The physiological responses of the rainbow trout to strenuous exercise: interactions of water hardness and environmental acidity

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Cannulated adult rainbow trout were subjected to 6 min of exercise stress in four different water conditions: hard water (≃140 mg/L as $CaCO_3$), control pH (≃7.5); artificial soft water (≃14 mg/L), control pH; hard water, acid pH (≃4.4); and artificial soft water, acid pH. Physiological changes and postexercise mortalities were monitored over a 12-h recovery period. The physiological responses to exercise stress were qualitatively similar in all treatments and are discussed in detail. At control pH, water hardness had minimal influence on the magnitude of physiological response and postexercise mortality. When fish were exercised in hard water at acid pH, the symptoms of postexercise acidosis were actually ameliorated slightly and there was no increase in mortality. However, acid exposure in soft water greatly exacerbated most of the postexercise disturbances and caused a doubling of mortality.

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Des truites arc-en-ciel portant une canule ont été soumises à 6 min de stress par exercice, en quatre stations différentes: eau dure équivalant à ≃140 mg/L de $CaCO_3$) et pH ≃7,5 (témoin), eau douce artificielle (≃14 mg/L) et pH témoin, eau dure et pH acide (≃4,4), enfin eau douce artificielle et pH acide. Les changements physiologiques et les taux de mortalité ont été enregistrés après l'exercice, durant une période de récupération d'une durée de 12 h. Les réactions physiologiques au stress sont semblables qualitativement dans toutes les situations et elles sont discutées en détail. Au pH témoin, la dureté de l'eau a une influence minimale sur l'amplitude des réactions physiologiques et sur la mortalité. Les poissons soumis au stress en eau dure à un pH acide manifestent des symptomes d'acidose améliorés et il n'y a pas d'augmentation de la mortalité. Cependant, l'exposition à l'acide en eau douce accentue les changements physiologiques et double la mortalité.

[Traduit par le journal]

Introduction

Strenuous activity causes large and complex disturbances in blood acid–base, metabolite, and electrolyte status in a variety of teleosts (cf. Jones and Randall 1978, for review). In the freshwater rainbow trout, *Salmo gairdneri*, we recently have analyzed these effects in detail (Turner *et al.* 1982). The responses included a profound acidosis of both metabolic and respiratory origin, a large rise in blood lactate and pyruvate concentrations, and fluctuations in major ion levels associated with both haemoconcentration and electrolyte movements between fluid compartments. Indeed, as has been known for many years (Black 1958), the physiological consequences of exhausting exercise may result in a delayed mortality of the fish several hours later. A comparison of blood parameters between trout which suffered such delayed mortality and those which survived after exercise revealed a significantly greater blood metabolic acidosis in the former, prior to death (Wood *et al.* 1983), although this effect itself did not seem large enough to be the proximate cause of death. It was suggested that the key toxic event was lethal acidosis within the intracellular compartment, an effect reflected in the sublethal extracellular acidosis.

Environmental acidification and the attendant loss of fish life is becoming an increasingly serious problem, both in Canada and abroad (Schofield 1976; Harvey 1979; Overrein *et al.* 1980; Haines 1981). A number of recent studies have examined the physiological responses of resting adult fish to water of low pH (cf. Fromm 1980; Haines 1981; Spry *et al.* 1981; Wood and McDonald 1982, for reviews). Many of the responses described are similar to those seen after strenuous exercise, e.g., blood acidosis, haemoconcentration, and disturbances in plasma electrolyte regulation. We recently have argued that the key toxic mechanism of acid lethality in resting fish is a cardiovascular disturbance primarily elicited by the failure of ionoregulation (Milligan and Wood 1982). In light of these similarities, it seemed likely that acid exposure and strenuous exercise should serve as potentiating and possibly additive stresses, a conclusion confirmed by 7-day lethality tests on fingerling trout (Graham and Wood 1981). At acid

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pH values between 3.4 and 4.2, exhausting exercise significantly reduced median survival time.

Another factor affecting survival time in acidic environments is water hardness (Lloyd and Jordan 1964; Leivestad *et al.* 1976; Brown 1981; Graham and Wood 1981). When hard water was diluted 10-fold with distilled water to produce an artificial soft water approximating the composition of natural soft water in acid-threatened Ontario Lakes (i.e., 14 mg/L as $CaCO_3$), the toxicity of H_2SO_4 to fingerling trout was increased. This occurred under both rest and exercise conditions (Graham and Wood 1981). In resting fish, water hardness had a profound effect on the physiological responses to acid exposure. Increased water hardness ameliorated the ion loss but elevated the extent of blood acidosis via direct effects on acid and ion fluxes through the gills (McDonald, Hobé, and Wood 1980; Wood and McDonald 1982; McDonald 1982; McDonald *et al.* 1982). The important element in the water hardness effect seems to be the $[Ca^{2+}]$ and not the other ion concentrations. Water hardness also influences resting blood acid–base status (McDonald, Hobé, and Wood 1980) and hydromineral balance (Potts and Fleming 1970, 1971; Cuthbert and Maetz 1972; Eddy 1975) in neutral environments.

Holeton and Stevens (1978) made observations on oxygen uptake as related to swimming speed in soft and extremely soft water for the Amazonian fish *Triportheus angulatus*. However, we are aware of no studies which compare the physiological responses of fish exercised in hard and soft water. It seems probable that the elevated diffusive gill permeability associated with low water $[Ca^{2+}]$ might exacerbate the acid–base, fluid volume, and ionoregulatory disturbances of strenuous activity, especially when combined with simultaneous acid stress. The aim of this study was to examine the influence of low water hardness and acid exposure, alone and in combination, on the physiological responses to strenuous exercise in the rainbow trout, and to evaluate the cause of any mortality which occurred.

Materials and methods

General

For comparative purposes, the experimental conditions have been chosen to duplicate as closely as possible those used for previous studies of exercise (Graham and Wood 1981) and acid effects (McDonald *et al.* 1980; McDonald and Wood 1981; Graham and Wood 1981; Wood and McDonald 1982; Milligan and Wood 1982) on rainbow trout from this laboratory.

Experimental animals and test water

Hatchery reared rainbow trout (200–400 g) from Spring Valley Trout Farm, Petersburg, Ontario, were acclimated to and tested in hard (≃140 mg/L as $CaCO_3$) or artificial soft water (≃14 mg/L) at 14 ± 1°C. Prior to acclimation, fish were held in hard water at seasonal temperatures, so acclimation for hard water tests was mainly a temperature adjustment, a 1°C/day change being employed until the test temperature was reached (Fry *et al.* 1946). For soft water acclimation, an additional period of at least 5 days was allowed; blood ion and acid–base status stabilizes within 4 days after a hard water to soft water transfer (McDonald *et al.* 1980). Fish were not fed during the acclimation period. Acclimation (pH = 6.5–8.0) and test waters (either control pH = 7.5 ± 0.1 or acid pH = 4.4 ± 0.1) of the two hardnesses were prepared as described by Graham and Wood (1981). H_2SO_4 was used as the acidifying agent, and complete decarbonation was effected by vigorous aeration at pH = 3.0 for 24 h (Lloyd and Jordan 1964) before adjustment to the final pH with NaOH. Periodic determination of major ions (Na^+, Cl^-, Ca^{2+}, and K^+) gave concentrations within the range reported by Graham and Wood (1981) for hard and soft water. Particular care was taken to prevent contact of test waters with any metal, thereby avoiding heavy metal contamination. The four different test water exposures were (*i*) hard water, control pH (n = 13); (*ii*) soft water, control pH (n = 11); (*iii*) hard water, acid pH (n = 7); (*iv*) soft water, acid pH (n = 9).

Following acclimation and prior to testing, all animals were anaesthetized in a solution of 1:15 000 MS-222 (methane tricane sulfonate: Sigma) and fitted with dorsal aortic catheters (Clay-Adams PE50 tipped with 22-gauge needles) implanted at the junction of the efferent branchial arteries (Smith and Bell 1964), then secured with silk sutures. The cannulae were filled with Cortland trout saline (Wolf 1963) heparinized at 50 i.u./mL (ammonium heparin, Sigma). After surgery, the fish were allowed to recover at least 24 h before experimentation in blackened Plexiglas boxes (4 × 7 × 30 cm) which restricted any liberal swimming movements. The boxes were flushed with acclimation water at 300–450 mL/min.

Exercise procedure

After the resting blood sample (see below) was withdrawn from the fish at control pH, the animal was transferred rapidly to a 500-L circular tank (diameter = 91 cm) filled with water of the appropriate composition and pH. Thus in the low pH tests, the start of exercise coincided with the start of acid exposure. Strenuous exercise in the form of burst swimming was induced by vigorously chasing the fish with a blunt prod for 6 min. The chasing procedure was continued for the full period even when the fish seemed to exhaust prematurely. By the end of 6 min, all trout seemed incapable of further burst performance; most remained still but some continued to swim slowly around the tank. The trout then was returned to a blackened Plexiglas box with water of the appropriate type and monitored over the following 12 h of recovery. It is important to note that this method for exercise did not control the amount of work administered to each fish beyond driving them to apparent exhaustion. Perhaps more importantly, the fright response to the exercise could not be quantified, but was assumed to be equal for each group since all exercise was done under identical conditions. The strenuous exercise protocol is therefore considered to be "exercise *stress*."

Blood sampling and analysis

The fish were sampled at the following times after exercise: 0 h (immediately after; never longer than 2 min), 0.5, 1, 2, 4, 8, and 12 h (if death did not occur earlier). These samples were compared with a resting sample (R) taken before the start of induced swimming activity while the animal was in acclima-

tion water at control pH. At each time, blood samples of 600 μL were withdrawn anaerobically from the dorsal aortic catheter into an ice-cold gas-tight Hamilton syringe and replaced with an equal volume of Cortland saline. The blood was analyzed for [lactate], pH, total CO_2 content (whole blood and plasma), haematocrit, [haemoglobin], plasma [protein], and plasma levels of sodium, potassium, and chloride ions.

A 100-μL aliquot of whole blood was used for lactate analysis and was placed in 200 μL of ice-cold perchloric acid (8% w/v) for deproteination. After centrifugation at 9000 *g* for 3 min, the supernatant was analyzed enzymatically for L-lactate using Sigma reagents and a micromodification of the procedure outlined in Anonymous (1977). Arterial blood pH (pHa) was measured on a 40-μL aliquot injected into a Radiometer microelectrode (type E5021) thermostatted to the experimental temperature; readings were taken from a Radiometer PHM 71 acid–base analyzer. Total CO_2 levels ($CaCO_2$) in whole blood and plasma were assayed on 50-μL aliquots by the micromethod of Cameron (1971); unknowns were bracketed by precision bicarbonate standards. Plasma was obtained by centrifuging 80 μL whole blood in sealed, heparinized microhaematocrit tubes (Radiometer) at 5000 *g* for 5 min. The haematocrit (Ht) was read directly from the tube which then was broken to allow anaerobic transfer of the plasma to the Cameron chamber via Hamilton syringe. To reduce response time, the P_{CO_2} electrode (Radiometer type E5036) in the Cameron chamber was thermostatted to 37°C; readings were taken from the PHM 71 analyzer. Arterial CO_2 tensions (Pa_{CO_2}) were calculated using a rearrangement of the Henderson–Hasselbalch equation:

$$[1] \quad Pa_{CO_2} = \frac{Ca_{CO_2}}{\alpha CO_2 (1 + \text{antilog pHa} - pk')}$$

where Ca_{CO_2} was measured in plasma, αCO_2 was the physical solubility of CO_2 in plasma, and pk' was the negative logarithm of the first apparent dissociation constant for carbonic acid. Values of αCO_2 and pk' at experimental temperature and pH were taken from tabulated mammalian data (Severinghaus 1965; Albers 1970) as complete data sets for fish blood are not available and were in good agreement with Wood, McDonald, and McMahon (1982).

Bicarbonate concentrations in whole blood and plasma were calculated as the difference between total CO_2 content and physically dissolved CO_2:

$$[2] \quad [HCO_3^-] = Ca_{CO_2} - (\alpha CO_2 \cdot Pa_{CO_2})$$

using the appropriate measurements of Ca_{CO_2}. The metabolic acid content (ΔH_m^+) of whole blood samples was calculated as:

$$[3] \quad \Delta H_m^+ = \Delta[HCO_3^-] - \beta(\Delta \text{pHa})$$

where $\Delta[HCO_3^-]$ and ΔpHa were the total measured changes in whole blood bicarbonate levels and pHa over any given sample interval and β was the nonbicarbonate buffer capacity of whole blood for that interval (see Wood *et al.* (1977) and McDonald, Boutilier, and Toews (1980) for detailed explanation of the formula). Summation of all ΔH_m^+ values signs considered, for each interval from the rest samples onwards, gave the total blood metabolic acid load at any time. The calculation of ΔH_m^+ was performed for whole blood rather than plasma because lactate also was measured in whole blood; this approach is preferred for quantitative analyses of blood H^+ ion balance (Piiper *et al.* 1972). Values of β for use in [3] were calculated from the blood haemoglobin concentration ([Hb]) using the relationship determined *in vitro* by Wood, McDonald, and McMahon (1982):

$$[4] \quad \beta = -1.073[Hb] - 2.48$$

Haemoglobin concentration was determined on 20-μL aliquots by the cyanmethaemoglobin method (Blaxhall and Daisley 1973) according to Anonymous (1978). Mean cell haemoglobin concentration (MCHC, i.e., grams Hb per millilitre of erythrocytes) also was estimated from the [Hb] measurement:

$$[5] \quad \text{MCHC} = \frac{[Hb]}{Ht}$$

Changes in MCHC have been reported along with the changes in [Hb] because the latter is greatly affected by sampling losses (McDonald, Hobé, and Wood 1980; Milligan and Wood 1982). MCHC is independent of sampling losses and provides a reliable index of erythrocyte swelling and (or) the mobilization of haemoglobin-poor erythrocytes into the circulation.

A 300-μL portion of the blood sample was centrifuged at 9000 *g* for 2 min and the plasma supernatant decanted. Total plasma protein concentration was determined with an American Optical Goldberg refractometer (Alexander and Ingram 1980). Plasma protein concentration also is affected by sampling, but to a much lesser extent than [Hb], and seems to provide a good index of changes in blood volume due to water shifts in and out of the vascular space (McDonald, Hobé, and Wood 1980; Milligan and Wood 1982; Wood and McDonald 1982). Plasma Na^+ and K^+ levels were determined by flame photometry (Eel Mark II) after appropriate dilution and swamping to eliminate interference effects. Chloride was determined by coulometric titration in a chloridometer (Radiometer CMT-10).

Treatment of data

Within each experimental treatment, data are reported as means ± 1 SEM (*n*) for those fish which survived throughout the 12-h recovery period. When values for fish which died are reported, these are shown as individual data points in figures, a different symbol being used for each fish within a treatment. In the soft water acid group, only two fish out of nine survived, so all data are shown as individual values. Data for the survivors of the hard water control pH treatment have been compared with responses to identical exercise regimes in soft water and in acid water, alone and in combination, which was the main focus of this paper.

Within each treatment, the paired Student's two-tailed *t*-test was used to compare means for survivors after exercise with the respective resting value. Differences in means between groups at the same sample time were assessed by the Student's unpaired two-tailed *t*-test. Differences in mortalities between exposure groups were assessed by the χ^2-test. All significant differences are noted at the 95% confidence level.

Results

Mortalities

In Table 1, the mortalities in each treatment over the 12-h postexercise period are compared with each other and with values from other studies in our laboratory on

TABLE 1. Percentage mortality over a 12-h period in cannulated rainbow trout exposed to various conditions

	Treatment	*N*	% mortality
I	Hard water, control pH, rest*	8	0%
	Hard water, acid pH, rest†	14	0%
	Soft water, control pH, rest*	6	0%
	Soft water, acid pH, rest‡	11	0%
II	Hard water, control pH, exercise§	13	38%
	Soft water, control pH, exercise§	11	45%
	Hard water, acid pH, exercise§	7	29%
III	Soft water, acid pH, exercise§	9	78%

NOTE: Mortalities in group II are significantly different ($p < 0.05$) from those in group I. Mortality in group III is significantly different from those in groups I and II.

*From McDonald, Hobé, and Wood (1980).
†From McDonald and Wood (1981).
‡From McDonald (1982).
§From present study.

similarly prepared trout exposed to virtually identical water conditions at rest. Exercise alone caused mortality to about 40% of the fish at control pH in both hard and soft water. The combination of acid exposure with strenuous exercise did not elevate this mortality in hard water, but approximately doubled it in soft water. With exercise alone, the majority of deaths occurred after 4 h of recovery, but when exercise was combined with acid exposure, the majority occurred prior to 4 h. Acid exposure alone caused no mortality to resting fish within 12 h in either hard or soft water.

The influence of low water hardness on postexercise responses

The physiological changes occurring after strenuous exercise at control water pH are compared for surviving animals from hard and soft water acclimated groups in Figs. 1, 2, 3, and Table 2. Prior to exercise, pHa was significantly lower in soft water trout (Fig. 1A). At rest, the levels of Pa_{CO_2} were very similar in the two groups (Fig. 1B), the pHa difference being mainly due to a lower plasma $[HCO_3^-]$ in the soft water fish (Fig. 1C). There were no other significant differences in any other recorded parameters at rest, though plasma ion and protein levels tended to be slightly lower (Fig. 3, Table 2) and MCHC slightly higher (Table 2) in soft water fish.

In general, the responses to exercise in soft water were very similar to those in hard water. Arterial pH decreased about 0.45 units over the exercise period (Fig. 1A) in concert with a sharp rise in Pa_{CO_2} (Fig. 1B) and a less dramatic decrease in plasma $[HCO_3^-]$ (Fig. 1C). Pa_{CO_2} returned to normal within 30 min, a more rapid correction than seen in hard water (Fig. 1B). However, $[HCO_3^-]$ continued to fall to about half its resting level at 1 h before slowly returning to normal by 8 h (Fig. 1C).

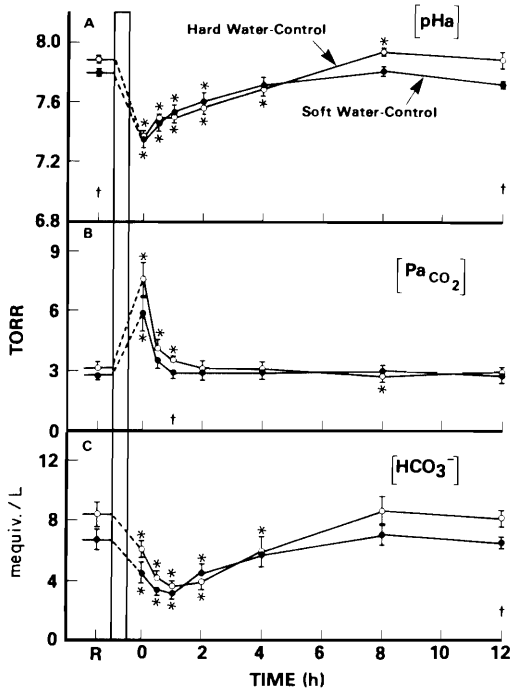

FIG. 1. Changes in (A) arterial pH (pHa); (B) arterial carbon dioxide tension (Pa_{CO_2}); and (C) arterial true plasma bicarbonate concentration ($[HCO_3^-]$) in surviving rainbow trout recovering from 6 min of strenuous exercise in either hard water (○, $n = 8$) at control pH or soft water (●, $n = 6$) at control pH (1 Torr = 133.32 Pa). Means ± 1 SEM. Bar represents period of exercise. R, resting sample taken under same conditions prior to exercise. * Signifies a significant difference ($p \leq 0.05$) within a treatment group from the corresponding resting value. *t* signifies a significant difference ($p \leq 0.05$) between treatment groups at a particular sample time.

At 12 h the original resting differences in pHa and $[HCO_3^-]$ between hard and soft water fish had been restored; because of reduced variability, the latter difference became significant. Blood [lactate] increased rapidly from negligible resting levels to about 9.5 mequiv./L by 0.5 h and then more slowly thereafter to a peak of about 13 mequiv./L at 2 h (Fig. 2A). Until that time, the [lactate] changes were identical to those seen in hard water fish. However, thereafter, [lactate] dropped at a much faster rate in soft water fish, returning to a value not significantly different from rest by 8 h. Blood metabolic acid load increased to the same extent as [lactate] immediately postexercise (0 h) but then stabilized at approximately 6 mequiv./L for the next hour (Fig. 2B) despite the continued rise in [lactate] to more than twice this value (Fig. 2A). By 8 h, the blood metabolic acid load had been eliminated. This correction of metabolic acidosis appeared slightly faster in the soft water fish, but in contrast to [lactate], the differences were not significant.

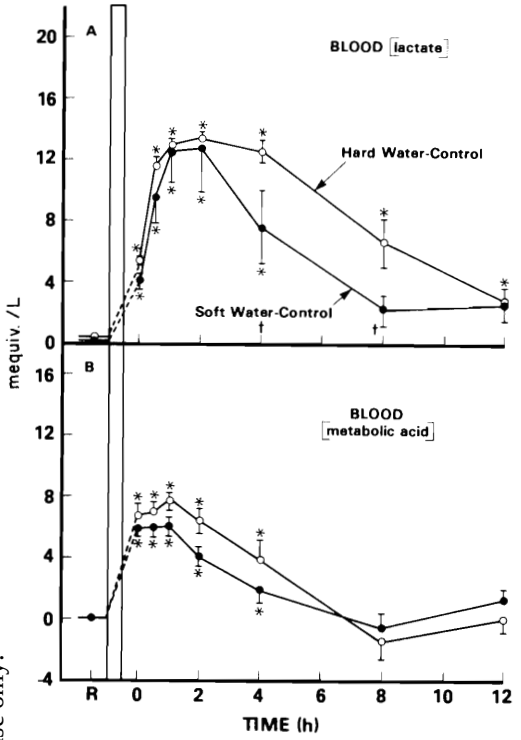

FIG. 2. Changes in (A) blood lactate concentration and (B) blood metabolic acid load in surviving rainbow trout recovering from 6 min of strenuous exercise in either hard water or soft water at control pH. Other details as in legend of Fig. 1.

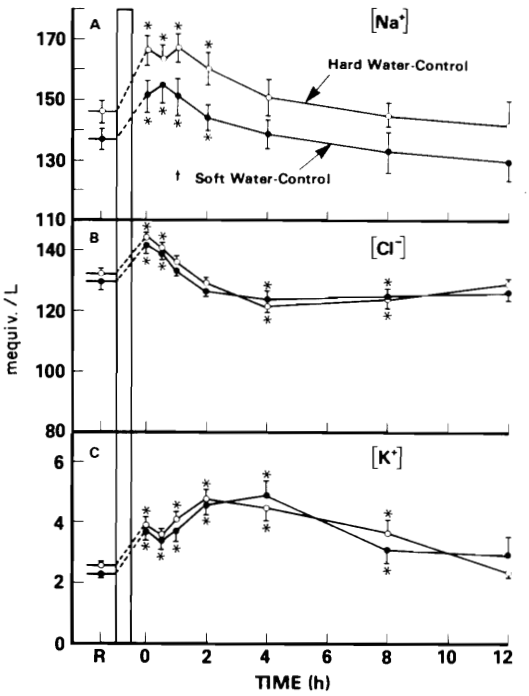

FIG. 3. Changes in (A) plasma sodium concentration; (B) plasma chloride concentration; and (C) plasma potassium concentration in surviving rainbow trout recovering from 6 min of strenuous exercise in either hard water or soft water at control pH. Other details as in legend of Fig. 1.

In soft water, plasma Na^+ and Cl^- levels both were significantly elevated by 10–15% during the 1st hour postexercise (Figs. 3A, 3B). $[Na^+]$ declined to resting levels by 4 h, while $[Cl^-]$ fell significantly below the resting level at 4 and 8 h, but returned to normal by 12 h. Plasma $[K^+]$ exhibited much larger changes, increasing by 70% immediately after exercise, then declining slightly, and finally peaking at more than twice the resting values at 4 h (Fig. 3C). Normal concentrations were restored by 12 h. All of these ionic changes were remarkably similar to those seen in hard water fish. The only significant difference was a lower $[Na^+]$ at 1 h postexercise in the soft water fish by enlargement of a nonsignificant difference already present at rest (Fig. 3A). Plasma protein concentration (Table 2) increased to the same extent as $[Na^+]$ and $[Cl^-]$ in the 1st hour after exercise and then slowly declined to below resting levels late in recovery due to sampling losses. MCHC declined slightly during the 1st hour, the decrease becoming significant by 30 min (Table 2). Hard water fish exhibited virtually identical changes in plasma [protein] but no change in MCHC after exercise, resulting in a significant difference between the two groups at 1 h (Table 2).

The overall picture therefore was one of great similarity between the response in hard and soft water at control pH, but including a few specific differences. This similarity also was reflected in the percent mortality data (Table 1) and in measurements from fish dying after exercise in the two groups. In brief, dying fish in both groups showed significantly greater blood metabolic acid levels and depressions in plasma $[Cl^-]$, $[HCO_3^-]$ and pHa relative to survivors at the same sample time; there were no other significant differences.

The influence of acid exposure on postexercise responses

When trout were exercised in hard water at pH ≃4.4 the general qualitative trends in survivors for all parameters (Figs. 4, 5, 6; Table 2) were similar to those seen in hard water at control pH, but there were a number of pronounced quantitative differences. In particular, the postexercise acidosis was not as large and resting pHa was restored more quickly (Fig. 4A), significant differences occurring from 0.5 through 4 h. This effect was due to a combination of slightly lower respiratory (i.e., Pa_{CO_2} elevation, Fig. 4B) and metabolic acidoses (i.e., ΔH_m^+ elevation, Fig. 5B), neither of which were significant by themselves. However, there was a secondary rise in the blood metabolic acid load at 12 h (Fig. 5B) which can be viewed as a direct effect of acid exposure

TABLE 2. Changes in plasma protein concentration, mean cell haemoglobin concentration (MCHC), and total blood hemoglobin concentration during recovery from strenuous exercise in surviving rainbow trout exposed to various water conditions (means ± SEM)

	Time postexercise							
	Rest	0 h	0.5 h	1 h	2 h	4 h	8 h	12 h
				Plasma [protein] (g/100 mL)				
Hard water, control pH (n = 8)	2.58 ±0.19	2.77* ±0.16	2.85* ±0.23	2.78* ±0.26	2.65 ±0.24	2.39 ±0.25	2.70 ±0.18	2.20* ±0.16
Soft water, control pH (n = 6)	2.43 ±0.20	2.71* ±0.24	2.70* ±0.26	2.46 ±0.25	2.25 ±0.23	2.02* ±0.20	1.82* ±0.23	1.78* ±0.18
Hard water, acid pH (n = 5)	2.86 ±0.40	3.26* ±0.48	3.24* ±0.45	3.06 ±0.43	2.55 ±0.34	2.44 ±0.33	2.34 ±0.22	2.40 ±0.28
Soft water, acid pH (n = 2)	1.60	2.40	2.70	2.60	2.55	2.50	2.20	2.10
				MCHC (g haemoglobin/mL RBC's)				
Hard water, control pH (n = 8)	0.25 ±0.02	0.25 ±0.02	0.25 ±0.01	0.23 ±0.01	0.24 ±0.01	0.22 ±0.02	0.28 ±0.04	0.23 ±0.01
Soft water, control pH (n = 6)	0.32 ±0.03	0.28 ±0.04	0.27* ±0.02	0.27† ±0.02	0.29 ±0.03	0.31 ±0.05	0.30 ±0.02	0.32 ±0.08
Hard water, acid pH (n = 5)	0.24 ±0.02	0.24 ±0.03	0.22 ±0.01	0.23 ±0.02	0.26 ±0.04	0.24 ±0.04	0.23 ±0.04	0.29 ±0.09
Soft water, acid pH (n = 2)	0.33	0.26	0.32	0.29	0.31	0.31	0.37	0.42
				Haemoglobin (g/100 mL blood)				
Hard water, control pH (n = 8)	5.48 ±0.47	7.08* ±0.51	7.26* ±0.64	6.35 ±0.34	5.75 ±0.28	4.84* ±0.37	3.32* ±0.50	2.63* ±0.17
Soft water, control pH (n = 6)	6.42 ±0.77	6.84 ±0.60	6.19 ±0.39	5.64 ±0.37	4.57† ±0.36	3.16*† ±0.30	2.23* ±0.36	1.81* ±0.42
Hard water, acid pH (n = 5)	7.00 ±1.18	7.50 ±1.29	7.40 ±1.11	6.90 ±1.28	6.00 ±1.24	4.50* ±1.10	3.50* ±0.97	3.40* ±1.10
Soft water, acid pH (n = 2)	5.50	5.35	6.65	5.98	5.45	4.68	3.65	2.90

NOTE: Because of the low n number, statistics were not calculated for the soft water, acid pH group.
*Significantly different ($p < 0.05$) from rest value in same group.
†Significantly different ($p < 0.05$) from corresponding value in hard water, control pH group.

unassociated with exercise (see Discussion). The elevation in blood [lactate] after exercise was also smaller than at control pH (Fig. 5A) and resting levels were regained more quickly.

Interpretation of the plasma ion data was complicated by the fact that preexercise resting levels were lower in the hard water acid group than in the hard water control group (Fig. 6), a difference which was significant for $[Cl^-]$ and $[K^+]$ but not $[Na^+]$. Prior to exercise the acid group was held at control pH and otherwise treated identically to the control group, so the reason for these differences is unknown. One possibility was a seasonal effect independent of acclimation, because the control experiments were performed in the spring – early summer while the acid tests were conducted in the fall. If the data are expressed as percentages of resting values, then all postexercise differences between the two groups in $[Na^+]$ (i.e., at 8 h) and $[Cl^-]$ (i.e., at 0, 0.5, 1, and 4 h) are eliminated but the $[K^+]$ elevation from 1 through 8 h remains significantly lower in the acid group. The effect on potassium, therefore, seems to show a real difference. Changes in plasma [protein] and MCHC were unaffected by acid exposure (Table 2).

Therefore, postexercise disturbances in hard water at acid pH were the same as, or smaller than those at control pH, a finding which agreed with the percent mortality data (Table 1). Similarly, the two fish which died after exercise in acidified hard water (Figs. 4, 5, 6) showed similar effects to those which died at control pH. Under the conditions of these experiments, there was no evidence that acid exposure and exercise were reinforcing toxic stresses in hard water.

The influence of low water hardness in combination with acid exposure on postexercise responses

In Figs. 7, 8, and 9, the postexercise responses of

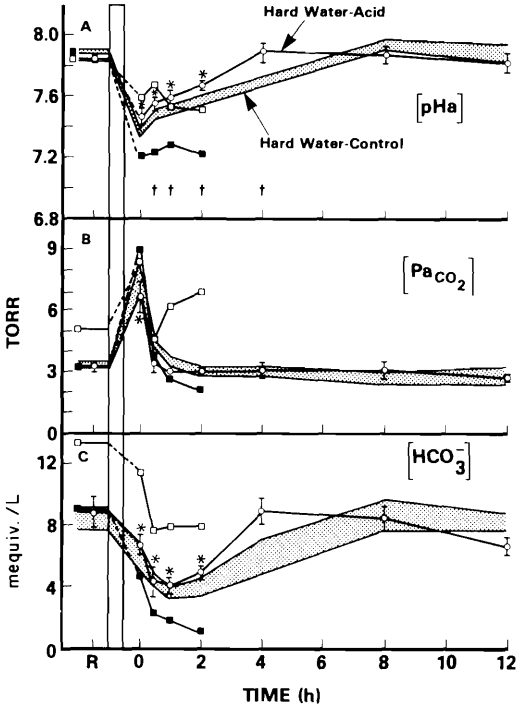

FIG. 4. A comparison of changes in (A) arterial pH (pHa); (B) arterial carbon dioxide tension (Pa_{CO_2}); and (C) arterial true plasma bicarbonate concentration ($[HCO_3^-]$) between trout recovering from 6 min of strenuous exercise in hard water at acid pH and those recovering in hard water at control pH. The hard water, control pH data are taken from Fig. 1 and are shown as a shaded band representing the means ± 1 SEM (n = 8). The hard water acid pH data are shown as means ± 1 SEM (○, n = 5) for survivors and as individual points for the two nonsurvivors. Bar represents period of exercise. R = resting sample taken in hard water at control pH prior to exercise or exercise plus acid exposure. * Signifies a significant difference ($p \leq 0.05$) within the hard water, acid group from the corresponding resting value. t signifies a significant difference ($p \leq 0.05$) between treatment groups at a particular sample time.

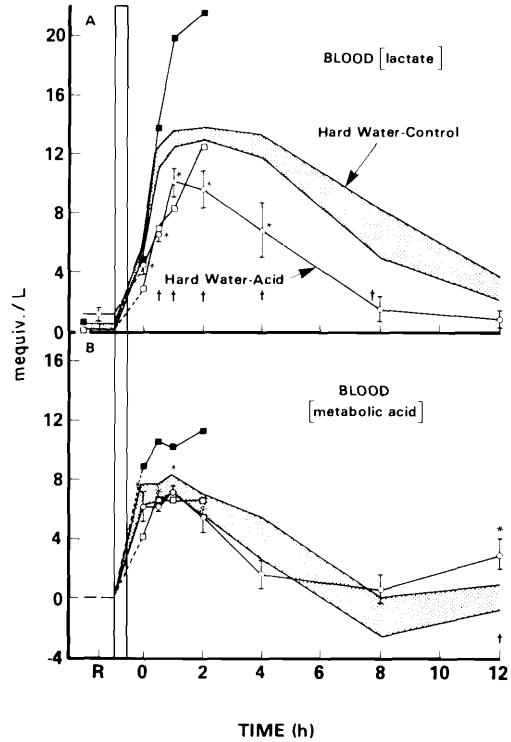

FIG. 5. A comparison of changes in (A) blood lactate concentration and (B) blood metabolic acid load between trout recovering from 6 min of strenuous exercise in hard water at acidic pH and those recovering in hard water at control pH. The hard water, control pH data are taken from Fig. 2 and are shown as a shaded band representing the means ± 1 SEM. Other details as in legend of Fig. 4.

trout in acidified soft water are compared with those of the surviving trout in the soft water of control pH. The soft water control group was used as the reference here as the preexercise acclimation conditions were identical, and resting values for all parameters were essentially the same in the two groups. If comparisons were to be made with the hard water control group, then the differences outlined below would be equally pronounced. The results provided clear evidence that acid exposure and exercise were reinforcing toxic stresses in soft water. Only two of the nine fish tested survived the entire experiment (Table 1) and most died before 4 h.

Major differences were seen in blood acid–base status. There were larger and more prolonged depressions of $[HCO_3^-]$ (Fig. 7C) and pHa (Fig. 7A) in the acid-exposed condition, the latter occurring despite generally reduced levels of Pa_{CO_2} (Fig. 7B). The Pa_{CO_2} reduction is interpreted as an attempted respiratory compensation for an elevated metabolic acidosis evidenced by the much greater levels of ΔH_m^+ (Fig. 8B). Blood lactate concentrations tended to be higher in the soft water acid group (Fig. 8A), though the differences were not as prominent as those for ΔH_m^+ (Fig. 8B).

Changes in plasma levels of Na^+ (Fig. 9A) and K^+ (Fig. 9C) were highly variable, but generally covered the same absolute range of values as in the control group. However, Cl^- concentrations in acid exposed fish fell consistently below those seen in control fish from about 1 h onwards (Fig. 9B). Even in the two survivors, $[Cl^-]$ remained depressed at 12 h. Postexercise changes in plasma [protein], and MCHC reported for these two survivors in Table 2 were representative of the acid soft water group as a whole. The MCHC remained within the control range while plasma protein levels were generally more elevated after exercise in acid water than in water of control pH.

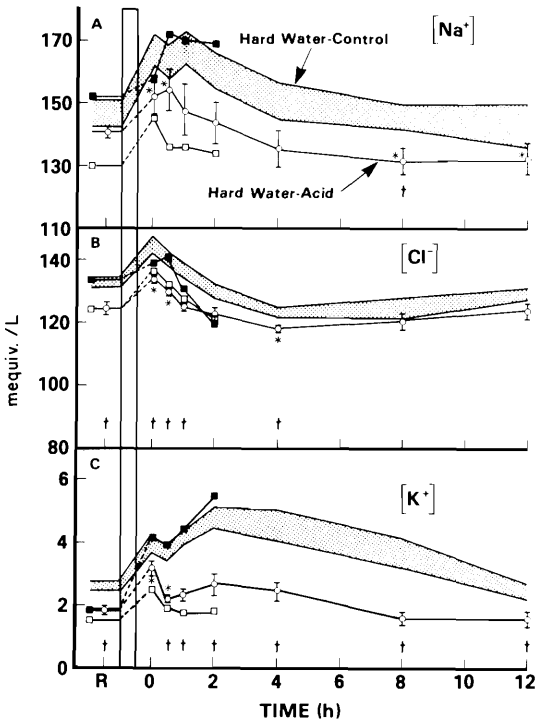

FIG. 6. A comparison of changes in (A) plasma sodium concentration; (B) plasma chloride concentration, and (C) plasma potassium concentration between trout recovering from 6 min of strenuous exercise in hard water at acidic pH and those recovering in hard water at control pH. The hard water, control pH data are taken from Fig. 3 and shown as a shaded band representing the means ± 1 SEM. Other details as in legend of Fig. 4.

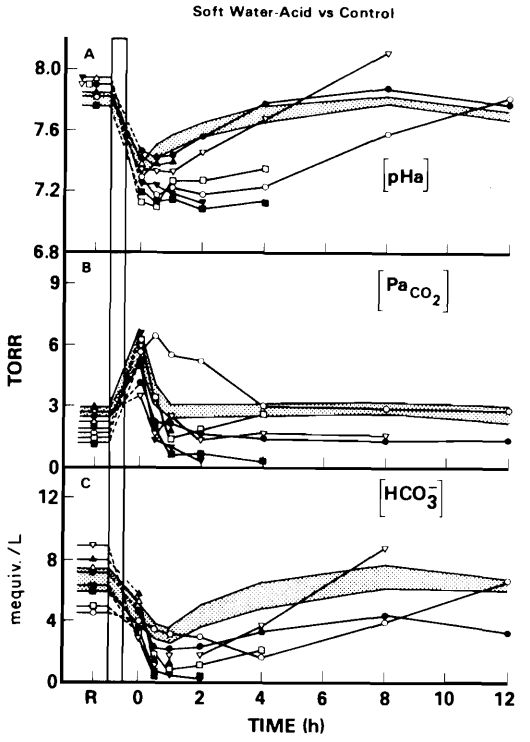

FIG. 7. A comparison of changes in (A) arterial pH (pHa); (B) arterial carbon dioxide tension (Pa_{CO_2}), and (C) arterial true plasma bicarbonate concentration ($[HCO_3^-]$) between trout recovering from 6 min of strenuous exercise in soft water at acid pH and those recovering in soft water at control pH. The soft water, control pH data are taken from Fig. 1 and are shown as a shaded band representing the means ± 1 SEM ($n = 6$). In the soft water, acid pH group, only two of the nine fish survived for the entire 12-h period, so all data are shown as individual points, a different symbol being used for each animal. Bar represents period of exercise. R = resting sample taken in soft water at control pH prior to exercise or exercise plus acid exposure.

Discussion

The responses of the rainbow trout to strenuous exercise stress in hard water at control pH have been interpreted in detail by Black (1957) and Black *et al.* (1959), and more recently by Turner *et al.* (1982). With some modifications, the same patterns have been seen in the other three water conditions examined here, so seem to represent fundamental responses to this treatment.

The blood pH depression (Fig. 1A) had two components which at 0 h were approximately equal in magnitude: a respiratory acidosis due to Pa_{CO_2} elevation (Fig. 1B), and a metabolic acidosis (Fig. 2B), reflected in $[HCO_3^-]$ loss (Fig. 1C). While the former was quickly corrected, the latter persisted for several hours. The metabolic acidosis resulted from H^+ ions produced with lactate by anaerobic metabolism in the largely glycolytic white muscle mass. The metabolic acid load in the blood stabilized at a level only about half that of the lactate anion (e.g., Figs. 2A, 2B). On the basis of perfusion experiments with an isolated preparation of the trout trunk (J. D. Turner and C. M. Wood, unpublished), this discrepancy has been explained as a differential release phenomenon, with further net proton efflux from muscle to blood (after exercise) being inhibited by the already low blood pH, while the lactate efflux continued. Nevertheless, less than 20% of the overall lactate and proton load appeared to be released into the bloodstream; the majority was thought to be retained in the muscle and undergo metabolic conversion *in situ*. Intracellular acidosis must, therefore, have been intense; indeed this has been proposed as the key toxic mechanism of postexercise mortality (Wood *et al.* 1983).

The high intracellular lactate load created an osmotic gradient moving water out of the ECF into the muscle cells. The resulting decrease in blood volume explains the postexercise rise in several plasma constituents: Na^+, Cl^- (Fig. 3), and plasma protein (Table 2). The elevation of plasma $[K^+]$ was far greater and more

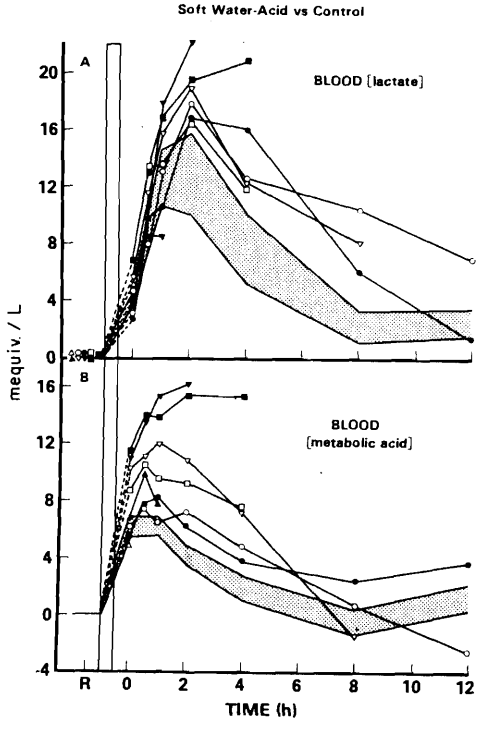

FIG. 8. A comparison of changes in (A) blood lactate concentration and (B) blood metabolic acid load between trout recovering from 6 min of strenuous exercise in soft water at acid pH and those recovering in soft water at control pH. The soft water, control pH data are taken from Fig. 2 and are shown as a shaded band representing the means ± 1 SEM. Other details as in legend of Fig. 7.

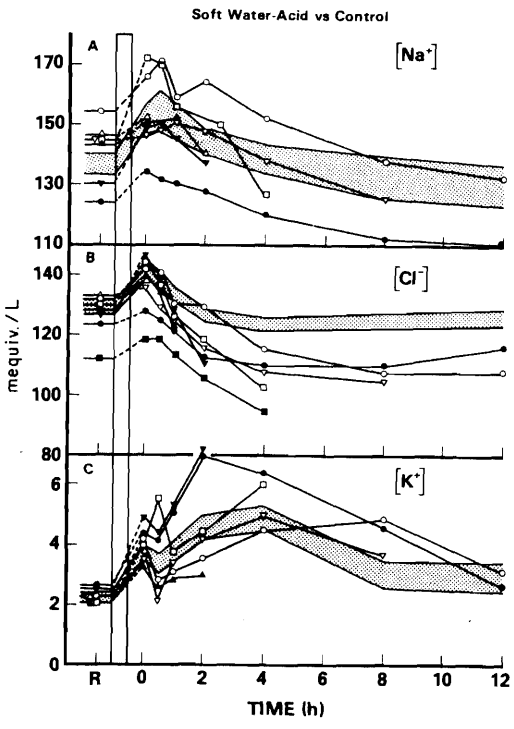

FIG. 9. A comparison of changes in (A) plasma sodium concentration; (B) plasma chloride concentration; and (C) plasma potassium concentration between trout recovering from 6 min of strenuous exercise in soft water at acid pH and those recovering in soft water at control pH. The soft water, control pH data are taken from Fig. 3 and are shown as a shaded band representing the means ± 1 SEM. Other details as in legend of Fig. 7.

prolonged than that of the other parameters (Fig. 3) and probably largely resulted from the migration of potassium out of muscle cells in response to intracellular acidosis (e.g., Ladé and Brown 1963). The fall of $[Cl^-]$ below resting levels late in recovery (Fig. 3B) was in some way coupled to the rise in [lactate] (Fig. 2A) and reflected the constraints of electroneutrality.

The responses to strenuous exercise stress in soft water were very similar to those in hard water. The minor differences in acid–base status (Fig. 1) and plasma $[Na^+]$ (Fig. 3A) during recovery most likely reflected the differences already present at rest. This finding opposes our original hypothesis that postexercise disturbances should be greater in soft water in view of the well known effect of low $[Ca^{2+}]$ in elevating membrane permeability and of exercise in increasing passive branchial fluxes of electrolytes and water (Wood and Randall 1973*a*, 1973*b*; Hofmann and Butler 1979). However, under more detailed scrutiny, the finding appears quite reasonable. Virtually all the studies showing these permeability effects on fish gills have dealt only with acute changes in water $[Ca^{2+}]$ or hardness (Potts and Fleming 1970, 1971; Cuthbert and Maetz 1972; Eddy1975; McWilliams and Potts 1978). There is very little information on the effects of water $[Ca^{2+}]$ after acclimation. According to Odulye (1975), differences in urine flow, indicative of differences in gill water permeability, persist after acclimation in the brown trout. However, recent work by McDonald (1982) and McDonald *et al.* (1982) on the rainbow trout has shown no pronounced differences in branchial Na^+ and Cl^- influx rates, efflux rates, net acid excretion rates, or urine flow rates between fish acclimated to $[Ca^{2+}]$'s of the hard and soft waters used in the present experiments. Furthermore, we are aware of no evidence that reduced water hardness interferes with exercise performance in acclimated fish. Indeed Waiwood and Beamish (1978) and Graham and Wood (1981) demonstrated that critical swimming speed was independent of water hardness in acclimated trout. Furthermore, Holeton and Stevens (1978) observed similar results with *Triportheus angulatus*. Enhanced prolactin secretion after acclimation to low water $[Ca^{2+}]$ was probably an important factor in these adjustments (e.g., Wendelaar Bonga and Van der Meij 1981).

A puzzling feature of the soft water versus hard water comparison at control pH was the more rapid return of blood lactate levels, and to a lesser extent blood metabolic acid levels, to resting values after exercise in soft water (Fig. 2). It was argued that this phase was dominated by metabolic processes removing lactate and protons from the blood in equivalent amounts (Turner *et al.* 1982). Perhaps acclimation to low water hardness in some way stimulated the activity of these metabolic processes.

Acute exposure to acid conditions in hard water did not exacerbate the physiological disturbances associated with exercise (Figs. 4, 5, 6) or increase short-term mortality (Table 1). Postexercise acidosis (Fig. 4A), blood [lactate] (Fig. 5A) and plasma [K^+] (Fig. 6C) elevations were actually lower than in the control animals. The most likely explanation is that the acid-exposed trout simply performed less swimming activity than the control group and thereby generated less lactate and protons in the intracellular compartment. While all fish were chased for the same time period and all exhibited apparent exhaustion, there was no guarantee that such treatment produced the same work loads. A method to ensure a uniform work load would employ a swim tunnel capable of generating a current sufficiently fast enough to force an adult trout to swim at burst performance levels. Indeed Graham and Wood (1981) have demonstrated that the critical swimming speed of fingerling rainbow trout ($\simeq$7.5 cm long) determined in a swim tunnel was reduced by acid exposure. However, it must be realized that the burst-type swimming performance occurring during the present experiments may be physiologically different than the performance put forth in attaining the critical swimming speed in a swim tunnel.

In hard water, the physiological effects of acid toxicity develop rather slowly (McDonald, Hobé, and Wood 1980; McDonald and Wood 1981; Milligan and Wood 1982; Ultsch *et al.* 1981). Thus, in the last sample of the present experiment (12 h), when exercise disturbances were completely corrected, typical hard water acid disturbances were just starting to appear. These included a significant elevation of the blood metabolic acid load (Fig. 5B), depression of the plasma [Na^+] (Fig. 6A), and the elevation of the plasma [protein] (Table 2) in some fish. This difference in time course from the exercise effects also probably contributed to the lack of interactive influence between exercise and acid stress in the present hard water tests.

The situation was very different in soft water. When acid exposure was combined with exercise in soft water, short-term mortality was greatly increased (Table 1), and physiological disturbances became more severe (Figs. 7, 8, 9, Table 2). Not only did the majority of trout die after this treatment, they died more quickly than those which succumbed after either exercise alone (Wood *et al.* 1983) or acid exposure alone (McDonald, Hobé, and Wood 1980) in soft water. Deviations in virtually all parameters were augmented relative to controls. As pointed out in the Introduction, many of these disturbances are qualitatively the same after either exercise or acid exposure alone though the key toxic mechanisms of action of the two treatments are thought to differ. Exercise is thought to kill by causing extreme intracellular acidosis which is reflected indirectly in extracellular pH depression, blood metabolic acid loading, plasma [K^+] elevation, and the accumulation of an unknown anion in the plasma (Wood *et al.* 1983). Acid expsoure is thought to kill by a failure of ionoregulation which precipitates haemoconcentration and cardiovascular disturbances (Wood and McDonald 1982; Milligan and Wood 1982). Inspection of data from dying fish in soft water under acid exposure only, exercise only, and the combined treatment of acid exposure and exercise proved highly informative. Quite clearly in the combined treatment deaths, deviations representative of intracellular acidosis (pHa, [HCO_3^-], [lactate], ΔH_m^+, [K^+]) were equal to or greater than those in the exercise only mortalities, while deviations representative of ionoregulatory failure and haemoconcentration ([Na^+], [Cl^-], plasma [protein] and [haematocrit]) were nowhere near as large as those in the acid only mortalities. We conclude that acid exposure potentiates the mechanism of exercise toxicity in soft water, rather than vice versa.

Unfortunately, the possible explanation is much less clear than the result. When resting trout were under environmental acid stress, low water hardness in fact tended to reduce branchial acid influx and the resulting accumulation of ΔH_m^+ in the blood by an unknown mechanism (McDonald, Hobé, and Wood 1980; Wood and McDonald 1982). It therefore seems unlikely that the greater metabolic acidosis after exercise in soft water fish exposed to acid could have resulted from that source. A more reasonable conclusion is that the greater acidosis resulted from greater metabolic acid production within the animal and (or) greater release from white muscle to blood. This was suggested by the higher blood levels of both lactate (Fig. 8A) and the unknown anion (see below) in these animals. However, it was argued earlier that acid exposure probably decreased exercise performance and therefore endogenous acid generation in hard water fish. There is no obvious reason why the same should not have been true in soft water fish; certainly Graham and Wood (1981) found a significant reduction in critical swimming speed at pH = 4.4 in soft water. We are left with the hypotheses that acid exposure perhaps interfered with branchial oxygen uptake and thus transport to the tissues (branchial mucous buildup? (Fromm 1980; Wood and McDonald 1982)), thereby forcing greater use of anaerobiosis during exercise, or that the release mechanism for

metabolic acid from muscle to blood was in some way affected. Clearly, further investigation is needed.

An interesting feature of the data was the much greater depression of plasma [Cl^-] after exercise in the soft water acid group (Fig. 9B). While it is tempting to ascribe the hypochloraemia to a direct effect of acid on ionoregulation, that was probably not the case, because [Na^+] broadly followed the control range (Fig. 9A). In the typical soft water ionoregulatory failure, [Na^+] and [Cl^-] are lost in equal amounts (McDonald, Hobé, and Wood 1980; McDonald 1982). The greater [Cl^-] depression in the exercised fish was more likely a passive consequence of the constraints of electroneutrality in the face of higher levels of both the lactate anion (Fig. 8A), and the unknown anion. As described by Wood *et al.* (1983), the latter can be estimated from the changes in the sum [$Na^+ + K^+$] − [$Cl^- + HCO_3^- +$ lactate$^-$]. Wood *et al.* (1983) found that this parameter increased markedly prior to postexercise death at control pH and suggested that the increase represented the anion of another organic acid. This could be a keto acid or a novel end product of anaerobic metabolism such as succinic acid (e.g., Johnston 1975*a*, 1975*b*; Smith and Heath 1980). In fish dying after exercise at acid pH in soft water, the unknown anion concentration rose to a level (15–25 mequiv./L) approximately three times that of the controls.

In summary, the results showed that fish performing exercise in acidified soft water suffer greater physiological disturbances and postexercise mortality when compared with other test exposures. The strenuous exercise procedure used in the present study is thought to produce results that closely duplicate those created by angling stress. Postrelease survival of angled fish may be seriously reduced in acidic natural waters of low hardness. Furthermore, the data indicated that physiological tests on resting fish may underestimate or misinterpret the toxic effects of acid in the wild where fish are intermittently very active.

Acknowledgements

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