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The pathophysiology of chloramine-T on rainbow trout gills

by

Mark D. Powell

**A thesis submitted to the School of Graduate Studies and Research,
University of Ottawa in partial fulfilment of the requirements
for the degree of Doctor of Philosophy in the
Ottawa-Carleton Institute of Biology.**



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THE PATHOPHYSIOLOGY OF CHLORAMINE-T ON RAINBOW TROUT GILLS

Doctor of Philosophy (1996), University of Ottawa (Biology)

Title: The Pathophysiology of chloramine-T on rainbow trout gills

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ABSTRACT

Chloramine-T is a widely used chemotherapeutic and prophylactic disinfectant in aquaculture for the control of gill diseases. Chloramine-T degrades in solution liberating sodium hypochlorite and the organic residue *paratoluenesulphonamide*. The goal of this thesis was to examine the pathophysiological effects of chloramine-T exposure on the respiratory, acid-base and ionoregulatory physiology of the rainbow trout gill. In particular, chloramine-T was used as a tool to probe the fundamental physiological processes of gas transfer, acid-base and ion regulation in response to branchial irritation. Exposure of rainbow trout (*Oncorhynchus mykiss*) to therapeutic and subtherapeutic concentrations of chloramine-T (9 mg.L⁻¹ and 2 mg.L⁻¹, respectively) caused acute respiratory and acid-base disturbances. These consisted of an increase in ventilation frequency and arterial blood PCO₂ (PaCO₂); arterial PO₂ (PaO₂) was unaffected. Exposure of fish to chloramine-T under hyperoxic and moderately hypoxic conditions suggested that CO₂ excretion was being impaired due to the secretion of mucus in response to irritation by hypochlorite. To verify that the apparent impediment to excretion was diffusional and not due to a reduced functional surface area for gas exchange, direct measurements of cardiac output, O₂ uptake, CO₂ excretion, dorsal and ventral aortic blood pressures were made. There were no changes in dorsal or ventral aortic pressures or in branchial or systemic vascular resistance. The perfusion convection requirement for CO₂, but not O₂, increased during exposure to chloramine-T even though there were consistent increases in cardiac output. Thus a greater blood flow than was achieved would be required to excrete an equivalent amount of CO₂ during chloramine-T exposure as under non-exposed (control) conditions. However, there was sufficient blood flow to maintain and even increase O₂ uptake.

Repeated intermittent exposure of fish to chloramine-T resulted in a reduction in the thickness of the gill epithelial diffusion barrier and a mucous cell hyperplasia. The consequences of these morphological changes on gas exchange were assessed using a graded hypoxic challenge. There was no effect of chloramine-T treatment on oxygen uptake rates but fish which had been pre-treated with chloramine-T maintained their P_{aO_2} at higher levels at 70-80 mmHg water PO_2 compared with controls. P_{aCO_2} levels were not significantly different between control and experimental animals even though chloramine-T treated animals had higher ventilation frequencies under pre-hypoxic conditions. This suggested that even though there was a reduction in the epithelial diffusion barrier of the gill, the presence of an increased mucous covering was a severe impediment to CO_2 excretion but not O_2 uptake.

Acute acid-base disturbances during chloramine-T exposure consisted of a metabolic alkalosis superimposed over a respiratory acidosis. A study of the net and unidirectional ionic and acid-base fluxes across the gill confirmed that chloramine-T exposure caused a reduction in the uptake of acidic equivalents. Acute Na^+ and Cl^- losses were also observed with chloramine-T exposure and were attributed to an increase in a transcellular rather than paracellular ionic efflux. Chloramine-T did not effect urine flow rate, glomerular filtration rate, renal Na^+ and Cl^- effluxes or renal clearance of Na^+ and Cl^- . Exposure of soft-water acclimated fish to chloramine-T did not cause increased ionic losses, consistent with the theory of transcellular rather than paracellular ionic losses. Ionic losses in both normal tap water and artificial soft water acclimated trout were eliminated by the addition of 0.1% (w/v) NaCl to the water.

Therefore, respiratory, acid-base and ionoregulatory disturbances due to therapeutic chloramine-T exposure of rainbow trout were not found to be pathological. However, the

effects of branchial irritation on gas transfer implicated the mucous coat as a major impediment to CO_2 excretion but not O_2 uptake under normal physiological conditions. This was probably due to an decrease in the diffusive conductance of the gill-water boundary layer. Ionic losses which were attributed to transcellular effluxes could be eliminated by the addition of NaCl to the water during chloramine-T exposure.

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ABBREVIATIONS

- α , Greek letter alpha
- β , Greek letter beta
- Δ , Greek letter delta
- BGD, bacterial gill disease
- CaO₂, arterial oxygen content
- CvO₂, venous oxygen content
- CaCO₂, arterial total carbon dioxide content
- CvCO₂, venous total carbon dioxide content
- CLT, chloramine-T
- DAP, dorsal aortic pressure
- DHBA, 3, 4-dihydroxybenzylamine
- DMSO, dimethylsulphoxide
- GFR, glomerular filtration rate
- Hb, haemoglobin
- Hct, haematocrit
- J_{in}^X , influx rate of X
- J_{out}^X , efflux rate of X
- J_{net}^X , net flux rate of X
- MS 222, Tricaine methanesulphonate (ethyl *m*-aminobenzoate)
- n, number of samples or individuals
- PEG, polyethylene glycol
- PO₂, partial pressure of oxygen
- PCO₂, partial pressure of carbon dioxide
- PaO₂, partial pressure of oxygen in arterial blood
- PaCO₂, partial pressure of carbon dioxide in arterial blood
- PwO₂, partial pressure of oxygen in water
- pHa, arterial pH
- p*TSA, *paratoluenesulphonamide*
- R_b, branchial vascular resistance
- R_s, system vascular resistance
- SEM or SE, standard error of the mean
- UFR, urine flow rate
- VAP, ventral aortic pressure

CHAPTER 1
GENERAL INTRODUCTION

An overview of gill physiology and pathology

Respiratory, acid-base and ionoregulatory physiology

As the primary site of gas exchange, the gill in most teleost fish species consists of a thin respiratory and ionoregulatory epithelium which overlays a sophisticated vasculature consisting of primary and secondary circuits (Olson 1991; 1996). Blood flow through the gills is such that blood and water move with respect to each other in a counter current fashion (Hughes 1984; Perry and McDonald 1991). The physical characteristics, gross and microscopic anatomy of teleost fish gills although varying between species follow essentially the same general plan and are extensively reviewed elsewhere (Laurent and Dunel 1980; Hughes 1984; Laurent 1984). However, of interest is the physiological interaction between the cellular components (e.g., the pavement cells, the chloride cells and the mucous cells) in the functioning of the gill as a respiratory as well as an acid-base regulatory and ionoregulatory organ.

1. Respiratory gas exchange

It is almost intuitive that the gills of fish are used for the uptake of oxygen from the surrounding water and the excretion of carbon dioxide and

other metabolic wastes (e.g., ammonia), an understanding of the physiological factors which dictate the efficiency and functionality of this are essential in order to appreciate the complexity of gas exchange across the gill. There is a logarithmic relationship between total gill surface area, filament length and lamellar area and body mass (Palzenberger and Pohla 1992). However, fish showing high metabolic activities tend to have a greater total gill surface area with respect to body mass (Palzenberger and Pohla 1992). The rate of gas exchange across the branchial epithelium (be it O₂ uptake or CO₂ excretion conventionally noted as MO₂ or MCO₂, respectively) are primarily dictated by the laws of diffusion and are thus mathematically defined as:

$$MO_2 = \frac{K_{O_2} \cdot A \cdot \Delta PO_2}{D} \qquad MCO_2 = \frac{K_{CO_2} \cdot A \cdot \Delta PCO_2}{D}$$

Where K_{O₂} or K_{CO₂} is the Krogh's permeation coefficient for diffusion of either O₂ or CO₂, A is the functional surface area of the gill, and D is the blood-water diffusion distance, in the case of the gill, primarily the respiratory epithelium. ΔPO₂ and ΔPCO₂ are the mean partial pressure gradients between the water and the blood for O₂ and CO₂, respectively and can be defined as:

$$\Delta PO_2 = 0.5(PaO_2 - PvO_2) - 0.5(PiO_2 + PeO_2)$$

$$\Delta PCO_2 = 0.5(PaCO_2 - PvCO_2) - 0.5(PiCO_2 + PeCO_2)$$

Where PaO_2 and $PaCO_2$ are the arterial partial pressures of oxygen and carbon dioxide, PvO_2 and $PvCO_2$ are the venous partial pressures of oxygen and carbon dioxide, respectively, PiO_2 and $PiCO_2$ are the partial pressures of oxygen and carbon dioxide in the inspired water, respectively, and PeO_2 and $PeCO_2$ are the partial pressures of oxygen and carbon dioxide in the expired water, respectively. Therefore gas exchange across the fish gill is dictated by the gill surface area, diffusion distance and partial pressure gradient such that perturbation of any of these parameters may impede O_2 uptake and/or CO_2 excretion (Piiper 1989; Perry and McDonald 1991).

The circulation of both blood (perfusion of the gills) and water (irrigation of the gills) means that convectional components also play an important role in the rates at which gases are exchanged across the gill. These perfusional components are primarily the rate of blood flowing through the gill (controlled by cardiac output) and the arterial-venous O_2 or CO_2 content difference (Perry and McDonald 1991). Thus O_2 uptake and CO_2 excretion can be defined as:

$$MO_2 = V_b (CaO_2 - CvO_2)$$

$$MCO_2 = V_b (CaCO_2 - CvCO_2)$$

Where V_b is the blood flow to the gills (in fish which have a single circulatory system this is cardiac output denoted as Q), CaO_2 and $CaCO_2$ are the arterial blood oxygen and carbon dioxide contents, respectively, CvO_2 and $CvCO_2$ are the venous blood oxygen and carbon dioxide contents, respectively. Similarly, ventilatory convection is determined by the rate of ventilatory water flow (dictated primarily by the frequency and amplitude of ventilation) (Piiper 1989; Perry and Wood 1989; Perry and McDonald 1991) and can be defined as:

$$MO_2 = V_w (CiO_2 - CeO_2)$$

$$MCO_2 = V_w (CiCO_2 - CeCO_2)$$

Where CiO_2 and $CiCO_2$ are the oxygen and carbon dioxide contents of the inspired water, respectively; CeO_2 and $CeCO_2$ are the oxygen and carbon dioxide contents of the expired water respectively and V_w is the water flow over the gills defined as:

$$V_w = V_f \cdot V_{svr}$$

Where V_f is the ventilation frequency and V_{svr} is the stroke volume of respiration which is dictated in part by the size of the buccal and opercular cavities (assuming a unidirectional ventilation flow typical of teleosts) and the amplitude (denoted as V_{amp}) of ventilation represented by the size of either jaw or opercular deflection. Thus variations in blood or water flow through or across the gill as well as diffusional limitations (discussed above) may severely impact upon the rate of gas exchange across the fish gill. Moreover, it is easy to appreciate the intimate association of diffusional and convective processes in the fish gill.

Factors such as the rate of oxygen loading/unloading, CO_2 transport within the blood and the blood haemoglobin/oxygen carrying capacity and affinity also influence gas transfer. Oxygen binding pigments (namely haemoglobin in fishes) are sensitive to pH (Bridges and Morris 1989). Reduced pH of the blood results in structural changes in haemoglobin reducing both its affinity for oxygen (the Bohr effect) as well as the number of active sites for oxygen binding (the Root effect) (Bridges and Morris 1989). To overcome these potential problems which may arise during periods of respiratory or metabolic acidosis, fish are able to optimise O_2 transport through a high degree of buffering within the erythrocyte using nucleotide

triphosphates and alkalization of the internal environment of the erythrocyte by activation of a Na^+/H^+ exchanger on the erythrocyte membrane (see reviews by Perry and Wood 1989; Nikinmaa 1992). The erythrocyte Na^+/H^+ membrane exchanger is under adrenergic control and is activated by plasma adrenaline and noradrenaline interaction with the β_1 adrenoreceptor (Nikinmaa 1992). Thus, catecholamines (namely adrenaline and noradrenaline) are released as a stress response to reductions in blood oxygen content (hypoxaemia) during hypoxia and in response to extracellular acidoses (Perry and McDonald 1991).

The controlled manipulation of the external environment has been a useful strategy for examining the physiological responses of fish which may occur in response to natural environmental perturbations. Hypoxia (lowered external O_2), hyperoxia (elevated external O_2) and hypercapnia (elevated external CO_2) have all been used singularly or in combination to dissect the various components of gas transfer in fish and thus help to elucidate the physiological processes. Exposure to acute hypoxia causes significant increases in ventilation volume (V_w) through increases in V_f and V_{ST} in an attempt to maximise O_2 uptake (Davis and Cameron 1971; Smith and Jones 1982). This occurs despite reductions in arterial PCO_2 and the subsequent

elevation in arterial pH (respiratory alkalosis) (see review by Perry and Wood 1989). However, hypoxia also results in a bradycardia (Fritsche 1990; Bushnell and Brill 1992) as well as decreases in stroke volume (Davis and Cameron 1971) and thus a reduction in cardiac output or alternatively stroke volume increases to maintain cardiac output (Fritsche and Nilsson 1989; Axelsson and Fritsche 1991). Under such circumstances, ventral aortic blood pressure is increased facilitating lamellar recruitment through increased perfusion of more distal gill lamellae and by changes in the blood flow pattern within individual lamellae (Booth 1978; 1979a,b). This occurs because of an increase in systemic vascular resistance. The net result is an increase in the functional surface area over which gas exchange can take place in the gill and hence maximising oxygen uptake.

External hypercapnia causes a reduction or transient reversal of the partial pressure gradient for CO₂ across the gills. Thus, CO₂ excretion is transiently reduced, eliminated or even reversed. Hypercapnia is accompanied by acid-base disturbances because the accumulation of CO₂ in the blood leads to an extracellular acidosis (respiratory acidosis). In turn, the acidosis causes a reduction in oxygen/haemoglobin affinity (Bohr effect) and in blood cell O₂ carrying capacity (Root effect). The reduction in O₂

transport leads to a hypercapnic hyperventilation (Smith and Jones 1982; reviewed by Perry and Wood 1989). This is in addition to the direct effect of hypercapnia in increasing ventilation. Cardiovascular responses to hypercapnia have been less extensively studied but include a bradycardia and a reduction of cardiac output. In addition, increases in dorsal and ventral aortic pressure through increases in systemic and branchial vascular resistance have been reported (Fritsche et al. in prep.). Increases in ventral and dorsal aortic blood pressure under hypoxia and hypercapnia are brought about through changes in systemic vascular resistance which is controlled in part by adrenergic neurons as well as the presence of circulating catecholamines (adrenaline and noradrenaline) (Fritsche and Nilsson 1990; Fritsche et al. in prep.). Intravascular adrenaline has been shown to also increase the volume of the branchial vascular space thus allowing more blood to flow through the branchial arterial circuit (see review by Nilsson 1984; Gardaire et al. 1991) and in concert with neurological pathways (innervation of the afferent lamellar arterioles: Donald 1984; 1987) allow for vasodilation and increased perfusion of the gill vasculature. The efferent filamental and lamellar vasculature is, however, primarily under neural cholinergic control (Bailly and Dunel-Erb 1986; Dunel-Erb et al. 1987) although some adrenergic

innervation has been described in *Cyprinus carpio* (Donald 1987).

Conversely, hyperoxia results in a suppression of ventilatory frequency and stroke volume thus resulting in elevations in arterial PCO_2 and subsequent respiratory acidosis. Thus, there is a rapid ventilatory adjustment of the acid-base status in hyperoxic fish (Thomas and Hughes 1982; Perry and Wood 1989).

2. Branchial ionic and acid-base regulation

The branchial epithelium plays an extensive ionoregulatory role. Epithelial ion transport mechanisms in fishes have been extensively reviewed (see Shuttleworth 1989; Evans 1991). In freshwater, the main ionoregulatory objective of the gill is the uptake of ions from the water whereas in saltwater the primary ionoregulatory function is the excretion of ions. The loss of ions in freshwater is minimised by the low epithelial permeability, a function of the deep epithelial tight junctions between adjacent epithelial cells. It is the “tightness” of the epithelium and integrity of tight junctions which maintains the low branchial permeability (Freda et al. 1991). This “tightness” is a function of the presence of calcium ions (McWilliams 1983; Marshall 1985). Exposure of fish to water low in calcium results in an acute loss of ions

(McDonald and Rogano 1986). However, this loss can be compensated for by the increase in the number of ionic uptake sites on the gill (Perry and Laurent 1989) which are thought to be under endocrine control with hormones such as prolactin being implicated to decrease epithelial ionic permeability (Wendelaar Bonga 1991) and cortisol for increasing ionic uptake sites and chloride cell proliferation (Perry and Laurent 1990).

The chloride cell has been implicated as the primary site of chloride exchange (probably via a band 3-like exchange protein located on the apical plasma membrane (Goss et al. 1992b; Sullivan et al. 1996)). Recently, it was suggested that sodium uptake may arise via an electrochemical gradient generated by a V-type H^+ ATPase located on the pavement epithelium (Lin and Randall 1991; Lin et al. 1994; Laurent et al. 1994; Sullivan et al. 1995;1996). Sodium uptake therefore occurs down an electrochemical gradient through sodium channels, the precise location of which has not yet been demonstrated but it is speculated that they may exist on the pavement epithelium (Goss et. al. 1992b).

In recent years models linking the compensation of acid-base disturbances with ionoregulatory function have been developed for freshwater fishes (see review by Heisler 1991) and have been supported by

morphological and physiological evidence (Goss et al. 1992a,b, Goss and Perry 1993). These models are based on the principle that strong ions (e.g., sodium and chloride) are exchanged for acidic and basic equivalents (e.g., H⁺ and HCO₃⁻) because of the need to maintain electroneutrality. Thus, acid-base disturbances within the fish are intimately linked to ionic fluxes across the gill epithelium.

The fish gill therefore is the site of convergence of respiratory, acid-base and ionoregulatory physiology and because of their intimate co-dependance, the disturbance of one system may also impact the others.

3. Branchial mucus

The mucous coat of the gills is comprised of numerous acidic glycosaminoglycans including chondroitin and heperan sulphates (Wasserman et al. 1972) and is particularly rich in sialic acid residues (Arillo et al. 1979; Lumsden and Ferguson 1994). In addition gill mucus has a positive electropotential with respect to the internal environment of the epithelium and higher ionic content than the surrounding water (Handy 1989). The material which is generally referred to as mucus is actually composed of two mucilaginous elements, the epithelial cell apical glycocalyx and the mucus

released from the mucous (goblet) cells of the gill epithelium. These can be seen morphologically as two distinct yet interspersed layers (Powell et al. 1992; 1993a; Lumsden et al. 1994). The dimensions of the glycocalyx differs between cell types (Powell et al. 1993b) and has been hypothesised to play a possible role in ionic transport (Powell et al. 1993a; 1994). The actual thickness of the mucous coat is not known but is believed to be at least 1 μm thick (covering the microridges of the gill pavement cells). Mucus has been shown to impair O_2 diffusion *in vitro* (Ultsch and Gros 1979) and it is clear that fish experience extensive mucous cell hyperplasia and an increased mucus covering of the gill during numerous environmental insults (see below) (Ferguson et al. 1992). However, relatively little is known about how this dynamic potential barrier to gas exchange affects the diffusive properties of the gill in response to brief chemical insult.

Gill diseases in aquaculture

Most finfish aquaculture production involves freshwater for at least part of the production cycle. For some species (e.g., carp, *Cyprinus carpio*, and channel catfish, *Ictalurus punctatus*) production is confined to freshwater environments. Anadromous euryhaline species such as trout and salmon are

spawned in freshwater where the eggs develop, the alevins hatch and parr (juveniles) develop. For many of the commercial trout species (e.g., rainbow trout, *Oncorhynchus mykiss*; brook trout, *Salvelinus fontinalis*; Arctic charr, *S. alpinus*) production can be continued in the freshwater environment. Parr of all salmonids (e.g., chinook salmon, *O. tshawytscha*; coho salmon, *O. kisutch* and Atlantic salmon, *Salmo salar*) including the aforementioned trout are able to acclimate to seawater under the appropriate environmental cues of temperature and photoperiod (Johnston and Saunders 1981; Zaugg 1981; McCormick et al. 1987). This stage in the lifecycle, known as smolting, results in increases in gill Na⁺, K⁺ ATPase activity and salinity tolerance corresponding to the natural seaward migration of the fish (Zaugg 1981; McCormick et al. 1987). Typically, the smolt are transferred to marine sea cages under which conditions for growth are optimised until market weight is reached (usually 2-3 years).

With the exception of severe disease epidemics, it is during the freshwater phase of the production cycle that the greatest losses occur. This is primarily due to fungal infection of eggs (e.g., Saprolegniosis caused by *Saprolegnia parasitica*), reduced hatching success and the occurrence of one or many bacterial (e.g., bacterial gill disease caused by *Flavobacterium*

branchiophila; columnaris disease caused by *Flexibacter columnaris*; furunculosis caused by *Aeromonas salmonicida*) and parasitic diseases (e.g., ichthyobodiasis, caused by *Ichthyobodo necator*, ich (also known as ick) caused by *Ichthyophthirius multifiliis*). Of perhaps the greatest significance to the freshwater salmonid aquaculture industry is bacterial gill disease (BGD).

The etiological agent of BGD has been identified as *Flavobacterium branchiophila* and was first successfully isolated and characterised biochemically by Wakabayashi et al (1980; 1989). Poor water quality, overstocking and improper management all exacerbate BGD and were once thought to be the initial cause of the disease (Post 1986). BGD remains one of the greatest limitations to freshwater aquaculture production (Speare and Ferguson 1989). Studies on the pathogenesis have shown an acute association of the bacteria with the epithelial surface of the gills (Kudo and Kimura 1983a; Speare et al. 1991a,b) which appears to cause epithelial cell necrosis and chronically, a hyperplasia and hypertrophy of the respiratory epithelium (Kudo and Kimura 1983b). Often associated with the disease is a characteristic "blebbing" of the pavement epithelial cells (Kudo and Kimura 1983a; Speare et al. 1991a,b) and a mucous cell hyperplasia (Ferguson et al. 1992). The blebbing is thought to be typical of the onset of epithelial necrosis

and is seen as a distortion and ballooning of the apical membrane of gill pavement cells under transmission electron microscopy (Speare et al. 1991a,b). Efforts to reproduce or horizontally transmit BGD in the laboratory have met with limited success (Kudo and Kimura 1983c; Ferguson et al. 1991). However, Ferguson and co-workers (1991) have successfully reproduced acute infections in rainbow trout using a strain originally isolated from brook trout (Ferguson et al. 1991).

The pathophysiology of BGD is still being investigated, however, experimental infections have induced a hypoxaemia and extracellular acidosis (P. Byrne pers. comm.) through respiratory dysfunction. Also associated with acute infections are hyponatraemia and hypochloraemia (Byrne et al. 1991) indicative of acute ionic losses.

Treatments for gill diseases

Due to the stochastic nature of gill disease outbreaks, disease management strategies rely on the use of static bath or flow-through chemical treatments. Such chemical treatments have included chloramine-T, formalin, salt, potassium permanganate, copper sulphate, chlorine, nitrofurans compounds, quaternary ammonium compounds to name but a few (Post

1986).

Chloramine-T is the most widely used therapeutic treatment for bacterial gill diseases (Thorburn and Moccia 1993; Smith et al. 1993) and has also been used effectively to treat *Ichthyophthirius multifiliis* infection in *Rasbora heteromorpha* and *Rutilus rutilus* (Cross and Hursey 1973).

Although not currently licensed by the United States Federal Department of Agriculture (USFDA) or Health Canada for use as a therapeutic for food fish in the United States or Canada, respectively, chloramine-T is extensively used as an "off label" product (Thorburn and Moccia 1993) and has been shown to be effective in treating natural outbreaks of BGD in hatcheries (From 1980; Bullock et al. 1991) as well as mixed gill infections (Ostland et al. 1995).

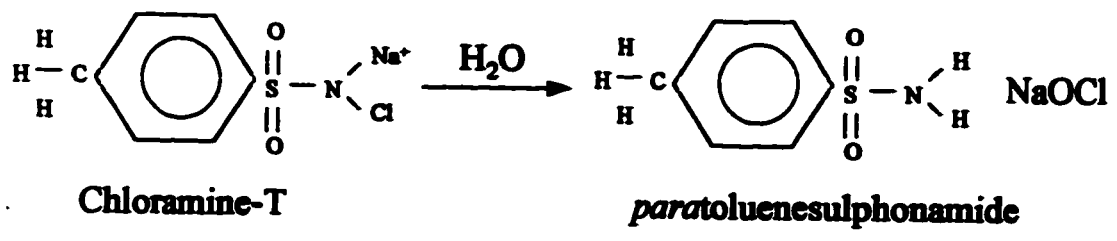
The use of chloramine-T either by itself or in conjunction with other chemical agents (e.g., formalin and salt) as a prophylactic disinfectant is a common practice (Thorburn and Moccia 1993). Fish are routinely treated with therapeutic concentrations of chloramine-T in an attempt to minimise the risk of disease or to keep bacterial numbers low thereby reducing the opportunity for a disease outbreak (Thorburn and Moccia 1993; Smith et al. 1993).

Chemistry and toxicology of chloramine-T, chlorine and chloramines

The synthesis and breakdown reactions of chloramine-T

Chloramine-T (*n*-sodium-4-*paratoluenesulphochloramide*) (Fig. 1-1) is synthesised from the precursor *paratoluenesulphonamide* by the addition of sodium hypochlorite. This reaction involves the formation of a covalent bond between the terminal nitrogen of the amide and the chlorine from hypochlorite. Sodium is associated with the amide by ionic attraction. Under anhydrous conditions, chloramine-T remains relatively stable. However it degrades upon exposure to air due to the presence of water vapour, giving the crystalline powder a faint smell of chlorine (Booth and McDonald 1988). Thus in solution, the ionic sodium dissociates freely and there is a nucleophilic substitution that results from the breaking of the nitrogen-chlorine bond giving rise to the residue *paratoluenesulphonamide* (*p*TSA) and the unstable chlorine radical. In the presence of hydroxyl ions this radical forms hypochlorous acid which dissociates into protons and the hypochlorite ion (OCl⁻). Since this digression is one side of an equilibrium reaction, the rate at which the nucleophilic substitution occurs is dictated by the consumption of chlorine in the formation of chloramines with nitrogenous and organic compounds.

Figure 1-1. Hydrolysis of chloramine-T to *paratoluenesulphonamide* and sodium hypochlorite.



The toxicity of chloramine-T

Chloramine-T has been used as an oxidant for many years and its uses are varied. For example it has been used as a bleaching agent in the pulp and paper industry (Hård and Bengtson 1968; Blomqvist et al. 1991), as a general disinfectant for animal husbandry and medical purposes (Booth and McDonald 1988), and as disinfectant tablets for the sanitation of drinking water (marketed under the synonym of Chlorazene). However, chloramine-T is a recognised allergen (Blomqvist et al. 1991) which elicits a type I hypersensitivity reaction resulting in rhinitis (Beck 1983; Hård and Bengtson 1968), contact urticaria (Dooms-Goossens et al. 1983) and even asthma (Dijkman et al. 1981) in humans.

Chloramine-T and the organic residue *p*TSA are readily taken up by the gills but are rapidly metabolised and excreted according to first order kinetics probably via the kidney (Dawson and Gingerich 1993). The toxicity of chloramine-T has been addressed in terms of LC_{50} for rainbow trout (*O. mykiss*), channel catfish (*I. punctatus*) and fathead minnow (*Pimephales promelas*) (Bills et al. 1988a). It was found that chloramine-T toxicity was increased at elevated temperatures, with reduced water pH and hardness (Bills et al. 1988a; Cross and Hursey 1973). However, concentrations and

durations that have been demonstrated to be effective for the treatment and control of bacterial gill disease (8.5-12 mg.L⁻¹: From 1980; Bullock et al. 1991) are well below the LC₅₀ for chloramine-T under any of the environmental conditions examined (Bills et al. 1988a, b). The amount of organic load in the water also affects the toxicity (in terms of LC₅₀) of chloramine-T (Bills et al. 1988b; Cross and Hursey 1973). Toxicity is decreased under conditions of high fish density and in the presence of faecal material and feed (Bills et al. 1988b). This likely reflects the rapid degradation of chloramine-T (shift in the equilibrium) as released chlorine complexes with organic and nitrogenous wastes to form less acutely toxic mono- and dichloramines or other more stable organic chloramines.

The use of chloramine-T as a prophylactic management tool was recently examined (Powell et al. 1994; G. Sanchez pers. comm.). Repeated 1 h treatments of juvenile rainbow trout at therapeutic doses have resulted in reduced growth rates (Powell et al. 1994; G. Sanchez pers. comm.). The decreased growth rate appears to be related to a reduced feed conversion efficiency (G. Sanchez pers. comm.). Moreover in one study, the incidence of a caudal necrosis (of unknown aetiology) was significantly prevalent in fish treated with 10-20 mg.L⁻¹ chloramine-T (Powell et al. 1994). Examination of

the gills of the fish from that study revealed an apparent chloride cell hyperplasia and a significant reduction in the number of mucous cells on the lamellar epithelium (Powell et al. 1995). The apical plasmalemma of chloride cells located at the base (basal) and along the lamellae (lamellar) of the gill was increased in a dose dependant manner. Since the chloride cell has been implicated as the site of chloride/bicarbonate exchange (Goss et al. 1992b; Sullivan et al. 1996), it was inferred that fish were compensating for an acid-base or ionic disturbance caused by chloramine-T exposure (Powell et al. 1995) which corresponded well with a dose dependant decrease in plasma Na^+ and Cl^- levels (Powell et al. 1994).

Toxicity of chlorine

1. Acute toxicity studies

The toxicity of chlorine and chloramines has been of great interest since they are environmental contaminants, and because they are used extensively as domestic water disinfectants (Davis and Roberts 1985) and industrial antibiofoulants (Mattice 1985; Fava et al. 1985). Both free chlorine and the ammonia complexed mono- and dichloramines show acute toxicity to fish and invertebrates although mono- and dichloramines are relatively less

acutely toxic than the free uncombined form (Heath 1977). The toxicity to total residual chlorine, mainly in terms of LC_{50} , has been extensively determined for numerous salmonid and non-salmonid species (Brooks and Seegert 1977; Johnson et al. 1977; Middaugh et al. 1977; Seegert and Brooks 1978; Hall et al. 1982; Brooks and Bartos 1984). In addition, the toxicity of combined forms of inorganic chlorine has also been investigated for a number of fish species. Primarily the LC_{50} to mono- and dichloramines have been examined (Johnson et al. 1977; Larson et al. 1977; Seegert et al. 1979; Tsai and McKee 1980; Brooks and Bartos 1984). In all cases the toxicity to combined forms of chloramines were lower than for exposure to free chlorine. Although LC_{50} studies are useful at determining acute and to a limited extent, long term toxicities, they rely on mortality as the endpoint and hence intermediate endpoints such as disruption to physiological processes, reproductive success and growth are not examined. In addition, studies are not always comparable as testing conditions are not always standardised.

2. Sublethal toxicity studies

Considerably fewer studies have examined the sublethal effects of chlorine or chloramines on fish. An acute decrease followed by a gradual

increase in haematocrit and haemoglobin, increases in methaemoglobin levels, intravascular haemolysis, increases in plasma proteins and reduced glutathione levels have been cited as common clinical pathological signs of chlorine toxicity in rainbow trout (*O. mykiss*) (Zeitoun 1977), coho salmon (*O. kisutch*) (Buckley et al. 1976), and fathead minnow (*Pimephales promelas*) (Grothe and Eaton 1975). Although there were significant increases in methaemoglobin concentration in all three studies, Zeitoun (1977) found at most a doubling of the control levels of methaemoglobin with exposure of rainbow trout to chlorine; Buckley et al. (1976) stated that methaemoglobin levels reduced functional haemoglobin levels by 8.2%, whereas Grothe and Eaton (1975) found levels reduced by 32%. Regardless, this reduction in the functional haemoglobin is of little pathological relevance since levels of methaemoglobin as high as 80% have been induced in studies examining nitrite toxicity with no pathological consequences in rainbow trout and Atlantic salmon (*S. salar*) (Eddy et al. 1983) and with little impact on oxygen delivery in exercising chinook salmon (*O. tshawytscha*) (Brauner et al. 1993). Intravascular haemolysis was probably related to increased fragility of erythrocyte membranes (Buckley et al. 1976) due to the oxidative effects of chlorine which would lead to peroxidation of the cell membranes.

Glutathione levels were thus concluded to be elevated in response to this oxidative stress (Zeitoun 1977).

3. Toxicity to gill epithelia

The effects of chlorine and chloramine exposure on the gills and respiratory-cardiovascular physiology of fish have been less thoroughly investigated. Bass et al. (1977) demonstrated that intermittent chlorination of rainbow trout (*O. mykiss*) and bluegill (*L. macrochirus*) resulted in lamellar fusion and hyperplasia of the respiratory epithelium of the gill. In addition increased numbers of mucous cells and the presence of mucoproteins (as stained positively by periodic acid-Schiff's stain) on the gill suggested increased mucus production and secretion (Bass et al. 1977). Similar findings have also been reported with marine fish species (Hose et al. 1983; Wiley 1983). Respiratory and cardiovascular disturbances in rainbow trout such as hypoxaemia, and bradycardia during acute exposure to pulses of sodium hypochlorite have been attributed to the impediment of gas transfer across the hyperplastic and mucus-covered gill epithelium (Bass and Heath 1977). In addition increases in the ventilation rate and coughing rate were also observed during chlorine exposure. Similar to other studies, methaemoglobin

levels were increased but of little physiological impact, being only 2.5% of the functional haemoglobin levels (Bass and Heath 1977) thus reinforcing the idea that methaemoglobinaemia is not pathognomonic for chlorine/chloramine toxicity.

Although less extensively examined, ionic disturbances have also been reported in aquatic organisms as a consequence of exposure to chlorine and chloramines (Scott 1983; Hose et al. 1983). The exact mechanisms by which these disturbances occur are not known although it has been speculated that they may arise from cellular damage through lipid peroxidation of either the branchial or renal epithelium by chlorine residuals (Hose et al. 1983).

Goals and objectives of the thesis

The primary goal of this thesis is to examine and characterise the pathophysiological responses of the rainbow trout gill to therapeutic levels of chloramine-T. It is hypothesised that branchial irritation from chloramine-T exposure will cause acute respiratory and acid-base disturbances such that the processes of gas transfer, are impeded. In understanding the pathophysiology of chloramine-T as a therapeutic on salmonid fish gills, it may be possible to make recommendations as to conditions under which chloramine-T may be

used with minimal physiological impact. The first part of the thesis examines the effects of acute (chapters 2-4) and long term (chapter 5) intermittent chloramine-T exposure on respiratory and acid-base physiology of the gill. In particular chloramine-T is used as a source of sublethal chlorine and an agent of branchial irritation and thus the physiological impact of chemical irritants and their effects on gas transfer in fish are examined. The second part of the thesis (chapter 6) examines the effects of chloramine-T exposure on the acid-base and ionic regulatory physiology of the gill. In addition the effects of chloramine-T exposure in ion poor, "soft" water are examined and the potential for combined treatment with NaCl (another therapeutic strategy for fish health management) at minimising ionic stress is evaluated (chapter 7). Thus this thesis is designed to address both the fundamental questions of the effects of chemical irritation on the basic physiology of the fish gill and, from a more applied perspective, the specific effects of a therapeutic prophylactic disinfectant which is used in the freshwater finfish aquaculture industry.

CHAPTER 2

RESPIRATORY AND ACIDBASE DISTURBANCES IN ARTERIAL BLOOD DURING EXOSURE TO CHLORAMINE-T, *PARATOLUENESULPHOAMIDE* AND HYPOCHLORITE.

INTRODUCTION

Despite widespread use as a disinfectant, therapeutic and prophylactic treatment in hatcheries, relatively little is known about the mechanism of action of chloramine-T or any physiological consequences of its treatment on fish. Similarly little is known about the effects of sublethal levels of chlorine (as either hypochlorite or mono- or dichloramine). Bass et al. (1977) demonstrated that sublethal chlorine pulses (0.4 and 0.5 mg.L⁻¹ total residual chlorine) caused an acute hypertrophy and hyperplasia of rainbow trout branchial epithelium and extensive mucus secretion. The same fish showed reduced arterial PO_2 , marked bradycardia and hyperventilation (Bass and Heath 1977). In a recent study (Powell et al. 1994), repeated intermittent exposure of rainbow trout to chloramine-T at therapeutic concentrations caused behavioural changes consistent with respiratory distress (i.e., fish crowded at the tank surface and appeared to hyperventilate). The same fish showed an apparent chloride cell hyperplasia and other morphological changes (increase in expression of the chloride cell apical plasma membrane) of their gills (Powell et al. 1995) similar to that seen during compensation of acid-base disturbances in freshwater fish (e.g., metabolic alkalosis) (Goss et

al. 1992 a,b; Goss and Perry 1993).

The present study was designed to assess the effects of a single pulse of chloramine-T at therapeutic concentrations and its primary degradation products, hypochlorite and *p*TSA on respiratory and acid-base variables of rainbow trout blood *in vivo*.

MATERIALS AND METHODS

Fish

The rainbow trout (*Oncorhynchus mykiss* Walbaum) used in this study were obtained from a commercial trout farm (Linwood Acres Trout Farm, Campbellcroft, Ontario) and acclimated to laboratory conditions for at least two weeks prior to use. Throughout the acclimation period and in subsequent experiments the fish were held under natural photoperiod in flowing, aerated and carbon filtered dechlorinated city of Ottawa water (<0.03 mg.L⁻¹ residual chlorine as determined by the N, N-ethyl-p-phenylenediamine ferrous titrimetric method (DPD method: Franson 1978) at 10°C containing 118.9 ± 2.4 μM Na⁺; 51.8 ± 0.9 μM Cl⁻; 365.8 ± 8.2 μM Ca²⁺ and 19.3 ± 0.5 μM K⁺ (mean ± SE) at pH 6.8.

Surgical procedures

Adult trout of mean mass 670.7 ± 15.8 g (mean \pm SE) were anaesthetised with tricaine methanesulphonate (MS 222) (100 mg.L^{-1}) and the gills constantly irrigated with the same oxygenated anaesthetic solution throughout the following surgical procedures. A polyethylene (PE 50: Clay Adams Inc.) catheter was implanted into the dorsal aorta according to the method of Soivio et al. (1975). Either the operculum was catheterised by drilling a small hole through the opercular bone approximately 1 cm from the trailing edge and a 50 cm length of PE 160 tubing heat-flared at one end inserted through the hole, or 1 cm^2 brass plate electrodes were sutured to the epithelium of each operculum. An incision was then made into the right flank just posterior to the head and the coeliac artery isolated and catheterised with PE 50 in both orthograde and retrograde directions (Thomas and LeRuz 1982). The wound was closed and the animal allowed to recover in fresh water in a black acrylic respirometer box with flowing water. All catheters were flushed and filled with non-heparinised Cortland's saline (Wolf 1963) prior to insertion. Following at least one day of recovery, the catheters were flushed with heparinised Cortland's saline (250 IU ammonium heparin: Sigma

Chemical Company, St. Louis Mo. USA) prior to experimentation.

In a second set of experiments rainbow trout of mean mass 206.5 ± 7.5 g (mean \pm SE) were anaesthetised as above and PE 50 catheters implanted into the dorsal aorta according to the method of Soivio et al. (1975). These fish were placed in individual black acrylic boxes with flowing fresh water and allowed to recover for 18-24 prior to chemical exposure.

Blood measurements

Extracorporeal circulation

Arterial PO_2 , PCO_2 and pH were measured continuously by drawing blood by peristaltic pump (flow rate $0.45 \text{ mL}\cdot\text{min}^{-1}$) from the coeliac artery and passing it over temperature controlled O_2 (Radiometer E5046), CO_2 (Radiometer E5036) and pH (Metrohm) electrodes arranged in series and connected to a Radiometer PHM 73 acid-base analyser. The blood was then returned to the coeliac artery. Dorsal aortic blood pressure and opercular pressure were monitored using Bell and Howell Type 4-327 I pressure transducers (Bell and Howell, Pasadena, California, USA) connected to a recording physiograph (Lafayette). Opercular impedance was monitored by

means of a customised impedance converter and amplifier. Analogue outputs from the acid-base analyser, physiograph and impedance converter were transformed into digital outputs using an analogue-digital interface (Data Translation Inc.) and relayed to a microcomputer. The output was recorded using customised data acquisition software (written by P. Thören, Göteborg, Sweden). Ventilation frequency was measured visually by counting buccal movements.

Plasma bicarbonate ($[\text{HCO}_3^-]$) concentrations were calculated from rearrangement of the Henderson-Hasselbalch equation (see below) using pH_a and PaCO_2 values measured with the extracorporeal circulation and constants from Boutilier et al. (1984). The non-bicarbonate buffer values for true plasma were calculated according to the regression equation given by Wood et al. (1982).

Henderson-Hasselbalch equation

$$\text{pH} = \text{pK} + \log_{10} \frac{\text{Total CO}_2 - (\alpha\text{CO}_2 \cdot \text{PCO}_2)}{\alpha\text{CO}_2 \cdot \text{PCO}_2}$$

$$\text{Total CO}_2 = (\text{antilog}(\text{pH}-\text{pK})+1) \cdot (\alpha\text{CO}_2 \cdot \text{PCO}_2)$$

$$[\text{HCO}_3^-] = \text{Total CO}_2 - (\alpha\text{CO}_2 \cdot \text{PCO}_2)$$

Fish were monitored in a stable resting state for 30 min. prior to chemical exposure. Fish were exposed to a 60 min. pulse of either chloramine-T (9 mg.L⁻¹ active ingredient, n = 7) (BDH Chemicals Inc. Toronto, Ont., Canada: Analytical grade Lot 106295/15998), *paratoluenesulphonamide* (*p*TSA) (9 mg.L⁻¹ active ingredient, n = 6) (Sigma Chemical Co. :Lot # 23H3595) dissolved in 0.0012% (v/v: final concentration) dimethylsulphoxide (BDH Chemicals Inc.), sodium hypochlorite solution (0.45 mg.L⁻¹, n = 7) (BDH Chemicals Inc.) or water (controls, n = 5). A stock solution of each chemical was made up using the same dechlorinated water as used in the fish holding system and metered into the inflowing water of the respirometer box via a Manostat Varistaltic AL series peristaltic pump (Manostat Inc. New York, USA) to yield the appropriate exposure concentration. Following exposure, fish were further monitored for an additional 90 minutes prior to termination of the experiment. Each fish received only one treatment chemical and treatments were conducted in randomised blocks.

Dorsal aorta catheterised fish

Prior to experimentation a 650 µL blood sample was withdrawn from each

fish. From this blood sample haematocrit was determined in duplicate using microcapillary tubes centrifuged at 10 000g for 10 min.; haemoglobin content determined spectrophotometrically using a commercial kit (Sigma Chemical Company); total oxygen content (CaO_2) determined according to the method of Tucker (1967). The remaining blood was centrifuged at 10 000g for 30s and the plasma frozen in liquid nitrogen and stored at -80°C for later determination of catecholamine levels. Catecholamines were extracted from the thawed plasma samples according to the method of Woodward (1982) and analysed using high performance liquid chromatography (Varian 9000 series system with electrochemical detection with an EG & G model 400 electrochemical detector) using DHBA (3,4 dihydroxybenzylamine: Sigma Chemical Co.) as an internal standard. Further blood samples were withdrawn immediately after exposure to a 60 min. pulse of each chemical (chloramine-T 9 mg.L^{-1} active ingredient, $n = 8$; *paratoluenesulphonamide* 9 mg.L^{-1} active ingredient, $n = 6$ dissolved in 0.012% v/v DMSO; sodium hypochlorite 0.45 mg.L^{-1} , $n=6$; or control $n = 7$) and a subsequent $650 \mu\text{L}$ blood sample withdrawn and analysed as above following a further 60 min. of recovery in freshwater.

Data handling and statistical analysis

For the purposes of comparing the responses of each measured variable to chemical treatment, a mean normalisation transformation was used to subtract the mean baseline from each successive data point. The end result is data expressed as a net change in each variable without violating assumptions of normality (Glantz 1987). The data points shown in the figures represent means at 15 min. intervals \pm standard error. As a consequence of this transformation means were statistically compared to baseline values using a repeated measures analysis of variance. *Post-hoc* analysis was performed with multiple Bonferoni corrected *t*-tests (Glantz 1987). Differences in the other measured blood parameters (e.g., CaO₂, haematocrit etc.) were analysed by repeated measures analysis of variance and differences isolated using Bonferoni corrected *t*-tests. P-values of 0.05 or less were considered to be significant.

RESULTS

Ventilation frequency (V_f)

Chloramine-T exposure (9 and 2 mg.L⁻¹) significantly increased V_f (Figs. 2-1 and 2-2). Ventilation frequency was also significantly elevated during

Figure 2-1. The change in ventilation frequency (V_t), arterial PCO_2 ($PaCO_2$), arterial PO_2 (PaO_2) and pH (pH_a) during exposure to 9 mg.L^{-1} chloramine-T (active ingredient) (filled circles). Chemical exposure period represented by the vertical bars. Control fish = open circles. * = significant from baseline zero. Error bars represent 1 SE.

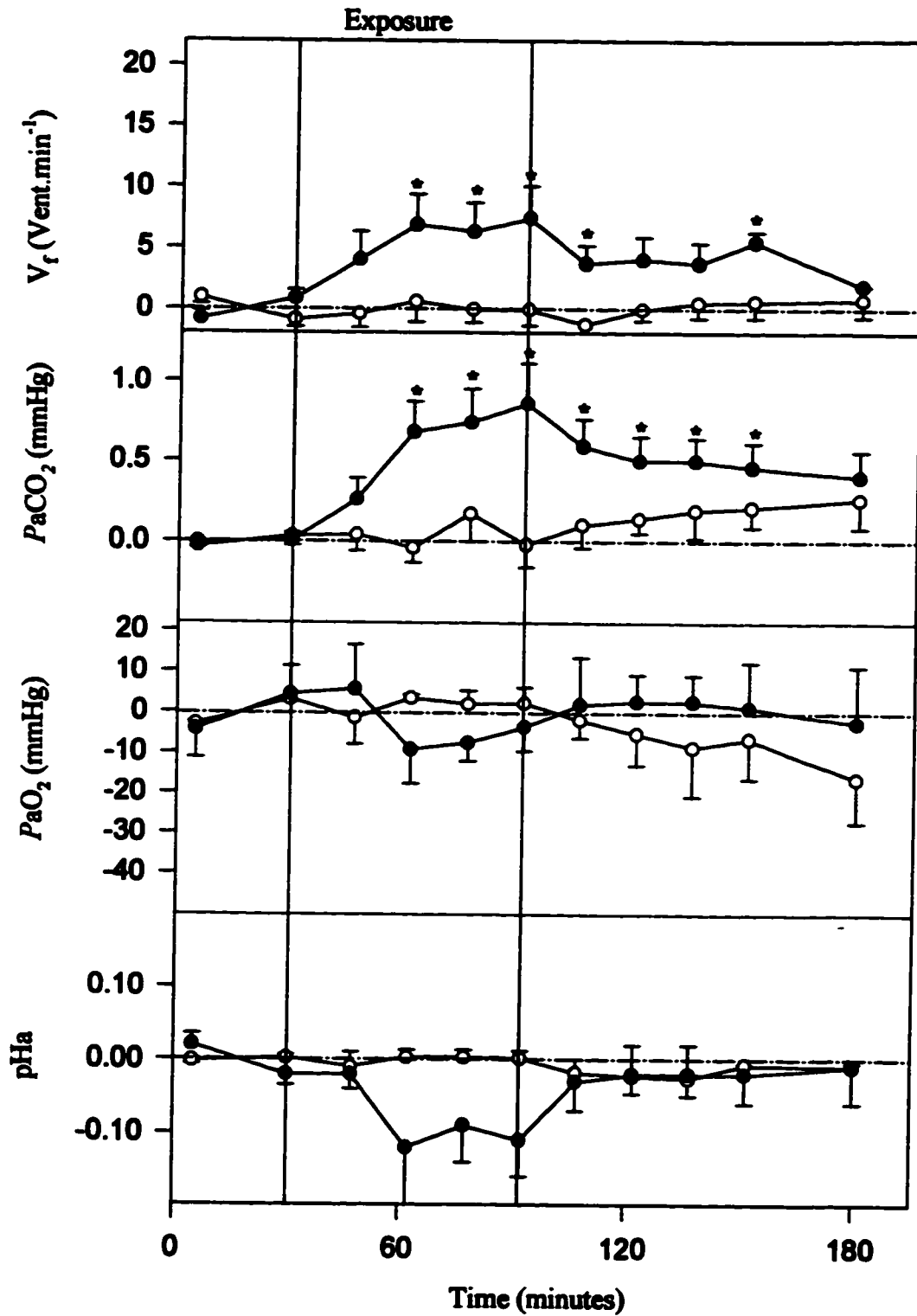
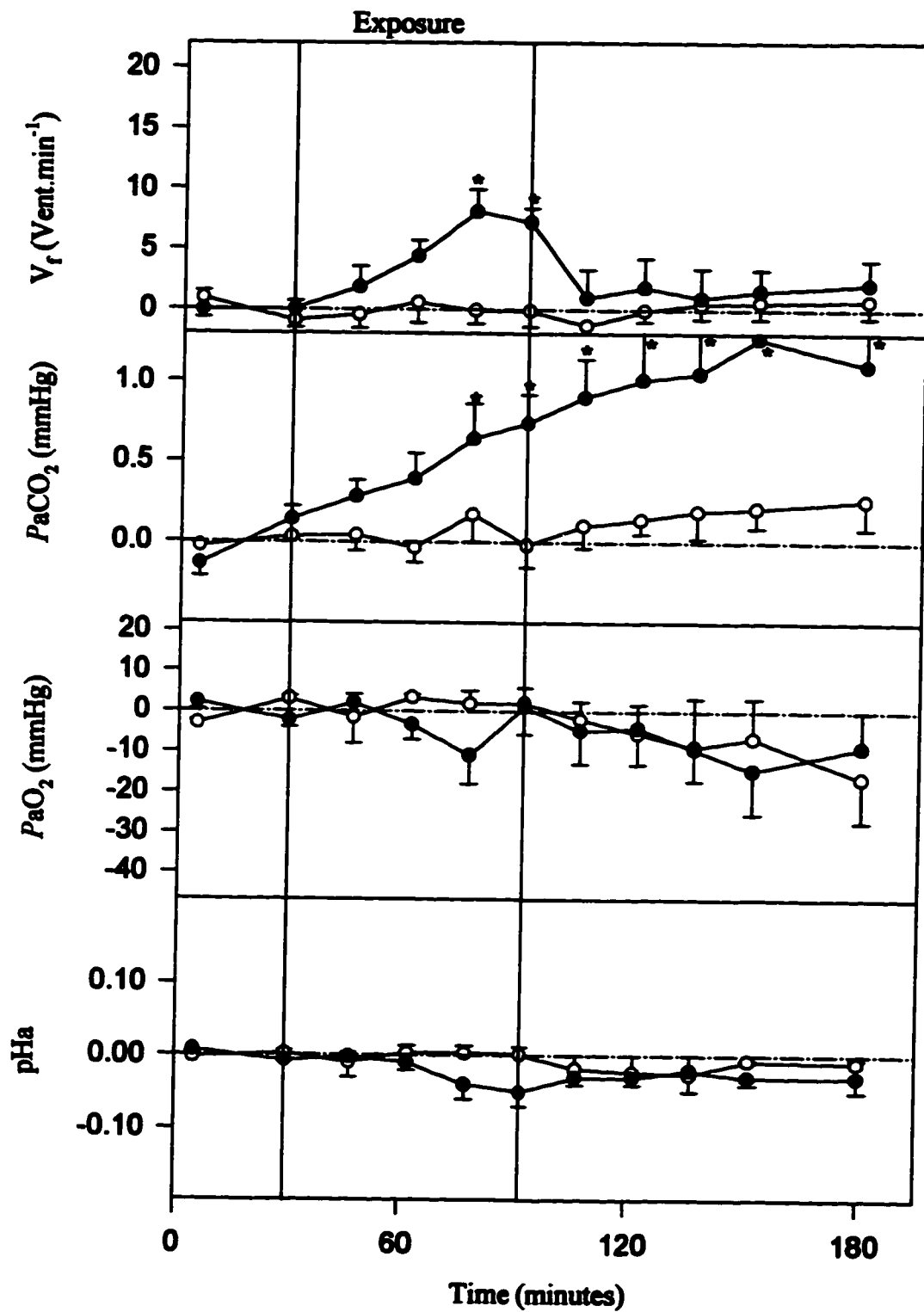


Figure 2-2. The change in ventilation frequency (V_f), arterial PCO_2 ($PaCO_2$), arterial PO_2 (PaO_2) and pH (pH_a) during exposure to 2 mg.L^{-1} chloramine-T (active ingredient) (filled circles). Chemical exposure period represented between the two vertical bars. Control fish = open circles. * = significant from baseline zero. Error bars represent 1 SE.



exposure to sodium hypochlorite (Fig. 2-3) but there was no significant effect of *p*TSA exposure on V_f (Fig. 2-4). Following the withdrawal of chloramine-T, V_f returned to baseline and control values within 15 min. (2 mg.L⁻¹ chloramine-T, Fig. 2-2) or within 90 min. (9 mg.L⁻¹ chloramine-T, Fig. 2-1). However, V_f remained significantly elevated in fish exposed to 0.45 mg.L⁻¹ sodium hypochlorite (fig. 2-3).

Arterial PCO_2 ($PaCO_2$)

Both concentrations of chloramine-T and sodium hypochlorite resulted in a significant increase in $PaCO_2$ during the exposure period (Figs. 2-1 to 2-3). At 9 mg.L⁻¹ chloramine-T, $PaCO_2$ declined to baseline levels within 90 min. of withdrawal (Fig. 2-1) However, at 2 mg.L⁻¹ chloramine-T, $PaCO_2$ levels remained significantly elevated upon withdrawal of chloramine-T (Fig. 2-2). Upon withdrawal of sodium hypochlorite, $PaCO_2$ levels remained significantly elevated during the 90 min. post-exposure period (Fig. 2-3). There was no significant change in the $PaCO_2$ levels in the blood of control fish or fish exposed to *p*TSA (Fig. 2-4).

Figure 2-3. The change in ventilation frequency (V_f), arterial PCO_2 ($PaCO_2$), arterial PO_2 (PaO_2) and pH (pH_a) during exposure to 0.45 mg.L^{-1} sodium hypochlorite (active ingredient) (filled circles). Chemical exposure period represented between the two vertical bars. Control fish = open circles. * = significant from baseline zero. Error bars represent 1 SE.

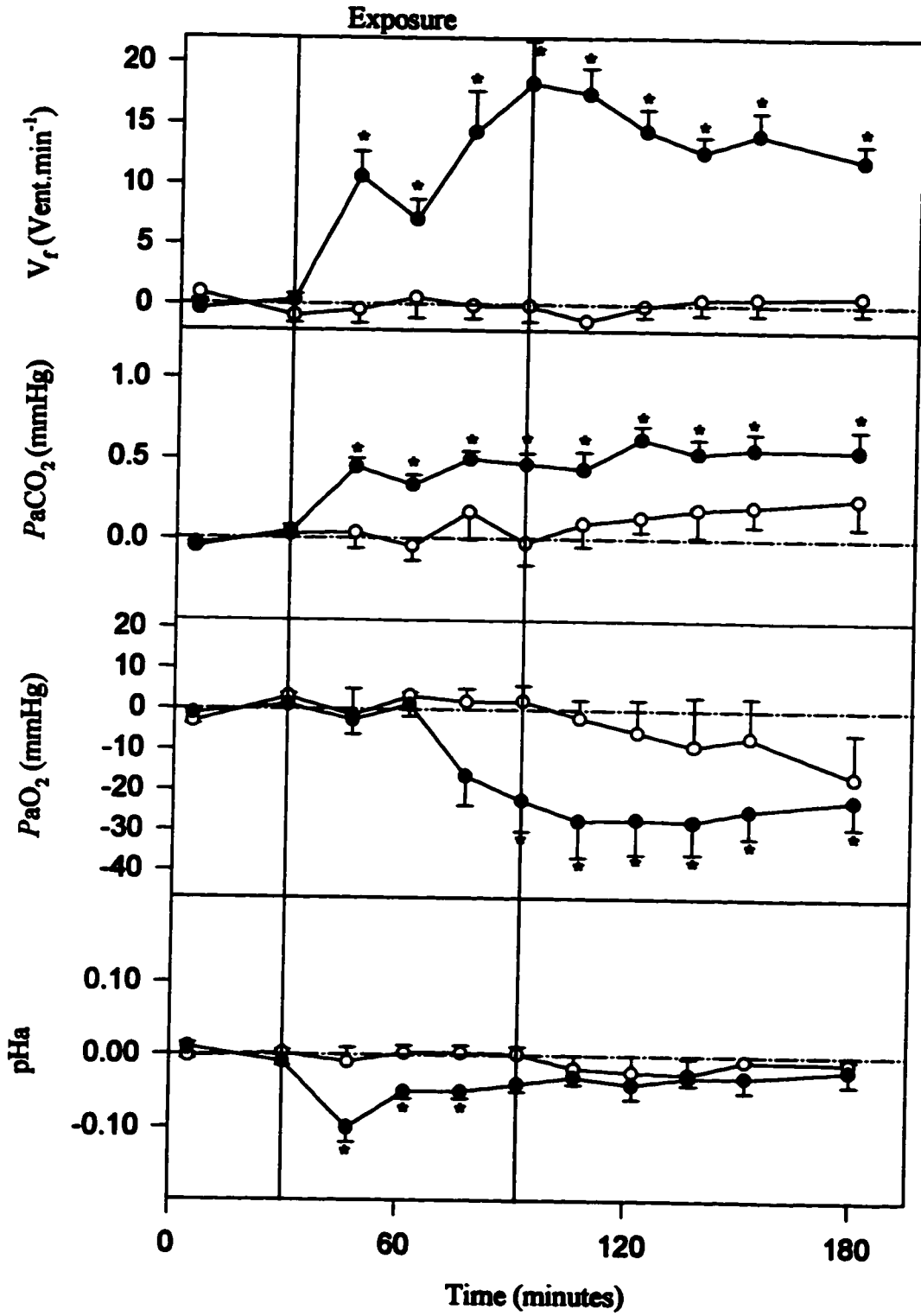
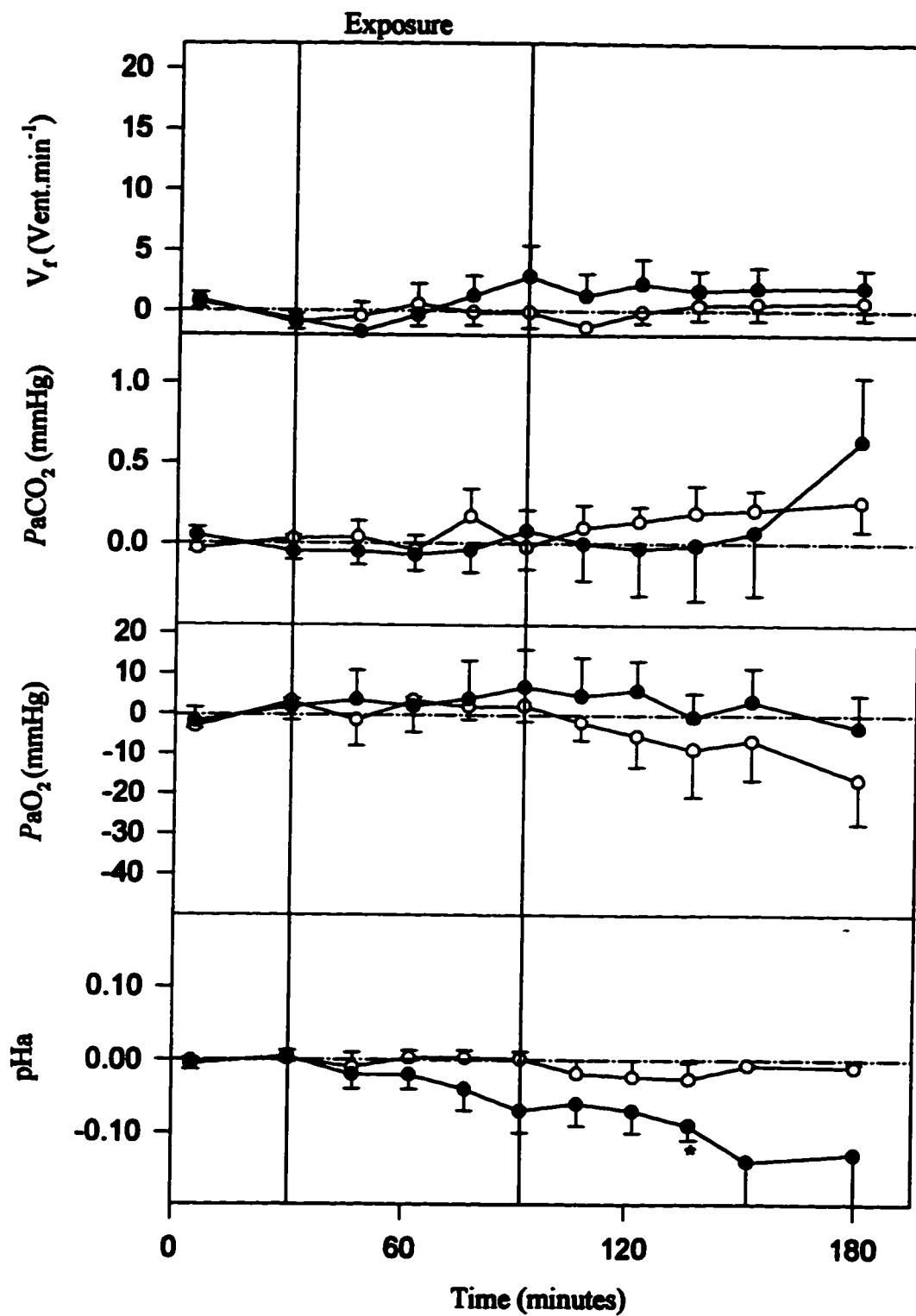


Figure 2-4. The change in ventilation frequency (V_f), arterial PCO_2 ($PaCO_2$), arterial PO_2 (PaO_2) and pH (pH_a) during exposure to 9 mg.L^{-1} *paratoluenesulphonamide* (*pTSA*) (active ingredient) (filled circles). Chemical exposure period represented between the two vertical bars. Control fish = open circles. * = significant from baseline zero. Error bars represent 1 SE.



Arterial PO_2 (PaO_2)

There was no significant effect of chloramine-T or *p*TSA exposure on the PaO_2 levels (Figs. 2-1, 2-2 and 2-4). However, exposure to 0.45 mg.L^{-1} sodium hypochlorite resulted in a significant decrease in PaO_2 which did not recover during the 90 min. post-exposure period (Fig. 2-3). There was no significant change in the PaO_2 of control fish over the experimental period.

Arterial pH (pH_a)

Although fish exposed to chloramine-T (at either concentration) appeared to show a decrease in arterial pH (Figs. 2-1 and 2-2) this was not statistically significant. However, fish exposed to 0.45 mg.L^{-1} sodium hypochlorite showed a significant decrease in pH_a during the exposure period which gradually recovered to pre-exposure values by the end of the experimental period (Fig. 2-3). Fish exposed to *p*TSA showed a gradual decrease in arterial pH during the experimental period and this was more pronounced throughout the recovery period (Fig. 2-4).

Other blood parameters

There were no significant differences between the pre-exposure values for any of the treatments with the exception of mean cellular haemoglobin concentration. However, the relevant comparisons are within treatment rather than between treatment groups (i.e., pre-exposure values compared to post-exposure values for the same treatment). There was no consistent significant effect of treatment on any blood parameter (Table 2-1). Despite significant decreases in haematocrit and haemoglobin concentration after 1 h. post-exposure to 2 mg.L⁻¹ chloramine-T there was no significant effect of exposure (to any of the chemicals in this study) on the O₂ specifically bound to haemoglobin (O₂/Hb) or mean cellular haemoglobin concentration (MCHC) (Table 2-1). Similarly there was no significant increase in plasma catecholamine levels in any of the treatments (Table 2-2).

Table 2-1. Mean (\pm SEM) oxygen content (CaO_2), haematocrit and haemoglobin (Hb) concentration, O_2 specifically bound to haemoglobin (O_2/Hb) and mean cellular haemoglobin concentration (MCHC) of rainbow trout arterial blood before and after exposure to chloramine-T (CLT), *paratoluenesulphonamide* (*p*TSA) or sodium hypochlorite (NaOCl).

Treatment	Pre-exposure	60 min. of exposure	1 h. Post-exposure
CaO_2 (mL O_2.100mL⁻¹blood)			
Control	6.83 \pm 0.69	5.86 \pm 0.65	5.08 \pm 0.72
CLT 2 mg.L ⁻¹	8.97 \pm 0.90	8.47 \pm 0.97	7.06 \pm 0.74
CLT 9 mg.L ⁻¹	10.70 \pm 0.99	9.34 \pm 0.93 ^a	7.89 \pm 0.53 ^a
NaOCl 0.45 mg.L ⁻¹	7.60 \pm 1.21	7.73 \pm 0.97	6.91 \pm 0.87
<i>p</i> TSA 9 mg.L ⁻¹	9.53 \pm 1.21	9.90 \pm 1.01 ^a	8.96 \pm 1.24
Haematocrit (%)			
Control	22.0 \pm 2.4	19.2 \pm 2.4	16.1 \pm 1.6
CLT 2 mg.L ⁻¹	22.2 \pm 0.9	20.5 \pm 1.3	18.1 \pm 0.8*
CLT 9 mg.L ⁻¹	27.3 \pm 2.4	25.1 \pm 2.1	23.0 \pm 1.7
NaOCl 0.45 mg.L ⁻¹	19.7 \pm 2.4	20.8 \pm 2.3	19.6 \pm 1.9
<i>p</i> TSA 9 mg.L ⁻¹	25.5 \pm 1.7	24.5 \pm 1.7	23.7 \pm 2.0

Table 2-1Continued

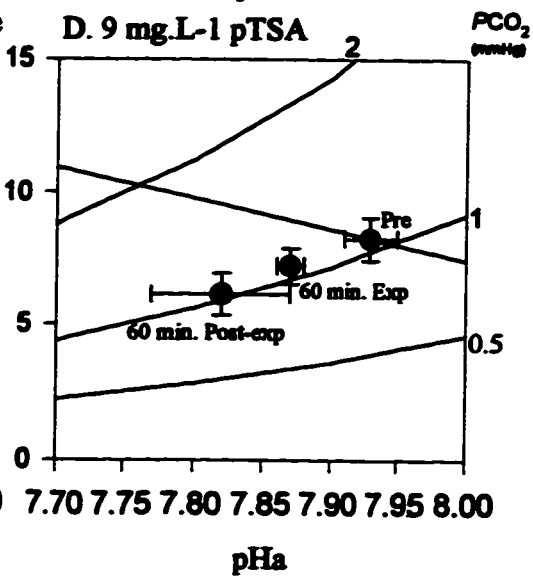
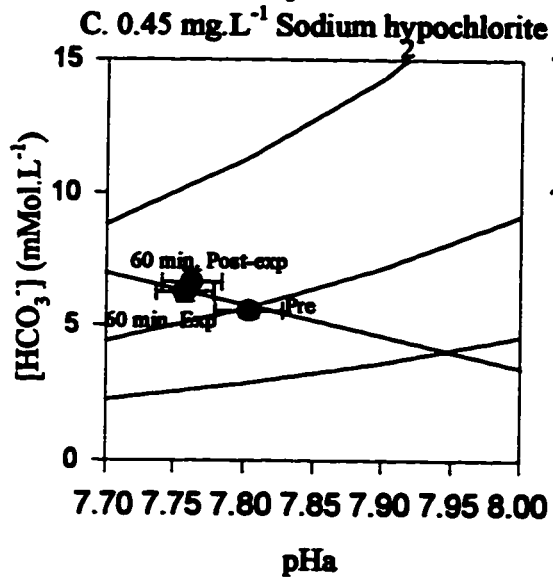
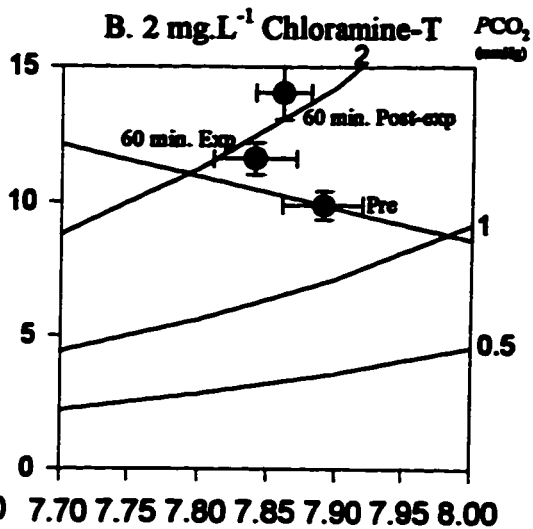
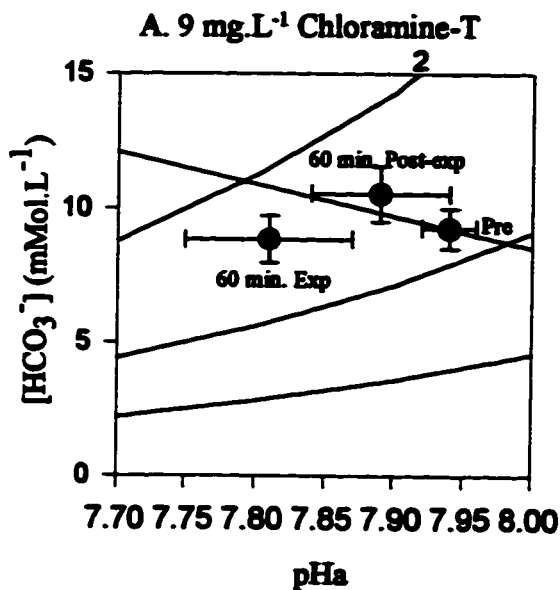
Treatment	Pre-exposure	60 min. of exposure	1 h. Post-exposure
Haemoglobin (g Hb.100 mL⁻¹ blood)			
Control	6.74 ± 0.77	5.76 ± 0.59	5.19 ± 0.60 [*]
CLT 2 mg.L ⁻¹	8.46 ± 0.46	7.81 ± 0.42	6.47 ± 3.75 [*]
CLT 9 mg.L ⁻¹	9.10 ± 0.77	8.79 ± 0.67 ^a	7.31 ± 0.52
NaOCl 0.45 mg.L ⁻¹	6.80 ± 0.92	7.35 ± 0.92	6.35 ± 0.70
pTSA 9 mg.L ⁻¹	9.77 ± 0.86	9.24 ± 0.71 ^a	8.10 ± 0.73 ^a
O₂/Hb (mL O₂/g Hb)			
Control	0.95 ± 0.06	0.92 ± 0.03	0.86 ± 0.05
CLT 2 mg.L ⁻¹	0.99 ± 0.08	1.01 ± 0.09	1.01 ± 0.09
CLT 9 mg.L ⁻¹	1.13 ± 0.08	1.02 ± 0.09	1.01 ± 0.04
NaOCl 0.45 mg.L ⁻¹	1.01 ± 0.10	0.98 ± 0.03	0.98 ± 0.08
pTSA 9 mg.L ⁻¹	0.92 ± 0.10	1.01 ± 0.07	1.01 ± 0.07
MCHC (g.mL⁻¹)			
Control	0.31 ± <0.01	0.30 ± 0.01	0.32 ± 0.01
CLT 2 mg.L ⁻¹	0.38 ± 0.02 ^a	0.38 ± 0.02 ^a	0.36 ± 0.01
CLT 9 mg.L ⁻¹	0.33 ± 0.01	0.35 ± 0.02	0.32 ± 0.01
NaOCl 0.45 mg.L ⁻¹	0.34 ± 0.01	0.35 ± 0.01 ^a	0.33 ± 0.01
pTSA 9 mg.L ⁻¹	0.38 ± 0.01 ^a	0.38 ± 0.01 ^a	0.34 ± 0.01

* significant from pre-exposure (p<0.05); ^a significantly different from control (p<0.05)

Table 2-2: Mean (\pm SEM) plasma catecholamine concentrations (nmol.L^{-1}) in rainbow trout arterial blood before and after exposure to chloramine-T (CLT), sodium hypochlorite (NaOCl) or *paratoluenesulphonamide* (*p*TSA).

Treatment	Pre-exposure	60 min. of exposure	1 h Post-exposure
Adrenaline			
Control	4.88 \pm 1.94	6.10 \pm 4.88	3.08 \pm 0.98
CLT 2 mg.L^{-1}	0.60 \pm 0.38	3.67 \pm 1.60	2.54 \pm 1.04
CLT 9 mg.L^{-1}	4.27 \pm 1.53	12.53 \pm 3.16	11.70 \pm 4.75
NaOCl 0.45 mg.L^{-1}	2.85 \pm 1.46	13.08 \pm 6.90	27.80 \pm 19.1
<i>p</i> TSA 9 mg.L^{-1}	5.20 \pm 4.63	6.14 \pm 3.23	16.19 \pm 4.41
Noradrenaline			
Control	2.96 \pm 1.52	1.71 \pm 1.14	3.69 \pm 1.42
CLT 2 mg.L^{-1}	1.21 \pm 1.00	1.53 \pm 0.92	1.04 \pm 0.64
CLT 9 mg.L^{-1}	4.90 \pm 1.41	4.46 \pm 1.44	7.25 \pm 2.14
NaOCl 0.45 mg.L^{-1}	1.28 \pm 1.28	8.94 \pm 5.26	7.49 \pm 2.58
<i>p</i> TSA 9 mg.L^{-1}	2.54 \pm 1.52	5.28 \pm 1.75	6.81 \pm 3.01

Figure 2-5. The relationship between plasma bicarbonate ($[\text{HCO}_3^-]$) and arterial pH for the blood of trout treated (filled circles) with 9 mg.L⁻¹ chloramine-T (A), 2 mg.L⁻¹ chloramine-T (B), 0.45 mg.L⁻¹ sodium hypochlorite (C) and 9 mg.L⁻¹ pTSA (D). Error bars represent 1 SE.



Acid-base status

It was evident from the pH-bicarbonate diagrams that exposure to chloramine-T resulted in an apparently mixed respiratory and metabolic acidosis at 9 mg.L⁻¹ chloramine-T. However, the point representing 60 min post-exposure was close to the buffer line (Fig. 2-5a). At 2 mg.L⁻¹ chloramine-T there was a respiratory acidosis superimposed over a metabolic alkalosis (Fig. 2-5b). However, fish exposed to 0.45 mg.L⁻¹ sodium hypochlorite exhibited a pure respiratory acidosis (Fig. 2-5c) whereas fish exposed to 9 mg.L⁻¹ pTSA exhibited a purely metabolic acidosis (Fig. 2-5c).

DISCUSSION

A striking feature of chloramine-T and hypochlorite exposure is the significant increase in ventilation frequency. However, despite this apparent hyperventilation, arterial PCO_2 was significantly elevated over baseline values. Generally, hyperventilation is associated with a lowering of arterial PCO_2 (Iwama et al. 1987). As a consequence of this it would be predicted that $PaCO_2$ should have decreased during the periods of increased ventilation. However, this did not occur. The fact that in this experiment $PaCO_2$ increased despite gill

hyperventilation suggests a change in the diffusive conductance of the gill which is impairing CO₂ excretion. A possible explanation for this is the secretion of mucus onto the gill surface thereby increasing the blood-to-water diffusion distance and/or unstirred boundary layer of the gill. This would, on one hand, cause a rise in PaCO₂ because the gill is believed to be diffusion limited with respect to CO₂ transfer (Cameron and Polhemus 1974; Malte and Weber 1985). However, on the other hand, the trout gill is principally perfusion limited with respect to O₂ transfer (Daxboeck et al. 1982; Randall and Daxboeck 1984; Malte and Weber 1985). Thus under normoxic conditions branchial oxygen uptake would not likely be limited by the thickness of the diffusion barrier, and hence PaO₂ was unaffected by chloramine-T (and presumably the release of mucus caused by chloramine-T). This notwithstanding, Bass and Heath (1977) using a similar concentration of sodium hypochlorite to that used in the present study, noted a lowering of PaO₂ associated with hypersecretion of mucus on the gill of repeatedly exposed rainbow trout (Bass et al. 1977). The argument above however, does not hold for hypochlorite since Bass et al. (1977) also noted acute morphological changes in the gill leading to a reduction in the gill surface area as well as diffusion distance. Nevertheless hypochlorite exposure caused an

elevation in $PaCO_2$ in this study and since this relationship between V_f and $PaCO_2$ was not observed with $pTSA$ exposure it strongly suggests that this hyperventilatory response and elevation in $PaCO_2$ is caused by the liberation of hypochlorite from chloramine-T. The apparent impairment of CO_2 excretion cannot be explained by chloramine-T induced inhibition of red blood cell (RBC) HCO_3^- dehydration because there was no effect of chloramine-T on the rate of RBC CO_2 excretion (unpublished results). Using the radioisotopic HCO_3^- dehydration assay of Wood and Perry (1991), RBC carbonic anhydrase (CA) activity was unaltered and this infers that inhibition of boundary layer CA (Wright et al. 1989) was also unaltered by chloramine-T and therefore not a factor in the elevation of $PaCO_2$.

Upon the withdrawal of chloramine-T and subsequent recovery of the fish, ventilation frequency decreased to baseline levels either within the first 15 min. (2 mg.L^{-1}) or over the subsequent 90 min recovery period (9 mg.L^{-1}). Over this recovery period, $PaCO_2$ levels in 2 mg.L^{-1} treated fish remained elevated and even continued to rise slightly whereas in the 9 mg.L^{-1} treated fish, $PaCO_2$ levels were gradually returned to baseline levels within 90 min. The $PaCO_2$ of fish treated with hypochlorite showed a persistent elevation despite the significantly

elevated ventilation frequency. This further suggests that elevation in arterial PCO_2 is caused by reduced gill conductance perhaps owing to mucus secretion. Where hyperventilation persists even after the withdrawal of chloramine-T (Fig. 2-1) and the stimulus for mucus secretion is removed, the PCO_2 levels were reduced to baseline levels. With the withdrawal of 2 mg.L^{-1} chloramine-T hyperventilation ceased almost immediately, the maintenance of hyperventilation which would lead to excretion of this excess CO_2 was not present, therefore CO_2 continued to build up in the blood because of increased mucous coat secreted onto the gill. With hypochlorite, the hyperventilation was sufficient to maintain $PaCO_2$ albeit at an elevated level. This hyperventilation may have been maintained as a consequence of the significant lowering of PaO_2 by 30 mmHg from approximately 97 mmHg to 68 mmHg (Fig. 2-3). This result is consistent with the findings of Bass and Heath (1977) where after a single 3 h pulse of peak concentration 0.4 mg.L^{-1} total residual chlorine, PaO_2 was reduced by approximately 60 mmHg compared with 30 mmHg after a 1 h pulse in our study. However, in the present study, the lowering of PaO_2 was insufficient to cause a significant depression of blood O_2 content. Thus the hyperventilation which occurred during chloramine-T exposure (no change in blood PaO_2 or content) or

hypochlorite (change in PaO_2 only) was unlikely induced by hypoxaemia (Smith and Jones 1982; see review by Shelton et al. 1986). Similarly, plasma catecholamine levels were not substantially elevated (see below) and could not have contributed to the hyperventilatory response (Randall and Taylor 1991). It is possible that the stimulus for ventilation originated in the central nervous system perhaps through stimulation of nociceptors by hypochlorite or chloramine-T.

There were no significant effects of treatment on total oxygen content, haematocrit or haemoglobin content. The decreases in haematocrit and haemoglobin were probably due to the removal of blood within a short period of time (1.95 mL within 3 h) and therefore likely were artefacts of repeated sampling. Similarly, there was no consistent effect of treatment on catecholamine concentrations. Although there were elevations of catecholamines in some individual fish (as represented by a high variance) (Table 2-2), the levels were considerably lower than those measured in the recovery of stressed fish (Gamperl et al. 1994) and probably of little physiological significance. Indeed, the levels of adrenaline and noradrenaline during exposure were substantially below those levels required to elicit red cell adrenergic responses (Tetens et al.

1988). This is reflected by the absence of any decrease in the mean cellular haemoglobin concentration which would be expected had there been any cell swelling due to an influx of water following β -adrenergic activation of the Na^+/H^+ exchange (Nikinmaa 1992).

In the extracorporeal circulation of trout, the arterial blood is in a state of acid-base disequilibrium (Gilmour et al. 1994) and therefore some small error may be associated with using values of $P_a\text{CO}_2$ and pH_a in equilibrium calculations. Nevertheless, since there was no significant acidosis with chloramine-T or elevation in catecholamine levels (which serve to enhance the disequilibrium (Gilmour et al. 1995)) these calculations can be made with acknowledgement that a small error in the estimates may exist.

Chloramine-T and perhaps hypochlorite appear to affect the acid-base status of trout. However, the exact nature of these effects is not clear. It was evident from this study that exposure to 9 mg.L^{-1} chloramine-T did not cause a significant acidosis (Fig. 2-5a). Hypochlorite on the other hand caused an entirely respiratory acidosis and $p\text{TSA}$ a metabolic acidosis. It may be that the effects shown by chloramine-T at 9 mg.L^{-1} may represent the combined effects of these chemicals. However, exposure to 2 mg.L^{-1} chloramine-T resulted in a

respiratory acidosis and a pronounced metabolic alkalosis with the withdrawal of chloramine-T (Fig. 2-5b). At present we can only speculate as to the causes of such disturbances. The development of a respiratory acidosis is consistent with our contention that chloramine-T and hypochlorite caused a hypersecretion of branchial mucus which was sufficient to impair CO₂ excretion. However, it would appear that there are metabolic components to the acid-base disturbances which probably reflect changes in branchial acid or base fluxes. We cannot discount the possibility that in fish treated with 9 mg.L⁻¹ chloramine-T or hypochlorite, metabolic compensation and a subsequent metabolic alkalosis was not complete by the end of the experimental period.

The acid-base disturbances seen in this study correlate well with the changes in chloride cell ultrastructure seen from rainbow trout which had been intermittently exposed to chloramine-T at similar concentrations (Powell et al. 1995). Gill chloride cells from intermittently exposed fish expressed a significantly greater proportion of the apical plasmalemma than control fish. Such a morphological response is similar to that seen in fish in which a metabolic alkalosis had been induced (Goss et al. 1992a,b; Goss and Perry 1993).

CHAPTER 3

**RESPIRATORY AND ACID-BASE DISTURBANCES IN ARTERIAL
BLOOD DURING EXPOSURE TO CHLORAMINE-T UNDER
HYPOXIA AND HYPEROXIA**

INTRODUCTION

Chloramine-T treatments are commonly carried out on fish which may be hypoxaemic due to the presence of bacterial gill diseases or the high stocking densities which are characteristic of commercial aquacultural practices. Supplemental oxygenation may be used as a partial therapy in diseased and/or heavily stocked tanks. The effects of therapeutic chloramine-T exposure under these conditions are not known.

A single pulse of chloramine-T at therapeutic (9 mg.L^{-1}) or subtherapeutic (2 mg.L^{-1}) levels under normoxic conditions was sufficient to impair CO_2 excretion without affecting O_2 uptake even though the fish were apparently hyperventilating (chapter 2). This response was attributed to a decrease in gill conductance induced by mucus secretion due to branchial irritation from hypochlorite. The aim of the present study was to examine the effects of chloramine-T treatment under moderately hypoxic and hyperoxic conditions to test the hypothesis that an acute pulse exposure to chloramine-T impairs gas transfer. Moderate hypoxia and hyperoxia were used as probes to dissect the effects of ventilation. Thus we would predict that under hypoxia, gill hyperventilation would attenuate the effects of chloramine-T on gas transfer

while under hyperoxia, hypoventilation would amplify the deleterious effects on gas transfer.

MATERIALS AND METHODS

Adult rainbow trout (*O. mykiss*) (N = 27) of mean weight 763.3 ± 30.5 g (mean \pm SE.) were purchased from a commercial farm (Linwood Acres Trout Farm, Campbellcroft Ontario) and acclimated to our laboratory for at least two weeks prior to use. Throughout the acclimation period and in all subsequent experiments the fish were held under a 12 h. light:12 h. dark photoperiod in flowing aerated and dechlorinated city of Ottawa water (residual chlorine levels were < 0.03 mg.L⁻¹ as determined by the DPD method (Franson 1978)) at 10°C containing 118.9 ± 2.4 μ M Na⁺; 151.8 ± 0.9 μ M Cl⁻; 365.8 ± 8.2 μ M Ca²⁺ and 19.3 ± 0.5 μ M K⁺ at pH 6.8.

Surgical procedures

Fish were anaesthetised with a sodium bicarbonate buffered 100 mg/L tricaine methanesulphonate (MS 222, Sigma Chemical Company, St. Louis, Mo. USA) solution and the gills were constantly irrigated with the same oxygenated

solution. Fish were implanted with PE 50 catheters in the dorsal aorta and coeliac arteries according to the methods of Soivio et al. (1975) and Thomas and LeRuz (1982), respectively as described in chapter 2. Brass electrodes (1 cm²) were sutured to the operculae to allow the monitoring of the opercular amplitude to verify changes in the visual observations on ventilation frequency as an indicator of hyper- or hypoventilation. The fish was placed in a black acrylic respirometer box supplied with flowing air saturated water and allowed to recover. The following day, the catheters were flushed once with Cortland's saline containing 250 IU ammonium heparin (Sigma Chemical Company) prior to experimentation.

Extracorporeal circulation

To avoid the effects of blood loss and possible anaemia which may result from repeated blood sampling and which may have affected ventilation patterns (Smith and Jones 1982), an extracorporeal blood circulation was employed. Moreover, changes in blood gases over such a relatively short period of chemical exposure require a sensitive and rapid technique for measurement. This is facilitated by the extracorporeal circulation (Thomas and LeRuz 1982) as

described in chapter 2. Dorsal aortic blood pressure was monitored from the saline filled dorsal aortic catheter using a Bell and Howell Type 4-327I transducer (Bell and Howell, Pasadena Ca. USA) to allow early detection of internal haemorrhage. A drop in blood pressure would indicate a possible internal haemorrhage and anaemia which might influence ventilation and blood gas measurements (Smith and Jones, 1982). Opercular impedance was monitored by means of a customised impedance converter and amplifier as in chapter 2. Analogue outputs from the acid-base analyser, pressure transducer and impedance converter were transformed into digital output using an analogue-digital interface (Data Translation Inc.) and relayed to a microcomputer using customised data acquisition software written by P. Thören, (Göteborg, Sweden). Ventilation frequency was monitored visually by counting buccal movements.

Total carbon dioxide content of the blood was determined from rearrangement of the Henderson-Hasselbalch equation (refer to chapter 2) using measured values of PCO_2 and pH from the extracorporeal circulation and constants from Boutilier et al. (1984). The non-bicarbonate buffer values for true plasma were calculated according to the regression equation given by Wood et al. (1982).

Experimental protocol

Fish were monitored in a stable resting state for 15 min under normoxic conditions. After this normoxic baseline period the water PO_2 (PwO_2) was lowered to 100 mmHg by bubbling nitrogen into a Plexiglas column filled with marbles (to increase surface area), the water from which supplied the respirometer box housing the fish. Alternatively the PwO_2 was raised to 430 mmHg by bubbling oxygen into the equilibration column. In both cases, steady state hypoxia or hyperoxia was achieved in approximately 15 minutes. Water PO_2 was measured using a Radiometer E5046 electrode and PHM 71 acid-base analyser interfaced with the microcomputer. Following the onset of hypoxia or hyperoxia the fish were monitored for 15 min under steady state conditions (hypoxic or hyperoxic baseline). Fish were exposed to 9 mg.L⁻¹ (n = 7 hypoxia; n = 9 hyperoxia) chloramine-T (active ingredient) (BDH Chemicals Ltd. Toronto, On.) or to no treatment chemical (control: n = 5 hypoxia, n = 6 hyperoxia). Chloramine-T was pumped by a Manostat Varistaltic AL series peristaltic pump (Manostat Inc. New York, NY) into the inflowing water system from a stock solution made up with the same water supplying the respirometer. The flow rates

of chloramine-T solution and system water were maintained so as to yield a final concentration of 9 mg.L^{-1} chloramine-T (active ingredient). Following a 60 min exposure to chloramine-T, the fish were monitored for a further 60 min before being returned to normoxic conditions by bubbling air through the equilibration column.

Data handling and statistical analysis

For the purposes of comparing the responses of each variable to chemical treatment under either hypoxia or hyperoxia, a mean normalisation transformation (as described in chapter 2) was employed. Thus results are expressed as net changes in a particular variable without violating assumptions of normality (Glantz 1987). The data points shown in the figures represent means at 15 minute intervals ± 1 standard error. Means were compared to baseline values using a repeated measures analysis of variance with Bonferoni-corrected multiple *t*-tests to isolate individual differences and points were analysed with a *t*-test between treatments (control and chloramine-T) (Glantz 1987). P-values of less than 0.05 were considered to be significant.

RESULTS

Ventilation Frequency (V_f)

Hypoxia (PwO_2 100 mmHg) was sufficient to significantly elevate the baseline V_f by approximately 7-8 ventilations. min^{-1} over normoxic baseline values prior to chloramine-T exposure (Fig. 3-1). However, under hypoxic conditions there was no significant effect of chloramine-T exposure on V_f .

Hyperoxia (PwO_2 430 mmHg) significantly suppressed baseline V_f by approximately 12-20 ventilations. min^{-1} from normoxic baseline values prior to chloramine-T exposure (Fig. 3-2). Under hyperoxic conditions all fish (chloramine-T exposed and controls) exhibited cyclical breathing, that is, a period of ventilation followed by a period of apnea lasting up to 20 seconds. Chloramine-T exposure resulted in an increase in V_f of approximately 10 ventilations. min^{-1} over the hyperoxic baseline period (Fig. 3-2). In control fish V_f was constant throughout the hyperoxic period (Fig. 3-2).

Blood gas tensions

Hypoxia caused a significant decrease in $PaCO_2$ of approximately 0.25

Figure 3-1. Net change in ventilation frequency (V_f), arterial PCO_2 ($PaCO_2$), arterial PO_2 (PaO_2) and pH (pH_a) during hypoxia ($PwO_2 = 100$ mmHg: area between vertical dotted lines) during exposure (area between solid vertical lines) to 9 mg.L^{-1} chloramine-T (Filled circles). Control (unexposed fish) represented by open circles. * $p < 0.05$ from normoxic baseline, + $p < 0.05$ from hypoxic baseline (prior to chloramine-T exposure).

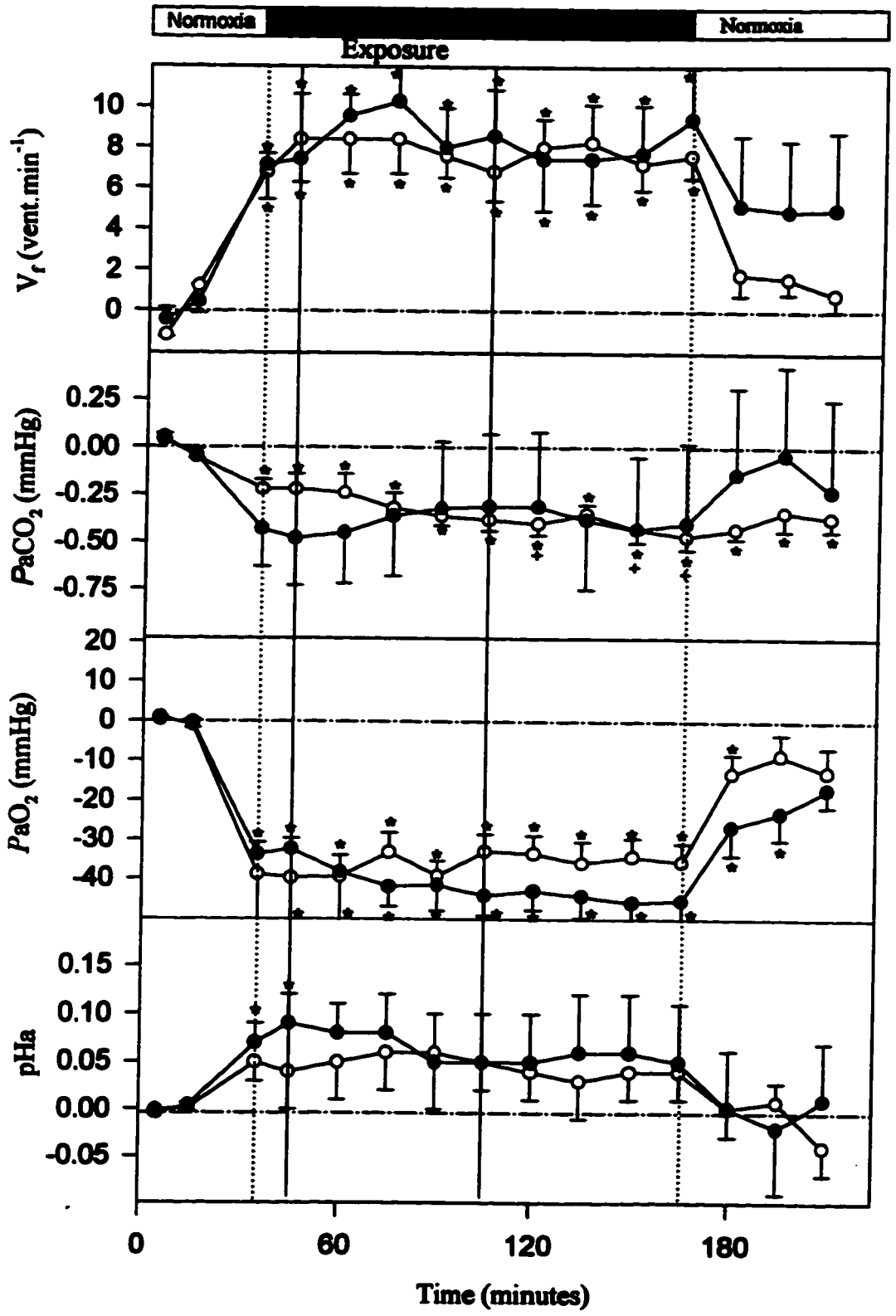
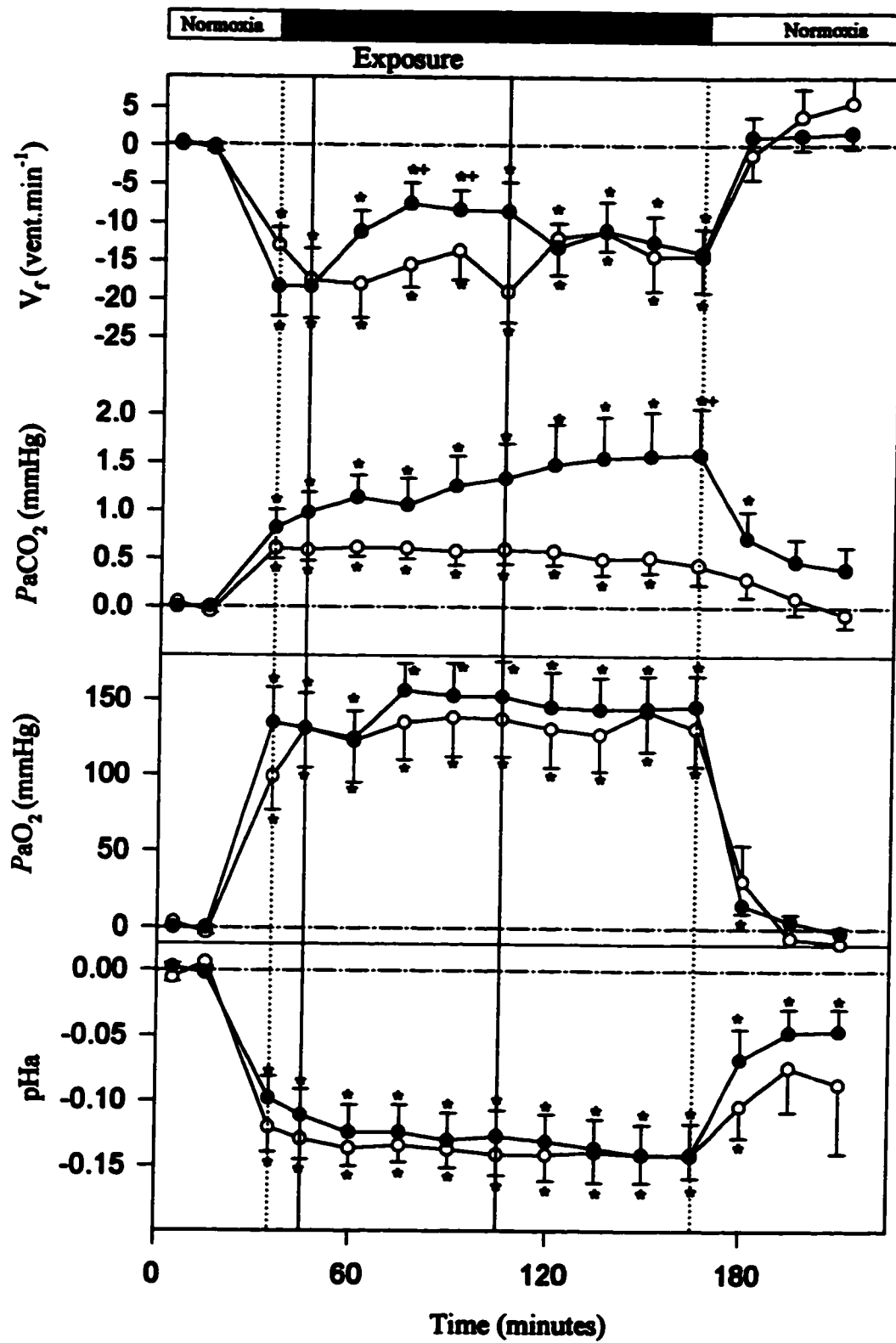


Figure 3-2. Net change in ventilation frequency (V_f), arterial PCO_2 ($PaCO_2$), arterial PO_2 (PaO_2) and pH (pHa) during hyperoxia ($PwO_2 = 430$ mmHg: area between vertical dotted lines) during exposure (area between solid vertical lines) to 9 mg.L^{-1} chloramine-T (filled circles). Control (unexposed fish) represented by open circles. * $p < 0.05$ from normoxic baseline, + $p < 0.05$ from unexposed control fish.



mmHg prior to experimental exposure to chloramine-T. However, chloramine-T exposure had no significant effect on arterial PCO_2 ($PaCO_2$). Hyperoxia resulted in a significant increase in $PaCO_2$ of approximately 0.6-0.75 mmHg prior to chloramine-T exposure. There was a significant difference between chloramine-T exposed fish and controls at 60 min. post-exposure (Fig. 3-2). There was a significant decrease in PaO_2 with hypoxia but no significant changes in PO_2 were measured during the period of chloramine-T exposure (Fig. 3-1). Hyperoxia, on the other hand, caused a significant increase in PaO_2 compared to the normoxic baseline period prior to experimental exposure. Arterial PO_2 remained constant throughout the exposure period, unaffected by exposure to chloramine-T. Similarly, following chloramine-T withdrawal, PaO_2 remained unchanged from the pre-exposure (hyperoxic baseline period) (Fig. 3-2).

Arterial pH (pHa)

Arterial pH was only slightly elevated over the normoxic baseline with the onset of hypoxia and remained relatively constant over the exposure period and subsequent hypoxic recovery period (Fig. 3-1). Hyperoxia, on the other hand, caused a significant decrease in pHa from the normoxic baseline and prior to

chloramine-T exposure but pHa was not affected by exposure to chloramine-T (Fig. 3-2).

Acid-base status

It was evident from the pH-bicarbonate diagrams that hypoxia resulted in a respiratory alkalosis prior to exposure to chloramine-T. Exposure to chloramine-T appeared to slightly reduce the magnitude of this alkalosis (Fig. 3-3). However, under hyperoxic conditions the fish experienced a respiratory acidosis prior to exposure to chloramine-T. Exposure to chloramine-T as well as following the subsequent withdrawal of the chemical resulted in the maintenance of the respiratory acidosis (Fig. 3-4).

DISCUSSION

Hypoxia causes significant increases in both ventilation stroke volume and frequency to increase ventilatory bulk water flow (Smith and Jones 1982; Randall 1982). In addition to this, there is also an increase in the perfusion of the gill by lamellar recruitment so as to increase the functional surface area over which gas exchange can occur (Randall 1982). Thus, during hypoxia trout

Figure 3-3. Relationship between plasma bicarbonate ($[\text{HCO}_3^-]$) and arterial pH from the blood from trout exposed to chloramine-T under pre-hypoxic (normoxic) and hypoxic ($P_{\text{wO}_2} = 100$ mmHg) conditions following 1 h exposure 9 mg.L^{-1} chloramine-T and following 1 h or withdrawal of chloramine-T (recovery). The curved isopleths represent the bicarbonate buffering capacity of the blood i.e., $[\text{HCO}_3^-]$ and pH at a given P_{CO_2} and the diagonal straight line represents the non-bicarbonate buffering capacity of the blood (buffer line). Migration of points along the non-bicarbonate buffer line is caused by increases or decreases in $[\text{HCO}_3^-]$ and pH representing a respiratory acidosis (increasing $[\text{HCO}_3^-]$, decreasing pH) or respiratory alkalosis (decreasing $[\text{HCO}_3^-]$, increasing pH).

9 mg.L-1 Chloramine-T under hypoxia (PwO_2 100 mmHg)

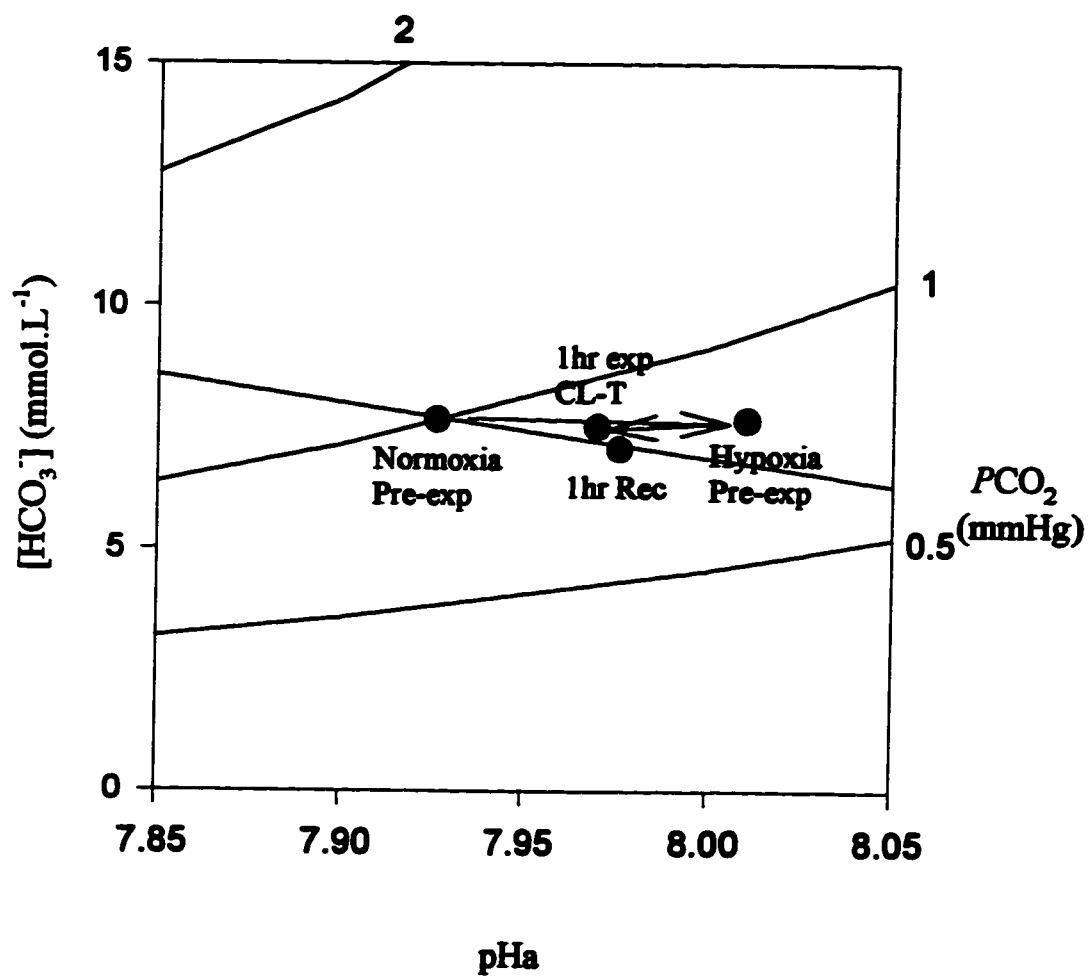
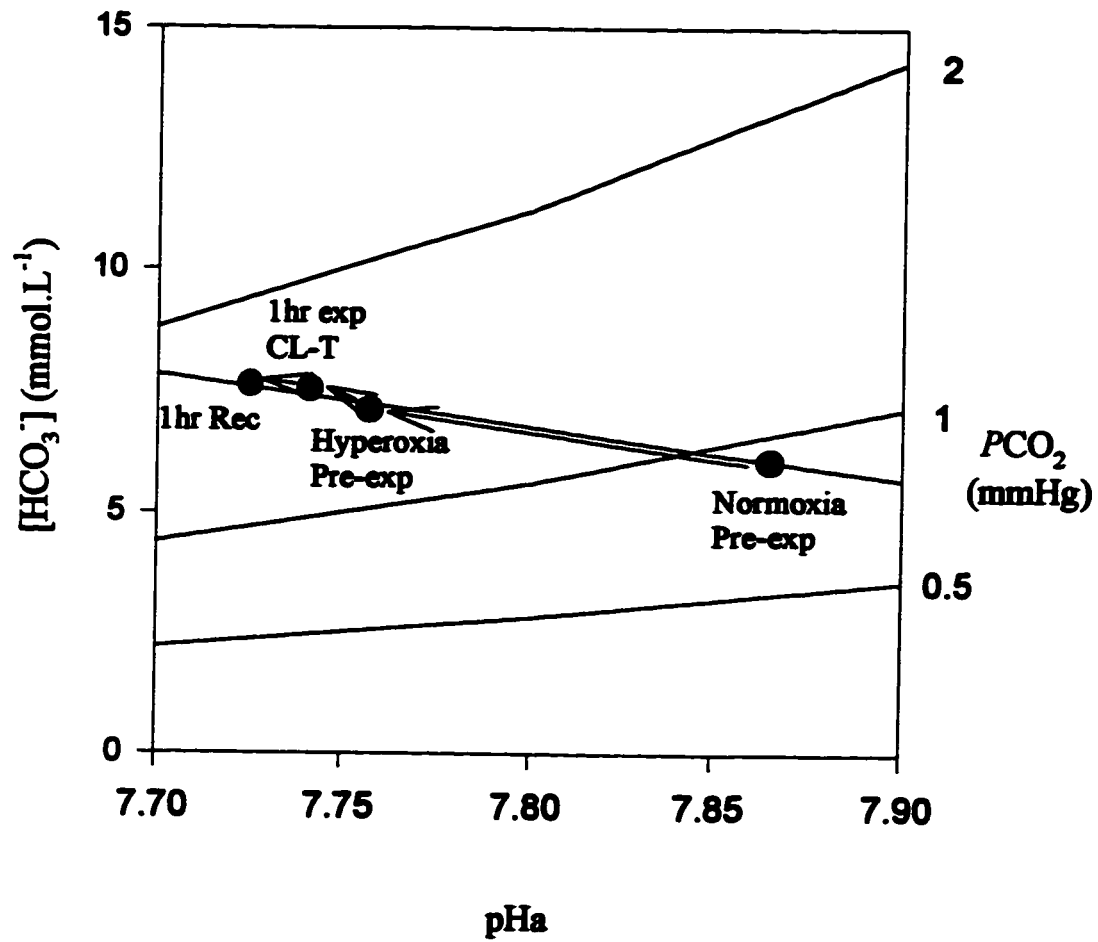


Figure 3-4. Relationship between plasma bicarbonate ($[\text{HCO}_3^-]$) and arterial pH from the blood from trout exposed to chloramine-T under normoxic, hyperoxic ($P_{\text{wO}_2} = 430 \text{ mmHg}$) pre-exposure, following 1 h exposure to 9 mg.L^{-1} chloramine-T and following 1 h of withdrawal of chloramine-T (recovery). For a detailed description of the isopleths and buffer lines refer to the legend of figure 3-3.

9 mg.L⁻¹ Chloramine-T under hyperoxia (*P*wO₂ 430 mmHg)



normally develop a respiratory alkalosis owing to the accelerated removal of CO₂ (Iwama et al. 1987). Exposure of hyperventilating fish to chloramine-T had no effect on any of the respiratory variables measured. This is in contrast to our previous study in which fish were exposed to chloramine-T under normoxic (air saturated) conditions (chapter 2). In that instance there was a rise in arterial PCO₂ attributed to a change in branchial conductance possibly caused by the secretion of mucus. Differences between the previous and the present study may be explained in terms of a functional reduction of the gill boundary layer due to hyperventilation and/or increase in bulk water flow over the gill (Randall 1982). Even with chloramine-T treatment which may have increased branchial mucus secretion (which under normoxic conditions reduced gill conductance (chapter 2), the partial pressure gradient was sufficiently maintained to overcome the effect of mucus secretion and therefore maintain the excretion of CO₂. Alternatively, increased water flow over the gills may have exerted a greater shear force and consequently stripped mucus from the gill surface. Oxygen uptake was not significantly affected under hypoxic conditions suggesting that increased ventilation was sufficient to maintain O₂ uptake as well as CO₂ excretion. Mucus has been demonstrated in vitro to impair O₂ diffusion (Ultsch

and Gros 1979). It is possible that at a lower water PO_2 , O_2 uptake may have been significantly impaired owing to increased branchial mucous secretion.

Hyperoxia on the other hand resulted in a reduced ventilation frequency and an elevation of $PaCO_2$. Exposure to chloramine-T significantly elevated ventilation frequency. Theoretically this would be expected to decrease arterial PCO_2 (Iwama et al. 1987). However, this was not the case. In fact $PaCO_2$ actually began to rise slightly which continued after the withdrawal of chloramine-T (Fig. 3-2). This further supports our suggestions that there was a reduction in the gill conductance, perhaps due to the secretion of mucus which impaired the diffusion of CO_2 across the epithelium but, did not affect the diffusion of O_2 (chapter 2).

As explained in chapter 2 the arterial blood is considered to be in a state of acid-base disequilibrium (Gilmour et al. 1994) and subsequently some small error may be associated with using values of $PaCO_2$ and pH in equilibrium calculations. Nevertheless, these calculations can still be made in the absence of large metabolic acidoses and elevated catecholamine levels which may serve to enhance the disequilibrium (Gilmour et al. 1995). Hypoxia resulted in a respiratory alkalosis prior to chloramine-T exposure. However, under hyperoxic

conditions there was the development of a respiratory acidosis which was maintained during chloramine-T exposure even though the fish showed increased ventilation. Thus CO₂ excretion must have also been impaired. Again the most likely candidate for this impairing of gas transfer is branchial mucus secreted in response to the irritating effects of hypochlorite from chloramine-T.

The rise in ventilation frequency in fish exposed to chloramine-T under hyperoxia suggested that chloramine-T (probably through the formation of hypochlorite) exerts a ventilatory stimulus independent of arterial oxygen tension or content (both remain high under hyperoxia). That such an apparently maladaptive response occurs is interesting and has been shown in response to hypochlorite and mixtures of hypochlorite and PCBs (Black and McCarthy 1990). In that study there was a decrease in oxygen uptake efficiency which was attributed to mucus secretion. However, unlike previous studies which have examined the respiratory response to high concentrations of hypochlorite (e.g., Bass and Heath 1977; Black and McCarthy 1990), the present study examined the effects of a less aggressive and more stable oxidising agent which presumably causes only minor irritation of the branchial membrane during a 60 min exposure period. The results of this and the pervious chapter suggest that branchial

irritation and presumably mucus secretion caused by chloramine-T has a severe impact on carbon dioxide exchange across the gill. Although oxygen uptake (in terms of arterial PO_2) is not as severely affected by branchial mucous secretion as caused by mild irritation by chloramine-T. However, it should be noted that previous studies (Bass and Heath 1977; Black and McCarthy 1990) did not measure carbon dioxide tensions. This study supports previous suggestions that CO_2 excretion at the gill is primarily limited by diffusive parameters whereas O_2 uptake would appear to be less sensitive to changes in the diffusive properties of the gill (Cameron and Polhemus 1974; Malte and Weber 1985). However, because CO_2 excretion is sensitive to ventilation, adjustments in breathing rates (and subsequent increase/decrease in bulk water flow) are sufficient to affect the extent to which branchial mucus (as stimulated by branchial irritation) may affect gas transfer in the fish gill.

From a practical perspective, the use of a single 60 min pulse of chloramine-T as a disinfectant appears to have little pathological effect on adult trout under either moderately hypoxic or hyperoxic conditions. Using chloramine-T as a tool to probe the response of the gill to mild irritation. In addition the use of hypoxia and hyperoxia have allowed us to dissect the

influence of ventilation on CO₂ excretion as it is affected by mucous secretion. Supporting the notion that CO₂ excretion appears to be primarily diffusion limited. It would appear from this study that chloramine-T exposure under moderately hypoxic conditions such as those which might be expected to occur under normal aquacultural conditions results in a lesser impact on the respiratory and acid-base status of the fish than under hyperoxic conditions. This implies that supplemental oxygenation may not be the best strategy when using chloramine-T commercially in heavily stocked tanks.

CHAPTER 4
CARDIO-RESPIRATORY EFFECTS OF CHLORAMINE-T
EXPOSURE

INTRODUCTION

In the previous two chapters it was shown that when exposed acutely to chloramine-T, trout show an increase in arterial PCO_2 under normoxic or hyperoxic conditions with no effect on arterial PO_2 . This rise in PCO_2 occurred even though the fish were hyperventilating in response to the chemical exposure (chapter 2). Hyperventilation, through exposure to moderate hypoxia eliminated any rise in arterial PCO_2 which occurred when fish were exposed to chloramine-T. Thus it was concluded that the irritating action of chloramine-T caused the secretion of branchial mucus which in turn impaired CO_2 excretion but did not hinder O_2 uptake. However, it was not known if during exposure to chloramine-T, blood flow through the gill was being reduced or if branchial vascular resistance was being reduced and resulting in a decreased functional surface area over which gas exchange could take place.

The aim of this study was therefore to examine the cardiovascular responses of rainbow trout during an acute exposure to a therapeutic concentration of chloramine-T. Parameters of particular interest were branchial and systemic vascular resistances, dorsal and ventral aortic pressures and cardiac output. It was hypothesised that if carbon dioxide excretion was being impaired

by diffusional processes alone, then there would be no effect of chloramine-T exposure on any of the cardiovascular parameters measured. Increased or decreased branchial vascular resistance would mean that the functional surface area of the gill was either increased or decreased, respectively.

MATERIALS AND METHODS

Fish

Adult rainbow trout (716.7 ± 34.8 g (mean \pm SE)) were purchased from Linwood Acres Trout Farm, Cambellcroft, Ontario and acclimated to laboratory conditions for at least 14 days prior to use. During the acclimation period, fish were maintained in dechlorinated city of Ottawa tap water under a natural photoperiod at 13-14°C and fed *ad libitum* on a commercial pelleted diet.

Surgical procedures

Fish were anaesthetised with an oxygenated dechlorinated city of Ottawa tap water with 100 mg.L⁻¹ tricaine methanesulphonate (TMS, Sindel Laboratories Ltd. Vancouver, BC.) solution buffered with sodium bicarbonate and the gills were constantly irrigated with the same solution throughout the following surgical

procedures.

A PE 160 (Clay Adams Inc.) catheter was implanted in to the buccal cavity to facilitate measurement of inspired water PO_2 . A catheter (PE 50) was implanted into the dorsal aorta according to Soivio et al. (1975). A small incision was made in the ventral surface anterior to the branchiostegal region, the pericardium was dissected and the ventricle and bulbus arteriosus exposed. A silicone catheter (I.D. 0.51 mm; O.D. 0.94 mm) was inserted into the bulbus using an internal trochar and attached to the bulbus wall using a small drop of cyanoacrylate adhesive (super glue; K. Olsen pers. comm.). The catheter was then connected to a length of polyethylene tubing (PE 60; Clay Adams). All catheters were filled with non-heparinised Cortland's saline (Wolf 1963) prior to insertion. A 3S or 4S ultrasonic flow probe (Transonics Systems Inc. Ithaca NY) was then attached non-occlusively around the bulbus and the incision closed with sutures. Finally, 1 cm² brass impedance electrodes were sutured to the trailing edges of the operculae. Fish were then allowed to recover in flowing freshwater overnight in 12.5L black acrylic respirometer boxes prior to experimentation. Following recovery, catheters were flushed with heparinised saline (25 IU.mL⁻¹ ammonium heparin: Sigma Chemical Company).

Dorsal aortic (DAP) and ventral aortic (VAP) pressures were measured from the saline filled dorsal aortic or bulbus catheters connected to UFI model 1050BP (UFI Morro Bay CA) pressure transducers calibrated against a water column. Blood flow through the ventral aorta was measured using the implanted ultrasonic flow probe connected to a Transonics T106 small animal blood flow meter. The flow probe was pre-calibrated in the factory and verified in the laboratory by pumping (using a peristaltic pump) a known flow rate of saline into the heart of a dead animal immersed in water. Inspired water PO_2 was measured continuously by drawing water by continuous siphon across a Radiometer E5046 oxygen electrode housed in a thermostated cuvette connected to a Cameron Instruments blood gas monitor (Cameron Instruments Co. Port Aransas, TX). The opercular impedance electrodes were connected to a customised impedance converter and preamplifier. The outputs from the pressure transducers, blood flow meter, and impedance converter were relayed to a data acquisition system (Biopac Systems Inc.) and data recorded using Acqknowledge 3.03™ software.

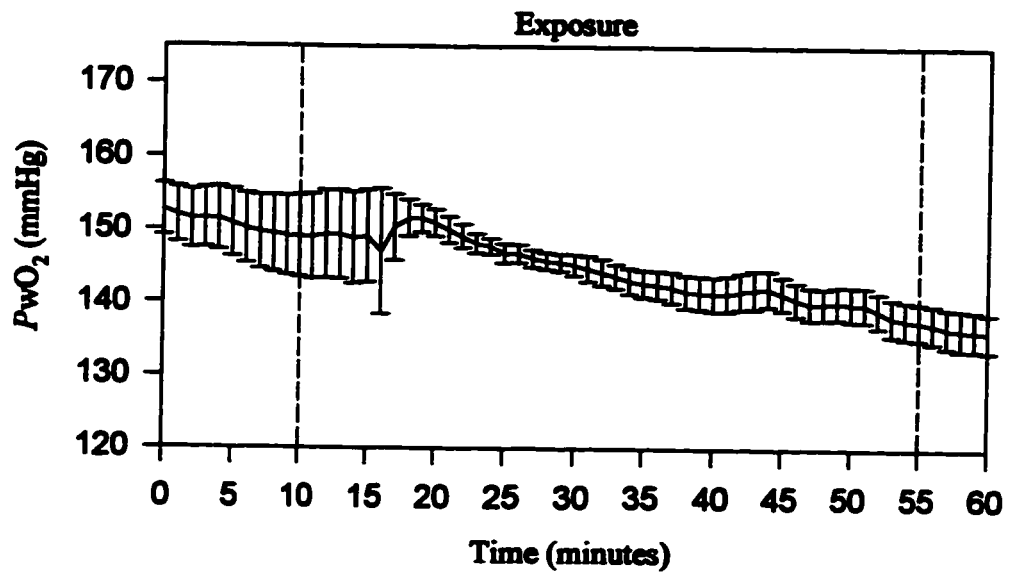
Experimental protocol

Arterial and venous blood samples (500 μ L) were anaerobically removed

via the dorsal aortic and bulbus catheters, respectively and replaced with twice the volume of saline to compensate for the blood volume loss. These were termed the pre-exposure samples. Haemoglobin concentration was determined on duplicate samples using a commercial spectrophotometric kit (Sigma Chemical Co.). Haematocrit was determined by drawing blood into duplicate microcapillary tubes and centrifuging at 10 000g for 10 min. Oxygen content of the blood was determined on duplicate 40 μL samples using an OxyconTM blood oxygen content analyser (Cameron Instrument Co.). The remaining blood was centrifuged at 10 000g for 30 s and duplicate 50 μL plasma samples analysed for total carbon dioxide content using a Corning 965 carbon dioxide analyser.

The water flow to the respirometer box was stopped and the boxes were aerated by means of an airstone to prevent the water in the respirometer from becoming hypoxic. Water PO_2 (PwO_2) levels did not fall below 135 mmHg (Fig. 4-1). Chloramine-T (9 $\text{mg}\cdot\text{L}^{-1}$ active ingredient, BDH Chemicals Ltd., Toronto Ontario: analytical grade lot No. 106295/15998) which had been pre-dissolved in 25 mL of the same water supplying the respirometer was then added to the box and the fish monitored for 45 minutes. The water flow was reinstated and a second 500 μL arterial and venous blood sample drawn from the dorsal

Figure 4-1. Water PO_2 (PwO_2) (mmHg) during a static exposure to chloramine-T (between vertical broken lines) (n = 11).



aortic and bulbus catheters and analysed as above. Controls consisted of fish which were not exposed to chloramine-T although 25 mL of dechlorinated tap water was added. A 45 min exposure was used because in previous studies (chapter 2) respiratory disturbances were observed within this time period.

Calculations and statistical analysis

Heart rate was determined automatically from the pulsatile ventral aortic pressure trace using the Acqknowledge™ software. Rates of oxygen uptake (MO_2) and carbon dioxide excretion (MCO_2) were calculated according to the formulae:

$$MO_2 \text{ (mmol.kg}^{-1} \cdot \text{h}^{-1}) = \frac{V_b(C_{aO_2} - C_{vO_2}) \cdot 60}{W \cdot 1000}$$

Where V_b is the measured blood flow at the time of blood sampling ($\text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$), C_{aO_2} is arterial oxygen content (mM), C_{vO_2} is venous oxygen content (mM), W is the weight of the fish (kg).

$$\text{MCO}_2 \text{ (mmol.kg}^{-1}.\text{h}^{-1}) = \frac{V_b(C_{a\text{CO}_2} - C_{v\text{CO}_2}) \cdot 60}{W \cdot 1000}$$

Where V_b is the measured blood flow at the time of blood sampling ($\text{ml.min}^{-1}.\text{kg}^{-1}$), $C_{a\text{CO}_2}$ is arterial carbon dioxide content (mmol), $C_{v\text{CO}_2}$ is venous carbon dioxide content (mmol), W is the weight of the fish (kg).

Branchial (R_g , $\text{cm H}_2\text{O.mL}^{-1}.\text{min}^{-1}$) and systemic (R_s , $\text{cm H}_2\text{O.mL}^{-1}.\text{min}^{-1}$) resistance were calculated from the ventral and dorsal aortic pressures and mass specific cardiac output traces in the Acqknowledge™ software according to the formulae:

$$R_g = \frac{\text{VAP-DAP}}{Q}$$

$$R_s = \text{DAP}/Q$$

Where VAP is the ventral aortic pressure ($\text{cm H}_2\text{O}$), DAP is the dorsal aortic pressure ($\text{cm H}_2\text{O}$), Q is mass specific cardiac output ($\text{mL.min}^{-1}.\text{kg}^{-1}$).

Data presented represent means \pm 1 standard error of the mean.

Differences between pre-exposure and post-exposure samples were compared using a paired *t*-test and comparisons between chloramine-T exposed and control fish tested using a two sample *t*-test (discrete data). Continuous data (cardiac

output, heart rate, VAP, DAP, R_g and R_g) were compared using a two way repeated measures analysis of variance and a Bonferoni corrected *t*-test to isolate differences. P values of less than 0.05 were considered to be significant.

RESULTS

There was no significant change in heart rate during exposure to chloramine-T although there was a high degree of variance in both the control and experimental (chloramine-T exposed) fish (Fig. 4-2a). Similarly, cardiac output was not changed significantly over the 45 minutes of exposure (Fig. 4-2b). Additionally, there was no significant effect of chloramine-T exposure on either dorsal (DAP) or ventral (VAP) aortic pressure (Fig. 4-3 a and b) or branchial or systemic resistance over the experimental period (Fig. 4-4 a and b). Ventilation frequency and amplitude also were not significantly altered over the experimental period during exposure to chloramine-T (Fig. 4-5a and b).

The cardiac output measured at the time of the removal of the post-exposure arterial and venous blood samples was significantly higher in the chloramine-T exposed fish compared with the cardiac output measured at the

Figure 4-2. Heart rate (beats.min⁻¹) (a) and cardiac output (mL.min⁻¹.kg⁻¹) (b) from trout during a static exposure to 9 mg.L⁻¹ chloramine-T (solid line) or controls (dotted line). Vertical broken lines represent the period in which the box was sealed and the chloramine-T was added.

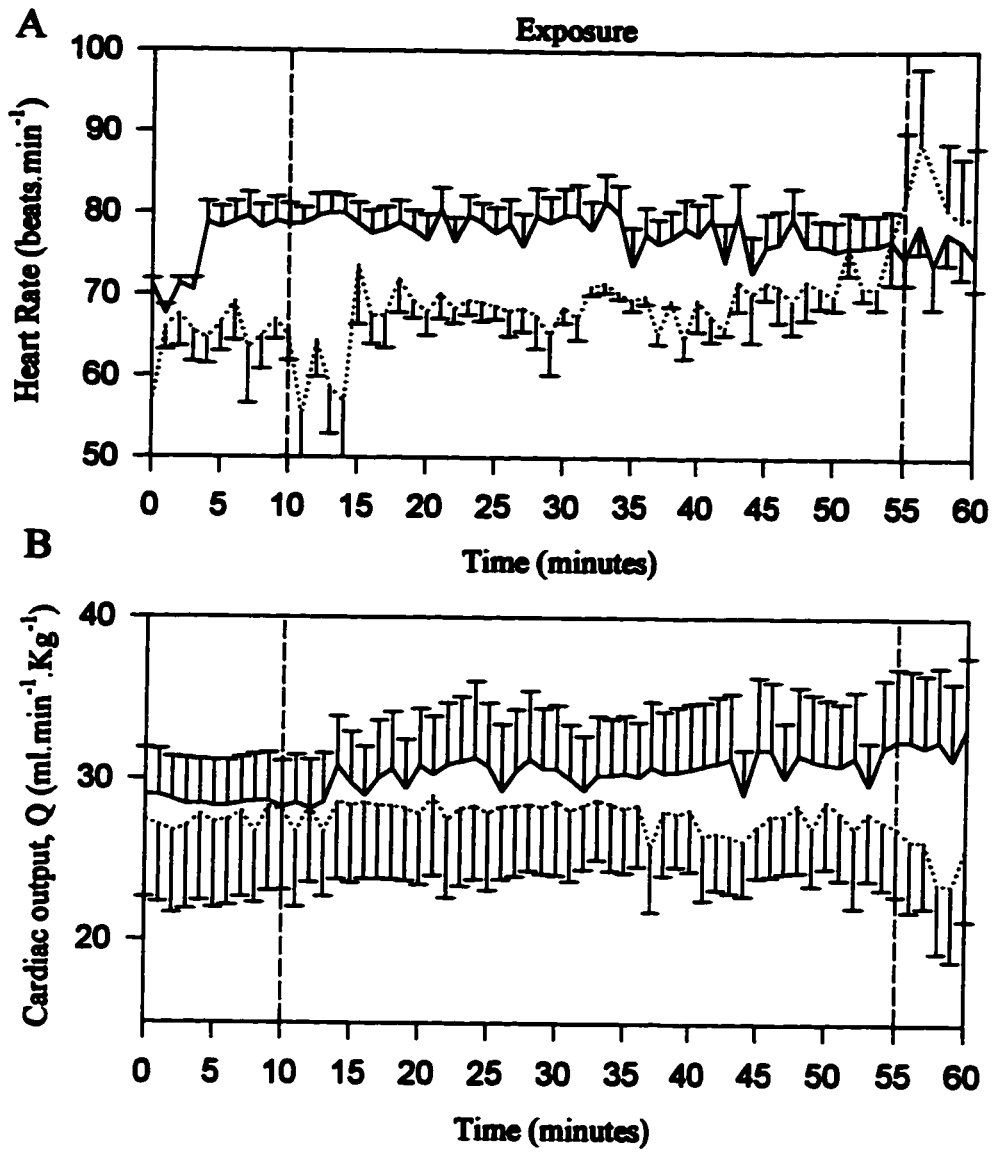


Figure 4-3. Mean dorsal (DAP) (a) and ventral (VAP) (b) aortic pressures (cm H₂O) from trout during a static exposure to 9 mg.L⁻¹ chloramine-T (solid line) or controls (dotted line). Vertical broken lines represent the period in which the box was sealed and the chloramine-T was added.

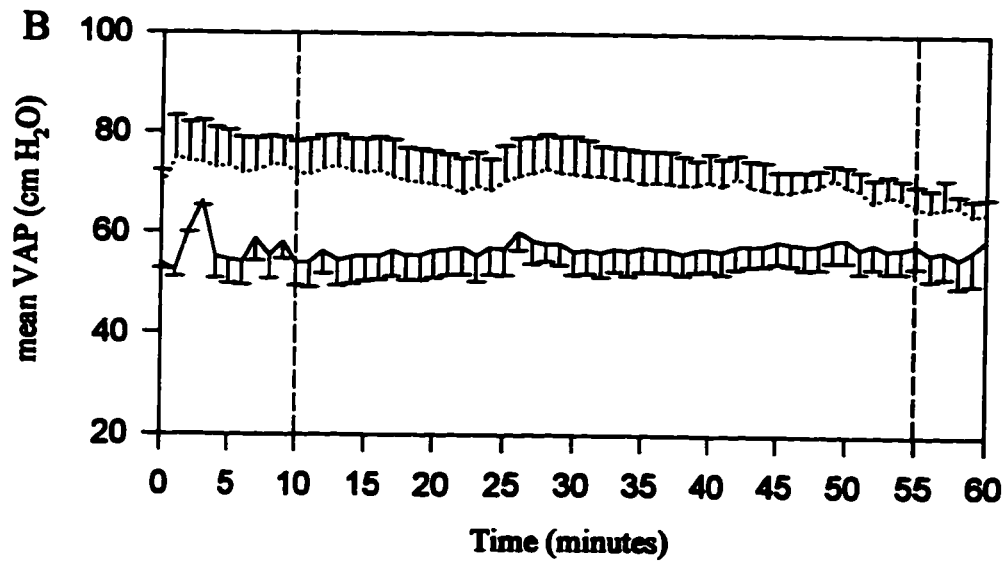
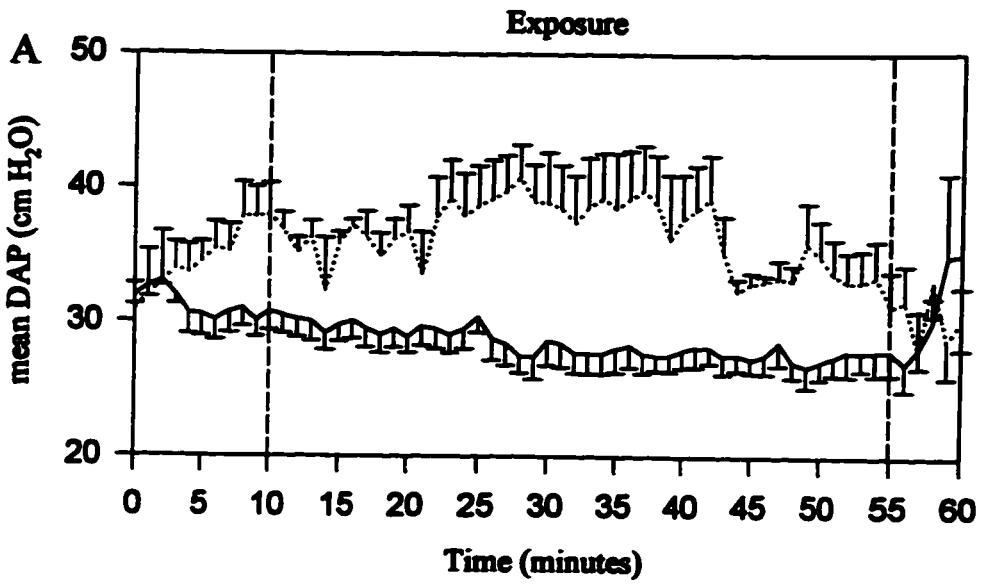


Figure 4-4. Mean branchial resistance, R_g (a) and mean systemic resistance, R_s (b) ($\text{cm H}_2\text{O}\cdot\text{mL}^{-1}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$) for trout exposed to $9 \text{ mg}\cdot\text{L}^{-1}$ chloramine-T (solid line) or controls (dotted line). Vertical broken lines represent the period in which the box was sealed and the chloramine-T was added.

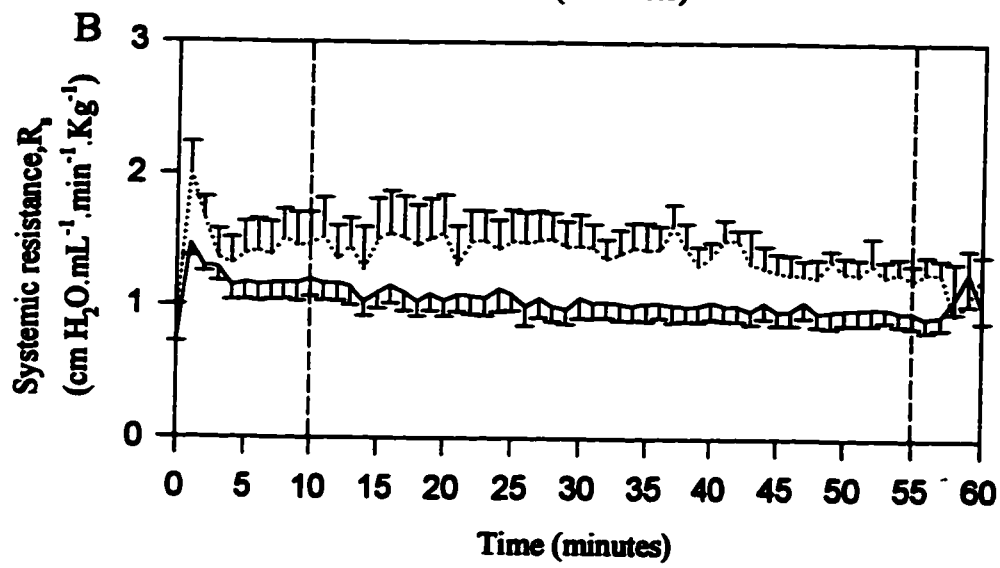
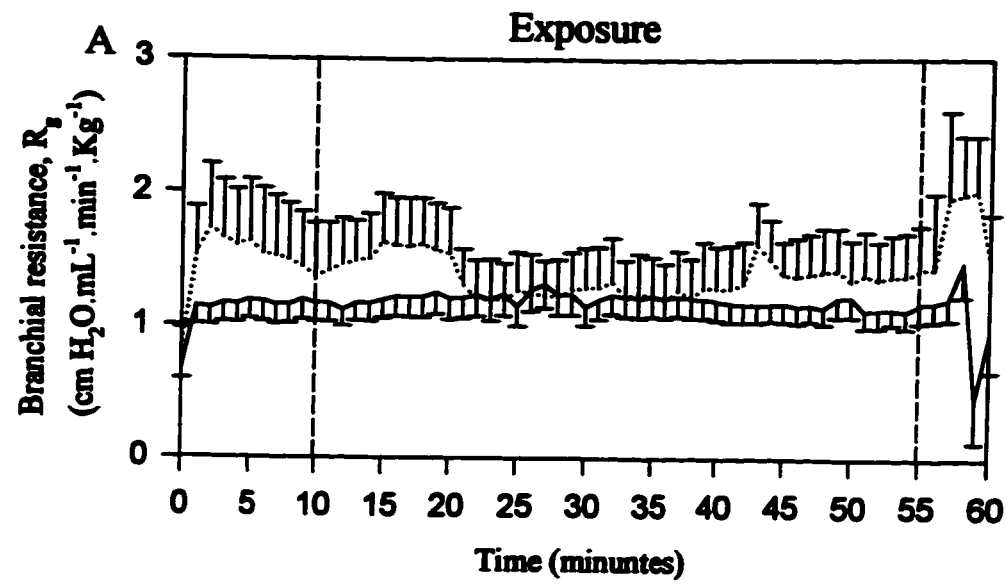


Figure 4-5. Mean ventilation frequency, V_f (ventilations.min⁻¹) (a) and mean ventilation amplitude, V_{amp} (cm opercular deflection) (b) for trout exposed to 9 mg.L⁻¹ chloramine-T (solid line) or controls (dotted line). Vertical broken lines represent the period in which the box was sealed and the chloramine-T was added.

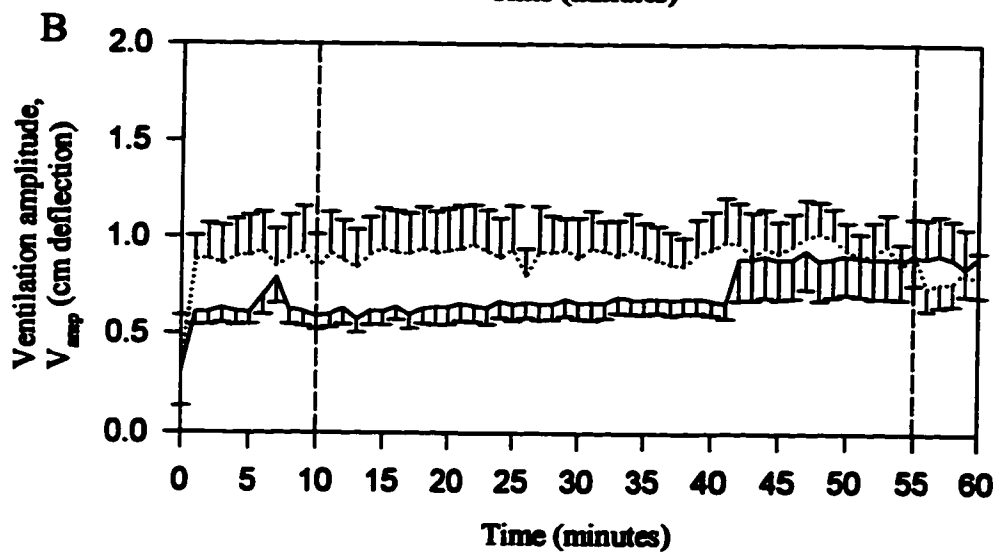
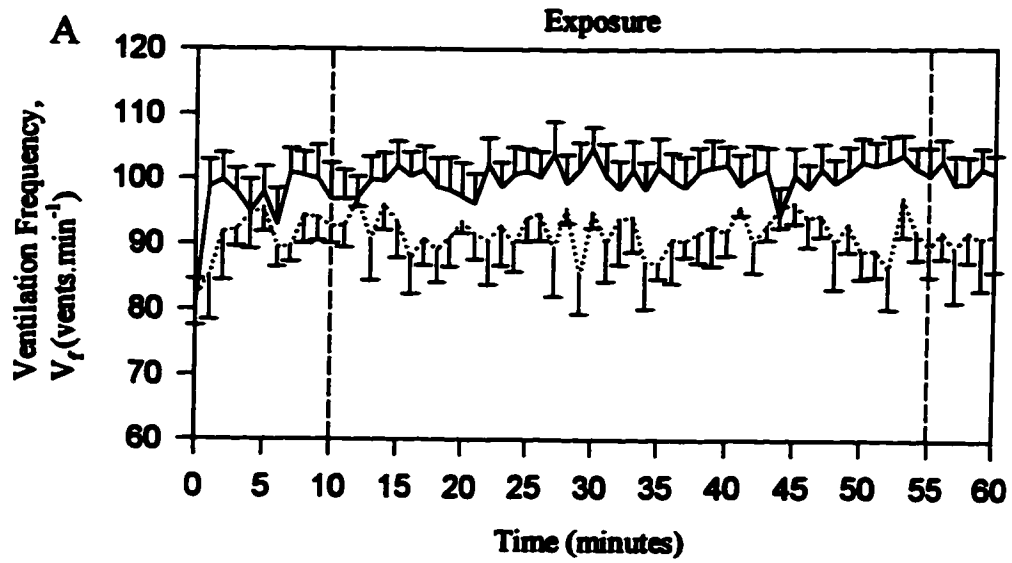


Table 4-1. Mean \pm SE values for cardiac output, Q (mL.min⁻¹.kg⁻¹); rate of oxygen uptake, MO₂ (mmol.Kg⁻¹.h); rate of carbon dioxide excretion, MCO₂ (mmol.kg⁻¹.h⁻¹); respiratory exchange ratio, R (MCO₂/MO₂); perfusion convection requirement for oxygen (mL.mmol⁻¹), Q/MO₂ and perfusion convection requirement for carbon dioxide, Q/MCO₂ (mL.mmol⁻¹) as determined from rainbow trout blood samples taken before and immediately following exposure to 9 mg.L⁻¹ chloramine-T.

	<u>Pre-exposure</u>		<u>Post-exposure</u>	
	<u>Control</u>	<u>Chloramine-T</u>	<u>Control</u>	<u>Chloramine-T</u>
Q	23.71 \pm 4.08	25.87 \pm 3.06	22.16 \pm 3.02	33.35 \pm 4.50*
MO ₂	2.83 \pm 0.48	3.24 \pm 0.38	2.88 \pm 0.46	4.17 \pm 0.37*~
MCO ₂	2.01 \pm 0.15	2.80 \pm 0.42	2.03 \pm 0.30	2.46 \pm 0.28
R	0.87 \pm 0.25	0.92 \pm 0.14	0.71 \pm 0.02	0.60 \pm 0.07
Q/MO ₂	12.03 \pm 5.52	8.61 \pm 1.23	8.83 \pm 1.89	8.14 \pm 0.88
Q/MCO ₂	11.75 \pm 1.88	10.31 \pm 1.40	12.20 \pm 2.49	15.38 \pm 2.52*

* represents a significant difference from the pre-exposure value

~ represents a significant difference from the control value

pre-exposure blood sampling (Table 4-1). However, there was no significant difference in cardiac output between the pre- and post-exposure control blood samplings (Table 4-1). Oxygen uptake rates were significantly elevated following exposure to chloramine-T (Table 4-1). There was no significant change in oxygen uptake rates in the control fish (Table 4-1). There were no significant differences in carbon dioxide excretion rates for either chloramine-T exposed or control fish (Table 4-1). Similarly, there was no significant difference in the respiratory exchange ratio between pre- and post-exposure measurements of oxygen uptake and carbon dioxide excretion rates for chloramine-T exposed or control fish (Table 4-1). The perfusion convection requirement for oxygen (Q/MO_2) was not significantly different between pre-and post exposure conditions for either chloramine-T exposed or control fish (Table 4-1). However, the perfusion convection requirement for carbon dioxide (Q/MCO_2) was significantly increased following exposure to chloramine-T compared with the pre-exposure condition (Table 4-1). There was no significant difference between the pre and post-exposure perfusion convection requirement for CO_2 in control fish (Table 4-1). Similarly, there were no significant differences between pre- and post-exposure samples for any of the blood parameters measured for either

Table 4-2. Mean \pm SE values for arterial and venous oxygen content (C_{aO_2} , C_{vO_2} ; mM), carbon dioxide content (C_{aCO_2} , C_{vCO_2} ; mM), haematocrit (Hct_a , Hct_v ; %), haemoglobin concentration ($[Hb]_a$, $[Hb]_v$; g.100 ml⁻¹), mean cellular haemoglobin concentration ($MCHC_a$, $MCHC_v$) and O_2 /haemoglobin ratio (O_2/Hb_a , O_2/Hb_v ; mol/mol) of blood sampled before and after exposure to 9 mg.L⁻¹ chloramine-T.

	<u>Pre-exposure</u>		<u>Post-exposure</u>	
	<u>Control</u>	<u>Chloramine-T</u>	<u>Control</u>	<u>Chloramine-T</u>
C_{aO_2}	4.64 \pm 0.53	3.44 \pm 0.43	4.77 \pm 0.63	3.20 \pm 0.35
C_{vO_2}	2.29 \pm 0.48	1.08 \pm 0.15~	2.51 \pm 0.66	0.91 \pm 0.20~
C_{aCO_2}	8.41 \pm 0.48	6.27 \pm 0.51~	7.99 \pm 0.57	7.40 \pm 0.49
C_{vCO_2}	9.95 \pm 0.63	8.15 \pm 0.60	9.57 \pm 0.66	8.74 \pm 0.60
Hct_a	29.62 \pm 1.69	20.94 \pm 1.96~	28.50 \pm 1.59	21.05 \pm 2.01~
Hct_v	29.87 \pm 1.74	20.89 \pm 2.15~	29.25 \pm 1.59	20.89 \pm 2.17~
$[Hb]_a$	9.53 \pm 0.89	7.38 \pm 0.96	9.38 \pm 0.50	7.09 \pm 0.92
$[Hb]_v$	10.20 \pm 0.74	7.44 \pm 0.92	9.45 \pm 0.35	6.79 \pm 0.91
$MCHC_a$	0.32 \pm 0.01	0.34 \pm 0.02	0.33 \pm 0.01	0.33 \pm 0.01
$MCHC_v$	0.34 \pm 0.01	0.35 \pm 0.02	0.32 \pm 0.01	0.32 \pm 0.02
O_2/Hb_a	3.10 \pm 0.14	3.05 \pm 0.12	3.18 \pm 0.33	2.63 \pm 0.29
O_2/Hb_v	1.41 \pm 0.29	0.93 \pm 0.07~	1.68 \pm 0.41	0.80 \pm 0.14~

~ represents a significant difference from the control value

~ represents a significant difference from the control value chloramine-T exposed or control fish (Table 4-2).

DISCUSSION

Exposure of fish to 9 mg.L⁻¹ chloramine-T did not significantly affect any of the cardiovascular parameters (Figs. 4-2 to 4-5). However, the point sample measurement of mass specific cardiac output was significantly elevated in fish which were exposed to chloramine-T. This apparent discrepancy may have been a result of these samples being taken outside of the period recorded with the data acquisition system. Nonetheless, there was a slight trend for increasing cardiac output during the exposure period even though significance was obscured by the interindividual variance (Fig. 4-2).

Ventilation frequency and amplitude were higher than previously measured (chapters 2 and 3) and probably resulted from the invasive nature of the surgery; ventilation frequency was not significantly increased during exposure to chloramine-T. This confirms the findings in chapter 3 where fish were exposed to chloramine-T under moderate hypoxia. In that case ventilation frequency was elevated due to the hypoxia but ventilation frequency was not

further increased as had been seen in chapter 2 by chloramine-T exposure.

Exposure to chloramine-T under hypoxic conditions did not significantly elevate arterial PCO_2 levels suggesting that CO_2 was probably not accumulating in the blood (i.e., CO_2 excretion rates were being maintained). The present study reaffirms these findings as there was no significant increase in either arterial or venous blood carbon dioxide content (Table 4-2) and CO_2 excretion rates were unaffected by chloramine-T exposure (Table 4-1).

Oxygen uptake rates, calculated from the point samples, were significantly increased over the period of exposure and were significantly higher in chloramine-T exposed fish compared to control fish. With no increases in ventral aortic pressure or gill resistance this increase in oxygen uptake could only have resulted through increased blood flow to the gill (cardiac output). The absence of any hypertension, in particular increases in VAP, during chloramine-T exposure reduces the likelihood of increased lamellar recruitment by perfusing more distal lamellae or by altering blood flow patterns within the gill (Booth 1978; 1979a,b). Carbon dioxide excretion does not, however, follow the same pattern as that of oxygen uptake. There was no significant increase in CO_2 excretion rates even though blood flow to the gill (cardiac output) was significantly elevated (Table 4-

1). As CO_2 is some 30 times more soluble in water than O_2 , if there was sufficient blood flow to allow an increase in O_2 uptake, then there should have been ample blood flow to facilitate CO_2 excretion if the two processes were governed by the same limiting factors. However, this was clearly not the case. Calculation of the perfusion convection requirement (blood flow per unit O_2 or CO_2 taken up or excreted, Q/MO_2 or Q/MCO_2 , respectively) showed that there was a significant increase in the perfusion convective requirement for CO_2 . This means that a greater blood flow than was achieved was required to excrete an equivalent amount of CO_2 after chloramine-T exposure as prior to exposure. Therefore the diffusive conductance of the gill must have been the major limiting factor to CO_2 excretion but not O_2 uptake. This supports the theory that O_2 uptake is primarily perfusion limited whereas CO_2 excretion is primarily diffusion limited (Cameron and Polhemus 1974; Daxboeck et al. 1982; Daxboeck and Randall 1984; Malte and Weber 1985).

In conclusion, chloramine-T results in only small increases in cardiac output which are sufficient to facilitate increased O_2 uptake. However, CO_2 excretion is maintained rather than increased as might be predicted from increased cardiac output. Since no significant changes in branchial vascular

resistance were measured it would appear that chloramine-T exposure does not result in any change in lamellar perfusion thereby changing the functional surface area for gas exchange. The reason for the apparent impediment of CO₂ excretion is probably due to the secretion of branchial mucus. This mucous covering reduces the diffusive conductance of the gill to CO₂ but does not impair O₂ uptake since it is not limited to the same extent by diffusion as is CO₂ excretion.

CHAPTER 5

RESPONSES TO A GRADED HYPOXIA FOLLOWING REPEATED

INTERMITTENT EXPOSURE TO CHLORAMINE-T

INTRODUCTION

Chloramine-T is used both therapeutically (to treat existing disease outbreaks) and prophylactically (as a disinfectant) for the treatment and management of bacterial gill diseases (BGD) (Thorburn and Moccia 1993). Under prophylactic conditions, fish which are not exhibiting clinical signs of BGD are exposed either occasionally or repeatedly on a regular weekly, twice weekly or biweekly basis. However, repeated exposure of rainbow trout to chloramine-T resulted in decreased growth rates (Powell et al. 1995; Sanchez pers. comm.) even though the concentrations of chloramine-T used were equivalent to those used therapeutically and well below the LC₅₀ for rainbow trout (Bills et al. 1988). As shown in chapters 2 and 3, the acute application of chloramine-T causes disturbances in CO₂ excretion across the gill, probably due to the secretion of branchial mucus which leads to disruption of the diffusive properties of the gill. In a previous study (Powell et al. 1994), fish which were intermittently exposed to chloramine-T for 1 h, twice per week on consecutive days exhibited dose dependent changes in chloride cell morphology and an apparent chloride cell hyperplasia as well as a general decrease in the number of branchial mucus cells (Powell et al. 1995). It was concluded that these

morphological changes probably resulted from repeated ionic or acid-base disturbances caused by chloramine-T insult (Powell et al. 1995). It is accepted that diffusive gas transfer processes across the gill are influenced by morphological variables including the thickness of the blood-to-water diffusion barrier as well as the functional lamellar surface area (Hughes and Morgan 1973; Randall and Daxboeck 1984). The morphological changes associated with repeated chloramine-T exposure as described by Powell et al. (1995) (chloride cell hyperplasia and possible thickening of the blood-to-water diffusion barrier) may have resulted in an impediment to gas exchange since hyperventilation and crowding at the water surface were reported during exposure to higher concentrations of chloramine-T (Powell et al. 1994).

The aim of the present study was to examine the effects of repeated exposure to chloramine-T on adult rainbow trout. It was hypothesised that the reduced growth rate reported by Powell et al. (1994) and Sanchez et al. (pers. comm.) may have arisen from changes in metabolic rate. Although chloramine-T causes acute respiratory and acid-base disturbances, it was hypothesised that repeat exposure to chloramine-T would result in morphological changes in the gill compensating for such disturbances. A graded hypoxia challenge was used

as a tool for examining the effects of morphological changes in the gill epithelium on gas exchange (Bindon et al. 1994; Greco et al. 1995).

MATERIALS AND METHODS

Fish and chloramine-T exposure

Adult rainbow trout (*O. mykiss* Walbaum) of mean weight 1077.7 ± 30.3 g (SE) were purchased from Linwood Acres Trout Farm, Campbellcroft, Ontario and acclimated to laboratory conditions for at least 1 month prior to experimentation. Throughout the acclimation period and subsequent periods of chloramine-T exposure, fish were held under natural photoperiod in flowing aerated and carbon-filtered, dechlorinated city of Ottawa tap water at 10°C. Residual chlorine levels were below detectable levels according to the DPD method (Franson 1978). Following acclimation the fish were divided into two groups and held in 300L square fibreglass tanks (12 fish per tank: 3.6 kg.m^{-3}) supplied with the same water as during the acclimation period. On alternate days the water supply to each tank was stopped and to one of the tanks 9 mg.L^{-1} chloramine-T (BDH Chemicals Inc. Mississauga Ont.) pre-dissolved in 25mL distilled water was added. To the other tank (control) 25 mL of distilled water

was added. Aeration was continued and the flow was reinstated after 1h of exposure. Throughout the exposure period free chlorine levels were periodically measured according to the DPD method (Franson 1978) and levels peaked at 0.2 mg.L⁻¹ which was equivalent to those measured by Bullock et al. (1991). Fish were fed to satiation on non-treatment days using a commercial pelleted diet. This treatment regime was repeated for 8 weeks prior to experimental use of the animals.

Surgical procedures

Fish were anaesthetised with tricaine methanesulphonate (MS-222) (100 mg.L⁻¹), and the gills were constantly irrigated with the same oxygenated anaesthetic throughout the following surgical procedures. Two polyethylene (PE50; Clay Adams Inc.) catheters were implanted in the dorsal aorta according to the method of Haman and Weber (1996). Brass 1 cm² electrodes were sutured to the trailing edge of the operculae (refer to chapter 2). The coeliac artery was cannulated according to the method of Thomas and LeRuz (1982) (refer to chapter 2). All catheters were flushed with Cortland's saline (Wolf 1963) prior to insertion. The animal was allowed to recover in a black acrylic respirometer box

with flowing fresh water which was first passed over a saturation column filled with marbles (to increase the surface area) into which air was bubbled. Following 24 h of recovery, the catheters were flushed with heparinised Cortland's saline (25 IU ammonium heparin; Sigma Chemical Company, St. Louis, Mo.) prior to experimentation.

Extracorporeal circulation

Continuous measurements of arterial blood PO_2 , PCO_2 and pH (PaO_2 , $PaCO_2$ and pHa , respectively) were made using the extracorporeal circulation as described in chapter 2. Opercular impedance was monitored by means of a customised impedance converter and amplifier. Analogue outputs from the acid-base analyser and impedance converter were transformed into digital outputs using an analogue digital interface (Data Translation Inc.) and relayed to a microcomputer where the output was recorded using a customised data acquisition software (AD DATA written by P. Thören, Göteborg, Sweden). Ventilation frequency was monitored visually by counting the number of opercular movements over a 30s period.

Graded hypoxia

The rate of oxygen uptake was determined by sealing the respirometer box and measuring the rate of O_2 consumption measured over a 15 minute period by siphoning water over a thermostatically controlled Radiometer E5046 oxygen electrode connected to a Radiometer PHM 72 mk2 acid-base analyser which was interfaced to the data acquisition system as described above. Water PO_2 (PwO_2) levels were not allowed to fall below 120 mmHg. Water in the box was circulated by means of a circulation pump which was maintained in an ice bath to prevent warming of the water in the respirometer. After 15 minutes the respirometer was opened and the water flow was reinstated. The fish was allowed to recover until ventilation and PaO_2 levels stabilised (approximately 30 min.). The fish were then subjected to a graded hypoxia by slowly mixing nitrogen gas with the air in a gas saturation column. This produced gradual hypoxic conditions with water PO_2 decreasing from 160 mmHg to 35 mmHg over a 20 min period. Blood samples (500 μ L) were taken from the dorsal aortic catheter at PwO_2 of 160, 100, 60 and 40 mmHg. The blood was centrifuged at 10 000g and the plasma decanted and frozen in liquid nitrogen and stored at -80°C for determination of plasma catecholamines (adrenaline and

noradrenaline). Catecholamines were later extracted from the thawed plasma samples according to the method of Woodward (1982) and analysed using high performance liquid chromatography (Varian 9000 series system with an EG & G model 400 electrochemical detector) using DHBA (3,4 dihydroxybenzylamine: Sigma Chemical Co.) as an internal standard. Following hypoxia the fish was returned to normoxic conditions (P_{wO_2} : 160 mmHg), killed with a blow to the head and the gill filaments from the second left gill arch removed and fixed in 2.5% glutaraldehyde buffered in 0.1M sodium cacodylate. A control and chloramine-T exposed animal were always tested on the same day in alternating order.

Histology and morphometry

Gill filaments from each fish were processed for light microscopy and embedding in either paraffin wax or Araldite/Epon resin. Tissue which was to be embedded in paraffin was first dehydrated in a graded series of ethanol, transferred to xylene then infiltrated and embedded in paraffin wax. Tissue which was to be embedded in resin was post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 1 h then dehydrated through a series of

graded ethanols, transferred to propylene oxide then infiltrated and embedded in Araldite/Epon resin.

Paraffin embedded tissue was sectioned at 6 μm and stained with periodic acid-Schiff (PAS) (Hinton 1990) to stain mucous cells. The number of mucous cells were determined by counting the number of PAS positive cells on individual randomly selected lamellar units defined as, the epithelium extending from the mid-point between adjacent lamellae, along the lamellar surface to the mid-point between the next adjacent lamella (Powell et al. 1995). Selection of the lamellae was made using a table of random numbers and rejecting each lamella up to that number determined from the random number table.

Resin embedded tissue was sectioned at 0.5 μm with a Reichert-Jung Ultracut E ultramicrotome and stained with toluidine blue in a 1% borax solution. Sections were then analysed on a Zeiss III compound microscope fitted with a Javelin CCD video camera. Images of each section (final magnification 2600x) were then digitally analysed using a Bioquant Hipad digitizer tablet and Bioquant Inc. image analysis software (R & M Biometrics Inc. Nashville, TN) with measurements being made using a Merz grid superimposed over the video monitor to ensure random orientation of the measurements on each section

(Weibel 1979). The parameters measured were: the thickness of the lamellar epithelium (blood-to-water diffusion path), the total width of the lamella and the interlamellar distance (distance between adjacent lamellae) (Hughes and Perry 1976). A total of 5 measurements for each parameter were made on 5 randomly selected non-contiguous fields on each of two independent sections from each fish. All cell counts and measurements were made using a randomised blind method in which the counter did not know whether tissues sections were from chloramine-T treated (experimental) or untreated (control) animals.

Statistical analysis

Comparisons were made between chloramine-T exposed and control fish using a *t*-test (discrete data) or a repeated measures two way analysis of variance with differences isolated using a Bonferoni corrected *t*-test (continuous data).

The fiducial limit was set at 0.05. Data are represented as means \pm 1 standard error of the mean (SEM).

RESULTS

There was no apparent difference in appetite between the treated and

experimental groups of animals during the exposure period; both groups appeared to eat the same amount nor were there any mortalities or disease which could be attributed to treatment. There was no difference in the rate of oxygen consumption between chloramine-T exposed and control fish (Fig. 5-1).

Responses to a graded hypoxia

Graded hypoxia caused significant reductions in arterial blood oxygen tensions (PaO_2) in both experiment (chloramine-T treated) and control (untreated) fish (Fig. 5-2). Chloramine-T exposed fish had a significantly higher PaO_2 compared with controls at PwO_2 of 80-70 mmHg (Fig. 5-2).

At PwO_2 of 40 mmHg the PaO_2 of chloramine-T-exposed fish was the same as that for controls (Fig. 5-2). There was no significant reduction in the arterial PCO_2 ($PaCO_2$) in either group of fish (chloramine-T treated or controls) during hypoxia nor were there any significant differences between treatments (Fig. 5-3).

Similarly there were no significant changes in arterial blood pH during exposure to the graded hypoxia nor were there any significant differences between treatments (Fig. 5-4). Plasma adrenaline levels were significantly elevated in both control and chloramine-T exposed fish at PwO_2 of 40 mmHg but there were

Figure 5-1. Mean oxygen uptake rate for rainbow trout following 8 weeks of intermittent exposure to chloramine-T (solid bar) compared with control (unexposed) trout (open bar).

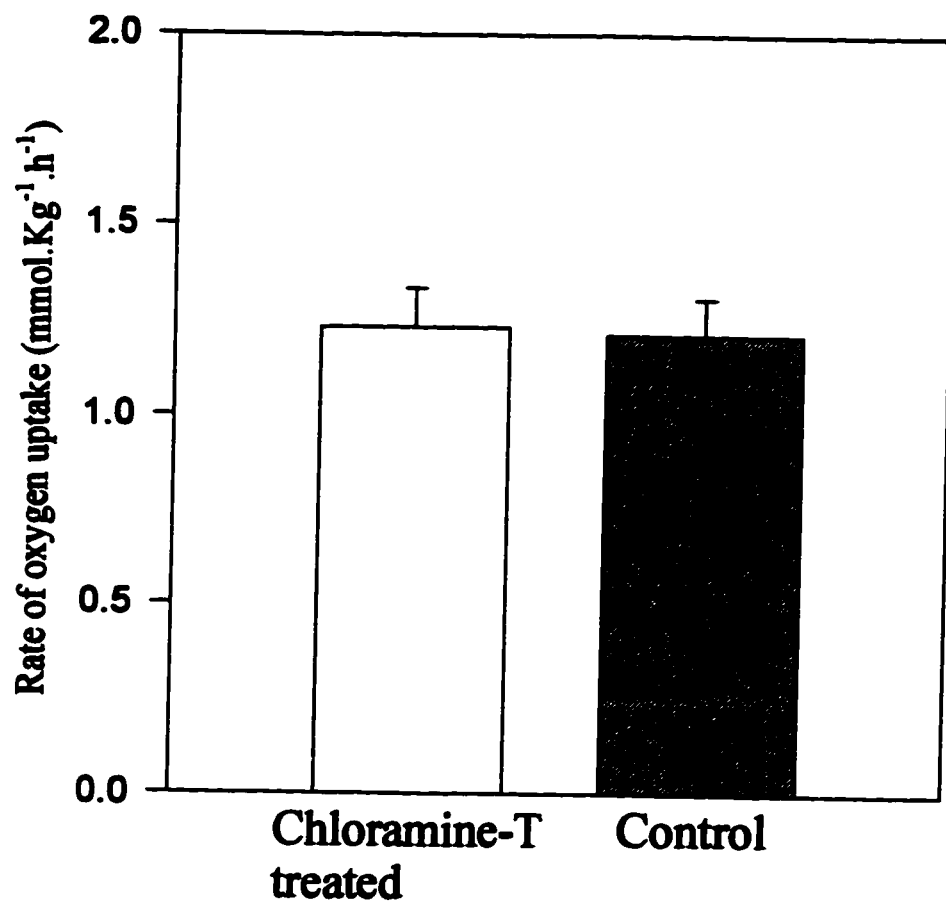


Figure 5-2. Arterial PO_2 (PaO_2) at a given water PO_2 (PwO_2) for rainbow trout following 8 weeks of intermittent chloramine-T exposure (open circles) and control (unexposed) fish (solid circles) during a graded hypoxia challenge.

***Represent significant difference between chloramine-T exposed and control fish**

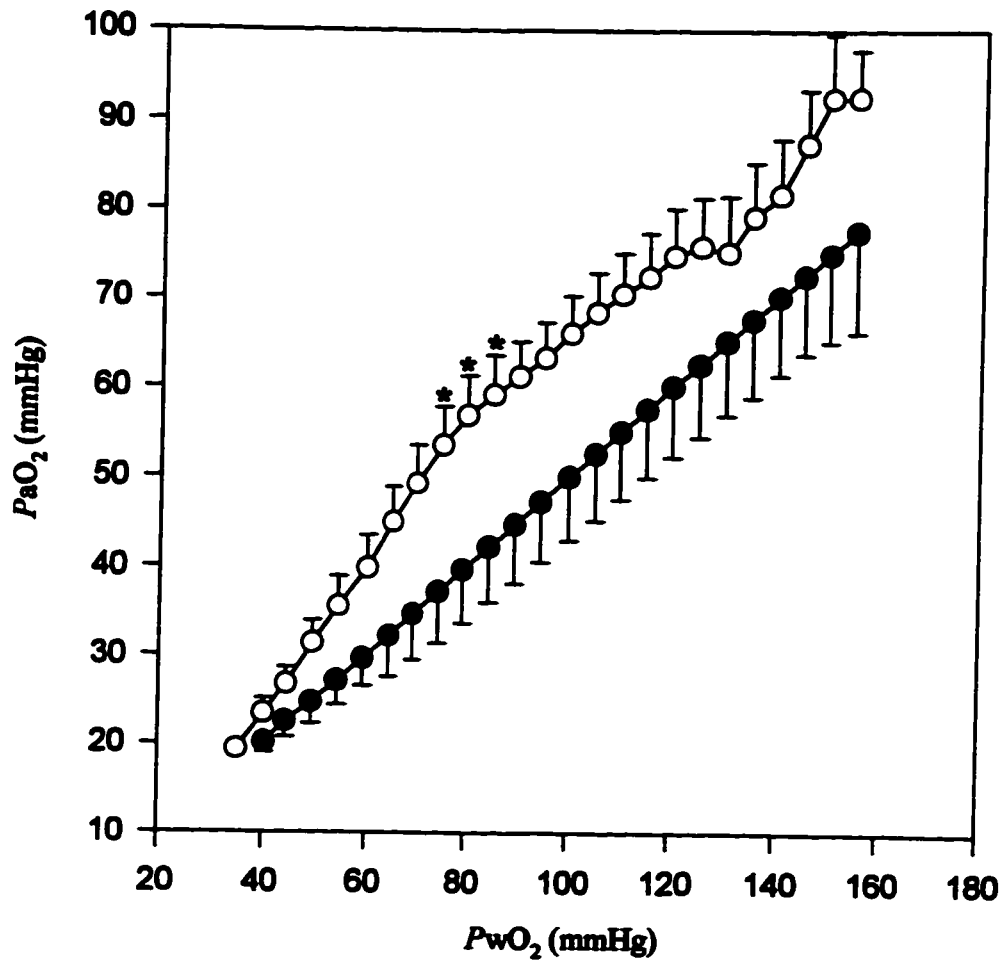


Figure 5-3. Arterial PCO_2 ($PaCO_2$) at a given water PO_2 (PwO_2) for rainbow trout following 8 weeks of intermittent chloramine-T exposure (open circles) and control (unexposed) fish (solid circles) during a graded hypoxia challenge.

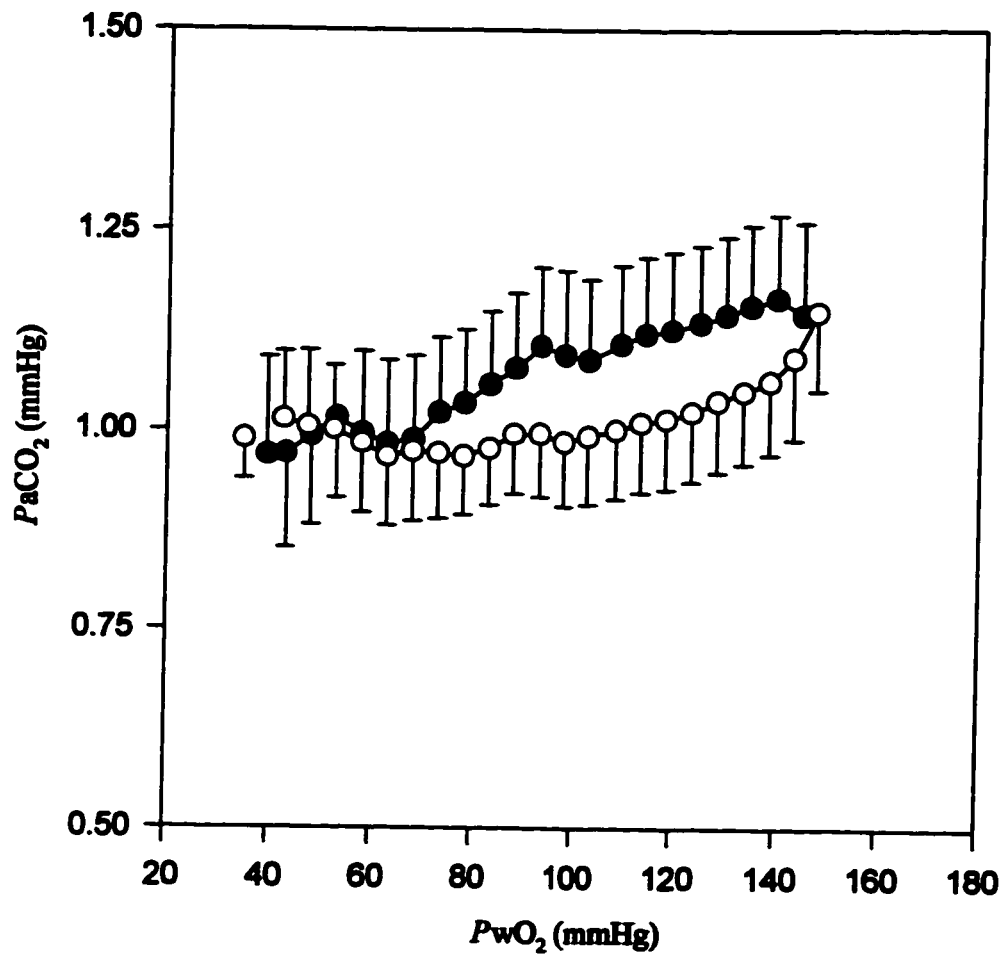


Figure 5-4. Arterial blood pH over a range of water PO_2 (PwO_2) during a graded hypoxia challenge of rainbow trout following an 8 week intermittent exposure to chloramine-T (open circles) or unexposed controls (solid circles).

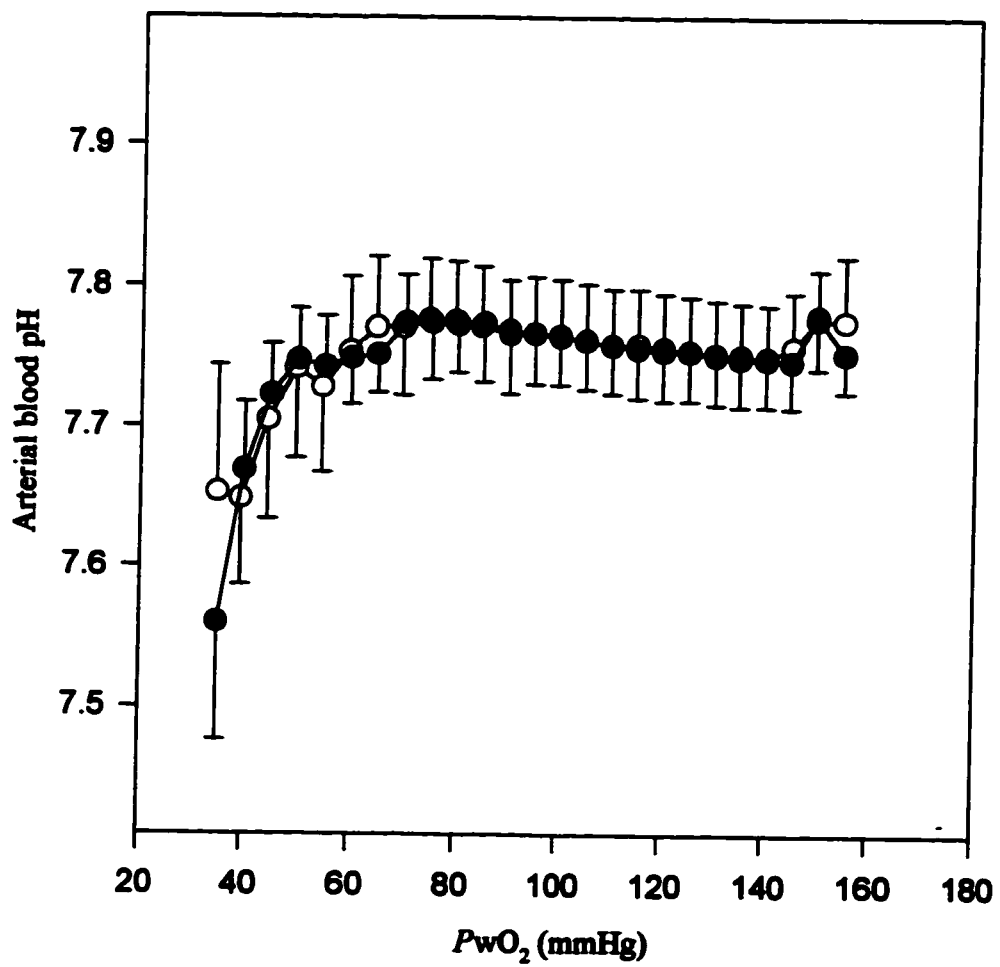
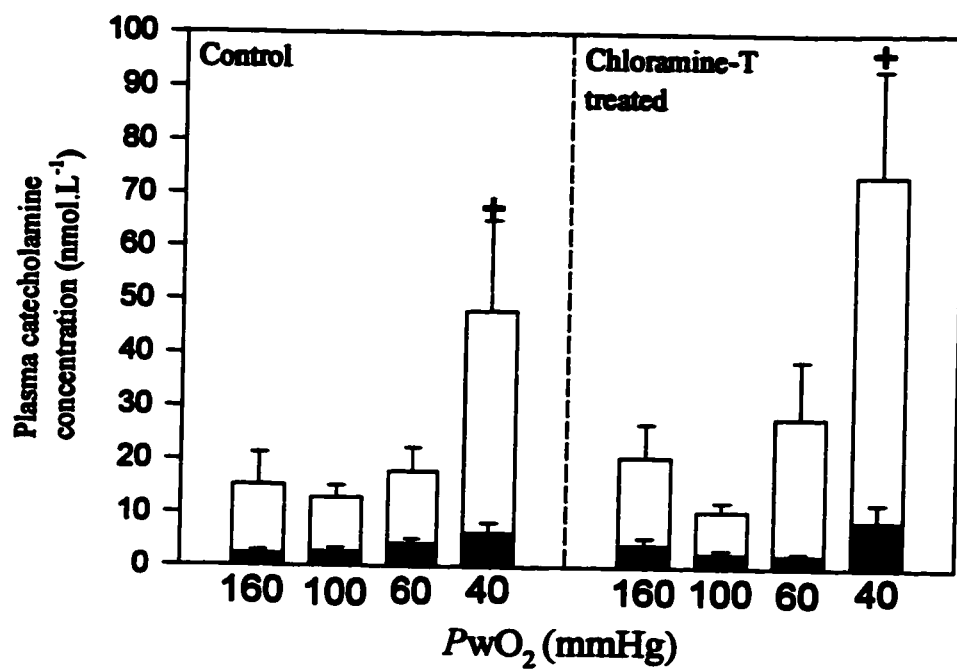


Figure 5-5. Plasma adrenaline (open bars) and noradrenaline (solid bars) levels in control and chloramine-T exposed (experimental) fish at different levels of hypoxia. +Represents values significantly different from the start of the graded hypoxia (normoxic condition: PwO_2 160 mmHg).



no significant differences between treatments (Fig. 5-5). Noradrenaline levels were not significantly elevated during the graded hypoxia in either group of fish (Fig. 5-5). Exposure to a graded hypoxia challenge did result in significant increases in the ventilation amplitude (V_{amp}) in both chloramine-T treated and control fish (Fig. 5-6). However, there were no significant differences in V_{amp} between chloramine-T exposed and control fish (Fig. 5-6). Ventilation frequency (V_f) on the other hand was significantly higher in chloramine-T treated fish at the beginning of the graded hypoxia challenge compared with controls (Fig. 5-7). As PwO_2 decreased, control fish significantly increased their breathing frequency whereas chloramine-T exposed animals did not (Fig. 5-7). There was no significant difference in V_f between chloramine-T exposed and control fish over the remaining duration of the graded hypoxia (Fig. 5-7).

Gill morphometry

There were significantly more PAS positive staining mucous cells on the gill lamellae of chloramine-T exposed fish compared with controls (Fig. 5-8). Morphometric analysis of the gill epithelium showed that chloramine-T exposed fish had a significantly reduced blood-to-water diffusion barrier (epithelial

Figure 5-6. Ventilation amplitude (V_{amp}) over a range of water PO_2 (PwO_2) during a graded hypoxia challenge of rainbow trout following an 8 week intermittent exposure to chloramine-T (open circles) or unexposed controls (solid circles). +Represent values significantly different from the start of the graded hypoxia.

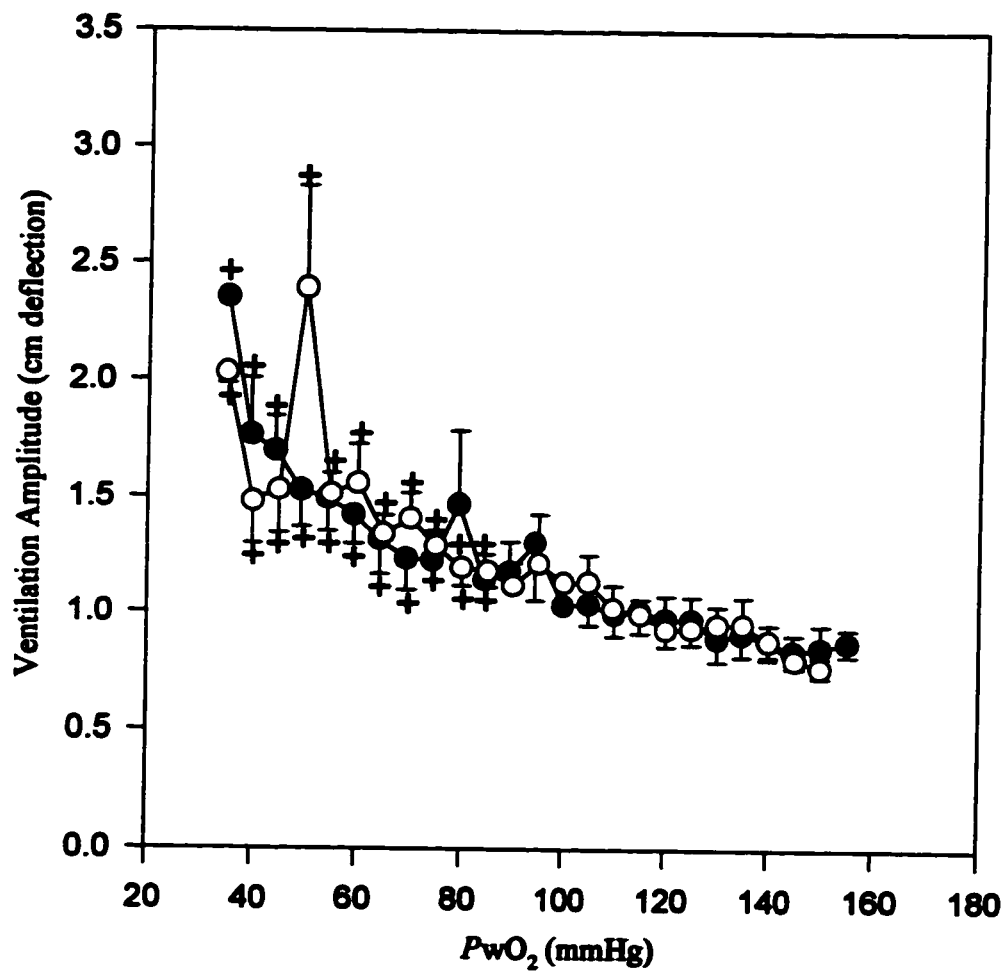


Figure 5-7. Ventilation frequencies (V_f) at different water PO_2 (PwO_2) for rainbow trout following 8 weeks of intermittent exposure to chloramine-T (solid bars) or unexposed controls (open bars). * Represents significant difference between chloramine-T exposed and control fish; + represents significantly different from the start of the graded hypoxia (normoxic conditions: PwO_2 160 mmHg).

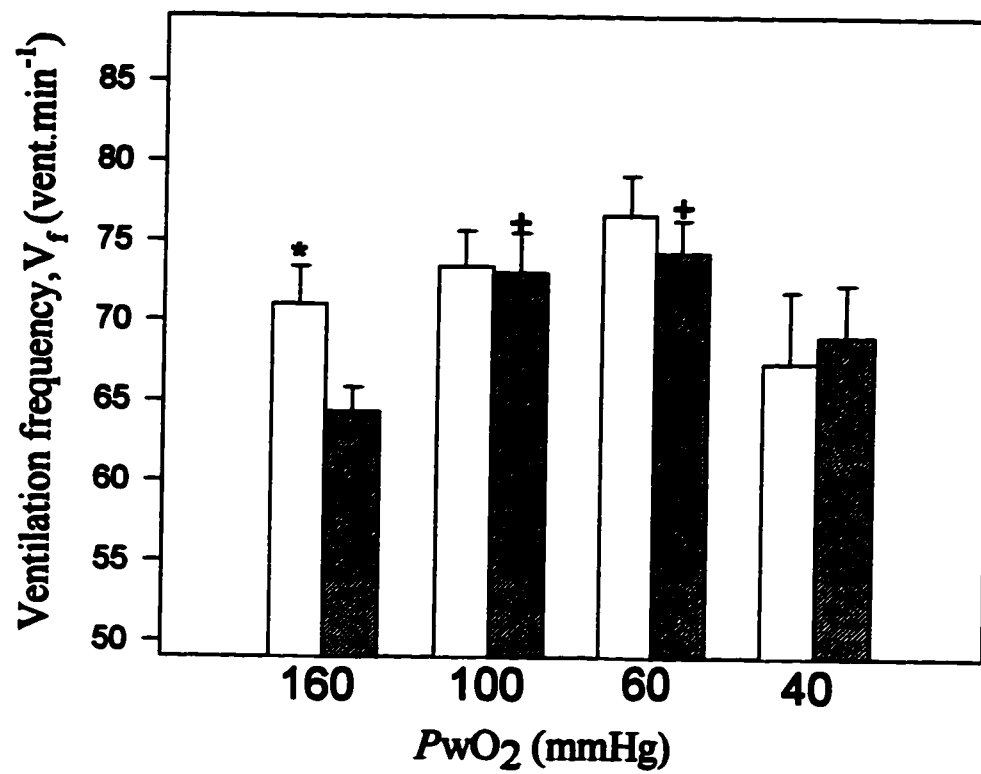


Figure 5-8. Mean number of PAS positive mucous cells on the lamellae of rainbow trout following 8 weeks of intermittent exposure to chloramine-T (open bar) or unexposed controls (solid bar). * Represents a significant difference between chloramine-T exposed and control fish.

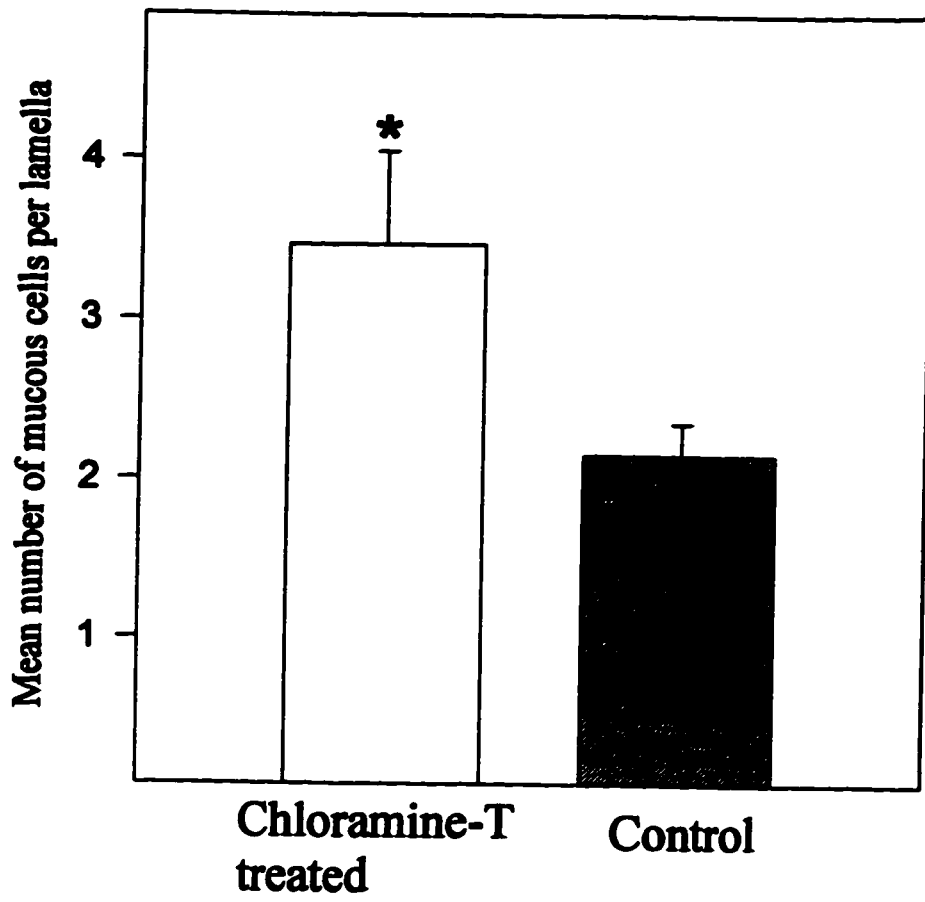
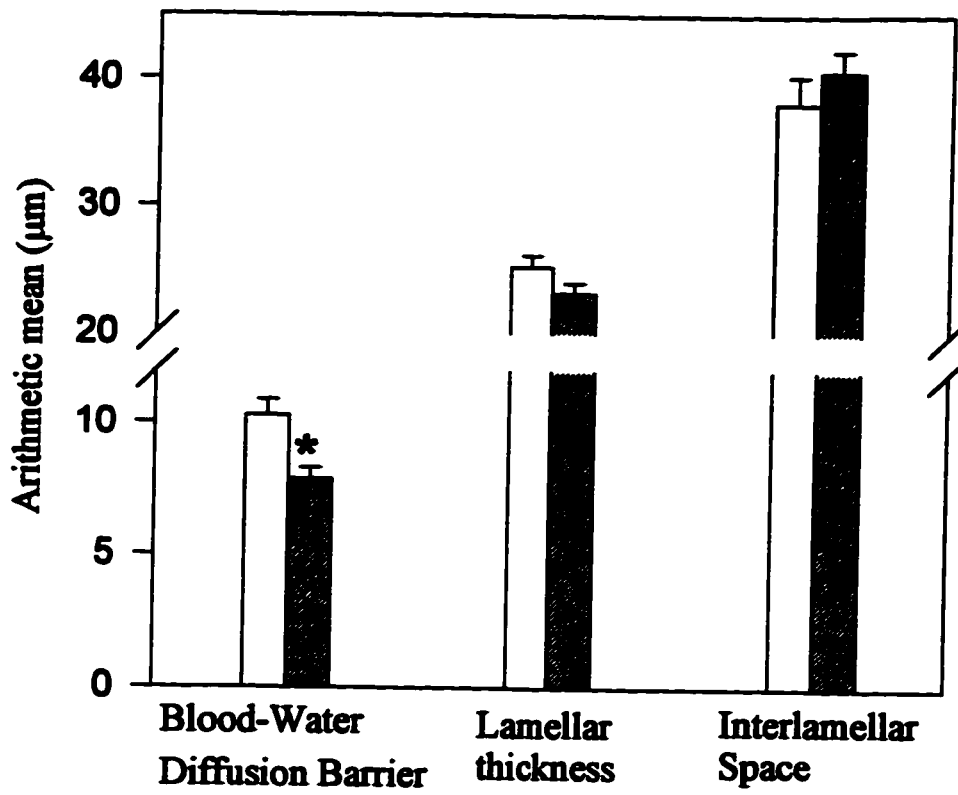


Figure 5-9. Morphometric measurements (arithmetic mean) of some physical dimensions of the gill lamellae of rainbow trout following 8 weeks of intermittent exposure to chloramine-T (solid bar) or unexposed controls (open bar).

*Represents a significant difference between chloramine-T exposed and control fish.



thickness) (Fig. 5-9). However, other morphometric indices measured, i.e., lamellar thickness and interlamellar distance were not significantly different between chloramine-T exposed and control fish (Fig. 5-9).

DISCUSSION

Repeated exposure to chloramine-T did not affect metabolic rate as determined by measuring oxygen uptake rate. The reduced growth rates described by Powell et al. (1994) and Sanchez et al. (pers. comm.) cannot, according to this study, be explained by a change in metabolic rate (oxygen uptake rates). The observation that chloramine-T exposed fish appeared to consume as much food as the controls concurs with the Sanchez et al. (pers. comm.) study and a similar previous observation (Powell et al. 1994) although Sanchez et al. (pers. comm.) noted a reduced feed conversion efficiency.

During the graded hypoxia challenge P_{aO_2} was higher in chloramine-T exposed fish compared with controls at P_{wO_2} of 70-80 mmHg. This was not due to differences in V_{amp} or V_f at these points. These two variables are the principle components of ventilation volume assuming that the size of the oral or branchial cavities remain constant. Interestingly, chloramine-T exposed fish did

not significantly increase their V_f whereas control fish did. The sporadically elevated PaO_2 in chloramine-T exposed fish can perhaps be explained in terms of diffusion. The epithelial diffusion barrier (blood to water diffusion distance) was significantly reduced in the chloramine-T exposed fish although there were significantly more mucous cells on the gills. It has been suggested that a thicker mucous coat may impair O_2 diffusion (Ultsch and Gros 1979). However, in the present study it would appear that the epithelium was the primary factor for determining the rate of O_2 diffusion across the gill down to a PwO_2 of 70 mmHg at which point the increased mucous coat provided increased resistance to O_2 diffusion and hence the steep decline in PaO_2 to control levels at PwO_2 of less than 70 mmHg. There was no significant difference in $PaCO_2$ between chloramine-T exposed fish and controls suggesting that although there was a reduction in the epithelial diffusion distance which would have facilitated CO_2 excretion, the increased mucous covering may have been sufficient to impede CO_2 excretion. This was reflected by the fact that arterial pH also did not change significantly over the course of the graded hypoxia. However at PwO_2 of less than 60 mmHg there was a trend (albeit not statistically significant due to the high variance between individual animals) for decreased blood pH which

probably arose, in part, as a consequence of a lactic acidosis due to an increase in anaerobic metabolism under hypoxia and the release of endogenous catecholamines (adrenaline and noradrenaline). Catecholamine release would have resulted in activation of the erythrocyte Na^+/H^+ exchanger and alkalization of the erythrocyte pH in an attempt to maximise O_2 transport (Perry and Wood 1989).

It has been suggested that the uptake of O_2 is perfusion limited (Daxboeck et al. 1982; Randall and Daxboeck 1984; Malte and Weber 1985) whereas CO_2 excretion is dependent on diffusive parameters (Cameron and Polhemus 1974; Malte and Weber 1985). In the present study the rate of oxygen consumption was the same between chloramine-T exposed and control fish yet the diffusion component of the gill (the blood to water barrier) was reduced which would provide reduced diffusive resistance. However, there was probably an increased mucous covering of the gill which has been suggested to impair O_2 diffusion (Ultsch and Gros 1979). Therefore O_2 uptake could only be maintained with increased water flow across the gill due to an elevated ventilation rate which meant that a sufficient O_2 partial pressure gradient for diffusion could be maintained. However, although there was decreased blood to water epithelial

diffusion barrier, CO₂ excretion (albeit not measured directly in this study) was probably maintained (as seen by *PaCO*₂) but not increased. This contradicts the prediction that increased ventilatory flow should lower *PaCO*₂ resulting in a respiratory alkalosis (Iwama et al. 1987). The reason for this was probably the increased amount of branchial mucus on the gill (as suggested by increased number of mucous cells). This would have effectively increased the gill-water boundary layer so impeding CO₂ excretion as discussed in chapter 3.

The morphological changes reported in the present study do not concur with previous observations on the effects of chloramine-T on rainbow trout gill epithelia: chloride cell hyperplasia, reduction in the number of lamellar mucous cells (Powell et al. 1995). However, these studies are not directly comparable for several reasons. First, the size of the fish and conditions under which they were held were different in the two studies; i.e., the present study used adult fish which were exposed on alternate days for at least 8 weeks whereas the previous study used juvenile fish which were treated only on two consecutive days per week for 4 weeks. Second, in the previous study, fish were sampled within 2 h of the final treatment. In the present study, surgical procedures meant that fish were tested and the gills sampled at least 24 h after an exposure. Third, the fish used

in the previous study had experienced a caudal necrosis during the exposure trial (Powell et al. 1994). Although unlikely, this may have confounded the results since these fish also showed reduced plasma Na^+ and Cl^- concentrations indicating ionic stress and hence perhaps an explanation for the chloride cell hyperplasia (Powell et al. 1994; 1995). In the present study there was no evidence of dermal erosion or reduced fin condition. It is also possible that fish of different sizes may respond differently to chloramine-T. Smaller fish are generally considered to have a higher mass specific metabolic rate (Cech 1990). It is possible therefore that smaller fish (like those used in the study by Powell et al. 1994; 1995) may have responded to ionic and/or acid-base disturbances by increasing the number of ionic uptake sites (chloride cell hyperplasia and increased chloride cell apical plasmalemma) at the expense of the mucous coat which may impede ionic uptake by trapping ions at the gill surface (Shephard 1989). Larger fish such as those used in the present study, however, protect the gills from branchial irritation by increasing the mucous coat on the gill and hence the mucous cell hyperplasia. To increase the number of chloride cells would be metabolically expensive. Although we report no increase in oxygen uptake rate in chloramine-T exposed fish, indicative of no difference in metabolic rate in the

present study, it is possible that the reduced growth rate exhibited by the fish in the previous study (Powell et al. 1994) may have arisen partially due to increased gill metabolism.

In conclusion, we have shown that repeated intermittent chloramine-T exposure does not appear to affect oxygen uptake rates in adult rainbow trout. Also, repeated chemical insult to the gill by chloramine-T resulted in a reduction in the blood to water diffusion barrier and a mucous cell hyperplasia. However, in spite of the mucous cell hyperplasia (and presumably increased mucous covering the gill), fish exposed to chloramine-T showed an increased P_{aO_2} level at 3 points compared with controls when challenged with a graded hypoxia. The reason for this is that at P_{wO_2} greater than 70-80 mmHg, the gills may not be ventilated at a maximal rate, but at P_{wO_2} of less than 70-80 mmHg, the transfer of O_2 is becoming diffusion limited as the driving force for diffusion (the partial pressure gradient) is reduced. Although the epithelial diffusion barrier (blood-to-water diffusion distance) was reduced, increased branchial mucus was further impeding oxygen uptake. However, P_{aCO_2} levels were not significantly reduced despite increased ventilatory frequency. This suggests that the reduction in the epithelial diffusion barrier may compensate for possible diffusive impediment of

gas transfer caused by increased branchial mucus.

CHAPTER 6
METABOLIC, ACID-BASE, AND IONIC FLUXES DURING
EXPOSURE TO CHLORAMINE-T

INTRODUCTION

Previous investigations into the potential use of chloramine-T as a safe prophylactic treatment have demonstrated that intermittent exposure of rainbow trout resulted in dose dependent reductions in plasma sodium and chloride levels (Powell et al. 1994). In the same fish there was an apparent hyperplasia of branchial chloride cells and an increase in the chloride cell apical plasma membrane (Powell et al. 1995). These morphological changes were consistent with other work where changes in chloride cell fractional surface area of the gill were correlated with change in ionic uptake (Goss and Perry 1993). In chapter 2 it was shown that acute exposure of rainbow trout to therapeutic concentrations of chloramine-T (9 mg.L^{-1}) caused respiratory and metabolic disturbances in the acid-base status of the fish. Specifically, chloramine-T exposure induced a metabolic alkalosis whereas exposure to the breakdown product *p*TSA caused a metabolic acidosis.

The aim of this investigation was to further examine the metabolic component of the acid-base disturbances caused by chloramine-T and its breakdown products (hypochlorite and *p*TSA). In addition, net and unidirectional whole body branchial and renal ion fluxes were examined as an

attempt to ascertain the mechanism (renal or branchial) by which these disturbances occurred.

MATERIALS AND METHODS

Fish

Rainbow trout (*O. mykiss*) were purchased from a commercial hatchery (Linwood Acres Trout Farm, Campbellcroft, Ontario) and acclimated to laboratory conditions for at least 3 weeks prior to use. During the acclimation period fish were held in 300 L rectangular fibreglass tanks and maintained at 10°C in aerated dechlorinated city of Ottawa water (Na^+ $118.9 \pm 2.4 \mu\text{M}$; K^+ $19.3 \pm 0.5 \mu\text{M}$; Ca^{2+} $365.8 \pm 8.2 \mu\text{M}$; Cl^- $151.8 \pm 0.9 \mu\text{M}$; pH 6.8). Residual chlorine levels were below those detectable using a N,N-ethyl-p-phenylenediamine ferrous titrimetric method (Franson 1978). Fish were fed on alternate days using a commercial pelleted diet. Food was withheld 24 h prior to experimental use.

Blood acid-base status and renal ionic fluxes

In order to examine the potential contribution of renal ionic effluxes to the whole body ion fluxes, fish were fitted with bladder and dorsal aortic catheters and exposed to 18 mg.L⁻¹ Chloramine-T.

Surgical procedures

Rainbow trout of mean weight (\pm SE) 251.4 \pm 14.6 g (n = 6) were anaesthetised with 80 mg.L⁻¹ MS-222 and fitted with dorsal aortic catheters (PE50 Clay Adams Inc.) according to the method of Soivio et al. (1975). The bladder was then catheterised with a heat-flared PE60 polyethylene catheter (Clay Adams Inc.) according to Curtis and Wood (1991). Fish were allowed to recover for 24 h in individual black acrylic boxes (3.2 L vol) with flowing aerated fresh water.

Experimental procedures

Following recovery from surgery, fish were injected with 0.5 mL ¹⁴C labelled polyethylene glycol ([¹⁴C]-PEG, molecular weight = 4000: Amersham) in Cortland's saline (Wolf 1963) to yield an activity of 0.3 mCi.100 g⁻¹ fish. The

catheter was then flushed with a further 0.25 mL of saline to ensure no residual PEG remained in the catheter. [^{14}C]-PEG was used as an extracellular marker to allow determination of glomerular filtration rate (GFR) as well as to determine the effects of chloramine-T exposure on branchial paracellular permeability. In a preliminary series of experiments it was determined that a stable level of [^{14}C]-PEG was achieved in the plasma after 12 h of the initial injection. Flux experiments were therefore conducted 12 h post-injection with [^{14}C]-PEG.

Water flow to the box was interrupted and the box sealed. Aeration and mixing of the water within the box was maintained by a curtain of bubbles provided from a perforated hose around the inner perimeter of the box. A 25 mL water sample was withdrawn to begin the flux period. After a period of 3.5 h a second water sample was removed and the water flow reinstated (pre-exposure flux). Urine was collected from the urinary catheters during the flux period. Following the pre-exposure flux the boxes were flushed for 1 h with fresh water. A second flux period was initiated with 18 mg.L^{-1} chloramine-T added to each box. Upon addition of the chloramine-T there was a 30 minute mixing period prior to taking the first water sample (25 mL). After 3 h a second 25 mL water sample was taken to end the flux.

Analysis of water, plasma and urine

A 500 μL blood sample was withdrawn from the arterial catheter at the beginning and end of the flux period and the blood pH determined using a Radiometer BMS Mk4 with a microcapillary G299A electrode. Arterial PO_2 (PaO_2) and O_2 content (CaO_2) were determined using a thermostatically controlled Radiometer E5046 electrode and an OxyconTM O_2 content analyser (Cameron Instruments Company, Port Aransas, Texas, USA), respectively. Haematocrit was determined from a sample of whole blood drawn into a microcapillary tube and centrifuged at 10 000g for 10 min. Haemoglobin content of the blood was determined with a commercial spectrophotometric assay kit (Sigma Chemical Company, St. Louis Mo.). The remaining blood was centrifuged at 10 000g and the plasma total CO_2 content (CaCO_2) was determined with a Corning 965 total CO_2 analyser. The red cells were then resuspended in non-heparinised Cortland's saline and re-injected into the fish. Arterial PCO_2 (PaCO_2) was calculated using a rearrangement of the Henderson-Hasselbalch equation with constants for pK'_{CO_2} from Boutilier et al. (1984):

$$\text{PCO}_2 = \frac{(1 + \text{antilog}(\text{pH} - \text{pK}'_{\text{CO}_2}))}{\text{Total CO}_{2\text{plasma}}}$$

A 100 μL sample of plasma was dispersed in 10 mL of fluor (Amersham ACS II) for the determination of plasma activity of [^{14}C]-PEG. Plasma was then diluted 1000x with deionised water and analysed for Na^+ and Cl^- as described above. A 5 mL sub-sample of water was mixed with 10 mL of fluor (Amersham ACS II) and the radioactivity of the water determined. Urine collected over the duration of the flux was diluted 100x with deionised water for determination of Na^+ and Cl^- as described above. A 1 mL sample of urine was mixed with 10 mL of fluor (Amersham ACS II) for determination of urine activity. Activity of the water, plasma and urine samples were determined using a Canberra Packard TR1000 liquid scintillation counter.

Whole body unidirectional ion fluxes

Experimental Protocol

Fish of mean weight (\pm SE) 27.0 ± 1.6 g were placed in individual 600 mL black acrylic boxes supplied with aerated flowing dechlorinated water 24 h prior to experimentation. To begin the flux, the water supply was stopped and the chamber was sealed. $^{22}\text{NaCl}$ (Amersham) or H^{36}Cl (Dupont) was added to each chamber to yield a final activity of $0.2 \mu\text{Ci}\cdot\text{mL}^{-1}$. Mixing and aeration of the

chamber were ensured using an air stone. Following a 30 min mixing period a 25 mL water sample was removed. After 3 h a second water sample was removed and the water flow was re-instated. This was called the pre-exposure (control) flux.

After flushing the boxes for 1 h with flowing fresh water a second flux (called the exposure flux) was carried out. Chloramine-T (2, 9 or 18 mg.L⁻¹ active ingredient, n = 9), sodium hypochlorite (0.2 mg.L⁻¹ active ingredient, n = 9) or *paratoluenesulphonamide* (*p*TSA) (9 mg.L⁻¹ active ingredient in 0.017% dimethylsulphoxide (v/v DMSO), n = 9) were added, where appropriate, to each box at the same time as the isotope. In a separate experiment it was determined that 9 mg.L⁻¹ chloramine-T resulted in 0.2 mg.L⁻¹ free chlorine (according to the DPD method, Franson 1978). This value is equivalent to other studies with chloramine-T (Bullock et al. 1991). The concentration of 9 mg.L⁻¹ was chosen for *p*TSA since this concentration was the maximum which could be derived from full degradation of 9 mg.L⁻¹ chloramine-T. The amount of isotope added varied between treatments in order to ensure a comparable specific activity. Control treatments consisted of a group of fish which were exposed to either distilled water (used to dissolve chloramine-T and dilute the sodium hypochlorite,

n = 9) or 0.017% DMSO (n = 9).

Water analysis

A 5 mL sub-sample of water was mixed with 10 mL aqueous liquid scintillation cocktail (fluor) (Amersham ACSII) and the radioactivity determined using an LKB Wallac 1215 Rackbeta liquid scintillation counter. A 5 mL sub-sample was then diluted 1:1 with deionised water and the Na⁺ content was determined using a Varian Spectra AA plus atomic absorption spectrophotometer. Alternatively a 1 mL sub-sample was analysed for Cl⁻ using a spectrophotometric assay (Zall et al. 1956). Total ammonium concentrations were determined using the salicylate-hypochlorite spectrophotometric method (Verdouw et al. 1978). Titratable alkalinity was determined according to the method of McDonald and Wood (1981).

Calculations and statistical analysis

Net and unidirectional ion fluxes were calculated according to the formulae:

$$J_{\text{net}} = \frac{[X]_O - [X]_T * V}{T * W}$$

Where $[X]_O$ is the concentration of ion X at the start of the flux (mM), $[X]_T$ is the concentration of the ion at the end of the flux period (mM), V is the volume of the flux chamber (L), T is the duration of the flux period (h) and W is the weight of the fish (g).

$$J_{\text{in}} = \frac{[(\text{cpm})_O/s - (\text{cpm})_T/s] * V}{SA * W * T}$$

Where $(\text{cpm})_O$ is the counts per minute at the start of the flux period, $(\text{cpm})_T$ is the counts per minute at the end of the flux period, W is the weight of the fish (g), T is the duration of the flux period (h), s is the sample volume (L), V is the flux chamber volume (L) and SA is the average specific activity of the sample, determined according to:

$$SA = \frac{(\text{cpm}_O + \text{cpm}_T) * 0.5}{([X]_O + [X]_T) * 0.5}$$

Unidirectional effluxes were determined according to Wood (1988):

$$J_{\text{out}} = J_{\text{net}} - J_{\text{in}}$$

Glomerular filtration rate (GFR) was calculated according to:

$$\text{GFR} = \frac{(\text{dpm}_{\text{PEG}})_{\text{urine}} * \text{UFR}}{(\text{dpm}_{\text{PEG}})_{\text{plasma}}}$$

Where $(\text{dpm}_{\text{PEG}})_{\text{urine}}$ is disintegrations per minute recorded in the urine, $(\text{dpm}_{\text{PEG}})_{\text{plasma}}$ is disintegrations per minute in the plasma, UFR is the urine flow rate ($\text{mL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$).

The renal clearance ratio (RCR) for Na^+ and Cl^- were calculated according to:

$$\text{RCR}_X = \frac{\text{UFR} * [\text{X}]_{\text{urine}}}{\text{GFR} * [\text{X}]_{\text{plasma}}}$$

Where $[\text{X}]_{\text{urine}}$ is the concentration of ion X in the urine (mM) and $[\text{X}]_{\text{plasma}}$ is the concentration of ion X in the plasma (mM). Pre-exposure and exposure fluxes and measurements were compared using a paired *t*-test and each flux was tested for significance from zero using a single *t*-test. The fiducial limit was set at 0.05.

RESULTS

Acid-base and respiratory data

Arterial PCO_2 levels did not increase significantly upon exposure to 18 $mg.L^{-1}$ chloramine-T (p-value = 0.085) neither was there a significant decrease in arterial pH although there were significant increases in both total CO_2 content of the plasma and calculated bicarbonate concentrations (Table 6-1). There was a significant decrease in PaO_2 during exposure to 18 $mg.L^{-1}$ chloramine-T. In addition, there was a small but significant decrease in CaO_2 during the pre-exposure flux period. However, there were no other significant effects of either the flux period or the exposure to chloramine-T on any of the respiratory variables examined (Table 6-2).

Renal flux studies

Exposure to 18 $mg.L^{-1}$ chloramine-T had no significant effect on plasma or urine Na^+ and Cl^- concentrations. Similarly, there was no significant effect of exposure on either urine flow rate or glomerular filtration rate. Renal efflux rates for Na^+ and Cl^- were also not statistically significant between pre-exposure and during exposure to 18 $mg.L^{-1}$ chloramine-T (Table 6-2). The renal clearance

Table 6-1: Mean \pm SE arterial carbon dioxide or oxygen tension (P_{aCO_2} , P_{aO_2} ; mmHg), arterial pH (pHa), total plasma carbon dioxide content ($CaCO_2$; mM) and plasma bicarbonate concentration ($[HCO_3^-]$; mM), arterial oxygen content (CaO_2 ; ml.100mL⁻¹), haematocrit (Hct; %), haemoglobin concentration ($[Hb]$; g.100mL⁻¹), O₂ specifically bound to haemoglobin (O₂/Hb) and mean cellular haemoglobin concentration (MCHC) at the start and end of a flux period for trout exposed to 18 mg.L⁻¹ chloramine-T. * = significant from start of flux.

	Pre-exposure		Exposure	
	Start	End	Start	End
P_{aCO_2}	1.60 \pm 0.16	1.70 \pm 0.23	1.29 \pm 0.15	1.81 \pm 0.25
pHa	7.95 \pm 0.07	7.92 \pm 0.05	8.01 \pm 0.04	7.98 \pm 0.05
$CaCO_2$	6.35 \pm 0.40	6.16 \pm 0.52	5.67 \pm 0.49	7.29 \pm 0.63*
$[HCO_3^-]$	6.25 \pm 0.39	6.05 \pm 0.51	5.59 \pm 0.49	7.17 \pm 0.62*
P_{aO_2}	91.14 \pm 7.24	85.76 \pm 4.50	100.00 \pm 6.45	87.67 \pm 5.24*
CaO_2	10.78 \pm 1.27	9.03 \pm 0.80*	9.54 \pm 0.68	7.53 \pm 0.92
Hct	27.75 \pm 2.09	26.75 \pm 2.19	28.00 \pm 2.22	26.25 \pm 2.27
$[Hb]$	8.69 \pm 0.62	9.24 \pm 0.80	9.08 \pm 0.73	8.71 \pm 0.78
O ₂ /Hb	1.14 \pm 0.12	0.94 \pm 0.05	0.99 \pm 0.05	0.82 \pm 0.05
MCHC	0.31 \pm 0.01	0.35 \pm 0.01	0.33 \pm 0.01	0.33 \pm 0.01

Table 6-2: Mean \pm SE plasma ($[X]_p$) and urine ($[X]_u$) concentrations, urine flow (UFR) and glomerular filtration rates (GFR), and renal ion effluxes (J^X_{out}), renal clearance ratios R_{CR_X} and branchial PEG efflux (J^{PEG}_{gill}) of trout exposed to 18 mg.L⁻¹ chloramine-T.

	$[Na^+]_p$ (mM)	$[Cl^-]_p$ (mM)	$[Na^+]_u$ (mM)	$[Cl^-]_u$ (mM)	UFR (mL.g ⁻¹ .h ⁻¹)	GFR (mL.g ⁻¹ .h ⁻¹)	J^{Na}_{out} (mmol.Kg ⁻¹ .h ⁻¹)	J^{Cl}_{out} (mmol.Kg ⁻¹ .h ⁻¹)
Pre-exposure	137.70 \pm 6.31	134.19 \pm 4.81	6.95 \pm 1.19	18.01 \pm 2.52	2.26 \pm 0.28	4.63 \pm 1.00	17.94 \pm 4.13	39.38 \pm 6.58
Exposure	156.90 \pm 7.63	154.23 \pm 5.93	8.79 \pm 1.78	17.16 \pm 3.25	2.27 \pm 0.04	4.55 \pm 0.82	23.36 \pm 6.83	44.84 \pm 15.20

continued	$R_{CR_{Na}}$	$R_{CR_{Cl}}$	J^{PEG}_{gill} (mmol.g ⁻¹ .h ⁻¹)
Pre-exposure	0.051 \pm 0.025	0.088 \pm 0.030	-0.021 \pm 0.011
Exposure	0.031 \pm 0.016	0.060 \pm 0.020	-0.013 \pm 0.009

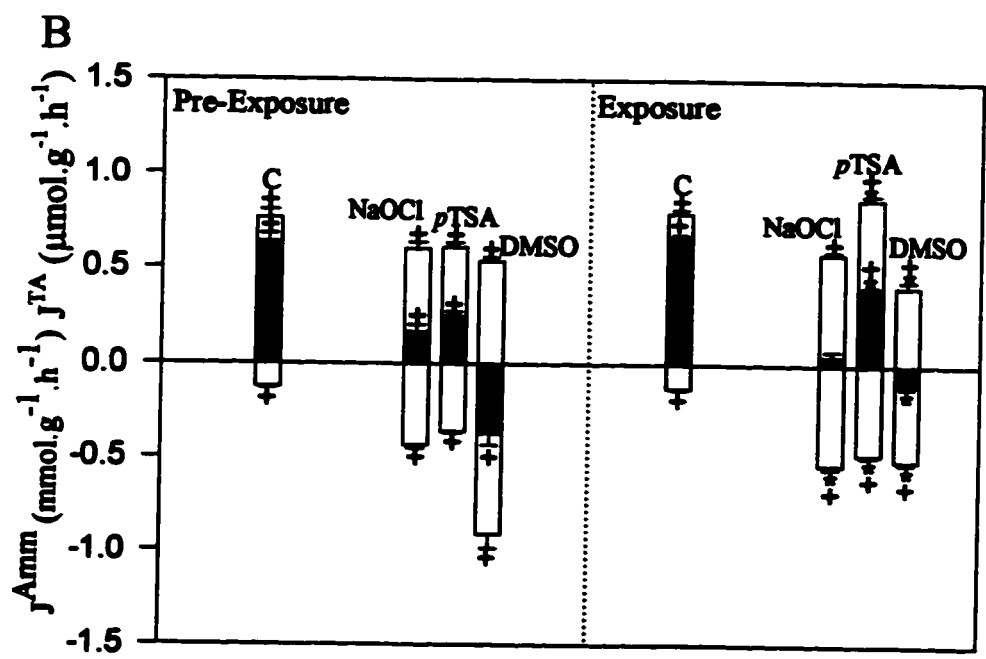
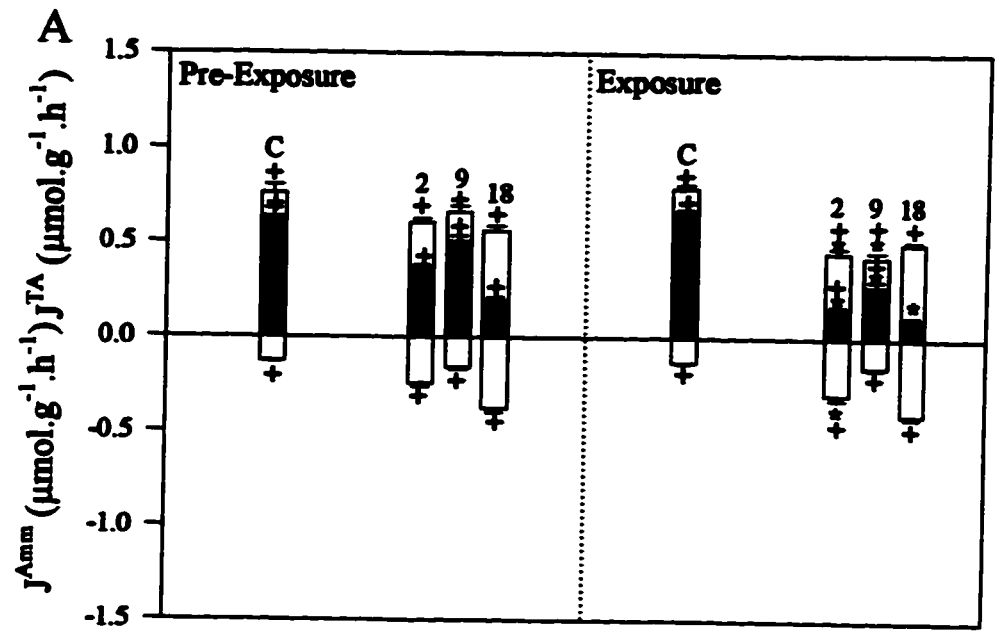
ratios for Na^+ and Cl^- were unaffected by exposure to chloramine-T (Table 6-2).

Similarly there was no significant increase in the branchial PEG efflux during exposure to 18 mg.L^{-1} chloramine-T (Table 6-2).

Whole body ion fluxes

Significant decreases in net acid uptake (i.e., net H^+ flux less positive) occurred at all three exposure concentrations of chloramine-T used compared with the pre-exposure flux (Fig. 6-1a). Similarly there was a decrease in the net titratable alkalinity flux for fish exposed to 2 and 9 mg.L^{-1} chloramine-T but this was not apparent at 18 mg.L^{-1} chloramine-T (Fig. 6-1a). Fish exposed to NaOCl also showed a decrease in net acid uptake, due in part, to increase in net ammonia excretion (Fig. 6-1b). Fish exposed to *p*TSA showed an increase in net titratable alkalinity flux as well as a significant increase in net ammonium excretion (Fig. 6-1b). The net effect was an increase in net acid uptake (a more positive net acid flux). There were no significant changes in the acid-base fluxes of control (unexposed) fish. However, there was a decrease in the net acid excretion in fish exposed to DMSO alone as well as significant decreases in net titratable alkalinity flux and net ammonium flux (Fig. 6-1b). Net acid fluxes

Figure 6-1. The effects of exposure to either control (C), 2, 9 or 18 mg.L⁻¹ chloramine-T (A) and its degradation products sodium hypochlorite (NaOCl), *paratoluenesulphonamide* (*p*TSA) and dimethylsulphoxide (DMSO) (B) on whole body flux rates of titratable alkalinity (J^{TA}), total ammonia (J^{Amm}) and net acidic equivalents ($J_{net}^{H^+}$). Positive values represent acidic equivalent uptake while negative values represent acidic equivalent excretion. Solid bars represent the net acidic equivalent flux ($J_{net}^{H^+}$) as the sum of J^{TA} and J^{Amm} , signs considered. Values are given as means \pm SE (n = 9) * significant difference from pre-exposure values, + represents fluxes which are significant from zero.



during the pre-exposure flux period were positive for all treatments except for fish which would be exposed to DMSO (Fig. 6-1b). Fish exposed to *p*TSA showed an increase in net titratable alkalinity flux as well as a significant increase in net ammonium excretion (Fig. 6-1b). The net effect was an increase in net acid uptake (a more positive net acid flux). There were no significant changes in the acid-base fluxes of control (unexposed) fish. However, there was a decrease in the net acid excretion in fish exposed to DMSO alone as well as significant decreases in net titratable alkalinity flux and net ammonium flux (Fig. 6-1b). Net acid fluxes during the pre-exposure flux period were positive for all treatments except for fish which would be treated with DMSO. This suggests that fish were in a state of acid uptake (i.e., reduced acid excretion) at the start of the experiment.

A significant increase in the net loss of Na^+ was measured in fish exposed to 18 mg.L^{-1} chloramine-T compared with pre-exposure values, but there were no significant changes in the net Na^+ flux at lower concentrations (Fig. 6-2a). Exposure to 9 mg.L^{-1} *p*TSA resulted in a significant increase in the Na^+ efflux which in turn resulted in a significant but negative net flux as compared with the pre-exposure values (Fig. 6-2b). There was also a significant increase in the

negative net Na^+ flux in fish exposed to NaOCl (Fig. 6-2b). Fish exposed to DMSO only showed a small but significant negative Na^+ net flux as compared with the small positive net flux during the pre-exposure period. There was no significant change in the unidirectional Na^+ fluxes (as compared with the pre-exposure flux period) for any of the chemical treatments (Fig. 6-2). Similarly, there were no significant changes in the net or unidirectional Na^+ fluxes of control (unexposed) fish during the exposure period (Fig. 6-2 a and b).

Exposure to chloramine-T caused significant increases in the net loss of Cl^- during exposure to 2 and 18 mg.L^{-1} concentrations (Fig. 6-3a). However, only in fish exposed to 18 mg.L^{-1} chloramine-T was a significant increase in the unidirectional efflux of Cl^- seen (Fig. 6-3a). NaOCl exposure also resulted in net Cl^- loss but no change in the unidirectional efflux of Cl^- (Fig. 6-3b). There was a significant increase in the negative net Cl^- flux upon exposure to *pTSA* (Fig. 6-3b). However, exposure to DMSO did not result in any significant changes in net Cl^- flux (Fig. 6-3b). In all treatments (Chloramine-T, *pTSA*, NaOCl or DMSO) there was no effect of exposure on Cl^- influx (Figs. 6-3a and b).

Figure 6-2. The effects of exposure to either control (C), 2, 9 or 18 mg.L⁻¹ chloramine-T (A) and its degradation products sodium hypochlorite (NaOCl), *paratoluenesulphonamide* (*p*TSA) and dimethylsulphoxide (DMSO) (B) on unidirectional (open bars) and net (solid bars) whole body Na⁺ flux. Positive values represent ionic uptake while negative values represent ionic losses. Values are given as means ± SE (n = 9) * significant difference from pre-exposure values, + represent fluxes which are significant from zero.

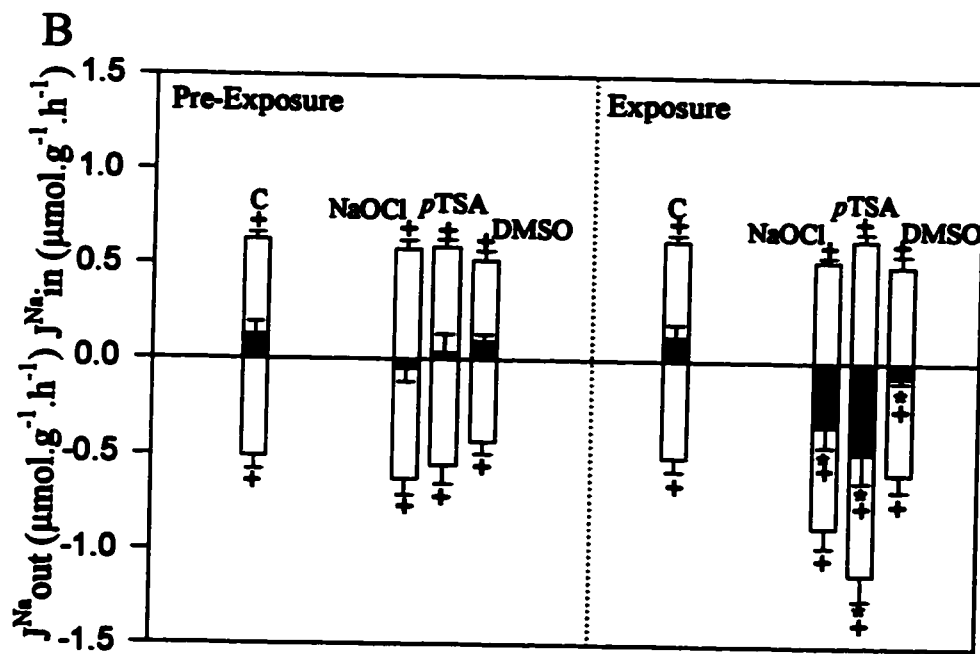
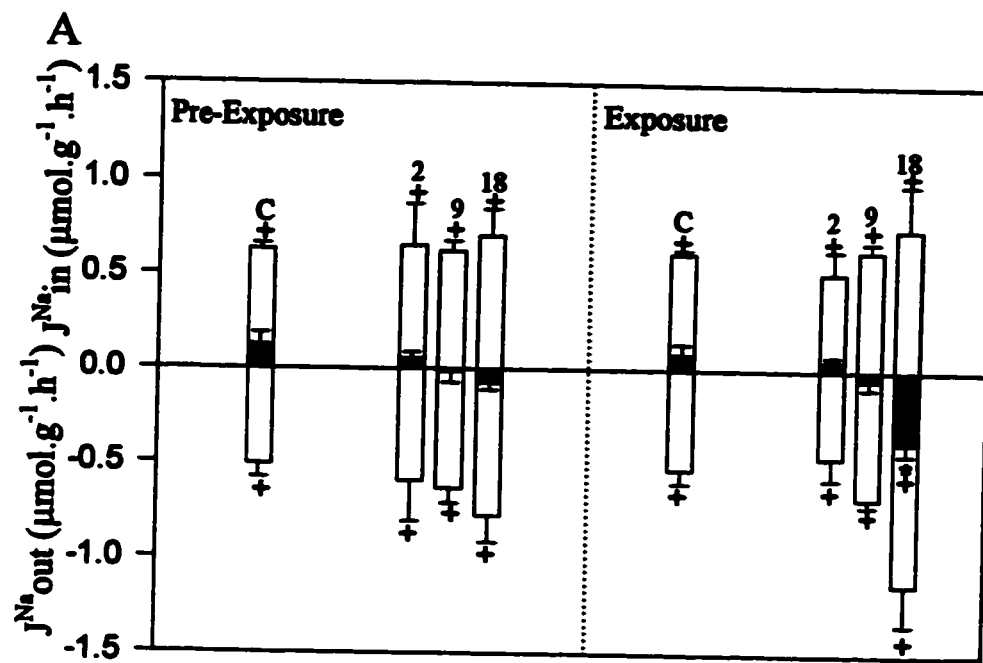
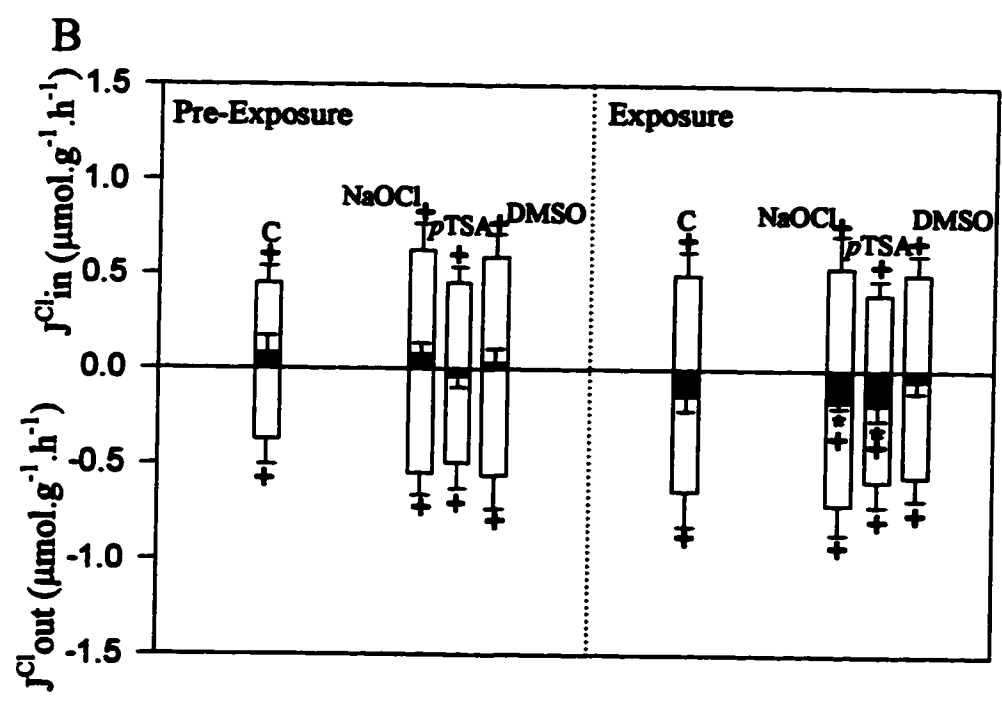
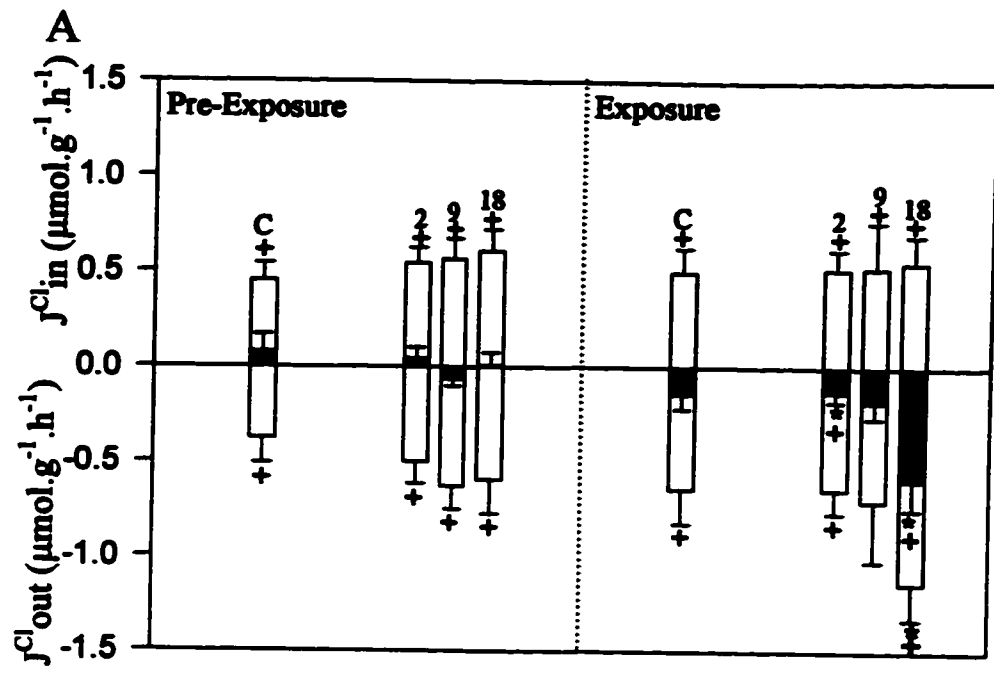


Figure 6-3. The effects of exposure to either control (C), 2, 9 or 18 mg.L⁻¹ chloramine-T (A) and its degradation products sodium hypochlorite (NaOCl), *paratoluenesulphonamide* (*p*TSA) and dimethylsulphoxide (DMSO) (B) on unidirectional (open bars) and net (solid bars) whole body Cl⁻ flux. Positive values represent ionic uptake while negative values represent ionic losses. Values are given as means ± SE (n = 9) * significant difference from pre-exposure values, + represents fluxes which are significant from zero.



DISCUSSION

It was important to first determine that a static exposure to chloramine-T resulted in arterial blood acid-base disturbances similar to those previously described in chapter 2 in which a flow-through exposure system was used. The significant rise in plasma total CO₂ content and calculated bicarbonate concentration were similar to those described in chapter 2. However, in the previous study a significant increase in PaCO₂ also was reported. Re-breathing of CO₂ and subsequent hypercapnia was evidently not a problem during the pre- and exposure period since there was no evidence of a hypercapnic acidosis occurring in the blood.

The metabolic alkalosis which occurs upon exposure to chloramine-T can be explained in terms of the branchial exchange of acidic and basic equivalents. Clearly the data show that there was a marked decrease in the net uptake of acid upon exposure to chloramine-T yet an apparent increase in net acid uptake upon exposure to *p*TSA and DMSO. Since *p*TSA had to be dissolved in DMSO prior to experimentation (due to the low solubility of *p*TSA) we cannot determine the effect of *p*TSA alone.

Differences in net ionic flux were not caused by the impediment of ionic

uptake mechanisms since ionic influx rates were unaffected by chloramine-T, NaOCl, *p*TSA or DMSO. Thus, altered net ionic fluxes which were observed were probably caused by alterations in ionic permeability of the gill epithelium. According to strong ion difference theory of acid-base balance, net acid excretion across the gill is set by the difference in the net flux of strong cations and anions (primarily Na⁺ and Cl⁻) (see Cameron and Iwama 1989 for review). Changes in the net fluxes of these ions would therefore be predictive of an acid-base disturbance (Cameron and Iwama 1989; Wood 1991). In this study, it was not possible to determine the strong ion difference directly because Na⁺ and Cl⁻ net fluxes were not determined on the same group of fish. There was an apparent excessive net loss of Cl⁻ relative to Na⁺ in fish exposed to chloramine-T and the opposite (greater net Na⁺ loss compared to net Cl⁻ loss) for *p*TSA. However, if the change in net Na⁺ or Cl⁻ flux are calculated (e.g., $J_{\text{net}}^{\text{Na}}_{\text{pre-exp}} - J_{\text{net}}^{\text{Na}}_{\text{exp}}$) and compared using a two sample t-test, then the net losses of Cl⁻ were significantly greater than Na⁺ at the 2 mg.L⁻¹ chloramine-T concentration only (p value = 0.05). Such an event would be expected to induce a metabolic alkalosis. Measurement of a decrease in net acid uptake in these same fish support this contention (Fig. 6-3a). Further, data from catheterised fish where significant

increases in plasma total CO₂ were measured without an accompanying decrease in pH confirm our suggestion of a metabolic alkalosis (Table 6-1). In chapter 2 it was shown that a single chloramine-T exposure of 1 h duration caused a mixed respiratory/metabolic acid-base disturbance consisting of a mild respiratory acidosis superimposed over a metabolic alkalosis (see chapter 2). This was particularly evident at the exposure concentration of 2 mg.L⁻¹. Net Na⁺ losses were significantly greater than net Cl⁻ loss for fish exposed to *p*TSA (but not for any other treatments). This would suggest the development of a metabolic acidosis which is supported by the fact that the same fish showed increased acid uptake (Fig. 6-1b). This is consistent with the data shown in chapter 2.

Exposure to hypochlorite, which is believed to be the primary disinfective component of chloramine-T resulted in increased net losses of both Na⁺ and Cl⁻. As with chloramine-T, there was a significant decrease in the net acid uptake supporting the idea of a metabolic alkalosis although this was not evident in terms of differential rates of ion loss. It has been shown that hypochlorite exposure causes an acute respiratory acidosis (chapter 2). However, here NaOCl exposures were for a longer period and at a lower concentration of NaOCl (0.2 mg.L⁻¹ for 3 h compared with 0.45 mg.L⁻¹ for 1 h as used in chapter 2).

Bladder catheterised fish were used to verify that the measured whole body fluxes primarily reflected branchial fluxes and to quantify the contribution from renal sources. The catheter method provided reliable estimates of urine flow rates and it was also possible to determine the ionic composition of the urine formed (Curtis and Wood 1991). To allow determination of glomerular filtration rate it was necessary to use an extracellular fluid marker. [^{14}C]-PEG was chosen as the marker of choice since it has been used in other studies with success (Curtis and Wood 1991; 1992). There was no effect of exposure to $18 \text{ mg}\cdot\text{L}^{-1}$ chloramine-T on the urine flow rate, glomerular filtration rate or on renal Na^+ and Cl^- effluxes. Therefore the possibility that differences in whole body ion fluxes were due to increased renal efflux can be eliminated.

The use of [^{14}C]-PEG as an extracellular marker allowed determination of branchial paracellular permeability. If altered whole body ion fluxes were not caused by increased renal efflux then chloramine-T must be acting on the gill or skin. There are two possible routes by which ions could be lost across epithelia, transcellularly and paracellularly (direct loss from the extracellular space across the epithelial tight junction). Assuming that [^{14}C]-PEG would not be transported into epithelial cells, measurement of radioactivity in the water would be directly

proportional to the rate of efflux across the epithelium by paracellular routes. There was no significant difference in the rates of [^{14}C]-PEG efflux across the gill with exposure to 18 mg.L^{-1} chloramine-T thus suggesting that altered ionic effluxes were not due to an increase in epithelial tight junction permeability. There was in fact a slight decrease in permeability which may be caused by a tightening of epithelial tight junctions. This suggests that the altered ionic effluxes at least with 18 mg.L^{-1} chloramine-T may have been due to transcellular processes. Chlorine (in the form of dissolved chlorine gas) has been demonstrated to cause membrane lysis and the leakage of macromolecules from bacterial cells as its primary mechanism of disinfection (Venkobacher et al. 1977). Thus it is possible that hypochlorite from chloramine-T degradation may exert a similar permeablising effect on the branchial epithelia of fish. This notwithstanding, it is recognised that PEG-4000 is a large molecule and it can only be concluded that molecules of this dimension and larger could not cross the epithelial tight junction in this study.

Irrespective of the route of ionic efflux, this study has demonstrated a differential loss of ions depending upon whether the fish was exposed to chloramine-T or its breakdown product *p*TSA. The reason for this differential

ion loss can only be speculated on at this point. However, chloramine-T is thought to elicit an acute secretion of mucus onto the gill which may impair gas exchange to varying degrees (Powell et al. 1994; chapters 2 and 3 of this thesis). Mucus is polyanionic and has the potential to trap cations such as Na^+ and Ca^{++} at the epithelial surface (Scot 1989; Shephard 1989). Thus if chloramine-T exposure resulted in an increase in branchial transcellular permeability, the secretion of mucus onto the gill would serve to trap cations such as Na^+ at the gill surface whereas anions such as Cl^- would be more readily lost to the environment. Hence it is conceivable that there would be a differential flux of ions. With *p*TSA there is not likely to be increased mucus secretion. In chapter 2 it was demonstrated that there was no effect of *p*TSA exposure on respiratory variables which could be explained by the absence of increased branchial mucus secretion above background/basal levels. The reason for greater Na^+ loss compared with Cl^- loss is interesting and difficult to explain although we cannot exclude the possibility that *p*TSA may affect renal function due to its structural similarity to parts of other antibacterial sulphonamides some of which such as sulphamerazine have been reported to cause renal lesions in cut-throat trout (*S. clarkii*) (Smith et al. 1973).

In conclusion, a static exposure to chloramine-T results in acid-base disturbances similar to those seen with flow-through treatments. In addition the metabolic alkalosis associated with chloramine-T exposure resulted in a decrease in whole body net acid uptake (acid excretion) whereas exposure to pTSA and DMSO resulted in increases in net acid uptake (acid uptake) resulting in a metabolic acidosis. These changes in the exchange of acidic and basic equivalents correspond with ionic losses which may be caused by increased transcellular permeability. Although relatively minor in terms of pathological effects, this work suggests that caution should be used when using chloramine-T disinfection either prophylactically or therapeutically when inorganic anions and cations may be limited such as in ion-poor (soft) waters given that under such conditions branchial permeability may be increased.

CHAPTER 7

IONIC FLUX DISTURBANCES DURING EXPOSURE TO CHLORAMINE-T: EFFECTS OF SOFT WATER ACCLIMATION

INTRODUCTION

Chloramine-T toxicity in terms of LC_{50} is low at the concentrations and durations and stocking densities used commercially (Bills et al. 1988a,b) but increases with reduced water hardness, reduced water pH and elevated temperature (Bills et al. 1988b). In the previous chapter, acute branchial ionic disturbances were demonstrated which probably occurred through increases in transcellular permeability of epithelial cells.

Water hardness affects the ionoregulatory physiology of the gill and acute branchial ionic losses have been demonstrated in fish exposed to calcium and sodium deficient water (McDonald and Rogano 1986; Perry and Laurent 1989). However, as the fish acclimate to reduced ion conditions, branchial permeability is reduced and the number of ionic uptake sites increases (McDonald and Rogano 1986; Avella et al. 1987; Perry and Laurent 1989). Thus fish held in ion poor environments (soft water) typically exhibit a greater number and larger chloride cells which may affect the physical dimensions of the gill resulting in possible respiratory compromise (Greco et al. 1995; 1996).

This study was initiated to investigate the effects of chloramine-T exposure on fish which were acclimated to soft (ion-poor) water conditions. It

was hypothesised that soft water acclimated fish would be more susceptible to acute ionic disturbances caused by chloramine-T. The addition of sodium chloride to the water was examined in combination with chloramine-T exposure as a potential method to counter ionic disturbances.

MATERIALS AND METHODS

Fish and acclimation

Rainbow trout (*O. mykiss*) were purchased from a commercial hatchery (Linwood Acres Trout Farm, Campbellcroft, Ontario) and acclimated to laboratory conditions for 2 weeks prior to experimental use in a 300 L rectangular fibreglass tank supplied with aerated dechlorinated city of Ottawa tap water at 10-11°C. Residual chlorine levels were below detectable limits using a DPD method (Franson 1978).

Following initial acclimation to laboratory conditions the population of fish were divided into two rectangular fibreglass tanks (300 L each) one receiving dechlorinated city of Ottawa tap water (TW), the other group was gradually acclimated to ion reduced, artificial soft water (ASW) conditions by gradually mixing dechlorinated tap water with water from a reverse osmosis unit.

Following a 7 day period of reducing water ion concentrations, fish were maintained at a reduced ionic concentration in the ASW for a further 14 days prior to their experimental use (Table 7-1). Water temperatures were maintained between 11 and 12°C throughout the acclimation and subsequent experimental periods (Table 7-1). Water ionic concentrations were determined from water samples collected from each (TW and ASW) tank using a Varian Spectra AA plus atomic absorption spectrophotometer. During the initial habituation and acclimation phases, fish were fed on alternate days on a commercial granular diet (Martin's Feed Mills Inc.). Fish in both groups appeared to consume approximately the same amount of feed. Feed was withheld for 24 h prior to experimental use.

Experimental flux protocol

Fish were placed in individual 600 mL black acrylic boxes supplied with either flowing dechlorinated tap water (TW) or artificial soft water (ASW) for 24 h prior to experimentation. Water in each box was aerated and mixed by means of an air stone. To begin the flux, the water supply was stopped and the

Table 7-1: Mean (\pm SEM) ionic composition of artificial soft water (ASW) and dechlorinated city of Ottawa tap water (TW) averaged over the 14 day acclimation period.

	Artificial soft water (ASW)	Tap water (TW)
Na ⁺ (mM)	0.043 \pm 0.003*	0.121 \pm 0.004
K ⁺ (mM)	0.005 \pm <0.001*	0.015 \pm <0.001
Ca ²⁺ (mM)	0.063 \pm 0.002*	0.351 \pm 0.004
Cl ⁻ (mM)	0.026 \pm 0.002*	0.141 \pm 0.002

* significantly different from tap water controls

chamber sealed. Either $^{22}\text{NaCl}$ (Amersham) or H^{36}Cl (Dupont) were added to each chamber to yield a final activity of $0.2 \mu\text{Ci.mL}^{-1}$. To ensure sufficient mixing and prevent hypoxia, aeration was continued. Following an initial 30 min mixing period, a 25 mL water sample was removed. After 3h a second water sample was removed and the water flow was re-instated.

After a 1 h period of flushing with flowing fresh water (either TW or ASW as appropriate), a second flux period (the exposure flux) was initiated. Chloramine-T (9 mg.L^{-1} active ingredient, $n = 6$: BDH Chemicals Inc. Mississauga, Ontario, Canada), or chloramine-T (9 mg.L^{-1}) and 17 mM NaCl (0.1% w/v) ($n = 6$: BDH Chemicals) were added, where appropriate, to each box at the same time as the isotope. A control group was also tested in which no chemical treatment other than distilled water (used to dissolve the chloramine-T and NaCl) was added ($n = 6$). The amount of isotope added varied between treatments in order to ensure a comparable specific activity. However, the specific activity in the chloramine-T + NaCl treatment was limited to 10% that of the other treatments.

Water analysis

A 5 mL sub-sample of water was mixed with 10 mL of aqueous liquid scintillation cocktail (fluor) (Amersham ACSII) and the radioactivity determined using an LKB Wallac 1215 Rackbeta liquid scintillation counter. A 5 mL sub-sample was then diluted 1:1 with deionised water and the Na^+ content determined using a Varian Spectra AA plus atomic absorption spectrophotometer. Alternatively a 1 mL sub-sample was analysed for Cl^- using a spectrophotometric assay (Zall et al. 1956).

Calculations and statistical analysis

Net (J_{net}^X) and unidirectional (J_{in}^X and J_{out}^X) ion fluxes were calculated according to the formulae given in chapter 6. Comparisons of net and unidirectional flux rates were made between the pre-exposure and exposure periods and between TW and ASW acclimated trout using either a paired or two sample *t*-test, respectively. In addition the change in each net flux from the pre-exposure and the exposure periods was calculated for each treatment and compared between TW and ASW acclimated fish using a two sample *t*-test. All means shown are ± 1 standard error of the mean. P values of less than 0.05 were

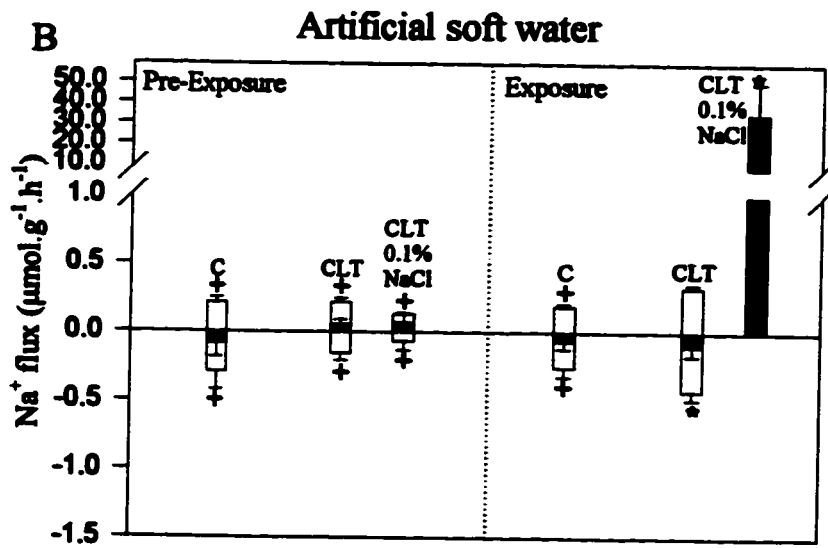
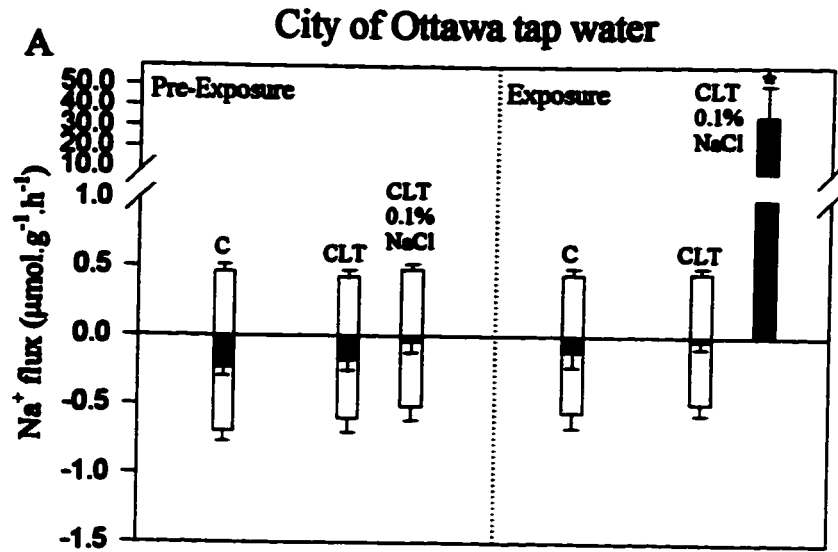
considered to be significant.

RESULTS

In fish acclimated to TW and exposed to chloramine-T alone, there was no significant difference in either Na^+ uptake or Na^+ efflux between the pre-exposure and the exposure flux periods (Fig. 7-1a). In control fish which were not exposed to any chemical, Na^+ influx and efflux and hence net flux during the exposure period were not significantly different from the pre-exposure flux period. Exposure of fish to chloramine-T and 0.1% NaCl (17 mM) resulted in highly significant increases in Na^+ influx which resulted in increases in a positive net flux since no efflux could be measured (Fig. 7-1a).

Fish acclimated to ASW showed a reduced Na^+ influx during the pre-exposure flux period as compared with the TW acclimated fish. There was a marked reduction in Na^+ efflux in the ASW acclimated fish during the pre-exposure period as compared with the TW acclimated fish. However, net fluxes were not significantly different between TW and ASW acclimated fish during the pre-exposure period. Exposure to chloramine-T alone resulted in a significant increase in Na^+ efflux, although the net flux was unaffected (Fig. 7-1b).

Figure 7-1. The effects of exposure to either control (C), 9 mg.L⁻¹ chloramine-T (CLT) or 9 mg.L⁻¹ chloramine-T and 0.1% NaCl (CLT 0.1% NaCl) on unidirectional (open bars) and net (solid bars) whole body Na⁺ flux in rainbow trout acclimated to dechlorinated city of Ottawa tap water (a) or artificial soft water (b). Positive values represent ionic uptake while negative values represent ionic losses. Values are given as means ± SE (n = 9), * significant difference from pre-exposure values, + significantly different from TW values.



Exposure to chloramine-T and 0.1% NaCl (17 mM) resulted in a highly significant increase in Na⁺ influx and net flux with no significant efflux measured (Fig. 7-1b).

There were no significant differences between the pre-exposure unidirectional or net Cl⁻ fluxes for ASW and TW acclimated fish. There was a reduced rate of Cl⁻ uptake in the ASW acclimated fish which were to be exposed to chloramine-T and NaCl during the pre-exposure (no chemical added) period compared with the TW acclimated group (Fig. 7-2a and b).

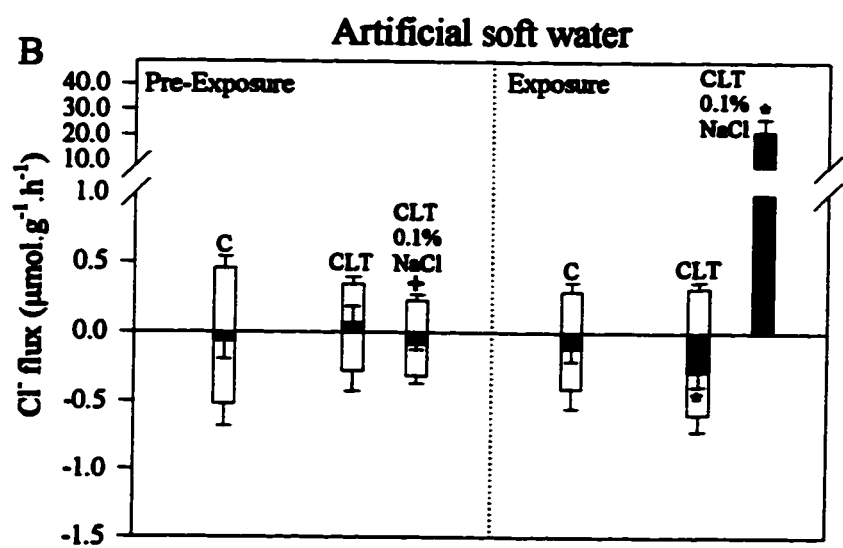
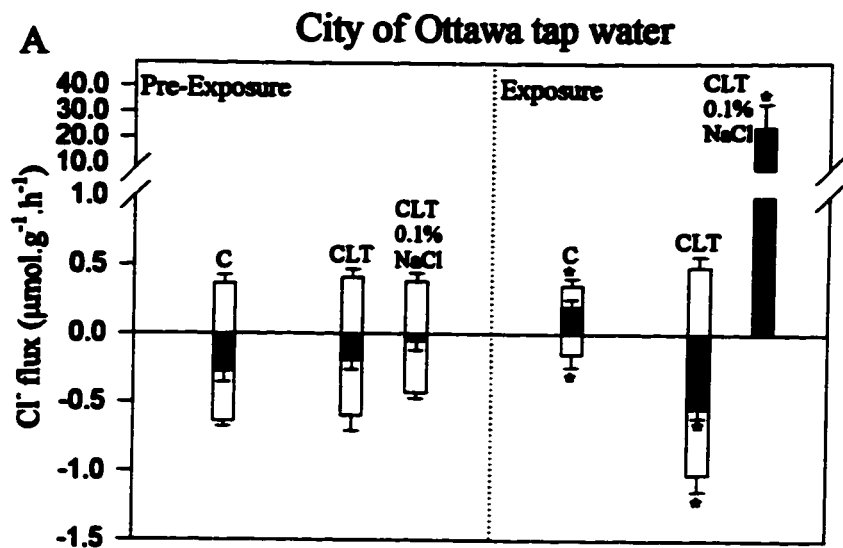
Tap water acclimated fish during the exposure flux period showed a significant reduction in Cl⁻ efflux and a significant increase in the positive net flux in the control group as compared with the pre-exposure period (Fig. 7-2a). Exposure to chloramine-T alone resulted in significant increase in chloride efflux but no significant increase in chloride uptake. This resulted in a significant increase in the negative net flux (Fig. 7-2a). Addition of 0.1% NaCl (17 mM) resulted in a significant increase in both the chloride influx and net flux with complete elimination of Cl⁻ efflux (Fig. 7-2a). Exposure to chloramine-T in ASW acclimated fish resulted in a significant and negative increase in net Cl⁻ flux although there were no significant changes in unidirectional Cl⁻ fluxes as

compared with the pre-exposure period (Fig. 7-2b). Addition of 0.1% NaCl (17 mM) with chloramine-T resulted in a significant increase in Cl⁻ uptake and diminishment of the efflux resulting in a significant positive increase in the net flux (Fig. 7-2b). There were no significant differences in the flux rates for control (no chemical) ASW acclimated fish during the exposure flux period. There was no significant difference in the change in the magnitude of the ion losses ($J_{\text{netpre-exposure}} - J_{\text{net exposure}}$) between either TW or ASW acclimated fish.

DISCUSSION

Fish which were acclimated to ion poor water (ASW) showed a reduced rate of Na⁺ efflux but not Cl⁻ efflux. Although acute exposure to ASW results in acute ionic losses through increased branchial permeability (McDonald and Rogano 1986; Perry and Laurent 1989), a tightening of the branchial epithelium (reduced permeability) gradually occurs during the acclimation process. The tightening is accompanied by an increase in chloride cells (Laurent and Hebibi 1989) which are implied to be the sites of Cl⁻ (Perry and Laurent 1989) and Ca²⁺ uptake (Perry et al. 1992). In the present study, the reduced branchial

Figure 7-2. The effects of exposure to either control (C), 9 mg.L⁻¹ chloramine-T (CLT) or 9 mg.L⁻¹ chloramine-T and 0.1% NaCl (CLT 0.1% NaCl) on unidirectional (open bars) and net (solid bars) whole body Cl⁻ flux in rainbow trout acclimated to dechlorinated city of Ottawa tap water (a) or artificial soft water (b). Positive values represent ionic uptake while negative values represent ionic losses. Values are given as means ± SE (n = 9), * significant difference from pre-exposure values, + significant difference from TW values.



permeability suggests that the ASW acclimated fish were fully acclimated to the ion poor conditions. This was evident since Cl^- uptake in the pre-exposure flux period was generally not significantly different from that in tap water fish and ionic effluxes were small. Although there were increased net losses of Cl^- upon exposure to chloramine-T in both TW and ASW acclimated fish, net flux and efflux rates were not significantly different between the two water types. If ion losses were due to increased permeability because of loss of tight junction integrity and hence increased paracellular flux, it may be expected that ionic losses during exposure to chloramine-T in ASW acclimated fish would be greater than TW fish. Moreover, it might be predicted that there would be losses of both Na^+ as well as Cl^- . This was clearly not the case suggesting that ionic effluxes were probably due to transcellular losses, perhaps through damage to the epithelial membrane by hypochlorite which is produced as a consequence of chloramine-T degradation (Booth and McDonald 1988). Hypochlorite has been demonstrated to disrupt bacterial cell membranes (Venkobacher et al. 1977). This is consistent with previous suggestions about the routes of ion losses with chloramine-T since no branchial efflux of the extracellular fluid marker ^{14}C -PEG could be measured (see chapter 6). It is unlikely that the ionic losses

demonstrated in both TW and ASW in this study are a result of the increased renal clearance of ions. Chapter 6 demonstrated that trout acutely exposed to 18 mg.L⁻¹ chloramine-T in dechlorinated city of Ottawa tap water showed no increase in glomerular filtration, urine flow, ionic renal efflux or renal clearance of ions. Although urine flow rates increase upon acclimation to ion poor water (McDonald and Rogano 1986), Perry and Laurent (1989) suggest that renal ionic effluxes are reputedly unaffected by acclimation to ion poor water. In the current study, negative net ion fluxes are therefore primarily reflective of branchial rather than renal ionic losses.

The addition of 17 mM (0.1% w/v) NaCl was used as a potential method for countering the effects of chloramine-T. Clearly the addition of NaCl was more than sufficient to overcome the ionic losses experienced during acute exposure to chloramine-T. Ionic uptake was significantly increased. It has been suggested that the rate at which ionic uptake mechanisms for both Na⁺ and Cl⁻ operate in the gills are limited by the availability of substrate (ions) (Goss et al. 1992). Therefore, with the addition of NaCl to the water, there was an increased availability of substrate and ionic uptake could proceed at J_{max}. However, no efflux could be measured. The reason for this was probably due to the

difficulties in measuring small changes in ionic flux in water of high ionic concentration (a 10 μmol change may be detectable when the background water ion concentration is measured in μmol s but not detectable when the background water ion concentration is measured in mmol s). Also the ionic efflux of the gill would be reduced somewhat because of the reduced concentration gradient due to the presence of NaCl in the water. In actual fact based on published values for J_{max} for Cl^- in rainbow trout (Goss and Wood 1991), it can be estimated that the addition of 0.130 mM NaCl (0.00075% w/v) would be sufficient to overcome the greatest net ionic losses in this study, i.e., those which occurred with chloramine-T alone in tap water (Fig. 7-2a). This may indeed be more beneficial since it would reduce the net NaCl accumulation which would occur with 17 mM (0.1% w/v) NaCl.

Combined treatments for therapy in aquaculture is a common practice. In addition to chloramine-T and formalin Thorburn and Moccia (1993) found that salt was a commonly used treatment on Ontario trout farms. The use of both chloramine-T and salt in a combined treatment may be a practical method for treating gill diseases both prophylactically as well as therapeutically. Not only may salt be useful as a therapeutic agent itself it remediates any acute ion losses

which may be associated with chloramine-T treatment, thereby minimising ionic stresses on the fish. However, some ionic losses may continue to occur following the withdrawal of the combined chloramine-T and salt therapy.

This study has shown that chloramine-T did not have a larger impact on net ionic fluxes in soft water acclimated fish as may have been predicted in terms of the LC_{50} toxicity data (Bills et al. 1988b). The increased toxicity of chloramine-T in soft water may therefore be due more to the lack of ionic substrate for the correction of ionic disturbances or due to respiratory impediment because of increased branchial mucus secretion.

CHAPTER 8
GENERAL DISCUSSION AND CONCLUSIONS

Respiratory and metabolic effects of chloramine-T

Chloramine-T at therapeutic concentrations does not have any severe pathological effects on the respiratory physiology of rainbow trout. This is in itself an important conclusion since although growth rates were shown to be suppressed with repeated exposure, no studies had previously examined whether metabolic rates or respiratory physiology were adversely affected. Interestingly, Powell et al. (1994) noted that during repeat exposure to chloramine-T, fish treated at the higher concentrations (namely 10 and 20 mg.L⁻¹) showed signs of respiratory distress, hyperventilation and crowding at the tank surface. This most likely occurred as a consequence of the chloride cell hyperplasia which those fish apparently exhibited (Powell et al. 1995). Chapter 2 clearly showed that PaO_2 levels were not compromised during exposure to chloramine-T. However, it was demonstrated in chapter 4 that oxygen uptake actually increased during exposure to chloramine-T because of increased blood flow to the gills. In chapter 5, however, repeated exposure to chloramine-T had no effect on oxygen uptake rate but there was a reduction of the blood-to-water diffusion barrier. This may have compensated for the mucous cell hyperplasia and subsequent impediment to oxygen uptake (if any) which it (the mucous cell hyperplasia) may have caused.

Reductions in growth rate (Powell et al. 1994; G. Sanchez pers. comm.) and reduction in food conversion ratio (G. Sanchez pers. comm.) were probably due to some alteration of metabolic partitioning rather than an overall effect on metabolic rate.

Although oxygen uptake was not adversely affected by exposure to chloramine-T, carbon dioxide excretion was. Fish are tolerant of high levels of carbon dioxide as has been demonstrated using external hypercapnia and hyperoxia as probes to induce respiratory acidosis or metabolic alkalosis (Goss et al. 1992b). The rise in $P_a\text{CO}_2$ and total CO_2 is non-lethal. The levels of CO_2 accumulation seen with exposure to chloramine-T were much lower than those which have been induced with hypercapnia or hyperoxia and thus are not likely to be of pathological importance. However, correction of the metabolic acid-base disturbances caused by chloramine-T which involve the transport of acidic and/or basic equivalents across the gill (see chapter 6) is potentially a large consumer of metabolic energy since acid-base correction involves the activation, upregulation and increased mRNA transcription and expression of H^+ -ATPases (Sullivan et al. 1995; 1996). Similarly acute ion loss caused by chloramine-T exposure (chapter 6 and 7) must also be compensated for by the activation of

ionic uptake mechanisms (Na^+ - H^+ linked uptake or $\text{Cl}^-/\text{HCO}_3^-$ exchange). These processes are similarly metabolically active. Thus there may be increased metabolism within the gill itself at the expense of other metabolic processes which may be involved in growth and hence a reduced growth rate, but no overall affect on metabolic rate (as determined by oxygen consumption, chapter 5).

Perfusion or diffusion limited gas exchange

It is apparent from the data presented in chapters 2, 3 and 4 that CO_2 excretion is affected to a greater extent by chloramine-T than O_2 uptake. How can this be if both processes (MO_2 and MCO_2) are dependant on diffusion as well as convectional and ventilatory processes as defined in chapter 1?

Moreover, since water has a higher capacitance for CO_2 than O_2 , why should CO_2 excretion be primarily diffusion limited but O_2 uptake not? The answer is not particularly complex as both processes (diffusion and convection) do indeed impact both O_2 uptake and CO_2 excretion and are not mutually exclusive.

Oxygen uptake primarily relies on the arterial-venous oxygen content difference and the PO_2 gradient as the driving for O_2 diffusion. As a result of the partial

pressure gradient for O_2 , oxygen diffusion occurs readily across the gill.

Increased blood flow through the gill would lead to increased oxygen uptake up to a point at which O_2 uptake would become diffusion limited. The process of carbon dioxide excretion in fish (as in other vertebrates) requires that bicarbonate (the main form in which carbon dioxide is transported in the blood) be transported into the erythrocyte via the band-3 anion exchanger located on the erythrocyte membrane in exchange for chloride. Once inside the erythrocyte bicarbonate is then hydrogenated by carbonic anhydrase, using protons which have been liberated from deoxyhaemoglobin. The result of this hydrogenation reaction is molecular CO_2 and water. The molecular CO_2 then can diffuse across erythrocyte membrane and the epithelium. Since this process requires that bicarbonate be transported into the erythrocyte, and that protons which are liberated from haemoglobin during oxygenation be made available for the hydrogenation of bicarbonate to molecular CO_2 , this process can only begin as the blood is transported through the lamellae. The rate at which bicarbonate can be transported into the erythrocyte is the rate limiting step in this process. Thus, as the blood passes through the gills, the functional window through which CO_2 can be excreted is reduced. Therefore, even a small impediment to diffusion

across this window will cause a reduction in the rate of CO₂ excretion although not appreciably affecting O₂ uptake. In this thesis it has been suggested that branchial mucus which was secreted in response to chloramine-T exposure was sufficient to limit the diffusion of CO₂ while not affecting O₂ uptake such that within the normal physiological range of the animal, O₂ uptake is predominantly perfusion limited whereas CO₂ is predominantly diffusion limited. It has also been shown that where the blood-to-water diffusion distance has been increased because of chloride cell hyperplasia due to either hormonal stimulation with ovine growth hormone and cortisol (Bindon et al. 1994) or acclimation to artificial soft (ion-poor) water (Greco et al. 1995), carbon dioxide excretion was impaired more than oxygen uptake (in terms of arterial PCO₂ and PO₂ levels). Using a graded hypoxia regime similar to that described in chapter 5, it was shown that an increase in the epithelial barrier thickness (blood-to-water diffusion barrier) resulted in an impediment of CO₂ excretion (Bindon et al. 1994; Greco et al. 1995). The role of branchial mucus in this model is indeed interesting. Irritation of the gill by chloramine-T under moderately hypoxic conditions allows for the maintenance of arterial PO₂ and the elimination of CO₂ (chapter 3). However, under hyperoxia, even though chloramine-T causes a

significant increase in ventilation frequency arterial PCO_2 in the blood increased. This suggests that chloramine-T caused an acute secretion of branchial mucus in response to chemical irritation. This mucous secretion was impairing CO_2 excretion by increasing the diffusive distance (decrease in the diffusive conductance) of the gill which in turn increases the boundary layer. Under hypoxia there was a sufficient partial pressure gradient between the bulk water flowing over the gill and the blood to maintain or increase CO_2 excretion rates. Under hyperoxia despite increases in ventilation, the boundary layer was the primary impediment to CO_2 excretion. Similarly this model holds if the data presented in chapter 5 is considered. Even though there was a reduction in the blood-to-water diffusion distance of the gill of chloramine-T treated fish, $PaCO_2$ levels were maintained at that of control fish even though the chloramine-T treated animal was hyperventilating which would predict a reduction in the $PaCO_2$ according to existing models of gas exchange (Iwama et al. 1987). This impediment to CO_2 excretion could be explained in terms of the increased number of mucous cells and (presumably) mucus on the gill. Arterial PO_2 levels in the blood were not significantly different from controls except at PwO_2 70-80 mmHg at which points O_2 uptake in chloramine-T treated fish must have

exceeded controls even though both groups were hyperventilating. At water PO_2 of less than 80 mmHg, the significant difference between chloramine-T treated and control fish was reduced because mucus was beginning to impair oxygen uptake (see above). Thus suggesting that acclimation to repeated chloramine-T exposure occurs with a reduction in blood-to-water diffusion distance of the gill. This reduction in gill epithelial thickness partially compensates for the increased boundary layer thickness caused by branchial mucous secretion.

Branchial mucus secretion in response to chloramine-T exposure

Throughout this thesis it has been frequently suggested that branchial mucus is the cause of the respiratory and acid-base disturbances. It is unfortunate that there are no reliable techniques available for quantitative measurement of the thickness of the gill mucous coat. Therefore, the evidence presented in this thesis has eluded to mucus as the cause of these physiological disturbances by a process of elimination. The changes observed in response to acute chloramine-T exposure were rapid, occurring within minutes and were unlikely to be due to significant morphological changes in the gill such as epithelial hyperplasia. Similarly, the disturbances in CO_2 excretion could be

attributed to changes in the functional surface area of the gill due to decreased branchial vascular resistance, but this was not the case. Also, repeated intermittent exposure to chloramine-T resulted in a mucous cell hyperplasia in the gills, which may be predicted given the repeated nature of the chemical insult to the gill. Therefore, although circumstantial, there is strong evidence to support the suggestion that mucus secretion onto the gill in response to chloramine-T exposure was the cause of the reduced diffusive conductance. It should be noted that several experiments required surgical procedures to be performed on both experimental and control animals. Tricaine methanesulphonate (MS 222, the anaesthetic used in these studies) is itself a branchial irritant and although experimental and control animals were subjected to the same conditions, it is possible that some of the effects on mucus secretion may reflect the combined effects of exposure to MS 222 and chloramine-T.

Remediation of the side effects of chloramine-T

Respiratory remediation

This research clearly indicates some of the pathophysiological effects of chloramine-T on trout gills. However, how can these effects be reduced or even

eliminated? Clearly, based on the data presented in chapters 2 and 3 with an absence of any hypoxaemia during exposure, supplementation of the water with oxygen would be unnecessary. If anything it may even be detrimental, as carbon dioxide continued to accumulate in the blood and would therefore have to be eliminated. However, the blood borne levels of CO₂ caused by chloramine-T exposure are of little pathological significance (see discussion above). Moderate hyperventilation may actually occur due to a moderate hypoxia, at the stocking densities used in commercial fish farms especially when treating with chloramine-T since chloramine-T increases blood flow in individual fish and thus oxygen uptake (chapter 4). As hyperventilation allows chloramine-T exposed fish to maintain low PaCO₂ levels in spite of branchial irritation and mucous secretion, supplemental aeration may not be necessary. However, it is important that the fish do not become hypoxaemic. If that were to occur then O₂ uptake may also be impaired during chloramine-T treatment. Therefore moderate aeration of the water during treatment would be more than sufficient to offset the risk of hypoxia.

Ionic remediation

The acid-base and ionic disturbances which chloramine-T exposure causes are potentially more of a problem to the fish than the respiratory disturbances. Net ionic losses mean that energy must be expended to recover the lost ions. Moreover, the route of ionic losses may be of great significance. Chloramine-T caused losses of both Na^+ and Cl^- by transcellular rather than paracellular pathways since the polyethylene glycol efflux (PEG-4000) was not increased upon exposure to chloramine-T (chapter 6). This suggests that cellular membrane damage is occurring upon exposure to chloramine-T and hence Na^+ and Cl^- permeability is increased. The sources of this damage can only be speculated upon although the release of reactive chlorine radical from the chloramine-T degradation, hydroxyl radicals from the photo-oxidation of hypochlorite or the oxidative effects of hypochlorite itself may be sufficient to cause peroxidation of the epithelial cell plasma membranes. Indeed, chlorine has been demonstrated to cause damage to bacterial cell membranes causing a leaching of intracellular materials (Venkobacker et al. 1977). This potentially pathological side effect of chloramine-T exposure could be overcome by the addition of NaCl to the water during treatment so reducing the gradient for ion

loss and increasing the ionic substrate for ionic uptake processes (chapter 7). Indeed the use of combined therapeutic and prophylactic treatments in aquaculture is a common practice (Thorburn and Moccia 1993). The combined use of salt and chloramine-T would prove beneficial as the NaCl would offset the toxic effects of chloramine-T. However, therapeutic concentrations of salt range up to 2% (w/v) are used industrially for treating gill diseases. Indeed, in chapter 7, 17 mM NaCl (0.1% (w/v)) was more than sufficient to offset the ionic disturbances in either Ottawa city tap water or artificial soft water. Concentrations of 0.1% NaCl were sufficient to prevent growth of the etiological agent of BGD, *Flavobacterium branchiophila* (Wakabayashi et al. 1980). However, NaCl has not been shown to be effective at either reducing bacterial cell numbers, increasing survival of diseased animals or preventing BGD outbreaks. Moreover, Na⁺ and Cl⁻ ions which are accumulated by the fish during salt treatment must be eliminated. Subsequently the use of a low concentration of salt (less than 0.1% w/v) in a combined treatment with chloramine-T would be sufficient to offset the ionic effects of chloramine-T yet minimising excessive ionic uptake. Saltwater has a lower solubility of O₂ than freshwater. Indeed fish which are acutely exposed to saltwater show increased ventilation and slight

reduction in arterial PO_2 , blood oxygen content and oxygen uptake rate, but no effect on $PaCO_2$ (Maxime et al. 1991). Therefore, fish during a combined treatment may be slightly hypoxic and increase their ventilation sufficiently to maintain CO_2 excretion and O_2 uptake and thus eliminate the rise in $PaCO_2$ as demonstrated in chapters 2 and 3. Thus the addition of salt with chloramine-T in a combined treatment may be an all round beneficial remedy for chloramine-T side effects counteracting both ionic as well as respiratory disturbances.

Toxicity and safety considerations

Based on previous LC_{50} studies chloramine-T was shown to be a relatively safe chemical for the treatment of fish in aquaculture (Bills et al. 1988a,b). Indeed acutely chloramine-T appears to have relatively little pathotoxicological effects on the respiratory, acid-base and ionoregulatory physiology of the gills, the target organ for treatment and disinfection. This is in contrast to other now commonly used disinfectants such as hydrogen peroxide (Johnston et al. 1993; Powell and Perry, in press) which has been given low regulatory priority by the USFDA. However, chloramine-T is still not officially approved as a therapeutic drug for aquaculture. The effects of repeated exposure to chloramine-T,

however, are of obvious concern to aquaculturists. Reduced growth and feed conversion (Powell et al 1994; G. Sanchez pers. comm.) are obviously detrimental although metabolic rates (in terms of oxygen uptake rates) are not significantly affected (chapter 5). Reduced growth is, however, not typical of other chemical disinfectants such as formalin which may even promote growth (Speare et al. 1996; Powell et al. 1996). Thus, chloramine-T may be a suitable candidate for occasional use as a disinfectant, but repeat prophylactic practices should consider other chemicals such as formalin. Nevertheless, chloramine-T is the treatment of choice on Ontario trout farms (Thorburn and Moccia 1993) and has been clearly demonstrated to be an effective treatment for BGD (From 1980; Bullock et al. 1991). Importantly from this thesis it can be concluded that treatment of uninfected individuals is relatively safe and that any toxic effects of chloramine-T on uninfected fish can be compensated for by the combined treatment with NaCl.

Future research

Although this thesis has clearly defined the key pathophysiological effects of chloramine-T on trout gill physiology it has only examined a small area in

which our knowledge of fish physiology is lacking. It is hoped that this section will highlight some of the areas which I feel have great potential for future research in this field.

Physiological toxicology of aquaculture therapeutants

There is very little information available as to the physiological effects or mechanisms of action of aquacultural therapeutants. In most cases chemicals are being used either through emergency drug release licence or unauthorised by veterinarians as "off label" treatments in an attempt to treat or prevent disease outbreaks. This is clearly an area for increased research. With knowledge of how topical chemotherapeutic agents such as chloramine-T work and their effects on the fish, we can devise potential strategies for remediating any adverse side effects as was demonstrated by this thesis. This is particularly true for hydrogen peroxide, a drug which has been readily accepted although there is some evidence that it may be acutely toxic to trout (Powell and Perry 1996, in press).

The physiological role of branchial mucus

In terms of understanding the role and impact of gill mucus on the boundary layer and its physiology, research is essentially halted until good estimates of the mucus thickness and turnover can be made. The former requires the use of fixation techniques which are resistant to the dehydration process of microscopy. Several of these have been developed for scanning electron microscopy of fish gills (Powell et al. 1992; Lumsden and Ferguson 1993), but estimates of the mucus coat thickness requires transmission electron microscopy. Powell et al. (1993) have made estimates as to the glycocalyx thickness of different gill cell types, but measurements of the mucous coat have eluded us to date. Secondly, estimates of mucus turnover would be useful in validating the physiological impact of branchial irritants and pathogens. This area is ripe for further investigation.

The biochemical physiology of chemical and biological gill irritation

The biochemical effects of chemical and biological irritants (drugs or pathogens) on gill cell physiology is another area where research is needed. It may be possible for instance to eliminate the transcellular net ion losses caused

by chloramine-T by supplementing the fish diet with antioxidants and thus reduce radical-induced membrane damage. All of these suggestions and questions which are raised from this thesis will, in time be answered, and will hopefully provide us with further insight into both the basic physiology of the fish gill as well as being practical contributions to fish physiology and biology.

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