GENETIC STUDIES OF TWO SPECIES OF INDIAN CATFISHES

ABSTRACT

ALKA (TANDON) PRAKASH
DEPARTMENT OF ZOOLOGY
SCHOOL OF LIFE SCIENCES
REGISTRATION NO. 406

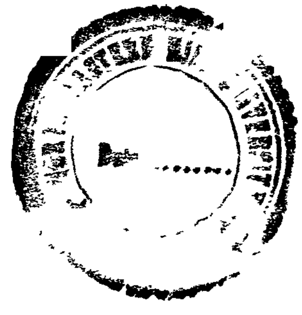
THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY

TO

NORTH-EASTERN HILL UNIVERSITY
SHILLONG, INDIA
APRIL, 1991

DS
597.520954
ALK

NEHU LIBRARY
A
A by
Date 17/10/2000
Class by
E
E by



Air-breathing fishes can use atmospheric oxygen directly for respiration, besides their gill-respiration. They are marketed alive and are also referred to as "live fishes". Both Clarias batrachus and Heteropneustes fossils are readily available in the North-eastern part of India and they fetch a high price due to their nutritive value.

The present genetic work on both these catfishes is biochemically oriented. It has been divided into various sections, including introduction, materials and methods, observations, discussion and concluding remarks. In each of these sections we have included the study of four dehydrogenases, that is, lactate dehydrogenase, malate dehydrogenase, alcohol dehydrogenase, and glucose-6-phosphate dehydrogenase, in at least six tissues from these fishes. Related work has been referred to in the introductory section.

Live fishes were sacrificed in the laboratory and fresh tissue samples from the kidney, liver, eye, muscle, brain, heart and gonads were used. All the experiments were carried out in a cold condition, below

4 degrees centigrade. In order to study the isozymes of the above mentioned enzymes the technique used was polyacrylamide gel electrophoresis followed by specific staining procedures. Distinct bands were obtained on the rod gels which were photographed and are presented in the various plates. Heat inactivation studies were also carried out. The bands were characterised and related to the genetics of these fishes.

Lactate dehydrogenase isozymes can be visualised on the gel due to the difference in their net charge. In most vertebrates this tetrameric enzyme is coded by two gene loci, LDH A and LDH B which synthesise two subunits A and B. The random association of these subunits yields five isozymes. The A subunit predominates in tissues under anaerobic glycolysis like the skeletal muscle and the B subunit in tissues with aerobic metabolism like the heart. Teleostean fishes possess a third locus the LDH C locus in addition to the LDH A and LDH B loci. The C subunit containing isozymes are synthesised by the neural tissues or the digestive tissues, predominantly in the liver. In *Clarius batrachus* and *Heteropneustes fossilis* at least three

LDH loci, the LDH A,B, and C loci, code for the observed isozyme patterns. Tissue-specific expression of the various isozymes is observed and a reverse mobility of the A and B isozymes is seen, as compared to most other vertebrates. In C-batrachus the cathodal-most kidney specific band, K , is seen to be present in the brain and heart tissues as well. This is distinct from the liver specific, C /F, band in its mobility. The K subunit differs distinctly from the C subunit, the former having a much higher molecular weight than the latter, as is observed from the detailed experiments in our present work. The expression of both the kidney -specific and the liver-specific bands in an individual gives us a clue to the idea that there might be more than three loci at play in this fish resulting in the unique pattern of the LDH isozymes observed therein.

Malate dehydrogenase is a dimeric enzyme catalysing the inter-conversion of malate to oxaloacetate in the Krebs cycle. It exists in two forms, the supernatant MDH(s-MDH) and the mitochondrial MDH(m-MDH). We have used the s-MDH which is known to be dimeric in nature and is coded by two gene loci, the MDH

A and the MDH B loci. Most tissues in our fishes exhibit a three band pattern along with the expression of some allelic forms. The kidney and liver tissues are sites of high MDH activity. There is evidence for the activity of an allele, the A', in these fish along with the A and B loci of MDH.

Alcohol dehydrogenase is primarily a liver-specific protein. It catalyses the interconversion of many alcohols to their corresponding aldehydes and ketones in the presence of NAD(H). In most fishes it is encoded by a single locus, its distribution varying from tissue to tissue. An overall analysis of this dehydrogenase in both Clarias batrachus and Heteropneustes fossilis shows the existence of at least two loci coding for the observed isozyme patterns. In C-batrachus three bands are scored in the eye tissue and in H-fossilis the maximum number of band phenotypes scored in the various tissues has been six. We have postulated two different models showing the phenotypic expression of various loci in our fishes. In C-batrachus two gene loci, A and B, code for the observed isozyme patterns whereas in H-fossilis an additional

allele, the A' gene, along with the A and B loci is responsible for the ADH activity observed in the various tissues of this fish.

Glucose-6-phosphate dehydrogenase catalyses the glucose-6-phosphate oxidation, providing NADPH for biosynthetic purpose, in the pentose phosphate pathway. G6PD isozymes in mammals are encoded in two separate loci, one locus being sex-chromosomally linked and the other being antosomal. The latter reacts more actively with galactose-6-phosphate and is now referred to as Hexose-6-phosphate dehydrogenase. Two forms of G6PD, called A and B, are also found in Clarias batrachus. The A fraction corresponds to G6PD and is observed in all the tissues, exhibiting high activity of this enzyme. The form corresponding to H6PD is observed only in the liver tissue. Here, G6PD is encoded by a single locus resulting in a single band as is observed in the various tissues of this fish. In Heteropneustes fossilis the brain and eye tissues exhibited a five band phenotype, whereas in other tissues G6PD activity was observed as a single band. In this fish G6PD may be a tetrameric enzyme resulting from the products of two codominant

autosomal gene loci. No sex differential G6PD activity was observed in both these fishes.

In the concluding remarks we have suggested what work can be done in the future, on the same lines, in order to confirm and fill the lacunae in the present work. This would involve a population genetic study related to the ecology of these fish.

We have also made a morphometric and meristic study on these fish. This has been included in Appendix I. This was done mainly because during the course of our research we came across some unique individuals of C. batrachus which had a distinct spotted appearance and a pinkish cream body colouration. Various morphometic parameters have been taken into account and regression equations of these parameters related to the entire body length have been presented in various tables. Length-weight relationships and the relative length of the gut of these fishes have also been analysed.

In Appendix II we have dealt with the karyotypic study of the chromosome complements of C.

batrachus and H. fossilis. There has been some controversy regarding their 2n number which has been confirmed in the present study. We have found that in C. batrachus, 2n=52 and in H. fossilis, 2n=56.

NEHU LIBRARY
Acc. No
Acc by
Date
Class by
Sub Head
Enter by
Transcribed

GENETIC STUDIES OF TWO SPECIES OF INDIAN CATFISHES

ALKA (TANDON) PRAKASH

DEPARTMENT OF ZOOLOGY

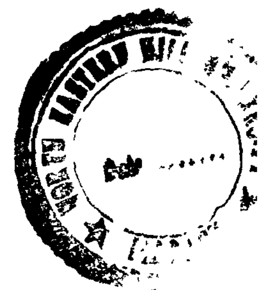
SCHOOL OF LIFE SCIENCES

REGISTRATION NO. 406

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

TO



NORTH-EASTERN HILL UNIVERSITY

SHILLONG, INDIA

APRIL, 1991

DS
597.520954
ALK

NEHU LIBRARY
A 102758
Date
Class by
Sub Heading
17/10/2000

PHONE :27760
GRAMS : NEHU

North-Eastern Hill University

Mayurbhanj Complex, Nongthymmai, Shillong-793014

Dean, Prof. K. Chatterjee, Dept. of Zoology
School of Life Sciences

11 th April, 1991

This is to certify that the thesis entitled "Genetic Studies of two species of Indian Catfishes", submitted by Mrs. Alka (Tandon) Prakash for the Degree of Doctor of Philosophy of the North-Eastern Hill University, Shillong (India), embodies the record of original investigations carried out under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of Ph. D. degree. This work has not been submitted for any Degree of any other University.

(K. Chatterjee)
Supervisor

Forwarded
A. Rajan Varman
9/4/91

Head
Department of Zoology
School of Life Sciences
North Eastern Hill University
Shillong

ACKNOWLEDGEMENTS

I wish to take this opportunity to express my deep sense of gratitude to my supervisor, Professor K. Chatterjee, Department of Zoology, Dean of School of Life Sciences, NEHU, for his guidance, aid and constant encouragement throughout the present work.

I am indebted to Dr. R.N. Sharan, Department of Biochemistry, NEHU, for extending his help and providing me assistance in the biochemical analysis of certain data.

I would like to thank Professor A. Raghuvarman, Head, Department of Zoology, NEHU, for providing me the necessary laboratory facilities during this study.

I would like to appreciate my seniors Dr. S.N. Datta and Dr. R.K. Bhattacharjee and my colleagues Moloy, Robin, Maitreyee, Pawanjit and Christopher, Cytogenetics laboratory, NEHU, for their invaluable help and generosity. My friends Jharna, Devjani and Shiv Prasad have also been a source of encouragement for me.

Thanks are due to Mr. R. Srivastava and Mrs. S. Das for typing the manuscript.

Finally I am grateful to my parents, brother and sister for their support and endurance and also to my husband, Dr. Sant Prakash, for his tremendous patience and inspiration throughout the course of my work.

A. Prakash.
11.4.91.

(ALKA PRAKASH neé TANDON)

CONTENTS

		Page No.
	FOREWORD	(i) - (ii)
1.	INTRODUCTION	1 - 20
	1.1 Lactate dehydrogenase	6 - 12
	1.2 Malate dehydrogenase	12 - 15
	1.3 Alcohol dehydrogenase	15 - 16
	1.4 Glucose-6-phosphate dehydrogenase	16 - 18
	1.5 Review of related biochemical work on Indian fishes	18 - 20
2.	MATERIALS AND METHODS	21 - 46
	2.1 Experimental animals	21 - 23
	2.2 Experimental methodology	23 - 32
	2.3 Separation of isozymes	32 - 34
	2.4 Heat inactivation	34 - 35
	2.5 Quantitative estimation of protein	35 - 38
	2.6 Partial purification of LDH from the kidney and liver tissues of <u>clarias batrachus</u>	38 - 46
3.	OBSERVATIONS	47 - 77
	3.1 Lactate dehydrogenase isozyme pattern	47 - 65
	3.2 Malate dehydrogenase isozyme pattern	65 - 72
	3.3 Alcohol dehydrogenase isozyme pattern	72 - 75
	3.4 Glucose-6-phosphate isozyme pattern	75 - 77
4.	DISCUSSION	78 - 134
	4.1 Lactate dehydrogenase	78 - 105
	4.2 Malate dehydrogenase	105 - 118
	4.3 Alcohol dehydrogenase	118 - 125
	4.4 Glucose-6-phosphate dehydrogenase	125 - 134
5.	CONCLUDING REMARKS	135 - 139
6.	REFERENCES	140 - 172
7.	FIGURES	173 - 193
8.	PLATES	194 - 201
	APPENDIX I	202 - 236
	APPENDIX II	237 - 247

List of Tables

		Page No.
Table 1.	Preferential inactivation of LDH isozymes by incubation in *Clarias batrachus*.	53
Table 2.	Preferential inactivation of LDH iso zymes by incubation in *Heteropneustes fossils.*	58
Table 3.	Values for the standard graph for protein assay	61
Table 4.	Values for the protein assay in the kidney tissue extract applied to the chromatography column	61
Table 5.	Values for the protein assay in the liver tissue extract applied to the chromatography column	61
Table 6.	Protein concentration in the kidney tissue fractions	63
Table 7.	Protein concentration in the liver tissue fractions	64
Table 8.	Comparative Rf values and molecular weights of the two cathodal LDH subunits found in the kidney (K) and liver (F/C) tissues of *Clarias batrachus*	66
Table 9.	Occurance of multiple electrophoretic forms of MDH in two major classes of vertebrates (Modified from Bailey *et al.*, 197Ø)	114
Table 1Ø.	Occurance of A and A' subunits of MDH in terms of number of individuals	116
Table 11.	Enzyme tissue specificity in *Clarias batrachus*	133
Table 12.	Enzyme tissue specificity in *Heteropneustes fossilis*	134

APPENDIX I

Table 1.	Morphometric analysis of *Clarias batrachus*	213
Table 1.1	Regression equations on morphometric parameters of *Clarias batrachus*	215
Table 2.	Morphometric analysis of *Clarias batrachus (S)*	216
Table 2.1	Regression equations on morphometric parameters of *Clarias batrachus* (S)....	217
Table 3.	Morphometric analysis of *Heteropneu stes fossilis*	218
Table 3.1	Regression equations on morphometric parameters of *Heteropneustes fossilis*..	219
Table 4.	Meristic counts of *Clarias batrachus*...	220
Table 5.	Meristic counts of *Clarias batrachus*(S)	222
Table 6.	Meristic counts of *Heteropneustes fossilis*	223

APPENDIX II

Table 1.	Chromosome complements of *C.batrachus* and *H. fossilis*	238
Table 2.	Karyomorphological studies of the chromosome complement of *Clarias batrachus*	244
Table 3.	Karyomorphological studies of the chromosome complement of *Heteropneus-tes fossilis.*	245

List of figures

		Page No.
Fig. 1	LDH isozymes in the tissues of Clarias batrachus	173
Fig. 2	LDH isozymes in the tissues of Heteropneustes fossilis	174
Fig. 3	Heat inactivation of LDH isozymes in the kidney tissue of Clarias batrachus.	175
Fig. 4	Heat inactivation of LDH isozymes in the liver tissue of Clarias batrachus.	176
Fig. 5	Isozymes observed in various fractions obtained by ammonium sulphate precipitation in the kidney tissue extract of Clarias batrachus	177
Fig. 6	Isozymes observed in various fractions obtained by ammonium sulphate precipitation in the liver tissue extract of Clarias batrachus	178
Fig. 7	Standard graph for protein estimation	179
Fig. 8	Eluent pattern of kidney tissue enzymes by DEAE cellulose chromatography	180
Fig. 9	Eluent pattern of liver tissue enzymes by DEAE cellulose chromatography	181
Fig. 10	Approximate molecular weight of the K and C subunits of LDH in Clarias batrachus	182

Fig. 11 Proposed model of LDH isozyme evolution (based on Holmes, 1972, view). The A type LDH is depicted as the original LDH. The B type was formed by a gene duplication of the A gene. The F(cod liver type), E(teleost eye type), C(mammalian and bird testes type) and K (the Clarias batrachus kidney type) genes are shown to have originated independently from the B type genes. 183

Fig. 12 MDH isozymes in the tissues of Clarias batrachus 184

Fig. 13 MDH isozymes in the tissues of Heteropneustes fossilis 185

Fig. 14 ADH isozymes in the tissues of Clarias batrachus 186

Fig. 15 ADH isozymes in the tissues of Heteropneustes fossilis 187

Fig. 16 Genetic models for ADH 188

Fig. 17 Genetic models for ADH (a) Clarias batrachus (b) Heteropneustes fossilis 189

Fig. 18 G6PD isozymes in the tissues of Clarias batrachus 190

Fig. 19 G6PD isozymes in the tissues of Heteropneustes fossilis 191

Fig. 20 Heat inactivation of G6PD isozymes in the liver tissue of Clarias batrachus 192

Fig. 21 Heat inactivation of G6PD isozymes in the brain tissue of Heteropneustes fossilis 193

List of Plates

		Page No.
Plate 1	The fish, *Clarias batrachus*	194
Plate 2	The fish, *Heteropneustes fossilis*	194
Plate 3	LDH isozyme pattern in the tissues of *Clarias batrachus*	195
Plate 4	LDH isozyme pattern in the tissues of *Heteropneustes fossilis*	195
Plate 5	Heat inactivation of LDH isozymes in the kidney tissue of *Clarias batrachus*	196
Plate 6	Heat inactivation of LDH isozymes in the liver tissue of *Clarias batrachus*	196
Plate 7	LDH isozyme pattern in the kidney and liver tissues of *Clarias batrachus*	197
Plate 8	Comparative LDH isozyme pattern in the kidney tissue extract, a mixture of kidney and liver enzyme extract and the liver tissue extract of *Clarias batrachus*	197
Plate 9	SDS-PAGE of the kidney and liver enzyme fractions	198
Plate 10	MDH isozyme pattern in the tissues of *Clarias batrachus*	199
Plate 11	MDH isozyme pattern in the tissues of *Heteropneustes fossilis*	199
Plate 12	ADH isozyme pattern in the tissues of *Clarias batrachus*	200
Plate 13	ADH isozyme pattern in the tissues of *Heteropneustes fossilis*	200
Plate 14	G6PD isozyme pattern in the tissues of *Clarias batrachus*	201

Plate 15 G6PD isozyme pattern in the tissues of *Heteropneustes fossilis* 201

APPENDIX I

Plate 1 The fish, *Clarias batrachus* (s) 236

APPENDIX II

Plate 1 Karyotype of *Clarias batrachus* 247

Plate 2 Karyotype of *Heteropneustes fossilis* 247

F O R E W O R D

Pisces are distinct in many ways and this is reflected in the enormous work done to study this class. Pisces arose in the Palaezoic era, over 400 million years ago. They have evolved significantly since then and are still in a state of active evolution and are held to be the most species rich class of vertebrates. Some 20,000 species of fish are known to inhabit various types of water bodies of which over a thousand species of fish occur in India.

Some freshwater fishes exhibit a unique environmental adaptation and can use the atmospheric air directly for respiration, besides their gill respiration. This mode of extra-branchial respiration enables them to thrive in waters deficient in oxygen, such as those of swamps and marshy areas where the usual gill-breathing fishes cannot survive. Due to this particular characteristic these fishes are called air-breathing fishes.

In the recent years these air-breathing fishes have caught the attention of fishery scientists because of their high demand. These fishes can make use of the oxygen depleted water bodies which cannot be easily made suitable for carp culture. They can also provide a considerable scope, to play

an important role in our rural economy and hence it becomes imperative to organised planned effort to render such fish production. In the present investigation an attempt has been made to fill up the existing lacunae in the biology and genetics of two air-breathing catfishes, Clarias batrachus and Heteropneustes fossilis. Moreover, information of this type may permit the development of successful economic ration for intensive catfish farming.

These fish are marketed alive like the murrels and koi (Anabas testudineus) and are also referred to as the 'live fishes'. They are known for their nutritive, invigorating and therapeutic qualities and are recommended by physicians as a part of the diet during convalescence. In some parts of our country, live fishes are preferred to major carps and are therefore in great demand and fetch a high price. Both Clarias batrachus and Heteropneustes fossilis are readily available in the North-Eastern part of India.

In the various classes of animals methods of taxanomic, cytogenetical and biochemical approach vary, imposed by the organisms themselves. Theoretically one would think that a

method of study would have equal significance in each biological division. However, it is not so. This is important and has to be remembered not only in dealing with various classes of organisms, but also with subdivisions down to the species and population level. In our present study we have adopted mainly a biochemical approach to analyse the genetic make up of both *Clarias batrachus* and *Heteropneustes fossilis*. During the course of research some cytogenetical and morphometric studies were also conducted. All this work is presented in the following chapters of this thesis.

1. INTRODUCTION

A consensus of estimates show that there are approximately 20,000 species of fishes living in the world today. This is almost half of all existing vertebrates with amphibians numbering about 2500, reptiles 6000, birds 8000 and mammals 4,500 species respectively (Lagler et al., 1962). Fishes are by far the largest class of vertebrates and show a far greater diversity of morphology and possibly of origin than do the other class of vertebrates. Despite the very large number of papers on general biology and taxonomy in the last few decades, fishes have not attracted as much attention as have the other classes of vertebrates. Most of the species studied are commercially important.

Looking at the overall picture of vertebrate evolution it is evident that genetic foundation which assumed the emergence of mammals and of man was laid at the very beginning, that is, at the stage of fish. Gershler's (1914) results of crosses between Xiphophorus and Platypoecilus, investigations on Zoarces and Lebistes (Schmidt, 1919), analysis of sex determination in Lebistes (Winge, 1930) and similar work on Oryzias by Aida (1930) are amongst the few examples of the

earliest work done in the field of genetics.

The molecular nature of the mechanisms for regulating gene function in air-breathing fishes have not been elucidated although the complexity in terms of the properties of their constituents as the physical structure of the genetic material is now known. Within a complex metazoan there are many different cell types, each of which represents the product of differentiative mechanisms transforming the zygote into the adult organism. The characteristic features of adult cells are ultimately attributable to their protein and more specifically enzyme complement, in terms of types of enzymes present and in the molecular homogeneity of a particular enzyme. Since genes for all the proteins found in an organism are present in each of the cell types, according to current biological dogma, it is of considerable interest to study their differential expression to gain insight into the factors controlling this expression. It has been observed that the relationship between certain enzymes and morphological differentiation is so close that often the appearance or disappearance of a specific enzyme is considered as a "biochemical marker" (Scholl and Anders, 1973).

A meaningful way to approach the overall problem of gene expression is to examine qualitative and quantitative aspects of a specific protein during the development of an organism. Isozymes, the multiple molecular forms of an enzyme, are particularly useful for such a study. These isozymes are generally found in species-specific and tissue-specific patterns. In addition, the relative proportions of the enzymes change in accordance with the state of differentiation of the tissue, the type of adult tissue and the tissue environment. This form of differential gene expression may endow an organism with a precise and refined control over metabolic events, as enzymatic requirements of different tissues fluctuate under varying metabolic stresses. It seems probable that both genetic and epigenetic factors regulate the expression of the enzyme in terms of its isozyme complement (Goldberg, 1969) and by comparing the properties of homologous proteins from different taxa one is in essence comparing their genes (Gorman et al., 1971).

Biochemists and physiologists have provided considerable information concerning the molecular machinery employed by species for adapting to environmental stress.

Utilising electrophoretic and histochemical techniques, population biologists have uncovered a large portion of protein variation within populations of the same species. These data have stimulated discussion concerning the role of natural selection in maintaining protein polymorphisms and the possible role of environmental factors. It is also asserted that allelic variability is a 'common' strategy for maximising population fitness in a variable environment (Powell, 1975). This hypothesis would predict restricted genetic variability in 'constant' environments.

Biochemical techniques designed to characterise and compare species on the basis of protein differences were started by Nuttal (1904). The technique of separating isozymes and isoallele forms of proteins by electrophoresis in starch and acrylamide gels was initiated in the late fifties (Smithies, 1955; Hunter and Markert, 1957) and further elaborated subsequently (Davis, 1964; Hubby and Lewontin, 1966). The refinement of these modern biochemical techniques of gel electrophoresis has enabled the genetic diversity, population structuring and evolutionary relationship of many fishes to be directly appraised (Allendorf and Utter, 1979;

Ferguson, 1980). Several other applications of biochemical genetic methods for the identification of fish populations have demonstrated the utility of electrophoresis as a technique for fisheries management (De Ligny, 1972; Utter _et al._, 1974; Thompson and Contin, 1980).

Electrophoretic screening of enzyme polymorphism has also been used as a successful adjunct to morphologically based systematics. When used in conjunction with a variety of accepted mathematical models of genetic similarity and divergence and dendrogram generating procedures, allozymic screening of large species populations at many distinct genetic loci enables a reasonable test of more classically derived phylogenies at the molecular level (Avise, 1974; Buth, 1984; Frankel, 1985). It has recently become apparent that while highly polymorphic loci permit relatively fine structured mapping of micro-evolutionary processes at the species level, slowly evolving loci, those that are highly conserved and exhibit limited polymorphism in natural populations, are quite useful in the solution of higher level or supraspecific systematic problems (Hedges, 1987).

Considerable knowledge has accrued concerning isozymes

and, therefore, in nature makes it an ideal system for studying all aspects of molecular and biochemical genetics. We have selected four dehydrogenases viz. lactate dehydrogenase, malate dehydrogenase, alcohol dehydrogenase and glucose-6-phosphate dehydrogenase for our present work.

1.1. Lactate dehydrogenase

Lactate dehydrogenase (LDH; E.C. 1.1.1.27) isozymes constitute a multigene family whose members are developmentally regulated (Lindsay, 1963; Zinkhàm *et al.*, 1963; Goldberg, 1963).

Apella and Markert (1961) dissociated LDH into four polypeptides which could be separated into two classes on the basis of charge in vertebrates. Each LDH isozyme is comprised of four polypeptide subunits which are assembled from two different kinds of polypeptides, A and B, synthesised under the control of two different genes (Markert, 1962; Cahn *et al.*, 1962; Shaw and Barto, 1963; Boyer and Fainer, 1963; Nance and Smithies, 1963; Dawson, 1964; Morrison and Wright, 1966). The A isozyme is found predominantly in tissues which may undergo

anaerobic glycolysis (e.g. skeletal muscle) whereas the B isozyme is the major form in tissues with an aerobic metabolism (e.g. heart; Wilson et al., 1963). The number and pattern of combination of the subunits of LDH show variations in vertebrates. Mammals show principally five tetramers and so do major vertebrates (Markert, 1963). These isozymes exist as homotetramers and as both, the homotetramers and the entire range of heterotetramers in vertebrates (Blanco et al., 1964).

In fish the lowest number of LDH isozyme phenotypes have been observed in flat fishes, showing only the A_4 band, followed by the fishes of Soleidae showing the A_4 and B_4 isozymes (Cahn et al., 1962; Markert and Faulhaber, 1965). The greatest number of LDH bands have been resolved electrophoretically for Salmonids, having as high as 27 bands (Odense et al., 1966).

Vertebrates also express a third type of LDH (Boyers et al., 1963; Nance et al., 1963; Shaw and Barto, 1963; Kraus and Neely, 1964). In mammals and birds this enzyme is expressed only in primary spermatocytes and has been referred to as LDH X

or C isozyme (Zinkham et al., 1964; Markert and Faulhaber, 1965; Goldberg, 1965; Daugherty, 1965). Many fish also have a third type of LDH which, unlike the C isozyme of warm blooded vertebrates, is not restricted to the primary spermatocytes. Most advanced teleosts limit the expression of this form of LDH to neural tissues such as the eye and the brain leading to the term E_4 (Whitt, 1968, 1969; Holmes and Markert, 1969; Markert and Ursprung, 1962). In Gadi-formes and Cypriniformes the liver is the tissue of choice for this isozyme (Kepes and Whitt, 1972; Whitt et al., 1973). It is also found as a minor component in blood, retina, brain and stomach (Shaklee et al., 1973). This form has been called the F_4 or liver specific LDH (Sensabaugh and Kaplan, 1972). In lower bony fish this LDH is expressed in heart, liver, gills, kidney and gonads (Markert et al., 1975).

Many polymorphisms at the LDH loci have been described in fish (Markert and Faulhaber, 1965; Hochachka, 1966; Odense et al., 1966, 1969; Lush and Cowey, 1968; Clayton and Gee, 1969; Hodgins et al., 1969; Utter, 1969).

With the advent of electrophoresis the study of

protein polymorphism was taken up and many natural populations were found to maintain a considerable level of protein variation. Scholl (1973) compared electrophoretic variation of enzyme proteins which are encoded in ten enzyme loci in platyfish and swordtails and observed a very low degree of structural variation of protein as compared to other congeneric species. Genetic variability for individual loci A, B and C, and the genes in particular, result in the diversity of LDH forms in hagfish, flatfish, salmonids, herring, cod, carp and hake (Lush, 1970; Wright and Atherton, 1970; Utter and Hodgins, 1971; Johnson, 1971; Dando, 1971; Sachko, 1971). Clayton and Franzin (1970) studied the genetics of multiple LDH isozymes in the muscle tissue of lake whitefish and found that the two distinct multimembered sets of LDH isozymes in lake whitefish were consistent with the previous observations of LDH in European coregonids (Klose et al., 1968; Ohno et al., 1968) and North American trout (Bailey and Wilson, 1968, Massaro and Markert, 1968; Ohno et al., 1968). This polymorphism in the LDH isozymes was observed as a consequence of gene duplication in the tetraploid species of salmonids.

Biochemical polymorphism at the LDH loci was also noted in Red sea bream by Taniguchi and Okada (1980). Some authors (Leslie and Vrijenhoek, 1977, Kirpichnikov and Muske, 1980) were of the opinion that protein polymorphism is largely determined by differences in species ecology.

The allelic LDH-B subunits found in rainbow trout livers were differentially designated as B' and B" (Tsuyuki and Williscroft, 1973). $H\kappa^{B}$ and $H\kappa^{A}$ (Bailey et al., 1976) or B' and B" (Utter and Hodgins, 1972). A direct correlation of B" allele was established with superior swimming endurance ability (Huzyk and Tsuyüki, 1974) and the observation of the convertive behaviour of the B"B" phenotype under low oxygen stress suggests an explanation for their poor swimming performance (Klar et al., 1979) and high mortality (Kao and Farley, 1978). Electrophoretic differentiation studies in four strains of domesticated rainbow trout show as much variability as in the wild type (Busack et al., 1979) leading to the conclusion that this variability may be a sample of a large amount of initial variability created by mixing trout from several sources to form the strain or it may be maintained by balancing selection.

Study on LDH allozymes in the nine-spined stickleback revealed the existence of three LDH loci (A, B and C) giving multiple isozymes but a reduction in B-gene expression and a widespread expression of A locus (Gesser and Poupa, 1973; Rooney and Ferguson, 1985). Variants at the LDH A, B and C loci have also been observed in Zoarces populations (Simonsen and Christiansen, 1985).

Analysis of the geographic distributions of electrophoretically detectable genetic variation can be a useful means for inferring the genetic structures of natural populations and for delineating taxonomic relationships (Allendorf and Utter, 1979). The geographic variation in LDH B gene frequencies of Fundulus heteroclitus may be due to the differences in temperature dependence and the pH dependence for pyruvate reduction found between the LDH-B allozymes which may in turn reflect a selective adaptation (Place and Powers, 1979). The kinetics of LDH in cardiac and skeletal muscle of an Antarctic teleost have been compared with a temperative teleost of comparable morphology and ecology. Dissimilarities at the biochemical level have been observed, which may be in response to the different temperature fluctuations (Fitch, 1988).

Lactate dehydrogenase is a slowly evolving gene cluster and is concerned with the evolution of the three main genes mentioned here. The evolutionary relationships among the LDH isozymes have been the subject of research and speculation in several laboratories over the last 20 years. Reviewing the various schemes presented by different authors we have also discussed the origin and evolution of the various LDH isozymes found in our fish species.

1.2. Malate dehydrogenase

Malate dehydrogenase (MDH; E.C. 1.1.1.37) is an enzyme of the citric acid cycle, which catalyses the oxidation of L-Malate to oxaloacetate, in the presence of NAD, which is reduced to NADH (Dey, 1984). MDH has been identified both in the mitochondria and in the cytoplasm of different tissues and occurs in two different fractions, the m-MDH and the s-MDH respectively. Both the fractions have similar molecular weight but they differ in amino acid compositions, cellular fractions, electrophoretic properties and kinetic properties (Siegel and Englard, 1960; Grimm and Doherty, 1961; Thorne and Cooper, 1964;

Wheat and Whitt, 1971). Each form is heterogenous and on electrophoresis gives several bands and sub-bands (Thorne <u>et</u> <u>al</u>., 1963). The s-MDH in conjunction with other lipogenic enzymes plays a key role in lipogenesis by generating NADH, which is utilised for lipid synthesis (Hulsman, 1962; Young <u>et</u> <u>al</u>., 1964; Skorkowski <u>et</u> <u>al</u>., 1980). Various evidences suggest that the MDH isozymes occur with identical subunits viz. A and B in the mitochondrial and the supernatant fractions and are under the control of separate genetic loci (Kitto <u>et</u> <u>al</u>., 1966; Bailey and Wilson, 1969; Wheat and Whitt, 1971; Place and Powers, 1978), a mechanism very different from that concerned in the heterogeneity of LDH. Despite a considerable difference in mobility a close similarity exists between the electrophoretic patterns of MDH of different animal species.

Various studies have revealed that MDH exists as a dimeric molecule (Davidson and Cortner, 1967; Bailey <u>et</u> <u>al</u>., 1970), with a molecular weight of about 60,000 Daltons (Murphey <u>et</u> <u>al</u>., 1967; Aspinwall, 1974). This enzyme is present in plants (Barron <u>et</u> <u>al</u>., 1961), micro-organisms (Yoshida, 1965) and animals, including fish (Grimm and

Doherty, 1961; Kitto and Kaplan, 1966; Kitto and Lewis, 1967; Bailey *et al.* , 1970; Whitt, 1970; Wheat and Whitt, 1971, ~~1972~~; Wilson *et al.*, 1973; Aspinwall, 1974; Place and Powers, 1978; Dey, 1984).

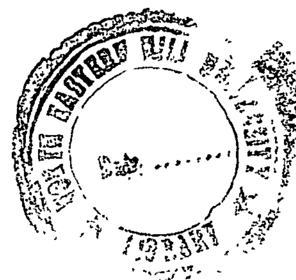
Supernatant malate dehydrogenase (s-MDH) has been studied in a diverse group of vertebrates, from agnathans to man. In fishes and amphibians three equally spaced, anodal bands of s-MDH are commonly observed (Bailey *et al.*, 1970). Wheat *et al.*, (1972) have demonstrated that s-MDH A and s-MDH B in sunfish are controlled by two unlinked loci. The salmonid fish are an interesting exception in that more than two s-MDH loci are present in these species (Aspinwall, 1974; Clayton *et al.*, 1975; Taggart *et al.*, 1981). Bailey *et al.*, (1970) have demonstrated the presence of duplicate loci encoding for the s-MDH B subunit of the king salmon, *Oncorhynchus*, which provides considerable evidence for duplicate genes (Ohno, 1970) encoding for the x-MDH A subunit in brown trout. In polyploid fishes of the genus *Hypentelium* five loci of MDH enzymes are expressed (Fisher *et al.*, 1980; Buth, 1983). Additional isozymes may result from allelic variation (Bailey, 1969, 1970; Whitt, 1970; Mann

and Vesting, 1970; Fujio and Kato, 1979; Busack et al., 1979) or from different conformational forms of the same enzyme (Kitto et al., 1966; Schecter and Epstein, 1968; Markert and Whitt, 1968). Many polymorphisms have been described for MDH B locus (Bailey et al., 1969; Richmond and Zimmerman, 1978; Place and Powers, 1978, 1979; Kirpichnikov and Muske, 1980; Philipp et al., 1981, 1983; Hines et al., 1983).

1.3. Alcohol dehydrogenase

In vertebrates alcohol dehydrogenase (ADH; E.C. 1.1.1.1.) is primarily a liver-specific protein found in parenchymal cells (Raiha et al., 1967) with minor activity in kidney, heart, lung and gut tissues. This enzyme is cytoplasmic and exhibits a somewhat broad substrate specificity (Cardemil, 1978), mediating the conversion of primary alcohols to aldehydes and secondary alcohols to ketones, utilising nicotinamide adenine dinucleotide (NAD^+) as the electron acceptor (Pietruszko et al., 1973). It is now well established that the active form of this enzyme is a dimer of about 80,000 Daltons and each monomer carries two zinc atoms with cystein residues as the primary ligands, and at

102758



least one zinc atom participates in the enzyme reaction (Branden et al., 1975).

ADH is regulated developmentally in all the vertebrates so far tested, appearing at the post fertilisation stages in fish (Hitzeroth et al., 1968; Frankel, 1980). Electrophoresis and specific histochemical techniques have demonstrated the presence of codominant allelic variation which may be used to identify various fishes (McAndrew and Majumdar, 1983).

As compared to the other enzymes, ADH has been the least utilised and studied group although it has all the potential for providing the necessary data for biochemical and genetical investigations as evident from the work of Frankel (1978, 1980, 1981, 1983).

1.4. Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49) catalyses the glucose-6-phosphate oxidation, providing NADPH for biosynthetic purposes in the pentose phosphate pathway (Bautista et al., 1984). The presence of G6PD isozymes in aquatic animals, described by several

authors (Hitzeroth *et al.*, 1968; Shatton *et al.*, 1971; Stegeman and Goldberg, 1971, 1972; Yamauchi and Goldberg, 1973; Nagayama *et al.*, 1975, Yamauchi, 1975; Yamauchi *et al.*, 1975; Cederbaum and Yoshida, 1976; Shaklee and Whitt, 1977; Philipps *et al.*, 1979; Hart and Pontier, 1982; Rodriguez-Segade and Freire, 1982) supports the hypothesis that this enzyme is crucial in the regulation of the metabolic flux of the pentose-phosphate pathway.

The ontogenic examination of G6PD in brown trout system was made by Yamauchi and Goldberg (1973) by which the parental types were shown to have distinguishable G6PD isozyme patterns and the hybrid patterns appeared to be the result of recombination of parental gene product (Yamauchi, 1975). Two G6PD isozymes with similar substrate specificities as that of mammals have been observed in lake trout (Stegeman and Goldberg, 1971). Genetic polymorphism at G6PD loci has also been demonstrated (Scholl, 1973).

In mammals, G6PD isozymes are encoded in two separate loci, one of them being sex-linked (Kirkman *et al.*, 1964) while the other is autosomal in origin (Shaw and Barto, 1965). An isozyme of G6PD having a high affinity for galactose-6-

phosphate has been detected in the liver of deer mouse, *Peromyscus maniculatus*, by Shaw and Barto (1965) and has since been found in other mammalian livers (Shaw, 1966; Ohno *et al.*, 1966; Shaw and Koen, 1968). This component migrates more cathodally than the sex-linked G6PD and stains equally well with hexose-6-phosphate as substrate and is therefore known as hexose-6-phosphate dehydrogenase (Ohno *et al.*, 1966; Shaw and Koen, 1968).

1.5. Review of related biochemical work on Indian fishes

The comparison of the arrays of isozymes including LDH, MDH, ADH and G6PD and their component polypeptides and the underlying genetic loci, in different groups and species of vertebrates seems likely to develop into an interesting exercise in comparative biochemical genetics. Though the knowledge of the occurrence of fish in India dates back to antiquity (Hora, 1956) not much work has been done as compared to the work done in other parts of the world. Chandrasekhar (1959) has studied the blood proteins of five Indian carps belonging to the family Cyprinidae. Das (1961) has analysed the blood biochemistry of three Indian carps.

Hussain and Siddique (1974) have made a comparative study on the tissue proteins of some catfishes. Menezes (1975) has analysed the eye lens and serum proteins of Sardinella sardinella longiceps electrophoretically. Krishnaja and Rege (1977, 1979) have made electrophoretic studies on the genetics of two species of Indian carps and their fertile hybrids. A study on seasonal size and a comparative study of plasma proteins of four air-breathing freshwater fishes has been made by Siddique (1977). G6PD and LDH activities in two air-breathing and two gill-breathing species of fish have been determined by Ramanujam and Ratha (1980). Basu et al., (1981) have studied the egg proteins in Notopterus notopterus and Mystus vittatus. Dhar and Chatterjee (1982) have made electrophoretic investigations on the protein variations in two species of Channa. The species specific patterns of LDH isozymes in the same genus were later reported by Chatterjee and Dhar (1985). Triveni and Rao (1986) have observed the tissue distribution and have characterised the LDH isozymes in two Cyprinids. Chatterjee et al., (1988) have reported a kidney-specific locus of LDH in Clarias batrachus. Tripathi and Shukla (1988) have made a comparative study on the skeletal muscle and liver LDH of

Clarias batrachus. Padhi and Khuda-Bukhsh (1989) have studied the LDH isozyme patterns in four species of *Mugil*. Krishna Rao *et al*., (1980) have given a survey of LDH isozymes in 52 species of teleosts, emphasizing the taxonomic significance of LDH-C gene expression. Bhattacharya *et al*., (1980) have described LDH as a genetic marker enzyme in *Tilapia mossambica*. Chatterjee (1989) has described the distribution pattern of esterases and LDH isozymes in some Indian air-breathing fishes.

The large variation among fish in the isozyme patterns of LDH, MDH, ADH and G6PD and the contrast between these patterns and those of mammals prompted us to undertake the present study, the primary object of which is to give genetic interpretations for patterns of electrophoretically detectable proteins mentioned here in the various tissues of two species of Indian catfish *Clarias batrachus* and *Heteropneustes fossilis*.

2. MATERIALS AND METHODS

2.1. Experimental animals

Two species of Indian catfishes, *Clarias batrachus* and *Heteropneustes fossilis* constitute the research specimens for the present study. They have a stout body, a flat head with a toad-like appearance and a broad mouth with four pairs of barbels. Their skin is slimy and scaleless. They are air-breathing, omnivorous and preferably bottom dwellers. Both these fishes were collected from ponds and rivers from Shillong and Gauhati.

2.1.1. *Clarias batrachus* (Linn)

Locally known as 'magur' and distributed throughout the inland waters of India, especially in the areas where water is turbid, this fish usually attains a length of about 28 cms or more. The head has a characteristic depression close to the eyes. The dorsal fin extends throughout the length of the trunk and the pectoral fins can be identified by the presence of finely serrated spines. The provision of an accessory respiratory organ, a dentritic apparatus attached to the gills, shows their amphibious mode of life.

They are nocturnal in habit and depend on small worms, midge larvae and micro-crustaceans for food (Plate 1).

Systematic position (Jhingran, 1974)

Class	:	Teleostomi
Subclass	:	Actinopterygii
Order	:	Cypriniformes
Division	:	Siluri
Suborder	:	Siluroidei
Superfamily	:	Siluroidae
Family	:	Clariidae
Genus	:	*Clarias*
Species	:	*batrachus*

2.1.2. *Heteropneustes fossilis* (Bloch.)

It is commonly known as 'singhi' and is found in all parts of India. The adults are dark brown in colour with two lateral yellowish bands and their young are reddish brown in appearance. They can be distinguished from *C. batrachus* because of their small head size, presence of a small dorsal fin and poisonous pectoral fin rays. They also possess a

characteristic respiratory air sac. They feed on worms, larvae, mussels and other small crustaceans (Plate 2).

Systematic position (Jhingran, 1974)

Class	:	Teleostomi
Subclass	:	Actinopterygii
Order	:	Cypriniformes
Division	:	Siluri
Suborder	:	Siluroidei
Superfamily	:	Siluroidae
Family	:	Heteropneustidae
Genus	:	*Heteropneustes*
Species	:	*fossilis*

2.2. Experimental methodology

Live fish were sacrificed in the laboratory and fresh tissue samples (kidney, liver, eye, muscle, brain, heart and gonads) were dissected out. These tissues were stored at -20°C and were used within 24 hours to reduce the risk of denaturation of the enzymes.

2.2.1. Homogenisation

The tissues obtained were blotted dry and weighed accurately. They were homogenised with 0.25 M sucrose solution (10% W/V) using an Umetrex electric homogeniser. The temperature was maintained below 4°C, throughout the process.

2.2.2. Extraction

The homogenate obtained was centrifuged at 15,000 x g for 15 minutes at 4°C to extract the enzyme solution. The pellet was discarded and the clear supernatant obtained containing the soluble proteins was taken up for further analysis.

2.2.3. Electrophoresis

When a particle of effective charge (Q) is forced to migrate in a viscous medium (liquid or gel) by the action of an electrical field (potential gradient, E) the phenomenon is generally referred to as electrophoresis (Maurer, 1971).

The driving force which acts upon the particle migrating with constant velocity is equal to the frictional resistance (F) which the particle must overcome in the medium, that is,

$$QE = F$$

The elctrophoretic mobility of a particle is defined as

$$m = \frac{d}{tE} = \frac{V}{E} = \frac{Q}{F} = \left(\frac{cm^2}{\text{Volt x Sec}}\right)$$

where 'd' is the migration distance of the particle in time 't', 'V' is the velocity and 'F' is the frictional resistance.

The ability of a protein molecule to migrate in an electric field depends primarily upon its net electric charge. Since this can be varied, within quite wide limits, by changing the pH of the medium, it is possible for a given protein to travel towards either electrode according to the conditions. The actual distances travelled by the various protein fractions in a given time are dependent largely on the strength of the electric field to which they are subjected. Care is exercised to avoid inactivation of the isoenzymes and local overheating. The work is carried out at 4°C.

The vertical disc electrophoresis system of Davis (1964) was employed with some modifications. The separating system is discontinuous with regard to pH value, buffer composition and gel pore size in which polycrylamide gel serves as the matrix. Disc electrophoresis is carried out in small columns of polyacrylamide gel consisting of three layers contained in suitable glass tubes. The three layers comprise of : (1) a large pore spacer or gel, (2) a small pore separation or running gel in which the sample constituents are separated out and (3) the sample solution. The gel tubes are vertically attached to two different reservoirs with the sample layer uppermost : Buffer is filled in both the reservoirs and care is taken to see that the upper end is submerged in the buffer in the upper chamber and the lower end is dipped in buffer contained in the lower chamber. Electrodes are placed in each reservoir and the polarity is set so that the sample ions migrate towards the small pore gel. A constant voltage is applied for a specific time. The gel is then removed and placed in a specific enzyme staining solution for a fixed period of time. Unbound dye is removed from the gel by washing in 7% acetic acid and then

the gel is preserved in a suitable solution. To minimize variations in isozyme patterns arising from extraneous factors, electrophoresis was carried out under strictly standardised conditions.

2.2.3.1. Reagents

These solutions are stored in dark bottles in a refrigerator and their shelf life is upto two months.

Reagent A : pH 8.9.

1 N HCl	48 ml
Tris (hydroxymethyl) methylamine	36.6 g
N, N, N, N - Tetramethyl ethylene-diamine (TEMED)	0.23 ml

The volume is made upto 100 ml with distilled water.

Reagent B : pH 6.7

I N HCl	48 ml
Tris	5.98 g
TEMED	0.46 ml
Distilled water	51.54 ml

Reagent C :

Acrylamide	10 g
Bis	0.8 g
Distilled water	100 ml

Reagent D :

Acrylamide	10 g
Bis	2.5 g
Distilled water	100 ml

Reagent E :

Riboflavin	4.0 g
Distilled water	100 ml

Reagent F :

Sucrose	40 g
Distilled water	100 ml

Reagent G :

Ammonium persulphate	0.14 g
Distilled water	100 ml

Stock buffer solution : pH 8.3

Tris	6.0 g
Glycine	28.8 g
Distilled water	1000 ml

For each reservoir, 10% strength of this stock solution is used.

Destaining solution :

Acetic acid	70 ml
Distilled water	1000 ml

2.2.3.2. Equipment used

The cylindrical glass gel tubes used were about 10 cm long with an inner diameter of 5 mm. These were fixed

vertically in the electrophoresis running chambers. The upper and the lower chambers were the buffer reservoirs with platinum electrodes dipping in the buffer. Link was maintained between the two chambers via the gel tubes. The other equipment included cables, a power supply (Systronic 604), gel tube stand, rubber caps for sealing one end of the gel tube, polymerising lamps, needle, syringe, test tubes etc.

2.2.3.3. Gel systems and their composition

7.7% separation gel was prepared just before use by mixing (V/V) the following reagents in fixed proportions

Reagent A	1 part
Reagent C	2 parts
Distilled water	1 part
Reagent G	4 parts

The spacer gel was prepared by mixing the following reagents in specific proportions :

Reagent B	1 part
Reagent D	2 parts

Reagent E	1 part
Reagent F	4 parts

2.2.3.4. Procedure

About 1.5 ml to 1.8 ml of the separation gel mixture was poured into the gel tubes which were fixed in the gel tube stand and were capped at bottom. Care was taken to prevent any air bubble in the gel column. A few drops of distilled water were carefully layered above the separation gel column. After polymerisation, the water from the top surface of the gel column was removed and 0.2 ml of the spacer gel mixture was poured carefully into the gel tubes above the separation gel. This was again carefully layered by a few drops of distilled water on the top surface and then left in fluorescence light for 30-40 minutes to polymerise.

The gel tubes were taken out from the stand and the water was removed carefully. They were then fixed vertically in the disc electrophoresis chamber as described by Davis (1964). About 100 μl of the test solution was then poured over the spacer gel column. The rest of the space in the gel

tube was filled with Tris-Glycine buffer (pH 8.3). The same buffer was then poured into the upper and the lower chamber to fill them completely. A drop of 1% Bromophenol blue indicator solution was mixed in the upper buffer chamber. This apparatus was placed at about 4°C and the two electrodes were connected to the electrophoresis power supply. The current strength was kept at 1.5 mA/gel tube for the first 15 mins and then raised to 3 mA/gel tube. A constant voltage mode of 220 V was applied. After the requisite running time, the power supply was cut off and the gel tubes were taken out from the apparatus. The gels were immediately removed from the gel tubes with the help of a long needle and water forced from a syringe and subjected to specific treatment to obtain the required isozyme patterns.

2.3. Separation of isozymes

Isozymes of an enzyme system, as it is known, are proteins of the same configuration but with a slightly different molecular weight. They can therefore be separated by electrophoresis due to their different electrophoretic mobilities.

The gels, after electrophoresis, were incubated in the specific stain for a fixed period of time. Gels incubated without the substrate served as controls. After the reaction the gels were washed and preserved in 7% acetic acid (V/V).

The following enzymes were investigated :

2.3.1. Lactate dehydrogenase (LDH; E.C. 1.1.1.27)

Buffer	:	Tris-Glycine buffer pH 8.3	
Stain	:	I M Tris-HCl, pH 8.0 - 2.5 ml	
		I M Lithium Lactate standard solution	0.5 ml
		NAD (B-Nicotinamide adenine dinucleotide)	80mg
		PMS (Phenazine methosulphate)	1.2 mg
		NBT (P-Nitroblue Tetrazolium chloride)	8.0 mg
		Distilled water	47 ml

The gels were incubated in the above solution at 37°C for 15 mins. The LDH bands obtained were violet in colour.

2.3.2. Malate dehydrogenase (MDH E.C. 1.1.1.37)

Buffer : Tris-Glycine pH 8.3

Stain : Same as LDH except that the Lactic acid substrate is replaced by 0.5 ml of I N Malic acid.

2.3.3. Alcohol Dehydrogenase (ADH; E.C. 1.1.1.1.)

Buffer : Tris-Glycine pH 8.3

Stain : The solution remains the same as for LDH except that the substrate is substituted by 1 ml Ethyl alcohol.

2.3.4. Glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49)

Buffer : Tris-Glycine pH 8.3

Stain :	0.05 m Tris HCl pH 8.0	50 ml
	Glucose-6-phosphate	40 mg
	NADP	20 mg
	PMS	0.6 mg
	NBT	15 mg

The gels are incubated in the above mixture at 37°C for 30 mins. The G6PD bands appear deep blue in colour.

2.4. Heat inactivation

All the four dehydrogenases were subjected to heat inactivation studies. The enzyme extract were taken in different test tubes and incubated at the various temperatures

for different periods of time. They were then cooled, filtered and subsequently subjected to electrophoresis and examined on gels as before.

LDH - heating from 50°-75°C for 5 mins and at 75°C for 15 mins.

MDH - heating at 50°C for 5 mins or more.

ADH - heating upto 30°C for 15 mins or more

G6PD - heating upto 60°C for 20 mins or more.

2.5. Quantitative estimation of protein

Estimation of protein is essential to get an approximation of the amount of sample required to be loaded for electrophoresis. Protein content in the various tissues was determined according to the Bio-Rad Protein assay method, which is a sample colorimetric assay for measuring total protein concentration. This method is based on the Bradford dye-binding procedure (Bradford, 1976).

2.5.1. Principle

The assay is based on the colour change of a dye in

response to various concentrations of protein (Bradford, 1976; Fazakas de st. Groth, 1963; Sednak and Grossberg, 1977; Reisner *et al.*, 1975; Pilgrim, 1977). In research applications this assay is preferred to the widely used lowry method because the absorbance of the dye-protein complex is more stable and the Bio-Rad assay does not require the initial timing necessary for other assays.

2.5.2. Procedure

I Bradford Reagent

Coomassie brilliant blue G-250 (SIGMA Chemical comp. USA)	100 mg
95% of Ethanol	50 ml

The dye is dissolved properly in a 250 ml conical flask. To this 100 ml of 85% (W/ V) phosphoric acid is added, mixed properly and then transferred into a dark bottle with a tight cap. It is stored refrigerated and can be used for a reasonably long period of time.

II Working solution

Bradford reagent	15 ml
Distilled water	85 ml

This is mixed thoroughly and filtered through Whatman number 1. filter paper. The filtrate is stored in a glass reagent bottle at room temperature. Its useful bench life is approximately two weeks.

2.5.3. Assay of protein

A Bovine serum albumin solution of 1.0 mg/ml concentration is prepared. Different tubes are numbered and taken with 0.00, 0.001, 0.002, 0.003, ... 0.01 ml of the protein solution in them. Their volume is increased to 0.1 ml by adding appropriate volumes of distilled water. To each tube 5.0 ml of the working solution is added. They are vortexed gently or inverted to mix properly but without frothing. These tubes are then incubated at 25°C in a water-bath for 5-10 mins. Their 'absorbance' is then read at 595 nm against the reagent blank between 5 min and 60 min. The protein concentration is determined after preparing a standard

curve. The stability of colour against time is recorded and that time period is fixed for future use.

2.6. Partial purification of LDH from the kidney and liver tissues of Clarias batrachus

The entire procedure for the partial purification of this enzyme was carried in cold conditions (4°C). 2 g of freshly cut kidney/liver tissue was taken and homogenised (using a kinetic homogeniser) in 2.0 ml of 50 mM sodium phosphate buffer at pH 7.0. This homogenate was centrifuged at 15,000 x g for 15 mins. to remove the cell debris.

2.6.1. Concentration by Ammonium sulphate precipitation

To the supernatant ammonium sulphate salt was added to 30% concentration with a constant slow stirring while adding the salt. This was allowed to stand for 60 mins. It was then spun again at 15,000 x g for 15 mins. The pellet was saved to check its protein content. To the supernatant, ammonium sulphate salt was added to 80% concentration, stirring slowly while adding the salt. This was left to stand for an hour,

then spun at 20,000 x g for 15 mins. The supernatant was saved. The pellet was taken and dissolved in a small volume of 10 mM Tris-HCl buffer at pH 8.5. This material was dialysed overnight in a large volume of the same buffer.

2.6.2. Chromatography

The dialysate obtained was spun at 15,000 x g for 15 mins to remove the particulate material. The supernatant was taken and the enzyme activity present in it was ascertained before applying it in the chromatography column.

Preparation of the DEAE cellulose column

To 10 g of DEAE cellulose

(a) 250 ml of 0.25 M NaOH solution was added with gentle mixing. This was left to stand for 30 mins.

(b) The NaOH solution was poured out and 250 ml of 0.25 M HCl was added. This was allowed to stand for 10 mins.

(c) The HCl was poured off and the DEAE cellulose was washed thrice with water.

(d) Steps (a) to (c) were repeated once again.

(e) 50 ml of starting buffer was added and it was left to stand for 30 mins.

(f) This was then packed carefully in the column.

0.01 M Tris-HCl (pH 8.4) containing 0.001 M mercaptoethanol was used as the starting buffer.

1 ml of enzyme preparation was loaded in the column. It was run and the enzyme fractions were collected at 2.5 mins. interval each. A voltage of 100 mV was applied along with a gradient of 0.1-0.35 M NaCl (total volume of 40 ml). Fractions with similar enzyme activity were pooled together. The fraction showing maximum enzyme activity was first tested to ascertain the amount of activity it contained and then used for SDS PAGE.

2.6.3. Slab-gel SDS PAGE

SDS PAGE was carried out on a slab gel according to the method described by Laemmli (1970), with slight modifications.

The following chemicals were used to prepare a slab gel :

Stock solution	Separating gel 10%	Stacking gel 3%
40% Monomer solution (T - 40/C - 2.5)	7.5 ml	1.0 ml
1.5 M Tris-HCl pH 8.8	9.6 ml	--
0.5 M Tris-HCl pH 6.8	--	1.5 ml
Water (Degas 10 mins)	12.6 ml	9.3 ml
10% SDS (Sodium dodecyl sulphate)	300 μl	120 μl
10% Ammonium persulphate	60 μl	60 μl
TEMED	15 μl	12 μl
Total volume	30 ml	12 ml

2.6.3.1. Procedure

The separating gel mixture was slowly poured in between two glass slabs held together by clips, with their three sides sealed. This gel was layered carefully by a few drops of

distilled water and left to polymerise for about 45 mins. Then the water was poured off and enough stacking gel mixture was poured (to about 1.0 cm height) above the separating gel system. The slot maker was inserted in between the glass slabs. This was left to polymerise for about 15-20 mins. The slot maker was then removed carefully and the apparatus was set up for electrophoresis at room temperature.

Running buffer - A 10 X buffer was prepared in the following manner :

Trizma (0.3%) - 30.0 g

Glycine (1.44%) - 144.0 g

SDS (0.1%) - 10.0 g

These were taken in 1000 ml of distilled, deionised water and dissolved completely.

Sample preparation - The test samples and the marker enzyme were separately mixed with the sample buffer in the ratio 1 : 1 (V/V) and boiled in closed vials for 5 mins in a boiling water bath.

Sample buffer

The sample buffer was prepared in the following way :

IM Tris-HCl pH 6.8	1 ml
1% SDS	4 ml
Glycerol	2 ml
Mercaptoethanol	1 ml
0.1% Bromophenol blue	0.5 ml
Distilled water	1.5 ml

All these constituents were mixed and the sample buffer was stored refrigerated.

50-100 ml of the prepared sample was then loaded in the various slots of the slab gel along with the marker enzyme in one slot, with the help of a micro-syringe and a long needle. The running buffer was filled in both the upper and the lower chambers of the electrophoresis apparatus, which were connected to the electrodes. The two chambers were linked via the slab gel. The electrodes were connected to a power pack. A constant current mode was applied at 27 for 20 mins. and this was raised to 55 after 20 mins. The voltage was

set at 300 and the power was set at 40. The slab gel was run for 6 hours after which the power was cut off.

The slab gel was carefully removed from between the glass slabs and was fixed in 20% Tricarboxylic acid for 10 mins. After fixation it was stained in the following staining mixture for 12-15 hours.

Stain

Coomassie Brilliant blue	0.4 g
Methanol	100 ml
Acetic acid	15 ml
Distilled water	100 ml

This mixture was filtered before use.

Destaining

The stained gel was then immersed in a destaining solution to remove excess of the stain picked up by the gel matrix. The destaining solution contained the following :

Glacial Acetic acid	70 ml
Methanol	300 ml
Distilled water	630 ml

Marker enzyme

SDS-6 (marker) Dalton Mark VI obtained from SIGMA was used. It contains 13.5 mg of a lyophilised mixture of the 6 proteins given below and Bromophenol blue tracking dye.

1. L - 4631 Lysozyme
   Approx. mol. wt. 14,300

2. L - 4756 Lactoglobulin
   approx. mol. wt. 18,400
   (subunit)

3. T - 9011 Trypsinogen PMSF Treated
   approx. mol. wt. 24,000

4. P - 1143 Pepsin
   approx. mol. wt. 34,700

5. A - 7642 Albumin, egg
   approx. mol. wt. 45,000

6. A - 7517 Albumin bovine

approx. mol. wt. 66,000

2.6.4. Calculations

The Rf values of the various bands observed on the slab gel were first calculated and then these values were plotted on semi log paper, and the molecular weights of the various subunits of kidney, liver LDH isozymes were determined.

3. OBSERVATIONS

3.1. Lactate dehydrogenase isozyme pattern

The lactate dehyrogenase enzyme (Lactate : NAD oxidoreductase, E.C. 1.1.1.27) is a tetrameric protein with a molecular weight of 140,000 in all vertebrates so far studied.

In most vertebrates the enzyme is coded by two gene loci (LDH - A and LDH- B) which synthesize the two subunits A and B. These two tetrameric subunits associate in the cytoplasm to yield five isozymes of the following composition, LDH-5 = A_4; LDH-4 = A_3B_1; LDH-3 = A_2B_2; LDH -2 = A_1B_3; and LDH-1 = B_4. The A_4 isozyme is found predominantly in tissues which may undergo anaerobic glycolysis (e.g. skeletal muscle) whereas the B_4 isozyme is the major form in tissues with an aerobic metabolism (e.g. heart). In addition, teleostean fishes have been shown to possess a third LDH locus, the LDH-C locus coding for LDH C_4/F_4 subunits. These C subunit containing isozymes are synthesized by neural tissues or in the digestive tissue, predominantly liver. These isozymes can be visualised electrophoretically because of the difference in their net charge.

3.1.1. Clarias batrachus

3.1.1.1. Tissue specificity

Gene products from various tissues have been scored to obtain a general LDH isozyme profile. The tissues used were chosen in order to show the differences in tissue regulation and to expose all the loci involved in the coding of LDH. These are described below :

Kidney

The kidney tissue exhibits the expression of three LDH loci. The two homopolymers and the three heteropolymers correspond to the A_4, A_3B_1, A_2B_2, A_1B_3 and B_4 isozymes resulting in the characteristic five banded LDH phenotype. In addition to these there is a characteristic cathodal band of high activity, which is designated as K_4 (Fig. 1; Plate 7). Two to three minor activity bands are present between B_4 and K_4 and are probably formed by hybridisation between these two close major bands, as shown by the heat inactivation study (Fig. 3). The B_4 band exhibits the highest activity in this tissue.

Heart

The classic five banded LDH pattern is obtained indicating that a prominent binomial degree of heterotetramer A-B hybridisation occurs in this tissue. Heat inactivation studies confirm that the characteristic cathodal band of high activity is the B_4 homotetramer (refer section 3.1.1.2.; Fig. 1). The existence of a reverse mobility of A and B subunits is confirmed by the presence of a highly active LDH B_4 at the cathodal end. A less active LDH K_4 band is also observed in the heart tissue (Plate 3). The heterotetramer A_1B_3 subunits shows the least active phenotype.

Muscle

This tissue exhibits a two banded phenotype. There is a distinct LDH A_4 high activity zone at the anodal end confirming once again the existence of reverse polarity of the A and B subunits (Fig. 1). The tissue also exhibits the least active B_4 (Fig. 1; Plate 3). The absence of heteropolymers between A_4 and B_4 is indicative of the loss of the ability of hybridisation between these two subunits.

Liver

This tissue has a striking similarity with the pattern observed in the heart tissue regarding the A_4 and B_4 activity (Fig. 1). B_4 activity is higher than that of A_4. The A_1B_3 subunit is the most prominent among the heterotetramers. In addition to these loci the liver tissue also exhibits a third locus, LDH-C coding for the C_4 activity which is less cathodal than the LDH-K_4 as found in the kidney tissue (Fig. 1; Plate 7). Faint indication of hybridisation between the cathodal B_4 and C_4 has been noticed.

Eye

This tissue shows the usual pattern of LDH bands as found in other vertebrates. All the five bands are resolved distinctly. This tissue shows the LDH homotetramers A_4 and B_4 and their heteropolymers A_1B_3, A_2B_2, A_3B_1 to be in their appropriate proportions (Fig. 1; Plate 3).

Brain

The total number of LDH isozymes observed is five. A_3B_1

subunit shows a higher activity than the other heterotetramers between A_4 and B_4. The homotetramers A_4 and B_4 show equal activity (Fig. 1; Plate 3). Occasional faint bands also appear at the cathodal end besides the five banded phenotypes.

Gonads

Both the male and the female reproductive tissues show a five banded LDH phenotype. The B_4 homotetramer exhibits the highest activity. The A_4, A_3B_1, A_2B_2 and A_1B_3 subunits are present with lesser activity (Fig. 1; Plate 3).

3.1.1.2. Thermostability test

Heat inactivation studies were performed to detect and confirm the LDH-A_4 and B_4 activity zones. LDH-A_4 has been observed to be more heat labile than B_4. The crude extracts incubated at 65°C - 70°C for 5 minutes show no activity at the anodal end corresponding to the A_4 homotetramer (Fig. 3 and 4; Plate 5 and 6). No LDH activity at the cathodal end is detected when the incubation temperature and time are raised to 75°C and 15 minutes, respectively. The former is confirmed

to be the A_4 isozyme and the latter the B_4 isozyme, respectively. This also confirms the existence of a reverse mobility of the A_4 and B_4 isozymes in this fish as compared to the other vertebrates.

The heat stability of the third locus LDH-C in relation to the LDH-A and LDH-B has also been tested in the liver tissue. The C_4 activity reduces when the extract is incubated at 65°C and ceases after A_4 when the extract is incubated at 70°C for 5 minutes (Fig. 4; Plate 6)

Another experiment has been performed to observe the heat tolerance of the kidney specific locus K_4 and its relation to the ubiquitous A_4 and B_4 subunits. The K_4 subunit has a similar heat stability pattern as that of C_4 LDH tetramers. Its activity reduces when incubated at 65°C for 5 minutes and finally ceases at 70°C for 5 minutes(Fig. 3; Plate 5). It is more heat labile than B_4, but less heat labile than A_4. Preferential heat inactivation of LDH isozymes by incubation is shown in Table 1.

3.1.1.3. Relative mobility of C_4 and K_4 homotetramers

The extracts from the liver and kidney tissues were

TABLE 1. Preferential inactivation of LDH isozymes by incubation in *Clarias batrachus*.

Temp °C for 5 mins.	A_4	A_3B_1	A_2B_2	A_1B_3	B_4	C_4 or F_4	K_4
Control	-	-	-	-	-	-	-
50	-	-	-	-	-	-	-
60	++	++++	+++	++	-	++	-
65	++++	++++	+++	++	-	+++	++
70	++++	++++	++++	++++	++	++++	++++
75	++++	++++	++++	++++	++++	++++	++++

- = No detectable inactivation
+ = Less than 50% inactivation
+++ = About 50% inactivation
+++ = Over 50% but not total inactivation
++++ = Total inactivation.

combined prior to electrophoresis. The results show that the C_4 homotetramer differs in mobility from the K_4 homotetramer, as they carry different charges. The former is distinctly less cathodal than the latter in electrophoretic migration (Plate 8).

3.1.2. *Heteropneustes fossilis*

3.1.2.1. Tissue specificity

Tissues from the heart, kidney, brain, liver, eye and skeletal muscle have been subjected to the LDH isozyme pattern analysis.

Kidney

Like *Clarias*, the kidney tissue exhibits a characteristic LDH subunit, the kidney specific K_4 isoenzyme. The activity of LDH-A_4 subunit is absent (Fig..2; Plate 4). The heterotetramers A_2B_2 and A_3B_1 also do not exhibit much activity. In addition the K_4 subunit shows the existence of hybridisation with the B_4 homotetramer (Plate 4). The total number of bands resolved are four.

Liver

This tissue has a close similarity with the kidney tissue. It also has reduced or no activity of the LDH-A locus. The B_4 is highly active, as is the third locus C. Both B_4 and C_4 appear as dark bands at the cathodal end and also exhibit a hybridised band between them (Fig. 2; Plate 4). The total number of bands resolved are four.

Eye

The total number of bands resolved are five. This tissue shows the presence of $LDHA_4$ and B_4 homotetramers and their heteropolymers A_1B_3, A_2B_2 and A_3B_1 in their appropriate proportions (Fig. 2). The only difference with other tissues is in the distance between B_4 and A_1B_3 isoenzymes. The space between these two bands is more (Fig. 2; Plate 4).

Muscle

In contrast to heart, the muscle tissue shows the

classic five banded LDH pattern. The LDH A_4 subunit shows the most activity, while the B_4 shows the least. Among the heterotetramers A_3B_1 is less active than A_2B_2 and A_1B_3 (Fig. 2; Plate 4). The muscle tissue exhibits reverse polarity.

Brain

This tissue resolved into a five banded pattern of uniform activity. There is an occasional faint resolution of a third locus C. The LDH A_4, A_1B_3, A_2B_2, A_3B_1 and B_4 are distinct and uniformly spaced (Fig. 2; Plate 4).

Heart

A six banded phenotype was observed in this tissue with uniform activity in the homotetramers A_3B_1, A_2B_2 and A_1B_3. The B_4 homotetramer is at its highest activity compared to that of the other isoenzymes. B_4 activity is higher than A_4 activity. The LDH-C locus is also active in this tissue and a distinct cathodal band is obtained which corresponds to the mobility of the liver specific C_4 band in this fish (Fig.

2; Plate 4). Like Clarias, this fish also shows the phenomenon of reverse mobility as observed during heat inactivation study.

3.1.2.2. Thermostability test

Heat inactivation study was carried out to detect the LDH A_4 and B_4 activity zones in this fish. LDH B_4 was found to be more stable than A_4. The crude extracts incubated at 70°C for 5 minutes showed no activity at the anodal end and a reduced activity zone at the LDH K_4 locus. An increase in time and temperature of incubation showed no cathodal activity. Therefore in the heart tissue, while observing the heat tolerance of LDH A_4 and B_4, the phenomenon of reverse mobility was detected which was confirmed by their activity observed in the muscle and other tissues as well. The most cathodal band observed in the liver (C_4), kidney (K_4) and heart is seen to be less heat labile than the A_4 homotetramer, but more heat labile than the B_4 homotetramer. Preferential heat inactivation of LDH isozymes by incubation is shown in Table 2.

TABLE 2. Preferential inactivation of LDH isozymes by incubation in *Heteropneustes fossilis.*

Temp °C	A_4	A_3B_1	A_2B_2	A_1B_3	B_4	C_4 or F_4	K_4
Control	-	-	-	-	-	-	-
50 for 5 mins.	-	-	-	-	-	-	-
60 for 5 min.	+	++	+++	++	-	++	+
70 for 5 min	++++	++++	++++	++++	+	++++	++++
75 for 15 min.	++++	++++	++++	++++	++++	++++	++++

- = No detectable inactivation
+ = Less than 50% inactivation
++ = About 50% inactivation
+++ = Over 50% but not total inactivation
++++ = Total inactivation.

3.1.3. Partial purification of LDH from the kidney and liver tissues of <u>Clarias batrachus</u>: It was carried out in the following way to obtain the results.

3.1.3.1. Concentration by Ammonium sulphate precipitation

To the supernatant obtained, from our tissue extract, after removing the cell debris, various concentrations of Ammonium sulphate were added in a series of tubes in order to determine the right amount of Ammonium sulphate required to concentrate the LDH enzyme activity. These fractions were dialysed overnight as described earlier and then spun to remove the precipitate. The different supernatant fractions obtained were run separate gel tubes (PAGE) to observe the LDH enzyme activity in each fraction. In the case of the kidney tissue it was seen that at 55% ammonium sulphate concentration almost all of the K_4 and A_4 isozymes were precipitated. The B_4 isozyme was seen to precipitate at about 70% ammonium sulphate concentration (Fig. 5 and 6).

Thus, in order to concentrate the enzyme in the extract from the kidney and liver tissues the residue obtained between

30% and 80% ammonium sulphate concentration was used after subsequent dialysis for further purification steps in each case.

The amount of protein present in the dialysed extract obtained was assayed by Bradford's method (1976), described earlier. The following results were obtained :

(i) The kidney tissue contained approximately 10 $\mu g/\mu l$ protein (from Table 4).

(ii) The liver tissue contained about 9 $\mu g/\mu l$ protein (from Table 5).

The LDH enzyme activity present in the above two extracts was observed on PAGE by active LDH staining. In the kidney tissue extract the K_4, B_4 and A_4 bands were present. In the liver tissue extract the C_4, B_4 and A_4 bands were darkly stained.

3.1.3.2. Chromatography

Different fractions of enzyme activity were collected in the fraction collector. Fractions with similar enzyme

TABLE 3: Values for the standard graph for protein assay.

Conc. value of the standard sample (BSA, Sigma) in µg	0.000	10.00	20.00	30.00	40.00	60.00	80.00	100.00
Absorbance	0.001	0.046	0.056	0.086	0.113	0.167	0.206	0.268

TABLE 4: Values for the protein assay in the kidney tissue extract applied to the chromatography column.

Sample (ml)	Water (ml)	Absorbance at 595 nm.
.01	.09	.221
.02	.08	.234
.03	.07	.259
.04	.06	.288
.05	.05	.299

TABLE 5: Values for the protein assay in the liver tissue extract applied to the chromatography column.

Sample (ml)	Water (ml)	Absorbance at 595 nm
.01	.09	.231
.02	.08	.243
.03	.07	.248
.04	.06	.250
.05	.05	.255

activity were pooled together and their protein assay was done. In the case of the kidney tissue four pooled fractions KI, KII, KIII, KIV) were obtained (Fig. 8) and for the liver tissue six pooled fractions (LI, LII, LIII, LIV, LV, LVI) were obtained (Fig. 9). Those fractions showing more protein concentrations, that is, the KII, KIII and LI and LII (Table 6 & 7 fractions, were first run on PAGE to see if LDH activity in these could be readily observed. Traces of LDH bands were observed in these fractions. All the fractions were then lyophilised in order to concentrate them.

3.1.3.3. Slab-gel SDS PAGE

The powdered form of these enzyme fractions were then resuspended separately in minimal volumes of Tris glycine buffer. These test samples were boiled with the sample buffer and then loaded in the SDS slab gel slots along with the marker enzymes. The pattern of the bands obtained is shown in Plate 9.

The Rf values for the probable bands indicating the K_4 and the C_4/F_4 subunits were calculated along with those

TABLE 6: Protein concentration in the kidney tissue fractions

Fraction	Sample (ml)	Water (ml)	Reagent (ml)	Absorbance of 595bn (nm)	Protein concentration (μg/ml)
Crude-extract (before chromatography)	0.010	0.040	2.5	0.284	
	0.015	0.035	2.5	0.277	7.565
	0.020	0.030	2.5	0.274	
	0.010	0.040	2.5	0.009	
KI	0.015	0.035	2.5	0.031	0.6
	0.020	0.030	2.5	0.041	
	0.010	0.040	2.5	0.105	
KII	0.015	0.035	2.5	0.115	2.893
	0.020	0.030	2.5	0.120	
	0.010	0.040	2.5	0.130	
KIII	0.015	0.035	2.5	0.137	3.57
	0.020	0.030	2.5	0.145	
	0.010	0.040	2.5	0.006	
KIV	0.015	0.035	2.5	0.013	0.392
	0.020	0.030	2.5	0.029	

TABLE 7: Protein concentration in the liver tissue fractions.

Fraction	Sample (ml.)	Water (ml)	Reagent (ml)	Absorbance at 595nm (cm)	Protein concentration (µg/ml)
Crude extract (before chromatography)	0.010	0.040	2.5	0.290	
	0.015	0.035	2.5	0.297	9.438
	0.020	0.030	2.5	0.313	
	0.010	0.040	2.5	0.072	
LI	0.015	0.035	2.5	0.101	2.103
	0.020	0.030	2.5	0.156	
	0.010	0.040	2.5	0.120	
	0.015	0.035	2.5	0.134	3.507
	0.020	0.030	2.5	0.159	
	0.010	0.040	2.5	0.000	
LIII	0.015	0.035	2.5	0.013	0.327
	0.020	0.030	2.5	0.031	
	0.010	0.040	2.5	0.023	
LIV	0.015	0.035	2.5	0.030	0.65
	0.020	0.030	2.5	0.053	
	0.010	0.040	2.5	0.500	
LV	0.015	0.035	2.5	0.010	0.0207
	0.020	0.030	2.5	0.010	
	0.010	0.040	2.5	0.035	
LVI	0.015	0.035	2.5	0.082	1.533
	0.020	0.030	2.5	0.095	

of the marker enzymes (Fig. 10). The standard line fitting was done with the help of a computor. Thus the approximate molecular weights of the K_4 and C_4/F_4 subunits were calculated from the standard line (Fig. 10; Table 8). The K subunit was seen to have an approximate molecular weight of 48.5 K and C subunit was seen to have an approximate molecular weight of 31.43 K.

3.2. Malate dehydrogenase isozyme pattern

Malate dehydrogenase (MDH; E.C. 1.1.1.37) is a dimer of molecular weight 60,000-80,000. It catalyses the interconversion of malate to oxaloacetate (Banaszak and Bradshaw, 1975; Darnall and Klotz, 1975) in the Krebs cycle. The MDH isozyme system in vertebrates consists of two main forms, the supernatant (s-MDH) and the mitochondrial (m-MDH). We have used the s-MDH which is composed mainly of two soluble cytoplasmic forms which join to form the heterodimer AB encoded by the two gene loci, MDH-A and MDH-B. The A_2 homodimer is cathodal in activity as compared to B_2 which is anodal. They also show differential tissue expression as B_2

TABLE 8: Comparative Rf values and molecular weights of the two cathodal LDH subunits found in the kidney (K) and liver (F/C) tissues of *Clarias batrachus*.

LDH Subunits	Rf values	Approximate molecular weight
K	$\frac{4.5}{7.9}$ = .570	48.5K
F/C	$\frac{3.3}{7.9}$ = .418	31.43K

is more restricted in its tissue activity whereas A_2 predominates in most tissues of both the fishes, *Clarias batrachus* and *Heteropneustes fossilis*.

3.2.1. *Clarias batrachus*

3.2.1.1. General profile

The tissues from the heart, kidney brain, eye, liver, skeletal muscle and gonads have been subjected to MDH isozyme pattern analysis. The minimum number of bands resolved were three which confirm the existence of the dimeric subunits MDH-A and MDH-B and their association to form the heterodimer A_1B_1 (Fig. 12; Plate 10).

3.2.1.2. Tissue specificity

The relative proportions of the three supernatant bands varies markedly from tissue to tissue. In the kidney and liver tissues the most anodal, MDH-B_2 shows an intense activity while in the muscle and heart tissues the cathodal

band, MDH-A_2 predominates; in the eye,gonadal and brain tissues all the three supernatant MDH isozymes occur in approximately equal amounts. The kidney and the liver tissues exhibit the highest MDH activity.

3.2.1.3. Allelic variation

Less common phenotypes (supernumerary bands) were also found which possessed additional isozymes produced by variants of subunits A and B, predominantly in the liver tissue of many individuals (Fig. 12). The electrohporetic mobilities and thermostability of these bands being more similar to the A_2 than the B_2 isozyme, it is assumed that these isozymes originated from a variant of subunit A which has been subsequently designated as A'. The presence of six banded MDH phenotype in the liver tissue appears to result from the combination of three types of subunits A, B and A', into the six theoretically possible dimers AA, AB, AA', BB, A'A' and A'B. The ratio of AA : AA' : A'A' appears to be constant in a number of tissues (heart, muscle, liver and kidney) even though the absolute concentration of the MDH isozyme varies widely in these tissues.

3.2.1.4. Thermostability test

The heat inactivation study shows that on incubation above 50°C for 5 minutes the MDH A_2 fraction decreases its activity and becomes totally inactive if the temperature is raised. It reconfirms the identification and position of both the MDH supernatant isozymes A_2 and B_2.

3.2.2. Heteropneustes fossilis

3.2.2.1. General profile

A similar MDH pattern analysis has been made in H. fossilis as in C. batrachus in order to obtain a comparative resolution in these air-breathing fishes. Seven tissues were analysed - kidney, liver, eye, skeletal muscle, brain, heart and gonads. All these tissues except the brain tissue show a minimum of three bands with varied proportions of A_2 and B_2 isozymes. A_2 isozyme activity at the cathodal end always predominates in all these tissues while the B_2 isozyme shows some significant activity in the kidney, heart

and gonadal tissues. These bands are equally spaced in all the tissues (Fig. 13; Plate 11).

3.2.2.2. Tissue specificity

H. fossilis exhibits a higher tissue specificity than C. batrachus. The brain tissue shows the least MDH activity and resolves into two bands on the gel. These bands correspond to the MDH isozymes A_2 and A_1B_1. This expression indicates that the homodimer at the anodal end, that is, B_2 is absent in this tissue.

The kidney, liver and the gonadal tissues show a higher cathodal activity of MDH bands than the other tissues. The kidney tissue also shows a high activity of the B_2 homodimer at a comparatively anodal position. The muscle tissue exhibits the three basic bands, A_2, A_1B_1 and B_2 distinctly. In the liver, eye, muscle and heart tissues the A homodimer activity is significantly higher than that of the B_2 homodimer. The total MDH activity exhibited by each of the tissues examined has a highly specific pattern.

3.2.2.3. Allelic variation

A maximum of six MDH isozymes phenotype was resolved as has previously been noted in Clarias. These six bands have been designated as AA, AA', AB, BB, A'B and A'A' encoded by the three MDH loci A, B and A'. Electrophoretic mobilities and thermostability studies show that the variants are more related to the subunit A and as in Clarias, we have again designated this locus as A'. Additional A variants differing in electrophoretic mobility from A' have also been detected (Fig. 13).

3.2.2.4. Thermostability test

As in *Clarias batrachus*, in *Heteropneustes fossilis* the MDH-A subunit is found to be more labile to heat than the B subunit. When the tissue extract is heated to 50°C for a maximum of 5 minutes the A_2 homodimer activity decreases and finally ceases at a higher temperature or at 50°C for a longer time. The B_2 homodimer activity diminishes above 60°C. This confirms the comparatively

cathodal and anodal position of A_2 and B_2 homodimers, respectively.

3.3. Alcohol dehydrogenase isozyme pattern

Alcohol dehydrogenase (ADH-1, E.C. 1.1.1.1.) is an NAD (H) dependent enzyme that catalyses the reversible interconversion of a vast number of alcohols and their corresponding aldehydes and ketones. This is primarily a liver specific protein with a substrate specificity and molecular weight around 40,000 (Vallee, 1985).

In fishes, ADH is almost confined to the liver tissue and the products of a single locus are also detected in some other tissues. The number, type and distribution varies from tissue to tissue. An overall analysis of this enzyme in both fishes shows the existence of its dimorphic nature as three banded phenotypes could be obtained.

3.3.1. Clarias batrachus

Seven tissues were used for ADH isozyme pattern

analysis - kidney, liver, eye, muscle, heart, brain and gonads. A zone of high activity at the cathodal end has been resolved in the kidney, liver and eye tissues. The other tissues show very faint activity of ADH. The maximum number of bands resolved is three, in the eye tissue, which confirms the dimorphic nature of the ADH isozyme subunits. These subunits are designated as ADH-A_2, A_1B_1 and B_2, starting from the anode. ADH-B_2 predominates in the kidney, liver and eye tissues, although minor activity has been resolved in other tissues including muscle, and testis (Plate 12). The tissue which failed to exhibit any significant activity was brain, hence this tissue has not been scored in our analysis Fig. 14 , depicts the array of ADH isozyme patterns in the various tissues.

3.3.2. Heteropneustes fossilis

The maximum number of ADH isozymes resolved has been six. A single zone of activity showing a single band at the cathodal end has been observed in tissues like kidney and heart. Three widely spaced bands have been observed in liver,

which confirms the dimorphic nature of the ADH isozymes and have been subsequently designated as ADH - A_2, A_1B_1 and B_2 (Fig. 15; Plate 13).

Alcohol dehydrogenase activity has been maximum in the liver tissue, although minor activity is observed in other tissues. The liver tissue has resolved into three dinstinct ADH isozyme bands of high activity. The tissues which failed to exhibit significant activity are the gonads and muscle. Hence these tissues have not been scored in our analysis.

The presence of a high number of ADH bands in the eye and brain tissues could be understood genetically by assuming that there are at least two structural loci A and B coding for the ADH dimers. The expression of three genes A, A' and B is observed, where A and A' are alleles. The isozymes are therefore designated as ADH A_2, AB, AA', B_2, A'B and A'_2. A_2 being most anodal. The formation of heterodimers between A_2 and A'_2 (AA') and B_2 & A' (A'B) also confirms the existence of the third ADH-A' gene.

3.3.3. Thermostability test

The identification of ADH A_2 and B_2 were made on the basis of thermostability tests. ADH A_2 was found to be more thermolabile than the latter. When incubated above 55°C for 5 mins. ADH A_2 showed diminished activity as compared to ADH B_2.

3.4. Glucose-6-phosphate dehydrogenase isozyme pattern

Glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49) is dimeric in nature and exists in just two forms without exhibiting the intermediate hybrid zone in most animals (Scholl, 1973). In mammals, G6PD isozymes are encoded by two loci, one being sex linked and the other autosomal. The autosomal one reacts more actively with Galactose-6-phosphate and is referred to as Hexose-6-phosphate dehydrogenase (H6PD). Two G6PD isozymes with similar substrate specificities as the mammalian G6PD isozymes have also been observed in the teleosts (Scholl, 1973). G6PD has been extensively studied and it can be used as a characteristic marker to identify different fishes.

3.4.1. *Clarias batrachus*

Seven tissues including kidney, liver, eye, brain, muscle, heart and gonads have been scored to study the G6PD isozyme pattern in this fish. All these tissues exhibited high G6PD activity. A maximum of two bands was observed in the liver, heart, muscle and gonadal tissues with the anodal band showing more activity than the cathodal one. A minimum of one band was consistently observed in each of the tissues. The eye and the brain tissue exhibited almost equal activity as observed on the gel. The heart and the kidney tissue were alike in their G6PD phenotypic expression (Fig. 18). Maximum activity was observed in the liver tissue, followed by slightly lesser activity in the kidney and heart tissues. Skeletal muscle showed the least G6PD activity (Plate 14). No sex differential activity was observed. The cathodal, minor activity, bands observed in the liver, heart, muscle and gonadal tissues stained more actively with galactose-6-phosphate substrate, confirming these to be the H6PD isozyme (Fig. 18).

3.4.2. *Heteropneustes fossilis*

In this fish also seven tissues including kidney, liver,

eye, muscle, brain, heart and gonads were scored for G6PD isozyme analysis. A maximum of five distinct, equally spaced, bands were resolved in both the eye and the brain tissues, identical in phenotypic expression. The rest of the tissues showed one band of G6PD isozyme activity. The liver and kidney tissues showed the highest activity of this isozyme exhibited by a single intense band on the gel. The least activity was seen in the skeletal muscle tissue. No sex differential activity was seen in this fish. The homologous G6PD phenotype with five closely spaced bands in the eye and brain tissues was a characteristic of this fish (Fig. 19: Plate 15).

3.4.3. Thermostability test

The heat inactivation study performed on G6PD isozymes show a reduced activity when incubated above 45°C for a duration of 5 minutes. The activity diminishes when either the temperature or the lenghh of incubation time are increased. The enzyme loses its total activity when the temperature is raised to 60°C (Fig. 20 and 21).

4. DISCUSSION

4.1. Lactate dehydrogenase

The LDH isozymes of vertebrates comprise one of the most informative and thoroughly studied multigenic enzyme systems known. It has been suggested that the molecular structure of this enzyme is an important determinant of pathways used for energy generation. It is reported to be present in almost all groups of vertebrates (Markert and Ursprung, 1974).

Lactate dehydrogenase (Lactate : NAD-oxido reductase, E.C. 1.1.1.27), which has a tetrameric structure and a molecular weight of 140,000 (Darnall and Klotz, 1975), catalyses the interconversion of lactate into pyruvate. In vertebrates the polypeptide subunits of LDH are encoded in at least two genetic loci whose alleles are codominantly expressed (Markert, 1962; Cahn _et al._, 1962; Shaw and Barto, 1963).

The genes coding for the LDH isozymes are developmentally regulated and differentially expressed (Lindsay, 1963; Fine _et al._, 1963). In all vertebrates there are two major LDH isozymes, A and B. The A isozyme predominates

in tissues which undergo anaerobic glycolysis, such as the skeletal muscle whereas the B isozyme is the major form found in tissues with an aerobic metabolism like the heart (Wilson et al., 1963). Some vertebrates also possess a third type of LDH, which in mammals and birds has been called LDH-X or the C isozyme and is expressed only in the primary spermatocytes (Goldberg, 1972; Zinkham et al., 1969). Many fish also have a third form of LDH which Markert et al., (1975) have also referred to as the C isozyme. Unlike the C isozyme of warm-blooded vertebrates, the C isozymes of fish are not restricted to primary spermatocytes. In the lower bony fishes (e.g. sturgeon) this LDH C isozyme is found in heart, gills liver, spleen, kidney and gonads. In the advanced teleosts its expression is limited to neural tissues such as the eye and brain, where it is called the E_4 isozyme (Lush et al., 1969; Whitt, 1970). In Gadiformes (e.g. cod), however, this isozyme is expressed in the liver tissue where it is referred to as the F_4 or the liver specific LDH (Sensabaugh and Kaplan, 1972).

Most fish appear to have either two or three major LDH isozymes. These two categories together with the five isozyme

fish, could all be produced through the activity of two genes, homologous to those producing the A and B subunits of mammalian LDH. If two such genes are indeed active then their polypeptide products are prevented in most fish from forming all possible tetrameric combinations. Presumably, the two isozyme fish make only the homopolymers A_4 and B_4; the three isozyme fish in addition, make one of the heteropolymers, presumably A_2B_2, and the five isozyme fish, make all the five tetramers, as do the mammals. However, among the five-isozyme fish only the whiting shows patterns suggesting a binomial distribution among the isozymes. The shad and the herring synthesise relatively small amounts of LDH-2 and LDH-4, indicating some restriction on the freedom of subunits to polymerize at random (Markert and Faulhaber, 1965).

Although polygenic control of LDH synthesis is the rule among fish, the fluke and related flatfishes are clear exceptions as these fish produce only one kind of subunit which polymerises to a single tetramer of LDH (Kaplan *et al.*, 1960, ~~1961~~). It was demonstrated (Cahn *et al.*, 1962; Wilson *et al.*, 1964) that this single variety of LDH possessed

immunochemical properties relating it closely to LDH extracted from the skeletal muscles of other fish, such as the tuna, but not to LDH from heart tissues. These data on flatfish were confirmed and extended by Daugherty (1965).

Bonavita and Gaurneri (1963) reported a three-isozymes fish as shown by electrophoretic analyses of brain tissues. Vessel and Bearn (1962) found six closely spaced isozymes on zymograms of carp tissues. Goldberg (1965) reported the results of his analyses on several species of trout, some trout species showed two or three isozymes, while others like the speckled trout exhibited nine regularly spaced bands which may have represented the activity of three non-allelic genetic loci.

The lactate dehydrogenase enzyme in a given organ may vary in different species. This is evident in the case of liver of most mammals where the rat, mouse and human livers contain the muscle type of the enzyme, whereas beef liver and liver of other ruminants contain primarily the heart type enzyme and the different LDH molecules may predominate at various times during the development of a given organ. It is

possible that particular molecular configurations are most efficient for given sets of metabolic conditions. While not much is known about the significance of LDH hybrids in the metabolism of a cell, it may be speculated that a tissue whose metabolism is intermediate between that of heart and the muscle tissue may function best with a mixture of both types of lactate dehydrogenases. At any rate, the evolution of a system in which there are two basic molecular units and a mechanism for altering the relative proportions of both the homopolymers and heteropolymers offers distinct adaptive advantage to the organisms. We may presume that such alterations in response to metabolic requirements have occurred with respect to two time scales - that of the evolution of species and ~~the~~ that of the development of an individual (Cahn *et al.*, 1962).

The LDH isozyme patterns observed in both our fish species, *Clarias batrachus* and *Heteropneustes fossilis* were distinctive with respect to their tissue-specificity, the mobility of the isozymes on the polycrylamide gel and their response to heat inactivation. The different tissues from both these fish were chosen so that all the loci encoding

the various LDH isozymes could be unravelled. In Clarias batrachus the five bands corresponding to the A_4, A_3B_1, A_2B_2, A_1B_3 and the B_4 isozymes are seen and in addition to these an intense characteristic cathodal band is observed which is designated as K_4 (Fig. 1; Plate 7). Hybridisation between K_4 and B_4 homopolymers is also observed in the form of minor bands. On the other band, in Heteropneustes fossilis also we find three different subunits though in this case the homopolymer, A_4, is absent. The heteropolymers A_2B_2 and A_3B_1 exhibit a comparatively lesser activity from that seen in C. batrachus. The position of the K_4 band is cathodal as in C. batrachus but the distance observed between the B_4 and the K_4 homopolymers is relatively lesser in H. fossilis, that is, this band is comparatively less cathodal. Since this extra cathodal band was first observed in the kidney tissue it has been named the kidney-specific or the K_4 band. The B_4 homotetramer exhibits the highest activity in this tissue as compared to the other LDH isozymes.

In the liver tissue we see the expression of all the three LDH loci A, B and C in the present work. The most prominant bands observed on the gel correspond to the A_4,

A_1B_3 and the C_4 (liver specific) isozymes. The other isozymes present show lesser activity. B_4 activity is higher than that of the A_4 isozyme. The C_4 band is cathodal to B_4 but this liver specific band is comparatively less cathodal to the K_4 band seen in the kidney tissue in C. batrachus. However, in H. fossilis the position of this C_4 band corresponds to that of the K_4 band.

The eye tissue exhibits all the five isozymes formed by the association of the two subunits A and B in their appropriate proportions in both the catfishes.

The muscle tissue characterised by anaerobic glycolysis shows an abundance of A_4 isozyme activity at the anodal end in both these air-breathing fishes. Our results are in agreement with those of Cahn et al., (1962), Markert and Ursprung, (1962), Dawson et al., (1964), Kaplan and Goodfriend, (1964). Both the A and B, LDH loci seem to be coding for the isozyme pattern observed in this tissue. In C. batrachus only the homopolymers A_4 and B_4 are observed. Probably the two subunits have lost the ability to polymerise and give rise to the heteropolymers in this fish. In H. fossilis five isozymes

are observed showing the homopolymers A_4, B_4 and the heteropolymers A_3B_1, A_2B_2 and A_1B_3. The A_3B_1 heteropolymer is more active than A_1B_3 and A_2B_2 isozymes.

The brain tissue exhibits high LDH activity in both the fishes. The A_4 and B_4 homotetramer show similar activity along with the heteropolymers A_3B_1, A_2B_2 and A_1B_3 regularly spaced in between them. Besides these five bands some extra cathodal bands are also resolved. In C. batrachus one of these bands corresponds to the C_4 and the other is seen between K_4 and C_4 in position,occasionally the K_4 isozyme is also seen. In H. fossilis the extra cathodal bands correspond to the K_4/C_4 position and a slightly less cathodal position indicating hybridisation between K_4 and B_4 homopolymers than the former. Thus in this tissue as well, we find at least three LDH loci, A, B, and C coding for the various isozymes.

In the heart tissue the B_4 isozyme activity has been found to be higher than the activity of the other isozymes of our fish. Similar results have been found in tissues receiving a constant supply of oxygen (Whitt, 1970 b). This

is justified by the fact that the heart tissue is an example of an aerobic type of tissue. This B_4 homotetramer is found to be cathodal in position as compared to the A_4 homotetramer. Both the subunits A and B are active and display the formation of their homotetramers and hybridise to form the three heterotetramers between them. In C. batrachus besides these five isozymes extra bands are observed at the cathodal end. The most cathodal isozyme corresponds to the K_4 homotetramer and the other corresponds to the C_4 isozyme. Faint activity between the C_4 and B_4 homotetramer is indicative of hybridisation between these two tetramers. In H. fossilis the most cathodal band observed corresponds to C_4/K_4 activity. Thus we see that in both these fishes at least three loci are present, coding for the various isozymes in the heart tissue.

The gonadal tissue showed more activity of the B subunits than the A subunits. A five isozyme pattern was observed in C. batrachus in both the tissues from the ovary and the testes.

The LDH isozyme patterns of the heart and skeletal muscle show that the fastest migrating isozyme (A_4) was

abundant in the muscle tissue. This confirms that both these fishes show a reversed mobility of the isozymes as compared to the other vertebrates. In one third of teleosts studied LDH appears with reverse mobility (Markert and Faulhaber, 1965; Markert *et al.*, 1975; Whitt, 1975; Whitt, 1983; Dhar and Chatterjee, 1982). In their review, de Almeida-Val, (1986) found that 30% of fish species have reverse LDH patterns. The thermostability tests performed show that the A_4 isozyme is more heat labile than the B_4 isozyme in *C. batrachus* and *H. fossilis*. This further confirms their reverse mobility with A_4 being the most anodal and B_4 being comparatively cathodal in position. Reverse mobility of LDH is also seen in some amphibians (Balek *et al.*, 1967).

The band spacing was also found to be characteristic of each of the two species of fish in the present analyses. Though in both *C. batrachus* and *H. fossilis* the space between A_4 and A_3B_1, A_3B_1 and A_2B_2 and A_2B_2 and A_1B_3 was almost equal, the space observed between A_1B_3 and B_4 was comparatively more. The space interval between the five isozymes on the whole was greater in *H. fossilis* than in *C. batrachus*. Thus the resolution of the bands showed a wider

separation of the isozymes in H. fossilis on the polyacrylamide gel. This suggests that the difference in charge between the A and B subunits is greater in H. fossilis than in C. batrachus Thus gels displaying the isozymes of the same tissue from the two species of fish could be recognised at a glance and could be used as an identifying factor for these fish resulting in species-specificity of LDH isozymes.

Tissue-specificity is also obvious from our results. The pattern of the LDH isozymes from among the various tissues of a single species is found to be variant and characteristic for each tissue. Besides this, the pattern of isozymes from the same tissue is also very specific in both the fishes. In C. batrachus in the kidney, (K_4), liver (C_4), brain (C_4), and heart (K_4 & C_4) tissues besides the A and B loci at least one additional locus seems to be at play. In H. fossilis too, in these four tissues an additional locus besides the A and B locus seems to be coding for the specific LDH isozyme patterns observed. Tissue-specificity is further strengthened by the differential action of each of these loci in the various tissues of these fish.

The pattern of LDH isozyme expression in H. fossilis

clearly indicates that the extra cathodal band seen to be active in the liver, kidney, brain and heart tissues is an expression of the C locus. This band is therefore designated as the C_4 isozyme. This C_4 isozyme is probably homologous to the liver-specific isozyme found in fishes of the family Cyprinidae and Gadoidae (Markert and Faulhaber, 1965; Klose et al., 1969; Odense et al., 1969; Utter and Hodgins, 1969; Lush, 1970; Kepes and Whitt, 1972; Numachi, 1972; Sensabaugh and Kaplan, 1972; Whitt et al., 1973 b; Wilson et al., 1973; Odense and Leung, 1975; Shaklee and Whitt, 1981). Such an expression of the C_4 isozyme is typical of nervous tissue or of liver, but never of both in the same species (Sensabaugh and Kaplan, 1972; Whitt et al., 1973 b). According to Markert et al., (1975), investigations employing immunochemical, genetic, physical and phylogenetic approaches have demonstrated that the eye-band LDH seen in many groups of teleosts and the liver-band LDH seen in other groups are encoded in the same basic locus, even though the isozymic products have somewhat different properties in these different groups. The liver-specific isozyme differs from the eye-specific isozyme in its electrophoretic mobility and its lability to thermal denaturation. Thus, in teleosts the

general trend in evolution is clearly for the specialisation of the C gene and restriction of its function to neural tissues, or for some fish to the digestive tissues (Markert et al., 1975; Whitt et al., 1975). Our report is the first on the presence of the C gene activity in Heteropneustes fossilis (Heteropneustidae). This report is in contrast to that presented by (dePanepucci et al., 1984; Krishna Rao et al., 1989).

In Clarias batrachus an even more interesting pattern of LDH isozymes is observed. Besides the liver-specific (C_4) isozyme, another more cathodal isozyme is expressed in the kidney, brain and heart tissues (K_4). This report on the presence of the liver-specific isozyme, an expression of the C gene activity, is also contradictory to the observations of de Panepucci et al., 1984; Krishna Rao et al., 1989 . This member of the family Clariidae has a highly species-specific pattern as exhibited by the K_4 isozyme. The C_4 and the K_4 isozymes are different in many respects. They vary in amount of charges they carry. The K_4 isozyme definitely carries more posstive charges than the C_4 isozyme (Plate 7).

An experiment was performed by running a mixture of

the kidney and the liver tissue enzymes in order to identify the K_4 and the C_4 isozymes as different bands on the polyacrylamide gel (Plate 8). The results confirm the comparatively cathodal position of the K_4 isozyme to the C_4 isozyme and that they are two distinct isozymes. Thus, it is probable that the gene locus responsible for controlling the synthesis of K_4 isozyme is different from the gene locus coding for the C_4 isozyme. To study this interesting phenomenon thermostability tests and further analyses by partial purification of the LDH enzyme from the kidney and the liver tissues of C. batrachus was carried out.

The LDH isozymes of C. batrachus have been shown to exhibit a hierarchy of susceptibility to inactivation by heat of the order $A_4 > C_4 > K_4 > B_4$. The A_4 isozyme is the most heat labile and has a marked difference with that of the B_4 isozyme, which is the least heat labile. But in the case of C_4 and K_4, they are both less heat labile than the A_4 isozyme and exhibit only a slight difference in the susceptibility to heat inactivation amongst them (Plate 5, Plate 6). Our results indicate that the activity of C_4 starts reducing at 65°C (time factor being constant) and

total activity is lost at 70°C. Whereas in the kidney enzyme, K_4, the activity ceases only at 70°C. Anyway we found that both the C_4 and K_4 isozymes lie in a range between A_4 and B_4 with respect to their thermal stability. In general the homotetramer B_4 from most vertebrates 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) with the exception of Rana pipiens (Kaplan, 1964; Goldberg and Wuntch, 1967) and Rhodeus amarus (Cyprinidae) (Hauss, 1975 a,b).

Ammonium sulphate fractionation of the kidney tissue extract shows that the K_4 isozyme is precipitated between 50% to 60% concentration of the salt, whereas the A_4 isozyme precipitated totally between 30% to 60% concentration of the salt (Fig. 5). The B_4 isozyme was precipitated between 40% to 70% of the salt concentration, thereby showing that the highest salt concentration was required to entirely precipitate this isozyme. Therefore we can say that ammonium sulphate precipitation was of the order $A_4 > K_4 > B_4$.

with increasing concentration of the salt. In the case of the liver tissue extract a similar observation was made and the C_4 isozyme was seen to precipitate at a concentration level between the A_4 and B_4 isozymes. Here also the precipitation was of the order $A_4 > C_4 > B_4$, with increasing salt concentration.

It can be said that the K_4 and the C_4 isozymes were definitely different from the A_4 and the B_4 isozymes with respect to their thermal stability as well as their ability to precipitate by ammonium sulphate fractionation. In both these respects the K_4 and the C_4 isozymes lie between the A_4 and the B_4 isozymes. In fact the K_4 and the C_4 isozymes seem to be more similar among themselves than to either A_4 or the B_4 isozymes.

On running the SDS-PAGE with the partially purified samples of both the kidney and the liver LDH enzyme various bands were obtained on the slab gel (Plate 9). Appropriate bands were chosen in both the cases in order to get an idea of the individual molecular weights of the K and C subunits. The specific band we chose as that of the K subunit was

from the KII enzyme fraction which had shown active staining of LDH on PAGE prior to SDS-PAGE. This band was not the same as the band obtained from the KI fraction which had shown no active LDH staining on PAGE showing that it did not have LDH activity. For the liver LDH enzyme, the particular band chosen to indicate the C subunit was from the LI fraction and was seen comparatively more intense than that observed in the crude ~~order~~ liver enzyme. Since we had loaded an equal amount of the enzyme in both the cases,this was confirmed to be the C subunit. From our results we have seen that the K subunit has an approximate molecular weight of 48.5 K and the C subunit has an approximate molecular weight of 31.43 K. These values should not be indicative for the individual molecular weights of these subunits as our enzyme preparation was not highly purified. In any case these values can definitely be used on a comparative basis. We can say for sure that the molecular weight of the K subunit is significantly higher than that of the C subunit. This may be one of the factors responsible for the comparatively slower anodal migration of the K_4 isozyme as compared to the C_4 isozyme on the polyacrylamide gel (Plate 7).

In general we can say that the resolution of the

isozyme patterns obtained for each of the enzymes studied was better when fresh tissues were used. If the tissues of the dissected fish were stored for more than 6 hours the enzyme activity decreased and a poorer resolution of the isozymes was observed on PAGE. This was important in the resolution of the K_4 and the C_4 isozymes of these fish.

The studies conducted by Altman and Robin (1969) on the pond turtle indicate that in a vertebrate capable of prolonged survival using anaerobic glycolysis as as the principal energy source, the enzyme LDH has unusual properties. These properties are consistent with the possibility that this enzyme is playing an important role in the ability of the animal to survive extreme hypoxia.

According to Junk (1984), fish are one of the best vertebrate groups for studying adaptive strategies to hypoxic environments, since they present alternative respiratory strategies, e.g. a swim-bladder acting as lungs (Arapaimagigas), stomach modification to capture air (Pterygoplichthys multriradiatus), a lip extension to increase the surface area (Colossoma macropomum; Brycon

cf. _melanopterum_), branchial cavities (_Symbranachus marmoratus_), and true lungs (_Lepidosiren paradoxa_).

The existence of these fish presenting alternative respiratory strategies in the same environment gives an ideal area for study from the ecological point of view. Many studies have shown that enzymes and isozyme systems are good instruments to answer questions concerning genetic, developmental, adaptive and evolutionary processes in these fish (Hochachka and Somero, 1973, 1984; Whitt, 1983, 1987; De Almeida-Val _et al._, 1990).

We propose that the activation of anaerobic metabolism in the K, L, B and H tissues is one molecular strategy used both by _Clarias batrachus_ and _Heteropneustes fossilis_. Probably this kind of adaptation strategy is occurring in these air-breathing species across their regulation gene systems, which can control the synthesis of LDH subunits A, and B & C in their various tissues.

Some investigators reported that the enzyme activity of a poikilotherm was increased during cold acclimation (Ekberg, 1962; Freed, 1965; Cooper and Ferguson, 1972).

According to Moon (1975), two basic isozyme strategies are found in the thermal adaptation of poikilotherms, i.e. (i) the "on-off" synthesis of a unique isozyme form and (ii) a complex isozyme system. In goldfish liver LDH, existence of the same five isozymes in the fish acclimated to different temperatures was indicated electrophoretically (Tsukuda, 1975; Yamawaki and Tsukuda, 1978). The isozyme pattern varies in the direction of increase of isozymes having adaptive quality to new ambient temeperature during acclimation (Yamawaki and Tsukuda, 1978).

The fish used for our present work were kept in a comparatively cooler environment than their natural habitat. It is tempting to suggest that the unique K_4 isozyme may thus have been synthesised in response to thermal adaptation in our fish, as this isozyme has not been observed by earlier workers (Krishna Rao et al., 1989).

Many studies have indicated that, besides the LDH A and B genes common to all vertebrates, fish possess additional LDH genes (Markert and Faulhaber, 1965; Klose et al., 1968; 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 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 the third LDH gene in fish, the C gene, has been clearly established by genetic studies (Whitt *et al.*, 1971; Vrijenhoek, 1972). According to Shaklee and Whitt (1981), the widespread existence of the two derived LDH C states (eye versus liver) throughout the advanced teleosts suggests that at a relatively early stage of evolution, chanellisation of the C gene was fixed into either of these two restricted modes.

Experiments conducted by Whitt (1968, 1969) demonstrate

that the B_4 and the E_4 isozymes are related and the E_4 isozyme is more closely related to the B_4 isozyme than to the A_4 isozyme. He was of the opinion that the LDH A and B loci of vertebrates have evolved from a single ancestral gene, as suggested by the similarity of amino acid sequence of the A and B polypeptide active sites, copolymerisation of the A and B polypeptides, and the postulated linkage of the A and B loci in trout (Markert, 1963; Morrison and Wright, 1966; Allison, 1968). Whitt (1969) thought that the LDH A locus may be more representative of the ancestral gene, from indirect evidences, one of the reasons being that the A gene is more active than the B gene in many vertebrate tissues, especially in flatfish, where the B gene function is restricted to only a few tissues (Markert and Holmes, 1969). The other reason was that the LDH A locus in teleosts appears to be less variable than the B locus, as is seen by the higher frequency of electrophoretic variants at the B locus (Markert and Faulhaber, 1965; Morrison and Wright, 1966; Odense et al., 1966; Holmes and Markert, 1969). Considerable divergence between the A and B loci has occurred which is reflected in the differences in total amino acid composition and peptide

patterns (Markert, 1963; Pesce et al., 1967) and the degree of immunochemical dissimilarity (Markert and Appella, 1963; Holmes and Markert, 1969; Markert and Holmes, 1969) between the A_4 and the B_4 isozymes. The E gene, according to these authors, arose from a duplication of the B gene&was followed by mutation and divergence through selection, though the E gene does not appear to have diverged as much from the B gene, as the A gene has from the B gene. Although the precise time of this duplication event is not known, the evolutionary survey of fishes by Horowitz and Whitt (1972) indicates that it must have occurred prior to the adaptive radiation of the teleosts.

In addition to possessing the eye band of LDH, the salmonids have duplicate,although slightly different,loci for the LDH A and the LDH B subunits (Holmes and Markert, 1969). These duplicated loci for LDH in salmonids can be explained to have arisen by a tetraploidisation event (Klose et al., 1968; Ohno et al., 1968; Ohno, 1970).

The liver-specific band of gadoids (Sensabaugh and Kaplan, 1972) and of cyprinids (Kepes and Whitt, 1972; Whitt

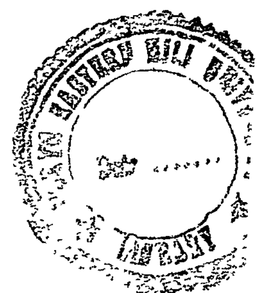
et al., 1973) is quite similar to the B_4 isozyme. The gene coding for the liver-band was derived during evolution from the gene encoding the B subunit of LDH (Klose *et al.*, 1969; Kepes and Whitt, 1972; Numachi, 1972; Sensabaugh and Kaplan, 1972; Whitt *et al.*, 1973; Wilson *et al.*, 1973).

Shaklee *et al.* (1973) believe that the eye-band of many teleosts and the liver-band of gadoid and cyprinid fishes are all the products of the third LDH locus, LDH-C. These authors state that all teleosts possess only three loci encoding LDH subunits - Ldh-A, Ldh-B and Ldh-C. The LDH loci observed in Salmoniformes and Cypriniformes are simply duplicated copies of these three basic loci.

The distinctive tissue-specific distributions and biochemical properties of the C subunit suggest that this particular gene product has been specifically moulded to occupy one or more specialised metabolic niches.

The co-expression of the Ldh-C locus with an anodal C_4 isozyme has been observed in some cichlid fish species (Perciformes, Cichlidae), in the eye, and with a cathodal L_4 isozyme in the liver. This fact might suggest and support

102758



the hypothesis of the presence of a fourth Ldh-locus in cichlids (Holt and Leibel, 1987).

In two species of fish it has been demonstrated that the Ldh-A and Ldh-C genes are connected (Morizot, 1983; Morizot and Siciliano, 1983), but in Salmoniformes the Ldh-B gene is not connected either with A or with C (Wright *et al.*, 1975, 1983).

The genes that code for LDH in teleosts are in a constant evolutionary process and one cannot assume that the specialised fishes lack evolutionary potential for LDH genes. However, the phylogenetic relationships of the various vertebrate LDH isozymes have been well established (Holmes and Markert, 1969; Markert and Holmes, 1969; Whitt, 1969; Holmes, 1972; Horowitz and Whitt, 1972; Shaklee *et al.*, 1975), but structural studies on rodent C-type LDHs (Chang *et al.*, 1979; Pan *et al.*, 1980) seem to contradict the accepted dogma. When the primary structures of mouse and rat C-type LDHs were determined in full (Pan *et al.*, 1983), comparisons of these sequences with the vertebrate A and B LDH isozymes revealed that the rodent C-type LDHs are

related equally distant from the A and B types (Li et al., 1983). The complete structure of the mouse A-type LDH protein and gene were determined (Li et al., 1985), which confirmed the prediction that the A and B LDH isozymes are more closely related to one another than they are to the mammalian C-type LDHs.

Recent work suggests that the C subunit has preceded the gene duplication which gave rise to the subunit A and B (Rehse and Davidson, 1986; Baldwin and Lake, 1987). Evidence that a C type isozyme was indeed the ancestral form of vertebrate LDH isozymes and that the C type LDHs of rodents and Atlantic cod are orthologous proteins resulting from speciation events rather than by independent gene duplications has been given by Rehse and Davidson (1986). They suggest that the genes coding the C type LDHs in vertebrates, during their evolution, seem to have come under the influence of different tissue regulators. These authors did not find any significant difference in the rates of evolution along the A, B or C lineages. If indeed their interpretation is incorrect then one would except to find more than three genetic loci for LDH in some vertebrates.

The data and analysis presented in this study, along with the other information already available from the earlier reports, point out the complexity of the LDH isozyme systems in fish. In our present study, we have seen that the C type LDH is expressed in the kidney, liver, heart and brain tissues, both in C. batrachus and H. fossilis. Here we are referring to all the vertebrate LDH isozymes which are not of the A or B form as C types, adopting the nomenclature advocated by Markert et al. (1975). Our interpretations are in agreement with the scheme presented originally by Holmes (1972). The liver-specific (F/C) and the kidney-specific (K) loci of LDH may thus have evolved independently from the B locus and each of these might be specialised for tissue-specific functions in our fish species.

Here it is worth mentioning that the heart tissue in Clarias batrachus shows isozymes corresponding to both the K_4 and C_4 bands of the kidney and liver tissues respectively. A similar expression is occasionally observed in the brain tissue of this fish. This interesting phenomenon strongly suggests that there may be more than three (A, B and C) loci

coding for the various LDH isozymes in this fish species. Fig. 11 shows our proposed model for LDH isozyme evolution, based on the acknowledged view of LDH isozyme evolution (Holmes, 1972; Markert et al., 1975; Holbrook et al., 1975).

4.2. Malate dehydrogenase

Malate dehydrogenase (MDH) catalyses the reversible oxidation of L-Malate to oxaloacetate with the concomitant reduction of NAD, and as mentioned earlier, is an essential component of aerobic metabolism. In the majority of fish species two main fractions of MDH have been observed, the mitochondrial (m-MDH) and the supernatant (s-MDH). Of these, the m-MDH fraction is less widely used due to experimental difficulties and it also exhibits lesser activity than the cytosolic MDH fraction. In order to study the genetic mechanism controlling this enzyme we have scored only the s-MDH fraction. This s-MDH isozyme has been reported to be dimeric in nature in fishes (Bailey et al., 1970; Whitt, 1970b ~~1977~~; Wilson et al., 1973; Frankel and Wilson, 1984). The two dimers are designated as MDH A_2 and MDH B_2 and encode for the MDH A and MDH B loci respectively. The random

association of A and B polypeptides result in the formation of the three, dimeric s-MDH isozymes, A_2, A_1B_1 and B_2.

MDH is likely to be a dimer in one kind of fishes (e.g. saury) and consists, like LDH, of four polypeptide chains in the other. The majority of fishes contain several MDH isozymes in their tissues. Their number sometimes exceeds nine (Numachi, 1970a, b) and in Atlantic salmon even attains fifteen (Wilkins, 1971). In the karyotype of salmon and whitefish the MDH isozymes are coded by no less than six different genes (Massaro, 1972). Polymorphism with regard to MDH is detected in populations of herring (Odense and Allen, 1971),chum and pink salmon (Slynko, 1971 a, b; Altukhov et al., 1972), saury hardtail, Distrema temminki (Numachi, 1970 a, b), Fundulus heteroclitus (Whitt, 1970), and in some others. A great number of species, however, are monomorphic with respect to this enzyme. In rainbow trout and chinook salmon,four MDH genes are found instead of two detected in herring and flatfish (Bailey et al., 1970).

Less complex patterns of MDH isozymes expression are observed in the two air-breathing fish, Clarias batrachus and

Heteropneustes fossilis, as might be expected from the dimeric structure of enzymatically active MDH molecules. The tissue-specific patterns observed in these fish appear to be similar when compared. Most tissues exhibit a three banded phenomenon along with the expression of some allelic forms which is discussed here in a later part. The position of MDH A_2 (cathodal) and B_2 (anodal) is confirmed by the thermostability tests.

Among all the tissues examined, the kidney tissue expresses the highest activity followed by the liver and heart or the gonadal tissues. While MDH B_2 appears uniformly in five of the tissues of Clarias, it exhibits the lowest activity in the brain and eye tissues of Heteropneustes (Fig. 13; Plate 11). MDH A_2 isozyme exhibits a relatively high activity in the cathodal region in all the tissues examined and shows differential expression in the tissues in both the fishes. The heterodimers formed show considerable variation depending upon the activity of MDH A_2 (Fig. 12; Plate 10). In Heteropneustes, A_2 activity is the least in the brain tissue and it predominates in the eye tissue. The band spacing is more close in the case of Clarias, while in

Heteropneustes the space between A_1B_1 heterodimer and the B_2 homodimer is more than that between A_1B_1 and A_2 bands. Due to the random association of MDH A and MDH B subunits tissue-specific patterns are expressed in these two fishes. Our results are thus in agreement with those of the other workers (Bailey *et al.*, 1970; Whitt, 1970; Wilson, 1973; Whitt *et al.*, 1977; Frankel and Wilson, 1984; Frankel, 1985) and this could be a possible confirmation of the existence of the three banded patterns of MDH in these two air breathing fishes.

None of the MDH bands obtained stain if NAD in the staining solution is replaced by NADP. This is therefore rules out the possibility that any of them might be malic enzyme, MOD (Scholl, 1973) rather than MDH activity bands.

The high activity of A_2 homodimer observed in the kidney tissue (Fig. 12 and 13), followed by that of the liver and muscle tissues in both the fishes raises the vital question as to whether tissue specificity in these cases is physiologically meaningful. The fact that this enzyme is involved in both glycogenesis and lipogenesis,as well as in

the transport of the reducing power during aerobic glycolysis (Bailey and Wilson, 1970), results in the expression of MDH isozymes to different extents in different tissues. It is possible that MDH A_2 and B_2 may have different metabolic roles which leads to tissue specificity. It may also be possible that in these air-breathing fish a functional specialisation of MDH A_2 has occurred in the kidney tissue. This is further supported by the expression of some allelic forms of A_2 (Fig. 12 and 13). Such observations have been made earlier in cold blooded vertebrates (Busack _et al._ 1979) which might also exist in our group of fishes.

In mammals, the brown adipose tissue is the most important site of lipogenesis and the activity of lipogenic enzymes are also very high in this tissue (Hahn, 1970; Klain and Hannon, 1977). In the rainbow trout, muscle is considered as the mosaic tissue, which is a blend of the red and the white tissue and it exhibits the highest activity of MDH as compared to the other tissues. In addition, the level of MDH in trout liver also supports the concept that in fish the liver tissue is an important site

of lipid synthesis and reserve (Dey, 1984). In *Clarias batrachus* and *Heteropneustes fossilis* besides the liver tissue, the kidney tissue also exhibits high MDH activity. Since fish do not have an appreciable brown adipose mass, it may be reasonable to suggest that their energy is probably located within these active tissues. The activity of MDH in fish liver and kidney tissues may also indicate the presence of a primitive metabolic pathway preceding the development of an active lipogenic adipose mass found in mammals.

The typical pattern of MDH activity observed during the course of this work may be related to several other factors as well. Hochachka (1968) has shown that lipogenesis in fish and in other poikilotherms is probably a basic requirement to environmental temperature adaptation. The association of allozymes and temperature has been extended in some cases to the demonstration of biochemical differences between genotypes (Koehn, 1969; Merritt, 1972; Powers and Powers, 1975). Studies of several enzymes in eastern North American populations of *Menidia beryllina* and *Cyrinodon variegatus* suggest that

the most polymorphic enzymes in these species vary in association with temperature (Johnson, 1974; Darling, 1976). Polymorphism for esterases and LDH in *Anoplarchus purpurescens* suggests a pervasive genetic adaptation to temperature in this species (Johnson, 1977). The temperature for enzyme substrate affinity in trout has been reported by Hochachka and Somero (1973), where a qualitative change in isozyme pattern is observed. Enhanced MDH activity in the cold-acclimated individuals of green sunfish has also been reported (Wilson *et al*., 1975). Somero (1973) and Wilson *et al*. (1974) state that the increase of glycolytic activity observed in warm-acclimated animals and increased oxidative activity in the cold-acclimated animals is perhaps best explained on the basis of oxygen availability rather than upon the direct effects of temperature.

The levels of isozymes in fish are also related to functional changes in developing organs and tissues (Champion *et al*., 1975). Moreover, the role played by hormonal/gonadal status in the physiological function of

fish has been well established (de Vlaming, 1975; Lapin, 1973).

In Clarias batrachus and Heteropneustes fossilis the low environmental temperature may be one of the possible reasons for the subtle difference in the isozyme pattern of MDH in the various tissues. This factor was involved because these fish were always kept in the laboratory for a few days before use. The temperature inside the laboratory was very low as compared to that found in the waters from where the fish were procured. Besides this there might be an increase in the oxidative activity in the tissues of these air-breathing fish because of low oxygen concentrations found in their ponds and ditches. Thus the typical tissue-specific expression of MDH in these catfish may be due to their unique metabolic behaviour. However, it may be stated that only after a detailed evaluation of all these parameters in relation to the MDH A_2 polymorphism along with other quantitative biochemical studies are thoroughly investigated, a possible conclusion on the characteristic tissue-specific expression of MDH in these fish can be established.

No sex differential activity of MDH has been observed, which suggests that the isozyme expression of MDH is autosomally inherited.

Variant MDH A subunit

Electrophoretic survey of the MDH isozymes in Clarias and Heteropneustes also revealed the fact that there exists an inconsistency in band number. Besides the three banded phenotypes which have been well established two or three additional bands have also been observed (Fig. 12 and 13; Plate 10 and 11). Such variations have been reported in a wide group of fishes (Table 9; Bailey et al., 1970) and also in some groups of amphibians (Chatterjee and Prakash, 1990), but in these air-breathing fishes in India this is probably one of the first reports.

These additional bands are highly cathodal, therefore, more close to MDH A locus. The six banded phenotype can be accounted for by the assumption that the gene for the A subunit is present in two allelic forms A and A'. The association of three gene products - A, B, and A' would

TABLE 9: Occurrence of multiple electrophoretic forms of MDH in two major classes of vertebrates (Modified from Bailey *et al.*, 1970).

Classes	Presence of 3 equally paced bands	Minimum number of bands
Fishes		
Eptatretus stouti	-	4
Squalus acanthias	+	8
Hydrolagus colliei	+	5
Acipenser medirostris	+	10
Acipenser transmontanus	+	6
Gashes callarias	-	3
Platichthys stellatus	+	4
Spirinchus sp.	+	4
Alosa sapidissima	+	4
Clupea harengus	+	4
Salmo sp.	+	4
Salvelinus sp.	+	4
Oncornynchus sp.	+	4
Clarias batrachus (present work)	+	3
Heteropneustes fossilis (" ")	+	3
Amphibians		
Bufo terrestris	+	3
Rana limnocharis	+	4
Ambystoma mexicanum	+	3
Typhlonectes compressicauda	+	3

produce the six dimers, AA, AA', AB, AB, A'B and A'A'. The electrophoretic spacing of the observed six bands fits this hypothesis (Fig 12 and 13).

This genetic interpretation is strengthened by the finding that the frequency of occurrence of A and the A' allele varies widely among the population and among the individuals (Table 10). In the majority of Clarias, individuals with A' subunit predominate while in Heteropneustes an equal proportion of AA and AA' or A'A' individuals could be observed. Both the liver and the kidney tissues have been scored for this purpose. It is thus unlikely that specific or non-specific somatic changes of a single polypeptide are responsible for the observed isozyme patterns. In order to elucidate the cellular origin of the various MDH bands in Fundulus, Whitt (1970) and Place and Powers (1978) found the MDH B_2 isozyme to be of mitochondrial origin. However, we will be using AA and A'A' nomenclature for the two major cathodally migrating forms until the relationship of these two forms is conclusively determined. Should they prove to be products of two separate loci and in the other major groups by other advanced techniques, they will then be

TABLE 10: Occurrence of A and A' subunits of MDH in terms of number of individuals.

Species	Type of subunit produced		
	A	A and A'	A'
Clarias batrachus	40	21	24
Heteropneustes fossilis	50	16	32

renamed as A and C loci, respectively.

The supernatant MDH A and B loci have arisen from duplication (Karig and Wilson, 1971; Wheat and Whitt, 1971; Wheat *et al*., 1971). Bailey *et al*. (1969, 1970) have biochemically analysed both the supernatant MDH A and MDH B isozymes of salmon and have found a close similarity between them in their kinetic properties, amino acid composition and immunochemical cross-reactivity. The good correlation between immunological similarity and sequence homology established for other enzymes (Prager and Wilson, 1971) suggests extraordinary homology between MDH A and MDH B. Furthermore, the structural similarity of the s-MDH A and B polypeptides in interspecific centrarchid hybrids is confirmed by the random assembly of these subunits into dimers both *in vivo* and *in vitro* (Wheat and Whitt, 1971; Wheat *et al*., 1971). The closely related duplicate gene loci encoding the supernatant MDH isozymes of fish are not closely linked and this absence of linkage between these loci may be related to the means of gene duplication and/or the specificity of their regulation (Wheat *et al*., 1971). This specificity of regulation in our fishes has probably given rise to the

allelic locus MDH A' which appears to be more similar to the MDH A locus than the MDH B locus with regard to its thermal stability and the number of positive charges carried by its phenotypic expression, that is, the homodimer A'A'.

4.3. Alcohol dehydrogenase

Alcohol dehydrogenase is primarily a liver-specific protein found in parenchymal cells (Raiha et al., 1967) but has also been reported to be present to a lesser extent in the kidney and stomach tissues of several species (Hitzeroth et al., 1968; Shaklee et al., 1974). This enzyme is cytoplasmic and exhibits a broad substrate specificity (Cardemil, 1978) mediating the conversion of primary alcohols to aldehydes and secondary alcohols to ketones (Pietruszko et al., 1973). It is known to be dimeric in the nature (Rossman et al., 1975) being encoded in the majority of vertebrates at a single gene locus (Hitzeroth et al., 1968; Shaklee et al., 1974; Levine and Haley, 1975; Frankel, 1978, 1980, 1981, 1983; Vallee, 1985; Keung et al., 1989).

A variety of ADH models (Fig. 16) can be used to explain the formation of the different ADH phenotypic patterns due to the dimeric nature of this enzyme. The activity of a single gene (Fig. 16 a) may exhibit either a single band or in case of an allelic variant three closely spaced bands on the gel. On the other hand when two genes are active then one may expect a three banded pattern with the bands relatively distant from each other, or a six or nine banded phenotype, or a four banded phenotype (Fig. 16 b).

Scandalios (1967) has produced evidence that the two isozymes of ADH in maize may represent the monomeric and dimeric forms of a single polypeptide, the heterozygote producing three bands of activity at the more cathodal position (suggesting a hybrid intermediate molecule), and only two bands at the anodal position. ADH isozymes in sorghum (*Sorghum bicolor*) have been analysed by Ellstrand *et al.*, (1983) and they have proposed a three-loci model as the best explanation of their observed patterns. They suggest that the evolution of *Sorghum* includes a gene duplication of the homologue of the Adh-1 locus in *Zea*.

ADH of Drosophila melanogaster is the product of the expression of a single gene (Grell et al., 1965). Four allelic variants of ADH from the fruitfly are well known and distinguished from each other (Thatcher, 1980). The allelic variants are designated according to their rate of mobility toward the anode, Adh^{UF} is an ultra-fast-moving variant, Adh^{F} a fast variant and Adh^{S} a slow variant (Johnson and Dennistone, 1964). Thirteen ADH variants have been found in Drosophila species (Chambers et al., 1984).

The expression of ADH isozymes in the clawed frog, Xenopus laevis, has been explained on the assumption of two structural genes designated as Adh-1 and Adh-2, coding for the Adh activity (Wesolowski and Lyerla, 1983). Here Adh-1 codes for three electrophoretically separable allelic variants capable of forming heterodimers with one another, the alleles being designated $adh\text{-}1^{a}$, $adh\text{-}1^{b}$, and $adh\text{-}1^{c}$ in descending order according to their products' anodal migration rates, and adh-2 codes for an ADH isozyme that does not form heterodimers with adh-1 products and comigrates with the $adh\text{-}1^{c}$ homodimeric product. The greatest number of isozymes observed from any given individual would be four,

while there could be six possible sites of ADH activity in this electrophoretic system. This model does not fit into the pattern observed in our fish, Heteropneustes fossilis, where five bands are observed at one time in both the eye and the brain tissues, in an individual. Prakash (1988) has explained the ADH isozymes observed in Rana limnocharis on the assumption that at least three genes code at two separate loci for its characteristic pattern [Fig. 16(b)(3)].

A single cathodal zone of species specific ADH, restricted in expression to extracts of liver, confirms to observations for ADH activity in many species of Cyprinidae (Frankel, 1978, 1983). Electropherograms of both Clarias batrachus and Heteropneustes fossilis differ to those observed in other fishes. Firstly, the ADH activity is not restricted to only the liver tissue but is also observed in the gonadal, kidney and eye tissues in our fish (Fig. 14 and 15). The tissue which failed to exhibit significant activity was the muscle tissue, which may probably reflect a comparatively low physiological requirement of ADH in this tissue. Secondly, the number of band phenotypes scored is more than one, both in Clarias batrachus and Heteropneustes

fossilis. The maximum number of ADH band phenotypes scored in a tissue in Clarias batrachus is three, which goes to prove the dimorphic nature of this enzyme. These subunits have accordingly been designated as A_2, A_1B_1 and B_2 as shown in our model (Fig. 17 a). These bands are resolved distinctly on the gel as they differ in their relative mobility due to the difference in the amount of charges they carry.

In Heteropneustes fossilis, the maximum number of band phenotypes resolved has been six. Of these, five bands have been observed in the eye and the brain tissue. Although a single zone of activity has been observed in the heart and the kidney tissues, the liver tissue exhibits three widely spaced bands (Fig. 15; Plate 13). This phenomenon can be explained by assuming that there are at least three structural genes A, B and A', which are coding for the ADH dimers. Here A and A' genes may be allelic in expression. The isozymes can therefore be designated as ADH A_2, AB, AA', B_2, A'B and A'_2 (Fig. 17 b). The presence of heterodimers between A' and B gene loci also confirms the existence of the third ADH gene, the A' gene, which may be formed by a point

mutation of the A gene resulting in allelic polymorphism at the A locus, in this fish.

A great deal of genetic polymorphism is seen in human liver ADH (Bosron _et al_., 1979; 1983; Yin _et al_., 1984). Most mammalian ADHs studied do not oxidise or oxidise secondary alcohols less efficiently than primary alcohols (Dafeldecker and Vallee, 1982; Ditlow _et al_., 1984; Julia _et al_., 1987; Fong and Keung, 1987, 1988). This has also been observed both in _Clarias batrachus_ and _Heteropneustes fossilis_ as in these fish, too, ethanol (substrate) is more readily oxidised as compared to methanol.

In most of the earlier reports on vertebrates, ADH activity is represented by a single zone in the cathodal region. Interestingly, besides high cathodal activity, additional active bands of ADH appear in _Clarias batrachus_ and _Heteropneustes fossilis_ which support the assumption that there may be an additional locus formed originally by the duplication of loci, giving rise to the A and B loci.

Looking at the overall expression of ADH in these air-breathing fishes it can be assumed that the ADH expression in these fishes is the product of more than one locus, which is again in contrast to the reports on other fishes (Philipp et al., 1979; Frankel, 1983; Frankel and Wilson, 1984; Vallee, 1985). Cruz et al., (1982) found a high activity of alcohol dehydrogenase in some carps, where anaerobic respiration predominates, and attributed this to an adaptation to survival in very low oxygen concentration; superimposing this hypothesis on the data obtained from our fish we can significantly correlate the high activity of ADH in *Heteropneustes fossilis* and to some extent in *Clarias batrachus*, to their anaerobic condition because these fish are obtained from ponds and oxygen depleted water bodies. Evidence of the high ADH activity as exhibited by the banding intensity is also correlated to the fact that these fish were under environmental stress exerted probably by the low oxygen content in the surrounding water.

Expression of additional bands of ADH and MDH phenotypes, both in *Clarias batrachus* and *Heteropneustes*

fossilis,have been observed alongwith the standard pattern. ADH phenotype also exhibits additional bands, therefore, the various reasons and hypotheses that we have given earlier with these other enzymes could also be applicable to this enzyme in these fish. The environmental oxygen perturbation which has significantly stressed the physiology of these fish may have resulted in the switching on of an additional regulatory gene (locus). Presence of this additional homodimer gene indicates that the changes are only at the transcriptional level. A detailed study on the expression of ADH isozymes with environmental correlates is required to draw any conclusive remark on the genetic status of these fish.

4.4. Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate; NADP oxidoreductase, E.C. 1.1.1.49) catalyses the glucose-6-phosphate oxidation, providing NADPH for biosynthetic purpose, in the pentose phosphate pathway. The regulation of this pathway includes questions for the

understanding of its operative activity *in vivo*. One of the metabolic regulation mechanisms developed by some animals resides in the existence of isozymes. The presence of G6PD isozymes in aquatic animals, described by several authors (Shatton *et al*., 1971; Stegeman and Goldberg, 1971, 1972; Yamauchi and Goldberg, 1973; Nagayama *et al*., 1975; Yamauchi, 1975; Cederbaum and Yoshida, 1976; Shaklee and Whitt, 1977; Phillip *et al*., 1979; Hart and Pontier, 1982; Rodriguez-Segade and Freire, 1982) supports the hypothesis that this enzyme is crucial in the regulation of the metabolic flux of the pentose phosphate pathway.

G6PD isozymes have been previously observed in mammals (Shaw and Barto, 1965; Shaw, 1966; Ohno *et al*., 1966). The mammalian G6PD isozymes are encoded in two separate loci, one locus is sex-chromosome linked (Kirkman and Hendrickson, 1963) and the other is autosomal (Shaw and Barto, 1965). The autosomally inherited mammalian isozyme reacts more actively with galactose-6-phosphate and is also referred to as "Hexose-6-phosphate dehydrogenase, H6PD" (Shaw and Koen, 1968). Two G6PD isozymes with similar substrate specificities as the

mammalian G6PD, have also been observed in trout (Stegeman and Goldberg, 1971) and in Xiphophorus (Scholl, 1973). The G6PD isozymes of Clarias batrachus and Heteropneustes fossilis are illustrated in Fig. 18 and Plate 14; Fig. 19 and Plate 15 respectively. The isozymes obtained are assumed to be homologous to the G6PD and H6PD isozymes of trout and other vertebrates. These bands have been labelled numerically from the anode to cathode (IUPAC-IUB Commission on Biochemical Nomenclature).

The two forms of G6PD, called A and B, are found in Clarias batrachus. The A fraction, corresponding to G6PD activity, was observed in all the tissues, with liver, heart and kidney tissues enhibiting the maximum activity and by contrast the muscle tissue showed the least. The form, corresponding to H6PD activity was observed as only a faint cathodal band in the liver tissue when glucose-6-phosphate was used as the substrate. This band stained more darkly with galactose-6-phosphate as the substrate.

In Heteropneustes fossilis the expression of the G6PD isozymes is more varied and tissue-specific. The

liver and the kidney tissues exhibited an intense band of activity. The muscle tissue showed only a negligible amount of activity as exhibited by a very faint band. The brain and the eye tissues were different from the other tissues, exhibiting a distinct five banded phenotype. No H6PD activity was observed when the gels were incubated in the medium containing galactose-6-phosphate as the substrate.

The extremely low activity of G6PD in the somatic muscle in both these fish suggests that the generation of NADPH from glucose oxidation via the hexose monophosphate shunt does not play an essential role in this tissue of fish. Similar observations have been made by Johnson (1975) in species of the atherinid genus Menidia, where G6PD activity was observed in the eye and liver tissues but no activity was seen in the muscle tissue. Similarly Fried et al.,(1969) found very high hepatic activity of G6PD in five species of teleosts indicating an active pentose shunt,but the muscle tissue exhibited negligible G6PD values.

High G6PD activity (generally indicative of an

active pentose shunt) in the livers of teleosts suggests that this tissue may be a major site of fatty acid synthesis at those phylogenetic levels preceeding the development of adipose tissue as a synthetically competent site. In preliminary studies with amphibians (*Rana pipiens*), appreciable hepatic G6PD activity has been encountered (Rosen *et al.*, 1968). Scholl and Anders (1973) have also reported the highest level of G6PD activity in the liver tissue in *Xiphophorus*.

In carps, G6PD occurs in a higher concentration in the red than in the white muscle (Bilinski, 1974). Similar results have been published by Bostrom and Johansson (1972), who worked on eel, *Anguilla*. These authors have suggested that the G6PD in red muscle is responsible for the generation of NADPH, essential for fatty acid biosynthesis. It is interesting to note that a contrasting result has also been observed in the muscle of cod (Bostrom *et al.*, 1973). It may be mentioned that we have used mixed white and red muscle as it was not possible to distinguish between the different muscle types in our fishes. Heart is known as an extreme red type of muscle. G6PD activity

observed in both Clarias batrachus and Heteropneustes fossilis exhibited a higher activity in the heart tissue than that observed in the somatic muscle tissue.

The molecular and genetic basis for teleost G6PD isozymes has still not been resolved entirely. In mammals the G6PD isozymes are encoded by two separate loci, one is sex-chromosome linked (Kirkman and Hendrickson, 1963) and the other is autosomal (Shaw and Barto, 1965). Scholl (1973) has assumed that the two isozymes which are described in Xiphophorus are homologous to the G6PD and H6PD of trout and other vertebrates and are inherited by two genes, the enzyme being dimeric in nature. Yamauchi and Goldberg (1973) found three zones in PAGE from liver extracts of river and sea trout. Cederbaum and Yoshida (1976) reported various regions with several bands in starch gel. They postulated two alleles of G6PD at one locus. These alleles were also detected in liver extracts of trout by Diebig et al. (1979). Allendorf et al. (1977) found tworecognisable forms of G6PD in rainbow trout which were probably encoded by a single locus, as in closely related salmonids. The two forms of G6PD observed in Clarias batrachus are assumed to be

homologous to those found in trout and other vertebrates. They are probably encoded by a single locus and the enzyme is dimeric in nature.

The resolution of G6PD into five isozymes in the brain and the eye tissues in Heteropneustes fossilis is strictly different from the pattern observed in Clarias batrachus. The electrophoretic mobility of these bands is identical in both these nervous tissues. This distinctive pattern distinguishes this fish from Clarias batrachus. The brain and eye lens electrophoretograms of G6PD in Cyprinus carpio also show a specific pattern which distinguishes it from other species. Here the molecule may be either in an active dimeric form or in a monomeric one, in dissociation equilibrium at the specified pH (AL - Hassan and Elias, 1988). A five banded pattern has been observed earlier by Yamauchi and Goldberg (1974) in both brook and lake trout, very similar to the one we see in Heteropneustes fossilis. These authors have also found nine forms of G6PD in splake trout. They have proposed that the G6PD isozymes are tetramers and the products of two codominant autosomal gene loci. Assuming a similar molecular

structure we can also explain the formation of the five isozymes in our fish. Since this distinct pattern is observed only in the two nervous tissues,there might be a selective increase in production of the distinct isozymes by these tissues. This may also be an indication of increased carbohydrate metabolism in these tissues.

There was no sex differential pattern in both our fish. This suggests that the influence of the X-linked locus is uniform in both the sexes and the G6PD enzyme behaves in the manner of autosomal inheritance.

The observed differences in the patterns of G6PD isozymes from these fish have put the question whether the enzyme activity appearing in the teleosts belongs to the same species of enzyme molecules as in the other higher vertebrates, nevertheless, the differences could be related to a distinct mechanism of the regulation of this enzyme.

TABLE 11 . Enzyme tissue specificity in *Clarias batrachus*.

Enzyme	Bands	Tissues						
		Kidney	Liver	Eye	Muscle	Brain	Heart	Gonads
LDH	A_4	++	+	+++	+++	+++	+++	+
	A_3B_1	++	+	++	++	++	++	++
	A_2B_2	++	+	++	-	++	++	++
	A_1B_3	++	++	++	-	++	++	++
	B_4	+++	+++	+++	+	++	+++	+++
	C_4	-	++	-	-	+	++	-
	K_4	++	-	-	-	-	++	-
MDH	A_2	++	++	++	++	+	++	++
	A_1B_1	++	+	++	++	+	++	++
	$A_1B'_1$	+	+	+	-	-	+	+
	B_2	+++	+++	++	+++	++	+++	++
	B'_2	+	++	-	-	-	-	-
	B'B	+	-	-	-	+	+	+
ADH	A_2	++	+++	++	+	-	-	+
	A_1B_1	+	-	+	-	-	+	+
	B_2	-	-	+	+	-	+	-
G6PD	A_2	+++	+++	+++	++	+++	+++	++
H6PD	A_2	-	+	-	+	-	+	-

+++ = very high activity
++ = high activity
+ = low activity
- = no activity

TABLE 12. Enzyme tissue specificity in *Heteropneustes fossilis*

Enzyme	Bands	Tissues						
		Kidney	Liver	Eye	Muscle	Brain	Heart	Gonads
LDH	A_4	+	-	++	+++	+++	++	+
	A_3B_1	+	-	++	++	++	+++	+
	A_2B_2	+	-	++	++	++	+++	+
	A_1B_3	+	+	++	++	++	+++	+
	B_4	+++	+++	++	++	++	+++	++
	C_4	++	++	-	-	-	-	-
MDH	A_2	++	+	-	++	-	++	++
	A_1B_1	++	+++	+	++	++	++	++
	$A_1B'_1$	-	-	+	-	-	++	-
	B_2	++	+++	+++	++	++	++	++
	B'_2	+	++	-	-	-	-	-
	B'B	+++	-	-	-	-	-	++
ADH	A_2	-	++	-	-	-	-	-
	A_1B_1	++	++	+	-	+	-	-
	A_1B_1	++	++	+	-	+	-	-
	B_1C_1	-	-	+	-	+	-	-
	B_2	-	+++	+	-	+	-	-
	C_2	-	-	+	-	+	-	-
G6PD	5	+++	+++	++	++	++	+++	+++
	4	-	-	++	-	++	-	-
	3	-	-	++	-	++	-	-
	2	-	-	++	-	++	-	-
	1	-	-	++	-	++	-	-

+++ = very high activity
++ = high activity
+ = low activity
- = No activity

5. CONCLUDING REMARKS

We have utilised the high resolving power of disc electrophoresis to dissect isozyme patterns of some dehydrogenases. The data were finally pooled to understand the genetics of the selected isozymes in the two air-breathing fishes viz. *Clarias batrachus* and *Heteropneustes fossilis*. Isozyme tissue-specificity is summarised in these two fishes in Table 11 and 12, respectively. It may be well to state here that fresh tissues processed under 4°C yielded the best results. Some isozymes e.g. the K_4 LDH could not be resolved either from stored tissue or those processed at room temperature.

It is accepted that LDH is coded at three different loci viz. A, B and C, each with allelic variations. The present investigation has revealed the presence of a fourth locus active mainly in the kidney. The controversy regarding the origin of the A, B and C genes has been considerably cleared by our discovery of the fourth LDH coding locus. We do admit that conclusive evidence has to be generated by studying their amino acid sequence and through immunochemical analysis. However, with our findings, it can now be stated

quite convincingly that the B and C genes originated from the A gene as claimed originally by Holmes (1972). We propose that the new gene also arose independently from the B gene in the same way.

Two malate dehydrogenase loci encoding cytosol enzymes are present in fish(Wheat et al., 1972; Clayton et al., 1973 b). In C. batrachus and H. fossilis also, two MDH loci, A and B, are responsible for the observed patterns. Due to the random association of the two subunits MDH A and MDH B, distinct tissue-specific patterns were observed in these two fishes. There is evidence of the existence of an allelic locus, the A' locus. A further insight into the understanding of the cathodally migrating MDH isozymes would help us to confirm whether or not these are the products of two separate loci.

Alcohol dehydrogenase is known to be mainly a liver-specific protein, encoded in the majority of fishes studied, at a single gene locus, as is obvious from the work of Philipp et al. (1979), Frankel and Wilson (1984) and Vallee (1985). On the contrary, we have proposed that at least two

gene loci, A and B, are responsible for the ADH activity observed in the present work in Clarias batrachus and Heteropneustes fossilis. In the latter there is evidence of an additional allelic locus the A' gene locus, along with the A and B genes, responsible for the characteristic pattern observed in the individuals of this fish. Different gene models have been postulated in Xenopus laevis (Wesolowski and Lyerla, 1983) and Rana limnocharis (Prakash, 1988) on the basis of two and three structural genes, respectively. Similarly we have proposed two different ADH models, one each for C. batrachus and H. fossilis. Our models are based on the action of two gene loci in C. batrachus and three genes A, A' and B in H. fossilis.

A detailed study relating to the correlation between these fishes and their environmental variability is required to obtain a complete picture of the regulatory genes responsible for the observed patterns of MDH and ADH activity in these air-breathing fishes.

The glucose-6-phosphate dehydrogenase enzyme gave no

evidence of sex-linked inheritance in C. batrachus and H. fossilis. In fishes the sex chromosomes are not heteromorphic and one cannot distinguish between the male and female karyotypes normally. The sex determining genes are distributed on the autosomes themselves. The same mechanism is reflected in their G6PD isozyme study and no sex differential activity is seen in these fishes. In C. batrachus the G6PD enzyme is encoded by a single gene locus. A characteristic five band pattern is observed in the eye and brain tissues of H. fossilis. In this fish G6PD isozymes may be tetramers encoded by two codominant autosomal gene loci, as is suggested by Yamauchi and Goldberg (1974) in trout species. In order to ascertain this, more elaborate research along biochemically analytical lines is required in the future. Only then one can finally draw a conclusion about the regulatory mechanism controlling the expression of the various isozymes observed in the present work both in Clarias batrachus and Heteropneustes fossilis.

Polymorphism at various isozyme loci exists in these air-breathing fishes. Population studies relating to the

Hardy-Weinberg law have to be conducted to obtain the allele frequencies. If the deviation of the resultant allele frequencies, from the expected values, is statistically significant, then forces like those of selection and environmental fluctuations must be acting upon the population. The various alleles could be existing in the population due to physiological stress viz. temperature increase or decrease, oxygen concentration, adaptive response to water conditions and so on, or in other words to increase the fitness of the species in their changing environment. The widespread heterozygosity for proteins provides both an excellent apportunity and a demand for further study of genetic and biochemical variation in terms of the environment. Much experimentation is necessary to clarify the direct and synergistic effects of different environmental factors, which was beyond the purview of the present work. The data provided here may serve as the background for such further research.

6. REFERENCES

* Aida, T. (1930) : J. Genetics 15, 1 - 16.

AL - Hassan, L.A.J. and Elias, N.H. (1988) : Isoenzymes of Cyprinids from the vicinity of Basrah in relation to classification. Biochemical Systematics and Ecology. 16, 2, 223-226.

Allendorf, F.W., Mitchell, N., Ryman, N. and Stahl,A. (1977): Isoenzyme loci in brown trout (Salmo trutta L): detection and interpretation from population level. Hereditas 86 : 179 - 190

Allendorf, F.W. and Utter, F.M. (1979) : In Fish Physiology, 8. W.S. Hoar, D.A. Randall & J.R. Brett, eds. 407 - 454, New York : Academic Press

Allison, W.S. (1968) : Structure and evolution of triosephosphate and lactate dehydrogenases. Ann. N.Y. Acad. Sci. 151, 180 - 189

Altman, M. and Robin, E.D. (1969) : Survival during prolonged anaerobiosis as a function of an unusual adaptation involving lactate dehydrogenase subunits. Comp. Biochem. Physiol. 30, 1179 - 1187.

Altukhov, Y.P., Salmenkova, E.A. Omelchenko, V.T., and Sachko, G.D. and Slynko, V.I. (1972) : Number of mono and polymorphous loci in the population of Tetraploid salmon species Oncorhynchus keta Walls. Genetika 8(2) : 67 - 75

Appella, E., Markert, C.L. (1961) : Dissociation of lactate dehydrogenase into subunits with guanidine hydrochloride. Biochem. Biophys. Res. Comm. 6, 171 - 176.

Aspinwall, N. (1974) : Genetic analysis of duplicate malate dehydrogenase loci in the pink salmon, Oncorhynchus gorbuscha. Genetics, 76 : 65 - 72.

Avise, J.C. (1974): Systematic value of electrophoretic data. Syst. Zool. 23, 465 - 481

Bailey, R.M. (1970) : A list of common and scientific names of fish from the United States and Canada. **Am. Fish. Soc., spec. Publ. 6, 150.**

Bailey, G.S., Cocks, G.T. and Wilson, C. (1969) : Gene duplication in fishes : Malate dehydrogenases of salmon and trout. **Biochemical and Biophysical Research Communications 34, 5, 605 - 612.**

Bailey, G.S., Tsuyuki, H., and Wilson, A.C. (1976) : The number of genes for lactate dehydrogenase in salmonid fishes. **J.Fish. Res. Board Can. 33:760-767**

Bailey, G.S. and Wilson, A.C. (1968) : Homologies between isoenzymes of fishes and those of higher vertebrates: evidence for multiple H_4 lactate dehydrogenase in trout. **J. Biol. Chem. 243, 5843 - 5853.**

Bailey, G.S., Wilson, A.C., Halver, J.E. and Johnson, C.L. (1970) : Multiple forms of supernatant malate dehydrogenase in salmonid fishes. Biochemical, Immunological and genetic studies. **J. Biol. Chem. 245, 5927 - 5940.**

Baldwin, J. and Lake, P.S. (1987) : Lactate Dehydrogenase Homopolymer of Hagfish heart and the single lactate dehydrogenase of Lampreys display greater Immunochemical similarity to LDH C_4 than to LDH B_4 of Teleost fish. **Journal of Experimental Zoology 242 (1) : 99 - 102.**

Balek, R.W., Snow, J. and Haduck, L. (1967) : Electrophoresis and heat inactivation of lactate dehydrogenase isozymes in Amphibia. **Life Sciences 6, 1035 - 1045.**

Banaszak, L.T. and Bradshaw, R.A., (1975) : Malate dehydrogenases. In. **The Enzymes (Edited by Boyer, P.D.), XI, 369 - 396. Academic Press, New York.**

Barron, E.J., Squires, C. and Stumps, P.K. (1961) : Fat metabolism in higher plants. XV. Enzyme synthesis of fatty acids by an extract of avocado mesocarp. J. Biol. Chem. 236, 2610 - 2614.

Basu, S.K Sahu, C.R. and Bose, A.K. (1981) . some biochemical patterns of egg proteins in Notopterus notopterus and Mystus vittatus (abst.) 4th All India Cong. Cytol. Genet. 36. 1981.

Bautista, J.M., Amando Garrido-Pertierra and Manuel Ruiz-Amil Purification and properties of two enzymatic forms of glucose - 6 - phosphate dehydrogenase from Dicentrarchus labrax L. liver. Comp. Biochem. Physiol. 77B, 4, 843 - 848

Bhattacharya, A., Sarkar, S.K., Basu, S.K. and Ganguly, (1989) :Lactate dehydrogenase as genetic marker enzyme in fish Tilapia mossambica. Indian Journal of Experimental Biology 27, 913 - 914.

Bilinski, E., (1974) : Biochemical aspects of fish swimming. In Biochemical and Biophysical Perspectives in Marine Biology. (Edited by Malins, D.C., and Sargent, J.R.) 1, 239 - 288, Academic Press, London.

Blanco, A., Zinkham, W.H. and Kupchyk, L. (1964) : Genetic control and ontogeny opf lactate dehydrogenase in pigeon testes. J. Exp. Zool., 156 : 137 - 152.

Bonavita, V. and Guarneri, R. (1963) : Lactic dehydrogenase isozymes in the nervous tissue. II. A comparative analysis in the vertebrates. J. Nerochem., 10: 743-753.

Bosron, W.F., Magnes, L.J., and Li, T-K. (1983) : Human liver Alcohol dehydrogenase : ADH. results from Genetic Indianapolis polymorphism at the ADH Gene Locus. Biochemical Genetics 21, 7/8, 735 - 744.

Bostrom , S.L., Fange R. and Johansson R.G. Enzyme activity patterns in gas gland tissue of the swim bladder of the cod (Gadus morrhua). Comp. Biochem. Physiol 43B, 473 - 478.

Bostrom, S.L. and Johansson, R.G. (1972) : Enzyme activity patterns in white and red muscle of the eel Anguilla anguilla at different developmental stages Comp. Biochem. Physiol. 42B, 533 - 542.

Bosron, W.F., Li, T.K., Dafeldecker, W.P. and Vallee, B.L. (1979) : Human liver Pi-alcohol dehydrogenase : kinetic and molecular properties. Biochemistry 18, 1101 - 1105.

Boyer, S.H., Fainer, D.C. and Watson-Williams, E.J. (1963) : Lactate dehydrogenase variant from human blood : Evidence for molecular subunits. Science, 141: 642-643

Bradford, M.M. (1976) : A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein - dye binding. Analyst. Biochem, 72, 248 - 254.

Branden, C.I., Jornvall, H., Eklund, H., and Furugren, B., (1975) : Alcohol dehydrogenase. In Boyer, P.D. (ed.), The Enzymes, vol. II. Oxidation - Reduction, Part A. 3rd ed., Academic Press, New York, 104 - 190.

Busack, C.A., Halliburton, R. and Gall, G.A.E. (1979) : Electrophoretic variation and differentiation in four strains of domesticated rainbow trout. (Salmo gairdneri). Can. J. Genet. Cytol. 21 : 81 - 94.

Buth, D.G. (1983) : Duplicate isozyme loci in fishes : origins, distribution, phyletic consequences, and locus nomenclature. In : Isozymes : current topics in biological and medical research, 10, 381 - 400. M.C. Rattazzi, J.C. Scandalios, and G.S. Whitt (eds.) Alan R. Liss Inc., New York, New York.

Buth, D.G. (1984): The application of electrophoretic data in systematic studies. Ann. Rev. Ecol. Syst. 15, 501 - 522.

Cahn, R.D., Kaplan, N.O., Levine and Zwilling, E. (1962) : Nature and development of lactic dehydrogenases. Science 13b, 962 - 969.

Cardemil, E. (1978) : Alcohol - oxidising enzymes from various organisms. Comp. Biochem. Physiol. 1978, 60B, 1 - 7.

Cederbaum, S.D., and Yoshida, A. (1976) : Glucose-6-phosphate dehydrogenase in rainbow trout. Biochem. Genet. 14, 245 - 258.

Chambers, K., Wilks, Ann, V. and Gibson, John, B. (1984) : Variation in the biochemical properties of the Drosophila Alcohol dehydrogenase allozymes. Biochemical Genetics 22, 1/2.

Champion, A.B., Soderberg, K.L. and Wilson, A.C. (1975) : Immunological comparison of azurins of known amino acid sequence. J. Molec. Evol. 5, 291 - 305.

Chandrasekhar, N. (1959) : Blood proteins of some Indian freshwater fishes. Proc. Ind. Acad. Sci., B. XLIX 377 - 385.

Chatterjee, K. (1989) : Cytotaxonomic and electrophoretic investigations on Indian air-breathing fishes. Fish Genetics in India, Edrs. Das and Jhingran (1989) : 83 - 99. Today and Tomorrow's Printers and Publishers, New Delhi - 110 005 (India).

Chatterjee, K. and Dhar, N.J. (1985) : Lactate Dehydrogenase Isozymes in the genus Channa : species-specific patterns. Biochemical Systematics and Ecology, 13, 4, 467 - 470.

Chatterjee, K., Joseph, P. and Tandon, A. (1988) :A new kidney-specific locus of lactate Dehydrogenase in the Indian catfish Clarias batrachus. Genome 30, 1, 32.45.15 XVIth International Congress of Genetics, Aug 20 - 27, 1988, Toronto, Canada.

Chatterjee, K., and Prakash, S. (1990) : Genetic variations in the natural populations of Rana limnocharis. Impacts of Environment on Animals and Aquaculture. (Eds. G.K. Manna and B.B. Jana), 251 - 256.

Chang, S.M.T., Lee, C.Y. and Li, S.S.L. (1979) : Structural relatedness of mouse lactate dehydrogenase isozymes, A_4 (muscle), B_4 (heart), and C_4 (testis). Biochem. Genet. 17, 715 - 729.

Clayton, J.W. and Franzin, W.G. (1970) : Genetics of multiple Lactate dehydrogenase isoenzymes in muscle tissue of Lake whitefish (Coregonus clupeaformis) J. Fish. Res. Bd. Can. 27 : 1115 - 1121.

Clayton, J.W. and Gee, J.H. (1969) : Lactate dehydrogenase isozymes in longnose and blacknose dace, (Rhinichthys cataractae and R. astratulus), and their hybrid. J. Fish. Res. Bd. Can 26, 3049 - 3053.

Clayton, J.W., Tretiak, D.N., Billeck, B.N. and Ihssen, P. (1975) : Genetics of multiple supernatant and into-chondrial malate dehydrogenase isozymes in rainbow trout (Salmo gairdneri). In Isozymes (Edited by Markert, C.L.) IV, 433 - 448. Academic Press, New York.

Cooper, S.C. and Ferguson, J.H. (1972) : The effect of cold acclimation upon glucose-6-phosphatase activity in two species of free-living nematodes-I. The effect of pH. Comp. Biochem. Physiol. 42A, 781-789.

Cruz, T.A., Thorpe, J.P. and Pultin, R.S.V. (1982) : Enzyme electrophoresis in Tilapia zillii: a pattern for determining biochemical genetic markers for use in Tilapia stock identification. Aquaculture 29, 311 - 329

Dafeldecker, W.P. and Vallee, B.L. (1982) : Alcohol dehydrogenase isozymes of horse liver. J. Prot. Chem. 1, 59 - 69.

Dando, R.P. (1971) : Lactate dehydrogenase polymorphism in the flatfish (Heterosomata). Extrait du Reports et Proces verbaux, 161 : 130 - 133.

Darling, J.S. (1976) : Electrophoretic variation in Cyprinodon variegatus and systematics of some fishes of the family Cyprinodontidae. Ph.D. thesis, Yale University, New Haven.

Darnall, D.W. and Klotz, I.M. (1975) : Subunit composition of proteins. Arch. Biochem. 166, 651-682.

Das, B.C. (1961) : Comparative study of the blood biochemistry of this species of Indian carp. Trans. Am. Fish Soc. 90 : 1 - 5.

Daugherty, W. (1965) : Analysis of the apparently single form of lactate dehydrogenase (LDH) found in the flatfish, Paralichthys dentatus. Am. Zool. 5,205

*Davidson, R.G. and Cortner, J.A., (1967) : Science, 157, 1569-1571.

Davis, B.J. (1964) : Disc electrophoresis; II method and application to human serum protein. Am. N.Y. Acad. Sci. 121: 404-427.

Dawson, C.E. (1964) : A revision of the Western Atlantic flatfish genus Gymnachirus (the naked soles.) Copeia 646-665.

Dawson, D.M., Goodfriend, T.L. and Kaplan, N.O. (1964) : Lactic dehydrogenases : functions of the two types. Science 143, 929-933.

*de Almeida-Val, V.M.F. (1986) : Lactato desidrogenase de duas especies de peixes da Amazonia, Mylossoma duriventris and Colossoma macropomum (characiformes): aspectos adaptativos. Ph. D. Thesis, Programa de Pos-Graduacao INPA/FVA, Manaus (AM), Brazil

de Almeida-Val, V.M.F., Schwantes, M.L.B., and Val,A.L.(1990) :LDH isozymes in Amazon Fish-I. Electrophoretic studies on two species from Serrasalmidae family: Mylossoma duriventtris and Colossoma macropomum. Comp. Biochem. Physiol. 95B, 1, 77-84.

Deibig, E., Mayer, J.N. and Glodek, P. (1979) : Biochemical polymorphism in muscle and liver extracts and in the serum of the rainbow trout Salmo gairdneri. Animal Bld. Grps. Biochem 10 : 165-174.

de Ligny, W. (1972) : Blood groups and biochemical polymorphism in fish. In : G. Kovacs and M. Papp (Editors), XIIth European conference on animal blood groups and biochemical polymorphism, 55-65. The Hague : Dr. W. Junk N.V. Publ.

de Panepucci, L.C.L., Schwantes, M.L.B. and Schwantes, A.R. (1984) : Loci that encode the lactate dehydrogenase in 23 species of fish belonging to the orders Cypriniformes, Siluriformes and Perciformes : Adaptive features. Comp. Biochem. Physiol. 77B, 867-876.

de Vlaming, V.L. (1975) : Effects of photoperiod - temperature regimes and pinealectomy on body fat reserves in the Golden Shiner : Fish. Bull. 73, 766-776.

Dey, A.C. (1984) : Distribution of malate dehydrogenase in different tissues of Rainbow trout (Salmo gairdneri) of different ages. Comp. Biochem, Physiol. 77B, 4, 675-678.

Dhar, N. and Chatterjee, K. (1984) : Electrophoretic investigations on Channa spp. I. Protein variation in Channa punctatus and Channa marulius (Pisces: Channidae). Perspectives in Cytology and Genetics 4: 149-152.

Ditlow, C.C., Holmquist, B., Morelock, M.M. and Vallee, B.L. (1984) : Physical and enzymatic properties of a classII alcohol dehydrogenase isozyme of human liver Pi-ADH. Biochemistry 23, 6363-6368.

Ekberg, D.R. (1962) : Anaerobic and aerobic metabolism in gills of the crucian carp adapted to high and low temperature. Comp. Biochem. Physiol. 5, 123-128

Ellstrand, N.C., Lee, J.M. and Foster, K.W. (1983) : Alcohol dehydrogenase isozymes in grain sorghum (Sorghum bicolor): Evidence for a gene duplication. Biochemical Genetics, 21, 1/2, 147-154.

*Ferguson, A. (1980) : Biochemical Systematics and Evolution. Glasgow: Blackie.

Fine, I.H., Kaplan, N.O. and Kuftinec, D. (1963) : Developmen -tal changes of mammalian lactic dehydrogenases. **Biochemistry, N.Y. 2, 116-121.**

Fisher, S.E., Shaklee, J.B., Ferris, S.D., Whitt, G.S. (1980): Evolution of five multilocus isozyme systems in the chordates. **Genetica 52/53: 73-86.**

Fitch, N.A. (1988) : Lactate Dehydrogenases in Antarctic and Temperate fish species. **Comp. Biochem. Physiol 91B, 4, 671-676.**

Fong, W.P. and Keung, W.M. (1987) : Substrate specificity of human class I alcohol dehydrogenase homo and heterodimers containing the beta-2 (oriental) subunit. **Biochemistry 26, 5726-5732.**

Frankel, J.S. (1978) : Gene activation of alcohol dehydrogenase in danio hybrids. J. **Hered. 69, 57-58.**

Frankel, J.S. (1980) : Expression of alcohol dehydrogenase during pearl danio development. J. Hered. 71:430-431.

Frankel, J.S. (1981) : Comparative analysis of Lactate Dehydrogenase isozymes in neural tissues of Poecilia (Poeciliidae). **Comp. Biochem. Physiol. 69B, 881-884.**

Frankel, J.S. (1983) : Lactate Dehydrogenase tissue specificity and characterisation in the Teleost genus Barbus. Comp. Biochem. Physiol 76B, 1, 103-105.

Frankel, J.S. (1985): Asynchronous expression of the alcohol dehydrogenase (EC 1.1.1.1) and the supernatant malate dehydrogenase (EC 1.1.1.37) loci during Barbus hybrid development (Cypriniformes, Teleostei). **Comp. Biochem. Physiol. B Comp.** Biochem. 81(3): 635-640.

Frankel, J.S., and Wilson, R.V. (1984) : Comparison of the spatial and temporal expression of supernatant malate dehydrogenase in Barbus hybrids (Cypriniformes, Teleostei.) Comp. Biochem. Physiol. 78B, 179-182.

Freed, J. (1965) : Changes in activity of cytochrome oxidase during adaptation of goldfish to different temperatures. Comp. Biochem. Physiol. 14, 651-659

Fried, G.H., Schreibman, M.F. and Kallman, K.D. (1969) : Enzymatic activities in tissues of Teleosts. Comp. Biochem. Physiol. 28, 771-776.

Fujio, Y. and Kato (1979) : Genetic variation in fish population. Bull. Jap. Soc. Sci. Fish 45: 1169-1178

*Gerschler, M.W. (1914) : Uber alternative Vererbung bei kreuzung von Cyprinodontiden - Gattungen. Z. Ind. Abst. Vererbgst. 12, 73-96.

Gesser, H. and Poupa, O. (1973) : The LDH system in the heart and skeletal muscle of fish: a comparative study. Comp. Biochem. Physiol 96B, 683-690.

Goldberg, E. (1963) : Lactate dehydrogenases in human testes. Science 139, 601-602.

Goldberg, E. (1965) : Lactate dehydrogenase in trout : Evidence for a third subunit. Science, 148: 391-392

Goldberg, E. (1969) : Ontogeny of lactate dehydrogenase isozymes in trout. In "Fish in Research" ed. Otto W. Neuhaus and John E. Halver) Academic Press, New York, London. 195-205.

Goldberg, E. (1972) : "Amino acid composition and properties of crystalline lactate Dehydrogenase X from Mouse Testes". Journal Biological chemistry 247(7): 2044-2048.

Goldberg, E. and Wuntch, T. (1967) : Electrophoretic and kinetic properties of Rana pipiens LDH isozymes. J. Exp. Zool. 165, 101.

---

*Gorman, G.C., Wilson, A.C. and Nakanishi, M. (1971) : Syst. Zool. 20, 167-185.

Grell, E.H., Jacobsen, K.B. and Murphy, J.B. (1965) : alcohol dehydrogenase in _Drosophila melanogaster_ : Isoenzymes and genetic variants. **Science 149: 80.**

Grimm, F.C. and Doherty, D.G. (1961) : Properties of the two forms of malic dehydrogenase from beef heart. **J. Biol. Chem, 236, 1980-1985.**

Hahn, P. (1970) : Fatty acid synthesis in brown and white adipose tissue and liver of the rate during develo--pment. **Physiol. Bohemoslov. 19, 369-375.**

Hart, N.H. and Pontier, P.J. (1982) : Ontogeny of glucose-6-phosphate and phosphogluconate dehydrogenases in _Brachydanio._ **Comp. Biochem. Physiol. 71B, 193-200**

*Hauss, R. (1975a) : Wirkungen der Temperatur auf Proteine, Enzyme und Isoenzyme aus Organen des Fisches _Rhodeus amarus_ Bloch. I. Einflu B der Adaptations termeratur auf die weibe Ruckenmuskulatur. **Zool. Anz. 194, 243-261.**

Hauss, R. (1975b) : Wirkungen der Temperatur aug Proteine, Enzyme und Isoenzyme aus Organen des Fisches _Rhodeus amarus_ Bloch.II. EinfluB der Adaptations temperatur auf des Gehirn. **Zool. Anz. 194, 262-278.**

Hedges, S.B. (1987) : Slow-evolving loci: an electrophoretic approach to higher-level systematic problems. **Am. Zool. 27, 84A.**

Hines, S.A., Philipp, D.P., Childers, W.F. and Whitt, G.S. (1983) : Thermal kinetic differences between allelic isozymes of malate dehydrogenase (Mdh-B locus) of Large-mouth bass, _Micropterus salmoides._ **Biochem. Genet. 21, 1143-1151.**

Hitzeroth, H., Klose, J., Ohno, S. and Wolf, U. (1968) : A synchronous activation of parental alleles at the tissue-specific gene loci observed on hybrid trout during early development. **Biochem. Genet. 1,287-300**

Hochachka, P.W. (1966) : Lactate Dehydrogenases in Poikilotherms : Definition of a complex isozyme system. Comp. Biochem. Physiol. 18,261-269.

Hockachka, P.W. (1968) : The nature of the thermal optimum for lungfish lactate dehydrogenase. Comp. Biochem. Physiol. 27, 609-611.

Hochachka, P.W. and Somero, G.N. (1973) : Strategies Biochemical Adaptation. Academic Press, New York.

Hochachka, P.W and Somero, G.N. (1984) : Biochemical Adaptation. Princeton University Press, Princeton, USA.

Hodgins, H.O., Ames, W.E. and Utter, F.M. (1969) : Variants of lactate dehydrogenase isozymes in sera of sockeye salmon (Oncorhynchus nerka.) J. Fish. Res. Board Can. 26, 15-19.

Holbrook, J.J., Lijas, A., Stendel, S.J. and Rossman, M.G. (1975) : Lactate dehydrogenase, p. 191-292. In P.D. Boyer (ed) The enzymes. Academic Press, New York, NY.

Holmes, R.S. (1972) : Evolution of lactate dehydrogenase genes. FEBS. Lett. 28: 51-55.

Holmes, R.S. and Markert, C.L. (1969) : Immunochemical homologies among subunits of trout lactate dehydrogenase isozymes. Proc. Nat'l Acad. Sci. U.S. 64, 205-210.

Holmes, R.S. and Scopes, R.K. (1974) : Immunological homologies among vertebrate-lactate dehydrogenase isozymes. Eur. J. Biochem. 43: 167-177.

Holt, R.W. and Leibel, W.S. (1987) : Co-expression of distinct eye-specific and liver-specific LDH isozymes in cichlid fish. J. Exp. 2001. 244(2) : 337-344.

Hora, S.L. (1956) : Fish paintings of third millenium B.C. from Nal (Baluchistan) and their Zoogeographic significance. Mem. Indian Mus. 14(2) : 78-84.

Horowitz, J.J., Whitt, G.S. (1972) : Evolution of a nervous system specific lactate dehydrogenase-isozyme in fish. J. Exp. Zool. 180, 13-32.

Hubby, J.L. and Lewontin, R.C. (1966) : A molecular approach to the study of genic heterozygosity in natural populations. Genetics 54(2) : 577-594.

Hulsman, W.C. (1962) : On the relationship between oxidation of malate and isocitrate and the synthesis of long chain fatty acids. Biochem. Biophys. Acta 62, 620-622.

Hunter, R.L. and Markert, C.L. (1957) : Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science 125, 1294-1295.

Hussain, A.V. and Siddique, A.Q. (1974) : Comparative studies on the tissue proteins of some catfishes. Mahasagar 7 (1 and 2): 53-59.

Huzyk, L. and Tsuyuki, H. (1974) : Distribution of LDH-B" gene in resident and anadromous rainbow trout (Salmo-gairdneri) from streams in British Columbia. J. Fish. Res. Bd. Can., 31, 106-108.

Jhingran, V.G. (1974) : Fish and Fisheries of India. Hindustan Publishing Corporation (India), Delhi. 2-69.

Julia, P., Farres, J. and Parex, X. (1987): Characterisation of three isoenzymes of rat alcohol dehydrogenase: distribution and physical and enzymatic properties. Eur. J. Biochem. 162, 179-189.

Junk, W.J. (1984) : Ecology of the "varzea" floodplain of Amazonian whitewater rivers. In the Amazon, Limnology and Landscape Ecology of a Mighty Tropical River and its Basic Monographiae Biologicae, 56, 215-243. W. Junk, Dordrecht, The Netherlands.

Johnson, M.S. (1971) : Adaptive Lactate dehydrogenase variation in the Crested Blenny, Anoplarchus. Heredity 27(2): 205-226.

Johnson, G.B. (1974) : Enzyme polymorphism and metabolism. Science 184, 28-37.

Johnson, M.S. (1975) : Biochemical Systematics of the Atherinid genus *Menidia*. Copeia, 4: 662-691.

Johnson, M.S. (1977) : Association of allozymes and temperature in the crested blenny *Anoplarchus purpurescens*. Mar. Biol. 41(2), 147-152.

Johnson, F.M. and Dennistone, C. (1964) : Genetic variation of alcohol dehydrogenase in *Drosophila melanogaster.* Nature 204: 906.

~~*German, G.C., Wilson, A.C. and Nakanishi, M. (1971) : Syst. Zool. 20, 167-185.~~

Kao, Y.H.J. and Farley, T.M. (1978) : Purification and properties of allelic lactate dehydrogenase isozymes at the B^2 locus in rainbow trout, *Salmo gairdneri*. Comp. Biochem. Physiol. 61B, 507-512.

Kaplan, N.O. (1964) : Lactate dehydrogenase-structure and function. In: subunit structure of proteins. Brookhaven Symposia in Biology 17, 131-153.

Kaplan, N.O., Ciotti, M.M., Hamolsky, M. and Beiber, R.E. (1960): Molecular heterogeneity and evolution of enzymes. Science 131, 392-397.

Kaplan, N.O. and Goodfriend, T.L. (1964) : Role of the two types of lactic dehydrogenase. Adv. Enz. Regul. 2, 203-212.

Karig, L.M. and Wilson, A.C. (1971) : Genetic variation in supernatant malate dehydrogenase of birds and reptiles. Biochem. Genet. 5, 211-221.

Kepes, K.L. and Whitt, G.S. (1972) : Specific lactate dehydrogenase gene function in the differentiated liver of cyprinid fish. Genetics 71 (no. 3/part 2), 529.

Keung, W.M., Ho, Y.W., Fong, W.P. and Lee, C.Y. (1989) : Isolation and characterisation of shrew (Suncus murinus) liver alcohol dehydrogenases. Comp. Biochem. Physiol. 93B, 1, 169-173.

Kirkman, H.N. and Hendrickson, E.M. (1963) : Sex-linked electrophoretic difference in glucose-6-phosphate dehydrogenase. Am. J. Human. Genet., 15: 241-249.

*Kirkman, H.N., McCurdy, P.R. and Naiman, J.L. (1964) : Cold Spring Harb. Symp. Quant. Biol. 29, 391.

Kirpichnikov, V.S. and Muske, G.A. (1980) : The adaptive value of biochemical polymorphisms in animal and plant populations. Genetica, 52/53: 183-193.

Kitto, G.B. and Kaplan, N.O. (1966) : Purification and properties of chicken heart mitochondrial and supernatant malic dehydrogenases. Biochemistry 5, 3966-3980.

Kitto, G.B. and Lewis, R.G. (1967) : Purification and properties of tuna supernatant and mitochondrial malate dehydrogenase. Biochem. Biophys. Acta 139, 1-15.

Kitto, G.B., Wassarmann, P.M. and Kaplan, N.O. (1966) : Enzymically active conformers of mitochondrial malate dehydrogenase. Proc. nat. Acad. Sci. 56, 578-585.

Klain, G.J. and Hannon, J.P. (1977) : Differential responses of rat brown and white adipose tissue to environmental or nutritional stress. Comp. Biochem. Physiol. 58B: 227-230.

Klar, G.T., Stalnaker, C.B., Farley, T.M. (1979): Comparative blood lactate response to low oxygen concentrations in rainbow trout, salmo gairdneri, LDH B^2 phenotypes. Comp. Biochem, Physiol. 63A, 237-240.

Klose, J., Wolf, K., Hitzeroth, H., Ritter, H., Atkin, N.B., Ohno, S. (1968) : Duplication of the LDH gene loci by polyploidisation in the fish order Clupeiformes. Humangenetik 5, 190-196.

Klose, J., Wolf, V., Hitzeroth, H., Ritter, H. and Ohno, S. (1969) : Polyploidization in the fish family Cyprinidae, order Cypriniformes II. Humangenetik, 7: 245-250.

Koehn, R.K. (1969): Esterase heterogeneity : dynamics of a polymorphism. Science, 163, 943-944.

Kraus, A.P. and Neely, C.L. Jr. (1964) : Human erythrocyte lactate dehydrogenase. Four genetically determined variants. Science, 145: 595-597.

Krishna Rao, M.R., Padhi, B.K. and Khuda-Bukhsh, A.R. (1989): Lactate dehydrogenase Isozymes in Fifty-two species of Teleostean Fishes: Taxonomic significance of LDH-C gene expression. Biochemical Systematics & Ecology: 17, 1, 69-76.

Krishnaja, A.P. and Rege, M.S. (1977) : Haemoglobin heterogeneity species of the Indian carp and their fertile hybrids. Ind. J. Exp. Biol. 15 (10): 925-926.

Krishnaja, A.P. and Rege, M.S. (1979) : Genetic studies on two species of the Indian carp Labeo and their fertile F_1 and F_2 hybrids. Ind. J. Exp. Biol. 17(3): 253-257.

Laemmli, U.K. (1970) : Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lagler, K.F. Bardach, J.E. and Miller, R.R. (1962) : Ichthyology. Published by John Wiley and Sons. New York, Chichester. Brisbane. Toronto.

Lapin, V.J. (1973) : Seasonal variations in the biochemical compostion of the organs and tissues of the White Sea flounder. J. Ichthyol. 13, 163-274.

Leslie, J.F. and Vrijenhoek, R.C. (1977) : Genetic analysis of natural populations of Poeciliopsis monacha: Allozyme inheritance and pattern of mating. J. Heredity, 68: 301-306.

Levine, J.P. and Hadley, L.R. (1975) : Gene activation of alcohol dehydrogenase in Japanese quail and chicken quail hybrid embryos. Biochem. Genet. 13: 435.

Li, S.S.L., Fitch, W.M., Pan, Y.C.E. and Sharief, F.S. (1983): Evolutionary relationships of vertebrate lactate dehydrogenase isozymes A_4 (muscle), B_4 (heart), and C_4 (testes). J. Biol. Chem. 258, 7029-7032.

Li, L.K., So, L. and Spector, A. (1985) : Membrane cholesterol and phospholipid in consecutive concentric sections of human lenses. J. Lipid. Res. 26, 600-609.

Lindsay, D.T. (1963) : Isozymic patterns and properties of lactate dehydrogenase from developing tissues of the chicken. J. Exp. Zool. 152: 75-89.

*Lush, I.E. and Cowey, C.B. (1968) : Biochem. J. 110, 33.

Lush, I.E., Cowey, C.B. and Knox, D. (1969) : The lactate dehydrogenase isozymes of twelve species of flatfish (Heterosomata). J. Exp. Zool 171: 105-118.

Lush, I.E. (1970): Lactate dehydrogenase isoenzymes and their genetic variation in coalfish (Gadus irrens) and cod (Gadus morhua). Comp. Biochem. Physiol. 32, 23-32.

Mann, K.G. and Vestling, C.S. (1970) : Isozymes of rat liver mitochondrial malate dehydrogenase: Evidence for the existence of non-identical subunits. Biochemistry 9: 3020.

Markert, C.L. (1962) : Isozymes in kidney development. In:Hereditary, developmental, and immunologic aspects of kidney disease. J. Metcoff, Ed. Northwestern University Press. Evanston III 1962). Proc 13th Ann. Conf. Kidney.

Markert, C.L. (1963) : Lactate dehydrogenase isozymes: dissociation and recombination of subunits. Science, N.Y. 140, 1329-1330.

Markert, C.L., Appella, E. (1963) : Immunochemical properties of lactate dehydrogenase isozymes. In: Antibodies to enzymes: a three component system. Ann. N.Y. Acad. Sci. 103, 915-929.

Markert, C.L. Faulhaber, I. (1965) : Lactate dehydrogenase patterns of fish. J. Exp. Zool. 159, 319-332.

Markert, C.L., Holmes, R.S. (1969): Lactate dehydrogenase isozymes of the flatfish, Pleuronectiformes: Kinetic molecular and immunochemical analysis. J. Exp. Zool. 171, 85-104.

Markert, C.L., Shaklee, J.B. and Whitt, G.S. (1975) : Evolution of a gene. Science (Wash., DC) 189: 102-114.

Markert, C.L. Ursprung, H. (1962) : The ontogeny of isozyme patterns of lactate dehydrogenase in the mouse. Dev. Biol 15, 363-381.

*Markert, C.L. and Ursprung, H. (1974) : Developmental Genetics. Prentice-Hall of India Private Ltd., New Delhi. p. 214.

Markert, C.L. and Whitt, G.S. (1968) : Molecular varieties of isozymes. Experientia 24, 977-991.

Massario, E.J. (1972) :Isozyme patterns of coregonine fishes: evidence for multiple cistrons for lactate and malate dehydrogenases and achromatic bands in the tissues of Prosobium cylindraceum (Pallas) and P. coulteri (Eigenmann a. Eigenmann). J. Exper. Zool. 179(2), 247-262.

Massaro, E.J. and Markert, C.L. (1968): Isozyme patterns of Salmonid fishes: Evidence for multiple cistrons for Lactate dehydrogenase polypeptides. J. Exp. Zool. 168: 223-238.

Maurer, H.R. (1971): Disc electrophoresis and related techniques of PAGE. Walter de Gruyter and Co. Berlin, 33rd Ed., 222.

McAndrew, B.J. and Majumdar, K.C. (1983) : Tilapia stock identification using electrophoretic markers. Aquaculture 3Ø, 249-261.

Menezes, M.R. (1975) : Electrophoretic studies on eye lens and serum proteins of Sardinella sardinella iougiceps Valenciennos. Mahasagar 8(3/4), 117-121

Merritt, R. (1972) : Geographic distribution and enzymatic properties of lactate dehydrogenase allozymes in the fathead minnow, Pimephales promelas. Am. Naturalis 1Ø6, 173-184.

Moon, T.W. (1975) : Temperature adaptation: isozymic function and the maintenance of heterogeneity. In Isozymes, Vol.2. Physiological function (Edited by Markert, C.L.), pg 2Ø7-22Ø. Academic Press, New York.

Morizot, D.C. (1983) : Tracing linkage groups from fishes to mammals. J. Hered. 74, 413-416.

Morizot, D.C. and Siciliano, M.J. (1983): Comparative gene mapping in fishes. In Isozymes: Current Topics in Biological and Medical Research. (Edited by Rattazzi, M.C., Scandalios, J.G., and Whitt, G.S.) 1Ø, 261-285. Alan R. Liss, New York.

Morrison, W.J. and Wright, J.E. (1966): Genetic analysis of three lactate dehydrogenase isozyme systems in trout : Evidence for linkage of genes coding subunits A and B. J. Exp. Zool. 163: 259-27Ø.

*Murphey, W.H., Barnaby, C., Lin, F.J. and Kaplan, N.O.(1967): J. Biol. Chem. 242, 1548.

Nagayama, F., Ohshima, H. and Takeuchi, T. (1975): Studies on the enzyme system of carbohydrate metabolism in fish-II. Purification of glucose-6-phosphate dehydrogenase and glucose dehydrogenase. Bull. Jap. Soc. Scient. Fish. 41, 1Ø69-1Ø74.

Nance, W.E., Claflin, A. and Smithies, O. (1963): Lactic dehydrogenase: Genetic control in man. Science, 142: 1Ø75-1Ø77.

Numachi, K. (1970a): Lactate and malate dehydrogenase isozyme patterns in fish and marine mammals. Bull. Jap. Soc. Sci. Fish. 36 (10). 1067-1075.

Numachi, K. (1970b): Polymorphism of malate dehydrogenase and genetic structure of juvenile population in saury Colabis saira. Bull. Jap. Soc. Sci. Fish. 36(12):1235-1241.

Numachi, K. (1972):Genetic control and subunit compositions of lactate dehydrogenase in Pseudorasbora parva. Japan J. Genet. 47, 193-201.

Nuttal, G.H.F. (1904): 'Blood immunity and blood relationship'. Cambridge University Press, London.

Odense, P.H., Allen, T.M. (1971): A biochemical comparison of some Atlantic herring populations. Rapp. Proc.-Verb. Reun. 161, 26.

Odense, P.H., Allen, T.M. and Leung, T.C. (1966): Multiple forms of lactate dehydrogenase and aspartate amino-transferase in herring (Clupea harengus L). Canad. J. Biochem., 44, 1319-1324.

Odense, J.P., Graymore, C.N. and Kissun, R.D. (1969): Multiplc forms of lactate dehydrogenase in the cod Gadus morhua L. Biochem. Genet. 3: 317-334.

Odense, P.H. and Leung, T.C. (1975): Isoelectric focusing on Polyacrylamide gel and starch gel electrophoresis of some gadiform fish lactate dehydrogenase isozymes. In Isozymes-III. Developmental Biology (Edited by Markert, C.L.) p. 485-501. Academic Press, New York.

Ohno, S. (1970): Evolution by Gene Duplication. Springer-Verlag, New York.

Ohno, S., Payne, H.W., Morrison, M., and Beutler, E.(1966): Hexose-6-phosphate dehydrogenase found in human liver. Science, 153: 1015-1016.

Ohno, S., Wolf, U., and Atkin, N.B. (1968): Evolution from fish to mammals by gene duplication. **Hereditas, 59**: 169-187.

Padhi, B.K. and Khuda-Bukhsh, A.R. (1989): Lactate dehydrogenase (LDH) isozyme patterns in four species of Mugil (Mugiligae: Pisces). Perspectives in Cytology and Genetics (eds. G.K. Manna and U. Sinha) 6: 125-129.

Pan, Y-C.E., Huang, S. Marciniszyn, J.P., Lee C-Y., and Li S-L.S. (1980): The preliminary amino acid sequence of mouse testicular lactate dehydrogenase. Hoppe-Seyler's Z. Physiol. Chem. 361: 795-799.

Pan, Y-C.E, Sharief, F.S. Okabe, M., Huang, S. and Li, S-L.S.(1983): Amino acid sequence studies on lactate dehydrogenase C4 isozymes from mouse and rat testes J. Biol. Chem, 258: 7005-7016.

Pesce, A., Fondy, T.P., Stolzenback, F., Castillo, F. and Kaplan, N.O. (1967): The comparative enzymology of lactic dehydrogenase-III. Properties of the H_4 and M_4 enzymes from a number of vertebrates. J. Biol. Chem. 242, 2151-2167.

Philipp, D.P., Childers, W.F., and Whitt, G.S. (1979): Evolution of patterns of differential gene expression: A comparison of the temporal and spatial patterns of isoenzyme locus expression in two closely related fish species (Northern largemouth bass, Micropterus salmoides salmoides and small mouth bass Micropterus delomeni) J. Expt. Zool., 210(3): 473-487.

Philipp, D.P., Childers, W.F. and Whitt, G.S. (1981): Management implications for different genetic stocks of largemouth bass (Micropterus salmoides) in the United States. Can. J. Fish. Aquat. Sci. 38, 1715-1723.

Philipp, D.P., Parker, H.R. and Whitt, G.S. (1983): Evolution of gene regulation: Isozymic analysis of patters of gene expression during hybrid fish development. In Isozymes: Current topics in Biological and Medical Research (Edited by Rattazzi, M.C., Scandalios, J.G. and Whitt G.S.) 1Ø, 193-237. Alan R. Liss, New York.

Pietruszko, R., Crawford, K. and Lester, D. (1973): Comparison of substrate specificity of alcohol dehydrogenase from human liver, horse liver and yeast towards saturated and Z-eomic alcohols and aldehydes. Arch. Biochem. Biophys., 159: 5Ø-56.

Pilgrim, L. (1977): "Protein Measurement", V. Georgia Research Reporter, 9,6.

Place, A.R. and Powers, D.A. (1978): Genetic bases for protein polymorphism in <u>Fundulus heteroclitus</u> (L.) I. Lactate dehydrogenase (LDH-B), malate dehydrogenase (MDH-R), glucosephosphate isomerase (GPI-B) and phosphoglucomutase (PGM-A). Biochem, Genet., 16(5/6): 577-591.

Place, A.R. and Powers, D.A. (1979): Genetic variation and relative catalytic efficiencies: Lactate dehydrogenase B allozymes of <u>Fundulus heteroclitus</u>. Proc. Natl. Acad. Sci. USA 76, 5, 2354-2358.

Plagemann, P.G.W., Gregory, K.F. and Wroblewski, F. (1961): Die elektrophoretisch trennbaren Lactat-dehydrogenasen des Sangetieres. III Einflub der Temperatur auf die Lactat-dehydrogenasen des Kaninchens. Biochem. 2. 334, 37-48.

*Plummer, D.T. and Wilkinson, J.H. (1963): Biochem. J. 87,423

Powell, J.R. (1975): Protein variation in natural populations of animals. 79-119 in Dobzhansky, T., Hecht, M.K. and Steere, W.C. (Eds.). Evolutionary Biology 8. Plenum, New York.

Powers, D.A. and Powers, D. (1975): Predicting gene frequencies in natural populations: a testable hypothesis. In: Isozymes. IV. Genetics and Evolution, 63-84. Ed. by C.L. Markert. New York: Academic Press.

Prager, E.M. and Wilson, A.C. (1971): The dependence of immunological cross-reactivity upon sequence resemblance among bird lysozymes. J. Biol. Chem. 246: 5978-5989.

Prakash, S. (1988): Genetic studies on Rana limnocharis Ph. D. Thesis, North-Eastern Hill University, India.

Raiha, N.C.R., Koshkinen, M. and Pikkarainen, P. (1967): Developmental changes in alcohol-dehydrogenase activity in rate and guinea-pig liver. Biochem. J. 103: 623-645.

Ramanujam, S.N. and Ratha, B.K. (1980): Glucose-6-phosphate Dehydrogenase (G6PDH) and lactate dehydrogenase (LDH) activities in two air-breathing and two gill-breathing species of fish - A comparative study. Proc. Indian Natn. Sci. Acad. B46, 2, 168-171.

Rehse, P.H. and Davidson, W.S. (1986): Evolutionary relationship of a fish C type lactate dehydrogenase to other vertebrate lactate dehydrogenase isozymes. Can. J. Fish. Aquat. Sci. 43(5): 1045-1051.

*Reisner, A.H., Nemes, P. and Bucholtz, C. (1975): Anal. Biochem., 64, 509.

Richmond, M.C. and Zimmerman, E.G. (1978): Effect of temperature on activity of allozymic forms of super -natant malate dehydrogenase in the red shiner, Notropis lutrensis. Comp. Biochem. Physiol. 61B, 415-419.

Rodriguez-Segade, S. and Freire, M. (1982): Partial purification and properties of three enzymatic forms of glucose-6-phosphate dehydrogenase from Mytilus edulis L. hepatopancreas. Comp. Biochem. Physiol. 72B, 117-121.

Rooney, C.H.J. and Ferguson, A. (1985): Lactate dehydrogenase isozymes and allozymes of the nine-spined stickleback Pungitius pungitius . (L) (Osteichthyes: Gasterosteidae). Comp. Biochem. Physiol. 81B, 3, 711-715.

Rosen, M., Fried, G. and Schaefer, C. (1968): A comparative study of the activities of alphaglycerophosphate dehydrogenase and lactic dehydrogenase in muscle of the mouse, rat, frog and American cockroach. Bio Science 18, 3Ø1-3Ø5.

Rossman, A.G., Liljas, A., Brandes, C.L. and Banaszak, L.J. (1975): Evolutionary and structural relationships among dehydrogenases. The Enzymes, Academic Press, 11: 61-1Ø2.

*Sachko, G.D. (1971): On the degree of polymorphism of the lactate dehydrogenase genes in fishes (Russ.) Nauchn. Soob. In-ta biol. morja. Vladyvostock 2, 19Ø-195.

*Scandalios, J.G (1967): Biochem. Genet. 1, 1-8.

*Schechter, A.N. and Epstein, C.J. (1968): Science 159, 997-999.

*Schmidt, J. (1919): J. Genetics 8, 147-153.

Scholl, A. (1973): Biochemical Evolution in the genus Xiphophorus (Poeciliidae, Teleostei). In "Genetics and Mutagenesis of fish" (Ed. J.H. Schnoder). 277-3ØØ.

Scholl, A. and Anders, (1973): Electrophoretic variation of enzyme proteins in Platyfish and Swordtails (Poeciliidae, Teleostei). Archiv. fur Genetik 46, 121-129.

Sedmak, J.J. and Grossberg, S.E. (1977): A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G25Ø. Anal. Biochem. 79: 544.

Sensabaugh, G.F. Jr., and Kaplan, N.O. (1972): A lactate dehydrogenase specific to the liver of gadoid fish. J. Biol Chem., 247: 585-593.

Shaklee, J.B., Champion, M. and Whitt, G.S. (1974): Developmental genetics of teleosts: a biochemical analysis of the lake chubsucker ontogeny. Devl. Biol. 38, 356-382.

Shaklee, J.B., Kepes, K.L. and Whitt, G.S. (1973): Specialised lactate dehydrogenase isozymes: the molecular and genetic basis for the unique eye and liver LDHs of teleost fishes. J. Exp. Zool. 185, 217-240.

Shaklee, J.B. and Whitt, G.S. (1977): Patterns of enzyme ontogeny in developing sunfish. Differentiation 9, 85-95.

Shaklee, J.B. and Whitt, G.S. (1981): Lactate dehydrogenase isozymes of Gadiform fishes: Divergent patterns of gene expression indicate a heterogenous taxon. Copeia 3, 563-578.

Shatton, J.B., Halver, J.E. and Weinhouse, S. (1971): Glucose (hexose-6-phosphate) dehydrogenase in liver of rain -bow trout. J. Biol. Chem. 246, 4878-4885.

Shaw, C.R. (1966): Glucose-6-phosphate dehydrogenase: homologous molecules in deer-mouse and man. Science 153, 1013-1015.

Shaw, C. and Barto, E. (1963): Genetic evidence for the subunit structure of lactate dehydrogenase isozymes. Proc. Natl. Acad. Sci. 50: 211-214.

Shaw, C.R. and Barto, E. (1965): Autosomally determined polymorphism of glucose-6-phosphate dehydrogenase in Peromyscus. Science, 148: 1099-1100.

Shaw, C.R. and Koen, A.L. (1968): Glucose-6-phosphate dehydrogenase and hexose-6-phosphate dehydrogenase of mammalian tissues. Ann. N.Y. Acad. Sci. 151, 149-156.

Siddique, N. (1977): Seasonal size and comparative study of plasma proteins of four air-breathing freshwater fishes. Proc. Ind. Acad. Sci. Sect. B 85(6), 384-390.

Siegel, L. and England, S. (1960): Malic dehydrogenase from acetone dried powders of whole beef heart muscle. Biochem. Biophys. Res. Commun. 3, 253-258.

Simonsen, V. and Christiansen, F.B. (1985): Genetics of Zoarces populations: XIV. variation of the lactate dehydrogenase isoenzymes. Hereditas 103(2): 177-185.

Skorkowski, E.F., Biegniewska, A., Aleksandrowicz, Z. and Swierczynski, J. (1980): Comparative studies on NADP-linked dehydrogenases in some tissues of fish and Crustaceans. Comp. Biochem. Physiol. 65B, 559-562.

*Slynko, V.I. (1971a) : Analysis of the malate dehydrogenase gene frequencies in the populations of Pacific salmon in the Sakhalin rivers (Russ.) Nauchn. Soob. In-ta biol morja. Vladyvostock 2, 212-214.

*Slynko, V.I. (1971b) : Polymorphism of the malate dehydrogenase isozymes in Pacific salmons (Russ.) Nauchn. soob. In-ta biol. morja, Vladyvostock 2, 207-211.

Smithies, O. (1955): Zone electrophoresis in starch gels: Group variation in the serum proteins of normal human adults. Biochem. J. 61: 629.

Somero, G.N. (1973): Thermal modulation of pyruvate metabolism in the fish Gillichthys mirabilis: The role of lactate dehydrogenases. Comp. Biochem. Physiol 44B, 205-209.

*St. Groth, F.D.S. et al. (1963):Biochim. Biophys. Acta, 71, 377.

Stegeman, J.J. and Goldberg, E. (1971): Distribution and characterisation of hexose-6-phosphate dehydrogenase in trout. Biochem. Genet. 5, 579-589.

Stegeman, J.J. and Goldberg, E. (1972): Properties of hepatic hexose-6-phosphate purified from brook trout and lake trout. Comp. Biochem. Physiol. 43B, 241-256.

Taggart, J., Ferguson, A. and Mason, F.M. (1981): Genetic variation in Irish population of brown trout (Salmo trutta L.): electrophoretic analysis of allozymes. Comp. Biochem. Physiol. 69B, 393-412.

Taniguchi, N. and Okada, Y (1980): Genetic study on the biochemical polymorphism in Red Sea Bream. Bulletin of the Japanese Society of Scientific fisheries.

Thatcher, D.R. (1980): The complete amino acid sequence of three alcohol dehydrogenase alleloenzymes (ADH N-11, ADH s and ADH uf) from the fruitfly Drosophila melanogaster. Biochem. J. 187: 875.

Thompson, H.C. Jr, Contin, R.F. (1980): Electrophoretic study of Atlantic bluefin tuna (Thunnus thynnus) from the eastern and western North Atlantic Ocean. Bull. Mar. Sci., 30(3), 727-731.

Thorne, C.J.R. and Cooper, P.M. (1964): Preparation of pig heart supernatant malate dehydrogenase. Biochim. Biophys. Acta 81, 397-399.

Thorne, C.J.R., Grossman L.I. and Kaplan N.O. (1963): Starch-gel electrophoresis of malate dehydrogenase. Biochim. Biophys. Acta 73, 193-203.

Tripathi, G. and Shukla, S.P. (1988): Comparative studies on skeletal muscle and liver lactate dehydrogenase of the freshwater catfish, Clarias batrachus. Biochemical Archives 4(1); 25-34.

Triveni, A. and P.R. Rao (1986): Tissue distribution and characterisation of lactate dehydrogenase isozymes in two fishes of the order Cypriniformes. Proc. Indian Acad. Sci. Anim. Sci. 95(2) 255-262.

Tsukuda, H. (1975): Temperature dependency of the relative activities of liver lactate dehydrogenase isozymes in goldfish acclimated to different temperatures. Comp. Biochem. Physiol. 52B, 343-345.

Utter, F.M. (1969): Lactate dehydrogenase isozymes of Pacific hake (Merluccius productus.) J. Exper. Zool. 172, 59-68.

Utter, F.M. and Hodgins, H.O. (1969): Lactate dehydrogenase isozymes of Pacific hake (Merluccius productus). J. Exp. Zool. 172, 59-67.

Utter, F.M. and Hodgins, H.O. (1971): Biochemical polymorphisms in the Pacific hake (Merluccius productus). Rapp. Proc. -Verb. Reun. 161, 87-89.

Utter, F.M. and Hodgins, H.O. (1972): Biochemical genetic variation at six loci in four stocks of rainbow trout. Trans. Am. Fish. Soc. 101: 494-502.

Utter, F.M., Hodgins, H.O. and Allendorf, F.W. (1974): Biochemical genetic studies of fishes: potentialities and limitations. In Biochemical and Biophysical Perspectives in Marine Biology (D. Malins, ed.), 1, 213-237 San Francisco: Academic Press.

Vallee, B.L. (1985): A novel approach to human ethanol metabolism: isozymes of alcohol dehydrogenase. Proc. Congr. Eur. Brew. Conv. 20, 65-90.

Vesell, E.S., and Bearn, A.G. (1962): Variations in the lactic dehydrogenase of vertebrate erythrocytes. J. Gen. Physiol., 45: 553-565.

Vrijenhoek, R.C. (1972): Genetic relationships of unisexual-hybrid fishes to their progenitors using lactate dehydrogenase isozymes as gene markers (Poeciliopsis, Poecilidae). The Americal Naturalist 106, 952: 754-765.

Wesolowski, M.H. and Lyerla, T.A. (1983): Alcohol dehydrogenase isozymes in clawed frog Xenopus laevis. Biochem. Genet. 21: 1003-1017.

Wheat, T.E. and Whitt, G.S. (1971): In vivo and in vitro molecular hybridisation of malate dehydrogenase isozymes. Experientia 27: 647-648.

Wheat, T.E., Childers, W.F., Miller, E.T. and Whitt, G.S. (1971): Genetic and *in vitro* molecular hybridisation of malate dehydrogenase isozymes in interspecific bass (*Micropterus*) hybrids. Anim. Blood. Grps. Biochem. Genet. 2, 3-14.

Wheat, T.E., Whitt, G.S. and Childers, W.F. (1972): Linkage relationships between the homologous malate dehydr--ogenase loci in teleosts. Genetics 70: 337-340.

Whitt, G.S. (1968): Developmental genetics of lactate dehydr--ogenase isozymes unique to the eye and brain of teleosts. Genetics 60, 237.

Whitt, G.S. (1969): Homology of lactate dehydrogenase Genes : E gene function in the Teleost nervous system. Science 166, 1156-1158.

Whitt, G.S. (1970a): Genetic variation of supernatant and Mitochondrial malate dehydrogenase isozymes in the Teleost *Fundulus heteroclitus*. Experientia 26/7, 4-736.

Whitt, G.S. (1970b): Directed assembly of polypeptides of the isozymes of lactate dehydrogenase. Arch. Biochem. Biophys., 138: 352-354.

Whitt, G.S. (1975): A unique lactate dehydrogenase isozyme in the teleost. In New Approaches to the Study of Vision in Fishes (Edited by Ali M.A.), 459-470. Plenum Press, New York.

Whitt, G.S. (1983): Isozymes as probes and participants in developmental and evolutionary genetics. In Isozymes: Current Topics in Biological and Medical Research (ed. by Rattazzi, M.C., Scandalios, J.G. and Whitt, G.S.) 10, 1-40 Alan R. Liss, New York.

Whitt, G.S. (1987): Species differences in isozyme tissue patterns: their utility for systematic and evolutionary analyses. In "Isozymes: Current Topics in Biological and Medical Research (Edited by Katazzi and Whitt.) 15, 1-26.

Whitt, G.S., Childers, W.F. and Cho, P.L. (1973): Allelic expression at enzyme loci in an interbridal hybrid sunfish. **J. Hered. 64, 55-61.**

Whitt, G.S., Childers, W.F., Wheat, T.E. (1971): The inheritance of tissue-specific LDH isozymes in interspecific bass (Micropterus) hybrids. **Biochem. Genet. 5(3), 257-273.**

Whitt, G.S., Miller, E.T. and Shaklee, J.B. (1973b): Developmental and biochemical genetics of lactate dehydrogenase isozymes in fishes. **In: Genetics and Mutagenesis of fish (Edited by Schroeder, J.H.), 243-276. Springer-Verlag, Berlin.**

Whitt, G.S. Philipp, D.P. and Childers, W.F. (1977): Aberrant gene expression during the development of hybrid sunfish (Perciformes Teleostei). **Differentiation, 9: 97-109.**

Whitt, G.S., Shaklee, J.B. and Markert, C.L. (1975): Evolution of the lactate dehydrogenase isozymes of fishes. **Isozymes: Genetic and Evol., Acad. Press 4: 381-400.**

Wilkins, N.P. (1971): Biochemical and serological studies on Atlantic salmon. (Salmo salar L.) **Rapp. Proc.-Verb. Reun. 161, 91-95.**

Wilson, A.C. (1973): Abnormal combination of molecular systems. **Mol. Biol. 80: 217-228.**

Wilson, A.C., Cahn, R.D. and Kaplan, N.O. (1963): Functions of the two forms of LDH in the breast muscle of birds. **Nature, Lond. 197, 331-334.**

Wilson, F.R., Champion, M.J., Whitt, G.S. and Prosser, C.L. (1975): Isozyme patterns in tissues of temperature acclimated fish. **In: Isozymes II. Physiological Function (edited by Markert, C.L.) 193-206. Acad. Press, New York.**

Wilson, A.C., Kaplan, N.O., Levine, L., Pesce, A., Reichlin, W. and Allison, W.S. (1964): Differential LDH isozyme expression amongst amphibian species. Fedn. Proc. Fedn. Am. Soc. Exp. Biol. 23: 1258-1266.

Wilson, A.C., Sarich, V.M. and Maxson, L.R. (1974): The importance of gene arrangements in evolution: evidence from studies on rates of chromosomal protein and anatomical evolution. Proc. Nat. Acad. Sci. USA, 71: 3028-3030.

Wilson, F.R., Whitt, G.S. and Prosser, C.L. (1973): Lactate dehydrogenase and malate dehydrogenase isozyme patterns in tissues of temperature-acclimated goldfish (Carassius auratus L.) Comp. Biochem. Physiol. 46B, 105-116.)

*Winge, O. (1930): J. Genetics. 23, 69-76.

Wright, J.E. and Atherton, L.M. (1970): Polymorphism for LDH and transferrin loci in brook trout populations. Trans. Amer. Fish. Soc. 99(1), 179-192.

Wright, J.E., Heckman, R. and Atherton, L.M. (1975): Genetic and developmental analysis of LDH isozymes in trout In: Isozymes-III. Developmental Biology (Edited by Markert, C.L.), 375-401. Acad. Press, New York.

Wright, J.E. Johnson, K., Hollister, A., and May, B. (1983): Meiotic models to explain classical linkage, pseudo -linkage and chromosome pairing in tetraploid derivative salmonid genomes. In Isozymes: Current Topics in Biological and Medical Research (Edited by Rattazzi, M.C., Scandalios, J.G. and Whitt, G.S.), 10, 239-260. Alan R. Liss, New York.

Wuntch, T. and Goldberg, E., (1970): A comparative physicochemical characterisation of lactate dehydrogenase isozymes in brook trout, lake trout and their hybrid splake trout. J. Exp. Zool. 174(3), 233-252.

Yamauchi, T. (1975): Glucose-6-phosphate dehydrogenase in brook, lake and splake trout: an isozymic and developmental study. **In:Isozymes III: Developmental Biology** Edited by Markert, C.L, 403415. Acad. Press, New York.

Yamauchi, T. and Goldberg, E. (1973): Glucose-6-phosphate dehydrogenase from brook, lake and splake trout: an isozymic and immunological study. **Biochem. Genet. 10, 121-134.**

Yamauchi, T. and Goldberg, E. (1974): Asynchronous expression of Glucose-6-phosphate dehydrogenase in splake trout embryos. **Developmental Biology 39, 63-68.**

Yamauchi, T., Stegeman, J.J. and Goldberg, E.(1975): The effects of starvation and temperature acclimation on pentose phosphate pathway dehydrogenase in brook trout liver. **Archs. Biochem. Biophys. 167, 13-20.**

Yamawaki, H. and Tsukuda, H. (1978): Significance of the variation in isozymes of liver lactate dehydrogenase with thermal acclimation in goldfish-I. Thermostability and temperature dependency. **Comp. Biochem. Physiol. 62B, 89-93.**

Yin, S.J., Bosron, W.F., Li, T-K., Ohnishi, K., Okuda, K., Ishii, H. and Tsuchiya, M. (1984): Polymorphism of human liver alcohol dehydrogenase: Identification of ADH_2 2-1 and ADH_2 2-2 phenotypes in the Japanese by isoelectric focussing. **Biochemical Genetics 22,** 1/2, 169-180.

Yoshida, A. (1965): Purification and chemical characterisation of malate dehydrogenase of Bacillus subtilis. **J. Biol. Chem. 240, 1113-1117.**

Young, J.W., Shrago, E. and Lardy, H.A. (1964): Metabolic control of enzyme involved in lipogenesis and gluconeogenesis. **Biochemistry** 3, **1687-1692.**

Zinkham, W.H., Blanco, A. and Kupchyk, L. (1963): Lactic dehydrogenase in Testis: dissociation and recombination of subunits. Science 142; 1303-1304.

Zinkham, W.H., Blanco, A. and Kupchyk, L. (1964): Lactate dehydrogenase in pigeon testes: genetic control by three loci. Science 144: 1353-1354.

Zinkham, W.H., Isensee, H. and Renwick, J.H. (1969): Linkage of lactate dehydrogenase B and C in pigeons. Science 164, 185-187.

* not seen in original.

NEHU LIBRARY
Acc. No 102758
Acc by Ajah
Date 22/6/95
Class by
Sub Heading by
Enter by
Inscribed

7. FIGURES

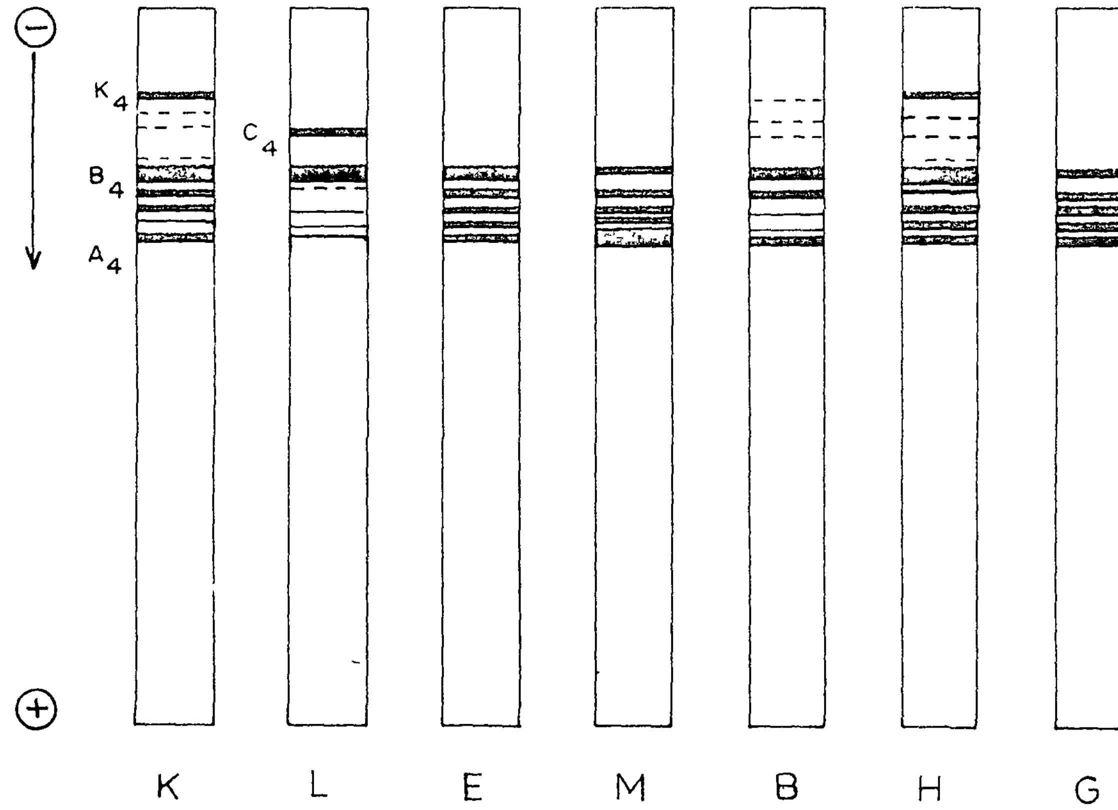


Fig. 1 : LDH isozymes in the tissues of *Clarias batrachus*.

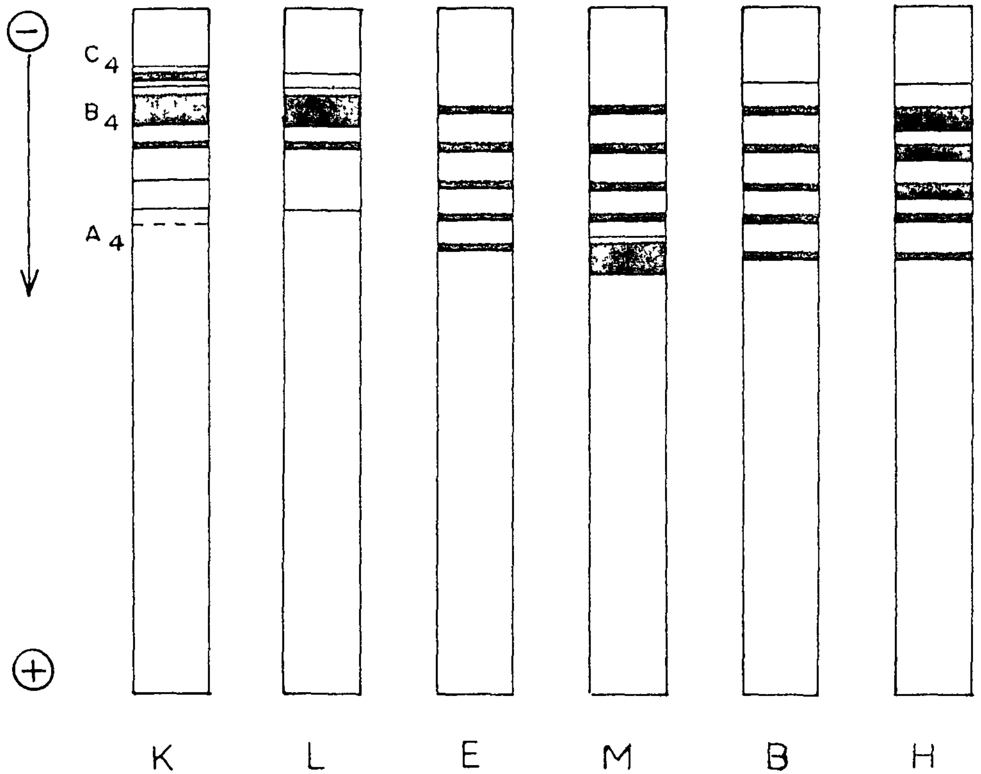


Fig. 2 : LDH isozymes in the tissues of *Heteropneustes fossilis*.

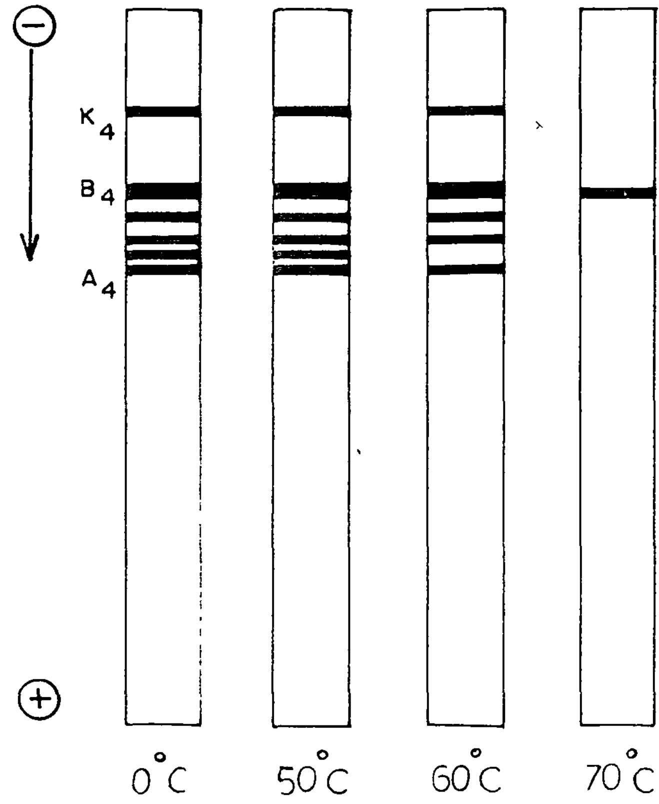


Fig. 3 : Heat inactivation of LDH isozymes in the kidney tissue of *Clarias batrachus*.

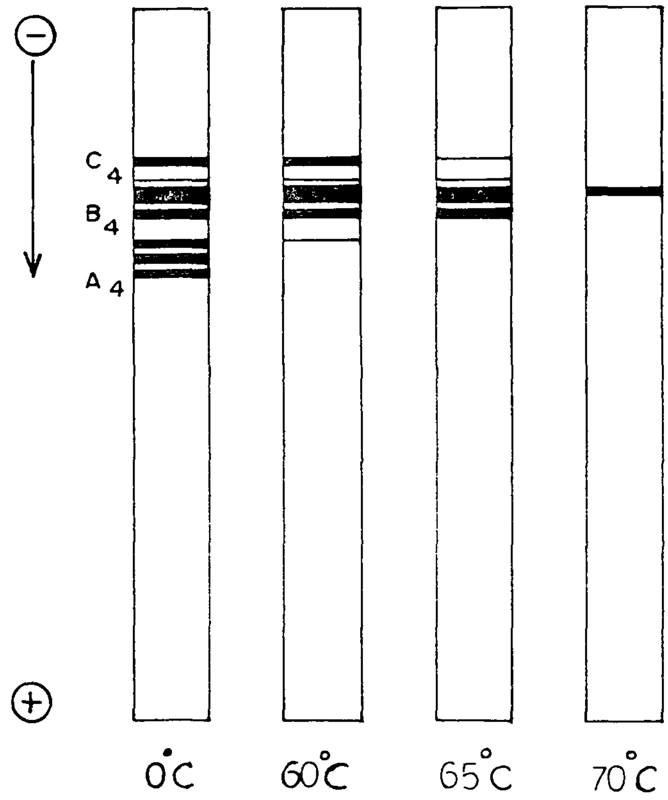


Fig. 4 : Heat inactivation of LDH isozymes in the liver tissue of *Clarias batrachus*.

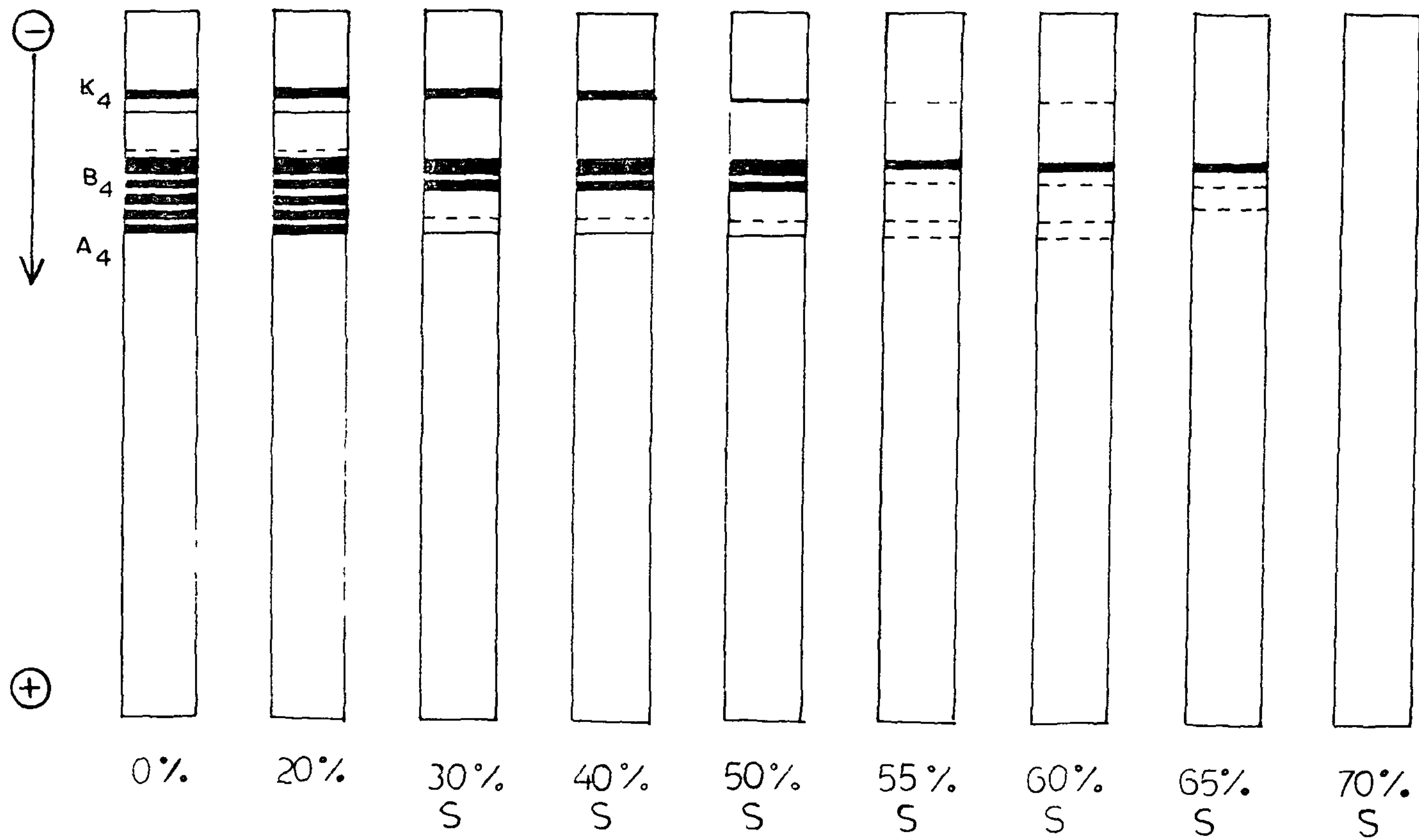


Fig. 5 : Isozymes observed in various fractions obtained by ammonium sulphate precipitation in the kidney tissue extract of *Clarias batrachus*.

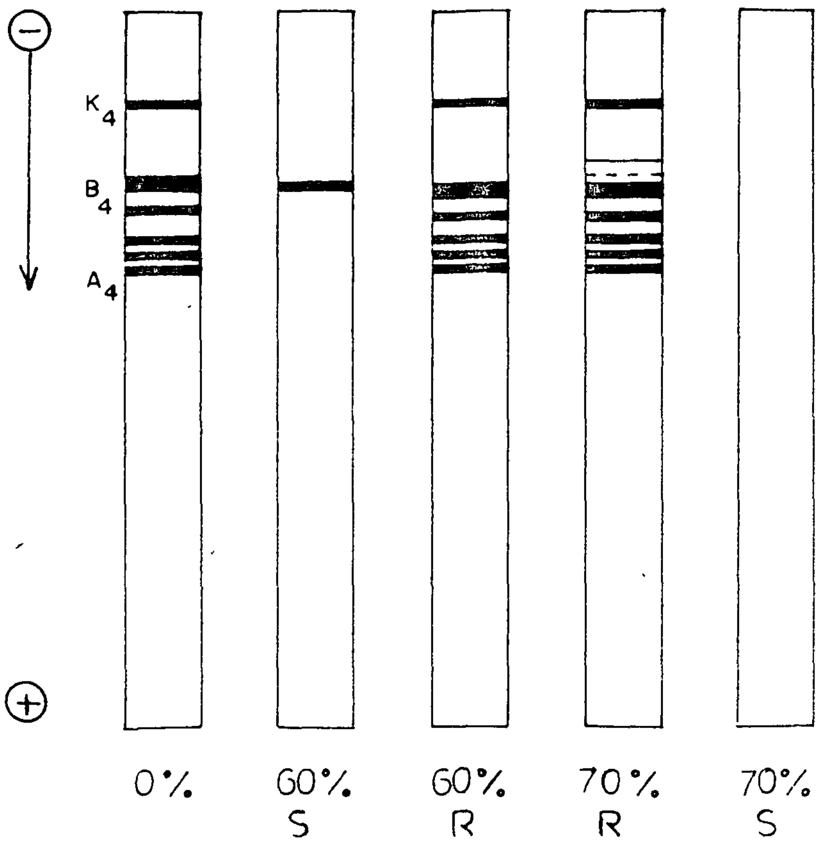


Fig. 6 : Isozymes observed in various fractions obtained by ammonium sulphate precipitation in the kidney tissue extract of *Clarias batrachus*.

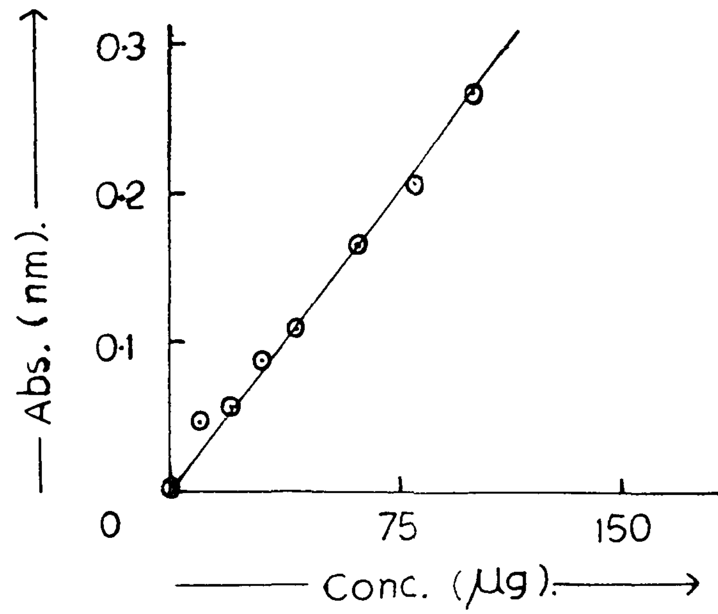


FIG. 7 : Standard graph for protein estimation.

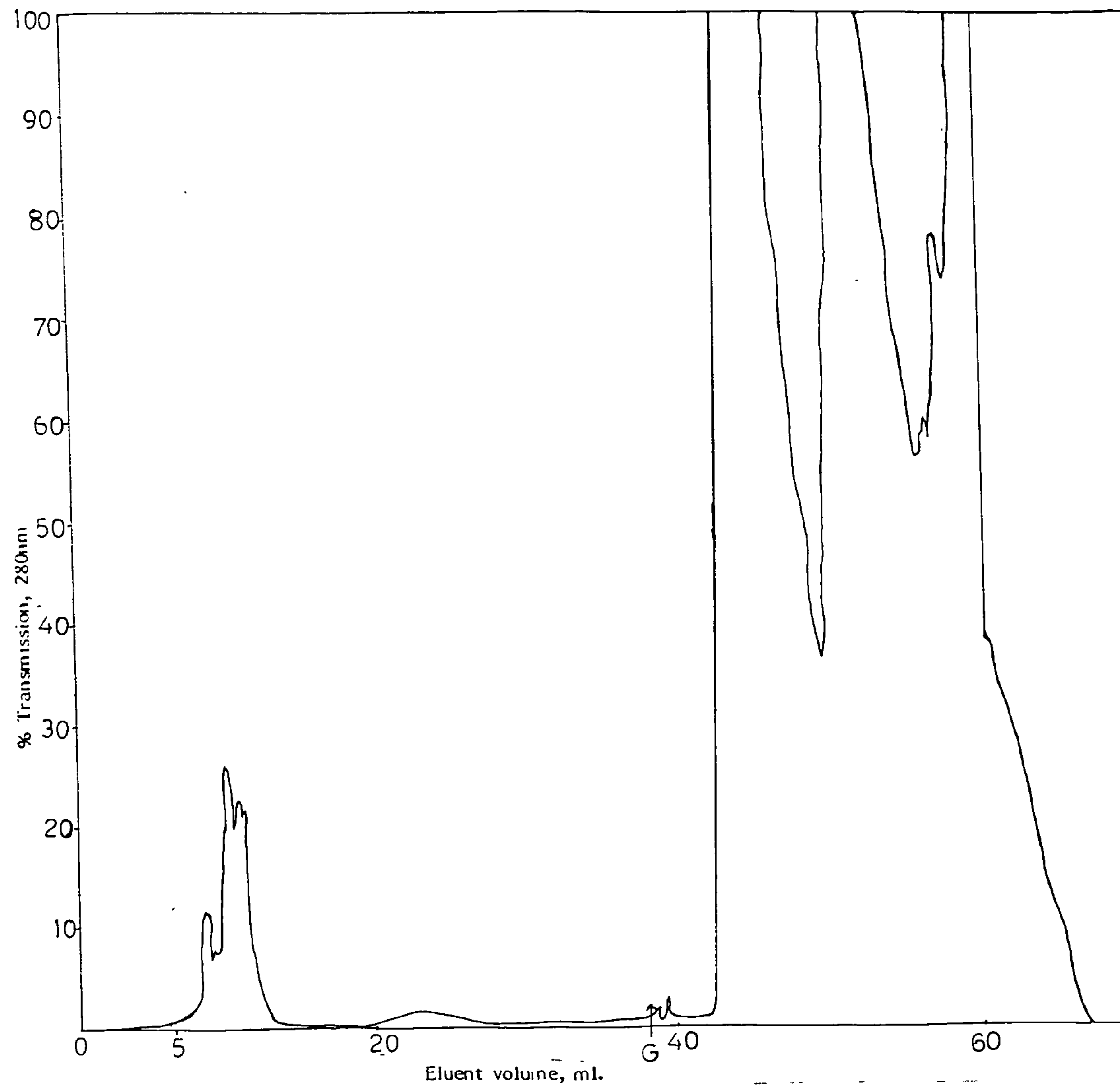


Fig. 8 : Eluent pattern of kidney tissue enzymes by DEAE cellulose chromatography.

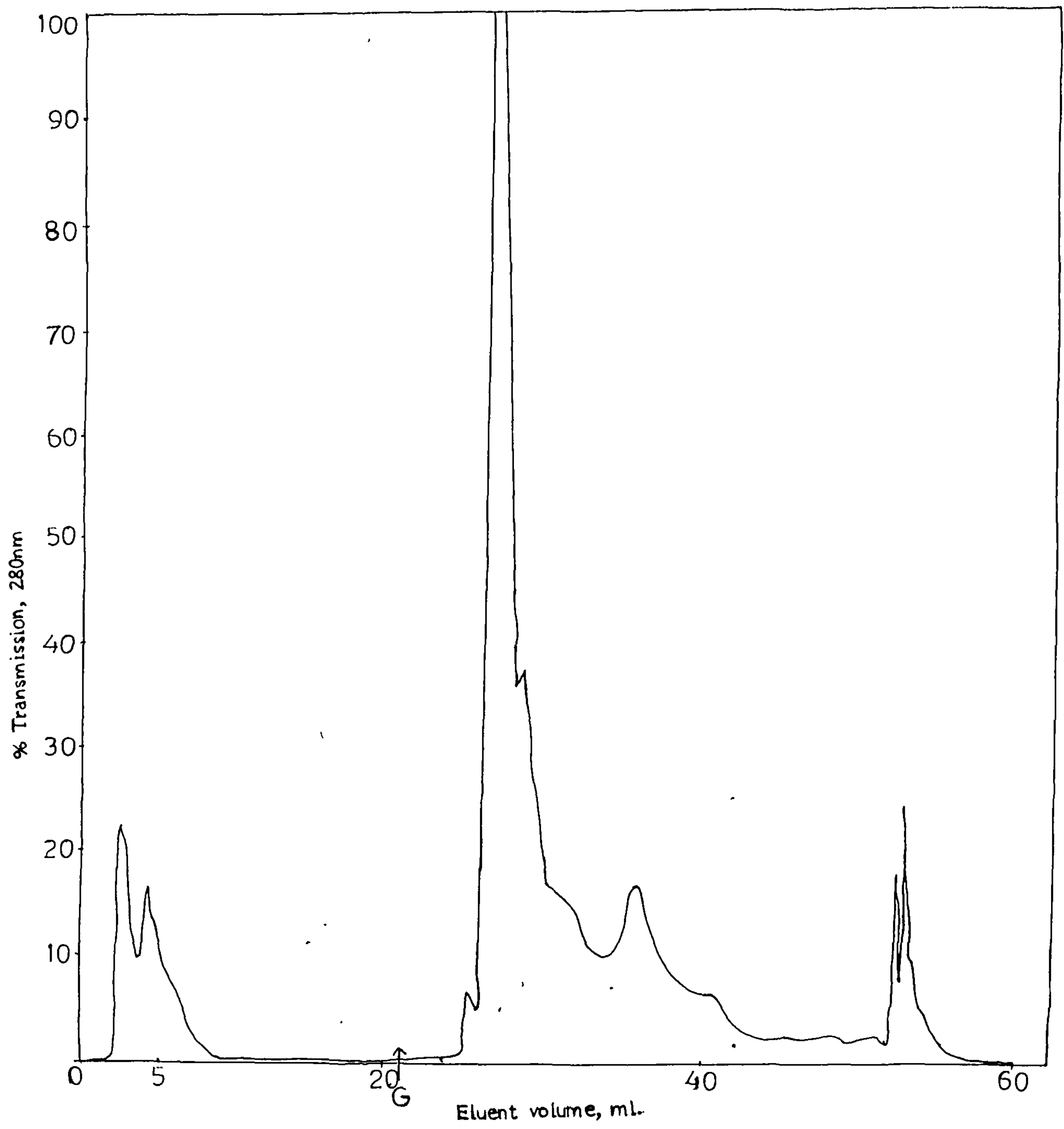


Fig. 9 : Eluent pattern of liver tissue enzymes by DEAE cellulose chromatography.

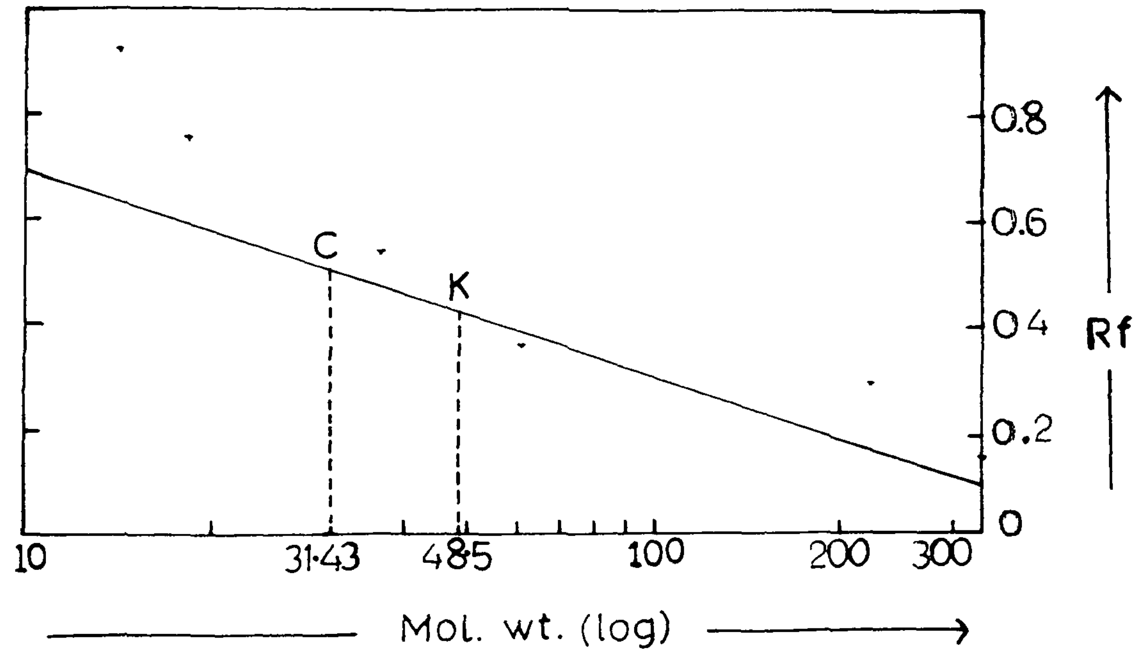


FIG. 10 : Approximate mol. wt. of the K and C subunits of LDH in *Clarias batrachus*.

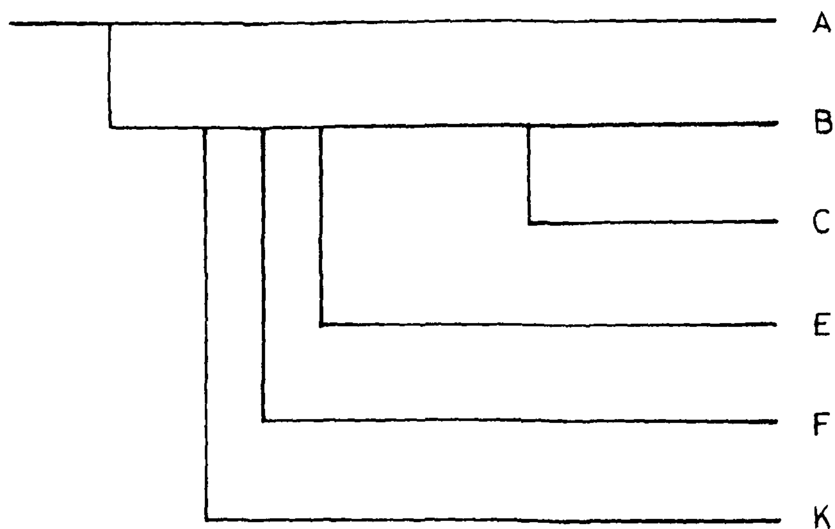


Fig. 11. Proposed model of LDH isozyme evolution (based on Holmes, 1972, view). The A type LDH is depicted as the original LDH. The B type was formed by a gene duplication of the A gene. The F (cod liver type), E (teleost eye type), C (mammalian and bird testes type) and K (the *Clarias batrachus* kidney type) genes are shown to have originated independently from the B type genes.

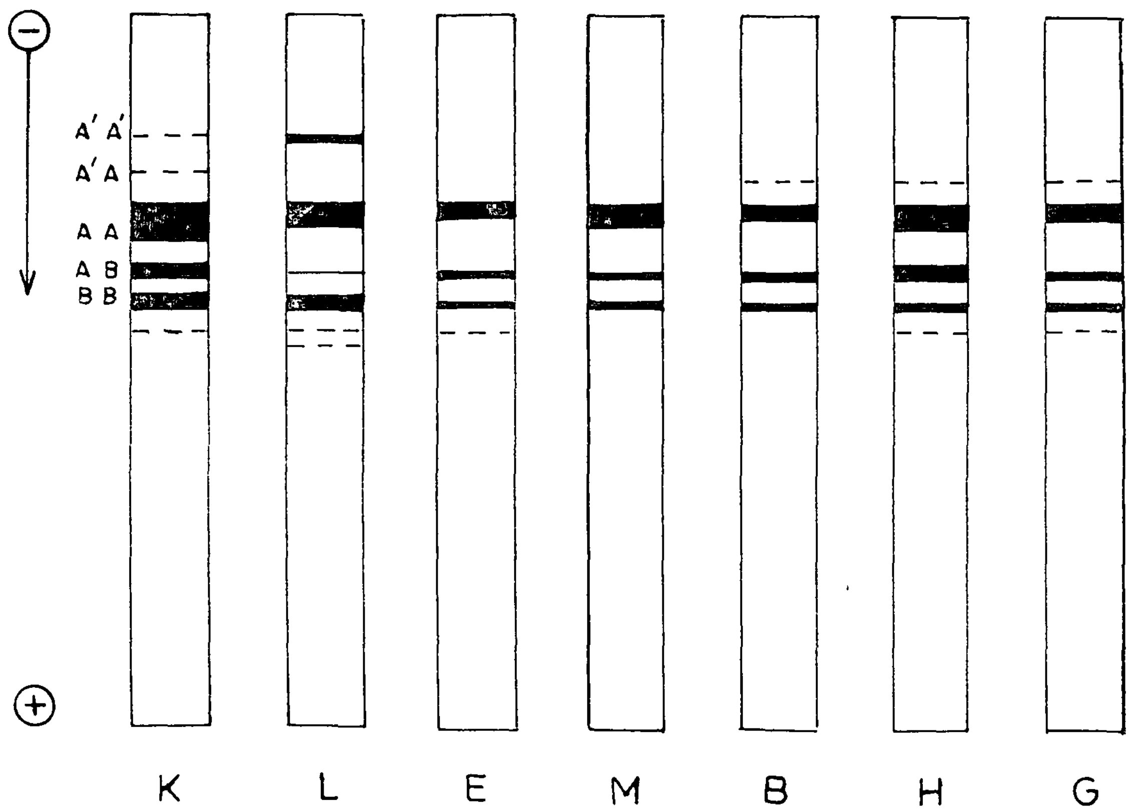


Fig. 12 : MDH isozymes in the tissues of *Clarias batrachus*.

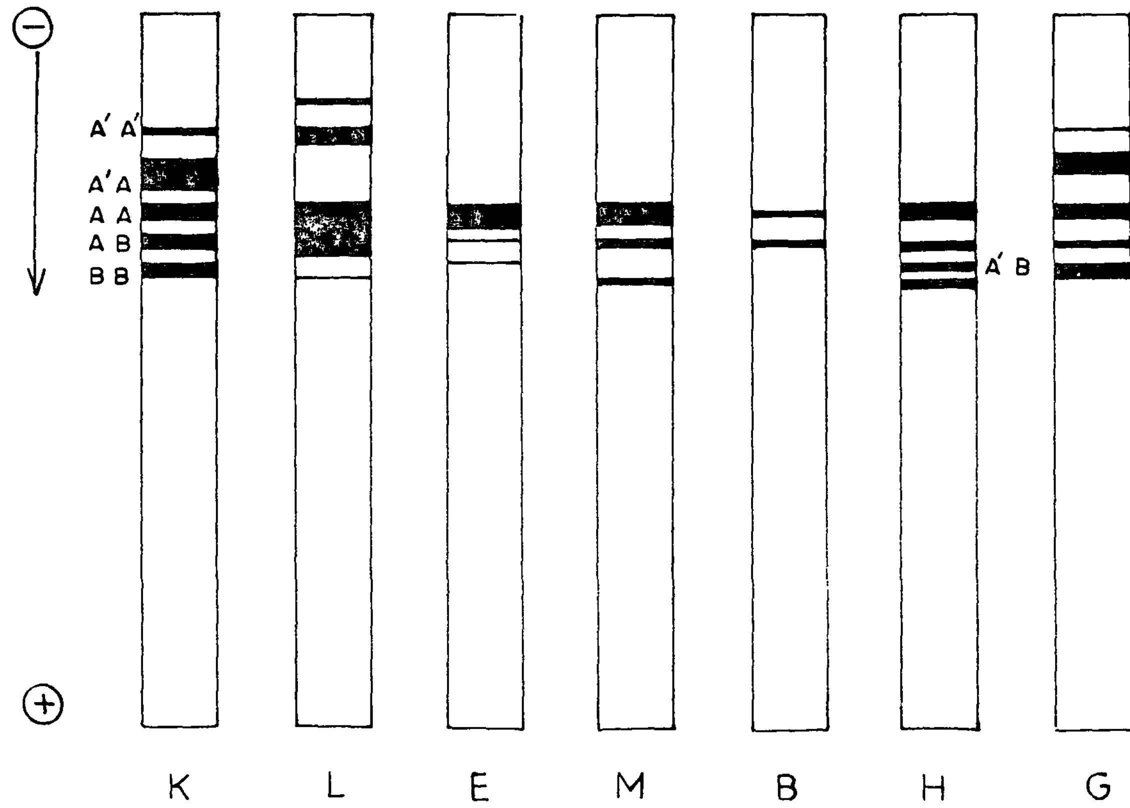


Fig. 13 : MDH isozymes in the tissues of *Heteropneustes fossilis*.

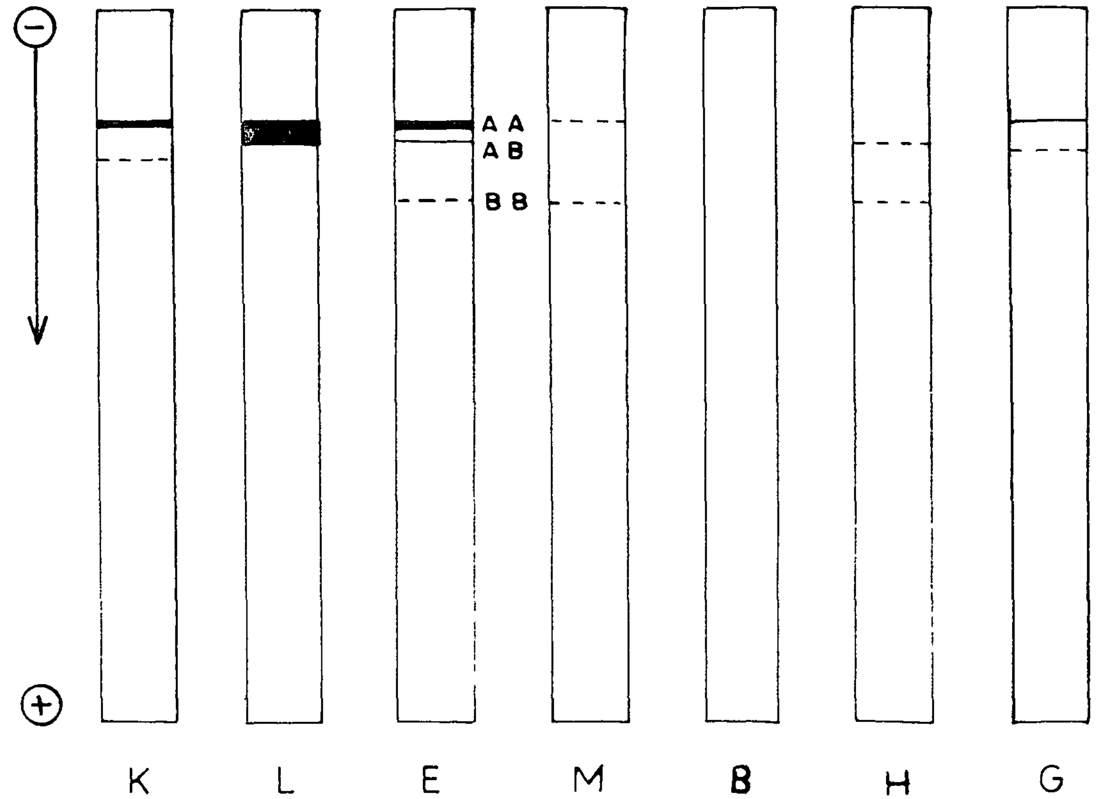


Fig 14 : ADH isozymes in the tissues of *Clarias batrachus*.

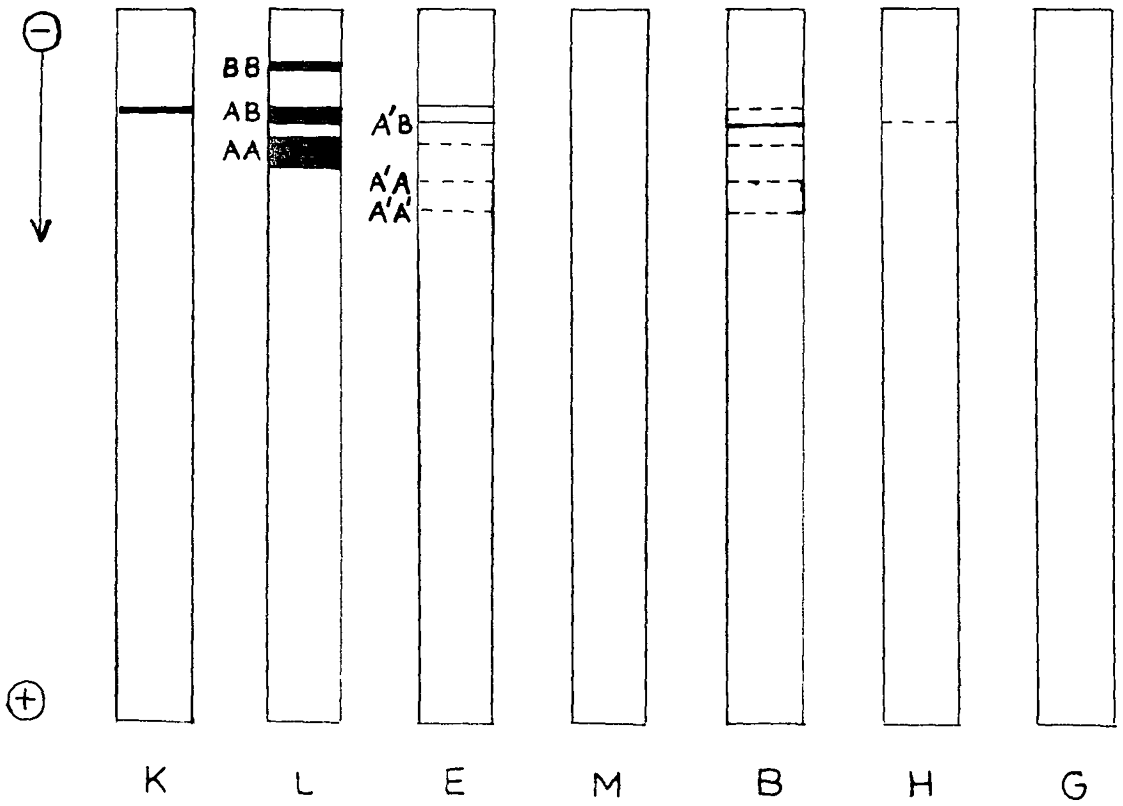


Fig. 15 : ADH isozymes in the tissues of *Heteropneustes fossilis*.

(a) Single gene models

AA

(1) With A gene

AA
A'A
A'A'

(2) With an allelic variant of A gene, (A and A')

(b) Two genes models

BB
AB
AA

(1) With A and B genes.

BB
AB
AA
A'B
A'A
A'A'

(2) With an allelic variant of A gene, and the B gene (A, A' and B)

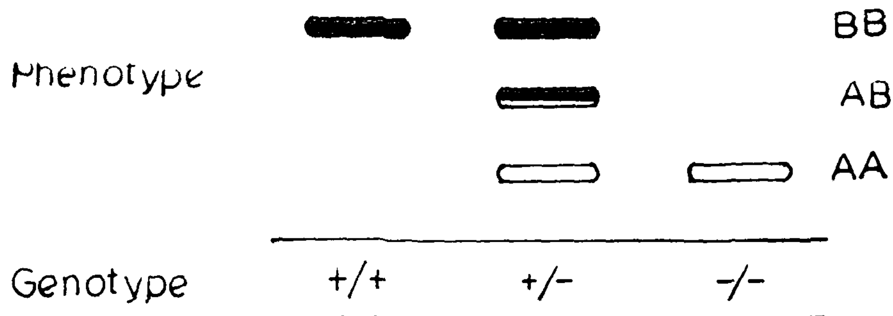
BB
AA
A'A
A'A'

(3) With allelic variant of A gene but with no heterodimer formation between A and B genes.

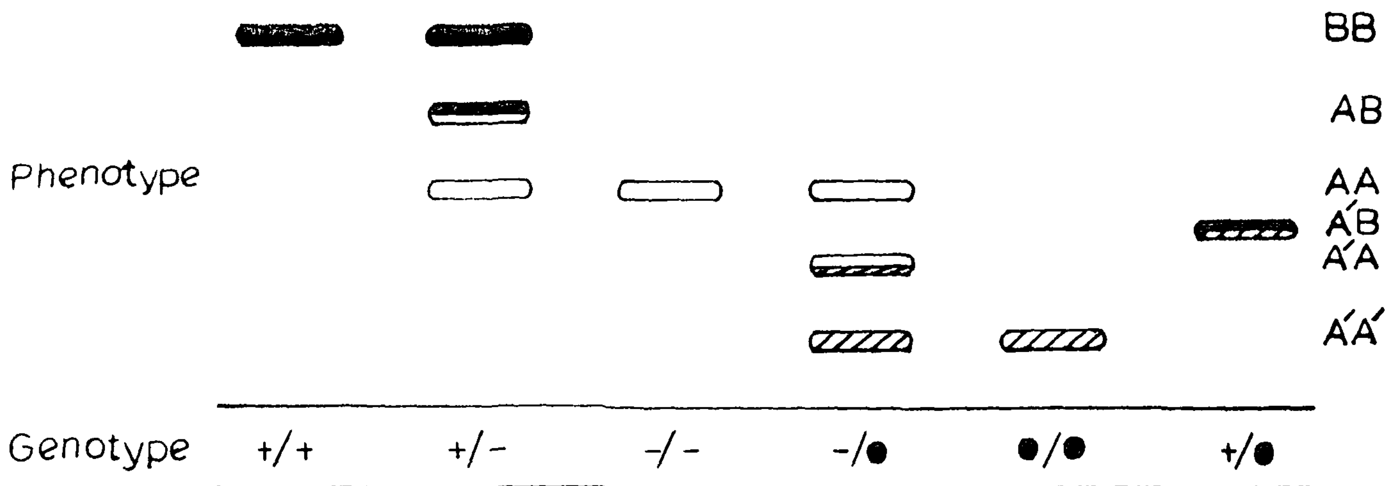
B'B'
BB'
AB'
BB
AB
AA
A'B
A'A
A'A'

(4) With allelic variants of A and B genes (A, A', B and B')

FIG. 16 : GENETIC MODELS FOR ADH.



(a) Clarias batrachus



(b) Heteropneustes fossilis

FIG. 17 : Genetic models for ADH

The gene + = B gene locus

- = A gene locus

● = A' gene (A locus)

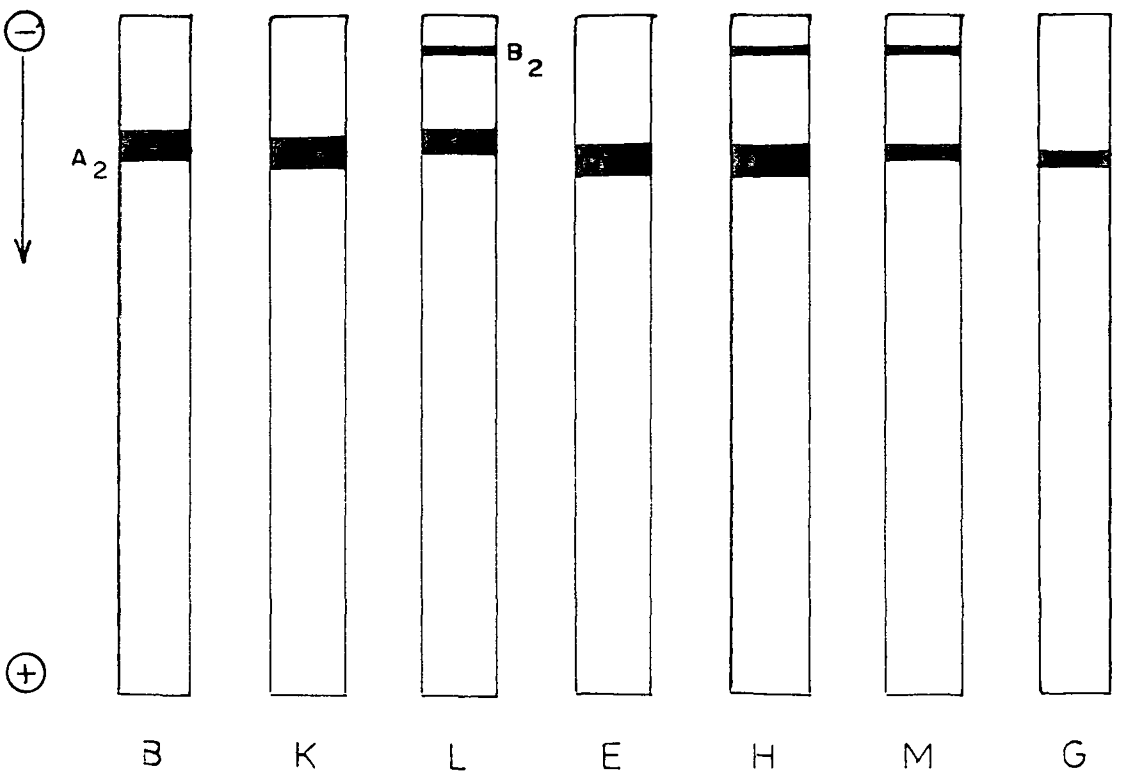


Fig. 18 : G6PD isozymes in the tissues of *Clarias batrachus*.

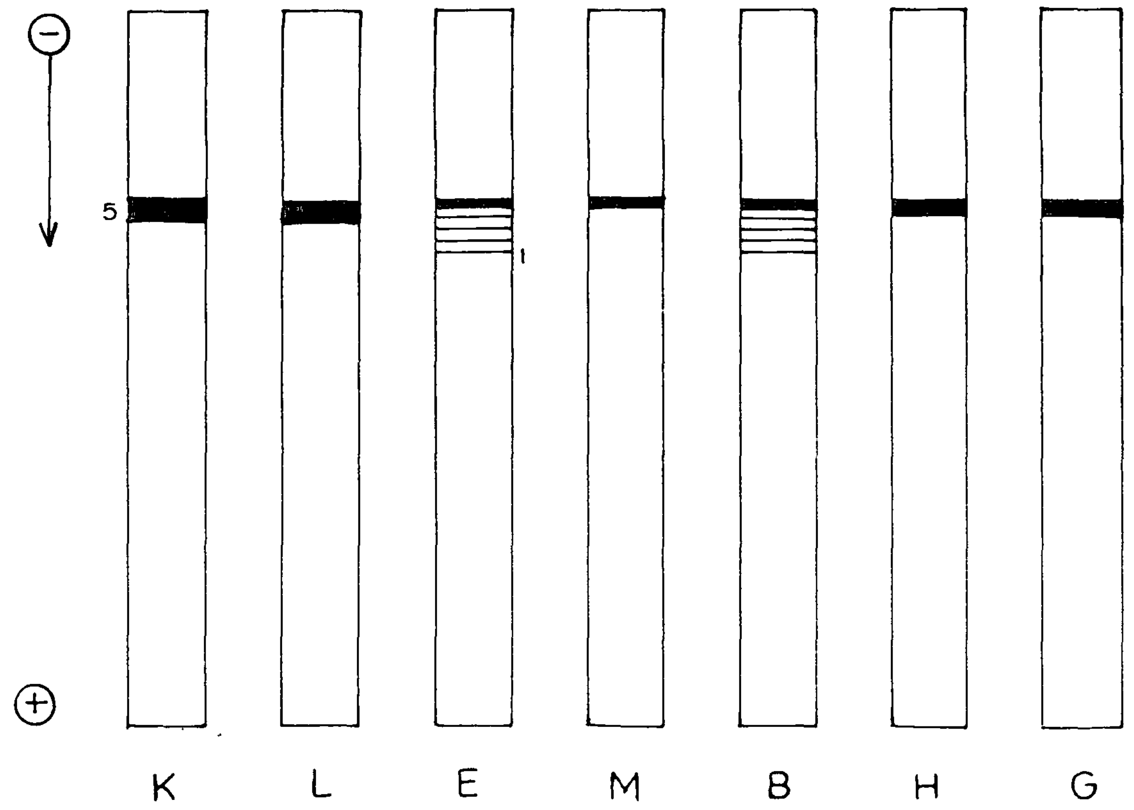


Fig. 19 : G6PD isozymes in the tissues of *Heteropneustes fossilis*.

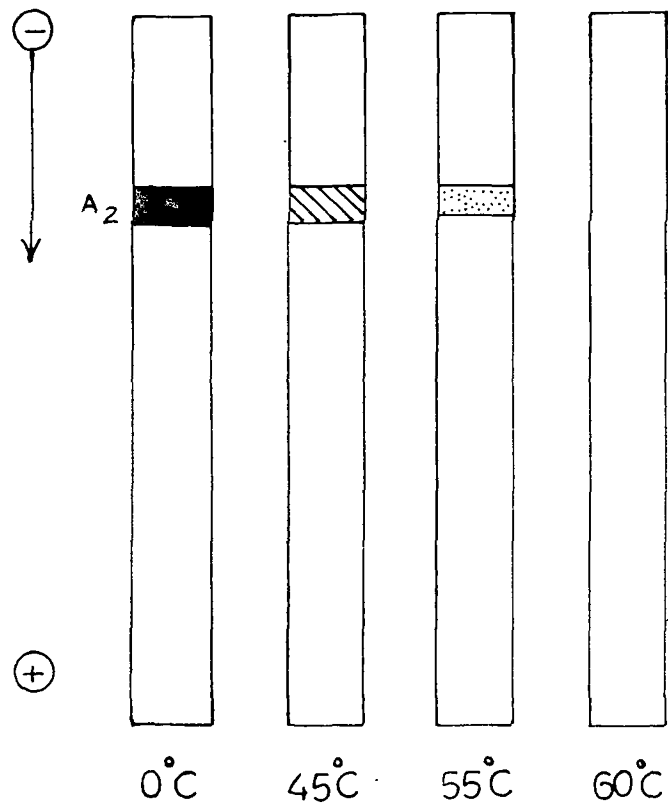


Fig. 20 : Heat inactivation of G6PD isozymes in the liver tissue of *Clarias batrachus*.

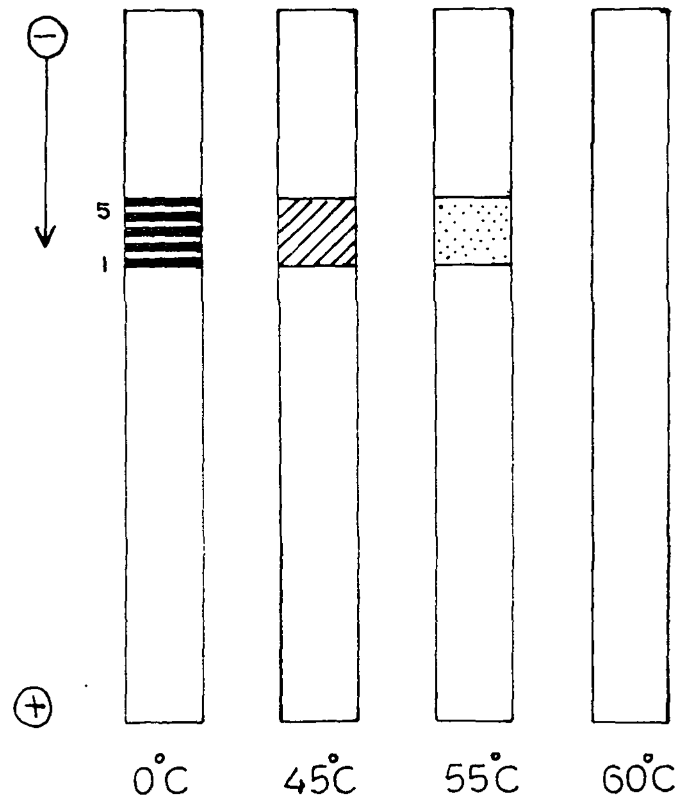


Fig. 21 : Heat inactivation of G6PD isozymes in the brain tissue of *Heteropneustes fossilis*.

8. PLATES

Plate 1 The fish, *Clarias batrachus*

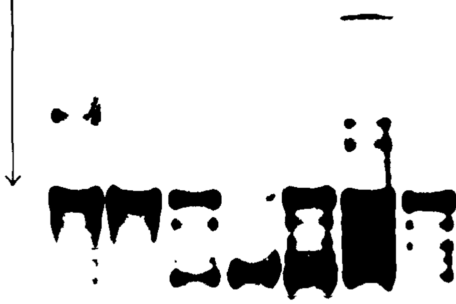
Plate 2 The fish, *Heteropneustes fossilis*

1

2

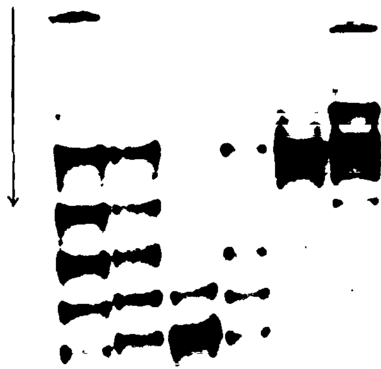
Plate 3 LDH isozyme pattern in the tissues of *Clarias batrachus*

Plate 4 LDH isozyme pattern in the tissues of *Heteropneustes fossilis*



K L E M B H G

3

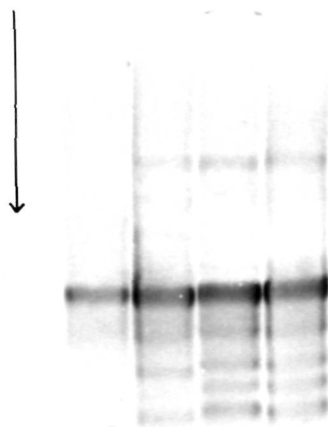


H B M E L K

4

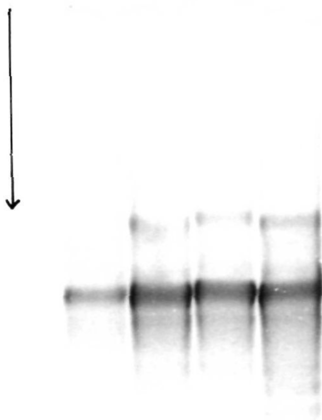
Plate 5 Heat inactivation of LDH isozymes in the kidney tissue of *Clarias batrachus*

Plate 6 Heat inactivation of LDH isozymes in the liver tissue of *Clarias batrachus*



70° 60° 50° 4°

5

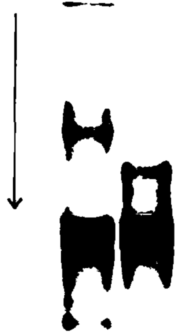


70° 65° 60° 4°

6

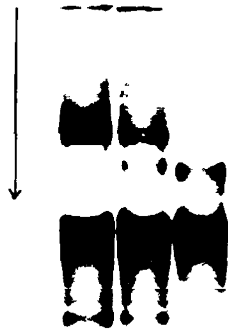
Plate 7 LDH isozyme pattern in the kidney and liver tissues of *Clarias batrachus*

Plate 8 Comparative LDH isozyme pattern in the kidney tissue extract, a mixture of kidney and liver enzyme extract and the liver tissue extract of *Clarias batrachus*



K L

7



K K+L L

8

Plate 9 SDS-PAGE of the kidney and liver enzyme fractions

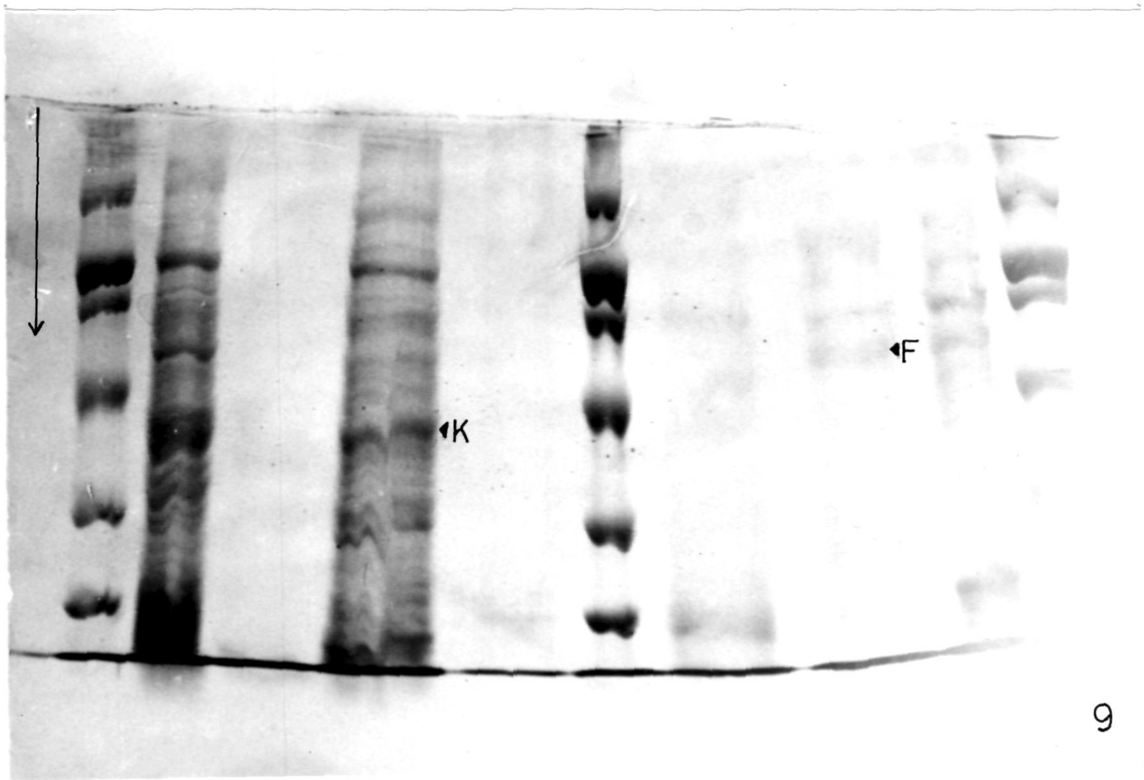
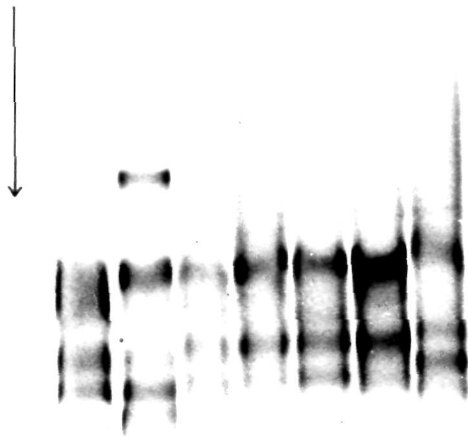

K
F
9

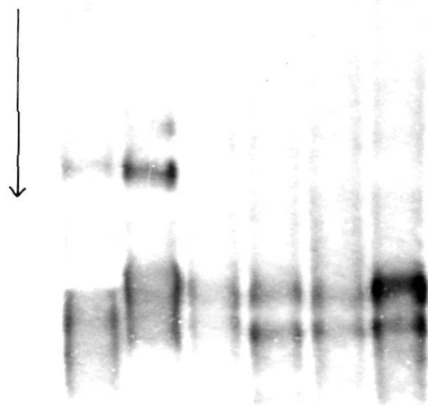
Plate 10 MDH isozyme pattern in the tissues of <u>Clarias batrachus</u>

Plate 11 MDH isozyme pattern in the tissues of <u>Heteropneustes fossilis</u>



K L E M B H G

10

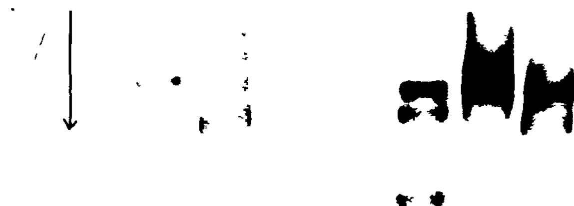


K L E M B H

11

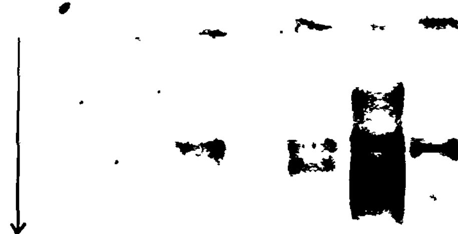
Plate 12 ADH isozyme pattern in the tissues of Clarias batrachus

Plate 13 ADH isozyme pattern in the tissues of Heteropneustes fossilis



G H B M E L K

12

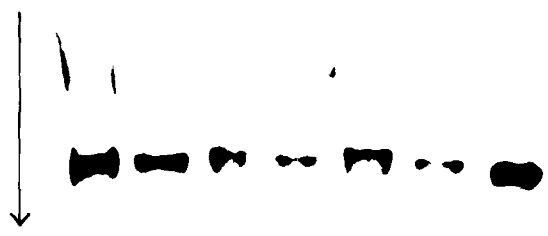


G H B M E L K

13

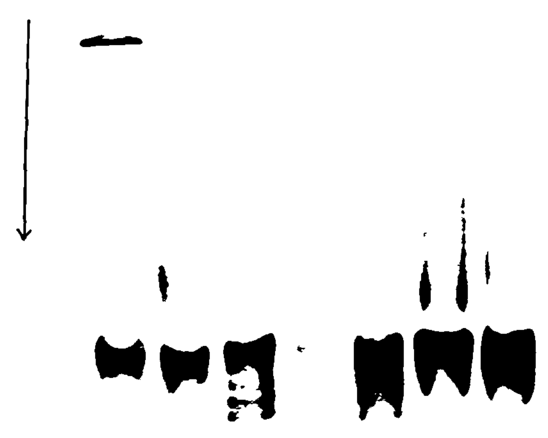
Plate 14 G6PD isozyme pattern in the tissues of *Clarias batrachus*

Plate 15 G6PD isozyme pattern in the tissues of *Heteropneustes fossilis*



K L E M B H G

14



G H B M E L K

15

A P P E N D I X . I

Fishes form an important part of the dietary protein. In order to increase the fish production we need to understand and gain knowledge of the biology of fishes. Since the air-breathing fishes, besides having high protein, high iron and low fat contents, have medicinal value, their increased production should be the aim of fishery scientists. In view of this, in the present study we have thrown some light on the morphometric characters, meristic counts and the length-weight relationship in two species of catfishes, *Clarias batrachus* and *Heteropneustes fossilis*.

While investigating on these fish we came across some unique specimens of *Clarias batrachus*. They were distinct from the usual grey coloured fishes in their characteristic spotted appearance. Their body was whitish pink or orangish in colour with dark black spots all over, including their barbels. Similar fish have been observed by Sen (1978) from Meghalaya.

Our study relating to these two, the usual and the unusual, types of *C. batrachus* specimens did not reveal any genetic differences among them, neither karyotypically nor

through biochemical analysis. We have designated, the spotted C. batrachus specimens as C. batrachus(S) in our present analysis.(Plate 1).

Material

The specimen, Clarias batrachus and Heteropneustes fossilis were obtained from Shillong and Gauhati. These fish fall under the category of air-breathing fish and can easily survive in ponds and ditches, or in shallow stagnant waters. These fish were collected over a period of about three years from March, 1986 to April, 1989.

The total length and weight of the fishes was recorded in their live condition and the rest of the morphometric characters and meristic counts were noted in the freshly killed specimen, within an hour of their sacrifice.

Eight individuals of Clarias were collected which were unique in their appearance and morphology. Their entire body is white or whitish pink in colour with black

spots. This distinct mottled appearance can easily differentiate them from the commonly occuring greyish black fish. These were grouped separately.

Morphometric measurements

The following morphometric measurements have been obtained according to the methods described by Lowe-McConnell (1971) :-

(1) Total length : It is measured from the tip of the snout to the tip of the tail.

(2) Standard length : It is the distance from the tip of the snout to the caudal base.

(3) Body depth : Vertical distance between the dorsal and ventral surface of the body at its greatest depth.

(4) Head length : It is measured from the tip of the snout to the posterior edge of the opercular bone.

(5) Head width : It is the greatest dimension with gill covers closed in normal position.

(6) Snout length : It is taken with a divider, from the anterior - most point on the snout to the front margin of the orbit.

(7) Post orbital length of the head : Distance from the posterior margin of the orbit to the end of the opercular bone.

(8) Sub orbital depth : Distance from the bony edge of the orbit to the suborbital or preorbital margin at its deepest point.

(9) Inter-orbital width : It is taken from the upper margin of the right orbit to the upper margin of the left orbit from the dorsal surface.

(10) Eye diameter : Distance from the anterior to the posterior margin of the eye in a straight line.

(11) Length of the lower jaw : It is the length of the mandible taken with one tip of the divider inserted in the posterior mandibular joint to give the maximum possible dimension.

(12) Length of the upper jaw : It is taken from the anterior-most point of the premaxillary to the posterior point of the maxillary.

(13) Gape width : It is the greatest transverse distance across the mouth opening, taken with the mouth closed.

(14) Pre-dorsal length : Distance from the tip of the snout to the first dorsal fin ray.

(15) Head depth : It is the perpendicular distance between the end of the nape to the ventral side of the head.

(16) Girth : It is the circumference of the body at its deepest point.

(17) Base length of the dorsal fin : Distance between the anterior and posterior end of the dorsal fin taken along its base.

(18) Base length of the anal fin : Distance between the anterior and posterior end of the anal fin taken along its base.

(19) Base length of the pectoral fin : Distance between the anterior and posterior end of the pectoral fin taken along its base.

(20) Base length of the pelvic fin : Distance between the anterior and posterior end of the pelvic fin taken along its base.

(21) Base length of the caudal fin : Distance between the anterior and posterior end of the caudal fin taken along its base.

(22) Distance between the pelvic fin and anal opening : It is measured from the posterior end of the pelvic fin to the anal opening.

(23) Distance between the pelvic fin and anal fin : It is measured from the posterior end of the pelvic fin to the anterior end of the anal fin.

Meristic characters

The following meristic counts were made according to

the methods given by Lowe-McConnell (1971) :-

(1) Number of rays in the dorsal fin.

(2) Number of rays in the pectoral fin.

(3) Number of spines in the pectoral fin.

(4) Number of rays in the pelvic fin.

(5) Number of rays in the anal fin.

(6) Number of rays in the caudal fin.

(7) Number of barbels.

All the above mentioned morphometric measurements and meristic counts were made only from the left side of the fish. Besides these parameters the colouration of the fish and their weight has also been recorded.

Regression analysis

In order to study the extent of association between any two variable morphometric characteristics the correlation coefficient has been determined and a regression approach has been made.

The equation for the true regression line is given by

the expression :

$$y = a + Bx$$

where y, one of the variables, is equal to the true y mean, x is the other variable equal to the true x mean. B is the true regression coefficient of y on x, and is estimated from the samples as b. The calculated regression coefficient b is determined by the following formula :

$$b = \frac{(x-\bar{x})\ (y-\bar{y})}{(x-\bar{x})^2}$$

The constant 'a' is then estimated by :

$$a = \bar{y} - b\bar{x}$$

$\bar{x}$ = mean of x and $\bar{y}$ = mean of y.

Length-weight relationship

The length-weight relationship has been calculated by allometric growth formula as used by Huxley (1924) in the

form of : $w = aL^b$; where w stands for weight, 'L' for length, 'a' and 'b' are constants. The equation has been transferred into a logarithmic expression as suggested by LeCren (1951) and has been expressed as :

$$\text{Log } W = \text{Log } a + b \text{ Log } L$$

The values of 'a' and 'b' were determined empirically.

The length-weight relationship during the present investigation has been studied with reference to the net weight of the fish, including their gut and gonads. The observed weights of the species were plotted against their observed standard length to examine the nature of the relationship. On converting the values to logarithms, the exponential relationship has been expressed as the linear equation described above. The regression of weight on length has been calculated by the method of "least squares".

Condition factor

Individual variations from the general length-weight

relationship have been studied under the general heading, "condition" (LeCren, 1951). Such changes in condition have been generally analysed by means of a 'condition factor' or 'Ponderal index', which has been calculated by using different formulae by various workers. However, in the present study, 'condition factor' has been determined by using the following formula (Hile, 1963; and Beckman, 1948) :

$$K = \frac{W \times 10^2}{L^3}$$

where, K = condition factor and W = weight of the fish. The number 10^2 is a factor used to bring the ponderal index (K) to near unity (Carlander, 1970).

Relative length of the gut

The guts were removed from the fish after dissection and were preserved in 5% formalin. They were later uncoiled, and their length measured. The ratio between the gut length and the total length of the fish (R.L.G.) has been calculated by adopting the following formula (Jacobshagen, 1913) :

$$R.L.G. = \frac{GL}{TL}$$

where, GL stands for the total length of the gut and TL is the total length of the fish.

Observations

Differences in the morphometric and meristic parameters between the males and females of both C. batrachus and H. fossilis are not well defined. However, it was observed that the females are always larger in size than the males and are numerically more in ratio. According to Nikolsky (1963), sex ratio varies considerably from species to species, but in most cases it is close to unity. Presence of more females in nature has been recorded by Hora and Mishra (1936) in Labeo dera. This phenomenon is probably related to the breeding biology of fishes.

1. Morphometry of Clarias batrachus

The mean values of the different morphometric measurements of this species have been presented in Table 1

TABLE 1 . Morphometric analysis of *Clarias batrachus*.

S.No.	Parameters	Mean (cm)	Mean % of total length	Range (cm)
1.	Total length	21.42	100	17.8 - 26
2.	Standard length	18.95	88.47	15.5 - 23.5
3.	Body depth	2.36	10.99	1.8 - 2.9
4.	Head length	4.67	21.82	4.2 - 6.0
5.	Head width	3.22	15.02	2.7 - 3.8
6.	Pre-dorsal length	5.84	27.25	5.0 - 6.4
7.	Head depth	1.71	8.00	1.5 - 2.0
8.	Girth	8.21	38.30	7.2 - 9.1
9.	Base length of dorsal fin	12.29	57.36	10.0 -13.2
10.	Base length of anal fin	8.86	41.34	7.5 -11.4
11.	Base length of pectoral fin	0.73	3.42	0.6 - 0.9
12.	Base length of pelvic fin	0.40	1.88	0.25-0.55
13.	Base length of the candal fin	1.51	7.05	1.0 - 2.3
14.	Distance between the pelvic fin and anal fin	1.53	7.16	1.2 - 1.9
15.	Dist. bet. tghe pelvic fin and anal opening	0.68	3.17	0.5 - 0.85
1.	Head length	4.67	100	4.2 - 6.0
2.	Snout length	1.46	31.12	1.1 - 1.8
3.	Post orbital length of head	3.59	76.89	3.1 - 4.4
4.	Sub orbital depth	1.00	21.41	0.8 - 1.3
5.	Inter orbital width	1.96	42.05	1.5 - 2.6
6.	Eye diameter	0.31	6.72	0.25-0.45
7.	Lower jaw length	0.83	17.76	0.6 - 1.1
8.	Upper jaw length	0.86	18.49	0.7 - 1.1
9.	Gape width	1.64	35.14	1.2 - 2.0

and the regression equations for the various morphometric characters studied are presented in Table 1.1.

2. Morphometry of Clarias batrachus (S)

The mean values of the different morphometric measurements of these individuals have been presented in Table 2 and the regression equations for these measurements are presented in Table 2.1.

3. Morphometry of Heteropneustes fossilis

The mean values of the different morphometric measurements of this species have been presented in Table 3 and the regression equations for these measurements are presented in Table 3.1.

4. Meristic counts of Clarias batrachus

The mean values and range of the meristic counts for this species have been presented in Table 4.

TABLE 1.1 . Regression Equations on morphometric parameters of *Clarias batrachus*.

S.No.	Parameters	Regression Equations	'r'	't'
1.	Total length (y) VS. Standard length (x)	y = 2.8173 + 0.9821 x	0.9805	21.17
2.	Total length (y) VS. Body depth (x)	6 =16.5916 + 2.1368 x	0.3089	1.38(N.S.)
3.	Total length (y) VS. Head length (x)	y = 1.6845 + 4.2379 x	0.7795	5.28
4.	Total length (y) VS. Head width (x)	6 = 1.0512 + 6.3268 x	0.6956	4.11
5.	Total length (y) VS. Pre-dorsal length (x)	6 =-0.0162 + 3.6807 x	0.8579	7.08
6.	Total length (y) VS. Head depth (x)	y =21.6387 + 0.0037 x	0.0003(N.S)	0.00127 (N.S.)
7.	Total length (y) VS. Girth (x)	6 = 4.6383 + 2.0652 x	0.5608	2.87
8.	Total length (y) VS. Base length of dorsal fin (x)	y = 4.6665 + 1.3676 x	0.8771	7.75
9.	Total length (y) VS. Base length of anal fin (x)	6 = 4.0226 + 1.9712 x	0.8979	8.65
10.	Total length (y) VS. Base length of pectoral fin (x)	y =10.8566 +14.7787 x	0.5749	2.98
11.	Total length (y) VS. base length of pelvic fin (x)	6 =15.5559 +14.6726 x	0.6125	3.29
12.	Total length (y) VS. base length of cana fin (x)	y =23.6284 +-1.3726 x	-0.3489	1.58(N.S.)
13.	Total length (y) VS. Dist. bet. pelvic fin & anal fin (x)	y = 7.4192 + 9.1928 x	0.7220	4.43
14.	Total length (y) VS. Dist.bet.pelvic fin & anal opening(x)	y =13.1117 +12.3224 x	0.5681	2.93
15.	Head length (y) VS. Head width (x)	y = 0.2876 + 1.3587 x	0.8120	5.90
16.	Head length (y) VS. Snout length (x)	y = 1.4709 + 2.2186 x	0.8474	6.77
17.	Head length (y) VS. Post orbital length of head (x)	y = 0.2897 + 1.2228 x	0.9233	10.20
18.	Head length (y) VS. Sub-orbital depth (x)	6 = 0.9522 + 3.7391 x	0.8753	7.68
19.	Head length (y) VS. Inter-orbital width (x)	y = 1.4563 + 1.6475 x	0.8566	7.04
20.	Head length (y) VS. Eye diameter (x)	y = 4.9116 +-0.6350 x	-0.1063 /	0.45(N.S.)
21.	Head length (y) VS. Lower jaw length (x)	y = 4.5652 + 0.1749 x	0.0819 (N.S.)	0.35(N.S.)
22.	Head length (y) VS. Upper jaw length (x)	6 = 3.9097 + 0.9306 x	0.3024	1.35(N.S.)
23.	Head length (y) VS. Gape width (x)	y = 3.6050 + 0.6578 x	0.4993	2.45

df = 104

P < .05

TABLE 2 . **Morphometric analysis of *Clarias batrachus (s)***

S.No.	Parameters	Mean (cm)	Mean % of total length	Range (cm)
1.	Total length	25.44	100	18.4 - 31
2.	Standard length	22.16	87.12	15.9 - 27.5
3.	Body depth	3.06	12.03	2.6 - 3.7
4.	Head length	5.66	22.25	4.2 - 7.0
5.	Head width	3.86	15.17	3.15 - 3.9
6.	Pre-dorsal length	7.36	28.93	4.9 - 9.7
7.	Head depth	2.44	9.59	1.8 - 3.1
8.	Girth	11.04	43.40	8.2 - 13.2
9.	Base length of dorsal fin	15.66	61.56	10.7 - 20.1
10.	Base length of anal fin	10.42	40.96	7.2 -12.7
11.	Base length of pectoral fin	1.13	4.44	0.65 - 1.5
12.	Base length of the pelvic fin	0.57	2.24	0.35 - 0.8
13.	Base length of candal fin	1.53	6.01	0.8 - 2.0
14.	Distance between pelvic fin and anal fin	1.7	6.68	1.3 - 2.0
15.	Distance between pelvic fin & anal opening	0.94	3.69	0.8 - 1.1
1.	Head length	5.66	100	4.2 - 7.0
2.	Snout length	1.81	31.98	1.3 - 2.3
3.	Post orbital length of head	4.34	76.68	3.0 - 5.5
4.	Sub orbital depth	1.33	23.50	1.0 - 1.6
5.	Inter-orbital width	2.62	46.29	1.9 - 3.3
6.	Eye diameter	0.45	7.95	0.4 - 0.55
7.	Lower jaw length	1.11	19.61	1.0 - 1.25
8.	Upper jaw length	1.05	18.55	0.7 - 1.25
9.	Gape width	1.87	33.04	1.5 - 2.2

TABLE 2.1 . **Regression equations on Morphometric parameters of *Clarias batrchus (s)***

S.No.	Parameters	Regression Equations	'r'	't'
1.	Total length (y) VS. Standard length (x)	y = 1.4746 + 1.0815 x	0.9945	16.4204
2.	Total length (y) VS. Body depth (x)	y = 6.9876 + 6.0302 x	0.7247	1.80(N.S.)
3.	Total length (y) VS. Head length (x)	y = 0.3361 + 4.4353 x	0.9900	12.15
4.	Total length (y) VS. Head width (x)	y =-8.7202 + 8.8498 x	0.9404	4.79
5.	Total length (y) VS. Pre-dorsal length (x)	y = 6.1585 + 2.6198 x	0.9852	9.95
6.	Total length (y) VS. Head depth (x)	y = 4.1979 + 8.7058 x	0.9448	4.99
7.	Total length (y) VS. Girth (x)	y = 1.1930 + 2.1963 x	0.9417	4.85
8.	Total length (y) VS. Base length of dorsal fin (x)	y = 4.4605 + 1.3397 x	0.9938	15.45
9.	Total length (y) VS. Base length of anal fin (x)	y = 2.4543 + 2.2059 x	0.9896	11.91
10.	Total length (y) VS. Base length of pectoral fin (x)	y =10.6419 +13.0957 x	0.9320	4.45
11.	Total length (y) VS. Base length of pelvic fin (x)	y = 9.6626 +27.6796 x	0.9778	8.08
12.	Total length (y) VS. Base length of caudal fin (x)	y =11.9853 + 8.7939 x	0.9122	3.86
13.	Total length (y) VS. Dist. bet. The pelvic fin & anal fin(x)	y = 1.19 +14.2647 x	0.9156	3.94
14.	Total length (y) VS. Dist.bet the pelvic fin & anal opening(x)	y=25.9361+ -0.5278x	-0.0156	0.027(N.S.)
15.	Head length (y) VS. Head width (x)	y = -2.1760 + 2.0300 x	0.9665	25.4
16.	Head length (y) VS. Snout length (x)	y = 0.5977 + 2.7969 x	0.9869	10.6
17.	Head length (y) VS. Post orbital length of head (x)	y = 0.9605 + 1.0828 x	0.9747	7.56
18.	Head length (y) VS. Suborbital depth (x)	y = 0.1326 + 4.1560 x	0.9569	5.71
19.	Head length (y) VS. Inter-orbital width (x)	y = 0.3723 + 2.0182 x	0.9893	11.75
20.	Head length (y) VS. Eye diameter (x)	y = 10.16 + -10 x	-0.6040	1.31(N.S.)
21.	Head length (y) VS. Lower jaw length (x)	6 = -1 + 6 x	0.6747(N.S.)	1.58(N.S.)
22.	Head length (y) VS. Upper jaw length (x)	y = 1.5153 + 3.9474 x	0.8485	2.78
23.	Head length (y) VS. Gape width (x)	y = -1.6895 + 3.9302 x	0.9845	9.72

df = 6

$p < .05$

TABLE 3. **Morphometric analysis of *H. fossilis*.**

S.No.	Parameters	Mean	Mean % of total length	Range (cm)
1.	Total length	15.47	100	14.2 - 17.7
2.	Standard length	13.82	89.33	12.5 - 15.8
3.	Body depth	1.95	12.57	1.4 - 2.75
4.	Head length	2.31	14.93	2.0 - 2.75
5.	Head width	1.78	11.47	1.4 - 2.1
6.	Pre-dorsal length	4.69	30.32	4.1 - 5.3
7.	Head depth	1.14	7.37	0.8 - 1.4
8.	Girth	4.92	31.8	3.8 - 6.4
9.	Base length of dorsal fin	0.46	2.97	0.35 - 0.6
10.	Base length of anal fin	8.01	51.78	6.0 - 9.7
11.	Base length of pectoral fin	0.34	2.20	0.25 - 0.65
12.	Base length of pelvic fin	0.25	1.62	0.2 - 0.3
13.	Base length of candal fin	0.62	3.98	0.5 - 0.8
14.	Distance between pelvic fin & anal fin	0.92	5.95	0.75 - 1.2
15.	Distanct bet. pelvic fin & anal opening	0.64	4.10	0.4 - 0.8
1.	Head length	2.31	100	2.0 - 2.7
2.	Snout length	0.55	23.59	0.35 - 1.0
3.	Post orbital length of head	1.58	68.18	1.3 - 2.2
4.	Sub-orbital width	0.52	22.29	0.35-0.65
5.	Inter-orbibal width	1.09	47.19	1.0 - 1.35
6.	Eye diameter	0.29	12.55	0.25 - 0.4
7.	Lower jaw length	0.51	22.08	0.4 - 0.75
8.	Upper jaw length	0.47	20.13	0.4 - 0.75
9.	Gape width	0.78	33.77	0.65 - 1.0

TABLE 3.1 . **Regression equations on morphometric parameters of *Heteropneustes fossilis.***

S.No.	Parameters	Regression Equations	'r'	't'
1.	Total length (y) VS. Standard length (x)	y = 0.7437 + 1.0656 x	0.9775	19.65
2.	Total length (y) VS. Body depth (x)	y =13.4686 + 1.0290 x	0.3164	1.42(N.S.)
3.	Total length (y) VS. Head length (x)	y =13.2296 + 1.0184 x	0.4748	2.29
4.	Total length (y) VS. Head width (x)	y = 9.6556 + 3.2758 x	0.5181	2.57
5.	Total length (y) VS. Pre-dorsal length (x)	y =13.2668 + 0.4907 x	0.3652	1.66(N.S.)
6.	Total length (y) VS. Head depth (x)	y =11.0638 + 3.8651 x	0.5358	2.69
7.	Total length (y) VS. Girth (x)	y =13.7591 + 0.3625 x	0.3789	1.74(N.S.)
8.	Total length (y) VS. Base length of dorsal fin (x)	y =11.1719 + 9.3438 x	0.6095	3.26
9.	Total length (y) VS. Base length of anal fin (x)	y = 8.8177 + 0.8305 x	0.6714	3.84
10.	Total length (y) VS. Base length of pectoral fin (x)	y =15.5030 +-0.0970 x	-0.0092	0.039(N.S.)
11.	Total length (y) VS. Base length of pelvic fin (x)	y =13.47 + 8 x	0.1459(N.S.)	0.626(N.S.)
12.	Total length (y) VS. Base length of condal fin (x)	y =10.0392 + 8.8306 x	0.6246	3.39
13.	Total length (y) VS. Dist. bet pelvic fin & anal fin (x)	y =10.2334 + 5.6919 x	0.6246	3.87
14.	Total length (y) VS. Dist. bet pelvic fin & anal opening(x)	y =12.8087 + 4.1911 x	0.5772	2.99
15.	Head length (y) VS. Head width (x)	y = 0.9537 + 0.7641 x	0.5490	2.79
16.	Head length (y) VS. Snout length (x)	y = 1.6706 + 1.1733 x	0.7617	4.99
17.	Head length (y) VS. Post orbital length of head (x)	y = 1.4696 + 0.5336 x	0.4744	2.29
18.	Head length (y) VS. Sub-orbital depth (x)	y = 2.1122 + 0.3840 x	0.1424(N.S.)	0.61(N.S.)
19.	Head length (y) VS. Inter-orbital width (x)	y = 0.1036 + 2.0242 x	0.8348	6.43
20.	Head length (y) VS. Eye diameter (x)	y = 1.0896 + 4.2083 x	0.7636	5.02
21.	Head length (y) VS. Lower jaw length (x)	y = 1.0947 + 2.3830 x	0.8557	7.02
22.	Head length (y) VS. Upper jaw length (x)	y = 1.5835 + 1.5624 x	0.6604	3.73
23.	Head length (y) VS. Gape width (x)	y = 0.5660 + 2.2359 x	0.8526	6.92

df = 68

$p < .05$

TABLE 4 . **Meristic counts of *Clarias batrachus*.**

S.No.	Parameters	Mean	Range
1.	Number of rays in the dorsal fin	61.5	54.66
2.	Number of rays in the pectoral fin	8.23	6-10
3.	Number of spines in the pectoral fin	1	Constant
4.	Number of rays in the pelvic fin	5.18	3-6
5.	Number of rays in the anal fin	45.64	38-53
6.	Number of rays in the caudal fin	13.77	11-17
7.	Number of barbels	8	Constant

5. Meristic counts of Clarias batrachus (S)

The mean values and range of the meristic counts of these individuals is presented in Table 5.

6. Meristic counts of Heteropneustes fossilis

The mean values and range of the meristic counts of this species have been presented in Table 6.

7. Length-weight relationship

7.1. Length-weight relationship in Clarias batrachus was calculated to be :

$$\text{Log } W = -1.3579 + 3.3048 \text{ Log } L \ (r = 0.6771)$$

The value of r is highly significant at 99% level of confidence and at 104 degrees of freedom.

7.2. The length-weight relationship in the individuals of Clarias batrachus(S) was observed to be :

TABLE 5 . Meristic counts of *Clarias batrachus (s)*

S.No.	Parameters	Mean	Range
1.	Number of rays in the dorsal fin	57.2	45-64
2.	Number of rays in the pectoral fin	8.8	8-9
3.	Number of spines in the pectoral fin	1	Constant
4.	Number of rays in the pelvic fin	6.2	6-7
5.	Number of rays in the anal fin	40	36-45
6.	Number of rays in the caudal fin	15.4	14-16
7.	Number of barbels	8	Constant

TABLE 6 . Meristic counts of *Heteropneustes fossilis.*

S.No.	Parameters	Mean	Range
1.	Number of rays in the dorsal fin	6.8	6-8
2.	Number of rays in the pectoral fin	6.2	6-7
3.	Number of spines in the pectoral fin	1.0	Constant
4.	Number of rays in the pelvic fin	6.0	Constant
5.	Number of rays in the anal fin	63.6	52-74
6.	Number of rays in the caudal fin	14.1	12-18
7.	Number of barbels	8	Constant

$$\text{Log } W = -2.1397 + 12.32 \text{ Log } L \; (r = 0.9404)$$

the value of r is highly significant at 99% level of confidence and at 6 degrees of freedom.

7.3. The length-weight relationship in *Heteropneustes fossilis* was calculated to be :

$$\text{Log } W = -0.6807 + 3.7914 \text{ Log } (r = 0.8226)$$

The value of r is highly significant at 99% level of confidence, with 68 degrees of freedom.

8. Condition factor

8.1. The value of condition factor in *Clarias batrachus* was calculated to be :

$$K = 1.02$$

8.2. The value of condition factor in the individuals of *Clarias batrachus*(S) was calculated to be :

$$K = 1.27$$

8.3. The value of condition factor in <u>Heteropneustes</u> <u>fossilis</u> was calculated to be :

$$K = 0.83$$

9. Relative length of the gut

9.1. The R.L.G. value was found to be 0.6615 in <u>Clarias</u> <u>batrachus</u>. Relation between the gut length (y) and the total length (x) was given by :

$$y = -1.6193 + 0.7546\,x \quad (r = 0.5919)$$

The value of r is highly significant at 99% level of confidence with 18 degrees of freedom.

9.2. The R.L.G. value was 0.7304 in the individuals of <u>Clarias</u> <u>batrachus</u>(S). Relation between the gut length (y) and the total length (x) was given by :

$$y = 4.3040 + 0.5612\,x \quad (r = 0.8841)$$

The value of r is fairly significant at 99% level of confidence, with 3 degrees of freedom.

9.3. The R.L.G. value was found to be 0.5326 in <u>Heteropneustes fossilis</u>. Relation between the gut length (y) and the total length (x) was given by :

$$y = -0.5045 + 0.5653\,x \quad (r = 0.2334)$$

The value of regression coefficient was found to be significant at 20% level of confidence and insignificant at higher levels of confidence, at 18 degrees of freedom.

It appears that most of the morphometric characters of <u>Clarias batrachus</u>(S) are more in length than those of <u>C. batrachus</u> individuals. (Table 1 and 2). Since the values obtained for both these fishes are different from each other, some differences obviously exist between these two fishes. The regression equations of the various parameters in relation to the total length (or head length) in <u>C. batrachus</u> and <u>C. batrachus</u>(S) are shown in Table 1.1 and 2.1 respectively. Most of the values of 'r' the regression coefficient are significant at 5% level indicating that there exists a significant correlation between the parameters in consideration. The 't' values are also mostly significant at 5% level indicating that the 'r' is

significantly different from zero. The negative 'r' values indicate that there is a negative correlation between the two parameters, that is, when one increases the other decreases.

Similarly Table 3 shows the morphometric analysis in _H. fossilis_ and Table 3.1 gives the regression equations on the various morphometric parameters. In this fish also, it appears that there exists a significant correlation between most of the parameters taken into account. The 't' values that are not significant at 5% level indicate that the 'r' value is not significantly different from zero.

One can expect intra-species variations in morphological features, due to the plasticity of ecological conditions (Schmidt, 1921; Vladykov, 1934; Lindsay, 1954; Fage, 1958). Polymorphic characters like body colouration cannot be taken as diagnostic features in distinguishing different species (Mayr, 1963).

Similarly meristic counts may or may not always be constant for a particular species because non-genetic variations of morphological characters are common in fish

(Barlow, 1961) and are always affected by environmental fluctuations during developmental stages (Lindsay, 1954; Barlow, 1961). According to Hubbs (1922) and Taning (1944) variation occurs in the number of rays in the unpaired fins in several species, which is also related to an adaptation to movement of water of various densities. The meristic counts of C. batrachus, C. batrachus(S) and H. fossilis are given in Table 4, 5 and 6 respectively. In C. batrachus(S) the number of rays in the pelvic fin and caudal fin in slightly higher than in C. batrachus and slightly lower in the anal fin than the latter.

It is the general expectation that the weight of fishes would vary as the cube of the length (Broady, 1945; Lagler, 1952; Rounsefel and Everhart, 1953; Brown, 1956). However, the length-weight relationship does not always obey the cube law in different fishes (Hile, 1936; LeCren, 1951; Martin, 1949; Sekharan, 1968; Jhingran, 1968; Bapat, 1970; Narasimhan, 1970; Chendar, 1975; Chatterjee et al., 1977; Mitra and Roy, 1977 - 1978). In C. batrachus, C. batrachus(S) and H. fossilis the value of exponent 'b' is higher than '3'. In C. batrachus(S) it exceeds this

value largely. Mitra and Naser (1987) have found that the allometric formula of Huxley (1925) is more applicable than the cube law in C. batrachus. Hughes et al.,(1974) while studying the effect of growth on gills and accessory respiratory organs of H. fossilis, have mentioned that the compressed body shape of the species is a probable cause of increase in the power function (b = 3.325). The value of the exponent (b) is generally influenced by numerous factors viz. seasonal fluctuations in environmental parameters, physiological conditions of the fish at the time of collection, sex, gonadal development and nutritive conditions of the environment of fishes (Sinha, 1973; Kaur, 1981; Dasgupta, 1982). According to Tesch (1971) the exponent 'b', value of '3' indicates that a fish grows isometrically.

It was seen that the value of condition factor K was higher in C. batrachus(S) than in C. batrachus and H. fossilis indicating that C. batrachus(S) has a better

'condition' than C. batrachus. The K value is influenced by the maturation of gonads, the food present in the alimentary canal and certain other environmental factors as suggested by Kaur (1981) and Dasgupta (1982).

According to Suyehiro (1941), Mookherji and Das (1945), AL-Hussaini (1949) and Das and Moitra (1958), there is a correlation between the food habits and the R.L.G. of fishes. The length of the gut is almost equal to, or in some cases less than the length of the fish in carnivores, while in herbivores it is longer, the increase being proportionate to the vegetable matter consumed. The R.L.G. in our fishes, in the present study, was found to be less than unity, reflecting their carnivorous food habit. There exists a significant correlation between the gut length and the total length of the fish, both in C. batrachus and C. batrachus(S) at 99% level of confidence. Whereas, in H. fossilis the correlation between these parameters was significant only at 20% level of confidence.

From the present study it appears that there are some morphological differences between C. batrachus and

C. batrachus(S). We conclude that these differences may have arisen due to variances in the environmental conditions of these fishes and are manifested mainly in the differences in their body colouration and size.

AL-Hussainy, A.H. (1949): On the functional morphology of the alimentary tract of some fishes in relation to differences in their feeding habits. Quart. J. Micr. Sci. 9(2): 190-240.

Bapat, S.K. (1970): Length-weight relationship of *Johnius dussumeiri* (Cuv. and Val.) Marathwada University J. Sci., 2: 189-194.

Barlow, G.W. (1961): Causes and significance of morphological variation in fishes. Syst. Zool. 10: 105-117.

Beckman, C.W. (1948): The length-weight relationship, factor for conversions between standard and total lengths, and coefficients of condition for seven Michigan fishes. Trans. Am. Fish. Soc. 75: 237-256.

Broady, S. (1945): Bioenergetics and Growth. Reinhold, New York.

Brown, M.E. (1957): The Physiology of Fishes. Vol. I, Acad. Pr., New York and London.

Carlander, D.K. (1970): Handbook of freshwater fishery biology. Vol. I. The Iowa State Univ. Press. Amer. Iowa.

Chatterjee, A., Siddiqui, A.Q. and Khan, A.A. (1977): Length-weight relationship of a carp, *Labeo bata* (Ham.). Proc. Indian Acad. Sci. (B), 86(3): 189-194.

Chondar, S.L. (1975): Length-weight volume relationship of *Gadusia chapra* (Ham.). J. Asiat. Soc., Calcutta, 17: 55-65.

Das, S.M. and Moitra, S.K. (1958): On the feeding types of fishes and the variations in the alimentary canal in relation to food. Journ. Ichthiol. Moscow, 10: 29-40.

Dasgupta, M. (1982): An investigation on the biology of Mahseers from the North-Eastern India. Ph. D. Thesis North Eastern Hill University.

Fage, L. (1958): Croissance, races, migrations. Traite de Zoologie de p.p. Grosse, Paris, Masson et Cie. Edit. 73: 1835-1849.

Hile, R. (1936): Age and growth of the cisco, Leucichthys artedi (Le Sueur), in the lakes of the north-eastern highlands, Wisconsin. Bull. U.S. Bur. Fish., 48: 211-317.

Hora, S.L. and Misra, K.S. (1936): Sexual dimorphism in the carp. Labeo dero (Ham.) Rec. Ind. Mus., 38:341-342.

Hubbs, C. (1922): Variation in the number of vertebrate and other meristic characters of fishes correlated with the temperature of water during development. Amer. Nat. 56.

Hughes, G.M., Singh, B.R., Guha, G., Dube, S.C. and Datta, J.S. (1974): Respiratory surface area of an air-breathing siluroid fish, saccobranchus (=Heteropneustes) fossilis in relation to body size. J. Zool. Lond., 172: 215-232.

Huxley, J.S. (1925): Constant differential growth ratios and their significance, Nature, Lond., 114: 895-896.

Jacobshagen, E. (1913): Untersuchungen uber das Darmsystem der Fische und Dipnoer. Jena. Z. Naturw., 49: 373-410.

Jhingran, A.G. (1968): The length-weight relationship and "K" factor of Gadusia chapra (Ham.) from the Ganga river system. Proc. Natn. Acad. Sci. India, 38 B: 249-263.

Kaur, S. (1981): Studies on some aspects of the ecology and biology of Channa gachua (Hamilton) and Channa stewartii (Playfair). Ph. D. Thesis, North-Eastern Hill University.

Lagler, K.F. (1952): Freshwater Fishery Biology. Wm. C. Brown Co., Dubugne, Iowa.

Le Cren, E.D. (1951): The length-weight relationship and seasonal cycle in gonad-weight and condition in the perch (Perca fluviatilis). J. Anim. Ecol., 20: 201-219.

Lowe-McConnell, R.H. (1971): Identification of freshwater fishes. In: Methods of Assessment of Fish production in Fresh waters. Ed. W.E. Ricker. Blackwell. Scientific Publications. Oxford and Edinburgh, 45-81.

Lindsay, C.C. (1954): Temperature controlled meristic variation in the Paradise Fish, Macropodus opercularis (L.) Can. J. Zool., 32: 87-98.

Martin, W.R. (1949): The mechanics of environmental control of body form in fishes. Univ. Toronto Stud. Biol. 58 (Publ. Ont. Fish. Res. Lab. 70):1-91.

Mayr, E. (1963): Animal species and Evolution. Harvard: Belknap Press.

Mitra, B. and Naser, M. (1987): Length-weight relationship in clarias batrachus (Linn.) Proc. Zool. Soc., Calcutta, 36: 29-35.

Mitra, B. and Roy, P. (1977-1978): On the relationship between length and weight of Puntius sophore (Ham.) J. Res., Visva-Bharati, 2: 9-16.

Mookerjee, H.K. and Das, R.K. (1945): Gut of carnivorous and herbivorous fishes in relation to their food at different stages of life. Proc. Indian Sci. Congr., 32(3):109.

Narasimhan, K.A. (1970): On the length-weight relationship and relative condition in Trichuirus lepturus (Linn.) Indian J. Fish., 17(1-2): 90-96.

Nikolsky, G.V. (1963) : The ecology of fishes. Academic Press, London and New York. 352.

Rounsefel, G.A. and Everhart, W.H. (1953): Fishery Science: Its methods and applications. John Wiley. New York.

Schmidt, J. (1921): Racial investigations. VII: Annual fluctuations of racial characters in Zoarces viviparous L. C.R. Lab., Carlasberg, 14: 1-20.

Sekharan, K.V. (1968): Length-weight relationship in Sardinella albella (Val.) and S. gibbosa (Bleak). Indian J. Fish., 15: 166-174.

Sen, T.k. (1978): Fishes of Assam with scientific, local and English names. Seafood Export J., 10(1): 63-67.

Sinha, A.L. (1973): Length-weight relationship of a freshwater catfish, Clarias batrachus (Linn.) Indian J. Zootomy 14(2): 97-102.

Sujehiro, Y. (1941): A study of the digestive system and feeding habits of fish. Jap. J. Zool., 10: 1-303.

Taning, A.V. (1944): Experiments on meristic and other characters in fishes. I. On the influence of temperature on some meristic characters in sea trout and the fixation period of these characters. Medd. Dan. Fisk. Havenders, 11(3): 1 - 66.

Tesch, F.W. (1971): Age and growth. In:Methods for assessment of fish production in Freshwater. (2nd ed.) Ed. W.E. Ricker. IBP Handbook No. 3. Blackwell Scientific Publications, Oxford and Edinburg, 93-102.

Vladykov, V.D. (1934): Environmental and taxonomic characters of fishes. Trans. R. Can. Inst., 20: 99-140.

Plate 1 The fish, *Clarias batrachus* (s)

A P P E N D I X I I

The knowledge of the diploid number, karyotypes and metrical data of metaphase chromosomes of fish are useful not only in clarifying their organisation and structure but also the organisation and structure of higher vertebrate chromosomes, since the origin of the genomes of the latter can be placed in some of the earlier piscine lines. Nevertheless, because of the methodological difficulties in this material their study has lagged far behind that of the other vertebrates.

There has been some controversy over the years, regarding the diploid number in the chromosome complements of C. batrachus and H. fossilis as shown in Table 1. In order to confirm the diploid number and the karyotypes of these fishes we carried out the present analysis.

Chromosome preparation

Adult individuals were injected intra-muscularly with 0.1% Bovine serum albumin solution (1 ml/100 g body weight) and 0.01% colchicine solution (1 ml/100 g body weight). The injected fish were kept in well aerated water

Table 1. Chromosome complements of *C. batrachus* and *H. fossilis*.

Clarias batrachus:

2n	Karyotype	no. of arms	Reference
52	52a	(52)	Nayyar 1966
52		58	Srivstava and Das 1968
50	16m+8sm+14st+12a	(74)	Rishi 1976
50		(74)	Rishi 1978

Heteropneustes fossilis:

2n	Karyotype	no of arms	Reference
58	58a	(58)	Nayyar 1966
56	18m+18sm+20a	(92)	Vasudevan et al 1973
56	4m+26sm+16a	(96)	Prasad et al 1974
56	18m+18sm+20a	(92)	Rishi 1976

and sacrified after 4 hours.

Cytological preparations were made from the kidney and gonads according to the air-dry technique employed by Dhar (1982). The tissues were first minced with a pair of scissors and then aspirated vigorously in tri-sodium citrate solution (1%) and allowed to stand for 45 minutes. This homogenous suspension was centrifuged at 1500-2000 rpm, for 15 minutes. The supernatant was discarded and the residue was fixed in 5 ml of Carnoy's fixative (acetic acid : absolute alcohol, 1 : 3 V/V) and again aspirated to obtain a homogenous solution. This was kept for 30 minutes. The suspension was again centrifuged at 1500-2000 rpm for 15 minutes and the residual cell mass obtained was resuspended in a minimal volume of the fixative. This was dropped (1 to 2 drops) on a slide, chilled previously in absolute alcohol, and ignited over a flame, dried and then stained in Giemsa solution.

Giemsa staining

The procedure as described by Chatterjee _et al_.

(1981) was followed with some modifications.

(i) Buffer A : m/15 disodium hydrogen ortho phosphate.

(ii) Buffer B : m/15 potassium dihydrogen ortho phosphate.

(iii) Working solution : Buffer A and B (1 : 1) pH 6.8 - 7.2.

(iv) Stain : 10 ml Giemsa solution (BDH) 90 ml working buffer solution. Time 15 to 20 minutes.

Chromosome analysis

All measurements were taken from metaphase plates. The following parameters were considered while studying the chromosome morphology.

(i) Length of the long arm of the chromosome

(ii) Length of the short arm of the chromosome

(iii) Arm-ratio or 'r' = $\frac{\text{Length of the long arm}}{\text{Length of the short arm}}$

(iv) Centromeric indices

$$= \frac{\text{Length of the short arm}}{\text{Length of the long arm}} \times 100$$

(v) Total length of the chromosome

(vi) % Relative length of the chromosome

$$\% \text{ RL} = \frac{\text{Total length of a chromosome}}{\text{Total length of the whole chromosome set}} \times 100$$

(vii) Chromosome type.

The position of the centromere was determined according to Levan _et al_. (1964) by dividing the long arm by the short arm to provide an arm ratio (r). If 'r' is 1.0 to 1.7, the centromere has been considered as median and the chromosome has been designated as 'm' type (metacentric); a 'r' of 1.7 to 3.0 reflects a submedian centromere and

the chromosome has been designated as 'sm' type (submetacentric). A 'r' between 3.0 to 7.0 indicates a subterminal centromere and the chromosome has been designated as 'st' type (subtelocentric). A 'r' of 7.0 to infinity indicates a terminal centromere and the chromosome has been referred to a 't' type (terminal).

The diploid number for both C. batrachus and H. fossilis have been determined by studying a large number of well spread metaphase complements from both the sexes. No heteromorphic sex chromosomes were found in these fish. The mean values of all measurements, taken from different chromosome sets, have been used in the tables for karyotypic studies.

Chromosome analysis of C. batrachus

To confirm the diploid chromosome number of this species, 120 well spread metaphase complements were studied. The total chromosome number varied from 48 to 52. The frequency of occurrence was as follows :

No. of chromosomes(2n)	48	50	52
No. of nuclei scored	3	18	99

A chromosome complement of 52 chromosomes was most frequent (82.5%) and has been confirmed to be the 2n number of this fish. Plate 1 shows the karyotype and Table 2 gives the karyomorphological analysis.

Chromosome analysis of *H. fossilis*

To confirm the diploid chromosome number of this fish, 112 well spread metaphase complements were studied. The 2n number varied from 54 to 58. The frequency of occurrence was as follows :

No. of chromosomes (2n)	54	56	58
No. of nuclei scored	6	101	5

The 2n number of 56 chromosomes was most frequent (90.2%) and has been confirmed to be the diploid chromosome number of this fish. Plate 2 shows the karyotype and Table 3 summarises the karyomorphological analysis.

Table 2. Karyomorphological studies of the chromosome complement of *Clarias batrachus*

Chromosome number	Length of the long arm (μ)	Length of the short arm (μ)	Total length (μ)	% relative length	arm ratio (r)	centromic indices	Chromosome type
1	3.28	1.72	5.00	6.18	1.91	52.44	Sm
2	2.97	1.56	4.53	5.60	1.90	52.53	Sm
3	2.98	1.41	4.39	5.43	2.11	47.32	Sm
4	2.81	1.56	4.37	5.41	1.80	55.52	Sm
5	2.66	1.26	3.91	4.84	2.13	46.99	Sm
6	2.03	1.72	3.75	4.64	1.18	84.73	m
7	2.19	1.41	3.60	4.45	1.55	64.38	m
8	2.29	1.25	3.54	4.38	1.83	54.59	Sm
9	2.34	0.94	3.28	4.06	2.49	40.17	Sm
10	2.08	0.98	3.06	3.79	2.12	47.12	Sm
11	2.04	0.78	2.82	3.49	2.62	38.24	Sm
12	2.13	0.68	2.81	3.48	3.13	31.93	St
13	1.88	0.98	2.86	3.54	1.92	52.13	Sm
14	1.86	0.94	2.80	3.46	1.98	50.14	Sm
15	1.46	1.30	2.76	3.41	1.12	81.25	m
16	1.41	1.35	2.76	3.41	1.04	95.75	m
17	1.36	1.34	2.70	3.33	1.02	98.53	m
18	1.36	1.33	2.69	3.33	1.02	97.79	m
19	1.88	0.80	2.68	3.32	2.35	42.55	Sm
20	1.34	1.32	2.66	3.26	1.02	98.51	m
21	2.56	.14	2.70	3.33	18.29	5.47	t
22	1.86	0.63	2.49	3.08	2.95	33.87	Sm
23	1.25	1.25	2.50	3.09	1.10	100	m
24	1.28	1.04	2.32	2.87	1.23	81.25	m
25	1.72	0.63	2.35	2.91	2.73	36.63	Sm
26	1.36	0.16	1.52	1.88	8.5	11.77	t

Table 3. Karyomorphological studies of the chromosome complement of *Heteropneustes fossilis.*

Chromosome number	Length of the arm (μ)	Length of the short arm (μ)	Total length (μ)	% relative length	arm ratio (r)	centromeric indices	Chromosome type
1	1.72	1.72	3.44	6.45	1.0	100	m
2	1.41	1.25	2.66	4.99	1.13	88.65	m
3	1.25	1.25	2.50	4.69	1.0	100	m
4	1.56	0.94	2.50	4.69	1.66	60.26	m
5	1.41	0.94	2.35	4.41	1.5	66.67	m
6	1.34	1.0	2.34	4.39	1.34	74.63	m
7	1.19	1.15	2.34	4.39	1.34	74.63	m
8	1.25	0.94	2.19	4.11	1.33	75.20	m
9	1.26	0.78	2.04	3.82	1.62	61.91	m
10	1.16	0.88	2.04	3.82	1.32	75.86	m
11	1.41	0.63	2.04	3.82	2.24	44.68	Sm
12	1.09	0.94	2.03	3.81	1.16	86.24	m
13	0.94	0.94	1.88	3.52	1.0	100	m
14	1.25	0.63	1.88	3.52	1.98	50.4	Sm
15	0.88	0.84	1.72	3.22	1.05	95.46	m
16	0.94	0.78	1.72	3.22	1.12	89.01	m
17	0.91	0.81	1.72	3.22	1.21	82.98	m
18	0.97	0.75	1.72	3.22	1.29	77.32	m
19	0.88	0.84	1.72	3.22	1.05	95.46	m
20	0.94	0.78	1.56	2.92	1.21	82.98	m
21	0.78	0.78	1.56	2.92	1.0	100	m
22	0.94	0.62	1.56	2.92	1.52	65.96	m
23	1.09	0.47	1.56	2.92	2.32	43.12	Sm
24	0.78	0.63	1.41	2.64	1.24	80.77	m
25	0.63	0.63	1.26	2.36	1.0	100	m
26	0.94	0.31	1.25	2.34	3.03	32.98	St
27	0.94	0.16	1.10	2.06	5.9	17.02	St
28	0.97	0.13	1.10	2.06	7.46	13.40	t

Nayyar. R.P. (1966): Karyotypic studies in thirteen species of fishes. Genetica 37: 78-92.

Prasad, R. and Manna, G.K. (1974): Somatic and germinal chromosomes of a live-fish, Heteropneustes fossilis (Bloch.) Caryologia 27(2): 217-223.

Rishi, K.K. (1976): Karyotypic studies on four species of fish. Nucleus, Calcutta 19(II): 95-98.

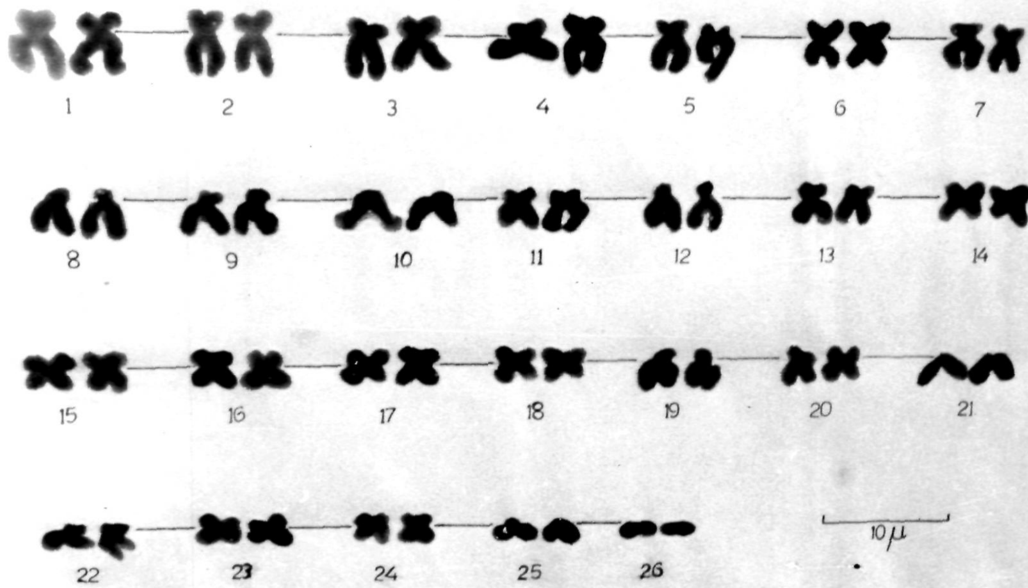
Rishi, K.K. (1978): Chromosomal homogeneity in two Indian catfishes. Geobios 5: 121-123.

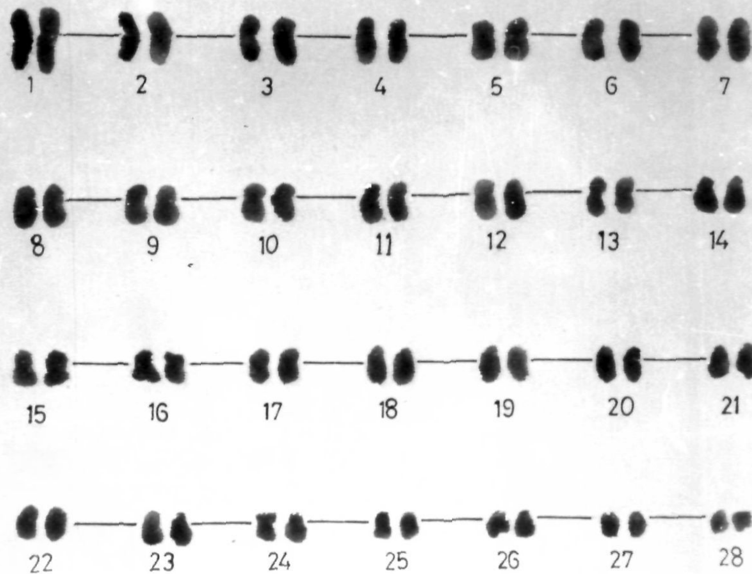
Srivastava, M.D.L. and Das, B. (1968): Somatic chromosomes of (Clarias batrachus (L.) (Clariidae: Teleostomi). Caryologia 21: 349-352.

Vasudevan, P., Rao, S.G.A. and Rao, S.R.V. (1973): Somatic and meiotic chromosomes of Heteropneustes fossilis. Curr. Sci. 42(12): 427-428.

Plate 1 Karyotype of <u>Clarias batrachus</u>

Plate 2 Karyotype of <u>Heteropneustes fossilis</u>


1 2 3 4 5 6 7
8 9 10 11 12 13 14
15 16 17 18 19 20 21
22 23 24 25 26
10μ
1


1 2 3 4 5 6 7
8 9 10 11 12 13 14
15 16 17 18 19 20 21
22 23 24 25 26 27 28
10 μ
2