar percentage of farms are using chloramine-T in trout hatcheries in Virginia, USA.

Toxicity of chloramine-T is increased at high temperatures, soft water (Schnick 1988) and acidic water (Cross and Hursey 1973); however the activity of chloramine-T and its toxicity is reduced when high organic loads are present in the tanks (Schnick 1988).

## 1.6 Stress in fish

Physical disturbances encountered in aquaculture such as the introduction of a therapeutic treatment in the tank water, handling and transport cause stress in fish and evoke a variety of responses that are mainly adaptive, but that can also be maladaptive (Barton and Iwama 1991). Normal physiological adaptations in fish such as smoltification also provoke a rise in the cortisol levels of fish (Barton et al. 1985; Shrimpton et al. 1994).

The term 'stress' is widely used to describe situations in which the fish fail to perform to their full potential. The fish's performance may be thus limited under aquaculture conditions, since the fish respond with a wide range of physiological and endocrinological changes which affect their ability to survive, grow, and reproduce (Pickering 1992). A definition of stress is that given by Brett in 1958 (Pickering 1993), in which stress is defined as:

> " a state produced by any environmental factor which extends the normal adaptive responses of an animal, or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced"

Most definitions of stress seem to agree that stress represents a reaction by fish to a stimulus and that this response may somehow alter the fish's homeostatic state (Barton and Iwama 1991).

### 1.6.1 The stress response

The effect of the stress response in an animal is initially beneficial, since it provides a physiological pathway which may be beneficial in responding to the stressor. For example, the pathway allows fish to mobilize energy by changing its metabolism from an anabolic to a catabolic state (Pickering 1992; 1993). However, when the stress is severe or prolonged, damaging side-effects appear which may contribute to delayed mortality in fish (Barton and Iwama 1991).

Teleost fish respond in a similar way to most, if not all, forms of environmentally related stress. It has been postulated that a stressed organism experiences three distinct

phases that have been termed the General Adaptation Syndrome (GAS): the first stage is an alarm reaction, characterized by a rapid physiological response, followed by a second stage of resistance, in which the organism adapts to or compensates for the altered conditions. If the stress is severe, compensation may not be possible and the organism reaches the third stage which is exhaustion (Barton and Iwama 1991). This mode of action is a generalization and may not be applicable to all situations. Another approach to defining temporal changes in stress response is the one taken by Moberg, who divides it into three stages: 1) recognition of a threat to homeostasis, 2) the stress response itself, and 3) the consequences of stress.

There are two main components of the neuro-endocrine system that are activated during the stress response: the sympathetico-chromaffin system and the hypothalamic-pituitary-interrenal (HPI) axis. The effect of the sympathetico-chromaffin system is the release of catecholamines (adrenaline, nor-adrenaline and dopamine), while the HPI axis releases corticosteroids (Pickering 1992).

### 1.6.2 Stress and the HPI axis

The hypothalamic-pituitary-interrenal axis is part of the fish's endocrine system and consists of a hierarchy of hormonal pathways. Neurosecretory cells in the hypothalamus - a specialized region in the ventral part of the brain - release the hormone corticotropin-releasing factor (CRF), which stimulates the pituitary gland to secrete another hormone, adrenocorticotropin (ACTH) into the blood. When ACTH reaches the interrenal cells of the anterior part of the fish kidney, these cells release one or more

corticosteroids into the blood. In salmonids the most plentiful and important corticosteroid is cortisol (Pickering 1987).

The HPI axis in teleost fish is activated in response to almost all forms of stress, and leads to an elevation in blood corticosteroid levels. The precise role that cortisol plays in this response is still the subject of much debate, but there is evidence that suggests that it promotes gluconeogenesis (Pickering 1987 and 1992) and lipolysis. Both of these are important factors in the mobilization of energy reserves in stressed fish. Other studies suggest that cortisol has an osmoregulatory role in fish (Pickering 1992), accelerating the re-establishment of osmotic and of ionic equilibrium during the recovery phase following stress.

### 1.6.3 Types of stress

Defining the stress event in terms of temporal characteristics is probably an artificial process of distinction. However, it is widely used in studies of the fish stress response.

#### *1.6.3.1 Acute Stress.*

Acute stress occurs when the stressor is removed before physiological compensation has been achieved (Sumpter et al. 1986). Generally the stress is of short duration: minutes or hours. Procedures common to the aquaculture industry, such as netting, grading, sorting, handling, prophylactic treatment, stocking, and hauling would be considered acute stressors (Barton et al. 1980; Barton and Iwama 1991; Pickering 1993).

The blood corticosteroid levels increase rapidly, but return to normal or basal levels within 24 hours (Pickering et al. 1982). The magnitude and rate of change of blood corticosteroid levels in stressed salmonid fish depend on the nature and severity of the stress, the species and the strain of fish (Pickering and Pottinger 1989), and the environmental temperature (Barton and Schreck 1987). This increase in the cortisol levels has been used as a quantitative index of the degree of stress experienced by the fish.

*1.6.3.2 Chronic Stress.*

A chronic stress event is one which is continuous over a long period of time, so that following the initial elevation of the cortisol levels, the fish acclimate, although at a reduced level of performance (Pickering 1987). Examples of chronic stressors associated with fish farming are overcrowding, variable water quality, social domination, and exposure to novel environments (Pickering 1993). The blood cortisol levels may be elevated for days or weeks, but acclimation eventually occurs (Pickering and Pottinger 1985). This can take as long as 25 days to be achieved, although a period of 7-10 days is more usual (Schreck 1981). Acclimation to chronic stress within a few days does not occur invariably, and this may lead to an increase in mortality due to common bacterial and fungal diseases (Pickering and Pottinger 1989).

### 1.6.4 Types of stress response

The severity of the physiological effects resulting from stress can be monitored using a wide array of biochemical and physiological procedures. There are three biological

levels in which these changes can be assessed: the primary, secondary or tertiary stress responses, according to the level of organization of the response (Barton and Iwama 1991).

*1.6.4.1 Primary stress response*

The primary stress response is readily assessed by monitoring the rise and fall of plasma cortisol or catecholamine concentrations. Methods to assess the primary stress response involve binding assays with radioactive tracers, e.g. radioimmunoassays (RIA) (Wedemeyer et al.1990). Catecholamines can be measured by using radio enzymatic prepackaged kits, thin layer chromatography (TLC), spectrofluorometry, and liquid chromatography (LC). However, most of these methods are quite expensive. Cortisol is the parameter of choice when assessing primary stress response, because of its responsiveness to stressors, ease of measurement, functional significance in physiological processes affecting fish health, abundant background literature on corticosteroids, assay specificity and economy, and cortisol's better stability than catecholamines during normal freezer storage (Wedemeyer et al. 1990; Barton and Iwama 1991).

*1.6.4.2 Secondary stress response*

Since the primary stress response causes physiological changes, these can be used as a measure of the stress response. These physiological changes are defined as secondary stress responses and occur as a result of the primary (i.e. endocrine) responses. These responses can be metabolic (e.g. plasma glucose, lactic acid, cholesterol), hematological (e.g. hematocrit, leucocrit, hemoglobin), hydromineral (e.g. plasma chloride, sodium,

potassium, protein), and structural (e.g. interrenal cell size, condition factor) (Wedemeyer et al. 1990; Barton and Iwama 1991; Iwama et al. 1995).

*1.6.4.3 Tertiary stress response*

When the effects of a stressor are reflected at the whole-animal level, these are referred as tertiary stress responses. These include changes in the metabolic rate, health, behaviour, growth, survival, and reproductive success. This usually means that unfavorable environmental conditions have exceeded the tolerance limits of fish (Wedemeyer et al. 1990). Most investigations with fish concentrate on measuring primary or secondary stress responses, or changes in individual performance. However, the effects of stress may be manifested at the population and community levels.

### 1.6.5 Adverse effects of stress

Fish have the capacity to respond physiologically to stress to overcome the disturbance imposed, but when the mechanisms are forced beyond their normal limits, the responses become detrimental to the fish's health (Barton and Iwama 1991). Some of the maladaptive consequences of stress are increases in the ion/water fluxes, increase in metabolic acidosis, decrease in the number of circulating lymphocytes (lymphocytopenia), decrease in immunocompetence, decrease in the reproductive capacity, and a decrease in the capacity for growth (Barton and Iwama 1991). A direct correlation between the cortisol levels and the increased susceptibility of fish to disease after exposure to heavy metal contaminated water has been reported (Carballo et al. 1995). Other consequences

of a suboptimal environment reflect in the fish performance and its capacity to grow and convert feed (Pickering 1993b), which highlights the importance of preserving the fish environment as stress-free as possible.

## 1.7 Growth in fish.

Growth is defined as any change in size or amount of body material, and can be measured as an incremental change or as a rate of change. Growth can be positive or negative, temporary or long lasting (Busacker et al. 1990). The performance of the fish is governed by its genetic potential and the environmental conditions in which it develops. Some environmental factors have a direct and major influence on growth rate and food conversion efficiency, and can reduce the performance of the fish (Pickering 1993b).

### 1.7.1 Quantification of growth

Changes in the weight or length are considered growth; if these changes are considered relative to the size of the fish they are termed relative growth. If the growth measurements are expressed in terms of a time interval, they constitute a growth rate. Growth can be quantified as follows:

*1.7.1.1 Length*

Body length is generally measured as total length or fork length. Length usually provides evidence for growth or lack of it. The advantage of this method is that length can be easily measured in the field or laboratory on live or preserved specimens (Busacker et al. 1990).

*1.7.1.2 Weight*

Busacker et al. (1990) considered that the change in mass is the most commonly used assessment of whole-body growth of fish. It is easy to measure and is a non-lethal procedure. When a fish grows, changes in weight are greater than changes in length, so that measurement of change in weight provides greater precision over short periods of time. There are different ways to assess weight directly: wet weight, dry weight, and ash weight. To assess changes in growth through time, specific growth rate is used to compare growth at intervals of less than a year, since in such short intervals it is considered that the young fish growth is exponential (Busacker et al. 1990; Hopkins 1992).

*1.7.1.3 Proximate analysis*

Proximate analysis is the determination of categories of compounds in mixture. It determines the tissue composition of a fish, reflecting its nutritional state and therefore is an indirect index of growth rate. Parameters that are considered include protein (with a molecular weight greater than 10,000), lipid and carbohydrate (glucose, glycogen, mucopolysaccharides). The protein, lipid and caloric contents of a fish are positively and strongly correlated with growth rate (Busacker et al. 1990).

### 1.7.2 Physiological and biochemical indices of growth

The physiological and biochemical indices of growth don't measure size, but the rate at which body tissue is elaborated. These methods include the measurement of protein synthesis, glycine uptake by fish scales, the RNA:DNA ratio, liver somatic index, and

condition factor (summarized by Busacker et al. 1990). The rate of protein synthesis is correlated with whole-body growth rate, the uptake of glycine is based on the assumption that the growth rate of the scale is proportional to the growth rate of the fish by providing a relative index of the rate at which protein is synthesized in the scales. The nucleic acid ratio serves as an index of protein synthesis and growth, because the DNA content is constant, while the RNA content varies with the rate of protein synthesis. The liver somatic index is an indicator of the nutritional state of the fish and its growth rate, while the condition factor is interpreted as a reflection of the nutritional state or well being of a fish and can be interpreted as an index of growth (Busacker et al. 1990).

### 1.7.3 Factors affecting growth

*1.7.3.1 Environmental factors.*

A wide range of environmental factors can affect growth in the fish, these range from the feed and feeding regime (Cho 1992; Hung et al. 1993), water quality (Johnston and Saunders 1981; Pickering 1993), lighting (Stefansson et al. 1990; Cho 1992; Sumpter 1992; Mortensen and Damsgård 1993; Jobling 1994), photoperiod (Jobling 1994), disturbances near the rearing tanks (Pickering 1993), stocking densities (Jørgensen et al. 1993; Bagley et al. 1994; Jobling 1994), water flow rates (Jørgensen et al. 1993; Bagley et al. 1994), acclimation procedures (Pickering 1993), to tank effect (Speare et al. 1995), temperature (Jobling 1994) and disease. Many of these factors adversely affect appetite and/or feed conversion efficiency. Appetite is the drive that initiates consummatory behaviour. It is important to maintain appetite in fish because of the need to ensure that

the feed regime is adjusted to maximize consumption, growth and conversion efficiency. Appetite can be suppressed by stress, oxygen, temperature, ammonia, aggression, and handling (Knights 1985) and since the food ingested will be insufficient, growth will be inhibited or cease completely (Hepher 1988).

*1.7.3.2 Sex*

Some species of fish show distinct differences in growth rate between sexes. In some species, like tilapias, the males grow faster, while in others, like carp and eels, the females grow faster than the male (Hepher 1988).

*1.7.3.3 Genetics*

Genetic characteristics may govern the capability of the fish to search for and utilize food, to compete for food with other fish, and the physiological utilization of the food. Inbreeding can cause morphological degeneration and a decrease in the growth rate, while cross breeding between different genotypes results in a better growth of the offspring than of any of the parents (Hepher 1988).

*1.7.3.4 Physiological state.*

The physiological well-being of the fish affects growth considerably. Fish suffering from diseases or parasites have a reduced growth or cease growing completely; stress also results in reduced growth. Growth may also be inhibited during gonadal maturation (Hapher 1988).

*1.7.3.5 Stress*

Most forms of environmental stress are reflected in fish in a reduction in growth rate, with important consequences for the aquaculture industry. As mentioned in section 1.6.2, cortisol is the main corticosteroid secreted as a response to a stressor. Cortisol has a catabolic action which is responsible for the mobilization of energy reserves by gluconeogenesis and lipolysis, whose net catabolic effect is a reduction in the growth rate (Pickering 1990). Therefore, the growth suppression observed is, in part, a consequence of the elevation of blood cortisol levels during stress (Pickering 1990).

## 1.8 Rationale for Research

The importance that salmonid culture has gained in recent years, and the economic impact that such an industry has in the creation of new employment options for Canadians, propels the drive to find new ways to optimize the production of marketable fish. The competitiveness of this industry on a world wide scale makes it necessary to find ways to reduce production costs. One of such ways is to reduce losses due to disease.

The special environmental characteristics that are found in the farming of fish may make them more susceptible to disease, since once one of the fish becomes sick, direct contact with its cohorts and the high densities maintained in tanks make it easy for pathogens to spread very rapidly. Many of these diseases are treated with chemicals, some of which are also used prophylactically to prevent the development or complications of a disease. This practice has been emerging in recent years in the Canadian salmonid industry, especially to prevent the appearance of bacterial gill disease, which provokes major losses in fingerlings and young fry. A chemical that has been used successfully in the combat and prevention of bacterial gill disease is chloramine-T. However, chloramine-T is not registered for use in Canada and its effects on healthy fish are poorly understood. Yet it is widely used in fish farms.

These facts have made it imperative to study chloramine-T following patterns and dosages used in the aquaculture industry, and to investigate the effects of this chemical on healthy fish when it is repeatedly applied prophylactically. This study helped to reveal some of the consequences that the use of this chemical may have on fish.

To do this, a series of experiments were carried out in which different parameters,

such as the primary, secondary and tertiary stress responses were evaluated, together with studies assessing the damage that this chemical may have in exposed tissues, namely gill and skin.

## 1.9 Objectives

1. Investigation of the primary and secondary stress responses of rainbow trout, after prophylactic exposure to chloramine-T.
2. Investigation of the tertiary stress response of rainbow trout to exposure to chloramine-T, at the level of growth.
3. Assessment of damage induced by chloramine-T to exposed tissues of rainbow trout, i.e. gills and skin.
4. Establishment of a technique for the preservation of the piscine mucous cover in skin and gill to permit ultrastructural evaluation.

# 2. EFFECTS OF A PROPHYLACTIC CHLORAMINE-T TREATMENT ON GROWTH PERFORMANCE AND CONDITION INDICES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)*

## 2.1 ABSTRACT

Chloramine-T is a commonly used therapeutic agent for the treatment of bacterial gill disease and related phenomena in the salmonid aquaculture industry. It is also commonly used as a prophylactic agent to prevent diseases in commercial salmonid hatcheries. Rainbow trout, *Oncorhynchus mykiss*, (avg. weight 98 g) were exposed to chloramine-T at 10 mg/L for 1 hour, twice weekly (a commonly used regime in the aquaculture industry) for 11 weeks. Fish were fed ad libitum without feed wastage throughout the 11 week trial. Body weight, specific growth rate, feed conversion index and appetite were assessed at the end of weeks 3, 6, 9 and 11 in exposed and control fish. Chloramine-T treatment was not associated with clinical disease or mortality. However, by the end of the trial the growth (based on body weight) of treated fish was significantly suppressed ($\delta$=7.3%) compared to controls. Growth suppression was attributed to a significant reduction ($\delta$=9.3%) of feed conversion efficiency in treated fish. Based on specific growth rates, chloramine-T has an early negative effect on growth. The effect diminished in later weeks, although was not completely lost, suggesting some degree of adaptation by the fish to the chemical agent.

*Editorial style conforms to the Journal of Aquatic Animal Health.

## 2.2 INTRODUCTION

A common problem encountered in the intensive early rearing of salmonids is topical infection of the skin and gills, involving a wide range of pathogens (Speare and Ferguson 1989). Treatment chemicals, such as chloramine-T, formalin, and others are frequently used on a prophylactic or metaphylactic basis (Thorburn and Moccia 1993), in addition to their use in treating outbreaks. Although the economic impact of infectious gill and skin diseases in salmonid aquaculture is yet to be quantified, the frequency of outbreaks combined with the often high morbidity and mortality during outbreaks strongly suggest the value of effective prevention methods. Presently, vaccines do not exist for any of the bacterial, protozoal, or fungal agents which account for the majority of the gill and skin diseases encountered in salmonid farming. In contrast, the previously mentioned chemical agents are generally effective in treating these pathogens. Prophylactic treatment regimes are gaining favour among fish farmers because of empirical observations that they reduce the frequency of disease outbreaks. Although the mechanism of prophylaxis is unknown when treatment chemicals are used intermittently, it is likely accomplished by reducing pathogen populations within the mucous biofilm of the fish surface, as well as in the tank environment.

Chloramine-T is an N-chloro sulfonamide. Its bactericidal and disinfecting properties in solution depend on the release of hypochlorous acid released when the N-Cl bond is partially hydrolyzed (Sheltmire 1962). Chloramine-T (sodium para-toluenesulphonchloramide) has been used to treat gill disease in salmonids and was shown

to be superior to earlier known therapeutants (From 1980). It is reasonably inexpensive and nontoxic (non-lethal) to rainbow trout in at least double the concentration needed to successfully treat gill disease (From, 1980). Ostland et al. (1995) have shown that when fish are treated for gill disease with chloramine-T, improved survival rates over the first four days of treatment are evident, which is not seen with standard formalin treatments. Bullock et al. (1991) reported that, in hatchery trials, a single treatment with 8.5 mg chloramine-T per liter provided effective control of bacterial gill disease. Farmers who use chloramine-T prophylactically treat fish more frequently (Thorburn and Moccia 1993), the effects of which have only recently been studied by Powell et al. (1994). Although chloramine-T is currently not a licensed fish therapeutic agent in North America, its efficacy (Bullock and Herman, 1991) has placed it on a priority list for approval in North America (Armstrong 1993). However, its present widespread use in the commercial fish farming industry (Speare and Ferguson 1989b; Smith et al. 1993; Thorburn and Moccia 1993) currently justifies investigation of its effect on fish production.

Powell et al. (1994) provided empirical evidence that exposure of rainbow trout juveniles to chloramine-T twice weekly for three weeks led to a dose-related reduction in growth rate and increased susceptibility to an undefined dermatitis associated with an altered skin biofilm which became dominated by *Pseudomonas* spp. and *Flavobacter* spp. Building on these findings, the current study specifically examines the effects of 1 hour biweekly exposures to chloramine-T (10 mg/L) on the growth, condition and health of juvenile rainbow trout (*Oncorhynchus mykiss*) during an 11-week controlled laboratory trial using conditions simulating aquaculture conditions.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Sample Population.

Size-graded diploid rainbow trout, (average weight and length of 98 g and 19.9 cm) were purchased from a commercial hatchery in Prince Edward Island, Canada. Following published recommendations (Busacker et al. 1990; Speare et al. 1995), these fish were acclimated for a period of 4 weeks to the trial conditions, including water quality, temperature, photoperiod, feed and feeding regime to which they would be exposed during the growth trial.

### 2.3.2 Assessment of weight and condition at the beginning of the growth trial.

At the end of the acclimation period, 102 arbitrarily selected fish were anesthetized in benzocaine solution (40 mg/L) and individually weighed, their fork length measured, and a binary evaluation was used to assess fin and eye condition. Fins were evaluated as normal (0) or damaged (1), based on grossly visible erosion, splitting, epithelial hyperplasia and/or deformation. Eyes were evaluated as normal (0) or damaged (1), based on opacity of the cornea or lens, intraocular hemorrhage, exophthalmia and/or enucleation.

### 2.3.3 System design and fish allocation.

Thirty-five fish were randomly allocated to each of 24, 100-L circular fiberglass tanks (Appendix A). The habitable volume of each tank was 78.0 L, yielding an initial stocking density of 45 kg/m$^3$. This stocking density was adjusted back to the initial levels

at the third week by removal of randomly selected fish. The water flow rate to each tank was 2.8 L/min. Relative to tank biomass the flow rates were 0.81 L/kg of fish/minute at the beginning of the trial and 0.55 and 0.51 L/kg of fish/minute for treated and control fish respectively at the end of the trial. The mid-tank dissolved oxygen (DO) levels in all tanks remained above 7.5 mg/L during the duration of the trial. Water temperature was 10°C.

### 2.3.4 Feeding.

Fish were fed ad libitum with one commercial feed (High Pro Grower, pellet size #3 and #4, Corey Feed Mills Ltd, Fredericton, NB). An increase in pellet size accommodated the increase in fish size after week 4. The feed's proximate analysis (dry weight basis), as provided by the manufacturer, is detailed in Appendix B.

During each week the fish were fed twice a day for five consecutive days, at 0900 and 1500 h. On day six they were fed once (0900) and were fasted on day seven. Feed was withheld the days that fish were weighed, but resumed the following day. Feeding fish twice daily is sufficient for optimal growth of rainbow trout (Alanära 1992). Feeding the fish 6 vs. 7 days a week does not inhibit growth (Cho 1992). Fish were carefully fed by hand and observed for satiation so that the amount of uneaten feed was negligible. The trial was 'single blind', since the people feeding the fish were unaware of treatment allocations. The sequence of tank feeding was changed weekly to avoid potential artifacts caused by feeding sequence on appetite or anticipatory stress. The amount of food consumed every Wednesday after the treatment with chloramine-T was recorded, as well as the overall feed intake in the week.

### 2.3.5 Allocation of treatments.

There were 12 treatment tanks and 12 control tanks. Results from a previous study (Speare et al. 1995) show that growth indices may be affected by tank location in the laboratory. Therefore, each exposure tank was paired with an adjacent control tank. In this way both tanks would be subjected to the same ambient effects. Exposure tanks were randomly selected after pairs were established. Specifically, 12 treatment tanks were paired with 12 control tanks. Of the 12 control tanks, 8 were assigned to be "sham" treatment tanks (i.e. water flow turned off and water added to simulate a treatment), and 4 were left completely undisturbed at the time of treatment. It was thought that the sham treatment control tanks were necessary because the act of turning water off for 1 h may affect growth performance - and this could be additive to the effect of the chemical agent itself.

### 2.3.6 Exposure to chloramine-T.

Fish were exposed to a 10mg/L dose of chloramine-T in a static bath (water was turned off for one hour after addition of the chemical). The inflow of the 8 sham treatment control tanks was also stopped for one hour to simulate conditions in the chloramine-T treated tanks whereas 4 other control tanks were left completely undisturbed. Exposures were conducted on Sunday and Wednesday each week for 11 weeks.

### 2.3.7 Sampling and collection of growth indices.

All fish were assessed during a single day at the end of weeks 3, 6, 9 and 11. All fish were netted and placed in a holding tank where they were anesthetized, weighed, measured and examined for signs of skin and fin damage or infection. They were then placed in a recovery tank for approximately 30 min, before being returned to their original tank. At the end of week 3, every third fish in a tank (n=11) was euthanized with an overdose of benzocaine, to maintain the initial stocking density. At the end of week 11, all fish were weighed, measured, evaluated and euthanized.

Growth indices measured or derived included: Body weight (Bw) in grams; mean weight (Mw); fish length (Fl) as fork length in cm; condition factor (K) was calculated as [weight/(length)$^3$] x 100; feed conversion index (FCI) = ($\Sigma$WFI/N)/MwT-Mwt, where $\Sigma$WFI = sum of food consumed during an interval of interest, MwT = final body weight for an interval of interest, Mwt = initial body weight for an interval of interest, N=number of fish; specific growth rate (SGR, %/day) = [(lnMwT - lnMwt)/(T-t)] x 100, where ln = natural logarithm, and T-t = number of days over which growth was evaluated. Appetite refers to feed consumed by fish weekly, expressed as a percentage (%) of the fish body weight. Feed intake following treatment with chloramine-T was recorded every Wednesday to investigate immediate effects of the treatment on appetite. For weeks in which a value for Bw had not been obtained directly from weighing fish, the Bw was estimated from the regression formula based on the SGR (Hopkins 1992) during the corresponding interval.

### 2.3.8 Statistics.

Statistical comparisons were made between values derived for treatment and control tanks. The unit of study in this trial was the tank of fish, not the individual fish. Growth performance indices were calculated for each tank of fish. Means and their standard errors (growth indices), or incidence (fin and eye scores) were then calculated for the groups of tanks assigned to each treatment. Before grouping data from the two control sets of tanks (sham treated and untreated), their performance was compared to each other and to the chloramine-T treated tanks using a one-way ANOVA. If no significant differences were noted between the two control groups, then they were combined into a single control group of twelve replicates. After grouping these controls, we compared treated tanks (12) to control tanks (12) using a single paired T-test for matched pairs.

For the fin and eye evaluation, a 2 x 2 contingency table and a $X^2$ test were used to compare the differences in the incidence of lesions between the treatments. For all statistical tests, differences were considered significant at the $\alpha$=0.05 level of probability. Statistical analyses were carried out using commercial software (MINITAB™Inc.). The growth trial was designed to achieve a power (1-ß) of > .80 to detect, as significant, a difference ($\delta$) of >15 g between treatment groups, with n=12, when $\alpha$=0.05 (Glantz 1992).

## 2.4 RESULTS

There were no significant differences in the flow rates or dissolved oxygen levels among all tanks. The behavior of the fish in treated and control tanks was similar, with

the exception that fish fed shortly after treatment with chloramine-T appeared to feed less aggressively. Otherwise, fish did not seem to be agitated in any way during or after the treatment periods.

Growth indices - Based on the ANOVA comparing treated tanks to sham controls and uninterrupted control tanks, there were no differences between the two types of controls. Therefore data for these two types of contests were combined and matched pairs T-tests were used, for each of the following performance indices. At the end of weeks three, six and nine control fish weighed significantly more than treated fish (Appendix C,1; Fig. 1). After 11 weeks fish treated with chloramine-T weighed less than the controls (209.1 g vs. 225.6 g), a difference ($\delta$) of 7.3% (16.6 g).

Condition index - No significant differences between the two groups of fish were detected at any interval during the growth trial (Appendix C,1).

Feed Conversion Index - The FCI for fish treated with chloramine-T was significantly higher (i.e. less efficient) than the FCI for control fish during the intervals of weeks 0-3 ($\delta$=21%), 4-6 ($\delta$=17%) and 1-11($\delta$=9.3%). (Appendix C,2; Fig.2). FCI was not significantly different for the intervals from week 7-9 or 10-11.

Specific Growth Rate - The SGR calculated for the entire 11 weeks was significantly lower in the treated fish when compared with control fish (0.98 vs. 1.08). In the first interval (weeks 0-3), control fish grew significantly faster than treated fish (Appendix C,2; Fig. 3). However, at the 4-6, 7-9 and 10-11 week intervals, the values in SGR for treated fish were lower but not significantly different from the SGR values for control fish (Appendix C,2).

Appetite - There was no significant effect of treatment on weekly feed consumption nor immediately following treatment (Appendix C,3; Fig 4), when intake was expressed on a percentage of body weight (%Bw) basis.

Fin and eye condition - Eye lesions were rarely encountered during the trial. A negligible number of fish developed corneal opacity. The frequency in treated and control fish was never significantly different (Appendix C,4).

The fin lesions observed were generally restricted to fin tips. By the end of week 3, the score for the dorsal fin (DF) of treated fish was significantly lower than for untreated fish ($x^2$=6.68; P=0.01), however no differences existed for adipose, caudal, anal, pelvic or pectoral fin scores. At the end of week 6, anal fin condition was significantly ($x^2$=4.84; P=0.05) lower for control vs. treated fish, however scores for all other fins were similar. By week 9, significant differences in the caudal and anal fin conditions were observed ($x^2$=28.684, P=0.05; $x^2$=6.174, P=0.05 respectively), with the control fish having lower scores than the treated fish. By the end of the trial, no significant differences existed between treated and control fish.

## 2.5 DISCUSSION

A number of methods have been used to determine the effects of chemicals on aquatic ecosystems and fish (Ensenbach and Nagel 1995) and specifically on fish held in captivity (Mallat 198?). Interpreting these results in the context of assessing the impact of a chemical or chemical group on aquaculture production must be done cautiously. Of

particular interest to fish farming are the effects of a chemical on mortality rate and somatic growth indices. Relatively few studies have examined the longer-term effects of a chemical or chemical treatment regimes on growth.

This study has shown that the pattern of intermittent exposure to chloramine-T, which is emerging in Canadian salmonid aquaculture (Thorburn and Moccia 1993), has a significant suppressive effect on growth, a finding which concurs with a trend noted earlier by Powell et al. (1994). These results also show that the growth suppression mechanism is through a deleterious effect on conversion efficiency, especially in the initial stages of the treatment regime, and not through adverse effects on appetite. In fact, despite a slightly greater feed intake by treated fish compared to controls during the first 3 weeks, their SGR was significantly less than for control fish.

In this study, a relatively long growth trial was used, spanning approximately 25% of the length of the production cycle and specifically focused on a production period during which considerable biomass increment occurs. In contrast, short-term growth trials are relatively common; however they are unable to detect compensatory growth (Hopkins 1992), a critical parameter to assess if the results of a growth trial are to be applied to aquaculture production models. To look for compensatory trends, growth indices were determined at several points through the trial, specifically to assess whether the impact of the chemical was mitigated by physiological compensation of the test animals to the exposure regime. With respect to growth, this comparison over time is best assessed through the SGR (Busacker et al. 1990; Hopkins 1992). In this trial, the greatest effect of the treatment, based on the SGR, occurred over the first three weeks, and then the effect

began to diminish throughout the remainder of the trial. This suggests that the treated fish began to accommodate to the effect of the treatment. Nevertheless, there was no period in which treated fish had a SGR equal to/higher than that of control fish; therefore there was no true compensatory growth phase in treated fish. Differences in weight would therefore be persistent, and in a production setting the overall growth of treated fish would continue to be limited.

Feed conversion efficiency is a critical issue to aquaculturists in a competitive industry where feed costs are a large component of the overall cost of production. Relatively few reports have advanced mechanisms by which chemical exposure limits conversion efficiency. As a result, the mechanisms advanced in this discussion to explain the negative effect of chloramine-T on conversion efficiency, are largely hypothetical. In general, FCI can be affected by problems relating to nutrient absorption (Reichenbach-Klinke 1975; Vignier et al. 1992), functional efficiency of the digestive system (Gerundo et al. 1991), diversion of energy via physiological events induced by stress (Pickering 1990), alteration of metabolism (Vignier et al. 1992), and loss and replacement of tissue and proteins (Powell et al. 1995).

Specific examples of the effects of chemical-treatment exposures on feed conversion of fish were reported by Vignier et al. (1992), who showed that Atlantic salmon (*Salmo salar*) parr exposed to crude oil had a decrease in conversion efficiency. Additionally, Reichenbach-Klinke (1975) demonstrated that malachite green blocked intestinal enzymes. Pickering (1990) advanced the hypothesis, based on a review of exposure trials, that exposure to treatment chemicals induces a stress response and the

evoked catabolic effects would directly influence FCI. In our study, the decrease in conversion efficiency may relate to a combination of several physiological pathways evoked by the treatment. Based on the review by Pickering (1990), it was reasonable to expect that the treatment stressed the fish and induced catabolic effects. Additionally, work by Powell et al. (1995) has shown that chloramine-T evokes a metaplastic response of gill epithelia which may be a response to acid-base and ionic disturbances. These findings suggest that energy may be diverted in treated fish towards cellular kinetics and compensatory ionoregulation.

The conversion efficiency of treated fish was negatively affected throughout the trial, suggesting a mechanism to explain the persistently reduced SGR throughout the trial. Furthermore, it points to the possibility that the basic physiological impact through which the chemical limits growth, could not be completely compensated for.

During this study fin and eye condition, especially corneal condition were evaluated, because we suspected that these tissues would be sensitive indicators of chemically induced epithelial damage. The previous study by Powell et al. (1994b) showed fin condition changes in chloramine-T treated fish, and Stoskopf (1993a&b) suggested that free chlorine at 0.002 ppm, a level lower than what is released during a standard chloramine-T treatment (Bullock et al 1991), is damaging to fish epithelium. Other researchers have also noted the sensitivity of the fish cornea to water-borne chemicals (Dukes 1975; Wester and Canton 1987). It is interesting, therefore, that by the end of the study period, there was no difference between treated and untreated fish with respect to fin or corneal deterioration. Therefore it cannot be concluded that this chemical

was directly damaging the host epithelial surfaces, at least at the macroscopic level.

Differences in containment facilities, water chemical characteristics, and endemic pathogen load may modify the response of fish to a treatment chemical. One important aspect to consider when treating or preventing disease, is that the chemicals used introduce a new factor to the environmental equation, with different effects for different chemicals (Branson 1993), and for different species (Holcombe et al. 1995). Some studies show a marked interaction between chemical exposure and disease incidence (Niimi 1990); others do not (Hetrick et al. 1934); and still others show resistance of chemically exposed fish to disease (Niimi 1990). The same likely holds true, in general, for the positive and negative effects of chemoprophylactic agents.

This study has demonstrated and confirmed previous results that repetitive, intermittent exposure to chloramine-T in a prophylactic regime at concentrations commonly used in the aquaculture industry, induces growth suppression. Hence it should thus be used with caution until the full range of effects caused are known. The main objective of this study was to assess the effect of chloramine-T on growth indices of healthy rainbow trout when used as a prophylactic agent, rather than to look at the effects when used as a therapeutic agent. These conclusions cannot be extrapolated to the effects that chloramine-T would have, when used as a treatment, on the growth indices of diseased or convalescent fish.

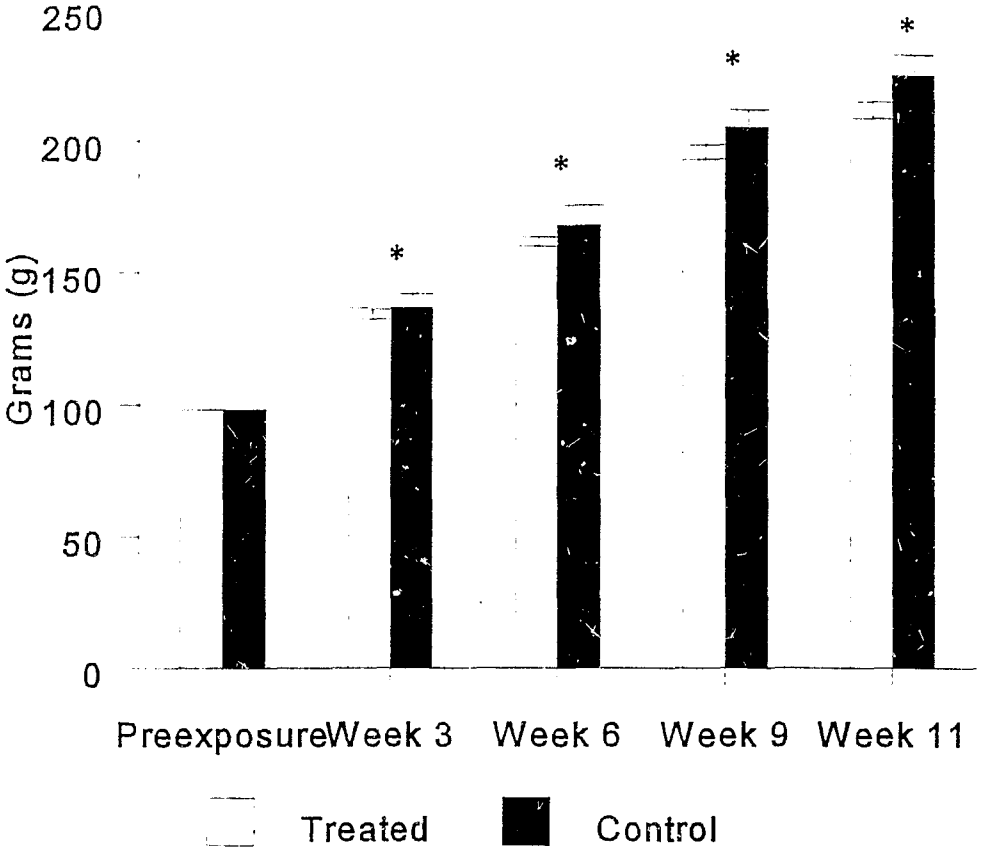


Fig. 1. Weight of fish in grams during the growth trial (Mean + SEM), * indicates significant difference.

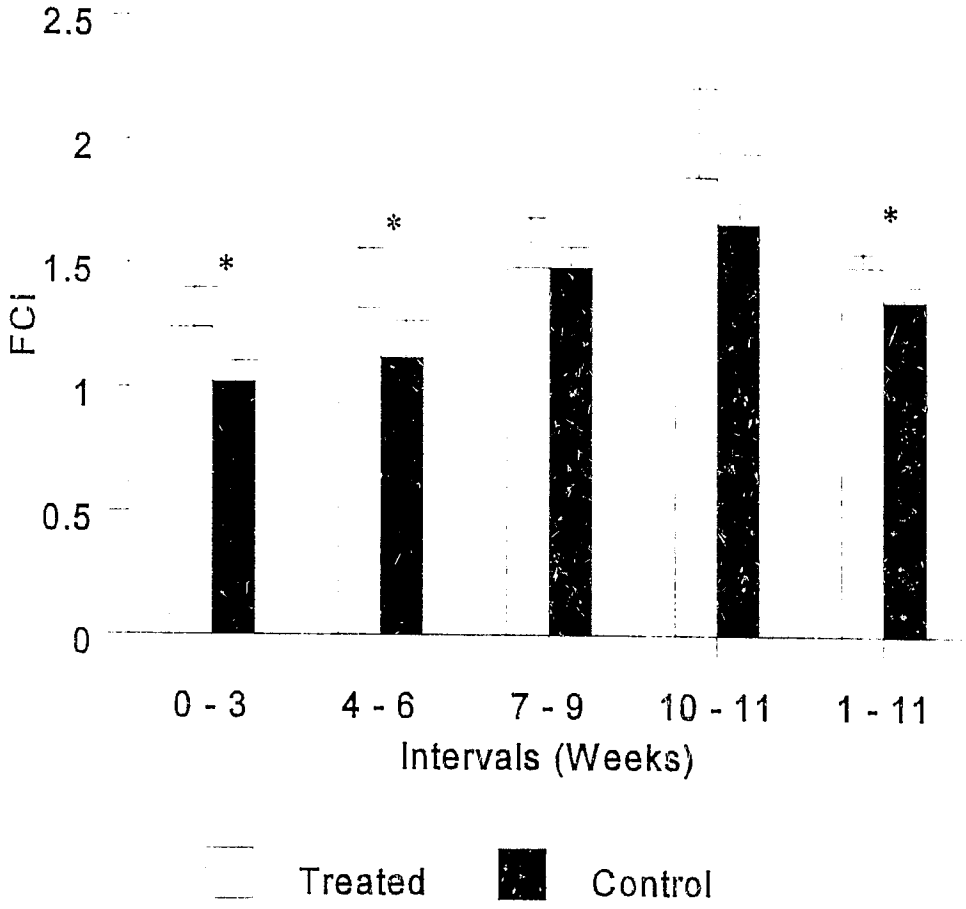


Fig. 2. Feed Conversion Index (FCI) for treated and control fish for the specified intervals during the growth trial (Mean + SEM), *indicates significant difference.

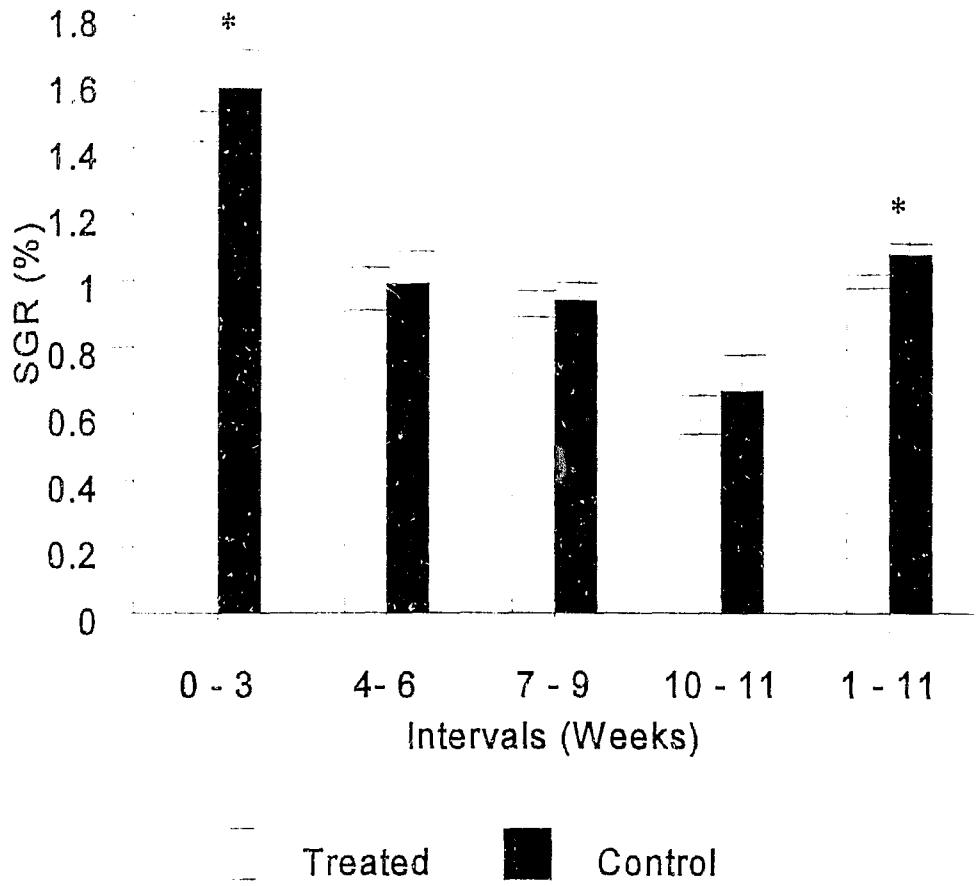


Fig. 3. Specific Growth Rate (SGR) for treated and control fish, for the specified intervals during the growth trial (Mean + SEM), * indicates significant difference.

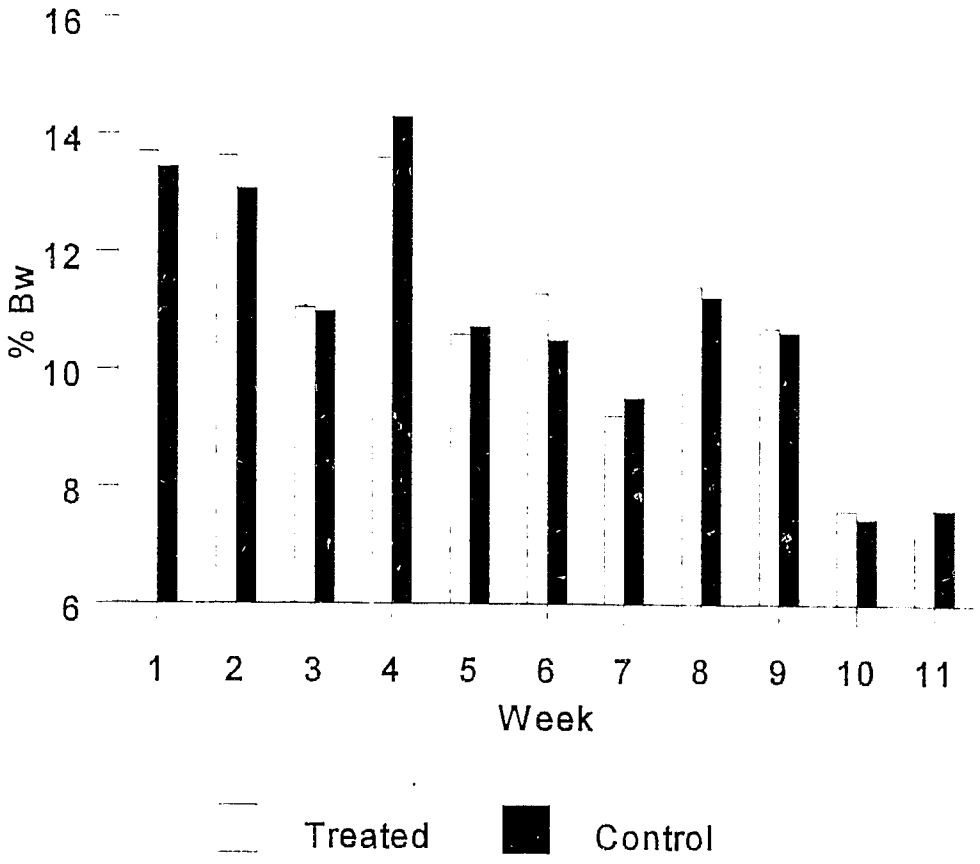


Fig. 4. Weekly Feed Intake for treated and control fish, expressed as the percentage of body weight (%Bw) during the growth trial. (Mean).

# 3. STRESS RESPONSE IN HEALTHY JUVENILE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AFTER REPETITIVE INTERMITTENT TREATMENT WITH CHLORAMINE-T AND FORMALIN.*

## 3.1 ABSTRACT.

Rainbow trout juveniles were subjected to an intermittent prophylactic regime of chloramine-T or formalin and the subsequent changes in the cortisol levels were determined at 1, 24 and 96 h post treatment. Exposure to 10 mg/L of chloramine-T or 0.2 ml/L formalin did not provoke a rise in the cortisol levels of treated fish from the baseline obtained at the beginning of the experiment. No significant differences were detected in the cortisol response between the treated and control groups in the different weeks.

Blood samples of rainbow trout juveniles, part of an accompanying growth trial, which were exposed to chloramine-T twice weekly, were obtained to measure the effects of the treatment on the hematocrit and plasma glucose, sodium and chloride levels. No evidence of a secondary stress response could be detected in the treated fish when they were compared to the controls. Hematocrit and plasma glucose, sodium and chloride remained within what are considered normal levels for resting or unstressed rainbow trout.

Chloramine-T does not seem to elicit a primary or secondary stress response in rainbow trout, when used at a 10 mg/L dose.

*Editorial style conforms to Journal of Aquatic Animal Health.

## 3.2 INTRODUCTION.

Stress, arising from husbandry practices inherent to intensive rearing of farmed fish (Tort et al.1994), is a cause of concern to fish farmers and fish health professionals, because it promotes disease and impairs growth (Pickering 1993). A new emerging husbandry practice in fish farming is the prophylactic use of chemicals to reduce the incidence of topical bacterial, protozoal, and fungal infections (Thorburn and Moccia 1993). Although the benefits of this approach to disease prevention are leading to a greater acceptance of this practice in aquaculture, the possible side effects that this may produce have not been adequately investigated (Branson 1993; Powell et al. 1994,1995). Two of the more frequently employed prophylactic agents are formalin and chloramine-T (Thorburn and Moccia 1993). These chemicals can be lethal to fish at high dose levels (From 1980, Howe et al. 1995) and in some cases mortality spikes occur after exposing fish to normal treatment levels (Southgate 1993). This raised the concern that prophylactic treatments may be an additional under-recognized source of stress to farmed fish. Of the range of chemical compounds available, chloramine-T is one of the most useful chemicals available to the fish farmer as it affects adversely myxobacteria, *Ichthyobodo, Trichodina, Ichthyophthirius* and *Gyrodactilus* (Scott 1993).

Stress has been biologically defined in terms of physiological responses at three levels: primary; secondary, and tertiary (Barton and Iwama 1991). During the primary stress response mediated by the activation of the hypothalamic-pituitary-interrenal (HPI) axis, plasma levels of catecholamines, ACTH and cortisol rise (Barton and Iwama 1991;

Pickering 1992). The HPI axis is stimulated in response to most forms of stress (Pickering 1993), and cortisol levels are generally used to investigate the primary stress response (Adams 1990). The functions of cortisol in teleost fish are varied and range from gluconeogenesis (Janssens and Waterman 1988) to stimulation of ionoregulatory processes that allow the fish to recover its ionic balance (Laurent and Perry 1990; Richman and Zaugg 1987). Cortisol, when compared to catecholamines and ACTH, is more easily detectable (Kjartansson et al. 1988) and its rise and fall is a relatively direct assessment of the severity and duration of the primary stress response (Wedemeyer et al. 1990). Since the resting or unstressed levels of circulating corticosteroids in fish are less than 30-40 ng/ml and ideally less than 5 ng/ml (Barton and Iwama 1991; Pickering and Pottinger 1989), an increase in this parameter is easily recorded. At the same time, the background literature describing the rise and fall of cortisol is considerable, the assay is fast and inexpensive, and cortisol is more stable in freezer storage. Determination of the circulating blood cortisol is highly useful in assessing adrenocortical function (Lowell and Dunn 1974).

Secondary stress responses result from the physiological effects of factors released during the primary stress response (Barton and Iwama 1991) and are measured by the assessment of a range of hematological parameters (Kjartansson et al. 1988). Barton and Iwama (1991) report that the secondary changes may be metabolic (plasma glucose, lactic acid), hematological (hematocrit, leukocrit, red blood cell count), ionoregulatory (plasma chloride, sodium, potassium), and structural (interrenal cell size, condition factor). Tertiary or whole animal responses are measured by parameters such as growth, metabolic

rate, and disease resistance.

To date there have been few studies addressing the effects of treatment chemicals when used to treat or prevent disease, on the stress response of fish. In a study by Pickering and Pottinger (1985), the prophylactic administration (1 daily pulse) of malachite green (2.27 mg/L) for 4 weeks caused a significant elevation of the blood cortisol levels of brown trout (*Salmo trutta)* within 1 hour of treatment. However, the treatment had no effect on the hematocrit, erythrocyte, thrombocyte, or lymphocyte counts in brown trout. However, in rainbow trout, malachite green causes a leucocytopenia (Hlavek and Bulkley 1980). In one of the first studies on the effects of formalin in rainbow trout, Wedemeyer (1971) reported a progressive hypochloremia after the fish were exposed to 0.20 ml/L formalin. The same effect was observed in steelhead exposed to 0.20 ml/L formalin (Wedemeyer and Yasutake 1974). Rostant et al. (1995) report that Atlantic salmon when exposed to formalin (0.25 $ml.L^{-1}.h^{-1}$, every third day for 5 weeks), had a prolonged increase in plasma cortisol. A more recent study, showed that when formalin was used in doses of up to 0.25 ml/L for 90 minutes every two weeks, there were no significant long term effects on electrolytes and blood hematocrit (Powell et al. 1996). There have been no studies addressing the interaction between chloramine-T and stress. Chloramine-T is known to release free chlorine in water and a report by Zeitoun (1977) demonstrated an increased hematocrit and plasma protein levels in rainbow trout after exposure to chlorine at different concentrations and times.

Studies to date suggested that repeated treatment with chloramine-T suppressed growth, decreased haematocrit levels, and caused a dose dependent decrease in plasma

sodium and chloride levels, without changing the plasma glucose concentrations (Powell et al. 1994). Powell et al. (1995) also reported that pre- and post-exposure hematological values of rainbow trout after a single pulse treatment with chloramine-T were not different.

Since the primary stress response is a good indicator of the immediate response of the fish to a potential stressor, and the secondary stress responses are a direct effect of the primary stress response (Casillas and Smith 1977), the objectives of the present study included the measurement of these two parameters, after exposure to formalin or chloramine-T, to help assess more accurately the magnitude of the physiological responses of rainbow trout in response to these agents.

## 3.3 MATERIALS AND METHODS.

### 3.3.1 Trial 1 (Primary stress indices).

Trial 1 was designed to evaluate the blood cortisol levels in rainbow trout after being intermittently exposed to treatment concentrations of formalin and chloramine-T once a week over a 4 week period.

*3.3.1.1 Sample population*: 120 diploid rainbow trout (*Oncorhynchus mykiss*), with an average weight of 182.80 g and an average length of 23.94 cm were used.

*3.3.1.2 System design*: The system consisted of 24, identical 78.0 L tanks (Appendix A). Groups of five fish were netted and randomly allocated to each tank by following a

generated series of random numbers (1-24). This resulted in a stocking density of 10 Kg/m$^3$. All tanks received water from a pumped well, at a flow rate to achieve a turnover time of 30 minutes. Water temperature was 10°C.

*3.3.1.3 Acclimation:* After allocation to tanks, the fish were maintained for 2 weeks, before the trial began and fed a commercial ration at 2% of the tank biomass daily. At the end of the acclimation period, to assess the uniformity of acclimation, the weights and lengths of the fish were taken from all the experimental fish, using the following procedure: Fish were anaesthetized in a solution of benzocaine dissolved in 95% ethanol, at 40 mg benzocaine/L of water in an aerated anaesthetic tank until the fish reached stage III, plane 1 of light anaesthesia (Brown 1993). Fish were then removed from the tank, placed on a ruler on top of an analytical balance (Mettler PJ 6000, Zurich, Switzerland) to record body weight and length. Fish were then returned to their experimental tanks for recovery. Condition factor [(K=weight/length$^3$) X 100] was assessed for the fish in the different treatments.

*3.3.1.4 Treatment and treatment groups*: Eight tanks of fish were randomly assigned to each treatment group: control (sham treatment), formalin, or chloramine-T. Each tank was treated once weekly, by turning the water flow off, and adding a diluted quantity of treatment chemical (or equivalent amount of water for sham treatments) for 1 h. Final tank concentrations were 0.20 ml/L for formalin treated tanks, and 10 mg/L for chloramine-T treated tanks. Water flow was turned on after 1 h to stop treatment.

*3.3.1.5 Sampling and blood collection to determine cortisol levels*: Blood was sampled from 15 fish after the acclimation period before any exposure to the chemicals or sham treatments. Specifically five fish (1 fish from each of 5 randomly selected tanks) within each treatment group were sampled, and their plasma used to determine baseline cortisol levels. After the beginning of the treatments, 4 fish from each treatment group (1 fish from each of 4 randomly chosen tanks) were sampled for blood at 1, 24 and 96 h after each treatment. For blood collection, fish were rapidly netted from their tank and anaesthetized in a solution of 40 mg/L benzocaine (Barton et al 1985). Blood (ca. 1 ml) was obtained from the caudal vein (Houston 1990) with a heparinized syringe and placed in a chilled heparinized vacutainer and maintained on ice until centrifuged ( Pickering et al. 1982; Sumpter et al. 1986). Care was taken that each blood sample was obtained within 3 min of removing the fish from its tank. The blood sample was separated into plasma from the red cells by centrifugation at 4°C (Pickering et al. 1982; Iwama et al. 1995). The plasma was stored at -20°C until analysis (Pickering and Pottinger 1983; Mazur and Iwama 1993).

*3.3.1.6 Radioimmunoassay*: The blood samples were analysed for plasma cortisol by radioimmunoassay (RIA) following the procedure recommended by the manufacturer of the Coat-A-Count™ Cortisol Kit (Diagnostics Products Corporation, Los Angeles, CA). This is a solid phase RIA, in which $^{125}$I-labelled cortisol 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 the reaction can be easily terminated and the antibody-bound fraction of the radio labelled cortisol isolated by decanting the supernatant. The radioimmunoassay

was performed at room temperature. All samples, including calibrators and controls supplied by the manufacturer, were analysed in duplicate and the final reported values are the mean of the two analyses (Appendix D).

*3.3.1.7 Control experiment for cortisol* - As a positive control for the study of the stress response of the fish, five adult rainbow trout and five juvenile brook trout (*Salvelinus fontinalis*) were subjected to acute stress. We used a handling stress which involved netting the fish and emersion for 90 sec (Barton and Iwama 1991; Barton and Schreck 1987). The fish blood was sampled 15 min, 24 h and 96 h post stress, and analysed for cortisol. Another subsample of "unstressed" fish (brook trout) was rapidly netted, anaesthetized, and blood sampled for cortisol assessment for direct comparison to our positive controls.

### 3.3.2 Trial 2 (Secondary stress indices).

To assess the development of secondary indicators of stress induced by chloramine-T treatment, a trial was developed as follows:

*3.3.2.1 Trial design and treatment exposure:* Twenty four tanks of diploid rainbow trout (avg. weight of 98 g) were used. A matched pairs design (based on tank location) was used, in which one tank of each pair was randomly assigned to a treatment (chloramine-T) or control tank (sham treatment). Chloramine-T tanks were treated (10 mg/L) twice weekly for 1 hour. Sham tanks were "treated" by turning off the water in 8 of the tanks,

and 4 of the control tanks were left undisturbed. Acclimation, assessment of weight, and system design were the same as in trial 1; however, a starting stocking density of 45 kg/m$^3$ was used, and fish were fed to satiation twice daily.

*3.3.2.2 Sampling and blood collection*: Blood was collected from treated and control groups of fish at the end of week three of the trial, during, and after a treatment with chloramine-T. Specifically, the fish were sampled just after the treatment began (time 0), and 2, 4, 8, 24, and 48 h after the treatment ended. Five fish from each group (i.e. one from each of 5 randomly selected tanks) were netted and their blood sampled. The procedure for netting, anesthetizing and blood drawing is the same as the one described above for the sampling of the cortisol levels. Plasma sodium, glucose, and chloride levels were determined with an automated discrete analyzer (Hitachi 911, Boehringer Mannheim) using Boehringer Mannheim reagents. Hematocrit was determined by the microhematocrit method.

### 3.3.3 Statistical analysis.

Trial 1: The null hypothesis tested was that treatment with chloramine-T or formalin did not elicit a significant rise in the blood levels of cortisol in rainbow trout, and therefore was not a stressor to the fish. This was tested through a one way ANOVA. Significant results were established at the $\alpha$=0.05 level. Analyses were made using MINITAB software.

Trial 2: In trial 2, the null hypothesis tested was that repeated intermittent treatment

with chloramine-T does not evoke hematological, metabolic or ionoregulatory changes in rainbow trout, when applied at 10 mg/L for 1 hour twice a week. Significant results were established at the $\alpha$=0.05 level. The results were tested through a one way ANOVA, using MINITAB computer software.

## 3.4 RESULTS

### Trial 1.

*Weights and condition factor (K) of fish*: The weights, lengths and K values of the fish subjected to the different treatments are shown in table I. At the beginning of the trial, the weights and lengths of the fish allocated to the different treatments (i.e. control, chloramine-T and formalin) did not exhibit a significant difference. At the end of the trial, no significant changes in the weights or lengths of the fish due to exposure to the different treatments were detected. However, the percentage of difference between the initial and the final weights among the different groups, showed that the fish treated with formalin had the smallest mean increment in weight ($\delta$=16.09%), while in the control and chloramine-T treated fish the differences were $\delta$=20.86% and 18.26% respectively. The condition factor of the fish at the end of the trial was not significantly different among the three groups of fish.

The baseline cortisol level determined for unstressed fish at the start of the experiment was 9.79±1.78 ng/ml which is within the range of the resting cortisol levels for fish (Barton and Iwama 1991). Compared with the baseline cortisol levels, the control

fish plasma cortisol levels (22.39±1.98 ng/ml) were significantly different (P=0.0003) 1 hour after the treatment started, and so were the cortisol levels for formalin treated fish (21.06±4.76 ng/ml; P=0.05). The levels were not significantly different for chloramine-T treated fish, however they were still elevated (15.69±2.53 ng/ml; P=0.078)( Fig 5).

At 24 h sampling, when each group was compared to the baseline values, no significant difference was found either in the control fish cortisol levels (7.12±1.7 ng/ml), formalin treated fish (6.74±1.85 ng/ml) nor the chloramine-T treated fish (14.64±3.2 ng/ml) and the baseline levels. No significant differences were detected between the experimental groups and the baseline 96 h after treatment: control fish (11.73±5.22 ng/ml), formalin treated fish (6.34±1.78 ng/ml), chloramine-T treated fish (15.03±4.61 ng/ml), (Fig.5).

The results for the control experiment are represented in Fig. 6. Both the rainbow trout and the brook trout had significant elevated plasma cortisol levels to 60.51±9.26 and 47.4±13.4 ng/ml respectively 15 minutes after a handling and emersion stress, when compared to the rainbow trout and brook trout baseline values. The cortisol levels remained significantly high for rainbow trout (62.28±1.81 ng/ml) and for brook trout (47.7±6.95 ng/ml) 24 hours after the acute stress, when compared to baseline values. The levels of cortisol were 45.7±15 and 36.61±9.07 ng/ml for rainbow trout and brook trout, respectively, 96 hours after the handling stress. The baseline cortisol levels in unstressed brook trout were 8.42±3.23 ng/ml, which is considered a normal or resting level of plasma cortisol in salmonids.

**Trial 2.**

*Hematocrit*: The haematocrit values were maintained within the normal value range for salmonids: 32-45% (Bowser 1993). However, chloramine-T treated fish showed a consistently higher haematocrit when compared to control fish, except for the sample taken at 24 h. No significant differences in the haematocrits of the experimental groups (treated and control) were detected at any of the sampling times (Fig. 7).

*Chloride:* There were no significant differences in the plasma chloride levels of the treated and control values in any of the sampling times. The chloride levels of the chloramine-T treated fish were consistently, although not significantly, lower than those of control fish at all times sampled. The values always remained at the low end of the normal range for plasma chloride: 120-147 mEq\L (Bowser 1993); however, the values for treated fish observed at 0, 4, and 48 h were lower than normal (115.4±11.06, 116.8±5.02 and 118.2±1.79 mEq\L, respectively) (Fig. 8).

*Glucose*: The plasma glucose levels between the experimental groups did not show any significant differences at any of the sampling times; nevertheless, the glucose levels for the chloramine-T treated fish tended to be lower than those of control fish. Fifty percent of all the values recorded for plasma glucose fell under what is considered 'normal' values (Bowser 1993). The levels in the control and experimental groups were basically the same. However, the plasma glucose levels of treated fish showed a significant ($P=0.02$) variation over the sampling time: there was a sharp rise in plasma glucose levels 48 h after treatment with chloramine-T, but this rise was also observed in the control fish (Fig. 9).

*Sodium:* There were no significant differences in the levels of plasma sodium at all sampling times among the three experimental groups. Furthermore, the plasma sodium levels in the treated fish did not fluctuate significantly over the sampling period of time. (Fig. 10).

## 3.5 DISCUSSION

The endocrine response of rainbow trout to a chemical prophylactic treatment with formalin or chloramine-T was examined. It appears that intermittent repeated exposure of fish to therapeutic concentrations of either chloramine-T once a week for four weeks was not sufficiently disturbing to elicit a significant deviation of the plasma cortisol levels from the levels found in control fish. Given the sensitivity of corticosteroids and their use as a standard measure of stress in fish (Kjartarsson et al. 1988; Adams 1990; Wedemeyer et al. 1990), it can be concluded that the treatment regimes were not stressful to rainbow trout.

Although rainbow trout did not exhibit a stress response to chloramine-T, the control experiment results, however, demonstrated that rainbow trout do respond to an acute stress and the levels of blood cortisol attained conform to those reported in the literature (Barton and Schreck 1987; Barton and Iwama 1991). It has been previously shown that the stress response duration is correlated to the duration of the stress (Pickering 1992). A stressor of 1 hour duration causes a rise in plasma cortisol levels that last at least 8 hours before they return to basal levels (Pickering 1992). This effect was not observed,

because basal cortisol levels for salmonids were found when the sampling was done 1 hour after treatment, regardless of the treatment used. In contrast Barton et al. (1980), showed a return to basal levels of cortisol in fish, 2 hour after a stressful event was reported.

Barton and Iwama (1991) summarized documented changes in plasma cortisol in fish resulting from aquaculture-related stressors. Although prophylactic chemical treatment is not included in this list, the high end levels of plasma cortisol reached in our study after treatment were comparable to those documented for rainbow trout when they are sampled without anaesthetic (i.e. 18 ng/ml), and lower than the levels reported for sampling with 125 mg/L tricaine methanesulphonate (i.e. 27 ng/ml). These levels remained within the range of what is considered resting (i.e. unstressed) levels of cortisol in salmonids.

Blood corticosteroids are a very sensitive indicator of stress in fish, and this sensitivity poses a problem when sampling fish: the act of removing a fish from a tank is sufficient to provoke an elevation of the cortisol levels of the remaining fish in the tank (Pickering et al. 1982). This was dealt in this experiment by sampling no more than one fish per tank, by restricting the netting operation to less than 90 seconds, since stressful stimuli (in this case, handling and confinement by netting) longer than 90 seconds causes a sharp rise in plasma cortisol, which attains a peak at 15 minutes and declines to basal levels over 2 hours (Barton et al. 1980), and by dealing with the experimental and control fish in an identical manner. Since the cortisol response in stressed fish depends on the level of the fish awareness (Schreck 1981), great care was taken in sampling the fish, so that the netting and anaesthetizing was done in the most rapid and efficient way to avoid

a rise in cortisol provoked by the sampling, and not by the treatment. Caudal vein puncture was used to sample blood, since this method causes less contamination by intracellular fluids than caudal transection (Høgåsen 1995) and blood samples were taken only once a day, at the same hour, to allow for consistency in the blood sampling, since elevated cortisol levels have been reported during the hours of darkness in rainbow trout (Baker and Rance 1980; Bry 1981; Zelnik and Goldspink 1981: cited by Pickering and Pottinger 1983).

In two cases, the cortisol levels of control fish were much higher that those of the formalin and chloramine-T treated groups. This probably reflects the role of handling stress, when the fish were netted from the tank and anaesthetized. These outliers (extreme observations that for one reason or other do not belong with the other observations or data) were removed from the final statistical analysis (Judd and McClelland 1989).

The lack of a stress response by rainbow trout to the treatment with chloramine-T was further reflected in the assessment of secondary stress response in which the hematocrit, as well as the levels of plasma chloride, sodium and glucose did not show a variation from the normal parameters. The secondary stress parameters help reveal conditions in the fish long before there is any outward manifestation of damage (McCay 1928), but after the primary rise in stress hormones. The most readily and frequently assessed parameter in fish blood is the haematocrit index (Houston 1990) which, with blood glucose levels, are significantly affected by stress in rainbow trout (Casillas and Smith 1977; Railo et al. 1985). Handling seems to elicit an elevation in plasma glucose while haematocrit does not seem to be appreciably altered in daily stressed fish (Barton et

al. 1987), although the haematocrit value may be increased as a cause of sampling stress (Railo et al. 1985). Blood levels of glucose and chloride are other frequently measured parameters to assess the secondary changes that occur during the stress response (Houston 1990). The methods used to measure these parameters tend to be simple and inexpensive (Wedemeyer et al. 1990). These blood parameters reflect a certain 'normal' haematological status for rainbow trout; any variance from this status can be used to assess specimen condition and diagnose induced stress (Houston 1990), even though changes in these parameters can arise from factors other than stress.

In the present study, the haematocrit values remained within the normal ranges reported for rainbow trout (Bowser 1993), although the levels in treated fish were always slightly above the control fish levels. This indicated that the fish were not suffering from nutritional deficiencies, disturbances caused by disease causing organisms, or other health related problems (Anderson 1990). Exposure to chemicals like chloramine-T, may cause fluctuations in the packed cell volume (PCV): anaemia or polycythemia. A chemical applied to the water may be considered a 'pollutant', and result in gill damage, which leads to internal hypoxia, which in turn may cause a swelling of the erythrocytes. This may account for the increase in haematocrit (Heath 1995). Another cause for an increase in the number of erythrocytes is the liberation of red blood cells from the spleen,which is induced by a contraction of the smooth muscle associated with the spleen (Perry and McDonald 1993). This helps to acutely increase the blood oxygen carrying capacity. A lack in increase in the hematocrit may indicate that the chemical did not cause enough gill damage to provoke hypoxia and thus a change in the hematocrit level. This may also indicate that

the hemoglobin levels in the blood and the blood oxygen-carrying capacity were not compromised (Perry and McDonald 1993) because of the treatment with chloramine-T, and the fish were not impaired by the treatment to meet metabolic oxygen requirements (Houston 1990). Our results of the changes in the haematocrit levels in rainbow trout as a result of treatment with chloramine-T are not consistent with previous results, in which a significant rise in the haematocrit level was observed in chlorine exposed fish (Zeitoun 1977) or where the haematocrits decreased significantly in both control and treated fish, after exposure to chloramine-T (Powell et al. 1994). Powell and Perry (1995) observed an acute decrease in the haematocrit level 1 hour after exposure to chloramine-T with values around 23% in fish exposed to 9 mg/L chloramine-T. We did not observe such a decrease. We did not find a rise in the haematocrit levels of treated fish, nor did we see a decrease, which is consistent with the results of Barham et al. (1980), where the haematocrit levels of oxytetracycline-treated rainbow trout did not differ from the values of a healthy, untreated control group.

When salmonids are subject to stressful treatments, substantial depression in plasma sodium and chloride levels which is not compensated until 24 h post stress have been widely reported (Postlethwaite and McDonald 1995). Specifically for chloramine-T, previous studies (Powell et al. 1994b), report a concentration dependent decrease in the levels of sodium and chloride in fish exposed to the chemical. The plasma chloride concentrations in the present experiment remained at pretreatment levels, in contrast to the studies noted above, and were not significantly different from levels in control fish. Similar results were obtained for plasma sodium concentrations. Hypochloremia can be

the result of gill chloride cell damage caused by the exposure to the chemical (Wedemeyer et al. 1991). It has been reported that treatment with chloramine-T causes significant changes in the chloride cell morphology and ultrastructure (Powell et al. 1995), which would in turn be reflected as a disturbance in the plasma levels of chloride and sodium. The same report also points out that chloramine-T might also cause epithelial degeneration on the gill and thus impair the normal ion regulation (Powell et al. 1994), since the mode of action of chlorine toxicity has been attributed to gill epithelial cell damage (Mitchell and Cech 1983). The absence of hypochloremia in this trial, suggests that there was no osmoregulatory compromise in the treated fish (Wedemeyer et al. 1991) caused by the chloramine-T or that a rapid compensation occurred.

Plasma glucose has been considered the most sensitive parameter in detecting sublethal stress responses (Wedemeyer et al. 1990; Fivelstad et al. 1995). Hyperglycaemia indicates a level to which an external stressor begins to cause physiological disturbances (Wedemeyer et al. 1990) when cortisol causes glucose mobilization from the fish liver (Barham et al. 1980). Results of the present study agree with what was previously reported by Powell et al. (1994) for chloramine-T treated fish, since any increase in the plasma glucose levels of treated fish was not observed. This reflects the results obtained in the first trial where a lack of elevated cortisol levels in the blood of chloramine-T treated fish was observed. This absence of a plasma glucose alteration means that the prophylactic treatment of the fish with chloramine-T does not stress the fish when used at 10 mg/L.

In summary, the results indicate that prophylactic treatment with chloramine-T apparently does not elicit a primary stress response in rainbow trout and thus the

subsequent secondary response is also absent. This indicates that rainbow trout physiological status was not compromised by the exposure to chloramine-T when applied in a 10mg/L dose.

Table I. Weights (g), length (cm) and K of fish in Trial 1.

| | Weight I N=40 | Weight F N=20 | K I N=40 | K F N=20 |
|---|---|---|---|---|
| Control | 177.60±12.32 | 224.30±41.25 | 1.27±0.05 | 1.39±0.06 |
| Chloramine-T | 185.7±11.56 | 227.20±52.1 | 1.33±0.08 | 1.38±0.02 |
| Formalin | 175.86±18.59 | 209.60±26.31 | 1.28±0.09 | 1.26±0.15 |

I: initial; F: final. Mean (+SD) of weights, lengths and K from fish are for the beginning of the trial.

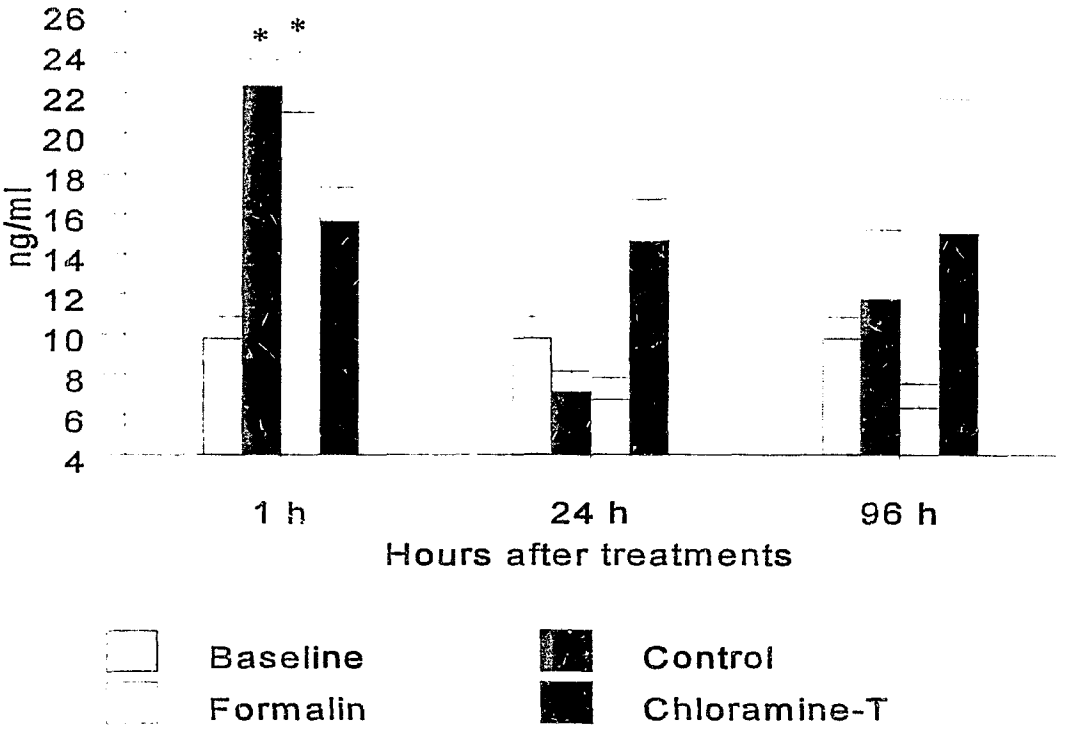


Fig. 5 Baseline and experimental cortisol levels in treated and control fish (Mean +SEM), * indicates significant difference..

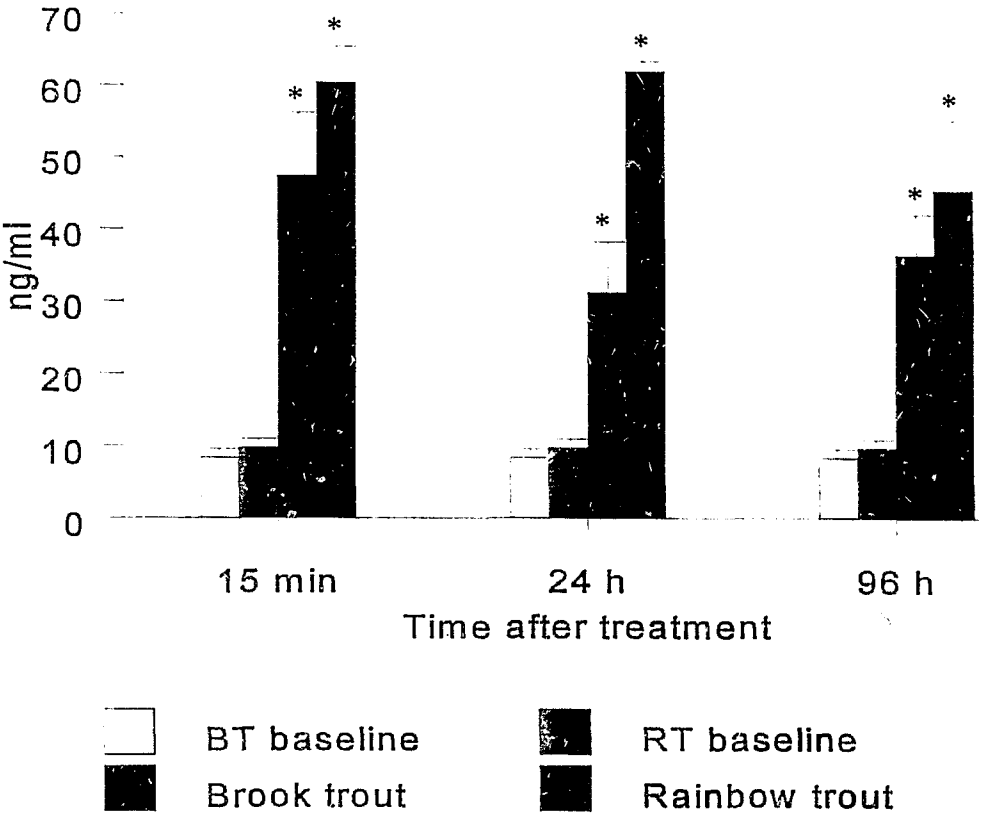


Fig. 6. Mean (+SEM) of cortisol levels (baseline and experimental) of brook trout and rainbow trout in the control experiment. Fish were stressed by handling (netting and emersion for 90 sec). BT: brook trout, RT: rainbow trout; * indicates significant difference from the baseline.

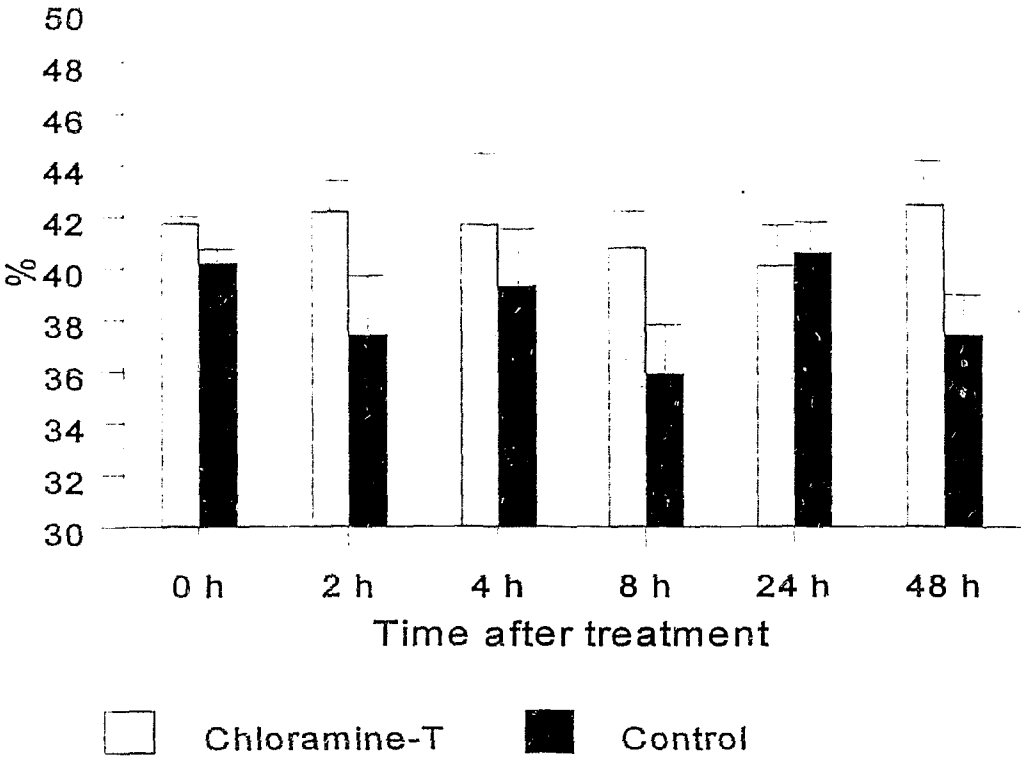


Fig. 7. Mean (+SEM) of the hematocrit in chloramine-T-stressed and control rainbow trout. Trial II.

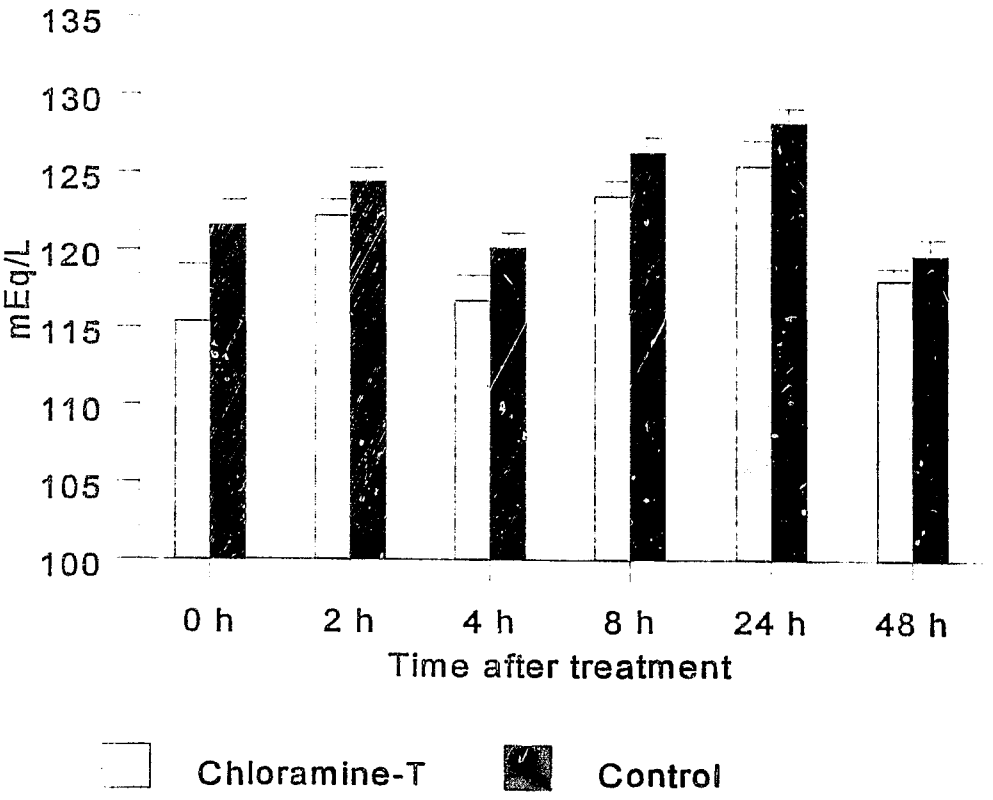


Fig. 8. Mean (+SEM) of plasma levels of chloride in chloramine-T-treated and control rainbow trout at different sample times after treatment. Tial II.

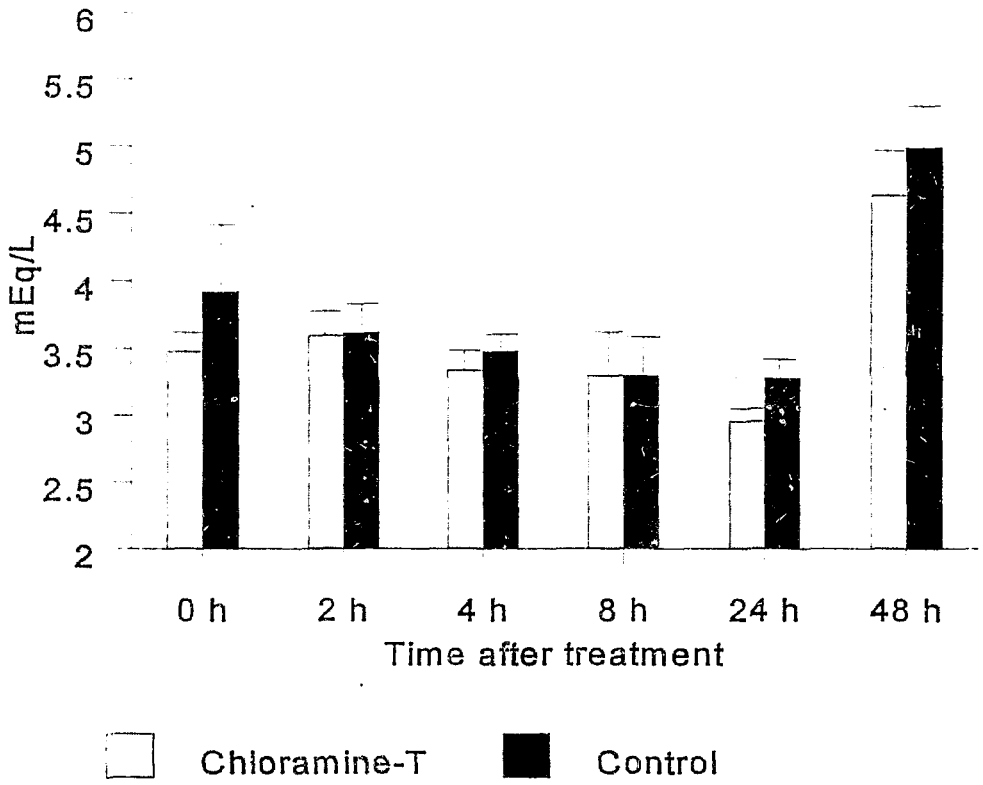


Fig. 9. Mean (+SEM) of plasma glucose levels in chloramine-T-treated and control rainbow trout at differen sample times after treatment. Trial II.

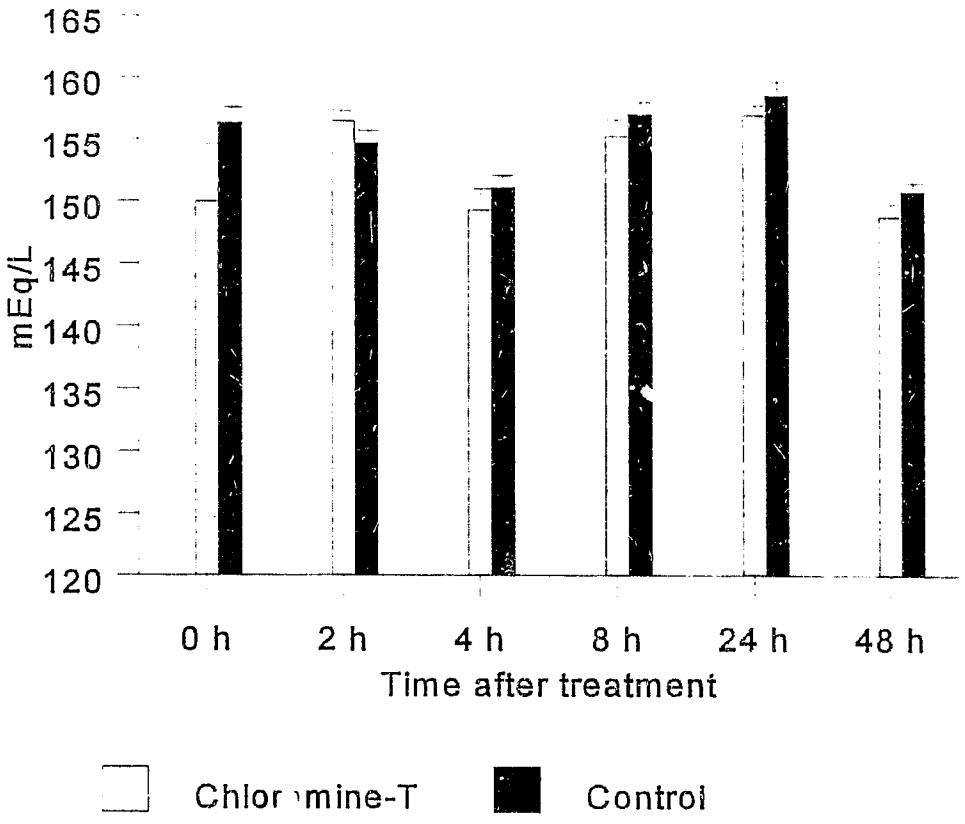


Fig. 10. Mean (+SEM) of plasma sodium levels in chloramine-T-treated and control rainbow trout. Trial II.

# 4. GILL STRUCTURAL CHANGES IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) ASSOCIATED WITH AN INTERMITTENT PROPHYLACTIC CHLORAMINE-T TREATMENT.*

## 4.1 ABSTRACT

This paper reports the structural and cellular changes observed in the gills of rainbow trout (*Oncorhynchus mykiss*) following exposure to an intermittent repetitive prophylactic treatment with chloramine-T (10 mg/L). The gill responses to chloramine-T were morphometrically assessed at three locations on the gill arch using light microscopy examination of tissues stained with hematoxylin and eosin and periodic acid-Schiff (PAS) + alcian blue pH2.5 (AB2). Chloramine-T did not cause an increase in the frequency of several typical changes of gills to toxicants. Specifically, there was no increase in lamellar edema, fusion, or inflammation, cellular changes (i.e. epithelial hyperplasia, hypertrophy, chloride cell metaplasia), or vascular lesions (i.e. pillar channel blood clots) in treated fish. Treatment was not associated with an increase in the number of lamellar mucous cells, however, the histochemical characteristics of the mucus produced in treated fish were significantly different from controls in terms of the generation of increased numbers of sialic-acid containing mucous cells, which were only rarely encountered in controls. Chloramine-T does not seem to act as a gill toxin and thus can be used as a prophylactic agent in the prevention of topical bacterial diseases.

*Editorial style conforms to the Journal of Aquatic Animal Health.

## 4.2 INTRODUCTION

The use of chemicals to prevent infectious diseases in cultured fish is an emerging practice in Canadian aquaculture (Thorburn and Moccia 1993). The chemicals used to prevent disease, were originally used in fish farming to treat infections. The effects of these chemicals, when used repeatedly on healthy populations, have been the object of relatively limited study. Chloramine-T (*N*-sodium-*N*-chloro-*para*-toluenesulphonamide) is currently used as a prophylactic and treatment agent for bacterial gill disease in salmonid hatcheries (Thorburn and Moccia 1993). Its effectiveness and low cost (From 1980) has made its use widespread in North American salmonid facilities (Smith et al. 1993; Thorburn and Moccia 1993; Powell et al. 1995).

Chloramine-T is a topical antiseptic applied to the fish usually in the form of a static bath (Herwig 1979). It dissociates freely in water into the hypochlorite ($OCl^-$) ion which later transforms into free chlorine (Sheltmire 1962) and *para*-toluenesulphonamide. The hypochlorous acid released is responsible for the bleaching and disinfecting properties of the chloramine solution (Sheltmire 1962). Free chlorine is a classically recognized toxin for many species of fish, acting through the interaction of the free radical on gill epithelial cells (Brooks and Bartos 1984). Despite this, the effects of chloramine-T, especially on the gill anatomy, have only been recently investigated. Of critical concern to aquaculturists is that recent research shows that this chemical, when applied at doses, durations, and frequencies similar to its use in salmonid aquaculture, significantly inhibits the growth of healthy rainbow trout (Powell et al. 1994) by inhibiting feed conversion efficiency (FCE)

(Chapter 2). In opposition to the possible benefits from prophylactic treatment, such as reducing the incidence and effects of disease, fish would take longer to reach a market weight (or seawater transfer weight if dealing with salmon). Additionally, feed costs, which constitute a large proportion of production costs for salmonids (Brannon and Klontz 1989), would be increased. Currently, it is not known how chloramine-T limits conversion efficiency. In Chapter 3 of this thesis, the hypothesis that treatment evokes a stress response was investigated. Based on our measurement methods, treatment was not demonstrably stressful and therefore we do not advance it as the factor limiting FCE.

An alternative explanation for the effects of chloramine-T on FCE is that the compound may be causing branchial damage, with growth suppression and FCE changes as a physiological consequence. The links between anatomical lesions and growth of fish have not been established; however, based on studies of mammalian production species, lung damage has been established as a cause of growth suppression of pigs in production settings (Dohoo and Montgomery 1996).

The gill epithelium is comparatively vulnerable to environmental toxins because it is in direct contact with the medium in which toxins are delivered (Wendelaar Bonga and van der Meij 1989), and has limited mechanisms of protection. Gill lesions due to toxins are predicted to have several physiological effects on fish due to the multiple functions of the gill. Specifically, it is the organ of gas exchange (Randall and Daxboeck 1984), ammonia excretion (Heisler 1984) and ion regulation (Evans 1993), and research exists to show the links between toxicant-induced gill damage and changes to these diverse physiological functions (Albassam et al. 1987).

Teleost branchial tissue responds in a fairly restricted, stereotypical fashion to diverse environmental toxins (Mallatt, 1985). Common lamellar changes include edema, fusion, and epithelial necrosis, hypertrophy, hyperplasia, and metaplasia (abnormal changes in the nature of a tissue) (Mitchell and Cech 1983; Mallatt 1985; Ewing et al. 1994). Hypothetically, some of these changes could be directly or indirectly involved in suppressing growth and FCE and, perhaps more importantly, may affect the ability of the gill to avoid colonization with endemic water-borne pathogens. In a previous study, it has been shown that rainbow trout treated with chloramine-T at 10 and 20 mg/L (i.e. 1-2 times the common treatment/prevention dose used in industry), developed lamellar edema, a reduction in the number of lamellar mucous cells, and an increase in the number of chloride cells (Powell et al. 1995). However, the previous study may have been complicated by the concurrent presence of elevated dissolved ammonia in the system during treatment (Powell et al. 1995). Additionally, the previous study spanned a relatively short duration time relative to the production cycle of the target animals and was not designed to mimic stocking densities usually encountered in a production setting.

The purpose of the current study was to assess the effects of chloramine-T on rainbow trout gill structure and mucous cell distribution and composition at the termination of an 11 week intermittent prophylactic regime, under management conditions simulating those in aquaculture. The dose level of chloramine-T (10 mg/L) was similar to that used in the aquaculture industry for the prevention of disease.

## 4.3 MATERIALS AND METHODS

*4.3.1 Fish and treatment allocation* - Rainbow trout with a mean weight of 98g were allocated to treatment tanks as previously described (Chapter 2) The initial stocking density was 45 kg/m3. The final design involved twelve treatment tanks matched to twelve control tanks, with pair matching based on previously observed location effects on growth.

*4.3.2 Feeding* - Fish were hand-fed ad libitum with two pellets sizes of one commercial feed: High Pro Grower #3 and #4 (Corey Feed Mills Ltd, Fredericton, N.B.). The proximate analysis of this feed is given in Appendix A.

*4.3.3 Exposure to chloramine-T* - 12 fish tanks were exposed to a dose of 10 mg/L chloramine-T in a 1-hour static bath. The application of the chloramine-T was done by predissolving 9.36 g of chloramine-T in 180 ml of distilled water and then mixing 15 ml of this solution with tank water to give the desired final concentration of chloramine-T in the tank. The water inflow was stopped for 1 hour to achieve the static bath. The water flow of 8 of the 12 control tanks was also stopped to simulate experimental conditions (sham treatment) and 4 control tanks were left undisturbed. Exposure to chloramine-T was done twice a week, on Wednesdays and Sundays, over an 11-week period.

*4.3.4 Sampling and tissue processing*- At the end of the companion study (11th week of the growth trial), 16 fish from each tank were arbitrarily netted and euthanized as a group

with an overdose of benzocaine (80 mg/L). All four gill arches of the left side were removed and fixed in neutral phosphate buffered 10% formalin. No more than 3 minutes elapsed from the moment the fish was euthanized until the gills were placed into fixative in order to prevent perimortem fixation artifacts (Speare and Ferguson 1989a). The second gill arches from 10 arbitrarily selected preserved specimens were placed in a histology cassette. Cassettes of tissue were then routinely processed for light microscopy: washed, dehydrated in graded concentrated ethyl alcohol (70% up to 100%), cleared in xylene and embedded in paraffin wax; 4-6$\mu$m-thick serial sections were made.

*4.3.5 Stains* - Samples were stained with either hematoxylin and eosin (H & E) to assess tissue damage and for quantification of the numbers of chloride cells, or periodic acid-Schiff ( PAS)-alcian blue pH2.5 (AB2) for mucous cell counts and to assess the mucus composition. These last two histochemical stains, belonging to methods of pre-lectin histochemistry (Ueda et al. 1994), were used to identify the main types of mucous glycoproteins. The combined PAS-AB2 stain yields a magenta colour if the mucins in the mucous cells are neutral, or a blue colour if they are acidic (Harris et al. 1973).

*4.3.6 Morphometric analysis* - Cellular composition and tissue structure changes in the gills were quantified by examination with a light microscope. Slides were coded and examined without knowledge of the treatment allocation. To determine the range of lesions and the overall status of the gill, three filaments from the second left gill arch from every fish were observed: the center filament, and a ventral and dorsal filament at a distance of

0.5 cm from the center filament. Filaments that were complete and well oriented were chosen. Morphometric indices based on a method of proportional morphometry developed for branchial tissue by Speare and Ferguson (1989a) were used. Specifically, each selected filament was evaluated for the following indices:

a) total number of lamellae suitable for quantification on the filament;

b) lamellar edema - percentage of lamellae with either visible separation of the two layers of epithelial cells and/or separation of epithelial cells from the flanges of the pillar cells involving at least 50% of a given lamella;

c) epithelial hyperplasia - the percentage of lamellae with one or more points of thickened areas of epithelial tissue, excluding those lamellae where this occurred at the lamellar tip;

d) lamellar fusion - the percentage of lamellae that were fused to adjacent lamellae;

e) lamellar inflammation - percentage of lamellae that had one or more clusters (>5 cells) of inflammatory cells;

f) interlamellar inflammation: percentage of zones between two adjacent lamellae that had clusters (>5 cells) of inflammatory cells;

g) "clavate" lamellae - percentage of lamellae with an accumulation of epithelial cells at or near the tip of the lamellae that increases the number of cell layers; this contrasts to index c in that it only occurs at the tip of the gill and imparts a club-shaped (= clavate) appearance;

h) blood clot - percentage of lamellae with one or more foci of accumulation of degenerate red blood cells (+/- fibrin) in the vascular channels of the lamella

with apparent rupture of the pillar cell, or pillar cell-endothelial cell junctions;

i) lamellar mid-section and tip width - the lamellar width (from the external cell membrane of the outer layer of epithelium from one side of the lamellae to the opposite) at the tip and mid-section of every fifteenth lamella starting from the one closest to the arch was measured (μm) using an ocular micrometer at 40x.

j) mucous cell numbers and composition - the number of positively stained mucous cells per lamellae (total cell count), and the number of mucous cells that stained blue (acidic or sialic-acid containing cells) or magenta (neutral cells) per lamellae was assessed by counting and evaluating all the mucous cells on the lamellae and dividing this value by the number of lamellae counted (adapted from Ferguson et al. 1992).

k) chloride cell numbers - the number of chloride cells was assessed by counting all chloride cells (ie. restricted to those whose apical-basal axis was perpendicular to the long axis of the lamellae, specifically to exclude those chloride cells in the interlamellar region) on ten arbitrarily selected lamellae.

*4.3.8 Statistical analysis* - The different indices were evaluated as follows: The values for each index from a given fish were expressed as the pooled mean from the three filaments evaluated. From this, a tank mean was calculated by pooling the means from the ten fish in the tank. A single paired T-test was used for each index (a-k, but not j) to test the null

hypothesis that there was no difference in the treated tanks compared to controls. To test the hypotheses that there were no differences in mucous cell numbers per lamellae or differences in mucous cell composition within and between treatment groups and between different locations on the gill , a two-way ANOVA was performed with treatment and gill location as factors. If significant differences were detected in these ANOVAs then an unpaired t-test was used as the post test for specific comparisons. An $\alpha$=0.05 was used for all statistical tests, and the analyses were done with a MINITAB (release 10.1) program.

## 4.4 RESULTS

The general qualitative appearance of the giils of treated and control fish were very similar. Based on morphometric assessment, there was no significant difference between treated and control tanks of fish for indices pertaining to proportion of lamellae that were fused or clavate (Table II; Figs. 14, 16), proportion of interlamellar zones which were inflamed (Table II), proportion of lamellae with blood clots (Table II; Fig, 17), the numbers of chloride cells per lamellae (Table IV), or the width of the tip sections of lamellae (Table III). However, we did find significant differences between treated and control tanks of fish for indices pertaining to the proportion of lamellae with either edema (Table II; Fig. 12), hyperplasia (Table II; Fig. 13), or infiltration with inflammatory cells (Table II; Fig. 15) with control tanks being more affected than treated tanks. The width of the middle sections of lamellae (Table III) was greater for fish in treated tanks compared to controls.

The ANOVAs on total mucous cell numbers and for the neutral mucin containing

cells, showed that no differences existed between treatment groups, or between locations on the gill within a treatment group (Table V). However, the ANOVA on sialic-acid (acidic) containing mucous cells showed that significant differences between treatments exist (treated fish have more cells per lamellae than controls), but not between locations within a treatment group, nor was there an interaction between treatment and location (Table V). For the treated groups there was no significant difference in the numbers of acidic compared to neutral mucous cells, but the ratio of acidic/neutral mucous cells increased. In the control fish there were significantly more neutral compared to acidic mucous cells, and in fact very few acidic cells existed (TableV). Location on the gill had no significant effect on the number or type of mucous cells in either the treated or control groups of fish. There was no significant interaction between the location and the mucus composition in either the control and treated fish. (Table V).

## 4.5 DISCUSSION

This study describes the changes observed in the morphology of the rainbow trout gill, after a long term trial (12 weeks) in which they were exposed to an intermittent chloramine-T regime at 10 mg/L. Chloramine-T can be considered a potential toxin due to its release in solution of free chlorine. Additionally, it has proven to be toxic to fish at relatively low applied concentrations in environments with minimal levels of divalent cations, low pH, and high temperatures (Sheltmire 1962; Mallatt 1985; Byrne et al. 1989; Bodesteiner et al. 1993; Powell et al. 1995). Free chlorine has been shown to cause a

hyperplastic response of the gill epithelia and hypersecretion of mucus in several teleost species (Zeitoun 1978, Brooks and Bartos 1984). However, the levels of chlorine used in the previous studies (1.09 mg/L or 0.25 - 0.64 mg/L respectively) are considerably in excess of that typically released during a standard chloramine-T treatment (i.e. 0.09 mg/L, Powell et al. 1995). Therefore these studies are not clear predictors of the range or severity of lesions associated with standard chloramine-T treatments in salmonid aquaculture. Except for the study by Powell et al. (1995), there are apparently no studies which specifically address the toxicity, and gill lesions associated with the standard use of chloramine-T in salmonid aquaculture.

Several of the findings were unexpected. Specifically, although it might have been predicted that repeatedly treating fish with chloramine-T would lead to chronic gill changes such as epithelial hyperplasia, lamellar edema, and cellular inflammatory infiltrates in the gill, the reverse was found. This type of branchial pathology is not restricted to toxicity situations but also frequently occurs in salmonids when kept in commercial settings of high stocking densities (Peters et al. 1984; Klontz et al. 1985). The pathogenesis is unknown, but may relate to the effects of waste metabolites produced by fish and discharged into tank water. Since fewer lesions were observed in chloramine-T treated fish, it is possible that chloramine-T therapy and the subsequent change in the mucus properties (acidification) resulted in a degree of protection of these fish to waterborne toxic metabolites. The reduction in epithelial hyperplasia and lamellar inflammation, combined with the absence of increased rates of other potential gill abnormalities (lamellar clubbing, fusion, interlamellar inflammation, pillar channel blood clots) in treated compared to control fish,

strongly suggests that chloramine-T, at the dose level used was not acting as a gill toxin.

An increased rate of lamellar edema was noted in the control compared to treated fish, in contrast to the work by Powell et al. (1995), in which lamellar edema was noted in chloramine-T treated fish. Whereas the index was significantly different between treatment groups in the present study, the actual differences in the values for each group were not large, and thus the differences are probably not physiologically significant. Although this index may relate to the increase in inflammatory cell infiltration of the lamellae in control fish compared to treated, it is also an index which is very prone to develop as a perimortem artifact (Speare & Ferguson 1989). Finally, in comparison to reports in which lamellar edema is cited as being physiologically significant (Mallatt 1985), the rates which were found in either group in the current study were quite small.

Chloride cells are located principally at the base of the lamellae on the filament epithelium (Eddy 1982). However, under certain conditions such as stress, toxicant exposure, and disease, chloride cells also develop along the lamellae of freshwater euryhaline salmonid fish (Eddy 1982; Laurent and Perry 1990; Laurent and Perry 1991; Ewing et al. 1994; Laurent et al. 1994; Powell et al. 1994a; Jürss and Bastrop 1995), probably due to a migration of chloride cells from the filament (Laurent et al. 1994). However, in the present study, the number of lamellar chloride cells was similar in treated and control fish in contrast to the shorter duration study by Powell et al. (1995). The discrepancy in results is interesting and could stem from the confounding variable of tank ammonia levels in the work by Powell et al. (1995), or, alternatively, could mean that the chloride cell number increase is not persistent during a longer exposure. Conceivably,

chloramine-T exposure may set into motion one of the mechanisms which results in chloride cell hyperplasia, but with repeated exposure over a longer duration, other compensatory physiological responses may occur which allow chloride cells numbers to return to normal. The latter hypothesis cannot be confirmed since the gills were only studied at the end of the trial.

Many water-borne toxins, gill diseases, and some therapeutic agents (e.g. formalin) produce a hyperplastic reaction of the branchial mucous cells (Mallatt 1985; Ferguson et al. 1992; Speare et al. in press). However, chloramine-T differs from this. Specifically, based on our index, no change in mucous cell numbers was demonstrated. Powell et al. (1995) quantified the mucous cells occurring within filament epithelium and found a significant reduction in mucous cell numbers after chloramine-T treatment. In combination with the current findings, chloramine-T treatment at standard dosages, may not be sufficiently irritating to the gill to induce an otherwise stereotypical gill response.

In addition to changes in numbers of mucous cells, the types of mucus and the relative amounts (Ueda et al. 1994) can also be affected by toxins and disease (Blackstock and Pickering 1982; Whitear 1986; Mittal et al. 1994; Ferguson et al. 1992). Treatment with chloramine-T led to an increase in sialic-acid containing mucous cells at the apparent expense of neutral-mucin containing mucous cells. Periodic-acid Schiff (PAS) stains neutral mucosubstances by the oxidation of vicinal hydroxyl groups (Mittal et al. 1994) in the carbohydrate fraction, followed by formation of colored complexes with the Schiff reagent; while alcian blue (pH 2.5) stains sulphate-free, sialic acid-containing mucins with a blue tint (Harris et al. 1973). AB2 thus distinguishes between neutral and acid

glycoproteins. The increase in the number of AB2 mucous cells in the treated fish can be accounted for by an increase in the number of sialic acid-rich glycoproteins (Harris et al. 1992), a response that has also been seen in rainbow trout infected with bacterial gill disease (Ferguson et al. 1992). This finding correlates well with the response of mammalian bronchial mucosa to certain forms of irritation, in which both the number of mucous cells and the degree of acidification of the mucus increase (Blackstock and Pickering 1982, Gail and Lenfant 1983).

The biological significance of the change in the type of mucus produced as a response to the exposure to chloramine-T remains speculative. Several hypothetical reasons for these changes have been given, and range from a change in the rate of mucus production, with some glycoproteins easier to produce than others (Mittal et al. 1994), to changes in the viscosity of the mucus (Ferguson et al. 1992). In mammals a decrease in the viscosity of the mucus has been correlated with the sialic acid content (Jones et al. 1973; Blackstock and Pickering 1982), which could increase the rate at which the mucus is moved over the gill surface, and also could alter the thickness of the mucous layer (Ferguson et al. 1992). This may help prevent the colonization of the epidermis by bacteria, fungi and parasites (Pickering and Macey 1977). The increase in the number of acid mucous cells in fish has also been thought to better help in the preservation of the fish health by preventing the proliferation of pathogenic microorganisms. Thus the mucus would act as an enhanced barrier to various environmental pathogens and prevent the colonization of the epidermis (Mittal et al. 1994). One can speculate that treating fish intermittently with chloramine-T could reduce rates of gill infection not only by directly interfering with bacterial numbers,

but also by increasing the protective potential of the gill mucus. This could explain the reduced rate of lamellar epithelial hyperplasia and lamellar inflammation in treated fish compared to controls, assuming that these changes could reflect a subclinical and undetected level of infection of the gill surface.

Table II. Mean (+SEM) percentage of gill lamellae that present structural changes.

| | Treated N=120 | Control N=120 | δ | P |
|---|---|---|---|---|
| Lamellar edema[1] | 8.55±1.36 | 11.50±1.20 | 2.95 | 0.05 |
| Lam. hyperplasia[2] | 8.66±0.78 | 10.60±0.74 | 1.94 | 0.03 |
| Lam. inflammation[3] | 1.95±0.25 | 2.75±0.30 | 0.80 | 0.02 |
| Clavate lamellae | 1.26±0.34 | 1.01±0.18 | 0.25 | 1.49 |
| Lamellar fusion | 0.69±0.13 | 0.64±0.13 | 0.05 | 0.79 |
| Interlam. Inflamm. | 0.11±0.07 | 0.06±0.03 | 0.05 | 0.26 |
| Blood Clot | 0.18±0.05 | 0.22±0.05 | 0.04 | 0.55 |

[1,2,3]Significant differences, δ difference.

Table III. Measurements of lamellar thickness. Mean (+SEM) width of the lamellae at the tip and middle section in μm.

| | Control<br>N=20 | Treated<br>N=20 |
|---|---|---|
| Tip | 16.6±0.83 [1] | 16.0±0.90 |
| Middle | 13.4±2.09 [1,2] | 14.5±0.57 [2] |

Tip: tip of lamellae; Middle: middle section of lamellae. [1,2]: significant differences.

Table IV. Mean (+SEM) number of chloride cells (x100) per lamellae in treated and control fish.

| Filament | Control N=120 | Treated N=120 | P |
|---|---|---|---|
| Central | 15.32±1.17 | 12.97±1.65 | 0.26 |
| Dorsal | 13.49±1.33 | 12.77±1.15 | 0.69 |
| Ventral | 14.70±1.34 | 14.10±1.64 | 0.78 |

Table V. Mean (+SEM) number of mucous cells (x100) per lamella, in treated and control fish with acid or neutral mucus content.

| | Control fish N=120 | | | Treated fish N=120 | | |
|---|---|---|---|---|---|---|
| Filament location | Acidic | Neutral | Total | Acidic | Neutral | Total |
| Central | 5.99±1.95[1,4] | 15.35±3.39[4] | 21.32±4.47 | 13.76±3.07[1] | 7.32±2.29 | 21.07±4.00 |
| Dorsal | 1.50±0.75[2,5] | 11.67±3.95[5] | 13.18±4.33 | 10.96±2.69[2] | 16.15±7.22 | 27.55±8.48 |
| Ventral | 2.92±1.0[3,6] | 9.60±2.36[6] | 12.52±2.61 | 9.44±1.57[3] | 12.65±2.39 | 22.08±2.38 |

Superscript: Values with similar superscript are significantly difference.

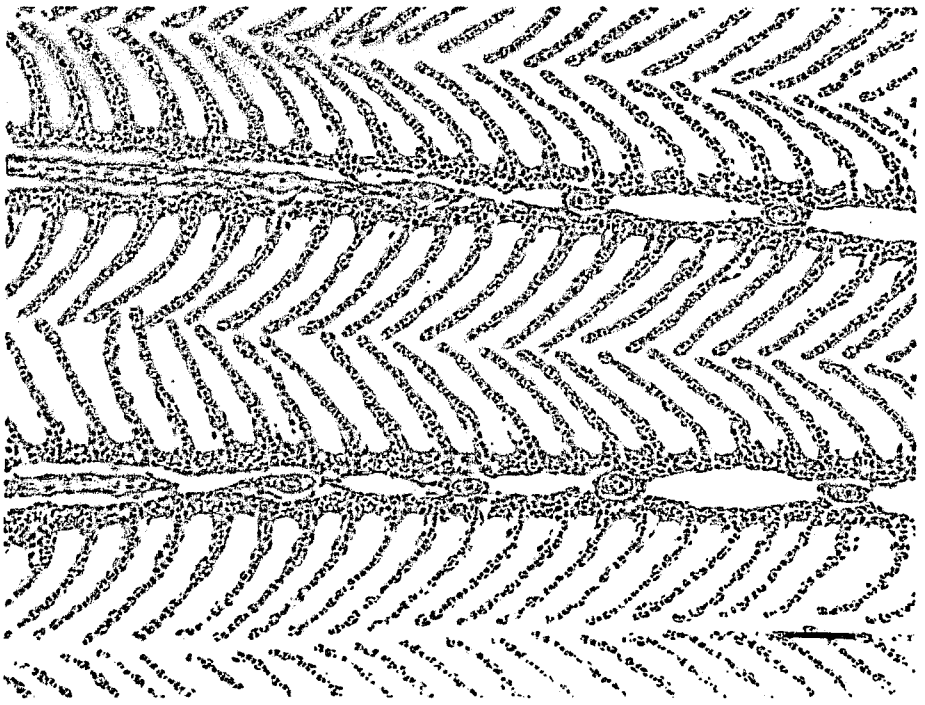

Fig. 11 Histological section of a normal rainbow trout lamellae. H&E. Scale bar = 130 μm.

Fig. 12 Histological section of a chloramine-T-treated fish showing lamellar edema. Note the visible separation of the two layers of epithelial and/or separation of epithelial cells from the flanges of the pillar cells (arrowhead), involving at least 50% of the lamellae. H&E. Scale bar = 85 μm.

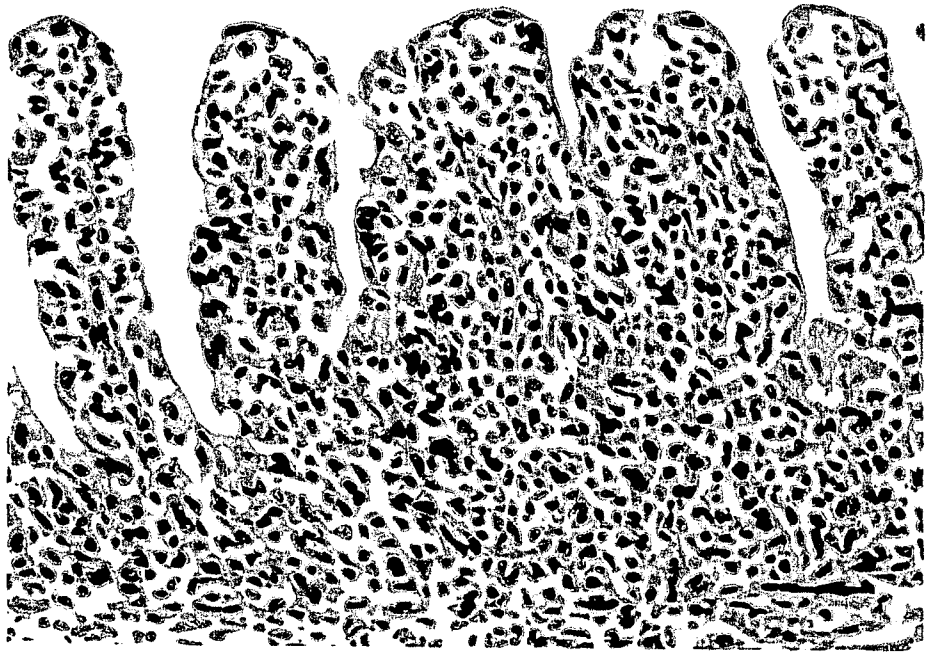

Fig. 13 Histological section of a chloramine-T-treated rainbow trout showing epithelial hyperplasia. Lamellae show one or more points of thickened areas of epithelial tissue (arrowhead). H&E. Scale bar = 20 μm.

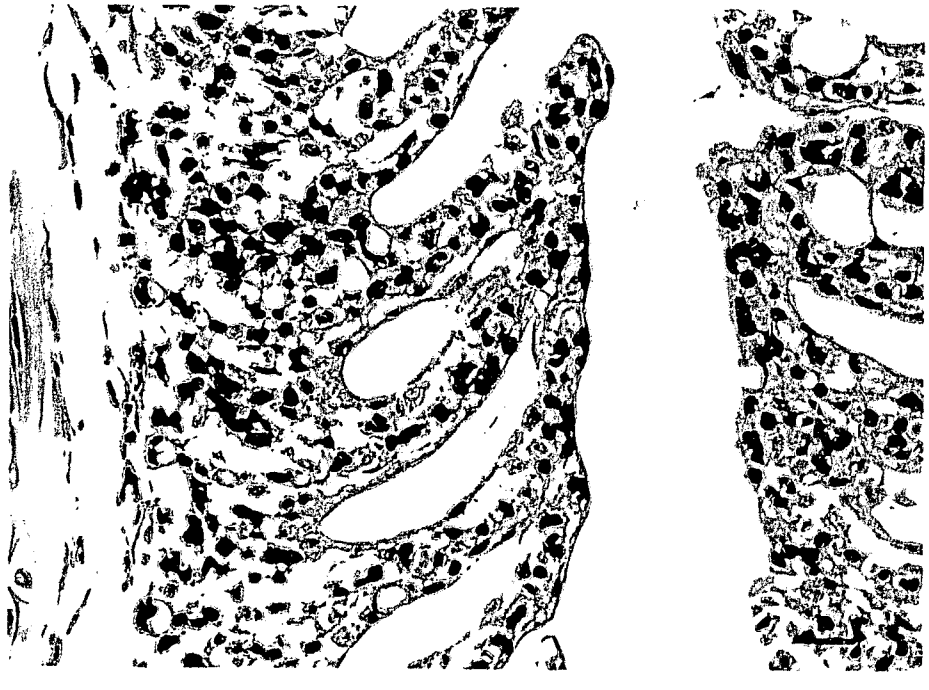

Fig. 14 Histological section of chloramine-T-treated rainbow trout gill showing lamellae fused to adjacent lamellae. H&E. Scale bar = 13 μm.

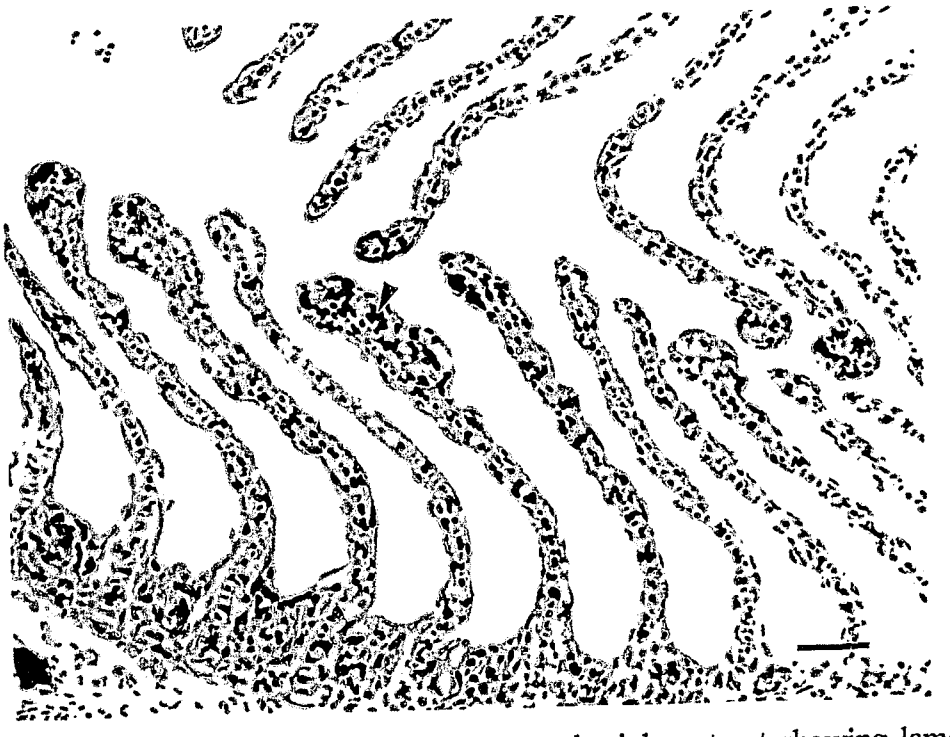

Fig. 15 Histological section of the gill of a control rainbow trout showing lamellar inflammation. Note the lamellae with one or more clusters (>5 cells) of inflammatory cells (arrowhead). H&E. Scale bar = 60 μm.

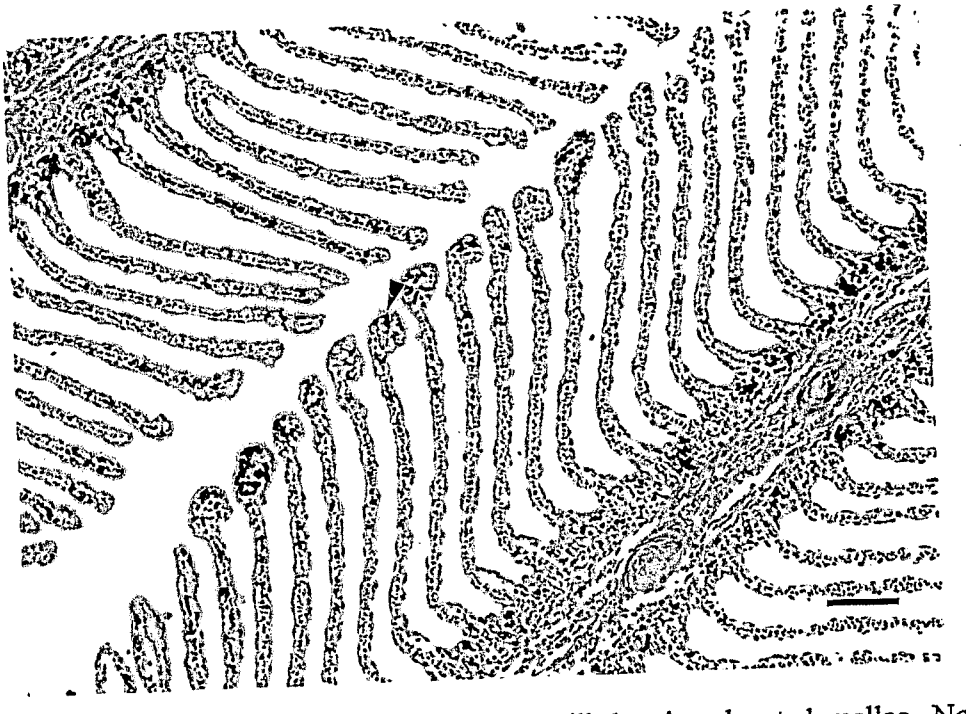

Fig. 16 Histological section of a control rainbow trout gill showing clavate lamellae. Note the accumulation of epithelial cells at or near the tip of the lamellae (arrowhead) that increases the number of cell layers. H&E. Scale bar = 90 μm.

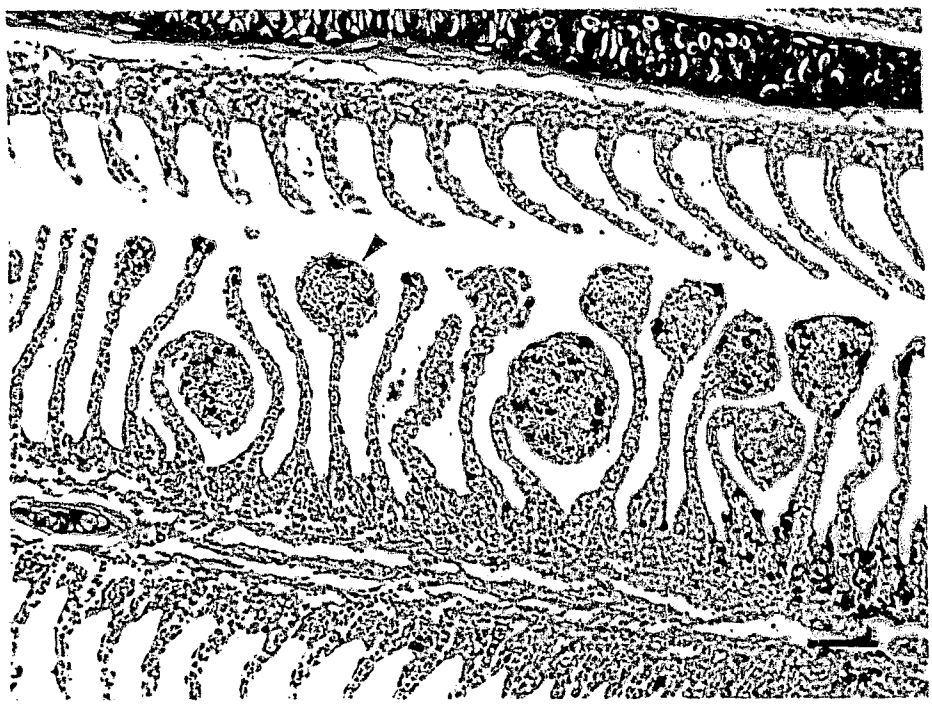

Fig. 17 Histological section of control rainbow trout gill showing blood clots. Lamellae present one or more foci of accumulation of degenerate red blood cells (+/- fibrin) in the vascular channels of the lamellae, with apparent rupture of the pillar cell (arrowhead). H&E. Scale bar = 90 μm.

Fig. 18 Histological section of a control rainbow trout gill showing metaplastic neutral mucous cells (arrowhead). PAS/AB2. Scale bar = 35 μm

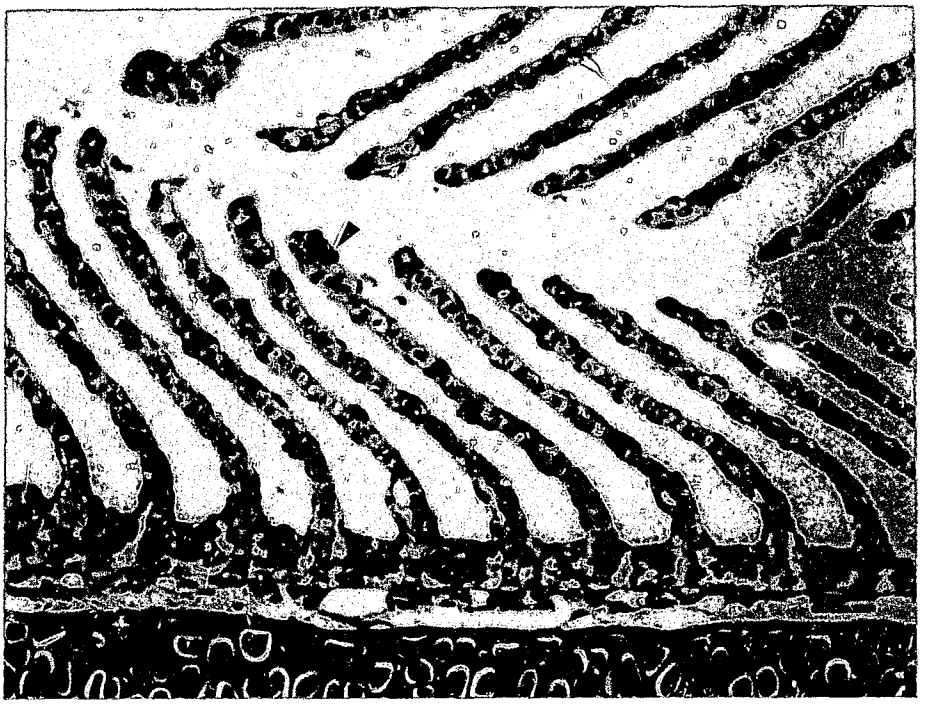

Fig. 19 Histological section of chloramine-T-treated rainbow trout gill showing lamellae with metaplastic sialic-acid containing mucous cells (arrowhead). PAS/AB2. Scale bar = 35 μm.

# 5. ADAPTATION OF A NON-AQUEOUS FIXATION REGIME TO STUDY EPIDERMAL AND GILL CHANGES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) REPEATEDLY EXPOSED TO FORMALIN AND CHLORAMINE-T.

## 5.1 ABSTRACT

Exposing rainbow trout to chloramine-T(10 mg/L) or formalin (0.2 ml/L) once a week for 4 weeks altered the epidermis by decreasing its thickness and increasing the number of electron dense vesicles in epithelial pavement cells. Formalin-treated fish's gill lamellae had an increase in the number of chloride cells and an accentuation of interstitial spaces between the two epithelial layers. Treatment did not cause a change in the mucous covering of the gills and skin, the degree of interdigitation of the epithelial basal lamina, the number of mucous cells/mm$^2$ or the electrondensity of the epidermal mucous cells mucosomes. Retaining the mucous covering of the gill and skin, for ultrastructural studies, is a common technical problem which was overcome by using 1% osmium tetroxide in a fluorocarbon solvent (FC-72™), a non aqueous fixative regime adapted from mammalian airway studies. With this method, a continuous, compact mucous coat of varying thickness along the skin and gill surfaces was retained. The non-aqueous fixative greatly enhanced the preservation of the skin mucous coat while providing excellent preservation of epidermal tissue. It can be concluded that chloramine-T can be used as a preventive agent in the control of topical diseases of fish, with little risk of causing a compromise in the osmorregulatory function or natural defense mechanism of the rainbow trout .

*Editorial style conforms to the Journal of Aquatic Animal Health.

## 5.2 INTRODUCTION.

Epidermal and branchial tissues of fish are multifunctional and complex (Henrikson and Matoltsy 1968; Hawkes 1983; Mallatt 1985; Rodger 1995) and are often the target site for waterborne pollutants (Mallatt 1985; Iger et al.1994; Sharples and Evan 1996). A component of both of these tissues is the mucus-rich biofilm surface, a structure which has been only minimally evaluated either in normal fish or those exposed to pollutants (Handy and Eddy 1991; Speare and Mirsalimi 1992; Shephard 1994; Powell et al. 1992; Lumsden et al. 1994). Biofilms are composed of microorganisms organized in viscous exopolysaccharide matrices associated with aquatic animal surfaces. Mucus is an important component of biofilms (Cheng et al. 1981). This biofilm, together with the glycocalyx (a carbohydrate rich peripheral zone on the outside surface of most eucaryotic cells, [Alberts et al. 1989]), is a critical interface with the environment. It facilitates a range of ionic interactions (Shephard 1994) and plays a protective role against infectious diseases, (Ingram 1980; Shephard 1993, 1994; Magariños et al. 1995) and environmentally-mediated damage (Yokote 1982; Hinton and Lauren 1990; Shephard 1993; Iger and Wandelaar Bonga 1994; Shephard 1994). Until recently, techniques to stabilize teleost mucus-rich biofilms did not exist. Hence, while cellular reactions in the gill and skin of fish during disease and pollutant exposure have been the subject of considerable study (reviewed by Mallatt 1985 [gill] ; Iger et al. 1992 [skin]), only a few studies (Speare and Mirsalimi 1992, Powell et al. 1992, Lumsden et al. 1994) have used techniques to stabilize and examine surface biofilms.

Histochemical analysis has revealed that the major components of piscine epidermal mucus are sialic acid-rich glycoproteins (Harris et al. 1972) containing sulphate and carboxyl radicals. The proteinaceous precursors are formed in the rough endoplasmic reticulum, and the carbohydrate moiety is synthetized by the Golgi cisternae of mucous cells (Whitear 1986a; Mittal et al. 1995). Mucus also contains immunoglobulins and other substances capable of reacting with infective organisms, thus providing the host with an immediate or first line of defence (Ingram 1980; Shephard 1994).

Chemicals are commonly used to treat and prevent diseases of commercially-reared salmonids (Thorburn and Moccia 1993). Two of the therapeutic agents more commonly used to treat diseases in fish are formalin and chloramine-T. Formalin is a mixture of 37% formaldehyde gas ($CH_2O$) dissolved in water, and stabilized with 10-15% methanol. It is effective in treating fish and eggs for external parasites, bacteria, and fungal infections (Howe et al. 1995). Chloramine-T (sodium paratoluenesulphonchloramide) is commonly used as the agent for treatment against bacterial gill and skin diseases (From 1980; Thorburn and Moccia 1993; Powell et al. 1994).

There are only a few studies on the effects of these chemicals on fish when used at therapeutic levels. The majority of the studies on formalin have dealt with the toxic effects at dosages above those used in aquaculture (Wedemeyer 1971; Smith and Piper 1972; Wedemeyer and Yasutake 1974; Speare et al. in press). Chloramine-T has not been studied as thoroughly. However, its active component, free chlorine, has received considerable attention (Zeitoun 1977, 1978). Other studies have shown that the gill is a target organ for the toxic effects of several therapeutic chemicals used in aquaculture (Mallat 1985; Byrne

et.al. 1989; Hinton and Lauren 1990; Lindesjöö and Thulin 1994). Unfortunately, many of these previously published studies cannot be used to predict outcomes of the therapeutic doses of chemicals used in aquaculture, because the dose levels studied exceeded dose levels used in aquaculture. For example, therapeutic levels of chloramine-T (Chapter 4) or formalin cause, at most, only minor gill changes (Speare et al. in press), compared to the severe damage caused by other compounds such as Zephiran (Byrne et al. 1989).

In aquaculture, the intensive rearing of fish frequently leads to infectious diseases in which pathogens colonize gill and skin surfaces (Speare and Ferguson 1989a; Armstrong 1993; Thorburn and Moccia 1993; Bullock et al. 1994). The mucous film and substances secreted into this film are widely believed to serve as the main defense against colonization and replication of surface pathogens (Hawkes 1974; Austin 1988). However, to date, studies on formalin and chloramine-T, either at toxic or therapeutic levels have not examined changes to the mucous biofilm, although a study by Powell et al. (1994a) examined the glycocalyx component of the biofilm during a chloramine-T treatment. Accordingly, it is critical to examine the effects of treatment chemicals on the mucous coat of fish and the adjacent epithelium, particularly when the chemicals are used repeatedly and fish are being reared at high stocking densities known to be a risk factor for topical infections (Noga 1996).

There is some evidence to suggest that exposure of fish to chemicals may affect the surface biofilm. For example, changes in the mucous cell numbers, shape, presence of electron dense mucosomes, reduction in the epidermal thickness, and folding of the basal lamina have been widely noted on the skin and gills of fish after exposure to a range of

chemicals (Mallatt 1985; Iger et al. 1988; Hinton and Lauren 1990; Iger and Wendelaar Bonga 1994; Iger et al. 1994a,b,c; Iger et al. 1995). Change in mucous cell content and number is likely to secondarily alter the biofilm. Currently, however, there are no published reports which describe the direct effects of chemicals on the mucous coat.

The first objective of this study was to assess qualitatively and quantitatively the effects of formalin and chloramine-T on mucus and tissue of exposed fish. Specific hypotheses under test included whether treatment chemicals affect skin thickness, mucous cells volume density (MCVv), and mucous cell number density (MCNv). In order to accomplish this, a protocol for the stabilization of the mucus-rich biofilm and fixation of skin and gill tissue for transmission and scanning electron microscopy using a non-aqueous fixative was developed. FC-72 is the commercial name of an inert perfluorocarbon that has an average molecular weight of 340, a boiling point of 56°C, a density of 1.68 g/cm$^2$ at 25°C and a vapour pressure of 232 torr at 25°C. Specifically, this non-aqueous fluorocarbon based method was compared to a conventional glutaraldehyde based regime.

## 5.3 MATERIALS AND METHODS.

*5.3.1 Development of a protocol for the preservation of piscine mucus* - Juvenile rainbow trout (20g and 21cm average weight and length) were euthanised using an overdose of TMS (tricaine methanesulfonate, Syndel Laboratories, Vancouver, B.C., Canada). Tissue sections (0.5 x 1.5 cm) from the anterior abdomen were excised and fixed by immersion in a processing vial containing a non-aqueous solvent, FC-72™ (3M, St. Paul, MN), with

dissolved 1% (w/v) osmium tetroxide (Marivac LTD. Halifax, N.S., Canada). This fluorocarbon is colourless, odourless, nonflammable and has an efficient removal time from the tissues during processing. After 90 minutes fixation, the tissues were washed in pure FC-72 2-5 times to remove any non-bound osmium tetroxide. Each rinse was 10 minutes in duration and care was taken to disturb the skin as little as possible. There was no postfixation. After this step, the tissues were routinely processed for transmission electron microscopy (TEM). Some of the tissue sections were fixed using a conventional method of fixation (glutaraldehyde followed by postfixation with osmium tetroxide), to compare the mucus and ultrastructural preservation with the non-aqueous method.

The processed tissues were thick sectioned at 0.5μm using an Ultracut E Microtome (Reichert Jung, Austria) and stained with 1% toluidine blue in a 1% sodium borate solution. Thick sections were examined with a Nikon 104 (Japan) light microscope to detect areas of interest before cutting 60 nm thin sections to examine at the electron microscope level. These sections were stained using 5% uranyl acetate in 50% ethanol and lead citrate (Sato). Thin sections were examined with a Hitachi H-600 transmission electron microscope.

### *5.3.2 Evaluation of effects of formalin and chloramine-T on the epithelium of rainbow trout.*

*5.3.2.1 Sample population* - 120 rainbow trout (average weight of 182.8 g and average length of 23.94 cm) were used. Five fish were randomly allocated to each of 24 replicate tanks (78L) and were acclimated for 2 weeks before the beginning of the experiment, at a stocking density of 10 kg/m$^3$. Fish were fed at 2% of their body weight once a day with a

commercial feed: High Pro Grower #4 (Corey Mills Ltd, Fredericton, NB). The water temperature was 10°C.

*5.3.2.2 Treatment* - The fish were divided into 3 experimental groups: Control, formalin and chloramine-T-treated. Eight tanks were randomly assigned to each treatment group. Both chemicals were used at recommended doses for prophylaxis, 10 mg/L chloramine-T and 0.2 ml/L formalin. Prophylactic treatment was performed once a week during 4 weeks and consisted of a 1 hour static bath with either one of the chemicals or a sham treatment (control) in which only water was added to the tank.

*5.3.2.3 Tissue sampling* - Fish were sampled 24 hours after each treatment. The sampling procedure was as follows: 1 fish was removed from each of 4 tanks of any given experimental group (n=4 for each treatment) and was euthanised with an overdose of benzocaine (80mg/L). A portion (0.5 cm x 2.0 cm) of skin was removed from the anterior abdominal region. This area has been shown to be particularly responsive with respect to changes in mucous cell concentration (Zuchelkowski et al. 1981). The gill filaments were obtained from the central region of the second left gill arch.

*5.3.2.4 Sample tissue processing* - The skin and gill samples were fixed and processed by the non-aqueous method described in section 5.3.1. The samples destined for TEM were cleared in propylene oxide, and embedded in Spurr's resin. Tissue samples to be used for scanning electron microscopy (SEM) were dehydrated, critical point dried and sputter

coated with gold palladium.

*5.3.2.5 Light Microscopic e. ion:* The semithin sections were evaluated for the following indices using an ocular micrometer:

a) skin thickness - mean distance from the basal lamina to the pavement cell surface (μm),

b) maximum mucous cell width (μm),

c) mucous cell numerical density (MCNv) - number of mucous cells per $mm^2$ = ($\Sigma$ #MC/S) / N, where #MC is the number of mucous cells, S is the sample area in $mm^2$, and N is the number of samples (ie. fish)

d) mucous cell volume density (MCVv) - percentage of epidermis occupied by mucous cells = $\Sigma$ %MC area / N ; where %MC area = (Total area occupied by mucous cells ( TAMC) in sample / sample area) x 100; TAMC= mean area of 5 biggest mucous cells x the number of mucous cells (in $mm^2$);

*5.3.2.6 Electron Microscopic qualitative evaluation of the skin and gill epithelium:*

Sections examined for TEM were used to evaluate:

a) Presence of the mucous layer,

b) Presence of high electron dense vesicles in the epithelial cells,

c) Folding of the basal lamina,

d) Presence of interstitial spaces in the lamellae,

e) Presence of chloride cells on the lamellae.

*5.3.2.7 Statistical evaluation* - The differences between the experimental groups and the controls for the different indices of section 5.3.2.5 were tested for statistical significance with a one way Analysis of Variance (ANOVA), using a MINITAB program, with and $\alpha$=0.05 level. Means and standard deviations (SD) were used throughout.

## 5.4 RESULTS

Overall preservation of the epidermis fixed with osmium tetroxide using fluorocarbon as a carrier was as good as with conventional fixatives, and efficiently preserved the mucous coat. In skin fixed in non-aqueous fixative there was a continuous layer of mucus in which the pavement cell microridges were immersed, contrasting with the pavement cell surface of glutaraldehyde-fixed skin which appeared 'naked' (Fig.20a). The mucous layer was more or less homogeneous in thickness and consisted of mostly finely fibrillar material consistent with the material enclosed in the mucosomes in the goblet cells (Fig. 20b; 21). The electron density of the mucous coat varied, and sometimes its thickness increased when foreign particles and what appeared to be cellular debris were present (Fig. 20b).

Skin and gills fixed in the fluorocarbon fixative showed an excellent preservation of the surface structure of the epithelial cells when examined by SEM. The fingerprint-like appearance of the microridges was readily apparent in sections where the mucous coat had been fractured and lost (Fig.22). The epidermal surface showed an extensive sheet of mucus over the microridges of the pavement cells. This cover was very smooth in

appearance and precluded the vision of the pavement cell microridges lying underneath (Fig. 22). On closer examination, the mucous cover seemed to vary in thickness (i.e. seen by SEM as a change in translucence), and when it thinned, the underlying microridge outline could be discerned (Fig. 23). An interesting feature found in the fish gill was that interlamellar spaces appear sometimes to be "bridged" by layers of mucus extending from one lamella to another (Fig. 24).

*Effects of Chloramine-T and Formalin on epidermis of rainbow trout.*

*Light Microscopy* - MCVv and MCNv did not vary significantly in any of the experimental groups (i.e. formalin or chloramine-T-treated, and controls) at any of the weeks when skin samples were taken (Table VI). Epidermal thickness and the width of the mucous cells was not significantly different at weeks 1, 2, 3. However, at week 4, a significant difference for the epidermal thickness, among the different experimental groups could be noted: the control fish had the thickest epidermis (mean 202.9 μm), whereas for formalin-treated fish it had a mean of only 126.1 μm thick, and for the chloramine-T-treated fish it was the thinnest (mean 110 μm). The maximum mucous cell width recorded in week 4, was 26.19 μm for control fish, with 21.56 and 20.50 μm for formalin and chloramine-T-treated fish respectively. An ANOVA did not show a detectable treatment dependent difference (P=0.07) in the width of the fish epidermis mucous cells from the three experimental groups.

*Electron Microscopy of the Epidermis* - The degree of folding of the basal lamina was not affected by the treatment used, with controls, formalin and chloramine-T-treated fish

having marked folding of the basal lamina (Fig. 25a,b). In control fish, a well preserved mucous cover was detected. The fibrillar nature of the mucous cover could be appreciated by comparing the mucus in the microridges and the mucus contained in the mucosomes, especially when the mucosomes were fixed in the process of breaking onto the surface of the skin (Fig. 21). The majority of mucosomes in the mucous cells were electron lucent. Differences in the density of the mucosomes did not seem to vary according to the treatment, with occasional mixtures of mucosomes of different densities present in the mucous cells from fish in all three experimental groups (Fig. 26).

Very few electron dense vesicles could be detected in the epithelial cells of control fish (Fig. 26), especially those cells beneath the surface (i.e. pavement) layer. Large numbers of these vesicles could be seen routinely in close proximity to the epithelial surface of formalin or chloramine-T-treated fish (Fig. 27, 28); these vesicles had various degrees of electron density. In the formalin-treated fish, the mucous coat showed different degrees of thickness. At times, it was greatly thinned and the only structure present on the tips of the microridges was the glycocalyx, but most often, a thick electron lucent mucous cover could be seen covering the epithelial surface, which at intervals appeared to be about to lift from the microridges, as noted by an even more lucent zone between the microridge tips and the rest of the cover (Fig. 20b). The same could be observed in the skin of fish treated with chloramine-T (Fig. 28), in which large numbers of electron dense vesicles could be seen in the pavement cells.

*Electron microscopy of the gills* - The gills of control fish showed excellent preservation. Little damage in the form of intercellular spaces between the two layers of epithelial cells was observed. A thick, electron-dense mucous coat was seen covering the lamellar epithelium in a continuous form. However, some fish exhibited only a thin mucous coat, concentrated on top of the microridges. Very few to no chloride cells were detected (Fig.29). In the fish from the group exposed to formalin, the most striking characteristic was the presence of a large number of chloride cells on the lamellar epithelium. These chloride cells showed microridges on their surface, which were always coated by a layer of mucus. The mucous cover was present over all the lamellar surface, with little variation in its thickness (Fig.30). Large interstitial spaces between the epithelial and the pillar cells were seen in the lamellae. The fish belonging to the chloramine-T exposed group exhibited a mucous coat with some variation in thickness. The mucous coat was comparatively electron dense and was continuous over the entire lamellar surface. Differences in the thickness of the mucous coat could not be detected over chloride and adjacent pavement cells. The presence of some interstitial spaces within the lamella was noted (Fig. 31).

## 5.5 DISCUSSION

In this study, a non-aqueous fixation protocol was adapted from a report on the stabilization of mucus of mammalian airways (Sims 1991). To our knowledge, it is the first time that it has been used to stabilize piscine epidermal mucus. Most of the fixation methods used for morphological description of the teleost skin are based on aqueous

fixatives, which wash away the mucous cover, although the addition of alcian blue (2%) to glutaraldehyde greatly enhances the labelling of the branchial mucous coat (Powell et al. 1992). It is relevant that it was found the piscine mucous coat of the skin and gills to be continuous. This strongly suggests that mucous secretions, as they emerge from a mucous cell, mix with secretions from other mucous cells. The coat which forms is therefore more likely to be consistent across an area of skin or gill with respect to amount, and mixture of carbohydrates. The close apposition of the mucus to the cell surface microridging observed are similar to that reported for teleost branchial mucus (Lumsden et al. 1994). The similarity between the mucus-epithelial interface for the gill and skin was not surprising considering the similarities in cell surface topography, and the presence of actively secreting mucous cells in the epithelium of both tissues.

The ultrastructural appearance of the mucous coat of the skin and gills was generally fibrillar. It is believed that the fibrillar appearance reflects the mucin 'bottle-brush' structure, with hundreds of small carbohydrate chains attached to the peptide part of the molecule (Lamblin and Roussel 1993).

The mucous coat preserved in the skin and gills by this method had a mean thickness of 0.6 μm. These results, which require more rigorous verification, may be subsequently refined to reflect topographic heterogeneity. However, these findings are generally similar to those reported by Lumsden et al. (1994) in which the mucous coat of the gill of trout, stabilized with rabbit antiserum to gill mucus, was reported to be less than 1μm.

This is the first report which specifically examined the mucous film after an

exposure to a disinfectant chemical. It has been previously thought that treatment, particularly with chloramine-T, strips the mucous coat of the fish, based on the observation of a mucous-film in the water of treated fish. It may be that treatment evokes a discharge of mucus, which is later replaced. This is important, particularly if these chemicals are used prophylactically, since loss of the mucous coat would likely promote the odds of infection. An interesting finding was the lack of a detectable bacterial or protozoal biota in the mucous coat. It is generally believed that the mucous coat protects the underlying epithelium by binding pathogens and facilitating their clearance. Previous bacteriological studies have shown that fish mucus generally contains some viable bacteria and therefore it was expected to detect them with ultrastructural examination. The fact that no bacteria were observed is quite interesting and supports a theory advanced by Crouse-Eisnor et al. (1985) that bacteriological studies of fish mucous have been biased by the use of techniques which do not exclude the bacterial biota of the environmental water which is collected along with mucus. The lack of bacterial flora in the fish must be taken as a preliminary observation, since the background bacterial flora in the water was generally quite low. If future studies in bacterially enriched environments show a similar result this suggests that the antibacterial substances which are known to exist in fish mucus (Sheppard 1993; 1994) successsfully eliminate or markedly diminish the numbers of bacteria which can exist in mucus. This suggests that the mucus of fish acts both biologically and mechanically to protect skin surfaces.

The use of an inert fluorocarbon as a carrier for osmium tetroxide was first described by Thurston et al. (1976), who proposed that the preservation of the mucous coat

using the fluorocarbon-osmium method appears to be due to rapid stabilization of mucus glycoprotein and glycosaminoglycan molecules. Since mucus is insoluble in fluorocarbon (Thurston et al. 1976), it is possible that it is retained in its normal position rather than undergoing dilution, as is the case when an aqueous fixative is used. The use of this non-aqueous fixative offers several other advantages: it seems to eliminate osmotic changes, possibly retaining more tissue proteins and lipids; it presents low solvent toxicities, has a stable chemical nature and is easy to handle in the laboratory (Thurston et al. 1976; Sims 1991). In addition, non-aqueous fixatives appear to result in excellent preservation of cytoplasmic, organellar and extracellular detail (Sims and Horne 1993). The results show that the use of a fluorocarbon-osmium fixative is effective in the preservation of the mucous coat on the skin and gills of fish, thus allowing for the investigation of epidermal mucus-related events in fish, e.g. the flow of mucus over the epithelial surface (Speare and Mirsalimi 1992), or the increase in mucous secretion recorded under toxic conditions that helps to prevent entry of toxicants into the fish (Kantham and Richards 1995), while at the same time allowing the assessment of biofilms and skin surface associated pathogens.

The changes observed in the skin of fish subjected to different chemical treatments showed that the exposure of rainbow trout to formalin and chloramine-T under laboratory conditions, simulating commercial rearing conditions, had some effects on the characteristics of the epidermis. Whether these are potentially detrimental or advantageous is not readily construed.

In the present trial, the MCN per $mm^2$ did not vary following exposure to formalin or chloramine-T. This is interesting, because changes to the number of mucous cells as a

response to environmental stressors, such as manure (Iger et al. 1988), wounding (Iger and Abraham 1990), parasites (Urawa 1992), acidified water and temperature elevation (Iger et al. 1994b, 1994c, 1994d) have been widely documented. These authors have suggested that environmental stressors stimulated mucous secretion leading to an exhaustion or reduction of mucous cell numbers. The results, (weeks 1-3) suggest that the treatments do not act in the manner of other stressors. The reduction of the absolute number of mucous cells on the skin by week 4 (both treatments) is a different phenomenon, since skin thickness was also reduced by this period. When mucous cell numbers were expressed proportionate to skin area, the results were not different from weeks 1-3.

An interesting observation in this study is the trend (that did not achieve significance) towards decrease in the size of the mucous cells at week four in chemically exposed fish. This could indicate that new mucous cells were being produced and so, were smaller. In general, mucous cells increase in size with maturation (Iger and Abraham 1990). This decrease in the size of the mucous cells, caused by exposure to chloramine-T and formalin, has not been (to the author knowledge) reported. However, the MCVv (i.e. percentage of the total area occupied by mucous cells in the skin samples) did not vary significantly among treatment groups, probably due to the decrease in the thickness of the skin at week four in the formalin and chloramine-T treated fish. This could mean that even if the area of the skin sample that was actually occupied by the mucous cells decreased (as compared to control, non-thinned skin), it still accounted for a similar percentage of a thinned epidermis, and no differences could be detected. The decrease in skin thickness in formalin and chloramine-T treated fish on the fourth week after the treatments started

was dramatic. This probably indicates that the rate of cell shedding surpassed cell replacement. The shedding of cells occurs following necrosis or apoptosis. Whereas necrosis of epithelial cells probably reflects the direct negative effects of a stressor, apoptosis is an indirect physiologically controlled cell death (Iger et al. 1994). An increased rate of cell shedding may indicate that the turnover rate of filament cells (and consequently pavement cells) is increased during adaptation to water treated with chemical compounds. Similar observations have been found in studies on epidermal cells of tilapia (Wendelaar Bonga et al. 1990), rainbow trout (Iger et al. 1994b), carp (Iger and Wendelaar Bonga 1994), bullhead catfish (Zuchelkowski et al.1985) exposed to acidified water, and in rainbow trout (Iger et al. 1994c) exposed to temperature elevation. An alternative is that the treatments are affecting the mitotic capabilities of the epithelial cells such that they do not keep up with normal, or respond to increased rates of cell loss.

The presence of abrupt interdigitations of the basal lamina in fish as a consequence of stress has been postulated by Iger et al. (1994c). However, in this trial, the incidence of basal lamina interdigitations in all groups of fish indicates that it was not a direct response to the chemical treatment. The presence of secretory vesicles of high electron density in challenged fish and their relative absence in control fish has been observed in the filament cells of fish exposed to a variety of stressors, including temperature (Iger et al. 1994c), acidified water (Iger et al. 1994 b), manure (Iger et al. 1988), and wounding (Iger and Abraham 1990). In the current study, both formalin and chloramine-T treatments induced the production of these high density vesicles. The presence of these vesicles indicates the activation of externally oriented defence mechanisms (Balm et al. 1995), believed to be

indicative of a general response to stress, specifically an elevation in the plasma cortisol levels (Iger et al. 1994b). However, in the present study these high density vesicles appeared even in the absence of an elevation of plasma cortisol (Chapter 3), so it is possible that the integument autonomically maintains the adjustments necessary for acclimation to the environmental stressors present, presumably via paracrine regulatory circuits (Balm et al. 1995). This increase in the high electron density vesicles appears primarily in the upper layers of the filamentary cells, mainly in the pavement cells. These secretory vesicles have been shown to possess peroxidase activity, their contents are released to the epithelial surface and contribute to the formation of the glycocalyx (Iger and Wendelaar Bonga 1994), which was well-maintained in our treated fish.

The presence of electron dense (serous) mucosomes in the mucous cells has been related to external stressors (Whitear 1986; Iger et al. 1994b). However, in this study, the electron density of the mucosomes was not a reflection of the treatment, as shown by their presence in control fish. A possible explanation could be the natural heterogeneity of the glycoproteins in the mucus (Mittal et al. 1995). It was particularly interesting to find that chemical treatment did not affect the thickness of the mucous coat of the gill or skin.

Lamellar edema, which was detected as enlargement of the intercellular tissue spaces, was a dramatic feature of the formalin treated fish and, to a much smaller extent, the chloramine-T treated fish. These structural changes suggest that there is a physiological compromise on the part of the fish (Kantham and Richards 1995; Powell et al. 1995), and agree with the effects of formalin on the gills of rainbow trout described in the work of Smith and Piper (1972). The visible increase in the number of chloride cells in formalin-

treated fish, suggests that an osmorregulatory imbalance was probably present, produced when the ion balance is disturbed by various types of water pollution (Wendelaar Bonga et al. 1990), since ion uptake and excretion from the environment is carried out by these cells (Smith and Piper 1972; Wendelaar Bonga et al. 1990; Powell et al. 1995). Chloride cell numbers increase, in general, as a response to stress (Jagoe et al. 1996). The increase in numbers of chloride cells present on the lamellar epithelium of chloramine-T-exposed fish was not as dramatic as that observed in the lamellae of formalin-exposed fish. These observations agree with the findings at the light microscopy level (Chapter 4). The presence of interstitial spaces within the lamella of chloramine-T-treated fish is an indication of a probable negative effect of the chemical treatment.

In summary, the adaptation of the non-aqueous fixation protocol greatly enhanced the preservation of the mucous coat on the skin and gills of fish. This technique can be used to fix specimens in the field, since it does not require the use of sophisticated equipment. With this technique the changes caused by chloramine-T at the ultrastructural level in the skin and gill of fish could be assessed. Chloramine-T causes a chronic thining of the epidermis of treated fish but does not affect the number of mucous cells, or causes a detachment of the mucous coat, and its effects on the gills were less dramatic than those caused by formalin.

It can be concluded that chloramine-T can be used as a preventive agent in the control of topical diseases of fish, with little risk of causing a compromise in the osmorregulatory function or natural defense mechanism of the rainbow trout.

Table VI. Effects of chloramine-T on morphometric parameters of rainbow trout abdominal skin. Mean±S.E.M.

| Parameter | Control N=20 | Formalin N=20 | Chloramine-T N=20 |
|---|---|---|---|
| MCN($mm^2$) | 500±102 | 574±136 | 458±49 |
| MCVv(%) | 27±6.5 | 21.5±9.8 | 15.6±6.1 |
| Skin thickness | 202.9±41.1 | 126.1±14.89 | 110±53.1 |
| Mucous cell width | 26.19±1.35 | 21.56±1.60 | 20.50±1.85 |

MCNv = Mucous cell number; MCVv = Mucous cell volume density; skin thickness and mucous cell width in μm.

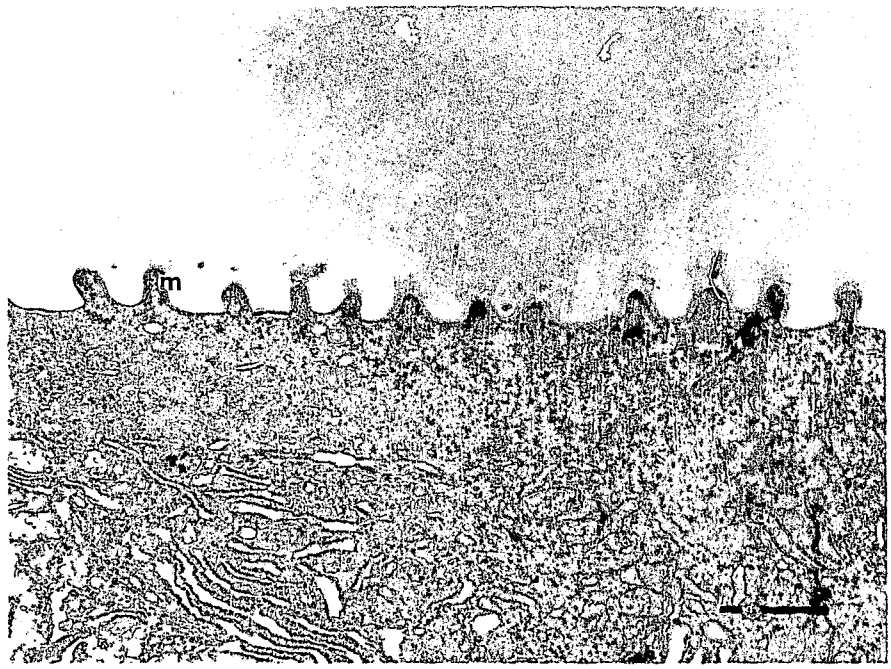


Fig. 20a. Transmission electron micrograph of the epidermis of a control fish (rainbow trout) fixed with a conventional aqueous fixative (glutaraldehyde). The glycocalyx and the mucous cover have been completely removed from the microridges (m). x 14 000; bar = 1 μm.

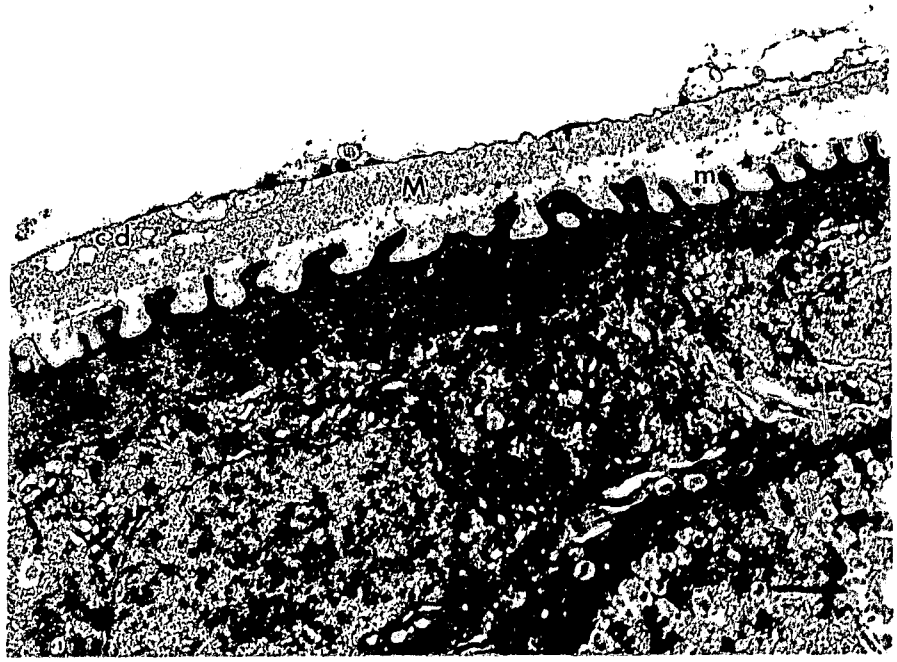


Fig. 20b. Transmission electron micrograph of the epidermis of a formalin-treated rainbow trout fixed with a non-aqueous fixative (fluorocarbon). Note the extensive mucous layer (M) covering the microridges (m). Cell debris (cd) can be seen trapped in the mucosal layer. x 9 600; bar = 1 μm.

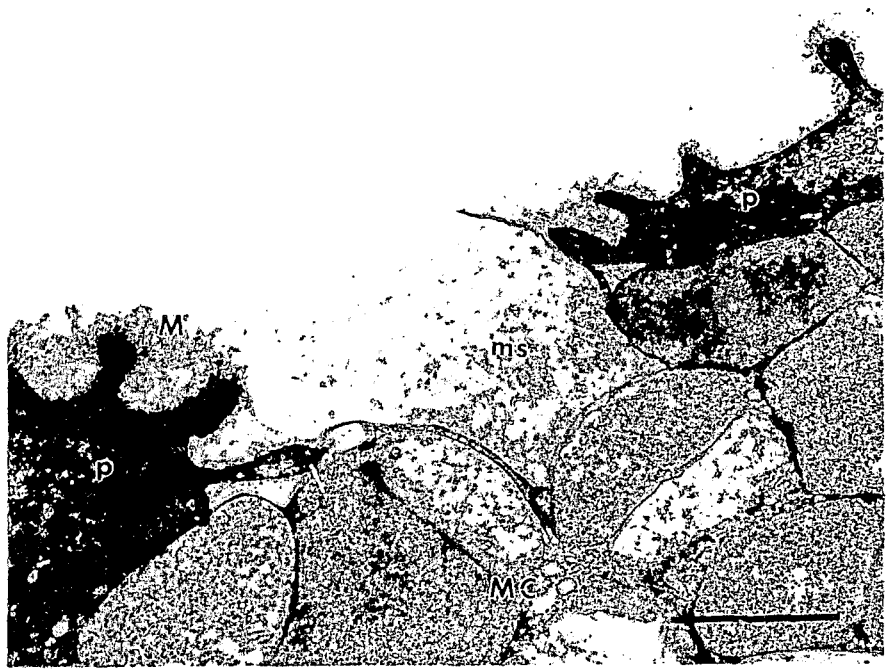


Fig. 21. Transmission electron micrograph of the epitdermis of a control rainbow trout, showing a mucous cell (MC) opening onto the epidermal surface. Note the similar nature of the mucosome (ms) content with that of the mucus (M) covering the microridges of the flanking pavement cells (p). x 22 000; bar = 1 μm.

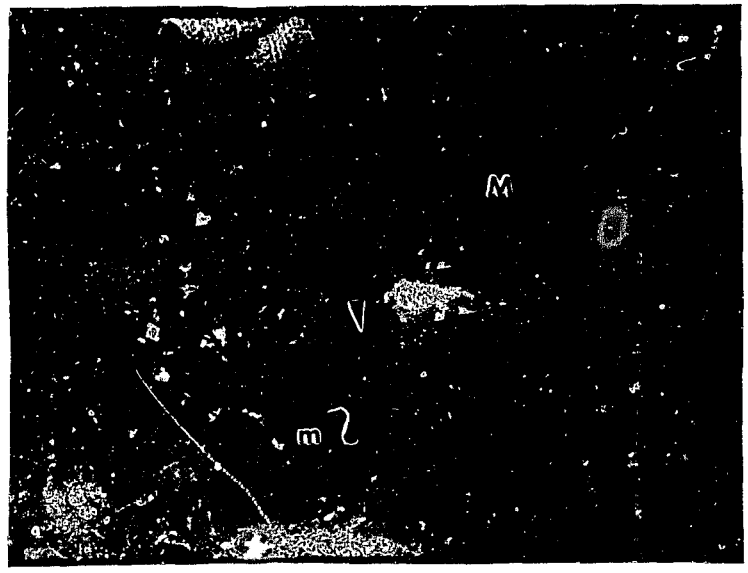


Fig. 22. Scanning electron micrograph of the epidermal surface of a control rainbow trout. Mucus (M) is seen as an extensive cover above the microridges (m), which look clearly preserved in the region were the mucus fractured and was lifted off (arrowhead). Note the smoothness of the mucous cover. x 1 000; bar = 10 μm.

Fig. 23. Scanning electron micrograph of the epidermal surface of a control rainbow trout showing thinning of the mucous cover that helps discern the microridges underneath (arrows). x 5 000.

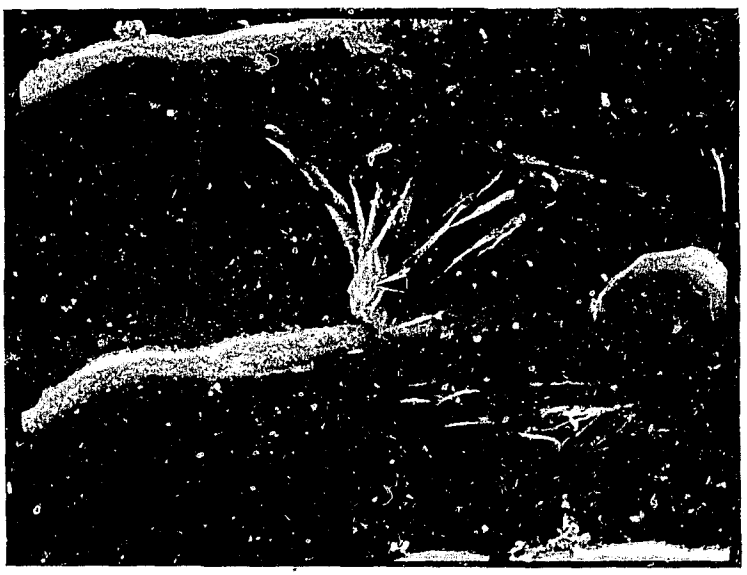

Fig. 24. Scanning electron micrograph of the epithelial surface of a control rainbow trout lamellae showing the mucous cover. The mucus (arrow) appears to peel off the lamellar surface, and to "bridge" with the mucus of adjacent lamellae. x 1 000; bar = 10 μm.

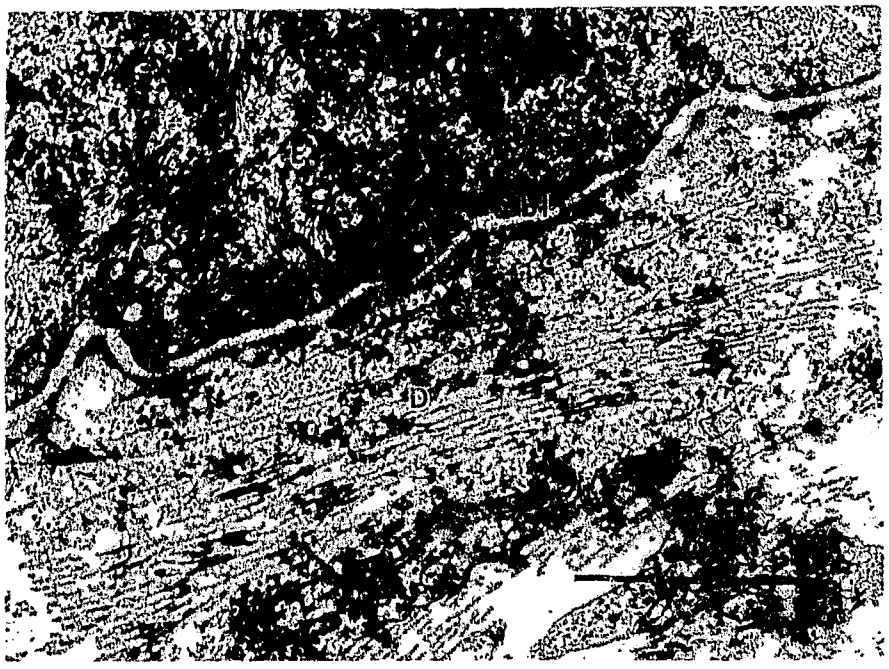


Fig. 25a. Transmission electron micrograph of the epidermis (E)/dermis (D) interface of a control rainbow trout. Basal lamina (bl) of a control fish showing its normal structure. x 32 000; bar = 1 μm.

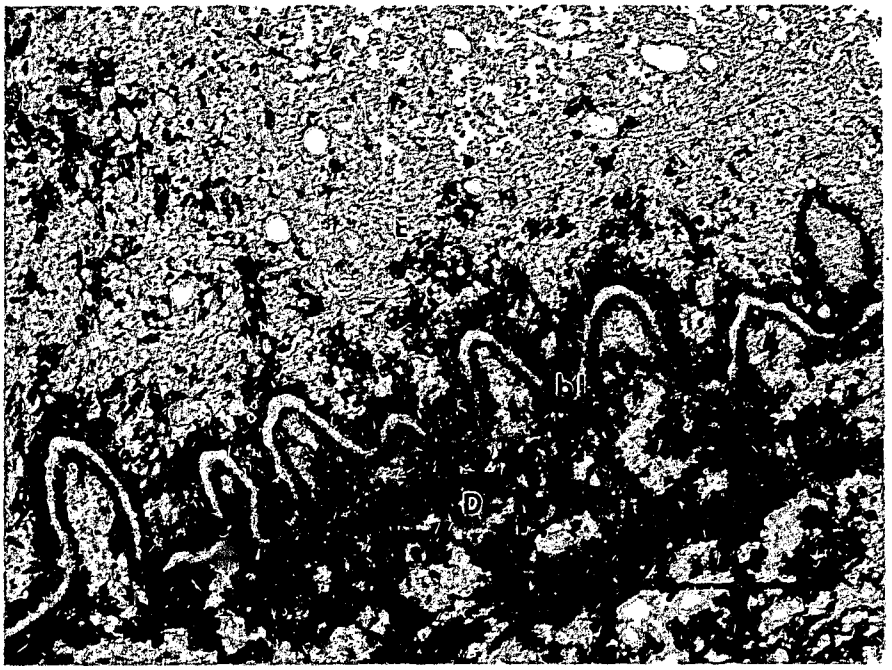


Fig. 25b. Transmission electron micrograph of the epidermis (E)/dermis (D) interface of a control rainbow trout. Note the heavily folded basal lamina (bl). x 22 000; bar = 1 μm.


Fig. 26. Transmission electron micrograph of the epidermis of a control rainbow trout, with a mucous cell (MC) showing electrondense mucosomes (arrowheads). Very few vesicles of high electron density can be seen in the pavement cell. x 6 400; bar = 1 μm.

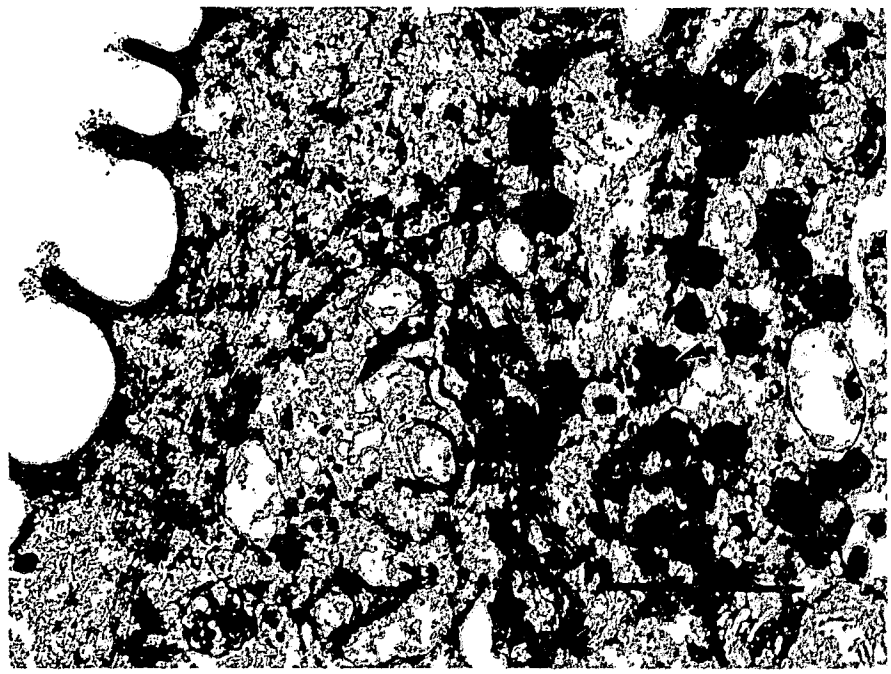

Fig. 27. Transmission electron micrograph of the epidermis of a formalin-treated rainbow trout. Note the presence of vesicles of high electron density (arrowheads) readily visible. x 27 200; bar = 1 μm.

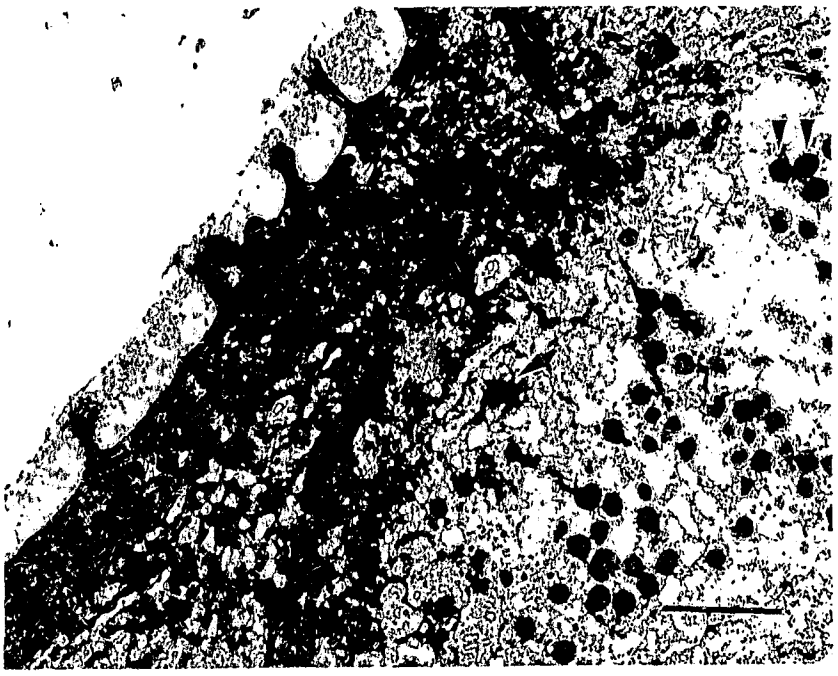

Fig. 28. Transmission electron micrograph of the epidermis of rainbow trout exposed to chloramine-T. Note the presence of large numbers of high electron dense vesicles (arrowheads). x 16 000; bar = 1 μm.

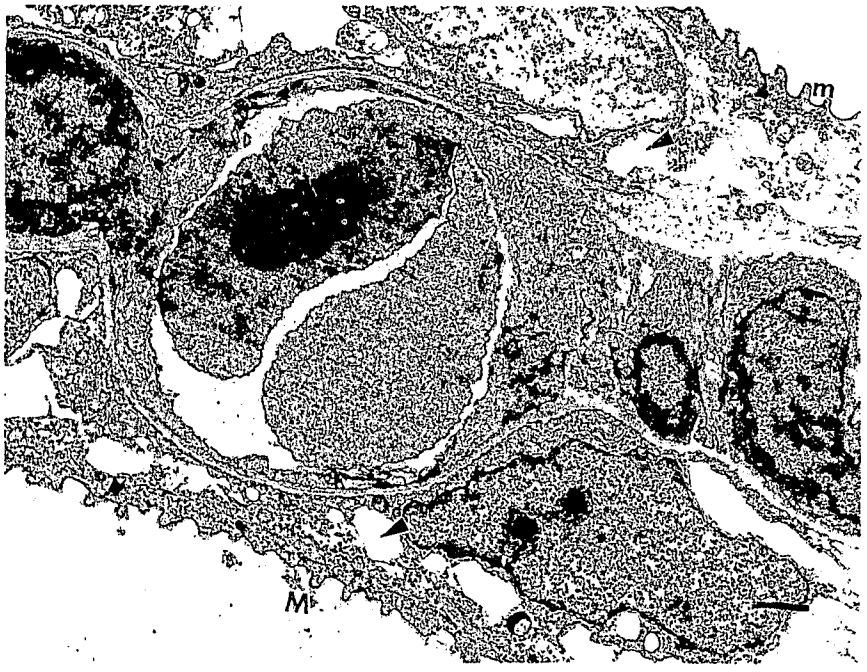


Fig. 29. Transmission electron micrograph of a lamella from a control fish. Note the mucus(M) preservation on the microridges(m) and overall good preservation of the lamellar structure. Very few interstitial spaces(arrowheads) can be seen. x 7 000; bar = 1 μm.

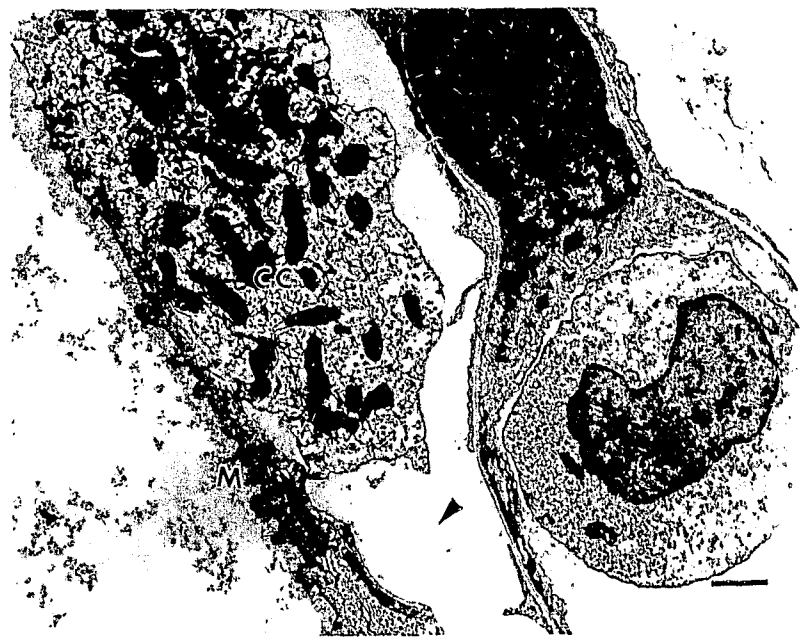


Fig. 3U. Transmission electron micrograph of a lamella from a formalin-treated fish. Note the presence of the chloride cell(CC) and big interstitial spaces (arrowhead). M (mucus). x 7 000; bar = 1 μm.

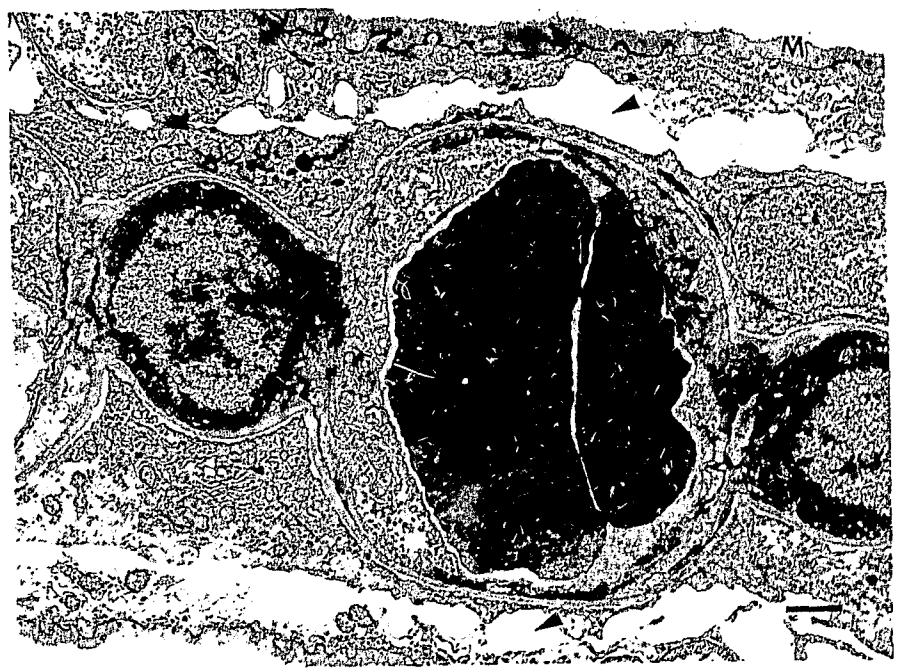


Fig. 31. Transmission electron micrograph of a lamella from a chloramine-T-treated fish. Note the presence of interstitial spaces (arrowheads), although not as large as the ones present in the formalin-treated fish. M (mucus). x 7 000; bar = 1 μm.

# 6. GENERAL DISCUSSION

Much of the research on therapeutic agents for farmed finfish has focused on their spectrum of activity and effectiveness in treating disease outbreaks. Considerably less research has been devoted to the pathophysiological effects which therapeutic agents may have on the target animals. Within this focus, it is important to consider the treatment of diseased animals and the prophylactic treatment of healthy animals as being very different.

The pathophysiological state of diseased animals needs to be considered during a treatment regime. The expected outcome is usually a change in the escalating mortality curve. However, when a treatment is being administered prophylactically, the target animals are presumed not to be pathophysiologically compromised. Therefore, the outcomes of interest differ compared to the treatment of diseased fish. Some of these outcomes, such as incidence of the disease that prompted the prophylactic treatment, incidence of other diseases as a result of the prophylactic treatment, or changes in the overall disease rates can only be assessed with on-farm studies spanning several production cycles. However, production efficiency indices, and pathophysiological changes related to the prophylactic treatment can be probed with laboratory studies designed to simulate production conditions. A laboratory study allows a better degree of control over environmental and management variables, as well as over the presence of disease, which could confound the research results on the farm. This thesis contains several studies that probed the effect of a prophylactic treatment with chloramine-T in simulated production settings on rainbow trout production indices, stress physiology, and pathology of two target organ systems (skin and gill). Because simulated

aquaculture conditions have been used, and the research animal is a commercial strain of an important farmed species, the results are relatively meaningful for aquaculture producers.

It is generally assumed that treating fish with a disinfectant chemical compound at sub-lethal levels is likely to be stressful, and that this stress will be reflected in the production economics. This is the assumed down-side of a prophylactic treatment, while the up-side is a reduction in disease incidence, which would have greater economic benefits. Chapter 3 specifically addressed this by examining the primary and secondary indices of the stress response of fish to routine chloramine-T or formalin bath treatments. Contrary to expectations, there was no evidence that either treatment evoked a stress response. The reasons for this are intriguing, especially in light of previous literature, specifically on formalin treatment, which cites treatment of rainbow trout (Barton and Iwama 1991) and Atlantic salmon (Rostant et al. 1994) as being stressful. One reason for this could be that the strain of trout that we were using may have had a blunted stress response, perhaps from farm-level genetic selection processes, but this hypothesis is weakened by demonstrating that the cortisol response of our fish to other forms of stress (i.e. handling) was intact. Alternatively, administration of treatment chemicals at the doses which are considered to be prophylactically effective, is generally not stressful to trout. It is important to notice that stocking density may play a role in the cortisol concentration, particularly overcrowding (Pickering 1987,1992, 1993a). The fish used in this study were kept at commercial stocking densities but this did not cause a change of the plasma secondary stress response. Further studies on different commercial strains of trout, with extension to other commercial salmonid species are warranted.

Growth performance indices, compared to stress indices, are a much more direct means of assessing the economically significant negative biological effects of a disinfectant based prophylactic regime. This was addressed in Chapter 2: significant reductions in growth rate occurred in fish on the chloramine-T prophylactic regime. This concurs with, and extends the preliminary findings of Powell et al. (1994b). Chloramine-T suppressed growth rates, mediated through an inhibited conversion efficiency rather than through appetite suppression, throughout a lengthy growth trial; this effect was most obvious in the early phase of the trial. There was no compensatory performance, and the treated fish were therefore unlikely to catch up with to untreated cohorts. This is a very serious problem from a practical point of view, since prophylactically treated fish would take longer to reach market size and consume more food to reach that weight. However, in a practical situation, aquaculturists would possibly use a prophylactic treatment regime only during a restricted period of high risk - such as treatment of juveniles during seasons of high risk for infectious gill and skin diseases. In this way, the production performance would be inhibited for a shorter duration. Additionally, if chloramine-T prophylaxis is effective in preventing gill disease, the negative effects which gill disease outbreaks would have on growth would be prevented, along with the replacement costs associated with mortalities.

Although this study shows that chloramine-T intermittent exposure reduces growth rate through feed conversion efficiency, an avenue through which to explore this effect has not yet been determined. Based on Chapter 3, stress does not appear to be a factor. A second mechanism explored was possible damage to skin and branchial tissues. It is known that free chlorine affects directly the epithelial cells, however, the effects of p-toluenesulfonamide and

its acute toxicity levels are not known. P-toluenesulfonamide is poorly taken up by the gills (Powell et al. 1995), but the presence of this compound in water during a chloramine-T treatment a variable that should be studied in the future. A number of differences were detected between treated and control fish, specifically an increase in the numbers of sialic-acid containing mucous cells in the gills of treated fish and a decrease in epidermal thickness. However, these changes are best thought of as modifications of the cellular composition of the gill and skin, rather than pathophysiologically significant lesions of the tissues. It is unlikely that the scope of the changes would have a dramatic bearing on growth rates, particularly when the literature on the effects of lung damage - for example pneumonia - on growth rates of other production animals are compared to our study (Dohoo and Montgomery 1996). This is specially important in the light of the analogy of both organs.

A non-aqueous fixation regime was successfully adapted to preserve the gill and skin mucus coat. Its advantages, pertinent to our study, included excellent preservation of cellular detail, glycocalyx, and mucous coat permitting observation with transmission and scanning electron microscopy. This technical advance was then used to yield the first ultrastructural observation of the effects of a treatment chemical on the external mucous biofilm of fish. A number of findings, relevant to fish health studies, emerged. These include the near absence of a bacterial component in the mucous coat of the test fish and the absence of a demonstrable effect of the disinfectant on the morphology of the mucous coat. The technique offers considerable opportunities for future research on the pathogenesis and treatment of topical infections of the skin and gill.

This study looked at the effects that a chemical agent used in the prevention of

bacterial topical diseases in fish. It involved an assessment of the effects from different angles, like a comprehensive study of the stress response and how this was related to the possible pathological effects that the chemical had on the epithelial surfaces. The conclusion of this study is that the use of chloramine-T as a prophylactic agent against topical bacterial diseases of fish seems to be relatively safe, since it does not cause a stress response, or dramatic changes in the ultrastructure of the skin and gills. The negative effects on growth are of concern, and open the posibility of investigating if chloramine-T causes changes at the intestine level that may impair nutrient uptake and thus decrease the feed conversion efficiency, since the results of this study point that the main effect that chloramine-T has on fish is in their ability to convert food. The role that p-toluenesulfonamide may have in this decrease of feed conversion efficiency warrants further study, particularly to see if the overall benefits of prophylactic treatment outweigh the associated costs.

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## APPENDIX B

Proximate Analysis of High Pro Grower™ fish feed[1]

| Component | Percentage |
|---|---|
| CrudeProtein (min) | 48.0% |
| CrudeFat (min) | 18.0% |
| CrudeFiber (max) | 2.5% |
| Sodium (actual) | 0.8% |
| Calcium (actual) | 1.5% |
| Phosphorous (actual) | 1.1% |
| VitaminA (min) | 6000IU/kg |
| VitaminD3 (min) | 4000IU/kg |
| VitaminE (min) | 250IU/kg |

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

## APPENDIX C

1. Mean body weight and condition index for control and chloramine-T-treated rainbow trout. N=12 tanks.

| | Mean body weight (g) | | | Mean condition index (K) | | |
|---|---|---|---|---|---|---|
| | Treated | Control | P | Treated | Control | P |
| Preexposure | 98±22.67 | 98±22.67 | | | | |
| Week 3 | 132.2±3.25 | 136.6±4.56 | 0.02 | 1.37±0.02 | 1.38±0.02 | 0.23 |
| Week 6 | 160.0±5.40 | 167.9±9.17 | 0.02 | 1.43±0.03 | 1.44±0.03 | 0.94 |
| Week 9 | 193.2±8.41 | 205.4±11.85 | 0.01 | 1.48±0.06 | 1.46±0.01 | 0.28 |
| Week 11 | 209.1±9.97 | 225.6±11.11 | 0.01 | 1.43±0.04 | 1.45±0.02 | 0.31 |

2. Feed Conversion Index (FCI) and Specific Growth Rate (SGR) for control and chloramine-T-treated rainbow trout. N=12 tanks.

| | Feed Conversion Index (FCI) | | | Specific Growth Rate (SGR %) | | |
|---|---|---|---|---|---|---|
| Weekly Interval | Treated | Control | P | Treated | Control | P |
| 0-3 | 1.24±0.03 | 1.02±0.01 | 0.01 | 1.42±0.14 | 1.58±0.157 | 0.01 |
| 4-6 | 1.32±0.30 | 1.12±0.19 | 0.03 | 0.91±0.18 | 0.99±0.13 | 0.28 |
| 7-9 | 1.49±0.22 | 1.49±0.13 | 0.98 | 0.89±0.10 | 0.94±0.09 | 0.25 |
| 10-11 | 1.86±0.42 | 1.67±0.36 | 0.25 | 0.54±0.18 | 0.67±0.17 | 0.17 |
| 1-11 | 1.50±0.07 | 1.36±0.09 | 0.01 | 0.98±0.06 | 1.08±0.06 | 0.01 |

3. Feed consumption for control and chloramine-T-treated rainbow trout. Mean weekly values and mean values immediately after treatment are expressed as percentage of the body weight of the fish. No significant differences were found between groups.

| | Weekly | | Immediately after Chloramine-T treatment | |
|---|---|---|---|---|
| | Treated Tanks n=12 | Control Tanks n=12 | Treated Tanks n=12 | Control Tanks n=12 |
| Week | | | | |
| 1 | 13.70±0.63 | 13.43±0.44 | 1.54±0.15 | 1.51±0.18 |
| 2 | 13.63±0.61 | 13.07±0.33 | 1.18±0.11 | 1.05±0.08 |
| 3 | 11.05±0.23 | 10.98±0.22 | 0.82±0.08 | 0.84±0.08 |
| 4 | 13.62±0.42 | 14.32±0.31 | 1.52±0.12 | 1.54±0.11 |
| 5 | 10.60±0.18 | 10.73±0.25 | 1.25±0.08 | 1.29±0.08 |
| 6 | 11.30±0.24 | 10.50±0.17 | 0.90±0.04 | 0.93±0.02 |
| 7 | 9.23±0.32 | 9.53±0.16 | 0.91±0.11 | 1.14±0.05 |
| 8 | 11.44±0.33 | 11.25±0.25 | 0.67±0.07 | 0.59±0.06 |
| 9 | 10.73±0.32 | 10.66±0.23 | 0.49±0.06 | 0.55±0.08 |
| 10 | 7.62±0.21 | 7.47±0.22 | 1.11±0.06 | 0.93±0.05 |
| 11 | 7.64±0.13 | 7.64±0.19 | 0.84±0.07 | 0.87±0.08 |

4. Fish eye evaluation of control and chloramine-T-treated rainbow trout. Mean% is the percentage of fish that showed signs of damage; $X^2$ is Chi-square; R is right eye; L is left eye. N=12 tanks in each group.

| | | Treated Mean % | Control Mean % | $X^2$ | P |
|---|---|---|---|---|---|
| Week | | | | | |
| 3 | R. | 0.48 | 1.19 | 1.253 | 0.263 |
| | L. | 0.00 | 0.71 | 2.968 | 0.085 |
| 6 | R. | 1.06 | 0.34 | 1.036 | 0.308 |
| | L. | 0.70 | 0.00 | 2.035 | 0.153 |
| 9 | R. | 0.70 | 0.34 | 0.349 | 0.554 |
| | L. | 0.00 | 0.34 | 0.988 | 0.320 |
| 11 | R. | 0.35 | 0.34 | 0.000 | 0.000 |
| | L. | 0.00 | 0.00 | 0.000 | 0.000 |

## APPENDIX D

Radioimmunoassay procedure for the assessment of cortisol levels in rainbow trout blood.

The Radioimmunoassay was performed at room temperature.

*1.* *a.*Four plain (12 x 75 mm) polypropylene tubes were labelled T (total counts) and NSB(nonspecific binding) in duplicate.
*b.* Twelve Ab-Coated tubes were labelled A to F in duplicate for the calibrators.

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

Contents of cortisol of each of the calibrators. Calibrators were in processed human serum.
*c.* Six Ab-Coated tubes were labelled L(low), M(medium), H(high) and were used as controls (L=77-125 nmol/L; M=274-370 nmol/L; H=683-907 nmol/L).
*d.* Ab-Coated tubes were labelled with the samples numbers.

*2.* 25µl of the A calibrator were pipetted into the tubes marked NSB and A. 25µl of each remaining calibrator, controls and fish serum sample were pipetted into the tubes prepared. The sample was pipetted directly to the bottom.

*3.* 1 ml of $^{125}I$ Cortisol was added to every tube and vortexed.

*4.* The tubes were incubated for 45 minutes at 37°C in a water bath.

*5.* The content of the tubes was decanted thoroughly and the tubes were placed in a gamma counter.

All samples (as well as calibrators and controls) were analysed in duplicate and the final lecture is the mean of the two duplicates.

## APPENDIX E

Non-aqueous fixation protocol for piscine epidermal mucus.

1. Immerse tissue sample in 1% $OsO_4$ dissolved in fluorocarbon solvent (FC-72)
2. Fix for 90 minutes at room temperature
3. Wash in pure fluorocarbon 2-5 times. Ten minutes per wash (last wash with distilled water).
4. Dehydrate tissue in increasing concentrations of ethanol up to 100% (2 changes of 10 minutes in each concentration).

For TEM:

5. Clear in propylene oxide (2 changes of 10 minutes)
6. Infiltrate in 50% propylene oxide / 50% Spurr's resin for 30 minutes
7. Infiltrate in 25 % propylene oxide / 50% Spurr's resin for 30 minutes
8. Infiltrate overnight in 100% Spurr's resin
9. Put tissues in flat moulds and polymerize overnight in a vacuum oven at 65-70°C

For SEM:

5. Citically point dry dehydrated tissues
6. Mount samples
7. Sputter coat critically point dried tissues with gold-palladium