AN EXAMINATION OF TWO REPORTED PROTEIN POLYMORPHISMS IN THREESPINE STICKLEBACK, GASTEROSTEUS ACULEATUS

by

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Abstract

Gasterosteus aculeatus L., the threespine stickleback is in morphological terms, an extremely variable species. It is found in a wide variety of habitats throughout the northern hemisphere. Forms of threespine stickleback are found in anadromous and freshwater situations. Taxonomic examinations concerning the distribution of various forms of stickleback have produced equivocal results.

Two stickleback protein polymorphisms have been reported in the literature. A hemoglobin polymorphism has been found in south European stickleback, and a muscle myogen polymorphism (enzyme: creatine kinase) has been found in stickleback from western Canada. The distribution of alleles in both these situations caused the researchers to hypothesize that the polymorphisms were related to the anadromous existences of certain forms of stickleback.

In this study I tested these hypotheses with populations from southwestern British Columbia, across a range of macrohabitat types. One population of sticklebacks sampled is, apparently, a relict anadromous population. This population provided the opportunity to test a population during (or after) selective pressure to adapt to a freshwater existence.

No evidence of the hemoglobin polymorphism was found, indeed some doubt was cast upon the European work due to technique aberrations. The muscle myogen polymorphism was present in a few populations, however, no basic environmental pattern emerged. The possibility of these alleles being selectively neutral is discussed.

Electrophoretic patterns of the hemoglobin and muscle myogen patterns provoked the suggestion that the various forms of stickleback do indeed belong to a single species.

The pattern of the muscle myogen polymorphism (creatine kinase) was found to be inconsistent with the dimeric structure generally reported for the enzyme.

The hypothesis relating the polymorphisms to anadromous existences must be rejected. Several alternative projects are suggested.

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Acknowledgements

Help comes in strange forms, to a graduate student, advice (hard to take), "know-how", patience, funds, conversation and most importantly criticism (also hard to take). It is obvious to me that my debt, to the many who helped me, is very great.

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CHAPTER I. GENERAL INTRODUCTION

Much scientific attention has been directed towards morphological variation in the species complex Gasterosteus aculeatus, the threespine stickleback.

Morphological features common to this fish such as plate number, spine number, spine length, mating colour, size, and vertebral number have been demonstrated to be extremely variable over a range of locations, (Hagen, 1967; McPhail, 1969; McPhail and Lindsey, 1970; Hagen and Gilbertson, 1972).

Fish with certain combinations of these morphological features have been given names by taxonomists, beginning with Cuvier and Valenciennes, (1829), (cited in Munzing, 1963,) for the sake of descriptive convenience. These forms of Gasterosteus aculeatus are described as leiurus, semi-armatus and trachurus, (Fig. 1). Leiurus is the unarmoured extreme, trachurus is the well armoured extreme, and semi-armatus is a moderate form between the two extremes. There has been some discussion with regard to the phylogenetic relationships among these forms, (Hagen, 1967; Miller and Hubbs, 1969; Hagen and McPhail, 1970), however no consensus has been reached. One thing is clear from this controversy: stickleback are not a typical species in the traditional sense. It should be noted that the forms do not satisfy Mayr's (1963) criterion for sub-species, as the populations

in British Columbia are not clearly geographically isolated.

With the hope that enzymatic work would clarify some of the phylogenetic relationships of the species, several workers have applied the technique of electrophoresis to populations of stickleback, (Aspinwall, pers. comm.; Hagen, 1967; Cucchi, 1969; and Jones, unpublished). From this work it appears that stickleback are invariable at the malate dehydrogenase and the alpha-glycerophosphate dehydrogenase loci, (Aspinwall, pers. comm.). Several other loci were examined by Aspinwall, with inconclusive results. Two polymorphisms have been reported in the literature.

Hagen (1967) reported that one of the muscle myogen proteins, later identified as creatine kinase (Gosselin-Rey et al.,1968) in the stickleback was variable and further stated that the variability could be used to distinguish between freshwater populations (leiurus), and anadromous populations (trachurus) in locations where the ranges of the two phases abutted. Callegarini and Cucchi (1968) reported a hemoglobin polymorphism which also appeared to correlate with freshwater and anadromous existences, in southern Europe.

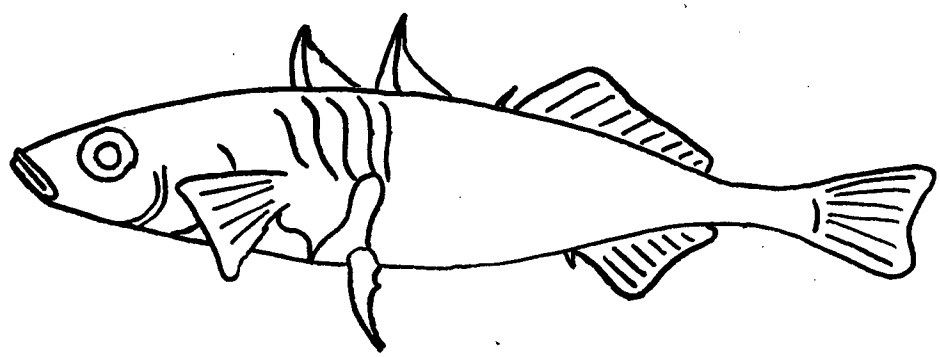
Although a definitive comparison between European populations and North American populations of Gasterosteus aculeatus has not been carried out it should be noted that the European sticklebacks electrophoretically studied by

FIGURE I

Three Forms of Gasterosteus Aculeatus

These three forms of Gasterosteus have been given names by taxonomists for descriptive convenience. Form A is called leiurus, B semi-armatus and C trachurus. Often this nomenclature is based upon the number of lateral armour plates; however there is a spectrum of forms between the extremes and the distinctions are often arbitrary. (Adapted from, McPhail and Lindsey, I970, and Hagen and Gilbertson, I972)

A.



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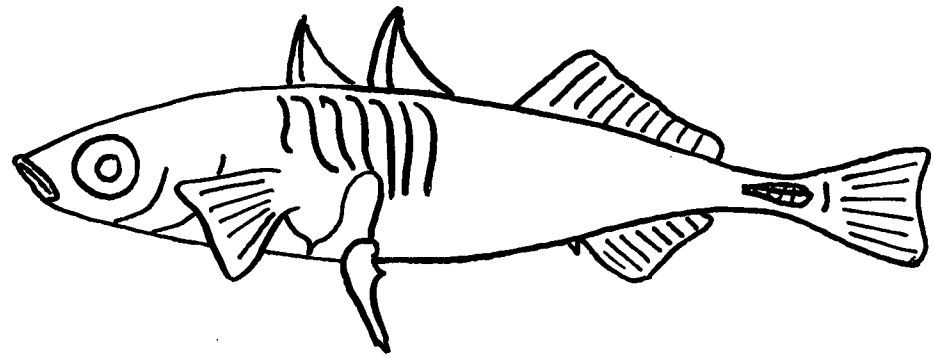

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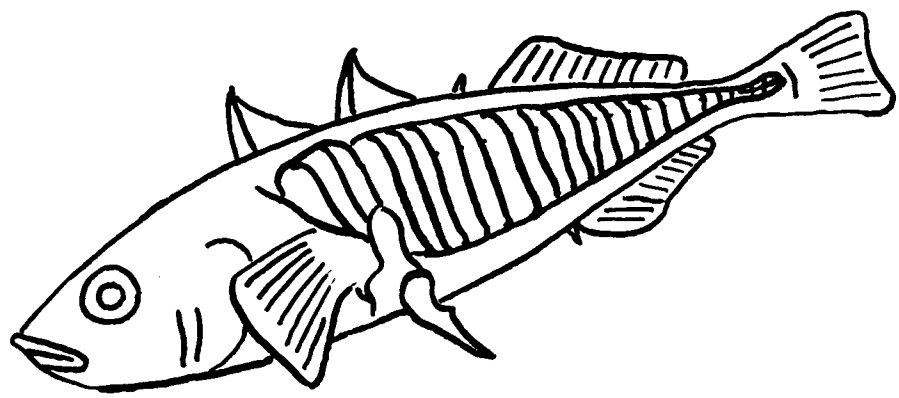


Italian researchers, resemble morphologically, the North American leiurus form. In Europe, the trachurus form is found along the northern coast (Scotland, Norway, U.S.S.R) and in the Black Sea area. The leiurus form is generally found in southwestern Europe (Spain, France, Italy, Albania, etc.) (Munzing, 1963).

Considering the unresolved controversy over the meaning of biochemical variability and the neutralist/neodarwinist arguments (Lewontin, 1974), it was interesting to note that the only known polymorphisms for this stickleback were both thought to be environmentally adaptive, thereby supporting the neodarwinist view. Basically, the neutralist view is that much of the enzymatic variability revealed by electrophoretic studies is irrelevant to the well being of the organism. The neodarwinist's (or selectionist's) view is that nearly all electroisozymes are individually selected units of heredity, that survive on the basis of their utility to the individuals they are found in.

An examination of the literature provides several alternatives accounting for variability patterns. Some of these alternatives do not necessarily reflect genetic differences between individuals. Obviously then, any research with phylogenetic implications must be performed in such a way that these confounding factors are neutralized. Some possibilities accounting for protein variability are as follows:

(1) Fish Age

Several groups of researchers have reported ontogenetic changes in fish hemoglobin. Wilkins and Iles (1966) for example, have found marked changes in the electrophoretic components of herring (Clupea harengus) with age. Similar situations exist in the Atlantic salmon (Salmo salar) (Koch et al., 1964), the roach (Rutilus rutilus), the rudd (Scardinius erythrophthalmus) (Perez and Maclean, 1974) and in the eelpout (Zoarus viviparus) (Hjorth, 1974). To compensate for this phenomenon, one must examine cases of variability to see that the distribution of polymorphisms is not dependent on age. In fish, determination of relative age does not present a great problem, as there is usually a relationship between length and age (within populations).

(2) Seasonal and Temperature Variability

Seasonal variability in concentrations of hemoglobin, hematocrit, and serum protein have been noted by Denton and Yousef (1975), in rainbow trout (Salmo gairdneri). Interestingly enough, it was discovered that this variation in concentrations took place independently of temperature and appeared to be in response to other environmental factors. Diet, metabolic adaptations and activity were suggested as possible explanations.

Baldwin and Hochachka (1970) have shown that rainbow trout (Salmo gairdneri) acclimated to different temperature

regimes exhibit different electrophoretic patterns of brain acetylcholinesterase. This is interesting since, in vitro, many enzymes have narrow temperature optimums where their efficiency is at a maximum.

(3) Environmental Adaptation

A most interesting example of apparent electrophoretic allozyme adaptation was cited by Powers, (1972). He demonstrated that two sympatric catostomid fish, Catostomis insignis and Catostomis clarkii exhibit hemoglobin variability that appears to coincide with micro habitat adaptation. Externally the fish are very similar, but C. clarkii is normally found in faster flowing sections of streams. C. clarkii has an extra electrophoretic hemoglobin component which when biochemically characterized is functionally independent of pH. C. insignis components are all functionally dependent upon pH. This dependence is termed the Bohr effect and is normally considered to be advantageous to the organism. So, why does C. clarkii have this pH independent component? Powers theorized that the extra component allowed C. clarkii to remain in the fast flowing water by maintaining some oxygen supply to muscle tissue, in spite of increased tissue acidity caused by the extra muscle action, thereby avoiding direct spatial competition with C. insignis. Biochemical evidence appears to support this theory. One would think that living in faster flowing water

would involve more muscular activity, thereby producing more lactic acid. Black (1958) has shown that when fishes are subjected to extreme exertion the build up of lactic acid in the blood can be great enough to overwhelm the buffer capacity of the blood and produce a pH change. Black also showed that this pH change lasted for fairly long periods of time (24 hours). Thus it would appear that a pH independent component would be extremely useful to an organism under these circumstances.

(4) Tissue Variability

An interesting pattern of protein variation involves variability among tissues within an individual organism. Scholl and Eppenberger, (1972) discussed the tissue variability of creatine kinase in a variety of fish species. Other researchers have illustrated the same phenomenon with respect to creatine kinase in frogs (Lyslova, 1971) and birds (Eppenberger, 1968).

Examining a specific type of tissue within individuals of a population can overcome confusion generated by this phenomenon.

(5) Historical Situations

Although it is a difficult concept to illustrate, several researchers have attempted to show differences based upon historical separations (neutral drift). Aspinwall (1974) has illustrated gene frequency differences between odd and even year classes of cycling pink salmon,

Oncorhyrchus gorbuscha. Pink salmon were ideal for this study because the year classes represent two individual demes. There is no migration between these populations. It is assumed that the two demes were formerly one, and have become separated by historical accident. Aspinwall also assumed that the selective forces on each year class have been constant. Most importantly, he illustrated that the moderate amount of migration between coastal rivers was great enough to keep the between year variability greater than the variability within a year over a wide geographical range.

Now, for the two reported protein polymorphisms in stickleback, both polymorphisms are reportedly associated with anadromous existences. Since both freshwater and saltwater populations are available in southwestern British Columbia, an ideal situation exists to test these hypotheses. In British Columbia the division between the distributions of the forms of stickleback are less clear than those previously mentioned in Europe. Normally the leiurus form is found in freshwater and the trachurus form is found in saltwater, however this distinction is a gross generalization. I felt that by designing the samples to cover the spectrum of morphological variation, salt or freshwater existences, and gross habitat types one could get a clear picture of the distribution of the polymorphisms within a relatively small geographical area.

Because there were differences in the techniques and histories of the two reported protein polymorphisms I have decided to report my examination of them independently in two individual chapters each with brief specific conclusions, followed by a final chapter of general discussion.

CHAPTER II. STICKLEBACK HEMOGLOBIN

1. Introduction

Italian researchers (Callegarini and Cucchi, 1968; Callegarini and Cucchi, 1969; Cucchi, 1969; Raunich et al., 1969; and Raunich et al., 1972) have examined a hemoglobin polymorphism in European stickleback.

This polymorphism involves three phenotypes A, AB and B (Fig.2). Phenotype A is found in non-migratory freshwater populations of stickleback from western Italy and southern Germany. Populations from the vicinity of Verona are also monomorphic for phenotype A. Phenotypes AB and B then become more common in populations as one approaches the Adriatic Sea, particularly in brackish water situations. Ultimately coastal populations around Venice and Ravena have a greater frequency of the Hb^B allele than the Hb^A allele.

Raunich et al., (1972) generated the hypothesis that the H^B allele was related to the brackish and salt water existence encountered by the migratory sticklebacks. They suggested that researchers from other coastal areas should investigate this hypothesis to see if the polymorphism was present in other migratory populations.

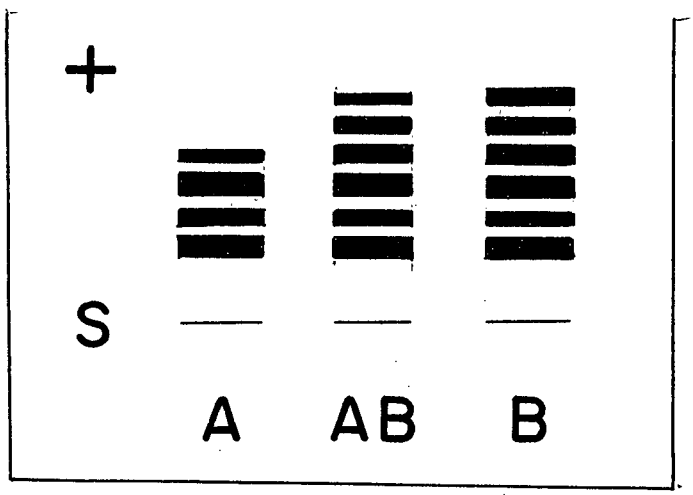
Several questions arose when I examined this work.

Firstly, was the polymorphism a genetic phenomenon?

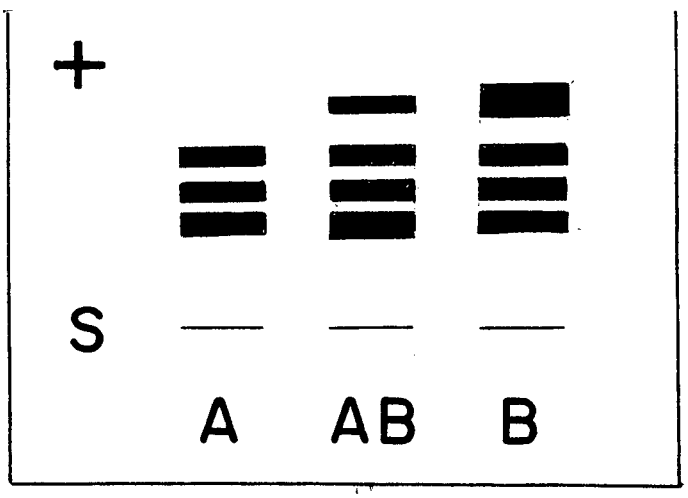
FIGURE 2

Patterns of the Reported Hemoglobin Polymorphism in Italy

The component patterns found in Italy are typified by 3 phenotypes, A,AB,and B.These phenotypes separate into different component patterns at different pH values. All bands are anodal,S is the starting point. British Columbia stickleback are typified by the A pattern in diagram (a),(Raunich et al.,1972).



(a) pH 8.6



(b) pH 3.5

The Italians suggested that it was. They conducted breeding experiments, the results of which suggested that the Hb^B allele was the product a single mutation. Their figures were so close to expected ratios that in fact statistics did not even have to be employed (an amazing fact considering other people's lack of success (McPhail, pers. comm.) at breeding experiment survivorship with western North American stickleback).

Secondly, they suggested that the presence of the polymorphism is related to the brackish and salt water existences of migratory populations. The question then arises, why weren't populations from the vicinity of Rome and Naples polymorphic? The Italian work states that morphological features were used to distinguish between migratory and stationary populations, and the Rome and Naples populations apparently fit the stationary population criterion. These distinguishing features were not specified.

The third question that arises is the question the Europeans pose. Do other populations of stickleback in coastal situations exhibit this protein polymorphism? Stickleback in southwestern British Columbia occupy a similar coastal situation, therefore, I set out to examine populations for the presence of the hemoglobin polymorphism. If the polymorphism was present I planned to examine its distribution on a micro-geographic scale to test the Italian hypothesis that the polymorphism was related to a brackish water existence.

2. Materials and Methods

(a) Field Techniques

The stickleback were collected with beach seines, pole seines and minnow traps. The fish were returned to the lab and kept alive until immediately before the electrophoresis was to begin. Mortality during storage was extremely low.

The fish were cold shocked then killed. Blood was extracted (Koehn, 1969) by making an incision in the anterior ventral region into the bulbous arteriosus. Blood was drawn into a pasteur pipet containing a small amount of 4% (w/v) sodium citrate solution. The sodium citrate acted as an anticoagulant. The blood was then centrifuged to separate the erythrocytes. This operation was performed three times at 0°C at about 5,000 r.p.m. The supernatant was replaced after each centrifugation. The erythrocytes were then lysed by adding a small volume of distilled water to the plug of packed cells. The cell particles were again centrifuged out leaving the hemoglobin in the supernatant.

(b) Electrophoretic Techniques

Electrophoresis was carried out utilizing Whatman #4 paper inserts in horizontal trays (after Selander and Yang, 1969(a)) with a 10.8% (w/v) starch gel.

Buffers used were similar to those used by Selander and Yang (1969(b)) for esterase electrophoresis. Specifically, the bridge buffer was composed of solution A and the gel

buffer was composed of solution B and A in a 9:1 ratio. Solution A was composed of 0.03 M lithium hydroxide (monohydrate) and 0.19M boric acid, pH 8.1; solution B was 0.008M monohydrate citric acid and 0.05M TRIS, pH 8.4.

Electrophoresis was carried out for 2.5 hours at 350 volts (and approximately 50 amps.) Gels were kept cool with cracked ice.

The gels were stained with the multi-purpose protein stain nigrosene (Brewer, 1970).

(c) Geographical Locations (Fig. 3)

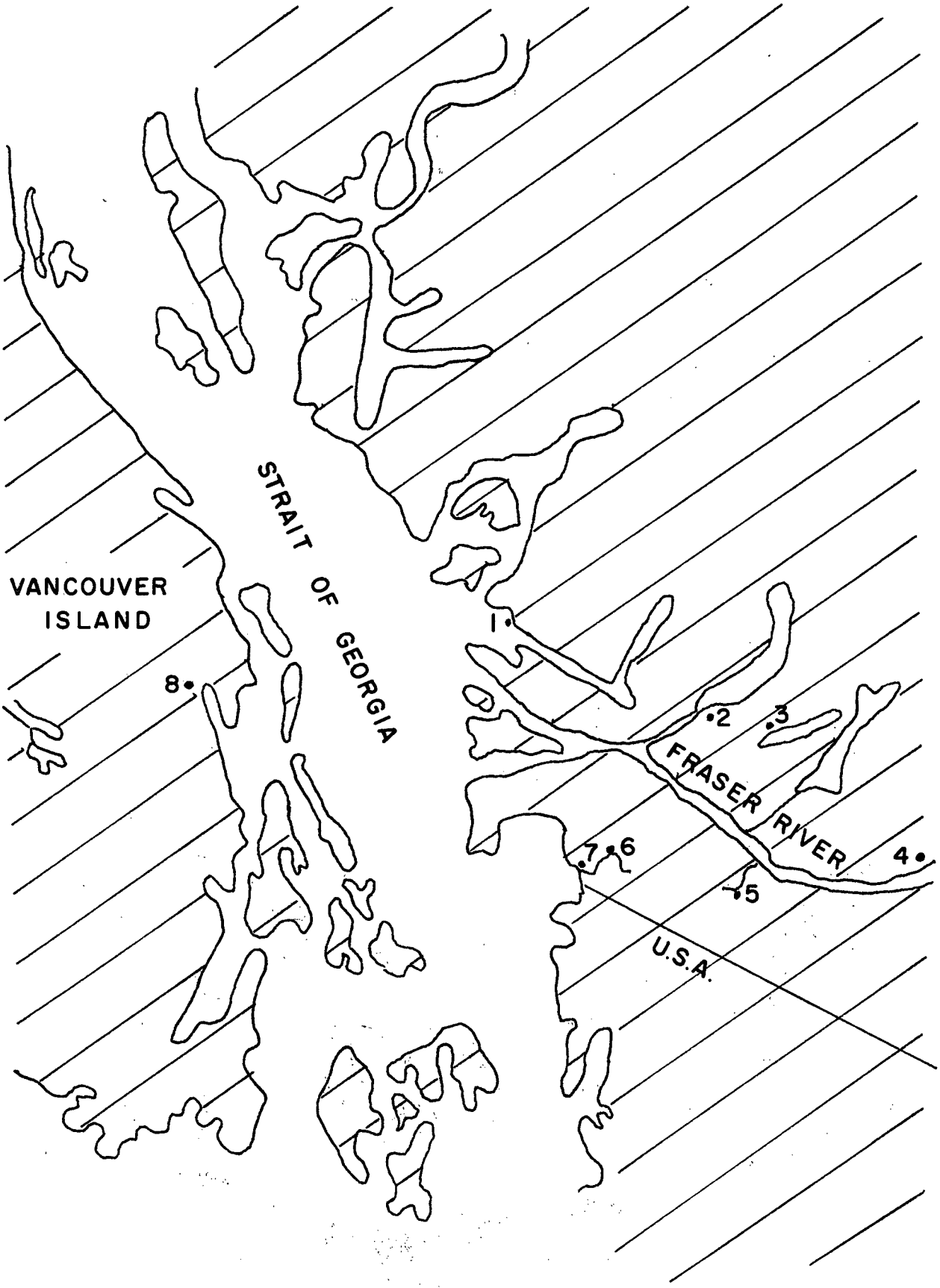
G. aculeatus of the trachurus form were collected, in salt-water (Burrard Inlet) north of the Fraser River's plume, migrating into freshwater to breed (Little Campbell River) south of the Fraser River, and from an unusual permenant freshwater situation (Lake Erroch).

The Lake Erroch fish are unusual because they completely resemble (phenotypically) the anadromous trachurus form of the fish and, yet are found in freshwater. It is very likely that this population is a marine relict similar to others found in the area, such as the relict population of long finned smelt, Spirinchus thaleichthys, found in nearby Harrison Lake, (Dryfoos, 1965). This population could be used to provide a check on any allozyme characteristic of either leiurus freshwater populations or trachurus anadromous populations, particularly if there was some evidence that the polymorphism was related to environmental adaptation.

FIGURE 3

Geographic Locations of the Stickleback Populations Sampled for the Hemoglobin Polymorphism

This map represents the south Vancouver Island, lower Fraser River region of British Columbia. Scale: 1 cm equals 9 km. Sample locations: 1. Burrard Inlet; 2. Marion Lake; 3. Mike Lake; 4. Lake Erroch; 5. Pemberton Ditch; 6. Little Campbell River (leiurus); 7. Little Campbell River (trachurus); and 8. Crystal Lake.


VANCOUVER
ISLAND
STRAIT OF GEORGIA
FRASER RIVER
U.S.A.
1
2
3
4
5
6
7
8

Freshwater G. aculeatus of the leiurus form were collected from Marion Lake and Mike Lake (Lower Mainland north of the Fraser); Pemberton Ditch (Lower Mainland south of the Fraser); and Crystal Lake (Vancouver Island). It should be noted that the Marion Lake fish were originally stocked with a Vancouver Island population from Chemainus Lake.

3. Results

The visual results of electrophoresis corresponded to the results achieved by staining the gels with nigrosene. That is, because hemoglobin is characteristically red; one could see the banding patterns without staining. Staining simply improved the resolution of the bands. Further, the agreement of patterns attested to the purity and composition of the hemoglobin.

The samples are summarized in Table 1.

No apparent variation was detected in any gels on the basis of age (length), sex, habitat or season. All gels except one (fig.4) illustrated the four bands characteristic of Italian phenotype A. This aberrant gel was produced from a sample of lake Erroch fish. Considering that several hundred other fish examined (trapped at the same time) did not exhibit any variation and the eight variable individuals were all formed on the same gel, it seems unlikely that the aberrant gel represented real genetic information. More will be said of this in chapter four.

Further experiments were performed varying the handling time of a single sample of blood. No variation could be generated within a reasonable amount of time (one hour). If samples were left for longer periods of time polymerization did occur but with no consistent pattern. Never were fewer than four components detected. Another possibility that I

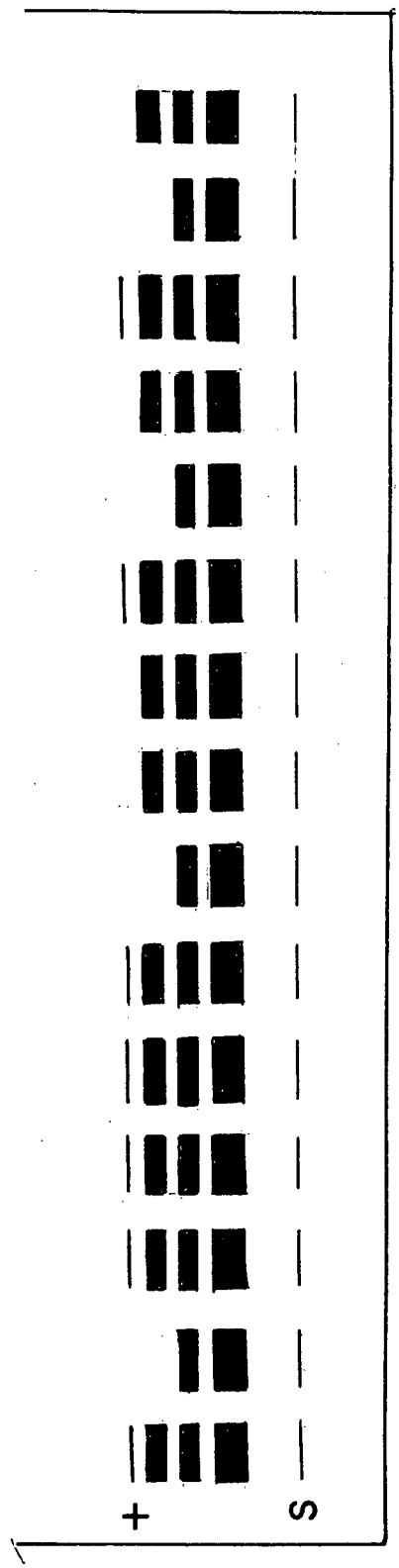
TABLE I. Summary of Hemoglobin Sample Locations and Results

POPULATION	SALTWATER OR FRESHWATER	FORM	HABITAT	SAMPLE SIZE	DATE	BLOOD TYPE
Burrard Inlet	saltwater	trachurus	coastal	32	Jan.1975	type A
Marion Lake	freshwater	leiurus	lake	90	July,1975	type A
Mike Lake	freshwater	leiurus	lake	13	Nov.1975	type A
Lake Errock	saltwater relict in freshwater	trachurus	lake	300	Jan.,May Oct.1975 June,1976	type A
Pemberton Ditch	freshwater	leiurus	stream	12	May,1976	type A
Little Campbell R.	freshwater	leiurus	stream	30	spring1975	type A
Little Campbell R.	saltwater	trachurus	coastal	105	spring1975	type A
Crystal Lake	freshwater	leiurus	lake	37	summer1975	type A

FIGURE 4

The Aberrant Gel

This figure is a diagramatic representation of the aberrant gel taken from a population of Lake Erroch fish. All bands were anodal, S is the starting point, pH 8.4. Four individuals were characterized by Hb with 2 components, four individuals had three components and seven individuals had four components(the typical type A pattern).


+
S

considered was that the four electrophoretic bands reflected the degree of oxygenation of the Hb molecule. Experiments using cyanide as a complexing agent (Sharp, 1973) showed this not to be the case.

4. Discussion and Conclusions

In one electrophoretic gel (15 fish) of Lake Erroch trachurus 8 individuals appeared to be aberrant. The banding pattern exhibited by these aberrant samples was similar to the Italian results (at pH 3.5).

Three things are unfortunate about this occurrence. Firstly, the fish are killed to remove the blood. Secondly, my own observations indicated that samples could not be stored. Thirdly, this incident occurred early in the experimentation and did not appear (at the time) to be unusual. The sum of those three facts, means that there are no second chances at determining the banding pattern of a specific individual.

Another researcher (Sharp, 1973) has reported transient situations occurring due to the innate instability of fish blood. Sharp (1973) recommends either carrying out electrophoresis immediately upon extraction of the blood from the live animals or generating met-hemoglobin by complexing the Hb molecule with cyanide. By carrying out both these alternatives I found that stickleback hemoglobin exhibits the same results when either of these alternatives are used.

Considering that only a few samples (eight) on one single gel (15 individuals) showed variation when over 200 individual samples from the same population immediately before and after the aberrant gel were monomorphic I concluded that some technical error had been made on that

particular day.

Obviously then, in British Columbia where salt-water trachurus, freshwater trachurus and freshwater leiurus all exhibit only phenotype A (Fig. 2), one must reject the general European hypothesis (that the polymorphism is related to an anadromous existence).

CHAPTER III. STICKLEBACK MUSCLE MYOGEN

1. Introduction

Hagen (1967) examined intensively, morphological, ecological and electrophoretic differences between a residential population of leiurus and an anadromous population of trachurus in the Little Campbell River in Southwestern British Columbia. Hagen illustrated that, in the Little Campbell River the two forms of stickleback are ecologically isolated. Transfer experiments were performed and showed that each form seemed to be well adapted to its own habitat and poorly adapted to the habitat of the other. Hagen concluded that the two forms in this particular habitat situation, fit Mayrs' (1963) criterion as individual species. Mayr (1970) agreed with Hagen's analysis.

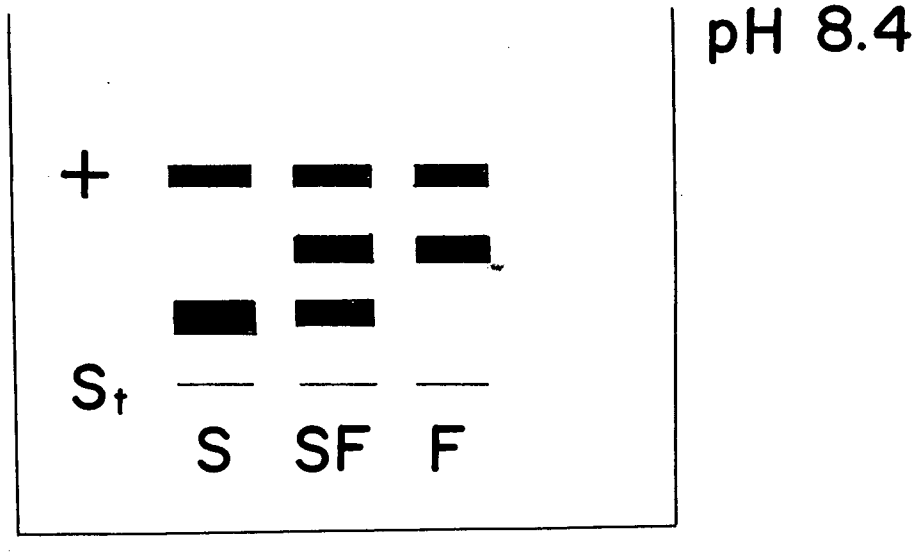
One of the characteristics Hagen utilized was the muscle myogen electrophoretic pattern (Fig. 5). He discovered that, as with body plates on fishes in the Little Campbell River; electrophoretic patterns could be used to distinguish each phase. In fact, Hagen felt that there was an absolute association between form and electrophoretic pattern.

Jones (unpublished) followed this work up by examining fish from Paxton Lake. He found that in this lake two forms appear to co-exist. He felt however, that they were ecologically isolated. Larson (1976) supports this view of the Paxton Lake stickleback. Larson illustrates that two ecologically

FIGURE 5

The Muscle Myogen Pattern

This figure is a diagramatic representation of the allozymic patterns of muscle myogen proteins. The polymorphic enzyme has been identified as creatine kinase. S_t is the starting point at pH 8.4. Type S is the slow allele, SF is the heterozygous allele and F is the fast allele.


pH 8.4
+
S_t
S
SF
F

different forms of stickleback inhabit the lake. One form is benthic, one limnetic. Further, it appears that this ecological separation is based upon inherent behavioral characteristics.

Jones utilized hybrid index scores with muscle myogen type as one of the characters, but Jones found that the precise distinction that Hagen was able to make did not exist. (Assuming one equates the limnetic and benthic forms described by Larson (1976) with the trachurus and leiurus forms described by Hagen (1967)). There is morphological evidence to support this assumption, (Larson, 1976). Instead Ives found that the benthic phase had a propensity for the F phenotype, whereas the limnetic type had a propensity for the S phenotype (Fig. 5).

A complication emerged when gene frequencies of the two populations were pooled. The pooled gene frequencies were found to coincide with Hardy-Weinburg frequencies. How two morphologically and behaviorally distinct populations can have a common gene with alleles at a Hardy-Weinburg frequency has not been satisfactorily explained.

Two important bits of information should also be considered with regard to the work in Paxton Lake. Firstly, Paxton Lake was dammed in 1956, effectively eliminating cutthroat trout, Salmo clarkii, the only significant predator of stickleback in the lake. Secondly, Hay and McPhail (1975) reported that sticklebacks prefer to mate with like

forms.

Accordingly, with the complications involved in the Paxton Lake system, it was plausible that Hagen's initial description of electrophoretic information was indeed accurate (i.e. trachurus exclusively one phenotype, leiurus the other) and that the Paxton Lake population was an aberrant population where premating isolating mechanisms had broken down between anadromous fish trapped by the dam and the resident population.

I therefore decided to test Hagen's initial assumption that the characteristic phenotype F (Fig. 5) typefied freshwater leiurus whereas the phenotype S typefied anadromous trachurus. Further, as Jones' work implied, there is often a difference between lifestyles of the two extreme phases. Higher plated animals tend to be limnetic planktivores whereas lower plated animals tend to be benthic browsers. Accordingly, to incorporate this aspect into the hypothesis, a range of populations from low to high (mean) plated was examined (in other words, to test if the polymorphism was related to lifestyle I assumed that increased plate number reflected an increased pelagic nature). Fish were also examined from a variety of gross habitat types. (Table 2)

It should be noted that Jones identified the polymorphic components as being the enzyme creatine kinase, as other researchers (Gosselin - Rey, et al., 1968) have

done with other fishes. This was performed using enzyme specific stains.

Creatine kinase is one of the enzymes which aids in the regeneration of ATP during muscular activity (Bergmeyer, 1974).

creatine phosphate + ADP $\longrightarrow$ creatine + ATP

TABLE II. A Summary of Muscle Myogen Population Locations, Forms and Habitats

POPULATION	FORM	SALT/FRESHWATER	HABITAT	POTENTIAL PREDATORS
Burrard Inlet	trachurus	saltwater	coastal	present
Mike Lake	leiurus	freshwater	bog lake	present
Lake Erroch	trachurus	freshwater	lake	present
Pemberton Ditch	leiurus	freshwater	stream	present
Little Campbell R.	leiurus	freshwater	stream	present
Little Campbell R.	trachurus	saltwater	coastal	present
Dougan Lake	leiurus	freshwater	small lake	present
Chemainus Lake	leiurus	freshwater	lake	probable
Mesachie Lake	semiarmatus	freshwater	large lake	present
Beck Lake	leiurus	freshwater	lake	present
Diver Lake	leiurus	freshwater	small lake	present
Brannen Lake	leiurus	freshwater	lake	present

2. Materials and Methods

(a) Field Techniques

Fishes were collected utilizing the methods previously described in Chapter II.

The fish were either killed immediately upon capture and frozen on dry ice or frozen after blood extraction. The samples were then stored until electrophoresis was carried out.

(b) Electrophoretic Techniques

At the appropriate time the samples were thawed and a block of muscle was removed from the left caudal region of each fish. Care was taken that only muscle was removed.

The muscle was mixed with an equal volume of 0.055M phosphate buffer at pH 7.5 and homogenized for approximately 10 seconds. The homogenate was centrifuged at 5000 r.p.m. for 5 minutes in a refrigerated centrifuge at 0°C. Paper inserts soaked in the supernatant were subjected to electrophores is in starch gel, as described in chapter II. The gels were subjected to 300 volts for two hours. Gels were stained with the all purpose protein stain nigrosene.

(c) Geographical Location

Fishes were collected from several locations in southwestern British Columbia. (Fig. 6)

Stickleback used were, of course, those collected for hemoglobin electrophoresis. Also, fishes from populations reflecting a cline of mean plate numbers, were collected from southeastern Vancouver Island (Table 3). The samples reflected a cross-section of forms and habitat types.

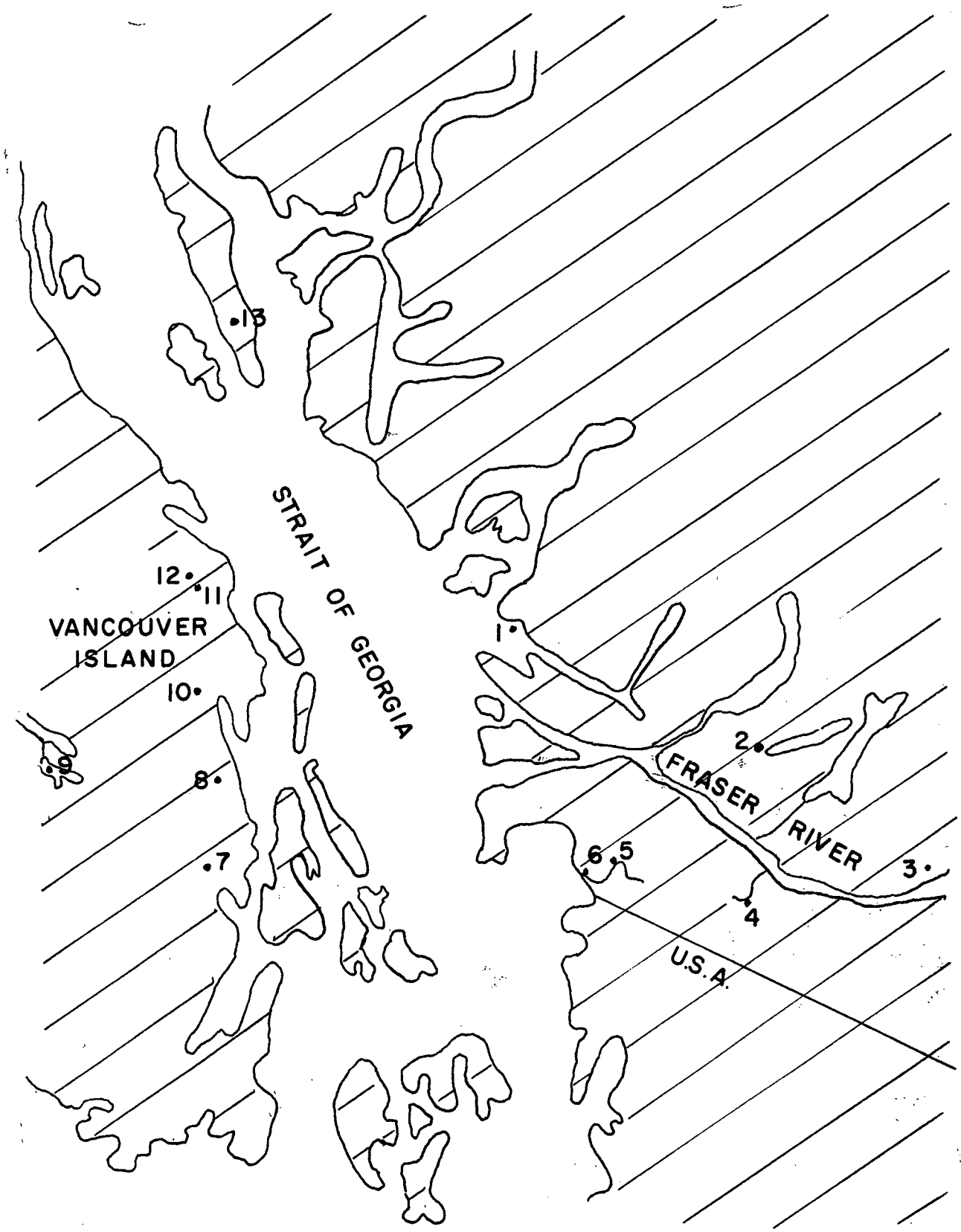
(d) Sample Storage

Samples were stored at -30°C. A small experiment was performed to test anecdotal information that samples could only be stored briefly. A sample of twelve fish was frozen in early October, 1975, thawed for electrophoresis one week later, refrozen, and then re-examined early September, 1976.

FIGURE 6

Geographical Locations of Stickleback Populations
Sampled for the Muscle Myogen Polymorphism

Scale: 1 cm equals 9 km. Sample locations: 1. Burrard Inlet; 2. Mike Lake; 3. Lake Erroch; 4.Pemberton Ditch; 5. Little Campbell River leiurus; 6. Little Campbell River trachurus; 7. Dougan Lake; 8. Chemainus Lake; 9. Mesachie Lake; 10. Beck Lake; 11. Diver Lake; 12. Brannen Lake; 13. Paxton Lake (studied by Jones and Larson).


13
STRAIT OF GEORGIA
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VANCOUVER
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U.S.A.

TABLE III. A Summary of Vancouver Island Populations Sampled

By assuming that increasing plate number per individual reflects increasing pelagic nature one can test the hypothesis that muscle myogen genotypes are related to lifestyle. Mean plate numbers per individual were previously determined by J.D. McPhail (pers.com.).

LOCATION	MEAN PLATE NO. PER INDIVIDUAL
Chemainus Lake	6.0
Dougan Lake	7.3
Beck Lake	10.0
Brannen Lake	11.2
Diver Lake	11.8
Mesachie Lake	14.5

3. Results

Banding patterns were similar to those observed by Jones (unpublished) and Hagen (1967), (Fig. 5). Variation occurred at a single locus with three banding patterns possible; SS, SF and FF.

Five of the twelve populations examined were polymorphic. In each of these cases the gene frequencies did not differ significantly from the Hardy-Weinburg frequencies, (Table 4). The five polymorphic populations were not characterized by any gross habitat type, (Table 4). Two creek populations, two lake populations, and one individual from an anadromous population were polymorphic.

Variation was not apparently based upon age (size), or season. (Fig. 7(a)(b)).

With regard to storage, experiments were performed re-examining refrozen samples after a year of storage. Results after one year were actually superior to the results initially obtained. In other words, initially ten individuals in a sample of twelve were clearly readable; one year later twelve samples were clearly readable with the latter situation reiterating the interpretation of the former ten results.

TABLE IV. Muscle Myogen Frequencies

Theoretical allele frequencies were calculated from the real data then expected Hardy-Weinberg frequencies were extrapolated. The resulting theoretical values were compared with the real frequencies using the Chi^2 test. Statistically there was no discrepancy between the real and theoretical values(figures in parentheses reflect expected values).

POPULATION	SAMPLE SIZE	S (SLOW)	S/F	F (FAST)	CHI^2 AND PROBABILITY
Burrard Inlet	32	32	-	-	
Lake Erroch	45	45	-	-	
Little Campbell R. (leiurus)	45	8(8.91)	24(22.23)	13(13.86)	0.28,P = .65
Little Campbell R. (trachurus)1975	34	33	1	-	
Little Campbell R. (trachurus) 1976	45	45	-	-	
Mike Lake	13	13	-	-	
Pemberton Ditch	12	7(7.44)	5(3.96)	-(0.48)	0.78, P = .40
Chemainus Lake	30	30	-	-	
Dougan Lake	28	12(12.88)	14(12.04)	2(2.80)	0.61, P = .45
Beck Lake	28	8(8.12)	14(14.00)	6(5.88)	0.004,P = .95
Diver Lake	30	30	-	-	
Brannen Lake	30	30	-	-	
Mesachie Lake	45	45	-	-	

FIGURE 7

Age and Season

Figure 7a.: Considering length to be synonymous with age within a given population(eg. Beck Lake) a statistical test was performed to see if there was any indication of a relationship between the muscle myogen polymorphisms and ontogenetic development. No relationship was found therefore it was concluded that the presence of the various protein types was independent of age.

Figure 7b.: Polymorphic gels were discovered at various times of year. This would seem to imply that the polymorphisms were not seasonally induced.

(A) ANALYSIS OF LENGTH (AGE) VERSUS GENOTYPE

GENOTYPE S (length in mm.)	GENOTYPE SF	GENOTYPE F
28.6	30.3	30.8
28.4	27.3	24.1
27.5	27.7	25.1
23.4	24.4	51.4
23.4	24.2	29.3
37.3	35.0	28.3
35.6	54.3	189.0
31.1	35.8	
235.3	34.7	
	36.1	$\bar{x}$ = 31.5
	37.1	
	38.4	
$\bar{x}$ = 29.4	34.2	
	30.7	
	470.2	$\bar{x}$ = 33.6

ANOVA

SOURCE	SUM OF SQUARES	DEGREES FREEDOM	MEAN SQUARE
BETWEEN MEANS	90.2	2	45.1
WITHIN GROUPS	1433.5	25	57.3

Test H_o: $\mu_1 = \mu_2 = \mu_3$, H_1 = not the same

Test statistic $F = \frac{Sm^2}{Sp^2}$, critical region $F < F_{.99(2,25)} = 5.6$

The value for F at Beck Lake is 0.8 therefore one must accept the hypothesis that the means are equal.

(B)

Time of year sample taken	polymorphic population
May 1976	Pemberton Ditch
July 1973	Paxton Lake
August 1976	Little Campbell R. leiurus
September 1976	Beck and Dougan Lake

4. Discussion and Conclusion

Obviously, Hagen's original assumption, that leiurus and trachurus are typefied by specific electrophoretic banding patterns, must be rejected for the species complex as a whole. Both the highest plated and lowest plated animals (i.e. ocean trachurus vs. Chemainus Lake leiurus) are electrophoretically similar. Indeed, even samples from the leiurus zone of the Little Campbell River do not completely reflect Hagen's original impression.

Further, using fish length within a population as an indication of fish age one must conclude that the polymorphism is not ontogenetic in nature. That is, the polymorphism is present in both small and large fishes.

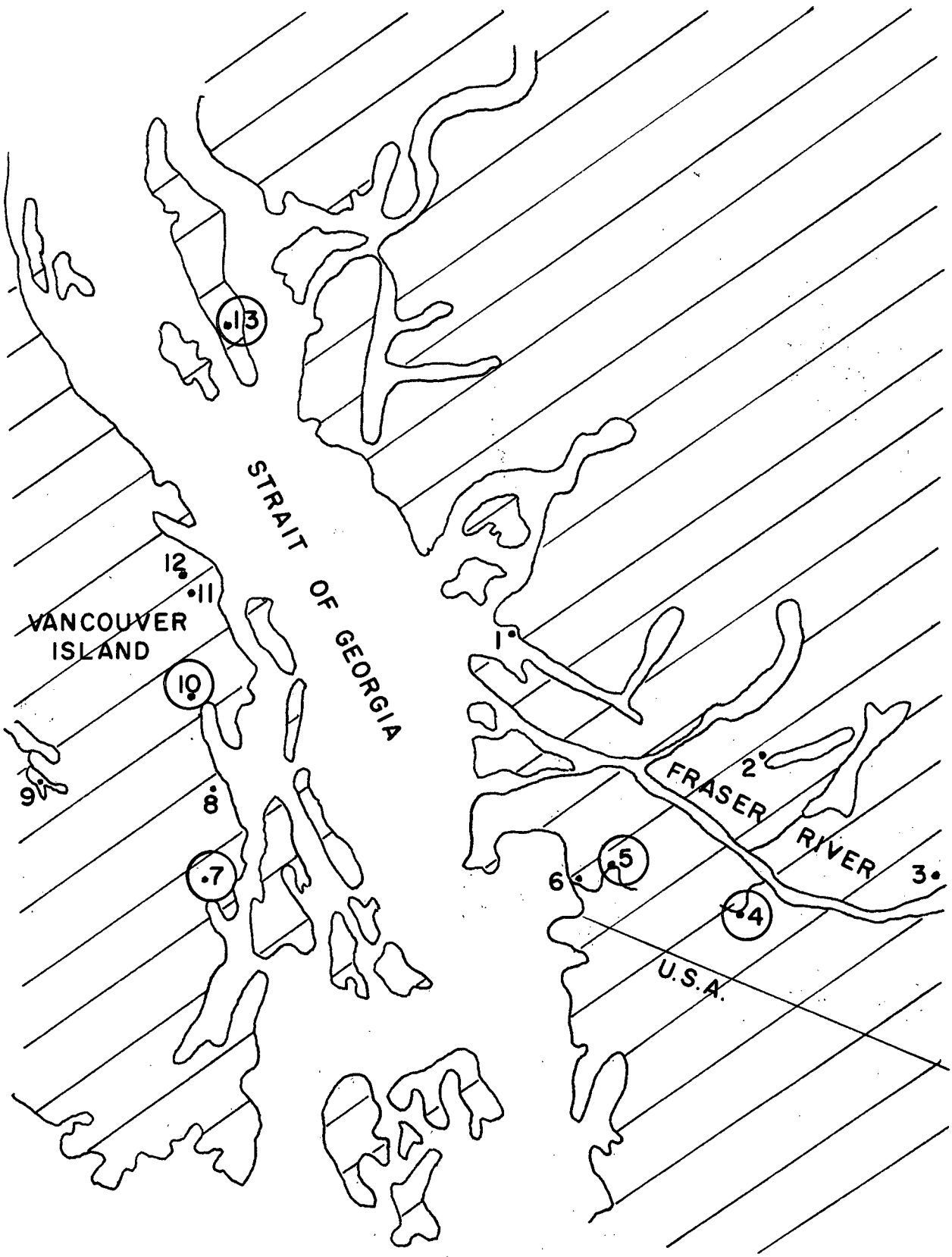
When Jones carried out his electrophoresis, fish smaller than 15 mm were completely homogenized. Researchers (Perriard et al., 1972) have shown enzyme variability between tissues, within a single individual. Indeed some researchers (Eppenberger et al., 1971; Lyslova, 1971) have found this very phenomenon occurring with the enzyme creatine kinase. By taking muscle tissue from the left caudal peduncle area this complication was avoided. Accordingly then, one can assume that the variability observed was real.

If one examines (Fig. 8) the geographical distribution of the protein polymorphism one notes there is no indication of the polymorphism reflecting ranges of individual demes of

FIGURE 8

Geographical Locations of the Polymorphic Populations

Populations 4, 5, 7, 10, and 13 (as well as one individual from the sample from population 6) were discovered to be polymorphic. The sites of these populations are circled.No geographic trend is apparent.


STRAIT OF GEORGIA
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FRASER RIVER
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Gasterosteus aculeatus. Also, considering altitude as a rough estimate of invasion time, older populations do not differ from the assumed parental populations. (ocean trachurus)

A comparison of gross habitat characteristics (i.e. stream, lake, salt-water, freshwater, etc.) with regard to the distribution of the polymorphism provides no obvious correlation.

CHAPTER IV. GENERAL DISCUSSION

My study contains results that are important at several different levels. This can be summarized in the following way.

(a) Stickleback Hemoglobin

Southwestern British Columbia Gasterosteus aculeatus have monomorphic hemoglobin. The transient polymorphism served, on a single occassion, casts some doubt on the European work, particularly when one considers the similarities between the transient pattern at pH 8.4 and the European results (Raunich et al., 1972) at pH 3.5. Obviously their work should be re-examined to double check their results. However, it would appear unlikely that their situation is a technique artifact, simply because the Europeans performed breeding experiments and found that the offspring ratios of the Hb polymorphism corresponded to a situation with variation in a single gene. It should be noted that only offspring were examined. It is clear, that if this aspect of stickleback genetics is to be persued it should be done in the Adriatic area, perhaps on a more physiological level.

As I mentioned in Chapter I, stickleback do not seem to fit traditional species paradigms. It would appear, therefore, that comparative genetic information among widely distributed populations has interest.

Some discussion (Avise, 1975) has occurred supporting the use of electrophoresis in systematics, particularly when several different loci are examined. Other researchers have compared species at a single locus. Tsyuki et al., (1963) and Sharp (1973) for example have compared hemoglobins among various related species. The former group examined hemoglobin molecules of seven salmonids of the northeastern Pacific Coast, and found that the banding patterns were very different for each species, both in terms of component number and shape. Ferguson (1974) considered several electrophoretic patterns (general proteins, esterases, creatine kinase and phosphoglucomutase) to suggest species status for three forms of a complex of coregonid fishes. These coregonid are extremely variable morphologically, with many of the variations, known to be environmentally induced.

With these precedents in mind, it appears that one could be justified in saying, the similarity among hemoglobin banding patters (anodal bands, three large, one small) of all freshwater (and some saltwater) European sticklebacks and all southern British Columbia sticklebacks provides reinforcing evidence that the populations in question belong to the same species.

(b) Stickleback Muscle Myogen

Two possibilities remain with respect to explaining the distribution of the creative kinase alleles in stickleback populations.

Firstly, it is possible that the polymorphism reflects adaptation to a subtle environmental character. This hypothesis is extremely difficult to test, and probably impossible to discount.

Secondly, the possibility also exists that the polymorphism is selectively neutral. There is some evidence to support this point of view. All polymorphic populations were in Hardy-Weinburg equilibrium. Admittedly the samples were small, however, with five polymorphic populations, all in Hardy-Weinberg equilibrium it would seem not implausible that the alleles are selectively neutral.

Further, Redfield et al., (1972) attempted a similar study examining a blood plasma polymorphism in blue grouse (Dendragapus obscurus) populations from a wide range of habitats on Vancouver Island. Results in the grouse situation were similar to those in stickleback except all populations examined contained the polymorphism. This result was cited as proof of selective maintenance. Study over a period of time, to see if gene frequencies, and locations of polymorphic stickleback populations change or remain stable, would clarify this situation.

Another interesting point is that stickleback are not the first fish reported to have a muscle myogen polymorphism. It has been stated in the literature (Morgan and Ulanowicz, 1976; Uthe and Ryder, 1970); that muscle myogen polymorphism is extremely rare. One is inclined to comment that muscle

myogen polymorphisms can be moved from the rare to the less than uncommon category since at least six species are known to have polymorphisms of this type. A comparision of these species (Table 5) illustrates the phylogenetic and environmental range encompassed by this polymorphism. (The polymorphism has not been positively identified as creatine kinase in all these examples).

Species exhibiting the polymorphism include saltwater fishes, freshwater fishes, river fishes, pelagic ocean fishes, piscivores and herbi vores. The only common factor among these species is that they are all teleosts, and this could be a reflection of the species sampled.

The creatine kinase polymorphism also emphasizes the lack of biochemical structural information across phylogenetic lines. Creatine kinase is a relatively well studied enzyme because of its association with the Duchenne type of muscular dystrophy (Bergmeyer, 1974). It has always been thought to have a dimeric structure (Kuby et al., 1962). This idea is not compatible with the stickleback polymorphism. If the enzyme was dimeric the heterozygous form (S/F) would have contained three bands instead of two (Fig. 9). It is remotely plausible that this dimeric structure could be masked by limitations of the technique on one occassion, however this multitude of examples provides many repetitions all implying non-dimeric structure. May (1975) also pointed out this monomeric nature of creatine kinase in the genus Onchorynchus. Many electrophoretic studies have pointed out

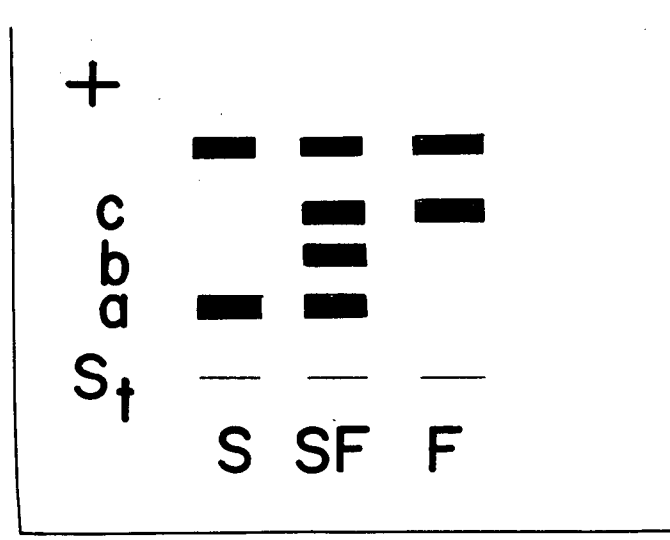
TABLE V. A Comparison of the Six Species Demonstrating a Similar Muscle Myogen Protein Polymorphism

SPECIES	LOCATION	HABITAT	ECOTYPE	FOOD	REFERENCES
Menidia menidia	Atlantic coast	estuarine areas	schooling	copepods shrimp mysids	Morgan and Ulanowicz,1976. Liem and Scott,1966
Catostomus catostomus	North America and Siberia	freshwater	bottom browser	amphipods insect larvae	McPhail and Lindsey, 1970. Tsuyuki et al.,1967.
Anoplopoma fimbria	North Pacific	saltwater	pelagic	piscivore	Tsuyuki and Roberts, 1969. Hart,1973.
Sebastodes elongatus	North America Pacific coast	saltwater	50-200 fathoms	-	Tsuyuki et al., 1969. Hart,1973.
Stizostedion vitreum	central and east North America	freshwater	pelagic schooler	piscivore	Uthe and Ryder, 1970. McPhail and Lindsey,1970.
Gasterosteus aculeatus	northern hemisphere	freshwater and anadromous	benthic/pelagic	ostracods larvae plankton	McPhail and Lindsey, 1970.

FIGURE 9

Hypothetical Pattern for Dimeric Quarternary Structure of the Enzyme Creatine Kinase

S_t represents the starting point. Homozygous S is represented by component a, homozygous F is represented by component c and heterozygous SF has both these components present plus component b (which would be a composite form of a and c).


+
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similar molecular incongruities but few researchers seem to have investigated further. For example comparisons of mammalian hemoglobin seems to have caused people to generalize the phylogenetic consistency of the quartenary structure of all proteins of all animals.

Consider the taxonomic arguments cited in this chapter with regard to hemoglobin. The same argument can be applied to the distribution of creatine kinase. The extreme forms of stickleback, leiurus and trachurus, were electrophoretically similar. That is, saline populations of trachurus were electrophoretically similar to the leiurus populations most remote from the ocean. This would also provide reinforcing evidence to support the view that leiurus and trachurus are members of the same species.

(c) Future Research

Consider the blood polymorphism in Europe. As I stated earlier, the authenticity of the polymorphism should be checked. Assuming the outcome of this check is positive, I would then perform the following bits of research.

The first operation I would perform would involve biochemically characterizing each electrophoretic hemoglobin component found in Gasterosteus aculeatus phenotypes A, AB and B (Fig. 2). This would either support the European hypothesis or suggest some other alternative hypothesis. Further, I would include a determination of the molecular weights of each of the components so that a clearer understanding of their structural nature might emerge. (Perhaps

even going as far as sequencing the protein).

Then, armed with a new hypothesis or reinforced old hypothesis I would perform transfer experiments. I would transport offspring of known parentage into salt, brackish and freshwater environments to determine whether or not the oxygen capacities were genetically predetermined or the result of acclimatization, (which would also be a genetic phenomenon). Guernsey and Puluhowich (1975) illustrated this latter phenomenon in blood oxygen capacities of the American eel (Anguilla rostrata) in salt, brackish and freshwater environments. A check for this phenomenon in British Columbia stickleback may also prove interesting. Would anadromous and freshwater sticklebacks (with the same electrophoretic components) have different oxygen capacities based upon electrophoretically undetectable genetic differences.

Another possible project could involve a biochemical comparison between the hemoglobin of the threespine stickleback (G. aculeatus) and the ninespine stickleback (Pungitius pungitius). Lewis et al., (1972) observed that in England these two species are often found together in the same habitats. They are also known to have virtually identical diets, (Hynes), 1950). The question arose, how do these species avoid competition? Lewis et al., observed that within those habitats, niche separation appeared to occur on the basis of oxygen availability. Pungitius pungitius

appeared to be more tolerant of low oxygen levels because of physiological adaptation. Since in Canada, areas exist where these species are found together and also areas exist where they are found alone (Scott and Crossman, 1973), it would appear that some interesting work could be carried out with respect to biochemical adaptation, and possibly (physiological) character displacement (Dobzhanski, 1970). Raunich et al., (1972) stated in Italy P. Pungitius hemoglobin is electrophoretically similar to the hemoglobin of non-migratory Gasterosteus aculeatus.

Now, the question arises what further work should be done with the creatine kinase polymorphism? If I were continuing in this research I would try to learn more about the *in vitro* biochemical characteristics of the creatine kinase components. I would establish pH optima, temperature optima pressure effects and molecular weights of the various components. This might answer the question whether or not the components are neutral, or give insight into their function, (if they have some subtle function). Further, as Scholl and Eppenberger (1972) have pointed out, electrophoretic components of creatine kinase vary among tissues within individual fishes. The question arises, Why? Why should muscle have one form of creatine kinase and brain, and teste and heart and stomach tissues all have other individual forms? Obviously, more knowledge on the

structural and biochemical characteristic levels may provide more insight into this question. Other questions arise in this regard. What is the function of creatine kinase in the brain? Do enzymes such as creatine kinase have multiple functions? Much more information is needed. The technique of electrophoresis will be utilized more effectively after questions like these have been answered.

Literature Cited

Aspinwall, N. 1974. Genetic analysis of North American populations of the pink salmon, Oncorhynchus gorbuscha, possible evidence for the neutral mutation-random drift hypothesis. Evolution 28: 295-305.

Avise, J.C. 1975. Systematic value of electrophoretic data. Systematic Zoology. 24: 465-481.

Baldwin, J., and P.W. Hochachka. 1970. Functional significance of isoenzymes in thermal acclimation: acetylcholinesterase from trout brain. Biochemical Journal 116: 883-887.

Bergmeyer, H.U.(Ed.) 1974. Methods of enzymatic analysis. Academic Press, New York and London: 784 - 797.

Black, E.C. 1958. J. Fisheries Research Board of Canada 15: p573.

Brewer, G.J. 1970. An introduction to isozyme techniques. Academic Press, New York.

Callegarini, C., and C. Cucchi. 1968. Polimorfismo emoglobinico in varie popolazioni di Gasterosteus aculeatus di bacini idrografici diversi. Annali Univ. Ferrara (Nuova Serie), 2: 31-36.

Callegarini, C. and C. Cucchi. 1969. Le emoglobine di Gasterosteus aculetus e di Gasterosteus pungitius (Teleostea gasterosteidae) Annali Univ. Ferrara (Nuova Serie), 2: 145-150.

Clemens, W.A., and G.V. Wilby. 1961. Fishes of the Pacific Coast of Canada. 2nd ed. Bull. Fish. Res. Bd. Canada 63. 443pp.

Cucchi, C. 1969. Analisi elettroforetica delle emoglobine di alcune specie di Teleostei. Istituto Lombardo (Rend. Sc.), B 103: 3-7.

Denton, J.E., and M.K. Yousef. 1975. Seasonal changes in the hematology of rainbow trout, Salmo gairdneri. Comp. Biochem. Physiol. 51(A): 151-153.

Dobzhanski, T. 1970. Genetics of the evolutionary process. Columbia University Press, New York and London. 505pp.

Dryfoos, R.L. MS, 1965. Life history and ecology of the longfin smelt in Lake Washington. Ph.D. Thesis. Coll. Fish., Univ. Wash., Seattle, Wash. 159pp.

Eppenberger, H.M. 1968. Comparative aspects of the multiple forms of creatine kinase, pp.231-242. In, Homologous enzymes and biochemical evolution, Ed. N.Thoai and J. Roche. Gordon and Breach, New York.

Eppenberger, H.M., A. Scholl and H. Ursprung. 1971. Tissue - specific patterns of creatine kinase (2.7.3.2.) in trout. FEBS Letters, 14: 317-319.

Ferguson, A. 1974. The genetic relationship of the coregonid fishes of Britain and Ireland indicated by electrophoretic analysis of tissue proteins. J. Fish Biol. 6: 311-315.

Gosselin-Rey, C., G. Hamoir and R.K. Scopes. 1968. Localization of creatine kinase in the starch-gel and moving boundary electrophoretic patterns of fish muscle. J.Fish. Res. Bd. Canada, 25(12): 2711-2714.

Guernsey, D.L., and J.J. Poluhowich. 1975. Blood oxygen capacities of eels acclimated to fresh-, brackish-, and salt-water environments. Comp. Biochem. Physiol. 52(A): 313-316.

Hagen, D.W. 1967. Isolating mechanisms in threespine sticklebacks (Gasterosteus). J.Fish. Res. Bd. Canada, 24(8): 1637-1692.

Hagen, D.W., and L.G. Gilbertson. 1972. Gasterosteus aculeatus L. in the Pacific Northwest, America. Evolution, 26: 32-51.

Hagen, D.W., and J.D. McPhail. 1970. The species problem within Gasterosteus aculeatus on the Pacific coast of North America. J. Fish. Res. Bd. Canada, 27: 147-155.

Hart, J.L. 1973. Pacific fishes of Canada. Bull. Fish. Res. Bd. Canada 180. 740pp.

Hay, D.E., and J.D. McPhail. 1975. Mate selection in threespine sticklebacks (Gasterosteus). J.Fish. Res. Bd. Canada, 53(4): 441-450.

Hjorth, J.P. 1974. Genetics of Zoarces populations. Hereditas 78: 69-72.

Hynes, H.B.N. 1950. The food of fresh-water sticklebacks (Gasterosteus aculeatus and Pygosteus pungitius), with a review of methods used in studies of the food of fishes. J. Anim. Ecol. 19: 36-58.

Koch, H.J.A., N.P. Wilkins, E. Bergstrom, and J.C. Evans. 1967. Further studies on multiple components of the haemoglobins of Salmo salar L. Meded. K. Vlaam. Acad. 29(7): 1-16.

Koehn, R.K. 1969. Hemoglobins of fishes of the genus Catostomus in Western North America. Copeia, 1969(1): 21-30.

Kuby, S.A., and E.A. Noltmann. 1962. ATP: Creatine transphosphorylase, p. 515, Vol. 6. In the Enzymes, ed. P.D. Boyer, H. Landy and K. Mysbach. Academic Press, New York and London.

Larson, G.L. 1976. Social behavior and feeding ability of two phenotypes of Gasterosteus aculeatus in relation to their spatial and trophic segregation in a temperate lake. Can. J. Zoo. 54(2): 107-121.

Lewontin, R.C., 1974. The genetic basis of evolutionary change. Columbia University Press, New York and London. 346pp.

Liem, A.H., and W.B. Scott. 1966. Fishes of the Atlantic Coast of Canada. Bull. Fish. Res. Bd. Canada 155. 485pp.

Lewis, D.B., M. Walkey and H.J.G. Dartnall. 1972. Some effects of low oxygen tensions on the distribution of the three-spined stickleback Gasterosteus aculeatus L. and the nine-spined stickleback Pungitius pungitius (L.). J. Fish Biol. 4(1): 103-109.

Lyslova, S.N. 1971. Temperature dependence of creatine kinase reaction rate. International Symposium Reinhardsbrunn, Mai 1971: 611-616.

May, B. 1975. Electrophoretic variation in the genus Onchorhynchus: methodology, genetic basis, and practical applications to fisheries research management. M.Sc. Thesis. Department of Fisheries, University of Washington, Seattle, Washington.

Mayr, E. 1963. Animal species and evolution. Harvard, Belknap Press, Cambridge, Mass.

Mayr, E. 1970. Populations, species and evolution. Harvard, Belknap Press, Cambridge, Mass. 453pp.

McPhail, J.D. 1969. Predation and the evolution of a stickleback (Gasterosteus). J. Fish. Res. Bd. Canada 26: 3183-3208.

McPhail, J.D., and C.C. Lindsey. 1970. Freshwater fishes of northwestern Canada and Alaska. Bull. Fish. Res. Bd. Canada 173: 381pp.

Miller, R.R., and C.L. Hubbs. 1969. Systematics of Gasterosteus aculeatus, with particular reference to intergradation and introgression along the Pacific Coast of North America: a commentary on a recent contribution. Copeia 1969: 52-69.

Morgan, R.P., and N.I. Ulanowicz. 1976. The frequency of muscle protein polymorphism in Menidia menidia (Atherinidae) along the Atlantic Coast. Copeia, 1976(2): 356-360.

Munzing, J. 1963. The evolution of variation and distributional patterns in European populations of the three-spined stickleback, Gasterosteus aculeatus. Evolution, 17: 320-332.

Perez, J.W., and N. Maclean. 1974. Ontogenetic changes in haemoglobins in roach, Rutilus rutilus (L.) and rudd, Scardinius erythrophthalmus (L.). J. Fish Biol. 6(4): 479-482.

Perriard, J., A. Scholl, and H.M. Eppenberger. 1972. Comparative studies on creatine kinase isozymes from skeletal-muscle and stomach of trout. J. Exp. Zoo. 182(1): 119-125.

Powers, D.A. 1972. Hemoglobin adaptation for fast and slow water habitats in sympatric catostomid fishes. Science, 177: 360-362.

Raunich, L. C. Callegarini and C. Cucchi. 1969. Il polimorfismo emoglobinico I: Gasterosteus aculeatus. Boll Zool. 36: 403.

Raunich, L., C. Callegarini and C. Cucchi. 1972. Ecological aspects of hemoglobin polymorphism in Gasterosteus aculeatus (Teleostea). 5th European symposium of marine biology. Piccin Publishers: 153-162.

Redfield, J.A., F.C. Zwickel, J.F. Bendell, and A.T. Bergerud. 1972. Temporal and spatial patterns of allele and genotype frequencies at the Ng locus in blue grouse (Dendragapus obscurus). Can. J. Zool. 50(12): 1657-1662.

Scholl, A., and H.M. Eppenberger. 1972. Patterns of the isozymes of creatine kinase in Teleostean fish. Comp. Biochem. Physiol. 42(B): 221-226.

Scott, W.B., and E.J. Crossman. 1973. Freshwater fishes of Canada. Bull. Fish. Res. Bd. Canada 184. 966pp.

Selander, R.K., and S.Y. Yang. 1969(a). Biochemical genetics and behavior in wild house mouse populations. In: contributions to behavior - genetic analysis. Ed. G. Lindzey and D.D. Thiessen. Appleton - Century - Crofts, New York.

Selander, R.K., and S.Y. Yang. 1969(b). Protein polymorphism and genic hoterozygosity in a wild population of the house mouse (Mus musculus). Genetics, 63: 653-667.

Sharp, G.D. 1973. An electrophoretic study of hemoglobins of some Scombroid fishes and related forms. Comp. Biochem. Physiol. 44(b): 381-388.

Tsuyuki, H., and E. Roberts. 1963. Species differences of some members of salmonidae based on their muscle myogen patterns. J. Fish. Res. Bd. Canada, 20(1): 101-104.

Tsuyuki, H., E. Roberts, R.H.Kerr, J.F. Uthe and L.W. Clarke. 1967. Comparative electropherograms of the family Catostomidae. J. Fish. Res. Bd. Canada, 24(2): 299-304.

Tsuyuki, H., E. Roberts, R.H. Lowes, W.Hadaway, and S.J. Westrheim. 1968. Contribution of protein electrophoresis to rockfish (Scorpaenidae) systematics. J.Fish. Res. Bd. Canada, 25(11): 2477-2501.

Tsuyuki, H., and E. Roberts. 1969. Muscle protein polymorphism of sablefish from the eastern Pacific Ocean. J. Fish. Res. Bd. Canada, 26(10): 2633-2641.

Uthe, J.F., and R.A. Ryder. 1970. Regional variation in muscle myogen polymorphism in walleye (Stizostedion vitreum vitreum) as related to morphology. J. Fish. Res. Bd. Canada, 27(5): 923-927.

Wilkins, N., and T. Iles. 1966. Haemoglobin polymorphism and its ontogeny in herring (Clupea harengus) and sprat (Sprattus sprattus). Comp. Biochem. Physiol. 17: 1141-1160.