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Physiological Adaptations of Rainbow Trout to Chronically Elevated Water pH (pH = 9.5)

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ABSTRACT Recent investigations have demonstrated that rainbow trout cope with acute high pH (pH > 9.0) exposure (lasting 3–8 days) through their ability to counteract high-pH-induced disturbances to ammonia excretion (J_{Amm}), acid–base homeostasis, and electrolyte balance. In the present investigation our goal was to establish how these physiological processes were modulated during chronic (28-day) high pH (pH = 9.5) exposure. Chronic high pH led to minimal mortality, and there were no long-term changes in stress indicators levels, such as cortisol or glucose. J_{Amm} was initially reduced by 40% at high pH but rapidly recovered and fluctuated around control rates, thereafter. Decreased J_{Amm} was associated with an initial 2.5-fold increase in plasma ammonia concentrations (T_{Amm}), followed by a return toward pre-exposure levels after 3 days. Overall, plasma T_{Amm} was slightly higher (40–80%) in the treatment fish, and this likely led to plasma P_{NH3}s that were sufficient to sustain J_{Amm} at high pH. White muscle T_{Amm} stores were also chronically elevated, by 50–100%. There was a transient, twofold elevation of J_{Urea} immediately following high-pH exposure, but by 3 days J_{Urea} had returned to control rates and stabilized thereafter. Plasma ion balance was well maintained at high pH, despite a chronic depression of Na^+ influx. Even though there was a persistent respiratory alkalosis at alkaline pH, blood pH was effectively regulated by a simultaneous metabolic acid load, which was not associated with increased lactic acid production. White muscle intracellular pH (pH_i) was unaltered during high pH exposure. We conclude that the long-term survival of rainbow trout in alkaline environments is facilitated by higher steady-state internal ammonia concentrations, the development of a sustained, compensatory metabolic acidosis which offsets decreased plasma P_{CO2}, and the effective regulation of plasma electrolyte balance.

Many eutrophic lakes undergo diurnal elevations in water pH that result from high rates of algal and macrophytic photosynthesis (Barica, '74, '90; Ayles et al., '76; Wetzel, '83). In many instances, water pH may exceed pH 9.0 and occasionally approach pH 10.0 (Barica, '74, '90; Ayles et al., '76). Such acute pH elevations may influence local populations of rainbow trout (*Oncorhynchus mykiss*), which now inhabit many eutrophic lakes in western North America (e.g., Barica, '74; Murray and Ziebell, '84; Prepas et al., '90). Some of these fish may also have to contend with chronically elevated water pH, because many eutrophic lakes are subject to long-term photosynthetically induced elevations in pH that may last for several days or weeks during the summer (Halstead and Tash, '82; Murray and Ziebell, '84; Prepas et al., '90). Attempts have also been made to establish rainbow trout populations in many western North American saline-alkaline lakes, that are permanently alkaline (pH 9.0–10.0) owing to high dissolved concentrations of HCO_3^- and $CO_3^=$ (e.g., Galat et al., '81, '85; Wilson et al., '94b). However, these planting efforts have only had limited success because of the rainbow trout's apparent inability to adapt to alkaline water (Kucera et al., '85; Coleman and Johnson, '88). In view of these facts, it is somewhat surprising that we know little about the physiological responses of rainbow trout to chronically elevated water pH. Accordingly, the goal of the present study was to describe how physiological processes were modulated during a prolonged (28-day) high-pH exposure regime.

A number of laboratory studies have suggested that acute high-pH exposure leads to inhibited ammonia excretion (Wright and Wood, '85; Wilkie and Wood, '91, '94, '95; Yesaki and Iwama, '92) and the disruption of internal acid–base and electrolyte balance (Heming and Blumhagen, '88; Lin and Randall, '90; Wilkie and Wood, '91, '95; Yesaki

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and Iwama, '92). In some instances, acute increases in pH have even been known to result in considerable mortality (Jordan and Lloyd, '64; Daye and Garside, '75; Yesaki and Iwama, '92). Since recent studies have suggested that adaptation to acute high pH (lasting 3–8 days) by rainbow trout is dependent upon the fish's ability to counter disturbances to nitrogenous waste excretion, acid–base balance, and ion regulation (Wilkie and Wood, '91, '95; Yesaki and Iwama, '92), the present study primarily focused upon these three areas.

High pH exposure blocks ammonia excretion across fish gills by decreasing the blood–gill water NH_3 gradient and/or inhibiting Na^+/NH_4^+ exchange processes (Cameron and Heisler, '83; Wright and Wood, '85). However, associated increases in blood total ammonia concentration likely led to the re-establishment of favourable blood–gill water NH_3 gradients that probably accounts for the recovery of J_{Amm} at high pH (Wilkie and Wood, '91, '95). The present investigation tested the hypothesis that ammonia excretion by rainbow trout in chronically alkaline water relied upon sustained elevations in steady-state internal ammonia levels. Accordingly, ammonia excretion patterns and internal storage, in the plasma and white muscle, were followed throughout the 4-week exposure to pH 9.5. Urea excretion patterns were also examined to test the alternate hypothesis that trout might increase their reliance upon urea excretion, to facilitate N-excretion, during chronic high pH exposure. Such a response has been reported in salmonids acutely exposed to higher pH (Wilkie and Wood, '91; Wilkie et al., '93) and in the alkaline-tolerant Cyprinid (*Chalcalburnus tarichi;* Danulat and Kempe, '92). One teleost, *Oreochromis alcalicus grahami,* which inhabits alkaline (pH 10) Lake Magadi, Kenya, excretes all of its nitrogenous waste as urea (Randall et al., '89; Wood et al., '89).

Acute exposure to alkaline pH can also lead to pronounced, sometimes lethal, reductions in plasma electrolytes (Na^+ and Cl^-) (Heming and Blumhagen, '88; Wilkie and Wood, '91; Yesaki and Iwama, '92), and these disturbances appear to be initiated by decreases in ion uptake across the gill epithelium (Wright and Wood, '85; Wilkie and Wood, '94). Long-term survival at high pH would, therefore, depend upon the efficient regulation of internal electrolyte balance and modifications in the patterns of branchial ion movements. To test this hypothesis, internal (plasma and white muscle) ion concentrations and branchial Na^+ fluxes (influx, outflux, net flux) were monitored throughout the experiment.

Eutrophic-alkaline environments (pH > 9.5) are often characterized by low levels of gaseous CO_2 due to the photosynthetic utilization of CO_2 by aquatic plants and algae (Halstead and Tash, '82; Wetzel, '83). Thus, the water may essentially act as a "CO_2 vacuum," which leads to CO_2 unloading across the gills (Johansen et al., '75), resulting in lowered blood CO_2 tension (respiratory alkalosis) and elevated blood pH (Lin and Randall, '90; Wilkie and Wood, '91; Yesaki and Iwama, '92). In the rainbow trout, acute high-pH-induced increases in blood pH are counterbalanced by the simultaneous generation of metabolic protons (metabolic acidosis) that appear to be the result of increased rates of lactic acid production (Wilkie and Wood, '91). To test the hypothesis that the long-term maintenance of blood acid–base balance at high pH was dependent upon a sustained metabolic acidosis, we measured the acid–base status and lactate concentrations in the plasma and white muscle of rainbow trout throughout this experiment.

MATERIALS AND METHODS

Experimental animals and set-up

Juvenile rainbow trout (*Oncorhynchus mykiss;* mean weight 63.4 ± 1.4 g; n = 104) were obtained from a local hatchery (Rainbow Springs, Thamesford, Ontario) during the summer months. Fish were then distributed equally to two 425-litre rectangular holding tanks, each receiving dechlorinated Hamilton City tapwater (composition: $[Na^+]$ = 0.6 mmol·litre^{-1}; $[Cl^-]$ = 0.8 mmol·litre^{-1}; $[Ca^{2+}]$ = 0.9 mmol·litre^{-1}; $[Mg^{2+}]$ = 0.4 mmol·litre^{-1}; $[K^+]$ = 0.03 mmol·litre^{-1}; titratable alkalinity = 2.0 mmol·litre^{-1}; pH = 7.9; temperature = 15°C) at rates of 1–2 l·min^{-1}. This rate of water replacement kept water total ammonia concentrations ($T_{Amm} = NH_3 + NH_4^+$) below 10 μmol·litre^{-1} and vigorous aeration maintained water P_{O2} levels above 130 torr. The fish were held under these conditions for 4 weeks prior to the start of the experiment. During this period the fish were fed, ad libitum, 3 times per week with commercial trout pellets (Martin Feed Mills). During the 4-week exposure period itself the fish were fed a minimum ration (approximating 1.0 percent body weight), every seventh evening to minimize the known effects that feeding has on nitrogenous waste excretion (Fromm, '63; Brett and Zala, '75). Had the fish been fed more frequently it would have ne-

cessitated higher rates of water replacement, to remove the additional nitrogenous waste, and made pH and temperature regulation very difficult.

The holding tanks also served as the experimental system during the 4-week high-pH exposure regime; both tanks (control pH = 7.9 and experimental pH = 9.5, respectively) received the same dechlorinated tapwater mentioned previously, but the experimental tank was maintained at pH 9.5 by the continual drop-wise addition of 1 N KOH. KOH addition resulted in water K^+ concentrations that approached 0.7 mmol·litre^{-1}. In earlier control experiments rainbow trout were exposed to K^+ (KCl) concentrations as high as 13 mmol·litre^{-1}. Importantly, it resulted in no mortality and only slight increases in plasma K^+ (plasma K^+ increased from 4.6 to 6.0 mmol·l^{-1}, Wilkie et al., '93). In addition K^+ is unlikely to be taken up across the gill because the water K^+ concentrations, at pH 9.5 in the present study, were well below levels that we have typically measured in trout plasma (e.g., 4.6 mmol·litre^{-1}, Wilkie et al., 1993). Thus, we are confident that the water K^+ concentrations used in the present study were not toxic to the fish.

Both tanks were subdivided into three sections by a coarse mesh screen that did not impede water flow. Section 1 (approximate volume = 75 litres) was the point of entry for the replacement water, which was continually added at 1–2 litres·min^{-1}. The fish were restricted to the much larger middle section 2 of the tank (volume = 300 litres), which also contained a constant level overflow with drainage to waste. Section 3 (volume = 50 litres) contained a thermometer, and in the case of the experimental tank, the pH monitoring electrode and the inflow for the base addition system. Base addition was regulated by a pH stat set-up that comprised a Radiometer TTT80 autotitrator connected to a PHM82 pH meter and a GK2401C pH electrode; when water pH dropped below 9.8, the autotitrator activated an electromagnetic control valve which regulated the drop-wise addition of KOH into the water from a 20-litre reservoir. Water from this part of the tank (section 3) was continually pumped to section 1 at a rate of 4 litres·min^{-1}, where it mixed with the replacement water and subsequently flowed into section 2 of the tank (where the fish were held) at a pH of 9.5. This set-up prevented the development of pH gradients that might have confounded data interpretation. Rainbow trout have been known to distribute themselves according to water pH where there is a gradient (Peterson et al., '89). For consistency, water in the control tank was recirculated in an identical manner. Water pH was independently monitored with a Radiometer pH meter and electrode. Water pH in section 2 was maintained at pH = 9.49 ± 0.03 (mean ± SEM) and 7.86 ± 0.01, in the experimental and control tanks, respectively.

At intervals throughout the experiment, subsamples of fish (n = 8) were removed from either the control or experimental tanks and placed in individual 3.0-litre darkened plexiglass flux boxes (described in McDonald, '83) housed in recirculating systems connected to the control and experimental tanks. Water was pumped from these tanks via a submersible pump and was distributed to each box via a flow-splitter at approximately 0.5 litres·min^{-1}. This water subsequently drained back into the appropriate tank. When water flow was cut off to the boxes for flux measurements (see below), it was necessary to manually monitor and adjust box water pH (9.5 to 9.7), at 30-min intervals using the independent pH meter and appropriate amounts of base (0.5 to 2.0 ml 1 N KOH; see Wilkie and Wood, '94).

Experimental protocol

Flux measurements, each on different groups of fish, were performed under pre-exposure conditions (pH = 7.9 in both tanks) and then at 3, 7, 14, 21, and 28 days of the exposure regime (pH = 9.5 in the experimental tank; pH = 7.9 in the control tank). At each measurement time, for each tank, 8 fish were taken from the tank, placed in their own individual flux boxes, and allowed to settle overnight (about 12 h) under flow-through conditions (0.5 litre·min^{-1}). This standard box acclimation protocol, for both control and experimental fish, likely mitigated any physiological effects that might have been associated with box stress. The next morning, flow to the boxes was cut off for approximately 3 h for the measurement of ammonia excretion (J_{Amm}), urea excretion (J_{urea}), and Na^+ influx (J^{Na}_{in}), outflux (J^{Na}_{out}) and net flux (J^{Na}_{net}) rates. Fifteen minutes prior to initiating this flux determination period, 4 μCi of $^{22}Na^+$ (New England Nuclear) was added to each flux box to allow the isotope to thoroughly mix with the water. After this equilibration period, 45-ml water samples were then withdrawn at 0, 1, 2, and 3 h of the flux determination period and water pH was adjusted and monitored as previously described. At the end of the flux period, the radioactive water was diverted to a waste container, to avoid contaminating the holding tank water, and the flow was re-established to the boxes.

To document the acute effects of high-pH exposure, an additional group of fish, previously acclimated to pH 7.9 water, were placed in flux boxes receiving the same pH 7.9 water, and allowed to acclimate overnight (12 h). The next morning water pH was increased to pH 9.5 and flux determinations were performed after 0–3 h, 8–11 h, and 24–27 h of pH 9.5 exposure. These data were then compared to pre-exposure measurements that were made in fish held at pH 7.9, which served as the controls. Since 12 h was required to allow the fish to acclimate to the flux boxes, it was not practical to perform simultaneous control measurements at 0–3 h, 8–11 h, or 24–27 h of high-pH exposure. Shortly after the 24–27 h flux measurements these fish were then sacrificed for blood sampling (see below for further details).

Approximately 5 h after flux determinations were performed, fish were sacrificed, one at a time, with an overdose of MS-222 (1.5 g·liter^{-1}) for blood and tissue sampling. MS-222 anaesthesia can lead to changes in plasma ion and cortisol concentrations (e.g., Laidley and Leatherland, '88). However, these authors suggested that if lethal doses of MS-222 are used, effects on cortisol concentration can be minimized because of the rapid induction of unconsciousness in the fish. Overdose with MS-222 in the present study generally led to complete immobilization of the fish within 1 min, and blood and white muscle samples were obtained within 30 sec following removal from the box. Control blood and muscle physiological parameters (e.g., blood ammonia, lactate, glucose, cortisol, ions; muscle ammonia, lactate, ions, water, and pH_i) were similar to control values reported in previous investigations (e.g., Milligan and Wood, '82; Pörtner et al., '90; Gamperl et al., '94; Wang et al., '94). The use of simultaneous control fish also mitigated any artifact that may have been brought about by MS-222 overdose.

Blood samples were taken at 3, 7, 14, 21, and 28 days, but muscle samples were only excised under pre-exposure conditions and at 7, 14, 21, and 28 days. An additional blood sample was taken after 1 day in a separate group of experimental fish to characterize the acute effects of high pH on blood parameters. After the fish were immobilized, following MS-222 addition to the box water, blood was immediately withdrawn via caudal puncture into an ice-cold, heparinized, gas-tight Hamilton syringe. Blood was immediately processed for haematocrit, haemoglobin, and lactate determinations, and the remainder was centrifuged at 10,000*g* for 3 min for separation of plasma. This plasma was then frozen in liquid N_2 and stored at –70°C for later determination of ammonia, cortisol, glucose, Na^+, and Cl^- concentrations. Separate aliquots of plasma were frozen for later measurements of plasma pH (pH_e = extracellular pH) and total CO_2 concentration. Immediately following blood sampling, white muscle samples were excised from the region between the adipose and dorsal fins, above the lateral line, and freeze clamped with liquid N_2–cooled aluminum tongs. The samples were temporarily stored in liquid N_2, and then at –70°C for later determination of the white muscle (WM) ammonia, lactate, Na^+ and Cl^- concentrations, water content, and intracellular pH (pH_i).

Analytical techniques and calculations

Water and blood parameters

Water ammonia concentration was determined via a micro-modification of the salicylate-hypochlorite assay (Verdouw et al., '78). Water urea was determined via the diacetyl monoxime method of Crocker ('67). Water and plasma Na^+ was determined using atomic absorption, and water $^{22}Na^+$ radioactivity was measured on 5-ml water samples mixed with 10 ml of aqueous counting scintillant (Amersham). Plasma Cl^- was determined via coulometric titration. Whole blood lactate (lactate dehydrogenase), plasma ammonia (glutamate dehydrogenase) and glucose (hexokinase/glucose-6-phosphate dehydrogenase) were all determined enzymatically using Sigma kits. Cortisol was determined via ^{125}I radioimmunoassay (ImmuCorp) measured on a Packard 5000 Series gamma counter. Blood haemoglobin concentration was determined by the cyanmethemoglobin method using Sigma reagents and standards.

Since blood samples obtained via caudal puncture represent mixed arterio-venous blood, plasma pH in the present study will be referred to simply as extracellular fluid pH (pH_e). Extracellular fluid pH and extracellular total CO_2 were determined simultaneously on plasma samples that had not been previously thawed. The pH_e was determined by injecting plasma into a thermostatted (15°C) capillary pH electrode [Radiometer (G297/G2)] that was connected to a Radiometer PHM72 pH meter. Total plasma CO_2 was determined on a Corning total CO_2 analyzer. As a check to ensure that freeze-thawing did not influence our measurements, we measured the pH and total CO_2 of fresh plasma samples and compared them to matching samples that were frozen and then thawed. We

found that freezing and thawing did not result in noticeable differences in either the total CO_2 content or pH of the plasma (C.M. Wood and R.S. Munger, unpublished observations).

White muscle parameters

White muscle intracellular pH (pH_i) was determined using the nitrilotriacetic acid–fluoride method of Pörtner et al. ('90) following pulverization of the muscle under liquid N_2 (see Wilkie and Wood, '95, for further details); the pH measurements were performed on the same capillary pH electrode set-up just described. White muscle ammonia, lactate, Na^+, and Cl^- concentrations were determined on white muscle extracts that had been ground to a fine powder under liquid N_2 and deproteinized in 9 volumes of 8% perchloric acid. Ammonia concentrations were determined on the Tris-neutralized extracts, according to the methods of Kun and Kearney ('71). White muscle Na^+, Cl^-, and lactate analyses were identical with those described for plasma. White muscle water content was established by oven-drying 200 mg of frozen white muscle to constant weight at 80°C.

Calculations

Ammonia (J_{Amm}) and urea excretion rates (J_{urea}) were calculated from the respective changes in water ammonia and urea concentrations during the flux period, the fish's weight, and the known box volume (see Wright and Wood, '85). Na^+ influx estimates (J^{Na}_{in}) were based on the disappearance of $^{22}Na^+$ from the water and the mean specific activity in the water during the flux period; net flux calculations (J^{Na}_{net}) were based on changes in cold (non-radioactive) Na^+ during the flux period, and Na^+ outflux (J^{Na}_{out}) was calculated as J^{Na}_{net} minus J^{Na}_{in} (see Wilkie and Wood, '94, for further details). Water, plasma, and white muscle P_{NH3} and NH_4^+ concentrations were determined from measurements of total ammonia and pH via manipulation of the Henderson-Hasselbalch equation and the appropriate solubility coefficients and pK_{Amm}, provided by Cameron and Heisler ('83). Plasma P_{CO2} (Pe_{CO2}) and HCO_3^- concentration ($[HCO_3^-]_e$) were calculated in an analogous manner using the solubility and pK′ constants supplied by Boutilier et al. ('84). The extracellular fluid metabolic acid-load (ΔH^+_m) was calculated with the following equation as outlined by Milligan and Wood ('86a,b):

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

Since the fish were sampled terminally, $[HCO_3^-]_1$ and $[HCO_3^-]_2$ represent simultaneous measurements of the mean extracellular fluid HCO_3^- concentrations, and pH_1 and pH_2 are the mean extracellular pHs in trout held at pH 7.9 and 9.5, respectively. Changes in extracellular lactate (ΔLac) were also based on mean values. β represents the mean blood buffer capacity, at each sample period, and was calculated from haemoglobin concentration using the regression formula derived by Wood et al. ('82) for rainbow trout blood. Mean cell haemoglobin concentration (MCHC), an index of red blood cell water content, was calculated as the haemoglobin concentration divided by the haematocrit (Milligan and Wood, '82).

Statistics

All data are expressed as means ± 1 SEM (N). Statistically significant differences between controls (pH 7.9) and experimental fish (pH 9.5) at the same sampling times in each experiment were determined via unpaired Student t-test (at $P < .05$), after first checking for homogeneity of variance by use of an F-test. In instances where this criterion was not satisfied, the more conservative Welch's approximate t-test was employed (Zar, '84). Data generated at 0–3 h, 8–11 h, and 1 day of high pH exposure were statistically compared to the respective pre-exposure (P) values via unpaired t-test because it was not possible to perform simultaneous control measurements at these times.

RESULTS

Survival and stress at high pH

Rainbow trout appear to survive readily at pH 9.5. As a result, only negligible mortality was observed (two fish died at 1 day = 4%) during the 28-day high-pH exposure period. There were no significant long-term changes in the quantity of stress indicators, such as plasma cortisol or glucose, following transfer to alkaline water. Plasma cortisol concentrations fluctuated around 3–5 $ng \cdot ml^{-1}$, while baseline glucose concentrations ranged from 3 to 5 $mmol \cdot liter^{-1}$ (Table 1). There was, however, an acute 130% increase in plasma glucose concentration after 1 day of high pH exposure (Table 1). Plasma cortisol was only slightly elevated at this time (Table 1).

Significant, long-term changes in blood lactate concentration were observed in those fish held at pH 9.5. Blood lactate concentrations approximated 0.46 $mmol \cdot liter^{-1}$ during the pre-exposure period, and exposure to pH 9.5 led to an

TABLE 1. Changes in plasma cortisol and glucose, and blood lactate concentrations of rainbow trout during 28 days at pH 9.5[1]

	Plasma glucose ($mmol \cdot l^{-1}$)		Plasma cortisol ($ng \cdot ml^{-1}$)		Blood lactate ($mmol \cdot l^{-1}$)	
	pH 7.9	pH 9.5	pH 7.9	pH 9.5	pH 7.9	pH 9.5
P	3.9 ± 0.2		5.5 ± 0.8		0.46 ± 0.04	
1 day	—	9.1 ± 1.8**	—	8.7 ± 1.6**	—	1.08 ± 0.19**
3 days	3.4 ± 0.1	5.0 ± 0.8	9.6 ± 1.1	4.0 ± 1.3*	0.59 ± 0.13	1.60 ± 0.47*
7 days	3.6 ± 0.3	4.8 ± 0.3*	5.8 ± 2.0	4.6 ± 0.9	0.47 ± 0.16	1.32 ± 0.18*
14 days	3.3 ± 0.1	3.6 ± 0.2	2.4 ± 0.2	3.0 ± 0.5	0.79 ± 0.08	1.03 ± 0.09*
21 days	3.7 ± 0.3	3.3 ± 0.6	3.4 ± 1.1	3.3 ± 0.5	1.20 ± 0.07	1.09 ± 0.19
28 days	4.3 ± 0.3	5.2 ± 0.5	3.5 ± 0.5	4.6 ± 1.2	0.96 ± 0.17	1.67 ± 0.29*

[1]Means ± SEM; n = 13 and 5–8 during the pre-exposure and experimental periods, respectively.
*Significantly different from simultaneous pH 7.9 (control) values ($P < .05$).
**Significantly different from pre-exposure (P) values ($P < .05$); test done only at 1 day as there were no simultaneous control values at these times.

acute 130% increase in blood lactate after 1 day; this elevation persisted for the first 3 days of exposure (Table 1). Blood lactate concentrations decreased slightly thereafter but were still significantly higher, by 30–150% at days 7, 14, and 28, than values measured in the simultaneous controls (Table 1). White muscle lactate concentrations did not appear to be affected by long-term high-pH exposure. White muscle lactate concentrations ranged from approximately 1.5 to 3.1 $mmol \cdot kg^{-1}$ wet weight in the control and treatment fish (data not shown).

Nitrogenous waste excretion and storage

Pre-exposure ammonia excretion rates (J_{Amm}) at control pH (7.9) averaged 340 $\mu mol\ N \cdot kg^{-1} \cdot h^{-1}$ (Fig. 1A). High pH exposure led to an initial 40% decrease in J_{Amm} during the first 3 h at pH 9.5 but by 1 day J_{Amm} had completely recovered and, thereafter, fluctuated between 175 and 300 $\mu mol\ N \cdot kg^{-1} \cdot h^{-1}$. The only exception was at 3 weeks, at which time J_{Amm} was significantly depressed to approximately 100 $\mu mol\ N \cdot kg^{-1} \cdot h^{-1}$. Similarly, J_{Amm} in control fish fluctuated between 200 and 300 $\mu mol\ N \cdot kg^{-1} \cdot h^{-1}$ during the 4-week experiment (Fig. 1A).

Initial urea excretion rates (J_{urea}), measured at pH 7.9, were about 36 $\mu mol\ N \cdot kg^{-1} \cdot h^{-1}$ (Fig. 1B). Exposure to pH 9.5 led to a 70–85% increase in J_{urea} during the 1st day of pH 9.5 exposure, but by 3 days, excretion rates had returned to pre-exposure levels and approximated rates measured in the control fish held throughout at pH 7.9 (Fig. 1B).

Pre-exposure plasma (= extracellular) T_{Amm} concentrations averaged 200 $\mu mol\ N \cdot litre^{-1}$, and exposure to pH 9.5 resulted in an acute 150% increase in plasma T_{Amm} after 1 day (Fig. 2). This was followed by a return of plasma T_{Amm} toward control levels over the next 2 days, but plasma T_{Amm} concentrations still tended to be slightly higher in the treatment fish, an effect which was significant on days 7, 14, and 28 (Fig. 2). These high-pH–induced increases in internal T_{Amm} were more pronounced in the white muscle. Pre-exposure ammonia concentrations in the white muscle approximated 750 $\mu mol\ N \cdot kg^{-1}$ wet weight. Exposure to pH 9.5 led to 50–133% higher white muscle T_{Amm} concentrations in the experimental fish (Fig. 3).

Haematological responses

Pre-exposure blood haematocrits where about 32%, and no changes in haematocrit were observed after 1 day of pH 9.5 exposure. After 3 days, however, blood haematocrits were about 28% greater in those fish held at pH 9.5 vs. pH 7.9. Haematocrit remained significantly elevated in the high pH exposure group, by about 25–35%, for the remainder of the experiment (Table 2). Despite these pronounced changes in haematocrit, the MCHC of fish exposed to pH 9.5 never significantly differed from that of the control fish. The MCHC fluctuated around 25 $g \cdot 100\ ml^{-1}$ throughout the experiments, with values ranging from 21 to 27 $g \cdot 100\ ml^{-1}$ (data not shown).

Internal Na^+ and Cl^- concentrations and Na^+ fluxes

Exposure to pH 9.5 had little effect on long-term maintenance of internal ion concentrations. Pre-exposure plasma Na^+ and Cl^- concentrations were 150 and 130 $mmol \cdot litre^{-1}$, respectively. This was followed by an acute 9% decrease in the plasma Na^+ concentration after 1 day at pH 9.5 (Table 2).

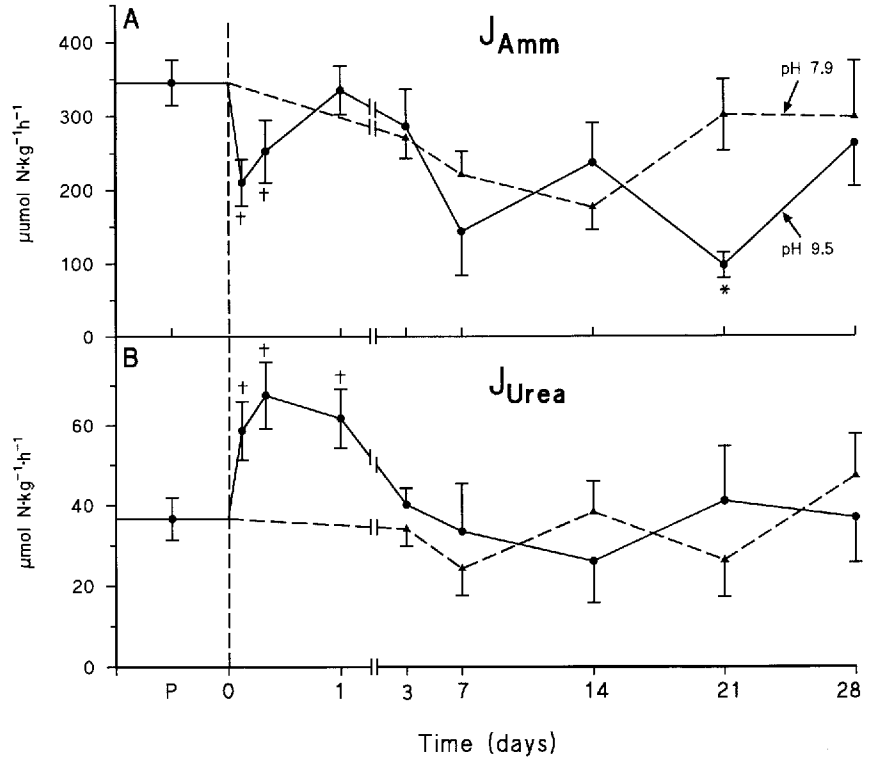

Fig. 1. **(A)** Ammonia excretion rates (J_{Amm}) and **(B)** urea excretion rates (J_{Urea}) in rainbow trout held at pH = 7.9 (broken line; triangles) or pH = 9.5 (solid line; circles) for 28 days. Means ± 1 SEM; n = 15 and n = 7–8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH = 7.9 (control) values, and the daggers specify significant differences between the rates at 0–3 h, 8–11 h, and 1 day of pH 9.5 exposure vs. pre-exposure (P) rates ($P < .05$).

Plasma Cl^- concentrations did not change significantly during this time. The initial disturbance to Na^+ balance was rapidly corrected, and there was no evidence of any longer-term disturbances to plasma Na^+ or Cl^- balance beyond the 1st day of high pH exposure. Generally, plasma Na^+ and Cl^- concentrations fluctuated around 140 mmol·litre^{-1} and 130 mmol·litre^{-1}, respectively, for the remainder of the experiment. This absence of significant disturbance to plasma ion balance was also reflected in the white muscle, where Na^+ and Cl^- concentrations ranged from 11 to 13 and 12 to 15 mmol·kg^{-1} wet weight, respectively (data not shown). White muscle water content was stable, about 81%, in fish held at both pHs (data not shown).

The maintenance of internal ion balance is interesting in view of the fact that Na^+ influx rates were persistently reduced, by 50–60%, during most of the high-pH exposure regime. Although these reductions were significant at 0–3 h, 1, 14, 21, and 28 days of pH 9.5 exposure (Fig. 4A,B) there were no significant changes in J^{Na}_{out} or J^{Na}_{net}, apart from elevated net losses at 28 days (Fig. 4A,B). There did appear to be increased net Na^+ losses during the first few hours of high pH exposure, but this trend was not statistically significant. Beyond 1 day it can generally be concluded that both groups of fish, control and high-pH exposed, were fairly close to "net Na^+ balance" for most of the experiment.

Acid-base balance

White muscle pH_i was stable during chronic high pH exposure. At no time did white muscle pH_i, which fluctuated around pH = 7.3, differ significantly from that measured in control fish (Fig. 5). Similarly, the pH of the extracellular fluid (pH_e = plasma pH) was also effectively regulated during chronic high pH exposure. The pre-exposure pH_e was approximately 7.8 in both groups of fish. The pH_e increased slightly in the control fish, to approximately pH 7.9, after 7 days and then stabilized in this group. The pH_e of those fish held at pH 9.5 was significantly

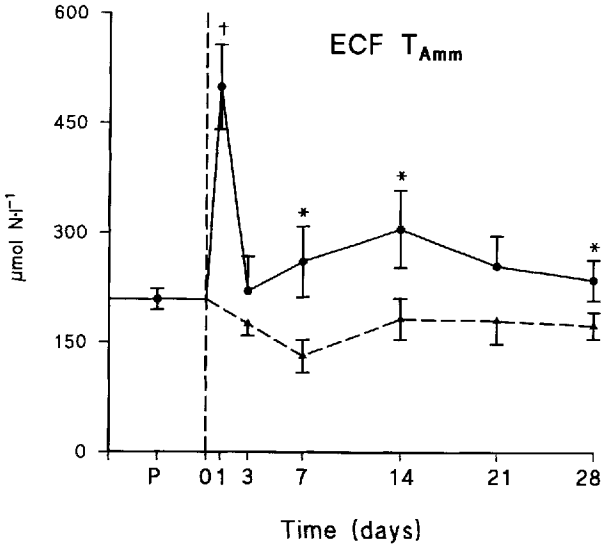

Fig. 2. Extracellular (plasma) total ammonia concentrations (T_{Amm}) in rainbow trout held at pH = 7.9 (broken line; triangles) or pH = 9.5 (solid line; circles) for 28 days. Means ± 1 SEM; n = 15 and n = 7–8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH = 7.9 (control) values. The dagger specifies a significant difference between values measured after 1 d vs. the pre-exposure (P) values. The level of significance is $P < .05$.

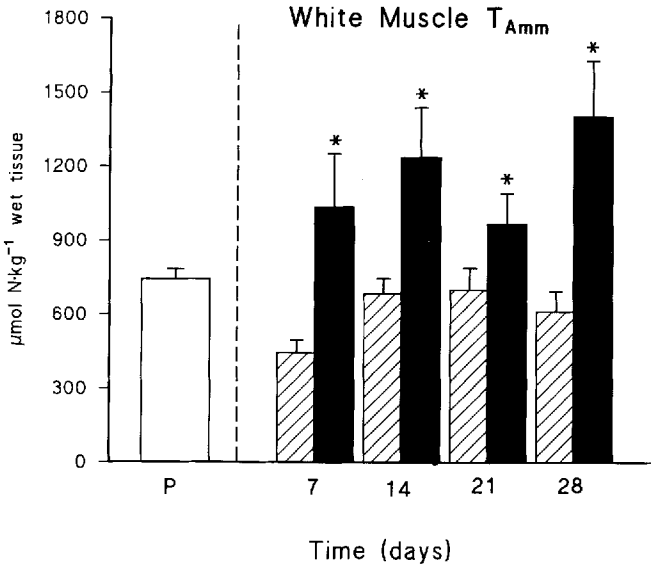

Fig. 3. White muscle total ammonia concentrations (T_{Amm}) in rainbow trout held at pH = 7.9 (hatched bars) or pH = 9.5 (solid bars) for 28 days. Means ± 1 SEM; n = 15 and n = 7–8 during the pre-exposure (P; open bar) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH = 7.9 (control) values ($P < .05$).

lower than the controls and fluctuated around pH 7.8 for most of the experiment (Fig. 5).

Despite the presence of a slight acidosis, the fish at pH 9.5 experienced a chronic respiratory alkalosis that was characterized by a persistent 30–40% reduction in extracellular fluid P_{CO2} (Pe_{CO2}; Fig. 6A). However, pH_e was maintained through a counterbalancing 30–50% reduction in extracellular HCO_3^- concentration ($[HCO_3^-]_e$; Fig. 6B). This decrease in extracellular HCO_3^- concentration resulted from a chronic metabolic acid load (ΔH^+_m) that approached 5 mmol·litre^{-1} from days 7 to 28 of the exposure (Fig. 6C). Changes in the blood lactate concentration (ΔLac), at these times, accounted for no more than 10% of this observed ΔH^+_m (Fig. 6C). Interestingly, the ΔH^+_m calculated over the first 3 days of high pH exposure closely approximated the estimates of ΔLac (Fig. 6C).

DISCUSSION

Survival and stress at high pH

Despite previous accounts of salmonid mortality at pH = 9.0 or greater (e.g., Eicher, '46; Jor-

TABLE 2. Changes in the blood haematocrit and plasma Na^+ concentration of rainbow trout during 28 days at pH 9.5[1]

	Blood haematocrit (%)		Plasma Na^+ (mmol·l^{-1})	
	pH 7.9	pH 9.5	pH 7.9	pH 9.5
P	32.3 ± 1.0		148.5 ± 1.0	
1 day	—	31.1 ± 1.3	—	135.0 ± 4.2**
3 days	28.8 ± 2.1	36.9 ± 2.3*	142.0 ± 2.6	137.8 ± 4.1
7 days	28.6 ± 2.5	38.9 ± 1.1*	145.7 ± 2.1	146.4 ± 4.2
14 days	26.4 ± 1.1	33.5 ± 2.1*	141.8 ± 1.9	144.8 ± 2.0
21 days	26.3 ± 1.9	34.0 ± 1.6*	137.8 ± 2.2	144.3 ± 3.3
28 days	27.4 ± 2.0	35.0 ± 1.9*	141.0 ± 1.6	136.1 ± 4.8

[1]Means ± SEM; n = 15–16 and 7–8 during the pre-exposure and experimental periods, respectively.
*Significantly different from simultaneous pH 7.9 (control) values ($P < .05$).
**Significantly different from pre-exposure (P) values ($P < .05$); test done only at 1 day as there were no simultaneous control values at these times.

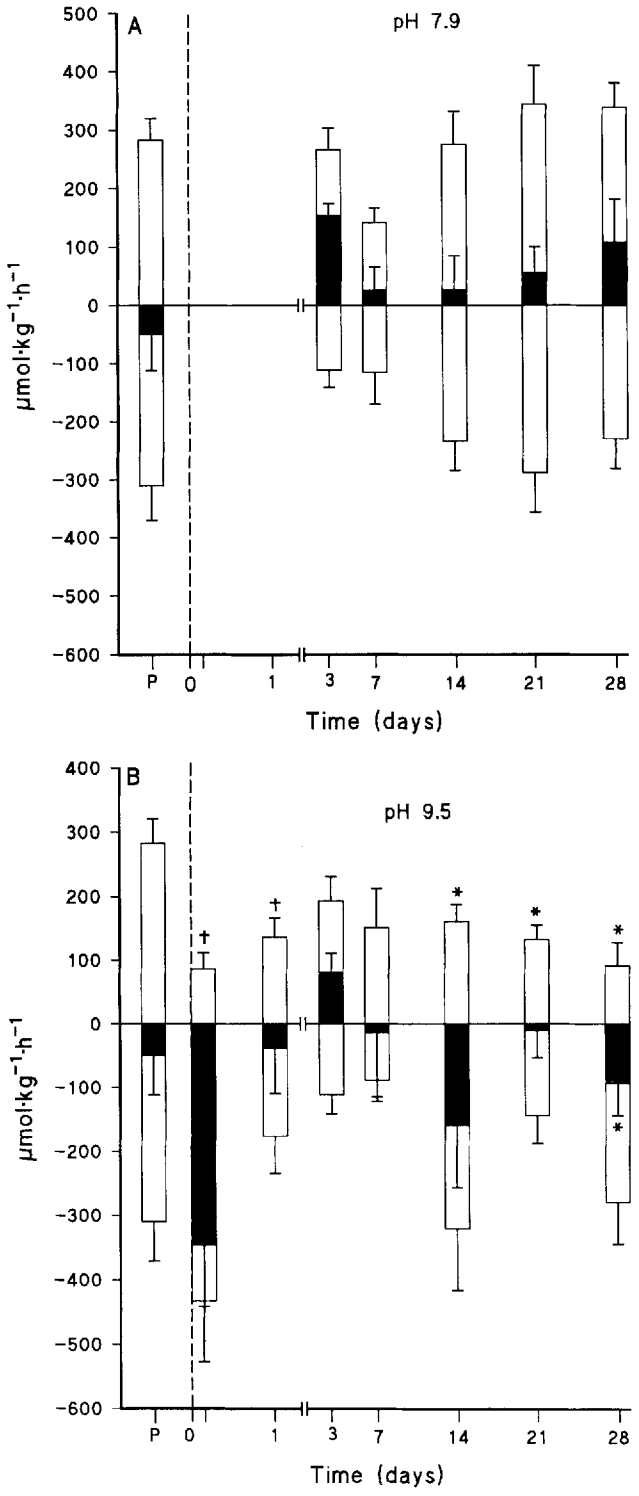

Fig. 4. Sodium influx (J^{Na}_{in}; upward facing open bars), outflux (J^{Na}_{out}; downward facing open bars) and net flux (J^{Na}_{net}; solid bars) rates of rainbow trout held at (**A**) pH = 7.9 or (**B**) pH = 9.5 for 28 days. Means ± 1 SEM; n = 14 and n = 7–8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences between the simultaneous pH = 7.9 (control; A) values and the pH 9.5 (B) values. In B, the daggers specify significant differences between the rates at 0–3 h and 1 day of pH 9.5 exposure vs. pre-exposure rates ($P < .05$).

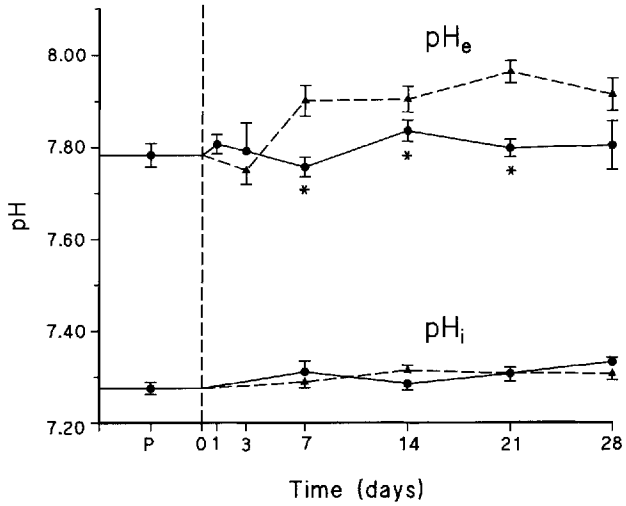

Fig. 5. Extracellular (plasma) (pH_e) and white muscle intracellular pH (pH_i) measurements in rainbow trout held at pH = 7.9 (broken line; triangles) or pH = 9.5 (solid line; circles) for 28 days. Means ± 1 SEM; n = 12–13 and n = 7–8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH = 7.9 (control) values ($P < .05$).

dan and Lloyd, '64; Daye and Garside, '75; Yesaki and Iwama, '92), the present investigation demonstrates that free-swimming rainbow trout are capable of long-term survival (28 days) at pH 9.5. However, the acute elevations in plasma glucose and, to a lesser extent cortisol, indicates that initial transfer to pH 9.5 imparts a significant short-term stress upon rainbow trout. The rapid return of these stress indicators, as well as the rapid correction of other physiological indices, to pre-exposure values supports arguments that rainbow trout quickly adapt to alkaline water. This adaptability is underscored by the long-term maintenance of plasma glucose and cortisol concentrations at levels that approximated those previously observed in similarly sampled, resting, unstressed trout (e.g., Woodward and Strange, '87; Barton and Iwama, '91; Pottinger and Pickering, '92; Gamperl et al., '94). However, slight elevations in blood lactate concentration may suggest that there was a persistent, albeit small, stress effect during chronic high pH exposure.

We previously suggested that high pH-induced acute elevations in blood lactate concentration reflect increased lactic acid production in the white

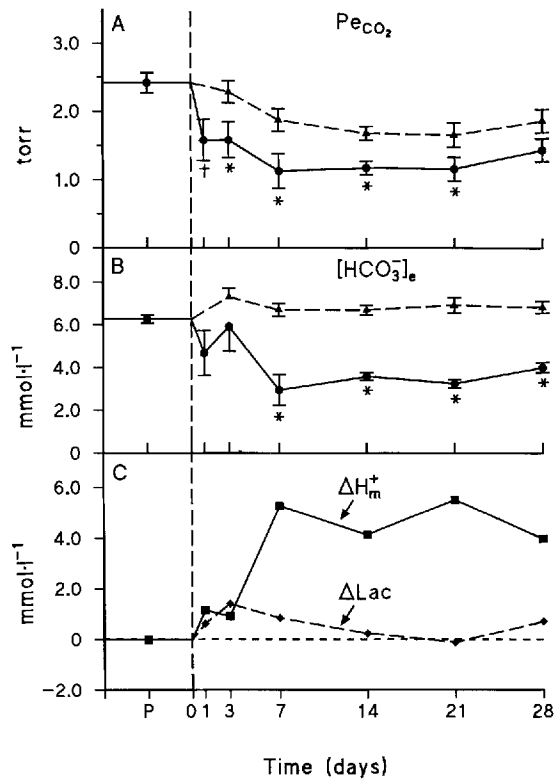

Fig. 6. Differences in extracellular (plasma) **(A)** P_{CO2} (Pe_{CO2}), and **(B)** HCO_3^- ($[HCO_3^-]_e$), and **(C)** metabolic acid load (ΔH^+_m) and lactate load (ΔLac) of rainbow trout. Analyses for A and B were done on rainbow trout held at either pH = 7.9 (broken line; triangles) or pH = 9.5 (solid line; circles) for 28 days. Calculations of ΔH^+_m in C were based on the respective mean $[HCO_3^-]_e$ and pH_e differences between the control vs. experimental fish; ΔLac was based on mean respective differences in whole blood lactate concentrations (see text for further details). Means ± 1 SEM; n = 12–13 and n = 7–8 during the pre-exposure (P) and experimental periods, respectively. Asterisks indicate significant differences from simultaneous pH = 7.9 (control) values ($P < .05$).

muscle, which, in turn, could supply metabolic protons to buffer increases in blood pH that are brought about by decreases in plasma P_{CO2} (Wilkie and Wood, '91, '95). Although, this may be the case in the first few hours–days of high pH exposure, the present study suggests this relationship breaks down beyond 3 days of high pH exposure (see below). More likely, the slight elevations in blood lactate imply that the fish were still moderately stressed in their alkaline environments and/or their basal energetic demands were increased. This latter hypothesis is supported by the chronic elevations of hematocrit that were observed in those trout chronically exposed to high pH and is discussed in more detail below.

Although internal ammonia levels increased during high pH exposure, the calculated ECF P_{NH3} was well below that, approximately 400 μtorr, known to contribute to high pH-induced or ammonia toxicity–induced mortality in salmonids (Yesaki and Iwama, '92; Wilkie et al., '93; R.W. Wilson and C.M. Wood, unpublished observations). Profound reductions in plasma Na^+ and Cl^- concentrations, which have been implicated in mortality at alkaline pH (Heming and Blumhagen, '88; Yesaki and Iwama, '92; Wilkie et al., '93), were also absent during chronic high pH exposure. Recently, in contrast to the present study, Yesaki and Iwama ('92) reported that rainbow trout exposed to soft ($[Ca^{2+}] = 0.03$ mmol·litre^{-1}), alkaline (pH 10.1) water suffered from high rates of mortality that were associated with pronounced ionoregulatory disturbances characterized by continual losses of plasma Na^+ and Cl^-. Unlike in the present and previous studies (Wilkie and Wood, '91, '95), they also reported that rainbow trout were unable to re-establish ammonia excretion, as reflected by persistent increases in plasma ammonia concentration, in the soft-alkaline water. When Yesaki and Iwama subsequently added Ca^{2+} to the water, however, it appeared to mitigate the disruptive influence that alkaline water had on the ammonia excretion and ionoregulatory capabilities of the fish, and they readily survived. Thus, the maintenance of water Ca^{2+} concentrations above the hardwater–softwater threshold of 0.4 mmol·litre^{-1} (Marier et al., '79) likely contributed to rainbow trout survival during chronic high-pH exposure in the present study. High water Ca^{2+} is also a key factor for trout survival in acidic waters (McDonald, '83). Clearly, more work needs to be done to account for the differential effects that hard-alkaline water and soft-alkaline water have on rainbow trout survival, especially as it pertains to ammonia excretion patterns and ion balance.

Nitrogenous waste excretion and storage

The initial inhibition of J_{Amm}, upon exposure to pH 9.5, followed by a rapid recovery to pre-exposure rates was consistent with earlier studies performed on the rainbow trout (Wilkie and Wood, '91, '94; Yesaki and Iwama, '92). Recovery of J_{Amm} was likely associated with the slight, but persistent elevations in plasma T_{Amm} that were observed. These elevations in T_{Amm} probably helped to drive J_{Amm} during long-term high pH exposure through the maintenance of favourable blood–gill water P_{NH3} gradients. This interpretation is supported by recent work on the high-pH-tolerant, Lahontan

cutthroat trout of alkaline Pyramid Lake, Nevada (Wright et al., '93; Wilkie et al., '94). Wright and colleagues ('93) demonstrated that elevated plasma T_{Amm} concentrations likely facilitated ammonia excretion by the cutthroat trout into its highly alkaline (pH 9.4) environment through the long-term maintenance of favourable blood–gill water P_{NH3} gradients. The dominant role that NH_3 diffusion plays in facilitating ammonia excretion in freshwater teleosts, at circumneutral pH, has also been demonstrated in a number of recent investigations (Cameron and Heisler, '83; Wright et al., '89; Wilson et al., '94a).

An induction in Na^+/NH_4^+ exchange (Wright and Wood, '85; Yesaki and Iwama, '92) probably played no role in the maintenance of J_{Amm} in the present study, and this interpretation is supported by the recent observations of Wilkie and Wood ('94) on rainbow trout. They reported that the complete recovery of ammonia excretion to pre-exposure rates, after 3 days of pH 9.5 exposure, was unaffected when Na^+ uptake was blocked with amiloride. Wilson et al. ('94a) have similarly suggested that Na^+/NH_4^+ exchange plays no role in facilitating J_{Amm} when environmental ammonia is elevated.

The maintenance of higher steady-state plasma T_{Amm} concentrations probably also accounted for the higher white muscle T_{Amm} levels that were observed during high pH exposure (Wilkie and Wood, '95). Retention of ammonia in the white muscle intracellular fluid (ICF) compartment is associated with extracellular (ECF) to white muscle loading. This loading occurs as a result of transiently elevated NH_4^+ electrochemical (F_{NH4}) and NH_3 partial pressure (ΔP_{NH3}) gradients from the ECF to white muscle ICF (Wilkie and Wood, '95). Under steady-state conditions, however, a favourable ECF to white muscle ICF F_{NH4} is thought to facilitate white muscle NH_4^+ loading, while NH_3 movements are in the opposite direction down the P_{NH3} gradient. As a result, net ammonia movements in the two directions are in approximate balance (Wright et al., '88; Wright and Wood, '88). Since respective estimates of F_{NH4} and ΔP_{NH3} were similar under control pH and alkaline conditions (approximated –40 mV and 50 μtorr, respectively) in the present study, we conclude that ECF to white muscle ICF ammonia distribution patterns reached a new steady state within the 1st week of high-pH exposure. Thus, it would appear that higher steady-state plasma ammonia concentrations not only facilitate J_{Amm} into alkaline water but also result in significant storage of ammonia in the white muscle compartment. This theory might also explain why white muscle ammonia concentrations are extremely high in the Cyprinid, *Chalcalburnus tarichi,* of highly alkaline (pH 9.8), Lake Van, Turkey (Danulat and Kempe, '92).

Unlike the Lake Magadi tilapia (*Oreochromis alcalicus grahami*), which exclusively excretes urea into its alkaline (pH 10) surroundings (Randall et al., '89; Wood et al., '89), it appears that chronic elevations in J_{urea} play no role in the long-term maintenance of nitrogenous waste excretion by rainbow trout during high pH exposure. However, J_{urea} was elevated during the 1st day or so of pH 9.5 exposure. This latter observation is consistent with previous acute high pH exposure experiments (Wilkie and Wood, '91; Wilkie et al., '93) and suggests that transiently elevated J_{urea} is a temporary nitrogenous waste detoxification response. Similar responses have been reported in the rainbow trout (Olson and Fromm, '71) and tilapia (*Oreochromis niloticus;* Wright, '93) following exposure to elevated ambient ammonia. We conclude that the rapid (8–48 h) re-establishment of J_{Amm} to pre-exposure rates makes chronic elevations of J_{Urea} unnecessary in the rainbow trout.

Haematological responses

The marked increases in haematocrit during high pH exposure implies that there were additional metabolic costs associated with high pH exposure. Higher haematocrit was not likely the result of a decrease in the ECF compartment volume or red blood cell swelling (cf. Milligan and Wood, '82, '86b), because no changes in mean cell haemoglobin concentration, muscle water, or muscle lactate concentrations were observed.

Chronic high pH exposure may well have increased the O_2 demands of the fish, leading to an increased blood O_2 carrying capacity via splenic red blood cell (RBC) expulsion and/or increased RBC production. Glycolytic flux may also have increased due to elevated concentrations of ammonia in body compartments such as the white muscle. Ammonia is a known stimulator of the key glycolytic enzyme, phosphofructokinase (Kuhn et al., '74), and it may stimualte glycolysis when fish are subjected to alkaline conditions. Indeed, such a response might explain why blood lactate concentrations are elevated under alkaline and hyperammonemic conditions (Wilkie and Wood, '91; Wilkie et al., '93; Wilson et al., '94a). Further studies, investigating the effects the high internal ammonia has on muscle metabolism, might help explain our observations and substantiate our hypothesis.

Internal Na^+ and Cl^- concentrations and Na^+ fluxes

During chronic high-pH exposure Na^+ uptake was, more or less, chronically depressed, but the similar plasma Na^+, as well as Cl^-, levels in the controls and experimental fish suggest that the trout were able to restore and maintain ion balance beyond 3 days of exposure to pH 9.5. These persistent reductions of J^{Na}_{in} seen at chronic high pH resemble those seen during chronic acid exposure, but unlike the present situation, low-pH-induced reductions of plasma Na^+ and Cl^- are not readily corrected (Audet et al., '88). In a previous investigation (Wilkie and Wood, '94), we demonstrated that 3 days of high-pH exposure resulted in persistently reduced Na^+ influx. The present study demonstrates that this reduction of J^{Na}_{in} persists for at least 28 days and may very well be permanent (Fig. 4B). In fact, the initial reduction in J^{Na}_{in} probably accounted for the significant decrease in plasma Na^+ that was seen after 1 day of high-pH exposure. The long-term regulation of internal Na^+ concentrations (Table 2), in the face of chronically reduced J^{Na}_{in}, must therefore have been due to chronically decreased J^{Na}_{out}. Such a trend is evident in the data (Fig. 4B) but is not supported statistically by the comparisons against the simultaneous measurements of J^{Na}_{out} in the control fish. However, it must be remembered that J^{Na}_{out} is an indirect calculation from two direct measurements ($J^{Na}_{out} = J^{Na}_{net} - J^{Na}_{in}$) and therefore incorporates greater error. The branchial morphological and hormonal basis of long-term adaptations in both Na^+ and Cl^- exchanges at high pH remains an important area for future research.

Acid–base balance

In the present study we wished to avoid the complicating effects of cannulation which appear to make trout more vulnerable to high-pH-induced mortality (Wilkie and Wood, '91). We therefore elected to sample the fish by rapid MS-222 overdose and caudal puncture, which obtained mixed arteriovenous blood. Previous work, employing arterial and venous catheters, has demonstrated that there is very little difference between arterial and venous blood pH in trout (Cameron and Heisler, '83; Currie and Tufts, '92). Our control estimates of ECF pH (pH_e = 7.78–7.94) agree closely with measurements obtained from cannulated fish in recent studies (arterial pH = 7.83; Wilkie and Wood, '91), as do our measurements of total CO_2.

In agreement with earlier studies (e.g., Wright and Wood, '85; Lin and Randall, '90; Wilkie and Wood, '91), rainbow trout chronically exposed to pH 9.5 developed a persistent decrease in Pe_{CO2} (i.e., respiratory alkalosis; Fig. 6A). Despite this respiratory alkalosis the fish were still able to effectively regulate extracellular pH through the development of a counterbalancing metabolic acidosis (Fig. 6B,C). Previous studies have indicated that metabolic acidosis in trout at high pH is associated with a lactacidosis (Wilkie and Wood, '91, '95; Wilkie et al., '93), which is thought to originate in the white muscle, where fourfold higher lactate concentrations have been measured during acute high-pH exposure (M.P. Wilkie, G.J.F. Heigenhauser, and C.M. Wood, unpublished data). In the present study, the first few days of pH 9.5 exposure were characterized by a metabolic acid load that appeared to be associated with increased blood lactate (Fig. 6C), but this relationship broke down by 7 days, suggesting that increased lactic acid production is only a short-term supplier of counterbalancing metabolic protons in alkalotic trout. There were also no long-term changes in white muscle lactate concentration. Therefore, the metabolic acidosis was likely associated with other physiological events that were not addressed in this study.

Potentially, differential modulation of branchial Na^+ and Cl^- exchange processes accounted for the acidosis at high pH (McDonald et al., '89; Goss et al., '92). However, the relatively stable plasma Na^+ and Cl^- concentrations, throughout most of the experiment, do not appear to support this theory. If long-term changes in Na^+ vs. Cl^- movements across the gill epithelium were associated with acidic equivalent retention (= base equivalent excretion) by the fish, they would have been accompanied by decreases in plasma Na^+ and/or increased Cl^- concentration. It should be noted that the magnitude of the metabolic acidosis, which was only about 5 mmol·litre^{-1}, would be reflected by similar changes in plasma Na^+ and Cl^- concentration. It would be difficult, however, to detect such subtle changes in plasma Na^+ and Cl^- against background concentrations of 130–140 mmol·litre^{-1} in the plasma. Thus, it is still possible that the metabolic acidosis was mediated by the gills, but other factors might also be involved. Further studies are required to elucidate the causes of the chronic metabolic acidosis in trout at high pH.

Despite the persistent reduction in extracellular P_{CO2}, white muscle pH_i was relatively stable throughout chronic exposure to pH 9.5. A meta-

bolic acidosis, similar to that seen in the ECF, may have contributed to acid–base regulation in the muscle, but an equally plausible explanation is that the higher buffer capacity of the white muscle, relative to the extracellular fluid (e.g., Milligan and Wood, '86b), buffered any decrease in Pi_{CO2} that would have occurred in this compartment.

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