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AMMONIA AND UREA EXCRETION IN LAHONTAN CUTTHROAT TROUT (*ONCORHYNCHUS CLARKI HENSHAWI*) ADAPTED TO THE HIGHLY ALKALINE PYRAMID LAKE (pH 9.4)

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Summary

Earlier studies have reported that acute exposure to alkaline pH strongly inhibits ammonia excretion in freshwater rainbow trout, but the Lahontan cutthroat trout thrives in Pyramid Lake, Nevada, at pH9.4. We investigated the rates and mechanisms of ammonia and urea excretion in this species in Pyramid Lake water to determine whether special strategies are employed to excrete nitrogenous wastes in an environment unfavourable for ammonia excretion. The majority of nitrogen wastes (N-wastes) were excreted as ammonia (56% through the gills, 10% through the kidney), while urea excretion accounted for 34% (32% gills, 2% kidney). Ammonia excretion was dependent on the NH_3 partial pressure gradient (ΔP_{NH_3}) across the gills and independent of Na^+ influx and acidification of the gill water boundary layer. Acute exposure to more alkaline water (pH10) decreased ammonia excretion by 52%, while exposure to neutral water (pH7.6) increased ammonia excretion by 200%. When fish were held in a 'closed system' for 8h, ammonia excretion decreased as water ammonia levels increased over the first 6h. However, after 6h a marked increase in ammonia excretion occurred which may have been associated with an increase in the P_{NH_3} gradient and/or activation of a carrier-mediated transporter. We conclude that Lahontan cutthroat trout, adapted to pH9.4 water, maintain N-waste excretion by modifying mechanisms common to other teleosts. These modifications include lower rates of ammonia excretion, a higher ratio of urea excretion to ammonia excretion, a higher rate of renal ammonia excretion, greater plasma pH and greater total ammonia level (increased plasma P_{NH_3}), which facilitate the diffusive excretion of NH_3 across the gills, and a lack of dependence of ammonia excretion on Na^+ influx.

Introduction

Ammonia excretion in aquatic animals is strongly dependent on environmental pH

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(Randall and Wright, 1989). In freshwater rainbow trout (*Oncorhynchus mykiss*), acute exposure to alkaline water (pH9.5) results in a severe inhibition of branchial excretion caused by a reduction or reversal of the blood-to-water P_{NH_3} gradient and a blockade of Na^+/NH_4^+ exchange (Wright and Wood, 1985). Urea excretion increases transiently and, by 48–72h, ammonia excretion recovers by mechanisms which are as yet unknown; nevertheless, plasma ammonia levels remain greatly elevated (Wilkie and Wood, 1991). Exposure to only slightly higher pH results in death (Jordan and Lloyd, 1964; Erichsen-Jones, 1964; Murray and Ziebell, 1984; Randall and Wright, 1989), while increased water hardness at high pH increases survival of trout (Yesaki and Iwama, 1992). The remarkable tilapia of Lake Magadi, Kenya (*Oreochromis alcalicus grahami*), survive in extremely alkaline water (pH10.0), but do not excrete ammonia. Instead, they detoxify ammonia and synthesize urea in the liver *via* the ornithine–urea cycle (Randall *et al.* 1989; Wood *et al.* 1989). Clearly, ammonia excretion in water above pH9 is a challenge and fish may utilize special mechanisms to maintain nitrogen excretion in severely alkaline environments. The Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*; Galat *et al.* 1985) native to Pyramid Lake, Nevada (pH9.4), not only survive at this alkaline pH, but grow to trophy size (record: 41lbs, 1925). Introductions of other salmonid species such as the rainbow trout, coho salmon (*O. kisutch*) and kokanee (*O. nerka*) have proved unsuccessful (Galat *et al.* 1985; Wheeler, 1987; Coleman and Johnson, 1988; Cerveri, 1990), suggesting that the Lahontan cutthroat has evolved physiological adaptations which permit it to tolerate Pyramid Lake's extreme environment. The composition of this terminal desert lake is unusual with a salinity of 4.4‰ and total carbon dioxide, sodium, chloride, potassium and magnesium levels 5–14 times greater than local fresh water (well water; see Table 1). Historically, the Lahontan trout spawned in the fresh water of the Truckee River, the only river flowing into the lake, but the diversion of much of its flow has rendered the breeding grounds inaccessible. Natural reproduction has essentially ceased and today the fishery is maintained by artificial propagation. Spawning fish return to a man-made stream where the eggs and sperm are air-stripped and dry-fertilized. The resulting fry are raised for approximately 12 months in well water in a hatchery. At 1 year of age, these fish are acutely transferred to ponds supplied with lake water to acclimate them for several weeks, prior to being stocked into the lake.

The objective of our study was to determine how nitrogenous wastes are excreted in Lahontan cutthroat trout adapted to Pyramid Lake water at pH9.4. In the first experiment, we measured the resting rates of ammonia and urea excretion *via* gills and kidney to test (a) whether absolute N-production rates were typical of salmonids; (b) whether a greater than normal proportion was excreted as urea; and (c) whether a greater than normal proportion was excreted by the kidney. The collection of urine in this experiment allowed analysis of urinary ionic composition, of interest in view of the reported abnormal histology of the kidney in Pyramid Lake trout (Galat *et al.* 1985). In the next set of experiments, we evaluated the possible role of the Na^+/NH_4^+ exchange mechanism in branchial ammonia excretion by exposing trout to the drug amiloride, a competitive Na^+ uptake blocker (Kirschner *et al.* 1973; Wright and Wood, 1985). In view of the Na^+-rich environment in which amiloride was used, we employed low-Na^+ artificial lake water to

confirm the result of this experiment. We also employed the carbonic anhydrase blocker acetazolamide to test whether acidification of the gill water boundary layer by CO_2 excretion played any role in branchial ammonia efflux (Wright *et al.* 1989). In the final series of experiments, we directly investigated the importance of NH_3 diffusion. The blood-to-water P_{NH_3} gradients were experimentally manipulated by acutely exposing trout to lake water of higher (pH10) and lower pH (pH7.6), and by holding fish in a 'closed system' for 8h in which water P_{NH_3} increased progressively.

Materials and methods

Experimental animals

All experiments were performed at the lakeside laboratory of the Pyramid Lake Fisheries, which kindly donated the fish and facilities used in this study. Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*; 100–281g) had been raised for approximately 1 year in well water (pH8.4; Table 1) and then transferred to large outdoor holding ponds served with flowing lake water (pH9.4; Table 1) 3–5 weeks prior to the present study, which was conducted in May, 1991. In the laboratory, the fish were held for an additional 5–10 days without food in flowing lake water at seasonal temperature (8–12°C). One day prior to experimentation, the animals were transferred to individual darkened acrylic flux chambers (McDonald and Rogano, 1986) supplied with lake water of the same temperature at $0.5\,l\,min^{-1}$, which kept water ammonia levels below $5\,\mu mol\,l^{-1}$. Mixing and air-saturated conditions were maintained by continuous bubbling of the perimeter of the flux box with air.

For some experiments, fish were surgically fitted with dorsal aortic (DA) (Soivio *et al.* 1972) or internal urinary bladder (Curtis and Wood, 1991) catheters while under MS-222 anesthesia. These fish were left to recover for 24–48h in their individual chambers prior

Table 1. *Chemical composition of well water, Pyramid Lake water and low-Na^+ artificial lake water*

Variable	Well water	Pyramid lake water	low-Na* lake water
pH	8.39	9.39	9.39
Total CO_2 ($mmol\,l^{-1}$)	4.18	19.60	18.36
Titration alkalinity to pH4 ($mmol\,l^{-1}$)	4.45	23.08	22.70
[Na^+] ($mmol\,l^{-1}$)	7.30	58.20	2.21*
[Cl^-] ($mmol\,l^{-1}$)	4.15	59.70	64.70
[K^+] ($mmol\,l^{-1}$)	0.22	2.90	8.30
[Ca^{2+}] ($mmol\,l^{-1}$)	1.25	0.15	0.34
[Mg^{2+}] ($mmol\,l^{-1}$)	0.68	5.61	6.25
[SO_4^{2-}] ($mmol\,l^{-1}$)	1.12	1.69	1.76
Total salinity (‰)	0.59	4.43	–

*Low-Na^+ (Na^+ replaced with choline). Values represent mean concentrations measured during experimental tests.

NO_3^- and phosphate levels were below detection ($<0.03 mmol\,l^{-1}$) in all samples.

to experimentation. Urine was collected into covered vials using a siphon of −300Pa. Urine was analyzed for ammonia, urea and a range of electrolytes. Blood samples (300 μl) were drawn anaerobically from the DA cannula and replaced with an equal volume of heparinized Cortland saline [Wolf, 1963; sodium heparin (Sigma); 100i.u.ml^{-1}]. Blood was analysed for pH (pHe), total plasma CO_2 content (C_{CO_2}) and total plasma ammonia (T_{amm}) and urea content (T_{urea}). At the time of blood sampling, the transepithelial potential (TEP) between body fluids and water was also determined. Three groups of experiments were performed.

Group 1: branchial versus *renal ammonia and urea excretion rates*

Trout (*N*=11) were fitted with urinary bladder catheters to allow separate measurement of branchial and renal fluxes (Wood, 1988). At the start of the experiment, inflow was stopped and water volume was reduced to approximately 3l so as to maximize the sensitivity with which changes in water ammonia and urea could be measured. Blank tests demonstrated that there was no loss of ammonia by volatilization to the air. Water samples (10ml) were taken at the start and every subsequent hour over a 3h period. Urine flow was collected over a 6–12h period. Samples were immediately frozen (−20°C) for analysis within 4 days.

Group 2: role of Na^+/NH_4^+ exchange and acidification of gill boundary layer

In these experiments, ammonia excretion was measured in fish over a 3h control period followed by a 3h experimental period. The control period was the same as described above (group 1 experiments), after which the box was flushed with fresh acclimation water and then changed to the experimental medium. This caused no apparent disturbance to the fish; changeover was complete within 5min. Water samples (10ml) were collected at the start of each 3h period and every subsequent hour for analysis of ammonia levels. Water pH was continuously monitored and maintained at pH9.4 by adding small volumes of 1mol l^{-1} KOH.

Four experiments were performed. In experiment i (amiloride), the fish were fitted with DA catheters; in experiments ii–iv, the fish were uncatheterized.

(i) Fish (*N*=9) were exposed in the external water to the drug amiloride ($C_6H_8ClN_7O$: Sigma, 10^{-4} mol l^{-1}), a sodium transport blocker, to determine the effects on ammonia excretion. Arterial blood samples and TEP measurements were taken at 0.5h and 2.5h of each 3h period. Amiloride was relatively insoluble in the alkaline lake water and it was necessary to add 0.4% ethanol to achieve complete solubility.

(ii) A control experiment was carried out in which fish (*N*=6) were exposed to lake water containing 0.4% ethanol.

(iii) Fish (*N*=8) were exposed to nominally sodium-free artificial lake water (low-Na^+ water) prepared by reconstituting deionized tapwater with salts to match approximately the composition of lake water. Sodium chloride was replaced with choline chloride. The measured composition of this low-Na^+ media is shown in Table 1. In spite of extensive flushing, it was impossible completely to eliminate the presence of Na^+ during the experimental period. Measured water Na^+ levels were 2.18±0.74mmol l^{-1} at the start and 2.24±0.78mmol l^{-1} at the end of the 3h test.

(iv) Fish (N=13) were exposed to the drug acetazolamide ($C_4H_6N_4O_3S_2$: Sigma, 1.6×10^{-3} mol l^{-1}), a specific inhibitor of carbonic anhydrase, in lake water.

Group 3: role of NH_3 diffusion

Three experiments were performed to determine the effects of changing water pH and blood-to-water ammonia gradients on ammonia and urea excretion rates.

(i) Changes in ammonia and urea excretion rates were measured in uncatheterized fish (N=5) exposed to lake water of pH10 prepared by titration with 1mol l^{-1} KOH, which raised the water K^+ level to approximately 13mmol l^{-1}. A 3h control period was followed by a 3h experimental period (pH10 water) and then a second 3h control period (i.e. recovery). Water sampling and flushing of the experimental chambers were performed as described above. Blank tests again demonstrated that losses of ammonia by volatilization over the 3h period were negligible, despite the high pH. As a precaution, water samples were acidified below pH7.0 immediately after collection, then frozen and later analyzed for ammonia and urea levels. Water pH was continuously monitored (mean=9.97±0.01) and adjusted, as necessary, with 1mol l^{-1} KOH.

(ii) Changes in ammonia and urea excretion rates and various blood variables were measured in cannulated fish (N=6) exposed to lake water of pH7.6. The experimental protocol was the same as described above for exposure to pH10 water, except that arterial blood samples and TEP measurements were taken at 0.5h and 2.5h of each 3h period. To avoid any possibility of hypercapnia in view of the high total CO_2 content of lake water, the experimental water was first decarbonated by acidification to pH4.0 with 6mol l^{-1} HCl and vigorous overnight aeration, then titrated back to experimental pH with 1mol l^{-1} KOH before introduction into the fish chambers. This reduced water total CO_2 levels to less than 1mmol l^{-1} and raised Cl^- and K^+ levels to about 83 and 7mmol l^{-1} respectively. Water pH was continuously monitored (mean=7.63±0.06) and adjusted with 1 mol l^{-1} HCl and 1mol l^{-1} KOH.

(iii) Changes in blood and water variables were monitored in cannulated fish (N=7) exposed to gradually increasing water ammonia and urea levels. This was accomplished by holding fish in a 'closed system' over an 8h period without flushing the box with fresh water. Water samples were collected hourly and blood samples and TEP measurements were taken at 0, 2, 4, 6 and 8h. Water pH was continuously monitored (mean=9.39±0.01) and adjusted with 1mol l^{-1} KOH and 1mol l^{-1} HCl.

Analytical procedures

Water pH was monitored with a glass combination electrode (Radiometer GK2401C) coupled to a Radiometer pHM 84 acid–base analyser. Water and urine ammonia levels were measured by means of the salicylate–hypochlorite assay (Verdouw *et al.* 1978) and urea levels by the diacetyl–monoxime method (Crocker, 1967) using commercial reagents (Sigma 535A). The uricase phosphotungstate assay (Henry *et al.* 1957) with commercial reagents (Sigma 680) was employed to check for the presence of uric acid in water. Urine cations were measured by atomic absorption and anions by HPLC using methods identical to those of Curtis and Wood (1991).

Whole-blood pH (pHe) was measured with a Radiometer E5021a 'gun' microelectrode regulated to the experimental temperature. Plasma was separated by centrifugation (2min at 13000 ***g***). Plasma C_{CO_2} was determined with a Corning digital CO_2 analyzer. Total plasma ammonia (T_{amm}) was assayed enzymatically with a diagnostic kit (Sigma 170A); total plasma urea ($T_{urea\text{-}N}$) was determined as above (Sigma 535A). Plasma urea values are expressed as $T_{urea\text{-}N}$ to account for two nitrogen molecules per urea molecule.

TEP between arterial blood and the environment was determined by means of 3mol l^{-1} KCl–agar bridges connected *via* Ag/AgCl electrodes to a high-impedance voltmeter (Perry and Wood, 1985). The reference electrode was placed in the water in the fish chamber and the measurement electrode was connected to the DA cannula. The system was zeroed by initially placing both electrode KCl agar tips in the water. The offset voltage was less than 2mV.

Calculations and statistics

The blood-to-water partial pressure gradient for NH_3 (ΔP_{NH_3}) was calculated from plasma and water total ammonia concentrations and pH using appropriate constants from Cameron and Heisler (1983) and equations outlined in Wright and Wood (1985), with one exception. In the present study, plasma P_{NH_3} levels were calculated from arterial values, whereas, in our previous study (Wright and Wood, 1985), arterial values were adjusted to account for higher ammonia levels in the venous blood. The blood-to-water electrochemical gradient for NH_4^+ (ΔNH_4^+) was calculated from plasma and blood NH_4^+ concentrations (see Wright and Wood, 1985) and TEP, according to the following equation:

$$\text{Net driving force for } NH_4^+ = \text{TEP} - E_{NH_4},$$

where

$$E_{NH_4} = \frac{\boldsymbol{R}T \ln [NH_4^+]_{water}}{ZF\,[NH_4^+]_{plasma}}$$

and $\boldsymbol{R}$, F, T and Z have their usual meaning.

Net fluxes of ammonia (J_{amm}) and urea (J_{urea}) were calculated as:

$$J^{net} = \frac{(T_i - T_f)V}{tW},$$

where T_i and T_f refer to initial and final concentrations of water ammonia or urea in μmol l^{-1}, V is the volume of the system in ml (corrected for sampling deficits), t is the elapsed time in h and W is the fish mass in kg. While J_{amm} was measured over 1 h intervals, J_{urea} was determined over 2 or 3h because changes in water T_{urea} were not large enough to be detected reliably over 1h.

Renal flux rates of ammonia, urea and electrolytes were calculated as the product of urine flow rate (UFR) and urinary concentration.

Values are reported as means ± 1 S.E.M. (N), where N represents the number of fish employed in the experiment. Student's two-tailed paired t-tests were used to evaluate the significance of differences between mean values ($P<0.05$).

Results

UFR and urinary ion excretion

Table 2 compares UFR and urinary excretion rates of Lahontan cuthroat trout in Pyramid Lake water with typical values for rainbow trout in fresh water, obtained with identical methods by Curtis and Wood (1991). Relative to rainbow trout, Lahontan cutthroat trout had lower UFR values and much higher excretion rates for most electrolytes, but especially ammonia, Na^+, Cl^-, Mg^{2+}, SO_4^{2-}, NO_3^- and PO_4^{2-}.

Branchial versus *renal ammonia and urea excretion rates*

Assuming that the sum of ammonia-N and urea-N represents total N-excretion in Lahontan cutthroat trout, overall rates were typically 100–200 $\mu mol kg^{-1} h^{-1}$ (Fig. 1). The excretion of uric acid, if it occurred, was undetectable (i.e. <15 $\mu mol\,N\,kg^{-1} h^{-1}$). The majority of N-waste was excreted across the gills as ammonia (56%, Fig. 1) but renal ammonia excretion was also important (10%). Urea-N excretion across the gills also accounted for a very significant portion (32%), while renal urea-N efflux contributed an additional 2%.

Role of Na^+/NH_4^+ exchange and acid gill boundary water layer

J_{amm} did not vary significantly over 3h in either control or experimental periods; therefore, average values for each 3h period are presented in Table 3.

It was necessary to pre-dissolve amiloride in ethanol, resulting in a final ethanol concentration of 0.4% in the experimental water. Therefore, initial control experiments were performed to test the effects of ethanol alone. Ammonia excretion was unchanged in control fish exposed to trace levels of ethanol (0.4%) in lake water (Table 3). Amiloride (10^{-4} mol l^{-1}), a Na^+ uptake blocker, had no effect on ammonia excretion when added to

Table 2. *Urine flow and urinary excretion rates of Lahontan cutthroat trout in Pyramid Lake water and rainbow trout in fresh water*

Variable	Lahontan cutthroat trout	Rainbow trout*
Urinary flow rate ($ml kg^{-1} h^{-1}$)	2.20±0.28 (11)	3.11±0.22 (19)
Urinary excretion rate		
Na^+ ($mmol kg^{-1} h^{-1}$)	88.37±18.85 (11)	22.14±2.45 (16)
K^+ ($mmol kg^{-1} h^{-1}$)	3.05±1.01 (11)	2.86±0.28 (16)
Ammonia ($mmol kg^{-1} h^{-1}$)	17.23±4.83 (11)	2.46±0.40 (16)
Ca^{2+} ($mmol kg^{-1} h^{-1}$)	3.36±0.60 (11)	4.20±0.68 (16)
Mg^{2+} ($mmol kg^{-1} h^{-1}$)	7.88±1.43 (11)	1.56±0.34 (16)
Cl^- ($mmol kg^{-1} h^{-1}$)	65.57±12.84 (11)	16.20±1.06 (16)
NO_3^{2-} ($mmol kg^{-1} h^{-1}$)	2.11±0.69 (7)	0.16±0.06 (16)
SO_4^{2-} ($mmol kg^{-1} h^{-1}$)	7.35±1.74 (11)	3.36±0.31 (16)
Phosphate ($mmol kg^{-1} h^{-1}$)	7.98±2.83 (9)	1.24±0.72 (16)
Urea ($mmol kg^{-1} h^{-1}$)	1.48±0.25 (11)	2.52±0.50 (15)

Means ± 1 S.E.M. (*N*).
*Rainbow trout data from Curtis and Wood (1991).

the water in the experimental period. Furthermore, ammonia excretion was unaffected by exposure to low-Na^+ lake water, indicating that Na^+-dependent ammonia excretion (Na^+/NH_4^+ exchange) is not an important component of net ammonia excretion in Lahontan cutthroat trout (Table 3).

Blood measurements made during the amiloride experiment (temperature=9°C) are summarized in Table 4. Blood pH was about 8.15, plasma C_{CO_2} about 8mmol l^{-1}, plasma T_{amm} between 190 and 270 μmol l^{-1}, plasma $T_{urea\text{-}N}$ about 30- to 50-fold greater than T_{amm}, and TEP slightly negative (about −5 mV) with respect to the environmental water. Plasma T_{amm} increased significantly during both control and experimental periods. None of these variables was affected by the amiloride treatment.

Acetazolamide (1.6×10^{-3} mol l^{-1}), a carbonic anhydrase inhibitor, was added to the water in the experimental period to test the importance of acidification of the gill water boundary layer. If carbonic-anhydrase-dependent CO_2 hydration in the gill epithelial boundary layer significantly lowers water pH near the gill surface, then acetazolamide should reduce acidification and inhibit ammonia excretion, at least temporarily. However, ammonia excretion was unchanged by the acetazolamide treatment, indicating that acidification of the boundary layer is not critical to the maintenance of ammonia excretion rates (Table 3).

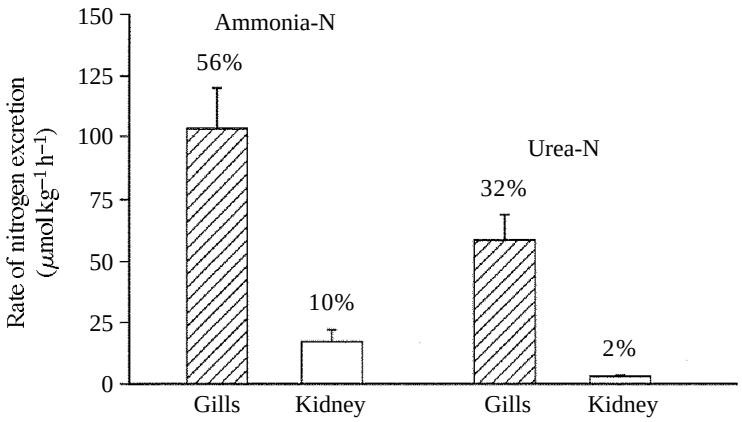

Fig. 1. The partitioning of ammonia and urea excretion between the gills and kidneys of Lahontan cutthroat trout adapted to lake water at pH9.4. Means ± S.E. (*N*=11).

Table 3. *Net ammonia excretion (μmol N kg^{-1} h^{-1}) during a 3h control period followed by a 3h experimental period*

Treatment	Control	Experimental
Control (0.4% ethanol)	72.1±7.7 (6)	64.7±6.9 (6)
Amiloride (10^{-4} mol l^{-1}) + 0.4% ethanol	105.8±15.1 (9)	107.3±12.0 (9)
Low-Na^+ lake water	116.3±16.6 (8)	109.7±14.1 (6)
Acetazolamide (1.6×10^{-3} mol l^{-1})	79.8±7.0 (13)	79.8±9.1 (13)

Means ± 1 S.E.M. (*N*).

Role of NH_3 diffusion

Exposure to pH10 water

If ammonia excretion is dependent on passive diffusion of NH_3, then one would predict that acute changes in external water pH would alter the rate of ammonia excretion. The ammonia excretion rate was significantly decreased (−52%) over 3h of exposure to pH10 water compared to the control value (Fig. 2A). When rates were compared on an hourly basis, however, the inhibition of ammonia excretion was significantly different from the control value only in the final hour. Ammonia excretion returned to control rates in the recovery period. Urea excretion was unaffected by the experimental treatment (Fig. 2B).

Exposure to pH7.6 water

Ammonia excretion increased threefold in response to exposure to pH7.6 water, with the greatest stimulation occurring during the first hour (Fig. 2C). In the subsequent recovery period, ammonia excretion returned to control rates. Urea excretion was unaffected by pH7.6 water (Fig. 2D).

Plasma T_{amm} decreased significantly during the experimental period, while water T_{amm} increased as a result of enhanced ammonia excretion rates (Fig. 3). The blood-to-water P_{NH_3} gradient (ΔP_{NH_3}) at 0.5 and 2.5h during the experimental period was unchanged. The estimated P_{NH_3} gradient (Fig. 4, see below) at 0h, however, may give a better indication of the immediate effects of pH7.6 water. The electrochemical gradient for NH_4^+ (ΔNH_4^+) was significantly decreased during pH7.6 exposure, with negative values in the last hour of the experimental period, indicating a reversal of the gradient (Fig. 3).

Fish became slightly acidotic during exposure to pH7.6 water (temperature=9.5°C), as pHe decreased by about 0.15pHunits (Table 5). Total CO_2 (C_{CO_2}) levels decreased by approximately 1mmol l^{-1}. During the recovery period, pH and C_{CO_2} were not significantly different from control values. TEP became more negative by about −4 mV during exposure to pH7.6; it returned to control values during recovery.

Table 4. *Arterial blood pH, plasma total CO_2, ammonia, urea levels and transepithelial potential (TEP) between body fluids and water during the control period and during treatment with amiloride* (10^{-4} mol l^{-1})

	Control		Amiloride	
Variable	0.5 h	2.5 h	0.5 h	2.5 h
pHe	8.129±0.036	8.167±0.033	8.106±0.023	8.153±0.031
C_{CO_2} (mmol l^{-1})	8.42±0.81	8.52±0.73	8.19±0.77	8.29±0.68
T_{amm} (μmol l^{-1})	168±12	255±22*	192±11	268±29*
$T_{urea\text{-}N}$ (mmol l^{-1})	8.15±1.45	8.22±1.31	7.82±1.58	7.45±1.27
TEP (mV)	−5.4±0.6	−5.1±0.3	−4.8±0.4	−4.5±0.4

Means ± 1 S.E.M. (N=8).

There were no significant differences between the control and amiloride means at corresponding times.

*Significantly different ($P<0.05$) from the corresponding 0.5h value.

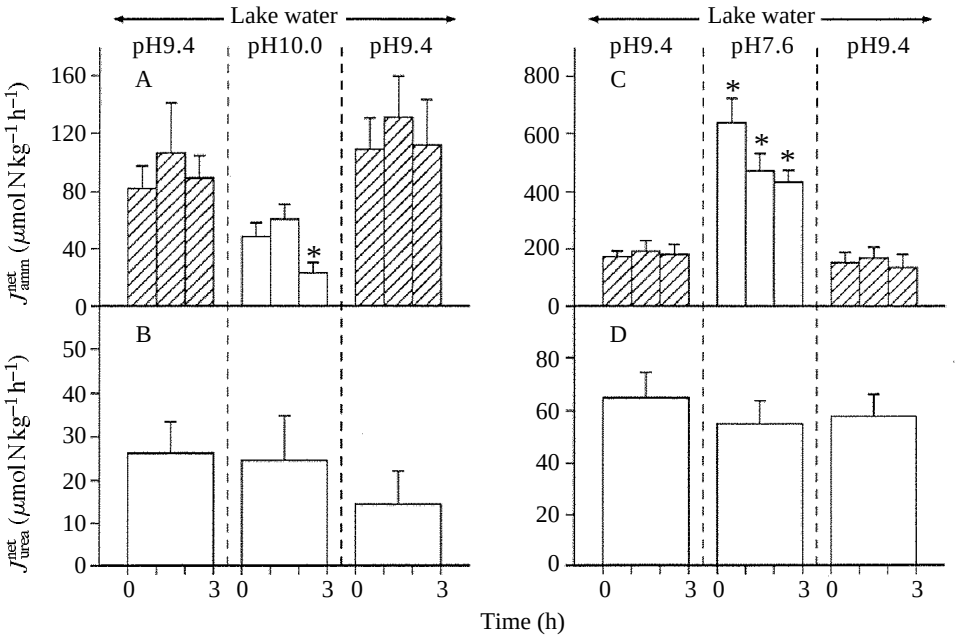

Fig. 2. The influence of altered water pH on ammonia (J_{amm}) excretion (A,C) and urea (J_{urea}) excretion (B,D) in Lahontan cutthroat trout. A 3h control period was followed by a 3h experimental period during which fish were exposed to either pH10 water (A,B; N=5) or pH7.6 water (C,D; N=6) and then recovered in lake water for a subsequent 3h period. Means + S.E. Asterisks denote a significant difference (P<0.05) from the corresponding control value. Water changeovers between treatments were complete within 5min.

Fig. 4 superimposes the calculated blood-to-water P_{NH_3} gradient on the hourly ammonia excretion rates. The P_{NH_3} gradients at 0h of the experimental and recovery period were estimated because it was apparent that the gradient at 0.5h (Figs 3 and 4) was not representative of the P_{NH_3} gradient immediately after exposure to water of a different pH. For example, blood samples taken 0.5h into the experimental period were already depleted of ammonia (Fig. 3) because of the rapid loss from the blood into the water. The estimated P_{NH_3} gradient at 0h of the experimental and recovery periods was conservatively calculated using the plasma P_{NH_3} level at 2.5h of the preceding period and the water P_{NH_3} value at 0h of the period in question. The estimated P_{NH_3} gradient at 0h of the experimental period (24.3±3.3mPa) is probably more representative of the initial gradient upon exposure to pH7.6 water, compared to the gradient at time 0.5h (7.5±0.9mPa). Similarly, the estimated P_{NH_3} gradient at 0h of the recovery period is lower (−5.6±2.1mPa) and therefore probably more realistic than the value at 0.5h (3.1±1.9mPa), in view of the low plasma T_{amm} at the end of the experimental period (Fig. 4). Changes in ammonia excretion rate appear to be associated with changes in the P_{NH_3} gradient when fish were initially exposed to pH7.6 water and recovery water (pH9.4). Between 0.5 and 2.5h of the experimental period, however, J_{amm} was significantly elevated despite a return of the P_{NH_3} gradient to control values. It may be

that carrier-mediated transport mechanisms were also involved in stimulating ammonia excretion during exposure to pH7.6 water.

Closed-system experiment

Ammonia excretion exhibited a step-wise decrease over the first 6h when fish were

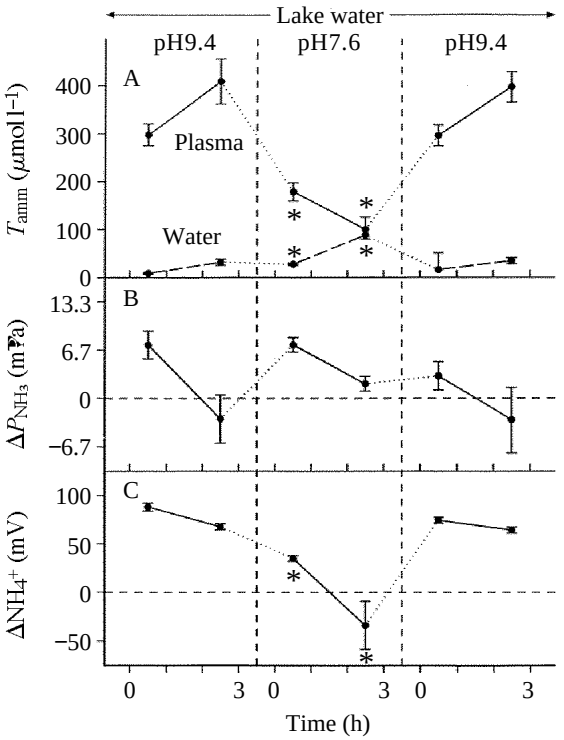

Fig. 3. The time course of changes in selected variables related to branchial ammonia excretion in Lahontan cutthroat trout exposed to pH7.6 water. (A) Total ammonia levels (T_{amm}) in plasma (solid lines) and water (dashed lines). (B) Calculated partial pressure gradient for NH_3 (ΔP_{NH_3}, blood-to-water) across the gills. (C) Calculated NH_4^+ electrochemical gradient (ΔNH_4^+, blood-to-water) across the gills. Means ± S.E. (N=6). Asterisks represent significant difference (P<0.05) from corresponding control value.

Table 5. *Arterial blood pH, plasma total CO_2 and transepithelial potential (TEP) across the gills during the control period and during exposure to pH7.6 water*

	Control		Experimental (pH7.6)		Recovery	
Variable	0.5 h	2.5 h	0.5 h	2.5 h	0.5 h	2.5 h
pHe	8.042±0.027	8.116±0.019	8.002±0.031	7.961±0.027*	8.104±0.019	8.156±0.017
C_{CO_2} (mmol l^{-1})	7.77±0.74	7.25±0.36	6.35±0.24*	6.16±0.41*	8.11±0.51	8.11±0.51
TEP (mV)	−5.7±0.4	−5.1±0.7	−9.7±1.0*	−10.3±1.9*	−6.2±1.0	−

Means ± 1 S.E.M. (N=6).
*Significantly different (P<0.05) from the corresponding value.

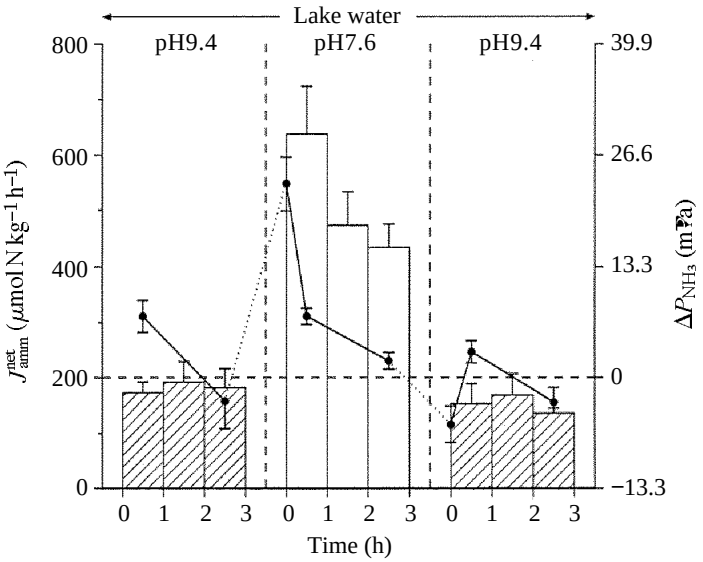

Fig. 4. The relationship between total ammonia excretion (J_{amm}, bars) and the NH_3 partial pressure gradient (ΔP_{NH_3} across the gills, line) in Lahontan cutthroat trout exposed to pH7.6 water. Means ± S.E. (N=6). P_{NH_3} values at 0h of the experimental and recovery periods were estimated as described in the Results.

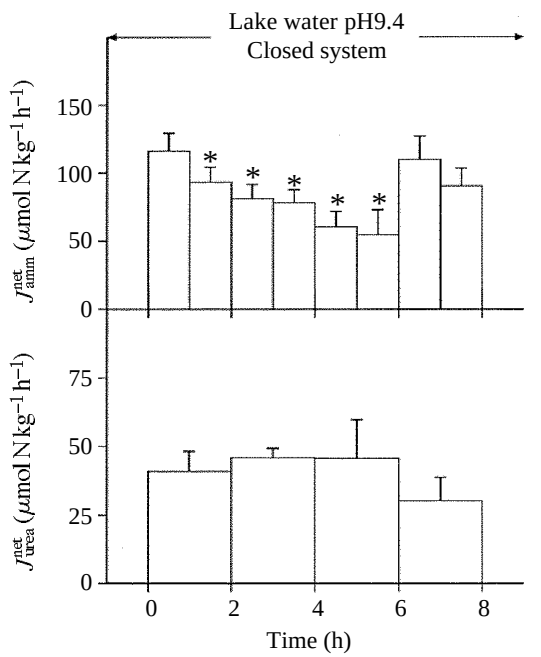

Fig. 5. The time course of changes in ammonia (J_{amm}) and urea excretion (J_{urea}) rates in Lahontan cutthroat trout held in a closed system for 8h. Means ± S.E. (N=7). An asterisk denotes a significant difference (P<0.05) from the control value (0–1h).

held in a ‘closed system’ (Fig. 5). From 6 to 8h, however, there was a sharp increase in ammonia excretion rates to levels comparable to initial excretion values (0–1h). As in other experimental treatments, urea excretion rates were not significantly changed (Fig. 5).

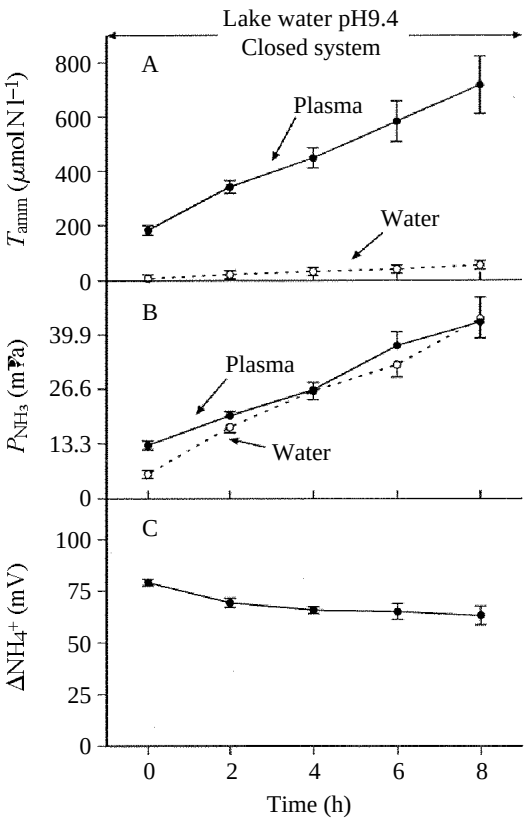

Fig. 6. The time course of changes in variables related to ammonia excretion in Lahontan cutthroat trout held in a closed system for 8h. (A) Total ammonia concentration (T_{amm}) in the plasma (solid line) and water (dotted line). (B) NH_3 partial pressure (ΔP_{NH_3}) in plasma (solid line) and water (dotted line). (C) NH_4^+ electrochemical gradient (ΔNH_4^+, blood-to-water) across the gills. Means ± S.E. (N=7). All values at 2–8h were significantly different (P<0.05) from control values (0h).

Table 6. *Acid–base status of fish in the ‘closed system’ experiment*

	Time				
Variable	0h	2h	4h	6h	8h
pHe	8.211±0.017	8.131±0.020*	8.132±0.014*	8.173±0.023	8.150±0.028
C_{CO_2} (mmol l^{-1})	8.3±0.8	8.1±0.8	7.5±0.7	7.1±0.5	7.1±0.8
TEP (mV)	−6±1	−6±1	−6±1	−8±1*	−7±1

Means ± 1 S.E.M. (N=7).
*Significantly different (P<0.05) from the control value at 0h.

Plasma T_{amm} increased in a linear manner, from 183±17 μmol l^{-1} at 0h to 716±106 μmol l^{-1} after 8h (Fig. 6). Likewise, water T_{amm} increased linearly, but accumulation after 8h was only 56±6 μmol l^{-1}. Plasma and water P_{NH_3} levels increased in parallel over the experimental treatment (Fig. 6). There was a small, progressive decrease in the electrochemical gradient for NH_4^+ over the 8h period from 79.2±1.6mV to 63.2±3.9mV.

There was a significant blood acidosis in the first 4h of the experiment (temperature 10°C; Table 6). Plasma C_{CO_2} was not significantly affected by the experimental treatment. TEP increased very slightly at 6h, but was not significantly different from the initial value by 8h.

Fig. 7 illustrates the blood-to-water P_{NH_3} gradient and ammonia excretion rates. Plasma P_{NH_3} levels were calculated based on blood samples collected at 0, 2, 4, 6 and 8h. Hourly plasma P_{NH_3} levels were estimated by linear interpolation. Over the first 5h of the 'closed system' experiment, the P_{NH_3} gradient and ammonia excretion rates both decreased over time. The marked increase in J_{amm} after 6h was associated with an increase in the mean P_{NH_3} gradient, but the data were highly variable.

Discussion

Partitioning of ammonia and urea excretion between the gills and kidneys

Most teleost fish excrete the majority of their nitrogen wastes as ammonia across the gills. Acute exposure to alkaline water (pH9.5) in rainbow trout inhibits ammonia excretion (Wright and Wood, 1985). One possible way to maintain nitrogen excretion in

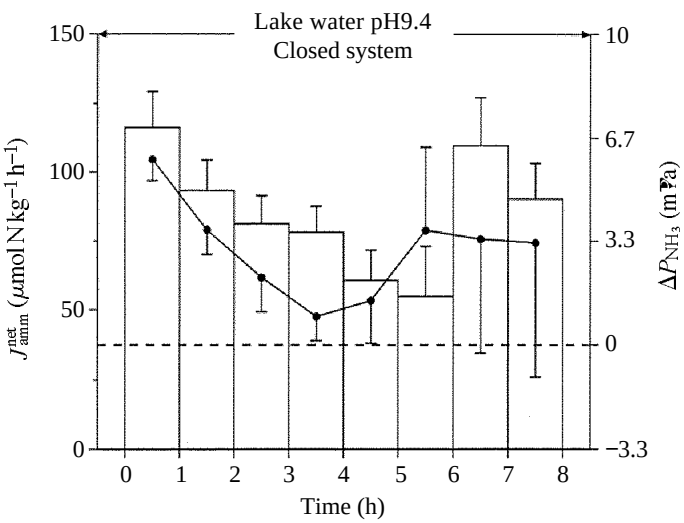

Fig. 7. The relationship between ammonia excretion (J_{amm}, bars) and the NH_3 partial pressure gradient (ΔP_{NH_3}, line) across the gills in Lahontan cutthroat trout held in a closed system over 8 h. Means ± S.E. (N=7). Hourly P_{NH_3} gradients were estimated as described in the Results. Refer to Fig. 5 for significant changes in J_{amm}. The P_{NH_3} gradients measured from 3–4h and from 4–5h were significantly different from the control value (0–1h) (P<0.05).

alkaline water would be to increase the proportion of nitrogen wastes excreted as urea. In freshwater rainbow trout, urea excretion accounts for approximately 13% of total nitrogen excretion, with the remainder being excreted as ammonia (Olson and Fromm, 1971; Wilkie and Wood, 1991). In contrast, the tilapia of Lake Magadi, *Oreochromis alcalicus grahami*, excrete 100% of their nitrogen wastes as urea, presumably an adaptation for survival in water of pH10 (Randall *et al.* 1989; Wood *et al.* 1989). Lahontan cutthroat trout adapted to pH9.4 water excreted 34% of their nitrogen wastes as urea (Fig. 1), whereas those adapted to neutral water excreted only 10% of their nitrogen wastes as urea (M. Wilkie, G. K. Iwama, P. A. Wright and C. M. Wood, in preparation). It is noteworthy that the absolute rates of urea excretion in the Lahontan cutthroat trout at pH9.4 were very similar to those reported by Wilkie and Wood (1991) for rainbow trout in neutral water. The relative rate of urea *versus* ammonia excretion, however, was much higher in the cutthroat trout because the absolute rate of ammonia excretion was about 50% lower compared to rates in the rainbow trout and about 70% lower compared to rates in Lahontan cutthroat trout acclimated to well water (M. Wilkie, G. K. Iwama, P. A. Wright and C. M. Wood, in preparation). Hence, one strategy for effectively excreting N-wastes in the alkaline waters of Pyramid Lake is to decrease the rate of ammonia excretion and to increase the relative proportion of nitrogen wastes excreted as urea. Urea excretion rate, however, was not regulated with acute changes in water pH (Fig. 2) or elevated water ammonia and urea levels (Fig. 5), although it increased significantly during longer-term exposure to pH10 in the accompanying study (Wilkie *et al.* 1993).

The pathway for urea synthesis in the tilapia *O. a. grahami* (Randall *et al.* 1989), the toadfish *Opsanus beta* (Read, 1971; Mommsen and Walsh, 1989), the freshwater air-breathing teleost *Heteropneustes fossilis* (Saha and Ratha, 1986) and higher vertebrates is the ornithine–urea cycle. The majority of teleosts, however, synthesize urea at relatively low rates through the metabolism of purines (uricolysis) or arginine. In the accompanying study, we have shown that Lahontan cutthroat trout are typical of other teleosts in that they have low activities of the ornithine–urea cycle enzymes but significant activities of uricase, allantoicase and allantoinase, the three enzymes involved in uricolysis (Wilkie *et al.* 1993).

In the freshwater rainbow trout, renal ammonia excretion accounts for only about 2% of total ammonia excretion, with the remainder excreted across the gills (McDonald and Wood, 1981; Wood, 1988; Curtis and Wood, 1991). In the present study, the renal component of ammonia excretion in Lahontan cutthroat trout was 14% (Fig. 1; 10% of total nitrogen excretion), a significantly greater value than that of the rainbow trout. Therefore, the greater role of the kidneys in ammonia excretion in Lahontan cutthroat trout on both an absolute and relative basis (see Table 2) may be another adaptation to maintain nitrogen excretion in a severely alkaline environment. Further work will be required to determine whether this difference results from a modification of active (e.g. ionic secretion, renal ammoniagenesis) or passive (e.g. filtration, diffusive secretion) mechanisms for renal ammonia excretion. Interestingly, there appears to be no accompanying modification of renal urea excretion (Table 2).

The lower UFR of Lahontan cutthroat trout relative to rainbow trout in fresh water (Table 2) clearly reflected the lower osmotic gradient for water entry at the gills in

Pyramid Lake water, which is equivalent to about 13% sea water (Table 1). Despite this lower UFR, the kidney made a much larger contribution to the excretion of most ions (Table 2). These higher excretion rates (for Na^+, Cl^- and Mg^{2+}) reflected the unusual saline conditions of Pyramid Lake, in which levels of all three ions are markedly elevated (Table 1), whereas the more normal excretion rates for Ca^{2+} and K^+ were correlated with the relatively low levels of these ions in the water. However, the higher rates of phosphate and NO_3^- excretion in the urine of Lahontan trout did not reflect water composition, for levels of these electrolytes were below detection in Pyramid Lake water. Finally, the higher SO_4^{2-} excretion is noteworthy, because Galat *et al.* (1985) correlated water SO_4^{2-} concentration with glomerular swelling, congestion and deposition of hyalin in the kidney tubules in Lahontan trout sampled from Pyramid and other alkaline lakes in the western United States. SO_4^{2-}concentration was only moderately elevated in Pyramid Lake water relative to most standard fresh waters (Table 1).

Mechanisms of ammonia excretion

In most teleosts, branchial ammonia excretion depends largely on the transepithelial NH_3 gradient (deVooys, 1968; Kormanik and Cameron, 1981; Cameron and Heisler, 1983; Holeton *et al.* 1983; Wright and Wood, 1985; Claiborne and Evans, 1988; Heisler, 1989). There is also evidence for a carrier-mediated transport of NH_4^+ *via* the electroneutral Na^+/NH_4^+ exchanger (Maetz and Garcia-Romeu, 1964; Kerstetter and Keeler, 1976; Payan, 1978; Wright and Wood, 1985), especially when the NH_3 gradient is directed inwards (Cameron and Heisler, 1983). In freshwater rainbow trout, acute exposure to alkaline water (pH9.5) strongly inhibited both ammonia excretion and Na^+ influx (Wright and Wood, 1985). Water Na^+ levels (Table 1) are much higher in Pyramid Lake water, which might facilitate Na^+/NH_4^+ exchange at high pH. Our results, however, indicate that the Na^+/NH_4^+ exchanger does not play a role in branchial ammonia excretion. Treatment with the Na^+ uptake blocker amiloride or exposure to low-Na^+ water had no effect on ammonia excretion (Table 3). In as much as our low-Na^+ water still contained about 2.2mmol l^{-1} of Na^+, we cannot eliminate the possibility that a high-affinity Na^+/NH_4^+ exchanger may have persisted. However, in freshwater rainbow trout acclimated to 0.6mmol l^{-1} Na^+, the K_m for Na^+ uptake was about 0.1mmol l^{-1} (Goss and Wood, 1991). K_m generally scales with acclimation Na^+ concentration, so the K_m for the present fish was probably about 10mmol l^{-1}, well above the concentration present in the low-Na^+ water.

Another possible strategy to facilitate branchial ammonia excretion in alkaline water would be to enhance the passive loss of NH_3 across the gills by acidification of the water near the gill surface. Carbonic anhydrase on the gill surface (Rahim *et al.* 1988) catalyses the hydration of excreted molecular CO_2 to HCO_3^- and H^+ and lowers the pH of the gill water boundary layer (Wright *et al.* 1986). Acetazolamide (1.6×10^{-3} mol l^{-1}), a carbonic anhydrase inhibitor, has been shown to reduce branchial ammonia excretion significantly in the freshwater rainbow trout (Wright *et al.* 1989). In contrast, acetazolamide had no effect on ammonia excretion rates in the present study (Table 3). Possibly, carbonic anhydrase is absent from the gill surface but, more likely, acidification

of the gill water boundary layer is relatively small because of the high buffering capacity of the Pyramid Lake water.

If ammonia excretion is mostly due to the passive loss of NH_3 across the gills, then NH_3 levels in the plasma must surpass water NH_3 levels to maintain the blood-to-water P_{NH_3} diffusion gradients. Plasma P_{NH_3} levels depend on plasma pH and T_{amm}. Control blood pH (about 0.3pHunits higher) and plasma T_{amm} (about 100 $\mu mol\,l^{-1}$ higher) were both moderately elevated in the Lahontan cutthroat trout (Tables 4–6, Figs 3, 6) compared to the rainbow trout in neutral water (Wright and Wood, 1985; Wilkie and Wood, 1991), ensuring a positive P_{NH_3} gradient across the gills despite the high external pH of 9.4 (Figs 3, 4, 6). If ammonia excretion is dependent on this P_{NH_3} gradient, then acute changes in water pH should alter branchial ammonia excretion. Ammonia excretion was inhibited by 50% in fish acutely exposed to pH10 water (Fig. 2). Upon exposure to more acidic water (pH7.6), ammonia excretion rates increased threefold (Fig. 2). There was a sharp increase in the blood-to-water P_{NH_3} gradient when fish were initially exposed to pH7.6 water (0h, Fig. 4), coincident with a peak increase in ammonia excretion rates (0–1h, Fig. 4). Ammonia was effectively 'washed out' of the fish during the experimental period (0h, Fig. 3A), resulting in a marked reduction in both the P_{NH_3} gradient and ammonia excretion rate in the initial hour of the recovery period. The observation that J_{amm} remained significantly elevated over control rates throughout the 3h of exposure to pH7.6 water, despite a return of the P_{NH_3} gradient to control values, implies that mechanisms in addition to NH_3 diffusion were employed. One possibility is that a carrier-mediated NH_4^+ mechanism was involved. Indeed, the increased availability of external H^+ may have facilitated NH_4^+ excretion *via* the putative H^+/NH_4^+ branchial transporter (Cameron, 1986). Clearly, further studies are required to identify the possible role of a Na^+-independent NH_4^+ transporter in these fish.

The NH_4^+ electrochemical gradient was inversely correlated with ammonia excretion in fish exposed to pH7.6 water (Fig. 3). These results confirm previous findings that passive loss of NH_4^+ is not a significant component of net ammonia excretion (Wright and Wood, 1985).

To investigate further the relationship between ammonia excretion and the P_{NH_3} gradient, fish were held in a closed system for 8h, resulting in a gradual build up of ammonia and urea levels in the water. There was a step-wise decrease in ammonia excretion in the first 6h of the experiment (Fig. 5), concomitant with a progressive increase in plasma total ammonia levels (Fig. 6) and a decrease in the P_{NH_3} gradient (Fig. 7). Surprisingly, ammonia excretion rates returned to 'control' rates (i.e. the 0–1 h value) after 6h, despite the fact that water total ammonia levels continued to rise. Moreover, the increase in ammonia excretion after 6h did not attenuate the accumulation of ammonia in the plasma. This may be due to changes in gill epithelial permeability or transport, in ammonia production rates, or to a redistribution of ammonia from intracellular stores to the extracellular compartment. The increase in ammonia excretion rates after 6h was associated with an increase in the P_{NH_3} gradient in some fish (Fig. 7). It may be that individual animals employed different mechanisms to restore ammonia excretion rates following several hours of depressed excretion while held in a closed system (Fig. 7). In some fish, a component of the increase in ammonia excretion after 6 h

may have been due to a stimulation of carrier-mediated NH_4^+ transport. Although ammonia excretion was independent of Na^+ influx under conditions of normal plasma and water ammonia (Table 1), it may be that the Na^+/NH_4^+ exchange plays a role in net ammonia excretion when blood T_{amm} is elevated. In this regard, Wilkie *et al.* (1993) report that, during chronic exposure of Lahontan cutthroat trout to pH10 water, ammonia excretion was initially inhibited but later recovered and increased above control levels, at a time when plasma T_{amm} was persistently elevated. Furthermore, Yesaki and Iwama (1992) showed that, when rainbow trout were acutely transferred from neutral soft water to alkaline (pH10) hard water, ammonia excretion rates decreased, while plasma T_{amm} increased. The addition of amiloride to the alkaline hard water completely eliminated ammonia excretion over a 3h period, suggesting that, under these conditions, ammonia was excreted through a Na^+-related mechanism (Yesaki and Iwama, 1992). There is also evidence in channel catfish (*Ictalurus punctatus*) that ammonia is excreted through an ion-exchange mechanism when water ammonia levels are elevated (Cameron, 1986). In Cameron's study, ammonia excretion was independent of Na^+, but appeared to be linked with H^+ efflux. Thus, the increase in ammonia excretion in cutthroat trout following 6h in a 'closed system' may be explained, in part, by an increase in the transepithelial P_{NH_3} gradient and/or the induction of a carrier-mediated NH_4^+ efflux mechanism.

Mechanisms of nitrogen excretion in the Lahontan cutthroat trout adapted to pH9.4 water

To conclude, the Lahontan cutthroat trout do not employ any unusual physiological strategies for nitrogen excretion in alkaline water, but instead have modified processes typical of other teleosts. These modifications include lower rates of total ammonia excretion, a higher rate of renal ammonia excretion and a greater percentage of total nitrogen wastes excreted as urea compared to fish in neutral water. Ammonia excretion in Lahontan cutthroat trout is primarily dependent on the blood-to-water P_{NH_3} gradient. To overcome elevated water P_{NH_3} levels, plasma pH and T_{amm} are slightly elevated compared to those of fish in neutral water. Changes in ammonia excretion with changes in water pH and ammonia levels were associated, in part, with the transepithelial P_{NH_3} gradient, but carrier-mediated NH_4^+ mechanisms may also play a role. Ammonia excretion is not dependent on Na^+ influx or acidification of the gill water boundary layer under control conditions. These modifications are apparently not energetically costly, as Lahontan cutthroat trout grow to trophy size and support an active sport fishing industry.

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