POPULATION STRUCTURE, BIOCHEMICAL ADAPTATION AND SYSTEMATICS IN TEMPERATE MARINE FISHES OF THE GENERA *ARRIPIS* AND *CHRYSOPHRYS* (PISCES: PERCIFORMES).

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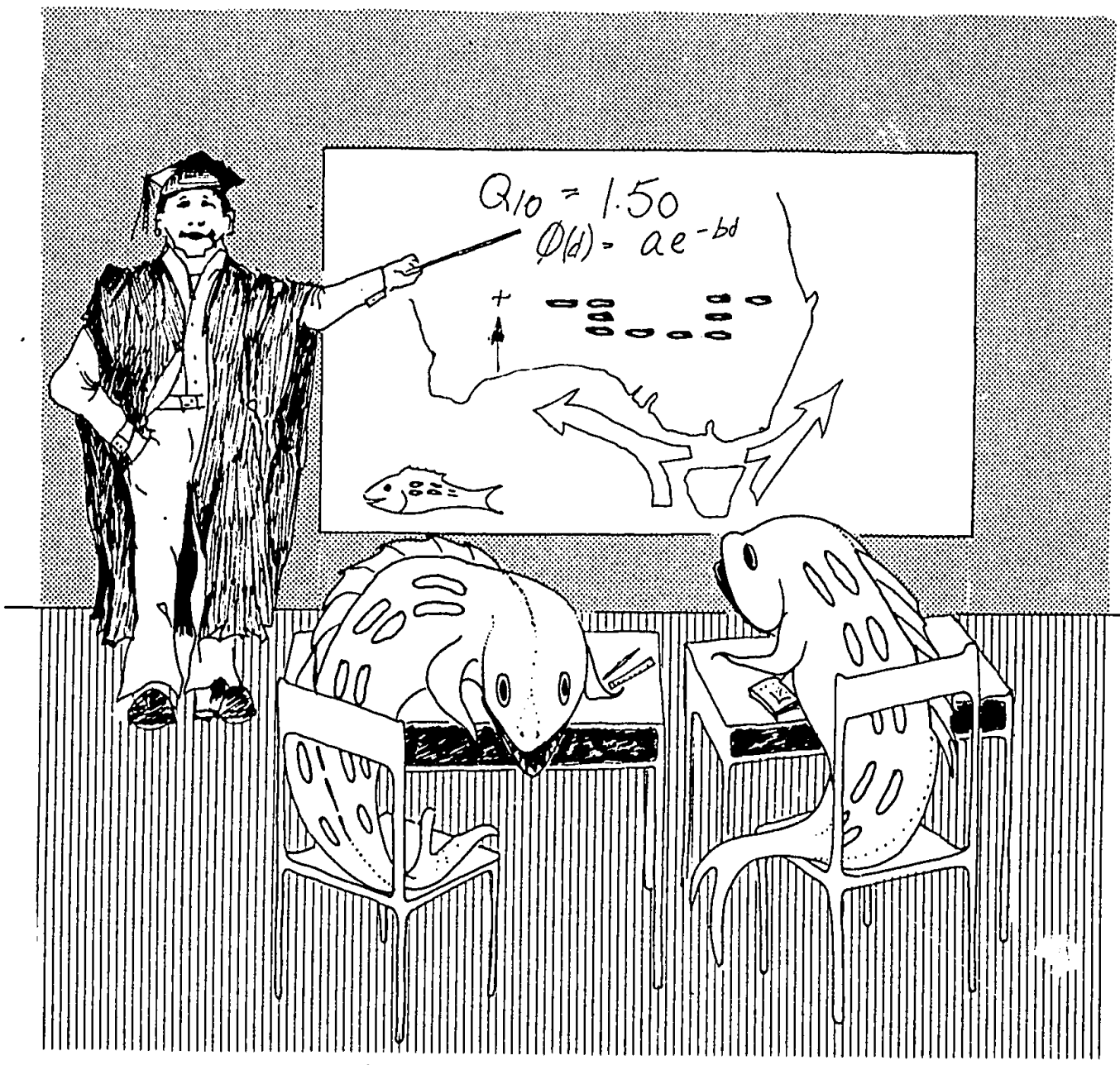
This thesis is the product of the author's original research except where specifically acknowledged.

M. MacDonald.

C. Murray MacDonald



"Is this guy for real !!?!"

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CONTENTS

ABSTRACT

Electrophoretic and heat stability data have been obtained from 22 enzymatic proteins to assess the amount and distribution of genetic variation in fishes of the temperate percoid genera *Arripis* and *Chrysophrys*.

Available biological and life history data have been reviewed, and indicate that *Arripis* species are mobile, migratory, pelagic fishes, with planktonic larvae and a comparatively short life span (up to 9 years), while *Chrysophrys* species are comparatively sedentary and demersal, have benthic larvae, and are long-lived (up to 60 years). The electrophoretic and heat stability data have been used to test the hypothesis that species with different life history characteristics exhibit different distribution patterns of genetic variation, and that these differences are the result of different adaptive strategies adopted by the organisms to cope with contrasting environmental regimes. The results generally agreed with predictions, although some alternative explanations for observed gene distributions could not be eliminated.

Electrophoretic data were also analysed using kinship and genetic distance techniques to provide information on the structure of snapper (*C. auratus*) and western salmon (*Arripis sp.*) populations in Australian waters. The results indicate that western salmon consist of a single breeding population extending across the southern Australian coastline, while at least five geographically distinct snapper populations were detected.

Enzyme kinetic studies were carried out on snapper isocitrate dehydrogenase (IDH) and salmon malic enzyme (ME) allozymes to determine whether or not functional differences existed in response to temperature fluctuation. It was found that differences did exist between IDH allozymes, but that the functional variation was correlated with heat stability variants rather than electrophoretic variants. The geographical distribution of snapper IDH variation was re-examined in the light of this result, and the relative contributions of random and selective processes to observed patterns were discussed. No functional differences in response to temperature fluctuations were detected for salmon ME electrophoretic or heat stability allozymes.

The observed functional characteristics of snapper IDH and salmon ME allozymes over known biological temperature ranges of the species were compared as indicators of biochemical adaptive strategies in organisms with different ecological requirements. The observed results agree with expectations based on the known biology of snapper and salmon, but further enzymes need to be assayed to confirm this pattern.

Taxonomy and evolutionary relationships within the genera *Arripis* and *Chrysophrys* have been examined using genetic distance and time-since-divergence estimates based on the electrophoretic data. Attempts have been made to reconstruct the evolutionary history of each genus on the basis of the genetic data, the current distribution of taxa, and information about past geomorphological events in key areas, such as Bass Strait.

Chapter 1

Introduction

1.1 General

One of the fundamental aims of biological research since the time of Charles Darwin has been to integrate information on the shape, distribution and ecological parameters of living organisms into the framework of current evolutionary theory. In recent years, the development of biochemical and molecular techniques for investigating the structure of genes and their products has resulted in a rapid advancement of the field of population genetics. Consequently, the question of relationships between genetic variability in natural populations and the environmental factors affecting these populations, has become a central topic of modern evolutionary biology, and one which many biologists believe can now be resolved.

Perhaps the most pervasive controversy arising from the discovery of extensive DNA and protein polymorphism in natural populations is the question of whether this molecular variation is physiologically meaningful, and hence able to contribute to adaptation via the process of natural selection, or whether such variation is evolutionary "noise" - i.e. without discernable effect on the fitness of organisms, and thus selectively neutral (Selander, 1976; and see section 3.1). An expansion of this central controversy has led to the definition of

problems in related areas. For example:-

a) What are the relative contributions of random and selective processes in determining the observed distribution of genetic variation in natural populations?

b) Can it be empirically demonstrated that specific polymorphisms have selective value - i.e. can it be shown that possession of one or other alternate forms of a gene affect the fitness of an organism in response to one or more environmental parameters?

c) Given that genotype/environment interactions occur, to what extent do observed patterns of genetic variation reflect adaptive "strategies" adopted by organisms to enhance fitness under prevailing environmental regimes?

d) What can the observed distribution of genetic variation in extant organisms tell the investigator about the evolutionary history of, and relationships between, these organisms?

These questions encompass current areas of general enquiry in the field of ecological population genetics, and provide a background for the specific problems to which this study is addressed.

1.2 The Marine Environment

Marine fishes were chosen for the present study primarily because of their intrinsic interest to the author. However, the marine environment and its resident organisms display an array of general and special properties which readily account for their recent and

prospective importance in analysing evolutionary processes.

Perhaps the most interesting property of a marine environment in the above context is that its effects on organisms are generally much more direct and unavoidable than corresponding interactions in terrestrial organisms. For example, while the superior heat-retaining capacity of water generally results in narrower ranges of temperature fluctuations in aquatic verses atmospheric environments, the persistent and often widespread nature of marine temperature regimes suggests that it is important for marine ectotherms to be adapted to prevailing conditions, as behavioural avoidence under these circumstances would be difficult. A similar argument can be made for other physical parameters, such as salinity, dissolved oxygen concentration and pressure.

The diversity of marine organisms and habitats is at least as extensive as that in a terrestrial environment, and in some situations (e.g. estuaries, lagoons, intertidal zones) the factors affecting the genetic structure of populations can be examined at the microgeographical level. In addition, many marine species are both abundant and densely distributed, and may be sampled extensively without seriously perturbing population structure and dynamics.

There are, however, some drawbacks in studying marine organisms for population genetic purposes. Perhaps the biggest problem is the apparent continuity of the physical marine environment and the associated difficulties of relating organisms to each other and to specific geographical locations - i.e. genetic populations cannot

be determined merely by observation. For these reasons the marine environment and its resident organisms are not readily amenable to manipulation, either in nature or in the laboratory. Another problem is that the marine environment is often hostile to man, making the collection of biological and genetic data a difficult prospect.

1.3 The Project

In this study electrophoretic techniques have been used to obtain information on the extent and distribution of protein polymorphisms within and between species of two genera of marine fishes - *Arripis* (family Arripidae) and *Chrysophrys* (family Sparidae). Both are percoid genera and both occur in the temperate coastal waters of southern Australia, but they differ from each other in a number of life history characteristics. For example, *Arripis* species are highly mobile, pelagic, migratory fishes with planktonic larvae, while *Chrysophrys* species are comparatively sedentary, demersal reef fishes with demersal larvae.

It is postulated that, as a result of these differences in life history characteristics, *Arripis* and *Chrysophrys* species will exhibit different distribution patterns (and possibly different amounts) of genetic variation, and that these differences reflect divergent "adaptive strategies" employed by the fishes to cope with contrasting ecological requirements. Various aspects of this hypothesis are explained in greater detail in later chapters. Electrophoretic data collected to test this

hypothesis were also used to provide information on the population structure of *Arripis* and *Chrysophrys* species in Australian waters, and on the possible evolutionary processes which may have led to the current phylogeny in each genus.

In chapter 2 are outlined the basic biology and broad ecological requirements of the Australian *Arripis* and *Chrysophrys* species selected for population genetic studies. The electrophoretic results obtained from the population studies are described in chapter 3. The data are analysed to provide information on the population structure of the species concerned, and the results are discussed in the light of the "adaptive strategies" hypothesis described above. In chapter 4 two polymorphic enzymes are chosen for kinetic analysis of the effects of temperature fluctuation on allozyme function. The results are discussed in terms of the adaptive value of allozyme variation. Predicted distributions based on the experimental results and on measured sea surface temperatures are compared to the observed allozyme distributions described in chapter 3. Electrophoretic data from all available *Arripis* and *Chrysophrys* taxa are compared in chapter 5 and, together with morphological and distributional information, an attempt is made to reconstruct the evolutionary history of each genus. Chapter 6 summarises the results obtained, and outlines some of the problems that need to be considered for the future conduct of population genetic studies.

1.4 Definitions

For the purposes of this study, I will define the term "stock" as meaning all those fishes present in a designated region or locality. The term "population" will be used to refer to a group of interbreeding fishes which can be distinguished in some way from other such groups, i.e. a Mendelian genetic population. A stock is therefore defined in a geographical sense, and may consist of individuals from one or more populations, while a population is defined in a genetic sense regardless of its distribution.

Chapter 2

Biology of The Fishes

2.1 Australian 'Salmon'

The Australian 'salmon', generally known as *Arripis trutta* (Bloch and Schneider, 1801) is a migratory marine pelagic fish endemic to the waters of the Australia - New Zealand region. It is in no way related to the true salmons (Order Salmoniformes) of the northern hemisphere, but is in fact a member of the monogeneric percoid family Arripidae (Order Perciformes). The common name 'salmon' was apparently applied to the Australian fish by early European colonists because of a superficial colour resemblance between juveniles of the Australian form and true salmonids (Malcolm, 1961, 1967).

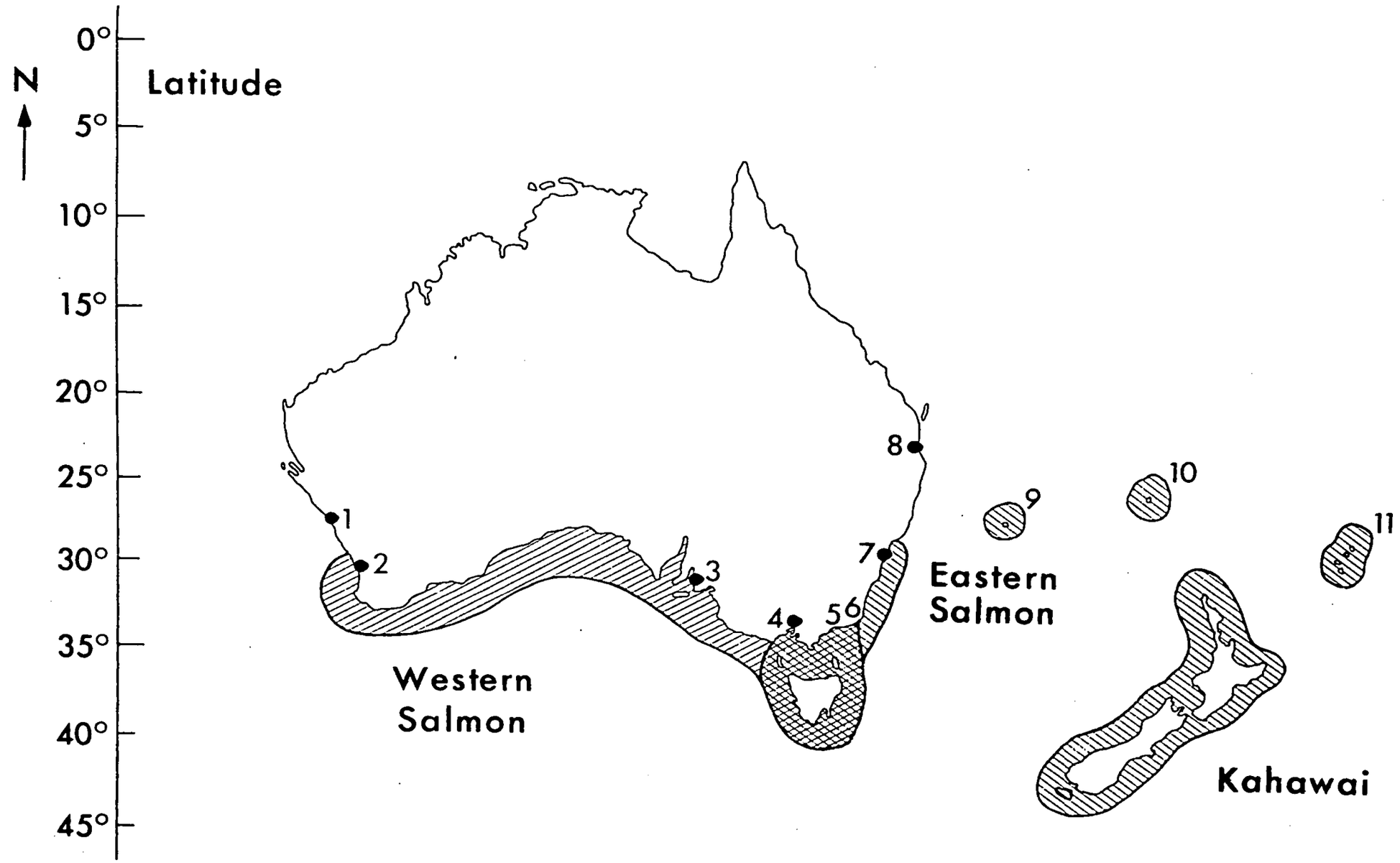
The Australian salmon has an inshore schooling habit, which results in dense concentrations of fishes appearing in shallow water along beaches or close to rocky outcrops. This habit has rendered the species vulnerable to intensive commercial exploitation and, subsequent to the establishment of a series of canneries in Australia between 1936 and 1949 (Malcolm, 1960, 1961), active beach seine fisheries have been established in Western Australia, South Australia, Victoria, New South Wales and Tasmania.

Adult salmon are typically dark bluish-green dorsally, pale yellow-green to white ventrally, and a mixture of green and reflective gold laterally. Juveniles commonly have longitudinal rows of eliptical golden-brown spots on

Figure 2.1

Distribution of members of the genus *Arripis* in the Australia-New Zealand region. The cross-hatched area around Tasmania indicates the overlap zone of eastern and western salmon in Australian waters. The ruff (*A. georgianus*) has a similar distribution to western salmon, except that it is not found in Tasmanian waters.

1 = Geraldton
2 = Perth
3 = Adelaide
4 = Melbourne (Port Phillip Bay)
5 = Gippsland Lakes
6 = Eden
7 = Sydney
8 = Brisbane
9 = Lord Howe Island
10 = Norfolk Island
11 = the Kermadec Islands


0°
5°
10°
15°
20°
25°
30°
35°
40°
45°
N
Latitude
1
2
3
4
5
6
7
8
9
10
11
Western Salmon
Eastern Salmon
Kahawai

the upper lateral portion of the body. Dorsal, anal and caudal fins are dark green-gray, pectoral fins yellowish-green, and pelvic fins hyaline. Malcolm (1966a) has recorded the most comprehensive morphological description to date, and the species has been figured by Fairbridge (1951).

2.1.1 Distribution

Australian salmon have been found around the entire southern Australian shoreline, extending seasonally up the east and west coasts to approximately 30°S (Fig 2.1). Development of commercial fisheries has stimulated scientific research on the species, and it soon became apparent that the Australian stocks consisted of at least two distinct breeding units. Fairbridge (1951) and Malcolm (1959) presented morphological and other evidence to distinguish between eastern and western forms, designated by Fairbridge as subspecies.

The western subspecies has been recorded as far north as Geraldton on the west coast of Western Australia, but it commonly occurs from the Perth area southwards throughout Western Australia and South Australia, and is the dominant subspecies in western Victoria (Malcolm 1961, 1966a). Western salmon are relatively common as far east as the Gippsland Lakes in Victoria, and an occasional specimen is caught in the Eden area of southern New South Wales. The eastern subspecies has been recorded as far north as Brisbane on the east coast of Queensland, but is normally found only from the waters of the Sydney area southwards. It is found throughout southern New South Wales, and is the dominant

subspecies in eastern Victoria (Malcolm 1961, 1966a,c). Eastern salmon are relatively common as far west as Port Phillip Bay in Victoria, and occasional specimens have been collected in South Australia and Western Australia.

The distributions of the two subspecies commonly overlap in central Victoria and around the entire Tasmanian coastline (Malcolm, 1966b; Stanley, 1978) and in these areas eastern and western salmon can be found in the same school of fishes. Later in the thesis (see Chapter 5) I will present evidence that these two taxa are in fact seperate species, thus confirming the tentative conclusion of Malcolm (1966a).

Salmon are abundant around the North and South Islands of New Zealand, where they are known as kahawai, and are also found in waters off Lord Howe Island, Norfolk Island and the Kermedec Islands (Fig 2.1). Specimens from the last three localities were not examined in this study, but Fairbridge (1951) reported morphological evidence which suggested the presence of separate breeding units in both New Zealand and Lord Howe Island waters. The taxonomy and evolutionary relationships of the three major salmon types around mainland Australia and New Zealand will be examined in greater detail in Chapter 5, but these groups will hereafter be referred to as western salmon, eastern salmon and kahawai respectively. Another arripid species *Arripis georgianus* is found in Australian waters, and its systematic relationship to *A. trutta* will be discussed in Chapter 5.

2.1.2 Reproduction

The spawning areas of the two types of Australian salmon are quite distinct - western salmon spawn in Western Australia and eastern salmon in southern New South Wales and eastern Victoria (Fig 2.2). In both types the strong inshore schooling habit is most apparent during coastal migrations to or from spawning areas. Studies of adult distribution and gonad maturation cycles (Malcolm 1960) indicate that the spawning phase of western salmon is between February and June, and that spawning can occur at any point west from Bremer Bay to Perth (Fig 2.1). However the peak months of gonad maturation/degeneration are April and May, and in these months most schools are sighted in the area between Cape Leeuwin and Busselton, indicating that this is the region of most intense spawning activity. There is evidence that some large male western salmon in South Australia complete the spermatogenic cycle in phase with spawning males in Western Australia, but failure to find sexually mature females in South Australia has led to the assumption that spawning does not occur in this area (Malcolm, 1960; personal observations).

The reproductive cycle of eastern salmon is less well defined. Available data on adult distribution and gonad maturation cycles (Stanley and Malcolm, 1977) suggest that spawning occurs from the Lakes Entrance region in eastern Victoria to areas just north of Bermagui in southern New South Wales, and that the spawning phase is November to April. However the most likely period of intensive spawning varies with area, and the data on peak gonad

Figure 2.2

Generalised pre-spawning migration paths and spawning grounds (shaded areas) of western and eastern salmon.

1 = Perth
2 = Busselton
3 = Cape Leeuwin
4 = Bremer Bay
5 = Esperance
6 = Lakes Entrance
7 = Bermagui
8 = Sydney
9 = Flinders Island


1
2
3
4
5
6
7
8
9
Western
Salmon
Eastern
Salmon

maturation/degeneration suggests intensive spawning periods of November to March near Bermagui, January and February near Eden, and December and January near Lakes Entrance.

Malcolm (1966a) has given a minimum length of 54cm length to the caudal fork (L.C.F.) for first sexual maturation in western salmon which, according to the growth data of Nicholls (1973), would correspond to specimens of 4+, 5+, and even 6+ years of age. Stanley and Malcolm (1977) specify approximately 39cm L.C.F. as the minimum maturation length of eastern salmon, corresponding to late 3+ or early 4+ year old fishes. No information is available on the spawning habits of kahawai, but Webb (1973a) found that kahawai gonads mature during spring (September to November) and gave a length at first maturity in both sexes of 52 - 54 cm total length (T.L.). According to the kahawai growth data of Eggleston (1975) this corresponds to 5+, 6+ and 7+ year old fishes. Eggleston states, however, that kahawai spawn from January to March and have a first maturation length of 35 - 40 cm L.C.F. - the equivalent of late 3+ and 4+ year old fishes. This agrees closely with the conclusions for eastern salmon, but as neither Webb nor Eggleston support their assertions with any quantitative data the situation remains to be clarified.

2.1.3 Migration

The differential distribution of adults and juveniles across the range of both western and eastern salmon and the limited extent of the spawning areas indicate that in

general there is a migration pattern of eggs, larvae and juveniles away from the spawning grounds, and of older fish back to these areas again.

There is no direct evidence of movement of young western salmon eastward across the Great Australian Bight, but the scarcity of juvenile salmon in bays and estuaries of Western Australia and the comparative abundance of 0+ year fishes in South Australia, western Victoria and parts of Tasmania indicate that such a movement probably occurs. Malcolm (1960) reports the presence of a year-round easterly continental shelf current along the southern coastline, with flow in this current reaching its greatest intensity from May to July. The easterly drift of eggs and larvae from an April/May spawning would be greatly aided by this current, but the arrival as early as August of young fishes in the eastern nursery areas at sizes of 4 - 10 cm L.C.F. (Nicholls, 1973) suggests that active swimming may also be a significant component of the easterly migration.

Juveniles up to 2+ years of age are generally resident in the shallow waters of bays and estuaries, but at this age they begin to leave sheltered waters, exhibit schooling behaviour and move along open coastline in a general westerly direction. Extensive tagging and age determination studies (Malcolm, 1960, 1966a; Nicholls, 1973; Stanley, 1978) have demonstrated the migration of older fishes from all eastern states back to Western Australia (Fig 2.2). Western salmon of more than 2+ years are rarely found in either Tasmania or the Gippsland Lakes region of Victoria. Schools of 3+, 4+ and 5+ year old

fishes become increasingly more common in western Victorian and South Australian waters, and specimens of up to 7+ years have been recorded in South Australia. Little is known about the distribution and movement of adults or juveniles along the central coastline of the Great Australian Bight, but tagging evidence suggests that the youngest fishes to reach Western Australia from eastern Australian nursery areas are 5+ years or older.

In the non-spawning phase the highest concentration of adult salmon is found on the south east coast of Western Australia between the Bremer Bay region and Esperence. In February resident adults, presumably together with schools that have arrived from eastern states, begin to migrate westward on a pre-spawning run. By April/May large numbers of salmon are concentrated in the area between Cape Leeuwin and Busselton. After spawning most schools then migrate back in an easterly direction to areas east of Bremer Bay. Some schools have also been observed moving north at this time as far as the Perth area.

The migration pattern for eastern salmon is essentially similar but less well defined than that of western salmon. Distribution of 0+ year juveniles mainly in Tasmanian and Victorian waters indicates a general migration southward and westward from the spawning areas on the south east tip of mainland Australia (Stanley, 1978; Malcolm, 1966c). Fishes up to 2 years of age generally inhabit shallow bays and estuaries. Then they begin to school on the open coastline. Tagging studies (Malcolm, 1966a; Stanley, 1978) show that older fishes (4+ years and more) begin to migrate away from northern and eastern

Tasmania - usually via the Flinders Island area - to eastern Victoria and southern New South Wales (Fig 2.2). There is also some evidence of movement of adult eastern salmon from New South Wales into eastern Victoria just prior to the spawning phase. However there is a more pronounced trend of movement north from the spawning grounds as far as the Sydney area, indicating a general dispersion of older adults in this direction after spawning.

The migration patterns of kahawai and of salmon found in waters off Lord Howe Island, Norfolk Island and the Kermedec Islands have not yet been described.

The frequent appearance and disappearance of both eastern and western salmon schools from shallow coastal waters during both spawning and non-spawning phases has led Malcolm (1960, 1966a) to suggest that adult and sub-adult salmon move offshore, a behaviour presumably related to pelagic feeding habits, and then back into shallow waters to "rest up" for a short period. Consequently it would be reasonable to expect salmon to be dispersed throughout continental shelf waters and perhaps beyond. However there are few confirmed sightings of offshore salmon, even after intensive fishing and research operations in Bass Strait and other areas over the past few years. Stanley (1978) also presents tagging data which suggests that, with the exception of the Bass Strait crossing, older salmon migrate to and from spawning areas along the coastline rather than directly through offshore waters. There are insufficient data to resolve this question at present.

2.1.4 Age and Growth

Eastern and western salmon of less than 50 mm L.C.F. have not yet been examined in detail. Consequently little is known of embryonic and larval development cycles other than the facts that the eggs float, the incubation period is about 40 hours, and at 4 days after hatching the larvae are about 4 mm long (Stanley and Malcolm, 1977).

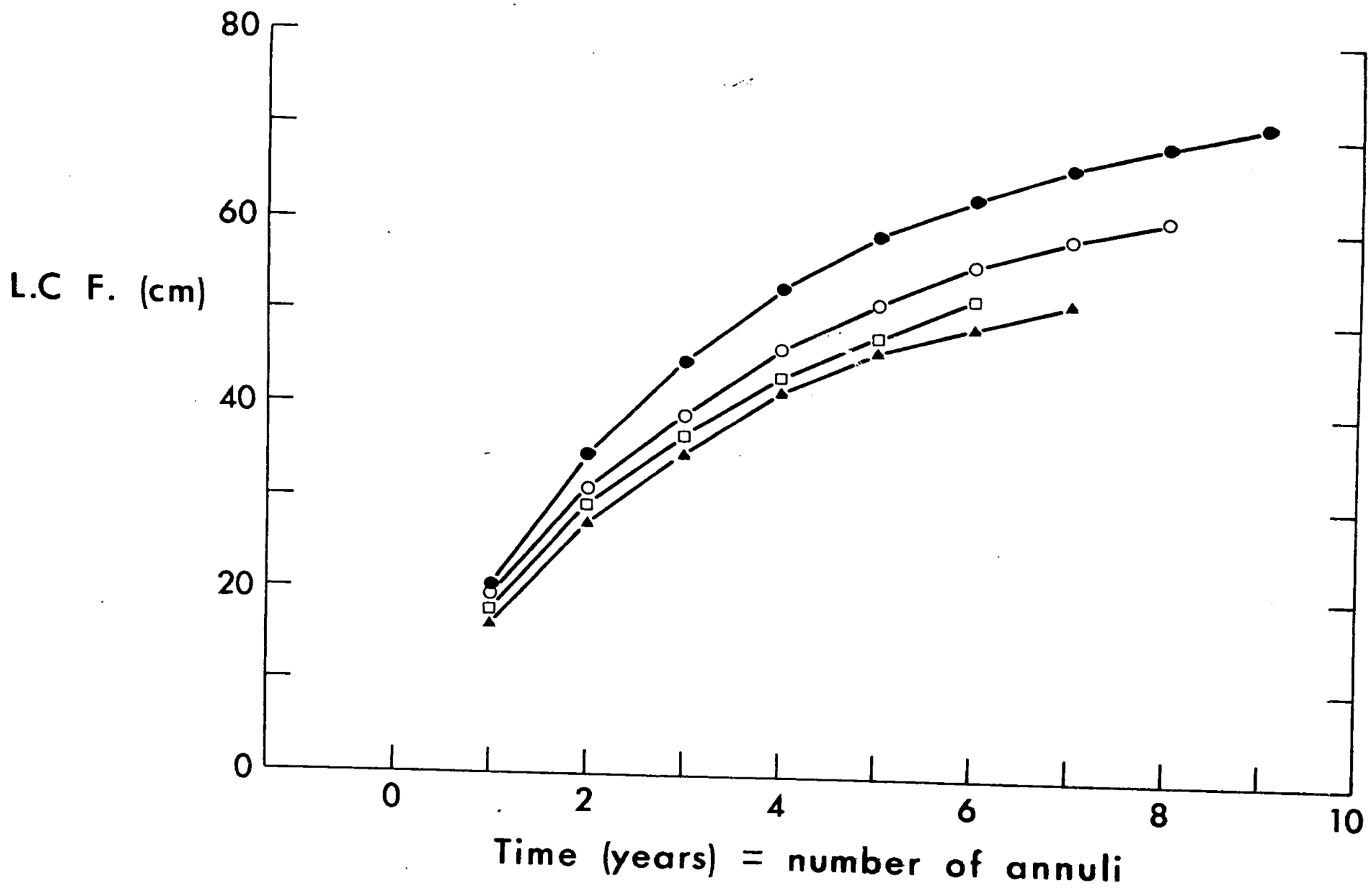
Growth rates of both eastern and western populations of salmon from the 50 mm juvenile stage onwards have been described by Nicholls (1973). Growth in the eastern salmon is uniform throughout its distribution. Adults of up to 7+ years have been recorded from scale ageing, at which time an average length of 52 cm L.C.F. and a weight of up to 3 kg have been attained.

Western salmon are generally larger at a given age than eastern salmon (Fig. 2.3). However growth rates vary considerably across the distribution of the western population. While salmon from Western Australia grow fastest, growth rates generally decrease with increasing distance from the Western Australian spawning grounds, until at the eastern end of the range they are similar to that of eastern salmon which inhabit the same waters. Adults of up to 9+ years have been recorded from Western Australia, at which time an average length of 71 cm L.C.F. and a weight of up to 6 kg have been attained.

Reported growth data for kahawai indicate rates similar to but slightly slower than those for eastern salmon (Eggleston, 1975). However kahawai appear to be longer lived than salmon, as scale readings of up to 10+

Figure 2.3

Mean growth curves for western salmon from Western Australia (●), South Australia (O) and Victoria (□), and for eastern salmon (▲). Redrawn from Nicholls (1973).


80
60
40
20
0
L.C F. (cm)
0
2
4
6
8
10
Time (years) = number of annuli

years and otolith readings of up to 22+ years (from the same group of specimens) have been recorded. Eggleston maintains that kahawai older than 5+ years or longer than about 40 cm L.C.F. cannot be reliably aged by counting scale annuli, and that otoliths should be used. It remains to be seen whether the difference in longevity between kahawai and the Australian salmons is real or can be largely accounted for by discrepancies in ageing techniques.

2.1.5 Food

Malcolm (1959, 1960, 1961) found that the eastern salmon is predominantly a plankton feeder, with the euphausiid *Nyctiphanes australis* Sars comprising the major part of its diet, while the western salmon is largely piscivorous, with the most common single dietry item being the pilchard, *Sardinops neopilchardus* (Steindachner). Although it is not made clear, this information presumably refers to older, migrating salmon, since Malcolm suggests that such food items indicate mostly offshore feeding and hence provide support for the inshore - offshore movement hypothesis. Thompson (1957), however, found that juvenile western salmon from an estuary on the west coast of Western Australia were also mainly piscivorous. Food preferences, as determined by percentage occurrence in stomach contents, indicated a diet of 80% small teleosts, 12% sea grass *(Zostera)* and 8% shrimps.

Recent studies of shallow tidal mudflats and seagrass

beds in Westernport Bay, Victoria (Robertson, 1977 and unpublished data) show that both eastern and western salmon juveniles have a diurnal movement pattern onto and away from such intertidal zones, presumably for feeding purposes. While stationed in these areas the two types of salmon occupy different parts of the water column, with eastern salmon generally remaining near the bottom, presumably in search of benthic dietry items, and western salmon actively traversing the midwater and sub-surface strata, possibly in pursuit of more mobile prey.

The concept of eastern salmon as plankton filter feeders in contrast to the more piscivorous western salmon is supported by the number and shape of gill rakers found in each type. Eastern salmon have an average of eight gill rakers more on the first branchial arch than do western salmon (Malcolm 1959, 1966a). Gill rakers in eastern salmon are also longer and more filamentous, making them more efficient as a filtering mechanism. The shorter, stubbier, and less numerous gill rakers in western salmon reflect a comparatively lower reliance on sifting of passive planktonic organisms as a food source.

An examination of stomach contents in juvenile kahawai from the Avon-Heathcote esturary, Christchurch, New Zealand (Webb 1973b) showed that over 90% of the diet consisted of equal quantities of small teleosts - mainly mullet, *Aldrichetta forsteri* and whitebait, *Galaxias maculatus* - and crustaceans, most of which comprised several species of crabs, plus unidentified

shrimps, isopods and euphausiids. Small amounts of mollusc, polychaete and algal remains were also detected. Information on the diet of older kahawai is not yet available.

2.1.6 Environmental Factors

There is virtually no information available for any of the salmon types concerning preferred and/or experienced ranges of environmental parameters, such as water temperature, pressure, salinity and dissolved oxygen concentration. Malcolm (1966a) stated that waters occupied by eastern and western salmon can be classified as warm temperate, with surface temperatures of between 14°C and 23°C in summer (February) and 10°C and 18°C in winter (August). Mean surface salinities in the same region vary between 26.0 and 35.0 parts per thousand, (°/oo). Juvenile salmon can be found almost to the limit of tidal waters in estuaries, indicating some tolerance to brackish waters. Lenanton (1977) has recorded western salmon from estuarine waters in Western Australia with salinity readings as low as 3.5 °/oo. However flushes of freshwater are not tolerated, and at such time juveniles are forced down to the lower reaches of estuaries or even out to sea. Good examples of this type of occurrence are seen at the mouth of the Murray River, South Australia, when the salt water barrages are opened to allow discharge of excess fresh water, and at the Gippsland Lakes, Victoria in time of flood.

The question of preferred and/or experienced

temperature ranges of Australian salmon will be discussed in greater detail in Chapter 4.

2.2 Snapper

In Australian waters snapper is the most common name given to *Chrysophrys auratus* (Bloch and Schneider, 1801), a species of the marine percoid family Sparidae (Order Perciformes) (Plate 2.1). The name "snapper" results from past confusion with the true snappers of the closely related family Lutjanidae. Sparids are generally known as sea breams or porgies (Eggleston, 1974), but in addition to the above misnomer *C. auratus* is also known as "cockney, pinkies, red bream and squire" - most of these terms applying to distinct life history stages of snapper.

The snapper is a predatory demersal fish, found from the shallow waters of estuaries and bays to the edge of the continental shelf at depths in excess of 200 metres. It is highly prized as a sport fish and as a table fish, and is the basis of extensive commercial and amateur fisheries in both Australia and New Zealand.

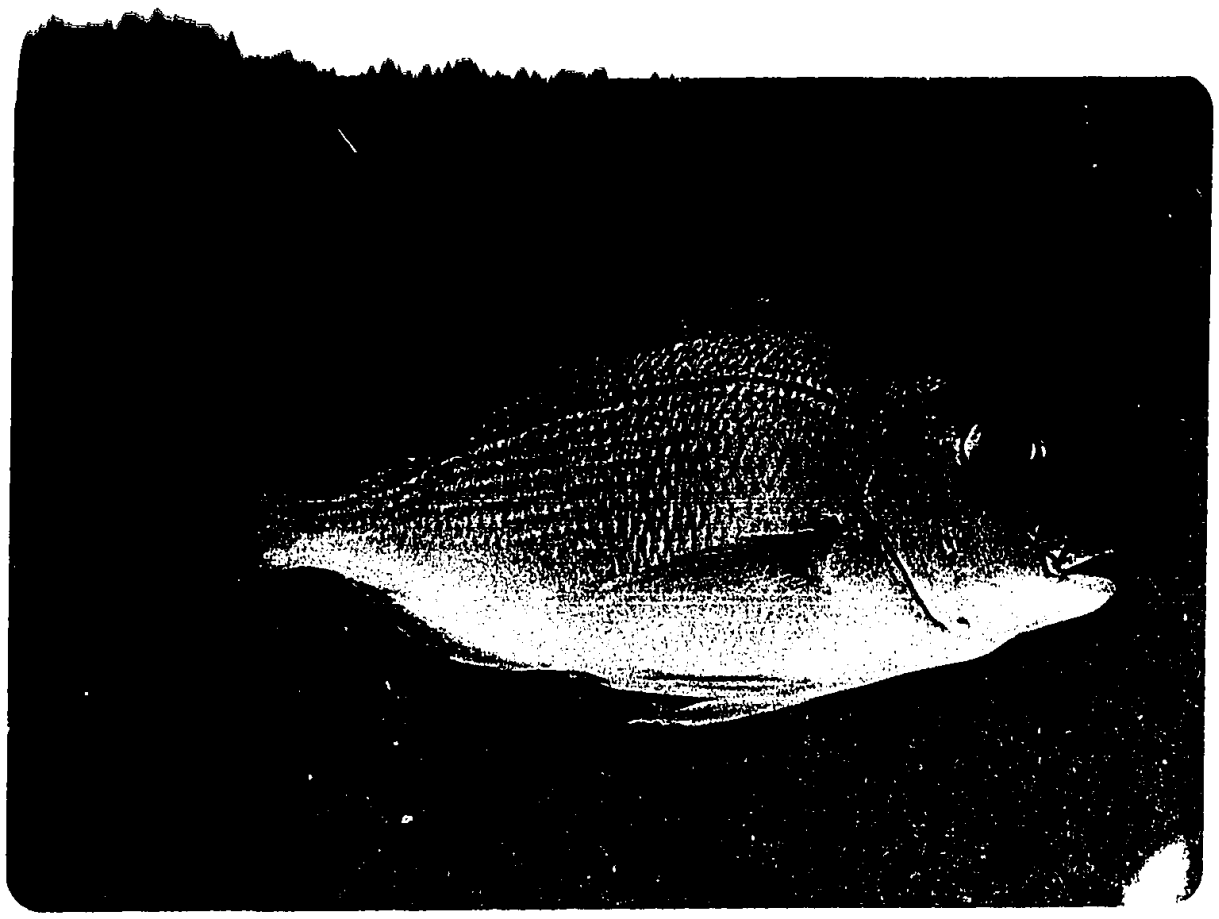
Adult snapper are typically red/brown on the head and upper half of the body, with numerous small irridescent blue spots. The sides are paler, and the belly is gray/white. In most cases the fins are also pale red. Juvenile snapper (up to 10 cm L.C.F.) have not developed the blue spots, but have a series of verticle red/brown bands around the body and tail. These bands disappear as the fish increases in size, and the blue

Plate 2.1

Australian snapper

(*Chrysophrys auratus*)

25 cm L.C.F.



spots become more prominent. During the breeding season large numbers of adult schooling snapper are caught which are much more brightly coloured, but in the same pattern as described above. However some adult fishes are caught in which there is virtually no red/brown colour at all, and the body is uniformly grey/white with faint blue spots. Many fishermen believe these two colour morphs represent distinct "races" of snapper, with the brightly coloured schooling snapper being seasonal spawning migrants and the paler snapper being year-round "residents". The colour variation may well be associated with spawning behaviour, with dietry fluctuations, or with the type of substratum upon which the fish is resident at the time, but there is no evidence from this or other studies that any genetic differences exist. Ripe adults of both types are found together (Colman 1972), and morphometric analyses have revealed no significant differences (Cassie, 1956a).

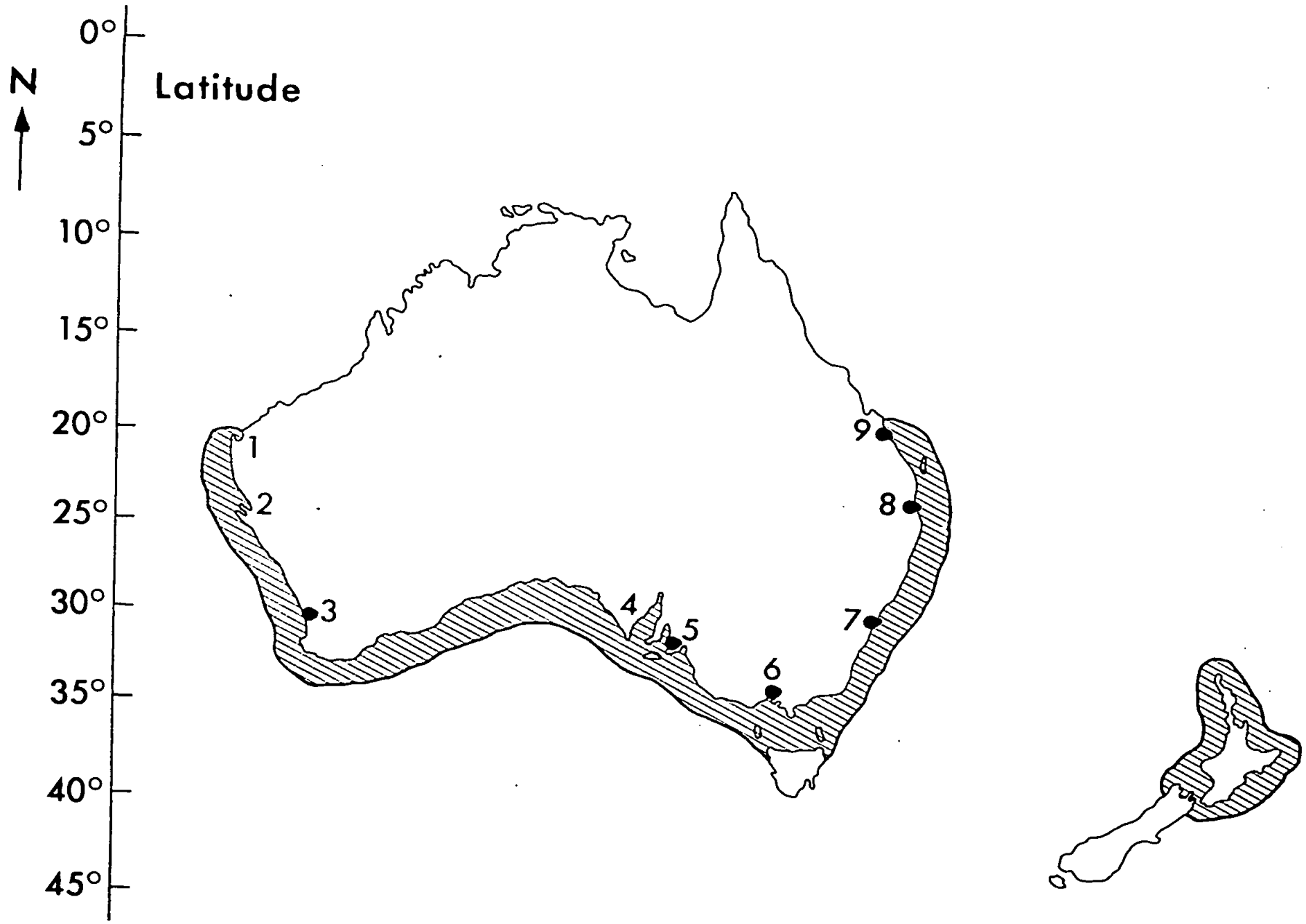
2.2.1 Distribution

Fishes of the genus *Chrysophrys* are found in warm temperate to subtropical continental shelf waters of the Indo-Pacific region (Fig 2.4 and 2.5). In Australian waters snapper are continuously distributed around the southern coastline from Exmouth Gulf in north-west Western Australia to about Rockhampton on the southeast coast of Queensland. While little is known of the relative abundance of snapper in different parts of this area, the fragmented nature of the commercial fishery - based

Figure 2.4

Distribution of snapper (Genus *Chrysophrys*) in Australian and New Zealand waters.

1 = Exmouth Gulf
2 = Shark Bay
3 = Perth
4 = Spencer Gulf
5 = Adelaide
6 = Melbourne (Port Phillip Bay)
7 = Sydney
8 = Brisbane (Moreton Bay)
9 = Rockhampton


N
Latitude
0°
5°
10°
15°
20°
25°
30°
35°
40°
45°
1
2
3
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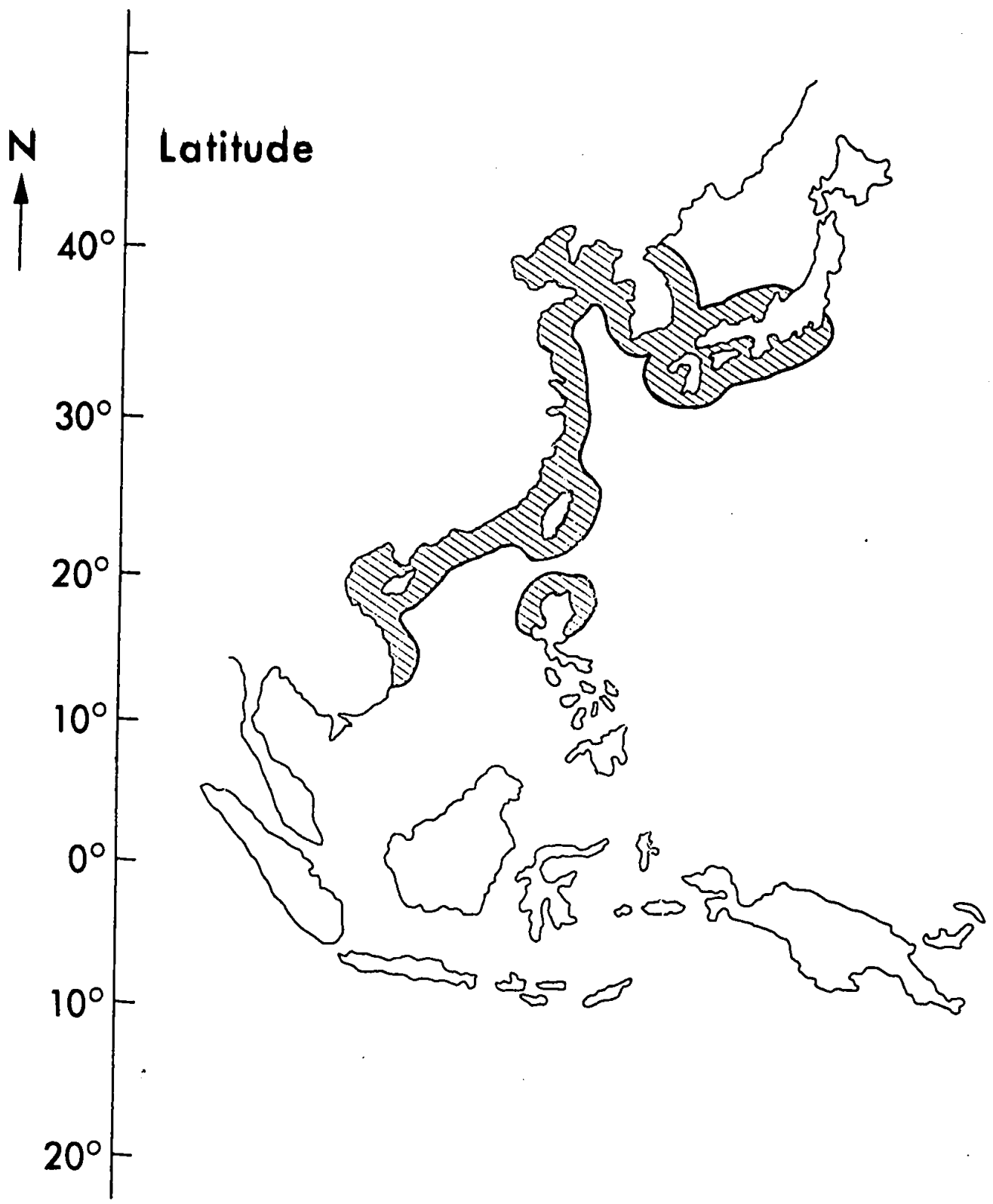
6
7
8
9

Figure 2.5

Distribution of the red sea bream (Genus *Chrysophrys*) in north-west Pacific waters.


N
Latitude
40°
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mainly on the seasonal occurence of schooling fishes - suggests that distribution is not uniform and that aggregations of snapper occur in preferred localities. This pattern persists even in areas where there is little or no commercial exploitation. Victorian coastal waters appear to form the southern limit for Australian snapper. The species is only occasionally reported from the north coast of Tasmania and is not found further south.

In New Zealand, snapper (also known as *C. auratus*) are commonly found around the entire North Island and around the northern tip of the South Island in the Tasman Bay region. According to Longhurst (1958) snapper are occasionally recorded down the east coast of the South Island as far as Banks Peninsula and down the west coast as far as Greymouth, but few such reports have been made in recent years. It is interesting to note that the southern limits of snapper in both Australia and New Zealand are very similar, suggesting a well defined limit of tolerance to a latitudinally associated environmental parameter. Water temperature is an obvious possibility, and will be discussed further in Chapter 4.

The only other member of the genus *Chrysophrys* is *C. major,* commonly known as red sea bream. This is a northern hemisphere species which is very similar to *C. auratus* in external morphology and colouration, despite a gap of 3,000 kilometers of tropical waters between the distributions of the two forms. Red sea bream are found in the South China Sea around the southern islands of Japan, the coastlines of Korea, China, Taiwan, Hong Kong, Vietnam, and the northernmost islands of the Phillipines.

This species has also been reported from near Bombay, India (Fowler, 1928) and from Hawaii (Fowler, 1900, 1922, 1928, 1929), but the authenticity of these reports is doubtful, since no one other than Fowler has made such recordings, and since 1929 there have been no reported sightings from those localities. The taxonomic and evolutionary relationships of northern and southern hemisphere *Chrysophrys* populations have been the subject of considerable debate, with opinions ranging from three species in the southern hemisphere and one to the north (Whitley, 1931; Yasuda and Mizuguchi, 1969), to a single species with northern and southern hemisphere "geographic" varieties (Fowler, 1933). This problem will be further discussed in Chapter 5, but fishes from the Australian and New Zealand region will hereafter be referred to as snapper, and those from the South China Sea as red sea bream.

2.2.2 Reproduction

There is little published information on the spawning habits of Australian snapper stocks. Observations on gonad development in specimens collected during this study (Table 2.1) suggest that gonad maturation, and presumably spawning activity, occur at various localities throughout the distribution of the species. This conclusion has been supported by communication with commercial fishermen from different areas who have observed surface schools of adult snapper engaged in spawning activity. Such activity consists

Table 2.1

Percentage distribution of gonad maturation stages in samples of Australian snapper. The code for gonad maturation follows that published by Crossland (1977a) for New Zealand snapper:-

0	-	Immature (juvenile)
1	-	Resting mature
2	-	Developing
3	-	Ripe and running ripe
4	-	Spent

Sample Location	n	Date	Length Range (L.C.F.)	Gonad Maturation Stages 0	1	2	3	4
Moreton Bay, Qld.	53	August 1979	13-24cm	100	-	-	-	-
Outside Moreton Bay, Qld.	14	June 1979	24-44	7	36	57	-	-
Off Sydney Heads, NSW	55	May 1977	23-50	31	67	2	-	-
Off Sydney Heads, NSW	33	July 1978	28-52	-	76	24	-	-
Mollymook, NSW	29	Dec. 1976	38-61	-	-	-	41	59
Port Phillip Bay, Vic.	72	Dec. 1976	33-81	-	-	17	79	4
Port Phillip Bay, Vic.	64	Nov. 1977	32-78	-	-	2	79	19
Cape Jervis, SA	37	March 1977	29-79	-	89	-	5	6
Cape Jervis, SA	57	Nov. 1977	50-82	-	2	75	21	2
Upper Spencer Gulf, SA	34	Nov. 1977	25-53	-	56	32	-	12
Upper Spencer Gulf, SA	70	April 1979	27-67	-	40	29	31	-
Lower Spencer Gulf, SA	32	Sep. 1978	29-45	-	81	19	-	-
Lower Spencer Gulf, SA	50	Nov. 1978	32-57	-	90	6	-	4
Shark Bay, WA	60	May 1978	35-66	-	23	72	5	-

mainly of a lethargic circular movement of the tightly bunched school and the discharge of copious quantities of ova and sperm into the water column. This type of spawning is usually observed in comparatively shallow waters (50 metres or less). There are however some areas where maturing adult snapper have been collected and where surface school spawning activity has not been observed. Whether spawning takes place mainly in midwater in these areas (Cassie, 1956a) is not known. Evidence from tagging studies in South Australia (Jones 1979) and in New Zealand (Cassie 1956a; Longhurst 1958; Crossland 1976) indicates that snapper do, however, undertake seasonal migrations into and away from shallow waters, and that the schooling phenomenon is associated with the congregation of fishes in a preferred spawning area.

Cassie (1956a) concluded from surface plankton sampling in Hauraki Gulf, New Zealand, that snapper probably don't spawn until the surface water temperature exceeds 18^{o}C. He also attributed the schooling of snapper at 25-30 metres in many areas to the presence of a 3^{o}C thermocline at about that depth. The fish could then rise through the thermocline to warmer waters to spawn.

The spawning period of Australian snapper appears to vary substantially over the species range. In southern New South Wales, Victoria, South Australia and south-west Western Australia snapper are predominantly late spring/summer breeders, with ripe gonads or spawning activity being observed from late October through until

early March (Lenanton, 1974; fishermen, pers. comm; and see Table 2.1). Snapper at the northern extremes of the Australian distribution appear to have adopted a winter spawning period. Adults collected from Shark Bay, Western Australia and from Moreton Bay, Queensland in late May and early June respectively were found to be in the process of gonad maturation (Table 2.1), and Bowen (1961) has suggested June to August as the spawning season in the Shark Bay area. Whether or not there is a clinal change in spawning period between these northern and southern extremes in response to changing temperature and photoperiod regimes (Girin and Devauchelle, 1978) is not clear. This matter will be discussed further in Chapter 3.

The peak spawning period of New Zealand snapper, determined mostly from studies on Hauraki Gulf stocks (Cassie, 1956a; Paul 1976; Crossland, 1977a), is November to February. Annual fluctuations in water temperature may vary the spawning period since ripe snapper have been observed as early as October and as late as March. Crossland (1977a) found from gonad maturation and oogenesis studies that snapper were serial spawners over the 3 to 3.5 - month breeding season. This type of spawning is thought to maximise fecundity in comparison with total spawners, and to provide the greatest chance in a variable environment that at least some eggs will hatch and develop under favourable conditions. The fecundity of Hauraki Gulf snapper showed a linear increase with length of fish, and single-season estimates of eggs spawned ranged from 83,000 at 25 cm to 6,164,000 at 50 cm

L.C.F. (Crossland, 1977b). Number of batches spawned per female in a season ranged from 6 to 59, and batch sizes ranged from 13,000 eggs at 25 cm to 104,000 at 50 cm L.C.F.

The spawning period of red sea bream, as with Australian snapper, appears to vary with latitudinal location. Ripe males and females have been observed in waters around Taiwan from December to late March (winter/early spring), with the peak spawning period suggested as being February and March (Huang et. al., 1974). In Japanese waters ripe adult red sea bream are apparent from April to late June (spring/early summer), with the spawning peak in early May to early June (Matsuura, 1972; Kittaka, 1977). Huang et. al.(1974) also found that red sea bream, in common with many sparid species, is amphisexual. Red sea bream is a protogynous sex reverser, with all 0+ and 1+ year old fishes having ovaries, some hermaphrodites with ovotestes present from 3+, onwards and only males or females from 7+ onwards. There is no available evidence to suggest that the closely related snapper from Australia and New Zealand is also amphisexual. Personal examination of snapper gonads has not revealed the presence of ovotestes, but it was found that specimens which had not yet achieved first gonad maturation were very difficult to sex, and most specimens less than 25 cm L.C.F. were merely labelled "juvenile". Studies of gonad histology are required to throw further light on this matter.

Winstanly (unpublished) suggests that snapper from Port Phillip Bay start to breed after they reach 27 cm

T.L. and 4 years old, but no data were presented to support this statement. Observations on New South Wales snapper (Fairbridge, 1943, unpublished) indicate a minimum at first maturity of 30 cm L.C.F. and 3 years of age - a considerable difference -, both in length of first maturity and in growth rate, compared to the preceeding statement. Jones (1979) found that snapper from Spencer Gulf, South Australia had a minimum spawning length of 31 cm T.L., although gonads of fishes between 25 cm and 30 cm T.L. did show some evidence of maturation. Jones also concluded that the minimum spawning age of snapper was 3 years. My own observations on gonad development show the onset of maturity in females from 25 cm L.C.F. upwards and in males from 27 cm, but the data are not comprehensive enough to detect geographical variation in age or size at first maturity. New Zealand snapper begin to exhibit small but ripe gonads at lengths between 20 and 24 cm L.C.F. (Cassie, 1956b; Paul, 1976). These lengths can correspond to 2+, 3+ or 4+ year classes, but Paul found from aging studies that most of the specimens undergoing first maturity were 3+ or 4+ years old.

2.2.3 Migration

Movements of Australian snapper during different phases of their life cycle are not well documented, but general patterns can be inferred from the distribution of size groups. Observations of spawning in comparatively shallow water, the presence of abundant small juveniles in sheltered bays and estuaries, and the predominance of

larger (≥ 35 cm L.C.F.) adults in catches from deeper, offshore waters suggest an inshore dispersal of juveniles, a gradual movement of adult fishes into deeper offshore waters, and a seasonal migration of spawning adults to preferred shallow water breeding grounds. There is evidence, however, that not all snapper follow this pattern. Tagging and age class distribution studies in Australia (Sanders, 1974; Jones, 1979) and in New Zealand (Paul, 1967; Crossland 1976) indicate that a large proportion of the schooling adults which congregate to spawn at a given location in any season consist of snapper which have either been resident in the area all year round or have moved away and returned to the same vicinity (in many cases to the exact same location) the next season. So it seems that not all adult snapper move out into deeper offshore waters, and of those that do it is not known what proportion participate in each season's spawning migration.

While the majority of tag returns from the above-mentioned studies revealed nett migration of less than 40 kilometers, there were some long distance returns which indicated that adult snapper are capable of substantial coastal migrations. The record to date for New Zealand is 416 km in 124 days along the east coast of the North Island (Paul, 1967). In Australia the longest recorded movement of a snapper is about 1400 km along the east coast, although it is likely that this distance was achieved over a period of more than one year (Sanders, 1974).

2.2.4 Age and Growth

The embryonic, larval and early juvenile periods (as defined in Balon, 1975) of Australian snapper are unknown. Cassie (1955) found that the time from spawning to hatching of New Zealand snapper eggs varied between 36 hours at 21°C and 45 hours at 18°C, and that the yolk sac of newly hatched embryos was absorbed by the fourth day after hatching - this being about the time the mouth first opens and the larvae begin to feed externally. These results closely parallel similar observations made on red sea bream (Sanders, 1975; Apostolopoulos, 1976). Cassie also observed that the newly hatched embryo floats at the surface for about 24 hours, but thereafter descends through the water column and prefers a demersal position for the rest of the yolk sac absorbtion period and the subsequent larval stages. Fukuhara (1969) found that red sea bream larvae grow fairly slowly in the first 22 days after hatching, reaching a total length of only 8.55 mm. Growth rapidly accelerates after this stage, with subsequent lengths attained averaging 17 mm at 30 days, 36 mm at 60 days and 74.5 mm at 120 days - by which time each individual is a fully formed juvenile.

The growth rate of snapper is highly variable throughout the species' range and appears to depend largely on the temperature regime and the quality and quantity of food available in a given locality (Godfriaux, 1969; Colman, 1972). Growth in New Zealand snapper has been described by Cassie (1956b), Longhurst (1958), Paul (1976) and Vooren and Coombs (1977), all of whom reported

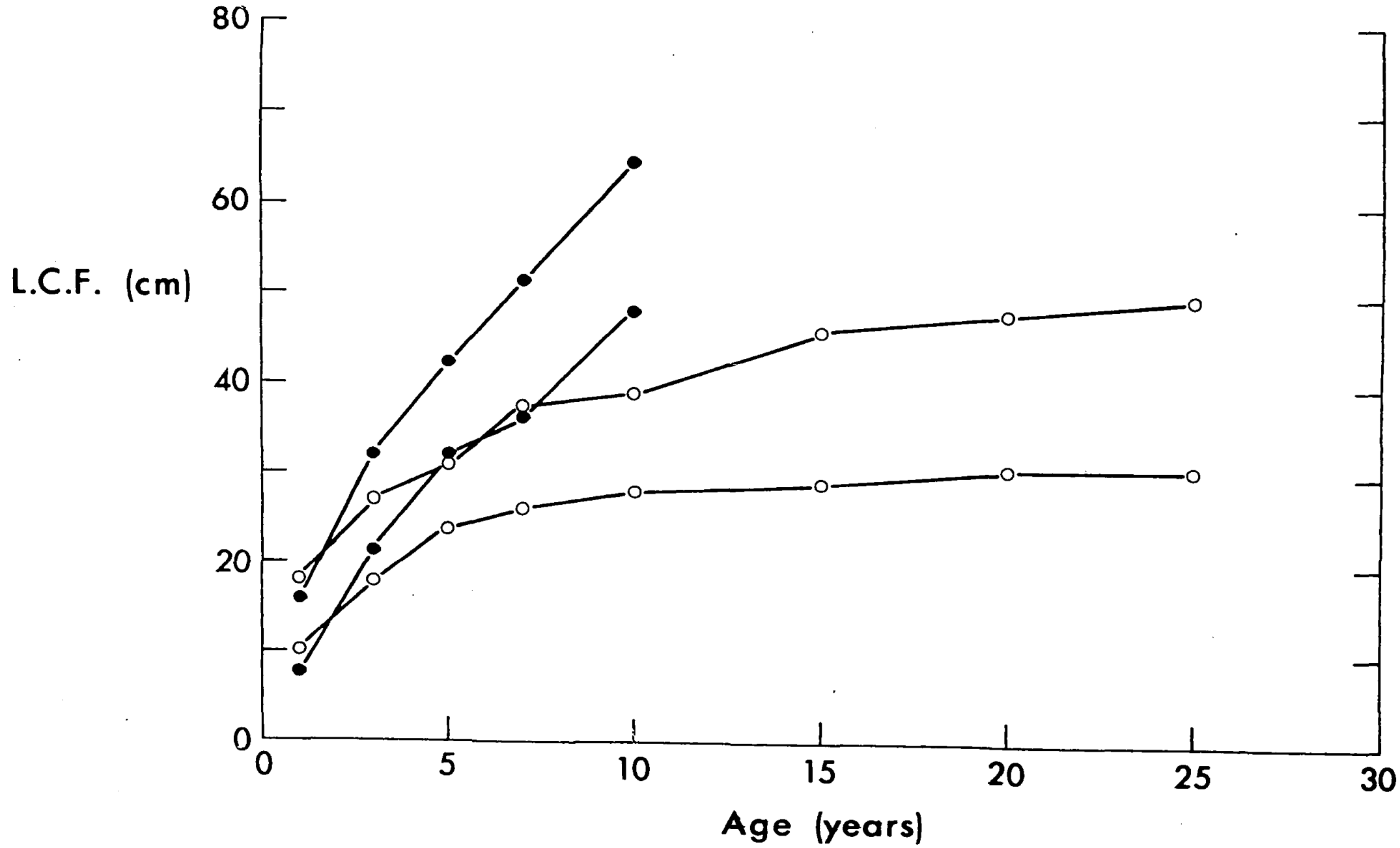
variation between local stocks. Figure 2.6 is a composite of these studies, showing clearly the flexibility in growth patterns within the species. Figure 2.6 also shows the pronounced allometry of the growth pattern, which is characteristic of a long-lived species. Lenanton (1974) reported growth rates of 1 year old snapper from Western Australia (11.0 to 14.5 cm L.C.F.) which were considered to be slightly faster than those of New Zealand fishes (10.2 to 12.7 cm) and which he attributed to the more northerly location of the Western Australian stock. Jones (1979) has constructed a growth curve for snapper from Spencer Gulf, South Australia which also indicates a much faster growth rate than New Zealand snapper.

There is, however, some evidence of considerable local variation in growth rates of Australian snapper (Sanders and Powell, 1979; M. Walker, K. Jones, pers. comm.), and it seems reasonable to assume that the Australian results are merely facets of a growth pattern equally as flexible as that in New Zealand.

The aging of snapper, whether by otolith reading, scale reading or length-frequency analysis, becomes very difficult in specimens of more than 5 years of age because of a rapid decline in growth rate. Despite this difficulty specimens from New Zealand have yielded otolith readings of up to 36 years (Paul 1976). Extrapolation of published growth curves for New Zealand snapper up to 70 cm L.C.F. indicates that ages in excess of 60 years can be attained. By contrast Jones (1979) estimated from scale readings that the age of a 107 cm T.L. (approx. 85 cm L.C.F.) snapper from South Australia was 19 years. The

Figure 2.6

The range of growth curves for New Zealand snapper (O) and red sea bream (●) based on data from Chang and Chen (1972), Paul (1976), and Vooren and Coombs (1977).


80
60
40
20
0
L.C.F. (cm)
0
5
10
15
20
25
30
Age (years)

growth rate of red sea bream appears to be a good deal faster than that of snapper (Fig 2.6), but a similar amount of local variation is also apparent (Chang and Chen, 1972). In view of the difficulties encountered in aging such long-lived species it is not clear whether the observed variation in growth patterns is a genuine difference within and between snapper and red sea bream populations, or a result of discrepancies in aging techniques.

2.2.5 Food

The snapper is an opportunistic demersal predator which occupies a very broad feeding niche and has a comparatively low diet selectivity. Godfriaux (1969, 1974) and Colman (1972) together list over 100 different food types identified from snapper stomach contents. Juveniles of 5 to 15 cm L.C.F. were found to have a diet consisting mainly of polychaete worms, small echinoderms and soft-bodied crustaceans - particularly mysids, amphipods and natant decapods. As the size of the snapper increases the frequency of polychaete worms and mysids drops away, and there is a switch towards longer, harder-shelled animals, such as crabs, echinoids, hermit crabs, molluscs and teleosts. Adult snapper in shallow inshore waters also take in a significant amount of pelagic euphasiids, but not those in deeper water. The reasons for this depth-specific selection is not clear, but is thought to be associated with avoidance of dietry overlap with another demersal predator, the tarakihi,

Cheilodactylus macropterus (Godfriaux, 1974). There appears to be a general decrease in the proportion of gut volume filled with food as the snapper get bigger. This is consistent with a gradual decrease in feeding activity of older fishes. There are also differences in the diet of snapper feeding at different depths and on mud versus silt substrates, but this is probably a function of the availability of food items rather than selective feeding. Snapper feed mainly by daylight, with peak feeding activity early in the morning and later in the afternoon. There is no significant variation in feeding habits between males and females. Adult snapper do not appear to suffer any significant predation. This, together with their diversity of diet and avoidance of food competition with other large teleosts (Godfriaux, 1970), may explain why snapper, wherever they occur, are more abundant than any other large demersal predator.

2.2.6 Environmental Factors

Snapper are confined to warm temperate and sub-tropical waters in Australia, with surface temperatures ranging from 18°C to 30°C in summer and 12°C to 20°C in winter. Reasons for the northern limit of the species' distribution are not obvious, although it seems reasonable to suggest that a greater diversity of reef fishes in tropical waters - particularly other sparids and members of the families Lutjanidae and Lethrinidae, which have similar ecological requirements - may present enough competition to displace snapper.

The southern limit of snapper distribution seems to be more closely tied to physiological requirements during reproduction and early development. As mentioned earlier, Cassie (1956a) concluded that a minimum surface water temperature of 18°C was required for snapper to commence spawning, and it is coincidental that the southernmost regular occurrences of snapper are in waters where the maximum summer temperature barely reaches 18°C. Temperature studies on the closely related red sea bream (Apostolopoulos, 1976) show that hatching rates of 70% and above can only be achieved at temperatures between 19°C and 29°C, and optimum conditions for larval survival were associated with temperatures between 19°C and 25°C. Tests of salinity conditions during the same study showed that similar rates of hatching and larval survival could only be achieved at salinity levels above 27.03 °/oo. It has also been shown that red sea bream eggs require water with a specific gravity of more than 1.023 to be able to float (Kittaka, 1977). This is an important point, as contact with the water surface is critical for the survival of eggs and embryos.

Information on the tolerance of snapper to changes in depth/pressure or dissolved oxygen concentration is not available.

Chapter 3

Electrophoretic Studies of Population Structure

3.1 Introduction

Investigations of the amount of genetic variation and its spatio-temporal characteristics in natural populations have assumed a central role in the development of current evolutionary theory. The last 15 years in particular have seen a proliferation of such studies, primarily through the application of electrophoretic techniques (Lodge, 1886; Smithies, 1955; Kohn, 1960), the use of histochemical staining methods to visualise bands of activity of specific enzymatic proteins (Hunter and Markert, 1957), and a realisation of the usefulness of these techniques in estimating the extent of protein variation (and hence genetic variation) in natural populations (Lewontin and Hubby, 1966; Harris, 1966; Johnson et al., 1966; Hubby and Throckmorton, 1965, 1968). Nevo (1978) compiled a list of some 250 animal and plant species that had been electrophoretically surveyed for genetic variation in natural populations by early 1976, and a survey of the literature since then suggests that the body of electrophoretic data has continued to increase at an exponential rate.

The discovery of substantially greater allelic variation at structural gene loci than was previously suspected has necessitated a reappraisal of concepts in population and evolutionary biology, and has led to enquiry and debate in three main areas:- (i) determination of the amount, extent

and nature of variation in different groups of organisms, (ii) identification and evaluation of the random and/or deterministic forces controlling the distribution of variation in space and time, and (iii) the role of structural protein variation in the evolutionary process. To provide an overview of the present "state of the art" in these topics is beyond the scope of this study, and the reader is referred to Powell (1975), Selander (1976) and Nevo (1978) for summaries of data on genetic variation in animal and plant species; to Lewontin (1974), Ayala (1976), Karlin and Nevo (1976), Grant (1977) and Dobzhansky et al. (1977) for reviews of experimental findings and conceptual developments; and to Wright (1969), Crow and Kimura (1970), Jacquard (1974), Nei (1975) and Endler (1977) for more theoretical and mathematical treatments. I will concentrate here on reviewing the contribution of studies on marine organisms, and fishes in particular, to the field of population genetics. It is necessary to begin, however, by dealing briefly with the general concepts that were used to formulate *a priori* hypotheses on, and subsequently interpret observed patterns of, genetic variation in the *Chrysophrys* and *Arripis* species studied in this thesis.

3.1.1 Maintenance of Genetic Variation in Natural Populations

In a reproductively isolated species mutation is the only source of new genetic variation in the structural component of the genome. There are, however, four major possible forces which can govern the distribution of genetic variation in space and time, namely mutation rates, gene flow, random genetic drift, and the phenotype/environment interactions

implicit in the term "natural selection".

The tendency for mutations to persist as allelic variation at a given gene locus is determined by the intrinsic rate of mutation (u) at the locus and the effective size (N_e) of the population into which the mutations are introduced. Thus the probability (P) that an individual chosen at random will be heterozygous at the given locus is defined by:-

$$P \simeq \frac{4N_e u}{4N_e u+1} \qquad \text{(Crow and Kimura, 1970)}$$

Mutation rates vary from organism to organism, and from one gene locus to another within the same organism, but most rates in eukaryotes range from 10^{-6} to 10^{-4} mutations per gene per generation (Ayala, 1976, Chapter 1). These rates for individual loci may seem low, but when multiplied by the number of genes in a genome (typically of the order of 10^4 in higher organisms) and the number of individuals in a population (e.g. average $N_e = 10^8$ in insect species) the potential for new variation in each generation is considerable.

Gene flow is the exchange of genes by sexual reproduction between interbreeding members of a species. Gene flow can be a random (e.g. passive dispersal of eggs or larvae in an aquatic environment), a directed (e.g. migration of juveniles and/or adults to feed or spawn), or even a selective process (e.g. non-random distribution of alleles associated with dispersal/migration capabilities). The relative importance of gene flow in determining patterns of genetic variation in a species will depend primarily on the distribution of the species and its life history characteristics - particularly its vagility. Passive gene flow (i.e. where allele

distributions are independant of dispersal/migration capabilities) will tend to promote a uniform spatial pattern of genetic variation wherever it occurs to any significant degree. Passive gene flow will normally operate in opposition to any force, whether random or directed, which tends to disrupt patterns of genetic variation. The patterns observed in natural populations will thus be manifestations of a dynamic equilibrium between these opposing forces.

Genetic drift describes the process whereby, through the accumulation of chance sampling accidents during the reproductive process, the pattern of genetic variation in a given population will change in a random fashion from generation to generation (e.g. change in frequency of alleles at a polymorphic locus). Because drift is a stochastic process, the magnitude of its effect is primarily dependant on the size of the population. In the absence of any other factors, we might expect to see appreciable variation over a few generations in the genetic structure of a population with a size of 10^2 or less, but very little change over the same time period in a population of magnitude 10^4 or more (Chakraborty and Nei, 1977). As mentioned above, any substantial passive gene flow between populations which have become genetically distinct through drift will tend to reduce this difference and minimise the possibility of it occuring again.

The invoking of random drift as an explanation for genetic differentiation, particularly between large populations, is based on the assumption that there are no selective differences between genotypes at a given locus (i.e. alleles are neutral with respect to one another). It has been

proposed that most allelic variation in structural loci, as detected by electrophoresis, is effectively neutral and that observed amounts and distributions of genetic variation are largely the result of time-dependant stochastic processes (Kimura, 1968; Kimura and Ohta 1971a, 1974). According to this "neutral" hypothesis mutations are constantly arising and most polymorphisms observed at a single point in time are merely transient, with each allele drifting to eventual extinction or fixation (Kimura and Ohta 1971b; Ohta 1974). The only type of selection event recognised under the neutral hypothesis is the removal of deleterious or lethal mutants from a population as they arise.

Natural selection is the process whereby interactions between the gene products, or phenotypes, of individual organisms and their environment renders them more or less "fit" than their conspecifics to contribute genetically to the next generation. This process relies on the existence of differences between the allelic products of gene loci which render them comparatively more or less adapted in a given environment. The concept of natural selection is one of the cornerstones of Darwinian evolutionary theory, and has been intuitively invoked on countless occasions to help explain the great diversity of organisms existing at present and in the fossil record.

There appears to be accumulating circumstantial evidence (e.g. Gillespie and Langley, 1974; Ohta, 1974; Kimura, 1976; Milkman, 1976a, 1978; Selander, 1976; Johnson, 1976) that the neutral hypothesis does not hold generally for protein variation and that a large proportion of electrophoretically detectable allozymic differences are of potential adaptive

significance (although to date selective coefficients have been measured only in laboratory populations). Wills (1973) has taken the extreme standpoint and suggests that all amino acid substitutions in proteins can potentially give rise to alleles with selective advantages or disadvantages, no matter how small the difference. He suggests that most mutations are unfavourable and are deleted, and that a very minor proportion are very favourable and quickly take over in a population. Most currently observed polymorphisms are attributed by Wills to the accumulation in time of allelic arrays with low selective differences or coefficients, and which are either being eliminated very slowly or are being maintained by selection in a balanced polymorphic state.

In view of the multitude of conflicting data and hypotheses published in recent years it seems most realistic to assume that both random and deterministic forces are operating to shape the pattern of genetic variation in a given population, but that the relative importance of these forces will vary in both space and time from genotype to genotype, from population to population and from species to species. For example in small populations Ohta (1974) points out that allelic selection coefficients (S) will be ineffectual, rendering the corresponding genotypes effectively neutral. It has been calculated that values of S required to overcome random genetic drift are of the order $S >> \frac{1}{4N_e}$, where N_e is the effective population size (Kimura and Crow, 1970; Milkman, 1978). As mentioned earlier passive gene flow will, under certain circumstances, also act as a unifying force in opposition to any selective pressures promoting change in patterns of genetic variation (Echelle et al., 1976; Jackson

and Pounds, 1979).

There are three main modes of selection that can influence the patterns of genetic variation in natural populations, namely directional selection, stabilising (or normalising) selection and diversifying (or disruptive) selection (Grant, 1977; Dobzhansky et al., 1977). Directional selection brings about a unidirectional change in the genetic composition of a population in response to a new or progressively changing environment, or to the advent of an allele with superior fitness. One of the best known examples of this type of selection is the ascendancy of a melanic form of the moth *Biston betularia* in response to pollution by industrial soot (Kettlewell, 1973). Under a new environmental regime the relative fitness values of genotypes will alter, and a formerly well adapted allele may be replaced in the population by another allele with superior fitness under the new conditions. Stabilising selection in a population well adapted to a given environment acts primarily to maintain optimum phenotypes and to eliminate occasional variants of lower fitness that may arise by mutation, gene flow or other random processes. This type of selection is probably the most common in nature, but is unspectacular and difficult to demonstrate, and is often overlooked. Diversifying selection occurs when a population is exposed to a heterogeneous environment, so that some habitats are favourable for some genotypes, and others for competing genotypes. In this situation the adaptedness of the population is enhanced by genetic diversity. The case of shell colour polymorphism in the European snail *Cepaea nemoralis* (Cain and Sheppard, 1954) is a good example. Seasonal fluctuations in frequency

of shell colour morphs reflect changing habitat colour patterns, which determine the relative fitness of morphs as camouflage against selective bird predation.

It has been proposed that most electrophoretically detected protein polymorphisms are responsive to environment-mediated selection and are maintained in natural populations by balancing selection - a form of stabilising selection (Richmond, 1970; Clarke, 1970; Prakash, 1973; Gillespie and Langley, 1974; Hedrick et al., 1976). The mechanism most commonly invoked to account for stable polymorphisms has been heterozygote superiority (also known as overdominance or heterosis), a situation where the heterozygous genotype has selective advantages over homozygous genotypes under most environmental conditions encountered by the population (e.g. Koehn, 1969). More recently Gillespie and Langley (1974) and Zouros (1976) have questioned the generality of this model, suggesting that in a majority of enzyme polymorphisms where potentially selective functional differences have been measured the heterozygous phenotype (including hybrid molecules in multimeric enzyme systems) is only intermediate in function and fitness compared to the homozygous phenotypes. The maintenance of a polymorphism under these conditions therefore requires a fluctuating environment, whereas the overdominance model will maintain a polymorphism in a uniform environment provided the heterozygous phenotype has maximum fitness.

Another type of balancing selection thought to contribute to the maintenance of genetic polymorphisms is frequency-dependant selection (Clarke, 1972), a situation where the relative fitness of a given allelic genotype is determined

largely by the proportion of individuals in the population that also have that particular genotype. Maximum selection for the genotype will occur when its frequency in the population is low and the habitat for which it has maximum fitness is under-utilised. Maximum selection against the genotype occurs when its frequency is high and it has saturated its preferred habitat, causing a spill-over into less favourable habitats. It has been suggested, for example, that this type of selection maintains a wing colour polymorphism in the butterfly *Papilio memnon* (Clarke and Sheppard, 1971), where Batesian mimicry appears to have been adopted to minimise bird predation.

It can be seen from the above discussion that spatially or temporally varying environments are theoretically able to maintain genetic polymorphisms. Hypotheses of causal relationships between genetic heterozygosity and some type of environmental variation date back to the early 1950's, and such concepts have been developed by Ludwig (1950), Levene (1953), Levins (1968), Selander and Kaufman (1973), Somero and Soulé (1974), Gillespie and Langley (1974), Johnson (1976), Valentine (1976), Ayala and Valentine (1979) and others. Most of these proposed environment/genotype associations are couched in terms of "adaptive strategies" to various aspects of the environmental regime, such as niche width or environmental grain, amplitude of environmental fluctuations, and predictability of environmental resources. Most of the evidence presented so far is equivocal. This problem will be further discussed in chapter 6 in relation to the results obtained in this thesis.

One commonly observed pattern of genetic variation in

natural populations is a cline or directional change in allele frequencies at a polymorphic locus over geographical distance. Clines across a species' distribution are often accompanied by a corresponding environmental gradient, suggesting that selection plays an important part in the differentiation of populations across the species' range, and that the geographic shape and amplitude of the cline are controlled by the interaction of selection and gene flow between differentiated populations (Endler, 1973, 1977; Nagylaki, 1975, 1976). There are, however, other possible explanations for observed clines. For example a cline may be merely a transient polymorphism which is the result of a favourable mutant progressively replacing superceded alleles across a species' range. Or a cline may form through secondary contact and gene flow between populations which had previously differentiated by random genetic drift in isolation from one another. The formation and shape of a cline can also be affected by the behaviour of the organism concerned. For example the adaptive significance of a polymorphism can be maximised by each individual actively selecting the habitat corresponding to optimal fitness of its specific genotype (Taylor, 1975; Casterlin and Reynolds, 1977). In practice it is very difficult to distinguish between these possibilities when describing clinal variation, particularly when the change in allele frequencies across the cline is small. It is therefore very important to know as much as possible about the ecological requirements and past history of a species in order to put observed patterns of clinal genetic variation in context.

One of the fundamental problems encountered during most

studies of genetic variation in natural populations is that the data can be explained on the basis of either random or deterministic theories. There are several major factors contributing to this dilema. Firstly, in natural populations it is extremely difficult - usually for logistic reasons - to obtain reliable estimates of effective population size, genic mutation rates and gene flow (dispersal and/or migration). Secondly, in studying environment/genotype interactions it is difficult to quantify environmental heterogeneity and to identify specific environmental parameters which may be affecting the organism most strongly. Thirdly, there is increasing support for the suggestion that very few loci in the genome of an organism are likely to yield measures of strong selection on an individual basis (Franklin and Lewontin, 1970; Soulé, 1973; Mitton and Koehn, 1975). Hedrick (1975) estimated that on average less than 50 loci per individual genome in a given environment might experience strong individual selection. He suggested that most loci undergo either epistatic selection as co-adapted gene complexes or stochastic perturbation as tightly linked chromosomal blocks - for example the chromosome III inversion polymorphisms in *Drosophila* (Dobzhansky, 1970) - and that individual polymorphisms may be selectively useful only in that they contribute to overall heterozygosity of the organism. Finally, any electrophoretic study of protein variation will be subject to inherent limitations of the technique. It has been calculated that on average only about 30% of all single amino-acid substitutions will lead to net surface charge differences detected by electrophoresis (Lewontin, 1974; King and Wilson, 1975) and that electrophoretic detectability may vary from

locus to locus (Johnson, 1973). In addition there is accumulating evidence (Milkman, 1976b) of extra alleles within a single electrophoretic mobility class (or electromorph) which are only detectable by methods such as thermal stability tests and molecular sieving. Whether electrophoretically cryptic variation is more or less important than surface charge variants in determining the adaptive contribution made to the organism by a given locus is not yet known, but the potential source of bias arising from the exclusive use of electrophoretic variation is obvious.

In spite of the above-mentioned difficulties Clarke (1975) has outlined a strategy for determining if an enzyme polymorphism is subject to selection. This strategy includes:- (1). Detection of alleles by as many different techniques as possible (2). Characterisation of structural and functional differences between alleles (3). On the basis of known differences, postulating selective factors and suggesting mechanistic relationships between selective factors and gene products (4). Testing the postulated mechanisms by experimentally manipulating environmental conditions to produce a predicted response and (5). Examining natural populations to determine whether or not the experimental model fits observed gene frequencies. Despite the heuristic appeal of this strategy and the promising results obtained from the application of this method to the alcohol dehydrogenose locus in *Drosophila melanogaster*, it is likely that the complete and successful application of the above scheme will be severely hampered by a lack of suitable polymorphic loci and of organisms amenable to experimental manipulation.

3.1.2 Genetic Variation in Marine Organisms

Studies in marine organisms which, to a certain extent, have been connected with the problem of genetic variability in relation to the environment, began a considerable time ago. Most of the early studies were on invertebrate taxa because of their accessability (low vagility and ease of capture) and ease of maintenance or manipulation under experimental conditions. For example Sexton and Clark (1936) observed that "recessive mutants" were quite abundant in populations of the amphipod *Gammarus chevreuxi* which had migrated from stable to ecologically variable environments, and from these results it was hypothesised (Bacci, 1954) that high frequency of recessive mutants would be a general feature in organisms occupying brackish water - a notoriously heterogeneous environment. These observations and predictions have not been supported by subsequent studies, and appear somewhat simplistic because of the lack of available methods for adequate genetic analysis at the time. Battaglia (1954, 1959) advanced the opposite hypothesis that genetic variability would be lower in brackish water populations than in marine populations, and supported this hypothesis with evidence from colour polymorphisms in the harpacticoid copepods *Porcellidium fimbriatum* and *Tisbe reticulata*. In *Tisbe* Battaglia was able to show that the colour polymorphism was genetically controlled, had an adaptive role, and was balanced - a considerable achievement at the time.

Studies on fish populations were begun somewhat later than those on invertebrates, possibly due to the logistic problems associated with collecting, maintaining and manipulating the generally more mobile vertebrate organisms.

In spite of attempts by von Toth (1932) to demonstrate racial differentiation of blood groups in herring, very few attempts were made to quantify genetic characters of fish species until the late 1950's (Marr, 1957; Cushing, 1964). Measurement of genetic variation in fish populations to a large extent originated from, and until the end of the 1960's was directed towards, problems in fisheries biology - particularly the need to identify distinct breeding populations in order to more fully understand the dynamics of abundance and distribution in commercially important fish species.

A review of studies in fish populations before 1970 (de Ligny, 1969) reveals that in a majority of cases blood group and serum protein polymorphisms were used as markers to determine the geographic distribution of populations. Some of the best known examples of this type of study are those carried out on the North Atlantic cod *Gadus morhua* using haemoglobin (Frydenberg et al., 1965), transferrin (Møller, 1966; Jamieson, 1970) and blood group polymorphisms (Møller, 1969); on the Atlantic herring *Clupea harengus* using transferrin and serum esterase systems (Naevdal and Haraldsvik, 1966; Naevdal and Danielsen, 1967; Naevdal, 1969); on Pacific skipjack tuna using blood groups (Sprague, 1963) transferrin (Barret and Tsuyuki, 1967; Fujino and Kang, 1968a) and serum esterases (Fujino and Kang, 1968b); and on the Pacific hake *Merlucius productus* using a transferrin system (Utter, 1969). It has been pointed out (Koehn, 1972), however, that the exclusive use of non-specific serum proteins in population studies can be misleading because these proteins are subject to non-inherited variation, and in many cases the observed patterns of variation are very difficult to type. Simonarson and Watts

(1969) found that serum esterase variation in populations of herring *(Clupea harengus)* can be at least partly attributed to the differential binding by the enzyme of small molecules such as sialic acid.

The advent of gel electrophoresis and its application to population studies of allozyme variation has greatly accelerated the amount of investigation into various aspects of evolutionary biology in marine organisms. This technique has been applied to numerous taxa of marine organisms to test hypotheses on genotype/environment interactions and on the evolutionary implications of patterns of variation. Summaries of population data on marine organisms over the past decade have been published by Selander and Johnson (1973), Powell (1975), Selander (1976) and Nevo (1978), but I will nevertheless cite a few specific studies which illustrate the types of questions to which electrophoretic data from marine organisms have been addressed.

One of the most common uses of electrophoretic data is for the estimation of amounts of genetic differentiation within and between closely related species, and subsequent inference of taxonomic and evolutionary relationships between the observed taxa. The literature is replete with studies of this type, but perhaps the most elegant examples from the teleost fauna are those on the Pacific salmon genus *Oncorhynchus* (Utter et al., 1973), the sunfish genus *Lepomis* (Avise and Smith, 1974), the pupfish genus *Cyprinodon* (Turner, 1974), and the atherinid genus *Menidia* (Johnson, 1975). King and Wilson (1975) and Wilson et al. (1977) have suggested that rates of protein and morphological evolution are largely independant of each other as they are controlled by separate

parts of the genome (i.e. structural loci verses regulatory loci). Supporting evidence, in the form of different amounts of morphological verses genetic divergence between species or subspecific populations, has been obtained from studies of two cyprinid fish species in California (Avise et al., 1975), the North American stickleback superspecies *Gasterosteus aculeatus* (Bell, 1976), Atlantic and Pacific species of the pomacentrid genus *Abudefduf* (Gorman and Kim, 1977), and North American and European species of the decapod lobster genus *Homarus* (Hedgecock et al., 1977). Mitton (1978), however, has produced data from the killifish *Fundulus heteroclitus* which supports the hypothesis that there is a positive relationship between heterozygosity at enzyme loci and developmental homeostasis. Mitton found that individuals heterozygous at any one of five polymorphic enzyme loci showed less morphological variance as a group than did homozygous individuals. This relationship was consistent in three separate populations and in both sexes.

Using a slightly different approach to the problem of the relationship between genetic variation and the evolutionary process, Avise and Ayala (1976) estimated genetic distances between species from ten genera of the speciose family Cyprinidae and compared them to genetic distances between eleven *Lepomis* species of the comparatively depauperate family Centrarchidae. The close agreement between average genetic distances within each of these two groups of similar evolutionary age suggested that genetic differentiation is a function of time since divergence rather than a function of the number of speciation events, and that the development of reproductive isolating mechanisms does not necessarily involve large amounts of change in structural genes.

A second recently expanding area in which the application of electrophoretic techniques has proved valuable is the attempt to identify factors which have given rise to and are maintaining observed patterns of genetic variation in natural populations. Lester (1979) has invoked panmictic gene flow through both larval dispersal and adult migration to account for genetic homogeneity of paeneid shrimps over distances of up to 1500 kilometers in the Gulf of Mexico. Random genetic drift due to dramatic population fluctuation and local extinction in a highly variable marginal environment is favoured by Vrijenhoek (1979) to explain genetic differentiation in stream populations of *Poeciliopsis monacha*. The pattern of differentiation in Canadian populations of lake whitefish *Coregonus clupeaformis* is explained by a theory of dispersal of genetically distinct post-glacial relict populations and a subsequent mixing of these isolates in some areas resulting in introgression of gene pools (Franzin and Clayton, 1977). A north-south cline in both overall heterozygosity and the allele frequencies of an esterase polymorphism has been reported for geographically isolated North American populations of longnose dace *Rhinichthys cataractae* (Merrit et al., 1978). It was concluded that this pattern arose through a progressive pattern of dispersal and loss of variability through founder effects.

By contrast Bell (1976) has invoked differential selection pressures to explain the genetic diversity found in local populations of the stickleback *Gasterosteus aculeatus*. Similar hypotheses have been used to explain spatial genetic differentiation in the North American eel *Anguilla rostrata* (Koehn and Williams, 1978) and in European populations of the

plaice *Pleuronectes platessa* (Beardmore and Ward, 1977).

Other approaches have been used to infer the adaptive significance of allelic variation at specific loci or sets of loci. One potentially sensitive method is to search for correlations between gene frequencies at homologous polymorphic loci in closely related sympatric species (Clarke, 1975). Such correlations would not be expected in the absence of selective forces and interspecific gene flow. The efficacy of this approach has been best demonstrated by species-pair comparisons of *Drosophila* (Ayala and Gilpin, 1974), but positive results have also been obtained from studies of the marine bivalve genus *Mytilus* (Koehn and Mitton, 1972) and the atherinid genus *Menidia* (Johnson, 1974). The biggest problem with this approach is difficulty in finding suitable sympatric species with homologous polymorphisms, and in this respect the marine environment, with great faunal diversity and comparatively more direct organism/environment interactions, appears most likely to provide the required conditions.

An increasingly popular method of infering the adaptive significance of genetic variation is to characterise some of the physiological and catalytic properties of allozymes in order to detect functional differences which are potentially subject to different selective pressures in a given environment. Results from this kind of work, together with observed correlations between gene frequency and the relevant environmental parameter(s), constitute substantial evidence that the locus examined is subject to selective forces. Studies of this kind are already numerous in the literature, and aquatic organisms which have proved amenable to this approach include the cyprinid sucker fish *Catostomus clarkii*

(Koehn, 1969), the freshwater sand shiner *Notropis stamineus* (Koehn et al., 1971), the fathead minnow *Pimephales promelas* (Merrit, 1972) and the killifish *Fundulus heteroclitus* (Mitton and Koehn, 1975; Place and Powers, 1979).

The effect of behavioural and other ecological interactions within and between species (e.g. competition and social hierarchies) can greatly influence population structure, and hence patterns of genetic variability, within a given species. Demonstrating the operation of such factors in populations of marine organisms is very difficult because of the logistic problems associated with measuring the required environmental and demographic parameters in an aquatic situation. Echelle et al. (1976) were able to show, however, that congeneric competition for territorial space was at least partially responsible for greater differentiation of allele frequencies between populations of the darter *Etheostoma spectabile* when it was sympatric with *E. radiosum*. *E. spectabile* is competitively excluded by its congener from certain areas of suitable stream habitat, creating spatial barriers to gene flow and allowing genetic drift or selective forces to differentiate gene frequencies. Simanek (1978) was also able to show that differences in average heterozygosity between southern U.S. populations of the live-bearing fish *Poecilia latipinna* were more closely correlated to the number of socially dominant males in the population than to other environmental or stochastic factors. The dominant males do most of the breeding in a population, so that fewer dominant males means less variability in the gene pool contributing to the next generation, and lower average heterozygosity.

Perhaps the most appropriate way of underlining the

usefulness of applying the electrophoretic technique to suitably chosen marine organisms is by referring to on-going studies of the bivalve mollusc genus *Mytilus* (Koehn and Mitton, 1972; Tracey et al., 1975; Koehn, et al., 1976; Milkman and Koehn, 1977; Koehn, 1978; Levinton and Lassen, 1978; Skibinski et al., 1978) and of a teleost, the eelpout *Zoarces viviparus* (see Christiansen and Simonsen, 1978 for a review of relevant studies). In both of these cases genetic variation - temporal, spatial and age-related - is seen in rich detail, and careful analysis of this detail has yielded not only information on the relative importance of various forces maintaining observed patterns of genic diversity, but also an insight into various aspects of the population structure and life history patterns of the species concerned.

3.1.3 Genetic Variation in Snapper and Western Salmon

In the absence of any previous genetic data on natural populations of Australian snapper and western salmon it is impossible to predict with any degree of certainty what patterns of genetic variation will be observed or how these patterns may be maintained. However there is a considerable amount of published literature in which correlations have been reported between degree and distribution of genic diversity, and the type of life history characteristics adopted by a species to cope with the demands of its environment (generally termed "adaptive strategies"). Valentine and Ayala (1978), while conceding that factors responsible for global patterns of genetic variability are still in dispute, present evidence to support the proposal

that in marine animals high genetic variability is correlated with high species diversity and temporal stability of the environment. Species under such conditions tend to be ecological specialists, and the high level of genetic variability may act to maintain adaptive flexibility in a functionally specialised genome while allowing fine-tuned adaptation to spatially differentiated micro-environments. Conversely, low genetic variability in species from areas of low species diversity is attributed to the higher fitness of functionally flexible gene products in organisms subject to harsh and temporally fluctuating environments.

An extra dimension to this model is that in general larger and more vagile organisms, which must be phenotypically more flexible to cope with an environment perceived as being unpredictably variable, have lower genetic variabilities than smaller and more sedentary organisms, which tend to perceive their environment as being coarse-grained or heterogeneous, but with some degree of temporal stability. Valentine and Ayala's model is a composite of ideas from a number of earlier models, such as the environmental grain and niche width/ variation hypotheses, and average heterozygosity estimates for 16 vertebrate and 27 invertebrate marine species show reasonable agreement with the predictions of the model. Given some knowledge of the distribution and life history characteristics of a species it is therefore possible to roughly assign this species to a position in the global hierarchy of genetic variability exhibited by the group of organisms to which the species is phylogenetically related.

Australian snapper and western salmon are both temperate water fishes, although the range of snapper extends slightly

further into low latitudes and that of western salmon into high latitudes. Given the pronounced latitudinal species diversity gradient found in teleosts and most other marine organisms (Schopf et al., 1978) both of the above species would be expected to fall into the middle to lower part of the range of average heterozygosities reported for fishes. In addition it might be expected that western salmon has lower levels of genetic variation than snapper because it is a highly mobile pelagic fish while snapper is a demersal and comparatively sedentary species. However these predictions might easily be confounded by stochastic effects, such as a loss of genetic variability when a population experiences a drastic reduction in size or during a recent speciation event. There is a possibility that the western salmon population, with a very restricted breeding area and a recent common ancestry with eastern salmon and kahawai, has been subjected to such stochastic effects. Comparative genetic data from eastern salmon and kahawai (see chapter 5) may throw some light on this question.

Consideration of the life history characteristics of western salmon and snapper is also useful in deducing the expected distribution patterns of any genetic variation detected in the two species. Western salmon has a well defined life history, including a restricted breeding season in a single area, long distance dispersal of eggs and larvae, a distinct juvenile nursery phase, and extensive migration of adults back to the spawning ground. Salmon also school by size and have a comparatively short life span (see chapter 2), so that a large number of schools consist of only one or two age classes. It therefore seems reasonable to expect that any

genetic differentiation in western salmon is likely to be manifested as year class differences due to differential survival of competing genotypes during the massive mortality event accompanying dispersal of eggs and larvae. An alternative but less likely pattern is the geographic differentiation of local stocks due to the imposition of a spatially heterogeneous selective regime on juveniles and sub-adults in the nursery areas.

By contrast the snapper is a comparatively sedentary demersal predator which seasonally aggregates in a number of preferred geographical areas to spawn serially over a two to three month period. Dispersal of eggs and larvae appears to be very limited and juveniles seek shelter in shallow, protected waters. While some adults undertake substantial migratory movements along the coastline, most tend to remain close to the area in which they were found as juveniles. It seems likely that under these conditions there will be substantially reduced gene flow across the species' distribution, and that any genetic variability is likely to take the form of differentiation of local populations - either in response to selective forces or to random genetic drift. Age-specific differences in allele frequencies may exist in response to temporal variation of selective regimes, but such effects will be very difficult to detect because of the long life span of the species (see chapter 2), the difficulty in ageing fish older than about 5 years, and the tendency of adult fishes of all ages to mix freely in spawning aggregations.

It is hoped that the ensuing electrophoretic studies will allow the above predictions to be tested, as well as providing some insight into population structure across the Australian

distribution of both snapper and western salmon.

3.2 Materials

3.2.1 Field Collection of Specimens

Sampling localities and associated collecting information are given for western and eastern salmon in Figure 3.1 and Table 3.1, and for Australian snapper in Figure 3.2 and Table 3.2. In all cases specimens were obtained by sampling the catches of commercial fishermen. Western and eastern salmon were collected using beach seines, ring nets, mesh or gill nets, and trolled handlines with fresh bait or artificial lures. Snapper were collected using hand lines, multiple-hook set lines, fish traps, mesh or gill nets, and demersal trawl nets. Most of the sampling was done personally, but some contribution was made by staff from Queensland University and the state fisheries authorities of Western Australia and South Australia, and by commercial fishermen (Table 3.1 and 3.2). The author gratefully acknowledges this assistance. Routine data collection on each fish sampled included fork length (L.C.F.), sex and state of gonad maturation. In the case of salmon the number of gill rakers on the first branchial arch were counted to distinguish between eastern and western forms. Where possible three to six scales (for ageing purposes) were taken from the shoulder region of a small subsample of fishes in each collection.

Figure 3.1

Location of collecting sites for sample sets of western and eastern salmon. See Table 3.1 for the sample code.

Australian Salmon

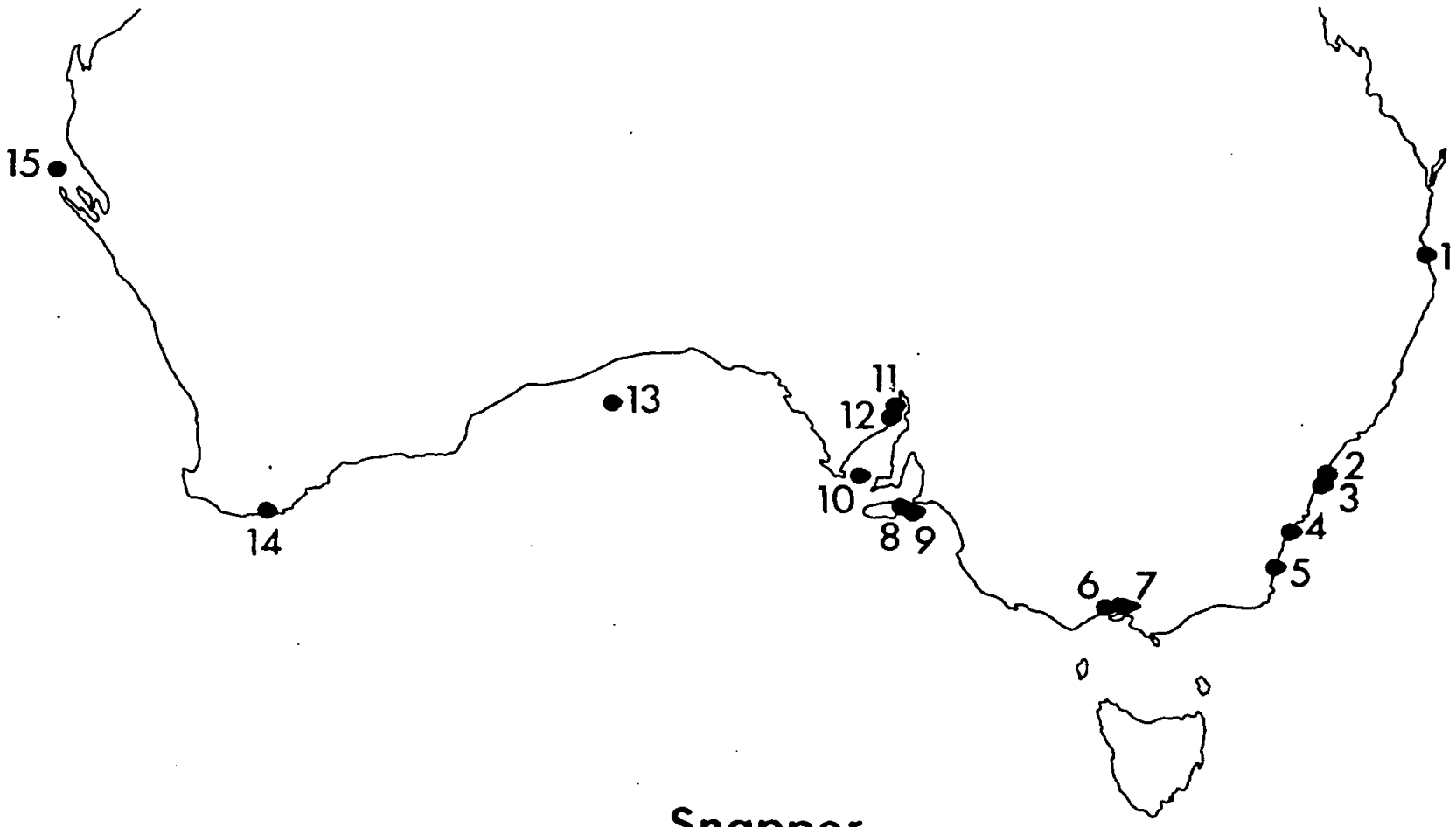
Table 3.1

Collection information for western and eastern salmon specimens electrophoretically surveyed for protein variation. * = specimens identified by gill raker counts as being eastern salmon.

Sample	Collecting Locality	Map Reference	Sample Size (n)	Collection Date	Length Range (LCF)	Capture Method	Sampled By
1	Port Phillip Bay, Vic.	38°21'S; 144°49'E	56	November 1976	23-38cm	beach seine	author
2	Murray River Mouth, SA	35°34'S; 138°53'E	45	October 1976	18-22	gill net	author
3	The Pages, SA	35°53'S; 138°23'E	55	November 1976	36-66	trolled handline	author
4	Cape Jervis, SA	35°38'S; 138°07'E	77	November 1976	22-38	trolled handline	author
5	Franklin Harbour, SA	33°45'S; 136°57'E	77	October 1976	16-25	ring net	author
6	Mt. Dutton Bay, SA	34°31'S; 135°25'E	50	October 1976	16-23	ring net	author
7	Venus Bay, SA	33°10'S; 134°38'E	51	October 1976	20-26	ring net	author
8	Bremer Bay, WA	34°21'S; 119°23'E	96	March 1977	51-68	beach seine	WA Department of Fisheries & W'life
9	Two Peoples Bay, WA	34°57'S; 118°11'E	73	March 1977	55-67	beach seine	WA Department of Fisheries & W'life
10	Bannister Head, NSW	35°20'S; 150°30'E	3*	January 1977	47-49	trolled handline	commercial fishermen
11	Twofold Bay, NSW	37°05'S; 149°53'E	3*	January 1977	44-49	trolled handline	author
12	Port Phillip Bay, Vic.	38°21'S; 144°49'E	13*	November 1976	28-36	beach seine	author
13	Five Mile Beach, Tas.	41°02'S; 146°55'E	64*	May 1977	32-46	beach seine	author

Figure 3.2

Location of collecting sites for sample sets of Australian snapper. See Table 3.2 for the sample code.



Snapper

Table 3.2

Collection information for Australian snapper specimens electrophoretically surveyed for protein variation.

Sample	Collecting Locality	Map Reference	Sample Size (n)	Collection Date	Length Range (LCF)	Capture Method	Sampled By
1	Moreton Bay, Qld.	27°07'S; 153°21'E	53	August 1979	13-24cm	hand line	Qld. Uni. staff
2	Off Sydney Heads, NSW	33°51'S; 151°20'E	55	May 1977	23-50	fish traps	author
3	Off Sydney Heads, NSW	33°51'S; 151°20'E	33	July 1978	28-52	fish traps	author
4	Bannister Head, NSW	35°20'S; 150°30'E	29	December 1976	33-61	hand line	commercial fishermen
5	Narooma, NSW	36°16'S; 150°08'E	33	March 1979	23-65	hand line	author
6	Port Phillip Bay, Vic.	38°18'S; 144°53'E	62	November 1976	36-81	set lines	author
7	Port Phillip Bay, Vic.	38°18'S; 144°53'E	64	November 1977	38-72	set lines	author
8	Backstairs Passage, SA	35°45'S; 138°08'E	37	March 1977	30-79	hand line	author
9	Backstairs Passage, SA	35°45'S; 138°08'E	57	November 1977	50-82	hand line	author
10	Rosalind Shoal, SA	34°58'S; 136°24'E	56	November 1978	29-64	hand line	S.A. Fisheries Division staff
11	Cowled Landing, SA	33°20'S; 137°30'E	33	November 1977	25-53	hand line	author
12	Cowled Landing, SA	33°20'S; 137°30'E	70	April 1979	27-67	hand line	A.N.U. staff
13	Great Aust. Bight, WA	33°12'S; 128°21'E	20	March 1978	31-71	trawl net	WA Department of Fisheries & W'life
14	Wilson's Inlet, WA	34°57'S; 117°22'E	18	May 1978	22-27	gill net	author
15	Shark Bay, WA	24°39'S; 113°12'E	60	May 1978	35-67	hand line	author

3.2.2 Tissue Extraction and Storage

Samples of liver tissue were dissected from each specimen within four to five hours of capture. These 2 to 3 cm^3 samples were placed in small capped plastic vials and snap frozen in a field container of liquid nitrogen at approximately -180°C. In most collecting areas the sexing of fish and dissection of liver tissue was readily facilitated by a mid-ventral incision to expose the visceral cavity. In some areas, however, the market quality of snapper would have been seriously reduced by readily visible incisions. In the interests of co-operation with the fishermen an alternative method was developed for extracting liver tissue. This method involved making an inconspicuous incision in membranous tissue underneath the gill arches, and probing behind the pectoral girdle into the visceral cavity to draw out some liver tissue. Specimens treated in this manner were also sexed by an alternative method which involved probing the anus, piercing the rectal wall, and drawing out some adjacent gonad material from the visceral cavity for examination.

Liver tissue samples were transported back to the laboratory either in liquid nitrogen or on dry ice, and were subsequently stored at -70°C until needed for electrophoretic or enzyme biochemical studies. Previous experience with the handling of fish tissues for electrophoretic studies (MacDonald, 1976, 1978) has shown that material stored in this manner will not show significant deterioration in enzyme activity or band resolution for at least two to three years. Results from

the present study have confirmed this finding.

3.2.3 Sample Size and Sampling Strategy

In planning a sampling survey of natural populations it is necessary to make an *a priori* decision about sample sizes. This decision will almost always represent a compromise between the practicality of sampling operations and the utility of the results obtained. An upper limit on sample sizes will be determined largely by available sampling resources and by reasonable expectations of successful collection given these resources. A lower limit on sample sizes will be determined by the maximum sampling error the investigator is prepared to tolerate when using statistical tests to discriminate between alternative hypotheses about the populations being sampled.

Sharp (1976), in assessing the usefulness of biochemical genetic data for stock identification and discrimination of pelagic mammals, listed the following statistical considerations as being important in deriving optimum sample size requirements:-

(a) the minimum difference in allele frequencies one is willing to accept as being biologically significant between two populations

(b) the maximum tolerated Type I error (α) i.e. the designated significance level for rejection of the null hypothesis that two samples were drawn from a single population

(c) the maximum tolerated Type II error (β). This statistical concept is usually expressed in the

form 1-β and is interpreted as the "power" of the statistical test (usually a χ^2 contingency test) to discriminate between the null and alternative hypotheses given a fixed value of α.

(d) the expected range of frequencies of a given allele in the population(s) to be sampled. The power of the statistical test to discriminate between allele frequencies is lowest in the .55 region and highest in the .90 region.

In the present study sample sizes of 50-70 individuals were initially decided upon, mainly because of practical limitations in the sampling operation. It was considered a desirable sampling strategy that, whenever possible, the collection from each area should represent the results of a single day's sampling, preferably from a single aggregation of fishes or from a single geographical point. It was hoped that this strategy would minimise the chances of obtaining confusing results by accidentally sampling a mixture of individuals from genetically distinct populations. The constraints of this strategy, together with the known efficiency of commercial fishing techniques, the uncertain availability and density of the fishes, and the time requirements of tissue dissection and data gathering for each specimen, made it unreasonable to expect sample sizes any larger than that mentioned above.

If a conventional significance level of $\alpha = .05$ is accepted, then extrapolation from tables showing the relationship between sample size and the variables listed above (Sharp, 1976) indicates that sample sizes of 50-70 will permit the differentiation of two populations with

allele frequency differences of magnitude .20 or more for common allele frequencies between .55 and .95, and differences of magnitude .10 or more for frequencies greater than .90 . Under these conditions, however, the discriminatory power of the test (1-β) is only .50 (or 50% certain). If, however, the range of observed common allele frequencies is restricted to .70 or above - as is the case for most of the sample estimates obtained for salmon and snapper in this study (see sections 3.4 and 3.5) - the discriminatory power of the statistical test is increased, and sample sizes of 50-70 will then permit differentiation of frequency differences of .20 or more with a power of 1-β = .80 (80% certainty).

It may be argued that in setting a lower limit of .20 as a significant difference in allele frequency between two populations, there is a risk that information will be lost by the failure to detect differences less than .20 but which nevertheless are valid population differences. It must be remembered, however, that sample estimates of allele frequencies are only an approximation of the actual frequencies in the populations sampled, and calculation of 95% confidence limits show that for sample sizes of 50-70 individuals and a frequency of, say, .75, the true value can lie anywhere within about ± .10. Even if the sample sizes are doubled to 100-140, the confidence interval is still about ± .075. The true difference in allele frequency between two populations may therefore be much less than that indicated by sample estimates, and the simplest way to minimise the possibility of rejecting the null hypothesis through this type of sampling error is to designate a larger

minimum significant difference in allele frequencies. Moreover, even if large sample sizes were used to demonstrate the statistical significance of allele frequency differences of .10 or less, the biological significance of such differences to the organism concerned would be questionable and would be difficult to demonstrate.

With these constraints in mind, sampling was undertaken at convenient sites throughout the Australian range of snapper and western salmon, with at least one sample of 50 or more eastern salmon for comparative purposes. Replicate samples of snapper were taken at irregular intervals from collecting sites in New South Wales, Victoria, South Australia and Western Australia (Table 3.2) to obtain measures of within-area sampling heterogeneity and to determine the temporal stability of any observed geographical patterns of genetic differentiation.

3.3 Methods

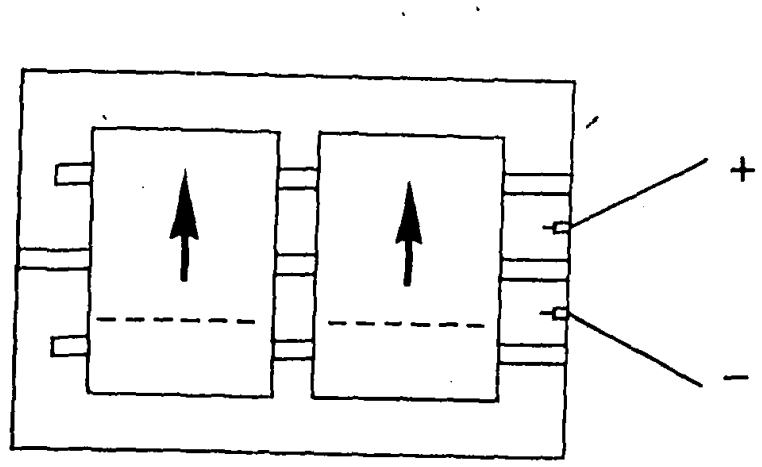
3.3.1 Electrophoretic Procedures

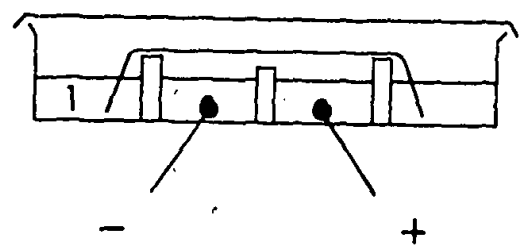
High voltage zone electrophoresis using 10cm x 17cm sheets of cellulose acetate gel supporting medium ("Cellogel" - Chemetron, Milan, Italy) was carried out in Gelman semi-micro electrophoresis tanks, each containing 400 ml of tank buffer (Figure 3.3). Electric current was supplied to the trays by Gelman constant voltage D.C. power packs (500 volts, 125 milliamps).

Small samples of frozen liver tissue were placed in glass vials on ice, and an equal amount by volume of cell-lysing

Figure 3.3

Diagram of the cellulose acetate electrophoresis apparatus used during the present study. The apparatus is shown both from above and in transverse section.





solution (0.2% β-mercaptoethanol in distilled water) was added to each sample. Samples were homogenised by masceration with a glass rod or, where necessary, by sonication. The samples were then centrifuged at 3,500 r.p.m. for 10 minutes, leaving the lysate containing the soluble proteins as a supernatant above the cell debris in the vial.

Cellogel sheets, stored in 30% methanol until used, were soaked in tank buffer solution for at least 15 minutes to remove alcohol from the gel. The sheets were then blotted to remove excess buffer and placed in the electrophoresis tanks. The sheets, with both ends immersed in the buffer, were held in position on tank bridges with magnetic metal grips. Individual protein samples were then applied to the porous side of the gel in a straight line using a draughtsman's pen and a ruler. A line usually consisted of 10 to 14 samples. The origin (point of application of the samples) was usually placed towards the cathodal end of the gel, as most of the enzymes surveyed in this study migrated towards the anodal end. When all samples had been applied the tanks were connected to the power packs and run at 250 volts and up to 0.8 milliamps/cm width of cellogel sheet for 70-150 minutes in a 5°C cold room. The length of an electrophoresis run depended on the time required to move a given enzyme about 5cm along the sheet from the origin. This distance was optimal in scoring comparative enzyme mobilities of different fishes.

When electrophoresis was terminated each cellogel sheet was removed from the tanks and immersed in a specific staining solution designed to visualise bands of activity of the desired enzyme. The sheets were then removed from the stain, blotted, and suspended horizontally in humid chambers to prevent the gel

drying out while bands of enzyme activity were staining up. When the bands were sufficiently resolved to be typed the staining reaction was terminated by immersing the cellogel sheet in 10% formalin. The gels were then stored at 5°C in 7% glycerol if required for future reference.

I have previously discussed the relative advantages of using cellulose acetate gels rather than other types of supporting media for electrophoretic procedures (MacDonald, 1976). The major reason is the speed and simplicity of the technique, which allows for the rapid screening of large numbers of samples giving the high quality resolution of phenotypes needed to undertake rigorous statistical analyses in population studies.

3.3.2 Buffers

A number of continuous tank buffers at various ion concentrations and pH levels were tested while developing methods for electrophoretically screening fish liver enzymes in this study. Buffers tested included tris citrate, tris borate, tris maleate, sodium barbitol, phosphate, and citrate-phosphate solutions. It was found that, for the enzymes surveyed, a buffer system of .05 molar tris maleate pH 7.8 gave the best results in most cases as a compromise between mobility/separation of allozymes and sharp definition of bands. Even when it was not the superior buffer system tris maleate still produced typable results, so it was eventually chosen as a standard buffer for screening all enzymes.

The tris maleate buffer consists of:-

50 mM. tris (hydroxy methyl) aminoethane (Sigma 7-9 grade)

1 mM. ethylenediamine tetraacetic acid (EDTA) (disodium salt, BDH reagent grade)

1 mM. magnesium chloride.

The pH is adjusted to 7.8 with a saturated solution of maleic acid (Fluka, reagent grade, Switzerland).

3.3.3 Enzyme Staining

The stain mixtures listed below were modified from Shaw and Prasad (1970), Brewer (1970), and Harris and Hopkinson (1977). All staining mixtures except that for esterase were made up to a volume of approximately 2 ml. Each 2 ml aliquot was poured onto a glass or perspex staining plate, and the cellogel sheets were stained by placing them face-down in the mixture for 5-10 seconds. A 2 ml aliquot was sufficient to stain four 10 cm x 17 cm gel sheets. Esterase stain mixtures were made up in quantities of 100 ml and each cellogel sheet was soaked in one of these aliquots until the bands were visualised.

The names used for enzymes are those recommended by the Nomenclature Committee of the International Union of Biochemistry (Enzyme Nomenclature, 1978), and the abbreviations follow the recommended format of Giblett (1976). The title and abbreviation of each enzyme is followed by its Enzyme Commission number.

Alcohol dehydrogenase (ADH) (E.C. 1.1.1.1)

1.2 ml	100 mM. tris-HCl (pH 8.0)
.2 ml	50% ethanol
.2 ml	oxidised nicotinamide-adenine dinucleotide (NAD) (10mg/ml)
.2 ml	phenazine methosulphate (PMS) (1mg/ml)
.2 ml	tetrazolium salt (MTT) (2mg/ml)

Glycerol-3-phosphate dehydrogenase (NAD^+) (GPD) (E.C. 1.1.1.8)

1.0 ml	100 mM. tris-HCl (pH 8.0)
.2 ml	glycerol-3-phosphate (sodium salt) (25mg/ml)
.2 ml	NAD (10mg/ml)
.2 ml	500 mM. magnesium chloride
.2 ml	PMS (as above)
.2 ml	MTT (as above)

Iditol (Sorbitol) dehydrogenase (SDH) (E.C. 1.1.1.14)

1.0 ml	100 mM. tris-HCl (pH 8.0)
.2 ml	sorbitol (40mg/ml)
.2 ml	sodium pyruvate (25mg/ml)
.2 ml	NAD (10mg/ml)
.2 ml	PMS
.2 ml	MTT

Lactate dehydrogenase (LDH) (E.C. 1.1.1.27)

1.2 ml	100 mM. tris-HCl (pH 8.0)
.2 ml	sodium lactate (25mg/ml)
.2 ml	NAD (10mg/ml)
.2 ml	PMS
.2 ml	MTT

Malate dehydrogenase (MDH) (E.C. 1.1.1.37)

1.2 ml	100mM. tris-HCl (pH 8.0)
.2 ml	sodium malate (25mg/ml)
.2 ml	NAD (10mg/ml)
.2 ml	PMS
.2 ml	MTT

'Malic' enzyme (ME) (E.C. 1.1.1.40)

1.0 ml	100 mM. tris-HCl (pH 7.6)
.2 ml	sodium malate (25mg/ml)
.2 ml	oxidised nicotinamide-adenine dinucleotide phosphate (NADP) (10mg/ml)
.2 ml	5 mM. manganese chloride
.2 ml	PMS
.2 ml	MTT

Isocitrate dehydrogenase ($NADP^+$) (IDH) (E.C. 1.1.1.42)

1.0 ml	100 mM. tris-HCl (pH 8.0)
.2 ml	sodium isocitrate (25mg/ml)
.2 ml	NADP (10mg/ml)
.2 ml	5 mM. manganese chloride
.2 ml	PMS
.2 ml	MTT

Phosphogluconate dehydrogenase (6PGD) (E.C. 1.1.1.44)

1.0 ml	100 mM. tris-HCl (pH 8.6)
.2 ml	6-phosphogluconic acid (sodium salt) (25mg/ml)
.2 ml	NADP (10mg/ml)
.2 ml	500 mM. magnesium cloride
.2 ml	PMS
.2 ml	MTT

Glucose-6-phosphate dehydrogenase (G6PD) (E.C. 1.1.1.49)

1.0 ml	100 mM. tris-HCl (pH 8.6)
.2 ml	glucose-6-phosphate (sodium salt) (25mg/ml)
.2 ml	NADP (40mg/ml)
.2 ml	500 mM. magnesium chloride
.2 ml	PMS
.2 ml	MTT

Galactose-6-phosphate dehydrogenase (Gal6PD) (E.C. ?)*

as for G6PD, but with galactose-6-phosphate as substrate

Glyceraldehyde-phosphate dehydrogenase (GAPD) (E.C. 1.2.1.12)

1.0 ml	100 mM. tris-HCl (pH 7.6)
10 µl	glyceraldehyde-3-phosphoric acid (in solution)
.2 ml	sodium pyruvate (25mg/ml)
.2 ml	NAD (10mg/ml)
.2 ml	sodium arsenate (1mg/ml)
.2 ml	PMS
.2 ml	MTT

Xanthine oxidase (XO) (E.C. 1.2.3.2)

1.4 ml	100 mM. tris-HCl (pH 7.6)
.2 ml	hypoxanthine (40mg/ml)
.2 ml	PMS
.2 ml	MTT

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*The enzyme catalysing the oxidation of galactose-6-phosphate is in fact a non-specific hexose-6-phospate ($NADP^+$) Oxidoreductase which is also visualised on gels stained for G6PD (Ruddle et al., 1968)

Superoxide dismutase (SOD) (E.C. 1.15.1.1)

1.6 ml	100 mM. tris-HCl (pH 8.0)
.2 ml	PMS
.2 ml	MTT

Aspartate aminotransferase (AAT) (E.C. 2.6.1.1)

1.2 ml	phosphate buffer (pH 7.0) (= 7.25gm $NaH_2PO_4.2H_2O$ and 2.70gm Na_2HPO_4 in 1 litre distilled H_2O)
.2 ml	aspartic acid (neutralised with sodium hydroxide) (50mg/ml)
.2 ml	α-ketoglutarate (sodium salt) (25mg/ml)
.2 ml	pyridoxal-phosphate (5mg/ml)
.2 ml	fast violet β (20mg/ml)

Adenylate kinase (AK) (E.C. 2.7.4.3)

1.0 ml	100 mM. tris-HCl (pH 8.0)
.2 ml	glucose (40mg/ml)
.2 ml	adenosine diphosphate (20mg/ml)
.2 ml	NADP (20mg/ml)
.2 ml	100 mM. magnesium chloride
5 µl	G6PD
5 µl	hexokinase
.2 ml	PMS
.2 ml	MTT

Phosphoglucomutase (PGM) (E.C. 2.7.5.1)

1.0 ml	100 mM. tris-HCl (pH 8.0)
.2 ml	glucose-1-phosphate (sodium salt) (25mg/ml) plus a trace of glucose-1,6-diphosphate
.2 ml	NADP (40mg/ml)
.2 ml	500 mM. magnesium chloride
10 µl	G6PD
.2 ml	PMS
.2 ml	MTT

Acetylesterase (EST) (E.C. 3.1.1.6)

35 mg	β napthyl acetate
50 mg	fast blue RR salt
100 ml	distilled H_2O

Guanine deaminase (GDA) (E.C. 3.5.4.3)

1.4 ml	phosphate buffer (as for AAT) or 100 mM. tris-HCl (pH 7.4)
.2 ml	guanine (25mg/ml - dissolved in sodium hydroxide)
40 µl	xanthine oxidase
.2 ml	PMS
.2 ml	MTT

Adenosine deaminase (ADA) (E.C. 3.5.4.4)

1.4 ml	phosphate buffer (as for AAT)
.2 ml	adenosine (25mg/ml)
40 µl	xanthine oxidase
10 µl	nucleoside phosphorylase
.2 ml	PMS
.2 ml	MTT

Fumarate hydratase (FUM) (E.C. 4.2.1.2)

1.2 ml	phosphate buffer (pH 7.5) (100ml = 16ml of 200 mM. $NaH_2PO_4.2H_2O$ and 84ml of 200 mM. Na_2HPO_4)
.2 ml	fumaric acid (25mg/ml - neutralised with sodium hydroxide)
.2 ml	NAD (10mg/ml)
5 µl	MDH
.2 ml	PMS
.2 ml	MTT

Mannosephosphate isomerase (MPI) (E.C. 5.3.1.8)

1.0 ml	200 mM. tris-HCl (pH 7.6)
.2 ml	mannose-6-phosphate (sodium salt) (25mg/ml)
.2 ml	NADP (10mg/ml)
.2 ml	200 mM. magnesium chloride
10 µl	G6PD
5 µl	glucosephosphate isomerase
.2 ml	PMS
.2 ml	MTT

Glucosephosphate isomerase (GPI) (E.C. 5.3.1.9)

1.2 ml	100 mM. tris-HCl (pH 8.0)
.2 ml	fructose-6-phosphate (sodium salt) (25mg/ml)
.2 ml	NADP (10mg/ml)
10 µl	G6PD
.2 ml	PMS
.2 ml	MTT

3.3.4 Terminology

Following the recommendations of Giblett (1976) a symbol has been assigned to every enzyme studied and written in non-italicised upper case letters (e.g. ADA for adenosine deaminase). The locus at which a protein is coded is given the same symbol, but in italics (e.g. *ADA*). Where there appears to be two or more loci coding for different electrophoretic forms of the same enzyme (isozymes), subscripts will be added to the symbols to distinguish these forms. For example LDH_A and LDH_B are two loci coding for different polypeptide subunits of the multimeric lactate dehydrogenase molecule, and AAT_S and AAT_M are supernatant and mitochondrial isozymes of aspartate aminotransferase. Allelic genes are identified by adding superscripts to the locus symbols (e.g. ADA^{100} and ADA^{113}). In this study the common allele at each polymorphic locus is designated 100, and each other allele is designated according to its proportional electrophoretic mobility compared to the common allele. Superscripts > 100 indicate a more anodally migrating allele. Genotypes will be indicated by pairs of superscripts, such as $ADA^{100/100}$ or $ADA^{100/113}$, and allelic phenotypes (allozymes) by non-italicised locus symbols followed by the appropriate allelic designation (E.G. ADA 100 or ADA 100-113).

While no breeding studies have been conducted, the genetic basis of observed electrophoretic variation has been inferred either by the close agreement of phenotype frequencies with Hardy-Weinberg expectations, or by comparison with published electrophoretic data on teleosts in which the heritability of phenotypes has been demonstrated. The enzymes

screened in this study were randomly chosen from the 50 to 60 enzymes which have been routinely surveyed in studies of natural populations over the past few years. The sole criterion for selection was ease of electrophoretic resolution of phenotypes.

3.4 Results - Salmon

3.4.1 Electrophoretic Patterns of the Enzymes

A total of 22 enzymatic proteins (section 3.3.3) were electrophoretically surveyed in nine sample sets of western salmon, totaling 580 individuals. The same enzymes were also surveyed in a total of 83 eastern salmon specimens from four sample sets (Table 3.1). From this survey an apparent 27 gene loci were scored for comparative electrophoretic mobility of their enzymatic products. Initial screening of loci for allelic polymorphisms was carried out using a sub-sample of 90 western salmon (10 from each sample set) and all of the available eastern salmon specimens. When allelic variation was detected at a particular locus all other western salmon specimens were screened to determine the geographic distribution of the observed variation.

At 15 out of 27 loci both eastern and western salmon exhibited identical monomorphic patterns of electrophoretic mobility. Isozymes in this category included ADA_2, GPI, PGM, Gal6PD, MDH_S, MDH_M, LDH_A, AAT_S, AAT_M, GAPD, XO, AK_2, GPD, $G6PD_1$ and $G6PD_2$. In addition, SOD was monomorphic in both western and eastern salmon, but with apparently fixed differences between the two forms - SOD 100 in western salmon and SOD 160

in eastern salmon. A similar situation was observed for LDH_B, with western salmon apparently fixed for LDH_B 100 and eastern salmon for LDH_B 87.

All of the above isozymes except MDH_S, LDH_A, LDH_B, AK_2, $G6PD_1$ and $G6PD_2$ exhibited single-banded patterns of staining activity after electrophoresis. MDH_S activity was expressed on cellogel as three clear bands in all individuals. Bailey et al. (1970), Utter et al. (1973) and Johnson (1975) postulate a dimeric subunit structure for NAD-dependant MDH in fishes. The MDH_S pattern in western and eastern salmon could be explained by postulating control by two loci, with the third band being an inter-locus heterodimeric isozyme. The two most anodal bands, however, show a considerable variation in staining intensity, and a more likely explanation for this pattern is the presence of some form of non-inherited variation in MDH molecular structure. Such possibilities include protein denaturation, interchangeable conformation states, and differential binding of cofactors or other molecules by the enzyme.

The LDH_A and LDH_B loci in salmon code for different polypeptide subunits which randomly associate to give the typical five-banded electrophoretic pattern of tetrameric LDH isozymes (Markert and Faulhaber, 1965). The LDH pattern for western salmon is more compact than that of eastern salmon because of the fixed allelic differences at the LDH_B locus.

The electrophoretic pattern of AK_2 in salmon consists of a strong cathodal band with a varying number of progressively less intense anodal bands in each sample. AK exhibits monomeric patterns of allelic variation in humans (Harris and Hopkinson,

1977) and fishes (Johnson, 1975), and the observed multi-band salmon AK_2 pattern can best be explained by one of the non-inherited types of variation described above.

G6PD activity in salmon consists of two widely spaced bands with a less intense intermediate band. The simplest interpretation of this pattern is to postulate genetic control at two loci, $G6PD_1$ and $G6PD_2$, with the intermediate band being an inter-locus heterodimeric isozyme. The quaternary structure of G6PD in fishes is not well documented, however, and an alternative explanation of the observed three-banded G6PD pattern in salmon is that G6PD is a tetramer which exhibits non-random subunit association. Enzyme biochemical studies are required to resolve this problem.

The staining of gels for ADA and GDA revealed two regions of activity in both cases. The faster migrating ADA_1 and GDA_1 bands were typeable, but the slower migrating cathodal ADA_2 and GDA_2 bands were very diffuse. Efforts to increase the resolution of bands of activity for these isozymes were unsuccessful, and they were consequently not scored.

Table 3.3 lists the ten remaining loci at which allozymic variation was observed in either western or eastern salmon. The eastern salmon sample of 83 is a composite of material collected from four different locations (see Table 3.1). Sample sizes at three of the four locations were not adequate to permit population studies, so the collections were combined to form a representative eastern salmon sample for comparison with data from western salmon samples.

Western salmon exhibited monomorphic single-banded electrophoretic patterns for IDH, SDH, ME and GDA_2, and in each of these cases the homozygous phenotype of the

Table 3.3

Allele frequencies for 10 polymorphic enzyme loci in samples of western and eastern salmon. For explanation of sample code numbers see Table 3.1.

Sample		1	2	3	4	5	6	7	8	9	E. Salmon (total)
n		56	45	55	77	77	50	51	96	73	83
Locus	Allele										
EST	107	.01	-	-	.01	.03	-	-	-	.03	-
	100	.90	.95	.96	.96	.93	.94	.95	.95	.88	.97
	93	.04	.03	.02	.01	.03	.03	.01	.03	.03	.03
	88	.05	.02	.02	.02	.01	.03	.04	.02	.06	-
IDH	113	-	-	-	-	-	-	-	-	-	.02
	100	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.98
FUM	100	.97	.98	1.0	.97	.99	1.0	.97	.96	1.0	1.0
	88	.03	.02	-	.03	.01	-	.03	.04	-	-
ADH	100	1.0	1.0	.99	1.0	.99	.99	1.0	.99	1.0	1.0
	40	-	-	.01	-	.01	.01	-	.01	-	-
6PGD	100	1.0	1.0	.98	.96	1.0	1.0	1.0	.97	.96	1.0
	89	-	-	.02	.04	-	-	-	.03	.04	-
SDH	100	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.70
	72	-	-	-	-	-	-	-	-	-	.30
ME	112	-	-	-	-	-	-	-	-	-	.03
	100	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.53
	93	-	-	-	-	-	-	-	-	-	.06
	75	-	-	-	-	-	-	-	-	-	.33
	68	-	-	-	-	-	-	-	-	-	.05
GDA_2	100	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.97
	84	-	-	-	-	-	-	-	-	-	.03
AK_1	100	1.0	.99	1.0	1.0	1.0	1.0	1.0	1.0	.99	1.0
	83	-	.01	-	-	-	-	-	-	.01	-
MPI	108	-	-	-	-	-	-	-	-	-	.02
	100	.99	1.0	1.0	1.0	1.0	1.0	1.0	1.0	.97	.75
	82	.01	-	-	-	-	-	-	-	.03	.23

common allele in eastern salmon had an identical mobility. The rare variant alleles IDH^{113} and GDA_2^{84} in eastern salmon were detected in heterozygous form only, with IDH exhibiting a typical three-banded dimeric pattern and GDA a two-banded monomeric pattern. A two-allele polymorphism was detected at the *SDH* locus, with all three phenotypes being observed. Heterozygotes exhibited the typical five-banded pattern of an enzyme with tetrameric subunit structure. Five alleles and 12 out of 15 possible phenotypes were detected at the *ME* locus (Plate 3.1), with heterozygotes again exhibiting the five-banded pattern of a presumed tetrameric molecule.

Rare heterozygous variants were found at the *FUM*, *ADH*, *6PGD*, AK_1 and *MPI* loci in western salmon, but the frequencies of these variants were so low that they were not detected in many of the geographical sample sets. ADH 100-40 and 6PGD 100-89 phenotypes exhibited three-banded dimeric patterns, while FUM 100-88 and MPI 100-82 revealed five-banded patterns and AK_1 100-83 two-banded patterns. Eastern salmon individuals had monomorphic single bands identical to the western salmon common allele phenotype at the *FUM*, *ADH*, *6PGD* and AK_1 loci, but were polymorphic at the *MPI* locus, containing the same two alleles as the western salmon samples plus a third rare variant, MPI^{108}.

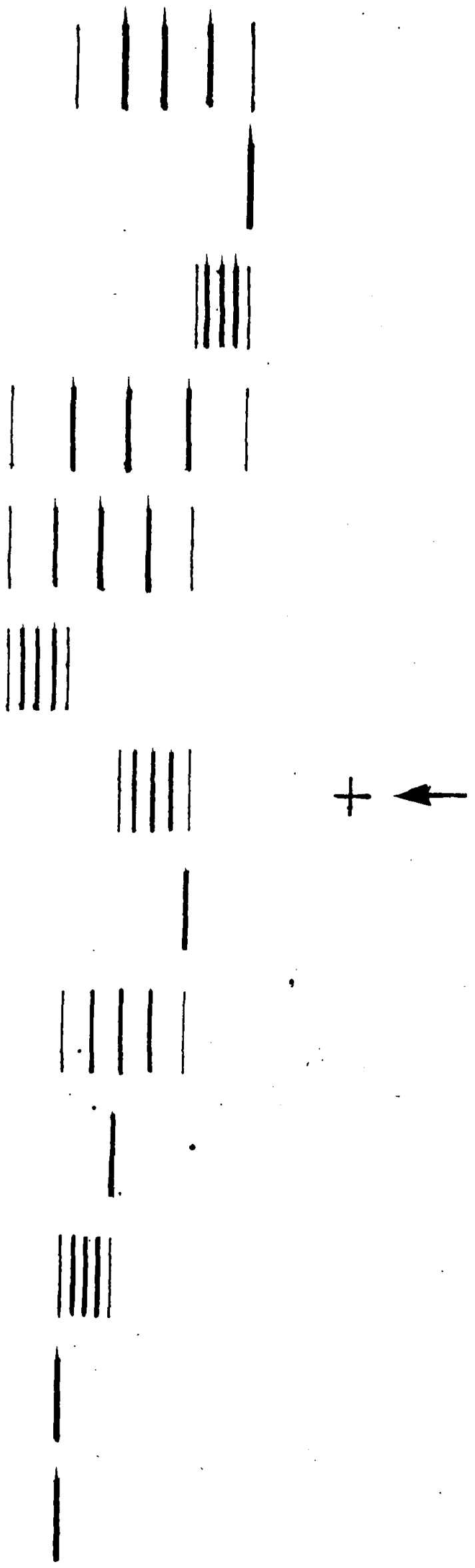
Of 27 loci examined in western salmon only *EST* exhibited a polymorphism in which variation was detected in all geographical sample sets, and in which the common allele frequency of some sample sets was below .95 - a conventionally accepted limit for a *bona fide* polymorphism.

Plate 3.1

Observed ME electrophoretic phenotypes in western and eastern salmon. From left to right the phenotypes are:-

ME 100 west.; ME 100 east.; ME 100/93; ME 93; ME 100/75; ME 75; ME93/75; ME 112/100; ME 112/75; ME 112/68; ME 75/68; ME 68; ME 100/68.

Note No photographic illustrations of gel phenotypes were possible because of deterioration of tissue samples due to a freezer failure.



Consequently *EST* was the only potentially useful genetic marker for studying population structure in western salmon. Four *EST* alleles and six out of ten possible phenotypes were detected (Plate 3.2). Scoring of EST phenotypes was complicated by the presence of secondary anodal bands of varying intensity in most individuals, resulting in two-band homozygous phenotypes and three- and four-band heterozygotes. The three-banded EST 100-93 phenotype occurs when the secondary band of the slower migrating allozyme is superimposed on the main band of the faster migrating allozyme. Simonarson and Watts (1969) have attributed such non-inherited variation in esterase systems to the binding by the enzyme of small molecules such as sialic acid.

Eastern salmon were also polymorphic for the *EST* locus, with a common allele electrophoretically identical to that in western salmon. Only one other rare variant, EST^{93}, was detected - an allele also shared with western salmon.

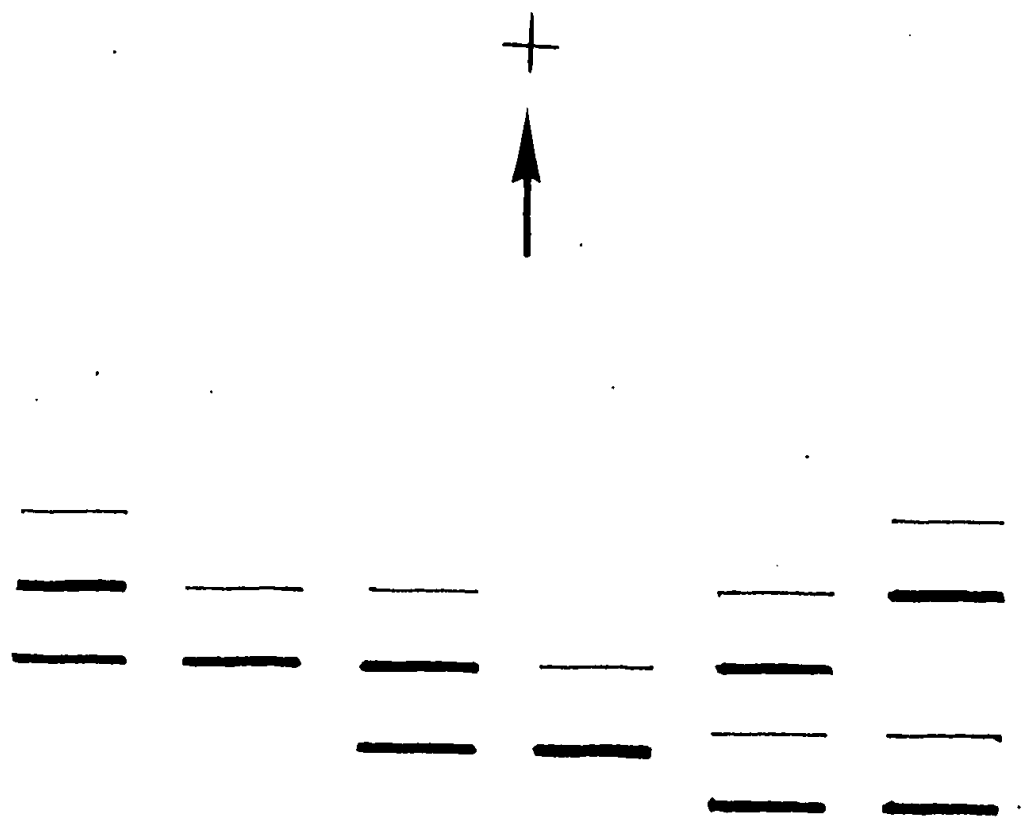
3.4.2 Amount of Genetic Variability

Of 27 enzyme loci screened for allelic polymorphism in western salmon six yielded electrophoretic variants. Only three loci (11.1 %) exhibited polymorphisms in which the frequency of the common allele for the combined samples was less than .99. These loci were *EST*, *FUM* and *6PGD*. Of these only *EST* (3.7 % of all loci) had a polymorphism with a common allele frequency of less than .95. Selander (1976) has reported an average of 30% loci polymorphic in 14 species of fishes on the basis that a polymorphism exists

Plate 3.2

Observed EST phenotypes in western salmon. From left to right the phenotypes are:-

EST 107/100; EST 100; EST 100/93; EST 93; EST 100/88; EST 107/88.



om

38;

when the common allele frequency (p) $\leq$ .99. Nevo (1978) has reported a much lower proportion of polymorphic loci (average 15.2%, range 0.0% to 56.0%) in 51 species of bony fishes, but direct comparison of these data with Selander's is difficult because it is not clear whether $p \leq .95$ or $p \leq .99$ was used as the criterion for a valid polymorphism. Whichever criterion or set of data is used, the western salmon result appears to be well below the average level of polymorphism for teleost fishes measured to date.

Perhaps the most informative single measure of the amount of genetic variation in a population is the overall incidence of heterozygosity (H). Heterozygosity can be measured as the mean proportion of heterozygotes per locus, or as the mean proportion of loci heterozygous in each individual. The estimates using either of these methods are the same but each method requires different sampling strategies, confidence statements and interpretations (Nei and Roychoudhury, 1974). The H estimate used here is mean heterozygosity per locus per individual - a combination of the above two methods. For each salmon sample set the total number of genotypes examined was obtained from the product of the sample size and the number of loci screened. H is thus the total number of observed heterozygotes as a proportion of total genotypes.

Mean estimated heterozygosity for the combined western salmon data is H = .008 ± .0003, with values for each of the individual samples ranging from H = .004 ± .0016 to H = .015 ± .0027 (Table 3.4). As with levels of polymorphism, the heterozygosity values for western salmon are well below the average for fishes (H = .078 ± .012) published by Selander (1976). They are towards the bottom of the range (.000 to .180,

Table 3.4

Estimates of mean heterozygosity per locus per individual (H) for western and eastern salmon samples, together with the standard error (S.E.) of each estimate.

Sample	n	H	S.E.
1	56	.009	± .0024
2	45	.005	± .0020
3	55	.004	± .0016
4	77	.009	± .0020
5	77	.006	± .0017
6	50	.004	± .0016
7	51	.006	± .0020
8	96	.010	± .0019
9	73	.015	± .0027
Total	580	.008	± .0003
Eastern Salmon	83	.049	± .0045

average = .051 ± .0169) reported by Nevo (1978), and also of the range (.005 to .180, average = .058 ± .006) reported by Powell (1975).

Eastern salmon, like western salmon were electrophoretically variable at six out of 27 loci (Table 3.3). However all six loci (22.2%) were polymorphic at the $p \leq .99$ level, and of these *SDH*, *ME* and *MPI* (11.1%) were variable at the $p \leq .95$ level. Mean heterozygosity for eastern salmon was H = .049 ± .0045. Polymorphism and heterozygosity estimates for eastern salmon are at least twice as high as those of western salmon for the same loci. The significance of this difference will be discussed in section 3.6 and in chapter 5.

3.4.3 Esterase - Genotype Distributions

Assuming a genetic basis for the electrophoretically detected EST variation in salmon, Table 3.5 lists the genotype distributions observed in samples of western and eastern salmon, together with the expected genotype distributions calculated according to the Hardy-Weinberg principle (Hardy, 1908). Comparison of observed and expected genotype distributions provides a measure of deviation from random mating expectations in a given population. Assessment of the type and magnitude of such deviations may in many cases provide clues concerning breeding structure of populations and/or forces acting on the observed genotypic array.

Testing for significant deviation from Hardy-Weinberg expectations is usually done by calculating a goodness-of-fit χ^2,

$$\text{i.e. } \chi^2 = \Sigma[(\text{Observed} - \text{Expected})^2/\text{Expected}]$$

Table 3.5

Observed (O) and expected (E) *EST* genotype distributions in each western salmon sample, for the combined western salmon data, and for the combined eastern salmon data. Smith's H and $\bar{H}$ are calculated on a simulated two-allele system obtained by bulking the rare alleles EST^{107}, EST^{93} and EST^{88}. * = significant deviation from Hardy-Weinberg expectations. For sample code see Table 3.1.

Sample		*EST* Genotypes						Smith's H	95% confidence intervals
		107/100	*100/100*	*100/93*	*93/93*	*100/88*	*93/88*		
1	O.	1	47	3	-	3	2	.027*	.051
	E.	1	46	5	-	4	-		.003
2	O.	-	41	1	1	2	-	.020*	.036
	E.	-	40	3	-	2	-		.004
3	O.	-	52	1	-	1	1	.017*	.027
	E.	-	51	2	-	2	-		.008
4	O.	2	70	2	-	3	-	-.002	.008
	E.	2	70	2	-	3	-		-.012
5	O.	5	67	2	1	2	-	.008	.024
	E.	5	66	4	-	2	-		-.007
6	O.	-	45	1	1	3	-	.017*	.033
	E.	-	44	3	-	3	-		.001
7	O.	-	46	1	-	4	-	-.002	.011
	E.	-	46	1	-	4	-		-.015
8	O.	-	86	6	-	4	-	-.003	.008
	E.	-	86	6	-	4	-		-.013
9	O.	4	56	5	-	8	-	-.013	.011
	E.	4	57	5	-	7	-		-.037
Total	O.	12	510	22	3	30	3	($\bar{H}$)	.011
	E.	11	507	29	1	31	1	.006*	.0008
East.	O		79	4	-			-.0005	.005
Salmon	E		79	4	-				-.006

and referring to the standard χ^2 tables with appropriate degrees of freedom to determine the probability of obtaining the observed value. The sensitivity of this test decreases, however, with decreasing numbers, and calculation of χ^2 values using expected numbers of less than 5 in any genotype class is likely to produce spurious significant results because of a disproportionate contribution of such classes to the overall χ^2 value (Fisher and Yates, 1963; Lewis, 1977).

The *EST* genotype data for salmon contain many expected classes which have values less than 5. Bulking of the rare EST^{107}, EST^{93} and EST^{88} alleles to simulate a two-allele polymorphism does not alleviate this problem, and further bulking of genotypes into homozygous or heterozygous classes renders the χ^2 goodness-of-fit test invalid through lack of degrees of freedom.

An alternative method for estimating deviations from Hardy-Weinberg expectations has been proposed by Smith (1970). The deviation estimate (commonly known as Smith's H) for a two-allele system is obtained from the formula:-

$$H = \frac{4n^2PQ-(2n-1)y}{4n(n-1)}$$

where n = sample size, P and Q = gene frequencies, and y = the observed number of heterozygotes. Smith proposed that this measure of deviation be judged significant if it exceeds twice its standard error - equivalent to a 95% confidence limit. A Smith's H value is therefore considered significant if its 95% confidence interval does not include the zero deviation point.

Smith's H has two major advantages over the χ^2 estimate of goodness-of-fit:- (a) Smith's H distinguishes between

positive and negative deviations, i.e. between excess homozygotes and heterozygotes. The χ^2 test merely registers a deviation. (b) Smith's H values for separate samples can be summed ($\bar{H}$) in a mathematically and biologically more acceptable manner than χ^2 values. For example χ^2 values are additive regardless of the direction of deviation, so that in a situation where both negative and positive deviations occur the combined χ^2 may be significant while the combined Smith's H may not.

Small but significant deviations in *EST* genotype distribution were recorded for western salmon samples from Port Phillip Bay, the Murray River mouth, the Pages, Mt. Dutton Bay, and for all the western salmon data combined (Table 3.5). Deviations within the individual samples appeared to be attributable to the unexpected presence of rare genotypes such as $EST^{93/93}$ and $EST^{93/88}$. However the most obvious difference between observed and expected genotypes in the combined data was the deficiency of $EST^{100/93}$ heterozygotes. This observation is even more interesting in view of the close agreement between observed and expected numbers of $EST^{107/100}$ and $EST^{100/88}$ heterozygotes. There are five possible explanations for the heterozygote deficiency:-

(a) EST 100-93 phenotypes were mis-identified as EST 93 homozygotes. There is a slight excess of $EST^{93/93}$ genotypes in the combined data, but not sufficient to account for the magnitude of the $EST^{100/93}$ deficiency.

(b) the heterozygote deficiency is the product of a Wahlund effect (Wahlund, 1928), i.e. sampling a number of isolated or partially isolated populations which have heterogeneous *EST* allele frequencies. This hypothesis is not

supported by the known life history characteristics of western salmon nor by the observed homogeneous *EST* allele frequencies (see section 3.4.4).

(c) there is a null allele (coding for no enzyme activity) in the western salmon *EST* system, and a proportion of apparent homozygous phenotypes are in reality null heterozygotes. This hypothesis cannot be discounted without breeding studies on the inheritance of electrophoretic patterns, but while some sample sets are out of Hardy-Weinberg equilibrium others are not, and a spatially heterogeneous *EST* null allele distribution is unlikely for reasons given in (b) above.

(d) a certain degree of inbreeding occurs due to consanguinous matings within cohorts of salmon that maintain their integrity (as a single school) from one generation to the next. Under these conditions one might expect that a decrease in heterozygosity would be observed in all classes of heterozygotes, but such is not the case with $EST^{107/100}$ or $EST^{100/88}$. Assortative mating has been ruled out as a possibility because of the non-selective, communal nature of reproductive events in western salmon and many other teleosts.

(e) the $EST^{100/93}$ genotype is at a selective disadvantage during at least part of the life cycle of the species, and a significant deficiency of these heterozygotes arises during the phases between spawning and maturity as a result of selective mortality. In the case of western salmon the most obvious time for such a selective event to occur is during the pelagic egg and larval stage, when very high mortality occurs. The postulation of heterozygous disadvantage poses theoretical problems concerning the maintenance of the polymorphism. These problems will be discussed in section 3.6.2.

A precise comparison of *EST* genotype distributions in different year classes of western salmon was not possible because of a lack of accurate ageing data on the specimens sampled. Examination of length frequency histograms, however, shows that in each sample one or more reasonably clear size modes are apparent (Figure 3.4). With the aid of growth curves for western salmon (Nicholls, 1973) and age/length keys (C.S.I.R.O. Division of Fisheries and Oceanography, unpublished data) it was possible to assign at least the smaller size modes to individual year classes. Some overlap in length distributions between year classes was unavoidable because of variation in growth rates both within and between areas. This overlap increased with age and it was impossible to distinguish individual size modes for fishes of 5+ years or more.

For the purposes of comparing *EST* genotype distributions the following length (L.C.F.) categories were established, each with a tentative year class assigned to it:-

1. 15cm - 24.9cm (Spawned 1975)
2. 25cm - 32.9cm (1974)
3. 33cm - 38.9cm (1973)

   39cm - 44.9cm no data
4. 45cm - 56.9cm (1970 and 1971)
5. 57cm and longer (1969 and before)

An examination of observed and expected *EST* genotypes in each of the year classes (Table 3.6) reveals that only the youngest (1975) juvenile year class deviates significantly from Hardy-Weinberg proportions, and that this deviation is due mainly to a deficiency of $EST^{100/93}$ heterozygotes. This result supports the hypothesis that the distribution of $EST^{100/93}$ is being affected by selective forces, and suggests

Figure 3.4

Length (L.C.F.) frequency histograms for the western salmon sample sets. See Table 3.1 for the sample code.

S = Sample

F = Frequency

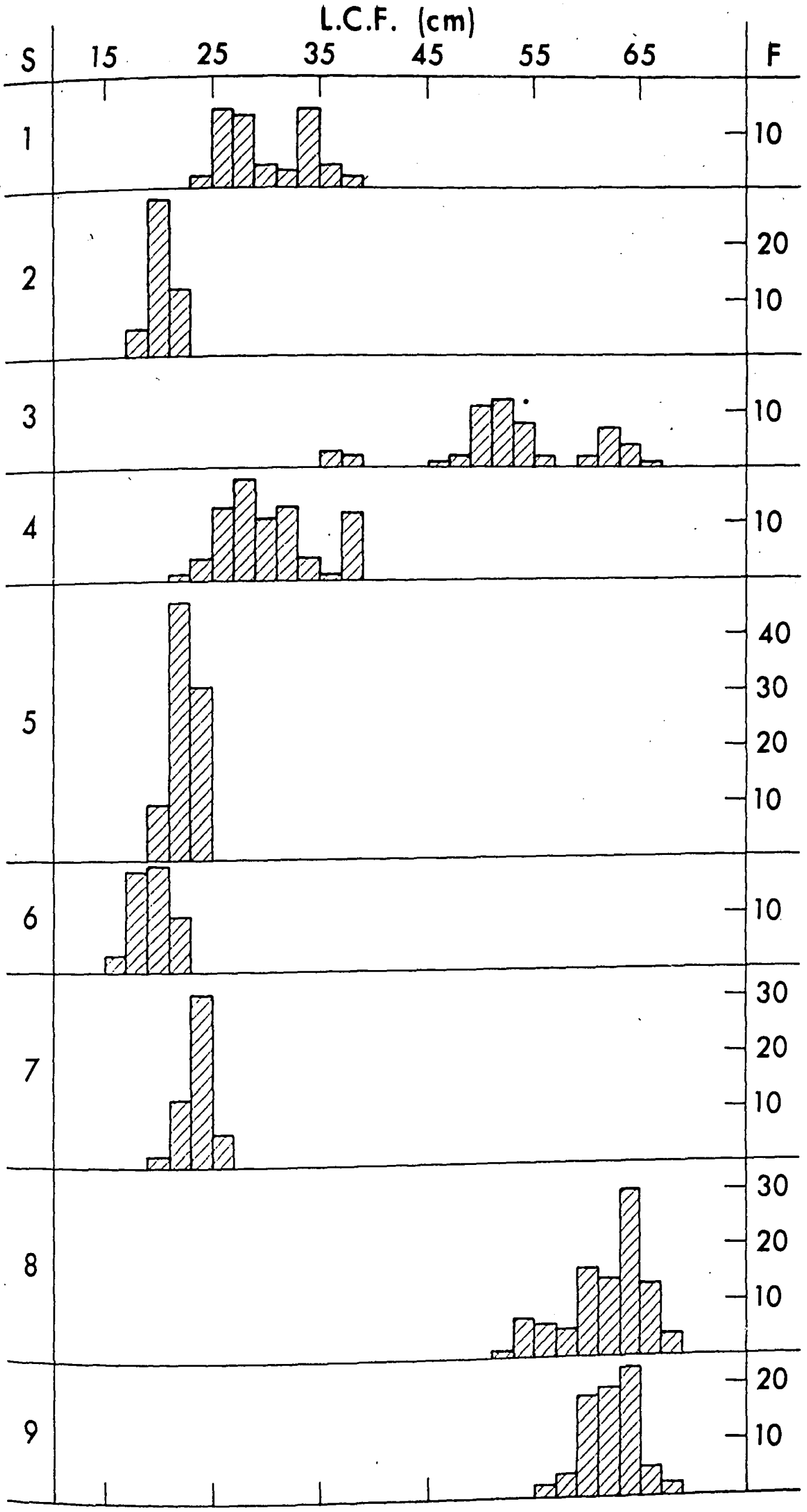

L.C.F. (cm)
S
15
25
35
45
55
65
F
1
10
2
20
10
3
10
4
10
5
40
30
20
10
6
10
7
30
20
10
8
30
20
10
9
20
10

Table 3.6

Observed and expected *EST* genotype distributions in different year classes of western salmon. See text in section 3.4.3 for year class code. * = significant deviation from Hardy-Weinberg expectations.

Year Class	n		$EST^{107/100}$	$EST^{100/100}$	$EST^{100/93}$	$EST^{93/93}$	$EST^{100/88}$	$EST^{93/88}$	Smith's H	95% Confidence Intervals
1	224	O.	5	199	6	3	11	-	.010*	.002
		E.	4	198	13	-	9	-		.018
2	95	O.	2	85	3	-	5	-	-.002	-.012
		E.	2	86	3	-	4	-		.008
3	42	O.	1	37	1	-	1	2	.018	-.005
		E.	1	36	3	-	2	-		.041
4	52	O.	-	47	2	-	2	1	.007	-.008
		E.	-	46	3	-	3	-		.022
5	167	O.	4	142	10	-	11	-	-.005	-.015
		E.	3	143	10	-	11	-		.005

that the selective event occurs within the first year of life. From the present data it was not possible to determine whether such selective events occur at irregular intervals and the heterozygote deficiency is carried through to maturity in the affected year class, or whether the $EST^{100/93}$ deficiency arises frequently in the early life history stages of year classes and is progressively counterbalanced with increasing age.

3.4.4 Esterase - Distribution of Genes

An initial comparison of *EST* allele frequencies in the western salmon samples indicates that the distribution of *EST* genes is homogeneous over the geographical range of the species. EST^{107} is present in some samples and not in others, but the average frequency of this allele for the combined data is so low (.01) that the observed heterogeneous distribution can be attributed to sampling error.

A χ^2 contingency test has been used to measure the homogeneity of allele frequency estimates from the western salmon samples. A contingency table is constructed by counting the numbers of each allele in each sample, and a χ^2 value is then generated to test the independance of rows and columns (Table 3.7). Each allele can be individually tested for homogeneity by combining all the other alleles to form a single alternative gene class. As with the χ^2 goodness-of-fit test, bulking of rare alleles will also help to avoid the statistical problems posed by low expected values in some classes. It can be seen from Table 3.7 that the distribution of EST^{100} is non-significant, and hence homogeneous (cf. $\chi^2_8 = 11.83$, probability (P) = .17). EST^{107}, EST^{93} and EST^{88} could not be

Table 3.7

Contingency table for χ^2 test of homogeneity of *EST*^{100} frequencies in western salmon samples. See Table 3.1 for sample codes. The χ^2 subscript indicates the number of degrees of freedom for the test.

Sample	2n		EST^{100}	EST^{Other}
1	112	O.	101	11
		E.	105	7
2	90	O.	85	5
		E.	84	6
3	110	O.	106	4
		E.	103	7
4	154	O.	147	7
		E.	144	10
5	154	O.	143	11
		E.	144	10
6	100	O.	94	6
		E.	93	7
7	102	O.	97	5
		E.	95	7
8	192	O.	182	10
		E.	179	13
9	146	O.	129	17
		E.	136	10
Total	1160		1084	76

cf. $\chi^2_8 = 11.83$ P = .17

tested individually because of low expected classes in some samples, but for each of these alleles the sample frequencies do not appear sufficiently variable to be considered significantly heterogeneous.

The distribution of *EST* alleles in each of the sexes was also considered. Examination of sex ratios (Table 3.8) revealed a significant excess of males in the Murray R. mouth and Franklin Harbour juvenile samples, and in the combined collection data (the biological implications of this result are discussed in chapter 6). A sample by sample comparison of the *EST* polymorphism in each sex was not possible because of small sample sizes, but a χ^2 homogeneity test of the combined data (Table 3.9) indicates that the distribution of the *EST* alleles in western salmon does not differ significantly from random with respect to sex.

A χ^2 homogeneity test of *EST* allele frequencies in each of the year class categories designated in section 3.4.3 indicates that, as with the comparison of sexes, the distribution of *EST* alleles in western salmon does not differ significantly between successive year classes (Table 3.10).

3.4.5 Kinship Analysis

There are a number of different types of genetic distance measure available for the comparison of local populations, subspecies, species and genera (Latter, 1973; Nei, 1973). The most widely used types are either descriptive measures which specify the degree to which populations are genetically different (e.g. Balakrishnan and Sanghvi, 1968; Hedrick, 1971; Rogers, 1972; Lakovaara et al., 1972), or measures of the rate

Table 3.8

Sex ratios of the individual and combined western salmon samples. See Table 3.1 for sample code.

Table 3.9

χ^2 homogeneity test between sexes for the combined western salmon *EST* allele frequency data.

Sample	Males	Females	n	χ^2_1	P.
1	23	33	56	1.79	.19
2	43	2	45	348.86	<.0001
3	34	21	55	3.07	.08
4	45	32	77	1.61	.23
5	52	25	77	8.33	.004
6	21	29	50	1.28	.27
7	24	27	51	.18	.72
8	56	40	96	2.67	.12
9	42	31	73	1.66	.22
Total	340	240	580	17.24	<.0001
Eastern Salmon.	40	43	83	.11	.75

Sex	2n		*EST*^{107}	*EST*^{100}	*EST*^{93}	*EST*^{88}
Females	480	O.	6	635	20	19
		E.	7	636	18	19
Males	680	O.	6	449	11	14
		E.	5	448	13	14
Total	1160		12	1084	31	33

cf. $\chi^2_3 = .83$ P. = .84

Table 3.10

A χ^2 homogeneity test of *EST* allele frequencies in different age classes of western salmon. For the year class code see text.

Year Class	2n		EST^{100}	EST^{Other}
1.	448	O.	420	28
		E.	419	29
2.	190	O.	180	10
		E.	176	14
3.	84	O.	77	7
		E.	78	6
4.	104	O.	98	6
		E.	97	7
5.	334	O.	309	25
		E.	312	22
Total	1160		1084	76

$\chi^2_4 = 2.04$ P. = .71

of mutational divergence between isolated populations (e.g. Nei, 1972; Latter, 1972). The most common use for these measures is in providing information on systematic and evolutionary relationships between taxa. Most species, however, are divided into a number of local populations, and observed patterns of intra-specific genetic variation can be influenced by the static aspects of population structure (e.g. breeding patterns and gene flow) as well as by dynamic evolutionary forces (e.g. mutation, drift and selection).

Morton et al. (1971) pointed out that descriptive measures of genetic distance between local populations are somewhat imprecise in that indices of distance may be arbitrarily derived and the resulting distance measures have no simple genetic or biological interpretation. Following concepts developed by Wright (1943, 1951) and Malécot (1948, 1959), Morton et al. proposed an alternative method of describing population structure - a measure known as the coefficient of kinship, ϕ. The kinship between two samples X and Y is defined as the probability that a randomly sampled gene from X will be identical by descent with a random allele from Y, and is estimated from the formula:-

$$\phi_{XY} = \frac{\Sigma\, q_{iX}\, q_{iY} - \Sigma\, Q_i^2}{1 - \Sigma\, Q_i^2}$$

where q_{iX} and q_{iY} are the frequencies of the i^{th} allele in samples X and Y respectively, and Q_i is the mean frequency of allele i over all the samples. The coefficient of kinship is similar in many respects to measures of inbreeding (Workman and Niswander, 1970), since it is effectively measuring the

co-divergence of two samples from the mean of the sample array. Under these circumstances only polymorphic gene loci are useful in contributing to kinship measures.

For a number of population samples taken over a geographic range the most succinct description of population structure is to plot pairwise coefficients of kinship against distance between samples. The theory of isolation by distance was pioneered by Malecot (1948, 1959), who defined the relationship between ϕ_{XY} and the Euclidean distance from X to Y. In practice one-dimensional distance is just as appropriate and, for finite geographic distances, it has been shown from observations on real populations and from simulation studies of artificial populations that:

$$\phi(d) \simeq ae^{-bd} \quad \text{(Morton et al., 1971)}$$

where d is the distance between samples, a is the mean *a priori* kinship within samples, and b is the logarithmic rate of change in space or time. This equation specifies a monotonic decreasing relationship between ϕ and distance, and in the absence of evolutionary forces such as selection, mutation and drift ϕ will be asymptotic towards zero with increasing distance.

When the number of populations sampled is finite some values of ϕ_{XY} will be negative because a proportion of samples will be less related than the average. In other words where ϕ_{XY} values of ≤ 0 are obtained two randomly sampled genes from X and Y are equally or more likely to be different than they are to be identical. For a regression of ϕ_{XY} with distance, the point at which the regression line reaches or passes

through $\phi = 0$ indicates the average distance within which some degree of inbreeding occurs, and can be considered the average size of the "neighbourhood" inhabited by an interbreeding population of the species investigated.

The fit of the Malécot equation to observed kinship data is dependant on assumptions implicit in the isolation-by-distance model (Harrison and Boyce, 1972). Firstly the observed patterns of genetic variation used as a basis for ϕ estimates are assumed to be in equilibrium in the populations sampled. Determining the spatio-temporal stability of polymorphisms is a problem faced by all models attempting to interpret patterns of genetic variation, and is not a specific limitation of the Malécot approach. While transient polymorphisms can usually only be detected by lengthy time-series studies, it appears that the assumption of equilibrium is at least reasonable in Australian snapper because of the short-term temporal stability of clinal variation at several loci in this species (see section 3.5.4).

Secondly the shape of the isolation-by-distance curve for a given species depends on the magnitude and variance of dispersal or migration and of other systematic forces such as selection (estimates of these parameters are incorporated in b, a constant in the Malécot equation). The fit of observed data to an expected regression will be good only if factors like migration and selection can be accurately estimated and are uniform over the sampled range of the species (Morton, 1975). It is my contention that in reality very few species will have patterns of genetic variation that fit the classic isolation-by-distance model. In the absence of empirical estimates of parameters such as migration and selection the

value of the Malécot approach lies in (a) providing a first approximation of kinship/distance relationships for an individual species and (b) using the differences between the observed and classic regressions to provide clues to the forces maintaining genetic variation in the given species.

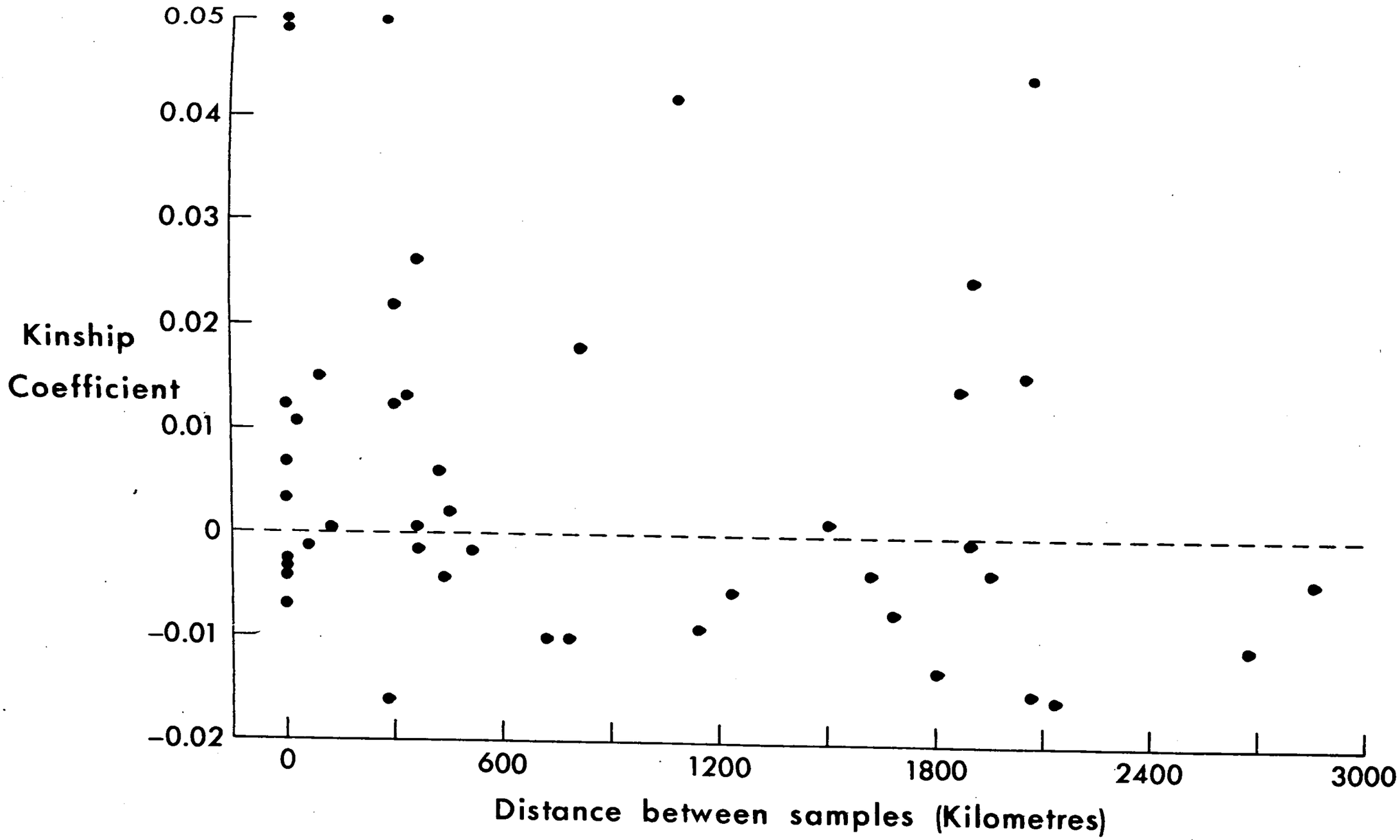
In the present study coefficients of kinship were computed using BIOKIN (Yee, 1973), a computer program developed by N.E. Morton and associates for the bioassay of kinship using sample estimates of allele frequencies. A plot of ϕ_{XY} against distance at the *EST* locus in the nine western salmon samples reveals a broad scatter of points on either side of the zero line throughout the distance range (Figure 3.5). For obvious reasons no attempt was made to fit a regression line, but the results nevertheless bring out two points:-

Firstly the distribution of ϕ_{XY} with distance as determined by a single locus is prone to high variance because of sampling error in the estimation of allele frequencies. This problem may be minimised by increasing sample sizes (and hence the precision of frequency estimates), or by estimating mean ϕ_{XY} over a large number of polymorphic loci. Estimates of ϕ_{XY} from each locus are independant in the absence of non-random associations of loci, but can be distorted by the influence of genetic drift or selection. The best way to avoid kinship estimates biased by these factors is to assay as many loci as possible (Morton, 1973).

Secondly, the comparatively even scatter of ϕ_{XY} values about the zero line in Figure 3.5 indicates that the probability of identity by descent of two genes sampled from any distance apart will be roughly equal. The absence of a

Figure 3.5

A comparison of western salmon EST kinship coefficients against distance between samples. The data points are generated by comparing each sample set against every other sample set.


0.05
0.04
0.03
0.02
0.01
0
−0.01
−0.02
Kinship
Coefficient
0
600
1200
1800
2400
3000
Distance between samples (Kilometres)

regression trend of the form $\phi(d) \simeq ae^{-bd}$ also suggests that there is no appreciable isolation by distance over the entire range of western salmon. These conclusions are consistant with the hypothesis that all western salmon stocks in southern Australian waters are part of a single panmictic breeding population.

3.5 Results - Snapper

3.5.1 Electorphoretic Patterns of the Enzymes

A total of 21 enzymatic proteins (section 3.3.3) from 26 presumed genetic loci were electrophoretically surveyed in 15 sample sets of Australian snapper, totaling 680 individuals (Table 3.2, Figure 3.2). The enzymes screened in snapper were the same as those surveyed in salmon, with the exception of G6PD, for which zones of activity on the gel were so diffuse as to be unscorable. 150 snapper (10 from each sample set) were used to initially screen loci for allelic polymorphisms, and all remaining specimens were scored for loci in which variation was observed.

Snapper were found to be electrophoretically monomorphic for 17 of the 26 loci studied. Isozymes in this category included GPD, MDH_S, MDH_M, ME, LDH_B, SOD, AK_1, AAT_S, AAT_M, $GAPD_1$, $GAPD_2$, XO, SDH, MPI, GDA_1, GDA_2 and 6PGD. All of these isozymes except LDH_B exhibited single-banded patterns of activity in liver tissue, with no evidence of non-inherited variation or inter-locus polypeptide subunit associations.

LDH activity was visualised as a two-banded pattern in all individuals except one. This result is unusual, as LDH

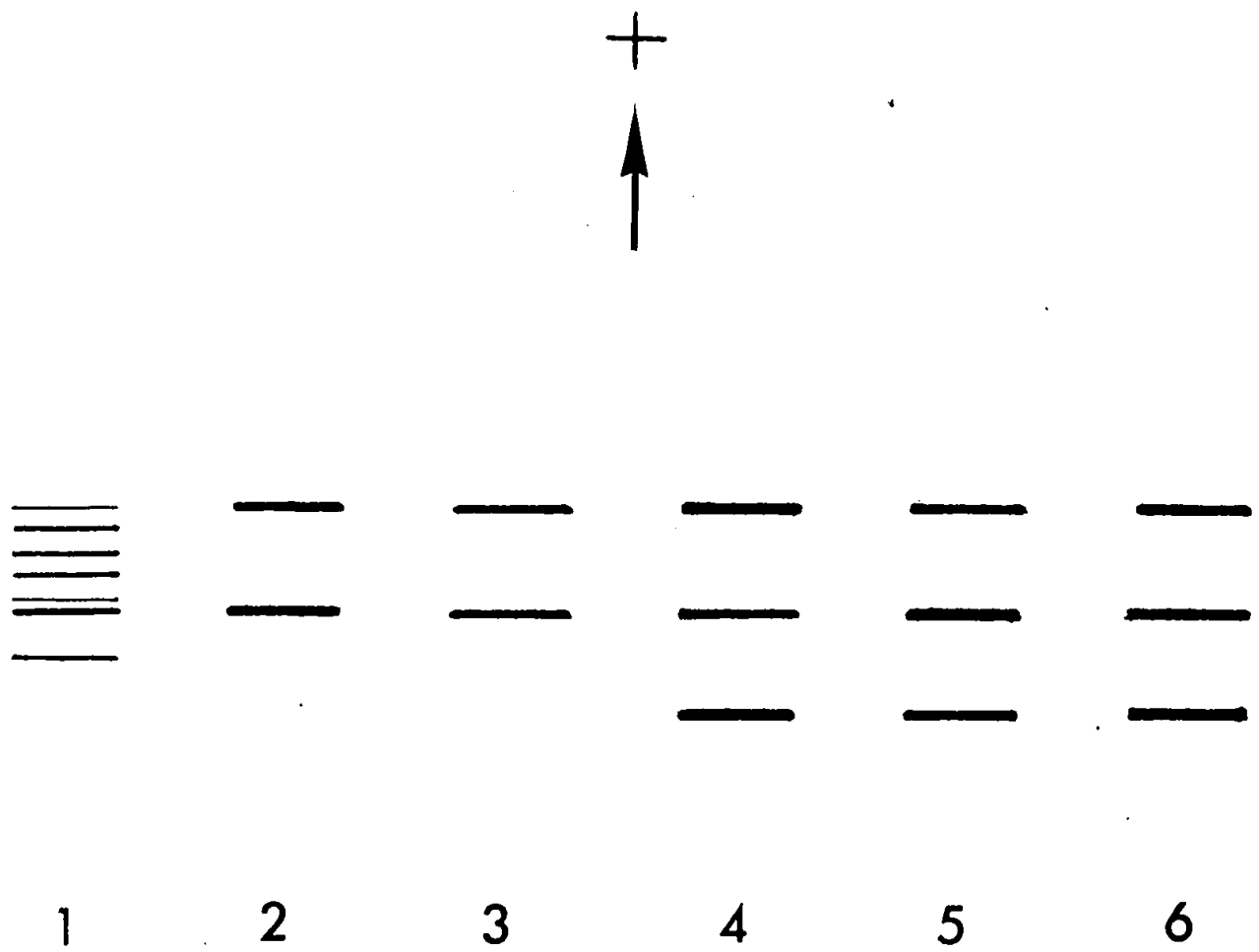
isozymes normally have a tetrameric subunit structure and form three- or five-band electrophoretic patterns in fish liver tissue as a result of random or restricted inter-locus heterotetramer formation (Markert and Faulhaber, 1965; Whitt, 1970). Comparisons of the Australian snapper (*Chrysophrys auratus*) with its closely related northern hemisphere congener, the red sea bream (*C. major*), reveal that *C. major* indeed has a three-banded LDH pattern in liver, suggesting genetic control by two loci, LDH_A and LDH_B, with the observed isozyme bands being LDH_{A4}, LDH_{A2B2} and LDH_{B4} respectively (Plate 3.3). The two bands in Australian snapper are electrophoretically identical to LDH_{A4} and LDH_{A2B2}, but no LDH_{B4} equivalent was detected.

Of the nine snapper loci at which electrophoretic variation was detected LDH_A and *Gal6P* exhibited extremely rare heterozygous phenotypes and could not be considered valid polymorphisms even at the $p \leq .99$ level. At both loci a single heterozygote was detected in the Shark Bay sample. The LDH_A heterozygote exhibited a typical five-banded tetrameric isozyme pattern, with the slower-migrating homotetramer of the rare allele almost identical in mobility with the LDH_{A2B2} inter-locus heterotetramer (Plate 3.3). The Gal6P heterozygote indicated a slower-migrating rare allele with a three-banded electrophoretic pattern. The quaternary structure of Gal6P in fishes is not known, but the observed heterozygote pattern suggests that the enzyme is a dimer in snapper.

The remaining seven variable loci were found to be polymorphic at the $p \leq .95$ level in one or more of the sample

Plate 3.3

Observed LDH phenotypes in Australian snapper (samples 1 to 3) and red sea bream (samples 4 to 6). Sample 1 is an $LDH_A^{100/85}$ heterozygote.


1
2
3
4
5
6

sets. Sample estimates of allele frequencies at each of these loci are given in Table 3.11. Six alleles were detected at the *EST* and *IDH* loci. EST heterozygotes were two-banded and 12 out of a possible 21 phenotypes were detected (Plate 3.4), while IDH heterozygotes displayed a typical three-banded dimeric electrophoretic pattern and only nine out of a possible 21 phenotypes were observed (Plate 3.5). Four alleles were detected at each of the *ADA*, *ADH*, *GPI* and *PGM* loci (Plates 3.6 to 3.9) with five, six, five and six phenotypes respectively observed out of a possible ten. ADA and PGM heterozygotes exhibited two-banded monomeric patterns while ADH and GPI heterozygotes appeared as typical three-banded dimers, with the intermediate heterodimer band more intensely stained than either homodimer.

A three-allele polymorphism was detected at the *FUM* locus (Plate 3.10). FUM activity was not always clearly defined on cellogel, and some form of non-inherited variation (as defined in section 3.4.1) is indicated by a secondary zone of less intense activity just anodal to the main band in homozygotes. Heterozygotes were nevertheless clearly detectable, and the multiple band pattern obtained supports the contention that FUM has a tetrameric subunit structure (Harris and Hopkinson, 1977).

3.5.2 Amount of Genetic Variability

Analysis of allelic variation at 26 enzyme loci in the combined Australian snapper samples shows that seven loci (26.9%) are polymorphic at the $p \leq .99$ level (Table 3.11). Of these seven loci four, *EST*, *IDH*, *ADA* and *GPI* (15.4%) are also

Table 3.11 Allele frequencies for seven polymorphic loci in Australian snapper

	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Locus	Allele															
EST	*117*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.01
	108	.04	.05	.06	-	.04	-	-	.07	.14	.08	.17	.16	.10	.14	.10
	100	.89	.89	.89	.93	.85	.96	.95	.86	.74	.79	.70	.72	.68	.67	.67
	95	.02	.04	-	.02	-	-	-	.03	.09	.08	.07	.04	.02	.14	.10
	90	.05	.02	.05	.05	.11	.04	.05	.03	.03	.05	.06	.08	.18	.03	.11
	83	-	-	-	-	-	-	-	.01	-	-	-	-	.02	.02	.01
IDH	*124*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.01
	114	.08	.10	.12	.09	.11	.15	.12	.20	.22	.20	.17	.18	.10	.22	.24
	111	-	.03	.02	.05	.01	.01	-	-	-	-	-	-	-	-	-
	100	.85	.80	.83	.83	.77	.73	.76	.70	.68	.72	.77	.71	.75	.56	.63
	80	.07	.06	.03	.03	.11	.11	.12	.10	.10	.08	.06	.11	.15	.22	.12
	70	-	.01	-	-	-	-	-	-	-	-	-	-	-	-	-
ADA	*124*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.01
	112	-	-	-	.03	-	-	-	-	-	-	-	-	-	-	.01
	100	.85	.85	.79	.76	.76	.80	.83	.93	.82	.88	.95	.83	.95	.81	.91
	88	.15	.15	.21	.21	.24	.20	.17	.07	.18	.12	.05	.17	.05	.19	.07

FUM	*120*	-	-	-	-	.03	-	-	-	-	-	-	.01	-	-	-
	100	.96	.96	.97	.90	.95	.95	.95	.97	.99	.99	.98	.99	.97	1.0	.95
	84	.04	.04	.03	.10	.02	.05	.05	.03	.01	.01	.02	-	.03	-	.05
ADH	*150*	-	-	-	-	-	-	-	.01	.01	.02	-	.01	-	-	.03
	125	-	-	-	-	-	-	-	-	-	.01	.09	.05	-	-	-
	100	1.0	1.0	1.0	1.0	1.0	.96	.98	.96	.94	.95	.91	.92	.95	.92	.93
	70	-	-	-	-	-	.04	.02	.03	.05	.02	-	.02	.05	.08	.04
GPI	*120*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.01
	108	-	.02	.02	-	.01	.02	.03	.01	.01	.03	.02	.04	.05	.03	.08
	100	1.0	.97	.98	.93	.97	.95	.95	.97	.97	.94	.95	.92	.92	.94	.88
	88	-	.01	-	.07	.02	.03	.02	.02	.02	.03	.03	.04	.03	.03	.03
PGM	*117*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.01
	110	.02	.04	-	.03	.02	.01	-	-	.04	.04	.02	.03	-	-	.01
	100	.98	.95	.98	.97	.92	.98	.98	1.0	.95	.96	.95	.96	.97	1.0	.97
	91	-	.01	.02	-	.06	.01	.02	-	.01	-	.03	.01	.03	-	.01

Plate 3.4

Observed EST phenotypes in samples of Australian snapper. From left to right the phenotypes are:-

EST 100; EST 108/100; EST 108; EST 117; EST 100/95; EST 95; EST 108/95; EST 100/90; EST 90; EST 108/90; EST 95/90; EST 100/83.

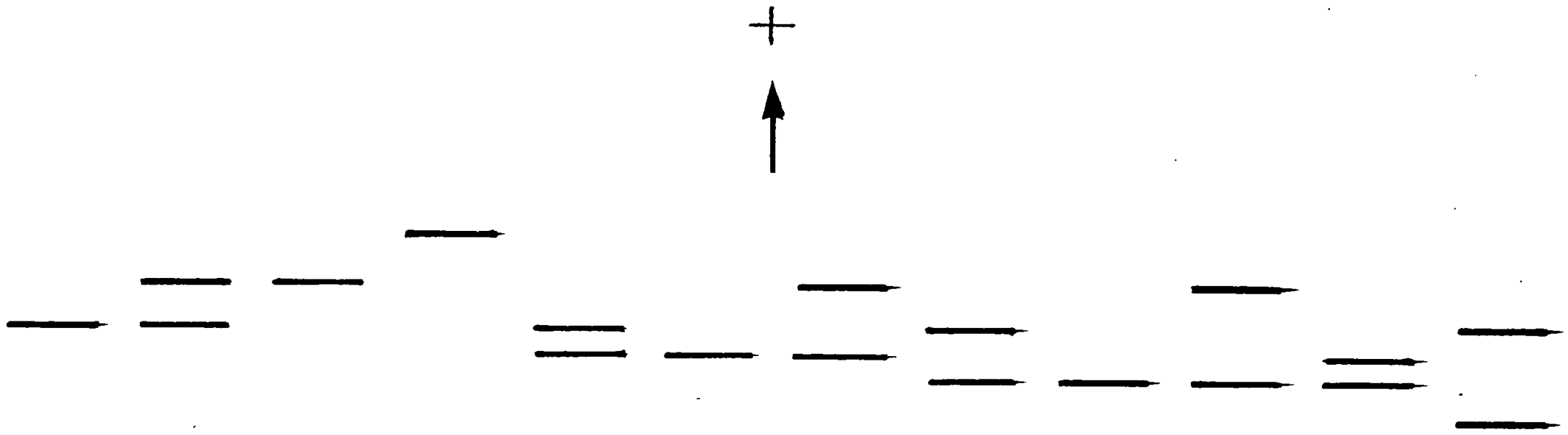


Plate 3.5

Observed phenotypes in samples of Australian snapper. From left to right the phenotypes are:-

IDH 100; IDH 111/100; IDH 114/100; IDH 114; IDH 124/114; IDH 100/80
IDH 80; IDH 114/80; IDH 100/70.

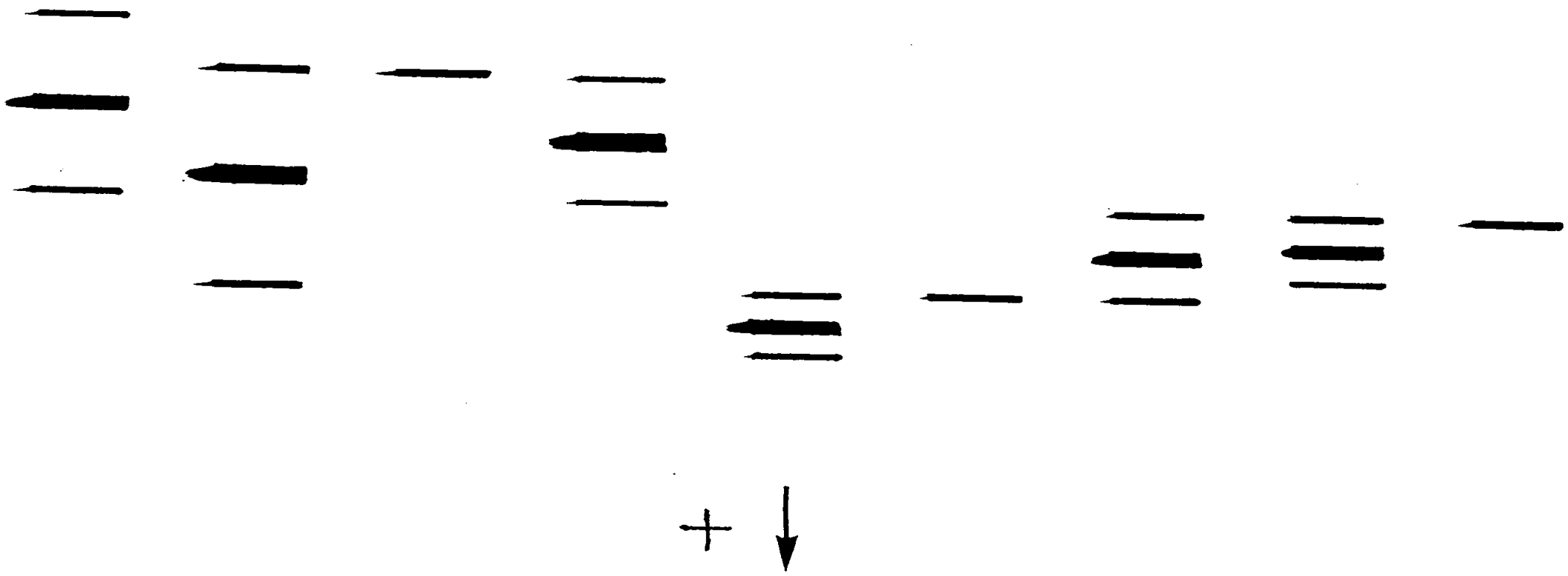


Plate 3.6

Observed ADA phenotypes in samples of Australian snapper. From left to right the phenotypes are:-

ADA 100; ADA 112/100; ADA 112/124; ADA 100/88; ADA 88.

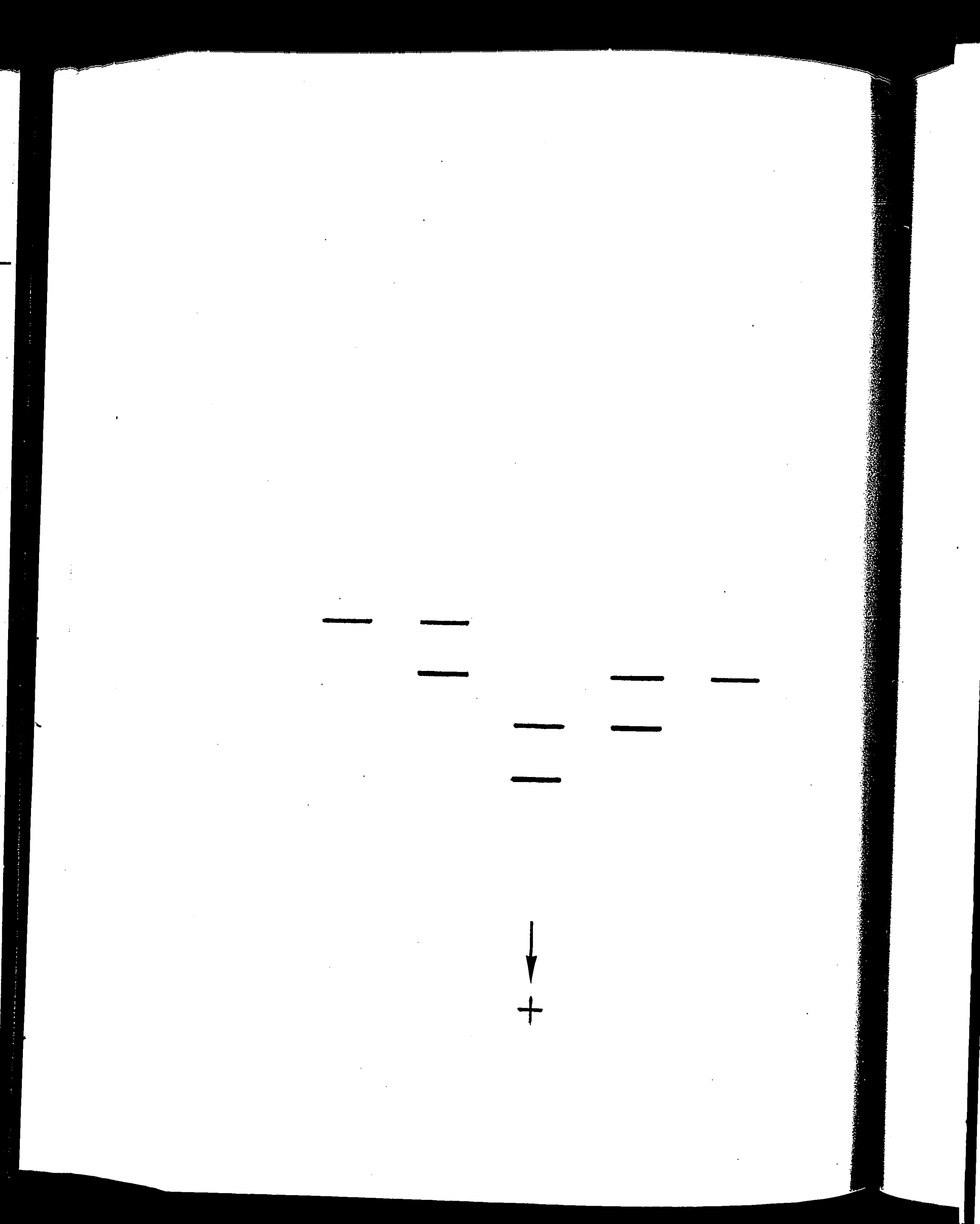


Plate 3.7

Observed ADH phenotypes in samples of Australian snapper. From left to right the phenotypes are:-

ADH 100; ADH 125/100; ADH 125; ADH 150/100; ADH 150; ADH 100/70.

Note:- the apparently large differences in mobility of these phenotypes is due to the fact that the allozymes separate near the origin of the gel.

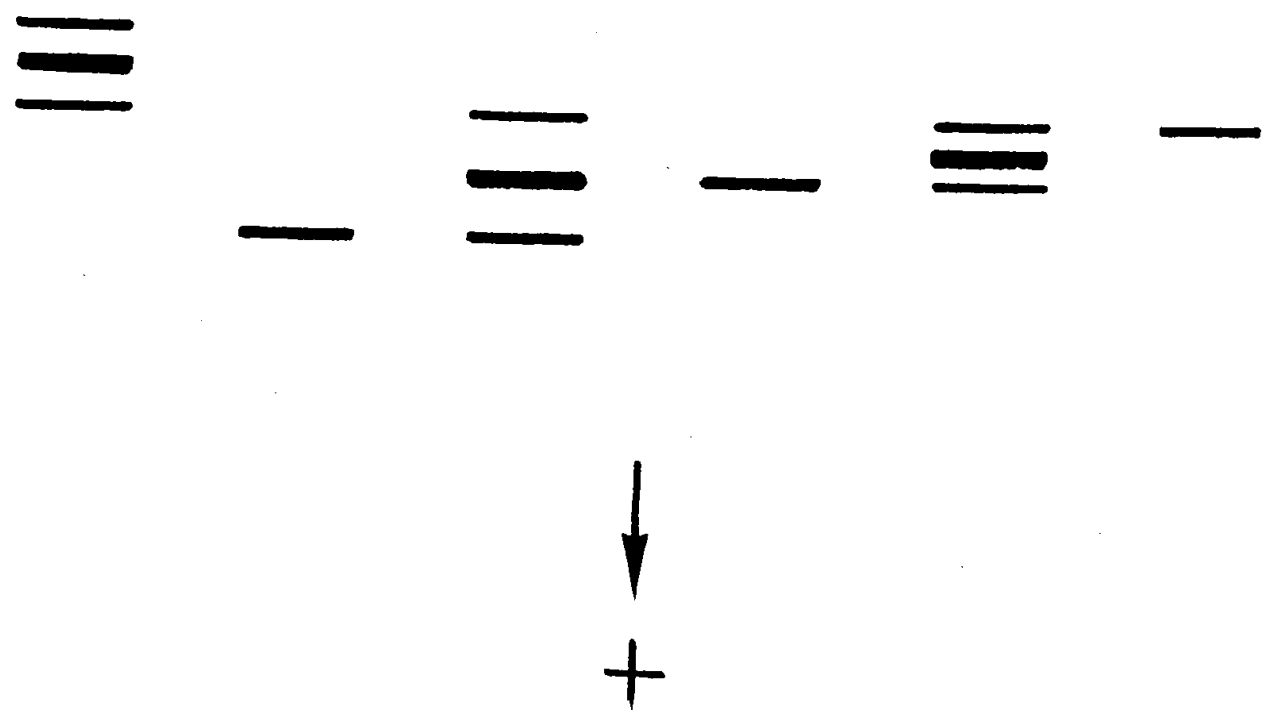


Plate 3.8

Observed GPI phenotypes in samples of Australian snapper. From left to right the phenotypes are:-

GPI 100; GPI 108/100; GPI 108; GPI 120/100; GPI 100/88.

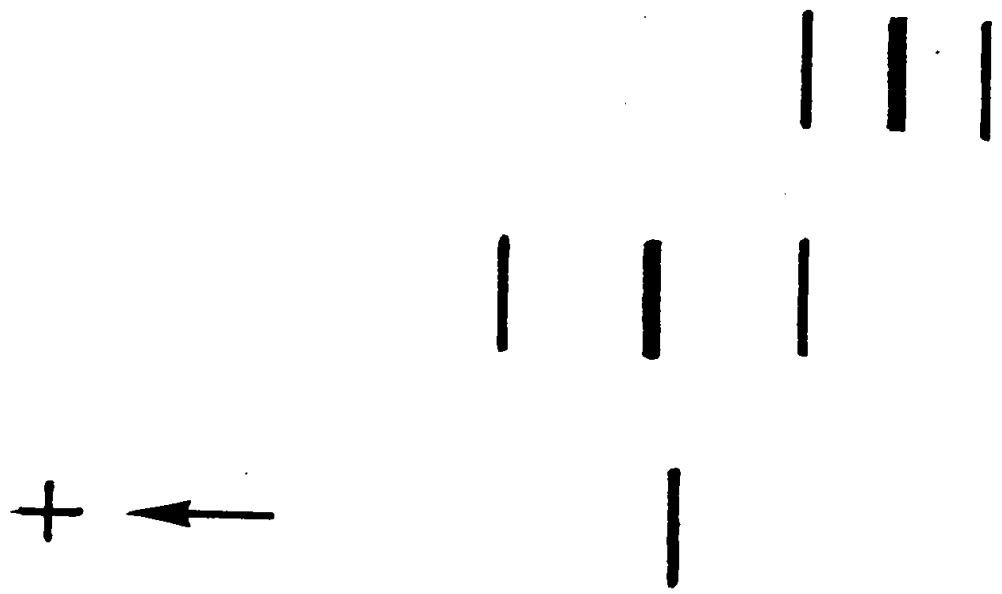


Plate 3.9

Observed PGM phenotypes in samples of Australian snapper. From left to right the phenotypes are:-

PGM 100; PGM 110/100; PGM 110; PGM 117/100; PGM 100/91; PGM 91.

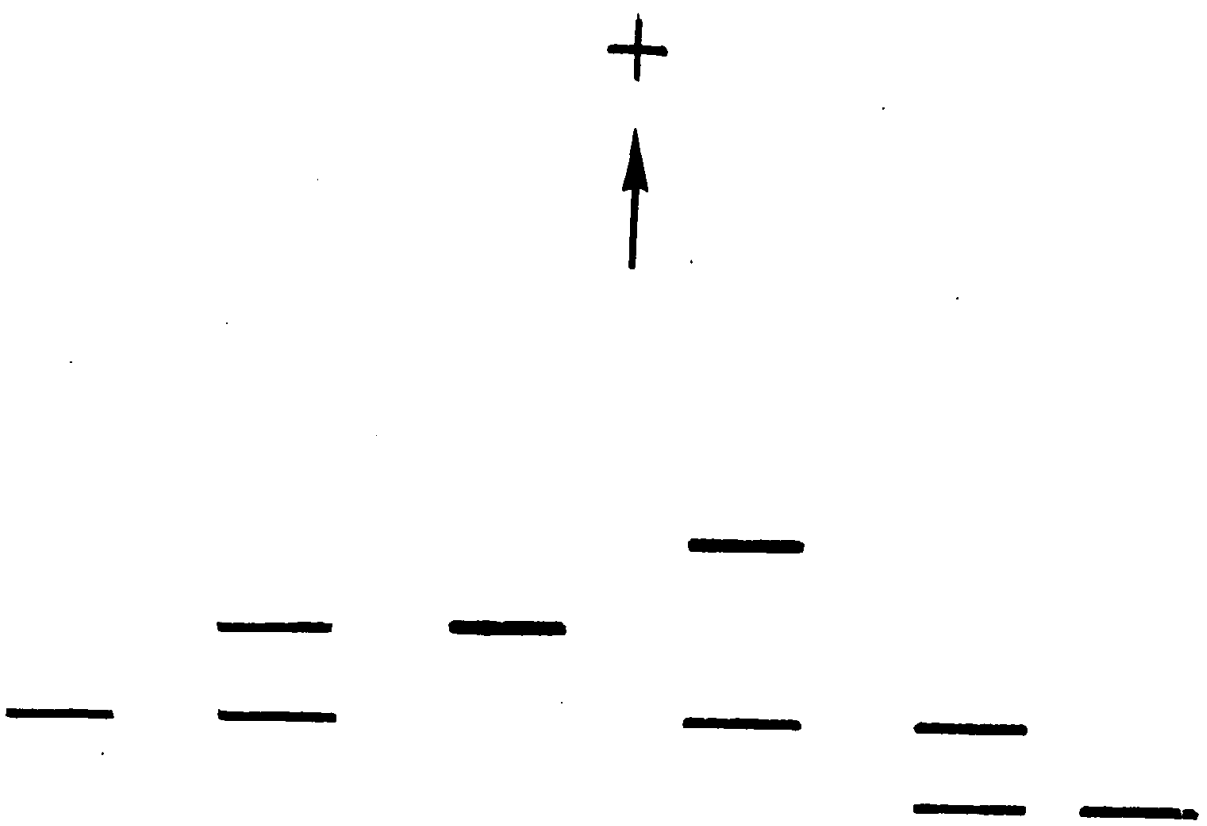
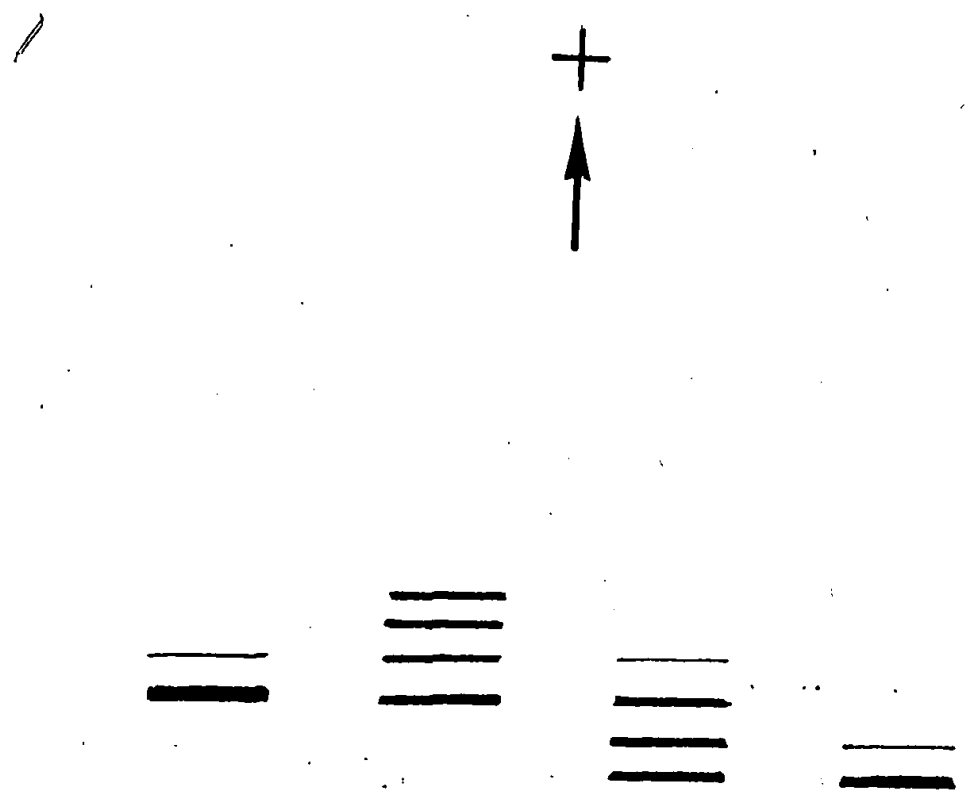


Plate 3.10

Observed FUM phenotypes in samples of Australian snapper. From left to right the phenotypes are:-

FUM 100; FUM 120/100; FUM 100/84; FUM 84.



polymorphic at the $p \leq .95$ level. The first percentage figure is substantially in agreement with the average of 30% loci polymorphic in fishes published by Selander (1976) and the second figure is almost identical to the average figure (15.2%) given for bony fishes by Nevo (1978), again suggesting that Nevo used $p \leq .95$ as a criterian for a valid polymorphism (see section 3.4.2). Studies of genetic variation in New Zealand snapper stocks (Smith et al. 1978) have yielded measures of 21.7% and 26.1% loci polymorphic ($p \leq .99$) from a total of 23 loci studied in two samples of about 85 individuals each. The results from the present study are similar, with proportions of polymorphic loci in each Australian sample ranging from 19.2% to 26.9%.

Mean heterozygosity estimates for the Australian snapper samples range from $H = .033 \pm .0044$ to $H = .061 \pm .0071$, with the combined data yielding an average of $H = .045 \pm .0016$ (Table 3.12). This value is a slight underestimate compared to the proportion of heterozygotes expected under random mating conditions ($H = .050 \pm .0018$), and reflects an overall excess of homozygous genotypes in some of the seven polymorphic loci typed (see section 3.5.3). The heterozygosity values for Australian snapper are close to, but marginally below, average values for fishes reported by Powell (1975; $H = .058 \pm .006$) and Nevo (1978; $H = .051 \pm .0169$).

Smith et al. (1978) have published heterozygosity estimates of $H = .079 \pm .034$ and $H = .084 \pm .035$ for two samples of New Zealand snapper. These values and the estimates obtained from Australian snapper are not directly comparable, because only 13 of the loci used to derive H values were scored in both studies. In addition there is a slight bias in the New Zealand

Table 3.12

Estimates of mean heterozygosity per locus per individual (H) from 26 loci in Australian snapper samples, together with the standard error (S.E.) of each estimate. The expected H estimate is calculated from Hardy-Weinberg proportions of heterozygotes.

Sample	n	H	S.E.
1	53	.033	± .0044
2	55	.040	± .0052
3	33	.035	± .0059
4	29	.048	± .0085
5	33	.047	± .0078
6	62	.041	± .0050
7	64	.036	± .0043
8	37	.033	± .0052
9	57	.051	± .0064
10	56	.048	± .0061
11	33	.041	± .0068
12	70	.057	± .0064
13	20	.048	± .0102
14	18	.049	± .0110
15	60	.061	± .0074
Total	680	Obs. .045	± .0016
		Exp. .050	± .0018

H estimates because the number of individuals typed from each sample varied from locus to locus. Mean heterozygosity per locus per individual calculated from the 13 loci common to both studies were H = .065 ± .0046 for Australian snapper and H = .075 ± .-052 for the combined New Zealand samples. These estimates are much closer than the previous comparison, but are still significantly different (cf. χ^2_1 = 4.11, P = .01). The implications of this result will be discussed in section 3.6 and in chapter 5.

3.5.3 Genotype Distributions

Assuming a genetic basis for electrophoretically detected variation at the seven polymorphic loci surveyed in Australian snapper (Table 3.11), observed genotype distributions were compared to those calculated from Hardy-Weinberg expectations in each sample and for the combined data. As with the salmon results (section 3.4.3), a χ^2 goodness-of-fit test could not be applied to the snapper genotype data, as even when rare alleles were combined to form a two-allele system, expected numbers in many genotype classes were less than 5.

Table 3.13 lists summed Smith's $\bar{H}$ estimates of deviations from random mating proportions at each locus in the combined snapper data set. The *ADA* and *GPI* loci show very close agreement between observed and expected genotype frequencies for both the individual snapper samples and the combined data. There is an apparently spurious significant excess of *FUM* homozygotes in the 1976 Port Phillip Bay sample, caused mainly by the unexpected presence of a rare $FUM^{84/84}$ individual. Smith's H values at all other samples are non-significant,

Table 3.13

Summed Smith's $\bar{H}$ estimates of deviations in genotype distributions at seven loci for the combined Australian snapper data. * = significant deviation from Hardy-Weinberg expectations.

Locus		Heterozygotes	Smith's $\bar{H}$	95% Confidence Intervals
EST	O.	169	.025*	.036
	E.	215		.014
IDH	O.	260	.016*	.030
	E.	284		.0008
ADA	O.	179	-.002	.008
	E.	178		-.012
FUM	O.	42	.0003	.003
	E.	42		-.002
ADH	O.	41	.007*	.010
	E.	52		.004
GPI	O.	64	-.00001	.031
	E.	65		-.023
PGM	O.	37	.002	.004
	E.	41		-.0002

however, and the observed and expected *FUM* genotype distributions in the combined data are in agreement within the limits of sampling error. A similar situation obtains at the *PGM* locus, where the Narooma and 1979 Cowled Landing samples exhibit homozygote excesses due to the unexpected presence of single $PGM^{91/91}$ and $PGM^{110/110}$ individuals respectively, but the summed Smith's $\bar{H}$ value is non-significant.

The *EST*, *IDH* and *ADH* loci all revealed significant overall deficiencies of heterozygous genotypes in the combined snapper data, but the pattern of the deviation is not the same in each of these loci. In the six-allele *EST* polymorphism all homozygote classes detected are excessive and all major heterozygote classes are deficient:-

EST Genotypes	Observed	Expected
117/117	1	-
117/100	-	2
108/108	17	4
108/100	55	84
100/100	481	458
100/95	37	47
95/95	5	1
100/90	58	65
95/90	2	4
90/90	7	2
100/83	4	3
108/95	8	4
108/90	5	6
Total	680	680

Only the Narooma, Backstairs Passage (Nov. 1977) and 1977 Cowled Landing collections showed significant deviations from Hardy-Weinberg proportions, but in seven out of 15 samples where deviations are observed the deviation is in the direction of heterozygote deficiency or homozygote excess. It will be shown in section 3.5.4 that EST^{100} allele frequencies are heterogeneous over the geographical range of Australian snapper, and the simplest explanation of the observed deficiency of heterozygotes is that there is a Wahlund effect due to sampling a number of localities where the snapper stock consists of a mixture of isolated or partially isolated populations with distinct *EST* allele frequencies. As with the salmon results, however, the possible presence of a null allele causing an apparent heterozygote deficiency cannot be ruled out without breeding studies.

Studies of the same *EST* locus in 12 sample sets (1341 individuals) of New Zealand snapper (Smith et al., 1978) have revealed a slightly different pattern of genotype distribution. No test was made on the combined data, but direct comparison of observed and expected genotype frequencies showed that, while excess homozygotes were evident in six out of 12 sample sets, three samples exhibited an excess of heterozygotes - including the only sample with a χ^2 value significant at the P = .05 level. An excess of heterozygotes is normally explained in terms of selective forces acting on genotypes to produce a net heterozygous advantage, and it may be that observed *EST* genotype distributions in samples of New Zealand snapper represent an array of outcomes produced by the opposing forces of heterozygous advantage and a Wahlund effect (EST^{100} allele

frequencies are also heterogeneous over the range of New Zealand snapper).

An examination of genotype distributions in different age classes of Australian snapper was not possible because of difficulties in ageing individuals, combined with a substantial variation of individual growth rates within and between areas (see chapter 2). Smith et al. (1978) arbitrarily divided each of their New Zealand snapper sample sets into three length classes - 20 to 29.5 cm, 30 to 39.5 cm, and 40 + cm. They found significant differences in genotype distributions between length classes in two out of 12 sample sets. In subsequent studies Smith (1979), with apparently improved ageing techniques, demonstrated a correlation between *EST* allele frequencies in different year classes and sea surface temperatures in the year that each class was spawned. He also found that the *EST* genotypes were in Hardy-Weinberg equilibrium for each year class, and suggested that temporal, rather than spatial, *EST* allele heterogeneity would account for an observed Wahlund effect in sample sets consisting of mixed year classes. This hypothesis seems to be a feasible alternative explanation for the Australian snapper *EST* results, but for the reasons given above could not be tested.

As with *EST*, there is a net deficiency of *IDH* heterozygotes in the combined Australian snapper data. An examination of observed and expected genotype distributions reveals, however, that the deviation is caused mainly by excesses of $IDH^{100/100}$ and $IDH^{114/114}$ homozygotes and a deficiency of $IDH^{114/100}$ heterozygotes:-

IDH Genotypes	Observed	Expected
124/114	1	-
124/100	-	1
114/114	29	17
114/111	-	1
114/100	132	158
114/80	22	20
111/100	9	7
100/100	385	373
100/80	95	96
100/70	1	1
80/80	6	6
Total	680	680

At the same time there is close agreement with Hardy-Weinberg proportions for genotypes involving the allele IDH^{80}. Of the individual sample sets only the 1976 Port Phillip Bay collection exhibited a significant heterozygote deficiency. This single significant result in 15 tests could be attributed to a Type II sampling error, but it is noteworthy that the 1977 Port Phillip Bay sample also exhibited a deficiency of heterozygotes, as did three other samples in which deviations were detected. The repeat result in Port Phillip Bay could be interpreted as the effect of inbreeding in a genetically isolated group of snapper that returns to the same spawning grounds year after year. However the stochastic effect of inbreeding is to reduce the proportions of all classes of heterozygotes (Crow and Kimura, 1970) and such a deficiency is not observed for $IDH^{114/80}$ and $IDH^{100/80}$ heterozygotes in the Port Phillip Bay samples, nor for

heterozygous classes at the *ADA* and *GPI* loci.

It will be shown in section 3.5.4 that IDH^{100} and IDH^{114} are significantly heterogeneous across the distribution of Australian snapper, while IDH^{80} frequencies are comparatively more stable. It seems reasonable, therefore, to explain observed deviations of snapper *IDH* genotype frequencies in terms of a Wahlund effect operating on genotypes involving IDH^{100} and IDH^{114}, but not on genotypes involving IDH^{80}. As with the *EST* locus, a lack of accurate ageing data made it impossible to determine whether the *IDH* heterozygote deficiency was due to spatial (breeding isolates) or temporal (year class) sources of heterogeneity in allele distributions.

Smith et al. (1978) found a close agreement between observed and expected *IDH* genotype frequencies in all samples of New Zealand snapper, but this result is not surprising in view of the uniformly high (p = .945 - .986) IDH^{100} allele frequencies obtained.

A significant deviation of ADH genotypes from Hardy-Weinberg proportions in the combined Australian snapper data (Table 3.13) is due mainly to an excess of $ADH^{125/125}$ homozygotes and a corresponding deficiency of $ADH^{125/100}$ heterozygotes:-

ADH Genotypes	Observed	Expected
150/150	1	-
150/100	7	9
125/125	4	-
125/100	6	14
100/100	633	628
100/70	28	29
70/70	1	-
Total	680	680

The excess of *ADH*$^{125/125}$ homozygotes can be traced to the two Cowled Landing samples from upper Spencer Gulf, South Australia - both of which exhibit highly significant Smith's H values. Table 3.11 shows that *ADH*^{125} was found only in the three Spencer Gulf samples, indicating the presence of an isolated breeding population resident in the Gulf. Under these circumstances a number of hypotheses, or a combination of them, can be invoked to explain the observed genotype deviations:-

(a) Mixed populations have been sampled due to migration of snapper into Spencer Gulf from other areas. This hypothesis is supported by recent tagging studies conducted by the South Australian Department of Agriculture and Fisheries (Jones, 1979).

(b) There is an appreciable measure of inbreeding in the distinct Spencers Gulf population. This possibility appears unlikely because of the size of the snapper stock as indicated by commercial catch statistics from the Gulf (Jones, 1979). However as nothing is known of the origin of the Spencer Gulf population, or whether it is further subdivided into smaller breeding isolates, the possibility of inbreeding effects cannot be ruled out.

(c) Selective forces operate on the *ADH* genotype array or on linked loci to produce a net heterozygote disadvantage during at least some phases of the life cycle in Spencer Gulf snapper. As with the *EST* and *IDH* loci it was not possible to test for temporal heterogeneity of *ADH* genotype distributions.

It can be seen from all of the above results that interpretation of genotype distributions in natural populations of fishes involves consideration of a number of alternate hypotheses, many of which cannot be rejected without much more extensive sampling and statistical analysis than was undertaken

here. Nevertheless heterozygote deficiency has been consistently observed in the Australian snapper samples, and the available evidence suggests that this pattern can best be explained by postulating a Wahlund effect as a result of sampling a mixture of differentiated geographical breeding populations.

3.5.4 Distribution of Genes

Examination of the allele frequencies for seven polymorphic loci screened in the 15 Australian snapper samples reveal a variety of distribution patterns (Table 3.11). A contingency χ^2 test of allele distributions at the *EST* locus (bulked into a three-allele system to avoid low expected numbers in some allele classes) produced a highly significant result (cf. χ^2_{28} = 120.51, $P < .0001$). The most obvious component of this spatial heterogeneity is a clinal decrease in the frequency of the common allele, EST^{100}, in samples starting from south-east Queensland and moving around the southern coastline to Shark Bay in north-west Western Australia (Figure 3.2 and Table 3.11). Homogeneity tests on individual alleles (see section 3.4.4) show that the EST^{100} distribution is highly heterogeneous (cf. χ^2_{14} = 100.39, $P < .0001$), as are the distributions of EST^{108} (cf. χ^2_{14} = 60.30, $P < .0001$) and EST^{90} (cf. χ^2_{14} = 32.59, $P = .005$). The other *EST* alleles were too rare to test individually. EST^{108}, EST^{95} and EST^{90} distributions are all clinal to some degree, but in a reverse direction to the EST^{100} cline.

The two Port Phillip Bay samples appear to differ from samples to the east and west in that they have the highest EST^{100} frequencies and, together with the small Bannister Head

collection, are the only samples in which EST^{108} was not detected. Based on the observed EST^{108} frequencies in other samples it is unlikely that the failure to detect this gene in specimens from Port Phillip Bay was due to random sampling error (cf. $\chi^2_1 = 25.03$, $P < .0001$). It therefore appears that gene flow from areas sampled to the east and west is sufficiently restricted to prevent the establishment of the EST^{108} allele in Port Phillip Bay snapper stocks.

A comparison of EST^{100} frequencies between duplicate sample sets from Sydney, Port Phillip Bay and Cowled Landing (upper Spencer Gulf, S.A.) shows close agreement in each case, and suggests a certain degree of temporal stability of the EST^{100} cline, at least over the period of the study. The two Backstairs Passage samples did not have similar EST^{100} frequencies ($p = .86$ and $p = .74$), but a possible explanation for this discrepancy can be found in the collection strategy adopted for the November 1977 sample set. This sample set is a composite of snapper collected on two consecutive days and from two locations approximately three kilometers apart. Estimates of EST^{100} frequencies in the two sub-samples were not significantly different because of the small numbers of specimens involved, but the estimate from one sub-sample agreed closely with the EST^{100} frequency of the March 1977 Backstairs Passage sample, while the other sub-sample was very similar to the two Cowled Landing samples. These observations, together with a significant observed deficiency of heterozygotes in the November 1977 Backstairs Passage sample (section 3.5.3) suggest that a mixed stock of snapper was sampled in November, but not in March.

Allele distributions at the *IDH* locus were also

significantly heterogeneous over the range of Australian snapper (cf. χ^2_{28} = 45.09, P = .02) and, as with the *EST* polymorphism, the most obvious component of the *IDH* variation is an east-west clinal decrease in the frequency of IDH^{100} (homogeneity χ^2_{14} = 32.58, P = .005). There is a compensatory reverse cline in frequencies of IDH^{114} (cf. χ^2_{14} = 27.60, P = .02), but the other major allele, IDH^{80}, does not show any discernable pattern of geographic variation, and in fact sample estimates of IDH^{80} frequencies are not significantly heterogeneous (cf. χ^2_{14} = 20.25, P = .13). Of the rare allele distributions IDH^{111} is notable in that, with the exception of a single heterozygote from Port Phillip Bay, it is found only in east coast snapper samples. Comparison of east coast samples with the rest shows that such a distribution is an unlikely result of random sampling (cf. χ^2_1 = 14.96, P < .001), and provides further evidence that westward gene flow in snapper is restricted around the south-eastern corner of the Australian coastline.

The *ADA* locus, with sample estimates of ADA^{100} ranging from .76 to .95, is as polymorphic as the *EST* and *IDH* loci, and has a significantly heterogeneous distribution of alleles over the range of the species (cf. χ^2_{14} = 30.50, P = .008). However there is no discernable geographic pattern of variation in the major alleles ADA^{100} and ADA^{88}, with frequency estimates appearing to fluctuate at random from sample to sample.

The *ADH* locus, while less polymorphic than the above three loci (Table 3.11), still has a significantly heterogeneous distribution of alleles (cf. χ^2_{14} = 37.60, P < .001). The two most striking components of this heterogeneity are the lack of any detected rare alleles in the east coast samples, and the presence of ADH^{125} only in samples taken from Spencer Gulf,

South Australia (Rosalind Shoal and Cowled Landing samples). These results represent further evidence that gene flow is restricted in a number of areas across the range of Australian snapper. There is also a discernable east-west cline in sample estimates of ADH^{100} frequencies, even though the observed range is only p = 1.0 to p = .91. Comparisons of allele frequencies in the four pairs of duplicate samples suggest that the ADH^{100} cline, as with those for EST^{100} and IDH^{100}, was temporally stable over the period of the study.

Of the remaining three polymorphic loci *FUM* and *PGM* had allele distributions which were homogeneous within the limits of sampling error (cf. χ^2_{14} = 21.76, P = .10 and cf. χ^2_{14} = 14.92, P = .41 respectively). The *GPI* polymorphism exhibited an overall heterogeneity of allele distributions (cf. χ^2_{14} = 27.58, P = .015). Most of this heterogeneity, however, can be attributed to the Shark Bay sample, which has a lower frequency of the common allele GPI^{100}. If this sample is excluded *GPI* allele distributions over the remaining snapper samples are homogeneous (cf. χ^2_{13} = 16.31, P = .24). Supporting evidence for the genetic distinctness of the Shark Bay sample is the unique presence in this sample of the rare alleles GPI^{120}, PGM^{117}, IDH^{124}, ADA^{124} and EST^{117} (see Table 3.11).

Table 3.14 lists the sex ratios in each snapper sample set and in the combined data. There is a significant excess of females in the Bannister Head sample and a significant excess of males in the Shark Bay sample, but overall the sex ratio is not significantly different from an expected 1 : 1 ratio. A sample by sample comparison of allele distributions in each sex was not carried out because of small sample sizes, but χ^2 homogeneity tests of the combined data shows that allele distributions at all polymorphic loci are random with respect to sex:-

Table 3.14

Sex ratios of the individual and combined Australian snapper samples. See Table 3.2 for sample code. * = samples in which individuals were not sexed, either because they were juveniles, or because of logistic problems.

Sample	Males	Females	n	χ_1^2	P.
1	-	-	-*	-	-
2	28	27	55	.02	.95
3	13	20	33	1.48	.26
4	4	25	29	15.21	.0001
5	-	-	-*	-	-
6	27	35	62	1.03	.31
7	30	34	64	.25	.64
8	19	18	37	.03	.86
9	26	31	57	.44	.52
10	26	30	56	.29	.62
11	14	19	33	.76	.39
12	34	36	70	.06	.81
13	9	11	20	.20	.66
14	-	-	-*	-	-
15	40	20	60	6.67	.01
Total	270	306	576	2.25	.14

Locus	χ^2	d.f.	P.
EST	.32	3	.96
IDH	.53	2	.78
ADA	.73	1	.42
FUM	.05	1	.89
ADH	.89	2	.64
GPI	1.14	2	.59
PGM	1.39	2	.53

Figure 3.6 shows the length distribution in each of the Australian snapper samples collected, and illustrates the difficulty encountered in trying to distinguish size modes which represent single year classes. Ageing of individual fish by counting scale annuli was attempted, but the results proved to be unreliable over a series of repeated counts, particularly for fish larger than about 30 cm LCF. Consequently no comparison of allele distributions in different age classes was possible.

In view of the similar clinal patterns of allele distribution observed at the *EST*, *IDH* and *ADH* loci a number of contingency χ^2 tests were carried out on these and the *ADA* locus to determine whether there was any non-random association of genotypes in pairs of loci:-

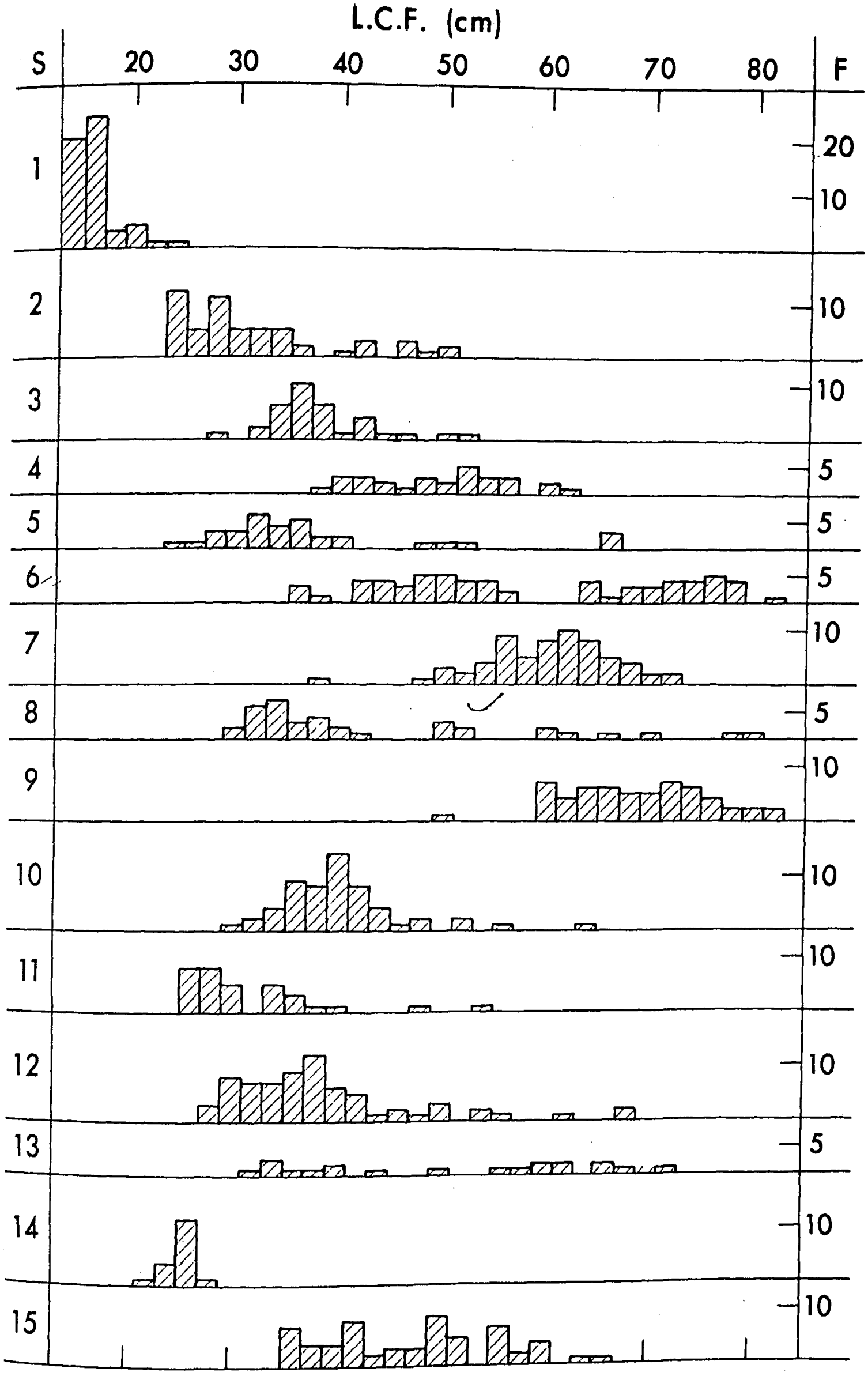
Loci	χ^2_4	P.
EST/IDH	3.27	.52
EST/ADH	2.48	.67
EST/ADA	1.43	.84
IDH/ADH	4.29	.37
IDH/ADA	.26	.98
ADH/ADA	.75	.93

Figure 3.6

Length frequency histograms for the Australian snapper sample sets. See Table 3.2 for the sample code.

S = Sample

F = Frequency


L.C.F. (cm)
S
20
30
40
50
60
70
80
F
1
20
10
2
10
3
10
4
5
5
5
6
10
7
5
8
10
9
10
10
10
11
10
12
5
13
10
14
10
15

The results indicate that genotypes at each of these four loci are distributed independently of each other. It must be noted, however, that genotype classes at each locus were bulked into a two-allele system to comply with statistical requirements, and the χ^2 test consequently has reduced power to detect associations between rare genotypes. In addition, the results suggest a lack of epistatic interaction between the loci analysed, but do not rule out the possibility that two or more of these loci may still be linked by being located on the same chromosome.

3.5.5 Kinship Analysis

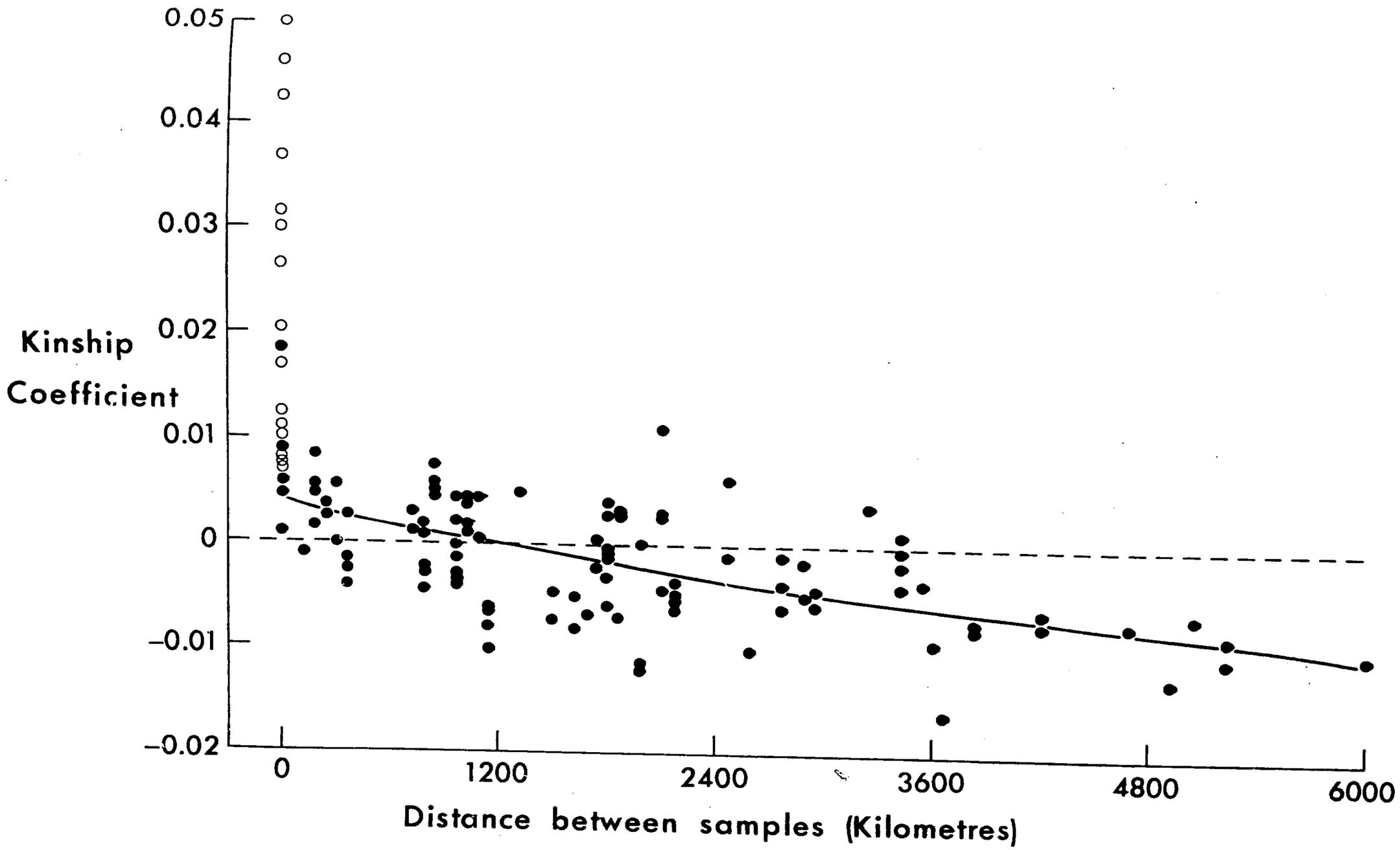
Average coefficients of kinship (as described in section 3.4.5) were computed for the seven polymorphic loci in Australian snapper samples, and plotted against geographical distance between samples (Figure 3.7). The regression line was fitted by computer using a second degree polynomial function. At the zero distance-between-samples mark most of the ϕ values, including all those greater than .02, are within-sample kinship estimates. The mean within-sample kinship estimate is .0212, with measures for each locus ranging from .0346 to .0121. Mean kinship coefficients between duplicate samples from the same location are much lower than within-sample estimates (.0007 to .0184), and may reflect either temporal variation in local patterns of genetic variation or - perhaps more commonly - random sampling error. The within-sample kinship estimates were excluded from the kinship/distance regression analysis, as they artificially bias the regression line towards higher relatedness near the zero distance mark,

Figure 3.7

Mean kinship coefficient values for pair-wise comparisons of snapper sample sets, plotted against geographical distance between the samples. The regression line is a line of best fit using a second degree polynomial function.

O = within-sample kinship coefficients

● = between-sample kinship coefficients


0.05
0.04
0.03
0.02
0.01
0
-0.01
-0.02
Kinship Coefficient
0
1200
2400
3600
4800
6000
Distance between samples (Kilometres)

and also influence the point at which the regression line meets the zero kinship line.

The regression line in Figure 3.7 intersects the zero kinship line at about the 1200 kilometers mark, and ϕ values then become more negative with greater distance. The consistently negative ϕ values at large distances are a reflection of clinal allele frequency distributions in the *EST*, *IDH* and *ADH* loci, and indicate that two genes sampled from extremes of the range of Australian snapper are more likely than not to be different. The decline of kinship to zero by 1200 kilometers indicates that this is the average length of coastline covered by an interbreeding population of Australian snapper.

It should be noted that kinship estimates and isolation-by-distance regressions are very sensitive to the sampling framework within which they are constructed. For example the present analysis is based on Australian snapper populations which have a continuous distribution around the southern coastline. If, however, we were to include some samples from the isolated and genetically distinct New Zealand snapper populations (Smith et al., 1978 and see chapter 5) the nett effect would be to increase kinship between Australian samples and thus increase estimates of the average size of the "neighbourhood" inhabited by an interbreeding population.

3.5.6 Topology of Kinship

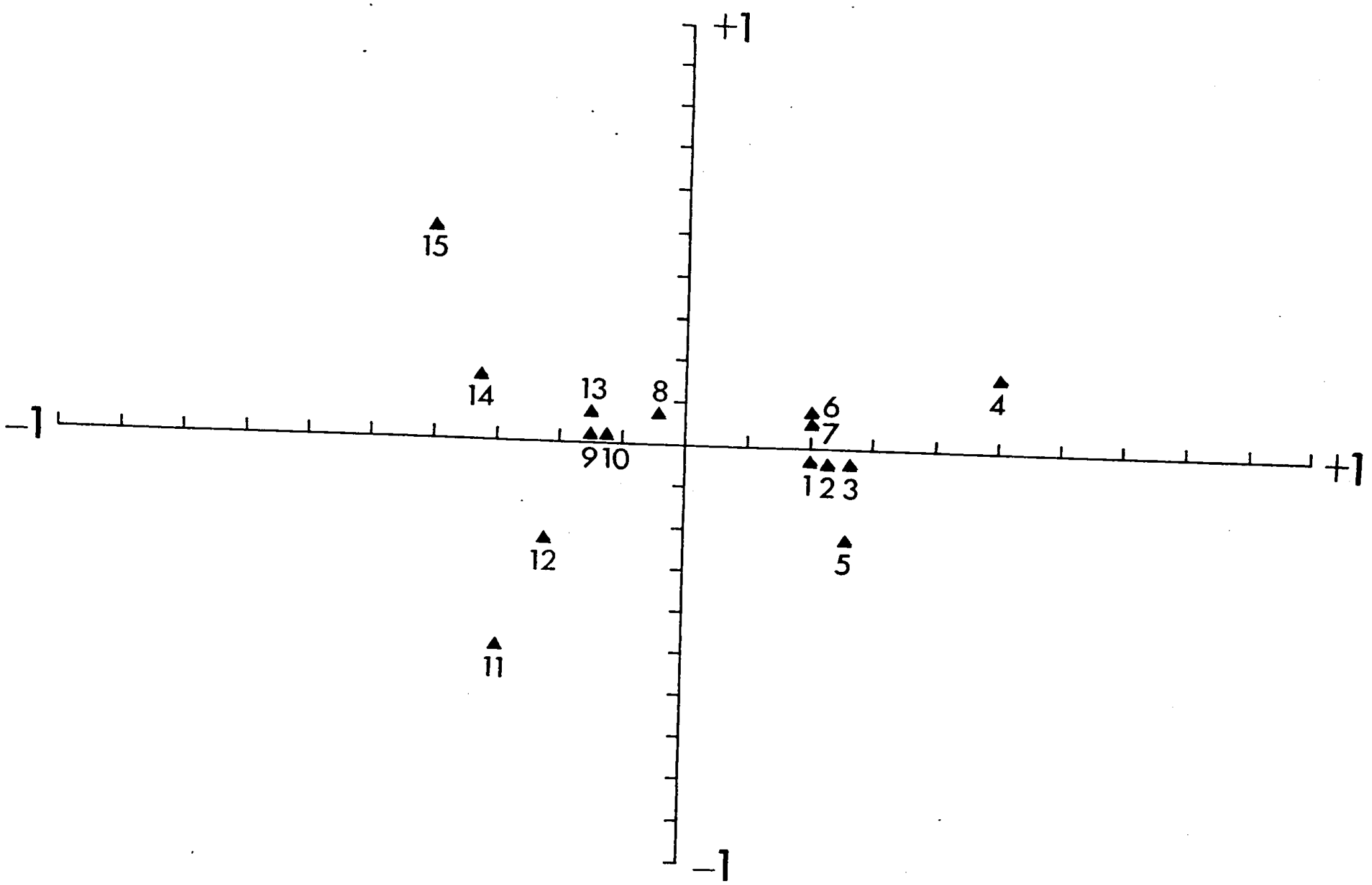
The plotting of a kinship/distance regression gives an indication of the degree of population structuring within the distribution of Australian snapper, but does not provide

information on the distribution of genetic variation or the relationships between individual sample sets. La Louel (1973) has developed a method for using ϕ values computed from the BIOKIN program (section 3.4.5) to visualise the topology of genetic variation over the range of the species studied. A principle components analysis is used, with a matrix of pairwise ϕ values between samples as input data. The computer program used in this study is PHEIGEN, developed by Lew (1973) following the concepts of La Louel. The genetic relationship between samples is visualised by partitioning observed variance into a number of components, representing these components as axes (eigenvectors) in an n-dimensional hypervolume, and plotting each sample according to its relative contribution (eigenvalue) to each component of variance.

In the case of Australian snapper 88.41% of observed variance in ϕ values is accounted for in the first two eigenvectors, and only these two axes are used to plot the samples (Figure 3.8). The nature of the PHEIGEN program makes it impossible to accurately correlate components of variance with specific external factors. It appears, however, that the component represented by the horizontal axis consists largely of geographic clines observed in allele frequencies at the *EST*, *IDH* and *ADH* loci, as the positions of samples along this axis are similar to the relative geographical locations from which the samples were obtained. The component of variance represented by the vertical axis is less easily described, but the positioning of the Shark Bay and upper Spencer Gulf samples at the extremes of this eigenvector suggest that the presence or absence of rare alleles in one or more loci largely determines the

Figure 3.8

Principle components analysis of the topology of genetic variation over the range of Australian snapper. The axes represent the principle components of variance (eigenvectors) and the data points are eigenvalues for each snapper sample set based on the pair-wise kinship coefficients given in Figure 3.7. See Table 3.2 for the sample code.


+1
15
14
13
8
6
7
4
−1
9 10
+1
1 2 3
12
5
11
−1

eigenvalues of each sample for this component.

3.5.7 Genetic Distance Analysis

An alternative method of visualising population structure within the distribution of a species is to obtain a descriptive measure of genetic distance between sample sets and plot these values against geographic distance between the samples. Genetic distance values between duplicate samples from a single location will provide a measure of intra-location heterogeneity. A measure of the average "neighbourhood" size of an interbreeding population can be obtained by observing where the genetic/geographic distance regression line intersects the upper limit of intra-location genetic distance values. Populations sampled further apart than the critical distance are thus genetically more divergent than can be explained by sampling error or local fluctuations.

Genetic distances (D) between snapper samples were based on allele frequencies at the seven polymorphic loci listed in Table 3.11 and were calculated using the following formula developed by Rogers (1972):-

$$D = \sqrt{\frac{1}{2}\sum_{i=1}^{m} (q_{iX} - q_{iY})^2}$$

where m = the number of alleles at a locus, and q_{iX} and q_{iY} = the frequencies of the i^{th} allele in samples X and Y respectively. Estimates from several loci are combined by calculating an average value of D. Other commonly used measures of genetic distance, such as that of Nei (1972), could

have been used, but comparisons by Hedrick (1975) and Schmitt (1977) have shown that Roger's D is highly correlated (>.90) with most of these other measures and is therefore equally as informative.

Figure 3.9 shows the regression of genetic distance against geographical distance in Australian snapper and it can be seen that the second degree polynomial regression line intersects the upper limit of intra-location genetic distance values at approximately the 900 kilometer mark. This is a fairly close approximation to the critical distance of 1200 kilometers derived from the kinship analysis, but for reasons outlined in section 3.4.5 the kinship/distance analysis is usually a more precise method of depicting population structure, and will be preferred here.

3.6 Discussion

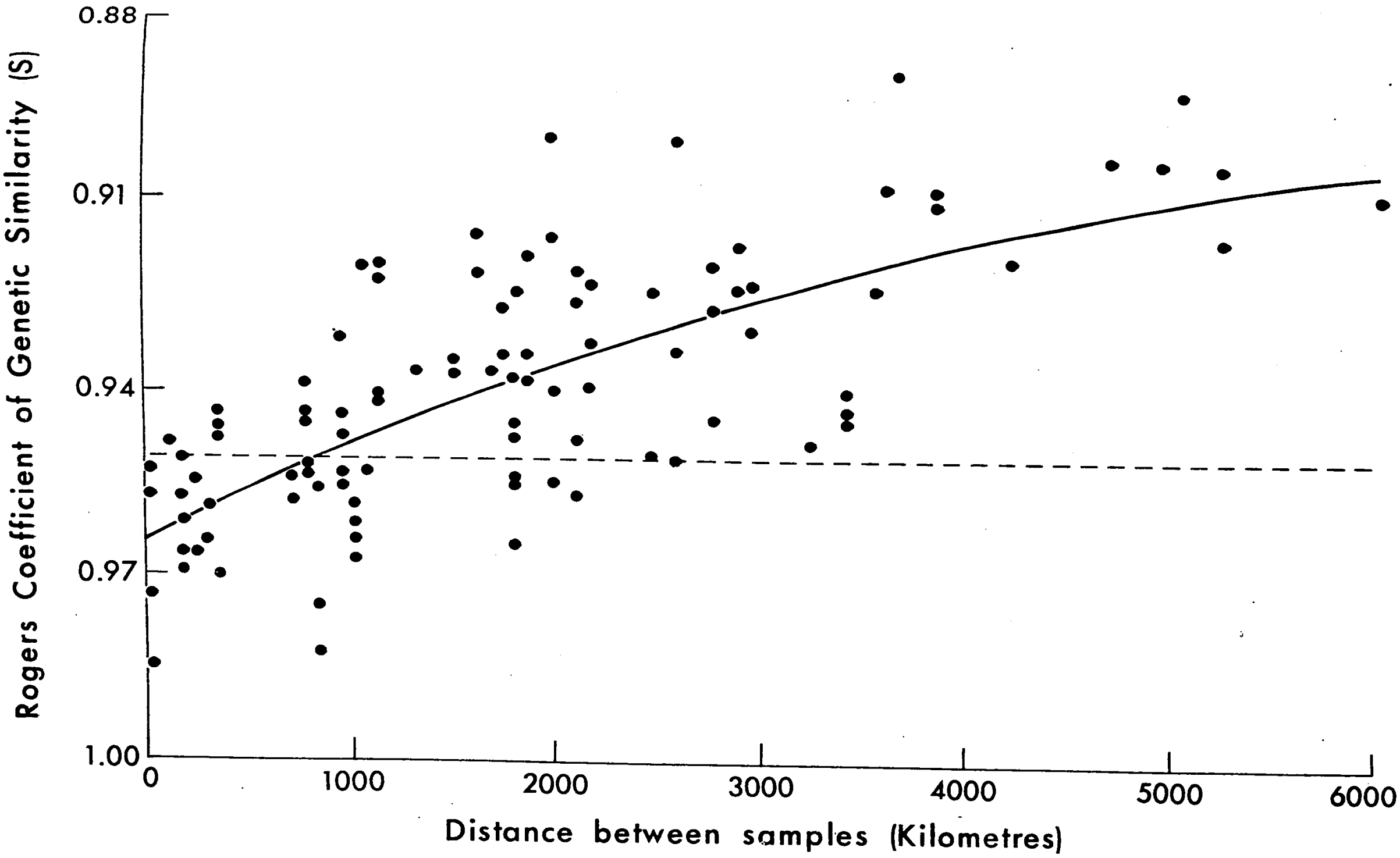
A comparison of the postulates made in section 3.1.3 and the subsequently obtained electrophoretic results indicates that observed patterns of genetic variation in western salmon and Australian snapper are substantially in agreement with patterns predicted from the life history characteristics of these species. There are, however, a number of reservations about this statement arising from alternative possible interpretations of the data. These are discussed below.

3.6.1 Heterozygosity

As expected, mean heterozygosity per locus per individual for Australian snapper fell into the middle to lower range of

Figure 3.9

Regression of Rogers S values for pair-wise comparisons of Australian snapper sample sets against geographical distance between samples. The broken line indicates the lower limit (.952) of observed Rogers S values for comparisons of sample sets from the same geographical location.


0.88
0.91
0.94
0.97
1.00
Rogers Coefficient of Genetic Similarity (S)
0
1000
2000
3000
4000
5000
6000
Distance between samples (Kilometres)

values for teleosts reported by Powell (1975) and Nevo (1978). The value for western salmon, however, is much lower (H = .008 ± .0003) than its latitudinal position and life history characteristics would predict, and only five teleost species were found by Powell and Nevo to have lower heterozygosity estimates.

It may be argued that low levels of variability in western salmon can be attributed to the choice of enzymes examined, and that those chosen happen to be essentially invariable in the taxonomic group to which western salmon belong. Differences between enzymes in the frequency with which they exist in the polymorphic state have been reported by many workers (see Koehn and Eanes, 1978 for a review), and have been variously attributed to the role of the enzyme in metabolism (Johnson, 1974, 1976; Gillespie and Langley, 1974; Zouros, 1975), the quaternary structure of the enzyme (Zouros, 1976; Ward, 1977; Harris et al., 1977), and the size of the constituent subunit(s) in the enzyme (Koehn and Eanes, 1977; Eanes and Koehn, 1978). Whatever the merits of these arguments, the enzymes examined in this study were chosen according to satisfactory electrophoretic results, a criterion which should be random with respect to enzyme-specific levels of polymorphism. In fact the 22 enzymes chosen had both single- and multi-substrate specificities, and consisted of representatives from the oxidoreductase, transferase, hydrolase, lyase and isomerase classes. Electrophoretic patterns also suggested that the enzymatic products of five loci were monomers, 13 were dimers, and nine were tetramers. Perhaps a more important point is that the same enzymes have been used in all species studied, so that any biases of the electrophoretic technique and the enzyme selection procedure are uniform.

Comparisons of genetic variability within and between the species studied are thus still valid.

The low heterozygosity estimate for western salmon cannot be considered to be characteristic of the family Arripidae, as H values for eastern salmon (.049 ± .0045) and tommy ruff *Arripis georgianus* (.041 ± .0176 - see chapter 5) are much higher. It is also unlikely that the western salmon result represents a major divergence in "adaptive strategy" with regard to enzyme function, as western and eastern salmon - despite their genetic distinctness at some loci (section 3.4.1) - have broadly overlapping life history characteristics and ecological requirements. The only remaining explanation is that the western salmon population has lost genetic variability during a comparatively recent population "bottleneck" - either because of a dramatic reduction in numbers, or as the result of a founder effect during speciation. This hypothesis will be discussed further in chapters 5 and 6. It is noteworthy, however, that in the absence of any apparent stochastic perturbations the heterozygosity estimates of eastern salmon and tommy ruff are in accord with expectations for temperate pelagic fishes (section 3.1.3).

There is a small but significant difference between heterozygosity levels in New Zealand and Australian snapper. There is also a discernable east-west increase in H values for each of the Australian snapper samples (Table 3.12), but most of the observed variation is within the limit of sampling error. The tenuous nature of these results preclude any further discussion at this point, but this evidence will be reviewed in the light of later information (chapter 5).

3.6.2 Variation Within and Between Populations

The lack of suitable polymorphic systems detected in western salmon samples made it difficult to test hypotheses concerning the distribution of variation in this species. In addition, sample estimates of allele frequencies for the one useful polymorphism at a liver *EST* locus were homogeneous over the entire range of the animal. The only detected variation was age-specific differences in *EST* genotype distributions, with an indicated deficiency of $EST^{100/93}$ heterozygotes in the youngest sampled year class (Table 3.6). This finding is in accord with the patterns of genetic variation predicted for western salmon (section 3.1.3), and the simplest explanation of the observed pattern is selective mortality of $EST^{100/93}$ individuals in the early life history phases of some year classes.

The data are not comprehensive enough to indicate the fate of deviations in $EST^{100/93}$ frequencies in given year classes, but invoking heterozygous disadvantage to explain $EST^{100/93}$ deficiencies raises two major points. Firstly it seems that a strong selective regime would have to be imposed on the *EST* genotypes (or on genotypes from linked loci) to produce the observed 50% deficiency of $EST^{100/93}$ heterozygotes in the 1975 year class (Table 3.6). However western salmon - in common with many teleosts - suffer very high mortality during the egg and larval phases of their life cycle, and the potential for temporal perturbation of gene and/or genotype distributions in a single breeding population is obvious, even when differences in relative fitness values are comparatively small. Selective events of this kind could explain observed

excesses of homozygotes in many teleost species (e.g. Utter et al., 1970; Koehn et al., 1971; Jameison et al., 1971; Mitton and Koehn, 1975; Le Clus, 1978; Smith, 1979), especially where deviations in genotype frequencies cannot be explained by the sampling of distinct populations.

Secondly the lower fitness of $EST^{100/93}$ heterozygotes implies the operation of disruptive selection, and in the absence of compensatory mechanisms should lead to either population subdivision or fixation of one of the two alleles. A stable polymorphism can be maintained, however, if there is a compensatory increase in heterozygosity with age, or if an excess of heterozygotes is produced in the next generation by a perturbed cohort, effectively counteracting heterozygote disadvantage. The alternatives cannot be assessed given the present data.

Observed deviations of genotype frequencies from Hardy-Weinberg expectations in Australian snapper were also in the direction of homozygote excess (Table 3.13), but the heterogeneous geographic distribution of *EST*, *IDH*, *ADA*, *ADH* and *GPI* alleles, and knowledge of the limited capacity for gene flow in this species, suggests that such deviations are more likely to be the result of a Wahlund effect in sampling genetically differentiated populations. This interpretation of the data is consistent with predictions made concerning the distribution of genetic variation in snapper (section 3.1.3), but it may not explain all - or even a majority - of the observed patterns. Smith (1978, 1979) has demonstrated year-class differences in *EST* gene and genotype frequencies in a single breeding population of New Zealand snapper and has correlated allele distributions with prevailing water

temperatures at the spawning grounds. This evidence suggests that the *EST* locus is directly or indirectly under selection, and advances temporal variation of gene and genotype frequencies as an alternative explanation of the observed deviations from Hardy-Weinberg proportions. Due to problems outlined in section 3.5.3 Australian snapper were not aged and year-class analysis of genetic variation was not carried out. Consequently it was not possible to assess the relative importance of spatial and temporal components of genetic variation in Australian snapper populations.

3.6.3 Geographic Variation and Population structure

Inferences regarding population structure in western salmon are necessarily limited by the lack of useful polymorphic markers. *EST* allele distributions are homogeneous over the range of the species, and all other loci are essentially monomorphic. Significant deviation of *EST* genotypes from Hardy-Weinberg expectations in some samples is more closely associated with year-class or temporal variation in selective regimes than spatial heterogeneity. While failure to detect variation is not a guarantee that variation is non-existent, the weight of evidence from morphological, ecological, tagging and genetic studies strongly supports the contention that western salmon are a single interbreeding population with a spawning area along the south-west tip of the Western Australian coastline and a distribution that extends right across the southern coastline of Australia.

The genetic data for Australian snapper is more comprehensive and thus more difficult to interpret. The

presence of significant spatial heterogeneity in the distribution of *EST*, *IDH*, *ADA*, *ADH* and *GPI* alleles suggests that there is restricted gene flow between a number of areas over the range of the species (Figure 3.2 and Table 3.11). Isolation-by-distance studies based on kinship estimates (Figure 3.7) indicate that on average an interbreeding population covers 1200 kilometers of coastline, but evidence from tagging studies (see chapter 2) and the distribution of rare alleles suggests that the actual range of some populations may vary considerably from this mean. The presence of a small isolate in Spencer Gulf, South Australia has already been described (section 3.5.4). By contrast there appears to be some gene flow along the entire east coast range of snapper. This observation is based on the homogeneity of allele frequency data in samples 1 to 5 (Table 3.2), and on long distance movements of adult snapper indicated by tagging studies in the early 1960's (Sanders, 1974). Results from the present study indicate that there could be at least five partially isolated genetic populations of snapper in Australian waters. Further work is needed to map the range of these populations in detail, but preliminary evidence suggests that samples from the east coast, Port Phillip Bay, Backstairs Passage, Spencer Gulf and Shark Bay are each representatives of seperate breeding units.

Discussion of the relative contribution of random and deterministic forces in maintaining observed polymorphisms in Australian snapper and salmon will be deferred until chapter 6 to allow for consideration of data arising from enzyme kinetic (chapter 4) and systematic (chapter 5) studies.

Chapter 4

Temperature Adaptation and Allozyme Function

"We may realistically anticipate learning more about whether alleles are adaptively neutral, or of how or when they exhibit advantages, by test tube studies than we may soon learn otherwise."

Lyn Throckmorton (1971)

4.1 Introduction

Having detected various geographical distribution patterns of electrophoretic allozymes in *Chrysophrys* and *Arripis* species (chapter 3), the question arises as to what processes are influencing these patterns. The possible mechanisms involved have been described in section 3.1.1, and from an evolutionary viewpoint it is of particular interest to assess the contribution made by natural selection. More specifically, can it be demonstrated that there are environment-mediated functional differences within an array of allozymes? If so, can it be shown that these potentially selective differences are realised in the form of variation in response to changing environmental regimes? Clarke (1975) has suggested an experimental scheme for tackling these questions (see section 3.1.1), and where possible this scheme has been followed in the present study. All but one of the requirements of Clarke's scheme have been at least partially satisfied, the exception being that it was not possible to test postulated allozyme/environment relationships by carrying out *in vivo* perturbation experiments to produce predicted responses.

One of the most difficult tasks faced in studies of

genotype/environment interactions is the detection of environmental components that are acting as selective agents on a given array of gene products. An *a priori* choice of which environmental variable(s) to measure is sometimes facilitated by observed correlations between allele distributions and a specific environmental component, or by differences between phenotypes which are so obvious as to immediately suggest a selective agent. Where such clues are absent the investigator can only make intuitive guesses as to the variables expected to have selective effects.

Ambient temperature was chosen here as a potentially selective environmental component for the following reasons:-

(a) As outlined in chapter 1, temperature is considered to be one of the most pervasive factors in the environment of aquatic ectotherms, and is likely to directly or indirectly influence the fitness of a large number of phenotypes in these organisms.

(b) The physiological properties of enzymes and enzyme-catalysed metabolic reactions are governed by certain basic laws of thermodynamics. It therefore seems reasonable to expect that functional differences between allozymes in response to temperature will be potentially selective in organisms that must compensate physiologically for external temperature fluctuations in order to maintain optimum metabolic rates.

(c) Although there is no obvious correlation between temperature and the geographic distribution of electrophoretic allozymes in Australian populations of snapper and 'salmon', the clinal variation at three loci in snapper (section 3.5.4) can be explained most simply as a response to a gradient in a

physical component of the environment. Temperature is still an obvious candidate, even though the relationship might be more subtle than is immediately apparent.

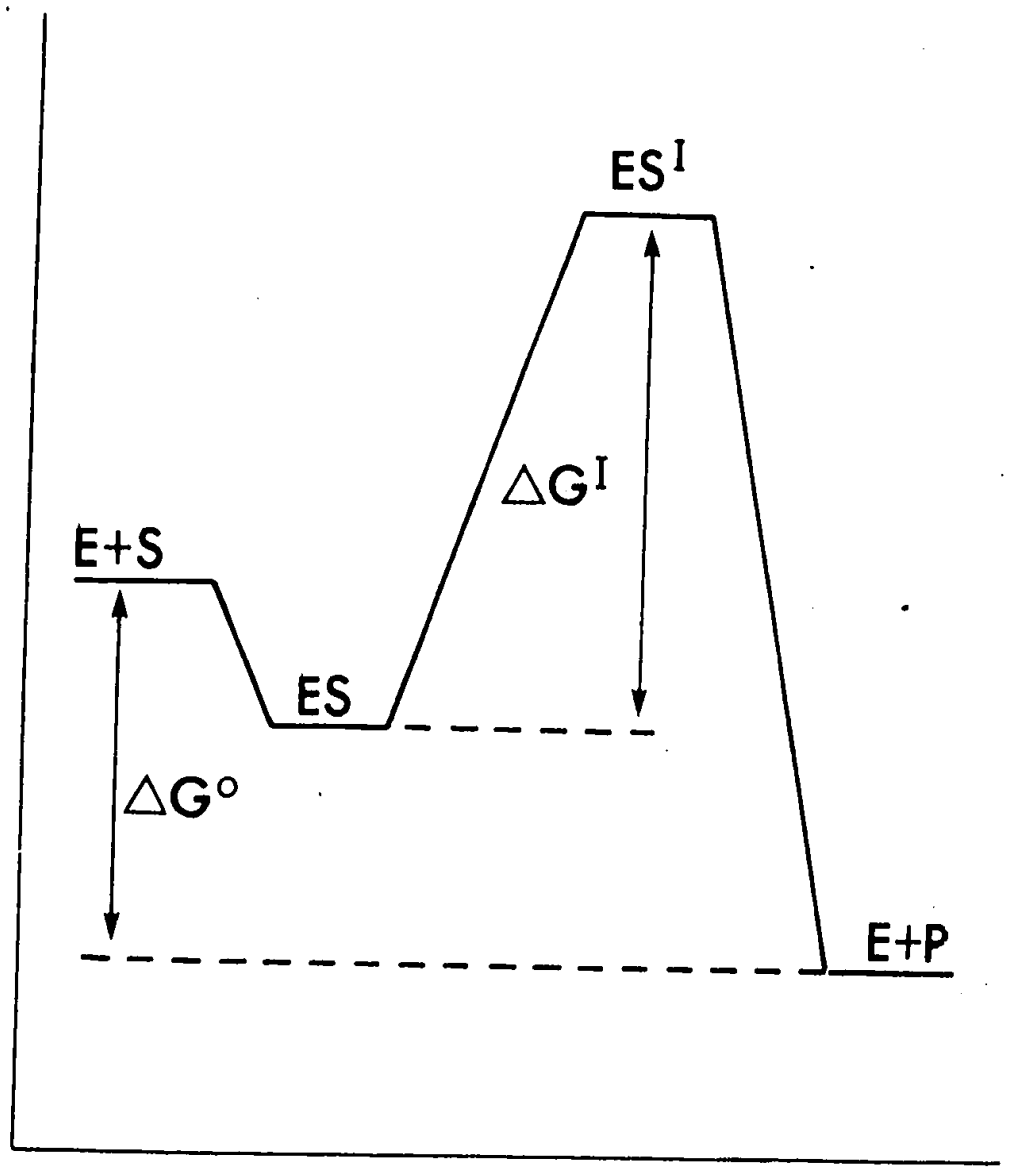
In considering temperature as a potentially selective environmental variable it is necessary to know something about the effects of this factor on enzyme function and on the rates of enzyme-catalysed reactions. It is also necessary to be aware of the "strategies" of biochemical adaption available to ectothermic organisms to compensate for temperature effects. I will briefly outline some of the theoretical aspects of enzyme/temperature interactions, citing mainly evidence obtained from studies on marine organisms to illustrate their considerable contribution to recent developments in this field.

4.1.1 Effect of Temperature on Reaction Rates

The rates at which the chemical reactions comprising metabolism occur are intrinsically dependant on the net difference in free energy (ΔG°) between the substrate(s) and product(s) of a reaction, and the magnitude of the "energy barrier" (ΔG^{I}) which needs to be overcome to activate the transition from substrate to product. ΔG° determines the thermodynomic feasibility of the reaction and ΔG^{I} the kinetic feasibility (Hochachka and Somero, 1973). Figure 4.1 illustrates these principles for a typical enzyme-catalysed reaction. An increase in cell temperature increases the average kinetic energy of the substrate molecules and increases the proportion that will have enough free energy to overcome the activation barrier and be converted to product. The relationship between temperature and reaction rate is defined

Figure 4.1

Diagramatic representation of free energy changes during a typical single-substrate enzyme-catalysed reaction. E = enzyme, S = substrate, P = product, ES = enzyme/substrate complex, ES^{I} = activated complex, ΔG^{o} = nett free energy change during the reaction, ΔG^{I} = energy needed to activate the ES complex.


Gibbs free energy (G)
E+S
ES
ES^I
ΔG^I
ΔG°
E+P
Reaction co—ordinates

by the Arrhenius Equation:-

$$k = Ae^{-Ea/RT}$$ (see Roberts, 1977)

where:-

k = the proportion of substrate molecules that have enough energy to overcome the activation barrier

T = absolute temperature

R & A = constants

Ea = the energy of activation required for the reaction to proceed.

The equation implies an exponential relationship between reaction rate and both temperature and activation energy, so that a small increase in temperature or a small decrease in Ea will result in a relatively large increase in reaction rate. It must be noted that Ea is only a measure of the enthalpy of activation, whereas ΔG^I includes both enthalpy and entropy changes during the activation process (Hochachka and Somero, 1973; Low et al., 1973). However entropy changes are difficult to measure, and for practical purposes Ea is usually considered a reasonable approximation of ΔG^I.

Empirical evidence confirms the exponential relationship between reaction rate and temperature. In the absence of any other source of perturbation most chemical reactions involving the breakage or formation of covalent bonds double or triple their rates with each 10°C increase in temperature. This relationship can be conveniently described as a temperature coefficient, Q_{10} (Hochachka and Somero, 1973) where:-

$$Q_{10} = \frac{\text{Reaction rate at T + 10°C}}{\text{Reaction rate at T°C}} = 2 \text{ to } 3$$

Reactions with Q_{10} values of less than 2 are therefore less heat sensitive than would be normally expected, and in the case of enzyme-catalysed reactions would be interpreted as having some mechanism for compensation of the temperature/rate effect. For more comprehensive reviews of the effect of temperature on reaction rates the reader is referred to Hochachka and Somero (1973), Morris (1974), Fersht (1977) and Roberts (1977).

4.1.2 Effect of Temperature on Enzymes

In the absence of a catalyst most metabolic reactions at physiological temperatures would not proceed at a rate fast enough to support life as we know it. Enzymic catalysts make use of the formation/breakage of weak non-covalent bonds to bind to substrate(s) and subsequently lower the energy barrier of the reaction by contributing chemical energy to the activation of transition state enzyme-substrate complexes. The activation energy (Ea) of the catalysed reaction is therefore lower than that of the uncatalysed reaction, and the reaction rate is considerably higher - hence the importance of catalysed metabolic reactions in sustaining living processes.

The "weak" bonds or interactions mentioned above consist of four main classes:- (a) van'der Waals' forces (b) hydrogen bonds (c) ionic (electrostatic) interactions and (d) hydrophobic interactions (Hochachka and Somero, 1973; Somero, 1975; Somero and Hochachka, 1976; Somero, 1978a). These weak bonds are of prime importance in determining the tertiary (i.e. shape or conformation) and quaternary (i.e. interactions between protein subunits) structure of enzymes,

and also the ability of enzymes to bind to substrate and other ligands. Because the energy required to form or break weak bonds (.3 to 8 kcal/mole - Somero and Low, 1977a) is very low compared to that for covalent bonds (30 - 200 kcal/mole - Barrow, 1961), the weak interactions are sensitive to temperature ranges encountered by living organisms and can readily be disrupted by small fluctuations within this range. Not all classes of weak bonds react in the same manner to temperature changes. For example hydrogen bonds and electrostatic interactions become increasingly more stable with reduction in temperature, whereas higher temperature enhances the strength of hydrophobic interactions (Brandts, 1967; Hochachka, 1974). Enzyme higher structure and function is therefore likely to be adversely affected by both high and low temperatures, the net result depending on the overall number and proportion of each type of weak bonds formed.

The effects of temperature on enzyme-catalysed reactions can therefore be best described in terms of a "compromise" between three main functional properties of enzymes:-
(a) structural stability (b) catalytic capacity and
(c) regulatory control of the reaction.

Most enzymes are thought to undergo rapid and reversible conformation changes during the binding and activation events in catalysis (Low and Somero, 1974). These conformation changes, which involve the formation and breakage of weak bonds, can contribute chemical energy to the activation of substrate and thus enhance the catalytic capacity of the enzyme (Low and Somero, 1975; Somero and Low, 1977b). The more conformationally "flexible" the enzyme, the greater its catalytic efficiency is likely to be. However the enzyme must

also maintain structural integrity in the face of fluctuating temperature to avoid denaturation. Under these circumstances the more conformationally "rigid" the enzyme molecule is the more thermostable it is. The properties of a given enzyme at a given temperature will thus be a compromise between opposing requirements.

At physiological substrate levels the ability of the enzyme to bind substrates, cofactors and other ligands can influence not only the catalytic efficiency of the enzyme but also its ability to respond to fluctuations in concentrations of ligands, i.e. the ability to regulate the flux of substrate(s) through the reaction (Somero, 1975, 1978a). The nature of enzyme-ligand weak bond interactions is determined primarily by the type and arrangement of amino acid residues at specific binding sites on the enzyme, but can also be influenced by the overall conformational shape of the enzyme molecule. For reasons given above, the more conformationally flexible an enzyme the better its binding abilities are likely to be. Enzyme-ligand interactions are thus a third aspect of the structure/function compromise of enzymes in response to temperature changes.

Clearly these three enzymic properties are closely interrelated, and modification of one is likely to affect the other two. However, the relative importance of these properties will differ between reactions and between organisms (Somero, 1975), and the hierarchical arrangement of these properties will determine the type of "adaptive strategy" adopted by the organism to compensate for the effect of temperature.

The genetic (and hence evolutionary) control of adaptive

properties of enzymes in response to temperature is inferred on the basis that protein primary structure (which is genetically coded) ultimately determines both the functional characteristics of an enzyme at a given temperature, and the range over which it is able to positively modulate metabolic reactions in response to temperature (Hazel and Prosser, 1974). The tertiary and quaternary structural integrity, catalytic capacity and ligand-binding abilities of an enzyme are a function of the number and type of weak bond interactions it is capable of forming and breaking. These weak bonds are in turn determined by the type and configuration of amino acid residues in the polypeptide chain(s) making up the enzyme molecule. Amino acid substitutions will therefore potentially alter the structural and functional characteristics of an enzyme under a given temperature regime, and produce variation which may be of selective value.

4.1.3 Adaptive Strategies

Having established the "goals" of enzymatic adaption to temperature (i.e. maintenance of structural integrity, adjustment of catalytic capacity to the cell's requirements for metabolic products, and adjustment of ligand-binding abilities to ensure maximum regulatory control of catalysis) we may next ask what methods or "strategies" are available to organisms for achieving these goals. For endothermic homeotherms this question is largely irrelevant, as the maintenance of a uniformly high cell temperature is likely to lead to the evolutionary development of enzymes with optimum characteristics at this temperature. It may be argued that

ectothermic poikilotherms (including most non-mammalian aquatic organisms) can achieve the same effect by behaviourally avoiding large fluctuations in ambient temperature. However, temperature is only one environmental parameter, and the benefits of behavioural regulation of this component must be assessed in the light of many other considerations. For example, the active selection of a favourable thermal regime may conflict with the requirements for obtaining food or for reproduction. This conflict can best be resolved if the organism develops physiological mechanisms which render it metabolically less sensitive to fluctuations in the prevailing thermal regime.

The differences between alternative biochemical strategies of adaptation to temperature are perhaps best explained in terms of the time period over which compensation occurs. Somero (1969), Hochachka and Somero (1973) and Hazel and Prosser (1974) have developed the concept of adaptation over three different time-periods:-

(a) instantaneous - where temperatures may vary considerably with little or no warning (e.g. movement of a fish across a thermocline, or the diurnal temperature fluctuations experienced by sessile intertidal invertebrates)

(b) adjustment over periods of acclimation - where adaptation to gradually changing temperatures takes a period of at least a week or more. The best illustration of this type of response is the acclimation of poikilotherms to seasonal extremes in ambient temperature

(c) adaptation over evolutionary time periods - for example the gradual change in thermal optima of metabolic reactions over many generations as a species adapts to a new

temperature regime.

One way in which an organism can compensate for the temperature/rate (Q_{10}) effect is by altering the concentration of enzyme in its cells so that the overall flux of metabolites through the reaction (measured as enzyme 'activity') is adjusted to desired levels. A review of studies up until 1974 (Hazel and Prosser, 1974, Table 2) has found that in tissues of many poikilothermic species a reduction in body temperature is accompanied by increases in measured enzyme activities in major metabolic pathways such as glycolysis, the Krebs or TCA cycle and the cytochrome c pathway. However, in some cases enzymes show no quantitative response or even an inverse response to low temperature acclimation (Hazel and Prosser, 1974, Table 3). This differential response of enzyme activities to temperature changes is attributed to a general "reorganisation" of metabolic requirements in organisms exposed to a new thermal regime (Somero and Hochachka, 1976). More recent studies, such as those on the teleosts *Lepomis cyanellus* (Shaklee et al., 1977) and *Blennius pholis* (Campbell and Davies, 1978), have confirmed the pattern reported by Hazel and Prosser.

There is some doubt, however, about the interpretation of enzyme activity estimates. Changes in activity can result from any of the following events:- (a) change in enzyme concentration (b) change in concentration of substrate or other ligands (c) qualitative alteration of catalytic properties in response to temperature (d) presence of multiple forms of the enzyme, each with optimum catalytic properties at different temperatures. There are only one or two publications in which activity changes during thermal acclimation have been clearly

shown to be due to change in enzyme concentration (e.g. studies by Sidell (1977) of the cytochrome c pathway in skeletal muscle of the sunfish *Lepomis cyanellus*). The evidence from other activity studies in support of a quantitative strategy of thermal adaptation must be considered equivocal.

The universal use of a quantitative strategy of thermal adaptation can be considered undesirable on a number of grounds. Firstly, change in enzyme concentration is unlikely to be a useful method of adjusting metabolic rates to instantaneous temperature fluctuations, because a genetic response is required to alter the rate of synthesis of either the enzyme itself or the modifying proteins which affect the structural stability/catalytic properties of the enzyme. For example Sidell et al. (1973) showed that the typical time course for enzyme activity changes in cold-acclimated goldfish (*Carassius auratus*) was three to four weeks. Secondly, varying the concentration of all enzymes in response to temperature changes may cause physiological problems in the cell, which has a finite capacity for solvency and for binding enzyme molecules to membranes (Atkinson, 1969; Somero and Hochachka, 1976). Thirdly, the benefits of an increase in enzyme concentration may be partially or entirely negated if the catalytic properties of the enzyme are reduced under a new thermal regime. In these circumstances it would seem more desirable over evolutionary time periods to develop enzymes with structural and/or catalytic properties which are optimal for the prevailing temperature regime.

Alternative strategies for metabolic adaptation to temperature in poikilotherms involve the "fine tuning" of enzymic properties to obtain optimum reaction rates in the

temperature range encountered by the organism. The strongest evidence for the adoption of such qualitative strategies comes from interspecific comparisons of the kinetic and structural properties of homologous enzymes. Comparisons between ectotherms and endotherms, and between aquatic poikilotherms adapted to different temperatures (e.g. antarctic verses tropical species), have revealed that in general the catalytic capacities of homologous enzymes increase in increasingly cold-adapted species. Lower energies of activation for reactions in cold-adapted species are thought to reflect an increased contribution by the enzyme to substrate activation in order to compensate for the lower average kinetic energy available at lower temperatures. Low et al. (1973) and Low and Somero (1974) observed this effect in a number of enzymes during comparative studies of lobster, cod, halibut, tuna, chicken and rabbit, as did Low and Somero (1976) when conducting temperature adaptation studies on muscle pyrurate kinase. There is little evidence, however, that individual organisms use changes in enzyme catalytic capacity to compensate for immediate or short term fluctuations in temperature, and it has been suggested (Hochachka and Somero, 1968, 1976; Somero, 1969, 1975, 1978a) that such changes are more important over evolutionary time scales.

Comparison of homologous enzymes between species has also shown a positive correlation between heat stability (as measured by thermal inactivation at high temperatures) and the temperature to which an organism is adapted (Somero, 1978). For example Johnston and Walesby (1977) have demonstrated this relationship for myofibrillar ATPases in 27 species of teleosts living at temperatures ranging from -2° to 40°C. It has been

found, however, that most enzymes undergo thermal denaturation at temperatures many degrees above the upper limit tolerated by the organism (Alexandrov, 1977). Preventing a loss of structural integrity is therefore not likely to be the major purpose for adjusting heat stability properties. Somero (1978a) has suggested that thermal stability is likely to co-vary with one or other of the catalytic properties of enzymes, once again emphasising the need to view temperature adaptation in enzymes as the results of co-evolution of structural and functional properties.

The adjustment of enzyme-ligand binding properties in response to temperature is a strategy that appears to be useful over any time scale of adaptation. The evolutionary conservation of an enzyme's affinity for ligands (as measured by the apparent Michaelis constant, K_m - see section 4.4.3) can be inferred from interspecific studies in which the maximum binding ability (lowest K_m values) of the enzyme occurs within the temperature range to which the species is adapted. Results of this type have been obtained from studies of LDH in teleosts (Hochachka and Somero, 1968; Valkirs, 1978) and of pyruvate kinase in vertebrates (Yancey and Somero, 1978). The value of the strategy of K_m alterations over evolutionary time scales is limited, however, unless accompanied by alterations in catalytic capacity of the enzyme. This is because a unilateral reduction of K_m will enhance the binding of ligands but may reduce the ability of the enzyme to respond to substrate fluctuations by forcing it to operate close to its maximum catalytic capacity (Hochachka and Somero, 1976; Somero, 1978a).

Perhaps a more important aspect of the relationship

between temperature and enzyme-ligand affinity is the potential role it can play in regulating the flux of substrates through metabolic reactions in the face of short-term or immediate fluctuations in ambient temperature. There is ample evidence in the recent literature of the general use of this adaptive strategy amongst poikilotherms (for reviews see Hochachka and Somero, 1973, 1976; Hazel and Prosser, 1974; Somero, 1978a), and it seems that of the various enzymic properties discussed earlier K_m modifications are of paramount importance in effecting rate compensation to short-term temperature fluctuations.

4.1.4 Multiple Enzyme Forms and Temperature Adaptation

There are basically two ways in which the rate and/or regulation of an enzyme-catalysed reaction can be maintained within acceptable limits over the entire range of temperatures encountered by an organism:-

(a) The enzyme may develop functional and structural properties which render the rate and/or regulation of the reaction comparatively insensitive to temperature over the entire physiological range of the organism. An enzyme with these attributes is considered "eurytolerant" (Hochachka and Somero, 1976; Somero and Low, 1977a) or "eurythermal" (Somero, 1978a).

(b) Two or more forms of the enzyme catalysing a specific reaction may be produced, each of which is "specialised" for optimal function over a small temperature range, but which in combination satisfy metabolic requirements over the entire temperature range of the organism. Included in this category

is the situation where the temperature tolerance of a reaction is enhanced by a polymorphism at a gene locus whose products modify or regulate the functional properties of the enzyme.

Which of these two options a species adopts will depend largely on the metabolic requirements and constraints imposed on the reaction catalysed by the enzyme in question, and also on the nature of the interaction between the enzyme and environmental parameters such as temperature. In view of the fundamental importance of metabolic processes to survival, it seems likely that the mechanisms employed by a poikilotherm to adjust its enzymes for optimal function in response to temperature (and other environmental parameters, such as pressure - see Somero, 1978b; Siebenaller and Somero, 1978; Childress and Somero, 1979) will determine a significant component of the total potential fitness of each individual. Information on temperature adaptation of enzyme function may therefore be useful as an indication both of the general adaptive strategy of a species, and of the possible relationships between environmental heterogeneity and overall genetic variability.

Multiple forms of a given enzyme can be synthesised in the cell either as the products of two or more gene loci (isozymes) or as allelic products of a single locus (allozymes). Qualitative function differences between isozymes in response to temperature have to date been clearly demonstrated only in the tetraploid salmonid, rainbow trout (*Salmo gairdneri*) (Baldwin and Hochachka, 1970; Hochachka and Lewis, 1970; Hochachka and Somero, 1973; Moon, 1974). Most other studies of the functional attributes of isozymes in fishes have revealed either no differences or quantitative

changes in response to temperature (e.g. Wilson et al., 1974; Somero, 1974). Somero (1978a) has suggested that the possession of functionally distinct isozymes is an adaptive strategy likely to be exhibited mostly by polyploid species, in which evolutionary adjustment of enzyme properties can be achieved at low cost by using a replicated genome to test the usefulness of new isozymic variants.

The importance of functionally distinct allozymes as a strategy for enhanced thermal tolerance in poikilotherms has been the subject of considerable recent debate (Somero, 1974, 1978a; Hochachka and Somero, 1976). Many attempts to infer thermal adaptation have been based on the demonstration of temperature-related clines in allele frequencies. Results of this kind have been produced from studies on the marine ectoproct *Schizoporella unicornis* (Schopf and Gooch, 1971), the crested blenny *Anoplarchus purpurescens* (Johnson, 1971, 1977) the atherinid genus *Menidia* (Johnson, 1974), the butterfly *Colias meadii* (Johnson, 1975), the barnacle *Balanus amphitrite* (Nevo et al., 1977) and other poikilotherms. This type of evidence is, however, only circumstantial, and in no sense actually demonstrates the operation of selection on the products of a polymorphic locus. The induction of allele or genotype frequency changes in laboratory populations in response to perturbations of the thermal environment is perhaps a more direct demonstration of the operation of selective forces, but again does not indicate where and how temperature is acting as a selective agent on the genome. For example, acclimation experiments on the killifish *Fundulus heteroclitus* (Mitton and Koehn, 1975) produced altered allele frequencies at several loci, but the superior tolerance of

highly heterozygous individuals suggested that in this case thermal adaptation was more likely to be the result of selection on multiple-locus phenotypes than on the phenotypic array of a single locus.

Kinetic studies of enzyme-catalysed reactions have been increasingly used in recent years to demonstrate temperature-related functional differences between allozymes. Distinct thermally-adapted allozymes have been found in the cyprinid fish *Catostomus clarkii* (Koehn, 1969), the sand shiner *Notropis stamineus* (Koehn et al., 1971), the minnow *Pimephales promelas* (Merrit, 1972), the killifish *Fundulus heteroclitus* (Place and Powers, 1977, 1979; Powers and Place, 1978), the vinegar fly *Drosophila melanogaster* (Wills et al., 1973; Miller et al., 1975; Alahiotus et al., 1977) and other poikilothermic species. The establishment of potentially selective allozyme differences, together with observed correlations between allele distributions and temperature gradients, constitutes a strong argument in favour of selection.

The interpretation of any data indicating the existence of functionally distinct allozymes must, however, be treated with some caution in view of recent evidence from studies on *Drosophila* (Cochrane, 1976; Finnerty and Johnson, 1979), mice (Dizik and Elliot, 1978) and *Colias* butterflies (Watt, 1977). These studies indicate that the shape and/or functional properties of the enzymatic products of a structural locus can be modified at the post-translation stage of production by a second locus (or loci). It is possible that allelic differences in electrophoretic mobility, heat stability and catalytic properties of enzymes may therefore be due to

polymorphisms at modifying loci rather than at the structural locus, although such a case has yet to be unequivocally demonstrated. Uy and Wold (1977) list 140 cases of co-valent post-translational modification reported to date, and argue that such occurences are common in nature. Most of these cases, however, are thought to be instances of regulatory modification of structural gene expression. While certain experimental and statistical techniques increase the likelihood of discrimination between presence or absence of polymorphic modifyers, this possibility cannot be completely discounted without amino acid sequencing data, and must be considered as an alternative explanation for what has been assumed to be allelic variation.

4.1.5 Experimental Design

Although it would be desirable to characterise the properties of as many enzymes as possible to obtain a more comprehensive picture of the adaptive strategies of Australian snapper and salmon towards temperature, the constraints of time and facilities have restricted the present study to one enzyme system for each species. The choice of enzymes will be discussed in section 4.1.6.

The experimental plan is based on that described by Clarke (1975), except that no pertubation studies were carried out to test postulated mechanistic relationships between allozyme distributions and temperature. The following experimental procedures were used:-

(a) Crude extracts of allozymes in a particular polymorphic system were assayed spectrophotometrically to

determine pH optima and the concentrations of substrate and coenzyme needed to produce maximum (V_{max}) and half-maximum reaction velocity at a given temperature.

(b) Each allozyme was assayed over a range of temperatures, including the physiological range of the organism, to determine the effect of temperature on enzyme activity at both V_{max} and physiological concentrations of substrate and cofactor.

(c) Allozymes were subjected to thermal inactivation at high temperatures in order to detect any differences in heat stability between electrophoretic classes, or between individuals within a single electrophoretic class.

(d) Specific activities of allozymes were roughly estimated by assaying the total soluble protein content of a crude extract and comparing it to the reaction velocity produced by the extract.

(e) Observed geographic distributions of electrophoretic alleles were reanalysed in the light of results from steps (a) to (d), with a view to providing clues concerning both the forces maintaining the observed polymorphisms and the type of adaptive strategy adopted by the organisms in response to temperature.

4.1.6 Choice of Enzymes

A. 'Malic' Enzyme (E.C. 1.1.1.40)

NADP-dependant malate dehydrogenase - also known as 'malic' enzyme (ME) - catalyses the reversible reaction:-

$$\text{malate} + \text{NADP} \xleftarrow{Mn^{++} \text{ or } Mg^{++}} \text{pyruvate} + CO_2 + \text{NADPH} + H^+$$

ME was chosen for kinetic studies in Australian salmon primarily because it is electrophoretically monomorphic in western salmon but polymorphic with five detected alleles in eastern salmon (section 3.4.1). Testing these different forms of ME for functional differences in response to temperature provides an opportunity to determine whether or not the two closely related and ecologically similar salmon species have evolved different adaptive strategies (as suggested by the electrophoretic data) for ensuring the adequate function of the ME reaction over their respective physiological temperature ranges.

Electrophoretic bands of ME activity were comparatively easy to score, and homozygous phenotypes for four of the five alleles in eastern salmon were available for kinetic analysis. In addition the ME catalysed reaction involves a direct oxidation/reduction of NADP and is therefore convenient to assay spectrophotometrically (see section 4.3.1).

The observed electrophoretic patterns in western and eastern salmon, and in other fishes, indicate that ME is a tetramer (Plate 3.1 and see Povey et al., 1975; Allendorf et al., 1977; Cross et al., 1979) and is coded at a single locus in liver. The intra-cellular location of ME in salmon liver is not known, but available evidence suggests that it is primarily cytoplasmic. Cross et al. (1979) have observed that isolated liver mitochondrial fractions from the salmonid fish *Salmo salar* yielded virtually no ME activity, in contrast to the high activity of the cytoplasmic fraction. Only a single, strong electrophoretic band of ME was detected in Australian salmon homozygotes, indicating that a similar situation may apply in this case.

The primary metabolic role of cytoplasmic ME in fishes is not clear, but is thought to be linked with the process of fatty acid synthesis (Baldwin and Reed, 1976). ME is one of only four enzymes that produce NADPH, which is used as the source of reducing power in the synthesis of fatty acyl CoA from acetyl CoA. ME is also part of the pyruvate-malate cycle, which is thought to be a means whereby acetyl CoA produced in the mitochandria can be transported into the cytoplasm where fatty acid synthesis occurs (Newsholme and Start, 1973). During the flux of metabolites through the pyruvate-malate cycle the two cytoplasmic NADP-dependant enzymes involved (ME and IDH) produce 50% of the NADPH requirements for reduction of acetyl CoA in the fatty acid synthetic pathway. ME and the other NADPH-producing enzymes may, therefore, fulfil a regulatory role in fat synthesis by controlling the availability of NADPH.

Studies on rainbow trout *Salmo gairdneri* (Dean, 1969), the blue crab *Callinectes sapidus* (Robert and Gray, 1972) and the blenny *Blennius pholis* (Campbell and Davies, 1978) indicate increased activity of NADPH-producing enzymes and increased levels of fat synthesis in animals acclimated to cold temperatures. Baldwin and Reed (1976) have postulated the presence of two forms of ME in rainbow trout liver which display different activities and regulatory properties in response to temperature changes. It therefore seems reasonable to investigate the possibility that the observed electrophoretic forms of ME in western and eastern salmon may indicate allozymes with a range of different properties in response to temperature fluctuations.

B. Isocitrate dehydrogenase (E.C. 1.1.1.42)

NADP-dependant isocitrate dehydrogenase was selected for enzyme kinetic studies in Australian snapper. It was found to be electrophoretically polymorphic, with clearly identifiable bands of activity and with all possible phenotypes of the major alleles (IDH^{100}, IDH^{114} and IDH^{80}) available for assay. IDH is also one of three variable enzymes which exhibit a clinal distribution of alleles in snapper around the Australian coastline. Clinal distributions of *IDH*, *EST* and *ADH* alleles suggest the possibility of an interaction between the allozyme products of these loci and an environmental gradient, but in each case a likely environmental parameter is not immediately obvious. NADP-IDH catalyses the reaction:-

$$\text{isocitrate} + \text{NADP} \xleftrightarrow{Mn^{++} \text{ or } Mg^{++}} \alpha\text{-keloglutarate} + \text{NADPH} + H^{+} + CO_2$$

This reaction involves a direct oxidation/reduction of NADP and is therefore easy to assay spectrophotometrically (see section 4.3.1).

As with ME in 'salmon', a single, strong electrophoretic zone of NADP-IDH activity was detected in snapper liver tissue, suggesting genetic coding at a single locus. The intra-cellular location of the enzyme in snapper is not known, but studies of NADP-IDH in liver tissue of trout (Wolf et al., 1970; Moon and Hochachka, 1971) failed to find activity in isolated mitochondrial fractions, suggesting that NADP-IDH is generally cytoplasmic in fish liver tissues. NADP-IDH activity in snapper liver is also much higher than that of NAD-IDH, which was difficult to detect using

electrophoretic techniques. This observation is consistent with those of Crabtree and Newsholme (1970), and Moon and Hochachka (1971), who reported NADP-IDH/NAD-IDH activity ratios of 10 to 100 for bird and mammal species, and up to 300 for fish species.

The primary metabolic role of cytoplasmic NADP-IDH is uncertain, but the enzyme is apparently connected with the pyruvate-malate cycle, and it also produces NADPH for use in the fatty acid synthesis pathway (Moon, 1972; Newsholme and Start, 1973; Moon and Storey, 1975). Geer et al. (1976) found that ME and NADP-IDH activities were induced in *Drosophila melanogaster* larvae and mammalian liver tissue by an acetate derivative of the Krebs or TCA cycle, were supressed by an excess of dietry lipids, but were not affected by high levels of dietry carbohydrates. This evidence supports the hypothesis of a major role for NADP-IDH in the fatty acid synthesis pathway.

Potentially adaptive functional differences in response to temperature have been reported for both isozymes (Moon and Hochachka, 1971) and allozymes (Redding and Schreck, 1979) of NADP-IDH in the trout *Salmo gairdneri*, and it seems reasonable to investigate the possibility that similar differences may exist between allozymes in the liver of Australian snapper.

4.2 Materials

Assays of ME and IDH activity were carried out using liver tissue from Australian salmon and snapper specimens collected for population studies as described in section

3.2.1. Tissues of specific individuals were selected at random depending on the electrophoretic phenotype required for analysis. As described earlier liver samples were snap frozen in liquid nitrogen at approximately -180°C and stored in the laboratory at -70°C to prevent loss of enzyme activity.

4.3 Methods

4.3.1 Preparation of Enzyme Extracts

Frozen liver and lysing solution were placed in a glass grinding tube in the ratio of approximately one part to five by volume, and mascerated on ice. The lysing solution consisted of 50 mM tris HCl pH 7.8 buffer, containing:-

0.05% β-mercaptoethanol (Sigma, Type I)
3.0 mM EDTA (BDH, AR grade)
1.0 µM NADP (Sigma grade, monosodium salt)

The lysing solution was stored at 5°C and was used within one week of preparation.

Homogenised samples were centrifuged in a refrigerated Sorvall SS-3 at 20,000 r.p.m. for 20 minutes. Aliquots of the resulting clear red supernatant fluid were transferred to glass vials and kept on ice until used.

4.3.2 Assay Procedures

Enzyme activity was assayed by following the reduction of NADP or oxidation of NADPH at 340 nm with a Gilford 2400

recording spectrophotometer. Constant temperatures (to within ± .5°C) were maintained during assays by coupling a Haake FK circulating water bath to the water jacket of the cuvette chamber. Reaction mixtures were made up to 1 ml in matched quartz cuvettes and changes in optical density (OD) per unit time during the reaction were continuously plotted on a chart recorder coupled to the spectrophotometer.

Each reaction mixture contained buffer, manganese ions, substrate, cofactor and crude enzyme extract. Stock solutions of reaction ingredients were kept on ice during the assay procedure. The type of buffer and concentration of substrate and cofactor used depended on the purpose of the assay (see sections 4.4 and 4.5). All reaction ingredients except the enzyme extract were initially added to the cuvette and incubated for five minutes at the desired temperature. During this period background OD levels were monitered, and any observed OD change subsequently used to correct activity estimates from the reaction assay. Background OD changes usually represented less than 3% of subsequent reaction measurements. The reaction was initiated by adding crude enzyme extract, and the reaction rate was measured over three minutes if linear kinetics were observed, or over the first 30 seconds if curved kinetics were obtained. Each activity value has been measured in units of ΔOD/minute, and is the average of duplicate assays.

The total protein content of crude enzyme extracts was estimated by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Equal quantities of enzyme extract from the same preparation were used for both activity assays and protein determinations, in order to simplify

calculations of specific activity for each individual.

4.3.3 Heat Stability Tests

Heat stability of allozymes was determined by comparing the activity of enzyme extracts subjected to a given high temperature with the activity of untreated enzyme from the same preparations. All assays were carried out under conditions of saturating substrate and cofactor. Enzyme extracts were placed in capped glass vials and immersed in a constant temperature water bath at high temperature for ten minutes. Heat-treated samples were then quickly cooled on ice to prevent further inactivation, and assayed at the selected physiological temperature, along with untreated samples, to obtain a measure of percentage enzyme inactivation at the treatment temperature.

Individual samples representative of the different electrophoretic allozymes were tested at a range of temperatures to obtain heat stability profiles. Other samples were then tested at one key temperature to determine which heat stability category they belonged to.

Heterozygous electrophoretic phenotypes of the snapper IDH polymorphism were tested for cryptic heat stability variants by combining electrophoretic and heat inactivation techniques. Samples from known IDH heterozygotes were electrophoresed as described in section 3.3.1. The electrophoresed gels were then enclosed in plastic wrapping (to prevent access by water) and immersed in a high temperature water bath for an empirically predetermined length of time. After heat treatment the gels were stained for IDH

activity (as described in section 3.3.3). Cryptic heat stability variation was determined by the presence or absence of differential thermal inactivation of allozyme bands within each individual sample.

4.4 Results - Malic Enzyme (ME) in Australian Salmon

4.4.1 Direction of the Reaction

Different metabolic roles for mitochondrial and cytoplasmic ME have been proposed on the basis of the relative rates of the forward (decarboxylation) and reverse (carboxylation) reactions (Frenkel, 1972; Baldwin and Reed, 1976). Relative rates of the forward and reverse reactions are also influenced by pH fluctuations (Hochachka and Mustafa, 1973). However, Young et al. (1964) suggest that, in liver tissue of mammals and birds, ME is localised in the cytoplasm and its primary role is to produce NADPH for the fatty acid synthesis pathway. Hochachka and Mustafa (1973) also found that the affinity for malate of ME from the adductor muscle of oysters was about thirty times that for pyruvate. They concluded that under most conditions ME functions in the direction of malate → pyruvate (i.e. decarboxylation).

Initial assays of both the forward and reverse ME reactions were carried out at 18°C (chosen as a median, and presumably optimal, temperature based on the known biological range of eastern and western salmon) and at pH 7.6 (similar to proven electrophoretic staining conditions). Assay conditions for the forward reaction were:-

50.0	mM	tris-maleate pH 7.6 (see section 3.3.2 for specifications)
2.5	mM	malic acid (SIGMA, crystaline monosodium salt)
0.1	mM	NADP (SIGMA grade, monosodium salt)
5.0	mM	manganous chloride ($MnCl_2$) (UNIVAR, AR grade)
10	µl	enzyme extract

and for the reverse reaction were:-

50.0	mM	tris-maleate pH 7.6
20.0	mM	pyruvic acid (SIGMA, Type II, monosodium salt)
0.125	mM	NADPH (SIGMA, grade III, disodium salt)
20.0	mM	sodium hydrogen carbonate ($NaHCO_3$) (Hopkin and Williams, AR grade)
5.0	mM	$MnCl_2$ (UNIVAR, AR grade)
10	µl	enzyme extract

In the absence of prior kinetic data on ME from Australian salmon the concentrations of reactants chosen above follow those used by Baldwin and Reed (1976) to assay trout liver ME under saturating substrate conditions. Reaction rates (ΔOD/minute) were obtained from homozygous individuals representing each of the alleles ME^{100}, ME^{93}, ME^{75} and ME^{68} in eastern salmon, and ME^{100} in western salmon (see Table 3.3 and Plate 3.2). The ratios of forward to reverse reaction velocity were:-

Species	Allele	Forward/Reverse reaction ratio
eastern salmon	ME^{100} east.	10.1
	ME^{93}	9.6
	ME^{75}	11.3
	ME^{68}	13.3

A ratio was not obtained for ME^{100} west. because there was no measurable enzyme activity during assay of the reverse reaction. These results indicate little differences in reaction ratios between the eastern salmon alleles, and they support the conclusion of Hochachka and Mustafa (1973) that malate → pyruvate is the dominant direction of the ME reaction. The reason for the comparatively low pyruvate → malate reaction rates is unknown, but could be attributed to artifacts of the *in vitro* assay system, to substrate or cofactor inhibition, to adverse pH conditions, or to unfavourable free energy changes during the reaction. Investigation of this problem is undoubtedly of importance in determining the metabolic role of ME in different tissues and intracellular locations, but the purpose of the present study is to detect temperature-related function differences between alleles, and only the malate → pyruvate reaction has been assayed extensively.

4.4.2 pH Optima

The tris-maleate system has buffering capacity over the pH range 5.0 to 9.0. 50mM tris-maleate was therefore chosen as the buffer to assay salmon ME activities over a range of

pH's. All assays were at 18°C and all buffer pH's were adjusted at this temperature. Concentrations of reactants were as given for the forward reaction (malate → pyruvate) in section 4.4.1. The results in Figure 4.2 show that all of the salmon ME alleles have pH optima of about 8.0 to 8.5 under *in vitro* assay conditions. In view of this result all further assays at 18°C were carried out at pH = 8.0.

There appear to be differences in levels of activity between ME 100 west., ME 100 east. and the other allozymes (Figure 4.2). It must be remembered, however, that these results have been obtained using 10 µl aliquots of crude extract in which the concentration of ME is unknown. The activity difference may, therefore, be an artifact of the extraction procedure. This problem will be discussed further in section 4.4.7.

4.4.3 Substrate/Velocity Curves

The ME reaction is a multi-substrate system, with both malate and NADP bound to the enzyme during catalysis. Experimental observations show that, when one substrate is held constant and the other varied, most multi-substrate systems obey saturation kinetics as described by the Michaelis-Menten equation:-

$$V = \frac{k_{cat}[E][S]}{K_m + [S]} \qquad \text{(see Fersht, 1977)}$$

where:-

$$k_{cat}[E] = V_{max}$$

Figure 4.2

Plots of reaction velocity against initial pH of the reaction mixture for the ME allozymes of eastern and western salmon. See section 4.4.2 for assay conditions.

● = ME 100 west.

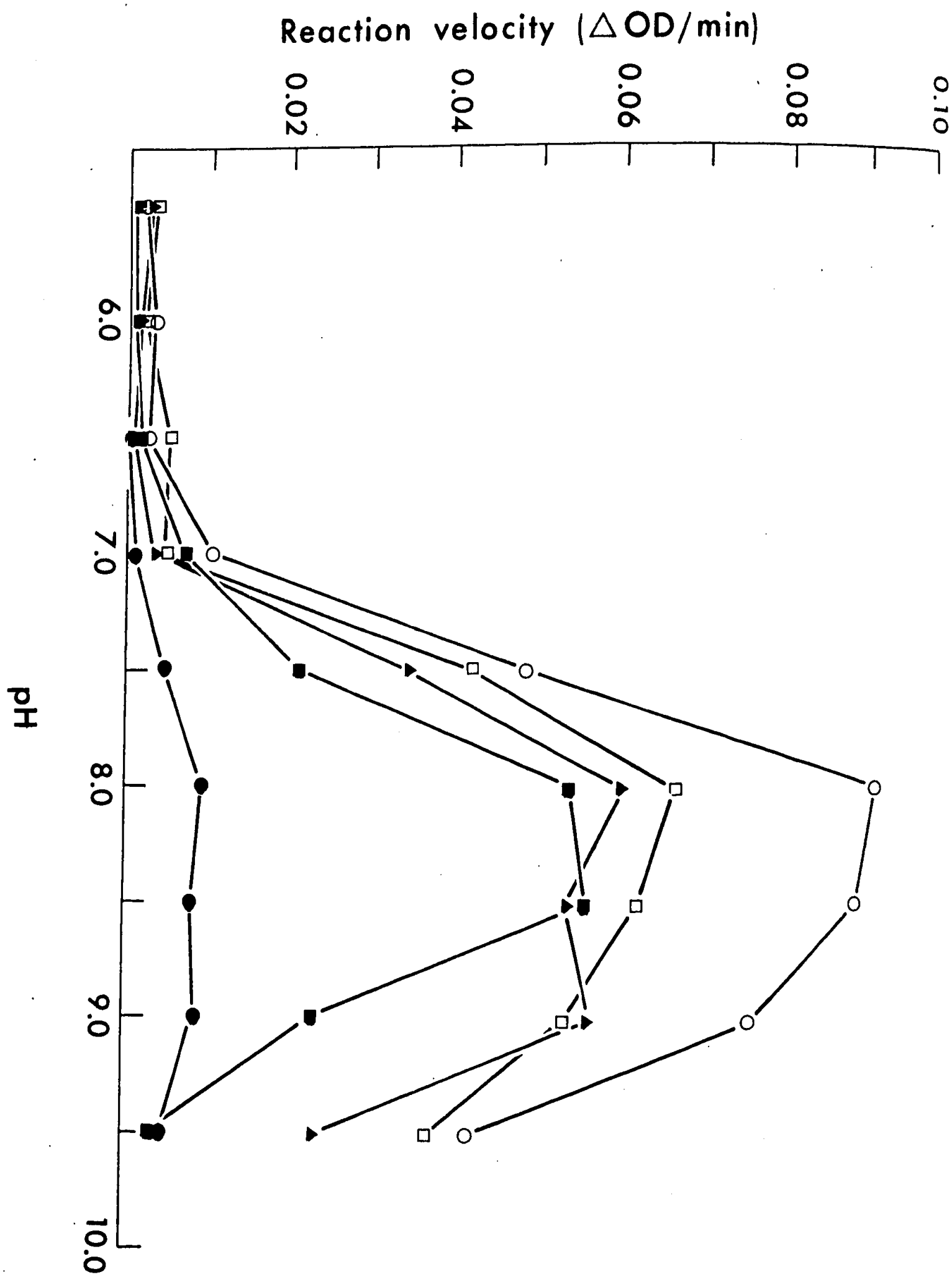
○ = ME 100 east.

■ = ME 93

□ = ME 75

▲ = ME 68

0.10


Reaction velocity (△OD/min)
0.02
0.04
0.06
0.08
0.10
pH
6.0
7.0
8.0
9.0
10.0

and:-

V = initial reaction velocity;

[E] = the concentration of enzyme;

[S] = the concentration of substrate;

k_{cat} = the intrinsic rate at which the enzyme can convert substrate to product;

K_m = the Michaelis constant ([S] at which $V = \frac{1}{2}V_{max}$), an approximate measure of affinity of the enzyme for the substrate;

V_{max} = the maximum reaction velocity achievable by a given amount of enzyme.

Under Michaelis-Menten kinetics a plot of V versus [S] will describe an inverted rectangular hyperbola, with V initially increasing rapidly in response to increases in [S], but becoming less sensitive to such increases until at saturating [S] a limiting velocity (V_{max}) is approached.

Plots of V/[malate] (Figure 4.3) and V/[NADP] (Figure 4.4) for individual samples representing each of the salmon ME allozymes show that saturation kinetics are observed for substrate concentrations up to approximately 3.2 mM malate and .3mM NADP. At concentrations higher than these the reaction rates of all allozymes decline, indicating the operation of some form of substrate inhibition, particularly in the case of malate. This observation suggests a possible metabolic control mechanism for the ME reaction in salmon, but except for differences in activity levels (see section 4.4.7), all allozymes assayed behaved in essentially the same manner, and the nature of ME substrate inhibition was not investigated in this study.

3.2mM malate and .3mM NADP were used as saturating

Figure 4.3

Plots of reaction velocity against final concentration of malate in the reaction mixture for the ME allozymes of eastern and western salmon. See Figure 4.2 for the plotting code. Assays were carried out at 18°C using 50 mM tris-maleate pH 8.0, 5 mM manganous chloride and .3 mM NADP.

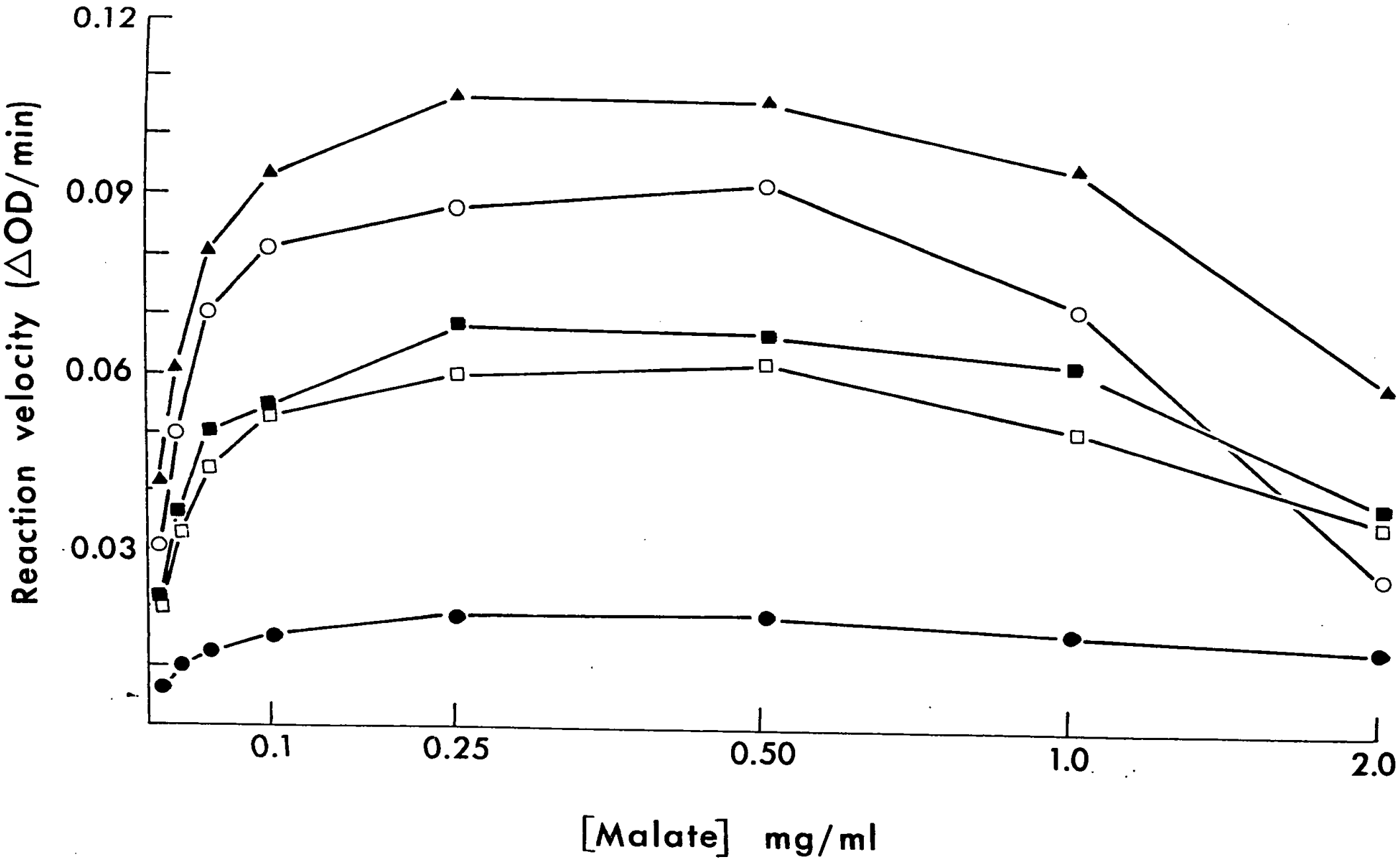
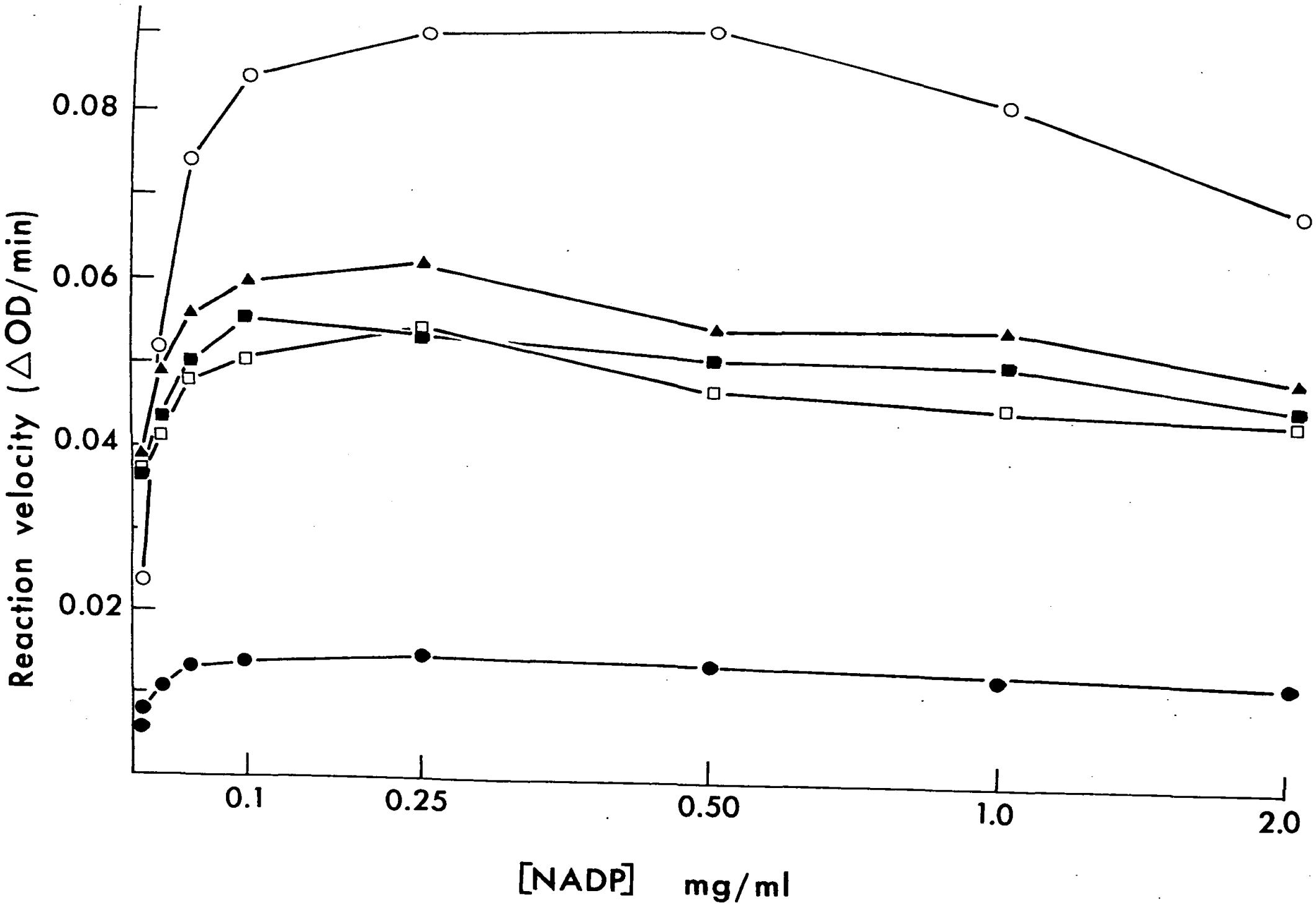

0.12
0.09
0.06
0.03
Reaction velocity (ΔOD/min)
0.1
0.25
0.50
1.0
2.0
[Malate] mg/ml

Figure 4.4

Plots of reaction velocity against final concentration of NADP in the reaction mixture for the ME allozymes of eastern and western salmon. See Figure 4.2 for the plotting codes. Assays were carried out at 18°C using 50 mM tris-maleate pH 8.0, 5 mM manganous chloride and 2.5 mM malate.


0.08
0.06
0.04
0.02
Reaction velocity (△OD/min)
0.1
0.25
0.50
1.0
2.0
[NADP] mg/ml

substrate concentrations for all subsequent assays in which maximum reaction velocities were desired.

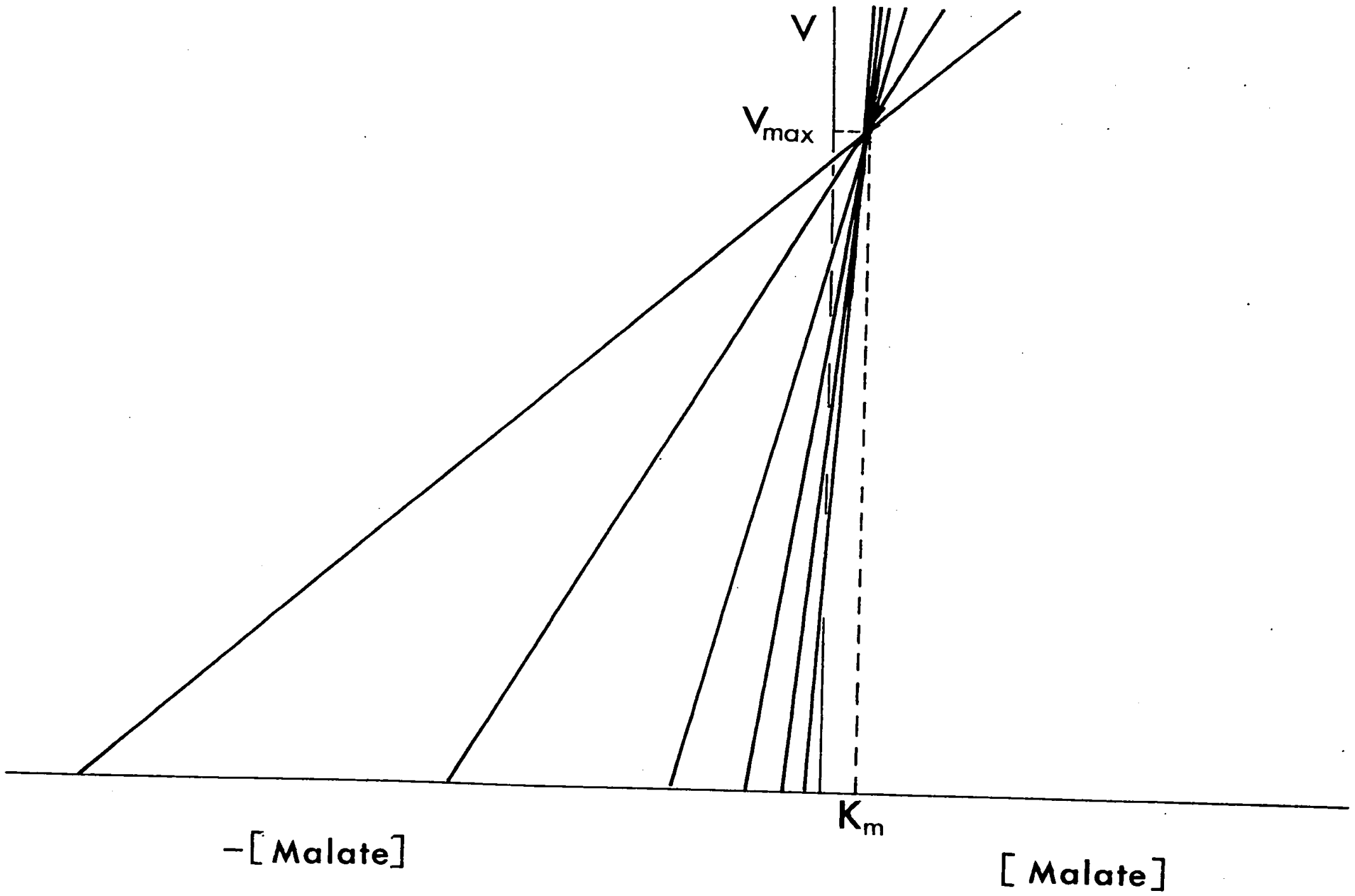
4.4.4 Enzyme - Substrate Affinity

In a closed reaction system the Michaelis constant (K_m) is a measure of equilibrium levels of enzyme-substrate complex versus levels of free enzyme, substrate and product. Michaelis-Menten kinetics assume that the rate of formation and dissociation of the enzyme-substrate complex is very much faster than the rate of conversion of the complex to product and free enzyme, so that K_m is a reasonable estimate of the dissociation constant (K_s) for enzyme and substrate only (Fersht, 1977). Under these circumstances K_m is defined as that concentration of substrate which produces a reaction velocity of ½ V_{max}, and is a measure of the affinity of the enzyme for substrate.

Apparent K_m [malate] and K_m [NADP] values were estimated for the salmon ME allozymes using the direct linear plot method of Eisenthal and Cornish-Bowden (1974). This method is illustrated in Figure 4.5 using experimental data from a homozygous ME 75 individual. The direct linear plot has advantages over other methods of K_m estimation (such as the Linweaver-Burke double reciprocal plot) in that it is simple to construct and provides an unbiased visual estimate of both (K_m, V_{max}) and the precision of the experimental observations. Cornish-Bowden and Eisenthal (1974) also show that non-parametric confidence limits can be placed on median estimates of K_m and V_{max} from direct linear plots, and that these confidence statements involve far less

Figure 4.5

Illustration of the direct linear plot method for estimating median K_m and V_{max} values (Eisenthal and Cornish-Bowden, 1974) using experimental kinetic data (as shown in Figure 4.3) from a homozygous ME 75 individual.


V
V_{max}
K_m
-[Malate]
[Malate]

sweeping assumptions about experimental error than those implicit in the least squares calculations of methods such as the Linweaver-Burke plots. The 95% confidence intervals in this study were calculated as a non-parametric rank order statistic, using a computer program (ENZYME 2) developed by Dr M.A. Adena and Dr G.K. Chambers at A.N.U. This statistic is known to be conservative, being equivalent to 3 - 5 standard errors of the mean in cases where calculations are based on ten or more lines intersection points.

Table 4.1 lists the median K_m estimations and approximate 95% confidence intervals of the salmon ME allozymes for both malate and NADP. In both cases it can be seen that the 95% confidence interval for each allozyme includes the median K_m values of all other allozymes, indicating that at 18°C enzyme-substrate affinities are homogeneous over the entire array of ME allozymes.

The results in Table 4.1 are based, however, on assays from only one homozygous representative per allele, and it would be desirable to have some estimate of the variance of apparent K_m values both within and between different allozyme classes. Such estimates have been obtained indirectly by assaying numerous crude enzyme extracts at saturating and K_m levels of substrates predetermined from section 4.4.3 and Table 4.1, and comparing values obtained from each individual for percentage activity at low vers s high substrate levels. Figure 4.6 shows the distribution of percentage activity values for 65 individuals from a number of ME allozyme classes. All of the eastern salmon individuals yielded values within the narrow range of 33 to

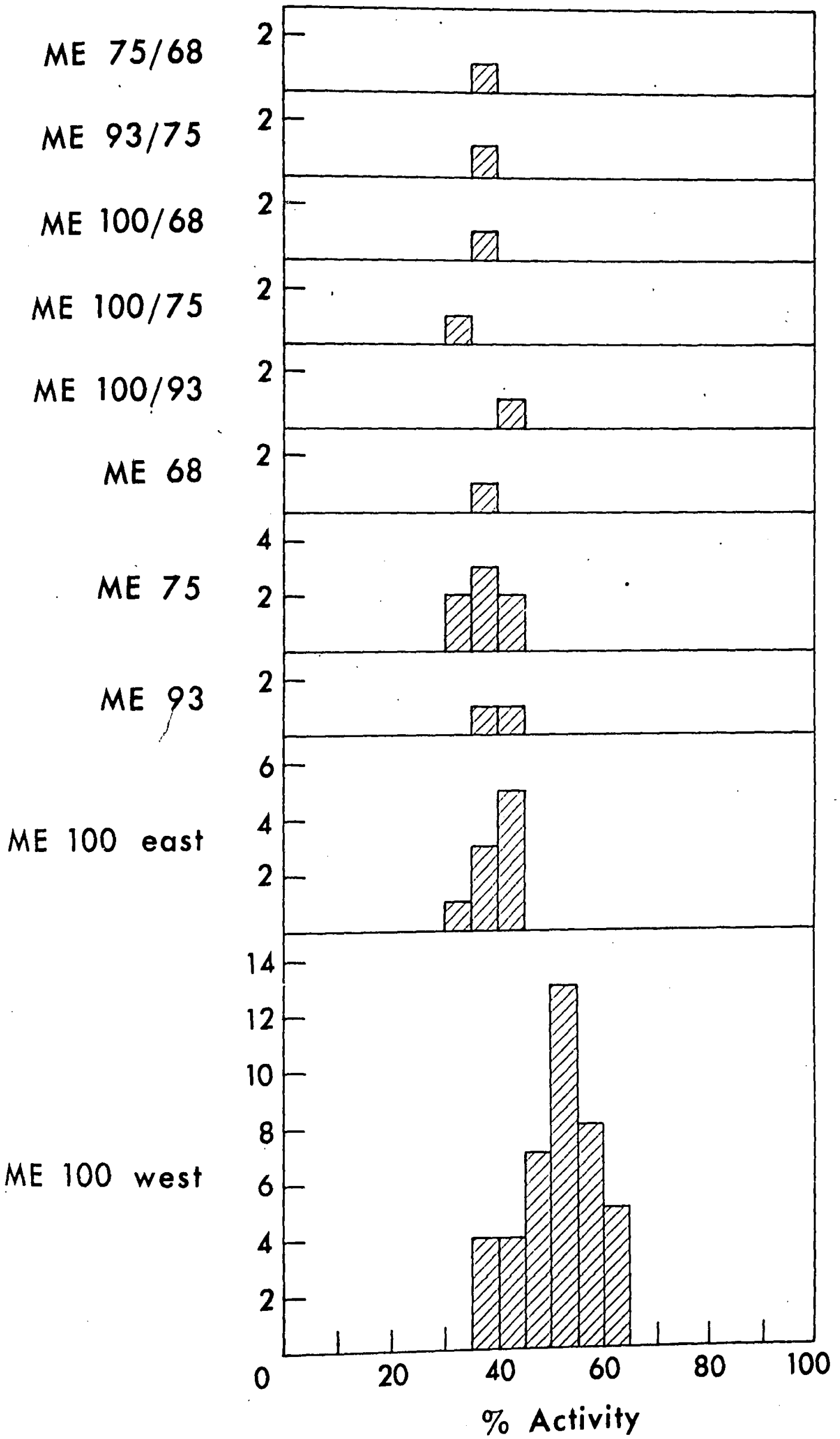
Table 4.1

Median K_m substrate levels (mg/ml) and approximate 95% confidence intervals for Australian salmon ME allozymes assayed at 18°C. Concentrations of .147 mM (.023 mg/ml) malate and .0107 mM (.009 mg/ml) NADP were chosen as apparent K_m substrate levels in all subsequent assays.

Substrate	Allozyme	Median K_m	95% Confidence Interval
Malate	ME 100 west.	.0237	.0200 - .0313
	ME 100 east.	.0220	.0184 - .0262
	ME 93	.0239	.0198 - .0278
	ME 75	.0232	.0218 - .0241
	ME 68	.0227	.0190 - .0263
NADP	ME 100 west.	.0083	.0061 - .0115
	ME 100 east.	.0108	.0048 - .0163
	ME 93	.0100	.0053 - .0149
	ME 75	.0089	.0066 - .0124
	ME 68	.0076	.0042 - .0123

Figure 4.6

Frequency histograms of percentage activity at apparent K_m versus saturating levels of substrate for 65 individuals representing most of the available ME electrophoretic phenotypes. Assays were carried out at 18°C using 50 mM tris-maleate pH 8.0 and 5 mM manganous chloride. K_m substrate levels used were .147 mM malate and .0107 mM NADP, while saturating levels were 3.2 mM malate and .3 mM NADP.


ME 75/68
ME 93/75
ME 100/68
ME 100/75
ME 100/93
ME 68
ME 75
ME 93
ME 100 east
ME 100 west
2
2
2
2
2
2
4
2
2
6
4
2
14
12
10
8
6
4
2
0
20
40
60
80
100
% Activity

45%, and although sample sizes for each allozyme class were not large enough for statistical analysis, it seems that percentage activity values, and by inference enzyme-substrate affinities, are homogeneous both within and between allozyme classes.

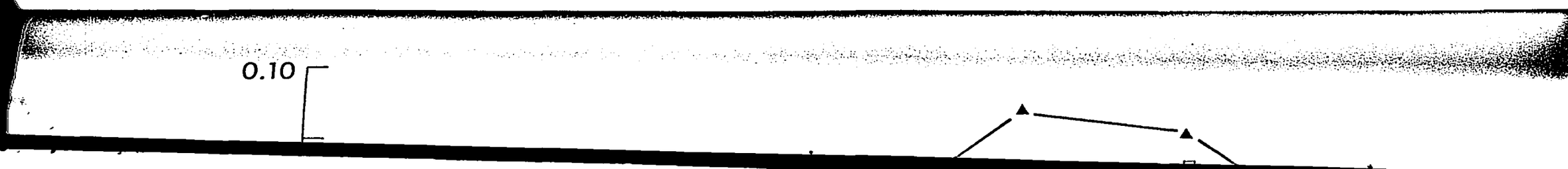
ME 100 west. individuals yielded a larger range of percentage activities (38 to 64%), possibly because a larger number of individuals were assayed, but the frequency histogram (Figure 4.6) appears to be unimodal, and there is little evidence for a heterogeneous distribution of values. Comparison of the ME 100 west. data with that for the combined eastern salmon allozymes does, however, indicate a bimodal distribution of percentage activity values, and a contingency χ^2 test confirms this (cf. $\chi^2_2 = 28.58$, $P < .001$). Assuming that all assays carried out at high substrate levels did in fact yield reaction velocities approximating V_{max}, it seems that eastern salmon ME allozymes generally produce lower percentage activity than do homologous western salmon ME extracts when assayed at .147 mM malate and .0107 mM NADP. It may be infered from this observation that at 18°C ME 100 west. has, on average, a higher affinity for malate and/or NADP than do the eastern salmon ME allozymes. This finding will be discussed further in section 4.6.

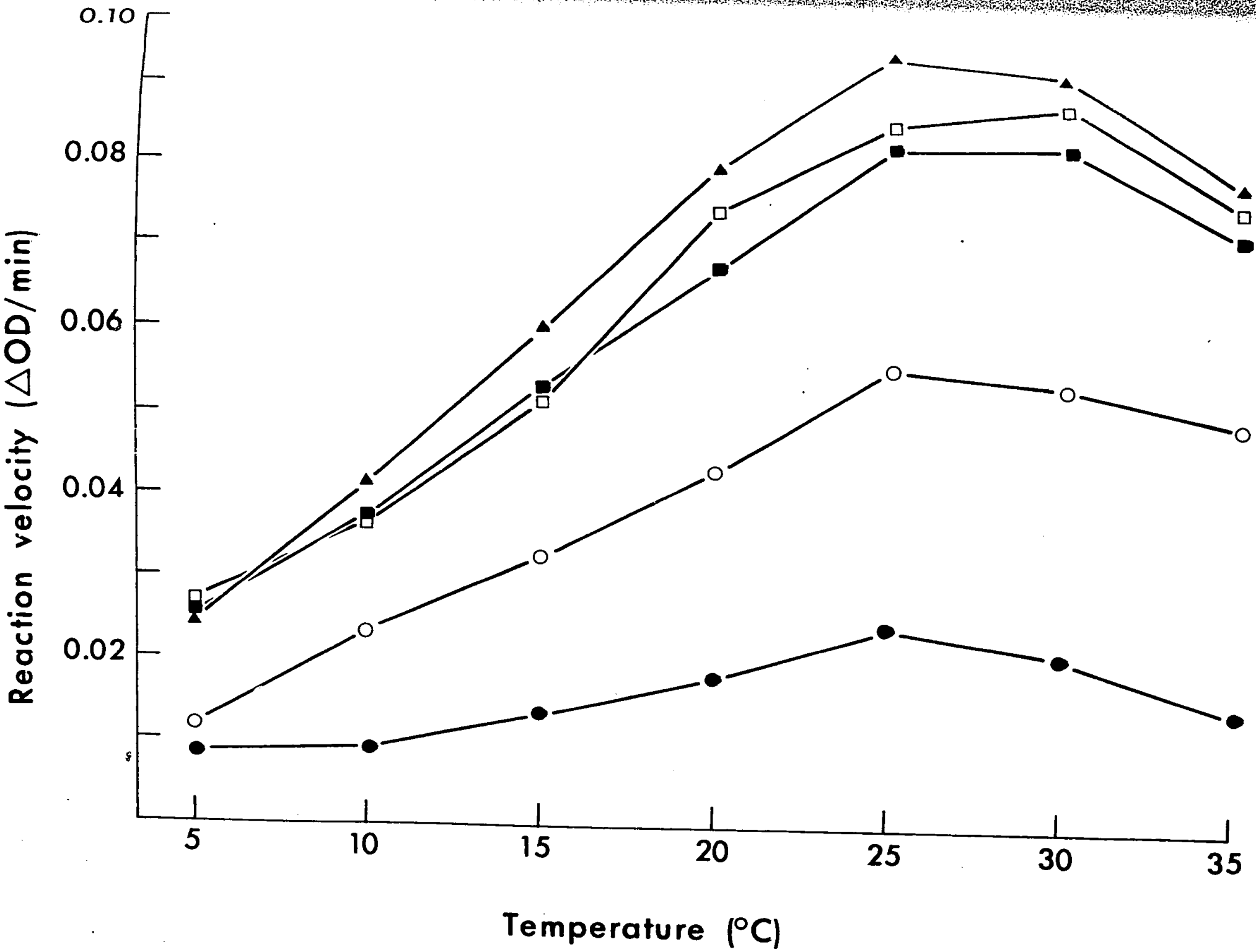
4.4.5 The Effect of Temperature on Reaction Velocities

Individual crude extracts of all western and eastern salmon ME allozymes were assayed at 5° intervals for temperatures between 5° and 35°C. Assays were carried out using both saturating (Figure 4.7) and approximate K_m

Figure 4.7

Saturation reaction velocities of the salmon ME allozymes when assayed at 5° intervals for temperatures between 5°C and 35°C. See Figure 4.2 for the plotting code. Assay conditions were :- 80 mM imidazole-HCl pH 8.0 (at 18°C), 5 mM manganous chloride, 3.2 mM malate and .3 mM NADP.




0.10
0.08
0.06
0.04
0.02
Reaction velocity (ΔOD/min)
5
10
15
20
25
30
35
Temperature (°C)

(Figure 4.8) levels of substrate. Assays at K_m levels of substrate were carried out on the assumption that these levels approximate *in vivo* intracellular concentrations, and therefore provide more realistic conditions for the testing of enzyme function against temperature.

The assumption that apparent K_m concentrations are equivalent to physiological substrate levels for all enzyme-catalysed reactions is contentious. Cornish-Bowden (1976) argues in favour of this hypothesis, concluding that:-

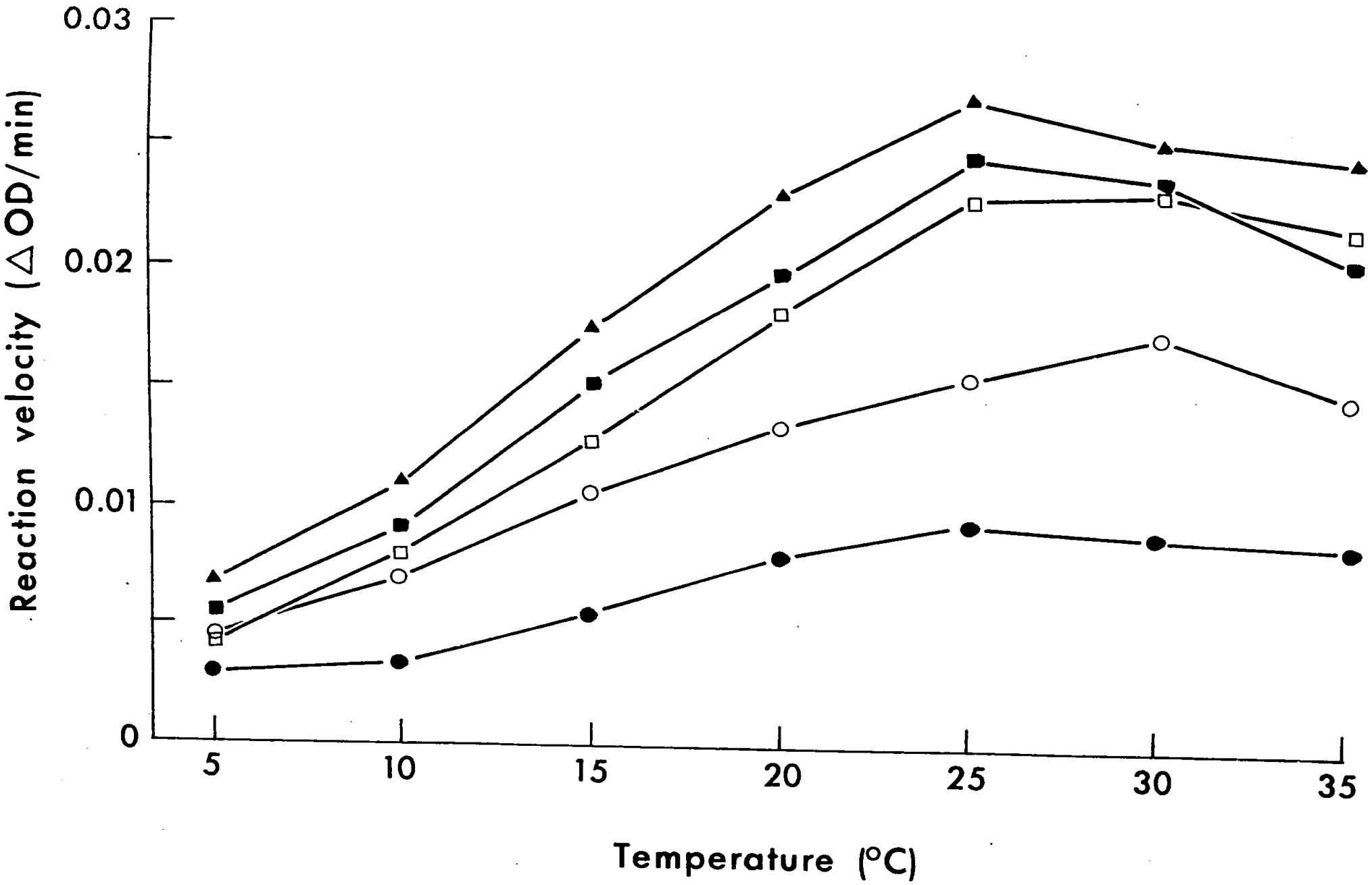
"If catalytic efficiency is considered solely from the point of view of maximising flux, then it follows that enzymes should have evolved to possess K_m values within one order of magnitude of the physiological concentrations of their substrate."

Fersht (1974, 1977) and Crowley (1975) favour the opposing hypothesis that maximum reaction rates are achieved by maximising the ratio k_{cat}/K_m, and also by increasing K_m values relative to [S]. Fersht (1977) found, however, that in a survey of 34 K_m values for enzymes of the glycolytic pathway over 50% were within one order of magnitude of measured physiological substrate concentrations, and over 90% were within two orders of magnitude. It seems, therefore, that the assumption of K_m substrate levels approximating physiological concentrations is reasonable, at least for enzymes with intracellular substrates.

An 80 mM imidazole-HCl buffer was chosen for temperature/velocity assays. The pH of this buffer varies with temperature in a manner similar to biological fluids (Yancey and Somero, 1978). The buffer was adjusted to pH 8.0 at 18°C, as described in section 4.4.2. Comparisons of

Figure 4.8

K_m reaction velocities of the salmon ME allozymes when assayed at 5° intervals for temperatures between 5°C and 35°C. See Figure 4.2 for the plotting code. Assay conditions were :- 80 mM imidazole-HCl pH 8.0 (at 18°C), 5 mM manganous chloride, .147 mM malate and .0107 mM NADP.


Reaction velocity (△ OD/min)
0.03
0.02
0.01
0
5
10
15
20
25
30
35
Temperature (°C)

ME activity from the same crude extract using either tris-maleate or imidazole-HCl revealed no discernable differences.

The results of the temperature/velocity assays are given in Figures 4.7 and 4.8. As with the data on pH optima (section 4.4.2) and substrate/velocity curves (section 4.4.3), there are differences in activity levels between the ME allozymes, probably due to different enzyme concentrations in the various crude extracts (see section 4.4.7). All ME allozymes show a virtually linear response to increasing temperature between 10° and 25°C at either saturating or K_m levels of substrate. The results at 5°C have been ignored because of potential bias due to technical difficulties in maintaining a constant low assay temperature. At temperatures greater than 25°C all allozymes begin to lose a significant proportion of their activity. It will be seen in section 4.4.6 that structural stability of all the ME allozymes is maintained at least up until 35°C, so the loss of activity after 25°C is more likely to be due to a deterioration in one or more functional properties of the enzyme, such as substrate binding ability or catalytic capacity.

The observed linear response of ME allozyme activities to temperature, instead of an expected monotonic increasing function (see section 4.1.1), indicates a moderate degree of positive compensation of reaction velocities. This effect is best illustrated by calculating Q_{10} values for assays at K_m substrate levels between 10° and 25°C:-

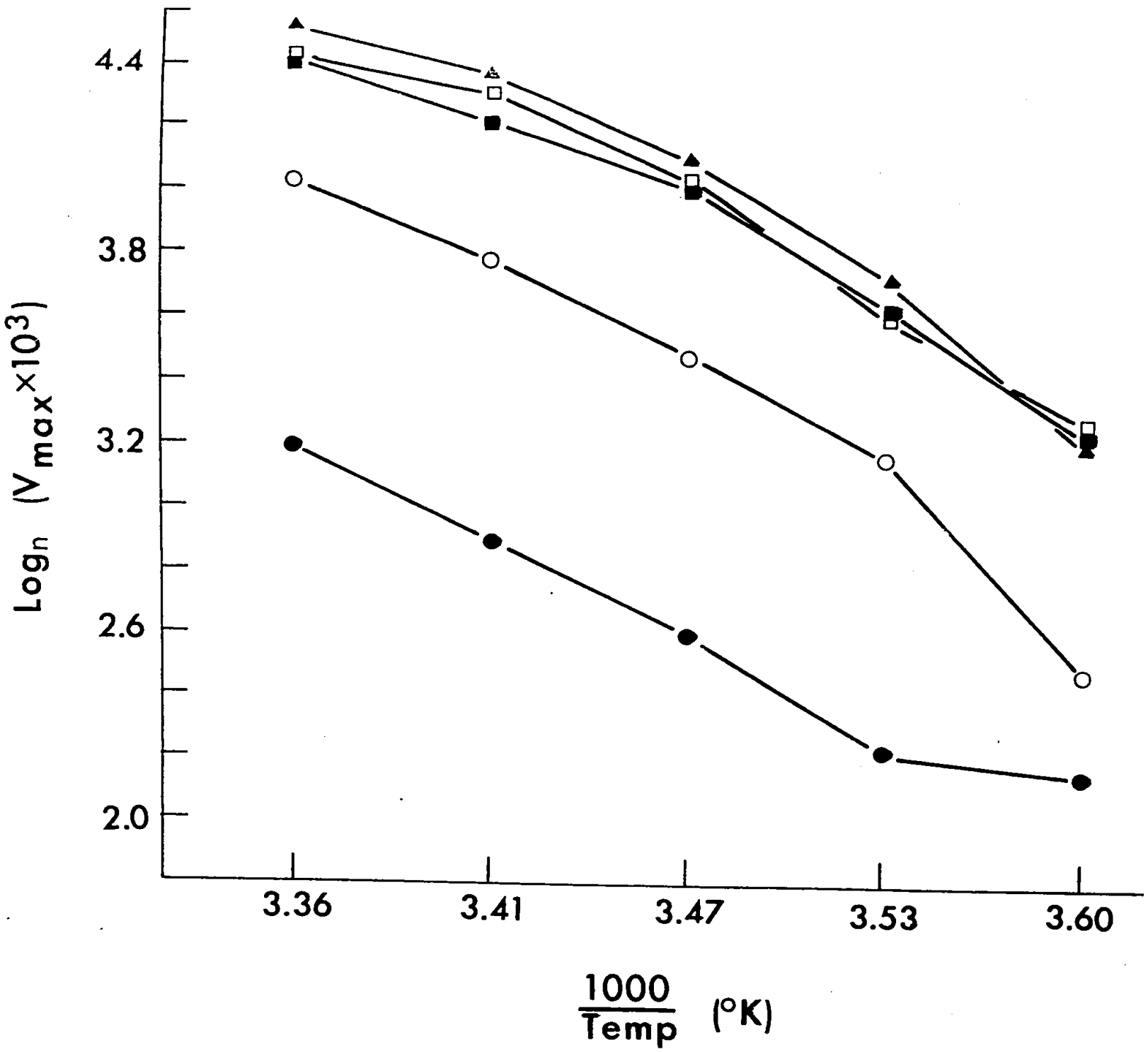
Allozyme	Q_{10} 10°-20°C	Q_{10} 15°-25°C
ME 100 west.	2.31	1.68
ME 100 east.	2.21	1.48
ME 93	2.18	1.62
ME 75	2.26	1.67
ME 68	2.10	1.55

It can be seen that Q_{10} values for the 15° - 25°C interval are substantially below those expected for a reaction in which no temperature compensation is found (Q_{10} = 2 to 3).

A comparison of Q_{10} values between allozymes reveals very little difference in the rate of increase of reaction velocities at either the 10° - 20°C or the 15° - 25°C intervals. This result suggests that all the salmon ME allozymes have a similar functional response to changes in temperature. Further evidence in support of this conclusion comes from Arrhenius plots of the energy of activation (E_a) for the reaction catalysed by each allozyme (Figure 4.9). E_a is calculated from the slope of the regression line, and it can be seen that the slopes of all lines are similar, at least over the first four data points (corresponding to 25°, 20°, 15° and 10°C respectively). The slight curve in all lines indicates that the catalytic capacities of the allozymes do change slightly in response to temperature, thus altering the amount of externally derived kinetic energy needed to activate the substrate. However this change is common to all the allozymes, and does not alter the conclusion of similar functional response to temperature.

Figure 4.9

Arrhenius plots of maximum reaction velocities (V_{max}) against temperature for the salmon ME allozymes. See Figure 4.2 for the plotting code. These plots are based on the data in Figure 4.7, and it has been assumed that reaction velocities obtained at saturating substrate levels are reasonable approximations of V_{max} values. Energy of activation (E_a) estimates are derived from the slopes of the regression lines.


4.4
3.8
3.2
2.6
2.0
Log$_n$ (V_{max} x10^3)
3.36
3.41
3.47
3.53
3.60
1000/Temp (°K)

4.4.6 Heat Stability

Extracts from the same individuals assayed for ME reaction velocity verses temperature in section 4.4.5 were also tested for heat stability using the methods described in section 4.3.3. Figure 4.10 shows the percentage activity remaining after ten minutes incubation at temperatures of 30° to 60°C. It is immediately apparent that the ME 100 west. extract has greater resistance to thermal inactivation than any of its eastern salmon ME homologues over the range of 35° to 55°C. The heat stability curves for the eastern salmon ME allozymes have similar shapes and are reasonably clustered when compared to the ME 100 west. individual but, as with the K_m calculations in section 4.4.4, it is desirable to test a number of individuals in each allozyme class to determine the precision of this result.

48°C was chosen as a key temperature to test ME heat stability in a large number of individuals because it was likely to provide the most obvious differences in percentage activity between different allozyme classes (see Figure 4.10). Extracts from a total of 64 individuals representing all available electrophoretic ME allozyme phenotypes were heat treated at 48°C, and the results are given in the form of a frequency histogram of percentage activities (Figure 4.11). These results confirm that the heat stability characteristics of all the eastern salmon ME allozymes are reasonably homogeneous, with activity values falling within the range 36 to 57%. However the ME 100 west. individuals appear to fall into three major classes of heat stability with percentage activity ranges of 0 to 24%, 43 to 61% and 84 to

Figure 4.10.

Heat stability profiles of the salmon ME allozymes over the temperature range 30° - 60°C. See Figure 4.2 for the plotting code. Heat stability is measured as percentage enzyme activity remaining after ten minutes incubation at the designated temperature. The vertical line indicates the key temperature (48°C) chosen for subsequent tests of ME heat stability in salmon individuals. Assays were carried out at 18°C using 50 mM tris-maleate pH 8.0, 5 mM manganous chloride, 3.2 mM malate and .3 mM NADP.

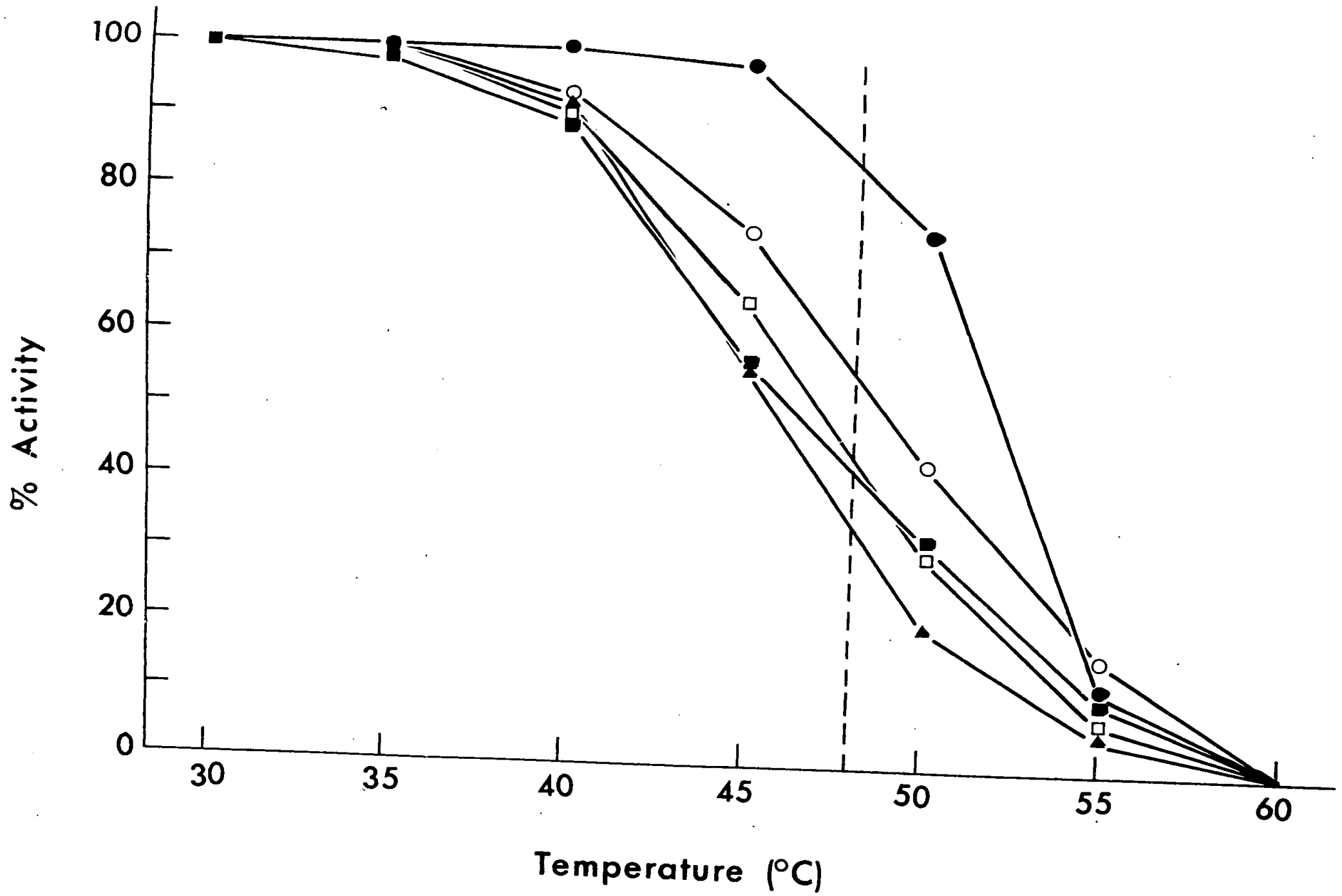
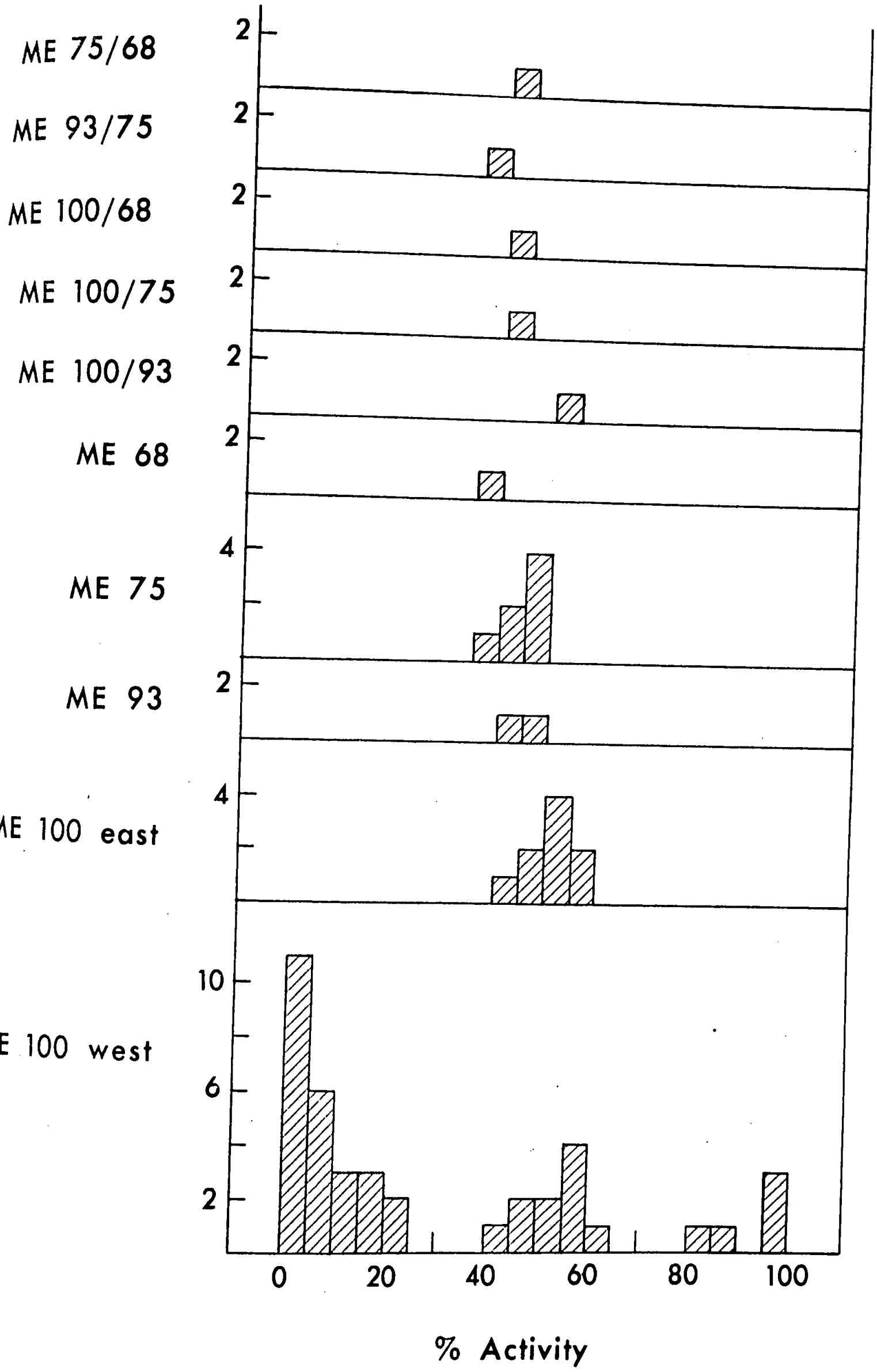

100
80
60
40
20
0
% Activity
30
35
40
45
50
55
60
Temperature (°C)

Figure 4.11

Frequency histograms of percentage activity after incubation at 48°C for 64 individuals representing most of the availible salmon ME electrophoretic phenotypes. Assay conditions were as given in Figure 4.10.


ME 75/68
ME 93/75
ME 100/68
ME 100/75
ME 100/93
ME 68
ME 75
ME 93
ME 100 east
ME 100 west
2
2
2
2
2
2
4
2
4
10
6
2
0
20
40
60
80
100
% Activity

100% respectively. The ME 100 west. individual used for heat stability tests in Figure 4.10 was chosen by chance from the latter class, which explains why it was more resistant than the eastern salmon ME allozymes to thermal inactivation.

Having established that there are at least three different heat stability classes within the ME 100 electrophoretic phenotype of western salmon, it is now of interest to determine whether or not these thermostability variants also display functional differences in response to temperature fluctuations. Although a genetic basis for the thermostability variants cannot be inferred from the present data (see section 4.6 for further discussion) the heat stability classes will be treated as allozymes for convenience, and will be labeled ME 100s, ME 100r/s and ME 100r for the heat sensitive, intermediate and heat resistant variants respectively. Individuals representing each of the thermostability variants were assayed at both saturating and K_m levels of substrate over the temperature range 5° to 35°C (Figures 4.12 and 4.13). The resulting velocity/temperature curves are very similar to those obtained for both the western and eastern salmon electrophoretic ME allozymes (Figures 4.7 and 4.8), as can be seen from the Q_{10} values calculated for assays at K_m substrate levels:-

Heat Stability Class	Q_{10} 10-20°C	Q_{10} 15-25°C
ME 100s	2.39	1.65
ME 100r/s	2.27	1.70
ME 100r	2.16	1.71

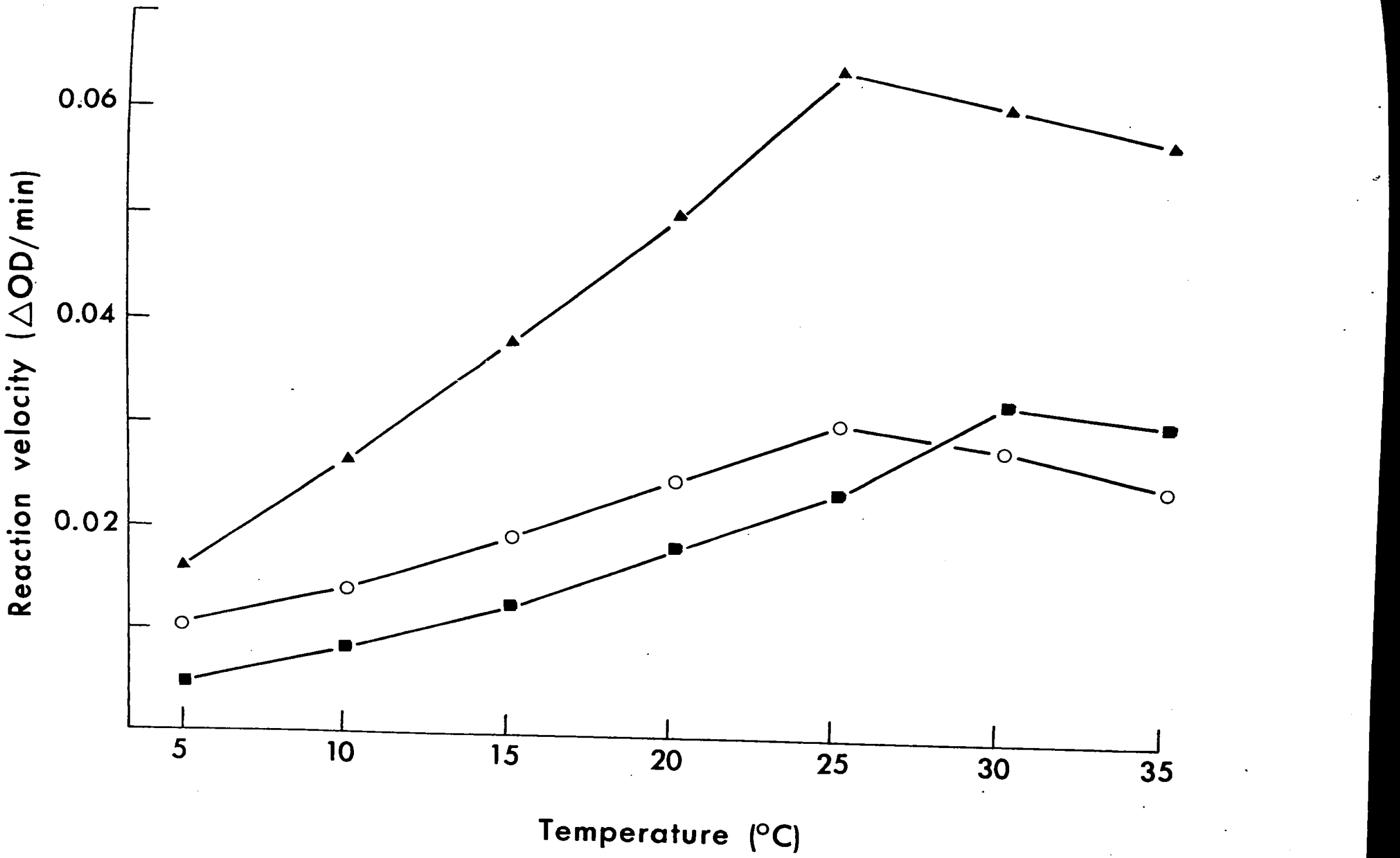
Figure 4.12

Saturation reaction velocities of the western salmon ME heat stability variants when assayed at 5° intervals for temperatures between 5°C and 35°C. Assay conditions were as given in Figure 4.7.

○ = ME 100r

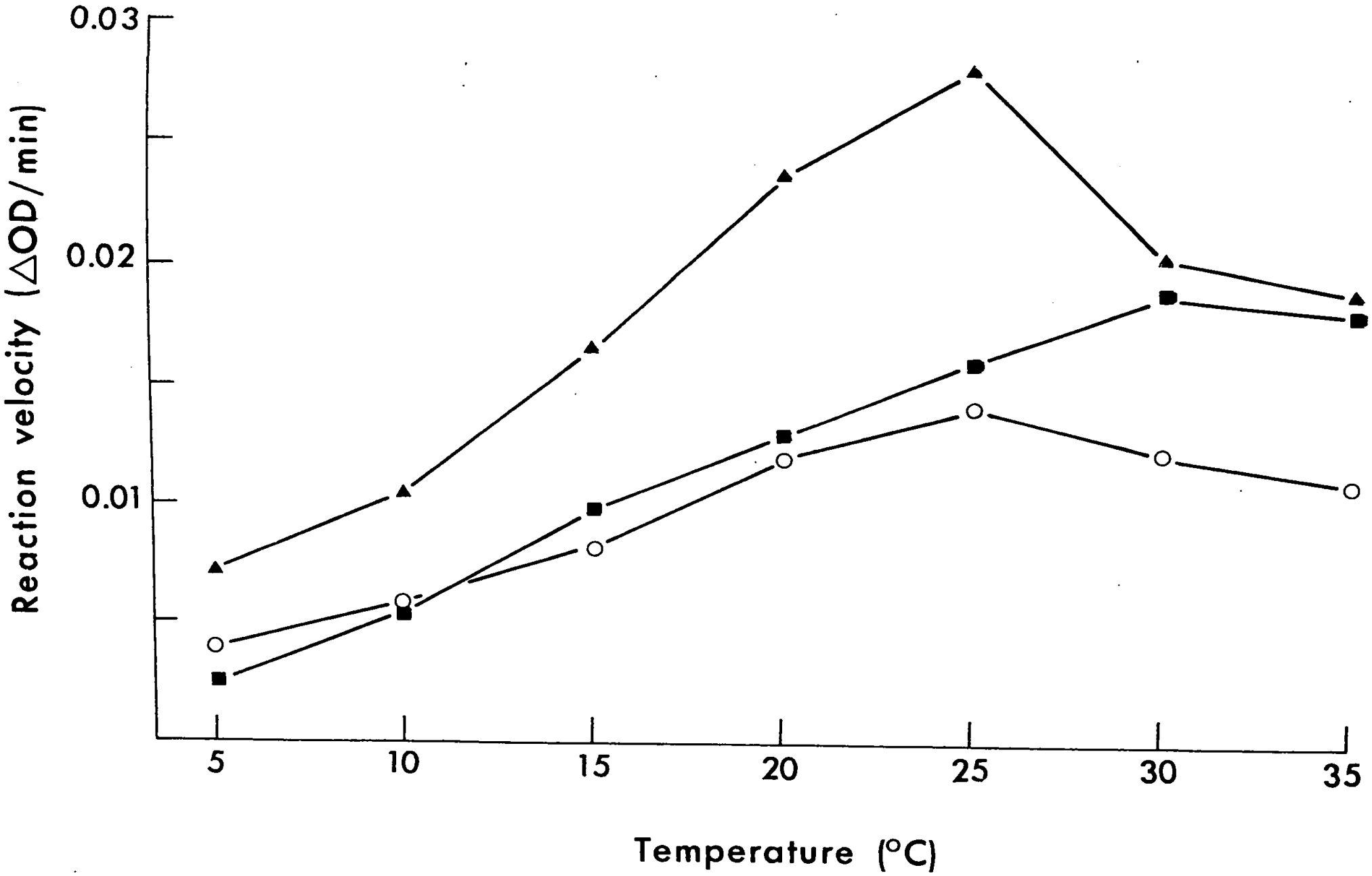
▲ = ME 100r/s

■ = ME 100s


0.06
0.04
0.02
Reaction velocity (ΔOD/min)
5
10
15
20
25
30
35
Temperature (°C)

Fugure 4.13

K_m reaction velocities of the western salmon ME heat stability variants when assayed at 5° intervals for temperatures between 5°C and 35°C. See Figure 4.12 for the plotting code. Assay conditions were as given in Figure 4.8.


0.03
0.02
0.01
Reaction velocity (ΔOD/min)
5
10
15
20
25
30
35
Temperature (°C)

It seems, therefore, that despite the existence of both electrophoretic and thermostability variants of ME in western and eastern salmon individuals, all forms of the enzyme display similar functional responses to temperature fluctuations over the range 10°C to 25°C.

4.4.7 Specific Activity

The substrate turnover rate or specific activity of an enzyme can only be measured if the concentration of enzyme in the reaction is known. This normally involves the purification of the enzyme and the addition of known quantities of pure extract to a controlled reaction system. When crude enzyme extracts are assayed the nearest approximation to specific activity that can be obtained is a measure of activity (in this case ΔOD/minute) per unit of total soluble protein in the crude extract. This is only a rough estimate, as the true specific activity of the enzyme may be masked by allosteric interaction with other proteins or by variation between individuals in the concentrations of total protein present. Differences in specific activity of crude enzyme extracts may also be due to the presence of allozymes with different catalytic efficiencies. The result from Arrhenius plots of E_a (Figure 4.9) suggest that this is not the case with the salmon ME allozymes, and it has been assumed that differences in specific activity are due largely to variation in the concentration of ME in the crude enzyme extracts.

65 individuals from all available electrophoretic and heat stability classes of ME in western and eastern salmon

were assayed at 18°C and saturating levels of substrate. Total soluble protein was determined for each crude enzyme extract and specific activities were calculated as ΔOD/ minute/mg protein. A frequency histogram of specific activities (Figure 4.14) shows that values for eastern salmon ME allozymes are broadly scattered over the range .353 to .985. Sample sizes of the allozymes are not adequate for statistical analysis, but it is apparent that the largest component of variation in specific activities is contributed by differences between individuals, rather than differences between allozyme classes.

ME 100 west. specific activity values are clustered in the range .031 to .222, and are obviously significantly less than the values for eastern salmon ME allozymes. This result explains the consistently lower activity levels obtained for western salmon during comparative assays for pH optima, substrate/velocity curves and temperature/velocity curves. It may be argued that these apparent differences in enzyme concentration are induced by geographical variations in the prevailing physical environment of the fishes. However a small proportion of both eastern and western salmon individuals assayed for specific activity were collected from the same school of fishes on the same day in Port Phillip Bay, Victoria (see Table 3.1). It seems, therefore, that the observed specific activity variation is a genuine difference between species. The implications of this result will be discussed in section 4.6.

A final test was carried out on ME 100 west. individuals to determine the relationship between specific activity and heat stability characteristics. A scatter diagram using

Figure 4.14

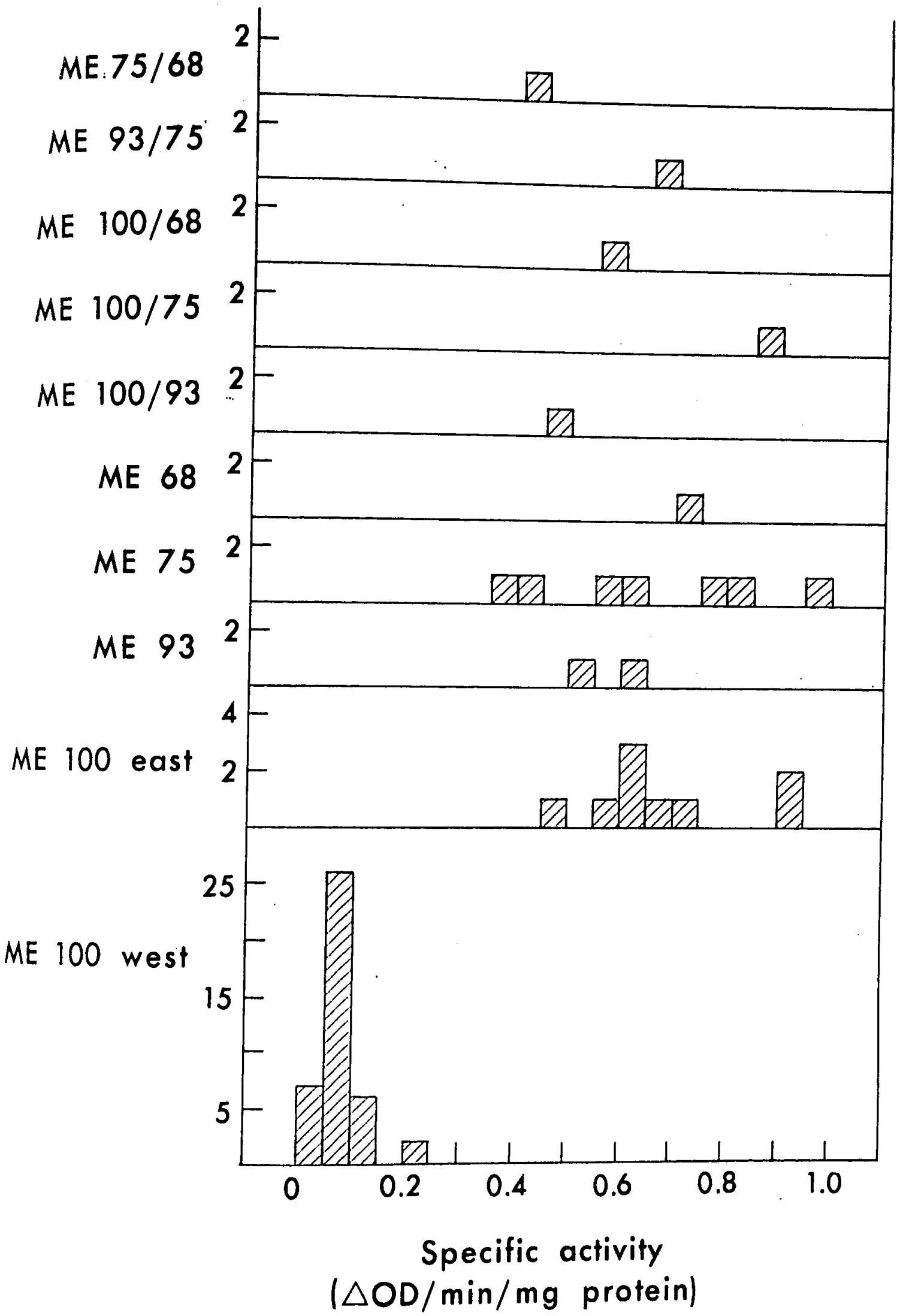
Frequency histograms of specific activity for 61 individuals representing most of the available Salmon ME electrophoretic phenotypes. Activity assays were carried out using saturating levels of substrate. Assay conditions were as given in Figure 4.11.

for

le

assays

strate.



these two parameters as axes (Figure 4.15) shows that there is no discernable correlation, and it can thus be inferred that there is no connection between the amount of enzyme in an extract and its heat stability characteristics.

4.5 Results - Isocitrate Dehydrogenose in Australian Snapper

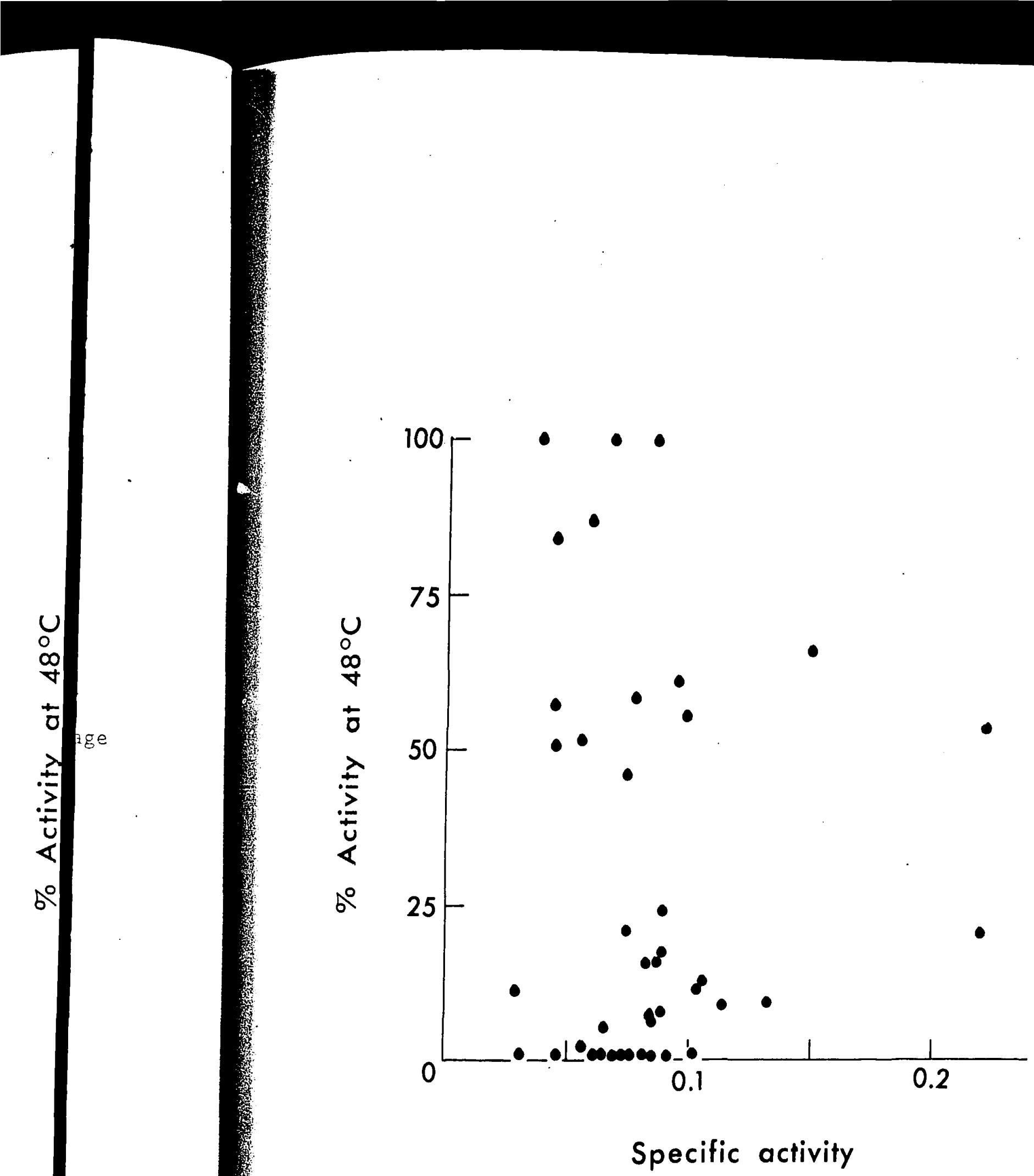
4.5.1 Direction of the Reaction

Randle et al. (1970) have shown that NADP-IDH catalyses a reversible equilibrium reaction in perfused rat heart tissue. The enzyme is product-inhibited by α-ketoglutarate (KGA) and NADPH in halophilic bacteria (Hochachka and Somero, 1973), and is generally thought to favour the decarboxylating (isocitrate → KGA) reaction when associated with the TCA cycle. The role of cytoplasmic NADP-IDH is less certain, but for reasons outlined in section 4.1.6 the reaction catalysed in this location is also thought to be mainly in the direction of NADPH production.

Initial assays of both the forward and reverse IDH reactions were carried out at 20°C (a median, and presumably optimal, temperature chosen on the basis of the known biological range of snapper in Australia) and pH 8.0 (as for proven electrophoretic assays - see section 3.3.3). Assay conditions for the decarboxylating reaction were:-

Figure 4.15

Scatter diagram of ME thermostability (percentage activity after treatment at 48°C) verses specific activity for crude enzyme extracts from 40 western salmon individuals.


% Activity at 48°C
100
75
50
25
0
0.1
0.2
Specific activity

50.0	mM	tris-maleate pH 8.0
5.0	mM	DL-isocitric acid (SIGMA, Type I, trisodium salt - contains approx. 50% threo-D_s-isocitrate)
0.2	mM	NADP
5.0	mM	$MnCl_2$
10	µl	enzyme extract

and for the carboxylating reaction were:-

50.0	mM	tris-maleate pH 8.0
20.0	mM	α-ketoglutaric acid (SIGMA)
0.25	mM	NADPH
20.0	mM	NaH_2CO_3
5.0	mM	$MnCl_2$
10	µl	enzyme extract

Homozygous representatives of the alleles *IDH*^{114}, *IDH*^{100} and *IDH*^{80} (see Table 3.11 and Plate 3.5) were assayed in both forward and reverse directions, but no enzyme activity was observed in any assay of the carboxylation reaction. There are a number of possible reasons for this result (as outlined in section 4.4.1), but for the purpose of this study the decarboxylation reaction was assumed to be kinetically more favourable and was used exclusively for temperature studies of the snapper IDH allozymes.

4.5.2 pH Optima

Homozygous IDH 114, IDH 100 and IDH 80 individuals were assayed for activity at pH values from 5.0 to 9.0 using the assay conditions given for the decarboxylation reaction described in section 4.5.1. All assays were at 20°C and all tris-maleate buffer pH's were adjusted at this temperature. Figure 4.16 shows that all three IDH allozymes have pH optima in the range 7.0 to 8.5 under *in vitro* assay conditions. Consequently a pH of 7.5 was chosen for all further assays at 20°C.

As with the salmon ME allozymes there are differences in activity levels between the three snapper IDH classes (Figure 4.16). These differences are again probably due to variation in the concentration of enzyme present in each of the crude extracts assayed.

4.5.3 Substrate/Velocity Curves

Plots of V/[isocitrate] (Figure 4.17) and V/[NADP] (Figure 4.18) for samples representing each of the three IDH allozymes show that Michaelis-Menten saturation kinetics are observed in all cases, although the approach to V_{max} is comparatively slower for NADP than for isocitrate measurements. There is no evidence of substrate inhibition over the range of concentrations used, and 3.5mM (1mg/ml) isocitrate and .2mM (.168 mg/ml) NADP were used in all subsequent assays where reaction velocities approximating V_{max} were desired.

Figure 4.16

Plots of reaction velocity against initial pH of the reaction mixture for the IDH allozymes of Australian snapper. See section 4.5.2 for assay conditions.

▲ = IDH 114

● = IDH 100

○ = IDH 80

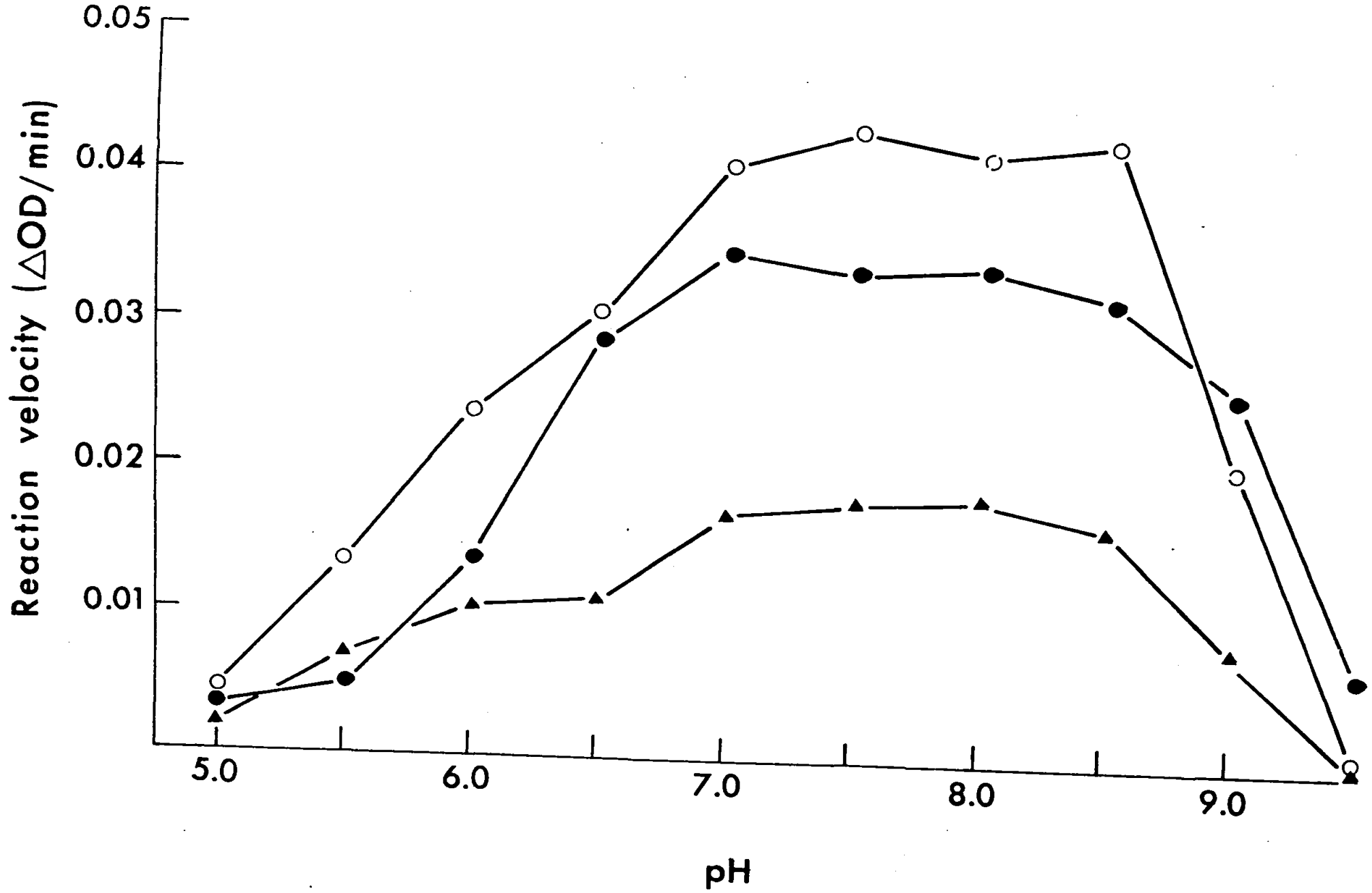

0.05
0.04
0.03
0.02
0.01
Reaction velocity (ΔOD/min)
5.0
6.0
7.0
8.0
9.0
pH

Figure 4.17

Plots of reaction velocity against final concentration of isocitrate in the reaction mixture for the IDH allozymes of snapper. See Figure 4.16 for the plotting code. Assays were carried out at 20°C using 50 mM tris-maleate pH 7.5, 5 mM manganous chloride and .2 mM NADP.

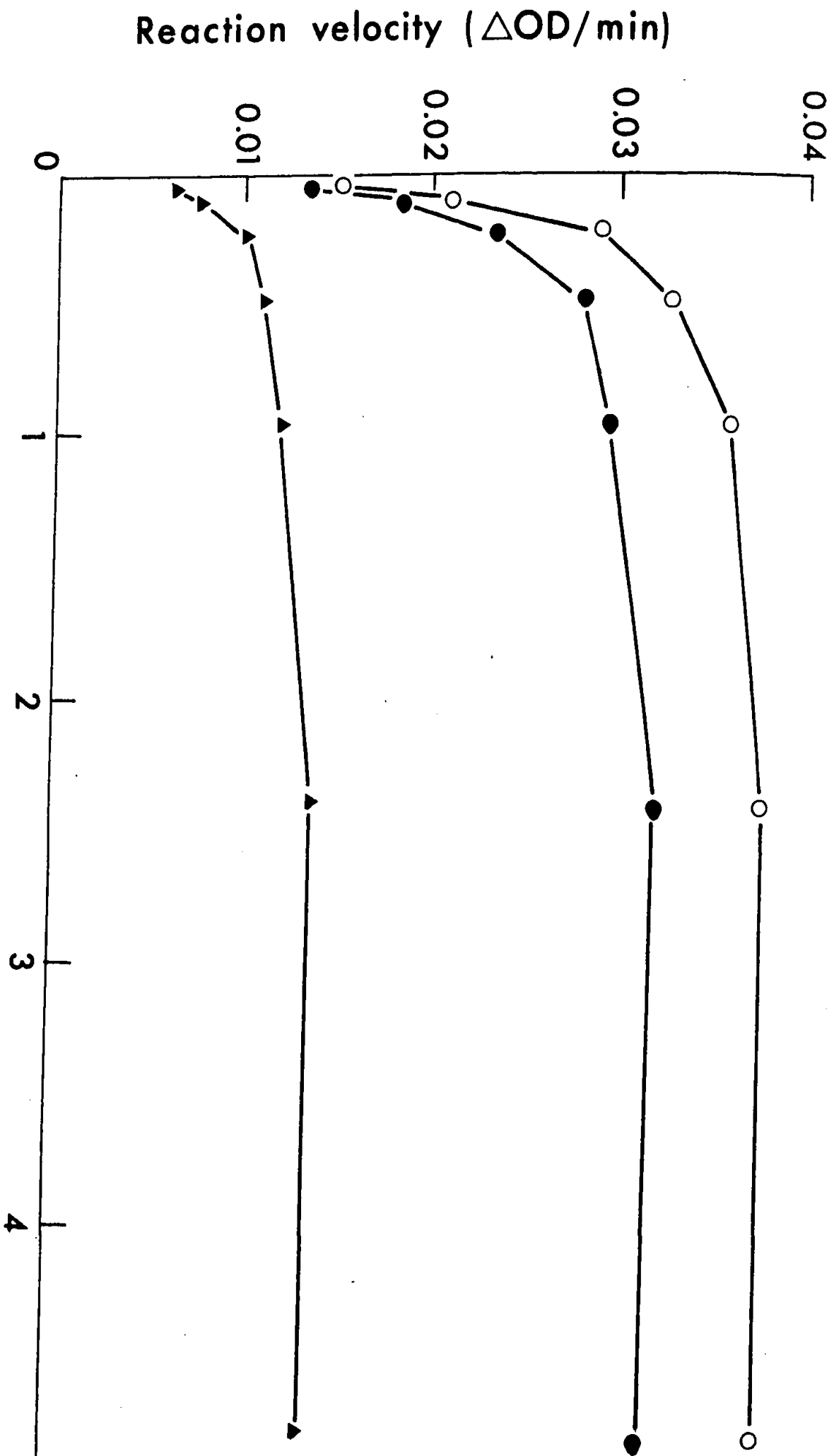
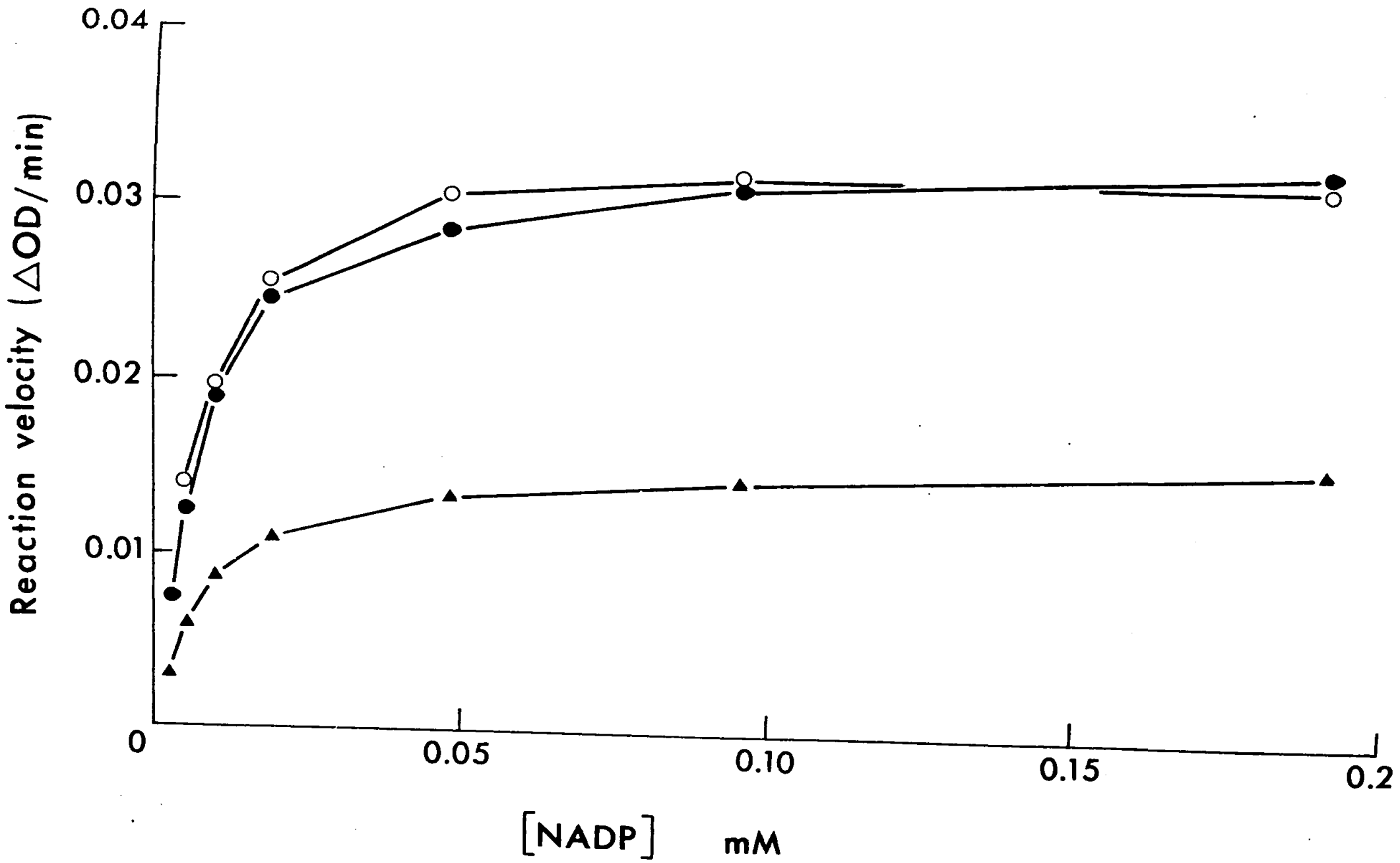

Reaction velocity (ΔOD/min)
0
0.01
0.02
0.03
0.04
1
2
3
4
5
[Isocitrate] mM

Figure 4.18

Plots of reaction velocity against final concentration of NADP in the reaction mixture for the snapper IDH allozymes See Figure 4.16 for the plotting code. Assays were carried out at 20°C using 50 mM tris-maleate pH 7.5, 5 mM manganous chloride and 5 mM isocitrate.


0.04
0.03
0.02
0.01
0
Reaction velocity (ΔOD/min)
0.05
0.10
0.15
0.2
[NADP] mM

4.5.4 Enzyme-Substrate Affinity

Direct linear plots (see section 4.4.4) of the substrate/velocity data in Figures 4.17 and 4.18 have provided median estimates of apparent K_m [isocitrate] and K_m [NADP] for each of the IDH allozymes. It can be seen from Table 4.2 that for both substrates the 95% confidence interval for each allozyme includes the median K_m values of the other two, suggesting that at 20°C there are no significant differences in enzyme-substrate affinity. This conclusion is supported by data from a frequency histogram of percentage activities at K_m versus saturating levels of substrate for 50 individuals representing all IDH electrophoretic phenotypes (Figure 4.19). All percentage activity values fall within the range 39 to 47%, and there is no evidence of heterogeneity either within or between allozyme classes. Consequently .06 mM isocitrate and .0075 mM NADP were used as approximate K_m levels of substrate for all further assays.

4.5.5 The Effect of Temperature on Reaction Velocities

Homozygous individuals representing each of the IDH allozymes were assayed at 5° intervals for temperature between 5° and 40°C, using both saturating and approximate K_m levels of substrate. The assays at saturating substrate levels (Figure 4.20) indicate that the IDH allozymes have similar functional responses to temperature, and Q_{10} values calculated from these assays suggest that there is very little temperature compensation occuring over the

Table 4.2

Median K_m substrate concentrations and approximate 95% confidence intervals for Australian snapper IDH allozymes assayed at 20°C. Concentrations of .06 mM isocitrate and .0075 mM NADP were used in all subsequent assays at K_m substrate levels.

Substrate	Allozyme	Median K_m (mM)	95% Confidence Intervals
Isocitrate	IDH 114	.0591	.0511 - .0889
	IDH 100	.0646	.0539 - .0886
	IDH 80	.0590	.0544 - .0758
NADP	IDH 114	.00744	.00713 - .00873
	IDH 100	.00802	.00619 - .00934
	IDH 80	.00764	.00465 - .00942

Figure 4.19

Frequency histograms of percentage activity at apparent K_m verses saturating levels of substrates for 50 individuals representing all IDH electrophoretic phenotypes. Assays were carried out at 20°C using 50 mM tris-maleate pH 7.5 and 5 mM manganous chloride. K_m substrate levels used were .06 mM isocitrate and .0075 mM NADP, while saturating levels were 3.5 mM isocitrate and .2 mM NADP.

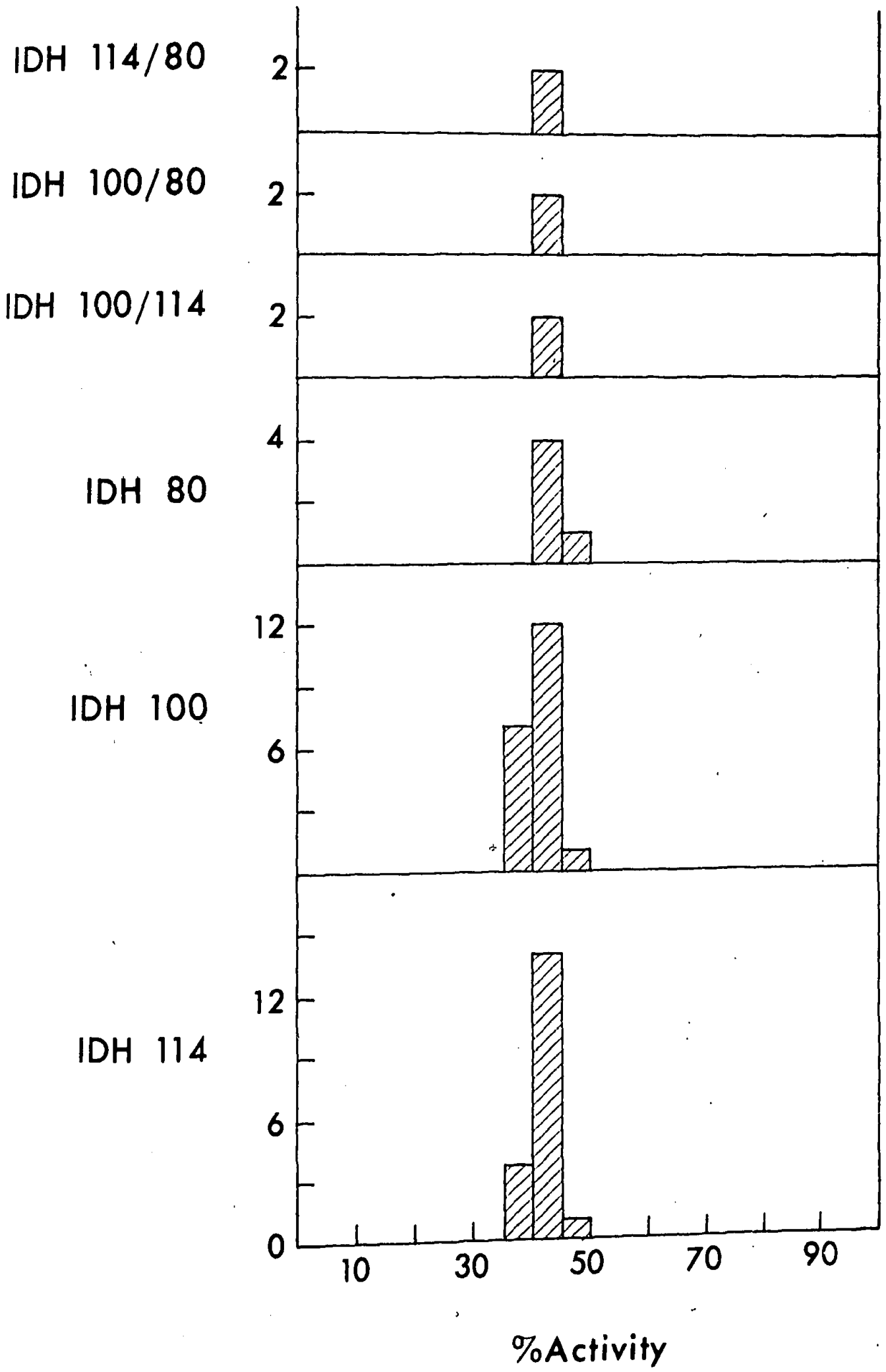
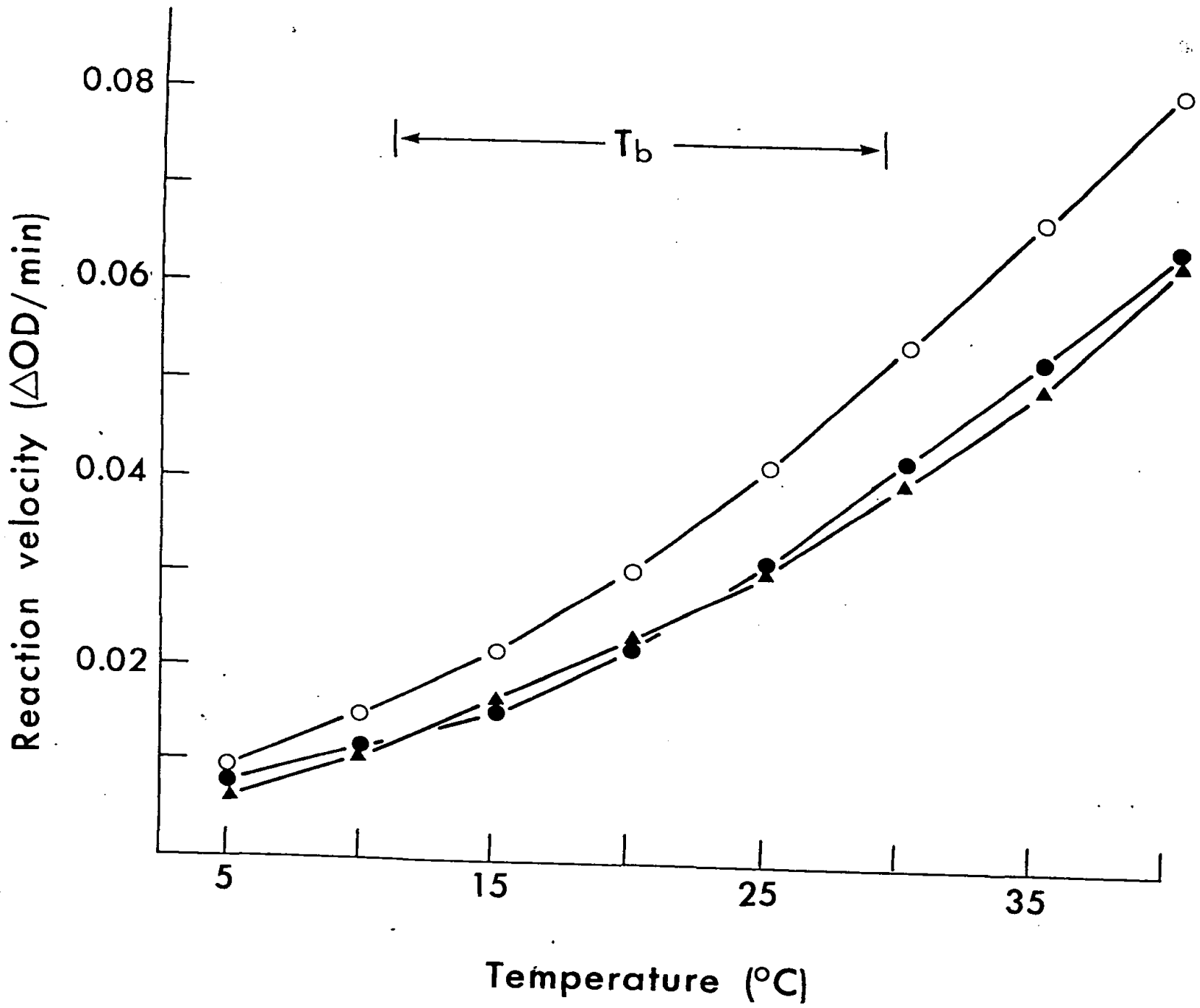

IDH 114/80
2
IDH 100/80
2
IDH 100/114
2
IDH 80
4
IDH 100
12
6
IDH 114
12
6
0
10
30
50
70
90
%Activity

Figure 4.20

Saturation reaction velocities of the snapper IDH allozymes when assayed at 5° intervals for temperatures between 5°C and 40°C. See Figure 4.16 for the plotting code. T_b = the range of surface temperatures recorded from waters where Australian snapper are known to occur. Assay conditions were :- 80 mM imidazole-HCl pH 7.5 (at 20°C), 5 mM manganous chloride, 3.5 mM isocitrate and .2 mM NADP.


0.08
0.06
0.04
0.02
Reaction velocity (ΔOD/min)
T_b
5
15
25
35
Temperature (°C)

biological range of Australian snapper:-

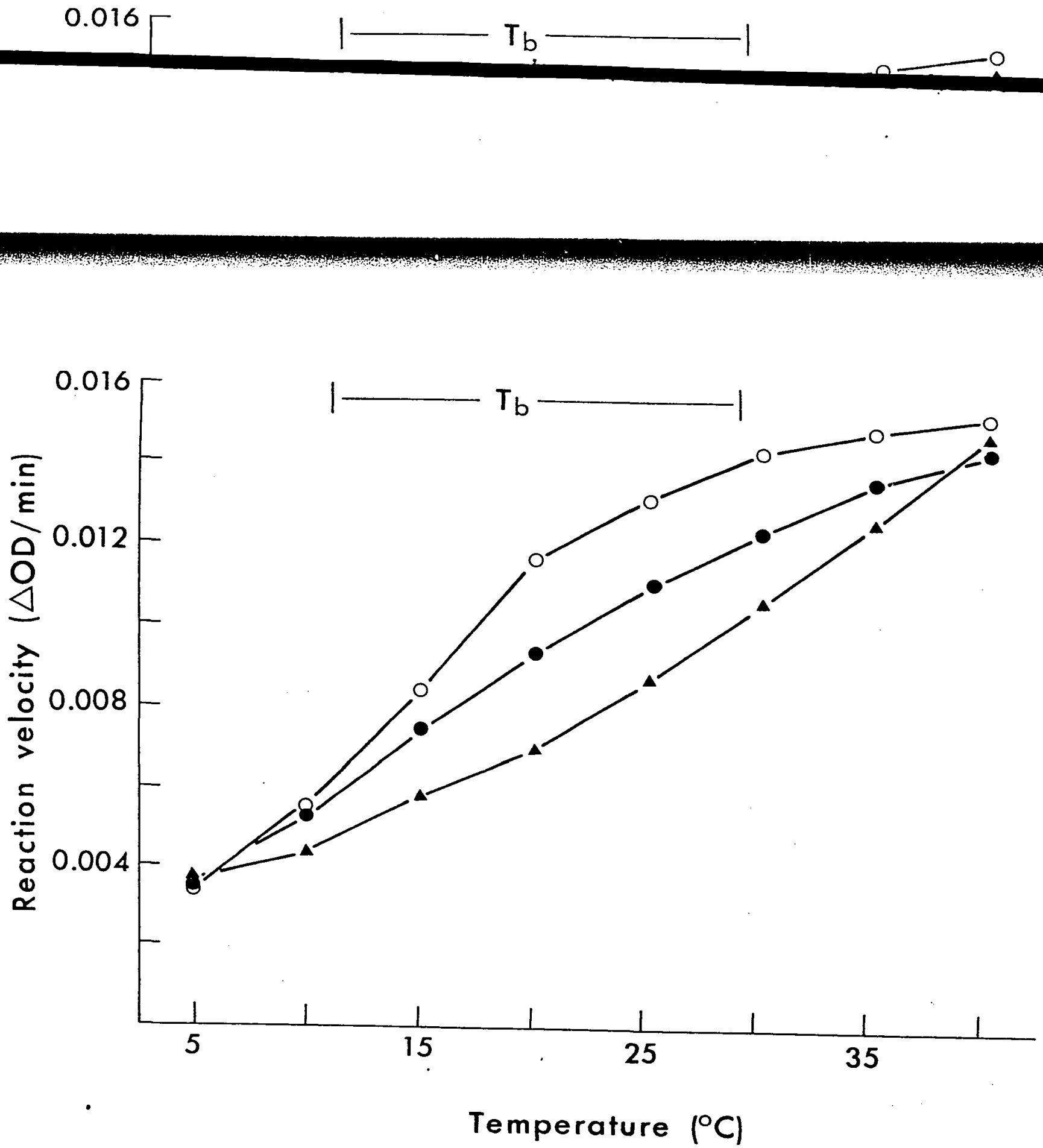
Allozyme	Q_{10} 10-20°C	Q_{10} 20-30°C
IDH 114	2.07	1.76
IDH 100	2.00	1.89
IDH 80	2.03	1.79

However when assays are done on the same enzyme extracts using K_m levels of substrate (Figure 4.21), the IDH allozymes behave differently compared to Figure 4.20 and compared to each other. It appears that there are functional differences in response to temperature between the IDH allozymes, but that at saturating levels of substrate these differences are masked by the ready availability of substrate. This result illustrates the point made earlier (section 4.4.5) that it is important to assay enzymes at presumed physiological as well as saturating substrate levels, in order to provide a biologically more realistic test of functional response to temperature or other environmental parameters.

Having found temperature-related differences in activity between the IDH allozymes it is logical to ask whether these differences can be attributed to any of the enzyme properties discussed in section 4.1.2. Variation in enzyme concentration between allozymes can be ruled out, because even if absolute differences in activity at a given temperature were due to variable concentrations of enzyme in the crude extracts a quantitative strategy cannot explain differences in the shapes of the activity/ temperature curves.

Figure 4.21

K_m reaction velocities of the snapper IDH allozymes when assayed at 5° intervals for temperatures between 5°C and 40°C. See Figure 4.16 for the plotting code. T_b = the range of surface temperatures recorded from waters where Australian snapper are known to occur. Assay conditions were :- 80 mM imidazole-HCl pH 7.5 (at 20°C), 5 mM manganous chloride, .06 mM isocitrate and .0075 mM NADP.


0.016
0.012
0.008
0.004
Reaction velocity (ΔOD/min)
T_b
5
15
25
35
Temperature (°C)

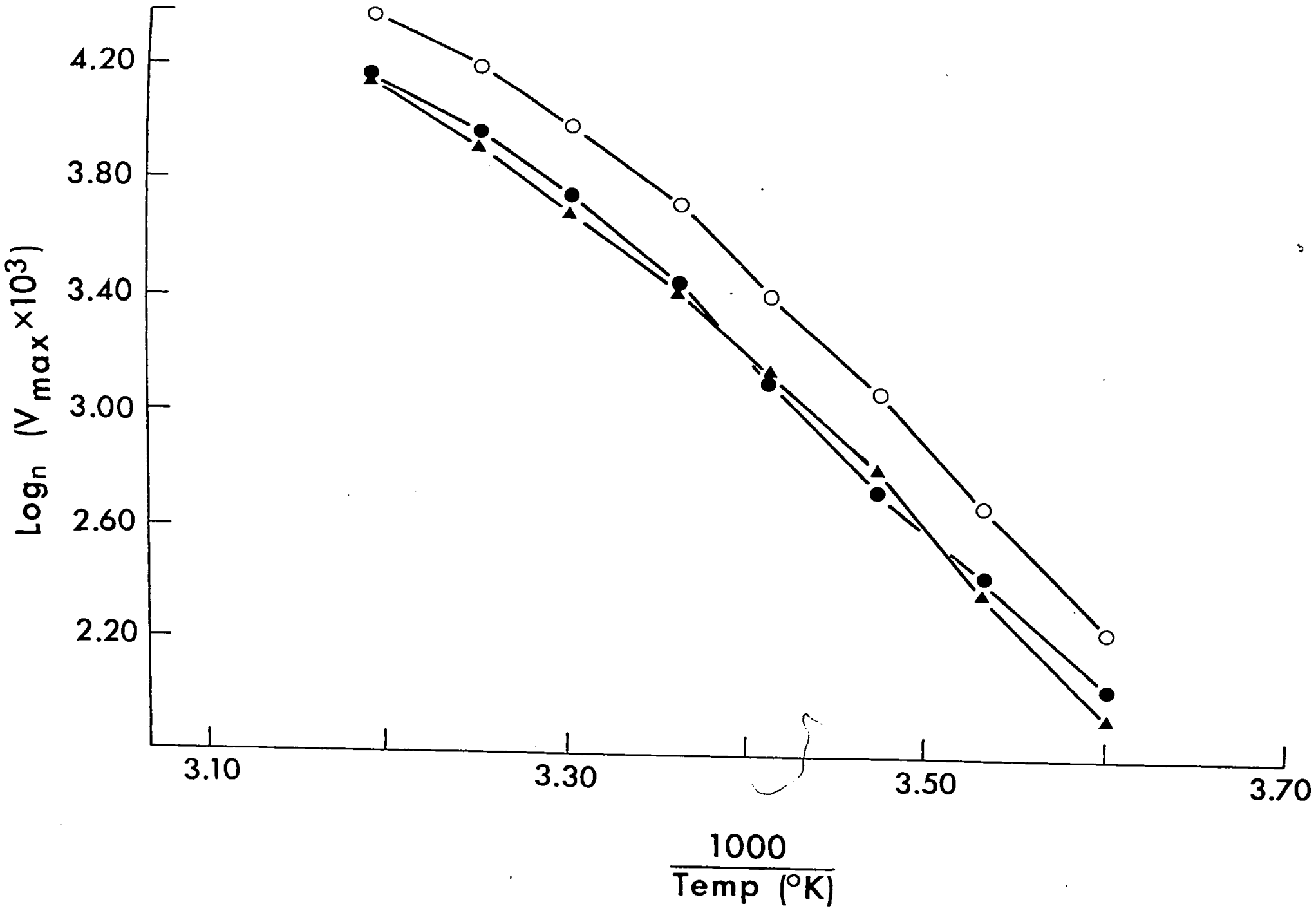
A second possibility is differential catalytic capacity of the IDH allozymes. Arrhenius plots of the energy of activation (E_a) for the reaction catalysed by each allozyme are given in Figure 4.22. E_a is calculated from the slope of the regression line. While the slopes of the lines (and thus E_a for the reactions) change slightly with temperature, all of them change in the same manner, and it can be inferred that at any given temperature the IDH allozymes have similar catalytic capacities.

We are left with differential enzyme-substrate affinities as the possible cause of functional differences between the IDH allozymes in response to temperature. While apparent K_m values for each IDH allozyme were not obtained over a range of temperatures during this study, the importance of enzyme-ligand binding ability can be inferred for two reasons. Firstly, as discussed in section 4.1.3, there is ample evidence in the recent literature to suggest that K_m modifications are of prime importance in effecting rate compensation to short-term or immediate temperature fluctuations. Secondly, the functional differences between the IDH allozymes were only observed at low substrate levels - levels at which the ability of the enzyme to bind substrate has a direct influence on the reaction velocity.

In attempting to explain the biological implications of the results in Figure 4.21 it is necessary to remove the bias arising from variation of enzyme concentrations in the crude extracts assayed. It has been found that the IDH allozymes have similar E_a's over the range 5° -

Figure 4.22

Arrhenius plots of maximum reaction velocities (V_{max}) against temperature for the snapper IDH allozymes. See Figure 4.16 for the plotting code. These plots are based on the data in Figure 4.20, and it has been assumed that reaction velocities obtained at saturating substrate levels are reasonable approximations of V_{max}. Energy of activation (E_a) estimates are derived from the slopes of the regression lines.


4.20
3.80
3.40
3.00
2.60
2.20
Log$_n$ (V_{max} x ×10^3)
3.10
3.30
3.50
3.70
1000
Temp (°K)

40°C, and similar K_m values at 20°C. However the IDH allozymes have different activities at 20°C in Figure 4.21, indicating quantitative differences between crude extracts. This problem can be overcome to some extent by converting all activities for each allozyme to a percentage of activity at 20°C (Figure 4.23). There is still some bias in the data arising from the use of different 20°C activity values for each allozyme as denominators, but the diagramatic representation obtained is nevertheless adequate for explaining the observed functional differences.

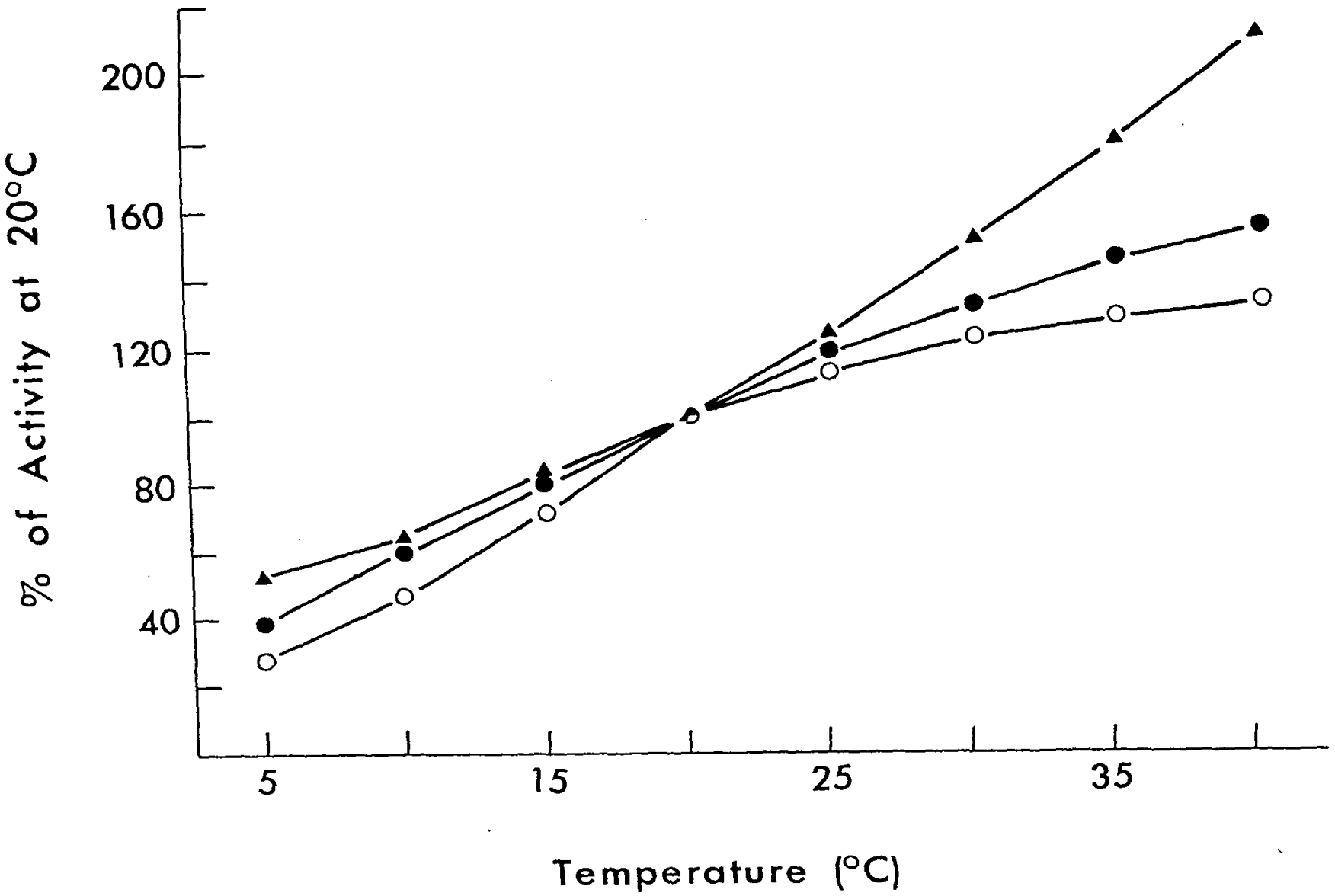
Q_{10} values for the data in Figure 4.21 are as follows:-

Allozyme	Q_{10} 10-20°C	Q_{10} 15-25°C	Q_{10} 20-30°C
IDH 114	1.55	1.48	1.31
IDH 100	1.77	1.48	1.32
IDH 80	2.14	1.57	1.19

It can be seen from these data and Figure 4.23 that IDH 114 exhibits a superior capacity for positive rate compensation at temperatures below 20°C, while IDH 80 is superior at temperatures above 20°C. IDH 100 has an almost linear response to temperature changes, and exhibits rate compensation abilities intermediate to the other allozymes over the temperature range assayed. Temperature/velocity assays were repeated using other representatives of the three IDH allozymes. Similar results were obtained, suggesting that the observed

Figure 4.23

Diagramatic representation of the comparative functional responses of the snapper IDH allozymes to temperature fluctuations. All reaction velocities in Figure 4.21 have been converted to percentages of activity at 20°C in order to reduce biases arising from quantitative differences between crude enzyme extracts.


% of Activity at 20°C
200
160
120
80
40
5
15
25
35
Temperature (°C)

variation in rate compensation ability is due to genuine functional differences between allozyme classes. Assuming that controlled flux of metabolites through the IDH reaction pathway is the desired objective, it seems that IDH 114 is best adapted to operate at temperatures toward the lower end of the biological range of snapper, while IDH 80 is best suited to the higher end. IDH 100, while less well modulated than IDH 114 and IDH 80 at their respective optima, has rate compensation abilities similar to these allozymes over the range 15° - 25°C, and has a uniform response to temperature over the entire thermal range of Australian snapper.

From the above results it is possible to postulate a mechanistic basis for differential selection of the snapper IDH allozymes based on their functional response to short-term temperature fluctuations. The implications of this hypothesis, and its credibility when compared to observed geographical distributions of snapper IDH allozymes, will be discussed in section 4.6.

4.5.6 Specific Activity

Specific activity estimates (as described in section 4.4.7) were obtained for 50 individuals representing all the available IDH electrophoretic phenotypes. Assays were at 20°C, using saturating levels of substrates. A frequency histogram of specific activity values (Figure 4.24) shows a range of .330 to .940, and indicates a broad distribution in all allozyme classes. Sample sizes for each class were not adequate for statistical analysis,

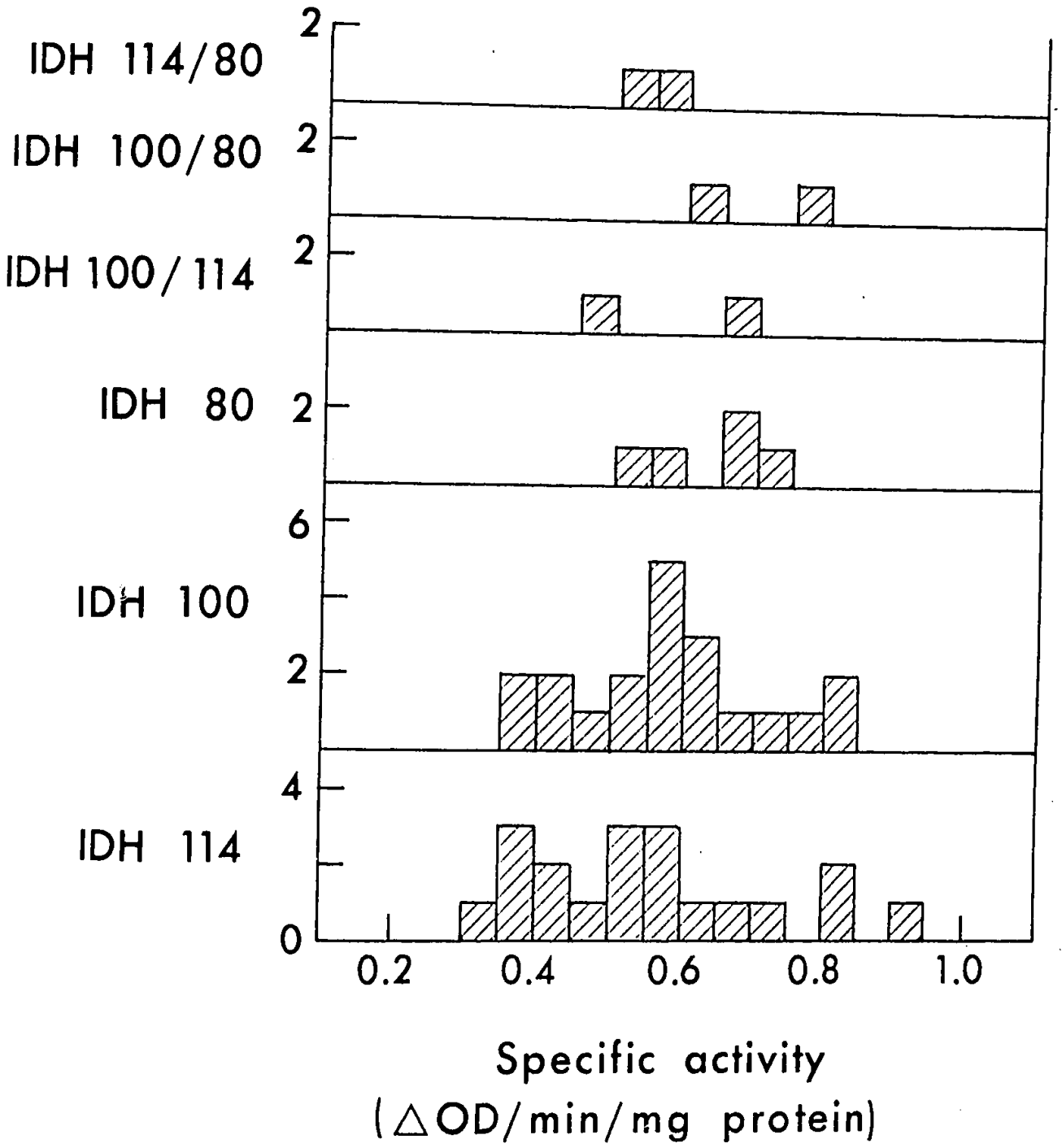
Figure 4.24

Frequency histograms of specific activity for 50 individuals representing all the IDH electrophoretic phenotypes. Activity assays were carried out at 20°C using saturating substrate levels. The assay conditions were as given in Figure 4.25.

retic

°C

tions



but it is apparent that the largest component of variation in specific activity values is differences within, rather than between, allozyme classes.

4.5.7 Heat Stability

The individuals assayed for IDH reaction velocity verses temperature in section 4.5.5 were also tested for heat stability by measuring percentage activity remaining after ten minutes incubation at temperatures from 30° to 60°C. Figure 4.25 indicates that the IDH 80 individual has superior resistance to thermal inactivation, particularly over the range 45° to 57°C. The IDH 100 individual has heat stability characteristics similar to IDH 80 up to about 43°C, but at higher temperatures is denatured at a faster rate. The IDH 114 individual is sensitive to thermal inactivation at all temperatures above 30°C, and, in this range, is denatured at a considerably faster rate than either IDH 100 or IDH 80.

In order to determine the general applicability of the above result 50°C was chosen as a key temperature for testing thermal inactivation, and 50 individuals representing all available IDH phenotypes were assayed for percentage activity after treatment at this temperature. A frequency histogram of these results (Figure 4.26) shows that there are indeed differences in heat stability between electrophoretic phenotypes, but that the observed variation is more complex than the initial thermostability tests indicated. IDH 100 and IDH 80 individuals appear to have a homogeneous distribution of heat stability values

Figure 4.25

Heat stability profiles of the snapper IDH allozymes over the temperature range 30° - 60°C. See Figure 4.16 for the plotting code. Heat stability is measured as percentage enzyme activity remaining after incubation for ten minutes at the designated temperature. Assays were carried out at 20°C using 50 mM tris-maleate pH 7.5, 5 mM manganous chloride, 3.5 mM isocitrate and .2 mM NADP.

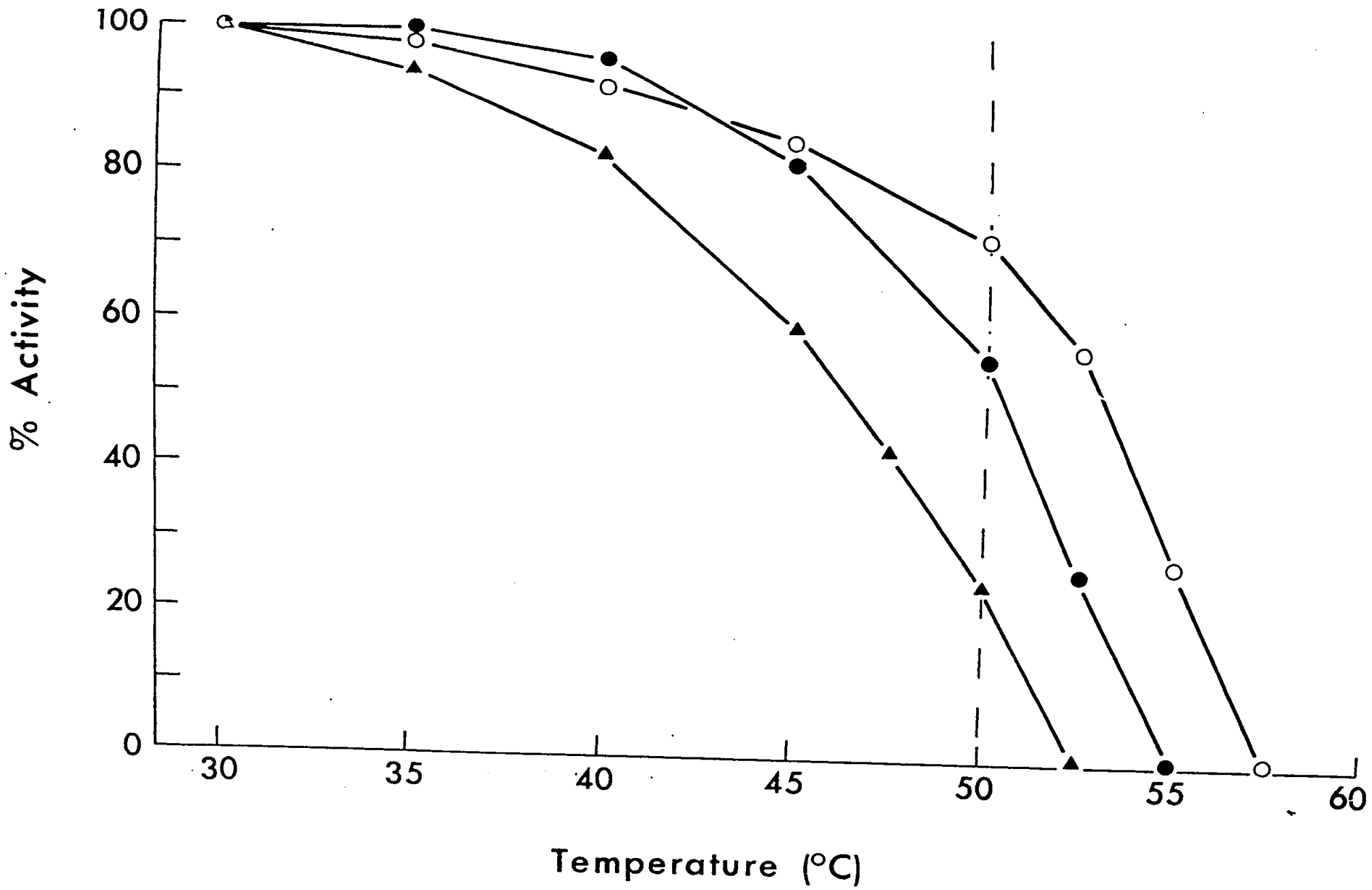

100
80
60
40
20
0
% Activity
30
35
40
45
50
55
60
Temperature (°C)

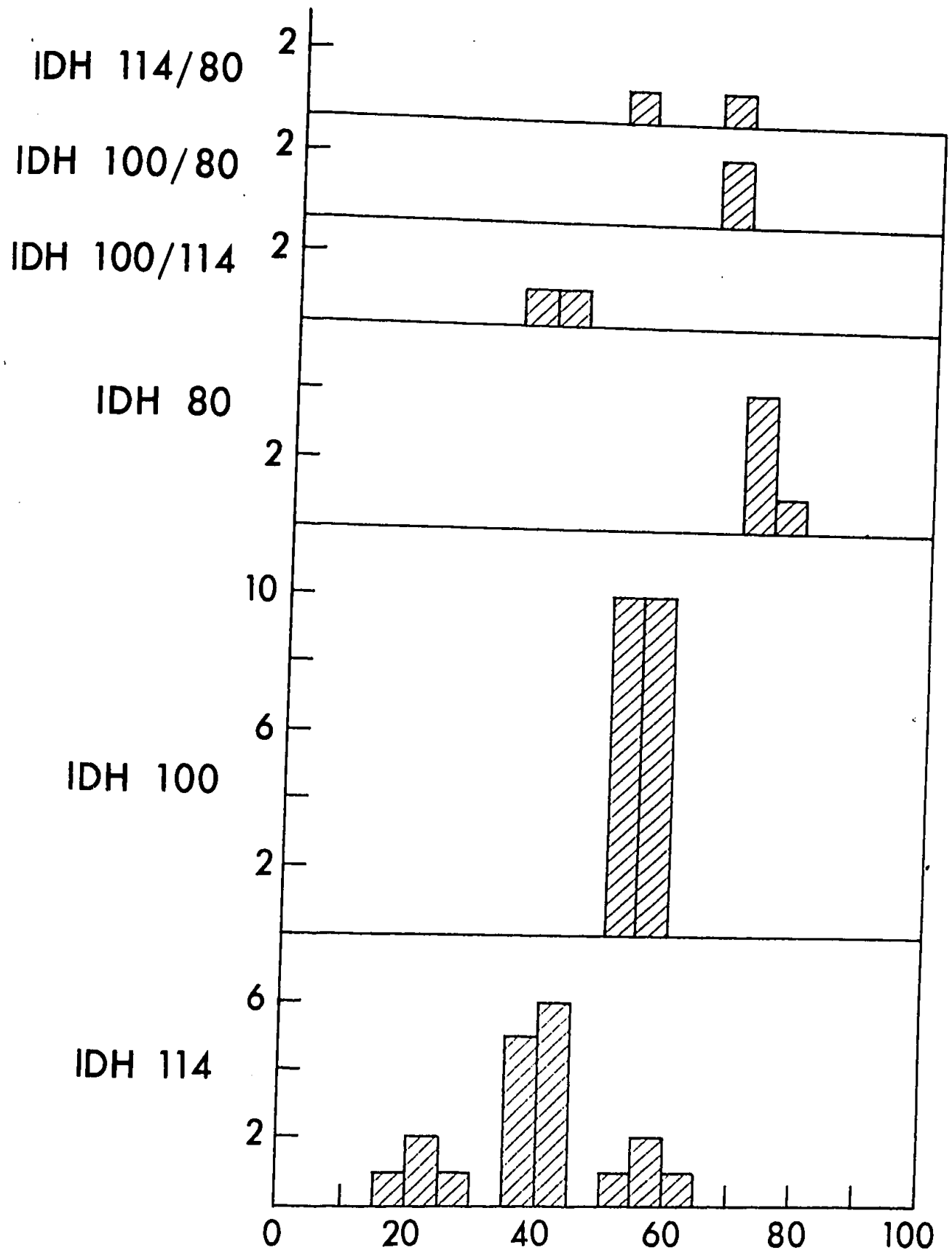
Figure 4.26

Frequency histograms of percentage activity after incubation at 50°C for 50 individuals representing all IDH electrophoretic phenotypes. Assay conditions were as given in Figure 4.25.

after

g all

were

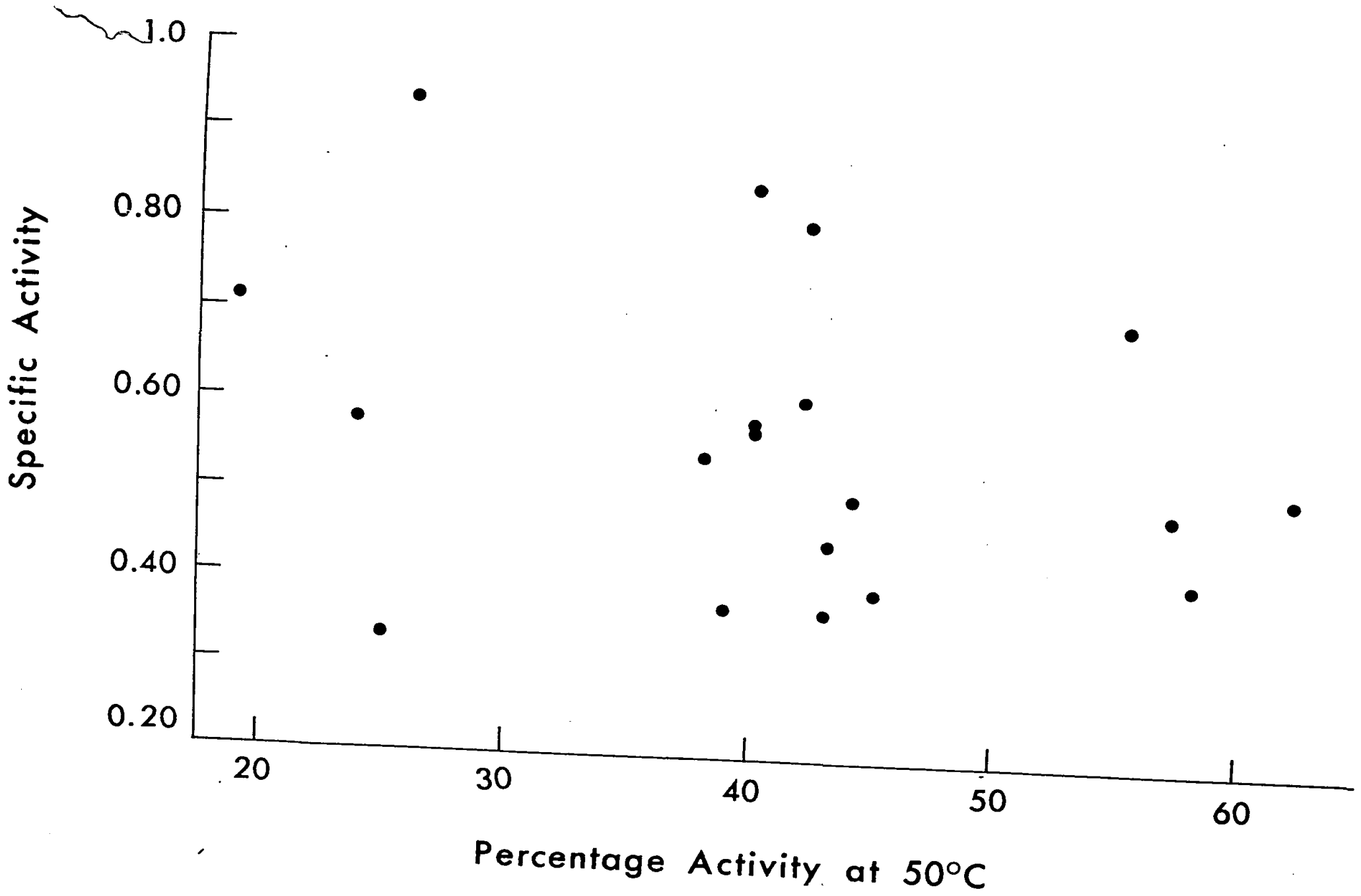


in the ranges 52% to 59% and 72% to 79% respectively. IDH 114 individuals exhibit three heat stability classes - a heat sensitive variant with a percentage activity range at 50°C of 19% to 26%, an intermediate type with a range of 38% to 45%, and a comparatively heat resistant type with a range of 55% to 62%. This last class has heat stability characteristics very similar to those of IDH 100. A scatter diagram of specific activity verses heat stability for the IDH 114 homozygotes (Figure 4.27) shows that there is no discernable relationship between the amount of enzyme in an extract and its resistance to thermal inactivation.

The genetic basis of snapper IDH thermostability variation cannot be unequivocally demonstrated using the present data but, for reasons given in section 4.6, is assumed to be the result of additional allelic variation at the *IDH* structural locus which is not detected by electrophoretic techniques. Under this assumption the three IDH 114 heat stability classes can best be explained as a cryptic two-allele thermostability polymorphism, with the intermediate class representing heat stability heterozygotes, and the comparatively heat resistant class having thermostability properties equivalent to those of IDH 100 homozygotes. Using the nomenclature devised for Australian salmon thermostability variants in section 4.4.6, the three IDH 114 classes will be labelled IDH 114s, IDH 114r/s and IDH 114r, while IDH 100 homozygotes will be labelled IDH 100r. IDH 80 homozygotes have electrophoretic and heat stability characteristics which are distinct from all other classes, and do not need to be relabelled.

Figure 4.27

Scatter diagram of IDH thermostability (percentage activity after treatment at 50°C) verses specific activity for crude enzyme extracts from 19 homozygous IDH 114 individuals.


1.0
0.80
0.60
0.40
0.20
Specific Activity
20
30
40
50
60
Percentage Activity at 50°C

It appears from the position of the IDH r/s class (Figure 4.26) that IDH thermostability heterozygotes have characteristics intermediate to those of their parent allozymes. Tests of individuals representing each of the available electrophoretic heterozygote classes (Figure 4.26) support this conclusion. The two IDH 100/114 individuals assayed fall within the expected percentage activity range (36% - 43%) of IDH 100r/114s heterozygotes, while the IDH 100/80 individuals conform to the expected values (62% - 69%) for IDH 100r/80 heterozygotes. Percentage activity values of 53% and 68% were obtained for the IDH 114/80 individuals, placing them in the IDH 114s/80 (45% - 53%) and IDH 114r/80 (63% - 71%) categories respectively.

It seems, therefore, that heat stability tests have revealed an additional allele within the IDH 114 electrophoretic class. As the IDH 114 individual chosen for velocity verses temperature assays in Figure 4.21 happened by chance to be heat-sensitive (IDH 114s), it is of interest to determine the functional response of IDH 114r individuals to temperature. It is also necessary to revise the geographical distribution of IDH alleles in Australian snapper to include the extra thermostability variation discovered. These results are described in the following sections.

4.5.8 Functional Response of IDH 114r to Temperature

Two IDH 114r individuals were selected for assays of reaction velocity at temperatures between 5° and 40°C.

Results similar to those in Figure 4.20 were obtained for assays at saturating levels of substrate. Assays at K_m substrate levels (Figure 4.28) produced velocity/temperature curves very similar in shape to that of the IDH 100r individual in Figure 4.21. Reaction velocities for the lower curve in Figure 4.27 were converted to percentages of activity at 20°C and compared to similar values for the IDH 100r individual in Figure 4.23. It was found that the regression lines were almost identical in shape (Figure 4.29), suggesting that IDH 114r and IDH 100r allozymes have similar functional responses to temperature fluctuations at low substrate concentrations. This finding also indicates that functional differences between the IDH allozymes are correlated with heat stability rather than electrophoretic variability (see section 4.6 for further discussion).

4.5.9 Revised Geographical Distribution of IDH Variation

All available electrophoretic IDH homozygotes from the snapper sample sets in Table 3.2 were tested for heat stability at 50°C. Electrophoretic heterozygotes were typed for heat stability variation using the post-electrophoresis heat treatment technique described in section 4.3.3. Table 4.3 lists allele frequencies which have been revised to include the extra IDH 114 heat stability variant.

A contingency χ^2 test shows that there is still significant heterogeneity in the overall geographic distribution of IDH alleles (cf. $\chi^2_{42} = 62.13$, $P = .02$),

Figure 4.28

K_m reaction velocities of two IDH 114r individuals when assayed at 5° intervals for temperatures between 5°C and 40°C. Assay conditions were as given in Figure 4.21.

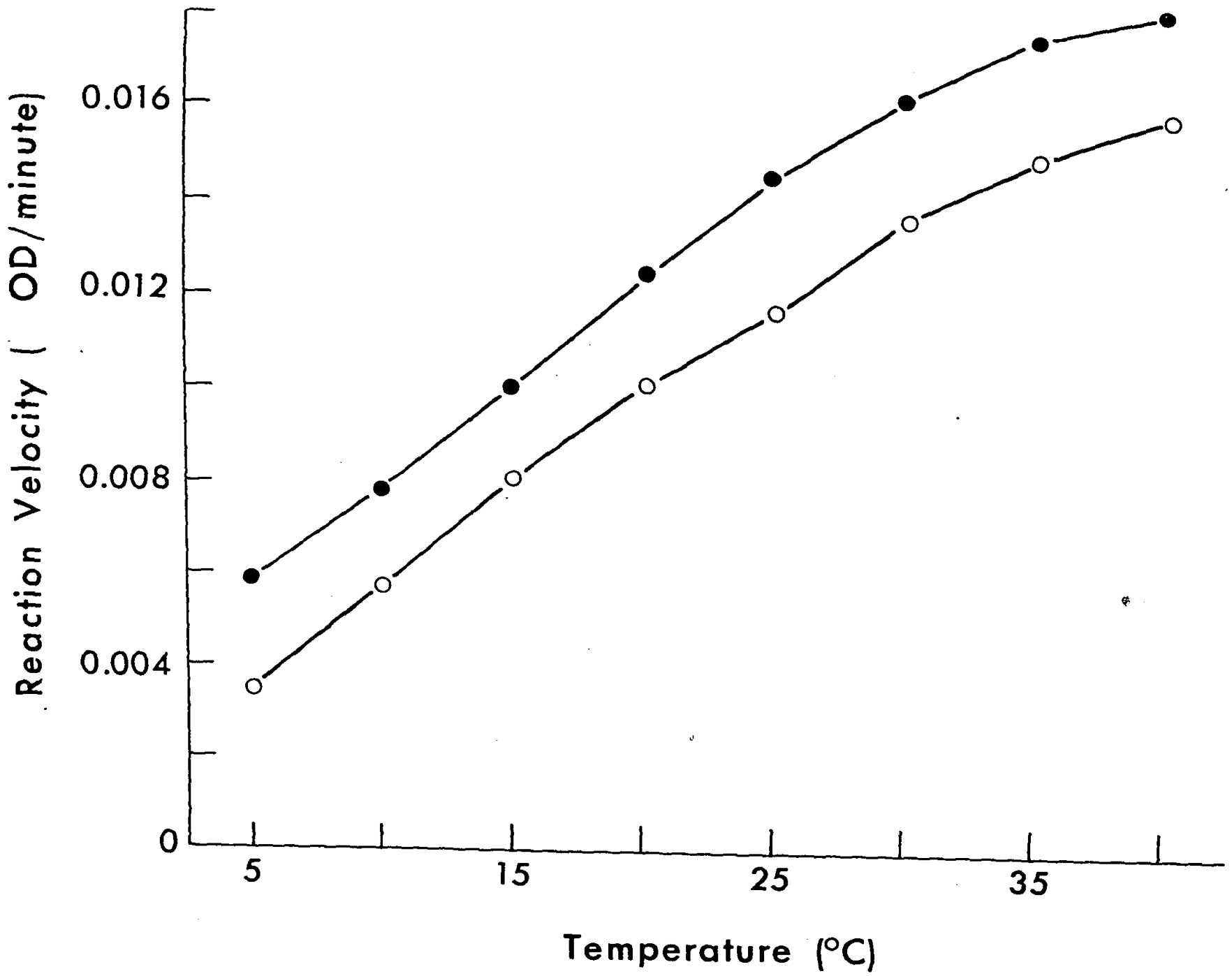

Reaction Velocity (OD/minute)
0.016
0.012
0.008
0.004
0
5
15
25
35
Temperature (°C)

Figure 4.29

Diagramatic representation of the functional responses of IDH 100r and IDH 114r allozymes to temperature fluctuations. ● = the IDH 100r individual from Figure 4.23, and O = the IDH 114r individual represented by the lower regression line in Figure 4.28. Reaction velocities have been converted to percentages of activity at 20°C in order to reduce biases arising from quantitative differences between crude enzyme extracts.

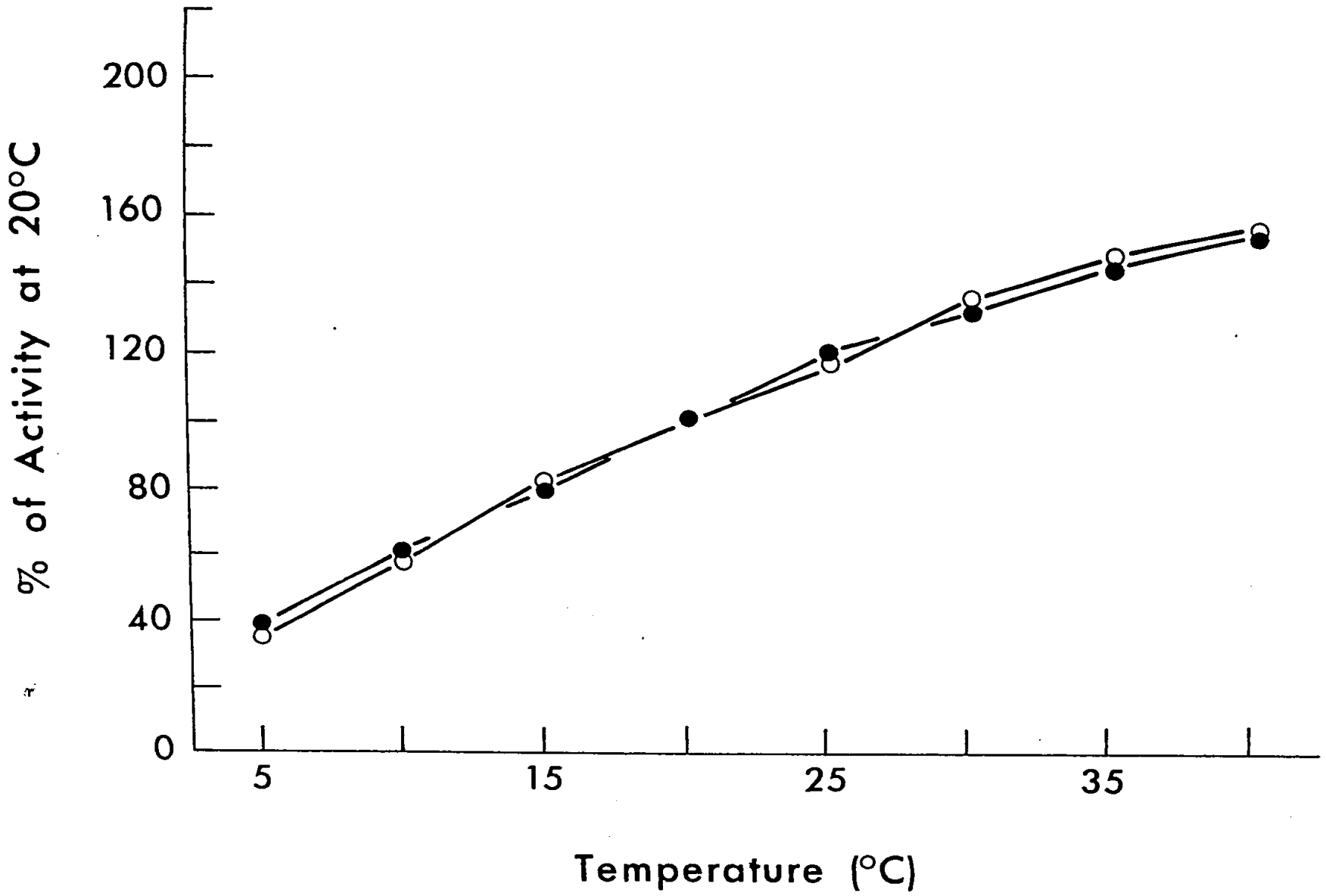

% of Activity at 20°C
200
160
120
80
40
0
5
15
25
35
Temperature (°C)

Table 4.3

Geographical distribution of IDH electrophoretic and heat stability allele frequencies in Australian snapper. See Table 3.2 for sample code.

Sample	2n	Allele				
		IDH^{114s}	IDH^{114r}	IDH^{100r}	IDH^{80}	Other
1	106	.028	.057	.849	.066	-
2	110	.018	.082	.800	.064	.036
3	66	.030	.091	.834	.030	.015
4	58	.034	.052	.828	.034	.052
5	66	.061	.045	.773	.106	.015
6	124	.089	.065	.733	.105	.008
7	128	.055	.063	.758	.124	-
8	74	.054	.149	.702	.095	-
9	114	.105	.114	.684	.097	-
10	112	.080	.116	.724	.080	-
11	66	.076	.091	.772	.061	-
12	140	.064	.114	.714	.108	-
13	40	.050	.050	.750	.150	-
14	36	.056	.167	.555	.222	-
15	120	.092	.150	.626	.124	.008

with clinal variation of IDH^{100r} frequencies (c.f. χ^2_{14} = 32.58, P = .005) being the major component of variance. The distribution of IDH^{80} is unaltered, and as was found in section 3.5.4, is homogeneous (cf. χ^2_{14} = 20.25, P = .13). Individual tests of IDH^{114s} (cf. χ^2_{14} = 15.68, P = .36) and IDH^{114r} (cf. χ^2_{14} = 19.73, P = .14) indicate that these alleles also have homogeneous distributions, contrary to the clinal distribution of electrophoretic allele IDH^{114} found in section 3.5.4.

If the snapper *IDH* data are rearranged to consist only of the heat stability alleles IDH^s, IDH^r and IDH^{80} (ie. IDH^{114r} and IDH^{100r} are combined and phenotypes involving IDH^{124}, IDH^{111} and IDH^{70} excluded) all significant geographical heterogeneity disappears. The combined data are homogeneous (cf. χ^2_{28} = 36.45, P = .15), as is an individual test of IDH^r (cf. χ^2_{14} = 20.26, P = .13). These results suggest that variation in snapper IDH heat stability characersitics is not associated with the clinal distribution of electrophoretic variants, and that thermostability allele frequencies are homogeneous over the range of Australian snapper and over the four-year period of this study.

The distribution of *IDH* heat stability genotypes was analysed using a Smith's H test of the deviation of observed numbers of heterozygotes from Hardy-Weinberg expectations (see section 3.4.3). Table 4.4 shows that all of the snapper sample sets and the combined data conform to Hardy-Weinberg expectations, within the limit of sampling error. This result supports the conclusion that IDH thermostability variants have a reasonably stable

Table 4.4

Smith's H estimates (with approximate 95% confidence intervals) of deviations from Hardy-Weinberg equilibrium of the snapper IDH thermostability genotypes. See Table 3.2 for the sample code.

Sample	Smith's H Value	Approximate 95% Confidence Intervals
1	-.0082	-.0317 .0154
2	-.0061	-.0264 .0143
3	-.0028	-.0228 .0173
4	-.0037	-.0277 .0204
5	-.0181	-.0612 .0249
6	.0286	-.0113 .0684
7	.0316	-.0054 .0686
8	.0068	-.0351 .0487
9	.0135	-.0294 .0563
10	-.0068	-.0430 .0294
11	.0137	-.0276 .0554
12	.0002	-.0339 .0343
13	.0145	-.0580 .0869
14	-.0163	-.1123 .0796
15	-.0160	-.0612 .0293
Combined Data	.0035	-.0067 .0137

stability

distribution throughout the range of Australian snapper.

4.6 Discussion

4.6.1 The Genetic Basis of Thermostability Variation

A number of heat stability classes were detected in both the salmon ME and snapper IDH enzymes assayed in this study. Similar studies in recent years have detected thermostability variants in a variety of enzymes from *Drosophila* (Bernstein et al., 1973; Singh et al., 1974, 1975, 1976; Thorig et al., 1975; Milkman, 1976b; Sampsell, 1977), from various species of gastropod and bivalve molluscs (Wilkins et al., 1978), and from the marine mussel *Guekensia demissa* (Gosling, 1979).

There are three possible explanations for thermostability variants within a single enzyme system:-

a) The variation is non-genetic, i.e. the heat stability of the enzyme in a given crude extract is a function of enzyme concentration and/or total protein rather than of genetically determined structural properties of the enzyme molecule. This possibility is not considered likely for the ME and IDH enzymes studied, because Figures 4.15 and 4.27 show no correlation between specific activity and heat stability of individual crude extracts. Comparisons between total protein content and heat stability of the same crude extracts also show no evidence of correlation.

b) The thermostability variants are phenotypic products of two or more alleles at the structural locus

under examination. This hypothesis cannot be unequivocally tested without data on the amino acid sequences of heat stability variants, or without breeding experiments, but can be inferred by eliminating other possible explanations.

c) The heat stability characteristics of an enzyme are influenced by the products of a second 'modifying' locus (see section 4.1.4). Under these circumstances heat stability variation could be due either to the presence/absence of the modifying protein, or to a polymorphism at the modifying locus. For example Rawls and Lucchesi (1974) have detected and mapped a modifying gene locus which alters the heat stability of NADP-IDH in *Drosophila melanogaster*.

Both salmon ME and snapper IDH thermostability variants have been assumed to be the result of allelic polymorphism at the structural locus, mainly for convenience of data analyses. Alternatives (b) and (c) above remain as equally possible explanations of variation in the ME system, but there is some circumstantial evidence to suggest that the IDH data is best explained by alternative (b).

Firstly, the detection of heat stability variation after electrophoretic separation of proteins (see sections 4.3.3 and 4.5.7) creates difficulties for the 'modifyer' hypothesis, because the modifying protein would need to be strongly bound to the enzyme for such a complex to survive extract preparation and the electrophoretic procedure. Secondly, if modifying proteins are affecting the heat stability characteristics of IDH extracts, then a

complicated pattern of linkage disequilibrium must be invoked to explain why IDH 100 and IDH 80 homozygotes have only one class of heat stability each, while IDH 114 individuals fall into three classes. Thirdly, if the IDH 114 heat stability variation is due to a two-allele polymorphism at the modifying locus, then only two classes of IDH 114 heat stability should be observed. The data in Figure 4.26 show three classes of heat stability, with the intermediate class apparently representing a heterozygous phenotype.

It seems, therefore, that alternative (c) can be largely discredited, and the simplest explanation of the IDH thermostability variation is that there is an additional allele at the *IDH* locus which is not detected by electrophoretic techniques.

4.6.2 Origin of *IDH* Allelic Variation

Figure 4.30 gives a diagramatic representation of the relationship between IDH electrophoretic and heat stability variants, and outlines the most parsimonious explanation of the derivation of the observed allelic array. IDH^{100} is the common allele in samples of both Australian and New Zealand snapper, and is the sole detected *IDH* allele in the northern hemisphere red sea bream (see chapter 5). Assuming that similar relationships exist between IDH electrophoretic and heat stability variants in New Zealand snapper and red sea bream, it seems likely that IDH^{100r} is the ancestral allele by virtue of its common possession by all members of the genus *Chrysophrys*.

Figure 4.30

A. Diagramatic representation of relationships between the IDH electrophoretic and heat stability variants.

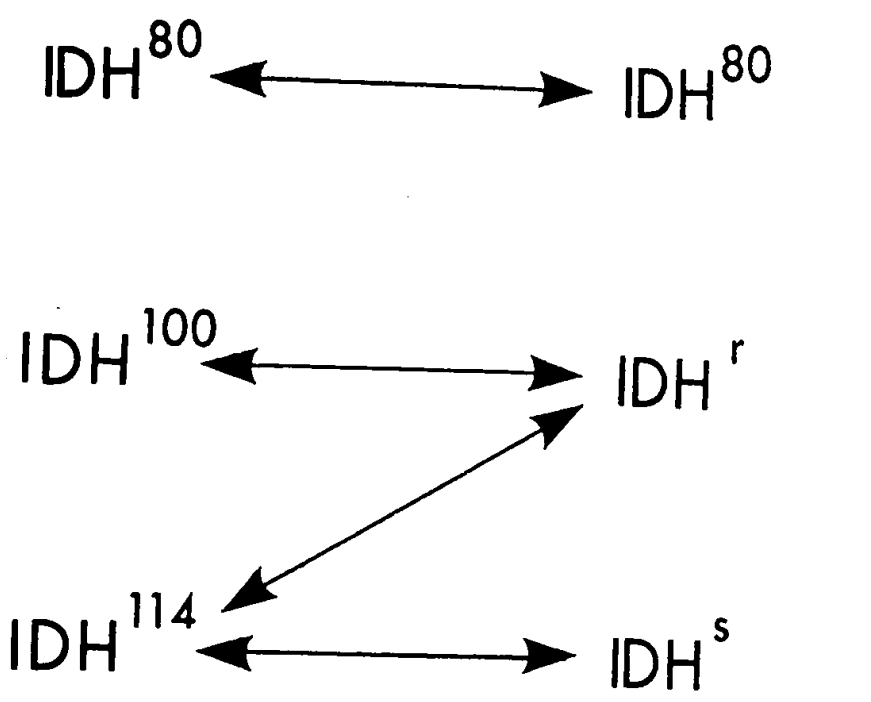
B. The most parsimonious explanation of the derivation of the Australian snapper IDH allelic array.

ips

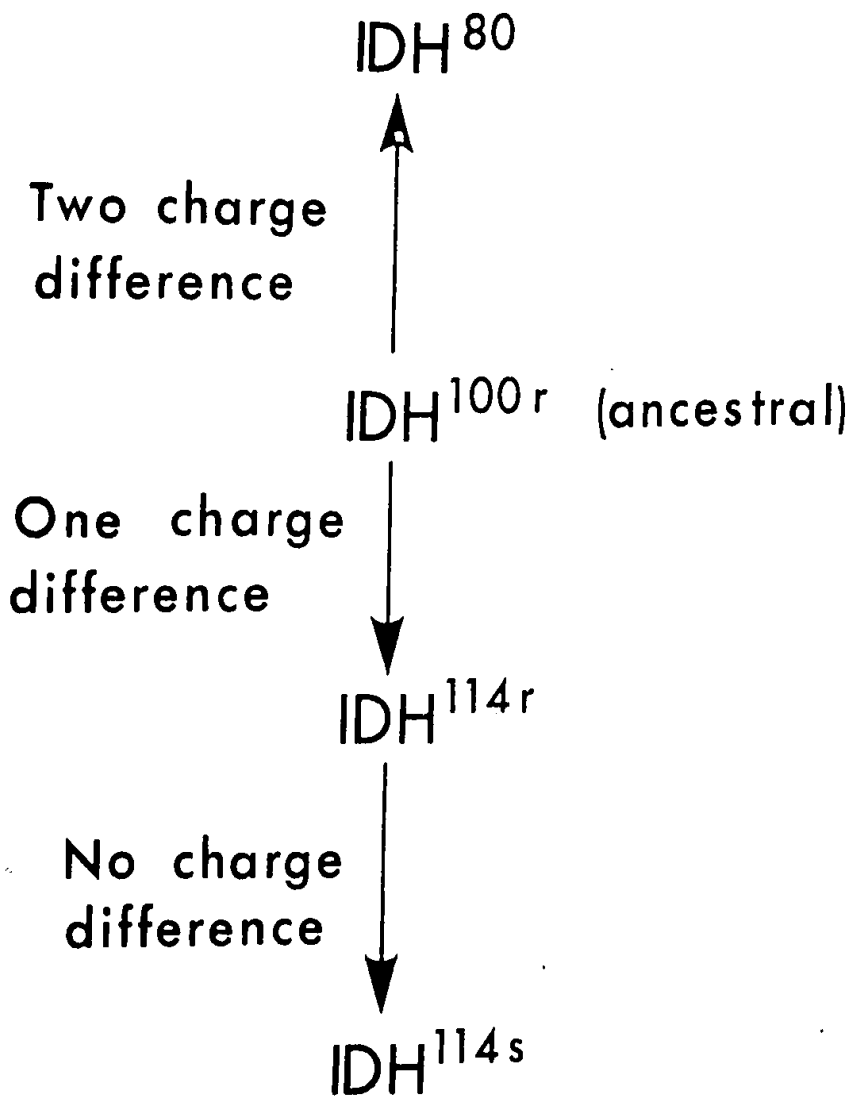
y variants.

array.

A



B



Electrophoretic patterns (Plate 3.5) suggest that IDH^{80} differs from IDH^{100r} by at least two charge differences. It is possible that the substitution(s) which produced altered electrophoretic mobility in IDH^{80} also produced its unique heat stability characteristics, but this hypothesis cannot be tested given the present data. By contrast it is clear that one or more amino acid substitutions giving rise to the electrophoretic allele IDH^{114} are independant of the processes giving rise to IDH^{s} from IDH^{r}. The simplest explanation for the origin of this allelic array is that IDH^{110r} gave rise to IDH^{114r} via a substitution that produced a single nett surface charge difference, and IDH^{114r} then gave rise to IDH^{114s} by means of one or more substitutions which did not produce a surface charge difference.

The distinct origins of electrophoretic and heat stability variation in at least some of the snapper *IDH* alleles make it easier to understand the incongruence of these two factors when they are correlated with functional differences between allozymes in response to temperature. This insight is also important in helping to explain the observed distribution of *IDH* alleles over the geographical range of Australian snapper.

4.6.3 Distribution of Snapper *IDH* Alleles

Of the combined array of *IDH* electrophoretic and heat stability alleles, IDH^{100r} displays a unidirectional decline in frequency in snapper samples taken from east to west around the Australian coastline. IDH^{80}, IDH^{114r} and

IDH^{114s} frequencies are all homogeneous within the limits of sampling error (section 4.5.9). Rearrangement of the *IDH* variation into purely electrophoretic classes indicates that both *IDH*^{100} and *IDH*^{114} have clinal distributions, while *IDH*^{80} is unaffected. When only heat stability variation is considered *IDH*^{r}, *IDH*^{s} and *IDH*^{80} all have homogeneous distributions throughout the range of Australian snapper.

Table 4.5 lists maximum and minimum surface temperatures recorded in various coastal waters around southern Australia in recent years. These data clearly show a decline in temperature with increasing latitude. In view of the different functional responses of the IDH thermostability allozymes to temperature (sections 4.5.5 and 4.5.8) it seems reasonable to predict that *IDH*^{80} should be found with increasing frequency in snapper populations from lower latitudes, while *IDH*^{s} should be more frequent in populations found in higher latitudes. *IDH*^{r} has uniform and intermediate rate compensation properties in response to temperature, and as a 'eurytolerant' allozyme would be expected to maintain a dominant distribution in all snapper populations.

Observed distributions of IDH thermostability allozymes do not fully support these predictions. *IDH*^{r} is the common allele in all sample sets, but *IDH*^{s} and *IDH*^{80} maintain homogeneous distributions despite changing thermal regimes. There are a number of possible reasons for this apparent anomaly:-

a) Surface temperature measurements are not an adequate representation of the thermal regime occupied

Table 4.5

Maximum and minimum surface temperatures recorded in the last 20 years from coastal waters in various localities around southern Australia. These data have been compiled from Vaux (1970), Gorshkov (1976), and from unpublished recordings provided by C.S.I.R.O. Division of Fisheries and Oceanography. The localities are listed in geographical order from the eastern to the western extremes of the range of Australian snapper.

Locality	Surface Water Temperature (°C.)	
	Minimum	Maximum
Fraser Island (Qld.)	20°	27°
Brisbane (Qld.)	19°	27°
Coffs Harbour (N.S.W.)	18°	25°
Sydney (N.S.W.)	15°	25°
Ulladulla (N.S.W.)	14°	23°
Eden (N.S.W.)	13°	22°
St. Helens (Tas.)	11°	20°
Port Phillip Bay (Vic.)	12°	23°
Port Macdonnell (S.A.)	13°	19°
Kangaroo Island (S.A.)	13°	22°
Lower Spencer Gulf (S.A.)	15°	22°
Upper Spencer Gulf (S.A.)	12°	26°
Streaky Bay (S.A.)	15°	20°
Albany (W.A.)	15°	21°
Cockburn Sound (W.A.)	17°	24°
Geraldton (W.A.)	18°	25°
Shark Bay (W.A.)	19°	27°
Exmouth (W.A.)	22°	28°

by the fishes. Snapper are a demersal species, and in deeper water may occupy a part of the water column with thermal characteristics very much different to those at the surface. It is also possible that snapper can behaviourally thermoregulate their environment, and are maintaining an apparently stable IDH polymorphism by actively selecting temperatures most suited to their genotype. Hall et al. (1978) have demonstrated active selection of preferred temperatures - even after acclimation to a different temperature - in the white perch *Morone americana*. This finding lends some weight to the above argument, but no further comment can be made until similar studies have been carried out on snapper.

b) Temperature, acting as a selective agent at the *IDH* locus, is not strong enough to significantly affect the overall fitness of individuals, and hence the distribution of alleles in response to a temperature gradient. The observed distribution may instead be the result of a stronger selective agent acting on the *IDH* alleles (e.g. salinity, dissolved oxygen content of the water, type and availability of food resources, or varying depth/pressure), or of non-random association with other loci at which selective agents are operating.

c) Selective differences between the *IDH* thermostability alleles strongly influence fitness from time to time, and a stable polymorphism is maintained by partial overdominance in a thermal environment which fluctuates more throughout the water column than the surface data would indicate. Under this scheme IDH^{80} and IDH^{S} would persist mainly in the heterozygous state, and

would be at an advantage compared to *IDH*^{r} only when extreme temperatures were encountered (see section 3.1.1 for discussion of this concept).

All of the above possibilities involve some measure of selection acting directly or indirectly on the *IDH* heat stability alleles. Another alternative is that the heat stability variants are selectively 'neutral' with respect to each other, and the homogeneous allele distributions are a product of stochastic processes. This alternative poses two problems, however. First, it ignores the evidence of the kinetic studies, which show that there are potentially selective functional differences between the IDH allozymes. Second, if the homogeneous thermostability allele distribution is a product of chance events, it is difficult to explain the heterogeneity in two of the *IDH* electrophoretic alleles other than by invoking selective forces. Bernstein et al. (1973) have proposed that at loci where a large array of alleles are detected it is likely that some will be maintained by selection and some by stochastic processes. This appears to be the case at the snapper *IDH* locus, but the question is which alleles are obeying which forces?

It may be argued that *IDH*^{100} and *IDH*^{114} have clinal distributions as a selective response to an unknown environmental gradient - and that the snapper *EST* and *ADH* polymorphisms exhibit similar responses. However clinal variation at these loci may also be attributed to the presence of transient alleles which arose in localised areas and are spreading throughout the species' range by stochastic processes.

It is clear that the snapper *IDH* polymorphism is more complex than was initially expected, and the question of maintenance of the polymorphism cannot be resolved given the data presently available. This unsatisfactory situation is often arrived at in similar studies of natural populations. Laboratory perturbation experiments with convenient organisms might validate postulated mechanistic relationships between allozymes and their environment by measuring selective coefficients, but this procedure was not feasible for the present material. However, the IDH kinetic data clearly indicate potentially selective functional differences in response to temperature, and a close correspondence of these differences with heat stability variation. Therefore the simplest explanation of the observed distribution of snapper IDH variation is that the thermostability alleles are being maintained by balancing selection (see section 3.1.1) according to their functional properties in response to temperature, while the electrophoretic variation is either responding to a different environmental parameter, or is in the process of spreading randomly throughout the range of Australian snapper.

4.6.4 Species Differences in Salmon ME Activity

The results of apparent K_m estimations (section 4.4.4) and specific activity measurements (section 4.4.7) for eastern and western salmon indicate that in western salmon a comparative increase in the catalytic efficiency of the ME system has been achieved by modifying the

enzyme molecule to obtain better substrate-binding abilities under physiological substrate conditions, thus reducing the amount of enzyme required to give the desired reaction rate. There may be some inherent dangers in adjusting K_m values without a corresponding alteration in catalytic capacity of the enzyme (see section 4.1.3), but it appears that in this case the advantages of greater efficiency at low substrate concentrations have outweighed a possible diminution of the enzyme's ability to control the flux of metabolites through the reaction pathway.

The reason for this divergence in strategies between the two closely related salmon species is not presently known, although it may be speculated that, as the two species have become ecologically most distinct in their dietry requirements, the different ME strategies are part of a general reorganisation of metabolic pathways in order to more fully utilise different food types.

4.6.5 Allozyme Function and Adaptive Strategies

Having obtained some information on allozyme function in response to temperature in Australian salmon and snapper, it is of some interest to examine the biochemical strategies of adaptation implied by these results, and to see whether or not these strategies fit in with previously described hypotheses (see chapter 3) concerning genetic variation and general ecological strategies of adaptation in the two groups.

The kinetic data on ME indicate that all salmon

individuals have reaction rates with a real and uniform ability to compensate for temperature fluctuations throughout the known range of the animal (10° - 25°C). An enzyme with such properties can be considered 'eurythermal', i.e. able to cope equally well with any temperature change within the physiological tolerance limits of the organism. Such a strategy for temperature compensation is consistent with the general adaptive strategy of a highly mobile pelagic fish species, which perceives its environment as fine-grained and unpredictably variable, and in which alleles that code for phenotypic plasticity would be at a selective advantage.

The snapper IDH data indicate a combination of strategies, with the eurythermal IDH^{r} predominant, but with the added presence of two rarer 'specialist' alleles - IDH^{s} and IDH^{80} - which are better suited to the task of temperature compensation, but only in narrow portions of the species' biological temperature range. The snapper's preferred biochemical strategy for IDH is consistent with a species which, while still reasonably mobile, is predominantly sedentary and demersal, and which is likely to perceive its environment as more coarse-grained but temporally more stable than that of Australian salmon. Under these circumstances it would be desirable to have arrays of specialist alleles to more fully exploit the environmental heterogeneity.

While the above results substantially support previously constructed hypotheses (see section 3.1.3), these conclusions must be treated with caution for two

major reasons. Firstly, only one enzyme system per species was assayed for kinetic data, and the results obtained may prove to be atypical when other loci are examined. This shortcoming of the experimental scheme was mentioned in section 4.1.5, and it is obviously desirable to characterise the catalytic properties of as many enzymes as possible to obtain a more comprehensive picture of the types of biochemical strategies adopted by a given species.

Secondly, both the salmon ME and snapper IDH systems contain some allozymes which do not exhibit unique functional responses to temperature fluctu ations. The products of four electrophoretic ME alleles in eastern salmon, and at least two heat stability alleles in western salmon, all respond in similar fashion to varying temperature. It is possible that at least some of the salmon ME allozymes are functional 'specialists' when subjected to fluctuations of an as-yet-undetected environmental parameter. This alternative is not consistent with the proposed strategy of phenotypic plasticity for Australian salmon. The conflict cannot be resolved unless the ME electrophoretic and heat stability alleles are assumed to be selectively neutral under all environmental conditions, and are therefore not contributing to the overall fitness of individual organisms.

In summary, if we accept the hypothesis of Bernstein et al. (1973) that both neutral and selected variants are likely to be found in large arrays of alleles, it is important to identify and characterise allelic products

which are undergoing selective maintenance, as this is likely to provide vital information concerning the interaction of organisms and their environments. The difficulties encountered in trying to demonstrate the presence of selectively maintained functional differences between salmon ME and snapper IDH allozymes do not detract from the considerable potential of enzyme biochemical studies for investigating the mechanistic links between the genetic attributes of organisms and their environment-mediated ecological attributes.

Chapter 5

Taxonomy and Evolutionary Relationships

5.1 Genus *Arripis*

5.1.1 Introduction

The genus *Arripis* is the sole member of the percoid family Arripidae, and is endemic to the temperate waters of the Australia-New Zealand region. To date it has been generally accepted that there are only two species in the genus - *Arripis trutta* (Australian 'salmon' or kahawai) and *A. georgianus* (ruff, tommy ruff, or Australian herring).

The taxonomic arrangement of the genus has been complicated, however, by the discovery of two apparently distinct breeding populations of *A. trutta* in Australian waters (Fairbridge, 1951; Malcolm, 1959; and see Chapter 2). Whitley (1951) erected a new subspecific name, *A.t. esper*, for the western population, and designated the eastern population as *A.t. marginata* after a description published by Cuvier and Valenciennes (1828, p.53). The validity of this revision is questionable, as will be shown in the following sections. Fairbridge (1951) and Malcolm (1966a) presented morphological evidence to suggest that salmon in Lord Howe Island and New Zealand waters were also distinct from the Australian populations. However, Malcolm (1961, 1966a) concluded that:-

> *"Since it has yet to be shown that the New Zealand, the Lord Howe Island, and the eastern subspecies stocks each represent separate subspecies, the name* Arripis trutta trutta *should be accepted for the entire Tasman Sea complex."*

Malcolm (1959) also stated that:-

> *"The* (eastern and western salmon) *populations probably represent true biological species, but until direct evidence of their reproductive isolation is obtained, it is best to consider them as subspecies."*

In an attempt to resolve some of the taxonomic confusion described above, electrophoretic data have been obtained from specimens of eastern and western salmon, New Zealand kahawai and ruff to determine the extent and nature of genetic divergence between these taxa. It was also envisaged that the electrophoretic data would provide some insight into the evolutionary relationships within the genus *Arripis*, and help to explain the current distribution of its members.

5.1.2 Materials and Methods

Liver tissue samples were obtained from 580 western salmon and 83 eastern salmon (see Table 3.1), from 20 New Zealand kahawai (collected by staff of the Fisheries Research Division, Wellington), and from 20 ruff (collected in Spencer Gulf, S.A.). Attempts to collect salmon samples from Lord Howe Island, Norfolk Island and

the Kermadec Islands (N.Z.) have so far been unsuccessful.

Collected liver tissue was frozen in liquid nitrogen and stored at -70°C in the laboratory. Samples were surveyed electrophoretically for mobility differences in 22 enzymatic proteins (as listed in section 3.3.3). The electrophoretic methods used were those described in section 3.3.

5.1.3 Results

The products of 27 presumed genetic loci were scored for allelic variation in the four *Arripis* taxa (Table 5.1).

A variety of genetic distance measurements have been described in the literature, any of which can be used to provide information on systematic and evolutionary relationships between taxa (see sections 3.4.5 and 3.5.7). Two of the more commonly used indices have been chosen for this study. These are Nei's genetic distance (D) - an estimate of the accumulated number of codon substitutions per locus since the time of divergence of two taxa (Nei, 1972), and Rogers' coefficient of similarity (S) - a measure of the mean geometric distance between allele frequency estimates over all loci surveyed (Rogers, 1972). Rogers' S values have been found to be similar to values derived from most other commonly used genetic distance indices (Hedrick, 1975; Schmitt, 1977), allowing comparison with a variety of values obtained for taxa at different stages of evolutionary divergence. Nei's D values are useful in that they can be combined with estimates of mean mutation rates at structural loci to

Table 5.1

Allele frequencies at 27 loci in four taxa of the genus *Arrtyia*. See section 3.3.3 for the full names of the enzymes. Abbreviations and allele codes follow the format described in section 3.3.4.

Locus	Allele	Western Salmon	Eastern Salmon	N.I. Kahawai	Puff
EST	107	.01	-	-	-
	101	.93	.97	.85	-
	99	.03	.03	.15	.913
	89	.03	-	-	.071
IDH	108	-	.01	-	-
	101	1.0	.99	1.0	1.1
ADA_2	104	-	-	.05	-
	101	1.0	1.0	.75	1.1
PGM	101	.99	1.0	1.0	1.1
	99	.01	-	-	-
ADH	101	.99	1.0	1.0	.95
	91	-	-	-	.05
	47	.01	-	-	-
GPI	101	-	-	-	1.1
	101	1.0	1.0	.95	-
	99	-	-	.05	-
PGM	106	-	-	.025	-
	101	1.0	1.0	.975	1.0
SOD	101	-	1.0	1.0	1.0
	100	1.0	-	-	-
6PGD	101	1.0	1.0	1.0	1.0
GPDH	101	.99	1.0	1.0	1.0
	94	.01	-	-	-
MDH_S	101	1.0	1.0	1.0	1.0
MDH_M	101	1.0	1.0	1.0	1.0
LDH_A	101	1.0	1.0	1.0	-
LDH_B	101	1.0	-	-	-
	97	-	1.0	1.0	1.0
AAT_S	101	1.0	1.0	1.0	.925
	98	-	-	-	.075

genus

nzymes.

bed in

Ruff
-
-
.925
.075
-
1.0
-
1.0
1.0
-
.95
.05
-
1.0
-
-
-
1.0
1.0
-
1.0
1.0
-
1.0
1.0
-
-
1.0
.825
.175

Locus	Allele	Western Salmon	Eastern Salmon	N.Z. kahawai	Ruff
AAT_M	*100*	1.0	1.0	1.0	1.0
SDH	*124*	-	-	-	1.0
	100	1.0	.70	.525	-
	72	-	.30	.475	-
GAPD	*100*	1.0	1.0	1.0	-
	50	-	-	-	1.0
ME	*112*	-	.03	-	-
	100	1.0	.53	.325	1.0
	93	-	.06	-	-
	75	-	.33	.65	-
	68	-	.05	.025	-
GDA_2	*111*	-	-	-	.975
	100	1.0	.98	1.0	.025
	84	-	.02	-	-
XO	*113*	-	-	.025	.975
	100	1.0	1.0	.975	.025
AK_1	*100*	.99	1.0	1.0	-
	83	.01	-	-	1.0
AK_2	*100*	1.0	1.0	1.0	-
	80	-	-	-	1.0
GPD	*112*	-	-	-	.75
	100	1.0	1.0	1.0	.25
MPI	*108*	-	.02	-	-
	100	.99	.75	1.0	1.0
	82	.01	.23	-	-
$G6PD_1$	*100*	1.0	1.0	1.0	1.0
$G6PD_2$	*100*	1.0	1.0	1.0	1.0

produce a crude time scale for the divergence of two taxa. Such estimates of time-since-divergence can then be compared to estimates inferred from other sources, such as the fossil record, the current distribution of taxa, and past geological and climatic events. Nei's D values have also been made statistically more meaningful by the development of methods for estimating sampling variance (Nei and Roychoudhury, 1974). It must be remembered, however, that time-since-divergence estimates are based on the assumption of neutral mutations and a constant rate of protein evolution. If selective agents are contributing to, or deleting, some of the genetic variation that occurs between taxa, then divergence estimates will be biased. This potential problem should be borne in mind when interpreting time-since-divergence estimates.

Table 5.2 summarises the genetic distance data obtained from electrophoretic survey of 27 loci in the four *Arripis* taxa, and Figure 5.1 is a dendrogram of the relationships between these taxa based on the Rogers' S values in Table 5.2. It can be seen that the ruff, *A. georgianus*, is quite distinct from the other taxa, yielding an average value of $\bar{S}$ = .527. This amount of divergence is considered sufficient to distinguish fish taxa at the species level (Ayala, 1975; Avise, 1976). More significantly, there are fixed allele differences at 5 loci (see Table 5.1) between ruff and eastern and western salmon, despite overlaps in the geographic distributions of these taxa. This apparent lack of gene flow between sympatric taxa is strong evidence that *A. georgianus* is a species separate from the other taxa.

Table 5.2

Matrix of Rogers' S (bottom) and Nei's D values ± Standard Error (top) for pairwise comparisons of taxa within the genus *Arripis*. These values are derived from allele frequency estimates at 27 loci (Table 5.1).

W.S
D values
E.S
ons of
N.Z
s are
R.
27 loci

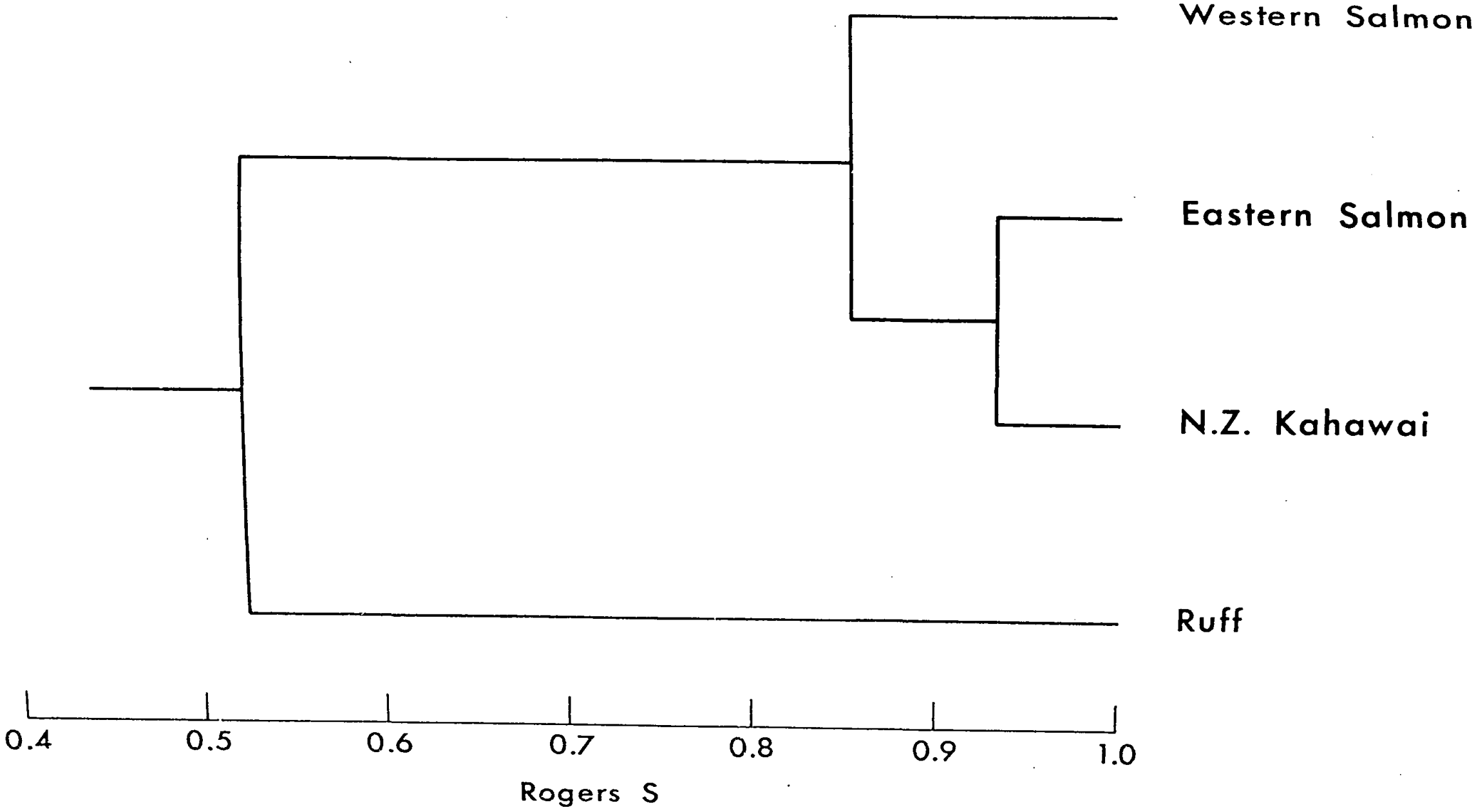
	Western Salmon	Eastern Salmon	N.Z. kahawai	Ruff
W.S.		.092 ± .011	.154 ± .015	.752 ± .039
E.S.	.878		.031 ± .005	.558 ± .031
N.Z.K.	.834	.933		.514 ± .030
R.	.463	.548	.570	

Figure 5.1

A dendogram of relationships between *Arripis* taxa based on the Rogers S values in Table 5.2. Mean S values for each dichotomy were calculated using the unweighted pair group average method of Sneath and Sokal (1973). Mean S values (and ranges) are as follows:-

Eastern salmon	V.	N.Z. Kahawai	=	.933
Western salmon	V.	Eastern salmon & Kahawai	=	.856 (.834 - .878)
Ruff	V.	The rest	=	.527 (.463 - .570)

Western Salmon



Morphologically ruff are also quite distinct from the other arripid taxa, as they grow to a much smaller maximum length (rarely more than 35 cm L.C.F.) compared to salmon (commonly 50 cm L.C.F. and upwards), and have distinctive coloration and markings (see Scott et al., 1974).

Western salmon has an average similarity value of $\bar{S}$ = .856 compared to eastern salmon and kahawai. This result alone would not be considered sufficient to establish a dichotomy at the specific level, particularly as these three taxa are virtually indistinguishable on the basis of external morphology. However western salmon is fixed for different alleles at the LDH_B and *SOD* loci compared to eastern salmon and kahawai. Eastern and western salmon have overlapping distributions in Victorian and Tasmanian waters, and are even found together in the same schools. This lack of gene flow between sympatric taxa is strong evidence in favour of the specific status of western salmon. In addition, Malcolm (1966a) has shown that the number of gill rakers in the first gill arch is a diagnostic character for this dichotomy, with western salmon having counts ranging from 25 to 31, while eastern salmon and kahawai counts range from 33 to 40.

Eastern salmon and kahawai are genetically the most similar of the arripid taxa, with a similarity value ($\bar{S}$ = .933) in the range expected for conspecific local populations (Ayala, 1975; Avise, 1976). These two taxa can, however, be considered as isolated breeding populations, as eastern salmon were found to be carrying

rare alleles not found in the kahawai sample, and vice versa. The best examples of this are at the ADA_2 locus, where ADA_2^{114} is present in kahawai at a frequency of .25 but absent in eastern salmon, and at the *MPI* locus, where MPI^{82} is present in eastern salmon at a frequency of .23 but absent in kahawai.

Malcolm (1966a) has shown that morphologically kahawai, eastern salmon and Lord Howe Island salmon are very similar, with characters such as dorsal fin ray counts and girth scale counts yielding slightly different mean values, but broadly overlapping distributions. Gill raker counts for Lord Howe Island salmon were substantially higher (range 37 - 42), but still overlapped the distributions for eastern salmon and kahawai.

It seems, therefore, that neither the morphological nor the genetic data support separation of Tasman Sea salmon stocks at a level higher than local populations. Eastern salmon and kahawai share common alleles at all 27 loci examined, and no diagnostic morphological features were detected. This situation may be altered when genetic studies are carried out on salmon from Lord Howe Island, Norfolk Island and the Kermadec Islands, but the above conclusions will be accepted for the present.

The electrophoretic data have also been used to compare overall levels of genetic variability between the *Arripis* taxa. Mean heterozygosities per locus per individual (see section 3.4.2) were calculated from the data in Table 5.1:-

Taxon	n	H	S.E.
Western salmon	580	.008	± .0003
Eastern salmon	83	.049	± .0045
N.Z. kahawai	20	.066	± .0110
Ruff	20	.041	± .0176

These figures indicate a much lower level of heterozygosity in western salmon compared to the other taxa. However, heterozygosity differences can be due to differences in allele frequencies, as well as differences in overall variability (Soulé, 1976), and it is perhaps more informative in this case to compare levels of polymorphism.

Table 5.1 shows that western salmon are polymorphic ($P \leq .95$) at only one out of 27 loci, compared to 3/27 for eastern salmon, 5/27 for kahawai and 4/27 for ruff. A similar number of electrophoretic alleles was detected in each taxon, but the sample size of western salmon is approximately seven times that of the next biggest sample, and it seems reasonable to conclude that western salmon would exhibit proportionately fewer alleles if similar numbers of specimens from other *Arripis* taxa were surveyed.

It seems, therefore, that western salmon have a much lower level of genetic variability - at least in the 27 loci surveyed - than do other *Arripis* taxa. The possible evolutionary implications of this result will be discussed in the next section.

5.1.4 Evolutionary Relationships within the Genus *Arripis*

Arripis species are members of the order Perciformes, a higher teleost taxon thought to have originated during the last great proliferation of fishes in the late Cretaceous - early Tertiary era (Gosline, 1971). This period coincides with estimates for the time of separation of the Australian and New Zealand plates, and the formation of the Tasman Sea (50 to 80 million years B.P. - Hayes and Ringis, 1972). There seems to be little doubt that the family Arripidae, because of its endemicity, originated in the Australasian region. However, there has been some debate recently over whether the distribution of the family in both Australian and New Zealand waters has resulted from geographical vicariance during the break-up of Gondwanaland, or from a subsequent trans-Tasman dispersal event (McDowall, 1978). There is no fossil evidence to support either hypothesis, but the greater diversity of *Arripis* species in Australian waters, the lack of any endemic New Zealand species, and the great mobility of all arripid fishes, suggest that Australia was the centre of origin of the genus, with subsequent dispersal of *A. trutta* to New Zealand.

The evolution and distribution of arripid species in Australian waters appears to have been primarily influenced by geomorphological events in the Bass Strait region. Both western salmon and ruff are distributed along the southern coastline from southwest Western Australia to Victoria and Tasmania, with their numbers rapidly declining towards the eastern end of Bass Strait.

Eastern salmon are found around southern N.S.W., Victoria and Tasmania, with their distribution terminating at the western end of Bass Strait.

Bass Strait is thought to have been dry land on at least three separate occasions since the beginning of the Quaternary (approximately 1.8 million years B.P.), due to the lowering of sea levels during glacial periods (Laseron, 1969; Keast et al., 1972; Bowler et al., 1976; Ollier, 1977). The most recent submergence occured at the end of the last glacial period - between 15,000 and 10,000 years B.P.

Reconstructions of sea surface temperatures during the height of the last glacial period (about 18,000 years B.P.) indicate that the waters around Bass Strait and southern Tasmania were about 4°C colder, on average, than they are today (CLIMAP, 1976). It is possible that these colder waters prevented movement of arripid species around southern Tasmania during glacial periods and, with the emergence of a land bridge across Bass Strait, the ancestral arripid stock was divided into two parts and underwent allopatric divergence. Speciation would then follow, either before or shortly after the establishment of a secondary contact zone in Bass Strait at the end of the glacial period.

The earliest speciation event in the genus *Arripis* appears to have been that which gave rise to ruff (*A. georgianus*) and to a common ancestor of the two Australian salmon species. The average genetic distance between ruff and the other arripid taxa is $\bar{D} = .608$. Assuming an average mutation rate of 10^7 years per substitution for

electrophoretically detected variation in structural loci (Nei, 1975), a crude estimate for the time since divergence of ruff and the salmon species is:-

$$t = 10^7 \times \bar{D} = 6.1 \text{ million years B.P. (range 5.8 - 6.7)}$$

If this estimate is valid, it places the divergence of ruff and salmon in the late Tertiary, long before the Quaternary glacial periods. Information about the geomorphology of the Bass Strait region in the Tertiary is scare, and no explanation of the events leading to the divergence and speciation of ruff and salmon will be attempted.

The time-since-divergence estimate for western salmon verses eastern salmon is:-

$$t = 10^7 \times .092 = 920{,}000 \text{ years (range .80 - 1.14 m.y.)}$$

This estimate suggests that ancestors of the western and eastern salmon species were isolated by a Bass Strait land bridge during one of the earlier Quaternary glacial periods, and that reproductive isolation was achieved prior to any subsequent glacial periods. The nature of the isolating mechanism is unknown, as there is no obvious reason why gene flow should not occur between extant populations of western and eastern salmon.

The low level of genetic variability in western salmon, compared to the other arripid taxa, suggests that this species has undergone a severe bottleneck in population numbers, either during or since its divergence

from eastern salmon. Nei et al. (1975) have shown that reduction in average heterozygosity during a bottleneck process depends not only on the effective size of the founding population, but also on the rate of population growth after the bottleneck. They also found that in general the time taken for a population to re-establish its equilibrium H value after bottleneck is roughly equivalent to the reciprocal of the mutation rate (assumed to be of the order of 10 million years in this case). Lack of information on the size of the western salmon founding population makes it impossible to estimate how many generations have passed since the bottleneck event. However, it is possible that the present low H value for western salmon was arrived at due to a founder effect associated with the allopatric divergence of the species some 920,000 years ago, and the species has not had sufficient time to accumulate new variation.

Of course there are other possible explanations for the lower level of genetic variability in western salmon. These explanations involve the selective maintenance of different numbers of alleles, and/or differences in population structure between taxa, leading to stochastic variation in the distribution of alleles. However, the similarities in life history characteristics and ecology of the *Arripis* taxa (see Chapter 2) render these alternatives less attractive than the bottleneck hypothesis.

The time-since-divergence estimate for eastern salmon verses kahawai is:-

$$t = 10^7 \times .031 = 310{,}000 \text{ years B.P.}$$

This estimate suggests that gene flow effectively ceased between eastern Australian and New Zealand salmon populations in the middle to late Pleistocene. Whether this date marks the first colonisation of New Zealand waters by migrating Australian fishes, or whether exchange of genes between already established populations ceased, is not clear given the present data.

5.1.5 Taxonomic Revision

It has been established that the genus *Arripis* consists of three recognised species; the ruff, *A. georgianus*; the western salmon - previously recognised only as the subspecies *A. trutta esper*; and a Tasman Sea complex which includes eastern salmon, kahawai, and possibly salmon from Lord Howe Island, Norfolk Island and the Kermadec Islands. *A. georgianus* remains as a valid species name, but there is some doubt about the validity of the name *A. esper* for western salmon. Whitley (1951) erected this name when it was first recognised that eastern and western salmon were distinct taxa. However, there are a number of earlier names applied to specimens from Victoria and Tasmania which may have priority (see Malcolm, 1966a), because it is possible that these specimens are juvenile western salmon. The type material for these names will have to be examined before this problem can be solved.

The Tasman Sea species includes the nominal

subspecies *A. trutta marginata* (eastern salmon) and *A. trutta trutta* (kahawai). Available morphological and biochemical data indicate that division at the subspecies level is not justified, and that the Tasman Sea complex should be provisionally regarded as a single species. According to the International Code for Zoological Nomenclature, the oldest available name for this species is *Arripis trutta* (Forster, MS in Bloch and Schneider, 1801) based on a specimen from Queen Charlotte Sound, New Zealand. A more detailed treatment of nomenclature and synonymies in the genus *Arripis* will be undertaken in subsequent publication of these results.

5.2 Genus *Chrysophrys*

5.2.1 Introduction

The genus *Chrysophrys* is a member of the large percoid family Sparidae, also known as porgies or sea breams. The name "snapper" is in fact a misnomer, as the *Chrysophrys* species are only distantly related to the true snappers of the family Lutjanidae. Sparids are generally sedentary demersal fishes, and are found mostly in the shallow tropical and sub-tropical waters of the world. The genus *Chrysophrys* is unusual in two respects:-

(a) It is a temperate genus in a mainly tropical family.

(b) It has an antitropical distribution, with representatives in the Australasian region and in

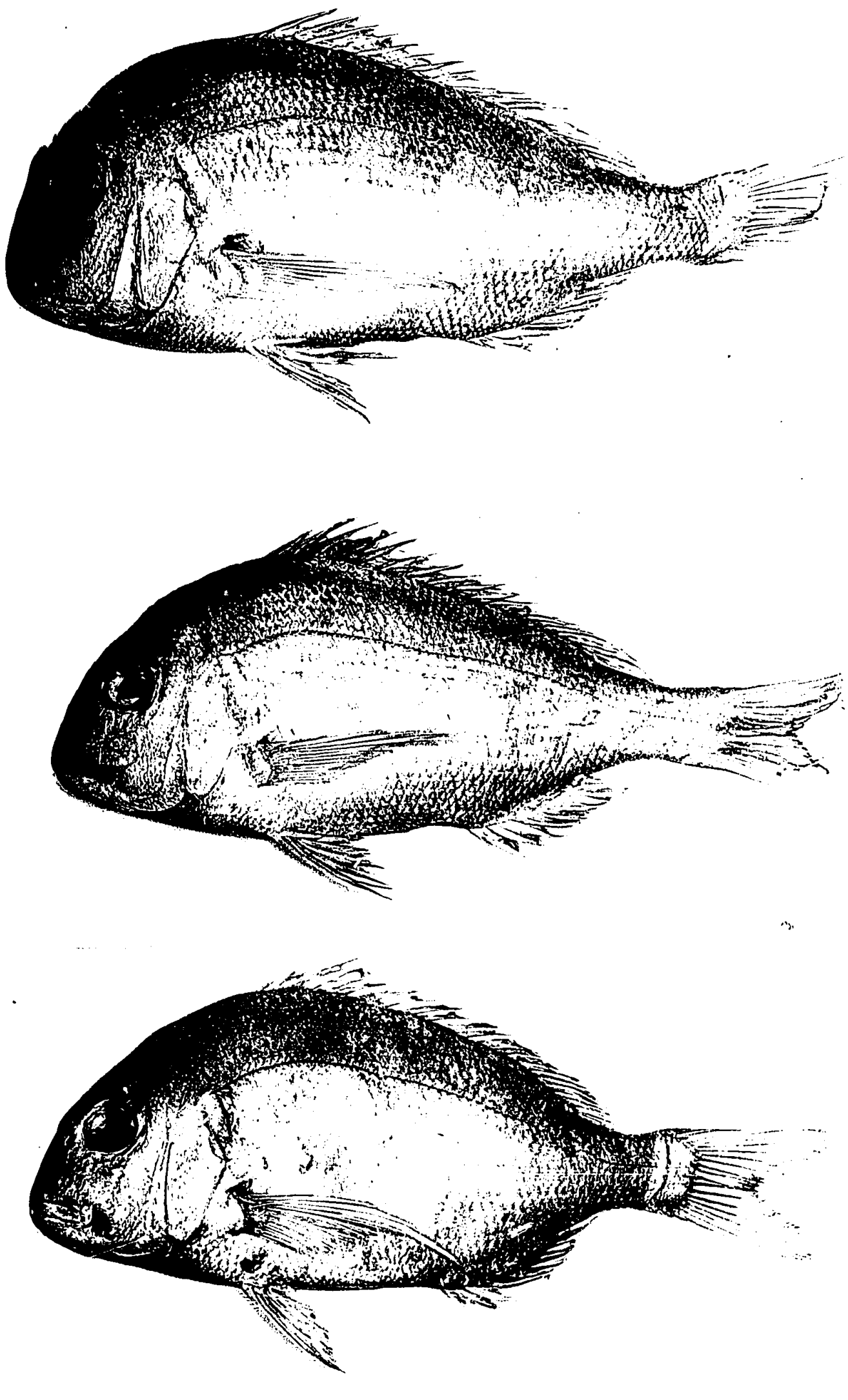
the South China Sea, but not in the intervening tropical waters. The morphological similarity between northern and southern hemisphere forms is striking (Plate 5.1).

Until recently members of the genus *Chrysophrys* had generally been included in the Atlantic and Mediterranean genus *Pagrus* (Cuvier and Valenciennes, 1830; Günther, 1859; Tenison-Woods, 1882; Akazaki, 1962; Manooch et al., 1976) or *Pagrosomus* (e.g. Stead, 1908; Fowler, 1928). Yasuda and Mizuguchi (1969b) showed that the Indo-Pacific *Pagrus* species were osteologically distinct from *Pagrus* species found in American and African waters, establishing the validity of the genus *Chrysophrys*.

Within the genus there is some confusion concerning the number of species present. Whitley (1931) nominated Western Australian snapper as *C. unicolor*, eastern Australian snapper as *C. guttulatus*, New Zealand snapper as *C. auratus* and red sea bream from the South China Sea as *C. major* (see Figures 2.4 and 2.5 for distribution of the genus). Fowler (1933) considered that the entire genus consisted of a single species, *C. auratus*, with no differentiation in the Australasian region, and the South China Sea representatives being merely a "geographical variety" of *C. auratus*. Yasuda and Mizuguchi (1969a) described a number of osteological and morphometric characters which they considered were sufficient to distinguish *C. auratus*, *C. unicolor* and *C. major* as separate species. There is some question about the usefulness of the characters measured in that study, and thus the validity of the conclusions reached. This

Plate 8.1

Frozen specimens of Australian snapper (top), New Zealand snapper (middle) and Taiwanese red sea bream (bottom), illustrating the morphological similarity of northern and southern hemisphere forms of *Chrysophrys*.



pectoral fin length (PFL), pelvic fin length (PelFL), length of the longest dorsal spine (LDS), head length (HL), snout length (SnL), post-orbital head length (PHL), eye width or orbit length (OL), upper jaw length (UJL), mandible length (ML), inter-orbital width (IW), and inter-nare width (INW). The meristic characters were:- number of dorsal spines (DS), dorsal soft rays (DR), anal spines (AS), anal rays (AR), pelvic spines (PelS), pelvic rays (PelR), pectoral rays (PR), branchiostegals (BST), scales in the lateral line (LL), scales in transverse series counted from the origin of the dorsal fin (ALL), and scales in transverse series counted from the front of the anal vent (BLL).

Proportions were obtained for each of the morphometric characters by comparing them against standard length or head length. The distributions of coefficients for each of the sample sets were then tested statistically for significant variation.

5.2.3 Electrophoretic Data

The products of 26 presumed genetic loci were scored for allelic variation in the Australian, New Zealand and Taiwanese samples (see Tables 3.11 and 5.3). Estimates of mean heterozygosity per locus per individual for the Australian sample sets are as given in Table 3.12. The estimate for the New Zealand sample is .051 ± .0046, and for the red sea bream sample is .075 ± .0063. These results are contrary to the findings of chapter 3, where the Australian H values of this study were compared to H values of .079 and .084 for New Zealand snapper

Table 5.3

Allele frequencies at 26 loci for Australian and New Zealand snapper and Taiwanese red sea bream. The enzyme and allele codes follow the format described in section 3.3.4. Full names of the enzymes are given in section 3.3.3.

* = see Table 3.11

\# = a rare allele was found only in the Shark Bay Australian snapper sample set. The Variation was detected in the form of a single heterozygous phenotype.

Locus	Allele	Australia	New Zealand	Taiwan
EST	*117*		-	-
	108		.34	.25
	100	*	.62	.55
	95		-	-
	90		.04	.18
	83		-	.02
IDH	*124*		-	-
	114		-	-
	111	*	-	-
	100		.97	1.0
	80		.03	-
	70		-	-
ADA	*124*		-	-
	112	*	-	-
	100		.80	.93
	88		.20	.07
FUM	*120*		-	-
	100	*	1.0	1.0
	84		-	-

Locus	Allele	Australia	New Zealand	Taiwan
ADH	*150*	*	-	.25
	125		-	-
	100		1.0	.57
	70		-	.18
GPI	*120*	*	-	-
	108		.28	-
	100		.72	1.0
	88		-	-
PGM	*117*	*	-	-
	110		-	-
	100		1.0	.88
	91		-	.12
MDH_S	*100*	1.0	1.0	.97
	86	-	-	.03
MDH_M	*100*	1.0	1.0	1.0
ME	*111*	-	.02	-
	100	1.0	.98	-
	89	-	-	1.0
LDH_A	*100*	1.0 (.99)#	1.0	1.0
LDH_B	*100*	-	-	1.0
	0	1.0	1.0	-
SOD	*100*	1.0	1.0	1.0
AK_1	*100*	1.0	1.0	1.0
AAT_S	*140*	-	-	.05
	100	1.0	1.0	.95
AAT_M	*100*	1.0	1.0	1.0
$GAPD_1$	*100*	1.0	1.0	1.0
$GAPD_2$	*100*	1.0	1.0	1.0
XO	*100*	1.0	1.0	-
	95	-	-	1.0
ADH	*115*	-	.03	-
	108	-	-	.98
	100	1.0	.97	-
	94	-	-	.02
MPI	*100*	1.0	1.0	1.0
Gal6PD	*100*	1.0 (.99)#	1.0	1.0
GDA_1	*107*	-	-	1.0
	100	1.0	1.0	-
GDA_2	*100*	1.0	1.0	1.0
6PGD	*114*	-	-	.13
	100	1.0	1.0	.87

(Smith et al., 1978). However, Smith et al. surveyed a number of loci not assayed in this study, and vice versa. The resulting difference in H estimates for New Zealand snapper illustrates how important the selection of loci is in providing a valid basis for comparison of heterozygosity values.

There are no obvious differences between the taxa in numbers of loci polymorphic ($P \leq .95$), with 4/26 in Australian snapper, 3/26 in N.Z. snapper and 5/26 in red sea bream. More alleles were detected in Australian snapper than in the other taxa, but as the sample size for Australian fishes was many times greater, this result is not surprising.

Table 5.4 shows Rogers similarity (S) and Nei genetic distance (D) estimates for pair-wise comparisons of the snapper and red sea bream samples. These estimates are based on the data in Tables 3.11 and 5.3. Although not shown, standard errors have been calculated for each of the Nei D estimates, and in all cases are less than 10% of the D value.

Figure 5.2 is a dendrogram of relationships between the *Chrysophrys* taxa based on the Rogers' S values in Table 5.4. It can be seen that the various Australian snapper samples are very similar genetically, with an average $\bar{S}$ value of .984, and a range of .970 to .996. It has been empirically determined (Ayala, 1975; Avise, 1976) that this is the level of genetic similarity found between conspecific local populations of a wide variety of vertebrates and invertebrates. It has been shown in Chapter 3 that the Australian snapper stock consists of

Table 5.4

Matrix of Rogers' $S_{,}$ (bottom) and Nei's D (top) values for pair-wise comparisons of sample sets of Australian and New Zealand snapper and Taiwanese red sea bream. See Table 3.2 for the Australian snapper sample code.

	Australian Snapper															N.Z.	Taiwan

Australian Snapper

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	N.Z. Snapper	Taiwan Red Sea Bream
1		.001	.001	.001	.001	.001	.001	.001	.002	.001	.002	.002	.002	.004	.004	.007	.240

Australian Snapper

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	N.Z. Snapper	Taiwan Red Sea Bream
1		.001	.001	.001	.001	.001	.001	.001	.002	.001	.002	.002	.002	.004	.004	.007	.240
2	.995		.001	.001	.001	.001	.001	.001	.002	.001	.002	.002	.002	.004	.003	.007	.242
3	.993	.993		.001	.001	.001	.001	.002	.002	.001	.003	.002	.003	.004	.004	.007	.241
4	.988	.989	.990		.001	.001	.001	.002	.003	.002	.004	.003	.004	.006	.005	.008	.245
5	.987	.989	.991	.986		.001	.001	.002	.002	.001	.003	.002	.003	.004	.003	.008	.244
6	.988	.989	.990	.988	.988		.001	.001	.002	.001	.003	.002	.003	.004	.003	.009	.245
7	.991	.991	.990	.988	.989	.996		.001	.002	.001	.003	.002	.003	.004	.003	.009	.244
8	.987	.988	.986	.979	.983	.987	.987		.001	.001	.001	.001	.002	.002	.001	.009	.242
9	.982	.985	.983	.977	.983	.985	.984	.987		.001	.001	.001	.002	.001	.001	.007	.243
10	.985	.988	.984	.980	.983	.986	.986	.991	.992		.001	.001	.001	.002	.001	.007	.242
11	.979	.982	.979	.973	.978	.979	.979	.986	.986	.988		.001	.001	.003	.001	.006	.239
12	.981	.984	.982	.978	.982	.984	.984	.985	.992	.991	.988		.001	.001	.001	.006	.242
13	.980	.981	.979	.973	.980	.980	.982	.986	.982	.985	.987	.985		.003	.001	.006	.240
14	.974	.975	.976	.970	.975	.980	.978	.980	.988	.984	.979	.986	.979		.001	.009	.247
15	.975	.977	.974	.971	.974	.978	.978	.984	.984	.986	.983	.985	.986	.982		.008	.245
NZ	.968	.966	.970	.964	.965	.963	.964	.961	.966	.965	.964	.969	.964	.965	.961		.241
RSB	.760	.758	.758	.751	.757	.752	.754	.759	.760	.760	.767	.761	.765	.755	.758	.760	

figure 5.)

A dendrogram of relationships between *Chrysophrys* taxa based on the Rogers S values in Table 5.4. Mean S values for each dichotomy were calculated using the unweighted pair group average method of Sneath and Sokal (1973). The shaded area represents the range of Rogers S values for comparisons between Australian samples. See Figure 3.2 for the sample code. Mean S values (and ranges) are as follows:-

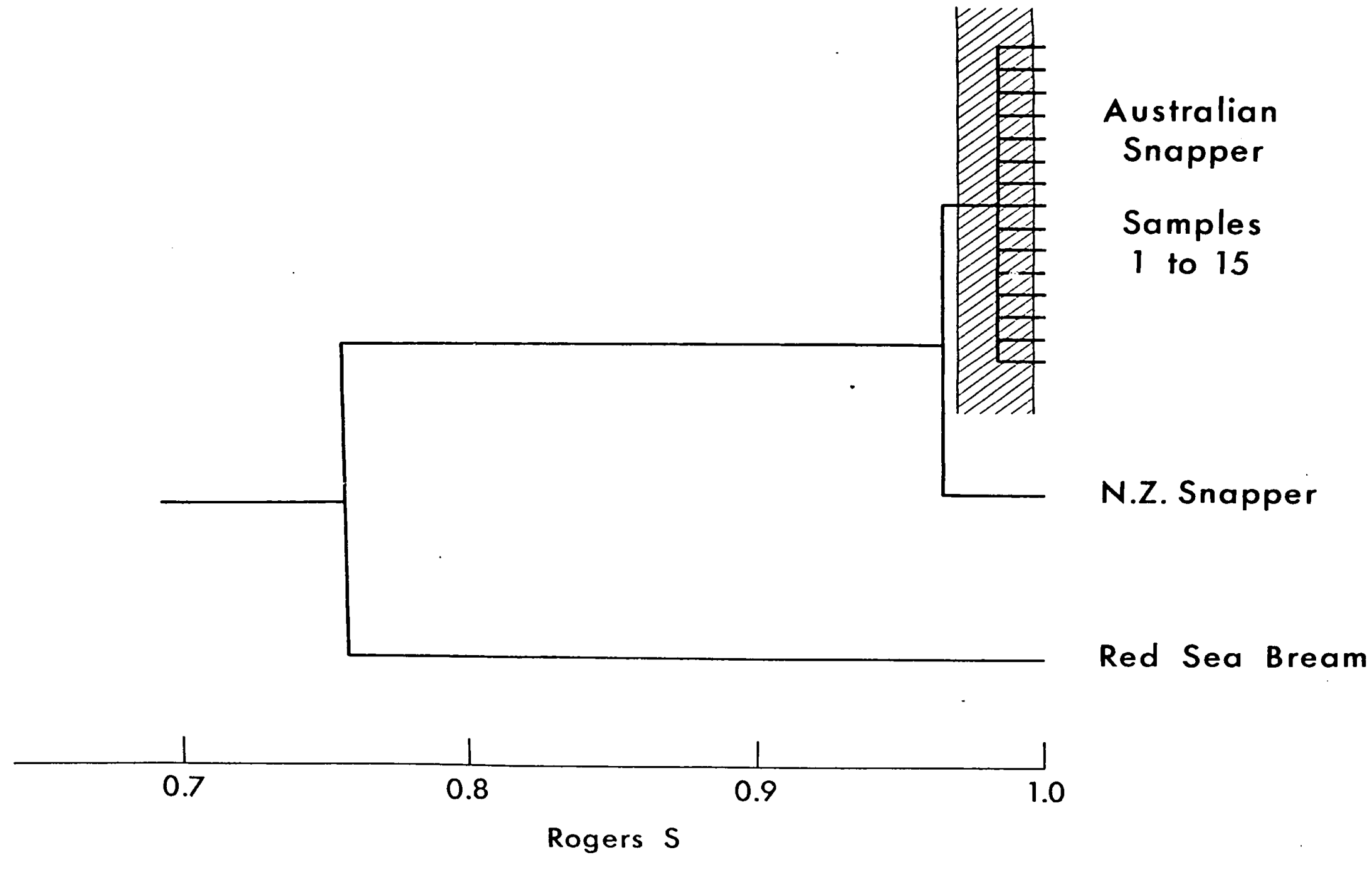
Between Australian Samples = .984 (.970 - .996)

Australian V. N.Z. Samples = .965 (.961 - .970)

Red Sea Bream V. the rest = .758 (.751 - .767)

Australian V. N.Z. Samples = .9[illegible] (.961 - .970)

Red Sea Bream V. The rest = .758 (.751 - .767)



a number of isolated or partially isolated breeding populations. Smith et al. (1978) have shown similar results for New Zealand snapper, with at least three genetically distinct local populations detected, and a Rogers $\bar{S}$ value of .98 for a comparison of sample sets from opposite ends of the North Island. Comprehensive biochemical data are not available for the red sea bream, but Taniguchi and Tashima (1978) found no statistically significant variation in the distribution of a serum esterase polymorphism in red sea bream around the southern coastline of Japan.

Figure 5.2 shows that the Australian and New Zealand sample sets have a mean $\bar{S}$ value of .965, with a range of .961 to .970. This level of similarity is only marginally below that for comparisons between the Australian samples. In addition, Australian and New Zealand snapper exhibit no fixed differences in electrophoretic mobility, and share common alleles at all 26 loci scored (Table 5.3). These results indicate that Australian and New Zealand snapper stocks are genetically distinct only at the level of conspecific local populations. The electrophoretic data do, however, indicate that the Tasman Sea is an effective breeding barrier for snapper. A comparison of the data in Table 5.3, and results from the electrophoretic survey of New Zealand snapper by Smith et al. (1978), reveal the following differences in allele frequency estimates:-

Locus	Frequency of Common Allele	
	Australian Snapper (15 sample sets - 680 fishes)	New Zealand Snapper (12 sample sets - 1399 fishes)
EST	.67 - .96	.47 - .73
IDH	.56 - .85	.95 - .99
ADH	.91 - 1.0	1.0
GPI	.88 - 1.0	.66 - .75
PGM	.95 - 1.0	1.0

The identical electrophoretic mobilities of alleles detected in the two different studies were verified by the exchange of reference phenotypes between laboratories across the Tasman. These results indicate that Australian and New Zealand snapper populations are maintaining distinct allele frequencies at a number of loci, and that gene flow across the Tasman is either very limited or absent. It is also possible that this genetic divergence between Australian and New Zealand snapper is maintained by selective forces in spite of trans-Tasman gene flow. However, migration of snapper across the Tasman seems unlikely in view of the life history characteristics of the species, and the hypothesis of allopatric divergence is preferred.

The northern hemisphere red sea bream is quite distinct from the southern hemisphere members of the genus *Chrysophrys*, with a mean genetic similarity of .758 and a range of .751 to .767. A comparison of this result with estimates of genetic similarity for other fishes (Ayala, 1975) and vertebrates (Avise, 1976), indicates that red sea bream and snapper are somewhere between the subspecies

and the species stage of evolutionary divergence. These northern and southern hemisphere *Chrysophrys* forms are allopatric, and the degree of reproductive isolation between them has not been established. However, there are fixed allelic differences at five out of 26 loci (Table 5.3), and Yasuda and Mizuguchi (1969a) have also described a diagnostic difference in skull morphology between northern and southern hemisphere forms (see next section). I consider this evidence sufficient to separate red sea bream and snapper at the species level. This taxonomic arrangement is in accord with popular usage in recent years.

5.2.4 Morphological Data

Yasuda and Mizuguchi (1969a) claim to have distinguished two southern hemisphere *Chrysophrys* species - *C. auratus* from New Zealand and *C. unicolor* from Western Australia - on the basis of differences in skull morphology and variation in two morphometric characters. While the red sea bream *(C. major)* can be readily distinguished from snapper by the presence of a bulbous swelling at the base of the supraoccipital crest, none of the osteological characters described by Yasuda and Mizuguchi show diagnostic differences between *C. auratus* and *C. unicolor*. Described variation in the cross-sectional shape of the supraoccipital crest is too subtle to be of taxonomic use, and this character is known to be variable between specimens collected from the same locality (W.S. Fairbridge, unpublished data). Apparent

differences in the body length at which the frontal bones fuse in juvenile snapper are also subtle (170 mm in *C. unicolor*, 200 - 250 mm in *C. auratus)* and cannot be considered taxonomically useful at the species level.

Yasuda and Mizuguchi have shown that regressions of head length and predorsal length against body length reveal variation between *C. unicolor* and *C. auratus* samples. It must be pointed out, however, that the range of measurements for the two nominal species overlap in every character examined, including the above two regressions. It may well be that statistically significant morphometric and meristic variation exists between snapper samples from different parts of the Australasian region, but it is also likely that such variation is due to divergence of local populations and/or the effects of different environmental regimes in different areas, rather than species differences (Barlow, 1961; Woodger, 1976).

Table 5.5 lists the mean and range of measurements for 17 morphometric and 11 meristic characters taken from five sample sets of Australian snapper. These data agree closely with that of Yasuda and Mizuguchi (1969a) where the same characters have been examined, and suggest that there is as much character-state overlap between Australian snapper samples as Yasuda and Mizuguchi found between the nominal species *C. auratus* and *C. unicolor*.

Table 5.6 is a summary of pairwise comparisons of the Australian snapper samples, showing in each case the proportion of morphometric and meristic characters found to exhibit significant variation in measurement

Table 5.5

Means and ranges of measurements of 17 morphometric and 11 meristic characters in five sample sets of Australian snapper. The character abbreviations are as described in section 5.2.2.

The samples are:-

Sample	Capture Location	Sample Size	Length Range (SL)cm.
1.	Off Sydney Heads, N.S.W.	33	24 - 46
2.	Port Phillip Bay, Vic.	60	34 - 65
3.	Backstairs Passage, S.A.	58	45 - 73
4.	Upper Spencer Gulf, S.A.	49	21 - 46
5.	Shark Bay, W.A.	45	48 - 60

Character	Mean and Range of Character States				
	1	2	3	4	5
5.	Shark Bay, W.A.		35		34 - 60

Character	Mean and Range of Character States				
	1	2	3	4	5
SL/BD	2.36 (2.17-2.68)	2.58 (2.34-2.86)	2.91 (2.56-3.32)	2.53 (2.23-2.79)	2.58 (2.21-2.89)
SL/DCP	9.18 (8.39-10.30)	9.60 (7.46-10.53)	10.23 (9.05-11.45)	9.21 (8.46-10.00)	9.42 (8.29-10.23)
SL/LCP	4.81 (4.51-5.26)	4.72 (4.11-5.52)	4.50 (4.12-4.87)	4.81 (4.46-5.24)	4.63 (4.15-5.11)
SL/PL	2.36 (2.18-2.54)	2.35 (2.18-2.53)	2.53 (2.30-2.72)	2.44 (2.28-2.61)	2.36 (2.19-2.50)
SL/DB	1.96 (1.82-2.06)	1.99 (1.83-2.15)	2.02 (1.91-2.19)	1.99 (1.88-2.16)	2.12 (1.89-2.26)
SL/AB	5.10 (4.76-5.66)	5.35 (5.00-6.02)	5.52 (5.11-6.19)	5.30 (5.00-5.75)	5.55 (5.19-6.12)
SL/PelFL	4.34 (3.94-5.03)	4.92 (4.46-5.70)	5.12 (4.60-6.48)	4.41 (3.97-4.92)	4.65 (4.24-5.04)
SL/PFL	2.73 (2.49-2.96)	3.01 (2.66-4.25)	3.02 (2.59-3.64)	2.82 (2.53-3.13)	2.82 (2.54-3.06)
SL/LDS	7.08 (6.19-9.44)	8.25 (6.80-11.21)	8.26 (6.74-10.86)	6.89 (6.00-9.12)	7.77 (6.30-10.42)
SL/HL	3.37 (3.10-3.65)	3.44 (3.26-3.74)	3.54 (3.02-3.80)	3.28 (3.00-3.61)	3.38 (3.17-3.54)
HL/SnL	2.19 (2.08-2.32)	2.05 (1.85-2.18)	2.06 (1.92-2.53)	2.21 (2.03-2.40)	2.11 (2.00-2.26)
HL/PHL	2.17 (2.08-2.25)	2.18 (2.07-2.31)	2.12 (2.03-2.44)	2.21 (2.09-2.29)	2.17 (2.06-2.29)
HL/OL	4.37 (3.80-4.82)	5.41 (4.16-6.34)	5.96 (5.07-7.00)	4.08 (3.39-5.31)	4.88 (4.25-5.70)
HL/UJL	2.49 (2.32-2.61)	2.43 (2.28-2.63)	2.39 (2.25-2.81)	2.46 (2.18-2.63)	2.35 (2.24-2.55)
HL/ML	2.40 (2.21-2.67)	2.36 (2.11-2.58)	2.37 (2.23-2.92)	2.42 (2.12-2.53)	2.30 (2.17-2.41)
HL/IW	3.02 (2.77-3.18)	2.85 (2.66-3.12)	3.04 (2.77-3.73)	3.46 (3.05-3.89)	3.16 (2.85-3.50)
HL/INW	5.27 (4.73-5.76)	4.35 (3.60-5.26)	4.90 (4.32-5.53)	5.34 (4.88-6.08)	4.86 (3.89-5.34)
DS	12 (12)	11.97 (11-12)	12 (12)	12 (12)	12 (12)
DR	11 (11)	11.00 (10-12)	11.00 (9-12)	11.03 (11-12)	10.97 (10-11)
AS	3 (3)	3 (3)	3 (3)	3 (3)	3 (3)
AR	9 (9)	9 (9)	8.98 (7-10)	9 (9)	9 (9)
PelS	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)
PelR	5 (5)	5 (5)	5 (5)	5 (5)	5 (5)
PR	15.00 (14-16)	14.89 (14-15)	14.92 (14-16)	14.94 (14-16)	14.91 (14-16)
LL	54.73 (52-58)	54.56 (50-59)	54.58 (50-58)	53.53 (50-56)	54.31 (50-57)
ALL	10.58 (9-12)	10.09 (9-12)	9.77 (9-11)	10.00 (9-11)	10.29 (9-11)
BLL	17.88 (17-19)	17.84 (16.21)	17.02 (16-18)	16.91 (16-18)	18.17 (17-21)
BST	6 (6)	6 (6)	5.98 (5-6)	6 (6)	6 (6)

distributions. The pairwise comparisons are based on specimens whose standard lengths fall within the range common to the two samples being compared. This selection procedure is designed to avoid bias in the data arising from characters which exhibit allometric growth patterns in relation to standard length. Because of differences in the length ranges of some of the snapper sample sets (e.g. 1 and 3, or 3 and 4 - see Table 5.5) a number of pairwise comparisons were omitted through lack of sufficient data for meaningful analysis. The data for each character was analysed orthogonally, with a non-parametric Kruskal-Wallis analysis of variance test (see Seigel, 1956) applied to all sample sets to determine whether or not heterogeneous character-state distributions existed. Where heterogeneity was detected, a non-parametric Mann-Whitney U test (see Seigel, 1956) was used to determine which sample sets were significantly different from each other.

The results in Table 5.6 show that comparisons of snapper samples from across the Australian range reveal significant variation ($P < .05$) in an average of 45% of the 28 characters examined, with morphometric characters contributing a far greater component of variation than meristic characters. This may seem a high figure for morphological divergence between conspecific local populations, but it must be remembered that the relative influence of genetic and environmental factors on the characters examined is unknown, and it may be that the morphological variation is to a large extent environmentally mediated. Closer inspection of the data

Table 5.6

Matrix of data for pairwise comparisons of Australian snapper sample sets, showing the proportion of morphometric and meristic characters found to exhibit significant variation in measurement distributions. Sample codes are given in Table 5.5. P=.05 and P=.01 indicate the level of Type I error designated in each case.

Samples Compared	Morphometric Characters (17)		Meristic Characters (11)		Total Characters (28)	
	P=.05	P=.01	P=.05	P=.01	P=.05	P=.01
1 v 2	.47	.24	.18	.09	.36	.18
1 v 4	.53	.35	.27	.18	.43	.29
1 v 5	.53	.41	0	0	.32	.25
2 v 3	.71	.65	.18	.09	.50	.43
2 v 5	.76	.59	.09	0	.59	.36
3 v 5	.76	.59	.18	.18	.54	.43

in Table 5.5 also shows that in most cases the character-state means for each sample set are comparatively close to one another, and indicates that a number of statistically significant results are due to leptokurtic character-state distribution - possibly an artefact of the measuring procedure. The biological significance of such results is debatable, and their taxonomic value is negligible.

The above results emphasise the point that traditional morphological methods are not always the most useful way of tackling taxonomic problems, and the electrophoretic data illustrate the usefulness of having different techniques available in order to determine the most powerful and productive taxonomic tools.

5.2.5 Evolutionary Relationships within the Genus

The genus *Chrysophrys* is placed in the order Perciformes, a higher teleost taxon thought to have originated during the last great proliferation of fishes in the late Cretaceous - early Tertiary era (Gosline, 1971). Perhaps the most interesting evolutionary aspect of the genus is its antitropical distribution, with morphologically very similar species found in temperate northern and southern Indo-Pacific waters, but not in the intervening tropical seas. There are three possible explanations for this type of distribution:-

a) The genus *Chrysophrys* is polyphyletic, i.e. the northern and southern hemisphere forms arose independantly from different ancestral sparid stocks,

and underwent convergent evolution in response to the requirements of similar niches in northern and southern hemisphere temperate coastal waters. This explanation seems unlikely in view of the high genetic similarity estimate ($\bar{S}$ = .758) for snapper and red sea bream. In order to support this hypothesis one must accept the unlikely possibility that most of the estimated 76% of structural loci with electrophoretically identical products achieved this situation purely by chance.

b) The genus *Chrysophrys* is monophyletic, and the current distribution is a result of fragmentation of a formerly continuous Indo-Pacific distribution - either because of climatic changes, or because of competitive displacement in tropical waters by other sparids or closely related percoid families.

c) The genus *Chrysophrys* arose either in southern or northern hemisphere temperate waters, and has managed to disperse across tropical waters (possibly during an Ice Age, when sea temperatures were generally lower) and establish populations in temperate waters of the opposite hemisphere.

Nei's genetic distance data in Table 5.4 show that red sea bream is distinct from Australian and New Zealand snapper at the level of about one codon substitution per four loci (Nei, 1972; and see section 5.1.3). Assuming an average mutation rate of 10^7 years per substitution for electrophoretically detected variation in structural loci (Nei, 1975), a crude estimate of time since divergence of snapper and red sea bream is:-

$t = 10^7 \times \bar{D} = 2.43$ million years B.P. (range 2.39 - 2.47)

If this estimate is valid, then the dichotomy between snapper and red sea bream originated in the late Tertiary, just before the first Quaternary glacial period, and well after the break up of Gondwanaland and the separation of Australia and New Zealand (50 - 80 million years B.P. - Hayes and Ringis, 1972). This evidence supports the hypothesis that an antitropical distribution of *Chrysophrys* species resulted from a relatively recent dispersal of fishes across the equator, possibly during the first Quaternary glacial period.

It is also interesting to note that red sea bream appear to have higher levels of heterozygosity than Australian or New Zealand snapper ($\bar{H}$ = .075 ± .0063 verses $\bar{H}$ = .045 ± .0016 and .051 ± .0046 - see section 5.2.3). While many explanations are possible (see section 5.1.3), these data may be interpreted to mean that red sea bream are an 'older' species than snapper (i.e. they have had a longer period of time in which to accumulate genetic variation). If this is so, then it is likely that the genus *Chrysophrys* originated in northern Indo-Pacific temperate waters and dispersed south across the equator to occupy temperate coastal waters in Australia and New Zealand.

Table 5.4 indicates that there is little genetic differentiation between Australian and New Zealand snapper, with an average $\bar{D}$ of .008. Based on this value, and a mutation rate of 10^7, an estimated time since divergence for Australian and New Zealand snapper is 80,000 years

B.P. (range 60,000 - 90,000). This seems to be too short a time framework to propose a recent trans-Tasman dispersal event, with subsequent establishment of a new population and build up of genetic variation to the level of the parent population. A more feasible alternative is that snapper were distributed in both Australian and New Zealand waters for many years before 80,000 B.P., but differentiation only began recently when events around this time resulted in the cessation of trans-Tasman gene flow and/or the selective maintenance of different allele distributions.

5.2.6 Taxonomic Revision

It has been established from biochemical and morphological evidence that the genus *Chrysophrys* consists of only two species, the northern hemisphere red sea bream, *C. major*, and a single snapper species distributed throughout the Australasian region. Previous reports of the presence of three southern hemisphere species - *C. auratus*, *C. unicolor* and *C. guttulatus* - have not been supported by the available biochemical and morphological evidence. According to the Rules of the International Code for Zoological Nomenclature, the oldest available name for the southern hemisphere snapper species is *Chrysophrys auratus* (Bloch and Schneider, 1801), based on a description of a specimen from New Zealand.

A more detailed treatment of nomenclature and synonymies will be undertaken in subsequent publication of these results.

Chapter 6

Concluding Remarks

Much of the discussion of results in relation to the hypotheses tested has been conducted in earlier chapters. It is nevertheless necessary to reiterate some points mentioned in order to assess the contribution made by this study towards the resolution of the broader problems of population biology outlined in chapter 1.

6.1 Processes Affecting the Distribution of Genetic Variation

The "selectionist verses neutralist" controversy has for the past 14 years dominated consideration of the role of molecular variation in the evolutionary process, and it seems we are still far from the goal of answering this fundamental question (see Selander, 1976). As with many other similar studies, attempts to determine the forces responsible for observed patterns of variation in *Arripis* and *Chrysophrys* species have not been completely successful. For example, geographical clines in allele frequencies have been detected in three snapper loci (*EST*, *IDH* and *ADH*), while allele frequencies at a fourth locus (A^{r4}) are distributed randomly over the Australian range of the species. It is difficult to explain this difference in allele distribution patterns as the result of random processes alone, but the data do not indicate which loci, if any, are subject to selective processes. This

problem has been encountered in earlier attempts to apply statistical models for the discrimination of selective verses random processes (Lewontin and Krakauer, 1973; Christiansen and Frydenberg, 1974).

Distributions of genotypes can sometimes be used to infer the operation of selective forces on a given locus. An example of this is the deficiency of $EST^{100/93}$ heterozygotes in samples of juvenile Australian 'salmon' (chapter 3). Although a number of alternative explanations are possible, this result has been interpreted as indicating differential mortality of EST genotypes in the early life history phase of the species. It is interesting to note that there is a significant bias of sex ratios towards excess males in juvenile year classes, but not in adult cohorts. Whether or not the EST polymorphism is sex-linked is unclear, but the deviations in sex ratio are further evidence of differential mortality in specific phases of the life history of salmon. However, attempts to interpret heterozygote deficiencies for several loci in snapper as evidence of selective processes are misleading, as observed geographical variation in allele frequencies indicate that the unusual genotype distributions are an artefact of the sampling procedure (i.e. a Wahlund effect - see section 3.6.2).

A more promising approach to the problem is that adopted in chapter 4, where environment-mediated functional differences between the products of allelic genes are experimentally demonstrated, and the distribution of variation in natural populations is compared to predictions based on empirical data. The activity assays

of snapper IDH allozymes are particularly interesting, because they indicate that observed functional differences in response to temperature fluctuation are more closely correlated to heat stability characteristics than to electrophoretic mobility. This is the first known report of such a result, and it illustrates the potential contribution that techniques other than electrophoresis can make to the detection of selectively important genetic variation.

The major current drawbacks of the enzyme kinetic approach are twofold. Firstly, for reasons given in chapter 3, many organisms and environmental parameters are not entirely amenable to the experimental scheme outlined by Clarke (1975). Secondly, even if physiologically important functional variation is demonstrated at a single locus, the effect of such variation on allele distributions is likely to be distorted or swamped on many occasions by selective processes acting at other loci (Wills et al., 1975; Mitton and Koehn, 1975). Nevertheless, the experimental approach offers the prospect of rigorous testing of genotype/environment interactions, and regardless of the outcome of the selectionist/neutralist controversy, such information will make a valuable contribution to ecological studies of organisms and their habitats.

6.2 Life History Characteristics, "Adaptive Strategies" and Genetic Variation

The electrophoretic data for western salmon and

Australian snapper support predictions that differences in the life history characteristics of these two species will be manifest in different distributions of genetic variation. Although polymorphisms were scarce in western salmon, the available EST data indicated a homogeneous distribution of alleles - a result consistent with expectations for a highly mobile pelagic fish with a single known spawning ground and a relatively short life span (6 - 9 years). The existence in snapper of geographical clines and heterogeneous distributions of rare alleles is also consistent with expectations for a comparatively sedentary fish with a long life span (up to 60 years) and demersal larvae. This correlation seems intuitively simple, as the life history characteristics of a species will to a large extent determine its vagility, and thus the nature of gene flow throughout its range. However, there is a surprising paucity of published studies in which specific *a priori* predictions of the distribution of genetic variation in species with contrasting life history characteristics are made and then tested.

It is recognised that the relationship between life history characteristics and distribution of genetic variation can be distorted by both selective and stochastic processes, but such occasions should in many cases be identifiable by the careful application of kinship and isolation-by-distance analyses (see chapter 3).

The correlation between observed electrophoretic and enzyme kinetic results, and the predictions based on differences in the "adaptive strategies" of salmon and snapper, are less convincing. Postulated relationships

between levels of genetic and environmental variability are numerous (see Valentine and Ayala, 1978), and have heuristic appeal in that they facilitate the integration of many aspects of population genetic and ecological theory. Unfortunately such relationships are generally not adequately defined, and thus cannot be rigorously tested.

Valentine and Ayala's (1978) model proposes a correlation between high genetic variability, high species diversity, temporal stability of the environment, and low vagility. This model was chosen for application in the present study because it is a composite of several earlier hypotheses, and it appears to have particular relevance for marine organisms.

Western salmon certainly have a lower level of genetic variability than do Australian snapper (see chapter 3) - a result which would be expected for a large, mobile organism which must be phenotypically flexible to cope with an environment perceived as being unpredictably variable. This expectation is also supported by enzyme kinetic data which indicate that salmon ME is a eurythermal enzyme, i.e. able to cope with any temperature within the physiological tolerance limits of the organism. Such a strategy for temperature compensation is consistent with the expected phenotypic plasticity of a large mobile organism in an unpredictably variable environment. By contrast snapper are genetically more variable, and have a number of functionally 'specialised' IDH allozymes to ensure optimum IDH activity over the entire temperature range of the organisms. This result is consistent with

expectations for a predominantly sedentary fish which perceives its environment as more coarse-grained but temporally more stable than that of Australian salmon (see section 3.1.3).

The above interpretation is, however, subject to a number of problems. Firstly, evidence from evolutionary studies in chapter 5 indicates that the low heterozygosity value for western salmon is most easily interpreted as a loss of genetic variability following a severe reduction in population numbers, either during or after divergence from eastern salmon. The closely related eastern salmon has at least as much genetic variability as Australian snapper. Secondly, the enzyme kinetic data are from only one enzyme system per species, and the interpretation of results in terms of "adaptive strategies" may well be altered if other enzymes are analysed. Thirdly, western and eastern salmon exhibit a number of electrophoretic and heat stability ME allozymes, and such variability is not consistent with an organism whose predicted strategy is to ensure the selection of a few alleles which code for phenotypic plasticity.

In spite of these and other problems, an investigation of the amount and distribution of genetic variation in species of the genera *Arripis* and *Chrysophrys* has been a worthwhile exercise for the following reasons:-

a) Analysis of protein variation at some 27 loci has provided data for the assessment of population structure in western salmon and Australian snapper, using both conventional methods and a technique (kinship analysis) not previously used on fishes.

These data are useful both in the applied fisheries context - i.e. as a basis for rational management of a living resource - and for more esoteric considerations of how life history characteristics affect the distribution of genetic variation in natural populations.

b) Experimental analysis of the functional adaptation of snapper IDH and sa[illegible] ME allozymes to fluctuating temperature has shown that structural variation in proteins can be correlated to functional differences and is not always electrophoretically detected. These results indicate that additional techniques are needed to fully assess the nature of protein polymorphisms in natural populations.

c) Electrophoretic data collected from all *Arripis* and *Chrysophrys* taxa ha clarified taxonomic arrangements and provided some insights into the evolutionary history of each genus, while morphological studies on the same taxa have in many cases failed to produce useful results.

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