

THE MAINTENANCE OF REPRODUCTIVE ISOLATION BETWEEN
HYBRIDIZING POPULATIONS OF THE PEAMOUTH CHUB, MYLOCHEILUS
CAURINUM AND THE REDSIDE SHINER, RICHARDSONIUS BALTEATUS

by

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September, 1968

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ABSTRACT

The modern species concept (Mayr, 1963) stresses the interbreeding within, and the reproductive isolation between, species. However, "interbreeding" is not strictly an intraspecific characteristic since numerous interspecific hybrids have been reported, especially among the temperate freshwater fishes. In this investigation, hybridizing (interbreeding) populations of the peamouth chub, Mylocheilus caurinum (Richardson), and the redbside shiner, Richardsonius balteatus (Richardson) from Stave Lake, British Columbia, were studied to determine: (1) if interbreeding between them was resulting in the swamping (lack of reproductive isolation) of their gene pools, and (2) if swamping was absent, what isolating mechanisms were operative?

To detect the presence or absence of swamping, two approaches were used. The first measured shifts in means of certain morphological characters for the two species within and outside the area of hybridization. The second determined the frequency of various hybrid generations. If swamping is not occurring, the frequency of hybrid backcross individuals should decrease as backcrossing continues. Both approaches indicated that swamping is absent between Mylocheilus and Richardsonius.

In the absence of swamping, isolating mechanisms between the two species were examined. Seasonal, temporal (diel), spatial, and ethological premating isolating mechanisms do not appear to be effective since Mylocheilus, Richardsonius, and their hybrids spawn at the same time and place within Devils Creek, a major spawning area in Stave Lake.

Egg and fry survival of crosses involving hybrid individuals were measured under experimental conditions to determine if they serve as postmating isolating mechanisms. F_1 hybrid males are partially sterile as demonstrated by the poor egg survival of crosses involving them. However, egg survival of hybrid females when backcrossed with Mylocheilus and Richardsonius males was comparable to the parental species' crosses. Thus, fertility is not considered an effective isolating mechanism. The experiments testing the fry survival of the parental species, reciprocal F_1 hybrids, and hybrid backcrosses to Mylocheilus (= BCM) yield a clue to what isolating mechanism prevents swamping. The fry survival of reciprocal F_1 hybrid fry do not differ significantly from Mylocheilus fry. In contrast, the survival of hybrid backcross to Mylocheilus fry is approximately 20 % less than either reciprocal F_1 hybrids or Mylocheilus fry after only 48 days rearing. Additionally, 11.5% of the surviving BCM fry possess gross abnormalities. Thus, hybrid inferiority is demonstrated experimentally.

Circumstantial evidence was also gathered from Stave Lake in 1967 which suggests the inferiority of BCM fry. Relative to Mylocheilus, BCMs were five times more abundant as fingerlings than as adults.

Little information was gathered on hybrid backcrosses to Richardsonius. They appeared scarce as fingerlings as well as adults.

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INTRODUCTION

The modern concept of "species" envisions them to be "... groups of actually or potentially interbreeding populations which are reproductively isolated from other such groups. " (Mayr, 1963). This concept stresses the interbreeding within, and the reproductive isolation between, species. Within the last thirty years, Dr. Carl. L. Hubbs and his colleagues demonstrated that interbreeding between supposedly valid species is common among freshwater fishes, and scores of hybrids are reported among the Catostomidae, Centrarchidae, and Cyprinidae. Is interbreeding between supposedly valid species reconcilable with the modern species concept (Dobzhansky, 1951; Sibley, 1961; Bigelow, 1965; and Hagen, 1967)? This question has interested evolutionists for many years and, consequently, they have made concerted efforts to understand the relationship between hybrids, hybrid zones, and reproductive isolation.¹ To clarify this relationship, mechanisms which maintain the genetic integrity (reproductive isolation) between two species must be understood. Mayr (1963) divides these into premating and postmating isolating mechanisms, a division based on the order in which they operate.

When two closely related, geographically isolated, populations re-establish contact, there are at least four possible outcomes: 1) the two

1)".. refers to the protective devices of a harmoniously coadapted gene pool against destruction by genotypes from other gene pools. " (Mayr, 1963).

populations do not interbreed because of effective premating isolating mechanisms, and so maintain the genetic integrity of the populations; 2) the two populations do interbreed but selection against the hybrids maintains the genetic integrity of the populations; 3) the two populations interbreed and certain recombinant types are at a selective advantage and, therefore, provide new genes to the gene pool(s) of one or both populations (introgression²). In such cases, reproductive isolation is maintained between the populations because the introgressed genes do not destroy--but increase--the harmony of the coadapted gene pools; or 4) the populations interbreed, fusing into a single population (swamping), and genetic integrity is lost.

According to the species definition of Mayr, the populations in situations (1), (2), and (3) have attained specific status. However, the distinction between (2), specific status, and (4), swamping, is difficult to make unless the populations have been observed for some time, or unless in the former case selection against the hybrids has been demonstrated.

The peamouth chub, Mylocheilus caurinum (Richardson), and the red-side shiner, Richardsonius balteatus (Richardson), are two common cyprinid fishes in British Columbia and the northwestern United States, and the two species coexist in many localities without interbreeding (Fig. 1). In fact, it is difficult to find large areas where their distributions do not overlap. Two such areas are Vancouver Island and the Sechelt Peninsula

2) Introgression is "... an infiltration of the germplasm of one species into that of another. " (Anderson and Hubricht, 1938).



Figure 1. The peamouth chub, Mylocheilus caurinum (above), redside shiner, Richardsonius balteatus (below), and their hybrid (middle). All three fish are in spawning coloration.

where only Mylocheilus is found. In one locality, Stave Lake, British Columbia, Mylocheilus and Richardsonius engage in extensive hybridization. The hybrid is also depicted in Figure 1. This hybridization is clearly demonstrated by a comparison of the anal fin ray distributions (Fig. 2) of the combined Mylocheilus-Richardsonius gene pools between Stave Lake (where hybrids occur) and adjacent Alouette Lake (where hybrids are absent). There is no overlap in anal ray number between Mylocheilus (7-8 anal rays) and Richardsonius (14-22 anal rays) in Alouette Lake, but in Stave Lake although the anal fin ray distribution is bimodal, there are many individuals with an intermediate number of anal rays (9-12). These individuals with 9 to 12 anal fin rays are hybrids.

Initially, it appeared that not only were the two species hybridizing in Stave Lake, but also that they were swamping. Circumstantial evidence supporting this view is: 1) the majority of spawning Mylocheilus in Stave Lake, the hybrid zone, are unusually small (≈ 120 mm) and resemble mature Richardsonius in size (≈ 100 mm). In contrast, spawning Mylocheilus from other areas, including the nearby (and at one time sympatric) Mylocheilus from lower Stave River, are usually much larger (≈ 200 mm); 2) Mylocheilus in the hybrid zone begin spawning approximately June 1, whereas populations outside the hybrid zone spawn much earlier. Mylocheilus from the Alouette and Stave Rivers spawn in late April or early May. Richardsonius spawn from late May to August in British Columbia (Carl, Clemens, and Lindsey, 1959). Both observations indicate that in the hybrid zone significant changes have occurred in the Mylocheilus population that bring

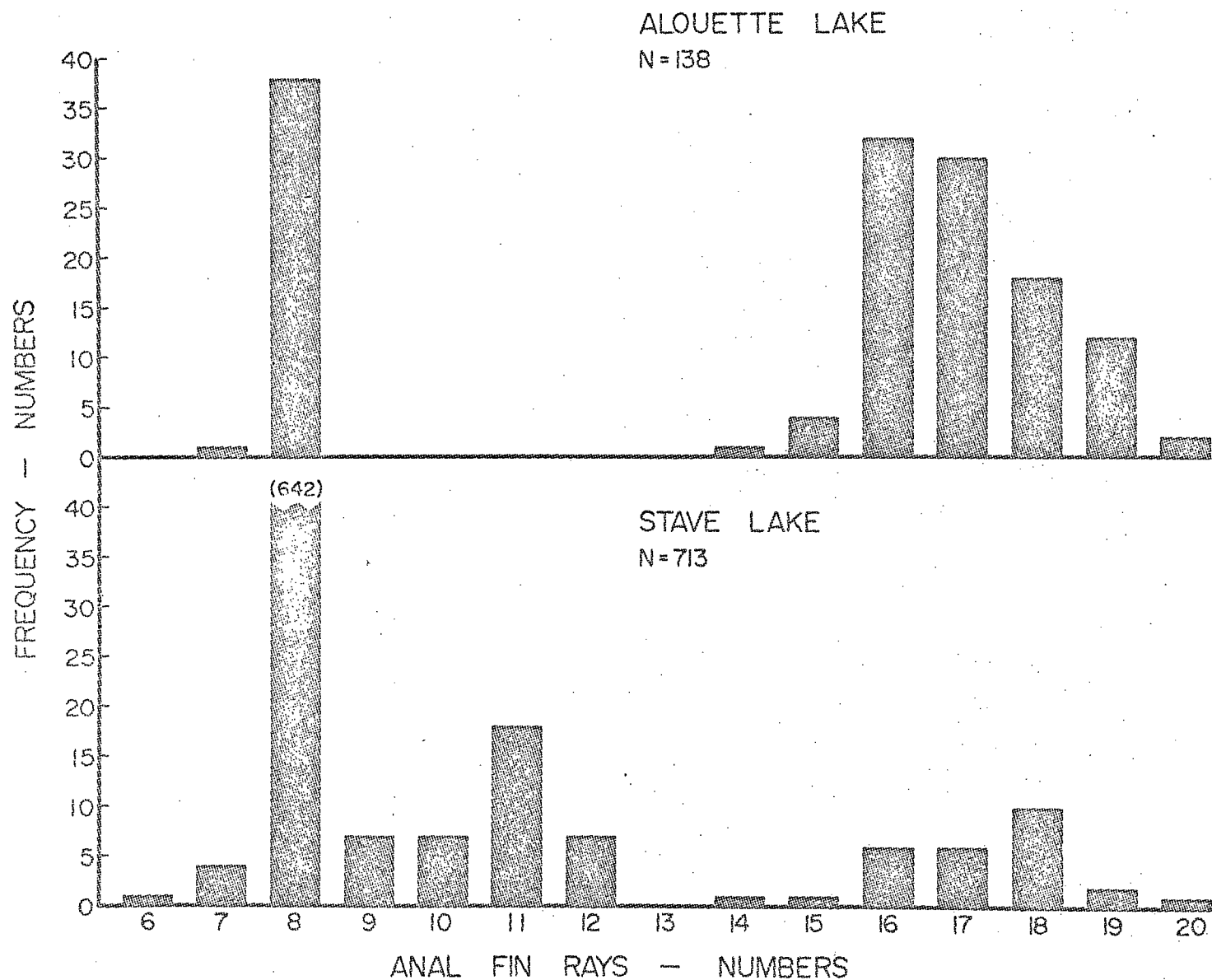


Figure 2. Anal fin ray distribution of the Mylocheilus-Richardsonius gene pool from Alouette and Stave Lakes (1967 adults). Samples were collected with 25 and 38 mm stretch mesh gill nets.

it closer to Richardsonius and suggest that swamping might be in progress.

The purpose of the present study was to determine which of the three hybrid situations described above best fits the Stave Lake populations of Mylocheilus and Richardsonius, and if reproductive isolation exists, to attempt to determine what isolating mechanism(s) are operative?

GENERAL MATERIALS AND METHODS

Description of Study Area

Stave Lake and one of its small tributary streams, Devils Creek, were the primary study areas (Fig. 3). Originally 19 km in length, the lake was enlarged to 30 km with the completion of hydroelectric facilities in 1924. The lake now consists of an upper portion, 19 km in length, and a lower portion of 11 km that corresponds to the flooded Stave River channel. The flooded portions contain large stands of submerged forest. Annual fluctuations in the lake level of 9 meters are not uncommon and certain tributary streams are markedly altered by these fluctuations. Stave Lake is an oligotrophic lake containing at least nine species of fish in addition to the aforementioned hybrids. They are: kokanee, Oncorhynchus nerka, three-spine stickleback, Gasterosteus aculeatus, cutthroat trout, Salmo clarkii, squawfish, Ptychocheilus oregonense, peamouth chub, Mylocheilus caurinum, redbside shiner, Richardsonius balteatus, largescale sucker, Catostomus macrocheilus, brown catfish, Ictalurus nebulosus, prickly sculpin, Cottus asper, and hybrids, Mylocheilus x Richardsonius.

Devils Creek, a small stream 2-4 meters wide, was used to observe spawning populations of Mylocheilus, Richardsonius, and their hybrids. Flowing into the lower portion of Stave Lake from Devils Lake, Devils Creek is ideally suited for observation because of its (1) small water volume, (2) good water clarity, and (3) short length (425 meters). These three factors make observations possible at any time throughout its length. Devils Creek also appears to be a major spawning area in the lower portion of Stave Lake.

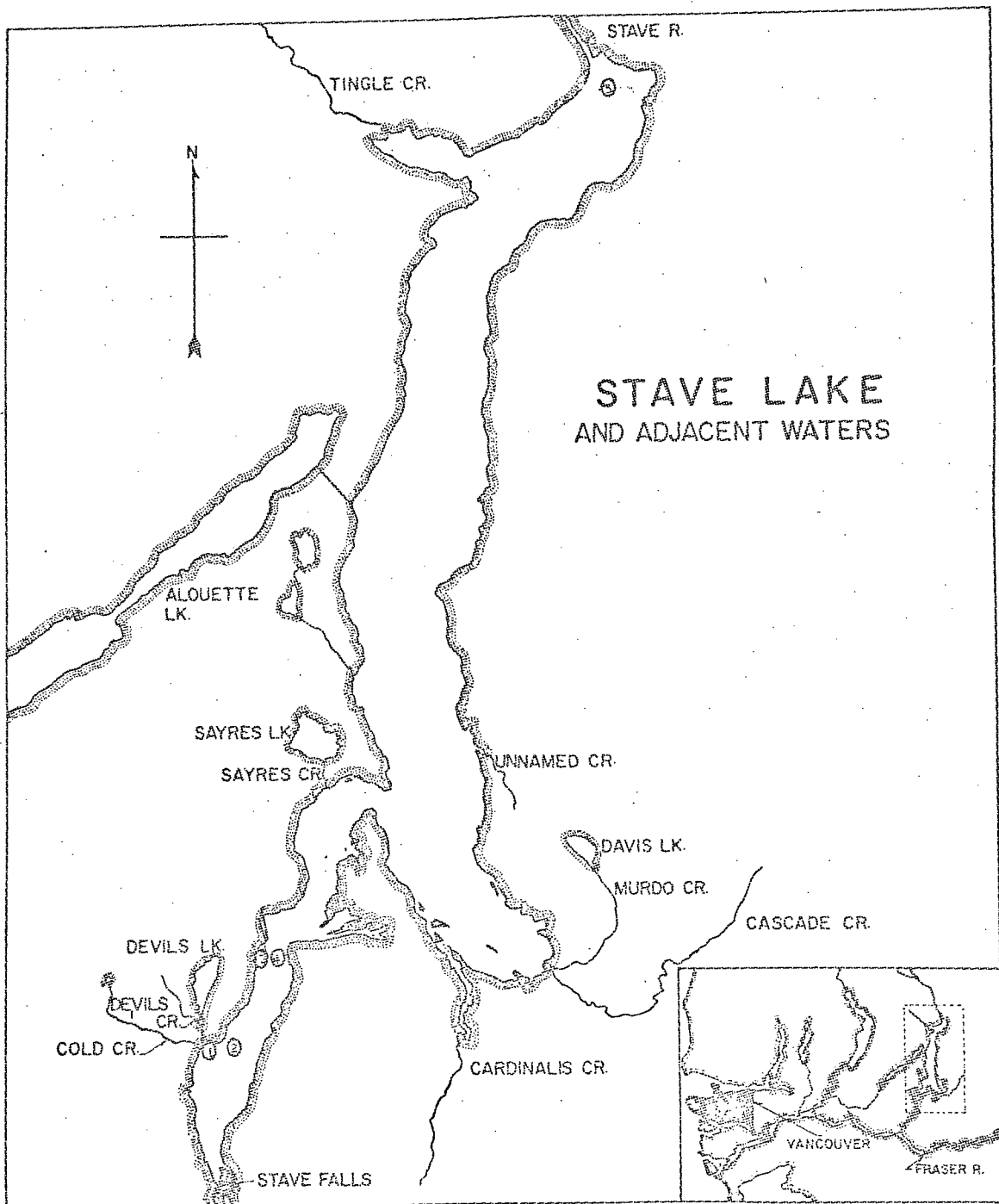


Figure 3. Stave Lake, British Columbia, and adjacent waters.

Sampling

Four regular sampling stations were established in Stave Lake to collect adult Mylocheilus, Richardsonius, and their hybrids (Fig. 3).

These were all in the lower portion of Stave Lake. However, some sampling was done at the extreme north end (upper portion) of the lake and hybrids were also abundant in this locality. Samples were taken by 19, 25, and 38 mm stretch mesh monofilament nylon gill nets varying in size from 2.4 x 15.2 m to 7.6 x 15.2 m. In most instances, sampling was done at night since day-time sampling was very ineffective. In addition to Stave Lake samples, adult Mylocheilus were sampled from Babine, Hatzic, Alouette, Ruby and Otter Lakes and the Stave River, while Richardsonius were sampled from Babine, Shea, and Alouette Lakes. No hybrid fishes (Mylocheilus x Richardsonius) are found in any of these locations.

Fingerling Mylocheilus, Richardsonius, and their hybrids were collected with a fine mesh dip net along the shores of the lower portion of Stave Lake. All collections of fingerlings were made during daylight hours. Heaviest concentrations of fingerlings appeared in the littoral areas adjacent to Devils and Rollie Creeks.

REPRODUCTIVE ISOLATION BETWEEN MYLOCHEILUS AND RICHARDSONIUS

Morphological Approach

Two approaches were used to determine if swamping is occurring between Mylocheilus and Richardsonius. The first, and more classical approach, attempts to measure the presence of genes of one species in populations of the other by morphological character comparisons. Significant differences in morphology between populations of each species within and outside the hybrid zone are taken as evidence for swamping. In Stave Lake obvious hybrids are excluded from the comparisons because they can not be considered as permanent members of either species.

A search was made for morphological characters which are widely divergent in the two species. Such characters are useful in determining if gene flow is occurring between Mylocheilus and Richardsonius. The characters used in this study are:

- 1) anal fin rays. Throughout its range, Mylocheilus typically possess 8 anal fin rays. Richardsonius' anal fin ray number is quite variable throughout its range and different populations possess different mean counts. According to Carl, Clemens, and Lindsey (1959) most British Columbia populations possess a mean number of 15 anal fin rays. In the hybrid zone, individuals which resemble typical Richardsonius possess 14-22 anal fin rays. The mode is either 16 or 17. Anal fin rays are not used to detect swamping, however, but as a tool to separate obvious hybrid individuals from members of the two parental species.

- 2) Lateral Line Scales. Mylocheilus possess 68 to 79 rows of lateral line scales while Richardsonius possess considerably fewer, from 54 to 67 rows (Carl, Clemens, and Lindsey, 1959).
- 3) Ratio of Predorsal to Prepelvic Length. The position of the dorsal fin relative to the pelvic fin is quite distinct in the two species. In Richardsonius, the dorsal fin is inserted considerably posterior to the insertion of the pelvic fins while in Mylocheilus it is inserted sometimes in line with, but more often slightly ahead of, the pelvic fin insertion. This relationship is quantified as the ratio of the predorsal to the prepelvic length.

All counts and measurements with the exception of the prepelvic length are in accordance with the procedures suggested by Hubbs and Lagler (1958). The prepelvic length is the straightline distance from the tip of the snout to the insertion of the outermost pelvic fin ray.

The distributions of lateral line scales and the predorsal/prepelvic length ratios are compared for populations of each species within and outside the hybrid zone. Samples of Mylocheilus outside the hybrid zone were collected from Vancouver Island and the Sechart Peninsula, while Richardsonius were sampled from Alouette Lake. In the comparisons, obvious hybrids are detected by their anal fin ray number and excluded.

Morphological-Biochemical Approach

The second approach, a combined morphological and biochemical one, attempts to measure the frequency of various hybrid types. If swamping

is occurring, backcross hybrids should be more abundant than F_1 hybrids, since many generations of hybrid backcrosses should exist in the population.

The inheritance of anal fin rays ascertained under laboratory conditions allows a reasonable separation of hybrids into two groups: (1) F_1 hybrids and 1st generation hybrid backcrosses and (2) 2nd and later generation hybrid backcrosses and the parental species. The former group, F_1 hybrids and 1st generation hybrid backcrosses, is separated into its component parts by two biochemical characters, the inheritances of which are determined by experimental crosses. The 2nd and later generation hybrid backcrosses of the latter group are separated from individuals of the parental species by these biochemical characters, also. The biochemical characters used, were chosen with care to include only proteins which (1) show genetic variability between--but not within--the two species, (2) possess codominance between alleles and (3) are not sex-linked. These requirements insure that any individual possessing alleles of both species for the biochemical characters in question, is detected.

Search for Biochemical Characters

Populations of Mylocheilus and Richardsonius from outside the hybrid zone were examined to detect biochemical characters whose genetic control is such that the loci in the two species possess homozygous, but different alleles. Enzymes, examined and discarded because they either show no allelic differences or share alleles between the two species are esterases, pseudocholinesterases, lactic dehydrogenase, succinic dehydrogenase, isocitric dehydrogenase, and glutamic dehydrogenase. Two proteins, a general muscle protein, and an enzyme, malic dehydrogenase show consistent differences between, but no variability within, the two species. These characters are used to separate F_1 hybrids and 1st generation hybrid backcrosses.

Electrophoretic Analysis

Samples of muscle are homogenized approximately 15 seconds in low ionic strength (0.055M) phosphate buffer, pH 7.45, until the homogenates possess a creamy texture. The ratio of muscle to phosphate buffer is 1:2. The homogenate is then centrifuged for 15 minutes at 37,000 x g and the supernatant collected. Starch gel electrophoresis of the supernatant is conducted at 180 volts for 2.25 hours using a micro method previously published (Tsuyuki et al., 1966). The general protein zones are stained with amido black 10B for three minutes. Malic dehydrogenase is stained for two hours in a solution containing (1) 23.3 ml of 0.1 M Tris buffer, pH 8.5, (2) 1.5 ml of 2 M malic acid, (3) 0.6 ml of 30 mg/ml nicotinamide adenine dinucleotide (NAD), (4) 0.12 ml of 5 mg/ml phenazine methosulfate,

and (5) 1.0 ml of 10 mg/ml nitro blue tetrazolium (Colowick and Kaplan, 1963).

Rearing of Experimental Animals

Artificial crosses of parental Mylocheilus, Richardsonius, reciprocal F_1 hybrids, and reciprocal hybrid backcrosses to both species were made from parents taken from Devils Creek in 1966, 1967, and 1968. The eggs were kept in a Heath incubator supplied with Devils Creek water. The water temperature in the incubator was the ambient temperature of the creek. Upon hatching, the fry were fed an identical diet regimen of Artemia nauplii, live plankton from Stave Lake, and frozen adult brine shrimp until they reached a sufficient size to permit electrophoretic analysis (35 mm). Prior to analysis, all progeny and the wild fish used as parents in the 1967 and 1968 crosses were frozen. Unfortunately, the carcasses of the parents used in the 1966 crosses were preserved in formalin which denatured their proteins.

Mylocheilus and Richardsonius Outside the Hybrid Zone

Muscle Proteins

At the concentration of the muscle protein extracts tested, several characteristic major and minor zones are present. The muscle proteins to be discussed are those major zones arbitrarily designated "1", "2", and "3" (Fig. 4). All individual Mylocheilus from populations outside the hybrid zone (Ruby, Hatzic, Alouette, and Babine Lakes and Stave River) display zone 3 whereas Richardsonius from outside the hybrid zone (Alouette, Shea, and Babine Lakes) possess exclusively zone 1, a zone of lesser anodal mobility (Table I, Fig. 4). No individuals outside the hybrid zone possess both zones 1 and 3, or 2.

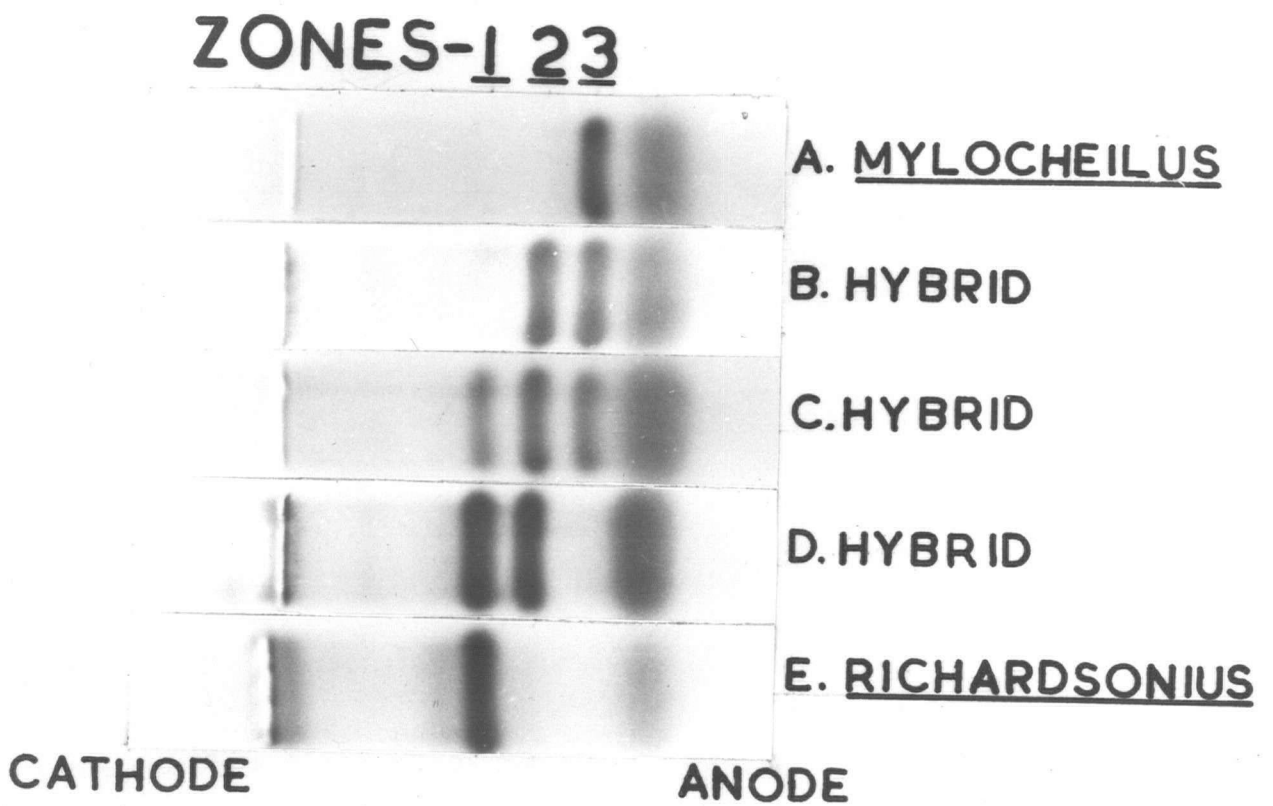


Figure 4. Starch gel electrophoresis of muscle proteins from Mylocheilus caurinum, Richardsonius balteatus, and their hybrids. Samples are from Stave Lake, British Columbia.

TABLE I. Muscle protein and malic dehydrogenase patterns of Mylocheilus and Richardsonius outside the hybrid zone (number of individuals).
 "M" = Mylocheilus; "R" = Richardsonius.

Area	Species	Muscle Proteins					Malic Dehydrogenase				
		Zone 1	Zones 1, 2	Zones 1, 2, 3	Zones 2, 3	Zone 3	Zone 3(5)	Zones 1, 2, 3	Zones 12345	Zones 3, 4, 5	Zones 1, 3, (5)
Ruby Lake, B.C.	M					35	35				
Stave River, B.C.	M					31	31				
Hatzic Lake, B.C.	M					25	25				
Alouette Lake, B.C.	M					27	38				
Babine Lake, B.C.	M					10					
Shea Lake, B.C.	R	25									25
Alouette Lake, B.C.	R	36									53
Babine Lake, B.C.	R	13									

Malic Dehydrogenase

Populations of Richardsonius exhibit a malic dehydrogenase pattern with three isozymes, designated zone "1", "3", and "5" (Fig. 5B). No variability is seen in the two populations examined: Shea and Alouette Lakes (Table I). Most Mylocheilus possess a single MDH isozyme with a mobility very similar to that of the "3" isozyme in Richardsonius. It is also designated as zone "3" (Fig. 5A,F). In some instances, however, the zone 3 isozyme in Mylocheilus continues weakly for some distance anodally and often terminates in what appears to be a second zone corresponding to that of zone 5 in Richardsonius (Fig. 5G). This zone is never present in Mylocheilus unless a considerable "slur" is also present, suggesting that it may be an artefact of the technique. It is not, therefore, accorded much weight in the subsequent analysis of hybrid individuals.

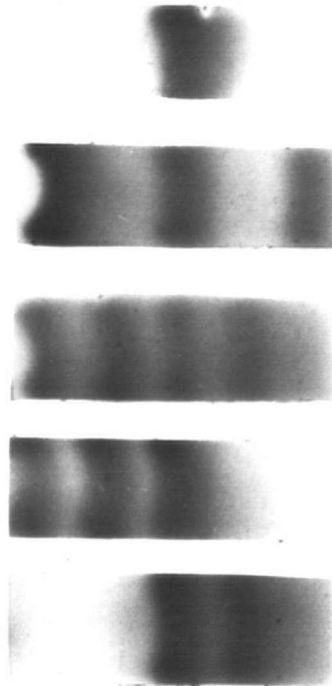
Inheritance of Biochemical Characters

Muscle Proteins

Crosses of Mylocheilus x Mylocheilus and Richardsonius x Richardsonius result in progeny with protein patterns characteristic of the respective parents (Table II). The F_1 progeny resulting from reciprocal crosses of Mylocheilus and Richardsonius produce a single electrophoretic pattern displaying zones 1 and 3 in addition to a unique intermediate zone, designated "2" (Fig. 4C). Reciprocal backcrosses of presumed F_1 hybrids to Mylocheilus (BCM)¹ produce offspring with three types of protein

1) For simplicity, some abbreviations are used in the text. They are: "M" = Mylocheilus, "R" = Richardsonius, " F_1 " = individual resulting from Mylocheilus x Richardsonius cross unless stated otherwise, "BCM" = F_1 hybrid backcross to Mylocheilus, "BCR" = F_1 hybrid backcross to Richardsonius.

ZONES 1 2 3 4 (5)



A. MYLOCHEILUS

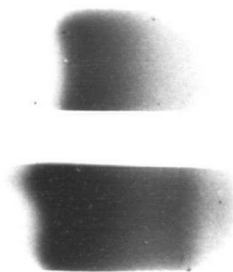
B. RICHARDSONIUS

C. HYBRID

D. HYBRID

E. HYBRID

ZONES 3 (5)



F. MYLOCHEILUS

G. MYLOCHEILUS

Figure 5. Starch-gel electrophoresis of malic dehydrogenase from Mylocheilus caurinum, Richardsonius balteatus, and their hybrids.

TABLE II. Muscle protein patterns of parents and progeny of crosses made in 1966 and 1967.

PROTEIN PATTERNS																							
Parents								Progeny (No. of individuals)															
Type of Cross Female Male		Zone Numbers						Zone Numbers															
		Female 1 2 3			Male 1 2 3			1	2	3	1	2	3	1	2	3	1	2	3				
								+			+			+	+	+	+			+	+		
<u>Mylocheilus</u>	<u>Mylocheilus</u>				+				+	40													
<u>Mylocheilus</u>	"Hybrid"				+	+	+	+	4	5			3										
"Hybrid"	<u>Mylocheilus</u>	+	+	+				+	6	22			14										
<u>Mylocheilus</u>	<u>Richardsonius</u>				+	+			40														
<u>Richardsonius</u>	<u>Mylocheilus</u>	+							+	40													
<u>Richardsonius</u>	"Hybrid"				*				*	4									5				
<u>Richardsonius</u>	"Hybrid"	+				+	+	+	1									1					
"Hybrid"	<u>Richardsonius</u>				*				*	7									9			5	
<u>Richardsonius</u>	<u>Richardsonius</u>	+				+												33					

* parental patterns from 1966 crosses not available.

patterns: a) the parental Mylocheilus pattern, zone 3, b) the pattern possessing zones 1, 2, 3, and c) a new pattern possessing only zones 2 and 3 and corresponding to that of Figure 4B. Reciprocal backcrosses of presumed F_1 hybrids to Richardsonius (BCR) also produce progeny with three types of protein patterns: a) the parental Richardsonius pattern, zone 1, b) the pattern possessing zones 1, 2, and 3 and c) a new pattern possessing only zones 1 and 2 and corresponding to that of Fig. 4D.

A genetic model explaining the inheritance of these proteins is depicted in Figure 6. The model proposes that zones 1 and 3 are each controlled by two loci, "A" and "B", each of which exists in two allelic forms. Mylocheilus is depicted as being homozygous for the genotype, $\frac{A}{A} \frac{B}{B}$, and Richardsonius homozygous for $\frac{A'}{A'} \frac{B'}{B'}$. While the model depicts loci A and B as being on separate chromosomes, they may, in fact, be located on the same chromosome but at least 50 map units apart.

Assumptions which are implicit in the model are:

- 1) Proteins detected as zones 1 and 3 are controlled by genes present in both parental species.
- 2) The products of the loci complex to form a dimer which is unique to each parental species.
- 3) Protein zone 2 represents a hybrid dimer composed of a polypeptide contributed from each of the parental species.
- 4) The polypeptides of protein zone 1 are electrophoretically indistinguishable as are those of protein zone 3.

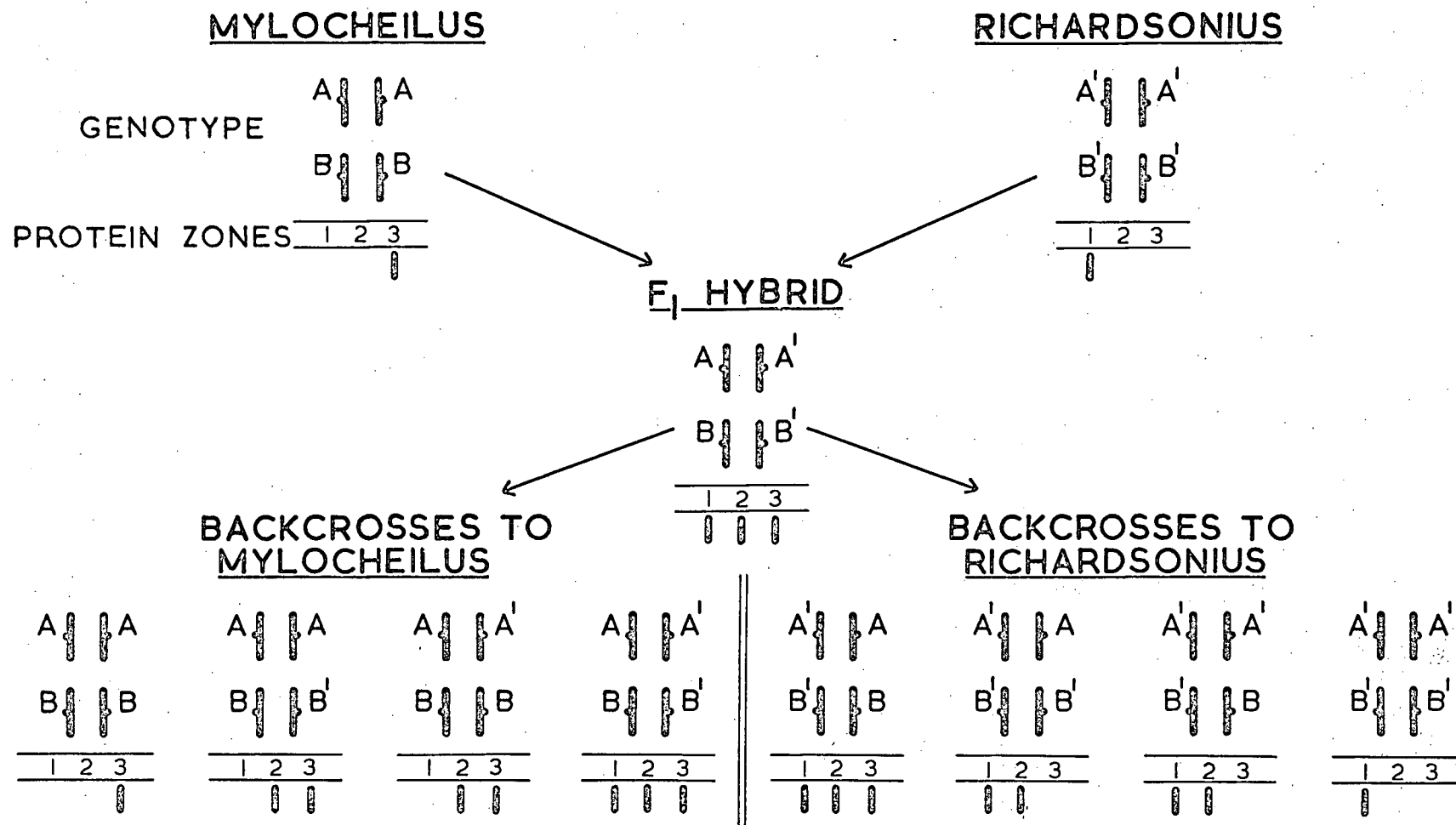


Figure 6. A genetic model for the inheritance of muscle proteins, zones 1 and 3, in Richardsonius and Mylocheilus, respectively.

The true breeding quality of artificial parental crosses is evidence for homozygosity of alleles A and B in Mylocheilus and A' and B' in Richardsonius. Further evidence is the lack of variation in these protein zones in either species outside the hybrid zone. The distribution of protein patterns in F₁ hybrid backcrosses to both species supports the idea of dual gene control. For example, in F₁ hybrid backcrosses to Mylocheilus a new type of pattern is found in addition to the expected parental (zone 3) and F₁ (zones 1, 2, 3) patterns. This pattern contains zones 2 and 3 but not zone 1 (Fig. 4B). If zone 2 is composed of subunits of zones 1 and 3, its presence indicates that only half of the components required to produce zone 1 are present. The half of the components of zone 1 which is present in this phenotype combines with some of the components of zone 3 to produce zone 2. Thus, only half of the genetic complement of Richardsonius is present in these phenotypes; the other half has segregated to another gamete. In presumed F₁ hybrid backcrosses to Richardsonius a similar situation occurs in which the new pattern possesses only zones 1 and 2 (Fig. 4D), indicating that only half of the components needed to produce the Mylocheilus zone 3 is present. In analogous fashion to the two-zoned BCM progeny, the components of zone 3 which are present combine with some of those of zone 1 to form zone 2.

If the model is correct, the F₁ hybrid backcross progeny to both species should possess one-zoned, two-zoned, and three-zoned patterns in a ratio of 1:2:1, respectively. This hypothesis is not rejected for either BCM progeny ($X^2 = 1.81$) or BCR progeny ($X^2 = 2.75 < X^2_{\alpha} = .05, df = 2 = 5.99$).

Although the sexes of artificially produced F_1 hybrids were not observed, there is no evidence that the protein zones are controlled by sex-linked genes, for reciprocal F_1 crosses produced only one protein pattern, namely, zones 1, 2, and 3. A discrepancy in the sex ratios of these crosses would not likely produce the above result since the sex ratio of natural hybrids do not differ significantly from 1:1 (See page 71).

The study of progeny produced under experimental conditions clarifies the type(s) of individual (hybrid, parental, etc.) associated with different muscle protein patterns. For example, individuals which possess only zone 1 may either be pure Richardsonius or hybrid backcrosses to Richardsonius while, similarly, those which possess zone 3 may either be pure Mylocheilus or hybrid backcrosses to Mylocheilus. The three-zoned pattern (Fig. 4, C) is possessed by the F_1 hybrid backcrosses to either species or the F_1 hybrid. The two types of two-zoned progeny, zones 1, 2 and zones 2, 3, are possessed only by hybrid backcrosses to Richardsonius and Mylocheilus, respectively.

Any of the above muscle protein patterns could be possessed by F_2 hybrids according to the genetic model presented above. However, it is assumed that they do not exist naturally as experimentally produced ones are extremely inviable whereas parental species' progeny and F_1 hybrids were raised, usually without difficulty.

Malic Dehydrogenase (MDH)

Parental crosses of Mylocheilus x Mylocheilus and Richardsonius x Richardsonius yield progeny which possess malic dehydrogenase patterns characteristic of the respective parents (Table III, Fig. 5). Progeny of reciprocal F_1 crosses possess a single pattern with zones 1, 2, 3, 4, (5). Four MDH patterns are observed when progeny of reciprocal presumed F_1 hybrid backcrosses to Mylocheilus are examined. They are: a) zone 3, the parental Mylocheilus pattern, b) zones 1, 2, 3, 4, (5), the F_1 hybrid pattern, c) zones 1, 2, 3 and d) zones 3, 4(5) (Fig. 5). Unfortunately, only two progeny from hybrid backcrosses to Richardsonius were reared in 1967. One possessed the typical Richardsonius pattern, zones 1, 3, (5) and the other, the F_1 hybrid pattern, zones 1, 2, 3, 4, (5). Because of the uncertainty regarding the existence of MDH zone 5 in Mylocheilus, the lack of hybrid backcrosses to Richardsonius, and the uncertain nature of MDH quaternary structure in other organisms, a detailed genetic analysis of this enzyme is not attempted. However, several aspects of the inheritance are apparent. First, codominance exists for the alleles controlling MDH since F_1 hybrids possess the zones of both Mylocheilus and Richardsonius. Additionally, two new zones, zones 2 and 4 are found in the F_1 hybrids, but neither is present in the parental species. Presumably, these new zones are hybrid MDH isozymes formed by the union of subunits from normally occurring isozymes in the two species. Second, there must be at least two loci, or groups of loci, controlling the synthesis of MDH in these species since

TABLE III. Malic dehydrogenase patterns of parents and progeny of crosses made in 1967.

Type of Cross Female Male		Malic Dehydrogenase Patterns							
		<u>Parents</u>				<u>Progeny</u>			
		Zone Numbers				Zone Numbers			
		Female		Male		3 (5)	1 2 3	3 4 (5)	1 2 3 4 (5)
		1 2 3 4 (5)		1 2 3 4 (5)					1 3 (5)
<u>Mylocheilus</u>	<u>Mylocheilus</u>	+		+		40			
<u>Mylocheilus</u>	Hybrid**	+		+	+	12	11	14	16
Hybrid**	<u>Mylocheilus</u>	+	+	+	+	7	7	11	17
<u>Mylocheilus</u>	<u>Richardsonius</u>	+		+	+				35
<u>Richardsonius</u>	<u>Mylocheilus</u>	+	+		+				40
<u>Richardsonius</u>	Hybrid**	+	+	+	+				1
<u>Richardsonius</u>	<u>Richardsonius</u>	+	+	+	+				33

** presumed F₁ hybrid

four phenotypes are observed in the presumed F_1 hybrid backcrosses to Mylocheilus. If a single locus is involved, only the parental Mylocheilus and the F_1 hybrid MDH patterns should be produced. Non-parental types of MDH in these backcrosses compose 44% of all progeny. The hypothesis that the ratio of parental to non-parental types in F_1 hybrid backcrosses to Mylocheilus is not significantly different from 1:1 is not rejected ($X^2 = 1.25 < X^2_{\alpha = .05, df=1} = 3.84$). If in fact, the hypothesis is true, the two loci or groups of loci are assorting completely independently.

As with the explanation of muscle protein inheritance, the sex was not determined for the F_1 hybrid progeny. However, sex-linked inheritance is not indicated since reciprocal F_1 hybrids both produce one and the same pattern.

The study of progeny produced under experimental condition clarifies the type(s) of individuals associated with the various MDH patterns. Those which possess the pattern, zones 3 (5) can be pure Mylocheilus or hybrid backcrosses to Mylocheilus while those which possess the zones 1, 3, (5) pattern can either be pure Richardsonius or hybrid backcrosses to Richardsonius. The zones 1, 2, 3, 4, (5) MDH pattern is possessed by reciprocal F_1 hybrids and presumed F_1 hybrid backcrosses to either species. The MDH patterns, zones 1, 2, 3 and zones 3, 4, (5) are unique to hybrid backcrosses to Mylocheilus. However, it is possible that they could be possessed by hybrid backcrosses to Richardsonius; it is not elucidated because only 2 individual hybrid backcrosses to Richardsonius were produced experimentally in 1967.

Linkage

If two biochemical characters are to be twice as useful as a single one in detecting hybrids they must be independent of one another; they must not be tightly linked. A chi-square test of independence of the two proteins was performed and the hypothesis that they are independent is rejected ($X^2 = 22.59 > X^2_{\alpha} = 0.05, df = 6 = 12.59$). An inspection of the data (Table IV) reveals that MDH pattern, zones 1, 2, 3, 4, (5), occurs much too frequently with muscle protein pattern, zones 2, 3, than would be expected if the two proteins are not linked. This cell(#6) contributes 44% of the significant chi-square value. Although it appears that the two proteins are partially linked, their combined value in detecting hybrids is not drastically reduced when compared to two completely independent or unlinked characters. For example, the probability of recognizing presumed F_1 hybrid backcrosses to Mylocheilus from those crosses shown in Table IV, assuming independence, is $p(\text{all cells}) - p(\text{cell 1} + \text{cell 10}) = 0.875$. Individuals of cells 1 and 10 are classified as pure Mylocheilus and F_1 hybrids, respectively. The proportion of presumed F_1 hybrid backcrosses to Mylocheilus actually observed is 0.852, a decrease of only 0.023 from the theoretical value.

TABLE IV. Correlation of malic dehydrogenase and muscle protein patterns of hybrid backcrosses to Mylocheilus. Numbers in parentheses are expected proportions for each cell assuming independence of inheritance of the two proteins. Encircled numbers are particular cells referred to in the text.

		Malic Dehydrogenase			
Muscle Proteins	Zones	3 (5)	1 2 3 4 (5)	3 4 (5)	1 2 3
	3	1 ^① (1/16)	3 ^② (1/16)	3 ^③ (1/16)	3 ^④ (1/16)
	2 3	2 ^⑤ (1/8)	15 ^⑥ (1/8)	5 ^⑦ (1/8)	5 ^⑧ (1/8)
	1 2 3	4 ^⑨ (1/16)	7 ^⑩ (1/16)	5 ^⑪ (1/16)	1 ^⑫ (1/16)

Inheritance of Anal Fin Rays; Separation of 2nd and Later
Generation Hybrid Backcrosses

The separation of 2nd and later generation hybrid backcrosses from 1st generation hybrid backcrosses and F_1 , F_2 , ... F_n hybrids permits an estimate of their abundance and, indirectly, an estimate of the selective advantage or disadvantage of various hybrid generations. In this study, the difficulty of separating the two groups is simplified by assuming that F_2 ... F_n hybrids do not exist in nature. Evidence supporting this assumption is the fact that of 632 eggs used in $F_1 \times F_1$ crosses in 1966, only 4 individuals were reared to the fingerling stage (≈ 50 mm). In essence, the task is reduced to the separation of 1st generation hybrid backcrosses and F_1 hybrids from all subsequent hybrid backcross generations.

The inheritance of a third character, anal fin rays, was studied in an attempt to effect a separation of these two groups. Anal fin rays were chosen as the character because 1) they are widely divergent in the two species; usually 8 in number in Mylocheilus, 14-22 in Richardsonius and 2) they display a polygenetic inheritance with no apparent dominance (Fig. 7).

Experimentally produced F_1 hybrids possess from 9 to 12 anal fin rays with approximately 93% possessing either 10 or 11 (Fig. 7). Very few F_1 hybrids possess 9(4.2%) or 12(2.1%) anal fin rays.

Backcrosses of presumed F_1 hybrids with 10, 11, and 12 anal fin rays, to Mylocheilus, mainly with 8, yield progeny with 7 to 11 anal fin rays, but with a large mode at 9(65.9%). Smaller peaks are at 8(15.2%) and at 10(17.1%). A cross of a hybrid male with 9 anal fin rays (determined to be at least a 1st generation hybrid backcross to Mylocheilus on the basis of its proteins) and a female Mylocheilus (8 rays) produced progeny with mainly

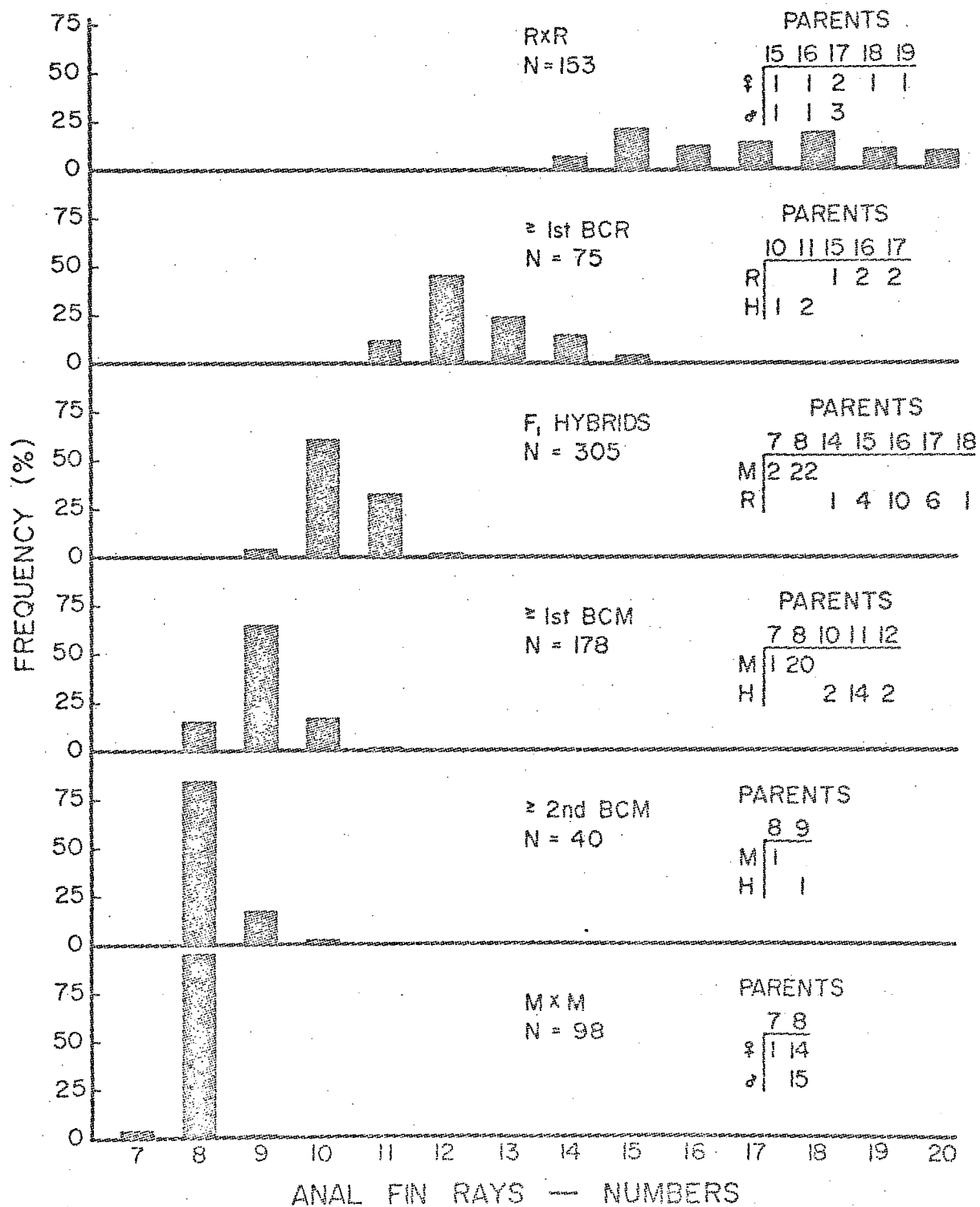


Figure 7. Anal fin ray distribution of 1966 and 1967 experimental crosses combined. Parents of crosses included on right side of graph.

8 anal fin rays (80%). Of the remaining progeny 17.5% possessed 9, and 2.5% possessed 10 anal fin rays. These progeny were at least 2nd generation hybrid backcrosses to Mylocheilus.

Presumed F_1 hybrid backcrosses to the other species, Richardsonius, yield progeny with anal fin rays ranging from 11 to 15. However, those individuals with 12 to 14 anal rays compose 84% of the BCR progeny. Unfortunately, no second generation BCRs were produced experimentally.

On the basis of anal fin ray inheritance, 8 anal fin rays were chosen as the dividing line; those with 8 or fewer are considered to be either pure Mylocheilus or at least 2nd generation hybrid backcrosses to Mylocheilus, while those with 9 to 11 anal rays are considered to be either 1st generation BCMs or F_1 hybrids. However, if the distributions for various crosses shown in Figure 7 are representative of those in nature, approximately 16% of the 1st generation BCMs would be classified in the wrong category; they would be classed as at least 2nd generation BCMs, therefore, producing an overestimate of the latter. On the other hand, approximately 20% of the 2nd generation BCMs would be classed as part of the population containing 1st generation BCMs and F_1 hybrids. Such sources of error cannot be eliminated from the method.

For hybrid backcrosses to Richardsonius, 15 anal fin rays were chosen as the dividing line; those with 15 or greater are classed as at least 2nd generation BCRs or pure Richardsonius, while those with 11 to 14 anal rays are classed as 1st generation BCRs or F_1 hybrids. Approximately 4% of the first generation BCRs would be misclassified as 2nd generation or later BCRs under this scheme. Unfortunately, 2nd generation BCRs

were not produced and consequently no estimate could be made of the individuals which would be misclassified as 1st generation BCRs.

Linkage of Biochemical and Morphological Characters

An important consideration in estimating the frequency of various hybrid backcross types is the non-linkage of either biochemical character with anal fin rays. Otherwise, an erroneous estimate may be obtained. For example, if those hybrid backcrosses to Mylocheilus with 8 anal fin rays always possess the parental Mylocheilus muscle protein and malic dehydrogenase patterns rather than hybrid patterns, most 2nd and later generation BCMs may not be detected, even when present. Consequently, possible linkage relationships between anal fin ray number and muscle protein or MDH patterns are investigated. Hybrid backcrosses to Mylocheilus were segregated into groups of 7+8 and 9 to 12 anal fin rays. The proportion of various patterns of muscle protein and MDH are then compared for the two groupings. The data, presented in Tables V and VI, show that for both muscle protein and MDH patterns, individuals with 7+8 anal fin rays do not differ significantly from those with 9 to 12 rays ($\chi^2_{\text{muscle proteins}} = 1.05 < \chi^2_{\alpha = .05, df = 2} = 5.99$; $\chi^2_{\text{MDH}} = 3.39 < \chi^2_{\alpha = .05, df = 3} = 7.81$). Linkage of the two biochemical characters with anal fin rays is, therefore, not found.

Unfortunately, insufficient numbers of progeny were reared and analysed to permit such a determination of linkage relationships between biochemical and morphological characters for hybrid backcrosses to

TABLE V. The proportion of various muscle protein patterns of presumed F_1 hybrid backcrosses to Mylocheilus in relation to anal fin ray number. Numbers in parantheses are expected values assuming homogeneity of the two distributions.

Muscle Protein Zones	Anal Fin Rays	
	7+8	9 to 12
1 2 3	4(3.53)	13(13.47)
2 3	4(5.40)	22(20.60)
3	3(2.08)	7(7.92)

TABLE VI. The proportion of various malic dehydrogenase patterns of presumed F_1 hybrid backcrosses to Mylocheilus in relation to anal fin ray number. Numbers in parentheses are expected values assuming homogeneity of the two distributions.

MDH Zones	Anal Fin Rays	
	7+8	9 to 12
1 2 3 4 (5)	3(5.19)	22(19.81)
1 2 3	2(1.66)	6(6.34)
3 4 (5)	3(2.70)	10(10.30)
3 (5)	3(1.45)	4(5.55)

Richardsonius. As a result, the assumption has to be made that the same linkage relationships hold for Richardsonius as they do for Mylocheilus.

Separation of F₁ Hybrids and 1st Generations Hybrid Backcrosses

Based on the inheritance of anal fin rays discussed in a prior section, an attempt is made to separate 2nd and later generation hybrid backcrosses from 1st generation hybrid backcrosses and F₁ hybrids. In this section, separation of the F₁ hybrids from the 1st generation hybrid backcrosses is based on the knowledge of the inheritance of muscle protein and MDH patterns.

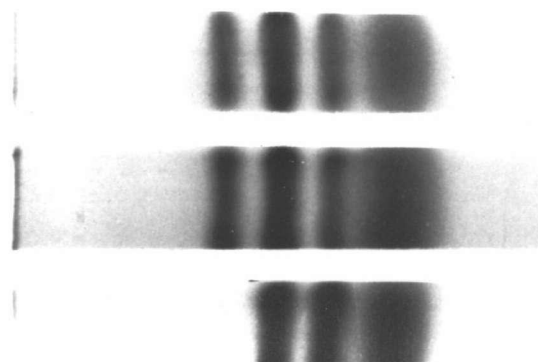
In certain cases, hybrid backcrosses can be identified from the analysis of a single protein because of the uniqueness of certain patterns (Fig. 8C). However, detection of many hybrid backcrosses results from an examination of the two proteins simultaneously (Fig. 8). For example, fish "B" of Figure 8 is classified a hybrid backcross to Mylocheilus because it possesses the F₁ hybrid muscle protein pattern but the Mylocheilus MDH pattern. The muscle protein pattern indicates its hybrid origin; the Mylocheilus MDH pattern indicates the unlikelihood of it being a F₁ hybrid since all F₁ hybrids possessed the zones 1, 2, 3, 4, (5) MDH pattern. Assuming F₂ hybrids do not exist in nature, this individual must be a BCM.

The classification of hybrid types based on the various combinations of muscle protein and MDH patterns is presented in Table VII. Individual

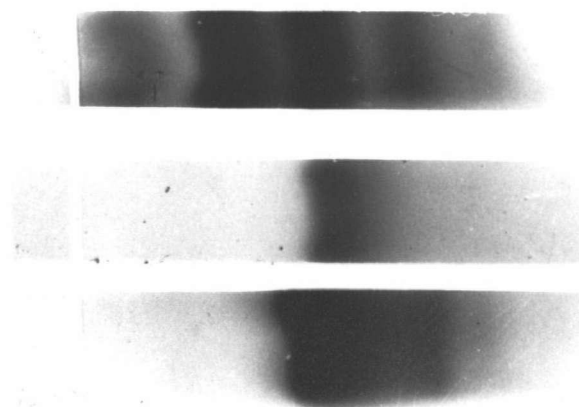
MUSCLE PROTEINS

MALIC DEHYDROGENASE

ZONES 1 2 3



ZONES 1 2 3 4 (5)



A.

B. ³⁷

C.

Figure 8. Electropherograms of muscle protein and malic dehydrogenase patterns from three natural hybrids from Stave Lake(1967 samples).

TABLE VII. Classification of hybrid types based on the combinations of muscle protein and malic dehydrogenase patterns. "F₁ hybrid" classification may include some hybrid backcrosses to either species because of assortment for F₁ hybrid patterns of both proteins simultaneously.

Hybrid Classification	Muscle Protein Zones		Malic Dehydrogenase Zones
"F ₁ Hybrids"	1, 2, 3	and	1, 2, 3, 4, (5)
Hybrid backcrosses to <u>Mylocheilus</u> (BCMs)	2, 3 3 3 3 1, 2, 3	and and and and and	any pattern 1, 2, 3, 4, (5) 1, 2, 3 3, 4, (5) 3
Hybrid backcrosses to <u>Richardsonius</u>	1, 2 1 1, 2, 3	and and and	any pattern 1, 2, 3, 4, (5) 1, 3, (5)

F₁ hybrids can not be identified with certainty because hybrid backcrosses to either species can assort for the F₁ hybrid muscle protein and MDH pattern simultaneously. In a like manner, hybrid backcrosses to either species can assort for the respective parental proteins simultaneously. Seven individuals with 9 anal fin rays possess parental Mylocheilus muscle protein and MDH patterns. Some of these individuals could be pure Mylocheilus, as individuals with 9 anal fin rays are occasionally found, even in allopatric populations. So, the number of hybrid backcrosses might be slightly underestimated.

Results

Predorsal/Prepelvic Length Ratios (D/P) and Lateral Line Scales of *Mylocheilus* and *Richardsonius* from Stave Lake and from Areas Outside the Hybrid Zone

Richardsonius from Alouette Lake, the major drainage system west of Stave Lake, were compared with those from Stave Lake, the hybrid zone. In both characters examined, lateral line scales and the D/P ratio, no significant differences are observed in the means of the two groups (Figs. 9 and 10, Table VIII). Similarly, Mylocheilus from Vancouver Island and the Seechelt Peninsula show no significant differences from those of the hybrid zone in the means of these characters (Figs. 9 and 10, Table VIII).

If the loci controlling the number of lateral line scales and the D/P ratio can be considered representative of the two genomes, swamping between Mylocheilus and Richardsonius does not appear in progress.

Composition of the Natural Hybrid Population in Stave Lake

On the basis of anal fin rays and two biochemical characters, an estimate was made of the various hybrids present in Stave Lake. Hybrids, in general, composed 6.1% of the combined Mylocheilus-Richardsonius gene pool in 1967. Of these hybrids, presumed F_1 s comprise 76.3%; presumed 1st generation hybrid backcrosses to Mylocheilus, 15.4%; and presumed 2nd or later generation hybrid backcrosses to Mylocheilus, 6.6% (Tables IX and X).

Presumed 1st and 2nd or later generation hybrid backcrosses to Richardsonius compose 1.7% and 0.0% of all hybrids, respectively.

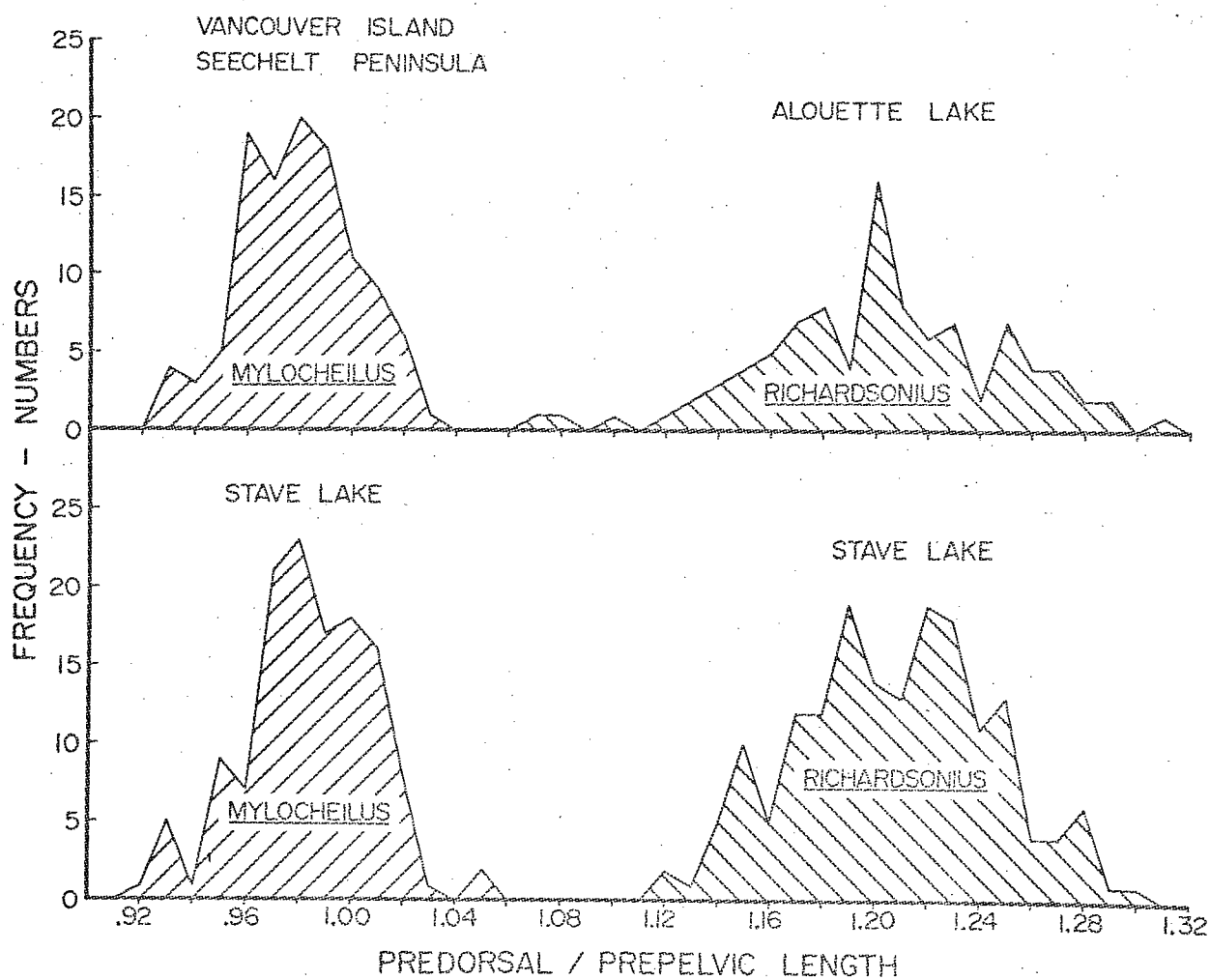


Figure 9. The ratio of predorsal to prepelvic length of Mylocheilus and Richardsonius within and outside the hybrid zone.

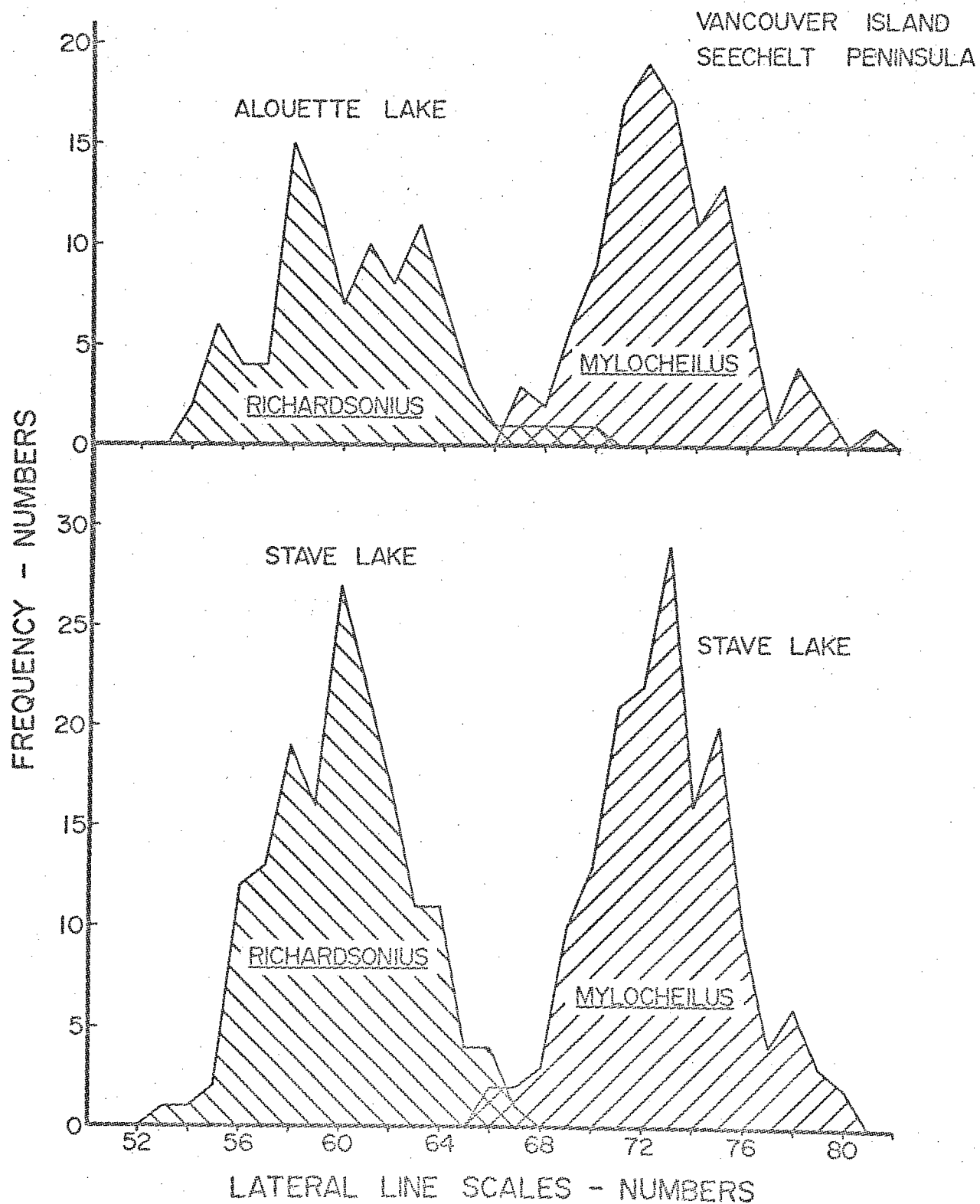


Figure 10. Lateral line scale distribution of *Mylocheilus* and *Richardsonius* within and outside the hybrid zone.

TABLE VIII. Comparison of morphological characters of Mylocheilus and Richardsonius from within and outside the hybrid zone.

Species	Area	Lateral Line Scales			D/P Ratios		
		\bar{X}	s^2	t	\bar{X}	s^2	t
<u>Mylocheilus</u>	Vancouver Island Sechelt Peninsula	72.7	7.1		0.980	0.0005	
<u>Mylocheilus</u>	Stave Lake	72.9	7.6	{ 0.43	0.984	0.0006	{ 1.55
<u>Richardsonius</u>	Alouette Lake	60.3	11.2	{ 0.51	1.203	0.0021	{ 0.98
<u>Richardsonius</u>	Stave Lake	60.1	7.5		1.208	0.0001	

** indicates significant "t" values, $\alpha = .05$.

TABLE IX. Muscle protein and malic dehydrogenase patterns of those individuals of the Mylocheilus-Richardsonius gene pools with ≤ 8 or ≥ 15 anal fin rays. Samples are from the hybrid zone and may include pure individuals of either species or 2nd and later generation hybrid backcrosses.

Zones =	Muscle Proteins					Malic Dehydrogenase				
	3	2, 3	1, 2, 3	1, 2	1	3, (5)	3, 4, (5)	1, 2, 3	1, 2, 3, 4, (5)	1, 3, (5)
<u>Mylocheilus</u>	249	-	-	-	-	248	1	-	-	-
<u>Richardsonius</u>	-	-	-	-	178	-	-	-	-	178

TABLE X. Composition of the Mylocheilus-Richardsonius gene complex of Stave Lake in 1967. Separation of hybrid types is based on the intertance of anal fin rays, a general muscle protein, and malic dehydrogenase. As shown in Table IX, 250 fish with ≤ 8 and 178 fish with ≥ 15 anal rays were examined to estimate the numbers of second or later generation hybrid backcrosses to Mylocheilus and Richardsonius respectively.

Type of Individual	Estimated Numbers	Percent of total	Percent of all hybrids
"Pure" <u>Mylocheilus</u>	1536.3	80.5	-
"Pure" <u>Richardsonius</u>	255.0	13.4	-
Presumed F ₁ Hybrids	89.0	4.7	76.3
Presumed 1st generation hybrid backcrosses to <u>Mylocheilus</u>	18.0	0.9	15.4
Presumed 2nd or later generation hybrid back- crosses to <u>Mylocheilus</u>	7.7	0.4	6.6
Presumed 1st generation hybrid backcrosses to <u>Richardsonius</u>	2.0	0.1	1.7
Presumed 2nd or later generation hybrid back- crosses to <u>Richardsonius</u>	0.0	0.0	0.0

These data indicate that F_1 hybrids are quite abundant in Stave Lake, while hybrid backcross individuals decrease in frequency beyond the first backcross generation. This suggests that they are at a selective disadvantage. Consequently, swamping is prevented between the two species; the two gene pools maintain their genetic integrity.

The hybrid population in 1966 represents 5.5% of the two gene pools combined. However, this estimate excludes any hybrids with 8 or less or 15 or greater anal fin rays because an electrophoretic analysis was not performed on these fish in 1966. If the above mentioned hybrids are subtracted from the 1967 hybrid population estimate, the two years data are comparable; the 1967 hybrid population is then estimated at 5.7%, an estimate very close to that obtained in 1966.

Isolating Mechanisms

In previous sections of the thesis it was determined that Mylocheilus and Richardsonius are reproductively isolated, even in the presence of extensive hybridization. If reproductive isolation truly exists, isolating mechanisms must be operative. This section of the thesis attempts to describe what those mechanisms are.

Materials and Methods

Observations

Spawning populations of Mylocheilus, Richardsonius, and their hybrids were observed in Devils Creek during the springs and summers of 1966 and 1967. In 1966, observations were made throughout the creek, while in 1967, they were concentrated in the lower section. This section was divided into 20 meter lengths when the lake level was very low (317 feet above sea level). The 20 meter sections begin at the lower log jam pool and terminate at the point where Devils Creek enters Stave Lake (Fig. 11). The lower section was 325 meters long at the beginning of observations on May 1, 1967. However, as the lake level rises the creek is inundated and decreases in length. An infra-red viewer was used with limited success for nocturnal observations in Devils Creek. With the viewer, the adults appear as silvery objects, the identity of which is difficult to ascertain except for the larger type of Mylocheilus. As a result the spawning behaviour of the two species and the hybrids could only be determined in a crude fashion. In order to estimate the proportions of the two species and the hybrids present in the creek during a spawning migration, a 25

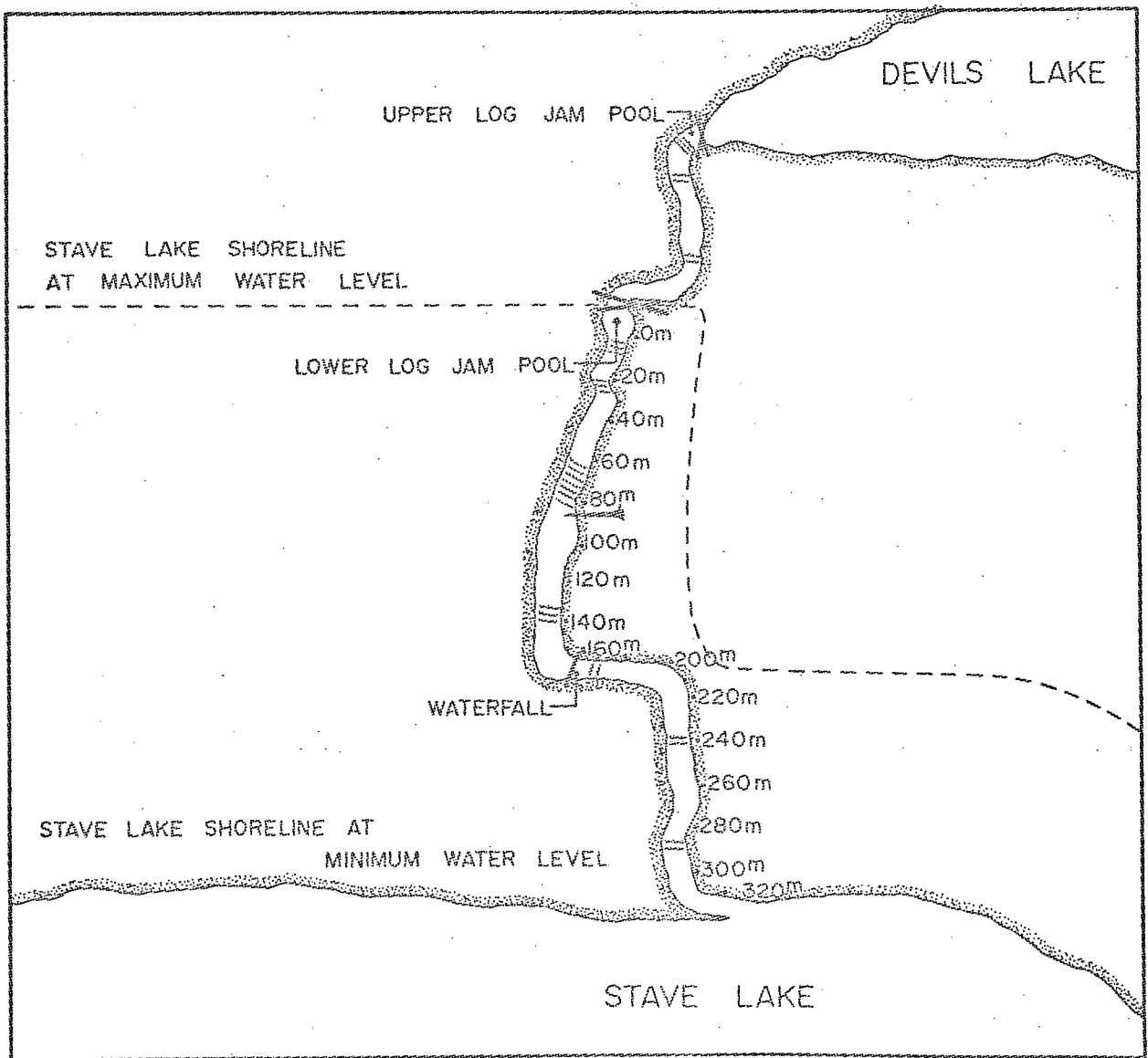


Figure 11. Diagram of Devils Creek, British Columbia.

and/or 38 mm stretch mesh gill net was placed across the mouth after the spawning fish had entered the creek. Later, after spawning, large numbers of fish were captured and identified as they returned to the lake. On some occasions spawning fish were sufficiently dense to permit sampling by hand.

Survival Experiments

Egg Survival to Hatching

Egg survival experiments were designed to test the viability of eggs from fertilization to hatching of various crosses involving Mylocheilus, Richardsonius, and their hybrids. Ripe males and females were collected from Devils Creek for use in the crossing experiments. Males were considered ripe when milt could be extruded manually. Transparency and adhesiveness of the eggs served as criteria for ripe females. 100-500 eggs were extruded from individual females into wetted plastic petri dishes. Several drops of milt were extruded from a male and mixed with the eggs. The eggs were water hardened for 15 minutes in a shallow tray containing Devils Creek water. Thereafter, the eggs were maintained until hatching in a Heath incubator supplied with flowing Devils Creek water. Dead eggs were usually removed daily from each lot to prevent fungal contamination of viable eggs. The ripe eggs of an individual female were divided into at least three lots and fertilized by the three types of males (Mylocheilus, Richardsonius, and hybrids). This practice serves to control the variability caused by using unripe females in the crosses.

The parents of all crosses made in 1967, but not in 1966, were frozen for subsequent electrophoretic analysis.

Survival of Yolk-sac Fry to Fingerlings

Yolk-sac fry of Mylocheilus, Richardsonius, reciprocal F₁ hybrids, and presumed F₁ hybrid backcrosses to Mylocheilus were reared under experimental conditions to determine their relative viabilities. Three days after hatching, 100 yolk-sac fry from each of the above-mentioned crosses were placed in identical plywood containers (50x50x50 cm) until they were 63 days old. One lot was reared for only 48 days. Most crosses had two replicates. Because of the high mid-summer temperatures of Devils Creek, flowing water from the colder creek adjacent to it supplied water to each rearing box. All experimental fry were maintained on equal quantities of identical diets of Artemia nauplii, live plankton from Stave Lake, and frozen adult brine shrimp. The fry from each lot were enumerated every 15-20 days. At the end of the experiment all fish were frozen for future morphological and biochemical examination.

Results

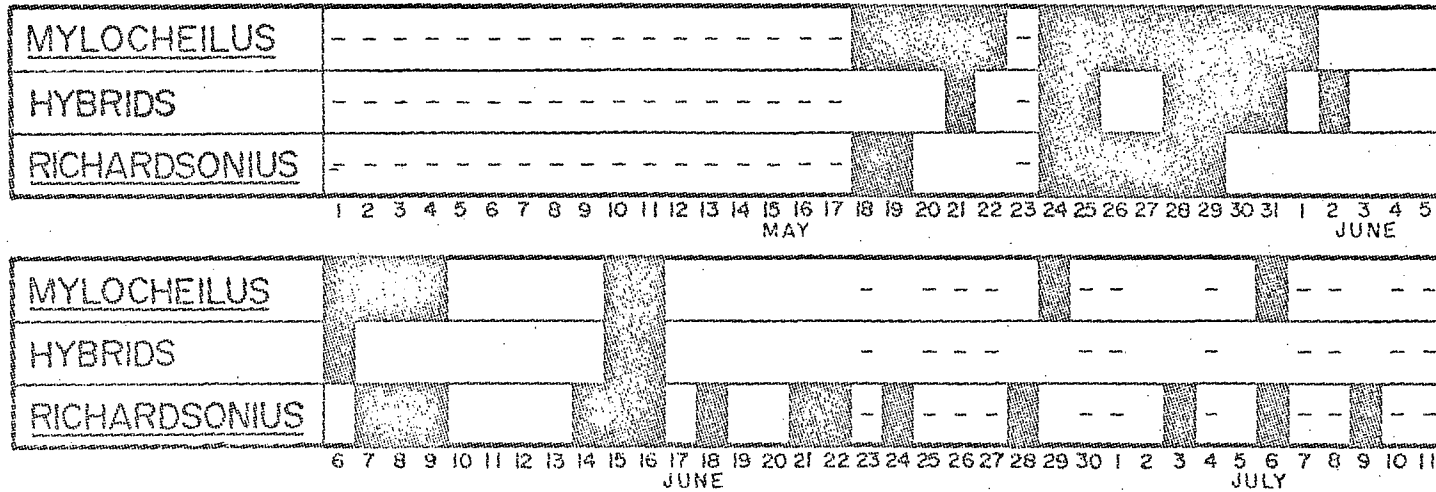
Premating Isolating Mechanisms

Seasonal Isolation

Observations on Devils Creek were conducted from May 18 to August 5 in 1966 and from May 1 to July 11 in 1967. Figure 12 graphically illustrates the time interval during which populations of Mylocheilus, Richardsonius, and their hybrids (in spawning condition) were present in Devils Creek. In 1966, Mylocheilus and Richardsonius were already present in low numbers when observations were begun on May 18. Hybrids were observed three days later on May 21, 1966. Although observations were begun on the first of May in 1967, fish in spawning coloration were not observed until May 17. It was apparent that qualitatively, at least, the spawning periods of the two species overlap to a great extent in Devils Creek. The spawning period of Richardsonius in 1966 and 1967 terminated on a later date than Mylocheilus. On a quantitative basis, it was estimated that the peak of spawning for Mylocheilus was June 16 in 1966 and June 13-15 in 1967 (Tables XI, XII). The spawning peak of Richardsonius was determined to be a week or so later. However, complete quantitative data were not collected.

Hybrid fishes are present in Devils Creek during most of the interval in which Mylocheilus, and during part of the interval in which Richardsonius, are present (Fig. 12). The peak of hybrid abundance appears to coincide closer to the peak of Mylocheilus abundance than the peak of Richardsonius abundance (Tables XI, XII).

1966



1967

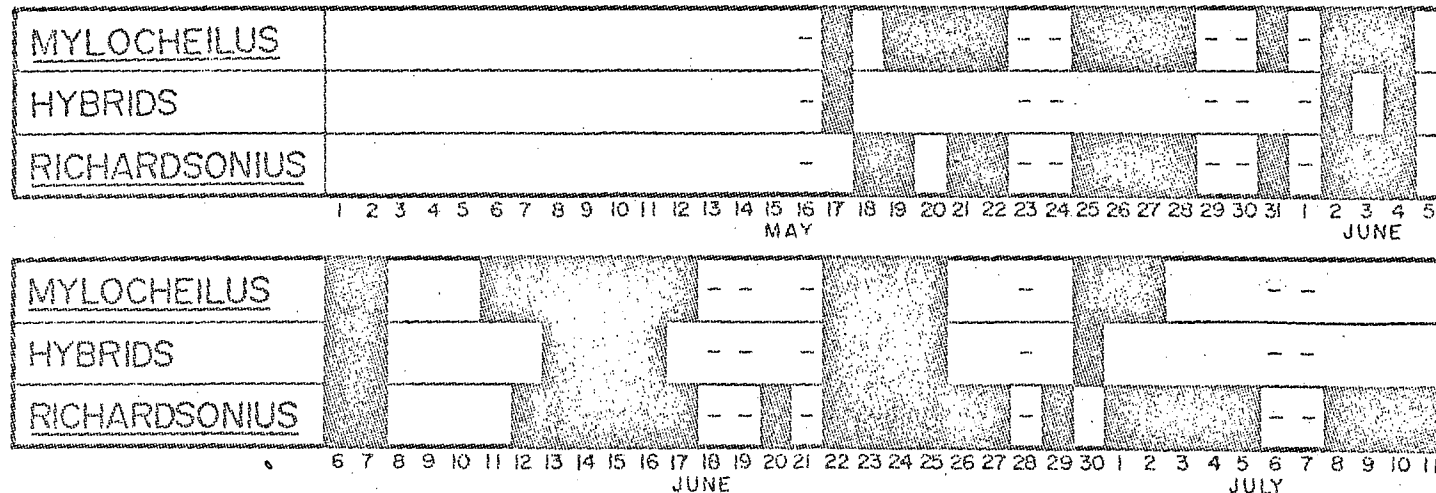


Figure 12. Seasonal distribution of Mylocheilus, Richardsonius, and their hybrids in Devils Creek for 1966 and 1967. Blackened areas are days when particular fish were present. Dashed areas are days when no observations were made.

TABLE XI. Gillnet catches of Mylocheilus, Richardsonius, and their hybrids from Devils Creek during the spawning season of 1966. **Includes one observation of fish sampled by hand. G = sexually mature fish, but not ripe; R = sexually mature, ripe fish; Sp = sexually mature, ripe fish which have deposited all or most of their gametes. Richardsonius are not represented in their true abundance because mainly 38mm stretch mesh gillnets were used. These allow most Richardsonius to escape.

		Mesh	<u>Mylocheilus</u>						Hybrids						<u>Richardsonius</u>								
		Size	$\sigma\sigma$			♀♀			$\sigma\sigma$			♀♀			$\sigma\sigma$			♀♀					
Location	Date	mm	G	R	Sp	G	R	Sp	G	R	Sp	G	R	Sp	G	R	Sp	G	R	Sp			
Lower Log																							
Jam Pool	5-21	38		2						1													
"	5-25	38		6			1																
"	5-30	38		2				1					1										
"	5-31	38		5			2	2		1													
"	5-31	19						1															
"	6-06	38		1			2							1									
"	6-07	38		1			3	2															
"	6-08	38					1	1	2														
"	6-09	38						1	1														
"	6-15	38		2			2	1		1			1										
"	6-16	38		20			2	3	1	1				1									
Riffle at																							
60' Creek																							
length	6-16	**		45			1	9	1	18				1			2						
Lower Log																							
Jam Pool	6-22	38								1													
Totals =			84			14 21 5			23			2 2 1			2								
			124						28						2			=			154		

TABLE XII. Gillnet catches of Mylocheilus, Richardsonius, and their hybrids from Devils Creek during the spawning season of 1967. G = sexually mature fish, but not ripe; R = sexually mature, ripe fish; Sp = sexually mature, ripe fish which have deposited all or most of their gametes.

Location	Date	Mesh Size mm	<u>Mylocheilus</u>						Hybrids						<u>Richardsonius</u>					
			♂♂			♀♀			♂♂			♀♀			♂♂			♀♀		
			G	R	Sp	G	R	Sp	G	R	Sp	G	R	Sp	G	R	Sp	G	R	Sp
Lower Log Jam Pool	5-17	38	9							1										
Devils Cr. Mouth	5-19	25	3																	
Lower Log Jam Pool	5-26	38	9																	
"	6-02	38	15							2					1					
Devils Cr. Mouth	6-02	38	19			8				5			1							
"	6-06	25	1																	
"	6-07	25	25							13					12		2			
"	6-13	25, 38	65			4	2	2		7			2	1	11		1			
"	6-14	25, 38	35			1	2	1	4	8			3	2	30		1	6		
Devils Cr.	6-15	25, 38	9			1	1	3		2				1	8		3			
Devils Cr. Mouth	6-16	25, 38	7							6					13		6	2		
"	6-17	25	4												12		9			
"	6-25	25	13			1		1		9			1	4	107		38	41		
"	6-26	38	1					1		2			1							
Totals =			215			15	24	11		55			8	8	1	193		60	49	
			265						71						303 = 639					

Spatial and Temporal (Diel) Isolation

An attempt was made to determine if Mylocheilus, Richardsonius, and their hybrids are spatially and or temporally (diel) segregated during spawning. Each evening from June 4 to June 17, 1967, the distribution of spawning fish from the lower section of Devils Creek was recorded. The data are graphically depicted in Figure 13. This figure illustrates the length of Devils Creek and the location of spawning fish in the creek on a given day. With few exceptions, the spawning of the two species occurs on the first riffle above the lake, regardless of the lake level. As the lake level rises during May, June, and July, therefore, decreasing creek length, spawning accordingly moves upstream. Interestingly, riffles on which spawning occurred during high lake levels in 1966, were not used in 1967 when the lake level was lower.

Both species of fish enter Devils Creek at approximately the same time each day. Richardsonius on the average may enter slightly earlier in the day, but it is difficult to estimate accurately. Fish begin entering Devils Creek as early as 2130 hours (Pacific Daylight Saving Time), although on some days they do not do so until 2245 hours. Spawning fish were observed on the first riffle as late as 0215 hours. Most fish have returned to the lake by daylight. In those situations where the lake level is very high, some fish move upstream after spawning and seek shelter in the deep pool at the lower log jam in Devils Creek (See Fig. 11).

Although it is quite apparent that Mylocheilus and Richardsonius are spawning on the same riffle at the same time, information on the behaviour

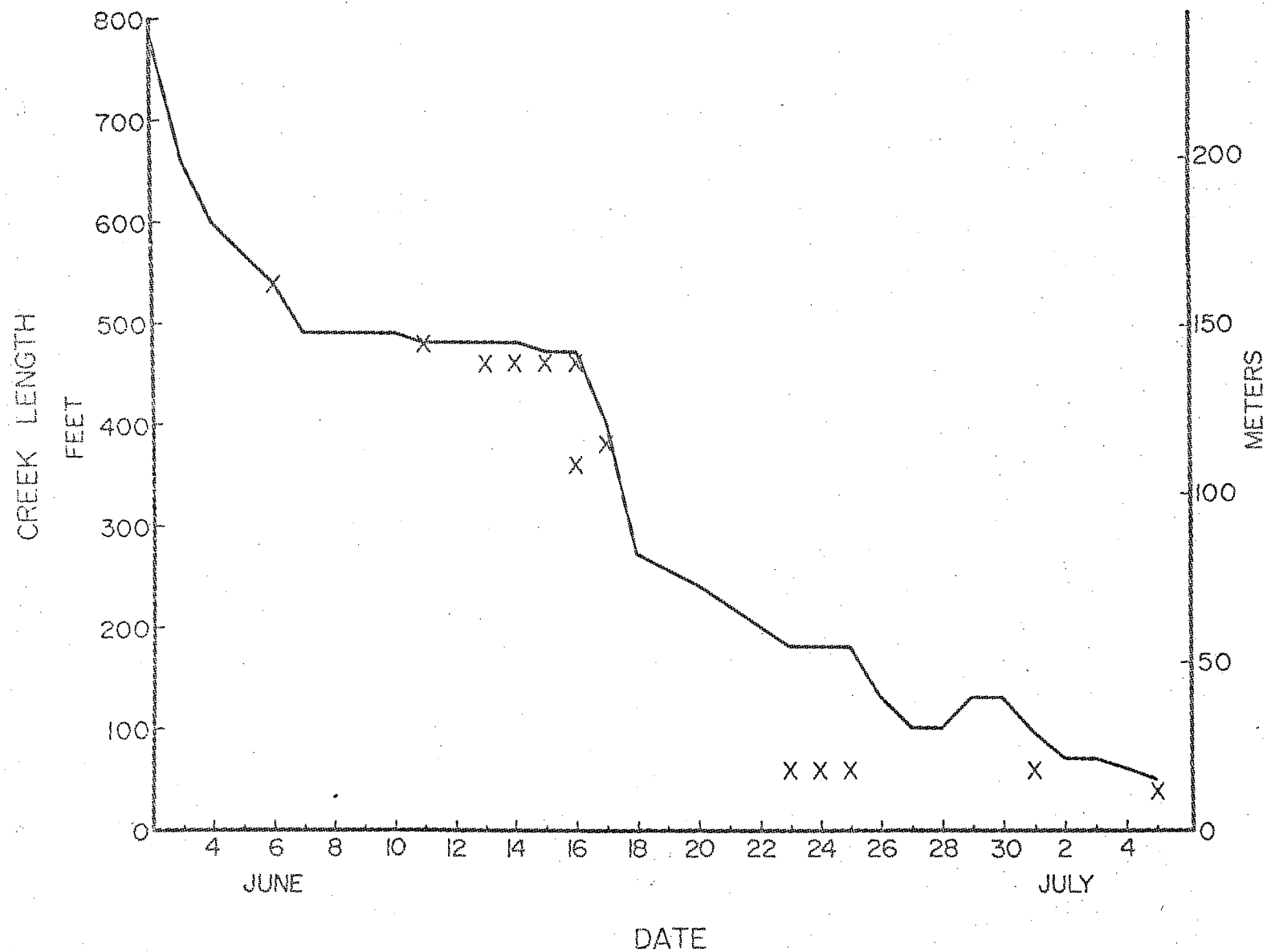


Figure 13. Relationship between location of spawning fish and the length of Devils Creek for particular dates in 1967. Crosses indicate creek locations of spawning fish.

of the hybrids was difficult to obtain since hybrids are difficult to recognize in the creek. However, two kinds of evidence suggest that hybrids, in fact, are not temporally or spatially segregated from the parental species during spawning. First, gill nets set below the riffle on which fish are spawning yield substantial numbers of hybrid fishes (Table XII). Since spawning fish are evident (with infra-red viewer) only on the first riffle above the lake during any given evening, hybrid fishes are likely spawning on the same riffle at the same time as are Mylocheilus and Richardsonius. Second, a sample of spawning fish collected by hand from a riffle area 18" square on June 16, 1966, revealed that 24.6% of them were hybrids (Table XIII).

As with the hybrids collected from the pelagic area of the lake, those hybrids in spawning coloration from Devils Creek appear to be primarily of a F_1 nature. Data on the anal fin ray distribution of hybrids collected from Devils Creek in 1967 are presented below:

<u>Anal Fin Rays</u>				
<u>Date</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>
June 7		3	10	
13	1	2	1	
15	1	5	8	
16			2	
17		2	4	
25	1	5	8	
26			3	
	3	17	36	0

TABLE XIII. Composition of a sample of the spawning population from Devils Creek at 2245 hours P.D.T., June 16, 1966. Spawning fish were collected by hand from an 18" square riffle area at the 20 meter creek length (see Figure 11).

	Females	Males	Total	Percentage Total
<u>Mylocheilus</u>	11	45	56	72.7
<u>Richardsonius</u>	0	2	2	2.7
Hybrid	1	18	19	24.6
Totals =	12	65	77	

Hybrids with 9 anal fin rays, thought on the basis of experimental crosses (Fig. 7) to be primarily hybrid backcrosses to Mylocheilus, are scarce. Only 3 out of 56 hybrids possessed such a number. In contrast, those with 11 anal fin rays are very abundant, suggesting that most of the hybrids are F_1 s.

Ethological Isolation

Information on the spawning behaviour of Mylocheilus, Richardsonius and their hybrids was difficult to obtain because spawning occurs on riffles during hours of darkness. Generally, it was determined that both species are mass spawners with many males attending a single female. Tables XI and XII reflect the great abundance of males over females. Whether F_1 hybrids are produced as the result of mis-matings between the two species or as the result of chance meeting of gametes of two properly mated species' groups was not determined. Likewise, spawning behaviour of hybrids was not observed but some inferences can be made. First, 23.8% of all hybrid females captured in Devils Creek were spent compared to 15.6% for Mylocheilus females, suggesting that hybrid females do deposit their ripe eggs. There is also evidence that hybrid eggs are fertilized and are viable at least to the "eyed" stage. On June 20, 1966, several hundred eggs spawned on the evening of June 16 were collected from the riffle at the 20 m creek length location. These eggs were reared in the laboratory until the resulting fry had their anal fin rays fixed in number. The distribution of anal fin rays from these fish is shown in Figure 14. Those fish with "9" anal fin rays presumably represent hybrid backcrosses

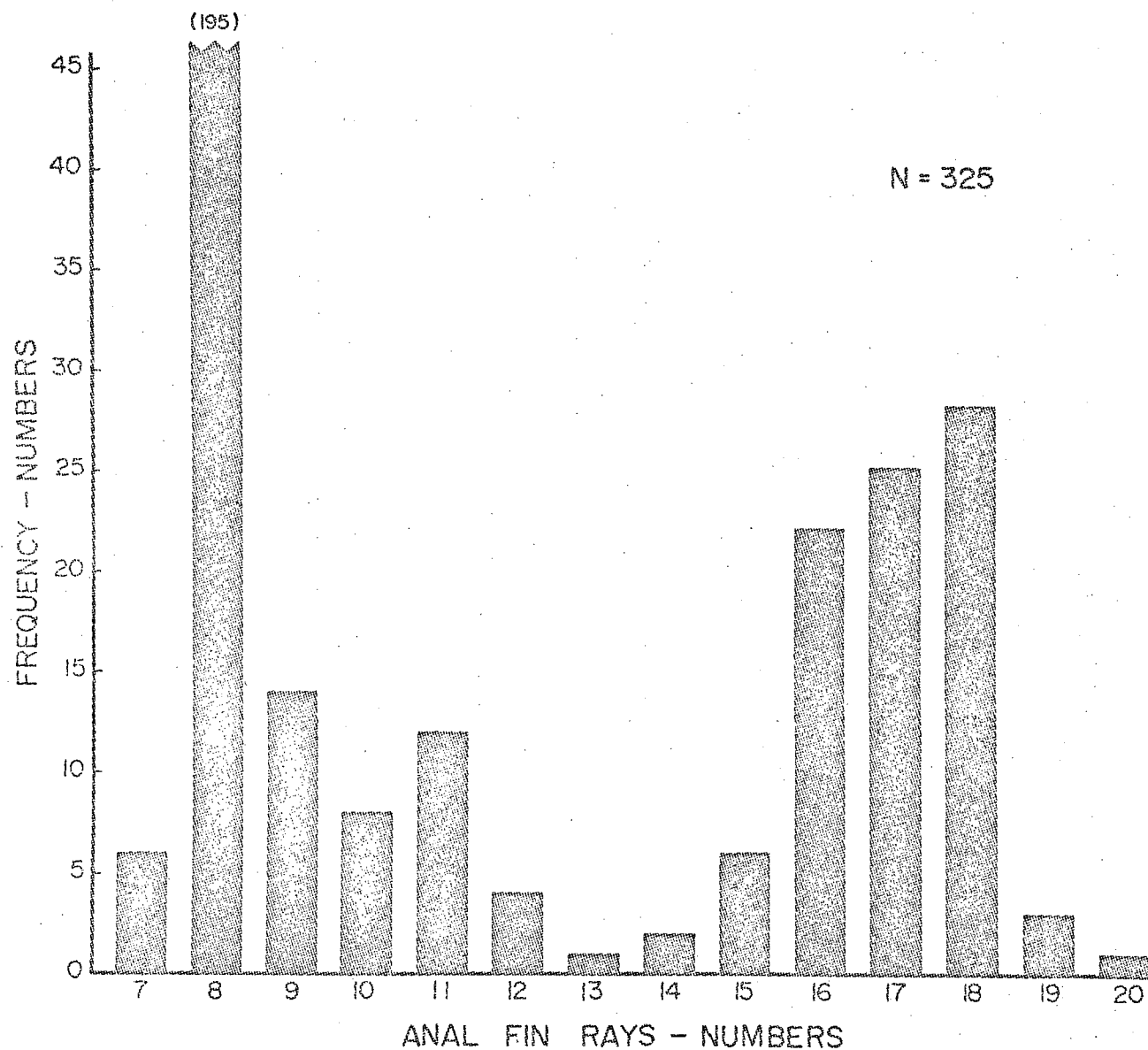


Figure 14. Anal fin ray distribution of fingerlings reared from eggs collected on June 20, 1966, from an 18" square section of the "20 meter" riffle of Devils Creek.

to Mylocheilus (See Fig. 7). Since hybrid males are, for the most part sterile, (to be discussed subsequently) these backcrosses are likely the result of hybrid ♀♀ x Mylocheilus ♂♂ crosses. If these inferences are correct, some hybrid females, at least, are able to complete spawning successfully.

The role that hybrid males play in the spawning aggregations is unknown.

Postmating Isolating Mechanisms

Egg Survival to Hatching

Hybrid sterility and inviability are considered to be important post-mating isolating mechanisms in many animal species. Consequently, they were studied in the two species under discussion to see what importance can be attributed to them. Eighty-two experimental crosses were made utilizing all combinations of Mylocheilus, Richardsonius, and presumed F₁ hybrid males and females. An analysis of variance for a 3 x 3 factorial design (Winer, 1962) was used to test for significant differences in mean survival between males and females. The level of significance in these tests, as in all others in this thesis, was .05. Clearly, male and female effects are significant ($F = 114.27$ for males, 35.19 for females $> F_{\alpha} = .05$, $df = 2, 73 = 3.15$). In view of significant "F" values for main effects (male, female), a Newman-Keuls test was used to test for significant differences between individual cell means in an ordered sequence (Winer, 1962). The cell means and tests of significance between them are given in Table XIV.

TABLE XIV. Mean egg survival of experimental crosses to hatching and tests of significance between ordered pairs of means using the Newman-Keuls procedure (Winer, 1962). 1966, 1967 and 1968 data combined.

	Cell Means		
	<u>Mylocheilus</u> ♀♀	Hybrid♀♀	<u>Richardsonius</u> ♀♀
<u>Mylocheilus</u> ♂♂	71.6	65.4	91.5
Hybrid♂♂	15.9	11.5	44.6
<u>Richardsonius</u> ♂♂	56.0	64.4	88.0
Newman-Keuls Test of Ordered Means			
Ordered Pairs	Mean Difference Between Ordered Pairs	Number of Steps Between Ordered Pairs	Critical Values of $S_{\alpha}q.95(r, 73)$
M♀H♂ and M♀M♂	55.7**	3	32.6
M♀H♂ and M♀R♂	40.1**	2	27.1
M♀H♂ and R♀H♂	28.7**	2	27.1
R♀R♂ and R♀H♂	43.4**	2	27.1
R♀M♂ and R♀H♂	46.9**	3	32.6
H♀H♂ and H♀R♂	52.9**	2	27.1
H♀H♂ and R♀H♂	33.1**	3	32.6
H♀H♂ and H♀M♂	53.9**	3	32.6
M♀M♂ and M♀R♂	15.6	2	27.1
M♀M♂ and R♀M♂	19.9	2	27.1
M♀M♂ and H♀M♂	6.2	2	27.1
M♀R♂ and H♀R♂	8.4	2	27.1
R♀R♂ and R♀M♂	3.5	2	27.1
R♀R♂ and M♀R♂	32.0	3	32.6
R♀R♂ and H♀R♂	23.6	2	27.1
H♀M♂ and H♀R♂	1.0	2	27.1
H♀M♂ and R♀M♂	26.1	3	32.6
H♀H♂ and M♀H♂	4.4	2	27.1

"M" = Mylocheilus, "R" = Richardsonius, and "H" = presumed F₁ Hybrid

** = significant

Crosses of Mylocheilus ♀♀ x Richardsonius ♂♂ and the reciprocal do not differ significantly in mean egg survival from pure crosses of either parental species.

Backcrosses of presumed F_1 hybrids to Mylocheilus give two significantly different results depending on which reciprocal is used. F_1 hybrid ♂♂ x Mylocheilus ♀♀ crosses produce a mean survival of 15.9% as compared to 71.6% for parental Mylocheilus crosses-- a significant difference. The reciprocal crosses, F_1 hybrid ♀♀ x Mylocheilus ♂♂, yield a mean survival of 65.4%, a mean survival which is not different from parental Mylocheilus crosses (71.6%).

Similarly, backcrosses of presumed F_1 hybrids to Richardsonius yield different results depending upon which reciprocal is used. Backcrosses of presumed F_1 hybrid ♀♀ x Richardsonius ♂♂ are not significantly different from parental Richardsonius crosses. However, backcrosses involving Richardsonius ♀♀ and presumed F_1 hybrid ♂♂ yield significantly poorer survival than parental Richardsonius crosses.

Presumed F_1 hybrids when cross mated produce the poorest mean survival of any cross, only 11.5%. Although an egg survival of 11.5% is not insignificant, the abnormality of newly hatched F_2 fry makes it unlikely that they exist in nature.

Of all crosses made, those which differ significantly always possess one thing in common: at least one of the crosses has a presumed F_1 hybrid as the male parent. However, within those crosses utilizing presumed F_1 hybrid males, better egg survival is obtained with Richardsonius

than with presumed F_1 hybrid or Mylocheilus females. This fact furnishes a clue as to the cause of the poor egg survival of the above crosses. If F_1 hybrid males are extremely sterile, they should not have been able to fertilize the eggs of Richardsonius females. But they do. This suggests that at least part of the mortality of Mylocheilus ♀♀ x presumed F_1 hybrid ♂♂ and presumed F_1 hybrid ♀♀ x presumed F_1 hybrid ♂♂ crosses may be attributed to genetic incompatibility or inviability.

However, there is some evidence that presumed F_1 hybrid males undergo abnormal spermatogenesis and gonadal development, leaving them partially sterile. The testes of these males are considerably smaller than those of either parental species. The quantity of milt which can be extruded is markedly reduced, and its transparency is great, in contrast to that from Mylocheilus or Richardsonius. Although the quantity of sperm in the milt does not appear reduced in presumed F_1 hybrids compared to either species, a microscopic examination of the milt reveals that the F_1 hybrid sperm is more variable in size.

There is also some evidence that the factor(s) in the F_1 hybrid male producing poor egg survival of $M\text{♀♀} \times H\text{♂♂}$ and $H\text{♀♀} \times H\text{♂♂}$ crosses is eliminated in at least some of the hybrid backcross to Mylocheilus males. In other words, it seems that assortment for the fertility and viability factors of Mylocheilus has occurred. The eggs of a single Mylocheilus female were split into five lots and fertilized with different males. The egg survival was as follows:

<u>♀</u>	<u>♂</u>	<u>Total Eggs</u>	<u># Eggs Surviving</u>	<u>% Survival</u>
M236	M239	1,695	1,379	81.4
"	R 241	640	413	64.5
"	R 242	878	767	87.3
"	H240	942	16	1.7
"	H238	871	803	92.2

"M" = Mylocheilus, "R" = Richardsonius, "H" = hybrid.

The data illustrate the great disparity between hybrid males. With hybrid male "240", egg survival is only 1.7%, but is 92.2% with hybrid male "238."

The muscle protein and MDH patterns of hybrid male "238" reveal that it is at least a first generation hybrid backcross to Mylocheilus. For hybrid male "240", the protein patterns are of a F_1 hybrid nature.

Survival of Yolk-sac Fry to Fingerlings

The survival of yolk-sac fry to the fingerling stage was examined under experimental conditions to discover whether hybrid individuals are inviable relative to the parental species. Such inviability can be a partial or complete postmating isolating mechanism. Two-way analyses of variance were used to test for differences in survival between crosses (Winer, 1962). Survival of Mylocheilus ♀♀ x Richardsonius ♂♂ crosses do not differ significantly from parental Mylocheilus crosses ($F = 0.001 < F_{\alpha=0.05, df=1,2} = 18.5$). Similarly, Richardsonius ♀♀ x Mylocheilus ♂♂ survival does not deviate significantly from parental Mylocheilus crosses ($F = 0.57 < F_{\alpha=0.05, df=1,2} = 18.5$). However, after 48 days of rearing, survival of hybrid backcrosses to Mylocheilus proves to be significantly lower than parental Mylocheilus crosses by approximately 20% ($F = 21.43 > F_{\alpha=0.05,$

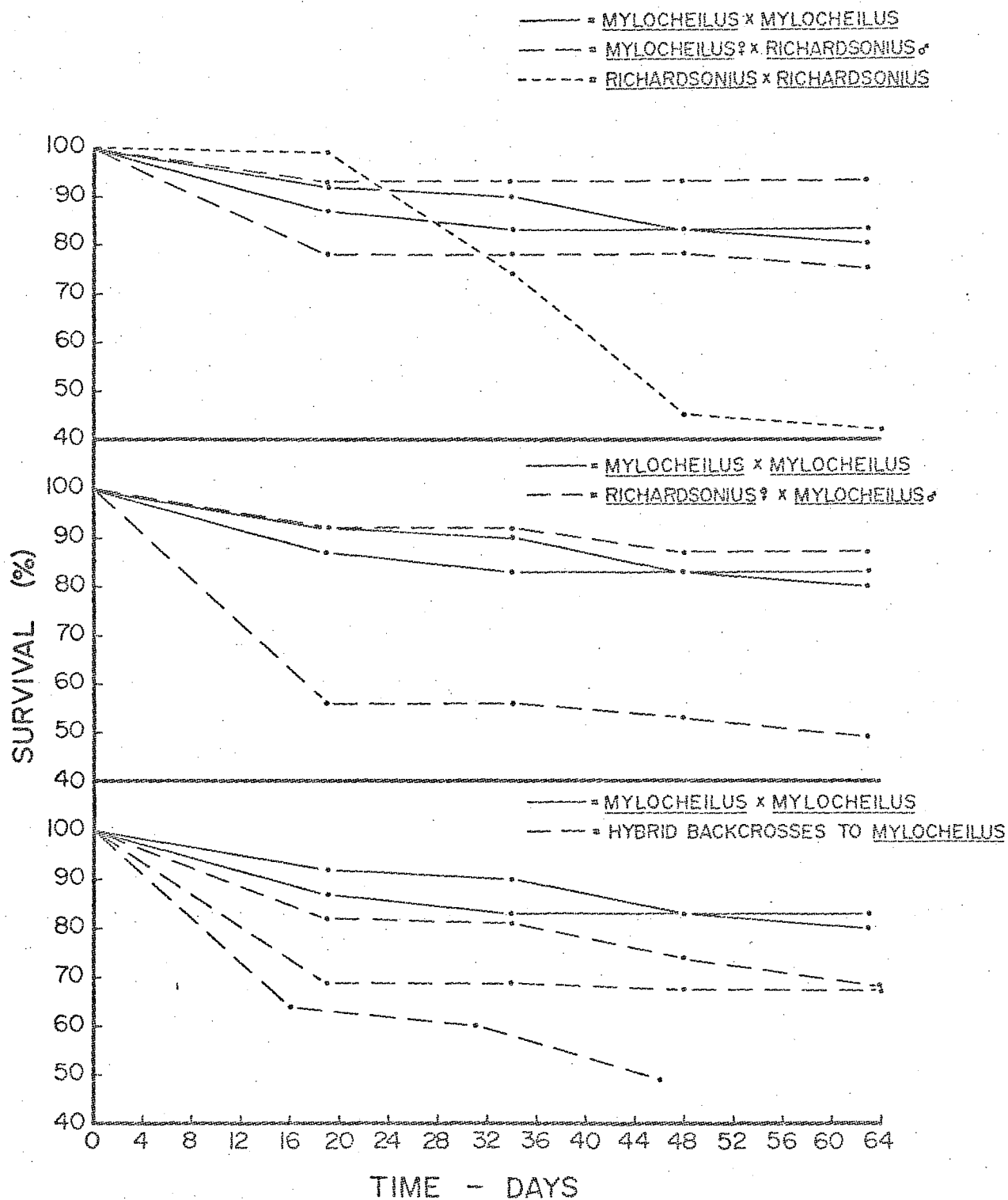


Figure 15. Survival of yolk-sac fry to the fingerling stage under experimental conditions.

df = 1, 13 = 4.67)(Fig. 15).

Only one lot of parental Richardsonius fry was reared because of the difficulty of procuring ripe females when the experiments were started. For an unknown reason, this lot has the poorest survival of any tested (Fig. 15).

An inspection of the data reveals that a considerable proportion of the total mortality (with the exception of the parental Richardsonius cross) is usually encountered by the first enumeration period (16 or 19 days). The rate of mortality subsequent to this is substantially lower. During the first interval, the switch-over from an endogenous to an exogenous food source may have accounted for the considerable mortality experienced. In sharp contrast, little mortality was suffered the first 19 days in the parental Richardsonius cross. Subsequently, however, the rate of mortality increased, producing the poorest survival of any lot. It is felt that the very poor survival of the Richardsonius lot is an anomaly. As a check Richardsonius fry were reared in the laboratory in 1968 along with F_1 hybrids. The survival of both was good and near equal.

In summary, the viability of presumed F_1 hybrid backcrosses to Mylocheilus is markedly poorer than Mylocheilus crosses or for either reciprocal F_1 hybrid crosses.

Abnormalities of Experimental Animals

Some fish, used in the experimental survival studies but not used for biochemical studies, were examined for obvious abnormalities at the termination of the experiment. The results are presented in Table XV. Progeny of parental Mylocheilus and reciprocal F_1 crosses are almost

TABLE XV. Gross abnormalities of fish used in the viability experiments from the yolk-sac fry to the fingerling stage.

<u>Female</u>	<u>Male</u>	<u>Number Examined</u>	<u>Number Abnormal</u>	<u>Type of Abnormality</u>
<u>Mylocheilus</u>	<u>Mylocheilus</u>	13	0	
<u>Mylocheilus</u>	<u>Mylocheilus</u>	112	0	
<u>Mylocheilus</u>	<u>Richardsonius</u>	88	1	
<u>Mylocheilus</u>	<u>Richardsonius</u>	2	0	
<u>Richardsonius</u>	<u>Mylocheilus</u>	75	0	
<u>Mylocheilus</u>	"Hybrid"	30	0	
"Hybrid"	<u>Mylocheilus**</u>	64	6	3 fish very small, 3 fish with bulging abdomens
"Hybrid"	<u>Mylocheilus</u>	37	6	2 fish with bulging abdomens 2 fish with light pigmentation 1 fish with light pigmentation and bulging abdomen 1 fish with deformed vertebral column

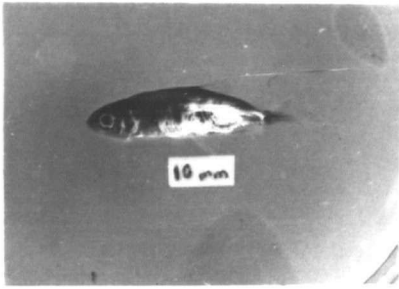
** some contamination of M♀ X H♂ crosses

free of gross abnormalities. In contrast, progeny of hybrid backcrosses to Mylocheilus possess many. For three such crosses, 11.5% of the individuals possessed distended abdomens, unusually light or dark body pigmentation, or deformed vertebral columns (Fig. 16). Although deformed fish are not considered as mortalities in the fry survival experiments, it is possible that such individuals would be selectively eliminated under natural conditions. Evidence that this occurs could have been determined if large numbers of adult hybrid backcrosses to Mylocheilus had been obtained, for they should contain many deformed individuals if selection has not operated against them.

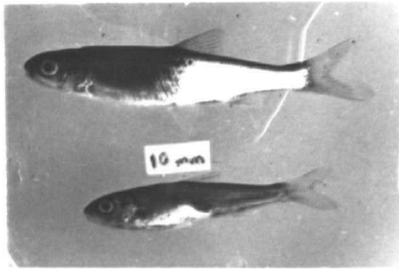
Hybrid backcrosses to Richardsonius were not considered because of a shortage of experimental animals.

Sex ratios of hybrid and parental species' populations

One hundred and nine hybrid individuals sampled from Stave Lake during the summer of 1967 were examined to detect any deviations from a 1:1 sex ratio which may serve as a postmating isolating mechanism. On the basis of biochemical characteristics (muscle proteins, MDH), the hybrids were divided into two groups, 1) presumed F_1 hybrids and 2) hybrid backcrosses to Mylocheilus. The sex ratios of the parental species' forms captured from Stave Lake (1" gill nets) were also examined and are shown below with the sex ratios of hybrid individuals.

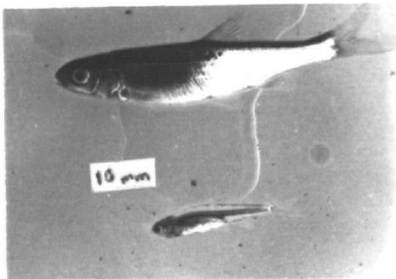


**A. DEFORMED VERTEBRAL
COLUMN**



B. NORMAL

C. DISTENDED ABDOMEN



D. NORMAL

E. ABNORMAL PIGMENTATION

Figure 16. Typical abnormalities of progeny from hybrid backcrosses to Mylocheilus.

	<u>Mylocheilus</u>	<u>Richardsonius</u>	Presumed <u>F₁ Hybrids</u>	Hybrid Backcrosses <u>To Mylocheilus</u>
Males	279	24	43	10
Females	212	100	46	8
Sex Ratio (♂♂ : ♀♀)	1:0.76	1:4.17	1:1.07	1:0.80
Chi-Square	9.14*	46.58*	0.10	0.22

* = significance at $\alpha = 0.05$

Surprisingly, both parental forms deviate significantly from a 1:1 sex ratio, but in different ways. For Mylocheilus, there are more males than females while the reverse is true for Richardsonius. Why these sex ratios are found is unknown, but possible factors responsible are: 1) an intrinsically abnormal sex ratio, 2) differential mortality of the two sexes subsequent to spawning, and 3) gill net selectivity. The sex ratios of spawning individuals of both species in Devils Creek heavily favor males.

Presumed F₁ hybrids and hybrid backcrosses to Mylocheilus from Stave Lake (1" gill nets) both possess sex ratios which are not significantly different from 1:1. Although the hybrid data are difficult to interpret in view of the sex ratios of the parental species, it is probably reasonable to conclude that the sex ratio of hybrids is not a postmating isolating mechanism between the two species.

Comparison of Fingerling and Adult Hybrid Fin Ray Distributions

The anal fin ray distributions of fingerling and adult hybrids were compared to obtain a rough estimate of hybrid vigor or hybrid inviability.

Based on the knowledge of the heritability of anal fin rays, those hybrids with 9 anal fin rays are presumed to be hybrid backcrosses to Mylocheilus, while those with 11 anal rays are presumed to be primarily F₁ hybrids. If F₁ hybrids and hybrid backcrosses to Mylocheilus are inferior, relative to the parental species, their frequency should decrease the older they become. Consequently, the frequency of those hybrids with 9 and 11 anal fin rays was compared relative to Mylocheilus as fingerlings and as adults (Fig. 17). The hybrids would have been compared to both parental species, but it was difficult to capture fingerling Richardsonius because of their small size. The data are as follows:

	Hybrids With 9 Anal Rays	Hybrids With 11 Anal Rays	Number of <u>Mylocheilus</u>	Ratio of the Number of Hybrids With 9 Anal Rays to <u>Mylocheilus</u>	Ratio of the Number of Hybrids With 11 Anal Rays to <u>Mylocheilus</u>
1966 Adults	7	18	647	0. 0108:1	0. 0278:1
1967 Adults	19	64	1, 544	0. 0123:1	0. 0415:1
1967 Finger-137 lings		79	2, 258	0. 0607:1	0. 0350:1

If it can be assumed that 1967 fingerling recruitment was qualitatively and quantitatively the same as for those years which produced the 1966 and 1967 adult populations, F₁ hybrids do not appear less abundant as adults than as fingerlings. On the other hand, hybrid backcrosses to Mylocheilus are approximately five times as abundant as fingerlings than as adults.

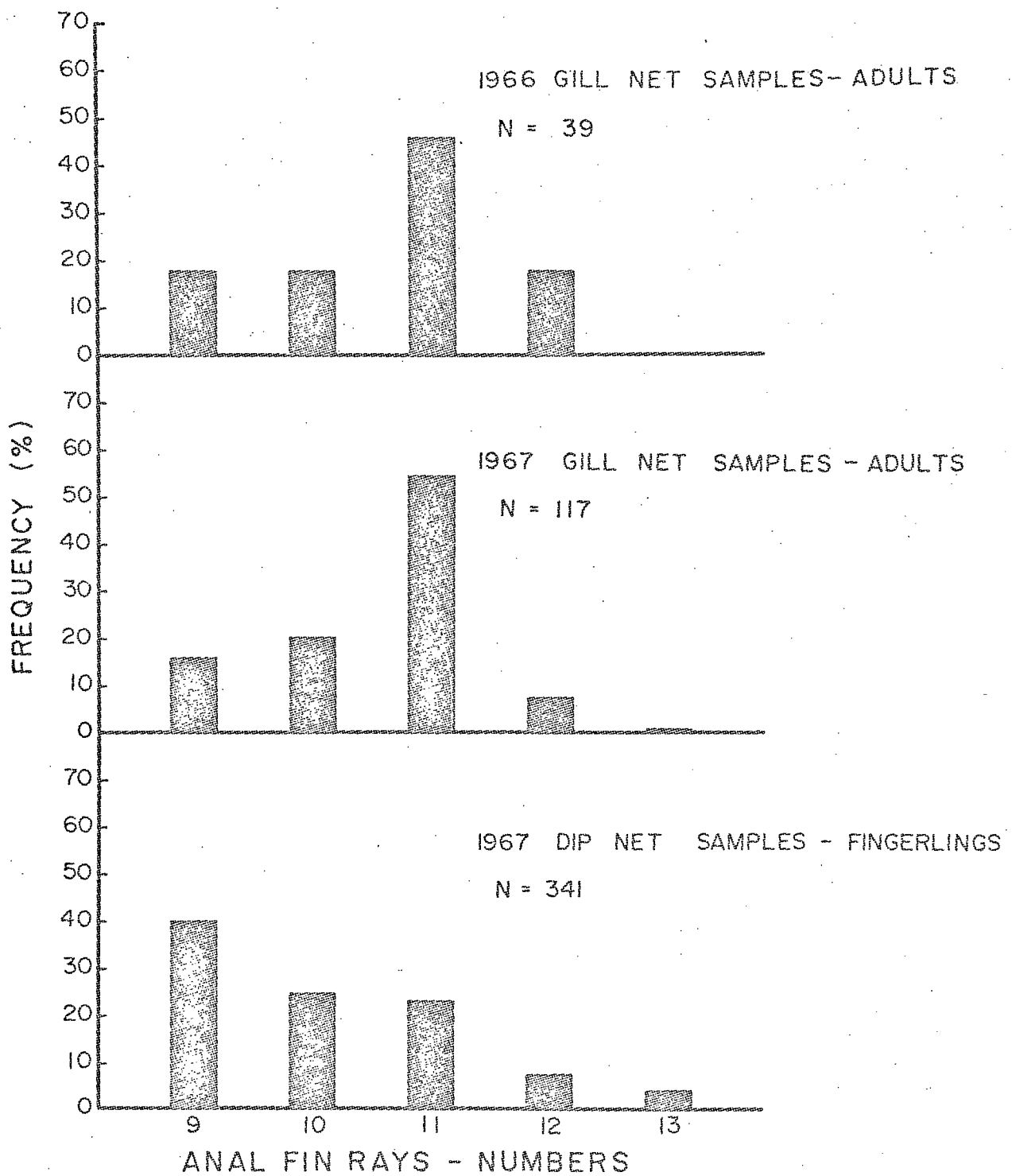


Figure 17. Distribution of anal fin rays from "9" to "13" for 1967 fingerlings and for 1966 and 1967 adults.

DISCUSSION

Introgression, Swamping, and Reproductive Isolation

Anderson and Hubricht (1938) defined introgression as "... an infiltration of the germplasm of one species into that of another. "

Stebbins (1959) states that there are three essential phases to this process: (1) the initial formation of the F_1 hybrids (2) their backcrossing to one or the other of the parental species, and (3) the natural selection of certain recombinant types. The first two phases are clear cut and, in most cases, the presence or absence of them is easily demonstrated. The third phase, however, is not easily demonstrated because the permanence of the recombinant types, a necessary conclusion if natural selection is favoring them, is difficult to establish. The selectively advantageous recombinants can possess one or innumerable alleles of the other species. The latter situation is known as swamping and this type of introgression, massive introgression, will be discussed first. The difficulty of determining the permanence of these recombinants (hybrid backcrosses) arises because phase 1 is constantly recruiting F_1 hybrids which in turn produce the 1st generation hybrid backcrosses or recombinants. Although all "introgressing" genes may be eliminated by the next generation, it will appear that they are permanent additions to the gene pool because they are continually being replenished. They are, however, transient. Backcross hybrids should not be considered as introgressants until their permanence has been established. Examples of hybrid backcrosses constantly being recruited but subsequently eliminated from the population

are the persistent hybrid zones between the cyprinids Gila orcuttii and G. mohavensis of southern California (Hubbs and Miller, 1943) and Acrocheilus aleutaceus and Ptychocheilus oregonense in British Columbia (Stewart, 1966).

Unless the frequency of various hybrid backcross generations can be determined, it appears difficult to determine if swamping is occurring when initial hybridization and backcrossing are present unless: (1) the swamping of the two gene pools is so near completion that it is unmistakable, or (2) the distributions of the two parental species for given characters are not stationary over time or space.

An attempt was made to differentiate between various backcross generations to determine the permanence or, inversely, the transience of "introgressing" genes in Mylocheilus and Richardsonius. Differentiation between 1st and 2nd or later generation hybrid backcrosses to Mylocheilus was achieved with a fair degree of precision because of the opportunity to make such crosses experimentally. Second generation hybrid backcrosses are scarce; only 1 was found in 249 supposedly pure Mylocheilus examined. For Richardsonius, even 1st generation hybrid backcrosses are scarce (1.7% of all hybrids), while no 2nd or later generation hybrid backcrosses were found in a sample of 178 supposedly pure Richardsonius.

Based on the frequency of "hybrid" protein patterns and the distribution of anal fin rays, the genes of one species which are "introgressing" into the gene pool of the other, appear to be eliminated with subsequent backcrossing. If the two proteins (≥ 4 loci) are considered representative of

the two genomes, it is concluded that swamping is not in progress.

Evidence for the absence of swamping between Mylocheilus and Richardsonius was also gathered from two morphological characters, lateral line scales and the ratio of the predorsal to the prepelvic length. Individuals from Stave Lake judged to be pure Mylocheilus and Richardsonius on the basis of anal fin rays were compared with populations where hybridization does not occur, or where it could not have occurred in the past because of the absence of one of the two species. No significant differences for either character are found between the means of the populations from within, and outside, the hybrid zone. In fact, Mylocheilus from the hybrid zone possess higher mean lateral line scale counts than allopatric populations, exactly opposite the expected result if Richardsonius genes for low scale numbers are introgressing into Mylocheilus.

Therefore, no evidence either morphologically or biochemically was found to suggest swamping between Mylocheilus and Richardsonius, even though hybrid backcrossing to Mylocheilus is not uncommon. Reproductive isolation is maintained.

Although the absence of introgression of several loci controlling biochemical and morphological characters is used as evidence against massive introgression or swamping between the two species, there is no evidence to refute the possibility that one, or two, or more, genes of each species could not have introgressed into the other. This possibility, though entirely plausible, is difficult to prove because one does not know if the supposedly introgressed character was already present in the introgressed

species at a low frequency. Directional selection of preexisting variability could then produce a result similar to introgression. Also, phenotypic modification of one morphological character to inadvertently resemble a similar one in another species cannot be ruled out. Either introgression, directional selection of preexisting variability, or phenotypic modification could explain the fact that the spawning time and breeding size of Mylocheilus approximates those of Richardsonius only in the hybrid zone.

Isolating Mechanisms

When both hybridization and reproductive isolation are present, it is clear that the hybrids between two species must be at a selective disadvantage. This inference has been made by many workers (Hubbs and Miller, 1943; Sibley, 1961; Mayr, 1963; Hagen, 1967; and Nelson, 1968). Often, the F_1 hybrids are vigorous but sterile (Hubbs, 1955) and this isolating mechanism prevents those genes from progressing any farther. However, many examples are known where the F_1 hybrids appear fully fertile (Hubbs and Strawn, 1956, 1957a; Hagen, 1967; and Nelson, 1968) and other isolating mechanisms must contribute to the elimination of their genes. Either the F_1 hybrids fail to mate or they do mate but the F_2 or hybrid backcross progeny are inviable. Evidence for hybrid inferiority in fishes other than hybrid sterility is meager. Many authors (Stewart, 1966; Hagen, 1967; Nelson, 1968) have been unable to demonstrate hybrid inferiority, an inferiority which must exist if reproductive isolation is to be maintained between hybridizing species. Hubbs (1958) has presented some of the best evidence for hybrid inferiority. F_1 hybrid backcrosses to Etheostoma spectabile and E. lepidum possess poorer survival to hatching and to the early larval stage than either parental species.

Isolating mechanisms effecting reproductive isolation between Mylocheilus and Richardsonius were examined generally in Stave Lake,

British Columbia, and in Devils Creek in particular.

Premating Isolating Mechanisms Between the Parental Species

Although the peak of spawning for Richardsonius occurs several days later than for Mylocheilus, the overlap between them is so great that seasonal isolation cannot be an effective barrier. Likewise, temporal and spatial factors are poorly developed isolating mechanisms in Devils Creek since both species usually spawn, beginning at dusk, on the first riffle above the lake. Also, eggs collected from an 18" square riffle area in Devils Creek yielded 11.8% hybrids, further evidence for the ineffectiveness of spatial and temporal mechanisms.

Data on ethological isolation between the two species is meager since night observation of riffles was near impossible. Only the presence or absence of fish could be detected. It was not discovered, therefore, whether F_1 hybrids were produced by mixed species aggregations or as the result of chance meeting of gametes from conspecific aggregations of each species.

F_1 Hybrid Viability

Data were collected on the survival of eggs to hatching of various crosses made under experimental conditions. Reciprocal crosses of Mylocheilus x Richardsonius (F_1 s) give good egg survival and are not significantly different from parental Mylocheilus or Richardsonius crosses. The survival of F_1 hybrids was also studied from the yolk-sac fry to the fingerling stage (approximately 60 days) under experimental conditions and the results, again, do not differ significantly from parental Mylocheilus crosses.

Some data were also gathered from the natural environment on the success of F_1 hybrids relative to that of Mylocheilus. If F_1 hybrids are ill-adapted to the environment, then their relative abundance should decrease between the fingerling and adult stages. To insure that mainly F_1 hybrids were included in this comparison, only hybrids with 11 anal fin rays were examined. The ratio of adult F_1 hybrids to adult Mylocheilus was 0.0278:1 in 1966 and 0.0415:1 in 1967, while the ratio of fingerling F_1 hybrids to fingerling Mylocheilus in 1967 was 0.0350:1. Assuming that the production of F_1 hybrids in 1967 was similar to those years which resulted in the 1966 and 1967 adult hybrid populations, it appears that, relative to Mylocheilus, they are not inferior in survival.

In conclusion, therefore, egg survival of F_1 hybrids under experimental conditions is comparable to that of the parental species; survival of yolk-sac fry to the fingerling stage is equal to Mylocheilus. Under natural conditions, there is no evidence that the survival of F_1 hybrids is any poorer than for Mylocheilus. The good viability of F_1 hybrids between Mylocheilus and Richardsonius is in agreement with the findings of Nelson (1968) for F_1 hybrids between two species of catostomids and for F_1 hybrids between two forms (species?) of Gasterosteus reported by Hagen (1967). Also, there is no evidence of hybrid vigor, as reported for many interspecific hybrids among fishes (i. e. Centrarchidae, Hubbs, 1955).

Spawning Behaviour of Hybrid Fishes

As in the case of the parental species the technical difficulties of observing at night limited observations of reproductive behaviour for

hybrid individuals. However, some inferences can be made from specimens and naturally spawned eggs collected from Devils Creek. Hybrids in spawning condition are abundant in gill nets set below the first riffle of Devils Creek during the spawning period; they composed 18.2% of all spawning fish in 1966 and 11.5% in 1967. The former figure is somewhat overestimated because the 38 mm gill nets used in 1966 discriminated against the smaller species, Richardsonius. Regardless of this discrepancy, the percentage of hybrids in spawning coloration in Devils Creek is high, and suggests the lack of temporal and spatial isolation between the hybrids and the parental species. Evidence corroborating the suggestion that hybrids are not spatially or temporally isolated from the parental species is that 24.6% of the fish collected from a spawning aggregation in an 18" square riffle area in Devils Creek were hybrids.

There is also a suggestion that at least some hybrid females are able to complete spawning successfully. The percentage of spent females (females that had probably deposited their eggs) is significantly higher for hybrids than for Mylocheilus. The presence of numerous BCM progeny from eggs collected from the spawning riffles of Devils Creek in 1966 and the numerous BCM fingerlings captured in the littoral zone of the lake in 1967 all indicate that some hybrid females do spawn and deposit their eggs in an adequate manner. These BCM progeny most likely originate from crosses between hybrid females x Mylocheilus males rather than from Mylocheilus females x hybrid males because hybrid males possess small quantities of abnormal milt and crosses involving them produce very poor egg survival.

From the scarcity of hybrids with 9 anal fin rays (3/56), a very large percentage of these spawning hybrids are presumed to be of F_1 origin. Apparently, adult hybrid backcrosses to Mylocheilus and Richardsonius are as scarce in the spawning populations as they are in samples taken from the pelagic areas of the lake.

In conclusion, there is no evidence that the spawning behaviour of hybrid females serves as an effective isolating mechanism. Except for the fact that hybrid males are not seasonally, temporally, or spatially segregated from the parental species, no information on their spawning behaviour is available. A possible ethological isolating mechanism between the species would be the homogamous mating of hybrids. Since such crosses are very unsuccessful, gene flow would cease. However, the spawning behaviour of the parental species--they both spawn in large, tightly packed, aggregations--would probably not allow hybrid males the exclusive use of a hybrid female.

F_1 Hybrid Fertility and Hybrid Backcross Viability

Egg survival experiments involving hybrid males and females were performed to ascertain their fertility relative to the parental forms. Crosses involving hybrid females, presumed to be F_1 s, and Mylocheilus and Richardsonius males are not significantly different in egg survival from parental Mylocheilus and Richardsonius crosses, respectively. Apparently, therefore, hybrid females are quite fertile. On the other hand, crosses of presumed F_1 hybrid males with females of both parental species yield poor egg survival, suggesting the partial sterility of these

males. Other evidence for the partial sterility of presumed F_1 hybrid males is the appearance of their milt; in contrast to males of the parental species, it is transparent and very reduced in quantity. The sperm, themselves, are more variable in size than those of Mylocheilus.

However, the better egg survival of hybrid backcrosses with Richardsonius than with Mylocheilus females suggests that genetic incompatibility may be a contributing factor in the poor egg survival of crosses involving F_1 hybrid males.

The mean egg survival of presumed F_1 hybrid ♀♀ x presumed F_1 hybrid ♂♂ crosses is the poorest of all crosses attempted. Additionally, these crosses characteristically produce abnormal fry. From 632 eggs, only 4 individuals were reared to the fingerling stage.

In one instance, a single Mylocheilus female was crossed with a Mylocheilus male, a hybrid backcross to Mylocheilus male, 2 Richardsonius males, and a hybrid male of presumed F_1 origin. Egg survival with the hybrid backcross to Mylocheilus male is 92.2% compared to 81.4% for the control (Mylocheilus ♂) and 1.7% for the presumed F_1 hybrid male. The milt of the BCM male was whitish in appearance and similar to males of both species. Obviously, assortment for parental Mylocheilus fertility factors had occurred. The proportion of hybrid backcrosses with restored fertility is probably a function of the number of genes by which it is controlled.

A statement should be made about the relationship of egg survival as measured experimentally to that under natural conditions. Egg survival

under experimental conditions is probably substantially better than natural survival. This is because of the way the eggs are fertilized and treated experimentally. The milt is placed directly on the eggs and the eggs subsequently submerged in a small quantity of water for about 15 minutes to water harden. Under the natural conditions of swift riffles, the sperm and eggs are probably in contact for only a very few seconds and therefore the probability of fertilization may be reduced. Dead eggs infected with fungus were usually removed from experimental lots every day, thus reducing the loss by contamination of otherwise viable eggs. In nature, fungus infected eggs attached to rocks in Devils Creek were very abundant. How important these two factors are in reducing the number of offspring produced by F_1 hybrid males is not known.

In conclusion, the fertility of F_1 hybrids is not an effective isolating mechanism between Mylocheilus and Richardsonius in Stave Lake. However, the partial sterility of F_1 hybrid males would certainly decrease the speed of swamping between the two species, if other isolating mechanisms were not effective.

Experimental data show that the survival from the yolk-sac fry to the fingerling stage is significantly less (approx. 20%) for hybrid backcrosses to Mylocheilus than for parental Mylocheilus individuals or for reciprocal F_1 hybrid individuals.

It should be understood that this 20% decrease in survival occurs over a period of only 48 days--- probably a small fraction of the normal life span. Consequently, such a differential mortality summed over the life span of

the hybrid backcrosses seems adequate to eliminate virtually all of them, thus insuring the maintenance of reproductive isolation between the two species. Besides this poorer survival, hybrid backcrosses to Mylocheilus contain more abnormal individuals (11.5%) than parental Mylocheilus or F_1 hybrid lots. If the 11.5% abnormal BCMs are considered as mortalities under natural conditions, as they probably should be, this further increases the differential mortality between them and Mylocheilus to 31%. Also, some selective factors (predation, disease, parasitism, etc.) acting against hybrid backcrosses in nature are probably absent from the experimental holding tanks. To my knowledge, with the exception of Hubbs' (1958) work on hybridization in Etheostoma, this is the only study where the inferred inviability of hybrid backcross progeny (even under laboratory conditions) has been demonstrated.

Some circumstantial evidence from the hybrid zone suggests that BCMs are selected against, relative to Mylocheilus. When the number of individuals with 9 anal fin rays, which are assumed to be primarily hybrid backcrosses to Mylocheilus, are compared as fingerlings and as adults, it is apparent that fingerling BCMs are approximately five times as abundant as adults. Unfortunately, only one year's data are available. However, if this year can be considered representative, then this is good evidence that BCMs are at a selective disadvantage relative to Mylocheilus. This type of information not only suggests that the BCMs are selected against, but also indicates what time interval in their lives selection against them is strong. The field data indicate that intense selection occurs between

the fingerling and the adult stage. In one way, this seems contradictory because laboratory experiments indicate that selection is strong from the yolk-sac fry to the fingerling stage. Perhaps, selection is intense during all stages of their lives. Because it is impossible (in this study) to identify BCMs before the fingerling stage, no measure of selection between the yolk-sac fry and fingerling stages could be obtained in nature.

Data on the viability of BCRs is meager. From the fingerlings captured in the littoral zone of the lake in 1967, there is no reason to suspect BCRs are numerous. Whether they are eliminated before the fingerling stage, or are never produced, is unknown.

What selective factors are operating to maintain the inferiority of hybrid backcrosses is unknown. As Hagen (1967) points out, a knowledge of the ecology of the two species is essential if one is to understand the selective factors operating against the hybrids. In this study, as in several others, mechanisms which effect reproductive isolation between two species are not the obvious ones of hybrid sterility or ethological isolation but appear to be the component ones associated with the inferiority of hybrid backcrosses, in part.

Cause(s) of Hybridization and Lack of Reinforcement

The excellent work of Carl Hubbs (1955 and many earlier papers) and his colleagues on hybridization in fishes reveals several generalizations about the process. One is the correlation between hybridization and disrupted environments. The cause(s) of hybridization in many cases is

apparently due to the alteration of the environment rather than to alterations in the genomes of the two hybridizing species. In Stave Lake, hybridization between Mylocheilus and Richardsonius is probably related to the enlargement of the original lake for hydroelectric purposes. The original facilities were constructed in 1911 with modifications in 1916 and 1923. Hybridization between the two species in Stave Lake has occurred at least since 1950, a period of 18 years.

Ronald Fisher (1930) initially proposed that natural selection acting against hybrid individuals would in turn act against those hybridizing individuals of each species, thus favoring conspecific matings. This "reinforcement of isolating mechanisms" would, with time, reduce or eliminate hybridization between two species. Much circumstantial evidence is present in the evolutionary literature of reinforcement of premating isolating mechanisms (Sibley, 1961). Likewise, Koopman (1950) has shown reinforcement of isolating mechanisms between Drosophila pseudoobscura and D. persimilis in the laboratory. However, there are also many recorded situations where hybridization has persisted for many years without reinforcement occurring. Hubbs (1961) presents evidence that hybrids between two forms of Siphateles obesus have existed for hundreds of years. Similarly, Nelson (1968) believes that Catostomus macrocheilus and C. commersonii have hybridized for several hundred years. In the present study, Mylocheilus and Richardsonius have hybridized for at least 18 years.

Various authors suggest factors governing the speed with which

reinforcement is completed. Sibley (1961) states that "The intensity of selection against the hybrids determines the speed and extent of reinforcement of the isolating mechanisms." (italics mine). Undoubtedly, the intensity of selection, " s " (Li, 1955), is important but frequently the intensity of selection is 1 (maximum intensity of selection against a given genotype) because the hybrids are sterile or inviable. Yet, hybridization persists. Equally important, it seems, is the genetic basis (number of genes and linkage) for the isolating mechanisms themselves. For example, reinforcement would be complete in one generation if the hybrids were sterile ($s=1$) and if the partially effective isolating mechanism(s) was controlled by a single, dominant, gene. On the other hand, many generations would be required before reinforcement could be completed if the isolating mechanism(s) was controlled multifactorially and with tight linkage. In such cases, those alleles which contribute to the inadequacy of the isolating mechanism(s) would be exposed to natural selection only intermittently. Consequently, their elimination from the gene pool(s) would be slow. This may be an explanation for the lack of reinforcement between the two hybridizing species of catostomids described by Nelson (1968). He believes that "... selection has not been severe enough to have prevented hybridization in any known locality." because a mismated individual has the opportunity to mate conspecifically in future years. However, female catostomids probably do not spawn more than two or three times (Geen et al., 1966). Thus the intensity of selection against hybridizing individuals would be .50 and .33, respectively, since all hybrids are eliminated as F_1 s. These intensities should be adequate (in the length of time that

hybridization has supposedly occurred) to produce reinforcement, if the genetic basis of the isolating mechanisms are not highly multifactorial. It seems clear that the intensity of selection is not the causative factor for the lack of reinforcement in the case of the hybridizing catostomids.

Moore (1957) suggests that lack of reinforcement persists between two hybridizing species because reinforced genotypes are at a selective advantage only in the hybrid zone. Allopatric populations, not possessing these genotypes, supply non-reinforced individuals to the hybrid zone via immigration, which subsequently hybridize. Because other isolating mechanisms (postmating) are also present, reproductive isolation is maintained and swamping prevented.

An alternative explanation for the lack of reinforcement in hybrid zones is the absence of genetic differences between those individuals of each species which do and those which don't hybridize. In such instances, there will be no basis on which reinforcing selection can operate. How plausible such an explanation is, may be known when the causes of hybridization are better understood.

In summary, the persistence of interspecific hybrids in the cyprinids, catostomids, and centrarchids can be interpreted as evidence for either: 1) the tight linkage between, and the large number of, genes controlling premating isolating mechanisms between species, 2) the immigration of non-reinforced genotypes into the hybrid zone (Moore's hypotheses), or 3) the lack of a genetic basis for hybridization. Since the intensity of selection is severe in many cases where fish species have hybridized, it

is probably of limited importance in the failure of reinforcement to occur.

For Mylocheilus and Richardsonius in Stave Lake, the lack of reinforcement may be attributed to any of the above explanations, save the one postulated by Moore. Non-reinforced genotypes probably cannot immigrate into the lake because of hydroelectric dams. A further reason for the absence of reinforcement between these two species in Stave Lake could be the short length of time reinforcing selection has been operating (18 years minimum). Whether this length of time is adequate again depends on the genetic control of the isolating mechanisms themselves. The intensity of selection is surely strong enough.

SUMMARY AND CONCLUSIONS

1. The peamouth chub, Mylocheilus caurinum (Richardson) and the redbreasted shiner, Richardsonius balteatus, (Richardson) are two closely related cyprinid fishes inhabiting the northwestern United States and British Columbia. While maintaining identities as species throughout most of their sympatric ranges, they engage in extensive hybridization in Stave Lake, British Columbia.
2. The purposes of this study were (1) to determine if reproductive isolation was being maintained between them and (2) if it was, what isolating mechanisms were responsible?
3. Two approaches were used to ascertain the presence or absence of reproductive isolation. The first, a morphological one, attempted to measure any shift in the means of morphological characters between populations within and outside the hybrid zone. Such a shift could be interpreted as resulting from introgression. For both characters used, no shifts in means were detected in either species, indicating that swamping was not in progress.
4. The second approach, a combined morphological-biochemical one, attempted to estimate the frequency of adult F_1 and various hybrid backcross generations. If swamping was in progress, adult hybrid backcross individuals should be more numerous than adult F_1 hybrids since there would be many more generations of them. Adult hybrid backcrosses to both Mylocheilus and Richardsonius were much less numerous than adult F_1 hybrids. Adult hybrid backcrosses to

Richardsonius were very scarce. Therefore, swamping was not suggested by this approach either.

5. In the absence of swamping, a search was made for isolating mechanism(s) effecting reproductive isolation between the species.
6. Devils Creek, a small tributary stream to Stave Lake, British Columbia, was used to investigate premating and postmating isolating mechanisms between the species.
7. The seasonal distributions of spawning Mylocheilus and Richardsonius, although not superimposed upon each other, overlap to a great extent. Spawning hybrids, mainly F₁s, were present throughout most of the spawning periods of both species, although they seemed somewhat more numerous during the early part of the spawning season.
8. Not only were both species and their hybrids not seasonally isolated, but also they usually entered Devils Creek at the same time--at dusk, or shortly thereafter. Most often they ascended Devils Creek to the first riffle above the level of the lake, regardless of the lake level. Therefore, combined temporal (seasonal and diel) and spatial factors did not appear to be effective premating isolating mechanisms.
9. Likewise, ethological isolation appeared poorly developed, if at all. Hybrid females (mainly F₁s) were numerous in the spawning populations and comprised at least an equal proportion of all spent females. Theoretically, they had deposited their eggs in an adequate manner. Evidence to this effect was the numerous hybrid backcross to Mylocheilus progeny (1) in naturally spawned eggs collected from Devils Creek and

(2) in naturally produced fingerlings collected in the littoral zone of Stave Lake. Since F_1 hybrid males have little reproductive potential (to be summarized subsequently), these hybrid backcross to Mylocheilus progeny probably arose from hybrid rather than Mylocheilus females.

10. Experiments were conducted under laboratory conditions to test egg survival. In general, egg survival was good for all possible combinations of Mylocheilus, Richardsonius, and presumed F_1 hybrids except for crosses involving the presumed F_1 hybrid male. Crosses involving this male yielded poor survival, partly because of hybrid sterility (small quantities of abnormal milt, variably sized sperm) but partly because of inviability factors. Crosses involving hybrid females and the parental species' males gave good survival. Although presumed F_1 hybrid males are at least partially sterile, swamping of the species would not be prevented, only slowed down, because the hybrid females would provide an alternate route.
11. The viability of fry to the fingerling stage was also tested under experimental conditions and gave the only clue for the absence of swamping. The survival of reciprocal F_1 fry was as good as for Mylocheilus. However, the hybrid backcross to Mylocheilus fry gave approximately 20% poorer survival than parental Mylocheilus fry. Additionally, the remaining fingerlings at the termination of the experiment possessed 11.5% abnormalities. While 20% less survival in itself would not prevent swamping, it should be

considered that such a differential occurred over a period of only 48 days. This study is one of the few in which the inferred inferiority of natural hybrids, other than sterility, has been demonstrated, even if done so under experimental conditions.

12. Introgression, swamping, and isolating mechanisms are discussed.

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