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ORIGINAL PAPER

Effects of lead nitrate on the activity of metabolic enzymes during early developmental stages of the African catfish, *Clarias gariepinus* (Burchell, 1822)

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Abstract Glucose-6-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH) and pyruvate kinase (PK) are key metabolic enzymes. G6PDH has been used as a biomarker of pollution-induced carcinogenesis in fish. LDH has been used as marker of lesions in toxicology and clinical chemistry, and PK catalyses the conversion of phosphoenol pyruvate to pyruvate, with regeneration of ATP. The effect of different concentrations of lead nitrate on the activity of these enzymes in two different early ontogenetic stages (embryonic and free embryonic stage) of the African catfish *Clarias gariepinus* was investigated. Embryo homogenates were used for measurements of G6PDH, LDH and PK activity spectrophotometrically at 340 nm and 25°C. The ontogenetic variations of the three enzymes during early ontogeny, from the 30 h to the 168 h post-fertilisation stage (PFS) (beginning of exogenous feeding), were studied. There was a significant decrease in activities of all three enzymes from 30 h-PFS to 96 h-PFS, followed by a significant increase in G6PDH and LDH. PK showed insignificant fluctuations in activity. Different patterns of enzyme activities were recorded due to exposure to different lead nitrate concentrations (100 μg/l, 300 μg/l and 500 μg/l). In the pre-hatching stage (30 h-PFS) the activity of the three enzymes increased at exposure to 100 μg/l lead nitrate and then decreased with increasing dose. In the post-hatching stages (48 h-PFS–168 h-PFS) G6PDH activity increased and LDH activity decreased with increasing lead concentrations. Unlike G6PDH and LDH, the PK enzyme fluctuated during the post-hatching stages and did not reveal a specific trend of response (increase or decrease) with increasing lead concentrations. Therefore, the measurement of G6PDH and LDH activities, but not PK activity, could be useful biomarkers of intoxication to reveal the embryotoxic potential of lead nitrate in fish embryos. The post-hatching stages of the African catfish are more sensitive than the pre-hatching stage (30 h-PFS) is, probably due to

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the protective capacity provided by the hardened chorion. The interaction and the main effects of age and lead doses were found to be highly significant, referring to the great impact of lead on these enzyme systems with increasing early development.

Keywords African catfish · Biomarkers · Enzyme activity · G6PDH · LDH Lead · Ontogenetic variation · PK

Introduction

In spite of their natural occurrence in the aquatic ecosystem, heavy metals represent a major environmental problem of increasing concern (Gill et al. 1990; Hunaiti and Soud 2000), and their monitoring has received significant attention in the field (Adham et al. 1999; Rashed 2001; Adham 2002; Pandey et al. 2003; Barnhoorn and van Vuren 2004) and under laboratory conditions (Christensen 1975; Gupta and Sastry 1981; Gilli et al. 2000; Almeida et al. 2001, 2002; Elumalai et al. 2002; Strmac and Braunbeck 2002; Long et al. 2003; Das et al. 2004a, 2004b). Lead (Pb) seems to lack biological functions and is very toxic to aquatic organisms, even in small doses (Sastry and Gupta 1980; Stouthart et al. 1994; Ahmed 1996; Lashein 1996). There have been many studies on the impact of Pb on adult fishes (Stouthart et al. 1994; Ahmed 1996), but very few studies have investigated the effect of Pb on larval stages (Lashein 1996; Mekkawy and Lashein 2003).

Biomarkers using aquatic species are important for detecting stressor components such as the presence of pollutants and changes in environmental factors. Biochemical and physiological indicators, such as enzymes, could be used (as biomarkers) to identify possible environmental contamination before the health of aquatic organisms is seriously affected (Jiminez and Stegeman 1990; Barnhoorn and van Vuren 2004) and to develop water quality indices (Powers 1989; Gayet et al. 1993; Zollner 1993; Melancon 1995; Pickering and Pottinger 1995). Such a biochemical approach has been advocated to provide an early warning of potentially damaging changes in stressed fish (Casillas et al. 1983). In toxicological studies of acute exposure, changes in concentrations and activities of some enzymes may reflect cell damage in specific organs (Casillas et al. 1983; Heath 1996).

The enzymes glucose-6-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH) and pyruvate kinase (PK) are metabolic key factors (Ocampos et al. 1987; Gill et al. 1990; Coppes 1992; Pelletier et al. 1994; Gilli et al. 2000; Kurutas and Tuncer 2000; Leopold and Loscalzo 2000; Long et al. 2003). It has been traditionally thought that G6PDH is a typical 'housekeeping' enzyme that is regulated solely by the ratio of NADPH to NAD (Kletzien et al. 1994; Tian et al. 1999). G6PDH has long been recognised as an antioxidant enzyme (Nogae and Johnston 1990; Pandolfi et al. 1995; Salvemini et al. 1999) and as a biomarker of pollution-induced carcinogenesis in fish (Winzer et al. 2001). It has been postulated that heavy metals have different effects on the activity of G6PDH in different fish species (Strik et al. 1975; Barnhoorn 1996).

The cytoplasmic enzyme LDH is widely used as marker of organ or tissue lesions in toxicology and in clinical chemistry (Das et al. 2004a). It has been used for demonstrating tissue damage in fish (Das et al. 2004a). In most cases of tissue damage, whether due to disease or toxic compound, the activity of LDH was reported to be significantly affected (Singh and Sharma 1998). LDH is a source of the oxidised coenzyme during the period of transient anaerobiosis or a reduced form of such coenzyme during aerobiosis (Coppes 1992). Therefore, LDH has also been used to be indicative of hypoxic conditions in the organism (Das et al. 2004a). Different metals seem to influence the activity of LDH (Christensen et al. 1982; Gill et al. 1990). G6PDH and LDH play an important role during glycolysis, and they have direct effects on the development of fish (Shaklee et al. 1974).

PK controls and catalyses the conversion of phosphoenol pyruvate (PEP) to pyruvate, while regenerating ATP (Gilli et al. 2000). Three forms of PK have been isolated in fish, and they show a variation in activity and occurrence between species. Pyruvate kinase is an enzyme that is

situated in the cytosol compartments in the cell and, therefore, is not as protected as the enzymes in the mitochondria are. Several authors (Randall and Anderson 1975; Ocampos et al. 1987; Isani et al. 1994) studied the inhibitory effect of divalent ions (Cu, Co, Be, Zn) on the activity of PK in fish. Metals such as copper, manganese and iron have been found to alter the activity of PK (Hansen et al. 1992; Barnhoorn 1996).

The altered activities of G6PDH, LDH and PK could be useful biomarkers of water pollution. If the organism is exposed to pollutants, these enzymes respond by activation or deactivation. The effect of heavy metals on enzyme activities has been described in fish (Jiminez and Stegeman 1990). Most of these studies addressed the impact of heavy metal exposure on the enzyme activities of juveniles (Almeida et al. 2001, 2002; Das et al. 2004a) and adult fishes (Jackim et al. 1970; Sastry and Gupta 1980; Gupta and Sastry 1981; Christensen et al. 1982; Gill et al. 1990; Bainy et al. 1996; Almeida et al. 2001, 2002; Barnhoorn and van Vuren 2004). However, only two articles (Christensen 1975; Das et al. 2004a) described the effect of chemicals on the enzyme activities of fish embryos. Although several researchers studied the effect of water pollution on the physiology and biochemistry of fish, no detailed information is available regarding the effects of lead on the activity of G6PDH, LDH and PK during early developmental stages of fish. Early developmental stages are generally considered to represent the most sensitive phases in the life cycle, being particularly sensitive to all kinds of low-level environmental influences (Hallare et al. 2005). Sensitivity of embryonic stages to chemical-induced adverse effects is based on the occurrence of developmental events, e.g. organogenesis (Honkanen 2004). Toxic stress may influence growth and developmental speed and may cause an increase in malformation and mortality. In addition to these parameters, other biomarkers can also be investigated in fish embryos (Luckenbach et al. 2001).

The African catfish *Clarias gariepinus* is commercially important and the most common fish species in Africa. This species was introduced into Europe, Asia and Latin America in the 1970s and has become one of the most important farmed catfish in the world (Verreth et al. 1993; Richter et al. 1995). Moreover, the African catfish has been used in fundamental research and toxicological studies (Nguyen and Janssen 2002), since it has a well-documented biology, short period of development, transparent eggs, and is easy to reproduce all year round (Degroot 1987; Volckaert et al. 1994). Therefore, this species was chosen as an excellent model for us to study embryotoxic effects induced by lead.

The aim of the present work was to study the ontogenetic variations in the activities of the three selected enzymes G6PDH, LDH and PK during early development (endogenous feeding) of the African catfish *Clarias gariepinus* and to use these early developmental stages as a model system to evaluate the effect of lead nitrate on the activity of these enzymes.

Materials and methods

Selection of adult specimens

Artificial reproduction of the African catfish, *C. gariepinus* took place in the hatchery of the Department of Fish Culture and Fisheries, Wageningen Agricultural University, The Netherlands. The criteria for the selection of spawners were those described by De Graaf and Janssen (1996). Females (1,000–1,500 g body weight) and males (1,000–3,000 g body weight) were fed on a commercial pellet diet (3% of body weight per day) and kept together in 800 l rectangular tanks containing tap water (conductivity 2,000 μs/cm; pH ≈ 7.5; oxygen 90–95% saturation; temperature 25°C; photoperiod 12:12 light:dark). The tanks were part of a recirculation system.

Induced ovulation and gametes collection

Male spawners were anaesthetised and subsequently killed with 400 mg/l MS-222 (tricaine methane sulphonate, Crescent Research Chemicals, Phoenix, Arizona, USA) buffered with 800 mg/l sodium bicarbonate. Afterwards, their testes were removed and the spermatozoa were pressed into a sterile dry Petri dish and immediately used for artificial reproduction. For the

collection of eggs, ovulation was artificially induced by intra-muscular injection of 4 mg/kg body weight carp pituitary suspension (Cps, Stoller Fisheries, Spirit Lake, Iowa, USA.). Between 11 h and 12 h after the injection, the fish were stripped and the eggs were collected in plastic containers; dry fertilisation was performed.

Exposure of embryos to lead nitrate

Fertilised eggs were divided into four groups: one control group (tap water; ca. 700 µs/cm and pH ≈8) and three groups exposed to 100 µg/l, 300 µg/l and 500 µg/l of lead nitrate. Exposure took place in 12 aquaria (20 cm × 20 cm × 5 cm), representing three replicates for each group. Pure lead nitrate [lead standard solution 1,000 mg/l, VWR (MERCK), Germany] was used for the exposure. Temperature was kept at 22–24°C.

Sampling

Exposure was started 6 h after fertilisation, and four embryos (for each enzyme) were collected at each sampling point, at the 30 h post-fertilisation stage (30 h-PFS), 48 h post-fertilisation stage (48 h-PFS), 96 h post-fertilisation stage (96 h-PFS), 144 h post-fertilisation stage (144 h-PFS), and 168 h post-fertilisation stage (168 h-PFS). Thus, intervals represented 24 h, 42 h, 90 h, 138 h and 162 h of exposure time respectively. Thirty hours PFS was the only pre-hatching stage, since the hatching process started 40 h after fertilisation.

Measurements of enzyme activities

Owing to the small size of the embryos, whole-body homogenates were used for the measurement of the enzyme activities. Homogenisation of the whole animal is a reliable method that provides a reasonable index of total enzyme activity in individuals whose body mass is predominantly composed of muscle tissue (Berges and Ballantyne 1991; Lemos et al. 2003). This procedure makes interpretation of the results more equivocal than investigations on older life stages when specific organ preparations can be used (Segner and Verreth 1995).

The activities of the selected enzymes were measured according to a modified protocol based on the method of Hardewig et al. (2004). The embryos were pulverised under liquid nitrogen, and approximately 100 mg of ground tissue powder was added to 5 vol. of buffer (50 mM Tris, pH 7.4, 1 mM ethylene diamine tetra-acetic acid (EDTA) and 2 mM $MgCl_2$). Tissue was homogenised briefly with an Ultra-Turrax (temperature was maintained at 4°C during homogenisation). The homogenate was centrifuged for 15 min at 10,000× *g* and 4°C, and the supernatants were used for the enzyme activity assay. Activities were determined in the supernatant with a plate reader (spectra Fluor Plus, Tecan) at a wavelength of 340 nm and at 25°C. G6PD was determined in extraction buffer containing 100 mM Tris/HCl (pH 7.6), 0.5 mM NADP, 3 mM glucose-6-phosphate, 6 mM $MgCl_2$ and 5 mM maleinimide. The reaction mixture contained 50 mM imidazole, 0. 2 mM pyruvate-Na and 0. 15 mM NADH for LDH. For PK, 50 mM imidazole, 5 mM ADP, 100 mM KCl, 10 mM $MgCl_2$, 0.15 mM NADH, 0.1 mM fructose 1–6, phosphate, 5 mM phosphoenol pyruvate and 25 U/ml LDH was used as extraction buffer. The catalytic activity of the selected enzymes was calculated in units per gramme protein (Bergmeyer et al. 1983).

Statistical analysis

The basic statistics, means, standard errors and ranges were estimated. The patterns of variation due to developmental stages and lead doses and their interaction were studied by two-way analysis of variance using the SPSS package (SPSS 1998) at the 0.05 significance level. Levene's test of equality of error variance of the dependent variables was applied, with rejection of the null hypothesis for raw, log-transformed and SQRT-transformed data. So, the homogeneity of variance was assumed for raw data. The model considered was: intercept + age + treatment + age × treatment (adjusted R^2 was 0.966, 0.965 and 0.943 for G6PDH, LDH and PK, respectively). A further design (age + treatment + age × treatment) exhibited a similar significant pattern of variations for the main effect of age and treatment factors

and their interaction ($P < 0.0001$) (adjusted R^2 was 0.993, 989 and 0.973 for G6PDH, LDH and PK, respectively). The pattern of variations was also recorded by one-way analysis of variance, revealing significant difference within the development stages and treatments ($P < 0.0001$); no homogeneity of variance, even with log and SQRT transformations, was recorded. The Tukey-HSD test was considered for multiple comparisons. Moreover, the Dunnett-*T* test was applied, measuring the control against other treatments in each developmental stage.

Results

In order to study the effect of different concentrations of lead nitrate on the activities of G6PDH, LDH and PK, we first had to study the ontogenetic changes in the activities of these enzymes during normogenesis.

The normal ontogenetic variations in enzyme activities

The activities of G6PDH, LDH and PK showed variability during the early embryonic stages (30 h-PFS–168 h-PFS) under normal conditions (Fig. 1). The pattern of the ontogenetic variation exhibited a significant ($P < 0.05$) decrease in the activities of all three enzymes till 96 h-PFS. After 96 h-PFS, the activities of G6PDH and LDH increased towards the higher stages (144 h-PFS–168 h-PFS). This increase was insignificant in the case of G6PDH and significant ($P < 0.05$) in the case of LDH (Fig. 1). PK activity (after 96 h-PFS) showed significant ($P < 0.05$) increase till 144 h-PFS and then a significant ($P < 0.05$) decrease till 168 h-PFS (Fig. 1). Therefore, the activities of all three enzymes decreased from 30 h-PFS to 96 h-PFS, followed by an increase in the activities of G6PDH and LDH. PK showed significant fluctuations in its activity (Fig. 1).

The enzyme activities after exposure to lead nitrate in comparison with normogenesis

The patterns of the enzyme activities of the different early ontogenetic stages under the influence of three different lead nitrate doses are presented in Tables 1, 2 and 3. In the groups exposed to 100 μg/l lead the activities of G6PDH and LDH were significantly lower ($P < 0.05$) till 96 h-PFS, then the activities were insignificantly higher till the 168 h-PFS stage in the case of G6PDH and significantly higher ($P < 0.05$) in the case of LDH (Tables 1 and 2). In the groups exposed to 300 μg/l lead the activities of G6PDH

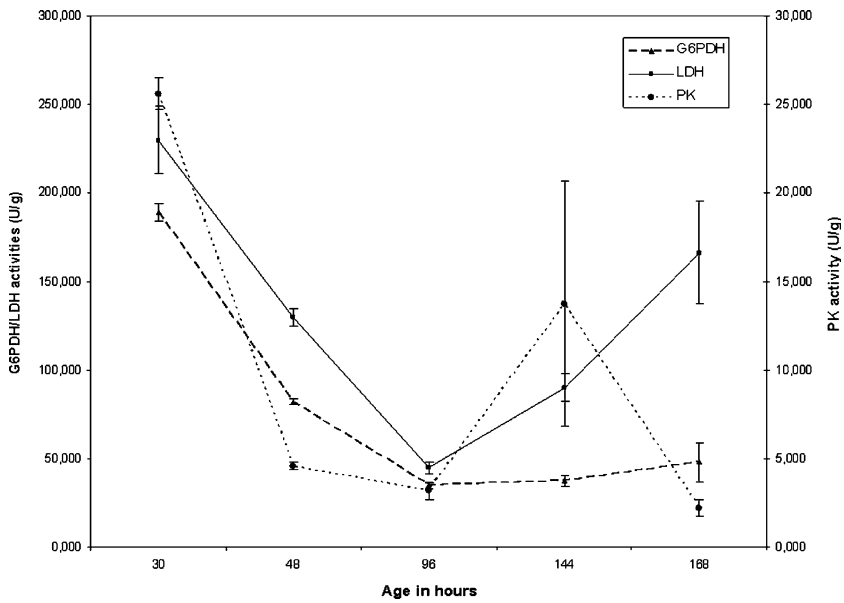

Fig. 1 Pattern of the activities of G6PDH, LDH and PK (mean ± SE) of the early developmental stages (during the period of endogenous feeding) of the African catfish *Clarias gariepinus* under controlled conditions

Table 1 Effect of different concentrations of lead nitrate on the activity of G6PDH (mean ± SE) during early developmental stages of the African catfish *Clarias gariepinus*. Values are in units per gramme protein

Embryonic stages*	Control	100 μg/l	300 μg/l	500 μg/l
30 h-PFS (pre-hatching)	189.2 ± 5.0 (158.1–195.0) a (A)	227.2 ± 6.8 (221.5–234.7) a (B)	149.6 ± 3.2 (145.4–152.0) a (C)	133.0 ± 14.4 (118.9–145.4) ab (C)
48 h-PFS**	81.8 ± 1.7 (79.3–82.6) b (A)	95.9 ± 11.5 (85.9–105.7) b (B)	104.1 ± 1.9 (102.4–105.7) b (BC)	109.9 ± 5.6 (102.4–115.7) a (C)
96 h-PFS	34.7 ± 1.9 (33.0–36.4) c (A)	55.4 ± 1.6 (52.8–56.2) c (AB)	67.8 ± 14.4 (59.5–89.2) c (B)	106.6 ± 23.3 (76.0–132.2) a (C)
144 h-PFS	37.2 ± 3.2 (33.0–39.7) cd (A)	52.9 ± 4.7 (46.2–56.2) c (AB)	69.4 ± 2.7 (66.1–72.7) c (B)	147.9 ± 18.2 (132.2–165.3) b (C)
168 h-PFS	47.9 ± 11.0 (33.0–56.2) d (A)	61.1 ± 9.5 (52.9–69.4) c (A)	104.1±7.9 (95.9–112.4) b (B)	162.8±1.7 (161.9–165.3) b (C)

* Embryonic stages showing similar lower case letters are insignificant within the treatment at 0.05 levels (vertical comparison). Treatments showing similar capital letters are insignificant within the embryonic stages at 0.05 levels (horizontal comparison)

** The hatching process started 40 h after fertilisation

Table 2 Effect of different concentrations of lead nitrate on the activity of LDH (mean ± SE) during early developmental stages of the African catfish *Clarias gariepinus*. Values are in units per gramme protein

Embryonic stages*	Control	100 μg/l	300 μg/l	500 μg/l
30 h-PFS (pre-hatching)	229.7 ± 19.2 (211.5–247.9) a (A)	295.0 ± 40.7 (234.7–323.9) a (B)	242.1 ± 7.3 (234.7–251.2) a (A)	244.6 ± 14.0 (228.1–257.8) a (A)
48 h-PFS**	129.7 ± 4.9 (125.6–135.5) b (A)	114.9 ± 14.6 (99.2–129.9) b (AB)	95.8 ± 2.7 (92.5–99.2) b (BC)	90.1 ± 11.9 (72.7–99.2) b (C)
96 h-PFS	44.6 ± 3.3 (42.9–49.6) c (A)	41.3± 1.9 (39.7–42.9) c (AB)	36.4 ± 2.7 (33.1–39.7) c (B)	35.5 ± 3.1 (33.1–39.1) c (B)
144 h-PFS	90.1 ± 7.8 (79.3–95.8) d (A)	65.3 ± 3.2 (62.8–69.4) c (B)	62.0 ± 3.2 (59.5–66.1) d (BC)	47.1 ± 12.8 (33.1–59.5) c (C)
168 h-PFS	166.1 ± 28.9 (132.2–191.7) e (A)	122.3 ± 9.7 (112.2–135.5) b (B)	109.1 ± 14.0 (95.9–128.9) b (BC)	81.8 ± 12.5 (72.7–99.2) b (C)

* Embryonic stages showing similar lower case letters are insignificant within the treatment at 0.05 levels (vertical comparison). Treatments showing similar capital letters are insignificant within the embryonic stages at 0.05 levels (horizontal comparison)

** The hatching process started 40 h after fertilisation

Table 3 Effect of different concentrations of lead nitrate on the activity of PK (mean ± SE) during early developmental stages of the African catfish *Clarias gariepinus*. Values are in units per gramme protein

Embryonic stages*	Control	100 μg/l	300 μg/l	500 μg/l
30 h-PFS (pre-hatching)	25.6 ± 0.9 (25.0–26.2) a (A)	27.6 ± 2.0 (26.2–29.1) a (A)	7.6 ± 1.3 (6.6–8.5) a (B)	3.9 ± 0.7 (3.5–4.4) ac (B)
48 h-PFS**	4.6 ± 0.2 (4.4–4.7) b (A)	8.5 ± 0.4 (8.2–8.8) b (B)	4.3 ± 0.2 (4.1–4.4) bc (A)	7.4 ± 0.2 (7.3–7.6) b (B)
96 h-PFS	3.2 ± 0.4 (2.8–3.5) b (A)	8.5 ± 0.4 (8.2–8.8) b (B)	7.2 ± 0.4 (7.0–7.6) a (B)	5.4 ± 0.4 (5.1–5.7) c (C)
144 h-PFS	13.7 ± 6.9 (8.8–18.6) ab (A)	3.0 ± 0.2 (2.8–3.2) c (A)	2.2 ± 0.4 (1.9–2.5) c (A)	2.8 ± 0.4 (2.5–3.2) a (A)
168 h-PFS	2.2 ± 0.4 (1.9–2.5) b (A)	2.8 ± 0.0 (2.8–2.8) c (A)	5.5 ± 0.7 (5.1–6.0) ab (B)	4.7 ± 0.4 (4.4–5.1) c (B)

* Embryonic stages showing similar lower case letters are insignificant within the treatment at 0.05 levels (vertical comparison). Treatments showing similar capital letters are insignificant within the embryonic stages at 0.05 levels (horizontal comparison)

** The hatching process started 40 h after fertilisation

and LDH decreased significantly ($P < 0.05$) till 96 h-PFS and increased significantly till 168 h-PFS (Tables 1 and 2). The activities of these two enzymes in the groups exposed to 500 μg/l lead decreased insignificantly in the case of G6PDH and decreased significantly ($P < 0.05$) in the case of LDH till 96 h-PSF. Thereafter, the activities of both enzymes showed significant increase ($P < 0.05$) till 168 h-PFS (Tables 1 and 2). The activity of PK decreased significantly ($P < 0.05$) from 30 h-PFS to 168 h-PFS in the group exposed to 100 μg/l lead nitrate. In the groups exposed to the other two concentrations (300 μg/l lead and 500 μg/l lead) the PK activity exhibited significant ($P < 0.05$) fluctuations towards increase and decrease (Table 3). In conclusion, the patterns of activity of the lead nitrate-treated embryos exhibited a pattern of enzyme activity similar to that of the untreated embryos (especially for G6PDH and LDH) but with significant variability in magnitude (Tables 1, 2 and 3).

Different patterns of enzyme activities were recorded within each embryonic stage due to the exposure to different concentrations of lead nitrate in comparison with the control. At 30 h-PFS (pre-hatching stage) the activities of the three selected enzymes exhibited similar patterns (Tables 1, 2 and 3). The activities increased significantly ($P < 0.05$), with the 100 μg/l lead dose recording the highest value of activity during the whole experiment, and then the activities of the three enzymes decreased concomitantly with the higher doses (Tables 1, 2 and 3). In the subsequent post-hatching stages (48 h-PFS–168 h-PFS) the activity of G6PDH increased significantly ($P < 0.05$) from the control up to 500 μg/l lead exposure (Table 1). The activity of LDH was significantly inhibited ($P < 0.05$) with increasing concentration of lead nitrate during all post-hatch stages (48 h-PFS–168 h-PFS) from the control to 500 μg/l lead (Table 2). The PK activity in each of the post-hatching stages (48 h-PFS–168 h-PFS) showed significant, and some times insignificant, fluctuations (increase and decrease) with increasing lead doses (Table 3). The same patterns of differences between the control and the lead nitrate-treated groups were also obtained when the Dunnett-*T* test was used (Table 4). In conclusion, in the 30 h-PFS, the activities of

Table 4 Comparison between the activities of G6PDH, LDH and PK in the control and in the other lead nitrate-treated groups during early developmental stages of the African catfish *Clarias gariepinus*, using the Dunnett-*T* test

Enzymes	30 h-PFS			48 h-PFS			96 h-PFS			144 h-PFS			168 h-PFS		
	100 μ/l	300 μ/l	500 μ/l	100 μ/l	300 μ/l	500 μ/l	100 μ/l	300 μ/l	500 μ/l	100 μ/l	300 μ/l	500 μ/l	100 μ/l	300μ/l	500 μ/l
G6PDH	*	*	*	*	*	*	–	*	*	–	*	*	–	*	*
LDH	*	–	–	–	*	*	–	*	*	*	*	*	*	*	*
PK	–	*	*	*	–	*	*	*	*	–	–	–	–	*	*

* Significant difference from the control

– No difference from the control

G6PDH, LDH and PK increased due to exposure to 100 μg/l lead and then decreased with increasing lead doses. After 30 h-PFS, the patterns of the activity under stress varied; G6PDH increased, LDH decreased and PK showed fluctuations.

Discussion

The normal ontogenetic variations in enzyme activities

There is no literature available concerning the pattern of ontogenetic metabolic enzyme activities in early developmental stages of fish before exogenous feeding. Only ontogenetic variations during development after the start of exogenous feeding have been studied (Somero and Childress 1985; Clarke et al. 1992; Segner and Verreth 1995). In the present work, the enzymatic activities of G6PDH, LDH and PK exhibited variability during the early embryonic stages of *C. gariepinus* (30 h-PFS–168 h-PFS), a period that included pre-hatching and post-hatching intervals. The patterns of variations exhibited significant decreases in the enzyme activities of all three enzymes studied till 96 h-PFS, followed by a relative overall significant increase in G6PDH and LDH activity; the activity of the latter enzyme increased faster than that of G6PDH. PK activity after 96 h-PFS showed significant fluctuation. The decrease of activities till 96 h-PFS of all three enzymes led us to the conclusion that the embryo in its early embryonic stages depended on the maternal enzymes. After this stage, the zygotic genes began to work, as expressed by the increasing activities of these enzymes (Mekkawy and Lashein 2003). A similar utilisation of the maternal G6PDH and LDH enzyme stores and their subsequent degradation before zygotic translation of mRNA was postulated by Mekkawy and Lashein (2003) for the grass carp, *Ctenopharyngodon idellus*. Those authors, on a genetic basis, concluded that the zygotic genes of the *C. idellus* embryo appear to be inactive up to the process of organogenesis. In *C. gariepinus* there is still such inactivation up to 96 h-PFS, during which time the major switching process between maternal and zygotic gene activation occurs. This means the utilisation, still, of maternal enzyme mRNA for a long period, in comparison with *C. idellus* (Mekkawy and Lashein 2003) and other fish species in which maternal proteins are synthesised up to the blastula stages, or even to gastrulation.

Increase of G6PDH and LDH activities from 96 h-PFS (2 days after hatching) onwards, before the onset of exogenous feeding (at 196 h-PFS of *C. gariepinus*), referred to the early substantial capacity for NADPH generation based on switch-on zygotic gene mechanisms (Segner and Verreth 1995). Segner and Verreth (1995) referred to such a capacity existing from the onset of exogenous feeding onwards, as evidenced by the increase of G6PDH activity with age. Many authors referred to the increase of LDH and G6PDH enzyme activities with increasing larval age of different fish species (Pelletier et al. 1995; Nathanailides 1996). However, Nathanailides (1996) referred to the decrease of LDH activity during development of Tilapia larvae. The PK activity, as an index of anaerobic potential (Forstner et al. 1983;

Hinterleitner et al. 1987; Kiessling et al. 1991), showed significant fluctuations at the onset of the zygotic gene mechanism (96 h-PFS).

The enzyme activities after exposure to lead nitrate

The ontogenetic pattern of activity of the lead nitrate-treated embryos exhibited a pattern of enzyme activities similar to that of the normal embryos but with a significant variability in magnitude. However, within each embryonic stage, in comparison with the control, different patterns of enzyme activities were recorded. At the 30 h-PFS, G6PDH activity increased at 100 μg/l lead exposure; with higher doses the activity decreased significantly in comparison with the control. In each of the other developmental stages (48 h-PFS–168 h-PFS) the G6PDH activity increased significantly compared to the control up to 500 μg/l lead exposure. A similar increase in G6PDH activity under stress was previously recorded (Wu and Lam 1997; Stephensen et al. 2000; Pandey et al. 2003; Rosety-Rodriguez et al. 2005). Pandey et al. (2003) recorded an increase in G6PDH activity in muscles, liver and gills of the Indian freshwater fish *Wallago attu* with increasing pollution in the river Yamuna. Stephensen et al. (2000) demonstrated that fish from polluted sites have high G6PDH activity due to the increased production of NADH for the detoxification process. Rosety-Rodriguez et al. (2005) also recorded that the activity of G6PDH increased in the blood of gilthead fish at the two high concentrations of malathion tested. Glutathione (GSH) serves to protect the cell against oxidative damage as it conjugates with compounds of exogenous and endogenous origin (Singhal et al. 1987). Thus, a decrease in the cellular level of GSH may cause accumulation of reactive oxygen species (ROS). The increase in G6PDH activity represents protection against elevated levels of ROS in cells exposed to an oxidant stress through the increased production of NADH (Stephensen et al. 2000; Leopold et al. 2003)

LDH is generally associated with cellular metabolic activity. Such activity is inhibited under stress, especially after exposure to heavy metals (Singh et al. 1974; Sastry and Gupta 1980; Gupta and Sastry 1981; Sastry and Rao 1984; Mishra and Shukla 1997; Fennouh et al. 1998; Singh and Sharma 1998; Almeida et al. 2001; Elumalai et al. 2002). In *C. gariepinus* at 30 h-PFS, the 100 μg/l lead dose led to a significant increase in LDH enzyme activity. No significant effects with higher doses of lead have been recorded in comparison with the control during this stage. In other embryonic stages (48 h-PFS–168 h-PFS) the enzyme activity significantly decreased concomitantly with the lead dose. In other words, the activity of LDH was significantly inhibited with increasing concentrations of lead nitrate. Sastry and Gupta (1980) postulated that inhibition of activity may be due to (1) ion imbalance or to (2) intracellular action of metal subsequent to initial plasma membrane damage. This inhibitory effect may also be due to the direct binding of the lead to the enzyme (Rajanna et al. 1990) and the formation of an enzyme-inhibitor complex (Singh and Sharma 1998). In consistence with our results, Mishra and Shukla (1997) reported that endosulfan (an insecticide) inhibited the activity of LDH in muscles and liver of the catfish *Clarias batrachus*. Elumalai et al. (2002) recorded inhibition in LDH activity in the crab *carcinus maenas* due to exposure to Cu, Cr or a mixture of both. In the same way Sastry and Rao (1984) and Almeida et al. (2001) recorded inhibition of the activity of LDH in the freshwater murrel *Channa punctatus* due to exposure to mercuric chloride and in Nile tilapia *Oreochromis niloticus* due to exposure to cadmium. In contrast to the present results, Das et al. (2004a) recorded an increase in the activity of LDH in the liver, gill, kidney, brain and serum of fingerlings of three species of the Indian major carp (*Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*) due to nitrite toxicity.

Most of the egg's carbohydrates are associated with the egg membrane and, therefore, are probably unavailable to the developing embryo, at least until hatching, the time of their release from that membrane (Mekkawy and Lashein 2003). However, intensive catabolism of carbohydrates commences at fertilisation, indicating that carbohydrates play an important nutritive role during initial cleavage (Diwan and Dhakad 1995). Such very early metabolism of carbohydrates is

reflected in the present work in terms of the decreased activity of LDH and the increased activity of the G6PDH enzyme system. It is supposed that the synthesis of metabolic enzymes through activation of maternal genes and internal carbohydrate metabolism is an adaptive or anti-oxidant mechanism in the embryo to tolerate the lead effects.

PK is a cytosolic enzyme that can be taken as indicator of the capacity of muscle tissue for anaerobic function (Berges and Ballantyne 1991; Lemos et al. 2003). In the present work the PK activity at the 30 h-PFS insignificantly increased at a lead dose of 100 μg/l and then sharply decreased. In each of the other embryonic stages (48 h-PFS–168 h-PFS), PK activity showed significant fluctuations with increasing lead doses. No data are available to explain such a specific trend of the activity of PK due to the exposure to different concentrations of lead nitrate.

Does the interaction between the two factors (age and lead nitrate dose) maximise the impacts on the enzyme activities? The interaction and the main effects of age and lead dose were found to be highly significant, referring to the great impact of lead on these enzyme systems with increase in age.

No specific response in the activities of all three enzymes has been recorded in the pre-hatching stage (30 h-PFS) after exposure to the lead nitrate doses. This means that the lead nitrate had a minute effect on the activities of these enzymes at this stage. In conclusion, the low susceptibility in the pre-hatching stages is most probably a consequence of the chorion, which seems to protect the embryo from a wide range of pollutants. Congruently, Osman et al. (unpublished data) recorded that lead has a negligible effect on the degree of DNA damage in the pre-hatching stage (30 h-PFS) in comparison with the post-hatching stages (48 h-PFS–168 h-PFS), due to the presence of the chorion, which protects the embryos. Mekkawy and Osman (2006) postulated earlier that the chorion of *C. gariepinus* started its hardening process 3 h after fertilisation, providing protection from lead 6 h after fertilisation. This hypothesis was first suggested by Nguyen et al. (1999) with regard to heavy metal accumulation but is now further supported by the findings on reduced genotoxicity in pre-hatching stages. The same result has been recorded by Honkanen (2004), suggesting that the presence of an egg shell in the pre-hatching stages seems to protect the embryos from a wide range of external stress factors during critical developmental events.

It could be demonstrated that the activities of G6PDH and LDH gave specific patterns of responses (G6PDH increased and LDH decreased) due to the exposure to different lead nitrate doses during the post-hatching stages (48 h-PFS–168 h-PFS). The PK enzyme did not give a specific trend of response (increase or decrease) to the exposure to the lead nitrate. In conclusion, the measurement of G6PDH and LDH activities, but not PK activity, could be useful biomarkers of lead pollution to reveal embryotoxic potential in fish; the post-hatching stages of the African catfish *C. gariepinus* are the most sensitive in early development.

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