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LOCI THAT ENCODE THE LACTATE DEHYDROGENASE IN 23 SPECIES OF FISH BELONGING TO THE ORDERS CYPRINIFORMES, SILURIFORMES AND PERCIFORMES: ADAPTATIVE FEATURES

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Abstract—1. Electrophoretic analyses of the lactate dehydrogenase isozyme patterns of 23 species of tropical fishes belonging to the orders Cypriniformes, Siluriformes and Perciformes indicates that at least two LDH loci—Ldh-A and Ldh-B are active. In the Perciformes species, in addition to these loci, the Ldh-C is also present, and restricted in its expression to the eye and brain.

2. Genetic variants were detected in the Ldh-A and Ldh-B loci of *Leporinus friderici* and in the Ldh-B locus of *Schizodon borelli* Cypriniformes.

3. An increase in the occurrence of the Ldh-BB′ and Ldh-B′B′ allotypes of *Leporinus friderici* was observed along the successive collects that started in 1980. This change in phenotypic frequency suggests that this polymorphism could be related to environmental conditions.

INTRODUCTION

The lactate dehydrogenase (EC 1.1.1.27) isozymes of fish have been the subject of many studies (Markert and Faulhaber, 1965; Bailey and Wilson, 1968; Klose *et al.*, 1968; Lush *et al.*, 1969; Markert and Holmes, 1969; Odense *et al.*, 1969; Utter and Hodgins, 1969; Numachi, 1970, 1972; Whitt, 1970; Sensabaugh and Kaplan, 1972; Markert *et al.*, 1975). The A and B genes of fish LDH have been shown to be homologous to those of higher vertebrates (Bailey and Wilson, 1968; Holmes and Markert, 1969) and presumably arose when a gene duplication event took place in a single ancestral form at about the time of the origin of the fish, roughly 500 million years ago (Markert *et al.*, 1975; Whitt *et al.*, 1975; Fisher *et al.*, 1980).

The existence of a third LDH gene in fish, now designated C, has been clearly established by genetics studies (Whitt *et al.*, 1971; Vrijenhoek, 1972). The tissue expression of the LDH C gene is very broad in the primitive bony fishes but is quite restricted in the more advanced teleosts (Shaklee *et al.*, 1973; Markert *et al.*, 1975). Most species of advanced teleosts express the C_4 homopolymer as a highly anodal isozyme restricted to the eye and brain tissues (Markert and Faulhaber, 1965; Massaro and Markert, 1968; Whitt, 1970, 1975). Alternatively many gadiform and ostariophysan fishes express the C_4 isozyme as a relatively cathodal isozyme predominating in the liver (Klose *et al.*, 1969; Sensabaugh and Kaplan, 1972; Kepes and Whitt, 1972; Numachi, 1972; Shaklee *et al.*, 1973; Whitt *et al.*, 1973). According to Shaklee and Whitt (1981), the widespread existence of the two derived LDH-C states (eye vs liver) throughout the advanced teleosts suggests that, at a relatively early stage of evolution, canalization of the C gene was fixed into either of these two restricted modes.

One unusual feature of the isozyme pattern of certain fish is that the usual electrophoretic mobility of the A and B subunits is reversed. In all mammals, birds, reptiles and amphibians the B subunit has the greatest net negative charge, and consequently the B_4 tetramer migrates more rapidly towards the anode during electrophoresis. The same is true for most fish, but a significant number have A and B subunits of just the opposite mobility (Markert and Faulhaber, 1965).

Although the precise metabolic function of different LDH isozymes has long been an unsolved problem, the physiological importance of these multiple forms has been inferred from their served tissue-specific distributions and related with aerobic requirements of the tissues. The B_4 isozyme, for example, is the predominant isozyme in aerobic tissues such as those of the heart and brain and is strongly inhibited by high pyruvate concentrations. The A_4 isozyme on the other hand, predominates in white skeletal muscle and is weakly, if at all, inhibited by pyruvate. This response to substrate has seemed particularly attractive in explaining the isozyme pattern of heart and skeletal muscles (Markert and Holmes, 1969). Also the apparent specialization of the two forms of the C_4 isozymes, eye band and liver band, in advanced teleosts strongly suggests that evolutionary pressure has molded each to accomplish specific and probably different metabolic functions (Shaklee *et al.*, 1973).

The significance of genetic variation has continued to be a controversial subject. While some population biologists ('neutralists') maintain that most genetic variants of enzymes are selectively equivalent, others ('selectionists') believe that polymorphisms are functionally nonequivalent and are maintained by natural selection. Polymorphism for all three loci of LDH has been widely shown in fish (Markert and Holmes,

1969; Whitt, 1969; Lush, 1970; Sachko, 1971; Shaklee *et al.*, 1973; Wilson *et al.*, 1973; Allendorf *et al.*, 1977; Klar and Stalnaker, 1979; Taggart *et al.*, 1981). Recent studies have linked different genotypes of LDH B with temperature, swimming endurance differences, different hatching times and hemoglobin function (Powers and Place, 1978; Powers *et al.*, 1979; Klar *et al.*, 1979; Di Michele and Powers, 1982a,b).

Thermostability has also been found to be a useful parameter for comparing LDH isozymes. In general the homotetramer B_4 from most animals is considerably more thermostable than is the A_4 homotetramer (Plageman *et al.*, 1961; Plummer and Wilkinson, 1963; Kaplan, 1964; Bailey and Wilson, 1968; Wuntch and Goldberg, 1970; Shaklee *et al.*, 1973).

The present paper describes the LDH isozyme patterns of 23 species of tropical fish in order to study the adaptive nature of protein heterogeneity found in ectotherms and to provide suitable material for the detailed biochemical comparison of polymorphic variants of LDH.

The number of loci present in different species were determined where possible, by observation of differential tissue expression of isozymes, thermostability tests and kinetic properties.

MATERIALS AND METHODS

Experimental animals

Specimens were taken from the Mogi-Guaçú river at Cachoeria de Emas and several reservoirs of the Paraná basin west and north of the state of São Paulo. The reservoirs described in the present report are Ilha Solteira, Água Vermelha, Marimbondo, Porto Colombia, Volta Grande, Jaguara, Graminha and Limoeiro. The fishes were caught with gill nets of two sizes (2.5 and 3.5 cm). Altogether 452 specimens were examined. Sampling data including orders families and species collected, are given in Table 1.

Preparation of tissues extracts

Tissues were dissected immediately after capture and kept in ice until they were brought to the laboratory for freezing (−15°C). The desired tissues, skeletal muscle, heart muscle, liver, eye and brain, were cut into small pieces and washed in cold sucrose solution 0.25 M. Tissues were homogenized with a three-fold vol of EBT 0.0071 M buffer, pH 8.7 (Boyer *et al.*, 1963) using a Potter–Elvehjem tissue grinder, at ice-cold temperature and then centrifuged at 19,000 ***g*** for 30 min at 4°C. The resulting supernatants were used for electrophoresis and for enzyme activity measurements.

Electrophoretic and staining techniques

Electrophoresis was carried out in horizontal gels containing 13% (w/v) corn starch prepared according to Val *et al.* (1981), using the Boyer *et al.* (1963) buffer system. A voltage of 5 V/cm was applied for 12–18 hr at 4°C. After electrophoresis, the starch gel was sliced lengthwise. The top slice of the gel was incubated in an LDH staining solution as described by Shaklee *et al.* (1973) in 0.5 M phosphate buffer pH 7.0 at 37°C in the dark. As a control for non-specific reductants or 'nothing dehydrogenase', the bottom slice of the gel was treated in the same way except that the substrate, lactate, was omitted from the staining solution. The extracts were treated with either NAD or β-mercaptoethanol to insure that the multiple LDH bands detected were not the product of conformational isozymes. To measure the effect of inactivation temperature (50 and 70°C) on the activity of skeletal and cardiac muscles, and eye LDH of isozymes and allozymes, the samples were

Table 1.A Some characteristics of fish lactate dehydrogenases

Fish species	Number of A–B tetramers	C subunit	Relative anodal mobility
Order Cypriniformes			
Family Characidae			
Astyanax bimaculatus	5	—	B > A
Astyanax fasciatus	5	—	B > A
Serrasalmus sp.	5	—	B > A
Family Erythrinidae			
Hoplias malabaricus	3	—	B > A
Family Anostomidae			
Leporinus friderici	5*	—	B > A
Leporinus elongatus	5*	—	B > A
Leporinus obtusidens	5*	—	B > A
Leporinus octofasciatus	5*	—	B > A
Leporellus vitattus	5*	—	B > A
Schizodon nasutus	5	—	B > A
Schizodon borelli	5*	—	B > A
Family Cynodontidae			
Galeocharax humeralis	5*	—	B > A
Galeocharax knerii	5*	—	B > A
Order Siluriformes			
Family Pimedlodidae			
Pimelodus maculatus	5	—	B > A
Family Loricariidae			
Hypostomus†			
(5 different species)		—	B > A
Order Perciformes			
Family Cichlidae			
Geophagus brasiliensis	2	yes	C > A > B
Tilapia rendalli	3	yes	C > B > A
Cichla ocelaris	2	yes	C > B > A
Family Scianidae			
Plagioscion sp.	3	yes	C > A > B

*These species usually present the third band duplicated.
†Classification of this species is underway.

incubated at these temperatures for different periods of time and cooled on ice. The heated homogenates were then centrifuged at 19,000 *g* for 30 min at 4°C and electrophoresed. The thermostability of these extracts was determined by heating 1 ml of each extract at 50°C for 2–70 min.

The LDH activity under several experimental conditions was assayed spectrophotometrically by measuring the initial rates of oxidation of $NADH_2$/min at 25°C. The change in absorbancy at 340 nm was measured in a Beckman 25 K spectrophotometer using a temperature-controlled cuvette holder. The assays were carried out in 0.05 M potassium phosphate buffer, pH 7.0 containing 1.3×10^{-4} M NADH and 3.3×10^{-4} M pyruvate. All reactions were carried out at least in duplicate and were initiated by the addition of a measured volume of extract.

The measure of the sensitivity of the heart and muscle LDH to substrate inhibition by pyruvate is expressed as the ratio of enzyme activity at low pyruvate concn (0.33 mM) to the activity at high pyruvate concn (10 mM). This rate is designated: L/H (Wilson *et al.*, 1963).

Nomenclature of LDH isozymes, subunit and structural genes are according to Markert (1962) and Shaklee *et al.* (1973).

RESULTS

Gel preparation, voltage, amperage and running times were carefully controlled in order to provide virtually identical electrophoretic conditions for all specimens screened. The tissues used were chosen in order to expose all the loci involved in the codification of LDH rather than to show differences in tissue regulation.

As shown in Table 1, as in all vertebrates, in each species analyzed were detected the A and B genes for LDH. The LDH C gene was detected only in both Perciformes families (Cichlidae and Scianidae) studied.

As in most vertebrates the muscle was characterized by a predominance of those enzymes which migrated less rapidly toward the anode, and the heart was characterized by a predominance of those enzymes which have the greater rate of mobility toward the anode. Exceptions were found in *Geophagus brasiliensis* and *Plagios cion* sp. (Cichlidae and Scianidae, Perciformes) (Fig. 1a and b), which presented a reversed pattern for LDH.

Within the orders Cyprinformes and Siluriformes we examined six families and were unable to detect either of the two patterns of expression of the C gene—an anodal isozyme in the retina or a cathodal isozyme in the liver.

The C_4 isozyme detected in species of Cichlidae and Scianidae appeared as an anodic band in the retina (Fig. 1, a and b).

Several approaches were taken to show homology between the *G. brasiliensis* LDH isozymes, which have a reversed pattern, with those of other fishes. The effect of heating (50°C) upon extracts of muscle, heart and eye LDH isozymes before electrophoresis is shown in Fig. 1b. The A_4 isozyme of this species, which has intermediate mobility between B_4 and C_4 isozymes more susceptible to heat inactivation than the B_4 and C_4 isozymes. The results of pyruvate inhibition expressed as L/H ratios, obtained with extracts of heart and eye were higher (heart = 2.87; eye = 2.64) than the ones obtained for muscle (1.91),

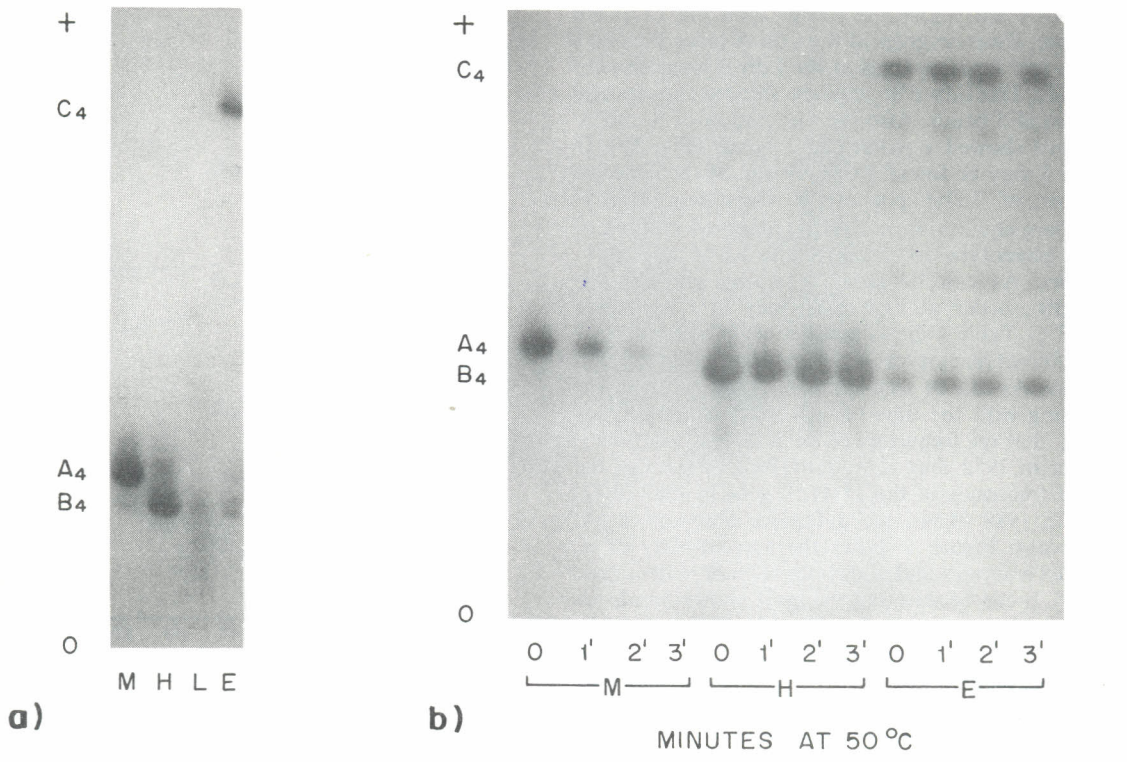


Fig. 1. Electrophoretic patterns of LDH from two species of Perciformes which show reverse isozyme patterns: (a) *Plagioscion* sp. (b) *Geophagus brasiliensis* showing the results of heat inactivation of its isozymes. M, skeletal muscle; H, heart muscle; L, liver; E, eye.

indicating that substrate inhibition is more pronounced for the heart and eye enzymes than it is for muscle LDH. So, on kinetic criteria, the LDHs of the heart and eye of this species appear to be composed primarily of B type subunits, more sensitive to high pyruvate levels. In contrast, muscle LDH is relatively insensitive to pyruvate inhibition, indicating a greater proportion of A type subunit. Of the two first extracts, *G. brasiliensis* heart is more sensitive to high pyruvate levels, indicating that it contains a higher proportion of B-type subunits.

The susceptibility of *G. brasiliensis* isozymes to high temperature (50°C) was examined. When the tissue extracts were exposed to 50°C for various time periods in the absence of substrate or coenzyme, then assayed at 25°C, the half-life of the skeletal muscle extracts was about 5 min while that of the heart and eye extracts was over 1 hr (Fig. 2). Because the muscle isozyme moves predominantly as a single component during electrophoresis, it might be expected to display simple kinetics of heat inactivation (Fig. 1b). Heart and eye isozymes, on other hand, might exhibit complex kinetics of heat inactivation, since by electrophoretic criteria they are a multicomponent system.

In estimating the proportion of loci that are polymorphic, a locus was not considered polymorphic if the commonest allele had a frequency greater than 0.95. Polymorphism for LDH was detected in two species, *Leporinus friderici* and *Schizodon borelli* (Anostomidae) (Fig. 3). In *L. friderici* 161 specimens were screened and genetic variants for the two loci, Ldh-A and Ldh-B, were found (Table 2). In the zymograms the LDH-AA′ allozyme showed to be slower than the LDH-AA and LDH-B′B′ faster than the LDH-BB isozyme. Heterozygotes at the Ldh-A locus showed 8 bands and heterozygotes at the Ldh-B locus showed 9 bands. The Ldh-B′B′ allotype showed the classical pattern of 5 bands. Also the association of A and A′ subunits showed a pattern of 5 bands (Fig. 3b). In Table 3 may be found a comparison of the expected number of LDH phenotypes (assuming Hardy–Weinberg equilibrium), with the observed number of LDH phenotype for each of the LDH loci in *L. friderici*. As can be seen, heterozygotes, allotype Ldh-BB′, occur at high frequency in this sample (49.07%). Table 4 shows the number of specimens of the different phenotypes obtained at different times of capture. As can be seen the phenotypic frequency is changing with the time of capture, and most interesting, that the frequency of the Ldh-B′B′ allotype is greater in 1982 than it was in 1980. In spite of the higher frequency of the LDH-B′ gene in 1982 (0.42) than in 1980 (0.34), the difference observed is not significant. Figure 4 shows the susceptibility of *L. friderici* isozymes and allozymes to high temperature (70°C). It can be seen that the most anodic bands are more stable to heat inactivation than the least anodic bands. Our results show patterns of 2, 3, 5 and more LDH bands in the different species screened (Table 1). In many species, frequently the third band, counting from anodal end, was duplicated (Fig. 5). As occurs in many teleosts, so also in our tissue specimens did secondary or satellite bands appear probably as a result of modifications of the LDH tetramers (Markert and Faulhaber, 1965; Lush, 1970).

DISCUSSION

A survey of LDH patterns in many fish reveals that they may contain one, two, three, four, five or a great many more electrophoretically distinguishable isozymes of LDH (Markert and Faulhaber, 1965). The fact that binomial distribution of five isozymes is not found in many fish indicates that the A and B subunits do not randomly associate in tetramers either because of the primary structure of the subunits or because epigenetic mechanisms operate to restrict subunit assembly (Markert, 1968).

The two-banded LDH pattern, as observed in *G. brasiliensis*, is formed probably by the presence of two homopolymeric isozymes and the absence of the expected heteropolymers. According to Ferris and Whitt (1978), the incompatibility to form heterodimers in heterozygous individuals at the creatine kinase A locus is probably caused by a temporal and/or spatial isolation of allelic Ck-A subunit synthesis and assembly. This hypothesis could be applied to LDH: heteropolymers exist *in vivo* but dissociate under electrophoretic conditions; heteropolymers exist *in vivo* but are rapidly degraded within the cells; heteropolymers do not form because of structural incompatibilities between the subunits; heteropolymers do not form because there is a temporal and/or spatial isolation of subunit synthesis and assembly.

According to Goldberg and Wuntch (1967) a three-banded pattern for LDH could be explained by the formation of homodimers which afterwards randomly associate to form homo- or heterotetramers.

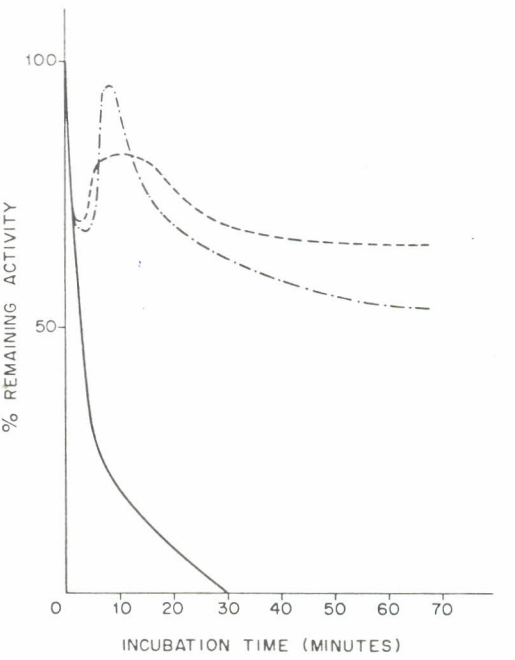
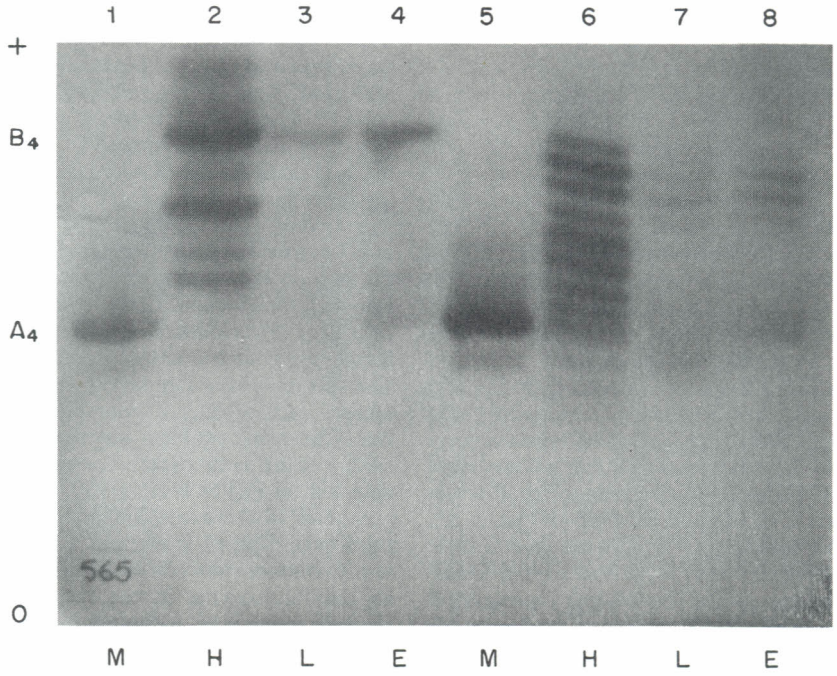


Fig. 2. Thermal inactivation of LDH from *G. brasiliensis* skeletal muscle (——), heart muscle (–.–) and eye (– – –) extracts at 50°C in 0.05 M phosphate buffer, pH 7.0. Initial pyruvate concentration 0.33 mM, initial NADH concentration 0.13 mM. Each point represents the average of at least 3 experiments.



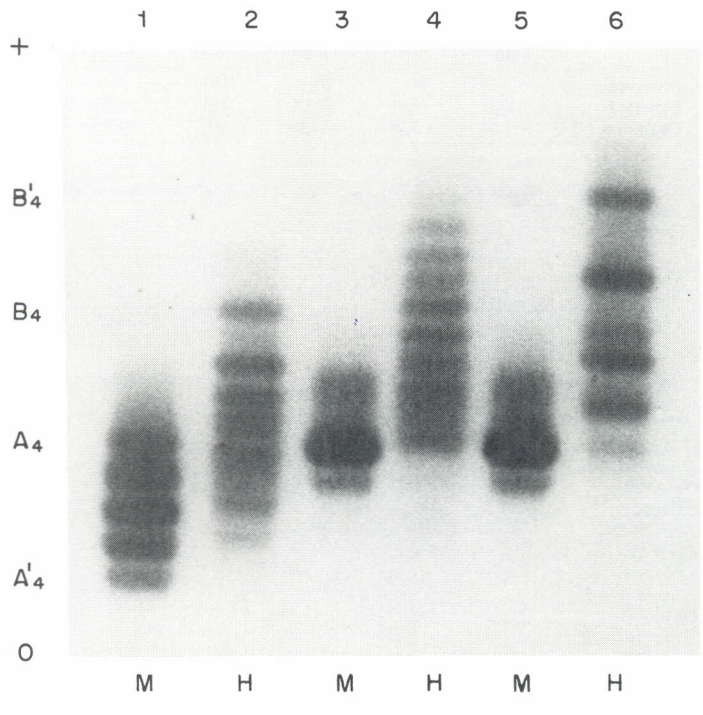


Fig. 3. LDH electrophoretic patterns observed in different tissues from: (a) *Schizodon borelli*: 1–4, AABB phenotype, 5–8, AABB′ phenotype; (b) *Leporinus friderici*: 1–2, AA′BB phenotype, 3–4, AABB′ phenotype, 5–6, AAB′B′ phenotype. M, skeletal muscle; H, heart muscle; L, liver; E, eye.

Table 2. Different LDH phenotypes found in *Leporinus friderici* (Anostomidae)

Phenotype	Number of individuals
AABB	58
AA′BB	4
AABB′	79
AAB′B′	20
Total	161

This kind of pattern was found in *Hoplias malabaricus* (Erythrinidae).

In many species (Table 1) the third band, counting from the anodal end, was duplicated. Lush (1970) attributes the splitting of the third band to the use of the Poulik (1957) buffer system, but this was not true in our case, since we did not use this one. This kind of duplication seems frequent in neotropical fish and has also been observed in species of *Semaprochilodus* Amazonian fish (Schwantes, personal observation).

Many studies have indicated that fish possess more than the LDH A and B genes common to all vertebrates (Merkert and Faulhaber, 1965; Klose *et al.*, 1968, 1969; Whitt, 1969, 1970; Odense *et al.*, 1969; Holmes and Markert, 1969; Lush *et al.*, 1969; Sensabaugh and Kaplan, 1972). Evolutionary surveys reveal that virtually all advanced teleosts synthesize the C_4 isozyme in retina or in liver tissues (Horowitz and Whitt, 1972; Markert *et al.*, 1975).

The electrophoretic data presented in this report indicate that the Ldh-C gene is not detected in the families Characidae, Erythrinidae, Anostomidae and Cynodontidae (Cypriniformes) and in Pimelodidae and Loricariidae (Siluriformes). Also Whitt and Maeda (1970) and Avise and Selander (1972) were unable to demonstrate the Ldh-C gene in *Astyanax mexicanus* and in other characids. However, Shaklee *et al.* (1973) and Whitt *et al.* (1975) described an LDH band corresponding to the C gene in Characidae. Toledo *et al.* (1978) described a C band in the eye and brain of three species of *Astya nax*, two of them being the same species that we studied, *A. bimaculatus* and *A. fasciatus*. Shaklee *et al.* (1973) described a band with intermediary electrophoretic mobility between the A_4 and B_4 homopolymers, corresponding to the Ldh-C gene in the family Anostomidae. In the catfishes, Pimelodidae and Loricariidae (Siluriformes), we were unable to detect the Ldh-C gene. The species of the family Loricariidae reveal a very unclear picture due to poor electrophoretic resolution. This same problem was noted by Shaklee *et al.* (1973) in the same order. The C gene, also, was not detected by Fisher *et al.* (1980) in this family.

Studies in the evolution of the regulation of the third gene, Lhd-C, show that advanced teleosts possess two main patterns of expression of the C gene, an anodal isozyme in the retina and/or cathodal isozyme in the liver. Although this general correlation of net charge with tissue distribution holds for most species, a few exceptions exist. Few species possess C_4 isozymes with intermediate net charges and/or tissue expression (Shaklee *et al.*, 1973; Whitt *et al.*, 1975). If the C_4 isozyme has the same electrophoretic mobil-

Table 3. Distribution of the phenotypes observed in *L. friderici* at the Ldh-B and Ldh-A loci

	BB	BB′	B′B′	Total	
Observed	62	79	20	161	
Expected	63.90	75.06	22.04	161	p(B) = 0.63
Deviation	−1.90	+3.94	−2.04	—	q(B′) = 0.37

$\chi^2_{[1]}{}^* = 0.452$

*At a probability level of 0.05, the $\chi^2_{[1]}$ value must be greater than 3.841 to be significantly different.

	AA	AA′	A′A′	Total	
Observed	157	4	—	161	p(A) = 0.99
Expected	157.79	3.19	0.02	161	q(A′) = 0.01

Table 4. Time of capture and distribution of the LDH phenotypes obtained from *L. friderici.*

	BB	*BB′*	*B′B′*	*Total*
January 1980				
Observed	13	24	3	40
Expected	15.41	19.62	4.97	
June 1980				
Observed	18	16	3	37
Expected	14.24	18.15	4.61	
January 1981				
Observed	7	16	—	23
Expected	8.85	11.29	2.86	
June 1982				
Observed	13	14	8	35
Expected	13.47	17.17	4.36	
January 1983				
Observed	11	9	6	26
Expected	10.01	12.76	3.23	
Total	62	79	20	161

$\chi^2_{[8]}{}^* = 16.39$

*At a probability level of 0.05, the $\chi^2_{[8]}$ value must be greater than 15.51 to be significantly different.

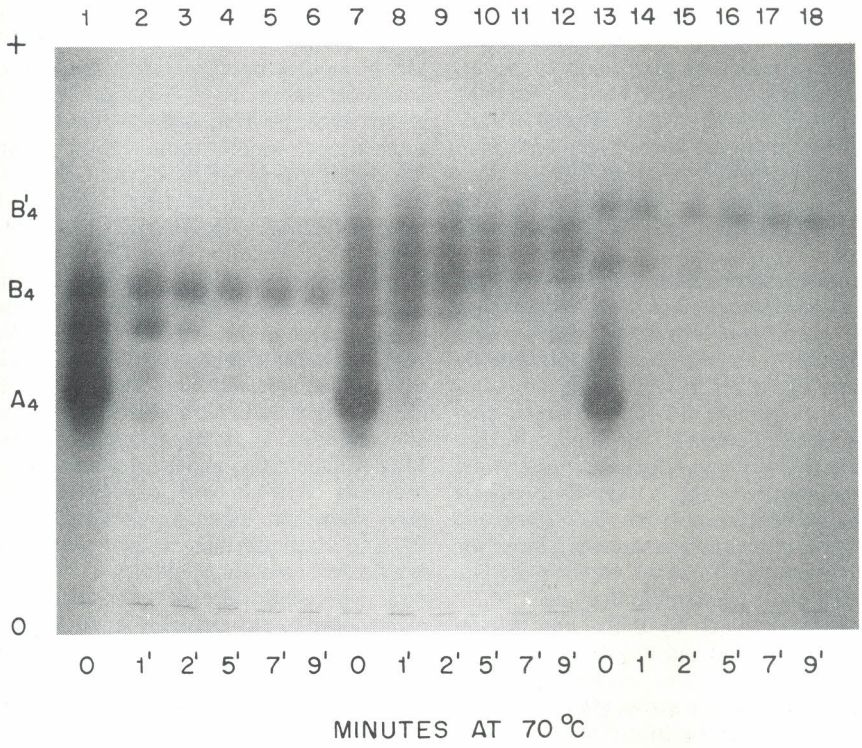


Fig. 4. Heat inactivation of a mixture of skeletal and heart muscle extracts of different phenotypes of *L. friderici*. 1–6, AABB phenotype; 7–12 AABB′ phenotype; 13–18, AAB′B′ phenotype.

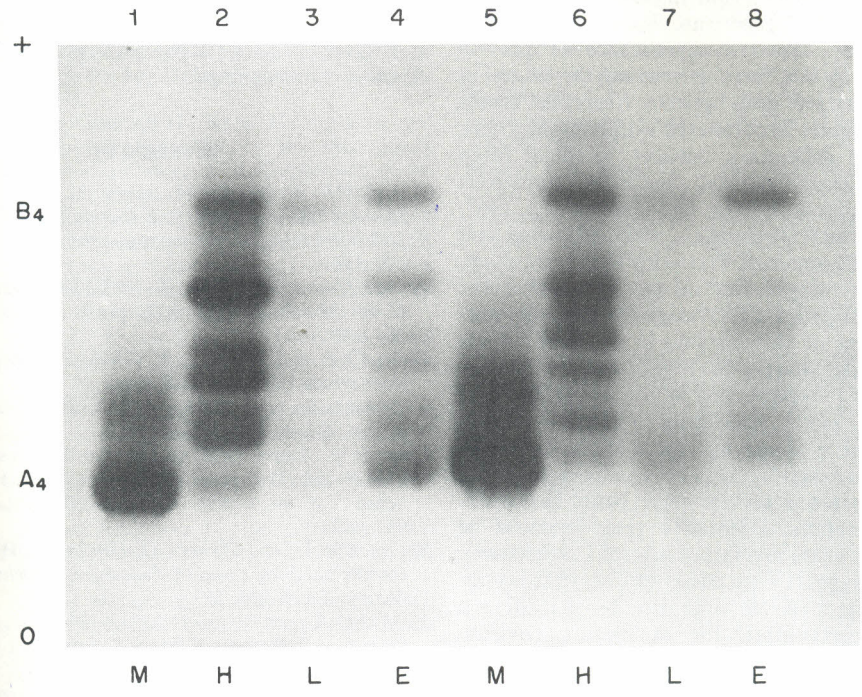


Fig. 5. Electrophoretic patterns of LDH from *Leporinus obtusidens* (1–4) and *Leporinus elongatus* (5–8), showing the duplication of the third band. M, muscle; H, heart; L, liver; E, eye.

ity as the other tetramers formed by the A and B subunits, it would be difficult to determine whether the C_4 isozyme is present or not in our patterns. Another and stronger possibility is that the retinal or liver specific LDH is lacking in these species. The C locus detected here, restricted to the retina and brain of four species of the order Perciformes, has also been detected by other authors in species of this order (Markert *et al.*, 1975; Toledo *et al.*, 1978; Fisher *et al.*, 1980).

In all the species screened here with the exception of *G. brasiliensis* and *Plagioscion* sp. (Perciformes), the B_4 isozyme was always more negatively charged than the A_4 isozyme. In an effort to deal more directly with the question of the reversibility of the isozymes in *G. brasiliensis*, thermostability and substrate susceptibility tests were undertaken. The thermostability tests made with *G. brasiliensis* tissue extracts show that the predominant isozyme in skeletal muscle is more susceptible to heat inactivation (50°C) than the predominant one in heart and eye showing a behavior similar to the A_4 isozyme of other vertebrates. The eye band was the most heat stable isozyme.

Similar to results obtained by Bailey and Wilson (1968) working on rainbow and brook trout LDHs, we detected the lowest L/H ratio in skeletal muscle extract of *G. brasiliensis*, which shows that its isozyme is inhibited in lesser degree by substrate excess than the predominant ones in eye and heart extracts. Again we observed for this isozyme the same behavior as that of the A_4 isozyme of other vertebrates. The reversed electrophoretic pattern which has been observed in *G. brasiliensis* and *Plagioscion* sp., may therefore be an incidental phenomenon of reduced significance when compared with the homologies that exist in other properties.

Polymorphism for LDH loci was found in two species, *Schizodon borelli* and *L. friderici*. According to Kirpichnikov (1973) there are three groups of facts elucidating the adaptative significance of protein polymorphism in fish. Among them are the 'clines' in gene frequencies, one gene-heterosis (increased fitness of the heterozygotes as compared with homozygotes), and functional differences between isoalleles often related to the environmental conditions. Numerous experiments support this last fact (Hochachka, 1967, 1968; Somero and Hochachka, 1969; Massaro and Booke, 1972; Powers and Place, 1978; Powers *et al.*, 1979). Markert emphasized in his works that the presence of numerous LDH isozymes provides normal functioning of this enzyme in different organs and tissues under sharply varying external and internal conditions (Markert and Whitt, 1968; Markert and Ursprung, 1971). According to Johnson (1974), the polymorphism of enzyme loci could be related to their metabolic function. Thus, the polymorphism of LDH loci detected in several fishes could be considered as a metabolic alternative since it could be responsible for some regulatory action. Furthermore, the author suggests that these polymorphisms increase the fitness of the organism by providing a metabolic compensation for fluctuating environments.

The Ldh-B locus has three electrophoretic phenotypes whose frequencies are consistent with the Hardy–Weinberg distribution. The two polymorphic loci, Ldh-A and Ldh-B, are tissue-specific in expression. The electromorphs encoded in alleles at these loci in natural populations of this species are more likely the result of the segregation of codominant alleles at single loci, and not the result of a post-translational modification. However, crosses were not performed to establish with certainty the genetic bases of the electrophoretic variants. Preliminary tests carried out with different phenotypes show that there is a correlation between thermostability and electrophoretic mobility. The fact that the genotypic frequency changes with the time of capture, resulting in an increase in the Ldh-B′B′ and Ldh-BB′ allotypes suggests that this polymorphism could be related with environmental conditions. The national program of alcohol production (Pro-alcool), which started in the late seventies, is greatly increasing pollution in the Magi-Guaçú river, and, as a consequence, probably changing environmental oxygen supply. If, as has been shown in other works (Powers *et al.*, 1979; Powers, 1980) the different genotypes for LDH-B are related to hemoglobin affinity to oxygen, this could well be a case of selection of the best-fitted genotype in response to the changing environment.

We believe that insight can be gained regarding the possibly adaptative nature of these protein polymorphisms by establishing the functional properties of each enzyme in relation to temperature conditions and hemoglobin function. This will be the subject of our future work.

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