ADAPTIVE LACTATE DEHYDROGENASE VARIATION IN THE CRESTED BLENNY, *ANOPLARCHUS**

M. S. JOHNSON†
College of Fisheries, University of Washington, Seattle, Washington

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1. Introduction

Gel electrophoresis of proteins has demonstrated much heterozygosity in populations (*e.g.* Harris, 1966; Lewontin and Hubby, 1966; Selander *et al.*, 1969). For population biologists, this finding is fortunate, for a readily available source of genetic variation can be called upon to test hypotheses. The choice of organisms for study can be made according to ecological suitability and theoretical relevance. This same high degree of genetic variation demands explanation, especially in view of theoretical problems of maintaining much heterozygosity (Kimura and Crow, 1964). Selective neutrality of much protein variation within and between species has been proposed (Kimura, 1968*a*, *b*; Crow, 1968; King and Jukes, 1969; Shaw, 1970), and would eliminate problems of genetic load. As Clarke (1970) points out, however, crucial tests of neutrality *v.* adaptiveness should come from population studies. While indications of adaptive roles for protein polymorphs are accumulating, very little of the environmental relationships has been revealed. Thus, widespread heterozygosity for proteins provides both an excellent opportunity and a demand for the study of genetic and biochemical variation in terms of the environment.

The crested blenny, *Anoplarchus*, offers excellent opportunities for such study. This fish is abundant and is easily captured in rocky intertidal areas of the western United States and Canada. Peden (1966) used a number of morphological criteria to distinguish two species of *Anoplarchus* which differ in their geographical and vertical distributions. *Anoplarchus insignis* tends to be more northern and in deeper water than *A. purpurescens*, the abundant form in Puget Sound. This dichotomy suggests selective forces which should be reflected in the genetic composition of the two species. The present study was initiated to consider enzyme variation in *Anoplarchus* against this background.

In particular, a lactate dehydrogenase (LDH) [EC 1.1.1.27] polymorphism in *A. purpurescens* was chosen for detailed study. LDH isozymes have been well studied, and their molecular and genetic bases are well understood (see Markert, 1968). Vertebrate LDH is tetrameric and usually consists of various combinations of two major subunit types. Coding of these subunits at separate autosomal loci has been demonstrated in man (see Vessell, 1965), deer mice (Shaw and Barto, 1963) and in trout (Morrison and Wright, 1966). A number of polymorphisms at these loci in fish have

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† Trainee, Federal Water Quality Administration. Current Address: Department of Biology, Yale University, New Haven, Connecticut 06520.

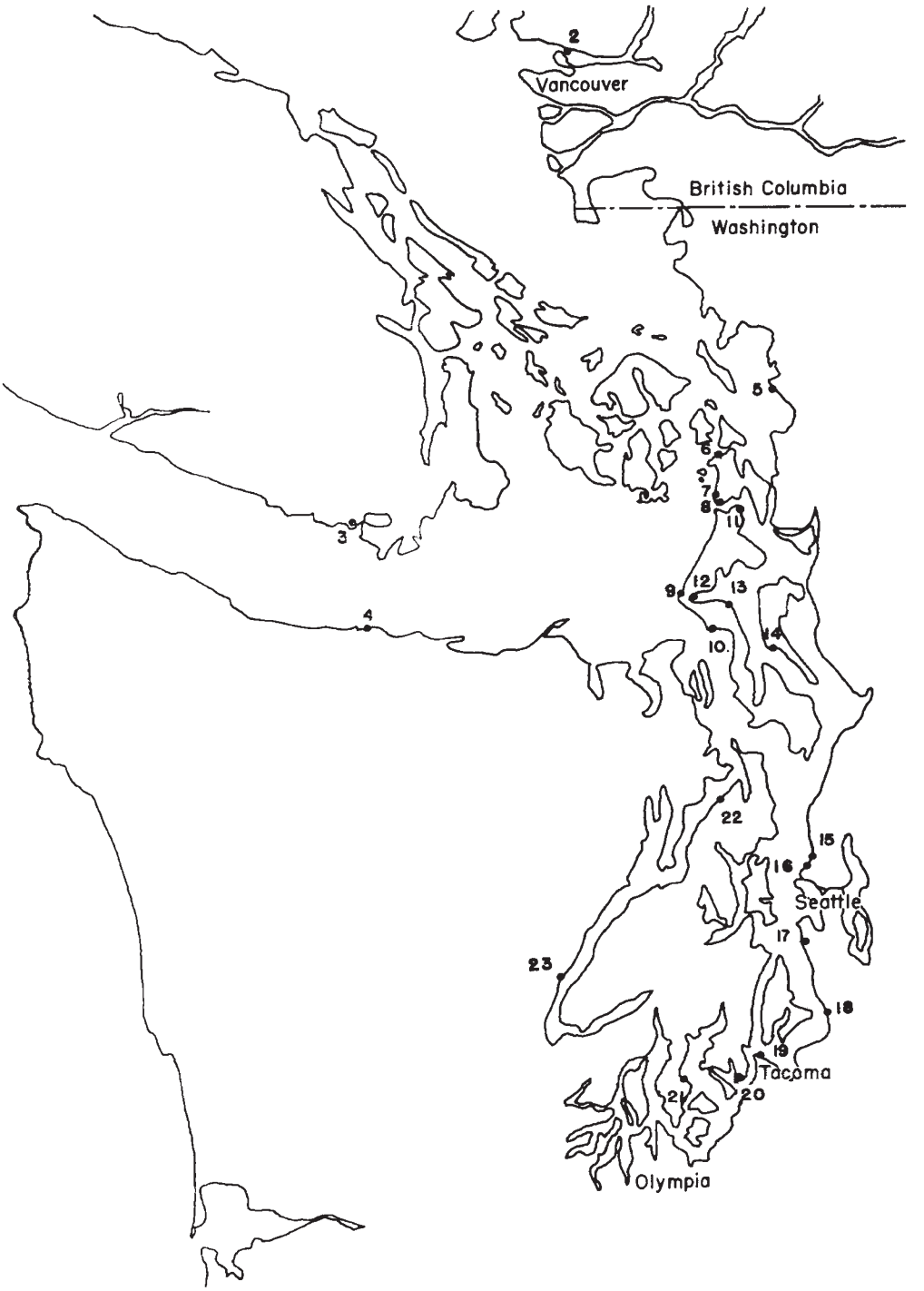


FIG. 1.—Sampling locations. Location numbers refer to table 1.

been described (Markert and Faulhaber, 1965; Hochachka, 1966; Odense *et al.*, 1966, 1969; Lush and Cowey, 1968; Clayton and Gee, 1969; Hodgins *et al.*, 1969; Utter, 1969). While these reports confirm the tetrameric and multiple subunit nature of fish LDH, they reveal nothing of the environmental relationships of the polymorphisms. In the present study, an attempt is made to relate the LDH polymorphism in *A. purpurescens* to the environment and to differences with *A. insignis*.

2. Materials and methods

(a) *Samples*

Population samples were taken between May 1969 and July 1970 at 23 localities in Puget Sound and the Strait of Georgia. Sampling sites are shown in fig. 1, with the exception of Quadra Island, British Columbia, which is approximately 100 miles north-west of Vancouver in the Strait of Georgia. All specimens were collected by hand beneath rocks at low tide. Samples were taken from as restricted an area as possible, usually less than 100 feet in diameter, and any replicate samples were taken from the same spot. The two species of *Anoplarchus* were identified according to Peden (1966).

(b) *Treatment of samples*

Samples were either frozen whole (at −20° C.) or held alive prior to use. Skeletal muscle was generally used for scoring, although other tissues were sometimes used for comparison. Protein extracts were obtained by incubating tissue samples in approximately two volumes of 2 per cent. phenoxyethanol (see Nakanishi *et al.*, 1969) for at least one hour. Samples kept in this solution in the refrigerator for three months continued to give readable LDH patterns.

(c) *Electrophoresis*

Horizontal starch gel electrophoresis was used to separate isozymes. The discontinuous lithium hydroxide–boric acid and Tris-citric acid buffer system described by Hodgins *et al.* (1969) was generally used. Gels were 14 × 22 × 0·6 cm. slabs, consisting of 14 per cent. starch (Connaught Medical Research Laboratories). Samples were applied on rectangles of Whatman No. 3 filter paper, approximately 20 samples per gel. The inserts were removed 15 minutes into the run. Electrophoresis was at 400 volts for approximately two hours. A tray of ice on top of the gel prevented overheating.

(d) *Staining*

LDH activity was demonstrated by painting the sliced surface of the gel with a solution of the following:

5·0 ml. 0·1 M lithium lactate in 0·1 M Tris (*p*H 8·3)
2·5 mg. diphosphopyridine nucleotide
0·5 mg. phenazine methosulfate
1·0 mg. p-nitrobluetetrazolium chloride.

After approximately 30 minutes in the dark at room temperature, the LDH isozymes were visible as dark blue bands on the whitish gel.

3. Results

(a) *Lactate dehydrogenase isozymes*

The LDH isozymes of *A. purpurescens* are shown in fig. 2, in which the presumed subunit composition of each band is shown. The patterns are similar to those of many teleosts (Markert and Faulhaber, 1965), in which combination of *A* and *B* subunits is nonrandom, producing three bands in tissues in which both subunits are present. The *A* subunit is the only type present in skeletal muscle, where its activity is the strongest. As in some other fishes (Markert and Faulhaber, 1965; Nakano and Whitely, 1965; Morrison and Wright, 1966), a third subunit, *C*, is restricted to eye tissue, and may form a hybrid molecule with the *B* subunit.

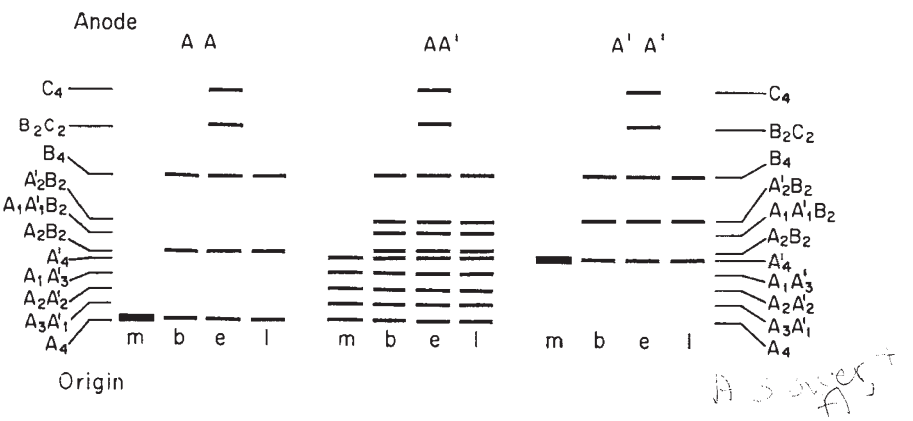


FIG. 2.—Lactate dehydrogenase isozymes of *Anoplarchus purpurescens*, showing variations in the *A* subunit in different extracts. *m* = skeletal muscle; *b* = brain; *e* = eye; *l* = whole larva.

Superimposed on this general isozyme pattern is variation in the *A* subunit. This is most simply demonstrated in muscle extracts, where single slow (*A*), single fast (*A'*), or series of five bands (*AA'*) are found. Whenever *A* and *A'* subunits occur together, there are five bands, which supports the assumption of a tetrameric structure of *Anoplarchus* LDH, and suggests a heterozygote, in which *A* and *A'* subunits combine randomly. That the combination is random is shown by the relative intensities of the bands. Within the heterozygtoes, the presumed $A_2A'_2$ band is darkest, while the homotetramers are faintest. The single bands of homozygotes are much more intense than the bands of heterozygotes.

Tissues in which the *B* subunit is present provide further clarification of the *A* subunit variation. The mobility of the A_2B_2 hybrid molecule reflects the different mobilities of *A* and *A'*. The heterozygote shows nine bands: A_4, $A_3A'_1$, $A_2A'_2$, $A_1A'_3$, A'_4, A_2B_2, $A_1A'_1B_2$, A'_2B_2, B_4. In all cases where more than one tissue from an individual were examined, the phenotype held true.

All three phenotypes were found in newly hatched larvae and in adults, regardless of sex or reproductive condition. Holding fish at temperatures from 1° to 24° C. for up to two months did not appear to influence scoring.

Freezing up to eight months and storage in phenoxyethanol did not influence mobilities.

To summarise, the LDH isozyme patterns of *A. purpurescens* indicate three subunit types with variation in the *A*, or muscle, subunit. Molecular evidence for an allelic interpretation for this variation is:

1. Only the expected numbers of bands are found.
2. The relative staining intensities are as expected.
3. Phenotypes hold true in all tissues scored for an individual.

The LDH isozymes of *A. insignis* are identical to those of *A. purpurescens*, except that *A. insignis* does not show the *A'* allele (149 individuals examined). Although identical mobility does not necessitate identity of primary structure, such identity is likely in very closely related species. Thus, it appears that the two species of *Anoplarchus* share an allele at the LDH-*A* locus, with *A. purpurescens* having an alternative allele.

(b) *Clutch analysis*

Anoplarchus spawns intertidally, the female guarding a clutch of 1000-3000 eggs. Thus, clutches for which female parents were known were readily available to verify segregation of *A* and *A'*. Each clutch was collected with its parent and held in a quart jar of sea water. Incubation temperatures were variable (8°-13° C.), but not unlike those experienced by eggs in the wild. Eggs were fanned by the females, and approximately three-fourths of the clutches hatched, varying in success from less than 10 per cent. to over 90 per cent. After hatching, a sample of larvae and the female were scored for LDH phenotype.

Although progeny lines are required for rigorous proof, the allelic nature of *A* and *A'* is supported by the production of 50 per cent. heterozygous progeny by heterozygous females (13 clutches, 624 larvae), and by the lack of *A'A'* offspring from *AA* females (30 clutches, 598 larvae) and *AA* offspring from an *A'A'* female (1 clutch, 62 larvae). However, the segregating clutches from homozygous females and the fully segregating clutches from heterozygous females deviate from the expected 1 : 1 and 1 : 2 : 1 ratios, with a general deficit of the *A'* allele. Since all clutches from heterozygous females include 50 per cent. heterozygotes, the apparent under-representation of *A'* in many clutches is probably not due to selection prior to scoring. A likely explanation of the skewed ratios is multiple matings. Since these clutches are from populations in which the frequency of *A'* is less than 30 per cent., the apparent under-representation of *A'* is as expected from multiple matings. Not enough is known of the breeding structure of *Anoplarchus* to verify this interpretation.

(c) *Geographical survey*

(i) *Species distribution.* *Anoplarchus purpurescens* is very abundant throughout the sampling region. *A. insignis*, however, is not common in this area, at least in the intertidal zone. The only location in Puget Sound where this northern species is abundant is the Tacoma Narrows, represented in the collections by Point Defiance and Point Fosdick. This area is characterised by rapid currents and much vertical mixing, and it is the coolest and least variable site in Puget Sound proper. Similar areas at Admiralty Head and

TABLE 1

Sample data for Anoplarchus purpurescens. *Location numbers refer to fig. 1*

Location	Date	N	Frequency of *A'*
Strait of Georgia, B.C.			
1. Francisco Point, Quadra Island	31.v.69	48	0·021
2. Stanley Park, Vancouver	30.v.69	106	0·038
Strait of Juan de Fuca			
3. Sooke, B.C.	1.vi.69	11	0·091
4. Tongue Point, Washington	16.vi.69	19	0·158
5. Larrabee State Park	19.vii.70	150	0·053
6. Washington Park, Anacortes	18.vii.70	114	0·083
7. Rosario Beach	1.vii.69	49	0·138
8. Bowman Bay, Desception Pass	29.vii.69	73	0·054
9. Point Partridge	23.v.70	67	0·082
10. Admiralty Head	28.vi.69	38	0·026
	23.v.70	28	0·072
Saratoga Passage, Puget Sound			
11. Hypus Point, Deception Pass	28.vi.69	46	0·054
	29.vii.69	87	0·121
12. San de Fuca, Penn Cove	15.vii.69	98	0·153
	5.ii.70	256	0·138
13. Snatellum Point	1.vii.69	51	0·147
14. Camano Island State Park	2.vii.69	49	0·174
Main Basin, Puget Sound			
15. Carkeek Beach, Seattle	4.v.69	298	0·154
	26.vii.69	99	0·162
	2.ii.70	72	0·236
	5.vi.70	148	0·189
16. Golden Gardens, Seattle	2.v.69	82	0·128
	7.iv.70	98	0·184
17. Lincoln Park Beach, Seattle	4.vi.69	18	0·167
18. Saltwater State Park	30.vii.69	29	0·155
19. Point Defiance Park, Tacoma	3.vi.69	24	0·083
	30.vi.69	26	0·135
	26.vii.69	131	0·195
	3.ii.70	58	0·172
	24.v.70	63	0·166
20. Point Fosdick	2.vi.69	27	0·166
21. Penrose Point State Park,	5.v.69	96	0·271
Carr Inlet	28.vii.69	41	0·305
	4.ii.70	123	0·228
	8.iv.70	153	0·271
	22.v.70	50	0·240
Hood Canal			
22. Kitsap Memorial State Park	29.vi.69	47	0·138
	10.iv.70	112	0·121
23. Lilliwaup	3.v.69	252	0·109
	4.iii.70	224	0·098

Deception Pass (one *A. insignis* found) did not produce the northern species, however. While collections for *A. insignis* are limited, they do provide a basis for comparison of the species at the Tacoma Narrows.

(ii) *Distribution of allelic frequencies.* As a first indication of environmental correlates, a geographical survey of the distribution of phenotypes was conducted. Samples are presented in table 1. None of the samples deviate significantly from genotype frequencies expected from Hardy-Weinberg equilibrium, which provides further support for the allelic interpretation of *A* and *A'*.

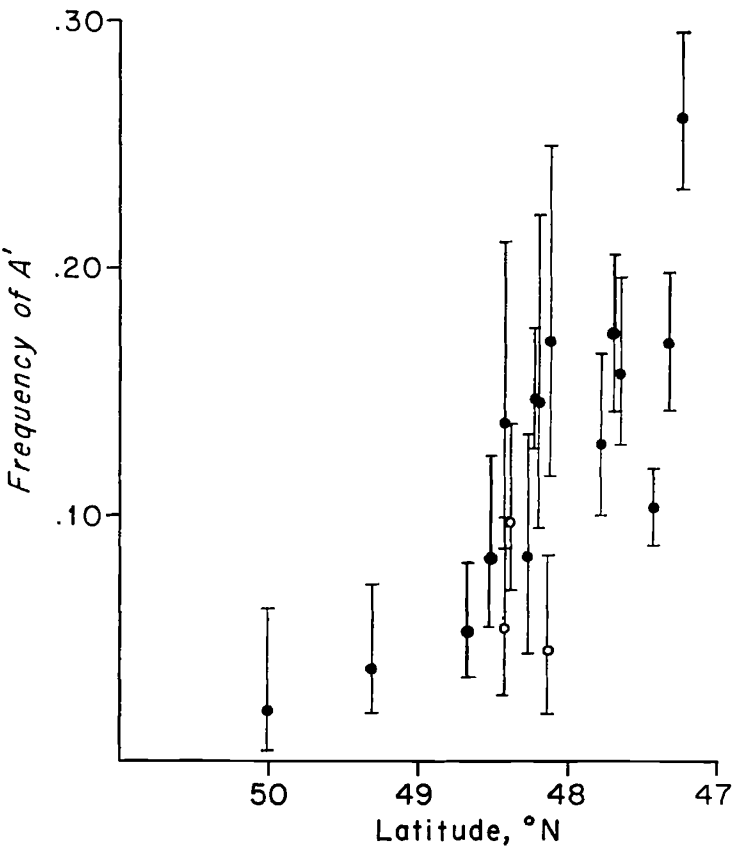


FIG. 3.—Latitudinal variation of *A'* frequency. Vertical lines are approximate 90 per cent confidence intervals. Open circles signify samples from entrances to Puget Sound.

The most important indication from the geographical data is an increase in the frequency of *A'* from north to south, shown in fig. 3. The frequency values for this plot are totals of all collections from a given site, and include sites with total samples greater than 40. Although there is heterogeneity within and between samples from a few locations, this does not affect the broad pattern, and will be dealt with later. From a frequency of less than 5 per cent. in the Strait of Georgia, *A'* rises to 13-17 per cent. in most of Puget Sound, and to 26 per cent. at Penrose Point in southern Puget Sound.

Figure 3 includes approximate 90 per cent. confidence intervals for the samples, indicating the significance of the general trend. The entrances to Puget Sound at Deception Pass and Admiralty Head have relatively low frequencies of *A'*, indicating a break in the cline, with a step up in *A'* frequency into Puget Sound.

Deception Pass and Admiralty Head are characterised by steep banks, rapid currents and much vertical mixing. This is in marked contrast to the quiet waters and gentle slopes of sites within Puget Sound. Only at Tacoma Narrows are similar conditions found within the Sound. While the frequency of *A'* here is not particularly low, this area does show peculiarities which will be considered later. The only other area with a relatively steep bank (but with very quiet waters) is Lilliwaup, on Hood Canal, where *A'* is at low frequency compared with other areas in the Sound.

To summarise, *A'* is at frequencies of less than 10 per cent. to the north and at the entrances to Puget Sound. Within most of Puget Sound, frequencies are 13-17 per cent., with a high of 26 per cent. at Penrose Point in southern Puget Sound.

(d) *Allelic frequency and depth*

Few areas in Puget Sound are suitable for a consideration of possible depth relationships of *A* and *A'*, primarily because most of the beaches have only a narrow band of suitable rocky habitat. However, Penrose Point has a long spit with intermittent rocky areas suitable for *Anoplarchus*. The spit forms a depth gradient and three sites were chosen along this gradient. Each site was less than 8 metres in diameter. Site H is the highest *Anoplarchus* habitat sampled, and is separated from the intermediate site I by 75 metres of beach free of *Anoplarchus*. Site J is 50 metres from I and is exposed only on the lowest tides. This site was collected only once, due to relative unexposure. A fourth site, K, is approximately 400 metres from H, towards the base of the spit. This area is not as well defined as the others, but is approximately intermediate in exposure to I and J. Site K forms a scattered, but continuous, rocky area down to the subtidal.

The Penrose Point samples are broken down according to individual sites in table 2. In May 1969, LDH-A' was 57 per cent. in the small sample from site H, significantly higher than at the other sites. Site I had an intermediate frequency, indicating an increase in LDH-*A'* frequency with increased exposure. An identical trend was found in April 1970, although the differences between sites are not in themselves significant. The February collection of sites H and K shows no correlation of *A'* with depth, site H showing a markedly lower *A'* frequency than in the springtime samples ($\chi^2_{(2)} = 9{\cdot}08$, $P < 0{\cdot}02$). Thus, while there appears to be a correlation of LDH-*A'* frequency with depth, the relationship is seasonal. Neither the depth nor seasonal comparisons indicate genotypic differences other than those due to gene frequency differences.

The seasonal variation in *A'* distribution is accompanied by variation in *Anoplarchus* abundance. *Anoplarchus* is most abundant at all Penrose Point sites in the winter. In the spring, there is a general reduction of numbers in the intertidal, but especially so at the more exposed sites. The site H samples (table 2) reflect this most clearly, because each collection included all available fish at this site. During the summer, no *Anoplarchus* are found at sites H and I, and numbers are reduced at K. Comparable observations

at noncollected sites indicate that this pattern is not solely due to collection pressure. Also, numbers at site H remained high after the February collection, dropping in March. Reappearance of large numbers of adults in the winter indicates that mortality is not the sole cause of low numbers in the summer. There appears to be a migrational component to the seasonal pattern. The resultant seasonal heterogeneity at the most exposed site suggests habitat preference differences associated with A and A'.

The 1969 samples at Quadra Island, San de Fuca, Carkeek and Lilliwaup were also divided as to upper and lower areas, although available habitat is more continuous at these sites. None showed significant heterogeneity, but each had a slightly higher frequency of A' in the upper area, consistent with the findings at Penrose Point.

TABLE 2

A′ frequency at Penrose Point sites. Sample sizes shown in parentheses

Date	Site H	Site I	Site J	Site K
5.v.69	0·571 (7)	0·303 (33)	0·235 (17)	0·237 (38)
28.vii.69	—	—	—	0·305 (41)
4.ii.70	0·232 (58)	—	—	0·223 (65)
8.iv.70	0·389 (9)	0·276 (74)	—	0·230 (76)
22.v.70	—	—	—	0·240 (50)

(e) *Allelic frequency over time*

During the spring and summer of 1969, more than one sample were taken from Hypus Point (Deception Pass), Carkeek, Point Defiance and Penrose Point, site K. The data are recorded in Table 1, and in each case there was at least a slight rise in A' frequency in the later collections, though the trend is not significant. That a change did occur at Point Defiance, at least, is indicated by the intermediate value of the second of three collections, along with the nearly significant difference between the first and third samples ($\chi^2_{(1)} = 3{\cdot}46$, $P < 0{\cdot}10$). The similar area at Hypus Point also shows a highly suggestive rise in A' frequency ($P < 0{\cdot}10$). No discernible genotype disequilibrium accompanied these changes. The apparent rise in A' frequency at Point Defiance and Hypus Point indicates a stronger correlation of relatively low frequencies of A' and areas of much vertical mixing in the spring of 1969 than at later dates.

Winter samples were taken at San de Fuca, Carkeek, Point Defiance and Penrose Point. With the exception of changes associated with depth mentioned in the previous section, no drop in A' frequency was found. At Carkeek, there was a sharp increase in A' ($\chi^2_{(1)} = 5{\cdot}63$, $P < 0{\cdot}02$).

Differences between 1969 and 1970 samples at a number of sites were sought. No changes were found, with the exception of the Seattle sites, which show an increase in A' ($\chi^2_{(2)} = 6{\cdot}99$, $P < 0{\cdot}05$). There are no obvious seasonal patterns in allelic frequencies applicable to all locations, but samples from some areas show particular types of heterogeneity. The rise in A' frequency at Seattle from 1969 to 1970, and the apparent rise during 1969 at Point Defiance and Hypus Point, suggest peculiarities associated with particular years, rather than general seasonal patterns.

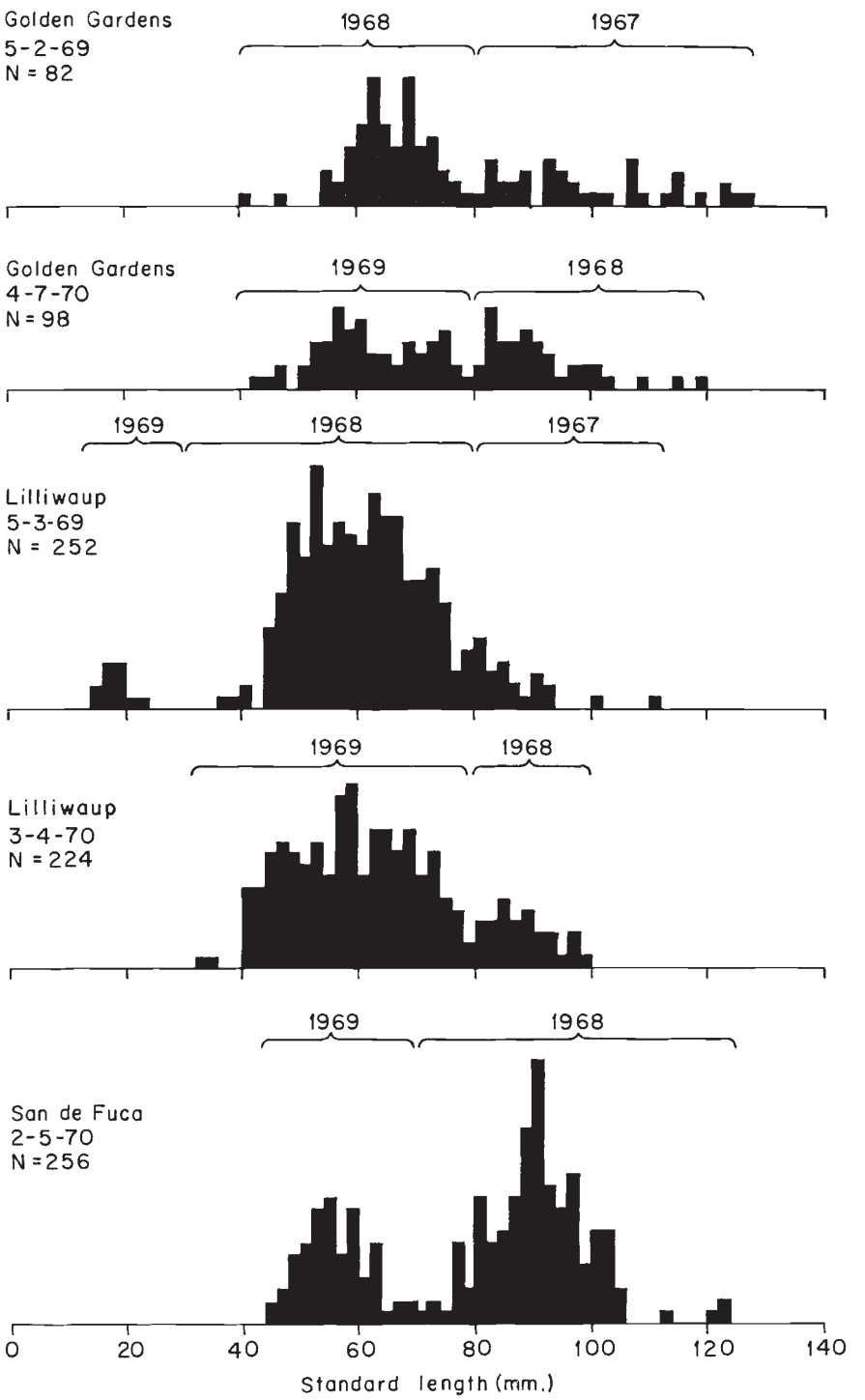
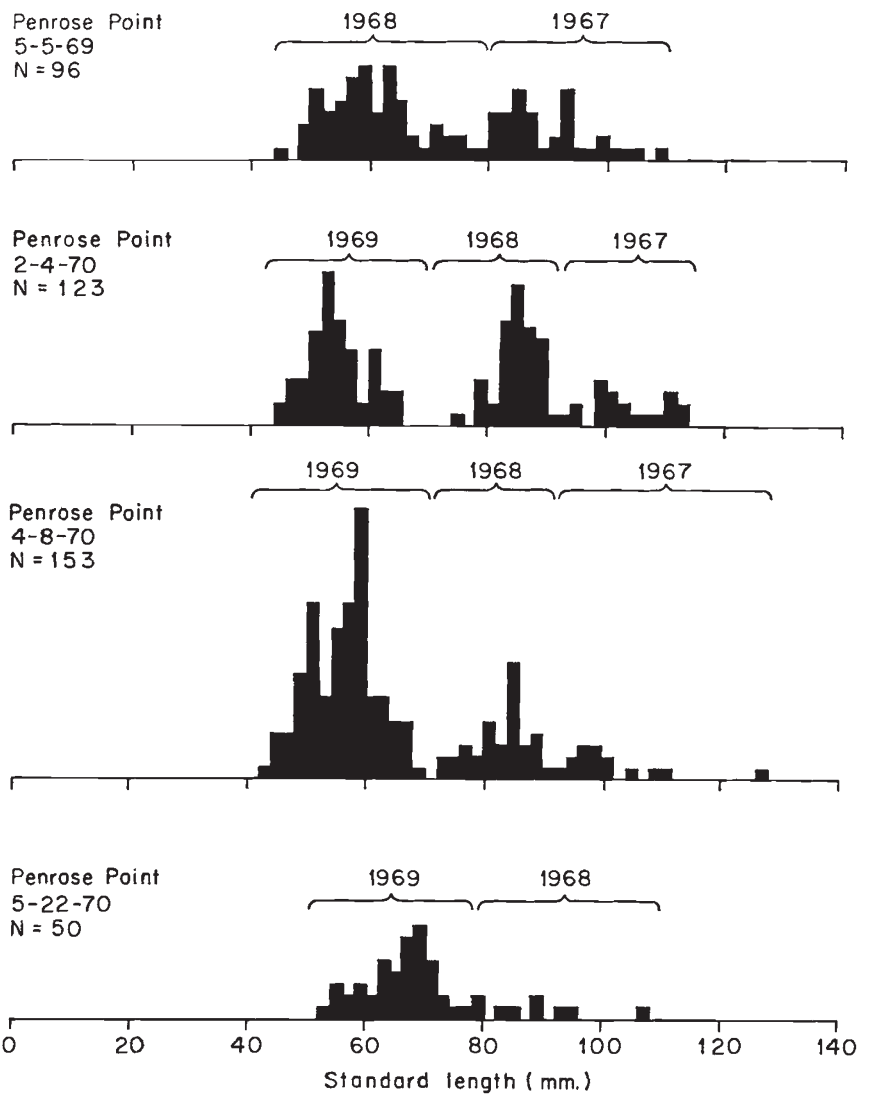


FIG. 4.—Size structure of samples used for age-group comparisons. Years above histograms refer to presumed year classes.

FIG. 4.—(*Continued*).



(f) *Sex and age comparisons*

(i) *Sex.* No sites showed heterogeneity between the sexes for either allelic frequency or genotype frequency, indicating that the LDH-*A* locus is autosomal.

(ii) *Age.* From most areas, winter and spring samples allow separation of age-groups through size distributions, while summer samples are unimodal. *Anoplarchus* breeds in February and March, and a few young of the year (approx. 20 mm. long) may be found intertidally early in the summer. However, only fish one year and older are abundant in the samples. The size distributions for samples allowing age class determination are shown in fig. 4, where the arbitrary division points and year classes are indicated.

Although there is overlap in some samples, the similarity of 1969 and 1970 size structures from each area increases confidence in the cutoff points. There appear to be three modes in the February and April 1970 samples from Penrose Point. Although overlap may be great, separation of two- and three-year old fish was considered preferable to lumping. For other collections, those fish two or more years old are designated two-year-olds, this age-group presumably predominating.

The data for each age-group are presented in table 3. The most striking

TABLE 3

Age-group comparisons for A′ frequency. Sample sizes shown in parentheses

Location	Year class	Year of collection: 1969	$\chi^2_{(1)}$	1970	$\chi^2_{(1)}$
Golden Gardens	1967	0·181 (22)	2·05	—	
	1968	0·096 (51)		0·141 (39)	1·54
	1969	—		0·212 (59)	
Penrose Point	1967	0·408 (38)	5·08*	0·250 (48)	
	1968	0·250 (54)		0·208 (130)	1·51
	1969	—		0·249 (211)	
Lilliwaup	1967	0·204 (22)	3·98*	—	
	1968	0·098 (223)		0·056 (36)	1·78
	1969	0·042 (12)		0·106 (188)	
San de Fuca	1968	—		0·141 (184)	0·04
	1969	—		0·132 (72)	
Combined		$\chi^2_{(3)}$ =	11·11*	$\chi^2_{(4)}$ =	4·87

* Significant at 0·05 level.

finding is from 1969, when the frequency of A' in the 1967 year class is nearly double that of the 1968 year class at all three locations ($\chi^2_{(3)} = 11{\cdot}11$, $P < 0{\cdot}02$). In 1970 there were no significant differences between age-groups, suggesting peculiarities between years rather than a general change in A' frequency with age. From Penrose Point and Lilliwaup, where the data are strongest, it is clear that the 1967 year class is the peculiar one, with a higher frequency of A' than the succeeding two year classes. This is not borne out at Golden Gardens. Since Seattle showed the only rise in A' frequency from 1969 to 1970, special factors may be involved here.

Although the recognition of the 1967 year class in 1970 at Penrose Point is especially suspect, the frequency of A' in that year class appears to have dropped to the level of the other age-groups ($\chi^2_{(1)} = 4{\cdot}86$, $P < 0{\cdot}05$). The 1968 year class did not change in allelic frequency over this period. The uniqueness of the high A' frequency in the 1967 year class in 1969 is further emphasised by the lower frequency in the 1969 year class. Since *Anoplarchus* begins breeding at age two (personal observations, based on size distributions), most of those born in 1969 were derived from 1967 year class parents, and the two groups show markedly different allelic frequencies.

(g) *Species comparison*

The general increase in *A'* from north to south and from less exposed to more exposed areas within the intertidal, parallels on a smaller scale the north-south and vertical distributions of the two species of *Anoplarchus* as *A. insignis* is the more northerly and deeper species. This suggests that the *A'* allele represents a significant difference between the species. Direct consideration of this possibility can be made at the Tacoma Narrows, the only area in Puget Sound where *A. insignis* was found to be abundant.

As indicated previously, there was a rise in *A'* frequency in Point Defiance samples from early June to late July 1969 (shown in fig. 5, *a*). For the same

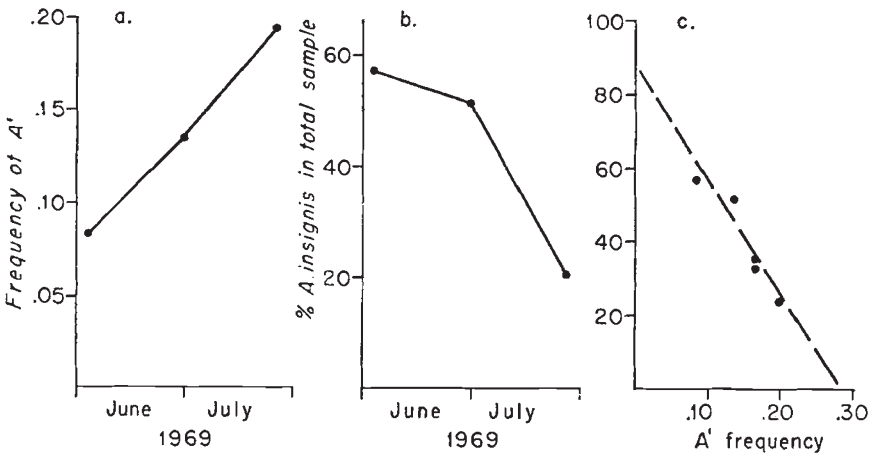


FIG. 5.—Species comparison at the Tacoma Narrows. (*a*) Change in *A'* frequency at Point Defiance in 1969. (*b*) Concurrent change in percent. *A. insignis* in total samples. (*c*) Regression of *A'* frequency against percent. *A. insignis* for all Tacoma Narrows samples containing both species.

dates, there was a decrease in the proportion of *A. insignis* in the total *Anoplarchus* samples (fig. 5, *b*). The collection at Point Defiance in February 1970 revealed no *A. insignis*. This may be due a higher tide, as *A, insignis* has been found only at the lowest exposed area of beach. The May 1970 sample included *A. insignis* in a proportion confirming the apparent negative correlation between *A. insignis* relative abundance and *A'* frequency. In fig. 5, *c* the frequency of *A'* is plotted against the proportion of *A. insignis* for all Tacoma Narrows samples (including Point Fosdick) that include both species. A very strong negative correlation is revealed ($r = -0{\cdot}958$, $P < 0{\cdot}01$). Thus, the sampling populations of the two species are more similar (lower frequency of *A'* in *A. purpurescens*) when the environment is more suitable for both (as judged by relative species abundance).

(h) *Environmental correlates*

The patterns associated with geography, intertidal depth, age-groups and species differences all demonstrate non-randomness of LDH-*A* allelic frequencies. An attempt has been made to determine possible environmental correlates with these patterns.

The rise in A' frequency from north to south immediately suggests temperature as a possible correlate, especially since intertidal areas are strongly influenced by atmospheric conditions. In Puget Sound, the discrepancy between air and water temperatures is generally greatest in the summer, and A' frequency broadly correlates with warmer summer conditions. Areas with steep slopes and much mixing should show less warming in the summer. Thus, the breaks in the cline at Deception Pass and Admiralty Head strengthen the correlation of A' with temperature.

A more detailed consideration is possible within Puget Sound, where oceanographic data are available. A comprehensive oceanographic survey in 1954 provides data for all areas for all seasons of that year (Barnes and Colias, 1956). Data from this single year have been used for comparison with allelic frequencies, since long-term averages are not available, and surveys in other years did not include all pertinent stations. A further limitation of the data is that values are for midchannel and may not adequately describe shore conditions.

In February 1954, the surface temperature-range between sites was less than 2° C. During August, however, the spread was greater than 7° C. A plot of A' frequency against August surface temperature reveals a positive correlation ($r = 0{\cdot}686$, $P < 0{\cdot}02$), supporting the broad correlation described above. Whereas the low frequencies of A' at the entrances to Puget Sound are well accounted for by this correlation, the low frequency at Lilliwaup (Hood Canal) seems exceptional.

Within Puget Sound there is a strong positive correlation between temperature and summer oxygen concentration, with the exception of Hood Canal which has high summer temperatures and low oxygen concentrations. Thus, a plot of A' frequency against summer oxygen concentrations yields a slightly stronger correlation than for temperature ($r = 0{\cdot}711$, $P < 0{\cdot}01$), accounting for the low frequency at Lilliwaup.

However, special features at Lilliwaup confound this apparent separation of the two correlates. Air temperatures during the winter and early spring are lower around Hood Canal than other areas of Puget Sound. The effects of this on inshore areas may not be well represented by the midchannel data. Also, *Anoplarchus* breeds a full month earlier in Hood Canal than at Penrose Point and Seattle. The eggs and larvae at Lilliwaup experience conditions similar to those of more northern areas, cooler than in most of Puget Sound. The correlations of A' frequency cannot be confidently separated.

The influence of air temperature on the intertidal is increased by tidal patterns within Puget Sound. In winter, when air temperatures are comparable to, or lower than, water temperature, the lowest tides are at night. In mid-March low tides switch to midday, and air temperatures are generally higher than water temperature. This combination, along with an increasing tidal range from March through June, enhances the warming effect of spring, and presumably results in a greater variance in temperature between high and low intertidal areas.

At Penrose Point, this seasonal pattern parallels patterns of *Anoplarchus* abundance and A' frequency at different heights within the intertidal. The winter to spring decrease in numbers and increase in A' frequency at the most exposed site, along with the increased variance of numbers and A' frequency between sites, correlates with an accentuated warming trend. Thus, the depth relationships provide a correlation of A' frequency and

temperature, paralleling that found from geographical comparisons. At low tide *Anoplarchus* are under rocks on damp substrate, rather than in pools, so oxygen availability should be higher at the more exposed sites. Since this should be so regardless of season, temperature appears to be the better correlate with the changes in *A'* frequency.

Special weather conditions in years affecting the *Anoplarchus* samples show additional correlation of *A'* frequency with temperature. The summer of 1967 was exceptionally hot in western Washington. The average high temperature at Seattle in August 1967 was more than 5° C. above normal: " In addition to being one of the warmest Augusts on record, this has been one of the warmest and driest summers . . ." (U.S. Environmental Services Administration, 1967). The combination of high temperature and little rain (less clouds, greater insolation) would have marked influence on inshore temperatures. Correlated with this exceptional summer is the high frequency of *A'* in the 1967 year class at Penrose Point and Lilliwaup. The summers following have been normal, and the frequency of *A'* in 1968 and 1969 year classes significantly lower.

The winter of 1968-69 was also abnormal, described as " the most severe winter in many respects since 1949-50 and one of the colder on record . . ." (U.S. Environmental Services Administration, 1969). That these extremely low temperatures are not the cause of the age-group differences is indicated by the common allelic frequencies of 1968 and 1969 year classes. However, the cold conditions early in 1969 may correlate with the apparently low frequencies of *A'* in earlier collections at Point Defiance and Hypus Point. The special nature of these sites is consistent with a lagged response to spring warming in areas of much vertical mixing. The after-the-fact nature of this correlation allows little confidence, but it is suggestively parallel to other results.

(i) *Temperature experiments*

In view of the very strong and consistent correlation of allelic frequencies with temperature, experiments were carried out to see if genotypes survive differentially according to temperature. Larvae of laboratory-hatched clutches from Penrose Point were used, as these are conveniently small and provide more suitable genotype frequencies than population samples. After hatching, larvae were held at 9·5° C. one to two days. At that time, approximately 50 active larvae from a single clutch were pipetted into each of two one-gallon jars of sea water. The jars were placed in controlled-temperature water baths, one at $16{\cdot}0 \pm 0{\cdot}2°$ C., the other at $4{\cdot}0 \pm 0{\cdot}5°$ C. Jars were aerated, and no food was provided. Dead individuals were collected at approximately 12-hour intervals, until at least half were dead. This took 2-3 days at 16° and 4-14 days at 4°. Individual larvae were scored, and the first half (as close as possible) to die in each jar was compared with the remaining half for genotype frequencies.

A' homozygotes were present in three clutches. Data on these clutches are arranged in table 4 to allow comparison of *A'A'* with each of the other genotypes. Small numbers necessitated use of Fisher's Exact Probability for these single-clutch comparisons. Combined probabilities were derived according to Fisher (1954). The clearest results are those for the *A'A'-AA* comparisons. At 4° C. all three clutches showed a relative advantage for

TABLE 4

Relative survival of larval genotypes at 4° and 16° C.

(*a*) Comparison of *AA* and *A'A'*

Clutch	Temp.	1st Half *AA*	1st Half *A'A'*	1st Half %*A'A'*	2nd Half *AA*	2nd Half *A'A'*	2nd Half %*A'A'*	P	Combined $\chi^2_{(4)}$
A	4	10	6	0·375	12	1	0·077	0·067†	12·86*
	16	12	0	0·000	8	5	0·394	0·024*	
B	4	6	3	0·333	12	1	0·077	0·149	6·07
	16	9	3	0·250	11	2	0·154	0·323	
C	4	10	7	0·412	9	2	0·182	0·155	5·48
	16	6	1	0·141	9	3	0·250	0·398	
								Combined $\chi^2_{(12)}$	= 24·31*
Total	4	26	16	0·381	33	4	0·108	$\chi^2_{(1)}$	= 7·83**
	16	27	4	0·129	28	10	0·263	$\chi^2_{(1)}$	= 1·92
								$\chi^2_{(2)}$	= 9·75**

(*b*) Comparison of *AA'* and *A'A'*

Clutch	Temp.	1st Half *AA'*	1st Half *A'A'*	1st Half %*A'A'*	2nd Half *AA'*	2nd Half *A'A'*	2nd Half %*A'A'*	P	Combined $\chi^2_{(4)}$
A	4	8	6	0·429	8	1	0·111	0·111	11·40*
	16	12	0	0·000	9	5	0·357	0·030*	
B	4	13	3	0·187	9	1	0·100	0·375	4·32
	16	11	3	0·214	14	2	0·125	0·308	
C	4	13	7	0·350	13	2	0·133	0·115	7·20
	16	12	1	0·077	10	3	0·231	0·249	
								Combined $\chi^2_{(12)}$	= 22·92*
Total	4	34	16	0·320	30	4	0·118	$\chi^2_{(1)}$	= 4·57*
	16	35	4	0·102	33	10	0·232	$\chi^2_{(1)}$	= 2·14
								$\chi^2_{(2)}$	= 6·71*

(*c*) Comparison of *AA* and *AA'*

Clutch	Temp.	1st Half *AA*	1st Half *AA'*	1st Half %*AA'*	2nd Half *AA*	2nd Half *AA'*	2nd Half %*AA'*	$\chi^2_{(1)}$	Combined $\chi^2_{(2)}$
A	4	10	8	0·440	12	8	0·400	0·03	0·11
	16	12	12	0·500	8	9	0·530	0·08	
B	4	6	13	0·685	12	9	0·428	2·63	2·63
	16	9	11	0·550	11	14	0·560	0·00	
C	4	10	13	0·565	13	2	0·133	7·09**	7·14*
	16	6	12	0·667	9	10	0·527	0·05	
D	4	8	9	0·530	10	5	0·333	1·25	3·72
	16	9	4	0·308	8	9	0·530	1·48	
E	4	7	17	0·709	12	7	0·368	4·97*	5·03†
	16	14	11	0·440	9	13	0·592	1·07	
F	4	15	6	0·286	12	8	0·400	0·43	1·87
	16	23	8	0·258	11	8	0·421	1·44	
G	4	19	8	0·296	21	5	0·192	0·77	0·82
	16	14	8	0·364	16	8	0·333	0·05	
								Combined $\chi^2_{(14)}$	= 21·33†
Total	4	75	74	0·497	92	44	0·324	8·57**	8·57*
	16	87	66	0·431	93	71	0·432	0·00	

† Significant at 0·10 level. * Significant at 0·05 level. ** Significant at 0·01 level.

AA ($\chi^2_{(6)} = 12{\cdot}96$, $P < 0{\cdot}05$). At 16° C. this trend is reversed in two of three clutches, significantly so in one. Weaker, but parallel results are seen for *A'A'*-*AA'* comparisons, with the homozygote at a relative disadvantage at 4° C., and at an advantage in two of three clutches at 16° C. Combination of probabilities for comparisons of *A'* homozygotes with the *A*-bearing genotypes shows *A'A'* to be the least fit genotype at 4° C. ($\chi^2_{(12)} = 23{\cdot}70$, $P < 0{\cdot}05$), and suggests the opposite at 16° C. ($\chi^2_{(12)} = 19{\cdot}06$, $P < 0{\cdot}10$).

The above clutches, plus another four, were used for *AA'*-*AA* comparisons (table 4, *c*). In six of seven clutches at 4° C., the homozygotes lived longer than the heterozygotes ($\chi^2_{(7)} = 17{\cdot}22$, $P < 0{\cdot}02$). At 16° C., the heterozygotes appeared at an advantage in five of seven clutches, though the results are very weak. While *A*-homozygotes clearly outsurvived the *A'*-bearing gentoypes at 4° C. ($\chi^2_{(13)} = 30{\cdot}18$, $P < 0{\cdot}01$), the data do not support the opposite at 16° C.

As an indication of the over-all pattern of relative genotype survival in the 4° C. and 16° C. groups, probability values at each temperature have been combined (table 4). From these values it is evident that the relative fitness of *A'A'* correlates with temperature, and the *AA*-*AA'* comparison is at least highly suggestive.

To summarise, under the experimental conditions the homozygotes clearly survived differentially in the direction expected from the field data. The heterozygotes showed intermediate fitness at 4° C., though not clearly at 16° C. Thus, the larvae, at least, can show differential survival correlated with temperature. In view of the strong field correlation of *A'* frequency with both temperature and oxygen, it is of note that the close correlation of these in the field would not be duplicated under experimental conditions.

4. Discussion

That populations contain much genetic variation has long been indicated by studies of quantitative inheritance, and this has been strikingly demonstrated by electrophoresis of proteins. The initial finding of polymorphisms at seven of 18 loci examined electrophoretically in *Drosophila pseudoobscura* (Lewontin and Hubby, 1966), plus the increased demonstration of large differences in amino acid sequences between species, have led to much discussion of the biological significance of protein variation. Evolutionists dealing with morphological characters, wary of suggestions of neutral genes, have proposed that protein variation must be adaptive (*e.g.* Simpson, 1964). That such an extrapolation from morphological characters may be unwarranted has led some to claim that most protein evolution is due to random fixation of neutral alleles (*e.g.* King and Jukes, 1969). As Cain (1951) very clearly stated some time ago, however, characters labelled " non-adaptive " are better labelled " uninvestigated " until the data are in. Of course, the same applies to presumed " adaptive " characters, and any useful generalisations must be based on data.

The critical test of functional significance must come from population studies, which are unfortunately scarce. Where evidence is available, however, it points to the adaptiveness of protein polymorphisms. Especially interesting is the relationship of transferrin allelic frequencies with population densities in two species of *Microtus* (Tamarin and Krebs, 1969). Semeonoff

and Robertson (1968) have found an apparently parallel pattern at an esterase locus in a third *Microtus* species. Koehn (1969) has found differential reaction rates of *Catostomus* esterase phenotypes at different temperatures, corresponding to a latitudinal cline in allelic frequencies of population samples. In caged populations of *Drosophila*, frequency dependent selection has been found at the Esterase-6 locus (Kojima and Yarbrough, 1967) and at an alcohol dehydrogenase locus (Kojima and Tobari, 1969). There are numerous other examples of clinal variation in allelic frequencies (*e.g.* Frydenberg *et al.*, 1965; O'Gower and Nicol, 1968) or constant frequencies over large areas (*e.g.* Prakash *et al.*, 1969). Although details are neded, the accumulating evidence indicates much pattern of allelic frequencies, which is not consistent with widespread neutral variation. It is also interesting that the more intensive the study, the stronger the evidence for natural selection.

The LDH polymorphism in *Anoplarchus* adds to the accumulating evidence against predominantly neutral protein variation. The north-south increase in A' frequency itself indicates selection, and the apparent breaks in the cline at recognisably different areas strengthen this indication. The close correlation of allelic frequencies with physical conditions in Puget Sound confirms the geographic pattern. The seasonal and depth relationships at Penrose Point, the age-group differences, the close correlation of allelic frequency with species composition at the Tacoma Narrows, and the apparent changes in frequencies at Point Defiance and Hypus Point in 1969 and at Seattle from 1969 to 1970, each point to non-neutrality of alleles. With the exception of the changes in allelic frequency in Seattle samples (for which no explanation is evident), all of the field data show a consistent correlation with temperature. Finally, the laboratory experiments show that, at least under some circumstances, differential mortality can occur in the direction expected if natural selection is operating.

The patterns in allelic frequencies found in the field are probably due both to natural selection and behaviour. Absence of data on migration in *Anoplarchus* precludes any critical evaluation of the relative effects of migration and selection in maintaining the cline. There is little doubt that habitat preference has at least sharpened some of the observed patterns. The seasonal and depth relationships at Penrose Point suggest local migrational differences associated with A and A'. The species-genotype correlation at the Tacoma Narrows could also result from behavioural patterns, as could the increase in A' frequency at Point Defiance and Hypus Point. Of course, behavioural differences between genotypes would themselves indicate functional differences between alleles.

The temperature experiments, while not to be directly extrapolated to natural conditions, confirm that there are differences between the genotypes that relate to temperature and survival. The age-group data provide more direct evidence that selection does in fact occur in the wild. The very high frequency of A' in the 1967 year class in 1969, the possible drop in that frequency in 1970 at Penrose Point, and the low frequency of A' in the 1969 year class, which was derived mainly from 1967 fish, all suggest that selection is strong.

A major reason for choosing *Anoplarchus* for study was the interesting distribution patterns of the sibling species, as shown by Peden (1966). The species are sympatric from the Aleutian Islands to Puget Sound, the southern

limit of *A. insignis*, with *A. purpurescens* extending south to central California. Throughout the area of sympatry, *A. insignis* tends to occur in deeper water than *A, purpurescens*, the more so towards the south. This distribution of *A. insignis* represents a common pattern of Pacific Coast fishes which suggests an important influence of temperature on fish distribution (Hubbs, 1948).

On a smaller scale, the geographic and vertical distribution of *A. purpurescens* LDH alleles parallels that of the two species. The close association of allelic frequency with relative occurrence of *A. insignis* at the Tacoma Narrows confirms the meaningfulness of this parallelism, and is further evidence of a correlation of allelic frequency with temperature. Of course, the fact that *A. insignis* was not found in cooler areas shows that temperature is not the only correlate with species distribution.

The adaptiveness of the LDH difference (the *A'* allele) between the species is interesting in light of taxonomic use of electrophoresis. While the data do not provide a geographical comparison, the patterns over time provide a parallel situation, showing greater similarity of the species when the environment is more suitable for sympatry. Such a finding is expected for characters adapted to physical parameters of the environment. Since uncritical evaluation of increased similarity in areas of sympatry might lead to a conclusion of introgression (*e.g.* see Miller and Hubbs, 1969), it is of obvious importance to understand the significance of taxonomic characters, including proteins.

The consistent correlation of *A'* frequency with temperature in the field and in the laboratory indicates a probable causal relationship. In view of its influence on enzyme kinetics and in view of its apparent role in *Anoplarchus* distribution, temperature is a reasonable selective factor. Work on muscle LDH has shown a relationship of environmental temperature and Michaelis constant in a number of fishes (Hochachka and Somero, 1968; Somero, 1969). Whether or not the correlation of *Anoplarchus* LDH phenotypes with temperature carries over to such biochemical differences remains to be determined, and such an analysis would certainly be a fruitful means of relating molecular function to the environment.

It would be naïve to presume temperature to be the only selective force affecting the *Anoplarchus* LDH polymorphism. The strong correlation with oxygen is suggestive of factors other than temperature operating on the polymorphism. In view of the important function of LDH in anaerobic metabolism in skeletal muscle, oxygen concentration is certainly a possible selective force. Of course, many factors will correlate with both oxygen and temperature, and much experimentation is necessary to clarify the direct and synergistic effects of different factors. The present data at least provide a background for such physiological and biochemical study.

The most important shortcoming of the present study is the lack of information on the mode of selection. While it is a reasonable assumption that the polymorphism is stable (Ford, 1964), even this has not been shown. In view of the problems of maintaining much variation through heterosis (Kimura and Crow, 1964), the method of selection is of critical importance. The present data provide no evidence either for or against heterosis, nor do they suggest other types of selection. For initial clues, perhaps a laboratory approach would be more fruitful than further field work on this question. The present study provides a basis for such an approach, as well as for inquiries on the fundamental biochemical differences between the alleles.

5. Summary

1. Starch gel electrophoresis revealed identical LDH patterns for the sibling species of *Anoplarchus*, with the exception that *A. purpurescens* has an alternative allele, *A'*, at the muscle LDH locus.

2. In Puget Sound and the Strait of Georgia, the *A'* allele increases in the 2-26 per cent. range from north to south, with relatively low frequencies at the turbulent entrances to Puget Sound, and a step up in frequencies into the Sound.

3. The *A'* allele is at relatively higher frequency at more exposed sites of the intertidal during the spring, but not in the winter.

4. The 1967 year class had a much higher frequency of *A'* than the succeeding two year classes.

5. At the Tacoma Narrows, where the two *Anoplarchus* species are sympatric, *A'* frequency was inversely correlated with the relative abundance of the northern species, *A. insignis*.

6. Laboratory experiments demonstrated differential survival of larval genotypes at high and low temperatures.

7. The laboratory experiments and nearly all of the field data show a consistent correlation of *A'* frequency with temperature, demanding an adaptive interpretation of the polymorphism and implicating temperature as one selective factor.

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