GENETIC VARIATION IN THE FISH *LIZA PARSIA* (HAMILTON--BUCHANAN)

DISSERTATION SUBMITTED BY

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C E R T I F I C A T E

This is to certify that this Dissertation is a bonafide record of work carried out by Shri PARAG B KARIA under my supervision and that no part thereof has been presented before for any other degree.

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P R E F A C E

The role of aquaculture in augmenting fish production is now well recognized. The selection of productive strains for successful culture and subsequent domestication in different environmental conditions is faced as a major problem by the aquaculturist. A genetic approach to the problem has assumed great importance. Fish genetics has gained priority since, in other problems inherent with a aquaculturist viz., culture techniques, nutrition, controlled spawning, seed production and mortality control, considerable progress has been achieved. Moreover, knowledge of the genetic make-up and variability of the wild as well as farmed fish stocks is a prerequisite for the management of genetic resources for genetic improvement.

In India, there is only meagre information on the genetic make-up and variation of marine fishes and shell fishes. Lack of standard research methods and techniques applicable here were the major constraints. Realizing the importance of application of genetic principles in the emerging aquaculture programmes in our country and the technical constraints involved in its promotion, the Centre of Advanced Studies in Mariculture, in consultancy with FAO experts facilitated the adoption of technical advice in the subject. The present work was initiated by this interaction.

This study was primarily aimed at standardizing the electrophoretic technique applicable for assessing the genetic variability. Subsequently an attempt at measuring the genetic variation of the fish Liza parsia was aimed at. L. parsia a common mullet found in the Cochin estuary contributes a thriving fishery in the estuaries and backwaters of India and is a candidate species of culture here. Isolated reports on biochemical genetics of these mullets are present, moreover they provide no information on their genetic variation (Sita Rama Reddy et al 1975). All this signify the need and approach underlined in the present study.

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INTRODUCTION AND REVIEW OF LITERATURE

Biochemical genetic studies using the technique of electrophoresis has been increasingly applied for fish study within the last two decades. Properly chosen electrophoretic variants reflect single gene differences at a particular locus. On the other hand "classical" morphological variants are controlled by more than one gene, allowing different genotypes to possess similar phenotypic expression. Moreover these "classical" variants are influenced by the environment making one question the genetic basis of the variant. The significance of biochemical genetic studies can be understood by its diverse application in fish genetic study (Wishard, Seeb and Utter, 1980), concerning the stock identification, species separation and hybridization. These studies have also helped to quantify the genetic variation within interspecies and intraspecies. Recently certain aspects as artificial hybridization, gynogenesis, polyploidy have become very significant in fish genetics of culturable species. Biochemical genetic studies have effectively assisted in assessing either the efficiency or influence of these phenomenon on fish. New light on gene-environment interaction is being facilitated by these studies. Last but not the least, biochemical genetic data in conjunction with chemical cytogenic techniques have played a major role in determination of both existence and mechanism of gene duplication (Utter, Hodgins & Allendorf, 1974). A rational and efficient use of biological resources requires a through knowledge of the amount and dis-

tribution of genetic variability within the species considered. Earlier studies were based on morphological characters, to assess genetic variability within a species. It was concluded that most of the species were subdivided into more or less genetically distinct subunits. To assess genetic variation based on morphological characters is difficult and tedious, (eg., Alm, 1949; Svardson, 1979) moreover the morphological differences between fish of different origin are caused by environmental factors and large fraction of evidence for genetic differences is circumstantial (Ricker, 1972; Thrope and Mitchell, 1981).

Biochemical genetic studies to assess the genetic variation has increased in the last decade with the use of biochemical markers through electrophoresis. Ferguson and Mason (1981) carried out electrophoretic work on reproductively isolated sympatric population of brown trout Salmo trutta and Riddel et al (1981) who worked on juveniles of Atlantic salmon and Ryman, (1979) show that some of the observed phenotypic differences can be explained on the basis of genetic differences as revealed by biochemical marker. Ryman (1983) worked on biochemical genetic variation on four salmonid species viz., Atlantic salmon (Salmo salar), brown trout (Salmo trutta), rainbow trout (Salmo gairdeneri), Sockeye salmon (O.nerka) show that considerably larger portion of the total gene diversity is found within population in the atlantic salmon and the rainbow trout as compared to the brown trout. Electrophoretic variation of 12 enzyme systems representing 26 loci in

four French strains of domesticated rainbow trout revealed large proportion of the variability in rainbow trout as compared to others (Guyomard, 1981). There is however an obvious lack of quantitative estimates of the magnitude of the genetic variation in the fish species. This is serious short coming from the perspectives of both conservation of genetic resources and the efficient use of existing genetic variation. It is frequently of little use to know that there is genetic variation without any information concerning the magnitude. (Ryman 1983). It is clear that such quantitative estimates would help in the identification of the different sources of genetic diversity which may have direct impact on choice for a strategy for an efficient use and conservation of genetic variability within different species (Ryman, 1983).

Inspite of the increasing interest in aquaculture, the study of quantitative genetics in fish still lies far behind that of farmed warm blooded animals. Most reported work is on C. carpio. Important breeding work done by Moav (1976), Kirpichinikov (1971); (1973), Golovinskay (1971), Schaperclaus (1961), on carps, bear indirect evidence for the existing genetic variability in those species. Utter, Allendorf, Hodgins (1973b) who worked on Rainbow trout and pacific salmon, revealed that there is greater gene diversity in former to the latter species.

Over the last decade there has been considerable amount of biochemical genetic work leading to differentiating species which share many idential morphological feature. Protein and

isoenzyme markers have been used and mostly the information on protein, lactate dehydrogenase and esterase that have been worked in the present study are present.

Identification of young salmonids is often not possible with morphological studies. Allendorf and Utter (1979) have worked on nine salmonid species of two genera and obtained isoenzyme patterns for creatine kinase and superoxide dismutase which are useful to identify the individuals to species level. This study has been particularly useful for differentiating out throat salmon and rainbow trout (S.clarki and S. gairdeneri) which often occur sympatrically and are morphologically very similar as juveniles.

Biochemical genetic studies have been extensively used to separate Tilapia species. Chen & Tsuyuki (1970) have described an electrophoretic method of species identification of four species of genus Tilapia, viz., T.mossambica, T.zilli, T.melanapleura and T.hornorum based on distinct muscle myogen and haemoglobin pattern in each species. Their lactate dehydrogenase zymograms could be used to differentiate the substrate spawners T.zilli and T.melanopleura from each other and from the other two mouth breeders T.mossambica and T.hornorum. Esterase zymograms could be used to differentiate the two month breedes from each other and from the other two substrate spawners which had identical esterase pattern. Biochemical markers from serum protein have been identified electrophoretically to distinguish between three economically important

Tilapia species viz. T.nilotica, T.aurea and T.vulcani (Avatalion et al, 1975 & 1976). Distinct esterase pattern of the surface mucus in different species of Tilapia have been obtained from electrophoretic studies, which could be used for their identification (Herzberg, 1978).

Scholl & Herzberg (1972) studied the lactate dehydrogenase isoenzymes of 17 species of south american Cichlids and have been grouped the same into six distinct groups which do not correspond to the conventional grouping by morphological classification. Electrophoretic examination of 27 protein loci in several morphologically distinct local races of cichlids in one of the American lakes revealed identical variation for these loci. This provided a basis for the conclusion that only one species with a single panmictic population is present is that lake (Sage & Selander, 1975).

Dotson & Graves (1982) biochemically identified with glucose3-phosphate dehydrogenase marker, the occurrence of a Bluefin tuna off Californian coast, not commonly found there. Differenting population of the tuna species within and between Pacific and Atlantic oceans has been facilitated via., electrophoretic studies of the erythrocyte antigen (Fujino, 1970). Biochemical genetic studies of the polymorphic loci of eye lens proteins in Thunnus thynnus, T.alalunga T.albana, have found to be characteristic for each species (Smith, 1965; Smith & Clemens 1973) variation of serum and liver esterases has been reported for several species of the tunnies with 3-4 alleles

for each locus. (Spargue, 1967; Fujino, 1970). Pacific skipjack was differentiated into characteristic east and west population and a dynamic migration, periodically, from west to east and vice versa was determined (Fujino, 1976).

Electrophoretic analysis of 4 species of Antartic fish (Notothenia rossii; N.reglecta, N.gibberifrons and chaenocephalus aceratus by Anderson (1982) reveal distinct electromorph frequencies at 10 enzyme loci for the 6 enzymes studied, in each of the four species. Dendogram of genetic distances provide supporting evidence for the classification of N.gibberifrons under a separate subgenus of Notothenia based on conventional morphological consideration (Anderson & Hureau, 1979).

The capability of managing a fishery on the basis of its subunit structure of the population is an objective that has generally eluded the biologists until recently because of the differences involved in defining the population. Conventional tagging, marking and morphological traits (Anas, Murai 1969) and relative mineral composition (Calaprice, 1971) have provided useful information, but are limited in that to defining a population on a genetic basis. Until recently the population structure of species was studied predominantly using quantitative morphological traits; unequivocal representation of population structure was obtained in many cases. With success in breeding in fishes and carrying out studies in inheritence of morphological characteristics, this problem was solved to a certain extent (eg., in eelpout zoarcea

viviparus by Smith, 1921). In carps it was simplified owing to the ease of breeding and controlled genetic experiments (Moav, 1976; Kirpichinikov, 1971 & 1973). However technical difficulties in breeding in the cod, herring and other species made analysis of population structure through these methods an insoluble tasks and studies pursued with such species revealed dubious results (See, Kirpichinikov, 1981). The use of techniques of biochemical genetics has led to a new and fruitful stage in fish population studies (Kirpichinikov, 1981). Wishard, Utter, Gunderson (1980) studied stock relationships of five commercially important rockfish (genus sebastes) species using biochemical genetic information of sixteen enzymes at twenty one loci developed through electrophoresis and determined eight stocks in total located in different places on the west coast of Canada and California. These result suggest management of the sub-population of these as separate stocks.

Electrophoretic investigation of the geographical distribution of variants is potentially a very powerful method for the analysis of population structure as has been proved in salmonids (Utter et al 1978).

Estimates of allelic frequencies at thirty loci in fourteen anadromous rainbow trout population of north west pacific revealed considerable genetic heterogenetty among the loci and indicated relationship previously not known. The total population was grouped into two taxonomic units which facili-

tated rational management of the fisheries there. (Allendorf, 1975, See Allendorf & Utter, 1979). Based on biochemical genetics variants in 3 species of Onchorynchus sp., Chinook salmon (O. twawytscha), Sockeye salmon (O. nerka), coho salmon (O. kisutch) and in rainbow trout (S. gairdeneri) of N.E. America their population structure was characterised (Utter et al 1973a). A common consequence of inbreeding in most cultured organisms including fishes is decreased viability and retardation of growth. Harmful consequences of inbreeding during fish reproduction have been noted by several authors (Kincaid, 1976b& 1976a; Kosswig, 1973; Allendorf & Utter, 1979); Kincaid, 1976) observed a slowing down process of 5 to 10% in growth rate upon tight inbreeding in rainbow trout. Inbreeding in carps has seen to cause a decrease in heterozygosity resulting in retardation of growth by 10 to 20% accompanied by decreased viability with increase in number of malformations (Moav & Wohlfarth 1968; Wohlfarth & Moav 1971). Biochemical genetic studies assumes importance in this context too, since through such studies, loss in heterogenity can be quantified.

Allendorf & Phelps (1980) have detected significant reduction in genetic variation in a hatchery stock of west slope cut throat salmon (S. clarki) in comparison with wild stock from which it was derived 14 years ago. Their conclusion were based on electrophoretic study at 35 loci of 17 enzymes. Their studies revealed that there is a (i) 57% reduction in proportion of polymorphic loci, (ii) 29% reduction in average number of allele per locus, (iii) 21% reduction

in average heterozygosity per individual: in the hatchery stock compared to wild. This was attributed to limited numbers of founders of hatchery stock and effect of genetic drift in the hatchery stock maintenance. Electrophoretic studies based on polymorphic loci in wild attantic salmon and in artificially reared fry, the progeny of 5 generations from the same wild stock population rendered reduction in proportion of polymorphic loci; reduction in general heterozygosity in the latter. (Cross and King, 1983). Vuorienen (1984) observed a loss of genetic variation in brown trout (S. trutta) hatchery stock founded 16 years ago in comparison to present wild stock. 2 enzyme loci out of the 7 originally polymorphic loci were found to be monomorphic. Electrophoretic studies reveal that loss of genetic variation due to inbreeding in hatchery stocks is particularly so in case of species with high fecundities like salmond fishes. (Ryman and Stahl, 1980).

Studies on the black sea-bream, Acanthopargrus schlegelli for determining genetic change in 1st and 2nd generation of its hatchery stock revealed no difference in heterozygosity between the 1st and 2nd generation suggesting inbreeding has little effect on 1st and 2nd generation (Tanguichi et al, 1983). However reduction in genetic variation from natural population and for 1st generation, of hatchery stock was observed, and a proposal to increase the number of contributing parents in the programme to propogate hatchery stock of the species to avoid influence of inbreeding was made. (Tanguichi et al, 1983).

Although inbreeding in general in harmful, it can be extremely useful in fish selection. By inbreeding stabilization of selective trait by increased homozygosity and augumented expression of several of them can be attained (See Kirpichinikov, 1981). The significance of gynogenesis by which this highly homozygous inbred lines could be produced and used to develop heterotic hybrids is well known. Biochemical genetic studies could provide data to assess the efficiency of such induced diploid gynogenesis. Genetic analysis of enzyme polymorphism in induced gymogenses in the plaice P.platessa demonstrates that the duplication of chromosome sets is caused by the suppression of formation of 2nd polar body at egg activation (Purdom et al, 1976) Recombination between loci and centromeres then leads to heterozygotes in breeds of diploid gynogenetic offsprings. By biochemical genetic studies the frequency of heterozygosity can be determined. This in turn gives the extent of such recombination, thus assessing the efficiency of induced or spontaneous diploid gynogenesis. The coefficients of inbreeding as determined by biochemical genetic studies in both induced and spontaneous gynogenetic diploids in the plaice (Pleuronetes platessa) are very close. Therefore in a simple gene of diploid gynogenesis there is scope for production of inbreed lines and lines and useful in eliminating several generations, of sibmatting required in inbreeding (Thompson et al, 1981).

Hybridization has been used in producing a wide variety of new genetic combinations. Increased production being realized in extensive and intensive fish farming with these hybrids

in well known (Bakos, 1979; Wohlfarth & Moav, 1972). Interspecific hybridization resulting in new kinds of social and feeding behaviour and better adaptation to environmental extremes in natural and controlled systems is well established in Salmonids (Chevaussus, 1979) in centrachids (Childers 1971) and in cyprinids (Wohlfarth et al, 1964; Moav & Wohlfarth 1966)

Biochemical genetic studies could be significant if applied to interspecies hybrids. To quote Utter, Hodgins and Allendorf (1974): "Some of the advantages offered by biochemical genetic methods for studies of intraspecific variation can be extended through studies of species hybrids because of the greater amount of genetic variation that exists between any two species than that exist with either of them". Suspected individual salmonids to be hybrids were determined electrophoretically whether or not they were indeed hybrids (Utter et al, 1973). Five species of salmonids of French population with the fry from specific crosses between them were electrophoretically analysed and hybrids, by PGI enzyme and anodal muscle proteins, were identified (Guyomard, 1978).

The possibility of natural hybridization between Etheostoma spectrabile and E. caeruleum at two Ohio location were investigated with starch-gel electrophoresis. No biochemical evidence for hybridization between the species was found (McLeod et al, 1980). By biochemical genetic studies identification of parent species in naturally occurring hybrids has been ascertained (Aspinwall & Tsuyuki, 1968) and in species

of genus Poeciliopsis (See Kirpichinikov, 1981). Electrophoretic study on the hybrid question of allelism and subunit composition of protein that are monomorphic in individual species but differing among them have been answered (Utter et al, 1973). There is also scope with these studies to identify the exotic portion of genome in hybrids which have been backcrossed and even intercrossed, as has been observed in salmonids (Utter et al, 1978).

That triploids may have higher growth rates (Purdom, 1976) is known. They may be preferred for other reasons too. Triploid F1 hybrid of grass carp are sterile and preferred over the diploid F1 hybrid which apart from providing same advantages have adverse affects on aquatic ecosystem. It is necessary to genetically analyse by biochemical methods the relative amounts of diploids and triploids in each hybrid programme (Magee & Philips, 1982). Electrophoretic procedures are known to have distinct advantage for investigation, of the genetic composition of hybrids wherein it could also determine ploidy and quantitatively determine parental allele dosage in individual hybrids. Biochemical genetic analysis on grass carp and big-head carp F1 hybrid and the parental species provide evidence to question the genetic status of the two parental species and shows occurrence of 100% triploidy in two instances and 44% in another hybrid production (Magee & Phillip 1982).

In fishes as in other groups of animals very little is

presently known concerning the extent to which genotype environment interaction of biochemical genetic variation can account for the amount of variation that has been described.

The potential usefulness of biochemical polymorphisms observed in most organisms thought to have direct bearing on natural selection and the environment could be extended considerably with a better understanding of the degree to which allele formation of different protein interact with components of the environment (See Utter, Hodgins, Allendorf, 1973; Mitton & Kohen, 1974) observed in Fundulus heteroclitus that significant differences in allelic frequencies and zygotic proportions of 12 polymorphic loci obtained electrophoretically, were associated with differences in environment. Isoenzyme studies in barnacles subjected to different temperatures naturally, show correlation between thermal and isoenzymic variation suggesting certain isoenzymes in barnacles are better adapted to higher temperatures by multiple variant strategy of thermal adaptation in which different variants function optimally at different temperatures (Nevo, Shimony & Libni, 1977) utilizing techniques of starch gel electrophoresis, levels of genetic variability in deep-sea teleosts of the Genus Sebastolobus was studied by Siebnaller (1978) to be lower than that in most fish. This was attributed to the physically stable and seasonless deep environment.

There are various causes of enzyme multiplicity and they may be divided into two categories namely, genetic causes

and post-translation causes. In turn there are two types of genetic multiplicity: firstly, multiple alleles at a single genetic locus and secondly, multiple genetic loci. It has been generally recognized that the Ldh isoenzymes detected in salmonid fishes are products of multiple genetic loci and some of them are derived from multiple alleles at a particular locus. (Utter, Allendorf & Hodgins, 1973). It is understood that multiplicity produced by multiple allelle is limited as two different alleles per diploid locus is the maximum possible genetic variation. However from one individual to another within the same species itself there may be considerable variation due to multiple alleles at various loci in the gene pool of the species. On the other hand multiple genetic loci in the absence of multiple alleles cannot account for differences between members of the same species as the same loci will be present in all members. But multiple genetic loci permit differences in isoenzyme profile both from one tissue to another and from one development stage to another even within the same tissue.

Enzymes may be subjected to post-synthetic alterations including addition of carbohydrate, limited proteolysis and the covalent modifications of aminoacid side chains - for eg., the aldolase in vertebrate skeletal tissues is encoded by a single locus only but one of the subunits A is subjected to post translation effect of deamination of an aspartic acid at the ?CO terminal to give A' which is detected electrophoretically

as an isoenzyme (after Rider & Taylor, 1980).

Apparent enzyme multiplicity may be due to artefacts also resulting from laboratory manipulations of cell and cell extracts. Liberation of proteolytic enzymes on cell disruptions and other effects of such unphysiological nature of enzyme assay could reveal artefacts but determined as isoenzymes.

In developmental process - a programme of selective gene expression operating on a constant pool of genetic information producing a complex organism from a single fertilized cell through tissue differentiation, each with a specific physical and metabolic characteristic, the latter facilitated by tissue specific isoenzyme patterns in the adult, essential to the diverse and integrated function of that adult - there is an obvious intricate series of changes which must occur.

Lactate dehydrogenase has proven to be an excellent gene marker for differential gene action during development (Markert, 1962; Cohn et al, 1962) and particularly so because of three distinct and homologous Ldh genes whose regulation is tissue specific (Markert & Faulhaber, 1965).

The appearance of one of the Ldh locus, the Ldh-E_4 during development occurs at time of structural and functional differentiation of retina and it seems to be coupled with differentiation of retina (Whitt, 1968).

Protein polymorphism among fishes has been extensively reported in the last two decades since improved techniques of

electrophoresis were developed in the mid-fiftees (Smithes, 1955) and subsequent to isoenzymes detected by Hunter and Markert (1957).

Proteins when composed of monomeric units give rise to a single band after electrophoresis in the case of homozygotes. Its isoenzyme with differential migration also appear as a single band but at different location in that homozygote. These two isoenzymes are codominantly expressed in the heterozygote with lesser intensities. Johnson et al, (1971) electrophoretically analysed PGM polymorphism in pacific ocean perch and detected three phenotypes; two (homozygotes) possessed one band either A or B at different positions and the third showing codominant expression of both of A & B (heterozygote).

Dimeric proteins reveal three bands in a heterozygote. Johnson, Utter, Hodgins (1972) observed that Tetrazoliumoxidase in the fishes of family scorpaenidae were expressed as three bands in the heterozygote and as a single band in the homozygotes. Proteins are rarely trimeric in nature but when so four bands are seen in a heterozygote. Tetrameric proteins are expressed as five bands in case of a heterozygote with only one band in case of the homozygotes; again if one of the genes is in heterozygous state upto 15 isoenzymes may be found in heterozygotes and 5 in the homozygotes. Lactate dehydrogenase system analysed electrophoretically in many fishes show this type of expression (Williscroft et al, 1970; Allendorf et al 1980; Shaklee et al, 1981; Magee et al, 1982; Beck et al, 1983).

Many systems have been formed based on the mobility or frequency of allelic variants detected by electrophoresis. Letters, numbers, and different apostrophes are used as symbols to describe these variants. Different workers have arbitarily used one or another system of nomenclature leading to confusion.

With the need for unambiguity in the nomenclature thereby to have an effective communication, a uniform system of nomenclature was suggested by Allendorf and Utter in 1979. To quote: "An abbreviation is chosen to designate each protein, when in italics these same abbreviation represent the loci coding for these proteins. In the case of multiple forms of the same enzyme a hyphenated numeral is included; the form with the least anodal migration is designated as one and the next two and so on. Allelic variants are designated according to the relative mobility. One allele (generally the most common) is arbitrarily designated as 100. This unit distance represents the migration distance of the isoenzyme coded for by this allele. Other alleles are then assigned a numerical value representing their unit distance. Thus an allele of the most cathodal lactate dehydrogenase locus coding an enzyme migrating one half of the distance as the common allele would be Ldh-1(50).

In the present study this system of nomenclature is adopted to designate the allelic variants.

Various electrophoretic methods for study of protein variations have been described. Starch gel electrophoresis

(Smithes, 1955) enhanced by the application of biochemical staining methods (Hunter & Markert, 1957) have simplified the study of protein variation. The polyacrylamide disc-gel electrophoresis method by Davis (1964) provides an alternate electrophoretic technique.

Subsequent workers on biochemical genetics have modified and described methods for similar studies. Electrophoretic methodology described by Utter, Hodgins and Allendorf, 1974 has been extensively used for studies in salmonids. Starch gel electrophoretic methods adopted for cichlids were given by Kornfield and Koehn (1975). Micro-starch gel electrophoresis has been described by Tsuyuki et al 1966.

Histochemical techniques of Siciliano & Shaw (1976) are very useful. Works of Ridgway et al (1970); Clayton et al (1972) describes specific buffer systems. Elaborate details of electrophoretic methods have been reviewed by Work & Work (1969); Brewer (1970) and Smith (1976), and Redfield and Salini (1980)

MATERIALS AND METHODS

Preliminary investigation revealed that standardisation of the certain methods in methodology was required prior to carrying out an experimental variable. Throughout the study the constant variable were the test animals, the basic polyacrylamide method.

The variables that had to be standardized for protein were -

1.1 Extraction

1.2 Amount of Tissue

1.3 Staining and Destaining procedures

1.4 Polyacrilamide Gel concentration

The variables that had to be standardized for isoenzyme studies were -

2.1 Extraction

2.2 Buffer system

2.3 Staining procedures

The experimental variables were -

3.1 Size classes

3.2 Tissues

3.3 Population

TEST ANIMAL : (Experimental subject)

Liza parsia, a euryhaline fish, called goldeneye-spot

mullet ('Kanambu' by the local fishermen of Kerala) is commonly found in the Cochin estuary and constitutes a thriving fishery in the estuaries and backwaters of India (Jhingran, 1982).

It grows to sizes of 400 mm. The minimum size at maturity are 120 mm. in males and 129 mm. in the case of females (Kurup & Samuel, 1983). Its fecudity varies from 2 to 6 lakhs in case of those found in Bengal (Sarojini, 1957) and 64,000 to 390,000 in the population found in the Cochin estuary (Kurup & Samuel, 1983).

The population of L.parsia in the Cochin estuary, on which the present study was made, spawn from Oct. to May, showing a peak spawning during December to April (Kurup and Samuel, 1983).

SAMPLING METHOD :

Live specimen were collected for the analysis. The adult specimens were purchased from the local chinese dipnet fishermen of the Vypeen barmouth region and juveniles were collected from the creeks of the same location. The larvae obtained by induced spawning of the adults of the same location were collected from the MPCL hatchery. All samples were transported in plastic transportation bags of 18 litres and 5 litres capacity. The bags were filled to two-thirds with the same sea water from where the samples were collected and the fish was transferred to the bags, then filled with oxygen

or air. Maximum time taken for transportation was only two hours. At the laboratory these specimens were transferred to perspex aquaria of 80 cm x 30 cm x 22 cm with fresh sea water of same salinity. The water was changed routinely and only live specimen were analysed. Preliminary investigation revealed that storage in deep freezer upto 1 week at 4°C gave equally good resolution for enzyme studies. However for protein separation, only live samples could be used since there was loss of bands on storing. The samples were homogenized in all types of glass homogenizers (after, Work & Work, 1966) with extraction buffer (Redfield & Salini, 1980) and centrifuged at 7,500 rpm for 20 minutes. All these procedures were carried out at lowered temperatures with the use of ice. All gels after electrophoresis were stored in 7% acetic solution, in test tubes.

ELECTROPHORETIC METHOD :

Polyacrylamide disc gel electroporesis method as described by Davis, 1964 was adopted. Stain and staining methods for enzymes were those described by Redfield and Salini (1980) for lactate dehydrogenase, sorbitol dehydrogenase, esterase and for acid and alkaline phosphatase, the methods by Brewer (1970) were used. Protein staining were from Davis, 1964.

STANDARDIZATION OF METHODOLOGY :

1. PROTEIN SEPARATION

1.1 EXTRACTION :

For extraction of protein three solvents were tested.

They were -

(a) double distilled water,

(b) grinding buffer pH 6.8,

(c) double distilled water with sucrose.

This was done to ascertain the better solvent giving maximum extraction without denaturation.

1.2 AMOUNT OF TISSUE :

Different amounts of Muscle tissue were tested to ascertain optimum quantity for good resolution without diffusion or trailing in gel and obtain perciptible intensities upon staining of separated bands. The different amounts were -

(a) 30 mg/ml. extracting solvent

(b) 60 mg/ml. extracting solvent

(c) 80 mg/ml. extracting solvent

1.3 STAINING AND DESTAINING PROCEDURES :

Various stains and destains, staining and destaining procedures, differing in time subjected to, were tried to determine the best method for obtaining stained bands with distinct margin and clear background.

The different stains tried were -

(a)	AMIDOBLACK	0.2% in double distilled water
(b)	Coomassie brilliant blue (Loba chemie)	0.2% in d.d.w.
(c)	- do -	0.25% in 5:5:1 ratio of methanol : H2O acetic
(d)	Kenacid blue (BDH)	0.25% in - do -
(e)	Kenacid blue (BDH)	0.2% in d.d.w.

The different destaining solutions tried were -

(a) Methanol : Water acetic (5:5:1) for stain 'c' & 'd'

(b) 7% acetic acid solution for other stain.

Staining times tried were - 4 Hrs, 10 Hrs, and 12 Hrs for stain 'b' and 'e'; ½ Hr for stain 'c' and 'd'; 1 Hr for stain 'a'. Destaining times tried were - 15 Mts for 'c' and 'd'; for other staining procedures, destaining was done till background was clean. The time varied from 3 to 4 Hrs.

1.4 POLYACRYLAMIDE GEL CONCENTRATION :

Various concentrations of acrylamide, a total of 12 combinations of acrylamide and bisacrylamide concentrations were analysed to obtain optimum'acrylamide - bisacrylamide ratio for best resolution.

The twelve combination of gels were -

Serial Number	Percentage of Acrylamide.	Percentage of bisacrylamide
1.	7	2
2.	7	2.5
3.	7	3.5
4.	7	4.5
5.	9	2
6.	9	2.5
7.	9	3.5
8.	9	4.5
9.	10	2.5
10.	10	3
11.	10	4
12.	10	5

2. ENZYME SEPARATION :

2.1 EXTRACTION :

Extraction of enzymes were tried using 3 solvents -

(a) double distilled water

(b) grinding buffer (Redfield & Salini, 1980) 1.21 g tris; 0.37 g (EDTA (Na_2) and 0.00153 NADP per litre of double distilled water: pH adjusted to 6.8 with HCl.

(c) 5 mM tris-HCl (pH 7.6) containing 1 mM M-2 Mercaptol ethanol for acid and alkaline phosphatases were used to avoid effect of EDTA in grinding buffer on Acph & Akph (Echetebu, 1980).

(d) Extraction of the enzyme LDH from the larvae of 2.5 mm size was done with 0.1 ml. equal volume of 30% dimethyl sulphanoxide in 0.05 M tris-HCl pH 7.8 and left overnight at 5°C (Anderson, 1982) No macceration was required.

2.2 BUFFERS :

The resolution of different enzymes were tested in six buffer systems using polyacrylamide gel electrophoresis, to determine the buffer giving best resolutions of the various enzymes analysed.

The buffer systems were -

Sl. No.	Buffer System	pH	GEL	Electrode	Author
1.	*Tris-Boric EDTA	9	Continuous 10.5g/l-Tris 0.39 g/l - EDTA (Na2)	Buffer 0.54g/l-Boric	Ayala et al 1972
2.	*Tris-Boric EDTA	8	25.44g/l Tris;9.276g/l Boric; 2.233g/l EDTA		c.f.Brewer, 1970
3.	Tris-Maleic EDTA	7.6	TRIS - 12·114 g/L; MALEIC(11.607g/L) EDTA - 3·72g/L $MgCl_2$ 2·03g/L		do-
4.	Tris-Citric		Discontinuous Buffer 1.09g/l-Tris 16.34g/l Tris 0.63g/l citric 9.45 g/l citric		Ayala et al 1972
5.	Tris-Glycine/Hcl	8.9	48ml/100ml -1N HCl 36.6g/100ml Tris	Tris-6g/l Glycine 28.8g/l	Davis, 1964
6.	Tris-citric LiOH-Boric	8.26	3.63g/lTris 1.05g/lCit. 10ml/elec-rode buffer	2.51g/l LLOH 18.54g/l Boric acid	Ferguson& Wallace, 1964

* Buffer 1 & 2 were used as Gel & electrode buffer in the ratios 1:100 as described in starch (Ayala et al 1972) and also 4:100 & 2:100 ratios were tried in the present study to compensate for dilution factor in gel preparation. Other buffers were used in ratios 100:40.

2.3 STAINING PROCEDURE :

The staining proceedures described by Redfield and Salini (1980) and Brewer (1980) were adopted. Staining buffer, tris-Hcl for lactate dehydrogenase was varied and tested with pH 7, 7.5, 8.5, 9.0.

3. EXPERIMENTAL VARIABLES :

3.1 SIZE CLASSES

The differences in protein expression were tested in -

	length (mm)	weight (gm)
(a) Juveniles	20 - 30	0.3 - 0.4
	30 - 40	0.4 - 1.5
(b) Adults	120 - 170	25 - 85

For enzyme the size classes tested were same as above including -

(c) Larvae	2.5 mm	1 mg

3.2 TISSUES :

The protein expression in different tissues were tested, only Muscle was tested in juvenile, and 4 tissues, viz., eye, liver, muscle and brain in adults.

The enzymes analysed were -

alcohol dehydrogenase (ADH, E.C. 1.1.1.1.), acid phosphatase (AP, E.C. 3.1.3.2.), alkaline phosphatase (AKPH, E.C. 3.1.1.2.), esterase (EST, E.C. 3.1.1.3.), lactate dehydrogenase (LDH, E.C. 1.1.1.27) and sorbitol dehydrogenase (SDH, E.C. 1.1.1.14.).

Enzymes were analysed in 6 tissues in adults viz., eye, muscle, heart, liver, kidney, brain and two tissues, muscle and eye in juveniles and the whole larvae.

3.3 POPULATION :

Juveniles and adults were collected from Vypeen barmouth area and nearby creeks. Of these 124 specimen were analysed. Larvae obtained from hartchery were derived from parents of the same population. These specimens were assumed to be taken from the same randomly mating population.

EXPRESSION OF PROTEIN BANDS :

The protein bands separated in the gel were given numbers starting from 1,2,3, ie. No.1 was given to that band closest to the origin and increasing numbers to bands towards the anodal region. These bands were also grouped into three systems, viz., I, II & III.

EXPRESSION OF ENZYME VARIATION :

The designation for gene loci and allelic variants encoding the enzymes surveyed are in accordance with the system

proposed by Allendorf and Utter (1979) Locus are designated with numbers for the dark distinct bands arising from origin towards anode. The common allele is designated as 100 and the others are designated in relation to this depending on their mobility.

SCORING ALLELIC FREQUENCY :

After obtaining the expression of a particular enzyme system and encating the loci, a number of specimen were analysed for the same enzyme system in the same tissue for any alleles if present at that loci and the frequency of that allele is estimated.

ESTIMATION OF GENETIC VARIATION :

AVERAGE HETEROZYGOSITY :

The average proportion of genome heterozygous per individual was estimated using the expression :

$$H = 1 - Pj^2 \qquad \text{Eq 1}$$

where PJ is the frequency of the jth allele at a locus (Selander & Johnson, 1973).

The frequency of the allele was estimated by the expression :

$$q\,B = \frac{2\Sigma(BB) + \Sigma(AB)}{2n} \qquad \text{Eq:2}$$

for (allele B) Ldh-2(125)

and

$$q\,A = \frac{2\Sigma(AA) + \Sigma(AB)}{2n}$$

for (allele A) Ldh-2(100)

Eq:3

HARDY-WEINBERG GENETIC MODEL (Stern, 1943)

The observed allelic frequency were compared with expected frequency obtained using an important genetic model, the Hardy-Weinberg Model -

$$P^2 (AA) + 2P_q (AB) + q^2 (BB) = 1 \qquad \text{Eq: 4}$$

where p and q are the frequencies of the alleles and AA, AB, and BB are the genotypes of individuals when two alleles of one and the same locus are codominant. Here AA and BB are homozygous and AB is heterozygous.

STATISTICAL TEST :

The differences between observed and expected values of allelic frequency were tested with the chi-square method.

χ^2 method

$$\left(\frac{\text{observed frequency} - \text{expected frequency}}{\text{Expected frequency}} \right)^2 \qquad \text{Eq: 5}$$

R E S U L T S

STANDARDIZATION OF METHODOLOGY

1. PROTEIN SEPARATION

1.1 EXTRACTION

Protein were resolved after extraction in the three solvents. The details of the separation are given in Table-1. From the Table it is seen that 9 bands are resolved in '1a' and their intensities are more when compared to '1b' which has only 4 bands. Bands show trailing in '1c'.

Extraction with just double-distilled water was found to be better and further protein extraction was done with double distilled water only.

1.2 AMOUNT OF TISSUE

Muscle protein was resolved using three different quantities of tissue viz. 30 mg/ml - 60 mg/ml and 80 mg/ml. The details of which are given in Table 2. It is seen from this that in '2a', protein is resolved into three systems - I, II and III which are faint and thinner in width. In '2c' the three protein systems are thick and intensely stained. In '2b' the three system with their component fraction are seen.

The differentiation of the system into different bands is not seen with 30 mg/ml tissue due to less protein and is not distinguishable with 80 mg/ml due to diffusion and merging of

TABLE 1

MUSCLE PROTEIN SEPARATION USING THREE DIFFERENT EXTRACTANTS

Serial Number	Extractant	No. of Bands	Intensities	Clarity of Bands & Gel
Ia	Double Distilled Water	9	xxxx	All Bands Clear & Distinct
Ib	Grinding Buffer	4	xx	Bands Lightly Stained
Ic	Double Distilled Water Sucrose	6	xxxx	Trailing in Gel Bands Appear Diffused with Merging of Closer Bands

xxxx INTENSE

xxx MEDIUM

xx LIGHT

x FAINT

TABLE 2

PROTEIN SEPARATION USING DIFFERENT QUANTITIES OF TISSUE FOR EXTRACTION TO OBTAIN THE OPTIMUM.

Serial Number	Amount of Tissue	No. of Systems	Bands & Expressed	Comment
		Number	System	
2a	30 mg/ml	3 Dark	I, II, III	Systems are Faintly Expressed; Bands thin
2b	60 mg/ml	6 Dark	Ia, Ib, IIa, IIb IIIa, IIIb	Systems I & II are in Intensities Expressing Components
2c	80 mg/ml	4 Dark	I, II, IIIa, IIIb	Systems I & II are Intensity Expressed & No Differentiation seen; Bands thick

bands. 60 mg/ of tissue / ml was the amount taken for all further protein analysis . The amount of protein in 0.04 ml. sample taken per tube was calculated to be 546/ug.

1.3 STAINING AND DESTAINING PROCEDURE

The results of the different staining and destaining procedures used are given in Table 3. The number of bands stained, their intensities and clarity of gel are tabulated.

The staining mixture giving optimum resolution are - 0.2% coomassie in d.d.w. and 0.25% Kenacid in methanol: Water : acetic (5:5:1)

The former was found useful and necessary in initial investigations as the light bands are distinct. However once the band pattern is familiarized with the latter was further used as the staining time involving ½ hour only was convenient and necessary for quicker confirmation of earlier results.

1.4 POLYACRYLAMIDE GEL CONCENTRATION

From the zymograms obtained (Fig.1, plate 1) in polyacrylamide gels of 12 varying combinations it was possible to arrive at the optimum acrylamide - bisacrylamide ratio needed for best resolution. The different bands in the system along with their intensities and positions obtained are given in Table 4.

From figure 1, plate 1 and Table 4 it is clearly evident that 10% acrylamide with 4% bisacrylamide gives the best resolution with maximum number of distinct protein bands

TABLE 3

MUSCLE PROTEIN SEPARATION TO DETERMINE APPROPRIATE STAINING METHOD & STAINING TIME

No.	Staining and Destaining Method	Duration of Staining (s) & Destaining (d)	No. of Bands	Intensities of Bands *	Band Gel Clarity **	Comment
1	Coomassie (LOBA) - Aqueous (b) 7%-Acetic (d)	S - 4 Hrs d - 2 Hrs	5	1,8,9,xxxx 4x, 7xx	Dark Bands Clear Gel - Sd	4 Lighter Bands not stained
2	Coomassie Aqueous (LOBA) - (s) 7%-Acetic (d)	S - 10 Hrs d - 2 Hrs	5	1,8,9 xxxx 4xx, 7xxx	Dark Bands Clear Gel - Sd	4 Lighter Bands not stained
3	Coomassie Aqueous (LOBA) - (s) 7%-Acetic (d)	s - 12 Hrs d - 2 Hrs	9	1,8,9 xxxx 2,3, 5,6xx 4xxx	All 9 Bands Clear Gel - Sd	9 Bands stained with
4	Kenacid - Aqueous (s) 7%-Acetic (d)	s - 5 Hrs d - 2 Hrs	5	1, xxxx	All Bands Clear Gel Diffused	Only 5 Bands Lighter Bands Clear - Gel Slightly Diffused

* XXXX INTENSE
XXX MEDIUM
XX LIGHT
X FAINT

** Sd - SLIGHTLY DIFFUSED

	Staining and Destaining Method	Duration of Staining (s) & Destaining (d)	No. of Bands	Intensities of Bands *	Band Gel Clarity **	Comment
5	Kenacid - Aqueous (s) 7%-Acetic (d)	s - 10 Hrs d - 2 Hrs	5	xxxx	All Bands Clear - Gel Diffused	Only 5 Bands Lighter Bands Clear, Gel Slightly Diffused
6	Kenacid - Methanol Water : Acetic (5:5:1) (s) 5:5:1 Mixture (d)	s - ½ Hr d - 20 Mts	8	1,4,7,8, 9xxxx 2,3,5xxx	2,3,5 Diffused Gel Clear	Lighter Bands Slightly Visible 6 seen
7	Kenacid Acetic Methanol : Water : Acetic 5:5:1 (s) 7%-Acetic (d)	s - ½ Hr d - 2 - 3 Hrs.	9	1,4,7,6, 9xxxx 2, 5 xxx 3 xx	2,5 Diffused Gel Slightly Diffused	7 Bands Clear 2 Slightly Diffused

s -- Staining

d -- Destaining

FIG. 1. MUSCLE PROTEIN SEPARATION USING DIFFERENT RATIOS OF ACRYLAMIDE AND BISACRYLAMIDE IN GEL

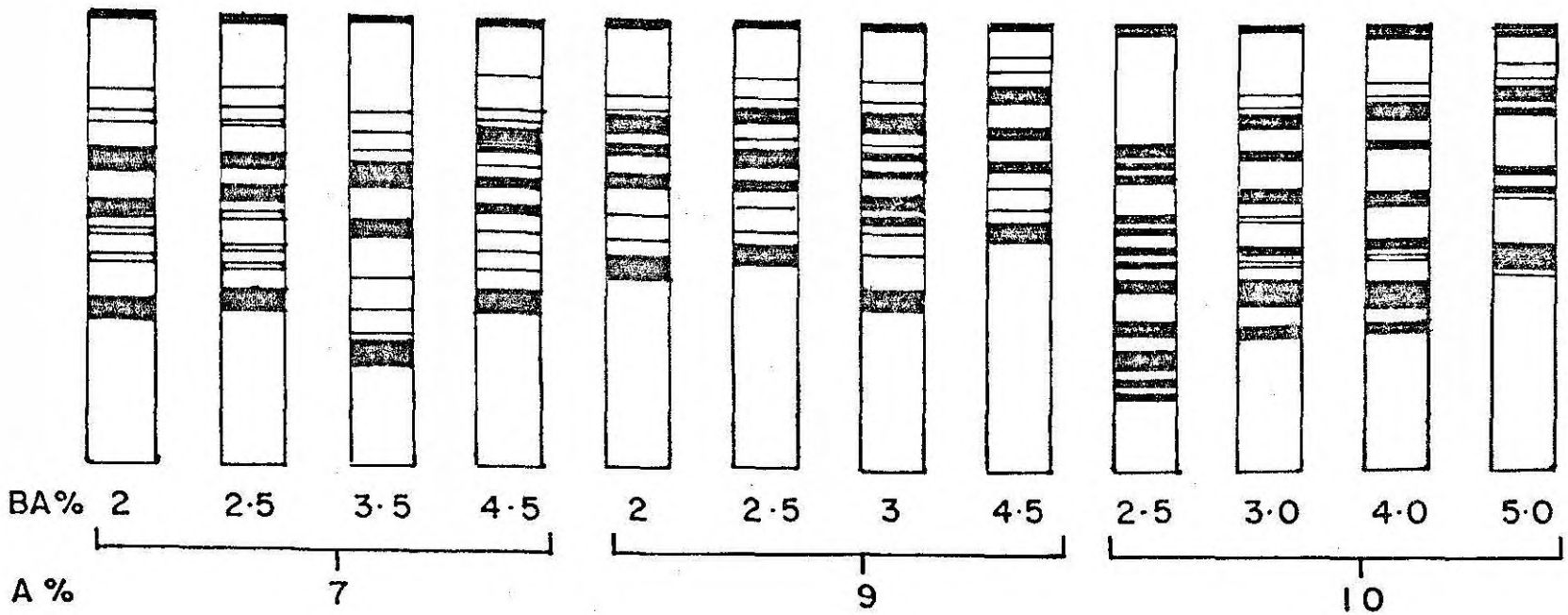
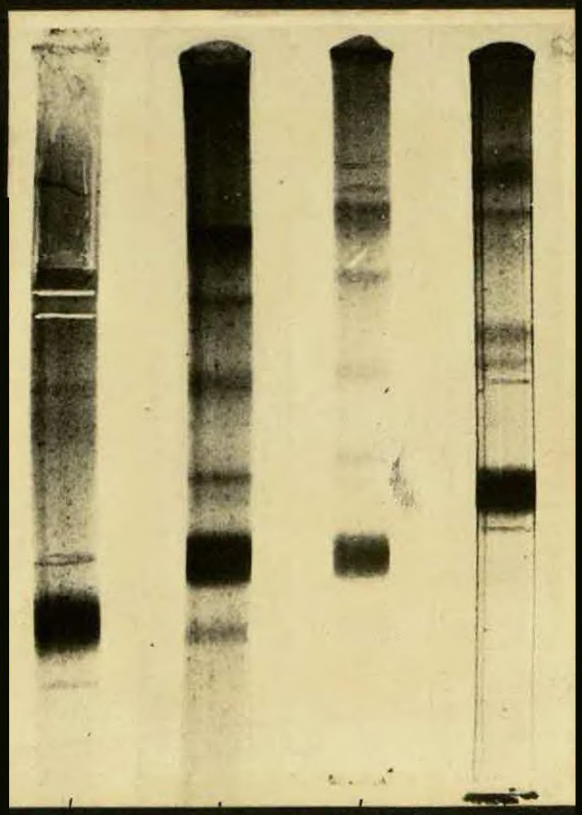


PLATE I

Muscle Protein separation with different Gel concentration:
A%-acrylamide; BA%-bisacrylamide



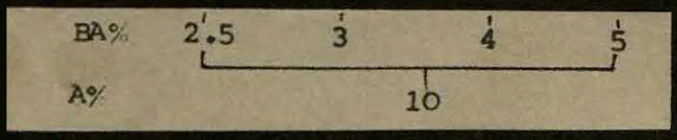


TABLE 4

PROTEIN SEPARATION USING DIFFERENT RATIOS OF ACRYLAMIDE AND BISACRYLAMIDE IN GEL FOR BEST RESOLUTION

Trial No.	Concentration of Acrylamide & Bisacrylamide %	No. of Bands & Systems Revealed		Position of Last Bands In System III	Dark Bands
		Bands	System		
1	7 - 2	9	I 1,2,3,4 II 5,6,7,8 III 9	III9 at 44 mm - 47 mm	I5 II6 III9
2	7 - 2.5	10	I 1,2,3,4,5 II 6,7,8,9 III 10	III10 at 42 mm - 45 mm	I5 II6 III10
3	7 - 3.5	10	I 1,2,3,4,5 II 6,7,8,9 III 10	III10 at 50 mm - 54 mm	I5 II6 III10
4	7 - 4.5	12	I 1,2,3,4,5 II 6,7,8,9,10,11 III 12	III12 at 42 mm 45 mm	I5 II8 III12
5	9 - 2	9	I 1,2,3,4 II 5,6,7,8 III 9	III9 at 37 mm - 40 mm	I4 II5,6 III9
6	9 - 2.5	9	I 1,2,3,4 II 5,6,7,8 III 9	III9 at 35 mm - 38 mm	I 4 II5,6 III9
7	9 - 3	12	I 1,2,3,4 II 5,6,7,8,9,10,11 III 12	III12 at 42 mm - 45 mm	I4 II6,7,8,9 III12
8	9 - 4.5	9	I 1,2,3,4 II 5,6,7,8 III 9	III9 at 31 mm - 34 mm	I4 II5,6 III9

Trial No.	Concentration of Acrylamide & Bisacrylamide %	No. of Bands & Systems Revealed		Position of Last Bands In System III	Dark Bands
		Bands	System		
9	10 - 2.5	13	I 1,2,3, 4,5 II 6,7,8, 9,10 III 11,12, 13	III11 at 51 mm 52 mm	I2,3,4 II5,6,7,8 9,10 III11,12,13
10	10 - 3.0	12	I 1,2,3, 4,5 II 6,7,8, 9,10 III 11,12	III11 at 40 mm 44 mm	I5 II6,7 III11,12
11	10 - 4.0	11	I 1,2,3, 4,5 II 6,7,8,9 III 10,11	III10 at 31 mm 45 mm	I5 II6,7 III10
12	10 - 5.0	11	I 1,2,3, 4,5 II 6,7,8, 9,10 III 11,12	III11 at 34 mm 38 mm	I5 II6,7 III10

separated in each system.

2. ENZYMES

2.1 EXTRACTION

Extraction of the enzyme Ldh was tested with double-distilled water and grinding buffer (pH 6.8). The zymogram (Fig.2) reveals the lactate dehydrogenase pattern of eye obtained after extraction in these two buffers respectively. It was seen that the intensity of band was very low with d.d.w. used as extraction. Therefore grinding buffer was used for all further extraction of lot as well as esterase. While investigating acid phosphatase and Akph another two extractants. Tris-Hcl (pH 7.6) and butanol were tested (~~see Section III 2-1~~). But as no proper resolution was obtained in either case with these two enzymes - no ascertainity can be made here.

2.2 BUFFERS

Six enzyme systems were analysed in eight buffer systems. Of these satisfied resolution could be obtained only for Ldh and Est.

Ldh : The comparative resolution of the Ldh system of the eye in the eight buffer systems tested, is tabulated (Table 5) wherein, the number of bands, their intensities, distinction, separation, migration rates, and running time are given. Zymograms of the resolved Ldh system in these different buffers are illustrated in Figure 3.

FIG. 2. RESOLUTION OF LDH AFTER EXTRACTION IN TWO SOLVENTS

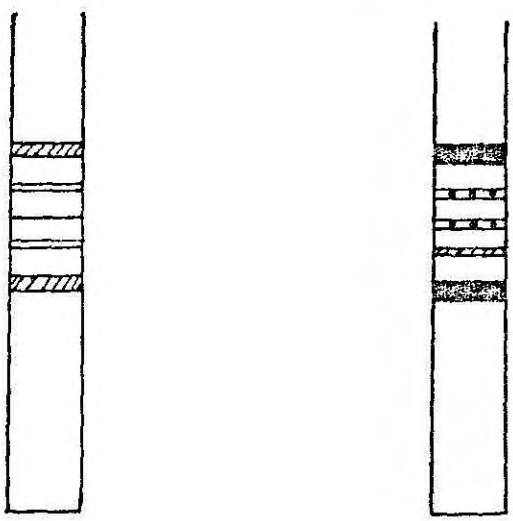


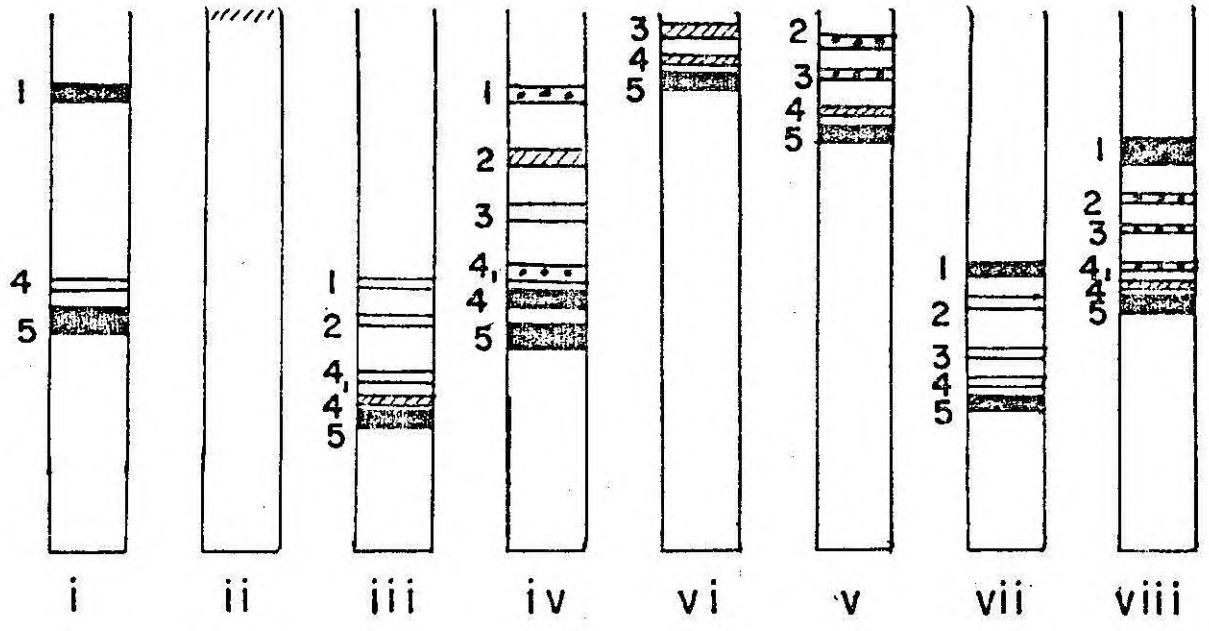
TABLE 5

LACTATE DEHYDROGENASE (Ldh) RESOLVED IN DIFFERENT BUFFER SYSTEMS

No	Buffer System	pH	No. of Bands	Migration	Time	Activity in Spacer Gel	Intensity of Bands	Separation	Distinction of Bands
1	Tris Boric EDTA 100:10 (E:G)	7	3	Moderate	40mts	Origin to First Band Diffused	1 xxxx	2nd, 3rd, 4th not resolved	Diffused Bands
2	Tris Boric EDTA 100:40 (E:G)	7	Nil	No Separation	40mts	Activity in spacer Gel	-	No Reslution	-
3	Tris Boric EDTA 100:20 (E:G)	8	5	Fast	40mts	Nil	1x, 3x, 5xxxx, 2x 4xx	Bands Too Close; However Complete	Clear
4	Tris Boric EDTA 100:40 (E:G)	8	6	Moderate	40mts	Nil	1xx, 4xxxx 2xxx, 5xxxx 3x	Good Reso-lution Bands Resolved with wide inter distance	Diffused Bands

No	Buffer System	pH	No. of Bands	Migration	Time	Activity in Spacer Gel	Intensity of Bands	Separation	Distinction of Bands
5	Tris Maleic EDTA	7.6	4	Slow	45mts	Moderate	2xx, 5xxxx 3xx, 4xxx	Good	Diffused Bands
6	Tris Citric	8.3	3	Slow	40mts	Moderate	3xxx, 4xxx 5xxxx	Good	Diffused
7	Tris Citric LiOH Boric	7	5	Fast	35mts	Nil	Light	Complete	Diffused
8	Tris Glycine Tris HCl	8.7 8.9	6	Moderate	90mts	Nil	1xxxx 2xx 3xxx 4xx 4xxx 5xxxx	Complete with sufficient inter distance	Clear Margin

FIG. 3. LACTATE DEHYDROGENASE SYSTEM OF THE EYE SEPARATED USING DIFFERENT BUFFER SYSTEMS



It is evident from Table 5 and Fig 3 that the Ldh system present in the eye is completely expressed in buffers (iii), (iv), (vii) & (viii). However diffused bands in buffer (iv) & (vii) and compressed bands in buffer (iii) are shortcomings to their efficiency. Tris-Hcl/Tris Glycine buffer (viii) not only resolved the Ldh system completely but also made it possible to obtain clear distinct bands without any diffusion.

This buffer was used for all further analysis of the system in other tissues, as well as for determination of ontological development of the enzyme; and for determinating the presence of any allele and its scoring.

Esterase : The comparative resolution of the esterase system from eight tissues viz., liver, muscle, heart, kidney, eye, spleen, brain, tried in eight different buffers are given in Table 6. It includes the extent of migration, intensity of bands, separation, and band distinction.

LIVER : The zymogram of the estrase system of liver separated using different buffer system is given in Fig.4. The expression of the whole system is observed with buffers (iv),(v), (vi),(vii) & (viii). With the other buffers, due to very fast or very slow migration the faster and slower components of the system are either lost or not separated, comparing buffers (iv),(vi),(vi),(viii) & (viii) the maximum number of hands are separated in buffers (v) & (viii) only. Tris-maleic

FIG. 4. ESTERASE IN LIVER RESOLVED USING DIFFERENT BUFFER SYSTEMS

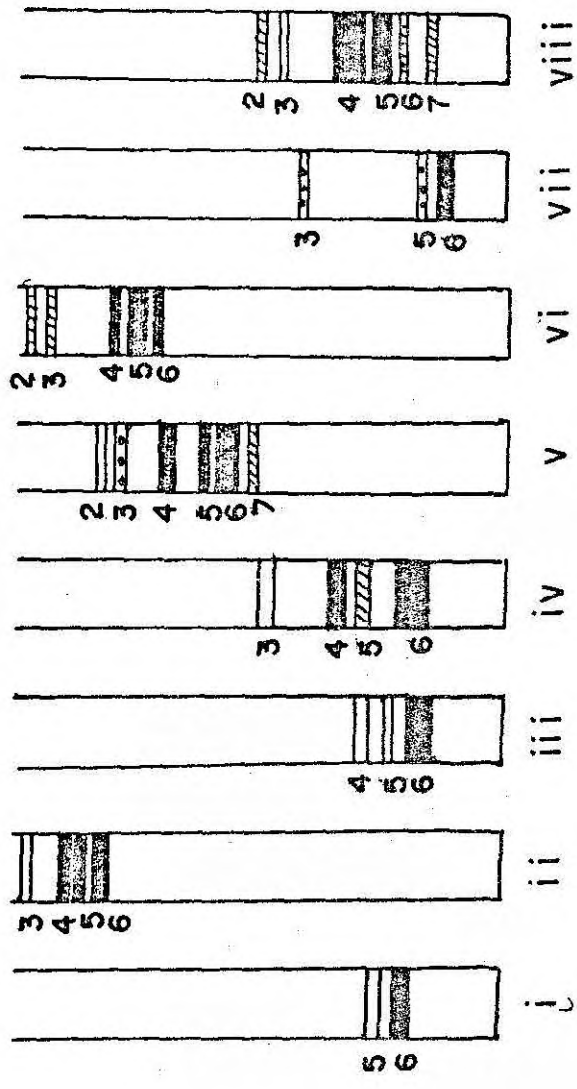


TABLE 6

ESTERASE SYSTEM RESOLUTION USING DIFFERENT BUFFER SYSTEMS

No	Buffer System	Number of Bands in different tissue	Migration	Time	Activity in space gel	Separation	Distinction of Bands	Intensity of Bands
i	Tris-Boric EDTA pH-7 100:10	L - 2 H - 3 M - 2 E - 2	Fast	40mts	Nil	Poor	Diffused	Faint activity in L,M,E bands seen after 1 hour In H-15 mts moderate activity
ii	Tris-Boric EDTA pH-7 100:40	L - 4 H - Nil S - 3 K - 3 M - 2 E - 3 B - 2	Slow	40mts	L - m H - i M - l E - M B - M K - I	Poor	Diffused	L - d M - d K - f E - f B - d
iii	Tris-Boric EDTA pH-8 100:10	L - 3 M - 3	Fast	40mts	L - m M - l	Good	Clear	L - l m - l
iv	Tris-Boric EDTA pH-8 100:40	L - 4 H - 2 M - 1 K - 3 S - 1	Fast	40mts	L - l H - l M - l K - l	Good	Clear	L - d H - d M - d K - d

No	Buffer System	Number of Bands in different tissue	Migration	Time	Activity in space Gel	Separation	Distinction of Bands	Intensity of Bands
V	Tris-Maleic EDTA pH-7.6	L-4, E-3 H-4, B-4 M-4, S-4 K-4	Medium	45mts	Slight activity in all tissue	Good	Clear	Dark for all tissues
Vi	Tris-Citric pH-8.31	L - 5 H - 2 M - 3	Slow	40mts	L - l H - I M - I	Poor	Diffuse	L - d H - d M - l
Vii	Tris-Citric LioH-Boric pH-7	L - 3 H - 1 M - 2	Fast	30mts	Nil	Good	Clear	L - l H - l M - l B - l
Viii	Tris-HCl Tris-Glycine	L - 6 M - 0 H - 0 B - 0	Fast	90mts	Nil	L - Good M, H, B - Nil	Clear	L - d

T I S S U E S

L - Liver
H - Heart
M - Muscle
E - Eye
K - Kidney
B - Brain

A C T I V I T Y

l - light
m - medium
i - intense

buffer (v) was found to be the best buffer as the enzyme was resolved well and migration was medium, whereas in buffer (viii) though resolution of system was complete, the migration was very fast, making it difficult to score allelic frequencies.

HEART : Esterase of the heart tissue was tested using these buffers except (iii). The maximum number of six bands where in 4 well separated bands were found only is buffer (v). No resolution was obtained in buffer (viii). See Fig 5 for the zymogram of the same.

MUSCLE : The zymograms (Fig.6) of muscle esterase resolved in all eight buffers reveal best resolution with medium migration in buffer (v) whereas in others either some of the isoenzyme were not resolved or were diffused.

KIDNEY : Only buffers (ii), (iv) & (v) were tested and expression of 5 isoenzymes in buffer (v) was obtained (See Fig.7).

EYE : Esterase in eye was attempted only in buffers (i),(ii) and (v). The number of bands resolved were 2, 3, 3 respectively. However in buffer (v) the separated bands were comparitively less diffuse and intensely stained (See Fig.8).

BRAIN : Zymograms of the brain esterase system resolved in buffers (ii),(v),(vii) (Fig.9) reveal 4 isoenzymes moderately stained obtained with buffer (v) (Tris-maleic).

SPLEEN : Expression of spleen esterase system was best in buffer (v) in comparison to two other buffers tried (ii) & (vi). (Fig.10).

FIG. 5. ESTERASE IN HEART RESOLVED USING DIFFERENT BUFFER SYSTEMS

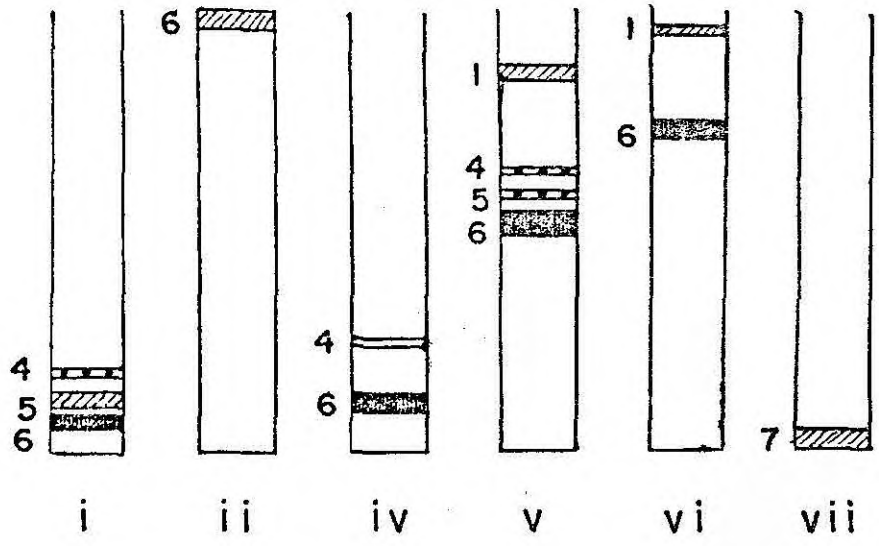
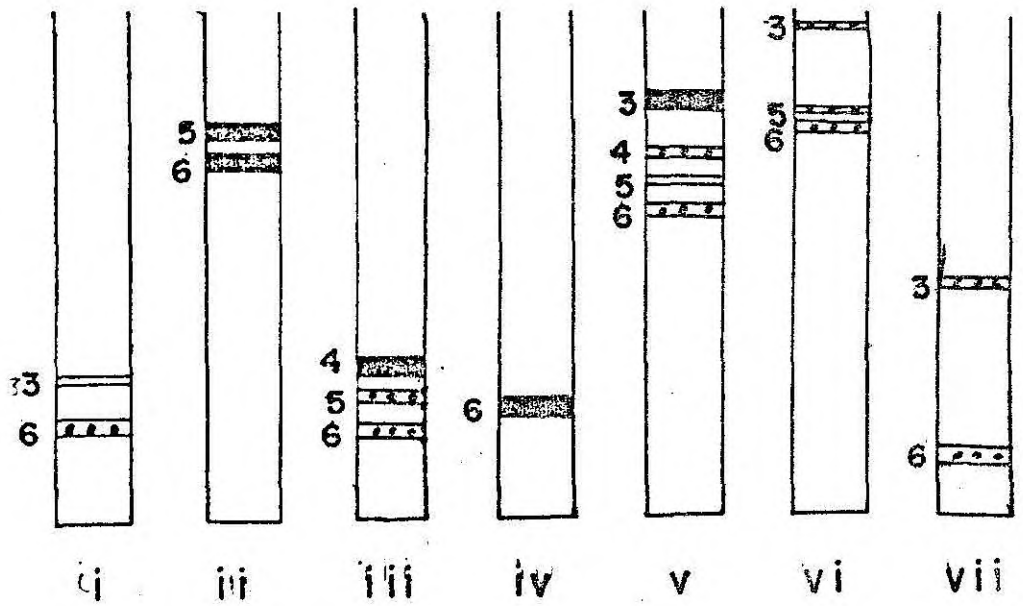


FIG. 6. ESTERASE IN MUSCLE RESOLVED USING DIFFERENT BUFFER SYSTEMS



ESTERASE IN KIDNEY & EYE RESOLVED USING DIFFERENT BUFFER SYSTEMS

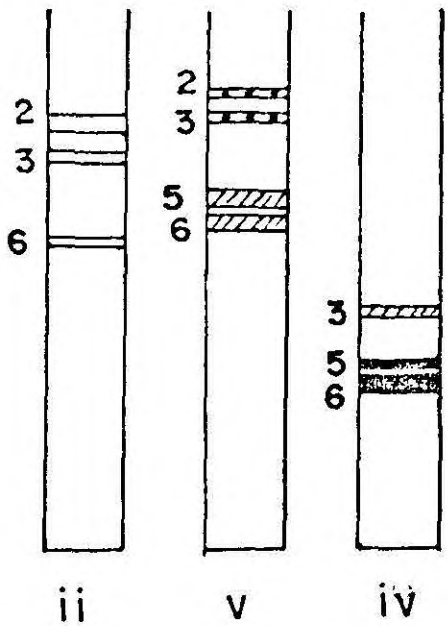


FIG. 7

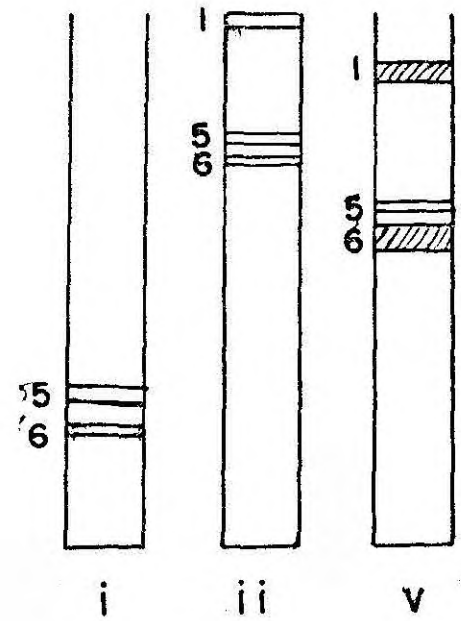


FIG. 8.

ESTERASE IN BRAIN & SPLEEN RESOLVED USING DIFFERENT BUFFER SYSTEMS

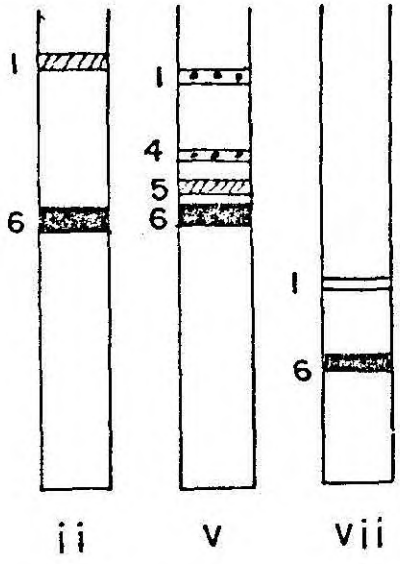


FIG. 9

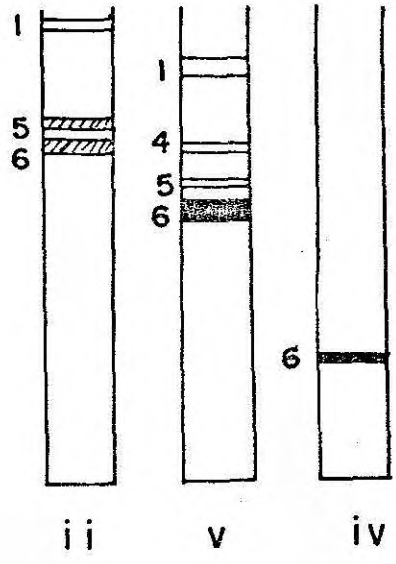


FIG. 10

From the resolution obtained for the esterase in the six tissues using eight different buffers - the buffer (v) Tris-Maleic EDTA (pH 7.6) was found to be the best. This buffer was further used for all isoenzyme analysis of the esterase system.

2.3 STAINING PROCEDURE

The zymograms of Ldh system of eye obtained using differential pH of tris-buffer during staining is given in Fig.11. Tris-buffer pH 7.5 was found to be optimum. The band intensity was less at pH 7 and faint expression of the 2nd locus, Ldh-2 with diffusion of bands was seen at pH above 7.5. Tris-Hcl buffer (pH 7.0) (Redfield & Salini 1980) was altered and this buffer at pH 7.5 was used for all staining purposes of Ldh analyses.

3. EXPRESSION OF MUSCLE PROTEIN IN JUVENILES AND ADULTS.

The muscle protein pattern of juveniles and adults are given in the zymogram (Fig.12). It is seen that there is no difference in protein pattern at the two life phases.

EXPRESSION OF PROTEIN IN DIFFERENT TISSUES

The protein pattern of different tissues is given in the zymogram (Fig.13). There are 17 bands in the eye, 18 bands present in the liver and 16 bands in the brain. Muscle has 11 bands; 5 bands in system I; 4 bands in System II, 2 band in System III.

FIG. 11. LDH SYSTEM OF EYE VISUALIZED WITH DIFFERENTIAL STAINING

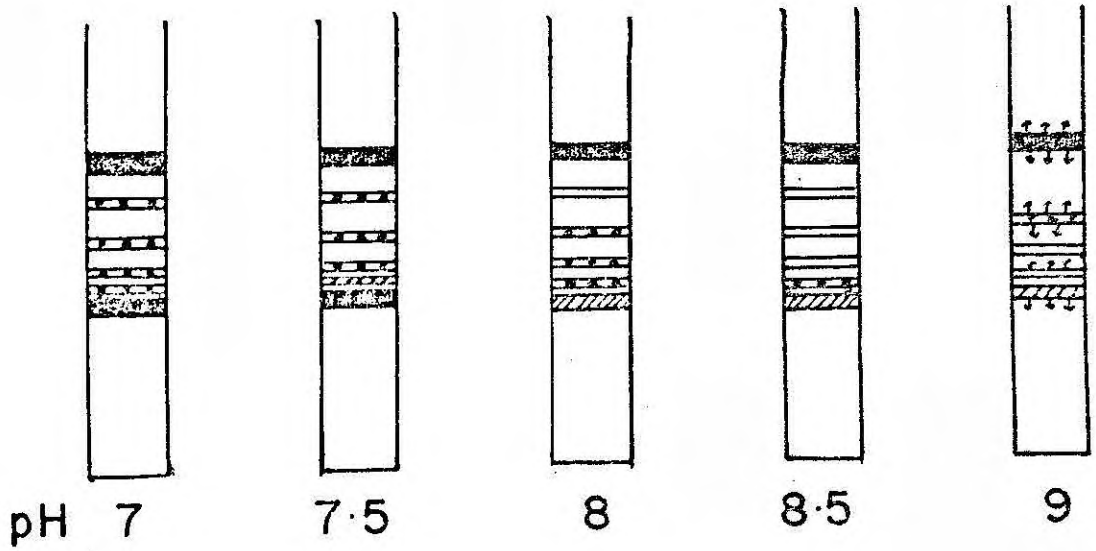
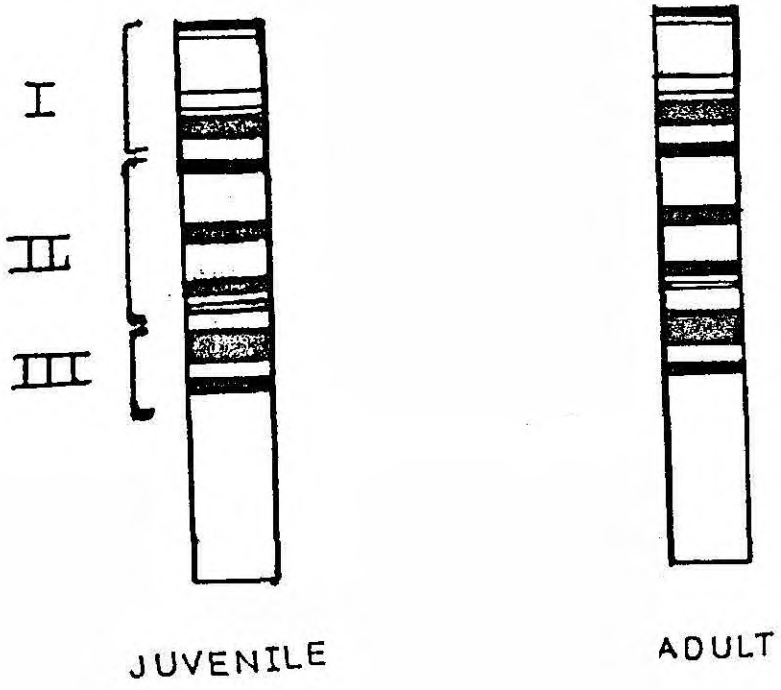


FIG 12 EXPRESSION OF MUSCLE PROTEIN IN JUVENILES & ADULTS



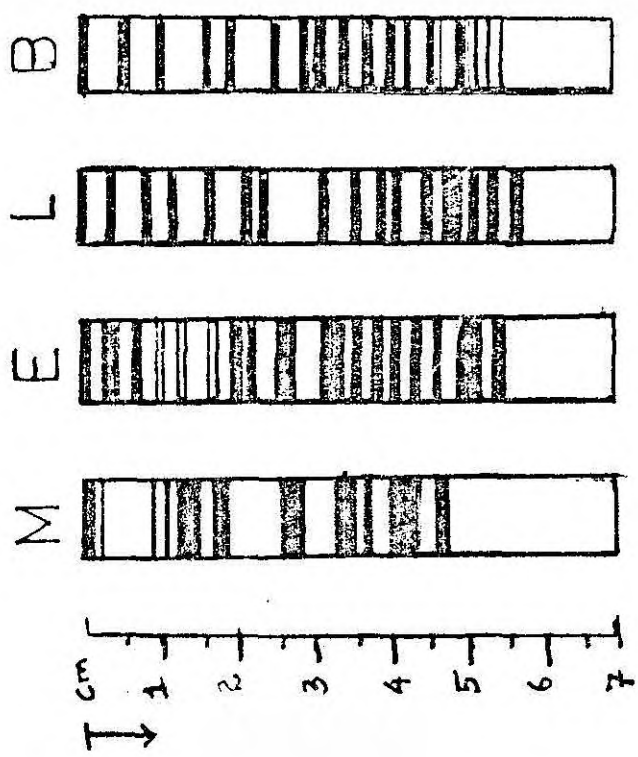


FIG. 13. EXPRESSION OF PROTEIN IN DIFFERENT TISSUES OF THE ADULT *LIZA PARSIA*

EXPRESSION OF LACTATE DEHYDROGENASE IN DIFFERENT TISSUES

The enzyme Ldh was extracted with the grinding buffer (pH 6.8) from six tissues viz., kidney, eye, liver, heart, muscle and brain, and resolved in Tris-Hcl: Tris-Glycine buffer. The details of the number of bands, of the Ldh system; band type; and their intensities in the different tissues are given in Table 7, Fig.14, Plate 2.

In the eye, two loci, Ldh-1 and Ldh-2 (A & B) were identified with a total of 5 isoenzyme (i, ii, iii, iv & v) bands (i) and (v) are dark being the tetrahomopolymers of A_4 & B_4 respectively. Bands (ii), (iii), (iv) are lighter due to hybridization of the products of the two loci to give the heteropolymers, A_3B_1, A_2B_2, A_1B_3.

All other tissues reveal the expression of the locus A_4 only with faint expression of the homopolymer B_4 in kidney, liver and heart.

In liver, the B_3A heteropolymer forming a third isoenzyme is also seen.

ONTOLOGICAL STUDY OF THE ENZYME LDH

The results of the electrophoretic analysis carried to determine whether any changes occur in the Ldh system during development are given in the zymogram (Fig.15, Plate 3). The Ldh system of the Muscle in the adult reveals full expression of locus A_4 and faint expression of locus B_4 with a heteropolymer A_3B_1. However the larvae of 2.4 mm reveal only the locus A_4.

TABLE 7

LDH EXPRESSION IN DIFFERENT TISSUES & VARYING BAND INTENSITIES

(RESOLVED IN TRIS-HCl/TRIS GLYCINE BUFFERS)

No.	Tissue	Band	1	2	3	4	5
1	Kidney	2	xxxx	-	-	-	x
2.	Eye	5	xxxx	xx	x	xxx	xxxx
3	Liver	3	xxxx	-	-	x	x
4	Heart	2	xxxx	-	-	-	x
5	Muscle	3	xxxx	-	x	-	x

FIG. 14. EXPRESSION OF THE LDH SYSTEM IN DIFFERENT TISSUES OF THE ADULT *LIZA PARSIA*

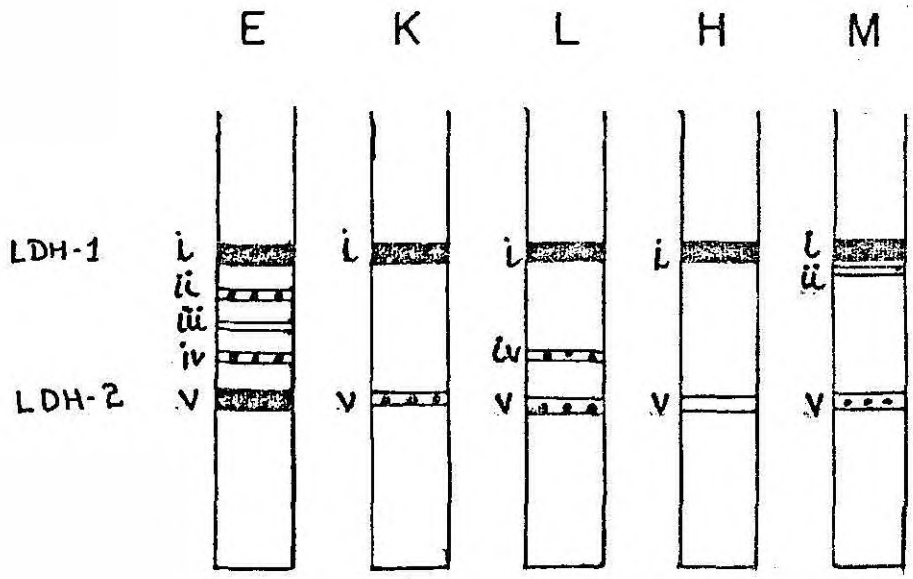
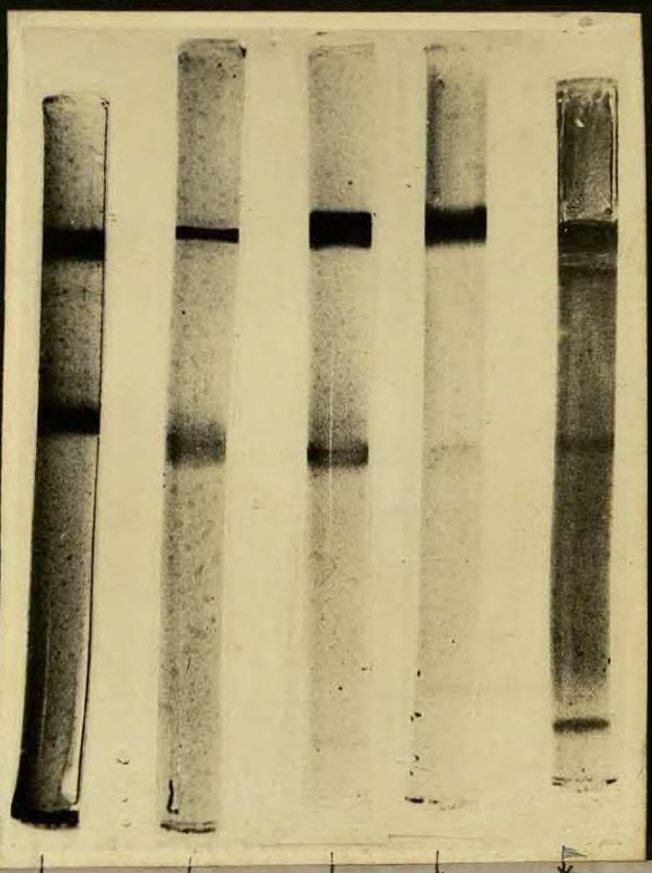


PLATE II



Expression of the LDH enzyme system in different tissues of the adult L. parsia.

FIG. 15. EXPRESSION OF MUSCLE LDH SYSTEM DURING DEVELOPMENT

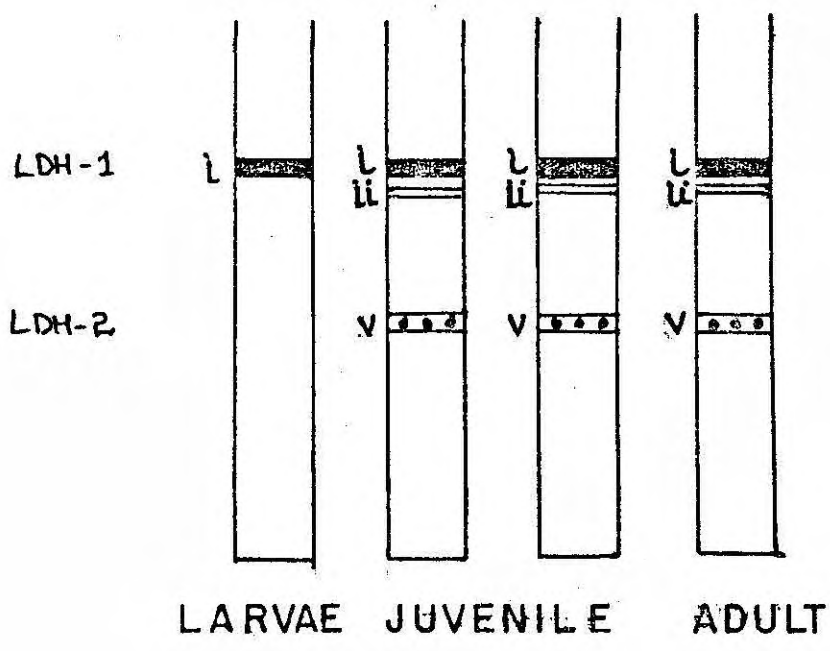
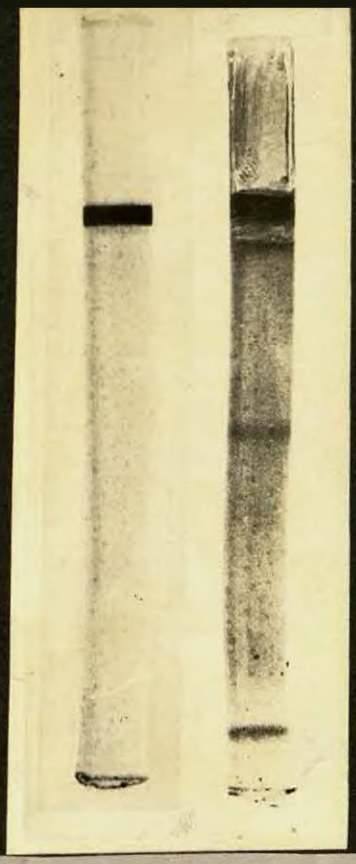


PLATE III



Expression of the Muscle LDH system during development:

The expression of Ldh system is expressed in juveniles of 24 mm itself.

From this it can be concluded that there is a change in Ldh system, during development. The inability to obtain larvae and juveniles of different stages between 2.4 mm and 24 mm impeded pinpointing the exact size at which the expression of locus Ldh-2 occurs.

EXPRESSION OF ESTERASE IN DIFFERENT TISSUES

Electrophoresis done with Tris.maleic buffer system to resolve and obtain the expression of the esterase isoenzyme system in six different tissues, viz., liver, kidney, heart, eye, muscle and brain are shown in zymogram (Fig.16); plate 3, The details of the number of bands expressed in different tissues and their intensities are tabulated in Table 8. The esterase bands were grouped into three systems according to their mobilities and intensities.

MEASURED GENETIC VARIATION :

A variety of measures can be used to express in a single statistic, the amount of genetic variation in a population. The overall incidence of heterozygosity was calculated after analysing 124 samples of Liza parsia, assumed to be collected from a random mating population, this being the most informative measure for a random mating population (See Kirpichinikov, 1980). From electrophoresis of the Ldh isoenzymes, the different genotypes occurring within the population and the frequency of

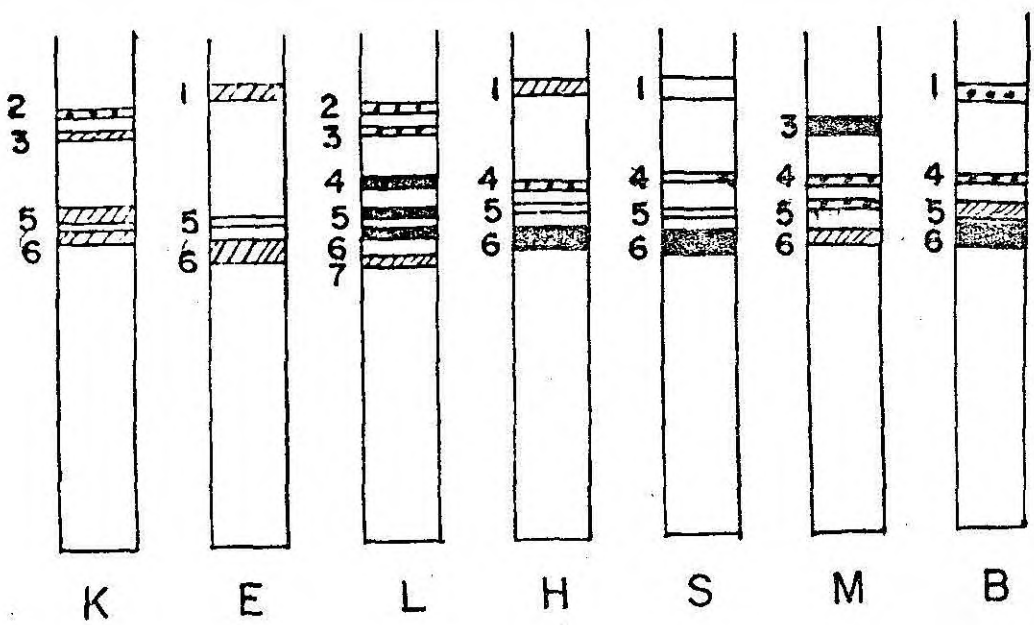
TABLE 8

ESTERASE (RESOLVED IN TRIS MALEIC BUFFER) ITS EXPRESSION IN DIFFERENT TISSUES AND BAND INTENSITIES

No.	Tissue	Band	1	2	3	4	5	6	7
1	Kidney	4	-	xx	xxx	-	xxx	xxx	-
2	Eye	3	xxx	-	-	-	x	xxx	-
3	Lever	6	-	xx	xx	xxxx	xxxx	xxxx	xxx
4	Heart	4	xxx	-	-	xx	x	xxxx	-
5	Spleen	4	x	-	-	x	x	xxxx	-
6	Muscle	4	-	-	xxxx	xx	xx	xxx	-
7	Brain	4	xx	-	-	xx	xxx	xxx	-

Note : xxxx - Intense
xxx - Medium
xx - Less
x - Faint
- - Not present

FIG. 16. EXPRESSION OF ESTERASE IN DIFFERENT TISSUES IN THE ADULT *LIZA PARSIA*



occurrence of those alleles were directly determined. In the Ldh isoenzyme system of the mullet Liza parsia two loci were identified in the eye. The slow migrating locus was termed Ldh-1, and the fast locus Ldh-2. Using the criteria that when the allelic frequency of the most common allele is less than 99% (< 0.99) that locus is polymorphic; it was found that the Ldh-2 was polymorphic and Ldh-1 monomorphic. In the polymorphic loci two alleles were observed, the most common being the slow one. Using the nomenclature of Allendorf and Utter, 1979; this allele was termed as Ldh-2(100) and the less common one as (PLATE IV; FIG 17) Ldh-2(125). The three genotypes observed were -

Genotype	Ldh-2(100/100) AA	Ldh-2(100/125) AB	Ldh-2(125/125) BB
Genotype Distribution	50	61	13
			n = 124

The observed allelic frequencies were tested for deviations from the expected values based on a simple genetic model, the Hardy-Weinberg model :

The observed allelic frequencies were determined from equations 2 and 3 as given in the section, Material and methods.

qA and qB values for the alleles Ldh-2(100) and Ldh-2 (125) are thus :

$$qA = 0.649$$

$$qB = 0.351$$

These observed allelic frequencies were substituted in the Hardy Weinberg Genetic Model (Eq.4) to obtain the expected genotypic frequency :

PLATE IV

Three Genotypes showing two alleles at the polymorphic loci, LDH-2:

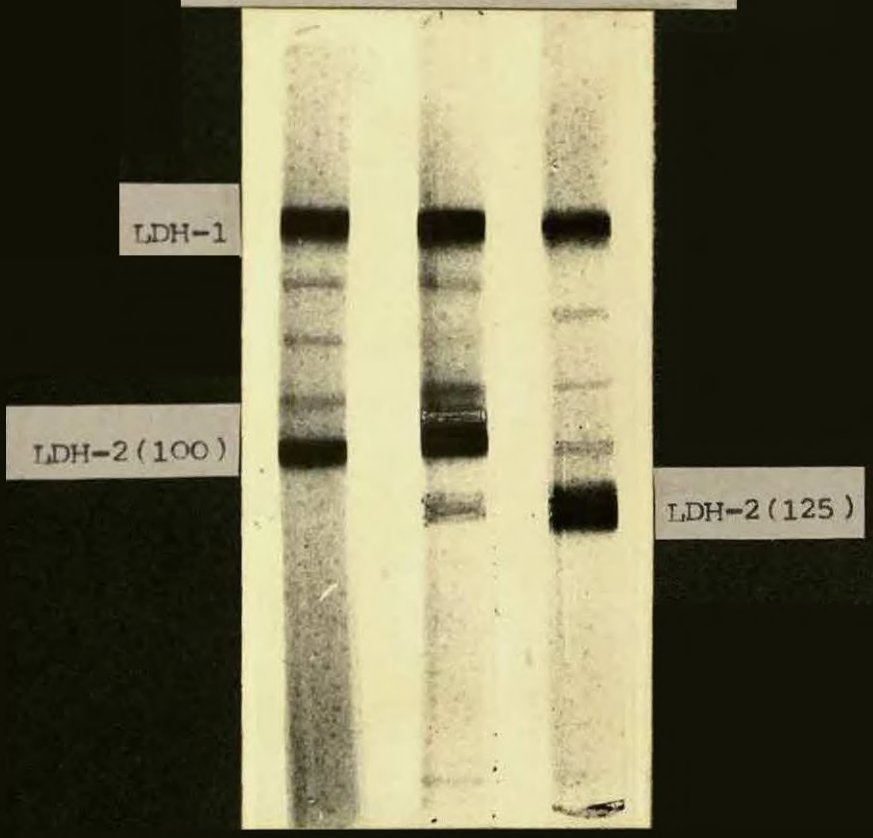
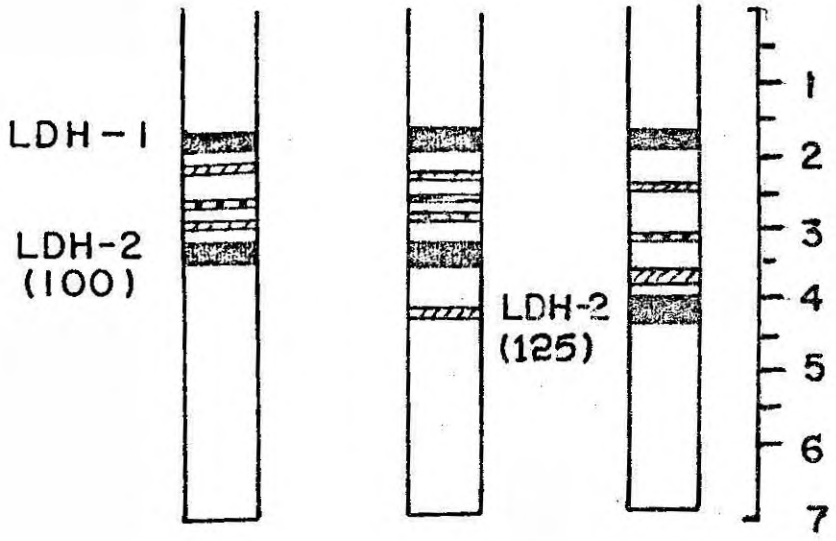


FIG. 17.

THREE GENOTYPES SHOWING TWO ALLELES AT THE POLYMORPHIC LOCI , LDH -2



AA = 0.421

AB = 0.456

BB = 0.123

The product of each of these expected genotypic frequencies with the number of samples (n = 124) analysed were calculated to give the expected genotype distribution with this n = 124. The value of the expected and observed values are shown below :

Genotypes	Expected Values	Observed values
AA	52.204	50
AB	56.544	61
BB	15.252	13

It is seen that the calculated values are very close to those actually determined. However to establish statistically whether the difference between observed and expected frequencies is not significant the chi-square test was used to test/the result. The chi-square test for these values, $\chi^2 = 0.7768$.

From chi-square tables of 2° of freedom this value is found to be less than 1 and the differences were established to be non-significant.

Heterozygosity :

The expected heterozygosity He , was then calculated from the tested allelic frequencies by the Eq:5, to be :

Expected (H_e)

Heterozygosity = 0.4556

The observed heterozygosity (H_o) was calculated from the genotypes observed :

No: of heterozygotes = 61

Total No: of samples = 124

Observed frequency of heterozygotes = $\frac{61}{124}$

Observed heterozygosity

Ho = 0.492

It is seen that there is not any significant difference between the observed and expected values of heterozygosity.

Observed heterozygosity is the average proportion of heterozygotes per locus, whereas expected heterozygosity is the average proportion of heterozygotes per locus predicted by the Hardy-Weinberg equilibrium.

D I S C U S S I O N

Electrophoretic methods for genetic analysis has been used successfully for fish by various workers (Utter, Hodgins and Allendorf, 1974; Kornfield & Koehn, 1975; Silciano & Shaw, 1976).

The polyacrylamide disc gel electrophoresis of Davis, 1964 was used by Vonwyl, 1979; Siva Prasad Rao, 1982) for lactate dehydrogenase separation. Buffer systems and staining techniques described by Silciano & Shaw, 1976; Ridgway et al, 1970; Clayton et al, 1972; Work & Work, 1966; Smith, 1976; and Brewer, 1970 have been used by several workers.

In the present study it was found that the following the procedures given by some of the above authors in studying the genetic variation of Liza parsia did not yield satisfactory results. Therefore certain modification were introduced and a combination of standard and modified procedures were studied.

Protein separation obtained after extraction in double-distilled water; grinding buffer (pH 6.8) and double distilled water with sucrose separately, reveal that double distilled water is the best extractant. Extracting protein in grinding buffer yielded fewer bands which are less intense. This may be due to the denaturation of proteins in the solution with pH values critical to its stability.

The enzyme lactate dehydrogenase was extracted in two solutions viz., double distilled water and grinding buffer (pH 6.8). The resolution of the enzyme using extract from the latter method was found to be better.

The amount of protein in the sample to be separated in polyacrylamide gel during electrophoresis is a critical factor (Davis, 1964). For protein separation in Liza parsia, which has an average of 22.75% total protein by wet weight (Elizabeth, per. comm., 1984); 60 mg/ml double distilled water gave the best resolution. The amount of protein in 0.04 ml of the sample to applied per tube was then calculated to be 546 /ug, and is the optimum concentration of protein sample for better clarity and resolution. Davis (1964) suggested not more than 200/ug to be used as sample in each tube. Work on protein separation in Scylla serrata ascertain the optimal concentration to be around 350-400/ug per tube for better clarity and resolution (c.f. Subhashini et al, 1981).

General proteins can be stained by various methods. These have been discussed by Work & Work (1969) and Smith(1976). Some of these methods were used and tried with certain modification. Commassie brilliant blue 0.20% in double-distilled water is easily prepared and used as a stain for proteins. Although the gels need to be stained for 12 Hrs, the bands obtained are distinct with a clear background after destaining in 7% acetic acid. This method was necessary for initial investigations of protein pattern. Kenacid (BDH) 0.25% in methanol:

water:acetic acid (5:5:1) solution stains for proteins in 20 minutes. The bands are perceptible after 10 minutes destaining in the same solution. This method was found to be convenient and necessary for quicker confirmation of earlier results.

Protein macromolecules vary in sizes and are separated best in optimum gel pore sizes. There is an interaction between bisacrylamide % and the total acrylamide used for the preparation of the gel that determines the minimum pore size. This is arrived at after redrawing the figure 1.1, pg 12 of Work & Work (1969). For most purposes 5% bisacrylamide gels are recommended by Work & Work (1969). However at 7% total acrylamide concentration 2.5% of bisacrylamide may be good (Davis, 1964). Experiments with serum proteins of male Scylla serrata with different gel concentrations of 5%, 6.3% and 7.7% show differential separation of distal and proximal protein fraction (Subhashini et al, 1981).

In the present study, with the results of 12 varying combinations of bisacrylamide and total acrylamide tested - 10% total acrylamide and 4% bisacrylamide was found to be the optimum ratio to provide pore sizes which resolve the protein into more fractions.

The protein pattern were obtained in muscle, liver, brain, and eye tissues. (Ref. Results). The pattern obtained in the muscle tissue could be divided into three systems depen-

ding on mobility and congregation of bands. System I is proximal to the origin with five bands. In the middle is the system II ~~ with four bands in each. The system III is distal to the origin, nearer to the anodal region and has 2 bands. There are 11 bands in total.

The muscle protein pattern of 5 species of grey mullets obtained by Herzberg & Pasteur (1975) reveal 9 bands in Liza provensalis; eight bands in Mugil cephalus; five bands each in L. aurata and L. saliens and nine bands in L. ramada. However, the authors divided the band pattern into four systems - A, B, C, D and moreover their results were obtained in 7.5% gel; not providing a basis for actual comparison. Eighteen bands are seen to be resolved in the liver tissue; sixteen in the brain and seventeen in the eye of L. parsia, in the present study.

Muscle protein pattern studied in juveniles of 24 mm - 30 mm size and in adults of 140 mm - 160 mm size collected from the same area revealed no differences (Ref. Results).

Studies on the juvenile and adults of 5 species of grey mullets by Herzberg and Pasteur (1975) showed differences in muscle pattern of juveniles and adults in each. All juveniles showed more intensities at their anodal most band or had an additional band in the anodal system. Results of other studies on ontological development of proteins are similar to the observation made here. No difference in muscle pattern were obtained between juveniles and adults of the rock fish, Sebastolobus alutus (Tsuyuki et al, 1968).

BUFFERS

The successful separation of isoenzymes depends on the control of certain variables. These include pH and temperature as well as the composition of the buffer, its ionic concentration and the characteristic of the enzyme. The buffer system should have a pH far away from the isoelectric point of the enzyme at which its electrical charge is neutral and gets precipitated. The pH of the buffer should also simultaneously stabilize the enzyme. The ionic strength also determines the mobility. The greater the ionic strength the slower the mobility of the charged molecules. However at reduced migration separation of the enzyme is always sharper.

The results of LDH and Esterase resolution tried in different buffers reveal that the choice of buffer could be very critical (refer Results).

The continuous buffer Tris-Maleic EDTA, pH 7.6 and discontinuous buffer Tris-HCl/Tris-Glycine, pH 8.9/8.6 were found to be the buffers for best resolution of Esterase and Ldh respectively.

Different buffer systems have been used by different workers for these enzymes. Table of buffers gives the details for comparison.

BUFFER - SYSTEMS USED BY OTHER WORKERS FOR ESTERASE AND LDH

S.No.	Buffer System	pH	Enzyme	Author(s)
1.	Tris-Citric Boric - NaOH (Poullik, 1957)	8.65/ 8.1	Est	Koehn, et al 1970
2.	Tris-Citric LiOH - Boric (Ridgway et al 1970)	8.6	Est, Ldh	Ridgway et al; Klar et al 1979
3.	Tris-Maleic - EDTA (Brewer, 1970)	7.6	Est	
4.	Tris-Citric LiOH - Boric (Ferguson & Wallace, 1961)	8.31/ 8.26	Est	* Thompson et al 1974 ** Mangaly & Jamieson, 1978
5.	Tris-HCl Tris-Glycine (Davis, 1964)	8.9 8.7	Ldh	Basasibwaki, 1975; Siva Prasad Rao et al
6.	Tris-Glycine (Gabriel 1971)	8.3	Est	Jen-Leih Wu et al, 1983
7.	Tris-Boric - EDTA	9	Ldh	Grant & Utter, 1980
8.	Tris-Citric	7	Ldh	Hodgins et al 1969

* the author has used the modified buffer by utter 1969

** these authors have used the same buffer, further modified from utter, 1969

ESTERASE ENZYME SYSTEM

Esterases is a term used to designate a few groups of enzymes varying in their function, with a wide range of substrate activity (Holmes & Whitt, 1970). This enzyme is a homopolymer, as a rule, and therefore isoenzymes are not considerable, except as observed in some fishes (Holmes & Whitt, 1970)

Esterase enzyme system was resolved in seven tissues viz., liver, muscle, kidney, heart, brain, spleen and eye using Tris-Maleic buffer (pH 7.6). Seven bands in total were identified. Four of these are expressed in kidney, heart, brain, muscle, and spleen. Eye has three bands and liver has six bands. These bands are grouped into 3 systems according to mobility, intensity and clarity of bands. Two bands are seen in the system I; in the II and III systems, three and one bands is seen respectively.

The tissues liver, heart and skeletal muscle analysed in the herring for esterase polymorphism show an expression of six to ten distinct bands grouped into 4 systems (Ridgway et al; 1970).

In the hake Merluccius merluccius three zones were identified. Zones I and II were intensely stained and were monomorphic. Zones II were composed of and showed polymorphism at one loci which possessed 3 alleles - F', F, S with allelic frequencies determined to be 0.003, 0.533, 0.464 respectively. (Mangaly & Jamieson, 1978).

Muscle esterase system in the pilchard Sardiniops ocellata revealed a total of six zones of activity. Each zone appeared as a single band or as group of bands. In the Zone II eleven bands were seen and were interpreted as the expression of eleven codominant alleles. (Thompson et al, 1974).

LACTATE DEHYDROGENASE SYSTEM

Isoenzymes of the lactate dehydrogenase system have been extensively studied in many animal species. In all these studies the isoenzymes were composed of four subunits (tetramers). The tetrameric structure was first reported in fish by Appella & Markert (1961). Lactate dehydrogenase isoenzymes in fish have been reported by several workers (Markert & Faulhaber, 1965); Clayton et al (1970); Kornfield et al (1982); Williscroft et al, (1970); Klar et al, (1979); Magee & Phillip, (1982); Taggart et al, (1980). The typical pattern seen from most vertebrate are presumably due to the tetrameric structure of enzymes, which is coded for by two separate structural genes whose monomeric products combine to form five isoenzymes represented as five bands on electrophoretic separation. (Markert, Shaklee & Whitt, 1975).

The lactate dehydrogenase isoenzyme system of the mullet, Liza parsia in the present study was identified to possess two loci - a slower migrating Ldh-1 and a faster migrating Ldh-2 locus. A total of five bands, the typical pattern, was observed. Bands A_4 and B_4 were dark being the homopolymers

of Ldh-1 and Ldh-2 loci respectively. The other three bands were lighter being the hybridised products of the two loci to give the heteropolymers A_3B, A_2B_2 and A_1B_3.

In the grass carp similar typical pattern with two loci were identified with a total of five isoenzymes (Magee et al, 1982).

Electrophoretic studies of lactate dehydrogenase from Hyla crucifer also showed the typical pattern composed of two loci (Keller & Lyerla, 1977).

However, McAndrew et al (1983), have observed in Tilapia sp., that the lactate dehydrogenase system in some tissues possess the two loci Ldh-1 and Ldh-2 with only one intermediate band, the A_2B_2 heteropolymer. The A_4 and B_4 loci of the lactate dehydrogenase system in the neutral tissues of Poecillia do not form heteropolymers due to genetically controlled restriction of subunit assembly (Frankel, 1981).

In the present study of the Lactate dehydrogenase system with its two loci, the fast locus Ldh-2 was found to be polymorphic as its allelic frequencies were less than 99% (<0.99) and the slower locus Ldh-1 was monomorphic. The polymorphic loci possessed two alleles Ldh-2(100) and the less common Ldh-2(125). The genotype observed were -

Genotype :	100/100 (AA)	100/125 (AB)	125/125 (BB)
Genotype frequency :	50 (Homozygote)	61 (Heterozygote)	13 (Homozygote)

The phenotype of the homozygotes AA, and BB possessed five isoenzymes each. The heterozygote AB possessed six isoenzymes. The sixth isoenzyme nearer to the anode was presumed to be the heteropolymer of the two codominant alleles the Ldh-2(100) and Ldh-2(125).

Theoretically 15 isoenzymes are to be found in the heterozygote if they possess 2 loci of a tetrameric enzyme with one of the locus to be polymorphic with two alleles. But many works on this enzyme system in different fishes show that not all 15 isoenzymes are active in the heterozygote (Hodgins et al, 1969; Morrison and Wright, 1966; Goldberg, 1966). Only nine bands were observed in the heterozygote of the lake-trout (Goldberg, 1966) and same maximum bands in were obtained in brooktrout (Morrison & Wright, 1966) and in sockeye salmon (Hodgins et al, 1969). This could be explained by the occurrence of isoenzyme overlapping; nonrandom combination of subunits and nonresolution of weak isoenzymes (Hodgins et al 1969)

In the case with the lactate dehydrogenase system of Liza parsia it is seen (ref. Results) that the distance between the two bands corresponding to the two alleles of the locus Ldh-2 are only a few millimeters apart. To obtain all the hybrid heteropolymers, if they were formed, is difficult to resolve within this distance over the gel. The hybrid heteropolymers of Ldh-1 with Ldh-2/Ldh-2(125) are also not seen, probably due to overlapping or low activity of the isoenzymes.

Therefore, it is suggested that the observed isoenzyme patterns result from very small differences in electrophoretic mobility between the sub-units coded by Ldh-2(100) and Ldh-2 (125).

Similar conclusions were made by Clayton & Franzin(1982) whose studies on lactate dehydrogenase isoenzyme in muscle tissue of lake white fish reveal five typical bands whereas in the heterozygote instead of fifteen bands only four were seen.

ALLELIC FREQUENCIES

In this study, with Liza parsia, polymorphic Ldh-2 locus of the eye tissue was analysed in 124 samples. It was found that 50 samples possessed the Ldh-2(100) allele; 13 samples the Ldh-2(125) allele and the rest 61 samples had the Ldh-2(100/125) codominant alleles. Expected frequencies calculated by the simple genetic model, the Hardy-Weinberg model were 0.421; 0.456; 0.123 respectively. Therefore the theoretically expected number of fish possessing these allele were equal to 52.204; 56.544; 15.252 respectively. These values are seen not to differ significantly from the observed values and is confirmed by the chi-square test which determined these differences between observed and expected as non-significant.

Fairbairn & Roff (1980) question the dependency on chi-square testing of genetic models of isoenzyme variability

without breeding data and claims that the power of the test to be very low with samples sizes to very much less than 200.

However many workers, whose work has been discussed in the introductory chapter ~~in the discussion~~ of this presentation, have used sample sizes as low as 3 to 15 numbers only (Anderson, 1982).

Lactate dehydrogenase isoenzyme and their polymorphic loci have been observed by many workers in fish. The number of loci, alleles if any, allelic frequencies and heterozygosities observed in some of the fishes have been tabulated. (see table)

Salmonids generally possess 5 distinct Ldh loci (see Utter et al, 1973). In rainbow trout only one loci was found polymorphic with two alleles (Williscroft et al, 1970) and with allelic frequencies as 0.96 and 0.04 (Utter et al, 1973). In Salmo clarki the cuthroat trout again, only one locus was polymorphic with two alleles having a allelic frequencies of 0.92 & 0.074 (Allendorff & Phelps, 1980). Eight salmonid species analysed in depth by Utter, Allendorf & Hodgins, 1973 reveal four species viz., Pink, Sockeye, Coho, Rainbow to possess a polymorphic Ldh locus with 2 alleles in each case. Significance of the occurrence of alleles could be presumed from some recent work. The closely related species of Sebastolobus (family Scorparidae) living at different depths, differ in sensitivity of muscle Ldh to pressure; in one of

the Sebastolobus species, S.attivelis found at lower depth a decrease in sensitivity is seen (Siebenaller, 1978; Siebenaller and Somero, 1978).

Kirpichinikov (1981) suggests that these two species of Sebastolobus possess different alleles for the Ldh locus and these alleles appear to code for allozymes with very different functional characteristics. The reasons for the presence of the alleles identified in Liza parsia at the Ldh-2 locus cannot be easily answered. Specific work on Ldh isoenzyme physiology, and function and their differences in expression due to the alleles could throw some light on the need of the same in Liza parsia, occuring in one and the same random mating population subjected to similar influences of environmental factors.

LDH EXPRESSION IN TISSUES

The expression of the Ldh system was determined in six tissues viz., eye, muscle, heart, liver and kidney. Two loci Ldh-1 and Ldh-2 are seen to be expressed intensely in the eye. In other tissues although the two locus are expressed the Ldh-2 has faint activity as revealed by less intense band at that region.

Generally it is known that Ldh locus fall into two types; heart-Ldh type and muscle-Ldh type. These two isoenzymes vary in their physiological function with respect to pyruvate inhibition. The M-Ldh type found in anaerobic conditions is

not inhibited by pyruvate facilitating easy conversion to lactic acid. The H-Ldh is inhibited by pyruvate concentrations and allows pyruvate to enter the Krebs oxidative cycle. This H-Ldh is found in aerobic conditions.

Keller et al, 1977 studied the Ldh in the spring peeper, Hyla crucifer revealing that the enzyme is coded for by separate heart and muscle genes. Extensive studies of Gadiformes fishes for the Ldh system by Shaklee & Whitt (1981), show a slower locus predominant in muscle and the faster locus being predominant in heart and a third locus predominates in liver. Ldh system identified in the grass carpand big-head carp by Magee et al (1982) reveal similar two loci, one predominating in heart and one in muscle.

In the present study though two loci were identified the slower loci Ldh-1 comparable to the Ldh-A, designated by the other authors, was equally expressed in all tissues. The faster locus Ldh-2 comparable to the Ldh-B was found predominant only in the eye tissue with faint expression in other tissues.

Similar findings were made by Jorgensen & Mustafa(1980) in the flounder, Platichthys flesus wherein the Ldh in heart and muscle both migrated with identical mobilities. Their kinetic studies on the same enzyme revealed similar behaviour of the two enzymes. The heart Ldh possessed the characteristic of the M-type Ldh with high apparent K_m values for pyruvate

and low subtrate inhibition at high pyruvate concentration and making the heart adapted to anoxic conditions.

The reason for predominance of M-type Ldh in the heart tissue in Liza parsia cannot be concluded unless such related kinetic studies as done in the flounder are conducted, however, a rational approach to reason out this observation could be made, with findings in the flounder, as a perspective. The need for predominance of M-type Ldh locus in the tissues of Liza parsia could be assumed to be based on the same grounds, as that are considered in the flounder; or that some other function of M-type Ldh enzyme attains a more significant role for the fish in its niche and necessiates the predominance of this type, of Ldh in all tissues. However it is to be noted that only correlated studies of gene expression, and its biochemical implication under the influence of the environment could ascertain these assumptions.

In addition to the two loci of the M and H type a third locus-C is characteristic solely in fishes (Markert et al (1975). The gene C also called L or E in the majority of teleosts is active in the eye retina. Whitt et al (1973) have shown it to be necessary for the rapid regeneration of the visual pigments, rhodopsin, or porphirin.

In the present study only two loci have been identified. There was no distinction between M-type Ldh and H-type Ldh as these two loci appeared with similar intensities and migrated

to same distances due to identical mobilities. In the eye an additional locus, Ldh-2 is present.

An evolutionary relationship of the three main Ldh genes of teleosts suggested a single ancestral gene for Ldh-A and Ldh-B with Ldh-E having derived from the Ldh-B by duplication and subsequent divergence (Whitt et al, 1973c). This was based on earlier observations revaling similar antigenic, physical and kinetic properties for retinal isoenzyme and the B_4 isoenzyme (Whitt 1969, 1970a). Some orders of teleosts such as anguilliformes, cyprniformes, siluriformes, and some families of gadiforms and clupeiformes lack retinal specific Ldh isoenzyme (Whitt et al, 1973c).

The Ldh-2 locus observed in Liza parsia could be functioning as the locus 'c' as most teleosts analysed have shown to possess this locus. However the work done by Whitt (1969), (1970a); Whitt et al (1973c) provide a basis for alternative suggestion that the retinal specific Ldh isoenzyme is lacking in this fish.

ONTOGENY OF LACTATE DEHYDROGENASE

After determining the expression of the lactate dehydrogenase system in the wild population of Liza parsia, a probe into the inheritance pattern of the system was initiated by analysing the male and female spawners contributing the parental expression. The larvae obtained from induced sprawning of these parents at the Marine Prawn Hatchery laboratory, were examined.

Incidentally, only one locus was expressed in the larvae of 2.5 mm size (fifth day post-hatching). This urged for further study of the ontogeny of the enzyme. However the larvae did not survive the critical phase of ten days post-hatching. Several attempts were made but proved futile. This impeded the study on this line. The initial purpose of study of inheritance pattern also could not be materialized. Instead the juveniles of size 24 mm, 30 mm, & 40 mm from the same wild population were examined and the expression of the Ldh system was observed as early as in sizes of 24 mm itself.

Although an ontological development existing in the Ldh system was confirmed, it was not possible to pinpoint the exact stage at which the expression of the system occurs, as the larvae and juveniles of sizes between 2.5mm and 24 mm were not procurable from the wild population too.

Several work on differential gene action for the Ldh enzyme during development have been made in fish (Nakano & Whiteley, 1965; Goldberg, 1966; Hitzeroth et al (1968); Whitt, 1970a, 1973c).

From these studies a revealation is made that the Ldh-E expression during teleost development occurs at the time of structural and functional differentiation of the retina. This observation tightly in turn suggests that E locus activation is probably coupled with the differentiation of the neural retina (Whitt 1968, 1970a). Such correlated studies in the case

of L.parsia with the observations made here and the determination of the actual time of appearance of mature visual apparatus, could be made to establish the function of the fast locus observed in the eye.

HETEROZYGOSITY

The heterozygosity values obtained on the basis of polymorphic loci was 0.492 and did not vary significantly from the expedted heterozygosity calculated from allelic frequencies and was 0.4556. These values of heterozygosity ($\bar{H}$) are quite high when compared to observed heterozygosities of three 3 species of trout. $\bar{H}$ values equal to 0.242; 0.293; 0.228 were determined for browntrout, rainbowtrout, and sockeye salmon respectively (Ryman, 1983). The values obtained in Liza parsia pertain to a single polymorphic loci Ldh-2. Most workers obtain the $\bar{H}$ values after averaging the values over a number of polymorphic loci.

Average heterozygosities values is an estimate of the total amount of genetic diversity within a population. This measure can provide an insight into the population history and structure and the amount of genetic interchange between population achieved by migration. It is however, necessary to analyse several loci, at least 20-25 loci and then determine the average $\bar{H}$. Interpretation with the statistic is only then possible, to arrive at its implications.

With this study an initiative has been made in assessing the genetic variation in the population of L.parsia found in the Cochin estuary.However this investgation confers only to a single polymorphic loci.Elaborate studies are at,atleast, 20-25 loci are necessary to quantify the magnitude of toatal genetic variation and mean Heterozygosity

SUMMARY

(1) The polyacrylamide gel electrophoretic methodology for protein and enzyme separation was standardized, and the reasons for these optimum conditions discussed.

(2) Protein patterns of the liver, muscle, eye, and brain tissues in the adult Liza parsia were obtained.

(3) Muscle protein of juveniles and adults were resolved. No difference in the pattern between juveniles and adult was observed.

(4) The expression of the esterase enzyme system was obtained in liver, kidney, heart, eye, muscle, and brain. 3 groups of esterase system with a total of six bands were identified.

(5) Lactate dehydrogenase isoenzyme expression was obtained in 5 tissues viz., eye, heart, muscle, liver, and brain. Two loci, Ldh-1 and Ldh-2 were identified with a typical 5 band isoenzyme pattern present in the eye tissue. In all other tissues the Ldh-1 was expressed with equal intensities and a faint expression of the Ldh-2 locus along with one or two interlocus hybrids were present.

(6) Ontological development of the Ldh isoenzyme system was detected. The system develops in the stage between

2.4 mm larvae and 24 mm juveniles. At the initial stages of post-hatching (2.4 mm) only the Ldh-1 locus is expressed.

(7) The faster locus Ldh-2 was found polymorphic with 2 alleles : Ldh-2(100) and Ldh-2(125). Their allelic frequencies were 0.649 and 0.341 which were not significantly different from the expected values.

(8) Average heterozygosity ($\bar{H}$) obtained from the analysis of the polymorphic loci Ldh-2, is 0.452 and is not significantly different from the expected value of $\bar{H} = 0.492$.

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