Journal of Fish Biology (1997) **51**, 795–806

Tilapia are able to withstand long-term exposure to low environmental pH, judged by their energy status, ionic balance and plasma cortisol

V. J. T. VAN GINNEKEN*, R. VAN EERSEL*, P. BALM†, M. NIEVEEN* AND G. VAN DEN THILLART*

**Animal Physiology, Institute of Evolutionary and Ecological Sciences (EEW), University of Leiden, Gorlaeus Laboratories, Einsteinweg 55, P.O. Box 9502, 2300 RA Leiden, The Netherlands and †Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands*

(*Received 15 March 1996, Accepted 6 May 1997*)

Tilapia *Oreochromis mossambicus* were exposed to water at pH 4·0 for 37 days. The water was acidified slowly over 6 h enabling the animals to acclimate and preventing damage of the gill epithelium. Additional stressors, e.g. aluminium ions and handling stress, were avoided. No mortality or decreased food consumption was observed during the exposure period. No significant changes were observed between the control and acid exposed groups for the energy rich compounds and related parameters, i.e. the adenylate energy charge, the pool of total adenine nucleotides, and the IMP load of white muscle and liver, indicating maintenance of homeostasis. Moreover, there were no significant differences between control groups and acidified groups at 3, 17 and 37 days for plasma sodium, chloride, cortisol and glucose, implying that ionic balance was maintained and that there was no activation of the pituitary–interrenal axis. It is concluded that tilapia can acclimate to water at pH 4·0 when the acidification rate is slow and additional stressors are avoided.

Key words: acid water; tilapia; long-term acidification; energy status; ionic balance; cortisol.

INTRODUCTION

Acid precipitation (acid rain, snowfall), primarily a consequence of emissions of industrial sources, is now prevalent in many parts of the world. The major acidifying components are SO_2, $(NO)_x$ and oxidation products of NH_3. As a consequence of the acidification, fish populations are vanishing, especially in poorly buffered areas like Scandinavia, Canada and the north-eastern U.S.A. (Beamish, 1976; Leivestad & Muniz, 1976). The main causes for the disappearance of fish populations are osmoregulatory stress, disturbances of the acid-base balance, increased gill diffusion distance resulting in hypoxia, reduced reproductive capacity, changes in predator–prey interactions, impaired growth, and an accumulation of aluminium and heavy metal ions in the acidified waters (Fromm, 1980). The resulting physiological and endocrinological changes observed in fish after exposure to water acidification can be studied systematically only in the laboratory. Exposure to sub-lethal acidity causes aberrant physiological functioning which can be indicated as the low pH syndrome, which includes: (1) recruitment of erythrocytes from spleen (Neville, 1979; Milligan & Wood, 1982); (2) increase of the size of the red blood cells (McDonald & Wood,

0022–1112/97/100795+12 $25.00/0/jb970480

1981); (3) increased level of haemoglobin (Nieminen *et al.*, 1982; Audet *et al.*, 1988); (4) temporarily increased level of lactic acid in plasma due to hypoxic stress (Murthy *et al.*, 1981; Ultsch *et al.*, 1981; Nieminen *et al.*, 1982); (5) hyperglycaemia (Nieminen *et al.*, 1982; Brown *et al.*, 1983; Lee *et al.*, 1983; Audet & Wood, 1988; Audet *et al.*, 1988; Tam *et al.*, 1987; Waiwood *et al.*, 1992) and increased levels of glycogen in the liver indicating stimulation of gluconeogenesis (Murthy *et al.*, 1981; Lee *et al.*, 1983); (6) increased levels of cortisol (Brown *et al.*, 1983, 1990; Tam *et al.*, 1987) or increased cortisol turnover rate (Balm, unpublished); (7) changes in the concentrations of adenosinetriphosphate (ATP), adenosinediphosphate (ADP), phosphocreatine (PCr), the adenylate energy charge (AEC) and the pool of total adenine nucleotides (TAN) in different tissues (Macfarlane, 1981; Haya *et al.*, 1985; Waiwood *et al.*, 1992). However, in a recent study on rainbow trout *Oncorhynchus mykiss* (Walbaum), exposure to pH 4·0 caused no major changes in several parameters which were presumed indicators of acid stress (Balm & Pottinger, 1993).

Balm & Pottinger's (1993) approach was different from other acidification studies. First, the water pH was lowered gradually enabling the animals to adapt to the acid conditions. Second, additional stressors like handling stress and high concentrations of aluminium, were avoided. The study of Balm & Pottinger (1993) was a semi-field study over a period of 2 weeks with rainbow trout concentrating on histological parameters, ionic balance, and indicators of the pituitary–interrenal axis. The new aspect of the present study is the additional measurement of the energy status of white muscle and liver tissue as indicators of stress under chronic acid conditions (37 days) at a low Ca^{2+} content of the water. In three previous studies, large differences were observed in energy status after acid exposure (Macfarlane, 1981; Haya *et al.*, 1985; Waiwood *et al.*, 1992). It was expected that when the acidification rate was slow and additional stressors were avoided, no effects would be observed on the energy status of tissues. The adenylate energy charge (AEC) is an indicator of the metabolic energy available to an organism from the adenylate pool at the time of sampling (Atkinson, 1968, 1972, 1977). In multicellular organisms as well as microorganisms in culture, the AEC lies within three ranges: between 0·8 and 0·9 for optimal conditions; 0·5–0·75 for limiting or perturbed conditions; and <0·5 for severe conditions (Ivanovici, 1979, 1980), and has been useful as an indicator of temperature and salinity stress (Ivanovici, 1980). This study uses the AEC as an indicator for acid stress.

This paper describes the effects of acidification on energy status, ionic balance, and plasma cortisol in tilapia chronically exposed to acid conditions (pH 4·0, low Ca^{2+}), and demonstrates that under very low additional stress levels no significant changes occur at pH 4·0.

MATERIALS AND METHODS

FISH

Mozambique tilapia *Oreochromis mossambicus* (Peters) with an average weight of approximately 60 g were held in 100-l aquaria, seven animals per aquarium, at the University of Nijmegen. Six groups were used, three control and three acid-exposed

groups. The following conditions were applied: 26° C, normoxic oxygen levels of 80–90% AS (air saturation), a 12 : 12 h light : dark regime, and daily feeding with Tetramin at 2% body weight day^{-1}, in a separate quiet room, where the fish observed human activity only twice a day for feeding. The fish were adapted to artificial soft water (pH 7·4) for 6 weeks prior to the experimental period. Artificial soft water was prepared by adding 0·44 mM NaCl, 0·86 mM $NaHCO_3$, 0·13 mM $MgCl_2$, 0·04 mM KCl, 0·2 mM $CaCl_2$ to demineralized water. Al_{total} was below the detection limit of 13·0 nmol l^{-1}. Water pH of the acid groups was reduced gradually to pH 4·0 over a period of 6 h. The pH of the water was maintained at pH=4·0 for 37 days with 1 mol l^{-1} H_2SO_4 by a titration system consisting of a pH meter (Consort P514) connected to a special low conductivity pH electrode (Russell CTL/LCW) and a Gilson peristaltic pump. The aquaria were aerated rigorously to eliminate CO_2 and to prevent hypercapnic conditions. On the sampling days the fish were anaesthetized in a solution of 3-amino-benzoate ethyl ester methanesulphonate (MS-222; Sigma, St Louis, MO, U.S.A.) buffered with $NaHCO_3$ at a final concentration of 200 ppm. To avoid handling stress, the solution of buffered MS-222 was pumped within 1 min with a peristaltic pump into the aquaria.

EXPERIMENTAL PROTOCOL AND SAMPLING PROCEDURE

A control and acid-exposed group were anaesthetized at the same time and sampled alternately. The time from anaesthetizing to blood sampling was 5 min while the time from start to finish blood sampling for control and acid exposed groups was 12 min. Blood was collected in EDTA/aprotinin (1·5 mg 3000 KIU^{-1} ml^{-1} blood; Sigma). Immediately afterwards the fish were killed by decapitation. White muscle (within 10–15 s) and liver tissue (within 30–40 s) was sampled and freeze-clamped between aluminium tongs, and cooled and stored in liquid nitrogen at −180° C until analysis. Frozen tissue was powdered in a grinder (type RMO; Retsch) with liquid nitrogen and 4·0 vol of perchloric acid (8%, v/v) in ethanol (40%, v/v) containing 4 mM NaF and 10 mM EDTA. The powder was stored for 10 min at −20° C in a centrifuge tube and then homogenized on ice with a high-speed mixer (type X 1020; Salm & Kip BV, Dottingen, Germany). The homogenate was stored for 30 min on ice and was further centrifuged (Sorvall RC-5B) for 20 min at 30 000 *g*. The extract was neutralized to pH 7·0 with 3 M potassium carbonate in 0·5 M triethanolamine. Finally, the extracts were separated into Eppendorf tubes and stored at −180° C (liquid nitrogen) until analysis.

ANALYTICAL METHODS

Metabolites

Haemoglobin content was measured in 20 µl blood using the cyanmethaemoglobin method (Boehringer Mannheim, Germany). Directly after blood sampling the blood was centrifuged (10 000 rpm for 5 min). The plasma was divided in Eppendorf tubes (10, 60 and 70 µl for cortisol, glucose, sodium and chloride respectively) and stored at −80° C for further analysis. For glucose measurements, 60 µl plasma were mixed with 540 µl 3% trichloric acid solution to precipitate plasma proteins and stored at −80° C. Glucose was determined by colorimetric assay (Sigma). Cortisol was measured by radio-immunoassay (Balm *et al.*, 1994). Plasma sodium and chloride levels were measured by flame photometric and colorimetric procedures (Technicon) respectively (Balm & Pottinger, 1993). Lactic acid was detected enzymatically according to the method of Hohorst (1970).

HPLC-analysis of nucleotides

Nucleotide analysis in the tissue extracts was based on the HPLC-method of Harmsen *et al.* (1982). The HPLC configuration consisted of a LKB 2248 pump with a Pharmacia LKB low pressure solvent mixer. The separation was performed on an anion-exchange column (200 × 4·6 mm) packed with 10 µm Partisal SAX (Whatman, Clifton, U.S.A.) operating at room temperature. A gradient elution system was used consisting of eluent A as 0·01 M H_3PO_4 adjusted to pH 2·85 and eluent B as 0·75 M KH_2PO_4 adjusted to pH 4·40. The gradient profile was 0% of B during the first 5 min followed by a

TABLE I. Parameters (means ± S.D.) measured in blood of tilapia groups sampled at 3, 17 and 37 days after exposure to pH 4·0; control groups (pH 7·6) were sampled at the same time

	3 days, control	3 days, pH=4·0	17 days, control	17 days, pH=4·0	37 days, control	37 days, pH=4·0
Hb (mM) (n=7)	nd	nd	4·70 ± 1·04	4·40 ± 0·55	4·55 ± 0·28	4·71 ± 0·33
Cortisol (ng/ml) (n=7)	157·4 ± 25·8	131·7 ± 27·7	101·6 ± 37·9	122·1 ± 50·9	155·3 ± 60·2	174·0 ± 46·7
Glucose (mM) (n=6)	3·08 ± 1·35	3·92 ± 1·35	2·81 ± 0·95	2·66 ± 1·21	3·83 ± 1·38	3·68 ± 1·64

*Significant difference with the corresponding control group ($P \leq 0{\cdot}05$).
nd, No data available.

linear gradient from 0 to 100% B for t=5–35 min, 100% B for t=35–40 min and 100% to 0% B for t=40–45 min. Detection was performed using an ultraviolet dual wavelength detector (LKB 2141 monitor) set at 256 for nucleotides and 210 nm for PCr detection. Quantification was performed using external standards.

STATISTICS AND CALCULATIONS

Data are presented as means ± S.E. Differences between groups were assessed by analysis of variance (ANOVA) against time. Per sample point differences of the treatment (acidification) were compared with a one-way ANOVA. Normality of the data and homogeneity of variances were checked by Kolmogorov–Smirnov and F_{max} tests, respectively. $P \leq 0{\cdot}05$ was considered as statistically significant.

From the nucleotides, the following parameters were calculated:

(1) total adenine nucleotides: TAN=[ATP]+[ADP]+[AMP];
(2) adenylate energy charge: AEC=([ATP]+$\frac{1}{2}$[ADP])/([ATP]+[ADP]+[AMP]);
(3) IMP load: IL=[IMP]/([ATP]+[ADP]+[AMP]).

RESULTS

No mortality, nor decreased food consumption was observed throughout 37 days in control and acid-exposed groups. Food was eaten within 1 min for both groups throughout the experimental period. No significant differences were observed between control and acid exposed groups for haemoglobin, cortisol, glucose (Table I) sodium and chloride (Fig. 1).

In white muscle the [phosphocreatine] (PCr) varied between 13 and 19·5 mM (Table II), the [adenosinetriphosphate] (ATP) was approximately 5 mM, while the adenylate energy charge (AEC) was in all cases 0·93 (Fig. 1). The [adenosinediphosphate] (ADP) was around 0·85 mM (Table II, [adenosine monophosphate] (AMP) remained below the detection limit of 3 μM, while the total adenine nucleotide pool (TAN) remained between 5·75 and 6·14 mM. The [inosinemonophosphate] (IMP) varied between 0·08 and 0·26 mM and the IMP-load (IL) between 0·01 and 0·04.

In liver tissue, the [PCr] was below the detection limit of 7 μM. The [ATP] in liver tissue was approximately eight times lower compared to white muscle, varying between 0·42 and 0·71 mM while the AEC varied between 0·67 and 0·79 (Fig. 1). The [ADP] was lower compared to white muscle, 0·41–0·60 mM while

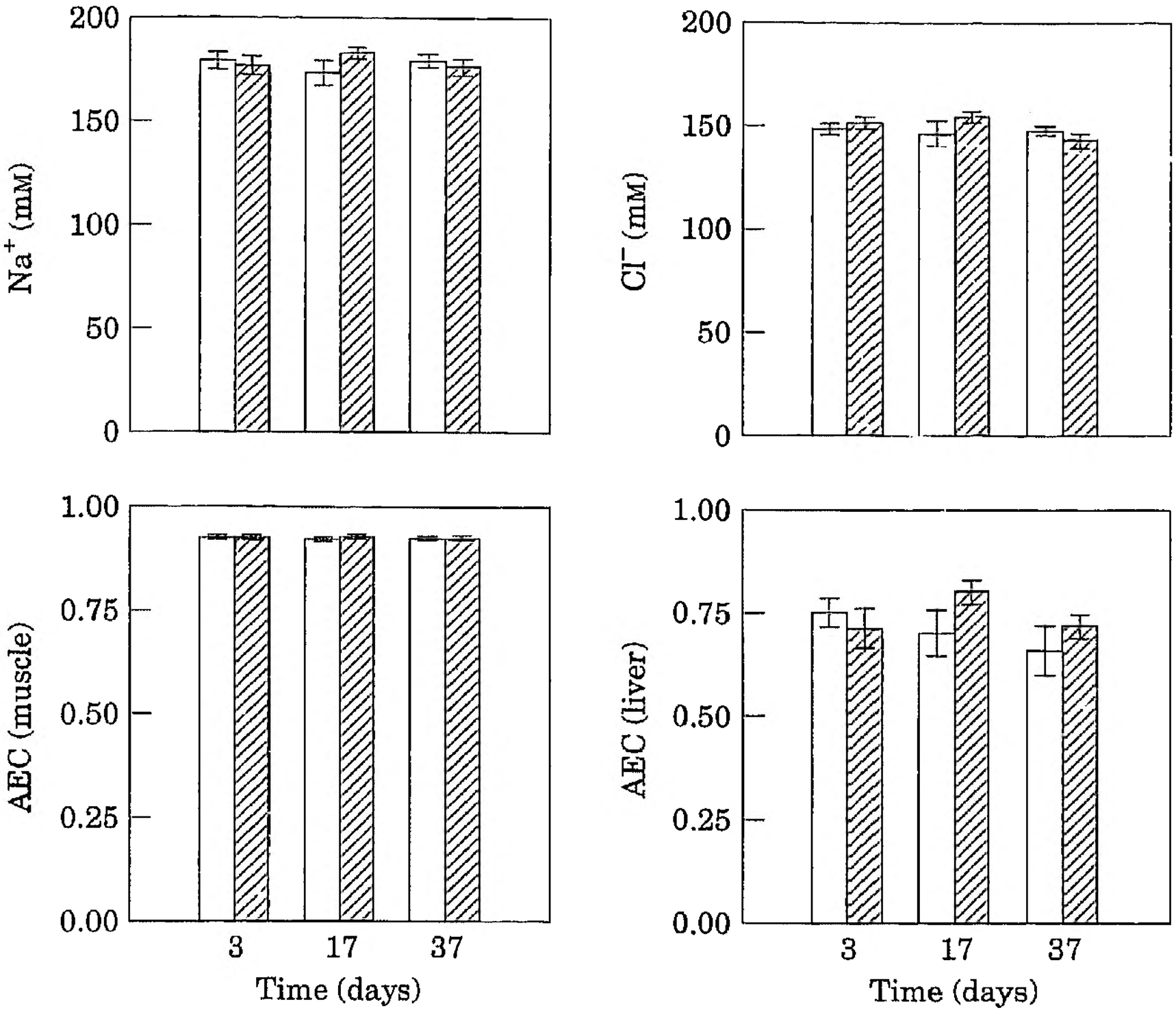

FIG. 1. The effect of long-term exposure to pH 4 on the adenylate energy charge (AEC) of white muscle and liver, and on the Na^+ and Cl^- plasma levels of tilapia. The exposure periods were respectively 3, 17, and 37 days. Shown are the values of the controls (□) and experimental groups (▨) ± S.E. (n=5 for each group); no statistically significant differences were observed between groups.

the [AMP] was between 0·79 and 0·83 mM (Table II). The TAN was fivefold lower compared to white muscle, varying between 1·03 and 1·30 mM. Finally, the [IMP] was lower in liver tissue compared to white muscle, in the range of 0·09–0·13 mM while the IMP-load showed a large variance between 0·07 and 0·29.

For all energy-rich compounds, notably the [ATP], [PCr] and the AEC, there were no significant differences between control and acid exposed groups for all the three sample points, 3, 17 and 37 days respectively (Table II, Fig. 1). Only for lactic acid in liver was there a significant difference between the control groups at days 3 (0·27 mM) and 37 (0·65 mM) (Table II). For all the other parameters no significant differences were observed between the three control groups.

DISCUSSION

There were no significant differences between any of the control or acidified groups, indicating that tilapia can adapt and survive acidified water conditions (pH 4·0) during 37 days and that no time effect was operative. There were no signs of a disturbed or impaired physiological condition resulting from acid exposure and no significant changes in haemoglobin, glucose, cortisol, sodium, chloride, and energy status in liver and white muscle. This is in contrast to most

TABLE II. Nucleotides (means ± S.D.) in white muscle and liver of tilapia groups sampled at 3, 17 and 37 days after exposure to pH 4·0; control groups (pH 7·6) were sampled at the same time

	3 days, control	3 days, pH 4·0	17 days, control	17 days, pH 4·0	37 days, control	37 days, pH 4·0
White muscle						
PCr	17·55 ± 5·37	19·52 ± 4·01	12·80 ± 2·48	17·94 ± 4·82	16·10 ± 2·01	15·72 ± 3·52
ATP	5·12 ± 0·47	4·92 ± 0·40	4·88 ± 0·46	4·92 ± 0·24	5·24 ± 0·32	5·18 ± 0·41
ADP	0·88 ± 0·16	0·83 ± 0·11	0·88 ± 0·12	0·83 ± 0·06	0·90 ± 0·12	0·85 ± 0·06
AMP	<3 μM	<3 μM	<3 μM	<3 μM	<3 μM	<3 μM
IMP	0·26 ± 0·24	0·14 ± 0·10	0·24 ± 0·15	0·08 ± 0·05	0·14 ± 0·08	0·14 ± 0·09
TAN	6·00 ± 0·59	5·75 ± 0·48	5·76 ± 0·49	5·75 ± 0·28	6·14 ± 0·42	6·02 ± 0·44
IL	0·04 ± 0·04	0·02 ± 0·02	0·04 ± 0·03	0·01 ± 0·01	0·02 ± 0·01	0·02 ± 0·02
Lactic acid	2·60 ± 0·75	2·33 ± 1·40	3·83 ± 0·93	2·35 ± 0·79*	3·47 ± 0·87	3·43 ± 1·58
Liver						
PCr	<7 μM	<7 μM	<7 μM	<7 μM	<7 μM	<7 μM
ATP	0·71 ± 0·35	0·66 ± 0·25	0·61 ± 0·34	0·62 ± 0·25	0·47 ± 0·24	0·42 ± 0·19
ADP	0·49 ± 0·14	0·60 ± 0·10	0·54 ± 0·11	0·41 ± 0·11	0·56 ± 0·11	0·53 ± 0·30
AMP	0·82 ± 0·16	0·79 ± 0·12	0·81 ± 0·11	0·83 ± 0·14	0·81 ± 0·18	0·79 ± 0·16
IMP	0·09 ± 0·07	0·09 ± 0·04	0·09 ± 0·07	0·12 ± 0·12	0·13 ± 0·12	0·13 ± 0·13
TAN	1·23 ± 0·44	1·30 ± 0·20	1·21 ± 0·41	1·03 ± 0·28	1·10 ± 0·18	1·04 ± 0·34
IL	0·09 ± 0·07	0·07 ± 0·03	0·09 ± 0·08	0·06 ± 0·06	0·17 ± 0·11	0·29 ± 0·36
Lactic acid	0·27 ± 0·03	0·33 ± 0·15	0·48 ± 0·23	0·32 ± 0·18	0·65 ± 0·29†	0·49 ± 0·38

*Significant difference with corresponding control group ($P \leq 0{\cdot}05$); †significant difference with control group 3 days.
Each group contained five animals. All values are expressed in mM, except for PCr in liver, AMP, EC and IL which are without dimension.

studies which report a general impairment of the physiology of fish by water acidification (Fromm, 1980; McDonald, 1983; Wood, 1989). An explanation for this discrepancy has been given recently by Balm & Pottinger (1993). They observed that rainbow trout exposed for 14 days to pH 4·0 survived without decreased food consumption: haematocrit and plasma protein were not affected and the pituitary–interrenal axis was not activated.

Only from electron microscopic examination of gill tissue was there evidence for an increased turnover rate of chloride cells and leucocyte infiltration in gills of acid-exposed fish (Balm & Pottinger, 1993). Lowering the pH gradually had enabled the trout to adapt to the acid conditions, preventing the gill damage which occurs at higher acidification rates (Stuart & Morris, 1985; Balm *et al.*, 1987; Wendelaar-Bonga *et al.*, 1990). This approach corresponds to the situation in nature (Henriksen *et al.*, 1984). Balm & Pottinger (1993) had avoided handling stress or additional stressors like aluminium ions and they confirmed that under these experimental conditions no significant activation of the interrenal axis or ion losses would occur. The present study confirms their hypothesis for tilapia after 37 days exposure to acidified water, for energy status, ionic balance and cortisol.

ENERGY STATUS

There are two major advantages for the AEC as a general index for stress. First, it is nonspecific and is generally applicable: and second, its response to environmental changes is rapid, from minutes for microorganisms (Wiebe & Bancroft, 1975) to 24 h for a mollusc (Wijsman, 1976). Changes of the AEC have been observed in several studies with perturbations in environmental conditions (salinity, temperature, exposure to hydrocarbons, and lack of oxygen) or with growth state. In the gastropod *Pyrazus ebeninus*, the AEC dropped below 0·8 when the external salinity was $\leq$17‰ at 20° C (Ivanovici, 1980): when the temperature was increased from 20 to 29° C the AEC dropped by 20% independent of salinity (Ivanovici, 1980). In both *Pyrazus ebeninus* and the bivalve *Trichomya hirsuta*, the AEC dropped significantly from control levels of 0·8–0·9 to 0·65–0·55 under reduced salinity, increased temperature and exposure to hydrocarbons (Ivanovici, 1979). In fish white muscle, the AEC remained stable or dropped little after hypoxia or anoxia exposure due to the buffering capacity of the PCr pool present (van den Thillart *et al.*, 1976; van den Thillart, 1980; Jorgensen & Mustafa, 1980; van Waarde *et al.*, 1983; van der Boon *et al.*, 1992; Caldwell & Hishaw, 1994). In liver, the situation is different due to the absence of the PCr pool. As a consequence, the ATP pool cannot be buffered via the creatine kinase reaction. Moreover, AMP conversion to IMP via the enzyme AMP desaminase is probably not operative in liver tissue (van den Thillart, 1980), which is supported by the high values of AMP found in liver tissue (Table II). Hence, AEC values were lower in liver tissue, and liver is more sensitive than is muscle to environmental stressors. In the present study, the AEC of white muscle and liver was unaffected, despite the sensitivity of the liver, so it is concluded that acidification has no direct or indirect effect (e.g. hypoxia) on this parameter.

It is reported that for the first 2–3 weeks exposure fish adapt to acidified conditions while after 37 days they reach a new steady state with respect to ionic

balance (Wendelaar-Bonga *et al.*, 1987; Audet *et al.*, 1988) and haematology (Audet *et al.*, 1988). However, at 3, 17 and 37 days in the present experiments there were no differences in the AEC between control and acid exposed groups.

As regards energy status, Macfarlane (1981) noted large changes in the AEC and TAN in Gulf Killifish *Fundulus grandis* Baird & Girard, after exposure to pH 5 and 4 for 96 h. Both parameters decreased in brain, gill, liver and muscle. Moreover, a decrease of [ATP] was observed in all tissues. Exposure of Atlantic salmon *Salmo salar* L., to pH 4·6 for 112 days resulted in an increased [ADP] and TAN in liver tissue (Waiwood *et al.*, 1992). AEC and PCr were lower in Atlantic salmon muscle after 15 days at pH 4·7 while ATP and TAN in muscle were lower after 62 days of exposure compared to controls (Haya *et al.*, 1985). However, additional stressors may be effective like smolting (Haya *et al.*, 1985; Waiwood *et al.*, 1992), handling stress (Macfarlane, 1981; Haya *et al.*, 1985) and large fluctuations in environmental conditions (Waiwood *et al.*, 1992). The latter stressor may have synergistic effects. Synergism was also observed in a recent *in vivo* ^{31}P-NMR study when Mozambique tilapia were exposed to acidification combined with hypoxia (van Ginneken *et al.*, 1996). Exposure to pH 4·0 for 12 h as a single stressor had no effects on phosphocreatine levels and the intracellular pH (pH_i) of the white muscle (as found by van Waarde *et al.*, 1990) while a combination of hypoxia and acidification had deleterious effects: 50% of the fish died during reoxygenation while the survivors showed a delayed recovery (van Ginneken *et al.*, 1996).

IONIC BALANCE

The observed values for chloride and sodium fell within the range of other studies (Balm & Pottinger, 1993; van Dijk *et al.*, 1993; Wendelaar-Bonga *et al.*, 1987). However, when Audet *et al.* (1988) exposed rainbow trout to pH 4·8, plasma sodium dropped slowly from 140 to 120 meq l^{-1} after 30–52 days and plasma chloride fell from 140 to 110 meq l^{-1} after 84 days. Probably the acidification rate was too abrupt or additional stressors were operative.

In Balm & Pottinger's (1993) study of acid exposed trout, chloride cells showed apoptosis, physiologically controlled cell death as described for tilapia (Wendelaar-Bonga *et al.*, 1990). There were, however, no indications for tissue necrosis in acid exposed animals (Balm & Pottinger, 1993), and it was suggested that the increased turnover rate of chloride cells in gill tissue (Wendelaar-Bonga *et al.*, 1990) was part of the adaptive regulatory response to acidified conditions enabling the fish to maintain ionic homeostasis (Balm & Pottinger, 1993). It is possible that such a response was also operative in our study.

GLUCOSE AND CORTISOL

In the acid-exposed groups, no hyperglycaemic response was observed: plasma glucose levels at 2·7–3·9 mM corresponded with previous reports for this species at 3·1 mM (van Waarde *et al.*, 1990). Hyperglycaemia has occurred upon water acidification in rainbow trout (Brown *et al.*, 1990), and in response to exogenous cortisol administration in roach *Rutilus rutilus* L. (Müller & Hanke, 1974) and eel *Anguilla japonica* Temminck & Schlegel (Chan & Woo, 1978). Therefore, it could be argued that the lack of a glycaemic response in the present fish reflected the lack of difference in cortisol levels between experimental groups. However,

these cortisol levels can hardly be considered basal. This was unexpected, since MS-222 anaesthesia was used partly to avoid the rapid sampling-associated elevation of cortisol (Balm *et al.*, 1994). It is suggested that either the animals perceived the MS-222 addition to the aquarium, or the interrenal stress response to sampling also occurs in anaesthetized animals. Consequently, conclusions regarding basal cortisol in the experimental groups would be tentative at best. Evidently though, the low pH treatment did not influence the magnitude of the sampling-associated cortisol response. Because stress responses are influenced by previous chronic stressful experiences associated with water quality (Barton *et al.*, 1985; Pickering & Pottinger, 1987), the data suggest that exposure to pH 4·0 for up to 37 days was not experienced as stressful by these fish. This is consistent with the measured metabolic and ionoregulatory parameters, which were in the control range in the acidified groups.

CONCLUSION

The study confirmed and extended the hypothesis of Balm & Pottinger (1993) that fish can acclimate to acidification alone if: (1) the acidification rate is slow, enabling the animals to mobilize adaptive mechanisms, such as a higher turnover rate of branchial chloride cells; and (2) additional stressors like handling and aluminium are eliminated. In the presence of additional stressors (biotic or abiotic), synergism may occur, explaining the results obtained with field studies.

VJTvG was supported by a grant of the Life Sciences Foundation (SLW), which is subsidized by the Netherlands Organization for Scientific Research (NWO), SLW-project no. 427024.

References

Atkinson, D. E. (1968). The energy charge of the adenylate pool as a regulatory parameter: interaction with feedback modifiers. *Biochemistry* **7,** 4030–4034.

Atkinson, D. E. (1972). The adenylate energy charge in metabolic regulation. In *Horizons of Bioenegetics* (San Pietro, A. & Gest, H., eds), p. 83. London: Academic Press.

Atkinson, D. E. (1977). *Cellular Energy Metabolism and its Regulation*. New York: Academic Press.

Audet, C. & Wood, C. M. (1988). Do rainbow trout (*Salmo gairdneri*) acclimate to low pH? *Canadian Journal of Fisheries and Aquatic Sciences* **45,** 1399–1405.

Audet, C., Munger, R. S. & Wood, C. M. (1988). Long-term sublethal acid exposure in rainbow trout (*Salmo gairdneri*) in soft water: effects on ion exchanges and blood chemistry. *Canadian Journal of Fisheries and Aquatic Sciences* **45,** 1387–1398.

Balm, P. H. M. & Pottinger, T. G. (1993). Acclimation of rainbow trout (*Oncorhynchus mykiss*) to low environmental pH does not involve an activation of the pituitary–interrenal axis, but evokes adjustments in branchial structure. *Canadian Journal of Fisheries and Aquatic Sciences* **50,** 2532–2541.

Balm, P. H. M., Lamers, A. & Wendelaar Bonga, S. E. (1987). Regulation of pituitary-interrenal axis activity in tilapia acclimating to low pH conditions. *Annales Société Royale Zoologique Belge* **117** (Suppl. 1), 343–352.

Balm, P. H. M., Pepels, P., Helfrich, S., Hovens, M. L. L. & Wendelaar Bonga, S. E. (1994). Adrenocorticotropic hormone (ACTH) in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). *General Comparative Endocrinology* **96,** 447–460.

Barton, B. A., Weiner, G. S. & Schreck, C. B. (1985). Effect of prior acid exposure on physiological responses of juvenile rainbow trout (*Salmo gairdneri*) to acute handling stress. *Canadian Journal of Fisheries and Aquatic Sciences* **42,** 710–717.

Beamish, R. J. (1976). Acidification of lakes in Canada by acid precipitation and the resulting effects on fishes. *Air and Soil Pollution* **6,** 501–514.

Boon, J. van der, de Jong, R. L., Thillart, G. E. E. J. M. van den & Addink, A. D. F. (1992). Reversed-phase ion-paired HPLC of purine nucleotides from skeletal muscle, heart and brain of the goldfish, *Carassius auratus* L. II. Influence of environmental anoxia on metabolite levels. *Comparative Biochemistry and Physiology* **101B,** 583–586.

Brown, S. B., Eales, J. G., Evans, R. E. & Hara, T. J. (1983). Interrenal, thyroidal and carbohydrate responses of rainbow trout (*Salmo gairdneri*) to environmental acidification. *Canadian Journal of Fisheries and Aquatic Sciences* **41,** 36–45.

Brown, S. B., MacLatchy, D. L., Hara, T. J. & Eales, J. G. (1990). Effect of low ambient pH and aluminium on plasma kinetics of cortisol, T_3 and T_4 in rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Zoology* **68,** 1537–1543.

Caldwell, C. A. & Hishaw, J. M. (1994). Nucleotides and the adenylate energy charge as indicators of stress in rainbow trout (*Oncorhynchus mykiss*) subjected to a range of dissolved oxygen concentrations. *Comparative Biochemistry and Physiology* **109B,** 313–323.

Chan, D. K. O. & Woo, N. Y. S. (1978). Effect of cortisol on the metabolism of the eel, *Anguilla japonica*. *General Comparative Endocrinology* **35,** 205–215.

Dijk, P. L. M. van, Thillart, G. van den & Wendelaar-Bonga, S. (1993). The influence of gradual water acidification on the acid/base status and plasma hormone levels in carp. *Journal of Fish Biology* **42,** 661–671.

Fromm, P. O. (1980). A review of some physiological and toxicological responses of freshwater fish to acid stress. *Environmental Biology* **5,** 79–93.

Ginneken, V. van, Thillart, G. van den, Addink, A. & Erkelens, C. (1996). Synergistic effect of acidification and hypoxia: *in vivo* ^{31}P-NMR study in fishes. *American Journal of Physiology* **271,** R1746–1752.

Harmsen, E., De Tombe, P. P. & de Jong, J. W. (1982). Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high-performance liquid chromatography. *Journal of Chromatography and Biomedical Application* **230,** 131–136.

Haya, K., Waiwood, B. A. & Eeckhaute, L. van (1985). Disruption of energy metabolism and smoltification during exposure of juvenile Atlantic salmon (*Salmo salar*) to low pH. *Comparative Biochemistry and Physiology* **82C,** 323–329.

Henriksen, A., Skogheim, O. K. & Rosseland, B. O. (1984). Episodic changes in pH and aluminium speciation kill fish in a Norwegian salmon river. *Vatten* **40,** 255–260.

Hohorst, H. J. (1970). *Methoden der enzymatischen Analyse*. Auflage Band II. Weinheim: Verlag Chemie.

Ivanovici, A. M. (1979). Adenylate Energy Charge: potential value as a tool for rapid determinations of toxicity effects. Proceedings of the Fifth Annual Aquatic Toxicity Workshop, Hamilton, Ontario, November 7–9 1978. *Fisheries and Marine Service Technical Reports* **862,** 241–255.

Ivanovici, A. M. (1980). The adenylate energy charge in the estuarine mollusc, *Pyrazus ebeninus*. Laboratory studies of responses to salinity and temperature. *Comparative Biochemistry and Physiology* **66A,** 43–55.

Jörgensen, J. B. & Mustafa, T. (1980). The effect of hypoxia on carbohydrate metabolism in flounder (*Platichthys flesus* L.). II. High energy phosphate compounds and the role of glycolytic and gluconeogenetic enzymes. *Comparative Biochemistry and Physiology* **67B,** 249–256.

Lee, R. M., Shelby, D. G. & Jezierska, B. (1983). Electrolyte balance and energy mobilization in acid-stressed rainbow trout (*Salmo gairdneri*) and their relation to reproductive success. *Environmental Biology of Fishes* **8,** 115–123.

Leivestad, H. & Muniz, I. P. (1976). Fish kill at low pH in a Norwegian river. *Nature* **259,** 391–392.

Macfarlane, R. B. (1981). Alterations in adenine nucleotide metabolism in the gulf killifish (*Fundulus grandis*) induced by low pH water. *Comparative Biochemistry and Physiology* **68B,** 193–202.

McDonald, D. G. (1983). The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri*, I. Branchial and renal net ion and H^+ fluxes. *Journal of Experimental Biology* **102,** 123–140.

McDonald, D. G. & Wood, C. M. (1981). Branchial and renal acid and ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH. *Journal of Experimental Biology* **93,** 101–118.

Milligan, C. L. & Wood, C. M. (1982). Disturbances in haematology, fluid volume distribution and circulatory function associated with low environmental pH in the rainbow trout, *Salmo gairdneri*. *Journal of Experimental Biology* **99,** 397–415.

Müller, R. & Hanke, W. (1974). The effects of adrenocortical hormones on osmomineral regulation and carbohydrate metabolism in the roach, *Leuciscus rutilus*. *General Comparative Endocrinology* **22,** 381.

Murthy, V. K., Reddanna, P., Bhaskar, M. & Govindappa, S. (1981). Muscle metabolism of freshwater fish, *Tilapia mossambica* (Peters), during acute exposure and acclimation to sublethal acidic water. *Canadian Journal of Zoology* **59,** 1909–1915.

Neville, C. M. (1979). Sublethal effects of environmental acidification on rainbow trout (*Salmo gairdneri*). *Journal of the Fisheries Research Board of Canada* **36,** 84–87.

Nieminen, M., Korhonen, I. & Laitinen, M. (1982). Effects of pH on the gill ATPase activity and blood composition of whitefish (*Coregonus peled*) and trout (*Salmo trutta fario*). *Comparative Biochemistry and Physiology* **71A,** 637–642.

Pickering, A. D. & Pottinger, T. G. (1987). Poor water quality suppresses the cortisol response of salmonid fish to handling and confinement. *Journal of Fish Biology* **30,** 363–374.

Stuart, S. & Morris, R. (1985). The effects of season and exposure to reduced pH (abrupt and gradual) on some physiological parameters in brown trout (*Salmo trutta*). *Canadian Journal of Zoology* **63,** 1078–1083.

Tam, W. H., Birkett, L., Markaran, R., Payson, P. D., Whitney, D. K. & Yu, C. K. C. (1987). Modification of carbohydrate metabolism and liver vitellogenic function in brook trout (*Salvelinus fontinalis*) by exposure to low pH. *Canadian Journal of Fisheries and Aquatic Sciences* **44,** 630–635.

Thillart, G. van den (1980). Anaerobic energy metabolism of goldfish, *Carassius auratus* (L). Influence of hypoxia and anoxia on phosphorylated compounds and glycogen. *Journal of Comparative Physiology* **136,** 45–52.

Thillart, G. van den, Kesbeke, F. & van Waarde, A. (1976). Influence of anoxia on the energy metabolism of goldfish, *Carassius auratus* (L). *Comparative Biochemistry and Physiology* **55A,** 329–336.

Ultsch, G. R., Ott, M. E. & Heisler, N. (1981). Standard metabolic rate, critical oxygen tension, and aerobic scope for spontaneous activity of trout (*Salmo gairdneri*) and carp (*Cyprinus carpio*) in acidified water. *Comparative Biochemsitry and Physiology* **67A,** 329–335.

Waarde, A. van, Thillart, G. van den & Kesbeke, F. (1983). Anaerobic energy metabolism of the european eel, *Anguilla anguilla* L. *Journal of Comparative Physiology* **149,** 469–475.

Waarde, A. van, Thillart, G. van den, Verhagen, M., Erkelens, C., Wendelaar-Bonga, S., Addink, A. & Lugtenburg, J. (1990). ^{31}P-NMR studies on acid-base balance and energy metabolism of acid-exposed fish. *Journal of Experimental Biology* **154,** 223–236.

Waiwood, B. A., Haya, K. & Eeckhaute, L. van (1992). Energy metabolism of hatchery reared juvenile salmon (*Salmo salar*) exposed to low pH. *Comparative Biochemistry and Physiology* **101C,** 49–56.

Wendelaar-Bonga, S., Flik, G. & Balm, P. (1987). Physiological adaptation to acid stress in fish. *Annales Société Royale Zoologique Belge* **117,** 243–254.

Wendelaar-Bonga, S. E., Flik, G., Balm, P. H. M. & Meij, J. C. A. van der (1990). The ultrastructure of chloride cells in the gills of the teleost *Oreochromis mossambicus* during exposure to acidified water. *Cell and Tissue Research* **259,** 575–585.

Wiebe, W. J. & Bancroft, K. (1975). Use of the adenylate energy charge ratio to measure growth state of natural microbial communities. *Proceedings of the National Academy of Sciences of the U.S.A.* **72,** 2112–2115.

Wijsman, T. C. M. (1976). Adenosine phosphates and energy charge in different tissues of *Mytilus edulis* under aerobic and anaerobic conditions. *Journal of Comparative Physiology* **107,** 129–140.

Wood, C. M. (1989). The physiological problems of fish in acid waters. In *Acid Toxicity and Aquatic Animals* (Morris, R., Brown, D. J. A., Taylor, E. W. & Brown, J. A., eds), pp. 125–152. Society for Experimental Biology Seminar Series, **34**. Cambridge: Cambridge University Press.