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LACTATE DEHYDROGENASE IN GUPPY FISH (*Poecilia reticulata*) AS A BIOMARKER FOR HEAVY-METAL POLLUTION IN FRESHWATER ECOSYSTEMS

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Abstract: Heavy-metal concentrations and allozyme variations were determined in females of guppy fish (*Poecilia reticulata*) populations collected from polluted and unpolluted sites. The concentrations of Cu and Fe were significantly ($P< 0.05$) higher in guppies collected from polluted drainage compared to the unpolluted population. Higher concentrations of Cu, Fe and Zn ($P< 0.05$) were found in the surface sediment, indicating contamination by the three metals in the polluted drainage. The insignificant difference ($P> 0.05$) in the Zn concentrations between the polluted and the unpolluted populations indicated that Zn, as a major essential metal, was regulated in this freshwater fish. Seven enzyme systems EST, G6PDH, LDH, MDH, PGI, PGM and SDH were tested. Only LDH (lactate dehydrogenase) was found to be a good biomarker for the contamination of Cu and Fe in *P. reticulata*. The zymogram of the unpolluted wild population showed the same monomorphic allele as the unpolluted domesticated guppies from a pet shop, thus, further confirming LDH in *P. reticulata* as a good biomarker of contamination by Cu.

KEYWORDS: *Poecilia reticulata*, allozyme, heavy metals, biomarker

Introduction

A consideration of the responses of biological systems to environmental contamination is of ecotoxicological concern. Hence, studies of pollution in the marine environment should not be based upon measurements of chemical and physical diagnostic parameters alone. Establishment of biomarkers on the biomonitors should be part of the bomonitoring studies nowadays. In fact, many studies have been carried out for the purpose of developing biomarkers for biological responses to environmental pollution (Depledge, 1993; Gillespie and Guttman, 1999).

Allozymes can be explained as variant forms of an enzyme that are coded by different alleles at the same locus (Steiner and Joslyn, 1979). Allozyme analysis has been the most commonly-used technique for quantifying population genetic structures for ecotoxicological purposes (Duan *et al.,* 2001). The allozyme structure is sensitive and it also differs according to the levels and types of pollutants. Changes in the structures of allozymes can be explored as potential biological monitoring systems to detect the levels of specific pollutants (Nevo et *al.,* 1986; Yap *et al.,* 2004, 2011; Yap and Tan, 2007; Knapen *et al.,* 2009). Several researchers have used allozyme polymorphism in fish for the biomonitoring studies (Chauhan *et al.,* 2007; Li *et al.,* 2009).

Guppy fish, *Poecilia reticulata*, (Family: Poeciliidae) is a tropical fish with a wide distribution and is found in great abundance in Malaysian freshwater ecosystems, such as in drains, ditches, and rivers (Yap *et al.,* 2008). The relationship between allozyme polymorphism and heavy-metal contamination in biomonitors has been reported in the literature for marine gastropods (Latvie and Nevo, 1981), blue mussel *Mytilus edulis* (Beaumont, 1991), green-lipped mussel *Perna viridis* (Yap *et al.,* 2004; Yap and Tan, 2007) and horseshoe crab *Carcinoscorpius*

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rotundicauda (Yap *et al.,* 2011). However, such a study in *P. reticulata* is lacking in the literature. Therefore, this paper aims to assess the possible relationship between heavy-metal concentrations and allozyme polymorphisms of guppy populations collected from polluted and unpolluted sites.

Materials and Methods

Sampling for guppy fish was conducted on 14th January 2009 at a clean pond site in Bukit Expo and a polluted main drainage channel, both located in the campus of Universiti Putra Malaysia (UPM), Serdang. Malaysia. No observable source of pollution was found at Bukit Expo and the surface-water parameters recorded *in-situ* were temperature (26.59°C), salinity (1.96 PSU) and conductivity (40584 µs/cm). On the other hand, the UPM Residential College (KMR) main drainage system is considered a polluted site due to several observable sources nearby including a busy bus stop, a restaurant and a residential college. The water parameters recorded *in-situ* were temperature (26.72°C), salinity (1.89 PSU) and conductivity (40007 µs/cm).

Along with the fishes, sediment samples were collected from both the sampling locations – 3.0 to 5.0 cm of the top layer of the surface sediment was collected and stored in polyethylene bags. The sediments were collected using a plastic spatula and brought back to the laboratory for metal analyses.

For allozyme analysis, muscle tissues were taken from guppy fishes. These tissue samples were immediately frozen to preserve their enzymatic activities. The samples were kept at -20°C until used for electrophoresis. Eleven females from each population were used for analysis. Frozen muscle tissues were homogenised manually in about 40 µL of distilled water or gel buffer solution (Steiner and Joslyn, 1979). Application of a continuous buffer system was often used for starch-gel electrophoresis, where the same buffer was

Table 1: Enzyme names and abbreviations, enzyme codes (E.C.), enzyme structures (ES), electrophoretic buffer system (BS), and stairing ingredients used for the allozyme study of *Poecilia reticulata*.

No.	Enzyme	E.C.	ES	BS	Ingredients
1.	α–Esterase (α-EST)	3.1.1.1	monomeric /dimeric	CA-7	0.02 g α- napthyl acetate; 0.04 g fast blue RR; 60 ml 0.5 M phosphate buffer; 2 ml acetone.
2.	Glucose 6-Phosphate Dehydrogenase (G6PDH)	1.1.1.49	dimeric	CA-7	0.06 g $NADP^+$; 0.004 g NBT; 0.04 gPMS; 10 ml 0.5 M tris-HCl buffer (pH7.1); 0.4 g Na_2 glucose 6 phosphate.H_2O.
3.	Lactate Dehydrogenase (LDH)	1.1.1.27	monomeric /dimeric /tetrameric	CA-7	0.027 g NAD^+;0.007 g PMS; 0.007 g NBT; 60 ml 0.2 M tris-HCl buffer (pH8.0); 12 ml 0.5M D-lactic acid.
4.	Malate Dehydrogenase (MDH)	1.1.1.37	dimeric	CA-7	0.02 g NAD^+; 0.004 g PMS; 0.06 g NBT ; 0.24 g DL-malic acid; 10 ml 0.5 M tris-HCl buffer (pH8.7); 34 ml DW.
5.	Phosphogluco-isomerase (PGI)	5.3.1.9	dimeric	CA-7	0.008 g $NADP^+$; 0.002 g PMS; 0.03 gNBT; 0.06 g fructose 6-phosphate; 15 µl G6PDH; 5 ml 0.5 M tris-HCl buffer (pH8.0); 1 ml 1 M $MgCl_2$; 17 ml DW.
6.	Phosphoglucomutase (PGM)	2.7.5.1	monomeric	CA-7	0.016 g $NADP^+$; 0.004 g PMS; 0.06 g NBT; 0.16 g Na glucose 1-phosphate; 60µl G6PDH; 10 ml 0.5 M tris-HCl buffer (pH8.0); 2 ml 1 M $MgCl_2$; 34 ml DW.
7.	Sorbitol Dehydrogenase (SDH)	1.1.1.14	dimeric	CA-7	0.02 g NAD^+; 0.004 g PMS; 0.006g NBT; 10 ml 0.5 M tris-HCl buffer (pH8.7); 34 ml DW.

used for both gel and electrode. Table 1 shows the buffer system and staining recipes for all the seven enzymes used for the allozyme study.

Each slice was stained for a particular allozyme according to the specifications which were modified from recipes of histochemical stainings (Kijimi *et al.,* 1988; Shaw and Prasad, 1970; Harris and Hopkinson, 1976). For the staining of Lactate Dehydrogenase (LDH) enzyme, the D-lactic acid was used instead of L-lactic acid (Sekiguchi, 1988). The gel was fixed in 7% acetate and 10% glycerol to stop the staining reaction.

The data were analysed with the assistance of BIOSYS-1 (Swofford and Selander, 1989) computer package. The analysis included the calculation of allelic frequencies.

For the heavy-metal analysis, the frozen guppy fish samples were thawed at room temperature. The samples were then separated into three partitions – head, visceral and tails – using a surgical knife and were dried in an oven at 60°C for 72 hours. The sediment samples were taken out of the polyethylene bag and dried in the same condition. The sediment samples were then sieved through a 63 µm stainless steel sieve and vigorously shaken to obtain homogeneity.

Both the fish and sediment samples were digested in digestion tubes. About 1.0 g fish-tissue sample were digested in 10mL of concentrated HNO_3 (Ana1aR grade, BDH 69%). As for the sediment samples, 0.5-1.0 g of dried samples were digested in 10 mL concentrated HNO_3 (Ana1aR grade, BDH 69%) and concentrated $HClO_4$ (Ana1aR grade, BDH 60%) in the ratio of 4:1. Samples were digested at 40°C for 1 hour and then at 140°C for the next 3 hours. Digested samples were then diluted with double-distilled water up to a volume of 40 mL and then filtered through a Whatman No. 1 (Filter speed: Medium; Whatman International Ltd. Maidstone, England) filter paper into an acid-washed polyethylene bottle for metal analysis.

All samples were analysed for Ni, Cd, Fe, Cu, and Zn using air-acetylene Perkin-Elmer™ flame atomic-absorption spectrophotometer Model AAnalyst 800 (AAS). Procedural blanks and quality-control samples made from the standard solution for each metal were analysed once for every five samples in order to check for accuracy.

T-test was applied to find the concentration differences of heavy metals in guppy fishes and sediments between the Bukit Expo and UPM main drainage (KMR). Statistical software, SPSS 12.0 for windows was used for data analysis purposes.

Results

Of the seven enzyme systems tested, namely α–Esterase (α-EST), Glucose 6-Phosphate Dehydrogenase (G6PDH), Lactate Dehydrogenase (LDH), Malate Dehydrogenase (MDH), Phosphoglucoisomerase (PGI), Phosphoglucomutase (PGM) and Sorbitol Dehydrogenase (SDH), only the enzyme LDH showed interesting electrophoretic banding patterns between the contaminated site (KMR) and the uncontaminated site (Bukit Expo). This particular enzyme migrated anodally. Two populations of *P. reticulata* were analysed to study the variation of LDH within and between populations.

The polluted fish population from KMR was found to be polymorphic with four individuals of AA phenotype and seven individuals of aa phenotype for LDH whereas the population from Bukit Expo was monomorphic for allele a (Figures 1a-1d). The polluted population at KMR showed 0.364 and 0.636 for the allelic frequencies of allele A and allele a respectively. Since there were no homozygous AA observed among all individuals in the Bukit Expo population, there was zero allelic frequency of allele A. The allelic frequency of allele a for LDH in the unpolluted population at Bukit Expo was 1.

To further investigate LDH is monomorphic for allele a in another unpolluted guppy population, one domesticated unpolluted guppy population was bought from a pet shop. The LDH assay showed a monomorphic banding pattern in this population. Hence, this result agreed with that of the unpolluted wild population collected

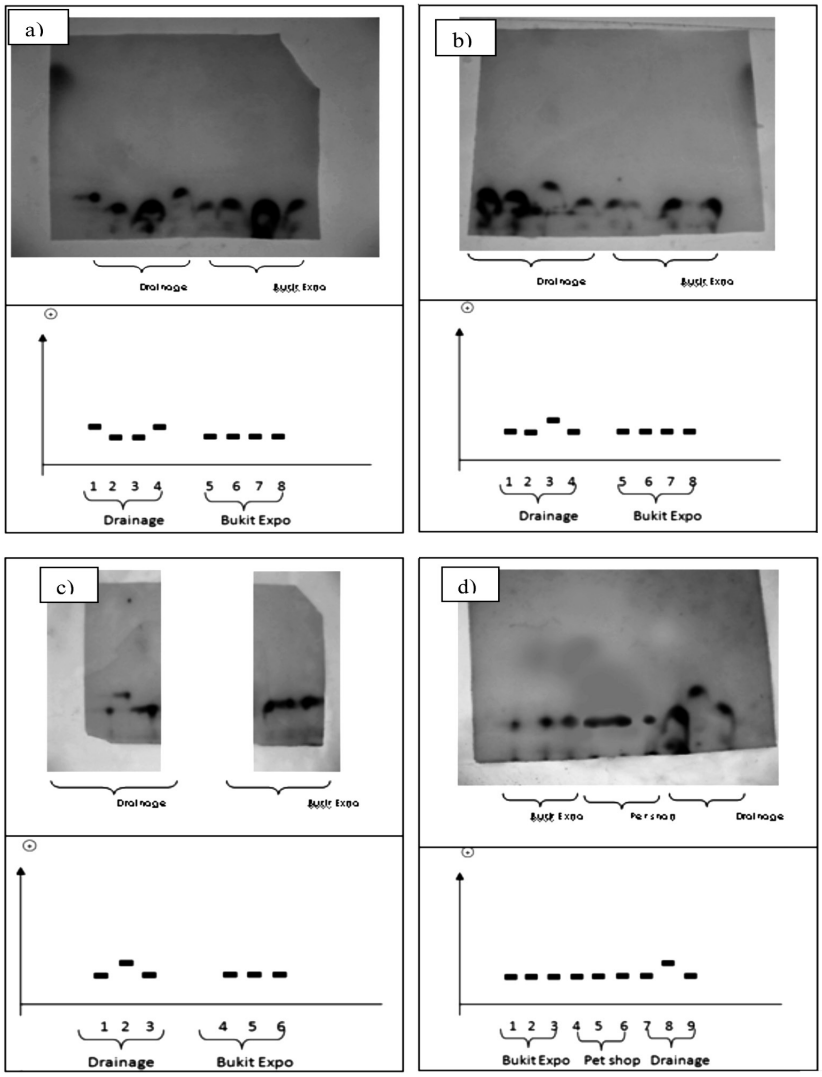

Figure 1: Zymogram of LDH in *Poecilia reticulata*; the photograph of LDH phenotypes; (a,b) the diagrammatic representation of LDH phenotypes observed (c,d). Note: Drainage= polluted drainage site at KMR.

from Bukit Expo. Both Bukit Expo and pet shop populations were monomorphic for the aa phenotype and no heterozygote was observed (Figure 1d).

The concentrations of Cd, Cu, Fe, Ni and Zn in the head, visceral and tails of two guppy populations are presented in Table 2. The concentrations of Cu and Fe were significantly ($P< 0.05$) higher in the three parts of the guppies collected from the polluted drainage at KMR when compared to the unpolluted population collected from Bukit Expo. While Cd, Ni and Zn concentrations in these two populations were not significantly ($P> 0.05$) different. The metal concentrations in the surface sediments from these two sites are given in Table 2. Significantly ($P< 0.05$) higher concentrations of Cu, Fe and Zn were also found in the surface

Table 2: Concentration (µg/g dry weight) of heavy metals in female guppy fish and surface sediments collected from the Bukit Expo Pond and the UPM Residential College (KMR) main drainage.

	Sites	Bukit Expo	KMR	Bukit Expo	KMR	Bukit Expo	KMR	Bukit Expo	KMR	Bukit Expo	KMR
Fish	Parts	Cd		Zn		Fe		Cu		Ni	
	Head	0.79	0.91	300.63	282.63	129	225*	4.35	10.81*	1.27	1.89
	Visceral	0.56	0.48	220.63	173.42	242	626.0*	12.93	15.26*	0.08	0.08
	Tail	1.10	1.05	187.10	182.95	31.5	61.9*	2.21	9.77*	0.20	0.08
Sediment		1.04	0.95	79.20	199.40*	1415	1505*	37.47	47.05*	15.81	16.75

Note: *= significantly higher at P< 0.05.

sediment, indicating contamination by the three metals at the polluted drainage at KMR. The insignificant (P> 0.05) concentrations of Zn in the guppy populations from both the polluted and unpolluted sites indicated that guppy had a good regulatory mechanism as well as tolerance to Zn exposure in their habitat as represented by the higher Zn level in the surface sediment at KMR. Another reason could be that the bioavailability of Zn to the biomonitor guppy was not significantly higher at KMR.

Discussion

The Cu and Fe concentrations in sediment samples and guppy fish samples from the polluted KMR drainage were significantly higher (P< 0.05) than those from the unpolluted Bukit Expo Pond. The guppy collected from the polluted site showed monomorphic LDH bands while the guppy from unpolluted site showed some polymorphic bands.

Interestingly, guppy fishes with similar morphologies which were bought from a pet shop were also monomorphic with LDH aa phenotypes. Guppy fishes in the pet shop were believed to be well taken care of and their ambient surrounding was considered unpolluted since the water quality in the aquarium was maintained and well controlled. Hence, the pet shop guppy fish's results further confirmed that polymorphism for the LDH enzyme system in guppy was a good biomarker of Cu contamination. However, this is because Cu is a well-known toxic metal at high concentrations in living organisms while Fe is not. Hence, it is believed that the high Cu exposure had promoted the polymorphism of the LDH enzyme in the polluted population. The present finding agreed with that reported by Mulvey *et al.* (2002) who found that the mummichog *Fundulus heteroclitus* (which belongs to same order Cyprinodontiformes as guppy) had significantly different LDH alleles at a heavily-polluted site when compared to unpolluted sites.

The polymorphic LDH alleles found in the polluted guppy population indicated selection of resistant LDH genotypes (Van Straalen and Timmermans, 2002) in response to Cu pollution. The LDH polymorphism of polluted guppy could be related to adaptation to environmental Cu contamination (Hummel and Patarnello, 1994; Gillespie and Guttman, 1999). It had been reported that marine animals were naturally selected for specific genotypes due to the effects of heavy metals (Lavie and Nevo, 1986; Ben-Shlomo and Nevo, 1988; Benton *et al.,* 1992; de Nicola *et al.,* 1993). These experiments have indicated both tolerance and sensitivity of allozyme genotypes to environmental stressors and pollutants. Heavy metals interacting with protein structures might alter the enzymatic properties and functions of different genotypes at a polymorphic locus. However, to our knowledge, how LDH interacts with other enzymes is still unknown and further studies are needed.

Conclusion

The LDH banding pattern of the unpolluted wild population was similar with that of the unpolluted domesticated guppies from a pet shop. On the contrary, the polluted guppy population showed polymorphism for LDH, thus, indicating that LDH was a good biomarker of Cu contamination based on this species.

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