Physiological Evidence of Acclimation to Acid/Aluminum Stress in Adult Brook Trout (Salvelinus fontinalis). 1. Blood Composition and Net Sodium Fluxes

C. M. Wood and D. G. McDonald
Department of Biology, McMaster University, Hamilton, Ont. L8S 4K1

C. E. Booth
Department of Biology, Eastern Connecticut State University, Willimantic, CT 06226-2295, USA

B. P. Simons
Department of Biology, McMaster University, Hamilton, Ont. L8S 4K1

C. G. Ingersoll
National Fisheries Contaminant Research Center, United States Fish and Wildlife Service, Columbia, MO 65201, USA

and H. L. Bergman
Fish Physiology and Toxicology Laboratory, University of Wyoming, Laramie, WY 82071, USA


Brook trout (Salvelinus fontinalis) adapt to chronic sublethal acid/Al stress. The accompanying acclimation confers greater resistance to short-term increases in Al and acidity. Adult trout were exposed in flowing soft water to eight combinations of pH (6.5, 5.2) x Ca^{2+} (25, 400 μequiv/L) x Al (0, 75, 150 μg/L = 0, 2.8, 5.6 μmol/L). After 10 wk, blood sampling by caudal puncture revealed no significant variations in osmolality, plasma protein, or hemoglobin and only minor differences (<15%) in plasma Na^+ and Cl^−. Overall, most electrolytes were higher in fish exposed to higher water Al and/or Ca^{2+}; only plasma Ca^{2+} was directly depressed by low pH. Hematocrit was raised by both low pH and elevated Al. When trout naive to both acid and Al were challenged with pH = 4.8, Al = 333 μg/L under flow-through conditions, there were large negative whole-body Na^+ fluxes and marked depressions of plasma Na^+ and Cl^−, hemococoncentration, and substantial mortality over 48 h. Prior exposure for 10 wk to pH = 5.2 plus either 75 or 150 μg Al/L prevented mortality and ameliorated or abolished these effects through a more rapid recovery of net Na^+ balance. Prior exposure to pH = 5.2 alone ameliorated these effects only slightly.

L’omble de fontaine (Salvelinus fontinalis) s’adapte à un stress chronique entraîné par des teneurs sublétales en acide et en Al; l’accoutumance concomitante lui confère une plus grande résistance à des augmentations à court terme de la teneur en Al et de l’acidité. On a exposé des ombles adultes à huit différentes combinaisons de pH (6.5 et 5.2), de teneurs en Ca^{2+} (25 et 400 μequiv/L) et de teneurs en Al (0, 75 et 150 μg/L = 0, 2.8 et 5.6 μmol/L) en eau douce à débit continu. Après 10 sem, l’échantillonnage du sang par ponction caudale n’a pas révélé de variations significatives de l’osmolalité, des niveaux de protéine dans le plasma ou du taux de l’hémoglobine, mais on a observé de légères différences (<15 %) des teneurs en Na^+ et Cl^− du plasma. En général, les niveaux de la plupart des électrolytes étaient plus élevés chez les poissons exposés à des teneurs élevées en Al et/ou en Ca^{2+} en milieu aquéux; seule la teneur en Ca^{2+} du plasma a été directement abaissée par un faible pH. Un faible pH et une teneur élevée en Al ont tous deux entraîné une augmentation du taux de l’hématocrite. Chez des ombles jamais exposés à un milieu acide ajouté d’Al et provoqués dans un milieu de pH 4.8 et de teneur en Al de 333 μg/L en débit continu, on a observé d’importants flux négatifs de la teneur en Na^+ dans tout l’organisme et des baisses marquées de la teneur en Na^+ et Cl^− du plasma et de l’hémococoncentration ainsi qu’une importante mortalité échelonnée sur 48 h. Une exposition préalable pendant 10 sem au pH 5.2 en présence de 75 ou 150 μgAl/L a prévenu cette mortalité et a amélioré ou éliminé ces effets par suite du rétablissement plus rapide de l’équilibre net du Na^+. L’exposition préalable au pH 5.2 n’a que légèrement atténué ces effets.

Received March 31, 1987
Accepted December 3, 1987
(J9209)

HOWEVER, IT IS LESS WIDELY APPRECIATED THAT FISH ARE NOW CHRONICALLY EXPOSED TO LOWER, BUT nevertheless POTENTIALLY HARMFUL, BACKGROUND LEVELS OF Al IN NATURAL SOFT WATERS IN MANY ACID-SENSITIVE REGIONS OF THE WORLD. FOR EXAMPLE, WRIGHT AND SNEVKIW (1978) FOUND AL AT 50–300 µg/L IN OVER 90% OF 700 LAKES IN SOUTHERN NORWAY, WHILE SCHOFIELD AND TROJNAR (1980) REPORTED A MEAN Al OF 111 µg/L IN 28 LAKES WITH SURVIVING BROOK TROUT (SALVELINUS FONTINALIS) POPULATIONS IN THE NORTH-EASTERN UNITED STATES. A RECENT SURVEY OF 810 LAKES IN EASTERN CANADA, MANY WITH REPRODUCING BROOK TROUT POPULATIONS, HAS REPORTED A MEAN Al OF 84 µg/L (KELSO ET AL. 1986). THE MEAN LAKE pH WAS ACIDIC (4.5–6.0) IN ALL OF THESE EXAMPLES. THESE FIGURES ARE FOR TOTAL Al; IN THE ABSENCE OF DETAILED WATER CHEMISTRY DATA, IT IS DIFFICULT TO ESTIMATE THE SIZE OF THE MONOMERIC FRACTION AND ITS TOXICITY TO FISH. NEVERTHELESS, THERE IS CONSIDERABLE EVIDENCE THAT TOTAL Al CONCENTRATIONS OF THIS MAGNITUDE CAN EXERT DAMAGING SUBLETHAL OR EVEN LEthal EFFECTS ON FISH WHICH HAVE NOT BEEN EXPOSED PREVIOUSLY TO Al (E.G. BAKER AND SCHOFIELD 1982; NEVILLE 1985). THEREFORE, FISH CHRONICALLY EXPOSED TO LOW LEVELS OF Al AND ACIDITY IN THE WILD MAY HAVE BECOME ACCLIMATED, INCREASING THEIR TOLERANCE TO THESE AND MORE TOXIC LEVELS. ALTERNATIVELY, THEY MAY HAVE BECOME WEAKENED AND SENSITIZED BY THESE LOW-LEVEL EXPOSURES.

LABORATORY EVIDENCE INDICATES THAT ACCLIMATION TO Al MAY OCCUR. ORR ET AL. (1986) DEMONSTRATED INCREASED RESISTANCE TO Al BY RAINBOW TROUT (SALMO GAIRDNERI) EXPOSED FOR 7–21 D TO Al AT 50% OF THE LC50 (AT CONSTANT pH = 5.2). IN BROOK TROUT, SIDDENS ET AL. (1986) REPORTED GREATER SURVIVAL OVER A 24-D PERIOD AT pH = 4.9 IF A GIVEN MAXIMUM LEVEL OF Al WAS PRESENTED CONTINUOUSLY, RATHER THAN INTERMITTENTLY, WHICH CAN BE INTERPRETED AS INDIRECT EVIDENCE OF ACCLIMATION. HOWEVER, NOTHING IS KNOWN ABOUT THE PHYSIOLOGICAL CHANGES WHICH MIGHT OCCUR DURING ACCLIMATION.

THE OVERALL OBJECTIVES OF THE PRESENT AND FOLLOWING TWO PAPERS (WOOD ET AL. 1988B; McDoNALD AND MILLIGAN 1988) WERE TO USE PHYSIOLOGICAL CRITERIA, ESPECIALLY IONOREGULATORY PARAMETERS, TO (I) CONFIRM THAT ADULT BROOK TROUT ADAPT TO SUBLETHAL Al AT pH = 5.2, (II) ASSESS WHETHER SUCH ADAPTATION confers ACCLIMATION, I.E. GREATER RESISTANCE TO SIMULTANEOUS INCREASES IN BOTH Al AND ACIDITY AS WOULD BE SEEN DURING EPISODIC ACID SURGES IN THE FIELD, (Iii) ASSESS WHETHER PRIOR EXPOSURE TO ACIDITY ALONE ALSO confers INCREASED RESISTANCE TO SUCH SURGES, AND (IV) UNDERSTAND THE MECHANISMS INVOLVED IN ANY SUCH INCREASED RESISTANCE. THIS FIRST PAPER FOCUSES ON PHYSIOLOGICAL CHANGES AFTER PROLONGED EXPOSURE (10 WK) TO SUBLETHAL ACID OR ACID/Al LEVELS, AND WHOLE-BODY Na+ FLUXES OF THESE SAME FISH DURING AN ACID/Al CHALLENGE (pH = 4.8, Al = 333 µg/L) WHICH IS IN THE LETHAL RANGE FOR NAIVE FISH (C.F. BOOTH ET AL. 1988). THE POSSIBLE INFLUENCE OF WATER Ca2+ ON THESE PROCESSES WAS ASSESSED USING LEVELS REPRESENTATIVE OF THE UPPER AND LOWER RANGES OF NATURAL SOFT WATER. AS FAR AS POSSIBLE, TEST CONDITIONS AND METHODS DUPLICATED THOSE USED IN OUR COMPANION STUDIES ON NAIVE BROOK TROUT (BOOTH ET AL. 1988; WOOD ET AL. 1988A).

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND HOLDING CONDITIONS

EXPERIMENTS WERE CONDUCTED AT THE FISH PHYSIOLOGY AND TOXICOLOGY LABORATORY, LARAMIE, WYOMING (ALTITUDE = 2200 m), WHICH PROVIDED FACILITIES FOR CONTINUOUS LONG-TERM EXPOSURES OF ADULT FISH TO DEFINED pH, Ca2+, AND Al CONDITIONS IN FLOWING ARTIFICIAL SOFT WATER. THE OVERALL EXPERIMENTAL PLAN WAS TO FIRST ACCLIMATE THE FISH TO SOFT WATER AND THEN TO EXPOSE SEPARATE GROUPS TO A VARIETY OF pH, Ca2+, AND Al COMBINATIONS FOR 10 WK. AT THE END OF THIS PERIOD, BLOOD WAS SAMPLED FROM A SUBSET OF THE FISH IN EACH GROUP TO EVALUATE THE EFFECTS OF THIS LONG-TERM SUBLETHAL EXPOSURE ON IONIC AND HEMATOLOGICAL PARAMETERS. OTHER FISH FROM SELECTED GROUPS WERE THEN CHALLENGED WITH A HIGHER Al/LOWER pH CONDITION (POTENTIALLY LEthal) FOR 48 H; Na+ FLUX RATES, MORTALITY, AND FINAL BLOOD PARAMETERS WERE MEASURED TO ASSESS RESISTANCE TO THIS MORE SEVERE CHALLENGE.

ARTIFICIAL SOFT WATER WAS GENERATED BY TREATMENT OF HARD WELL WATER (COMPOSITION IN TABLE 1) WITH SEDIMENT FILTRATION, NaCl SOFTENING, REVERSE OSMOSIS, AND SEPARATE BED DEIONIZATION (WATER SYSTEM DESIGNED BY CONTINENTAL WATER SYSTEMS, DENVER, COLORADO). THE pH WAS ADJUSTED TO 6.5 WITH KOH AND THE WATER WAS THOROUGHLY MIXED AND THEN DELIVERED TO INDIVIDUAL HEAD TANKS, WHERE THE pH WAS LOWERED TO THE DESIRED LEVEL WITH H2SO4. THE CARBONATE ALKALINITY OF THIS WATER AT pH = 6.5 WAS ABOUT 2.5 mg/L. ALL pH ADJUSTMENTS WERE MADE WITH LEEDS AND NORTHRUP pH CONTROLLERS (pH ANALYZER/CONTROLLER MODEL NO. 7083). LASTLY, THE REQUIRED LEVELS OF CaCl2 AND AlCl3 WERE ADDED VIA MARION BOTTLES TO CONTINUOUS-FLOW SERIAL DILUTERS, FROM WHERE THE WATER WAS DELIVERED TO THE EXPOSURE TANKS, AND THEN TO WASTE. THE TANKS WERE ROUND 340-L FIBERGLASS CHAMBERS SERVED WITH A FLOW OF 1.9 L/min, PROVIDING 8 VOLUME ADDITIONS PER DAY. EACH TANK CONTAINED 25 FISH AND REPRESENTED ONE OF THE EIGHT pH/Ca2+/Al COMBINATIONS WHICH WERE TESTED (TABLE 1). TWO TANKS (50 FISH) WERE ALSO RUN WITH HARD WELL WATER, WHICH DIFFERED PRINCIPALLY IN ITS HIGHER Ca2+ AND pH LEVELS (TABLE 1).

TEST ANIMALS WERE 18- to 20-MO-OLD BROOK TROUT (MEAN WEIGHT = 170 g), OBTAINED FROM CLINE'S TROUT FARM (BOULDER, COLORADO) AND INITIALLY ACCLIMATED FOR 34 D TO ARTIFICIAL SOFT WATER (pH = 6.5, Ca2+ = 25 µequiv/L). THE EXPOSURE PERIOD STARTED ON MAY 15, 1985, AND WAS APPROXIMATELY 10 WK (ACTUAL RANGE 64–85 D). THE pH WAS GRADUALLY REDUCED FROM ACCLIMATION (6.5) TO THE DESIRED LEVEL OVER 3 D, AND THEN, Al ADDITION WAS STARTED 6 D LATER (DAY 0 OF EXPOSURE). TO PREVENT MORTALITY, THE SET pH IN THE ACIDIC TREATMENTS WAS RAISED FROM 5.0 TO 5.2 ON DAY 16. DETAILED GROWTH DATA WERE RECORDED ON ALL FISH AND ARE REPORTED IN MOUNT ET AL. (1988).

EXPERIMENTAL TEMPERATURE WAS 11 ± 1°C, CONDUCTIVITY RANGED FROM 40 TO 115 µS/cm, DEPENDING ON THE PARTICULAR pH/Ca2+/Al COMBINATION, AND D ISSOLVED O2 WAS MAINTAINED ABOVE 60% SATURATION. FISH WERE FED 1% BODY WEIGHT/DAY FLOATING TROUT CROW (Purina No. 5106) AND TANKS WERE SYPHONED DAILY. PHOTOPERIOD WAS ADJUSTED BIVARIOUS TO FOLLOW THE NATURAL CYCLE FOR LARAMIE, WYOMING. WATER pH WAS MEASURED DAILY (ORION ROSS COMBINATION ELECTRODE NO. 810200 ON A SARGENT WELCH LS pH METER) AND OTHER PARAMETERS WEEKLY (Al, Ca2+, Na+, Cl−, SO42−) ON SAMPLES TAKEN DIRECTLY FROM THE TANKS.

BLOOD SAMPLING

ON DAY 68, SIX FISH WERE SELECTED AT RANDOM FOR BLOOD SAMPLING FROM EACH OF THE NINE EXPOSURE CONDITIONS (EIGHT pH/Ca2+/
<table>
<thead>
<tr>
<th>pH</th>
<th>Ca\textsuperscript{2+} (μequiv/L)</th>
<th>Al (μg/L)</th>
<th>pH</th>
<th>Ca\textsuperscript{2+} (μequiv/L)</th>
<th>Total Monomeric</th>
<th>Inorganic monomeric</th>
<th>Na\textsuperscript{+} (μequiv/L)</th>
<th>Cl\textsuperscript{-} (μequiv/L)</th>
<th>SO\textsubscript{4}\textsuperscript{2-} (μequiv/L)</th>
<th>Mortality (%)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>400</td>
<td>0</td>
<td>6.35 ± 0.03</td>
<td>342</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>222</td>
<td>431</td>
<td>47</td>
</tr>
<tr>
<td>6.5</td>
<td>25</td>
<td>0</td>
<td>6.48 ± 0.03</td>
<td>28</td>
<td>(&lt;1 - 7)</td>
<td>(&lt;1 - 5)</td>
<td>(&lt;1 - 7)</td>
<td>(73 - 453)</td>
<td>(400 - 451)</td>
<td>(16 - 106)</td>
</tr>
<tr>
<td>5.2\textsuperscript{a}</td>
<td>400</td>
<td>0</td>
<td>5.19 ± 0.05\textsuperscript{b}</td>
<td>337</td>
<td>(&lt;1 - 7)</td>
<td>3</td>
<td>3</td>
<td>(75 - 433)</td>
<td>(90 - 96)</td>
<td>(&lt;10 - 106)</td>
</tr>
<tr>
<td>5.2\textsuperscript{a}</td>
<td>25</td>
<td>0</td>
<td>5.25 ± 0.06\textsuperscript{b}</td>
<td>29</td>
<td>(&lt;1 - 6)</td>
<td>3</td>
<td>(&lt;1 - 6)</td>
<td>(70 - 466)</td>
<td>(408 - 490)</td>
<td>(13 - 206)</td>
</tr>
<tr>
<td>5.2\textsuperscript{a}</td>
<td>400</td>
<td>75</td>
<td>5.12 ± 0.05\textsuperscript{b}</td>
<td>341</td>
<td>(&lt;1 - 7)</td>
<td>3</td>
<td>(&lt;1 - 7)</td>
<td>(71 - 452)</td>
<td>(76 - 99)</td>
<td>(10 - 202)</td>
</tr>
<tr>
<td>5.2\textsuperscript{a}</td>
<td>25</td>
<td>75</td>
<td>4.95 ± 0.05\textsuperscript{b}</td>
<td>26</td>
<td>(&lt;1 - 6)</td>
<td>3</td>
<td>17</td>
<td>(64 - 469)</td>
<td>(411 - 493)</td>
<td>(14 - 206)</td>
</tr>
<tr>
<td>5.2\textsuperscript{a}</td>
<td>400</td>
<td>150</td>
<td>5.13 ± 0.05\textsuperscript{b}</td>
<td>339</td>
<td>(&lt;1 - 7)</td>
<td>134</td>
<td>4</td>
<td>(73 - 457)</td>
<td>(96 - 114)</td>
<td>(19 - 202)</td>
</tr>
<tr>
<td>5.2\textsuperscript{a}</td>
<td>25</td>
<td>150</td>
<td>5.07 ± 0.06\textsuperscript{b}</td>
<td>29</td>
<td>(&lt;1 - 7)</td>
<td>134</td>
<td>4</td>
<td>(70 - 470)</td>
<td>(437 - 504)</td>
<td>(99 - 206)</td>
</tr>
<tr>
<td>Hard water</td>
<td>7.8 ± 0.01</td>
<td></td>
<td>(2440 - 2680)</td>
<td>(&lt;1 - 4)</td>
<td>(139 - 187)</td>
<td>(133 - 157)</td>
<td>(20 - 87)</td>
<td>(71 - 460)</td>
<td>(107 - 115)</td>
<td>(19 - 206)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Intended pH was originally 5.0 but was raised to 5.2 on day 16. The means over the entire exposure period are reported for 60 daily measurements ± 1 SE.

\textsuperscript{b} Mortality out of 25 fish in each treatment, except in hard water, where there were 50 fish.
Al combinations plus hard water). Each fish was netted individually into a bucket containing 40 mg MS-222/L (Sigma) to induce light anaesthesia. After 3 min, a 500-μL blood sample was rapidly withdrawn into a heparinized syringe (rinsed with 500 IU ammonium heparin/L (Sigma) by blind puncture of the caudal vein. Fish were returned to their holding tanks for a minimum 1-wk recovery prior to use in any other experiments (Wood et al. 1988b; McDonald and Milligan 1988). Blood samples were analyzed for hematocrit (Ht), hemoglobin (Hb), and plasma levels of Na+, Cl−, K+, Ca2+, osmolality, and total protein.

At the end of the 48-h challenge experiments (see below), blood from surviving fish was sampled as described above, except that the anaesthetic was added directly to the closed flux box to minimize disturbance. These blood samples were analyzed for Ht and plasma levels of Na+, Cl−, and total protein. Thus, these parameters can be compared before and after the 48-h challenges for fish in each of the eight pH/Al exposure conditions.

**Flux and Challenge Experiments**

Between days 64 and 85, six fish from each of the eight pH/Al exposure conditions were transferred to individual flux boxes (cf. Booth et al. 1988) which received a continuous flow (−0.5 L·kg⁻¹·min⁻¹) of water identical in composition to that of their exposure condition. Water PO₂ was maintained above 115 Torr, PCO₂ levels below 1 Torr, and HCO₃⁻ levels ranged from 50 μequiv/L at pH = 6.5 to 0 at pH = 4.8. After a 24-h settling period, the flow rate and inflow pH were adjusted to maximize detection of inflow versus outflow differences in Na⁺ while simultaneously maintaining the measured pH in the box within 0.05 unit of the intended value. After a further 4–6 h, water samples were taken for measurement of Na⁺ flux rates by the Fick principle (cf. Booth et al. 1988). These measurements represented net Na⁺ exchange under the exposure condition and therefore were the control values for the subsequent challenges. The inflowing water was then changed to the challenge condition.

The challenge condition was pH = 4.8, Al = 333 μg/L at the same Ca²⁺ (25 or 400 μequiv/L) as that in the exposure condition; this combination was previously shown to be lethal in the range for naive brook trout (Booth et al. 1988). In addition, six fish from each of the zero-Al exposure conditions (pH = 6.5, Ca²⁺ = 25 or 400 μequiv/L) were challenged with pH = 4.8, Al = 0 μg/L to discern the effect of acidity alone. Na⁺ flux measurements were taken at 4, 8, 12, 24, 30, 36, and 48 h during all challenges. Immediately after the 48-h determinations, blood samples were drawn from surviving fish as described above. Inflow pH and flow rate were adjusted throughout the experiment to maintain the desired conditions. Water was not recirculated but, rather, led to waste after leaving the chambers to minimize speciation changes in Al. Al and Ca²⁺ levels were monitored daily in inflowing and outflowing water; deviations from intended values were less than 20%.

**Analytical Techniques and Calculations**

Analytical methods were identical to those described in Booth et al. (1988) and Wood et al. (1988a) with the following exceptions. Plasma Na⁺ and water Na⁺ and Ca²⁺ levels were determined on a Perkin–Elmer model No. 2380 atomic absorption spectrophotometer. Water Al was measured with an associated high-temperature graphite furnace (Perkin–Elmer model No.

![FIG. 1. Plasma (A) Na⁺, (B) Cl⁻, (C) total osmolality, (D) Ca²⁺ and (E) K⁺ in the blood of brook trout after 10 wk of exposure to eight different combinations of pH, Ca²⁺, and Al in soft water, or to hard water (HW) at circumneutral pH (7.8) (cross-hatched). Values in the two exposures at circumneutral acidity (pH = 6.5) are stippled. Means ± 1 SEM for N = 6 in each cell. On the right are tabulated the variables which significantly affected (p < 0.05) the measured parameters overall, either individually or as interactive effects. Lines underscore subsets of means among which there were no significant differences; means not underscored with the same line were significantly different from one another (p < 0.05).](http://example.com/fig1.jpg)

HGA-400). Al was speciated into total, monomeric, and inorganic monomeric after the method of LaZerte (1984). Water Cl⁻ and SO₄²⁻ levels were measured using a Dionex ion chromatograph (model No. 2110) with an AS 40 column. Plasma osmolality was determined by vapour pressure osmometry (Wescor model No. 5100B).

Net whole-body Na⁺ flux rates (J Na⁺, whole body) were calculated by the Fick principle from the measured difference between inflow and outflow Na⁺ levels and the flow rate through the box. Cumulative Na⁺ losses over 48 h were estimated as the areas under curves relating J Na⁺ and time for individual fish. Mean cell Hb concentration (MCHC) was calculated as the ratio of Hb and Ht measurements for individual fish.
FIG. 2. (A) Ht, (B) Hb, (C) mean cell Hb concentration (MCHC), and (D) plasma protein concentration in the blood of brook trout after 10 wk of exposure to eight different combinations of pH, Ca\(^{2+}\), and Al in soft water, or to hard water (HW) at circumneutral pH. Other details as in legend to Fig. 1.

Treatment of Data

Data have been reported as means ± 1 SE (N) for each group (i.e. exposure condition). One-way analysis of variance was used to assess differences between groups; when the F value indicated significance, Duncan's (1955) multiple range test was applied to discern specific differences. Two-way analysis of variance (pH × Ca\(^{2+}\) at Al = 0; Al × Ca\(^{2+}\) at pH = 5.2) was employed to detect specific pH, Ca\(^{2+}\), Al, and interaction effects across the entire set of exposure conditions. Differences in J_{\text{on}} Na\(^{+}\) from zero and changes in blood parameters within individual groups as a result of the 48-h challenges were evaluated by Student's two-tailed t-test, unpaired design. A 5% significance or protection level was used throughout.

Results

Exposure Conditions and Mortality

While agreement between intended and actual values of water chemistry was generally good, mean Ca\(^{2+}\) at the higher concentration was about 15% below planned, and mean pH was up to 0.2 unit different from planned levels (Table 1). This partly reflected the resetting of intended acidic pH from 5.0 to 5.2 on day 16. The variation in Na\(^{+}\) reflected cycling of the water-softening and deionizing systems. During the flux and challenge trials of this and the following paper (Wood et al. 1988b), the fluctuation was reduced, yielding a Na\(^{+}\) level of 50–100 μequiv/L in different experiments, which varied less than 10% during any individual experiment. Speciation analysis revealed that almost all added Al was in the monomeric form in the tanks, but only about 25–33% of this was inorganic, due to complexation by organic wastes excreted by the fish. Mortality occurred only in the exposures at acidic pH with low Ca\(^{2+}\) (Table 1) and ceased shortly after pH was reset to 5.2 on day 16. As this mortality was low, the effect of “selection” on the subsequent physiological data was minimal. Growth was depressed in these same treatments (see Mount et al. 1988).

Effects of pH/Ca\(^{2+}\)/Al Exposures on Blood Composition

Ten weeks of exposure to a range of pH, Ca\(^{2+}\), and Al caused significant variation in plasma Na\(^{+}\) levels (Fig. 1A). The absolute differences were rather small, ranging from a high of 153 ± 2 mequiv/L (N = 6) (at pH = 5.2, Ca\(^{2+}\) = 400 μequiv/L, Al = 75 μg/L) to a low of 133 ± 2 mequiv/L in the same condition at low Ca\(^{2+}\) (i.e. pH = 5.2, Ca\(^{2+}\) = 25 μequiv/L, Al = 75 μg). While there was no direct pH effect, both elevated Ca\(^{2+}\) and elevated Al acted alone to significantly raise plasma Na\(^{+}\). These relationships were complicated by significant pH × Ca\(^{2+}\) and Ca\(^{2+}\) × Al interactions. In addition, plasma Na\(^{+}\) was significantly higher in hard water than in either of the soft waters at circumneutral pH. Similar significant variation was seen in plasma Cl\(^{-}\) levels, but to a much lesser extent (Fig. 1B). Overall there was a significant Ca\(^{2+}\) × Al interaction.

Despite these differences in the two major ionic constituents of plasma, total osmolality did not vary significantly across exposure conditions, staying at a relatively uniform level of 305–325 mosmol/L (Fig. 1C). Interestingly, plasma protein, which contributes the colloidal fraction of total osmotic pressure, was similarly unaffected (Fig. 2D).

Plasma Ca\(^{2+}\) levels varied significantly among exposure conditions in a pattern somewhat similar to that of Na\(^{+}\) (compare Fig. 1D and 1A). On a relative basis, these differences were much greater (~50%) than those for Na\(^{+}\) (~15%), plasma Ca\(^{2+}\) ranging from a low of 4.5 ± 0.1 mequiv/L (at pH = 5.2, Ca\(^{2+}\) = 400 μequiv/L, Al = 0 μg/L) to a high of 6.7 ± 0.5 mequiv/L (at pH = 5.2, Ca\(^{2+}\) = 400 μequiv/L, Al = 75 μg/L). Overall, plasma Ca\(^{2+}\) was significantly depressed by low pH and raised by elevated Al. Interestingly, water Ca\(^{2+}\) itself did not exert a significant influence on plasma Ca\(^{2+}\), and there were no hardwater versus softwater differences at circumneutral pH.

Plasma K\(^{+}\) concentrations were also significantly affected by exposure condition, varying by ~50% from 2.3 ± 0.1 mequiv/L (at pH = 6.5, Ca\(^{2+}\) = 25 μequiv/L) to 3.3 ± 0.2 μequiv/L (at pH = 5.2, Ca\(^{2+}\) = 400 μequiv/L, Al = 150 μg/L) (Fig. 1E). The dominant influence was a stimulation by elevated Al; while there were no direct pH or Ca\(^{2+}\) effects, significant pH × Ca\(^{2+}\) and Ca\(^{2+}\) × Al interactions were seen. Hardwater versus softwater differences at circumneutral pH were not significant.

Neither Hb (9.4–10.5 g/100 mL; Fig. 2B) nor plasma protein concentration (4.7–5.9 g/100 mL; Fig. 2D) was significantly affected by the exposure conditions. However, Ht varied considerably from a low of 31 ± 1% (in hardwater) to a high of 46 ± 3% (at pH = 5.2, Ca\(^{2+}\) = 25 μequiv/L, Al = 0 μg/L) (Fig. 2A). Overall, Ht was significantly and separately raised by both low pH and elevated Al, and there was a Ca\(^{2+}\) × Al interaction.

interaction. In addition, Ht was significantly higher in both of the soft waters at circumeutral pH than in hardwater. Calculated MCHC virtually mirrored these changes (compare Fig. 2C and 2A), although the overall effects were limited to a Ca\textsuperscript{2+} × Al interaction.

Effects of Exposure Conditions on Na\textsuperscript{+} Fluxes and Mortality during Challenge

When tested in the water of their exposure condition (i.e. control measurements), all groups except one maintained a $J_{\text{net Na}^+}$ which was not significantly different from zero (Fig. 3, 4), indicating that the fish were in whole-body Na\textsuperscript{+} balance after 10 wk. The single exception was the group exposed to pH = 5.2, Ca\textsuperscript{2+} = 25 μequiv/L, Al = 75 μg/L, where the net flux was significantly negative (Fig. 3D). However, as these same trout were later able to restore zero balance even in the presence of the acid/Al challenge, we are inclined to dismiss this initial imbalance as an experimental artifact, perhaps reflecting disturbance during the control measurements.

In the two groups held at pH = 6.5 (in the absence of Al), challenge with low pH = 4.8 alone resulted in a shift in $J_{\text{net Na}^+}$ to moderately negative values, although the net flux became significantly different from zero at 0–8 h and 36–48 h only (data not shown). These changes were small relative to those seen with pH = 4.8 plus Al = 333 μg/L, indicating that the toxic effect of the latter challenge was mainly due to Al.

At the lower Ca\textsuperscript{2+} level (25 μequiv/L), the acid plus Al challenge resulted in marked Na\textsuperscript{+} losses which persisted throughout the 48-h period in fish previously naive to both Al and low pH (i.e. exposed to pH = 6.5, Al = 0 μg/L; Fig. 3A). Indeed, four of these six fish died during the final 12 h of the challenge. In the trout previously exposed to low pH alone (i.e. pH =

---

**Fig. 3.** Net whole-body Na\textsuperscript{+} fluxes with the environment ($J_{\text{net Na}^+}$) of brook trout after 10 wk of exposure to four different combinations of pH and Al at low Ca\textsuperscript{2+} (25 μequiv/L) and during a 48-h challenge with pH = 4.8, Ca\textsuperscript{2+} = 25 μequiv/L, Al = 333 μg/L. The exposure conditions were maintained during the control measurements. Means ± 1 SEM for $N = 6$ in each cell, except at 48 h where $N$ was reduced by the number of deaths tabulated. Asterisks indicate mean values of $J_{\text{net Na}^+}$ which were significantly different from zero ($p < 0.05$).

**Fig. 4.** Net whole-body Na\textsuperscript{+} fluxes with the environment ($J_{\text{net Na}^+}$) of brook trout after 10 wk of exposure to four different combinations of pH and Al at high Ca\textsuperscript{2+} (400 μequiv/L) and during a 48-h challenge with pH = 4.8, Ca\textsuperscript{2+} = 400 μequiv/L, Al = 333 μg/L. Other details as in legend to Fig. 3.
5.2, Al = 0 μg/L), virtually identical Na⁺ losses occurred during the challenge and one of the six fish died (Fig. 3B). However, prior exposure to low levels of Al (75 or 150 μg/L) in addition to low pH conferred increased resistance. Initial Na⁺ loss rates during the challenge were similar to those in trout naive to Al, but zero net Na⁺ balance was restored by 24 or 30 h (Fig. 3C, 3D). Furthermore, none of these fish died.

At the higher Ca²⁺ level (400 μequiv/L), the overall trends were similar but less clear due to variability between animals, and the toxicity of the challenge appeared to be reduced (Fig. 4). Again, Na⁺ loss rates during the challenge were least in the fish previously exposed to pH = 5.2 plus Al = 75 or 150 μg/L, and there were no mortalities in these groups.

When the data were analyzed in terms of cumulative whole-body Na⁺ losses over 48 h for individual fish (Fig. 5), losses were greatest (~4000 μequiv/kg) in the groups naive to both low pH and Al and least (~1700 μequiv/kg) in the groups previously exposed to pH = 5.2, Al = 75 μg/L. There was no discernible effect of water Ca²⁺ on cumulative Na⁺ loss.

Effects of Exposure Conditions on Blood Composition after Challenge

There were significant variations in some blood parameters as a result of the exposure conditions alone (Fig. 1, 2), so significant variations after the 48-h challenges would be expected regardless of challenge effects. The changes in blood composition resulting from the challenge are the more meaningful parameters and are presented in Fig. 6. At the lower Ca²⁺ level (25 μequiv/L), plasma electrolyte changes were in general accord with the Na⁺ flux data of Fig. 3 and 5A. Thus, a 48-h challenge with low pH = 4.8 alone had no effect on plasma Na⁺ and Cl⁻ concentrations in naive fish, but a challenge of the same group with pH = 4.8 plus Al = 333 μg/L caused highly significant decreases in both parameters (Fig. 6A). These effects of the acid/Al challenge were attenuated in fish previously exposed to pH = 5.2, Al = 0 μg/L and pH = 5.2, Al = 150 μg/L and abolished in the group exposed to pH = 5.2, Al = 75 μg/L. Decreases in plasma Cl⁻ levels were generally greater than those of Na⁺. Ht and plasma protein concentration fell after challenge of naive fish with low pH = 4.8 alone, but increased greatly when this same group was challenged with pH = 4.8 plus Al = 333 μg/L (Fig. 6C). In agreement with the Na⁺ and Cl⁻ changes, these hemococoncentration effects were attenuated by prior exposure to pH = 5.2, Al = 150 μg/L and abolished by prior exposure to pH = 5.2, Al = 75 μg/L. However, in disagreement with the electrolyte data, hemococoncentration was also prevented in the group previously held at pH = 5.2, Al = 0 μg/L.

At the higher Ca²⁺ level (400 μequiv/L), effects of prior exposure on changes in blood parameters during the challenges were less clear, reflecting the same variability seen in the Na⁺ flux data of Fig. 4 and 5B. Nevertheless, there were large decreases in plasma Na⁺ and Cl⁻ (Fig. 6B) and accompanying increases in Ht and plasma protein levels (Fig. 6D) in the naive fish under acid/Al challenge. Prior exposure to either low pH = 5.2 alone or pH = 5.2 plus 75 or 150 μg/L Al either reduced or abolished these effects.

Discussion

Water Chemistry

The present and following papers are the first physiological studies on fish chronically exposed to acid and Al for a prolonged period under defined softwater conditions. For acid stress alone, it is well documented that the water quality parameter of key importance in modifying both toxicological and physiological responses is Ca²⁺ (e.g. Brown 1981, 1983; Wood 1988), which was controlled closely in the present experiments (Table 1). The fluctuations in water Na⁺ during the 10 wk of exposure probably had minimal influence on the overall results because they occurred simultaneously in all exposure tanks as the water treatment system cycled. Furthermore, Brown (1981) found little effect of water Na⁺ over the range of variation noted here on the toxicity of acid to brown trout (Salmo trutta). During the challenge experiments, the variation was greatly reduced to levels representative of very dilute natural soft water (50–100 μequiv/L). Based on branchial Na⁺ transport studies in these fish (McDonald and Milligan 1988), the influence of this small variation on the present results would have been negligible. Total Al levels were well controlled during both exposure and challenge experiments, although the contribution of the inorganic monomeric fraction was rather variable, probably due to variable complexation by organics originating from food and excreta in the water. Al speciation was not measured during the challenges, but the faster water turnover and the lack of feeding here probably resulted in a greater inorganic monomeric fraction, which would tend to increase toxicity.

Effects of pH/Ca²⁺/Al Exposures on Ionoregulatory Parameters

Ten weeks of exposure to a range of sublethal pH/Ca²⁺/Al conditions induced significant variation in plasma electrolyte
concentrations (Fig. 1). For Na\(^+\) and Cl\(^-\), the absolute sizes of these chronic effects were rather small. Indeed, the total variation among the different exposure conditions was within the normal range which we (Booth et al. 1988) and other workers (e.g. Houston et al. 1971; McCormick and Naiman 1984) have observed for brook trout maintained at circumneutral pH in either hard or soft water. Furthermore, there was no significant variation in plasma osmolality (Fig. 1C), of which Na\(^+\) and Cl\(^-\) are the major constituents. This is possibly explained by the mobilization of other osmolytes such as glucose and taurine during long-term exposures (Fugelli and Vislie 1982; Giles et al. 1984; Scherer et al. 1986).

Qualitatively, chronic effects on Na\(^+\) and Cl\(^-\) regulation were very different from acute effects. Thus after 10 wk, there was no significant overall influence of low pH, elevated Al had a significant positive influence on plasma Na\(^+\) (Fig. 1A), and the fish were in net Na\(^+\) balance in most conditions (Fig. 3, 4). The acute effect of all the acid and acid/Al conditions (i.e. pH = 5.2, Al = 0–150 \(\mu g/L\)) would have been an initial loss of Na\(^+\) and Cl\(^-\) across the gills and depression of plasma levels, followed by a partial recovery over the next few days due mainly to reduction of the unidirectional efflux components (Booth et al. 1988). Taken together, these observations suggest that the recovery process continues during chronic exposure, resulting in adaptation to the sublethal stresses.

There have been a number of previous studies on the chronic ionoregulatory effects (2 wk to 3 mo) of low pH exposure alone (4.5–6.0) on other salmonids (rainbow trout, brown trout, Atlantic salmon (Salmo salar)), each of which is more acid sensitive than brook trout (Grande et al. 1978). Recovery of plasma Na\(^+\), Ca\(^2+\), and osmolality was at best partial in all investigations (McWilliams 1980; Lee et al. 1983; Saunders et al. 1983; Johnston et al. 1984; Giles et al. 1984; Lacroix et al. 1985; Weiner et al. 1986; Brown et al. 1986). Thus the present study with brook trout is the first to show complete recovery of these parameters, at least at pH = 5.2. However, in the same species at a more severe pH (\(\leq 4.6\)), Leivestad et al. (1976) found that plasma Na\(^+\) and Cl\(^-\) were still depressed after 1 yr of exposure, suggesting that complete recovery may be blocked by a threshold somewhere between 5.2 and 4.6.

The significant depression of Ca\(^2+\) levels caused by chronic exposure to low pH alone in the present study (Fig. 1D) has been observed previously in other salmonids (Wood and McDonald 1982; Scherer et al. 1986; Weiner et al. 1986). The mechanism is unknown. The depressions occur despite the minimal effects of acute acid exposure on Ca\(^2+\) exchange with the environment (Höbe et al. 1984; Booth et al. 1988) and the lack of chronic effects of low pH on whole-body Ca\(^2+\) dynamics in brook trout (Rodgers 1984). As impairment of Ca\(^2+\) regulation has been implicated in reproductive failure during long-term acid stress, this is clearly an important area for further investigation.

The present study is the first to examine the ionoregulatory effects of long-term acid plus Al exposure. The results, which show a significant supportive effect of Al on plasma Na\(^+\), Ca\(^2+\), and K\(^+\) levels (Fig. 1), are unexpected in view of the normally
toxic action of Al on ionoregulation. They suggest that low levels of Al may actually be beneficial during chronic acid exposure. At present, we can only speculate that this reflects some sort of recovery " overshoot," a view supported by subsequent studies on Na+ uptake kinetics in these same fish (McDonald and Milligan 1988). Alternatively or additionally, the small fraction of Al present as Al3+ at pH = 5.2 may have exerted a Ca2+ -like action in stabilizing branchial permeability. Schofield and Trojanar (1980) and Baker and Schofield (1982) have suggested that this action is the basis of the protective effect of Al seen at somewhat lower pH's.

There was no chronic influence of any of the pH/Ca2+/Al exposures on either blood Hb or plasma protein levels (Fig. 2B, 2D). Thus, O2 and CO2 transport capacity, blood buffering capacity, and plasma oncotic pressure were probably little affected. Nevertheless, there were substantial variations in Ht due to variations in MCHC (Fig. 2A, 2C). The two most likely explanations are erythrocytic swelling and/or a greater fraction of Hb-poor erythrocytes (e.g., reticulocytes) in the circulation under chronic sublethal stress. Both effects are seen during acute lethal acid stress (Milligan and Wood 1982). The more dilute intracellular environment of swollen erythrocytes will improve the O2 affinity of the blood (Nikinmaa 1986).

Effects of Exposure Conditions on Responses to Challenge

In terms of both mortality and net Na+ loss rates, Wyoming trout naive to acid and Al responded similarly to the naive Ontario trout of our companion studies (Booth et al. 1988), although the challenge protocols were slightly different. As before, all the mortality and the major part of the Na+ losses were clearly attributable to the Al component of the exposure at pH = 4.8, Al = 333 μg/L. The resultant reductions in plasma ions and increases in Ht and plasma protein levels due to internal fluid shifts were also similar to those seen in the Ontario trout (Booth et al. 1988; Wood et al. 1988a). Thus there did not appear to be significant differences in sensitivity associated with the differences in fish stocks, water quality, and altitude.

We have characterized the branchial ion loss response of naive brook trout to acid/Al challenge as comprising two phases (Booth et al. 1988). Over the first 6–12 h, there is an initial "shock" phase of high salt loss due to both an inhibition of active uptake and a more important stimulation of diffusive efflux. By 24 h, this is replaced by a phase of partial "recovery" in which the diffusive efflux is returned to control levels or lower, while the inhibition of active uptake largely persists.

In the present study, there was no detectable effect of the pre-exposure condition on Na+ loss rates during the initial shock phase (Fig. 4, 5). There was, however, a clear acceleration of the recovery phase in trout previously exposed to lower levels of Al (75 or 150 μg/L) at pH = 5.2, Ca2+ = 25 μequiv/L (Fig. 3C, 3D). Thus by 24–30 h, the animals had returned to zero Na+ balance, resulting in a halving of the total Na+ loss over 48 h (Fig. 5A) and a prevention of mortality during this period. Plasma Na+ and Cl- depressions and hematological changes were similarly attenuated (Fig. 6A, 6C). Prior exposure to Al = 75 μg/L appeared to be slightly more effective than 150 μg/L in conferring this increased resistance. At the higher Ca2+ = 400 μequiv/L, the effects were less clear due to variability among fish. Nevertheless the overall pattern as shown by net Na+ flux rates (Fig. 4), cumulative losses (Fig. 5), and plasma changes (Fig. 6B, 6D) was similar to that at low Ca2+. In total, these results indicate that chronic exposure to low levels of Al and acidity induced physiological acclimation in the traditional sense — increased resistance of a more severe exposure.

The results with prior exposure to low pH = 5.2 alone were equivocal. Mortality during acid/Al was reduced but not prevented, Na+ loss rates remained high (Fig. 3B, 4B, 5), yet plasma Na+ and Cl- and hematological changes were reduced or abolished (Fig. 6). The protective effect seemed greater at the higher Ca2+. These results are perhaps to be expected inasmuch as most of the toxic effect of the challenge was due to the Al component, to which these fish were still naive. Any increased resistance could be explained by the fact that a major action of Al is similar to that of acid alone (ionoregulatory impairment) and that morphological changes in the gills are somewhat similar during chronic sublethal acid exposure as during acid plus Al exposure (see below) (Tietge et al. 1988). However, our more detailed physiological studies indicate that prior exposure to acid alone does not provide significant acclimatory resistance to acid plus Al stress (Wood et al. 1988b; McDonald and Milligan 1988).

Several studies have described dramatic morphological changes in the gills associated with chronic exposure to sublethal acid/Al stress (Chevalier et al. 1985; Karlsson-Norgren et al. 1986a; 1986b; Tietge et al. 1988). These include deformation and fusion of lamellae, epithelial detachment, a general swelling of the lamellae due to epithelial hyperplasia, white blood cell infiltration of the lymphatic spaces, increased prevalence of "dense" cells, and most notably a gross proliferation of chloride cells, many of which may contain Al inclusion bodies. Blood to water diffusion distance clearly increases. Based on these descriptions, one might speculate that the acclimatory resistance to acid/Al challenge results from the increased diffusion distance limiting passive ion efflux, while the greater number of chloride cells limits the inhibition of active uptake. However, this scenario predicts that Na+ losses should have been reduced primarily during the initial shock phase and not as observed during the later recovery phase. Perhaps in Al-acclimated fish, the recovery phase is different, involving an activation of the greater uptake capacity of the proliferated chloride cells (cf. McDonald and Milligan 1988) or a more effective reduction of the passive permeability.

In summary, the present results indicate that brook trout adapt to chronic sublethal acid/Al stress. At least at the Al levels employed here, the fish demonstrate acclimation rather than sensitization, in accord with the results of Orr et al. (1986) on rainbow trout. The acclimation has a definite physiological basis reflected in ionoregulatory parameters. We suggest that this acclimation process allows fish to survive in the wild under conditions where they might otherwise succumb. Specifically, fish chronically exposed to low levels of Al in mildly acidic soft water may possess increased resistance to short-term increases in Al and acidity associated with episodic "surges."

Acknowledgements

This work was supported by a contract ("Lake Acidification and Fisheries," RP-2346-01) from the Electric Power Research Institute, Environmental Assessment Department, through a subcontract from the University of Wyoming. We thank Dr. J. Mattice, EPRI project manager, for his advice and encouragement and the staff of the Fish Physiology and Toxicology Laboratory, University of Wyoming, for their assistance and hospitality.
References


