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Stress protein Hsp70 response of Nile Tilapia *Oreochromis niloticus niloticus* (Linnaeus, 1758) to induced hypoxia and recovery

Die Bildung des Stressproteins Hsp70 bei *Oreochromis niloticus niloticus* (Linnaeus, 1758) nach induzierter Hypoxie und anschließender Erholung

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Zusammenfassung: An 28 Nilbarschen (*Oreochromis niloticus niloticus*) wurde die Bildung des Hitzeschockproteins Hsp70 unter Hypoxie untersucht. 24 Fische wurden in einem System, in dem Warmwasser zirkulierte, einer induzierten Hypoxie ausgesetzt, vier Fische dienten als Kontrolle. Die Versuchstiere wurden nach 45 min und 2 h, 4 h, 6 h und 8 h nach Zufuhr von Wasser höheren Sauerstoffgehaltes untersucht. In der Leber war der Hsp70-Gehalt während des Stresses am höchsten, unterschied sich jedoch nicht signifikant von demjenigen der Fische, die sich vom Stress erholt hatten. In den Kiemen war er ebenfalls zunächst hoch, sank aber signifikant mit zunehmender Erholungszeit. Durch dieser Stressprotein-Reaktion kann *O. n. niloticus* wahrscheinlich die täglich stark schwankenden Sauerstoffkonzentrationen in Teichanlagen tolerieren.

Schlüsselworte: *Oreochromis niloticus*, gelöster Sauerstoff, Umweltstress, Fisch, Hitzeschockprotein

Summary: We examined the effects of hypoxia on the Hsp70 response in Nile Tilapia (*Oreochromis niloticus niloticus*). Twenty four fish were exposed to induced hypoxia in a warm-water recirculating system, four were used as controls. Fish were investigated at 45 min and 2h, 4h, 6h, and 8h after the transfer to a higher oxygen level. In the liver the Hsp70 level was highest during stress, but did not differ significantly from stress recovered fish. In the gill it was highest during stress, but decreased significantly with ongoing recovery. The stress protein response of tilapia may play a role in survival under conditions of high diurnal dissolved oxygen variations in pond systems.

Key words: *Oreochromis niloticus*, dissolved oxygen, environmental stress, fish, heat shock protein

1. Introduction

Direct effects of a stressor on fish are usually metabolic and affect cellular components such as enzymes and membranes or impair vital functions such as respiration, while indirect effects are expressed primarily in the food uptake rates (Adams 1990). Environmental stresses, such as heat shock or hypoxia/anoxia may be commonly faced by many organisms in the aquatic environment. To modulate the effect of those stressors, organisms

are capable of synthesizing a group of proteins, known as stress or heat-shock proteins (Hsps). It is generally assumed, that heat shock proteins protect the organism from the influence of temporary extreme environmental conditions (Feder and Hofmann 1999). Stress proteins of the family Hsp70 have been found to contribute to correct protein folding. Hence, they may stabilize proteins during stress, for instance during high temperature or low oxygen levels (Heads et al. 1995, Cumming et al. 1996). Furthermore, they can act as a biochemical marker for stress effects resulting in impaired protein integrity, called proteotoxicity (Sanders 1993). However, only few studies have focused on the stress protein response of fish to anoxia or hypoxia (Airaksinen et al. 1998, Gamperl et al. 1998). Currie and Tufts (1997), for instance, showed that rainbow trout *Oncorhynchus mykiss* red blood cells were capable of proteins synthesis during two hours of anoxia but did not increase Hsp70 synthesis.

All these studies were conducted on the cellular level only. However, data on stress protein responses in fish following hypoxia are missing, even though low oxygen levels often occur in aquaculture ponds including tilapia pond cultures. Therefore we studied the stress protein Hsp70 response of Nile Tilapia *Oreochromis niloticus* after hypoxia in a laboratory environment.

2. Materials and methods

2.1. Experimental design

The experiment was conducted at the Department of Aquaculture Systems and Animal Nutrition, University of Hohenheim, Germany. A warm-water recirculation system with water temperatures at 23±0.1°C was connected to aquaria and filtering system. A constant photoperiod of 12 h light and 12 h dark was maintained. No critical values were detected for ammonia (< 1.0 ppm) and nitrite (< 0.1 ppm). A total of 28 fish with an average body weight of 122±36 g was used. The control group (n = 4) was placed in aquaria with a dissolved oxygen level (DO) of 7.5 mg/l over a period of 10 days. The other fish were kept for the same time period and were exposed to diurnal dissolved oxygen variations, as they appear often in the pond cultures (night and early morning: < 1 mg/l O_2, day: > 5 mg/l O_2). After 10 days, when oxygen levels in the aquarium reached hypoxic conditions (0.85 mg/l O_2), the control group and four fish of the experimental group were sacrificed and the liver and gills were frozen in liquid nitrogen. Thereafter, the remaining 20 fish were transferred to aquaria with dissolved oxygen levels of 7.5 mg/l like the control group had been exposed to. The fish (n = 4, each randomly selected) were dissected 45 min, 2 h, 4 h, 6 h and 8 h after the transfer to the higher oxygen level. Both liver and gills were frozen in liquid nitrogen.

2.2. Stress protein analysis

The frozen samples of liver and gill from the controls (n = 4) and the treatments (n = 4 each) were individually homogenized with an Ultra-Turrax tissue homogeniser for 5 s in a pH 7.4 buffered extraction solution (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl, 5% SDS, 2% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA),the volume of which was adjusted to the sample's weight) and the homogenate subsequently centrifuged (10 min, 20,000 g at 4°C). The total protein concentration in each supernatant was determined by the method of Bradford (1976). Equal amount of dissolved protein (20 µg) were separated by SDS-PAGE (12% acrylamide-bisacrylamide) for 20 min at 80 V and 120 min at 120 V, transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience), and the membrane blocked for 2 h with 50% horse serum in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris, pH 7.5). The nitrocellulose membrane was incubated with a primary antibody

against Hsp70 (mouse anti-human, Dianova, Germany) at room temperature overnight. Subsequently, the filter was washed for 5 min with TBS. The secondary antibody (peroxidase-conjugated goat anti-mouse IgG, Dianova, Germany) was added for 2 h at room temperature. After repeated washing for 5 min in TBS, the antibody was visualized by 4-chloro(1)naphthol. The grey scale value intensity of the Hsp70 bands in the immunoblots were quantified by densitometric image analysis (Herolab E.A.S.Y., Germany). For this system, a standard curve relating the different weights of supernatant Hsp70 and signal density was established by Schill et al. (2002). Each gel contained a prestained protein ladder (Benchmark, GibcoGRL, Life Technologies, Gaithersburg, MD, USA). Additionally, gels were stained with Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA) after the transfer procedure to ensure complete protein transfer.

2.3. Statistics

Differences in the Hsp70 levels of the fish between the control and the treatments were statistically tested using the Duncans Multiple Range Test (DMRT) at significance level of $p < 0.05$ with the aid of STATISTICA Statsoft Inc. 5.1, 1997. Results are given as Mean ± SD. The levels of statistical significance used in each analysis was $p < 0.05$ (significant, *).

3. Results

The results are summarised in figure 1 (above) for the liver samples from *O. niloticus* and in figure 1 (below) for the gill samples from the identical fish. The level of Hsp70 in the liver tended to be reduced over the observed time. Hypoxic fish showed a level of more than 43,000 (arbitrary grey scale intensity), while the same parameter declined to a level of nearly 28,000 after 8 h acclimatisation in water

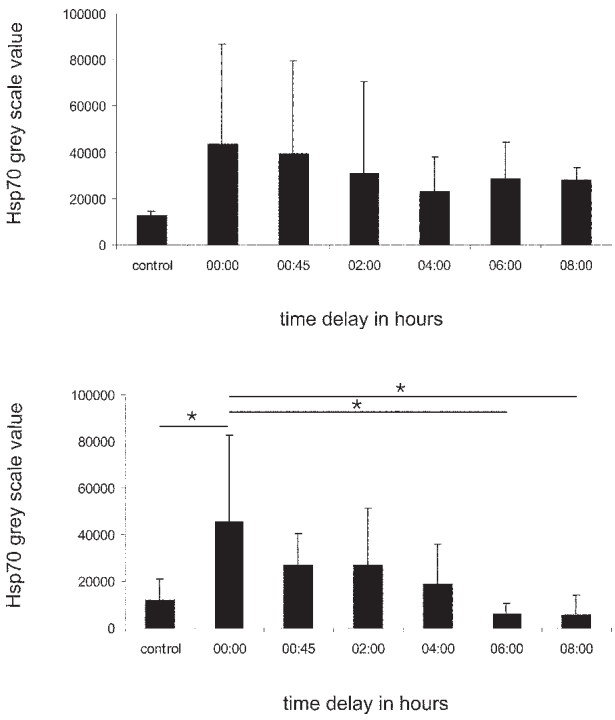

Fig. 1: Hsp70 level (mean ± SD) in the liver (above) and the gills (below) of *O. n. niloticus*. Results for controls, hypoxic (00:00) and post stressed animals (00:45-08:00); n = 4 for all columns. * significant differences at $p < 0.05$.

Abb. 1: Hsp70-Gehalt (Mittelwert und Standardabweichung) in der Leber (oben) und in den Kiemen (unten) von *O. n. niloticus*. Ergebnisse für die Kontrollen, für die Tiere unter Hypoxie (00:00) und nach unterschiedlicher Erholung (00:45-08:00); n = 4 für alle Säulen. * signifikante Unterschiede bei $p<0,05$.

containing higher DO levels. Hsp70 levels decreased linearly with a coefficient of determination (R^2) of 0.98 after 4 hours post stress and a R^2 of 0.67 after 8 h post stress. However, all data obtained for the liver (including the control) were not significantly different (Fig. 1 above).

The measured levels of Hsp70 in the gills of respective fish were significantly reduced ($p<0.05$) after 4 h acclimation to ambient dissolved oxygen levels in the water (Fig. 1 below). The level of Hsp70 was also significantly lower ($p < 0.05$) in the control group than in the hypoxic group. A linear decrease of Hsp70 level was observed after 4 h ($R^2 = 0.84$) and 8 h ($R^2 = 0.92$), respectively.

At 45 min post stress, the Hsp70 level in the liver was still about the same as in the hypoxic group, while the Hsp70 level in the gill tended to be reduced. However, this observed stress protein decrease in the gills was statistically significant after 6 h of recovery and further on, only.

4. Discussion

Stress proteins mitigate the impact of stress in individuals and these mechanisms are becoming well understood at the level of model proteins, but less is known at the level of the cell, tissue, organ and whole organism (Feder and Hofmann 1999). Therefore studies of stress protein response in organs with high metabolic activity, like liver and gills, are of high interest. In this study, we provided the first evidence that Hsp70 acts as a biochemical marker linked to hypoxia-caused proteotoxicity in Nile Tilapia facing long term hypoxic conditions.

The r-value, which describes the goodness-of-fit of the stress response distribution, decreased in tilapia with longer time delay after the removal of the stressor in observed liver samples. Additionally, the variability in Hsp70 levels increased tremendously, both in liver and gill samples, after tilapia faced oxygen depletion. This might be due to an increase in individual variation at non-optimal conditions. This is furthermore supported by Dietz and Somero (1994), and Köhler et al. (2001), who also found wide variations of Hsp levels in fish. Physical handling, however, did not increase Hsp70 levels in the liver of rainbow trout (Vijayan et al. 1997). Faust et al. (2004) showed high intra-specific variations in the activity of lactate dehydrogenase (LDH) in rainbow trout hearts to short term hypoxia.

The gills exhibited significant differences in the Hsp70 levels between control, hypoxic, and recovered fish. However, they are the primary organ to be affected by hypoxia and, therefore, the proteotoxic effect was likely to occur in more prominent way as in the liver. The present study points out only an initial but pertinent information on aspects of the response of fish to environmental stresses, such as hypoxia. Our results should encourage further research in this topic of environmental stress physiology since long term studies in the field suggested seasonal changes in heat shock response of fish (Dietz and Somero 1992, Köhler et al. 2001). Furthermore, constant conditions and single stressors rarely occur in nature (Heath 1987) and field trials are therefore vital to provide in-depth knowledge in stress physiology of fish.

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