The influence of gradual water acidification on the acid/base status and plasma hormone levels in carp

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Carp (Cyprinus carpio) fitted with arterial catheters were subjected to gradual water acidification (from pH 7-6 to 4-0 in 4 h), and then monitored for 48 h. The measured blood parameters showed little or no disturbance; there was no ionoregulatory disturbance, no hyperglycemia, increase in plasma lactate, hypoxemia, or swelling of red blood cells. Only a slight transient decrease of plasma pH was found. Plasma catecholamine levels remained at control level. The plasma cortisol concentration showed a transient increase, and was returned to the control level 24 h after beginning water acidification.

Our results contrast with literature data on carp where exposure to pH 4-0 caused severe blood electrolyte disturbance. We conclude that the rate of water acidification (gradual in our study as compared to acute in the literature) rather than the pH level itself determines the early effects of water acidification.

Key words: catecholamines; cortisol; carp; acid/base regulation; acid water.

1. INTRODUCTION

The physiological effects of acid water on fish have been studied intensively over the last 15 years (McDonald, 1983a; Howells, 1984; Wood, 1989). From these studies, it became clear that the key toxic mechanism of pure acid stress is disturbance of electrolyte balance at the gills, and not internal acidosis. A net H⁺ ion influx is dependent on the relative movements of strong cations and anions. Thus, as is discussed by Wood (1989), water acidification can only lead to internal acidosis as a result of ionoregulatory failure. The ionoregulatory failure may also trigger circulatory collapse which finally causes death (Wood & McDonald, 1982).

Ultsch et al. (1981) found, in carp, that two instant step changes in water pH (from 7-4 to 5-1 and from 5-1 to 4-0) resulted in declining plasma sodium and chloride concentrations and a progressive reduction of arterial pH. A further decrease of the pH to 3-5 finally led to the death of the animals within 24 h. Other studies with various fish species have produced similar results (McDonald, 1983a; Howells, 1984; Wood, 1989).

Using tilapia (Oreochromis mossambicus Peters), Wendelaar Bonga et al. (1987) showed that an immediate drop in water pH to pH 4-0 (the rate of water acidification was 18 pH units per hour) can lead to substantial structural damage of the branchial epithelium. However, at a lower rate of water acidification (3-0 pH units per hour and lower) these effects were not seen. A lower rate of water acidification is more environmentally relevant, because in nature water...
acidification is gradual, not immediate (Seip & Tollan, 1978; Henriksen et al., 1984). Thus, studies on fish experiencing gradual acidification may give a better insight into the acid stress tolerance of the species concerned.

In the present study, we wanted to investigate whether the disturbances in electrolyte and acid/base balance reported by other authors after acute acidification would take place when the acidification proceeded at a rate sufficiently low (0-9 pH unit per hour) to prevent epithelial damage. The blood parameters we measured included arterial extracellular pH, total CO₂, CO₂ tension, O₂ tension, haematocrit, and haemoglobin concentration; the plasma parameters measured were lactate, glucose, sodium, chloride, potassium, and total calcium. In addition, plasma cortisol and catecholamine levels were measured.

II. MATERIALS AND METHODS

Carp (300–700 g) of both sexes were obtained from the O.V.B., Lelystad, The Netherlands and were held for at least 6 months in dechlorinated and well aerated local tap water of 20±1°C. The fish were kept on a 14:10 LD photocycle and fed daily with commercial trout pellets.

At least 3 weeks before the start of the experiments the fish were transferred to four times diluted local tap water. For this, 1 vol of metal-free tap water was mixed with 3 vols of demineralized (by ion exchangers) water. The end concentrations were: Na⁺ 0.83, Cl⁻ 0.92, Ca²⁺ 0.68, K⁺ 0.07, Mg²⁺ 0.16, NO₃⁻ 0.03, HCO₃⁻ 1.11, SO₄²⁻ 0.29, SiO₂ 0.06 mmol l⁻¹. Al₆⁺ was below the detection level of 6.7 nmol l⁻¹. Water composition was similar to our earlier experiments (Balm, 1986; van Waarde et al., 1990). During the acclimation period and throughout the experiments, the fish were kept in this water at 20±0.5°C. To acclimate the fish to the confinement conditions of the experimental period, they were kept in separate chambers (26×26×35 cm). Through a perspex partition the carp could keep in visual contact with each other. In the acclimation chambers the water pH was maintained at 7.6 by a pH stat device. No metal parts were used in this setup. Because preliminary experiments showed that trout pellets cause too much pollution of the water, fish were fed daily with cichlid food in flake form (Lapis, Europet, Nürnberg, Germany); feeding was suspended at least 24 h before cannulation. Experiments were performed over the period October–December.

For catheterization (Soivio et al., 1972, 1975), 48 h before the experiments, a fish was anaesthetized in a Tris-buffered (pH 7) 100 mg l⁻¹ MS222 solution. During surgery the gills were irrigated with a well aerated Tris-buffered 75 mg l⁻¹ MS222 solution (18±0.5°C). After operation, the fish was placed in a narrow PVC chamber with constantly flowing aerated water (0.5 l min⁻¹). A pH stat device (METROHM 605/614/655) titrating 1 mol l⁻¹ NaOH was used to maintain the water pH at 7.6.

In this study there were two groups of fish: a control group (n=4) and an acid group (n=5). In the acid group, blood samples were taken at 3 h (t=−3 h) and 30 min (t=−0.5 h) before water acidification (Fig. 1). Between t=0 h and t=4 h the water pH was gradually lowered from pH 7.6 to pH 4.0, with 0.5 mol l⁻¹ H₂SO₄ and a pH stat device. During this acidification period, the Pco₂ level of the water was kept low by vigorous aeration. Further blood samples were taken at 2 h (pH = 5.8), 4 h (pH = 4.0), 7 h, 24 h and 48 h after the start of the acidification. In the control group, blood samples were taken at the same intervals (Fig. 1). The pH stat device was used throughout the experiment to keep the water pH at 7.6. The pH electrodes (Russell CTL/LCW for low conductivity) employed were specially designed to be used in solutions of low ionic strength.

Samples (750 μl) of whole blood were anaerobically withdrawn into ice-cold gas-tight Hamilton syringes via the dorsal aortic catheter. The blood was replaced by saline (Wolf, 1963). Blood samples were analysed for arterial extracellular pH (pHₐ), total CO₂ (Tco₂), CO₂ tension (Pco₂), O₂ tension (Po₂), haematocrit, haemoglobin concentration, and plasma levels of lactate, glucose, sodium, chloride, potassium, total calcium, adrenaline, noradrenaline, and cortisol.
To measure the water $P_{CO_2}$, an air-tight syringe was filled with water from the setup. After 4 min of constantly flushing the cell of a Radiometer bloodgas analyser (BMS3 M2), thermostatted to the experimental temperature and connected to a Radiometer PHM 71, the water $P_{CO_2}$ was read. Arterial $pH$, $P_{CO_2}$ and $P_{O_2}$ were determined together by injecting 130 µl whole blood into the Radiometer bloodgas analyser. Total CO$_2$ was measured using a modified form of the method of Cameron (1971). A spike (100 µl 10 mm NaHCO$_3$), two standards (50 µl 10 mm and 50 µl 5 mm NaHCO$_3$) and two blood samples (50 µl each) were injected into the Cameron chamber successively. The two standards were used to calculate the $T_{CO_2}$ of the blood samples, using a linear regression. In this calculation, a compensation was made for the volume driven out by each sample. From reference measurements the accuracy was found to be ±0.1 mmol l$^{-1}$. Plasma lactate, glucose, and total calcium were measured using Sigma (St Louis, MO) kits. Plasma sodium, potassium, and chloride concentrations were measured using an autoanalyser and a flame photometer.

Immediately after blood sampling, 250 µl whole blood was added to 10 µl of a preservative mix (90 mg ml$^{-1}$ EGTA plus 60 mg ml$^{-1}$ glutathione in 3.6% KOH), plasma was separated by centrifugation, immediately frozen in liquid nitrogen, and stored at −80°C. In these plasma samples, catecholamines were measured, applying high performance liquid chromatography (De Potter et al., 1987). These measurements were performed by Universitaire Instelling Antwerpen, 2610 Wilrijk, Belgium. Plasma cortisol was determined by radioimmunoassay (RIA) as described by De Man et al. (1980).

Data were expressed as means ± s.e.m. The data were tested for significance ($P \leq 0.05$) with the Student’s two-tailed $t$-test, paired or unpaired as appropriate. For two parameters (plasma cortisol and blood $P_{O_2}$) a log transformation of the data prior to testing was used to increase the homogeneity of the variances.

### III. RESULTS

In both the control and the acid group there were no mortalities during the experiment. In the control group, significant changes in blood parameters were only a transient increase in blood $P_{O_2}$ [Fig. 2(d)] and a decrease in haematocrit [Fig. 2(e)] due to blood sampling.
Water $PCO_2$ levels were monitored during the acidification period (Fig. 1). Before acidification the water $PCO_2$ was 0.08 kPa, at $t=1.5$ h the $PCO_2$ was maximal (0.32 kPa), at $t=2$ h it was 0.23 kPa and at $t=4$ h it was 0.16 kPa.

In the acid-treated fish, the $PH_c$ [Fig. 2(a)] reached a minimum at $t=2$ h (it decreased from 7.73 to 7.55). A slightly higher $PH_c$ was measured 2 h later, although the difference with the control group was still significant. After 2 days of acid exposure the blood $pH$ was completely back to control level. In addition to a transient insignificant increase, the $TCO_2$ [Fig. 2(b)] tended to rise which led to a significant difference at $t=48$ h. The increase in the blood $PCO_2$ [Fig. 2(c)] just after the start of the acidification coincided with a (not significant) increase in $PO_2$ [Fig. 2(d)]. While the $PO_2$ was back at the control level at $t=4$ h, the $PCO_2$ remained at a slightly elevated level throughout the experiment. The difference with the controls was significant at $t=4$ h and $t=48$ h. In the control group, the
TABLE I. The effect of gradual water acidification (between $t = 0$ h, pH 7.6 and $t = 4$ h, pH 4.0) on the plasma concentrations of lactate, glucose, sodium, chloride, potassium and total calcium in carp. Means ± s.E.M. *, Significant difference ($P \leq 0.05$) between control and acid group at a particular sample time

<table>
<thead>
<tr>
<th>Time</th>
<th>-3 h</th>
<th>-0.5 h</th>
<th>2 h</th>
<th>4 h</th>
<th>7 h</th>
<th>24 h</th>
<th>48 h</th>
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<tr>
<td></td>
<td>Plasma lactate (mmol l$^{-1}$)</td>
<td></td>
<td></td>
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<tr>
<td>Acid group</td>
<td>1.26 ±0.26</td>
<td>1.11 ±0.08</td>
<td>0.80 ±0.08</td>
<td>1.03 ±0.12</td>
<td>1.82 ±0.50</td>
<td>1.08 ±0.10</td>
<td>1.15 ±0.27</td>
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<td>Control group</td>
<td>0.96 ±0.11</td>
<td>0.96 ±0.10</td>
<td>1.05 ±0.15</td>
<td>1.18 ±0.22</td>
<td>1.17 ±0.15</td>
<td>1.09 ±0.05</td>
<td>1.45 ±0.27</td>
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<tr>
<td></td>
<td>Plasma glucose (mmol l$^{-1}$)</td>
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</tr>
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<td>Acid group</td>
<td>1.4 ±0.4</td>
<td>1.6 ±0.9</td>
<td>0.8 ±0.3</td>
<td>1.6 ±0.6</td>
<td>1.0 ±0.2</td>
<td>2.2 ±0.7</td>
<td>1.5 ±0.3</td>
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<tr>
<td>Control group</td>
<td>1.2 ±0.5</td>
<td>1.1 ±0.4</td>
<td>1.1 ±0.4</td>
<td>0.7 ±0.3</td>
<td>0.5 ±0.2</td>
<td>0.8 ±0.3</td>
<td>0.4 ±0.2</td>
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<td>Plasma Na$^{+}$ (mmol l$^{-1}$)</td>
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<tr>
<td>Acid group</td>
<td>139.9 ±2.1</td>
<td>142.2 ±2.5</td>
<td>137.4 ±5.4</td>
<td>147.1 ±3.1</td>
<td>146.4 ±5.2</td>
<td>138.0 ±1.5</td>
<td>138.4 ±5.6</td>
</tr>
<tr>
<td>Control group</td>
<td>132.7 ±3.8</td>
<td>139.4 ±3.4</td>
<td>138.6 ±5.1</td>
<td>142.9 ±3.3</td>
<td>145.8 ±7.0</td>
<td>142.9 ±1.9</td>
<td>131.4 ±4.9</td>
</tr>
<tr>
<td></td>
<td>Plasma Cl$^{-}$ (mmol l$^{-1}$)</td>
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<tr>
<td>Acid group</td>
<td>139.8 ±5.1</td>
<td>135.8 ±6.5</td>
<td>134.5 ±4.6</td>
<td>144.0 ±3.9</td>
<td>143.2 ±9.0</td>
<td>139.7 ±4.7</td>
<td>136.5 ±10.1</td>
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<tr>
<td>Control group</td>
<td>123.1 ±5.5</td>
<td>133.8 ±7.7</td>
<td>130.8 ±8.2</td>
<td>137.4 ±0.9</td>
<td>140.1 ±7.6</td>
<td>145.2 ±4.9</td>
<td>135.8 ±7.5</td>
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<td></td>
<td>Plasma K$^{+}$ (mmol l$^{-1}$)</td>
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<tr>
<td>Acid group</td>
<td>2.68 ±0.09</td>
<td>2.29 ±0.22</td>
<td>2.36 ±0.14</td>
<td>2.68 ±0.26</td>
<td>3.63 ±0.51</td>
<td>2.87 ±0.10</td>
<td>4.41 ±1.3</td>
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<td>Control group</td>
<td>2.86 ±0.40</td>
<td>3.03 ±0.13</td>
<td>2.48 ±0.14</td>
<td>2.41 ±0.07</td>
<td>2.71 ±0.24</td>
<td>3.10 ±0.41</td>
<td>2.84 ±0.17</td>
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<tr>
<td></td>
<td>Plasma Ca$^{2+}$ (mmol l$^{-1}$)</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Acid group</td>
<td>2.64 ±0.20</td>
<td>2.07 ±0.36</td>
<td>2.80 ±0.24</td>
<td>2.40 ±0.37</td>
<td>2.77 ±0.20*</td>
<td>2.86 ±0.10*</td>
<td>3.04 ±0.20</td>
</tr>
<tr>
<td>Control group</td>
<td>2.24 ±0.24</td>
<td>2.09 ±0.20</td>
<td>2.24 ±0.20</td>
<td>—</td>
<td>1.96 ±0.18</td>
<td>2.06 ±0.03</td>
<td>2.43 ±0.35</td>
</tr>
</tbody>
</table>
haematocrit [Fig. 2(e)] declined up to \( t = 4 \) h and then remained fairly constant until the end of the experiment. In the acid group, a similar initial reduction was seen, but the decrease of the haematocrit was less severe and more transient, and restoration followed after 7 h. The mean cellular haemoglobin concentration [MCHC, Fig. 2(f)] was constant within each group. Compared to the acid group, the control group had a slightly higher MCHC throughout the experiment. This difference between groups was significant at \( t = 24 \) h.

Plasma lactate, glucose, sodium, and chloride did not differ between the acid group and the control group (Table I). Compared to the control group, plasma
potassium was lower in the acid group at $t = -0.5$ h, and the plasma total calcium was higher in the acid group at $t = 7$ h and $t = 24$ h (Table I).

No significant changes were observed in the plasma adrenaline [Fig. 3(a)] or noradrenaline concentrations [Fig. 3(b)]. The values observed in the acid group remained close to the pre-experimental levels at $t = -3$ h and $t = -0.5$ h. As a reaction to the water acidification, the plasma cortisol levels in the acid group showed a transient increase [Fig. 3(c)]. Between $t = 2$ h and $t = 4$ h, as the water pH decreased from 5.8 to 4.0, the cortisol concentration rose threefold and remained at this level up to $t = 7$ h; 17 h later cortisol was similar to control level.

### IV. DISCUSSION

In this study, cannulated carp were exposed to sublethal levels of water acidity. Gradual acidification to pH 4.0 led to only minor transient changes in the measured blood parameters and did not cause any mortality in the 2-day experimental period. Thus, carp are able to survive water of pH 4.0 for 48 h, without major physiological disturbances. In addition, in the first month after their return to the holding tanks, there was no mortality among the experimental fish.

These findings contrast with observations of Ultsch et al. (1981) on the same species. In those experiments, lowering of the water pH abruptly to 4.0 caused a decline of plasma ions, and a progressive reduction of arterial blood pH. A 100% mortality within 24 h was observed at pH 3.5. There are a few differences in experimental conditions and in the protocol between our study and the study of Ultsch et al. The difference in acidification procedure is important. The immediate pH decrease Ultsch used contrasts with our procedure of gradual acidification. Using tilapia, Wendelaar Bonga et al. (1987) found that a sudden drop in water pH (the rate of water acidification was equivalent to 18 pH units per hour), led to substantial structural damage of the branchial epithelium, which included dying of most of the chloride cells, and to severe loss of blood electrolytes. At a lower rate of water acidification (3 pH units per hour and lower) these effects were not seen. This is in agreement with a recent study with carp (S. Wendelaar Bonga, unpubl. obs.), in which a slightly reduced plasma osmolarity after gradual, and a significantly lower plasma osmolarity after acute water acidification was found. Another factor which might have caused a much more severe disturbance in electrolyte status and acid/base balance in Ultsch’s experiment is additional stress: the step change from pH 7.4 to 5.1 was reported to excite the animals. No obvious signs of excitement were observed during our acidification procedure. Balm (1986) showed that handling stress in tilapia in acid water impaired osmoregulation whereas the same type of handling had no effect under control conditions. The $[\text{Ca}]_{\text{tot}}$ in the experimental water was four to five times higher in Ultsch’s experiment ([Ca$^{2+}$] $2.8-3.5 \text{ mmol l}^{-1}$) than in our experiment. The marked depression in plasma pH, found by Ultsch, in comparison to our results, is in agreement with results of Wood (1989) who found that depressions in plasma pH were greatest at the highest water Ca$^{2+}$ and were linearly reduced as Ca$^{2+}$ falls. High water calcium levels are known to be protective against ionoregulatory disturbances (McDonald, 1983b). Thus, in the study by Ultsch et al. (1991), the ionoregulatory disturbances were substantial despite the high water $[\text{Ca}]_{\text{tot}}$. 
To avoid hypercapnic conditions during the water acidification, we used a four-compartment system, which was constantly vigorously aerated. To check if our setup was successful in keeping water $P_{CO_2}$ at control level, it was monitored during the experiment. Despite our precautions, the water $P_{CO_2}$ rose and reached a maximum at $t = 1.5$ h of 0.32 kPa. Claiborne & Heisler (1984) found in carp that mild hypercapnia for 2 h ($P_{CO_2} \approx 1.0$ kPa) increased arterial blood $P_{CO_2}$ (from 0.64 to 1.5 kPa), decreased plasma pH (from 7.87 to 7.63) and elevated plasma $[HCO_3^-]$ (from 14 to 16.5 mmol l$^{-1}$). So we assume that the increased blood $P_{CO_2}$ of 0.25 kPa at $t = 2$ h was mainly due to the elevated water $P_{CO_2}$ at this point in time. This is possibly the reason for at least part of the decrease in the blood pH$_c$. The increased blood $P_{CO_2}$ was not caused by hypoventilation since water acidification caused a transient rise in oxygen uptake in carp (P. van Dijk, G. van den Thillart & S. Wendelaar Bonga, unpubl. obs.).

Erythrocyte recruitment due to spleen contraction is probably responsible for the less pronounced decrease in haematocrit (between $t = -0.5$ h and $t = 48$ h) in the acid group compared to the control group. These new RBCs obviously did not have an effect on the mean cellular haemoglobin concentration. The MCHC was constant throughout the experiment, also indicating the absence of red cell swelling or shrinkage. Similar to the spleen contraction, the red cell swelling is under adrenergic control. In vitro results in carp (Salama & Nikinmaa, 1988) have shown that the adrenergic-induced red cell swelling only occurred when either the plasma pH was below 7.5 at normal $P_{O_2}$ or when at pH 7.5 the $P_{O_2}$ of the incubation was decreased to 30 mmHg or below. In the present study these conditions were never met, which explains the absence of red blood cell swelling.

Moreover, in the present study the catecholamines remained at control level. A rise of catecholamines is typically associated with the alarm phase of the stress response. The absence in our experiment is an indication that our experimental procedure was not very stressful for the animals. The measured plasma concentrations are well within the range known from the literature for cannulated fish at rest (Ristori & Laurent, 1985; Primmett et al., 1986; Butler et al., 1986, 1989). It is possible that our sampling protocol did not detect a short transient rise in adrenaline and/or noradrenaline. Tang & Boutilier (1988) found that plasma catecholamine levels peaked immediately after exhaustive exercise in rainbow trout, and then decreased rapidly over a subsequent 40-min recovery period. Boutilier et al. (1986) suggested that in rainbow trout, plasma catecholamines increase in response to the rate of decrease of blood pH. We found a moderate decrease in pH$_c$ of 0.2 units. In teleosts, due to the Bohr and Root effect, an acidosis can cause hypoxemia. Perry et al. (1989) demonstrated that in rainbow trout, hypoxemia rather than blood acidosis is the proximate stimulus causing the release of catecholamines into the circulation during acute hypercapnic acidosis. From our data, we would not expect hypoxemia to have occurred since measured pH$_c$ values were too high to cause a Root effect (Pelster & Weber, 1990), and the $P_{O_2}$ of the blood did not decrease at any point. From this it follows that the plasma catecholamine concentration must have remained at control levels throughout the experiment. Interestingly, with trout, Witters et al. (1991) who likewise reduced water pH gradually (from pH 6.8 to 5.0 in 3–4 h), also found no significant difference in plasma catecholamine concentration before and after water acidification. Ye et al. (1991), however, also with rainbow trout but
using acute water acidification, did find increases in plasma catecholamine concentrations. It seems plausible to view the acute form of water acidification as an additional stressor, which, in combination with the low pH, causes the plasma catecholamine concentrations to rise. Even though plasma catecholamines remained at control level, we cannot exclude other factors such as changes in turnover rate of catecholamines. We have some evidence for tilapia (P. Balm, P. van Dijk, G. van den Thillart & S. Wendelaar Bonga, unpubl. obs.) that fish exposed to acid water (3 days at pH 4.0) have a lower turnover rate of catecholamines.

Although gradual reduction of the water pH did not noticeably influence electrolyte and acid/base balance of the extracellular fluid, this does not imply that the reduction was not experienced as a stressor. In fact, the reverse is indicated by the transient peak in the plasma cortisol concentration. Such a transient increase is typical for the primary response of fish to stress, including acid stress. Goss & Wood (1988) found in rainbow trout that acid stress (pH = 4.8) caused a transient increase in plasma cortisol concentration. Cortisol was back at control levels after 1 day, a finding similar to that of the present study. Combined acid and aluminium stress (pH = 4.8, Al = 112 µg 1⁻¹), however, caused permanent high plasma cortisol levels until death (Goss & Wood, 1988). The return of the plasma cortisol concentration to the control level does not necessarily imply that cortisol dynamics returned to control values. Balm (1986) concluded that after 2 days of acid stress the turnover rate of cortisol in tilapia is increased. He came to this conclusion because he found an increased release of cortisol by the headkidneys even though plasma cortisol concentrations were similar to those of the controls. Cortisol stimulates the Na⁺-dependent ATPase activity of the chloride cells, which promotes active Na⁺-uptake (Mayer-Gostan et al., 1987). The fact that plasma cortisol was elevated while plasma sodium was not decreased might mean that a rise in sodium efflux was compensated for by an increase in active sodium uptake.

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