Prolactin and Acid Stress in the Teleost Oreochromis (formerly Sarotherodon) mossambicus

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Prolactin is probably implicated in the control of physiological adaptation to sublethal acid stress in tilapia. Exposure of fish to water at pH 3 caused death within 72 hr, which was associated with pronounced hemodilution. At pH 4 mortality was low, although a substantial and rapid decrease in plasma osmolality, plasma Na\(^+\), and plasma total Ca was observed. These effects were at least partly due to increased permeability of the gills for water and ions. After a few days at pH 4, restoration of plasma osmolality, Na\(^+\), and total Ca was noticeable. Control levels were reached after 5 days for Ca, and after 10 days for osmolality, Na\(^+\), and branchial osmotic water permeability. Prolactin secretion increased markedly during acid exposure, as was established by morphometrical and biochemical methods. In tilapia, administration of prolactin is known to raise plasma osmolality, Na\(^+\), and plasma total Ca. This hormone further has been shown to reduce branchial osmotic water permeability. It is concluded therefore that the restoration of plasma electrolytes and branchial osmotic water permeability during chronic acid stress are causally connected with the observed stimulation of prolactin secretion.

Many aquatic organisms are exposed to high acidity levels due to acid rain. In fish, acute exposure to water with an acidity lower than pH 4 usually leads to death within some hours or days. Although respiratory problems may contribute to this high mortality, the main cause of death is considered the high loss of ions, mainly through the gills (Muniz and Leivestad, 1980a; McDonald, 1983a, b; Milligan and Wood, 1982). Acute exposure to water of pH 4 or 5 may also be lethal for fish. However, the survival time is usually longer, although often with notable disturbances of the ionic composition of the body fluids. And, although a substantial decline in fish populations has been reported for such areas, fish are still present in naturally acidified water up from pH 4 (Creasner, 1930; Leivestad et al., 1976; Muniz and Leivestad, 1980a; Harvey, 1980). For trout, natural populations are known that have developed tolerance to acid stress (Gjedrem, 1976; McWilliams, 1982).

The physiological mechanisms that enable fish to live in acid water are not well known. Since water and ion balance is under endocrine control, hormones are undoubtedly closely implicated in the process of adaptation to acid water. However, reports on the endocrine system during acid exposure are scarce. Most attention has been focused on cortisol (Ashcom, 1979). We have studied the role that prolactin may perform during the first weeks of adaptation of the tilapia Oreochromis mossambicus to water of sublethal pH. Prolactin was chosen because it is, with cortisol, one of the main osmoregulatory hormones in freshwater fish. The ionic disturbances caused by low pH are supposed to be caused mainly by increased permeability of the gills to ions (McWilliams and Potts, 1978; McDonald, 1983a, b). This supposition has been corroborated by the observation that acid tolerance in trout is associated with low Na\(^+\) permeability (McWilliams, 1982). The control of the branchial permeability to water and ions is one of the main functions of prolactin in fish, and therefore its involvement in adaptation to acid stress is indicated. Administration of mammalian prolactin reduces the permeability of the gills to water and ions.
in several species, including tilapia (Dhar-mamba and Maetz, 1972; Wendelaar Bonga and Van der Meij, 1981). This action of prolactin contributes to the maintenance of plasma osmolality and plasma ion concentrations.

In this paper we report the effects of acid stress on prolactin cell activity and on some physiological parameters controlled by prolactin: plasma osmolality, Na\(^+\), and total Ca, and the osmotic water permeability of the gills. This report is part of our studies on the environmental control of prolactin secretion in fish.

**MATERIALS AND METHODS**

Sexually mature male *Oreochromis mossambicus* averaging 12 cm in length and 20 g in weight were obtained from laboratory stock. The fish were kept in 100-liter freshwater aquaria at 25\(^\circ\)C on a 12-hr photoperiod, and were fed throughout the experiments with Tetramin tropical fish food. The fish were transferred to experimental tanks about 4 days before acidification of the water. Direct transfer to tanks with acid water appeared to result in a much higher mortality, apparently due to the combination of handling stress and acid stress.

The experimental tanks contained circulating tap water [Ca\(^2+\) concentration: 0.8 mM; for ionic composition see Wendelaar Bonga and Van der Meij (1981)]. The concentration of nitrogenous wastes was monitored daily in the first week of exposure (when the ammonia concentration usually rises rapidly), and every third day in the remaining part of the experimental period, and was kept at a low level ([NH\(_4^+\) < 0.5 mg/liter) by replacing part of the water. At the first day of the experimental period (Day 0) the pH of the water was reduced to pH 5.4, or 3, respectively, by adding H\(_2\)SO\(_4\). Adjustments of the pH was followed by titration with NaOH or H\(_2\)SO\(_4\). The final sulfate concentration never surpassed 2 mM.

At the end of the experimental period fish were slightly anesthetized and blood was collected from the caudal blood vessels. Plasma osmolality and plasma Na\(^+\) and total Ca levels were determined by atomic absorption spectrophotometry.

**Branchial osmotic water uptake rates.** Water inflow rates were determined in isolated gill arches, after the method described by Ogawa et al. (1973), but without equilibration. The two most rostral pairs of gill arches were incubated for 30 min in well-aerated tap water (4 molosmol/liter; pH 7.4). After incubation and freeze-drying the dry weight of the gill arches was determined and the water uptake per mg gill water calculated.

Water inflow rates were expressed as milliliters per 100 ml gill water per osmole of osmotic gradient between gill fluid compartments and incubation medium per minute. The osmotic gradient at the start of the incubation was taken as the difference between the plasma osmolality of the fish concerned and the osmolality of the incubation fluid. During incubation, the gradient lowered because of osmotic water uptake and loss of ions (mainly Na\(^+\), K\(^+\), and Cl\(^-\)). The decrease due to osmotic water inflow was corrected according to Lock et al. (1981). The net ion outflux was estimated from the difference in Na\(^+\), K\(^+\), and Cl\(^-\) content of the incubation fluid before and after incubation. The total increase of these ions correlated well with the increase in osmolality of the incubation fluid, but could be determined with greater accuracy. The ion concentrations were determined by atomic absorption spectrophotometry. After incubation the percentage loss of Na\(^+\) from the gills was calculated. The gill arches were dissolved in concentrated HNO\(_3\) for 1 hr at 60\(^\circ\)C and, after neutralization of this solution with ammonia, the ion concentration was determined and the percentage of Na\(^+\) lost during incubation calculated.

**Estimation of prolactin cell activity.** For light and electron microscopy the pituitary glands were fixed as described previously (Wendelaar Bonga and Van der Meij, 1980), dehydrated, and embedded in Spurr's resin. For light microscopy 1-\(\mu\)m-thick sections were stained with toluidine blue and the volumes of the cells determined as described previously (Wendelaar Bonga, 1978). For determination of the total volume of all prolactin cells per animal, the rostral pars distalis (RPD; "prolactin lobe"), that can easily be distinguished under a dissection microscope, was carefully separated from the pituitary gland. The volumes of the total pituitary gland and of its prolactin lobe were calculated by measuring their water displacement in a microcapillary of known diameter, under a dissection microscope.

To determine the incorporation rate of \([\text{H}]\)lysine, the prolactin lobes were preincubated for 30 min at 22\(^\circ\)C in Dulbecco's Modified Eagle's Medium (MDM), with 1.25 mM CaCl\(_2\) and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes; 310 mosmol/l), and subsequently incubated in 100 \(\mu\)M MDM for 4 hr at 22\(^\circ\)C in a metabolic shaker (40 Hz) with 10 \(\mu\)Ci \([\text{H}]\)lysine (sp act 60 Ci/mol; New England Nuclear Corp., Boston, Mass.). Lobes and media were subjected to SDS–gel electrophoresis as described by Wendelaar Bonga et al. (1983). The slab gels were fixed and sliced, and the slices counted in a Rackbeta LSC liquid scintillation counter. Parts of the gels were silver stained after Morissey (1981) and scanned with a Bio-Rad Model 1650 densitometer.

**Statistics.** The data were statistically analyzed with Student's \(t\) test (two-sided; \(\alpha = 5\%\)).
RESULTS

1. Effect of Water pH on Plasma Osmolality

Acute exposure of fish to acid water of pH 3 resulted in high mortality. Less than 15% survived longer than 48 hr, and all died within 72 hr. After 4 hr, when the first fish died, plasma osmolality was already reduced significantly \( (P < 0.001) \) and the drop in osmolality continued until the end of the experiment (Fig. 1). At pH 4, however, mortality was low. Less than 10% died during the 6 weeks of the experimental period, most of them in the third and fourth weeks. At pH 5, mortality was not significantly different from that of the controls (less than 5%). Around 50 fish per group were examined. At both pH 4 and 5 plasma osmolality dropped abruptly during the first 24 hr \( (P < 0.001 \) and \( P < 0.01 \), respectively; Fig. 1). The reduction was more severe at pH 4. After the first day, a gradual increase followed in both groups. After 10 days at pH 5 and 30 days at pH 4 plasma osmolality had returned to control levels (Fig. 1). Water at pH 4, which was considered to represent sublethal stress to the fish, was selected for further experiments.

2. Plasma \( \text{Na}^+ \) and Plasma Total Ca at pH 4

Exposure to water of pH 4 led to a significant drop \( (P < 0.01 \) after 24 hr) in plasma \( \text{Na}^+ \), that was followed by an almost complete recovery in the following weeks. The changes in plasma \( \text{Na}^+ \) ran parallel to those of plasma osmolality (Fig. 2). Plasma Ca showed a similar pattern, but the initial decrease \( (P < 0.01 \) after 24 hr) was followed by an increase that was more pronounced than that of plasma osmolality or \( \text{Na}^+ \). Plasma total Ca levels were slightly, although not significantly, higher than those of controls already after 5 days.

3. Branchial Permeability to Water and Ions

When freshly isolated gill arches were incubated in fresh water (pH 7.4) for the determination of the osmotic water uptake rates, ions were lost from the gills of acid-stressed fish in a higher rate than from the gills of control fish. In the 30-min incubation period, 8.7 ± 0.6% of the total initial \( \text{Na}^+ \) content of the gills was lost, whereas this percentage amounted to 24.8 ± 4.9 \( (P < 0.001) \), 16.3 ± 3.1 \( (P < 0.01) \), and 12.3 ± 2.9 for gills of fish exposed to pH 4 for 10 hr, 10 days, and 30 days, respectively. This difference implies that the osmotic gradient between the gill fluid compartments and the incubation fluid decreased more in the gills of acid-exposed fish than in the gills of the controls. Table 1 shows that the osmotic water uptake rates—these data were corrected for the differences and changes in the osmotic gradients during the
TABLE 1

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<tr>
<th>Exposure time</th>
<th>Branchial osmotic water inflow rate</th>
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<tr>
<td></td>
<td>Branchial water inflow rate</td>
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<tr>
<td>10 hr</td>
<td>1.32 ± 0.13</td>
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<tr>
<td>10 days</td>
<td>1.23 ± 0.26</td>
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<tr>
<td>30 days</td>
<td>1.28 ± 0.24</td>
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<tr>
<td>pH 4</td>
<td>2.11 ± 0.27*</td>
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<td>1.48 ± 0.19*</td>
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Note. Values are ml H2O inflow/100 ml gill water/osmol/min of fish adapted to fresh water (controls, pH 7.4) or acidified freshwater (pH 4) for 10 hr, 10 days, or 30 days; means ± SD of 10 fish per group.

* Significantly different from controls (P < 0.001).
** Significantly different from pH 4, 10 hr (P < 0.01).

measurements—were significantly increased after 10 hr of acid stress; after 10 days, the osmotic water uptake rate of the gills of acid-exposed fish was no longer significantly different from that of the controls.

4. Prolactin Cell Activity

Size of the rostral pars distalis. The prolactin cells of tilapia, like most other teleost fish, are confined to the rostral pars distalis of the pituitary gland. This lobe consists almost exclusively of prolactin cells interspersed with some nongranulated stellate cells. The RPD also contains adrenocorticotropic (ACTH) cells, but the volume fraction of these cells is negligible. Any substantial volume change of the RPD may be ascribed to changes in prolactin cell volume. Figure 3 showed that the volume of the RPD, expressed as percentage of the pituitary gland volume, displayed a quite pronounced increase during exposure of fish to water to pH 4, especially during the first 10 days (P < 0.001). The other parts of the pituitary gland did not change noticeably in size (data not shown here).

Prolactin cell size. The growth of RPD was paralleled by an increase in volume of the prolactin cells (Fig. 3). However, the increase in cell volume of about 40% (P < 0.01) was less than the increase in RPD volume. This nearly doubled in 4 weeks. Therefore growth of the prolactin cells was apparently accompanied by cell proliferation.

Prolactin cell structure. Ultrastructural examination of prolactin cells of fish exposed for 10 days to water of pH 4 showed marked changes that point to enhanced secretion of prolactin (Figs. 3–5). The extent of the granular endoplasmic reticulum and the volume of the mitochondria per cell increased significantly (P < 0.05). The extent of the Golgi system did not increase significantly, but the percentage of Golgi areas showing signs of active secretion, namely secretory granules in the process of budding off from the Golgi lamellae, almost doubled (P < 0.01). Signs of granule release by exocytosis were also more frequently observed in cells of the acid-exposed fish.

[^3H]Lysine incorporation rate. Freshly dissected prolactin lobes that were incubated in a medium with [^3H]lysine showed uptake of the label and incorporation in prolactin. Part of this labeled and therefore apparently newly synthesized prolactin was released during the incubation period. This was concluded from the autoradiograms prepared after SDS–gel electrophoresis of homogenates of the lobes as well as of the trichloro-acetic acid (TCA)-precipitated fraction of the incubation medium. Two bands, with apparent molecular weights of around 20,000 and 21,500, which are typical for tilapia prolactin (Wendelaar Bonga et al., 1983), were found. In prolactin lobes of fish exposed for 10 days to pH 4, the total amount of labeled prolactin recovered from prolactin lobes and medium was more than twice that of controls, which points to highly stimulated prolactin synthesis (Fig. 6). The data further show that the amount of labeled prolactin released in the incubation medium increased for the lobes from the acid-stressed fish. However, these data do not properly reflect total release of prolactin during the incubation period, since during incubation both labeled, newly synthesized, prolactin and unlabeled prolactin were released. The latter fraction represents prolactin that had been synthesized before the start of the incubation.
tometry of silver-stained SDS gels, which revealed labeled as well as unlabeled prolactin, showed that the release of prolactin by the prolactin lobes of fish from acid water was increased by almost a factor of three (Fig. 7).

DISCUSSION

1. Low pH and Plasma Electrolytes

Acute exposure to water of pH 3 proved to be lethal for tilapia. Observations on trout (Daye and Garside, 1975, 1977) and perch (Lyons, 1982) have also shown that acute exposure to pH levels below pH 4 is critical for fish. Field observations have revealed that fish hardly occur in water with a pH lower than 4 (Harvey, 1980). However, comparison of laboratory data with field observations is complicated by the circumstance that in natural waters other factors associated with low pH, especially high aluminum concentrations, may contribute significantly to the deleterious effects of acid water (Fromm, 1980; Muniz and Leivestad, 1980b). The high mortality of tilapia at pH 3 was associated with a sudden and severe drop in plasma osmolality. Losses of plasma electrolytes have been commonly observed in acid-stressed fish. It reflects disturbed osmoregulation, and this is considered by several authors the major cause of death under lethal pH stress (Jozuka and Adachi, 1979; Muniz and Leivestad, 1980a; Lyons, 1982; Milligan and Wood, 1982; Nieminen et al., 1982; McDonald, 1983a, b).

Exposure of tilapia to sublethal acid stress (pH 4 or 5) also led to a pronounced drop in plasma electrolytes. However, it was less than at pH 3 and already after 5 days a tendency became noticeable that plasma osmolality, Na⁺, and total Ca levels increased. In the course of the following weeks almost normal levels were found. Thus, tilapia show an adaptive response to acid stress. Restoration of plasma electrolytes, as observed in tilapia, has to our knowledge not been reported for other species. This may be due to the short duration of most experiments (varying from one or more hours to several days). But in acid-stressed brown trout plasma chloride levels
Figs. 4, 5. Prolactin cells of fish exposed for 10 days to water of pH 4 (Fig. 4) or pH 7.4 (controls, Fig. 5). At pH 4 prolactin cells are enlarged and show more granular endoplasmic reticulum (ger) and more extensive Golgi areas (Ga); 11,000 ×.
remained low for at least 7 days (Muniz and Leivestad, 1980a). In acid-stressed brook trout, plasma Na$^+$ showed a fall that continued throughout the 5-day experimental period, although the Na$^+$ uptake, after a severe inhibition in the first few hours of acid exposure, was partially restored in the hours thereafter (Ashcom, 1979). In brown trout, recovery of Na$^+$ influx was complete after 10 days at pH 6, but only 20% at pH 4.6, and even absent at pH 4.0 (McWilliams, 1980a, b).

2. Low pH and Gill Permeability

Our data on the water uptake rates of gills incubated in vitro show that the uptake rate per unit of osmotic gradient, and thus the osmotic water permeability of the gill surface (Ogawa et al., 1973; Wendelaar Bonga et al., 1983), is significantly increased in the first day of acid exposure. The high net efflux of ions from the gills observed in the same experiments indicates that the permeability to ions is also increased substantially, although this may be partly due to a reduction of the active uptake of ions. In trout, exposure to low pH was shown to stimulate passive ion efflux (Packer and Dunson, 1970; Ashcom, 1979; McWilliams and Potts, 1978; McDonald, 1983a). Such changes in ion permeability may account for most of the drop in plasma electrolytes following acid exposure (Ashcom, 1979; McDonald, 1983a). In trout, increased urinary losses also contribute to the reduction of plasma electrolytes (McDonald, 1983a).

Our data on tilapia show that an initial marked increase in gill permeability is followed by a return to control levels after about 10 days. Probably the recovery of normal branchial permeability after 10 days is causally related to the concurrent restoration of plasma electrolyte levels. Such a relationship was also found in a natural population of acid-tolerant brown trout. Acid tolerance, as reflected by reduced ion effluxes during acid exposure, appeared to
be associated with low branchial permeability to Na\(^+\) and H\(^+\) (McWilliams, 1982).

3. Low pH and Prolactin Secretion

Exposure of tilapia to low pH leads to a pronounced increase in prolactin cell activity, as estimated by two different procedures. The morphometrical results—increases in volume of the rostral pars distalis, prolactin cell and nuclear volume, extent of granular endoplasmic reticulum, mitochondria, and Golgi areas—all point to an increased capacity of the fish to secrete prolactin. The prolactin cells of these fish were the largest we ever found in our experiments with tilapia. The higher incidence of visible signs of granule formation in the Golgi areas and of exocytosis of secretory granules further demonstrate that synthesis and release of prolactin are considerably increased in the acid-stressed fish. The high rates of synthesis of prolactin displayed by freshly dissected pituitary lobes of fish from acid water further support this conclusion.

Our earlier studies on the prolactin cells in *O. mossambicus* have shown that osmolality and the concentrations in the ambient water of divalent ions (Ca\(^{2+}\) and, to a lesser extent, Mg\(^{2+}\)) are the most important factors that determine the rate of prolactin secretion in vivo (Wendelaar Bonga and Van der Meij, 1980, 1981; Wendelaar Bonga et al., 1983). In freshwater with relatively low Ca\(^{2+}\) and Mg\(^{2+}\) levels, prolactin secretion is high. When the osmolality of the water is increased by adding NaCl, prolactin cell activity is reduced, until a minimum is reached in water that is isosmotic with the blood (around 300 mosmol/liter). Under such conditions the osmotic water fluxes across the gill epithelium are almost zero and branchial passive fluxes of Na\(^+\) and Cl\(^-\) are very low. A reduction in osmotic water fluxes and passive ion fluxes is also effected by high concentrations of Ca\(^{2+}\) and, to a lesser degree, by Mg\(^{2+}\). Since in the same concentration range these ions specifically reduce prolactin secretion, we have concluded that the effects of ambient osmolality and ambient Ca\(^{2+}\) and Mg\(^{2+}\) levels on the prolactin cells are mediated by the influence of these factors on the branchial fluxes for water and ions (Wendelaar Bonga and Van der Meij, 1980, 1981). We have postulated that any factor that alters the passive fluxes of monovalent ions and water across the gills will also influence prolactin secretion. The pH of the water is such a factor; in tilapia reduction of the normal pH of the water increases the permeability of the gills to water and ions, and in addition stimulates prolactin secretion.

The reduction of the branchial osmotic water permeability to almost normal values that followed the initial increase may be effected by prolactin. Administration of ovine prolactin in tilapia (Wendelaar Bonga and Van der Meij, 1981) and some other fish species (Ogawa *et al.*, 1973; Ogawa, 1977) reduces the osmotic water permeability of the gills, when measured with the same *in vitro* procedure used in this study. Prolactin administration has also been shown to reduce the passive Na\(^+\) fluxes in tilapia (Dharmamba and Maetz, 1972). The restoration of plasma osmolality and Na\(^+\) that follows the initial reduction in the acid-stressed fish, is likely related partly to the reduction of the gill permeability and can therefore be considered an indirect effect of the enhanced prolactin secretion. Cortisol may also contribute to the restoration of normal plasma electrolyte levels, by stimulating the active uptake of Na\(^+\). We found enhanced plasma cortisol levels in acid-stressed tilapia (unpublished observations). Similar results have been reported for acid-stressed brook trout (Ashcom, 1979).

Prolactin may also be responsible for the rapid restoration of plasma calcium and the slight but significant hypercalcemia that was observed in the second and third weeks of acid exposure. In tilapia, prolactin stimulates the active uptake of calcium through
the gills and has a hypercalcemic action (Wendelaar Bonga and Flik, 1982; Wendelaar Bonga et al., 1983). We have connected this calcium-mobilizing property of prolactin with its reducing effect on gill permeability (Wendelaar Bonga et al., 1983). Permeability of cellular membranes is directly related to the amount of calcium and, to a lesser extent, magnesium bound to membrane phospholipids (Schoffeniels, 1967; Ebel and Günther, 1980). For brown trout gills, McWilliams (1983) has shown that, 

- Calcium but also by increasing the calcium
- Calcium from the gill surface. He further showed that, in vitro at low pH levels, the surface-bound calcium is lost less rapidly from the gills of an acid-tolerant strain than from the gills of a nontolerant strain of brown trout. The tolerance to acid stress was further associated with gill permeability to Na⁺ and H⁺ (McWilliams, 1982).

Such a reduction might be effected by prolactin, that could act not only by mobilizing calcium but also by increasing the calcium binding capacity of the gill membranes.

REFERENCES


McWilliams, P. G., and Potts, W. T. W. (1978). Effects of pH and calcium concentrations on gill poten-


