Environmental acidification triggers oxidative stress and enhances globin expression in zebrafish gills

Jessica Tiedke, Ceyda Cubuk, Thorsten Burmester *

Institute of Zoology and Zoological Museum, University of Hamburg, Martin-Luther-King-Platz 3, D-20146 Hamburg, Germany

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ABSTRACT

Animals in many aquatic ecosystems must cope with changing environmental parameters, such as temperature, oxygen availability or pH. We have investigated the molecular responses to acidification in the gills and body of zebrafish (*Danio rerio*) by means of quantitative real-time PCR. Expression levels of typical stress genes and genes for antioxidant defense were strongly enhanced in gills, and to lesser extents in the body, suggesting that acidification leads to oxidative stress. Surprisingly, the globins were found to be among the most prominent stress–responsive proteins in our study. Myoglobin showed the strongest response of all investigated genes in the gills, as confirmed by Western blotting. These findings agree with the role of globins in oxidative energy metabolism, but may also hint at a specific function in antioxidative defense.

1. Introduction

Freshwater ecosystems are the habitat for about one third of the global vertebrate diversity [1]. Many freshwater environments suffer from multiple stressors like seasonal or spatial fluctuations of temperature, oxygen, or pH [2]. On the global scale, acidification is an acute problem of many freshwater ecosystems, mainly arising from anthropogenic activities [3,4]. Acidification has already resulted in reductions of fish stocks in several freshwater systems [4]. The low buffering capacity of fish blood, due to a low bicarbonate concentration, makes fishes particularly sensitive to acidification [5]. The pH of the extracellular fluid is mainly adjusted by the highly energy consuming efflux of H^+ and HCO_3^- along with Na^+ and Cl^- influxes over the gill epithelium [5]. The gills are a multi-functional organ and the main side that is affected by fluctuations in the aquatic environment [5,6].

The disturbance of the cellular homeostasis typically leads to a stress response, which is triggered by defective macromolecules [7]. This defense reaction is essential for a temporary increase of stress tolerance and the protection of the cellular homeostasis. Although our knowledge about universality in the response to different stressor is still incomplete, it is well known that certain parts of the cellular stress response, such as molecular chaperones, antioxidants or apoptosis enzymes, are inducible by a broad range of environmental challenges [7,8].

It has been demonstrated in several fish species that the acclimation to environmental stressors like pollution lead to an increase in the production of reactive oxygen species (ROS), mainly arising from the respiratory chain [9,10]. ROS are highly reactive molecules that participate in several signalling pathways [11]. Excessive ROS production, however, interferes with the redox state and leads to oxidative stress, which causes damage of DNA, protein, and membranes [12]. ROS defence is mainly accomplished by superoxide dismutases, catalases, and glutathione peroxidases [13]. Recently, it has been suggested that globins are also involved in ROS defence [14–17]. These small metallo-proteins play an important role in respiration, but may also have other functions. In addition to hemoglobin and myoglobin (Mb), neuroglobin (Ngb) [18], cytoglobin (Cygb) [19] and globin X (GbX) [20] are known in teleost fish.

The objective of this study was to characterize the impact of environmental acidification on the fish gills. We focused on selected stress-responsive genes. In addition to the evaluation typical stress genes, specific attention was devoted to the globins. The understanding of the molecular mechanisms that are involved in a specific stress response shed light on the basis of stress tolerance in fish species and the specific role of globins.

2. Materials and methods

2.1. Experimental design

An inbred strain of zebrafish *Danio rerio* (Hamilton, 1822) (Cypriniformes) was obtained from the Max-Planck Institute for

* Corresponding author. Address: Institute of Zoology and Zoological Museum, Biocenter Grindel, University of Hamburg, Martin-Luther-King-Platz 3, D-20146 Hamburg, Germany. Fax: +49 40 42838 3937.
E-mail address: thorsten.burmester@uni-hamburg.de (T. Burmester).

Developmental Biology (Dr. Brigitte Walderich, Tübingen, Germany). Fishes were kept in 50 l aquaria at 24–26 °C with 12.5 h/11.5 h day/night light cycles. Fish were fed daily with *Artemia* sp. and fasted 24 h prior to the experiments. During treatment, the zebrafish fasted and the water was renewed every 24 h. For acidification experiments, tap water was buffered with 1 mM 2-morpholinoethansulfonic acid and adjusted to pH 4.0 with HCl. Groups of male fishes ($n = 4$) were exposed to acidification (24, 72 and 120 h) in 6 l tanks. The experiments were repeated three times and a total of twelve specimens were used for each period. After treatment, gill arches of each group ($n = 4$) were pooled. Tap water (mean pH 7.4 ± 0.19) served as a control and experiments were performed in parallel in separate tanks.

2.2. RNA extraction

Zebrafish were anaesthetized on ice and the gills arches of each fish were removed. Gill arches and the remaining carcasses were rinsed in sterile PBS. For each single experiment, a pool of gill pairs from four individuals was used. Tissue samples were grinded to a fine powder with liquid nitrogen using a mortar and pestle. Extraction of total RNA was performed with peqGOLD Trifast™ (Peqlab, Erlangen, Germany) according to the manufacturer's instruction. Total RNA was purified with the RNeasy® Mini kit (Qiagen, Hilden, Germany), including an on-column digestion with RNase-free DNase (Qiagen). The quality and integrity of total RNA was assessed spectrometrically and by gel electrophoresis.

2.3. Molecular cloning and sequencing

Reverse transcription reactions were performed with pooled RNA of each group resulting in three cDNA samples from each acidification period (24, 72, and 120 h). cDNA was synthesized using SuperScript™ III RT (Invitrogen, Karlsruhe, Germany) and oligo-(dT)18 oligonucleotide primers according to the manufacturer's protocol. Coding sequence fragments (80–200 bp) of each gene of interest were amplified using gene specific primers (Supplemental Table 1). After cloning into pGEM–T easy (Promega, Mannheim, Germany), the cDNA fragments were sequenced by a commercial service (GATC Biotech, Konstanz, Germany).

2.4. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) experiments were carried out on an ABI 7300 Real-Time PCR System with the ABI Power SYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany) employing a two step protocol. For each PCR reaction, we used cDNA equivalent to 37.5 ng total RNA. To avoid amplification of genomic DNA we used intron-spanning oligonucleotides (10 μm) constructed on sequences withdrawn from NCBI and ENSEMBL databases (Supplemental Table 1). Quantification was performed using a standard cycling protocol (95 °C 10 min, 95 °C 15 s, 60 °C 15 s; 72 °C 30 s). Primer specificity was checked by a dissociation curves analysis with a temperature range of 60–95 °C. Seventeen putatively stress regulated genes and two potential reference genes were investigated. The PCR reactions with each cDNA were performed as technical triplicates resulting in nine data points for each gene in each period (24, 72, and 120 h). Standard curve reactions using recombinant plasmids as templates were run as duplicates.

2.5. Analyses of expression data

The qRT-PCR data were first evaluated by ABI 7300 Sequence Detection Software 1.3.1 (Applied Biosystems). Amplification efficiency was determined by the slope of the standard curve. Evaluation of expression levels was performed using the $\Delta\Delta CT$ method [21] and further analysed with IBM SPSS Statistics 20. Nine data points (three biological × three technical replicates) were used for comparison of mRNA levels between acidification and control experiments. The distribution of variables and the equality of variances were determined using the Kolmogorov–Smirnoff test and Levene's test. In case of homogenic variances, one-way ANOVA followed by Dunnett's t-test was applied, in the case of heterogeneity a one-way ANOVA using the Games-Howell correction for post hoc analysis was performed ($n = 9$; d.f. = 36; $P < 0.05$).

## 2.6. Protein extraction and Western blotting

Protein levels of Mb and Ngb in gills was determined by Western blotting. Acidification experiments were run as described above. We pooled the gill pairs of three individuals from each experimental condition. Whole gill arches were homogenized by sonication in 20 mM HEPES, pH 7.0, with Complete™ protease inhibitor (Roche Diagnostic, Mannheim, Germany). After centrifugation, the supernatants were collected and protein concentrations were determined by the BCA method [22]. 30 μg of whole protein extract from each treatment was separated on a 13% SDS–PAGE and transferred on a PVDF membrane using the semidry method. The membrane was blocked with 2% bovine serum albumin in TBS for 1 h and subsequently incubated in blocking solution containing diluted primary antibody (1:500 *D. rerio* anti-Mb rabbit antiserum or 1:20 *D. rerio* anti-Ngb rabbit antiserum [23]). Detection was performed using a horseradish-peroxidase-conjugated mouse anti-rabbit antibody (1:10,000) (Dianova, Hamburg, Germany) in combination with the chemiluminescent AceGlow™ (Peqlab). The images were scanned and imported into the ImageJ 1.46r (http://imagej.nih.gov/ij/). Protein levels were estimated by analyses of grey values. For statistical evaluation, all acidification treatments were pooled. Significance levels were estimated by Student's T-test.

3. Results

3.1. Effects of environmental acidification on mRNA levels in zebrafish

We monitored the response to acidification employing genes that mirror different cellular functions (Supplemental Table 2). Groups of four male fishes were challenged with pH 4 for 24, 72 or 120 h; three independent experiments were performed ($n = 3$). We investigated the effects on the gills, which are the first target organs of environmental acidification, as well as the impact on the remaining bodies. Differences between groups were analysed by ANOVA. Two putative reference genes (*Actb, Rplp0*) were tested, the mRNA levels of *Rplp0* were not affected by the experimental conditions and used as reference. Most of the other investigated genes respond to the acid environment of pH 4 with enhanced mRNA levels in the gills (Fig. 1), but also in the remaining fish tissues (Fig. 2).

The molecular chaperones (*Hsp70, Hsp90a, Ppia*) showed a rapid increase in gills. All three chaperones displayed a similar pattern of expression changes, with the strongest response after 24 h (up to 7.6-fold for *Hsp70*). In the fish bodies, the response was less pronounced, with *Ppia* showing the strongest increase of mRNA levels (Fig. 1A). The levels of *Parp* and *Top2*, two enzymes which are involved in DNA repair, had divergent responses. While *Top2* mRNA were strongly elevated for all the periods in both the gills and bodies (Figs. 1B and 2B), *Parp* mRNA levels did not change in the gills, and were mildly elevated in the bodies only at 72 h (3.1-fold). We also tested two genes putatively involved in apoptosis. The levels of *Casp3*, which plays a key role in the control of apoptosis [24], were significantly enhanced in the gills after 72 and 120 h (Fig. 1C).
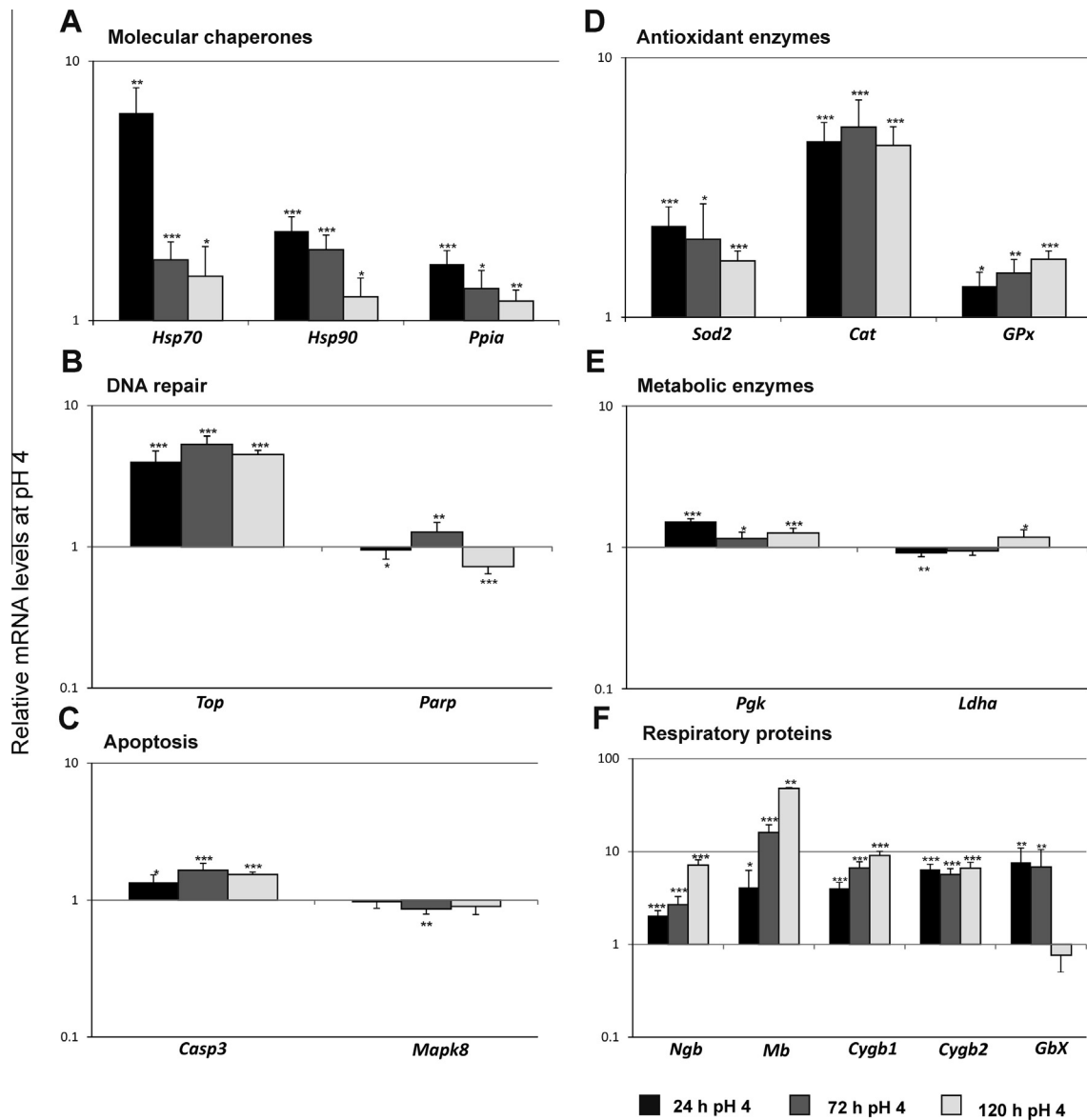
Fig. 1. mRNA levels of different sets of genes involved in basic cell functions at 24 h (black), 72 h (dark grey) and 120 h (light grey) acidification at pH 4 in gills of zebrafish. The y-axis has a logarithmic scale. Bars indicate standard deviation. ***$P < 0.0005$; **$P < 0.005$; *$P < 0.05$.

By contrast, the levels of *Mapk8*, an enzyme that is required for TNF-alpha-induced apoptosis [25], was essentially unaffected.

Gene expression analyses of three antioxidant enzymes (*Sod2*, *Cat*, *Gpx*) revealed essentially the same patterns in the gills and the bodies (Figs. 1D and 2D). In the gills, the *Sod2* mRNA increased 2.2-fold after 24 h and then slightly declined until the end of the measurements at 72 h. The same pattern was found in the bodies. By contrast, the levels of catalase and *Gpx* mRNA increased gradually over time, at least in the gills (Fig. 1E). The enzymes of the aerobic and the anaerobic metabolism, *Ldha* and *Pgk*, respectively, were found unaffected by acidification in the gills, while their mRNA levels slightly increased in fish bodies (Fig. 2E).

We specifically tested the changes of five globin genes: *Mb*, *Ngb*, *Cygb1* and *2* and *GbX*. Notably, the globins showed similar trends in the gills, but divergent regulation in the remaining fish tissues (Figs. 1 and 2E). In the gills, all globins respond to the acidification by a strong increase of mRNA levels, with the exception of *GbX*. *Mb* displayed the strongest response and was found after 72 h to be

elevated up to 42-fold compared to the controls. In the bodies, *GbX* showed the strongest response, while the levels of *Mb* did not significantly change.

3.2. Changes in globin protein expression

We specifically tested protein levels of Mb and Ngb in response to acidification. Proteins were extracted from the gills and pools of three pairs of gill arches were applied per sample ($n = 3$). Because no appropriate antibody was available to detect a putatively non-regulated house-keeping gene, total protein levels were measured and used as loading control. We found elevated protein levels of Mb after acidification (Fig. 3A). The strongest response was after 72 h. The mean increase of Mb protein levels was 8.2+/−3.3-fold ($P = 0.03$). Ngb protein levels also showed an about 1.5+/−0.3-fold increase and the highest Ngb protein levels were detected after 120 h (Fig. 3B). However, the increase was not significant ($P = 0.06$).
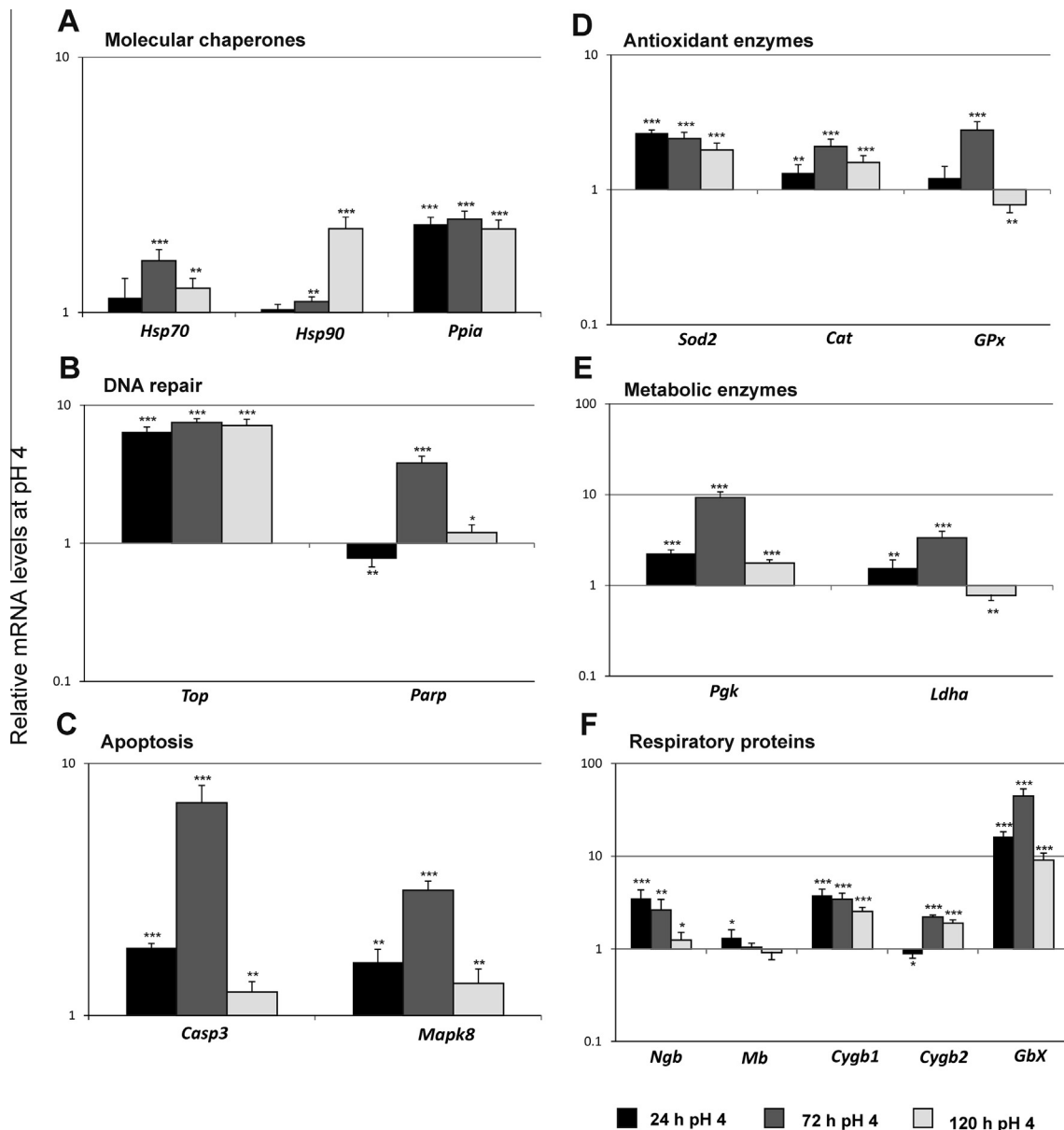
Fig. 2. mRNA levels of different sets of genes involved in basic cell functions at 24 h (black), 72 h (dark grey) and 120 h (light grey) acidification at pH 4 in in remaining bodies of zebrafish. The *y*-axis has a logarithmic scale. Bars indicate standard deviation. ***$P < 0.0005$; **$P < 0.005$; *$P < 0.05$.

4. Discussion

4.1. Acidification triggers cellular stress response

Environmental stress leads to the disruption of the homeostatic equilibrium [7]. Previous studies demonstrated that molecular chaperones, such as Hsps, are induced by various stressors such as toxins or heavy metals in fish [26,27]. *Hsp70* displayed the strongest response, particularly at acute acidification (Fig. 1A). It assists folding of nascent proteins and refolding of damaged or aggregated proteins, while Hsp90 is mainly involved in the maturing processes of protein kinases and steroid hormone receptors [28]. These results demonstrate that acidification initiates the molecular stress response in gills. The induction of chaperone-expression in the remaining fish tissue was less pronounced, which may be explained by the gills being the main site of the interaction with the acidic environment. In addition, the bicarbonate buffer system of the blood, secure acid–base balance and homeostasis

[5]. Cyclophilin A (Ppia) is a molecular chaperone with peptidyl-prolyl isomerase activity (Ppiase) and is responsible for the acceleration of the rate limiting steps of protein folding [29]. Like the Hsps, cyclophilins respond to many types of stressors, such as thermal stress, UV irradiation or pH changes [30]. This agrees with the enhanced cyclophilin levels in the gills and bodies (Figs. 1A and 2A).

4.2. Acidification induces ROS defence

The formation of ROS is the most prominent effect of stress and causes oxidative damage of macromolecules. Under physiological conditions, the mitochondrial respiratory chain generates small amounts of ROS as byproduct of complex I [31], The proximal mitochondrial ROS is superoxide (O_2^-), which is converted into the less toxic hydrogen peroxide (H_2O_2) by SOD. H_2O_2 is further decomposed into H_2O and O_2 by peroxidases, such as catalase
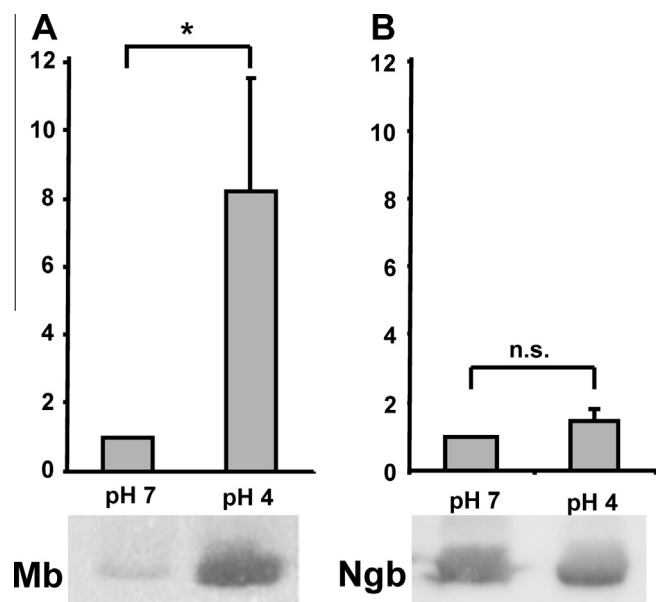
Fig. 3. Response of Mb and Ngb protein in gill tissue of zebrafish to acidification. The fishes have been adapted to pH 4 for 24, 72 and 120 h. Each lane was loaded with 30 μg of total protein. (A) Mb was detected by anti-zebrafish-Mb serum at the predicted mass of 15.6 kDa. (B) Ngb was detected using anti-zebrafish-Ngb serum in the range of the predicted mass of 17.7 kDa. Bars indicate standard deviation.

and glutathione peroxidases [31]. ROS generation may exceed adequate levels under stressful conditions [11].

In zebrafish gills acidification causes significant increases in the levels of antioxidant enzymes Sod2, Cat and Gpx (Fig. 1D) and at a lower level in the remaining tissue (Fig. 2D). A pH-dependent induction of oxidative stress has already been demonstrated in the mollusk *Crassostrea virginia* [32]. In that study, the exposure to elevated environmental P_{CO_2} levels (causing low pH) leads to enhanced levels of oxidative stress associated proteins such as Cu,Zn-SOD and peroxiredoxins.

We consider it unlikely that the acid itself causes ROS production. Rather an increase in the aerobic metabolism of the respiratory chain may lead to the formation of ROS. A higher level of energy production is likely required for the enhanced ion exchange across the gill surface as well as the synthesis of stress-related proteins such as epithelial exchangers etc. This leads to an increase of O_2 consumption, which is, in turn, responsible for the formation of ROS. A higher oxygen consumption rate in response to environmental acidification has already been observed in the crustacean *Daphnia pulex* [33].

We also found increased mRNA levels of the two metabolic enzymes *Pgk* and *Ldha* in the fish bodies (Fig. 2E). A recent study on the effects of hypercapnia on the Mediterranean fish *Sparus aurata* found enhanced activity levels of Ldh and enzymes from glycolysis in red muscle and heart [34]. This suggests that a shift from aerobic to anaerobic metabolism has occurred. The authors argued that low blood pH in hypercapnic fish might lead to a decrease of oxygen affinity of hemoglobin, resulting in decreased oxygen supply. Since we also found enhanced mRNA levels of respiratory proteins, one might also argue that zebrafish suffer from tissue hypoxia as an effect of environmental acidification.

4.3. The specific role of globins

Surprisingly, the globins were found to be among the most prominent stress–responsive proteins. The interaction of ROS with heme-proteins was initially first seen with Hb [35]. More recently,

it has been suggested that also Mb, Ngb, and Cygb might also play an important role in ROS scavenging [36,37]. The most pronounced response to acidification was observed for Mb mRNA in the gills (Fig. 1), which was confirmed on the protein level by Western blotting (Fig. 3). While in mammals, *Mb* is almost exclusively found in the cardiac and skeletal muscles, in zebrafish and other cyprinids it is additionally expressed in the gills, brain, liver and kidney [38–40]. Mb is considered a respiratory protein that supplies the mitochondria in muscle cells with O_2 [41], but it is unknown whether it has the same function in the non-muscle tissues of fish. Studies employing *Mb*-deficient mice revealed that Mb protects cardiac muscle against bioactive ROS [14,42]. A similar protective function is also conceivable for the gills, where *Mb* mRNA and protein were significantly induced upon acidification (Figs. 1 and 3). The same argument may also be true for the other globin types, which also increased upon acidification. In the literature, Ngb and Cygb have been related to ROS protection [15–17]. In this context, it should be noted that Ngb, which is a neuronal protein in vertebrates, was found in the mitochondria-rich chloride cells in the zebrafish gills [23]. Thus globins by be instrumental in the response of the fish to oxidative stress, as induced by acidification. On the other hand, if all globins (including Ngb, Cygb and GbX) are actually involved in respiration, their higher expression levels in response to acidification may be related to an enhanced O_2 consumption due to the higher energy demands. Both hypotheses of globin functions – ROS defence and respiration – are in line with the observation that *Ngb* and *Mb* levels increase upon hypoxia [43].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.104.

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