1998

The use of isoelectric focusing to assess the genetic diversity of the cyprinid fishes: Rhinichthys cataractae and Rhinichthys atratulus in West Virginia

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The use of Isoelectric Focusing to Assess the Genetic Diversity of the Cyprinid Fishes: *Rhinichthys cataractae* and *Rhinichthys atratulus* in West Virginia.

Thesis submitted to
The Graduate School of
Marshall University

In partial fulfillment of the
Requirements for the Degree of
Master of Science
in Biological Sciences

by

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Marshall University
Huntington, West Virginia
July 1998

This thesis was accepted on September 30,1998, as meeting the research requirement for the Master's Degree.

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ACKNOWLEDGEMENTS

First, I would like to thank God for everything. I would like to thank Dr. Michael Little, Professor of Biological Sciences at Marshall University, for his tremendous help in making this thesis possible. Thanks to Dr. Marcia Harrison and to Dr. Ron Gain of Marshall University for being on my Thesis Committee. Special thanks to my father, Dr. Nurul A. Miah, to my mother, Mrs. Meherun N. Miah, and to my brother, Tariq Miah for immeasurable support, encouragement, dedication, and for believing in me. Thanks also to Dr. Anwar Chowdhury for his support. This study was supported by a grant by NASA.

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ABSTRACT

In this study, isoelectric focusing is used to separate proteins of *Rhinichthys cataractae* and *R. atratulus* and conventional staining is used to identify alleles of six genes. The objective of this work is to determine whether these methods can effectively assay isozymes that may be used to establish the relationship between anthropogenic stress and genetic diversity. Alleles for LDH-A, LDH-B, EST-A, EST-B, EST-C, and EST-D loci were identified for two species of fish, *R. atratulus* and *R. cataractae*. Both fish species are known to be sensitive to anthropogenic stress and known to be widely distributed in stressed environments. Alleles were identified as the isoelectric points for proteins identified as homopolymers. Isoelectric points were calculated relative to protein standards using the regression equations y= 3.8943X+2.49 and y= -1.7277X+6.07 for 3-9 and 5-8 isofocusing gels, respectively. LDH-A focused in the more basic region of the gel, and the LDH-A homopolymer was identified as darkest and widest band produced in the basic region of muscle tissue. The LDH-B homopolymer focused in the more acidic region of the gel. The results were as follow: pI values for *R. atratulus*, EST-A were 3.82 for muscle, 3.47 for head, and 3.08 for heart tissues. The pI values for *R. atratulus*, EST-B were 4.13 for muscle, 4.06 for head, and 3.55 for heart tissues. The pI values for *R. atratulus*, EST-C were 4.84 for muscle, 4.84 for head, and 4.33 for heart. The pI value for *R. atratulus*, LDH-A was 5.34. The pI values for *R. atratulus*, LDH-B alleles were 5.37 and 5.18. The pI values for *R. cataractae*, EST-A were 4.17 for head and heart. The pI value for *R. cataractae*, EST-B was 4.41. The pI value for *R. cataractae*, LDH-A was 5.37. The pI values for two LDH-B alleles in *R. cataractae* were 5.39 and 5.13. Overall, Isoelectric focusing was found to be an accurate and efficient method for the analysis of isozyme diversity of *R. cataractae* and *R. atratulus*.

INTRODUCTION

Statement of purpose:

The Blacknose dace, *Rhinichthys atratulus*, and Longnose dace, *R. cataractae* are common cyprinid fishes in the upland streams of West Virginia. The two species are morphologically and ecologically similar and occur sympatrically in streams of the Greenbrier, New, Gauley, Cheat, and Monongehala River systems (Stauffer *et al.* 1995). Both species are sensitive to human activity and may have experienced a decrease in genetic diversity in response to anthropogenic stress. In this work, isoelectric focusing is used to separate proteins of these two species and conventional staining is used to identify alleles of six common genes that may be used in an assessment of genetic diversity.

The Blacknose dace, *R. atratulus* , is similar to the Longnose Dace in identification, distribution, and habitat (Figure 1). *Rhinichthys atratulus* is a cyprinid minnow found mostly in the northern streams of North America where it occurs from Nova Scotia west throughout the Great Lakes and upper Mississippi River drainages and

Figure 1. *Rhinichthys atratulus*


1 mm

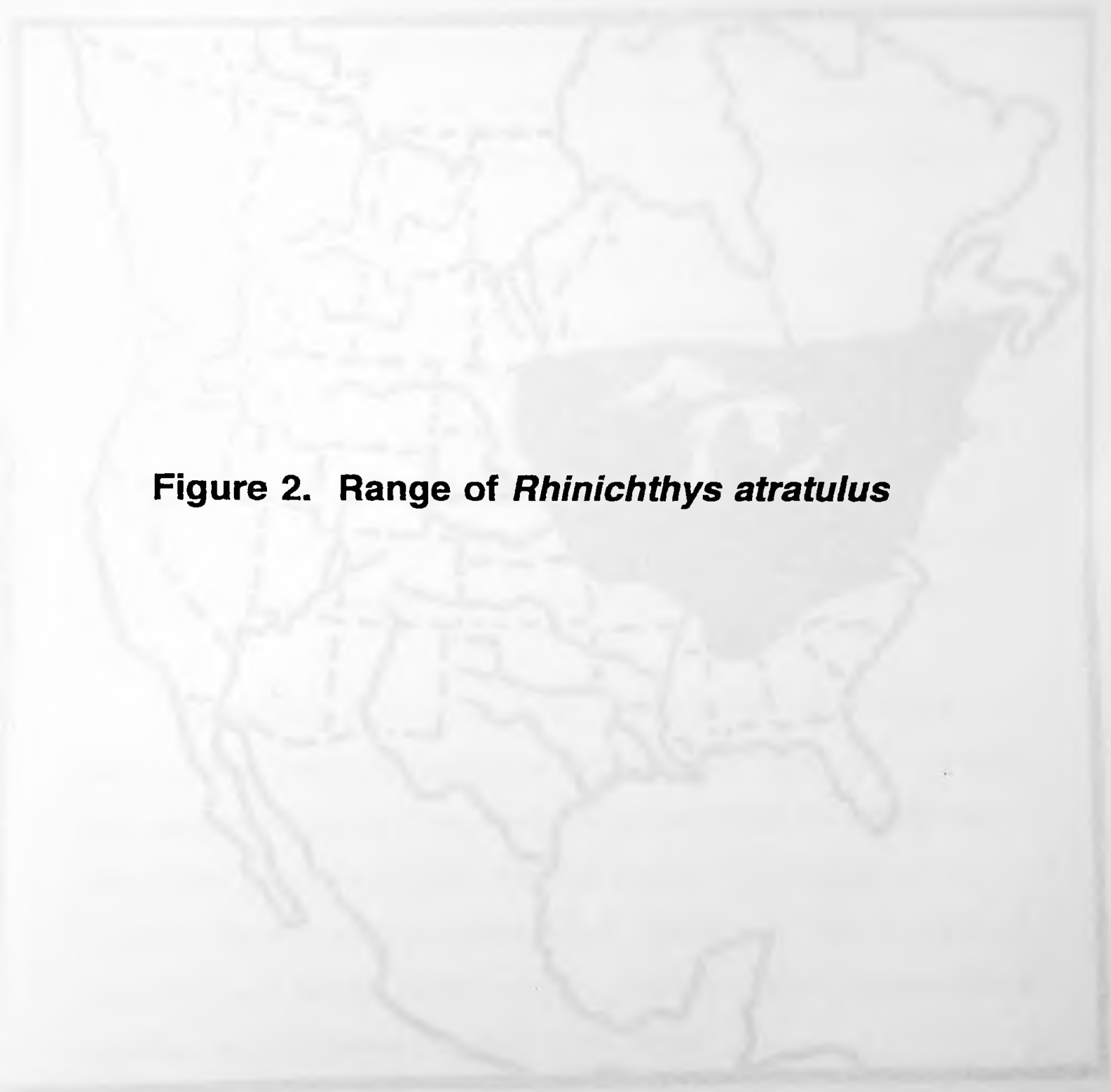


Figure 2. Range of *Rhinichthys atratulus*

south to Tennessee, Georgia, and Alabama (Bragg 1980) (Figure 2.) It inhabits small, high gradient streams and feed primarily on terrestrial insects, beetles, snails, water mites, and stoneflies. The fish is usually found in small, cool mountain streams over gravel or boulder substrates (Stauffer *et al.* 1995).

Rhinichthys atratulus is a highly variable species consisting of three distinct subspecies. *Rhinichthys a. atratulus*, *R. a. obtusus*, and *R. a. meleagris* are distinguished primarily by differences in male nuptial coloration but can also be distinguished by individual morphometric and meristic characters. Nuptial coloration of males and scale counts are used for separation of *R. a. obtusus* from *R. a. atratulus* (Matthews et al. 1982). All three subspecies have recently been reported from West Virginia.

The longnose dace, *R. cataractae* , is a cyprinid minnow widely distributed in headwater streams of North America (Figure 4.) It is most abundant in tributaries of the Great Lakes, throughout the Appalachian Mountains, and in the northern Rocky Mountains. The longnose dace is typically found in small or medium-sized streams with high gradients. It functions as a benthic feeder preying primarily on aquatic insects such as mayflies and caddisflies (Stauffer et al. 1995).

Figure 3. ***Rhinichthys cataractae***


1 mm

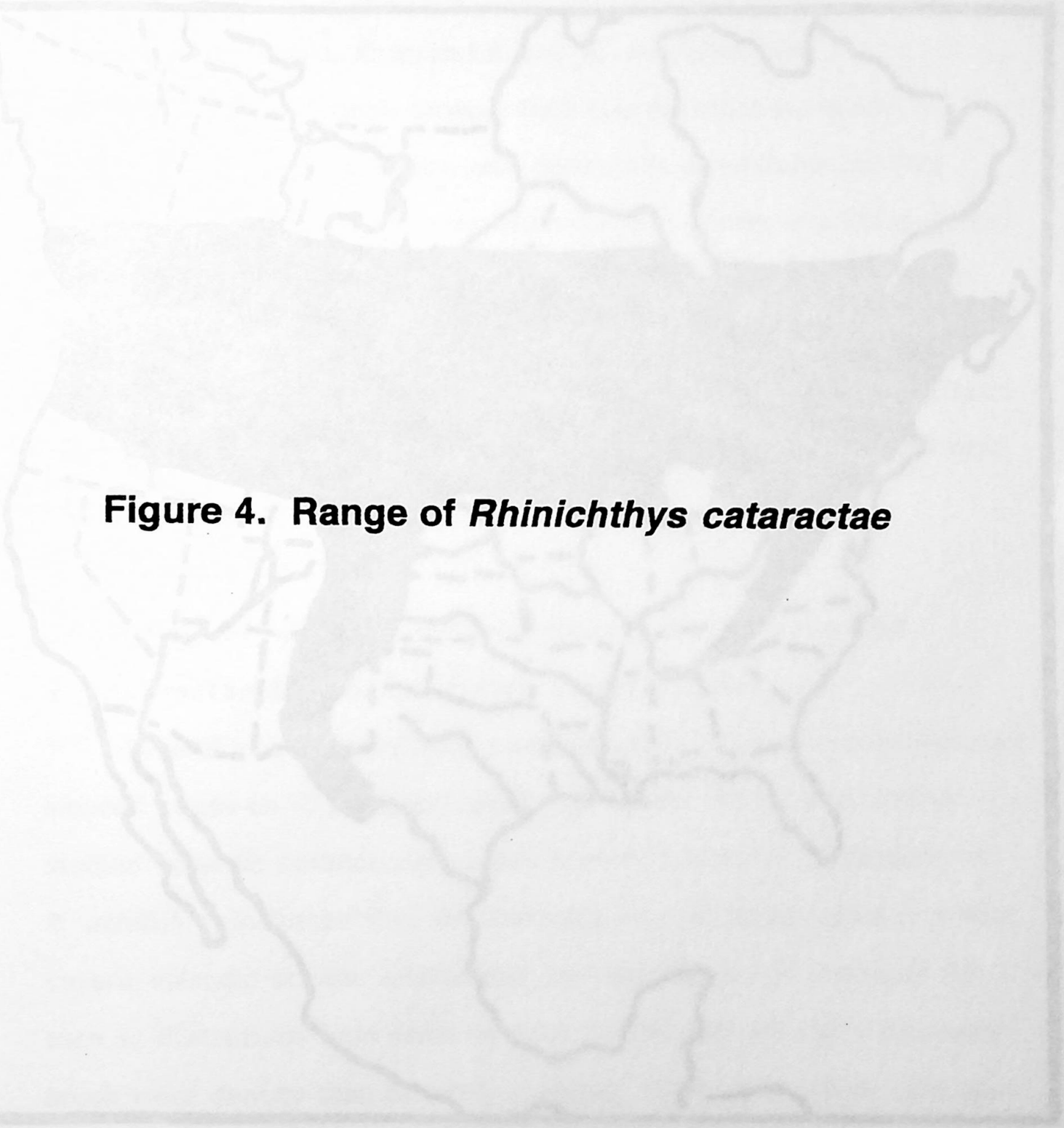


Figure 4. Range of *Rhinichthys cataractae*

In West Virginia, *R. atratulus* and *R. cataractae* are subjected to anthropogenic stress. Both species occur in streams affected by acid mine drainage, acid deposition, and siltation and are appropriate subjects for an investigation of the affects of anthropogenic stress on genetic diversity (pers. com, Dan Cincotta, WV Department of Natural Resources). These events may have repeatedly reduced population size and as a consequence, reduced genetic diversity. To date, the effects of anthropogenic stress on genetic diversity of West Virginia fishes has been little studied.

Sensitivity of cyprinid fishes including R. *atratulus* and *R. cataractae* to environmentally induced stress:

Several investigators have examined the effects of environmentally induced stress on *R. atratulus* and *R. cataractae.* Baker *et al.* (1996) studied episodic acidification on fish in several streams. Bioassays of *R. atratulus* indicated that fish mortality was positively correlated with severe episodic stream acidification and eliminated acid sensitive fish such as *R.atratulus* from some streams. Acidic streams also had a lower brook trout density than nonacidic streams. Baker *et al.* 1996, concluded that episodic acidification can have long-term effects on fish

communities in small streams. Van Sickle *et al.* (1996) studied episodic acidification of small waterways. Brook trout (*Salvelinus fontinalis*), sculpins (*Cottus cognatus and C. bairdi*), and Blacknose dace (*R. atratulus*) were studied. Bioassays during acidic episodes and periods of low levels of acidification indicated that fish had significantly higher mortality during episodic events. Differences between mortality rates were not significant for populations subjected to periodic acidic treatment compared to those continuously exposed to acidification (Van Sickle *et al.* 1996).

Newman and Dolloff (1995) examined the behavior of *R. atratulus* in an acidified laboratory stream. The objective of this study was to determine the ability of the fish to avoid depressions in the ambient pH and recognize and use a neutral-pH microhabitat refuge during acute reductions. Fish avoided areas of higher stream acidification and feeding was suppressed during periods of high acidification. Both blacknose dace and brook trout avoided the acid pulse (ambient pH reduced from 7.2 to 5.1) by sheltering in the pH - neutral refuge.

Pinder and Morgan (1995) studied the relationship of habitat and pH

among the distribution of cyprinid in the streams of Maryland. Streams with low ANC (acid neutralizing capacity) lacked cyprinids. The water in these streams had a pH of 5.30 or less. Population size of cyprinid fishes varied in high ANC value streams (pH of 6.49 or higher) and was not highly correlated with level of acidification. The water with lower ANC showed higher quantities of inorganic monomeric aluminum and low amounts of dissolved calcium. They concluded that in Appalachian streams of Maryland, cyprinid distributions are affected by factors related to surface water acidification.

Dennis *et al.* (1995) studied the conditions of *R. atratulus* in the Shenandoah National Park over the maximum life span of 3.25 years. They hypothesized that environmental stress may negatively effect sodium concentrations in freshwater fish. A constant labeled as "K" was established as a ratio of weight standardized to length. This value was used to estimate the health of individuals in a population that had been subjected to stress. *Rhinichthys atratulus* in low ANC habitats had lower K values than other populations. During summer base flow conditions, mean whole body sodium concentrations of adult *R. atratulus* maintained in cages were found to be highest in the low ANC stream and lowest in the high ANC stream. Hence, whole body sodium concentrations and ion

regulation were highly associated with K and ANC values. The metabolic cost of this ionoregulatory over-compensation may necessitate the diversion of energy from somatic growth and explain the poorer condition of fish from such waters.

Simonin *et al.* (1993) investigated the response of Blacknose dace and brook trout to the acidification of Adirondack streams. Mortality of both species was greatest after episodic surges in precipitation and snow melts. *Rhinichthys atratulus* was more sensitive to these conditions than brook trout. Survivorship was highest for fish that had been in streams for 15 to 24 days prior to acidification and had acclimated to acidification prior to storm events. Extended periods of poor water quality resulted in fish mortality and may be more important to native populations than short acidic episodes.

Separation of enzymes by starch gel and IEF electrophoresis:

Enzymes are proteins that have either an overall negative or positive charge based on their specific amino acid composition. They consist of a single tertiary structure or may have quartenary structure resulting from the combination of different subunits. The union of protein subunits may produce enzymes with dimer, trimer, or tetramer quartenary structure.

Enzymes made up of several subunits may consist of tertiary structures from a single locus or multiple gene loci (Hillis and Moritz 1997). Electrophoresis can identify enzymes by forming enzyme specific bands on a gel, hence separating the variants. In an electrical field, proteins tend to move towards the pole of the opposite charge. The distance of migration is effected by the size and /or charge of the protein. Overall differences in charge can be used to separate isozymes of any enzyme system.

Isoelectric focusing (IEF) is a technique that separates proteins according to net charge only. Proteins migrate through an electrochemical ampholyte pH gradient in polyacrylamide gels. When an electric potential is created through a power source, proteins move to their isoelectric point (pI value). The pI value is the location on the gel where the pH of the gel produces a protein with no net charge. Isozymes may be identified as their pI value.

Most genetic analyses currently use starch gel electrophoresis to isolate isozymes. An example of this is the experiment by Kopp et al. (1995) where fish exposed to acid were studied. In starch gels, the most common allele is arbitrarily given a value of 100 and all other alleles of that gene are given values proportionate to the migration of the 100

allele. Thus an allele that migrates 4/5's of the total migration of the 100 allele is identified as allele 80 (Hillis and Moritz 1996). Although this method has been used extensively in genetic analysis for almost 50 years, it has one serious deficiency. Alleles can only be identified relative to distance migrated. Isozymes, therefore, have no absolute value that can be easily transferred from one study to another. This deficiency can be rectified, if reference tissue extracts are maintained for future reference. This is a serious problem in environmental studies which may take place over an extended time period and may exceed the time interval during which reference materials are maintained.

Characteristics of isozymes studied in this work:

Thorough studies on lactate dehydrogenase show that it is visible as four polypeptide chains (a tetramer). Five isozyme bands correspond to the five possible tetramer combinations of two kinds of subunits (Clayton *et al.* 1969). Kaplan (1964) also relates the catalytic properties of the isozymes to the subunit composition of tetramer isozymes in specific tissues. Lactate Dehydrogenase mediates the conversion of lactate and oxidized NAD+ to pyruvate and reduced NAD+. Typically in heart tissue, the LDH-B gene primarily produces pyruvate and in skeletal muscle tissue,

the LDH-A gene primarily produces lactate.

Esterases detect the sensitivity of fish species to pollution. Highly active esterase (EST) allozyme causes an individual to gain resistance to stress. In other words, the molecular basis of high esterase activity in resistant individuals is high esterase enzyme titer, due to amplification (increase in copy number) of the [EST] gene.

Studies of genetic diversity of cyprinid fishes including *R. atratulus* and *R. cataractae*:

Clayton and Gee (1996) studied lactate dehydrogenase in *R. cataractae* and *R. atratulus* and their hybrid. Five isozymes matching with the tetramers of the muscle (LDH-A) and heart (LDH-B) subunits were identified for both species. The heart type subunits were mostly visible among the longnose dace isozymes. Also, they detected "multiple band pattern" (hence, can show "two kinds of heart subunits") among the first generation offspring. Two alleles of LDH-A (muscle) were detected in *R. cataractae.*

Market and Faulhaber (1965) examined LDH isozymes of 30 species of fishes and found only 3 which produced all five possible HM tetramers.

Dace heart extract produced anodal bands that stain more intensely than the cathodal bands as do most birds and mammals. Ten out of 30 fish species produced LDH bands with reversed heart-muscle LDH mobility.

Merritt *et al.* (1978) studied genetic variability in *R. cataractae.* Genetic variability was measured for 21 loci from 13 populations. North-south clines in heterozygozity and *est-1* gene frequencies were noted in the Connecticut River [USA] system. Evidence indicated that these clines resulted from drift and founder effect and that random processes were the primary determinants of gene frequency in southern Connecticut River populations of *R. cataractae* (Merritt *et al.* 1978). Most protein polymorphism in similar headwater fish species probably represents effectively neutral variation. Gene frequency in these populations was determined primarily by stochastic, not deterministic, processes (Merritt *et. al.* 1978).

Rhinichthys cataractae may be involved in the production of a nominal species, *R. bowersi*. This species may have been produced by hybridization of *R. cataractae* and the chub, *Nocomis micropogon.* A comparison of *R. bowersi* with its putative parents in 16 morphometric and meristic characteristics by a hybrid index indicates it is intermediate in 6 characters, closer to *R. cataractae* in 3 and closer to *N. micropogon*

in 2 (Stauffer *et al.* 1979).

Goodfellow *et al.* (1982) examined isozymes of *Campostoma anomalum* , *R. cataractae* , and their F-1 offspring. Forty-four enzymatic loci of serum proteins and muscle were identified. Of the 44 loci scored, acid phosphatase, alkaline phosphatase, esterase, alpha glycerol, phosphate dehydrogenase, malate dehydrogenase and phosphoglucomutase showed hybrid inheritance patterns. Banding patterns for all mixtures of parental species were identical to those observed in the hybrid.

The relationship between environmental stress and genetic diversity:

A number of investigators have examined the relationship between increasing anthropogenic stress and decreasing genetic diversity. Kopp *et al.* (1995) quantified indicators of environmental tolerance among fish populations exposed to acid deposition. Starch-gel electrophoresis was used to observe the genetic structure of *R. atratulus* and four other fish species. Their tolerance to acid - stressed (low, pH, high Al) habitats varied significantly among species. Among these fish, they found that heterozygosity, polymorphism, and acid tolerance were not consistent. As predicted from previous works, H (heterozygosity) was high in central mud

minnow populations and low in Blacknose dace. Results suggest that any relationship between genetic variability and stress tolerance may lack predictive power when applied to specific species and specific stressors.

Fore *et al.* (1995) tested the hypothesis that genetic diversity of the stoneroller, *C. anomalum* , was reduced by anthropogenic stress. Stoneroller populations from seven Ohio streams were examined. Samples were taken at, below, and above point sources of various pollutants. Allele and genotype frequencies were significantly different at the point source compared to sites upstream and downstream. They concluded that genetic diversity is an indicator of water quality that may detect changes in population size and genetic diversity.

Fore *et al.* (1995) examined genetic diversity of populations of *Pimephales notatus* from 15 Ohio streams. The purpose of this study was to determine whether genetic diversity of *P. notatus* could serve as an effective biomarker of exposure to anthropogenic stressors by comparing genetic measures with other biological indicators of water quality. The Ohio Environmental Protection Agency (Ohio EPA) is currently using two biocriteria: the Index of Biotic Integrity (IBI) and the Invertebrate Community index (ICI). These indices are based on structural and functional ecological attributes of biological communities

(Fore *et al.* 1995). Seven enzyme loci were studied. It was found that genetic diversity was not related to IBI or ICI, but the range of ICI and IBI numbers were small. The value IBI could be predicted by studying the proportion of individuals who had the absence of esterase locus 3. The IBI declined as the number of individuals lacking esterase locus 3 increased. This study suggested that allele and genotype frequencies may have been impacted without affecting overall species diversity (Fore *et al.* 1995).

Isoelectric focusing is a method that has been little used in studies of genetic diversity relative to environmental stress. However, this method may prove to be a more effective and efficient mechanism for the analysis of isozyme diversity than conventional starch gel electrophoresis. Isoelectric focusing may prove to be particularly useful in long term studies of the effects of anthropogenic stress on the genetic structure of populations.

In this study, I determine whether isoelectric focusing can effectively assay isozymes that may be used in future environmental studies. Isozymes from *R. atratulus* and *R. cataractae* are isolated by isoelectric focusing. Both fish species are known to be sensitive to anthropogenic stress and known to be widely distributed in stressed environments. Isoelectric focusing gels are stained by conventional

methods for six commonly studied loci, LDH-A, LDH-B, EST-A, EST-B, EST-C, and EST-D.

MATERIALS AND METHODS

A minimum of 30 *R. cataractae* and *R. atratulus* were collected by electrofishing in Seneca Creek, Glady Fork, Horseshoe Run, and Middle Fork River. Specimens were labeled according to date, species, and stream and immediately frozen on dry ice. Fish were stored in the laboratory at -70 C.

Tissue Preparation:

Fish were thawed and head, heart, and muscle tissues removed. Hearts were removed through an incision in the ventral surface of the fish and transferred into a microcentrifuge tube containing 2 ml of extraction buffer. Head and approximately 2 g of muscle tissues were each removed from the fish and also stored in 2 ml of extraction buffer. All tissues were ground in extraction buffer and stored on ice. Tissue homogenates were then centrifuged at 10,000 X g for 20 minutes at 4^{o}C. Supernatants were removed and stored at -70^{o}C.

Isoelectric Focusing:

Five microliters of each sample were loaded onto 0.75 mm acrylamide IE Phastgels (Pharmacia Biotech). The separations were

carried out on a Phast System Isoelectric Focusing System programed to 10 minutes at 1000 Volts, then at 2000 Volts and 7 Watts. After the separations, the gels were placed in plastic trays where appropriate stains for each enzyme system were added as follow:

LDH: 50mL 0.2M Tris (pH 8.0), 8mL 1.0M lithium lactate (pH 8.0), 1mL 10mg/mL NAD, 1mL 5mg/mL NBT, 1mL 5mg/mL PMS

EST: 50mL 0.2M Tris (pH 7.0), 3mL alpha napthyl acetate solution, 0.05g fast blue BB salt

(NAD = Nicotinamide adenine dinucleotide
NBT = Nitro-blue tetrazolium
PMS = Phenazine methosulfate)

Gels were stained in a 37°C incubator without light for 45 minutes. After staining, solutions were drained and gels rinsed with 100 ml of distilled water. Gels were then emersed in 2% glycerol solution for one hour. Gels were air dried at room temperature and laminated onto typing paper. The gels were scanned with a Hewlett Packard scanner for amplification of banding patterns.

Alleles of each locus were identified as the pI value (isoelectric point) of homopolymers. Each pI value was calculated relative to a standard curve generated from protein standard mixture with pI values of 5.1, 5.9, 6.6, 6.8, and 7.2. After isoelectric focusing this lane was cut from the gel and stained by standard silver staining (obtained from Sigma kit AG-25). The standard was then fixed twice (5 min.) with 100 ml tricarboxylic acid and washed with distilled water followed by the addition of 100 ml 5% glutaraldehyde (Pharmacia) in sodium acetate. After 10 minutes a second rinsing followed by the addition of 100 ml silver equilibrator (Sigma). After 10 minutes, it was placed in 100 ml developer (Sigma). The gels were put in a stop buffer of 100 ml 2% acetic acid for 5 minutes. Finally, distilled water was used to rinse the gel and then 2% glycerol was added. The gel was laminated onto typing paper after drying.

pI value determination:

Gels were scanned and isoelectric points for each isozyme found by using a standard curve (Figure 6.) Each standard curve was calculated by Exel 6.0 using known pI values. (Figure 7).

Figure 5. Standard Curve for 3-9 gels

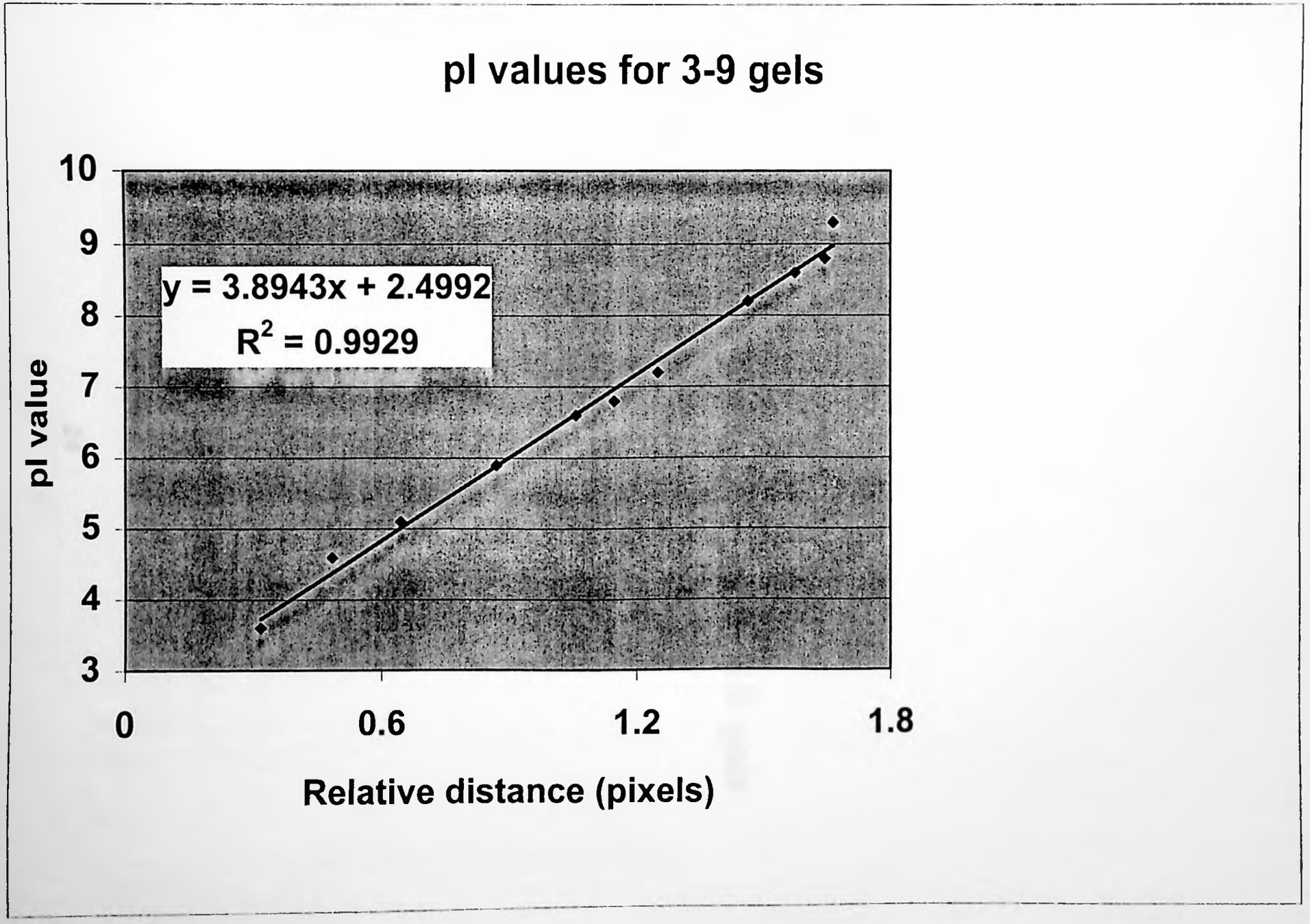
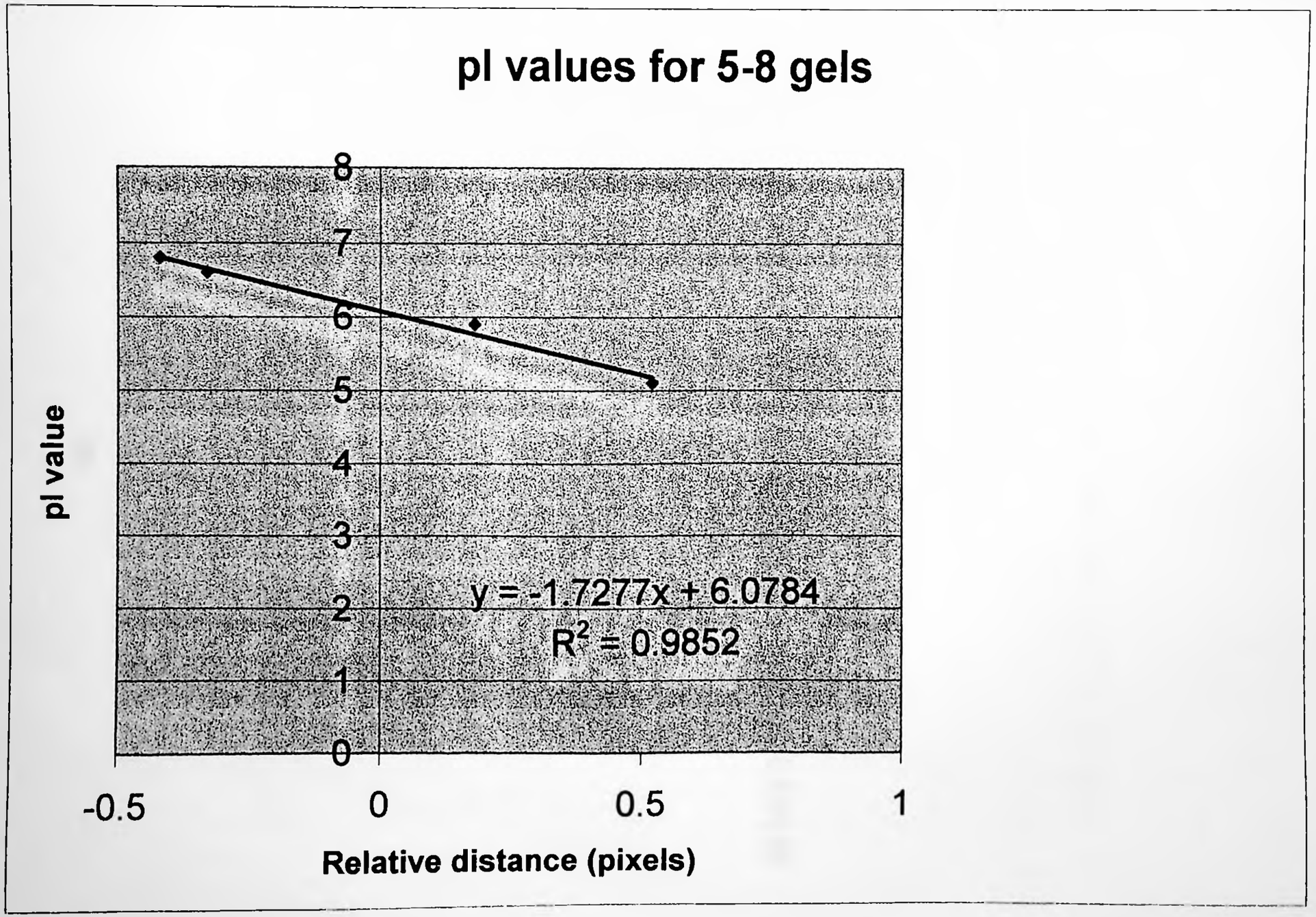

pI values for 3-9 gels
y = 3.8943x + 2.4992
R2 = 0.9929
pI value
10
9
8
7
6
5
4
3
0
0.6
1.2
1.8
Relative distance (pixels)

Figure 6. Standard Curve for 5-8 gels


pI values for 5-8 gels
pI value
8
7
6
5
4
3
2
1
0
y = -1.7277x + 6.0784
R2 = 0.9852
-0.5
0
0.5
1
Relative distance (pixels)

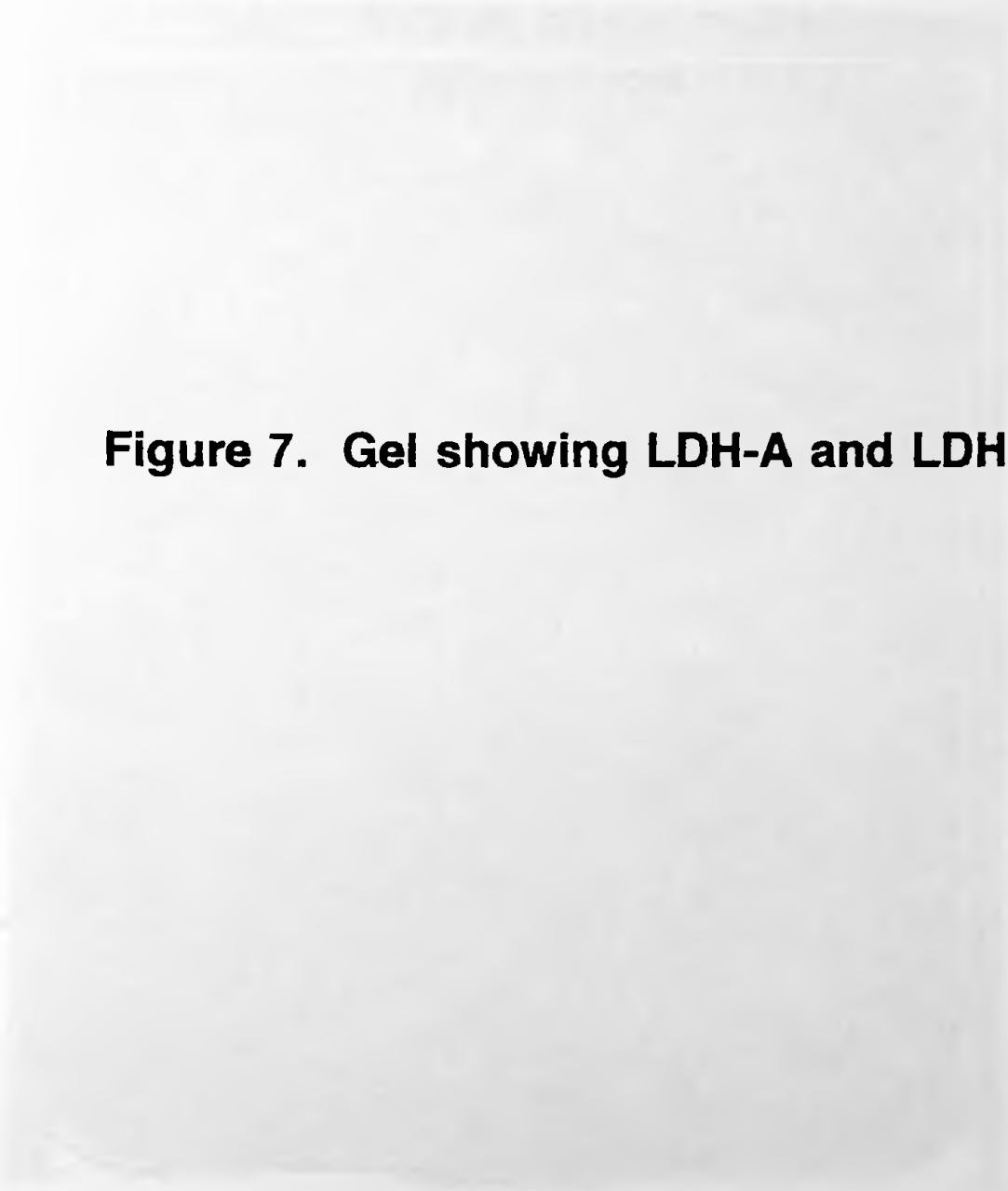


Figure 7. Gel showing LDH-A and LDH-B

LDH-B
LDH-A
pH 5
pH 8

Figure 8. Gel showing EST A, EST B, EST C, and EST D

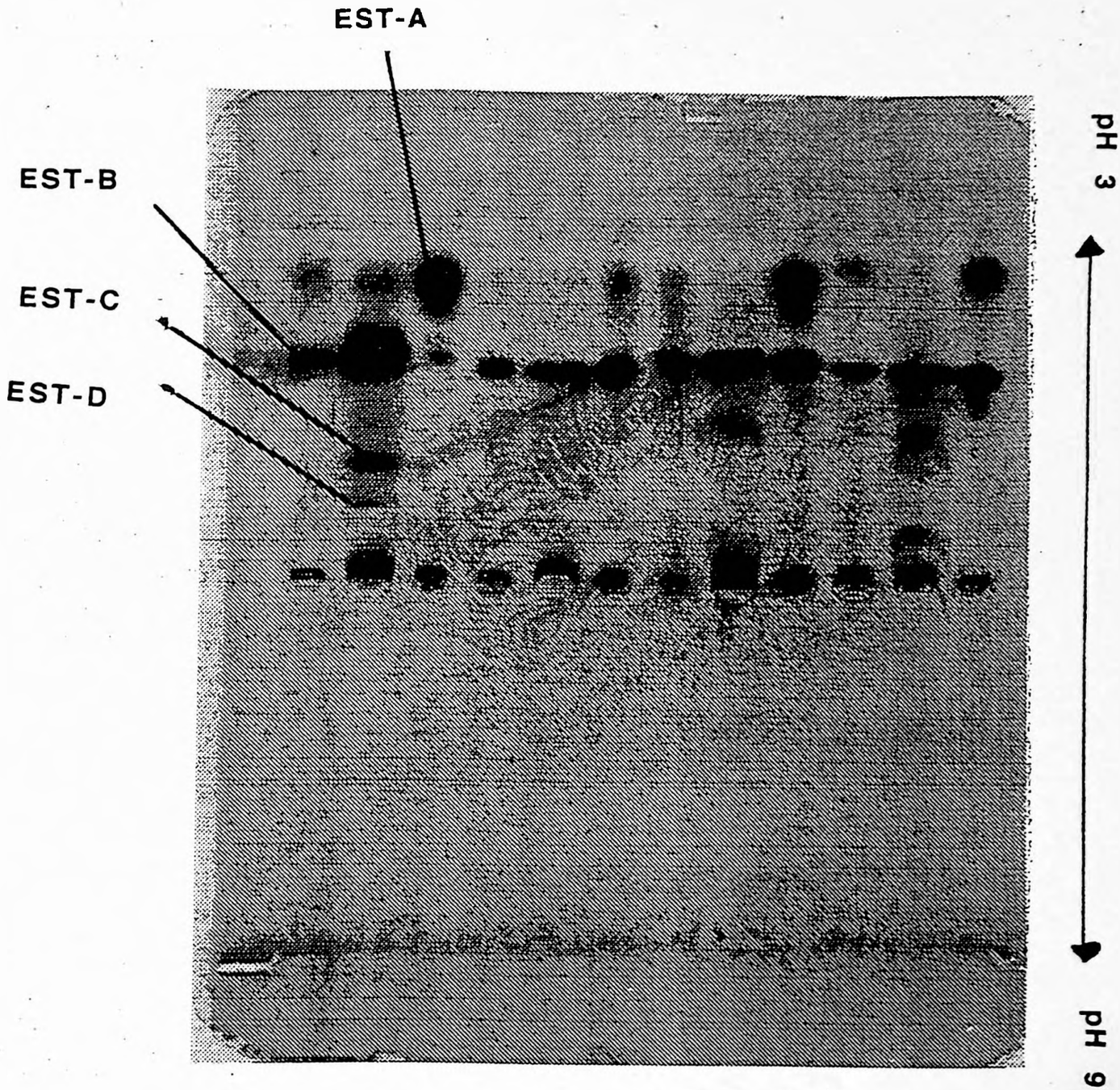

EST-A
EST-B
EST-C
EST-D
pH 3
pH 9

Figure 9. Regression equations for pI Values 5-8 and 3-9

5-8 Gels **y=-1.7277X + 6.0784**

3-9 Gels **y=3.8943X + 2.4992**

RESULTS

LDH

Isozymes for LDH-A and LDH-B were identified for 31, 62, and 22 *R. cataractae* collected from Horseshoe Run, Seneca Creek, and Glady Fork, respectively. Isozymes for these loci were also identified from 10, 7, 55, and 11 *R. atratulus* from Seneca Creek, Glady Fork, Horseshoe Run and Middle Fork, respectively. All isozymes were separated on Pharmacea 5-8 pH gels. The pI values for both loci and both species were calculated using the regression equation of y=-1.7277X + 6.0784.

All populations of *R. atratulus* were fixed for a single allele at the LDH-A locus with a pI value of 5.37 (Table 1). As expected in tissues that express two alleles from two loci as a tetramer, three heteropolymers were evident between the homopolymers. The system indicated a high level of resolution by producing two distinct isozymes for the HHMM heteropolymer.

Most *R. cataractae* produced a single allele for LDH-A with a pI value of 5.37. Two *R. cataractae* from Glady Fork expressed patterns

consistent with homozygosity for a second allele at the LDH-A locus. One fish showed some heteropolymers as double bands of protein. This is a pattern normally expressed in heterozygous fishes. However, isozyme patterns were not sufficiently resolved in this specimen to clearly identify this specimen as heterozygous.

The pI values for *R. cataractae* and *R. atratulus* LDH-B alleles were calculated using the y=-1.7277X-6.0784. Alleles of each locus were identified as the pI value. Each pI value was calculated relative to a standard curve. The standard curve generated from protein standards with pI values of 5.1, 5.9, 6.6, 6.8, and 7.2 (Figure 6). Two *R. cataractae* alleles were found with pI values of 5.39 and 5.13. The 5.39 allele was more abundant and maybe homologous to the LDH-B100 allele identified in other studies. All populations of *R. atratulus* expressed a single allele for LDH-B with a pI value of 5.34. This allele appears to be homologous to the 5.39 pI value.

Esterases

Esterases from loci A, B, C and D were extracted from 31 *R. cataractae* collected from Horseshoe Run, 62 *R. cataractae* from

Seneca Creek, and 22 *R. cataractae* collected from Glady Fork. The EST bands were clearly visible on each gel. These esterases were also extracted from 10 *R. atratulus* collected from Seneca Creek,7 from Glady Fork, 55 from Horseshoe Run, and 11 from the Middle Fork River.

The pI values for *R. atratulus* were calculated from the regression y=3.8943X + 2.4992. *Rhinichthys atratulus* expressed a single allele for Esterase A. For the muscle, the pI value was found to be 3.82. The pI Value for the head was 3.47 and for the heart it was 3.08. (Table 1).

The pI values for *Rhinichthys atratulus* was also found using the regression equation y=3.8943X + 2.4992 for Esterase B. The pI value for the muscle was found to be 4.13, head pI value is 4.06, and for the heart, it is 3.55. (Table 2).

There were eleven *R. atratulus* obtained from Middlefork. Eight of them produced esterases. There was one heterozygous pattern among *R. atratulus* from Middlefork. For EST A and EST C, patterns for both were same in head and muscle. The Esterase C pI value for the muscle was found to be 4.84, for the head the pI value was also 4.84, and for the heart, it was 4.33. (Table 3).

Seven *R. atratulus* were collected from Glady. All 7 produced

esterases. All esterase from heart tissue show a single band at the same distance as EST-C. Three of these fish had bands below EST-C.

Of fifty-five *R. atratulus* caught from Horseshoe, all produced esterases. Four of the fish show bands below Esterase C. Of 10 *R. atratulus* caught from Seneca, 8 produced esterase B in heart tissue with the same pI value as esterase C.

The pI values for the *R. cataractae* were calculated using the regression equation of y=3.8943X + 2.4992 for Esterase A. The pI value was found to be 4.17 for the heart and head tissues.

There were 31 *R. cataractae* collected from Horseshoe Run. All fish produced esterase. Of those fish, clear esterase patterns could be distinguished for six fish that had bands for Esterase B in head tissue and eleven fish that had bands only for Esterase B in heart tissue. The regression equation y=3.8943X + 2.4992 was used. The pI value was found to be 4.41 for head tissue. (See Table 5).

Sixty two *R. cataractae* were collected from Seneca Creek. Of these fish, 38 produced distinct esterase bands. 19 produced no esterase in heart and 19 produced esterase B with a single allele.

There were 22 *R. cataractae* collected from Glady. Thirteen of

these fish produced distinct esterase bands. Five produced single esterase bands in the heart tissue.

Table 1. pI Values for *Rhinichthys atratulus* using Regression equation y=3.8943X+2.4992 (Esterase A).

	MUSCLE	HEAD	HEART
Mean Distance in Pixels	0.34	0.25	0.15
pI Values	3.82	3.47	3.08

Table 2. pI Values for *Rhinichthys atratulus* using Regression equation y=3.8943X+2.4992 (Esterase B).

	MUSCLE	HEAD	HEART
Mean Distance in Pixels	0.42	0.40	0.27
pI Values	4.13	4.06	3.55

Table 3. pI Values for *Rhinichthys atratulus* using Regression equation y=3.8943X+2.4992 (Esterase C).

	MUSCLE	HEAD	HEART
Mean Distance in Pixels	0.60	0.60	0.47
pI Values	4.84	4.84	4.33

Table 4. pI Values for *Rhinichthys cataractae* using Regression equation y=3.8943X+2.4992 (Esterase A).

	HEAD	HEART
Mean Distance in Pixels	0.43	0.43
pI Values	4.17	4.17

Table 5. pI Values for *Rhinichthys cataractae* using Regression equation y=3.8943X+2.4992 (Esterase B).

	HEAD
Mean Distance in Pixels	0.49
pI Values	4.41

Table 6. pI Values for *Rhinichthys cataractae* using Regression equation y=-1.7277X+6.0784 (LDH-A).

pI Value	5.37

Table 7. pI Values for *Rhinichthys cataractae* using Regression equation y=-1.7277X+6.0784 (LDH-B).

Mean Distance in Patel	[illegible]	[illegible]
pI Value	5.39	5.13

Table 8. pI Values for *Rhinichthys atratulus* using Regression equation y=-1.7277X+6.0784 (LDH-A).

[illegible]	[illegible]
pI Values	5.34

Table 9. pI Values for *Rhinichthys atratulus* using Regression equation y=-1.7277X+6.0784 (LDH-B).

[illegible]	[illegible]	[illegible]
pI Values	5.37	5.18

DISCUSSION

Isoelectric focusing and accessment of anthropogenic stress:

In this study, the ability of IEF to efficiently separate and label alleles was examined for six loci from two species of Appalachian fishes. The effectiveness of IEF focusing was determined by comparing results from IEF with those previously reported from studies using starch gel electrophoresis. Alleles of LDH-A, LDH-B, and four esterase loci were identified for *R. atratulus* and *R. cataractae*. The pI values for each allele were determined by regression analysis relative to protein standards with known pI values. Because few investigators have used IEF to separate isozymes, the ability of this method to reliably identify alleles is poorly understood.

If IEF efficiently separates isozymes, it may be used to measure effects of human activity on genetic diversity of Appalachian fishes. Simonin et al. (1993) and other investigators have related such factors as environmental acidification and metal toxicity to reduced survival and growth rates. However, the relationship between environmental deterioration and genetic loss has been little studied.

Investigators have hypothesized that continuous environmental

stress measurably changes the genetic diversity of fish populations. Kopp et al., (1993) exposed five species of fish, including *Rhinichthys atratulus*, to low pH, high aluminum, soft water. *Rhinichthys atratulus* was identified as an environmentally sensitive species with low heterozygosity. It is suggested that any relationship between genetic variability and stress tolerance may lack predictive power when applied to specific species and specific stressors.

Investigators have related frequent genetic drift events with reduction in allelic diversity or associated stress with increased heterozygosity. Fore et al., (1995) examined eight variable loci from the stoneroller, *C. anomalum*, and found significantly different levels of genetic diversity between sites upstream and downstream from point sources of contaminants. However, changes in allelic frequencies and numbers of alleles were not consistent with values from the Index of Biotic Integrity or Invertebrate Community Index and values from these indices were not considered to be predictive of genetic diversity.

Comparison of IEF and starch gel electrophoresis:

The protein polymers produced from LDH-A and LDH-B loci have been

studied for many fishes. During starch gel electrophoresis,homozygous LDH-A (AAAA) homopolymer is the most cathodal of LDH proteins. The LDH-B homopolymer the most anodal homopolymer and heteropolymers (BBBA, AABB, AAAB) appear as bands between LDH A and LDH B. Therefore, a total of 5 bands are observed when fish are homozygous for both loci. If a fish is heterozygous for one locus and homozygous for another, 17 bands are produced; fish heterozygous for both loci produce 36 bands (Hillis and Moritz, 1995).

Clayton and Gee (1969) studied Lactate Dehydrogenase isozymes in *R. cataractae* and *R. atratulus* and their hybrid. Five isozymes were found after electrophoresis of the muscle and heart tissue. Among the first generation *R. cataractae* , two kinds of heart subunits were observed. Of 30 species of fishes, Market and Faulhaber (1965) found only 3 which produced all five possible HM tetramers (Clayton et al. 1969). This research mainly proved that there is more than one A gene present as well as more than one B gene present among these fish population.

Beck *et al.* (1984) used isoelectric focusing to determine LDH patterns in carp species and their hybrids. Female grass carps were

crossed with male bighead carps. Three genes for LDH (A, B, C) were found. The C locus was active only in the liver tissue. This study showed that there is the presence of the B2 gene, and not just one B locus/gene. Isoelectric focusing revealed extra LDH bands in liver extract between the A4 and C4 isozymes.

Cyprinid fishes produce homopolymers and heteropolymers in muscle, brain, and heart tissues. These isozymes contain polypeptides from both LDH-A and LDH-B are identified as heteropolymers. *R. atratulus* and *R. cataractae* are cyprinid fishes. Cyprinid fishes produce polypeptides from both LDH-A and LDH-B in the head, heart, and muscle tissue (Clayton and Gee, 1969). Consequently, a minimum of five bands are found in LDH gels if a fish is homozygous for both loci and more than five bands are produced if a fish is heterozygous for either locus.

In this study, a single allele was detected for LDH-A and two alleles for LDH-B for both *R. atratulus* and *R. cataractae* . If IEF effectively separated these three alleles, then three basic isozyme patterns should be detected: homozygous for A and B1 with five bands; homozygous for A and B2 with 5 bands, and homozygous for A and heterozygous for B with 17 bands. The distribution of alleles at the B locus should follow

Hardy-Weinberg proportion. Among all *R. atratulus*, only a single allele was found for A. If all the genes were found to be A1, then it can be said that it's fixed for that allele. If any A2 gene were found then it is named heterozygous. LDH A is mostly found in the muscle. It is not common in the heart and LDH B is dominant in the heart.

Among *R. cataractae* populations in this study, a B2 allele (heterozygous) was present in three fish. *Rhinichthys cataractae* numbers 303, 307, and 308 were found to fall in this category. It was observed that when B2 was present, it appeared above B1 and hence, there were more than the usual five bands on the gel. In addition, these three fish had a downward shift of all bands due to the presence of B2. All other *R. cataractae* had the five bands produced from one A1 and one B1 band.

Three LDH loci are expressed in fishes. LDH-A is expressed in all tissues but is predominant in muscle. The gene LDH-B is also expressed in all tissues but is predominant in heart tissue. The gene LDH-C is expressed predominantly in the eye and brain (Phipp *et al.* 1983). The gene LDH is tetramer that contains polypeptides from either LDH-A or LDH-B producing a homopolymer or can contain polypeptides from more than one locus producing heteropolymers. A fish is homozygous for both loci produces five bands: two homopolymers from LDH-A and LDH-B (AAAA and

BBBB) and three heteropolymers (AAAB, AABB, and ABBB) containing polypeptides of both loci (Hillis and Moritz, 1996).

In this study, esterase gels showed genes A, B, C, and rarely D. Gene A was at the acidic end with genes B, C, D sequentially positioned towards the basic end of the gel. Esterase B showed two different patterns (one that resembled a "dice pattern" and the other had a unique pattern where the middle band was much higher up). The EST B gene was found only in heart tissue. Among the *R. atratulus* populations only, the heart (EST B) were found at the same level as EST C. If in any of the fish, two EST B isozymes were found, it was assumed that the fish was heterozygous due to the presence of allelic differences.

All esterases focused in the anodal end of the gel and produced isozymes indicative of genes A, B, and C. In order of focusing distance esterases focused sequentially from A to D as pH of the pI value increased. In both species, tissue specific patterns were found. Esterase A was the most anodal migrating esterase and was expressed in head and muscle tissues. Esterase B was present only in heart tissue. Esterase C was expressed in head and muscle tissue. All esterases found in the area nearest the cathodal area below esterase C was identified as esterase D. Two staining systems were used. Bands were more intense when alpha

napthyl acetate was used as a substrate. Beta napthyl acetate produced pink and lighter bands that could not always be resolved.

Isoelectric focusing has been found to be a consistent and reasonable method to separate esterase and LDH isozymes. This method was particularly useful in the separation and labeling of esterases of loci A, B, and C. However, clear patterns of heterozygosity expected for alleles of LDH-B were not apparent. Until heterozygous individuals are clearly identified and their allelic patterns tested for Hardy-Weinberg proportion, the usefulness of this method will remain in question.

CONCLUSION

Overall, Isoelectric focusing was found to be an effective and efficient method for the analysis of isozyme diversity of *Rhinichthys cataractae* and *Rhinichthys atratulus*. Whether IEF can effectively assay isozymes was determined here that may be used in future environmental studies. Both of these fish are known to be sensitive to anthropogenic stress found by previous studies. IEF gels were stained successfully by conventional methods for 5 common loci, LDH-A, LDH-B, EST-A, EST-B, and EST-C.

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