THE PHYSIOLOGICAL ADAPTATIONS OF TWO SALMONIDS, THE RAINBOW TROUT (*Oncorhynchus mykiss*) AND THE HIGH pH TOLERANT LAHONTAN CUTTHROAT TROUT (*Oncorhychus clarki henshawi*), TO HIGHLY ALKALINE ENVIRONMENTS

By

MICHAEL PATRICK WILKIE, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

McMaster University

DOCTOR OF PHII.OSOPHY (1994) McMASTER UNIVERSITY

(Department of Biology) Hamilton, Ontario

TITLE: The Physiological Adaptations of Two Salmonids, the Rainbow trout (*Oncorhynchus mykiss*) and the High pH Tolerant Lahontan Cutthroat Trout (*Oncorhynchus clarki henshawi*), to Highly Alkaline Environments.

AUTHOR: Michael Patrick Wilkie, B.Sc. (McMaster University)

SUPERVISOR: Professor Chris M. Wood

NUMBER OF PAGES: xxviii, 327

THE PHYSIOLOGY OF SALMONIDS IN HIGHLY ALKALINE ENVIRONMENTS

ABSTRACT

Efforts to stock salmonids into alkaline waters have had little success. Accordingly, this thesis examined the survival and physiology of two salmonids, the rainbow trout (RBT; *Oncorhynchus mykiss*) and the high pH tolerant Lahontan cutthroat trout (LCT; *Oncorhynchus clarki henshawi*) experimentally exposed to alkaline pH. Exposure of RBT (acclimated at pH 8.0) to pH 9.5, for up to 4 weeks, led to minimal mortality and was characterized by transiently inhibited ammonia excretion rates (J_{Amm}), and chronically elevated plasma and white muscle total ammonia stores ($T_{Amm} = NH_3 + NH_4^+$). Elevated plasma T_{Amm} facilitated recovery of J_{Amm} by maintaining positive NH_3 gradients across the gill epithelium. Temporarily elevated urea excretion rates prevented internal T_{Amm} from reaching toxic levels while J_{Amm} was inhibited. A chronic respiratory alkalosis (decreased blood P_{CO2}) rapidly developed at high pH, but a counterbalancing metabolic acidosis (decreased blood $[HCO_3^-]$), which initially resulted from elevated white muscle lactic acid production, stabilized blood pH after 1 day. Transient and chronic decreases in gill Cl^- transporter (J^{Cl}_{max}) and Na^+ transporter (J^{Na}_{max}) number, respectively, resulted in decreased Cl^- and Na^+ uptake and led to reduced plasma $[Cl^-]$ and $[Na^+]$. A complete recovery of J^{Cl}_{max} (by 3 days) and counterbalancing reductions in gill Na^+ losses, re-established internal ion balance beyond 3 days. In contrast, the LCT (reared at pH 8.4) never before exposed to alkaline pH, rapidly adapted to the alkaline (pH 9.4) waters of Pyramid Lake, Nevada, their natural adult habitat. These adaptions included permanently reduced basal ammonia production which was reflected

in chronically lowered J_{Amm} and minimal, temporary increases in plasma T_{Amm}. Chronic elevations in plasma NH_3 were not required for maintenance of J_{Amm} in the LCT. The LCT also underwent a chronic respiratory alkalosis and a transient metabolic alkalosis but increases in gill chloride cell exposure quickly mitigated the continual base load by facilitating HCO_3^- excretion. No disturbances to internal ion balance were observed. The responses of LCT to challenge in more alkaline (pH 10) lake water resembled those of RBT at pH 9.5 (15°C) and greatly increased plasma NH_3 and reduced plasma ion concentrations, resulted in 50 % mortality by 72 h. In conclusion, alterations in N-waste metabolism and gill functional morphology facilitate salmonid survival at high pH. However, the ability of LCT to rapidly make these adjustments, with minimal disturbance to its pre-exposure physiological status, enables it to survive in an environment which is unsuitable for other salmonids.

ACKNOWLEDGEMENTS

It is probably difficult, if not impossible, for one to complete their Ph.D. studies without the generous support and encouragement of others. In this regard I have been very fortunate to have worked in McMaster's Department of Biology for the last several years and I would like to express my thanks to everyone who helped make my stay at McMaster both enjoyable and productive.

Firstly, I would like to thank my supervisor, Chris Wood, for giving me the opportunity to work on a fascinating project, despite my "colourful" undergraduate background. His sound advice, patience, understanding and keen intellect, regarding not only my project, but other broader issues, is sincerely appreciated.

I would also like to thank the members of my supervisory committee, Gord McDonald, George Heigenhauser and Mike O'Donnell, for their continuous encouragement and constructive suggestions and criticisms over the last few years. I am also grateful to my "surrogate" committee, which included Drs. Rick Playle, Rick Gonzalez, Rod Wilson, Scott Reid, Pat Chow-Fraser and Peter Part, for their constructive comments and for listening to, and usually logically dismantling, many of my arguments (Scientific arguments too).

I am also grateful to Drs. Patricia Wright and George Iwama; their contributions and support (and patience) on the Lahontan cutthroat trout project were invaluable. I also thank Drs. John Lott and Greg Goss, and Derek Flannigan and Klaus Schultes, for providing valuable instructional and technical guidance regarding the treatment and analysis of gill tissue samples for electron microscopy. I am also grateful to Pat Hayward, Barb Reuter and the rest of the office staff for their never-ending generosity and assistance.

I would also like to thank Tony Stea, Randy Lauff, Yuxiang Wang, Marjorie Patrick, Erica Hawkins and Nathalie Patenaude for their valued friendship, advice, tolerance (at times) and "off the wall" humour. The friendship and assistance of Steve Munger, my alternate mentor, was invaluable. The comradery and friendship that I enjoyed with all of my other lab, office and softball teammates, including Steve Alexander, Christer Hogstrand, Jacqui Dockray, Tyler Linton, Annika Salama, Shaheen Zia, Russ Ellis, Rod Rhem, Monica Scarabello, Paul Wilson, Chris Schutz, Brian Driscoll and many others will not be forgotten. The patience and support of Dr. Bruce Tufts is also greatly appreciated.

I would also like to recognize the contributions of my parents, Gord and Mary, who provided constant encouragement, as well as financial aid (to bail me out of the financial messes I usually found myself in), over the course of my undergraduate and graduate studies. I would also like to thank the Mitton family for their support and endless hospitality.

Finally, I would like to thank Cindy Mitton, whose constant love, understanding, advice, and occasional "kicks-in-the-butt", allowed me to "keep my head above water" during difficult times.

THESIS ORGANIZATION AND FORMAT

In consultation with my supervisory committee, it was decided that this thesis would be organized in the "Open-faced" format recently approved by McMaster University. Accordingly, this thesis comprises six chapters which are manuscripts that have been published, accepted for publication, submitted for publication, or are to be submitted to peer review scientific journals. A seventh chapter (Chapter 1), provides a general introduction and synthesis of the results and interpretations presented in the aforementioned chapters. A model to describe salmonid physiology at high pH is presented in the final chapter (Chapter 8). Additional, related work is presented in appendix one.

Chapter 1: General Introduction and Synthesis of Findings.

Chapter 2: Nitrogenous waste excretion, acid-base regulation, and ionoregulation in rainbow trout (*Oncorhynchus mykiss*) exposed to extremely alkaline water.

Authors: M.P. Wilkie and C.M. Wood (referred to as Wilkie and Wood, 1991).

Date accepted: January 31, 1991.

Journal: Physiological Zoology 64:1069-1086 (1991).

Comments: Data were generated exclusively by M.P.W., under the supervision of C.M.W.

Chapter 3: Recovery from high pH exposure in the rainbow trout: Ammonia wash-out and the restoration of blood chemistry.

Authors: M.P. Wilkie and C.M. Wood (referred to as Wilkie and Wood, 1994b).

Date accepted: Provisionally accepted on August 18, 1994.

Journal: Physiological Zoology.

Comments: Data were generated exclusively by M.P.W. under the supervision of C.M.W.

Chapter 4: Modulation of branchial ion movements by rainbow trout at high pH.

Authors: M.P. Wilkie, P. Laurent and C.M. Wood [referred to as Wilkie, Laurent, and Wood, unpublished data (Chapter 4)].

Comments: This study has not yet been submitted to a scientific journal. It is part of a collaborative project with Dr. Pierre Laurent, Centre National de la Recherche Scientifique, Strasbourg, France. The portion of the study presented here describes high pH induced changes in branchial Cl^- and Na^+ fluxes and links them with changes that occur in the fish's ion and acid-base balance. This section of the work was performed exclusively by M.P.W., under the supervision of C.M.W., and is presented here in its entirety. The second part of the study examines the possible linkage of ion flux events with modifications in trout gill morphology at high pH. Dr. Laurent is currently performing the morphological analyses in Strasbourg; this latter data will not be presented in this thesis.

Chapter 5: The physiological adaptations of the Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*), following transfer from well water to the highly alkaline waters of Pyramid Lake, Nevada (pH 9.4).

Authors: M.P. Wilkie, P.A. Wright, G.K. Iwama and C.M. Wood (referred to as Wilkie *et al.*, 1994).

Date accepted: October 1, 1993.

Journal: Physiological Zoology 67:355-380 (1994).

Comments: The major portion of this work were performed by M.P.W. under the supervision of C.M.W., with minor contributions and considerable logistical support from G.K.I. and C.M.W. P.A. Wright performed all the enzymatic analyses.

Chapter 6: The physiological responses of the Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*), a resident of highly alkaline Pyramid Lake, Nevada (pH 9.4), to challenge at pH 10.

Authors: M.P. Wilkie, P.A. Wright, G.K. Iwama and C.M. Wood (referred to as Wilkie *et al.*, 1993).

Date accepted: October 13, 1992.

Journal: Journal of Experimental Biology 175:173-194 (1993).

Comments: The major portion of this work were performed by M.P.W., with help and supervision from C.M.W. P.A. Wright performed all the enzymatic analyses. G.K.I. provided considerable logistical support.

Chapter 7: Nitrogenous waste excretion, acid-base regulation, an ionoregulation in rainbow trout (*Oncorhynchus mykiss*) exposed to extremely alkaline water.

Authors: M.P. Wilkie, H.E. Simmons and C.M. Wood (referred to as Wilkie *et al.*, 1995).

Date submitted: May, 1994.

Journal: Fish Physiology and Biochemistry.

Description: Data were generated exclusively by M.P.W., with important technical contributions by H.E. Simmons, under the supervision of C.M.W.

Chapter 8: A model to describe salmonid physiology at high pH.

Appendix 1: The effects of extremely alkaline water (pH 9.5) on rainbow trout gill function and morphology.

Authors: M.P. Wilkie and C.M. Wood (referred to as Wilkie and Wood, 1994a).

Date accepted: August 29, 1993.

Journal: Journal of Fish Biology 45:87-98 (1994).

Comments: This publication is pertinent to this thesis and contains data that were exclusively generated by M.P.W., under the supervision of C.M.W. However, the gill morphological data were obtained by M.P.W. as part of his required research project in a graduate course in electron microscopy that was taught by Dr. J.N.A. Lott.

Therefore, these data are not included as a chapter in this thesis, but only as an appendix.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 7

CHAPTER 8

APPENDIX 1

LIST OF TABLES

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 1

GENERAL INTRODUCTION AND SYNTHESIS OF FINDINGS

BACKGROUND

Numerous eutrophic lakes and ponds undergo diurnal and seasonal increases in water pH due to decreases in water CO_2 concentration that accompany high rates of algal and/or macrophytic photosynthesis (Jordan and Lloyd, 1964; Wetzel, 1983; Barica, 1974, 1990; Murray and Ziebell, 1984; Hansen *et al.*, 1991). Decreases in the concentration of this weak acid often lead to water pH's that exceed pH 9.0 (Wetzel, 1983; Barica, 1974; 1990) and occasionally approach pH 10.0 (Wetzel, 1983; Hansen *et al.*, 1991). Such photosynthetically induced pH surges have been directly implicated, or cited as contributing factors, in a number of fish kills (Eicher, 1946; Jordan and Lloyd, 1964; Barica, 1974; Alabaster and Lloyd, 1980). In addition to these transiently alkaline lakes, there are many permanently alkaline lakes which contain healthy fish populations and which are characterized by high concentrations of dissolved HCO_3^- and $CO_3^=$ salts. For instance, Pyramid Lake, Nevada, has concentrations of dissolved HCO_3^- that approach 16 $mmol \cdot l^{-1}$ and a resultant pH of 9.2 (Galat *et al.*, 1981). Lake Magadi, Kenya, which is fed by $NaHCO_3$ and Na_2CO_3 rich volcanic springs, has total [HCO_3^- + $CO_3^=$] concentrations approximating 180 mM and a resultant pH of 10 (Reite *et al.*, 1974; Maetz and De Renzis, 1978; Johansen *et al.*, 1975; Wood *et al.*, 1989), while the waters of Lake Van, Turkey, the world's largest "soda" lake, approximate

pH 9.8 (Danulat and Selcuk, 1992).

Attempts to stock salmonids, such as rainbow trout, brown trout (*Salmo trutta*), kokanee (*Oncorhynchus nerka*), and coho salmon (*Oncorhynchus kisutch*), into a number of permanently alkaline North American lakes, including Pyramid Lake (Nevada), Lake Omak (Washington) and Green Lake (British Columbia) have met with only limited success due to high levels of post-stocking stress and mortality (Kucera *et al.*, 1985; Coleman and Johnson, 1988; Yesaki, 1990). In general, very little is known about the physiological responses of salmonids exposed to these conditions. However, gradual acclimation to alkaline pH (Jordan and Lloyd, 1964; Murray and Zeibell, 1984) and increased water hardness (Yesaki and Iwama, 1992) both appear to significantly enhance rainbow trout survival at high pH.

The effectiveness of the recently developed eutrophication-mitigation technique, known as ($CaCO_3$ + CaOH) liming, depends upon elevations in water pH. These increases in water pH, sometimes approaching pH 10.0, lead to the formation of $CaCO_3$ which simultaneously forms complexes with orthophosphate (eg. phosphorous), an essential nutrient for aquatic macrophytes and algae (Wetzel, 1983). This co-precipitation effectively removes the orthophosphate from the water column and makes it unavailable to aquatic autotrophs (Avnimelnech, 1980; Prepas *et al.*, 1990; Murphy *et al.*, 1990). This technique is also employed to prevent summer and winter fish kills, that result from dissolved O_2 depletion following algal bloom crashes (Barica, 1974; Ayles *et al.*, 1976). Since artificial increases in water pH could actually confound efforts to preserve fish populations, a basic understanding of how salmonids respond to acute and chronic elevations in environmental pH would be useful to both limnologists and fishery managers.

SALMONID SURVIVAL AT HIGH pH

Few studies have examined salmonid survival and physiology in alkaline environments ($pH \geq 9.0$). Most previous studies on altered environmental pH have focussed on how environmental acidification affects fish survival and physiology, due to the ongoing problem of acid rain (see McDonald, 1983a; Wood, 1989 for reviews). Most of the previous work at alkaline pH has been toxicological, rather than physiological (see Alabaster and Lloyd, 1980 for review). These toxicological studies have demonstrated that warmwater fish, such as largemouth bass (*Micropterus salmoides*), goldfish (*Carassius aurtatus*), sunfish (*Lepomis gibbosus*) and bluegills (*Lepomis macrochirus*) may tolerate pH's as high as 10.5 (Wiebe 1931; Sanborn, 1945). Work on the brook trout (*Salvelinus fontinalis*), however, indicated that these coldwater fish are less tolerant to alkaline pH with upper lethal limits of approximately 9.8 (Daye and Garside, 1975). Work on the rainbow trout is less conclusive. Jordan and Lloyd (1964) reported that rainbow trout were able to tolerate pH 9.8 for 15 days but significant mortality has been reported at pH's between pH 8.8 and 10.1 (Jordan and Lloyd, 1964; Alabaster and Lloyd, 1980; Heming and Blumhagen, 1988; Yesaki and Iwama, 1992). Murray and Ziebell (1984) reported significant mortality following abrupt introduction of rainbow trout into pH 9.5 water but this effect was eliminated when the fish were gradually introduced (acclimated) to elevated pH. Thus, factors such as acclimation history (Jordan and Lloyd, 1964; Murray and Zeibell, 1984), as well as water quality (Yesaki and Iwama, 1992), may account for the differential responses of salmonids to high pH. Clearly, these fish are capable of at least limited survival at alkaline pH but little is known about the acute (< 3 days) or chronic (> 3 days) physiological responses that enable fish to tolerate alkaline pH or ultimately lead to death.

SALMONID PHYSIOLOGY AT HIGH pH

The limited physiological data available suggest that fish exposed to high pH suffer from three main types of physiological disturbance: blockade of ammonia excretion (Cameron and Heisler, 1983; Wright and Wood, 1985; Lin and Randall, 1990; Yesaki and Iwama, 1992; Wright, 1993), increased blood pH (alkalosis; Johansen *et al.*, 1975; Wright and Wood, 1985; Yesaki and Iwama, 1992), and severe disturbances to internal ion balance (Heming and Blumhagen, 1988; Yesaki and Iwama, 1992).

Nitrogenous Waste Excretion and Metabolism

Ammonia chemistry will be briefly reviewed to illustrate why high pH affects ammonia excretion (J_{Amm}) by fish. In solution, ammonia exists as ionized NH_4^+ or unionized NH_3. The sum of NH_4^+ + NH_3 comprises the total ammonia concentration (T_{Amm}) of a solution, with a dissociation constant (pK'_{Amm}) of approximately 9.5 (at 15°C):

$$NH_4^+ + H_2O \rightleftarrows NH_3 + H_3O^+ \quad (1)$$

Since the classic work of Smith (1929) it has been widely accepted that ammonia-N constitutes at least two-thirds of the nitrogenous waste (N-waste) produced by ammoniotelic teleosts and that 90 % J_{Amm} is via the gill epithelium, while the remainder is excreted via renal pathways or across the skin (see Wood, 1993 for review). However, the mechanism(s) by which ammonia is excreted across the branchial (gill) epithelium remains a subject of considerable controversy (See Heisler, 1990; Wood, 1993 for recent reviews). For instance, Cameron and Heisler (1983) and Heisler (1990) have argued that transbranchial NH_3 diffusion, down favourable blood-water P_{NH3} gradients (ΔP_{NH3}), predominates under normal environmental circumstances, while other workers have proposed that branchial Na^+/NH_4^+ exchange, in which Na^+ uptake is coupled to NH_4^+ extrusion is the important mechanism (Maetz and

Garcia-Romeu, 1964; Maetz, 1973; Wright and Wood, 1985; McDonald and Milligan, 1988; Yesaki and Iwama, 1992). Since, the pK'_{Amm} approximates 9.5, elevations in water NH_3 concentration invariably accompany increases in external pH (Emerson *et al.*, 1975; Cameron and Heisler, 1983). Hence, short-term increases in environmental pH (pH 9.5; 3 h) are thought to inhibit J_{Amm} by reducing the blood to gill water boundary layer P_{NH3} diffusion gradient (Cameron and Heisler, 1983; Wright and Wood, 1985; Wright *et al.*, 1989) . Wright and Wood (1985) have also suggested that high pH may inhibit J_{Amm} through direct affects on the Na^+/NH_4^+ exchanger, because Na^+ uptake at the gill tends to fall during alkaline exposure. There is little information to describe how longer term high pH exposure (> 3 h) affects J_{Amm} patterns in rainbow trout. The fact that some fish are capable of excreting ammonia against P_{NH3} gradients that would normally oppose J_{Amm} (Maetz, 1972, 1973; Cameron and Heisler, 1983; Wright and Wood, 1985; Heisler, 1990) and that salmonids can tolerate long periods of elevated ambient ammonia (Burrows, 1964; Rice and Stokes, 1974; Thurston *et al.*, 1984; Solbe and Shurben, 1989) suggest that rainbow trout might re-establish J_{Amm} during more prolonged high pH exposure. The increases in plasma ammonia that accompany high pH exposure (Wright and Wood, 1985; Lin and Randall, 1990) may lead to the re-establishment of favourable blood-gill water ΔP_{NH3} and ultimately account for the recovery of J_{Amm} during chronic high pH exposure. Thus, a major focus of this thesis was to follow ammonia excretion patterns during longer term high pH exposure (3 h to 4 weeks) and dissect the mechanisms by which ammonia is excreted, if at all, under such conditions.

The resultant retention of ammonia, due to high pH-induced blockade of J_{Amm} (Wright and Wood, 1985; Lin and Randall, 1990), could also ultimately result in ammonia toxicity

and death. Ammonia primarily affects the central nervous system and its stimulatory affects on neurons lead to hyperexcitability and increased ATP consumption (Hillaby and Randall, 1979; Arillo *et al.*, 1981). This effect, in combination with a simultaneous impairment of oxidative metabolism, ultimately exhausts the animals ATP stores and leads to coma and eventually death (Arillo *et al.*, 1981). These potentially harmful effects might be circumvented, however, by storing the ammonia in relatively inert tissues, such as the white muscle, until conditions are again conducive to J_{Amm}. Since, the white muscle intracellular compartment comprises approximately 45 % of the fish's total weight (Stevens, 1968; Milligan and Wood, 1986a,b) it constitutes a vast potential ammonia reservoir in trout. The possibility that the white muscle played such a role during high pH exposure was investigated in the present series of investigations, by following changes in the white muscle T_{Amm} concentration, as well as extracellular fluid (ECF = plasma) T_{Amm} levels, during acute and chronic high pH exposure.

Another strategy to avoid ammonia toxicity would be to convert N-waste to alternate, less toxic end-products, such as urea (Saha and Ratha, 1987, 1989; Randall *et al.*, 1989; Walsh *et al.*, 1990) or glutamine (Levi *et al.*, 1975; Arillo *et al.*, 1981). These alternate N-waste products might then be stored internally or be excreted by the animal during the period of inhibited J_{Amm}. Urea excretion (J_{Urea}) is one such strategy employed by teleosts such as the gulf toadfish (*Opsanus beta*; Walsh *et al.*, 1990) and the air-breathing catfish (*Heteropneustis fossilis*; Saha and Ratha, 1987, 1989) under conditions, such as air exposure, that prevent normal branchial J_{Amm}. In fact, the tilapia (*Oreochromis alcalicus grahami*) of Lake Magadi, Kenya, which thrives in that lakes' alkaline (pH 10.0) waters, is completely ureogenic and excretes all of its N-waste as urea (Randall *et al.*, 1989; Wood *et al.*, 1989). Rainbow trout

are also known to elevate urea excretion rates during prolonged exposure to high environmental ammonia (Olson and Fromm, 1971). As yet, no study has examined the potential role that elevated J_{Urea} plays in nitrogenous waste excretion ($J_{Waste-N}$) in trout at high pH. Accordingly, the excretion patterns and internal storage of urea by rainbow trout were followed during acute and chronic high pH exposure.

Acid-base Balance

High pH-induced acid-base disturbances can be respiratory (decreased arterial blood P_{CO2}; Pa_{CO2}) or metabolic (increased metabolic HCO_3^-) in origin (Johansen *et al.*, 1975; Wright and Wood, 1985; Heming and Blumhagen, 1988). An increase in water pH, to approximately 9.5, shifts the CO_2 dissociation curve in water far to the right, and ultimately results in large reductions in the water concentration of gaseous CO_2 (Wetzel, 1983). This decrease in water P_{CO2} creates a CO_2 "vacuum" that results in extremely low values of blood P_{CO2} (Johansen *et al.*, 1975). This high pH-induced respiratory alkalosis, has been observed in rainbow trout (Wright and Wood, 1985; Lin and Randall, 1990) but metabolic alkaloses have also been reported (Heming and Blumhagen, 1988). Therefore, a goal of this thesis was to further characterize the internal acid-base status of rainbow trout during acute and chronic high pH exposure and establish what, if any, compensatory adjustments took place. Potential adjustments might include increased lactic acid production, which is known to be stimulated by decreases in the blood P_{CO2} in mammals (Bock *et al.*, 1932; Eichenholz *et al.*, 1962; Takano, 1968; Garcia *et al.*, 1971) and/or differential changes in net Na^+ vs. Cl^- movements across the gill epithelium (McDonald *et al.*, 1989) resulting in the movement of acidic or basic equivalents.

It is now well established, according to the Strong Ion Difference theory and the

constraints of electroneutrality (Stewart, 1983), that on a net basis, Na^+ and Cl^- are taken up across the branchial epithelium for acidic (eg. H^+, NH_4^+) and basic equivalents (eg. HCO_3^-, OH^-), respectively (see McDonald *et al.*, 1989; Wood, 1991 for reviews) and that modulation of Na^+ vs. Cl^- transbranchial movements plays a key role in the maintenance of internal acid-base homeostasis following environmentally or experimentally induced acid-base disturbances. These disturbances included abrupt temperature change (Cameron, 1976), low pH (McDonald *et al.*, 1983), acid or base infusion (Goss and Wood, 1990b; 1991), hypercapnia (Cameron, 1976; Cameron and Iwama, 1987) and hyperoxia (Wood *et al.*, 1984). Wright and Wood (1985) and Wood (1989) measured unidirectional Na^+ and Cl^- movements the across gill epithelium during very short-term alkaline exposures, but they made no attempt to establish what role these movements might play in correcting high pH-induced internal acid-base disturbances. Accordingly, a major focus of this thesis was to examine how Na^+ and Cl^- influx, outflux and net fluxes were altered in rainbow trout during both short and long-term high pH exposure and establish how these ion movements affected acid-base homeostasis, as well as internal ion balance.

Maintenance of Internal Ion Balance

Reductions in plasma Na^+ and Cl^-, leading to internal fluid shifts, red blood cell swelling and ultimately circulatory failure have been implicated as the primary cause of death in salmonids exposed to acidic pH (Milligan and Wood, 1982). In some cases, mortality at high pH may be attributable to similar decreases in plasma Na^+ and Cl^- concentration (Heming and Blumhagen, 1988; Yesaki and Iwama, 1992). To date there is very little information that describes how these disturbances are initiated, let alone corrected by fish during high pH exposure. Wright and Wood (1985) have reported decreased Na^+ influx

during short-term high pH exposure in rainbow trout. Wood (1989) has reported similar findings for Cl^- influx, as well as increases in Na^+ and Cl^- outflux during high pH exposure. However, neither of these studies examined longer term responses. Therefore, an important goal of this thesis was to establish whether high pH-induced alterations in ion flux patterns and internal electrolyte status occurred during more prolonged alkaline exposure.

In the last few years, considerable data has accumulated that indicates gill chloride cells (CC; also referred to as mitochondria rich cells) are probably the site of branchial Cl^- uptake, via Cl^-/HCO_3^- exchange, and possibly the site of Na^+ uptake, via Na^+/H^+ exchange, in freshwater teleosts (Laurent *et al.*, 1985; Perry and Laurent, 1989; Perry *et al.*, 1992). For example the fractional surface area (FSA) of exposed CCs on the gill epithelium increases when fish are exposed to dilute environments (Laurent and Dunel, 1980; Laurent *et al.*, 1985; Perry and Laurent, 1989; Laurent and Perry, 1990) or softwater (Perry and Wood, 1985). It has also been shown by Goss and colleagues (1992a,b) that experimentally induced internal alkalosis, leads to increases in branchial CC FSA. These increases in CC FSA may help regulate blood pH by facilitating higher rates of base removal through elevations in the number of Cl^-/HCO_3^- exchangers. Furthermore, decreases in branchial CC FSA during acidosis have led to the suggestion that the gill pavement cells, rather than the CC, are the more important sites of Na^+ uptake in freshwater teleosts (Goss *et al.*, 1992a). Clearly, alterations in branchial CC number and/or surface area during exposure to high environmental pH might provide fish with a valuable mechanism by which they could correct high pH-induced internal ion and acid-base disturbances. Appendix 1 (Wilkie and Wood, 1994a) addresses these possibilities.

THE HIGH pH TOLERANT LAHONTAN CUTTHROAT TROUT

The Lahontan cutthroat trout (LCT) of Pyramid Lake, Nevada is an unusual salmonid which appears particularly suited for survival in alkaline water (pH 9.4; Wright *et al.*, 1993). Formerly, the LCT thrived in Pyramid Lakes' alkaline waters and supported a huge commercial and sport fishery (Wheeler, 1987; Coleman and Johnson, 1988; Cerveri, 1990). These fish also grew to legendary sizes; the world record cutthroat trout, weighing 18.6 kg, was caught in Pyramid Lake in 1925 (Coleman and Johnson, 1988). Prior to the mid-1900's, the adults of this lacustrine salmonid returned annually to spawn in the freshwater of the Truckee River, and young fish spent most of their juvenile life there before migrating into Pyramid Lake. Unfortunately, frequent droughts and the diversion of the Truckee River, which was the only water input into this terminal lake, for agricultural and municipal uses, led to continual declines in the lakes' water levels and eventually made the productive spawning beds of the Truckee inaccessible to mature LCT. By 1944 the original Pyramid Lake strain of LCT was declared extinct but in the early 1960's the fishery was revived through the initiation of a vigorous trout planting program (Coleman and Johnson, 1988). Despite the remarkable success of this program, the LCT is still designated as a threatened species due to the paucity of successfully reproducing populations (Williams *et al.*, 1989). The eggs and sperm required for this stocking program are obtained from mature adults that are netted as they migrate up an artificial spawning channel adjacent to the Pyramid Lake fish hatchery. The resultant progeny are then hatchery reared in well water (pH 8.4) for 1 year, after which they are introduced into the lakes' alkaline waters.

The Lahontan cutthroat trout readily adapts to the highly saline and alkaline water of Pyramid Lake and does not exhibit the visible signs of stress (eg. changes in behaviour or

body pigmentation) or suffer the degree of mortality that was seen when attempts were made to stock other salmonids such as the rainbow trout, brown trout (*Salmo trutta*), coho salmon (*Oncorhynchus kisutch*), brook trout (*Salvelinus fontinalis*), and Yellowstone cutthroat trout (*Oncorhynchus clarki bouvieri*) into the lake (Coleman and Johnson, 1988). Due to the LCTs' remarkable ability to adapt and thrive in Pyramid Lake, experiments were designed to assess what physiological adaptations distinguished this fish from other salmonids, such as the rainbow trout, that were unable to adapt to Pyramid Lake water. Physiological parameters, similar to those described above, were measured to assess how ammonia and urea excretion, acid-base and internal ion balance were affected immediately following transfer, and after prolonged residence, in Pyramid Lakes' alkaline waters.

Continued diversion of the water of the Truckee River and frequent drought may not only lower lake water levels but also concentrate Pyramid Lakes' alkaline salts and potentially result in increased water pH (Galat *et al.*, 1981, 1983). To assess how this might affect resident LCT, an experiment was designed that monitored the animals physiological responses and mortality during 3 days of exposure to a further elevation of lakewater pH to 10.

SUMMARY OF OBJECTIVES

Keeping the previous framework in mind, the goals of this thesis were to:

1. Characterize the nature of the acute (3 days) physiological responses and adaptations of rainbow trout to high pH (pH 9.5) by focussing upon the effects that alkaline water had on N-waste excretion, acid-base status and internal ion balance (Chapter 2).

2. Examine the ability of rainbow trout to recover from acute (2 days) high pH exposure upon return to water of normal pH (pH 8.0) by following ammonia storage and excretion patterns, acid-base balance, plasma ions and arterial blood P_{O_2} during a 48 h post-exposure period (Chapter 3).

3. Characterize how high pH exposure altered Na^+ and Cl^- influx and outflux patterns across the rainbow trout branchial epithelium and utilize saturation kinetic analysis to elucidate how changes in gill ion transporter number (J^{ION}_{max}) and/or affinity (K^{ION}_m) initiated, and possibly corrected, high pH-induced changes in transbranchial ion movements (Chapter 4).

4. Describe the acute and long-term physiological responses that permitted the high pH tolerant Lahontan cutthroat trout to readily adapt to the highly alkaline waters (pH 9.4) of Pyramid Lake, Nevada by focussing upon adjustments in N-waste excretion, acid-base balance, ionoregulation and gill morphology following transfer into the

alkaline lakewater from pH 8.4 well water (Chapter 5).

5. Describe the physiological responses of lakewater adapted Lahontan cutthroat trout to challenges at higher pH (pH 10) and describe how these responses either permitted survival or ultimately lead to death (Chapter 6).

6. Determine if rainbow trout were capable of surviving for prolonged periods (28 d) at high pH (pH 9.5), to describe the physiological responses that either permitted the fish survive or ultimately led to death, and to compare these observations to those made on the high pH tolerant Lahontan cutthroat trout (Chapter 7).

7. Construct an integrative model that describes the physiological responses and adaptations of rainbow trout to highly alkaline water (Chapter 8).

SYNTHESIS OF FINDINGS

RAINBOW TROUT

Survival and Stress at High pH

Although rainbow trout were able to survive in alkaline freshwater (pH 9.5) for 3 days (acute exposure), this acute exposure still resulted in 40 % mortality. However, the additional stresses imposed upon the fish by catheterization, blood sampling and confinement (in flux boxes), may have made the fish more susceptible to mortality at high pH. This hypothesis was verified in separate chronic (28 d) high pH exposure experiments, with uncannulated, freely swimming fish, where total mortality was less than 5 %. Although primary stress parameters, such as cortisol and glucose, were not examined during acute high pH exposure, analyses on blood samples taken from fish between 3 d and 28 d of chronic exposure yielded plasma glucose and cortisol concentrations that approximated those of resting, unstressed rainbow trout (Woodward and Strange, 1987). No changes in secondary stress parameters, such as hematocrit and hemoglobin, were seen during chronic high pH exposure at 10°C. However, chronic exposure at 15°C led to pronounced 30-50 % increases in hematocrit and hemoglobin, without corresponding changes in mean cell hemoglobin concentration. These data suggest that high pH exposure at 15°C is more energetically demanding than at 10°C. The stable blood Pa_{O_2} before, during and after high pH exposure, suggests that O_2 uptake was not impaired and therefore, did not account for the elevations of hemoglobin and hematocrit seen at 15°C. Furthermore, O_2 delivery to the tissues was not likely impaired because white muscle ATP and creatine phosphate concentrations were stable after 2 days of pH 9.5 exposure. Thus, rainbow trout, not subjected to additional exogenous

stressors, can readily survive for prolonged periods at high pH but the exposure is more energetically demanding at warmer temperatures.

Nitrogenous Waste Excretion and Metabolism

In agreement with previous studies (eg. Cameron and Heisler, 1983; Wright and Wood, 1985) acute high pH exposure led to initial decreases in ammonia excretion rates (J_{Amm}) and corresponding increases in plasma total ammonia (T_{Amm}). These initial reductions in J_{Amm} might have resulted from blockade of Na^+/NH_4^+ exchange or decreases in the blood-to-gill boundary layer water P_{NH3} gradient (ΔP_{NH3}). Ammonia excretion generally recovered to pre-exposure rates by 24-48 h and chronic increases in blood T_{Amm} likely facilitated this recovery and sustained J_{Amm} during long-term (28 d) high pH exposure by maintaining elevations of the blood-to-gill boundary layer ΔP_{NH3}. The possibility of Na^+/NH_4^+ exchange at high pH seems unlikely since Wilkie and Wood [1994a (Appendix 1)] demonstrated that inhibition of Na^+ influx (J^{Na}_{in}), with the Na^+ channel blocker amiloride, had no corresponding effect on J_{Amm}. Further experiments, exploring the potential interactions between J^{Na}_{in} and J_{Amm}, corroborated these findings and saturation kinetics experiments also revealed no correlation between increased Na^+ uptake rate vs. J_{Amm} at high pH. The possibility of H^+/NH_4^+ exchange at high pH seems unlikely because of the paucity of H^+ in alkaline water and NH_4^+ diffusion seems unlikely in a freshwater teleost (Heisler, 1990).

Inhibited J_{Amm} (Chapter 2) led to an "ammonia deficit" that approximated 4 500 umol $N \cdot kg^{-1}$ during the first 48 h at pH 9.5. Only a small proportion (3 %) of this deficit, however, could be explained by ammonia storage in the extracellular fluid space. Transiently elevated urea excretion rates (J_{Urea}) accounted for about 20 % of this ammonia deficit and probably served as an ammonia detoxification mechanism by ensuring that minimal rates of

N-waste excretion took place while J_{Amm} was reduced. However, elevations of J_{Urea} did not persist beyond 3 days in fish chronically exposed to pH 9.5, although the percentage contribution of J_{Urea} to $J_{Waste-N}$ increased from approximately 10 % to about 25 %, during chronic exposure at 10°C.

To establish the fate of the "missing ammonia" an experiment was designed that followed J_{Amm} patterns through 48 h at pH 9.5 and a subsequent 48 h recovery period at pH 8.0. The ammonia deficit incurred by the fish during this experiment was fully repaid by 24 h of high pH exposure. The first 12 hours of the recovery period were characterized by an ammonia wash-out of approximately 5 000 umol·kg^{-1}, with 5-fold elevations of J_{Amm} during the first few hours. The ammonia wash-out, in the absence of an ammonia deficit, suggested that the fish actually increased the metabolic production of ammonia during the 48 h pH 9.5 exposure period. Subsequent experiments suggested that at least 40 % of the surplus T_{Amm} originated in the white muscle intracellular fluid (ICF), where T_{Amm} concentrations were about 2.5-fold greater in fish held at pH 9.5 for 48 h.

These high levels of white muscle T_{Amm} were likely due to the initial increases in plasma (= ECF) ammonia, that resulted from inhibited branchial J_{Amm}, and the subsequent generation of ECF to white muscle ICF P_{NH3} and NH_4^+ electrochemical gradients (F_{NH4}) that favoured white muscle ICF ammonia loading. White muscle T_{Amm} was also persistently elevated through 28 d of chronically elevated pH, at 10°C and 15°C. These chronically greater white muscle T_{Amm} concentrations probably reflected the higher plasma T_{Amm} concentrations that were required to drive J_{Amm} during prolonged alkaline exposure. Furthermore, the presence of inwardly directed F_{NH4} and outwardly directed P_{NH3} gradients, favouring ECF to white muscle ICF NH_4^+ loading and NH_3 movement in the opposite

direction, respectively, suggested that white muscle ICF to ECF NH_4^+ and NH_3 distributions had returned to steady state conditions after 7 d at pH 9.5. These gradients resembled those seen during pre-exposure conditions and were similar to gradients previously measured in resting fish (Wright and Wood, 1988; Wright *et al.*, 1988). Thus, these experiments discount the presence of a true ammonia deficit and suggest that rainbow trout actually increase ammonia production during acute high pH exposure at 15°C. Furthermore, they also emphasize the importance of the white muscle in serving as an acute and long-term "ammonia reservoir" when plasma T_{Amm} increases as a result of inhibited branchial J_{Amm}. This ability to store ammonia in the white muscle would be of benefit to fish not only during high pH exposure but also under conditions where environmental ammonia concentrations are elevated, such as would occur following the turnover of eutrophic lakes or with anthropogenic ammonia deposition (Barica, 1974; Wetzel 1983).

Ammonia excretion patterns and metabolism were also markedly affected by differences in water temperature. Predictably, pre-exposure J_{Amm} was lower at 10°C than at 15°C but chronic high pH exposure at 10°C resulted in chronically depressed J_{Amm}, whereas long-term exposure at pH 9.5 and 15°C was characterized by an initial inhibition, then complete recovery of J_{Amm} that was sustained throughout the remainder of the experiment. These latter observations are consistent with the recovery of J_{Amm} that has been observed during acute high pH experiments at 15°C. The lowered J_{Amm}, as well as decreased absolute plasma and white muscle ammonia concentrations, at pH 9.5 and 10°C suggest the fish had chronically reduced basal ammonia production. Such a reduction may have occurred via lowered basal N-metabolism, increased reliance on other substrates (eg. fatty acids, carbohydrates) for energy production, and/or excretion of alternate N-waste product(s), such

as trimethylamine oxide (TMAO) or glutamine.

Acid-Base Regulation

Acute high pH exposure led to a pronounced respiratory alkalosis that was characterized by a persistent decrease in arterial P_{CO2} (Pa_{CO2}) and resultant 0.2 unit increases in pH_a. The onset of a delayed metabolic acidosis, that was reflected by a metabolic acid load (ΔH^+_m) of approximately 3 mmol·l^{-1}, led to a stabilization of pH_a, at a new, slightly elevated set-point. Elevations in blood lactate closely followed increases in ΔH^+_m and suggested that increases in glycolytic flux, leading to greater lactic acid production, provided the H^+ that was responsible for this partial acid-base compensatory response. Subsequent experiments indicated that this ECF lactacidosis originated in the white muscle compartment where the ICF concentrations of lactate were 4-fold greater in fish held at high pH for 48 h.

All of the resultant white muscle ΔH^+_m, associated with the white muscle lactate load, was lost to the ECF and/or water and was reflected by a white muscle H^+_m deficit of approximately -1.5 mmol·kg^{-1}. This deficit completely accounted for the ECF ΔH^+_m and the remainder of the white muscle H^+_m deficit, about 1 mmol·kg^{-1}, was presumably lost across the gills. In fact, indirect estimates of H^+ excretion rates across the gill, based upon $J^{Cl}_{net} - J^{Na}_{net}$ calculations, suggested that the fish had lost approximately 0.8 mmol·kg^{-1} of H^+_m via this route during the first 48 h of pH 9.5 exposure. With current technology it was not possible to directly measure acid excretion rates into alkaline water.

Potentially the slight, 0.05 unit elevations in white muscle intracellular pH (pH_i) and/or chronically elevated white muscle T_{Amm} might have stimulated glycolytic flux (eg. Eichenholz, 1962, Takano, 1969; De Loecker, 1964) and accounted for elevated lactate concentration in the white muscle ICF. The fact that white muscle ATP and creatine

phosphate stores, as well as Pa_{O2} were stable during the 48 h high pH exposure regime rule out hypoxemia as a causative factor for elevated lactic acid production.

The chronic respiratory alkalosis seen at 2 days persisted through 28 d of chronic high pH exposure. However, rather than operating at a slightly elevated set-point pH, the development of a more severe metabolic acidosis, characterized by a ΔH^+_m of approximately 5 mmol·l^{-1}, led to a chronic 0.1 unit depression in ECF pH (pH_a). White muscle pH_i measurements, from 7 to 28 days, indicated that there were no apparent disturbances to white muscle ICF acid-base homeostasis.

Although blood lactate levels were slightly elevated throughout the chronic high pH exposure regime they accounted for less than the 10 % of the ECF ΔH^+_m. Furthermore, white muscle lactate concentrations approximated the simultaneous measurements made in the control fish. This suggests that energetically expensive increases in lactic acid production only serve as a temporary method of acid-base regulation; other longer term methods of pH_a regulation are necessary for long-term survival at high pH.

Since, acid-base balance and ionoregulation are inextricably linked, a number of workers have suggested that the modulation of transbranchial ion movements plays a key role in the correction of disturbances to acid-base homeostasis (eg. McDonald *et al.* 1989; Goss *et al.* 1992b). In fact, the persistent depression of Na^+ influx (J^{Na}_{in}), seen in rainbow trout during acute and chronic high pH exposure was due to a decrease in affinity of the branchial Na^+ carrier, and may have served to retain H^+, in the face of chronically lowered Pa_{CO2}. However, the aforementioned indirect estimates of net H^+ movements ($J^{H}_{net} = J^{Cl}_{net} - J^{Na}_{net}$) during acute exposure, suggest that branchial ion movements played no role in the correction of acid-base disturbances and may have, in fact, exacerbated acute high pH induced alkalosis.

Thus, the issue of branchial mediated acid-base correction during high pH exposure remains unresolved and warrants further research.

Maintenance of Internal Ion Balance

Acute exposure to pH 9.5 clearly disturbed electrolyte balance in rainbow trout. These disturbances were generally characterized by 5-10 % reductions in plasma Na^+ and Cl^- concentration after 24-48 h at pH 9.5. Thereafter, plasma Na^+ and Cl^- concentrations stabilized; in fact, no reductions in plasma, or white muscle, Na^+ or Cl^- were evident during chronic high pH exposure. Subsequent radio-tracer ($^{22}Na^+$, $^{36}Cl^-$) experiments, designed to characterize these acute high pH-induced ionoregulatory disturbances, suggested that these initial reductions in plasma electrolytes at high pH were solely due to decreases in J^{Na}_{in} and J^{Cl}_{in}. Only small, insignificant, changes in ion outflux (J^{Na}_{out} and J^{Cl}_{out}) were observed over the first 24 h of pH 9.5 exposure. However, the initial net Cl^- (J^{Cl}_{net}) losses were corrected by 48-72 h via a complete recovery of J^{Cl}_{in} to pre-exposure rates. Gradual reductions in J^{Na}_{out}, which were significant by 72 h, counterbalanced the chronic reductions in J^{Na}_{in} and ultimately re-established net Na^+ balance (ie. $J^{Na}_{net} = 0$). Interestingly, this chronic suppression of J^{Na}_{in} persisted throughout 28 d of chronic high pH exposure. Although no significant reductions in J^{Na}_{out} were observed during chronic high pH, it is likely that decreases in branchial Na^+ permeability offset this chronic J^{Na}_{in} reduction and allowed the fish to maintain internal Na^+ concentration at pre-exposure levels.

Saturation kinetic analyses at high pH revealed that transient 50 % reductions in J^{Cl}_{max}, and more pronounced 70 % reductions in J^{Na}_{max}, accounted for the initial reductions in J^{Cl}_{in} and J^{Na}_{in}, respectively. The complete restoration of J^{Cl}_{max} to pre-exposure rates accounted for the restoration of J^{Cl}_{in} and suggested that initial high pH-induced reductions in branchial Cl^-

transporting capacity were rapidly corrected. Two-substrate kinetic analyses (Goss and Wood, 1991) also suggested that the presence of a high pH-induced metabolic acidosis, reflected by lowered plasma HCO_3^- concentration, likely impeded J^{Cl}_{in} by limiting internal counterion supply for branchial Cl^-/HCO_3^- exchange. However, the analyses also revealed increased actual Cl^- transporter capacity by 24 h, which would have counteracted the lack of internal HCO_3^-. This elevation in Cl^-/HCO_3^- exchange capacity may have resulted from increased insertion of apical Cl^-/HCO_3^- proteins and/or greater exposure of branchial chloride cells (CC), the putative site of the Cl^- uptake in freshwater teleosts. In fact, Wilkie and Wood [1994a (Appendix 1)] reported elevated branchial CC fractional surface area, via increased CC density and individual surface area, in rainbow trout exposed to pH 9.5 for 3 d.

Initial reductions in J^{Na}_{in} also resulted from decreased transporter capacity (ie. J^{Na}_{max}). The gradual recovery of J^{Na}_{in} to pre-exposure rates was due in part to a partial recovery of J^{Na}_{max} to 60 % of control rates but further recovery was circumvented by a dramatic increase in K^{Na}_m. These data suggest that the high pH-induced reductions in Na^+ uptake capacity were followed by a gradual recovery of Na^+ transporter number. Complete recovery of J^{Na}_{in}, however, was precluded by a persistent decrease in transporter affinity for Na^+. Two-substrate analyses of Na^+ uptake patterns generally supported these interpretations, and demonstrated that a lack of internal counterion, H^+, masked an almost complete recovery of Na^+ transporter numbers to pre-exposure levels. The elevation of K^{Na}_m may have been an attempt to retain H^+, to counter the fish's tendency to lose acid (gain base) during high pH exposure (see above).

THE HIGH pH TOLERANT LAHONTAN CUTTHROAT TROUT

Lahontan cutthroat trout (LCT), that had been raised in pH 8.4 well water and never previously exposed to alkaline (pH 9.4) Pyramid Lake water, readily adapted to the lake's extreme alkalinity and suffered no mortality following transfer. This ability to adapt to lakewater was underscored by the absence of any changes in plasma glucose concentrations and the rapid adjustments that were observed in a variety of physiological parameters, including N-waste excretion, acid-base balance and ionoregulation.

Introduction into alkaline lakewater resulted in an immediate, and chronic, 70 % reduction in J_{Amm} that persisted for up to 5 weeks, and possibly indefinitely (ie. 2 years). Reduced J_{Amm} was accompanied by a 30 % elevation of plasma T_{Amm} but this latter effect was corrected by 3 days and subsequently stabilized thereafter. These results strongly suggest that basal ammonia production by LCT is dramatically reduced following transfer into alkaline Pyramid Lake water. Furthermore, it resembles the responses seen in rainbow trout during high pH exposure at 10°C. As previously suggested for the rainbow trout, this decreased ammonia production might have been due to the excretion of an alternate N-waste product, such as urea or glutamine, and/or reductions in amino acid deamination (ie. basal N-metabolism). The role of urea for maintenance of $J_{Waste-N}$ in Pyramid Lake was examined, but no compensatory increases in the contribution of this potential alternate N-waste product were observed. However, the percentage contribution of J_{Urea} to total $J_{Waste-N}$ increased from approximately 10 % in well water to 20-30 % in Pyramid Lake.

The immediate inhibition of J_{Amm}, following well water to lake water transfer, was probably due to a decrease in the blood-to-gill water ΔP_{NH3}. Although the high buffer capacity of Pyramid Lake water precludes boundary layer acidification (Wright *et al.*, 1993),

the higher pH of the lake water (close to pK_{Amm}) would have increased the proportion of NH_3 in the gill boundary layer water and therefore, decreased the blood-to-gill water ΔP_{NH3} and ultimately J_{Amm}. Unlike the earlier observations made on the rainbow trout, the rapid correction, and long-term maintenance, of plasma T_{Amm} at pre-exposure values suggest that chronically elevated internal ammonia concentrations are not necessary to facilitate J_{Amm} following transfer into Pyramid Lake. Furthermore, chronically reduced ammonia production rates, avoided such potentially toxic increases in plasma T_{Amm}. However, plasma P_{NH3}'s were about 2-3 fold greater in LCT, both before and after transfer, than in similarly sized rainbow trout held in freshwater. This maintenance of higher basal plasma P_{NH3}, under all conditions, probably facilitates the minimal J_{Amm} observed in the alkaline environment and suggests that the fish is pre-adapted for life in Pyramid Lake.

LCT also experienced an immediate, chronic respiratory alkalosis following transfer into Pyramid Lake and a simultaneous, transient metabolic alkalosis. The latter was corrected after 1 d in lake water, but unlike the situation seen in the rainbow trout, this compensation was not related to the development of a lactacidosis. Rather the LCT appeared to counteract the continual base load exerted upon it in lake water through dramatically increased gill CC FSA, which presumably facilitated continual HCO_3^- removal via Cl^-/HCO_3^- exchange. The relatively stable plasma HCO_3^- concentrations seen in fish that had lived in lakewater for up to 2 years, despite the presence of large inwardly directed electrochemical gradients favouring HCO_3^- and OH^- (= H^+ loss) uptake, supports this conclusion. In contrast, the greater CC FSA seen in rainbow trout following high pH exposure (Appendix 1) is likely related to maintenance of J^{Cl}_{in} in the face of chronically reduced internal HCO_3^-. Increased CC FSA might also explain the absence of ionoregulatory disturbances in LCT, following lakewater

transfer, but most likely the high concentrations of Na^+ and Cl^- in Pyramid Lake mitigated any potential ion imbalances.

The abilities of the LCT in Pyramid Lake to decrease basal ammonia production and counteract continual base loads,are key adaptations that enable this fish to survive in its harsh environment at pH 9.4. However, with continued diversion of Pyramid Lake's water inflow and the threat of drought, it is possible that the concentrations of alkaline HCO_3^- and $CO_3^{=}$ salts will increase further and lead to future elevations in lakewater water pH. Accordingly, Lahontan cutthroat trout were subsequently challenged in Pyramid Lake water raised to pH 10.

The responses of the LCT challenged at pH 10 were remarkably similar to those observed in the rainbow trout challenged pH 9.5. Exposure to pH 10 lake water led to a transient 50 % reduction in J_{Amm} over the first few hours of exposure but J_{Amm} had completely recovered by 24 hours; in fact J_{Amm} was significantly elevated during the last 36 h of the experiment. The initial inhibition of J_{Amm} resulted in marked, 2-fold increases in plasma T_{Amm} and unlike the situation following transfer into pH 9.4 lakewater, there was no tendency for plasma T_{Amm} to return to pre-exposure values. This persistent elevation in plasma T_{Amm} probably facilitated the recovery of J_{Amm} at pH 10 by re-establishing favourable blood-to-gill water ΔP_{NH3}. The temporary doubling of J_{Urea} during pH 10 exposure resembled the transient stimulation seen in the rainbow trout at high pH and supports the supposition that increased J_{Urea} at high pH serves to ameliorate potentially toxic elevations of internal ammonia by facilitating some N-waste excretion during periods of inhibited J_{Amm}.

In contrast to the situation following transfer of the LCT into lakewater, the challenge at pH 10 was characterized by the fish's marked inability to regulate acid-base balance. This

was reflected by the development of a more profound respiratory alkalosis and a more severe metabolic alkalosis. This latter disturbance was probably related to the development of much larger inwardly directed electrochemical gradients favouring OH^- (= H^+ loss) and $CO_3^{=}$ uptake. Although Pa_{CO2} stabilized, albeit at a new lower set-point, the severity of the metabolic alkalosis progressively worsened and by 48 h pH_a was about 0.3 units higher. Increases in blood lactate, similar to that seen in the rainbow trout, suggested that increased lactic acid production might have prevented even more pronounced increases in pH_a. The pH 10 challenge also led to dramatic ionoregulatory disturbances which were characterized by 10 % reductions in plasma Na^+ and Cl^- concentration by 48 h. These disturbances were partially reflected in the white muscle ICF, where Cl^- concentration was depressed by about 30 %.

Finally, exposure to pH 10 led to mortality in 50 % of the LCT by 72 h. Fish that ultimately died had much higher plasma P_{NH3}'s and suffered from more severe reductions in plasma Na^+ and Cl^- concentrations. Thus, these more susceptible LCT died from a combination of high pH-induced ammonia toxicity and ionoregulatory failure when exposed to pH 10 Pyramid Lake water.

Rainbow trout are capable of survival in alkaline waters, although it appears more physiologically demanding then it is for the Pyramid Lake Lahontan cutthroat trout. Rainbow trout survival at high pH is dependant upon: chronic elevations in internal ammonia to facilitate J_{Amm}; persistent metabolic acidosis to counter high pH-induced respiratory alkalosis; and correction of acute internal electrolyte imbalances via changes in gill ion transport capacity. In contrast, the Lahontan cutthroat trout quickly adapts to alkaline Pyramid Lake water through: reduced metabolic ammonia production, reflected by chronically lowered J_{Amm} and minimal, transient increases in internal ammonia; a rapid induction of branchially

mediated HCO_3^- excretion, likely via elevated CC fractional surface area; and an absence of disturbances to internal electrolyte balance. In conclusion, alterations in N-waste metabolism and gill functional morphology mitigate acute high pH-induced physiological disturbances in salmonids exposed to alkaline environments. However, the ability of the Lahontan cutthroat to rapidly make these adjustments, with minimal disturbance to its pre-exposure physiological status, allows this fish to thrive in an environment which is unsuitable for other salmonids.

CHAPTER 2

NITROGENOUS WASTE EXCRETION, ACID-BASE REGULATION AND IONOREGULATION IN RAINBOW TROUT (Oncorhynchus mykiss) EXPOSED TO EXTREMELY ALKALINE WATER.

ABSTRACT

Rainbow trout (Oncorhynchus mykiss) survived in alkaline freshwater (pH = 9.5) for 72 h, though the exposure rendered the fish more susceptible to mortality from other causes. At pH 9.5 ammonia excretion (J_{Amm}) was initially blocked, and total plasma ammonia levels increased. However, J_{Amm} steadily recovered thereafter; by 48 h control rates were re-established and plasma total ammonia stabilized at 6 times control. The initial blockade of J_{Amm} was associated with a reversal of the blood to bulk water P_{NH3} gradient. Paradoxically, the continued depression of J_{Amm} until 48 h occurred despite the presence of favourable blood to water gradients for passive NH_3 and NH_4^+ diffusion. An increase in urea excretion helped sustain waste N excretion in the face of inhibited J_{Amm}. A respiratory alkalosis (decreased Pa_{CO2}, increased pHa) occurred initially, but was partially counteracted by a metabolic acidosis (decreased plasma HCO_3^-) which stabilized pHa at about 8.0 throughout the exposure. Increases in blood lactate, without marked changes in arterial O_2 tension, suggested that an activation of glycolysis occurred which was not caused by hypoxemia. Plasma Na^+ and Cl^- levels decreased by about 7 percent during the first 24 h of exposure, but stabilized thereafter.

INTRODUCTION

Most investigations of environmental pH effects on the physiology of fish have focussed upon increased acidity, reflecting the ongoing problem of environmental acidification (reviewed by McDonald 1983a; Wood 1989). Only a few have examined the effects of an alkaline environment (Jordan and Lloyd 1964; Johansen, Maloiy, and Lykkeboe 1975; Eddy, Bamford, and Maloiy 1981; Wright and Wood 1985; Heming and Blumhagen 1988; Playle and Wood 1989; Randall *et al.* 1989; Wood *et al.* 1989; Lin and Randall 1990). Studies on the rainbow trout (Oncorhynchus mykiss) have suggested that this species is unable to tolerate high pH (9.0-10.0) for more than a few hours or days (Jordan and Lloyd 1964; Heming and Blumhagen 1988; Randall and Wright 1989). However, in the wild, the related cutthroat trout (Oncorhynchus clarki henshawi) thrives in highly alkaline lakes of comparable pH (Galat *et al.* 1985). The goals of the present study were to determine whether or not the rainbow trout can survive an acute exposure to pH = 9.5 for a 3 day period, and to identify the associated physiological mechanisms that either permit adaptation or result in mortality. Our study focussed on three areas: nitrogenous waste excretion, acid-base balance, and ionoregulation.

Acute exposure to alkaline pH is known to inhibit branchial ammonia excretion (Wright and Wood 1985; Randall and Wright 1989; Lin and Randall 1990). This phenomenon is probably due to a reduction of the diffusion gradient for NH_3 (ΔP_{NH3}) across the gills when the environmental pH approaches pK_{Amm} (about 9.50 at 15 C; Cameron and Heisler 1983), and to an inhibition of branchial Na^+ influx/NH_4^+ efflux exchange (Wright and Wood 1985). However, fish are still able to excrete some ammonia-N in the face of opposing P_{NH3} gradients (Maetz 1972, 1973; Payan 1978; Cameron and Heisler 1983; Wright and

Wood 1985; Cameron 1986; Claiborne and Evans 1988). This raises the possibility that recovery of Na^+/NH_4^+ exchange or induction of other mechanisms (see Evans and Cameron 1986) might support adequate rates of ammonia-N excretion over the longer term. A more unusual strategy has been identified in a tilapia (Oreochromis alcalicus grahami) which thrives in the highly alkaline water (pH $\simeq$ 10) of Lake Magadi, Kenya. This fish excretes all waste nitrogen as urea rather than ammonia (Randall *et al.* 1989; Wood *et al.* 1989). To address these possibilities, the present study examined rates of ammonia-N (J_{Amm}) and urea-N excretion (J_{Urea}) and plasma levels of these two waste products in trout throughout the exposure to alkaline pH.

At high pH, the water outside the gills becomes an essential "vacuum" for P_{CO2} due to diffusion trapping of CO_2 as HCO_3^- or $CO_3^=$ (Johansen *et al.* 1975). Decreases in arterial CO_2 tension (Pa_{CO2}) and resulting increases in arterial pH (pHa) ("respiratory alkalosis") are known to occur during the first few hours of exposure (Wright and Wood 1985; Lin and Randall 1990). Over the longer term, increases of plasma HCO_3^- and additional increases in pHa ("metabolic alkalosis") might result from losses of H^+ and/or entry of HCO_3^-, OH^-, or CO_3^{2-} at the gills. Alternately, some form of metabolic compensation might be implemented. Heming and Blumhagen (1988) reported elevated plasma HCO_3^- and pHa levels after one day of high pH exposure, which were apparently corrected on subsequent days in surviving trout. These possibilities were evaluated by following arterial acid-base status throughout the exposure in the present study.

Ionoregulatory failure is the major cause of death in fish acutely exposed to acidic environments (pH = 4.0-4.5) due to inhibition of active influx and stimulation of diffusive efflux of Na^+ and Cl^- at the gills (reviewed by McDonald 1983a; Wood, 1989). Similar

effects have been observed during acute exposure to alkaline pH (Wright and Wood 1985; Heming and Blumhagen 1988; Wood 1989), raising the possibility that considerable ionoregulatory disturbance might occur during longer term exposure. Accordingly, the levels of plasma Na^+ and Cl^- were monitored in the rainbow trout exposed to high pH in the present study.

MATERIALS AND METHODS

Experimental Animals and Set-up

Adult rainbow trout (Oncorhynchus mykiss = Salmo gairdneri; 346.5 $\pm$ 8.6 g; n = 105) of both sexes were obtained from Spring Valley Trout Farm, New Dundee, Ontario, held in moderately hard dechlorinated Hamilton city tapwater (Ca^{2+} = 1.0, Mg^{2+} = 0.4, Na^+ = 0.6, Cl^- = 0.8, titration alkalinity = 2.1 mmol.L^{-1}; hardness = 140 mg.l^{-1} as $CaCO_3$) at 6° - 15°C and were fed twice weekly with commercial trout pellets (Martin Feed Mills Inc.). One to two weeks prior to experiments, batches of 20 fish were transferred to a 560 l "Living Stream" (Frigid Units Inc.) that was supplied on a flow-through basis with the same water at 15 $\pm$ 1°C. During this acclimation period, the fish were starved to remove the influence of feeding on nitrogen metabolism (Fromm 1963).

Approximately 48 h prior to experimentation, the fish were fitted with dorsal aortic catheters (Soivio, Westman, and Nyholm 1972) while under MS-222 anaesthesia (1:10 000 dilution; Sigma) and then transferred to one of seven darkened plexiglass flux boxes in a 200 L, continuously flowing recirculating system (15 $\pm$ 0.2°C). Each box received a flow of about 2 L.min^{-1}. Water in the reservoir tank of the system was continually replaced at approximately 2 L.min^{-1}, which led to water total ammonia levels (T_{Amm}) of about 10 umol.L^{-1}. The flux boxes, described by McDonald (1983b), comprised an inner chamber containing the fish, and an outer chamber containing most of the water volume (approximately 5 L). Vigorous aeration of the outer chamber maintained circulation during periods when the box was operated as a closed system for flux determinations.

The head tank of the recirculating system was fitted with a Radiometer GK2401C combination pH electrode connected to a PHM 82 meter and TTT80 autotitrator. This pH-

stat maintained the desired water pH in the face of continual acidification due to CO_2 production by the fish and the replacement addition of fresh water. Activation of the titrator opened an electro-magnetic valve (Nacon Industries) which allowed 1.0 N NaOH to flow in a drop-wise fashion into the vigorously aerated head tank. Control water pH was 8.15 $\pm$ 0.04 and while experimental pH was kept at 9.50 $\pm$ 0.03 as measured in the boxes using an independent Radiometer GK2401C electrode and PHM 72MK2 meter. NaOH addition by the titrator during the pH = 9.50 experimental period resulted in an elevation of water Na^+ from the control level of 0.6 mmol.L^{-1} to approximately 4.2 mmol.L^{-1}. Simultaneously, the water Ca^{2+} declined from 1.0 mmol.L^{-1} to about 0.15 mmol.L^{-1}, associated with an obvious precipitate which accumulated in the system. When the flux boxes were closed systems for flux determinations, the initial pH was set by the pH-stat, but thereafter each box was manually titrated at 15 min intervals with 1.0 N NaOH, using the independent electrode and meter.

Experimental Protocol

Flux determinations (3 h) for J_{Amm} and J_{Urea} were performed under control conditions (pH = 8.15, after 48 h recovery from surgery) and at 0 - 3 h, 8 - 11 h, and approximately 24 h, 48 h, and 72 h exposure to pH = 9.50. At the start of the 0 - 3 h flux, the boxes were flushed with pH 9.50 water for 10 min to quickly raise the pH from 8.15 to 9.50, and then closed. Water samples (15 mL) were taken at 0 h, 1 h, 2 h, and 3 h of each flux period and analyzed for T_{Amm} and urea. For J_{Amm}, fluxes were calculated from changes in water T_{Amm} over the first hour only, in order to minimize the known influence of elevated water T_{Amm} on the measured rate (Wright and Wood 1985). Typically, water T_{Amm} rose to no more than 40 umol.L^{-1} over this first hour. Had we instead chosen to calculate J_{Amm} values from the T_{Amm}

changes over the full 3 h periods, absolute rates would have been lower but none of the conclusions would have been qualitatively altered. The much smaller J_{Urea} values were measured over the full 3 h period, because changes in water urea concentration were too small to measure reliably over 1 h periods. We have no information on whether J_{Urea} values are influenced by the levels of water T_{Amm} which accumulated. Between flux periods, the boxes were opened to the pH-statted recirculating system and operated in this open mode until the next determination. Several flux tests were run with known T_{Amm} but no fish present to check for ammonia loss at pH 9.50; this was negligible.

Blood samples (450-550 uL, drawn anaerobically via the catheters into ice cold, gas tight Hamilton syringes) were taken immediately prior to the start of all flux periods except the first experimental period. Sampling at this time avoided disturbance during flux determinations while providing measurements of plasma T_{Amm} and acid-base status only 30 minutes before the mid-point of the J_{Amm} measurements, before water T_{Amm} had increased to any great extent. This prevented overestimation of plasma T_{Amm} levels which might be caused by progressive elevation of plasma T_{Amm} levels due to box closure during the flux period. P_{NH3} (ΔP_{NH3}) and NH_4^+ ($\Delta[NH_4^+]$) diffusion gradients from plasma to bulk water were calculated based on the blood measurements prior to the flux period, and the water levels measured at the start of the flux period. The diffusion gradients reported for the first one hour of exposure to pH 9.5 are based upon the control blood sample, because it is unlikely that blood parameters would change during the first few minutes of exposure to high pH.

Blood samples were analyzed for hematocrit (Ht), arterial whole blood pH (pHa), O_2 tension (Pa_{O2}), lactate, true plasma total CO_2 (Ca_{CO2}), plasma T_{Amm}, urea, Na^+, and Cl^- concentrations. To avoid excessive removal of blood, not all parameters were measured in all

fish; rather, different experimental series focussed on different combinations of parameters. After sampling, remaining blood from the P_{O2} electrode was returned to the fish, together with sufficient Cortland saline (Wolf 1963) to restore the blood volume.

Analytical Techniques and Calculations

Analytical methods were identical to those employed by Wright and Wood (1985), with the following exceptions. Pa_{O2} was measured with a Radiometer E5046 electrode thermostatted to 15°C; Ca_{CO2} was measured with a Capnicon (Cameron Instruments Inc.); plasma and water urea were measured by the diacetyl monoxime method of Crocker (1967); lactate was measured by the L-lactate dehydrogenase method/NADH method as described by Turner, Wood, and Clark (1983); plasma Na^+ was measured by atomic absorption (Varian AA1275); and plasma Cl^- was measured by coulometric titration (Radiometer CMT10).

J_{Amm} and J_{Urea} were calculated over 1 h and 3 h flux periods respectively from measured concentration changes in the water, the known volume of water in the flux box, and the fish's body weight. Pa_{CO2} and arterial plasma HCO_3^- were calculated from pHa and Ca_{CO2} using appropriate solubility coefficients (αCO_2), pK' values from Severinghaus (1965) as tabulated by Boutilier, Heming and Iwama (1984), and standard manipulations of the Henderson-Hasselbalch equation. Plasma and water NH_3, NH_4^+, P_{NH3}, and diffusion gradients from blood plasma to water were similarly calculated from T_{Amm} and pH measurements in each medium, appropriate solubility coefficients (αNH_3) and pK_{Amm} values from Cameron and Heisler (1983), and the Henderson-Hasselbalch relationship. All relevant equations are given by Wright and Wood (1985). The net load of acidic equivalents ("metabolic acid load", H^+m) in the blood at each sample time was calculated from changes in pHa, HCO_3^-, and Ht (as an index of non-HCO_3^- buffer capacity), as outlined by McDonald, Hobe, and Wood

(1980).

One important difference from Wright and Wood (1985) is that all reported blood plasma values are based on the measured arterial value alone. Ventral aortic concentrations were not measured. Therefore the diffusion gradients were calculated from arterial blood plasma to bulk water, and are undoubtedly lower than if a mean arterial-venous figure had been used. In view of the large changes observed in plasma T_{Amm} during the experiment, we were not confident in applying the 1.33x correction factor developed by Wright and Wood (1985) to estimate the mean arterial-venous T_{Amm} levels.

All results have been expressed as means $\pm$ 1 SEM (n) where n represents the number of animals contributing data to the mean. Paired Student's t-test (2-tailed; $P < 0.05$) was used to determine the significance of changes, with each animal serving as its own control.

RESULTS

Survival

Approximately 40% of the fish tested died during the 72 h exposure to pH 9.50. In most, though not all cases these mortalities could be attributed to experimental mishap - *eg.* overt bleeding, abnormally low Ht, blood clots, pH-stat or aeration failure. However, it was our impression that the fish were much less resistant to experimental disturbance at pH 9.50 than at pH 8.15 As a check, 10 cannulated trout were subjected to an identical confinement and blood sampling regime in the experimental system, but water pH was kept at 8.15. None died despite very low haematocrits in several fish. To confirm that the exposure to pH 9.5 did not itself induce mortality, four separate series of experiments, using a total of 24 uncannulated fish were performed. Only one of these fish died during 72 h exposure to pH 9.50 in the experimental system. In a subsequent study (M. P. Wilkie and C. M. Wood, unpublished observations) conducted in the same water quality at pH 9.5 with free-swimming, routinely fed trout, there was no mortality during a 5 week exposure. We conclude that exposure is not in itself toxic, but does render the fish more susceptible to other stresses.

Ammonia Excretion

Rainbow trout exhibited a control J_{Amm} of approximately 220 umol-N.kg^{-1}.h^{-1} at pH 8.15 (fig. 1A). Immediately upon exposure to pH 9.50, ammonia excretion was blocked and there was a short-lived net uptake of ammonia. By 8 h, a net efflux of ammonia resumed, but this remained significantly lower than control at both 8 h and 24 h. J_{Amm} continued to recover with time; control rates were re-established at 48 h and 72 h. Arterial plasma T_{Amm} was about 100 umol.L^{-1} under control conditions (fig. 1B). Plasma T_{Amm} had increased about 4-fold by 8 h and almost 6-fold by 24 h, in accord with the inhibition of J_{Amm}, and then

remained at this level for the duration of the exposure.

At pH 8.15, ΔP_{NH3} was not significantly different from zero (fig. 1C). However, in the first hour of exposure to pH 9.50, estimated ΔP_{NH3} declined to -400 uTorr, explaining the negative J_{Amm} at this time. By 8 h, ΔP_{NH3} had increased markedly to almost +150 uTorr, and remained there through 72 h. The recovery of J_{Amm} did not appear to be completely correlated with the establishment of the elevated P_{NH3} gradient, because J_{Amm} was still significantly lower at 8 and 24 h, while ΔP_{NH3} had rebounded by 8 h (compare figs. 1A and 1C). At pH 8.15, the $\Delta[NH_4^+]$ was about + 60 u.nol.L^{-1} (fig. 1D). Upon initial exposure to pH 9.5, $\Delta[NH_4^+]$ was unchanged, but by 8 h and 24 h had increased to about 6 and 12 times the control level respectively, stabilizing thereafter.

Control J_{Urea} at pH 8.15 was approximately 32 umol-N.kg^{-1}.h^{-1}, accounting for only about 12% of total waste-N excretion (fig. 2A). J_{Urea} increased significantly to 53 umol-N.kg^{-1}.h^{-1} after 8 h alkaline exposure, at which time it represented 45% of total waste-N excretion. J_{Urea} remained significantly elevated through 72 h (fig. 2A), but its relative contribution to total waste-N excretion progressively decreased towards control levels as J_{Amm} recovered (fig. 1A). Plasma urea-N was about 5000 umol-N.L^{-1} at pH 8.0 (fig. 2B), or about 50-fold higher than plasma ammonia-N (fig. 1B). Throughout most of the exposure to pH 9.5, plasma urea-N did not change; it was significantly lower than control only at 48 h (fig. 2B).

Blood Acid-Base Status

Blood acid-base disturbances are displayed on a pH-HCO_3^- diagram (fig. 3). A significant respiratory alkalosis was seen at 8 h exposure to pH 9.50, reflected in a rise in pHa from 7.83 to 7.97. Pa_{CO2} declined from 3.1 to 1.4 Torr and plasma HCO_3^- fell from 7.5 to 5.1 mmol.L^{-1}. Arterial pHa stabilized after this initial increase, despite a further

progressive decline in Pa_{CO2} to 0.7 Torr by 72 h. This stabilization was attributable to a concurrent metabolic compensation (see Discussion) reflected in a marked, continual decline in plasma HCO_3^- to 2.5 mmol.L^{-1} by 72 h. The calculated metabolic acid load (ΔH^+m) rose progressively during alkaline exposure, reaching 3.3 mmol.L^{-1} by 72 h (fig. 4A).

Control blood lactate was approximately 0.5 mmol.L^{-1} at pH 8.15 (fig. 4B). After 8 h in alkaline water, lactate increased by about 3-fold, and after 24 h by about 6-fold. Up to 24 h, lactate and ΔH^+m were similar, but thereafter, lactate declined while ΔH^+m continued to rise (fig. 4A,B). However lactate remained significantly elevated above the control level through 72 h of alkaline exposure. This increase in blood lactate was not correlated with any marked disturbance of arterial blood oxygenation. Pa_{O2}, approximately 105 Torr at pH 8.15, exhibited a slight but significant decline after 8 h at high pH (fig. 4C). However, Pa_{O2} did not deviate significantly from the control value for the remainder of the exposure period.

Ionoregulation

Control Na^+ and Cl^- levels in blood plasma were approximately 143 and 138 mmol.L^{-1} (fig. 5). By 24 h exposure to pH 9.5, these declined by about 6% and 8% respectively, but there was little further change during the remainder of the experiment.

DISCUSSION

Survival

Rainbow trout can survive at pH 9.5, but the exposure clearly disturbs internal homeostasis of ammonia, acid-base status, and ions, and renders the fish more susceptible to mortality from other causes. It is likely that experimental stresses (catheterization, blood sampling, confinement *etc.*) were responsible for unexplained fatalities and contributed to the higher rates of mortality at comparable pH reported by Jordan and Lloyd (1964), Heming and Blumhagen (1988) and Randall and Wright (1989). In the latter two studies, mortality was attributed to ionoregulatory disturbance and ammonia toxicity respectively. The trout of Heming and Blumhagen (1988) exhibited much larger decreases in plasma electrolytes despite a less severe alkaline exposure (pH 8.7), presumably due to differences in the experimental conditions or fish stocks used, because water chemistry was comparable in the two studies. Randall and Wright (1989), based on the work of Lin and Randall (1990), reported that trout died when plasma T_{Amm} built up to only about 20% of the levels observed in surviving trout of the present study. However, firm conclusions are difficult because the alkaline exposure was more severe (pH 10.0), the water chemistry very different (extremely soft water elevated in NaCl and $CaCl_2$), and the trout were loaded with the additional stress of ventilation masks. Whatever the exact cause of death, it is clear from all three investigations that water pH's around 9.5 are marginal for the survival of Oncorhynchus mykiss. This probably explains the failure of stocking efforts for rainbow trout in alkaline lakes where the Lahontan cutthroat trout thrives (Galat *et al.* 1985).

Nitrogenous Waste Excretion

Acute exposure to pH 9.50 caused an immediate blockade of J_{Amm}, in agreement with

the earlier work of Wright and Wood (1985) conducted in the same water quality. However, the present study demonstrates that with continued high pH exposure, J_{Amm} gradually returned to control levels and plasma T_{Amm} stabilized at about 6 times the control concentration. Wright and Wood (1985) attributed the immediate blockade of J_{Amm} partially to an inhibition of branchial Na^+/NH_4^+ exchange, and partially to a reversal of the P_{NH3} gradient from blood plasma to bulk water. In the present study, this gradient became highly negative during the first hour of high pH exposure, explaining the transitory net uptake of ammonia by the fish. The subsequent rise in the P_{NH3} gradient to highly positive values (due to the build-up of plasma T_{Amm} and rise in pHa) would favour NH_3 diffusion from blood plasma to water, and may have played a role in the recovery of J_{Amm}. However, this does not appear to be the complete explanation, because the recovery of J_{Amm} was slower than the elevation of ΔP_{NH3}. At present, we can only speculate as to additional mechanisms involved in the recovery of J_{Amm}. Possibilities include: (i) activation of Na^+/NH_4^+ exchange (Cameron and Heisler 1983; note the stabilization of plasma Na^+ after the first 24 h of alkaline exposure); (ii) activation of H^+/NH_4^+ exchange (Cameron 1986); (iii) re-acidification of the gill boundary layer through increased CO_2 or H^+ production (Wright, Randall, and Perry 1989); (iv) increased simple diffusive efflux of NH_4^+ favoured by the marked rise in the $[NH_4^+]$ gradient (McDonald, Tang, and Boutilier 1989). All should be amenable to experimental test in future investigations.

During high pH exposure, trout were able to tolerate a sustained increase in plasma T_{Amm} to more than 600 umol.L^{-1}, yielding a P_{NH3} of more than 250 uTorr, both well above the normal physiological range. However, these levels are much lower than those measured in the plasma of trout dying from acute injections of ammonium salts (Hillaby and Randall

1979). They are also lower than levels in the plasma of seawater-adapted trout surviving for 24 h in elevated ambient ammonia (pers. comm., R.W. Wilson). Thus rainbow trout can tolerate abrupt short term increases in plasma T_{Amm}, but it remains an open question whether or not they can tolerate such levels indefinitely.

The inhibition of J_{Amm} by alkaline exposure resulted in an "ammonia debt" of about 4500 umol-N.kg^{-1} before normal rates were resumed at 48 h. Based on measured changes in plasma T_{Amm}, storage in the extracellular fluid volume (~0.25 L.kg^{-1}; Milligan and Wood 1986) amounted to only 3% of this figure, suggesting that ammonia-N production was inhibited, redirected to other end-products, or that ammonia was stored elsewhere. While we have no information on ammonia production rates, the measured increase in urea-N excretion certainly accounted for about 20% of the "ammonia debt". Conversion of ammonia to relatively non-toxic glutamine, as observed in the brain of goldfish exposed to high ambient ammonia (Levi *et al.* 1974), is another possibility. Intracellular white muscle ammonia levels increase by up to 7000 umol.kg^{-1} after exercise in trout (Mommsen and Hochachka 1988; Wright and Wood 1988), so this is one likely storage site.

The elevated J_{Urea} during high pH exposure ensures that some nitrogenous waste excretion occurs while J_{Amm} is inhibited. This may prevent acute ammonia toxicity. The response is similar to that exhibited by trout exposed to high ambient ammonia, which similarly inhibits J_{Amm} (Olson and Fromm 1971). This small increase in J_{Urea} is reminiscent of the strategy (complete ureotelism) employed by the tilapia endemic to the pH 10 water of Lake Magadi, Kenya (Randall *et al.* 1989; Wood *et al.* 1989). In mammals, urea synthesis is favoured by internal alkalosis (Bean and Atkinson 1984). However, in contrast to mammals and the Lake Magadi tilapia (Randall *et al.* 1989) both of which have an active ornithine urea

cycle (OUC), the OUC appears to be non-functional (absent, repressed, or incomplete) in most teleost fish (Mommsen and Walsh 1989). The toadfish (Opsanus sp. Read 1971) is the only other exception. In the rainbow trout, some or all of the enzymes may be present, though in very low levels (Huggins, Skutsch, and Baldwin 1969; Rice and Stokes 1974; Chiu, Austic, and Rumsey 1986). Therefore, the increased J_{Urea} at high pH in the trout was probably via the standard urea production pathways of uricolysis or arginase activity (Mommsen and Walsh 1989), rather than to an induction of OUC activity.

Blood Acid-Base and Ion Regulation

The blood alkalosis upon initial exposure to high pH water ("a P_{CO2} vacuum"; Johansen *et al.* 1975) was due solely to the decrease in Pa_{CO2}, consistent with Wright and Wood (1985) and Lin and Randall (1990). Over 72 h, there appeared to be no ability to restore Pa_{CO2} to normal levels. In contrast to Heming and Blumhagen (1988), there was no rise in plasma HCO_3^- at 24 h. Analysis of the acid-base data on a pH-HCO_3^- diagram (fig.3; see Wood, McMahon, and McDonald 1977, for details) reveals two clear components to the response. The initial phase of "respiratory alkalosis" (titration along the non-HCO_3^- buffer line by decreased Pa_{CO2}) was followed after 8 h by a progressive "metabolic" compensation (loss of HCO_3^- base = gain of acidic equivalents) which prevented any further rise in pHa. Had this compensation not occurred, pHa would have exceeded 8.20 by 72 h, in contrast to the observed stabilization at pH 7.97.

The rise in blood lactate was similar in magnitude to ΔH^+m, over the first 24 h of alkaline exposure. This suggest that activation of glycolysis might have contributed to this metabolic compensation of acid-base balance. Either the rise in systemic pHa or the rise in internal T_{Amm} levels may have played a role in this activation. In mammalian red blood cells,

for example, glycolysis is stimulated by alkalosis alone (de Loecker 1964), while NH_4^+ is reported to specifically activate phosphofructokinase, a key regulatory enzyme in glycolysis (Kuhn *et al.* 1974). Notably, the rise in ΔH^+m and lactate occurred in the absence of external or internal hypoxia. The measured levels of Pa_{O2} remained well above those needed to saturate the arterial blood (Eddy 1971). Measurements of lactate production and turnover rates are required in future studies to confirm that glycolysis contributes to acid-base control in trout exposed to alkaline environments.

Plasma Na^+ and Cl^- stabilized after the first 24 h of decline at levels well above those associated with mortality in trout exposed to acid water (McDonald 1983a; Wood 1989). While ion flux rates were not measured, this suggests that the initial inhibition of branchial Na^+ and Cl^- uptake, seen upon exposure to pH 9.5 in previous studies (Wright and Wood 1985; Wood 1989), does not continue for long. The recovery of Na^+ balance may have played a role in the recovery of ammonia excretion via Na^+/NH_4^+ exchange. The reason for the much larger electrolyte declines in the trout of Heming and Blumhagen (1988) exposed to a more moderate alkaline pH (8.7) is unknown.

In conclusion, rainbow trout can survive at pH 9.50 for at least 72 h, though marked disturbances occur in ammonia excretion, acid-base balance, and ionoregulation which may render the fish more susceptible to death from other causes. Several adaptations occur, including a rapid increase in urea excretion, a subsequent re-establishment of ammonia excretion, a metabolic compensation opposing respiratory alkalosis, and a limitation of plasma ion losses. Recently, Yesaki (1990) has shown generally similar effects in rainbow trout exposed to pH 10, in very hard water. Further studies are needed to characterize the longer term response, and to determine the physiological and biochemical mechanisms that permit

these adaptations to take place.

Fig. 1. The influence of severe alkaline exposure (pH = 9.5) upon (A) ammonia excretion (J_{AMM}), (B) plasma total ammonia concentration (T_{AMM}) in rainbow trout, (C) the calculated arterial blood to bulk water partial ressure gradient for NH_3 (ΔP_{NH3}) and (D) the calculated arterial blood to water NH_4^+ concentration gradient ($\Delta[NH_4^+]$) across the gills of rainbow trout. Means $\pm$ 1 SEM: $n > 20$ for the control, 1 h, 8 h, and 24 h periods, n = 9 - 11 at 48 h, and n = 7-9 at 72 h. Asterisks indicate significant differences from control values ($P < 0.05$).

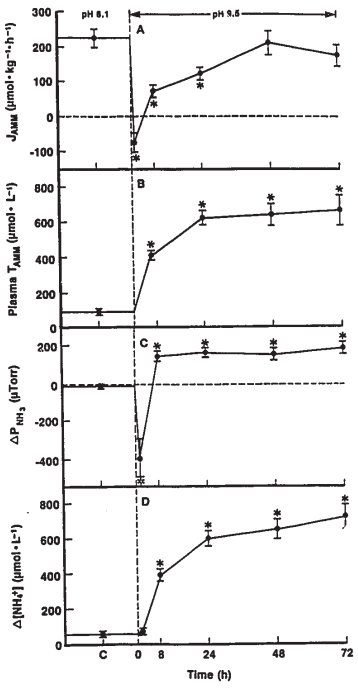


Fig. 2. The influence of severe alkaline (pH = 9.5) exposure upon (A) urea excretion (J_{urea}) and (B) plasma urea concentration in rainbow trout. Means $\pm$ 1 SEM; $n > 20$ for the control, 3 h, 8 h, and 24 h periods for both J_{urea} and plasma urea; n = 26 at 48 h and n = 19 at 72 h for J_{urea}; n = 12 at 48 h and n = 7 at 72 h for plasma urea. Asterisks indicate significant differences from control values ($P < 0.05$).

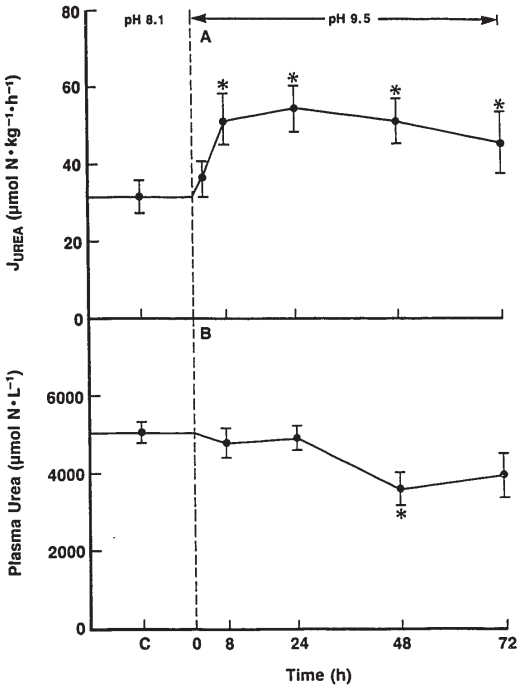


Fig. 3. pH-HCO_3^- diagram showing changes in arterial blood acid-base parameters (pHa, Pa_{CO2}, and plasma $[HCO_3^-]_e$) over time during exposure of rainbow trout to extremely alkaline water (pH = 9.50). Means ± 1 SEM. For all three parameters, n > 20 during the control, 8 h, and 24 h periods, n = 13 at 48 h, and n = 9 at 72 h. All parameters were significantly different form control values at 8, 24, 48, and 72 h.

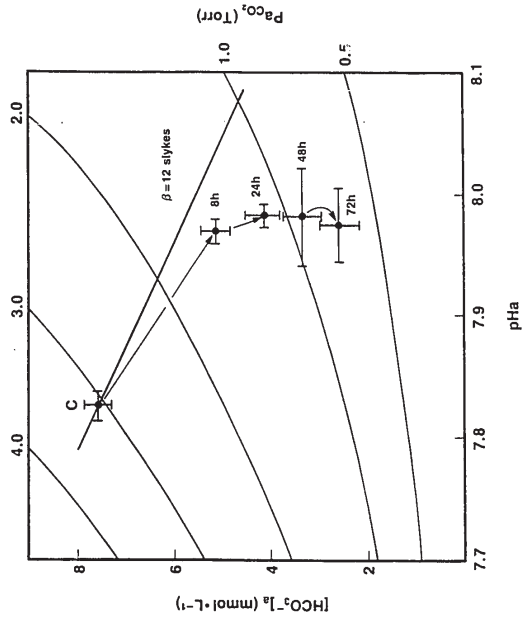


Fig. 4. The influence of severe alkaline exposure (pH = 9.5) upon (A) blood metabolic acid load (ΔH^+m), (B) blood lactate concentration and (C) arterial P_{o2} (Pa_{o2}). Means ± 1 SEM; n > 15 for the control, 8 h, and 24 h periods for all 3 parameters; n = 13 at 48 h and n = 9 at 72 h for ΔH^+m; n = 11 at 48 h and n = 7 at 72 h for blood lactate; n = 8 at 48 h and n = 5 at 72 h for Pa_{o2}. Asterisks indicate significant differences from control values ($P < 0.05$).

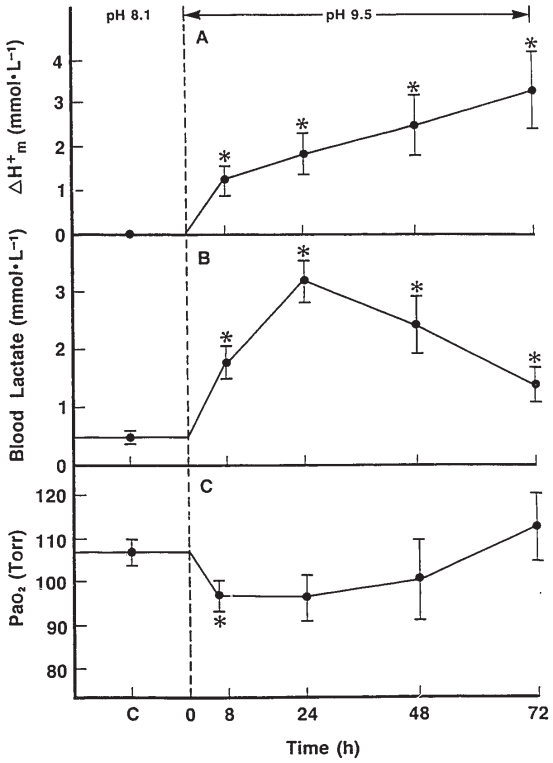
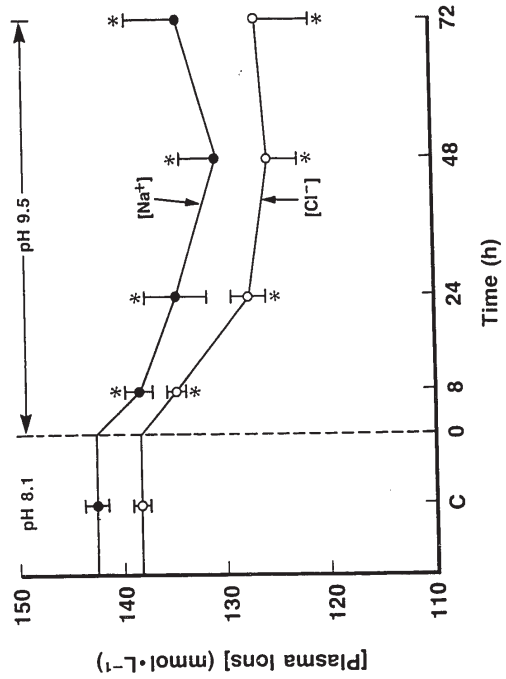


Fig. 5. The influence of severe alkaline exposure (pH = 9.5) upon plasma Na^+ and Cl^- concentrations in rainbow trout. Means ± 1 SEM. For both Na^+ and Cl^-, $n > 20$ during control, 8 h and 24 h periods; $n = 14$ at 48 h and $n = 9$ at 72 h for plasma Cl^-; $n = 13$ at 48 h and $n = 9$ at 72 h for plasma Na^+. Asterisks indicate significant differences from control values ($P < 0.05$).



CHAPTER 3

RECOVERY FROM HIGH pH EXPOSURE IN THE RAINBOW TROUT: AMMONIA WASH-OUT AND THE RESTORATION OF BLOOD CHEMISTRY.

ABSTRACT

The physiological responses of rainbow trout were followed during 48 h of high pH (pH 9.5) exposure and a further 48 h of recovery at pH 8.0. High pH exposure temporarily inhibited ammonia excretion (J_{Amm}) and led to a 6-fold increase in plasma ammonia (T_{Amm}). By 24 h at high pH J_{Amm} had returned to pre-exposure (control) rates but plasma T_{Amm} remained elevated. The fish also developed a transient metabolic alkalosis (increased metabolic base) and a sustained respiratory alkalosis [decreased plasma CO_2 tension (Pa_{CO2})]. Plasma Na^+ and Cl^- concentrations were reduced by 5 % after 48 h at pH 9.5. An "ammonia wash-out", of about 5000 umol·Kg^{-1}, occurred during the first 12 h of the recovery period, with 5-fold elevations in J_{Amm} during the first few hours. This ammonia wash-out was accompanied by a return of plasma T_{Amm} to pre-exposure concentrations after 3 h. The amount of excess T_{Amm} excreted by the fish during the wash-out was about 50-fold greater than extracellular fluid (ECF) T_{Amm} stores. Subsequent experiments indicated that the white muscle (WM) intracellular fluid (ICF) compartment had stored at least 40% of the excess T_{Amm}. The T_{Amm} concentrations in this compartment were 2.5-fold greater in fish held at pH 9.5 for 48 h. Estimates of the plasma (= ECF) and WM ICF pH, P_{NH3} and NH_4^+ levels

indicated the development of favourable ECF:ICF electrochemical gradients for NH_4^+ uptake, and P_{NH3} gradients for NH_3 uptake by the WM, during the initial period of high pH exposure, with a partial return towards steady state by 48 h. Thus, the WM serves as an "ammonia reservoir" for rainbow trout when plasma T_{Amm} increases due to temporary reductions in branchial ammonia excretion. Increased WM lactate concentration, suggested that this compartment also supplies metabolic H^+ to the ECF for extracellular compensation of high-pH-induced respiratory alkalosis. The rapid return of other physiological indices such as pH_a, Pa_{CO2}, and plasma Na^+ and Cl^-, to control levels during recovery (within 3-8 h), as well as constant arterial Pa_{O2}, suggests that high pH induced physiological disturbances are reversible and that there was no high pH induced gill histopathology during such short term exposures.

INTRODUCTION

Fish may encounter temporary elevations in environmental pH due to the photosynthetic removal of CO_2 in eutrophic waters (Jordan and Lloyd 1964; Barica 1974; Wetzel 1983). Permanently alkaline lakes, which are the result of high local concentrations of dissolved alkaline salts (eg. $NaHCO_3$, $Ca(HCO3)_2$) also occur in many regions (eg. Johansen, Maloiy, and Lykkeboe 1975; Galat *et al.* 1981; Danulat and Kempe 1992). Previous investigations with rainbow trout have indicated that exposure to highly alkaline water ($pH \geq 9.0$) results in an inhibition of ammonia excretion and resultant increases in plasma ammonia (Wright and Wood 1985; Wilkie and Wood 1991; Yesaki and Iwama 1992), the development of respiratory and/or metabolic alkalosis (Wright and Wood 1985; Heming and Blumhagen 1988; Lin and Randall 1990; Wilkie and Wood 1991; Yesaki and Iwama 1992) and ionoregulatory disturbances characterized by decreases in plasma Na^+ and Cl^- concentration (Heming and Blumhagen 1988; Wilkie and Wood 1991, 1994; Yesaki and Iwama 1992). Wilkie and Wood (1991) demonstrated that rainbow trout are capable of counteracting these disturbances in a variety of ways. For instance, they reported a full recovery of ammonia excretion during 72 h of high pH exposure, stabilization of plasma Na^+ and Cl^- at slightly reduced levels, and a stabilization of blood pH at an elevated value that was related to the development of a simultaneous metabolic acidosis.

One question that has received little attention is the ability of fish to recover from short term alkaline exposure (ie. 2 to 3 d) upon return to water of normal pH. Many investigators have reported that highly alkaline water results in histological changes to the gill epithelium and other tissues (Eicher 1946; Jordan and Lloyd 1964; Daye and Garside 1976; Galat *et al.* 1985; Wilkie *et al.* 1994; Wilkie and Wood 1994). Changes in gill structure

could alter branchial gas, ion and acid-base exchange processes not only during high pH exposure but also following re-introduction into a circumneutral pH environment. Accordingly, we followed ammonia excretion patterns, acid-base status, plasma electrolytes and arterial P_{O2} in rainbow trout that had been re-introduced into circumneutral pH (pH 8.0) water after 48 h of exposure to pH 9.5.

Previously, Wilkie and Wood (1991) observed increased blood lactate concentrations in rainbow trout that were experiencing a respiratory alkalosis during exposure to pH 9.5. It was, therefore, suggested that increased lactic acid production accounted for the development of a counterbalancing metabolic acidosis, which eventually stabilized blood pH. Accordingly, the potential contribution that elevated WM lactic acid production made to this compensatory process was investigated in the present study by determination of WM lactate concentrations, in conjunction with simultaneous measurements of extracellular acid-base status and white muscle ATP, creatine phosphate and arterial O_2 tension, in fish exposed to pH 9.5 for 48 h.

Wilkie and Wood (1991) observed, that despite a full recovery of J_{Amm} by 48 h of high pH, approximately 3600 umolN·Kg^{-1} of T_{Amm} that should have been excreted by rainbow trout during this first 2 days of high pH exposure remained missing, even after taking elevated urea-N excretion into account. They suggested that this missing ammonia-N was either directed to other end-products, such as glutamine, that nitrogenous waste production was reduced and/or that ammonia was stored in a body compartment other than the extracellular fluid. In the present study we investigated the latter hypothesis.

To establish if the missing ammonia was retained by the fish during high pH exposure, we followed ammonia excretion rates for 48 h after the fish had been returned to circumneutral pH. We hypothesized that if rainbow trout were storing ammonia, re-

introduction into circumneutral pH would result in an elevated ammonia excretion that would allow the fish to repay its "ammonia deficit".

Since, the white muscle intracellular compartment (ICF) comprises approximately 45 % percent of the trouts total body weight (Stevens 1968; Milligan and Wood 1986a,b) it constitutes the largest potential ammonia reservoir in the fish. Thus, in a separate series of experiments, we investigated whether there were significant elevations in the white muscle ICF ammonia content and/or changes in intracellular pH in rainbow trout held at high pH for 48 h. Such a strategy would appear advantageous because it would have lower metabolic cost relative to strategies that might include conversion of ammonia to an alternate waste product and it would not affect the metabolic scope of the fish through reductions in its overall metabolic rate.

MATERIAL AND METHODS

Experimental Animals and Set-up

Rainbow trout (*Oncorhynchus mykiss*; mean weight = 290.5 ± 12.9 g, n = 22) were obtained from Spring Valley Trout Farm (Petersburg, Ontario) and held in moderately hard (140 $mg \cdot l^{-1}$ as $CaCO_3$), dechlorinated, 15°C Hamilton tapwater (Composition: $[Na^+]$ = 0.6, $[Cl^-]$ = 0.8, $[Ca^{++}]$ = 0.9, $[Mg^{++}]$ = 0.15, titration alkalinity = 2.0 $mmol \cdot L^{-1}$) for no more than 3 months. The fish were starved approximately 1 week prior to surgery, at which time they were anaesthetized with MS-222 (1:10 000 dilution; Sigma), fitted with chronic indwelling dorsal aortic catheters (Sovio, Westman, and Nyholm 1972) and then allowed to recover 48 h in individual darkened plexiglass flux boxes (volume = 3.0 L; see McDonald and Rogano 1986 for details), supplied with the same pH 8.0 tapwater at approximately 0.5 $L \cdot min^{-1}$ and 15°C. The boxes received their water from a mixing head tank served by an inflow of tapwater (5.0 $L \cdot min^{-1}$) and fitted with a pH stat set-up. The water subsequently drained into each of the 8 flux boxes *via* a flow-splitter and was then flushed to drain. A flow-through system was employed to minimize changes in water Ca^{++} concentration that occur when hard Hamilton tapwater is raised to pH 9.5. Wilkie and Wood (1991) reported that water Ca^{++} concentrations declined by 80 % during a 72 h high pH exposure regime in a re-circulating system that had partial water replacement. In contrast, in the present study, water $[Ca^{++}]$ concentrations were maintained above 0.5 $mmol \cdot L^{-1}$. The pH stat set-up consisted of a Radiometer GK2401C pH electrode and PHM 84 pH meter connected to a TTT80 autotitrator; when pH dropped below 9.65, the autotitrator signalled an electromagnetic control valve (Nacon Industries) which regulated the drop-wise addition of 1N KOH into the head tank, from a 15 L KOH reservoir. Resultant water K^+ concentrations did

not exceed 0.7 $mmol \cdot L^{-1}$. Previous experiments have shown that water K^+ concentrations in this range cause no apparent physiological effects in rainbow trout (Wilkie *et al.* 1993; Wilkie and Wood 1994).

Experimental Protocol

Part I: Recovery of Physiological Status Following High pH Exposure

Ammonia excretion rates (J_{Amm}) and changes in blood chemistry were determined in 7 rainbow trout initially held at pH 8.0 (pre-exposure period), then exposed to pH 9.5 for 48 h, and finally returned to pH 8.0 for 48 h. Ammonia excretion rates were determined at pH 8.0, and at 0-1 h, 8-9 h, 24-25 h and 48-49 h of pH 9.5 exposure, and at continuous 30 minute intervals over the first 3 hours of the post-exposure period , as well as at 8-9 h, 12-13 h, 24-25 h and 48-49 h after re-introduction into circumneutral pH. Flux determination periods were initiated when flow to the boxes was cut-off, and lasted for 30-60 minutes. Vigorous aeration ensured thorough mixing and oxygenation. Water ammonia levels usually approached 50 $umol \cdot L^{-1}$ by the end of the one hour flux periods. During the 0-3 h post-exposure period, greatly elevated J_{Amm} necessitated flushing the boxes every 60 minutes, to prevent water ammonia concentrations from reaching levels that might inhibit ammonia excretion (Cameron and Heisler 1983; Wright and Wood 1985; Wilkie and Wood 1991). Water pH control during the closed flux period at high pH was also complicated by CO_2 addition to the box *via* aeration and CO_2 excretion by the fish, which drove water pH down. Accordingly, pH was maintained *via* manual monitoring of box pH with an independent pH meter (Radiometer PHM 72) and electrode (GK2401C) and successive 0.5 ml additions of 1N KOH to the box every 30 minutes or when pH dropped below pH 9.5.

Blood samples were taken during the pre-exposure period, at 8 h and 48 h of pH 9.5

exposure, and at 3 h, 8 h and 24 h of the post-exposure period. To minimize the effects that box closure might have had on blood parameters, such as ammonia, we elected to take blood samples 30-60 minutes prior to flux determinations (cf. Wilkie and Wood 1991; Wilkie *et al.* 1993). Blood samples (700 uL) were drawn through the dorsal aortic catheter into an ice-cold, heparinized, gas-tight Hamilton syringe and replaced with an equal volume of Cortland saline (Wolf 1963). Whole blood was immediately analyzed for arterial pH (pH_a), hematocrit, lactate and arterial O_2 tension (Pa_{O2}). Blood used in Pa_{O2} determinations (approximately 150 uL) was re-infused into the fish. Remaining unused blood was centrifuged at 10 000 g and the plasma used for immediate determination of plasma total CO_2 and protein concentration; the remainder was frozen for later determination of plasma total ammonia (T_{Amm} = NH_3 + NH_4^+), Na^+ and Cl^- concentrations. Simultaneous water samples were taken for determination of inspired P_{O2} and pH.

Part II: Ammonia storage and Lactate Production in the White Muscle Compartment

To elucidate the potential role that white muscle might play as a reservoir for excess internal ammonia and a source of metabolic H^+, 12 rainbow trout of similar size and age were cannulated as previously described and exposed to pH 9.5 (N = 6, experimental group) or pH 8.0 (N = 6, controls) for 48 h. Blood samples (700 uL) were taken immediately prior to high pH exposure (control) and at 8, 24 and 48 h of pH 9.5 exposure, and analyzed for pH_a, Pa_{O2} and T_{Amm}, as previously described. At the end of the 48 h exposure regime, the fish were sacrificed with an overdose of MS 222 (1.5 $g \cdot L^{-1}$). Death resulted in less than a minute, at which time the fish were removed from the box and a "filet" of white muscle excised from the trunk, above the lateral line and between the adipose and dorsal fins. The tissue was immediately freeze-clamped with liquid N_2 cooled aluminum tongs, stored initially

in liquid N_2, and subsequently at -70°C until processed for determination of intracellular pH (pH_i), T_{Amm}, lactate, ATP and creatine phosphate concentrations.

Analytical Techniques and Calculations

Analytical techniques for water total ammonia concentrations (T_{Amm} = NH_4^+ + NH_3), plasma T_{Amm}, hematocrit, whole blood lactate, pH_a, Pa_{O2}, and plasma Na^+ and Cl^- were identical to those described by Wilkie and Wood (1991). Plasma total CO_2 was measured on a Corning total CO_2 analyzer and plasma protein was measured by refractometry (Alexander and Ingram 1980).

Ammonia excretion rates were calculated from the change in water T_{Amm} during the flux period, the known box volume and the fish's weight (see Wright and Wood 1985). The P_{NH3} and NH_4^+ levels were calculated by using the Henderson-Hasselbalch equation, and employing the appropriate solubility coefficients and pK_{Amm} values provided by Cameron and Heisler (1983). The P_{CO2} and HCO_3^- concentrations in arterial blood plasma were determined in a similar manner using the appropriate pK' and solubility coefficients provided by Boutilier, Heming and Iwama (1984). The net load of acidic equivalents ("metabolic acid load", $\Delta[H^+_m]$) was calculated from changes in pH_a, HCO_3^- and hematocrit (as an index of blood buffer capacity) as outlined in McDonald, Hobe and Wood (1980).

Measurements of white muscle total ammonia were made on tissue that was ground to a fine powder under liquid N_2 and deproteinized in 10 volumes of 8 % perchloric acid. The ammonia concentration of the extract was determined, after neutralization with TRIS buffer, by the methods of Kun and Kearney (1971).

White muscle pH_i was determined using the method of Portner *et al.* (1990). Briefly, white muscle was ground to a fine powder under liquid N_2; 100 mg of the powder was then

added to approximately 400 ul of ice cold metabolic inhibitor consisting of 6 mmol·L^{-1} nitrilotriacetic acid and 150 mmol·L^{-1} potassium fluoride. The sample was subsequently stirred with a liquid N_2 cooled needle, vortexed and subsequently centrifuged at 10 000 g for 30 seconds. The supernatant was then immediately analyzed on the same pH micro-electrode set-up as for pH_e (pH_e) determination. White muscle lactate, ATP and creatine phosphate was determined on freeze-dried white muscle that was deproteinized in 60 volumes of 6 % perchloric acid. The neutralized extracts were then enzymatically analyzed for lactate (lactate dehydrogenase; Bergmeyer, 1983), ATP (hexokinase and glucose-6-phosphate dehydrogenase; Trautschold *et al.*, 1985) and creatine phosphate (creatine kinase; Heinz and Wieber, 1985).

White muscle total ammonia concentration was converted to umol·L^{-1}ICF from umol·g^{-1} wet weight, according to the following expression:

$$\text{ICF } T_{Amm} = (\text{wet } [T_{Amm}] - \text{ECFV} \cdot \text{plasma } [T_{Amm}])/\text{ICFV}$$

where ICFV and ECFV are the respective white muscle intracellular and extracellular fluid volumes for rainbow trout white muscle at 15°C reported by Milligan and Wood (1986a); wet $[T_{Amm}]$ and plasma $[T_{Amm}]$ are the wet white muscle and plasma ammonia concentrations, respectively. White muscle P_{NH3} and $[NH_4^+]$ were estimated in an identical manner to calculations used for extracellular (plasma) determinations of P_{NH3} and $[NH_4^+]$.

Calculations used to convert white muscle lactate, ATP and creatine phosphate concentrations to mmol.L^{-1} ICF were analagous to those used for white muscle ammonia.

Statistics

All results are expressed as means $\pm$ 1 SEM (N = 6-7). Statistically significant differences in Part I of the study, amongst the pre-exposure regime, exposure regime and the post-exposure regime were determined with repeated measures analysis of variance followed

by a Bonferroni post-test at $P \leq 0.05$. An unpaired 2-tailed students t-test was used to test for statistical significance ($P \leq 0.05$) in Part II of the study after first establishing homogeneity of variance using an F-test.

RESULTS

Part 1: Recovery of Physiological Status following pH 9.5 exposure

Ammonia Metabolism

Ammonia excretion rates of rainbow trout held in pH 8.0 water (pre-exposure) were approximately 240 umol·Kg^{-1}·h^{-1}. Over the first hour of high pH, J_{Amm} was reduced 80% to 44 umol·Kg^{-1}·h^{-1} (Fig. 1). This was followed by a gradual recovery of J_{Amm}, which by 8 h returned to levels that were 50% of the pre-exposure values; by 24-48 h J_{Amm} had returned to values not significantly higher than pre-exposure rates (Fig. 1). A dramatic increase in J_{Amm} was observed after the trout were returned to circumneutral pH water (pH 8.0; Fig. 1). Relative to J_{Amm} at 48 h, J_{Amm} was approximately 3-fold higher during the first hour of the post-exposure period and was 5 times greater than pre-exposure rates. J_{Amm} gradually declined, from approximately 1200 umol·Kg^{-1}·h^{-1} over the first hour of the post-exposure period, to 760 umol·Kg^{-1}·h^{-1} after 3.0 hours (Fig. 1). At 8 h J_{Amm} was still significantly elevated and by 12-24 h it reached levels comparable to the pre-exposure rates (Fig. 1).

In accordance with the initially inhibited J_{Amm} at pH 9.5, plasma T_{Amm} increased 5.5-fold, from a pre-exposure concentration of 60 umol·L^{-1} to 330 umol·L^{-1} by 8 h, with a further increase to approximately 400 umol·L^{-1} at 48 h (Fig. 2A). The levels of circulating unionized ammonia (NH_3) increased to a greater relative extent than plasma T_{Amm} during high pH exposure because of the development of a simultaneous blood alkalosis (see below). The pre-exposure P_{NH3} was 20 uTorr. By 8 h at pH 9.5, however, it had increased 9-fold to approximately 180 uTorr, with a further increase to about 270 uTorr at 48 h, reflecting the progressive alkalosis that developed (Fig. 2B). Re-introduction into circumneutral pH led to a rapid recovery of plasma T_{Amm}; by 3 h post-exposure plasma T_{Amm} had returned to pre-

exposure concentrations of approximately 50 umol·L^{-1} (Fig. 2A), thereafter stabilizing at about 30 umol·L^{-1} at 8 h and 24 h. Plasma P_{NH3} levels were also rapidly restored to pre-exposure levels by 3 h (Fig. 2B). At all times during the experiment, NH_4^+ (not shown) constituted more than 97 % of the plasma T_{Amm}, and therefore followed virtually identical trends to those in T_{Amm} (Fig. 2A).

Plasma Ions

By 48 h of high pH exposure plasma Na^+ and Cl^- were 5 % lower than the pre-exposure concentrations of 143 and 136 mmol·L^{-1}, respectively (Fig. 3). Re-introduction into circumneutral pH led to a re-establishment of plasma Cl^- concentrations after only 3 h while plasma Na^+ returned to pre-exposure values at 8 h. Plasma protein concentrations fluctuated around 2 g·100 ml^{-1} and did not change during either the exposure or post-exposure period, despite repetitive blood sampling (data not shown).

Acid-Base Status

Upon exposure to alkaline pH, the rainbow trout underwent a combined metabolic and respiratory alkalosis. At 8 h, this alkalosis was characterized by a 0.15 unit elevation in pH_e, a $\Delta[H^+_m]$ of -2.68 mmol·L^{-1} (ie. a "metabolic base load") and a 40% lower CO_2 tension (Pa_{CO2}) of approximately 1.6 torr (Fig. 4A,B,D). By 48 h, the metabolic component of the alkalosis was corrected, in association with a 3-fold elevation in blood lactate concentration, but pH_e was further elevated to about 8.17 due to a further decline in Pa_{CO2} to 0.45 Torr. No changes in Pa_{O2} were observed (Fig. 4C; note the Pa_{O2} data at 8 h were lost due to P_{O2} electrode failure).

Acid-base status was rapidly restored during the post-exposure period. Arterial pH had returned to pre-exposure values only 3 h after the trout were returned to circumneutral

pH, whereas Pa_{CO2} remained slightly depressed and ΔH^+_m remained close to zero (Figs. 4A,B,D). Interestingly, blood lactate was still significantly elevated at this time (Fig. 4D). By 8 h, however, Pa_{CO2} and blood lactate concentration had returned to levels not significantly different from pre-exposure values. Again, no changes in blood O_2 tension were observed (Fig. 4C).

Part II: Ammonia Storage and Lactate Production in the White Muscle Compartment

The plasma T_{Amm} concentrations, P_{NH3} levels, and acid-base status of fish exposed to pH 9.5 in Part II of the study (Fig. 5, Table 1) exhibited similar changes to those observed in Part I, though ammonia levels peaked at 8 h rather than 48 h and absolute elevations were smaller (Figs. 5A,B). There were no significant changes in control fish held at pH 8.0. The build-up of plasma T_{Amm}, during pH 9.5 exposure, coincided with a 2.2 fold elevation in white muscle T_{Amm} concentrations that approached 3 000 umol·L^{-1} ICF (Fig. 5A). Fish held at pH 8.0 had white muscle T_{Amm} concentrations of approximately 1250 umol·L^{-1} ICF (Fig. 5A). The development of a significant intracellular alkalosis (pH_i elevation of 0.09 units, Table 1) contributed to the 2.8-fold elevation in white muscle P_{NH3} in the fish exposed to pH 9.5 (Fig. 5B).

White muscle lactate concentrations were approximately 2.4 mmol.L^{-1} ICF in fish held at pH 8.0 but increased almost 4-fold to 8.8 mmol.L^{-1} ICF after 48 h at pH 9.5 (Fig. 6A). Arterial P_{O2} approximated 110 Torr in control fish and did not change after 48 h at pH 9.5 (Fig. 6B). Similarly, white muscle ATP and creatine phosphate were unaffected by high pH exposure and approximated 10 and 35-40 mmol.L^{-1} ICF, respectively (Fig. 6B)

DISCUSSION

Ammonia Metabolism and Storage in the White Muscle

Previously, Wilkie and Wood (1991) observed, that despite full recovery of ammonia excretion by 48 h of a 72 h high pH exposure regime, approximately 3600 umol·Kg^{-1} of ammonia that should have been excreted by rainbow trout were unaccounted for. They suggested that this "ammonia deficit" resulted from either reduced ammonia production (ie. lower metabolic rate), conversion of the ammonia to other waste products, or storage in other body compartments. In the present study, recovery of J_{Amm}, by 24 h of pH 9.5 exposure and slight elevation at 48 h, suggests that there was no reduction in ammonia production during the 2 day exposure. In fact, the high post-exposure "ammonia wash-out", which approximated 5000 umol·Kg^{-1} (Fig. 7), suggests that there may have been increased ammoniogenesis, that contributed to increased internal T_{Amm} levels during high pH exposure. The rapidity of the wash-out also implies that ammonia was not converted to an alternate end-product to any great extent, although there may have been a small stimulation of urea production (Olson and Fromm 1971; Wilkie and Wood 1991; Wilkie *et al.* 1993; Wright 1993) or glutamine production (Levi *et al.* 1974; Arillo *et al.* 1981). These possibilities should be investigated in more detail.

The post-exposure wash-out of ammonia is reminiscent of that seen in fish recovering from exhaustive exercise (Milligan and Wood 1986a,b). The ammonia wash-out following exercise results from increased white muscle NH_3 production via increased adenylate breakdown (Driedzic and Hochachka 1978). In the present study, the ammonia wash-out was likely related to the initial high pH-induced blockade of ammonia excretion at the gill and possibly elevated ammoniogenesis during and after high pH exposure. Accordingly, internal

(plasma) T_{Amm} levels likely increased until an outward P_{NH3} gradient between the plasma and the water, most likely the unstirred boundary layers of the gills (Wright, Randall and Perry 1989; Wright, Iwama and Wood 1993), was achieved enabling J_{Amm} to return to pre-exposure rates. The Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*) of alkaline Pyramid Lake, Nevada, similarly tolerates higher internal levels of T_{Amm} and P_{NH3}, to facilitate J_{Amm} in alkaline environments (Wright *et al.* 1993; Wilkie *et al.* 1994).

Wilkie and Wood (1991) suggested other potential mechanisms of ammonia excretion by rainbow trout at pH 9.5 including: increased branchial Na^+/NH_4^+ exchange (Maetz 1972, 1973; Cameron and Heisler 1983) or H^+/NH_4^+ (Cameron 1986) exchange. The former possibility now appears unlikely since Wilkie and Wood (1994) recently demonstrated, using amiloride and radiotracers ($^{22}Na^+$), no link between Na^+ uptake and NH_4^+ excretion by rainbow trout at high pH. The possibility of H^+/NH_4^+ exchange seems unlikely because alkaline water is, by definition, deficient in available protons for such an antiporter. It is possible, however, that increased CO_2 excretion and/or enhanced activity of putative proton pumps on the branchial epithelium (Avella and Bornancin 1989; Lin and Randall 1993) might supply H^+ to an unstirred boundary layer for such a process, or for increased "diffusion trapping" of NH_3. A final possibility is that NH_4^+ diffusion, due to elevated NH_4^+ concentration, leads to recovery of J_{Amm} (Goldstein, Claiborne and Evans 1982; McDonald and Prior 1988; Heisler 1990), though the bulk of evidence at present weighs against an important role for NH_4^+ diffusion in freshwater fish (Wood 1993).

The theory governing ammonia distribution across fish white muscle cell membranes has been clearly established (Wright, Randall and Wood 1988; Wright and Wood 1988; Tang, Lin and Randall 1992). In brief, the membranes appear to be freely permeable to both NH_3

and NH_4^+. Both the transmembrane pH gradient ($pH_i < pH_e$; eg. Table 1) acting on NH_3 diffusion and the electrical gradient (membrane potential negative inside by 70 to 90 mV; Hagiwara and Takahashi 1967; Hidaka and Toida 1969) acting on NH_4^+ diffusion favours higher intracellular concentrations of T_{Amm} than the extracellular concentrations. The electrical gradient appears to have the larger influence, such that under steady state conditions, the distribution ratio $[T_{Amm}]_i/[T_{Amm}]_e$ is closer to that dictated by the electrical gradient (25 to 35) than to that dictated purely by the pH gradient (3-6). For example, under control conditions in Part II of the present study, the distribution ratio was about 24 (Table 1). As a result, there is a standing P_{NH3} gradient from intracellular to extracellular compartment of about 70 uTorr (eg. Fig. 5B; Table 1) and a small NH_4^+ electrochemical gradient of about -6 mV in the opposite direction (Table 1). As a result there is a continuous steady state shuttle whereby NH_3 diffuses from ICF to ECF, and NH_4^+ from ECF to ICF along partial pressure and electrochemical gradients, respectively.

Given this situation, as T_{Amm} built up in the ECF during the first 24 h at high pH (Figs. 2,5), in the face of inhibited branchial excretion but continuing production by the liver, it would automatically accumulate simultaneously in the white muscle ICF until a new steady state developed. The analysis in Table 1 illustrates that early in the exposure (eg. 8 h), the P_{NH3} gradient would likely be reversed so as to favour NH_3 entry into the ICF, while the electrochemical gradient for NH_4^+ entry would be greatly increased. Later on (eg. 48 h), their gradients return towards control values as a new steady state is approached. Note that the gradients and distribution ratio had not fully returned to control values in the fish in Part II, suggesting that a new steady state had not been fully achieved by 48 h (Table 1). Because the new steady state distribution ratio will be largely determined by the membrane potential,

the increase in $[T_{Amm}]_i$ is far greater than that in the $[T_{Amm}]_e$. Assuming that the total white muscle ICFV constitutes 45 % of the fish's body weight (Stevens 1968; Milligan and Wood 1986a), and the same $[T_{Amm}]_i/[T_{Amm}]_e$ relationship held in the fish of Part I as in Part II, then T_{Amm} retention in the white muscle would explain about 40 % of the observed post-exposure ammonia wash-out of approximately 5 000 umol·Kg^{-1}. The remainder was likely due to similar retention of T_{Amm} in other tissue compartments and/or elevated production. The amount stored in the total ECFV (0.25 L·Kg^{-1}; Milligan and Wood 1986a) was no more than 100 umol·Kg^{-1}, or about 5 % of the observed wash-out.

Although plasma P_{NH3} and white muscle intracellular P_{NH3} were dramatically elevated during pH 9.5 exposure, they did not appear to be toxic to the fish. The rapid return of plasma P_{NH3}, and presumably white muscle P_{NH3}, to pre-exposure levels, following re-introduction into circumneutral pH water (Fig. 2), supports our earlier arguments that fish modulate internal T_{Amm} levels in accordance with external environmental conditions. The plasma P_{NH3}'s of Part I, though elevated, are still likely below the toxic thresholds for rainbow trout and are comparable to those observed by Wilkie and Wood (1991) (approximately 300 uTorr) in trout that experienced negligible mortality at pH 9.5 and about 25% lower than levels observed in salmonids suffering mortality in alkaline or hyperammonemic environments (eg. Yesaki and Iwama 1992; Wilkie *et al.* 1993; R.W. Wilson and C.M. Wood, unpublished observations). In conclusion, it is apparent that the white muscle is capable of storing much of the ammonia that was retained by the fish as a result of the initial high pH-induced inhibition of ammonia excretion and/or generated through increased rates of ammoniogenesis. In terms of environmental relevance, the ability of fish to store ammonia in the white muscle compartment would benefit the animal not only at high

pH, but also when it was subjected to high environmental ammonia concentrations, such as would occur following the turnover of eutrophic water bodies or as a result of anthropogenic ammonia deposition (Barica 1974; Wetzel 1983).

Restoration of Acid-Base and Ion Balance

The respiratory alkalosis observed during the high pH exposure has been observed in a number of previous studies on trout (Wright and Wood 1985; Lin and Randall 1990; Wilkie and Wood 1991; Yesaki and Iwama 1992) and the metabolic alkalosis in several others (Heming and Blumhagen 1988; Yesaki and Iwama 1992; Wilkie *et al.* 1993, 1994). Wilkie *et al.* (1994) and Wilkie and Wood (1994) postulated that correction of the alkalosis resulted in part from increased branchial Cl^-/HCO_3^- exchange that was brought about by observed increases in the fractional surface area of branchial chloride cells. Significant metabolic production of lactic acid also likely contributed to the stabilization of blood pH as observed in the present and previous studies (Wilkie and Wood 1991; Wilkie *et al.* 1993). In fact, the increases in WM lactate observed here support the supposition that most of the metabolic acid appearing in the ECF originated in the WM ICF (Fig. 6A), and was not therefore, the result of branchial HCO_3^- extrusion. The absence of any change in Pa_{O2}, ATP or creatine phosphate suggest that this increase in WM lactate production was *not* associated with hypoxemia. The observed intracellular alkalosis and/or elevated white muscle T_{Amm} concentrations may have led to this lactacidosis by stimulating glycolytic flux in this compartment. In fact, respiratory alkalosis is known to lead to lactacidosis in mammals (Bock *et al.* 1932; Eichenholz *et al.* 1962; Takano 1968; Garcia *et al.* 1971) and elevated NH_4^+ is known to stimulate phospho-fructokinase (PFK), a key glycolytic flux generating enzyme (Kuhn *et al.*, 1974).

The potential contribution that white muscle lactic acid production made to the extracellular metabolic acidosis can be quantified by constructing metabolic H^+ (H^+_m) and lactate budgets for the WM ICF space, the ECF space and the water. Such an analysis, using the methods outlined by Milligan and Wood, 1986a,b) was used here. Values for the white muscle intracellular space, extracellular space and each compartments respective non-bicarbonate buffering capacity were also obtained from Milligan and Wood (1986a,b). Accordingly, this analysis suggested that virtually all of the ECF acidosis arose from metabolic H^+ production in the white muscle compartment (Fig. 8). In fact the WM developed a H^+_m deficit of approximately 1.5 mmol·Kg^{-1} body weight, while the ECF had a H^+_m surplus that approximated 0.6 mmol·Kg^{-1} body weight. Presumably the remaining H^+_m was lost across the gills to the water (Fig. 8A). The lactate load in the WM, about 2.9 mmol·Kg^{-1} body weight, was more than sufficient to account for the WM H^+_m deficit. The ECF lactate load, less than 0.5 mmol·Kg^{-1} body weight, probably reflected "overflow" lactate from the WM ICF. It is unlikely that much lactate was lost across the gills (Milligan and Wood 1986a) (Fig. 8B).

Re-introduction of the fish into circumneutral pH led to rapid re-establishment of blood acid-base status. Only Pa_{CO2} was still significantly reduced 3 hours after return to circumneutral pH; all other acid-base parameters had returned to pre-exposure values. Surprisingly, despite the continued significant elevation of blood lactate, there was no post-exposure acidosis (ie. "overshoot") as Pa_{CO2} moved towards pre-exposure levels. However, the continued significant depression of Pa_{CO2} at 3 hours may have contributed to the normalization of blood pH at this time.

As in previous studies (Heming and Blumhagen 1988; Wilkie and Wood 1991; Yesaki

and Iwama 1992; Wilkie *et al.* 1993), the trout in the present study experienced significant reductions in plasma Na^+ and Cl^- by 48 h of high pH exposure. Since trout suffering mortality at high pH experienced severe ionoregulatory disturbances (Yesaki and Iwama 1992; Wilkie *et al.* 1993) it may be symptomatic of direct high pH induced damage to the gill epithelium. Indeed, Daye and Garside (1976), studying brook trout (*Salvelinus fontinalis*), reported severe histological changes to the gills, including mucus cell hypertrophy and epithelial lifting. Galat *et al.* (1985) and Wilkie *et al.* (1994) also observed chloride cell hyperplasia in Lahontan cutthroat trout inhabiting alkaline lakes. Wilkie and Wood (1994) have reported similar responses in chloride cell morphometry in rainbow trout following high pH exposure. The fact that no changes in Pa_{O2} were observed in the present or previous high pH exposure studies (Wilkie and Wood 1991; Wilkie *et al.* 1993, 1994) suggests high pH induced alterations in gill morphology do not impede gas exchange and are likely adaptive, rather than pathological. The rapid post-exposure restoration of the rainbow trouts' plasma ion, acid-base and plasma ammonia balance to pre-exposure levels supports this theory. It should be kept in mind, however, that factors such as water hardness, temperature and ionic composition play an important role in determining a fish's ability to tolerate environmental stressors such as elevated environmental pH (Yesaki and Iwama 1992).

In conclusion, despite numerous previous investigations detailing the toxicity of alkaline environments to teleosts (eg. Eicher 1946; Jordan and Lloyd 1964; Daye and Garside 1975, 1976; Alabaster and Lloyd 1980; Murray and Ziebell 1984; Heming and Blumhagen 1988; Yesaki 1990; Yesaki and Iwama 1992; Wilkie *et al.* 1993) it appears that the physiological disturbances initiated by exposure to pH 9.5 are readily reversible in rainbow trout. Although physiological disturbances occur across the gill epithelium, the fact that Pa_{O2}

remains constant prior to, during and after alkaline exposure suggests no impairment of oxygen uptake and hence, no gill histopathology, resulted. The rapid restoration of acid-base, ion and ammonia balance in the plasma, during the post-exposure period, supports this conclusion. During the period of initial exposure to high environmental pH, increased plasma P_{NH3} enables the fish to re-establish a positive P_{NH3} diffusion gradient across the gill epithelium and ultimately results in re-establishment of ammonia excretion rates despite continued high pH exposure. Greater plasma T_{Amm} also leads to the development of ECF:ICF gradients that favour NH_3 and NH_4^+ uptake into the white muscle compartment such that far more T_{Amm} accumulates in the muscle ICFV than in the ECFV after 48 h of pH 9.5 exposure. Storage of ammonia in the tissues probably accounts for much of the prolonged ammonia wash-out observed during the post-exposure period. Finally, elevations in white muscle lactic acid production appear to make a significant contribution to rainbow trout extracellular acid-base regulation during acute high pH exposure.

TABLE 1: Estimates of the White Muscle Intracellular: Extracellular P_{NH3}, NH_4^+ Electrochemical Gradients and ICF:ECF Ammonia Distribution Ratios in Rainbow Trout Held at pH 9.5 or pH 8.0.

	pH 8.0	pH 9.5	
White Muscle ICF		8 h	48h
pH_i	7.164 ± 0.029	—	7.255 ± 0.018*
T_{Amm} (umol·L^{-1})	1316.1 ± 107.7	—	2944.8 ± 524.8*
[NH_4^+] (umol·L^{-1})	1311.8 ± 107.5	—	2933.0 ± 522.7*
P_{NH3} (uTorr)	86.7 ± 6.5	—	238.4 ± 43.4*
ECF (Plasma)			
pH_e	7.840 ± 0.016	8.018 ± 0.011*	8.026 ± 0.024*
T_{Amm} (umol N·L^{-1})	59.5 ± 6.38	330.5 ± 49.9*	241.1 ± 34.1*
[NH_4^+] (umol N·L^{-1})	58.6 ± 6.3	323.0 ± 49.0*	235.6 ± 33.5*
P_{NH3} (uTorr)	18.4 ± 1.9	151.0 ± 18.8*	111.7 ± 12.7*
E_{NH4} (mV)	-77.2 ± 4.4	-35.3 ± 3.6*^	-61.1 ± 7.3*
$^bF_{NH4}$ (mV)	-5.8 ± 4.4	-47.7 ± 3.6*^	-21.9 ± 7.3*
ICF P_{NH3} - ECF P_{NH3} (uTorr)	68.3 ± 7.8	-62.1 ± 23.8*^	126.8 ± 48.8
$[T_{Amm}]_i/[T_{Amm}]_e$	24.0 ± 3.9	4.3 ± 0.6*^	13.4 ± 2.2*

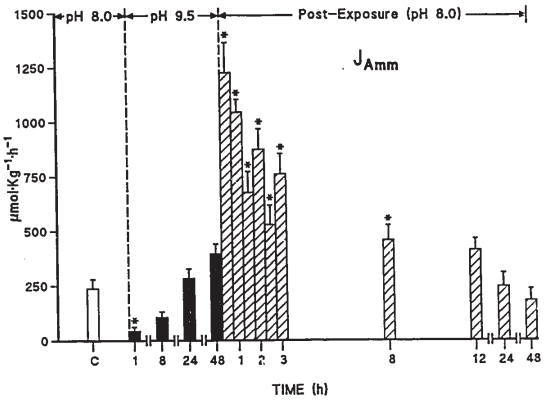
* For these calculations white muscle pH_i and T_{Amm} values at 8 h were assumed to be the same as values in the control (pH 8.0) muscle samples.

^{b} $F_{NH4} = TEP - E_{NH4}$; where the transepithelial potential is assumed to be -83 mV (see Wright, Randall and Wood 1988).

* A negative F_{NH4} indicates an inwardly directed gradient for NH_4^+.

* indicate significant differences from control (pH 8.0) values ($P < 0.05$; N=6).

Fig. 1. Rates of ammonia excretion in rainbow trout prior to pH 9.5 exposure (pre-exposure; pH 8.0), during 48 h of pH 9.5 exposure and during 48 h following pH 9.5 exposure (post-exposure; pH 8.0). Means ± 1 SEM; N = 7. Asterisks indicate significant differences from pre-exposure (control) values ($P < 0.05$).



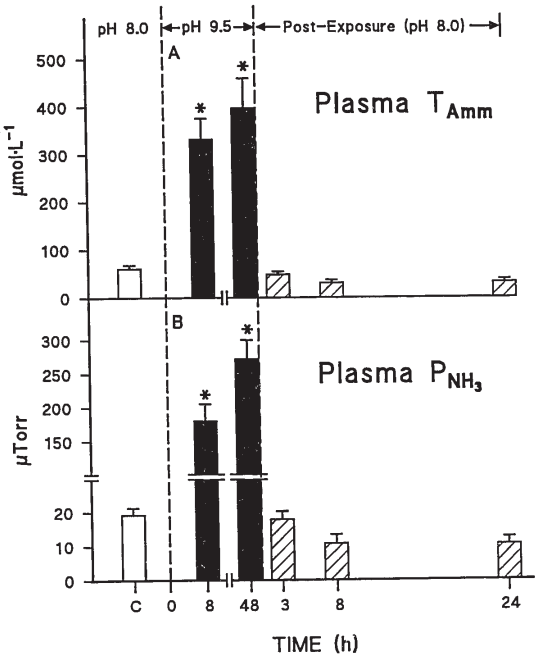


Fig. 2. Changes in (A) plasma total ammonia (T_{Amm}) concentration and (B) plasma NH_3 partial pressure (P_{NH3}) of rainbow trout, prior to pH 9.5 exposure (pre-exposure; pH 8.0), during 48 h of pH 9.5 exposure and during 48 h following pH 9.5 exposure (post-exposure; pH 8.0). Means ± 1 SEM; N = 7. Asterisks indicate significant differences from pre-exposure (control) values ($P < 0.05$).

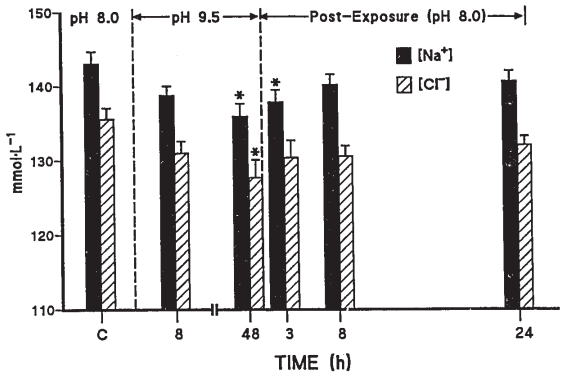


Fig. 3. Changes in the plasma Na^+ (solid bars) and Cl^- (hatched bars) concentrations of rainbow trout prior to pH 9.5 exposure (pre-exposure; pH 8.0), during 48 h of pH 9.5 exposure and during 48 h following pH 9.5 exposure (post-exposure; pH 8.0). Means ± 1 SEM; N = 7. Asterisks indicate significant differences from pre-exposure (control) values ($P < 0.05$).

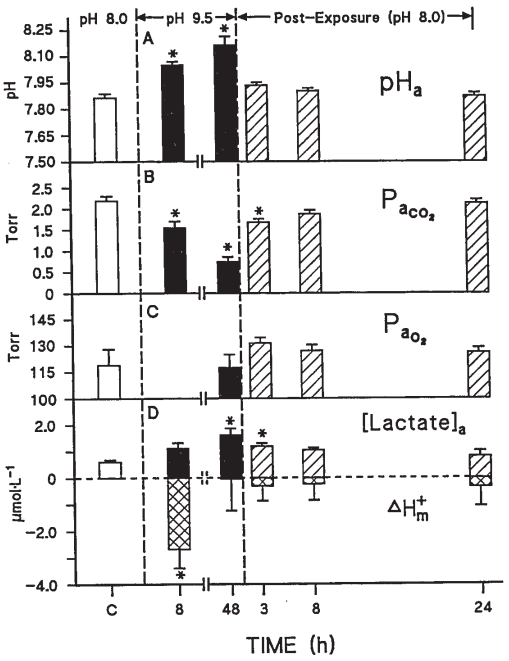


Fig. 4. Changes in the (A) arterial blood pH (pH_a); (B) arterial plasma CO_2 tension (Pa_{CO2}); (C) arterial blood O_2 tension (Pa_{O2}) and (D) metabolic acid load (ΔH^+_m; downward directed cross hatched bars) and whole blood lactate concentration (upward directed bars) of rainbow trout prior to pH 9.5 exposure (pre-exposure; pH 8.0), during 48 h of pH 9.5 exposure and during 48 h following pH 9.5 exposure (post-exposure; pH 8.0). Means ± 1 SEM; N = 7. Asterisks indicate significant differences from pre-exposure (control) values ($P < 0.05$).

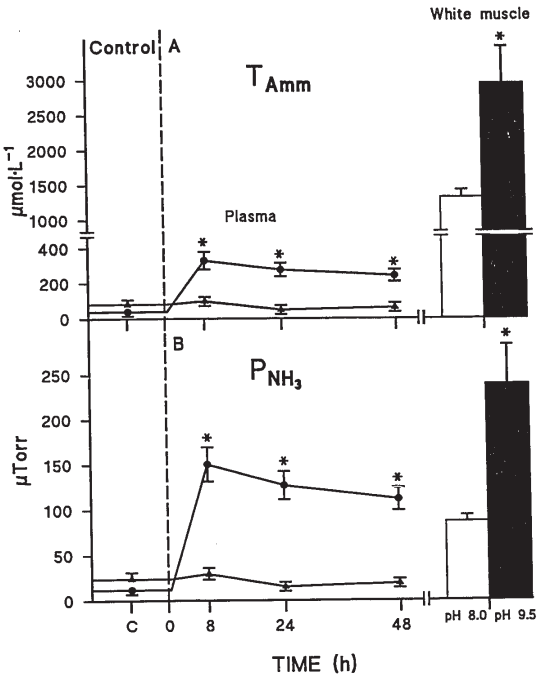


Fig. 5. Plasma (= extracellular fluid; line graphs) and terminal white muscle intracellular fluid (bar graphs) (A) T_{Amm} concentrations, and (B) P_{NH3}, of fish held at pH 8.0 (▲ and open bar, respectively) or exposed to pH 9.5 (● and solid bar, respectively) for 48 h. Means ± 1 SEM; N = 7 for plasma values and N = 6 for white muscle intracellular measurements. Asterisks indicate significant differences between fish exposed to pH 9.5 *vs.* those held at pH 8.0 (controls; $P < 0.05$).

Fig. 6. Terminal white muscle intracellular fluid (bar graphs) (A) lactate concentrations, and (B) ATP and creatine phosphate concentrations (mmol·L^{-1} ICF), and (B) arterial blood P_{O2} (Torr) of fish held at pH 8.0 (open bar) or exposed to pH 9.5 (solid bar, respectively) for 48 h. Means ± 1 SEM; N = 6. Asterisk indicates significant differences between fish exposed to pH 9.5 *vs.* those held at pH 8.0 (controls; $P < 0.05$).

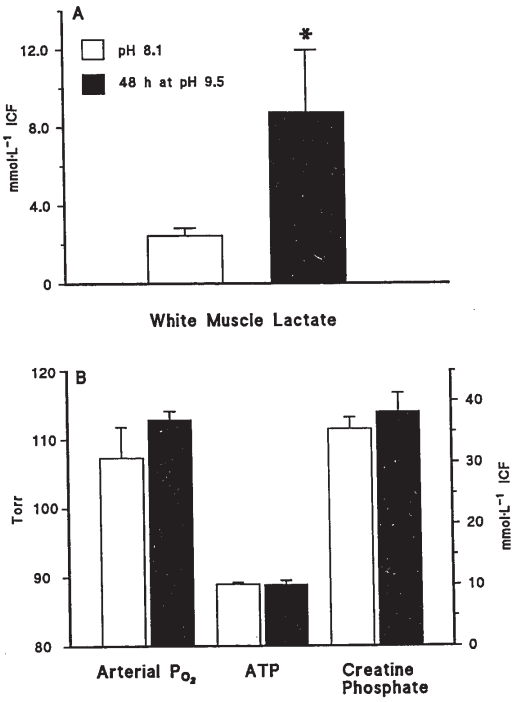
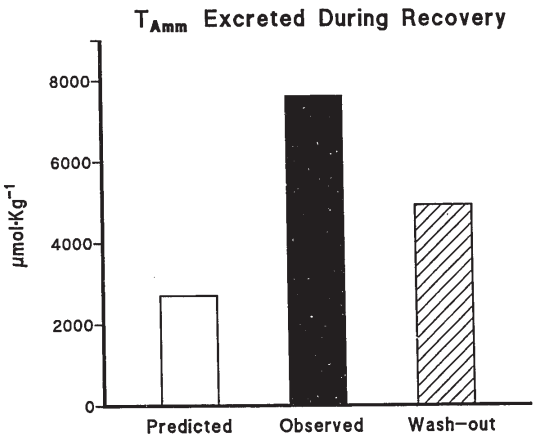


Fig. 7. Predicted (open bar) and observed amounts of T_{Amm} (solid bar) excreted by rainbow trout during the first 12 h of recovery from high pH (pH = 9.5) exposure. The predicted T_{Amm} assumes the pre-exposure J_{Amm} continued during the 12 h recovery period. The ammonia wash-out (hatched bar) is the observed T_{Amm} excreted - the predicted T_{Amm} excreted. Estimate based on mean ammonia excretion rates of 7 fish.



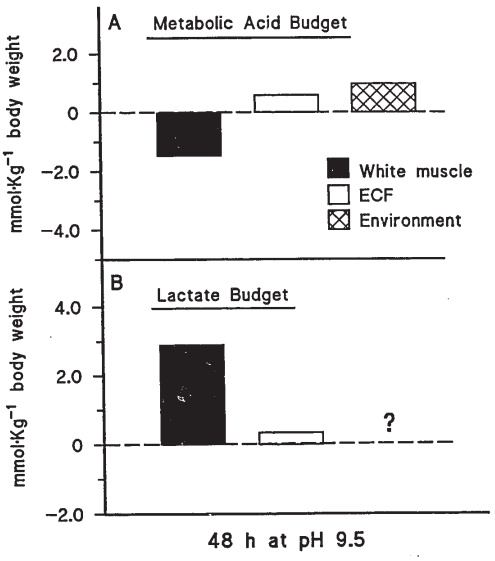


Fig. 8. Estimates of the (A) metabolic acid budget (H^+_m) and (B) lactate budget for the white muscle intracellular fluid space (WM ICF; solid bars), the extracellular fluid space (ECF; open bars) and the environment (water; cross hatched bars) for rainbow trout held at pH 9.5 for 48 h. Estimates based on respective mean differences in the acid-base status of rainbow trout held at pH 9.5 vs. pH 8.0 as described in Milligan and Wood (1986a,b). Estimates were based on the respective compartment fluid volumes and non-bicarbonate buffering capacities provided by Milligan and Wood (1986a,b).

correlated with Na^+ influx. Thus, rainbow trout regulate internal ion balance at high pH through differential modification of Cl^- vs. Na^+ transbranchial movements. Currently, electron microscopy is being used to establish if the ammonia excretion and ion flux patterns reported here are associated with alterations in trout gill ultrastructure.

CHAPTER 4

MODULATION OF BRANCHIAL ION MOVEMENTS BY RAINBOW TROUT AT HIGH pH.

ABSTRACT

Exposure of rainbow trout to highly alkaline (pH = 9.5) water resulted in initial 60-70% reductions in Cl^- and Na^+ influx (J^{Cl}_{in} and J^{Na}_{in}, respectively), but only affected outflux (J^{Cl}_{out} and J^{Na}_{out}) to a small degree. J^{Na}_{in} remained depressed, but J^{Cl}_{in} recovered by 3 d of alkaline exposure. The initial reductions in J^{Cl}_{in} and J^{Na}_{in} resulted in respective net Cl^- and Na^+ losses (J^{Cl}_{net} and J^{Na}_{net}) of 150-200 umol·kg^{-1}·h^{-1}. Both J^{Na}_{net} and J^{Cl}_{net} were restored by 72 h. Saturation kinetic analysis revealed transient 50 % reductions in J^{Cl}_{max} and more sustained 70 % decreases in J^{Na}_{max}, which accounted for decreases in influx. A complete recovery of J^{Cl}_{max} to pre-exposure levels, ultimately led to re-establishment of J^{Cl}_{in} by 72 h. Internal Cl^- concentrations decreased by about 10 % over the first day of exposure but stabilized thereafter. Despite chronically reduced J^{Na}_{in}, plasma Na^+ concentration was relatively stable. This was due to the gradual development of a counterbalancing reduction in J^{Na}_{out}, which was significant by 72 h. In contrast to the kinetics of Cl^- uptake, a persistently depressed J^{Na}_{max} (by 33 %), and a more pronounced decrease in transporter affinity (4-fold increase in K^{Na}_m) accounted for the persistent reduction in J^{Na}_{in}. Initial reductions in ammonia excretion (J_{Amm}) were followed by a gradual recovery of J_{Amm}, to control values by 72 h, but were not

INTRODUCTION

The basic physiological responses of teleosts to highly alkaline environments (pH > 9.0) have been well characterized over the last several years (eg. Johansen *et al.*, 1975; Randall *et al.*, 1989; Danulat and Kempe, 1992; Wright *et al.*, 1993). In salmonids most attention has focussed on the high pH induced inhibition of branchial ammonia excretion [J_{Amm}; Wright and Wood, 1985; Wilkie and Wood, 1991, 1994a (Appendix 1),b; Yesaki and Iwama, 1992; Wilkie *et al.*, 1993; Wright *et al.*, 1993], decreases in plasma Na^+ and Cl^- concentration (Heming and Blumhagen, 1988; Wilkie and Wood, 1991, 1994b; Yesaki and Iwama, 1992; Wilkie *et al.*, 1993) and the development of profound respiratory and/or metabolic alkalosis (Wright and Wood, 1985; Heming and Blumhagen, 1988; Lin and Randall, 1990; Wilkie and Wood, 1991; Yesaki and Iwama, 1992; Wilkie *et al.*, 1993, 1994). In some cases, mortality at high pH appears to be at least partially attributable to severe depressions of plasma electrolytes (Yesaki and Iwama, 1992; Wilkie *et al.*, 1993) and survival appears to be dependant upon restricting Na^+ and Cl^- losses to the first 24-48 h of high pH exposure (Wilkie and Wood, 1991). Despite this fact, little is known about how ionoregulatory disturbances are initiated, let alone corrected by salmonids exposed to alkaline conditions, and how they may interact with acid-base disturbances. Wilkie and Wood [1994a (Appendix 1)] reported that reductions in Cl^- and Na^+ uptake might account for some net losses of Na^+ and Cl^- during 72 h of high pH exposure. They did not, however, resolve if changes in Cl^- and Na^+ outflux also contributed to and/or corrected high pH-induced ionoregulatory disturbances. Accordingly, the goal in Part I of this investigation was to build on these earlier observations, and characterize how Cl^- and Na^+ influx and outflux patterns were altered during exposure (72 h) of rainbow trout (*Oncorhynchus mykiss*) to high pH (pH

9.5).

The differential influx rates of Cl^- and Na^+ across the rainbow trout gill epithelium, reported by Wilkie and Wood [1994a (Appendix 1)] raised the possibility that modulation of Na^+ and Cl^- movements contributed to acid-base regulation at high pH. Such compensatory responses have previously been reported in fish subjected to a variety of acid-base insults including abrupt temperature change (Cameron, 1976), low pH (McDonald *et al.*, 1983), acid or base infusion (Goss and Wood, 1990b, 1991), hypercapnia (Cameron, 1976; Cameron and Iwama, 1987) and hyperoxia (Wood *et al.*, 1984). Direct measurements of net acidic equivalent flux across the gills at high pH cannot be performed with current technology because of the tendency of HCO_3^- (negative acidic equivalents) to precipitate out of solution at high pH, thereby interfering with standard titration (McDonald and Wood, 1981) and "ΔHCO_3^-" (Heisler, 1984) methods. It is now well established, however, according to the Strong Ion Difference theory and the constraints of electroneutrality (Stewart, 1983), that Cl^- and Na^+ are taken up across the gills in exchange for basic equivalents (= acid uptake) and acidic equivalents (= base uptake), respectively (McDonald *et al.*, 1989; Wood, 1991). Accordingly, net H^+ movements were indirectly estimated by examining the differences between net Cl^- and net Na^+ movements during the 72 h high pH exposure regime.

In part II we investigated the mechanisms by which changes in branchial J^{Cl}_{in} and J^{Na}_{in} took place during high pH exposure. To accomplish this, saturation kinetic analyses were performed under control conditions and at various times during 72 h exposure to pH 9.5. The protocol used closely followed that employed by Goss and Wood (1990a) and was designed to establish if the maximal transport rates (J^{ION}_{max}) or transporter affinity (K^{ION}_m), or both were altered during exposure to pH 9.5. Accordingly, kinetic curves for Na^+ and Cl^-

uptake (influx) were generated by measuring the respective ion uptake rates at progressively higher external NaCl concentrations. Changes in J^{ION}_{max} are thought to reflect changes in either the number of transport sites and/or the availability of the appropriate internal acidic or basic equivalent counterion (H^+, NH_4^+, HCO_3^-, OH^-), while changes in K^{ION}_m are thought to reflect changes in the accessibility or avidity of the transporters for the external substrates (Na^+, Cl^-; Goss and Wood, 1991).

Ultimately, the saturation kinetics data, presented here in its entirety, are to be correlated with changes that are observed in the gill morphology of these same fish. This companion study is a collaborative project with Dr. Pierre Laurent of the Centre National de la Recherche Scientifique (CNRS), Strasbourg, France. Dr. Laurent will perform the morphological analyses, which will not be presented here.

Although Wright *et al.* (1993) and Wilkie and Wood [1994a (Appendix 1)] have provided evidence in favour of passive NH_3 diffusion as the dominant mechanism of ammonia excretion during high pH exposure, this area is still a subject of considerable debate (see Wood, 1993 for review). For instance, Yesaki and Iwama (1992) have presented pharmacological evidence, using amiloride, suggesting the presence of Na^+/NH_4^+ exchange in rainbow trout at pH 10. In contrast, Wilkie and Wood [1994a (Appendix 1)] found that amiloride treatment, while inhibiting J^{Na}_{in}, has no effect on J_{Amm} at pH 9.5. Thus, a secondary goal of the current investigation was to re-examine the potential relationship between Na^+ influx and ammonia excretion under alkaline conditions.

METHOD AND MATERIALS

Experimental Animals and Set-up

Rainbow trout (*Oncorhynchus mykiss*; mean weight 229.0 $\pm$ 5.7 g; n = 40) of both sexes were obtained from Spring Valley Trout Farm, Petersburg, Ont., in September 1993 and allowed to acclimate to hard dechlorinated Hamilton tapwater (Composition: $[Na^+]$ = 0.6, $[Cl^-]$ = 0.8, $[Ca^{++}]$ = 0.9, $[Mg^{++}]$ = 0.4, titratable alkalinity = 2.0 $mmol \cdot l^{-1}$) for a minimum of six weeks. Exactly one week prior to experimentation the fish were transferred in batches of 10, from the 6-10°C holding tank, to a temperature acclimation tank, which paralleled the experimental temperature of 13-15°C. The fish were starved at this time to minimize the known effects that feeding has on ammonia metabolism and excretion patterns (Fromm, 1963; Brett and Zala, 1975). Two days prior to experimentation 8 of the fish were placed into individual, darkened plexiglass flux boxes that received flowing water at 0.5 $l \cdot min^{-1}$. The boxes were part of a "flow-through" experimental system, fitted with a pH-stat, which has been described in detail previously (Wilkie and Wood, 1994b). A flow-through system was employed to mitigate unavoidable decreases in water Ca^{++} that occur at pH 9.5 (Wilkie and Wood, 1991; 1994b). Water Ca^{++} concentration approximated 0.4-0.6 $mmol \cdot l^{-1}$ during the high pH exposure regime in the present study, above the reference limit for "hard" water of 0.4 $mmol \cdot l^{-1}$. (Marier *et al.*, 1979). The pH-stat set-up was only used during high pH exposure regimes; the untreated, dechlorinated tapwater (pH 8.0) served as the control media in Parts I and II of the study. The pH-stat set-up comprised a Radiometer TTT80 autotitrator, PHM84 pH meter and a GK2401C macro-pH electrode; the latter monitored head tank water pH. When the water pH dropped below 9.65, the autotitrator activated an electromagnetic control valve which regulated the drop-wise addition of 1 N KOH into the vigorously aerated

head tank. The pH of water entering the boxes, from the head tank, was periodically monitored with an independent PHM82 pH meter and GK2401C pH electrode and approximated 9.56 $\pm$ 0.02 during high pH regimes. Resultant water K^+ concentrations during the high pH exposure regimes never exceeded 1.0 mmol·l^{-1}; K^+ concentrations in this range are not known to elicit any detectable physiological responses in salmonids [Wilkie *et al.*, 1993; Wilkie and Wood, 1994a (Appendix 1)].

When water flow was cut off to the boxes during high pH flux determinations, continuous CO_2 addition to the box by the fish and, to a lesser extent by aeration, continually drove water pH down. This necessitated manual monitoring of water pH at 30 minute intervals; when water pH dropped below 9.50 an appropriate amount (0.5 to 2.0 ml) of 1 N KOH was added to the water through small openings in the box lid. The flux boxes used in this study were slightly modified versions of those described in detail by McDonald and Rogano (1986). Briefly, the boxes comprised an aerated chamber which was well aerated, thereby ensuring thorough mixing, and a connecting inner chamber that contained each fish. Boxes used in Part I were approximately 3.0 L in volume; those used in Part II were about 2.0 l. The shorter flux determination periods in Part II necessitated the use of smaller box volumes for more accurate resolution of differences in water radioactivity, ion and ammonia concentrations. The boxes were fitted with "mykiss mikro-siphons" that consisted of a 3-way stopcock, to which a syringe could be attached or detached, and a 10 cm length of tygon tubing which led into the inner chamber of the box. Since the boxes were covered and the syringes were fitted on an externally mounted valve, the fish were presumably unaware of the researchers' presence when water samples were taken.

Experimental Protocol

Part I: Unidirectional Fluxes of Na^+ and Cl^- During High pH Exposure.

Flux rates were determined on 7 rainbow trout initially held at pH = 7.9 and then exposed to pH 9.5 for 75 h. Flux measurements were made at pH 8.0 (control), and after 0-3, 8-11, 24-27, 48-51 and 72-75 h of alkaline exposure. At these times ammonia excretion and Na^+ and Cl^- influx, outflux and net flux rates were determined. Twenty minutes prior to the initiation of flux determinations, the boxes were fitted with the mykiss mikro-siphons and flow to the box was cut off. Approximately 4 uCi of $^{24}Na^+$ and 10 uCi of $^{36}Cl^-$ were added to the box water and allowed to equilibrate for 15 minutes. After equilibration, a 10 ml water sample was withdrawn for pH determination, followed by a 45 ml water sample for later determination of $^{24}Na^+$ and $^{36}Cl^-$ radioactivity, "cold" (non-radioactive = stable isotope) Na^+ and Cl^- concentrations and water ammonia concentration. The process was repeated at 1 h and 3 h, after which the boxes were re-opened to the flow-through system. At the end of the final flux determination (72-75 h) the fish were sacrificed with an overdose of freshly prepared MS 222 solution (1.5 g·l^{-1}; Syndel) which was buffered to pH 7.0 with several pellets of KOH. Blood samples (1 ml) were then drawn into a Hamilton syringe via caudal puncture, centrifuged at 10 000 g and the plasma frozen for later determination of cold $^{24}Na^+$ and $^{36}Cl^-$ radioactivity, plasma Na^+ and Cl^- concentrations.

Part II: High pH Induced Changes in Cl^- and Na^+ Uptake Kinetics.

Saturation kinetics analysis were performed using a protocol developed by Goss and Wood (1990a,b) in which the water Cl^- and Na^+ concentrations were sequentially increased, and the Cl^- or Na^+ influx rates measured at each respective concentration. Accordingly, estimates of the apparent maximal transport rates (J^{ION}_{max}) and the affinity of the transporters

(K^{ION}_m) for Cl^- and Na^+ were established.

Kinetic analyses were performed at pH 8.0 (control) and after approximately 10 h, 1 day and 3 days of exposure to pH 9.5. A separate batch of 6-7 fish was used at each time period. For each kinetic determination, the total flux period generally lasted for 6 h and water ammonia concentrations never exceeded 100 umol·l^{-1}. Determinations of Na^+ and Cl^- influx were made over five, 30 minute intervals in which the nominal water NaCl concentrations were 80, 200, 350, 600 and 1 200 umol·l^{-1}, respectively. A final flux determination, at 2 200 umol·l^{-1} of NaCl, lasting 60 minutes, was necessary to detect small changes against the high absolute activities of $^{24}Na^+$ and $^{36}Cl^-$ in the box at the start of this final flux period.

Accurate determination of ion uptake kinetics across fish gills necessitates the use of NaCl free water so that external Na^+ or Cl^- concentrations can be easily and accurately manipulated during the uptake kinetic experiments (eg. Shaw, 1959; Goss and Wood, 1990a). It is important, however, to ensure that other water characteristics, such as Ca^{++}, Mg^{++} and titratable alkalinity are maintained because changes in water Ca^{++} and/or Mg^{++} may elicit changes in branchial transepithelial potential (Goss and Wood, 1990a) and/or Na^+ and Cl^- permeability (McDonald and Rogano, 1986). Such changes could disturb normal branchial ion influx rates. Accordingly, NaCl free water was prepared by first passing dechlorinated Hamilton tapwater through a de-ionizing canister, collecting the water and then adding back the appropriate amount of Ca^{++} ($CaCO_3$ salt) and Mg^{++} ($4MgCO_3{\cdot}Mg(OH)_2{\cdot}4H_2O$ salt) so that it mimicked the composition of the Hamilton tapwater (see Goss and Wood, 1990a for further details). The composition of the nominally NaCl free water prepared for the current experiment was as follows: [Na^+] = 2 umol·l^{-1}; [Cl^-] = 1.5 umol·l^{-1}; [Ca^{++}] = 0.9 mmol·l^{-1};

[Mg^{++}] = 0.3 mmol·l^{-1}; titratable alkalinity = 2.57 mmol·l^{-1}.

Each flux series was preceded by a thorough flushing of each flux box (3/4 replacement X 7) with the NaCl free water at control (pH 8.0) or experimental pH (pH 9.5). A 10 ml water sample was then taken to check pH, and fifteen minutes prior to each flux determination, a known amount of radiolabelled (4 uCi $^{24}Na^+$·ml^{-1}, 2 uCi $^{36}Cl^-$·ml^{-1} see below) NaCl was taken from a 1 mmol·l^{-1} NaCl stock solution and added to each box to yield the appropriate external NaCl concentration. Water samples (10 ml + 45 ml, respectively) were then taken at the start and end of each 30 minute (or 60 minute) flux. At the end of each flux period, the water removed due to sampling (110 ml) was then replaced with an equal volume of NaCl free water (at the appropriate pH and temperature) and the appropriate volume of radiolabelled NaCl required for the next flux determination period. This process was subsequently repeated at each nominal NaCl concentration. The radiolabelled NaCl was taken from the same 1 mmol·l^{-1} stock solution during each experimental series to ensure constant water specific activity (Goss and Wood, 1990a) between different sample periods. Following the final 60 minute flux determination period, water flow was briefly re-established to each box after which the fish were sacrificed and sampled as previously described. Plasma in this series was analyzed for $^{24}Na^+$ and $^{36}Cl^-$ radioactivity, "cold" Na^+ and Cl^- concentration, total ammonia and lactate concentrations. Gill samples were also excised from the fish that had been held at pH 8.0, and at pH 9.5 for 10 h and 3 days. These tissues were fixed and processed for analyses in the aforementioned companion study.

Preliminary experiments indicated that the levels of $^{24}Na^+$ radioactivity, utilized in Part I, over 3 h flux determination periods, were not high enough to obtain adequate resolution of changes in radioactivity when shorter flux periods (30 minutes) were used during

Na^+ uptake kinetics experiments. Furthermore, due to the fact that kinetics experiments were more prolonged (ie. 6 h), than the unidirectional flux study, there was more radioactive decay of the $^{24}Na^+$ prior to measurements of radioactivity. Accordingly, through, "trial and error" preliminary experiments, it was determined that approximately 4 $uCi \cdot ml^{-1}$ of $^{24}Na^+$ was required for accurate determination of J^{Na}_{in} during the kinetics experiments. The levels of $^{36}Cl^-$ (2 $uCi \cdot ml^{-1}$) used were also sufficient.

Analytical Techniques and Calculations

Unidirectional Fluxes and Ammonia Excretion Rates

Rates of Na^+ and Cl^- uptake were calculated from decreases in water radioactivity over the course of each flux determination period, the known box volume and weight of the fish according to the following formula (outlined in Wood, 1988):

$$J^{ION}_{in} = \frac{(CPM_i - CPM_f)}{MSA} \cdot \frac{Volume}{W \cdot T} \quad (1)$$

where: J^{ION}_{in} represents J^{Na}_{in} or J^{Cl}_{in}; CPM_i and CPM_f are the respective $^{24}Na^+$ and $^{36}Cl^-$ ($CPM \cdot ml^{-1}$) at the start and end of each flux determination period; MSA is the mean water specific activity ($CPM \cdot umol^{-1}$); Volume refers to flux box water volume (~ 3.0 l or 2.0 l minus the fish's weight); W and T represent fish weight (kg) and flux duration (h), respectively. Back-flux correction was not necessary as the internal specific activity, based on plasma measurements, was never greater than 10 % of the water specific activity (Wood, 1988).

Estimates of J^{Na}_{net} and J^{Cl}_{net} were based on differences in the "cold" (non-radioactive) Na^+ and Cl^- concentrations in the water according to the following formula:

$$J^{ION}_{net} = \frac{([ION]_i - [ION]_f) \cdot Volume}{W \cdot T} \quad (2)$$

where: J^{ION}_{net} represents J^{Na}_{net} or J^{Cl}_{net}; $[ION]_i$ and $[ION]_f$ represent the concentrations of cold Na^+ or Cl^- at the start and end of each flux period and Volume, W and T have the same meanings as previously stated.

Net ion movements (J^{ION}_{net}) are the sum of the influx (J^{ION}_{in}) and outflux rates (J^{ION}_{out}). Accordingly, the latter were calculated according to the following expression:

$$J^{ION}_{out} = J^{ION}_{net} - J^{ION}_{in} \quad (3)$$

Ammonia excretion rates were calculated from changes in water total ammonia (T_{Amm}) concentrations using an equation analogous to 2.

Since $^{24}Na^+$ emits both gamma and beta rays while $^{36}Cl^-$ is a pure beta emitter, samples were first analyzed (in triplicate) for total gamma counts on a Packard 5000 series gamma counter. Since $^{24}Na^+$ has a very short half-life ($t_{1/2}$ = 15 h) and $^{36}Cl^-$ a very long half-life ($t_{1/2} = 3.1 \times 10^5$ years), the samples were then allowed to decay for at least 40 $^{24}Na^+$ half-lives, at which time the gamma counts were exhausted. Samples were then prepared for measurements of $^{36}Cl^-$ activity by scintillation counting; 10 ml of aqueous counting scintillant (ACS fluor; Amersham) were added to each 5 ml water sample. The samples were then left in the dark overnight to minimize chemiluminescence after which $^{36}Cl^-$ CPM were determined on a 1217 Rackbeta scintillation counter. The concentrations of cold Na^+, in water and plasma, were determined via atomic absorption (Varian 1275 AA). Cold Cl^- concentrations in water were determined by the mercuric thiocyanate assay (Zall *et al.*, 1956) and in plasma by coulometric titration (Radiometer CMT10 chloridometer). Water T_{Amm} concentrations were determined via a micro-modification of the salicylate-hypochlorite assay (Verdouw *et al.*,

1978). Plasma ammonia and lactate concentrations were determined enzymatically (glutamate dehydrogenase and lactate dehydrogenase, respectively) with commercial kits (Sigma).

Saturation Kinetics

Data generated in Part II of the study indicated that both Na^+ and Cl^- influx followed first order saturation kinetics. Accordingly, Na^+ or Cl^- influx rates could be predicted by the Michaelis-Menten relationship:

$$J^{ION}_{in} = \frac{J^{ION}_{max} \cdot [ION]_o}{K^{ION}_m + [ION]_o} \quad (4)$$

Where: J^{ION}_{max} represents the apparent maximal Na^+ or Cl^- uptake rate; $[ION]_o$ is the external Na^+ or Cl^- concentration and K^{ION}_m is an inverse index of the affinity of each respective transporter for Na^+ or Cl^-. Specifically, K^{ION}_m represents the external ion concentration at which J^{ION}_{in} is exactly equal to 50 % of J^{ION}_{max}. The curves displayed in Figs. 5 and 7, were fitted according to the Michaelis-Menten equation (Eqn. 4). Estimates of J^{ION}_{max} and K^{ION}_m were determined on individual fish by Eadie-Hofstee regression analysis (Michal, 1983), where J^{ION}_{in} was plotted against $J^{ION}_{in}/[ION]_o$; the y-intercept and the negative slope of the relationship yielded estimates of J^{ION}_{max} and K^{ION}_m, respectively. Eadie-Hofstee regression analysis was used, rather than traditional Lineweaver-Burke plots, because the former is said to be more resistant to departures from linearity (Goss and Wood, 1990a).

Statistics

All data are expressed as means $\pm$ 1 SEM (n). Data generated in Part I were analyzed by repeated-measures analysis of variance (ANOVA); if significant differences were detected, the ANOVA was followed by a Bonferroni post-test. Data generated in part II were tested by a simple one-way ANOVA and the Tukey-Kramer post-test. Statistical significance

was at the $P < 0.05$ level.

RESULTS

Part I: Unidirectional Movements of Na^+ and Cl^- Across the Gill Epithelia.

At control pH 8.0 J^{Cl}_{in} and J^{Na}_{in} were both about +220 umol·kg^{-1}·h^{-1}, and equal to J^{Cl}_{out} and J^{Na}_{out}; thus these fish were in ion balance as net movements of both Na^+ and Cl^- approximated zero (Figs. 1, 2). Over the first 3 h of exposure to pH 9.5, J^{Cl}_{in} was reduced by 60 percent. Thereafter, J^{Cl}_{in} gradually recovered and by 72 h had returned to control rates (Fig. 1). Surprisingly, J^{Cl}_{out} did not change following transfer to pH 9.5. The only exception was at 24 h of pH 9.5 exposure; at this time J^{Cl}_{out} was about 1.7-fold greater than the control rates (Fig. 1). Reduced J^{Cl}_{in} over the first 24 h resulted in net Cl^- losses that approximated -200 umol·kg^{-1}·h^{-1}. By 48 h, the gradual recovery of J^{Cl}_{in} led to re-establishment of J^{Cl}_{net} to rates which were not significantly different from control values (Fig. 1).

Exposure to pH 9.5 resulted in a chronically depressed J^{Na}_{in} (Fig. 2), in contrast to J^{Cl}_{in}. Initially J^{Na}_{in} decreased by 70% but was followed by a slight recovery at 8 h, to 100 umol·kg^{-1}·h^{-1} at which time J^{Na}_{in} stabilized at a level about 50 % lower than controls (Fig. 2). No significant changes in J^{Na}_{out} occurred during the first 24 h at pH 9.5. Regardless, chronically reduced J^{Na}_{in} resulted in net Na^+ losses of about -150 to -200 umol·kg^{-1}·h^{-1} over this same time period. A gradual reduction in J^{Na}_{out}, which was significant by 72 h, counterbalanced reductions in J^{Na}_{in} and limited net Na^+ losses to the first 24 h or so of exposure. By 48 h the fish had re-established Na^+ balance (Fig. 2).

The differences between J^{Cl}_{net} and J^{Na}_{net} provided an indirect index of net acidic equivalent movements (J^{H}_{net}) during the experiment (cf. McDonald *et al.*, 1989; Wood, 1991). Under control conditions and during the first 8 h at high pH, J^{H}_{net} approximated zero but at 24 h and 72 h an excess of Cl^- over Na^+ loss indicated that there were net acidic equivalent

losses across the gill epithelium of about -70 and -100 umol·kg^{-1}·h^{-1}, respectively (Fig. 3). At 48 h, however, J^{Cl}_{net} - J^{Na}_{net} ($\sim J^{H}_{net}$) was not significantly different from controls.

Ammonia excretion patterns mirrored results of previous high pH experiments with rainbow trout [Wilkie and Wood, 1991, 1994a (Appendix 1),b]. Under control conditions J_{Amm} was about -135 umol·kg^{-1}·h^{-1} and was subsequently reduced by 70 % during the first few hours at pH 9.5. The J_{Amm} gradually recovered towards control rates and by 24 h was no longer significantly different from controls, stabilizing around 160 umol·kg^{-1}·h^{-1} (Fig. 4).

Part II: High pH Induced Changes in Cl^- and Na^+ Uptake Kinetics

At pH 8.0 and 9.5, Cl^- uptake closely followed first order saturation kinetics (Figs. 5,7). High pH exposure resulted in a temporary downward shift in the Cl^- kinetics curve after 10 h, which was characterized by Cl^- uptake rates that were about 50-60 % lower than the respective control measurements at pH 8.0. Subsequent recovery was evident: the kinetics curves at 1 and 3 days of pH 9.5 exposure virtually overlapped and were characterized by J^{Cl}_{in} rates that were not significantly different from the corresponding control estimates (Fig. 5). Eadie-Hofstee regression analysis provided estimates of the respective J^{Cl}_{max} and K^{Cl}_{m} for each curve. The control J^{Cl}_{max} approximated 360 umol·kg^{-1}·h^{-1}. Fish held at pH 9.5 for 10 h had a mean J^{Cl}_{max} that was significantly lower, by about 50 %, than the control J^{Cl}_{max} estimate. After 1 day and 3 days of high pH, however, J^{Cl}_{max} returned to 300 umol·kg^{-1}·h^{-1}, which was not significantly different from the control values (Fig. 6A). High pH exposure led to no change in the K^{Cl}_{m} relative to the pH 8.0 value of 310 umol·l^{-1} (Fig. 6B).

Na^+ uptake also exhibited first order saturation kinetics at both control and high pH. After 10 h at pH 9.5, there was a pronounced downward shift in the Na^+ kinetics curve which was characterized by 60-70 % lower Na^+ influx rates. Fish held for 1 day and 3 days

at pH 9.5 exhibited kinetic curves that were shifted back towards the control curve, but despite this partial correction, J^{Na}_{in} was still 40-50% lower than the control measurements (Fig. 7). Eadie-Hofstee analysis revealed a J^{Na}_{max} of 480 umol·kg^{-1}·h^{-1} at pH 8.0. After 10 h at pH 9.5, however, J^{Na}_{max} was 70 % lower than the pH 8.0 value and was still 46 % and 33 % below control J^{Na}_{max}, after 1 and 3 days at high pH, respectively (Fig. 8A). The K^{Na}_{m} at pH 8.0 approximated 88 umol·l^{-1}, remained unaltered after 10 h, and increased slightly after 1 day of pH 9.5 exposure. By 3 days, however, a marked loss of affinity was apparent; the K^{Na}_{m} was about 375 umol·l^{-1}, a value about 4.3-fold greater than the pH 8.0 estimate (Fig. 8B).

Analysis of plasma samples, taken from the fish at the end of each kinetics experiment, revealed that Cl^- concentrations were about 137 mmol·l^{-1} in control fish. Exposure to high pH, however, resulted in a significant 10 % decline in plasma Cl^- levels after only 1 day; no further change was observed at 3 days (Table 1). Plasma Na^+ concentrations approximated 150 mmol·l^{-1} at control pH. Exposure to pH 9.5 led to less pronounced, non-significant, decreases in plasma Na^+ concentration that approximated 4 % by 3 days (Table 1).

Plasma lactate concentration was significantly elevated by high pH exposure. Under control conditions plasma lactate concentration was about 1.1 mmol·l^{-1} but it was 33 % higher in fish sampled after 1 d and 3 d at pH 9.5 (Table 1).

Ammonia excretion rates during the kinetics exposure regime showed qualitatively similar trends to those observed in Part I of this study. At pH 8.0 J_{Amm} was approximately 125 umol·kg^{-1}·h^{-1}. Fish that had been exposed to pH 9.5 for 10 h exhibited J_{Amm}'s that were 90 % lower than control J_{Amm}. By 1-3 days, however, J_{Amm} had recovered and was about 70

umol·kg^{-1}·h^{-1} and not significantly different from J_{Amm} at pH 8.0 (data not shown).

Fish sampled at pH 8.0 had plasma total ammonia concentrations (T_{Amm}) of 85 umol·l^{-1}. The initial inhibition of J_{Amm} during the first few hours of pH 9.5 was reflected in 4-fold and 7-fold elevations in T_{Amm} at 10 h and 1 day of pH 9.5 exposure, respectively. After 3 days, plasma T_{Amm} was still 3.5-fold greater than control values (Table 1).

DISCUSSION

Unidirectional Na^+ and Cl^- Movements

The net ion movements measured in the present study represent the sum of branchial and renal ionoregulatory processes. However, essentially all ion uptake is via the gill epithelium in rainbow trout (see McDonald, 1983 for review), and greater than 90 % of whole body ion losses occur across this same surface under most circumstances (McDonald and Wood, 1981), therefore the whole body ion influxes, outfluxes and net fluxes measured here are considered to be largely branchial fluxes.

Exposure of salmonids to highly alkaline water leads to significant, sometimes lethal, ionoregulatory disturbances which are characterized by lowered plasma Na^+ and Cl^- concentration (Heming and Blumhagen, 1988; Wilkie and Wood, 1991, 1994a,b; Yesaki and Iwama, 1992; Wilkie *et al.*, 1993). Wright and Wood (1985) and Wood (1989) reported immediate reductions in J^{Na}_{in} and J^{Cl}_{in}, respectively, upon exposure of rainbow trout to pH 9.5, but made no longer term measurements. Wilkie and Wood [1994a (Appendix 1)] measured Na^+ and Cl^- uptake over a 72 h pH 9.5 exposure regime, similar to the present one, and reported chronic and temporary reductions in J^{Na}_{in} and J^{Cl}_{in}, respectively. Outflux was not measured, however. In the present investigation, initial exposure of rainbow trout to highly alkaline water (pH 9.5) clearly interfered with Na^+ and Cl^- influx but had surprisingly little effect on respective outfluxes. The present data, regarding ion influx at high pH, are in general agreement with the aforementioned studies (eg. Wright and Wood, 1985; Wood, 1989; Wilkie and Wood, 1994a) but previous observations regarding outflux are contradictory. For instance, Wright and Wood (1985) observed no stimulation of Na^+ outflux during 3 h of pH 9.5 exposure while Wood (1989) reported 2-3 fold elevations in Na^+ and Cl^-

losses under identical experimental conditions. The data generated here tend to support the findings of Wright and Wood (1985) and suggest that stimulation of net Cl^- and Na^+ losses, during the first 24 h at high pH, were due solely to reduced branchial Na^+ and Cl^- influx.

The flux rates measured here were certainly sufficient to explain the reductions in plasma Cl^- and Na^+ concentrations observed in previous (high pH exposure) investigations (Wilkie and Wood, 1991, 1994b), as well as the marked decrease in plasma Cl^- concentration, and the slight non-significant reduction in plasma Na^+ levels observed in the present study. Model calculations, using the J^{Cl}_{net} and J^{Na}_{net} rates measured over the first 24 h of pH 9.5 exposure in the present study, suggest that the fish should have lost approximately 4.8 and 4.4 $mmol \cdot kg^{-1}$ body weight of Cl^- and Na^+, respectively. These losses would constitute 14.6 % and 10.5 % decreases in the animals' total exchangeable Cl^- and Na^+ pool, respectively (Cl^- pool = 32.9 $mmol \cdot kg^{-1}$ and Na^+ pool = 42.05 $mmol \cdot kg^{-1}$; Wood, 1988) and would be reflected by plasma Cl^- and Na^+ concentrations of approximately 116.7 and 134.6 $mmol \cdot l^{-1}$, respectively. The plasma Cl^- and Na^+ concentrations observed at 24 h were actually 5.0 % and 9.0 % higher than these predicted values. This observation might be attributable to compensatory shifts of Cl^- and Na^+ from the intra-to-extracellular fluid compartments or shifts of water (haemoconcentration) in the opposite direction.

To a certain extent, high pH induced ionoregulatory disturbances resemble those seen in teleosts at low pH. For instance, acid-exposure (pH 4.0 to 4.8) leads to similar reductions in Cl^- and Na^+ influx (Packer and Dunson, 1970; Maetz, 1973; McWilliams, 1980a,b; McDonald *et al.*, 1983; Wright and Wood, 1985; Audet *et al.*, 1988) but initial elevations in Na^+ and Cl^- outflux have also been reported (Packer and Dunson, 1970, 1972; McWilliams, 1982; McDonald *et al.*, 1983). The severity of low pH induced ionoregulatory disturbances

is exacerbated in waters of low Ca^{++} concentration (McDonald *et al.*, 1980, 1983; Marshall, 1985). There is evidence that water Ca^{++} concentration also has profound effects at high pH. Yesaki and Iwama (1992) have demonstrated that high pH-induced (pH 10) disturbances to plasma electrolyte status are more pronounced in soft alkaline vs. hard alkaline water. Although, Yesaki and Iwama did not measure Na^+ and Cl^- movements, it seems likely that the paucity of Ca^{++} in the high pH soft-water environment led to greater inhibition of ion influx and/or stimulated diffusive ion losses leading to greater overall net Na^+ and Cl^- losses. The mortality in high pH soft-water, reported by Yesaki and Iwama, may have been attributable, in part, to ionoregulatory failure but they also reported greater elevations of plasma total ammonia with resultant NH_3 concentrations approaching toxic levels (R.W. Wilson and C.M. Wood, unpublished observations, Wilkie *et al.*, 1993). Thus, the maintenance of water Ca^{++}, above the hardwater-softwater threshold of 0.4 $mmol \cdot l^{-1}$ (Marier *et al.*, 1979), in the present investigation was likely an important factor allowing adaptation of the rainbow trout to alkaline pH.

Previous experiments in Hamilton tapwater (Wilkie and Wood, 1991, 1994b) have shown that high pH induced reductions in plasma Na^+ and Cl^- concentration are generally restricted to the first 48 h of exposure after which stabilization occurs, indicating that the fish have re-established Na^+ and Cl^- balance (ie. $J^{ION}_{net} = 0$). The present investigation demonstrates that complete restoration of branchial Cl^- influx to control rates, after 1-2 days of high pH exposure, accounts for the re-establishment of Cl^- balance. Recovery of Na^+ balance ($J^{Na}_{net} \simeq 0$), on the other hand, is due to reductions in transbranchial Na^+ outflux which counterbalance chronically lowered J^{Na}_{in} by 48-72 h.

Re-establishment of Na^+ balance, at low pH, similarly results from reductions in Na^+

outflux and generally occurs over the first few hours or days of the exposure (McDonald *et al.*, 1980, 1983; Audet *et al.*, 1988). The reduction in Na^+ outflux reported here is consistent with the low pH observations and the time course of this corrective response suggests hormonal influences play a role. The most logical candidate is prolactin, which is thought to play a key role in teleost adaptation to dilute and acidic environments (Wendelaar Bonga *et al.*, 1984).

Unlike J^{Na}_{out}, J^{Cl}_{out} did not change in the present study. This finding resembles previous observations, made by Audet *et al.* (1988), at sublethal acid-pH (pH 4.8). The opposite response, reduced J^{Cl}_{out} relative to J^{Na}_{out}, has been observed following the transfer of trout to dilute media (Perry and Laurent, 1989). Together these observations support previous claims (eg. Goss *et al.*, 1992a,b) that fish are capable of independent modulation of Cl^- and Na^+ outflux, in addition to influx, when they experience internal ion or acid-base imbalances as a result of environmental insults.

The role that the differential modulation of Na^+ vs. Cl^- movements across the branchial epithelium plays in regulating acid-base balance is now well established (Cameron, 1976; Perry *et al.*. 1981; McDonald *et al.*, 1983, 1989; Wood *et al.*, 1984; Cameron and Iwama, 1987; McDonald and Milligan, 1988; McDonald and Prior, 1988; Goss and Wood, 1990a,b; Goss *et al.*, 1992b) and can be described in terms of the Strong Ion Difference (SID) theory and the constraints of electroneutrality (Stewart, 1983). Wood (1989) and McDonald *et al.* (1989) have neatly summarized how modulation of trans-branchial Na^+ and Cl^- movements, both the influx and outflux components, affects fish acid-base balance. The basic premise is that net branchial Na^+ and Cl^- uptake must be accompanied by the excretion of an acidic equivalent (H^+ or NH_4^+) or a basic equivalent (OH^- or HCO_3^-), respectively.

Accordingly, the difference between J^{Cl}_{net} and J^{Na}_{net} yields estimates of net H^+ flux.

The observation that net losses of Cl^- and Na^+ were virtually identical during the first 8 h of high pH exposure, suggests there was no corresponding net H^+ movement across the branchial epithelium (J^{H}_{net}) and therefore no branchially mediated internal metabolic acid-base disturbance. This thesis is supported by previous experiments which have demonstrated that rainbow trout exposed to high pH experience a pure respiratory alkalosis (decreased Pa_{CO2}; Wilkie and Wood, 1991) over the first 8 h of exposure. At 24 h, however, when net losses of Cl^- exceeded Na^+ losses (Compare figs. 1 and 2), J^{H}_{net} was outwardly directed implying a net acid loss (= base uptake) to the animal (Fig. 3). Similarly, at 72 h there also appeared to be a net acid-loss, despite the fact that J^{Cl}_{net} and J^{Na}_{net} were not significantly different from control values. A conservative interpretation of these results is that modulation of net Na^+ and Cl^- movements, at the very least, played no role in correcting high pH induced blood alkalosis, and may have actually contributed to it. Experiments by Wilkie and Wood (1994b) suggest that increased white muscle lactic acid production, generated excess metabolic acid that was subsequently excreted to the ECF during a 48 h high pH exposure regime. Indeed, plasma lactate was significantly elevated in the fish held at pH 9.5 for 10 h, 24 h and 72 h in Part II of this study. Conceivably, "extra" metabolic acid, generated through greater white muscle lactic acid production, might have been excreted across the gills and be reflected by an increased J^{Cl}_{net} - J^{Na}_{net} difference. In fact, Wilkie and Wood (1994b) estimated that these "extra" metabolic acid losses approximated 1 $mmol \cdot kg^{-1}$ body weight over the first 48 h at pH 9.5. Interestingly, the indirect estimates of H^+ excretion calculated here suggest the present fish lost approximately 0.8 $mmol \cdot kg^{-1}$ body weight of metabolic acid during the first 48 h at pH 9.5. Thus, it appears that rainbow trout may produce excess metabolic H^+ at high pH,

which is subsequently unloaded across the gill epithelium.

Recent findings have suggested that J_{Amm} at high pH is facilitated through elevations in internal ammonia that generate favourable blood to water NH_3 diffusion gradients (Wright *et al.*, 1993; Wilkie *et al.*, 1994; Wilkie and Wood, 1994b). The possibility of Na^+/NH_4^+ exchange, espoused by numerous researchers (eg. Maetz and Garcia-Romeu, 1964; Maetz, 1973; Wright and Wood, 1985; McDonald and Milligan, 1988; Yesaki and Iwama, 1992) does not appear tenable for rainbow trout exposed to highly alkaline Hamilton tapwater for two reasons. Firstly, there was no correlation between J^{Na}_{in} and J_{Amm} in the trout held at high pH in the present investigation. This is underscored by the observation that by 24 h J_{Amm} had fully recovered while J^{Na}_{in} was still 50 % lower than control rates (Compare Figs. 2 and 4). Secondly, in previous experiments, amiloride induced blockade of Na^+ uptake after 75 h of pH 9.5 exposure, following complete recovery of J_{Amm} to control rates, had no effect on ammonia excretion rates [Wilkie and Wood, 1994a (Appendix 1)]. Possible paracellular NH_4^+ diffusion (McDonald and Prior, 1988) at high pH also seems improbable because, unlike fish in saltwater, where paracellular NH_4^+ diffusion may be quantitatively important for N-waste excretion (Goldstein *et al.*, 1982; Evans and More, 1988), freshwater fish have relatively impermeable branchial tight junctions (see McDonald, 1983 for review). Decreases in the blood-water diffusion distance and/or greater lamellar surface area, however, might have increased the diffusive capacitance of the gill to NH_3 (Laurent and Hebibi, 1989) and accounted for the recovery of J_{Amm} at high pH.

Na^+ and Cl^- Uptake Kinetics

Saturation kinetic analysis at control pH yielded an estimate of J^{Cl}_{max}, approximately 350 $umol \cdot kg^{-1}h^{-1}$, which is in general agreement with previous values generated for rainbow

trout *in vivo* (McDonald and Rogano, 1986; Goss and Wood, 1990a) and with *in situ* (anaesthetized, artificially irrigated) preparations (Kerstetter and Kirschner, 1972). However, our estimates of K^{Cl}_{m}, approximately 300 $umol \cdot l^{-1}$, agreed with Kerstetter and Kirschners' *in situ* estimates, but were 2-fold higher (ie. affinity lower) than *in vivo* estimates made by Goss and Wood (1990a). We have no explanation for this discrepancy, but model calculations using the Michaelis-Menten relationship (eqn. 4), the external Cl^- concentration of Hamilton tapwater and the calculated K^{Cl}_{m} and J^{Cl}_{max} values, yielded a J^{Cl}_{in} (210 $umol \cdot kg^{-1}h^{-1}$) which agreed closely with the control J^{Cl}_{in} measurements of Part I (220 $umol \cdot kg^{-1}h^{-1}$). The estimates of J^{Na}_{max} and K^{Na}_{m}, of approximately 480 $umol \cdot kg^{-1}h^{-1}$ and 88 $umol \cdot l^{-1}$, respectively, were in close agreement with a number of previous *in vivo* investigations, including Goss and Wood (1990a,b) and Lauren and McDonald (1987). The K^{Na}_{m} estimates made here are somewhat lower (ie. affinity higher) than estimates made *in situ* (eg. Perry *et al.*, 1985; Kerstetter *et al.*, 1970; Avella, *et al.*, 1987, Avella and Bornancin, 1989).

The significant decrease in J^{Cl}_{max}, after 10 h at pH 9.5, suggests that the initial reductions in J^{Cl}_{in}, observed in Part I, were solely a result of a transient decrease in transporter numbers that was corrected after only 1 day at pH 9.5. The absence of any change in K^{Cl}_{m} indicates that there was no change in transporter affinity for Cl^-. This observation also precludes any competitive inhibition of the Cl^- transporter by OH^- ions (Michal, 1983). In all likelihood, alkaline water effectively decreased branchial Cl^- transporting capacity through direct inactivation of the transport proteins. The return of J^{Cl}_{max} to control values by 3 d, reflecting a re-establishment of Cl^- transporting capacity, accounted for the complete recovery of J^{Cl}_{in} observed during pH 9.5 exposure.

Saturation kinetic analysis of Na^+ uptake patterns indicated that the greatest reduction

in J^{Na}_{max} occurred at 10 h of pH 9.5 exposure, which corresponds with the time frame of minimal Na^+ uptake reported in Part I (Fig. 2). The gradual recovery of J^{Na}_{in}, to 50 % of control rates, was due in part to a partial recovery of J^{Na}_{max} to about 60 % of control rates but further recovery was circumvented by the dramatic increase in K^{Na}_{m} observed at 72 h. The basic interpretation of these results is that high pH brought about a rapid decrease in Na^+ transporter number followed by a gradual return of Na^+ transport capacity towards control rates. Decreased transporter affinity for Na^+ after 3 days of high pH exposure, however, precluded complete recovery of Na^+ influx. Decreased J^{Na}_{max} may have been due to non-competitive inactivation of Na^+ transporters through the direct affects of the alkaline water (Wright and Wood, 1985).

Kirschner (1988) has asserted that 1-substrate ion uptake kinetic analyses are overly simplistic if the animal in question is undergoing a change in internal acid-base status. As mentioned earlier, high pH exposure results in the development of a respiratory alkalosis and a simultaneous, counteracting metabolic acidosis, both of which can decrease internal HCO_3^- and H^+ concentration by up to 70 % and 40 %, respectively (eg. Wright and Wood, 1985; Lin and Randall, 1990; Wilkie and Wood 1991, 1994b; Wilkie *et al.* 1993). Potentially, this lack of internal counterion, for each respective Cl^-/HCO_3^- and Na^+/H^+ exchanger, could be reflected by reduced Cl^- and Na^+ uptake rates, respectively, during high pH exposure. Furthermore, evidence that Na^+ uptake could be linked to an electrogenic proton pump (Avella and Bornancin, 1989; Lin and Randall, 1993) raises the possibility that estimates of K^{Na}_{m} and J^{Na}_{max} are not truly indicative of true Na^+ transporter avidity or number (Potts, 1994).

Goss and Wood (1991) recently developed and calibrated a two-substrate kinetic

model which relates the gill transport of Cl^- and Na^+ to arterial blood acid-base status in rainbow trout living in Hamilton tapwater (Fig. 9). In brief, this graphical analysis can be used to separate the influence of changes in internal counterion availability (ie. HCO_3^- for Cl^- uptake and H^+ for Na^+ uptake) from changes in transporter numbers. Changes in the availability of internal counterion only, without changes in transporter numbers, results in shifts along the regression lines, whereas decreases in transporter numbers alone result in upward deviations, and *vice versa*. In Fig. 9, we have employed the kinetic data from the present study and the arterial acid-base data of Wilkie and Wood (1991) on similarly sized trout of the same genetic stock to examine the ion transport responses to alkaline exposure.

Under control conditions, $1/J^{Cl}_{max}$ vs. $1/[HCO_3^-]_a$ estimates agreed closely with the control data of Goss and Wood (1991; Fig. 9A). At 10 h, however, the mean data point shifted to the right and deviated upwardly from the regression line suggesting that a combination of decreased internal $[HCO_3^-]$ and decreased transporter number accounted for initial reductions in J^{Cl}_{max} (Fig. 9A). At 1 d and 3 d however, these points were shifted even more dramatically to the right, reflecting the chronic high pH-induced metabolic acidosis (decreased $[HCO_3^-]_a$) that developed. The downward deviation of these points (Fig. 9A) is consistent with an increase in the number of branchial Cl^-/HCO_3^- exchangers. Thus, it appears that initial high pH-induced reductions in J^{Cl}_{in} were due to a combination of decreases in transporter number and a lack of available internal HCO_3^-, to drive branchial Cl^-/HCO_3^- exchange. Persistently reduced HCO_3^-, would have prevented full recovery of J^{Cl}_{max} but this detrimental effect appears to have been offset by a <u>real</u> increase in Cl^-/HCO_3^- transporter numbers. Such a counterbalancing effect might have been elicited through increases in the apical insertion of Cl^-/HCO_3^- proteins (Goss *et al.*, 1992b) and/or increased chloride cell (CC)

fractional surface area; the latter of which is the purported site of Cl^-/HCO_3^- exchange (eg. Perry and Laurent, 1989; Perry *et al.*, 1992; Goss *et al.*, 1992a,b, 1994). This interpretation is supported by the 4-fold increases in CC FSA that have been observed in rainbow trout exposed to pH 9.5 for 72 h [Wilkie and Wood 1994a (Appendix 1)]. Similar branchial modifications have also been reported in the Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*) of alkaline Pyramid Lake, Nevada (Galat *et al.* 1985; Wilkie *et al.* 1994). The time course of the recovery of J^{Cl}_{max} suggests hormonal influences were important. One potential candidate is cortisol, which is known to elicit increases in Cl^- influx and chloride cell surface area and number (see Laurent and Perry, 1991 for review).

A similar 2-substrate analysis was performed to describe Na^+ uptake kinetics at high pH. The present control $1/J^{Na}_{max}$ vs. $1/[H^+]_a$ estimate was again in good agreement with the control data of Goss and Wood (1991; Fig. 9B). At 10 h, however, $1/J^{Na}_{max}$ vs. $1/[H^+]_a$ shifted slightly to the right and deviated dramatically upwards from the regression line. This dramatic upward deviation implies that there was a major reduction in Na^+/H^+ exchange site number, in addition to counterion limitations (decreased internal H^+ due to high pH-induced respiratory alkalosis). Interestingly, at 1 d and 3 d, the mean data points returned towards the regression line, while still maintaining a small rightward shift, suggesting that transporter numbers were restored while internal substrate limitation persisted due to chronic alkalosis. The persistent reduction of J^{Na}_{in} at high pH and normal levels of external Na^+ concentration, however, appears to have primarily resulted from the 4-fold greater K^{Na}_m (Fig. 8B).

Goss and Wood (1990a,b) found that trout were capable of increasing K^{ION}_m (ie. decreasing affinity) to counteract acid-base disturbances, this raises the possibility that increased K^{Na}_m was an attempt to retain H^+, to counter the tendency to gain base (lose acid)

during high pH exposure (eg. Fig. 3). It is unlikely that Na^+ influx was competitively inhibited by OH^- due to the differential charges of both species. Alternatively, increased K^{Na}_m may be simply a reflection of high pH-induced changes in the gill architecture.

Recently, Goss *et al.* (1992a,b) have suggested that while Cl^- uptake is localized to chloride cells, Na^+ uptake might actually take place across the respiratory cells. If Cl^- and Na^+ are truly taken up across two specific cell types (CC and respiratory cells,respectively), with markedly different architectures, then high pH induced alterations in gill morphology might differentially affect the structure and/or accessibility of each ion's respective transporter. For instance, factors such as diffusion distance or mucus secretion might impede Na^+ access to Na^+ channels but have little effect on Cl^- access to Cl^-/HCO_3^- exchangers and therefore, result in increased K^{Na}_m but not K^{Cl}_m. Alternatively, if Na^+ uptake is truly linked to an electrogenic proton pump (Avella and Bornancin, 1989; Lin and Randall, 1993), then these high pH-induced increases in K^{Na}_m might simply be due to reduced epithelial cell Na^+ conductance and/or result from changes in electrical potential across the gill epithelial cell apical membranes (Potts, 1994).

Table 1: Plasma Ions, Lactate and Ammonia Concentrations Following High pH (pH 9.5) Exposure in Part II.

	pH 8.0	pH 9.5		
	(Control)	10 h	1 day	3 days
$[Na^+]$ ($mmol \cdot l^{-1}$)	150.5 ± 0.8	144.1 ± 2.3	146.8 ± 3.0	144.1 ± 1.6
$[Cl^-]$ ($mmol \cdot l^{-1}$)	136.6 ± 1.2	128.3 ± 1.9	123.1 ± 5.7*	122.9 ± 3.3*
[Lac] ($mmol \cdot l^{-1}$)	1.12 ± 0.02	1.22 ± 0.05	1.60 ± 0.17*	1.65 ± 0.11*
$[T_{Amm}]$ ($umol \cdot l^{-1}$)	84.7 ± 13.8	358.4 ± 81.5*	577.9 ± 65.0*	285.0 ± 59.4*

Figure 1: The influence of 72 h of se·ere alkaline exposure (pH =9.5) upon branchial Cl^- influx (J^{Cl}_{in}: upward facing bars), outflux (J^{Cl}_{out}: downward facing bars) and net movements (J^{Cl}_{net}: shaded bars) in rainbow trout. Means ± 1 SEM; n= 7. Asterisks demonstrate statistical significance ($P < 0.05$) from control measurements at pH 8.0.

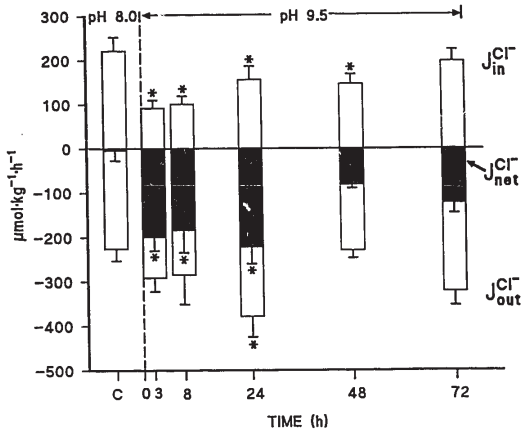


Figure 2: The influence of 72 h of severe alkaline exposure (pH =9.5) upon branchial Na^+ influx (J^{Na}_{in}: upward facing bars), outflux (J^{Na}_{out}: downward facing bars) and net movements (J^{Na}_{net}: shaded bars) in rainbow trout. Means ± 1 SEM; n= 7. Asterisks demonstrate statistical significance ($P < 0.05$) from control measurements at pH 8.0.

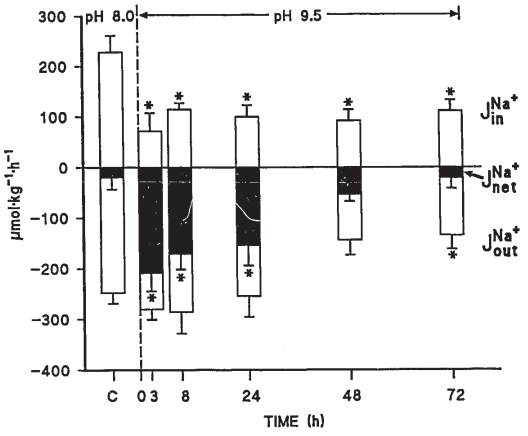


Figure 3: The influence of 72 h of severe alkaline exposure (pH =9.5) upon an indirect estimate of net branchial proton movements ($J^{H}_{net} = J^{Cl}_{net} - J^{Na}_{net}$) in rainbow trout. Means ± 1 SEM; n= 7. Asterisks demonstrate statistical significance ($P < 0.05$) from control measurements at pH 8.0.

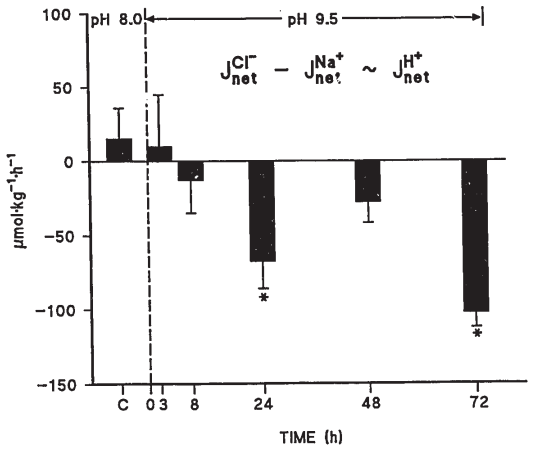


Figure 4: The influence of 72 h of severe alkaline exposure (pH =9.5) upon branchial ammonia excretion (J_{Amm}) in rainbow trout. Means ± 1 SEM; n= 7. Asterisks demonstrate statistical significance ($P < 0.05$) from control measurements at pH 8.0. Note: Unlike previous investigations, where ammonia excretion was expressed as a positive value, J_{Amm} is expressed as a negative value to indicate its outward movement and to be consistent with conventions used to express trans-branchial ion movements.

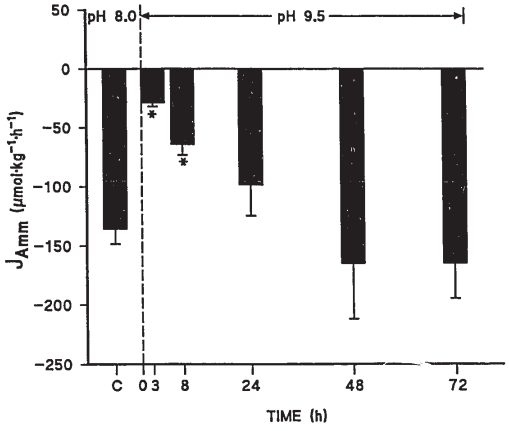


Figure 5: Saturation kinetic analysis of Cl^- uptake (J^{Cl}_m) vs. external Cl^- concentration by different groups of rainbow trout sampled at control pH (pH 8.0; circles; n = 7) or after 10 h (solid triangles; n =7), 1 d (diamonds; n = 6), and 3 d (stars and broken line; n = 7) of pH 9.5 exposure. The data are expressed as means ± 1 SEM.

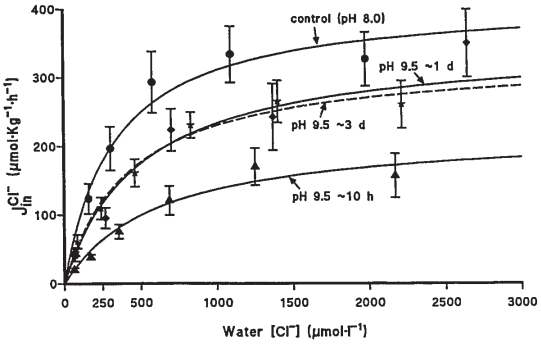


Figure 6: Eadie-Hofstee regression analysis of the data presented in Figure 5 showing differences in the apparent maximal Cl^- transport rate (J^{Cl}_{max}) and inverse of Cl^- transporter affinity (K^{Cl}_m) of rainbow trout sampled at control pH (n = 7) or after 10 h (n =7), 1 d (n = 6), and 3 d (n = 7) of pH 9.5 exposure. The data are expressed as means ± 1 SEM and asterisks indicate statistically significant differences from pH 8.0 values ($P < 0.05$).

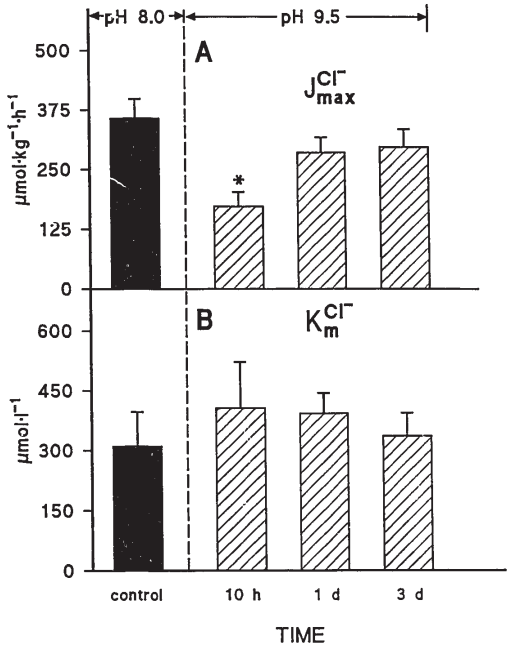


Figure 7: Saturation kinetics analysis of Na^+ uptake (J^{Na}_{in}) vs. external Na^+ concentration by different groups of rainbow trout sampled at control pH (pH 8.0; circles; n = 7) or after 10 h (solid triangles; n =7), 1 d (diamonds; n = 6), and 3 d (stars and broken line; n = 7) of pH 9.5 exposure. The data are expressed as means ± 1 SEM.

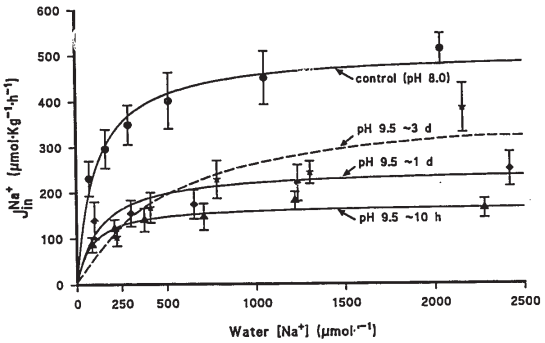
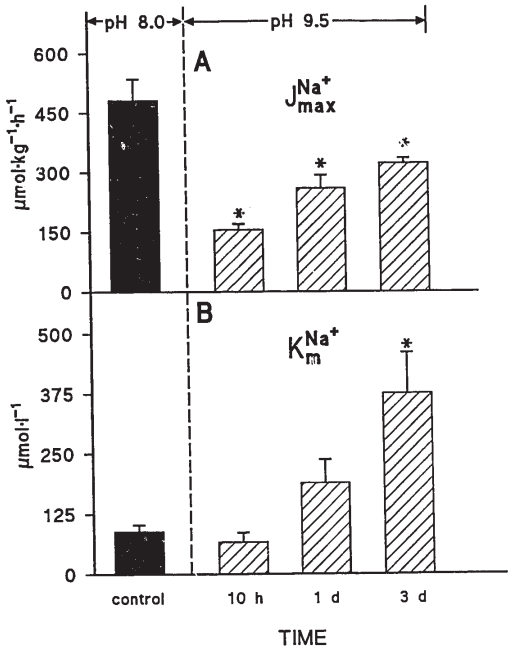


Figure 8: Eadie-Hofstee regression analysis of the data presented in Figure 5 showing differences in the apparent maximal Na^+ transport rate (J^{Na}_{max}) and inverse of Na^+ transporter affinity (K^{Na}_m) of rainbow trout sampled at control pH (pH 8.0; n = 7) or after 10 h (n =7), 1 d (n = 6), and 3 d (n = 7) of pH 9.5 exposure. The data are expressed as means ± 1 SEM and asterisks indicate statistically significant differences from pH 8.0 values ($P < 0.05$).



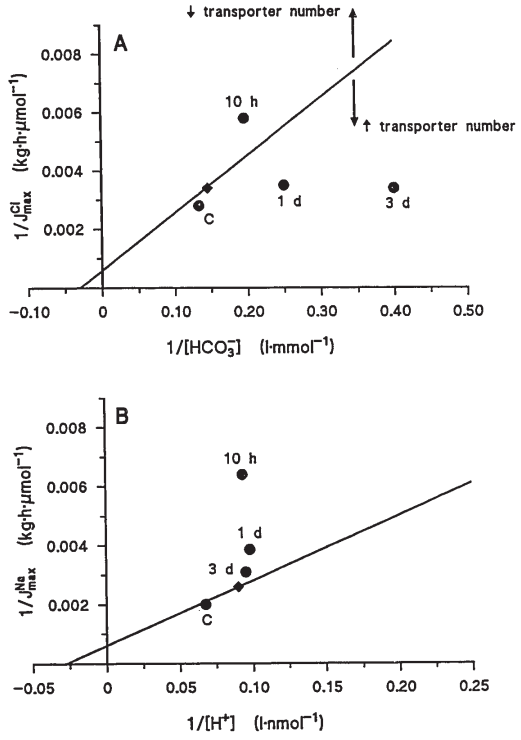


Figure 9: Two-substrate kinetic analysis, employing the methods of Goss and Wood (1991), for analysis of the relative roles of internal substrate (counterion) availability and transporter number in altering the *apparent* J^{Cl}_{max} and *apparent* J^{Na}_{max}, respectively, for Cl^- and Na^+ uptake in rainbow trout; $[HCO_3^-]_a$ and $[H^+]_a$ represent arterial plasma HCO_3^- and H^+ concentrations, respectively. The regression lines represent data collected by Goss and Wood (1991), following imposition of various internal acid-base disturbances to rainbow trout in Hamilton tapwater. For clarity, only the control data points of Goss and Wood (1991; diamonds) are presented. Circles represent the apparent J^{Cl}_{max} and J^{Na}_{max} estimates presented in Figs. 6 and 8 of the present study plotted against corresponding measurements, at the same time, in identically exposed trout by Wilkie and Wood (1991). Upward or downward deviations (vertical arrows) away from the regression line represent *true* changes in transporter number, while changes in internal substrate are reflected by movements along the regression line. See text for further details.

CHAPTER 5

THE PHYSIOLOGICAL ADAPTATIONS OF THE LAHONTAN CUTTHROAT TROUT (Oncorhynchus clarki henshawi), FOLLOWING TRANSFER FROM WELL WATER TO THE HIGHLY ALKALINE (pH 9.4) WATERS OF PYRAMID LAKE, NEVADA.

ABSTRACT

Salmonids experience severe disturbances in the excretion and internal regulation of ammonia, acid-base balance, and ionoregulation when challenged with alkaline pH. We followed the responses of a high pH tolerant salmonid, the Lahontan cutthroat trout (Oncorhynchus clarki henshawi) for 72 h after transfer from pH 8.4 well water into the alkaline water (pH 9.4) of Pyramid Lake, Nevada. Fish which had been living in Pyramid Lake for 3 weeks, 5 weeks and 2 years, were also examined. A combined metabolic and respiratory alkalosis (negative ΔH^+_e and decreased Pa_{CO2}, respectively) occurred initially. The metabolic component was corrected within 24 h but the respiratory component persisted for up to 5 weeks. Transfers also resulted in an immediate 70% reduction in ammonia excretion (J_{Amm}) and a 30 % increase in plasma ammonia (T_{Amm}). T_{Amm} was corrected within 3 days but J_{Amm} remained depressed indicating reduced ammonia production rates. Because urea excretion (J_{Urea}) did not change, the contribution of J_{Urea} to total N-excretion increased from 10% in well water to 25% in fish acutely and chronically exposed to lake water. Liver

enzyme activities indicated that the pathway for urea production was uricolysis, not the ornithine urea cycle. Branchial chloride cell fractional surface area increased in lake water and this may have counteracted the base load by promoting base equivalent excretion via Cl^-/HCO_3^- exchange. Plasma Na^+ and Cl^- levels were slightly higher in Pyramid Lake water. We conclude that the Lahontan cutthroat trout are able to survive in Pyramid Lake's alkaline environment because of their ability to reduce ammonia production, thereby avoiding chronic elevation of plasma T_{Amm}, and their ability to control blood acid-base and ionic status under alkaline conditions.

INTRODUCTION

Wilkie and Wood (1991) recently described the physiological responses of rainbow trout (Oncorhynchus mykiss) following transfer from circumneutral (pH 8.1) to alkaline (pH 9.5) water . Substantial disturbances occurred in nitrogenous waste excretion, blood acid-base balance and ionic status; none of which were fully corrected within 72 h. Other studies on rainbow trout have documented similar effects (Wright and Wood 1985; Heming and Blumhagen 1988; Lin and Randall 1990; Yesaki and Iwama 1992). Natural acute exposure to such a high pH is, however, a very unusual circumstance for this species.

We examined the physiological responses of a related salmonid, the Lahontan cutthroat trout (LCT; Oncorhynchus clarki henshawi) following transfer to pH 9.4. Acute alkaline exposure is part of the LCT's "natural" life-cycle. The first year of life is spent in well water (pH 8.4), following which the fish are moved abruptly into the highly alkaline (pH 9.4) and moderately saline waters (4.4 ppt) of Pyramid Lake, Nevada (Table 1). Attention has recently shifted to this species (Wright, Iwama, and Wood 1993; Wilkie *et al.* 1993) because it exhibits unusual tolerance to high pH and thrives in highly alkaline lakes throughout the northwestern United States (Trotter 1991). Attempts to stock other salmonids, such as coho salmon (Oncorhynchus kisutch), kokanee (Oncorhynchus nerka), brown trout (Salmo trutta), and rainbow trout into these lakes have failed (Galat *et al.* 1985; Kucera, Koch, and Marco 1985; Coleman and Johnson 1988).

The Lahontan cutthroat trout is now designated as "threatened" due to a paucity of successfully reproducing populations (Williams *et al.* 1989). Historically the Pyramid Lake LCT spawned and passed through the juvenile lifestages in the fresh water environment of the Truckee river which feeds into Pyramid Lake. Prolonged drought and water diversion have

made this river inaccessible to spawning condition LCT for many years, however (Galat *et al.* 1981, 1985; Kucera *et al.* 1985; Coleman and Johnson 1988). Indeed, by 1944 the original Pyramid Lake LCT was declared extinct. A vigorous stocking program, where juvenile LCT are reared for 1 year in well water prior to introduction into Pyramid Lake's alkaline waters, has revived the lake's cutthroat trout fishery (Coleman and Johnson 1988).

The minimal mortality experienced by LCT following transfer into Pyramid Lake (Coleman and Johnson 1988; D. Mosely and P. Wagner, personal communication) suggests that these fish are able to rapidly correct, or resist, the physiological disturbances observed in other salmonids at high pH. The purpose of the present investigation was to determine the physiological characteristics that allow LCT to adapt to alkaline Pyramid Lake water (pH 9.4) and to establish the time course of these adaptations. We followed the responses of naive LCT, which had never been exposed to high alkalinity, through a 72 h acute exposure to Pyramid Lake water. Analyses focussed on nitrogenous waste excretion, acid-base balance, and ionoregulation. Apart from the fact that this study was performed in the field using the well water and alkaline lake water available on site, methods closely duplicated those of our earlier laboratory study on O. mykiss (Wilkie and Wood 1991).

In addition, we investigated long term adaptions by studying fish which had been in lake water for 3 weeks, 5 weeks and 2 years (returning spawners). In light of emerging evidence on the possible importance of urea production as an adaptation to high pH (Wood 1993), we also measured hepatic activities of uricolytic and ornithine-urea cycle (OUC) enzymes. Similarly, in view of recent findings on alterations in branchial chloride cell surface area in response to acid-base challenge (Goss *et al.* 1992b), we looked for changes in the surface morphometry of these cells following transfer to alkaline lake water. Galat *et al.*

(1985) reported apparent chloride cell hyperplasia in Lahontan cutthroat trout living in Pyramid Lake.

MATERIAL AND METHODS

All experiments were performed at the lake-side laboratory of Pyramid Lake Fisheries during May and June, 1991. We followed responses for 72 h following exposure to Pyramid lake water in 1 year old Lahontan cutthroat trout (Oncorhynchus clarki henshawi; n = 8) of both sexes. The mean weight of these fish was 243.5 $\pm$ 16.6 g (SEM). Fish were kept indoors for 3 weeks prior to the experiments in 500 L fibreglass holding tanks served with flowing hatchery well water. The chemistry of this well water (Table 1; moderately high HCO_3^- and pH) reflects its origin from deep desert wells. The fish had been hatchery-reared in the well water for the first year of their lives and were exposed to highly alkaline, and moderately saline, Pyramid lake water for the first time during our experiments (Table 1).

Separate groups of 1 year old, well water-reared LCT were sampled 3 weeks [202.3 $\pm$ 17.5 g (n = 9)] and 5 weeks [242.4 $\pm$ 9.2 g (n = 13)] after exposure to lake water. Data are also reported for 3-4 year old cutthroat trout [500.0 $\pm$ 16.3 g (n = 6)] that had been free living in Pyramid Lake for approximately 2 years. These latter trout were netted as they migrated up an artificial spawning channel. All fish from these three groups were held in 500 L tanks provided with flowing lake water for at least 1 week prior to experimentation. All fish were starved a minimum of 1 week to minimize variation in nitrogen-metabolism (Fromm 1963).

Two days prior to sampling, fish were fitted with chronic indwelling dorsal aortic catheters (Soivio, Westman and Nyholm 1972) under MS 222 anaesthesia (1:10 000 dilution; Sigma) and immediately placed in darkened, well aerated (P_{O2} = 125-130 Torr), acrylic flux boxes (McDonald 1983). Water ammonia levels were less than 5 μmol $N{\cdot}L^{-1}$ when the boxes were operated as open systems. Mean water temperature during the study of the acute

responses was 10.4 ± 0.4 °C. The mean well water pH for this study was 8.35 ± 0.010 and the mean lake water pH was 9.36 ± 0.006. For the longer term comparisons, the water temperature, at the time of sampling, was 7.5 °C (pH = 9.41 ± 0.005) for the fish sampled after 3 weeks of exposure to lake water (3-week fish), 9.5 °C (pH = 9.39 ± 0.003) for those sampled after 5 weeks (5-week fish) and 10.0 °C (pH = 9.38 ± 0.008) for those sampled after 2 years (2-year fish).

Well aerated water was distributed to each flux box at 0.5 $L \cdot min^{-1}$ via a flow-splitter. Two 500 L central reservoirs continuously served the flux boxes with an excess of either well water (pH 8.4) or lake water (pH 9.4). pH was monitored with a Radiometer GK 2401C combination electrode and PHM 72 pH meter.

Experimental Protocol for Acute Response Experiments

Experimental methods followed those of Wilkie and Wood (1991). Water samples (15 mL) were taken at 0 h, 1 h, and 3 h of each period. J_{Amm} and J_{Urea} were determined first in well water, then at 0-3 h, 8-11 h, 24-27 h, 48-51 h and 72-75 h of exposure to Pyramid Lake water. With the exception of the 0-3 h period of lakewater exposure, blood samples were taken 30 minutes prior to box closure. This was done to minimize disturbance to the fish and to ensure that truly representative J_{Amm} and J_{Urea}'s were measured (cf. Wilkie and Wood 1991; Wilkie *et al.* 1993). Blood samples (1 mL) were drawn into two heparinized 500 μL gas-tight Hamilton syringes via the arterial catheter. Arterial pH (pH_a) and O_2 tension (Pa_{O2}) were measured immediately; blood used for the latter (approximately 200 μL) was re-infused into the fish. Cortland's saline was then infused to replace blood lost due to sampling and to maintain the internal ionic and osmotic status (Wolf 1963). Aliquots of whole blood were saved for later analysis of hemoglobin (20 μL) and lactate content (100 μL). The remainder

was centrifuged and a small amount of plasma (50 μL) decanted for immediate determination of plasma total CO_2 and protein concentration. The remaining plasma (400 to 500 μL) was frozen for later analysis of T_{Amm}, urea, Na^+ and Cl^-, glucose and cortisol. Water P_{O2}, pH and total CO_2 concentration in each box were also measured at the time of blood sampling. To establish how ammonia excretion was achieved in Pyramid Lake's highly alkaline environment, the blood-to-water partial pressure gradients for NH_3 (ΔP_{NH3}) and concentration gradients for NH_4^+ ($\Delta[NH_4^+]$) were estimated using the measured pH values and T_{Amm} values in plasma and water (Wright and Wood 1985; Wilkie and Wood, 1991). In a few fish, the transepithelial potential (TEP) across the gills between the arterial blood and the environment was also measured using methods identical to those described by Perry and Wood (1985). Determination of TEP allowed us to estimate the trans-branchial electrochemical gradients for OH^- (H^+), HCO_3^- and $CO_3^=$.

Fish were sacrificed after 72 h with an overdose of MS 222 (1.5 $g.L^{-1}$) and the second left gill arch excised for morphometric analysis of branchial chloride cell surface area. In addition, the livers were quickly extracted, freeze-clamped, and stored in liquid N_2 for later determination of ureagenic enzyme activity. A control group, kept in well water, was similarly sacrificed and sampled.

Our analytical techniques and the calculations used to estimate nitrogenous waste excretion rates, water and blood chemical parameters, and electrochemical gradients are described by Wilkie and Wood (1991), Wright *et al.* (1993) and Wilkie *et al.* (1993).

Experimental Protocol for Long term Comparisons

Water and blood samples were taken essentially as described above. Only single samples were taken from the 3-week fish and 2-year fish. These fish were then sacrificed for

gill and liver excision. Tissue samples were not extracted from the 5-week fish.

Gill Sampling Techniques and Analysis

The methods used in this study are based on those of Laurent and Perry (1990) and Goss, Laurent and Perry (1992a). The gill filaments were trimmed from the excised second left gill arch in small pieces (approximately 10 filaments per piece), rinsed in ice-cold, 0.15 $mol \cdot L^{-1}$ Na^+ cacodylate buffer and then fixed in 5 % glutaraldehyde for 60-70 minutes. After fixation, pairs of filaments, joined at the septum, were dissected away from one another and washed 3 times with ice cold buffer and refrigerated at 4 °C for several hours. The paired filaments were then taken through a partial ethanol dehydration series (30 %, 50 % and 70 % ethanol) and shipped back to McMaster University in 70 % ethanol. Subsequently, the gills were completely dehydrated in 95 % and absolute ethanol and then taken through two successive baths (2 minutes each) of 1,1,1,3,3,3-hexamethyldisilazane (Aldrich) and air dried. The paired filaments were then mounted on aluminum stubs, sputter-coated and viewed on a ISI-DS130 dual stage scanning electron microscope at 2000 times magnification. At least 8 non-contiguous fields (approximately 2500 μm^2 per field), along the trailing edge of a filament, were randomly photographed for each fish. The individual surface areas of chloride cells (CC) in each field were subsequently determined with a Graphic Master digitizing tablet (Numonics) and an accompanying software program (Sigma Scan; Jandel Scientific). Chloride cell fractional surface area (CC FSA) and CC density were calculated from the estimates of individual CC surface areas and the total filamental surface areas measured per fish. The filamental epithelium was used for morphometry, rather than the lamellar epithelium, because the paired filaments could be mounted parallel to the face of the aluminum stub. This made the flat, relatively uniform surface of the trailing filamental

epithelium, accessible for examination with the scanning electron microscope. Furthermore, the gill lamellae are less appropriate for such analysis because the undulating nature of their topography makes viewing more difficult and measurements prone to error (Goss *et al.* 1992a).

Statistics

All data are expressed as means ± 1 SEM (n). For the 72 h acute lake water exposure experiment, each animal served as its own control and paired, two-tailed t-tests were used to determine statistical significance ($P < 0.05$). For long term exposures, data for fish held in lake water was compared to data generated for fish held in well water. Therefore, an F-ratio was calculated to test for homogeneity of variance, followed by an unpaired, 2-tailed t-test to determine significant differences ($P < 0.05$). Ureagenic enzymes and gill morphometric data were evaluated by Analysis of Variance and subsequent paired contrasts ($P < 0.05$) were made by a Tukey-Kramer Honestly Significant Difference Test using a commercially available statistics package (SAS JMP; SAS Inc. 1989).

RESULTS

Nitrogenous Waste Excretion

Lahontan cutthroat trout in well water exhibited a J_{Amm} of approximately 330 μmol $N \cdot kg^{-1} \cdot h^{-1}$. This was reduced nearly 70% during the first hour of exposure to alkaline lake water; depression of J_{Amm} persisted throughout the 3 day exposure (Fig. 1A). The associated initial 30 % rise in plasma T_{Amm}, to about 300 μmol $N \cdot L^{-1}$ seen at 8 to 24 h (Fig. 1B) was no longer evident by 72 h (Fig. 1B). Urea excretion rates remained stable around 40 μmol $N \cdot kg^{-1} \cdot h^{-1}$ for the duration of the three day exposure (Fig. 1A). As a result, the percentage contribution of J_{Urea} to total nitrogen excretion ($J_{total-N}$) increased from 10% in well water to approximately 25% in lake water. Plasma urea levels were between 5500 and 6500 μmol $N \cdot L^{-1}$ for 24 h following exposure to lake water, but then declined to approximately 4000 μmol $N \cdot L^{-1}$ after 48 h.

In well water, both the blood-water NH_3 (ΔP_{NH3}) and NH_4^+ ($\Delta[NH_4^+]$) gradients were outwardly directed (approximately 25 μTorr and 165 μmol $N \cdot L^{-1}$, respectively; Figs. 1C,D) and were in accordance with the increase in plasma T_{Amm} (Fig. 1B). After 8 h of exposure to lake water both ΔP_{NH3} and $\Delta[NH_4^+]$ increased significantly to approximately 100 μTorr and 290 μmol $N \cdot L^{-1}$, respectively (Figs. 1C,D). The greater relative increase in ΔP_{NH3} reflected a marked elevation in blood pH. Thereafter, ΔP_{NH3} and $\Delta[NH_4^+]$ gradually declined in parallel with the fall in plasma T_{Amm}, and by 72 h, neither was significantly different from control values.

ΔP_{NH3} in fish sampled at 3 and 5 weeks were not significantly different from well water values. Similarly, $\Delta[NH_4^+]$, approximately 180 μmol $N \cdot L^{-1}$ in these fish, was also similar to that seen in well water. Fish sampled after 2 years in lake water had significantly

elevated ΔP_{NH3} and $\Delta[NH_4^+]$ (approximately 70 μTorr and 280 μmol $N \cdot L^{-1}$, respectively).

The reduction in J_{Amm} seen upon initial exposure to lake water persisted for an extended period. Fish sampled after 3 and 5 weeks exposure to lake water exhibited J_{Amm}'s of only about 100 μmol $N \cdot kg^{-1} \cdot h^{-1}$. These values are the same as those measured over the first hour at pH 9.4. The J_{Amm} of the 2-year trout was not significantly different from the rates observed in well water fish, however (Fig. 2A).

Despite the persistent depression of J_{Amm} in lakewater adapted fish, plasma T_{Amm} was not persistently elevated. Rather T_{Amm} remained relatively stable, from 72 h onwards in all groups. The 2-year fish, however, showed elevated plasma T_{Amm} (Fig. 2B). Plasma P_{NH3} was about 50 μTorr in the well water fish and not significantly different from values measured in fish exposed to lakewater for 72 h, 3 weeks or 5 weeks. Blood P_{NH3} was, however, significantly higher in the 2-year fish (Fig. 2C).

J_{Urea} was not significantly different from pre-exposure values in any of the groups (Fig. 2D). The percentage contribution of J_{Urea} to $J_{total-N}$ was, however, 2-3 fold higher in all groups of lakewater adapted fish.

Ureagenic Enzyme Activities

The activity of the key regulatory enzyme in the OUC pathway, carbamoyl phosphate synthetase III (CPS III), was just above the level of detection (Table 2). Activities of glutamine synthetase, ornithine carbamoyl transferase, and argininosuccinate synthetase were relatively low compared to teleosts with a functional OUC pathway (Randall *et al.* 1989; Mommsen and Walsh 1989). The activity of arginase, which hydrolyzes dietary arginine to ornithine and urea (see Wood 1993 for review) was typical for a teleost (Mommsen and Walsh 1991; Table 2). None of these enzymes were altered by exposure to lake water.

Significant uricolytic enzyme activity was present in Lahontan cutthroat trout (Table 2). The activities of uricase and allantoicase were within the range of values reported for fish by Cvancara (1969) and Goldstein and Forster (1965), respectively. Quantitative differences in activity were also observed between the groups of trout examined; uricase activity was 2-fold greater in the 3-week fish, but was significantly lower in the 2-year fish. Relative to the well water-reared trout, allantoicase activity was significantly lower in the 2-year fish (Table 2).

Blood Parameters

No significant changes in Pa_{O2}, plasma glucose, or cortisol were observed after 72 h of exposure to lake water (Table 3). Plasma protein levels remained stable over the first few hours of exposure to lake water but declined by 20 % after 48-72 h of exposure (Table 3). Blood hemoglobin had declined by almost 50% after 72 h. These latter effects are largely explicable as the consequences of repetitive blood sampling.

Fish sampled after 3 weeks and 5 weeks in lake water had plasma protein, hemoglobin and Pa_{O2} levels that were comparable to fish in well water. Glucose concentrations were depressed after 5 weeks in lake water but were elevated in fish that had been residing in the lake for 2 years. Plasma protein levels were depressed in the latter group. Plasma cortisol concentrations were variable (135 to 280 $ng \cdot mL^{-1}$) in all groups (Table 3).

Transepithelial Potential

Transepithelial potentials were -6. $\pm$ 1.7 mV in well water fish and significantly lower than values (-3.2 mV) measured in trout exposed to lake water for 5 weeks (Table 4). Estimates of the electrochemical gradients for OH^- indicated that the inwardly directed

gradient for OH^- (= outwardly directed electrochemical gradient for H^+) increased 5-fold in lake water (Table 4). Furthermore, the outwardly directed electrochemical gradients for HCO_3^- and CO_3^{2-} in well water, were reversed following transfer into lake water and resulted in large inwardly directed gradients for these basic anions (Table 4).

Acid-Base Balance and Ionoregulation

LCT in well water had a pH_a of 7.9 (Fig. 3A), a Pa_{CO2} of 1.8 Torr (Fig. 3B), and a plasma $[HCO_3^-]_a$ of approximately 6.7 $mmol \cdot L^{-1}$. By 8 h, following transfer into lake water, the fish rapidly underwent a combined respiratory and metabolic alkalosis which was characterized by a 0.2 unit increase in pH_a, a 30% reduction in Pa_{CO2}, no change in $[HCO_3^-]_a$, and a ΔH^+m of approximately -4 $mmol \cdot L^{-1}$ (ie. a metabolic base load; Figs. 3A,B,C). The partial correction and subsequent stabilization of pH_a at approximately pH 8.05, after 24 h, was the result of a stabilization of Pa_{CO2} and the elimination of the metabolic base load (Figs. 3A,B,C). These alterations in blood acid-base status occurred without alterations in blood lactate concentration (Fig. 3C). In the longer term exposure groups, blood acid-base status remained very similar to that attained by 24-72h and was characterized by chronic respiratory alkalosis (Fig. 4).

A progressive 6-8% increase in plasma Na^+ and Cl^- was observed during the first 72 h of exposure to lake water (Fig. 5A). This trend towards greater plasma Na^+ and Cl^- was still seen at 3 weeks. At 5 weeks and 2 years, levels had decreased slightly, but even at the latter time, the elevation in Cl^- above the levels in well water adapted fish remained significant (Fig. 5B).

Gill Structure and Chloride Cell Morphometry

The filamental surface of the LCT gill was predominantly composed of micro-ridged

pavement (or respiratory) cells and contained varying numbers of villous chloride cells which appeared in openings of the respiratory epithelium (Figs. 6A,B,C). Mucous cells were rarely seen. Qualitatively, the relative absence and small size of filamental epithelial CCs in well water fish is apparent in Fig. 6A. Following transfer into lake water, the exposure of CC's on the filamental surface became much more pronounced through changes in both individual cell surface areas and cell densities (Figs. 6B,C). Similarly, the lamellar gill surface of well water adapted fish had very few CC's. Exposure to lake water led to changes in lamellar CC exposure and density that paralleled those observed on the filamental epithelium (Figs. 6D,E,F).

Chloride cell fractional surface area (CC FSA) was 4-fold greater after 3 days exposure to lake water and 10-fold and 20-fold higher after 3 weeks and 2 years in lake water, respectively (Fig. 7A). Differences in CC FSA between the naive trout and those exposed to lake water for 3 days were due to the 2-fold greater individual CC surface area combined with a 2-fold greater CC density (Figs. 7B,C). The higher CC FSA observed in fish exposed to lake water for 3 weeks and 2 years were solely due to greater individual CC surface areas (Fig. 7B).

DISCUSSION

Survival and Stress

The absence of mortality following acute transfer of Lahontan cutthroat trout into Pyramid Lake water illustrates how readily these fish adapt to the highly alkaline environment. This is underscored by the rapid adjustments that we observed in a variety of physiological parameters including nitrogenous waste excretion, ionoregulation and acid-base regulation. Background cortisol levels were, however, relatively high in all exposure groups (Table 3). This may be attributed to the handling, confinement and catheterization of a wild trout (Woodward and Strange 1987; McDonald and Milligan 1992). Accordingly, we believe that transfer into lake water was not unduly stressful. The absence of any increase in plasma glucose, following acute lake water exposure (Table 3), supports our conclusion.

Nitrogenous Waste Excretion

The initial reduction of J_{Amm}, immediately observed following transfer into lake water, parallels that seen in Oncorhynchus mykiss exposed to pH 9.5 water (Wilkie and Wood 1991). Unlike the rainbow trout, however, there was no tendency for J_{Amm} to return to pre-exposure levels in the LCT (Figs. 1A, 2A). We estimate that approximately 16,000 μmol $N \cdot kg^{-1}$ of waste ammonia was unaccounted for during the first 72 h at pH 9.4. Plasma T_{Amm} levels (Fig. 1B) suggest that virtually none (< 1%) of the "missing" ammonia was stored in the extracellular space. The fact that J_{Amm} remained depressed indefinitely while plasma T_{Amm} eventually returned to, and remained at, pre-exposure levels (Figs. 1B, 2B) suggests that LCT were either excreting another N-waste product(s) and/or amino acid deamination rates were permanently reduced.

The possibility that LCT were excreting another N-waste product is intriguing and

deserves further investigation. Absolute rates of J_{Urea} did not change following transfer to lake water (Figs 1A, 2D), so urea did not fulfil this role. Potential alternate waste products include trimethylamine oxide (TMAO), glutamine, creatine, creatinine or purines such as uric acid (see Forster and Goldstein 1969; Mommsen and Walsh 1992). In a related study (Wright *et al.* 1993), however, we were unable to detect any uric acid excretion in LCT which had been exposed to lake water for about 4 weeks and which exhibited rates of J_{Amm} and J_{Urea} very similar to those of the present study.

The alternate possibility, that of reduced use of amino acids as an energy source and reduced endogenous ammonia production also deserves further investigation (Walton and Cowey 1982; Van Waarde 1983). The persistent respiratory alkalosis (Figs. 3,4) may have altered metabolic enzyme activities, and led to a greater reliance on other fuels such as glycogen and/or fatty acids.

Pyramid Lake water's greater salinity (approximately 60 $mmol \cdot L^{-1}$ NaCl; Table 1) may also have accounted for the some of the persistent reduction in J_{Amm}. Ammonia production may have declined in accord with an overall reduction in metabolic rate as a result of lower ionoregulatory and osmoregulatory costs in the more isotonic environment (Rao 1968). Brett (1979) noted that growth rates generally increase with salinity in euryhaline fish, such as the cutthroat trout. A greater proportion of amino acids may have been incorporated and retained in structural protein, rather then deaminated. Indeed, the rapid growth of the LCT in Pyramid Lake's alkaline/saline waters is legendary (Coleman and Johnson 1988).

The initial inhibition of J_{Amm} upon exposure of LCT to lake water was similar to that observed in rainbow trout exposed to comparable pH, and it is likely that similar explanations apply (Wright and Wood 1985; Lin and Randall 1990; Wilkie and Wood 1991; Yesaki and

Iwama 1992). These include a decrease in the ΔP_{NH3} between the blood and gill boundary layers that drives the diffusive efflux of NH_3, and/or an inhibition of Na^+/NH_4^+ exchange. Although the blood-bulk water ΔP_{NH3} increased rather than decreased upon exposure to alkaline pH (Fig. 1C), this value will be differentially affected by the presence or absence of boundary layer acidification and associated diffusion trapping of NH_3 as NH_4^+ in the gill water (Randall and Wright 1989). Acidification of gill water by CO_2 and possibly H^+ efflux across the gills is well established in rainbow trout in fresh water (Lin and Randall 1990; Playle and Wood 1989) and this phenomenon clearly augments J_{Amm} (Wright, Randall and Perry 1989). It is likely that the same phenomenon occurs in the LCT in well water. Blockade of CO_2 hydration and boundary layer acidification in gill water by acetazolamide clearly reduces J_{Amm} in rainbow trout (Wright *et al.* 1989). In other words, boundary layer acidification increases the blood-boundary layer ΔP_{NH3} above the blood-bulk water ΔP_{NH3} and effectively augments J_{Amm}. In the highly buffered waters of Pyramid Lake, however, boundary acidification is probably reduced or non-existent. As a result, gill boundary layer pH will approach the pH of the bulk water, and therefore approximately 50 % of the excreted ammonia will exist as NH_3 in the gill water. This would effectively reduce the blood-boundary layer ΔP_{NH3} below the blood-bulk water ΔP_{NH3} and result in reduced J_{Amm}. Indeed, Wright *et al.* (1993) found no effect of acetazolamide treatment on J_{Amm} in LCT adapted to Pyramid Lake water. They also found that Na^+-free water and amiloride treatment did not effect J_{Amm}, suggesting that Na^+/NH_4^+ exchange does not occur once the animals are adapted to Pyramid Lake water. In contrast, amiloride treatment consistently reduces J_{Amm} in rainbow trout in fresh water (Kirschner, Greenwald and Kerstetter 1973; Wright and Wood 1985).

Wright *et al.* (1993) did not examine the LCT in well water, but they concluded that

maintenance of a large blood-to-water ΔP_{NH3}, via chronically elevated pH_e and plasma T_{Amm}, was probably the most important factor sustaining the (limited) J_{Amm} in lake water. The fact that the arterial blood P_{NH3} in LCT in well water was similar to values observed after 3 weeks and 5 weeks in lake water (approximately 56 μTorr: Fig. 2C) suggests that this species was "pre-adapted" to living in a high pH lake. Such a pre-adaptation would likely be selected for, as would an ability to chronically reduce ammonia production rates, in a salmonid which has a long evolutionary history in alkaline lakes (Trotter 1991).

Ureagenic Enzyme Activities

Urea excretion did not account for the missing waste-N. Its persistence at high pH and its greater percentage contribution to N-excretion appears to maintain minimal rates of $J_{Waste-N}$. The presence of significant activities of enzymes involved in uricolysis in both well water fish and those adapted to Pyramid Lake for 3 weeks or 2 years suggests that the majority of urea production resulted from uricolysis (Table 2). Danulat and Kempe (1992) observed very high rates of urea excretion in the cyprinid Chalcalburnus tarichi, endemic to highly alkaline Lake Van, Turkey (pH 9.8) and suggested that much of this excretion was due to hydrolysis of arginine, catalyzed by hepatic arginase. We found hepatic arginase activity was about 16-fold higher than that reported by Danulat and Kempe (1992) but equal to values reported by Chiu, Austic and Rumsey (1986) for fingerling rainbow trout. Arginine is an essential amino acid for teleosts (Forster and Goldstein 1969) and since the fish in the present study had been starved prior to experimentation, it seems unlikely that there would have been sufficient arginine flux through arginase to sustain urea production.

The OUC pathway contribution to urea synthesis was insignificant in cutthroat trout, as CPS III and other enzyme activities in the cycle were negligible or very low. This

observation is consistent with work performed on other salmonids (Chiu, Austic and Rumsey 1986; Huggins, Skutch and Baldwin 1969) and the high pH tolerant cyprinid Chalcalburnus tarichi (Danulat and Kempe 1992).

Acid-Base Balance and Ionoregulation

The respiratory alkalosis accompanying high pH exposure persisted indefinitely (Figs. 3B, 4B), but the metabolic alkalosis was corrected within 24 h (Figs. 3C,4C). The long term control of metabolic acid base status is interesting in view of the fact that large electrochemical gradients, favouring losses of metabolic acid (protons) or gains of metabolic base, developed following transfer to high pH (Table 4). The presence of significant inwardly directed electro-chemical gradients for HCO_3^- and $CO_3^{=}$ can be attributed to the unusually high concentrations of these ions in Pyramid Lake water (Galat *et al.* 1985; Table 1). Despite these exogenous factors, the similar levels of plasma HCO_3^- in well water LCT and those residing in Pyramid Lake for up to 2 years suggests that these fish are in a steady state with respect to long term acid-base status. One possible mechanism of acid-base control is the increased production of metabolic acid, via increased lactic acid production (Eichenholz *et al.* 1962). Such a response has been observed in rainbow trout at pH 9.5 and in LCT in pH 10 water (Wilkie and Wood, 1991; Wilkie *et al.* 1993). We observed no such response in this study, however. Another possibility is that the persistent external base load was counteracted by a stimulation of gill Cl^-/HCO_3^- exchange. Increased Cl^-/HCO_3^- exchange might have been augmented by branchial chloride cell proliferation, as has been demonstrated in other teleosts subjected to alkalotic disturbances in systemic acid-base status (see Goss *et al.* 1992a, 1992b).

We suggest that the greater CC FSA found in lakewater adapted LCT (Fig. 7) is linked to long term acid-base regulation in an environment that exerts a continual base load on

the fish. The rapidity (24 h) of the correction of the metabolic alkalosis, following transfer into Pyramid Lake water, does not argue against a branchial mechanism of metabolic acid-base control. Indeed, Goss *et al.* (1992a) demonstrated that changes in branchial morphology occur 6 hours after the initiation of acid-base disturbances. Therefore, it is quite possible that increased CC FSA, and associated Cl^-/HCO_3^- exchange, accounted for the correction of the alkalosis after only 24 h of lake water exposure. Further evidence in support of branchial acid-base regulation by the LCT was the presence of a significant correlation between pH_e and CC FSA (CC FSA = $(1.15 \times 10^6)(pH_e) - (9.16 \times 10^6)$; $r = 0.585$, $P<0.05$). Galat *et al.* (1985) reported that LCT, living in a variety of alkaline lakes, had considerable CC hyperplasia and suggested that it was correlated to the sum of external HCO_3, $CO_3^{=}$ and Cl^-. Our results corroborate these findings.

Ionoregulatory failure, characterized by 15-20 % decreases in plasma Na^+ and/or Cl^-, has been cited as a potential contributing factor in the deaths of rainbow trout exposed to high pH (Heming and Blumhagen 1988; Wilkie and Wood 1991; Yesaki and Iwama 1992). In contrast, the LCT exhibited slight increases in plasma Na^+ and Cl^- following transfer to lake water (Fig. 5). The high salinity of Pyramid Lake water may account for this difference. The increase in branchial CC FSA accompanying lake water adaptation (Figs. 6,7), however, may have also have prevented ionoregulatory disturbance in these fish (Laurent, Hobe and Dunel-Erb 1985; Laurent and Perry 1990; Perry, Goss and Laurent 1992).

CONCLUSIONS

Unlike other salmonids, Lahontan Cutthroat trout readily adapt to the extreme pH of Pyramid Lake by making a number of unique physiological adjustments. The apparent persistence of reduced J_{Amm} in lakewater adapted fish suggests that this species adapts to

alkaline lake water by decreasing endogenous ammonia production. This allows the fish to rapidly correct internal ammonia levels and prevents NH_3 from reaching toxic levels. Furthermore, the LCT also rapidly corrects, and continues to regulate, its metabolic acid-base status, despite the presence of large inwardly directed electrochemical gradients for basic equivalents. Increases in branchial CC FSA may actually augment this acid-base regulation, and also prevent plasma ion dilution, through the modulation of Na^+ and Cl^- uptake at the gill.

Table 1: Typical Chemical Composition of Well Water (pH 8.4) and Pyramid Lake Water (pH 9.4).

	WELL WATER	PYRAMID LAKE WATER
pH[1]	8.35	9.36
$[H^+]$ ($\mu mol \cdot L^{-1}$)	4.40×10^{-3}	0.43×10^{-3}
$[OH^-]$ ($\mu mol \cdot L^{-1}$)	0.66	6.70
P_{CO_2} (Torr)	0.78	0.26
$[HCO_3^-]$ ($mmol \cdot L^{-1}$)	4.35	13.80
$[CO_3^{=}]$ ($mmol \cdot L^{-1}$)	0.04	4.97
Titration Alkalinity[2] ($mmol \cdot L^{-1}$)	4.45	23.08
$[Na^+]$ ($mmol \cdot L^{-1}$)	7.30	58.20
$[Cl^-]$ ($mmol \cdot L^{-1}$)	4.15	59.70
Total Salinity ($g \cdot L^{-1}$)	0.59	4.43

[1] Measured at 10.4°C.

[2] Titration alkalinity to pH = 4.0.

Table 2: Activities of Ornithine Urea Cycle Enzymes and Uricolytic Enzymes (means ± 1 SEM; n) in Lahontan Cutthroat Trout Living in Well Water or Pyramid Lake Water.

OUC ENZYMES[1]	Well Water	Lake Water	
		3 WEEKS	2 YEARS
Glutamine synthetase	0.43 ± 0.08 (6)	0.35 ± 0.07 (8)	0.57 ± 0.08 (6)
Carbamoyl Phosphate synthetase III	0.01 ± 0.01 (4)	0.02 ± 0.01 (4)	0.03 ± 0.01 (6)
Ornithine Carbamoyl transferase	0.03 ± 0.01 (6)	0.03 ± 0.00 (8)	0.03 ± 0.00 (6)
Argininosuccinate synthetase	0.05 ± 0.01 (5)	0.04 ± 0.00 (5)	0.05 ± 0.01 (5)
Arginase	38.51 ± 2.99 (6)	40.96 ± 3.92 (8)	40.76 ± 6.32 (6)
Uricolytic ENZYMES			
Uricase	0.92 ± 0.19 (6)	1.83 ± 0.23* (7)	0.51 ± 0.11* (6)
Allantoinase	1.70 ± 0.27 (6)	1.61 ± 0.22 (8)	0.78 ± 0.27 (6)
Allantoicase	0.66 ± 0.09 (6)	0.48 ± 0.08 (8)	0.28 ± 0.10* (6)

[1] Activities are expressed as $\mu mol \cdot g^{-1}$ wet liver tissue$\cdot min^{-1}$, except CPS III, which is expressed as $\mu mol \cdot g^{-1}$ mitochondria$\cdot h^{-1}$.

* Significantly different from fish held in well water ($P < 0.05$).

* Significantly different from fish held in lake water for 3 weeks ($P < 0.05$).

Table 3: Arterial Blood and Plasma Measurements Taken from Lahontan Cutthroat in Well Water (pH 8.4) and Over the Initial 72 h Exposure to Lake Water and After 3 Weeks, 5 Weeks, and 2 Years Exposure to Lake Water.

Media	Plasma Protein ($g \cdot 100\ mL^{-1}$)	Hemoglobin ($mg \cdot 100\ mL^{-1}$)	Glucose ($mmol \cdot L^{-1}$)	Cortisol ($ng \cdot mL^{-1}$)	Pa_{CO_2} (Torr)
Well Water					
1 year	4.1 ± 0.2	12.0 ± 0.9	6.3 ± 0.7	254.5 ± 26.7	97.6 ± 7.5
Pyramid Lake					
8 h	4.0 ± 0.2	10.0 ± 0.6	5.1 ± 0.4	281.3 ± 6.3	99.1 ± 2.7
24 h	3.6 ± 0.2	8.8 ± 0.8*	5.2 ± 0.4	146.3 ± 28.4	104.9±5.4
48 h	3.2 ± 0.2*	7.9 ± 0.7*	5.0 ± 0.4	146.3 ± 28.6	98.8 ± 8.8
72 h	3.2 ± 0.2*	6.1 ± 0.7*	4.8 ± 0.3	157.6 ± 22.4	99.8 ±4.0
3 weeks	3.9 ± 0.2	8.9 ± 0.9*	6.7 ± 0.8	135.8 ± 39.3	90.9 ±6.9
5 weeks	3.9 ± 0.2	8.0 ± 0.5*	3.3 ± 0.6*	248.8 ± 25.5	88.7 ±4.0
2 years	2.5 ± 0.1*	N/A	9.4 ± 0.8*	277.1 ± 43.3	69.3±14.3

* Significantly different from well water values ($P < 0.05$).

Table 4: Electrochemical Gradients for Acid-base Relevant Ions Between the Blood and Water of Lahontan Cutthroat Trout in Well Water (pH 8.4; n = 3) or Adapted to Lake Water (pH 9.4; n = 13) for 3 Weeks.

ION	TEP	Nernst Potential		F_{ION}[1,2]	
	Well Water	Lake Water	Well Water	Lake Water	Well Water	Lake Water
OH^- (H^+)	-6.2 ±-1.7	-3.2 ± 0.4*	-19.8 ± 1.5	-70.6 ± 1.4*	+13.6 ± 1.7	+67.4 ± 1.3*
$CO_3^{=}$	-6.2 ± 1.7	-3.2 ± 0.4*	+33.2 ± 2.6	-38.6 ± 1.5*	-39.4 ± 4.3	+35.5 ± 1.3*
HCO_3^-	-6.2 ± 1.7	-3.2 ± 0.4*	+12.4 ± 1.5	-16.7 ± 2.2*	-18.6 ± 3.1	+13.6 ± 2.0*

* Significantly different from well water values ($P < 0.05$)

All data expressed in mV.

[1] F_{ION} = TEP - Nernst Potential

[2] A positive (+) F_{ION} indicates an inwardly directed electrochemical gradient for anions.

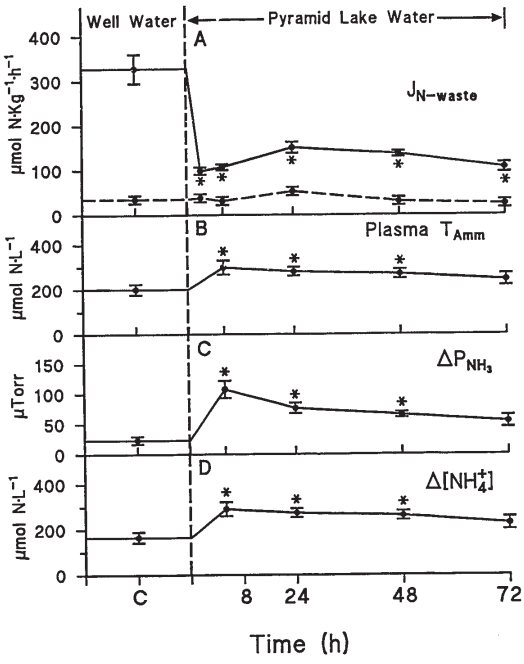


Fig. 1. Changes in (A) ammonia excretion (solid line) and urea excretion (dashed line); (B) plasma total ammonia (T_{Amm}); (C) the calculated arterial blood to water NH_3 partial pressure gradient (ΔP_{NH_3}); and (D) the calculated arterial blood to water NH_4^+ concentration gradient (ΔNH_4^+) of Lahontan cutthroat trout, following transfer into alkaline Pyramid Lake water (pH 9.4) from well water (pH 8.4). Means ± 1 SEM; n = 7. Asterisks indicate significant differences from well water values ($P < 0.05$).

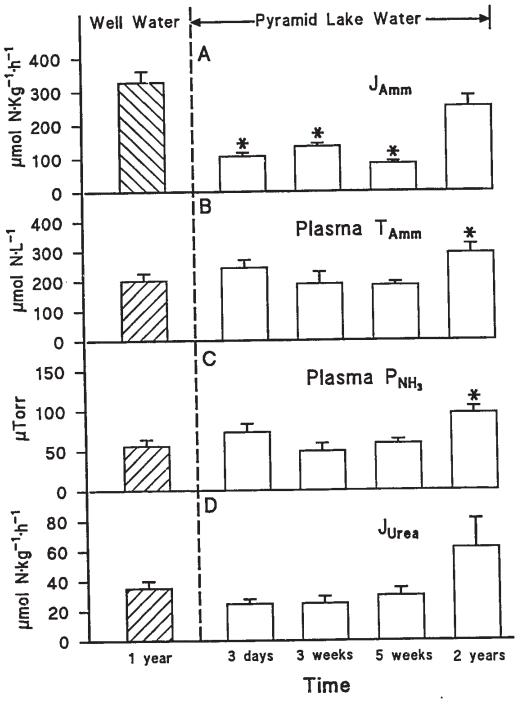


Fig. 2. The influence of long-term exposure to alkaline Pyramid Lake water (pH 9.4) upon (A) ammonia excretion (J_{Amm}); (B) plasma total ammonia concentration (T_{Amm}); (C) the partial pressure of NH_3 in arterial blood; and (D) urea excretion (J_{Urea}) by Lahontan cutthroat trout previously reared in well water (pH 8.4). Means ± 1 SEM; n = 7 in well water and after 3 days at pH 9.4; $n \geq 8$ after 3 weeks; n = 13 after 5 weeks; and $n \geq 6$ after 2 years exposure to lake water. Asterisks indicate significant differences from well water values ($P < 0.05$).

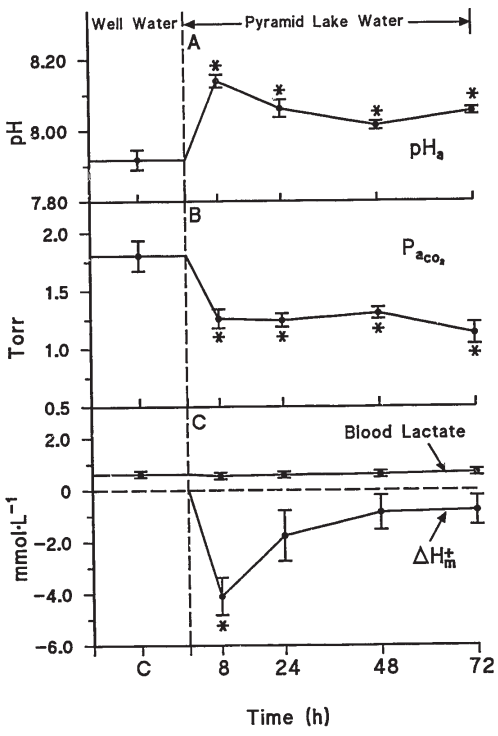


Fig. 3. Changes in (A) arterial pH (pH_a); (B) arterial carbon dioxide tension (Pa_{CO_2}); and (C) blood lactate and metabolic acid load (ΔH^+_m) of Lahontan cutthroat trout following transfer into alkaline Pyramid Lake water (pH 9.4) from well water (pH 8.4). Means ± 1 SEM; n = 7. Asterisks indicate significant differences from well water values ($P < 0.05$).

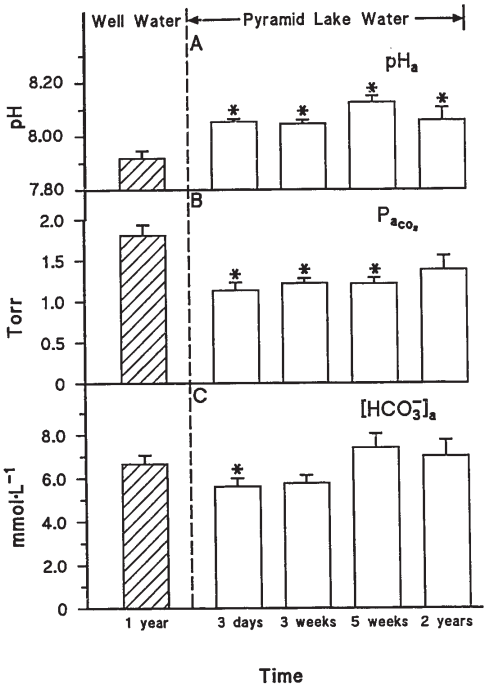


Fig. 4. The influence of long-term exposure to alkaline Pyramid Lake water (pH 9.4) upon (A) arterial blood pH (pH_a); (B) arterial CO_2 tension (Pa_{CO_2}); and (C) arterial HCO_3^- ($[HCO_3^-]_a$) in Lahontan cutthroat trout previously reared in well water (pH 8.4). Means ± 1 SEM: n = 7 in well water and after 3 days in lake water; n = 8 after 3 weeks; n = 13 after 5 weeks; and n = 6 after 2 years exposure to Pyramid Lake water. Asterisks indicate significant differences from well water values ($P < 0.05$).

Fig. 5. (A) Change in plasma Na^+ and Cl^- concentrations in Lahontan cutthroat trout over the first 72 h following exposure to alkaline Pyramid Lake water (pH 9.4) and (B) after 3 days, 3 weeks, 5 weeks and 2 years in lake water. Means $\pm$ 1 SEM; n = 7 in well water (pH 8.4) and after 3 days in lake water; n = 8 after 3 weeks; n = 13 after 5 weeks and n = 6 after 2 years exposure. Asterisks indicate significant differences from well water values ($P < 0.05$) for Na^+ and Cl^-, respectively.

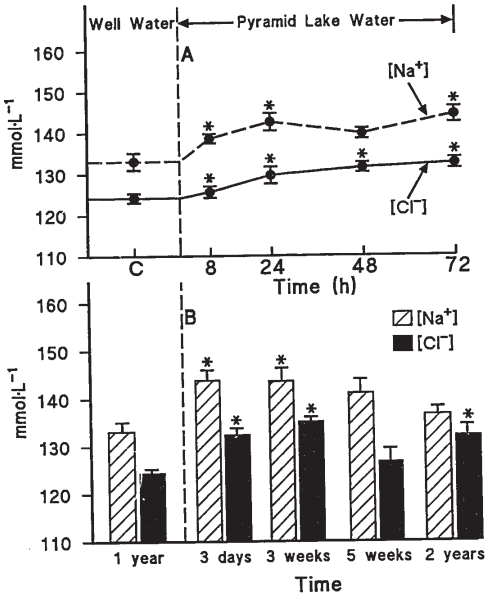
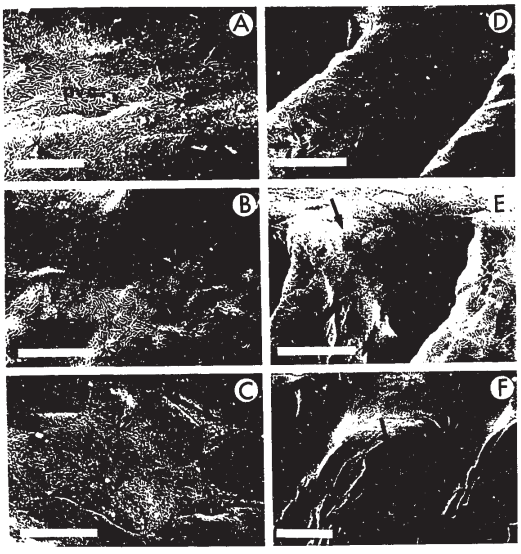


Fig. 6. Representative scanning electron micrographs of the filamental epithelium (A,B,C) and lamellar epithelium (D,E,F) of Lahontan cutthroat trout reared in well water (pH 8.4; A,D) or alkaline Pyramid Lake water (pH 9.4) for 3 days (B,E) or 3 weeks (C,F). Note the increased density of chloride cells (indicated by arrows) on the filamental and lamellar epithelium of fish exposed to lake water for 3 days and the larger chloride cells after 3 weeks vs 3 days of exposure to lake water. pvc Pavement Cell; Bar = 20 micrometers.



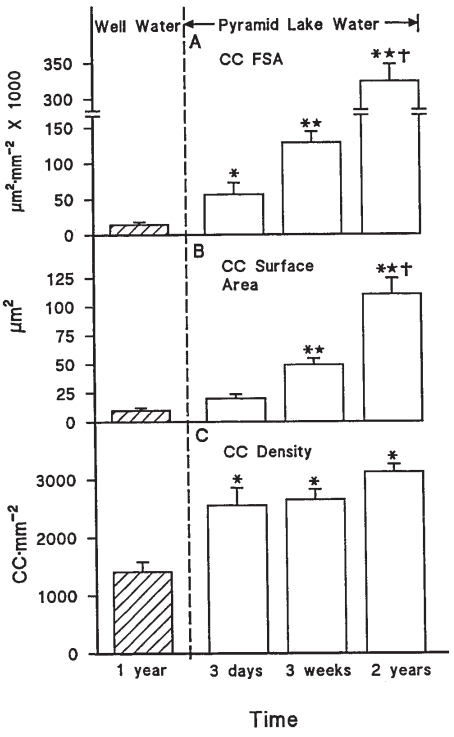


Fig. 7. Differences in branchial (A) chloride cell fractional surface area (CC FSA); (B) mean individual chloride cell surface area; and (C) chloride cell density of Lahontan cutthroat trout exposed to well water (pH 8.4) or Pyramid Lake water (pH 9.4) for 3 days, 3 weeks or 2 years. Asterisks indicate significant differences from well water values; stars indicate significant differences from 3 days exposure to Pyramid Lake water; daggers indicate significant differences from 3 weeks exposure to lake water ($P < 0.05$).

CHAPTER 6

THE PHYSIOLOGICAL RESPONSES OF THE LAHONTAN CUTTHROAT TROUT (*Oncorhynchus clarki henshawi*), A RESIDENT OF HIGHLY ALKALINE PYRAMID LAKE (pH 9.4), TO CHALLENGE AT pH 10.

ABSTRACT

Dessication of Pyramid Lake, Nevada has led to continued increases in the lake's alkalinity (currently pH 9.4) that may threaten the resident Lahontan cutthroat trout population. In this study, Lahontan cutthroat trout were challenged with more alkaline water (pH 10). The objectives were to describe physiological responses which may permit survival or lead to death in future potential environmental conditions, and to cast further light on the mechanisms of nitrogenous waste excretion, acid-base regulation, and ionoregulation in this unusual salmonid. Ammonia excretion (J_{Amm}) was reduced 50% in the first few hours, but had fully recovered by 24 h and exceeded control by 36-48 h. A sustained, two-fold elevation of plasma ammonia may have facilitated the recovery of J_{Amm} by increasing the blood-to-water P_{NH3} diffusion gradient and NH_4^+ electrochemical gradient. Urea excretion (J_{Urea}) almost doubled at 24-48 h of pH 10 exposure. Activities of ornithine-urea cycle enzymes in the liver were very low, and there was no induction at pH 10. However all three enzymes of the uricolytic pathway were present, and allantoicase activity increased significantly at pH 10, a possible explanation for elevated J_{Urea}. Increased liver glutamine

synthetase activity at pH 10 is consistent with a possible ammonia detoxification mechanism. A combined respiratory (decreased Pa_{CO2}) and metabolic (gain of basic equivalents) alkalosis developed at pH 10 and resulted in a 0.25 unit increase in arterial blood pH. Electrochemical gradients for $CO_3^=$ and OH^- entry and H^+ efflux all increased, but the gradient for HCO_3^- entry decreased to zero. Blood lactate increased without marked changes in arterial O_2 tension, suggesting that increased lactic acid production contributed to acid-base control. Plasma Na^+ and Cl^- decreased and K^+ increased during pH 10 exposure. Survival at pH 10 was relatively poor; >50% died after 72 h exposure. Greatly elevated plasma P_{NH3} and depressed plasma Na^+ and Cl^- levels in non-surviving trout suggest that a combination of ammonia-toxicity and ionoregulatory failure led to death in susceptible cutthroat trout.

INTRODUCTION

The Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*) appears to be uniquely adapted to the highly alkaline waters (pH 9.4) of Pyramid Lake, Nevada, thriving under conditions which are toxic to other salmonids (Galat *et al.*, 1985; Coleman and Johnson, 1988). A full description of the lake's chemical composition is presented in Table 1 of Wright *et al.* (1993). Exposure of other salmonids to water pH's in this range has been reported to cause problems in acid-base regulation, ionoregulation, and ammonia excretion (Wright and Wood, 1985; Heming and Blumhagan, 1988; Randall and Wright, 1989; Lin and Randall, 1990; Wilkie and Wood, 1991; Yesaki and Iwama, 1992).

The preceding study (Wright *et al.*, 1993) demonstrated that branchial ammonia excretion rates (J_{Amm}) are rather low in the Lahontan cutthroat in Pyramid lakewater at pH 9.4, and that a number of compensatory physiological adjustments have been made to cope with the situation. J_{Amm} is facilitated by relatively high plasma pH and total ammonia (T_{Amm}) levels, which allow the maintenance of a positive P_{NH3} diffusion gradient from blood to water across the gills despite the high external pH. Renal ammonia excretion is relatively high, and urea accounts for a larger proportion of nitrogenous waste excretion than normally seen in salmonids. Acute exposure (3h) to pH 10 severely depresses branchial ammonia excretion without altering urea excretion, a result attributed to the reduction in the P_{NH3} gradient.

These observations raise the questions how, and indeed whether, this species can withstand longer term exposure to more alkaline pH. The question is not just of academic importance; higher water pH's may threaten the survival of the Pyramid Lake cutthroat trout population. The lake is terminal and drains only by evaporation but its water levels are not being maintained due to the diversion of much of its only freshwater inflow, the Truckee River.

Continuing restriction of this inflow plus ongoing drought may lead to further increases in pH by concentrating alkaline HCO_3^- and $CO_3^{=}$ salts (Galat *et al.*, 1981, 1983). Indeed, Pyramid Lake's current pH of 9.4 is approximately 0.2 units higher than in the early 1980's (Vigg and Koch, 1980; Galat *et al.*, 1981, 1983, 1985).

Accordingly, the goal of the present investigation was to describe the physiological responses that either permit survival or result in death when Lahontan cutthroat trout are challenged with higher pH (pH 10) for several days. In light of past reports (above), the study focused on acid-base regulation, ionoregulation, and nitrogenous waste excretion. We were particularly interested to see if ammonia excretion recovered during a longer term (72 h) challenge at pH 10, if there were compensatory increases in urea excretion, if lactic acid production occurred as a mechanism for acid-base regulation in the face of alkalosis, and if plasma Na^+ and Cl^- levels declined. All these responses have been seen in a recent study of rainbow trout (*Oncorhynchus mykiss*) exposed to pH 9.5 (Wilkie and Wood, 1991).

Another major objective of the study was to quantify the hepatic enzymes associated with urea production in the Lahontan cutthroat trout. The traditional view has been that the ornithine urea cycle (OUC) is not expressed in teleosts and that ureagenesis occurs mainly by uricolysis. However, several clear exceptions have now been identified (Read, 1971; Saha and Ratha, 1987, 1989; Randall *et al.*, 1989; Mommsen and Walsh, 1989). In one of these cases, the Lake Magadi tilapia (*Oreochromis alcalicus grahami)*, active ureagenesis by the OUC appears to be associated with the high environmental pH (pH 10) in which the fish normally lives (Wood *et al.*, 1989; Wright et al, 1990). A related tilapia endemic to neutral water (*Oreochromis nilotica*; Wood *et al.*, 1989) and the rainbow trout (Wilkie and Wood, 1991), both of which are thought to lack the OUC, also increased urea production upon

exposure to high environmental pH. These observations raise the possibility that OUC activity may be induced by alkaline conditions. We therefore measured the key hepatic enzymes of the OUC and uricolysis in the Lahontan cutthroat trout under "control" conditions (pH 9.4) and after 72 h exposure to pH 10.

METHODS AND MATERIALS

Experimental Animals and Set-up

One year old Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*; 242.2 $\pm$ 9.2 g, N = 21) of both sexes were obtained from Pyramid Lake Fisheries, Nevada and held in Pyramid lakewater at pH 9.4. Fish origin and holding conditions were identical to those described by Wright *et al.* (1993). The trout were fitted with indwelling dorsal aorta catheters (Soivio *et al.*, 1972), under MS-222 anaesthesia, and allowed to recover 72h in darkened flux chambers. Water flow to each chamber was approximately 0.5 $L.min^{-1}$, on a flow-through basis and water Tamm levels never exceeded 5 umol $N.L^{-1}$. Experimental temperature was 9.5 $\pm$ 0.3 °C. Thirteen trout were subjected to pH 10 exposure, and an additional 8 fish were sacrificed for tissue samples under control conditions.

For exposures to pH 10, Pyramid lakewater was pumped first into a 50 L central reservoir fitted with a pH-stat that consisted of a Radiometer GK2401C combination pH electrode connected to a PHM82 pH meter and a TTT80 autotitrator. The system controlled an electromagnetic valve (Nacon Industries) which regulated the dropwise flow of 2 N KOH into the vigorously aerated reservoir. This addition of KOH resulted in water K^+ concentrations of approximately 13 $mmol.L^{-1}$ at pH 10 in comparison to the normal level of 2.9 $mmol.L^{-1}$ at pH 9.4. The water was then pumped to each fish box at 0.5 $L.min^{-1}$ from which it overflowed to waste. Mean control water pH was 9.380 $\pm$ 0.004 and experimental

water pH was 9.990 $\pm$ 0.005, as measured in the boxes with an independent electrode and meter. When flux boxes were operated as closed systems at pH 10, for determination of ammonia and urea excretion rates, pH was initially set by the pH-stat but CO_2 excretion by the fish drove water pH down. This made it necessary to continually monitor pH and adjust it with 2 ml additions of 2N KOH at 1 h and 2 h. As a result, water K^+ increased by 2.7 $mmol.L^{-1}$ to approximately 15.7 $mmol.L^{-1}$ by the end of each flux period.

Experimental Protocol

Flux determinations of J_{Amm} and J_{Urea} were performed under control conditions at pH 9.4 and at 0-3 h, 8-11 h, 24-27 h, 36-39 h, 48-51 h and 72-75 h of pH 10.0 exposure. Water samples (15 ml) were taken at 0 h and 3 h of each flux period, immediately acidified with 2N HCl to prevent NH_3 loss, frozen and later analyzed for Tamm and urea. Since one molecule of waste-nitrogen (N) is excreted in one molecule of NH_3 and 2 molecules of waste-N are excreted in a molecule of urea, ammonia and urea excretion rates were expressed in umol $N.kg^{-1}.h^{-1}$. These rates were measured over one 3 h interval from concentration differences between the start and end of the flux period (see Wright *et al.*, 1993). Typically, water Tamm never exceeded 50 umol $N.L^{-1}$ over 3 h. Between flux periods, boxes were opened to the flow-through system.

Thirty minutes prior to each flux determination, 1.0 ml blood samples were drawn into two 500 ul, heparinized, gas-tight Hamilton syringes, for determination of arterial blood O_2 tension (Pa_{O2}), pH (pH_a), hematocrit, hemoglobin, and lactate, and plasma Tamm, urea, total CO_2 (Ca_{CO2}), Na^+, Cl^-, K^+, glucose, cortisol, and protein. In addition, water samples for determination of inspired P_{O2} (P_{IO2}) and total CO_2 were taken. Branchial transepithelial potential (TEP) was measured while blood samples were being processed. Plasma was

separated by centrifugation (2 min at 13,000 g) and frozen for later analysis of Tamm, urea, glucose, cortisol, Na^+, Cl^-, and K^+. Hematocrit was determined by centrifugation (5 min at 500 g) and plasma Ca_{CO2} and protein were determined with plasma decanted from the hematocrit tubes. At the completion of sampling, blood used for determination of Pa_{O2} (normally 200 ul) was re-infused into the fish, along with sufficient Cortland saline (Wolf, 1963) to re-establish blood volume.

Blood sampling preceded the flux determinations by 30 min to minimize disturbance to the fish and ensure that blood composition was not influenced by box closure. Had blood sampling occurred at the middle or end of the flux period, blood acid-base status and plasma ammonia might have been altered due to build-up of Tamm in the water (*cf.* Wilkie and Wood, 1991; Wright *et al.*, 1993). Diffusion gradients for NH_3 (ΔP_{NH3}) were calculated from the blood measurements taken prior to the flux and the water measurements at the very start of the flux period, before any ammonia build-up in the water had occurred (Wilkie and Wood, 1991). Diffusion gradients calculated for the first hour of exposure to pH 10 were based upon the control blood sample, because it was unlikely that blood parameters would change during the first few minutes at pH 10. Estimates of the blood-to-water gradient for P_{O2}, P_{CO2}, H^+, OH^-, HCO_3^-, and $CO_3^{=}$ (see below) were determined from water and blood samples obtained simultaneously.

The 6 fish which were still alive at 75 h were sacrificed with an overdose of MS-222 (1.5 $g.L^{-1}$). White muscle and liver samples were freeze-clamped in liquid nitrogen and stored at -70°C for later analysis of white muscle ions and ureagenic enzymes, respectively. For purposes of comparison, white muscle and liver samples were taken in an identical fashion from 8 trout which had been similarly cannulated and blood sampled at pH 9.4. The

blood data from these fish were not significantly different from the control data from the experimental series and so have not been reported.

Analytical Techniques for Water and Blood

Methods for water and plasma ammonia, urea, pH, P_{O2}, and total CO_2 determinations were identical to those described by Wilkie and Wood (1991) and Wright *et al.* (1993). Plasma Na^+ and K^+ were measured via atomic absorption (Varian 1275) and plasma Cl^- by coulometric titration (Radiometer CMT10). White muscle Na^+, Cl^-, K^+ and water were determined by techniques outlined in Wood and LeMoigne (1991). Glucose (hexokinase/glucose-6-phosphate dehydrogenase), lactate (lactate dehydrogenase), and hemoglobin (cyanmethemoglobin) were determined with commercial kits (Sigma). Cortisol was measured by (^{125}I) radio-immunoassay (ICN Biomedicals Inc.) using standards diluted to the protein concentrations found in trout plasma and read on a Packard 5000 Series Gamma Counter. Plasma protein was determined by refractometry (Alexander and Ingram, 1980).

Arterial P_{CO2} (Pa_{CO2}), HCO_3^- ($[HCO_3^-]_a$), and $CO_3^{=}$ ($[CO_3^{=}]_a$) were calculated from Ca_{CO2} and pH_a using the Henderson-Hasselbalch equation, appropriate solubility co-efficients and pK_1' and pK_2' values outlined in Boutilier *et al.* (1984) and Skirrow (1975). Water P_{CO2}, HCO_3^-, and $CO_3^{=}$ were determined from total water CO_2 and pH in a similar fashion, using constants at the appropriate chlorinity and salinity from Skirrow (1975). Water and blood P_{NH3} and $[NH_4^+]$ were calculated from the pH and Tamm values as outlined by Wright and Wood (1985) and Wright *et al.* (1993) using appropriate constants from Cameron and Heisler (1983). Water and blood H^+ concentrations were based on pH measurements in the two media. Accordingly, water and blood OH^- concentrations were calculated from H^+ concentrations and the temperature-corrected ionization constant for water (CRC, 1984). The

net metabolic acid load to the blood plasma (ΔH^+m) was calculated from changes in blood pH, plasma HCO_3^-, and hemoglobin, using procedures outlined by Turner *et al.* (1983). Mean cell hemoglobin concentration (MCHC) was simply the blood hemoglobin divided by blood hematocrit (Turner *et al.*, 1983).

Transepithelial potential across the gills was measured and estimates of the electrochemical driving force (F_{NH4}) for NH_4^+ diffusion from blood to water calculated as described by Wright *et al.*(1993). Estimates of the electrochemical forces for other ions (F_{HCO3}, F_{CO3}, F_{OH}, F_H) were calculated in an analogous manner.

Analytical Techniques for Ureagenic Enzymes

OUC and uricolytic enzymes were measured on liver samples which had been stored at -70°C. Enzyme activities are given in umoles of substrate converted to product per gram of liver tissue fresh weight in 1 min at 22°C under saturation conditions, with one exception. CPS activity is given in umoles per gram of mitochondria per hour. Appropriate control experiments were conducted to validate the specificity and linearity (with tissue amount and time) of each assay. Tissue was prepared by homogenizing liver samples in a 1:10 (weight:volume) solution of ice-cold HEPES buffer (50 $mol.l^{-1}$, pH 7.5), using a hand-held glass homogenizer. The protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was added to each liver homogenate sample (approx. concentration 0.1 $mmol.l^{-1}$). Tissue homogenates were kept on ice and used within 15 min. Uricolytic enzymes - uricase (Brown et al. 1966), allantoinase (Takada and Noguchi, 1983), allantoicase (Brown et al. 1966), and some OUC enzymes - glutamine synthetase (Webb and Brown, 1980), ornithine carbamoyl transferase and arginase (Mommsen et al. 1983) - were assayed spectrophotometrically using methods described previously. Modified methods were used for the other OUC enzymes (below).

Carbamoyl phosphate synthetase (CPS) was assayed by a radiotracer technique after isolation of mitochondrial fragments from frozen liver samples by differential centrifugation (Moyes et al. 1986). The mitochondrial pellet was resuspended and sonicated in a solution containing glycerol (50%), potassium phosphate buffer (20 $mmol.l^{-1}$, pH 7.4), β-mercaptoethanol (5 $mmol.l^{-1}$), ethylenediaminetetraacetic acid (EDTA, 0.5 $mmol.l^{-1}$), and bovine serum albumin (0.02%). Two additional protease inhibitors, leupeptin and aprotinin, were added to each tissue sample (10 $ug.ml^{-1}$). Mitochondrial homogenate (25 ul) was added to 375 ul of a reagent mixture containing ATP (8 $mmol.l^{-1}$), creatine phosphate (10 $mmol.l^{-1}$), $MgSO_4$ (13 $mmol.l^{-1}$), KCl (40 $mmol.l^{-1}$), dithiothreitol (2 $mmol.l^{-1}$), ornithine (10 $mmol.l^{-1}$), N-acetyl glutamate (NAG, 2 $mmol.l^{-1}$), L-glutamine (10 $mmol.l^{-1}$), $NaHCO_3$ (1 $mmol.l^{-1}$ cold + 0.4 uCi ^{14}C-$NaHCO_3$), HEPES (50 $mmol.l^{-1}$, pH 8.0), ornithine carbamoyl transferase (0.3 U), and creatine kinase (20 U). Samples were incubated for 60 min at 22°C and the reaction terminated by addition of 50 ul of trichloroacetic acid (35%). Unincorporated ^{14}C-CO_2 was removed by shaking samples in a fume hood for 60 min. Samples were counted in 4 ml of scintillation fluor (Scintiverse BD, Fisher) after neutralization with $NaCO_3$.

CPS III is a mitochondrial enzyme in fish associated with the inner mitochondrial membrane (Mommsen and Walsh, 1989). To separate possible contamination with cytosolic CPS II, activity was measured with (CPS II & CPS III) and without (CPS II) NAG, in the presence of the substrate, glutamine. Total CPS III activity was calculated as the difference in enzyme activity in the presence and absence of NAG. Initial experiments showed that the addition of ammonia (the key substrate for the mammalian-type CPS I enzyme) to the assay medium had no effect on total CPS activity and ammonia was excluded from subsequent assays.

Argininosuccinate synthetase activity was assayed spectrophotometrically in a multi-step procedure. First, citrulline and aspartate were converted to argininosuccinate by endogenous argininosuccinate synthetase. Second, argininosuccinate was converted to arginine by exogenous argininosuccinate lyase. Third, exogenous arginase converted arginine to urea and ornithine, and finally, urea was metabolised to ammonia by the addition of exogenous urease. The amount of urea formed (umoles.g liver tissue^{-1}.min^{-1}) should be directly proportional to the formation of arginine. Liver tissue was sonicated in 1:2 (w:v) solution of ice-cold HEPES buffer (50 mmol.l^{-1}, pH 7.5). The reagent mixture contained potassium phosphate (50 mmol.l^{-1}, pH 7.5), citrulline (1 mmol.l^{-1}), aspartate (3 mmol.l^{-1}), $MgSO_4$ (2 mmol.l^{-1}), argininosuccinate lyase (0.3 U), arginase (100 U), adenosine triphosphate (ATP 1 mmol.l^{-1}), and an ATP regenerating system, creatine phosphate (5 mmol.l^{-1}) and creatine kinase (8 U). Double-strength reagent mixture (50 ul) was combined with equal volumes of liver homogenate and the reaction was terminated after 60 min at 22°C with 10 ul of $HClO_4$ (70%). A sample (100 ul) of neutralized supernatant was incubated (60 min at 22°C) with 25 ul of a urease solution (5 mg.ml^{-1}). Ammonia concentration was determined on 50 ul of deproteinized supernatant (Verdouw et al. 1978).

Statistics

All data are expressed as means $\pm$ 1 SEM (N) where N = the number of animals contributing data to the mean. Each animal served as its own control, so the paired two-tailed Student's t-test was used to evaluate the significance of changes observed, whereas the comparable unpaired test was used to evaluate differences between independent means ($P < 0.05$). An F-test was used to determine homogeneity of variance between independent means.

When this latter criteria wasn't satisfied a Mann-Whitney non-parametric statistical test was used to test for statistical significance.

RESULTS

Survival

After 72 h at pH 10, 12 of 13 fish were alive, but over the next 4 h, 50% of the remaining fish died. These results suggest that Lahontan cutthroat trout are incapable of surviving a pH = 10 challenge for more than a few days.

Nitrogenous Waste Excretion

At pH = 9.4, J_{Amm} was approximately 85 umol N.kg^{-1}.h^{-1}. During the first 3 h at pH 10, J_{Amm} dropped significantly by about 50% (Fig. 1A). This depression was short-lived; by 8 h J_{Amm} had recovered and by 36 and 48 h was significantly elevated by 25 percent, to approximately 110 umol N.kg^{-1}.h^{-1} (Fig. 1A). Over the last 24 h of exposure, J_{Amm} dropped slightly and was no longer significantly elevated (Fig. 1A). The initial inhibition of J_{Amm} led to elevated plasma Tamm. After 8 h at pH 10, plasma T_{Amm} increased about 2-fold from resting levels of 185 umol N.L^{-1}, thereafter stabilizing at approximately 400 umol N.L^{-1} (Fig. 1B).

The arterial blood to bulk water gradient for NH_3 diffusion (ΔP_{NH3}) was about 50 uTorr at pH 9.4 and fell only slightly upon initial exposure to pH 10 (Fig. 2A). However, by 8 h ΔP_{NH3} had increased almost 4-fold to 180 uTorr and by 72 h, reached 240 uTorr (Fig. 2A). There was also a strong electrochemical gradient of about +100 mV for NH_4^+ diffusion out of the fish at pH 9.4 (Fig. 2C). The contribution of the slightly negative TEP (-3 mV) to this gradient was negligible (Fig. 2B). F_{NH4} increased to +145 mV at 8 h, largely due to the two-fold elevation in plasma NH_4^+, while TEP increased by 1mV (Fig. 2C). By 48 h, F_{NH4} had stabilized at approximately +130 mV while TEP had returned to control levels (Figs. 2B,C).

At pH 9.4, J_{Urea} was approximately 30 umol N.kg^{-1}.h^{-1}, increased slightly over the first 8 h of exposure to pH 10 (Fig. 3A), and was significantly elevated to 50 - 60 umol N.kg^{-1}.h^{-1} by 24 - 48h (Fig. 3A). At 72 h, J_{Urea} dropped to about 45 umol N.kg^{-1}.h^{-1} (Fig. 3A). Plasma urea fell slightly during exposure to pH 10 from about 6000 umol N.L^{-1} under control conditions to about 5000 umol N.L^{-1} at 72 h (Fig. 3B).

Ureagenic Enzymes

To investigate the origin of the increased J_{Urea}, the major hepatic enzymes of both the OUC and uricolytic pathway were measured. Amongst the uricolytic enzymes (Table 1), only allantoicase increased significantly, by approximately 50%, in fish exposed to pH 10. There were no significant changes in the activities of two other enzymes, uricase and allantoinase. In the OUC (Table 2), the key regulatory enzyme carbamoyl phosphate synthetase (CPS III), and ornithine carbamoyl transferase (OTC) both had very low activities at pH 9.4, and the latter was essentially undetectable at pH 10, suggesting that the OUC was not involved in urea production in the Lahontan cutthroat trout. Levels of the other OUC enzymes were also relatively low and did not change as a result of exposure to pH 10. However, the activity of glutamine synthetase, which is not part of the OUC but rather converts glutamate and NH_3 to the substrate glutamine, increased significantly by approximately 150% at pH 10 (Table 2).

Acid-Base Balance

Arterial pH was approximately 8.1, Pa_{CO2} about 1.2 Torr, and $[HCO_3^-]_a$ about 7.4 mmol.L^{-1} in Lahontan cutthroat trout at pH 9.4 (Fig. 4). After 8 h at pH 10, the trout exhibited a significant metabolic alkalosis (negative ΔH^+_m, Fig. 5A) characterized by a significant rise in pH_a (Fig. 4A) and a slight increase in $[HCO_3^-]_a$ (Fig. 4C). By 24h, this was compounded by a respiratory alkalosis as Pa_{CO2} fell significantly (Fig. 4B). The combined

respiratory and metabolic alkalosis was more or less stable thereafter, with mean pH_a at about 8.35, mean Pa_{CO2} at about 0.75 Torr, ΔH^+_a at about -3 mmol.l^{-1} and $[HCO_3^-]_a$ unchanged from control levels.

Blood lactate increased steadily from about 1.0 mmol.L^{-1} at pH 9.4 to more than 3 mmol.l^{-1} after 48 h at pH 10 (Fig. 5B). Increased blood lactate was not due to hypoxia; inspired P_{O2} never dropped below 125 Torr and Pa_{O2} was stable through 48h at approximately 90 Torr (Fig. 5C). The significant depression of Pa_{O2} at 72 h may have been due to complicating factors that were associated with the imminent onset of death (Fig. 5C).

The acid-base composition of the water (Table 3) was markedly altered by titration to pH 10 with KOH; [OH$^-$] and [H$^+$] increased and decreased by 4-fold, respectively. Water P_{CO2} (0.25 Torr) was close to atmospheric P_{CO2} at pH 9.4, but fell by 75% at pH 10; at the same time [HCO_3^-] was reduced by 50% and [$CO_3^{=}$] increased more than 2-fold. These changes, in combination with alterations in blood acid-base status (Fig. 4), resulted in a small but significant reduction in the blood-water P_{CO2} gradient (Fig. 6A) and larger changes in the electrochemical gradients for OH$^-$, H$^+$, HCO_3^- and $CO_3^{=}$ (Fig. 6B). F_H was strongly positive at pH 9.4, and became significantly more positive during exposure to pH 10, favouring the outward flux of H$^+$. At pH 9.4, the electrochemical driving forces were also positive for, in ascending order, HCO_3^-, $CO_3^{=}$, and OH$^-$, favouring the influx of these anions across the gills. F_{CO3} increased slightly and F_{OH} (same as F_H) increased to a greater extent upon exposure to pH 10. However, the gradient for HCO_3^- entry was virtually eliminated by exposure to pH 10 because of the accompanying decrease in water HCO_3^- concentration (Table 3). The electrochemical gradients for all these ions remained stable from 8 through 72 h of pH 10 exposure (Fig. 6B).

Ionoregulation and Hematological Indicators

At pH 9.4, plasma Na$^+$ and Cl$^-$ were 141 mmol.L^{-1} and 126 mmol.L^{-1}, respectively (Fig. 7A). After a slight non-significant increase during the first 8 h at pH 10, Na$^+$ and Cl$^-$ gradually declined in an approximately equimolar fashion, a significant 10% depression occurring by 48 - 72 h. Plasma K$^+$ was approximately 2.9 mmol.L^{-1} at pH 9.4. After 48 h at pH 10, K$^+$ had significantly increased by 1.0 mmol.L^{-1} and by 72 h had reached 5.3 mmol.L^{-1} (Fig. 7B). White muscle samples taken from fish which survived 72 h exposure to pH 10 exhibited similar water, Na$^+$, and K$^+$ levels to those taken from control fish at pH 9.4 (Table 4). However muscle Cl$^-$ levels were significantly depressed by about 30% (Table 4).

Despite repetitive blood sampling, plasma protein was stable at approximately 4 g.100 ml^{-1} over the first 48 h at pH 10. A small but significant decrease was observed at 72 h (Fig. 7C). Blood hemoglobin (not shown) fell to a greater extent (from 8.0 to 4.9 g. 100 ml^{-1}), but still rather less than expected based on the amount of blood sampled and standard estimates of blood volume in salmonids (*eg.* Olson, 1992). These observations, together with the small increases in Na$^+$ and Cl$^-$ at 8 h (Fig. 7A) suggest that a slight hemoconcentration accompanied exposure to pH 10. This hemoconcentration was not due to shift of plasma fluid into the red blood cells. Indeed, mean cell hemoglobin concentration (MCHC) increased significantly at pH 10 (Fig. 7D), suggesting that the red blood cells had shrunk about 13% by 72 h.

Plasma Glucose and Cortisol

Plasma glucose was 3.3 mmol.L^{-1} at pH 9.4 and then doubled after 8 h at pH 10; thereafter it returned to control values and more or less stabilized (Table 5). No significant changes in plasma cortisol were observed (Table 5).

DISCUSSION

Nitrogenous Waste Excretion

In agreement with Wright *et al.* (1993), ammonia excretion was initially inhibited during exposure to pH 10. The diffusion gradient for NH_3 (ΔP_{NH3}) dropped only slightly, but it must be remembered that this was measured only at the very start of exposure before any ammonia built up in the water. It is highly likely that ΔP_{NH3} fell to a much greater extent as the 3 h period progressed. Surprisingly, recovery of J_{Amm} did not result in a reduction of plasma T_{Amm}. However, this chronic elevation in T_{Amm} did result in increased driving gradients for both NH_4^+ and NH_3 diffusion, with the latter augmented by the persistent alkalosis. These increased gradients may have facilitated passive excretion of NH_3 (Cameron and Heisler, 1983) or NH_4^+ (McDonald and Prior, 1988) at pH 10. However, the possible activation of Na^+/NH_4^+ exchange (Wright and Wood, 1985) or H^+/NH_4^+ exchange (Cameron, 1986) cannot be discounted.

The Lahontan cutthroat trout also increased J_{Urea} while at pH 10, similar to the response of the rainbow trout at pH 9.5 (Wilkie and Wood, 1991). The gradual decrease in plasma urea-N at pH 10 suggests possible excretion of existing urea stores but repetitive blood sampling may have contributed to the trend. Furthermore, each fish excreted an additional 326 umol of urea-N over the 72 h experiment but the total decreases in plasma urea-N only amounted to 20 % of this value. Several other studies have shown that fish, even those lacking the OUC, excrete urea when environmental conditions impede ammonia excretion (Olson and Fromm, 1971; Saha and Ratha, 1987, 1989; Wood *et al.*, 1989; Walsh *et al.*, 1990). This appears to be particularly true when environmental pH is high (Wood *et al.*, 1989; Wilkie and Wood, 1991). Increased urea production by uricolysis (see below) may

have resulted from increased *de novo* purine synthesis occurring as a result of increased plasma T_{Amm} and perhaps glutamine (see Holmes, 1978 for review).

Ureagenic Enzyme Activity

It is unlikely that elevated J_{Urea} was due to increased OUC activity because the key regulatory enzyme CPS III exhibited barely detectable activity at both pH 9.4 and at pH 10. CPS III activities were also several orders of magnitude lower than those observed in two actively ureagenic teleosts, *Opsanus tau* (Read, 1971; Mommsen and Walsh, 1989) and *Oreochromis alcalicus grahami* (Randall *et al.*, 1989). Moreover, the activities of three other OUC enzymes did not change.

Uricolysis probably accounted for the majority of J_{Urea} under control conditions (pH 9.4) and at pH 10. Higher allantoicase activity at pH 10 suggests that increased flux through the uricolytic pathway led to the elevation in J_{Urea} at this pH. Another possible explanation for increased J_{Urea} could be elevated arginase activity, but the fish in this study had been starved so it seems unlikely that breakdown of dietary arginine led to the observed increase in J_{Urea}.

Glutamine synthetase (GS) catalyzes conversion of glutamate and NH_4^+ to glutamine for use by CPS III in the OUC. However, the 2-fold elevation in GS activity observed in this study was probably related to ammonia detoxification rather than N-waste excretion. Liver glutamine concentrations have been reported to increase in rainbow trout (Arillo *et al.*, 1981), goldfish (Levi *et al.*, 1974) and carp (Pequin and Serfarty, 1968) exposed to elevated water ammonia levels or infused with NH_4Cl. Cutthroat trout at pH 10 were similarly "ammonia-loaded", so the response probably served to decrease hepatic ammonia levels rather than to channel glutamine into the OUC.

Acid-base Balance

In normal Pyramid lakewater (pH 9.4), Lahontan cutthroat trout are in a state of chronic respiratory alkalosis characterized by low Pa_{CO2} and high pH_a, approximately 50% lower and 0.3 units higher, respectively, than values reported for other salmonids in circumneutral water (Fig. 4; *cf.* Perry *et al.*, 1981; Cameron and Heisler, 1983; Wright and Wood, 1985; Wilkie and Wood, 1991). Low Pa_{CO2} reflects the fact that the high pH water outside the gills acts as a "P_{CO2} vacuum" due to diffusion trapping of CO_2 as HCO_3^- or $CO_3^{=}$ (Johansen *et al.*, 1975).

The combined respiratory and metabolic alkalosis observed at pH 10 was the result of a number of factors. The respiratory alkalosis developed because water P_{CO2} was reduced further at pH 10 (Table 3), thereby increasing the "P_{CO2} vacuum" and reducing Pa_{CO2} a further 40 % by 24-48 h. Analysis of electrochemical gradients suggested that increased driving forces for $CO_3^{=}$ and OH^- entry and H^+ efflux accounted for the metabolic alkalosis. Surprisingly, F_{HCO3} decreased to a value not significantly different from zero. Thus, HCO_3^- entry was not a causitive factor in the development of the metabolic alkalosis.

The appearance of lactate in the bloodstream suggests that increased lactic acid production helped neutralize the metabolic base load, thereby allowing pH_a to stabilize by 48 h. Wilkie and Wood (1991) observed a very similar phenomenon in rainbow trout suffering a pure respiratory alkalosis when exposed to pH 9.4 and mammals also increase lactic acid production in response to respiratory alkalosis (Bock *et al.*, 1932; Eichenholz *et al.*, 1962; Takano, 1968; Garcia *et al.*, 1971). Such increases in blood lactate might be attributable to an increased tissue:blood lactate gradient that could result from either increased glycolytic flux or decreased pyruvate oxidation in the tissue (Ward *et al.*, 1982). Increased lactate

production was probably not the result of catheter induced stress because cutthroat trout that underwent an identical sampling protocol, following transfer into Pyramid Lake water at pH 9.4, experienced no increases in blood lactate (Wilkie *et al.*, 1994). Furthermore, Wood *et al.* (1982) have shown that anemia leads to no change in blood lactate levels in rainbow trout.

Ionoregulation and Hematological Indicators

The decrease in plasma Na^+ and Cl^- experienced by the cutthroat trout at pH 10 was similar to decreases observed in rainbow trout at high pH (Heming and Blumhagen, 1988; Wilkie and Wood, 1991; Yesaki and Iwama, 1992). Possible explanations for these observations include decreased branchial uptake and/or increased diffusive efflux of Na^+ and Cl^- (Wright and Wood, 1985; Wood, 1989; Wilkie and Wood, 1994a). Clearly further studies are needed to describe the movements of Na^+ and Cl^- at pH 10.

At low environmental pH, branchial ion losses are known to cause hemoconcentration due to red blood cell swelling brought on by an osmotic redistribution of water into intracellular compartments (see Wood, 1989). However, increased MCHC indicates that the red blood cells actually shrank. Moreover, there was no increase in muscle water content or loss of muscle Na^+ and K^+, unlike the response of trout to low pH (Wood, 1989). The lower white muscle $[Cl^-]$ observed at pH 10 is perplexing and should be studied further.

Mortality, Toxic Mechanisms, and Prognosis

The >50% mortality observed after 72h of pH 10 exposure demonstrated that Lahontan cutthroat trout are severely affected by this highly alkaline environment; the survival of the species in Pyramid Lake appears threatened if lake pH increases to this range in future years. This upper pH limit approximates values (9.8 - 10.2) reported in other salmonids (Erichsen Jones, 1964; Jordan and Lloyd, 1964; Daye and Garside, 1975; Murray and

Ziebell, 1984; Randall and Wright, 1989; Yesaki and Iwama, 1992). Thus, despite the fact that the Lahontan cutthroat trout presently thrives under conditions (pH 9.4, unusual water chemistry) unfavourable for other salmonids (Galat *et al.*, 1985; Coleman and Johnson, 1988), it does not appear to have an unusually high tolerance to alkaline pH when exposed acutely.

As to the mechanism of mortality, several possibilities associated with the experimental protocol itself can likely be eliminated. A similar experimental regime at pH 9.4 caused no mortality (Wilkie *et al.*, 1994). Increased environmental K^+ (13-15.7 $mmol.L^{-1}$; due to the use of KOH to increase system pH), which may have contributed to increased plasma K^+ was probably not involved. The trout's plasma K^+ concentration of 5.3 $mmol.L^{-1}$ at 72 h was similar to measurements on healthy salmonids (McDonald and Milligan, 1992). As an additional check, we exposed 8 similarly sized rainbow trout to 13 $mmol.L^{-1}$ KCl for 72 h. Plasma K^+ in the rainbow trout increased only slightly from 4.6 to 6.0 $mmol.L^{-1}$ and none of these fish died. Toxicity due to waterborne NH_3 at high pH can also be eliminated. Water NH_3 concentrations observed at pH 10 never exceeded 4.1 $umol.L^{-1}$ and were well below the 96 h LC50 for cutthroat trout (approximately 30 umol $NH_3.L^{-1}$; Thurston *et al.*, 1978) and rainbow trout (50 umol $NH_3.L^{-1}$; USEPA, 1985).

Analysis of data taken at 48h (before any mortality occurred) from subsequent survivors and non-survivors proved instructive (Table 6). This analysis suggests that a combination of internal ammonia toxicity and ionoregulatory failure, possibly accentuated by the alkalosis, led to mortality.

At 48h, plasma pH_a rose to almost 8.4 in non-survivors, but this was not significantly higher than the mean in survivors (Table 6). These pH_a values are amongst the highest ever

recorded for salmonids, equalling or slightly exceeding those seen during $NaHCO_3$ infusion (Goss and Wood, 1990) and $NaHCO_3$ exposure (Perry *et al.*, 1981). However, mortality did not occur in any of these studies and therefore, it seems unlikely that high pH_a was the direct cause of death. However, T_{Amm}, P_{NH3} and $[NH_4^+]$ were all significantly higher in those Lahontan trout which eventually suffered mortality (Table 6). There is published evidence to suggest that NH_4^+ may be directly toxic to fish (Hillaby and Randall, 1979; Smart, 1978). However, elevated plasma $[NH_4^+]$ was probably not the direct cause of death in the present study because higher NH_4^+ levels were measured in rainbow trout surviving under alkaline conditions and dying under high environmental ammonia conditions (Table 6). Elevated plasma P_{NH3} was probably the toxic moiety of ammonia in the cutthroat trout at pH 10. The plasma P_{NH3} of 378 uTorr in cutthroat trout which eventually died approached the 96 h LC50 for waterborne P_{NH3} in cutthroat trout (approximately 530 uTorr; Thurston *et al.* 1978) and approached plasma levels (461 uTorr) measured in rainbow trout that died shortly after 5 h exposure to 2 $mmol.L^{-1}$ Tamm (Table 6).

Ionoregulatory failure may also have contributed to death. Plasma Na^+ and Cl^- concentrations were both significantly depressed in non-surviving trout (Table 6), approaching levels known to contribute to mortality in acid-stressed trout (see Wood, 1989 for review).

To conclude, the responses of the Lahontan cutthroat trout, normally living at pH 9.4 in Pyramid Lake, to exposure to pH 10, were remarkably similar to those of the freshwater rainbow trout transferred from pH 8.1 to 9.5 (Wilkie and Wood, 1991). An inhibition and subsequent recovery of J_{Amm}, an activation of J_{Urea}, probably via uricolysis, a marked blood alkalosis partially compensated by lactic acid production, and a decrease in plasma electrolytes were the most notable responses. A combination of ammonia toxicity and

ionoregulatory failure led to mortality in susceptible cutthroat trout. Ironically, increased plasma Tamm, which facilitated J_{Amm} at pH 10, also caused toxic increases in blood P_{NH3}. Clearly, if the pH of Pyramid Lake continues to climb its population of Lahontan cutthroat trout will be threatened.

Table 1. Uricolytic enzyme activity (means ± 1 SEM; N) in Lahontan cutthroat trout in control (pH 9.4) water or exposed to pH 10 water for 72 h.

ENZYMES[1]	pH = 9.4	pH = 10.0
Uricase	1.83 ± 0.23 (7)	1.63 ± 0.14 (6)
Allantoinase	1.61 ± 0.22 (8)	0.99 ± 0.16 (6)
Allantoicase	0.48 ± 0.08 (8)	0.70 ± 0.03* (6)

[1] Activities are expressed as umol.g^{-1} liver tissue.min^{-1}.

* Significantly different from control fish ($P < 0.05$).

Table 2. Ornithine-urea cycle enzyme activity (means ± 1 SEM; N) in Lahontan cutthroat trout in control (pH 9.4) water or exposed to pH 10 water for 72 h.

ENZYMES[1]	pH = 9.4	pH = 10.0
Glutamine synthetase	0.35 ± 0.04 (8)	0.85 ± 0.17* (6)
CPS III	0.02 ± 0.01 (4)	0.02 ± 0.01 (4)
OTC	0.03 ± 0.00 (8)	0.00 ± 0.00 (8)
Argininosuccinate synthetase	0.04 ± 0.00 (5)	0.05 ± 0.00 (5)
Arginase	40.96 ± 3.92 (8)	52.26 ± 2.87 (6)

[1] Activities are expressed as umol.g^{-1} liver tissue.min^{-1}, except for CPS III, umol.g^{-1} mitochondria.h^{-1}

* Significantly different from control fish ($P < 0.05$).

Table 3. Acid-base composition of Pyramid Lake water.

	pH = 9.4	pH = 10.0
pH	9.38	9.99
$[H^+]$ (umol.L^{-1})	0.41 x 10^{-3}	0.10 x 10^{-3}
$[OH^-]$ (umol.L^{-1})	7.18	29.6
P_{CO2} (Torr)	0.25	0.06
$[HCO_3^-]$ (mmol.L^{-1})	14.10	7.55
$[CO_3^{=}]$ (mmol.L^{-1})	5.01	11.81

Table 4. White muscle ions and water content (means ± 1 SEM; N) in Lahontan cutthroat trout in control (pH 9.4) water or exposed to pH 10 water for 72 h.

[ION]	pH = 9.4	pH = 10.0
$[Cl^-]^1$	20.2 ± 1.8	14.5 ± 0.4*
	(7)	(5)
$[Na^+]^1$	9.3 ± 0.8	8.6 ± 0.8
	(7)	(5)
$[K^+]^1$	125.9 ± 2.3	123.9 ± 2.6
	(7)	(5)
H_2O^2	74.21 ± 0.59	72.51 ± 0.83
	(7)	(5)

[1] Concentrations expressed as $mmol.kg^{-1}$ wet tissue.

[2] White muscle water content expressed as percent H_2O.

* Significantly different from control fish ($P < 0.05$).

Table 5. Plasma glucose and cortisol (means ± 1 SEM; N) in Lahontan cutthroat trout in control water (pH 9.4) and during 72 h exposure to pH 10 Pyramid Lake water.

Time (h)	Lake water pH	Glucose ($mmol.L^{-1}$)	Cortisol ($ng.ml^{-1}$)
Control	9.4	3.3 ± 0.6	248.8 ± 25.5
8	10.0	6.8 ± 0.9*	255.3 ± 23.2
24	10.0	3.5 ± 0.8	278.9 ± 36.7
48	10.0	4.1 ± 1.1	269.5 ± 46.7
72	10.0	4.1 ± 0.7	205.4 ± 30.5

* Significantly different from control plasma samples taken prior to pH 10 exposure.

Table 6: Key hematological parameters (mean ± 1 SEM) in surviving and non-surviving cutthroat trout after 48 h at pH 10, in rainbow trout after 48 h at pH 9.5 and in rainbow trout exposed to 2 $mmol.L^{-1}$ T_{Amm} for 5 h.

	Cutthroat trout		Rainbow trout	
	Survivors at pH 10.0 (N = 6)	Non-survivors at pH 10.0 (N = 6)	pH = 9.5 [1]Exposure	2 $mmol.L^{-1}$ [2]T_{Amm}
T_{Amm} ($umol.L^{-1}$)	331 ± 43	498 ± 55*	640	1325
P_{NH3} (uTorr)	231 ± 46	378 ± 40*	295	461
$[NH_4^+]$ ($umol.L^{-1}$)	318 ± 41	478 ± 53*	632	1302
pH_a	8.310 ± 0.05	8.376 ± 0.03	7.982	7.889
$[Na^+]_a$ ($mmol.L^{-1}$)	137.9 ± 3.2	123.5 ± 3.5*	130.8	149.2
$[Cl]_a$ ($mmol.L^{-1}$)	128.5 ± 3.0	102.8 ± 4.8*	125.8	135.6

[1] From Wilkie and Wood (1991).

[2] R.W. Wilson and C.M. Wood (unpublished results).

* Significantly different from surviving cutthroat trout ($P < 0.05$).

Fig. 1. Influence of a pH 10 challenge on (A) ammonia excretion (J_{Amm}) and (B) plasma total ammonia concentration (T_{Amm}) in Lahontan cutthroat trout acclimated to pH 9.4 Pyramid Lake water. Values are means ± 1 SEM, N = 13 for the control, 3 h, 8 h, 24 h and 36 h periods; N = 12 at 48 h; N = 10 for plasma T_{Amm} and N = 7 for J_{Amm} at 72 h. Asterisks indicate significant differences from control (pH 9.4) values ($P < 0.05$).

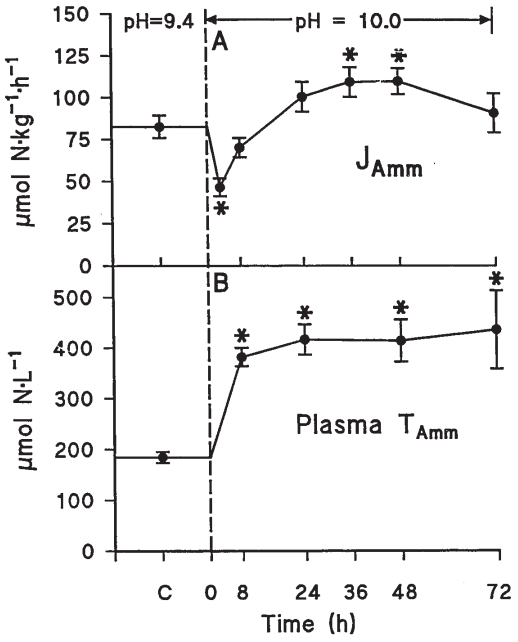


Fig. 2. Influence of a pH 10 challenge on the (A) blood-to-water NH_3 gradient (ΔP_{NH_3}), (B) transepithelial potential (TEP) and (C) electrochemical gradient for NH_4^+ (F_{NH_4}) of Lahontan cutthroat trout acclimated to pH 9.4 Pyramid Lake water. Values are means ± 1 SEM, n = 13 for control, 3 h, 8 h, 24 h and 36 h periods; n = 12 at 48 h and n = 10 at 72 h. Asterisks indicate significant differences from control (pH 9.4) values ($P < 0.05$).

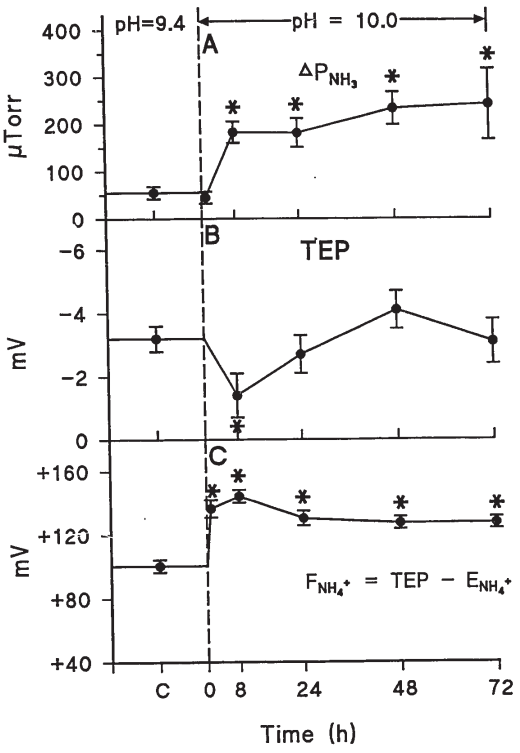


Fig. 3. Influence of a pH 10 challenge on (A) urea excretion rates (J_{Urea}) and (B) plasma urea concentration in cutthroat trout acclimated to pH 9.4 Pyramid Lake water. Values are means ± 1 SEM, N = 13 for the control, 3 h, 8 h, 24 h and 36 h periods; N = 12 at 48 h; N = 10 for plasma urea and N = 7 for J_{Urea} at 72 h. Asterisks indicate significant differences from control (pH 9.4) values ($P < 0.05$).

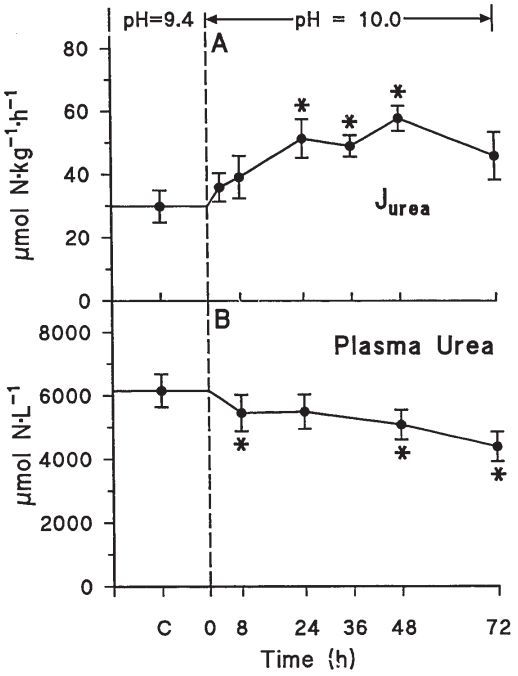


Fig. 4. Influence of a pH 10 challenge on (A) arterial pH (pHa), (B) arterial CO_2 partial pressure (Pa_{CO2}) and (C) arterial HCO_3^- concentration ($[HCO_3^-]_a$) in cutthroat trout acclimated to pH 9.4 Pyramid Lake water. For further details refer to Fig. 2.

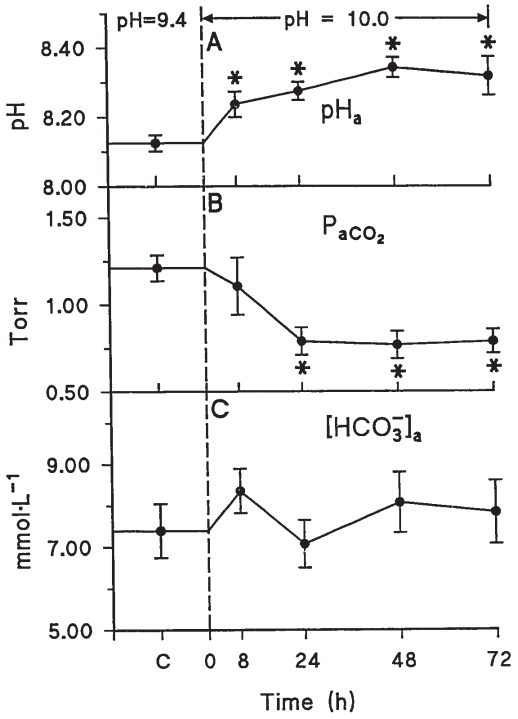


Fig. 5. Influence of a pH 10 challenge on (A) metabolic acid load (ΔH_m^+), (B) blood lactate and (C) inspired P_{O2} (P_{IO2}; solid line) and arterial P_{O2} (Pa_{O2}; dashed line) in cutthroat trout acclimated to pH 9.4 Pyramid Lake water. For further details refer to Fig. 2.

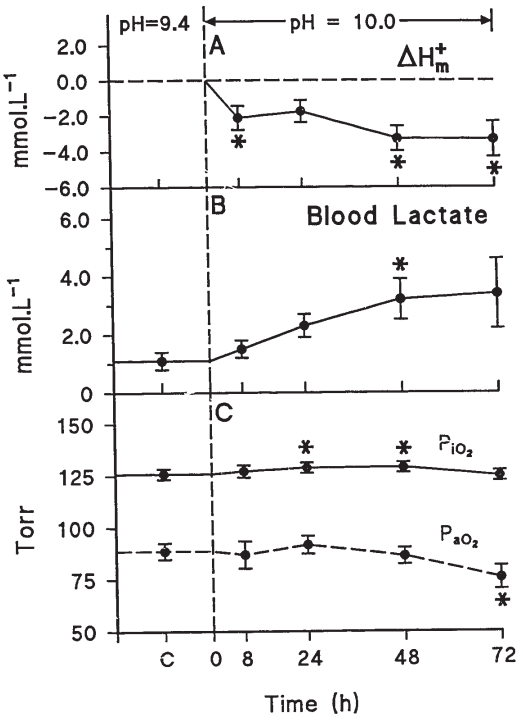


Fig. 6. Influence of a pH 10 challenge on (A) the blood-to-water CO_2 partial pressure gradient (ΔP_{CO_2}) and (B) the blood-to-water electrochemical gradients for H^+ and OH^- (F_H and F_{OH}, respectively; dashed line), HCO_3^- (F_{HCO_3}; solid line), and $CO_3^{=}$ (F_{CO_3}; dotted line) in cutthroat trout acclimated to pH 9.4 Pyramid Lake water. For further details refer to Fig. 2.

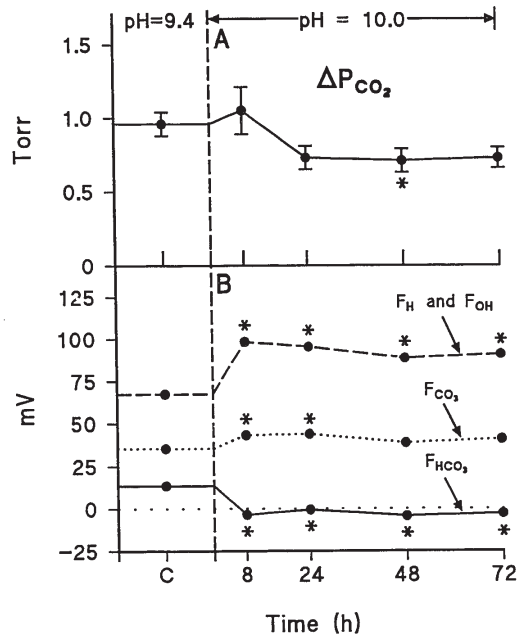
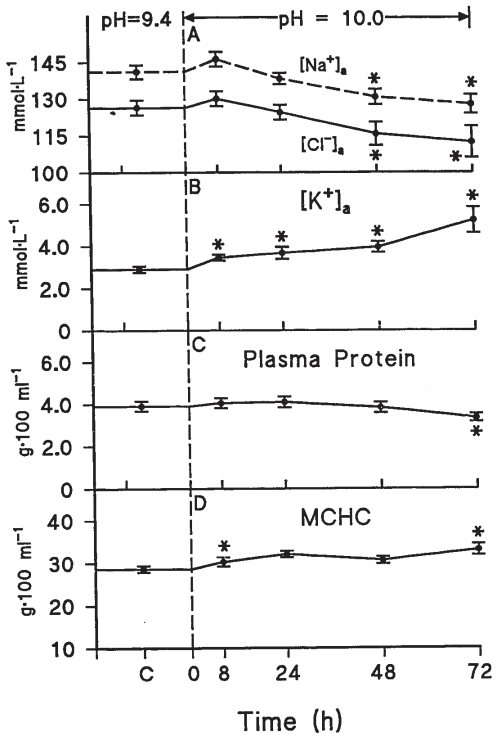


Fig. 7. Influence of a pH 10 challenge on (A) plasma Na^+ (dashed line) and Cl^- (solid line), (B) plasma K^+, (C) plasma protein and (D) mean cell hemoglobin concentration in cutthroat trout acclimated to pH 9.4 Pyramid Lake water. For further details refer to Fig. 2.



CHAPTER 7

PHYSIOLOGICAL ADAPTATIONS OF RAINBOW TROUT TO CHRONICALLY ELEVATED WATER pH (pH = 9.5).

ABSTRACT

Rainbow trout suffered negligible mortality during 4 weeks of exposure to pH 9.5 in separate experiments at 10°C (no deaths) or 15°C (4 % mortality). Ammonia (J_{Amm}) and urea excretion rates (J_{urea}) and plasma and white muscle ammonia (T_{Amm}) were all lower at 10°C; this was probably a reflection of lower basal N-metabolism in the cooler environment. Exposure to pH 9.5 at 10°C led to a chronic, 70 % reduction in ammonia excretion rates (J_{Amm}). At 15°C J_{Amm} was initially reduced by 35 % but rapidly recovered and fluctuated around control levels, thereafter. At both 10°C and 15°C, plasma ammonia was chronically elevated by 40-80 % and 70-100 %, respectively, and white muscle T_{Amm} was chronically elevated, by 50-100 %. There were no long term changes in J_{urea} or plasma urea at pH 9.5, at either 10°C or 15°C, although there were marked reductions in white muscle urea concentration. No changes in plasma cortisol or glucose were observed. Blood lactate was elevated, by 30-75 % at both temperatures, a response likely related to acid-base regulation at high pH rather than stress. Blood hematocrit and hemoglobin increased at 15°C, but not at 10°C, and there were no corresponding changes in mean cell hemoglobin concentration. This suggests that high pH exposure at 15°C placed greater metabolic demands on the fish,

necessitating increased blood O_2 carrying capacity. Unidirectional Na^+ influx was chronically inhibited at 15°C, but no high pH-induced changes in plasma or white muscle Na^+ and Cl^- concentration were observed at either temperature. At 15°C, extracellular metabolic acidosis (decreased HCO_3^-) counteracted a chronic high pH-induced respiratory alkalosis (decreased P_{CO2}) and led to chronically lowered extracellular pH; white muscle intracellular pH was unaltered. Thus, rainbow trout readily survive at alkaline pH but differences in temperature qualitatively alter the trout's physiological responses to this environmental stressor.

INTRODUCTION

The Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*) of Pyramid Lake, Nevada (pH = 9.2-9.4; Galat *et al.* 1985; Coleman and Johnson 1988; Wright *et al.* 1993) readily adapt and grow to large sizes in its native alkaline surroundings. However, most studies regarding salmonid exposure to alkaline environments (pH > 9.0) have reported significant mortality after only a few hours or days (Eicher 1946; Jordan and Lloyd 1964; Daye and Garside 1975; Heming and Blumhagen 1988; Yesaki 1990; Wilkie and Wood 1991; Yesaki and Iwama 1992). Jordan and Lloyd (1964) observed significant mortality for rainbow trout (*Oncorhynchus mykiss*) exposed to pH 9.5 over 15 days but reported increased survival if the fish were previously acclimated to a moderately alkaline pH (8.4). Murray and Zeibell (1984) substantiated these findings, and Yesaki and Iwama (1992) demonstrated that greater water Ca^{++} concentrations enhance survival of rainbow trout at pH 10. Wilkie and Wood (1991) reported partial mortality in rainbow trout exposed to pH 9.5 for 3 days but these fish were cannulated (via dorsal aortic catheters). As yet, no detailed study has focussed on the physiological adaptations that might permit rainbow trout to tolerate alkaline pH for prolonged periods (> 15 days). Accordingly, the goal of the present investigation was to assess the mortality and physiology of freely swimming rainbow trout, exposed to pH 9.5 for 1 month, that were not subjected to the additional stress imposed by surgically implanted dorsal aortic catheters. Separate exposures were conducted at 10°C and 15°C to assess the possible effects of temperature on the responses.

Alkaline water inhibits ammonia excretion (J_{Amm}) in salmonids (Cameron and Heisler 1983; Wright and Wood 1985; Lin and Randall 1990; Wilkie and Wood 1991, 1994a,b; Yesaki and Iwama 1992; Wright *et al.* 1993; Wilkie *et al.* 1993; 1994), urea excretion (J_{urea})

transiently increases, but after 6-48 h J_{Amm} is fully re-established and J_{urea} declines (Wilkie and Wood 1991, 1994a,b). This recovery is apparently related to increased plasma total ammonia (T_{Amm}), which re-creates favourable blood-gill water P_{NH3} gradients that drive J_{Amm} (Wright *et al.* 1993; Wilkie *et al.* 1994). Accordingly, we sought to determine if ammonia and urea excretion patterns changed, and whether internal T_{Amm} was chronically elevated, in rainbow trout over 28 d of high pH exposure.

Cortisol and glucose are well known primary stress indicators in fish (see Barton and Iwama 1991 for review). The immunosuppressive and proteolytic effects of cortisol could confound trout adaptation to high pH by increasing the fish's sensitivity to infection (Barton and Iwama 1991) and/or elevating circulating ammonia (Audet *et al.* 1988), respectively. Hence, an objective of the present investigation was to follow changes in plasma glucose and cortisol during chronic high pH exposure. Hemoglobin and hematocrit were also monitored throughout the experiment; changes in these secondary stress parameters could also provide an indirect index of the animals' metabolic demands at high pH.

Disturbances to internal ion balance, due to inhibited Na^+ and Cl^- uptake and/or increased ion outflux, at high pH are partially ameliorated by 3 d [Wilkie and Wood 1991, 1994a; M.P. Wilkie, P. Laurent, and C.M. Wood, unpublished data (Chapter 4)]. It is not known, however, if trout are able to restore and maintain internal ion balance during more prolonged high pH exposure. In the present study, plasma and white muscle Na^+ and Cl^- concentrations at both temperatures, as well as Na^+ influx, outflux, and net flux rates at 15°C, were assessed throughout the experiment.

The high pH-induced respiratory alkalosis, experienced by rainbow trout over 3 d at pH 9.5, is partially offset by a simultaneous lactacidosis (Wilkie and Wood 1991; Wilkie and

Wood 1994b) that appears to originate in the white muscle (Wilkie and Wood 1994b). However, it is not known whether such an energetically expensive physiological adjustment could persist for more than a few days. Therefore, extracellular acid-base status, as well as white muscle and blood lactate concentrations were examined regularly during the 28 d experiment at 15°C. In addition, the white muscle intracellular pH (pH_i) was examined to determine if previously observed, acute increases in white muscle pH_i (Wilkie and Wood 1994b) persisted beyond the first few days of high pH exposure.

METHODS AND MATERIALS

Experimental Animals and Set-up

Two groups of juvenile rainbow trout (*Oncorhynchus mykiss*; mean weight 95.9 ± 3.0 g; n = 200) were obtained from 2 local hatcheries in the fall of 1991 (Group 1; n = 96; Spring Valley Trout Farm, Petersburg, Ontario) and summer of 1992 (Group 2; n = 104; Rainbow Springs Hatchery, Thamesford, Ontario). Group 1 fish were used for the 10°C experiment and Group 2 for the 15°C experiment. Within each group, fish were distributed equally to two rectangular holding tanks (Living Stream; water volume = 425 l), each receiving dechlorinated Hamilton City tapwater (composition: $[Na^+]$ = 0.6; $[Cl^-]$ = 0.8; $[Ca^{++}]$ = 0.9; $[Mg^{++}]$ = 0.4; $[K^+]$ = 0.03; titratable alkalinity = 2.0 mmol·l^{-1}; pH = 7.9; temperature = 10°C, group 1 or 15°C, group 2) at rates of 1-2 l·min^{-1}. This rate of water replacement kept water ammonia concentrations below 10 umol·l^{-1} and vigorous aeration and mixing maintained water P_{O2} levels above 130 torr. The fish were acclimated under these conditions for 4 weeks prior to the start of the experiment. During this acclimation period the fish were fed, *ad libitum*, 3 times per week with commercial trout pellets (Martin Feed Mills). During the 4 week exposure period itself the fish were fed a minimum ration (approximating 1.0 % body weight), every seventh evening to minimize the known effects that feeding has on nitrogenous waste excretion (Fromm 1963; Brett and Zala 1978).

The holding tanks also served as the experimental system during the 4 week high pH exposure regimes; both tanks (control pH = 7.9 and experimental pH = 9.5, respectively) received the same dechlorinated tapwater mentioned previously, but the experimental tank was maintained at pH 9.5 by the continual drop-wise addition of either 1 N NaOH (in the 10°C experiment) or 1 N KOH (in the 15°C experiment; see below for explanation). Both tanks

were sub-divided into 3 sections by a coarse mesh screen that did not impede water flow. Section 1 (approximate volume = 75 l) was the point of entry for the replacement water, which was continually added at 1-2 l·min^{-1}. The fish were restricted to the much larger middle section 2 of the tank (volume = 300 l), which also contained a constant level overflow with drainage to waste. Section 3 (volume = 50 l) contained a thermometer for temperature monitoring and, in the case of the experimental tank, the pH monitoring electrode and the inflow for the base addition system. Base addition was regulated by a pH stat set-up that comprised a Radiometer TTT80 autotitrator connected to a PHM82 pH meter and a GK2401C pH electrode; when water pH dropped below 9.8, the autotitrator activated an electromagnetic control valve (Nacon Industries) which regulated the drop-wise addition of 1 N NaOH or 1 N KOH into the water from a 20 l Nalgene reservoir. Water from this part of the tank (section 3) was continually pumped to section 1 at rate of 4 l·min^{-1}, where it mixed with the replacement water and subsequently flowed into section 2 of the tank (where the fish were held) at a pH of 9.5. This set-up prevented the development of pH gradients that might have confounded data interpretation. Rainbow trout have been known to distribute themselves according to water pH where there is a gradient (Peterson *et al.* 1989). For consistency water in the control tank was re-circulated in an identical manner. Water pH was independently monitored with either a Cole-Parmer glass pH electrode and a Markson digital hand-held pH meter (Model 88) or a Radiometer PHM72 pH meter and GK2401C pH electrode. Water pH in section 2 was maintained at pH = 9.45 ± 0.12 (mean ± SEM) and pH = 9.49 ± 0.03 in the 10°C and 15°C experiments, respectively. Water pH's in the respective control tanks were 7.88 ± 0.01 and 7.86 ± 0.01 at 10°C (10.3 ± 0.1°C) and 15°C (15.3 ± 0.1°C).

At intervals throughout the experiment, sub-samples of fish (n = 8) were removed from either the control or experimental tanks and placed in individual 3.0 l darkened plexiglass flux boxes (described in Wilkie and Wood, 1994b) housed in recirculating systems connected to the control and experimental tanks. Water was pumped from these tanks via a submersible pump and was distributed to each box via a flow-splitter at approximately 0.5 $l \cdot min^{-1}$. This water subsequently drained back into the appropriate tank. When water flow was cut-off to the boxes for flux measurements (see below), it was necessary to manually monitor and adjust box water pH (9.5 to 9.7), at 30 minute intervals using the independent pH meter and appropriate amounts of base (0.5 to 2.0 ml of 1 N NaOH or KOH; see Wilkie and Wood, 1991, 1994b).

Experimental Protocol

Protocol at 10°C

Measurements, each on different groups of fish (n = 8), were performed under pre-exposure conditions (pH = 7.9 in both tanks) and then at 3, 7, 14, 21 and 28 days of the exposure regime (pH = 9.5 in the experimental tank; pH = 7.9 in the control tank). At each measurement time, for each tank, 8 fish were taken from the tank, placed in their own individual flux boxes, and allowed to settle overnight under flow-through conditions (0.5 $l \cdot min^{-1}$). The next morning, flow to the boxes was cut-off for 3 h for the measurement of ammonia flux (J_{Amm}) and urea flux (J_{urea}) rates. Water samples (30 ml) were taken at 0, 1 and 3 h of the flux period and water pH was adjusted and monitored as previously described. At the end of the flux period, flow was re-established to the boxes. Approximately 5 h later, the fish were sacrificed with an overdose of MS-222 (1.5 $g \cdot l^{-1}$) for blood and water tissue sampling. Blood samples were taken at all times, but muscle samples only under pre-

exposure conditions and 7 and 28 days. Immobilization of the fish occurred within 1 minute of MS-222 addition, after which blood was immediately withdrawn via caudal puncture into an ice-cold, Na^+ heparinized, gas tight Hamilton syringe. Blood was immediately processed for hematocrit, hemoglobin, and lactate determinations, and the remainder centrifuged at 10 000 G for 3 minutes for separation of plasma. Plasma protein was determined immediately by refractometry, and the remaining plasma frozen in liquid N_2 and stored at - 70°C for later determination of ammonia, urea, cortisol, glucose, Na^+ and Cl^- concentration. Immediately following blood sampling, white muscle samples were excised from the region between the adipose and dorsal fins, above the lateral line, and freeze clamped with liquid N_2-cooled aluminum tongs. The samples were temporarily stored in liquid N_2, and then at -70°C for later determination of the white muscle (WM) ammonia, urea, lactate, Na^+ and Cl^- concentrations.

Protocol at 15°C

The protocol at 15°C was virtually identical to that at 10°C, but with the additional sampling of white muscle at 14 d and 21 d, and the additional measurement of plasma pH and total CO_2, white muscle intracellular pH (pH_i) and water content, and Na^+ influx (J^{Na}_{in}), outflux (J^{Na}_{out}) and net flux (J^{Na}_{net}) rates at all times. The latter necessitated the use of 1 N KOH, rather than NaOH, to maintain water pH at pH 9.5; otherwise continual addition of NaOH would have resulted in background water Na^+ levels that approached 2 $mmol \cdot l^{-1}$ making accurate determination of changes in "cold" (non-radioactive Na^+) concentration and $^{22}Na^+$ radioactivity very difficult. The Na^+ flux measurements were determined in conjunction with ammonia and urea flux measurements and involved the addition of 4 uCi of $^{22}Na^+$ (New England Nuclear) to each flux box 15 minutes prior to initiating the flux

determination period. After this equilibration period, 45 ml water samples were then withdrawn at 0, 1, 2 and 3 h of the flux determination period. In order to document the acute effects of high pH exposure, Na^+ fluxes, J_{Amm} and J_{urea} were also measured in separate batches of experimental fish at 0-3 h and 24-27 h (1 day) of high pH exposure.

Analytical Techniques and Calculations

Water and Blood Parameters

Water ammonia concentration was determined via a micro-modification of the salicylate-hypochlorite assay (Verdouw *et al.* 1978). Water and blood urea was determined via the diacetyl monoxime method of Crocker (1967) using commercial reagents (Sigma). Water and plasma Na^+ was determined using atomic absorption (Varian 1270). Water $^{22}Na^+$ radioactivity was measured on 5 ml water samples mixed with 10 ml of aqueous counting scintillant (ACS; Amersham). Plasma Cl^- was determined via coulometric titration (Radiometer CMT10 Chloridometer). Whole blood lactate (lactate dehydrogenase), plasma ammonia (glutamate dehydrogenase) and glucose (hexokinase/glucose-6-phosphate dehydrogenase) were all determined enzymatically using Sigma kits. Cortisol was determined via ^{125}I radioimmunoassay (Immunocorp) measured on a Packard 5000 Series gamma counter. Plasma protein was determined via refractometry and hemoglobin was determined by the cyanmethemoglobin method using Sigma reagents and standards.

Since blood samples obtained via caudal puncture represent mixed arterio-venous blood, plasma pH in the present study will be referred to simply as extracellular fluid pH (pH_e). Extracellular fluid pH and extracellular total CO_2 were determined simultaneously on plasma samples that had not been previously thawed. The pH_e was determined by injecting plasma into a thermostatted (15°C) capillary pH electrode [Radiometer (G297/G2)] that was

connected to a PHM72 pH meter (Radiometer), and total plasma CO_2 was determined on a Corning model 965 total CO_2 analyzer.

White Muscle Parameters

White muscle intracellular pH (pH_i) was determined using the nitrilotriacetic acid-fluoride method of Portner *et al.* (1990) following pulverization of the muscle under liquid N_2 (see Wilkie and Wood, 1994b for further details); the pH measurements were performed on the same capillary pH electrode set-up just described. White muscle ammonia, lactate, urea, Na^+ and Cl^- concentrations were determined on white muscle extracts that had been ground to a fine powder under liquid N_2 and deproteinized in 9 volumes of 8 % perchloric acid. Ammonia concentrations were determined on the TRIS neutralized extracts, according to the methods of Kun and Kearney (1971), while urea was determined on unneutralized extracts via a micro-modification of the diacetyl monoxime method of Crocker (1967). White muscle Na^+, Cl^- and lactate analyses were identical to those described for plasma. White muscle water content was established by oven-drying 200 mg frozen pieces of white muscle to constant weight.

Calculations

Ammonia (J_{Amm}) and urea excretion rates (J_{urea}) were calculated from the respective changes in water ammonia and urea concentrations during the flux period, the fish's weight and the known box volume (see Wilkie and Wood, 1991). Na^+ influx estimates (J^{Na}_{in}) were based on the disappearance of $^{22}Na^+$ from the water (see Wilkie and Wood 1994a for details) and the mean specific activity in the water during the flux period; net flux calculations (J^{Na}_{net}) were based on changes in cold (non-radioactive) Na^+ during the flux period and Na^+ outflux (J^{Na}_{out}) was calculated as J^{Na}_{net} minus J^{Na}_{in}. Water, plasma and white muscle P_{NH3} and NH_4^+

concentrations were determined from measurements of total ammonia and pH via the Henderson-Hasselbalch equation and the appropriate solubility co-efficients and pK_{Amm}, provided by Cameron and Heisler (1983). Plasma P_{CO2} (Pe_{CO2}) and HCO_3^- concentration ($[HCO_3^-]_a$) were calculated in an analogous manner using the solubility and pK' constants supplied by Boutilier *et al.* (1984). The extracellular fluid metabolic acid-load (ΔH^+_m) was calculated with the following equation as outlined by Milligan and Wood (1986a,b):

$$\Delta H^+_m = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta(pH_1 - pH_2) \quad (1)$$

Since the fish were sampled terminally, $[HCO_3^-]_1$ and $[HCO_3^-]_2$ represent simultaneous measurements of the mean extracellular fluid HCO_3^- concentrations and pH_1 and pH_2 are the mean extracellular pH's in trout held at pH 7.0 and 9.5 respectively. Changes in extracellular lactate (ΔLac) were also based on mean values. β (beta) represents the blood, non-bicarbonate, buffer capacity and was calculated using the regression formula derived by Wood *et al.* (1982) for rainbow trout blood:

$$\beta = -1.073 \text{ X } [Hb] - 2.48 \quad (2)$$

where [Hb] represents the blood hemoglobin concentration.

Mean cell hemoglobin concentration (MCHC) was calculated as the hemoglobin concentration divided by the hematocrit (Milligan and Wood, 1982).

Statistics

All data are expressed as means $\pm$ 1 SEM (N). Statistically significant differences between controls (pH 7.9) and experimental fish (pH 9.5) at the same sampling times in each experiment were determined via unpaired students t-test (at $p < 0.05$) after first checking for

homogeneity of variance by use of an F-test. In instances where this criteria was not satisfied, the more conservative Welch's approximate t-test was employed (Zar 1984). Statistical comparisons for data generated at 0-3 h and 1 d of high pH exposure at 15°C were via unpaired t-test to the respective pre-exposure (P) values, because simultaneous control measurements were not made at these times.

RESULTS

Survival

Uncannulated rainbow trout appear to survive readily at pH 9.5. Alkaline exposure resulted in no mortality over 28 days exposure at 10°C and minimum mortality (2 fish died at 1 day = 4 %) at 15°C.

Nitrogenous Waste Excretion and Storage

Pre-exposure ammonia excretion rates (J_{Amm}) at control pH (7.9) approximated 180 umol $N{\cdot}kg^{-1}{\cdot}h^{-1}$ at 10°C and 330 umol$N{\cdot}kg^{-1}{\cdot}h^{-1}$ at 15°C (Fig. 1). High pH exposure at 10°C led to a rapid 77 % decrease in J_{Amm} that persisted for the entire 4 week experiment. During this time period J_{Amm} in the control fish gradually declined to approximately 100 umol $N{\cdot}kg^{-1}{\cdot}h^{-1}$ (Fig. 1A). High pH exposure at 15°C led to an initial 40 % decrease in J_{Amm} during the first 3 h at pH 9.5 but by 1 day J_{Amm} had completely recovered and thereafter, fluctuated between 175 and 300 umol $N{\cdot}kg^{-1}{\cdot}h^{-1}$. The only exception was at 3 weeks, at which time J_{Amm} was significantly depressed to approximately 100 umol $N{\cdot}kg^{-1}{\cdot}h^{-1}$. Similarly, J_{Amm} in control fish fluctuated between 200 and 300 umol $N{\cdot}kg^{-1}{\cdot}h^{-1}$ (Fig. 1B).

Pre-exposure plasma T_{Amm} concentrations at 10°C (approximately 150 umol $N{\cdot}l^{-1}$) were about 30 % lower than at 15°C. Exposure to pH 9.5 at 10°C resulted in a chronic 40-80 % elevation of plasma T_{Amm} relative to the controls, and was significant at all times except 14 days (Fig. 2A). A similar trend at 15°C was characterized by an acute 3-fold increase in plasma T_{Amm} after 1 day of high pH exposure. This was followed by a return of plasma T_{Amm} towards control levels over the next 2 days but plasma T_{Amm} was still 70-100 % greater in the treatment fish, an effect which was significant on days 7, 14 and 28 (Fig. 2B).

These high pH-induced increases in internal T_{Amm} were more pronounced in the white muscle. The pre-exposure ammonia concentrations approximated 350 and 750 umol $N{\cdot}kg^{-1}$ wet weight, at 10°C and 15°C respectively. After 7 and 28 days at 10°C the treatment fish, held at pH 9.5, had white muscle T_{Amm} that were 2-fold greater than levels found in the fish held at pH 7.9 (Fig. 3A). White muscle ammonia concentrations were also chronically elevated, by 1.5 to 2.0-fold, at all sample times in the fish held at pH 9.5 and 15°C (Fig. 3B).

Initial urea excretion rates (J_{urea}), measured at pH 7.9, were about 16 umol $N{\cdot}kg^{-1}{\cdot}h^{-1}$ at 10°C and about twice as high under similar conditions at 15°C. Exposure to pH 9.5 at 10°C led to no significant change in J_{urea}, although there was a tendency for J_{urea} to decrease as the experiment progressed; by 4 weeks J_{urea} in both tanks was 50 % lower than the rates measured during the pre-exposure period (Table 1). At 15°C J_{urea} increased by 70-80 % during the first day of pH 9.5 exposure but by 3 days, excretion rates had returned to pre-exposure levels and approximated rates measured in the control fish held throughout at pH 7.9 (Table 1).

Plasma urea concentrations at 10°C approximated 3800 umol $N{\cdot}l^{-1}$ during the pre-exposure period and gradually declined thereafter. Plasma urea concentrations were significantly lower, by about 35 %, in the experimental fish after 2 and 4 weeks of pH 9.5 exposure at 10°C. At 15°C, pre-exposure plasma urea concentrations were about 3000 umol $N{\cdot}l^{-1}$. With only one exception, a significant elevation at 3 days, plasma urea concentrations did not differ between the control and treatment fish (Table 1).

Interestingly, pre-exposure white muscle urea concentrations (approximately 750 and 600 umol $N{\cdot}kg^{-1}$ wet weight at 10°C and 15°C respectively), at both temperatures, were about 80 % lower than the respective plasma urea concentrations (Table 1). Exposure to pH 9.5

at 10°C led to significant 75 % decreases in white muscle urea content after 7 and 28 days and 50 % decreases at 15°C, which were significant at 7, 14, and 21 days (Table 1).

Lactate, Glucose and Cortisol

At 10°C blood lactate approximated 450 umol·l^{-1} during the pre-exposure period and increased by about 30 % throughout exposure to pH 9.5, an effect significant at 3, 14, and 21 days. However, blood lactate never exceeded 640 umol·l^{-1} during high pH exposure at 10°C (Table 2). Qualitatively similar results were observed at 15°C, although the absolute blood lactate concentrations, under control conditions, were substantially greater (460-1200 umol·l^{-1}) at the warmer temperature. Similarly, the absolute lactate elevation was larger at 15°C, reaching levels as high as 1700 umol·l^{-1}. The fish held at pH 9.5 and 15°C for 3 days had blood lactate concentrations that were 170 % greater than controls. At 14 and 28 days blood lactate concentrations were 30 % and 75 % greater, respectively, than controls (Table 2).

White muscle lactate concentrations ranged from approximately 1400 to 2600 umol·l^{-1} at 10°C and 1500 to 3100 umol·kg^{-1} wet weight at 15°C. White muscle lactate concentrations did not appear to be affected by high pH exposure (Table 2).

At 10°C plasma cortisol fluctuated around 10 ng·ml^{-1} but concentrations were about 50 % lower at 15°C (Table 3). Plasma cortisol concentrations did not increase when the trout were exposed to pH 9.5 at either temperature. Baseline plasma glucose concentrations were about 2-fold higher at 10°C (approximately 8 mmol·l^{-1}) than at 15°C (approximately 4 mmol·l^{-1}) under pre-exposure conditions. Glucose tended to fall during exposure to pH 9.5 at 10°C, an effect significant on days 7 and 14, and to rise very slightly at 15°C, an effect which was significant only on day 7 (Table 3).

Plasma Protein, Hematocrit and Hemoglobin

Plasma protein, hematocrit, and hemoglobin levels were all about 33 % higher at 10°C than at 15°C (Table 4, Fig. 4A). High pH exposure had no apparent effects on plasma protein at either temperature. At 10°C, hemoglobin concentrations and hematocrit were unaffected by pH 9.5 exposure (Table 4; Fig. 4A). Predictably there were no differences in mean cell hemoglobin concentration (MCHC) during the experiment (Fig. 4C). However, the situation was markedly different at 15°C. At 15°C hemoglobin initially increased by 50 % after 3 days of pH 9.5 exposure but dropped slightly over the next few days. It remained significantly elevated in the high pH exposure group, by about 35 %, for the remainder of the experiment (Table 4). Fish held at pH 7.9 exhibited hematocrits that ranged from 26-32 %, but hematocrit was permanently elevated, by about the same relative proportion as hemoglobin, in the fish held at pH 9.5 (Fig. 4B). Despite these dramatic changes in hematocrit and hemoglobin concentration the MCHC of fish exposed to pH 9.5 at 15°C never significantly differed from that of the control fish (Fig. 4D).

Internal Na^+ and Cl^- Concentrations and Na^+ Fluxes

Exposure to pH 9.5 had surprisingly little effect on long-term maintenance of internal ion concentrations. Plasma Na^+ and Cl^- concentrations approximated 133 and 121 mmol·l^{-1}, respectively at 10°C and were about 5-10% lower than values measured at 15°C. Interestingly, at 10°C plasma Na^+ and Cl^- were significantly elevated by 5-12 % at 7, 14 and 21 days of exposure. At 15°C there were no high pH-induced changes in plasma Na^+ and Cl^- balance (Table 5).

White muscle ion balance, was similarly unaffected by high pH exposure. At 10°C white muscle Na^+ and Cl^- concentrations were about 7 to 10 mmol·kg^{-1} wet weight, while at

15°C, the values ranged from 11 to 15 mmol·kg^{-1} wet weight (Table 5). White muscle water percentage, approximately 81 %, was stable in fish held at both pH's (data not shown).

The maintenance of internal ion balance is interesting in view of the fact that Na^+ uptake rates, assessed only at 15°C, were chronically reduced, by 50-60%, during high pH exposure. Although these reductions were significant at 0-3 h, 1, 14, 21 and 28 days of pH 9.5 exposure (Fig. 5A,B) there were no significant changes in J^{Na}_{out} or J^{Na}_{net}, apart from elevated net losses at 28 days (Fig. 5A,B). In general, the control fish (pH 7.9) were in net positive Na^+ balance throughout the experiment, while the trout chronically exposed to high pH stayed at about zero balance.

Acid-base Balance

Internal acid-base status was only assessed during the 15°C experiment. White muscle pH_i was remarkably stable during chronic high pH exposure; at no time did pH_i, which fluctuated around pH = 7.3, differ significantly from that measured in control fish (Fig. 6).

In contrast to the white muscle, major changes in the animals' extracellular acid-base status were observed. The pre-exposure extracellular pH (pH_e) was approximately 7.78 (Fig. 6). The pH_e of the control fish increased thereafter, fluctuating between 7.84 and 7.94 for the remainder of the experiment. In contrast to the control fish, pH_e was consistently lower, by approximately 0.1 units, throughout the exposure at pH 9.5, a difference which was significant for the first 3 weeks (Fig. 6). Paradoxically, this relative acidosis developed despite the simultaneous presence of a marked respiratory alkalosis (decreased Pe_{CO2}) that was characterized by persistent 30-40 % reductions in plasma P_{CO2} (Fig. 7A). Plasma HCO_3^- concentrations during high pH exposure were 33-50 % lower than respective control concentrations (Fig. 7B). Subsequent estimates of the metabolic acid-load (ΔH^+_m), based on

mean differences in pH_e and extracellular HCO_3^- concentration between the control and high pH groups (see Methods), indicated a chronic ΔH^+_m of about 5 mmol·l^{-1} from 7 days onward (Fig. 7C). Changes in the extracellular fluid lactate concentration (ΔLac), at these times, accounted for no more than 10 % of this observed ΔH^+_m (Fig. 7C).

DISCUSSION

Survival

Despite numerous accounts of salmonid mortality at pH=9.0 or greater (Eicher 1946; Jordan and Lloyd 1964; Daye and Garside 1975; Murray and Zeibell 1984; Heming and Blumhagen 1988; Wilkie and Wood 1991; Yesaki and Iwama 1992), the present investigation clearly demonstrates that free-swimming, non-cannulated rainbow trout are capable of long-term survival (1 month) at pH 9.5. Exposure to pH 9.5 appeared to be less physiologically demanding in cooler 10°C water, than at 15°C. This interpretation is supported by the increased hematocrit, hemoglobin, lactate and higher internal ammonia levels observed at 15°C during high pH exposure. Although internal ammonia levels increased during high pH exposure at 10°C and 15°C, the resultant ECF P_{NH3} at both temperatures were well below those, approximately 350 utorr, known to contribute to high pH-induced or ammonia toxicity-induced mortality (Yesaki and Iwama 1992; Wilkie *et al.* 1993; R.W. Wilson and C.M. Wood, unpublished observations). High pH-induced decreases in plasma Na^+ and Cl^-, which have been implicated in mortality at alkaline pH (Yesaki and Iwama 1992; Wilkie *et al.* 1993), were not observed at either 10°C or 15°C. The maintenance of water Ca^{++}, above the hardwater-softwater threshold of 0.4 $mmol \cdot l^{-1}$ (Marier *et al.* 1979), during both exposure regimes, may have played a role in preventing potential internal ion disturbances in the present study. In fact, Yesaki and Iwama (1992) have demonstrated high water Ca^{++} is a key factor for trout survival at high pH.

Nitrogenous Waste Excretion and Storage

Lowered overall N-metabolism in the 10°C *vs.* 15°C environments, probably accounted for the lower pre-exposure J_{Amm} observed in the cooler waters (Maetz 1972; Brett and Groves 1979). At 15°C, the initial inhibition of J_{Amm}, upon exposure to pH 9.5, followed by a rapid recovery to pre-exposure rates is consistent with earlier studies performed on the rainbow trout (Wilkie and Wood 1991, 1994a,b). Previously, it has been argued that increased plasma T_{Amm}, associated with the initial blockade of branchial J_{Amm}, ultimately facilitated recovery of ammonia excretion at pH 9.5 through re-establishment of favourable blood-gill water P_{NH3} gradients (Wilkie and Wood 1991, 1994b; Wright *et al.* 1993; Wilkie *et al.* 1994). Putative Na^+/NH_4^+ exchange does not appear to be a factor in the recovery of J_{Amm} in high pH Hamilton tapwater (Wilkie and Wood 1994a). The present study similarly suggests that long-term maintenance of J_{Amm} is also dependant upon chronic elevations of plasma T_{Amm}. This adaptation is similar to that employed by the high pH tolerant Lahontan cutthroat trout of Pyramid Lake, Nevada (Wright *et al.* 1993; Wilkie *et al.* 1994). Although the Lahontan cutthroat trout and the present rainbow trout at 10°C, had chronically reduced J_{Amm}, the maintenance of higher internal T_{Amm} was probably necessary to maintain positive blood-gill water ammonia gradients to drive minimal J_{Amm} (Wright *et al.* 1993; Wilkie *et al.* 1994).

Retention of ammonia in the white muscle (WM) ICF compartment is associated with ECF-WM ICF ammonia loading, down the transiently elevated ΔNH_4^+ electrochemical (F_{NH4}) and ΔP_{NH3} gradients, from the ECF to WM ICF, that quickly develop following acute high pH exposure (Wilkie and Wood 1994b). Under steady state conditions, however, a favourable ECF-WM ICF F_{NH4} is thought to favour WM NH_4^+ loading but NH_3 movements are in the opposite direction down NH_3's ΔP_{NH3} gradient (Wright *et al.* 1988; Wright and Wood 1988) such that net ammonia movements in the two directions are in approximate balance. Estimates of the F_{NH4} and ΔP_{NH3} gradients in the present study, approximately -40

mV and 50 utorr, respectively, were identical during both control and high pH regimes. This suggest that the fish quickly reached a new steady state with respect to their internal ammonia distribution after 1 week of alkaline exposure. This observation also supports the supposition that the fish maintained elevated steady state ECF and WM T_{Amm} concentrations in order to maintain J_{Amm} during chronic alkaline exposure. Similarly, the very high levels of plasma and white muscle ammonia found in the Cyprinid, *Chalcaburnus tarichi*, of highly alkaline (pH 10.1), Lake Van, Turkey (Danulat and Kempe 1992), might be a key factor in this fish species ability to excrete ammonia into its harsh environment.

Although, similar arguments apply at 10°C, the chronic high pH-induced reduction of J_{Amm} in cooler water probably reflected lowered basal N-metabolism. However, the possibility that other N-waste products, such as glutamine or trimethylamine oxide (see Forster and Goldstein 1969 for review), are being excreted at high pH cannot be discounted. The fact that plasma and WM T_{Amm} concentrations were 50 % lower, than levels measured at 15°C, supports the former suggestion. Changes in the animals' substrate preferences, that resulted in lowered rates of amino acid oxidation and greater reliance upon glycogen and fatty acid oxidation might account for lowered internal ammonia production rates. For instance, enzymatic studies by Guderly and Gawlicka (1992) have suggested that rainbow trout in cold water increase their reliance on fatty acid metabolism for energy production. Experiments utilizing O_2, CO_2, and T_{Amm} respirometry might shed more light on these vastly different responses in N-metabolism to high pH at 10°C vs. 15°C. Interestingly, these reductions in J_{Amm} in the cooler water closely resemble those seen the high pH tolerant Lahontan cutthroat trout and suggest that reductions in ammonia production might facilitate trout survival in alkaline environments.

Chronic high pH exposure did not lead to long term increases in J_{urea} at either 10°C or 15°C; although J_{urea} was elevated during the first day or so of exposure at 15°C. This latter observation is consistent with previous acute high pH exposure experiments (Wilkie and Wood 1991; Wilkie *et al.* 1993a) and suggest that transiently elevated J_{urea} is a temporary nitrogenous waste detoxification response that ensures some N-waste excretion takes place until internal ammonia stores and excretion rates achieve new steady state levels. Similar observations have been reported for rainbow trout exposed to elevated ambient ammonia (Olson and Fromm 1971). At 10°C, however, the percentage contribution of J_{urea} to overall $J_{waste\text{-}N}$ ($J_{waste\text{-}N} = J_{Amm} + J_{urea}$) increased 2-3 fold from approximately 8 % under control conditions to 20-30 % at pH 9.5, an effect reminiscent of the response exhibited by the Lahontan cutthroat trout upon exposure to alkaline Pyramid Lake water (Wilkie *et al.* 1994). This strategy also resembles that employed by *C. tarichi* in its highly alkaline surroundings (Danulat and Kempe 1992).

Surprisingly, plasma urea was about 2-3 times greater than muscle urea when compartmental concentrations were expressed per litre of extracellular fluid and intracellular fluid, respectively (assuming a white muscle intracellular fluid volume of approximately 0.689 $l \cdot kg^{-1}$ wet muscle; Milligan and Wood 1986b). Most previous studies have indicated that urea is passively distributed across fish white muscle membranes with an ECF:ICF ratio of 1:1 (eg. Forster and Goldstein 1976; Wood *et al.* 1989). The present investigation implies either active ICF-ECF urea transport and/or limited white muscle membrane urea permeability. The high pH induced reductions in white muscle urea content, at both temperatures (Table 1), may reflect reduced urea production by the fish or, as previously suggested, conversion of N-waste to other end-products.

Hematological Responses

Glucose and cortisol concentrations were unchanged throughout the exposure to pH 9.5, approximating levels seen in unstressed and resting trout (Woodward and Strange 1987), which suggests the fish adapted readily to their highly alkaline surroundings. Chronic elevations in blood lactate, however, during most of the high pH exposure regime at both 10°C and 15°C, imply that the fish had greater energetic demands. Furthermore, the marked increases in hematocrit and hemoglobin, which are regarded as secondary stress indicators (Barton and Iwama 1991), during high pH exposure at 15°C but not at 10°C, implies that high pH exposure was more costly in the warmer water. These changes, without corresponding changes in MCHC, at 15°C suggest that high pH either inhibited O_2 uptake and/or increased the O_2 demands of the fish which necessitated splenic red blood cell (RBC) expulsion and/or increased RBC production. Previous experiments, indicating unaltered arterial P_{co2} and white muscle ATP and creatine phosphate levels in rainbow trout at high pH likely rule out an O_2 delivery problem (Wilkie and Wood, 1994b). However, greater internal concentrations of ammonia, leading to ammonia induced hyper-excitability (Smart 1978; Arillo *et al.* 1981) and possible activation of the glycolytic enzyme, phosphofructokinase (Kuhn *et al.* 1974), might explain the present observations in trout exposed to pH 9.5 at 15°C. Internal ammonia levels may not have been high enough to elicit similar responses at 10°C.

Ion and Acid-base Regulation

During chronic high pH exposure at 15°C, Na^+ uptake was chronically depressed but the similar plasma Na^+, as well as Cl^-, levels in the controls and experimental fish suggest that these non-cannulated trout were able to restore and maintain ion balance beyond 3 days of exposure to pH 9.5. These persistent reductions of J^{Na}_{in} seen at chronic high pH resemble

those seen during chronic acid exposure, but unlike the present situation, low pH induced reductions of plasma Na^+ and Cl^- are not readily corrected (Audet *et al.* 1988). This maintenance of internal ion balance at high pH is underscored by the relatively stable white muscle Na^+ and Cl^- concentrations observed at both 10°C and 15°C. By 3 d of exposure to pH 9.5, restoration of Cl^- balance has been achieved through re-establishment of J^{Cl}_{in}, while restoration of Na^+ balance has occurred not by recovery of J^{Na}_{in}, which remains depressed, but rather by a decrease in J^{Na}_{out} [M.P. Wilkie, P. Laurent, and C.M. Wood, unpublished data (Chapter 4)]. The present study demonstrates that this reduction of J^{Na}_{in} persists for at least 28 days, and may very well be permanent (Fig. 5B). The long-term regulation of Na^+ balance which allows maintenance of stable internal Na^+ concentrations (Tables 5 and 6), in the face of chronically reduced J^{Na}_{in}, must therefore be due to chronically decreased J^{Na}_{out}. Such a trend is evident in the data (Fig. 5B) but is not supported statistically by the comparisons against the simultaneous measurements of J^{Na}_{out} in the control fish. However, it must be remembered that J^{Na}_{out} is an indirect calculation from two direct measurements ($J^{Na}_{out} = J^{Na}_{net} - J^{Na}_{in}$) and therefore incorporates greater error. The branchial morphological and hormonal basis of long term adaptations in both Na^+ and Cl^- exchanges at high pH is an important area for future research.

Blood sampling by chronic cannulation is the technique of choice for measuring blood acid-base status in fish. However, in the present study we wished to avoid complicating effects of cannulation which appear to make trout more vulnerable to high pH-induced mortality (Wilkie and Wood, 1991). In any event, it would have been impractical to maintain free-swimming cannulated fish for 28 days. We therefore elected to sample fish by rapid MS-222 overdose and caudal puncture, which obtained mixed arterial-venous blood. Wood *et al.*

(1989), working on small rainbow trout (1 to 6 g), reported that blood pH was considerably underestimated (by up to 0.5 pH units) if sampling was via caudal puncture. However, the measurements of control extracellular pH reported here (pH_e = 7.78 to 7.94), on much larger fish (100 g), agree closely with arterial pH estimates obtained via dorsal aortic catheter in previous studies (pH_e = 7.83; Wilkie and Wood 1991), as do measurements of plasma total CO_2. The fact that blood sampling took place rapidly, following MS 222 overdose, was likely an important factor that ensured truly representative blood samples were obtained.

In agreement with earlier studies (eg. Wright and Wood 1985; Lin and Randall 1990; Wilkie and Wood 1991, 1994b; Wilkie *et al.* 1994) the rainbow trout chronically exposed to pH 9.5 at 15°C developed a persistent decrease in Pe_{co2} (ie. respiratory alkalosis; Fig. 7A). However, this alkalosis was offset by the development of a more severe metabolic acidosis that actually resulted in lowered pH_e (Figs. 6 and 7). Previous studies have indicated that metabolic acidosis in trout at high pH is associated with increased blood lactate (Wilkie and Wood 1991, 1994b). It is thought that this lactacidosis originates in the WM compartment, where 4-fold greater lactate concentrations have been measured, after 2 days of exposure to pH 9.5 (Wilkie and Wood 1994b). Curiously, in the present study WM lactate was not elevated in the fish exposed to high pH at 10°C or 15°C, despite persistent elevations of blood lactate (Table 7). Regardless, increases in blood lactate concentration only accounted for a small percentage of ΔH^+_m (Fig. 7C). Perhaps altered branchial Na^+ vs. Cl^- flux patterns, leading to net acidic equivalent retention, accounted for this acid-base overcompensation. In fact, the constant suppression of Na^+ uptake during high pH exposure suggests that there may have been some branchial mediated acidic equivalent retention; experiments designed to

evaluate long-term changes in net branchial [Cl^- - Na^+] fluxes (eg. McDonald *et al.*, 1989; Wood, 1989) would shed more light on this question.

Despite the persistent changes in extracellular acid-base status, white muscle pH_i was relatively stable throughout the chronic exposure to pH 9.5. Tang *et al.* (1992) recently demonstrated that white muscle intracellular P_{co2} (Pi_{co2}) is in passive equilibrium with extracellular P_{co2} (Pe_{co2}) in trout. Therefore, the present fish likely had chronically reduced WM Pi_{co2} that was presumably offset by a metabolic acid load. The mechanisms by which such a compensatory response would occur remain a mystery but it could be related to overall increases in basal metabolism during high pH exposure. Long-term maintenance of extracellular and white muscle intracellular ion and acid-base status was likely a key adaptation that permitted the fish in the present study to survive at pH 9.5 for 4 weeks. Furthermore, similar ion and long-term acid-base regulation is likely a key factor that enables the Lahontan cutthroat trout to function in its highly alkaline environment.

Table 1: Urea excretion rates (J_{urea}) and internal urea concentrations of rainbow trout during 28 days at pH 9.5 at 10°C or 15°C.

	Urea Excretion Rates (umol N·kg⁻¹·h⁻¹)		Plasma Urea (umol N·l⁻¹)		White Muscle Urea (umol N·kg⁻¹ wet weight)	
	pH 7.9	pH 9.5	pH 7.9	pH 9.5	pH 7.9	pH 9.5
10°C						
P	16.3 ± 1.8		3819 ± 250		764 ± 63	
3 d	20.4 ± 2.4	15.3 ± 2.1	3218 ± 312	2787 ± 324	———	———
7 d	16.4 ± 5.4	16.4 ± 1.2	3135 ± 190	2650 ± 131	940 ± 89	209 ± 36*
14 d	13.5 ± 0.2	12.1 ± 1.6	3069 ± 189	2087 ± 160*	———	———
21 d	9.0 ± 1.7	10.7 ± 1.5	2673 ± 250	2032 ± 205	———	———
28 d	8.2 ± 2.2	6.4 ± 1.4	3151 ± 485	1917 ± 107*	617 ± 93	223 ± 33*
15°C						
P	36.6 ± 5.3		2990 ± 217		590 ± 75	
0-3 h	———	58.6 ± 7.4†	———	———	———	———
1 d	———	61.7 ± 7.4†	———	———	———	———
3 d	34.0 ± 4.3	40.1 ± 4.1	2065 ± 156	3236 ± 360*	———	———
7 d	24.2 ± 6.6	33.4 ± 11.9	2307 ± 323	3302 ± 403	347 ± 31	232 ± 27*
14 d	38.2 ± 7.7	26.0 ± 10.2	2305 ± 191	2168 ± 348	427 ± 54	257 ± 16*
21 d	26.3 ± 9.0	41.0 ± 13.6	2675 ± 279	2387 ± 238	464 ± 50	216 ± 34*
28 d	47.1 ± 10.5	36.8 ± 11.1	2585 ± 326	2653 ± 548	484 ± 67	326 ± 18

* Significantly different from simultaneous pH 7.9 (control) values ($p < 0.05$).

† Significantly different from pre-exposure (P) values ($p < 0.05$); test done only at 0-3 h and 1 day as there were no simultaneous control values at these times. Means ± SEM; n=11-16 and 5-8 during the pre-exposure (P) and experimental periods, respectively.

Table 2: Changes in blood and white muscle lactate concentrations of rainbow trout during 28 days at pH 9.5 at 10°C or 15°C.

	Blood Lactate (umol·l⁻¹)		White Muscle Lactate (umol·kg⁻¹ wet weight)	
	pH 7.9	pH 9.5	pH 7.9	pH 9.5
10°C				
P	470 ± 20		2010 ± 266	
3 d	383 ± 45	570 ± 51*	———	———
7 d	498 ± 51	640 ± 84	2640 ± 680	1740 ± 280
14 d	441 ± 42	608 ± 52*	———	———
21 d	337± 35	423 ± 33*	———	———
28 d	439 ± 59	603 ± 84	2130 ± 540	1410 ± 130
15°C				
P	458 ± 40		2764 ± 768	
3 d	590 ± 130	1600 ± 470*	———	———
7 d	470 ± 60	1320 ± 180*	1530 ± 120	2260 ± 310
14 d	790 ± 80	1030 ± 90*	2160 ± 260	3140 ± 570
21 d	1200 ± 70	1090 ± 190	2920 ± 280	2250 ± 240
28 d	960 ± 170	1670 ± 290*	2800 ± 240	2390 ± 230

* Significantly different from pH 7.9 (control) values ($p < 0.05$). Means ± SEM; n=10-15 and 6-8 during the pre-exposure (P) and experimental periods, respectively.

Table 3: Changes in plasma cortisol and glucose concentrations of rainbow trout during 28 days at pH 9.5 at 10°C or 15°C.

	Plasma Cortisol (ng·ml⁻¹)		Plasma Glucose (mmol·l⁻¹)	
	pH 7.9	pH 9.5	pH 7.9	pH 9.5
10°C				
P	11.3 ± 0.8		7.9 ± 1.2	
3 d	9.0 ± 1.1	10.3 ± 2.5	7.3 ± 1.9	6.8 ± 0.6
7 d	11.0 ± 0.9	9.9 ± 1.3	8.8 ± 1.0	5.4 ± 0.6*
14 d	9.9 ± 1.2	6.9 ± 0.9	8.0 ± 1.4	5.1 ± 0.4*
21 d	8.0 ± 0.6	7.2 ± 1.3	6.3 ± 0.6	5.5 ± 0.3
28 d	8.7 ± 1.4	5.7 ± 0.7	6.6 ± 1.8	5.5 ± 0.6
15°C				
P	5.5 ± 0.8		3.9 ± 0.2	
3 d	9.6 ± 1.1	4.0 ± 1.3*	3.4 ± 0.1	5.0 ± 0.8
7 d	5.8 ± 2.0	4.6 ± 0.9	3.6 ± 0.3	4.8 ± 0.3*
14 d	2.4 ± 0.2	3.0 ± 0.5	3.3 ± 0.1	3.6 ± 0.2
21 d	3.4 ± 1.1	3.3 ± 0.5	3.7 ± 0.3	3.3 ± 0.6
28 d	3.5 ± 0.5	4.6 ± 1.2	4.3 ± 0.3	5.2 ± 0.5

* Significantly different from pH 7.9 (control) values ($p < 0.05$). Means ± SEM; n=13-15 and 6-8 during the pre-exposure (P) and experimental periods, respectively.

Table 4: Hematological indices in rainbow trout during 28 days at pH 9.5 at 10°C or 15°C.

	Hemoglobin Concentration (g·100 ml⁻¹)		Plasma Protein (mg·100ml⁻¹)	
	pH 7.9	pH 9.5	pH 7.9	pH 9.5
10°C				
P	9.0 ± 0.3		4.2 ± 0.1	
3 d	9.0 ± 0.6	8.9 ± 0.4	4.0 ± 0.2	4.2 ± 0.2
7 d	10.0 ± 0.3	9.2 ± 0.7	4.1 ± 0.2	4.0 ± 0.2
14 d	8.8 ± 0.3	9.0 ± 0.5	4.3 ± 0.2	3.9 ± 0.2
21 d	7.9 ± 0.4	8.9 ± 0.3*	3.8 ± 0.2	3.6 ± 0.1
28 d	7.2 ± 0.5	7.5 ± 0.3	3.5 ± 0.3	3.5 ±0.1
15°C				
P	6.9 ± 0.4		3.3 ± 0.1	
3 d	6.3 ± 0.3	9.6 ± 0.4*	2.5 ± 0.1	3.6 ± 0.3*
7 d	6.3 ± 0.5	8.7 ± 0.4*	2.6 ± 0.2	3.2 ± 0.2
14 d	6.0 ± 0.3	7.9 ± 0.4*	2.0 ± 0.2	2.4 ± 0.3
21 d	7.0 ± 0.4	9.3 ± 0.5*	2.2 ± 0.2	2.7 ± 0.2
28 d	6.6 ± 0.5	8.7 ± 0.4*	2.4 ± 0.3	2.4 ± 0.3

* Significantly different from pH 7.9 (control) values ($p < 0.05$). Means ± SEM; n=15-16 and 7-8 during the pre-exposure (P) and experimental periods, respectively.

Table 5: Changes in the plasma Na^+ and Cl^- concentrations of rainbow trout during 28 days at pH 9.5 at 10°C or 15°C.

	Plasma [Na^+] ($mmol \cdot l^{-1}$)		Plasma [Cl^-] ($mmol \cdot l^{-1}$)	
	pH 7.9	pH 9.5	pH 7.9	pH 9.5
10°C				
P	132.9 ± 1.6		121.4 ± 2.3	
3 d	136.0 ± 3.0	138.3 ± 2.1	124.0 ± 3.8	130.6 ± 1.9
7 d	129.1 ± 1.2	137.3 ± 0.8*	115.7 ± 1.3	131.3 ± 1.8*
14 d	127.5 ± 2.3	134.5 ± 0.9*	121.2 ± 3.0	132.2 ± 1.4*
21 d	133.9 ± 2.0	139.3 ± 0.7*	124.5 ± 2.4	132.1 ± 1.0*
28 d	136.1 ± 2.9	136.1 ± 0.9	125.2 ± 2.8	129.2 ± 2.3
15°C				
P	148.5 ± 1.0		130.3 ± 1.0	
3 d	142.0 ± 2.6	137.8 ± 4.1	129.2 ± 1.7	125.2 ± 6.2
7 d	145.7 ± 2.1	146.4 ± 4.2	130.3 ± 2.7	132.3 ± 2.3
14 d	141.8 ± 1.9	144.8 ± 2.0	130.1 ± 2.0	129.9 ± 1.9
21 d	137.8 ± 2.2	144.3 ± 3.3	125.4 ± 3.3	132.2 ± 1.5
28 d	141.0 ± 1.6	136.1 ± 4.8	128.7 ± 1.5	122.6 ± 4.5

* Significantly different from pH 7.9 (control) values ($p < 0.05$). Means ± SEM; n=12-14 and 5-8 during the pre-exposure (P) and experimental periods, respectively.

Table 6: Changes in the white muscle Na^+ and Cl^- concentrations of rainbow trout during 28 days at pH 9.5 at 10°C or 15°C.

	White Muscle [Na^+] ($mmol \cdot kg^{-1}$ wet tissue)		White muscle [Cl^-] ($mmol \cdot kg^{-1}$ wet tissue)	
	pH 7.9	pH 9.5	pH 7.9	pH 9.5
10°C				
P	7.9 ± 0.3		9.1 ± 0.4	
7 d	7.7 ± 0.7	8.4 ± 0.4	8.0 ± 0.5	9.4 ± 0.4
28 d	7.4 ± 0.5	8.9 ± 0.3*	8.6 ± 0.5	10.3 ± 0.5
15°C				
P	13.4 ± 1.0		14.8 ± 1.1	
7 d	11.4 ± 1.1	12.0 ± 0.3	11.5 ± 1.2	12.5 ± 0.6
14 d	12.1 ± 0.8	12.5 ± 1.0	13.6 ± 1.0	14.4 ± 0.9
21 d	12.3 ± 0.9	11.2 ± 0.7	14.1 ± 1.2	12.9 ± 0.9
28 d	13.4 ± 1.0	10.8 ± 0.7*	14.9 ± 1.1	11.5 ± 0.9

* Significantly different from pH 7.9 (control) values ($p < 0.05$). Means ± SEM; n=9-15 and 5-8 during the pre-exposure (P) and experimental periods, respectively.

Figure 1: Ammonia excretion rates (J_{Amm}) in rainbow trout held at pH=7.9 (broken line; triangles) or pH=9.5 (solid line; circles) for 4 weeks at either (A) 10°C or (B) 15°C. Means ± 1 SEM; n = 15-16 and n = 7-8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH=7.9 (control) values and the daggers specify significant differences between the rates at 0-3 h and 24 h of pH 9.5 exposure *vs.* pre-exposure (P) rates ($p < 0.05$).

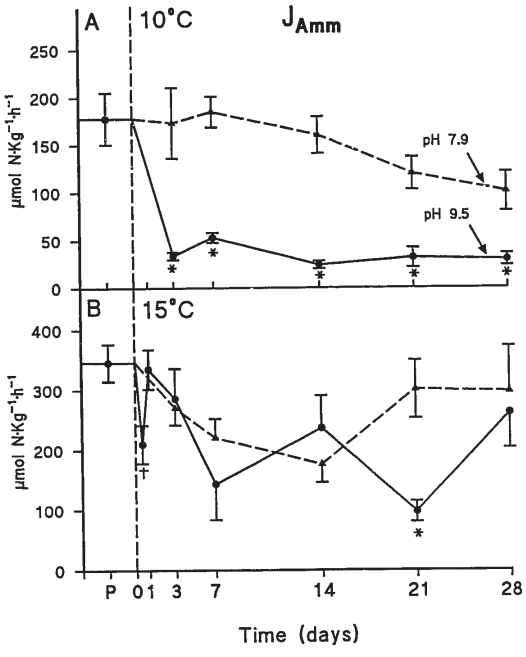


Figure 2: Extracellular (plasma) total ammonia concentrations (T_{Amm}) in rainbow trout held at pH=7.9 (broken line; triangles) or pH=9.5 (solid line; circles) for 4 weeks at either (A) 10°C or (B) 15°C. Means ± 1 SEM; n = 15 and n = 6-8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH=7.9 (control) values ($p < 0.05$).

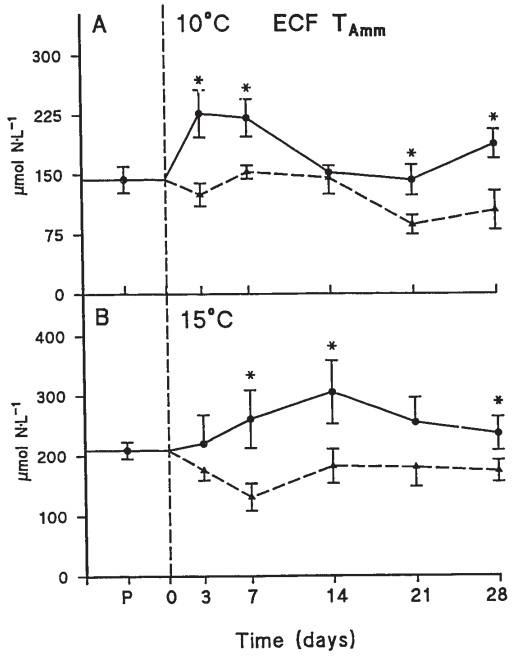
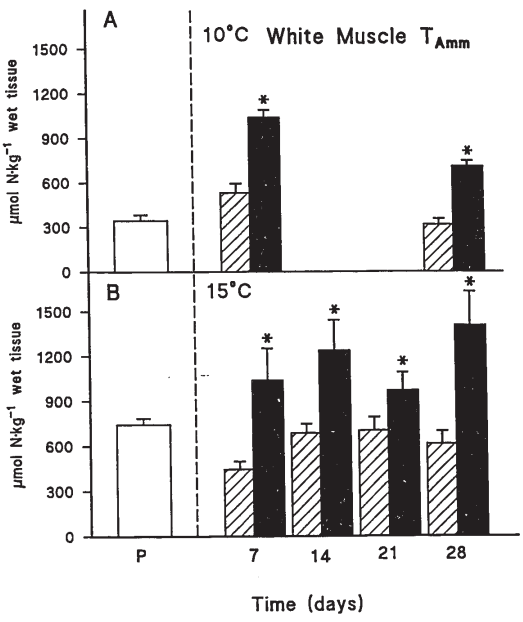


Figure 3: White muscle total ammonia concentrations (T_{Amm}) in rainbow trout held at pH=7.9 (open and hatched bars) or pH=9.5 (solid bars) for 4 weeks at either (A) 10°C or (B) 15°C. Means ± 1 SEM; n = 10-15 and n = 7-8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH=7.9 (control) values ($p < 0.05$).



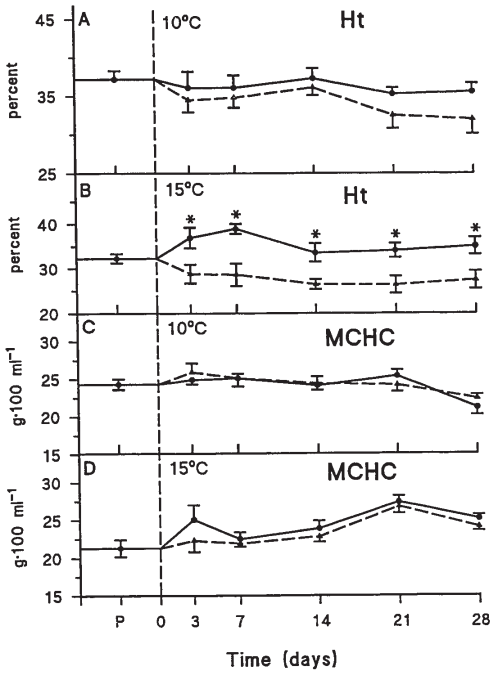


Figure 4: Changes in (A) blood hematocrit (Ht) at 10°C and (B) at 15 °C, and the (C) calculated mean cell hemoglobin concentration (MCHC) at 10°C and (D) at 15 °C of rainbow trout held at either pH=7.9 (broken line; triangles) or pH=9.5 (solid line; circles) for 4 weeks. Means ± 1 SEM; n = 15-16 and n = 7-8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH=7.9 (control) values ($p < 0.05$).

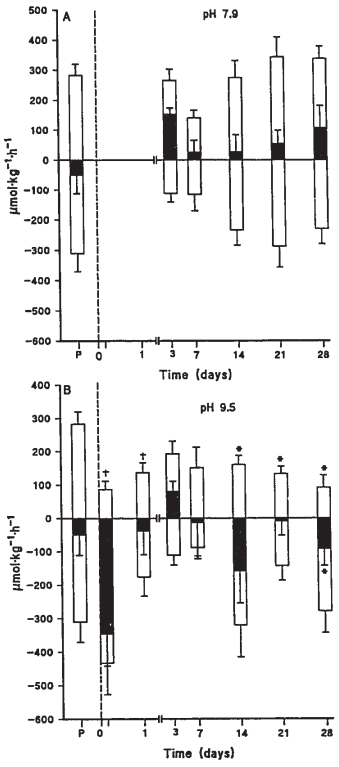


Figure 5: Sodium influx (J^{Na}_{in}; upward facing open bars), outflux (J^{Na}_{out}; downward facing open bars) and net flux (J^{Na}_{net}; solid bars) rates of rainbow trout held at (A) pH=7.9 or (B) pH=9.5 for 4 weeks at 15°C. Means ± 1 SEM; n = 14 and n = 7-8 during the pre-exposure (P) and experimental periods, respectively. *Asterisks indicate significant differences from simultaneous* pH=7.9 (control) values and the daggers specify significant differences between the rates at 0-3 h and 24 h of pH 9.5 exposure *vs.* pre-exposure rates ($p < 0.05$).

Figure 6: Extracellular (plasma) pH (pH_e) and white muscle intracellular pH (pH_i) measurements in rainbow trout held at pH=7.9 (broken line; triangles) or pH=9.5 (solid line; circles) for 4 weeks at 15°C. Means ± 1 SEM; n = 12-13 and n = 7-8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH=7.9 (control) values ($p < 0.05$).

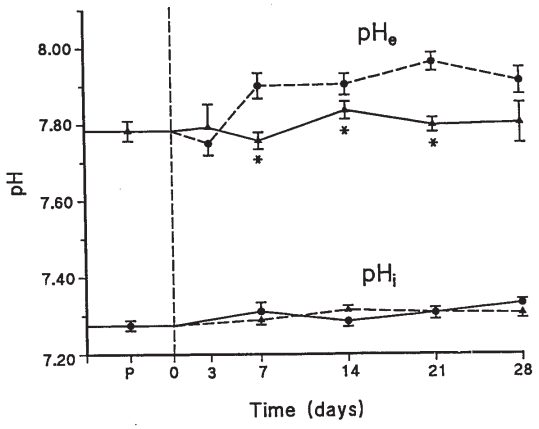
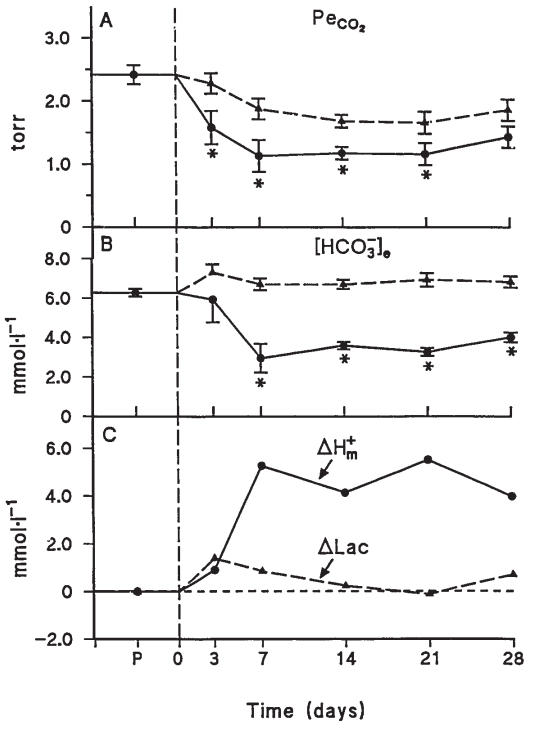


Figure 7: Differences in extracellular (plasma) (A) P_{CO2} (Pe_{CO2}); and (B) HCO_3^- ($[HCO_3^-]_e$); and (C) metabolic acid load (ΔH^+_m) and lactate load (ΔLac) of rainbow trout. Analyses for (A) and (B) were done on rainbow trout held at either pH=7.9 (broken line; triangles) or pH=9.5 (solid line; circles) for 4 weeks at 15°C. Calculations of ΔH^+_m in panel (C) were based on the respective mean $[HCO_3^-]_e$ and pH_e differences between the control vs. experimental fish; ΔLac was based on mean respective differences in whole blood lactate concentrations (see text for further details). Means ± 1 SEM; n = 12-13 and n = 7-8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH=7.9 (control) values ($p < 0.05$).



CHAPTER 8

A MODEL TO DESCRIBE SALMONID PHYSIOLOGY AT HIGH pH.

Exposure of salmonids to high pH leads to physiological disturbances and adaptations that involve the white muscle intracellular compartment (ICF), the blood and extracellular fluid (ECF), the gill and its unstirred boundary layers, and the water. Accordingly, a simplified integrative model (Fig. 1) is presented for rainbow trout that describes physiological processes in these regions under circumneutral pH (pH 8.0) and high pH (pH 9.5) conditions at 15°C. Under steady state conditions, at pH 8.0, CO_2 produced in the white muscle and other tissues, and carried in the plasma and red blood cells largely as HCO_3^-, is excreted down favourable transbranchial P_{CO2} gradients, to the gill's unstirred boundary layers (NB. also referred to as the unstirred gill water). Some of this CO_2 is subsequently hydrated in the boundary layers, via a carbonic anhydrase catalyzed reaction, to HCO_3^- and H^+ (Wright *et al.*, 1986), while the remainder diffuses directly into the bulk water (Fig. 1A). The H^+ produced via this hydration converts excreted NH_3 to NH_4^+ (diffusion trapping), and thereby maintains a steady-state P_{NH3} diffusion gradient that facilitates J_{Amm}. J_{Amm} may also take place via Na^+/NH_4^+ exchange, where NH_4^+ is thought to replace H^+ on branchial Na^+/H^+ antiporters (Fig. 1A). Internally, it appears that metabolically produced ammonia is distributed between the muscle and ECF according to the white muscle ICF-to-ECF NH_3 diffusion and NH_4^+ electrochemical gradients (F_{NH4}). Under steady state conditions NH_4^+

moves into the white muscle ICF down its inwardly directed F_{NH4}, while NH_3 moves in the opposite direction down favourable P_{NH3} gradients (Fig. 1A). Steady state Na^+ and Cl^- losses (outflux) are counterbalanced by continual influxes of Na^+ and Cl^- (J^{Na}_{in}, J^{Cl}_{in}) via branchial Na^+/H^+ and Cl^-/HCO_3^- exchange, respectively (Fig. 1A).

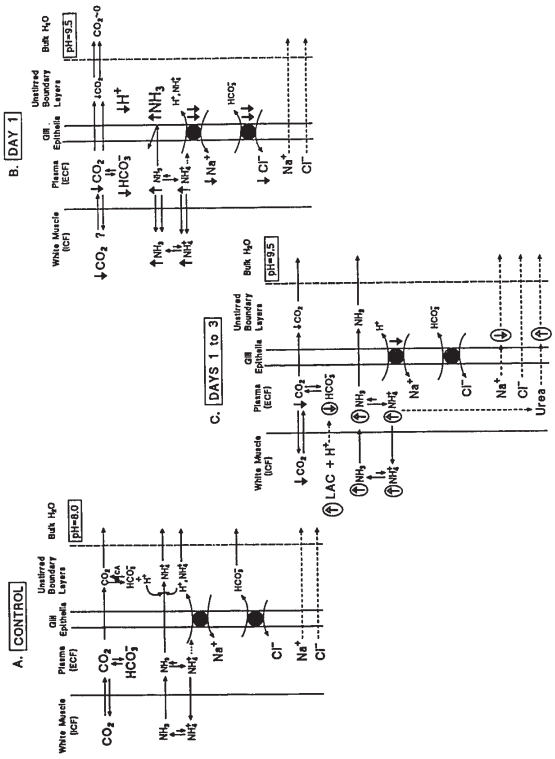
At high pH, the P_{CO2} diffusion gradient across the gill epithelia, facilitating CO_2 excretion, is significantly enhanced due to the virtual depletion of gaseous CO_2 in the bulk water, an effect which should be a least partially reflected in the boundary layer water as well. Accordingly, accelerated losses of CO_2 across the respiratory surfaces of the gill result in the development of a respiratory alkalosis, that is characterized by decreased plasma P_{CO2} and HCO_3^-, and increased extracellular pH (pH_e; approximates arterial pH) and white muscle intracellular pH (pH_i; Fig. 1B). Such increases in boundary layer pH, towards the pK'_{Amm} ($pK'_{Amm} \simeq 9.5$) elevate the NH_3 concentration in this region and reduce the transbranchial P_{NH3} gradient that drives J_{Amm}. Ultimately, this factor, and possibly inhibited Na^+/NH_4^+ exchange, lead to reduced J_{Amm} at high pH (Fig. 1B). The resultant ECF retention of NH_4^+ and NH_3 enhance the ECF-to-white muscle ICF F_{NH4} and reverse the ICF-to-ECF P_{NH3} gradient, respectively, and lead to a net uptake of ammonia by the white muscle (Fig. 1B). Decreased Na^+ and Cl^- uptake, due to high pH-induced reductions in Na^+/H^+ and Cl^-/HCO_3^- transporter number respectively, account for initial decreases in plasma Na^+ and Cl^- concentration (Fig. 1B).

After approximately 1-3 days at high pH rainbow trout return to a new steady state that is characterized by a stabilization of pH_e, at a new elevated set point, and re-establishment and maintenance of pH_i at pre-exposure levels. This stabilization appears to result from the development of a simultaneous metabolic acidosis that is partially attributable

to increased white muscle lactic acid production and subsequent extrusion of the metabolic H^+ to the ECF (Fig. 1C). Persistently elevated steady state ECF and white muscle ammonia concentration, characterized by a return of the white muscle ICF-to-ECF F_{NH4} and P_{NH3} gradients to pre-exposure status, leads to the re-establishment of transbranchial P_{NH3} gradients that facilitate J_{Amm}. Transient increases in J_{Urea} ensure that minimal N-waste excretion takes place while J_{Amm} is inhibited (Fig. 1C). Despite persistently reduced J^{Na}_{in}, internal Na^+ concentrations are maintained through counterbalancing decreases in transbranchial Na^+ outflux. Internal Cl^- concentrations are re-established through a recovery of Cl^-/HCO_3^- exchange capacity (Fig. 1C).

The Lahontan cutthroat trout encounters similar disturbances to acid-base balance and ammonia excretion following transfer to Pyramid Lake (pH 9.4). In addition to a chronic, uncompensated respiratory alkalosis, the fish also undergoes a transient metabolic alkalosis. This alkalosis is counteracted through increases in branchial chloride cell mediated HCO_3^- extrusion. Rather than increase its internal ammonia concentrations, to potentially toxic levels, this salmonid chronically depresses J_{Amm} through either decreases in N-metabolism and/or excretion of an alternate N-waste product. A similar high pH adaptive response is seen in the rainbow trout but only during alkaline exposure in cooler (10°C) water. The Lahontan cutthroat trout experiences no internal ion imbalances following transfer to Pyramid Lake. Thus, an ability to counteract high pH-induced disturbances to acid-base balance, ammonia excretion and internal ion concentration enable salmonids to survive in alkaline environments.

Fig. 1. A model to describe rainbow trout physiology under (A) CONTROL (pH 8.0) conditions, (B) during DAY 1 of pH 9.5 exposure, and (C) between DAY 1 and DAY 3 of pH 9.5 exposure. Open-headed arrows are indicative of high pH-induced changes in the fish's physiological status (B and C). Encircled, open-headed arrows specify physiological adaptations to highly alkaline water which generally occur between days 1 to 3 of exposure (C). See text for further details.



APPENDIX 1

The Effects of Extremely Alkaline Water (pH 9.5) on Rainbow Trout Gill Function and Morphology.

Journal of Fish Biology (1994) 45, 87-98

by

Michael P. Wilkie and Chris M. Wood

Department of Biology

McMaster University

1280 Main Street West

Hamilton, Ontario

L8S 4K1

Telephone: (905) 525-9140

FAX:(905) 522-6066

Running Title: High pH and its effects on trout gills.

Abstract

Rainbow trout that were held under control conditions, at pH 8.0, in moderately hard Hamilton tapwater, had Cl^- and Na^+ influx rates (J^{Cl}_{in} and J^{Na}_{in}, respectively) of 270 and 300 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, respectively. Exposure to pH 9.5 water led to an immediate 67 % decline in J^{Cl}_{in} and a 45% reduction in J^{Na}_{in} at 0-1h. Influx rates declined further and by 4-5 h the net decreases in both J^{Cl}_{in} and J^{Na}_{in} approximated 80%. By 24 h J^{Cl}_{in} had recovered to rates not significantly different from those at pH 8.0; while J^{Na}_{in} only partially recovered and remained about 50 % lower than control measurements through 72 h. The complete recovery of J^{Cl}_{in} and partial recovery of J^{Na}_{in} may have been related to a four-fold greater branchial chloride cell (CC) fractional surface area observed in rainbow trout exposed to pH 9.5 for 72 h. Ammonia excretion (J_{Amm}) was about 170 umol $N \cdot kg^{-1} \cdot h^{-1}$ at pH 8.0 but was initially reduced by 90 % over the first hour of high pH exposure. J_{Amm} rapidly recovered and by 24 h it had returned to pre-exposure levels. This recovery tended to parallel the partial recovery of J^{Na}_{in}. However, subsequent addition of amiloride (10^{-4} M) to the water at 75 h led to no change in J_{Amm}, despite a 50 % reduction in J^{Na}_{in}. Thus, it does not appear that there is a linkage between Na^+ influx and the recovery of ammonia excretion under highly alkaline conditions.

Key Words: Chloride Cells, Ammonia excretion, amiloride, High pH, Na^+ influx, Cl^- influx

Introduction

Numerous eutrophic lakes and ponds experience diurnal and seasonal rises in water pH as a result of high rates of algal photosynthesis (Wetzel, 1983; Hansen *et al.*, 1991). Until recently, little was known about the physiology of fish in alkaline environments, despite the fact that water pHs approaching 10 are common in eutrophic waters (eg. Jordan and Lloyd, 1964; Wetzel, 1983; Murray and Ziebell, 1984; Barica, 1974,1990). Disturbances in internal ion homeostasis, acid-base balance and nitrogenous waste (N-waste) excretion have been commonly observed in salmonids exposed to high pH (pH $\geq$ 9.0; Wright and Wood, 1985; Heming and Blumhagen, 1988; Wood, 1989; Lin and Randall, 1990; McGeer *et al.*, 1991; Wilkie and Wood, 1991; Yesaki and Iwama, 1992; Wilkie *et al.*, 1993; Wright *et al.*, 1993; Wilkie *et al.*, 1994). Wilkie and Wood (1991) suggested that the persistent decrease in plasma Na^+ and Cl^- seen in rainbow trout (*Oncorhynchus mykiss* Walbaum) exposed to pH 9.5 for 3 days was due to a decrease in ion influx and/or increased diffusive efflux of ions across the gill epithelium. Previously, in acute studies, Wright and Wood (1985) and Wood (1989) had shown reduced Na^+ and Cl^- influx in rainbow trout exposed to pH 9.5 for 3 h, whereas a number of longer term studies indicated that rainbow trout are capable of surviving at high pH for days or even weeks (Jordan and Lloyd 1964; Murray and Ziebell, 1984; Wilkie and Wood, 1991; Yesaki and Iwama, 1992). Such long-term survival at high pH would be dependent upon the animals' capability to regulate ion influx and/or diffusive ion losses so as to re-establish internal Na^+ and Cl^- balance. In the present investigation we employed radiotracers ($^{22}Na^+$ and $^{36}Cl^-$) to determine the quantitative changes in unidirectional Na^+ and Cl^- influx throughout the first 72 h exposure of rainbow trout to pH 9.5.

Branchial ammonia excretion is severely inhibited at high water pH (Wright and Wood, 1985; Yesaki and Iwama, 1992) but completely recovers after 24-48h in rainbow trout acutely exposed to pH 9.5 (Wilkie and Wood, 1991). It is often argued that NH_4^+ can substitute for H^+ on the branchial Na^+/H^+ exchanger and that a significant proportion of ammonia can be excreted via Na^+/NH_4^+ exchange on the branchial epithelium (eg. Maetz and Garcia Romeu, 1964; Maetz, 1972, 1973; Cameron and Heisler, 1983; Wright and Wood, 1985, McDonald and Milligan, 1988). Wilkie and Wood (1991) suggested that the recovery of ammonia excretion (J_{Amm}) by rainbow trout during high pH exposure might have been due to increased Na^+/NH_4^+ exchange. This hypothesis was tested in the present study by attempting to correlate changes in J_{Amm} with those observed for branchial Na^+ influx. The Na^+ transport inhibitor, amiloride, was then used to block Na^+ influx to establish if Na^+ influx was related to J_{Amm} after adaptation to high pH.

The importance of branchial chloride cells (CC) for ionoregulation in freshwater teleosts is now well established (Laurent *et al.*, 1985; Avella *et al.*, 1987; Perry and Laurent, 1989; Laurent and Perry, 1990; Perry *et al.*, 1992) but until recently, little was known about the potential role that the CC played in regulating acid-base homeostasis. Recent work by Goss and colleagues (1992*a*,*b*) suggests that changes in the exposed fractional surface area of branchial chloride cells (CC) leads to differential influx rates for Cl^- and Na^+. To investigate the potential role that branchial CCs played during exposure to high pH, we utilized scanning electron microscopy to measure the individual surface areas, densities and exposed fractional surface areas of branchial CCs in rainbow trout that were held either at pH 8.0 or pH 9.5 for 3 days.

Materials and Methods

Experimental Animals and Set-Up

Adult rainbow trout (*Oncorhynchus mykiss* Walbaum; mean weight = 278 g) were obtained from Spring Valley Trout Farm, Petersburg, Ontario and maintained in moderately hard, dechlorinated Hamilton city tapwater (composition: Ca^{++} = 1.0 mmol.l^{-1}; Mg^{++} = 0.3 mmol.l^{-1}; K^+ = 0.05 mmol.l^{-1}; Na^+ = 0.6 mmol.l^{-1}; Cl^- = 0.8 mmol.l^{-1}; titration alkalinity = 2.1 mmol·l^{-1}; hardness = 140 mg·l^{-1} as $CaCO_3$; pH ≃ 8.0). The fish were acclimated to the experimental temperature of 13-16°C for one week prior to experimentation, during which time they were starved to remove the effects that feeding might have on nitrogen metabolism (Fromm, 1963). Two days prior to sampling the fish were transferred to one of seven, darkened, 5 l, plexiglass flux boxes (see McDonald, 1983 for details) contained in a 200 l recirculating system. Water flow to each box was approximately 1.0 l·min^{-1} when the boxes were opened to the re-circulating system. When the boxes were operated as closed systems during flux determinations (see below), internal aeration maintained water P_{O2} above 130 torr and also provided vigorous mixing for flux measurements.

The incoming, dechlorinated Hamilton tapwater served as the control media (mean pH = 8.06 ± 0.04). The mean experimental pH of 9.54 ± 0.05 was maintained via a pH-stat set-up. Water pH was continuously monitored with a Radiometer GK2401C combination pH electrode connected to a PHM 82 pH meter; and a TTT 80 autotitrator (Radiometer). The latter controlled a solenoid valve which regulated the drop-wise addition of 1 N KOH into the well-mixed head tank. Water then flowed from the head tank (at pH 8.0 or pH 9.5) into the flux boxes, which drained to a lower reservoir and subsequently pumped back up to the head tank. Continuous additions of KOH were necessary because water replacement into the

system (approximately 3 l·min^{-1}) and CO_2 production by the fish titrated water pH downward (see Wilkie and Wood, 1991 for further details). Titration with KOH led to water K^+ concentrations that approximated 0.7 mmol·l^{-1}.

Experimental Protocol and Analytical Techniques

Part I: Cl^- and Na^+ Influx

The rates of unidirectional Cl^- and Na^+ influx (J^{Cl}_{in} and J^{Na}_{in}, respectively) and ammonia excretion (J_{Amm}) were determined at pH 8.0 and at 0-1 h, 4-5 h, 8-9h, 24-25 h, 48-49 h, 72-73 h and at 75-76 h of pH 9.5 exposure. Ten minutes prior to the start of each flux determination water flow to each box was cut off and 4 μCi·ml^{-1} of ^{22}Na (New England Nuclear) and 10 μCi·ml^{-1} of ^{36}Cl (ICN Biomedicals Inc., Canada) was added to each box to permit sufficient mixing of the isotope. For the 75 h sample period, 10^{-4} M of amiloride (Na^+ salt; Sigma) was added, along with the isotope, 10 minutes prior to the start of the flux period, to determine if potential decreases in J^{Na}_{in} were associated with changes in J_{Amm} (cf. Wright and Wood, 1985). At time 0 and 60 minutes, 50 ml water samples were taken for later determination of total gamma and beta counts, "cold" Cl^- and Na^+ concentrations and total ammonia concentrations. Due to the short duration of each flux period, we were unable to obtain adequate resolution of changes in cold Na^+ and Cl^- concentrations in the water and thus, were unable to estimate net ion fluxes. These short flux periods were necessary to minimize potentially interfering changes in water chemistry caused by the fish's metabolism (eg. decreases in water pH, increases in water ammonia) during the period of box closure .

^{36}Cl is a pure beta emitter, while ^{22}Na emits both beta and gamma rays. Accordingly, each sample was analyzed for total activity (on a 1217 Rackbeta scintillation counter) and then for gamma activity alone (ie. ^{22}Na; on a Packard 5000 series gamma counter). The counts

(beta only) attributable to ^{36}Cl were determined by subtracting ^{22}Na counts from the total counts, after correcting for the different counting efficiencies of ^{22}Na on the two machines. Total water Cl^- was determined via coulometric titration (Radiometer CMT10 chloridometer) and Na^+ via atomic absorption (Varian 1275 AA). Water ammonia was determined using a micro-modification of the salicylate-hypochlorite assay described by Verdouw *et al.* (1978). J^{Cl}_{in} and J^{Na}_{in} were calculated using the following formula:

$$J^{Cl}_{in} \text{ or } J^{Na}_{in} = \frac{(CPM_i - CPM_f)}{MSA} \cdot \frac{Volume}{W \cdot T} \quad (1)$$

Where: CPM_i and CPM_f are the respective beta counts (CPM·ml^{-1}) at the start and end of each flux determination period; MSA is the mean specific activity of either $^{36}Cl^-$ or $^{22}Na^+$ relative to the respective cold concentrations of each ion in the water (CPM·$umol^{-1}$); Volume refers to the volume of water in the flux box (≃ 3.0 l); W and T are the weight (kg) of each animal and the duration of each flux period (h), respectively. Back-flux correction was not necessary as internal specific activity never exceeded 10% of external specific activity for either Na^+ or Cl^- (see Wood, 1988 for details).

J_{Amm} was calculated using the following formula (also see Wright and Wood, 1985):

$$J_{Amm} = \frac{([Amm]_i - [Amm]_f) \cdot Volume}{W \cdot T} \quad (2)$$

Where: $[Amm]_i$ and $[Amm]_f$ are the respective concentrations of total water ammonia at the start and end of each flux period; Volume, W and T have the same meanings as previously stated.

Part II: Chloride Cell Morphometry and Morphology

The analysis was based on the methods of Laurent and Perry (1990) and Goss *et al.* (1992*a*). Briefly, separate batches of rainbow trout were held in flux boxes (described above) and exposed to pH 8.0 (controls) or pH 9.5 water for 72 h. At this time the fish were sacrificed, one at a time, with an overdose of MS 222 anaesthetic (Sigma; 1.5 $g.l^{-1}$). The second left gill arch was excised and the gill filaments trimmed from the arch in small pieces, rinsed in ice cold 0.15 $mol \cdot l^{-1}$ Na^+ cacodylate buffer (BDH), then fixed in 5% glutaraldehyde for approximately 70 to 80 minutes. After fixation, pairs of filaments, still joined at the septum, were dissected away from one another, washed 3 times with buffer, and then stored at 4°C for several hours. The paired filaments were partially dehydrated via an ethanol series (35 %, 50 %, 70 %) and then stored in 70 % ethanol for several days. The gills were subsequently dehydrated in 95 % and absolute ethanol and then taken through two successive baths (2 minutes each) of 1,1,1,3,3,3-hexamethyldisilazane (Aldrich) and air dried. The paired filaments were then mounted onto aluminum stubs, sputtered coated with gold and subsequently viewed on a ISI-DS130 dual stage scanning electron microscope at 2000 times magnification. The region of analysis was located on the trailing edge of the filament and next to the septum. Approximately, six non-contiguous fields (approximately 2500 μm^2 per field) were photographed per filament. A total of 18 fields per fish were subsequently analyzed. Individual CC surface area was determined with a MOP-3 digitizing system (Carl Zeiss, Inc.). Chloride cell fractional surface area (CC FSA) and CC density were subsequently determined from the individual CC surface areas and total filamental surface area examined. The filamental epithelium, rather than the lamellar epithelium, was used for determination of CC surface area because the former is more appropriate for morphometry.

The paired filaments can be mounted parallel to the face of the aluminum stub. As a result, the flat and relatively uniform surface topography of the filamental epithelium is readily accessible for viewing via the scanning electron microscope. The undulating nature of the gill lamellae also makes the lamellar surface less suitable for viewing and the morphometrical measurements more vulnerable to error (see Goss *et al.*, 1992*a*).

Statistics

All data have been expressed as means $\pm$ 1 SEM (N) where N is the number of animals sampled. The statistical significance of differences with respect to Cl^-, Na^+, and ammonia fluxes was determined via a paired t-test ($P < 0.05$) with the Bonferroni correction procedure for multiple comparisons. The significance of differences in gill morphometrical data were determined via an unpaired t-test ($P < 0.05$), after first performing an F-test to check for homogeneity of variance.

Results

Cl^- and Na^+ Influx and Ammonia Excretion

Rainbow trout, held at control pH (pH 8.0), had Cl^- and Na^+ influx rates of 270 $umol \cdot kg^{-1} \cdot h^{-1}$ and 300 $umol \cdot kg^{-1} \cdot h^{-1}$, respectively (Figs. 1 and 2). Chloride influx was dramatically reduced by 67% over the first hour at high pH (pH 9.5). By 4 h, the inhibition of J^{Cl}_{in} reached 83% to 45 $umol \cdot kg^{-1} \cdot h^{-1}$. However, by 8 h Cl^- influx rates started to increase again, and by 24 h had returned to a level not significantly different form pre-exposure values. At this time, J^{Cl}_{in} was approximately 210 $umol \cdot kg^{-1} \cdot h^{-1}$ and by 72 h it had recovered further, to approximately 260 $umol \cdot kg^{-1} \cdot h^{-1}$ (Fig. 1).

Na^+ influx, on the other hand, was reduced by 45 % during the first hour of high pH exposure and declined further so that by 4 hours it was 80 % lower than control measurements. Despite a slight recovery of Na^+ influx over the next few hours, J^{Na}_{in} never fully recovered and fluctuated around 150 $umol \cdot kg^{-1} \cdot h^{-1}$ for the remainder of the 72 h exposure (Fig. 2).

Analysis of terminal plasma samples revealed that plasma Cl^- and Na^+ concentrations approximated 116 $mmol \cdot l^{-1}$ and 138.9 $mmol \cdot l^{-1}$, respectively. Neither value was significantly different from concentrations measured in fish that were held at pH 8.0 (data not shown).

Ammonia excretion was about 180 $\mu molN \cdot kg^{-1} \cdot h^{-1}$ at control pH and decreased by 90 % during the first hour of high pH exposure but gradually recovered; by 24 h J_{Amm} had returned to 150 umol $N \cdot kg^{-1} \cdot h^{-1}$, a value not significantly different from the pre-exposure rate (Fig. 3). To establish if there was a relationship between J^{Na}_{in} and J_{Amm}, the Na^+ influx blocker amiloride, was added to the water at 75 h. Despite a 50 % reduction in J^{Na}_{in}, there was no effect of amiloride on J_{Amm} (compare Figs. 2 and 3).

Chloride Cell Morphometry

Examination of the branchial filamental epithelium of rainbow trout held at pH 8.0 *vs.* pH 9.5 for 72 h suggested that the differential alterations in Na^+ and Cl^- influx might be explained by high pH induced increases in branchial CC fractional surface area. Those exposed to pH 9.5 for 72 h had 4-fold greater CC fractional surface areas of about 280 000 $\mu m^2 \cdot mm^{-2}$ (Fig. 4A). This higher CC FSA was due to the 2-fold greater CC surface area and 2-fold greater CC density in the fish held at high pH (Figs. 4B, 4C). The individual CC surface area in these fish approached 50 μm^2 and CC density was approximately 6000 $CC \cdot mm^{-2}$.

The greater CC FSA in the pH 9.5 fish is readily apparent in the representative scanning electron micrographs that are presented in Figs. 5A and 5B. Note the larger surface area and increased numbers of individual CCs in the fish that were held at pH 9.5. Furthermore, the CCs of the treatment fish were often aggregated, whereas CCs in the control fish were more or less solitary. The surface morphology of visible filamental CCs varied widely in fish at high pH. For instance, there were qualitative differences in the appearance and density of apical microvilli both amongst and within individual fish; frequently CCs that appeared stunted or worn, were in close association with CCs that possessed numerous, prominent apical microvilli (Figs. 5A, 5B).

Discussion

Previous studies have reported persistent, sometimes lethal, reductions in plasma Cl^- and Na^+ concentrations in rainbow trout exposed to alkaline water (pH $\geq$ 9.5; Heming and Blumhagen, 1988; Wilkie and Wood,1991; Yesaki and Iwama, 1992). The present study suggests that some of these reductions might have been caused by an initial inhibition of branchial Cl^- and Na^+ influx. However, the ability of rainbow trout to survive at pH 9.5 for up to 5 weeks (Wilkie and Wood, 1991; Wilkie *et al.*, 1995), suggests that the fish are able to offset these initial ionoregulatory disturbances. It appears that the fish in the present study were effectively regulating their internal ionic status because plasma Cl^- and Na^+ levels were normal. Furthermore, these levels approximated to values that were previously reported for rainbow trout that survived at pH 9.5 for 72 h (Wilkie and Wood, 1991). The present study suggests that some correction of internal Cl^- and Na^+ balance might have resulted from complete re-establishment of J^{Cl}_{in} and partial recovery of J^{Na}_{in}. Future studies should focus on how these two parameters are modulated during long term high pH exposure and also establish if there are significant reductions in the efflux components of Cl^- and Na^+.

The 4-fold increases in CC FSA, observed at pH 9.5 in the present study, are probably indicative of an adaptive response of salmonids to highly alkaline environments. Elevations in CC surface area and/or number have previously been observed in Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*) acutely exposed to, or living in highly alkaline Pyramid Lake, Nevada (pH = 9.4; Galat *et al*, 1985; Wilkie *et al.*, 1994). Potentially, the increased CC FSA, observed in the present study, might have accounted for the complete recovery of J^{Cl}_{in} and the partial recovery of J^{Na}_{in} observed at high pH. Studies on fish in ion poor water indicate that both Na^+ and Cl^- influx are positively correlated with increases in

branchial CC FSA (Laurent *et al.*, 1985; Avella *et al.*, 1987; Perry and Laurent, 1989). However, when acid-base disturbances are experimentally induced, the correlation between J^{Na}_{in} and CC FSA breaks down while that between J^{Cl}_{in} and CC FSA is accentuated (Goss *et al.*, 1992*a,b*). Indeed, Goss and colleagues have suggested that only J^{Cl}_{in} is directly mediated via the CC's, whereas J^{Na}_{in} may occur through the pavement ("respiratory") cells of the branchial epithelium.

Based on our earlier work (Wilkie and Wood, 1991) it is highly likely that the rainbow trout exposed to pH 9.5 in the present study were suffering from a respiratory alkalosis (see also Wright and Wood, 1985; Lin and Randall, 1990; Yesaki and Iwama, 1992). It is therefore possible that increased CC FSA was also related to the maintenance of acid-base homeostasis. Goss and colleagues (1992*a,b*) have suggested that increased CC FSA, induced in rainbow trout and bullheads (*Ictalurus nebulosus*) via metabolic alkalosis, leads to a stimulation of Cl^- influx and a relative decrease in Na^+ influx which results in a net excretion of basic equivalents (= retention of acidic equivalents). The differential recovery of Cl^- and Na^+ influx in the present study (Figs. 1 and 2) may be explained by the fact that increased CC FSA would favour Cl^- influx over Na^+ influx, and therefore a net excretion of basic equivalents according to the theory of Goss and colleagues (1992*a,b*). Indeed, the blood acid-base measurements of Wilkie and Wood (1991) indicated a compensatory loss of basic equivalents from the blood stream by the third day of exposure to pH 9.5. The possibility that changes in CC FSA differentially affects Cl^- and Na^+ uptake, to offset alkalosis in trout at high pH, is an intriguing possibility that deserves further study.

High pH exposure induces elevations in internal NH_4^+ (eg. Wright and Wood, 1985; Lin and Randall, 1990; Wilkie and Wood, 1991; Yesaki and Iwama, 1992) and accordingly,

several researchers have proposed that NH_4^+ can replace H^+ as the internal counterion for the Na^+/H^+ exchanger under these conditions (Wilkie and Wood, 1991; Yesaki and Iwama, 1992). In the present study, the observation that both Na^+ influx and ammonia excretion (J_{Amm}) declined and recovered (though J^{Na}_{in} only partially) over a similar time frame might suggest a functional linkage between these two fluxes (compare Figs. 2 and 3). However, Na^+/NH_4^+ exchange seems unlikely at high pH because addition of the Na^+ uptake blocker amiloride to the water at 75 hours had no effect on J_{Amm}, despite a 50 % reduction in J^{Na}_{in} (Compare figs. 2 and 3). These observations are in contrast with those of Yesaki and Iwama (1992) who reported that addition of amiloride to pH 10 water inhibited J_{Amm} by rainbow trout. Unfortunately, rates of J^{Na}_{in} were not determined by Yesaki and Iwama. At least in alkaline Hamilton tapwater, it does not appear that NH_4^+ is a counterion for Na^+.

It is likely that simultaneous increases in blood pH and ammonia led to a steady rise in the blood P_{NH3} of the rainbow trout exposed to high pH (eg. Wilkie and Wood, 1991; Yesaki and Iwama, 1992; Wright *et al.*, 1993). Potentially, this may have increased the blood to water P_{NH3} diffusion gradient and accounted for the recovery of J_{Amm} that was observed in this study and those previously mentioned. Future studies should address this possibility in more detail.

In summary, the differential recovery of Cl^- influx, relative to Na^+ influx, observed following exposure of rainbow trout to pH 9.5, may have been related to greater branchial CC FSA. Furthermore, the present study suggests that NH_4^+ is not a counterion for Na^+ under alkaline conditions. Accordingly, future investigations should attempt to deduce the mechanisms behind high pH-induced alterations in ion influx patterns and also establish how such changes affect overall ionic balance, ammonia excretion, and acid-base regulation, in

rainbow trout exposed to alkaline pH. Determining the role that branchial chloride cells play in mediating ion influx at high pH may also provide clues that describe how some salmonids are able to tolerate alkaline environments indefinitely (Galat *et al.*, 1985; Wilkie *et al.*, 1994).

Acknowledgements

We thank Drs. G. Goss and J. Lott, and Mr. K. Schultes, for instruction and guidance. D. Flannigan, T. Yount and H. Simmons provided excellent technical help. This work was supported by an NSERC operating grant to CMW and an Ontario Graduate Scholarship to MPW.

Fig. 1. The influence of severe alkaline exposure (pH = 9.5) upon branchial Cl^- influx (J^{Cl}_{in}) in rainbow trout. Means ± 1 SEM; n = 7. Asterisks indicate statistically significant differences from influx rates measured at pH 8.0 ($P < 0.05$).

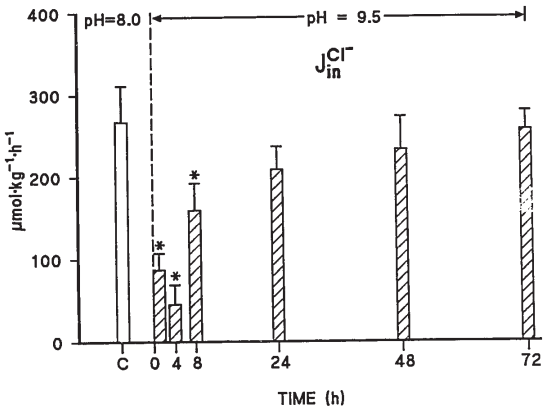


Fig. 2. The influence of severe alkaline exposure (pH = 9.5), and subsequent amiloride treatment at 75 h (solid bar), upon branchial Na^+ influx (J^{Na}_{in}) in rainbow trout. Means ± 1 SEM; n = 7. Asterisks indicate statistically significant differences from rates measured at pH 8.0 ($P<0.05$). Dagger indicates that the changes in J^{Na}_{in} following amiloride treatment, were significantly different from J^{Na}_{in} at 72 h ($P < 0.05$).

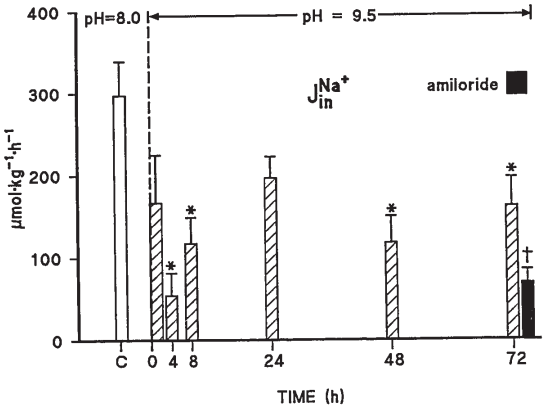


Fig. 3. The influence of severe alkaline exposure (pH = 9.5), and subsequent amiloride treatment at 75 h (solid bar), upon the rates of ammonia excretion (J_{Amm}) by rainbow trout. Means ± 1 SEM; n = 7. Asterisks indicate statistically significant differences from J_{Amm} at pH 8.0 ($P < 0.05$).

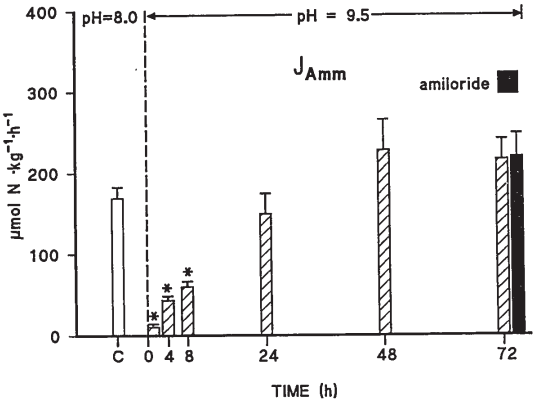
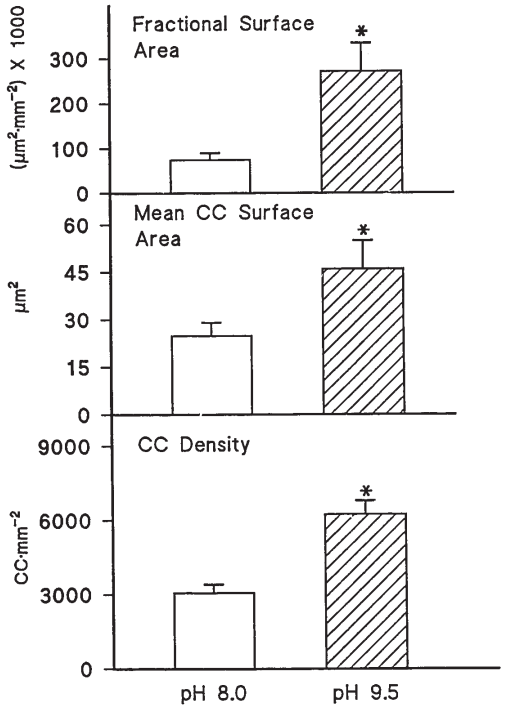


Fig. 4. Differences in branchial (A) chloride cell fractional surface area (CC FSA); (B) mean individual chloride cell surface area; and (C) chloride cell density of rainbow trout exposed to pH 8.0 or pH 9.5 dechlorinated Hamilton tapwater. Means ± 1 SEM; n = 4 at pH 8.0 and n = 5 at pH 9.5. Asterisks indicate significant differences from pH 8.0 values ($P < 0.05$).



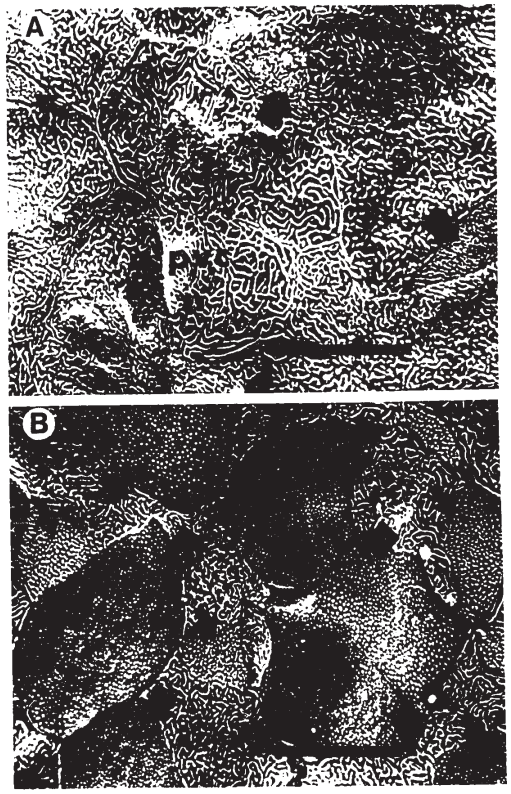


Fig. 5. Representative scanning electron micrographs of the filamental epithelium of rainbow trout exposed to pH 8.0 (A) or pH 9.5 (B) dechlorinated Hamilton tapwater. Note the greater density and individual surface areas of CCs (indicated by arrows) in the pH 9.5 trout. Also, note the differences in ornamentation of the CCs and the tendency of CCs to form aggregations in the pH 9.5 fish. pvc = Pavement cell; Bar = 10 micrometers. Magnification: 2650 X (A), 2850 X (B).

LITERATURE CITED

Alabaster, J.S., and R. Lloyd. 1980. Extreme pH value. In Water Quality criteria for freshwater fish. 2nd ed. Butterworth Scientific. London.

Alexander, J.B. and G.A. Ingram. 1980. A comparison of five of the methods commonly used to measure protein concentrations in fish sera. Journal of Fish Biology 16:115-122.

Arillo, A., C. Margiocco, F. Melodia. P. Mensi, and G. Schenone. 1981. Ammonia toxicity mechanism in fish: studies on rainbow trout (*Salmo gairdneri* Rich.). Ecotoxicology and Environmental Safety 5:316-328.

Audet, C.E., R.S. Munger, and C.M. Wood. 1988. Long term sublethal acid exposure in rainbow trout (*Salmo gairdneri*) in softwater: effects on ion exchanges and blood chemistry. Canadian Journal of Fisheries and Aquatic Sciences 45:1387-1398.

Avella, M., A. Masoni, M. Bornancin, and N. Mayer-Gostan. 1987. Gill morphology and sodium influx in the rainbow trout (*Salmo gairdneri*) acclimated to artificial freshwater environments. Journal of Experimental Zoology 241:159-169.

Avella, M. and M. Bornancin. 1989. A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). Journal of Experimental Biology 142:155-175.

Avnimelnech Y. 1980. Calcium carbonate-phosphate surface complex in calcerous systems. Nature 288:255-257.

Ayles, B.G., J.G.I. Lark, J. Barica, and H. Kling. 1976. Seasonal mortality of Rainbow trout (*Salmo gairdneri*) planted in small eutrophic prairie lakes of central Canada. Journal of the Fisheries Research Board of Canada 33:647-655.

Barica, J. 1974. Extreme fluctuations in water quality of eutrophic fish kill lakes: effect of sediment mixing. Water Research 8:881-888.

Barica, J. 1990. Ammonia and nitrite contamination of Hamilton Harbour, Lake Ontario. Water Pollution Research Journal of Canada 25:359-386.

Barton, B.A. and G.K. Iwama. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annual Review of Fish Diseases 1:3-26.

Bean, E. J., and D. E. Atkinson. 1984. Regulation of the rate of urea synthesis in liver by extracellular pH: a major factor in pH homeostasis in mammals. Journal of Biological Chemistry 259:1552-1559.

Bergmeyer, H.L. Methods of Enzymatic Analysis. 1983. Academic Press, New York.

Bock, A.V., D.B. Dill, and H.T. Edwards. 1932. Lactic acid in the blood of resting man. Journal of Clinical Investigation. 11:775-788.

Boutilier, R.C., T.A. Heming, and G.K. Iwama. 1984. Physicochemical parameters for use in fish respiratory physiology. Pages 401-430 in W.S. Hoar and D.J. Randall, eds. Fish Physiology. Volume 10A. Academic Press, New York.

Brett, J.R. 1979. Environmental factors and growth. Pages 599-675 in W.S. Hoar, D.J. Randall, and J.R. Brett, eds. Fish Physiology. Volume 8. Academic Press, New York.

Brett, J.R. and C.A. Zala. 1975. Daily pattern of nitrogen excretion and oxygen consumption and of sockeye salmon (*Oncorhynchus kisutch*) under controlled conditions. Journal of the Fisheries Research Board of Canada 32:2479-2486.

Brett, J.R. and T.D.D. Groves. 1979. Physiological energetics. Pages 279-352 in W.S. Hoar, J.R. Brett and D.J. Randall (eds). Fish Physiology, Volume VIII. Academic Press, New York.

Brown, G.W. Jr., J. James, R.J. Henderson, W.N. Thomas, R.O. Robinson, A.L. Thompson, E. Brown, and S.G. Brown. 1966. Uricolytic enzymes in liver of the Dipnoan *Protopterus aethiopicus*. Science 153:1653-1654.

Burrows, R.E. 1964. Effects of accumulated excretory products on hatchery reared salmonids. U.S. Fish. Wild. Serv. Bur. Sport Fish. Wild. Res. Rep. 66:1-12.

Cameron, J.N. 1976. Branchial ion uptake in arctic grayling: resting values and effects of acid-base disturbance. Journal of Experimental Biology 64:711-725.

Cameron, J.N. 1986. Responses to reversed NH_3 and NH_4^+ gradients in a teleost (*Ictalurus punctatus*), an elasmobranch (*Raja erinacea*), and a crustacean (*Callinectes sapidus*): evidence for NH_4^+/H^+ exchange in the teleost and the elasmobranch. Journal of Experimental Zoology 239:183-195.

Cameron, J.N. and N. Heisler. 1983. Studies of ammonia in the trout: physico-chemical parameters, acid-base behaviour and respiratory clearance. Journal of Experimental Biology 105:107-125.

Cameron, J.N., and G.K. Iwama. 1987. Compensation of progressive hypercapnia in channel catfish and blue crabs. Journal of Experimental Biology 133:183-197.

Cerveri, D. 1990. Pyramid Lake: Legends and Reality, 2nd ed. Nostalgia Press, Elko, Nevada.

Chiu, Y. N., R. E. Austic, and G. L. Rumsey. 1986. Urea cycle activity and arginine formation in rainbow trout (*Salmo gairdneri*). Journal of Nutrition 116:1640-1650.

Claiborne, J. B., and D. H. Evans. 1988. Ammonia and acid-base balance during high ammonia exposure in a marine teleost (*Myoxocephalus octodecimspinosus*). Journal of Experimental Biology 140:89-105.

Cleveland Rubber Company. 1984. Handbook of Chemistry and Physics, 65th edition. R.C. Weast editor. Cleveland Rubber Company Press Inc., Boca Raton, Florida.

Coleman, M.E., and V.K. Johnson. 1988. Summary of Management at Pyramid Lake, Nevada, with emphasis on Lahontan cutthroat trout, 1954-1987. American Fisheries Society Symposium 4:107-115.

Crocker, C. L. 1967. Rapid determination of urea nitrogen in serum or plasma without deproteinization. American Journal of Medical Technology 33:361-365.

Cvancara, V.A. 1969. Comparative study of liver uricase activity in freshwater teleosts. Comparative Biochemistry and Physiology 28:725-732.

Danulat, E., and S. Kempe. 1992. Nitrogenous waste excretion and accumulation of urea and ammonia in *Chalcalburnus tarichi* (Cyprinidae), endemic to the extremely alkaline Lake Van (Eastern Turkey). Fish Physiology and Biochemistry 9:377-386.

Danulat, E., and B. Selkuk. 1992. Life history and environmental conditions of the anadromous *Chalcalburnus tarichi* (Cyprinidae) in the highly alkaline Lake Van, Eastern Anatolia, Turkey. Archive fur Hydrobiologie.

Daye, P.G. and E.T. Garside. 1975. Lethal levels of pH for brook trout, *Salvelinus fontinalis* (Mitchill). Canadian Journal of Zoology 53:639-641.

Daye, P.G. and E.T. Garside. 1976. Histopathologic changes in surficial tissues of brook trout, *Salvelinus fontinalis* (Mitchill), exposed to acute and chronic levels of pH. Canadian Journal of Zoology 54:2140-2155.

De Loecker, W.C.J. 1964. The influence of ammonium ions on glycolysis in red blood cells. Clinica Chimica Acta 10:157-164.

Driedzic, W.R., and P.W. Hochachka. 1978. Metabolism in fish during exercise. Pages 503-543 in W.S. Hoar and D.J. Randall, eds. Fish Physiology Vol. VII. Academic Press. New York.

Eddy, F. B. 1971. Blood gas relationships in the rainbow trout *Salmo gairdneri*. Journal of Experimental Biology 55:695-711.

Eddy, F. B., O. S. Bamford, and G. M. O. Maloiy. 1981. Na^+ and Cl^- effluxes and ionic regulation in *Tilapia grahami*, a fish living in conditions of extreme alkalinity. Journal of Experimental Biology 91:349-353.

Eichenholz, A., R.O. Mulhausen, W.E. Anderson, and F.M. MacDonald. 1962. Primary hypocapnia: a cause of metabolic acidosis. Journal of Applied Physiology 17:283-288.

Eicher, G.J. 1946. Lethal alkalinity for trout in waters of low salt content. Journal of Wildlife Management 10:82-85.

Emerson, K., R.C. Russo, R.E. Lund, and R.V. Thurston. 1975. Aqueous ammonia calculations: Effect of pH and temperature. Journal of the Fisheries Research Board of Canada 32:2379-2383.

Erichsen Jones, J.R. (ed.). 1964. Acids and alkalis: pH tolerance limits. Pages 107-117 in Fish and River Pollution. Butterworths, London.

Evans, D.H., and K.J. More. 1988. Modes of ammonia transport across the gill epithelium of the dogfish pup (*Squalus acanthias*). Journal of Experimental Biology 138:375.

Evans, D. H., and J. N. Cameron. 1986. Gill ammonia transport. Journal of Experimental Zoology 239:17-23.

Forster, R.P., and L. Goldstein. 1969. Formation of excretory products. Pages 313-350 in W.S. Hoar and D.J. Randall, eds. Fish Physiology. Volume I. Academic Press, New York.

Forster, R.P. and L. Goldstein. 1976. Intracellular and osmoregulatory role of amino acids and urea in marine elasmobranchs. American Journal of Physiology 230:925-931.

Fromm, P.O. 1963. Studies on renal and extra-renal excretion in a freshwater teleost, *Salmo gairdneri*. Comparative Biochemistry and Physiology. 10:121-128.

Galat, D.L., E.L. Lider, S. Vigg, and S.R. Robertson. 1981. Limnology of a large deep North American terminal lake, Pyramid Lake, Nevada, U.S.A. Hydrobiologia 82:281-317.

Galat, D.L., G. Post, T.J. Keefe, and G.R. Boucks. 1983. Histological Changes in the gill, kidney, and liver of Lahontan cutthroat trout (*Salmo clarki henshawi*) living in lakes of different salinity-alkalinity. Technical report submitted to the United States Bureau of Indian Affairs, Phoenix, Arizona.

Galat, D.L., G. Post, T.J. Keefe, and G.R. Boucks. 1985. Histological changes in the gill, kidney and liver of Lahontan cutthroat trout, *Salmo clarki henshawi*, living in lakes of different salinity-alkalinity. Journal of Fish Biology 27:533-552.

Garcia, A.C., Y.L. Lai, B.A. Atterby, and E.B. Brown Jr. 1971. Lactate and pyruvate accumulation during hypocapnia. Respiration Physiology 12:371-380.

Goldstein, L., and R.P. Forster. 1965. The role of uricolysis in the production of urea by fishes and other aquatic vertebrates. Comparative Biochemistry and Physiology 14:567-576.

Goldstein, L., J.B. Claiborne, and D.H. Evans. 1982. Ammonia excretion by the gills of two marine teleost fish: The importance of NH_4^+ permeance. Journal of Experimental Zoology 219:395-397.

Goss, G.G. and C.M. Wood. 1990a. Na^+ and Cl^- uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout I. Responses to environmental hyperoxia. Journal of Experimental Biology 152:521-547.

Goss, G.G. and C.M. Wood. 1990b. Na^+ and Cl^- uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout II. Responses to bicarbonate infusion. Journal of Experimental Biology 152:549-571.

Goss, G.G. and C.M. Wood. 1991. Two-substrate kinetic analysis: a novel approach linking ion and acid-base transport at the gills of freshwater trout, *Oncorhynchus mykiss*. Journal of Comparative Physiology B 161:635-646.

Goss, G.G., P. Laurent, and S.F. Perry. 1992a. Evidence for a morphological component in acid-base regulation during environmental hypercapnia in the brown bullhead (*Ictalurus nebulosus*). Cell Tissue Research 268:539-552.

Goss, G.G., S.F. Perry, C.M. Wood, and P. Laurent. 1992b. Mechanisms of ion and acid-base regulation at the gills of freshwater fish. The Journal of Experimental Zoology 263:143-159.

Goss, G.G., C.M. Wood, P. Laurent, and S.F. Perry. 1994. Morphological responses of the rainbow trout to (*Oncorhynchus mykiss*) gill to hyperoxia, base ($NaHCO_3$) and acid (HCl) infusions. Fish Physiology and Biochemistry. In Press.

Guderly, H. and A. Gawlicka. 1992. Qualitative modification of muscle metabolic organization with thermal acclimation of rainbow trout, *Oncorhynchus mykiss*. Fish Physiology and Biochemistry 10:123-132.

Hagiwara, S. and K. Takahashi. 1967. Resting and spike potentials of skeletal muscle fibres of salt-water elasmobranchs and teleost fish. Journal of Physiology 190, 499-518.

Hansen, A.M., J.V. Christensen, and O. Sortkjer. 1991. Effect of high pH on zooplankton and nutrients in fish-free enclosures. Archiv fur Hydrobiologie 123:143-164.

Heinz, F., and Wieber, H. 1985. Creatine phosphate. Pages 507-514 in H.U. Bergmeyer ed. Methods of Enzymatic Analysis Volume VIII. Metabolites 3. Lipids, Amino Acids and Related Compounds, 3rd ed. VCH Publishers, Deerfield Beach, Florida.

Heisler, N. 1984. Acid-base regulation in fishes. Pages 315-401 in W.S. Hoar and D.J. Randall Fish Physiology Volume 10A. Academic Press, New York.

Heisler, N. 1990. Mechanisms of ammonia elimination in fishes. Pages 137-151 in J.P. Truchot and B. Lahlou eds. Animal Nutrition and Transport Processes. 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects. Comparative Physiology. Basel, Karger.

Heming, T.A., and K.A. Blumhagen. 1988. Plasma acid-base and electrolyte status of rainbow trout exposed to alum (aluminum sulphate) in acidic and alkaline environments. Aquatic Toxicology 12:125-140.

Hidaka, T., and N. Toida. 1969. Biophysical and mechanical properties of red and white muscle fibres in fish. Journal of Physiology 201:49-59.

Hillaby, B. A., and D. J. Randall. 1979. Acute ammonia toxicity and ammonia excretion in rainbow trout (*Salmo gairdneri*). Journal of the Fisheries Research Board of Canada 36:621-629.

Holmes, E.W. 1978. Regulation of purine synthesis de novo. Pages 21-41 in W.N. Kelley and I.M. Weiner eds. Handbook of Experimental Pharmacology Volume 51: Uric Acid. Springer Verlag, New York.

Huggins, A. K., G. Skutsch, and E. Baldwin. 1969. Ornithine-urea cycle enzymes in teleostean fish. Comparative Biochemistry and Physiology 28:587-602.

Johansen, K., G.M.O. Maloiy, and G. Lykkeboe. 1975 A fish in extreme alkalinity. Respiration Physiology 24:163-171.

Jordan, D.H.M., and R. Lloyd. 1964. The resistance of rainbow trout (*Salmo gairdneri* Richardson) and roach (*Rutilus* (L.)) to alkaline solutions. International Journal of Air and Water Pollution 8:405-409.

Kerstetter, T.H., and L.B. Kirschner. 1972. Active chloride transport by the gills of rainbow trout (*Salmo gairdneri*). Journal of Experimental Biology 56:263-272.

Kerstetter, T.H., L.B. Kirschner, and D. Rafuse. 1970. On the mechanisms of ion transport by the irrigated gills of rainbow trout (*Salmo gairdneri*). Journal of General Physiology 56:342-359.

Kirschner, L.B., L. Greenwald, and T.H. Kerstetter. 1973. Effect of amiloride on sodium transport across body surfaces of freshwater animals. American Journal of Physiology 224:832-837.

Kirschner, L.B. 1988. Basis for apparent saturation kinetics of Na^+ influx in freshwater hyperregulators. American Journal of Physiology 254:R984-R988.

Kucera, P.A., D.L. Koch, and G.F. Marco. 1985. Introductions of Lahontan cutthroat trout in Omak Lake, Washington. North American Journal of Fisheries Management 5:296-301.

Kuhn B., G. Jacobasch, C. Gerth, and S. M. Rapoport. 1974. Kinetic properties of the phosphofructokinase from erythrocytes of rats and rabbits. European Journal of Biochemistry 43:437-442.

Kun, E. and E.B. Kearney. 1971. Ammonia. Pages 1802-1806 in H.U. Bergmeyer ed. Methods of Enzymatic Analysis, Volume 4. Academic Press, New York.

Lauren, D.J., and D.G. McDonald. 1987. Acclimation to copper by rainbow trout, *Salmo gairdneri*: physiology. Canadian Journal of Fisheries and Aquatic Sciences 43:1488-1496.

Laurent, P. and S. Dunel. 1980. Morphology of gill epithelia in fish. American Journal of Physiology 238:R147-R159.

Laurent, P., H. Hobe, and S. Dunel-Erb. 1985. The role of environmental sodium chloride relative to calcium in gill morphology of freshwater salmonid fish. Cell Tissue Research 240:675-692.

Laurent, P., and N. Hebibi. 1989. Gill morphometry and fish osmoregulation. Canadian Journal of Zoology 67:3055-3063.

Laurent, P., and S.F. Perry. 1990. Effects of on gill chloride cell morphology and ionic uptake in freshwater trout, *Salmo gairdneri*. Cell Tissue Research 259:429-442.

Laurent, P., and S.F. Perry. 1991. Environmental effects on fish gill morphology. Physiological Zoology 64:4-25.

Levi, G., G. Morisi, A. Coletti, and R. Catanzaro. 1974. Free amino acids in fish brain: Normal levels and changes upon exposure to high ammonia concentrations *in vivo*, and upon incubation of brain slices. Comparative Biochemistry and Physiology 49A:623-636.

Lin, H.L., and D.J. Randall. 1990. The effect of varying water pH on the acidification of expired water in rainbow trout. Journal of Experimental Biology 149:149-160.

Lin, H., and D.J. Randall. 1993. H^+-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. Journal of Experimental Biology 180:163-174.

McDonald, D. G. 1983a. The effects of H^+ upon the gills of freshwater fish. Canadian Journal of Zoology 61: 691-703.

McDonald, D. G. 1983b. The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri*. I. Branchial and renal net ion and H^+ fluxes. Journal of Experimental Biology 102:123-140.

McDonald, D. G., H. Hobe, and C. M. Wood. 1980. The influence of calcium on the physiological responses of the rainbow trout *Salmo gairdneri*, to low environmental pH. Journal of Experimental Biology 88:109-131.

McDonald, D.G. and C.M. Wood. 1981. Branchial and renal net ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH. Journal of Experimental Biology 93:101-118.

McDonald, D.G., R.L. Walker, and P.R.H. Wilkes. 1983. The interaction of low environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri*. II. Branchial ionoregulatory mechanisms. Journal of Experimental Biology 102:141-155.

McDonald, D.G. and M.S. Rogano. 1986. Ion regulation by the rainbow trout, *Salmo gairdneri*, in ion-poor water. Physiological Zoology 59:318-331.

McDonald, D.G. and C.L. Milligan. 1988. Sodium transport in the brook trout, *Salvelinus fontinalis*: effects of prolonged low pH exposure in the presence and absence of aluminum. Canadian Journal of Fisheries and Aquatic Sciences 45:1606-1613.

McDonald, D.G. and E.T. Prior. 1988. Branchial mechanisms of ion and acid-base regulation in the freshwater rainbow trout, *Salmo gairdneri*. Canadian Journal of Zoology 66:2699-2708.

McDonald, D.G., Y. Tang, and R.G. Boutilier. 1989. Acid and ion transfer across the gills of fish: mechanisms and regulation. Canadian Journal of Zoology 67:3046-3054.

McDonald, D.G., and C.L. Milligan. 1992. Chemical properties of the blood in Fish. Pages 55-133 in W.S. Hoar and D.J. Randall, eds. Fish Physiology. Volume XII. Academic Press, New York.

McGeer, J.C., L. Baranyi, and G.K. Iwama. 1991. Physiological responses to challenge test in six stocks of coho salmon (*Oncorhynchus kisutch*). Canadian Journal of Fisheries and Aquatic Sciences 48:1761-1771.

McWilliams, P.G. 1980a. Effects of sodium uptake in Norwegian brown trout (*Salmo trutta*) from an acid river. Journal of Experimental Biology 88:259-267.

McWilliams, P.G. 1980b. Acclimation to an acid medium in the brown trout *Salmo trutta*. Journal of Experimental Biology 88:269-280.

McWilliams, P.G. 1982. The effects of calcium on sodium fluxes in the brown trout, *Salmo trutta*, in neutral and acid media. Journal of Experimental Biology. 96:439-442.

Maetz, J. 1972. Branchial sodium exchange and ammonia excretion in the goldfish, *Carassius auratus*. Effects of ammonia loading and temperature changes. Journal of Experimental Biology 56:601-620.

Maetz, J. 1973. Na^+/NH_4^+, Na^+/H^+ exchanges and NH_3 movement across the gill of *Carassius auratus*. Journal of Experimental Biology 58:255-275.

Maetz, J., and F. Garcia Romeu. 1964. The mechanism of sodium and chloride uptake by the gills of a fresh-water fish, *Carassius auratus* II. Evidence for NH_4^+/Na^+ and HCO_3^-/Cl^- exchanges. Journal of General Physiology 47:1209-1227.

Maetz, J. and G. De Renzis. 1978. Aspects of the adaptation of fish to high external alkalinity: comparison of *Tilapia grahami* and *T. mossambica*. Pages 213-228 in K. Schmidt Neilsen, L. Bolis, and S.H.P. Maddrell (eds.). Comparative Physiology: Water, Ions and Fluid Mechanics. Cambridge University Press. Cambridge, U.K.

Marier, J.H., L.C. Neri, and T.W. Anderson. 1979. Water hardness, human health and the importance of magnesium. National Research Council of Canada, Environmental Secretariat, Publication number NRCC 1758:1-119.

Marshall, W.S. 1985. Paracellular ion transport in trout opercular epithelium models osmoregulatory effects of acid precipitation. Canadian Journal of Zoology. 63:1816-1822.

Michal, G. 1983. Determination of Michaelis constants and inhibitor constants. Pages 86-104 in H.U. Bergemeyer (ed.) Methods of Enzymatic Analysis Volume 1. Verlag Chemie, Weinham, Germany.

Milligan, C.L. and C.M. Wood. 1982. Disturbances in haematology, fluid volume distribution and circulatory function associated with low environmental pH in the rainbow trout, *Salmo gairdneri*. Journal of Experimental Biology 99:397-415.

Milligan, C.L., and C.M. Wood. 1986a. Tissue intracellular acid-base status and the fate of lactate after exhaustive exercise in the rainbow trout. Journal of Experimental Biology 123:123-144.

Milligan, C.L., and C.M. Wood. 1986b. Intracellular and extracellular acid-base status and H^+ exchange with the environment after exhaustive exercise in the rainbow trout. Journal of Experimental Biology 123:93-121.

Mommsen, T.P., C.J. French, and P.W. Hochachka. 1983. Sites and patterns of protein and amino acid utilization during the spawning migration of salmon. Canadian Journal of Zoology 58:1785-1799.

Mommsen, T. P., and P. W. Hochachka. 1988. The purine nucleotide cycle as two temporally separated metabolic units: A study on trout muscle. Metabolism 37:552-556.

Mommsen, T. P., and P. J. Walsh. 1989. Evolution of urea synthesis in vertebrates: the piscine connection. Science 243:72-75.

Mommsen, T.P., and P.J. Walsh. 1991. Urea synthesis in fishes: evolutionary and biochemical perspectives. Pages 137-163 in P. Hochachka and T.P. Mommsen, eds. The Biochemistry and Molecular Biology of Fishes. Elsevier Science, Publications.

Mommsen, T.P., and P.J. Walsh. 1992. Biochemical and environmental perspectives on nitrogen metabolism in fishes. Experientia 48:583-593.

Moyes, C.D., T.P. Moon, and J.S. Ballantyne. 1986. Oxidation of amino acids, Krebs cycle intermediates, fatty acids and ketone bodies by *Raja erincacea* liver mitochondria. Journal of Experimental Zoology 237:119-128.

Murphy, T.P., E.E. Prepas, J.T. Lim., J.M. Crosby, and D.T. Walty. 1990. Evaluation of calcium carbonate and calcium hydroxide treatments of prairie drinking water dugouts. Lake and Reservoir Management 6:101-108.

Murray, C.A. and C.D. Ziebell. 1984. Acclimation of rainbow trout to high pH to prevent stocking mortality in summer. Progressive Fish Culturalist 46:176-179.

Olson, K.R., and P.O. Fromm. 1971. Excretion of urea by two teleosts exposed to different concentrations of ambient ammonia. Comparative Biochemistry and Physiology 40A:999-1007.

Olson, K.R. 1992. Blood and extracellular fluid volume regulation: role of the renin angiotensin system and atrial natriuretic peptides. In W.S. Hoar and D.J. Randall, eds. Fish Physiology. Volume XII. Academic Press, New York.

Packer, R.K. and W.A. Dunson. 1970. Effects of low environmental pH on blood pH and sodium balance of brook trout. Journal of Experimental Zoology 174:65-71.

Packer, R.K. and W.A. Dunson. 1972. Anoxia and sodium loss associated with the death of brook trout at low pH. Comparative Biochemistry and Physiology A. 41: 17-26.

Payan, P. 1978. A study of the Na^+/NH_4^+ exchange across the gill of the perfused head of the trout (*Salmo gairdneri*). Journal of Comparative Physiology B 124:181-188.

Pequin, L. and A. Serfaty. 1968. La regulation hepatique et intestinale del'ammonieemie chez la carpe. Arch. Sci. Physiol. 22:449-559.

Perry, S.F., M.S. Haswell, D.J. Randall and A.P. Farrell. 1981. Branchial ionic uptake and acid-base regulation in the rainbow trout, *Salmo gairdneri*. Journal of Experimental Biology 92:209-303.

Perry, S.F., C.E. Booth and D.G. McDonald. 1985. Isolated perfused head of rainbow trout II. Ionic fluxes. American Journal of Physiology 249:R255-R261.

Perry, S.F., and C.M. Wood. 1985. Kinetics of branchial calcium uptake in the rainbow trout: effects of acclimation to various external calcium levels. Journal of Experimental Biology 116:411-433.

Perry, S.F. and P. Laurent. 1989. Adaptational responses of rainbow trout to lowered external NaCl concentration: contribution of the branchial chloride cell. Journal of Experimental Biology 147:147-168.

Perry, S.F., G.G. Goss, and P. Laurent. 1992. The interrelationships between gill chloride cell morphology and ionic uptake in four freshwater teleosts. Canadian Journal of Zoology 70:1775-1786.

Peterson, R.H., K. Coombs, J. Power, and U. Paim. 1989. Responses of several fish to pH gradients. Canadian Journal of Zoology 67:1566-1572.

Playle, R. C., and C. M. Wood. 1989. Water chemistry changes in the gill microenvironment of rainbow trout: experimental observations and theory. Journal of Comparative Physiology B 159:527-537.

Portner, H.O., R.G. Boutilier, Y. Tang, and D.P. Toews. 1990. Determination of intracellular pH and P_{co2} after metabolic inhibition by fluoride and nitrilotriacetic acid. Respiration Physiology 81:255-274.

Potts, W.T.W. 1994. Kinetics of Na^+ uptake in freshwater animals: a comparison of ion-exchange and proton pump hypotheses. American Journal of Physiology 266:R315-R320.

Prepas, E.E., T.P. Murphy, J.M. Crosby, D.T. Walty, J.T. Lim, J. Babin, and P.A. Chambers. 1990. Reduction of phosphorous and chlorophyll *a* concentrations following $CaCO_3$ and $Ca(OH)_2$ additions to hypereutrophic Figure Eight Lake, Alberta. Environmental Science and Technology 24:1252-1258.

Randall, D.J., and P. A. Wright. 1987. Ammonia distribution and excretion in fish. Fish Physiology and Biochemistry 3:107-120.

Randall, D.J., and P. A. Wright. 1989. The interaction between carbon dioxide and ammonia excretion and water pH in fish. Canadian Journal of Zoology 67:2936-2942.

Randall, D.J., C.M. Wood, S.F. Perry, H. Bergman, G.M.O. Maloiy, T.P. Mommsen, and P.A. Wright. 1989. Urea excretion as a strategy for survival in a fish living in a very alkaline environment. Nature 337:165-166.

Rao, G.M.M. 1968. Oxygen consumption of rainbow trout (*Salmo gairdneri*) in relation to activity and salinity. Canadian Journal of Zoology 46:781-786.

Read, L. J. 1971. The presence of high ornithine-urea cycle enzyme activity in the teleost *Opsanus tau*. Comparative Biochemistry and Physiology 39B:409-413.

Reite, O.B., G.M.O. Maloiy and B. Aasehaug. 1974. pH, salinity and temperature tolerance of Lake Magadi *Tilapia*. Nature 247:315.

Rice, S. D., and R. M. Stokes. 1974. Metabolism of nitrogenous wastes in the eggs and alevins of rainbow trout, *Salmo gairdneri* Richardson. Pages 324-337 in J. H. S. Blaxter, ed. The early life history of fish. Springer-Verlag, Berlin, Heidelberg, New York.

Saha, N., and B.K. Ratha. 1987. Active Ureogenesis in a freshwater air-breathing teleost, *Heteropneustes fossilis*. Journal of Experimental Zoology. 241:137-141.

Saha, N. and B.K. Ratha. 1989. Comparative study of ureogenesis in freshwater air-breathing teleosts. Journal of Experimental Zoology 52:1-8.

Sanborn, N.H. 1945. The lethal effects of certain chemicals on freshwater fish. Canning Trade 67:26.

SAS Institute. 1989. SAS JMP user's guide. SAS Institute, Cary, North Carolina.

Severinghaus, J. W. 1965. Blood gas concentrations. Pages 1475-1487 in W.O. Fenn and H. Rahn, eds. Handbook of physiology, Sect. 3 Volume 2. American Physiological Society, Washington, D.C.

Shaw, J. 1959. The absorption of Na^+ ions by the crayfish, *Astacus pallipes*, Lereboullet. I. The effect of external and internal sodium concentrations. Journal of Experimental Biology 36:126-144.

Skirrow, G. 1975. The dissolved gases - carbon dioxide. Pages 1-192 in J.P. Riley and G. Skirrow, eds. Chemical Oceanography. Vol. 2(2). Academic Press, New York.

Smart, G.R. 1978. Investigations of the toxic mechanisms of ammonia to fish gas-exchange in rainbow trout (*Salmo gairdneri*) exposed to acutely lethal concentrations. Journal of Fish Biology 12:93-104.

Smith, H.W. 1929. The excretion of ammonia and urea by the gills of fish. Journal of Biological Chemistry 781:727-742.

Soivio, A.K., K. Westman, and K. Nyholm. 1972. Improved method of dorsal aortal catheterization: haematological effects followed for three weeks in rainbow trout. Finnish Fish Research 1:11-21.

Solbe, L.G. and D.G. Shurben. 1989. Toxicity of Ammonia to early life stages of rainbow trout (*Salmo gairdneri*). Water Research 23:127-129.

Stevens, E.D. 1968. The effect of exercise on the distribution of blood to various organs in rainbow trout. Comparative Biochemistry and Physiology 25:615-625.

Stewart, P.A. 1983. Modern quantitative acid-base chemistry. Canadian Journal of Physiology and Pharmacology 61:1444-1461.

Takada, Y. and T. Noguchi. 1983. The degradation of urate in liver peroxisomes. Journal of Biological Chemistry 258:4762-4764.

Takano, N. 1968. Role of hypocapnia in the blood lactate accumulation during acute hypocapnia. Respiration Physiology 4:32-41.

Tang, Y., H. Lin, and D.J. Randall. 1992. Compartmental distribution of carbon dioxide and ammonia in rainbow trout at rest and following exercise, and the effect of bicarbonate infusion. Journal of Experimental Biology 169:235-249.

Thurston, R.V., R.C. Russo, and C.E. Smith. 1978. Acute toxicity of ammonia and nitrite to cutthroat trout fry. Transactions of the American Fisheries Society 107:361-368.

Thurston, R.V., R.C. Russo, R.J. Luedtke, C.E. Smith, E.L. Meyn, C. Chakaoumakos, K.C. Wang, and C.J.D. Brown. 1984. Chronic toxicity of ammonia to rainbow trout. Transactions of the American Fisheries Society 113:56-73.

Trautschold, I., W. Lamprecht, and G. Schweizer. 1985. ATP: UV method with hexokinase and glucose-6-phosphate dehydrogenase. Pages 346-357 in H.U. Bergmeyer ed. Methods of Enzymatic Analysis Volume VII. Lipids, Amino Acids and Related Compounds, 3rd ed. VCH Publishers, Deerfield Beach, Florida.

Trotter, P. 1991. Cutthroat trout. Pages 236-265 in J. Stolz and J. Schnell, eds. Trout. Stackpole Books, Harrisburg, Pa.

Turner, J. D., C. M. Wood, and D. Clark. 1983. Lactate and proton dynamics in the rainbow trout (*Salmo gairdneri*). Journal of Experimental Biology 104:247-268.

United States Environmental Protection Agency. 1985. Ambient water quality criteria for ammonia. Report no. EPA 440 / 5 -85 -001.

Van Waarde, A. 1983 Aerobic and anaerobic ammonia production by fish. Comparative Biochemistry and Physiology 74B:675-684.

Verdouw, H., C.J.A. van Echted, and E.M.J. Dekkers. 1978. Ammonia determination based on indophenol formation with sodium salicylate. Water Research 12:399-402.

Vigg, S.C. and D.L. Koch. 1980. Upper lethal temperature range of Lahontan cutthroat trout in waters of different ionic concentration. Transactions of the American Fisheries Society 109:336-339.

Walsh, P.J., E. Danulat, and T.P. Mommsen. 1990. Variation in urea excretion in the gulf toadfish, *Opsanus beta*. Marine Biology 106:323-328.

Walton, M.J., and C.B. Cowey. 1982. Aspects of intermediary metabolism in salmonid fish. Comparative Biochemistry and Physiology 73B:59-79.

Ward, G.R., J.R. Sutton, N.L. Jones, and C.J. Toews. 1982. Activation by exercise of human skeletal muscle pyruvate dehydrogenase *in vivo*. Clinical Science 63:87-92.

Webb, J.T. and G.W. Brown Jr. 1980. Glutamine synthetase: assimilatory role in liver as related to urea retention in marine chondrichthyes. Science 208:293-295.

Wendelaar Bonga, S.E., J.C.A. Van Der Meij, and G. Flik. 1984. Prolactin and acid stress in the teleost *Oreochromis* (formerly *Sarotherodon*) *mossambicus*. General and Comparative Endocrinology 55:323-332.

Wetzel, R.G. 1983. Limnology 2nd edition. W.B. Saunders Company. Philadelphia, PA.

Wheeler, S.S. 1987. The Desert Lake: The Story of Nevada's Pyramid Lake. The Caxton Printers. Caldwell, Idaho.

Wiebe, A.H. 1931. Note on the exposure of several species of pond fishes to sudden changes in pH. Transactions of the American Microscopy Society 50:380-383.

Wilkie, M.P., and C.M. Wood. 1991. Nitrogenous waste excretion, acid-base regulation, and ionoregulation in rainbow trout (*Oncorhynchus mykiss*) exposed to extremely alkaline water. Physiological Zoology 64:1069-1086.

Wilkie, M.P., P.A. Wright, G.K. Iwama and C.M. Wood. 1993. The physiological responses of the Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*), a resident of highly Pyramid Lake (pH 9.4), to challenge at pH 10. Journal of Experimental Biology 175:173-194.

Wilkie, M.P., P.A. Wright, G.K. Iwama and C.M. Wood. 1994. The physiological adaptations of the Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*), following transfer from fresh water to the highly alkaline waters of Pyramid Lake, Nevada (pH 9.4). Physiological Zoology. 67:355-380.

Wilkie, M.P., and C.M. Wood. 1994a (Appendix 1). The effects of extremely alkaline water (pH 9.5) on rainbow trout gill function and morphology. Journal of Fish Biology 45:87-98.

Wilkie, M.P., and C.M. Wood. 1994b. Recovery from high pH exposure in rainbow trout: ammonia wash-out and the restoration of blood chemistry. Physiological Zoology. In Press.

Williams, J.E., J.E. Johnson, D.A. Hendrickson, S. Contreras-Balderas, J.D. Williams, J. Navarro-Mendoza, D.E. McAllister, and J.E. Deacon. 1989. Fishes of North America Endangered, Threatened, or of Special Concern: 1989. Fisheries 14:2-20.

Wolf, K. 1963. Physiological salines for fresh water teleosts. Progressive Fish Culturalist. 25:135-140.

Wood, C.M. 1988. Acid-Base and ionic exchanges at the gills and kidney after exhaustive exercise in the rainbow trout. Journal of Experimental Biology 136:461-481.

Wood, C.M. 1989. The physiological problems of fish in acid waters. Pages 125-152 in R. Morris, E.W. Taylor, D.J.A. Brown, and J.A. Brown, eds. Acid Toxicity and Aquatic Animals. Cambridge University Press, Cambridge, United Kingdom.

Wood, C.M. 1991. Branchial ion and acid-base transfer in freshwater teleost fish: Environmental hyperoxia as a probe. Physiological Zoology 64:68-102.

Wood, C.M. 1993. Ammonia and urea metabolism and excretion. Pages 379-425 in D.H. Evans, ed. The Physiology of Fishes. Cleveland Rubber Company Press, Boca Raton, FLA.

Wood, C. M., B. R. McMahon, and D. G. McDonald. 1977. An analysis of changes in blood pH following exhausting activity in the starry flounder, *Platichthys stellatus*. Journal of Experimental Biology 69:173-185

Wood, C.M., D.G. McDonald, and B.R. McMahon. 1982. The influence of experimental anemia on blood acid-base regulation *in vivo* and *in vitro* in the starry flounder (*Platichthys stellatus*) and the rainbow trout (*Salmo gairdneri*). Journal of Experimental Biology 96:221-337.

Wood, C.M., M.G. Wheatly and H. Hobe. 1984. The mechanisms of acid-base and ionoregulation in the freshwater rainbow trout during environmental hyperoxia and subsequent normoxia. III. branchial exchanges. Respiration Physiology 55:175-192.

Wood, C. M., S. F. Perry, P. A. Wright, H. L. Bergman, and D. J. Randall. 1989. Ammonia and urea dynamics in the Lake Magadi tilapia, a ureotelic teleost fish adapted to an extremely alkaline environment. Respiration Physiology 77:1-20.

Wood, C.M., and J. LeMoigne. 1991. Intracellular acid-base responses to environmental hyperoxia and normoxic recovery in rainbow trout. Respiration Physiology 86:91-113.

Woodward, J.J., and R.J. Strange. 1987. Physiological stress responses in wild and hatchery-reared rainbow trout. Transactions of the American Fisheries Society 116:574-579.

Wright, P.A. 1993. Nitrogen excretion and enzyme pathways for ureagenesis in freshwater tilapia (*Oreochromis niloticus*). Physiological Zoology 66:881-901.

Wright, P.A., and C.M. Wood. 1985. An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockade. Journal of Experimental Biology 114:329-353.

Wright, P.A., T. Heming, and D.J. Randall. 1986. Downstream changes in water flowing over the gills of rainbow trout. Journal of Experimental Biology 126:499-512.

Wright, P.A. and C.M. Wood. 1988. Muscle ammonia stores are not determined by pH gradients. Fish Physiology and Biochemistry 5:159-162.

Wright, P.A., D.J. Randall, and C.M. Wood. 1988. The distribution of ammonia and H^+ between tissue compartments in lemon sole (*Parophrys vetulus*) at rest, during hypercapnia and following exercise. Journal of Experimental Biology 136:149-175.

Wright, P. A., D. J. Randall, and S. F. Perry II. 1989. Fish gill water boundary layer: a site of linkage between carbon dioxide and ammonia excretion. Journal of Comparative Physiology B 158:627-635

Wright, P.A., S.F. Perry, D.J. Randall, C.M. Wood, and H. Bergman. 1990. The effects of reducing water pH and CO_2 on teleost fish adapted to an extremely alkaline environment. Journal of Experimental Biology 151:361-369.

Wright, P.A., G.K. Iwama and C.M. Wood. 1993. Ammonia and urea excretion in Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*) adapted to highly Pyramid Lake (pH 9.4). Journal of Experimental Biology 175:153-172.

Yesaki, T. 1990. The survival and physiology of rainbow trout in alkaline hardwater. M.Sc. thesis. University of British Columbia.

Yesaki, T.Y., and G.K. Iwama. 1992. Some effects of water hardness on survival, acid-base regulation, ion regulation and ammonia excretion in rainbow trout in highly alkaline water. Physiological Zoology 65:763-787.

Zall, D.M., M.D. Fisher, and Q.M. Garner. 1956. Photometric determination of chlorides in water. Analytical Chemistry 28:1665-1678.

Zar, J.H. 1984. Biostatistical Analysis, 2nd ed. Prentice Hall, Englewood Cliffs, New Jersey.