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Divalent cations enhance ammonia excretion in Lahontan cutthroat trout in highly alkaline water

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The Lahontan cutthroat trout lives under highly alkaline and saline conditions in Pyramid Lake, Nevada (pH 9·4; 0·2 mmol l^{-1} Ca^{++}; 7·3 mmol l^{-1} Mg^{++}). These experiments were conducted to study the possible roles of water Ca^{++} and Mg^{++} concentrations on ammonia excretion in the Lahontan cutthroat trout under highly alkaline conditions. The basic protocol of the experiments was to determine ammonia excretion rates during the following three exposure periods (each of 3-h duration) in sequence: (a) in normal lake water; (b) in soft lake water with the divalent cation concentrations reduced; and (c) in the soft lake water with either Ca^{++} or Mg^{++} (or no divalent cations added) added back at the appropriate lake water concentration. The soft-water exposure caused a significant reduction in ammonia excretion to about half of the control (original lake water) levels. When either Ca^{++} or Mg^{++} was added to the soft water in the third exposure period, the ammonia excretion rates were increased more than twofold back to lake water levels.

Key words: alkaline lakes; Lahontan cutthroat trout, *Oncorhynchus clarki henshawi*; ammonia excretion; divalent cations; calcium; magnesium.

INTRODUCTION

Ammonia is produced at greater rates than urea in most teleosts. Ammonia excretion (Jamm) occurs predominantly across the gill epithelium in the dissolved gas form (NH_3), although significant quantities can be excreted in the ionic form, NH_4^+. It has been shown in acute laboratory experiments, lasting several hours, that water pH has a dramatic effect on total Jamm in fishes (Randall & Wright, 1989); Jamm is inhibited in alkaline water (pH 9·5), due to a reversal of the ammonia partial pressure (PNH_3) gradient, and possibly also a blockade of branchial Na^+/NH_4^+ exchange (Wright & Wood, 1985). Elevating water pH above 9·5 has been shown to be toxic to fish (Erichsen-Jones, 1964; Murray & Siebell, 1984; Randall & Wright, 1989; Yesaki & Iwama, 1992). Under more chronic conditions, Wilkie & Wood (1991) showed in rainbow trout *Oncorhynchus mykiss* (Walbaum) exposed to pH 9·5, a recovery of ammonia excretion rate after 48–72 h, and that there was a transient increase in urea excretion. The mechanisms by which such recovery occurs are not yet fully understood, though a restoration of the outwardly directed PNH_3 gradient seems to play an important role.

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In contrast to the results of the laboratory experiments described above with rainbow trout, there are natural fish populations that live under highly alkaline conditions. A species of tilapia *Oreochromis alcalicus grahami* (Boulenger) of Lake Magadi, in Kenya live in waters of pH 10. They detoxify ammonia and produce urea in the liver through the ornithine urea cycle (Randall *et al.*, 1989; Wood *et al.*, 1989). The Lahontan cutthroat trout *Oncorhynchus clarki henshawi* Richardson in Pyramid Lake, Nevada, in the United States thrives in waters of pH 9·4 (Galat *et al.*, 1985). In a study of nitrogen excretion in four species of fish from Pyramid Lake (McGeer *et al.*, 1994), we reported that Jamm in the Lahontan cutthroat trout accounted for 85% of total nitrogen excretion; a level generally higher than in the other three species (about 70% in the tui chub *Gila bicolor* (Girard), Tahoe sucker *Catostomus tahoensis* Gill & Jordan, and the cui-ui *Chasmistes cujus* Cope) studied. The balance of nitrogen excretion in the Lahontan cutthroat trout is urea (McGeer *et al.*, 1994; Wright *et al.*, 1993; Wilkie *et al.*, 1993), which is produced through uricolysis and not through the ornithine urea cycle (McGeer *et al.*, 1994; Wilkie *et al.*, 1993). Our studies suggest that Lahontan cutthroat trout maintain total Jamm in the highly alkaline waters of Pyramid Lake by modification of mechanisms found in other ammoniotelic teleosts living in more pH-neutral waters. Relative to those fishes, Lahontan cutthroat trout tolerate higher blood ammonia levels; have a higher proportion of nitrogenous waste excretion as urea; have higher rates of renal total ammonia excretion; and have higher plasma pH (Wright *et al.*, 1993; McGeer *et al.*, 1994; Wilkie *et al.*, 1994). The elevated plasma pH and Tamm levels increase the PNH_3 gradient from blood to water, thus maintaining the diffusion of NH_3 from the fish in spite of the alkaline conditions (Wright *et al.*, 1993).

Many of the alkaline lakes in North America that support fish populations are considered hard-water lakes because of high concentrations of Ca^{++} (see Yesaki & Iwama, 1992). It has been shown that elevating the concentraton of Ca^{++} reduced the associated stress and enhanced ammonia excretion in rainbow trout exposed to highly alkaline (pH 10) water (Yesaki & Iwama, 1992). Pyramid Lake is relatively saline, with high concentrations of Na^{+}, Cl^{-}, and Mg^{++}, but low concentrations of Ca^{++} (see Table I). The present experiments were designed to test the hypothesis that the divalent cations in the water play an important role in ammonia excretion in Lahontan cutthroat trout in Pyramid Lake. The experimental design involved the measurement of ammonia excretion in response to the manipulation of water Ca^{++} and Mg^{++} concentrations. Haematocrit (Hct), blood acid base status and gas tensions, ventilation rates, and total protein concentration were measured to describe the physiological response of the fish to the experimental conditions. Plasma cortisol concentration was used as an indicator of general stress in the fish.

MATERIALS AND METHODS

The experiments were conducted at the laboratory facilities of Pyramid Lake Fisheries, in Sutcliffe, Nevada. Details of the lake water are given in Table I. Lahontan cutthroat (160–245 g) which had been reared for approximately 1 year in well water (pH 8·4, see Wright *et al.*, 1993 for other chemical characteristics), were transferred to large

TABLE I. Chemical characteristics of Pyramid Lake water

Variable	Lake water	Soft water
Temperature	9·2	9·3*
P_{O_2} (mm Hg)	130·0	128·7*
pH	9·39	9·64*
Total CO_2 (mmol l^{-1})	19·6	19·4*
P_{CO_2} (Torr)	0·26	
$[HCO^-_3]$ (mmol l^{-1})	13·8	5·0*
$[CO^=_3]$ (mmol l^{-1})	4·97	
Titration alkalinity	23·08	
Total salinity (‰)	4·43	
$[Na^+]$ (mmol l^{-1})	58·2	
$[Cl^-]$ (mmol l^{-1})	59·7	
$[Ca^{++}]$ (mmol l^{-1})	0·21*	0·12*
$[Mg^{++}]$ (mmol l^{-1})	7·32*	0·79*
$[SO^=_4]$ (mmol l^{-1})	1·69	

Data from Wright *et al.*, 1993; Wilkie *et al.*, 1994; and *this study.

TABLE II. Details of treatments 1–4

Treatment	Protocol	Fish preparation
1	Lake–soft–lake (LSL)	Cannulated fish
2	Lake–soft–soft (LSS)	Uncannulated fish
3	Lake–soft–soft plus Ca^{++} (LSS+Ca^{++})	Uncannulated fish
4	Lake–soft–soft plus Mg^{++} (LSS+Mg^{++})	Uncannulated fish

outdoor holding ponds receiving lake water (pH 9·4) for 3–5 weeks prior to the commencement of these experiments. All experimental fish were transferred to 2200 l indoor tanks (20–30 fish $tank^{-1}$) receiving lake water. There was a period of 5–10 days between the transfer to indoor tanks, and the time when the fish were used for experiments.

PROTOCOL

Two sets of experiments were conducted. In the first set of experiments, Jamm measurements were made with cannulated fish exposed to treatment 1 below [Table II; Fig. 1(a)]. The second series of experiments comprised the measurement of Jamm in uncannulated fish [treatments 2, 3 and 4 below, Fig. 1(b), (c) and (d)]. Blood samples were collected and analysed to describe the physiological status of the fish. In each experiment, the fish were exposed to the three water qualities in sequence.

The term lake (L) refers to lake water, and soft (S) refers to lake water with much of the Ca^{++} and Mg^{++} removed by precipitation (Table I). Because the lake water was nearly saturated with divalent cations, we manipulated its ionic content by reducing the concentration of divalent cations. Raising the pH of the lake water to about 13 with KOH caused the formation of a white precipitate. Approximately 4 kg of KOH was added to 2200 l of lake water to increase the pH to 12·95. Although the water K^+ concentration was not measured, theoretically this would have increased the water K^+ concentration by about 32 mmol l^{-1}. This was done in one of the indoor 2200-l tanks. The solid precipitate was allowed to settle to the bottom of the tank, and 1000 l of the

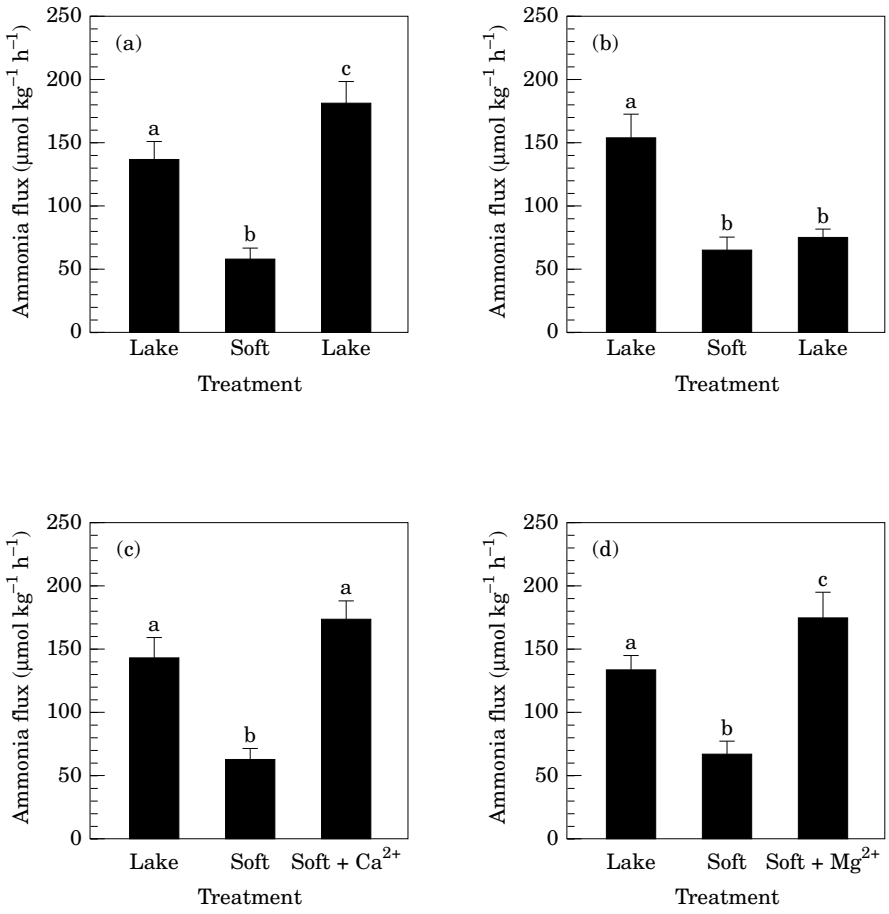

FIG. 1. Total ammonia excretion rates in Lahontan cutthroat trout exposed for 3-h periods in Pyramid Lake water (lake); to lake water with much of the Ca^{++} and Mg^{++} removed by precipitation (soft); and then recovered in lake water [(a) $n=12$ for each mean], soft water [(b) $n=8$ for each mean], or in soft water with either Ca^{++} [(c) $n=8$ for each mean] or Mg^{++} [(d) $n=8$ for each mean] added as $CaCl_2$ and $MgCl_2$ salts, respectively. See text for details. Data shown as means ± S.E. Superscripts of different letters indicate statistically significant differences among means ($P \leq 0{\cdot}05$).

clear supernatant was transferred to another tank and then adjusted to the temperature and pH (with HCl) of the lake water; this was used as the soft water. Ca^{++} (0·09 mmol l^{-1}) and Mg^{++} (6·53 mmol l^{-1}) were added to that soft water as $CaCl_2$ and $MgCl_2$ salts, respectively, to bring their respective concentrations back up to normal lake water levels. Temperature and pH of the resulting water were adjusted and maintained at lake values (Table I).

The following procedures were followed for both cannulated and uncannulated fish. Fish were not fed for 5–10 days in the laboratory prior to experiments. The day before each experiment, individual fish were placed in darkened aerated perspex chambers, similar to those described by McDonald & Rogano (1986), receiving a flow of about 0·5 l min^{-1} which kept ammonia levels below 5 μmol l^{-1}. Excretion rates were measured in the following way. Flow was stopped and the water level set to about 4 l. About 15 ml of water was collected at the beginning (0 h) and at the end (3 h) of each exposure period for the determination of pH and total ammonia concentration. Water samples were acidified to trap all ammonia in ionic form, and frozen for later analysis of ammonia concentration. Changes in water Tamm (μmol l^{-1}) over the 3-h exposure

period were multiplied by the container water volume (l), and divided by fish weight (kg) and excretion time (h) to determine Jamm (μmol kg^{-1} h^{-1}). Each experiment was conducted over a 3-day period and all exposure periods were 3 h in duration. On day 1, the lake water Jamm measurement was conducted. On day 2, the water source was changed over to soft water 6 h prior to the determination of Jamm. The water source was changed back to lake water (treatment 1), soft water (treatment 2) or to one of the divalent cation-supplemented soft-water conditions (treatments 3 or 4) approximately 2 h after the end of the flux period on day 2. On day 3, the final Jamm was determined in one of the four water qualities above.

In order to understand the physiology of changes which occurred in response to soft-water exposure, the physiological response of the fish to one of the experiments (LSL) was described by blood sampling with chronic indwelling catheters and analysis of selected variables. The fish were fitted wtih chronic catheters in the dorsal aorta by the method of Soivio *et al.* (1972) under general anaesthesia with buffered MS-222. Fish were allowed to recover for 24–48 h between surgery and the start of the experiment. Blood sampling was conducted 1 h prior to the 0-h water sample and 1 h after the 3-h water sample on each day. Blood samples were collected for the analysis of acid-base status (pH; HCO_3^- concentration, [HCO_3^-]; and the partial pressure of CO_2, P_{CO_2}), Hct, the partial pressure of O_2 (P_{O_2}), and plasma concentrations of ammonia, total protein, Na^+, Cl^-, K^+, Ca^{++} and Mg^{++}, and cortisol. Ventilation frequency was also recorded, by counting the total number of opercular cover movements within intervals of 15 or 30 s, and calculating the breaths min^{-1}.

ANALYTICAL PROCEDURES

Water pH was monitored with either a Beckman portable pH meter with combination-electrode, or a Radiometer system with glass combination-electrode (GK 2401C) and PHM84 acid-base analyser. The two methods were cross-validated. Water ammonia concentration was determined by the salicylate–hypochlorite method (Verdouw *et al.*, 1978). Blood pH was measured on whole blood using a glass capillary electrode (Radiometer E5021a) calibrated with precision phosphate buffers at the temperature of the experimental animals. Whole blood P_{O_2} was measured with a Radiometer glass electrode, thermostatted to the ambient water temperatures, and calibrated with air-saturated water and a zero standard (sodium bisulphite). Haematocrit was determined after centrifugation of whole blood in glass capillary tubes for 2 min at 13 000 *g* in a microhaematocrit centrifuge. The plasma from the capillary tubes was used to determine the concentrations of total CO_2 with a Corning digital CO_2 analyser. Total protein concentration of the plasma was estimated by a refractometer (Alexander & Ingram, 1980). Total ammonia concentration (Tamm) of the plasma was determined enzymatically (Sigma 170A). Plasma concentrations of Na^+ and Cl^- were measured with a Corning 410C flame photometer and with a Haake Buchler digital chloridometer, respectively. Other plasma cation concentrations were determined by ion chromatography (Shimadzu Model HIC-6A, Shimadzu Corp., Kyoto, Japan). Plasma cortisol concentration was determined by radio-immunoassay (Clinical Assay, Massachusetts).

CALCULATIONS AND STATISTICS

Calculations for blood HCO_3^- and P_{CO_2} were based on the Henderson–Hasselbalch equation, using the appropriate constants from Boutilier *et al.* (1984). Plasma NH_4^+ concentrations and PNH_3 tensions were calculated with the Henderson–Hasselbalch equation and from the equation:

$$PNH_3 = (Tamm - NH_4^+) \times \alpha NH_3$$

using arterial pH and Tamm, and where αNH_3 is the solubility of ammonia. Blood to water gradients were simply calculated by the difference between blood and water concentrations for each variable. The changes in blood Tamm, NH_4^+, and NH_3 over each 3-h exposure period in the LSL experiment were calculated in order to relate these blood changes with the Jamm data. Statistical analyses were conducted on the absolute

differences in each variable between 0 and 3 h. One-way analyses of variance, with Tukey's tests were used to discern statistical significance in the differences among means. A fiducial limit of $P \leq 0{\cdot}05$ was used in all tests. The data are presented as means ± S.E., with the number of fish indicated by (*n*).

RESULTS

The soft-water treatment resulted in significant inhibition of ammonia excretion to about half of the control levels in all experiments (Fig. 1). The addition of lake water, or either calcium or magnesium restored the Jamm back to control levels, whereas keeping the fish in soft water maintained the reduced Jamm rates (Fig. 1). Blood ammonia concentrations, as well as blood-to-water ammonia gradients in the LSL experiment increased during each 3-h exposure period (Table III). The changes in blood-to-water gradients for NH_4^+ and PNH_3 were significantly higher during the soft-water exposure period, compared to either lake-water exposure periods (Table III).

Blood pH showed a consistent trend to increase during each exposure period, but the only statistically significant increase was during the soft water exposure period (Table IV). There were no significant differences in P_{CO_2} tensions or HCO_3^- concentrations among any of the sample times. While ventilation frequencies were similar among all samples, blood P_{O_2} tensions in the first samples of both the soft- and final lake-water exposures were higher than all other samples (Table IV). Among the measured plasma ions, the only significant differences were in plasma K^+ concentrations which were greater during the soft-water exposure than either lake-water exposures (Table IV).

The Hct of fish in the LSL experiment declined continuously from about 25 to 7%, with the largest declines occurring between the initial lake- and soft-water exposure periods and during the soft-water exposure period (Table IV). Mean plasma protein concentrations also declined, from an initial value of 82·5 to 58·0 mg ml^{-1} over the course of the experiment (Table IV). Although cortisol concentrations also showed consistently increasing trends during each of the three exposure periods, none of the differences within each exposure period was significant. Plasma cortisol concentrations were significantly higher during soft-water exposure than both lake-water exposure periods (Table IV).

DISCUSSION

Highly alkaline lakes that support ammoniotelic teleost species have typically hard water. Yesaki & Iwama (1992) showed that water Ca^{++} plays an important role in ammonia excretion in rainbow trout. Exposure of rainbow trout to highly alkaline soft water, in that study, inhibited Jamm and the addition of Ca^{++} restored Jamm. In the present study, the addition of either Ca^{++} or Mg^{++} back to the levels present in normal Lahontan Lake water caused similar effects in Lahontan cutthroat trout (Fig. 1). Since the normal lake water levels of both ions were reconstituted by these additions, more than 70 times more Mg^{++} was added than Ca^{++}. While this may suggest that Ca^{++} had a more potent effect than Mg^{++} in enhancing Jamm, this remains equivocal since only one concentration of each ion was tested: i.e. lower concentrations of

TABLE III. Plasma ammonia concentration and blood-to-water ammonia gradients in Lahontan cutthroat trout, with chronic indwelling catheters, exposed for the following 3-h exposure periods in sequence: Pyramid Lake water (lake); to lake water with much of the Ca^{++} and Mg^{++} removed by precipitation (soft); and then recovered in lake water (recovery)

	Lake 0	Lake 3	Soft 0	Soft 3	Recovery 0	Recovery 3
Plasma ammonia concentration						
Tamm (μmol l^{-1})	164·49 ± 11·43 (5)[a]	291·26 ± 22·80 (5)[b]	181·30 ± 8·75 (5)[a]	379·15 ± 24·52 (5)[c]	179·01 ± 11·26 (5)[a]	379·43 ± 33·65 (4)[c]
NH^+_4 (μmol l^{-1})	160·97 ± 11·42 (5)[a]	282·87 ± 21·48 (5)[b]	175·21 ± 8·36 (5)[a]	367·96 ± 23·33 (5)[c]	175·42 ± 11·24 (5)[a]	372·05 ± 32·92 (4)[c]
PNH_3 (μTorr)	66·15 ± 3·99 (5)[a]	157·74 ± 24·94 (5)[b]	114·56 ± 11·45 (5)[b]	210·53 ± 29·32 (5)[c]	67·43 ± 2·89 (5)[a]	138·73 ± 16·33 (4)[b]
Blood-to-water gradients						
Tamm (μmol l^{-1})	148·86 ± 10·19 (5)[a]	257·45 ± 20·72 (5)[b]	163·84 ± 7·79 (5)[a]	353·13 ± 22·55 (5)[c]	155·81 ± 10·38 (4)[a]	341·47 ± 29·63 (4)[c]
NH^+_4 (μmol l^{-1})	150·09 ± 10·53 (5)[a]	257·39 ± 19·81 (5)[b]	163·18 ± 7·69 (5)[a]	347·48 ± 21·80 (5)[c]	157·07 ± 10·73 (5)[a]	343·57 ± 29·90 (4)[c]
PNH_3 (μTorr)	− 20·11 ± 7·33 (5)[a]	6·19 ± 24·01 (5)[a]	15·85 ± 8·58 (5)[a]	109·82 ± 28·26 (5)[b]	− 20·78 ± 6·97 (4)[a]	− 33·61 ± 7·42 (4)[a]

0 and 3 refer to the blood samples taken just before and just after each 3-h exposure period. See text for details. Data shown as means ± S.E. (*n*). Superscripts of different letters indicate statistically significant differences among means ($P \leq 0{\cdot}05$).

TABLE IV. Plasma concentrations of ions, cortisol, and total protein, as well as blood acid-base status, partial pressure of oxygen, haematocrit, and ventilation rates in Lahontan cutthroat trout; fish, with chronic indwelling catheters, were exposed for the following 3-h exposure periods in sequence: Pyramid Lake water (Lake); to lake water with much of the Ca^{++} and Mg^{++} removed by precipitation (soft); and then recovered in lake water (recovery)

	Lake 0	Lake 3	Soft 0	Soft 3	Recovery 0	Recovery 3
Ions (mmol l^{-1})						
Na^+	139·71 ± 4·30 (7)^a	144·00 ± 3·11 (9)^a	135·67 ± 4·69 (9)^a	137·22 ± 2·13 (9)^a	131·33 ± 3·83 (9)^a	131·89 ± 3·67 (9)^a
K^+	1·74 ± 0·34 (7)^a	2·02 ± 0·26 (9)^a	3·15 ± 0·22 (9)^b	3·79 ± 0·32 (9)^b	2·30 ± 0·13 (9)^{ab}	2·64 ± 0·26 (8)^a
Cl^-	104·17 ± 3·91 (7)^a	104·96 ± 6·57 (9)^a	97·36 ± 6·82 (9)^a	103·92 ± 5·49 (9)^a	103·16 ± 10·16 (9)^a	100·75 ± 4·48 (8)^a
Ca^{++}	3·32 ± 0·67 (7)^a	3·19 ± 0·60 (9)^a	3·53 ± 0·63 (9)^a	2·35 ± 0·35 (8)^a	3·87 ± 0·32 (9)^a	2·81 ± 0·50 (8)^a
Mg^{++}	3·54 ± 0·87 (7)^a	4·37 ± 0·95 (9)^a	4·98 ± 0·96 (9)^a	3·78 ± 0·93 (8)^a	4·86 ± 0·83 (9)^a	3·75 ± 0·87 (8)^a
pH	8·057 ± 0·034 (13)^a	8·143 ± 0·026 (13)^a	8·158 ± 0·016 (7)^a	8·208 ± 0·014 (5)^a	8·072 ± 0·020 (5)^a	8·155 ± 0·021 (5)^a
P_{CO_2} (mmHg)	1·48 ± 0·17 (13)^a	1·25 ± 0·07 (13)^a	1·39 ± 0·10 (7)^a	1·18 ± 0·05 (5)^a	2·08 ± 0·48 (5)^a	1·34 ± 0·27 (5)^a
Bicarbonate (mmol l^{-1})	6·52 ± 0·55 (13)^a	7·16 ± 0·35 (13)^a	8·13 ± 0·27 (7)^a	7·96 ± 0·34 (5)^a	9·54 ± 1·80 (5)^a	7·72 ± 1·37 (5)^a
Plasma cortisol (ng ml^{-1})	163·0 ± 1·96 (13)^a	218·0 ± 2·16 (12)^a	249·2 ± 2·75 (11)^b	280·0 ± 2·07 (10)^b	151·9 ± 1·93 (9)^a	198·0 ± 2·79 (9)^{ab}
Haematocrit (%)	24·7 ± 2·6 (13)^a	19·5 ± 1·8 (13)^b	12·5 ± 1·3 (13)^c	10·3 ± 1·2 (13)^{cd}	7·1 ± 1·6 (13)^d	7·0 ± 1·5 (7)^{cd}
Total protein (mg ml^{-1})	82·5 ± 3·3 (13)^a	77·8 ± 5·4 (13)^a	69·3 ± 3·4 (13)^b	67·2 ± 2·4 (13)^b	58·7 ± 5·4 (9)^b	58·0 ± 4·7 (8)^b
P_{O_2} (mmHg)	82·8 ± 5·3 (13)^a	81·2 ± 4·1 (13)^a	96·1 ± 2·8 (12)^b	89·0 ± 2·5 (10)^{ab}	98·1 ± 3·4 (10)^b	86·5 ± 2·6 (9)^{ab}
Ventilation (breaths min^{-1})	69 ± 4 (13)^a	79 ± 3 (13)^a	86 ± 5 (13)^a	80 ± 5 (13)^a	67 ± 8 (13)^a	73 ± 8 (13)^a

0 and 3 refer to the blood samples taken just before and just after each 3-h exposure periods. See text for details. Data shown as means ± S.E. (*n*). Superscripts of different letters indicate statistically significant differences among means ($P \leq 0{\cdot}05$).

Mg^{++} which may have had similar effects as the Ca^{++} were not tested. The mechanisms by which these divalent cations enhance ammonia excretion in this as well as other trout species are still not understood.

There are several passive and active mechanisms that may be a part of explaining our observations. Although there is the possibility that the unequal pre-flux acclimation times may have affected the subsequent flux rates, this possible effect was not evidently significant. Evidence for this can be seen in the second experiment (LSS) in which the excretion rate in the intermediate flux period (soft water), which received the shortest acclimation time of 6 h, was similar to the excretion rates in the final soft-water exposure which had an overnight pre-flux acclimation period (Fig. 1). The divalent cations presumably acted on branchial processes of ammonia excretion. Possible increase in the thickness of the mucus layer at the gill surface may have provided a more stable acidic boundary layer in which conversion of NH_3 to NH_4^+ would have been enhanced, and in which a higher blood to water NH_3 gradient would have been maintained. Wright *et al.* (1993) showed the importance of the maintenance of blood to water NH_3 gradients, and the lack of dependence on Na^+ influx, in total ammonia excretion in Lahontan cutthroat trout. If a thicker mucus layer on the gills did exist with higher divalent cation concentrations in the water, it is unlikely that it occurred to an extent that gas transport was affected, as blood P_{O_2} was maintained, and even slightly increased with soft-water exposure as well as with the second hard-water exposure, at similar ventilation rates. It is also possible that higher divalent cation concentrations may have stimulated active proton excretion rates, such that a more acidic boundary layer resulted. Lin & Randall (1992) showed that the electrogenic proton pump on the trout gill was stimulated by high concentrations of plasma cortisol and water Ca^{++}, but not in the presence of high water sodium concentration. There was no correlation between plasma cortisol and ammonia excretion rates in this study. It is unknown whether the Na^+ concentration of Pyramid Lake water, which is high for freshwater lakes, is high enough to attenuate the stimulatory effect of high water Ca^{++} concentration. While theoretically increased acidification of the boundary layer would enhance ammonia excretion in Lahontan cutthroat trout as described above, it is also likely that the highly buffered water of the lake would minimize or severely reduce the existence of a significantly acid boundary layer. It is also possible that some carrier-mediated NH_4^+ transport is activated under hard-water conditions. These possible mechanisms warrant further study in the future.

The increases in blood ammonia levels during each 3-h exposure period occurred concomitantly with the increases in water ammonia concentrations such that outward gradients were maintained over the 3-h period. There was a consistent trend for Jamm to be higher in the recovery exposure period compared to the initial lake-water exposure period (Fig. 1). Furthermore, there were consistently higher increases in both blood ammonia concentrations as well as in blood-to-water gradients for the recovery periods compared to the initial lake-water exposure periods (Table III). This supports the possibility that the inhibition of Jamm during the soft-water exposure increased body stores (e.g. white muscle) of ammonia, and that the increases in plasma levels of Tamm and NH_4^+ during the final recovery period in lake water reflected increased

mobilization of ammonia from tissue stores. Wilkie & Wood (1995), reported an inhibition of ammonia excretion in rainbow trout by raising water pH from 8 to 9·5. In that study, they found Tamm concentrations of white muscle from fish at pH 9·5 to be 2·5 times higher than fish at pH 8·0. Plasma Tamm levels returned to control levels only after 3 days, when this ammonia washout period during recovery was over (Wilkie & Wood, 1995). This long ammonia washout period is evidence against this possibility of ammonia build-up in the muscle tissues as a result of the inhibition of ammonia excretion in this expeirment because the blood ammonia levels, only 6 h after switching the water supply from hard lake water, at the beginning of the soft-water exposure period were not different from the initial levels in the control lake water (Table IV). This suggests that either the washout was particularly quick in this experiment, or that such a build up of tissue stores did not occur. Although it is possible that the higher plasma K^+ concentrations (Table IV) during the soft and early recovery exposure periods were associated with the possible movement of ammonia stores (as NH_4^+, see Wright, 1995) from the muscle intracellular compartment, into the blood, and then out into the water, it is perhaps more likely that they were caused mainly by the theoretically higher K^+ levels (see Materials and Methods) in the soft water from the addition of KOH to cause the precipitation of ions out of solution.

Somewhat different in pattern to the Tamm and NH_4^+ response to soft-water exposure, but reflecting the pattern seen in the Jamm data, plasma PNH_3 tensions increased greatly with soft-water exposure and returned to initial control levels during recovery (Table III). There was a very large and significant increase to blood-to-water PNH_3 during the soft-water exposure, over the other exposure periods (Table III). The mechanisms that inhibit Jamm in soft water probably have significant effects on NH_3 flux. These data point to the importance of plasma PNH_3 tensions and blood-to-water PNH_3 gradients in Jamm. Although Jamm can be linked to Na^+/NH_4^+ exchange in highly alkaline water (Wright & Wood, 1985; Yesaki & Iwama, 1992), there is growing evidence that Jamm is dependent to a large part on PNH_3 gradients and not on NH_4^+-linked excretion (Wright *et al.*, 1993; Wilson *et al.*, 1994; Wilkie *et al.*, 1996). Thus, it is possible that water concentrations of divalent ions may alter gill permeability such that PNH_3 flux is affected. These data support the possibility that it is through NH_3 flux that the acute changes in Jamm take place.

It is not clear why the blood-to-water PNH_3 gradients were negative at the beginning of both lake-water exposures. One possible reason for the negative calculated gradients in the early part of the exposure period is that the calculation of PNH_3 gradients involved the use of arterial blood, which would have had lower PNH_3 partial pressures than venous blood. Accurate estimation of such gradients requires both arterial as well as venous PNH_3 partial pressures. It is also possible that such negative PNH_3 gradients were artifacts of using bulk water instead of using boundary layer water, which probably would be more acidic, to estimate water PNH_3 and to calculate those gradients. We have observed significant increases in Jamm and blood-to-water PNH_3 gradients with reduction in water pH (Wright *et al.*, 1993) in Lahontan cutthroat trout. While it may be unlikely that there was a large pH difference between the laboratory layer water and the bulk water in such a highly alkaline and buffered system, this possibility warrants further study.

All of the plasma cortisol concentrations were very high relative to those one might expect in resting trout (Barton & Iwama, 1991). While these data could be considered to reflect a generally stressed state in all the experimental animals, plasma cortisol concentrations close to these values have been reported for trout under control, or resting conditions in black perspex boxes (Gamperl *et al.*, 1994). The values here are consistent with those we reported previously for Lahontan cutthroat trout (Wilkie *et al.*, 1994) where fish were transferred from well to lake water. We did not measure any increase in blood glucose concentration, typical of the generalized stress response, and speculated that while cannulation and holding these wild fish in our perspex experimental chambers elevated plasma cortisol concentrations, the fish may not have been unduly stressed. Our data showing resting values, and no large changes, in blood ion concentrations, acid-base and blood gas status, and ventilation rates supports the possibility that the fish were not unduly stressed during these experiments. Furthermore, control ammonia excretion rates are in agreement with those reported previously for Lahontan cutthroat trout at Pyramid Lake, under similar experimental conditions (McGeer *et al.*, 1994; Wright *et al.*, 1993; Wilkie *et al.*, 1993). Temperature, nutritional state, time of day, and developmental stages (e.g. non-stress levels of cortisol increase 10-fold during smolting in anadromous salmonids) are some factors that can influence resting cortisol levels (Barton & Iwama, 1991). The increase in plasma cortisol concentration during soft-water exposure suggests that the hypothalamus–pituitary–interrenal axis was stimulated by some factor associated with soft-water exposure.

There were several physiological responses that were consistent over the time course of the entire experiment, and apparently independent of the treatments. The declines in both Hct and total protein concentration were probably a function of repeated blood sampling and replacement with saline. Such a procedure would have aided in maintaining blood ion concentrations, as seen in the data, but the lack of protein in the physiological saline used probably diluted the blood with respect to that variable. It seems clear that ventilatory factors did not influence PNH_3 tensions. Ventilation rates did not change, and blood P_{O_2} and P_{CO_2} tensions were generally maintained throughout this experiment, with the exception of blood P_{O_2} which increased slightly at the beginning of the soft-water exposure as well as the recovery in lake water. Maintenance of blood P_{O_2} in alkaline water has been reported by several studies (Wilkie & Wood, 1991, 1995; Wilkie *et al.*, 1993, 1994).

The treatment protocol of removing divalent cations by precipitation may have an ecological and physiological relevance to the Lahontan cutthroat trout in Pyramid Lake. There have been reports of a lake-wide precipitation phenomenon known as a ‘ whiting ’ which starts at a focal point, such as an algal bloom, and spreads over the entire lake (Galat *et al.*, 1981). The precipitates, which can amount to 55 000 tons in a single event, apparently are divalent carbonates that settle to the bottom of the lake. It is evident from the differences in lake water and the soft water which we created through a similar precipitation that those precipitates are most likely $MgCO_3$ in that the molar equivalents in Mg^{++} and HCO_3^- match. The natural whitings would presumably soften the water as we have done experimentally in the present study. One exception would perhaps be that of water pH which was maintained at lake levels in this study, but may tend

to be relatively acidic in the soft-water phase during and following such whitings. Our findings suggest that while the decreased divalent cation concentration(s) of the water during whitings may inhibit ammonia excretion, this may be offset to some degree by the enhancement of ammonia diffusion from blood to water, by the probably acidic nature as well as the lowered buffering capacity of the resulting soft lake water.

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