Comparative genetics of rainbow trout from the geothermally heated Firehole River, Wyoming
by Paul Wayne Fisher

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Zoology
Montana State University

Abstract:
Rainbow trout from warm and cool sections of the Fire-hole River and a hatchery strain were compared by starch gel electrophoresis. No statistically significant deviations from Hardy-Weinberg expectations were observed at any of the 25 loci examined. Nor were there any statistically significant differences in allele frequencies between populations, although the rare allele at three loci (EST-S, LDH-4 and MDH-1) were not observed in the hatchery strain. The implications of these findings are discussed and the possibility of a loss of cold acclimatory ability among the Firehole River rainbow trout is proposed.

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Approved:

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Head, Major Department

Graduate Dean

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ABSTRACT

Rainbow trout from warm and cool sections of the Firehole River and a hatchery strain were compared by starch gel electrophoresis. No statistically significant deviations from Hardy-Weinberg expectations were observed at any of the 25 loci examined. Nor were there any statistically significant differences in allele frequencies between populations, although the rare allele at three loci (EST-S, LDH-4 and MDH-1) were not observed in the hatchery strain. The implications of these findings are discussed and the possibility of a loss of cold acclimatory ability among the Firehole River rainbow trout is proposed.

INTRODUCTION

The effects of temperature on aquatic poikilotherms has received much attention over the years. The body temperature of these organisms generally stays within 1C of the ambient temperature (Hazel and Prosser, 1974), yet they are able to stabilize their metabolic rate while habitat temperatures fluctuate. This compensation is the result of a variety of physiological and biochemical adjustments which occur over three distinct time courses: 1) instantaneous compensations; 2) adjustments which require a period of acclimation (or acclimatization), usually several weeks in duration; and 3) those compensations which occur on an evolutionary time scale, involving many generations (Somero, 1969). The mechanisms of the first two have been well studied, primarily because they are amenable to laboratory manipulation. The third, however, has received comparatively little attention because of the time required. The Firehole River of Yellowstone National Park provides a unique opportunity to study the long term effects of thermal enrichment on populations of rainbow (Salmo gairdneri) and brown (S. trutta) trout.

The Firehole River is wholly contained within Yellowstone National Park, originating as a small, cold stream near Madison Lake (Figure 1). The mean monthly temperatures

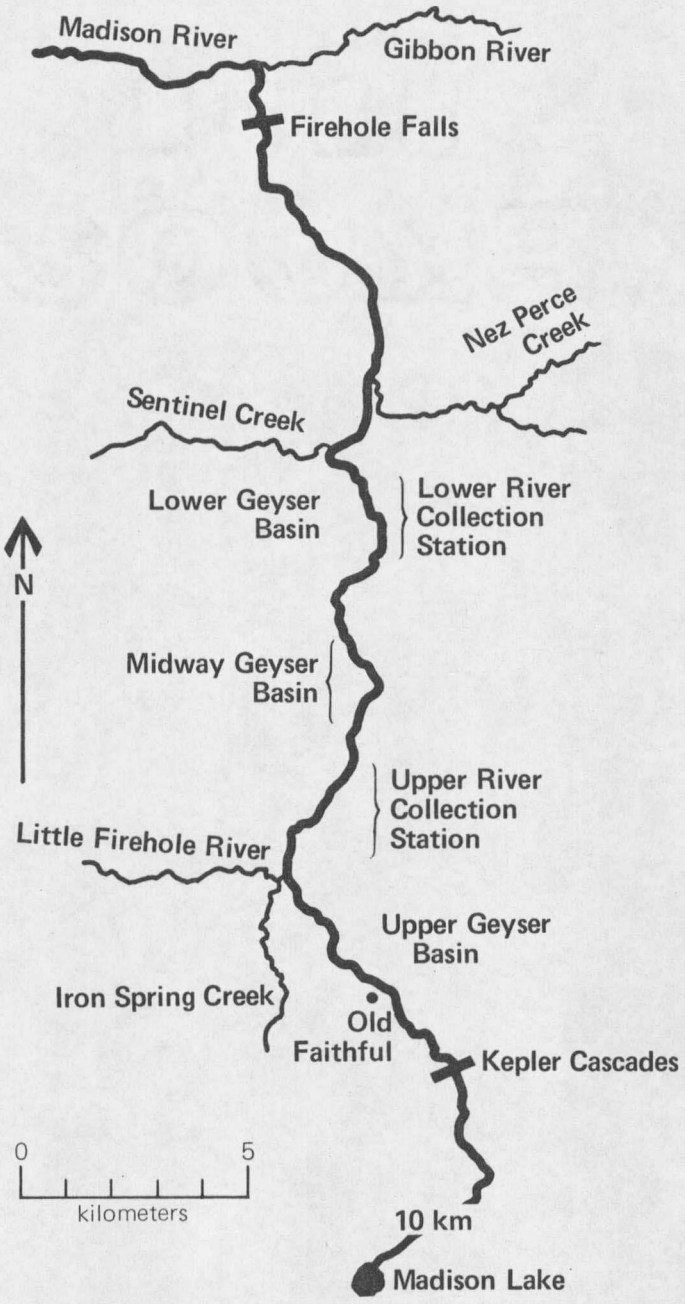

Figure 1. Map of the Firehole River, Yellowstone National Park, Wyoming with location of collection stations.

of the river between Madison Lake and Kepler Cascades vary from 2C in January to 14C in July, typical of other mountain streams in the area (Burkhalter, 1979). Below Kepler Cascades the river drains three major geothermal areas. The first is the Upper Geyser Basin which includes Old Faithful geyser. Thermal input from this basin raises the water temperature by approximately 10C. Further downstream the river runs through the Midway and Lower Geyser Basins which raises the average monthly water temperatures, in the area immediately below the Lower Geyser Basin, to 12C in January and 26C in July with an observed maximum of 29.5C. Thermal inflow from these and several smaller geothermal features account for 20-40% of the total non-runoff discharge, resulting in a temperature increase of approximately 11C and an increase in mineral (Na^+, HCO_3^-, Cl^-, and SiO_2) content with an accompanying increase in primary production and benthic insect production (Armitage, 1958; Boylen and Brock, 1973).

The Firehole River was barren of fish until 1889 when stocking of rainbow, brown and brook (Salvelinus fontinalis) trout was initiated (Benson et al., 1959). Stocking was discontinued in 1955 and the populations have been selfsustaining and capable of supporting a sport fishery since that

time. Today brook trout are mainly restricted to the area above Kepler Cascades where they constitute 85% of the fish population with brown trout making up the remainder (Kaya, 1978a). Immediately below Kepler Cascades, which forms a barrier to upstream movement, brown trout predominate with a small percentage of rainbows. As the water temperature increases downstream the proportion of rainbow trout increases, reaching 75% in the warmest reaches of the river just below the Lower Geyser Basin. All of the Firehole River fish are isolated by Firehole Falls which forms a barrier to upstream movement of fish from the Madison River system (Kaya, 1978a).

Kaya (1977, 1978b) and Kaeding and Kaya (1978) found fish inhabiting the thermally enriched sections of the river to have some interesting characteristics. Both rainbow and brown trout show two growth checks per year in this area, one in midwinter due to low temperatures, the other in late summer, apparently due to the elevated temperatures. Both species also gain a distinct growth advantage over trout in the unheated sections of the river, presumably because of favorable temperatures in early spring and enhanced food availability. Reproductive success of brown trout is low in the hottest sections of the river with a small percentage of the population developing mature gonads and many of these

showing subsequent gonadal regression. Rainbow trout, however, have been shown to reproduce successfully in the warmest areas. Eggs taken from redds in the vicinity of the Lower Geyser Basin all proved to be of rainbow trout. The Firehole rainbow trout are now a fall spawning population. Gonads begin to enlarge in August and reach full maturity in November with spawning taking place in November and December. Since all of the hatchery strains used in stocking the river were spring spawners, a shift in spawning time has apparently been selected for within the Firehole population. Juvenile and fingerling rainbow trout from the Firehole and two hatchery strains were tested for thermal tolerance. When acclimated to high temperatures (21 or 24.5C) no difference was observed between the groups, all of them having an upper incipient lethal temperature of 26.2C. This is interesting in that the average monthly temperature of the Firehole River in July is 26C with an observed maximum of 29.5C, well above the measured incipient lethal temperature, yet no recent fish kills have been observed. At intermediate and low acclimation temperatures (17-5C), the Firehole fish did show a higher thermal tolerance than either hatchery strain.

It was these observations that stimulated interest in studying the genetics of the rainbow trout inhabiting the Firehole River. It was proposed that the Firehole fish would be either a specialized population uniquely adapted to the thermally enriched environment, in which case they would be expected to have a relatively low average heterozygosity ($\bar{H}$), or that they would represent a highly plastic, very eurythermal population with a high average heterozygosity. Brown (1978) measured $\bar{H}$ for these rainbow trout and found them to be heterozygous at 20% of the 25 loci surveyed giving an $\bar{H}$ value of 0.049. $\bar{H}$ values for the majority of 41 other native and hatchery rainbow trout populations fall within one standard error of this figure, indicating that the average heterozygosity of the Firehole population is typical of this species. In the course of his study Brown found one locus, esterase-1, to be out of Hardy-Weinberg equilibrium, with a deficiency of heterozygotes. Several explanations for this were presented: lumping samples from two different subpopulations, with different allele frequencies, into one population; selection against the heterozygotes; or misinterpretation of the genetic basis of the polymorphism; but none of these were confirmed.

Adaptation of poikilotherms to a new thermal regime will undoubtedly include qualitative enzymic changes (Somero, 1969). Mechanisms to adjust metabolic rate and regulatory functions to elevated temperatures could include shifts in maximum enzyme-substrate affinity (minimum K_m), increased conformational stability, adjustment of activation energy and ligand binding, and compensations for interaction with an altered lipid and ionic milieu (Alexandrov, 1967; Hochachka and Somero, 1968, 1973; Hazel and Prosser, 1974). Many of these compensations can be expected to be associated with changes in the primary structure of enzymic proteins.

Several techniques have been developed for the detection of differences in primary structure. Of these, electrophoresis has become the method of choice because of the specificity, simplicity of visualization of gene products, and capacity to utilize crude extracts in small quantities (Brewer, 1970). The method separates proteins primarily on the basis of electrical charge, but starch gel electrophoresis has the added advantage of also separating proteins, to some extent, according to size and conformation. This is a result of the pores of the starch gel matrix being approximately the same size as protein molecules, giving the matrix a sieving effect resulting in increased resolution.

Coyne (1976) has shown that the use of a variety of buffer systems can increase the resolving power of electrophoresis. The pH of the buffer influences the net electrical charge of the protein, the greater the charge the faster the rate of migration. The ionic strength of the buffer also influences migration. Low ionic strength allows faster migration of macromolecules, higher voltage drop and less heat production, while high ionic strength buffers produce less diffuse bands. The association of ions in the conducting medium with uncharged groups of the protein to form charged complexes may also affect migration and resolution.

The Ennis Hatchery Rainbow Strain was used as a comparative hatchery stock for the Firehole populations because: 1) it was derived from rainbow trout from the Ennis Fish Hatchery which supplied many of the rainbow trout used in stocking the Firehole River, so they should share many common alleles with the Firehole populations; and 2) it has undergone selection for fall spawning and therefore has been subjected to similar, even if artificial, selection pressure. These were the only fish available from this hatchery because of a temporary closure. The strain has been maintained at the Beulah, Wyoming U.S. National Fish Genetics Laboratory.

Electrophoresis was used in this study to look for genetic differences between rainbow trout inhabiting warm and cool sections of the Firehole River and the Ennis Hatchery Strain. Correlation of interpopulation genetic variation with habitat temperature may indicate a mechanism of biochemical adaptation.

MATERIALS AND METHODS

Rainbow trout were collected by D.C. electrofishing from two sections of the Firehole River. The cool, upper river station extended for approximately 3 km. between the Upper and Midway Geyser Basins (Figure 1). Samples of 30 and 19 fish were taken from this area in October 1978 and October 1979. The warm, lower river station was another 3 km. section of river within the Lower Geyser Basin approximately 2 km. upstream from Sentinel Creek. Thirty fish were collected at this station in August 1978. All fish were measured for total length, to the nearest mm., and samples of muscle, liver, heart and eye were immediately frozen on dry ice. Tissue samples were later transferred to a freezer and stored at -50C. Blood samples were obtained by bleeding directly from the heart with a Pasteur pipette. Blood samples were stored on ice until returned to the

laboratory where they were centrifuged at 5000 g's for 15 minutes to separate serum and cells. The serum was transferred to a separate vial and both fractions were stored at -50C.

Twenty fish of the Ennis Hatchery Strain were obtained from the U.S. National Fish Genetics Laboratory in Beulah, Wyoming. Blood samples were taken and separated before shipping whole carcasses on dry ice. Upon receipt of the fish, tissue samples were taken as above and stored, along with the blood samples, at -50C.

Approximately 0.5 g pieces of muscle, liver, heart and eye were ground in a glass, hand tissue homogenizer with an approximately equal volume of grinding solution (0.1M Tris, 0.001M EDTA, 10^{-5}M NADP, 10^{-5}M ADP, pH 7). Homogenates were spun at 12,100 g's for 20 minutes in a Sorval RC2-B refrigerated centrifuge. The supernatants were decanted into clean vials and stored at -50C.

Gels for electrophoresis were made by cooking and stirring 70 g of starch (Electrostarch lot #307 or Sigma Hydrolyzed Potato Starch) and 500 ml. of buffer (Appendix B) in a one liter sidearm flask. After reaching a full boil the gels were evacuated for one minute then poured into a 23 x 15 cm. form, made with 13 mm. high plexiglass borders

clamped to a 6 mm. thick glass plate. After cooling to room temperature the gels were covered with plastic wrap and refrigerated. Gels were always used within 24 hours.

Whatman, number three filter paper wicks (1.5 x 0.5 cm.) were soaked in the tissue supernatants or blood serum, any excess was blotted off, and aligned in a slit cut in the gel approximately 5 cm. from one edge. Wicks soaked in bromophenol blue marker dye flanked the samples. After loading, the two halves of the gel were held together with elastic bands. The gel was placed on electrode trays containing buffer, with several layers of cloth toweling providing contact with the gel, and the appropriate voltage was applied using a Heathkit IP-17 variable power supply. After 15 minutes the wicks were removed and electrophoresis continued until the marker dye reached the anodal end of the gel. Throughout the run the gels were cooled with trays of ice placed directly on top of each gel.

Gels were cut using a flat blade guided by 1.5 mm. thick plexiglass strips. Four or five slices, discarding the top slice, could be obtained from each gel. Each slice was stained for specific enzyme activity (Appendix C) for one-half to 12 hours in the dark. Reagent grade chemicals for stains were obtained from the Sigma Chemical Company,

St. Louis, MO or the Aldrich Chemical Company, Milwaukee, WI. When the zymograms were visible the slices were rinsed in distilled water and fixed in a 5:5:1 solution of methanol, water and acetic acid.

Notes of the electrophoretic pattern were made and a photograph taken of each slice. Genetic interpretation of the zymograms was based on previously published inheritance studies of rainbow trout and other salmonids. Statistical comparision of genotype distributions with those expected from Hardy-Weinberg equilibrium were made by chi square analysis. Comparison of allele frequencies between populations were done by a 2x3 or 3x3 contingency chi square test (Snedecor and Cochrane, 1967; Spiess, 1977).

The system of nomenclature used was adapted from Allendorf and Utter (1979). In this system each protein is named by a three letter abbreviation. If the abbreviation is italicized it represents the gene coding for that protein. If several loci code for proteins of similar function they are sequentially numbered according to their electrophoretic migration from cathode to anode. For example, AAT-1 is a more cathodal migrating protein than AAT-2. Multiple alleles at a single locus are numbered parenthetically with the most common allele being assigned the number 1.00. Less common

alleles are given values denoting their electrophoretic mobility compared to the most common allele, thus, LDH-1 (1.00) is the most common allele at the LDH-1 locus, while LDH-1 (0.50) would be a less common allele whose product migrates half as far as LDH-1 (1.00).

RESULTS

The number of loci coding for each protein was determined from zymogram patterns and the literature. The electrophoretic phenotype of each enzyme system examined is described below.

Aspartate Aminotransferase, E.C. 2.6.1.1, AAT: AAT exists in cytoplasmic and mitochondrial forms. The cytoplasmic form is a dimer coded for by two loci in salmonids (AAT-3 and AAT-4), (Allendorf et al., 1975; Allendorf and Utter, 1976; and Lynch and Vyse, 1979). These two loci interact to form three anodal migrating isozymes. The mitochondrial form is also a dimer coded for by two loci, (AAT-1 and AAT-2), but no heterodimer is formed resulting in two cathodal bands. This five banded pattern was observed in all liver samples surveyed. AAT-4 is not expressed in muscle or heart, consequently only one cytoplasmic isozyme is formed in these tissues.

Alcohol Dehydrogenase, E.C. 1.1.1.1, ADH: ADH is presumed to be a dimer coded for by a single locus (Allendorf et al., 1975). It was visualized as a single cathodal band for all liver samples examined.

∝-Glycerol-Phosphate Dehydrogenase, E.C. 1.1.1.8, AGP: Engel et al. (1971) showed AGP to be coded for by three loci in rainbow trout. In this study, as in Allendorf et al. (1975), only two loci were visualized, AGP-1 which was monomorphic and AGP-2 which segregated for two alleles, AGP-2 (1.00) and AGP-2 (0.50). Both loci interact to form dimeric isozymes which migrate cathodally in buffer system A. A three banded zymogram was seen for doubly homozygous fish muscle, while individuals heterozygous at AGP-2 produce a five banded pattern (Figure 2).

Esterase, E.C. 3.1.1.1, EST: Liver extracts produce three zones of esterase activity corresponding to three loci, EST-1, EST-2 and EST-3, (Allendorf et al., 1977) EST-1 had three alleles, EST-1 (0.90), EST-1 (1.00) and EST-1 (1.10), resulting in six single and double banded electrophoretic phenotypes in the most cathodal zone (Figure 3). EST-2 was found to be monomorphic producing a single band of activity. EST-3 had very low activity and could not be consistently resolved so it will not be considered in this discussion.

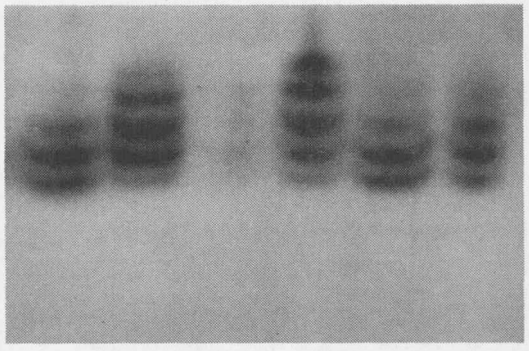

Figure 2. α-Glycerol-phosphate dehydrogenase zymogram from muscle extracts with position of the presumed homodimers. All lanes show AGP-1 homodimer.

Lane--1, 3, 5 and 6 AGP-2 (1.00) homozygotes
--2 and 4 AGP-2 (1.00/0.50) heterozygotes

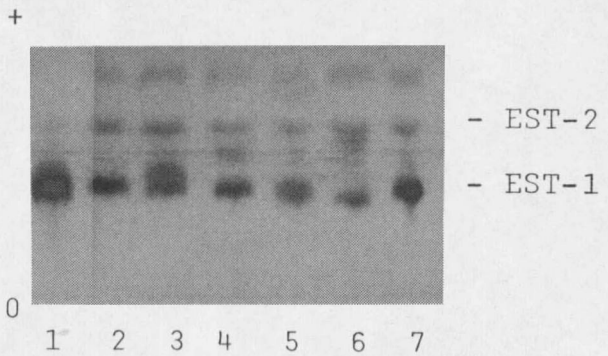

Figure 3. Esterase zymogram from liver extracts. All lanes show EST-2 activity.

Lane--1 and 5 EST-1 (0.90/1.00) heterozygotes
--2, 4 and 7 EST-1 (1.00) homozygotes
--3 EST-1 (1.00/1.10) heterozygote
--6 EST-1 (0.90) homozygote

A fourth locus is active in blood serum (Nyman and Shaw, 1971). EST-S was found to have two alleles, EST-S (1.00) and EST-S (0.80), resulting in a two banded pattern in heterozygotes (Figure 4).

Lactate Dehydrogenase, E.C. 1.1.1.27, LDH: LDH is a tetramer which is coded for by at least five loci in rainbow trout (Wright et al., 1975). LDH-1 and LDH-2 are most strongly expressed in muscle. They were both monomorphic but interact with each other to produce a five banded zymogram (Figure 5). Liver tissue expresses only one locus, LDH-4, which was polymorphic having two alleles (LDH-4 (1.00) and LDH-4 (0.60). Homozygotes produce a single band of activity while heterozygotes produce a five banded pattern. LDH-3, a monomorphic locus, and LDH-4 are both found in heart. Migration of the LDH-3 homotetramer is intermediate between LDH-4 (1.00) and LDH-4 (0.60) resulting in a compressed five banded pattern in individuals homozygous at both loci. LDH-3 expression was obscured in heart extracts of fish heterozygous at LDH-4. LDH-5 codes for a very rapidly migrating isozyme which is expressed in nervous tissue. Best visualization of this locus was in retinal homogenates where it produced a single invariant band in all individuals examined.

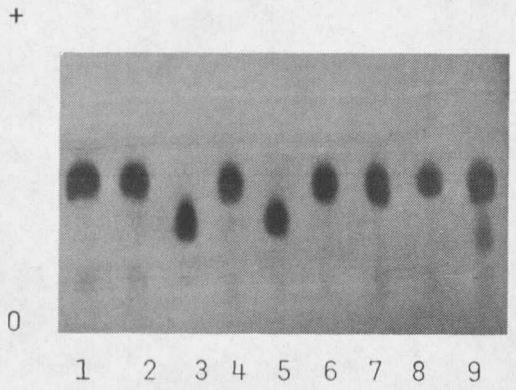

Figure 4. Esterase zymogram from blood serum.

Lane--1, 2, 4, 6, 7 and 8 EST-S (1.00) homozygotes
--3 and 5 EST-S (0.80) homozygotes
--9 EST-S (1.00/0.80) heterozygote

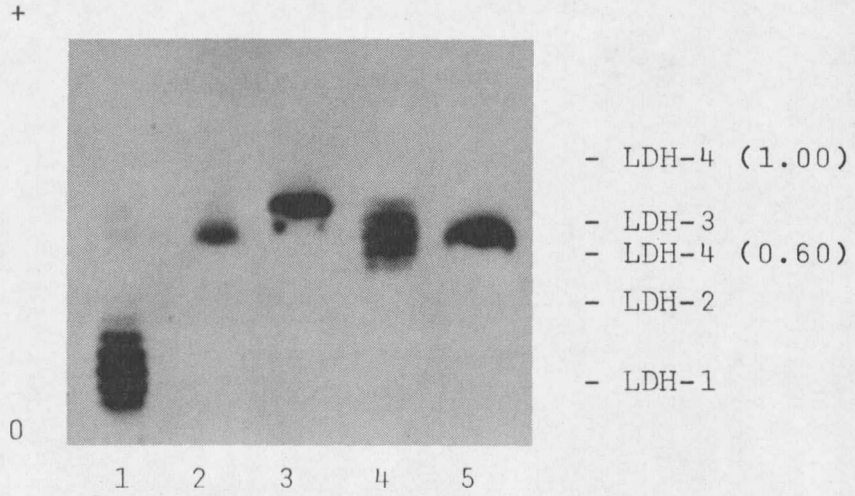

Figure 5. Lactate dehydrogenase zymogram with position of the presumed homotetramers indicated.

Lane--1 muscle, LDH-1 and LDH-2
--2 and 5 heart, LDH-3
--3 liver, LDH-4 (1.00) homozygote
--4 liver, LDH-4 (1.00/0.60) heterozygote

Malate Dehydrogenase, E.C. 1.1.1.37, MDH: There are at least three loci which code for cytoplasmic MDH (Bailey et al., 1970; Allendorf et al., 1975). MDH-2 and MDH-3 are best expressed in muscle while MDH-1 is most strongly expressed in liver. MDH-2 and MDH-3 where found to be monomorphic for the same electrophoretic allele, resulting in a single band of activity. MDH-1 interacts with MDH-2 and MDH-3 in liver producing a three banded pattern in triply homozygous fish (Figure 6). MDH-1 (1.00/0.50) heterozygotes produce a five banded pattern.

Peptidase, E.C. 3.4.1.2, PEP: Two invariant bands for this system were observed in muscle extracts. In the absence of variation the number of loci controlling the enzyme cannot be determined. For this study, as was done for brown trout (Allendorf et al., 1977) a two locus system has been assumed.

Phosphoglucose Isomerase, E.C. 5.3.1.9, PGI: PGI is a dimer coded for by three loci in rainbow trout (Avise and Kito, 1973). The products of these three loci interact to produce a six banded zymogram in triply homozygous fish. This was the pattern observed in all muscle samples examined.

Superoxide Dismutase, E.C. 1.15.1.1, SOD: The use of tetrazolium dies in staining solutions often results in the

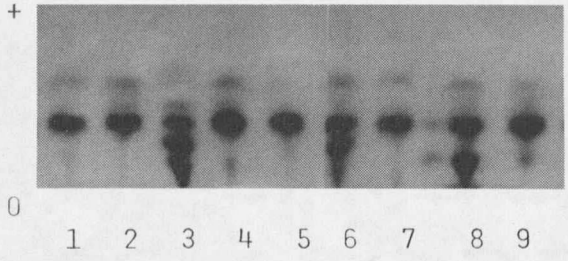

Figure 6. Malate dehydrogenase zymogram from liver extracts with position of the presumed homodimers indicated. All columns show MDH-2 and 3 homodimer.

Lane--1, 2, 4, 5, 7 and 9 MDH-1 (1.00) homozygotes
--3 and 6 MDH-1 (1.00/0.50) heterozygote
--8 MDH-1 (0.50) homozygote

nonspecific staining of the entire gel, especially in the presence of light. SOD is visualized as accromatic spots on this light blue background.

SOD is a dimer coded for by a single locus segregating for as many as three alleles in rainbow trout (Utter and Hodgkins, 1972; Diebig et al., 1979). In the populations studied here, two alleles, SOD (1.00) and SOD (0.70), were observed with homozygotes producing a single band and heterozygotes producing a three banded pattern.

Xanthine Dehydrogenase, E.C. 1.2.3.2, XDH: XDH was expressed as a single invariant band in the populations examined here as it is in most salmonid populations (Allendorf et al., 1975, 1977; Kristianson and McIntyre, 1976; Lynch and Vyse, 1979). In the absence of variation the number of loci controlling a protein cannot be determined, so the simplest model of a single locus has been assumed.

The allele frequency, the observed and expected genotype distributions (assuming Hardy-Weinberg equilibrium) and the number of individuals surveyed for each locus are listed in Tables 1, 2 and 3. The tissue expression and preferred buffer system for best resolution are listed in Appendix A.

Table 1: A list of the monomorphic loci with the number of individuals examined from each population.

Locus	LFR[1]	UFR	EHS	Locus	LFR	UFR	EHS
AAT-1	29	43	20	LDH-5	17	32	20
AAT-2	29	43	20	MDH-2	28	33	20
AAT-3	29	43	20	MDH-3	28	33	20
AAT-4	29	43	20	PEP-1	24	28	19
ADH	29	35	20	PEP-2	24	28	19
AGP-1	28	38	20	PGI-1	28	38	20
EST-2	29	28	20	PGI-2	28	38	20
LDH-1	28	38	20	PGI-3	28	38	20
LDH-2	28	38	20	XDH	28	35	20
LDH-3	30	40	20				

[1]LFR = Lower Firehole River
UFR = Upper Firehole River
EHS = Ennis Hatchery Strain

Table 2: Loci which were polymorphic in at least one of the populations examined.

Locus and Population	Observed Genotype Dist. homo / hetero / homo (Expected Genotype Dist. assuming Hardy-Weinberg equilibrium)	Frequency of the Most Common Allele	P[1]
AGP-2			
LFR[2]	0 / 1 / 27 (0.01 / 0.99 / 27.00)	0.982	0.900
UFR	0 / 6 / 32 (0.24 / 5.53 / 32.23)	0.921	0.900
EHS	0 / 4 / 16 (0.20 / 3.60 / 16.20)	0.900	0.900
EST-S			
LFR	0 / 1 / 29 (0.01 / 1.00 / 28.99)	0.983	0.900
UFR	3 / 4 / 28 (0.71 / 8.58 / 25.71)	0.857	0.250
EHS	0 / 0 / 20 (0.00 / 0.00 / 20.00)	1.000	0.900
LDH-4			
LFR	0 / 1 / 28 (0.01 / 0.97 / 28.02)	0.983	0.900
UFR	0 / 6 / 29 (0.26 / 5.50 / 29.24)	0.914	0.900
EHS	0 / 0 / 20 (0.00 / 0.00 / 20.00)	1.000	0.900

Table 2 (cont.)

Locus and Population	Observed Genotype Dist. homo / hetero / homo (Expected Genotype Dist. assuming Hardy-Weinberg equilibrium)	Frequency of the Most Common Allele	P
MDH-1			
LFR	1 / 1 / 26 (0.08 / 2.86 / 25.06)	0.946	0.500
UFR	0 / 1 / 31 (0.01 / 1.01 / 31.00)	0.984	0.900
EHS	0 / 0 / 20 (0.00 / 0.00 / 20.00)	1.000	0.900
SOD			
LFR	8 / 12 / 3 (8.53 /10.95 / 3.52)	0.609	0.750
UFR	6 / 13 / 1 (7.81 / 9.38 / 2.81)	0.625	0.100
EHS	3 / 7 / 6 (2.64 / 7.72 / 5.64)	0.594	0.750

[1]The probability of exceeding the deviation of the observed genotype distribution from the expected.

[2]LFR = Lower Firehole River
UFR = Upper Firehole River
EHS = Ennis Hatchery Strain

Table 3: Results for the three allele EST-1 locus.

Population	Observed Genotype Dist. SS / SM / MM / MF / FF / SF (Expected Genotype Dist. assuming Hardy-Weinberg equilibrium)	Allele Frequencies S/M/F	P[1]
LFR[2]	2 / 5 / 16 / 3 / 2 / 1 (0.86 / 6.88 / 13.81 / 5.52 / 0.55 / 1.38)	0.172/0.690/0.138	0.750
UFR	2 / 5 / 13 / 5 / 3 / 0 (0.72 / 5.80 / 11.58 / 7.06 / 1.07 / 1.77)	0.161/0.643/0.196	0.100
EHS	0 / 5 / 6 / 5 / 2 / 2 (0.61 / 3.85 / 6.05 / 6.05 / 1.51 / 1.95)	0.175/0.550/0.275	0.950

[1]The probability of exceeding the deviation of the observed genotype distribution from the expected.

[2]LFR = Lower Firehole River
UFR = Upper Firehole River
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DISCUSSION

The fish of the Firehole River have been exposed to an elevated thermal regime for as long as 90 years and have been completely isolated from fish of the Madison River system by Firehole Falls. Migration within the river also appears to be restricted. Kaya (1978a) marked 500 fish in the vicinity of the Lower Geyser Basin and did not recover any of the marked fish above the Midway Geyser Basin one month later. Burkhalter (1979) observed that Excelsior Geyser, in the Midway Basin, produces a thermal gradient which nearly extends across the entire river. Whether this forms an impassable barrier to fish is not known but it could reduce migration between the upper and lower Firehole populations. Although gene flow between the Firehole populations may have been minimal since the cessation of stocking in 1955, no statistically significant genetic differences were found at any of the 25 loci surveyed. There were also no statistically significant differences between the Firehole populations and the Ennis Hatchery Strain.

All loci were found to be in compliance with Hardy-Weinberg expectations, but agreement of genotype distributions with the square law does not prove compliance with Hardy-Weinberg conditions (Spiess, 1977). For example, if the fitness values for the genotypes AA (W_1), Aa (W_2) and

aa (W_3) form a geometric progression ($1:r:r^2$) the population will appear to be in Hardy-Weinberg equilibrium even though it is experiencing selective pressures. A balance between such factors as inbreeding, selection or migration can also imitate Hardy-Weinberg conditions; for instance, inbreeding coupled with overdominance can cause a population to fit the square law. Consequently, while deviation from the square law demands explanation, compliance does not exclude the influence of selection, non-random mating, genetic drift, or migration.

EST-1, which was found to be out of Hardy-Weinberg equilibrium by Brown (1978) has been determined to be a three allele system. In the early stages of this study, before resolution of the third allozyme, the medially migrating allele, EST-1 (1.00) was scored as a null allele resulting in a heterozygote deficiency similar to that observed by Brown. Once the third allele was resolved consistently this locus also conformed to Hardy-Weinberg expectations.

The differences in allele frequency between the populations were also found to be within statistical limits. The greatest differences were observed at the EST-S locus (P=0.500) If different selective pressures are indeed acting

on these populations, at these loci, divergent allele frequencies would be expected after 24 years of separation (Prakash et al., 1969; Spiess, 1977).

It is possible that differences in allele frequency have been overlooked due to the limitations of electrophoresis. Electrophoresis is able to resolve only those changes in primary structure which result in a change in the net electrical charge of a protein. Changes of this type are estimated to account for only 30% of all amino acid substitutions (Shaw, 1965, 1970). Thus, the loci examined may possess variation which was not recognized. Due to the staining techniques available, electrophoresis is also limited to the study of structural genes which code for enzymes with fairly high activities or for protein products which are found in high concentration, such as some of the serum proteins. The technique does not allow for the investigation of regulatory loci which may play a major role in the thermal adaptation of poikilotherms.

Reproduction is both physiologically and behaviorally complex, so the coordinated action of many genes are probably involved. Adaptation to a new annual cycle of reproduction may involve selection at both structural and regulatory loci. Since rainbow trout have been in the

Firehole River for less than 90 years, the new reproductive cycle is most likely the result of selection on previously existing genetic potential and not a function of the accumulation and selection of new mutations. This assumption is supported by the fact that a shift to fall spawning has been successfully selected for in several hatchery strains of rainbow trout, including the Ennis Hatchery Strain.

While the genetic potential to change reproductive strategy appears to have been present in the Firehole population, this does not seem to be the case in regard to thermal tolerance. Rainbow trout from the Firehole River and two hatchery strains showed no difference in upper incipient lethal temperature when acclimated to high temperatures (Kaya, 1978b). A population of bluegills (Lepomis macrochirus) was found to have an elevated critical thermal maximum and death point after 13 years of exposure to thermal effluent from a nuclear power plant (Holland et al., 1973). Thus, thermal enrichment of the environment may impose sufficient selective pressure on a population of poikilotherms to raise its thermal tolerance if the genetic potential for such a change is present within the population. An electrophoretic survey of 20 loci in this bluegill population did not reveal any differences in allele frequencies

as compared to populations inhabiting thermally unenriched ponds (Yardley et al., 1974).

Temperature acclimation or adaptation of poikilotherms involves almost every aspect of their physiology and biochemistry, resulting in a major metabolic reorganization (Hochachka and Somero, 1971, 1973; Somero and Hochachka, 1971; Hazel and Prosser, 1974; Shaklee et al., 1977). Increases in temperature are associated with a decrease in pH, and an increase in lipid saturation. Temperature increases also affect the concentrations of electrolytes and the relative role of various metabolic pathways in intermediary metabolism. The enzyme complement of an organism must compensate for these changes to stabilize metabolic rates and regulatory functions.

Bluegills, as well as most teleosts, have developed a complement of eurytolerant enzymes, each one capable of maintaining its functional integrity over the entire temperature range of the species (Somero, 1975; Shaklee et al., 1977). These enzymes adjust their kinetics to compensate for temperature changes through changes in relative enzyme concentrations, concentrations of allosteric effectors, adjustment of the affinity for these allosteric effectors

(K_i, K_a) and conformational changes (Hochachka and Somero, 1973; Moon, 1975; Wilson et al., 1975).

Salmonids on the other hand exploit a different strategy of adaptation. They produce a series of stenotolerant isozymes rather than a single eurytolerant enzyme (Hochachka and Somero, 1968; Somero and Hochachka, 1971; Hazel and Prosser, 1974). Each isozyme is coded for by a separate locus and is only produced under limited thermal conditions. For example, acetylcholine esterase from the brain of rainbow trout exists in two isozymic forms. When a fish is acclimated to 17C only the warm isozyme is produced. If acclimated to 2C only the cold form is found, while both isozymes are produced at 12C (Baldwin and Hochachka, 1970). Each of these isozymes is uniquely adapted to the cellular environment, and has kinetic properties suited to its particular temperature range.

It is believed that salmonids are able to exploit this strategy because they are recent tetraploids (Ohno et al., 1965; Ohno, 1970). Tetraploidy results in the duplication of all genetic loci. These redundant loci are free to accumulate random mutations, sometimes resulting in functionally different but related isozymes. Because tetraploidy duplicates the entire genome it does not result in

problems of gene dosage effects, regulatory limitations or increased unequal corssing-over associated with tandom duplications. It is, however, restricted to organisms which do not possess a well established chromosomal sex determining mechanism.

The adaptation of enzymes to temperature is often measured by their K_m-temperature relationship because of the critical importance of enzyme-substrate affinity on reaction rate and regulatory function (Hochachka and Somero, 1968). The eurytolerant enzymes of most teleosts are characterized by a consistently low and stable K_m throughout the entire temperature range of the species (Somero, 1969, 1975; Baldwin, 1971; Hazel and Prosser, 1974). The stenotolerant isozymes of salmonids typically have narrow U-shaped K_m-temperature curves indicating a rapid decline in enzyme-substrate affinity outside the narrow temperature range of the isozyme (Hochachka and Somero, 1971, 1973; Somero, 1975). The warm and cold isozymes of an enzyme system tend to have their minimum K_m points 5-10C apart, near the lower end of their respective temperature ranges, but the curves overlap to ensure proper function over a continuous temperature range. Because each isozyme is functional over only part of the organism's temperature range, salmonids have developed

regulatory mechanisms to control the expression of each isozyme.

The absence of electrophoretic differences observed here and by Yardley et al. (1974) may be due to different causes because of differences in the strategies of enzyme adaptation between trout and bluegill. Extension of the thermal tolerance of bluegills may be accomplished by changes other than altering the primary structure of various enzymes, such as changes in the effect of allosteric modulators. An increase in the thermal tolerance of rainbow trout would require either the development of a new stenotolerant isozyme or the development of a eurytolerant isozyme. Either of these changes would probably require a change in primary structure of the enzyme. Developments of this type have apparently not occurred in the Firehole River trout. In the absence of the introduction of a new, adaptive isozyme in the Firehole fish, all three of the populations would be expected to express the warm isozyme of most enzyme systems. Water temperatures during sampling of the Firehole rainbow trout exceeded 16C and the Ennis Hatchery Strain was maintained at 11C (Kinkaid, personal communication), thus all the fish were acclimated to temperatures

within the range of most warm isozymes (Somero and Hochachka, 1971).

Although the differences in allele frequencies between the populations of this study were not statistically significant, the rare allele at several loci, EST-S, LDH-4, and MDH-1, was not observed in the Ennis Hatchery Strain. This may be due to the sample or loss of the allele from the Ennis Hatchery Strain as a result of selection or inbreeding. Alternatively, the rare allele may have been contributed to the Firehole populations by another hatchery strain used in stocking the river.

Sentinel Creek (Figure 1) appears to be the only significant cold water refuge available to fish in the lower reaches of the river (Kaya et al., 1977). There does not seem to be any cold springs or other refuge present (Burkhalter, 1979). Use of Sentinel Creek is primarily by fish from downstream areas which follow the temperature gradient it creates. The lower river collection station was well above Sentinel Creek; so fish from this area probably did not take advantage of this refuge.

The increased thermal tolerance of rainbow trout from the Firehole River, when acclimated to low temperatures (Kaya, 1978b) and the distribution of rainbow trout, mainly

in the thermally enriched sections of the river below the Upper Geyser Basin (Kaya, 1978a), may indicate a loss of the ability to acclimate to low temperatures. This could involve loss of the cold isozymic form of some enzymes or the regulatory mechanisms controlling their expression. As long as the fish stayed in the geothermally influenced areas of the river, where water temperatures do not fall below 12C, there would be relaxed selective pressure to maintain the cold form of most enzymes. It would be interesting to test the critical thermal minimum of the Firehole rainbow trout and to compare these populations for isozyme expression after a period of cold exposure.

The observations discussed above suggest that the rainbow trout inhabiting the lower reaches of the Firehole River, where the water temperature in July averages 26C, are existing at the limits of their thermal tolerance. The periods when temperatures exceed the measured upper incipient lethal temperature of 26.2C, for these fish, may be brief enough not to be fatal. Burkhalter (1979) found these very high temperatures occur primarily on clear hot summer afternoons and that the low night time temperatures, clear night skies, low relative humidity and consistent breezes

of the area tend to moderate water temperatures, affording the fish a recovery period each night.

Although no recent fish kills have been observed, two were reported in the 1960's (McClelland, 1960; Roeder, 1961). This supports the assumption that trout in this area are near their upper thermal limit. Water temperature at the time of one of these kills was recorded as high as 32.6C but how long these temperatures persisted is not known.

Future study of the Firehole River trout populations should include an analysis of enzyme systems from brain tissue. The central nervous system has been shown to be one of the most sensitive areas to thermal stress and has been implicated in playing a critical role in the early stages of thermal death (Prosser and Brown, 1961; Baslow, 1967; Baldwin and Hochachka, 1970). Confirmation of the suggestion that the Firehole rainbow trout have lost the ability to acclimate to cold temperatures would be very interesting. This should include measurement of the lower incipient lethal temperature and comparison of isozyme expression with a strain of rainbow trout known to have cold acclimatory ability.

APPENDIX

Appendix A. List of loci with tissue expression and preferred buffer system for best resolution.

Locus	Tissue Expression					Preferred Buffer
	Liver	Muscle	Heart	Eye	Serum	
AAT-1	X	X	X			G
AAT-2	X	X	X			G
AAT-3	X	X	X			G
AAT-4	X					G
ADH	X					any
AGP-1		X				A
AGP-2		X				A
EST-1	X					B,G
EST-2	X					B,G
EST-S					X	D,F
LDH-1		X				B,E,F,G
LDH-2		X				B,E,F,G
LDH-3			X			B,E,F,G
LDH-4	X					B,E,F,G
LDH-5				X		B,E,F,G
MDH-1	X					G
MDH-2	X	X				G
MDH-3	X	X				G
PEP-1		X				B,H
PEP-2		X				B,H
PGI-1		X				F,G
PGI-2		X				F,G
PGI-3		X				F,G
SOD	X					F,G
XDH	X					B,F,G

Appendix B. Buffer systems.

Buffer System		pH	Voltage
A) Clayton (Clayton and Tretiak, 1972)			
Electrode:	0.04 M Citrate	6.1	250V
Gel:	0.002 M Citrate	6.0	
Both buffers are pH adjusted with N-(3-aminopropyl)-morpholine.			
B) ASA (Nelson et al., 1977)			
Electrode:	0.1 M Tris (hydroxymethyl) aminomethane (Tris) 0.1 M NaH_2PO_4	8.1	170V
Gel:	Dilute electrode buffer 1:9 with water.		
C) AMPDA (Nelson et al., 1977)			
Electrode:	0.25 M Tris 0.07 M Citrate	7.6	250V
Gel:	Dilute electrode buffer 1:15 with water.		
D) ARG (Nelson et al., 1977)			
Electrode:	0.1 M Tris Buffer is pH adjusted with acetic acid.	8.3	300V
Gel:	Dilute electrode buffer 1:9 with water.		
E) Lithium Hydroxide (Selander et al., 1971)			
Stock Sol'n A:	0.03 M LiOH 0.19 M Borate	8.1	300V
Stock Sol'n B:	0.05 M Tris 0.008 M Citrate	8.4	
Electrode:	Stock Sol'n A		
Gel:	1:9 mixture of Stock Sol'n A and B.		

Buffer System		pH	Voltage
F) Tris-Borate-EDTA (Selander et al., 1971)			
Electrode:	0.5 M Tris 0.65 M Borate 0.02 M Disodium ethylendiamine tetraacetate (EDTA)	8.0	200V
Gel:	Dilute electrode buffer 1:9 with water.		
G) Continuous Tris-Citrate (Selander et al., 1971)			
Electrode:	0.223 M Tris 0.086 M Citrate	6.3	200V
Gel:	0.008 M Tris 0.003 M Citrate	6.7	
H) Ridgeway (Ridgeway et al., 1970)			
Electrode:	0.06 M LiOH 0.3 M Borate	8.3	250V
Gel:	0.03 M Tris 0.005 M Citrate	8.0	
I) Staining Buffer (Allendorf et al., 1977)			
0.2 M Tris-HCl		8.0	

Appendix C. Staining solutions adapted from Allendorf et al. (1977).

Abbreviations: NAD = B-nicotinamide adenine dinucleotide

NADP = B-nicotinamide adenine dinucleotide phosphate

NBT = Nitro blue tetrazolium

PMS = Phenazine methosulfate

Enzyme	Stain	
AAT	Pyridoxal-5'-phosphate	1 mg
	L-aspartic acid	200 mg
	α-ketoglutaric acid	100 mg
	Fast blue BB salt	300 mg
	0.2 M Tris-HCl, pH 8.0	50 ml
ADH	NAD	10 mg
	NBT	10 mg
	PMS	5 mg
	95% ethanol	10 ml
	Ridgeway gel buffer	50 ml
AGP	NAD	10 mg
	NBT	10 mg
	PMS	5 mg
	DL-α-glycerophosphate	1 g
	Ridgeway gel buffer	50 ml
EST	Fast Garnet GBC salt	40 mg
	1% naphthyl-propionate in 1:1 acetone and water	5 ml
	Ridgeway gel buffer	50 ml

Enzyme	Stain	
LDH	NAD	10 mg
	NBT	10 mg
	PMS	5 mg
	DL-lactic acid (60% syrup)	1 ml
	Ridgeway gel buffer	50 ml
MDH	NAD	10 mg
	NBT	10 mg
	PMS	5 mg
	DL-malic acid (1.0 M, pH 7.0)	10 ml
	Ridgeway gel buffer	50 ml
PEP	O-dianisidine	50 mg
	L-leucyl-L-alanine	80 mg
	Snake venom	10 mg
	$MgCl_2$	50 mg
	Peroxidase	500 u
	0.2 M Tris-HCl, pH 8.0	10 ml
	water	40 ml
PGI	NADP	10 mg
	NBT	10 mg
	PMS	5 mg
	$MgCl_2$	10 mg
	Na-fructose-6-phosphate	25 mg
	Glucose-6-phosphate Dehydrogenase	10 u
	Ridgeway gel buffer	50 ml
SOD	NBT	10 mg
	PMS	10 mg
	Ridgeway gel buffer	50 ml
	(expose to light)	
XDH	NAD	10 mg
	NBT	10 mg
	PMS	5 mg
	Hypoxanthine	20 mg
	Ridgeway gel buffer	50 ml

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