



Genetic variation in the rainbow trout population in the Firehole River
by David Lawrence Browne

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

Montana State University

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Abstract:

Genetic variability was estimated in the rainbow trout population in the Firehole River in Yellowstone National Park by means of gel electrophoresis. The heterozygosity of 25 loci was used to estimate total heterozygosity, which was found to be typical of other rainbow trout populations. A deficit of heterozygotes exists at one locus (EST-1); possible reasons, for this are discussed.

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IN THE FIREHOLE RIVER

by

DAVID LAWRENCE BROWNE

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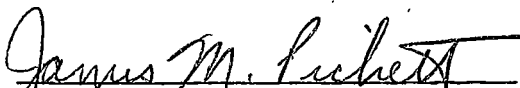
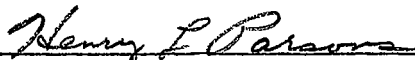
in

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TABLE OF CONTENTS

	Page
VITA	ii
ACKNOWLEDGMENTS	iii
LIST OF TABLES	v
LIST OF FIGURES	vi
ABSTRACT	vii
INTRODUCTION	1
MATERIALS AND METHODS	7
RESULTS	9
DISCUSSION	23
APPENDIX	31
LITERATURE CITED	39

LIST OF TABLES

Table	Page
1. Table of results	20

LIST OF FIGURES

Figure	Page
1. A Map of the Firehole River showing the collection area below the Midway Geyser Basin	5
2. Products of <u>PGI-1</u> , <u>PGI-2</u> and <u>PGI-3</u> , interacting to form dimers, cause six-banded zymograms	12
3. LDH zymograms of liver tissue and heart tissue	14
4. MDH zymograms of liver tissue	16
5. A gel stained for G6PD activity has light, bleached spots at the sites of SOD activity	18
6. Comparison of $\bar{H}$ between the Firehole population and 41 others	27

ABSTRACT

Genetic variability was estimated in the rainbow trout population in the Firehole River in Yellowstone National Park by means of gel electrophoresis. The heterozygosity of 25 loci was used to estimate total heterozygosity, which was found to be typical of other rainbow trout populations. A deficit of heterozygotes exists at one locus (EST-1); possible reasons for this are discussed.

INTRODUCTION

One of the keystones of evolutionary theory is the existence of heritable variation within and among populations. In Darwin's day, careful and often exhaustive anatomical comparisons documented variation, but the inheritability of such variation was unpredictable. This made absolute measurements of genetic variability impossible, and no mathematical treatments of selection and population structure could be devised.

The gene theory led to a more quantitative way of describing populations and the selective forces brought to bear upon them. It was seen that two or more alleles may exist in a population for several reasons:

1. When an allele is in the process of being replaced by another because of selection pressures, both can be found in the population. This is a transient polymorphism.
2. Two alleles that confer comparable fitness upon their carriers (neutral alleles) may coexist awhile. Random fluctuations in allele frequency will eventually cause one allele to be lost, so this is also a transient polymorphism.
3. When heterozygotes are more fit than homozygotes (overdominance), a stable polymorphism will be maintained.

4. Migration can maintain polymorphisms when selection pressures vary throughout the range of a population.

The modern recognition that proteins are the specific products of one or a few genes apiece has allowed direct and absolute measurements of genetic variation. Theoretically, any protein separation technique can be used to discern allelic variation, but gel electrophoresis has become the method of choice (Shaw, 1965; Selander, 1976; Gottlieb, 1971). It has the advantages of being sensitive to small differences in primary structure (amino acid substitutions), achieving separations from crude homogenates, and being inexpensive.

Electrophoresis exploits the fact that when charged particles or bodies are put into an electric field, they experience a force which is proportional to their charge. Proteins in solution are no exception. Several amino acids have ionizable groups which may be charged; at physiological pH's (6-8.6) most proteins carry a negative charge. The important point is that allelic products of different charge will experience different forces in an electric field, will thus migrate at different rates, and will separate. To minimize diffusion, the proteins are made to migrate through a gel slab or other matrix. A variety of polymeric materials can be used as matrixes. Starch gels, which are cheap and effective, were used in this study.

Protein-specific stains are used to visualize the position of individual gene products in a gel (Hunter and Markert, 1957).

Generally, conversion of an enzymic substrate to a product is coupled chemically to production of an insoluble dye. The location of the enzyme is revealed by spots of color in the gel.

There are limitations to both electrophoresis and staining procedures. Foremost among these is the inability to detect amino acid substitutions which do not alter electrophoretic mobility (Shaw, 1965). Also, the choice of proteins surveyed may not represent a random sampling of the genome. Proteins amenable to electrophoresis are soluble enzymes of high activity, and staining methods are most easily devised for dehydrogenases and enzymes which produce reduced products. To measure accurately electrophoretic variability, care must be taken to survey as wide a variety of enzymes, and as many, as possible (Nei and Roychoudhury, 1974). If these precautions are taken, observed electrophoretic variation will be indicative of the overall heterogeneity within a population (Lewontin and Hubby, 1966).

Electrophoretic studies have been done on many types of organisms. The most common way of expressing variability is $\bar{H}$, the average proportion of heterozygotes at all loci surveyed. A review by Powell (1975) lists estimates of $\bar{H}$ for 38 species of fish which range from 0.005 to 0.18. Among salmonids, Allendorf and Utter (1977) have estimated the following average values of $\bar{H}$: O. corhynchus gorbusha, 0.039; keta, 0.045; O. kisutch, 0.015; O. nerka, 0.018; Ontschawytscha,

0.035; Salmo apache, 0.0; S. clarki, 0.063 (coastal) and 0.023 (interior); S. gairdneri, 0.060; S. salar, 0.024.

Many of these studies had one of two goals. Some investigators (for example, Lynch, 1977) have clarified relationships within a group of related species or subspecies. Others attempted to correlate habitat or selection with allele frequencies or $\bar{H}$ (Christiansen, et al., 1975; Frydeberg, et al., 1973; Merritt, 1972). Cause-and-effect is not easily demonstrated, though; this is the goal of many population geneticists. Toward this end, knowledge of the genetic structure of marginal or peripheral populations will be useful (Prakash, et al., 1969). The variability maintained in unusual environments, either measurably dynamic or demonstrably static, will also be interesting.

One such marginal population living in a well defined environment is the Salmo gairdneri (rainbow trout) population in the upper stretches of the Firehole River in Yellowstone National Park (Fig. 1). A fifteen kilometer section of the river, isolated from the lower river by the large Firehole Falls, receives large amounts of geothermally heated water which raises the water temperature about 10C°. As documented by Kaya (1977) and Kaeding and Kaya (1978) this is marginal habitat for rainbow trout. No fish were endemic to the river above Firehole Falls. Planting began in 1889 and continued sporadically until 1955. Rainbow trout were recruited into a breeding population during that time. The reproductive schedule of this population is

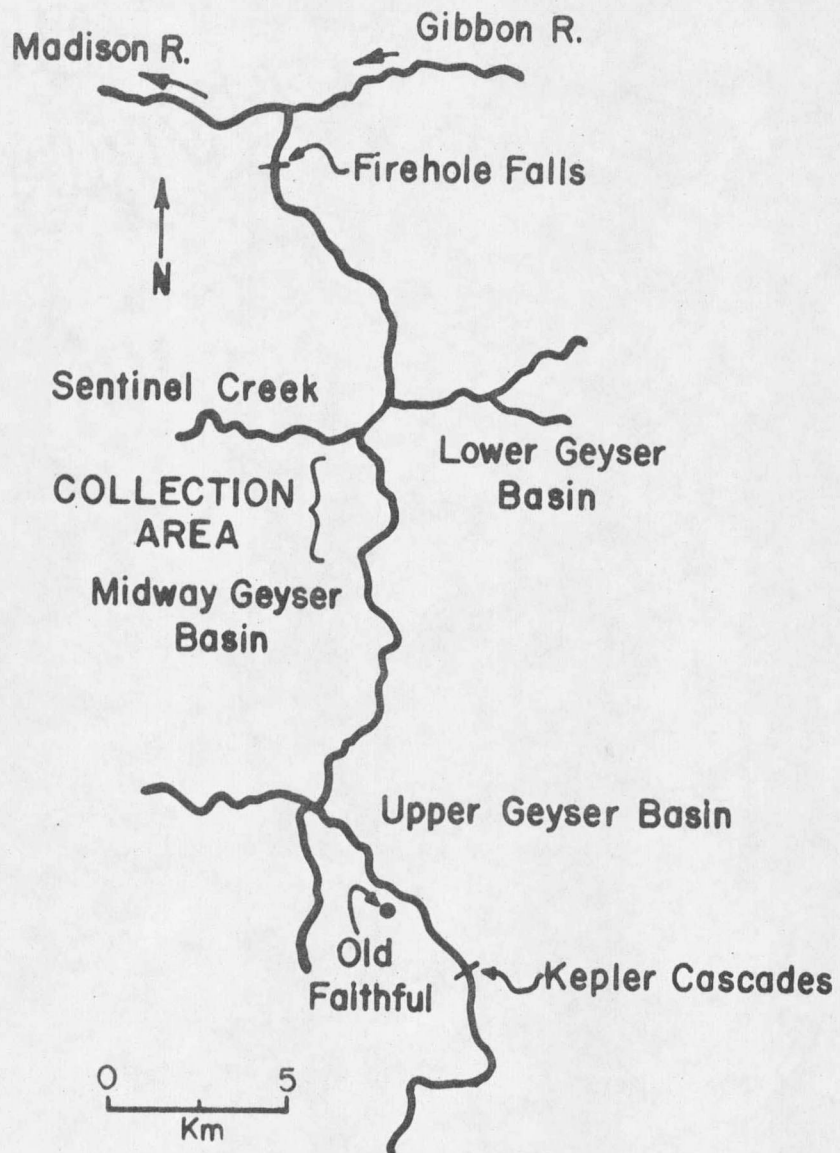


Figure 1. A map of the Firehole River showing the collection area below the Midway Geyser Basin

unusual (Kaya, 1977) because, unlike other rainbows which are spring spawners, these fish spawn in November and December. This behavior not only avoids the unfavorable water temperatures of the normal spawning season; it allows rapid growth of the young all winter, equivalent to a full year's growth in a colder regime.

The purpose of this study was to estimate variability in this population of rainbow trout by means of an electrophoretic survey. Numerous similar studies of rainbows and other salmonids provided guidelines and methodology for the survey. The genetic interpretation of electrophoretic patterns was made on the basis of previously published reports. It is hoped that this preliminary genetic survey may aid in understanding the genetic basis of the unique biology of this population.

MATERIALS AND METHODS

Rainbow trout were collected by electrofishing or angling. After weighing and measuring the fish, tissue samples (liver, muscle, heart, and eye) were frozen on dry ice. Samples were later transferred to a freezer and stored at -50° until used.

Pieces of liver or heart approximately 0.5 g were ground in a glass tissue homogenizer with an approximately equal amount of grinding solution (0.1 M Tris, 0.001 M EDTA, 10^{-5} M NADP, pH 7.0). Muscle and eye tissue were ground with about 0.5 ml grinding solution per gram of tissue. Homogenates were spun in a blood centrifuge at top speed (4000 rpm) for 15 minutes. The supernatants were pipetted to clean 2 ml tubes and kept at -50° when not in use.

Gels were made by cooking and stirring 75 g starch (Electro-starch) and 500 ml buffer (Appendix) in a sidearm flask. After the solution boiled, it was evacuated for 1 minute, then poured into a 17 x 23 cm form made by clamping plexiglass border strips to a thick glass plate. When the gel cooled to room temperature, it was covered with plastic wrap and stored at least 8 hours in a refrigerator. Gels were used within 2 days.

A slit was cut the length of the aged gel about 5 cm from one edge. Thick (1 mm) filterpaper wicks (1/2 x 2 cm) were soaked in the sample supernatants and placed in the slit. Two wicks soaked in Bromophenol Blue, a dark dye, flanked the samples.

Gels were set on electrode trays containing buffer (Appendix). The gaps between the electrodes and the gel edges were bridged by several layers of soaked cloth towels, and the proper voltage was applied across the gel with a Heathkit IP-17 power supply. After electrophoresing for 15 minutes, when the dye had moved about 0.5 cm, all wicks were removed and the slit was held tightly closed with rubberbands. A pan with 3-4 kg ice was set on top of the gel, and electrophoresis was continued for $3\frac{1}{2}$ - 4 hours or until the marker dye approached the anodal edge of the gel.

One-sixteenth inch thick plexiglass strips were used to guide a metal blade through the gels to obtain three or four flat slices per gel. Each slice was stained for a different enzymatic activity. (The staining solutions used are given in the Appendix). When the zymograms were visible (15 minutes to overnight, but usually 3 to 4 hours) the slices were rinsed with water, then fixed in a methanol/water/acetic acid (5:5:1) solution. The fixed gel was usually photographed, after written notes were made of the electrophoretic pattern of each sample.

RESULTS

I have used the terminology of Prakash, et al. (1969) to report my results. A gene is named for its product by using an italicized abbreviation. Thus, LDH codes lactate dehydrogenase, PEP codes peptidase, etc. If several genes produce proteins of similar function they are numbered starting with the most cathodal protein. Thus, LDH-1 codes for a more cathodal protein than LDH-2 does. Finally, when multiple alleles are found at a locus, they are named parenthetically with numbers denoting their comparative mobility. One hundred is assigned to be the mobility of the most common allele. Thus, LDH-1 (100) is the most common allele at the LDH-1 locus; LDH-1 (50) would be a less common allele whose product migrates only half as fast.

Monomorphic Proteins

Alcohol Dehydrogenase, 1.1.1.1, (ADH): ADH, a dimer, is one of the few proteins commonly studied which migrates cathodally. It is thought to be controlled by a single locus (Allendorf, et al., 1975). The enzyme was expressed by a single invariant band in 31 samples from the Firehole River.

α -Glycerol-phosphate dehydrogenase, E.C. 1.1.1.8, (AGPD): AGPD is reported to be coded by two loci, AGPD-1 and AGPD-2 (Allendorf, et al., 1975, Engle, et al., 1971) because an invariant three-banded (fixed

heterozygote) pattern can be observed on Gel F. Using this buffer system, and buffers A and D, I was unable to consistently visualize products of more than one locus. AGPD-1 was represented by a single invariant band in 33 fish samples.

Fumarase, E.C. 4.2.1.2., FUM: Two fumarase loci are reported in Salmo trutta (Allendorf, et al., 1977) and the results of this study are also best interpreted as evidence of two loci. Two invariant bands of activity were visible in gels of 40 samples. Both FUM-1 and FUM-2 are expressed in muscle tissue, in contrast to the reported expression in S. trutta.

Glucuronidase, E.C. 2.3.1.31, GUS: Fifty-two liver samples had glucuronidase activity. Allendorf, et al. (1977) reported that two loci are expressed in liver tissue of S. trutta, one of which is also expressed in muscle tissue. I was not able to detect glucuronidase activity in muscle, and found only single invariant bands from liver, indicative of a single monomorphic locus.

Leucine aminopeptidase, E.C. 3.4.1.1, LAP: In the 20 muscle samples tested, this enzyme was expressed as three invariant bands. This fixed heterozygosity is caused by fixation at two individual loci. Allendorf, et al. (1977) report the expression of a single locus in muscle and a separate locus in liver.)

Peptidase, E.C. 3.4.1.2, PEP: A single invariant band of peptidase activity was found from 57 muscle samples. Evidence of a second locus (Allendorf, et al., 1977) was not found in muscle, liver, or eye extracts.

Phosphoglucose isomerase, E.C. 5.3.1.9, PGI: PGI, a dimer, is coded by three genes whose products interact to produce six bands of activity from samples of triply homozygous rainbow trout muscle (Avisé and Kitto, 1973). This pattern (Fig. 2) was observed in 44 muscle samples.

PGI-1 is not active in liver tissue: 35 samples each gave rise to three invariant bands of activity.

Xanthine dehydrogenase, E.C. 1.2.3.2, XDH: This enzyme is invariant in most if not all salmonoid populations (Kristiansson and McIntyre, 1976; Allendorf, et al., 1975). The Firehole population is not exceptional. Twenty samples each produced a single invariant band of activity.

Polymorphic Proteins

Esterase, E.C. 3.1.1.1, EST: Three zones of esterase activity were found on most gels, caused by EST-1, EST-2 and EST-3 (Allendorf, et al. 1977). Of the three esterases EST-1 was the most active. Of 99 samples scored, 65 were homozygous for the common allele, EST-1(100). Zymograms from 10 fish had a more cathodal band of EST-1(85). Twenty-four samples produced both bands and were scored as heterozygotes.

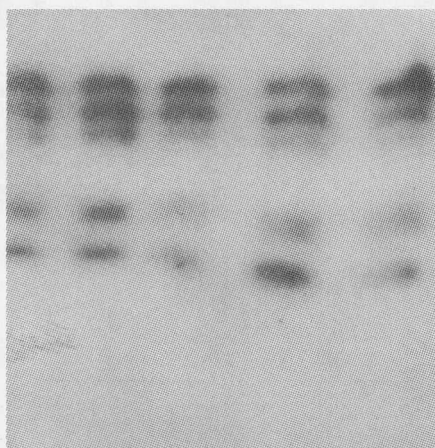


Figure 2. Products of PGI-1, PGI-2 and PGI-3, interacting to form dimers, cause six-banded zymograms.

The middle bands, products of EST-2, were invariant in 65 fish.

Only one gel retained EST-3 activity. Of the 27 liver homogenates scored, 21 had a single common band of activity. One fish, however, produced a single more cathodal band, designated EST-3(90). Five zymograms had the two-banded pattern expected of heterozygotes.

Lactate dehydrogenase, E.C. 1.1.1.27, LDH: LDH-1 and LDH-2, also called LDH-A and LDH-A', are both expressed in muscle tissue (Wright, et al., 1975). Their products interact to form tetrameric LDH molecules, giving rise to five bands of activity in doubly homozygous muscle zymograms. This was the pattern seen in all 52 muscle samples surveyed. No variant alleles were detected.

LDH-4 is the only LDH gene expressed in liver tissue. Of the 78 livers sampled, 71 had a single band of activity, from LDH-4(100). Another allele, LDH-4(70), is also present in the Firehole population. Six animals were shown to be heterozygous by their five-banded liver zymograms. One fish was homozygous for LDH-4(70).

Heart tissue expresses both LDH-3 and LDH-4. Since the mobility of LDH-3(100) is nearly the same as the mobility of LDH-4(70), the zymograms of double homozygous (LDH-3 and LDH-4) hearts are very like the zymograms of liver from LDH-4(70/100) heterozygotes (Fig. 3). The heart zymograms of LDH-4(70/100) fish were five-banded as well, but the bands were not equally intense. The heart of the LDH-4(70) homozygote produced only a single band of activity. Ten-banded



Figure 3. LDH zymograms of liver tissue (lanes 1-4) and heart tissue (lanes 5-7). Liver expresses LDH-4 only; heart expresses LDH-3 and LDH-4. The genotypes of the samples are:

Lanes 1 and 4: LDH-4(100)/LDH-4(100),
 Lane 2: LDH-4(70)/LDH-4(70),
 Lane 3: LDH-4(100)/LDH-4(70),
 Lane 5: LDH-3, LDH-4(100)/LDH-4(100),
 Lane 6: LDH-3, LDH-4(70)/LDH-4(70),
 Lane 7: LDH-3, LDH-4(100)/LDH-4(70).

Notice the unequal intensity of the bands in lane 7.

patterns, which would be expected of fish heterozygous at LDH-3, were not found. Thus, LDH-3 is monomorphic.

LDH-5 is expressed in eye tissue (Horowitz and Whitt, 1972; Whitt and Horowitz, 1970). Its product was expressed as a single invariant band in all 37 eye samples tested.

Malate dehydrogenase, E.C. 1.1.1.37, MDH: There are at least three genes that produce cytoplasmic MDH in rainbow trout. MDH-2 and MDH-3 (also called MDH-B and MDH-B') are expressed in muscle tissue (Bailey et al., 1970). However, since the products of the common alleles of the genes have identical electrophoretic behavior (and may be identical) doubly homozygous fish samples produce a single band of activity. This was the pattern seen in muscle samples from the Firehold River.

MDH-1 is most strongly expressed in liver tissue, where its products interact with those of MDH-2 and MDH-3. Three-banded zymograms are produced by triply homozygous fish livers. MDH-1 heterozygotes are recognized by the three-banded pattern which is found in the MDH-1 homodimeric region of a gel (Fig. 4). Nine such heterozygotes were found among 45 liver samples.

Phosphoglucumutase, E.C. 2.7.5.1, PGM: Three genes code monomeric PGM enzymes in rainbow trout (Roberts, et al, 1969) and other salmonids (Utter and Hodgins, 1970; Kristiansson and McIntyre, 1976) producing three zones of activity on some gels. The most anodal form, PGM-3,

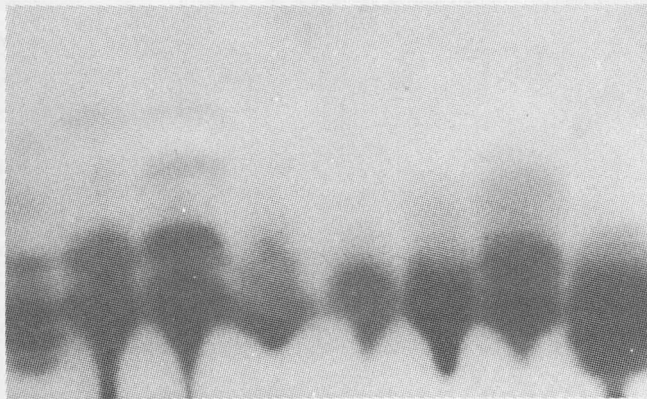


Figure 4. MDH zymograms of liver tissue. Lanes 1 and 8 are from MDH-1(100/50) heterozygotes; the rest are from MDH-1(100/100). MDH-2 and MDH-3 are expressed more strongly in some samples than others.

often has low activity and is not detected. This was the case in my samples. Of the two gene products detected, PGM-2 was invariant in 51 fish and produced a single band. PGM-1 was polymorphic: 19 samples produced the two-banded zymogram expected of heterozygotes. Thirty fish were homozygous for the common allele PGM-1(100) and two fish expressed only the less common allele PGM-1(65).

Both genes are expressed in muscle and liver, but activity is greatest in muscle.

Superoxide dismutase, E.C. 1.15.1.1, SOD: Enzymatic oxidations which are accompanied by the reduction of NAD^+ or NADP^+ are most commonly detected by adding one of several tetrazolium dyes to the staining solution. These dyes not only turn dark blue at the site of NADH and NADPH evolution, but nonspecifically stain the entire gel a light blue, especially in the presence of light.

The reduced (blue) tetrazolium is a substrate for the dimeric enzyme SOD. This enzyme bleaches out spots in the blue-cast gel (Fig. 5). It is polymorphic in rainbow trout (Cederbaum and Yoshida, 1972) including the Firehole population. Of 50 fish genotyped, 20 were heterozygous on the basis of their three-banded zymograms. Twenty other fish were homozygous for SOD(100), the cathodal product. Ten fish produced only the anodal product, SOD(140).



Figure 5. A gel stained for G6PD activity (dark spots) has light, bleached spots at the sites of SOD activity. Lanes 1-3 are from SOD(100/100) homozygotes. Lane 4 and lanes 11-14 are from SOD(100/140) heterozygotes. Lane 9 is from a SOD(140/140) homozygote.

Other Protein Systems

Several enzyme systems were not included in the estimate of variation in the Firehole population.

Esterase: As can be seen from Table 1, the observed EST-1 phenotypes are not in a Hardy-Weinberg ratio. Esterases are notorious for expressing nongenetic variability (Utter, et al., 1974) so this system was not included in the computation of $\bar{H}$.

Glucose-6-phosphate dehydrogenase, E.C. 1.1.1.49, G6PD: G6PD zymograms have recently been shown to express nongenetic differences between molecules (Cederbaum and Yoshida, 1976). G6PD mobility is influenced by NADP binding and possibly other factors. Conversion between different states during electrophoresis may account for the poor resolution of the various forms.

My gels had three zones of G6PD activity. The most anodal zone was a large diffuse region. No variability was detected in this region of the gels. The two cathodal forms were more distinctly resolved. Forms with differing mobility were evident, but though the protein is thought to be a dimer only one- or two-banded patterns were seen, characteristic of monomeric proteins when the patterns are representative of genetic variability.

Table 1. Table of results.

Monomorphic systems:

<u>ADH</u>	N = 31	<u>PEP</u>	N = 57
<u>AGPD</u>	N = 33	<u>PGI-1</u>	N = 44
<u>FUM-1</u>	N = 40	<u>PGI-2</u>	N = 44 (muscle)
<u>FUM-2</u>	N = 40		N = 35 (liver)
<u>GUS</u>	N = 52	<u>PGI-3</u>	N = 44 (muscle)
<u>LAP-1</u>	N = 20		N = 35 (liver)
<u>LAP-2</u>	N = 20	<u>XDH</u>	N = 20

Polymorphic systems:

Locus	Observed genotype dist. (homo/hetero/ homo)	$\bar{h}$	Expected genotype dist. (assuming Hardy-Weinberg equilibrium)	P*
<u>EST-1</u>	65/24/10	0.242	60/34/5	0.015
<u>EST-2</u>	N = 65	0		
<u>EST-3</u>	21/5/1	0.185	20.5/6/0.5	0.75
<u>LDH-1</u>	N = 52	0		
<u>LDH-2</u>	N = 52	0		
<u>LDH-3</u>	N = 37	0		
<u>LDH-4</u>	71/6.1	0.077	70.25/7/54/.21	0.20
<u>LDH-5</u>	N = 37	0		
<u>MDH-1</u>	36/9/0	0.200	36.45/8.1/.45	0.80
<u>MDH-2</u>	N = 45	0		
<u>MDH-3</u>	N = 45	0		
<u>PGM-1</u>	30/19/2	0.373	30.63/17.79/2/59	0.90
<u>PGM-2</u>	N = 51	0		
<u>SOD</u>	20/20/10	0.400	18/24/8	0.55

*P is the probability of exceeding the deviation of the observed dist. from the expected.

$\bar{h} = 0.049$

25 loci total (excluding EST-1)

In 15 of 36 fish scored, G6PD-1 was expressed as two bands. Fourteen fish expressed only the cathodal band: the other 7 expressed only the anodal band. Assuming the two-banded patterns indicate heterozygosity, these numbers are in a Hardy-Weinberg ratio ($\chi^2 < 0.65$, $P = 75\%$).

G6PD-2 expression is qualitatively similar but unlinked to G6PD-1 expression. Ten fish expressed two bands, 12 fish only had the cathodal band, and 10 fish only had the anodal band. These numbers are not in a Hardy-Weinberg ratio ($\chi^2 = 4.6$, $P = 10\%$).

Since there is confusion regarding the number of genes coding G6PD and their relation to observed zymograms, and since some data does not represent a Hardy-Weinberg ratio, this system was not included in the computation of $\bar{H}$.

Isocitrate dehydrogenase, E.C. 1.1.1.42, IDH: There are two tissue-specific IDH's in trout, IDH-A in liver and IDH-B in muscle (Allendorf and Utter, 1973; Reinitz, 1977; Engel, et al., 1975). IDH-A has been shown to be coded by two genes. The interpretation of zymograms of this system is difficult for two reasons. First, one locus is evidently monomorphic; the other is highly polymorphic, with multiple alleles in many populations, but the two loci share an identical allele. Thus, discrimination between the genotypes AAAA' and AAA'A' is difficult.

Also, though the protein is known to be a dimer, some investigators have observed only two-banded zymograms from fish known to be heterozygous (Allendorf and Utter, 1973; Reinitz, 1977). Other investigators have reported five- and six-banded patterns. Since I observed only 1 five-banded pattern in 88 samples (the rest had one, two or three bands) it is not clear what genotype should be assigned the three-banded gels.

The primary drawback to measuring heterozygosity at these loci was the feeble activity of this enzyme. I could find no conditions which allowed satisfactory visualization of this system.

IDH-B, expressed in muscle, is also reportedly controlled by two loci which are not linked to the IDH-A loci (Reinitz, 1977). The IDH zymograms obtained from muscle are also not typical of dimeric enzymes. Since the activity of this enzyme was as weak as that of IDH-A, no measurement of heterozygosity was possible.

Calculation of $\bar{H}$

In Table 1, observed phenotypic distributions are compared with Hardy-Weinberg expectations, and the fraction of heterozygotes, $\bar{h}$, is listed for each locus. The average of all $\bar{h}$'s (with the exclusions noted above) is $\bar{H}$. $\bar{H}$ for the Firehole population is 0.049.

DISCUSSION

The Salmo gairdneri population in the Firehole River is polymorphic at 20% of the 25 loci surveyed. These 25 loci are a fairly even mix of multiple substrate (7), regulatory (8), and nonregulatory (10) enzymes. It is important to include enzymes of all these types in population surveys because nonregulatory enzymes are relatively less variable in many organisms, including fish (Powell, 1975). Enzymes which act on a variety of substrates, and regulatory enzymes whose activities are modulated by nonsubstrate materials, must maintain proper activity through a range of conditions. The mixture of two, three, five or more forms of enzymes present only in heterozygotes may confer higher fitness than the homozygous proteins (Koehn, 1969). Stable polymorphisms maintained by heterozygote advantages would not be as common among nonregulatory enzymes, whose activities are usually simply related to substrate concentrations (Johnson, 1974).

The most commonly studied enzyme, though, is LDH, a nonregulatory enzyme. Many species are polymorphic at one of the LDH loci. Allele frequencies vary from one population to another, and from one location to another in large populations. When they vary with latitude, selection by the temperature of the climate is often suspected. Johnson (1971) raised larval crested blennies (Anoplarchus) at different temperatures and found differential survival dependent upon genotype and temperature. To study the functional basis of selection

on LDH, Merritt (1972) collected Pimephales from several latitudes. Two LDH alleles were present in his samples. The allele common in the north is rare in the south, and above 25° it's homogeneous product has a much higher K_m for pyruvate than proteins containing the common southern subunit, as expected if temperature plays a selective role.

A similar enzymatic study concerning temperature was done with serum esterase of Catastomid fishes (Koehn, 1969). Again, there appear to be "northern" and "southern" alleles. Sera from fish homozygous for the common southern allele were inactive at 0°, but activity increased at higher temperatures. The northern protein is active at 0° but nearly inactive at 37°. At intermediate temperatures sera from heterozygous fish are more active than sera from either homozygote.

Enzyme K_m 's do not just vary with respect to temperature. Different substrates have different K_m 's, and allosteric modulators affect substrate binding. Heterosis could be favored throughout ranges of substrate composition and allosteric modulator concentrations just as it is throughout ranges of temperature in the case of Pimephales LDH.

Polymorphisms were found in all three groups of enzymes. The multiple-substrate SOD and EST enzymes are highly variable in trout and also other fish (Powell, 1975). Multiple alleles coding PGM, a regulatory enzyme whose activity is increased by small amounts of glucose-1, 6-diphosphate, were described by Roberts (1969). The

nonregulatory liver isozymes MDH-1 and LDH-4 are polymorphic in other rainbow populations (Allendorf, et al., 1975; Utter and Hodgins, 1972).

$\bar{H} = 0.049$ (SE = 0.023) in this population, a value similar to those of other rainbow populations. Allendorf and Utter (1975) have listed $\bar{H}$ for 41 populations of native and hatchery rainbows, mostly steelhead. $\bar{H} = 0.060$ in the resident populations, with an exceptional value of 0.020 at the University of Washington hatchery. An earlier study (Utter, et al., 1973) of three populations reared at that hatchery measured $\bar{H}$ values of 0.034, 0.036, and 0.038.

The sampling variance of $\bar{H}$ is called $V(\bar{H})$ (Nei and Roychoudhury, 1974):

$$V(\bar{H}) = \frac{V(h)}{r}$$

where r is the number of loci examined and $V(h)$ is the variance of h , estimated by

$$V(h) = \frac{r}{k=1} \frac{(h_k - \bar{H})^2}{r - 1} = 0.0133$$

in the Firehole population. $V(\bar{H})$ is thus:

$$V(\bar{H}) = \frac{0.0133}{25} = 0.0053.$$

The standard error (SE) of $\bar{H}$ is the square root of $V(\bar{H})$:

$0.0053 = .023$, which is about 47% of $\bar{H}$. This large standard error is caused by the uneven distribution of h values used to calculate $V(h)$.

Eighty percent of the loci surveyed are monomorphic with $h = 0.0$;

20% have scattered h values much greater than $\bar{H}$. This causes $V(h)$ to

be large in spite of the large number of loci examined. To reduce the standard error to 20% of $\bar{H}$ at least 60 loci would have to be examined.

Figure 6 depicts the values of $\bar{H}$. Allendorf and Utter (1977) measured in the 41 populations of rainbow trout they surveyed, grouped into 0.5% classes. The variability of the heterozygosity of the 14 loci examined is evident. The distribution of $\bar{H}$'s is not caused by sampling variance because the same 14 loci were surveyed in each population. The different $\bar{H}$ values may or may not be randomly distributed within this small section of the genome; $\bar{H}$ values of the total genomes cannot be presumed to have the same distribution. This is why estimates of heterozygosities of total genomes have such large standard errors, and comparisons of populations are not usually significant. In particular, the Firehole River value of $\bar{H}$ is shown above Allendorf and Utter's data, with its standard error. Most of the other populations are within one SE of the Firehole value, and all but two are within two SE. The standard error that would arise from computing total $\bar{H}$ from just 14 loci is certainly larger than the Firehole standard error, though it is not calculable from the published data. No significant differences in $\bar{H}$ among these populations can be measured on the basis of 14 or 24 loci, so no general conclusions can be reached about the effects of population size, isolation, and heat on total genetic variability.

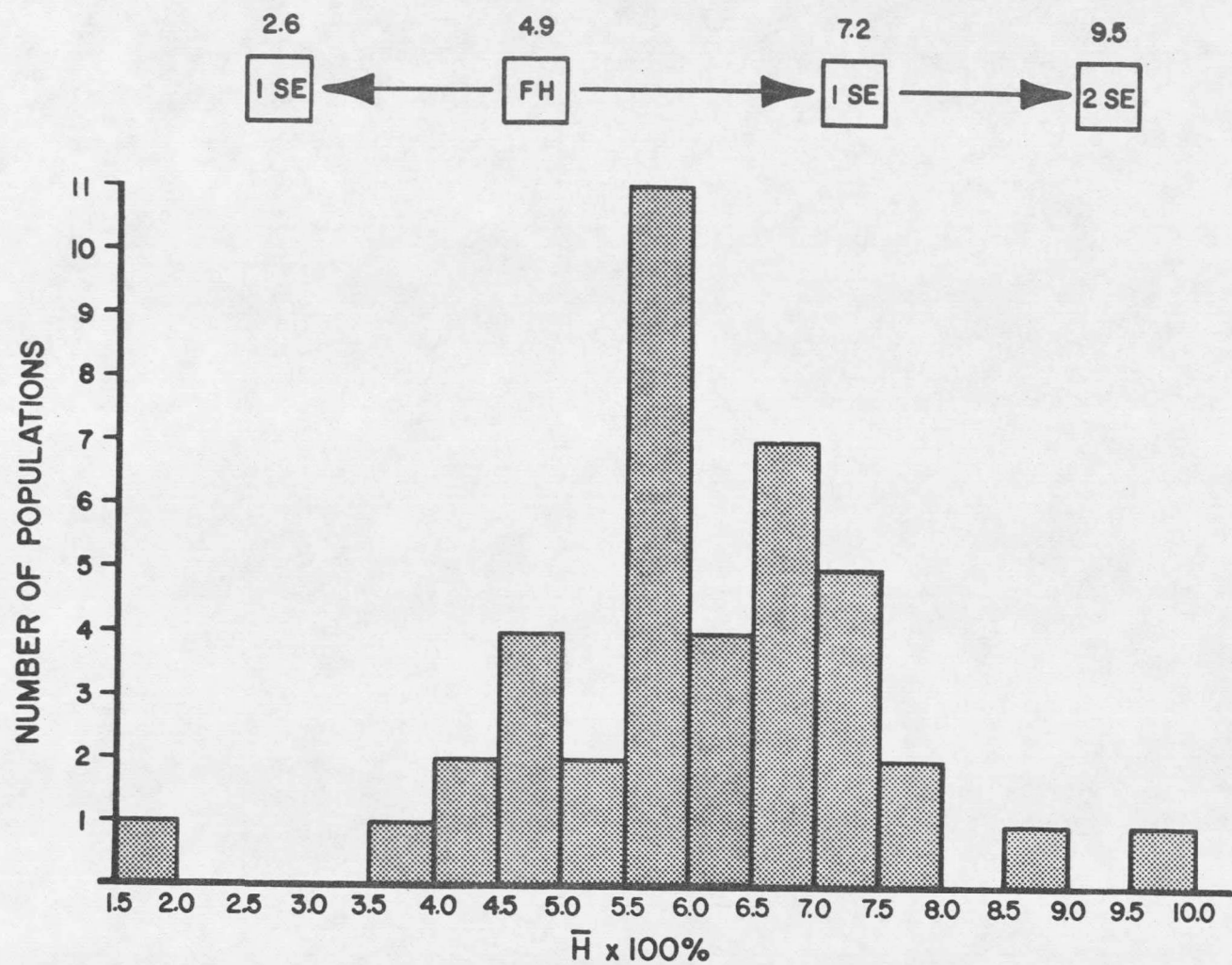


Figure 6. Comparison of $\bar{H}$ between the Firehole population and 41 others.

Variability at individual loci is well documented, but it's maintenance will have to be explained on a case-by-case basis. The enzymology of allelic proteins, as well as their distribution, was used to explain LDH and EST polymorphisms, as previously mentioned. Geographical variation on a continental scale was shown to be caused by temperature. Temperature is the most commonly studied selective force because it is easily manipulated in the lab, and easily altered by man's damming, irrigating, and power generating activities. The effects of heated discharges from power generators have become particularly important recently. Allele frequencies in some species are evidently unchanged by heated waters. Bass (Micropterus salmoides), mosquito fish (Gambusia affinis), and bluegill (Lepomis macrochirus) in heated Savannah River waters are no different than nearly populations in unheated waters (Yardley, et al., 1974). Some species have definitely been affected by thermal effluents, though. A population of Fundulus heroclitus living in heated portions of Long Island Sound has assumed genetic and morphological characteristics of populations living a thousand miles south of it (Mitten and Koehn, 1975). $\bar{H}$ has increased in this population, and comparisons between age classes showed that multilocus heterozygotes have increased survival, so the polymorphisms are maintained by selection.

Esterases, which are among the most variable enzymes in the animal kingdom (Powell, 1975), have particularly been implicated in

thermal adaptation. Geographical clines of allele frequencies have been related to temperature in catostomid fishes (Koehn, 1969), Notropus stramineus (Koehn, et al., 1971), salmonids (Nyman and Shaw, 1971), and Zoarces viviparus (Frydeberg, et al., 1973). Stable polymorphisms are maintained by selection favoring heterozygotes; allele frequency clines are caused by clines of selection pressure (which are in turn caused by temperature clines).

Selection favoring heterozygotes is not the only way temperature can affect EST allele frequencies. Nyman (1975) measured the frequencies of serum esterase alleles in populations of ruff (Gymnocephalus cernua) where a nuclear generator discharges heated water, and at unaffected sites nearby. Ruff migrate from brackish coastal water to spawning sites in the adjacent fresh waters. The heated discharge enters along one of these migration routes. The common EST allele was present at a frequency of 0.6 in control populations, but at 0.9 in fish migrating into heated waters. Sometimes the generating station did not operate, and then the allele frequency returned to 0.6.

The Firehole rainbow trout population has a significant deficit of EST-1 heterozygotes. One explanation for this could be that these trout behave somewhat like ruff. Spawning beds at different sites in the Firehole have different water temperatures, and allele frequencies of fish on these beds may be influenced. When two or more

subpopulations with different allele frequencies are sampled together, as one population, an apparent deficiency of heterozygotes is found.

Other explanations of the EST-1 heterozygote deficiency are possible. Utter, et al. (1973) excluded esterases from calculations of $\bar{H}$ in salmonids because they were unable to get repeatable results with these systems. It is possible that some Firehole rainbows were incorrectly genotyped, but their zymograms were repeatable. The genetic basis of esterase variation has been shown for Zoarces and Gymnocephalus (Christiansen, et al., 1973; Nyman, 1969) but the assumption of a single diallelic EST-1 locus in trout may be incorrect.

A third explanation for the EST-1 heterozygote deficiency may be the simplest: heterozygotes die. This possibility has a precedent in the Kaloe Cove populations of Zoarces (Christiansen, et al., 1974). The two EST-3 alleles in this population are evidently incompatible. At birth, a deficiency of heterozygotes exists, and this deficit becomes more pronounced in older age classes. No age class differences are found in the FH trout, but selection against zygotes could account for this.

More extensive sampling is needed to determine which of these explanations is correct. Since none of the other polymorphisms appear to be involved in the thermal adaptation of these trout, and their overall variability is not significantly different than that of other populations, EST-1 would be a good candidate for further, more intensive study.

APPENDIX

APPENDIX

Buffer Systems

Buffer System	pH	Voltage	Reference
A. Ridgeway			
Electrode: 0.06 <u>M</u> LiOH	8.3	250 V	Ridgeway, et al. 1970
0.3 <u>M</u> Borate			
Gel: 0.03 Tris	8.0		
0.005 <u>M</u> Citrate			
B. Poulik			
Electrode: 0.3 <u>M</u> Borate	8.2	250 V	Selander, et al. 1971
Gel: 0.076 <u>M</u> Tris	8.7		
0.005 <u>M</u> Citrate			
C. Lithium Hydroxide			
Stock Sol'n A: 0.03 <u>M</u> LiOH	8.1	350 V	Selander, et al. 1971
0.19 <u>M</u> Borate			
Stock Sol'n B: 0.05 <u>M</u> Tris	8.4		
0.008 <u>M</u> Citrate			
Electrode: Stock Sol'n A			
Gel: 1:9 mixture Stock Sol'n A and B			

Buffer Systems	pH	Voltage	Reference
D. Tris-Borate-EDTA			
Electrode: 0.5 <u>M</u> Tris	8.0	200 V	Selander, et al. 1971
0.65 <u>M</u> Borate			
0.02 <u>M</u> EDTA (disodium ethylenediamine tetraacetate)			
Gel: Dilute electrode-buffer 1:9 with water.			
E. Continuous Tris-Citrate			
Electrode: 0.223 <u>M</u> Tris	6.3	170 V	Selander, et al. 1971
0.086 <u>M</u> Citrate			
Gel: 0.008 <u>M</u> Tris	6.7		
0.003 <u>M</u> Citrate			
F. Wolf Acidic Phosphate			
Electrode: 0.04 <u>M</u> Na ₂ HPO ₄	6.5	250 V	Wolf, et al. 1970
0.06 <u>M</u> NaH ₂ PO ₄			
Gel: Dilute electrode buffer 1:9 with water.			

Tissues and buffers used to resolve the examined enzyme systems

Enzyme	Tissues				Buffers
	Liver	Muscle	Eye	Heart	
ADH	X				A,B
AGPD		X			A,D,F
EST	X				A,C
FUM		X			A,B
G6PD	X		X		A,B,E,F
GUS	X				A
IDH	X	X			C,E,F
IDH	X	X	X	X	A,B,C
MDH	X	X	X		A,B,E,F
PEP		X			A
PGI		X			A,B
PGM	X	X			A,B,C
SOD	X				B,D
XDH	X				B

Staining Solutions

Abbreviations: NAD = β -nicotinamide adenine dinucleotide

NADP = β -nicotinamide adenine dinucleotide phosphate

NBT = p -nitro blue tetrazolium

Enzyme	Stain	
ADH	50 ml	Ridgeway Gel Buffer (see above)
	10 mg	NAD
	10 mg	NBT
	5 mg	PMS
	10 ml	90% ethanol
AGPD	50 ml	Ridgeway Gel Buffer
	10 mg	NAD
	10 mg	NBT
	5 mg	PMS
	1 g	LD- α -glycerophosphate
EST	50 ml	Ridgeway Gel Buffer
	5 ml	1% α -naphthyl-acetate in 1:1 acetone/water
	150 mg	Fast Blue BB salt
FUM	10 ml	0.2 M Tris, pH 8.0
	40 ml	water
	10 mg	NAD
	10 mg	NBT
	5 mg	PMS
	400 mg	Fumaric acid
	300 units	Malate Dehydrogenase

Enzyme	Stain	
G6PD	10 ml	0.2 <u>M</u> Tris, pH 8.0
	40 ml	water
	10 mg	NADP
	10 mg	MgCl ₂
	10 mg	NBT
	5 mg	PMS
	200 mg	Glucose-6-phosphate
GUS	50 ml	Wolf Acidic Phosphate Electrode Buffer
	10 mg	Naphtol-A-B-glucoronide
	10 mg	GBC Fast Garnet salt
IDH	10 ml	0.2 <u>M</u> Tris, pH 8.0
	40 ml	water
	10 mg	NADP
	10 mg	MgCl ₂
	10 mg	NBT
	10 mg	MTT tetrazolium
	5 mg	PMS
LAP	200 mg	DL-isocitric acid
	50 ml	0.13 <u>M</u> Tris, 0.043 <u>M</u> citrate, pH 7
	10 mg	L-leucyl- β -naphtylamide
	10 mg	MgCl ₂
	20 mg	Black K salt
LDH	50 ml	Ridgeway Gel Buffer
	10 mg	NAD
	10 mg	NBT
	5 mg	PMS
	25 ml	0.5 <u>M</u> DL-lactic acid

Enzyme	Stain	
MDH	50 ml	Ridgeway Gel Buffer
	10 mg	NAD
	10 mg	NBT
	5 mg	PMS
	25 ml	0.5 <u>M</u> DL-Na-Malate, pH 7
PEP	10 ml	0.2 <u>M</u> Tris, pH 8.0
	40 ml	water
	50 mg	O-dianisidine
	80 mg	L-leucyl-L-alanine
	10 mg	snake venom
	50 mg	MgCl ₂
	500 units	Peroxidase
PGI	50 ml	Ridgeway Gel Buffer
	10 mg	NADP
	10 mg	MgCl ₂
	10 mg	NBT
	5 mg	PMS
	25 mg	Na-fructose-6-phosphate
PGM	10 units	Glucose-6-phosphate Dehydrogenase
	10 ml	0.2 <u>M</u> Tris, pH 8.0
	40 ml	water
	10 mg	NADP
	10 mg	MgCl ₂
	10 mg	NBT
	5 mg	PMS
	300 mg	K-glucose-1-phosphate
	0.5 mg	α -D-glucose-1,6-diphosphate
	50 units	Glucose-6-phosphate Dehydrogenase

Enzyme	Stain	
SOD	50 ml	Ridgeway Gel Buffer
	20 mg	NBT
	5 mg	PMS
	expose to light	
XDH	50 ml	Ridgeway Gel Buffer
	10 mg	NAD
	10 mg	NBT
	5 mg	PMS
	20 mg	Hypoxanthine

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