Nature and Time Course of Acclimation to Aluminum in Juvenile Brook Trout (*Salvelinus fontinalis*). I. Physiology

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Chronic exposure (up to 42 d) of juvenile brook trout (*Salvelinus fontinalis*) to sublethal aluminum at pH 5.2 resulted in a damage/repair acclimation phenomenon. The damage developed rapidly (within 24 h), was centered at the gills, and was characterized by substantial accumulation of Al, a corresponding reduction of gill sialic acid content (a measure of gill mucus), and inhibition of branchial Na^+ transport. The corresponding internal effects of this initial damage were losses of electrolytes, hemoconcentration, and impaired tissue O_2 delivery (as indicated by elevated lactate). Repair was characterized by progressive reduction of gill Al, restoration of sialic acid content, recovery of Na^+ transport, and reduction in hemoconcentration and lactate levels. Accompanying the recovery was progressive development (by day 10 onwards) of increased resistance (i.e. acclimation) to acutely lethal Al. This acclimation was characterized by a reduction in both the rate of mortality and in the magnitude of physiological disturbances relative to control (i.e. Al naive) fish. The increased short-term resistance translated to greatly improved survivorship and correspondingly diminished physiological impact in the face of chronically elevated Al levels (2 wk at <300 μg Al/L). The acclimation process clearly resulted from specific changes at the gills.

L'exposition chronique jusqu'à 42 jours d'ombles de fontaine juvéniles à des concentrations sublétales d'aluminium dans un milieu de pH 5,2 a donné lieu à un phénomène d'acclimation suivant un cycle perturbations/rétablissement. Les perturbations, qui se sont présentées en deçà de 24 h, touchaient surtout les branchies. Elles étaient caractérisées par une importante accumulation d'Al, une baisse correspondante de la teneur en acide sialique dans les branchies (une quantification du mucus des ouïes) et l'inhibition du transport du Na^+ dans les branchies. Les répercussions internes correspondantes de ces perturbations initiales concernaient la perte d'électrolytes, l'hémoconcentration et la réduction de l'apport d'O_2 dans les tissus (comme l'ont révélé les teneurs élevées en lactate). Le rétablissement était caractérisé par une diminution graduelle de la teneur en Al dans les branchies, le rétablissement de la teneur en acide sialique, le rétablissement du transport du Na^+ et une baisse de l'hémoconcentration et des teneurs en lactate. L'élaboration progressive à partir du dizième jour d'une résistance accrue (c'est-à-dire l'acclimatation) à des teneurs létales aiguës d'Al suivait le processus de rétablissement. Cette acclimatation était caractérisée par une baisse du taux de mortalité et de l'ampleur des perturbations physiologiques par rapport aux poissons témoins qui n'avaient jamais été exposés à l'Al. Cette résistance accrue à court terme s'est traduite par un taux de survie nettement amélioré et une incidence physiologique diminuée porportionnellement en présence de teneurs en Al élevées de façon chronique (2 sem à moins de 300 μg Al/L). Le processus d'acclimatation était clairement le résultat de modifications particulières survenues dans les branchies.

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An important correlate of environmental acidification is elevation in dissolved aluminum; levels in excess of 100 μg/L have been reported in lakes and rivers still supporting fish populations (Wright and Snekvik 1978; Schofield and Trojnar 1980; Kelso et al. 1986). This has prompted investigators to question the impact on fish of chronic sublethal Al exposure. Studies to date are generally agreed that prolonged (i.e. >1 wk to several weeks) exposure to sublethal Al conveys increased resistance to short-term increases in Al (Guthrie 1982; Orr et al. 1986; Siddens et al. 1986; Wood et al. 1988a, 1988b; McDonald and Milligan 1988), i.e. fish acclimate to Al. Furthermore, this is an effect specifically of Al rather than the low pH's which invariably accompany elevated Al (Wood et al. 1988a, 1988b). The Al acclimation takes the form of a reduced rate of mortality in the face of lethal Al levels (Guthrie 1982; Wood et al. 1988a, 1988b), an increase in the toxic threshold to Al (specifically, an increase in the 96-h, LC_{50}; Orr et al. 1986), and a reduction in the magnitude of physiological disturbances (Wood et al. 1988a, 1988b; McDonald and Milligan 1988).

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The physiological effects of Al (impaired ionoregulation and gas exchange) indicate that the gills are the main target organ. Thus, we hypothesize that the acclimation process arises from specific biochemical, physiological, and/or morphological changes in the gills. In our previous studies, we showed that Al acclimation led to an improvement of ionoregulatory function in the face of Al challenge; specifically, lower net Na^+ losses during elevated Al and a more rapid recovery of net Na^+ balance (Wood et al. 1988a, 1988b). The increased resistance of Na^+ regulation was, at least in part, due to changes in branchial active Na^+ transport; fish maintained at low pH (pH 5.2) in the presence of Al had a higher rate of branchial Na^+ uptake than fish maintained at pH 5.2 alone or those maintained at pH 6.5 (McDonald and Milligan 1988). These findings were obtained on brook trout (*Salvelinus fontinalis*) exposed 10 wk to Al; presumably the Al adaptation process was complete by this time.

Thus the objective for this and the companion study (Mueller et al. 1991) was to examine both the time course and the nature of acclimation to Al in brook trout. The focus of the present study was upon various measures which either directly or indirectly assess the physiological state of the gills (gill mucous and Al content, Na^+ transport activity, body electrolytes, lactate, and hematology) whereas Mueller et al. (1991) focus specifically on changes in gill morphology.

Methods

Experimental Animals and Holding Conditions

Experiments were conducted at the Fish Physiology and Toxicology Laboratory, Laramie, WY, which provided facilities for continuous exposure of fish to defined pH, Ca^{2+}, and Al conditions in flowing artificial soft water. Artificial soft water (composition in Table 1) was generated by treatment of hard well water ($Na^+ = 0.3$ mequiv/L, $Ca^{2+} = 2.6$ mequiv/L) with sediment filtration, NaCl softening, reverse osmosis, and separate bed deionization. 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 H_2SO_4. 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). The required levels of $CaCl_2$ and $AlCl_3$ were added via Mariotte bottles to continuous-flow serial diluters. From here the water was delivered to the exposure tanks, and then to waste. The tanks were round, 800-L fiberglass chambers served with a flow of 4 L/min, providing 7 volume additions per day.

Test animals were juvenile brook trout (2–13 g) obtained from Cline's Trout Farm, Boulder, CO, and initially maintained with periodic feeding in hard well water. Prior to softwater acclimation, the fish were fin clipped for later separation into three groups and then transferred to soft water for an acclimation period of 14 d. Experimental temperature was 10 ± 1°C. Fish were fed floating feed (Purina Trout Chow) at 1% body weight/day and tanks were siphoned daily to remove organic debris. Photoperiod was adjusted biweekly to follow the natural cycle for Laramie. Water chemistry was measured daily on samples taken directly from the tanks.

Experimental Protocol

Brook trout were exposed to one of three acclimation conditions (Group I, Table 1): normal soft water (pH 6.5, Ca^{2+} = 25 μequiv/L, Al = 0; 6.5/0 group), low-pH soft water (pH 5.2, Ca^{2+} = 25 μequiv/L, Al = 0; 5.2/0 group), and low-pH soft water + Al (5.2/Al group) for up to 42 d. One tank was used for each exposure and each tank started with 400–600 fish. The initial Al level for the 5.2/Al group was 150 μg/L but this

TABLE 1. Nominal exposure conditions and measured water chemistry (means and ranges) during 24-d chronic exposures. Group I data are for exposures shown in Fig. 2. All levels were reduced from 150 to 75 μg/L on day 3 (see Fig. 3). Group II data are for exposures conducted 6 mo later (see Fig. 3).

Nominal			Measured					
pH	Ca^{2+} (μequiv/L)	Al (μg/L)	pH	Temperature (°C)	Ca^{2+} (μequiv/L)	Total Al (μg/L)	Na^+ (μequiv/L)	Cl^- (μequiv/L)
				Group I				
6.5	25	0	6.60 (6.35–6.82)	9.1 (9.0–9.6)	28 (23–32)	0.9 (<1–3.7)	43.9 (39.6–47.4)	43.4 (40.9–45.7)
5.2	25	0	5.13 (4.97–5.32)	9.2 (8.9–9.6)	26 (22–29)	1.4 (<1–3.7)	43.9 (40.0–47.9)	45.1 (40.9–57.8)
5.2	25	150 (0–3 d)	5.26 (5.20–5.37)	9.0 (9.0)	22 (22–23)	164.5 (157.4–171.6)	50.0 (49.2–50.9)	64.0 (61.5–66.3)
5.2	25	75 (3–24 d)	5.16 (5.01–5.23)	9.2 (9.0–9.5)	27 (23–29)	68.2 (55.6–78.3)	42.6 (39.1–44.4)	48.8 (48.2–50.2)
				Group II				
6.5	25	0	6.48 (6.02–6.79)	10.2 (10.0–10.5)	36 (33–38)	0.0	56.5 (49.2–61.3)	57.5 (46.3–70.0)
5.2	25	0	5.29 (5.08–5.61)	10.3 (10.0–10.5)	37 (32–40)	0.0	58.3 (46.1–67.4)	57.5 (48.0–66.6)
5.2	25	75	5.12 (51.0–5.37)	9.9 (9.5–10.1)	34 (29–38)	70.2 (65.0–75.2)	56.1 (45.7–63.9)	63.8 (51.3–73.3)
5.2	25	150	5.09 (4.91–5.39)	9.9 (9.5–10.0)	34 (28–38)	145.2 (140.8–148.0)	65.2 (47.4–75.7)	81.5 (61.5–92.2)

was reduced on day 3 to 75 μg/L to reduce mortalities and was maintained at 75μg/L thereafter (Table 1, Group I). To assess the effect of this first 3-d exposure to a variable Al regime, a second acclimation experiment (Group II, Table 1) was conducted 6 mo later. Brook trout (8–46 g) were obtained from the same source and acclimated to soft water for 14 d. They were then divided into four exposure groups: 6.5/0 ($N=60$), 5.2/0 ($N=60$), 5.2/75 ($N=100$), and 5.2/150 ($N=100$) and maintained under these conditions for up to 10 d. Fish ($N=10$ from each group) were sampled at periodic intervals (days 1, 2, 4, 7, and 10; e.g. see Fig. 3) and analyzed for whole-body electrolytes and gill Al.

To assess the nature and time course of acclimation in Group I fish, animals were periodically sampled from each acclimation condition for measurements of physiological state or for challenge with higher Al levels. Physiological measures included whole-body electrolytes (Na^+, Cl^-), whole-body lactate, hematology (hematocrit and plasma protein), gill chemistry (sialic acid and Al content), and branchial Na^+ fluxes (influx, J_{in}, and net flux, J_{net}). Resistance to challenge was assessed on the basis of cumulative mortality over time and by the magnitude of physiological disturbances in survivors at 24 h.

From day 0 to 24 of the acclimation experiment, 52 fish from each acclimation group were removed at each sampling time (days 1, 2, 4, 7, 10, 13, 17, and 24; eg. see Fig. 2). Sixteen of these fish were used for measurements of Na^+ uptake; half were subsequently returned to their respective acclimation tank and the other half killed for measurements of whole-body electrolytes. Another 10 fish were immediately killed and a blood sample obtained by caudal puncture using a modified 100-μL Hamilton gas-tight syringe. Hematocrit and plasma protein were determined on this blood sample. The second gill arch from the right side was taken for histological examination (see companion study by Mueller et al. 1991) and the remaining gill basket was weighed, frozen, and later digested for gill Al and sialic acid determination. A third group of 10 fish from each acclimation group was transferred to a single challenge tank (pH 5.2, nominal Al = 1000 μg/L) and cumulative mortalities recorded over time until all fish were dead. An estimate of the time to 50% mortality (LT_{50}) was determined by log-probit analysis for each group exposed to the challenge. A fourth group of 16 fish from each acclimation group was challenged simultaneously with the same condition; survivors at 24 h were used for either (i) determinations of whole body electrolytes and lactate or (ii) measurements of hematocrit, plasma protein, gill Al and sialic acid content, and gill histology.

A final challenge experiment was begun on day 28 of the acclimation experiment. Here, a total of 90 fish from each of the 6.5/0 and 5.2/Al groups were transferred in groups of 15 to one of six exposure tanks (pH 4.8 at 0, 100 or 300 μg Al/L; pH 5.2 at 0, 100 or 300 μg Al/L). Fish were maintained for 2 wk under these conditions and cumulative mortalities recorded over time. At the end of the 2 wk, up to 10 survivors from each group in each tank were then measured for whole-body electrolytes, hematology, and gill Al.

Analytical Techniques and Calculations

Whole-body lactate and electrolytes

After a killing blow to the head, fish were grasped immediately with aluminum tongs and freeze-clamped at −80°C in liquid N_2 (~5 s), weighed, and homogenized in 30 mL of ice-cold 8% perchloric acid in a food chopper (Black & Decker HC 20). The homogenate was allowed to stand at 4°C for 2 h and then centrifuged at 900*g* for 10 min. Duplicate 100-μL aliquots of the supernatant were analyzed enzymatically for lactate (L-lactate dehydrogenase/NADH method; Loomis 1961; Sigma reagents). Triplicate 100-μL aliquots of supernatant were measured for Na^+ by AAS (Perkin Elmer atomic absorption spectrophotometer, model 2380) following 1:100 dilution in deionized water. Triplicate 200- to 400-μL aliquots were measured for Cl^- directly by coulometric titration with a Radiometer CMT-10 chloridometer.

Blood measurements

Blood samples (50–200 μL) obtained by caudal puncture were centrifuged in heparinized microcapillary tubes at 5000*g* for 5 min. Percent red cell volume (hematocrit) was measured and plasma drawn off for immediate analysis of protein. Plasma protein was determined with an American Optical Goldberg refractometer (Alexander and Ingram 1980).

Gill aluminum and sialic acid content

Gills (whole branchial baskets minus the second arch on the right side) were digested in 0.1 N H_2SO_4 (5.5. mL acid/g gill tissue) at 80°C overnight. Triplicate aliquots (10–25 μL) of this digest were analyzed for Al with the pyrocatechol violet method (Dougan and Wilson 1974) using a standard curve prepared from a digest of gill tissues collected on day 0 from 6.5/0 animals, i.e. animals that had not been exposed to Al.

Duplicate 25-μL aliquots of the gill digest were analyzed for sialic acid content using the thiobarbituric method of Warren (1963). This assay is used as a convenient method for estimating the mucous content of the gills (e.g. Arillo et al. 1979). Sialic acids are the main acidic component of fish mucus (Harris et al. 1973). Standard curves were prepared using *N*-acetylneuramic acid (NANA, obtained from Sigma). According to Zuchelkowski et al. (1985), NANA is the most common form of sialic acid produced in fish mucous cells.

Na^+ uptake

Na^+ transport was assessed by examining the relationship between unidirectional Na^+ uptake (J_{in}^{Na+}) and water Na^+ concentration ($[Na^+]$) at pH 6.5. Fish were transferred from their respective acclimation tanks (16 from each group) to individual 250-mL chambers containing normal soft water (pH 6.5, Al = 0; Table 1). Each chamber was immersed in a 10°C water bath and was fitted with an air line which provided aeration and mixing. Each acclimation group was exposed to four Na^+ levels ($N=4$ at each level; 0.45, 0.75, 1.45, and 2.85 mequiv/L) and Na^+ fluxes measured for 1 h. Na^+ was added as NaCl to each chamber along with $^{22}Na^+$. The amount of $^{22}Na^+$ was varied so that specific activity remained constant at 0.9 μCi/mequiv (1 μCi = 37 kBq). Water samples (14 mL) were taken 5 min after Na^+ addition (to allow for mixing) and again at the end of 1 h. Water samples were analyzed in duplicate for $[Na^+]$ by AAS and for $^{22}Na^+$ activity by scintillation counting (5 mL of water + 10 mL of ACS fluor) in a Beckman model LS9000 scintillation counter.

J_{in}^{Na+} (nanoequivalents per gram per hour) was determined according to the following formula:

$$J_{in}^{Na+} = \frac{(cpm_i - cpm_f) \times vol}{time \times wt \times SA}$$

where cpm is the concentration of radiotracer ($^{22}Na^+$) in the medium (counts per minute per millilitre) at the beginning (*i*)

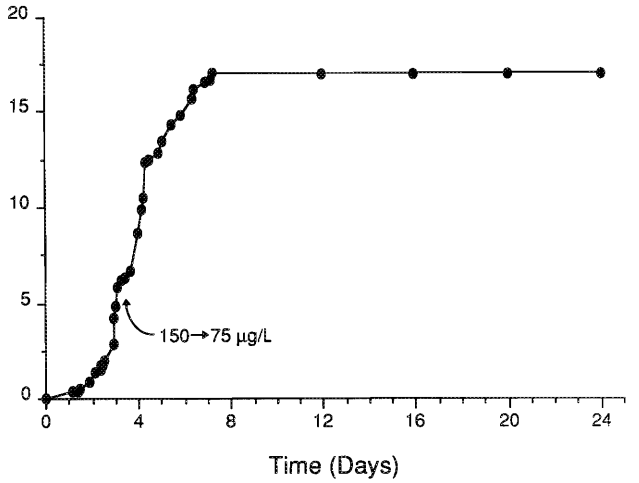

FIG. 1. Cumulative mortality during chronic Al exposure at pH 5.2 in juvenile brook trout at 10°C ($N = 600$ on day 0). On day 3, water Al levels were decreased from 150 to 75 µg/L (indicated by arrow).

and end (f) of the measurement period, vol is the volume (millilitres) of the flux chamber, SA is the radiospecific activity of the external medium (cpm per nanoequivalent), time is in hours, and wt is the animal's body weight (grams).

Net Na^+ flux (J_{net}^{Na+}, (nanoequivalents per gram per hour) was determined from changes in Na^+ concentration in the water:

$$J_{in}^{Na+} = \frac{([Na^+]_i - [Na^+]_f) \times vol}{time \times wt}.$$

Treatment of Data

Data have been reported as means ± 1 SE for each group (i.e. each exposure condition). One-way analysis of variance was used to assess differences among groups; when the F value indicated significance, Fisher's protected least significant difference (PLSD) was applied to discern specific differences (Winer 1971, p. 201). Estimates of LT_{50} were computed by log-probit analysis of time/percent effect data. Nomographic methods outlined in Litchfield (1949) and Litchfield and Wilcoxon (1949) were used to compute 95% confidence limits for the log-probit regressions. LT_{50}'s were considered significantly different when the confidence limits did not overlap. A 5% significance level was used throughout.

Results

"Sublethal" Al Exposure

An Al level of 150 µg/L (nominal, see Table 1) was chosen for the Al acclimation study, since we had previously shown (Wood et al. 1988a) that this level was sublethal to adult brook trout but would still evoke the acclimatory response. Furthermore, initial 48-h range-finder tests confirmed sublethality to juveniles. However, once the acclimation began, initial mortalities were unacceptably high; they had reached 4% by day 3 and were continuing (Fig. 1). Consequently, there was a risk of biasing this experiment by selection for hardier individuals rather than examining acclimation per se. Accordingly, the Al level was reduced from 150 to 75 µ/L on day 3 (indicated by arrow in Fig. 1). Although mortalities continued to occur, they eventually leveled out at 17% by day 7 of the exposure. In contrast, no mortalities occurred in the two other exposures, 5.2/0 and 6.5/0.

The control or 6.5/0 fish, while showing no consistent change in any measured parameter over time, did show a daily variation of up to 10% from overall means. Mean values for each measured parameter computed for the 24-d control period (1 ± SEM, $N = 80$) are shown on the broken lines in Fig. 2. To facilitate the comparison between the control and treatment groups (5.2/0 and 5.2/Al), and to correct for the daily variation, all measured variables on the treatment groups have been expressed as a difference from control fish *measured on the same day*.

Fish exposed to Al (150 → 75 µg/L) at pH 5.2 showed significant physiological disturbances relative to controls (Fig. 2): a depression in whole-body Na^+ and Cl^- of nearly equal magnitude (Fig. 2A and 2B); an increased hematocrit and plasma protein concentration (Fig. 2C and 2D), due likely to transcellular fluid shifts and hemoconcentration resulting from the electrolyte losses; and a significant elevation in whole-body lactate (Fig. 2E), probably resulting from impaired O_2 delivery to tissues. These physiological disturbances developed quickly, reached a peak at about 4 d of Al exposure, and thereafter gradually abated. Body Na^+ and Cl^- levels stabilized at about 80% of controls by 24 d of exposure but were still significantly depressed. Similarly, there was still a significant hemoconcentration evident. In contrast, body lactate levels had returned to control levels by day 17 of Al exposure.

Over the first 24 h of Al exposure, gill Al levels rose sharply, reaching a peak of 251 ± 30 µg/g wet gill tissue, and thereafter declining to a steady-state level of about 40 µg/g by day 10 (Fig. 2F). Correlated with the high gill Al levels was a significant depression in gill sialic acid content. Over the first week of the exposure, sialic acid levels were consistently about 35% less than controls: 1.68 ± 0.13 ($N = 38$) versus 2.55 ± 0.19 ($N = 40$) µmol/g wet gill tissue in the 5.2/Al and 6.5/0 groups, respectively. After 1 wk the sialic acid content of the gills of the 5.2/Al group increased to control levels, and thereafter the two groups were not significantly different from one another.

The fish exposed to pH 5.2 alone also showed some significant disturbances, indicating that they too were stressed. These consisted of a depression in body electrolytes late in the exposure period (Fig. 2A and 2B), some elevation of plasma protein (Fig. 2D) but not of hematocrit (Fig. 2C), and a significant elevation of body lactate, but only on one day (day 12; Fig. 2E). Not surprisingly, gill Al was no different than controls in this group, and was not significantly different than zero. Gill sialic acid content also remained virtually identical to controls at a mean level of 2.65 ± 0.12 ($N = 67$) µmol/g throughout the 24-d exposure.

The variable Al regime over the first few days of this experiment prompted the second series of exposures (Group II, Table 1) where the effects of the two Al levels (75 and 150 µg/L) were compared at pH 5.2 (Fig. 3). The main finding of this experiment was that exposure to constant 75 µg/L produced the same disturbance to body electrolytes as the exposure to pH 5.2 alone (Fig. 3A and 3B), i.e. this suggests that Al = 75 µg/L was below the threshold required to produce an Al-specific effect. In contrast, the constant 150 µg/L exposure produced the same disturbance as the 150 → 75 exposure (Fig. 3A and 3B). This suggests that with above-threshold Al levels, the critical period is the initial stages of the exposure. Accompanying the 150 → 75 and 150 exposures was substantial

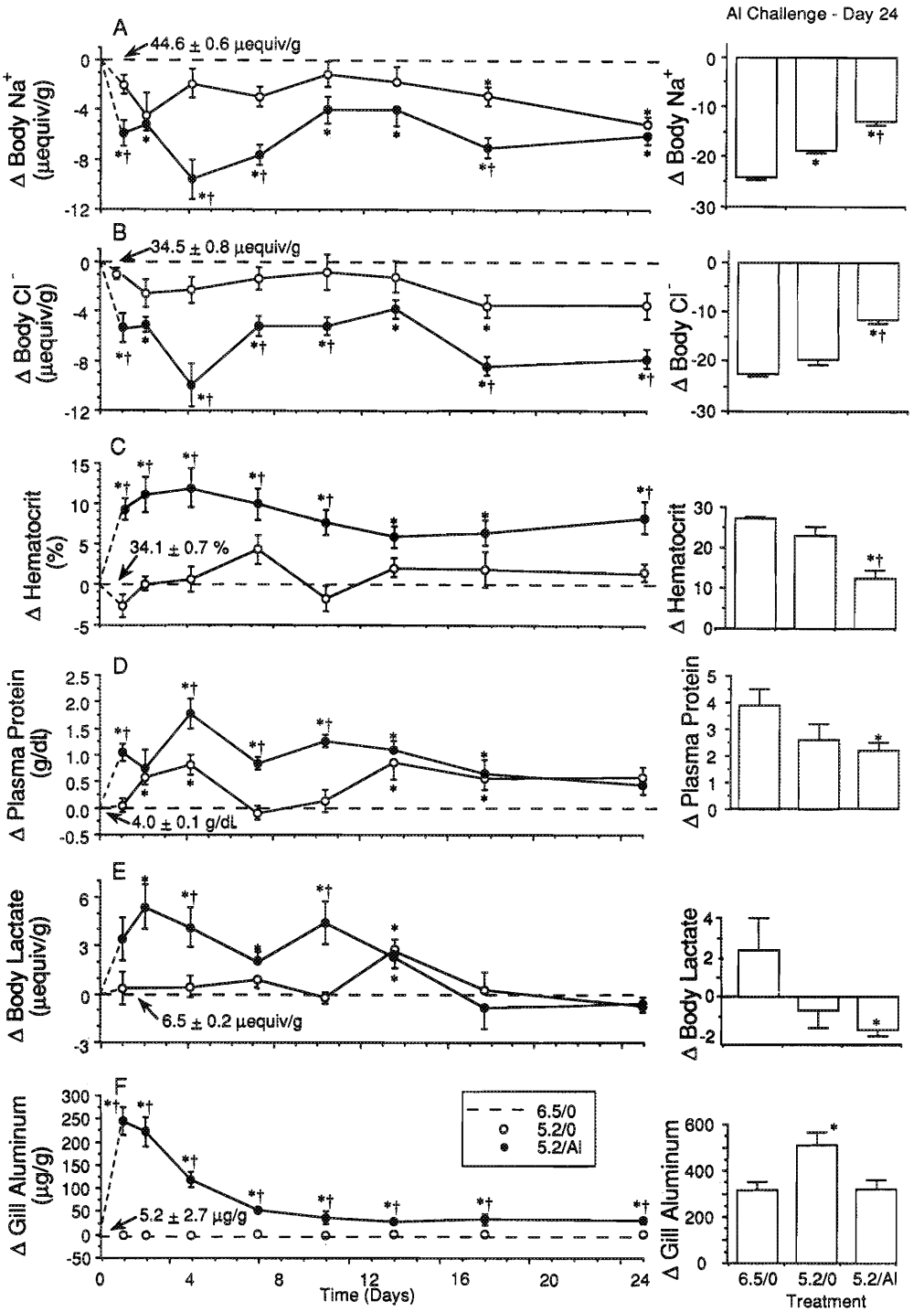

FIG. 2. (A) Whole-body Na^+, (B) whole-body Cl^-, (C) hematocrit, (D) plasma protein, (E) whole-body lactate, and (F) gill Al during 24 d of chronic exposure to Al (150 → 75 µg/L, left-hand panel) and following 24 h of acutely lethal Al challenge started on day 24 (right-hand panel; challenge = pH 5.2, 746 µg Al/L). Values on the broken lines are the averages for the whole 24-d period for the 6.5/0 (control) group (means ± 1 SE $N = 80$). Daily means (± SE) for 5.2/0 fish (open circles) and 5.2/Al fish (closed circles) are expressed as the difference from the mean for control fish measured on the same day. In the Al challenge (right-hand panel), values indicated are the differences (mean ± 1 SE) from the chronic exposure measurements on the same group made 24 h earlier. Asterisks indicate means (± 1 SE, $N = 8$–10) significantly different ($p < 0.05$) from 6.5/0; crosses indicate means significantly different from 5.2/0.

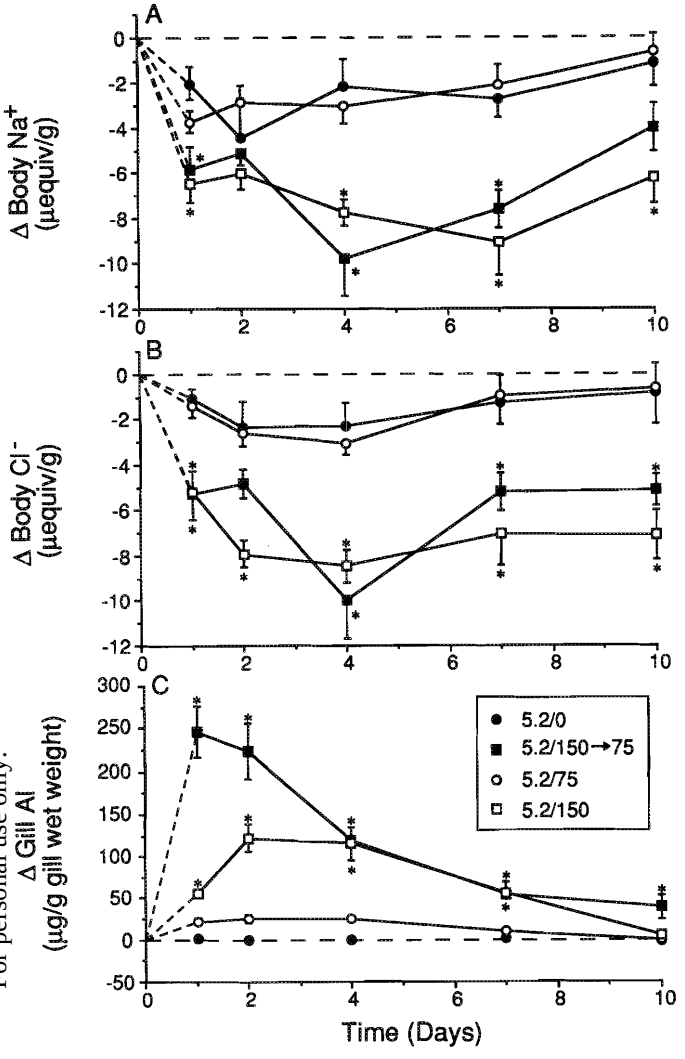

FIG. 3. (A) Whole-body Na^+, (B) whole-body Cl^-, and (C) gill Al during 10 d of acclimation to pH 5.2 and four different Al regimes (0, 75, 150 → 75, and 150 μg/L). Closed symbols are data from the first chronic exposure experiment taken from Fig. 2; open symbols are data from the second chronic exposure experiment conducted 6 mo later (Group II, Table 1). All values are mean differences from controls (6.5/0) measured on same day (± 1 SE, $N = 8$–10). Asterisks indicate means significantly different ($p < 0.05$) from corresponding means in the experiments conducted at the same time (i.e. ● versus ■, ○ versus □).

initial accumulation of Al on the gills (Fig. 3C). In contrast, there was only trace accumulation in the fish at constant 75 μg/L, further reinforcing the notion that this level of Al was below threshold (Fig. 3C). For some unknown reason, the initial gill in the 150 → 75 group was greater than in the constant 150 group. Nonetheless, by day 4, both groups had virtually identical Al levels, and thereafter, gill levels declined more or less in parallel in both groups, despite the continued presence of Al in the water.

Na^+ Transport Activity

In control (6.5/0) fish measured at pH 6.5, there was a linear relationship between J_{in}^{Na+} and external [Na^+] over the tested range of 0.4–2.8 mequiv Na^+/L for each of the eight separate occasions over the 24 d that measurements were made (Fig. 4).

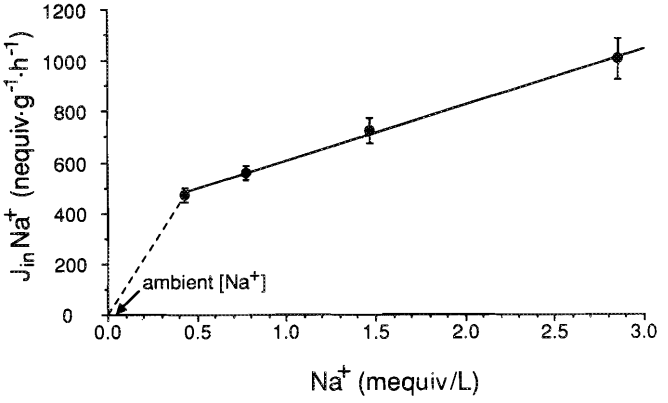

FIG. 4. Relationship between J_{in}^{Na+} and external [Na^+] in juvenile brook trout at pH 6.5. Each value is the mean (± 1 SE, $N = 32$) of measurements made on eight separate occasions during the 24-d chronic exposure experiment on groups of four different fish on each occasion. Line fitted by least squares regression; $J_{in} = 219 \cdot Na^+ + 387$, $r = 0.998$. Arrow indicates the [Na^+] level to which all fish were acclimated (0.04 mequiv/L).

In previous studies on brook trout (McDonald and Milligan 1988), we found a similar linear relationship. This linearity means that Na^+ transport activity cannot be expressed in the more conventional terms of saturation kinetics (i.e. maximum transport rate (J_{max}) and transport carrier affinity (K_m)). However, it remains possible that a saturable component exists in the untested range of 0.0–0.4 mequiv Na^+/L which encompasses ambient [Na^+] for these fish (0.04 mequiv/L; Table 1).

In the 5.2/0 and 5.2/Al groups, there were also linear relationships between J_{in}^{Na+} and [Na^+] although with different elevations. Thus, for the sake of simplicity, only the J_{in}^{Na+} measurements at the lowest [Na^+] (0.4 mequiv/L) have been plotted (Fig. 5A). The effect of Al exposure was an immediate and significant inhibition of Na^+ uptake (to about 50% of controls) by 24 h of exposure which gradually but almost completely recovered over the subsequent 23 d. Because the measurements of J_{in}^{Na+} in Al-exposed fish were made at pH 6.5 *without Al in the water*, this initial inhibition probably resulted from accumulated physiological damage to the gills. Similarly, the gradual recovery can be taken as evidence of repair of that damage.

No similar damage appears to have resulted from the pH 5.2 exposure. Over the first 10 d of pH 5.2 exposure, J_{in}^{Na+} was virtually identical to controls (Fig. 5A); thereafter, it was significantly higher than controls. The latter corresponds to the period where the 5.2/0 animals were showing significant depression of body Na^+ (Fig. 2A). This suggests that net Na^+ losses resulting from pH 5.2 exposure specifically stimulated the Na^+ uptake mechanism when the animals were briefly exposed to pH 6.5

Examination of J_{net}^{Na+} in each group ($N = 16$ at each measurement period; Fig. 5B) reveals the same basic trends as seen in the J_{in}^{Na+} measurements at the lowest Na^+ ($N = 4$; Fig. 5A). For the 6.5/0 group, there was very little variation with time in J_{net}^{Na+}, and overall, these fish exhibited a slight net uptake of Na^+, averaging 103 ± 76 nequiv·g^{-1}·h^{-1}, i.e. the animals were essentially in Na^+ balance. The other two groups showed significantly different responses over the first 10 d of exposure. The 5.2/0 group showed a greater net uptake relative to controls (Fig. 5B), while the 5.2/Al group showed a significant loss of Na^+ relative to controls (Fig. 5B), although they had lost the

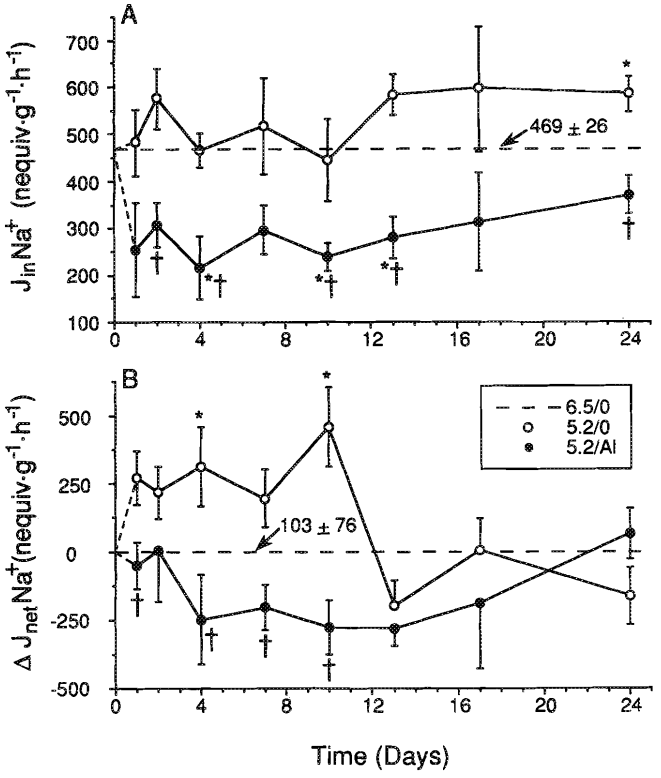

FIG. 5. (A) J_{in}^{Na+} measured at Na^+ = 0.4 mequiv/L (means ± 1 SE, $N = 4$); (B) average J_{in}^{Na+} relative to controls (broken line) measured at Na^+ = 0.4–2.8 mequiv/L (means ± 1 SE, $N = 16$). Asterisks indicate means significantly different ($p < 0.05$) from 6.5/0; crosses indicate means significantly different from 5.2/0.

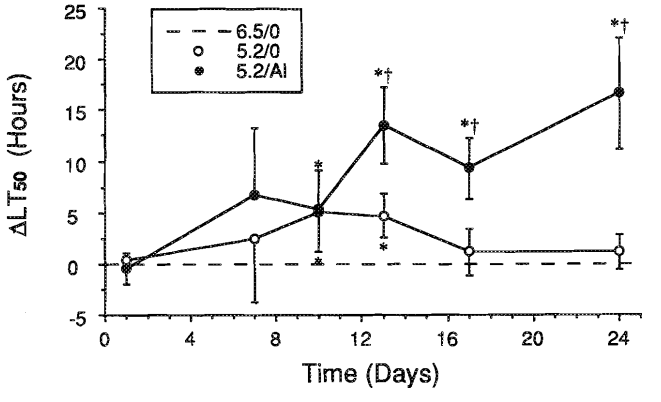

FIG. 6. Estimated LT_{50}'s in brook trout exposed to acutely lethal Al (607–1020 μg Al/L at pH 5.2). Open symbols indicate 5.2/0 fish and closed symbols indicate 5.2/Al fish, expressed as a difference (mean ± 95% confidence limits) from 6.5/0 fish measured on the same day. Asterisks indicate means significantly different ($p<0.05$) from 6.5/0; crosses indicate means significantly different from 5.2/0.

most Na^+ of the three groups (Fig. 2A and 2B). Clearly, the 5.2/0 group had retained the ability to restore electrolyte losses upon pH 6.5 exposure whereas that ability was significantly impaired in the 5.2/Al group.

Al Challenge during Acclimation

The Al challenge conditions were nominally 1000 μg Al/L at a pH of 5.2 for 48 h but, in fact, the actual mean Al value

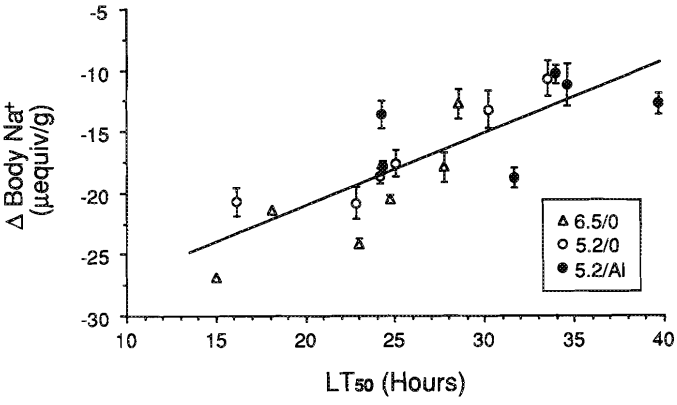

FIG. 7. Relationship between LT_{50} and loss of body Na^+ over the first 24 h of exposure in 24-h survivors for all three groups of fish. Data taken from all six challenge experiments (see Fig. 6). Line fitted by least squares regression; $\Delta NA^+ = 0.63 \cdot LT_{50} - 33.5$, $r = 0.835$.

for individual challenges varied from 610 to 1020 μg/L. Consequently, there was a corresponding variation in the LT_{50}'s, ranging, for example, in controls from 15 ± 1.3 to 33.8 ± 2.2 h. Since all three groups were exposed to Al in the same tank and thus to the same challenge conditions, there were similar variations in LT_{50} in the other two groups. Therefore, in order to clarify the differences amongst the groups, the LT_{50}'s for the 5.2/0 and 5.2/Al groups have been expressed relative to the controls (Fig. 6).

After 24 h of Al acclimation, the 5.2/Al group responded identically to challenge as controls, but from day 10 onward showed progressively greater Al resistance until day 24, where their LT_{50} was about 1.7-fold greater than controls. This was an effect primarily of Al, as the LT_{50}'s of the 5.2/0 group were virtually identical to controls throughout the acclimation period except for slightly higher values at days 10 and 12.

Not surprisingly, the Al challenge had a severe impact on physiological state as assessed from animals alive at 24 h. The degree of disturbance evident at 24 h was directly correlated with LT_{50}. This was evident in all three acclimation groups, as illustrated by the relationship between LT_{50} and Na^+ losses at 24 h (Fig. 7). This relationship emphasizes that electrolyte losses were the main cause of death by Al challenge, not only for the 6.5/0 group but for the 5.2/0 and the 5.2/Al groups as well.

A detailed view of the physiological effects of challenge is shown in Fig. 2 (right-hand bars); this is the challenge experiment started on day 24 of the acclimation ($LT_{50} = 23 \pm 1.5$ h for 6.5/0 fish). By 24 h of this challenge, fish from the 6.5/0 group had lost about 50% of their body electrolytes (Fig. 2A and 2B), leading to a pronounced hemoconcentration (doubling of both hematocrit and plasma protein; Fig. 2C and 2D) impaired O_2 delivery (50% increase in body lactate; Fig. 2E). These disturbances were accompanied by a substantial accumulation of gill Al (Fig. 2F). In this challenge, gill Al was 317 ± 34 μg/g wet gill tissue ($N = 4$), but this was not a typical value for the 6.5/0 group. The typical value was, in fact, much higher, averaging 798 ± 62 μg/g overall ($N = 34$). The accumulation of Al on the gills was similarly correlated with a significant depression in sialic acid content, as noted earlier during Al acclimation, but the depression was more substantial, to about 36%, on average, of control levels (0.98 ± 0.13 ($N = 34$) versus 2.73 ± 0.15 μmol/g ($N = 65$)).

The increased Al tolerance of the 5.2/Al fish by 24 d of chronic Al exposure was evident in significantly lower electrolyte losses resulting from the Al challenge compared with controls and correspondingly smaller hematological and lactate disturbances (Fig. 2A–2E). These fish, nonetheless, accumulated a virtually identical amount of Al on their gills (Fig. 2F) and there was a similar depression in gill sialic content.

Admittedly, the less severe loss of electrolytes in the 5.2/Al fish could be at least partly attributed to the fact that they started off the challenge with less to lose (Fig. 2A and 2B). Similarly, the 5.2/0 group started with less Na^+ and lost less relative to controls, yet showed no greater Al resistance after 24 d of exposure. However, it should be emphasized that in all three groups the physiological measurements were made on survivors taken at 24 h into the challenge experiment. About 50% of the animals in the 6.5/0 and 5.2/0 groups had already died prior to this sampling, while none had died in the 5.2/Al group. Consequently, the 6.5/0 and 5.2/0 groups would have been heavily selected for resistant individuals, while no such selection had occurred for the 5.2/Al fish. Thus, the effects of the Al challenge on the 6.5/0 and 5.2/0 population as a whole were likely much more severe than those indicated by the measurements in Fig. 2 whereas the 5.2/Al measurements were more representative.

Final Al Challenge

Although chronic sublethal Al exposure increased resistance to lethal Al over the short term, the question that remained was whether the 5.2/Al fish had acquired any long-term tolerance of Al. This was assessed in the final challenge experiment which began on day 28 by exposing fish from the 5.2/Al and 6.5/0 groups to various pH/Al combinations for a period of 14 d.

Mortality was dramatically less in the group preexposed to Al (Fig. 8A). Indeed, only 2 of 90 5.2/Al fish died over the 2-wk period compared with 34 of 90 6.5/0 fish. Mortality was correlated with Al in the latter and Al was more toxic at pH 5.2 than at pH 4.8. The biggest differences between the two groups were seen at the highest Al (300 μg/L) which caused only 2 mortalities out of 30 in the 5.2/Al group versus 28 out of 30 in the 6.5/0 group.

Correspondingly, there was a much greater physiological impact of Al on the 6.5/0 group. Net Na^+ losses (Fig. 8B) were directly correlated with mortality, and the increases in hematocrit were, in turn, correlated with the depression in body Na^+ (Fig. 8C). Accumulation of Al on the gills was generally greater at pH 5.2 than at pH 4.8 in relation to the greater toxicity of the former (Fig. 8D).

The 5.2/Al group, in contrast, actually showed some recovery in body Na^+ and hematocrit after 2 wk at pH 5.2 and 0 Al. This recovery was not complete, but rather was to levels identical to those of the 6.5/0 group exposed to 5.2/0. Nonetheless, their responses to elevated Al and lowered pH were considerably attenuated compared with the 6.5/0 group. The attenuation was most apparent at 4.8/300 where the loss in Na^+, the increase in hematocrit, and the increase in gill Al were less than half those seen in the 6.5/0 group. Again, it should be noted that the actual differences were probably greater, given the sampling bias discussed above. In this exposure, mortalities for the 5.2/Al and 6.5/0 groups were 7 and 87%, respectively; the measurements for the 6.5/0 group were based on two hardy survivors only.

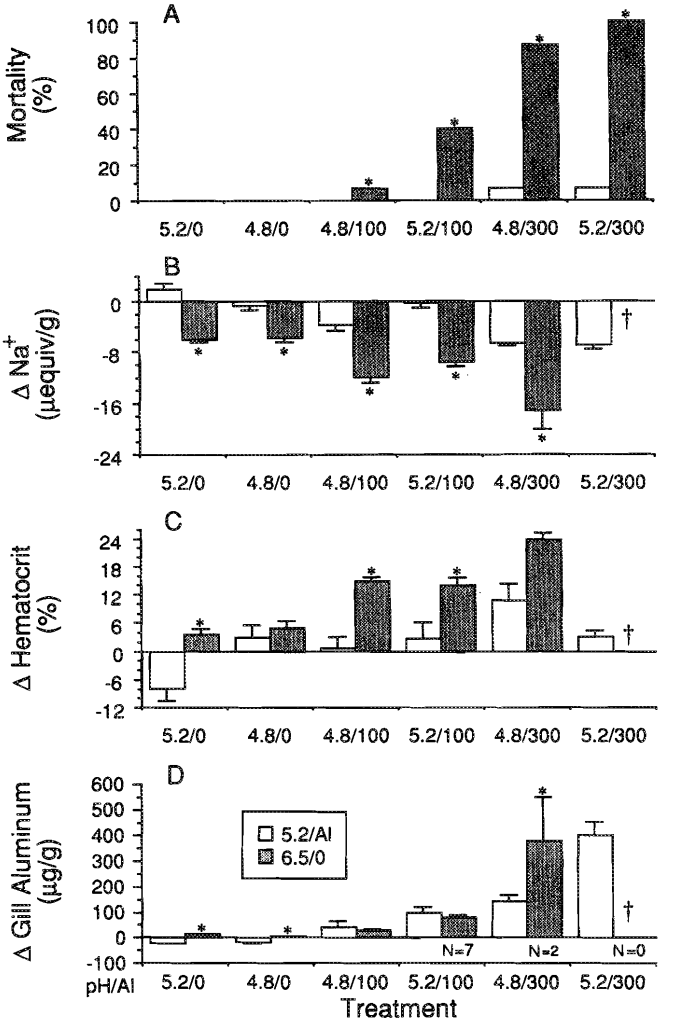

FIG. 8. (A) Percent mortality, (B) body Na^+, (C) hematocrit, and (D) gill Al in brook trout exposed 2 wk to low pH/Al (nominal values indicated). This is the final challenge experiment started on day 28 with 15 fish in each chronic exposure group. Open bars are 5.2/Al fish; shaded bars are 6.5/0 fish. Bars in Fig. 8B–8D indicate mean difference from the value in the same group prior to challenge (± 1 SE, $N = 10$ except where noted). Asterisks indicate significantly different ($p < 0.05$) means from 5.2/Al; Crosses indicates no survivors remaining after the 2-wk exposure.

Discussion

This study confirms and extends previous studies (Wood et al. 1988a, 1988b; McDonald and Milligan 1988) in finding that brook trout acclimation to Al. After only 10 d of exposure to largely sublethal Al, juveniles showed significantly increased resistance to lethal Al consisting of an increased LT_{50} and reduced physiological responses. The nature of the responses suggests that the acclimation most likely arose from changes at the gills. Questions that are thus important to answer concern the specific mechanism of action of Al at the gills and the precise nature of the adaptive changes that are brought about by sublethal Al exposure.

Mechanism of Toxic Action of Al

In previous brook trout studies (Booth et al. 1988), we established that in the initial (i.e. "shock") phase of Al exposure,

net ion losses arose primarily from large increases in diffusive ion fluxes across the gills, attributed to Al-induced increases in gill ionic permeability. Later on (in the "recovery" phase), as diffusive ion losses declined, the persistent inhibition of Na^+ and Cl^- uptake made a progressively greater contribution to the continuation of net ion losses or, in the present context, to the failure to completely recover preexposure electrolyte levels (Fig. 2A and 2B).

Clearly, the initial shock phase was very important in the present study, for it was during the first 24–48 h of the exposure to 150 μg Al/L that most of the physiological disruption (Fig. 2 and 3) and morphological damage (Mueller et al. 1991) took place. Earlier, Wood and McDonald (1987) and Booth et al. (1988) proposed that this initial phase was characterized by Al binding and/or precipitation at gill surfaces, specifically to anionic residues in mucous and outerfacing membranes. This action presumably displaced bound Ca^{2+}, weakened tight junctions, increased ionic permeability, and deformed and lifted the lamellar epithelium, causing increased exposure of binding sites, further gill Al accumulation, and further gill damage. In the present study, as shown previously (Booth et al. 1988), the gill Al accumulation was dramatic; 251 μg/g by 24 h of Al exposure (Fig. 2F) which represents a 1700-fold concentration over Al in the water (0.15 μg/mL). Correlated with the accumulation of Al was a 35% decline in gill sialic acid content. This is most readily interpreted as indicating a decline in gill mucous content, for in Salmonidae, sialic acid is the major cationic component of epithelial mucous (Harris et al. 1973) and its concentration is directly proportional to mucous cell density (Pickering 1974).

A decline in mucous content is unexpected given that hypertrophy and hyperplasia of gill mucous cells is often a correlate of Al exposure (e.g. Muniz and Leivestad 1980; McCahon et al. 1987) and, indeed, is a common response to gill irritants in general (Mallatt 1985). Nonetheless, if Al stimulated mucous secretion and sloughing to the extent that it exceeded mucous production, then sialic acid content would decline even in the face of mucous cell proliferation. Another possibility is that sialic acid content also declined out of proportion to mucous content. Zuchelkowski et al. (1985) reported increased synthesis of neutral sulfomucins (which do not contain sialic acid) in response to acid stress in catfish (*Ictalurus nebulosus*).

Whatever the changes in mucus, the gills did not continue to accumulate Al, nor did they remain leaky. By day 4, net ion losses appeared to have ceased and thereafter must have been reversed, as some recovery of Na^+ and Cl^- balance was evident (Fig. 2A and 2B). This recovery was accompanied by the removal of Al from the gills (Fig. 2F), restoration of sialic acid content, and almost complete recovery of branchial Na^+ transport (Fig. 5) and removal of the respiratory effect, as evidenced by the recovery of whole-body lactate levels (Fig. 2E). This was not simply restoration of the preexposure state, however, as development of resistance to Al was coincident with this recovery.

Mechanism of Acclimation to Al

So the question is "What specific changes have occurred in these gills so that they now better resist the toxic effects of Al?" The removal of Al from the gills during continued sublethal exposure (Fig. 2F and 3C) is perhaps the most important observation of the present study and may provide at least a partial answer to this question. The gills became less attractive to Al. Reid et al. (1991) have made a qualitatively similar observation on rainbow trout (*Oncorhynchus mykiss*) exposed to sublethal Al (27 μg/L) at the same pH (5.2). These findings argue for either an increased turnover rate of mucus at the gills, reducing the amount of Al held on the surface at any one time, or a decrease in the actual Al-binding affinity of the gill surface. Indeed, both mechanisms are likely. In rainbow trout, the extraction of Al from the water passing over the gills far exceeds the net accumulation of Al on the gills under these conditions, suggesting that the majority is sloughed off with mucus (Playle and Wood 1989). The companion histological examination (Mueller et al. 1991) of gills from brook trout in the present study demonstrates that gross hypertrophy and proliferation of branchial mucous cells coincided with the development of acclimation. Furthermore, Al-acclimated brook trout showed negligible hyperventilation during challenge, in contrast with naive fish, so less Al would be brought into contact with the gills (Walker et al. 1991). The in vitro studies of Reid et al. (1991) showed that quantitative and qualititative changes occurred in the cation-binding activity of the gills of rainbow trout during acclimation to Al. Specifically, these consisted of an increased binding affinity for Ca^{2+}, a decreased binding affinity for Al, and a decline in the potency of Al at displacing Ca^{2+}. These changes effectively reduced the surface activity of Al and led to about a 50% reduction of gill Al accumulation during Al challenge.

Unfortunately, the evidence that Al-acclimated brook trout actually accumulated less Al on the gills during challenge is, at best, marginal (Fig. 2F and 8D), in contrast with the clear results of Reid et al. (1991) on rainbow trout. While we cannot eliminate a true difference in mechanism between the two species, it seems more probable that the sampling bias during challenge, discussed earlier, prevented clear demonstration of the expected lower gill accumulation in acclimated brook trout. The actual propensity of the gills for Al is significantly lower in brook trout than in rainbow trout. For example, when both species were exposed to the same condition (48 h at pH 4.8, 111 μg Al/L), gill Al levels in the brook trout were only half those in the rainbow trout (D. G. McDonald and C. M. Wood, unpubl. results). This lower Al-binding activity in the brook trout may, in fact, underlie the intrinsically greater Al tolerance of this species.

In the Al-acclimated brook trout, the gills accumulated apparently similar amounts of Al as in unacclimated fish, but Al was clearly less reactive. Unfortunately, our physiological observations do not reveal the specific nature of these adaptive changes at the gills. They suggest repair of the initial damage rather than adaptation. Furthermore, the repair was not even complete over the period when acclimation was clearly established (days 10–24; Fig. 6), as evidenced by only partial correction and continued disturbance of Na^+ transport (Fig. 5), electrolyte levels, lactate levels, hematology (Fig. 2), and gill morphology (Mueller et al. 1991). Nonetheless, whatever the changes in the gills, the final challenge (Fig. 8) suggests that they convey relatively long-term tolerance of elevated Al. Consequently, in a field situation, fish chronically exposed to sublethal levels of Al might be able to survive otherwise lethal levels of Al during an episodic surge lasting perhaps as long as 2 wk.

Thus, we conclude that the adaptive response of the gills involves subtle but profound changes in the surface chemistry of the gills which may involve changes in rates of synthesis and/or turnover of membrane proteins, other proteins or lipids,

and/or de novo synthesis of new versions of these ligands with altered properties. Finally, we are left to ponder the metabolic costs and compromises associated with such a process, whether the adaptive changes convey resistance to any other toxicants, and how long the adaptive changes persist when Al is removed from the water. Questions such as these are, of course, not only applicable to Al but generally to chronic exposure of fish to sublethal toxicants.

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