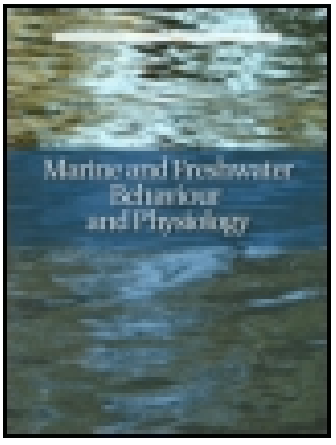

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Metabolic and molecular responses in Nile tilapia, Oreochromis niloticus during short and prolonged hypoxia

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Metabolic and molecular responses in Nile tilapia, *Oreochromis niloticus* during short and prolonged hypoxia

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The strictly aquatic breathing Nile tilapia, *Oreochromis niloticus* is an extremely hypoxia-tolerant fish. To augment our understanding of the effects of hypoxia on anaerobic glycolysis in the Nile tilapia, we studied the effect of short-term for 1 day (trial 1) and long-term for 30 days (trial 2) hypoxia on a selected glycolytic enzymes activity and mRNA expression in liver and white muscle. The hypoxic oxygen concentrations used in the two trials were 2, 1, and 0.5 mg O_2 L^{-1} for comparison with a control normoxic group 8 mg O_2 L^{-1}. The activity of phosphofructokinase (PFK), pyruvate kinase (PK), and lactate dehydrogenase (LDH) in liver and white muscle except liver LDH decreased in trial 1 and increased in trial 2. Assessments of mRNA levels in trial 1 revealed that *PFK* was downregulated and *LDH* was upregulated in liver and white muscle, while *PK* fluctuated between upregulation in liver and downregulation in white muscle. Meanwhile, *PK* and *LDH* were upregulated while *PFK* was similar to control values in both tissues in trial 2. Comet assay results demonstrated an increase in DNA damage that was directly proportional to increasing hypoxic concentrations. This damage was more pronounced in trial 1. This suggests that the Nile tilapia cope better with long-term hypoxic conditions, possibly as an adaptive response.

Keywords: Nile tilapia; *Oreochromis niloticus*; hypoxia; PFK; LDH; PK; enzymatic activity; gene expression

Introduction

Environmental hypoxia is a common challenge that many aquatic organisms experience in their habitat (Gracey et al. 2011). Many intensive fish culture systems are subjected to a lack of dissolved oxygen (DO) in the water due to high fish density, feeding practices, algal blooms, and elevated temperatures that reduce the availability of oxygen. This hypoxia can affect growth, food consumption, and the physiological state of fishes by impairing energy metabolism in fish tissues (Foss et al. 2002). Hypoxia can moreover lead to high rates of mortality of fish in aquatic systems depending upon their degree of hypoxia tolerance (Martínez et al. 2011).

Depending on the severity of hypoxia and its duration, exposure to hypoxia elicits multiple behavioral, biochemical, physiological, and molecular responses in fish

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(Richards 2011). Responding to hypoxia usually requires metabolic reprogramming so that energy-demanding processes are regulated to match available energy reserves (Gracey et al. 2011). A number of studies reported conflicting results concerning the effect of hypoxic exposure upon glycolytic enzyme activity. Several reports have indicated an increase in the glycolytic enzyme activity in liver and white muscle of common carp, *Cyprinus carpio* (Zhou et al. 2000) and liver of killifish, *Fundulus heteroclitus* (Kraemer & Schulte 2004) in hypoxic exposure, which presumably augment the capacity of fish tissues for anaerobic energy production. Other studies reported a decrease or no change in enzyme activity (Driedzic et al. 1985; Almeida-Val et al. 1995). The degree and direction of enzyme adjustments generally depend upon the duration and severity of hypoxia (Almeida-Val et al. 1995; Johansen et al. 2006) as well as the tissue and species studied (Chippari-Gomes et al. 2005; Dalziel et al. 2005; Martinez et al. 2006; Martínez et al. 2009).

While phosphofructokinase (PFK) is an important control point in the glycolytic pathway since it is one of the irreversible steps and has key allosteric effectors (Lushchak et al. 1998; Martínez et al. 2011), pyruvate kinase (PK) is a final-stage enzyme in glycolysis, which generates the substrates ATP and pyruvate for anaerobic and aerobic metabolism. Meanwhile, lactate dehydrogenase (LDH), an important glycolytic enzyme in biological systems, is the terminal enzyme of anaerobic carbohydrate metabolism that is responsible for the conversion of pyruvate to lactate, the end products in the process of glycolysis, when the amount of oxygen is limited (Elcock & McCammon 1996).

The molecular response of fish to hypoxia tends to be more sensitive and usually occurs earlier than those at higher levels of biological organization (Zhang et al. 2009) have not been studied extensively. Examples are the euryoxic fish goby, *Gillichthys mirabilis* (Gracey et al. 2001), the zebra fish, *Danio rerio* (Ton et al. 2003), the Japanese medaka, *Oryzias latipes* (Ju et al. 2007), the killifish (Flight et al. 2011), and the gulf killifish, *Fundulus grandis* (Everett et al. 2012). Although the changes in enzymes activity required for metabolic reorganization during hypoxia are often regulated by the differential expression of mRNA (Semenza et al. 1996), very little is known about the molecular mechanisms underlying the various physiological adaptations of aquatic organisms in response to hypoxia. On the other hand, hypoxia-induced DNA damage and apoptosis have been studied extensively in many mammalian cell lines (Thompson 1998; Bras et al. 2005) but there are only a few studies using whole animal models such as sea horses *Hippocampus reidi* (Negreiros et al. 2011) and common carp (Mustafa et al. 2011).

In North Africa and the Near East, Egypt is by far the dominant producing country (92% of the total for the region) and is considered as the second biggest tilapia producer after China (FAO 2006). Because tilapia is commonly cultured in heavily fertilized ponds where community respiration is similar to or exceeds oxygen production by phytoplankton, the fish is capable of surviving dawn DO concentration of less than 0.5 mg L^{-1}, levels considerably below the tolerance levels for most other cultured fish (Teichert et al. 1997; Stickney 2000; Lim et al. 2006). However, the fish growth becomes depressed when concentration fall chronically below 1–2 mg L^{-1} (Teichert et al. 1997). The aim of the present study was to evaluate and link the effects of short-term for 1 day (trial 1) and long-term for 30 days (trial 2) hypoxia on the glycolytic activity of the enzymes PFK, PK, and LDH, the mRNA expression in Nile tilapia liver and white muscle tissues as well as to examine DNA damage induced by hypoxic conditions in both tissues.

Materials and methods

Fish

Nile tilapia juveniles were obtained from a fish hatching pond in Fowa city (Kafr El-Sheikh Governorate, Egypt). The fish were transported in oxygenated cellophane bags at mid-October. Fish of nearly equal size (10 ± 1.2 g) were randomly distributed in 24 glass aquaria of 40-L size (60 × 35 × 40 cm). Five fish were placed in every aquarium giving a rearing density of 1.25 g L^{-1} in order not to exceed, by the end of the experiment, the upper rearing density limit of the fish for experimental testing (2.5 g L^{-1}). Fish were acclimated to laboratory conditions for one week. Each glass aquarium was equipped with a continuous aeration system, biological filter, and thermostatic-controlled heater. The water temperature was steadily increased from 18 °C, by 2 °C every 2 days, to reach the desired temperature 26 ± 0.5 °C, over 10 days. This is the optimum temperature giving maximal growth of Nile tilapia (Mishrigi & Kubo 1978). Fish were acclimated to that temperature for 4 weeks. On alternate days, 50% of the aquarium water was exchanged with dechlorinated water, which was previously adjusted to 26 °C. The fish were fed with commercially available pellets (25.2% protein, 5% lipids, 5.7% carbohydrate and fibers with total energy 2505 kcal kg^{-1}) at a rate of 3% of their body weight, once daily, at 10:00 am during all experimental periods. Water conditions were as follows: temperature, 26 ± 0.5 °C; pH 7.2 ± 0.3; ammonia, less than 0.25 mg L^{-1}; nitrate, less than 20 mg L^{-1}; and nitrite, less than 2 mg L^{-1}.

Experimental design

Following laboratory thermal acclimation, fish were classified into two groups; each was subdivided into four subgroups with 15 fish. These were cultivated in oxygen saturations 8.0, 2, 1, and 0.5 mg O_2 L^{-1}. The experiment was either terminated after 1 day (trial 1) for short-term hypoxia exposure or 30 days (trial 2) for prolonged hypoxia exposure. The oxygen concentrations were chosen to mimic possible hypoxic conditions faced by the fish in its natural habitat (Teichert et al. 1997; Mallya 2007). To maintain the desired hypoxic conditions in each aquarium, nitrogen gas and atmospheric air were pumped into water using nitrogen gas pressure regulator (GH-591, Zhejiang, China). To ensure that the fish were not gasping at the surface to artificially increase oxygen uptake, the treated aquaria were covered with suitable glass plates during the exposure time. The levels of DO were monitored continuously using a DO meter (HI 9142 Hanna, Italy).

Tissue sampling

Fish were sacrificed at the end of each trial. White muscle samples were taken dorsal to the lateral line, avoiding red muscle contamination as well as tissue squeeze, and the right lobe of the liver was excised. Both tissues were dissected on ice, weighed, frozen in liquid nitrogen, and stored at −45 °C for biochemical analysis. For RNA extraction, 20 mg of liver and white muscle tissue were immediately submerged in collection vessels containing 200 µL of RNA later Stabilization Reagent (Qiagen, Germany) to preserve the RNA. Samples were incubated overnight at 2–8 °C before transfer to −20 °C until analysis. For comet assay, approximately 1 g of tissue sample was required; hence samples from 6 replicate fish (from the same group) were taken, pooled, frozen in liquid nitrogen and stored at −20 °C until analyzed.

Extraction of preparations for enzyme assay

Tissue samples were homogenized in buffer containing 20 mM HEPES, pH 7.6, 1 mM EDTA, and 30 mM β-mercaptoethanol using Omni international homogenizer (USA), then delivered to a 1.5 mL Eppendorf tube and centrifuged in cooling centrifuge (Hettich, Germany) at 6000 × *g* for 15 min at 4 °C. The yielded supernatant was used immediately to estimate the glycolytic enzyme activity.

Enzyme assays

PFK, PK, and LDH were assayed kinetically according to the protocols of Bergmeyer (1981). For each enzyme in each tissue, the concentrations of substrates, cofactors, and linking enzymes were optimized to give maximal activity. The activity was measured by monitoring the absorbance of NADH at 340 nm (extinction co-efficient of NADH = 6.22 mM^{-1} cm^{-1}) using a thermostated UV/VIS Spectrophotometer (Jenway 6505, UK) at 25 °C. The reaction was started by the addition of an appropriate amount of the cytosolic fraction to 1 mL of the reaction mixture. Conditions of the enzyme assays were optimized with respect to substrate concentrations on the bases outlined by Foster and Moon (1986). The final reaction mixtures for enzyme activity were as follows:

(1) PFK (E.C., 2. 7. 1. 11): 65 mM imidazol–HCl buffer (pH 7.4), 1 mM EDTA, 30 mM β-mercaptoethanol, 6 mM fructose6-phosphate, 0.15 mM NADH, 2 mM ATP, 100 mM KCl, 10 mM $MgCl_2$ and 1 μmL^{-1} aldolase (Ald, E.C., 4.1.2.7), 10 μmL^{-1} glycerol-3-phosphate dehydrogenase (G-3-PDH, E.C., 1.1.1.8), and 29 μmL^{-1} triose phosphate isomerase (TPI, E.C., 5.3.1.1).
(2) PK (E.C., 2. 7. 1. 40): 65 mM imidazol–HCl buffer (pH 7.4), 1 mM EDTA, 30 mM β-mercaptoethanol, 2.5 mM PEP, 0.15 mM NADH, 3 mM ADP, 10 mM $MgCl_2$, 50 mM KCl, and 0.375 (liver) or 1.5 (muscle) μmL^{-1} L-LDH (E.C., 1. 1. 1. 27).
(3) LDH (E.C., 1. 1. 1. 27): 65 mM imidazol–HCl buffer (pH 7.4), 1 mM EDTA, 30 mM β-mercaptoethanol, 1 mM pyruvate, and 0.15 mM NADH.

RNA extraction

RNA was extracted using Norgen purification kit (Thorold, Canada) according to the manufacturer's instructions. To control for inter-individual variation, pooled RNA from six fishes was assessed at each time point. Total RNA was solubilized in RNase-free water and quantified by measuring optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio >1.80. The quality and integrity of total RNA were assessed by inspection of the ribosomal RNA bands (18S and 28S) in ethidium bromide-stained 1% agarose gels under ultraviolet (UV) light and electrophoresis of the RNA confirmed that it was intact. In order to eliminate residual genomic DNA in the total RNA preparation, all RNA samples were treated with RNase-free DNase kit (Fermentas, USA).

RT-PCR

One microgram of RNA were reverse transcribed from each sample with oligo-dt primer and First Strand cDNA Synthesis Kit (RevertAid™ H Minus, Fermentas, USA) in a

total volume of 20 µL, according to the protocol provided by the supplier. To control for genomic contamination, negative control samples were performed using RNA that had not be reverse transcribed.

For *PFK* (accession no. DQ066870.1), *PK* (accession no. DQ066876.1), and *LDH* (accession no. EU313200.1) genes, PCR primers were designed using the Primer3 program (accessible at http://www–genome.wi.mit.edu/cgi–bin/primer/primer3 www.cgi) on the base of their nucleotide sequences available on GenBank. *β-actin* amplification was used as the housekeeping gene in semi-quantitative RT-PCR analysis based on published sequences, AA566386 (Choi 2004). Products obtained using 20 cycles of amplification were within the linear range of signal amplification and allowed titration of the amount of template to be subsequently used in order to obtain consistent amounts of products between samples. The adjusted cDNA volumes were then used in the succeeding PCR reactions with gene specific primers for *PFK*, *PK,* and *LDH*. Oligonucleotide primers were synthesized commercially by Metabion International AG (Martinsried/Deutschland). In a final volume of 20 µL, 2 µL (~0.1 µg) of cDNA was then amplified using 2 µL of dNTP (2.5 mM each), 2 µL of 10× PCR Buffer, 0.5 µL (5u μL^{-1}) Taq™ DNA polymerase (Intron biotechnology, Korea), 0.1 µL (10 pmol) forward primer, 0.1 µL (10 pmol) reverse primer, and 13.3 µL sterilized distilled water. Thermal cycling parameters were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of amplification (94 °C for 40 s for DNA denaturation, 55–62 °C for 40 s annealing temperatures (see Table 1), extension at 72 °C for 1 min), and final extension at 72 °C for 5 min. DNA amplifications were carried out on a Techne, TC3000 thermal cycler (UK). Primers sequences, expected amplicon size, and annealing temperature are listed in Table 1.

Following amplification, PCR products were electrophoresed at 90 V in ethidium bromide-stained 1% agarose gels and DNA bands were visualized using long wavelength UV illumination and photographed using a gel documentation system (UV products, Ltd, Cambridge, UK). The level of expression of different bands was analyzed by an ImageJ gel analysis program (Abramoff et al. 2004). This relies on comparing the density of each target gene band of hypoxic treatment with the corresponding control normoxic band relative to positive control *β-actin* band. To check for the right amplification of the desired fragments, PCR products were purified (Jena Bioscience GmbH Kit, Germany) and sequenced (Macro GeN Company, South Korea).

Detection of DNA damage by comet assay

The comet assay was performed according to the protocol recommended by Singh et al. (1988) and Tebbs et al. (1999). Briefly, 1 g of liver or muscle samples was minced, transferred to 1 mL ice-cold PBS, and homogenized in ice. A 100 µL of cell suspension was mixed with 600 µL of low-melting agarose (0.8% in PBS) and 100 µL of this mixture was spread on agarose precoated slides. The coated slides were immersed in lysis buffer (0.045 M tris borate EDTA, TBE, pH 8.4, containing 2.5% sodium dodecyl sulfate, SDS) for 15 min. The slides were placed in electrophoresis chamber containing only TBE buffer. Following electrophoresis (2 V cm^{-1}, 2 min, 100 mA), gel staining was performed with Et Br (20 µg mL^{-1}) at 4 °C. The DNA fragment migration patterns of 100 cells, for each tested hypoxia level as well as normoxia control, were evaluated with a fluorescence microscope with excitation filter 420–490 nM using a Komet 5 image analysis software (Kinetic Imaging Ltd., Liverpoo1, UK) linked to a CCD

Table 1. List of all primers used, together with PCR amplification conditions.

Target	Acc. No. (GenBank)	Sequence (5′–3′) (S: Sense, A: Anti-sense)	Expected PCR product size	Source	Ta (Annealing temperature °C)
PFK	DQ066870.1	S: TCGACATTCGGGACCTAGAG A: CTTCCTGCAGTCGAACACG	170 bp	This study	55
PK	DQ066876.1	S: CCGTAAGGCTGCAGACGTGCA A: ATCTGCGCACGCCCTCATGG	104 bp	This study	57
LDH	EU313200.1	S: GGCACTAACCTCGACTCTGC A: CCTTCCAGTTCTCTGGGTCA	202 bp	This study	62
β-actin	AA566386q	S: TGGCATCACACCTTCTATAACGA A: TGGCAGGAGTGTTGAAGGTCT	139 bp	Choi (2004)	58

camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring:

(1) Tail Length: the distance of DNA migration from the body of the nuclear core, used to evaluate the extent of DNA damage.
(2) Tail Moment: the product of the tail length and the fraction of total DNA in the tail. It incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail).
(3) Tail % DNA = 100 − Head % DNA.

Statistical analysis

Graph Pad Prism 5.0 software (Graph Pad Software, Inc., San Diego, CA, USA) was used for the statistical analyses in this study. The statistical evaluation of all data was done using one-way analysis of variance to check the effect of hypoxia. The significance of difference owing to this effect was evaluated using multiple comparisons Dunnett's test (compare all vs. controls). p values ≤0.05 were regarded as statistically significant.

Results

Enzymatic activity

Maximal activities of the selected glycolysis enzymes in liver and muscle tissues of the Nile tilapia after short- and long-term exposure to hypoxia or normoxia are presented in Table 2 and Figures 1, (for liver) and 2 (for muscle).

Short-term exposure to hypoxia caused decrease in the activities of PFK, PK, and LDH in both liver and white muscle except for liver LDH. In the liver, the activity of PFK and PK showed significant decrease in fish cultivated at 2, 1, and 0.5 mg O_2 L^{-1} at $p \leq 0.05$. The activity of LDH, on the other hand, showed significant increase in all tested hypoxic groups except at the highest oxygen concentration 2 mg O_2 L^{-1} which revealed no significant change. Regarding to white muscle, PFK, PK, and LDH activity was significantly decreased at 2, 1, and 0.5 mg O_2 L^{-1} at $p \leq 0.05$.

Long-term exposure to hypoxia caused increase in the activities of PFK, PK, and LDH in both liver and white muscle. In liver, the activity of both of PFK and PK showed significant increase in all tested hypoxic groups, except at the lowest oxygen concentration 0.5 mg O_2 L^{-1} which showed no significant change. In muscle, a significant increase in PFK and LDH activity was observed at 2, 1, and 0.5 mg O_2 L^{-1}, while, PK activity showed no significant change in 2, 1, and 0.5 mg O_2 L^{-1}.

Gene expression

Semi-quantitative RT-PCR method was used to detect the expression of *PFK*, *PK,* and *LDH* of the Nile tilapia that reflects the changes in transcription levels (mRNA abundance) of these genes in both liver and white muscle after exposure to both short- and long-term hypoxia in comparison with the normoxic group. When the viability of the purified RNA samples was analyzed via the amplification of *β-actin*, all samples from the control as well as the hypoxic fish presented detectable quantities of *β-actin* mRNA

Table 2. Effect of exposure to hypoxia (2, 1, and 0.5 mg O_2 L^{-1}) on PFK, PK, and LDH activities (μmol min^{-1} g^{-1} wet weight tissue) in liver and white muscle of Nile tilapia for 1 day and 30 days.

Control		One day			Thirty days		
mg O_2 L^{-1}	8	2	1	0.5	2	1	0.5
Liver							
PFK	1.23 ± 0.13	0.95 ± 0.20*	0.93 ± 0.21*	0.85 ± 0.057*	2.06 ± 0.45*	1.96 ± 0.44*	1.35 ± 0.43
PK	3.68 ± 1.17	2.35 ± 0.38*	2.04 ± 0.40*	3.98 ± 1.53	7.22 ± 1.17*	5.92 ± 1.53*	3.98 ± 1.53
LDH	8.20 ± 0.46	8.23 ± 1.44	9.81 ± 1.46*	11.26 ± 1.95*	14.75 ± 2.54*	20.88 ± 1.79*	22.64 ± 3.94*
Muscle							
PFK	3.12 ± 0.14	2.43 ± 0.17*	2.14 ± 0.61*	1.66 ± 0.96*	6.96 ± 1.24*	6.82 ± 0.78*	5.02 ± 1.12*
PK	48.95 ± 6.93	35.37 ± 7.79*	27.89 ± 2.89*	25.08 ± 3.93*	57.53 ± 11.22	55.52 ± 11.12	53.11 ± 4.03
LDH	638.91 ± 102.5	165.15 ± 45.5*	144.74 ± 45.7*	82.72 ± 31.87*	1521 ± 175.0*	1560 ± 128.3*	1144 ± 76.78*

Notes: Each reading represents Mean ± SD of 6 fish.
*indicates the difference in hypoxia groups against control group ($p \leq 0.05$).

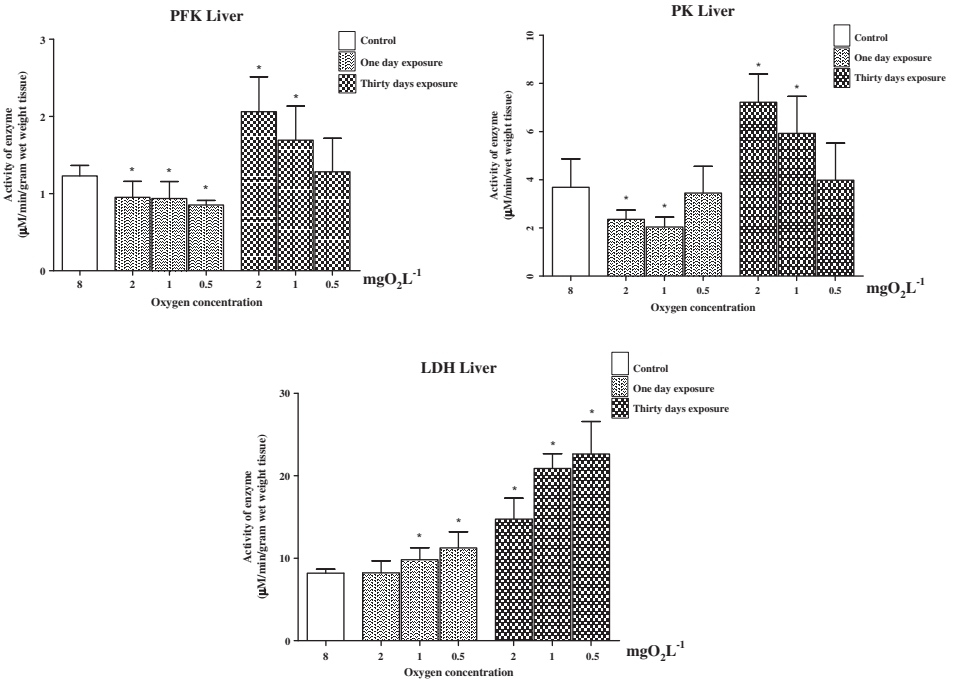

Figure 1. Effect of hypoxia (2, 1, and 0.5 mg O_2 L^{-1}) exposure on the activity of PFK, PK, and LDH (µmol min^{-1} g^{-1} wet weight tissue) in liver of *Oreochromis niloticus* for 1 day and 30 days.
Note: Each reading represents Mean ± SD of 6 fish and asterisks indicate significant change in hypoxia groups against control group (*$p \leq 0.05$).

(139 bp fragment) showing an acceptable integrity to amplification as well as a successful first strand cDNA preparation. Furthermore, no amplification product could be detected from any of the negative control specimens, which demonstrated that any contaminating DNA did not amplify using the selected pairs of primers.

Sequence analysis of each of the amplified PCR products of *PFK*, *PK,* and *LDH* confirmed the right amplifications of the desired fragments. Amplicons sequences revealed 100% identity to the published Nile tilapia gene sequences (data not shown).

Changes in transcription levels of Nile tilapia *PFK*, *PK,* and *LDH* genes in liver and white muscle after exposure to both short- and long-term hypoxia in comparison to the normoxic group and to the housekeeping gene, *β-actin*, are presented in Figures 3 and 4, respectively. In the liver of Nile tilapia following short-term hypoxic exposure, mRNA expression of *PFK* was decreased by 58, 48, and 34% at 2, 1, and 0.5 mg O_2 L^{-1}, respectively. Meanwhile, the transcript level of *PK* was remarkably upregulated by 112% and 87% at 2 and 1 mg O_2 L^{-1}, respectively, but it was similar to that characteristic of normoxia at 0.5 mg O_2 L^{-1}. The transcription level of *LDH* was however increased by 6, 11, and 18% at 2, 1, and 0.5 mg O_2 L^{-1}, respectively. In muscle after short-term hypoxic exposure, the mRNA levels of the *PFK* and *PK* were decreased. The mRNA level recorded 17, 54, 67% and 21, 17, 21% decrement at 2, 1, and 0.5 mg O_2 L^{-1}, for *PFK* and *PK*, respectively. On the other hand, the mRNA level of *LDH* recorded a slight increase by 1, 11, and 5% at 2, 1, and 0.5 mg O_2 L^{-1}, respectively.

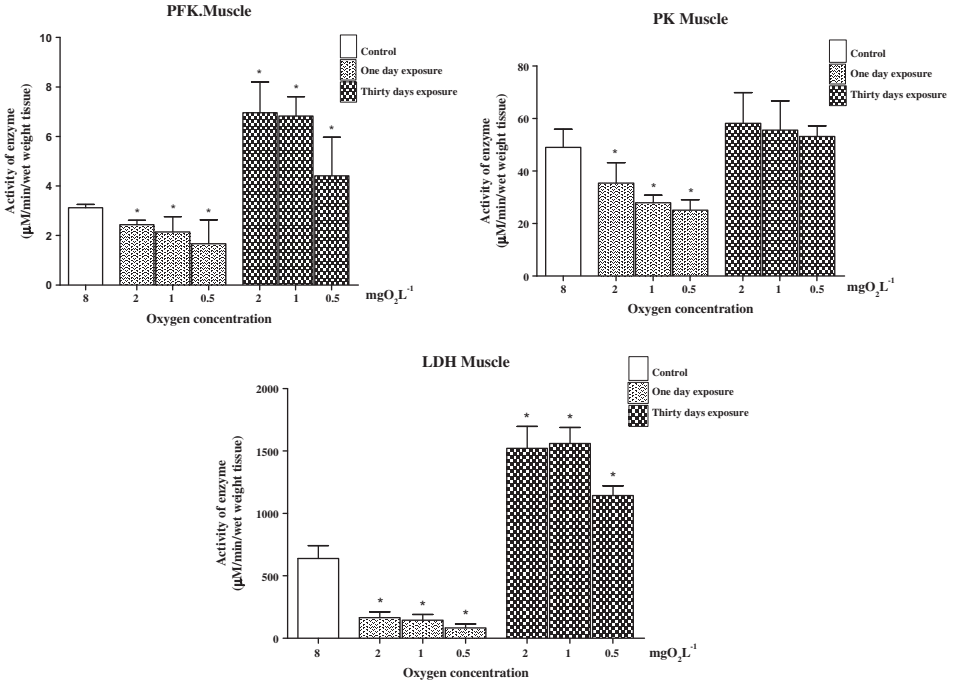

Figure 2. Effect of hypoxia (2, 1, and 0.5 mg O_2 L^{-1}) exposure on the activity of PFK, PK, and LDH (µmol min^{-1} g^{-1} wet weight tissue) in white muscle of *Oreochromis niloticus* for 1 day and 30 days.
Note: Each reading represents Mean ± SD of 6 fish and asterisks indicate significant change in hypoxia groups against control group (*$p \leq 0.05$).

In liver, following long-term hypoxic exposure, *PFK* expression was similar to normoxic level at 2 mg O_2 L^{-1} or slightly increased by 5 and 7% at 1 and 0.5 mg O_2 L^{-1}, respectively. While, the transcription level of *PK* was noticeably increased at all the tested oxygen levels as it recorded 119, 183, and 50% at 2, 1, and 0.5 mg O_2 L^{-1}, respectively. Meanwhile, *LDH* transcription level was increased by 29, 37, and 43% at 2, 1, and 0.5 mg O_2 L^{-1}, respectively. In muscle, while, no change in mRNA level of *PFK* has been observed in fish at all the tested oxygen concentrations, *PK* mRNA level was slightly increased by 10% at 2 mg O_2 L^{-1}, 20% at 1 mg O_2 L^{-1}, and 13% at 0.5 mg O_2 L^{-1}. The mRNA level of *LDH* was also slightly increased by 18, 8, and 14% at 2, 1, and 0.5 mg O_2 L^{-1}, respectively, following long-term hypoxic exposure.

The relationship between enzyme activity and expression

Relating PFK, PK, and LDH enzyme activity and expression resulted in three obvious patterns (Figure 5). The first is characterized by the existence of an excellent correlation between tested glycolytic enzyme activity and mRNA expression. In this case, both activity and mRNA levels coordinately go up (e.g. liver LDH after both short- and long-term or liver and muscle PK following long-term hypoxia exposure) or down (e.g. liver and muscle PFK or muscle PK after short-term hypoxia exposure). The second pattern, when enzyme activity is higher than the corresponding gene expression, as it is

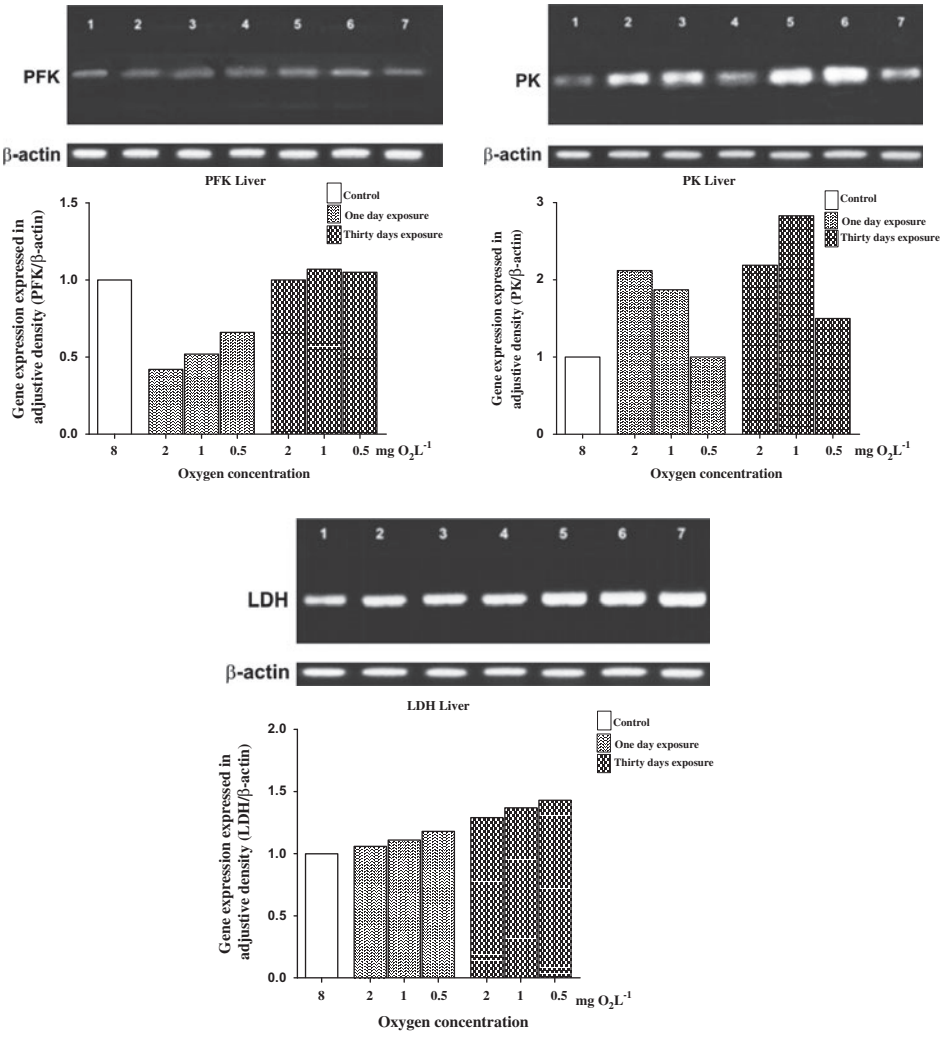

Figure 3. Effect of hypoxia (2, 1, and 0.5 mg O_2 L^{-1}) exposure on gene expression of PFK, PK, and LDH in liver of *Oreochromis niloticus* for 1 day and 30 days.

the case for liver and muscle PFK and muscle LDH following long-term exposure. The third pattern, when increases in mRNA levels were recorded with no equivalent increase in enzyme activity. This applies to liver PK and muscle LDH after short-term exposure.

Comet assay

To assess DNA damage due to hypoxia, a comet assay was performed. The results showed that hypoxic exposure induced an increase in DNA damage that was indicated by an increase in tail length, tail DNA%, and tail moment in both liver and white muscle following both short- and long-term exposure at all the tested oxygen concentrations. DNA damage was noticeably increased in a concentration dependent manner. A

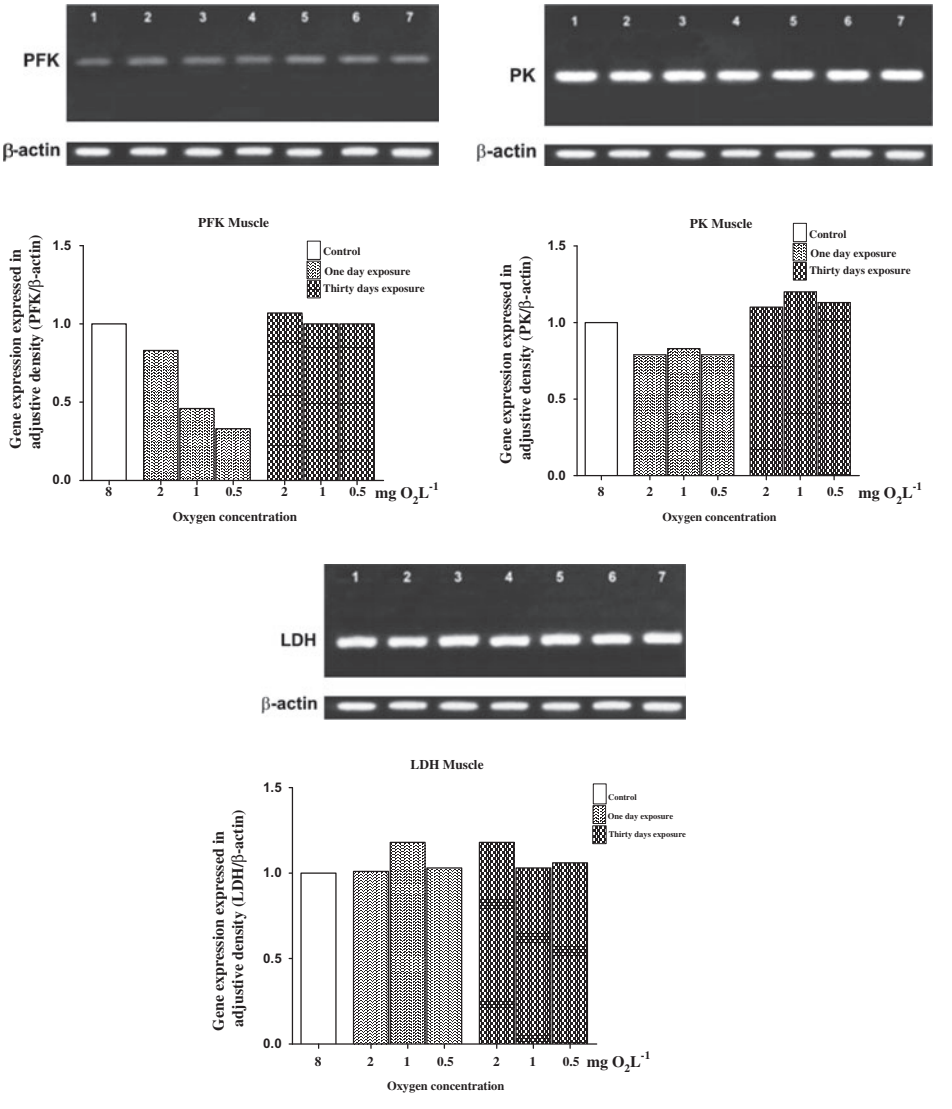

Figure 4. Effect of hypoxia (2, 1, and 0.5 mg O_2 L^{-1}) exposure on gene expression of PK, PFK, and LDH in muscle of *Oreochromis niloticus* for 1 day and 30 days.

hypoxia effect was more pronounced in short-term exposure than in long-term. The tail moment was chosen to present the results because it facilitates comparison among the tested groups. The tail moment was increased by 14.70, 15.59, and 19.23 μm at 2, 1, and 0.5 mg O_2 L^{-1}, respectively, in the liver following 1 day of hypoxic exposure while it was increased by 10.82, 12.11, and 14.35 μm at 2, 1, and 0.5 mg O_2 L^{-1}, respectively, following 30 days of exposure compared to the normoxic group (2.89 μm) (Figure 6 and Table 3). Likewise, in muscle, the tail moment was increased by 12.32, 14.26, and 17.67 μm at 2, 1, and 0.5 mg O_2 L^{-1}, respectively, following 1 day while it was increased by 6.25, 8.55, and 12.78 μm at 2, 1, and 0.5 mg O_2 L^{-1}, respectively,

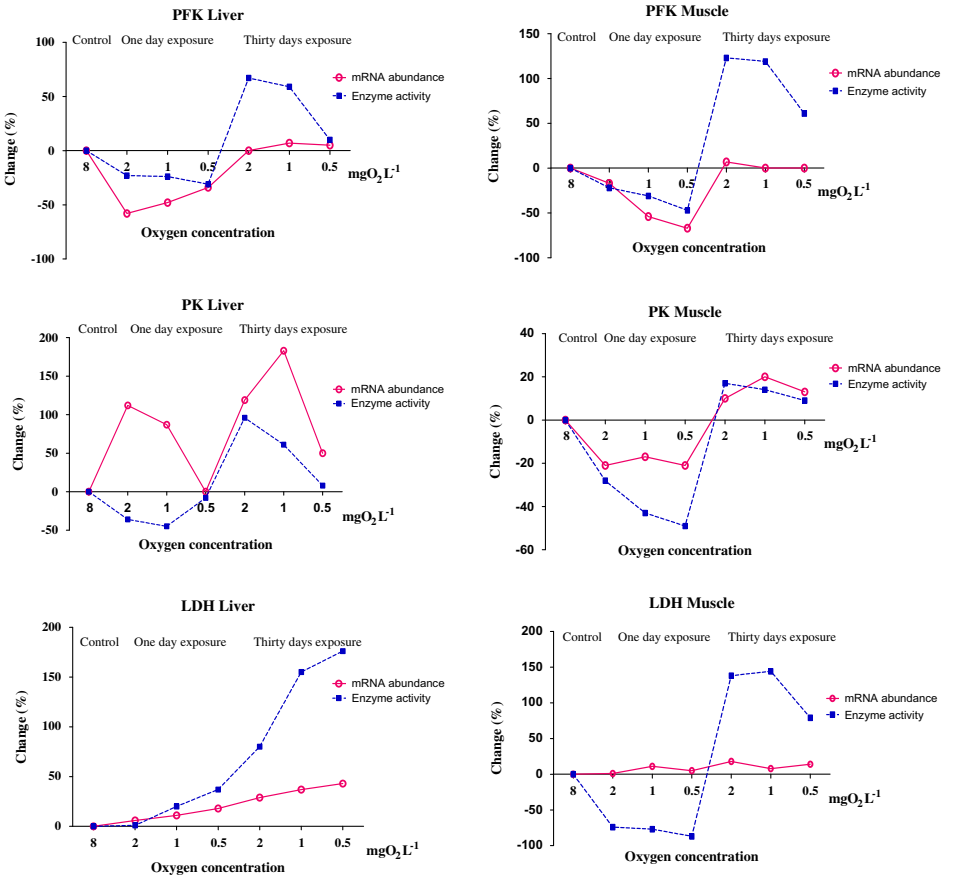

Figure 5. (Colour online) Correlation between mRNA abundance and enzyme activity in PFK, PK, and LDH liver and muscle over time of exposure to hypoxia (2, 1, and 0.5 mg O_2 L^{-1}) in *Oreochromis niloticus*.

following 30 days of exposure compared to the normoxic group (2.22 µm) (Figure 6 and Table 3).

Discussion

The metabolism of fishes is dependent on the availability of ambient oxygen. In hypoxic or anoxic states, the metabolic depression applies well to fish, where aerobic metabolism of fish decreases and the fish rely upon anaerobic glycolysis for energy production (Virani & Rees 2000). The role of the glycolytic pathway in these states is therefore critical (Lushchak et al. 1998). In the present study, the maximal activities of the glycolytic PFK, PK, and LDH enzymes were taken as representative for energy production in liver and white muscle of Nile tilapia subjected to both short- and long-term hypoxia. The results indicated that both exposures can differently affect the enzymes of carbohydrate metabolism in the fish. In liver, following short-term hypoxic exposure, the specific activities of PFK and PK decreased, while LDH activity was elevated at all oxygen concentrations compared to normoxia. In accordance,

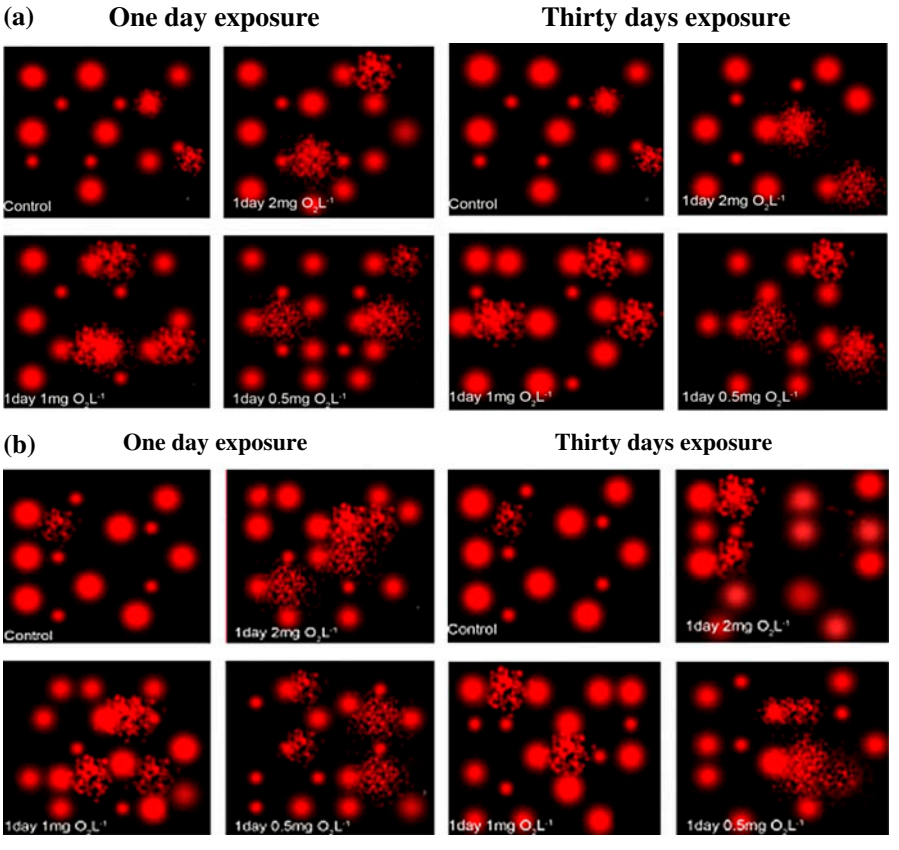

Figure 6. (Colour online) Photomicrographs representation of DNA damage, using comet assay, in liver (a) and white muscle (b) of *Oreochromis niloticus* after 1 day and 30 days from exposure to hypoxia in control, 2, 1, and 0.5 mg O_2 L^{-1}. In control, the majority of cells have normal nuclei without tails, revealing scarce detectable DNA damage. Cells from hypoxic groups displayed comets with various tail lengths and percentages demonstrating DNA damage.

similar reduction in PFK and PK enzymes activity was noticed in the liver of the teleost fish sea scorpion, *Scorpaena porcus* (Lushchak et al. 1998). This may be consistent with a primary function of this organ in glucose export during hypoxia in order to provide fermentative fuel to other vital organs. In agreement with this notion, it has been suggested that during short-term hypoxia, catecholamines regulate glucose availability in rainbow trout by inhibiting PK in the liver that suggests an activation of gluconeogenesis and an inhibition of glycolysis (Wright et al. 1989). On the other hand, the elevation in liver LDH activity is in accordance with Kraemer and Schulte (2004) who reported a significant increase in liver LDH activity of the teleost killifish after 3 days of hypoxic exposure. In contrast, no changes in liver LDH activity of the common estuarine fish *Leiostomus xanthurus* was shown after exposure to different levels of hypoxia for 12 h (Cooper et al. 2002), and a slight decrease in LDH activity in Nile tilapia exposed for 10 h to severe hypoxic exposure (Ishibashi et al. 2002). The current liver LDH elevation may indicate that although this enzyme plays a critical role in anaerobic metabolism in muscle, it is also involved in the process of gluconeogenesis by catalyzing the conversion of lactate into pyruvate in liver, which

Table 3. Comet assay parameters obtained by image analysis in liver and white muscle of Nile tilapia after 1 and 30 days of hypoxic exposure.

Groups	Tailed (%)	Untailed (%)	Tails length (μm)	Tail DNA (%)	Tail moment
Liver					
Control	4	96	1.67	1.73	2.89
1 day 2 mg	11	89	3.91	3.76	14.70
1 day 1 mg	13	87	4.05	3.85	15.59
1 day 0.5 mg	15	85	4.82	3.99	19.23
30 days 2 mg	8	92	3.21	3.37	10.82
30 days 1 mg	10	90	3.52	3.44	12.11
30 days 0.5 mg	11	89	3.91	3.67	14.35
Muscle					
Control	4	96	1.54	1.44	2.22
1 day 2 mg	8.6	92.94	3.61	3.95	12.32
1 day 1 mg	9.4	91.96	3.58	3.44	14.26
1 day 0.5 mg	11.5	89.95	4.52	3.91	17.67
30 days 2 mg	4.9	96.91	2.56	2.44	6.25
30 days 1 mg	6.8	94.92	3.12	2.74	8.55
30 days 0.5 mg	5.11	95.89	3.98	3.21	12.78

can then be used for glucose production. This may be consistent with the primary function of liver in glucose export during hypoxia for metabolism by extra-hepatic tissues such as heart and brain, which are important in the maintenance of an organism's homeostasis (Panepucci et al. 2001; Martínez et al. 2011).

In muscle, following short-term hypoxic exposure, all the tested glycolytic enzyme activities were decreased in fish cultivated at all hypoxia exposures. Similarly, muscle LDH and PK activities were decreased in the two Amazonian cichlids *Symphysodon aequifasciatus* and *Astronotus crassipinis* exposed to hypoxia (Chippari-Gomes et al. 2005). This reduction in muscle glycolytic enzyme activity may be due to reduction in fish activity and locomotion during hypoxia (Wannamaker & Rice 2000; Martinez et al. 2006). Some other studies on fish muscles have showed either increased activity in PFK and LDH (Lushchak et al. 1998) or normal activity in PK and LDH (Cooper et al. 2002; Chippari-Gomes et al. 2005). Following long-term hypoxic exposure, the maximal activities of all of the examined enzymes were elevated in both liver and muscle at all tested oxygen concentrations. In agreement, previous studies reported that exposure to long-term hypoxia increases liver LDH of the killifish during the first 28 days of exposure up to here to hypoxia (Greaney et al. 1980), PFK, PK, and LDH of tench *Tinca tinca* during the first 42 days of exposure to hypoxia (Johnston & Bernard 1982) and gulf killifish after 4 weeks from exposure to hypoxia (Martinez et al. 2006). Although PK and PFK enzymes are specific to catabolic reactions (glycolysis) under hypoxia, LDH enzyme is shared between catabolic and anabolic reactions (glycolysis and gluconeogenesis) and increased activities of all these enzymes imply a futile cycle whose net result is ATP turnover (Martinez et al. 2006). Increased activity of the examined enzymes in the white muscle following long-term hypoxia is in accordance with Johnston and Bernard (1982) and Martínez et al. (2011) who found that activity of PFK in skeletal muscle of tench, exposed to hypoxic water for 6 weeks and the African fish *Barbus neumayeri* exposed to hypoxia for 4 weeks, were higher than normoxic fish. This reflected an adaptation response for enhanced anaerobic glycolysis during exposure to hypoxic water. In contrast, some previous studies reported that exposure to long-term hypoxia either decreases PFK, PK, and LDH activities in skeletal muscle of gulf

killifish after 4 weeks from exposure to hypoxia (Martinez et al. 2006) or have no change on LDH activity of killifish during hypoxic for 28 days (Greaney et al. 1980).

Although previous studies revealed that a number of transcripts differentially regulated by hypoxia (e.g. Flight et al. 2011; Everett et al. 2012), there is a lack of information on hypoxia responses of fish at the molecular level, especially the analysis of the differential expression pattern of glycolytic *PFK* and *PK* genes. The results of the present study revealed that *PFK* mRNA levels were decreased in liver after short-term hypoxic exposure. No changes in the gene expression, however, were observed after long-term hypoxic exposure at all the tested oxygen concentrations. In contrast previous studies have shown an increase in *PFK* mRNA levels in blastulae embryonic (CAB) cell line of the goldfish, *Carassius auratus* after 0, 3, 6, 24, 48, and 72 h of hypoxia exposure (Zhong et al. 2009), in *G. mirabilis* after 48 h of exposure to hypoxia (Gracey et al. 2011) and in human liver cell culture (Caco-2 cells) after 7–9 days exposure of hypoxia (1% O_2) (Carrière et al. 1998). The current analysis of *PK* and *LDH* mRNA expression revealed an upregulation of these genes in liver following both short- and long-term hypoxic exposure and the increase in *LDH* mRNA level was relative to the increase in both of the exposure time and the level of hypoxia. In parallel, mRNA levels for *LDH-A*, one of the predominant patterns of LDH isozymes, was elevated in liver of the teleost fish *G. mirabilis* in consequence with duration of hypoxia exposure (8, 24, 72, and 144 h) (Gracey et al. 2001) and over 124 h (Gracey et al. 2011). Such increase was attributed to maintaining a continuous rate of anaerobic glycolysis, since this is a regular response in animals exposed to deep and prolonged hypoxia. In addition, *PK* expression was proved to be induced by exposure of Hep3B cells to 1% O_2 or chemical hypoxia mimetics, such as cobalt chloride or desferrioxamine (Semenza et al. 1994). In contrast to our results, some other studies have shown a downregulation of *PK* mRNAs in liver cell culture after exposure to hypoxia (1% O_2) (Carrière et al. 1998; Rissanen et al. 2006). This indicated that oxygen has a direct regulatory effect on metabolism of hepatocyte culture. On the other hand, no change in the expression of hypoxia-responsive genes (e.g. *LDH-A* or *-B*) was noticed in liver of the hypoxia-tolerant killifish after exposure to 15 h of severe hypoxia (Richards et al. 2008).

The present results revealed that muscle *PFK* and *PK* mRNA levels were decreased in Nile tilapia after short-term hypoxic exposure in comparison to normoxic group. In contrast, some other studies have detected a slight upregulation of *PFK* and *PK* expression in both cultured skeletal muscle cells of mouse under hypoxic (1% O_2) conditions for 4, 6, and 12 h (Dehne et al. 2007) and skeletal muscle of *G. mirabilis* after exposure to environmental hypoxia at 0.8 mg O_2 L^{-1} for 124 h (Gracey et al. 2011), respectively. The transcriptional activation of glycolytic genes during hypoxia was attributed to the binding of HIF-1α transcription factor (Semenza et al. 1996), which accumulates during hypoxia (Jiang et al. 1996). In addition, a number of other transcription factor families, e.g. NF-kB, AP-1, p53, and Myc, are also known to affect hypoxic gene expression. Several of these factors affect transcription directly and others modulate the effects of the HIF transcription factors (Kenneth & Rocha 2008). More recently groups of microRNAs (miRNAs) have been demonstrated to affect hypoxic gene expression (Kulshreshtha et al. 2007; Rocha 2007; Kenneth & Rocha 2008). While providing no information regarding the expression of *PFK* in muscle following long-term hypoxic exposure, our results revealed that muscle *PFK* mRNA did not change while muscle *PK* mRNA levels were increased at all tested oxygen concentrations after long-term hypoxic exposure. In agreement with this result, an increase of *PK* expression was detected in cultured mammalian myoblast cells after exposure to long-term hypoxia

(Webster 1987). In contrast, Le Moullac et al. (2007) indicated that *PK* mRNA levels were decreased in muscle of *Crassostrea gigas* (Mollusca) during prolonged hypoxia for 20 days. In the present study, we have found that muscle *LDH* mRNA levels were somewhat similar to that of the normoxic group following both short- and long-term exposures. In accordance, no changes in the expression of *LDH-A* were detected in muscle of either the hypoxia-tolerant killifish after exposure to 15 h of severe hypoxia (Richards et al. 2008) or adults Oscar fish, *A. crassipinis* after hypoxia or anoxia (Almeida-Val et al. 2011). This suggests that skeletal muscles have developed other mechanisms of tolerance and do not depend exclusively on gene regulation to survive hypoxic episodes. In an extensive review, Nikinmaa (2002) suggested that the possible oxygen tensions leading to the stabilization of HIF-1 protein have been insufficiently studied and that they may vary between species and cell types. If this is true, the stabilization or decrease in *LDH-A* expression could be explained as a result of this process (Almeida-Val et al. 2011). Furthermore, muscle-type tissues have an already high glycolytic capacity that may be more than adequate to match the reduced metabolic demands of hypoxia, which was suggested by Gracey et al. (2001) to explain the difference in the tissue expression of glycolytic enzymes.

Since there is no simple relation that could explain the association between enzyme activity and expression, it is always challenging to establish definitively the molecular mechanisms responsible for particular patterns of metabolic enzyme levels seen between animals or physiological states. With respect to fish, little is known about how glycolytic enzyme activity relates to their mRNA expression. The present results suggest three relationship patterns between enzyme activity and mRNA expression during hypoxia. The first pattern, where both activity and mRNA level, in parallel, go up (e.g. liver LDH after both short-term and long-term hypoxia or liver and muscle PK following long-term hypoxia exposure) or down (e.g. liver and muscle PFK or muscle PK after short-term hypoxia exposure). Similar association was described for liver LDH-B mRNA and the protein it codes in gulf killifish exposed to ~13.5% air saturation for 4–48 h (Crawford et al. 1999; Everett et al. 2012) and considered to be generally true for many other metabolic genes (Rees et al. 2011). This good correlation may be best explained by transcriptional regulation where mRNA is a good predictor of specific enzyme activity (Haas et al. 2005) for a number of reasons including mRNA stability that is similar to the *in vivo* protein half-life (Laberge et al. 2009). The second pattern implies poor association, where increases in mRNA levels were recorded with no equivalent increase in enzyme activities, as in the case of liver PK and muscle LDH after short-term exposure. This may be explained by lower translation efficiency of the corresponding gene transcripts, shorter protein half-life or faster organelle turnover. Iwata et al. (2000) and Kong et al. (2000) noticed a large increase in the levels of gene expression that were not congruent with changes in activity of ornithine–urea cycle enzymes in crowded gulf toad fish. Greenbaum et al. (2003) suggested that such poor correlations are due to complicated post-transcriptional and/or post-translational mechanisms as well as differential *in vivo* protein half-lives. Furthermore, it has been previously suggested that glycolytic enzymes may be regulated at all stages of protein synthesis from transcriptional regulation to post-translational modifications (Moyes & LeMoine 2005). The third pattern is illustrated when enzyme activity is higher than the corresponding gene expression, as it is the case for liver and muscle PFK following long-term exposure. Such a relationship has been shown previously by Yang and Somero (1996) who found that LDH activity increased with body mass while *LDH* mRNA levels did not parallel this change in white muscle of barred sand bass

Paralabrax nebulifer. The same findings were reported by Burness et al. (1999) studying citrate synthase (CS) activity and expression in rainbow trout following differing in size and Dalziel et al. (2005) relating the differences in CS activities of homologous muscles of tuna and billfish to transcriptional regulation. This was attributed to the greater half-life of the protein in the hypoxic animals, such that less mRNA was needed to maintain the enzyme activity or to greater translational efficiency in hypoxic fish, such that more protein was produced per unit transcript. In this specific case, the tissue could thus achieve an increase in activity without a remarkable change in gene transcription (Moyes & LeMoine 2005).

The results of the comet assay revealed that hypoxic exposure induced DNA damage in both liver and white muscle following both short- and long-term hypoxic exposure at all the tested oxygen concentrations compared to the normoxic group. The increase in DNA damage was directly proportional to the increasing in hypoxia concentration and acute hypoxia induced greater damage. Poon et al. (2007) investigating the response of the intact liver of common carp to hypoxia, 42 days at 0.5 mg O_2 L^{-1}, realized an extensive DNA damage in liver cells, especially during the first week of exposure. The authors attributed the results to cellular mechanisms that seemed to be directed towards preventing apoptosis in the face of DNA damage and promoting DNA repair. Likewise, Ahmed et al. (2011) exposing *Oreochromis mossambicus* liver, gills, and blood tissues to the genotoxic sodium arsenite for 48, 96, and 192 h, observed a decline in DNA damage in all the tissues after 192 h of exposure. Our findings also indicated that liver was more vulnerable to hypoxic effect than muscle where the latter appears to be relatively hypoxia tolerant compared to the liver as evidenced by the reduction in percentage of DNA tail and tail moment. Comparable results were reported by Al-Salahy (2006) who found that hypoxia significantly increased DNA fragmentation in liver but not in white muscle of the catfish *Clarias gariepinus* exposed to hypoxic water (from 5.1 to 0.6 mg O_2 L^{-1}).

In conclusion, hypoxia caused overall biochemical and molecular changes in Nile tilapia, which reflected on both enzyme activity and expression. The results obtained verified that while limited variations were evidenced among the tissues examined and to a slightly higher extent among the enzymes examined, pronounced changes were recorded between short- and long-term exposures. It seems likely that Nile tilapia cope better with long-term hypoxic conditions, possibly as an evolved adaptive response. To better understand glycolytic enzyme regulation during hypoxia, mechanisms that underlie their control need to be further elucidated, including information on mRNA and protein stability. In addition, to explain the different patterns in gene expression relative to enzyme activity during hypoxia, future studies should examine possible transcription factors in the promoter regions of the glycolytic genes beside post-transcriptional control of these enzymes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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