Long-Term Sublethal Acid Exposure in Rainbow Trout (*Salmo gairdneri*) in Soft Water: Effects on Ion Exchanges and Blood Chemistry

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Audet, C., R. S. Munger, and C. M. Wood. 1988. Long-term sublethal acid exposure in rainbow trout (*Salmo gairdneri*) in soft water: effects on ion exchanges and blood chemistry. Can. J. Fish. Aquat. Sci. 45: 1387–1398.

Long-term sublethal acid exposure (3 mo, pH 4.8) in adult rainbow trout (*Salmo gairdneri*) acclimated to artificial soft water (Ca^{2+} = 50, Na^+ = 50, Cl^- = 100 $\mu eq \cdot L^{-1}$) caused transient net losses of Na^+ and Cl^-. Net flux rates of both ions were returned to control levels after 30–52 d of acid exposure through a new equilibrium between unidirectional influx and efflux, where both were lower than control rates. K^+ balance remained negative and Ca^{2+} balance at zero throughout the exposure. No changes in net acidic equivalent flux occurred, indicating the absence of acid–base disturbance, but ammonia excretion increased over time. Muscle K^+, Na^+, and Cl^- fell and Ca^{2+} increased. Plasma Na^+, Cl^-, and osmolality decreased, while plasma protein, glucose, and blood hemoglobin increased during the first few weeks of acid exposure. Plasma K^+ and Ca^{2+} did not change. General stabilization of plasma parameters occurred in concert with the stabilization of Na^+ and Cl^- flux rates, but no recovery to control levels was observed for any of them. We conclude that despite this stabilization at a new steady state, rainbow trout were physiologically affected in a deleterious manner by chronic sublethal acid exposure in soft water.

Chez la truite arc-en-ciel (*Salmo gairdneri*) acclimatée à de l'eau douce à faible teneur ionique (Ca^{2+} = 50, Na^+ = 50, Cl^- = 100 $\mu eq \cdot L^{-1}$), l'exposition prolongée à pH acide sous-létal (3 mo, pH 4,8) a causé, dans un premier temps, des pertes nettes de Na^+ et de Cl^-. Les flux nets des deux ions ont été rétablis après 30 à 52 jours d'exposition, ceci à travers un nouvel équilibre des flux unidirectionnels d'entrée et de sortie, les deux étant plus faibles que chez les contrôles. Le flux net de K^+ s'est maintenu négatif et celui de Ca^{+2} près de zéro tout au long des 3 mo d'exposition. Les flux nets d'équivalents acides n'ont pas été affectés, indiquant que l'exposition à l'acidité n'a pas créé de déséquilibre acido-basique. Cependant, l'excrétion d'ammoniaque a augmenté en fonction du temps d'exposition. Dans le muscle, on a observé des pertes de K^+, de Na^+ et de Cl^- mais une augmentation du Ca^{+2}. Durant les premières semaines d'exposition, le Na^+, le Cl^- et l'osmolalité plasmatiques ont décru alors que les concentrations plasmatiques de protéines, de glucose et la concentration d'hémoglobine sanguine augmentaient. Le K^+ et le Ca^{+2} plasmatiques n'ont pas été affectés. Plusieurs paramètres plasmatiques se sont stabilisés de concert avec la stabilisation des flux de Na^+ et de Cl^- mais aucun retour aux niveaux contrôles n'a été observé. Nous concluons, qu'en dépit du fait qu'un nouvel équilibre physiologique ait été atteint durant l'exposition prolongée à un pH acide sous-létal, la truite arc-en-ciel est physiologiquement affectée par l'acidité lorsque ce stress est appliqué en eau douce à faible teneur ionique.

Received November 19, 1987
Accepted May 11, 1988
(J9500)

Reçu le 19 novembre 1987
Accepté le 11 mai 1988

Acidification of freshwater habitats in Canada, as well as in numerous other northern countries, now endangers many natural fish populations. There have been many studies on the toxic *lethal* effects of acute acid exposure in fish. In salmonids, it is now generally recognized that the toxic mechanism of acute lethal exposure (pH < 4.5) results from ionoregulatory disturbance (reviewed by Leivestad et al. 1976; Muniz and Leivestad 1980; Wood and McDonald 1982; Howells et al. 1983; McDonald 1983a; Wood 1988a). This conclusion may only be true when fish are held in soft water (low ionic strength and calcium content), but such conditions are representative of freshwater habitats threatened by the acidification process.

On the other hand, the effects of chronic *sublethal* acid exposure (pH 4.5–6.0) are not so well known, and no clear pattern has yet emerged. Some studies seem to indicate that adaptation to such an environment is possible (e.g. McWilliams 1980; Fraser and Harvey 1984; Leino and McCormick 1984; Wood et al. 1988a; Sadler and Lynam 1986), while others have demonstrated chronic ionoregulatory disturbance, growth impairment, increased levels of stress indicators, and/or a reduction of reproductive capacity (e.g. Menendez 1976; Lee et al. 1983; Saunders et al. 1983; Brown et al. 1984; Giles et al. 1984; Johnston et al. 1984; Rodgers 1984; Haya et al. 1985; Lacroix 1985; Scherer et al. 1986; Tam and Payson 1986; Weiner et al.

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1986; Jones et al. 1987; Lacroix and Townsend 1987; Tam et al. 1987; Wood et al. 1988c). The situation is further complicated by the fact that only a few of these studies (Saunders et al. 1983; Fraser and Harvey 1984; Haya et al. 1985; Johnston et al. 1984; Lacroix 1985; Weiner et al. 1986; Lacroix and Townsend 1987; Wood et al. 1988a, 1988c) were performed in soft water (i.e. $[Ca^{2+}] < 200$ $\mu eq \cdot L^{-1}$); the remainder were performed in either hard water or water of unstated composition.

In view of the fact that large areas of natural soft water in eastern Canada are now acidified to the pH 4.5–6.0 range (Kelso et al. 1986; Minns and Kelso 1986), there is a clear need to improve our knowledge on the physiological effects of long-term sublethal acid exposure in soft water. The work presented here is part of an overall study on the ionoregulatory, endocrine, and branchial morphological responses of adult rainbow trout (*Salmo gairdneri*) to long-term (12 wk) sublethal acid exposure (pH 4.8) in flowing soft water. The artificial soft water used was chosen to duplicate the composition of typical acid-threatened lakes on the Canadian Shield. The present paper describes unidirectional Na^+ and Cl^- fluxes and net fluxes of Na^+, Cl^-, Ca^{2+}, and K^+, titratable acid, ammonia, and acidic equivalents measured periodically in the same animals throughout the 12 wk of exposure. The goal was to test whether ionoregulatory disturbance, and not acid–base disturbance, was the major consequence of long-term sublethal acid exposure, as is the situation during short-term lethal exposure (see Wood 1988a for review), and to characterize the nature of any disturbances which occurred. We also looked at the general internal physiological status of these fish to see if physiological steady state was achieved during chronic acid exposure.

Methods

Fish Holding and Acid Exposure

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 first acclimated for 2–4.5 mo to artificial soft water (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). None of the fish were in breeding condition.

ASW was generated by dechlorination of tap water, followed by deionization using a reverse osmosis system (Culligan Aqua-Clear MP1000), and final supplementation of the product with $CaCl_2$ and NaCl stock solutions via a peristaltic pump. Approximately 30 fish were kept in a 450-L tank during ASW acclimation and during each experiment. ASW was delivered via a continuous flowing system providing 90% replacement of the tank volume every 24 h, as calculated from the nomogram of Sprague (1973). The fish were fed once a week to satiation with trout pellets (40% floating trout feed grower pellets, Martin Feed Mills Limited). In view of the limited volume of our ASW supply, this feeding regime was selected as one adequate to maintain body weight (see Results) while minimizing contamination of the holding water. The time of feeding was not kept constant relative to sampling periods, but the fish were not fed in the 48 h prior to any experiment or sampling.

After at least 2 mo of acclimation to ASW at pH ≈ 6.5, the pH was lowered to 4.8. Acidified water was obtained by titration of the flowing soft water with 0.5 N H_2SO_4 using a Radiometer titrator (TT80), pH meter (pHM 82), and combination glass-reference electrode (GK2401C) which opened and closed a magnetic valve to deliver acid to a header tank. The soft water in the header tank was vigorously bubbled with air and passed through a gas stripping column prior to entering the fish holding tank in order to prevent P_{CO_2} elevations. Water pH in the holding tank was continuously recorded on a chart recorder via an independent pH meter (Fisher 119) coupled to a Cole-Palmer (5658-10) combination glass-reference electrode. Water samples were taken weekly during the two first experiments and daily for the other three to monitor Na^+ and Ca^{2+} levels. Control ASW pH varied within ±0.3 unit, acidified ASW pH within ±0.1 unit, and Ca^{2+} and Na^+ concentrations within ±9 $\mu eq \cdot L^{-1}$.

Experimental Design

The basic experimental protocol of 2–4.5 mo of ASW acclimation at pH ≈ 6.5 followed by up to 12 wk of pH 4.8 exposure was repeated five times in experiments starting November 1985, April 1986, October 1986, January 1987, and June 1987. This repetition of experiments was necessary because of our limited supply of ASW. The first experiment focussed on repetitive flux measurements in the same 12 fish throughout the 12-wk period (control, 5 h, and 1, 2, 4, 7, 11, 16, 23, 30, 52, and 81 d). Terminal blood samples were taken on day 81. Other fish from this same experiment were utilized in the challenge experiment described by Audet and Wood (1988) after day 81. The other series provided fish for terminal blood and tissue sampling before (control) and at various times during the 12-wk exposure (4 h and 1, 3, 8, 22, 50, and 81 d). Not all experimental times were sampled in each series, but control samples were taken 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 five series were pooled.

A simultaneous exposure of the fish to ASW at neutral pH was not run in parallel to each of the series, in view of the relatively long period allowed for ASW acclimation, and the fact that the experiment was repeated five times. We kept control fish for period varying from 2 to 4.5 mo in ASW and no significant differences were found between our five groups. While trout do not acclimate instantaneously to soft water, adjustment is complete within several weeks (McDonald et al. 1980; McDonald and Rogano 1986). Therefore, we did not expect that additional weeks in ASW would bring any significant changes in the status of the fish.

Flux Measurements

In order to minimize variation and perform accurate backflux correction of the radioisotopic uptake (see below), it was essential that the same 12 fish be tested at each time of flux measurements. Thus the trout used for these measurements were individually coded for identification with plastic wires anchored through the dorsal fin.

At least 24 h prior to a flux determination, the fish were transferred to individual flux boxes of the type described by McDonald (1983b). These consisted of a transparent inner chamber which confined the fish and a dark outer one (with transparent lid) which contained the majority of the water volume (≈7 L). An airlift pump at the rear of the inner chamber recirculated the water and maintained oxygen saturation. Mix-

ing was further aided by perimeter aeration of the outer box. During the adjustment period, each box received a constant flow of water (1.5 L·min^{-1}) at the appropriate pH. All flux measurements were performed at times between 1100 and 1600 to minimize the influence of diurnal rhythms. At the beginning of the experiments, flow of water through the box was stopped (aeration and temperature being maintained), the volume set to a precise figure, and 5 μCi (185 kBq) of $^{22}Na^+$ and 5 μCi of $^{36}Cl^-$ (New England Nuclear) added to each box. We allowed 30 min for the mixing of radioisotopes and then started sampling (time 0). Every hour for 5 h, 60 mL of water was sampled from each box. From these 60 mL, different aliquots were prepared: 10 mL for titratable alkalinity (kept at 5°C and analyzed within 12 h), 2 × 5 mL for gamma counting, 2 × 5 mL for scintillation counting, and 10 mL to which we added one drop of concentrated Aristar HNO_3 before storage at 5°C for subsequent Cl^- and cation measurements. The rest of the water was used for immediate pH measurement (Radiometer PHM82 meter plus GK2401C electrode), frozen at −20°C, and later used for ammonia determination. The water pH of each box was therefore maintained at the experimental value by adding an appropriate volume of 0.02 N H_2SO_4 once every hour. All flux measurements were based on average values for the 5 h of each measurement period.

The net fluxes (J_{net}) of Na^+, Cl^-, K^+, and Ca^{2+} were given by the changes of the total amount of ions in the water volume. J_{in} for Na^+ and Cl^- was measured by the disappearance of radioisotopes from the water, while J_{out} for Na^+ and Cl^- was given by the subtraction of J_{in} from J_{net}. Net losses by the fish have a negative sign and net gain a positive sign. The different equations were as formulated in Hōbe et al. (1984) except for J_{in}, as we had to apply a significant backflux correction. As the same fish were subjected to radioisotope flux measurements 12 times during the 81-d exposure, they accumulated significant amounts of radioactivity internally. The efflux of this radioactivity ("backflux") during subsequent flux measurement periods would thereby tend to reduce the calculated J_{in} values below real values. We therefore applied the backflux correction equation of Maetz (1956):

$$(1)\quad J_{in} = \frac{V_{ext}\cdot((C_i - C_f) - SA_{int}\cdot(X_i - X_f))}{SA_{ext}\cdot W\cdot t}$$

where C_i and C_f are initial and final radioactivities in the water (counts per minute (cpm) per millilitre) during the flux period in question, X_i and X_f are the initial and final concentration in the water (microequivalents per millilitre) of the ion in question, SA_{int} and SA_{ext} are the mean internal and external specific activities (cpm per microequivalent) over the period (see equations 2 and 3), V_{ext} is the volume of the system (millilitres) corrected for sampling deficits, t is the elapsed time (hours), and W is the body weight (kilograms). SA_{ext} and SA_{int} are given by

$$(2)\quad SA_{ext} = \frac{1}{2}\cdot\left(\frac{C_i}{X_i} + \frac{C_f}{X_f}\right)$$

$$(3)\quad SA_{int} = \frac{\Sigma C_i + \Sigma C_f}{2\cdot V_{int}\cdot X_p}$$

where ΣC_i and ΣC_f are the summated $^{22}Na^+$ or $^{36}Cl^-$ radioactivity (cpm) accumulated by the fish from the water at the start and end of the flux period, respectively, V_{int} is the internal radiospace, and X_p is the plasma concentration of the ion in question. V_{int} was taken as 280.35 mL·kg^{-1} for Na^+ and 252.65 mL·kg^{-1} for Cl^-, from the measurements of Wood (1988b) in rainbow trout. Plasma ion levels for each fish at any time were based on regression lines between the control value and the measured terminal value.

In order to estimate ΣC_i and ΣC_f, it was necessary to calculate the amount of radioactivity lost since the previous determination. We used the formula

$$(4)\quad \text{cpm lost} = (\text{cpm}\cdot\text{fish}^{-1})\cdot e^{(-J_{out}^{Na^+}\cdot W\cdot SA_{int}\cdot h)}$$

where cpm·fish^{-1}, $J_{out}^{Na^+}$, and SA_{int} were as measured at the end of the previous flux period.

At the end of the experiment (day 81), we measured SA_{int} for Na^+ and Cl^- in the terminal plasma samples for comparison with the value calculated by the above procedures (equations 3 and 4). For $SA_{int}^{Na^+}$, we did not find any difference between the calculated and the measured values. However, we underestimated the $SA_{int}^{Cl^-}$, calculated values being 56% of measured. Model calculation based on these discrepancies indicate that the maximum possible error would have occurred on day 16 (8% underestimate of Cl^- influx); errors on other days would have been far less. We therefore consider these possible errors relatively unimportant.

Water cations (Na^+, K^+, and Ca^{2+}) were measured by atomic absorption (Varian 1275-AA). Cl^- was measured by coulometric titration (Buchler chloridometer) or by a colorimetric method based on the formation of ferric thiocyanate proportional to the chloride concentration in the water sample (Zall et al. 1956). $^{22}Na^+$ was measured by gamma counting (Nuclear Chicago model 1085), and combined $^{22}Na^+$ and $^{36}Cl^-$ cpm were determined by liquid scintillation counting (LKB 1217, Rackbeta). $^{36}Cl^-$ cpm were obtained by subtraction after correcting for differences in efficiency of $^{22}Na^+$ counting by the two instruments.

Total ammonia measurements utilized the salicylate–hypochlorite reaction of Verdoux et al. (1978). Titratable alkalinity was measured as previously described by McDonald and Wood (1981). Values were corrected for 0.02 N H_2SO_4 addition to the flux boxes during the experiments. The net titratable acid flux was calculated from the change in titratable alkalinity. The net flux of acidic equivalents was calculated as the sum of the net titratable acid and the net ammonia flux, signs considered. As McDonald and Wood (1981) pointed out, the method does not distinguish between ammonia measurement in the NH_3 and NH_4^+ forms, nor between the net excretion of acidic equivalents and the net uptake of basic equivalents, or vice versa. Fortunately, this does not matter in terms of the net acid–base budget of the fish.

Blood and Tissue Chemistry

Blood and tissue samples at various times during the 12-wk exposure were taken from fish in the other four experimental series and on day 81 from the first series. Each experiment started with about 30 fish. At sampling, fish were individually anesthetized in 0.01% MS-222 (pH adjusted to 6.5 or 4.8 as appropriate with KOH), and blood was withdrawn immediately by caudal puncture. At some sample times (control and 1, 3, 8, and 81 d), white muscle samples were also excised from the epaxial mass below the dorsal fin. All samples were taken at times between 1330 and 1700 to minimize the influence of diurnal rhythms.

Plasma was separated by centrifugation (10 000 × g for 2 min). Plasma cations (Na^+, K^+, and Ca^{2+}) were measured

by atomic absorption (Varian 1275-AA), Cl^- by coulometric titration (Radiometer CMT10 chloridometer), osmolality by vapor pressure osmometry (Wescor 5100B), and plasma proteins by refractometry (American Optical TS meter). The calibration of the refractometer had earlier been validated against the Biuret method for plasma protein (Sigma procedure No. 540). Hemoglobin and glucose were measured using commercial kits (Sigma procedures No. 525 and No. 16-UV). Hematocrit was measured by centrifugation (5000 × *g* for 5 min) and mean cell hemoglobin concentration (MCHC) as the ratio of hemoglobin concentration to hematocrit.

Muscle water content was determined by drying to a constant weight at 85°C. The same tissue samples were subsequently analysed for Na^+, K^+, Ca^{2+}, Cl^-, and Mg^{2+} by instrumental neutron activation using the facilities of the McMaster Nuclear Reactor (cf. Spry et al. 1988 for methodology).

Statistical Analysis

In the flux series, for which the same 12 fish were always used, the number of fish decreased from 12 to 7 due to mortality in the two last sampling periods (52 and 81 d). However, for the sampling days preceding day 52, we did not find any significant differences between the fish which survived through the end of the experiment versus the ones that died (*t*-test). The data from all fish were therefore pooled and used for subsequent analysis.

Data have been routinely expressed as mean ± SEM (N), where N represents the number of fish. All the data were analysed by nonbalanced one-way ANOVA, $p \leq 0.05$ (time of exposure tested as the source of variation), followed by a Tukey–Kramer test of comparison of means (Sokal and Rohlf 1981) with $\alpha = 0.05$. Normality of the data and homogeneity of variances were checked by Kolmogorov–Smirnov and F_{max} tests, respectively. All the data were normally distributed; however, in some cases, transformations were required to obtain homogeneity of variances. Thus, data for $J_{out}^{Cl^-}$ and titratable acid flux were transformed as $(x + 325)^2$ and $\log_{10}(x)$, respectively, plasma Cl^- and plasma glucose as x^4 and $1/x$, respectively, and data for muscle Ca^{2+} and K^+ as $\log_{10}(x)$ and x^4, respectively, prior to ANOVA. Arithmetical means ± SEM are shown on the figures. All tried transformations failed to obtain homogeneity of variances for $J_{net}^{Ca^{2+}}$, acidic equivalents flux, and muscle Na^+. In these cases, ANOVA was applied for overall effects and then the Games and Howell test of comparison of means, designed for heterogeneous variances, was applied according to Sokal and Rohlf (1981).

Results

Mortalities

From the 12 rainbow trout used for flux measurements in the first experimental series, 4 died between the fourth and the seventh week of acid exposure and 1 more between the seventh and the twelfth week. Five other fish from the first series died between the fourth and the twelfth week. There were no mortalities in the subsequent four experimental series.

Ion Exchanges

The main effect of sublethal acid exposure (pH 4.8) in adult rainbow trout was a transient ionoregulatory disturbance reflected in net losses of both Na^+ and Cl^- (Fig. 1). ANOVA showed that J_{in} and J_{out}, as well as J_{net}, for both Na^+ and Cl^- were influenced by the length of sublethal acid exposure. Net Na^+ and Cl^- fluxes became negative and significantly different from the control levels on the first day of exposure, but were completely restored after 30–52 d of acid exposure. However, this was achieved through a new equilibrium between J_{in} and J_{out}, rather than by a return of J_{in} and J_{out} to control levels.

Disturbance of $J_{net}^{Na^+}$ occurred very rapidly (Fig. 1a), with a changeover to negative balance in the first 5 h of acid exposure. The net loss of Na^+ was the direct consequence of a 70% inhibition of J_{in} over first 16 d of exposure. A partial recovery of $J_{in}^{Na^+}$ subsequently occurred, but $J_{in}^{Na^+}$ was still significantly below the control values even after 81 d of exposure. Net Na^+ loss caused by this inhibition of $J_{in}^{Na^+}$ was partly compensated by a ≈40% decrease of $J_{out}^{Na^+}$ (Fig. 1a). J^{Na^+} was significantly reduced in the first 5 h of acid exposure without any subsequent significant recovery over 81 d of acid exposure. The recovery of Na^+ net flux observed during chronic acid exposure was therefore the result of a partial recovery of $J_{in}^{Na^+}$ coupled with a persistent inhibition of $J_{out}^{Na^+}$.

Overall variations of $J_{net}^{Cl^-}$ were similar to those observed for Na^+ (Fig. 1b). Significant reduction occurred by 24 h and $J_{net}^{Cl^-}$ flux remained negative through 23 d of acid exposure. At day 30, net flux became positive and no longer different from the control. The negative net flux was the result of a significant inhibition of $J_{in}^{Cl^-}$ (Fig. 1b). Within 5 h of exposure, $J_{in}^{Cl^-}$ was reduced by half and was kept around 30% of the control value during the first days of exposure. Recovery occurred gradually and $J_{in}^{Cl^-}$ was equal to about 75% of control value from 30 d of exposure through the end of the experiment. Changes in $J_{out}^{Cl^-}$ (Fig. 1b) were less marked than in $J_{out}^{Na^+}$, a significant reduction from control value being observed only at 2, 16, and 81 d of chronic acid exposure.

No significant variations were observed for either Ca^{2+} or K^+ net fluxes. On average, fish were in balance for their Ca^{2+} exchange with the environment, overall mean Ca^{2+} fluxes being equal to 1.2 ± 3.94 $\mu eq \cdot kg^{-1} \cdot h^{-1}$ (133). The overall mean of K^+ fluxes was equal to −15.1 ± 0.9 $\mu eq \cdot kg^{-1} \cdot h^{-1}$ (134). There was no major effect of acid exposure on net fluxes of acidic equivalents (Fig. 2). Overall ANOVA indicated a significant treatment effect but the a posteriori test showed a significant difference only between day 81 and day 2, with neither being different from the control value. Although the net balance of acidic equivalents was little affected, this was not true for the separate components of that measurement. Thus, ammonia excretion continuously rose with time, increasing by 75% after 81 d of acid exposure compared with the control value (Fig. 2). The story for titratable acidity was less clear although the general trend was similar to that for ammonia excretion. A transient decrease of titratable acid uptake was found at 2 d. Significant increases occurred after 52 and 81 d of acid exposure.

Blood and Tissue Chemistry

The plasma ion data confirmed the results of whole-body flux measurements. Almost all measured parameters were affected by chronic acid exposure, but except for plasma glucose levels, all these deviations from control values stabilized before the end of the experiment. However, in no case was there any recovery to the control values.

Plasma Na^+ and Cl^- levels underwent major changes during chronic acid exposure. After 8 d, plasma Na^+ concentration was significantly reduced, and stabilized around 86% of the control value after 22 d of acid exposure (Fig. 3a). A similar

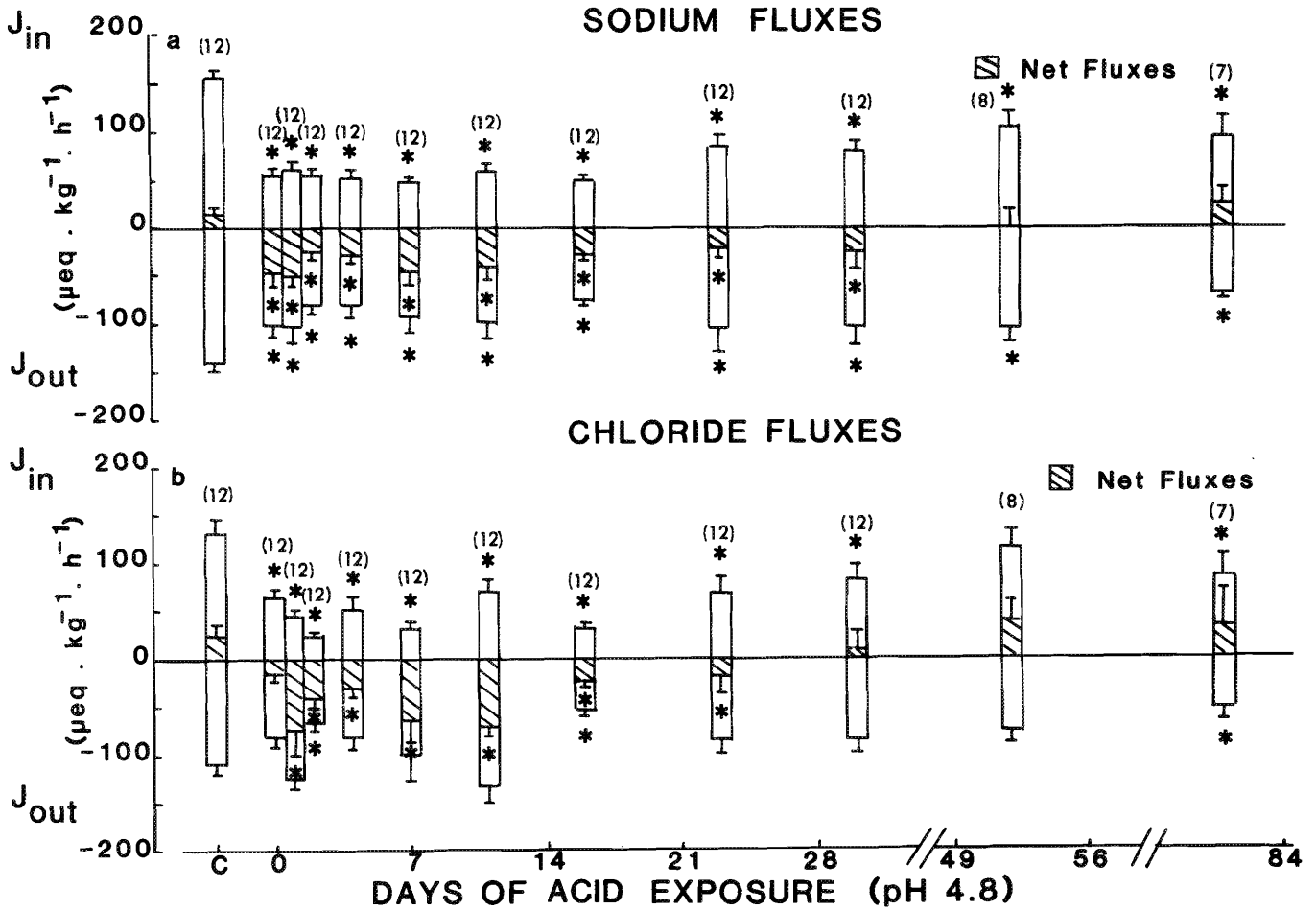

FIG. 1. (a) Sodium and (b) chloride fluxes during long-term sublethal acid exposure in rainbow trout. Significant effects of the length of acid exposure occurred for each set of data (ANOVA, $p \leq 0.01$: $J_{net}^{Na^+}$, $J_{in}^{Na^+}$, $J_{net}^{Cl^-}$, $J_{in}^{Cl^-}$, and $J_{out}^{Cl^-}$; $p \leq 0.05$, $J_{out}^{Na^+}$). Means ± SEM; numbers in parentheses indicate the number of fish; asterisks indicate the days of exposure for which the results are significantly different from the control values. The complete results of the mean comparison test are presented below. The numbers represent the days of exposure, and single lines underscore days between which there was no significant difference ($p \leq 0.05$). C = control experiment prior to acid exposure.

$J_{net}^{Na^+}$	1	0	7	11	4	16	2	30	23	52	C	81
$J_{in}^{Na^+}$	7	16	4	0	2	11	1	30	23	81	52	C
$J_{out}^{Na^+}$	C	23	52	30	1	0	11	7	4	3	16	81
$J_{net}^{Cl^-}$	1	11	7	2	4	16	23	0	30	C	81	52
$J_{in}^{Cl^-}$	2	16	7	1	4	23	11	0	30	81	52	C
$J_{out}^{Cl^-}$	11	1	C	30	7	4	23	0	52	2	16	81

pattern occurred for plasma Cl^- (Fig. 3b). The final Cl^- decrease was slightly greater (around 20% of the initial level) than for Na^+. Plasma levels of Ca^{2+} (Fig. 3c) were not affected by chronic acid exposure, while only a transient increase of plasma K^+ (Fig. 3c) was observed during the first hours of acid exposure.

A short-term 5% decrease of plasma osmolality occurred in the first hours of acid exposure (Fig. 4a). A significant drop of similar magnitude was also observed after 8 d. Thereafter, osmolality remained constant at a level about 20 mosm·kg^{-1} below control. This was a smaller relative and absolute decrease than in plasma Na^+ and Cl^- levels (Fig. 3a, 3b). The plasma protein concentration changed in the opposite direction (Fig. 4b). From day 1 onwards, plasma protein levels were significantly elevated by 20–30% throughout the period of acid exposure, except at day 50 where the results were anomalous, perhaps due to the low sample number. Plasma glucose increased dramatically over time, being significantly different from the control group after only 4 h of exposure and reaching threefold to fivefold the control level at days 22–81 (Fig. 4c).

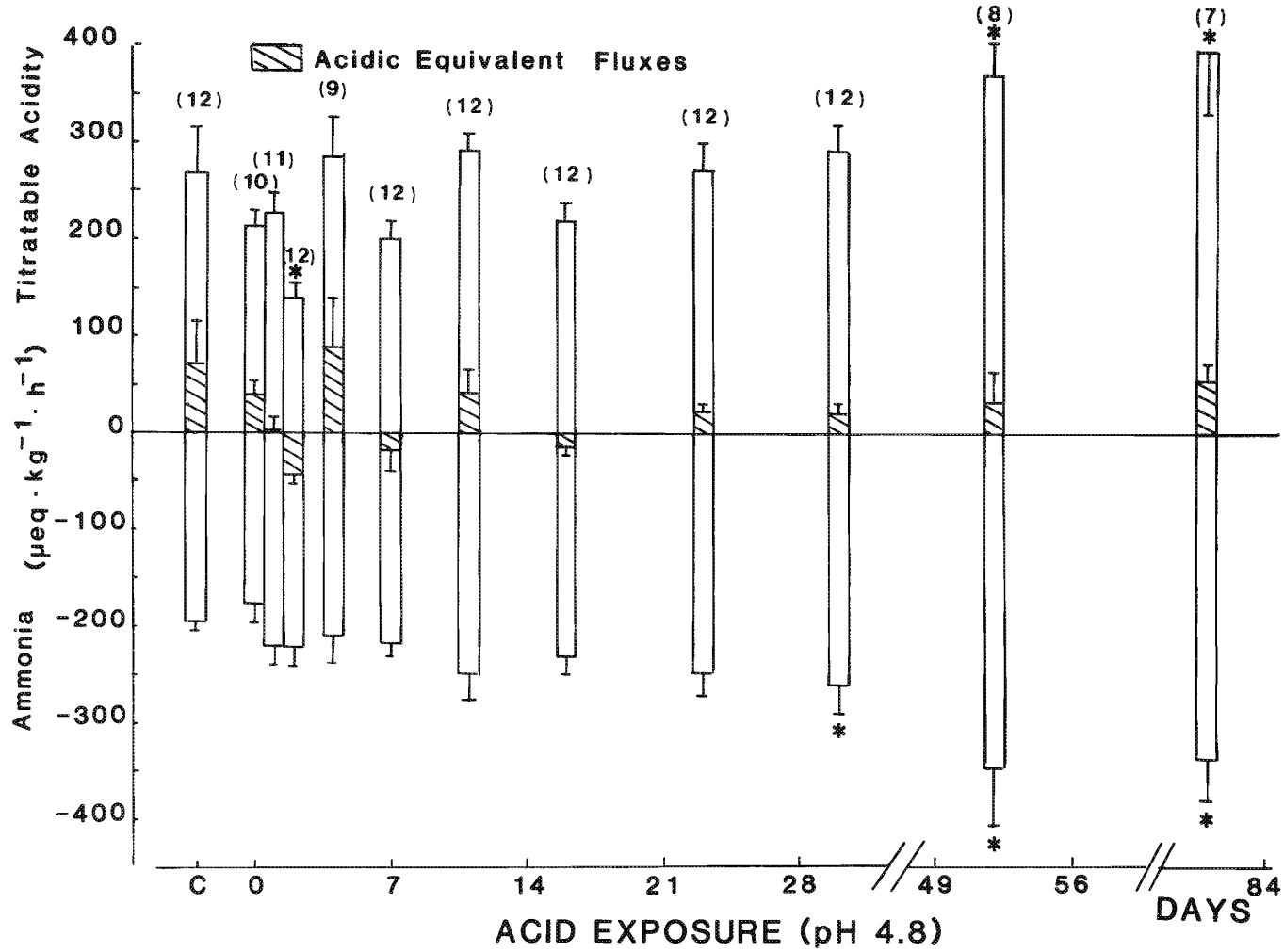

FIG. 2. Net acidic equivalent, ammonia, and titratable acidity fluxes during long-term sublethal acid exposure in rainbow trout. Significant effects of length of acid exposure were found for each parameter (ANOVA, $p \leq 0.01$). See legend to Fig. 1 for other details.

$J_{net}^{Amm.}$ 52 81 30 11 23 16 2 1 7 4 C 0

$J_{net}^{Tit.\ ac.}$ 2 7 16 0 1 C 30 23 4 11 52 81

J_{net}^{H+} 2 7 16 1 30 23 52 11 0 81 C 4

Hemoglobin concentration in the blood increased by about 25% under chronic acid exposure (Fig. 5a). A first increase was observed at day 3 and a second one at day 22 after which hemoglobin concentration remained relatively constant through the end of the experiment. Rather surprisingly, there were no significant effects on hematocrit (Fig. 5b) which remained stable at an overall average of 36.0 ± 0.8% (90) through the whole experiment. The net result of these changes was an increase of the MCHC index during chronic acid exposure (Fig. 5c). Such changes in MCHC suggest either a shrinking of red blood cells or an increase in their hemoglobin content.

Ion concentrations in epaxial white muscle changed over time under chronic acid exposure (Table 1). The main muscle tissue electrolyte, K^+, was not affected during the first week of acid exposure but was reduced by 20% after 81 d. Mg^{2+} concentration was not affected. Significant effects of the length of exposure on muscle Na^+ and Cl^- were found, both falling by 40–50% by day 81. However, the a posteriori test demonstrated a significant difference only for Cl^-. Muscle Ca^{2+} was stable during the first week, but increased by 67% after 81 d of exposure.

No significant effects of chronic acid exposure on either weight (overall mean of 0.239 ± 0.005 kg (88)) or white muscle water content (overall mean of 79.9 ± 0.2% (50)) were observed.

Discussion

The present study is the first to assess the chronic physiological responses of adult salmonids to long-term *sublethal* acid exposure in soft water under controlled laboratory conditions. The whole-body flux measurements showed that ionoregulatory disturbance is the main toxic effect of such exposure. No disturbance of net acidic equivalent exchange with the environment is associated with such conditions, and therefore, internal acidosis should not occur. This confirms results already

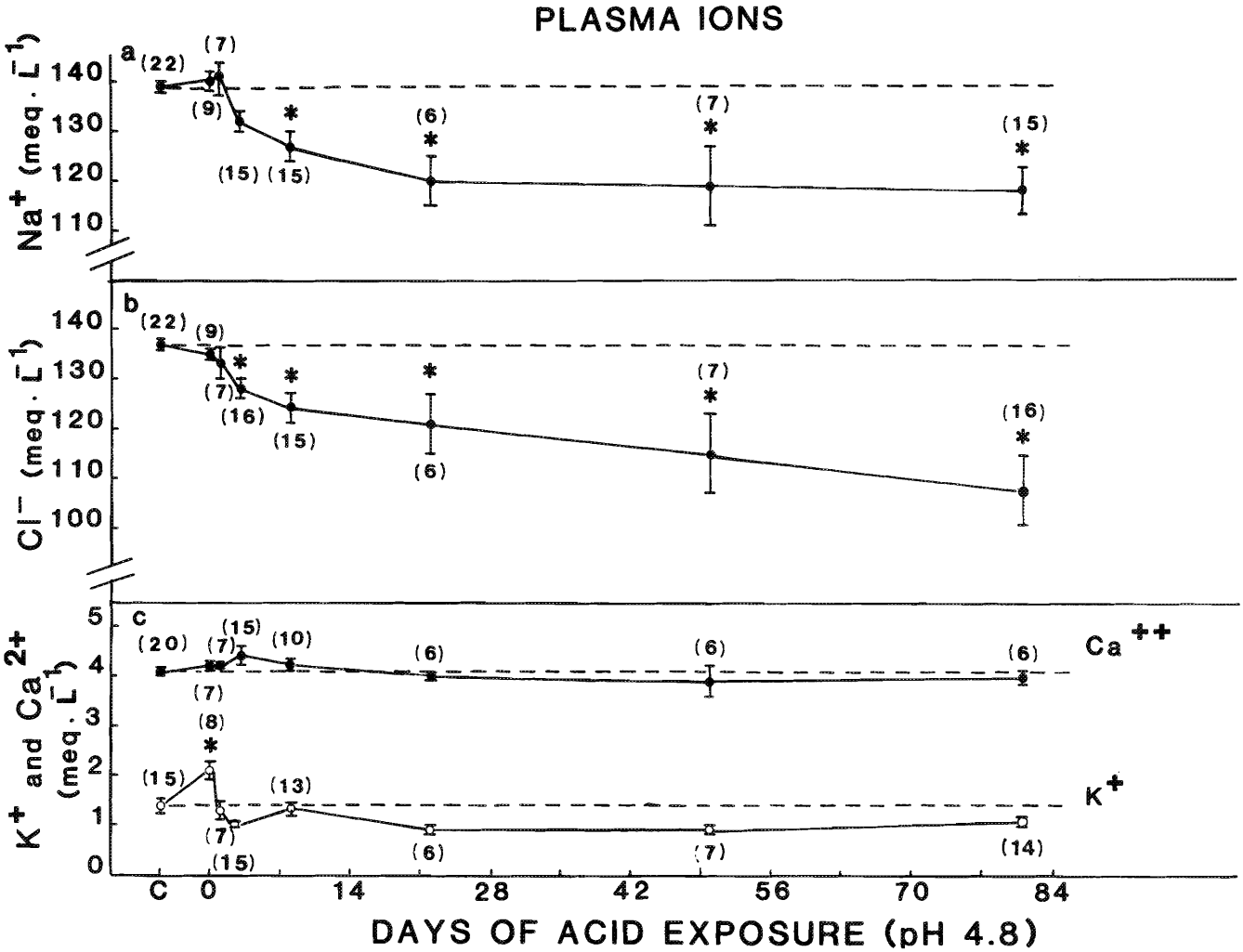

FIG. 3. Plasma levels of (a) sodium, (b) chloride, and (c) potassium and calcium during long-term sublethal acid exposure in rainbow trout. Significant effects of length of acid exposure were found for Na^+, Cl^-, and K^+ (ANOVA, $p \leq 0.01$) but not for Ca^{2+}. See legend to Fig. 1 for other details.

Plasma Na^+	50	81	22	8	3	C	0	1
Plasma Cl^-	81	50	22	8	3	1	0	C
Plasma K^+	22	50	3	81	1	8	C	0

obtained under acute *lethal* acid exposure in rainbow trout in soft water (reviewed by Wood 1988a). It has been demonstrated previously that gill fluxes account for the major part of ion losses (around 80%) during lethal exposure (McDonald and Wood 1981; McDonald 1983b; McDonald et al. 1983). Therefore, it is reasonable to believe that the major portion of this disturbance of ionic exchange during sublethal exposure is also located at the gill site.

Both the intensity and the nature of ionoregulatory disturbance observed under chronic acid exposure are different from those under acute lethal exposure in rainbow trout. Under sublethal acid exposure (pH 4.8), $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ are only partially inhibited, while under lethal acid conditions (pH 4.0–4.5), J_{in} of both are almost completely inhibited (McDonald et al. 1983; Audet and Wood 1988). Moreover, a partial recovery of influx occurred under chronic acid exposure, and alterations in J_{out} (see below) were rather different from those seen under acute exposure to more severe pH.

While functional gill models suggest that the chloride cells are the most likely sites of active ionic uptake in freshwater fish (e.g. Evans 1982), this has remained controversial, with other investigators believing that the lamellar respiratory cells are the important transporting cells (e.g. Girard and Payan 1980; Payan et al. 1984). However, more recent work points strongly to the chloride cells as the major sites of active uptake (Perry and Wood 1985; Avella et al. 1987). Na^+/NH_4^+,H^+ and Cl^-/HCO_3^-,OH^- exchanges are thought to occur at the apical border of these cells. It has been proposed (reviews by McDonald 1983a; Wood 1988a) that inhibition of sodium uptake during acid exposure could be the result of a H^+ competition with Na^+ for the transport site and/or a titration of negative charges in Na^+-specific channels, thereby restricting access of the ion to its transport system. By either mechanism, the inhibition of Na^+ uptake would be concentration dependent, thereby explaining the only partial effect of pH = 4.8 relative to more complete inhibition in previous studies at pH = 4.0–

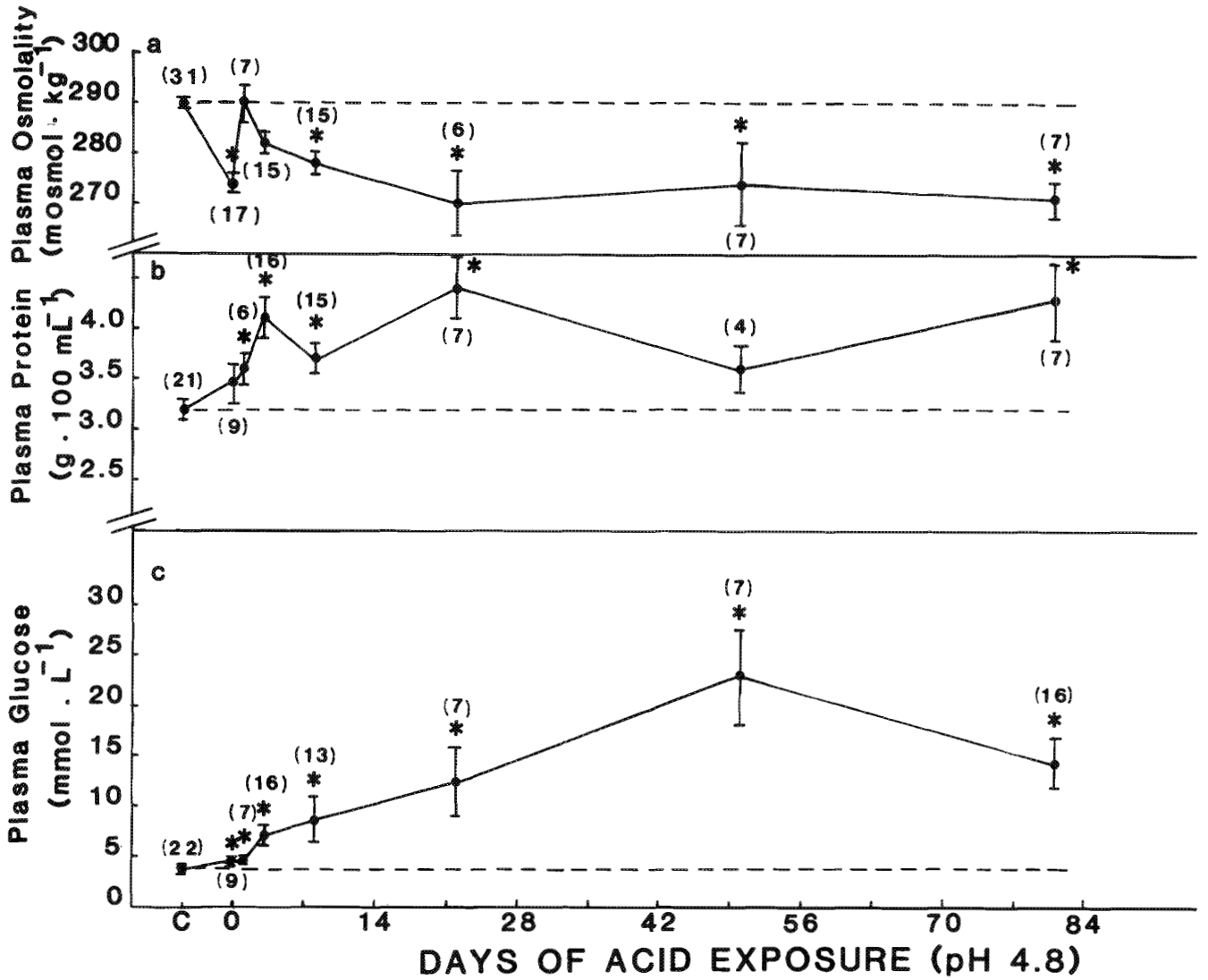

FIG. 4. (a) Plasma osmolality, (b) plasma protein concentration, and (c) plasma glucose concentration during long-term sublethal acid exposure in rainbow trout. Significant effects of length of acid exposure were found for all parameters (ANOVA, $p \leq 0.01$). See legend to Fig. 1 for other details.

Plasma osmolality	22	81	0	50	8	3	C	1
Plasma protein	C	0	50	1	8	3	81	22
Plasma glucose	50	81	22	8	3	1	0	C

4.5. Unfortunately, no satisfactory explanation is yet available for the inhibition of chloride uptake at low pH.

The partial recovery of Na^+ and Cl^- uptake during chronic exposure is particularly interesting. McWilliams (1980) reported a complete recovery of $J_{in}^{Na^+}$ in brown trout (*Salmo trutta*) chronically exposed to a very mild acidity (pH = 6.0) in hard water. Possible explanations include adaptative changes in the affinities of the carriers for Na^+ and Cl^- and/or increases in the amount of carrier available. The recent study of Avella et al. (1987) on rainbow trout suggests that $J_{in}^{Na^+}$ is closely correlated with the abundance of chloride cells in the gills. Leino and McCormick (1984) reported both a proliferation of chloride cells and a change in chloride cell morphology during chronic exposure of fathead minnows (*Pimephales promelas*) to pH = 5.0–5.5 in moderately hard water. It is possible that a similar phenomenon was responsible for the partial recovery of $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ in the present study. Nevertheless, the recovery was insufficient to restore the internal levels of these ions, which at best were stabilized, not corrected.

An important observation is the ability of the fish to reduce $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ immediately and maintain this reduction during chronic sublethal acid exposure. This is different from acute acid stress where net Na^+ and Cl^- losses were mainly the consequence of tremendous initial increases of J_{out} which then decreased over time (for a review, see Wood and McDonald 1982; Wood 1988a). The decrease of J_{out} observed under sublethal acid exposure could be the consequence of a decrease of membrane permeability through endocrine modulation. Wen-

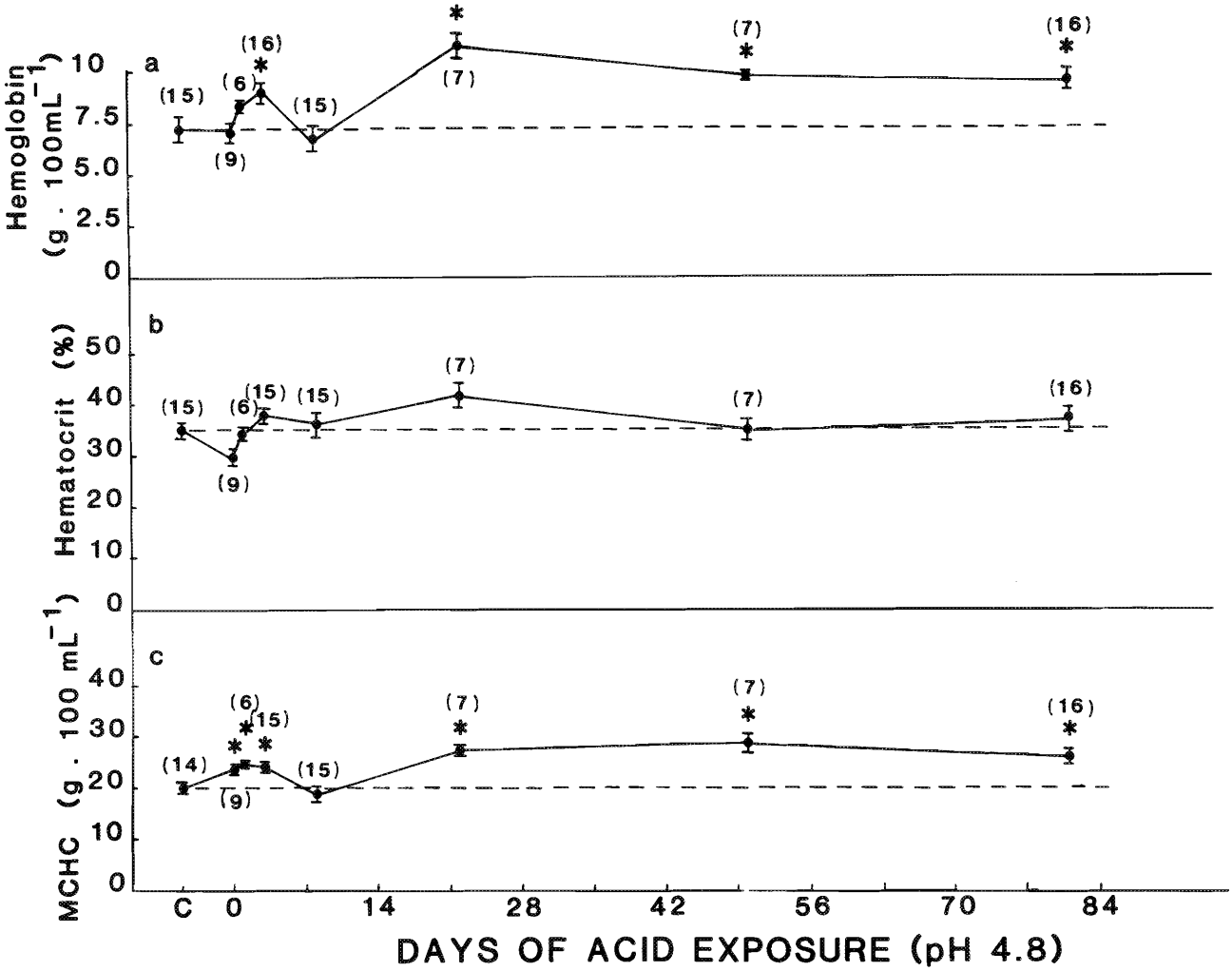

FIG. 5. Blood hematology during long-term sublethal acid exposure in rainbow trout, (a) Hemoglobin concentration; (b) hematocrit; (c) MCHC. Significant effects of length of acid exposure were found for hemoglobin and MCHC (ANOVA, $p \leq 0.01$) but not for hematocrit. See legend to Fig. 1 for other details.

Hemoglobin	8	0	C	1	3	81	50	22
MCHC	8	C	0	3	1	81	22	50

TABLE 1. Ion concentrations (meq·kg^{-1} wet weight) in muscle tissue of rainbow trout during long-term sublethal acid exposure. Means ± SEM; number of fish was always 7; mean values from the same column but not followed by a common letter were significantly different (ANOVA, $p \leq 0.05$; Tukey test, $\alpha = 0.05$).

Length of acid exposure (d)	K^+	Mg^{2+}	Na^+	Cl^-	Ca^{2+}
Control	136.0 ± 1.96 ab	24.0 ± 0.54 a	7.8 ± 0.42 a	10.5 ± 0.60 a	4.3 ± 0.61 a
1	140.5 ± 2.54 a	23.7 ± 0.28 a	6.5 ± 0.43 a	8.9 ± 0.43 ab	4.5 ± 0.35 a
3	127.3 ± 1.49 bc	24.6 ± 0.49 a	6.2 ± 0.44 a	7.7 ± 0.42 bc	4.8 ± 0.59 a
8	132.1 ± 3.17 ab	23.9 ± 0.59 a	6.1 ± 0.15 a	8.3 ± 0.44 ab	4.2 ± 0.30 a
81	109.0 ± 8.57 c	22.4 ± 1.45 a	4.9 ± 1.01 a	5.5 ± 1.04 c	11.4 ± 1.75 b

delaar Bonga et al. (1984a, 1984b) reported that tilapia exhibited an increased synthetic activity of prolactin cells following acute acid exposure. The principal effect of prolactin in freshwater fish is a decrease of cell membrane permeability to water and ions in integuments (see Nicoll 1981 for review). McWilliams (1980) also suggested, based on changes in transepithelial potential measurements, that the success of brown trout to acclimate to a mildy acidic environment should be largely dependent on reduced gill membrane permeability.

There is an alternative hypothesis to explain the decrease of $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ in response to sublethal acid exposure. de Renzis (1975) and Wood et al. (1984) proposed that some Cl^-/Cl^- and Na^+/Na^+ exchanges (exchange diffusion) are present in the gill transport cells. Inhibition of $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$

associated with an equal decrease in $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ could reflect an inhibition of the exchange diffusion component (Na^+/Na^+ and Cl^-/Cl^-) at the gills by external acidity. By this scenario, the fraction of $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ which persisted, and which later showed partial recovery, would be largely as Na^+/NH_4^+, H^+ and Cl^-/HCO_3^-, OH^- exchanges, respectively, while the fraction of $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ which persisted would occur largely by simple diffusion. More detailed kinetic, endocrine, and structural work will be required to determine whether either or both of these hypotheses (permeability reduction and/or exchange diffusion inhibition) are correct.

No major changes in net acidic equivalent flux were found during acid exposure. This confirms that acidosis is not a problem for fish under sublethal acid stress in ASW typical of natural acidic waters (e.g. Wood 1988a). By strong ion difference theory (Stewart 1983), acidic equivalent uptake is a dependent variable constrained by an excess of strong cation loss over strong anion loss. In rainbow trout, this occurs only during acid stress in water of high calcium content (McDonald 1983a, 1983b; McDonald et al. 1983; Wood 1988a). Under low calcium concentrations, as in the present study, chloride loss tends to equal or exceed sodium loss, thereby preventing acidic equivalent uptake.

Ammonia excretion as NH_4^+ is thought to be linked with Na^+ uptake (reviewed by Evans 1982). Wright and Wood (1985) showed that acute acid exposure reduced ammonia efflux by only 30% in rainbow trout but completely inhibited sodium influx, indicating that under acid conditions, NH_3 diffusion must account for the major part of ammonia excretion. At low pH, water becomes an almost infinite sink for NH_3 (by instantaneous interconversion to NH_4^+), so the P_{NH_3} gradient for NH_3 diffusion is greatly elevated. This effect probably contributed to the increase in ammonia excretion observed during our experiment and seen in several other studies under more severe exposure once the initial inhibition was attenuated (reviewed by Wright and Wood 1985). However, ammonia can only be excreted as fast as it is produced, so the long-term progressive increase in ammonia excretion must have had a metabolic basis. The cause is probably increased protein catabolism. Such an hypothesis is supported by our observation of a substantial increase of plasma cortisol which lasted throughout the 81 d of chronic acid exposure in rainbow trout (C. Audet and C. M. Wood, in prep.). It is surprising that in the first hours, no inhibition at all of ammonia efflux was observed in concordance with the immediate partial inhibition of Na^+ uptake. One possible explanation is that inhibited NH_4^+ efflux (via Na^+/NH_4^+ exchange) was stoichiometrically replaced by increased NH_3 efflux (due to the improved diffusion gradient). An alternate explanation is that Na^+/Na^+ exchange was inhibited more by pH = 4.8 than was Na^+/NH_4^+ exchange, which would support the argument about exchange diffusion presented earlier. Again, more data are needed to resolve this point.

The plasma ions and osmolality data followed the trend expected from the flux measurements. There were substantial decreases in plasma Na^+ and Cl^- during the period of negative net fluxes, and the timing of their stabilization agreed extremely well with the recovery of J_{net}. The larger decrease of plasma Cl^- over plasma Na^+ was in accord with more negative net fluxes of Cl^- than Na^+ in the first weeks of pH 4.8 exposure. As plasma osmolality stabilized fairly rapidly compared with plasma ions, with lesser absolute and relative decreases, other parameters must be involved in the maintenance of stable plasma osmotic pressure. Plasma glucose is certainly one of them. Impaired iono-osmoregulation was also found in rainbow trout (Giles et al. 1984) and Arctic char (*Salvelinus alpinus*) (Jones et al. 1987) following 22 and 14 d of sublethal acid exposure in hard water. Other studies pointed to similar ionoregulatory disturbances on a long-term basis during sublethal exposure of various durations in soft water: brook trout, pH 4.6, 1 yr (Muniz and Leivestad, as reported by Leivestad et al. 1976); juvenile Atlantic salmon (*Salmo salar*), pH 4.2–4.7, 4 mo (Saunders et al. 1983); white sucker (*Catostomus commersoni*) and pumpkinseed (*Lepomis gibbosus*), pH < 5.0, 19 d (Fraser and Harvey 1984); Atlantic salmon, white sucker, and alewife (*Alosa pseudoharengus*), pH 4.9–5.2, fish captured in the wild (Lacroix 1985); juvenile Atlantic salmon, pH 4.8–5.6, 50 d, field conditions (Lacroix and Townsend 1987). In contrast, Wendelaar Bonga et al. (1984a) showed recovery of plasma osmolality and almost complete restoration of plasma Na^+ in tilapia after 40 d of exposure to pH 4 or 5 in hard water. McWilliams (1980) also showed a recovery of plasma ions after 40 d of exposure of brown trout to very mild acid conditions (pH 6.0) in hard water. Wood et al. (1988a, 1988b, 1988c) showed no significant changes in plasma electrolytes in brook trout (*Salvelinus fontinalis*) kept for 10 wk at pH = 5.2 or 10 d at pH 4.8 in soft water. Thus, there appear to exist considerable species differences in the ability to adapt to sublethal acid exposure.

No significant decrease of plasma K^+ over time was observed, while the overall mean K^+ net flux was negative. The drop in muscle K^+ without simultaneous changes in muscle water points to an intracellular source for this loss. In a recent field study on long-term sublethal acid exposure in juvenile Atlantic salmon, Lacroix and Townsend (1987) similarly found no changes in plasma K^+ levels. However, plasma K^+ tended to increase through loss from the intracellular fluid in several previous studies on acute acid stress (McDonald and Wood 1981; Hōbe et al. 1984; Stuart and Morris 1985).

No changes in Ca^{2+} fluxes or plasma Ca^{2+} were observed, but muscle Ca^{2+} was dramatically increased after 3 mo of sublethal acid exposure. The meaning of this observation is unclear, although it is known that acid exposure interferes with calcium homeostasis and causes a mobilization of Ca^{2+} from bone in tilapia (Wendelaar Bonga et al. 1987). Previous studies employing more severe acid exposure (pH 4.0–4.5) reported responses ranging from no change in muscle Ca^{2+} (Neville 1979; Sadler and Lynam 1986) to significant increases (Hōbe 1987).

Increased plasma protein concentrations have been almost universally observed in previous studies on acute acid exposure and probably result from changes in plasma volume secondary to ionic disturbances, as discussed by Milligan and Wood (1982). A similar explanation probably applies to the chronic increase in plasma protein observed in the present study. Previous acute studies have also found an increase in hemoglobin levels comparable with that of the present investigation, again resulting from plasma volume contraction. However, the chronic increase in MCHC index is exactly opposite to that seen in any previous acute study. MCHC usually decreases greatly, the expected stress response indicative of cell swelling (Soivio and Nikinmaa 1981) and ionic dilution (Milligan and Wood 1982). The apparent shrinkage during chronic sublethal exposure may be viewed as a mechanism to prevent viscosity increases associated with hemoconcentration; however, its cause is unknown. This effect was not seen during chronic acid exposure of brook trout to pH = 5.2 in soft water (Wood et al. 1988a), but McWilliams (1980) found a decrease in hematocrit

(MCHC not measured) in brown trout chronically exposed to pH = 6.0 in hard water.

The chronic increase in blood glucose level agrees with previous studies in both hard water (Brown et al. 1984; Jones et al. 1987; Scherer et al. 1986; Tam et al. 1987) and soft water (Wood et al. 1988c). Both this plasma glucose elevation and the increased ammonia excretion were probably associated with the high plasma cortisol levels observed during the present study (C. Audet and C. M. Wood, in prep.). High cortisol levels are generally associated with glucose mobilization, stimulation of gluconeogenesis, and protein catabolism (Leach and Taylor 1980, 1982). These data indicate a maintained high level of stress in our fish chronically exposed to low water pH.

In summary, the general conclusion of this study is that rainbow trout are physiologically affected in a deleterious manner by chronic sublethal acid exposure. They undergo large changes in their internal physiological status, most of which are associated with disturbances in ionic exchange with the environment. However, the fish stabilize their internal condition after a few weeks of exposure, reaching a new physiological equilibrium without exhibiting any recovery back towards control levels. What is the biological meaning of such a new steady state? The following paper (Audet and Wood 1988) assesses whether this condition represents true physiological acclimation, in the sense of increased resistance to a more severe exposure.

Acknowledgements

We thank J. LeMoigne, G. G. Goss, and V. Cavdek for their assistance. The research was supported by an NSERC strategic grant in environmental toxicology to C.M.W.

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