Branchial Morphological and Endocrine Responses of Rainbow Trout (*Oncorhynchus mykiss*) to a Long-Term Sublethal Acid Exposure In Which Acclimation Did Not Occur

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Changes in branchial morphology and in plasma cortisol, adrenaline, and noradrenaline were quantified throughout an 81-d exposure of rainbow trout (*Oncorhynchus mykiss*) to sublethal acidity (pH 4.8) in artificial soft water and after a 5-h acid challenge (pH 4.0) of naive fish and 81-d acid-preexposed fish. Changes in branchial morphology at pH 4.8 were generally very mild and characterized by slight increases in filamental mucous cells and decreases in lamellar mucous cells. Chloride cell numbers and branchial Na^+–K^+- and total ATPase activities did not change. The filamental epithelium thickened, but the water–blood diffusion distance in the lamellae decreased during chronic exposure. Cortisol was significantly elevated throughout whereas catecholamines exhibited relatively little response. Response to acute pH 4.0 challenge was similar in naive and 81-d acid-exposed fish: epithelial damage, increase in visible mucous cells, loss of chloride cells by necrosis, and high cortisol levels but no changes in lamellar or filamental epithelial thickness, diffusion distance, ATPase activities, or catecholamine levels. Previously reported physiological data from these same trout demonstrated that sensitization rather than acclimation had occurred. Therefore, these observations support the view that acclimation does not occur in the absence of significant branchial damage and repair.

Les changements morphologiques au niveau des branchies ainsi que les variations de cortisol, d'adrénaline et de noradrénaline plasmatiques ont été quantifiés chez la truite arc-en-ciel (*Oncorhynchus mykiss*) au cours d'une exposition de 81 jours à pH acide sous-létal (pH 4,8) dans une eau à faible teneur ionique ainsi qu'après une exposition de 5 h à pH 4,0, chez des poissons n'ayant jamais été exposés à l'acidité et chez des poissons pré-exposés à l'acidité durant 81 jours. Les changements morphologiques au niveau des branchies étaient généralement faibles et caractérisés par une légère augmentation des cellules à mucus sur les filaments branchiaux et une diminution sur les lamelles branchiales. Nous n'avons observé aucun changement du nombre de cellules à chlorure, ni de l'activité Na^+–K^+ et ATPasique totale branchiale. L'épaisseur des filaments a augmenté mais la distance de diffusion lamellaire a décru au cours de l'exposition chronique. Le cortisol est resté significativement plus élevé durant toute la période d'exposition alors qu'il n'y a eu que très peu de réponse au niveau des catécholamines. La réponse à pH 4,0 a été similaire chez les poissons pré-exposés pour 81 jours et chez ceux non pré-exposés à l'acidité: dommages épithéliaux, augmentation du nombre visible de cellules à mucus, perte de cellules à chlorure par nécrose, concentrations plus élevées de cortisol mais aucun changement significatif au niveau de l'épaisseur des épithéliums lamellaires et filamentaux, de la distance de diffusion, des activités ATPasiques ou des niveaux de catécholamines. Des données précédemment publiées et provenant des mêmes poissons indiquaient une sensibilisation plutôt qu'une acclimatation. Les présentes observations supportent donc l'hypothèse que l'acclimatation ne se produit pas en l'absence de dommages branchiaux significatifs et de réparation subséquente.

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The present investigation is part of an overall study on the ionoregulatory, endocrine, and branchial morphological responses of adult rainbow trout (*Oncorhynchus mykiss*) to chronic acid exposure (81 d) at pH 4.8 in artificial soft water (ASW) and the effects of such an exposure on the responses to a further acute acid stress (5 h at pH 4.0). Particular care was taken to duplicate the water chemistry characteristic of typical acid-threatened softwaters in eastern North America and to measure the various ionoregulatory, morphological, and endocrine parameters on the same individuals. The first part of our study (Audet et al. 1988) showed that chronic acid exposure produced a reduction of branchial influx (J_{in}) and efflux rates (J_{out}) and a negative net flux (J_{net}) of the two major plasma electrolytes (Na^+ and Cl^-). On a long-term basis, J_{net} values were restored to approximately control levels but plasma Na^+ and Cl^- concentrations remained depressed, glucose remained elevated, and hematological parameters remained disturbed. Thus, there was stabilization at a new steady state, but no recovery. The second part (Audet and Wood 1988) demonstrated that these chronically exposed fish suffered more extreme Na^+ and

Cl^- losses and other physiological disturbances than did naive fish when challenged with a more severe acid stress. Thus, there was sensitization rather than acclimation.

The first objective of the present work was to quantitatively characterize possible morphological and enzymatic alterations in the gills. Our immediate goal was to see whether or not these correlated with observed changes in branchial Na^+ and Cl^- fluxes and internal physiology during chronic and acute acid exposures. We also measured primary endocrine stress indicators (plasma cortisol and catecholamines) to better understand their role in the observed responses. Both agents have been implicated in the control of branchial transport function (Wood 1991) and cortisol in the control of branchial morphology (Perry and Wood 1985; Laurent and Perry 1990). We were also particularly interested in whether a sublethal exposure which did not induce either recovery or acclimation was associated with significant histopathological damage.

Recently, McDonald and Wood (1992) have proposed that branchial mechanisms of acclimation are a function of damage repair. By this hypothesis, for a toxicant to induce acclimation, it must first cause significant structural damage to the gill epithelium. While such damage is traditionally seen as a correlate of acutely lethal acid stress (Mallatt 1985; McDonald et al. 1991), several recent studies have suggested that chronic sublethal exposure to pure acidity, without significant involvement of metals, may cause little or no disruption of gill morphology and histology in salmonids (Jagoe and Haines 1983; Karlsson-Norrgren et al. 1986; Evans et al. 1988; Mueller et al. 1991). Other chronic studies do not agree (Daye and Garside 1976; Tietge et al. 1988; Laurent and Perry 1991). However, none of these investigations examined whether acclimation occurred. The present study, in combination with Audet et al. (1988) and Audet and Wood (1988), is therefore the first to assess gill morphological responses and to test for acclimation simultaneously.

Methods

Exposure and Sampling

This study was performed concurrently with those reported in two previously published papers (Audet et al. 1988; Audet and Wood 1988) which provide complete information on fish holding and experimental protocols. Key details are repeated here. The whole study was conducted in flowing artificial soft water to which the trout had been acclimated for 2–4.5 mo prior to testing. The study was divided into two main parts: long-term sublethal acid exposure (81 d at pH 4.8) and acute severe acid stress (5 h at pH 4.0), hereafter described as "acid challenge." Acid challenge was imposed either on fish kept under circumneutral conditions (pH ≈ 6.5, "naive" fish) or on fish which had been previously exposed to long-term sublethal acid conditions (3 mo at pH 4.8, "acid-preexposed fish"). Adult rainbow trout (200–300 g) of both sexes were obtained from a hardwater source (Spring Valley Trout Farm, Petersburg, Ontario) and initially held in Hamilton tap water (Ca^{2+} = 1800, Na^+ = 600, Cl^- = 800 $\mu eq \cdot L^{-1}$). Stock and experimental fish were kept at 15 ± 1°C under a 24 h light photoperiod. The fish were acclimated for 2–4.5 mo to ASW (Ca^{2+} = 50, Na^+ = 50, Cl^- = 100 $\mu eq \cdot L^{-1}$; pH 6.5) before being used as either controls (no acid exposure) or experimentals (chronic acid exposure or pH 4.0 challenge). None of the fish were in breeding condition. ASW production, fish density, feeding schedule, water acidification, and pH recording procedures are fully described in Audet et al. (1988).

The basic experimental protocol of 2–4.5 mo of ASW acclimation at pH ≈ 6.5 followed by up to 81 d of pH 4.8 exposure was repeated five times to yield the blood and tissue chemistry data reported by Audet et al. (1988). The morphological and endocrine results presented here for the long-term acid exposure come from three of these experiments starting in April, October, and January. Not all experimental times were sampled in each series, but control samples were taken at the start of every series. Simultaneous exposure of the fish to ASW at neutral pH was not run in parallel to each of the series in view of the long period allowed for initial acclimation to ASW in every experiment. As expected, given the constant temperature and photoperiod regimes, no significant differences among series were found in controls or at common sample times. Therefore the data from the different series were pooled. The pH 4.0 challenge experiments reported here were run at the end of the 81-d exposure which had been started in October, i.e. in early January. At this time, challenge exposures of naive and acid-preexposed fish were conducted *simultaneously*. Samples were taken from naive fish (pH 6.5) and acid-preexposed fish (pH = 4.8) both before and immediately after 5 h of exposure to pH 4.0.

In the 81-d sublethal acid exposure study, trout were sampled under control conditions (C) and at 1, 3, 8, 22, 50, and 81 d of exposure to pH = 4.8 and, in the challenge experiment, after 5 h of exposure to pH 4.0. At sampling, fish were individually anaesthetized in 0.01% MS-222 (pH adjusted to 6.5, 4.8, or 4.0 as appropriate with KOH). Blood was withdrawn immediately by caudal puncture and plasma obtained by centrifugation (10 000*g* for 2 min). An aliquot of plasma was immediately frozen at −80°C in liquid nitrogen for later determination of cortisol and catecholamines. After caudal puncture, the second gill arch of the left side of the fish was clamped at ventral and dorsal margins; the middle part was sectioned and put in 4% formaldehyde – 5% glutaraldehyde fixative solution buffered with sodium cacodylate (0.1 M, pH 7.2). A catheter was then introduced into the bulbus arteriosus and the gills were perfused with ammonium heparin (2500 $IU \cdot mL^{-1}$) diluted in Cortland saline solution (Wolf 1963) to "wash out" the red cells. The second gill arch of the right side was then sectioned and frozen in liquid nitrogen for later Na^+–K^+-ATPase activity determination.

Cortisol was measured by ^{125}I radioimmunoassay (New England Nuclear). Adrenaline and noradrenaline were determined on alumina-extracted plasma samples using high-performance (pressure) liquid chromatography (Waters 510 pump, reverse phase C-18 column) with electrochemical detection (Waters M460 ECP). All plasma hormone concentration determinations were in duplicate. For each fish, Na^+–K^+-ATPase activity was measured on gill homogenate by the method of Stagg and Shuttleworth (1982) and protein content by the method of Lowry et al. (1951). Average total gill ATPase activity and Na^+–K^+-ATPase activity are reported both per gram of gill tissue and per milligram of protein. Gill tissues were kept immersed in the fixative for a maximum period of 2 wk. They were then postfixed in 1% osmium, stained with uranyl acetate, dehydrated in ethanol, and embedded in Spurr. When embedded, tissues were oriented in order to obtain sagittal sections. Semithin sections (0.5 μm) were prepared and stained with toluidine blue. For controls and long-term sublethal acid-exposed fish (81 d), ultrathin (60–80 nm) sections were stained with lead citrate and chloride cells were examined in a Philips 300 electron microscope.

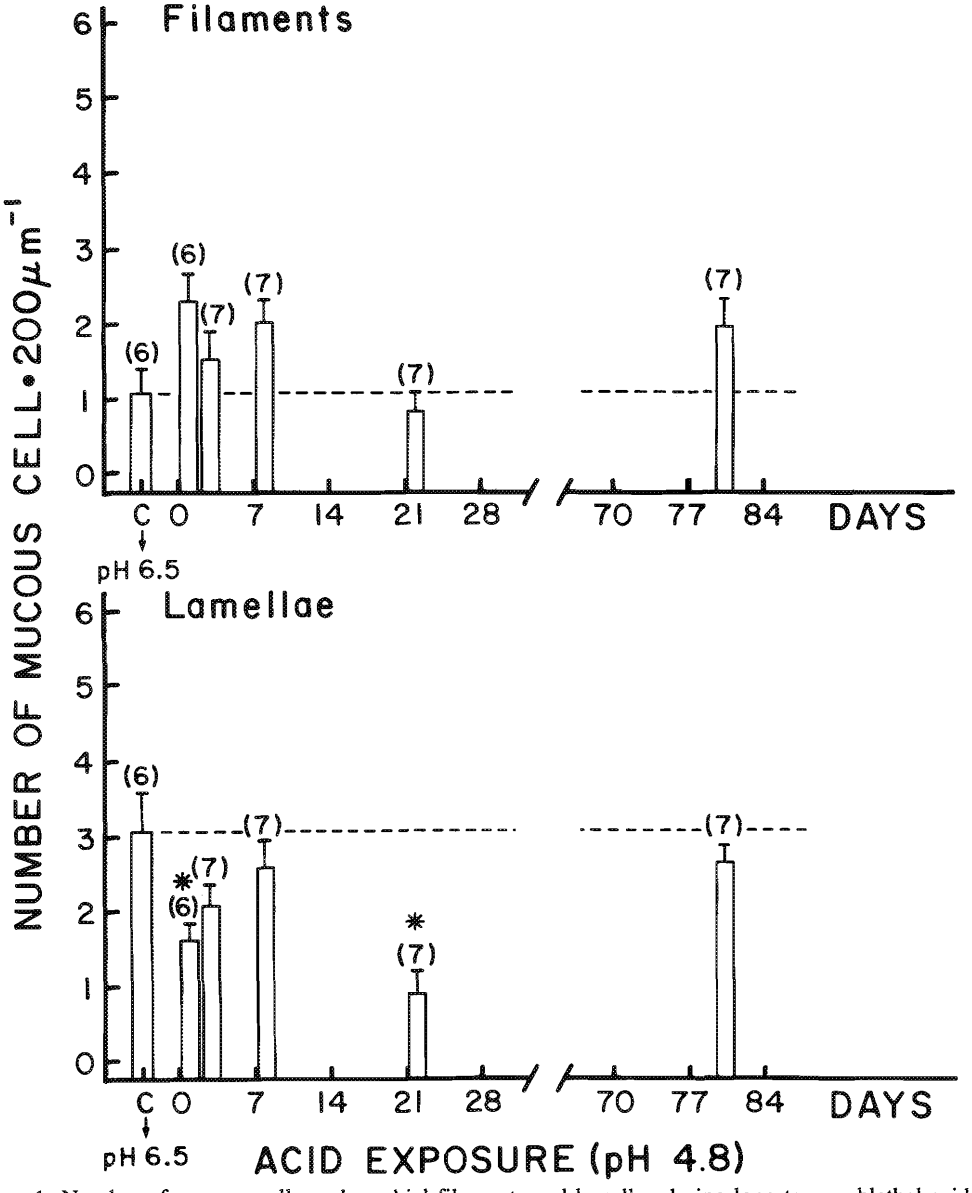

FIG. 1. Number of mucous cells on branchial filaments and lamellae during long-term sublethal acid exposure in rainbow trout. Means ± SEM; numbers of fish in parentheses. C = control ASW acclimated fish (pH 6.5). Asterisks represent significant differences from controls (level indicated by broken line). Results of the mean comparison tests are presented below: numbers represent the days of exposure, and single lines underscore days between which there was no significant difference:

Filaments:	22	C	3	81	8	1
Lamellae:	22	1	3	8	81	C

Histological Measurements

Histological measurements were done on a Leitz light microscope coupled with a Zeiss Videoplan image analysis system (MOP-40). A photomicrograph of a Merz grid was transferred onto a transparency which was fixed to the screen of the image analyzer. The system was calibrated using a stage micrometer (Leitz). The different parameters measured were interlamellar distance, thickness of filament, thickness of filamental epithelium, thickness of secondary lamellae, thickness of lamellar epithelium, water–blood diffusion distance, numbers of chloride and mucous cells on the filament, and numbers of chloride and mucous cells on the lamellae.

Interlamellar distance was measured at the base of lamellae on three different filaments per fish, employing 10 measurements per filament (total of 30 measurements per fish). For thickness of filament and secondary lamellae as well as thickness of their epithelia and diffusion distance, the points from which measurements were initiated were randomly chosen by the use of a Merz grid (Hughes and Perry 1976). Thickness of filament and thickness of filamental epithelium were determined on three filaments, with three measurements on each (total of nine measurements per fish). Thickness of secondary lamellae, thickness of lamellar epithelium, and water–blood diffusion distance were measured on 10 lamellae from three filaments per fish. As thickness of lamellar epithelium and

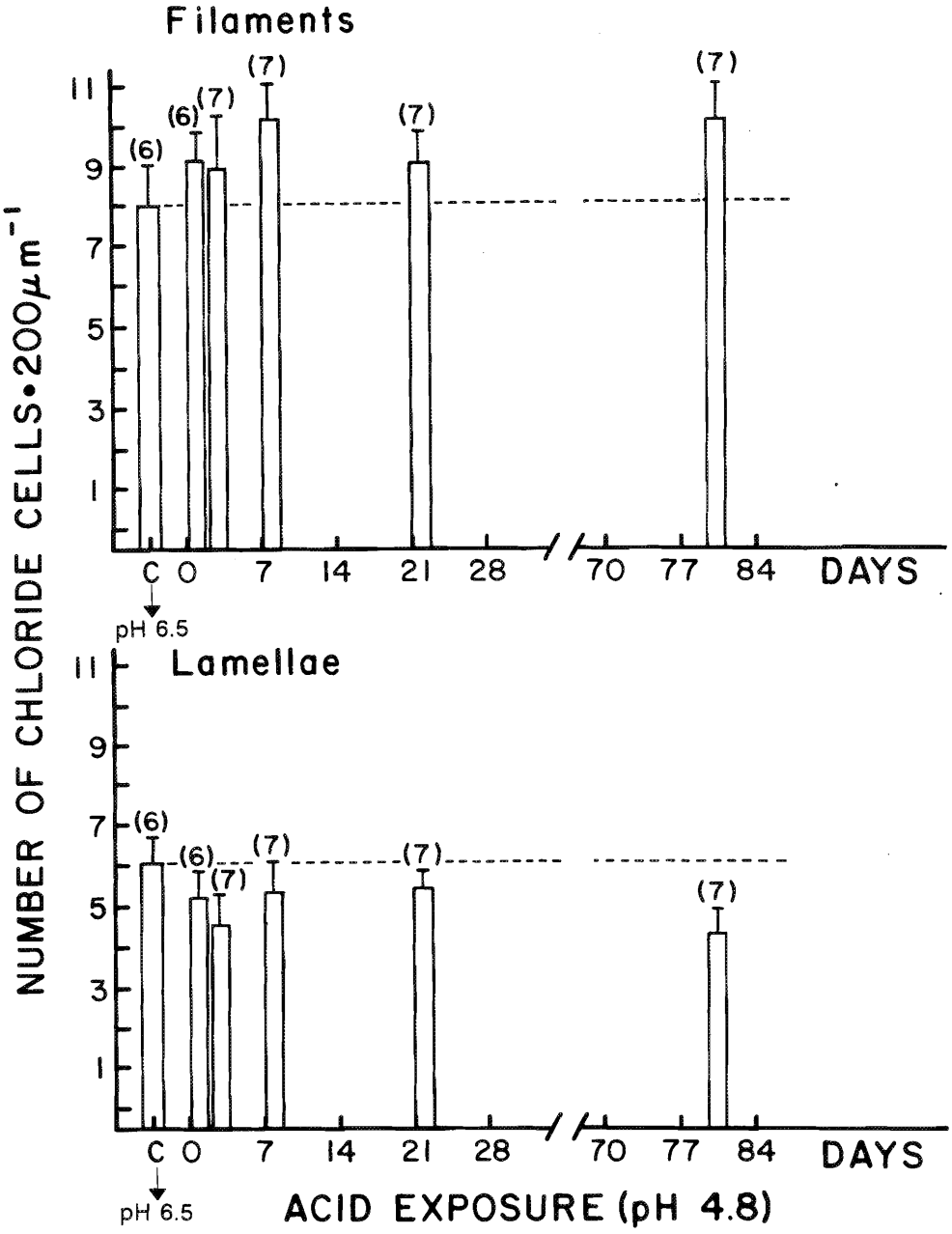

FIG. 2. Number of chloride cells on branchial filaments and lamellae during long-term sublethal acid exposure in rainbow trout. Differences not significant. See legend to Fig. 1 for other details.

water–blood diffusion distance were measured on high magnification (1125×), the field of our Merz grid did not cover the entire surface of a secondary lamella. Therefore, to ensure representative data for these two parameters, we took three different measurements per secondary lamella: one in the basal area, the second in the middle area, and the third one at the apical level. The points from which these measurements were initiated were randomly chosen by the use of the Merz grid. In total, thickness of the epithelium of secondary lamellae and water–blood diffusion distance were both obtained from 90 different measurements compared with 30 for thickness of secondary lamellae. Both mucous and chloride cells were counted on 10 secondary lamella from three filaments per fish. Length of the tissue sections covered was measured and the number of cells then reported per 200 μm. Each fish provided only one value for each parameter, this value being the average of all measurements of that parameter in that fish (9–90 depending on the parameter).

Statistics

Data have been routinely expressed as mean ± SEM (n) where n represents the number of fish. The data for long-term sublethal acid exposure were analyzed by nonbalanced one-way ANOVA, $p \leq 0.05$ (time of exposure tested as the source of variation), followed by a Tukey test of comparison of means adjusted for unequal n (T') (Sokal and Rohlf 1981) with $\alpha = 0.05$. All other statistical comparisons were done by t-test ($p \leq 0.05$). Data were tested for normality and for homogeneity of variances (Kolmogorov–Smirnov test and F_{max} test, respectively). All the data were normally distributed; however, in some cases, transformations were required to obtain homogeneity of

variances. Thus, data for cortisol and adrenaline were transformed as $\log_{10}(x)$ prior to ANOVA, while data for noradrenaline were transformed as $1/\sqrt{x}$.

Results

Long-Term Sublethal Acid Exposure

Sublethal acid exposure produced only slight changes in gill morphology. Except for chloride cell numbers, significant treatment effects (ANOVAs, $p < 0.05$) were observed on all measured parameters. However, these changes were often transient and/or of small magnitude.

Significant variations in the number of mucous cells were observed on both filaments and lamellae. The number of mucous cells tended to be higher at pH 4.8 than at pH 6.5 (ASW controls) on filaments, while the reverse tendency was observed on lamellae (Fig. 1). On days 1 and 22, significantly lower counts of mucous cells were observed on lamellae compared with control fish. No significant changes in the number of chloride cells followed the pH 4.8 exposure (Fig. 2).

Interlamellar distance increased significantly following 1 d of exposure to pH 4.8 but was not significantly different from controls throughout the remainder of exposure (Table 1). The thickest filaments were found on days 3, 8, and 81, in association with generally thicker filamental epithelia at these times (Table 1). Significant treatment effects (ANOVA) were also found for thickness of both secondary lamellae and lamellar epithelium, but these changes were too small to be detected by a posteriori tests (Table 1). The net effect of these small variations, however, was reflected in the water–blood diffusion distance measurements, with a tendency for reduced diffusion distance throughout the period of acid exposure (Table 1). This narrowing of diffusion distance was most pronounced on day 22 when a significant 25% decrease was observed compared with the diffusion distance in ASW control and day 3 fish.

Light microscopy observations revealed that apical hyperplasia (Fig. 3a) and club deformations (Fig. 3b) were frequent features in ASW control fish as well as in pH 4.8 exposed fish. Most of the lamellar mucous cells were concentrated at the tip of lamellae (Fig. 3c). In contrast, lamellar chloride cells were found all along the lamellae in controls and in fish exposed to pH 4.8 for 1, 3, and 8 d (Fig. 3d and 3e). However, they were more concentrated at the base of lamellae after 22 and 81 d of sublethal acid exposure (Fig. 3f and 3g). Additional qualitative observations of chloride cells in control and day 81 fish were carried out by transmission electron microscopy. No marked differences were present between chloride cells of the two groups. However, apoptosis (cf. Wendelaar Bonga and van der Meij 1989) was more frequent in ASW control fish, and the presence of rough endoplasmic reticulum was more prominent in long-term acid-exposed fish than in controls.

No significant changes in total gill ATPase activity or in Na^+–K^+-ATPase activity were observed during this experiment. The overall average values (controls and long-term acid-stressed fish) obtained were, for total gill activity, 368 ± 14.8 (50) μmol $PO_4 \cdot g$ gill tissue$^{-1} \cdot h^{-1}$ and 4.9 ± 0.29 (48) μmol $PO_4 \cdot$mg protein$^{-1} \cdot h^{-1}$, and for Na^+–K^+-ATPase activity, 61 ± 4.1 (51) μmol $PO_4 \cdot g$ gill tissue$^{-1} \cdot h^{-1}$ and 0.8 ± 0.05 (49) μmol $PO_4 \cdot$mg protein$^{-1} \cdot h^{-1}$.

Following transfer to pH 4.8, plasma cortisol increased to a maximal level on day 3 and stayed high until the end of the experiment (Table 2). Adrenaline and noradrenaline, although highly variable, also reached a maximum concentration on day 3 (Table 2). On other days, plasma concentrations were not significantly elevated, and by day 81, noradrenaline levels were equal to and adrenaline levels were significantly below those of control fish.

Acute Severe Acid Challenge

Changes in identifiable mucous and chloride cell numbers were the most prominent effects of pH 4.0 challenge on gill morphology. In naive fish, a significant increase in the number of visible mucous cells on both filaments and lamellae followed pH 4.0 challenge for 5 h (Fig. 4). In fish preexposed to pH 4.8 for 3 mo, the rise was only significant on lamellae. However, when the two pH 4.0 challenge groups were compared, no significant difference was found between them. Both naive and chronically acid-preexposed fish showed a significant decrease of approximately 35% in the number of chloride cells on filaments following 5 h of challenge at pH 4.0 (Fig. 4). Many chloride cells appeared necrotic and in the process of lifting out of the epithelia. Final numbers of chloride cells on filaments

TABLE 1. Effect of long-term sublethal exposure on gill morphometry. Results are presented as mean ± SEM (n), n indicating the number of fish. Results of the a posteriori tests of comparison of means are indicated by superscript letters a and b. Means with the same superscript letter are not significantly different from one another. Asterisks represent significant differences from controls (C).

Day	Interlamellar distance (μm)	Thickness of filaments (μm)	Thickness of filamental epithelium (μm)	Thickness of secondary lamellae (μm)	Thickness of lamellar epithelium (μm)	Diffusion distance (μm)
C (pH 6.5)	21.6 ± 0.95^{a} (6)	88.9 ± 7.97^{a} (6)	28.0 ± 2.26^{ab} (6)	14.9 ± 0.67^{a} (6)	4.1 ± 0.28^{a} (6)	5.6 ± 0.30^{b} (6)
1	27.3 ± 1.87^{b}* (6)	85.1 ± 11.75^{a} (6)	21.8 ± 3.06^{a} (6)	12.6 ± 0.90^{a} (6)	3.4 ± 0.40^{a} (5)	4.4 ± 0.39^{ab} (5)
3	21.9 ± 1.01^{ab} (7)	157.9 ± 16.98^{b}* (7)	40.9 ± 4.01^{b} (7)	15.1 ± 0.63^{a} (7)	3.9 ± 0.23^{a} (7)	5.4 ± 0.30^{b} (7)
8	23.5 ± 1.12^{ab} (7)	119.8 ± 16.6^{ab} (7)	34.4 ± 3.33^{ab} (7)	14.1 ± 0.64^{a} (7)	4.1 ± 0.30^{a} (7)	4.8 ± 0.26^{ab} (7)
22	20.3 ± 0.95^{a} (7)	71.06 ± 7.64^{a} (7)	22.7 ± 2.95^{a} (7)	12.5 ± 0.45^{a} (7)	3.0 ± 0.20^{a} (7)	4.2 ± 0.23^{a}* (7)
81	22.4 ± 1.39^{ab} (7)	118.6 ± 11.91^{ab} (7)	28.1 ± 2.79^{ab} (7)	15.0 ± 0.62^{a} (7)	3.8 ± 0.24^{a} (7)	5.1 ± 0.23^{ab} (7)

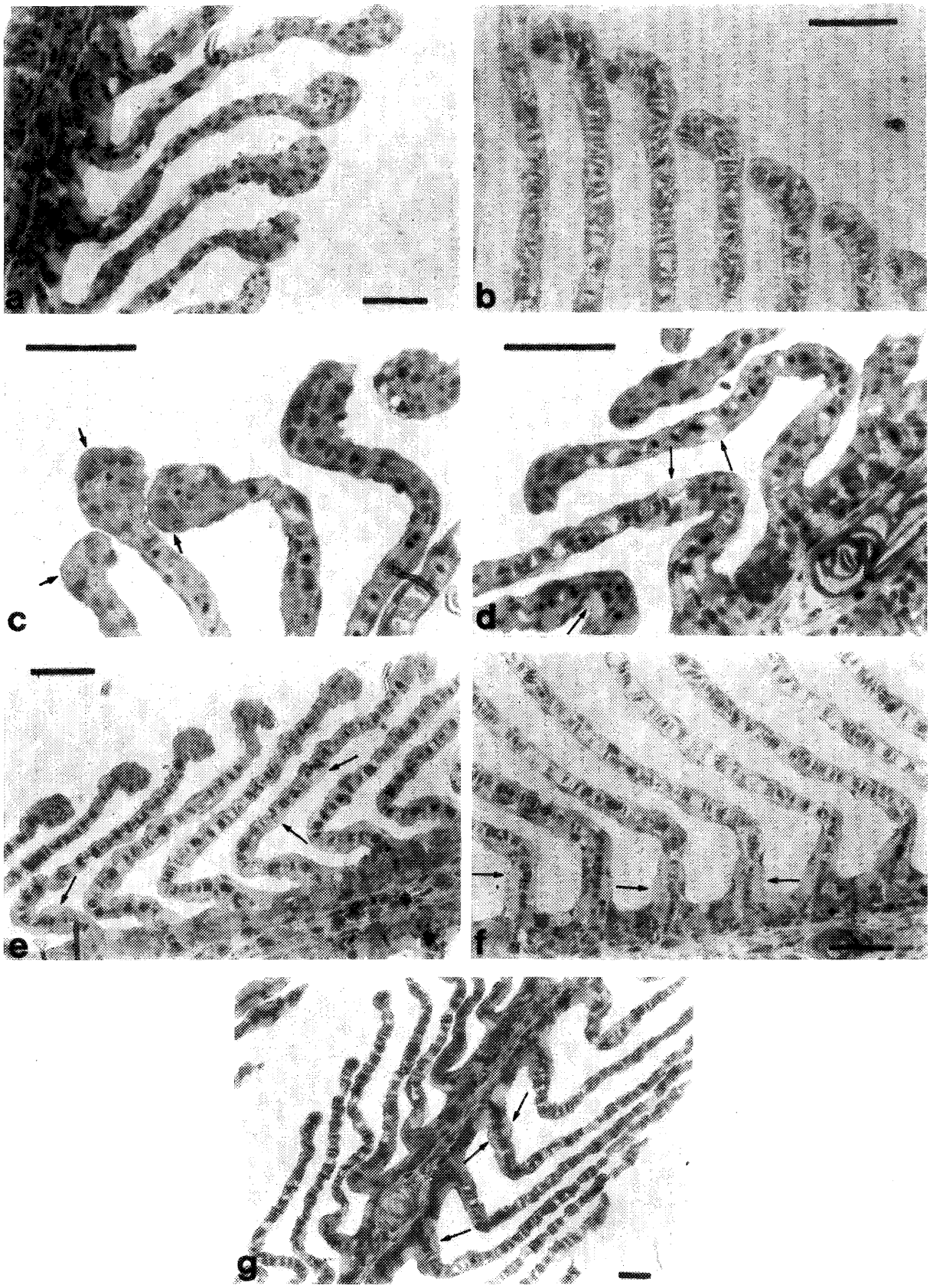

FIG. 3. Effects of long-term sublethal acid exposure on gill morphology in rainbow trout. (a) Apical hyperplasia, ASW control; (b) club deformation, pH 4.8, day 81; (c) mucous cells on lamellar tips, ASW control; (d) lamellar chloride cells in ASW control; (e) lamellar chloride cells, pH 4.8, day 3; (f) lamellar chloride cells, pH 4.8, day 81; (g) lamellar chloride cells, pH 4.8, day 22. Short arrows: mucous cells; long arrows: chloride cells; scale bars = 50 μm.

TABLE 2. Effect of long-term sublethal acid exposure on plasma cortisol and catecholamines. Results are presented as mean ± SEM (n), n indicating the number of fish. Results of the a posteriori tests of comparisons of means are indicated by superscript letters a to e. Means with the same superscript letter are *not* significantly different from one another. Asterisks represent significant differences from controls (C).

Day	Cortisol ($ng \cdot mL^{-1}$)	Adrenaline ($nmol \cdot L^{-1}$)	Noradrenaline ($nmol \cdot L^{-1}$)
C (pH 6.5)	42.6 ± 5.5^{ab} (23)	19.8 ± 3.67^{bcd} (20)	9.0 ± 1.54^{bcd} (20)
1	52.9 ± 17.2^{abc} (17)	8.7 ± 2.66^{ab} (7)	12.9 ± 1.83^{a*} (7)
3	128.1 ± 25.4^{d*} (16)	57.5 ± 24.50^{e*} (16)	22.8 ± 8.30^{ab} (16)
8	84.3 ± 17.9^{cd*} (7)	12.4 ± 3.80^{ab} (7)	6.3 ± 1.61^{cde} (7)
22	65.9 ± 2.2^{bcd} (5)	12.3 ± 2.64^{abc} (5)	3.2 ± 0.26^{e} (5)
50	121.8 ± 37.0^{d*} (7)	41.1 ± 19.95^{ce} (7)	8.7 ± 2.02^{bcde} (7)
81	96.1 ± 26.0^{cd*} (7)	9.4 ± 4.59^{a*} (7)	7.7 ± 3.73^{de} (7)

were similar between the two groups challenged with pH 4.0. Acute pH 4.0 challenge also produced a decrease of almost 50% in chloride cell numbers on the secondary lamellae of naive fish. This effect was not seen upon pH 4.0 challenge in the acid-preexposed fish, but this group already exhibited somewhat lower chloride cell numbers on lamellae. As with filamental chloride cells, no significant difference was found on lamellar chloride cell numbers between the two groups of fish challenged with pH 4.0 (Fig. 4).

A significant 42% increase in interlamellar distance was observed in naive fish challenged with pH 4.0 (Table 3). The response was similar to that seen after exposure of naive fish to pH 4.8 for 1 d (Table 1). This effect did not occur when acid-preexposed fish were challenged with pH 4.0. The difference between the two pH 4.0 groups was significant. The 5-h challenge at pH 4.0 did not induce any changes in thickness of the filaments or secondary lamellae, thickness of their epithelia, or water–blood diffusion distance in either naive or acid-preexposed fish (Table 3).

Light microscopy demonstrated the presence of apical hyperplasia in both pH 4.0 challenged groups, similar to that in the naive and long-term acid-preexposed fish. The lamellar epithelium was often damaged and separated in fish challenged with pH 4.0 (Fig. 5a and 5b). Severe lamellar swelling was observed in two of the naive fish challenged with pH 4.0 (Fig. 5c). This damage to the lamellar epithelium may have impaired cell type identification, leading to artificially reduced cell numbers. Nevertheless, as noted above, lamellar mucous cell numbers were significantly increased in this group. This increase was caused by an increased number of visible mucous cells along the lamellae (Fig. 5d) instead of only at the tips as seen at pH 4.8.

Again, no significant differences in total gill ATPase activity or gill Na^+–K^+-ATPase activity resulted from severe acid exposure in either treatment group. Values were, for total gill activity, 342 ± 22.0 (28) $\mu mol\ PO_4 \cdot g\ gill\ tissue^{-1} \cdot h^{-1}$ and 4.8 ± 0.47 (28) $\mu mol\ PO_4 \cdot mg\ protein^{-1} \cdot h^{-1}$, and for Na^+–K^+-ATPase activity, 59 ± 4.0 (28) $\mu mol\ PO_4 \cdot g\ gill\ tissue^{-1} \cdot h^{-1}$ and 0.8 ± 0.08 (28) $\mu mol\ PO_4 \cdot mg\ protein^{-1} \cdot h^{-1}$.

Naive fish exposed to pH 4.0 exhibited a threefold increase in their plasma cortisol concentration (Fig. 6). In acid-preexposed fish, the transfer to pH 4.0 did not significantly increase plasma cortisol concentration, but levels were already very high in these fish. There was no significant difference in plasma cortisol between the two pH 4.0 challenged groups. No significant differences were observed in plasma concentrations of adrenaline and noradrenaline following the pH 4.0 exposure in either naive or acid-preexposed fish; average plasma adrenaline concentration was 25.7 ± 8.39 (5) $nmol \cdot L^{-1}$ in challenged naive fish and 18.6 ± 7.2 (5) $nmol \cdot L^{-1}$ in challenged acid-preexposed fish, while plasma noradrenaline concentration was 17.6 ± 6.17 (5) and 11.7 ± 2.28 (5) $nmol \cdot L^{-1}$ in the two challenged groups, respectively.

Discussion

The most important finding of the present study is that only minor changes in gill morphology occurred during long-term sublethal acid stress. These results are consistent with some previous field and laboratory studies on salmonids. In particular, Lacroix et al. (1990) found more or less unchanged gill features in feral brook trout (*Salvelinus fontinalis*) and juvenile Atlantic salmon (*Salmo salar*) exposed naturally to a range of different pH and Al concentrations in softwater streams of Nova Scotia. In that study, the high organic carbon concentrations of the water apparently protected against Al toxicity, and sublethal physiological effects similar to those observed by Audet et al. (1988) were attributed to acidity alone. Karlsson-Norrgren et al. (1986) reached a similar conclusion with respect to the lack of branchial morphological damage accompanying extended exposure of brown trout (*Salmo trutta*) to pH 5.5 plus Al in humate-enriched softwater. The studies of Jagoe and Haines (1983), Evans et al. (1988), and Mueller et al. (1991) on Sunapee (*Salvelinus alpinus aquassa*), rainbow trout, and brook trout, respectively, also indicated minimal changes in gill morphology in fish exposed to pure sublethal acid stress.

In contrast, a number of other studies on both salmonid (Daye and Garside 1976; Tietge et al. 1988; Brown et al. 1990a; Jagoe and Haines 1990; Laurent and Perry 1991) and nonsalmonid species (Leino and McCormick 1984; Leino et al. 1987; Wendelaar Bonga et al. 1990) have reached a different conclusion, even though acidity alone was thought to be the major or sole stressor. These investigations have documented a variety of pronounced structural changes in the gills (chloride cell proliferation and/or degeneration, mucous cell proliferation, hyperplasia of the lamellar and/or filamental epithelia, increases in the blood–water diffusion distance). Of these, only slight increases in filamental mucous and chloride cell numbers and a variable thickening of the filamental epithelium were seen in the present investigation, and the blood–water diffusion distance in the lamellae actually declined. The reasons for this disagreement are unclear but may include differences in the severity of acid exposure, water chemistry (especially hardness), species, and the involvement of metals or other toxic agents in those studies showing pronounced effects.

The present results certainly support the hypothesis of McDonald and Wood (1992) that at the branchial level, significant structural damage is a necessary prerequisite of acclimation and that acclimation is associated with damage repair. Furthermore, they agree with the majority of the literature (reviewed by Audet et al. 1988; Audet and Wood 1988; Wood 1989) which indicates that acclimation to acidity alone does not occur or is at best equivocal. However, this does not deny the

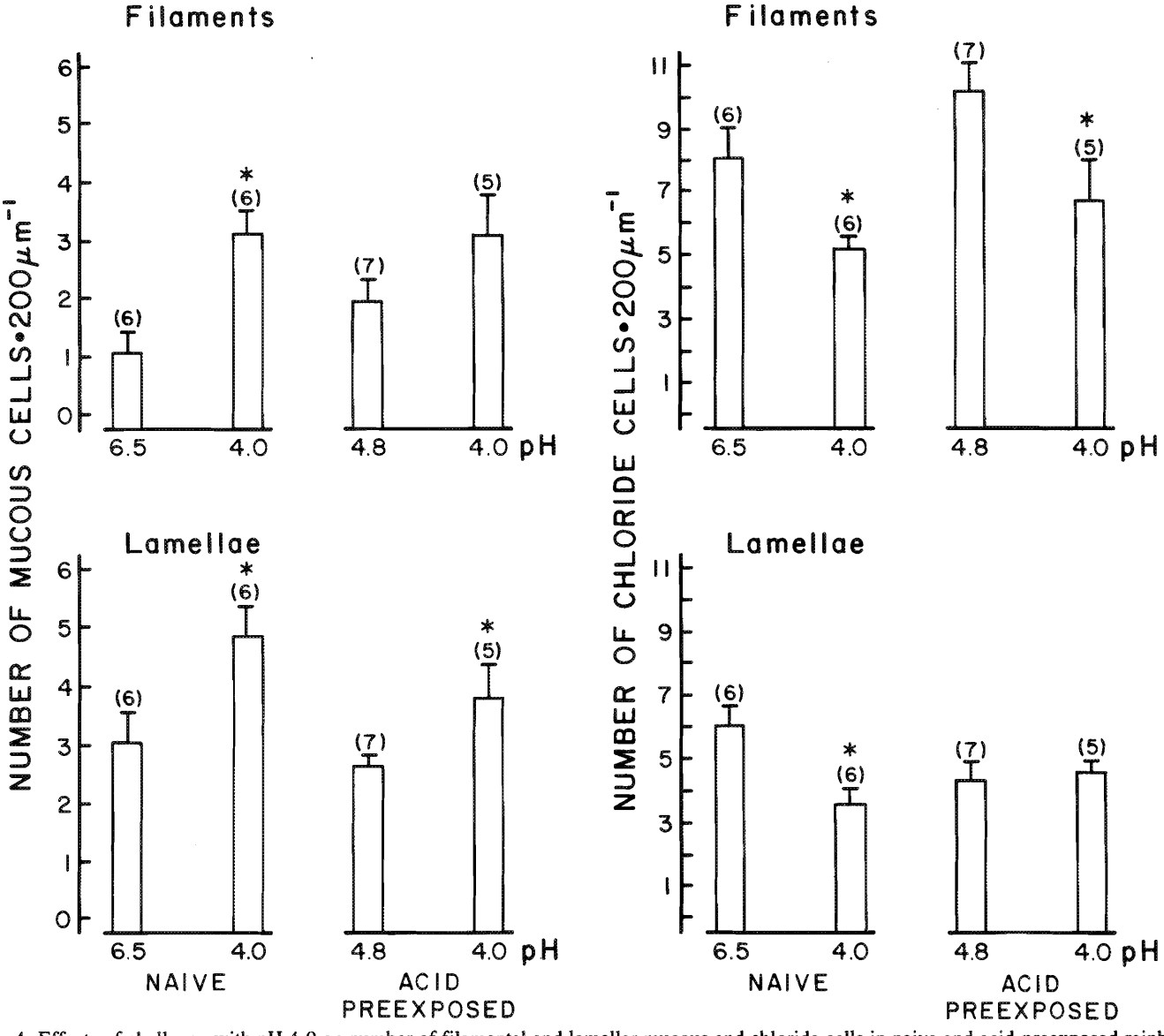

FIG. 4. Effects of challenge with pH 4.0 on number of filamental and lamellar mucous and chloride cells in naive and acid-preexposed rainbow trout. Asterisks represent significant effects ($p \leq 0.05$) of pH 4.0 challenge in naive fish (column 1 versus column 2) or in acid-preexposed fish (column 3 versus column 4). Numbers of fish in parentheses.

possibility of acclimation to acidity, given the right set of circumstances. Indeed, we would predict that acclimation should have occurred in those studies listed above where significant damage repair was seen during exposure. However, as challenge tests were not performed in any of these studies, this remains hypothetical. Nevertheless, it is noteworthy that the tilapia (*Oreochromis mossambicus*), which is much more resistant to low pH than the rainbow trout, exhibited substantial initial gill damage followed by pronounced repair, most prominently a proliferation of chloride cells, during exposure to pH 4.5 (Wendelaar Bonga et al. 1990). At the same time, complete restoration of plasma electrolytes occurred (Wendelaar Bonga et al. 1984). Such complete recovery in the continued presence of the stressor is often indicative of acclimation (McDonald and Wood 1992).

In the present study, there was no recovery of plasma Na^+ and Cl^- during chronic exposure to pH 4.8, but rather an eventual stabilization at a reduced level. There was, however, a partial restoration of the initially inhibited J_{in} components, while the initially inhibited J_{out} components remained low, such that the J_{net} values for both ions returned to control levels by day 81. By analogy to other studies (Leino and McCormick 1984; Avella et al. 1987), we originally hypothesized that the partial J_{in} recovery was the result of an increased number of transport sites via an increase in chloride cell numbers (Audet et al. 1988). The current observations show this hypothesis to be false. No significant changes in chloride cell numbers were observed on either filaments or lamellae, although their distribution on the lamellae was altered. There was a tendency for concentration of chloride cells at the base of the lamellae after chronic sublethal exposure.

Control animals at pH 6.5 already exhibited numerous chloride cells on lamellae, probably resulting from the softwater acclimation employed to duplicate realistic field conditions. Various authors (Laurent et al. 1985; Perry and Wood 1985; Avella et al. 1987; Spry and Wood 1988; Laurent and Hebibi 1989; Perry and Laurent 1989; Laurent and Perry 1991) have proposed that the high number of chloride cells found on lamellae of trout exposed to soft water is an adaptive response to enhance active ion influx in a dilute environment. In the present

TABLE 3. Comparison of gill morphology before and after a 5-h challenge at pH 4.0 between fish never exposed to sublethal acid stress (naive, pH 6.5) and fish preexposed for 81 d to pH 4.8.

Gill parameter	Naive fish		Acid-preexposed fish	
	Before	After	Before	After
Interlamellar distance (μm)	21.6 ± 0.95 (6)	27.6 ± 2.10[a] (6)	22.4 ± 1.39 (6)	19.1 ± 1.37[b] (5)
Thickness of filaments (μm)	88.9 ± 7.97 (6)	96.5 ± 8.39 (6)	118.6 ± 11.89 (7)	119.6 ± 17.12 (5)
Thickness of filamental epithelium (μm)	28.0 ± 2.26 (6)	30.8 ± 1.39 (6)	28.1 ± 2.79 (7)	28.2 ± 3.51 (5)
Thickness of secondary lamellae (μm)	14.9 ± 0.67 (6)	15.2 ± 0.79 (6)	15.0 ± 0.62 (7)	15.3 ± 0.61 (5)
Thickness of lamellar epithelium (μm)	4.1 ± 0.27 (6)	4.0 ± 0.17 (6)	3.8 ± 0.24 (7)	3.6 ± 0.21 (5)
Diffusion distance (μm)	5.6 ± 0.30 (6)	5.6 ± 0.23 (6)	5.1 ± 0.23 (7)	5.2 ± 0.29 (5)

[a]Significant effect ($p \leq 0.05$) of pH 4.0 challenge in naive fish.
[b]Significant difference between naive and acid-preexposed fish following challenge with pH 4.0.

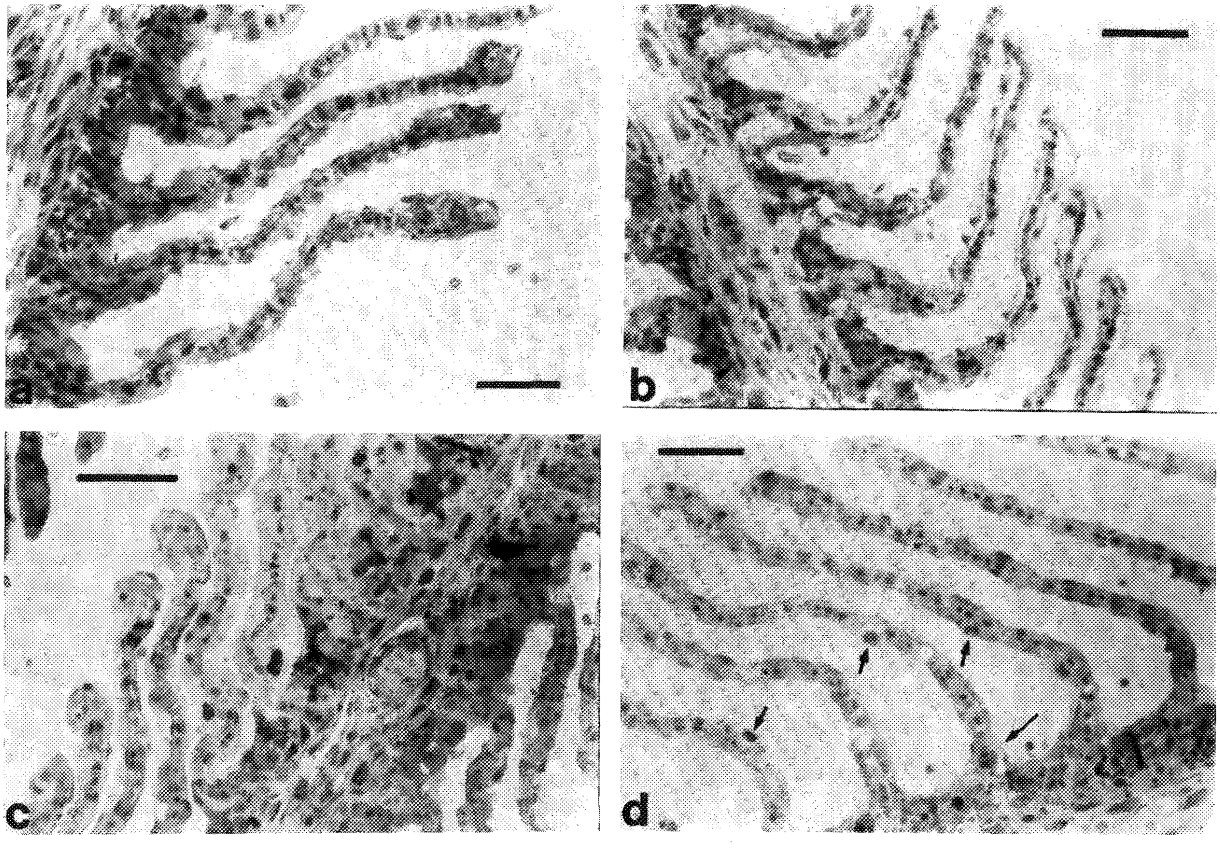

FIG. 5. Effects of acute pH 4.0 challenge on gill morphology in rainbow trout. (a and b) Damage to the lamellar epithelium following pH 4.0 challenge in acid-preexposed fish; (c) swelling of the lamellar epithelium following pH 4.0 challenge in naive fish; (d) lamellar mucous cells following pH 4.0 challenge in naive fish. Short arrows: mucous cells; long arrows: chloride cells; scale bars = 50 μm.

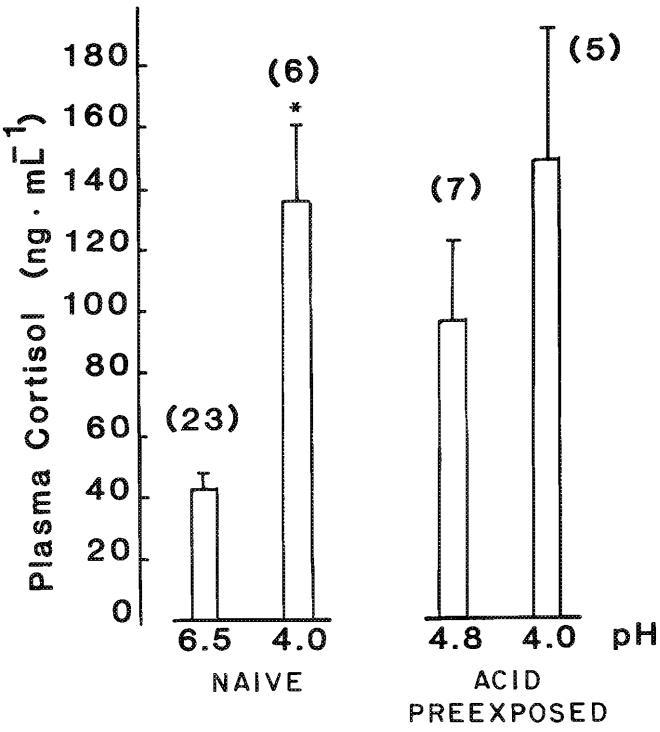

FIG. 6. Effects of pH 4.0 challenge on plasma cortisol concentration in naive and acid-preexposed rainbow trout. Asterisk represents a significant effect ($p \leq 0.05$) of pH 4.0 exposure in naive fish (column 1 versus column 2). Numbers of fish in parentheses.

experiment, we speculate that the softwater-acclimated trout had already fully recruited the potential offered by chloride cell proliferation to increase ion uptake.

The question then arises: what mechanism(s) could explain the partial recovery of influx observed in chronically exposed rainbow trout? There was no evidence of increased enzymatic activity of chloride cells following long-term sublethal acid exposure, at least as indicated by Na^+–K^+-ATPase activity. A decrease in Na^+–K^+-ATPase and/or total ATPase activity in the gills has been observed in several other studies on acid-exposed salmonids (Nieminen et al. 1982, pH 5.0; Saunders et al. 1983, pH 4.2–4.7; Staurnes et al. 1984, pH 5.0). Electron microscopy also provided no definitive evidence for an increase in chloride cell activity in the present study. Apical pits appeared to be absent from the chloride cells in both groups, in sharp contrast with the observations of Leino and McCormick (1984), Leino et al. (1987), and Wendelaar Bonga et al. (1990) on fathead minnow (*Pimephales promelas*) and tilapia chronically exposed to sublethal low pH. However, there was one subtle difference in ultrastructure between the chloride cells of control and 3-mo acid-exposed trout. Apoptosis (degeneration preceding physiologically controlled cell death; cf. Wendelaar Bonga and van der Meij 1989) seemed to be more frequent in control fish, and prominent rough endoplasmic reticulum more frequent in experimental fish. This may correlate with the slightly greater chloride cell numbers observed on the filaments and at the base of the lamellae in long-term acid-exposed trout. Cells in this position are thought to be younger (Conte and Lin 1967; Zenker et al. 1987). Therefore the actual number of "healthy," fully functioning chloride cells may have been greater in long-term acid-exposed fish. Whether this factor alone was responsible for the partial recovery of J_{in} values remains unresolved.

In the present study, the fish reduced $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ immediately upon exposure to pH 4.8 and maintained this reduction throughout the 3-mo experiment (Audet et al. 1988). We originally hypothesized that this response to sublethal pH could be the consequence of a decrease of membrane permeability through endocrine modulation and/or the inhibition of the exchange diffusion component (Na^+/Na^+ and Cl^-/Cl^- exchange) at the gills. In the model presented by McDonald and Prior (1988) on branchial exchanges in rainbow trout, simple passive effluxes (not involving exchange diffusion) would be achieved largely through tight junctions on paracellular channels. This aspect of membrane permeability was not surveyed by the present study. However, thickness of the filaments did tend to increase under chronic acid exposure, although the response was not immediate. Thickening probably resulted from the proliferation of undifferentiated epithelial cells, as chloride cell numbers did not change and alterations in mucous cell numbers did not parallel the changes in filamental thickness. Filamental thickening has been documented in other salmonid species exposed to low pH with or without parallel Al contamination (Chevalier et al. 1985; Evans et al. 1988; Brown et al. 1990a; Jagoe and Haines 1990). Brown et al. (1990a) interpreted the response as a way to reduce ion permeability. However, in the present study, this would likely have only a small impact considering the much larger exchange surface offered by lamellae and the fact that diffusion distance on lamellae actually decreased under chronic acid exposure. We conclude that inhibition of exchange diffusion, which would likely have no morphological correlate, was probably the more important contributor to the immediate and sustained reductions in J_{out} values.

The decrease in blood–water diffusion distance in the lamellae during chronic acid exposure was surprising. Such a decrease would tend to facilitate instead of limit passive ion fluxes. However, it is noteworthy that Laurent and Hebibi (1989) observed the same phenomenon after transfer of rainbow trout to ion-poor water. These investigators suggested that the response served to facilitate the transcellular diffusive efflux of NH_3 at a time when Na^+/NH_4^+ exchange was likely inhibited. Low environmental pH is also thought to inhibit Na^+/NH_4^+ exchange (Wright and Wood 1985). The present fish exhibited a sustained increase in NH_3 efflux, apparently a diffusive NH_3 efflux, throughout acid exposure (Audet et al. 1988).

Increased numbers of mucous cells on filaments and lower numbers on lamellae were observed during chronic exposure to pH 4.8. Mucous cell hyperplasia and hypertrophy on filaments have been reported previously in acid-stressed salmonids (Daye and Garside 1976; Jagoe and Haines 1990; Laurent and Perry 1991). McDonald (1983) suggested that increased mucous production during acidification may decrease the loss of ions by passive diffusion.

The major physiological and minor morphological adjustments which occurred under chronic sublethal acid exposure did not confer increased tolerance to acute acid challenge (pH 4.0). Indeed, increases in both $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ were almost twice as large as in naive fish (Audet and Wood 1988). In general, the morphological changes seen after 5 h of acute acid exposure in both groups were similar and in accord with those observed in many previous studies employing severe acid challenge (e.g. Daye and Garside 1976; Jagoe and Haines 1983; Mallatt 1985; McDonald et al. 1991). Damage was not as severe as in some of these studies. Nevertheless, the general epithelial damage, the loss of identifiable chloride cells by necrosis, and the increase in visible mucous cells were the expected

responses. Chloride cells were clearly the most damaged cell type, many being lifted out from the epithelia and difficult to identify under the light microscope. This lifting may be the cause of the increased interlamellar distance observed in naive fish after a 5-h challenge at pH 4. However, increased interlamellar distance was also recorded after 1 d of exposure to pH 4.8 without simultaneous observation of chloride cell necrosis or lifting. Furthermore, interlamellar distance did not increase when acid-preexposed fish were challenged with pH 4.0, even though these fish had a greater concentration of chloride cells at the base of the lamellae. These observations suggest that an additional mechanism must be involved.

Plasma cortisol is considered a good primary stress indicator and plasma glucose a good secondary stress indicator in fish (Donaldson 1981; Wedemeyer and McLeay 1981). In long-term acid-exposed fish, the plasma cortisol concentration rose and stayed elevated for the whole duration of the experiment, in accord with the prolonged elevation in blood glucose (Audet et al. 1988). These observations indicate that the fish remained under a high level of stress throughout the exposure and are in accord with the lack of acclimation. Previous investigations have produced conflicting results about the cortisol response to chronic sublethal acid stress. Most studies have shown a transient rise in cortisol levels (reviewed by Wendelaar Bonga and Balm 1989), while others have shown that the increases were of longer duration (Brown et al. 1984, 1986a, 1986b, 1990b; Tam et al. 1987, 1988; Whitehead and Brown 1989). Tam et al. (1988) also showed that acid exposure results in hypertrophy and activation of corticotropes and interrenal cells. In the present study, the sustained increase in cortisol probably contributed to both the sustained elevation in NH_3 excretion and the hyperglycaemia observed during chronic sublethal acid exposure (Audet et al. 1988). Cortisol is also reported to cause chloride cell proliferation on the gills, thereby increasing ion uptake (Perry and Wood 1985; Laurent and Perry 1990). The present morphological results do not concur; however, it is possible that elevated cortisol levels were important in maintaining chloride cell numbers more or less unchanged and in promoting their transport function in the face of potential inhibition by acidity.

Catecholamines are generally agreed to raise blood glucose (Mazeaud and Mazeaud 1981) and have an as yet controversial role in the control of branchial ion transport (Wood 1991). Concentrations increased only transiently (day 3) during chronic exposure in contrast with the sustained changes in plasma cortisol, glucose, ions, and branchial Na^+ and Cl^- fluxes. Similarly, catecholamines exhibited little response to acute pH 4.0 challenge, in contrast with the other parameters. While this suggests that catecholamines were relatively unimportant in the observed phenomena, it must be noted that control levels were high and the data variable (cf. Witters et al. 1991) in the present study. Catecholamine release is known to occur extremely rapidly when fish are disturbed (Mazeaud and Mazeaud 1981); the present data were probably confounded by the stress of capture, anaesthesia, and caudal sampling such that small variations due to acid exposure would have been undetectable. However, Witters et al. (1991), using a cannulation approach to avoid sampling disturbance, saw no change in plasma adrenaline or noradrenaline when rainbow trout were exposed to pH 5.0 for 2 d. Similarly, Ye et al. (1991) found that catecholamine levels did not change in rainbow trout exposed to pH 4.0 (for up to 72 h) until immediately before death, when they increased abruptly. There is clearly a need for longer term studies on this question employing sampling by cannulation.

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