Physiological Evidence of Acclimation to Acid/Aluminum Stress in Adult Brook Trout (*Salvelinus fontinalis*). 2. Blood Parameters by Cannulation

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Brook trout (*Salvelinus fontinalis*) exposed for 10 wk to sublethal acid (pH = 5.2) plus Al (150 μg/L) in flowing soft water (Ca^{2+} = 25 μequiv/L) did not exhibit chronic respiratory disturbance or elevated stress indices, as revealed by sampling of arterial blood gases, acid–base status, glucose, and cortisol via an indwelling catheter. Acclimation occurred, which prevented mortality and greatly attenuated the disturbances of respiratory, acid–base, and stress parameters normally seen upon challenge with more severe acid (pH = 4.8) plus Al conditions (333 μg/L) for 3 d. Ionoregulatory, fluid volume, and hematological disturbances were similarly reduced. Higher water Ca^{2+} (400 μequiv/L) slightly delayed but did not prevent this suite of toxic responses in naive fish. These disturbances did not occur in naive fish challenged with acid alone (pH = 4.8). However, long-term adaptation to acid alone (pH = 5.2) resulted in elevated glucose and cortisol levels and offered no protection against the more severe acid plus Al challenge. Thus the acclimation was to Al rather than to acidity itself, and low levels of Al may be beneficial to fish under chronic acid stress.

Des ombles de fontaine (*Salvelinus fontinalis*) exposés pendant 10 sem à des concentrations sublétales d'acide (pH = 5,2) et à de l'Al (150 μg/L) en eau douce à débit continu (Ca^{+2} = 25 μequiv/L) n'ont pas montré de perturbations respiratoires chroniques ou un stress élevé après échantillonnage à l'aide d'une sonde à demeure des gaz sanguins artériels, de l'équilibre acidobasique, du glucose et du cortisol. On a observé une accoutumance qui a éliminé la mortalité et a grandement atténué les perturbations des paramètres respiratoires, de l'équilibre acidobasique et du stress, normalement observées après provocation dans un milieu à teneurs en acide (pH = 4,8) et en Al (333 μg/L) plus élevées pendant 3 d. De même, les perturbations ionorégulatoires, hématologiques et volumétriques des fluides ont été réduites. Une teneur aqueuse en Ca^{+2} plus élevée (400 μequiv/L) a légèrement retardé cette séquence de réactions toxiques chez les poissons naïfs mais ne l'a pas éliminée. Ces perturbations n'ont pas été observées chez les poissons naïfs provoqués dans un milieu acide seulement (pH = 4,8). Toutefois, une adaptation à long terme à un milieu acide seulement (pH = 5,2) a entraîné des niveaux élevés de glucose et de cortisol et n'a pas offert de protection contre la provocation plus violente à l'acide ajouté d'Al. Ainsi, l'accoutumance ne concerne que l'Al et non l'acidité même et de faibles teneurs en Al peuvent être bénéfiques pour les poissons faisant l'objet d'un stress à l'acide chronique.

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In the preceding study, we demonstrated that brook trout (*Salvelinus fontinalis*) exposed for 10 wk to a range of sublethal pH, Ca^{2+}, and Al conditions show adaptation as manifested by regulation of plasma ions and hematology (Wood et al. 1988a). However, stress indices such as blood glucose or cortisol (Donaldson 1981; Wedemeyer and McLeay 1981) were not measured, so it was not possible to conclude whether sublethal stress persisted despite adaptation of ionoregulatory function. Respiratory and acid–base parameters were also not assessed. Morphological observations on the gills of trout exposed for extended periods to sublethal acid/Al conditions have shown a general thickening of the lamellar epithelium and associated increase in the water to blood diffusion distance (Chevalier et al. 1985; Tietge et al. 1988; Karlsson-Norrgren et al. 1986a, 1986b). Such changes might well cause a chronic limitation of respiratory gas exchange and associated disturbance of acid–base balance, thereby contributing to sublethal stress. Therefore the first objective of the present study was to determine whether chronic respiratory limitation and/or sublethal stress occurred in these fish after 10 wk of exposure. Cannulation was employed for blood sampling (cf. Wood et al. 1988b), permitting repetitive determination of blood gases, acid–base status, glucose, cortisol, and other parameters with minimal disturbance to the fish.

The preceding study also demonstrated that 10 wk of exposure to sublethal levels of Al and acid together resulted in acc-

limation, as evidenced by greater survival and smaller ionoregulatory disturbances during a more severe acid plus Al challenge (Wood et al. 1988a). However, respiratory disturbance and acidosis are also important components of the lethal response to acid/Al (Rosseland 1980; Neville 1985; Wood et al. 1988b). We anticipated that such disturbances might well be worsened in trout already burdened with thickened lamellar epithelia as a result of long-term sublethal exposure (Tietge et al. 1988). Therefore the second objective of the present study was to employ chronic cannulation to assess whether respiratory, acid–base, and stress responses to a more severe challenge were exacerbated as a result of long-term sublethal exposure, or rather were attenuated by acclimation. By permitting repetitive blood sampling, these experiments allowed a more detailed examination of the time course of blood responses to challenge in the various exposure groups. They therefore clarified the role of water Ca^{2+} and answered the question whether prior exposure to sublethal acidity alone also provided acclimatory resistance to combined acid/Al challenge, for the results of the preceding study (Wood et al. 1988a) were equivocal on this point.

Materials and Methods

Experimental Animals, Exposures, and Challenge Conditions

Adult brook trout were exposed for 10 wk to different combinations of pH, Ca^{2+}, and Al in flowing artificial soft water at the Fish Physiology and Toxicology Laboratory, Laramie, Wyoming (altitude = 2200 m). Details on fish origin, holding, and water chemistry have been described in the preceding paper (Wood et al. 1988a). The overall experimental plan was first to examine blood parameters (by arterial cannulation) while the fish remained in their exposure water in order to detect whether chronic respiratory effects and/or sublethal stress occurred after 10 wk of exposure. These same trout were then challenged with a higher Al/lower pH condition (potentially lethal) for 3 d. During this period, blood was repetitively sampled via the catheter to assess the nature and time course of respiratory and other disturbances and the extent of acclimatory resistance offered by the prior exposure conditions.

Fish were exposed for 10 wk to four different combinations of pH, Ca^{2+}, and Al (Table 1). These combinations were chosen to assess the effects of low Ca^{2+} (25 μequiv/L) versus higher Ca^{2+} (400 μequiv/L) within the softwater range, the effects of exposure to low pH (5.2) alone, and the effects of combined exposure to low pH (5.2) plus Al (150 μg/L). Fish from each exposure were then subjected to the more severe combined acid (pH = 4.8) plus Al (333 μg/L) challenge. In addition, fish from the pH = 6.5, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L exposure were challenged with low pH (4.8) alone to discern the effects of acidity by itself.

Arterial catheters were implanted via the caudal artery as described previously (Wood et al. 1988b). The preexposure water was used for anaesthesia (50 mg MS-222/L, Sigma) on the operating table, and the pH was held at the appropriate level by addition of KOH. After cannulation, the fish were transferred to individual flux boxes served with a continuous flow (~0.5 $L \cdot kg^{-1} \cdot min^{-1}$) of water *identical in composition to that of their exposure condition* (cf. Wood et al. 1988a). The P_{O_2} of the inflowing water was maintained above 115 torr, P_{CO_2} below 1 Torr and temperature at 11 ± 1°C.

After 48 h of recovery, a blood sample was taken from each fish while it remained in its exposure water to determine chronic effects on blood parameters. The measurements from this sample also served as the *control* values for the subsequent challenges. The water was then changed to the appropriate challenge condition (Table 1) and samples taken at 4, 18, 28, 42, and 66 h, if death did not occur earlier. Observations for mortalities were continued until 72 h.

All blood samples (700 μL) were withdrawn anaerobically from the arterial catheters into gas-tight, ice-cold Hamilton syringes and immediately replaced by reinfusion of an equal volume of nonheparinized Cortland saline (Wolf 1963). Samples were analyzed for arterial O_2 tension (Pa_{O_2}), pH (pHa), red blood cell intracellular pH (RBC pHi), total O_2 in whole blood (Ca_{O_2}), total CO_2 in whole blood (Ca_{CO_2}) and true plasma, hematocrit (Ht), hemoglobin (Hb), lactate, and glucose, and plasma levels of Na^+, Cl^-, K^+, Ca^{2+}, total protein, and cortisol.

Analytical Techniques and Calculations

Analytical techniques were identical to those described by Wood et al. (1988b), but with the following additional measurements. Plasma cortisol was determined on 20-μL samples using a Corning ^{125}I radioimmunoassay kit. RBC pHi was measured by the freeze-thaw red cell lysate method of Zeidler and Kim (1977), as validated for trout cells by Milligan and Wood (1985). The same Radiometer pH microelectrode (type E5021) was employed for the lysate as for the whole-blood pHa measurement, and the red cell pellet was obtained from the sample centrifuged at 9000 × *g* for 2 min to separate plasma for ions and cortisol. Ca_{O_2} was determined by means of a Lex-O_2-Con analyzer (Lexington Instruments), using a sample volume of

TABLE 1. Intended exposure and challenge conditions for each experimental group, and mortalities and median survival times during each 72-h challenge.

Exposure			Challenge				
pH	Ca^{2+} (μequiv/L)	Al (μg/L)	pH	Ca^{2+} (μequiv/L)	Al (μg/L)	Mortality (during 72-h challenge)	LT50 (h) (with 95% CL)
6.5	25	0	4.8	25	0	0/6	>72
6.5	25	0	4.8	25	333	6/6	34.1 (19.9 – 52.0)
6.5	400	0	4.8	400	333	5/6	53.0 (38.1 – 73.7)
5.2	25	0	4.8	25	333	6/7	31.6 (21.2 – 54.5)
5.2	25	150	4.8	25	333	0/6	>72

50 μL and the recalibration procedure described by Wood et al. (1979).

The following parameters were calculated as described by Wood et al. (1988b): Pa_{CO_2}, plasma bicarbonate concentration (HCO_3^-), mean cell Hb concentration (MCHC), and the concentration of metabolic H^+ added to the whole blood (ΔH^+m). As Ca_{O_2} was measured directly in the present study, Hb-bound O_2 per unit Hb ($[O_2]/[Hb]$ was calculated as

$$(1)\quad [O_2]/[Hb] = \frac{Ca_{O_2} - (Pa_{O_2} \cdot \alpha O_2)}{[Hb]}$$

where αO_2 represents the tabulated value (Boutilier et al. 1984) for O_2 solubility in trout plasma at the experimental temperature. The inverse of the ratio of the final measured plasma protein concentration to the initial value in each fish was employed as an index of the change in plasma volume, as described by McDonald et al. (1980).

Treatment of Data

Median survival times (LT50) and their 95% confidence limits were calculated by standard log/probit analysis and nomographic methods (Litchfield 1949; Litchfield and Wilcoxin 1949). Other data have been generally expressed as means ± 1 SE (N) for each group. Comparisons amongst values taken when the fish were still under their four different exposure conditions, or amongst final values after challenge, were performed by one-way analysis of variance, followed by Duncan's (1955) multiple range test when the F value indicated significance.

As discussed previously (Wood et al. 1988b), it is difficult to accurately portray mean responses for physiological data when members of the group die at different times as a consequence of the experimental treatment. This was a problem for three of the challenge treatments in the present study where mortality was 6/6, 5/6, and 6/7, but not for two others where survival was 100% over 72 h. Therefore, the approach adopted was to show a few key parameters as plots of individual data in groups exhibiting mortality and as means ± 1 SEM in groups exhibiting 100% survival. For all other parameters, data were summarized as means ± 1 SEM for final values, where the final value represented either the 66-h measurement or the last measurement prior to death. In the three groups exhibiting mortality, even one of the two survivors appeared close to death at 72 h as evidenced by laboured breathing, poor colouration, and disturbed physiological parameters. Thus for these groups, the final means were representative of the blood status shortly prior to death and therefore comparable with the "terminal" data of Wood et al. (1988b). In all groups, there was a paired design, so Student's two-tailed paired t-test ($p \leq 0.05$) was used for comparisons between the control values taken under the exposure condition and the intervening or final values taken under the challenge condition.

Results

Chronic Respiratory and Stress Effects of pH/Ca^{2+}/Al Exposures

When fish were examined in the conditions to which they had been exposed for 10 wk, there were no significant differences in Pa_{O_2}, $[O_2]/[Hb]$, or Pa_{CO_2}, suggesting that neither sublethal acid alone nor acid plus Al caused chronic respiratory problems, at least under resting conditions (Table 2). Indeed, blood lactate was significantly lower in fish exposed to sublethal acid plus Al than in those held at pH = 6.5, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L. There were significant variations in blood acid–base status, but these resulted from

TABLE 2. Respiratory, acid–base, and stress parameters in the arterial blood of brook trout after 10 wk of exposure to various pH/Ca^{2+}/Al conditions. Means ± 1 SEM.

	Exposure conditions			
	pH = 6.5 Ca^{2+} = 25 μequiv/L Al = 0 μg/L N = 12	pH = 6.5 Ca^{2+} = 400 μequiv/L Al = 0 μg/L N = 6	pH = 5.2 Ca^{2+} = 25 μequiv/L Al = 0 μg/L N = 7	pH = 5.2 Ca^{2+} = 25 μequiv/L Al = 150 μg/L N = 6
Pa_{O_2} (Torr)	65.6 ± 3.0	76.4 ± 3.9	62.8 ± 3.8	68.7 ± 6.0
$[O_2]/[Hb]$ (μmol/g)	58.2 ± 1.2	56.7 ± 2.0	63.0 ± 1.3	61.0 ± 1.1
Pa_{CO_2} (Torr)	2.28 ± 0.12	2.63 ± 0.19	1.99 ± 0.24	2.83 ± 0.24
$[HCO_3^-]$ (mequiv/L)	9.74 ± 0.88	6.84 ± 0.56[a]	8.34 ± 1.11	7.82 ± 0.65
pHa	7.957 ± 0.036	7.769 ± 0.025[a]	7.914 ± 0.084	7.795 ± 0.048
RBC pHi	7.405 ± 0.025	7.375 ± 0.018	7.437 ± 0.038	7.367 ± 0.022
MCHC (g/mL)	0.294 ± 0.006	0.324 ± 0.013	0.279 ± 0.009	0.291 ± 0.021
Lactate (mequiv/L)	1.07 ± 0.10	0.78 ± 0.06	0.78 ± 0.13	0.59 ± 0.15[a]
Glucose (mmol/L)	3.98 ± 0.36	4.47 ± 0.35	5.83 ± 0.67[a]	5.07 ± 0.44
Cortisol (ng/mL)	156.3 ± 19.7	162.3 ± 34.9	312.5 ± 44.7[a]	148.8 ± 23.4[b]

[a]Significantly different ($p < 0.05$) from comparable value at pH = 6.5, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L.
[b]Significantly different ($p < 0.05$) from comparable value at pH = 5.2, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L.

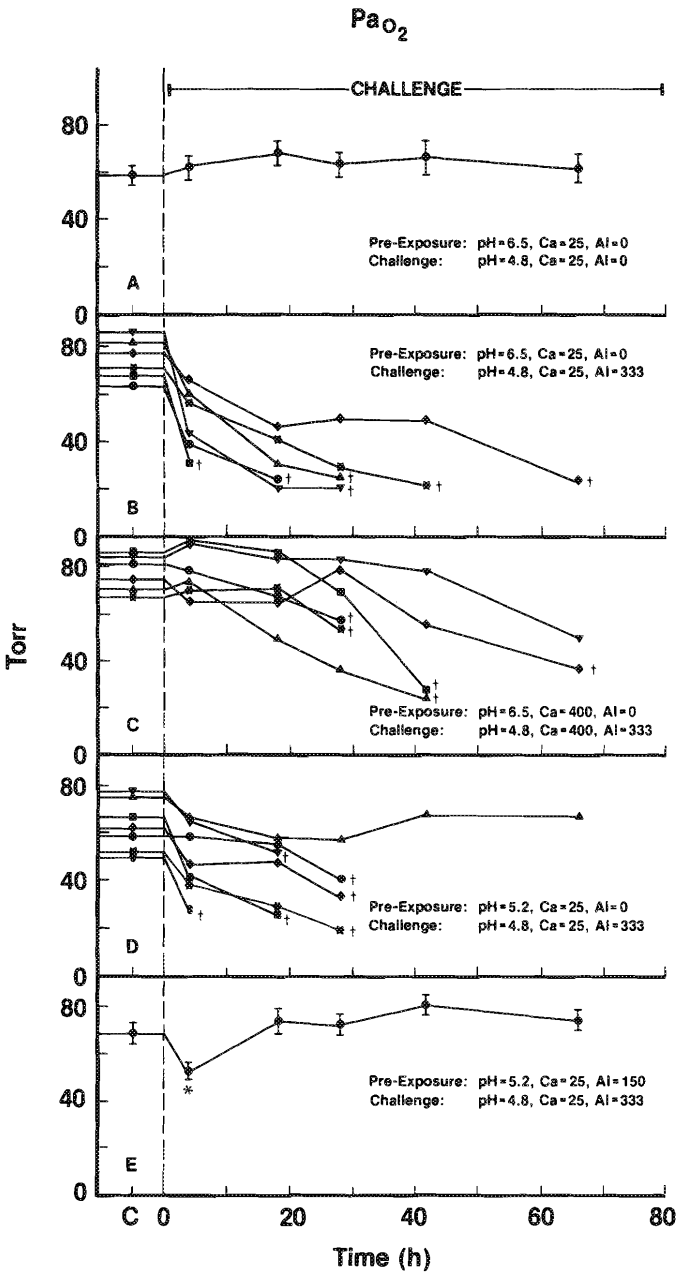

FIG. 1. Changes in Pa_{O_2} in brook trout challenged for 3 d with (A) acid alone (pH = 4.8) or (B–E) acid plus Al (pH = 4.8, Al = 333 μg/L) in flowing artificial soft water. The fish had been previously exposed to various pH/Ca^{2+}/Al conditions for 10 wk (Ca^{2+} = μequiv/L; Al = μg/L). Data are means ± 1 SEM (N = 6) in groups with 100% survival until 72 h and are for individual fish (N = 6–7) in groups with 83–100% mortality until 72 h († signifies death). A different symbol is used for each fish. Asterisks indicate means significantly different ($p < 0.05$) from the control mean taken from these same fish in their exposure water prior to challenge.

differences in water Ca^{2+} levels rather than from sublethal acid or Al exposure (Table 2). Thus, plasma HCO_3^- and pHa were significantly lower at the higher water Ca^{2+} level. RBC pHi and MCHC remained constant in the face of this variation (Table 2). Hb, plasma protein, and ions (Na^+, Cl^-, Ca^{2+}, and K^+) were also unaffected by exposure to acid alone or acid plus Al (data not shown). However, Na^+, Cl^-, and Ca^{2+} levels were all significantly elevated at the higher water Ca^{2+} concentration.

Despite the absence of respiratory and ionoregulatory effects, significant elevations in both blood glucose and cortisol indicated a chronic stress response in the fish exposed to pH = 5.2, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L (Table 2). Interestingly, this was not seen at the same pH in the presence of 150 μg Al/L, suggesting that under these circumstances, Al may have exerted a protective action.

Effects of Exposure Conditions on Mortality during Challenge

The survival data alone clearly indicated the toxicity of the challenges to the various groups (Table 1). Fish that were naive to both acid and Al did not die when challenged with acid alone (pH = 4.8 at Ca^{2+} = 25 μequiv/L) for 72 h, but fish from the same exposure all died when challenged with acid *plus* Al (333 μg/L). Exposure and acid/Al challenge at the higher Ca^{2+} = 400 μequiv/L did not appreciably affect mortality and only slightly extended LT50. The one survivor at 72 h appeared to be close to death. Prior exposure to sublethal acid alone also had no ameliorative effect on mortality or LT50 during the acid/Al challenge, although the sole survivor at 72 h appeared to be reasonably healthy. However, prior exposure to sublethal acid plus Al (pH = 5.2, Ca^{2+} = 25 μequiv/L, Al = 150 μg/L) had a dramatic protective effect, for there was no mortality during the 72-h challenge with pH = 4.8, Ca^{2+} = 25 μequiv/L, Al = 333 μg/L (Table 1).

Relative to noncannulated Wyoming fish subjected to identical exposure regimes and 48-h challenges, these mortality rates were comparable or slightly greater (Wood et al. 1988a). Relative to cannulated Ontario fish tested in comparable challenges (Wood et al. 1988b), these rates were essentially identical.

Effects of Exposure Conditions on Respiratory and Acid–Base Responses to Challenge

The Pa_{O_2} data were representative of respiratory and acid–base responses in general and therefore have been shown in detail in Fig. 1. In fish naive to both acid and Al at Ca^{2+} = 25 μequiv/L, challenge with acid alone (pH = 4.8) had no effect on Pa_{O_2} (Fig. 1A), but challenge with acid plus Al (pH = 4.8, Al = 333 μg/L) caused a rapid and dramatic fall in Pa_{O_2} to values typical of venous blood prior to death (Fig. 1B). Prior exposure and challenge at the higher Ca^{2+} level only slightly delayed this effect (Fig. 1C), while prior exposure to acid alone (pH = 5.2) offered no protective influence at all (Fig. 1D). In contrast, fish previously exposed to acid plus Al showed a transitory fall in PaO_2 after 4 h, followed by a complete return to resting values for the remainder of the challenge period (Fig. 1E).

Very similar trends were seen for other respiratory parameters (Table 3). Thus, final values of $[O_2]$/[Hb] were significantly depressed in all groups challenged with acid plus Al, except for the group previously exposed to pH = 5.2, Al = 150 μg/L. Pa_{CO_2} increased in mirror image to the decreases in Pa_{O_2} during challenge, again with the exception of the trout previously exposed to acid plus Al. Here, Pa_{CO_2} actually decreased below resting levels by the final sample of the challenge period. Challenge of naive fish with acid alone exerted no significant respiratory or acid–base effects (data not shown).

If decreases in arterial blood oxygenation are severe enough to impair O_2 delivery to the tissues, then the production of lactic acid by anaerobic metabolism may occur. Blood lactate meas-

TABLE 3. Final challenge parameters in the arterial blood of brook trout exposed to various pH/Ca^{2+}/Al conditions for 10 wk. The fish were challenged with pH = 4.8, Al = 333 μg/L at the exposure Ca^{2+} level; the tabulated final values represent either the 66-h measurements (for survivors) or the last measurement prior to death (for mortalities).

	Exposure conditions			
	pH = 6.5 Ca^{2+} = 25 μequiv/L Al = 0 μg/L *N* = 6	pH = 6.5 Ca^{2+} = 400 μequiv/L Al = 0 μg/L *N* = 6	pH = 5.2 Ca^{2+} = 25 μequiv/L Al = 0 μg/L *N* = 7	pH = 5.2 Ca^{2+} = 25 μequiv/L Al = 150 μg/L *N* = 6
$[O_2]$/[Hb] (μmol/g)	45.0 ± 5.1[a]	41.2 ± 5.6[a]	50.8 ± 4.4[a]	59.3 ± 1.2[b]
Pa_{CO_2} (Torr)	3.87 ± 0.40[a]	2.94 ± 0.36	3.12 ± 0.40[a]	2.34 ± 0.21[a,b]
$[HCO_3^-]$ (mequiv/L)	8.96 ± 1.10	4.74 ± 0.71[a,b]	7.60 ± 0.92	5.98 ± 0.47[a,b]
pHa	7.715 ± 0.008[a]	7.567 ± 0.046[a,b]	7.742 ± 0.045[a]	7.762 ± 0.015[b]
RBC pHi	7.431 ± 0.037	7.352 ± 0.051	7.428 ± 0.041	7.380 ± 0.026
ΔH^+m (mequiv/L)	3.90 ± 0.55[a]	4.12 ± 0.80[a]	2.15 ± 0.60[a]	1.20 ± 0.35[a,b]
MCHC (g/mL)	0.234 ± 0.017[a]	0.221 ± 0.018[a]	0.231 ± 0.007[a]	0.271 ± 0.018[b]
Relative plasma volume (%)	74.4 ± 2.2[a]	70.3 ± 2.0[a]	76.1 ± 1.9[a]	92.2 ± 5.6[b]
Cortisol (ng/mL)	322.0 ± 51.3[a]	265.9 ± 47.8[a]	292.2 ± 73.4	215.2 ± 39.1[a]

[a]Significant change ($p < 0.05$) with respect to the control measurements taken from these same fish in the exposure water prior to challenge, as tabulated in Table 2. By definition, the control value for ΔH^+m is 0 and for the relative plasma value is 100%.
[b]Significantly different ($p < 0.05$) from comparable value of fish exposed to pH = 6.5, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L.

urements clearly indicated that this was the case (Fig. 2). Lactate did not change when naive fish were challenged with acid only (Fig. 2A), but increased in concert with falling Pa_{O_2} during combined acid/Al challenge (Fig. 2B; cf. Fig. 1). This lactate mobilization was not appreciably altered by either higher water Ca^{2+} levels (Fig. 2C) or prior exposure to low pH alone (Fig. 2D), but was prevented in the fish previously exposed to both stressors (Fig. 2E), confirming the protective action of this treatment.

Acid plus Al challenge induced a significant blood acidosis in fish naive to both stressors at Ca^{2+} = 25 μequiv/L; pHa fell by ~0.25 unit (Table 3). In terms of standard acid–base terminology (Davenport 1974), this acidosis was of compound "respiratory" and "metabolic" origin, for Pa_{CO_2} increased (respiratory component) and there was a significant accumulation of metabolic H^+ (i.e. positive ΔH^+m). These two influences would be expected to exert opposing effects on plasma HCO_3^-, which did not change. Prior exposure and challenge at the higher Ca^{2+} level slightly delayed but did not prevent these effects, although here the metabolic component was dominant, resulting in a significant fall in HCO_3^- (Table 3). The final pHa in these fish was also substantially lower than in other groups (Table 3), but this reflected the fact that the starting pHa prior to challenge was also lower (Table 2); the actual fall in pHa was comparable. Prior exposure to acid alone did not ameliorate either the respiratory or metabolic components of acidosis during combined acid/Al challenge (Table 3). In contrast, the fall in pHa during challenge was entirely prevented by prior exposure to sublethal acid plus Al. In these fish, a significant respiratory alkalosis (fall in Pa_{CO_2}) counteracted a small metabolic acidosis (ΔH^+m), resulting in unchanged pHa (Table 3). In general, measured increases in blood lactate (Fig. 2) were large enough to account for the metabolic component (ΔH^+m) in all but the last group.

In contrast with extracellular pH (pHa), intracellular pH (pHi) in the RBC's was extremely well regulated during acid/Al challenge (Table 3). There were no significant variations at any time in RBC pHi, either within or between groups, despite substantial decreases in pHa and increases in Pa_{CO_2} under some conditions.

In summary, these data provide physiological evidence that prior exposure to sublethal acid plus Al confers upon respiratory and acid–base functions almost complete acclimatory resistance to a more severe acid plus Al challenge for 72 h. The blockade of respiratory gas diffusion across the gills and accompanying compound acidosis seen in naive fish are clearly prevented. However, prior exposure to sublethal acid alone provides no such resistance. Higher water Ca^{2+} levels slightly delay but do not prevent these deleterious respiratory and acid–base disturbances.

Effects of Exposure Conditions on Other Physiological Responses to Challenge

In general, trends for other physiological parameters were in accord with those for respiratory and acid–base measurements. Thus, blood glucose, a sensitive stress indicator (Wedemeyer and McLeay 1981), did not change when naive fish were challenged with pH = 4.8 alone (Fig. 2A), but increased markedly in all three groups in which combined acid/Al challenge induced disturbances of gas exchange (Fig. 2B, 2C, 2D). Prior exposure to sublethal acid plus Al prevented the glucose response (Fig. 2E).

Plasma cortisol, another index of stress (Donaldson 1981), proved to be an even more sensitive indicator. Cortisol did not change over 66 h of exposure to pH = 4.8 alone (Fig. 3), but increased within 4 h of combined acid/Al challenge in naive fish at both low and high Ca^{2+} levels. These increases were

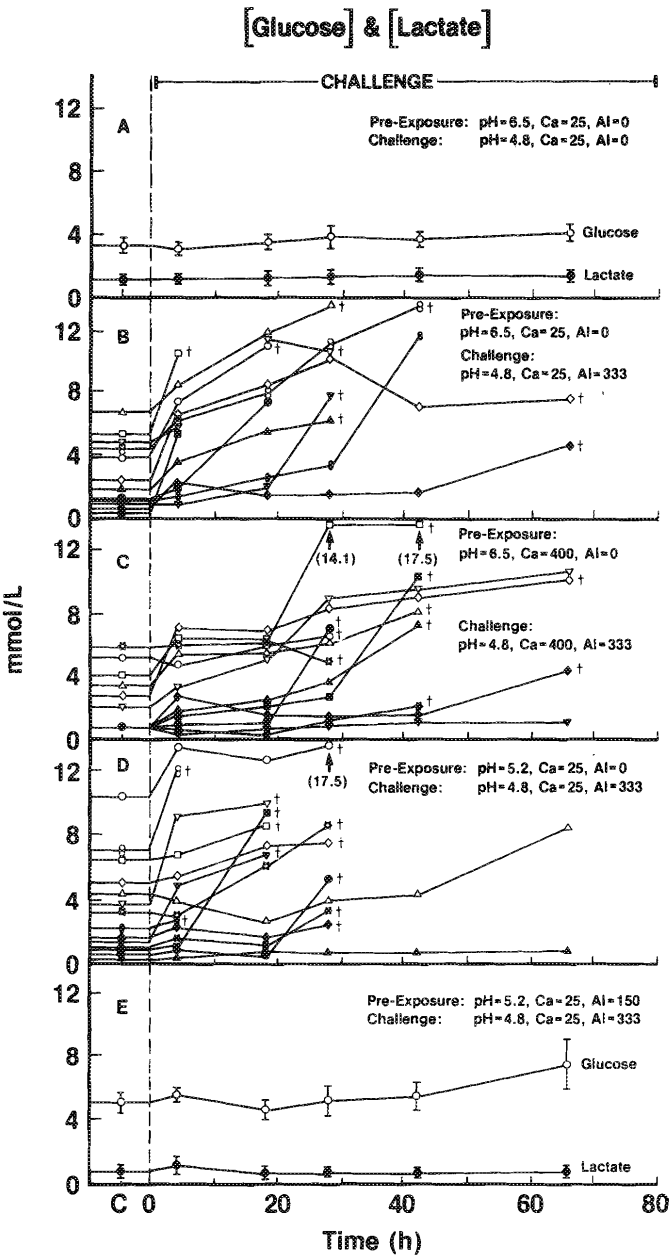

FIG. 2. Changes in glucose (open symbols) and lactate (solid symbols) concentrations of arterial blood in brook trout challenged for 3 d with acid alone (pH = 4.8) or acid plus Al (pH = 4.8, Al = 333 μg/L) in flowing artificial soft water. Other details as in legend to Fig. 1.

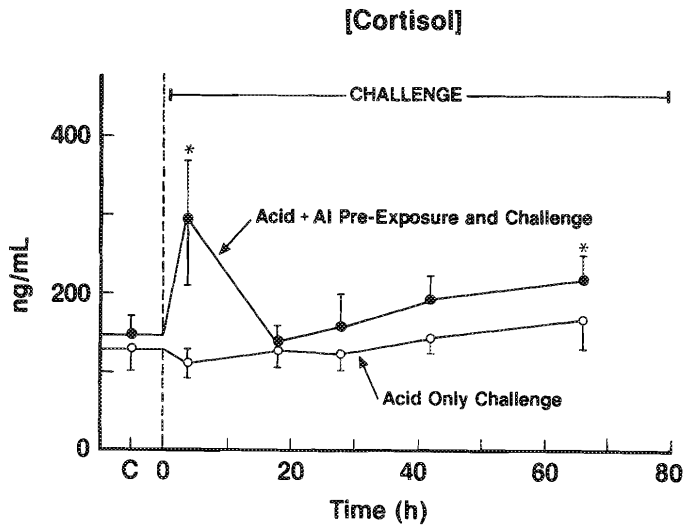

FIG. 3. Comparison of changes in plasma cortisol levels of brook trout under two different exposure/challenge treatments. Open symbols represent a group pre-exposed to pH = 6.5, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L for 10 wk and then challenged with pH = 4.8, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L. Solid symbols represent a group pre-exposed to pH = 5.2, Ca^{2+} = 25 μequiv/L, Al = 150 μg/L for 10 wk and then challenged with pH = 4.8, Ca^{2+} = 25 μequiv/L, Al = 333 μg/L. Means ± 1 SEM (N = 6). Asterisks indicate means significantly different ($p < 0.05$) from the control mean taken from these same fish in their exposure waters prior to challenge.

maintained until the final samples (Table 3). Cortisol did not increase in the group previously exposed to sublethal low pH alone. However, this is not surprising inasmuch as cortisol levels were already elevated by a comparable amount in these fish as a result of the exposure itself, prior to challenge (Tables 2, 3). Interestingly, cortisol did increase initially during challenge in fish previously exposed to both stressors (Fig. 3). This rise was coincident with the transitory fall in Pa_{O_2} at this time (4 h; Fig. 1E) and then disappeared as Pa_{O_2} returned to control levels. However, by the final sample (66 h), cortisol was again elevated in this group (Fig. 3), suggesting that a second stress response was starting, perhaps triggered by ionoregulatory disturbance (see below).

Plasma Na^+ and Cl^- levels fell slightly in naive fish challenged with pH = 4.8 alone, although the changes were significant only at the final sample (Fig. 4A). Challenge with acid plus Al provoked much more rapid decreases in both ions; Cl^- fell to a greater extent than Na^+ (Fig. 4B), probably because of the accompanying rise in lactate (Fig. 2B). While fish at the higher water Ca^{2+} level started with higher Na^+ and Cl^- concentrations, this did not prevent comparable falls during acid/Al challenge (Fig. 4C). Prior exposure to sublethal acid alone was also ineffective in preventing this decline (Fig. 4D). Prior exposure to both acid and Al greatly attenuated the ionoregulatory disturbance, although plasma Na^+ and Cl^- still decreased significantly at the final sample (Fig. 4E). Nevertheless, these changes were no more serious than those in naive fish exposed to acid alone (Fig. 4A).

There were no significant changes in plasma Ca^{2+} as a result of challenge, but K^+ increased by ~50% in fish challenged with acid plus Al (data not shown). Again this response was not seen in the group previously exposed to both acid and Al.

Apparent shifts in fluid volume distribution also occurred during challenge. Plasma volume (as calculated from plasma protein changes; McDonald et al. 1980) did not change significantly in naive fish challenged with acid alone, but decreased by ~25% as a result of combined acid/Al challenge (Table 3). This contraction of plasma volume was not altered by either higher water Ca^{2+} levels or prior exposure to pH = 5.2 alone, but was prevented by prior exposure to pH = 5.2, Al = 150 μg/L. MCHC, usually considered an index of RBC swelling (Milligan and Wood 1982), showed almost identical trends (Table 3). Thus there was an apparent fluid shift into the red cells in association with the decrease in plasma volume, an effect which was prevented by prior exposure to acid plus Al.

In summary, these data corroborate the conclusions based on respiratory and acid–base measurements and indicate that general stress responses, ionoregulatory problems, and fluid volume shifts occur in tandem with gas exchange and acid–base disturbances during acid/Al challenge. Therefore, acclimation

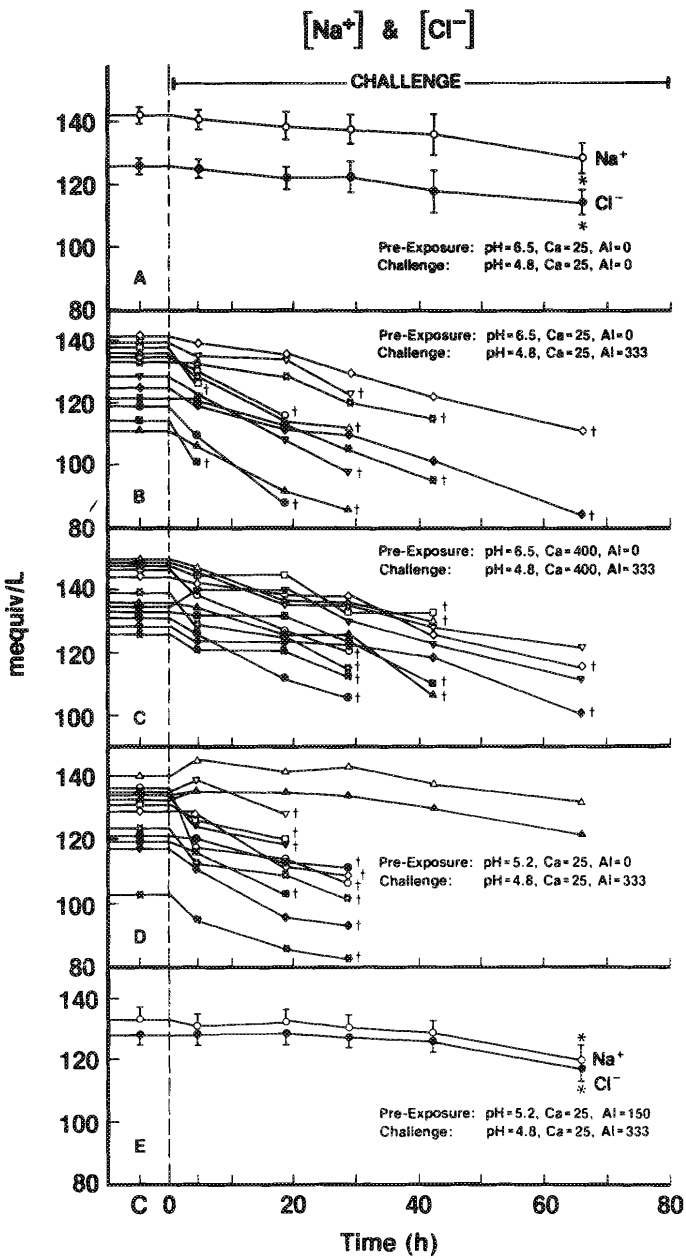

FIG. 4. Changes in the plasma Na^+ (open symbols) and Cl^- (solid symbols) concentrations of arterial blood in brook trout challenged for 3 d with acid alone (pH = 4.8) or acid plus Al (pH = 4.8, Al = 333 μg/L) in flowing artificial soft water. Other details as in legend to Fig. 1.

to sublethal acid plus Al eliminates not just some, but virtually all short-term physiological responses to more severe combined challenge with the two stressors. Prior exposure to sublethal acid alone is ineffective, while higher Ca^{2+} levels slightly delay but do not prevent the suite of toxic responses. Finally, the small disturbances in plasma Na^+, Cl^- (Fig. 4E), and cortisol (Fig. 3) seen at 66 h in the acclimated fish suggest that acclimatory resistance may not last indefinitely.

Discussion

Effects of pH/Ca^{2+}/Al Exposures on Respiratory and Stress Indices

The blood gas, acid–base, and lactate measurements from cannulated fish (Table 2) demonstrated that 10 wk of sublethal acid or acid plus Al exposure had no chronic respiratory effect, at least in resting animals. Thus if there was a thickening of the lamellar epithelium which increased diffusive resistance of the gills (see the Introduction), it was compensated by other factors such as lamellar recruitment or ventilatory adjustments (cf. Wood and Perry 1985). However, it remains possible that the capacity for increasing O_2 and CO_2 exchange during exercise could have been reduced, for this was not tested. Acute ventilatory effects of acid and acid plus Al exposure have been documented (Hargis 1976; Neville 1979, 1985; Rosseland 1980; Walker et al. 1988), but there is little previous information on long-term responses. Giles et al. (1984) found a significantly elevated ventilation rate in rainbow trout (*Salmo gairdneri*) held for 22 d at pH = 4.9 or below, but interpretation was confounded by elevated P_{CO_2} in their test waters (cf. Neville 1979). We are aware of no exercise studies on this topic.

The presence of elevated plasma cortisol and blood glucose after 10 wk of exposure to pH = 5.2, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L suggests that a chronic stress response occurred (Table 2). These findings are in general agreement with those of several studies on rainbow trout exposed to sublethal acidity (pH = 4.7 – 5.7) for 5 – 42 d (Brown et al. 1984; Barton et al. 1985; Brown et al. 1986a, 1986b) and lake whitefish (*Coregonus clupeaformis*) held at pH = 4.1 – 5.0 for 14 d (Scherer et al. 1986). In contrast, Lee et al. (1983) found no change in cortisol in rainbow trout maintained at pH's as low as 4.1 for 14–21 d, but they measured the hormone by a fluorometric technique, rather than by radioimmunoassay.

A unique finding of the present study is that the presence of Al = 150 μg/L at this same pH = 5.2, Ca^{2+} = 25 μequiv/L prevented this stress response (Table 2). This further supports our contention, previously argued on the basis of plasma ion measurements only, that low levels of Al may actually be beneficial during chronic acid exposure (Wood et al. 1988a). Again, we speculate that this could result from either greater chloride cell proliferation (Tietge et al. 1988) leading to more effective recovery or a stabilizing action of Al^{3+} on branchial permeability (Schofield and Trojnar 1980). However, it must also be noted that Al was not protective but rather detrimental to the growth of the present fish under acidic conditions (Mount et al. 1988).

A number of other parameters sampled by cannulation in the present study (plasma ions, protein, and hematology) duplicated those sampled by caudal puncture from a wider range of exposures in Wood et al. (1988a). In general, there was good agreement between the two studies, and the same explanations should apply. However, plasma Ca^{2+} was substantially higher in the presence of higher water Ca^{2+} in the present study, a difference which was not seen in the caudal puncture study. While this effect is not unexpected, it could have been exaggerated had there been a disproportionate number of females in the group, for vitellogenesis was just starting by the time of these experiments (D. R. Mount, unpubl. results). Unfortunately, the animals were not sexed in the present study.

Responses to Challenge in Naive Fish

Qualitatively, the physiological responses of cannulated Wyoming brook trout to challenge with pH = 4.8 alone or pH = 4.8, Al = 333 μg/L resembled those of similarly exposed Ontario fish, and mortality rates were virtually identical (Wood et al. 1988b). This agrees with the results of ion flux studies on noncannulated fish which also showed comparable acid/Al

sensitivities in the two populations (Booth et al. 1988; Wood et al. 1988a). Thus in both groups, the effects of pH = 4.8 alone were negligible. In contrast, the effects of pH = 4.8 plus 333 μg/L Al were traumatic, involving ionic depletion, inhibition of respiratory gas exchange, hemoconcentration, acid–base dysfunction, and death. The causative mechanisms have been discussed in detail by Wood et al. (1988b); in brief, they all relate to the interaction of Al with the branchial epithelium.

Quantitatively, there were two notable differences in the responses of the Wyoming fish. Firstly, the extent of respiratory disturbance upon combined acid/Al challenge at Ca^{2+} = 25 μequiv/L (Fig. 1, 2; Table 3) was much larger and comparable with that seen in Ontario fish at Ca^{2+} = 400 μequiv/L. The most likely explanation is the difference in inspired Po_2 (~120 versus ~150 Torr) resulting from the difference in altitude (2200 versus 100 m), although differences in fish stocks or water quality cannot be excluded. The other discrepancy was in blood glucose, which increased greatly in the Wyoming fish prior to death (Fig. 2), but not in the Ontario fish. Glucose mobilization has not been assessed in previous studies on Al, but has been widely documented in fish acutely exposed to severe acid stress (e.g. McDonald 1983; Lee et al. 1983; Brown et al. 1984, 1986b; Barton et al. 1985; Scherer et al. 1986). The difference is probably due to the fact that the Ontario fish were starved for at least 7 d prior to test, while the Wyoming fish were fed up until the day of cannulation.

Two measurements unique to the present study were RBC pHi and plasma cortisol. RBC pHi was remarkably invariant in the presence of extracellular acidosis during acid plus Al challenge (Tables 2, 3). It is now clear that increased plasma catecholamines during acidotic stress in fish may play a critical role in stabilizing RBC pHi, thereby protecting the intracellular milieu of Hb for O_2 transport (Nikinmaa 1986). We therefore suggest that significant catecholamine mobilization occurred in the present fish. Circulating catecholamine levels have not yet been reported in any acid or acid/Al study on fish, although strong pharmacological evidence exists that they are elevated during severe acid challenge in rainbow trout (Milligan and Wood 1982).

Corticosteroids are the other group of hormones commonly mobilized during stress (Wedemeyer and McLeay 1981; Donaldson 1981). Rapid increases in plasma cortisol levels during acute, severe acid exposure (pH = 4.0 – 4.5) have been documented in brook trout (Mudge et al. 1977; Ashcom 1979) and rainbow trout (Adams et al. 1985), but at higher pH's (>4.7), the response took more than a week to develop in rainbow trout (Brown et al. 1984). This is in accord with the present results, where there was no increase in cortisol over 66 h of acute exposure to pH = 4.8 (Fig. 3) but a doubling after 10 wk of chronic exposure to pH = 5.2 (Table 1). Additionally, the present results show that the presence of Al (333 μg/L) at pH = 4.8 induces a rapid rise in plasma cortisol during acute exposure. We have seen a similar response in rainbow trout acutely exposed to Al = 112 μg/L at pH = 4.8 (Goss and Wood 1988). A possible benefit of cortisol mobilization in these circumstances may be its documented effect in stimulating chloride cell proliferation on the secondary lamellae (Doyle and Epstein 1972; Perry and Wood 1985).

Effects of Exposure Conditions on Responses to Challenge

The present results show clearly that 10 wk of exposure to sublethal acid plus Al (pH = 5.2, Ca^{2+} = 25 μequiv/L, Al = 150 μg/L) induces acclimatory resistance to a more severe acid plus Al challenge, while exposure to sublethal acid alone (pH = 5.2, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L) does not. This reinforces the conclusion with respect to Al acclimation of the accompanying investigation (Wood et al. 1988a), which was based only on Na^+ flux and plasma ion data. The present data additionally demonstrate protection against respiratory problems, acidosis, and hemoconcentration, as well as attenuation of the stress response signalled by glucose and cortisol elevation. The results also clarify the situation (i.e. lack of acclimation) with respect to the effect of prior exposure to acid alone, which had been equivocal previously, and show that higher water Ca^{2+} levels slightly delay but do not prevent the toxic responses to acid plus Al challenge. Note that these experiments did not test whether prior exposure to acid alone induced greater resistance to challenge with acid alone, which is a separate issue.

These experiments provide physiological confirmation of the LC50 data of Orr et al. (1986) on rainbow trout who demonstrated that prior exposure to low levels of Al (87 and 154 μg/L at pH ≃ 5.2) provides increased resistance to higher levels (~1.8-fold increase in LC50 from ~175 to ~315 μg/L at pH ≃ 5.2 for both exposures). Our studies covered approximately the same range of concentrations, but with a more resistant species (Grande et al. 1978). Fish chronically exposed to low levels of Al in the wild will clearly possess greater resistance to short-term increases in Al associated with episodic events (snowmelt and rainstorm runoff). However, it is unclear whether this increased resistance represents true tolerance, i.e. whether it will allow the animals to survive indefinitely under the more severe condition. The results of Orr et al. (1986) suggest that it should, for they continued their tests for 6 d and were able to document increases in "incipient" LC50 levels, implying an enhancement of long-term survival. However, our Al-acclimated fish, while showing an absence or rapid correction of any initial disturbances during challenge, did exhibit the apparent start of a stress response at 66 h, manifested as decreased plasma Na^+ and Cl^- (Fig. 4E) and elevated cortisol levels (Fig. 3). Much longer challenges will be required to test whether the increased resistance is of finite duration.

We have suggested that the protection against ion loss offered by Al acclimation could involve a greater uptake capacity of the proliferated chloride cells or a more effective reduction of passive permeability during acid/Al challenge (Wood et al. 1988a). It is difficult to see how such mechanisms could also prevent interference with O_2 and CO_2 diffusion across the gills. Perhaps the explanation is a more general one in that chronic sublethal exposure alters the nature of gill surface ligands for Al and/or the pH of the branchial microenvironment, so as to reduce Al accumulation during the challenge. This in turn might prevent mucification, inflammation, epithelial separation, and the associated increase in diffusion distance. We hypothesize that Al-acclimated trout accumulate less Al on the gills during a more severe challenge and that the morphological response of the branchial epithelium is attenuated. These topics are currently under investigation in our laboratories.

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