THERMAL SELECTION AT AN ENZYME LOCUS IN POPULATIONS OF THE RED SHINER, NOTROPIS LUTRENSIS, RECEIVING HYPOLIMNION EFFLUENTS FROM A RESERVOIR

THESIS

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Genetic variation was examined at 19 loci encoding enzymatic and general proteins Notropis lutrensis from the Brazos River in Texas. The thermal regime of the Brazos River below Possum Kingdom Reservoir is altered due to the release of water from the hypolimnion. Summer water temperatures fluctuate as much as 7°C. Levels of heterozygosity at the malate dehydrogenase-2 locus were correlated with the degree of water temperature fluctuation at each locality. The isozymes from three homozygous patterns of supernatant malate dehydrogenase (Mdh-1, Mdh-2) exhibited different activities at different experimental temperatures.

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CHAPTER I

INTRODUCTION

The use of electrophoresis for examining allozymic variation at loci controlling regulatory enzymes and other proteins has demonstrated that the level of genetic variation in natural populations is high (Harris, 1966; Lewontin and Hubby, 1966; Selander and Johnson, 1973; Selander and Kaufman, 1973; Selander, 1976). Furthermore, electrophoretic techniques have provided a basis for understanding interspecific and intraspecific genetic variation and have produced a great interest in the maintenance of genetic polymorphisms. Two opposing views have developed concerning the nature of high levels of genetic variation, i.e., (1) that allozymic variants are functionally identical and are selectively neutral (Kimura, 1968, 1969; King and Jukes, 1969), and (2) that genetic variation is maintained by some type of balancing selection (Johnson, 1971, 1972, 1973; Clarke, 1970; Ayala, 1972; Ayala and Anderson, 1973; Selander and Kaufman, 1973).

Several arguments have been developed against the hypothesis of selective neutrality. Johnson (1972) found in several natural populations with a high number of alleles at a given locus a greater "evenness" of allelic frequencies

than expected if selection were not operating. Analysis of extensive data collected on Drosophila sp. support the hypothesis of Gillespie and Kojima (1968) that enzymes with external substrates are more polymorphic than enzymes with internal substrates, and Johnson (1973) proposed that those enzymes important in metabolic regulation are sensitive to selective.

Many studies support the view that polymorphisms are a result of adaptive strategies to environmental variability (Johnson, 1973; Selander and Kaufman, 1973; Gillespie and Langley, 1974; Bryant, 1976; Valentine, 1976). Levinton (1973) found that the degree of polymorphisms in several species of mollusks decreased with depth of burial in the intertidal sediment, since the environment becomes less variable with increase in depth. Levins (1968) proposed that the degree of polymorphism is a genetic response to environmental grain. A course-grained environment, i.e., an environment that is uncertain and variable will result in selection for organisms with a more plastic genome than environments which are fine-grained, i.e., environments that are more predictable and less variable (Bryant, 1974; Gillespie, 1974).

McNaughton (1974) stated that temperature is probably an important factor in enzyme evolution, and several studies appear to support this contention. For instance, Koehn and

Rasmussen (1967) and Koehn (1969) found that allelic frequencies of the esterase enzymes in the fish, Catostomus clarkii, could be correlated to variation in ambient temperatures. In the fathead minnow, Pimiphales promelas, Merritt (1972) demonstrated enzyme-substrate affinities of lactate dehydrogenase responded preferentially to different temperatures, and that a north-south cline in gene frequencies existed for this enzyme. Thermal effects on enzyme activity have also been reported by Hochachka and Somero (1968), Somero (1969), Somero and Hochachka (1971), and Powers (1972). These studies indicate that an inverse relationship exists between temperature and enzyme-substrate affinity in response to a change in temperature. On an evolutionary basis, allelic substitutions may create changes in activation energies in the enzymes involved. It appears that such biochemical differences between isozymes would have selective importance. Structure-function relationships in relation to environmental parameters need to be determined to solve this problem. The possible adaptive advantages of polymorphic proteins can only be realized when the biochemical properties of these proteins are related to the appropriate environmental parameters.

Therefore, additional investigations are necessary to determine the relative roles of selection or random processes (neutrality) in structuring the genomes of natural

populations. This study involved analysis of genetic variation in the red shiner, Notropis lutrensis, to determine if the variation observed is indicative of selection.

N. lutrensis is an important forage fish (Hubbs, 1954) and was selected as an experimental organism due to its small size and abundance. The red shiner's range extends from Wyoming to Illinois and southward along the Mississippi River to northern Mexico (Hubbs and Strawn, 1956). N. lutrensis matures as a yearling, spawns in lakes and streams and feeds on algae, insects, and crustaceans (Koster, 1957; Cross, 1958). Hubbs et al. (1953) reported that N. lutrensis is usually found in pools but is not excluded from faster flowing water.

The study was located on the Brazos River below Morris Sheppard Dam, constructed in 1941, in Palo Pinto Co., Texas. Periodic release of water from the hypolimnion of the reservoir results in unpredictable water flow and in altered temperature regimes downstreams.

The objectives of my research on N. lutrensis were to determine (1) the allelic frequencies and levels of heterozygosity at 19 loci for several populations to determine if the populations could be differentiated genetically, and (2) the "relative fitness" of the isozymes comprising enzymes with heterogeneous allelic frequencies. The "relative fitness" of the isozymes constituting the enzyme

malate dehydrogenase was determined since preliminary results indicated that the allelic frequencies varied between populations of N. lutrensis.

CHAPTER II

MATERIALS AND METHODS

Study Area

Specimens (n=446) of Notropis lutrensis were collected from the Brazos River below Morris Sheppard Dam, constructed in 1941. The Brazos River has a watershed of 41,700 square miles, and the river below Morris Sheppard Dam is constantly supplied with 10-15 cfs leakage from the hypolimnion of Possum Kingdom Reservoir (Cloud, 1973). Hydroelectric power is generated for supplemental energy to a metropolitan population of approximately 750,000.

The locations of the four collection sites on the Brazos River were (1) 1 km below Morris Sheppard Dam on Texas State Highway 16, (2) 30 km below Morris Sheppard Dam on Texas State Highway 4, (3) 57 km below Morris Sheppard Dam on U.S. Highway 180, and (4) 121 km below Morris Sheppard Dam on Interstate 20 (Fig. 1). All collection localities were in Palo Pinto County, Texas, except the fourth site, which was in Parker County, Texas. Temperature data were collected at the time of each collection, using a YSI Model 54 Oxygen/ Temperature Probe. Additional, yearly data were provided by K. W. Stewart.

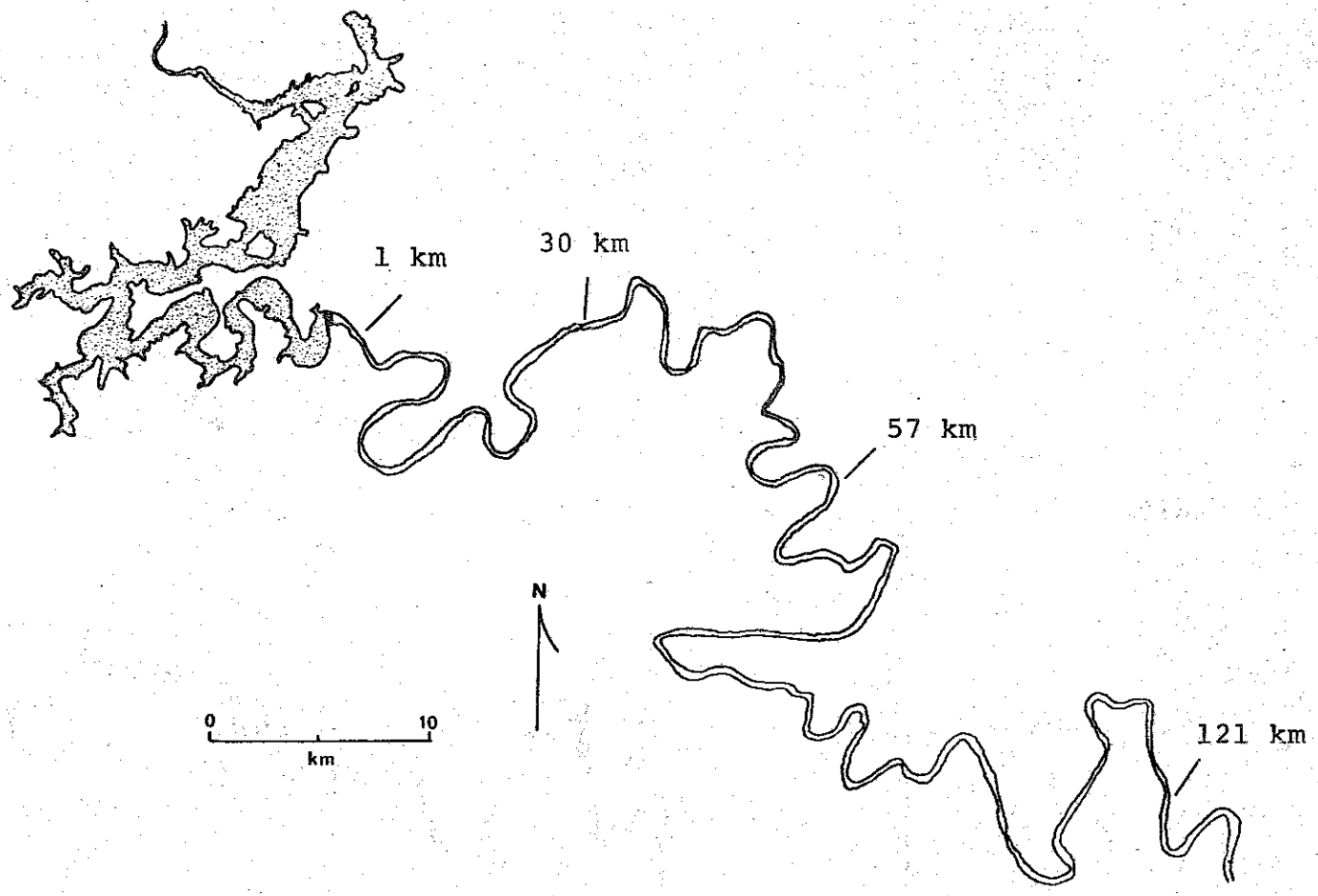


Figure 1. Collecting localities for four populations of Notropis lutrensis on the Brazos River below Possum Kingdom Reservoir.

Species Studied

Fish were collected using a 3-m, 0.32-cm mesh seine which allowed collection on all size classes of N. lutrensis. After collection fish were placed in buffer (0.1 M Tris HCl, 0.001 M EDTA, pH 7.0), frozen over dry ice in the field, returned to the laboratory, and stored at -15°C. Within 48 hrs after collection, each fish was weighed, homogenized in an equivalent amount of 0.1 M Tris HCl, 0.001 M EDTA, pH 7.0 buffer, centrifuged for 15 min, and immediately stored at -15°C.

Allozyme Analysis

Horizontal starch-gel electrophoresis was utilized to determine allelic frequencies and levels of heterozygosity for the four populations of N. lutrensis. The gel mold consisted of a glass plate (152 mm x 220 mm x 6 mm) and four plexiglass strips held in place with petroleum jelly. After the gel was poured into the mold, a plexiglass plate was used to cover the gel until it hardened. All gels were prepared from a 12% suspension of hydrolysed starch (Sigma Chem. Co.). Suspended starch was poured into the buffer heated to boiling in a 1,000-ml, round-bottom flask. The solution was then shaken and simultaneously degassed with an aspirator for approximately 1 min. After the gels cooled, insertion lines for the samples were formed by cutting the gels into two slightly unequal halves.

Homogenized samples were absorbed onto no. 3 filter paper and blotted. Fifteen samples could be placed along the insertion line on each gel. Generally, a sample of 30 specimens from each population was electrophoresed for each of the various proteins.

The electrophoretic apparatus consisted of two trays (35 mm x 8 mm x 6 mm), with a 304-mm no. 22 platinum wire in each tray. Gels were placed across the parallel trays, and sponge cloths were used as bridges between gels and the electrode buffer. Electrophoresis was conducted in a controlled chamber between 0-10°C.

A total of 10 enzymes encoded by 15 loci and general proteins encoded by four loci were scored for each population using four buffer systems. Glutamate oxalate transaminase (Got-1) was electrophoresed on Buffer System A (gel buffer: 76 mM Tris HCl, 5 mM citric acid, pH 8.65; electrode buffer: 300 mM boric acid, 60 mM NaOH, pH 8.1) of Ayala et al. (1974) as modified by Avise et al. (1974). Malate dehydrogenase (Mdh-1, Mdh-2, Mdh-3), lactate dehydrogenase (Ldh-1, Ldh-2), and indophenol oxidase (Ipo-1) were run on Buffer Systems B (gel buffer: 0.008 M Tris HCl, 0.003 M citric acid, pH 6.7; electrode buffer: 0.223 M Tris HCl, 0.086 citric acid, pH 6.3) (Selander et al., 1971). Phosphoglucose isomerase (Pgi-1, Pgi-2), isocitrate dehydrogenase (Idh-1), phosphoglucomutase (Pgm-1), phosphogluconate dehydrogenase (Pgd-1)

were electrophoresed on Buffer System C (gel buffer: 9 mM Tris HCl, 3 mM citric acid, 0.08 mM EDTA, pH 7.0; electrode buffer: 135 mM Tris HCl, 45 mM citric acid, 1.30 mM EDTA, pH 7.0) of Ayala et al. (1972) as modified by Avise et al., (1974). Buffer System D, (Selander et al., 1971), was used for electrophoresis of leucine amino peptidase (Lap-1), esterases (Est-1, Est-2), and general proteins (Pr-1, Pr-2, Pr-3, Pr-4) (gel buffer: 1:9 ratio of solutions A and B. Solution A: 0.003 M lithium hydroxide, 0.09 boric acid, pH 8.1; Solution B: 0.05 M Tris HCl, 0.008 M citric acid, pH 8.4. Electrode buffer: solution A).

Stains for Pgd-1, Pgi-1, Pgi-2, Ipo-1 and Pgm-1 were prepared according to Ayala et al., (1974), while all other proteins were stained according to Selander et al., (1971). Gels were fixed in 45 parts methanol and 55 parts acetic acid and distilled water. Coding of genotypes was done by assigning the fastest migrating allele as a, the next fastest as b, etc., with the assumption of homology across species.

Gene frequencies and proportion of heterozygotes were calculated for all loci. Heterozygosities were not considered for genotypes with alleles having frequencies of ≤ 0.05 when occurring in the heterozygous condition.

Analysis of Temperature in Isozyme Activities of Malate Dehydrogenase

The isozymes comprising malate dehydrogenase in Notropis lutrensis exist in two forms: (1) a supernatant form found in the cytoplasm encoded by two loci (Mdh-1, Mdh-2) and (2) a mitochondrial form encoded by a single locus (Mdh-3). It has been well documented that both forms exist as dimers (Chilson et al., 1966; Whitt, 1970; Wheat et al., 1971; Davidson and Cortner, 1967), and that the two loci of the supernatant forms are a result of gene duplication (Karig and Wilson, 1971). When both supernatant forms are in the homozygous condition, random combining of differently-charged subunits results in the formation of three isozymes. Heterozygotes formed between allozymic forms are represented by six isozymes.

To determine activities of the various isozymes of Mdh-1 and Mdh-2 loci, in relation to temperature, those isozymes represented by the homozygous condition were used. For the three homozygous genotypes demonstrated for the Mdh-1 and Mdh-2 loci in N. lutrensis, ff was designated the fastest migrating pattern, mm was designated the next fastest, and ss was designated the slowest migrating pattern. A minimum of five replicates of each of the three homozygous genotypes was electrophoresed, except the ff pattern, for which only three replicates were used. After electrophoresis was completed, the gel was sliced in half,

and one half was incubated for 45 min at one of five experimental temperatures (10, 15, 20, 25, and 30°C) in an environmental chamber; the other half (control) was incubated at 30°C. Stain and a cellulose acetate plate (Titan III Helena Laboratories) were placed over the gel. A glass plate placed on top of the cellulose acetate plate insured proper mirror-image staining. Stained plates were fixed in a 5% solution of acetic acid and distilled water. Replicate isozyme patterns were scanned on a Helena Laboratories Quick Quant II Densitometer with a 525-nm filter. Each isozyme was recorded as percent activity of the combined three isozymes, constituting 100%, using the Mdh-1 homodimer (common to all genotypes) to standardize against concentration differences.

CHAPTER III

RESULTS

Electrophoretic Results

Nineteen loci were examined from at least 30 genomes from each locality, with the exception of the Mdh-2 locus where 326 animals were analyzed. Of the 19 loci examined, nine were monomorphic with the same allele fixed in all populations. These loci were Pgi-2, Lap-1, Got-1, Ldh-2, Ipo-1, Pt-1, Pt-2, Pt-3, and Pt-4.

Malate Dehydrogenase

Malate dehydrogenase was demonstrated to be encoded by three loci. All isozymes comprising the enzyme migrated anodally, with the mitochondrial form (Mdh-3) having the slowest migration (Fig. 2). Localities reported below reflect distances of sample from Morris Sheppard Dam.

Malate Dehydrogenase-1. The Mdh-1 locus was monomorphic in populations from 10 km, 30 km, and 57 km. The 121-km population was weakly polymorphic with two alleles, the common b allele and a rare allele, a (Table 1).

Malate Dehydrogenase-2. The Mdh-2 locus was the most polymorphic of the three Mdh loci, with three alleles segregating from most populations (Table 2). All six possible

Table 1. Allelic frequencies for the polymorphic loci, proportion of loci polymorphic per population (P), proportion of loci polymorphic per individual (H) in four populations of N. lutrensis.

Protein locus and allele	Locality			
	1-km	30-km	57-km	121-km
Isocitrate dehydrogenase				
Idh-1a	1.00	1.00	1.00	0.93
Idh-1b	----	----	----	0.07
Phosphoglucose isomerase-1				
Pgi-1a	0.08	0.07	0.02	0.10
Pgi-1b	0.87	0.93	0.98	0.90
Pgi-1c	0.05	----	----	----
Phosphoglucose mutase-1				
Pgm-1a	0.10	0.17	0.06	0.13
Pgm-1b	0.90	0.83	0.92	0.87
Pgm-1c	----	----	0.02	----
6-Phosphogluconate dehydrogenase				
6Pgd-1a	0.15	0.05	0.10	----
6Pgd-1b	0.78	0.95	0.90	1.00
6Pgd-1c	0.07	----	----	----
Malate dehydrogenase-1				
Mdh-1a	0.02	0.02	0.03	0.05
Mdh-1b	0.98	0.98	0.97	0.95
Malate dehydrogeanse-2				
Mdh-2f	0.06	0.07	0.06	0.02
Mdh-2m	0.75	0.65	0.68	0.77
Mdh-2s	0.09	0.28	0.26	0.21
Malate dehydrogenase-3				
Mdh-3a	0.07	0.03	0.07	0.05
Mdh-3b	0.93	0.97	0.93	0.95
Esterase-1				
Es-1a	0.02	0.18	0.18	0.07
Es-1b	0.25	0.80	0.69	0.42
Es-1c	0.73	0.02	0.13	0.48
Es-1d	----	----	----	0.03

Table 1 (Continued)

Protein locus and allele	Locality			
	1-km	30-km	57-km	121-km
Esterase-2				
Es-2a	0.72	0.42	0.71	0.58
Es-2b	0.28	0.50	0.27	0.42
Es-2c	----	0.08	0.02	----
Lactate dehydrogenase-1				
Ldh-1a	0.72	0.83	0.77	0.80
Ldh-1b	0.28	0.17	0.23	0.20
(P)	47.37	31.58	36.84	36.84
(H)	9.94	8.35	9.54	9.39

Table 2. Genetic variation at the malate dehydrogenase-2 locus for four populations of N. lutrensis.

Location	No. of Individuals	Allelic Frequencies f	m	s	Effective Number of Alleles	H%
1 km below Reservoir	48	0.06	0.75	0.19	1.66	39.85
30 km below Reservoir	154	0.07	0.65	0.28	1.98	48.05
57 km below Reservoir	93	0.06	0.68	0.26	1.87	38.71
121 km below Reservoir	31	0.02	0.77	0.21	1.57	35.48

homozygous and heterozygous genotypes were observed (Fig. 2). In all populations, the m allele was most common, with frequencies ranging from 0.65 at the 30-km locality to 0.77 at the 121-km locality, while the s allele was the next most common, with frequencies ranging from 0.09 at the 1-km locality to 0.28 at the 30-km locality. The f allele was least common, with frequencies of 0.06 from the 1-km and 57-km populations, 0.07 from the 30-km population, and 0.02 from the 121-km population. The f allele was not found in two additional populations located on the Brazos River or in seven populations of N. lutrensis from the Trinity River. Goodness of fit tests of the observed to the expected zygotic frequencies demonstrated that the four populations are in Hardy-Weinberg equilibrium (Appendix I).

Malate Dehydrogenase-3. The Mdh-3 locus was weakly polymorphic with two alleles, a and b, occurring at the 1-km, 30-km, 57-km, and 121-km populations, with the frequency of the b allele ranging from 0.93 to 0.97. However, the 30-km population was monomorphic for the b allele.

Other Proteins

Isocitrate Dehydrogenase-1. The Idh-1 locus was monomorphic for the a allele in most populations, except the 121-km population, where the rare b allele had a frequency of 0.07 (Table 1).

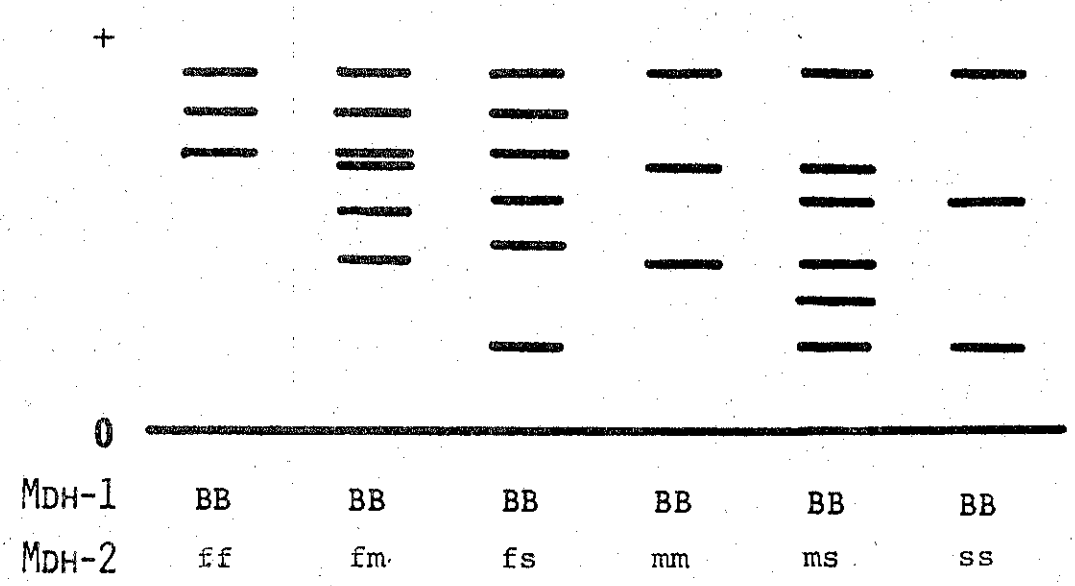


Figure 2. Representation of electrophoretic variation in supernatant malate dehydrogenase (Mdh-1, Mdh-2) in Notropis lutrensis.

Phosphoglucose isomerase-1. The Pgi-1 locus was polymorphic with three alleles in the 1-km population, with the b allele occurring at a frequency of 0.87. All other populations were weakly polymorphic, with two alleles at the Pgi-1 locus. The common allele, b, exhibited frequencies ranging from 0.90 to 0.98.

Phosphoglucomutase-1. The Pgm-1 locus was polymorphic with two alleles. The common b allele had frequencies of 0.90, 0.83, and 0.87 from the 1-km, 30-km, and the 121-km populations, respectively. The 121-km population was polymorphic, with a rare allele, c, having a frequency of .02.

6-Phosphogluconate Dehydrogenase-1. The 6-Pgd-1 locus was polymorphic with three alleles at the 1-km locality, the common b allele having a frequency of 0.78. The 30-km and 57-km populations were polymorphic, with two alleles, the b allele exhibiting frequencies of 0.95 and 0.90, respectively. The 121-km population was monomorphic, with the b allele fixed.

Esterase-1. At the Es-1 locus, the c allele was the most common in the 1-km and 121-km populations, with frequencies of 0.78 and 0.48, respectively. In the 30-km and 57-km populations, the b allele was the most common, with respective frequencies of 0.80 and 0.69.

Esterase-2. At the Es-2 locus, the 1-km and 121-km populations were polymorphic, with the a allele having frequencies of 0.58 and 0.72 respectively. A third allele, c,

was present in the 30-km population, with a frequency of 0.08, and at the 57-km locality, with a frequency of 0.02.

Lactate Dehydrogenase-1. The Ldh-1 locus was polymorphic with two alleles. The common b allele ranged in frequency from 0.72 at the 121-km population to 0.83 at the 57-km population.

Genetic Variation

Total levels of heterozygosity (H) for all proteins from the 1-km, 30-km, 57-km, and 121-km populations were similar with values of 9.39%, 9.54%, 8.35% and 9.97%, respectively. Mean $\overline{H}$ for the four populations was 9.17% (Table 1). Proportion of loci polymorphic per population (P) ranged from 31.58% at the 57-km population to 47.37% at the 121-km population, with a $\overline{P}$ of 38.16.

Levels of Heterozygosity and Daily Temperature Changes on the Brazos River

A summary of annual temperature data collected for three sites on the Brazos River indicates that both the mean and upper extreme water temperatures are lower immediately below the reservoir, and annual variation in water temperature at this site is also lower (Table 3). Temperature showed the greatest fluctuation a short period of time after the release of water from Morris Sheppard Dam. Since water is continuously leaked from the hypolimnion of the 45-m dam,

Table 3. Temperature parameters for three localities on the Brazos River below Possum Kingdom Reservoir.

Site	Distance Below Dam	Mean Annual H_2O Temp.	Range	SD	Greatest Daily Temp. Fluctuation
1	1 km	15.6 C	11-20 C	3.31	4.0 C
2	30 km	19.2 C	11-29 C	6.39	7.0 C
3	57 km	18.7 C	10-29 C	7.30	1.7 C

the drop in temperature below the dam is about 4°C. The most severe temperature change during power generation occurs at the second site, 30 km below the dam, where as much as 7°C drop in temperature may occur. Records from a constant temperature recorder located at the 30-km locality indicate that water temperature in August may drop from 29 to 22°C over a period of only 4 hrs. Further downstream the temperature change is not as great, with daily temperature fluctuations of approximately 1.7 and 1°C at the 57-km and 121-km localities, respectively.

A comparison of levels of heterozygosity at the Mdh-2 locus in individual samples taken, with maximum temperature change with power generation reveals two features: (1) heterozygosity varies directly with maximum change in water temperature (Figure 3). The highest level of heterozygosity (48.05%) occurred 30 km below the dam where the temperature change may be as great as 7°C in summer months. At 121 km below the dam where H=35.48%, maximum temperature change is approximately 1°C. An analysis of variance comparing the effect of variable temperature change on levels of heterozygosity demonstrated a significant F_S ($P<0.01$) (Appendix II) and a significant correlation coefficient of 0.85. (2) Levels of heterozygosity do not correlate with distance from the dam, i.e., the change is not clinal. Thus, levels of heterozygosity do not decrease with distance from

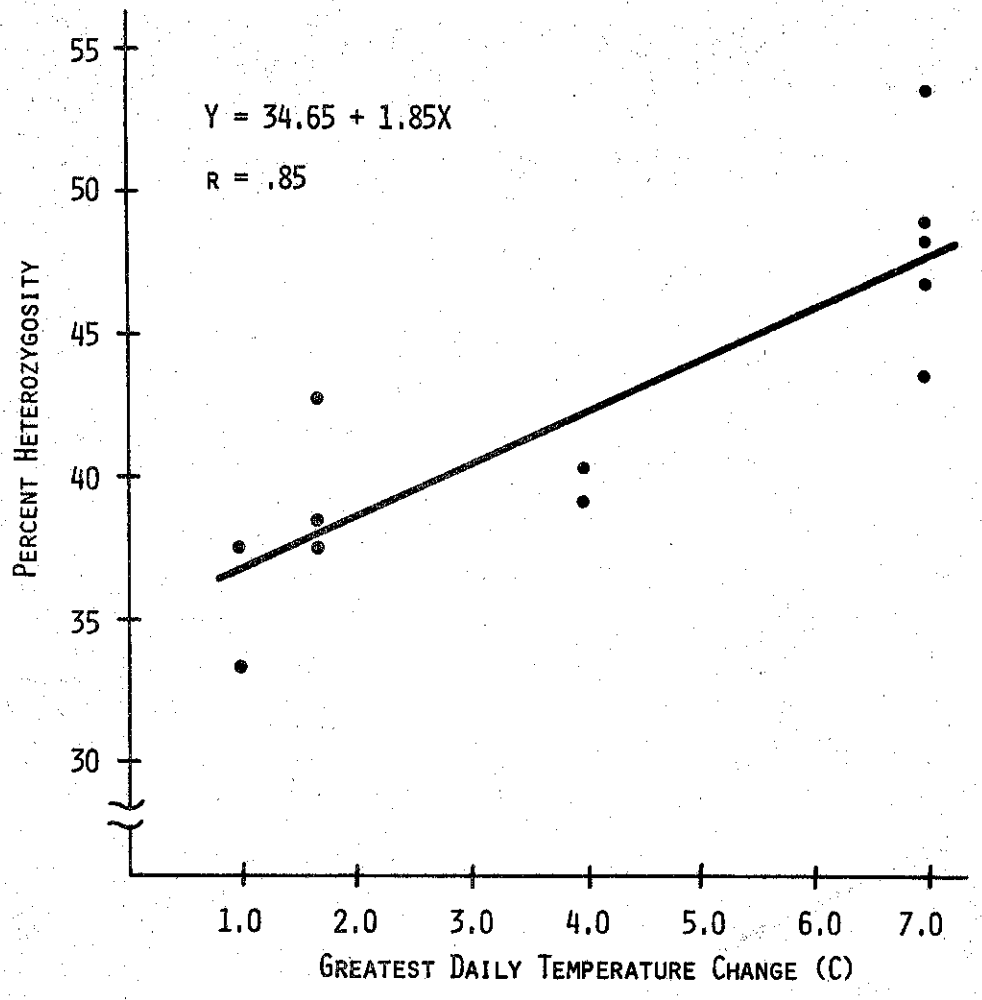


Figure 3. Linear regression of levels of heterozygosity against greatest daily temperature change at collection sites for N. lutrensis.

the dam, rather heterozygosity increases between 1 and 30 km below the dam, and then decreases progressively.

Malate Dehydrogenase Isozyme Activities

An analysis of activity of the Mdh isozymes incubated at five experimental temperatures demonstrated that the various isozymes are functionally different (Figs. 4, 5, 6). The Mdh-1 homodimer, common to all genotypes, exhibited an increase in activity from 28% at 10°C to 38% at 30°C. Heterodimers in the ff and mm patterns also showed a general increase in activity from 20-30% between 10°C and 30°C. The heterodimer from the ss pattern exhibited an increase in activity from 20-50% between 10 and 30°C. However, striking differences in activity were exhibited by the slowest migrating isozymes for each of the patterns, and the ANOVA revealed that these differences are significant ($F_{.05(2,71)} = 19.10$). In general, the three slow isozymes had the following patterns of activity: (1) activity of the slowest isozyme of the ff electromorph increased from about 40% at 10°C to 95% at 20°C and gradually declined to 65% at 30°C; (2) activity of the slowest isozyme of the mm electromorph was generally less than that of the slowest ff and ss isozymes, and showed a slight increase in activity over the temperature range of 55% to 65%, with greatest activity at 25°C; (3) activity of the slowest isozyme of the ss electromorph exhibited about 55% of the total activity at 10°C and

Figure 4. Percent activities at five temperatures for three isozymes comprising the ff pattern for malate dehydrogenase (Mdh-1, Mdh-2).

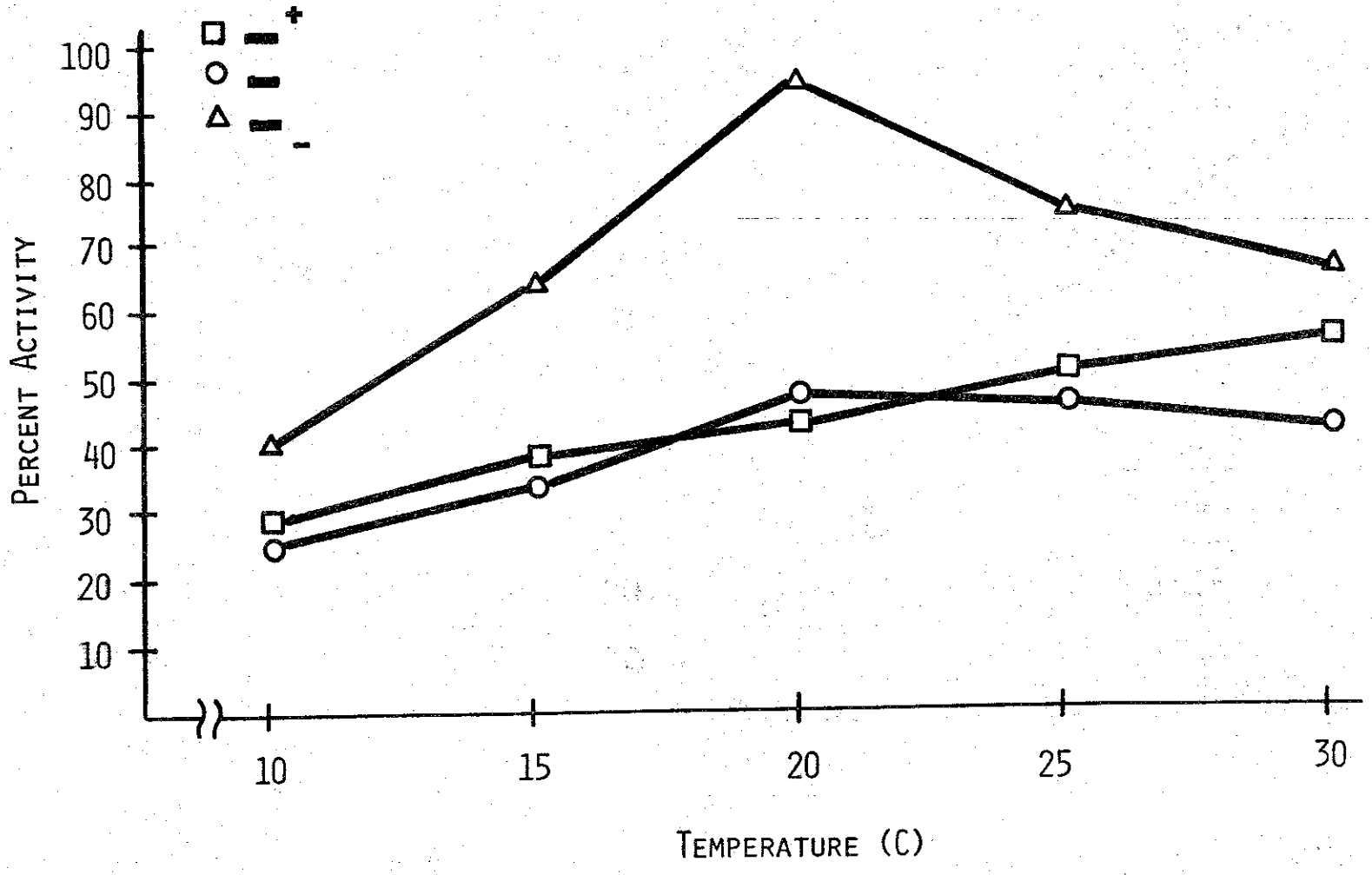


Figure 5. Percent activities at five temperatures for three isozymes comprising the mm homozygous pattern for malate dehydrogenase (Mdh-1, Mdh-2).

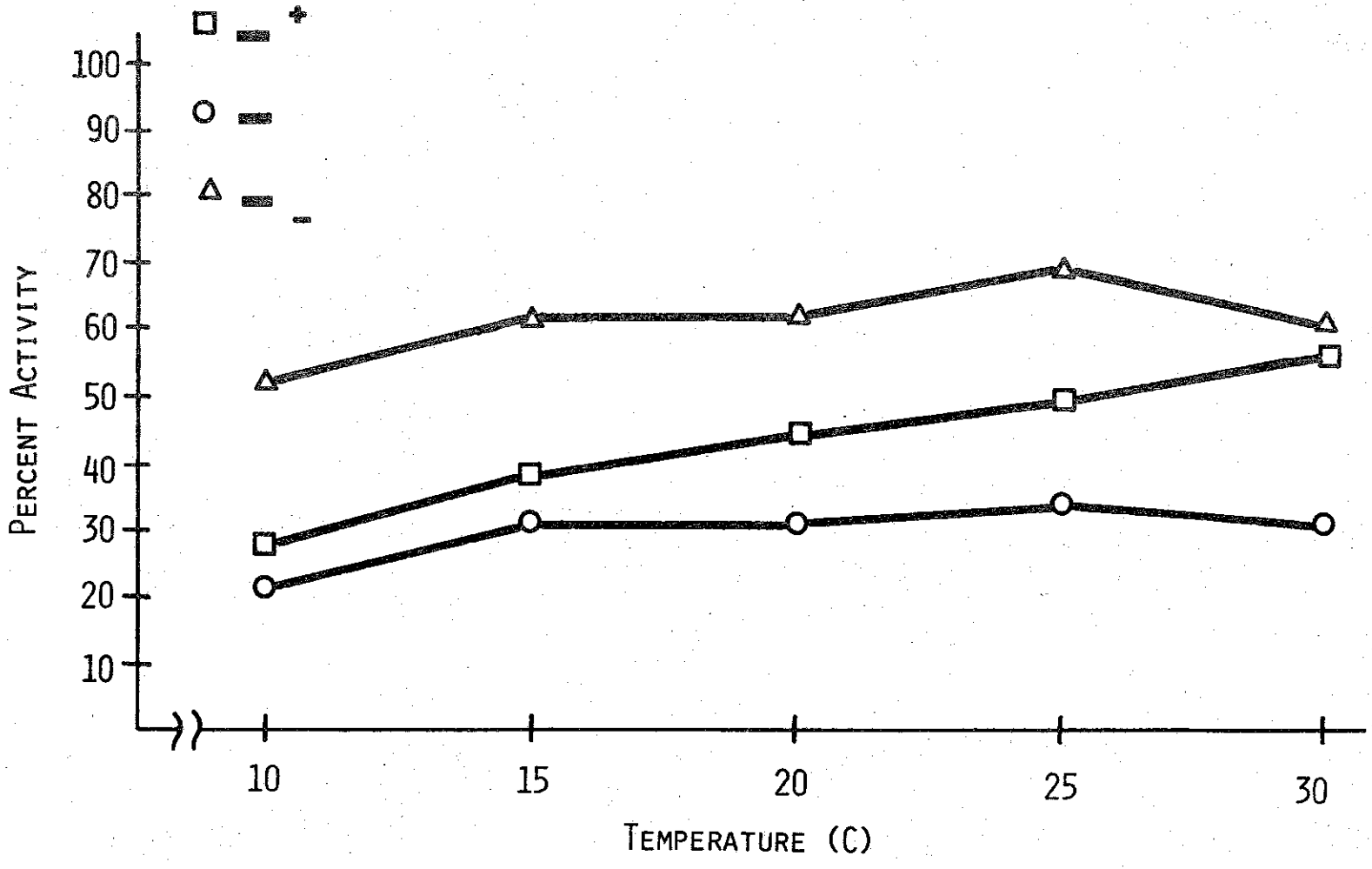


Figure 6. Percent activities at five temperatures for three isozymes comprising the ss homozygous pattern for malate dehydrogenase (Mdh-1, Mdh-2).

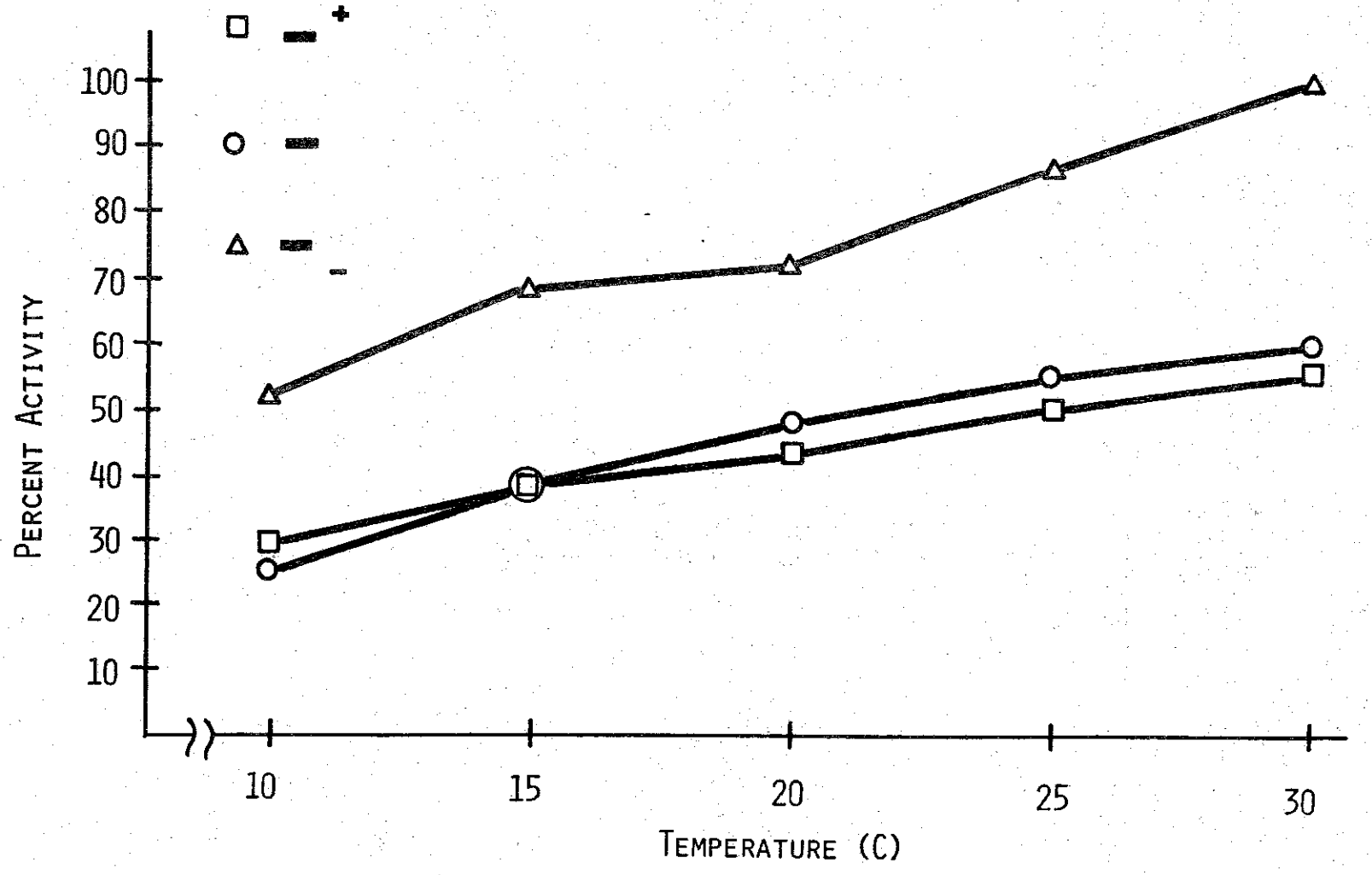
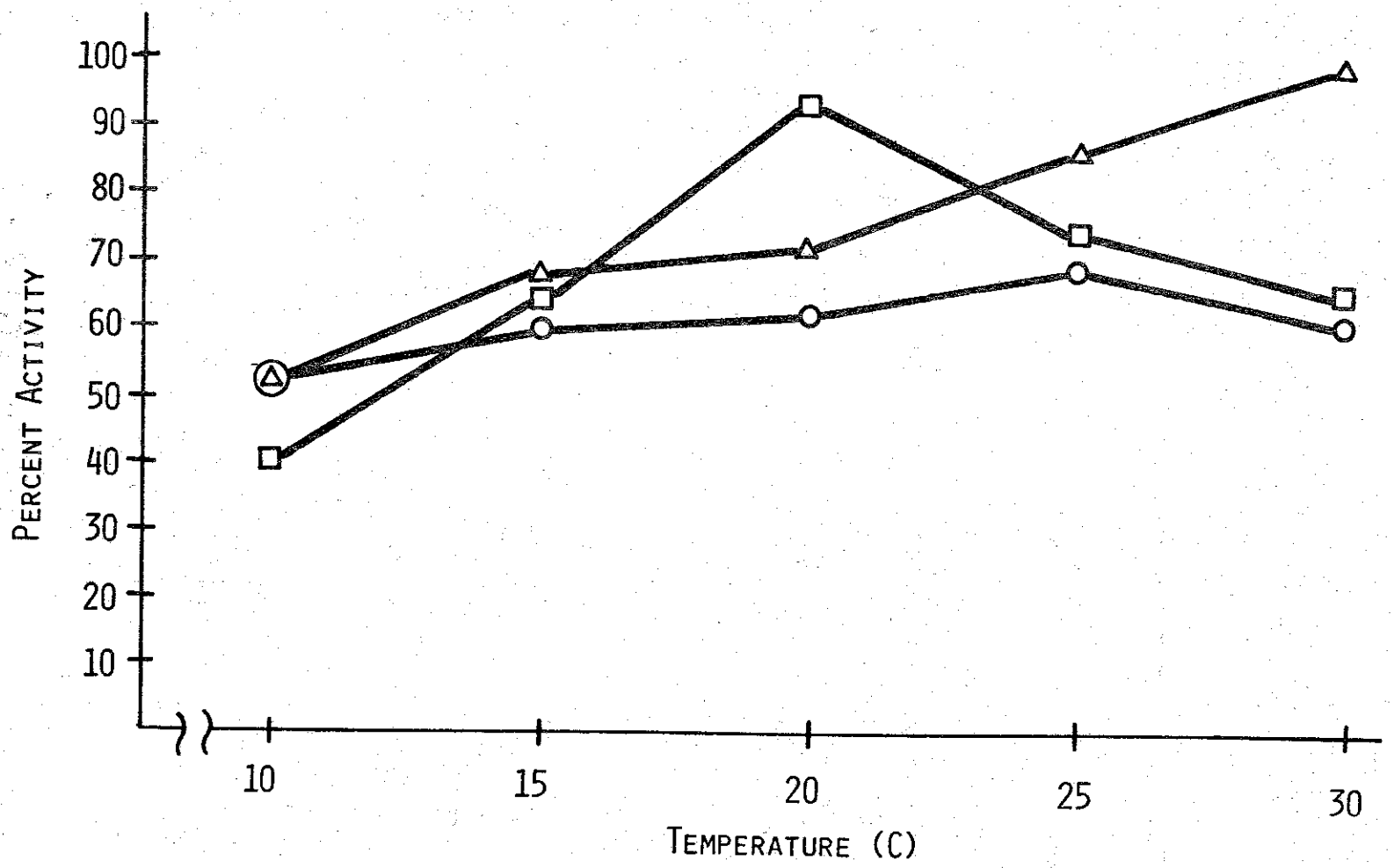


Figure 7. Percent activities at five temperatures for slowest migrating isozymes from the three homozygous genotypes for malate dehydrogenase (Mdh-1, Mdh-2); □ = the ff pattern isozyme, O = the mm pattern isozyme, Δ = the ss pattern isozyme.



reached an optimal activity of 100% at 30°C (Fig. 7). Therefore, three different thermal optima exist for the three slow electromorphs, i.e., 20°C for ff, 25°C for mm, and 30°C for ss.

CHAPTER IV

DISCUSSION

Since the evolution of deoxyribonucleic acid, subsequent evolution has occurred primarily by modification of already-existing components of DNA. An average sequence of 1000 nucleotides are needed to code for the "typical" protein in a eukaryotic genome consisting of 1.5×10^6 such nucleotide sequences. The origination of proteins requires a concomitant increase in the genome. It seems unlikely that all proteins have arisen de novo; rather it is more probable that gene duplication, followed by mutation, has allowed the formation of proteins with varying structure and function (Markert et al., 1975). Many proteins such as phosphoglucose isomerase (Avise and Kitto, 1973), glucose-phosphate isomerase (Dando, 1974; Whitt et al., 1976), cytochrome c, the α and β subunits of hemoglobin, and lactate dehydrogenase have been demonstrated to have arisen via gene duplication (Markert et al., 1975).

Likewise, the two loci of the supernatant malate dehydrogenase (s-Mdh) have been demonstrated to have arisen from gene duplication (Bailey and Wilson, 1970; Whitt, 1970; Karig and Wilson, 1971). Duplication has been documented in other fish (Bailey and Wilson, 1970; Wheat et al.,

1971; Numachi et al., 1973, 1973; Avise and Selander, 1972). Malate dehydrogenase exists as a dimer, therefore, with one allele segregating from the Mdh-1 locus, and three alleles segregating from the Mdh-2 locus in N. lutrensis, three homozygous patterns each consisting of three isozymes, and three heterozygous patterns each consisting of six isozymes result. Similarly, six genotypes for s-Mdh were demonstrated in the walleye, Schizostedion vitreum (Clayton et al., 1971) and in the Mexican tetra, Astyanex mexicanus (Avise and Selander, 1972).

The structural variability in s-Mdh patterns in N. lutrensis is accompanied by functional differences between isozymes with respect to temperature. In homozygotes, the slowest migrating isozymes always exhibited the greatest activity over the other isozymes comprising the s-Mdh patterns. The slowest migrating isozymes from each genotype also exhibited different thermal optima. Therefore, heterozygous combinations between any two s-Mdh patterns resulted in the formation of six isozymes, each with a different thermal optimum. Populations of N. lutrensis are highly polymorphic at the Mdh-2 locus, with a mean level of heterozygosity in Brazos River populations of 40.4%. Furthermore, levels of heterozygosity can be demonstrated to be correlated to the degree of daily fluctuation in water temperatures for each locality.

Levels of heterozygosity observed increased from the 1-km population to the 30-km population, followed by a reduction in heterozygosity at the 121-km population. This pattern of variation is not typical of a cline. For instance, fish heterozygous for the f and s alleles at the Mdh-2 locus have six different s-Mdh isozymes, each with an optimum activity somewhere between 20 and 30°C. It is not surprising that the highest proportion of such Mdh-2 heterozygotes were found at the 30-km population where daily water temperatures fluctuate the greatest, 7°C. Furthermore, the ff homodimer appears to be a "cold-water" isozyme and occurred in frequencies greater than 0.05 only in populations within 121 km of Morris Sheppard Dam, where cold water is released continuously from the hypolimnion.

Several other studies have demonstrated functional differences in enzymes in relation to temperature in attempts to explain the genetic composition at the locus controlling that enzyme. For instance, Koehn et al., (1971) investigated heterozygote deficiency in populations of Notropis stramineus and demonstrated temperature differences in isozymes comprising from the Es-1 and Es-2 loci. Es-1 isozymes in the heterozygous condition exhibited maximum activity at higher temperatures, while the Es-1 isozymes in the homozygous condition exhibited maximum activity at lower temperatures. These authors concluded

that the observed functional differences were indicative of selection, wherein the heterozygotes were at an advantage at higher environmental temperatures, and the homozygotes were at an advantage at lower environmental temperatures. In tobacco plants, De Jong (1973) demonstrated changes in relative isozyme intensities and shifted migration rates of some isozymes of malate dehydrogenase in response to changes in temperature and day-length. He believed that these changes were adaptive in nature, since certain isozymes performed more efficiently under different environmental parameters. McNaughton (1974) discriminated populations of cattail, Typha latifolia, on the basis of malate dehydrogenase thermostabilities, activation energies, and activity levels. T. latifolia, which has a broad climatic range, may owe its success to the ability to evolve different climatic genotypes as directed by natural selection.

My study on N. lutrensis indicates that selection has been an important factor in the maintenance of genetic variation at the Mdh-2 locus. The degree of heterozygosity at the locus appears to be adaptively important in relation to the degree of thermal heterogeneity at the collection sites; an increase in the degree of thermal heterogeneity was accompanied with an increase in the level of heterozygosity at the Mdh-2 locus. This supports Levins (1968) hypothesis

that genetic variation should increase with environmental variability. Since functional differences exist among the s-Mdh isozymes, heterozygous individuals are able to provide the required, variable response to immediate temperature changes. Therefore, heterotic balancing selection in populations of N. lutrensis has acted to maintain higher levels of heterozygosity at the Mdh-2 locus in populations from habitats with greater temporal heterogeneity.

Demonstration of the importance of human activity and alteration of natural ecosystems in genomic modification is not without precedence, and discussion of the classical examples is unnecessary here. However, with the advent of allozyme analysis, these studies have broadened in scope. The effects of thermal alteration on aquatic ecosystems, as produced by the U. S. Atomic Energy Commission's Savannah River Plant (SRP), has been the topic of numerous investigations. For over 20 years, effluents from the reactors have been directly discharged into the surrounding ecosystem, causing thermal regimes to range from normal to temperatures exceeding 50°C (Gibbons and Sharitz, 1974). This thermal heterogeneity, as created by human activity, has greatly affected the structure of this aquatic ecosystem. The most obvious effect was a reduction in species diversity (Parker et al., 1971). The effects of such severe thermal alterations were also examined from the

standpoint of genetic selection. The Malate dehydrogenase-1 locus in populations of largemouth bass, Micropterus salmoides, demonstrated changes in allelic frequencies in response to different temperature regimes. In thermally affected regions, the Mdh-1^a allele occurred at high frequencies, while the Mdh-1^b allele exhibited high frequencies in regions with a normal thermal regime. This indicates that Mdh-1^a allele had a selective advantage in abnormal thermal conditions, while the Mdh-1^b allele has a selective advantage under normal thermal conditions (Yardley et al., 1974). Furthermore, fish from a post-thermal habitat exhibited intermediate allelic frequencies, perhaps returning to normal conditions.

Nyman (1975) examined the effects of thermal effluents from the Oskarshama Nuclear Power Station (OKG) in Sweden, constructed in 1972. The major emphasis was on changes in migratory patterns and genetic composition in populations of ruff fish, Gymnocephalus cernua. Within the period of 1 year, fish migrating into areas with a high thermal regime exhibited high frequencies for the "fast" allele encoding from a serum esterase locus, while fish leaving the thermally altered area demonstrated lower frequencies for this allele. Furthermore, the population of ruff fish immediately outside the area with high thermal regimes exhibited an increase in the frequency of the most anodal

allele, presumably an effect from immigrant fish. Examination on the effects of the OKG (Nyman, 1975) and on the effects of the SRP (Yardley et al., 1974) indicate that short-term evolutionary changes may occur as a result of drastic thermal alterations.

The current study also reflects the effects of environmental heterogeneity, as created by man, on genetic composition. Morris Sheppard Dam was completed in 1941, with impoundment beginning at the same time. From an evolutionary standpoint, the genetic variation in s-Mdh as established herein has evolved rather recently. N. lutrensis spawns throughout the spring and summer in a variety of conditions (Miller and Robinson, 1973). From observations of the occurrence of breeding males at the collection sites, preliminary data indicate that N. lutrensis reproduces at least twice and perhaps three times a year. Therefore, a maximum of only 108 generations were needed for the evolution of the observed genetic variation at the Mdh-2 locus. Apparently, drastic changes in thermal regimes were not required for selection to act on this locus, and thermal heterogeneity on the Brazos River, as created by human disturbances, appears to be the primary factor involved with genetic variation at the Mdh-2 locus in populations of N. lutrensis.

APPENDIX I

km Below Reservoir	N		Frequencies FF	FM	FS	MM	MS	SS	X^2	P
1	48	OBS	0	3	3	28	13	1	4.35	.43
		EXP	.2	4.5	1.1	27.0	13.5	1.7		
30	154	OBS	2	11	8	67	55	11	3.37	.50
		EXP	.9	14.9	6.4	64.9	55.2	11.7		
57	93	OBS	1	4	5	48	27	8	7.02	.15
		EXP	.3	7.5	2.8	43.4	32.8	6.2		
121	31	OBS	0	0	1	19	10	1	2.66	.60
		EXP	0	.9	.3	18.4	10.0	1.4		

APPENDIX II

Source of Variation	DF	SS	MS	F_s
Among Temperature groups	3	295.8094	98.6031	10.09**
Linear Regression	1	269.1972	269.1972	20.23*
Deviations from regression	2	26.6122	13.3061	
Within groups	8	78.1522	9.7690	
Total	11	373.9626		

* = 0.05<P<0.01
** = 0.01<P<0.001

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