



Isozymes of malate-and lactate dehydrogenase of *Astyanax bimaculatus* as biomarkers of environmental impact

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ABSTRACT

The activity of certain isozymes may serve as biomarkers of specific physiological conditions of living organisms. The present work aimed to evaluate malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) isozymes from the hepatic, branchial and renal tissues of the fish *Astyanax bimaculatus* as biochemical markers of environmental changes in the Una River Basin (Taubaté, SP, Brazil). For this study, the specimens were collected in water bodies located in three sampling sites of the basin, called P1 (Itaim stream), P2 (a stream along the Remédios municipal road) and P3 (a small lake near the Dr. José Luiz Cembranelli municipal road). Physicochemical analysis of the water from these sites indicated that P1 was the less polluted site, P2 presented high levels of electrolytes in water, and P3 presented the worst water quality among the sampling sites. The isoenzymes were separated by native-PAGE and identified by their activity on the polyacrylamide gel. The results indicated that four isozymes of MDH occur in the liver of these fish, and the activities of MDH-1 and MDH-4 were detected only in fish from P2, suggesting that these isoforms have potential as biomarkers of the presence of high levels of electrolytes in water. In the kidney, MDH-1 was detected in fish from P2 and P3, but it was not detected in those from P1, suggesting that the presence of this isozyme may be a biomarker of low-quality water. The gill MDH and LDH isozyme profiles of all tissues examined showed some similarities between individuals from the three collection sites, indicating that they are not suitable as biomarkers of the environmental conditions of these sites.

Keywords: *Astyanax bimaculatus*, enzymes, lambari-do-rabo-amarelo, water pollution.



Isozimas de malato desidrogenase e de lactato desidrogenase de *Astyanax bimaculatus* como biomarcadoras do impacto ambiental

RESUMO

A atividade de determinadas isozimas pode ser biomarcadora de condições fisiológicas específicas dos organismos. O presente trabalho teve como objetivo avaliar as isozimas de malato desidrogenase (MDH) e de lactato desidrogenase (LDH) provenientes dos tecidos hepático, branquial e renal do peixe *Astyanax bimaculatus* como marcadoras bioquímicas de mudanças ambientais na Bacia Hidrográfica do Rio Una (Taubaté, SP, Brasil). Para isso, os espécimes desse peixe foram coletados em corpos d'água localizados em três locais da bacia, denominados P1 (ribeirão Itaim), P2 (córrego à beira da Estrada Municipal de Remédios) e P3 (lagoa próxima à Estrada Municipal Dr. José Luiz Cembranelli). Análises físico-químicas da água desses três locais indicaram que P1 foi o local menos poluído, P2 apresentou altos níveis de eletrólitos e P3 apresentou a pior qualidade da água entre os três. As isoenzimas foram separadas por meio de eletroforese em condições não-desnaturantes e identificadas pela sua atividade no gel de poliacrilamida. Os resultados indicaram que ocorrem quatro isozimas de MDH no fígado, sendo que as atividades de MDH-1 e MDH-4 foram detectadas somente nos peixes originários de P2, sugerindo que essas isoformas apresentam potencial como biomarcadoras da presença de níveis elevados de eletrólitos na água. No rim, a atividade de MDH-1 não foi detectada em peixes de P1, mas esteve presente em peixes de P2 e P3, sugerindo que a presença dessa isozima pode ser biomarcadora de águas de baixa qualidade. Os perfis de isozimas de MDH branquial e de LDH de todos os tecidos examinados apresentaram algumas semelhanças entre indivíduos provenientes dos três locais de coleta, indicando que não são adequados como biomarcadores das condições ambientais presentes nesses locais.

Palavras-chave: *Astyanax bimaculatus*, enzimas, lambari-do-rabo-amarelo, poluição da água.

1. INTRODUCTION

Isozymes, or enzyme isoforms, are gene products of different protein coding genes. Isozymes originate from multiple gene *loci* that encode structurally distinct polypeptide chains. Isoforms may catalyze the same reaction, but differ in molecular weight, their kinetics, substrate affinity, number of subunits and amino acid sequences, electrical charge, or electrophoretic mobility. Isozyme analysis can be performed with chemical separation of proteins by non-denaturing native polyacrylamide gel electrophoresis (native PAGE). Due to different electrophoretic mobilities of isozymes, different band patterns (zymograms) are obtained. The zymograms are visualized using an enzyme-specific staining mixture, which includes substrate, cofactor and oxidized salt (Al-Huqail and Abdelhaliem, 2015). Isozymes can reside in different cellular compartments and could have different functions (*e.g.*, cytosolic versus mitochondrial forms of malate dehydrogenase or superoxide dismutase) (Storey, 2015).

Malate dehydrogenase (MDH) catalyzes the reversible oxidation of malate to oxaloacetate, reducing NAD^+ to NADH. Cytosolic (cMDH) and mitochondrial (mMDH) MDH are found in vertebrates and invertebrates. Both isozymes are produced in cytosol from distinct genes, but mMDH is subsequently exported to the mitochondrial matrix (Basaglia, 1989; Musrati *et al.*, 1998). Mitochondrial MDH is a Krebs cycle enzyme, while the cytosolic isoform reduces oxaloacetate in the cytoplasm. Both isozymes participate in the malate-aspartate shuttle system, ensuring the exchange of reducing equivalents between the cytoplasm and the mitochondrial matrix. MDH is also involved in gluconeogenesis and lipogenesis (Nelson and Cox, 2014).

Furthermore, three or six MDH isozymes were found in fish (De Luca *et al.*, 1983; Monteiro *et al.*, 1991; Farias and Almeida-Val, 1992).

Lactate dehydrogenase (LDH) catalyzes the reversible reduction of pyruvate to lactate, oxidizing NADH to NAD⁺. LDH is the terminal enzyme of anaerobic glycolysis and contributes to continued ATP production by glycolysis because the reaction permits NAD⁺ regeneration. Previously it was thought that the enzyme was present only in the cytoplasm, but now there is robust evidence that the enzyme is also found in the mitochondria (mLDH). Lactate can be transferred to the mitochondrial matrix via a lactate transporter protein (MCT1) and oxidized there to pyruvate by mLDH, which provides pyruvate to oxidative processes and to gluconeogenesis in the matrix (Storey, 2015; Meshcheryakova *et al.*, 2016). LDH is composed of A and B subunits, that forms homo- or heterotetramers in fish and higher animals. Five isozymes of LDH have been found, but salmonids and other tetraploid fish species express 14-16 isozymes. (Meshcheryakova *et al.*, 2016). Amazon fish express 3 LDH isoforms (de Almeida-Val *et al.*, 1990), and brain and eye tissues of *A. bimaculatus* express 4 isozymes (Toledo Filho *et al.*, 1978).

Activity levels of isoforms can be modulated by the environmental conditions where fish live. In the ocular and muscular tissues of the carp *Cirrhinus mrigala* and in the cardiac tissue of another carp *Labeo rohita*, MDH isozymes showed different patterns of electrophoretic mobility when fish from different collection sites (non-wastewater and wastewater ponds) were compared, suggesting that pollutants can regulate the expression and/or activity of these isozymes (Manna and Chakraborty, 2012). In microalgae *Porphyridium cruentum* (red alga), *Scenedesmus incrassatulus* (green alga) and *Trachydiscus minutus* (yellow-green alga) cultured in medium with and without nitrogen (N) or subsequently resupplied with N to the starved cells, the results showed different numbers of expressed and active MDH isozymes (Gigova and Ivanova, 2015). The number of MDH isozymes of leaves was also changed in maize cultivated under salt stress when compared with control plants (Eprintsev *et al.*, 2011). The fingerlings of the fish *Mugil cephalus* can express two isozymes of MDH, but one of them disappears when the fish is exposed to a specific nickel concentration in water (Nagarjuna and Mohan, 2017). In humans, LDH isozymes named LDH4 and LDH5 are predominantly expressed by tumor tissues and have been proposed as a diagnostic marker for endometrial cancer (Galal and Meleis, 2016) or for refractory *Mycoplasma pneumoniae* pneumonia (Liu *et al.*, 2018). Therefore, MDH and LDH isozymes' expression and activity have potential as biomarkers, and the aim of this work was to investigate the isozymes of both enzymes as candidate biomarkers of environmental change in the Una river basin, located in São Paulo state, Brazil. These enzymes were extracted from hepatic, branchial and renal tissues of the fish *Astyanax bimaculatus*, locally called lambari-do-rabo-amarelo, abundant in the Una river (Honorio and Martins, 2018).

2. MATERIALS AND METHODS

2.1. Sample collection

Specimens of *A. bimaculatus* were caught with a line and hook and/or fishing net (size 20) in the Una river basin between December 2013 and March 2014. Fish capture was authorized by the Ministry of Environment (SISBIO, No. 41097-1) and the Committee for Ethics in Animal Research of University of Taubaté (CEUA/UNITAU No. 013/2013). The collection sites were localized in the municipality of Taubaté (SP) at (1) Itaim stream (P1), on the property of Fazenda Piloto (an experimental farm) of the University of Taubaté (S 23°01'38,3", W 45°30'31,6"); (2) Remédios (P2), in a stream located along the municipal road Remédios (S 23°03'26.4", W 45°30'2.5") close to a field with rice cultivation; (3) Ipiranga (P3), in a small lake near a pasture for cattle and close to the municipal road Dr. José Luiz Cembranelli (S 23°01'49,4", W 45°30'16,7"). A map of the sampling sites was published in previous studies

(Batista *et al.*, 2014; Ribeiro *et al.*, 2015). Fish were immediately anesthetized with 0.1% (w/v) benzocaine, killed by decapitation and dissected. Hepatic, branchial and renal tissues were stored at -86°C.

2.2. Tissue homogenates

The tissues were homogenized in a Potter-Elvehjen using 50 mM Tris-HCl buffer (pH 7.4), in the proportion 1:5 (w/v) and then subcellular structures were disrupted by a sonic dismembrator (Model 100, Fisher Scientific). The homogenates were centrifuged at 14,000 x g (Eppendorf 5810R) for 10 minutes. Supernatants were used for electrophoresis.

2.3. Protein quantification

Total protein was determined by the Bradford method (1976) using bovine serum albumin (BSA) as a standard.

2.4. Electrophoresis and activity staining

Non-denaturing polyacrylamide gel electrophoresis (native PAGE) was carried out with 2.5% stacking gel and 7.0% separating gel, in a buffer containing 25mM Tris and 192mM glycine. After pre-running for 1h, a sample volume containing 10µg of tissue total protein and 10.0% glycerol was loaded onto staking gel and the electrophoretic run was performed at 100V for 3.5h at 4°C (Davis, 1964). The electrophoretic conditions used for branchial LDH and MDH were slightly different from the standard normally adopted in this procedure since 9.0% separating gel was used instead of 7%, and the run time was 4h.

After electrophoresis, the activity of MDH was revealed by incubating polyacrylamide gel in 168mM Tris-HCl buffer (pH 7.9) containing 210mM L-malate, 300µM NAD⁺, 26µM nitro-blue tetrazolium (NBT) and 86µM phenazine methosulfate (PMS). The gel was incubated in the dark at 37°C for at least 2h. For LDH detection, the composition of solution for gel incubation was the same, except that malate was substituted by 200mM lactate (Lin and Somero, 1995).

For the negative control, after electrophoresis, the gel was incubated with all reagents but without the substrate. No bands were detected, indicating that unspecific dehydrogenases did not contribute to band formation.

3. RESULTS AND DISCUSSION

The physicochemical analysis of the water of P1, P2 and P3 at the time of fish capture has already been published (Batista *et al.*, 2014; Ribeiro *et al.*, 2015). These data indicated that: (1) the less impacted site was P1; (2) the hardness was the highest and the conductivity of the P2 aquatic environment (198.7 µS cm⁻¹) was 1.9 times higher than in P1, and 1.6 times higher than in P3, suggesting that P2 fish were exposed to high levels of electrolytes from an impacted environment (Cetesb, 2009); (3) P3 presented the highest values of chemical oxygen demand (COD), indicating abundance of organic matter in the water, and presented higher values of apparent color, turbidity, nitrate and nitrite among the three sampling sites. Further analysis of the waters indicated that P2 had higher levels of calcium, chloride, iron, manganese and magnesium in comparison with P1 and P3. These analyses also showed that the aluminum level and *Escherichia coli* contamination is higher in P3 than in P2 or P1 (Batista *et al.*, see preceding paper in this journal).

Four isozymes of MDH were detected in livers of *A. bimaculatus* (Figure 1A). MDH-1 is the isozyme that first migrated toward the positively charged anode and its presence as a faint band was detected only in the fish from P2. These fish also expressed two other isoforms of

MDH (MDH-2 and MDH-4), and the fish “e” also presented MDH-3. Fish from P1 presented activity of MDH-2 or MDH-3. In fish from P3, only MDH-2 was clearly detected in all of them. When total activity of liver MDHs measured by spectrophotometric methods was compared among fish collected at the three sites, no statistically significant difference was found (Ribeiro *et al.*, 2015). Nevertheless, the present data indicated that hepatic MDH of the fish from P2 showed a distinct pattern of isozyme expression when compared with fish from P1 and P3. Therefore, for detection of environmental changes, the analysis of the isozyme expression of hepatic MDH could be more sensitive than measurements of enzyme total activity. The pattern of isozyme expression showed by fish from P2 has potential as a biomarker of environments containing high levels of electrolytes. Water from P2 presented the highest values of hardness at the time of fish capture (Batista *et al.*, 2014), and further analysis indicated high levels of calcium and magnesium in its water when compared with P1 and P3 (Batista *et al.*, see preceding paper in this journal). Because P2 was localized in an agricultural area used for rice and other cultures, liming may be the origin of these minerals. In fish, iono- and osmoregulatory mechanisms require an energy supply that is mainly obtained by carbohydrates metabolized by the liver (Tseng and Hwang, 2008). Therefore, the expressions of MDH-1 and MDH-4 that were clear for fish from P2 may be involved with energy production and/or gluconeogenesis in order to respond to increased energy requirement for survival in this environment.

In gills (Figure 1B), MDH-1 was detected as a faint band in fish from P1 (individual “d”), P2 (“e” and “f”) and P3 (“i”). All fish presented MDH-2 and MDH-3. MDH-4 was not detected in fish from P3 and in the individual “c” from P1. A band at the top of the gel could be observed in all wells, however this band may be protein aggregates containing MDH. Right below the top, MDH-5 was more clearly detected in fish from P1 (“c” and “d”), P2 (“e”, “f” and “g”) and P3 (“k”). Because some individuals from different sites showed similar patterns of isozyme expression (“c” and “k” or “d” and “e”), it can be concluded that environmental differences did not produce a clear distinctive pattern of gill isoenzyme expression.

In kidneys (Figure 1C), MDH-1 was detected as a faint band in all fish from P3 and in the individual “e” and “g” from P2. MDH-2 was present in almost all fish, except in fish “c” and “j” from P1 and P3, respectively. The fish “c” and “j” showed MDH-3 and only the fish “c” showed MDH-4. The most peculiar characteristic of MDH isozymes of kidney is the undetectable levels of MDH-1 in fish from P1. Therefore, the expression of MDH-1 may be related to environmental changes of P2 and P3, and the expression or activity of this isozyme was not required in the less impacted area (P1). However, two fish from P2 (“f” and “h”) also did not show MDH-1, indicating that kidney MDH-1 expression is not a clear biomarker for environmental conditions of the sampling site P2. Nevertheless, de Almeida *et al.* (2016) found that MDH-1 of muscle of *A. bimaculatus* from P3 increased about twice in comparison with P1 and P2. Therefore, muscle MDH-1 and kidney MDH-1 could be candidate biomarkers of environmental conditions at P3.

LDH-1 was the isozyme that first migrated toward the anode (Figure 2). Three isoforms of LDH were detected in livers of *A. bimaculatus*, and all fish from the three sampling sites presented LDH-1 (Figure 2A). The activity of LDH-3 was higher in fish “h” from P3 when compared with all other fish, except for fish “g” that showed LDH-1, -2 and -3 with similar staining intensity of the bands. Only LDH-1 was clearly detected in fish from P1 and in the fish “f” from P2. More fish from P1 were also analyzed and only LDH-1 was detected in their livers (results not shown). Therefore, it may be possible that high expressions of LDH-2 and -3 were not required for fish from P1 because of environmental conditions at this site, which was the least impacted among sampling sites. However, because one fish from P2 (“f”) showed a similar band pattern to fish from P1, more investigations are necessary to confirm this hypothesis.

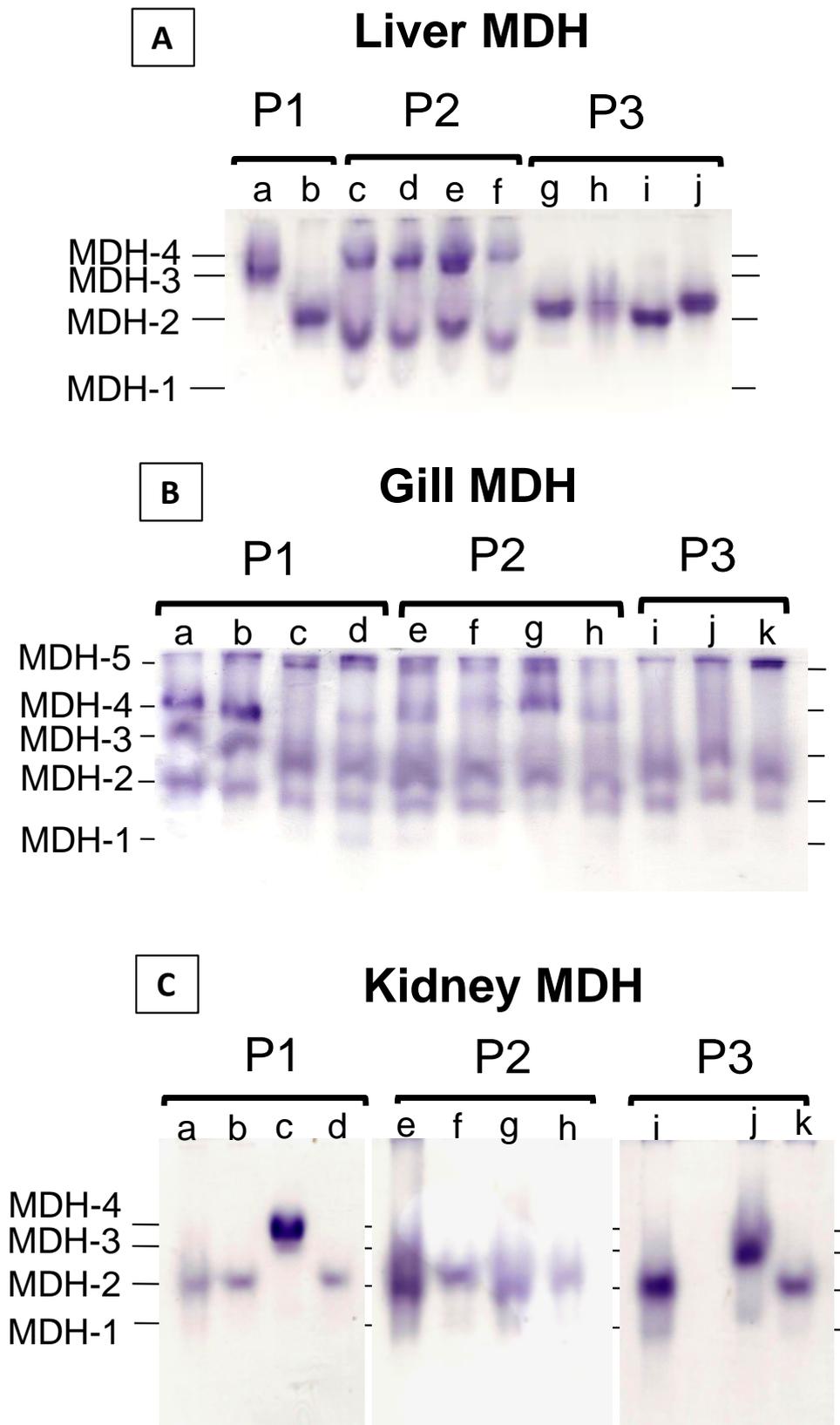


Figure 1. Activity of MDH isozymes of different individuals (a-k) extracted from the liver, gills and kidney of *A. bimaculatus* collected at 3 sites (P1, P2 and P3). Equal letters (a-k) between liver, gill, and kidney trials do not necessarily indicate the same individuals.

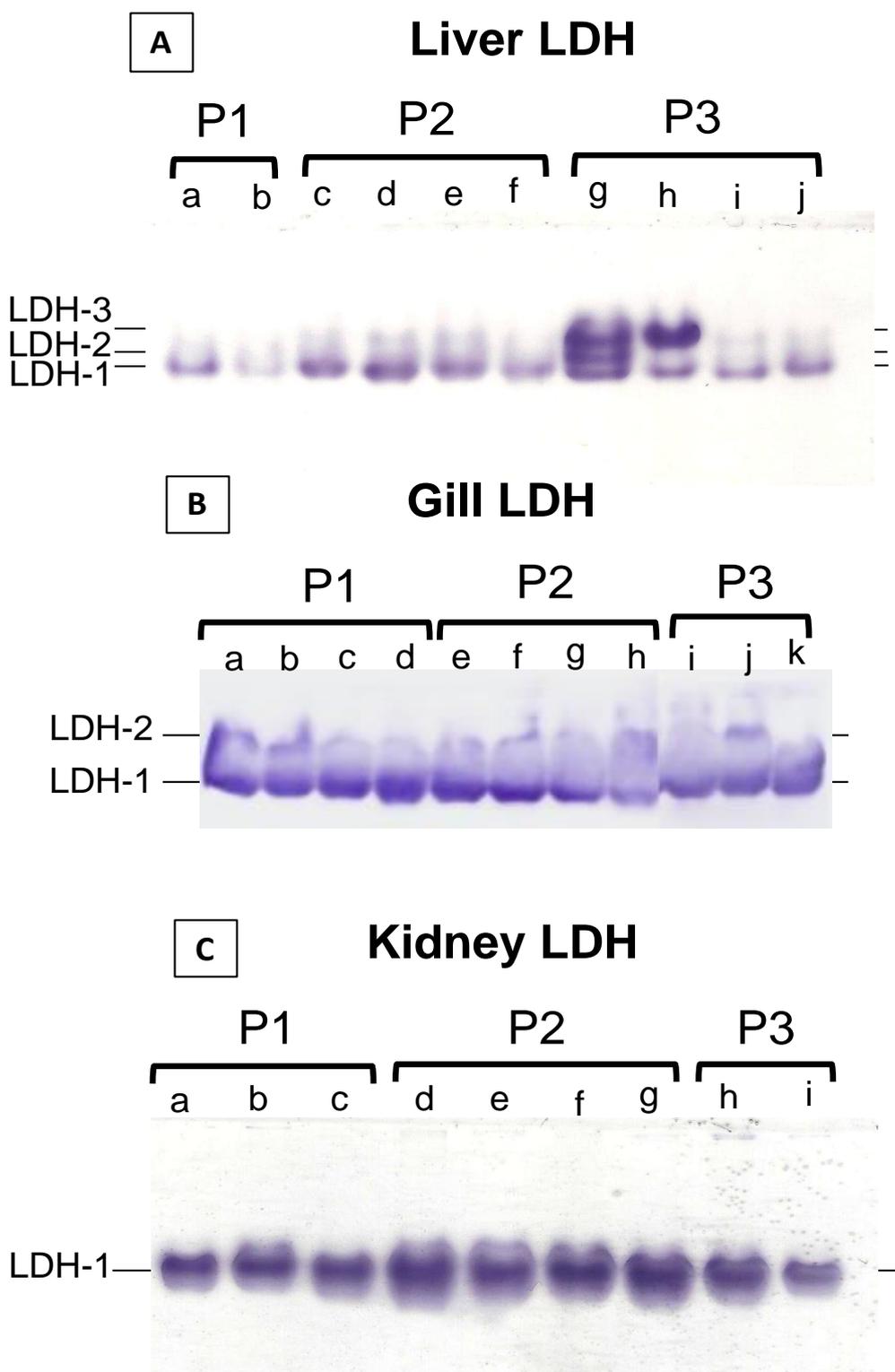


Figure 2. Activity of LDH isozymes extracted from the liver, gills and kidney of different individuals (a-k) of *A. bimaculatus* collected at 3 sites (P1, P2 and P3). Equal letters (a-k) between liver, gill, and kidney trials do not necessarily indicate the same individuals.

Two isoforms of gill LDH were detected (Figure 2B). LDH-1 bands were more intensely stained than LDH-2 in all fish except for fish “h”. Fish from different sampling sites showed similar staining patterns for the bands (e.g., “a”, “b”, “f” and “j”). Therefore, there are no clear and distinctive patterns of gill LDH isozyme activity in fish from the three sites.

Only one LDH was detected in the kidney of *A. bimaculatus*, independent of sampling site (Figure 2C). In addition, total activity of LDHs of kidneys was not influenced by sampling site (Batista et al, see preceding paper in this journal). Hence, kidney LDH is not a suitable biomarker for prevailing environmental conditions at P1, P2 and P3.

Many factors affect LDH activity in fish, such as temperature and oxygen levels in water (Panepucci *et al.*, 2000), starvation (Lowery and Somero, 1990), presence of metals in water (Reddy, 2012; Teodorescu *et al.*, 2012) pesticides (Moreira *et al.*, 2010) and herbicides (Xu *et al.*, 2015). The activity of LDH isoforms can be regulated by rising levels of pyruvate that inhibit a specific isoform but does not affect other types of them. Enzyme acetylation, ubiquitination, SUMOylation, methylation and phosphorylation are mechanisms that regulate LDH in some organisms (Storey, 2015). Therefore, LDH is sensitive to various factors and several mechanisms control its activity. The level of total LDH activity or isozyme activities could be distinct among the fish living in the same body of water because they could be under different physiological conditions due to disease and starvation. Hence, in general, LDH isoforms of *A. bimaculatus* could be signals of energy metabolism changes in the tissue but may not always indicate a cause, and the enzyme is an unspecific biomarker, if analyzed alone without other analyses.

4. CONCLUSIONS

The simultaneous expression of MDH-1 and MDH-4 in the liver of *A. bimaculatus* has potential as a biomarker in freshwater environments containing high levels of electrolytes such as calcium and magnesium. The activity of MDH-1 of the kidney appears to be related to P3, the site that presented the worst water quality. MDH isozymes from gills, as well as LDH from liver, gills and kidneys did not show potential as biomarkers of specific environmental conditions that prevailed in the sampling sites of the Una River basin.

5. ACKNOWLEDGMENTS

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