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Transient Increase in Chloride Cell Number and Heat Shock Protein Expression (hsp70) in Brown Trout *(Salmo trutta fario)* Exposed to Sudden Temperature Elevation

Heike Schmidt[1,*], Horst Posthaus[1], André Busato[2], Thomas Wahli[1], Willy Meier[1] and Patricia Burkhardt-Holm[1,3]

[1] Centre of Fish and Wildlife Health, Institute of Animal Pathology, University of Berne, CH-3012 Berne, Switzerland
[2] Institute of Animal Breeding, University of Berne, CH-3012 Berne, Switzerland
[3] Interdisciplinary Centre for General Ecology, University of Berne, CH-3012 Berne, Switzerland

* Corresponding author

The native cold-adapted brown trout (*Salmo trutta fario*) is often the subject of biomonitoring field studies. Groups of trout were exposed to a sudden temperature rise, from 8 °C to 19 °C for two hours, and thereafter set back to 8 °C. Gill samples of control animals, of fish after the exposure period, and after 24 and 48 hours of recovery at a temperature of 8 °C were examined histologically, immunohistochemically, electron microscopically, and by Western blot analysis. By means of immunohistochemistry and electron microscopy, an increase of chloride cells was observed after the temperature elevation. During the recovery period the number of chloride cells decreased.

Western blot analysis for stress proteins (hsp70), widely used as a biomarker for environmental stress, was performed from skin and gill. Whereas in the gill both isoforms, the constitutive and the heat inducible form, of hsp70 were detected in all groups, in the skin the control animals only showed the constitutive form. After two hours of exposure both isoforms were visible. An increased expression of hsp70 could be demonstrated in both organs after the exposure. Comparison of the hsp70 values between gill and skin showed tissue-specific differences during the recovery period. In the gill hsp70 rapidly decreased, while in the skin the level remained elevated over the whole observation period.

When hsp70 is used as a biomarker in field studies, the fast and organ-specific reaction in the gill and skin of brown trout has to be taken into consideration.

Key words: Biomarker / Cell stress / Ecology / Environment / Tissue specific reaction.

Introduction

In fish the gill epithelium is the site of respiratory gas transfer, ion regulation, acid-base balance, and excretion of nitrogenous products. It is composed of two distinct epithelial surfaces, the filament or primary epithelia and the lamellar or secondary epithelia. The primary and secondary epithelium consists of five kinds of epithelial cells: pavement cells, mucous cells, chloride cells, neuro-epithelial cells and undifferentiated cells (Laurent and Dunel, 1980; Laurent and Perry, 1995; Sardet *et al.*, 1979). Chloride cells play a role in the acid-base regulation (Perry, 1997). Ultrastructurally they are characterised by the abundance of mitochondria, an extensive tubular system, and sub-apical vesicles (Perry, 1997). Chloride cells are rich in Na^+,K^+-ATPase, which is mainly located in the intracellular basolateral tubules (Flik and Perry, 1989; Perry, 1997; Witters, 1996). Under conditions of disturbed ion regulation, such as exposure to soft water or toxicants, or after cortisol treatment, chloride cells proliferate on both the filament and the lamellae (Flik and Perry, 1989; Mallat, 1985; Wendelaar Bonga and Lock, 1992). Although the effect of different stressors on the chloride cells has been investigated, a heat stress related alteration of chloride cells has not yet been described. Since the native cold-adapted brown trout (*Salmo trutta fario*) is often the subject of biomonitoring in field studies, such an alteration could have consequences for the use of the gill as an indicator organ.

Under physiological conditions low amounts of highly conserved proteins, called stress or heat shock proteins (hsps), are synthesised by all nucleated cells (Ashburner, 1982). These proteins have essential functions as molecular chaperones, which assist in the intracellular transport and folding of newly synthesised proteins (Gething and Sambrook, 1992; Hartl, 1996). Intracellular accumulation of aberrant proteins may be provoked by exposure to stressful environmental conditions such as elevated temperature, anoxia/hypoxia, heavy metals, and xenobiotics (Brown *et al.*, 1995; Cairo *et al.*, 1985; Dietz and Somero, 1993; Nover, 1991; Sanders, 1993; Williams *et al.*, 1996). As a result, the level of hsp induction increases and regular protein synthesis is repressed (Hightower, 1980; Lindquist, 1986). Hsps take part in repairing and protecting cellular proteins from stressor-induced damage to limit protein aggregation, while severely damaged proteins are targeted for ubiquitin-tagging and proteolysis (Hartl, 1996; Parsell and Lindquist, 1993). In addition to an established

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role of hsp70 and other hsps in protein (re)folding, heat shock proteins may also be involved in other aspects of cell function (Rother *et al.*, 1997; Tamura *et al.*, 1997). The 70 kDa family of heat shock proteins is represented by two major members, a highly stress inducible protein of 68 kDa and a constitutively expressed protein of 70 kDa (also called hsc70) (Abukhalaf *et al.*, 1994).

The aim of our study was to test the suitability of three unrelated criteria, namely specific structural alterations, the number and the distribution of chloride cells in the gill, and the induction of hsp70 in gills, to demonstrate reactions of the gill epithelium to acute environmental stress. To allow a direct comparison between the hsp70 level of gill and skin, samples of skin were included in this study. These two organs were chosen because they are directly and continuously exposed to environmental irritants.

Results

Structural alterations

Neither immediately after temperature shock nor during recovery was a change in the architecture of the gill filaments and the lamellae seen. Histological sections of the gill stained with haematoxylin and eosin showed no obvious alterations in the control nor in the experimental groups (data not shown). Occasionally mitotic figures could be seen in primary epithelium cells of all experimental groups, however no differences in the amount between these groups was evident.

Chloride Cells

After immunohistochemical labelling with an antibody recognising the Na^+,K^+-ATPase, positively labelled cells were present in the gill epithelium of all groups. In the control group the positive cells were mainly located in the primary epithelium, in the inter-lamellar regions, and at the junctions between the filaments and lamellae. The chloride cells were found singularly or, less frequently, in groups of two to three cells. In the secondary epithelium single chloride cells could occasionally be found, mainly at the base of the lamellae.

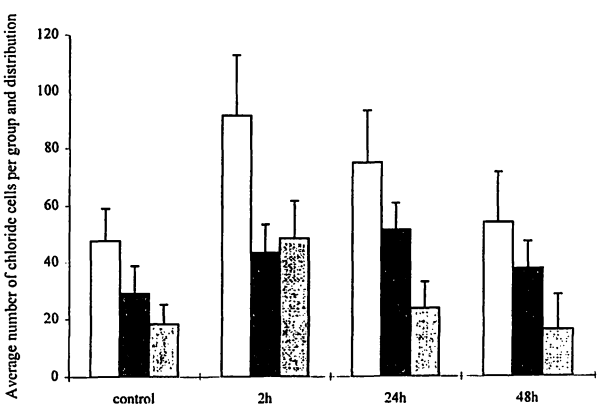

Fig. 1 Time Course of Chloride Cell Number in the Gill of Brown Trout after a Sudden Temperature Rise (Two Hours) and during Recovery Period.
Average number of chloride cells on both, filament and lamellae, per group (white bar) and average number of chloride cells per group and distribution (filament: black bar, lamellae: gray bar). See text for further details.

After two hours of elevated temperature the total amount of Na^+,K^+-ATPase positive cells had increased significantly (Figure 1). This was mainly due to the significantly elevated number of these cells in the secondary epithelium, whereas changes in number of Na^+,K^+-ATPase positive cells in the primary epithelium were less pronounced (Figure 1, 2, a, b). In contrast to the control, after exposure the chloride cells in the primary epithelium were arranged in clusters. Neither changes in nuclear morphology nor mitotic figures were observed in Na^+,K^+-ATPase positive cells.

The electron microscopical examination confirmed these findings. After two hours of exposure an increased number of cells characterised by an ovoid shape, a round nucleus, a cytoplasmic matrix of low electron-density, and small, round mitochondria, and a loosely branched tubular system were present in the basal layer of the secondary lamellae (Figure 2, d). This morphological appearance corresponds to that of mature chloride cells, which are characterised by their abundance of mitochondria, a large ovoid nucleus and an extensive tubular system (Figure 2, c). Therefore we assumed these cells to be immature chloride cells.

After a recovery period of 24 hours the total amount of Na^+,K^+-ATPase positive cells decreased but still remained elevated compared to the control group. With regard to the localisation, the number of positive cells in the primary epithelium was still increased whereas in the secondary epithelium it decreased approximately to the control level (Figure 1). Electron microscopically, only few cells could be identified as immature chloride cells. The majority of the chloride cells was differentiated and located in the apical layer of the primary and secondary epithelium.

After a recovery period of 48 hours the number of Na^+,K^+-ATPase positive cells was comparable to that of the controls (Figure 1). The number of positive cells in the primary and in the secondary epithelium was lower than in the 24 hours samples.

The χ^2-Test indicated a significant difference in the distribution of chloride cells between groups by localisation. The analysis of variance indicated significant group effects on cell numbers and a linear contrast revealed a significant difference between control and all treatments. The pairwise comparison of means showed significant differences between all groups except group 2 (2 hours exposure) to 3 (24 hours recovery). The model exhibited good fit ($R^2 = 0.58$). Divided into the two distributions, filament and lamellae, significant group effects and significant differences between the control and all treatments could be found. The pairwise comparison of means showed significant differences between groups 1 (control) and 3 for the chloride cells on the filament and between group 2 and all other groups for the cells on the lamellae. The models ac-

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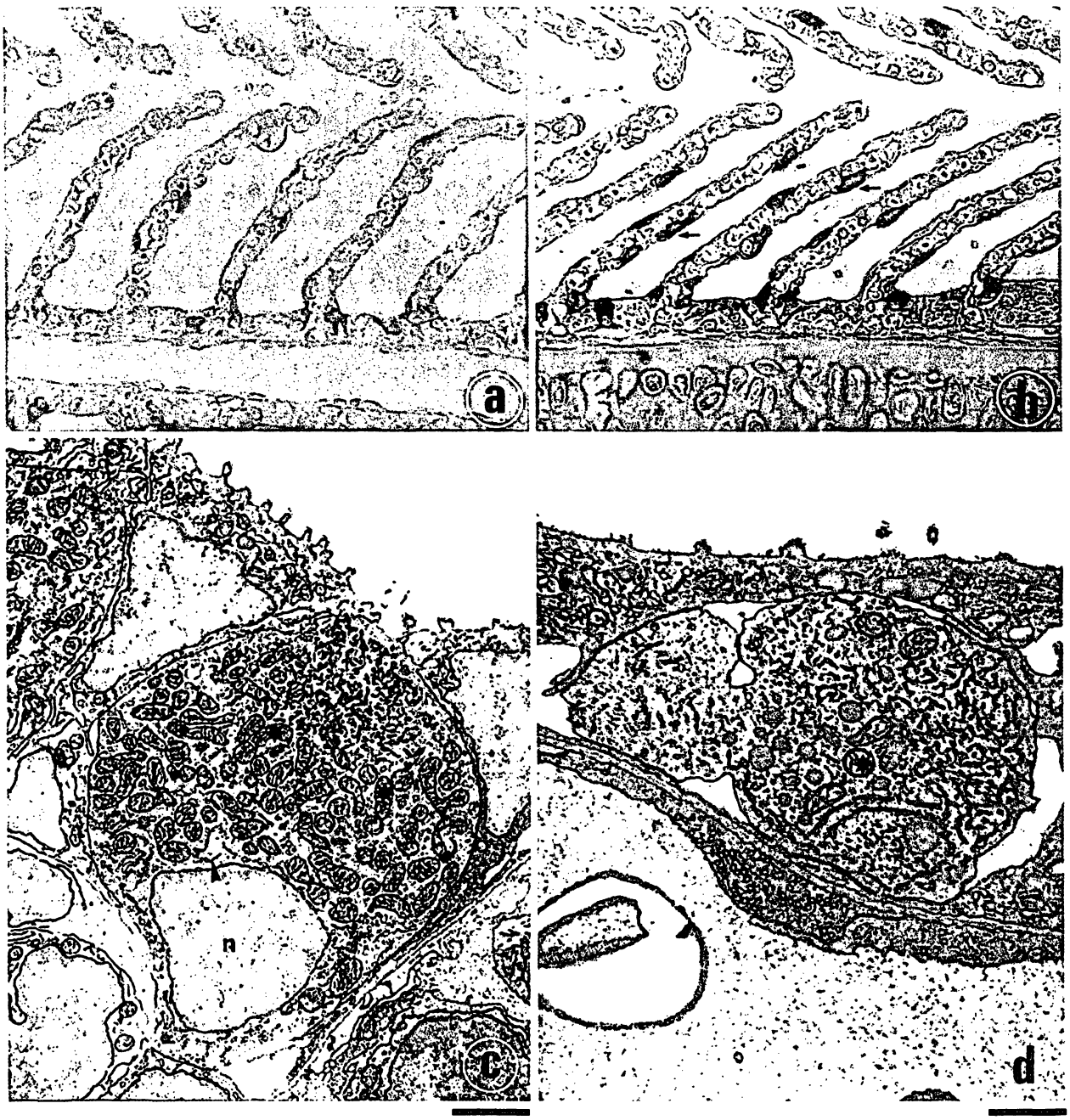

Fig. 2 Light and Electron Microscopical Analysis.
Micrographs of gill sections labelled with the mouse monoclonal antibody IgGα5, raised against the α subunit of the Na^+-K^+-ATPase. (a) Control animal, (b) gill section of an animal after the two hour exposure period. After exposure to the elevated temperature more chloride cells were localised on the lamellae (arrow); Representative transmission electron microscopical photograph of (c) a mature chloride cell characterised by the abundance of mitochondria (*), a large nucleus (n) and an extensive tubular system (arrowhead) (scale bar: 1 μm) and of (d) an immature chloride cell characterised by an ovoid shape, a cytoplasmic matrix of low electron density, small round mitochondria (*), and a loosely branched tubular system (arrowhead) (scale bar: 1 μm).

counted for 71% and 85% of the variation of chloride cells on the filament and the lamellae respectively (R^2 = 0.71, 0.85). In none of the examined groups were significant differences found between the three localisations tested (basal, middle and apical part of the primary lamella).

Expression of hsp70 and Variation between Tissues

Western blots probed with the antibody against hsp70 revealed two protein bands of the hsp70 family in the region of 70 kDa in the gill of brown trout of all groups. Both isoforms were detected as faint bands in the control animals. After the two hours of high temperature exposure, the expression of the highly inducible member of hsp70 had in-

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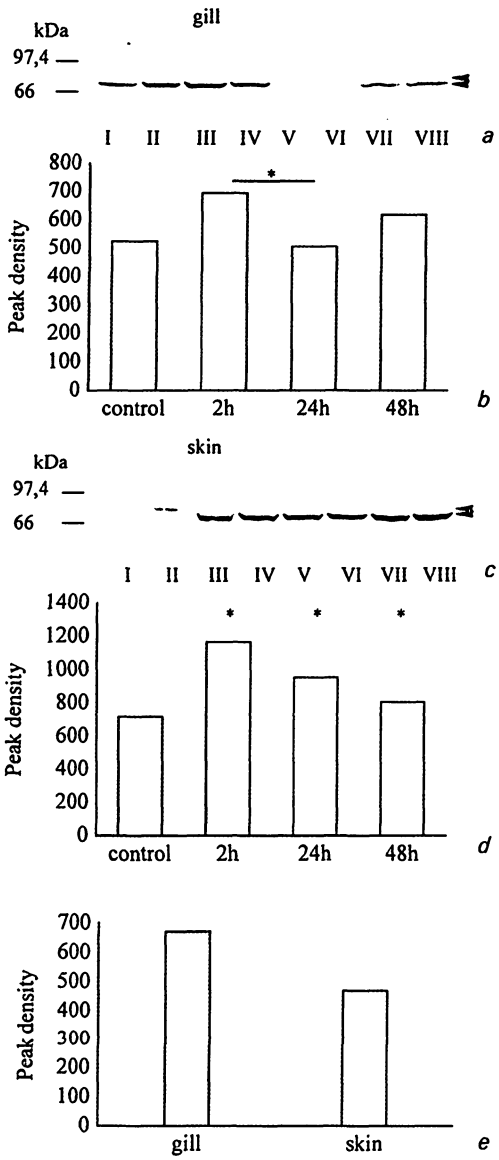

Fig. 3 Western Blot Analysis of Gill and Skin Lysates Developed with a Monoclonal Anti-Bovine Brain hsp70 Antibody.
Western blot of gill samples of two representative animals per group: in all samples two bands are visible (arrows), however, the band of hsc70 is often hardly visible, lanes I – II: control group, lanes III–IV: group after temperature elevation, lanes V–VI: recovery group (24 hours), lanes VII–VIII: recovery group (48 hours) (a); time course of the hsp70 levels in the gill epithelium of all animals after temperature elevation and during recovery, mean values of five animals per group, statistically adjusted for 6 gels per organ, * indicates significant differences from control value ($p < 0.05$) (b); Western blot analysis of skin lysates, samples of two representative animals per group: in the control group only one band is visible, in the experimental groups two bands are visible, the intensity of the hsc70 band decreased (arrows), therefore, the hsc70 band is often hardly visible, lanes I–II: control group, lanes III – IV: group after temperature elevation, lanes V–VI: recovery group (24 hours), lanes VII–VIII: recovery group (48 hours) (c); time course of the hsp70 levels in the skin epithelium of all animals, mean values of five animals per group, statistically adjusted for 7 gels per organ, * indicates significant differences from control value ($p < 0.05$) (d); Western blot analysis of gill and skin samples of all control animals, direct comparison of the hsp70 level between control groups, gill and skin (e).

creased (Figure 3, a, b). The highest amount was found directly after the exposure period. During recovery the protein level decreased significantly. The amount of hsp70 after 24 hours of recovery was lower than in the control group. Afterward the protein concentration increased again, slightly above the level of the controls. Results of the densitometric analysis are shown in Figure 3, b. The analysis of variance indicated a significant difference of hsp70 expression between treatment groups. A linear contrast comparing all treatments with the control did not indicate a significant difference and pairwise comparison of means revealed only a significant difference between groups 2 and 3. The model exhibited good fit ($R^2 = 0.82$).

In the skin samples of the control animals only the band at 70 kDa of the constitutive member (hsc70) was detected . In all exposed animals however the highly inducible isoform of hsp70 was present, whereas the intensity of the band of hsc70 decreased. (Figure 3, c, d). In contrast to the results in the gill the level in the skin remained elevated over the whole period of recovery. The highest accumulation was found after two hours of exposure followed by a slight decrease in the 24 hour samples. The level after 48 hours of recovery was comparable to that of the 24 hour samples. Pairwise comparison of means revealed significant differences between the control group and all experimental groups (Figure 3, d). The model accounted for 95% of the variation of hsp70 ($R^2 = 0.95$).

Figure 3, e illustrates the direct comparison of the hsp70 levels in the gill and the skin of control animals. These results show that under physiological conditions the stress protein amount normalised for protein content is clearly higher in the gill than in the skin.

Discussion

We investigated the effect of a short term temperature elevation on specific structural alterations, on the number and distribution of chloride cells in the gill, and on the induction of hsp70 in gills of brown trout.

Immunohistochemical labelling with the monoclonal antibody against the Na^+,K^+-ATPase is a specific tool for the identification of chloride cells (Witters *et al.*, 1996). In our study we demonstrated an increase in the total amount of Na^+,K^+-ATPase positive cells as a consequence of a sudden temperature elevation. This was mainly due to the significantly elevated number of these cells in the secondary epithelium, whereas changes in number of Na^+,K^+-ATPase positive cells in the primary epithelium were less pronounced. After two hours of heat stress there was a clear increase in the number of immature chloride cells in the basal layer. There is evidence that even immature chloride cells are labelled by the monoclonal antibody used (Burkhardt-Holm, 1997). Positively labelled cells showed no mitotic figures. This confirms results reported by Laurent and Dunel (1980) that immature chloride cells develop from undifferentiated basal cells. Other studies also described increased numbers of chlo-

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ride cells on the lamellae after changes of environmental conditions (Flik and Perry, 1989; Mallat, 1985; Wendelaar Bonga and Lock, 1992). Activation of these cells in our experiment might be induced by the effect of endogenous cortisol and catecholamine secretion provoked by the sudden temperature rise. After longterm treatment with cortisol chloride cell proliferation in gill epithelia and altered ionic uptake in freshwater teleosts were found (Flik and Perry, 1989; Laurent and Perry, 1990; Perry *et al.*, 1992). Acute effects of cortisol on chloride cells were not investigated so far but it is known that cortisol may take many hours to exert its full effect on osmoregulatory function in the gill epithelia (Eddy, 1981). In contrast, elevation of the levels of circulating catecholamines is a more immediate and dramatic response of fish to a wide variety of stressors (Wendelaar Bonga, 1993). Acute stress goes along with catecholamine release resulting in disturbed acid-base balance, ion losses, and osmotic water uptake across the gill (Wendelaar Bonga and Lock, 1992). Morgan and Potts (1995) found a direct effect of adrenoreceptor agonists on the influx of Na^+ and Cl^- across the branchial epithelium. Therefore catecholamine release could result in secondary effects on chloride cells, such as proliferation.

After 24 hours of recovery a higher amount of differentiated chloride cells was present in the branchial epithelium. This corresponds to results of Mallatt (1985), who concluded that cellular differentiation generally requires 12–24 hours to become evident.

A second effect of temperature shock was a rapid increase of hsp70 levels in gill and skin. In all gill samples two bands were visible. After the temperature elevation the amount of the highly stress inducible form of hsp70 increased. The perturbations seen in the expression levels in the gill during recovery can be part of a rebound effect following the sudden replacement to the original temperature. Lindquist (1986) postulated that as long as cells or organisms are maintained at high temperatures, hsps are the primary product of protein synthesis. When they are returned to normal temperature, normal protein synthesis gradually resumes (Lindquist, 1986). In cell cultures of chinook salmon embryo cells and rainbow trout fibroblasts, the maximum hsp70 expression was reached after two hours of temperature elevation. It decreased to control levels after 10 to 24 hours of recovery (Gedamu *et al.*, 1983, Mosser *et al.*, 1986). This pattern is in accordance with the results from the gill presented in this study.

The skin samples of the control animals only showed the band of the constitutive member (hsc70). After the exposure period the intensity of this band decreased slightly whereas in the gill samples of all groups two bands were present.

In contrast to the results of the gill the stress protein level in the skin remained at an elevated level for the rest of the observation period, confirming previous observations (Burkhardt-Holm *et al.*, 1998). We conclude that the expression of the different members of the hsp70 family and the heat shock response is tissue-specific in the investigated organs. Tissue-specific patterns of hsps have also been reported in other species and organs (Abukhalaf *et al.*, 1994; Dietz and Somero, 1993; Dyer *et al.*, 1991). However, to our knowledge, this is the first study revealing a tissue-specific pattern during recovery from a short temperature shock. The rapid downregulation of hsp70 in the gill suggests that regeneration in the gill occurs faster than in the skin. This corresponds to the observation that the mitotic activity and the cell renewal rate of gill epithelium is higher than that of skin epithelium (Bullock *et al.*, 1978; Chretien and Pisam, 1986). In addition, the constitutive hsp70 level is higher in the gill than in the skin. The gill, as a membranous gas- and ion-exchange organ, requires highly effective mechanisms to maintain and regain membrane structural integrity, which protects it against constantly changing conditions, i.e. temperature, gases, ions, pH, etc. The gill epithelium consists of only two cell layers, which may require more potent mechanisms of the single epithelial cell than in the multilayered epidermis.

Brown trout prefer a temperature range between 12° and 17 °C (Ferguson, 1958), but water temperatures approaching 20 °C and more are not uncommon during the summer months in their habitats. In our study the fish were transferred rapidly to the higher water temperature and back to the original tank. Whether the hsp70 level reacts similarly under gradually changing conditions has to be tested. Fader *et al.* (1994) found large seasonal variations in the stress protein expression in the liver of brown trout under natural conditions. Therefore it is important to distinguish between the influence of an artificial stressor and the physiological acclimatisation response of fish to environmental fluctuations.

Materials and Methods

Experimental Design

Twenty brown trout (*Salmo trutta fario*) approximately one year old were selected from the stock culture located at the Fish Disease Laboratory, University of Berne. After acclimatisation for five months at 8 °C the fish were randomly assigned to one control and three experimental groups of five animals each. The controls were euthanised in buffered 3-aminobenzoic acid ethyl ester (MS-222, Sandoz LTD, Basle, Switzerland) without any treatment and the remaining animals were transferred for two hours into a tank containing well-aerated tap water at 19 °C. One group of animals was euthanised immediately after the two hour exposure period and two other groups after 24 and 48 hours of recovery in water at 8 °C (group 3 and 4). Pieces of skin cut laterally below the dorsal fin and the gills were taken from each fish and processed for further investigations.

Light and Electron Microscopy

Samples of gills were fixed in Bouin solution for 24 hours, routinely processed for histology and cut into 5 μm thick sections, which were stained with haematoxylin and eosin. For electron microscopy, samples were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, for 2 hours and treated as described previously (Burkhardt-Holm *et al.*, 1998). Ultrathin sections were

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stained with alkaline lead citrate and examined with an electron microscope (Zeiss EM 902).

Immunohistochemistry

To suppress the endogenous peroxidase activity, unstained mounts of gills were exposed to H_2O_2 (3%, 15 min), blocked in 0.1% bovine serum albumin (BSA) for 20 min and incubated in a moist chamber for one hour at room temperature with a mouse monoclonal antibody, raised against the α subunit of the avian Na^+,K^+-ATPase (Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, Iowa City, USA). For visualising, a Histo-SP kit AEC -mouse (Zymed, South San Francisco, CA, USA) was used. Following a 10 min wash with phosphate-buffered saline (PBS) the sections were labelled with a biotinylated second antibody for 10 min, washed again and incubated with peroxidase-labelled streptavidin for 10 min. After a 5 min rinse in PBS the sections were stained with the substrate for 5 min and finally rinsed with aqua bidest. Negative controls were performed with PBS instead of the primary antibody.

The distribution and amount of Na^+,K^+-ATPase positive cells were measured by light microscopic examination. One gill section from each animal was examined. On each slide two randomly selected areas were investigated. The chloride cells were counted in three localisations on each area, namely the basal part, the middle part, and the apical part of the filaments. On each localisation 20 randomly selected lamellae were examined. Additionally, the distribution of chloride cells on the filament, including the junctions between filament and lamellae, and the lamellae were differentiated. The counts were repeated four times.

Protein Immunoblots

Samples of skin and gill were homogenised on ice in lysis buffer [1% NP40, 66 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8.0), 2 μg/ml Leupeptin, 2 μg/ml Aprotinin, and 1 mM PMSF]. Homogenates were centrifuged twice at 15000 *g* for 30 min at 4 °C. The total protein concentration in each sample was determined using the bicichoninic acid (BCA) method (Pierce, Rockford, IL, USA). The lysates were dissolved in 6 × SDS sample buffer (Laemmli, 1970) and heated at 95 °C for 5 min. Equal amounts of protein were separated on a one-dimensional 7% polyacrylamide gel under standard denaturing conditions according to the method of Laemmli (1970). A 70 kDa heat shock protein from bovine brain (Sigma, St. Louis, MO, USA) was used as standard. The proteins were then electroblotted onto nitrocellulose membranes and incubated overnight at 4 °C with 1 μl/ml of a mouse monoclonal antibody against bovine brain hsp70 (Sigma, St. Louis, MO, USA), which detects both isoforms of hsp70. Following washes in TBS-T the blots were incubated with a 1:3000 dilution of the second antibody, horseradish peroxidase-conjugated anti-mouse IgG (Amersham International plc, Amersham, UK) for one hour, and visualised on preflashed X-ray film with a Super Signal™ Substrate Western Blotting (Pierce, Rockford, Illinois, USA).

20 fish were examined, six gels were carried out for gill samples, seven gels for skin samples. Quantification of both isoforms of hsp70 was carried out using a Molecular Imager and Imager Densitometer with associated Molecular Analyst®/Macintosh software (Bio-Rad Laboratories, Hercules, CA, USA). Band density determinations were only made from bands of the same immunoblot to ensure uniformity. A direct comparison between the two organs was possible exclusively for the control group where samples of both organs were included in the same gel.

Statistical Analysis of hsp70 and Chloride Cell Number

The hsp70 expression between treatment groups was assessed using a two-way analysis of variance. The band density was used as the response variable and the explanatory variables included the treatment groups and the gel number in order to account for the variation of average immune response between gels.

The numerical difference of chloride cells between control- and treatment groups was assessed by a three-way analysis of variance. The explanatory variables included the treatment group, the fish number, the localisation of the cells within the filament, or the distribution within filament and lamellae, and the batch number of the section. Preplaned comparisons between means were performed using specific linear contrasts and pairwise comparisons of means were done using Bonferroni adjustments. Agreement between models and data was assessed by evaluation at R-square values (R^2). The difference in frequency distributions of chloride cells between groups and localisations was evaluated with χ^2-tests. The level of significance of all statistical tests was set at 5%.

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