ABSTRACT

The effects were studied on short-term and chronic acid exposure (1 day - 3 months) on prolactin secretion and on several parameters affected by prolactin in tilapia. The short-term effects are not only dependent on the pH level but also on the rate of acidification. Rapid acidification to pH 4.0 leads to necrosis of the integumental epithelium, causes severe damage of the skin and high plasma electrolyte losses, and impairs the response of the prolactin cells. At a lower rate (0.05 pH unit/min) necrosis hardly occurs and the drop in plasma electrolytes is minimal. Nevertheless, branchial permeability to water and ions increases and, probably as a direct response, prolactin secretion increases threefold. During chronic exposure branchial permeability and plasma electrolyte levels are normal, but prolactin secretion remains high. Thickness and mucocyte density of the epidermis are increased, which may represent a structural correlate of the reducing effect of prolactin on the permeability to water and ion of this epithelium. Chronic exposure to pH 4.0 does not increase the resistance of the fish to a further decrease of water pH.

INTRODUCTION

The effects of acidification of the ambient water on fish are multiple and complicated. Laboratory observations indicate that disturbed acid-base balance and water- and ion regulation are the primary deleterious effects, that may be lethal under conditions of severe acid stress (McWilliams, 1960; McDowall, 1983; Wendelaar Bonga et al., 1984a). From field studies it has been concluded that it is not increased mortality of adult fish, but recruitment failure caused by reduced reproductive activity in combination with limiting hatching success that are the main causes of the reduction and often disappearance of fish populations from acidified water (Maine, 1981; Frenette and Dodson, 1984).

This discrepancy between the conclusions from laboratory and field studies are partially caused by differences in the parameters determined, and partially by differences in the conditions investigated. Most field studies are dealing with the effects of chronic but sublethal acidification, and attention is usually concentrated on population structure and reproductive success. However, under field conditions the effects of acidity of the water are difficult to separate from those of other variables such as the presence...
of toxic metals. Moreover, disturbances caused by capture of the fish hamper the proper evaluation of plasma electrolyte balance or the acid base status.

The evaluation of these physiological parameters has been the aim of most of the laboratory studies. These usually concern short-term experiments to analyse the effects of acute exposure of fish to low pH. Exposure time is often too short not only to determine effects on growth and reproduction, but also for evaluation of the process of physiological acclimation. It may take weeks and possibly months before new equilibria have been fully established. This implies that to date only the first stages of acclimation to acid stress have been evaluated adequately. We therefore have started experiments of two months and more to study acclimation in the cichlid teleost *Oreochromis mossambicus* (tilapia). Originating from Africa, tilapia has been introduced in tropical areas of most continents. It is successfully used for fish farming, although frequently in heavily polluted and acidified water. It has the great advantage over most other fish species that its whole life cycle can be studied in the laboratory.

From earlier studies we have concluded that prolactin is one of the primary hormones controlling physiological acclimation to acid water in tilapia and probably many other fish species. The hormone has multiple effects. These include control of plasma osmolarity, sodium and calcium levels, permeability of the integument to water and ions, the active uptake of calcium via the chloride cells, and secretion of mucus by the integument (Loretz and Bern, 1982; Wendelaar Bonga and Meis, 1981; Flik et al., 1986). Prolactin is a slow-acting hormone, probably because its action on water and ion regulation involves the stimulation of growth and differentiation of epidermal cells, including the filament containing cells, chloride cells and mucocytes.

In the present chapter we report on the effects of exposure periods up to three months on prolactin secretion, and on various parameters known to be effected by prolactin: plasma electrolytes, osmotic permeability of the gills to water, calcium fluxes of the gills, thickness of the skin epithelium and the density of mucocytes. Effects on growth and reproduction are also reported. Finally, to investigate whether or not a two-month acclimation period increases the resistance of the fish to a further drop in water pH, we compared the capacity of the acid-acclimated fish and unexposed control fish to cope with a severe, almost lethal drop in water pH. Data on sodium fluxes of acid exposed tilapia are reported separately (Flik et al., 1987).
MATERIALS AND METHODS

Adult sexually mature tilapia Oreochromis mossambicus from laboratory stock were kept in well aerated freshwater tanks as described in Wendelaar Bonga et al., 1984a. Methods for microscopy, estimation of prolactin secretion, determination of plasma osmolarity and electrolyte levels and branchial osmotic water uptake rates have been described in the same paper or in Wendelaar Bonga et al., 1984b. For branchial calcium fluxes, see Flik et al., 1985. Epithelial height and density of mucocytes of the skin epidermis were determined in light-microscopical sections as described in Wendelaar Bonga and Meis (1981). HgSO₄ was used for water acidification.

RESULTS

1. Prolactin secretion.

Effects of short-term exposure (0-10 days). The most prominent phenomenon occurring in the pituitary gland of acid stressed tilapia is the marked activation of the prolactin cells. The first indication is a massive degranulation of the prolactin cells during the first hours following acidification of the water. The prolactin cells increase substantially in size (Fig. 1) and in number during the first two weeks at pH 4.0. A marked increase of the granular endoplasmic reticulum becomes noticeable within a few days (Wendelaar Bonga et al., 1984a). The total size of the rostral pars distalis (prolactin lobe) almost doubles. This increase is caused by the increase in size and number of the prolactin cells, which comprise most of the prolactin lobe.

Fig. 1. Effect of exposure of male tilapia to water of pH 4.0 on prolactin cell volume (n=6), and on prolactin synthesis as estimated in vitro by determining the 3H-lysine incorporation rate into prolactin of freshly dissected prolactin lobes (100%: controls from water of pH 7.3; n=3-4). Means ± S.E.
A minor part of the increase is caused by proliferation and hyperplasia of the ACTH cells (Wendelaar Bonga and Balm, 1987). The structural changes shown by the PRL cells reflect a dramatic rise in prolactin synthesis and release. This can be demonstrated when the freshly dissected prolactin lobes of acid exposed fish are incubated in a medium containing tritiated amino acids. After a standard incubation procedure (Wendelaar Bonga et al. 1981a) the rate of prolactin synthesis - a direct reflection of the in vivo rate of prolactin secretion at the time of capture - can be estimated after gel electrophoresis of homogenates of the prolactin lobes and of the incubation media by autoradiography and densitometry of the bands representing prolactin. The results show a dramatic rise of prolactin synthesis and release during the first day of acid exposure. The rate of increase of prolactin synthesis is much higher than that of cell volume (Fig. 1). The rate of prolactin synthesis is inversely related to water pH (Fig. 2). Fish exposed for 10 days to water of pH 4.0 show a threefold increase over control fish (Fig. 2). The increase in the rate of release of prolactin from the prolactin lobes is similar to that of prolactin synthesis (results not shown). However, the intensity of the response of prolactin secretion to water acidification is not only dependent on water pH, but also on the rate of reduction of water pH at the start of the experiment. A threefold increase in water pH is only obtained after reduction of the water pH at a rate of 0.05 pH unit per min. or lower. When the rate is accelerated to 0.3 pH unit per min. - this leads to substantial damage of the branchial epithelium, see below - the short term response of the prolactin cells is less intense, and the increase in the rate of prolactin synthesis is only twofold (Fig. 2). Apparently, if the drop in water pH is too severe, the endocrine response is hampered.

Fig. 2. Effect of exposure of male tilapia for 10 days to water pH 4.0 or 4.5 on prolactin synthesis as estimated in vivo by determining the 3H-lysine incorporation rate into prolactin of freshly dissected prolactin lobes (controls, water pH 7.3: 100%, n=3-4). The fish were adapted to acid water at a rate of about 0.05 pH unit.min⁻¹; 4.0° fish adapted at a rate of about 0.3 pH unit.min⁻¹. Means ± S.D.
Effects of long-term exposure (11-80 days). After ten days at pH 4.0 the volume of the individual prolactin cells maintains a high level until day 80. The rate of prolactin synthesis per animal shows a slight tendency to decrease, but at day 80 it is still not significantly different from the high level observed at day 10.

2. Plasma electrolytes.

Effects of short-term exposure. During the first days at low pH plasma osmolality and electrolyte levels tend to decline. However, the severity of this decline is not only dependent on water pH (Wendelaar Bonga et al., 1984a), but also on the rate of reduction of water pH. At a rate of about 0.3 pH unit or more per minute the reduction in water of pH 4.0 can be 10% or more (Wendelaar Bonga et al., 1984a). At a rate of 0.05 pH unit or less it is only a few percent and often not statistically significant (Table 1). At this rate normal plasma osmolality and electrolyte levels are usually restored within a few days, whereas at the high rates of acidification restoration does occur, but it may take several weeks before control levels are obtained, especially at pH levels below 4.0 (Wendelaar Bonga et al., 1984a).

Effects of long-term exposure. Irrespective of the initial rate of reduction of the pH, normal plasma osmolality and sodium and calcium levels are found, forty (Wendelaar Bonga et al., 1984), sixty (Table 1) or eighty days (results not shown) of exposure to pH 4.0.

Table 1. Effect of exposure to water of pH 4.0 on plasma osmolality, sodium and total calcium levels and osmotic permeability of the gills to water of tilapia; unexposed fish were from water of pH 7.3; n=5; means ± S.D.

<table>
<thead>
<tr>
<th>Days of exposure to pH 4.0</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasma osmolality (mosmol/L⁻¹)</td>
<td>314 ± 6</td>
<td>302 ± 7</td>
<td>318 ± 9</td>
<td>313 ± 11</td>
</tr>
<tr>
<td>plasma Na⁺ (mM/L⁻¹)</td>
<td>138 ± 11</td>
<td>132 ± 9</td>
<td>138 ± 7</td>
<td>143 ± 13</td>
</tr>
<tr>
<td>plasma total Ca (mM/L⁻¹)</td>
<td>2.94 ± 0.08</td>
<td>2.79 ± 0.09</td>
<td>3.09 ± 0.11</td>
<td>3.05 ± 0.10</td>
</tr>
<tr>
<td>branchial osmotic water permeability*</td>
<td>1.25 ± 0.17</td>
<td>1.05 ± 0.23*</td>
<td>1.36 ± 0.15</td>
<td>1.41 ± 0.21</td>
</tr>
</tbody>
</table>

*) ml H₂O perfused/100 ml gill water/osmol/min during incubation in distilled water, pH 4.0; *') significantly different, P < 0.01.
3. Branchial osmotic permeability to water.

The osmotic permeability to water of the gills was estimated by determining the water inflow rate upon incubation of freshly isolated gills in distilled water at the same pH as the fish concerned (for more details see Ogawa et al., 1973; Wendelaar Bonga et al., 1981(a)). Exposure to water of pH 4.0 leads to a rapid increase of the water permeability of the gills, but control values are found within days, and these are maintained for at least 60 days at low pH (Table 1).

1. Branchial calcium fluxes.

Acid water exposure initially leads to loss of bone calcium in tilapia, although the extent is determined by the rate of pH decrease. At the slow rates of acidification mentioned above it is often hardly noticeable. After a rather abrupt change, high bone calcium losses have been observed in the first weeks of exposure to water of pH 4.0. Restoration occurred in the following weeks (Wendelaar Bonga and Dederen, 1985). Losses were higher (up to 15%) in the scales than in the skeletal bones (6-8%). The losses are most likely caused by increased branchial permeability to Ca\(^{2+}\), and to reduction of the active uptake of Ca\(^{2+}\) across the gill epithelium.

In tilapia, Ca\(^{2+}\) exchange with the water is almost exclusively limited to one gill area (Flik et al., 1985). In growing fish as used in our experiments, the influx (mostly ATP-dependent, probably transcellular active uptake) surpasses the efflux of Ca\(^{2+}\) (passive, probably paracellular outward diffusion). This results in a net uptake of Ca\(^{2+}\), which suffices skeletal growth and metabolic needs of calcium (Flik et al., 1985; Fig. 3). Abrupt exposure of fish to pH 3.0 results in a dramatic reduction of the influx and a similarly dramatic increase of Ca\(^{2+}\)-efflux (Fig. 3). The resulting net loss of calcium explains the initial drop in plasma calcium as well as the decalcification of bone that occur in tilapia exposed to acidified water.

\[ F_{\text{net}} = F_{\text{in}} - F_{\text{out}} \]

Fig. 3. Ca\(^{2+}\)-flux rates after transfer of male tilapia to water of pH 3.0. In- and efflux rates were determined simultaneously by exposing \(^{45}\)Ca-labeled fish (74 KBq g\(^{-1}\) fish, intraperitoneally injected 4 days prior to the experiment) to \(^{44}\)Ca-containing water. \(^{45}\)Ca radioactivity in water and plasma samples was determined after decay of \(^{44}\)Ca. Mean ± S.D. of four fish of about 20 g body weight.
We have no data on calcium fluxes after long-term exposure. However, since after a few weeks plasma calcium as well as bone calcium density return to normal values, it is clear that the fish rapidly regain a positive calcium balance. Whether this is due to reduced efflux, restoration of influx or - this is most likely - both, remains to be established.

5. The epithelium of the skin.

Effects of short-term exposure. We have examined the epithelium covering the gills and the abdominal part of the skin. In both areas, exposure to acid water induces necrosis of the superficially located cells of the skin, including mucocytes and, in the gills, the chloride cells. However, at a given pH, the extent of the necrosis is dependent on water pH as well as on the rate of reduction of water pH. After reduction to pH 4.0, at the high rate mentioned above, many cells, including most of the chloride cells, are killed. In most parts of the skin, which are covered by six to ten cell layers, the epithelium remains visibly intact, except for the upper one or two layers. In the lamellae of the gills, however, which are usually covered by only one cell layer, this may lead to substantial damage, and often disruption of the epithelial structure. In the skin epithelium the necrotic cell layers are sloughed off after a few days and become replaced by cells not noticeably different from the control condition. The epithelium increases in thickness and contains many mucocytes.

When the water pH is reduced to pH 4.0 at a lower rate, necrosis is occasionally observed. Disruption of the epithelium of the branchial lamellae does not occur, and the structure of most of the chloride cell is not noticeably affected. The mucocytes in gills and abdominal skin show signs of increased release activity. The height of the abdominal skin epithelium as well as the number of mucocytes increase significantly during the first ten days of acid exposure (Fig. 4).

![Fig. 4. Epithelial height and number of mucocytes as determined light microscopically in cross-sections of the abdominal epidermis. Fish were exposed to water of pH 4.0; control values: pH 7.3 (day 0). Rate of reduction of water pH: about 0.05 pH unit/min.](image-url)
Effects of long-term exposure. After the first ten days, only minor changes occur in the skin epithelium. In the gills, the number of chloride cells and mucocytes remains higher than in controls, although there is a tendency to decrease. In the abdominal skin, epithelial height and number of mucocytes remain at the elevated level reached already at day 5 (Fig. 4).

Immediately after reduction of water pH to pH 4.0, irrespective of the rate of reduction, growth ceases and reproductive activity is no longer observed. During long-term exposure, growth resumes but at a 50% lower rate than under control conditions. No spawning occurs in the observation period of 80 days, although tilapia are successive spawners with a spawning cycle in the controls of 28.2 ± 6.0 days (a group of 7 males and 8 females). In the ovaries of the acid exposed fish large scale atresia was observed.

7. Resistance to increased acid stress.
Groups of fish exposed for 10 or 60 days to water of pH 4.0 were transferred to water of pH 3.2 (rate of pH reduction: 0.05 pH unit per min.). After 18 h of exposure, this leads to only minor changes in plasma electrolyte levels and there is no mortality. In the fish pre-exposed to water of pH 4.0, plasma electrolyte losses are substantial, in particular the loss of calcium, and mortality is high, irrespective of the length of pre-exposure time at pH 4.0 (Table 2).

### Table 2. Effect of exposure to water of pH 3.2 for 18 h on plasma osmolarity, sodium and total calcium, and on survival of male tilapia pre-exposed to water of pH 4.0 for 0 (fish from water of pH 7.3), 10 or 60 days; n=6-12; survival percentage refers to males and females, n=12-16; means ± S.D.

<table>
<thead>
<tr>
<th>Days of pre-exposure to pH 4.0</th>
<th>0</th>
<th>10</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasma osmolarity (mosmol.L⁻¹)</td>
<td>305 ± 11 (-15%)</td>
<td>268 ± 13 (-16%)</td>
<td>271 ± 9 (-15%)</td>
</tr>
<tr>
<td>plasma Na⁺ (mM.L⁻¹)</td>
<td>131 ± 6 (-15%)</td>
<td>113 ± 8 (-15%)</td>
<td>120 ± 7 (-15%)</td>
</tr>
<tr>
<td>plasma total Ca (mM.L⁻¹)</td>
<td>2.74 ± 0.12 (-7%)</td>
<td>2.40 ± 0.09 (-25%)</td>
<td>2.35 ± 0.10 (-30%)</td>
</tr>
<tr>
<td>survival (%)</td>
<td>100</td>
<td>40</td>
<td>46</td>
</tr>
</tbody>
</table>

*Significantly different (P < 0.05) from control values (presented in Table 1).
Evidence is growing that many hormones are involved in the response of fish to acid stress. We have recently reviewed the literature (Wendelaar Bonga and Balm, 1987) and have concluded that several pituitary cell types - in particular prolactin cells, ACTH cells and MSH cells - as well as the interrenal cells, the adrenocortical homologue of teleosts, become activated. ACTH and MSH both are involved in the control of interrenal cells. The pituitary-interrenal axis in fish probably plays a similar role in the physiological response to many types of stress as the pituitary-adrenal axis in higher vertebrates (Mazeaud and Mazeaud, 1981). Cortisol, the major mineralocorticoid of teleosts is undoubtedly of importance in the primary response of fish to acid stress. Transient, but high increases of cortisol have been reported for trout and tilapia (Brown et al., 1984; Balm, 1986). Cortisol promotes tissue catabolism, which leads to higher glycemia and increased nitrogen excretion, and stimulation of the Na⁺-dependent ATPases of the chloride cells, which promotes active Na⁺ uptake. A comparable response of the pituitary-interrenal axis is known to occur after other types of stress, e.g. hypoxia, exposure to heavy metals, temperature change, exhaustive exercise or handling of fish. These types of stress all induce disturbances of hydromineral regulation and acid base balance in fish, similar as exposure to acid water.

In addition to cortisol, prolactin is an important osmoregulatory hormone in fish (Loretz and Bern, 1982). As mentioned in the Introduction, this hormone controls the permeability of the integument, in particular its epithelial layer, to water and ions. Although prolactin, unlike cortisol, is not generally considered a hormone participating in the primary stress response of fish, it certainly is of major importance for adaptation to acid stress. The secretion of prolactin is greatly stimulated immediately after water acidification (Wendelaar Bonga et al., 1986a,b; Balm 1987). The drop in plasma electrolytes, caused by increased permeability of the gills to water and ions, is considered the stimulus for this response. The present data show that prolactin secretion remains at a very high level throughout the 80-days experimental period, long after the initially decreased plasma osmolarity and ion levels returned to normal. We suggest that this restoration, and the subsequent maintenance of normal plasma electrolyte levels during chronic exposure to acid water, is at least partially effected by the chronically elevated secretion of prolactin. This is indicated by the present observation that the osmotic permeability of the gills to water returns to normal within 10
days in acid water. Further evidence is provided by the apparent restoration of the calcium balance of tilapia after several weeks in acid water (Wendelaar Bonga and Decteren, 1986). Both in- and efflux of calcium are controlled by prolactin in this species (Flik et al., 1985). From our study on the sodium balance of tilapia in acid water we conclude that the positive balance observed during prolonged acid stress is caused by the prolactin controlled low branchial Na⁺ efflux rather than by the cortisol controlled active uptake of Na⁺ from the water (Flik et al., 1987). Prolactin further stimulates growth and differentiation of the epidermal cells, including the mucocytes, in gills and skin (Wendelaar Bonga and Meis, 1982). The increased height of the skin epithelium and density of mucocytes, that continued throughout the experimental period, further reflect increased prolactin secretion during acid stress. These phenomena may represent the structural correlate of prolactin's action on the epidermal permeability to water and ions.

Increased ion losses across the gills generally occur after acidification of the water. Subsequent reduction of the loss rates and restoration of plasma ion levels have been reported, although there are marked species-specific differences (McDonald 1983; McDonald et al., 1983; Krout and Dunson, 1985). Prolactin may therefore contribute to this reduction of ion losses also in other species than tilapia. However, stimulation of active ion uptake has also been reported e.g. for trout and mudminnow (McDonald et al., 1983; Flik et al., 1987) and thus the involvement of cortisol is also indicated. Most likely, prolactin and cortisol act synergistically in the restoration and maintenance of ion homeostasis in acid water. However, whereas prolactin seems to dominate in tilapia, in other species, such as the mudminnow, cortisol may play the leading role: in contrast to tilapia, the positive sodium balance of the mudminnow is effected by increase of the Na⁺-influx rather than by reduction of the Na⁺-efflux (Flik et al., 1987).

Our data show that the effects of acute exposure to acid water are not only dependent on the pH level, but also to a remarkable extent on the rate of reduction of water pH. A high rate of reduction reduces necrosis of the outer cell layers of the epidermis and this may have devastating effects on the gills. It also obviously hampers the endocrine response at critical pH levels. Most fishes are more sensitive to acid stress than tilapia, and the rate of reduction of water pH that is harmful for other species may be correspondingly lower. This experimental parameter is hardly ever mentioned in literature. The present study indicates, however, that it is essential for evaluation not only of the effects of water pH in short-term laboratory
experiments but also of the consequences of acid spills of sudden snow melts on fish populations in natural waters.

Finally, we conclude from our observations that chronic exposure to acid water does not enhance the resistance of fish to a further reduction of water pH. Swarts et al. (1985) came to the same conclusion for rainbow trout, although in their experiments the pretreatment lasted only 5 days. Apparently, the fish only compensate the effects of acid water, but are unable to acclimate successfully. This is also indicated by observations that fish pre-exposed to acid water are less resistant to handling stress (Barton et al., 1985) or exhaustive exercise (Graham and Wood, 1981). During the present experiments growth was reduced and reproduction stopped completely. These and similar observations (Mount, 1973; Frenette and Dodson, 1981; Frenette and Payson, 1986) are in line with the above conclusion that acclimation does not occur. Apparently, successful adaptation as has been reported for acid-resistant wild strains of some species of fish, develops very slowly. This may be genetic adaptation rather than physiological acclimation. For fish an episodic drop in water pH seems more dangerous in water that is chronically acidified than in neutral water.

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sodium flow in the tilapia Oreochromis mossambicus and the East American mudminnow Umbra pygmaea (Submitted).


