Cellular responses of the skin and changes in plasma cortisol levels of trout (Oncorhynchus mykiss) exposed to acidified water

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Abstract. The skin structure and the plasma cortisol levels of trout, Oncorhynchus mykiss, were examined during 7 days of exposure to water of pH 5. By day-4 and -7 the thickness of the epidermis was significantly (0.05) less in acid exposed fish than in controls, and degenerative cells were common in the upper epidermal layers. Many epidermal cells exhibited signs of necrosis, and by day-7 many apoptotic cells were also present. Secretory vesicles of high electron density were abundant in the filament cells of the 3-4 outermost layers of epidermis, and intercellular spaces had increased. Mitotic figures occurred throughout the epidermis, with the exception of the outermost cell layer. Mucous cells became elongated after day-1, and later, newly differentiating mucous cells could be seen close to the skin surface, and many mucocytes contained mucosomes of high electron density. Rodlet cells were occasionally seen. Chloride cells appeared similar to those of control fish. Many leucocytes, mainly macrophages and lymphocytes, had penetrated the epidermis via the highly undulating basal lamina, and at day-7 numerous apoptotic lymphocytes were found. In the dermis, melanosomes became dispersed in the cytoplasmic extensions of melanocytes which were present in the epidermis of all acid-exposed fish. Iridocytes were rare after day-4, while fibroblasts were abundant and secreted large amounts of collagen. After 1 day of exposure to acidified water, a significant (0.05) elevation of the plasma cortisol level had occurred, but this subsequently declined, and had returned to control values by day-7. The changes in skin structure, however, remained throughout the whole exposure period.

Key words: Skin – Epidermis – Necrosis – Mucous cells – Cortisol levels – Leucocytes – Water acidification – Oncorhynchus mykiss (Teleostei)

Introduction

Anthropogenic acidification of fresh water has adverse effects on fish as well as on other aquatic organisms. Severe exposure may eventually lead to death, and thus to the disappearance of fish populations from many lakes and rivers—a well-documented phenomenon in the salmonids of North-America and Europe. Even a moderate reduction in water pH may be a stressor to some fish species. In several reports sublethal exposure has been shown to cause disturbances in water and ion balance, an elevation of plasma cortisol levels, and a suppression of somatic growth and reproduction (Goss and Wood 1988; Wendelaar Bonga and Dederen 1986; Mackett et al. 1992). The ionoregulatory failure is mainly caused by dysfunctioning of the branchial epithelium, and intensive research has been carried out to evaluate the histopathological changes in the gills under the impact of acid water (Wendelaar Bonga et al. 1990; Freda et al. 1991; Balm and Pottinger 1993).

The skin is another tissue directly exposed to the water and waterborne pollutants, and might thus be expected to react to acid water. Unlike the gill epithelium, the impact of an acidic environment on this tissue has received little attention, although it is metabolically very active and responds rapidly to a wide variety of stressors (Whitear 1986; Iger 1992). Piscine skin morphology is well documented, but information about the effects of acid exposure is sparse (Iger and Wendelaar Bonga 1994). We have previously studied the effects of stressors such as heavy metals, thermal elevation or wounding on the skin of fish, and have reported a common pattern of changes, including increased secretory activity and cell death by apoptosis in both epidermis and dermis (Iger et al. 1992; Iger et al. 1994; Iger and Abraham 1990). We have also shown that similar changes could be induced by injection of ACTH (Iger 1992), thus indicating that changes may be mediated by corticosteroid (cortisol) secretion. In the present study, the ultrastructural changes in skin induced by exposure of rainbow trout to acid water, are examined and correlated with plasma cortisol levels.
Materials and methods

Thirty-four male and female trout, *Oncorhynchus mykiss*, weighing 69±4 g (mean±SEM), were kept in 2 groups for an acclimation period of 20 days in artificial fresh water (demineralized water with the following salts in mmol/L⁻¹: 3.8 NaCl; 0.8 CaCl₂; 0.2 MgSO₄; 0.335 NaHCO₃; 0.06 KCl) at pH 7.5, and a temperature of 12°C. One group was maintained in water of neutral pH, whereas in the second group, the water pH was gradually lowered (over 3 h) to pH 5, by continuously adding diluted H₂SO₄. Demineralized water was used to avoid possible interaction between H⁺ and metal ions such as Al or Cu, which are present in Nijmegen tap water. Throughout the experiment, a period of 7 days, the pH of the water was adjusted automatically with pH-stat equipment (Consort, Belgium). The water of both groups was continuously circulated, well aerated, and filtered (Eheim filter 2034, EHEIM, Germany).

Skin and blood samples were taken from fish anesthetized with 2-phenoxy-ethanol (1:3000; Fluka Chemicals), at day-1, -4 and -7. Three to 5 fish were used from each group at each sample. For electron microscopy, pieces of skin (about 3x3x0.5 mm) were taken from the dorsal part of the head and treated as previously described (Iger et al. 1993). Thin sections were cut and used for morphometric analysis. The following measurements were taken:

1. Epidermal thickness: 3 photographs at low magnification [(×100)] were taken of each skin sample. The thickness of the epidermis was measured on ×4 photo enlargements. Areas with taste buds were excluded from this analysis.
2. Number of vesicles of high electron-density: sections were scanned and vesicles counted in 7 pavement cells per fish, with a 5 cell interval between measured cells. Necrotic and apoptotic cells were excluded from this analysis.

For the measurement of plasma cortisol levels, blood samples were removed by puncture of the caudal blood vessels on day-0 (just before the water acidification) and day-1, -4, and -7, after acidification. Plasma cortisol levels were determined by RIA, as described by Balm et al. (1994).

The Mann Whitney *U* test was used to determined differences between controls and experimental fish samples. A *P*<0.05 was accepted as level of significance.

Results

Controls

Skin. The ultrastructure of rainbow trout skin was similar to that already reported for other teleosts (Whitaker 1986; Iger et al. 1994). Briefly, the epidermis was 120–145 µm thick and was composed of filament-containing epithelial cells (filament cells), with the outermost layer differentiating into pavement cells, characterised by folding of the apical membrane into microridges. Necrotic or apoptotic pavement cells were very scarce. Oval shaped mucous cells, containing electron-lucent mucosomes, were common, and lymphocytes and Merkel cells were also occasionally observed. Chloride cells were rare. In the dermis there were different types of pigment cells, i.e., melanocytes, xanthocytes and iridocytes, among the fibroblasts and loosely arranged collagen fibers. In the deeper dermal zones capillaries were present.

Cortisol levels. The plasma cortisol levels remained below 5 ng.ml⁻¹ throughout the experiment (Fig. 13).

Acid-exposed fish

Epidermis. In these specimens the thickness of the epidermis had significantly (*P*<0.05) decreased by day-4 (Fig. 14), and by day-7 had thinned out to approximately 90 µm. Depressed areas, probably representing sites where degenerate pavement cells had been shed, were commonly seen on the skin surface. Rodlet cells (Fig. 12), not found in control fish, were present in the outer epidermal layers from day-4 onwards. Chloride cells and Merkel cells were rare. The basal lamina was highly undulated (Fig. 1).

Necrotic pavement cells (swollen cells with disrupted membranes, electron-lucent cytoplasm and fragmentation of nuclear heterochromatin) were common in all samples (Fig. 2), and most contained numerous vesicles of high electron-density. On day-7, apoptotic pavement cells (shrunken cells showing condensation of cellular components, agglutination of heterochromatin, and loss of junctional complexes between the cells) were also found (Fig. 3). These cells mostly contained electron-lucent vesicles. While normal pavement cells exhibited a pronounced glycocalyx cover at their apical membrane, degenerative (necrotic as well as apoptotic) pavement cells had lost their cover. The non-degenerative pavement cells showed signs of increased synthetic and secretory activity, as was reflected by their content of well developed rER, Golgi areas, and many vesicles of high electron density that were occasionally fused with the apical membrane. The number of vesicles of high electron density was significantly (*P*<0.05; Fig. 15) higher in acid-exposed trout than in control fish. By day-4, similar characteristics of increased activity were found also in the filament cells of the 3–4 outermost epidermal layers. By the end of the experiment (day-7) such phenomena were restricted to the 2–3 outermost layers, and the intercellular spaces between these filament cells were...
enlarged, but the tight junctions between the pavement cells remained intact (Fig. 5). Interestingly, filament cells adjacent to necrotic pavement cells developed microridges (Fig. 4) and tight junctions before reaching the surface. Mitotic filament cells, not detected in control fish, were frequently observed after day-4, and occurred throughout the epidermis, but mainly in the outer layers.

At day-1, most mucous cells were found close to the skin surface and were elongated (Fig. 6) rather than oval-shaped. At day-4, such cells were rare, and from this time onwards, mucous cells with extensive rER and mucosomes of high electron density were more common (Fig. 7). From day-4 onwards, a mucous layer occasionally remained attached to the epidermal surface in acid-exposed fish, whereas in controls this layer disappeared during fixation. At day-7, newly differentiated mucous cells were noticed in the outer layers of the epidermis, occasionally adjacent to the pavement cells, rather than in their normal location, i.e., close to the basal filament cells. Apoptotic mucous cells (Fig. 8) that had not yet released their content were found after 7 days.

Macrophages and lymphocytes were common in the dermis and penetrated the epidermis. At day-1 these leucocytes were located mainly in the inner layers of the epidermis. Later they were found throughout this tissue. Epidermal macrophages appeared very active, with well-developed rER and Golgi areas, many vesicles (probably lysosomes) and several phagosomes. Frequently, lymphocytes were found adjacent to mucous cells, a phenomenon not observed in control fish. Many apoptotic leucocytes were observed. This phenomenon was first noted on day-4, and was common by day-7. Apoptotic lymphocytes (Fig. 9) were found mainly in the outer epidermal layers, while apoptotic granulocytes (mainly basophils and eosinophils) were common in the dermis.

**Dermis.** From day-4 onwards, many fibroblasts were found near the basal lamina. They appeared very active
and contained abundant rER, large Golgi areas and many small vesicles at the periphery of the cells. The vesicles occasionally fused with the cell membrane. After 7 days the outer dermis was filled up with randomly arranged collagen fibers.

Throughout the whole period pigment granules were dispersed in the cytoplasmic extensions of melanocytes, rather than in the cell bodies as in the controls. These extensions, restricted to the dermal zone in control fish, penetrated into the basal layers of the epidermis within 24 h, and in later samples, were found all through the epidermis (Fig. 10), up to one layer from its surface. In both dermis and epidermis many extensions of melanocytes were apoptotic. Such extensions were mostly found in macrophages (Fig. 11), although they could also be found inside filament- and mucous cells. From day-4 onwards, iridocytes were rare, and degenerate iridocytes were never seen.

**Cortisol levels.** At day-1 and day-4 the plasma cortisol levels of acid-exposed trout were significantly ($P<0.05$; Fig. 13) higher than in control fish, but by day-7 the cortisol level was similar to that of the controls.

**Discussion**

The present results demonstrate that exposure of rainbow trout to acidic water under laboratory conditions had substantial and prolonged effects on the epidermal and dermal skin layers, and a transient effect on plasma cortisol levels. Most of the changes observed in the skin have also been found after exposure to other stressors (see below), and therefore may represent a general response to stressors. This response may be partially under the control of cortisol, because the plasma levels of this hormone were elevated shortly after the initial exposure. In addition, some changes in the skin might be interpreted as direct effects of the low water pH (e.g., necrosis of pavement cells), or as local adjustments of this tissue (e.g., formation of ridges in filament cells deeper to the layer of the pavement cells).

The elevation of plasma cortisol levels, as we observed in rainbow trout at day-1 and -4, has frequently been reported for fish under stress (Pickering and Pöttinger 1989), and has also been reported earlier for exposure of fish to acid water. The transient nature of the elevation is in line with the conclusion of Goss and Wood (1988) that in rainbow trout a persistent rise in plasma cortisol is observed only during severe stress, such as may be induced by acid water containing aluminium. These authors noted a significant elevation of plasma cortisol in rainbow trout after 4 and 7 h of exposure to water of pH 4.8. However, Balm and Pöttinger (1993) were unable to find a rise in cortisol level of rainbow trout sampled after 4 h, and after 2, 7 and 14 days of exposure to pH 4.0. On the other hand, in brook trout plasma cortisol levels became elevated after 4–21 days in water of pH 4.2 (Mackett et al. 1992), and in carp exposed to water of pH 4.0 and an undetectable aluminium concentration (detection level: 6.7 nmol.L$^{-1}$), an elevated plasma
cortisol level was recorded to have lasted for less than 24 h (Van Dijk et al. 1993).

Epidermal thickness decreased in acid-exposed trout, indicating that, during the 7 days of exposure, the rate of cell shedding surpassed the rate of cell replacement, even though mitotic cells were commonly found in the acid exposed fish. The increased rate of cell shedding and mitosis indicate that the turnover rate of filament cells (including the pavement cells) is increased during adaptation to water of low pH. Similar conclusions have been drawn for the epidermal cells of the gills of tilapia and the skin of carp exposed to acid water (Wendelaar Bonga et al. 1990; Iger and Wendelaar Bonga 1994). Shedding of epithelial cells has also been reported for acid-exposed bullhead catfish (Zuchelkowski et al. 1985). We have made a distinction between necrotic pavement cells, reflecting accidental cell death, and apoptotic cells, reflecting physiologically controlled cell death (Wyllie 1981). The necrotic cells noted in the present study probably reflect the direct negative effects of low water pH. The apoptotic cells appeared only after 7 days, which may be the result of exhaustion of highly activated pavement cells, and thus, an indirect effect of acidification on the ageing of these cells. Necrosis and apoptosis have also been reported in gill epithelium of tilapia, and in the epidermis of carp exposed to acidified water (Wendelaar Bonga et al. 1990; Iger and Wendelaar Bonga 1994).

The development of ridges noted in deep lying filament cells is interesting because normally these structures are characteristic only of the outermost cell layer of pavement cells (Uehara 1988). In a previous experiment we found development of ridges in cells of comparable location in epidermal tumor-like structures of lead-exposed carp (Iger 1992). In both cases the development of ridges is accompanied by enlargement of the intercellular spaces at the apical region of these cells. The loose connection between these cells, in particular at their apical pole, might have triggered the formation of the ridges. These cell layers showed well developed tight junctions, as did normal pavement cells. This contrasts with the observation of Freda et al. (1991) on the gills of rainbow trout from acid water. They observed shortening or opening of the tight junctions of the branchial epithelium.

The significant increase recorded in electron-dense vesicles in pavement cells of acid-exposed trout may reflect the elevation in plasma cortisol levels since filament cells are stimulated by administration of ACTH (Iger et al. 1992). This is supported by the finding of Marshall (1979) that the number of “mucous vesicles” in filament cells, probably identical with the secretory vesicles observed in this study, was increased by exogenous cortisol. In previous studies we have demonstrated that these vesicles show peroxidase activity and contribute to the formation of the glycocalyx (Iger et al. 1993; Iger and Wendelaar Bonga 1994).

The stimulation of mucus secretion by rainbow trout skin in acid water is similar to that reported for e.g., acid-exposed brown bullhead, carp and tilapia (Zuchelkowski et al. 1985; Wendelaar Bonga et al. 1990; Iger and Wendelaar Bonga 1994), and to that of fish exposed to many pollutants (Iger et al. 1988; Iger 1992), or fish suffering from parasitic infection (Urawa 1992). In the present study, the mucous cell response of the skin to acid water can be classified into the following phases: firstly, intensive migration of mucous cells towards the skin surface, reflected by the appearance, after 24 h, of many elongated rather than goblet-shaped cells. This phase probably corresponds to the phase of hypertrophy of mucous cells in male bullhead fish exposed to acid water (Zuchelkowski et al. 1986). Secondly, exhaustion of the skin mucus supply. This has not been reported before for acid-exposed fish, but has been recorded in fish exposed to other stressors. In fish kept in manured water, for example, the skin becomes almost depleted of epidermal mucous cells after 3 days (Iger et al. 1988). Bulluck et al. (1978) report that the generation time for epidermal cells of plaice is 108 h, which might explain why rainbow trout, after 4 days of acid-exposure, have insufficient newly differentiated mucous cells to compensate for the discharged mucus. The third phase, the acclimation phase, is characterised by the restoration of the mucous cell population, and by the synthesis and secretion of electron-dense mucosomes. The latter have been previously reported in the skin of fish stressed by infection of ectoparasites (Blackstock and Pickering 1980), by water containing heavy metals, or during social interactions or temperature elevation (Iger 1992; Iger et al. 1992; Iger et al. 1994). Their appearance under the impact of a wide range of stressors, therefore, indicates that this phenomenon is part of a general stress response.

Electron-dense mucosomes probably have a more serous composition than the normal electron-lucent mucosomes (Whitear 1986), and this is also indicated by the observation that epidermal mucous cells in acid-stressed brown bullhead show increased PAS-reactivity, a characteristic of neutral or basic mucus material (Zuchelkowski et al. 1985). A more basic mucus content has also been recorded in the serous mucous cells (acidoophilic granular cells) of trout infected by parasites (Blackstock and Pickering 1980). However, since mucus is initially synthesized as basic or neutral glycoproteins (Mittal et al. 1980), an increased PAS-reactivity could also reflect a predominance of newly differentiated mucous cells, and an increased turnover of this cell-type (Iger et al. 1988; Iger and Wendelaar Bonga 1994). The presence of a mucus layer attached to the epidermal surface after fixation of the skin from acid-exposed trout may indicate that its composition has changed, although Fromm (1980) has suggested that this might be caused by coagulation in low pH conditions. The appearance of newly differentiated mucous cells close to the skin surface in acid treated fish has not previously been reported for rainbow trout.

The invasion of the epithelms by lymphocytes and macrophages in response to low pH conditions, noted in rainbow trout, has also been observed in carp exposed to acidified water (Iger and Wendelaar Bonga 1994), lead (Iger 1992), or after wounding of the skin (Iger and Abraham 1990), and in trout exposed to elevated temperature (Iger et al., 1994). It is also well known from the branchial epithelium, as a response to water acidifi-
dispersion induces darkening of the skin, a phenomenon exposed to thermal pollution (Iger 1992; Iger et al. 1992; Iger and Abraham 1990; Iger et al. 1983; Iger and Abraham 1990; Iger et al. 1992; Iger and Abraham 1990; Iger et al. 1992). The phagocytic activity of macrophages, observed in the present study, indicates the presence of damaged epithelial cells in the deeper epidermal layers. Superficial degenerated cells are sloughed off into the water (Wendelaar et al. 1990; Iger and Wendelaar Bonga 1994). In human skin, the migration of lymphocytes to the skin produces a sequence of deformations in the basal lamina (Warnel and Hull 1984), and the undulations of the trout basal lamina, observed after 24 h of acid-exposure, might have a similar origin.

The apoptosis of leucocytes, particularly lymphocytes, might be related to the elevated cortisol levels. Maule et al. (1989) have shown that elevated cortisol levels decrease the immune response and disease resistance of chinook salmon. Our finding that the elevation in cortisol levels is followed by apoptotic processes in lymphocytes indicates that cortisol stimulates lymphocyte apoptosis in fish as it does in mammals (Schwartzman and Cidlowski 1993).

Cortisol induces chloride cell proliferation in the branchial epithelium of rainbow trout (Perry and Wood 1985), but in the present experiment elevated cortisol was not accompanied by a noticeable increase in skin chloride cells. It would seem therefore, that a chloride cell response in rainbow trout is restricted to the gills. The lack of a skin response cannot be ascribed to the short duration of the experiment, because new chloride cells become noticeable within 3–4 days of exposure to acid water in the epidermis of carp, Cyprinus carpio (Iger and Wendelaar Bonga 1993), and in the gill epithelium of tilapia, Oreochromis mossambicus (Wendelaar Bonga et al. 1990) and of swordtails, Lebistes reticulatus, transferred to sea water (Chretien and Pisam 1986).

The peripheral migration of dermal fibroblasts and intense deposition of collagen fibers observed in this zone are known to be induced by a variety of stressors (Bucke et al. 1983; Iger and Abraham 1990; Iger et al. 1992; Iger et al. 1994). In carp, this activity is stimulated by ACTH (Iger et al. 1992), and in the rainbow trout, therefore, may be induced by the elevated cortisol levels, and may have a protective function. The random arrangement of the collagen fibers is indicative of their rapid synthesis, as has been suggested by Whitear (1990).

Dispersion of melanosomes into the cytoplasmic extensions of the melanocytes also is a phenomenon associated with exposure to a variety of stressors, and has been reported in carp skin after exposure to low pH or heavy metals, in catfish experiencing social stress, and trout exposed to thermal pollution (Iger 1992; Iger et al. 1992; Iger et al. 1994; Iger and Wendelaar Bonga 1994). The dispersion induces darkening of the skin, a phenomenon which in Atlantic salmon and the common carp can be induced by administration of ACTH (Langdon et al. 1984; Iger 1992), and may similarly represent a cortisol-controlled general response to stress conditions.

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