J. exp. Biol. ***175****, 173–194 (1993)*
Printed in Great Britain © The Company of Biologists Limited 1993

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

MICHAEL P. WILKIE*, PATRICIA A. WRIGHT†, GEORGE K. IWAMA‡ and CHRIS M. WOOD*

Pyramid Lake Fisheries, Star Route, Sutcliffe, Nevada 89510, USA

Accepted 13 October 1992

Summary

Desiccation of Pyramid Lake, Nevada, has led to continued increases in the lake's alkalinity (currently pH9.4) that may threaten the resident Lahontan cutthroat trout population. In this study, Lahontan cutthroat trout were challenged with more alkaline water (pH10). 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 by 50 % in the first few hours, but had fully recovered by 24h and exceeded control values by 36–48h. A sustained, twofold elevation of plasma ammonia concentration may have facilitated the recovery of J_{amm} by increasing the blood-to-water ammonia partial pressure diffusion gradient (ΔP_{NH_3}) and NH_4^+ electrochemical gradient. Urea excretion (J_{urea}) almost doubled at 24–48h of pH10 exposure. Activities of ornithine–urea cycle enzymes in the liver were very low and there was no induction at pH10. However, all three enzymes of the uricolytic pathway were present, and allantoicase activity increased significantly at pH10, a possible explanation for the elevated J_{urea}. Increased liver glutamine synthetase activity at pH10 is consistent with a possible ammonia detoxification mechanism. A combined respiratory (decreased Pa_{CO_2}) and metabolic (gain of basic equivalents) alkalosis developed at pH10 and resulted in a 0.25 unit increase in arterial blood pH. Electrochemical gradients for CO_3^{2-} and OH^- entry and H^+ efflux all increased, but the gradient for HCO_3^- entry decreased to zero. Blood lactate level increased without marked changes in arterial O_2 tension, suggesting that increased lactic acid production contributed to acid–base control. Plasma Na^+ and Cl^- levels decreased and K^+ level increased during pH10 exposure. Survival at pH10 was relatively

*Present address: Department of Biology, McMaster University, Hamilton, Ontario, Canada, L8S 4K1.

†Present address: Department of Pathology, University of Guelph, Guelph, Ontario, Canada, N1G 2W1.

‡Present address:Department of Animal Science, University of British Columbia, Vancouver, British Columbia, Canada V6T 2A2.

Key words: cutthroat trout, high pH, urea, uricolysis, ornithine–urea cycle, ammonia, ionoregulation, acid–base balance, lactate, ammonia toxicity, *Oncorhynchus clarki henshawi.*

poor: more than 50% of the fish died after 72h exposure. Greatly elevated plasma P_{NH_3} 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*, Gill and Jordan) appears to be uniquely adapted to the highly alkaline waters (pH9.4) 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 values 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 Lake water at pH9.4 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}), which allow the maintenance of a positive P_{NH_3} 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 pH10 severely depresses branchial ammonia excretion without altering urea excretion, a result attributed to the reduction in the P_{NH_3} gradient.

These observations raise the questions of 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 values 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 owing 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^{2-} 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 1980s (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 (pH10) for several days. In light of past reports, the study focused on acid–base regulation, ionoregulation and nitrogenous waste excretion. We were particularly interested to see whether ammonia excretion recovered during a longer-term (72h) challenge at pH10, whether there were compensatory increases in urea excretion, whether lactic acid production occurred as a mechanism for acid–base

regulation in the face of alkalosis and whether plasma Na^+ and Cl^- levels declined. All these responses have been seen in a recent study of rainbow trout (*Oncorhynchus mykiss*) exposed to pH9.5 (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 (pH10) 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 (*Oncorhynchus mykiss*, 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 (pH9.4) and after 72h of exposure to pH10.

Materials and methods

Experimental animals and set-up

One-year-old Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*; 242.2±9.2g, N=21) of both sexes were obtained from Pyramid Lake Fisheries, Nevada, and held in Pyramid Lake water at pH9.4. 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 for 72 h in darkened flux chambers. Water flow to each chamber was approximately $0.5\,l\,min^{-1}$, on a flow-through basis and water T_{amm} never exceeded $5\,\mu mol\,N\,l^{-1}$. Experimental temperature was 9.5±0.3°C. Thirteen trout were subjected to pH10 exposure and an additional eight fish were killed for tissue samples under control conditions.

For exposures to pH10, Pyramid Lake water was pumped first into a 50l 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 $2mol\,l^{-1}$ KOH into the vigorously aerated reservoir. This addition of KOH resulted in water K^+ concentrations of approximately $13mmol\,l^{-1}$ at pH10 in comparison to the normal level of $2.9mmol\,l^{-1}$ at pH9.4. 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±0.004 and experimental water pH was 9.990±0.005, as measured in the boxes with an independent electrode and meter. When flux boxes were operated as closed systems at pH10, 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 monitor pH continually and to adjust it with 2ml additions of $2mol\,l^{-1}$ KOH at 1h and

2 h. As a result, water K^+ increased by 2.7mmol l^{-1} to approximately 15.7mmol 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 pH9.4 and at 0–3h, 8–11h, 24–27h, 36–39h, 48–51h and 72–75h of pH10.0 exposure. Water samples (15ml) were taken at 0h and 3h of each flux period, immediately acidified with 2mol l^{-1} HCl to prevent NH_3 loss, frozen and later analyzed for T_{amm} 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 one molecule of urea, ammonia and urea excretion rates were expressed in μmol 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 T_{amm} never exceeded 50 μmol N l^{-1} over 3h. Between flux periods, boxes were opened to the flow-through system.

Thirty minutes prior to each flux determination, 1.0ml blood samples were drawn into two 500 μl, heparinized, gas-tight Hamilton syringes for determination of arterial blood O_2 tension (P_{aO_2}), pH (pHa), haematocrit, haemoglobin and lactate, and plasma T_{amm}, urea, total CO_2 (C_{aCO_2}), Na^+, Cl^-, K^+, glucose, cortisol and protein. In addition, water samples for determination of inspired P_{O_2} (P_{IO_2}) and total CO_2 were taken. Branchial transepithelial potential (TEP) was measured while blood samples were being processed. Plasma was separated by centrifugation (2min at 13000 ***g***) and frozen for later analysis of T_{amm}, urea, glucose, cortisol, Na^+, Cl^- and K^+. Haematocrit was determined by centrifugation (5min at 500 ***g***) and plasma C_{aCO_2} and protein were determined on plasma decanted from the haematocrit tubes. At the completion of sampling, blood used for determination of P_{aO_2} (normally 200 μl) 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 30min to minimize disturbance to the fish and to 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 T_{amm} in the water (Wilkie and Wood, 1991; Wright *et al.* 1993). Diffusion gradients for NH_3 (ΔP_{NH_3}) 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 pH10 were based upon the control blood sample, because it was unlikely that blood variables would change during the first few minutes at pH10. Estimates of the blood-to-water gradient for P_{O_2}, P_{CO_2}, H^+, OH^-, HCO_3^- and CO_3^{2-} (see below) were determined from water and blood samples obtained simultaneously.

The six fish that were still alive at 75h were killed 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 eight trout which had been similarly cannulated and blood

sampled at pH9.4. 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_{O_2} 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 haemoglobin (cyanmethaemoglobin) were determined with commercial kits (Sigma). Cortisol was measured by ^{125}I radioimmunoassay (ICN Biomedicals Inc.) using standards diluted to the protein concentrations found in trout plasma and analysed on a Packard 5000 Series gamma counter. Plasma protein was determined by refractometry (Alexander and Ingram, 1980).

Arterial P_{CO_2} (Pa_{CO_2}), HCO_3^- ($[HCO_3^-]_a$) and CO_3^{2-} ($[CO_3^{2-}]_a$) were calculated from Ca_{CO_2} and pHa using the Henderson–Hasselbalch equation, appropriate solubility coefficients and pK_1' and pK_2' values outlined in Boutilier *et al.* (1984) and Skirrow (1975). Water P_{CO_2}, $[HCO_3^-]$ and $[CO_3^{2-}]$ 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_{NH_3} and $[NH_4^+]$ were calculated from the pH and T_{amm} 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 haemoglobin, using procedures outlined by Turner *et al.* (1983). Mean cell haemoglobin concentration (MCHC) was simply the blood haemoglobin divided by blood haematocrit (Turner *et al.* 1983).

Transepithelial potential across the gills was measured and estimates of the electrochemical driving force (F_{NH_4}) for NH_4^+ diffusion from blood to water were made as described by Wright *et al.* (1993). Estimates of the electrochemical forces for other ions (F_{HCO_3}, F_{CO_3}, 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 μmoles of substrate converted to product per gram of liver tissue fresh mass in 1min at 22°C under saturation conditions, with one exception: CPS activity is given in μmoles 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 (w:v) solution of ice-cold Hepes buffer (50mmol l^{-1}, pH7.5), using a

hand-held glass homogenizer. The protease inhibitor phenylmethylsulphonyl fluoride (PMSF), was added to each liver homogenate sample (approximate concentration 0.1mmol l^{-1}). Tissue homogenates were kept on ice and used within 15min. 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 (see below).

Carbamoylphosphate 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 (20mmol l^{-1}, pH7.4), β-mercaptoethanol (5mmol l^{-1}), ethylenediaminetetraacetic acid (EDTA, 0.5mmol l^{-1}) and bovine serum albumin (0.02%). Two additional protease inhibitors, leupeptin and aprotinin, were added to each tissue sample (10 μg ml^{-1}). Mitochondrial homogenate (25 μl) was added to 375 μl of a reagent mixture containing ATP (8mmol l^{-1}), creatine phosphate (10mmol l^{-1}), $MgSO_4$ (13mmol l^{-1}), KCl (40mmol l^{-1}), dithiothreitol (2mmol l^{-1}), ornithine (10mmol l^{-1}), *N*-acetyl glutamate (NAG, 2mmol l^{-1}), L-glutamine (10mmol l^{-1}), $NaHCO_3$ (1mmol l^{-1} cold+0.4 μCi [^{14}C]$NaHCO_3$), Hepes (50mmol l^{-1}, pH8.0), ornithine carbamoyl transferase (0.3units) and creatine kinase (20units). Samples were incubated for 60min at 22°C and the reaction was terminated by addition of 50 μl of trichloroacetic acid (35%). Unincorporated [^{14}C]CO_2 was removed by shaking samples in a fume hood for 60min. Samples were counted in 4ml of scintillation fluor (Scintiverse BD, Fisher) after neutralization with $NaHCO_3$.

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 and 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 (μmol g^{-1} livertissuemin^{-1}) should be directly proportional to the formation of arginine. Liver tissue was sonicated in a 1:2 (w:v) solution of ice-cold Hepes buffer (50mmol l^{-1}, pH7.5). The reagent mixture contained potassium phosphate (50mmol l^{-1}, pH7.5), citrulline (1mmol l^{-1}), aspartate (3mmol l^{-1}), $MgSO_4$ (2mmol l^{-1}), argininosuccinate lyase (0.3 units), arginase (100 units), adenosine triphosphate (ATP

1 mmol l^{-1}) and an ATP regenerating system, creatine phosphate (5mmol l^{-1}) and creatine kinase (8 units). Double-strength reagent mixture (50 μl) was combined with equal volumes of liver homogenate and the reaction was terminated after 60min at 22°C with 10 μl of $HClO_4$ (70%). A sample (100 μl) of neutralized supernatant was incubated (60min at 22°C) with 25 μl of a urease solution (5mg ml^{-1}). Ammonia concentration was determined on 50 μl of deproteinized supernatant (Verdouw *et al.* 1978).

Statistics

All data are expressed as means ± 1 S.E.M. (*N*) where *N* is 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 was not satisfied, a Mann–Whitney non-parametric statistical test was used to test for statistical significance.

Results

Survival

After 72h at pH10, 12 of 13 fish were alive, but over the next 4h, 50% of the remaining fish died. These results suggest that Lahontan cutthroat trout are incapable of surviving a pH10 challenge for more than a few days.

Nitrogenous waste excretion

At pH9.4, J_{amm} was approximately 85 μmol N kg^{-1} h^{-1}. During the first 3h at pH10, J_{amm} dropped significantly by about 50% (Fig. 1A). This depression was short-lived; by 8h J_{amm} had recovered and at 36 and 48h was significantly elevated by 25%, to approximately 110 μmol N kg^{-1} h^{-1} (Fig. 1A). Over the last 24h of exposure, J_{amm} dropped slightly and was no longer significantly elevated (Fig. 1A). The initial inhibition of J_{amm} led to elevated plasma T_{amm}. After 8h at pH10, plasma T_{amm} increased about twofold from resting levels of 185 μmol N l^{-1}, thereafter stabilizing at approximately 400 μmol N l^{-1} (Fig. 1B).

The arterial blood to bulk water gradient for NH_3 diffusion (ΔP_{NH_3}) was about 6.7mPa at pH9.4 and fell only slightly upon initial exposure to pH10 (Fig. 2A). However, by 8 h ΔP_{NH_3} had increased almost fourfold to 24mPa and by 72h reached 32mPa (Fig. 2A). There was also a strong electrochemical gradient of about +100mV for NH_4^+ diffusion out of the fish at pH9.4 (Fig. 2C). The contribution of the slightly negative TEP (−3 mV) to this gradient was negligible (Fig. 2B). F_{NH_4} increased to +145mV at 8h, largely due to the twofold elevation in plasma NH_4^+ concentration, while TEP increased by 1mV (Fig. 2C). By 48h, F_{NH_4} had stabilized at approximately +130mV while TEP had returned to control levels (Fig. 2B,C).

At pH9.4, J_{urea} was approximately 30 μmol N kg^{-1} h^{-1}. It increased slightly over the first 8h of exposure to pH10 (Fig. 3A) and was significantly elevated to 50–60 μmol N kg^{-1} h^{-1} by 24–48h (Fig. 3A). At 72h, J_{urea} dropped to about

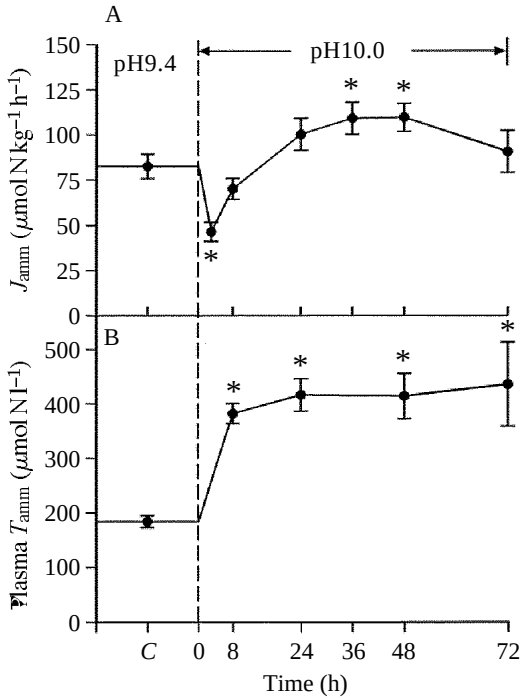

Fig. 1. Influence of a pH10 challenge on (A) ammonia excretion (J_{amm}) and (B) plasma total ammonia concentration (T_{amm}) in Lahontan cutthroat trout acclimated to pH9.4 Pyramid Lake water. Values are means ± 1 S.E.M., N=13 for the control, 3h, 8h, 24h and 36h periods; N=12 at 48h; N=10 for plasma T_{amm} and N=7 for J_{amm} at 72h. Asterisks indicate significant differences from control (pH9.4) values (P<0.05). *C*, control period.

45 μmol N kg^{-1} h^{-1} (Fig. 3A). Plasma urea fell slightly during exposure to pH10 from about 6000 μmol N l^{-1} under control conditions to about 5000 μmol 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 pH10. There were no significant changes in the activities of two other enzymes, uricase and allantoinase. In the OUC (Table 2), the key regulatory enzyme carbamoylphosphate synthetase (CPS III) and ornithine carbamoyl transferase (OTC) both had very low activities at pH9.4, and the latter was essentially undetectable at pH10, 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 pH10. However, the activity of glutamine synthetase, which is not part of the OUC but converts glutamate and NH_3 to the substrate glutamine, increased significantly by approximately 150% at pH10 (Table 2).

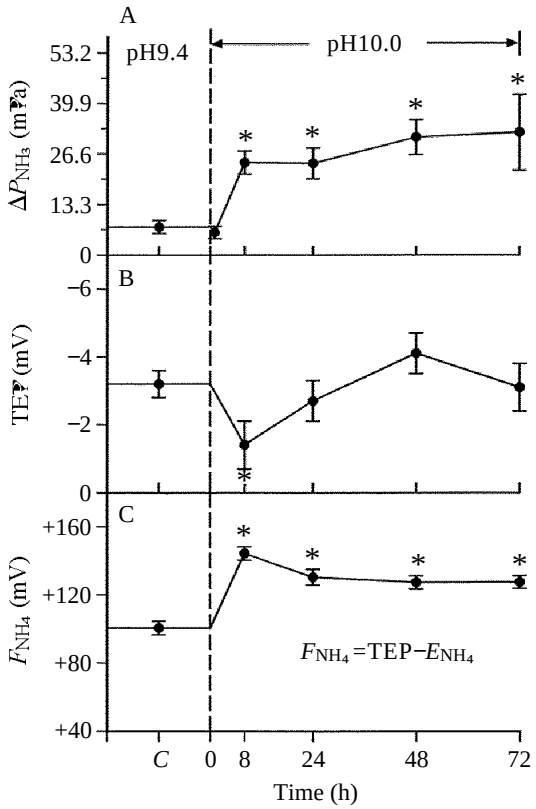

Fig. 2. Influence of a pH10 challenge on (A) blood-to-water NH_3 gradient (ΔP_{NH_3}), (B) transepithelial potential (TEP) and (C) electrochemical gradient for NH_4^+ (F_{NH_4}) of Lahontan cutthroat trout acclimated to pH9.4 Pyramid Lake water. Values are means ± 1 S.E.M., N=13 for control, 3h, 8h and 24h periods; N=12 at 48h and N=10 at 72h. Asterisks indicate significant differences from control (pH9.4) values (P<0.05). *C*, control period.

Acid–base balance

Arterial pH was approximately 8.1, $P_{a_{CO_2}}$ about 159Pa and $[HCO_3^-]_a$ about 7.4mmol l^{-1} in Lahontan cutthroat trout at pH9.4 (Fig. 4). After 8h at pH10, the trout exhibited a significant metabolic alkalosis (negative ΔH_m^+, Fig. 5A) characterized by a significant rise in pHa (Fig. 4A) and a slight increase in $[HCO_3^-]_a$ (Fig. 4C). By 24h, this was compounded by a respiratory alkalosis as $P_{a_{CO_2}}$ fell significantly (Fig. 4B). The combined respiratory and metabolic alkalosis was more or less stable thereafter, with mean pHa at about 8.35, mean $P_{a_{CO_2}}$ at about 100Pa, ΔH_m^+ at about -3 mmol l^{-1} and $[HCO_3^-]_a$ unchanged from control levels.

Blood lactate concentration increased steadily from about 1.0mmol l^{-1} at pH9.4 to more than 3mmol l^{-1} after 48h at pH10 (Fig. 5B). Increased blood lactate was not due to hypoxia; inspired P_{O_2} never dropped below 16.7kPa and $P_{a_{O_2}}$ was stable for 48h at approximately 12kPa (Fig. 5C). The significant depression of $P_{a_{O_2}}$ at 72h may have been due to complicating factors associated with imminent death (Fig. 5C).

The acid–base composition of the water (Table 3) was markedly altered by titration to pH10 with KOH; $[OH^-]$ and $[H^+]$ increased and decreased by fourfold, respectively. Water P_{CO_2} (33.3Pa) was close to atmospheric P_{CO_2} at pH9.4, but fell by 75% at pH10; at the same time $[HCO_3^-]$ was reduced by 50% and $[CO_3^{2-}]$ increased more than twofold. These changes, in combination with alterations in blood acid–base status

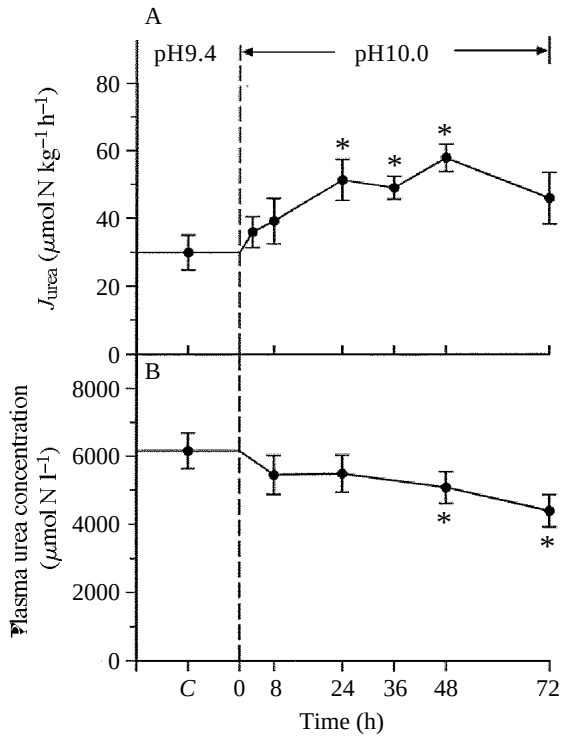

Fig. 3. Influence of a pH10 challenge on (A) urea excretion rates (J_{urea}) and (B) plasma urea concentration in cutthroat trout acclimated to pH9.4 Pyramid Lake water. Values are means ± 1 S.E.M., N=13 for the control, 3h, 8h, 24h and 36h periods; N=12 at 48h; N=10 for plasma urea and N=7 for J_{urea} at 72h. Asterisks indicate significant differences from control (pH9.4) values (P<0.05). *C*, control period.

Table 1. *Uricolytic enzyme activity in Lahontan cutthroat trout in control (pH9.4) water or exposed to pH10 water for 72 h*

Enzymes	pH9.4	pH10.0
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)

Means ± S.E.M. (*N*).
Activities are expressed as μmol g^{-1} livertissuemin^{-1}.
*Significantly different from control fish (P<0.05).

(Fig. 4), resulted in a small but significant reduction in the blood-to-water P_{CO_2} gradient (Fig. 6A) and larger changes in the electrochemical gradients for OH^-, H^+, HCO_3^- and CO_3^{2-} (Fig. 6B). F_H was strongly positive at pH9.4 and became significantly more positive during exposure to pH10, favouring the outward flux of H^+. At pH9.4, the

Table 2. *Ornithine–urea cycle enzyme activity in Lahontan cutthroat trout in control (pH9.4) water or exposed to pH10 water for 72 h*

Enzymes	pH9.4	pH10.0
Glutamine synthetase	0.35±0.04 (8)	0.85±0.17* (6)
CPS III	0.02±0.01 (4)	0.02±0.01 (4)
Ornithine carbamoyl transferase	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)

Means ± S.E.M. (*N*).

Activities are expressed as $\mu mol\,g^{-1}$ livertissuemin^{-1}, except for CPS III, which is expressed as $\mu mol\,g^{-1}$ mitochondria h^{-1}.

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

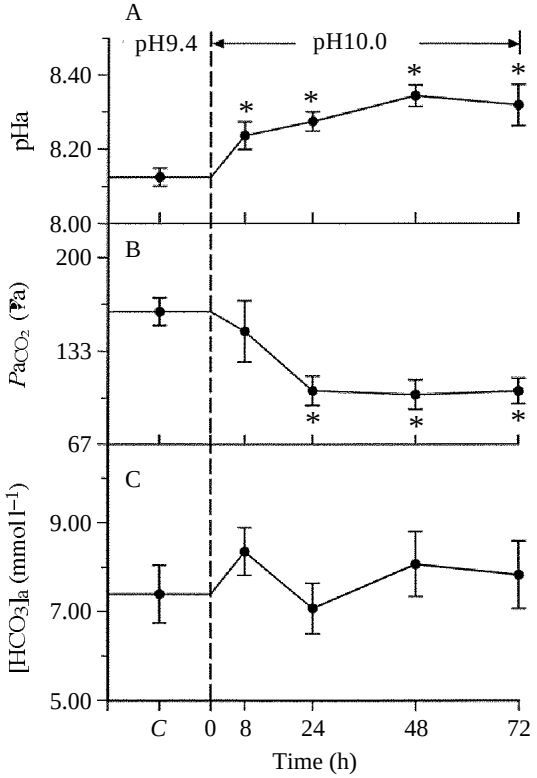

Fig. 4. Influence of a pH10 challenge on (A) arterial pH (pHa), (B) arterial CO_2 partial pressure (P_{aCO_2}) and (C) arterial HCO_3^- concentration ($[HCO_3^-]_a$) in cutthroat trout acclimated to pH9.4 Pyramid Lake water. For further details refer to Fig. 2.

electrochemical driving forces were also positive for, in ascending order, HCO_3^-, CO_3^{2-} and OH^-, favouring the influx of these anions across the gills. F_{CO_3} increased slightly and F_{OH} (same as F_H) increased to a greater extent upon exposure to pH10. However, the gradient for HCO_3^- entry was virtually eliminated by exposure to pH10 because of the accompanying decrease in water HCO_3^- concentration (Table 3). The electrochemical gradients for all these ions remained stable from between 8 and 72h of pH10 exposure (Fig. 6B).

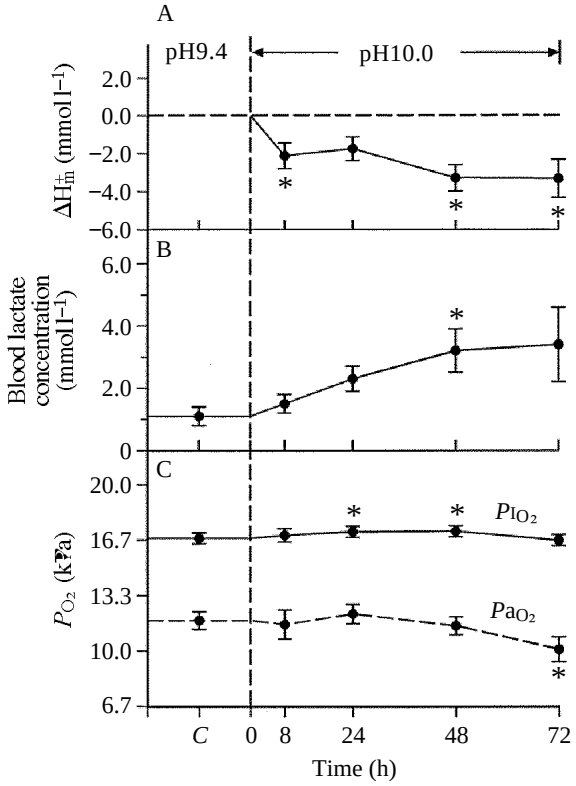

Fig. 5. Influence of a pH10 challenge on (A) metabolic acid load (ΔH_m^+),(B) blood lactate and (C) inspired P_{O_2} (P_{IO_2}; solid line) and arterial P_{O_2} (P_{aO_2}; dashed line) in cutthroat trout acclimated to pH9.4 Pyramid Lake water. For further details refer to Fig. 2.

Table 3. *Acid–base composition of Pyramid Lake water*

	pH9.4	pH10.0
pH	9.38	9.99
$[H^+]$ (μmol l^{-1})	0.41×10^{-3}	0.10×10^{-3}
$[OH^-]$ (μmol l^{-1})	7.18	29.6
P_{CO_2} (Pa)	33.3	8.0
$[HCO_3^-]$ (mmol l^{-1})	14.10	7.55
$[CO_3^{2-}]$ (mmol l^{-1})	5.01	11.81

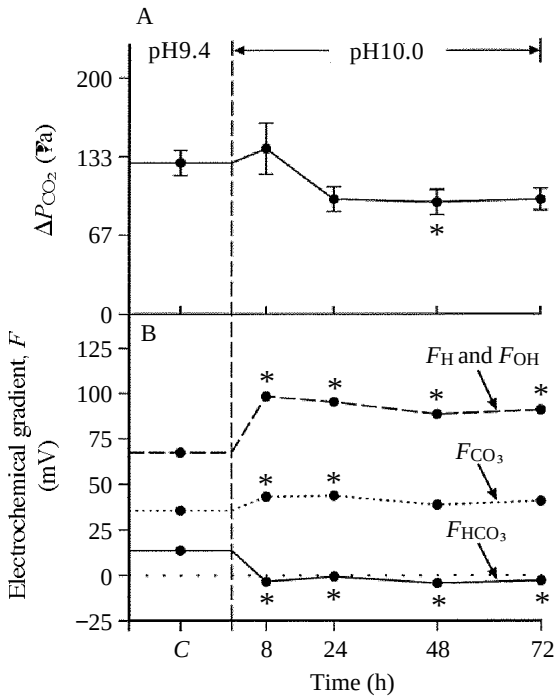

Fig. 6. Influence of a pH10 challenge on (A) blood-to-water CO_2 partial pressure gradient (ΔP_{CO_2}) and (B) blood-to-water electrochemical gradients for H^+ and OH^- (F_H and F_{OH}, respectively; dashed line), HCO_3^- (F_{HCO_3}; solid line) and CO_3^{2-} (F_{CO_3}; dotted line) in cutthroat trout acclimated to pH9.4 Pyramid Lake water. For further details refer to Fig. 2.

Ionoregulation and haematological indicators

At pH9.4, plasma Na^+ and Cl^- were 141mmol l^{-1} and 126mmol l^{-1}, respectively (Fig. 7A). After a slight non-significant increase during the first 8h at pH10, Na^+ and Cl^- concentrations gradually declined in an approximately equimolar fashion, a significant 10% depression occurring by 48–72h. Plasma K^+ was approximately 2.9mmol l^{-1} at pH9.4. After 48h at pH10, K^+ concentration had increased significantly by 1.0mmol l^{-1} and by 72h had reached 5.3mmol l^{-1} (Fig. 7B). White muscle samples taken from fish which survived 72h of exposure to pH10 exhibited similar water, Na^+ and K^+ levels to those taken from control fish at pH9.4 (Table 4). However, muscle Cl^- levels were significantly depressed by about 30% (Table 4).

Despite repetitive blood sampling, plasma protein was stable at approximately 4g 100ml $^{-1}$ over the first 48h at pH10. A small but significant decrease was observed at 72h (Fig. 7C). Blood haemoglobin (not shown) fell to a greater extent (from 8.0 to 4.9 g 100ml $^{-1}$), but still rather less than expected based on the amount of blood sampled and standard estimates of blood volume in salmonids (Olson, 1992). These observations, together with the small increases in Na^+ and Cl^- at 8h (Fig. 7A), suggest that a slight haemoconcentration accompanied exposure to pH10. This haemoconcentration was not due to a shift of plasma fluid into the red blood cells. Indeed, mean cell haemoglobin

concentration (MCHC) increased significantly at pH10 (Fig. 7D), suggesting that the red blood cells had shrunk by about 13% by 72h.

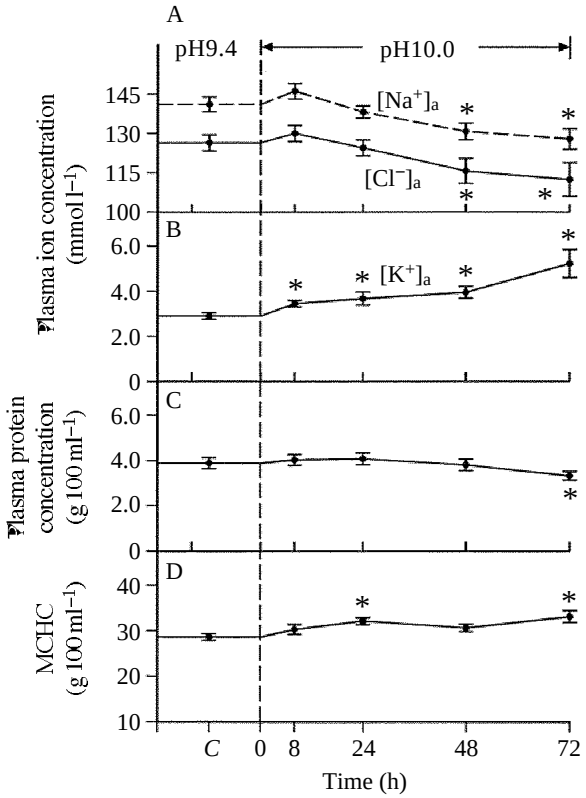

Fig. 7. Influence of a pH10 challenge on (A) plasma Na^+ (dashed line) and Cl^- concentration (solid line), (B) plasma K^+ concentration, (C) plasma protein concentration and (D) mean cell haemoglobin concentration (MCHC) in cutthroat trout acclimated to pH9.4 Pyramid Lake water. For further details refer to Fig. 2.

Table 4. *White muscle ion and water content in Lahontan cutthroat trout in control (pH9.4) water or exposed to pH10 water for 72 h*

Constituent	pH9.4	pH10.0
$[Cl^-]$	20.2±1.8 (7)	14.5±0.4* (5)
$[Na^+]$	9.3±0.8 (7)	8.6±0.8 (5)
$[K^+]$	125.9±2.3 (7)	123.9±2.6 (5)
H_2O	74.21±0.59 (7)	72.51±0.83 (5)

Values are mean ± S.E.M. (*N*).
Concentrations expressed as mmol kg^{-1} wet tissue.
White muscle water content expressed as percentage H_2O.
*Significantly different from control fish ($P<0.05$).

Table 5. *Plasma glucose and cortisol levels in Lahontan cutthroat trout in control water (pH9.4) and during 72h of exposure to pH10 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

Values are mean ± S.E.M. (*N*).
*Significantly different from control plasma samples taken prior to pH10 exposure.

Plasma glucose and cortisol

Plasma glucose was $3.3 mmol\,l^{-1}$ at pH9.4 and doubled after 8h at pH10; thereafter, it returned to control values (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 pH10. The diffusion gradient for NH_3 (ΔP_{NH_3}) 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_{NH_3} fell to a much greater extent as the 3h 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 pH10. 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 pH10, similar to the response of the rainbow trout at pH9.5 (Wilkie and Wood, 1991). The gradual decrease in plasma urea-N at pH10 suggests possible excretion of existing urea stores, but repetitive blood sampling may have contributed to the trend. Furthermore, each fish excreted an additional 326 μmol of urea-N over the 72h 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 levels (see Holmes, 1978, for a 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 pH9.4 and at pH10. 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 (pH9.4) and at pH10. Higher allantoicase activity at pH10 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 twofold 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 pH10 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 Lake water (pH9.4), Lahontan cutthroat trout are in a state of chronic respiratory alkalosis characterized by low $P_{a}CO_2$ and high pHa, approximately 50% lower and 0.3 units higher, respectively, than values reported for other salmonids in circumneutral water (Fig. 4; Perry *et al.* 1981; Cameron and Heisler, 1983; Wright and Wood, 1985; Wilkie and Wood, 1991). Low $P_{a}CO_2$ reflects the fact that the high-pH water outside the gills acts as a 'P_{CO_2} vacuum' owing to diffusive trapping of CO_2 as HCO_3^- or CO_3^{2-} (Johansen *et al.* 1975).

The combined respiratory and metabolic alkalosis observed at pH10 was the result of a number of factors. The respiratory alkalosis developed because water P_{CO_2} was reduced further at pH10 (Table 3), thereby increasing the 'P_{CO_2} vacuum' and reducing $P_{a}CO_2$ by a further 40% after 24–48h. Analysis of electrochemical gradients suggested that increased driving forces for CO_3^{2-} and OH^- entry and H^+ efflux accounted for the metabolic alkalosis. Surprisingly, F_{HCO_3} decreased to a value not significantly different from zero. Thus, HCO_3^- entry was not a causative 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 pHa to stabilize by 48h. Wilkie and Wood (1991) observed a very similar phenomenon in rainbow trout suffering a pure respiratory alkalosis when exposed to pH9.4, 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 concentration 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 pH9.4, experienced no increases in blood lactate (M. P. Wilkie, P. A. Wright, G. K. Iwama and C. M. Wood, unpublished results). Furthermore, Wood *et al.* (1982) have shown that anaemia, in the range observed in this study, leads to no change in blood lactate levels in rainbow trout.

Ionoregulation and haematological indicators

The decreases in plasma Na^+ and Cl^- concentrations experienced by the cutthroat trout at pH10 were 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; M. P. Wilkie and C. M. Wood, unpublished results). Clearly, further studies are needed to describe the movements of Na^+ and Cl^- at pH10.

At low environmental pH, branchial ion losses are known to cause haemoconcentration 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 pH10 is perplexing and should be studied further.

Mortality, toxic mechanisms and prognosis

The greater than 50% mortality observed after 72h of pH10 exposure demonstrated that Lahontan cutthroat trout are severely affected by this highly alkaline environment; the survival of the species in Pyramid Lake appears to be 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 (pH9.4, 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.

Several possible causes of mortality associated with the experimental protocol itself can probably be eliminated. A similar experimental regime at pH9.4 caused no mortality (M. Wilkie, P. A. Wright, G. K. Iwama and C. M. Wood, unpublished results). Increased environmental [K^+] (13–15.7mmoll^{-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 5.3mmoll^{-1} at 72h was similar to measurements on healthy salmonids (McDonald and Milligan, 1992). As an additional check, we exposed

eight similarly sized rainbow trout to 13mmol l^{-1} KCl for 72h. Plasma K^+ in the rainbow trout increased only slightly from 4.6 to 6.0mmol 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 pH10 never exceeded 4.1 μmol l^{-1} and were well below the 96h LC_{50} for cutthroat trout (approximately 30 μmolNH_3 l^{-1}, Thurston *et al.* 1978) and rainbow trout (50 μmolNH_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 death.

At 48h, plasma pHa rose to almost 8.4 in non-survivors, but this was not significantly higher than the mean in survivors (Table 6). These pHa 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, death did not occur in any of these studies and, therefore, it seems unlikely that high pHa was the direct cause of death. However, T_{amm}, P_{NH_3} and $[NH_4^+]$ were all significantly higher in those Lahontan trout that eventually died (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_{NH_3} was probably the toxic moiety of ammonia in the cutthroat trout at pH10. The plasma P_{NH_3} of 50.4mPa in cutthroat trout which eventually died approached the 96h LC_{50} for waterborne P_{NH_3} in cutthroat trout (approximately 70.6mPa; Thurston *et al.* 1978) and approached plasma levels

Table 6. *Key haematological variables in surviving and non-surviving cutthroat trout after 48h at pH10, in rainbow trout after 48h at pH9.5 and in rainbow trout exposed to 2 mmol l^{-1} T_{amm} for 5 h*

	Cutthroat trout		Rainbow trout	
	Survivors at pH10.0 (N = 6)	Non-survivors at pH10.0 (N = 6)	pH9.5 exposure†	2 mmol l^{-1} T_{amm}‡
T_{amm} (μmol l^{-1})	331±43	498±55*	640	1325
P_{NH_3} (mPa)	30.7±6.1	50.4±5.3*	39.3	61.5
$[NH_4^+]$ (μmol l^{-1})	318±41	478±53*	632	1302
pHa	8.310±0.050	8.376±0.026	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

Values are mean± S.E.M. (N).
†From Wilkie and Wood (1991).
‡R. W. Wilson and C. M. Wood (unpublished results).
*Significantly different from the value for surviving cutthroat trout (P<0.05).

(61.5 mPa) measured in rainbow trout that died shortly after 5h exposure to 2 mmol l^{-1} T_{amm} (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 death in acid-stressed trout (see Wood, 1989, for a review).

To conclude, the responses of Lahontan cutthroat trout, normally living at pH9.4 in Pyramid Lake, to exposure to pH10, were remarkably similar to those of the freshwater rainbow trout transferred from pH8.1 to pH9.5 (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 electrolyte concentrations were the most notable responses. A combination of ammonia toxicity and ionoregulatory failure led to death in susceptible cutthroat trout. Ironically, increased plasma T_{amm}, which facilitated J_{amm} at pH10, also caused toxic increases in blood P_{NH_3}. Clearly, if the pH of Pyramid Lake continues to climb its population of Lahontan cutthroat trout will be threatened.

Thanks to the staff of the Pyramid Lake Fisheries, especially P. Wagner, D. Mosely, L. Carlsen and N. Vucinich, for their hospitality and invaluable assistance during our stay at Pyramid Lake. Dr G. Wedemeyer, of the United States Fish and Wildlife Service, provided valuable guidance and support. Excellent technical assistance was provided by C. Mazur, J. McGeer and R. Ellis. Crucial mechanical assistance was provided by Certified Transmission, Salt Lake City, Utah. We are indebted to Dr R. W. Wilson and T. Yesaki for access to unpublished data. This study was supported by NSERC Operating Grants to C.M.W., G.K.I. and P.A.W. and an NSERC International Collaborative Grant to G.K.I and C.M.W.

References

ALEXANDER, J. B. AND INGRAM, G. A. (1980). A comparison of five of the methods commonly used to measure protein concentrations in fish sera. *J. Fish Biol.* **16**, 115–122.

ARILLO, A., MARGIOCCO, C., MELODIA, F., MENSI, P. AND SCHENONE, G. (1981). Ammonia toxicity mechanism in fish: studies on rainbow trout (*Salmo gairdneri* Rich.). *Ecotox. Environ. Safe.* **5**, 316–328.

BOCK, A. V., DILL, D. B. AND EDWARDS, H. T. (1932). Lactic acid in the blood of resting man. *J. clin. Invest.* **11**, 775–788.

BOUTILIER, R. C., HEMING, T. A. AND IWAMA, G. K. (1984). Physicochemical parameters for use in fish respiratory physiology. In *Fish Physiology*, vol. XA (ed. W. S. Hoar and D. J. Randall), pp. 401–430. New York: Academic Press.

BROWN, G. W., JR, JAMES, J., HENDERSON, R. J., THOMAS, W. N., ROBINSON, R. O., THOMPSON, A. L., BROWN, E. AND BROWN, S. G. (1966). Uricolytic enzymes in liver of the Dipnoan *Protopterus aethiopicus. Science* **153**, 1653–1654.

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. *J. exp. Zool.* **239**, 183–195.

CAMERON, J. N. AND HEISLER, N.(1983). Studies of ammonia in the trout: physico-chemical parameters, acid–base behaviour and respiratory clearance. *J. exp. Biol.* **105**, 107–125.

CLEVELAND RUBBER COMPANY. (1984). *Handbook of Chemistry and Physics*, 65th edition (ed. R. C. Weast). Boca Raton, Florida: Cleveland Rubber Company Press Inc.

COLEMAN, M. E. AND JOHNSON, V. K.(1988). Summary of management at Pyramid Lake, Nevada, with emphasis on Lahontan cutthroat trout, 1954–1987. *Am. Fish. Soc. Symp.* **4**, 107–115.

DAYE, P. G. AND GARSIDE, E. T. (1975). Lethal levels of pH for brook trout, *Salvelinus fontinalis* (Mitchill). *Can. J. Zool.* **53**, 639–641.

EICHENHOLZ, A., MULHAUSEN, R. O., ANDERSON, W. E. AND MACDONALD, F. M. (1962). Primary hypocapnia: a cause of metabolic acidosis. *J. appl. Physiol.* **17**, 283–288.

ERICHSEN JONES, J. R. (ed.) (1964). Acids and alkalis: pH tolerance limits. *Fish and River Pollution*. London: Butterworths. pp. 107–117.

GALAT, D. L., LIDER, E. L., VIGG, S. AND ROBERTSON, S. R. (1981). Limnology of a large deep North American terminal lake, Pyramid Lake, Nevada, U.S.A. *Hydrobiologia* **82**, 281–317.

GALAT, D. L., POST, G., KEEFE, T. J. AND BOUCKS, G. R. (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., POST, G., KEEFE, T. J. AND BOUCKS, G. R. (1985). Histological changes in the gill, kidney and liver of Lahontan cutthroat trout, *Salmo clarki henshawi*, living in lakes of different salinity–alkalinity. *J. Fish Biol.* **27**, 533–552.

GARCIA, A. C., LAI, Y. L., ATTBERY, B. A. AND BROWN, E. B., JR (1971). Lactate and pyruvate accumulation during hypocapnia. *Respir. Physiol.* **12**, 371–380.

GOSS, G. G. AND WOOD, C. M. (1990). Na^+ and Cl^- uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout. II. Responses to bicarbonate infusion. *J. exp. Biol.* **152**, 549–571.

HEMING, T. A. AND BLUMHAGEN, K. A. (1988). Plasma acid–base and electrolyte status of rainbow trout exposed to alum (aluminum sulphate) in acidic and alkaline environments. *Aquat. Toxicol.* **12**, 125–140.

HILLABY, B. A. AND RANDALL, D. J.(1979). Acute ammonia toxicity and ammonia excretion in rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Bd Can.* **36**, 621–629.

HOLMES, E. W. (1978). Regulation of purine synthesis *de novo*. In *Handbook of Experimental Pharmacology*, vol. 51, *Uric Acid* (ed. W. N. Kelley and I. M. Weiner), pp. 21–41. New York: Springer Verlag.

JOHANSEN, K., MALOIY, G. M. O. AND LYKKEBOE, G. (1975). A fish in extreme alkalinity. *Respir. Physiol.* **24**, 163–171.

JORDAN, D. H. M. AND LLOYD, R.(1964). The resistance of rainbow trout (*Salmo gairdneri* Richardson) and roach (*Rutilus rutilus* (L.)) to alkaline solutions. *Int. J. Air Water Pollut.* **8**, 405–409.

LEVI, G., MORISI, G., COLETTI, A. AND CATANZARO, R. (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. *Comp. Biochem. Physiol.* A **49**, 623–636.

LIN, H. L. AND RANDALL, D. J. (1990). The effect of varying water pH on the acidification of expired water in rainbow trout. *J. exp. Biol.* **149**, 149–160.

MCDONALD, D. G. AND MILLIGAN, C. L. (1992). Chemical properties of the blood in Fish. In *Fish Physiology*, vol. 12 (ed. W. S. Hoar and D. J. Randall). New York: Academic Press. (in press).

MCDONALD, D. G. AND PRIOR, E. T. (1988). Branchial mechanisms of ion and acid–base regulation in the freshwater rainbow trout, *Salmo gairdneri*. *Can. J. Zool.* **66**, 2699–2708.

MOMMSEN, T. P., FRENCH, C. J. AND HOCHACHKA, P. W. (1983). Sites and patterns of protein and amino acid utilization during the spawning migration of salmon. *Can. J. Zool.* **58**, 1785–1799.

MOMMSEN, T. P. AND WALSH, P. J. (1989). Evolution of urea synthesis in vertebrates: the piscine connection. *Science* **243**, 72–75.

MOYES, C. D., MOON, T. P. AND BALLANTYNE, J. S. (1986). Oxidation of amino acids, Krebs cycle intermediates, fatty acids and ketone bodies by *Raja erincacea* liver mitochondria. *J. exp. Zool.* **237**, 119–128.

MURRAY, C. A. AND ZIEBELL, C. D. (1984). Acclimation of rainbow trout to high pH to prevent stocking mortality in summer. *Progve Fish Cult.* **46**, 176–179.

OLSON, K. R. (1992). Blood and extracellular fluid volume regulation: role of the renin angiotensin system and atrial natriuretic peptides. In *Fish Physiology*, vol. 12 (ed. W. S. Hoar and D. J. Randall). New York: Academic Press. (in press).

OLSON, K. R. AND FROMM, P. O. (1971). Excretion of urea by two teleosts exposed to different concentrations of ambient ammonia. *Comp. Biochem. Physiol.* A **40**, 999–1007.

PEQUIN, L. AND SERFATY, A. (1968). La regulation hépatique et intestinale del'ammoniéemie chez la carpe. *Archs Sci. Physiol.* **22**, 449–559.

PERRY, S. F., HASWELL, M. S., RANDALL, D. J. AND FARRELL, A. P. (1981). Branchial ionic uptake and acid–base regulation in the rainbow trout, *Salmo gairdneri. J. exp. Biol.* **92**, 289–303.

RANDALL, D. J., WOOD, C. M., PERRY, S. F., BERGMAN, H., MALOIY, G. M. O., MOMMSEN, T. P. AND WRIGHT, P. A. (1989). Urea excretion as a strategy for survival in a fish living in a very alkaline environment. *Nature* **337**, 165–166.

RANDALL, D. J. AND WRIGHT, P. A. (1989). The interaction between carbon dioxide and ammonia excretion and water pH in fish. *Can. J. Zool.* **67**, 2936–2942.

READ, L. J. (1971). The presence of high ornithine–urea cycle enzyme activity in the teleost *Opsanus tau*. *Comp. Biochem. Physiol.* B **39**, 409–413.

SAHA, N. AND RATHA, B. K. (1987). Active ureogenesis in a freshwater air-breathing teleost, *Heteropneustes fossilis. J. exp. Zool.* **241**, 137–141.

SAHA, N. AND RATHA, B. K. (1989). Comparative study of ureogenesis in freshwater air-breathing teleosts. *J. exp. Zool.* **52**, 1–8.

SKIRROW, G. (1975). The dissolved gases – carbon dioxide. In *Chemical Oceanography*, vol. 2 (ed. J. P. Riley and G. Skirrow), pp. 1–192. New York: Academic Press.

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. *J. Fish Biol.* **12**, 93–104.

SOIVIO, A. K., WESTMAN, K. AND NYHOLM, K.(1972). Improved method of dorsal aortal catheterization: haematological effects followed for three weeks in rainbow trout. *Finn. Fish Res.* **1**, 11–21.

TAKADA, Y. AND NOGUCHI, T. (1983). The degradation of urate in liver peroxisomes. *J. biol. Chem.* **258**, 4762–4764.

TAKANO, N. (1968). Role of hypocapnia in the blood lactate accumulation during acute hypocapnia. *Respir. Physiol.* **4**, 32–41.

THURSTON, R. V., RUSSO, R. C. AND SMITH, C. E. (1978). Acute toxicity of ammonia and nitrite to cutthroat trout fry. *Trans. Am. Fish Soc.* **107**, 361–368.

TURNER, J. D., WOOD, C. M. AND CLARK, D. (1983). Lactate and proton dynamics in the rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* **104**, 247–268.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY. (1985). Ambient water quality criteria for ammonia. Report no. EPA 440/5-85-001.

VERDOUW, H., VAN ECHTED, C. J. A. AND DEKKERS, E. M. J. (1978). Ammonia determination based on indophenol formation with sodium salicylate. *Water Res.* **12**, 399–402.

VIGG, S. C. AND KOCH, D. L. (1980). Upper lethal temperature range of Lahontan cutthroat trout in waters of different ionic concentration. *Trans. Am. Fish Soc.* **109**, 336–339.

WALSH, P. J., DANULAT, E. AND MOMMSEN, T. P.(1990). Variation in urea excretion in the gulf toadfish, *Opsanus beta. Mar. Biol.* **106**, 323–328.

WARD, G. R., SUTTON, J. R., JONES, N. L. AND TOEWS, C. J. (1982). Activation by exercise of human skeletal muscle pyruvate dehydrogenase *in vivo. Clin. Sci.* **63**, 87–92.

WEBB, J. T. AND BROWN, G. W., JR (1980). Glutamine synthetase: assimilatory role in liver as related to urea retention in marine chondrichthyes. *Science* **208**, 293–295.

WILKIE, M. P. AND WOOD, C. M. (1991). Nitrogenous waste excretion, acid–base regulation and ionoregulation in rainbow trout (*Oncorhynchus mykiss*) exposed to extremely alkaline water. *Physiol. Zool.* **64**, 1069–1086.

WOLF, K.(1963). Physiological salines for fresh water teleosts. *Progve Fish Cult.* **25**, 135–140.

WOOD, C. M. (1989). The physiological problems of fish in acid waters. In *Acid Toxicity and Aquatic Animals* (ed. R. Morris, E. W. Taylor, D. J. A. Brown and J. A. Brown), pp. 125–152. Cambridge: Cambridge University Press.

WOOD, C. M. AND LEMOIGNE, J. (1991). Intracellular acid–base responses to environmental hyperoxia and normoxic recovery in rainbow trout. *Respir. Physiol.* **86**, 91–113.

WOOD, C. M., MCDONALD, D. G. AND MCMAHON, B. R. (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*). *J. exp. Biol.* **96**, 221–337.

WOOD, C. M., PERRY, S. F., WRIGHT, P. A., BERGMAN, H. L. AND RANDALL, D. J. (1989). Ammonia and urea dynamics in the Lake Magadi tilapia, a ureotelic teleost fish adapted to an extremely alkaline environment. *Respir. Physiol.* **77**, 1–20.

WRIGHT, P. A., IWAMA, G. K. AND WOOD, C. M. (1993). Ammonia and urea excretion in Lahontan

cutthroat trout (*Oncorhynchus clarki henshawi*) adapted to the highly alkaline Pyramid Lake (pH9.4). *J. exp. Biol.* **175**, 153–172.

WRIGHT, P. A., PERRY, S. F., RANDALL, D. J., WOOD, C. M. AND BERGMAN, H. (1990). The effects of reducing water pH and CO_2 on teleost fish adapted to an extremely alkaline environment. *J. exp. Biol.* **151**, 361–369.

WRIGHT, P. A. AND WOOD, C. M.(1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockade. *J. exp. Biol.* **114**, 329–353.

YESAKI, T. Y. AND IWAMA, G. K. (1992). Some effects of water hardness on survival, acid–base regulation, ion regulation and ammonia excretion in rainbow trout in highly alkaline water. *Physiol. Zool.* **65**, 763–787.