THE POPULATION GENETICS, TAXONOMY AND ECOLOGY OF SOME BRITISH AND AUSTRALIAN HYDROBIID SNAILS WITH PARTICULAR REFERENCE TO THE COLONISING PROSOBRANCH POTAMOPYRGUS JENKINSI (SMITH)

BY

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ABBREVIATIONS USED IN THE TEXT

TERM IN FULL	ABBREVIATION
Potamopyrgus jenkinsi strain A	P.jenkinsi A
" " " B	" " B
" " " C	" " C
(see section IV - 1 - 1 for definition of 'strain')	
α - naphthyl acetate	α - n - a
β - naphthyl acetate	β - n - a
naphthol - AS - acetate	n - AS - a
α - naphthyl proprionate	α - n - p
β - carboxy-choline iodide	β - c-c - I
α - naphthyl butyrate	α - n - b
α - naphthyl laurate	α - n - l
5 - bromo-indoxyl acetate	5 - Br-ind - a
indoxyl acetate	ind - a.

A B S T R A C T

Polyacrylamide gel electrophoresis has been used to study protein polymorphisms in populations of British and Australasian Hydrobiid snails. Zymograms obtained from parthenogenetic populations were interpreted genetically by a technique based on the biochemical and physical properties of the allozyme bands.

Phylogenetic relationships between Hydrobiid species have been computed from the electrophoretic data using an index of genetic distance. The British species Potamopyrgus jenkinsi was found to consist of at least three genetically distinct strains (A, B and C) which correspond to the morphological strains recognised by previous authors. P. jenkinsi has been shown to be more closely related to the New Zealand species P. antipodarum than to the native British Hydrobiids (Hydrobia ulvae, H. neglecta and H. ventrosa) or to the Australian species P. nigra. It is thus suggested that P. jenkinsi was introduced into Europe from New Zealand.

Differences in the distribution and levels of electrophoretic variation have been shown to be related to the reproductive strategies of Hydrobiid populations. Variation in the sexually reproducing species is distributed evenly between and within populations whereas variation in wholly or partly asexual species is distributed between weakly polymorphic or monomorphic populations. A large scale electrophoretic survey of British populations of P. jenkinsi (strain A) revealed a virtual absence of variation both within and between populations. In the course of this survey a diet-induced esterase was discovered.

Levels of heterozygosity were greater in asexual Hydrobiid populations than in sexual populations with the highest level occurring in P. jenkinsi.

An attempt has been made to relate levels of genetic variation to physiological ecology by means of experiments designed to investigate the behaviour and survival of Hydrobiids in eighteen different combinations of temperature and salinity. It is suggested that P. jenkinsi A is a 'generalist' relative to other fresh water animals but a 'specialist' relative to other British Hydrobiids. Evidence is presented suggesting that the distribution of British Hydrobiids is determined by the availability and stability of physical environmental resources. Differences in the physiological tolerances of P. jenkinsi strains A and B were sufficient to account for the differences in their geographic distributions.

Relationships between genetic variation and ecological strategy in P. jenkinsi are discussed in the light of evidence from other colonising species.

I N T R O D U C T I O N

"Darwin's survival of the fittest is really a special case of a more general law of survival of the stable" - Richard Dawkins in 'The Selfish Gene'.

Recent evolutionary theory tends to regard the organism as a vehicle for enhancing the stability and thus the survival of the genome, buffering the delicate strands of genetic information against the vagaries of the physical and biotic environment. The diversity of life may be viewed as a spectrum of different strategies that have evolved to ensure the survival and reproduction of countless different units of genetic information both in the past and in the present.

Individuals possess certain heritable characteristics which determine the homeostatic response of their population to environmental change. Such 'adaptive strategies' of populations appear to be related to the manner in which the component individuals experience their environment. A population may evolve a strategy lying somewhere between two extremes represented by:

A. Genetic 'tracking' of environmental fluctuations by short-term rapid evolution such that the mean fitness per individual remains reasonably constant. This strategy must, however, involve an element of lag between the onset of the fluctuation and the evolutionary response by the population. We might expect tracking to be pursued by populations of small relatively immobile organisms which, in a physiological sense, are minimally buffered against the environment. Such organisms (e.g. colonising plants and small invertebrates) are said by Levins[2] to experience their environment as 'coarse-grained'; the individual is faced with a high degree of temporal and/or spatial environmental uncertainty.

B. Response to environmental change by means of physiological 'plasticity' of individuals within the population. Plasticity is common in, for example, large perennial plants and, perhaps taken to its extreme, in large homeothermic animals. Such organisms quickly respond to potentially de-stabilising fluctuations by means of homeostatic mechanisms controlling growth and metabolism. They may be thought of as experiencing their environment as 'fine-grained'; the tolerance of the individual is unlikely to be

exceeded by environmental conditions; to the individual, the environment is predictable.

The adaptive strategy pursued by a population (or species) may well be dependent on the level of variation present.

Fisher[3] in 1930 formulated his Fundamental Theorem of Natural Selection as "the ability of a population to adapt genetically to its surroundings is a function of the level of variation present in the population". The theorem has been supported experimentally by Ayala[4] using pure and hybrid lines of Drosophila. We might therefore expect that populations exhibiting strategy A above will be more variable than those exhibiting strategy B. Selander and Kaufman[5] have provided strong support for this hypothesis by comparing levels of variation in invertebrates (coarse-grained strategists) with those of vertebrates (fine-grained strategists).

Crucial to the genetic response of a population to environmental change is its reproductive strategy since this may affect, amongst other things, factors such as population size and the level of genetic variation and consequently evolutionary rate.

Williams[6] gives an excellent review of reproductive strategies and their genetic implications; a resumé of the most common forms is given below.

Perhaps the most widespread strategy in eukaryotes is sexual reproduction where recombination and segregation generate a plethora of different genotypes within a population (determining phenotypes with different fitnesses) with each round of mating.

Two main theories have been advanced for the selective advantage of sex.

The first is concerned with the potential evolutionary rate of sexual populations. The traditional view is that these populations can evolve faster than asexual populations and will in the long run be the evolutionary 'survivors'. This, the 'long-term explanation' of Maynard Smith[7], has been severely criticised by Williams[8] on the grounds that it invokes group selection and since group selection appears to be

confined to a few specific situations (i.e. kin selection) in higher animals, the theory is probably not generally applicable. Moreover, Maynard Smith has shown theoretically that sex will accelerate evolution only if populations are larger than 1/10 μ (where μ = rate of favourable mutation per locus per generation) and that in an infinite population, sex will accelerate evolution by a factor equal to the number of different loci at which favourable mutations can occur.

Williams[6] has offered a second explanation for the selective advantage of sex, namely that since sexually reproducing individuals produce highly variable progeny, there is a greater immediate chance that some will have high fitness in future environmental conditions. Maynard Smith[7] has criticised this, the 'short-term' explanation on the grounds that environmental fluctations must be large enough to threaten extinction for sexual reproduction to have an immediate advantage over asexual reproduction.

Some aspects of sexual reproduction appear to be disadvantageous relative to asexual (apomictic) reproduction; sexual reproduction results in substantial genetic load, the disruption of highly fit genotypes and the presence in the population of reproductively wasteful males. Maynard Smith[7] has pointed out that this latter factor is less important in situations where both parents care for the progeny.

Eshel and Feldman[9] consider that these seemingly disadvantageous factors far outweigh the apparent advantages of sex and thatasexual and sexual populations evolve at much the same rate. Williams[6] suggests that this may be of primary importance in the understanding of the evolutionary role of sex; the advantages of sexual reproduction may lie in its retardation of adaptation and in the limitation of the attainable precision of adaptation imposed by recombinational load.

Other reproductive strategies include distinct and genetically very different forms of parthenogenesis.

The least common and perhaps the least understood form of parthenogenesis is automixis, a form of degenerate sexual reproduction. Diploidy is usually restored by fusion of haploid muclei after meiosis, although factors such as premeiotic doubling of chromosomes and suppression of recombination (e.g. in some Lepidoptera) may complicate the genetic

consequences of this strategy. Suomalainen[10] provides a clear review of the complexities involved in such oogenesis. In all cases no exchange of gametes takes place between individuals; all are female.

Many types of automixis may be regarded as an extreme form of inbreeding; deleterious recessives may be rapidly exposed to selection leading to fixation at most loci. Heterozygosity may be reduced to a level maintained by mutation rate alone and we might expect complete individual homozygosity although overall levels of polymorphism within and between populations may remain high.

The common and closely related form of degenerate sexuality, self-fertilisation, is essentially a form of automixis with similar theoretical genetic consequences.

Since all individuals in an automictic population are female, the intrinsic rate of increase is potentially double that of sexual females producing the same number of offspring (assuming a 50:50 sex ratio in the latter). This phenomenon is common to all parthenogens and a further, related characteristic of such organisms is the ability to found a colony from a single individual at any stage of development.

Perhaps the most widespread type of parthenogenesis is apomixis, reproduction without males and without a meiotic division during oogenesis.

Apomixis is the most efficient way by which a highly fit genotype may be preserved and reproduced since, in the absence of segregation and recombination, there is no genetic load and copying fidelity of the parental genome is only affected by mutation rate. New (mostly recessive) mutations are unlikely to be rapidly expressed (although mitotic recombination may occur), particularly if the organism is poly-ploid; parthenogenetic populations incorporate advantageous mutations more slowly than comparable sexual populations.[10]

For these reasons, we might expect variation to be restricted or absent in apomictic populations and consequently their genetic response to environmental change to be minimal.

These theoretical restrictions to the evolutionary response of

parthenogenetic organisms to major and unpredictable environmental fluctuation led geneticists such as Fisher[3], Darlington[11] and White[12] to regard such species as evolutionary 'dead-ends' doomed to extinction; their study was considered to be largely irrelevant to our understanding of long-term evolutionary processes.

But many parthenogenetic organisms such as aphids[13], weevils[10], water-fleas[14], dandelions[15] and some fish[16, 17] are successful and apparently persistent species, often dominant in successionally immature ecosystems and widely distributed geographically. Many are highly successful colonisers and indeed we might, apart from the genetic considerations outlined above, expect this to be so for fecundity reasons.

We are thus faced with an apparent paradox posed by the theoretical evolutionary 'dead-end' view of apomixis presented by conventional evolutionary dogma and the contrasting widespread success of apomictic species in the real world.

It was this paradox that prompted Suomalainen to begin his early studies[18] on the extent and nature of variation in natural populations of parthenogenetic weevils. Using morphological markers he demonstrated that such populations were indeed monomorphic or weakly polymorphic but that there appeared to be considerable variation between apomictic weevil populations of the same morphospecies from different geographic areas. He concluded that the amount of variation present within a morphospecies of apomictic weevil was sufficient to provide considerable evolutionary potential. Morphological markers in these species were however too few to enable large-scale estimates of variation to be made.

In the mid-1960's a powerful new method for estimating levels of genetic variation was provided with the development of gel electrophoresis/ histological staining techniques by Harris[19], Lewontin and Hubby[20]. These techniques allowed large-scale estimates of variation to be made for species with few morphological markers (e.g. molluscs and annelids) and for large numbers of similar loci to be surveyed in different species. Reviews of such surveys have been compiled for various purposes by, for example, Selander and Kaufman[5], Johnson[21], Selander[22], Ward[23] and Koehn and Eanes[24].

Absolute levels of variation derived from these surveys must be

treated with caution since electrophoresis detects markers that are an extremely small and biased subset of the array of proteins constituting the whole organism. Only soluble proteins are detectable and of these only a small proportion contribute to overall estimates of variation. We know very little of the levels of variation associated with, for example, the insoluble structural proteins; these may prove to have very different levels of variation relative to the small globular enzymes usually detected by electrophoresis. Certainly enzyme molecular structure has an effect on levels of variation of the soluble enzymes;[25] non-regulatory enzymes are far less variable than regulatory or variable-substrate enzymes; monomers are more variable than polymers.[26]

The detection of alleles by electrophoresis relies on the principle that a mutation at a particular locus results in an alteration in the net charge of the gene product. It is generally assumed that the charge on the protein molecule is altered by the substitution of a different amino-acid due to a base-pair substitution (or other mutational rearrangement of the DNA such as inversion, deletion or insertion). Gene products from different loci may have very different molecular sizes and are thus separated on gels. The alteration of the overall charge of a mutant gene product results in an altered mobility relative to that of the unaltered allele.

Salient points here are that not all mutations result in an altered gene product due to the redundancy of the genetic code and that not all amino-acid substitutions result in changed charge states since several amino-acids have similar or no charge. In the most recent estimates of the proportion of mutations that are detectable by electrophoresis, Maruyama and Kimura[27] calculate that 25% should be detectable.

A further difficulty associated with electrophoretic surveys is that, in organisms for which breeding data is unobtainable (such as apomictic parthenogens and those organisms which cannot be easily reared in the laboratory), null alleles (gene products which do not stain on gels) may be wholly undetectable; this inevitably results in under-estimation of levels of variation in such organisms.

Variation which is non-genetic in origin (epi-genetic variation) may also result in misleading interpretations of zymograms. Few electrophoretic studies have been specifically designed to investigate the

possible presence of epi-genetic factors but Oxford[28] and more recently Gill[29] have reported such effects on molluscan isozymes.

Despite the above shortcomings of electrophoretic techniques, estimates of absolute levels of variation are remarkably consistent (see below); electrophoresis is probably most reliable for comparative work where surveys of variation are performed by assaying similar loci using the same electrophoretic method for each population.

Perhaps the most controversial subject in current population genetics is the adaptive significance of electrophoretic variation. If such variation is maintained largely by stochastic processes and not by natural selection then the regulation of levels of variation by reproductive strategy cannot be considered an adaptive strategy and we must look elsewhere for the adaptive roles of different modes of reproduction. An outline of the existing evidence for and against the maintenance of this and other variation by natural selection is given below.

Firstly, let us consider the selective maintenance of visible variation. The most powerful evidence for this has come from the classic studies of stable and transient polymorphisms, mainly those of animals. Cain and Sheppard's[30] work on colour polymorphisms in Cepaea, Kettlewell's[31] studies of melanism in the Lepidoptera and Allison's[32] discovery of the link between haemoglobin polymorphisms and malaria in man demonstrate convincingly that selection is responsible for maintaining visible variation in these populations. Perhaps the most convincing feature of these and other studies is that the functional significance of the markers in the wild can be identified and, by careful observation and experimentation, the selective co-efficients of at least some can be estimated.

The problem with electrophoretic markers is that, in the vast majority of cases, their functions are unknown although recently Beranek and Oppernoorth[33] have provided evidence linking the RAE (resistance associated esterase) locus in the aphid Myzus persicae with organophosphorus insecticide detoxification.

However, since our most convenient access to genetic variation in natural populations is by using gel electrophoresis recent attempts to resolve the mechanisms by which variation is maintained have concentrated on this technique.

Two main and opposing hypotheses have been advanced to explain levels of both visible and electrophoretic variation in natural, sexually reproducing populations; the 'selective' hypothesis founded on the early theoretical work of Muller[34] and Dobzhansky[35] and the 'neutral' hypothesis put forward more recently by Crow and Kimura[36] in an attempt to explain the high levels of variation revealed by electrophoresis.

The selectionists have two rather different theoretical bases for their views. The earlier theory was put forward by Muller[34] who considered that, for most loci, selection tends to favour fixation of the most fit allele and that the majority of heterozygosity can be explained as being merely the transient state of selection for another, more fit, allele. Muller reached this conclusion after examination of the theoretical implications of genetic load on highly heterozygous individuals and predicted that only a few loci might qualify for maintenance of their variability by some form of balancing selection.

This view was challenged by, for example, Dobzhansky[35] and Wallace[37] who considered it unlikely that there was a single most fit allele at most loci in a population and that because most loci would be in selective equilibrium, balancing selection would maintain the majority in the heterozygous state. This 'balance hypothesis' predicts that only a few loci would be at fixation; in particular those associated with, for example, central metabolic functions might be found in a highly conserved homozygous state.

Both the above hypotheses invoke natural selection as the major force determining the maintaining levels of genetic variation in natural populations.

Evidence for selectionist interpretations has been provided by correlations between gene frequencies and environmental variables. For example, Johnson[38, 39] in a study of the Crested Blenny Anoplarchus has found associations between allele frequencies and temperature gradients. McKechnie et. al.[40] in a large-scale study of Euphydryas butterfly populations obtained highly significant associations between several environmental parameters and electrophoretic allele frequencies. Other studies such as those of Schopf and Gooch[41] and Johnson et. al.[42] have revealed similar correlations.

It must be remembered however that until our understanding of the metabolic functions of isozymes improves, we cannot determine whether such correlations are causally linked and such evidence is largely circumstantial.

Crow and Kimura[36] argued from a rather different standpoint, namely that those mutations most likely to survive in a population are probably selectively neutral and that, as such, they could be maintained in an array of polymorphic states by a process akin to Wright's genetic drift, ultimately reaching fixation. Thus most variation might be explained as having little evolutionary significance but merely as the product of random effects on neutral alleles.

There is an increasing body of evidence that tends to refute this 'neutral' hypothesis. Predictions from the hypothesis with respect to the variability of enzyme type and the general levels of variation in populations do not fit with the evidence from natural and experimental populations.[43, 44] The neutral hypothesis also predicts that loci in geographically separated populations of a species should lose their identity and diverge. Ayala et. al.[45] and Prakash et. al.[46] have shown that the identity of such loci is generally conserved and Slatkin[47] has recently shown that this is unlikely to be due to gene flow. This is supported by McKechnie et. al.[40] who also eliminated gene flow as a factor in maintaining allele frequency similarities in isolated populations of Euphydryas butterflies.

Ohta[48] has recently proposed an extension of the neutral hypothesis which overcomes some of these difficulties. His model acknowledges selective effects on electrophoretic alleles but does not invoke balancing selection as the major force maintaining such variation. This, the mutation-equilibrium theory, proposes that variation may be maintained by a balance between the mutation rate to slightly deleterious alleles (which appear as low frequency electromorphs at different integer positions on gels according to Ohta and Kimura's[49] 'step' model) and selection against these alleles. Biochemical heterogeneity between alleles (in terms of their hydrolysis rates using natural substrates) has indeed been demonstrated by Danford and Beardmore,[50] supporting the contention that differential selection between alleles is at least possible.

Excellent reviews of the state of the selectionist/neutralist debate

up until 1974 are given by Lewontin[51] and Johnson[52]; both agree that on balance most evidence supports selective models.

One implication of the selective hypothesis is that the overall level of electrophoretic variation in a population should be adaptive.

Consequently many recent contributions to the debate have taken the form of attempts to test the suggestion of Dobzhansky[53] and more recently Van Valen[54] that the overall level of polymorphism in a population represents an adaptive strategy maximising population fitness in an heterogeneous environment. Those populations exploiting a wide resource spectrum (or 'niche') should show greater genetic variability than those which are habitat specialists. Babbel and Selander,[55] in an electrophoretic study of four species of flowering plant and Nevo[56] have recently produced evidence in support of this concept but an electrophoretic survey of the brachiopod Frieleia halli undertaken by Ayala and Valentine[57] appears to refute the theory.

A major problem with such studies is that estimates of environmental parameters are frequently made by subjective, rather intuitive methods and as such are somewhat crude. Moreover, the differences in levels of variation between populations may be so small that the technique becomes insensitive. We need to look at populations having very different levels of variation and heterozygosities, preferably from the same species.

Since these levels may theoretically be affected by the reproductive strategy of a population it is of interest to compare levels of electrophoretic variation in natural populations having very different reproductive strategies.

In absolute terms electrophoretic surveys of loci in sexually reproducing populations have revealed levels of genetic variation an order of magnitude greater than those previously considered likely (for reviews see Selander[23] and Powell[58]); most species are polymorphic at about 30% of their loci and have a mean heterozygosity of around 10%. In view of the sampling bias inherent in the technique, such levels are remarkable for their consistency. Variation in the vast majority of sexual populations is distributed evenly both within and between populations with genotype frequency distributions usually conforming to Hardy-Weinberg expectations.

In contrast, electrophoretic surveys of apomictic populations (mainly insects) by, for example, Suomalainen,[59] Saura[60] and Lokki[61] have revealed rather different patterns of polymorphism to those in sexual populations. Levels of variation within apomictic species are usually similar to those found in closely related sexual species[61] but variation is distributed in a different way. Individual populations are either monomorphic or weakly polymorphic with the bulk of variation represented by differences between populations.

Due to the lack of recombination and segregation inherent in apomixis, recessive mutations are rarely exposed to selection. Consequently such mutations may accumulate as heterozygotes. Given sufficient time populations might be expected to become highly heterozygous and indeed this is generally the case. Lokki[62] has modelled this accumulation and has shown that there is a maximum value of functioning heterozygosity which is attained after approximately u^{-1} generations (u = mutation rate). Populations may also originate with high heterozygosity due to hybridisation; parthenogenesis is often an evolutionary response to the 'meiotic barrier' caused by hybridisation.

Patterns of polymorphism similar to those outlined above have been recognised in apomictic populations of grasshoppers,[63] cockroaches,[64] aphids[13] and water-fleas.[14]

The origin of this variation in apomictic species has been shown to be due to two processes:

i) the polyphyletic origin of populations giving multiple clones in a particular geographic area. This can be due either to multiple introductions of divergent clones into the area such as those found in populations of the colonising cockroach Pycnoscelus surinamensis[64] or sympatric multiple origin of parthenogenesis such as that suggested for the bagworm moth Solenobia triquetrella by Lokki.[59]

ii) divergence due to the accumulation of mutations and chromosomal re-arrangements such as that suggested by Suomalainen and Saura[65] for apomictic populations of the weevil Otiorrhynchus scaber.

Both these processes may be simultaneously or singly responsible

for producing genetically divergent clones each adapted to a particular geographic and/or biotic environment.

Since most of the apomictic organisms that have been studied have persisted in the study areas for a very long time (in the case of some weevil species probably since the last Ice Age[65]), we are looking at the genetic structure of such populations after the above evolutionary processes have occurred. Little is known of the transient processes involved during clonal divergence. Direct evidence might be provided by the study of species that have recently invaded new ranges.

One such species is the aquatic operculate prosobranch snail, Potamopyrgus jenkinsi (Smith); thought to be the only European mollusc to reproduce parthenogenetically.[66] No cytological evidence of meiosis has been found in this species[67, 68] and only a single male has been found in over one hundred years of collecting by enthusiastic malacologists.[69] It is therefore reasonable to assume that reproduction is apomictic; further evidence supporting this contention is presented in the present work.

P. jenkinsi is an extremely prolific and common species and, since its first appearance in Britain in the mid-nineteenth century, has successfully colonised a wide range of habitat types throughout Britain and Europe.[70, 71] Indeed, it is frequently the dominant organism in immature freshwater ecosystems[72] and has been recorded from virtually every freshwater habitat type except those in montane regions. In brackish water P. jenkinsi is sometimes found co-existing with one or more of the obligate sexually reproducing Hydrobia species. In Britain and Europe this latter genus is represented by three species; H. ventrosa Montagu, H. ulvae Pennant and H. neglecta Muus, all of which are confined to brackish or sea water.

The taxonomic status of P. jenkinsi is currently uncertain. Few population studies have been carried out on this species but Warwick[73] has recognised three morphologically distinct strains (his A, B and C) which seem to be ecologically separated by salinity.[74] Winterbourn[75] and more recently Simpson[76] have compared shell form and body pigmentation of these strains and question their distinctness, suggesting that they in fact form part of continuous variation within the species. The electrophoretic evidence presented here fully supports Warwick's division of

P. jenkinsi and the strains are accordingly referred to as P. jenkinsi A, P. jenkinsi B and P. jenkinsi C in the text.

The species was originally[77] placed in the genus Hydrobia (= Paludestrina) but is now recognised as having much closer affinities (e.g. in the anatomy of the reproductive system) to the Australasian genus Potamopyrgus and is thought by Winterbourn[78] to be conspecific with either P. antipodarum (Gray) from New Zealand or the Australian species P. nigra (Quoy and Gaimard).

Both these latter species are interesting since populations are often found containing a proportion of parthenogenetic females;[78, 79] in P. antipodarum the proportions of parthenogenetic females in various populations appear to be constant (for up to 8 years, i.e. about 30 generations) irrespective of fluctuations in population size,[80] suggesting that sex ratio in this species may be adaptive.

Ecologically, P. antipodarum and P. nigra are found in a wide range of aquatic habitats[78] and are often highly successful colonisers of both freshwater and brackish habitats.

In the Hydrobiidae, therefore, we have a number of species whose modes of reproduction range from wholly asexual (P. jenkinsi) through mixed asexual/sexual (P. antipodarum, and P. nigra) to obligate sexual (the Hydrobia) species.

The work described in this thesis was designed to investigate the nature and extent of genetic variation in populations of these species having different reproductive strategies and to attempt to answer the following questions:

i) In what way are the levels and patterns of variation in such populations adaptive?
ii) Is the nature of such variation related to colonising ability?
iii) What are the general taxonomic relationships between the British and Australasian Hydrobiidae?
iv) In particular, is P. jenkinsi closely related to the Australasian species of Potamopyrgus and if so which one?

Since morphological markers are few in these species, estimates of their relative levels of genetic variation were prepared from large-scale

electrophoretic surveys of similar loci in each population. As is frequently the case in such surveys, the interpretation of the genetic significance of the zymograms was hampered by a paucity of breeding data, particularly for the wholly apomictic populations of Potamopyrgus where crosses could not be set up. Many workers attempt to solve this problem either by reference to loci in closely related sexually reproducing species or by guesswork based on the recognition of 'typical' electrophoretic patterns. For various reasons (see Chapter III) these approaches were found to be inappropriate to the present work. Consequently bands were assigned to loci on the basis of homologies in their physical and biochemical properties; the method is similar to that used by Oxford[81] in an attempt to classify the esterases of the land snails Cepaea nemoralis and C. hortensis.

The use of electrophoretic markers in surveys of variation within closely related species allows the calculation of estimates of the genetic distance between them to be made. The application of such techniques to the taxonomic problems associated with P. jenkinsi is presented in Chapter VI, together with a full discussion of the validity of such estimates.

A further advantage of using electrophoretic methods is that survey results may be compared with the bulk of data upon which current debates on the origin, nature and maintenance of genetic variation in natural populations are focussed. The results of the Hydrobiid surveys undertaken in the present work are discussed relative to other data and in the light of the effects of reproductive strategy on overall levels and distribution of genetic variation.

The relationship between variation and ecological strategy (in particular that of colonising ability) is discussed and investigated experimentally by estimating the relative resource utilisation curves for temperature and salinity tolerance of British species of Hydrobia and Potamopyrgus having different reproductive strategies (see Chapter VII).

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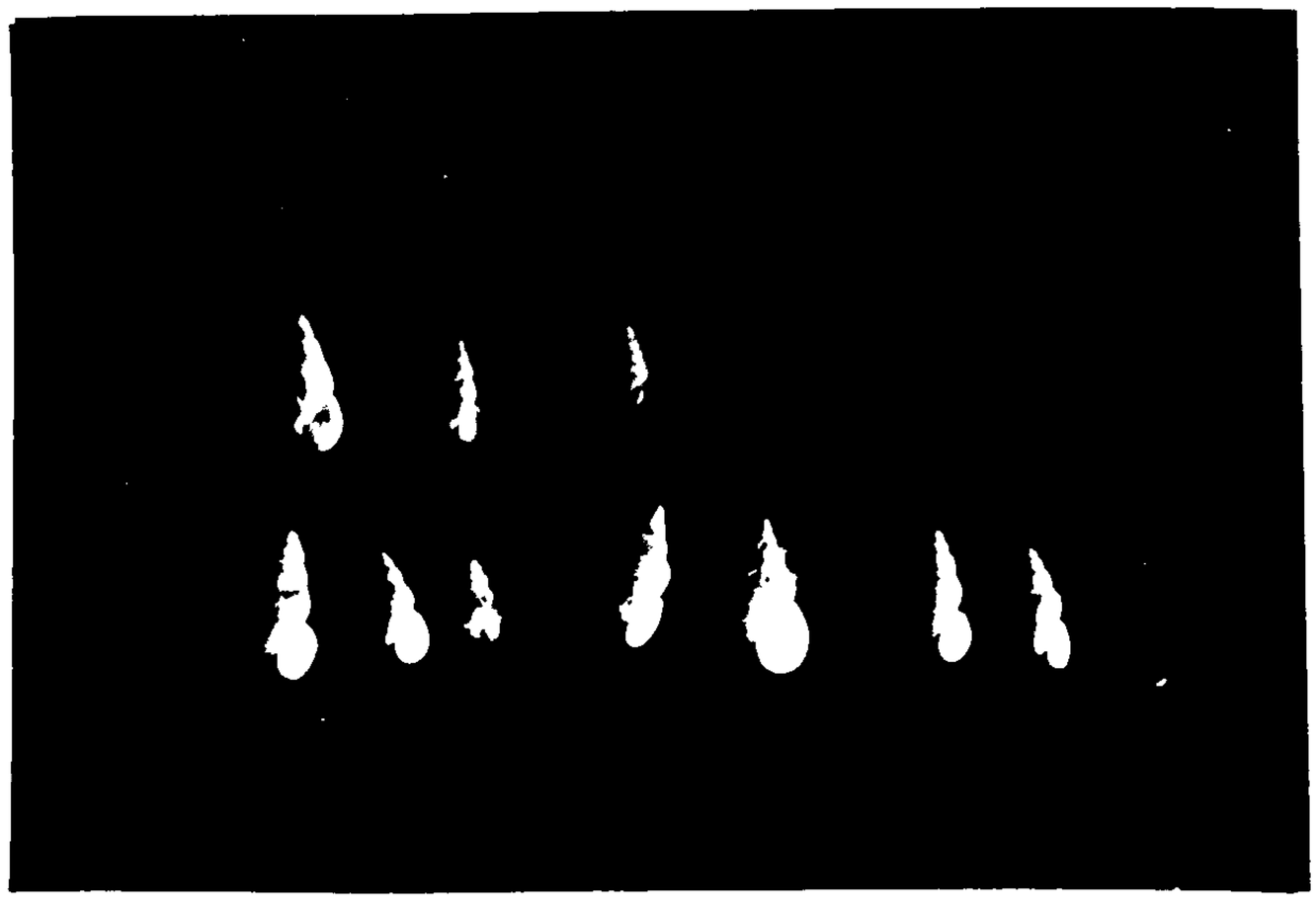
A B C



D E F G H J K

Plate 1 : Shells of species used in the study;

A - Hydrobia ulvae - from popln. 56

B - Hydrobia neglecta - from popln. 62

C - Hydrobia ventrosa - from popln. 50

D - P.jenkinsi strain A - from popln. 11

E - P.jenkinsi strain B - from popln. 1

F - P.jenkinsi strain C - keeled specimen from poln. 35

G - P.antipodarum - smooth-shelled specimen from popln.45

H - P.antipodarum - strongly keeled specimen from popln. 45

J - P. nigra - smooth-shelled specimen from popln. 40

K - P. nigra - Keeled specimen from popln. 42

CHAPTER I : MATERIALS AND METHODS

The methods described in this chapter only refer to general techniques used in this study. The more specialised techniques associated with, for example, molecular weight determination are described in the appropriate chapters.

I - 1 Snail Collection and Recording

Samples of population of snails were obtained both by myself and by persons to whom requests were sent. Wherever possible, details such as the site grid reference, date of sampling and the general physical and biotic nature of the site were recorded. Samples of water were obtained from several sites and the chloride concentration estimated by titration against N/35.5 $AgNO_3$ solution using lead chromate indicator. Chloride concentration in mg/l was calculated using the formula

$$Cl = \frac{1000}{x} y$$

Where x = volume (ml) of sample titrated and y = volume (ml) N/35.5 $AgNO_3$ needed to just change the indicator from yellow to red.

A list of the sampling locations together with the details of each site can be found in the Appendix and a selection of shells is illustrated in Plate 1. Snails for electrophoresis were taken randomly from the samples. The remaining snails were then placed in a stock tank reserved for that population. Stock tanks were 30 cm x 30 cm x 10 cm clear plastic boxes (fitted with lids) containing aerated (10 days) tap water or an appropriate dilution of sea-water obtained from Scarborough, North Yorkshire. Snails were maintained on a diet of boiled, dried lettuce. During the winter months, lettuce was often covered with a residue (possibly an insecticide) toxic to the snails and leaves were, therefore, thoroughly washed (30 minutes) in hot tap water before boiling. No significant mortality occurred in laboratory stocks fed on lettuce treated in this way. Pieces of chalk were added to the tanks and appeared to help the snails lay down a strong shell, making them more resistant to handling.

Larger stock tanks from which large numbers of snails could be obtained (for e.g. molecular weight studies 0 see section II - 3) were started from single population samples placed in plastic bins (60 cm x 30 cm x 30 cm). These cultures were maintained in a similar way to the smaller stocks mentioned above.

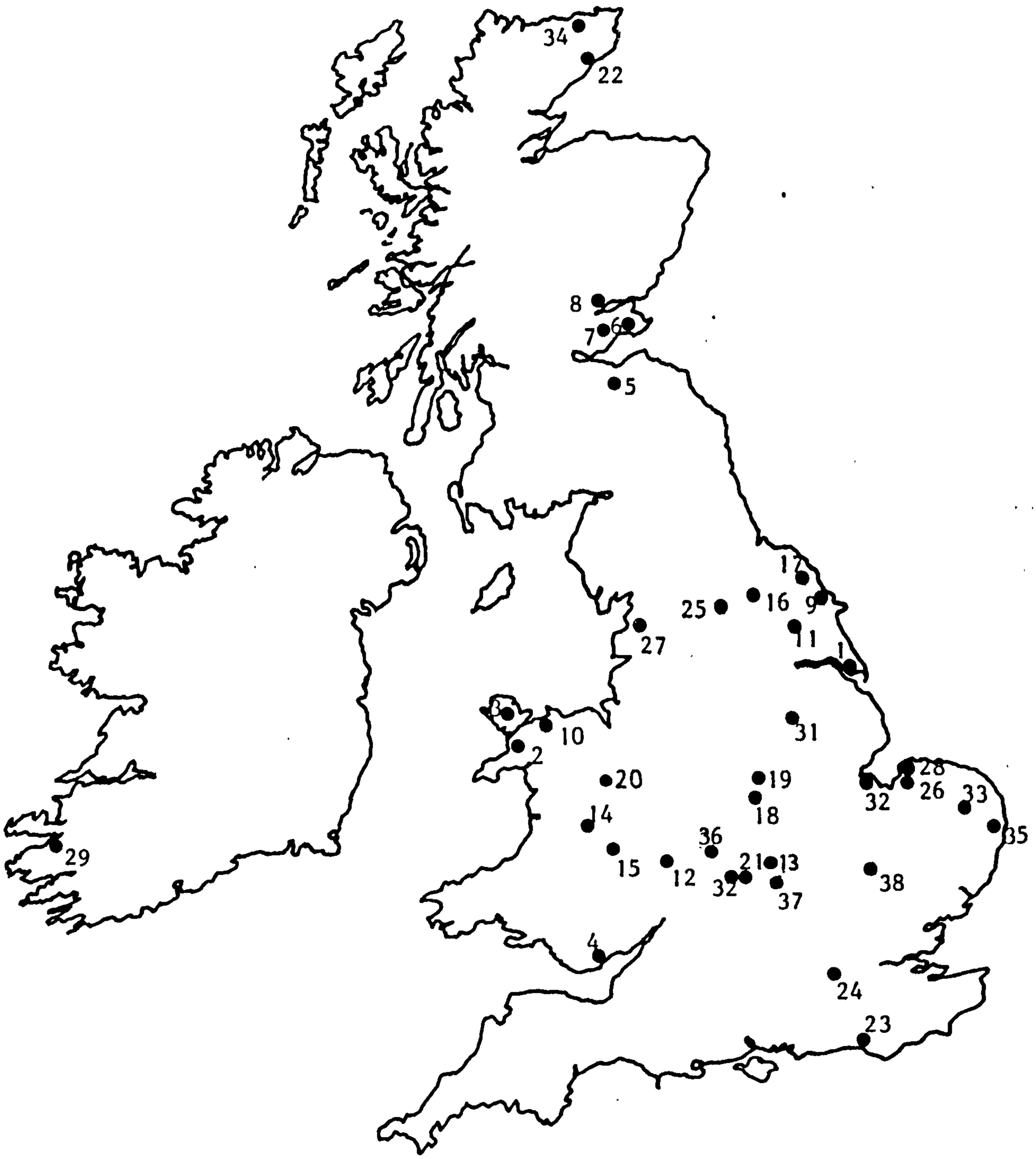


Fig.1 : Sampling sites of Potamopyrgus jenkinsi in Britain

I - 1 - 1 P. jenkinsi (Smith)

Populations of P. jenkinsi were sampled at 38 locations throughout England, Scotland and Wales. One Irish population was sampled and snails from a population in Brittany were collected by M. J. Winterbourn. Specific locations are given in the Appendix and illustrated in Fig. 1. All were lowland sites which differed widely in climate and general ecology. P. jenkinsi was usually found in flowing water although some coastal populations were collected from still water. Snails were collected with a pond-net or by picking out stones from the water-course and removing the attached animals. Samples of more than 30 individuals were collected from 33 of the sites. Snails were maintained in the laboratory as described in section I - 1.

Having first established that specimens were P. jenkinsi (using Macan[1]), samples were examined for the presence of males by looking for the presence of a penis on the head. All samples of P. jenkinsi contained only females, usually with young in the brood pouch.

Shell morphology and body pigmentation was found to be of three distinct types, corresponding to the strains described by Warwick[2] as A, B and C. All the snails from inland, fresh water sites had a thin, usually encrusted, shell which was slenderer and longer than that of snails from coastal, brackish site. Mantle pigmentation in freshwater specimens varied from almost colourless to dark grey. Snails always had the same pigmentation within a population. Brackish-water specimens had very dark grey mantles with a black spot near the eye and the shells were thicker and more compact than those of fresh-water snails. The snails from Burgh Castle, Suffolk (popln. 35) resembled the brackish-water snails but shells were smaller with a pronounced keel and the body pigmentation was a 'patchy' grey. All populations (except that from Brittany - see below) were found to be monomorphic for one of these three morphological types.

Using Warwick's criteria, all the freshwater specimens were classified as strain A (referred to as P. jenkinsi A in the text), the two samples from brackish-water as strain B (P. jenkinsi B) and the snails from Burgh Castle as strain C (P. jenkinsi C). Shells of snails from the latter site were uniform in shape and not the 'slender and stout' mixture described by Warwick for strain C. The Brittany population contained both strain A and strain C.

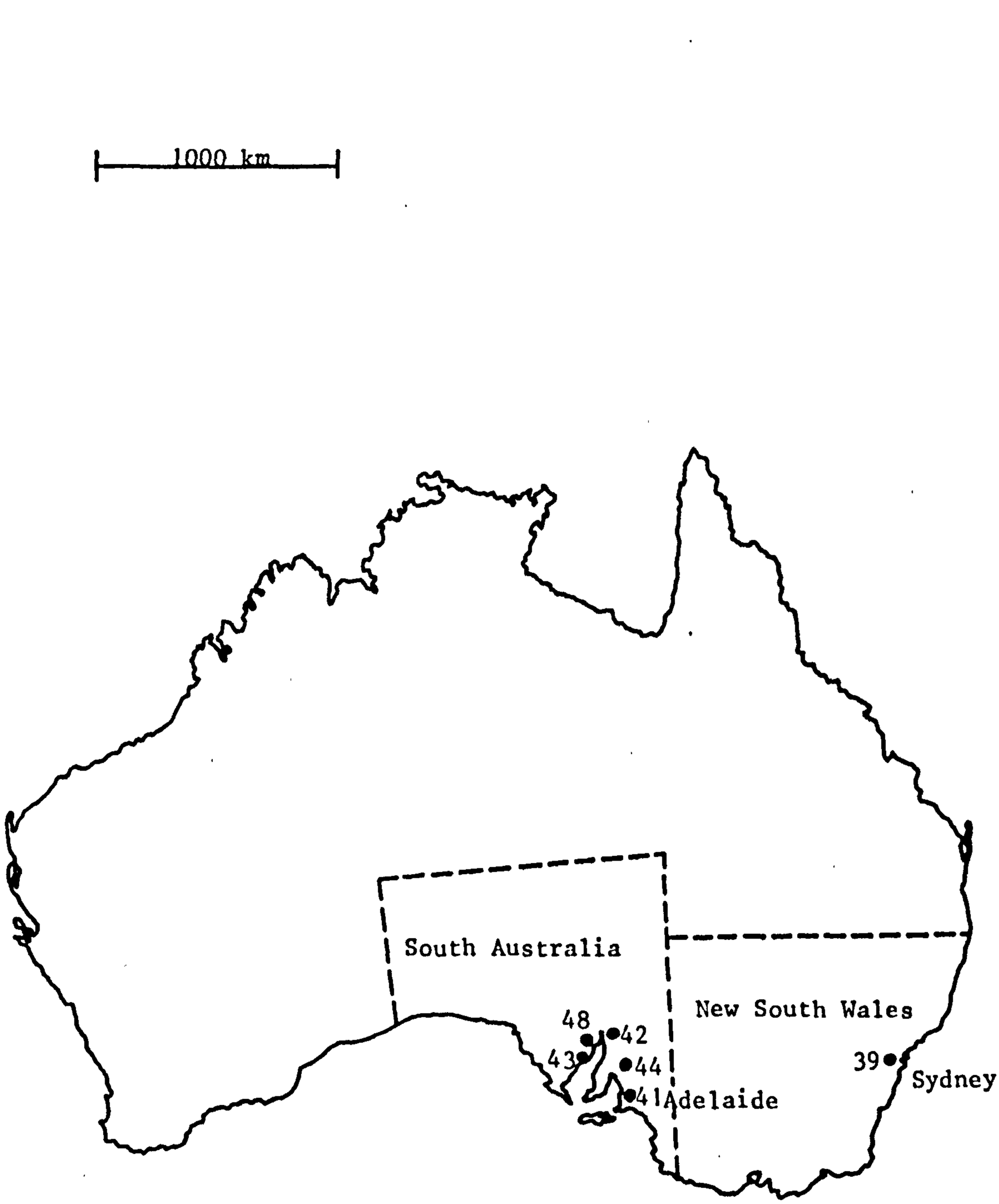


Fig.2 : Sampling sites of the Australian species *Potamopyrgus nigra*

I - 1 - 2 The Australasian species

Samples of two populations of the New Zealand species Potamopyrgus antipodarum (Gray) were obtained from inland fresh-water sites in North Island. The snails were similar in shell morphology and body pigmentation to P. jenkinsi A but were larger and some individuals were more tumid (resembling P. jenkinsi B in shell morphology). A high frequency of shell ornamentation (48% of individuals in the Waikato River population and 73% in the Campus Lake) was present, consisting of a spiny keel on the last three whorls of adult snails.

A small sample of P. estuarinus Winterbourn was obtained from a saline lagoon in South Island, New Zealand. The snails superficially resembled P. antipodarum but, like the British Hydrobia ulvae (Pennant) had no brood pouch. No pigmentation was evident on the tentacles (unlike H. ulvae which has a black spot at the tip) and the body was an even grey colour. The sex ratio in this species was approximately 1 : 1.

The Australian species P. nigra (Quoy and Gainard) is taxonomically rather loosely defined[3] but samples of snails considered by Prof. W. D. Williams (University of Adelaide) to be of this species were obtained from six sites near Adelaide. A further sample of P. nigra was obtained from a population at Narrabeen near Sydney.

Snails from these locations (Fig. 2) were similar to P. jenkinsi A in shell morphology and body pigmentation. Males (up to 10%) were present in some populations (40, 41, 43 and 48) and absent in others (see Appendix). Less than 10% of the snails had ornamented keels, all populations contained some snails with ornamented shells.

Many snails in the six Adelaide samples arrived in a distressed condition probably as a result of low temperature and pressure in the aircraft hold. They were immediately frozen and stored at -20° C until needed for electrophoresis.

I - 1 - 3 British species in the genus Hydrobia

Populations of Hydrobia ulvae, H. ventrosa (Montagu) and H. neglecta Muus were sampled from various sites in Britain. Site salinities were measured whenever possible and snails were maintained in the laboratory in an appropriate dilution of sea-water. Site details are given in the Appendix.

I - 2 Methods of Extraction

I - 2 - 1 Preparation of extracts from single snails

Individual whole snails were placed in 6 mm. diameter x 6 mm. deep cylindrical depressions in perspex blocks. 0.2 ml of 40% sucrose solution was added at 5°C and the snail homogenised using a closely fitting rotating glass rod mounted in a Gallenkamp variable speed stirrer. The method was similar to that used by Johnson[4] for preparing extracts of Drosophila. The homogenate was allowed to settle for five minutes and the supernatant fluid then withdrawn for electrophoresis. Sufficient extract for several electrophoresis runs was produced. Extracts were stored by placing the (labelled) perspex blocks covered with plastic 'cling' film into a deep freeze at -20°C.

I - 2 - 2 Preparation of mass extracts

Fifty adult snails were placed in a 10 ml glass homogeniser tube with 5 ml of 40% sucrose at 5°C. Homogenisation was achieved using a rapidly rotating glass rod and the sample allowed to settle for ten minutes. The supernatant fluid was carefully withdrawn using a bulb pipette and placed in a vial for storage at -20°C. No detectable change in staining intensity of the enzymes used in the study occurred after up to three months' storage.

I - 3 Electrophoresis of Snail Extracts. The Continuous pH Slab System

Extracts were run on starch gel and polyacrylamide gel under continuous and discontinuous pH conditions using several different gel and electrode buffers. Two methods were eventually chosen giving a large number of bands with good resolution.

For the population surveys a continuous pH polyacrylamide vertical slab gel system was chosen as the apparatus allowed the separation of up to 50 individual extracts on the same gel - an important factor enabling large numbers of snails to be electrophoresed quickly and allowing cross-reference between extracts on the same gel to be easily made.

For molecular weight studies the disc electrophoretic method was used (see section I - 4).

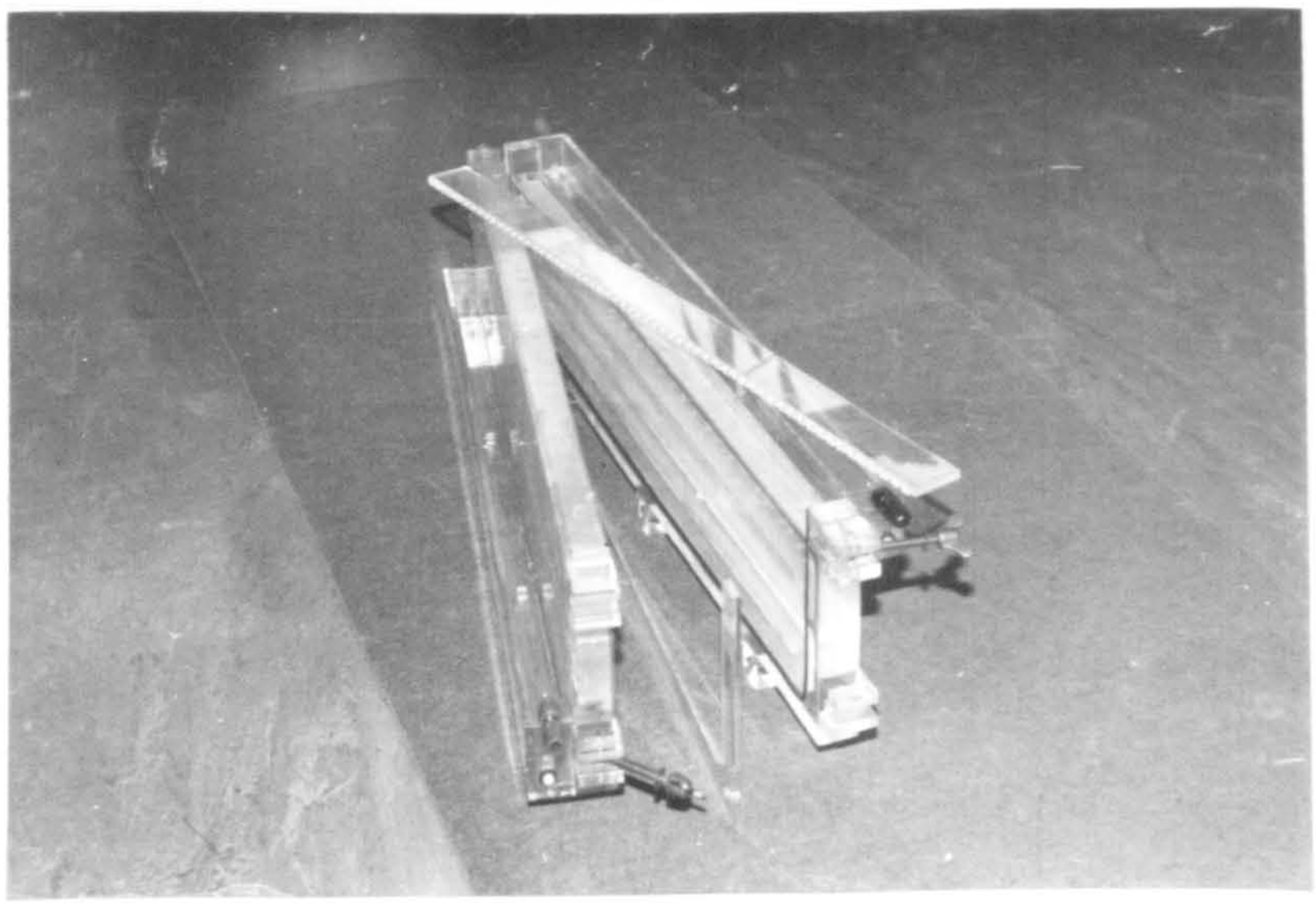


Plate 2 : Apparatus used for vertical slab electrophoresis. The 'comb' used to form sample wells is lying on top of the watercooled plates which have been separated to reveal the 6mm spacer.

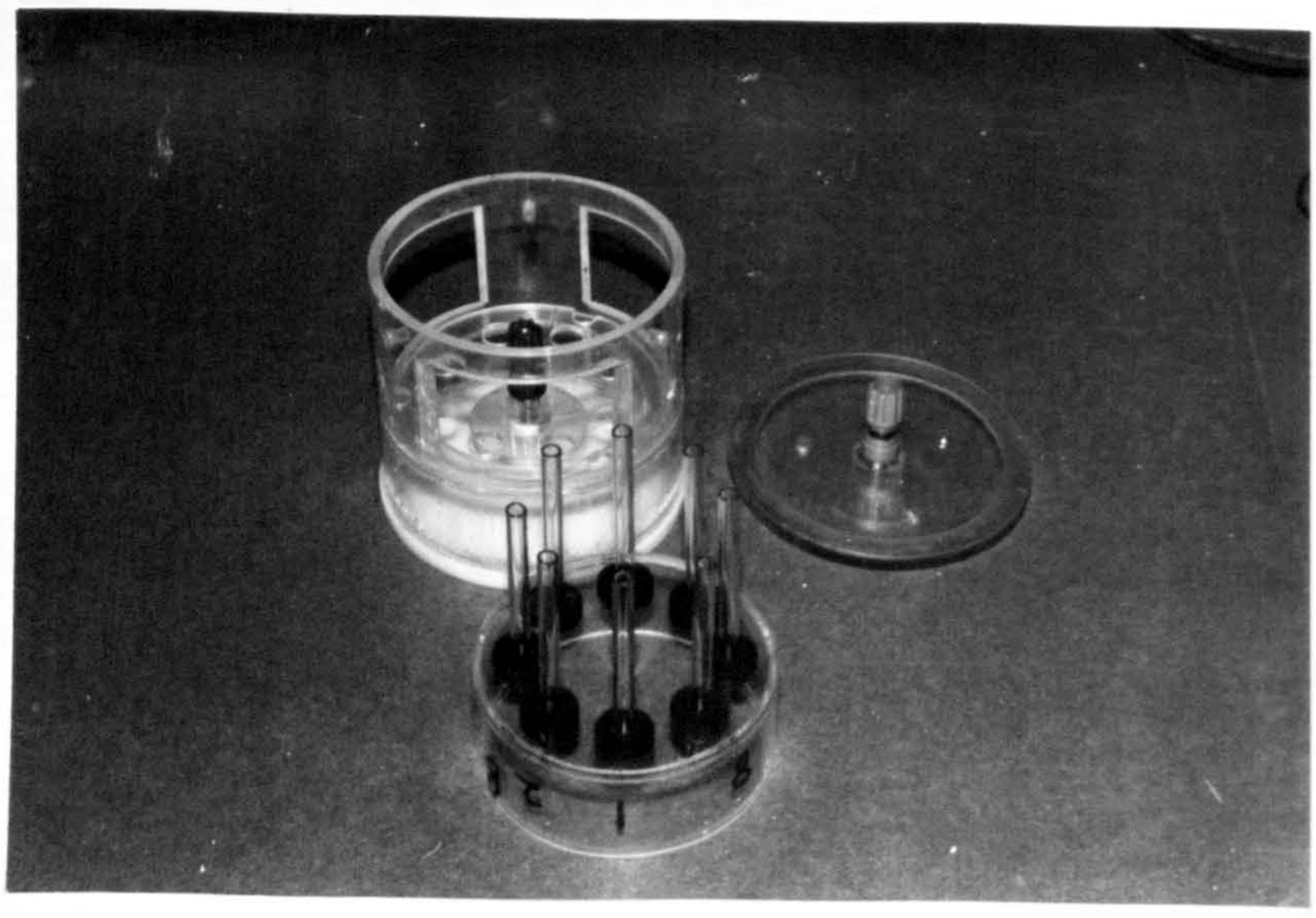


Plate 3 : The disc electrophoresis apparatus. The upper buffer compartment has been removed and inverted to show the 6mm tubes in which gels are formed and run.

I - 3 - 1 Apparatus

An electrophoresis tank based on a design described by Roberts and Jones[5] was made by the workshop staff of the University of York. The design was modified by making the tank twice as long as the original.

The apparatus basically consisted of two vertical water-cooled perspex plates separated by a 6 mm perspex spacer (see Plate 2). Contact with the electrode buffers was made directly with the gel in the top (cathode) compartment and via a dialysis membrane between the bottom of the gel and the anode compartment. Electrodes were made from platinum wire which spanned the entire length of each buffer compartment. The apparatus was contained within a fan-ventilated case fitted with a hinged lid. All cooling connections and drains were fitted with copper earthing blocks and the case was equipped with double safety switches so that it was not possible to open the lid with the power supply switched on. Power was supplied by a 'Locarte' voltage-regulated power pack.

The plates were cooled by cold tap water; no evidence of gel warming was found under the electrophoretic conditions used.

I - 3 - 2 Preparation of Vertical Slab Gels

Stock solutions used in the preparation of gels were based on those described by Smith.[6] The following stocks were made up and stored at 5°C in one litre dark glass bottles.

i) Stock Solution A: 45.5 grm tris (hydroxymethyl) aminomethane (SIGMA) + 20 grm glycine per litre of solution. When diluted for the preparation of gels and electrode buffer the solution pH was 9.5.

ii) Stock Solution B: 1.4 grm ammonium persulphate per litre of solution.

iii) Stock Solution C: For the preparation of 7.5% gels, 300 grm acrylamide and 8 grm bis - N,N' - methylenebis-acrylamide (SIGMA) were dissolved in 800 ml distilled water and the solution made up to one litre. For 5% gels 200 grm acrylamide and 5.33 grm bis per litre of solution were used.

The apparatus needed a total of 500 ml of gel solution to prepare a single gel. This was made up by thoroughly mixing 125 ml of solution A with 125 ml of solution C. 0.4 ml of TEMED (N,N,N',N', - tetramethylethylenediamine) was added to 250 ml of solution B in a one litre beaker and the A + C mixed solution added. The solution was quickly and thoroughly stirred with a glass rod and poured into the assembled apparatus. The sample well former (the 'comb' in Plate 2) was clipped into place, ensuring that no air bubbles were trapped beneath the teeth, and the gel left to polymerise for at least 50 minutes. Sargent[7] has reported the inhibition of acrylamide polymerisation by oxygen. The comb was, therefore, made so as to fit closely between the plates and no problems with inhibition by oxygen were encountered. The comb was left in the gel until extracts were ready for electrophoresis thus preventing drying out of the top surface.

I - 3 - 3 Electrophoretic method

Extracts were absorbed into 3 mm x 6 mm sample wicks cut from Whatman No. 1 filter paper. The cooling water was turned on, the comb carefully removed, and the wicks placed in the wells making sure that no air bubbles were trapped beneath. A space was usually left between groups of ten wicks to allow the gel to be cut into sections (see section I - 3 - 4).

Electrode buffer was prepared by diluting 250 ml of stock solution A to one litre with distilled water. This dilution gave a final buffer concentration and pH (9.5) identical to that of the gel. 500 ml of electrode buffer was added to the anode compartment and 500 ml added slowly and carefully (in order to disturb the wicks as little as possible) to the cathode compartment. If dehydrogenases were to be stained for, then 12.5 mg. NAD were dissolved in the cathode buffer (see section I - 5 - 3).

When the cathode compartment was filled, the safety case was quickly closed and the fan ventilator switched on. A voltage of 150 to 200V was then applied to give a running current of 200 mA. It was found that a run time of four hours under these conditions gave adequate resolution of the isozymes studied. The exception to this was in the resolution of the most cathodal bands (in e.g. Potamopyrgus - see section IV - 3 - 1) where gels were run for eight hours.

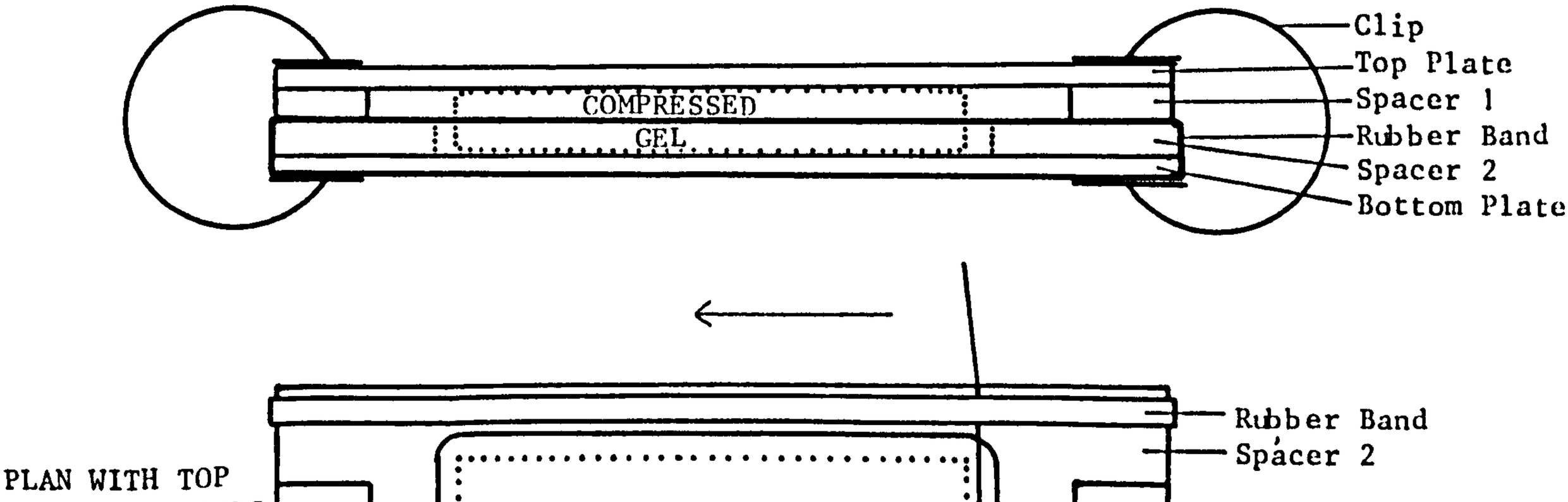


Fig.3 : A simple apparatus for slicing polyacrylamide slab gels (modified from Henderson[8])

I - 3 - 4 Gel slicing

Starch gels were found to give poor resolution of bands relative to acrylamide. However, one of the advantages of using starch is that gels can be conveniently cut into several slices which can then each be stained for a different enzyme system.

Acrylamide gel is, unlike starch, elastic at the gel concentrations used. This property is convenient when handling gels, reducing breakage, but when slicing is attempted the gel simply deforms and then splits randomly. It has been reported by Henderson[8] that compression of the gel reduces its elasticity and allows controlled slicing to be performed. An apparatus was devised for slicing (Fig. 3) which consisted of a 6 mm plate glass base and top with perspex spacers placed between them. Pressure was applied by two 'Bulldog' paper clips.

Slicing was performed by placing the gel section (usually eleven sample wells long and wetted with electrode buffer) between the plates and attaching the clips making sure that the top and bottom plates were parallel. Nylon fishing line (Bayer) of 1 kg breaking strain was then pulled through the gel in the direction shown. The apparatus was then disassembled, the gel slices carefully separated from each other, and each slice placed in an appropriate staining solution. Up to three slices could be obtained from a 6 mm slab gel using spacers of different thicknesses.

I - 4 Electrophoresis of Snail Extracts. The Discontinuous pH Disc Gel System.

I - 4 - 1 Apparatus

The apparatus used was based on the 'disc' system described by Davis and illustrated in Plate 3. The electrophoretic tank consisted of two cylindrical perspex buffer compartments, the anodal placed below the cathodal. Gels were prepared in either 5 mm diameter or (in order to electrophorese 0.05 ml extracts) 2.5 mm. diameter x 75 mm glass tubes. Longer (110 mm) tubes were sometimes used (e.g. to resolve close bands) by the simple expedient of placing corks between the upper buffer compartment and the perspex spacer. Each apparatus holds only eight gels making the technique unsuitable for large-scale population surveys. However, a front is formed during electrophoresis enabling accurate measurements of R_f values to be made. This, together with the fact that

several concentrations of gel can be included in each run, makes the technique suitable for the molecular weight determination described in section III - 3).

I - 4 - 2 Preparation of disc gels and electrode buffer

The stock solutions given below were made up according to the recipes in Davis.[9] Solutions were stored at 5°C in 250 ml dark glass bottles and no loss of quality was found after storage for several months except in the case of the ammonium persulphate solution which deteriorated after six weeks' storage. This was freshly prepared at six-monthly intervals.

i) Stock solution A: 48 ml MHC1 + 36.6 grm tris + 0.23 ml TEMED made up to 100 ml with distilled water.

ii) Stock solution B: 5.98 grm tris + 0.46 ml TEMED + approx. 48 ml MHC1 (pH adjusted to 6.7 by titrating with MHC1) to 100 ml with distilled water.

iii) Stock solution C: 30 grm acrylamide + 0.8 grm bis made up to 100 ml with distilled water. This solution gave a final gel concentration of 7.5% acrylamide. For 5% gels 20 grm acrylamide + 0.533 grm bis/100 ml solution were used.

iv) Stock solution D: 10 grm acrylamide + 2.5 grm bis to 100 ml with distilled water.

v) Stock solution E: 4 mg riboflavin made up to 100 ml with distilled water.

Gels were prepared in two parts; an upper 'spacer' gel and a lower 'separating' gel. This system was found to give resolution as good as the more complex system described by Sargent and better than by using the separating gel alone. Tubes were stoppered lightly at one end and placed vertically in a rack. A mark was made with a felt-tip pen 1 cm from the unstoppered end.

24 ml of lower gel solution was prepared by thoroughly mixing 3 ml solution A, 6 ml of the appropriate solution C, 3 ml distilled water and 12 ml ammonium persulphate solution. The mixture was pipetted into the tubes using a 25 ml disposable plastic syringe and was sufficient to fill 16 tubes to the mark. A layer of distilled water was then applied to the top surface by means of a hand-operated spray gun.

This layer ensured that polymerisation was uninhibited by oxygen (section I - 3 - 2) and that the top surface of the polymerised gel was flat and uniform. The tubes were left for one hour at room temperature to allow polymerisation to take place. Tubes were placed out of direct sunlight as a high light level seemed to promote the formation of gas bubbles which distorted the gel.

After polymerisation, the water layer was flicked off and the gel surface dried with tissue paper. Upper gel solution was prepared by mixing one part (by volume) of B, two parts of D and one part of E with four parts of distilled water. The solution was usually prepared in batches of 24 ml and stored at 5°C in a 250 ml dark glass bottle. 0.15 ml of upper gel solution was pipetted into each tube containing lower gel and then covered with a water layer as before. Tubes were placed 10 cm from a 40 watt fluorescent light and photo-polymerised until the appearance of slight opacity indicated that they were ready for use. Tubes prepared in this way were either used immediately or stored at 5°C for up to three days. It was found that the water layer tended to diffuse through the gel during storage giving erratic electrophoretic results. Tubes to be stored, therefore, had the water layer removed as before and were wrapped in 'cling film' to prevent drying out. Similar samples applied to fresh gels and to those stored in this way for five days gave identical electrophoretic patterns.

Electrode buffer was prepared by dissolving 0.3 grm of tris and 1 grm of glycine in 500 ml of distilled water for each apparatus. The buffer differs from that used by Davis (which contained 1.44 grm glycine and 0.3 grm of tris/500 ml) and was found to give better resolution of bands in extracts of Potamopyrgus. The buffer was cooled to 5°C before use.

I - 4 - 3 Electrophoretic method

The stoppers were carefully removed from the tubes and a mark made on the glass with a felt-tip waterproof pen 5 cm from the upper gel/lower gel junction (8 cm for gels in 11 cm tubes). Tubes were then inserted into the upper buffer compartment grommets (see Plate 3) and the space left in the tube by the rubber stopper filled with electrode buffer. 250 ml of electrode buffer was placed in the lower buffer compartment and the apparatus assembled making sure that no air bubbles were trapped beneath the tubes. The upper compartment was

filled with 200 ml electrode buffer and a few drops of bromophenol blue solution were added to mark the front during electrophoresis.

Extracts in 40% sucrose (section I - 2 - 1) were layered on to the upper gel surface using 1 ml plastic syringes. 0.1 ml of extract was used for quantitative studies. Extracts were added carefully to avoid disturbance by air bubbles and vibration. The inclusion of a gel containing no extract showed that no cross-contamination occurred when this method was used. When the extracts had been added to all eight gels the lid containing the cathode was carefully placed in position and the apparatus placed in a refrigerator at 5°C. Voltage was applied (using a Heathkit constant-voltage power pack) and adjusted to give a current of 24 ma (3 ma per tube). An initial voltage of 200 V was required and this dropped to about 180 V during a run, indicating that the resistance of gels changed during a run. The rate of migration of the front varied with the extract applied indicating that differences in extract composition (protein concentration, lipid content, etc.) affect ion flow.

When the front in a particular tube had reached the distance mark, the power was briefly switched off, the tube removed and a rubber stopper placed in the grommet to prevent buffer loss. The current was automatically reduced by the constant voltage facility in the power pack as gels were removed. In this way all tubes could be run to the same distance, making inter-tube comparisons visually simple. Run time was shown to have no effect on banding patterns by running the same extract on four gels for twice as long as normal (80 mins. at 1.5 ma per tube) and comparing these with the same extract run under normal conditions (40 minutes at 3 ma per tube).

Gels were removed from the tubes by inserting a thin 5 cm hypodermic needle between the glass and the gel and, whilst squeezing distilled water out of the syringe, moving the needle around the circumference of the gel. This was done with the distal end of the tube under the surface of distilled water in a plastic dish. With practice, gels could be freed from the tubes quickly and without damage. Gels were then placed in appropriate staining solutions.

After use, tubes were washed thoroughly by brushing the inside surface in hot soapy tap water. They were then rinsed successively with hot tap water and distilled water containing a few drops of detergent. This procedure gave even layering of water on to

the gel surface and prevented gels sticking in the tubes. The tubes were air-dried over a heater and stored in a dry, closed container.

I - 5 Staining Procedures

Gels were stained for general proteins or for iso-enzymes where the following general principle was used:-

$$\text{enzyme} + \text{substrate} \underset{\text{(if any)}}{\overset{\text{cofactors}}{\rightleftharpoons}} \text{product}$$

$$\text{product} \downarrow + \text{soluble dye-coupler} \rightarrow \text{insoluble dye product}$$

Co-factors may be metal ions such as Mg^{2+} and Ca^{2+} or co-enzymes such as NAD. The insoluble dye products appear as bands at the sites of enzyme activity. The intensity of the bands is dependent on the rate of the forward reaction which in turn depends on enzyme activity, enzyme concentration, substrate concentration, pH and temperature. Substrate concentration was usually in excess and the temperature was constant during staining. In most staining procedures the substrate and the dye-coupler were both present in the staining solution (a simultaneous coupling reaction). Staining was carried out in 10 ml test tubes (for disc gels) or in flat dishes (for slab gels).

In order to avoid local depletion of substrate and dye-coupler the tubes were inverted periodically and the dishes placed on a constant-speed horizontal shaker.

I - 5 - 1 Esterases

The general principle behind the detection of esterases is that they are able to hydrolyse the ester bond of an (artificial) substrate releasing a product able to combine with a diazonium salt to give an insoluble dye product. Substrates are commonly α or β-naphthyl esters which release α- or β-naphthol respectively. These combine with the diazonium salt (Fast Garnet GBC (Gurr) was used in the present study) giving an insoluble purple (α-naphthol) or red (β- naphthol) azo dye.

The basic staining solutions contained:

20 mg naphthyl ester (Sigma) dissolved in 0.5 ml acetone.
100 mg Fast Garnet GBC
100 ml 0.2 M sodium phosphate buffer pH6.

The general esterase stain giving the greatest number of bands contained a mixture of α-naphthyl acetate, β - naphthyl acetate and α -naphthyl proprionate (20 mg of each/100 ml of solution). The stain was used for the large-scale surveys described in chapters 4 and 5. Additional substrates were used to determine the substrate specificities of esterases; the use of these is described in section III - 1 - 1.

The phosphate buffer was chosen from a number of standard buffers as it gave a higher hydrolysis rate with most substrates. A stock solution was prepared by dissolving 27.36 grm $Na_2HPO_4 \cdot 12H_2O$ and 8.8 grm $NaH_2PO4 \cdot 2H_2O$ and making up to 2 litres with distilled water.

Gels and staining solutions were kept at room temperature (usually 22°C) in the appropriate containers and periodically inspected. When the gel was optimally stained it was placed in 30% ethanol to stop the reaction and fix the bands.

I - 5 - 2 Acid and Alkaline Phosphatases

The staining method for acid phosphatase was based on that given by Shaw and Prasad.[10] Gels were stained in the following solutions:-

20 mg α-naphthyl acid phosphate (Sigma)
100 mg Gast Garnet GBC (Gurr)
50 mg $MgCl_2 \cdot 2H_2O$.
100 ml sodium acetate buffer pH5.

The acetate buffer was prepared by dissolving 28.1 grm CH_3COONa and 6.87 ml glacial acetic acid in distilled water and making the solution up to 2 litres. 1 grm of $MgCl_2H_2O$ was added and the pH adjusted to 5 with acetic acid. Magnesium was found to be an essential cofactor for the activity of Hydrobiid phosphatases.

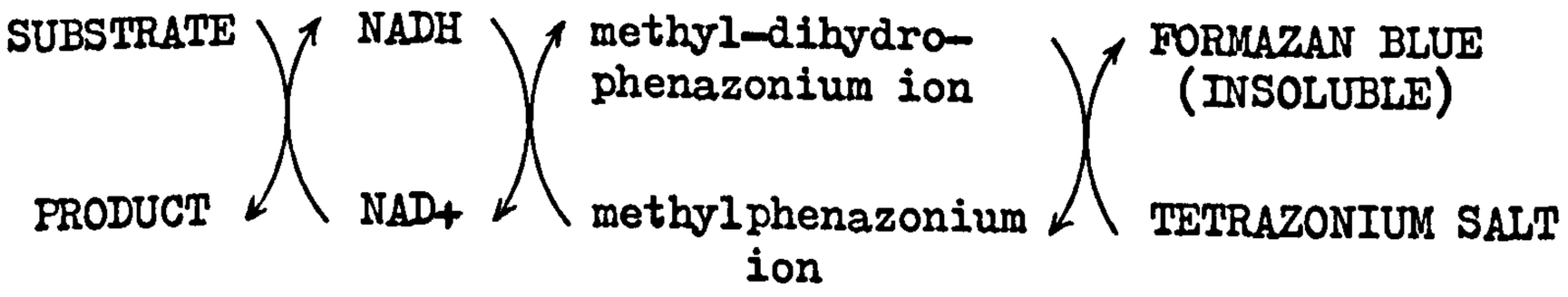
Alkaline phosphatases were stained for using a pH 8.5 0.2 phosphate buffer in place of the acetate buffer used above. Staining procedure was similar to that used for esterases

(section I - 5 - 1) and the reaction was stopped using 30% ethanol as before.

When extracts of P. jenkinsi A and Hydrobia ventrosa were run on 7.5% disc gels and the gels stained for alkaline phosphatase as above, the zymograms obtained were identical to those seen on gels stained for acid phosphatase, although staining was less intense. Extracts run on 5% gels also gave the same banding patterns when stained for acid or alkaline phosphatases and the alkaline phosphatases had the same molecular weights (see section III - 3) and were probably the same isozymes. In view of the higher hydrolysis rate under acid (pH5) conditions, gels were only stained for acid phosphatase in subsequent work.

I - 5 - 3 Dehydrogenases

Detection of dehydrogenases on gels relies on the reduction of a tetrazolium salt to formazan; an insoluble blue dye. The reaction is driven by a hydrogen ion from the dehydrogenation of the substrate by the enzyme. The hydrogen ion is transferred to the tetrazolium salt by NAD and phenazonium methosulphate (PMS).



The substrate is usually the sodium or potassium salt of an organic acid or an alcohol. A more complete account of the theory behind dehydrogenase staining is given by Wilkinson.[11]

'Standard' enzyme staining methods (developed for mammalian and Drosophila[13] studies) do not work for extracts of all organisms.[14,15] Enzyme staining depends on factors such as protein concentration, substrate specificity and pH. Most authors only publish details of successful procedures and consequently information on the suitability of staining procedures for particular phylads of organisms is sparse. This is unfortunate as much time and energy is wasted on unsuccessful techniques and much information is lost on the possible metabolic significance of staining failures.

In order to assess the suitability of dehydrogenase stains

for the study material various stains were tried on disc gels prepared by running mass extracts of P. jenkinsi A, H. ventrosa, H. ulvae and a Drosophila melanogaster extract (prepared from approximately 200 flies homogenised in 20 ml 40% sucrose as described in section I - 2 - 2). Since cofactor requirements may differ between enzymes, each stain was tried with NAD and NADP added together. Gels were stained in different pH conditions by using buffer solutions of pH 6, 7, 8, and 9.

The staining solutions were based on the general recipe:

a) 500 mg. of substrate (if a solid) or 1 ml (if a liquid) was dissolved in buffer at 5°C. If the acid form of the substrate was used then sufficient solid $HaCO_3.H_2O$ was added to adjust the pH to that of the buffer.

b) 20 mg NAD + 20 mg NADP (Sigma)

c) 20 mg NBT (Gurr)

d) 5 mg PMS (Sigma)

made up to 100 ml with cold (5°C) buffer and stored in the dark until needed.

Staining solutions were prepared during electrophoresis runs and never stored for more than 30 minutes. Incubation was carried out at 22°C for 50 mins. Buffer solutions were prepared as follows:

pH6: 1.368 grm $Na_2HPO_4.12H_2O$ + 0.44 grm $NaH_2PO_4.2H_2O$ in 100 ml solution.

pH7: 1.791 grm $NaHPO_4.12H_2O$ + 0.78 grm $NaH_2PO_4.2H_2O$ in 100 ml solution.

pH8: 1.695 grm $Na_2HPO_4.12H_2O$ + 0.042 grm $NaH_2PO_4.2H_2O$ in 100 ml solution.

pH9: 0.2 m tris/HC1

Electrophoresis was performed using the discontinuous pH method described in section I - 4). The results of the experiment are summarised in Table 1. Only MdH was found to stain satisfactorily in the Hydrobiids although Drosophila extracts stained normally (Dickinson and Sullivan was referred to for the expected Drosophila patterns) except in the case of glucose-6-phosphate dehydrogenase where no bands were detected.

TABLE 1 : Results of staining for dehydrogenases at different pH values without adding cofactors to the electrode buffer.

Isozyme	Substrate	pH	Mass extract			Dros.	Dros. Exp.*
			P.j.	H.u.	H.v.		
Lactate dehydrogenase	Lactic acid (+ $Na_2 CO_3$)	6	-	-	-		
		7	-	-	-	1 band	1 band
		8	+/-	-	-	1 band	
		9	-	-	-	1 band	
Alcohol dehydrogenase	iso-propyl alcohol	6	-	-	-		
		7	-	-	-	2 bands	up to 9
		8	-	-	-	3 bands	bands
		9	-	-	-	3 bands	
Malate dehydrogenase	malic acid (+ $Na_2 CO_3$)	6	-	-	-		
		7	+/-	+	+	2 bands	up to 3
		8	+	+	+	3 bands	bands
		9	+	?	?	?	
Hexose-6-dehydrogenase	glucose-6-phosphate	7	-	-	-	-	up to 2
		8	-	-	-	-	bands
Isocitrate dehydrogenase	Isocitric acid (+ $Na_2 CO_3$)	7	-	-	-	1 band	up to 3
		8	-	-	-		bands
Xanthine dehydrogenase	Sodium xanthate	7	-	-	-	2 bands	2 bands
		8	-	-	-		
Glutamate dehydrogenase	Glutamic acid (+ $Na_2 CO_3$)	7	-	-	-	3 bands	multiple
		8	-	-	-		bands
Fumarase	Fumaric acid (+ $Na_2 CO_3$)	7	-	-	-	3 bands	-
		8	-	-	-		

Key: + bands of medium intensity
+/- bands of light intensity
- no bands visible
? diffuse region of stain.

* Expected number of bands from Dickinson and Sullivan[16].

Subsequent to these attempts at staining for dehydrogenases, oxygen inhibition of dehydrogenases was reported.[17] The experiment was re-run using buffer solutions of pH7 and 8 and staining the gels in the dark in a vacuum flask. Single bands appeared on gels of P. jenkinsi A stained for Ldh and Adh; no bands appeared on the H. ulvae or H. ventrosa gels although Drosophila gels stained as before. No extra bands appeared on gels stained for Mdh.

Wilkinson[11] has reported inhibition of dehydrogenases by pyruvate, presumably an important negative feed-back control in the citric acid cycle. A set of eight disc gels was prepared by electrophoresis of a P. jenkinsi A extract and four gels were stained for Idh, Xdh, Ldh and Mdh as before (without vacuum) at pH8. The remaining four were stained with staining solutions to which 0.1 grm hydrazine sulphate (a pyruvate inactivator) per 100 ml of solution had been added. Mdh stained as before (? 3 bands) and Ldh stained as a single band (in the same position as in the vacuum flask experiment) on the gel stained with the inclusion of hydrazine sulphate. No bands appeared on gels stained for Xdh or Idh.

To investigate the effect of including the cofactor in the gel or the buffer (Manwell[18]) an experiment was devised in which NAD (25 mg/l) was added to the upper buffer compartment of one apparatus, the lower compartment of another and to the gel solutions during the preparation of a third. Extracts of P. jenkinsi A, H. ventrosa, H. ulvae and Drosophila were run as before on each apparatus and the gels stained for Mdh with NAD included in the staining solution. Mdh activity in all gels was increased by the addition of NAD either to the gels or to the upper buffer solution. Subsequently, 25 mg NAD/100 ml of buffer was routinely added to the cathodal buffer in both disc and slab electrophoresis when gels were to be stained for Mdh, Ldh or Adh. Addition of NAD to the cathodal buffer was tried before staining for Idh, Xdh, fumarase and G-6-Pdh, with no success.

It was thought that the lack of success experienced in staining for dehydrogenases might have been due to powerful non-specific proteases (lysozymes) hydrolysing dehydrogenases during the extraction procedure. This possibility was investigated by mixing Drosophila extracts with equal volumes of the snail extracts and electrophoresing the mixtures after they had stood at room temperature (approx 22°C) for 30 minutes. A control of Drosophila extract + an equal volume of

distilled water was used. The gels were stained for fumarase as before (Table 1) and three bands appeared on the experimental gels and on the control in the same positions as before. It is, therefore, unlikely that proteases are responsible for the lack of dehydrogenase activity found in the Hydrobiid extracts.

Wool[19] has recently reported similar difficulties in staining dehydrogenases in extracts of the parthenogenetic aphid Myzus persicae. These difficulties may indicate either a fundamental difference in metabolism between Drosophila (and mammals) and these organisms - an unlikely explanation - or that the conditions for optimal activity of these enzymes lie outside the scope of the methods tried.

I - 5 - 4 Tetrazolium oxidase (TO)

Gels stained for dehydrogenases have a blue background stain due to the light-induced deposition of formazan. TO bands appeared as clear zones against this background as TO oxidises the tetrazolium salt (NBT) in these areas, preventing the formation of formazan. Bands were seen more easily (on disc gels) when the gels were briefly exposed to daylight. Although TO was visible on slab gels, resolution was poor and TO was not routinely scored in the population surveys. TO is now thought to be due to superoxide dismutase (SOD).[20]

I - 5 - 5 Amylase (Amyl)

A technique for detecting amylase was developed based on the enzymatic hydrolysis of starch.

Starch incorporated into the gel solutions tended to (rather unpredictably) migrate out of the polymerised gel during electrophoresis. Robson[21] has recently reported that inclusion of starch in acrylamide gels on which Asellus extracts were run led to the formation of (artifactual) multiple bands probably due to the formation of enzyme-substrate complexes.

A more satisfactory method was devised in which gels were soaked, after electrophoresis, in a 1% starch solution at room temperature for two hours. Gels were then washed thoroughly with two changes of distilled water and flooded with iodine vapour. Clear

amylase bands appeared against a blue background. Bands were too diffuse to score on slab gels but resolution was good on disc gels.

I - 5 - 6 Peroxidase (Perox)

The usual substrates used in peroxidase stains are benzidine and o-dianisidine; both are highly dangerous carcinogens. Liu and Gibson[22] have developed a method using eugenol (2 - methoxy - 4 - allyl-phenol) and hydrogen peroxide which is much safer to use. Peroxidase in the presence of hydrogen peroxide catalyses the formation of free radicals of eugenol which condense to form a white precipitate. The precipitate is intensely fluorescent under UV light.

Gels were soaked after electrophoresis in a solution prepared by dissolving 75 mg eugenol in 100 ml 0.05 m phosphate buffer (pH6) to which 10 ml of 30% w/v H_2O_2 was added just before use. After soaking, gels were placed under short-wave UV light and bluish fluorescent bands were clearly visible. The positions of bands relative to the front were quickly measured using a clear plastic rule as the fluorescence faded after about ten minutes. Peroxidase was only clearly resolvable on disc gels.

I - 5 - 7 General Protein

Gels were stained in a Coomassie Blue/trichloro-acetic acid (TCA) staining solution based on that described by Sedmark and Grossberg[23] for staining mammalian proteins.

A stock staining solution was prepared by first dissolving 1.1 grm of Coomassie Blue G 250 (Gurr) in 330 ml methanol and stirring for 30 minutes. The solution was poured into 670 ml distilled water and mixed well. 130 grm TCA were added and dissolved by stirring for a further 30 minutes. The solution was filtered (Whatman No. 1) and stored in a dark glass bottle at room temperature.

Gels were first fixed in 20% TCA W/V/20% V/V isopropanol solution for 15 minutes, and then transferred to the stain for 2 hours at 40°C. Destaining was carried out in an 8:3:1 mixture of water: ethanol:glacial acetic acid. The destaining mixture was freshly made up as ethyl acetate formed on storage making the solution less efficient. Destaining usually took between 10 and 15 hours at room temperature (22°C) and was judged to be complete when blue bands were clearly visible

against a light blue background. Destaining was stopped by transferring gels to 30% glycerol solution.

I - 5 - 8 Stains (other than dehydrogenases) tried without success

The following stains were tried on disc gels prepared by running extracts of P. jenkinsi A, H. ulvae and H. ventrosa. No bands (or faint uninterpretable bands) resulted from these methods:-

ENZYME	BUFFER	RESULT	REFERENCE
Catalase	0.05 m phosphate pH7	No zones	Brewer[24]
Leucine amino peptidase	0.5 m tris/ maleic acid pH6	Diffuse zone in P.jenkinsi	Shaw & Prasad[10]
Hexokinase	tris/HC1 pH8	No zones	Shaw & Prasad[10]
∝-glucuronidase	0.2 m phosphate pH6.9	No zones	Coles[25]
∝-glucopyranos-idase	0.2 m phosphate pH6.9	No zones	Coles[25]
sulphatase	0.2 m phosphate pH6	Vague zone in P.jenkinsi	Shaw & Prasad[10]
Peroxidase	0.1 m citric acid pH5	No zones	Coles[25]
Phosphogluco-mutase	0.1 m tris/ HC1 pH7.1	No zones	Spencer et. al.[26]
Hexokinase	0.1 m tris/ HC1 pH7	No zones	Shaw & Prasad[10]

I - 6 Storage of Gels

I - 6 - 1 Disc Gels

Disc gels were normally stored at room temperature in 30% ethanol in stoppered 10 ml glass test-tubes. Providing the ethanol covered the entire gel and the tubes were placed in the dark, no deterioration in band intensity or resolution occurs for several years.[27] Gels stored in this way shrink due to loss of buffer into the ethanol but a comparison of gels run with the same (deep-frozen at -20°C) extract before and after storage for 30 days (by which time maximum shrinkage had occurred) showed that shrinkage was uniform over the whole gel and that no change in R_f values (of esterases) (R_f - see section II - 1 - 1) had occurred. Gels stained for general protein were stored in 30% glycerol in the same way as those stored in ethanol.

I - 6 - 2 Slab gels

Slab gels were removed after 3 hours from the 30% ethanol used to stop the staining reaction (section I - 5 - 1) and placed in 500 ml 70% ethanol in a plastic dish fitted with a lid. The dish was placed on a horizontal shaker for 2 hours and the 70% ethanol then discarded. 500 ml of absolute ethanol was then added and the dish shaken for a further 12 hours. The dehydrated gels were removed and air-dried.

The technique produced hard opaque 'gels' which were easily labelled and handled. The gels shrank to about a quarter of their original size during dehydration with no change in the R_m (see section II - 1 - 1) values of bands. Faint bands were intensified during the process and were more easily scored on dehydrated gels. No noticable loss in intensity of bands was apparent after 2 years' storage in the dark.

I - 7 Photography of gels

Gels were photographed using 35 mm Kodak Plus-X pan-chromatic film in a camera mounted above a horizontal copy table. Disc gels were usually removed from their storage tubes for photography and placed on a Kodak "Cold-light" light box to provide back-illumination.

Dehydrated slab gels were placed on the copy table and illuminated from above using four "Photoflood" bulbs arranged around the gel. Shutter speeds and aperture settings were determined by using the built-in light meter on the camera.

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CHAPTER II : ISOZYME AND PROTEIN PATTERNS OF SOME BRITISH AND AUSTRALIAN HYDROBIIDAE

II - 1 General Banding Patterns

Electrophoresis of snail extracts on 7.5% slab and disc gels stained as described in section I - 5 gave the banding patterns depicted in Figs. 4 - 12.

No estimate of variation between individuals is included; each figure has been built up by pooling data from all individuals sampled from a species in order to develop a system of nomenclature for the bands. Not all of the bands depicted in Figs. 4 - 12 were necessarily found in any one snail. Bands have been included which were specific to the specialised substrates used in section III - 1 - 7. Electrophoresis of mass extracts (section I - 2 - 2) was used to compile some of the banding patterns (see section VI - 2) and, due to the 'dilution effect' described in section III - 1 - 3), rare bands may be absent from these figures.

II - 1 - 1 Recording of Banding Patterns

Two methods of recording the positions of bands were used:-

i) R_f value: applicable only to gels run with discontinuous buffer systems, the R_f ('relative to the front') value is the distance moved by a band relative to the distance moved by the front (a region of changing pH and negatively charged small molecules rendered visible by the inclusion of bromophenol blue in the cathodal buffer).

ii) R_m value: the distance moved by a band relative to the distance moved by some other, arbitrarily chosen, reference band. Bands running in front of the reference band have an R_m value greater than one. Continuous pH gels do not develop a front during electrophoresis and so only R_m values can be calculated for bands separated by this system.

The band used as a reference for the calculation of R_m was Est. 9 in *P. jenkinsi A.* The band appeared with a high frequency in populations of the *Potamopyrgus* species (Chapter IV) and was clearly

Fig. 4: Composite banding patterns found in populations of P. jenkinsi A.

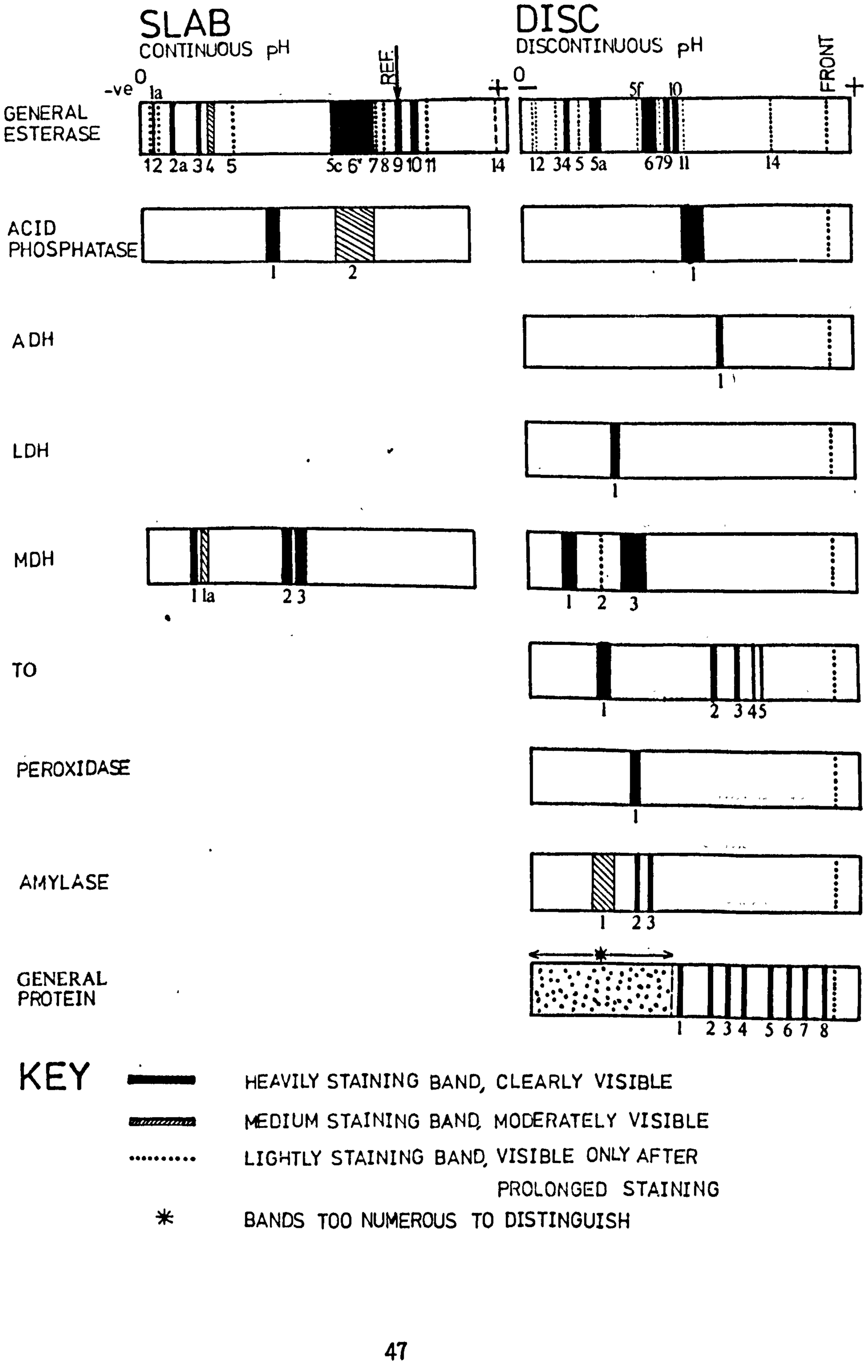


Fig. 5: Composite banding patterns found in populations of P. jenkinsi B.

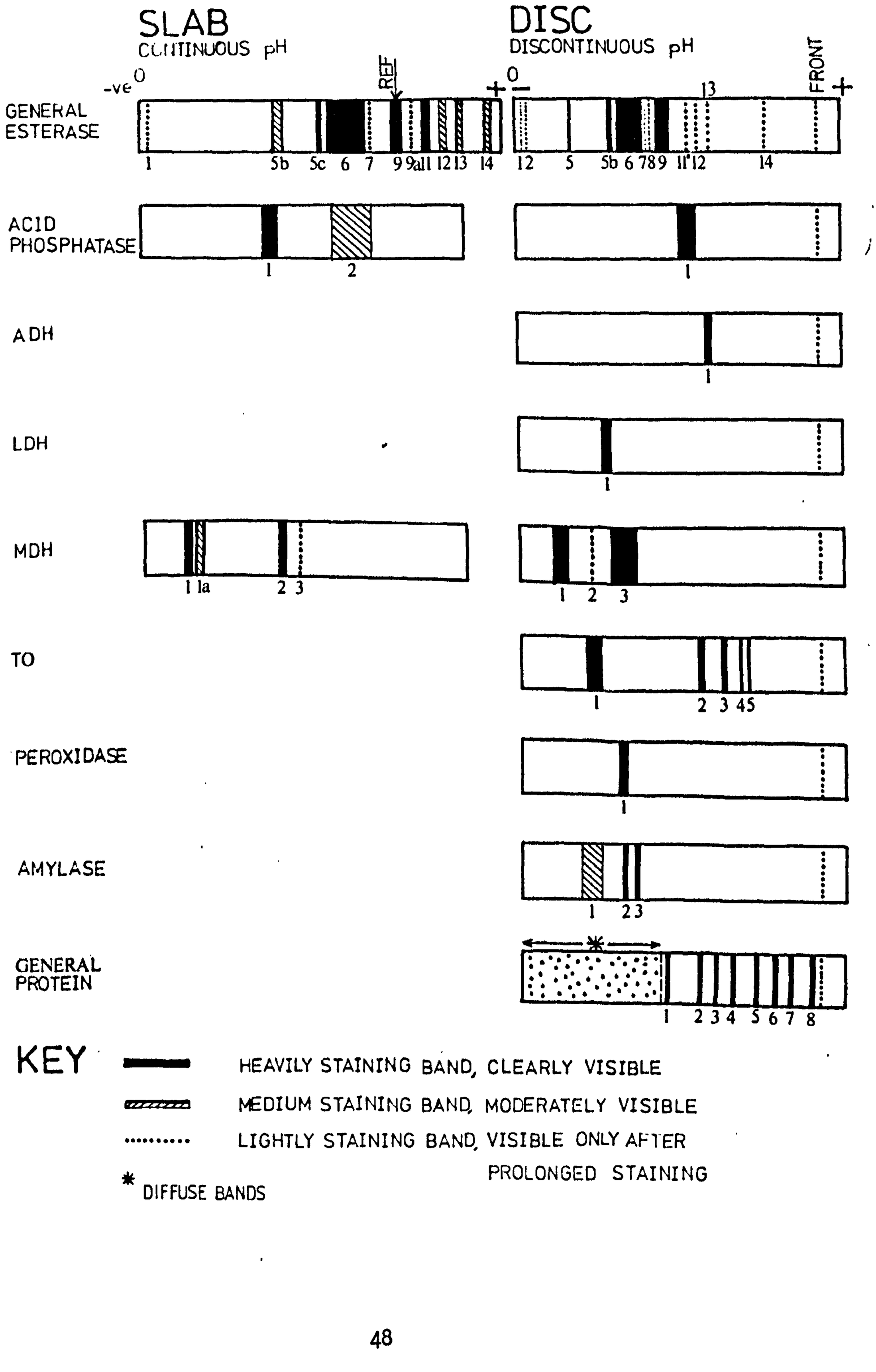


Fig. 6: Composite banding patterns found in populations of P. jenkinsi C.

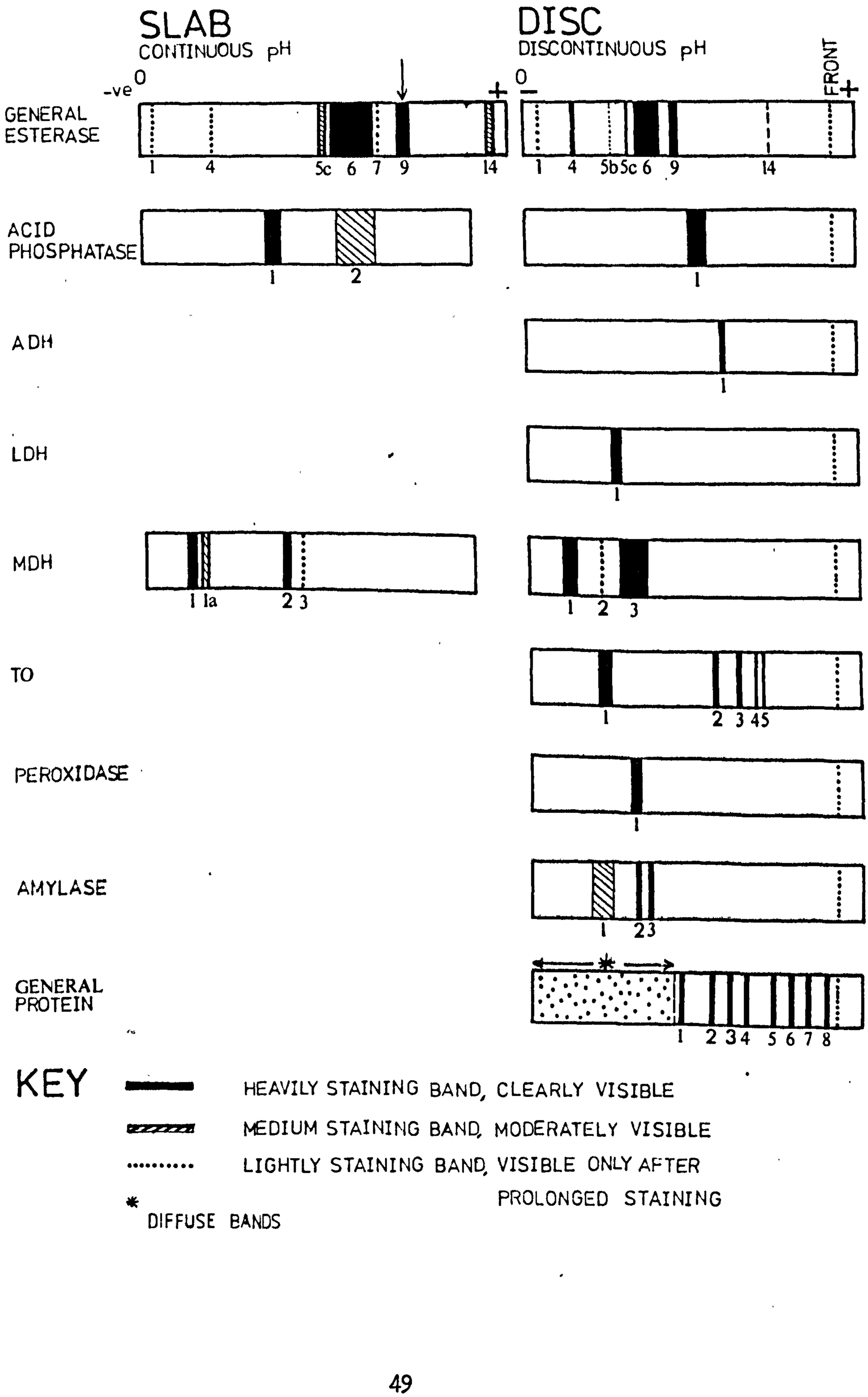


Fig. 7: Composite banding patterns found in populations of P. antipodarum.

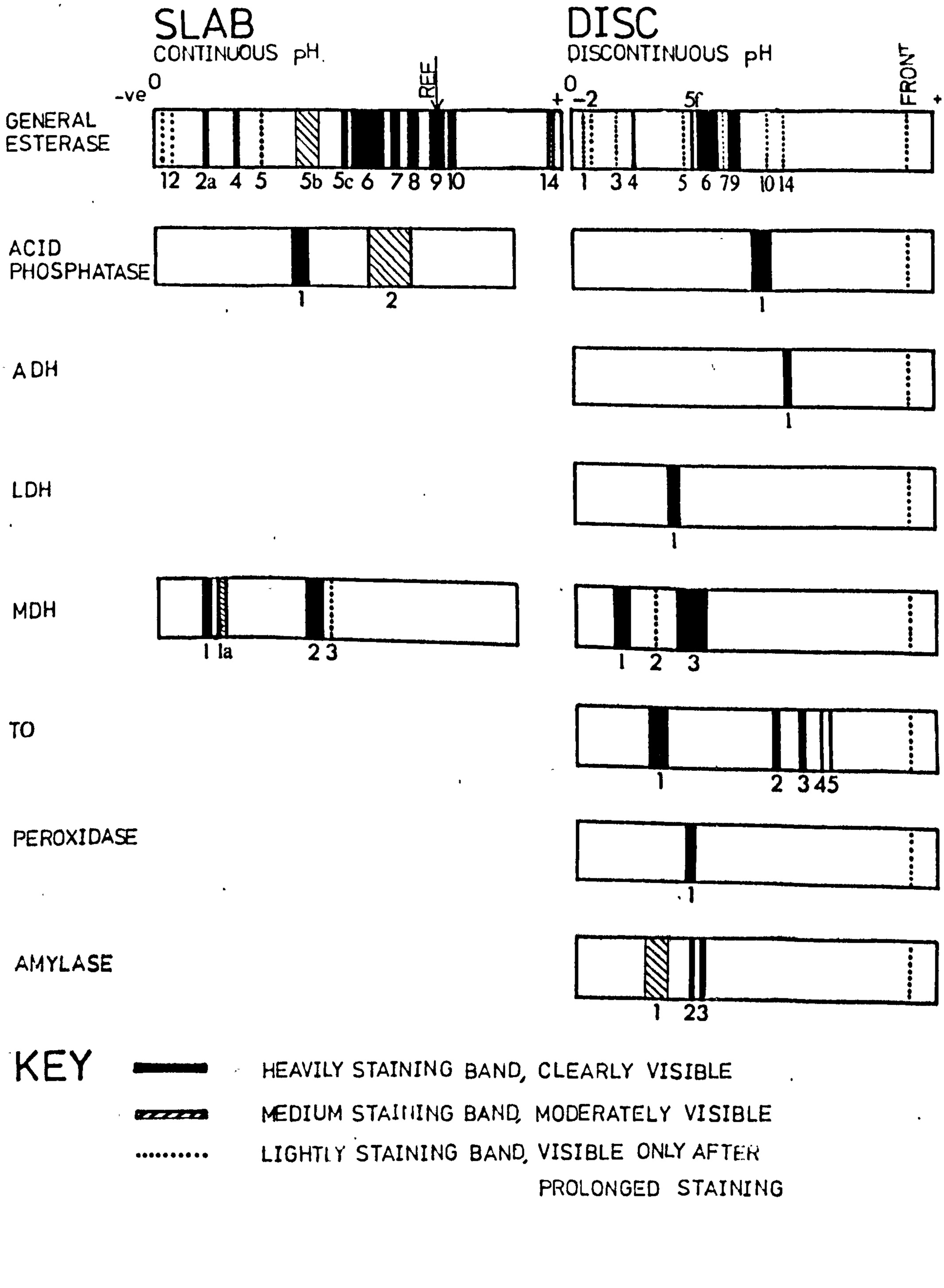


Fig. 8: Composite banding patterns found in populations of P. nigra.

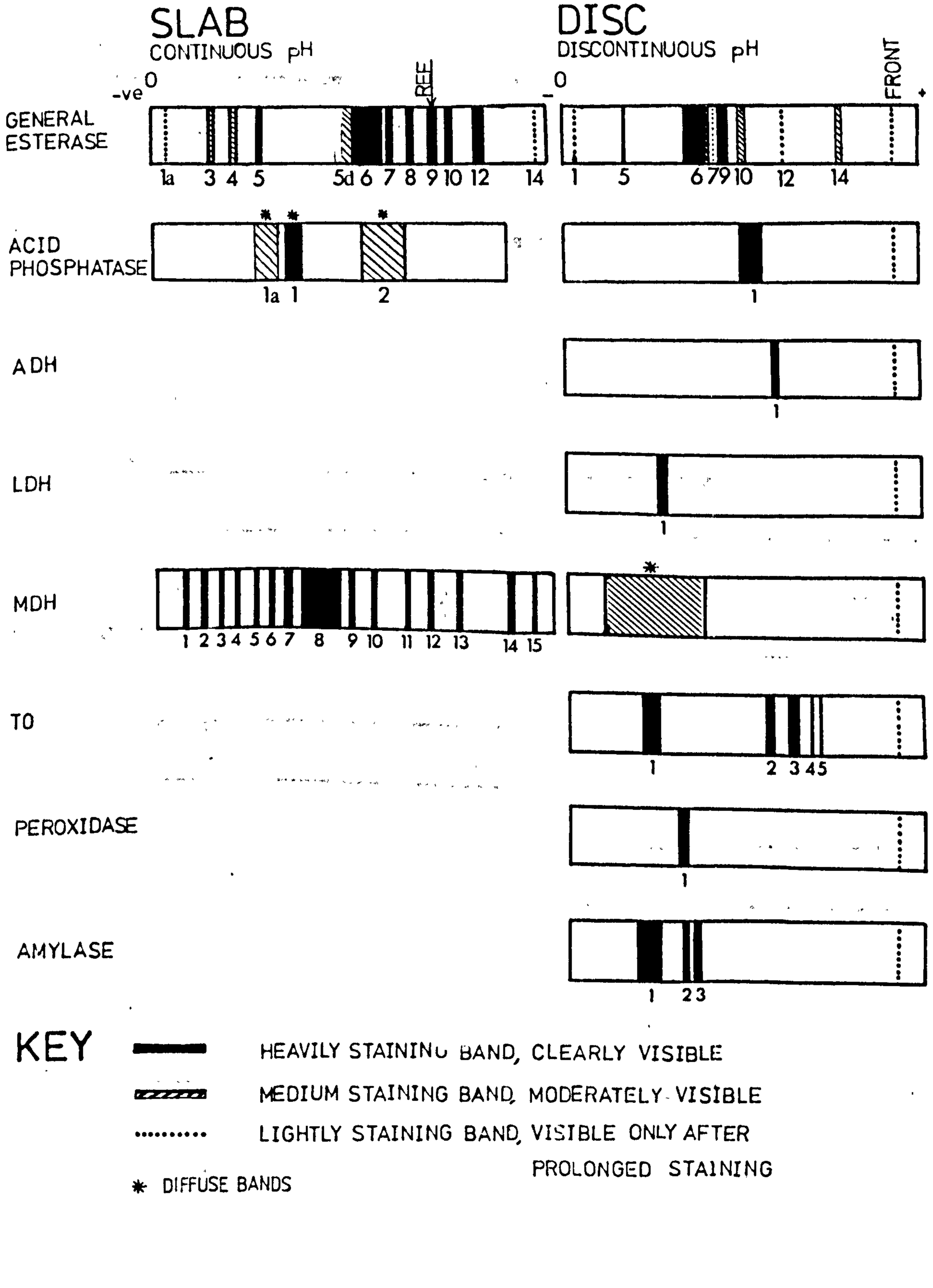


Fig. 9: Composite banding patterns found in populations of P. estuarinus.

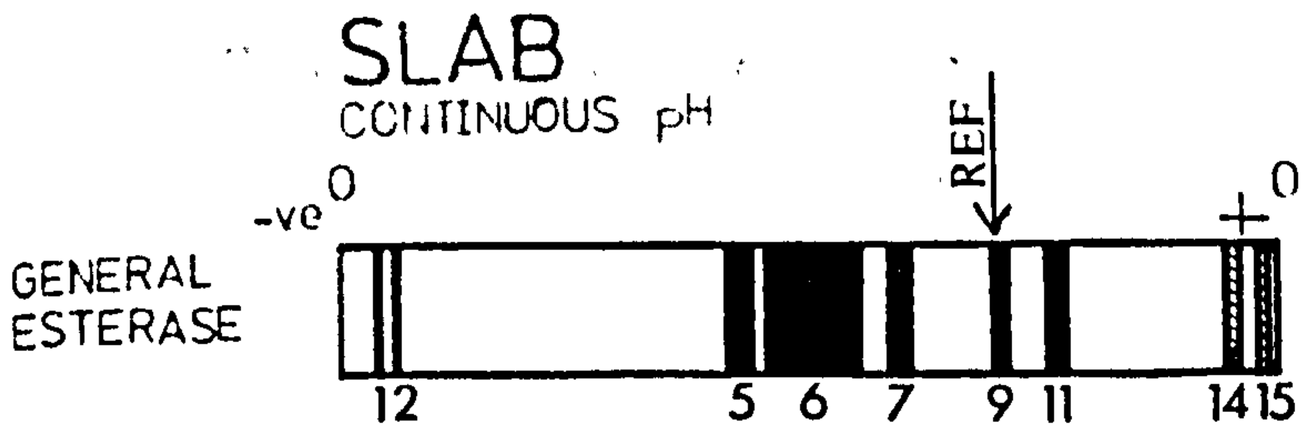
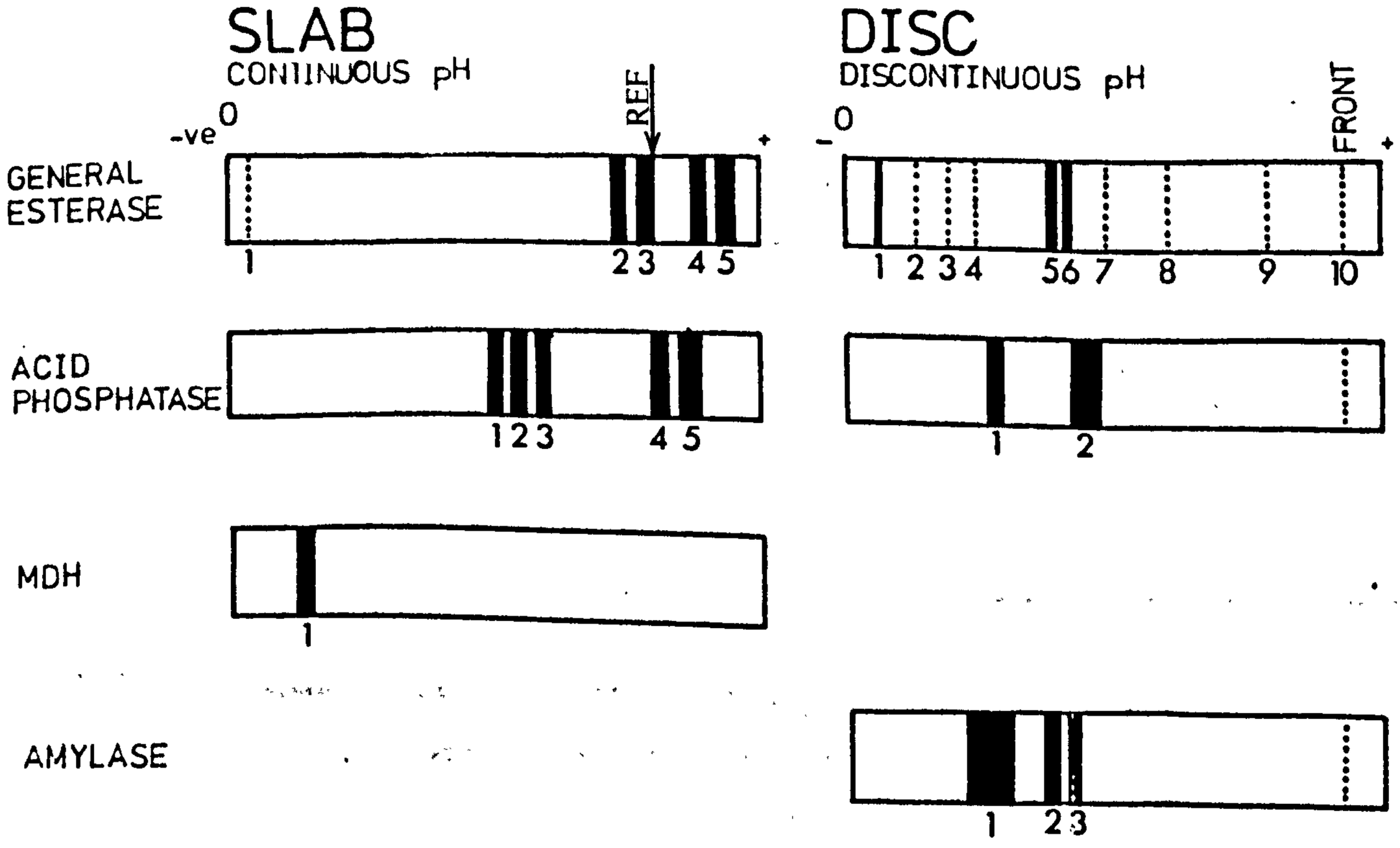


Fig. 10: Composite banding patterns found in populations of H. ventrosa.



KEY

HEAVILY STAINING BAND, CLEARLY VISIBLE

MEDIUM STAINING BAND, MODERATELY VISIBLE

LIGHTLY STAINING BAND, VISIBLE ONLY AFTER PROLONGED STAINING

Fig. 11: Composite banding patterns found in populations of H. ulvae.

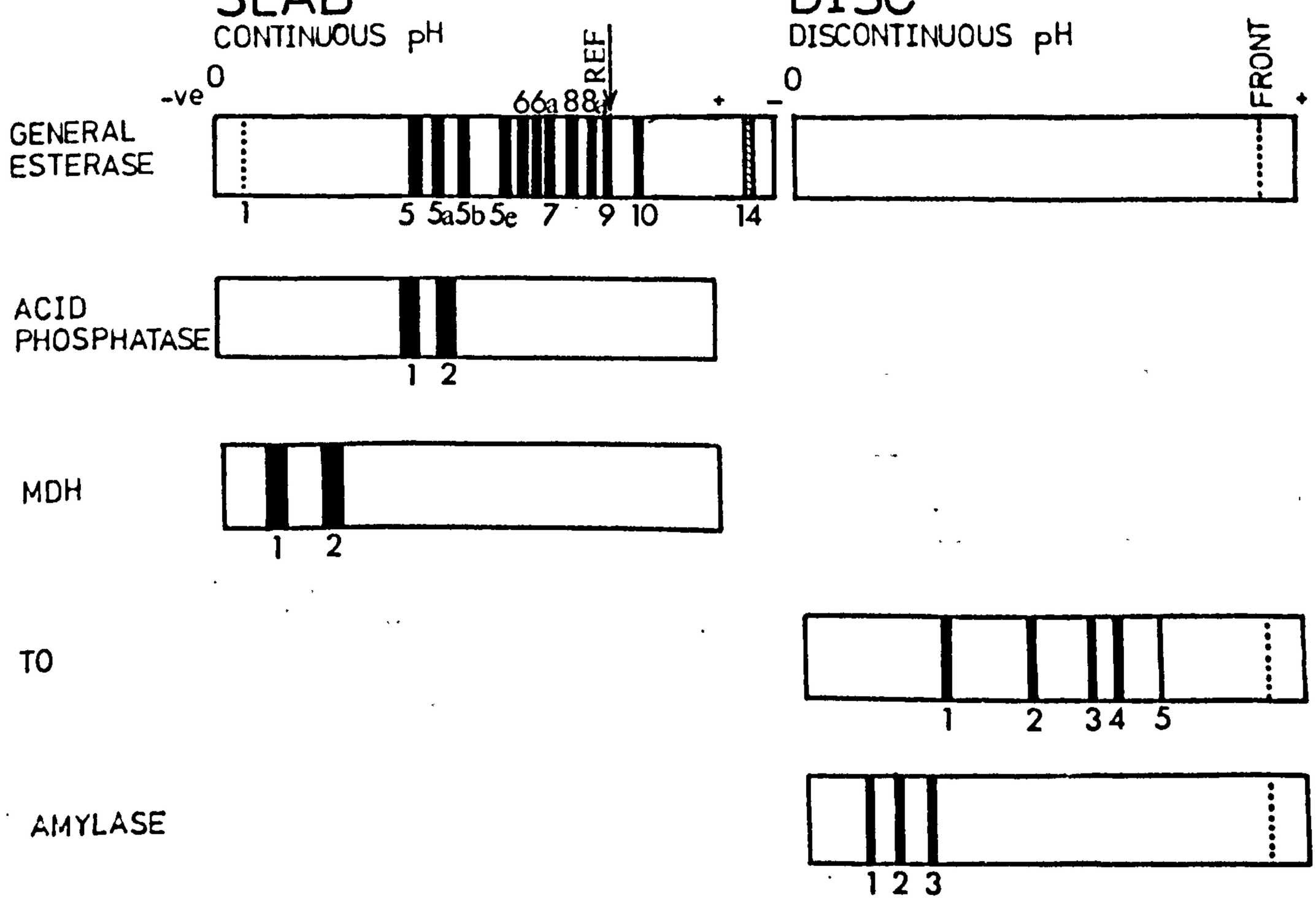
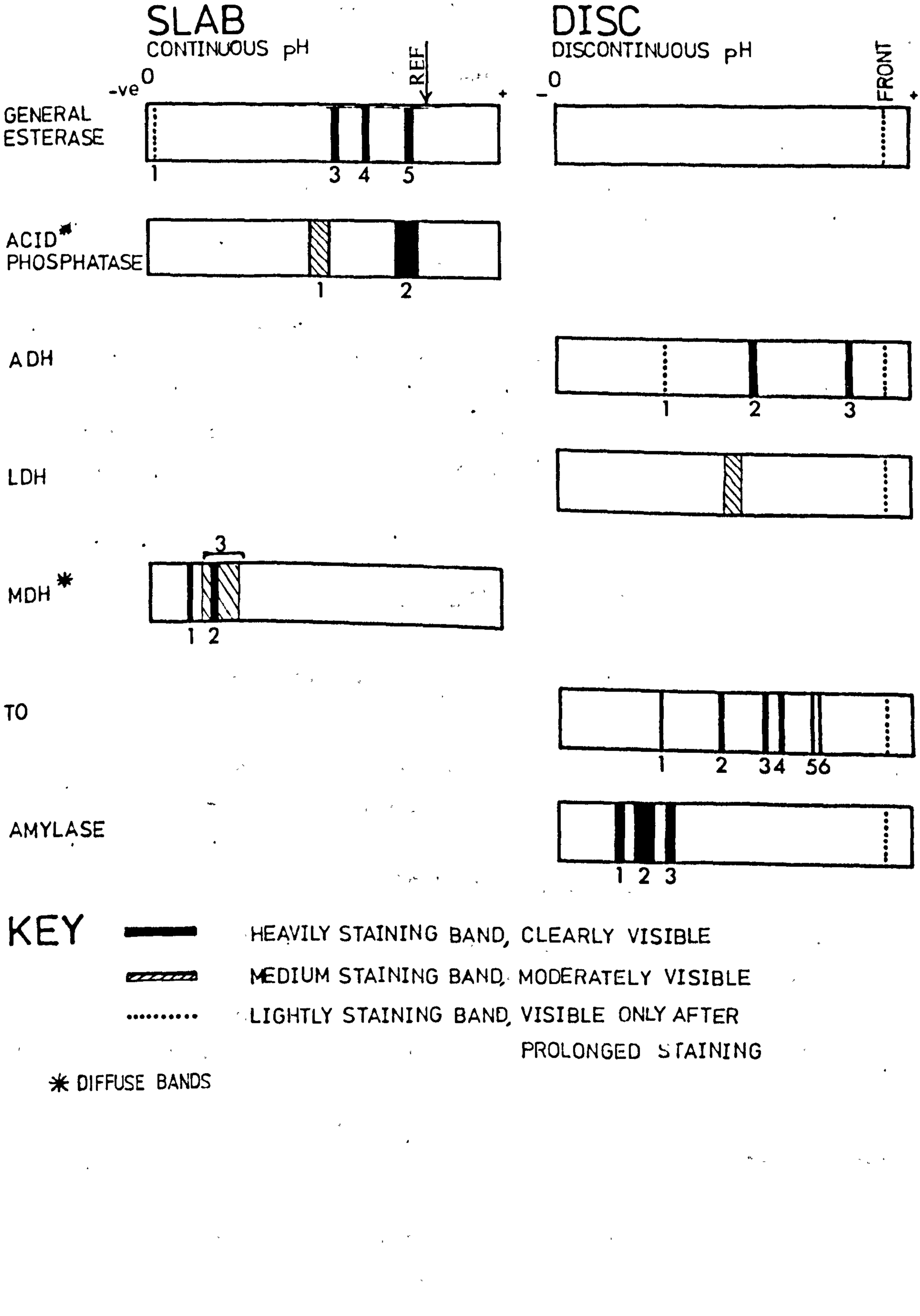


Fig. 12: Composite banding patterns found in populations of H. neglecta.



visible on slab gels stained for general esterase (section I - 5 - 1). Samples lacking Est. 9 (e.g. some Hydrobia populations) were run on the same gel as samples of Potamopyrgus containing the band. Errors caused by differences between gels were avoided by using this method.

Relative mobilities of bands resulting from stains other than esterase were estimated by slicing the slab gel (section I - 3 - 4) and staining one slice for esterase and the other slices for the desired isozyme. The position of Est. 9 was measured and used as a reference for bands appearing on the other slices. These measurements were made by placing gel slices on the Kodak illuminator before they were dehydrated in order to avoid errors due to the unequal shrinkage of slices (stained in different ways) during dehydration.

The positions of bands on dehydrated slab gels were measured by placing a graduated rule against the gel and reading off the relevant measurements to the nearest 0.5 mm. Disc gels were removed from their storage tubes, placed on the illuminator, and the positions of bands and the front measured in the same way.

R_m and R_f values were then calculated from:-

$$R_m = \frac{\text{Distance of study band (mm) from the origin}}{\text{Distance of reference band (mm) from the origin}}$$

and

$$R_f = \frac{\text{Distance of the study band (mm) from origin}}{\text{Distance of the front (mm) from origin}}$$

Where the origin was the junction between upper and lower gels for disc gels and the bottom of the sample well for slab gels.

II - 1 - 2 Statistics calculated for R_m and R_f values

Whenever possible, mean R_m and R_f values were calculated from at least ten measurements taken from gels run at different times in order to include variation caused by minor differences in running conditions. Fewer measurements were available for rare bands.

Mean R_m and R_f values for the bands shown in Figs. 4 - 12 are given in Tables 2 - 10. 95% confidence limits were estimated by multiplying the standard error of the mean by the appropriate t ($p = 0.05$) value. For sets of data where $n > 30$, $t = 1.96$ was used; for smaller sets the value of $t_{(n-1)}$ was used.

Continuous pH						Discontinuous pH					
Band		Rm	n	95% CL	C*	Band		Rf	n	95% CL	C*
Est.	1	0.0412	30	0.0012	0.0842	Est.	1	0.0321	30	0.0020	0.1762
	1a	0.0571	2	-	-		2	0.0482	30	0.0026	0.1550
	2	0.0729	30	0.0018	0.0679		3	0.1107	12	0.0035	0.0512
	2a	0.1430	2	-	-		4	0.1482	26	0.0016	0.0279
	3	0.2253	12	0.0062	0.0437		5	0.1857	18	0.0016	0.0170
	4	0.2746	14	0.0086	0.0550		5a	0.2410	8	0.0054	0.0277
	5	0.3537	18	0.0048	0.0271		5f	0.3857	3	0.0215	0.0304
	5c	0.6711	20	0.0081	0.0346		6	0.4196	30	0.0097	0.0652
	6*	0.7562	30	0.0140	0.0518		7	0.4536	24	0.0080	0.0422
	7	0.8431	30	0.0090	0.0299		9	0.4804	30	0.0032	0.0185
	8	0.9110	30	0.0055	0.0170		10	0.5089	30	0.0027	0.0151
	9	1.000	30	-	-		11	0.5321	30	0.0078	0.0408
	10	1.0640	30	0.0056	0.0147		14	0.8220	5	-	-
	11	1.1074	30	0.0123	0.0310	AP	1	0.5554	30	0.0071	0.0355
	14	1.3793	21	0.0069	0.011	Mdh	1	0.1339	30	0.0015	0.0320
Acid pH	1	0.4968	30	0.0040	0.0225		2	0.2375	12	0.0059	0.0397
	2	0.8274	30	0.0150	0.0506		3	0.3429	30	0.0033	0.0271
Mdh	1	0.1705	30	0.0019	0.0305	TO	1	0.2393	10	0.0045	0.0264
	2	0.5432	30	0.0057	0.0291		2	0.6000	6	0.0317	0.0529
	3	0.6054	30	0.0134	0.0619		3	0.6768	8	0.0280	0.0510
							4	0.7286	8	0.0341	0.0575
							5	0.7518	8	0.0259	0.0423
						Perox	1	0.3411	4	0.0193	0.0407
						Amyl	1	0.2357	4	-	-
							2	0.3464	5	-	-
							3	0.3893	5	-	-
						GP	1	0.4875	3	-	-
							2	0.5893	4	-	-
							3	0.6446	4	-	-
							4	0.6929	4	-	-
							5	0.7821	4	-	-
							6	0.8429	4	-	-
							7	0.8964	4	-	-
							8	0.9589	4	-	-
						Adh	1	0.6357	4	-	-
						Ldh	1	0.2893	4	-	-

TABLE 2:

Potamopyrgus jenkinsi A:

Relative positions of bands on 7.5% gels: see test for details of statistics.

Band		Continuous pH Rm	n	95% CL	C*
Est.	1	0.0274	30	0.0012	0.1230
	5b	0.5432	30	0.0092	0.0471
	5c	0.6711	30	0.0078	0.0323
	6*	0.7521	30	0.0092	0.0342
	7	0.8430	30	0.0096	0.0318
	9	0.9988	30	-	-
	11	1.1179	15	0.0207	0.0336
	12	1.1953	15	0.0278	0.0422
	13	1.2500	5	-	-
	14	1.3667	18	0.0247	0.0364
A.P.	1	0.5032	30	0.0116	0.0618
	2	0.8295	30	0.0130	0.0423
Mdh	1	0.1684	30	0.0028	0.0448
	2	0.5368	30	0.0073	0.0326
	3	0.6063	30	0.0116	0.0515

Band		Discontinuous pH Rf	n	95% CL	C*
Est.	1	0.0214	30	0.0014	0.1820
	2	0.0433	30	0.0018	0.1132
	5	0.1786	18	0.0038	0.0425
	5b	0.3393	22	0.0077	0.0513
	5f	0.3879	19	0.0134	0.0715
	6	0.4089	29	0.0069	0.0444
	7	0.4321	18	0.0133	0.0617
	8	0.4464	30	0.0058	0.0351
	9	0.4875	30	0.0047	0.0260
	11	0.5711	9	-	-
	12	0.6003	9	-	-
	13	0.6334	7	-	-
	14	0.8149	9	-	-
A.P.	1	0.5678	18	0.0174	0.0615
Adh	1	0.6304	7	0.0194	0.0333
Ldh	1	0.2875	8	0.0195	0.0811
Mdh	1	0.1339	21	0.0047	0.0775
	2	0.2357	14	0.0060	0.0438
	3	0.3429	14	0.0092	0.0466
TAO	1	0.2429	15	0.0139	0.1035
	2	0.5929	15	0.0183	0.0560
	3	0.6714	15	0.0234	0.0632
	4	0.7268	15	0.0167	0.0415
	5	0.7536	15	0.0167	0.0400
Perox	1	0.3339	4	-	-
Amyl	1	0.2357	5	-	-
	2	0.3411	5	-	-
	3	0.3804	5	-	-
G.P.	1	0.4857	4	-	-
	2	0.5929	4	-	-
	3	0.6446	4	-	-
	4	0.7018	4	-	-
	5	0.7821	4	-	-
	6	0.8429	4	-	-
	7	0.8964	4	-	-
	8	0.9643	4	-	-

TABLE 3:

Potamopyrgus jenkinsi B:

Relative positions of bands on 7.5% gels:

see text for details of statistics.

Band		Continuous pH Rm	n	95% CL	C*	Band		Discontinuous pH Rf	n	95% CL	C*
Est.	1	0.0379	30	0.0059	0.4221	Est.	2	0.0515	5	-	-
	4	0.2653	28	0.0068	0.0666		4	0.1536	21	0.0069	0.0993
	5c	0.6700	4	-	-		5	0.1826	23	0.0071	0.0898
	6*	0.7610	30	0.0108	0.0382		5b	0.2768	14	0.0098	0.0612
	7	0.8431	22	0.0164	0.0441		5c	0.3000	28	0.0037	0.0320
	9	0.9977	30	-	-		5e	0.3518	8	0.0192	0.0654
	14	1.3559	13	0.0206	0.0253		5f	0.3851	2	-	-
A.P.	1	0.4989	15	0.0132	0.0482		6	0.4286	30	0.0076	0.0472
	2	0.8232	24	0.0117	0.0337		9	0.4875	30	0.0091	0.0499
Mdh	1	0.1705	30	0.0043	0.0669		14	0.8113	5	-	-
	2	0.5347	30	0.0142	0.0712	A.P.	1	0.5604	18	0.0607	0.2177
	3	0.5979	30	0.0164	0.0734	Adh	1	0.6315	5	-	-
						Ldh	1	0.2878	5	-	-
						Mdh	1	0.1339	8	-	-
							2	0.2393	8	-	-
							3	0.3446	8	-	-
						TAO	1	0.2393	4	-	-
							2	0.6036	4	-	-
							3	0.6750	4	-	-
							4	0.7321	4	-	-
							5	0.7536	4	-	-
						Perox	1	0.3393	4	-	-
						Amyl	1	0.2375	5	-	-
							2	0.3446	5	-	-
							3	0.3893	5	-	-
						G.P.	1	0.4964	4	-	-
							2	0.5892	4	-	-
							3	0.6464	4	-	-
							4	0.6964	4	-	-
							5	0.7804	4	-	-
							6	0.8393	4	-	-
							7	0.8911	4	-	-
							8	0.9571	4	-	-

TABLE 4:

Potamopyrgus jenkinsi C:

Relative positions of bands on 7.5% gels: see text for details of statistics.

Band	Continuous pH Rm	n	95% CL	C*
Est. 1	0.0211	21	0.0021	0.2162
2	0.0632	21	0.0053	0.1855
2a	0.1789	13	0.0056	0.0520
4a	0.2884	14	0.0069	0.0416
5	0.3721	8	0.0187	0.0602
5b	0.5474	12	0.0272	0.0783
5c	0.6737	12	0.0137	0.0320
6*	0.7579	28	0.0275	0.0936
7	0.8463	26	0.0035	0.0103
8	0.9094	13	0.0347	0.0631
9	0.9948	5	-	-
10	1.0526	7	0.0082	0.0084
14	1.4130	16	0.0075	0.0100
A.P. 1	0.4973	14	0.0054	0.0187
2	0.8337	17	0.0271	0.0632
Mdh 1	0.1685	30	0.0017	0.0276
2	0.5474	21	0.0467	0.1873
3	0.6063	8	0.0764	0.1546

Band	Discontinuous pH Rf	n	95% CL	C*
Est. 1	0.0229	30	0.0008	0.1587
2	0.0421	22	0.0038	0.2061
3	0.1286	9	0.0112	0.1132
4	0.1821	14	0.0034	0.0319
5b	0.3379	16	0.0043	0.0240
5f	0.3571	21	0.0054	0.0322
6	0.4018	28	0.0189	0.1211
7	0.4500	14	0.0079	0.0303
9	0.4839	30	0.0066	0.0365
10	0.5244	3	0.0578	0.0444
14	0.8256	24	0.0218	0.0626
A.P. 1	0.5643	8	0.0074	0.0157
Adh 1	0.6357	4	-	-
Ldh 1	0.2875	4	-	-
Mdh 1	0.1357	10	0.0031	0.0323
2	0.2321	14	0.0039	0.0290
3	0.3393	14	0.0091	0.0463
TAO 1	0.2446	2	-	-
2	0.5911	2	-	-
3	0.6732	2	-	-
4	0.7286	2	-	-
5	0.7536	2	-	-
Perox 1	0.3393	2	-	-
Amyl 1	0.2464	2	-	-
2	0.3482	2	-	-
3	0.3804	2	-	-

TABLE: 5

Potamopyrgus antipodarum :

Relative positions of bands

on 7.5% gels:

see text for details of statistics.

Band	Continuous pH Rm	n	95% CL	C*
Est. 1a	0.0463	11	0.0094	0.3025
3	0.2353	22	0.1260	0.1212
4	0.3030	6	0.3150	0.0991
5	0.3847	22	0.0489	0.2865
5d	0.6968	24	0.0093	0.0316
6*	0.7726	30	0.0237	0.0821
7	0.8484	26	0.0061	0.0179
8	0.9263	9	0.0158	0.0222
9	1.0061	30	-	-
10	1.0631	13	0.0185	0.0288
12	1.1705	5	0.0891	0.0613
14	1.3621	28	0.0311	0.0588
A.P. 1a	0.4042	8	-	-
1	0.4989	9	-	-
2	0.8253	11	-	-
Mdh 1	0.0956	3	-	-
2	0.1618	4	-	-
3	0.2218	6	-	-
4	0.2758	6	-	-
5	0.3453	2	-	-
6	0.4042	2	-	-
7	0.4632	4	-	-
8	0.5789	2	-	-
9	0.6905	3	-	-
10	0.7708	5	-	-
11	0.8947	6	-	-
12	0.9726	8	-	-
13	1.0800	3	-	-
14	1.2611	7	-	-
15	1.3474	9	-	-

Band	Discontinuous pH Rf	n	95% CL	C*
Est. 1	0.0215	15	0.0015	0.1231
5	0.1839	16	0.0029	0.0294
6	0.3946	16	0.0073	0.0349
7	0.4485	19	0.0083	0.0382
9	0.4839	20	0.0072	0.0319
10	0.5199	4	0.0225	0.0312
12	0.6091	5	0.0427	0.0565
14	0.8122	11	0.0485	0.0888
A.P. 1	0.5714	11	0.0259	0.0677
Adh 1	0.6286	5	-	-
Ldh 1	0.2893	5	-	-
Mdh +	-	-	-	-
TAO 1	0.2446	1	-	-
2	0.6036	2	-	-
3	0.6750	2	-	-
4	0.7268	2	-	-
5	0.7554	2	-	-
Perox 1	0.3411	1	-	-
Amyl 1	0.2411	1	-	-
2	0.3536	1	-	-
3	0.3929	1	-	-

TABLE 6:

Potamopyrgus nigra :
Relative positions of bands on 7.5% gels: see text for details of statistics.

Band		Continuous pH Rm	n	95% CL	C*
Est.	1	0.0611	14	0.0078	0.2141
	2	0.0703	14	0.0084	0.2068
	5c	0.6216	10	0.0397	0.0893
6*	6	0.7091	1	-	-
	6a	0.7537	9	0.0649	0.1121
	7	0.8648	13	0.0249	0.0477
	9	0.9985	7	-	-
	11	1.1080	11	0.0164	0.0221
	14	1.3780	13	0.0530	0.0637
	15	1.4321	4	0.0456	0.0200

TABLE: 7

Potamopyrgus estuarinus :
Relative positions of bands on 7.5% gels: see text for details of statistics.

Band		Continuous pH Rm	n	95% CL	C*
Est.	1	0.0432	30	0.0010	0.0626
	2	0.9280	5	0.0168	0.0146
	3	0.9948	30	0.0042	0.0112
	4	1.0830	30	0.0082	0.0204
	5	1.1875	21	0.0096	0.0117
A.P.	1	0.6293	4	0.0513	0.0512
	2	0.6875	6	0.0291	0.0403
	3	0.7500	7	0.0372	0.0537
	4	1.0208	9	0.0527	0.0671
	5	1.1042	21	0.0176	0.0350
Mdh	1	0.1711	30	0.0021	0.0334

TABLE 8:
H. ventrosa :
Relative positions of bands on 7.5% gels:
see text for details of statistics.

Band		Discontinuous pH Rf	n	95% CL	C*
Est.	1	0.0625	16	0.0013	0.0376
	2	0.1351	16	0.0026	0.0355
	3	0.2000	8	0.0055	0.0329
	4	0.2554	9	0.0044	0.0224
	5	0.3982	20	0.0052	0.0277
	6	0.4375	26	0.0047	0.0264
	7	0.5180	3	-	-
	8	0.6430	2	-	-
	9	0.8480	2	-	-
A.P.	1	0.3040	4	-	-
	2	0.4820	3	-	-
Amyl	1	0.2770	2	-	-
	2	0.4050	2	-	-
	3	0.4455	2	-	-

Band		Continuous pH Rm	n	95% CL	C*
Est.	1	0.0732	12	0.0034	0.0739
	5	0.5000	12	0.0352	0.1107
	5a	0.5597	12	-	-
	5b	0.6231	30	0.0147	0.0633
	5c	0.7115	30	0.0148	0.0557
	6	0.7692	30	0.0075	0.0532
	6a	0.8077	10	0.0254	0.0446
	7	0.8654	30	0.0129	0.0399
	8	0.9038	12	0.0225	0.0391
	8a	0.9615	1	-	-
	9	0.9986	17	0.0235	0.0457
	10	1.0769	9	0.0332	0.0401
	14	1.3462	22	0.0270	0.0453
A.P.	1	0.4865	1	-	-
	2	0.5946	40	0.0171	0.0901
Mdh	1	0.1354	30	0.0036	0.0722
	2	0.2713	12	0.0078	0.0458

Band		Discontinuous pH Rf	n	95% CL	C*
TAO	1	0.3036	4	-	-
	2	0.4821	4	-	-
	3	0.6071	4	-	-
	4	0.6696	4	-	-
	5	0.7589	4	-	-
Amyl	1	0.1340	2	-	-
	2	0.1950	2	-	-
	3	0.2700	2	-	-

TABLE 9:

H. ulvae :
Relative positions of bands on 7.5% gels; see text for details of statistics.

Band		Continuous pH Rm	n	95% CL	C*
Est.	1	0.0556	30	0.0046	0.2236
	3	0.6672	22	0.0090	0.0303
	4	0.7667	30	0.0089	0.0311
	6	0.9278	26	0.0096	0.0256
A.P.	1	†	-	-	-
	2	†	-	-	-
Mdh	1	0.1458	8	0.0047	0.0388
	2	†	-	-	-
	3	†	-	-	-

TABLE 10:

H. neglecta :
Relative positions of bands on 7.5% gels; see text for details of statistics.

† bands too diffuse to measure accurately.

Coefficients of variation[1] ($C^* = \frac{\text{standard deviation}}{\text{mean}}$)

were calculated for each set of R_m and R_f values. C* represents the proportion of variation about each mean, a quantity which might be expected to be similar for each band. There was, however, an increase in C* for bands at the cathodal end of gels perhaps representing a decrease in the accuracy of measuring the distance run by slower bands. It is an empirical fact that, when using the same measurement technique, small measurements are likely to be less accurate than larger measurements.[2] There may, however, be some other explanation of this trend related to e.g. the rate at which molecules of different size enter the gel.

II - 1 - 3 Resolution of bands having similar mobility

Bands were sometimes difficult to distinguish for two reasons:

i) their similar mobilities on the same gel (e.g. Est. 1 and Est.2 in P. jenkinsi A on 7.5% gels - Fig. 4).

ii) their similar mobilities on different gels (e.g. between individuals of H. ventrosa possessing only Est. 2 and those having only Est. 3). This was particularly a problem with rare bands where fewer measurements of mobility made estimates of R_m or R_f less reliable.

In the first case, the presence of two or more close bands on 7.5% gel was usually revealed by electrophoresis of the extract on a lower concentration (usually 5%) gel or by using a longer run time.

The second problem was easily resolved by mixing extracts from the two individuals and running the mixture on 7.5% or 5% gels. If the bands in question really were different isozymes then, on staining, they appeared as separate entities.

In some cases, notably the more cathodal zones staining for general protein and the Est. 6* region of Potamopyrgus species, bands were too numerous to be resolved by the above methods (see section III - 3 - 7) for techniques used to resolve the Est. 6* region).

II - 1 - 4 Direction of migration

The possibility of bands migrating cathodally under the

electrophoretic conditions used in the study was investigated by running extracts of all species on disc and slab gels with the polarity of the apparatus reversed. Staining for general esterase, acid phosphatase and Mdh showed that no bands had migrated cathodally.

II - 1 - 5 Nomenclature

Bands were numbered from the cathode to the anode according to the system adopted by Hunter et. al.[3] and many subsequent authors. Bands which migrated to approximately similar positions on 7.5% gels were assigned the same number. This does not necessarily imply that they are similar in properties other than their mobility under the electrophoretic conditions used. Bands discovered subsequent to the development of a numerical system for a particular banding pattern were assigned a number with a letter suffix (e.g. Est. 5a in P. jenkinsi A).

A particular problem was the naming of the Est. 6 region on Potamopyrgus gels. This region, later shown (section III - 3 - 7) to be composed of several overlapping bands, often appeared as a diffuse wide band and is referred to in the text as Est. 6*. The R_m of Est.6* was measured from the estimated midpoint of the diffuse zone.

CHAPTER II : REFERENCES

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CHAPTER III : SOME BIOCHEMICAL AND PHYSICAL PROPERTIES OF THE ESTERASES OF BRITISH AND AUSTRALASIAN SPECIES OF POTAMOPYRGUS

Segregation and recombination do not occur in organisms such as P. jenkinsi because of the absence of a meiotic division during egg maturation (apomixis). It is, therefore, not possible to determine the formal genetics of markers by the usual methods of setting up crosses in sexually reproducing organisms or by isolating virgin females in automictically reproducing organisms and scoring progeny for segregation ratios.

Estimates of genetic variation in asexual and sexual populations are often made[1,2,3] by assigning bands to particular loci on the basis of their reaction to one substrate at one pH and their mobility at one gel concentration. The hazards of this approach have been exposed by e.g. Johnson,[4] who revealed the presence of multiple alleles at the ∝-glycerophosphate dehydrogenase locus of Colias butterflies (where previously only two had been detected) by the simple expedient of running extracts on gels of different acrylamide concentration. As Colias is sexually reproducing he was able to confirm the true allelic nature of bands at this locus by scoring the progeny from crosses and obtaining segregation ratios. Similar cases of multiple over-lapping bands have been reported for Drosophila isozymes.[5,6] A further confounding factor in the analysis of banding patterns may be the presence of null alleles; a heterozygote for a visible allele and a null allele may be wrongly scored as a homozygote for the visible allele. Null alleles have been shown to be relatively common in e.g. insect populations[7] but their possible presence has been frequently ignored in estimating variation in a population.[8] Errors in interpretation due to unsuspected multiple alleles and null alleles inevitably lead to under-estimation of the true amount of variation within a population.

A more serious error of such 'armchair' genetics is the assignment of alleles to the wrong loci resulting in an incorrect estimate of heterozygosity. Electrophoretic loci in parthenogenetic organisms have, in the past, been identified by reference to presumed homologous loci in closely related sexually-reproducing populations, a method which gives no information on alleles found only in the parthenogenetic population. In several large electrophoretic surveys from which estimates of variation have been made, no information on how

alleles were identified is given - presumably guesswork was used. Guesswork based on the easily recognised banding patterns produced by multimeric isozymes is probably fairly reliable but many enzymes are monomers (e.g. about a third of isozymes in man are monomeric[9]). Esterases are a particularly monomeric class of isozyme often used in electrophoretic surveys, and indeed none of the esterases in the present study was found to be multimeric.

Oxford[10] has shown that for esterases in the land snails Cepaea nemoralis and C. hortensis, the molecular weights of allelic products from the same locus are essentially the same and that allelic products from different loci often have different molecular weights. Similarly, the five alleles that Johnson[4] found at the α-glycerophosphate dehydrogenase locus in Colias had closely similar molecular weights. This is to be expected if it is assumed that different allelic products (proteins) from the same locus differ by only a few amino-acids - a difference in molecular weight that is undetectable by electrophoretic methods. Mutational events resulting in more drastic modification of molecular structure will probably result in a non-functional gene product which may be selected out of the gene pool or remain as a null allele. The argument also applies to chromosomal structural re-arrangements such as inversions - there is no direct evidence that base-pair substitution constitutes the major source of natural genetic variation.

Oxford has also investigated the biochemical properties[11] and the formal genetics[12] of the esterases of Cepaea nemoralis and it is evident from his data that (visible) alleles shown by breeding experiments to be at the same locus had similar biochemical properties. Conversely, the biochemical properties of alleles at different loci were usually dissimilar. Alleles at the same locus are usually of similar and characteristic staining intensity and colour when exposed to different artificial substrates simultaneously. Esterase loci may produce enzymes such as carbonic anhydrases, proteases, sulphatases, lipases, etc.[15] When these enzymes hydrolyse, say, a mixture of α-n-acetate and β-n-acetate they give rise to bands having colours and densities characteristic of the enzyme type (e.g. carbonic anhydrases will stain red and lipases purple in the above example).

Biochemical similarity between alleles at the same locus also occurs in many enzyme systems in Drosophila[13] and in the mosquito Anopheles punctipennis[14] and often forms the basis of guesswork genetic

analysis of electrophoretic banding patterns. When allelic isozymes are visible on gels stained using artificial substrates they rarely appear with significantly lower activity then others at the same locus. This is not always the case, however; Wright[16] has shown that alleles at the Es-6 locus in Drosophila differ in their activity and more recently, Danford and Beardmore[17] have demonstrated that alleles at this locus differ in their reactions to several naturally occurring substrates. However, many artificial substrates (particularly acetates and methyl-substituted esters) are hydrolysed equally by the two alleles at this locus.

The impression gained from large-scale surveys of the esterases of mammals,[18] birds,[19] frogs,[20] and insects[21] is that the reactions of alleles at a particular locus to artificial substrates (usually α- and β-naphthyl acetates) are remarkably similar. Lowered activity of an allele is usually expressed as invisibility (a null allele) when these substrates are used.

These empirical observations on the physical and biochemical characteristics of allelic products present a method by which loci may be at least tentatively distinguished and bands assigned to them with more confidence than if genetic interpretation is done by guesswork. Such 'finger-printing' of bands by means of their properties also presents a method by which homologous bands separated by different electrophoretic systems may be recognised. Regions in which bands are thought to overlap (e.g. the Est.6* region in Potamopyrgus) may also be resolved if bands have different reactions to particular substrates.

In summary, for species where breeding data is unobtainable, the determination of the physical and biochemical properties of isozymes, although time-consuming and laborious, may be a useful aid in the genetic analysis of electrophoretic banding patterns. In this chapter the substrate specificities, inhibition characteristics and relative molecular weights of the esterases of the Potamopyrgus species are determined and these properties used in order to identify homologies between bands both within and between species. Homologous bands within species are assumed to be alleles and loci are accordingly distinguished. Homologous loci between species are identified in the same way.

III - 1 The Reactions of Esterase Bands to Various Substrates

III - 1 - 1 Substrates used

As many esterase substrates as were readily available from Sigma Limited were used in the study. A list of these, together with abbreviations used in the text, is given below.

1.	α-naphthyl acetate	α-n-a
2.	β-naphthyl acetate	β-n-a
3.	naphthyl -AS- acetate	n-AS-a
4.	α-naphthyl proprionate	α-n-p
5.	β-carboxy choline iodide	β-c-c-I
6.	α-naphthyl butyrate	α-n-b
7.	β-naphthyl butyrate	β-n-b
8.	α-naphthyl laurate	α-n-l
9.	5-bromo-indoxyl acetate	5-br-ind-a
10.	indoxyl acetate	ind-a

III - 1 - 2 Preparation of Staining Solutions

10^{-3} M solutions of these substances were prepared by dissolving the appropriate weight (of solids) or volume (of liquids) in 100 ml of the 0.2 M pH 6 phosphate buffer described in section I - 5 - 1. Substrates usually dissolved easily if a few drops of acetone were added to the substance before addition of the buffer solution. α-n-laurate was rendered soluble only by the prior addition of 5 ml propylene glycol and 5 drops of acetone. The addition of 5 ml propylene glycol and 5 drops of acetone to the general esterase staining solution described in section I - 5 - 1 resulted in no inhibition (compared to a control with no propylene glycol) of esterase bands on slab gels prepared by electrophoresis of an extract of P. jenkinsi A.

III - 1 - 3 Extracts used

As homogenisation of single snails gave an insufficient volume of extract for comparative studies, mass extracts were prepared as in section I - 2 - 2. Homogenates of 200 adult snails in 20 ml 50% sucrose were made for each Potamopyrgus species. The snails came from the following populations:-

P. jenkinsi A - Popln. 11

P. jenkinsi B - Popln. 28

P. jenkinsi C - Popln. 35 (lab clone)

P. antipodarum - Popln. 45

P. nigra - 42 (100) + 40 (100) (100 from popln. 40 + 100 from popln. 42).

These extracts were also used for the inhibitor study (section III - 2) and for molecular weight determinations and were stored at -20°C.

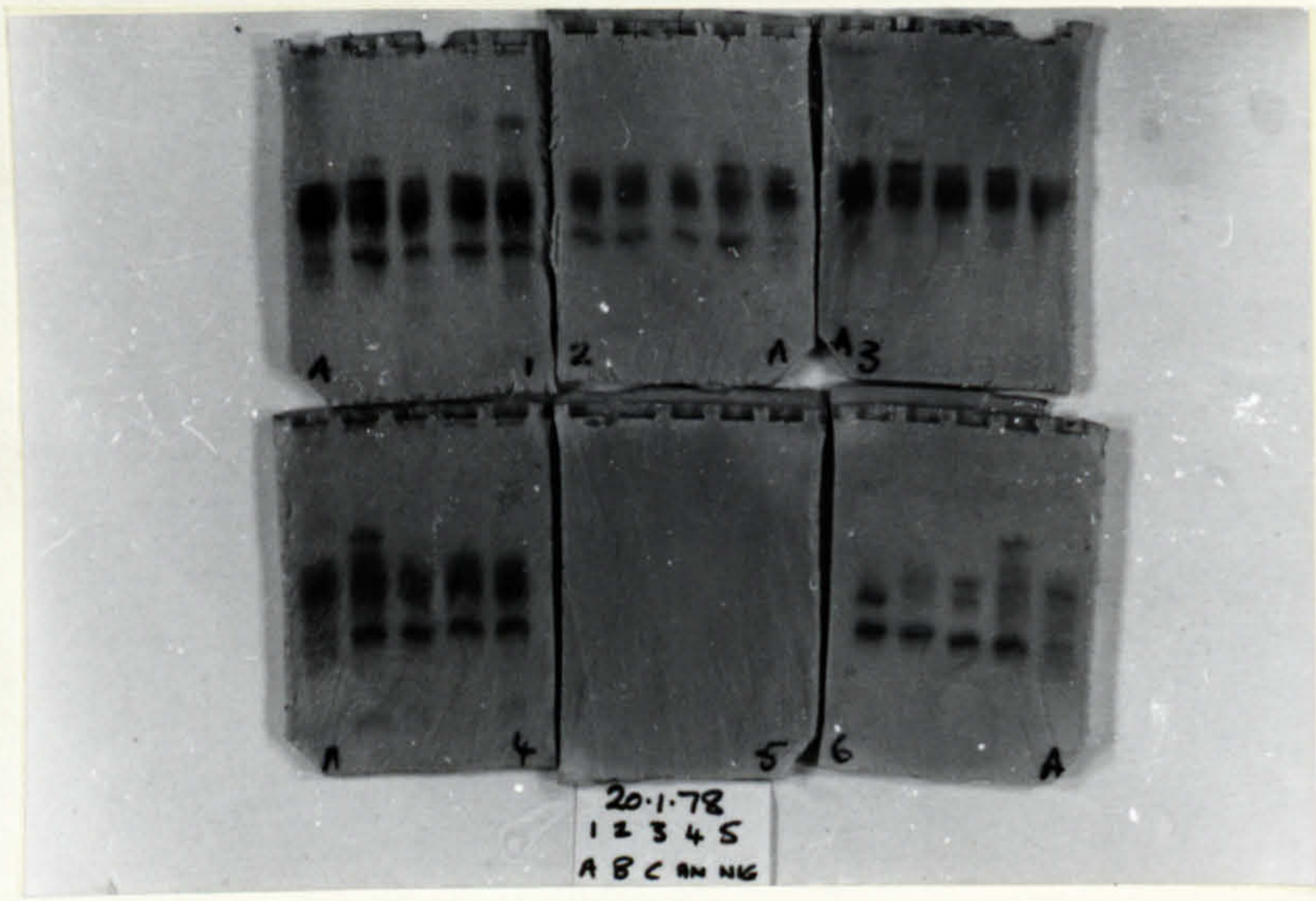
In preparing mass extracts, rare bands (such as Est.3 and 4 in P. nigra and Est. 5b in P. antipodarum) are inevitably diluted by those snails in the population sample which do not possess them. For this reason, some esterases were difficult to detect on the gels. This "dilution effect" was an insignificant problem in extracts of P. jenkinsi as variation in this species was virtually absent; only Est. 1a (see section IV - 3 - 1) was undetectable in mass extracts of P. jenkinsi A.

III - 1 - 4 Electrophoresis

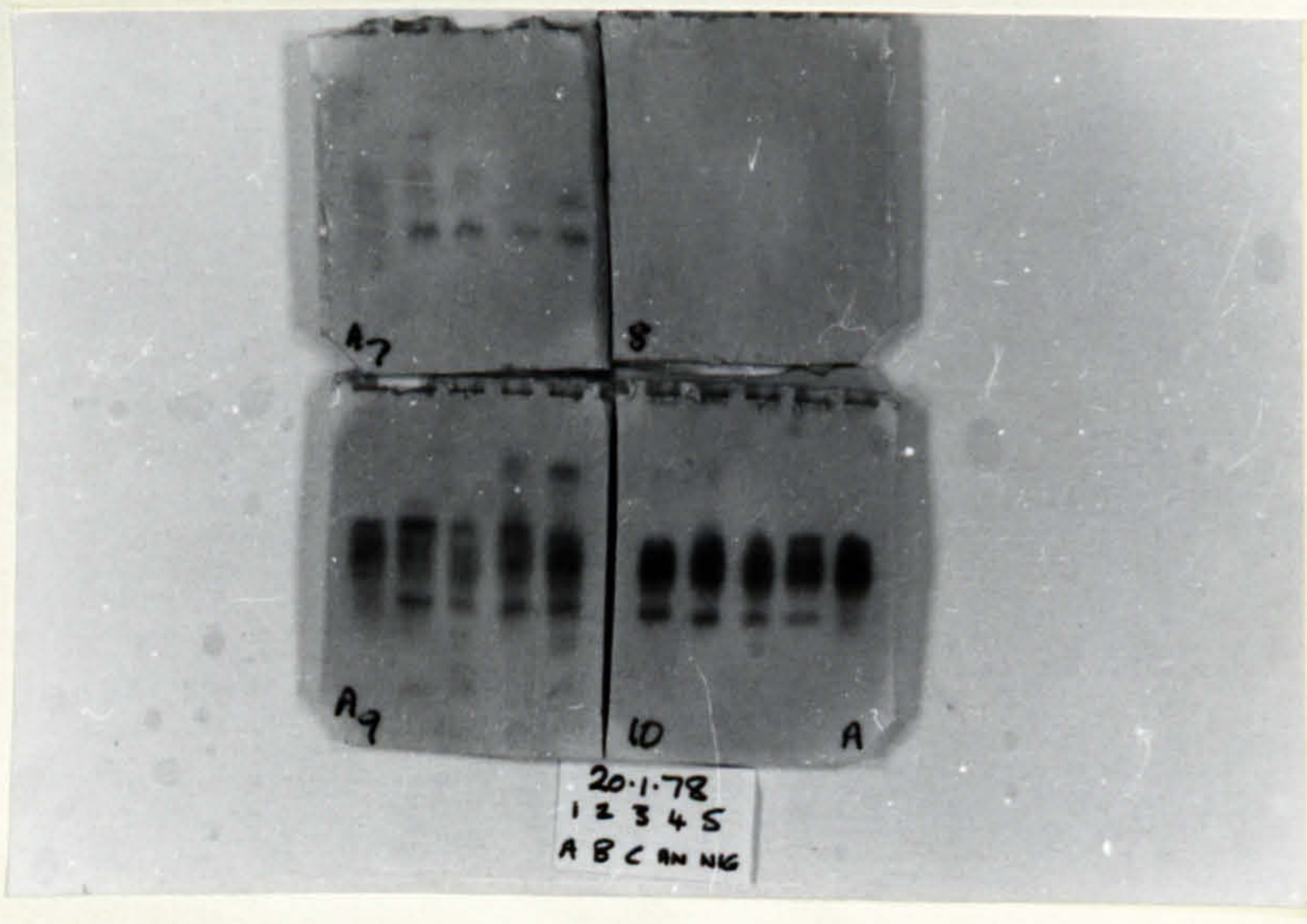
Samples absorbed into wicks were arranged on 60-slot slab gels (section I - 3) in ten groups of five wicks (one extract of each species per group). After electrophoresis, the gel was cut into sections each containing a group of five species. Each section was then sliced (section I - 3 - 4) into two. The method gave two replicates for each staining solution from the same electrophoresis run. One replicate was placed in staining solution containing the test substrate and the other in a staining solution containing α-n-acetate (to provide a scoring reference gel - see section III - 1 - 6).

III - 1 - 5 Staining and Storage of Gels

Gels were incubated in the appropriate staining solution for 30 mins. at 25°C enabling a comparison of the hydrolysis rates of different substrates to be made for a particular esterase band. Gels were placed in staining solutions in plastic dishes on a fixed-speed horizontal shaker. After 30 minutes they were removed and immediately placed in 30% ethanol to stop the reaction. Bands were scored visually (section III - 1 - 6) and dehydrated for storage as described



Plates 4 and 5 : Gel slices stained using various substrates as described in section III-1-5. The order of the extracts on the slices is P.jenkinsi A, P.jenkinsi B, P.jenkinsi C, P. antipodarum and P.nigra but this is reversed in some cases (eg. gel 2). The position of the P.jenkinsi A extract is indicated by an 'A'. Substrates are numbered according to Table 10a .



in section I - 6 - 2.

III - 1 - 6 Scoring of Band Intensity

Estimates of hydrolysis rate were visually scored on a subjective scale similar to that used by Stephen and Chaldelin[22] and Oxford[11]. The reference used was Est. 9 of P. jenkinsi B stained with α-n-a. This heavily staining band was represented by ++; bands of medium intensity by +; those of low intensity by +/- and no detectable activity by -. Bands which were just visible on some gels but not on replicates were scored as ? + or ? +/-.

Visual estimates of hydrolysis rates were not entirely reliable due to the fact that different substrates produced different coloured dyes. α-naphthyl substrates on hydrolysis release α-naphthol which when coupled with Fast Garnet produces a purple dye. β-naphthyl substrates produce a red dye from β-naphthol and substrates containing the indoxyl group gave a blue-black dye. An effort has been made to correct for this difference in colour by visually estimating the intensity of the bands only and by scoring bands from monochrome photographs of gels prepared by using panchromatic film (see section III - 1 - 7 for details). Experience showed that the latter method was more consistent and it was adopted for routine use.

Bands were identified by measuring the mobility of the band in question relative to that of Est. 9 (P. jenkinsi A). Est. 9 was clearly visible on all of the gel slices except those stained using n-AS-a where the positions of bands were measured relative to Est. 5c in P. jenkinsi B.

III - 1 - 7 Substrate specificities of esterases run on slab gels

Plates 4 and 5 show a set of dehydrated slab gels stained with various substrates. Panchromatic film has been used to reduce the effect of different dye colours on staining intensity. Bands on gels 2 and 7 were red; other bands were purple or blue/black (in the case of indoxyl-substrates). The film was however slightly less sensitive to red so that bands on gels 2 and 7 appear slightly less intense on the plates than on the actual gels. The contrast between the bands and the gel background was slightly enhanced by photography in the case of red-stained gels. Visual estimates of staining intensities relative to that of Est. 9 of P. jenkinsi A(stained with

TABLE 10a:

KEY to Symbols used in TABLES 11 - 20

Substrates

1	α - n - acetate
2	β - n - acetate
3	naphthol - AS - acetate
4	α - n - proprionate
5	β - carboxy choline iodide
6	α - n - butyrate
7	β - n - butyrate
8	α - n - laurate
9	5 - bromo-indoxyl acetate
10	indoxyl acetate

Reactivity

++	heavily-staining band
+	medium-staining band
+/-	lightly staining band
-	band not visible
?	reaction unknown
? + AND ? +/-	reaction not repeatable

Substrate	BAND NUMBER														
	1	2	2a	3	4	5	5c	6	6a	7	8	9	10	11	14
1	+	+	+	+	? +/-	? +/-	+	++	?	+/-	+/-	+	+	+/-	-
2	-	-	-	-	-	-	+	+	?	+	-	+	+	?	-
3	-	-	+	+	-	-	+	++	?	+	+	-	-	-	-
4	+	+	+/-	+/-	-	-	+	++	?	+	+	+	+	+/-	+/-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	+/-	+/-	-	+	?	+/-	+/-	+/-	+/-	+/-	-
7	-	-	-	-	-	-	-	+/-	?	+/-	+/-	+/-	+/-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	+/-	+/-	-	-	+	++	++	+	?	+/-	+/-	-	+/-
10	-	-	-	-	-	-	++	++	?	++	+	+/-	+/-	-	-

TABLE 11: Reactions of the Esterases of Potamopyrgus jenkinsi A to various substrates.

Substrate	BAND NUMBER 1	5b	5c	6	6a	7	9	11	12	13	14
1	+	+/-	+	+	?	+	++	+/-	+/-	+/-	-
2	-	? +	+	+	?	+	++	-	-	-	-
3	-	+/-	+	+	?	+	+/-	-	-	-	-
4	+	+	+	+	?	+	++	-	-	-	+/-
5	-	-	-	-	-	-	-	-	-	-	-
6	-	+	+/-	+/-	?	+/-	++	-	-	-	-
7	-	+/-	?	+/-	?	+/-	+	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-
9	+	+/-	+	+/-	?	+/-	+	+/-	+/-	? +/-	+
10	-	-	+	++	-	++	+	-	-	-	-

TABLE 12: Reactions of the Esterases of Potamopyrgus jenkinsi B to various substrates.

	BAND NUMBER							
Substrate	1	4	5c	6	6a	7	9	14
1	+	+/-	+	+	?	+	+	-
2	-	-	+/-	+	?	+/-	+	-
3	-	-	+	+	?	+/-	-	-
4	-	-	+/-	+	?	+	++	+/-
5	-	-	-	-	-	-	-	-
6	-	+/-	-	+	?	+	++	-
7	-	-	?	?	?	-	+	-
8	-	-	-	-	-	-	-	-
9	-	-	+	+	?	+	+	+
10	+	-	?	++	?	++	+	-

TABLE 13: Reactions of the Esterases of Potamopyrgus jenkinsi C to various Substrates.

	BAND NUMBER													
Substrate	1	2	2a	4a	5	5b	5c	6	6a	7	8	9	10	14
1	+	+	? +/-	? +/-	+/-	-	+	+	?	+	+/-	++	+/-	-
2	-	-	-	-	-	? +/-	+	+	?	+	+/-	++	?	-
3	-	-	-	-	-	-	+	+	?	+	?	-	?	-
4	+	+	-	-	? +/-	? +/-	+	+	?	+	?	++	?	+/-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	?	+	?	+/-	-	++	-	-
7	-	-	? +/-	? +/-	-	-	-	?	?	?	?	+	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	+	?	++	+/-	?	+	+/-	++	-	+/-
10	+	+	-	-	+/-	-	+	++	?	++	+	++	-	-

TABLE 14: Reactions of the Esterases of Potamopyrgus antipodarum to various substrates.

	BAND NUMBER											
Substrate	1a	4	5	5d	6	6a	7	8	9	10	12	14
1	+		+	+	+	?	+	+	++	+/-	+/-	-
2	-	-	-	+	+	?	+	+	+	-	-	-
3	-	-	-	+	+	?	+	-	-	-	-	-
4	+	-	+	+	+	?	+	+/-	++	-	-	+/-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	-	? +/-	-	-	-	?	+	-	++	-	-	-
7	-	-	-	-	-	?	+	-	+	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	+	+	+	?	+	?	++	+/-	+/-	+/-
10	+	-	+/-	+/-	++	?	++	+	++	-	-	-

TABLE 15: Reactions of the Esterases of Potamopyrgus nigra to various Substrates.

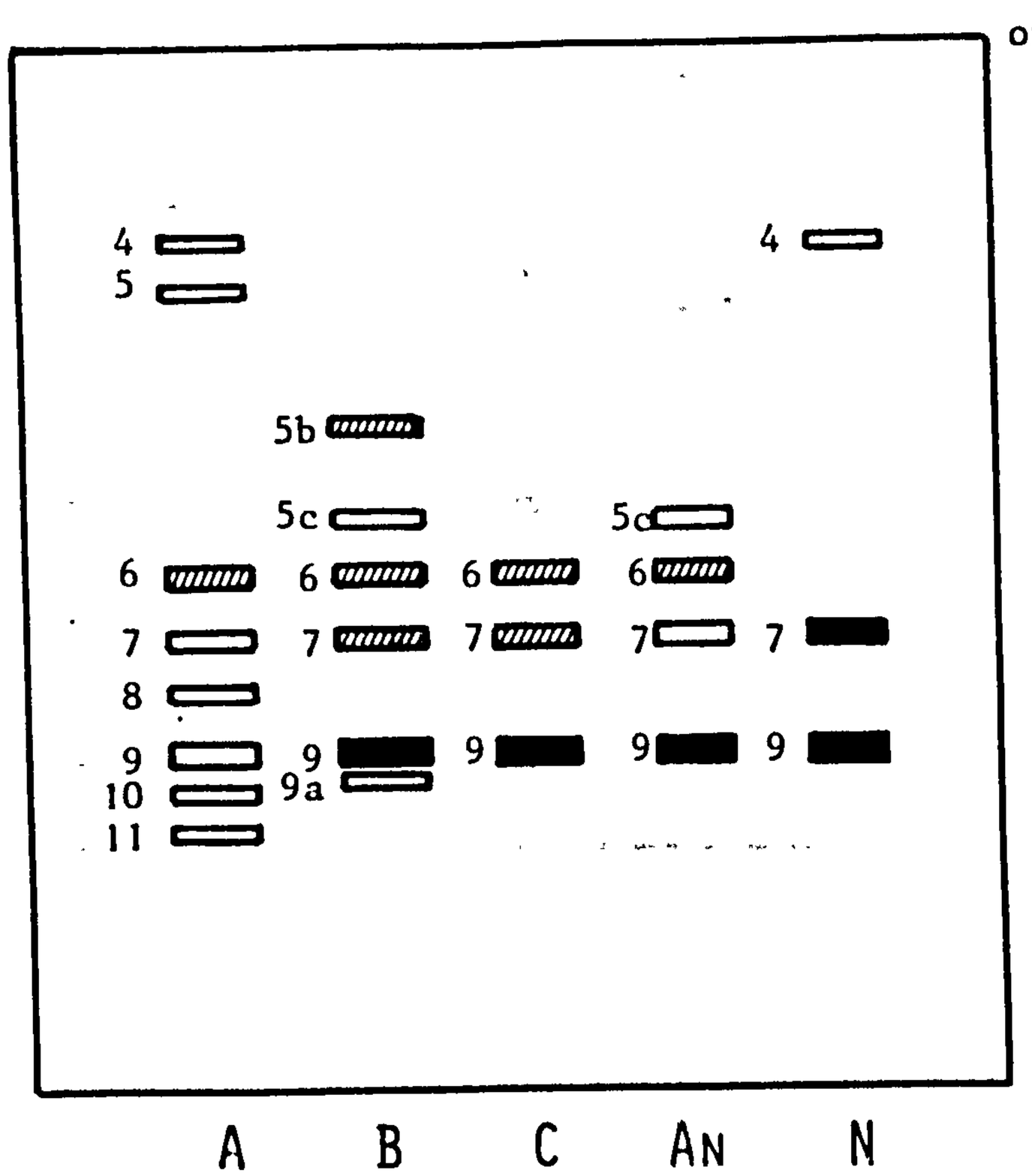


Fig.13 : Esterase phenotypes of five mass extracts of Potamopyrgus species ; continuous pH slab gel stained using ∝ - n - butyrate.

A - P.jenkinsi A

B - P.jenkinsi B

C - P.jenkinsi C

An - P.antipodarum

N - P.nigra

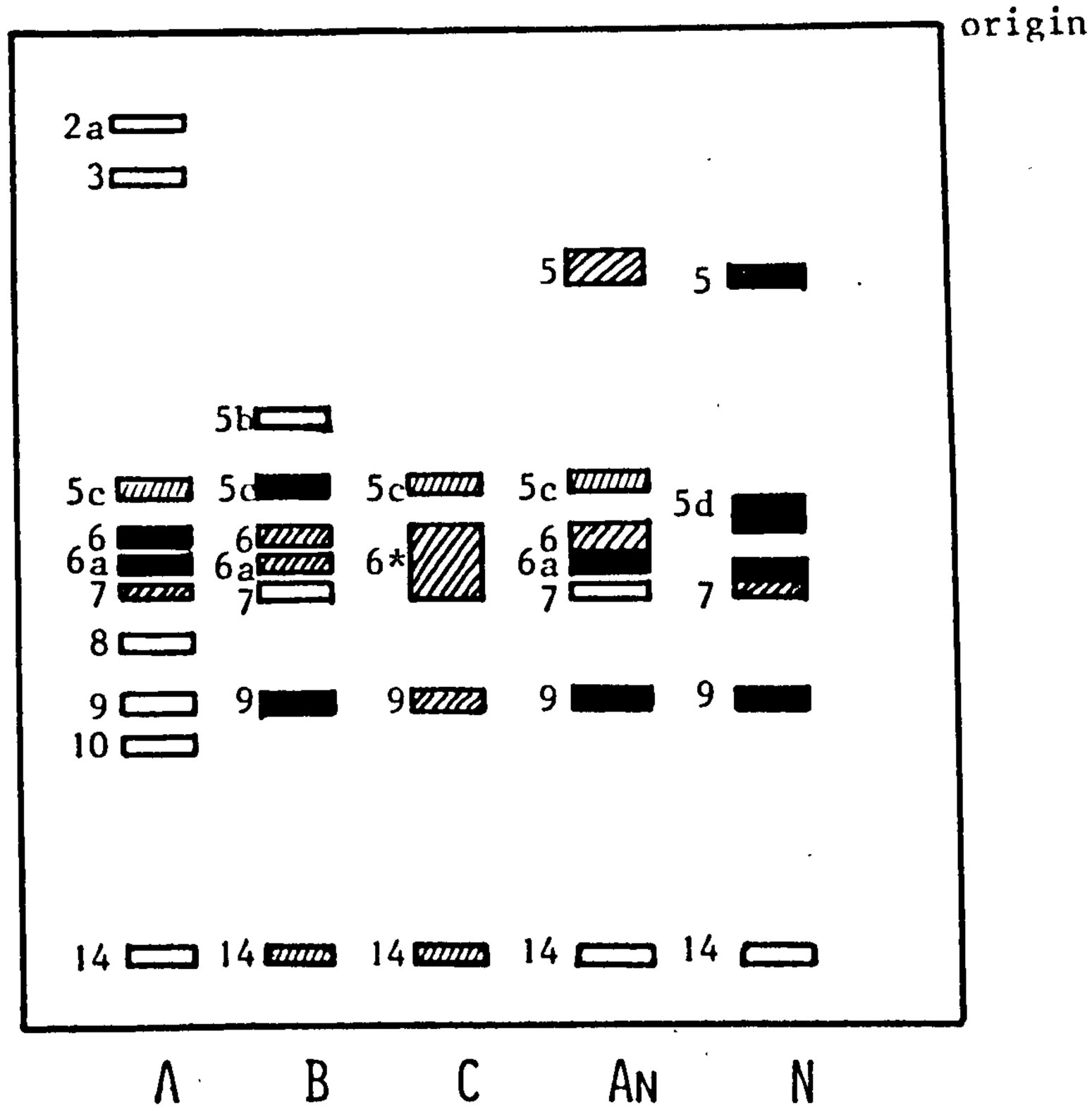


Fig.14 : Esterase phenotypes of five mass extracts of Potamopyrgus species; continuous pH slab gel stained using 5 - br - ind - acetate.

A - P.jenkinsi A

B - P.jenkinsi B

C - P.jenkinsi C

An - P.antipodarum

N - P.nigra

α-n-a) are presented in Tables 11 to 15.

The maximum staining intensity for most bands was given with α-n-a and α-n-p substrates. No bands were visible on gels stained using β-c-c-I or α-n-l and staining was faint on gels stained using β-n-b. β-c-c-I is a substrate for vertebrate cholinesterases and therefore none of the esterases in Potamopyrgus extracts were homologous with these enzymes. Oxford[11] found that the esterases of Cepaea nemoralis and Cepaea hortensis were also unable to hydrolyse β-c-c-I and α-n-l.

Esterases 1 and 2 were specific for α-n-a, α-n-p and ind-a only and no other bands showed a similar pattern of specificity. Esterases 2a and 3 of P. jenkinsi A each had the same "fingerprint". Esterases 2a and 4a of P. antipodarum had different substrate specificities to Est. 2a and 3 of P. jenkinsi A but both were specific for α-n-a and β-n-b only. Esterases 4 and 5 of P. jenkinsi A were found to be specific for α-n-b (they appeared on one set of gels stained with α-n-a but the result was not repeatable), although they were usually just visible on gels stained with the general esterase mixed substrates. Est. 4 of P. nigra was also found to be specific for α-n-b but was not homologous with the proprionate-specific Est. 5 of P. nigra. Est. 5 in P. nigra was biochemically similar to Est. 5 in P. antipodarum. Est. 5b in P. antipodarum was rare in the Waikato River population (section IV - 6 - 1) used to prepare the mass extract and was, therefore, diluted by snails not having 5b (see section III - 1 - 3). The band was faintly visible on some gels stained using α-n-a, α-n-p and 5-br-ind-a but the results were not always repeatable. Est. 5b may be homologous to Est. 5b in P. jenkinsi B but the evidence is not conclusive.

Gels stained with α-n-b and 5-br-ind-a revealed clearly defined bands in the Est. 6* region. Diagrammatic representations of some of the gels shown in Plate 4 (gel 6: α-n-b) and Plate 5 (gel 9: 5-br-ind-a) are given in Figs. 13 and 14. α- and β-n-b revealed three bands (Est. 5c, 6 and 7) with Est.6 absent in P. nigra and Est.5c just visible in P. jenkinsi B and P. antipodarum. The gel stained with 5-br-ind-a revealed the probable presence of a fourth band (Est. 6a) between bands 6 and 7 (R_m = 0.79). The band was most easily seen in the P. jenkinsi B extract staining with the same intensity as Est. 6. Est. 6a is presumably specific for substrates other than butyrate esters and may well be responsible for the merging of Est.6

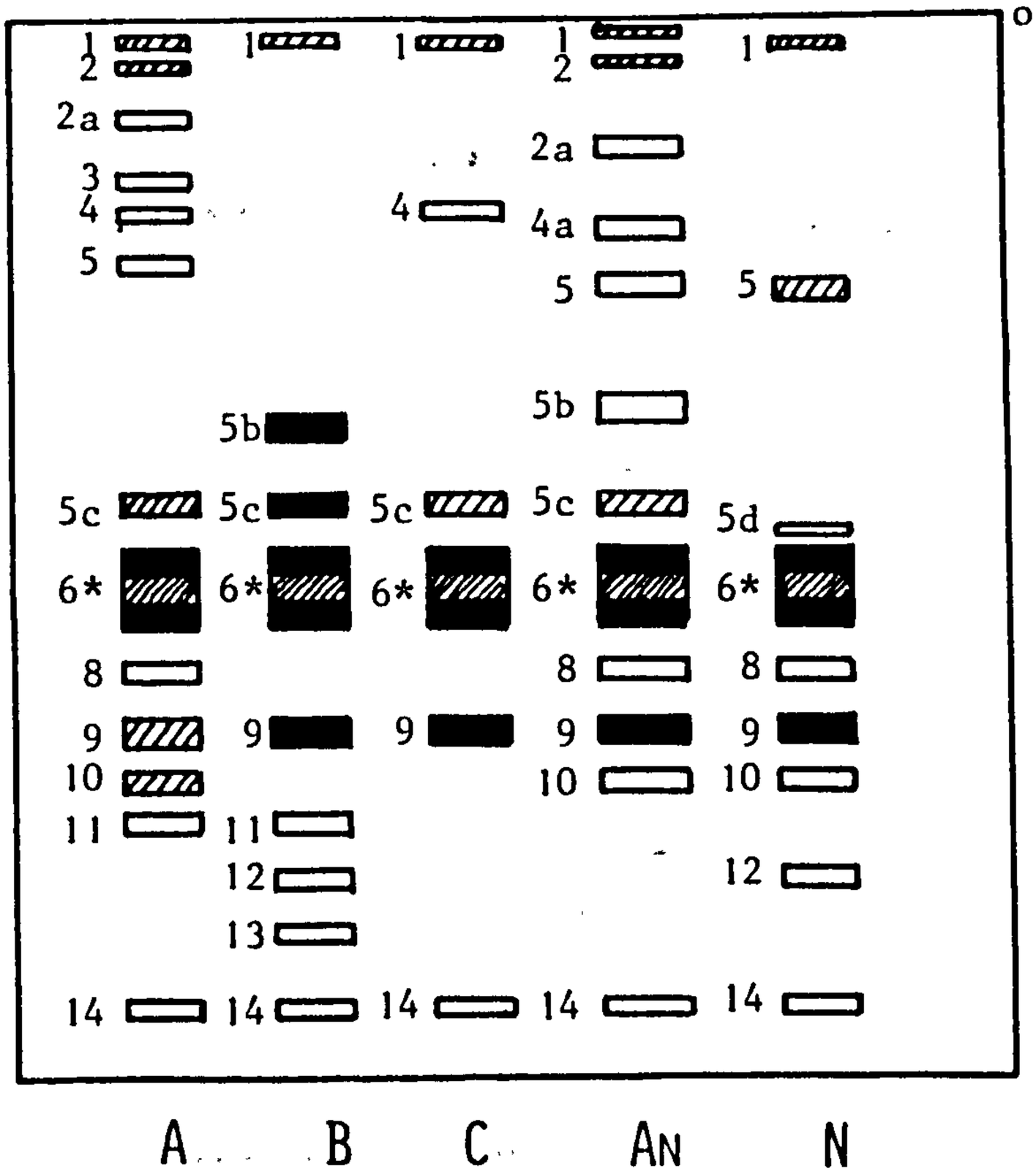


Fig.15 : Esterase phenotypes of five mass extracts of Potamopyrgus species; continuous pH slab gel stained using ∝ - n - acetate and ∝ - n - proprionate.

A - P.jenkinsi A

B - P.jenkinsi B

C - P.jenkinsi C

An - P.antipodarum

N - P.nigra

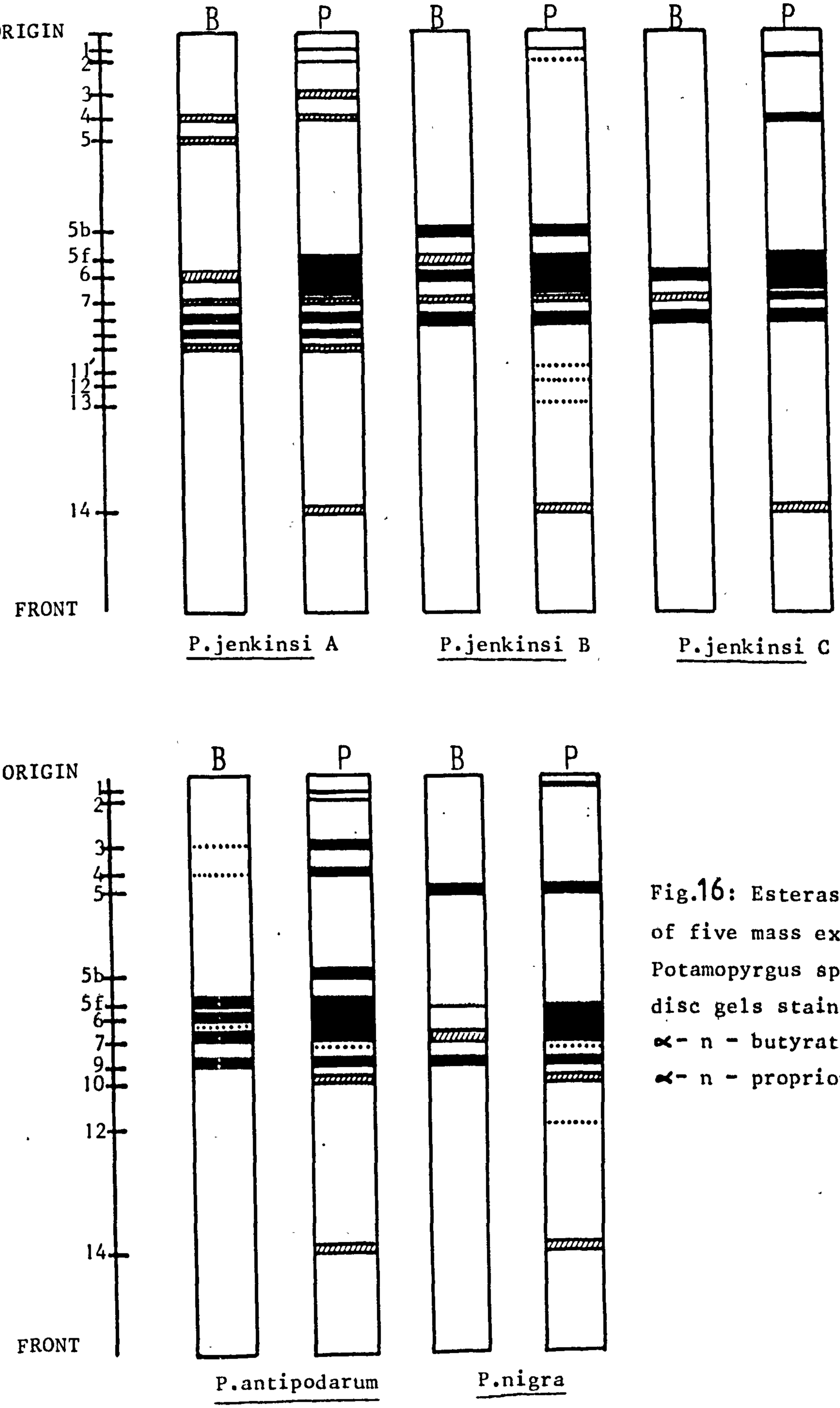


Fig.16: Esterase phenotypes of five mass extracts of Potamopyrgus species ; 7.5% disc gels stained using ∝- n - butyrate (B) and ∝- n - proprionate (P).

and Est.7 seen on gels stained with general esterase substrates (Fig. 15). It is also possible that at least one other band was staining up with Est. 6a and overlapping with Est. 5c on gels stained using 5-br-ind-a.

Est. 6 did not appear in P. nigra extracts stained with α-n-b although the band was clearly visible in the other four extracts. Est. 7 however stained heavily in P. nigra extracts suggesting that if Est. 6 and 7 were possibly allelic, P. nigra populations were largely homozygous for Est. 7 and populations of the other species were largely heterozygous at this locus.

The simplest interpretation of the above data is that four bands are in the Est. 6* region, two specific for α-n-butyrate (Est. 6 and Est. 7) and two not specific for this substrate (Est. 5d and 6a in P. nigra and Est. 5c and 6a in the other species). Confirmation of the structure of this region was obtained during molecular weight studies (section III - 3) and during a survey of the Gaywood River population of P. jenkinsi A where one individual which lacked Est. 5c and 6 showed a faint band at R_m 0.79.

Est. 9, the band used as the reference in measuring R_m values, had similar substrate affinity in all extracts except P. jenkinsi A where some activity was detected with n-AS-a. Esterase 10 in P. jenkinsi A had the same substrate specificity as Est. 9 in this extract. Esterases 11, 12, and 13 in P. jenkinsi B and 10 and 12 in P. nigra hydrolysed α-n-a and 5-br-ind-a only; the rare Est. 10 in P. antipodarum may have had similar properties but the protein concentration was too low to enable repeatable results to be obtained.

Esterase 14 was specific for α-n-p and 5-br-ind-a in all extracts and was most active in extracts of P. jenkinsi B and C.

III - 1 - 8 Substrate specificity of Esterases on disc gels

In view of the clear resolution of the Est. 6* region obtained by staining slab gels with α-n-b (section III - 1 - 7) and in order to be able to recognise bands on both slab and disc gels, sets of disc gels (11 cm) were prepared by running the same mass extracts as before. One set was stained using α-n-b and the other with α-n-p. The results are illustrated in Fig. 16.

The three bands on butyrate-stained P. jenkinsi C gels and the two fast bands on butyrate-stained P. nigra gels are assumed to be bands Est. 6, 7 and 9 (P. jenkinsi C) and 7 and 9 (P. nigra) seen on butyrate-stained slab gels. The possibility of these bands being in different relative positions on the two electrophoretic systems is considered to be unlikely since:

i) On butyrate-stained P. nigra gels the presumed Est. 6 band in P. jenkinsi C was absent on disc and slab gels.

ii) Est. 9 was characteristically heavily staining relative to Est. 7.

Five bands appeared in the Est. 6* region on butyrate-stained P. jenkinsi A disc gels compared with six bands on slab gels. Est. 6, 7 and 9 were in similar positions to those on P. jenkinsi C and the two faster bands were assumed to be Est. 10 and Est. 11 seen on slab gels. Est. 8 was not seen on disc gels but a faint band was detected in this region (overlapped by Est. 9) during molecular weight determinations (section III - 3). Esterases 6, 7 and 9 were present on butyrate-stained P. jenkinsi B disc gels together with two slower bands, the slower staining more heavily than the faster. These were thought to be Est. 5b and 5c on slab gels as the slower band appeared on proprionate-stained P. antipodarum disc gels (Est. 5b showed faintly on P. antipodarum disc gels stained using α-n-p). Butyrate-stained P. antipodarum disc gels showed four bands (Est. 5c, 6, 7 and 9) in the Est. 6* region.

P. jenkinsi A gels stained using α-n-b showed two bands in the Est. 4 region and those stained with α-n-p showed two different slow bands. Disc gels stained for general esterase show three bands in this region (the centre band staining heavily) probably as a result of both these overlapping systems staining up. This banding pattern is typical of a dimeric heterozygote and would certainly have been scored as such if the substrate specificity study had not been undertaken.

Bands 3 and 4 also appeared as heavily staining bands on proprionate-stained P. antipodarum disc gels. A single band (? 4 on slab gels) appeared on proprionate-stained P. jenkinsi C disc gels and a single band (?5 on slab gels) in this region on both butyrate and proprionate-stained P. nigra disc gels. Bands in this region present

a problem because although Est. 3 and Est. 4 on P. antipodarum disc gels appear to be equivalent to Est. 2a and 4a on slab gels (they have the same biochemical properties), their relative positions were different in the two electrophoretic systems.

On slab gels, bands 2a and 3 in P. jenkinsi A were slower than 2a and 4a in P. antipodarum and had different substrate specificities whereas on disc gels Est. 3 and 4 (the probable equivalents of 2a and 3 on slab gels) in both species had the same mobility and substrate specificity. If these bands represent alleles at the Es-2 locus they would be scored as homologous between species on the disc system but not so on slab gels.

In the Est. 1 region bands seemed to be equivalent to those on slab gels apart from in P. jenkinsi B where two bands (Est. 1 and Est. 2) appeared on disc gels and only one on slab gels. The faster of the two bands was faintly staining and may represent an artifact or possibly, post-translational modification of Est. 1 similar to that reported for the Es-1 locus of Cepaea nemoralis and C. hortensis by Oxford, (see section IV - 3 - 1). However, if the latter was the case, then Est. 2 should have appeared on slab gels (although the slab system is a little less sensitive than the disc system).

Bands which ran faster than the Est. 6* region consisted of the faintly-staining Est. 11, 12 and 13 in P. jenkinsi B, Est.12 in P. nigra and the proprionate-specific Est. 14 in all extracts. Est.11 in P. jenkinsi B ran faster than Est. 11 in P. jenkinsi A suggesting that these bands might not be homologous although they have the same mobility on slab gels.

III - 2 Inhibition Properties of the Esterases

Augustinsson[23] and Pearse[24] demonstrated in the early 1960's that many classes of chemical compound inhibit vertebrate esterases and that such inhibition characteristics could be used to classify these enzymes into acetyl-, aryl-, carboxyl- and cholin-esterases. Subsequent work by e.g. Bulmer and Fisher[25] and Ecobichon[26] has confirmed this classification for vertebrates but recent studies by Oxford[11] and Stephen and Cheldelin[22] have shown that the vertebrate esterase classification cannot be used for invertebrate esterases. The data presented in this section were not used to attempt to classify

the esterases in this way but were obtained in order to provide additional information on which to distinguish loci in general and the Est. 6* region in particular.

III - 2 - 1 Inhibitors used

Many of the compounds used are highly toxic to living organisms due to their ability to inhibit esterases and other enzymes concerned with vital aspects of metabolism. The organophosphorus compound (E600) is particularly dangerous (it is a potent cholinesterase inhibitor) and was handled with due respect.

The inhibitors employed were chosen by reference to the work of Oxford[11] and Ecobichon[26] and were:

INHIBITOR	WORKING CONCENTRATION
E600 (diethyl-p-nitrophenyl phosphate)	10^{-3} M
pCMB (parachloromercuribenzoate)	10^{-4} M
urea	1 M
silver nitrate	10^{-1} M
acetazolamide	10^{-3} M
copper sulphate	10^{-2} M
quinine sulphate	10^{-3} M
sodium fluoride	10^{-2} M
sodium sulphate	2.5×10^{-3} M
EDTA (disodium ethylenediamine tetra-acetic acid)	10^{-3} M

All the solutions were made up in 0.2 M phosphate buffer (pH6 - section I - 5 - 1) with the exception of $CuSO_4$ and $AgNO_3$ which reacted with the buffer and were dissolved in distilled water instead. Solutions were stored for up to one month in a refrigerator and then unused solutions discarded. E600 solutions were made up fresh within one hour of use as Bulmer and Fisher[25] and Oxford[11] report loss of potency of solutions of related compounds after prolonged storage.

III - 2 - 2 Extracts used

The extracts were the same mass extracts used in section III - 1 - 3; they had been stored at -20° C for two weeks.

	1	2	2a	3	4	5	5c	6*	7	8	9	10	11	14
E 600 10^{-3}M	−	−	−	−	−	−	−	−	−	−	−	−	−	−
E 600 10^{-4}M	−	−	−	−	−	−	−	+/−	−	−	−	−	−	−
EDTA	+	+	−	−	−	−	+	+	+	−	+/−	+/−	−	−
pCMB	+	+	+	+	−	−	+	+	+/−	−	+/−	+/−	−	−
UREA	+	+	−	−	−	−	+	+	−	−	−	−	−	−
ESERINE	−	−	−	−	−	−	−	+	−	−	−	−	−	−
$AgNO_3$	−	−	−	−	−	−	−	−	−	−	−	−	−	−
ACETAZOLAMIDE	+	+	−	−	−	−	+	+	+	+	+	+	+/−	+/−
$CuSO_4$	+/−	+/−	−	−	−	−	+	+	+	+	+	+	−	−
QUININE SULPHATE	+/−	+/−	−	−	−	−	+	+	+	+	+	+	+/−	+/−
NaF	+	+	+/−	+/−	−	−	+	+	+	+	+	+	−	−
Na_2SO_4	+	+	−	−	−	−	+	+	+	+	+	+	−	−
CONTROL	+	+	+/−	+/−	+/−	+/−	+	++	+	+	+	+	+/−	+/−

TABLE 16: Reactions of the Esterases of Potamopyrgus jenkinsi A to various inhibitors

TABLE 17: Reactions of the Esterases of Potamopyrgus jenkinsi B to various inhibitors.

	1	5b	5c	6*	7	9	11	12	14
E600 10^{-3}M	-	-	-	-	-	-	-	-	-
E600 10^{-4}M	-	-	-	+/-	-	-	-	-	-
EDTA	+	-	+	+	+/-	+/-	-	-	-
pCMB	+/-	-	+	+	+/-	+/-	-	-	-
UREA	+/-	-	+	+/-	-	-	-	-	-
CONTROL	+	+/-	+	++	+	++	+/-	+/-	+

TABLE 18: Reactions of the Esterases of Potamopyrgus jenkinsi C to various inhibitors.

	1	4	5c	6*	7	9	14
E600 10^{-3}M	-	-	-	-	-	-	-
E600 10^{-4}M	-	-	-	+/-	-	-	-
EDTA	+	-	+	+	+/-	+/-	-
pCMB	+/-	-	+	+	+/-	+/-	-
UREA	+/-	-	+/-	+/-	-	-	-
CONTROL	+	+/-	+	++	+	++	+

TABLE 19: Reactions of the Esterases of Potamopyrgus antipodarum to various inhibitors.

	1	2	2a	4a	5	5b	5c	6*	7	8	9	10	14
E600 10^{-3}M	–	–	–	–	–	–	–	–	–	–	–	–	–
E600 10^{-4}M	–	–	–	–	–	–	–	+/–	–	–	–	–	–
EDTA	+	–	–	–	+/–	–	+	+	+/–	–	+/–	–	–
pCMB	+/–	–	–	–	+/–	–	+	+	+/–	–	+/–	–	–
UREA	+/–	–	–	–	–	–	+	+/–	–	–	–	–	–
CONTROL	+	+/–	+/–	+/–	+/–	+/–	+	++	+	+/–	++	+/–	+/–

TABLE 20: Reactions of the Esterases of Potamopyrgus nigra to various inhibitors.

	1	5	5d	6	7	8	9	10	12	14
E600 10^{-3}M	–	–	–	–	–	–	–	–	–	–
E600 10^{-4}M	–	–	–	+/–	–	–	–	–	–	–
EDTA	+	+	+	+	+/–	–	+/–	–	–	–
pCMB	+/–	+/–	+	+	+/–	–	+/–	–	–	–
UREA	+/–	–	+	+/–	–	–	–	–	–	–
CONTROL	+	+	+	++	+/–	+/–	++	+/–	+/–	+

III - 2 - 3 Electrophoresis

Replicate slab gels were prepared as described in section III - 1 - 4. Each five-extract section was sliced in two; one slice was placed into an appropriate inhibitor/staining solution and the control slice into distilled water/staining solution.

III - 2 - 4 Staining using inhibitors

Experimental gel slices were pre-incubated by soaking in 100 ml of the appropriate inhibitor solution for 30 minutes at 25°C prior to the addition of substrates (20 mg each of α-n-a, α-n-p and β-n-a dissolved in 5 drops of acetone) and 100 mg of solid Fast Garnet. Control gels were pre-incubated in distilled water. Gels pre-incubated in $CuSO_4$ and $AgNO_3$ were removed from the inhibitor solution and, after quickly washing with distilled water, placed in the usual general esterase staining solution (section I - 5 - 1). This was an attempt to ensure that reactions between the inhibitor and staining solution were minimised.

Incubation with the stain was continued for a further 30 minutes and gels were then fixed in 30% ethanol in the usual way.

III - 2 - 5 Scoring band intensity

Bands were visually scored for intensity by reference to the same band on control gels. Scoring was based on the subjective scale described in section III - 1 - 6. A + in Tables 16 to 20 indicates activity and not inhibition.

III - 2 - 6 Results of inhibitor studies

Tables 16 to 20 summarise the results obtained using the above methods. Acetazolamide, copper sulphate, quinine sulphate, sodium fluoride and sodium sulphate had little or no effect on P. jenkinsi A gels apart from slightly lowering the overall staining intensity so as to render faint bands (Est. 2a, 3, 4, 5 and 11) undetectable. Silver nitrate produced a grey background against which no bands were visible. Eserine produced an orange stain on the background similar to that reported by Ecobichon[26] and Oxford[11]. Only Est. 6* was visible on these gels; other bands were possibly represented by diffuse esterase stains on other parts of the gel.

For these reasons, the above inhibitors were left out of later runs.

E600 lowered the overall staining intensity even at the lowest concentration used (10^{-4} M), where only Est. 6* was just visible. EDTA and pCMB had similar effects, inhibiting Est. 2a, 3 (EDTA only), 4, 5, 8, 11 and 14 completely and Est. 7, 9 and 10 partially in P. jenkinsi A; Est. 5a, 9 (partially), 12 and 14 in P. jenkinsi B; Est. 4 and 9 (partially) and 14 in P. jenkinsi C; Est. 2, 2a, 4a, 5b 7 (partially), 10 and 14 in P. antipodarum and 9 (partially), 10, 12 and 14 in P. nigra.

Only Est. 1, 2 and 5c were completely uninhibited by urea in P. jenkinsi A and only Est. 5c (5d in P. nigra) was uninhibited in the other extracts. Est. 6* was partially inhibited by urea in all extracts.

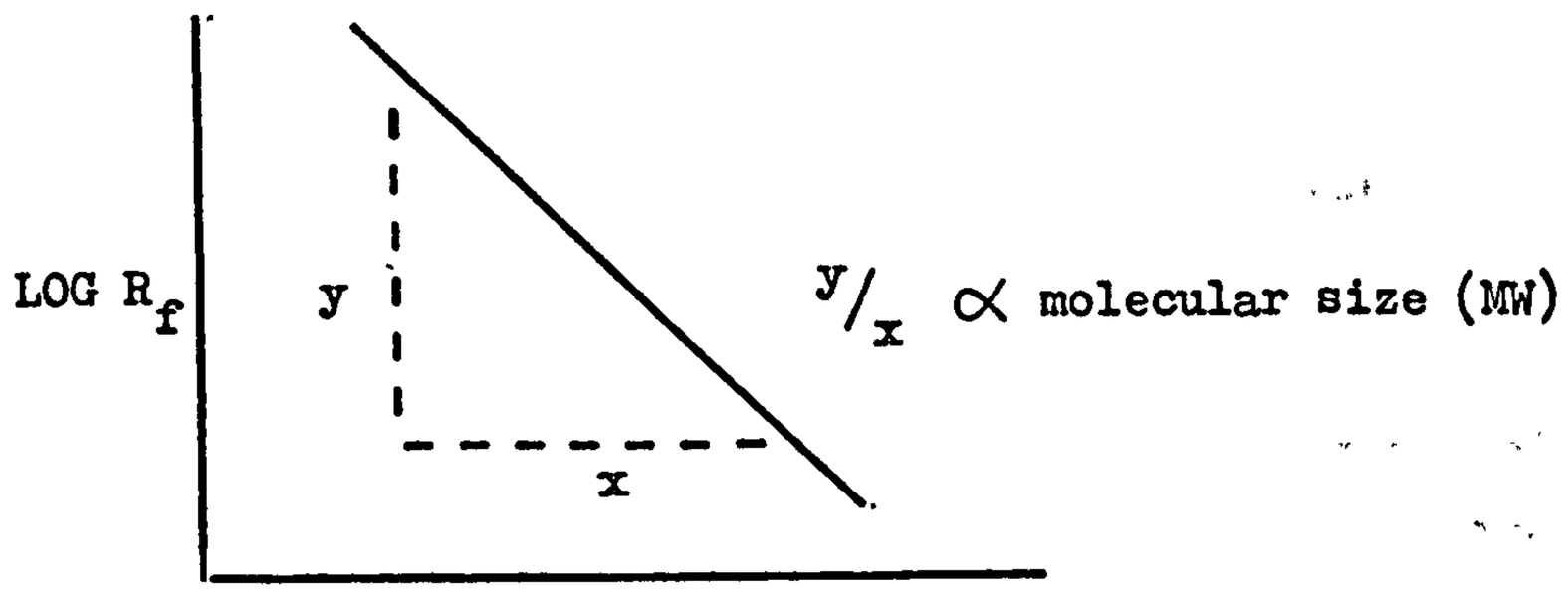
No inhibitor provided sufficient differential inhibition of the bands making up Est. 6* to add to the information obtained from substrate specificity studies. The inhibition results were generally less informative than substrate specificity since many of the inhibitors caused an overall lowering in staining intensity and did not seem to be specific for any particular band. Some bands did, however, appear to have homologous inhibition properties, in particular Est. 2a and 3 in P. jenkinsi A which were slightly activated by PCMB. The inhibition data generally supported the homologies between bands described in section III - 1 - 7 (particularly homologies between bands in different species).

III - 3 The Relative Molecular Weights of the Esterases

An electrophoretic method for estimating the molecular weights of proteins was first described by Smithies[27] using starch gel and later developed by Zwaan[28] and Parish and Marchalonis[29] for use on the acrylamide gels.

The method relies on the principle that molecules are exponentially retarded as pore size decreases, allowing separation of molecular charge from molecular size; both of which affect electrophoretic mobility. The slope of the straight line obtained by plotting log R_m of a band at various gel concentrations is thus directly proportional to the molecular size of the protein (or other charged molecule) making up the band. Molecular size of globular proteins,

(e.g. isozymes) is generally linearly related to molecular weight.[28]



GEL CONCENTRATION

A "Ferguson"[31] plot of this type (usually the R_f is multiplied by 100 to avoid negative logarithms) may be used to estimate the absolute molecular weight of the band by comparing the slope with that obtained by running a number of "standard" proteins (having molecular weights estimated by other methods e.g. ultra-centrifugation) on a range of gel concentrations. However, Oxford[10] has pointed out that estimates of absolute molecular weight obtained in this way have very wide confidence limits unless the standard plot is constructed using a large number of proteins. Relative molecular weights obtained by comparing slopes (without reference to a standard slope) are, on the other hand, estimated much more accurately.

The method has an advantage over other methods of estimating molecular weight in that estimates of the slopes of mixed proteins may be obtained simultaneously. Hedrick and Smith[32] developed the method for use on crude enzyme preparations such as the extracts described in the present work. In order to do this it is ideally necessary to be able to recognise bands in all gel concentrations. In practice, this is relatively easy for mixtures containing few proteins and has been used by e.g. Kingsbury and Masters,[33] Oxford[10] and, more recently, Johnson,[34] to investigate the molecular sizes of allelic products. More complex mixtures of proteins lead to problems of poor resolution and the overlapping of bands such as those described in section III - 3 - 5.

The technique is also limited to proteins larger than 10,000 daltons. Jeffrey[35] has shown that Ferguson plots are linear down to this molecular size, but investigation of slopes below this by Gonenne and Lebowitz[36] have shown that the plot is non-linear. None of the Potamopyrgus esterases was estimated to have a molecular

size of less than 10,000 daltons (see section III - 3 - 6).

III - 3 - 1 Preparation of gels

Sets of gels were prepared, each set consisting of eight gels having final acrylamide concentrations of 4, 5, 6, 7, 8, 9, 10 and 11 per cent. Discontinuous pH gels were used as the formation of a front during electrophoresis allows accurate measurements of relative mobilities of bands to be made. Several sets were usually produced at the same time using acrylamide solutions prepared by diluting a 12% final concentration solution C (prepared by dissolving 48 grams acrylamide and 1.28 grm bis in warm distilled water and making up to 100 ml with distilled water). Dilution was carried out according to Table 20a to give a final volume of 4 ml solution C for each gel concentration. This, when made up as described in section I - 4 - 2, gave a final volume of 16 ml of lower gel solution per gel concentration - enough for ten sets of gels.

TABLE 20a : Dilutions of 12% acrylamide solution

Final gel concentration	ml 12% soln. C	ml distilled water
4	1.34	2.66
5	1.66	2.34
6	2.00	2.00
7	2.34	1.66
8	2.66	1.34
9	3.00	1.00
10	3.34	0.66
11	3.66	0.34

Gellation times were found to be similar for all gel concentrations used.

III - 3 - 2 Extracts used

The extracts described in section III - 1 - 3 were used for the molecular weight determinations. These were stored at -20°C between electrophoresis runs. A mass extract prepared in the same way using snails from the Campus Lake population of *P. antipodarum* (popln. 46) was used to estimate the slopes of Est. 6 and 6a. This population lacked Est. 7 which normally obscures these two bands.

III - 3 - 3 Electrophoresis

Electrophoresis was performed as described in section I - 4 - 3 using eight different concentrations of gel in each run. 0.1 ml of extract was applied to the upper gel surface and gels were removed when the front had reached a mark (5.5 cm for 'short' gels and 8 cm for 'long' gels - see section I - 4 - 1) made on the tube with a waterproof pen. Lower concentration gels ran faster than those of high concentration.

III - 3 - 4 Staining

After electrophoresis, gels were removed from the tubes and the front marked by cutting the gel with a razor blade.

Sets of gels were originally stained using the general esterase stain (section I - 5 - 1) but as the resultant banding patterns were too complex to interpret (individual bands could not be followed between gels), other sets were stained using α-n-b substrate. This necessarily gave less information as fewer bands are butyrate specific. Some sets of gels were also stained using α-n-p (e.g. P. jenkinsi A and C and P. antipodarum) in order to obtain data on e.g. Est. 3 and 4 in P. jenkinsi A and Est. 5 in P. jenkinsi C - bands which are proprionate-specific. After staining, gels were fixed in 30% ethanol and bands scored before being photographed.

III - 3 - 5 R_f measurements

It was not possible to measure R_f values at all gel concentrations for all bands visible on 7.5% gels stained for general esterase because:

i) Only gels stained with α-n-b (or, in some cases, α-n-p) were used in the measurement of R_f values (see section III - 3 - 4).

ii) Faint bands (e.g. Est. 7) became too diffuse to measure in lower gel concentrations.

iii) Bands move with different relative speeds in different concentrations of acrylamide and overlapping frequently occurred. Where overlapping bands were of similar widths, the R_f was assumed

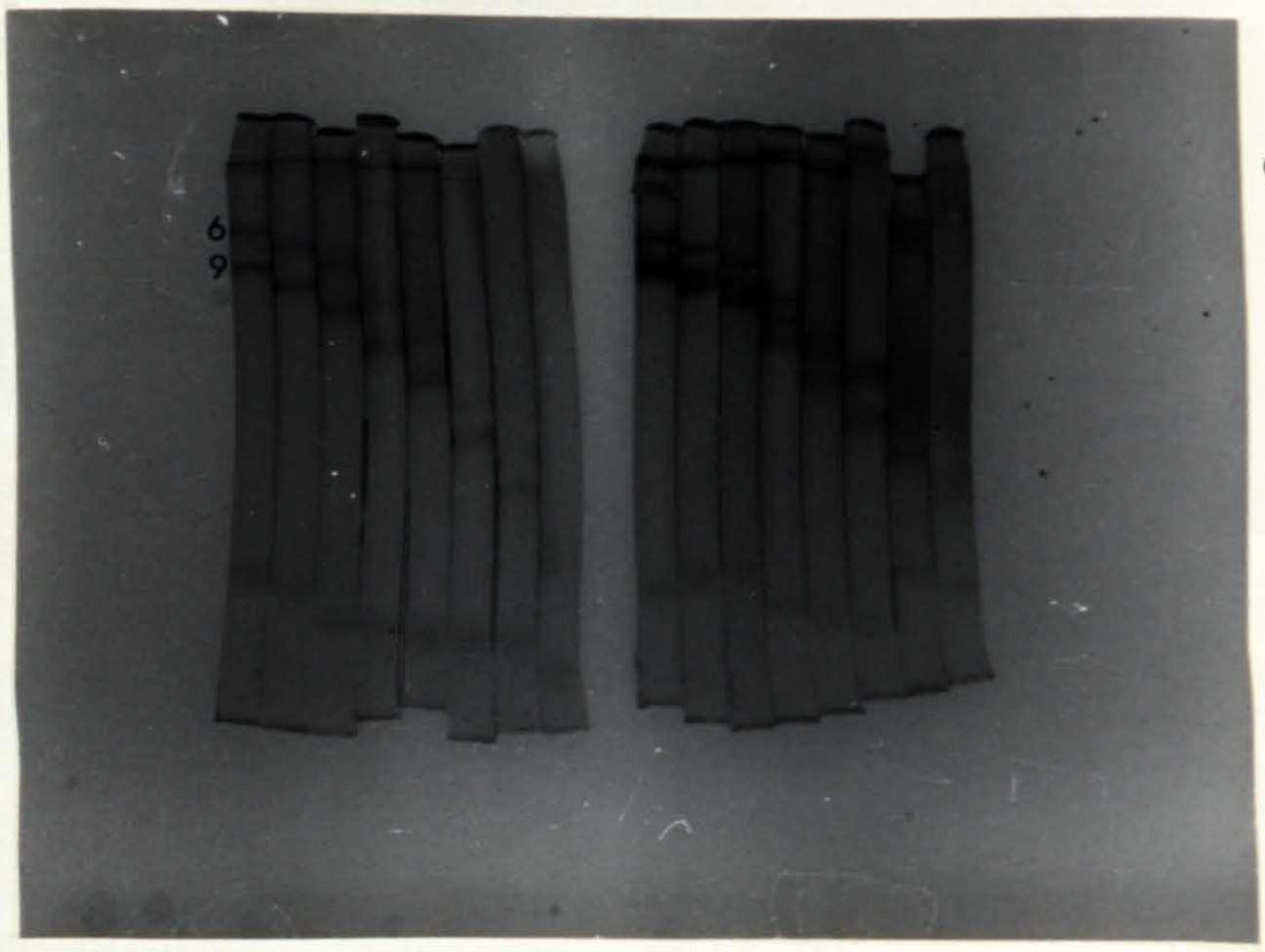


Plate 6 : Electrophoresis of a mass extract of the Campus Lake population (46) of P.antipodarum on sets of 4 to 11% disc gels. The left-hand set was stained using ∝ - n - b and the set on the right using ∝ - n - p. Esterases 6 and 9 stain heavily with ∝ - n - b and Esterases 6, 6a and 9 with ∝ - n - p.

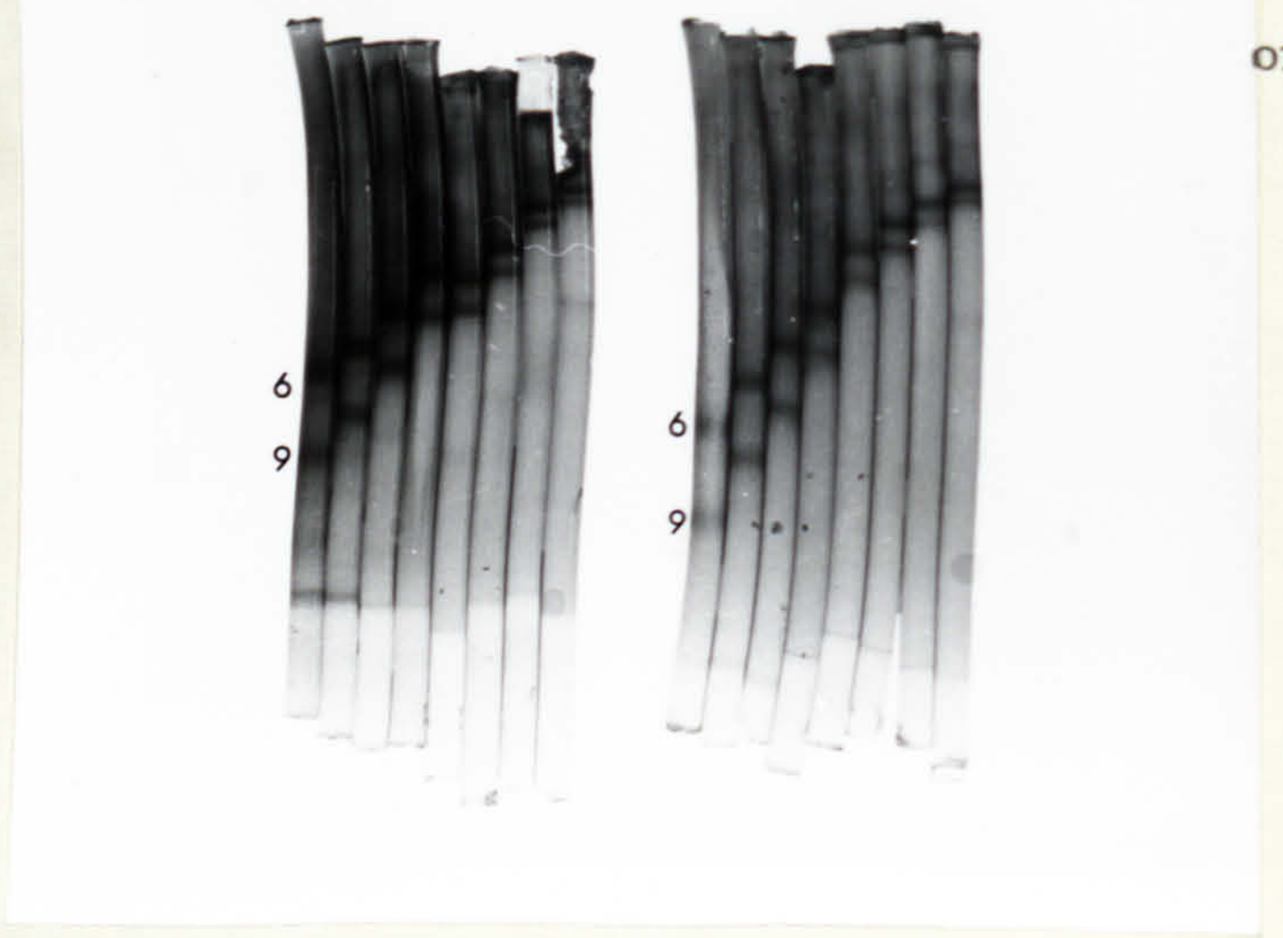


Plate 7 : Replicate sets of 4 to 11% disc gels on which an extract of P.jenkinsi C has been run, stained for esterase using ∝ - n - b. The difference in the slopes of bands 6 and 9 can be clearly seen.

to be the same for each; where one band was narrower only the R_f of the wider band was recorded.

iv) Rare bands occurring in P. antipodarum (e.g. Est.5b) and P. nigra (e.g. Est. 3 and Est. 4) were sometimes difficult to detect due to the dilution effect described in section III - 1 - 2. Est. 5b was, however, highly active towards α-n-p and an estimate was obtained for this band. The dilution effect did not affect extracts prepared from monomorphic populations, (e.g. P. jenkinsi A) as bands in these populations occur in all individuals

Consequently the number of R_f measurements used to estimate the slopes of the log R_f vs gel concentration plot varied for different bands. No slope was estimated from fewer than five points (gel concentrations); usually each point was estimated from at least two replicate R_f measurements. Plates 6 and 7 show sets of gels stained for butyrate-specific and proprionate-specific esterases.

III - 3 - 6 Estimation of slopes

Slopes and regression statistics were obtained by plotting $\log_{10}$ 100 R_f values against gel concentration (% acrylamide) using the University of Michigan regression procedure on the DEC-10 computer at York. The procedure produces a visual plot of the data together with the statistics associated with each regression line. Slopes and 95% confidence limits are presented in Table 21. Confidence limits are those generated for the mean slope and are considered to be legitimate as only the relative slopes are to be compared. Oxford[10] has pointed out that if absolute molecular weights are to be calculated, confidence limits need to be set using methods of 'inverse prediction' described by Sokal and Rohlf.[37]

Slopes were compared by first looking for charge/size isomers (described by Hedrick and Smith[32]) e.g. those bands which remained equidistant in all gel concentrations in a particular extract (e.g. Est. 9 and Est. 10 an Plate 8). These isomers differ in charge only, unlike other bands which differ in both size and charge since they have obviously different slopes (see Est. 6 and Est. 9 on Plate 7). Molecular size (and therefore weight) relationships between bands

P. jenkinsi A

Est	Mean Slope$_2$ (x 10^2)	95% CL
1	30.20	± 3.702
2	29.80	± 3.145
3	5.18	± 0.680
4	7.14	± 0.425
5	6.94	± 1.071
5f	6.58	± 0.460
6	6.72	± 0.577
7	6.58	± 0.268
8	8.98	± 0.420
9	7.18	± 0.187
10	6.92	± 0.261
11	7.08	± 0.244

P. jenkinsi B

Est	Mean Slope$_2$ (x 10^2)	95% CL
1	25.50	± 4.610
5b	9.20	± 0.586
5f	6.52	± 0.292
6	6.68	± 0.537
6a	6.39	± 0.303
7	6.63	± 0.611
9	7.16	± 0.149

P. jenkinsi C

Est	Mean Slope$_2$ (x 10^2)	95% CL
1	24.10	± 4.254
4	6.78	± 0.478
5f	6.29	± 0.501
6	6.52	± 0.317
6a	6.27	± 0.816
7	6.69	± 0.583
9	7.12	± 0.299

P. antipodarum

Est	Mean Slope$_2$ (x 10^2)	95% CL
1	23.84	± 2.252
5b	9.38	± 0.434
5f	6.61	± 0.208
6	6.78	± 0.189
6a	6.30	± 0.577
7	6.73	± 0.565
9	7.09	± 0.116

P. nigra

Est	Mean Slope$_2$ (x 10^2)	95% CL
1	26.00	± 3.377
5	9.03	± 1.341
5f	6.02	± 0.239
7	6.81	± 0.436
8	9.08	± 0.892
9	6.97	± 0.230
10	6.81	± 0.288

TABLE 21: Slopes of Log_{10} (100 R_f) vs gel concentration plots for the Esterases of Potamopyrgus ssp.

within an extract were visually deduced in this way.

Suspected homologies between bands in different extracts were confirmed by comparing slopes using the statistical routine described by Quenouille[38] for the simultaneous comparison of regression coefficients (using a variance ratio test). Data were initially tested for homogeneity using Bartlett's test. A specimen calculation for the Est. 9 bands in the five extracts is given in Appendix II. Similar comparisons of other slopes (all data sets were homogeneous) gave the following F values.

BANDS	F value	df
Est. 1 (&2 in P. jenkinsi A)	1.62	6,83
5f (=5c on slab gels)	0.97	5,134
6	0.53	5,166
6a	2.55	4,48
7	1.67	5,114
9	2.40	5,156

F values are consistent with the hypothesis that, within a data set, the slopes of the bands did not significantly differ (p 0.95). It is, therefore, legitimate to estimate the mean slope of bands within a particular locus by combining data from each extract. These values are given in Table 21.

In terms of absolute molecular weight, the estimates for the Est.1 bands were extremely high and were equivalent to approximately 6×10^5 daltons (using the standard calibration curve given by Oxford[10]). This suggests that these bands are probably polymeric and might explain why the Est. 1 band in P. jenkinsi B appeared as two bands on disc gels and one on slab gels (section III - 1 - 8). The molecule could have been disrupted by the change in pH at the front during disc electrophoresis resulting in the production of two distinct entities.

III - 3 - 7 Homologies between esterases and their assignment to loci

It has been shown in section III - 1 - 8 that most esterases on disc gels could be recognised on slab gels. Bands on slab gels were, therefore, assigned relative molecular weights on this basis. The most likely relationships between bands based on their substrate specificities, inhibition properties and molecular weights are depicted in Fig. 17 and Table 22. Molecular weight estimates were not

TABLE 22: Assignment of esterase bands to loci on the basis of their biochemical and physical properties.

LOCUS	MAIN SUBSTRATES	MAIN INHIBITORS	REL.MOL.WT.	P.j.A	P.j.B	P.j.C	P.anti	P.nigra
1	∝-n-a, ∝-n-p	E600	26.6	1,2	1	1	1,?2	1
2	∝-n-a, n-AS-a	EDTA, UREA	-	2a,3	-	-	2a, 4a	-
3	∝-n-b	All	6.95	4,5	-	4	-	4
4	indoxyl-a	E600, UREA	-	-	-	-	5	5
4a	∝-n-p	All	9.29	-	5b	-	5b	-
5	not ∝-n-b	E600	6.37	5c,6a	5c,6a	5c,6a	5c,6a	5d,6a
6	∝-n-b	E600	6.68	6,7	6,7	6,7	6,7	6,7
8	∝-n-a, indoxyl-a	All	9.03	8	-	-	8	8
9	∝-n-a, indoxyl-a	E600, UREA	7.07	9,10	9,9a	9	9	9
10	∝-n-a	All	6.94	11	-	-	10	10, 12
11	∝-n-a, indoxyl-a	All	-	-	11,12	-	-	-
12	∝-n-p, indoxyl-a	All	-	14	14	14	14	14

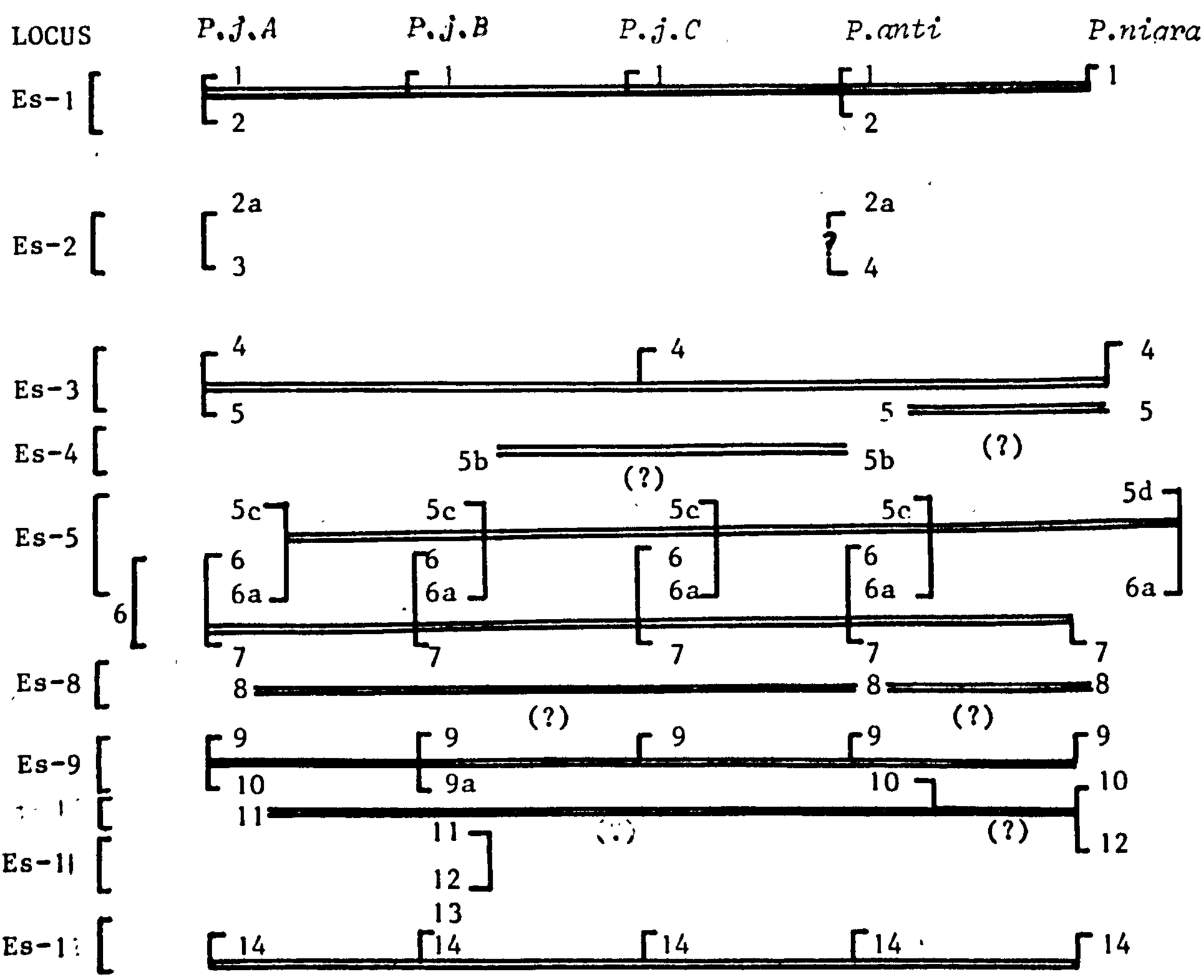


Fig.17 : Homologies between *Potamopyrgus* esterases based on their biochemical and physical properties.

available for some bands (section III - 3 - 5) and homologies between these and other bands were treated cautiously as they are based on their mobility on 7.5% gel and biochemical properties only. Relationships between such bands are marked (?) on Fig. 17.

It is possible to identify pairs of bands within a species which had unique biochemical and physical properties. Bands were also found which could not be paired with any other band within a species (e.g. Est. 11 in P. jenkinsi A). Homologies between pairs of bands and single bands between species are represented by double lines on Fig. 17.

For the reasons given at the beginning of this chapter, it was assumed that a group of homologous bands represented alleles at a particular locus - the locus is characterised by the biochemical and physical properties of its gene products. Bands which could not be paired within a species presented a problem as they may represent either a locus heterozygous for the visible allele or the visible/null heterozygote. In the absence of breeding data, the problem could be partially resolved by reference to differences in staining intensity of the single band in population surveys. It is reasonable to assume, for example, that the following banding patterns at a locus in five individuals:

1	2	3	4	5

theoretically represents genotypes

null/null	null/A	A/A	null/null	null/A
1	2	3	4	5

Similarly, if two visible alleles (A and B) have different mobilities and are segregating with a null allele, the following theoretical banding patterns may result:

phenotype	A					
	B					
individual		1	2	3	4	5	6
genotype		A/B	A/A	B/B	B/null	A/null	null/null

Patterns such as these were found in populations of P. antipodarum (e.g. Est. 14 in Fig. 19) and P. nigra (e.g. Est. 1 in Fig. 20), possibly as a result of occasional rounds of sexual reproduction in these largely parthenogenetic populations. Allele

frequencies (including those of the null) are determined in this way in chapter IV, but due to possible errors caused by e.g. differences in staining time and temperature they should be treated with caution.

The presence of null alleles in P. jenkinsi populations is impossible to detect due to the complete absence of segregation. When calculating heterozygosity and genetic distances in chapter VI, it is assumed that single bands in P. jenkinsi represent loci which are homozygous for a visible allele. As some null alleles may have been present at these loci, this assumption almost certainly resulted in an under-estimate of the proportion of heterozygotes per locus.

In summary, the approach used in this chapter to the problem of the genetic interpretation of esterase banding patterns in Potamopyrgus species has provided a basis on which to assign bands to loci which is better than the simple guesswork often used. However, due to the practical and theoretical problems associated with the method, and in order to confirm the interpretation of, particularly, P. jenkinsi esterases, it would be desirable to set up crosses between sexually-reproducing individuals from species (such as P. antipodarum) having loci homologous with those of P. jenkinsi.

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CHAPTER IV : ELECTROPHORETIC VARIATION IN BRITISH AND AUSTRALASIAN SPECIES OF POTAMOPYRGUS

IV - 1 Electrophoresis

Where sufficient numbers of adult snails were available, at least thirty from each population were individually electrophoresed, (see Table 23). Snails were normally electrophoresed within one week of arrival in the laboratory; one sample of P. jenkinsi C (Popln. 35) had been maintained by Mr. T. Warwick (University of Edinburgh) in a large laboratory tank for an unknown period.

Electrophoresis was performed on continuous pH slab gels as described in section I - 3. Whenever possible, all snails from a population were electrophoresed on the same gel. Gels were sliced (section I - 3 - 4) and slices stained for general esterase (section I - 5 - 1), acid phosphatase (section I - 5 - 2) and Mdh (section I - 5 - 3). Relative mobilities of bands were measured using Est. 9 as a reference band (see section II - 1 - 1).

IV - 2 Statistical treatment of genotype frequencies

In this and the following chapter, observed genotype frequencies are tested, where appropriate, for their goodness-of-fit to Hardy-Weinberg expectations by using Chi-squared (χ^2) tests.

Williams[1] has pointed out that Pearson's approximation to

$$\chi^2 = \sum (O-E)^2/E$$

is less robust than the maximum likelihood estimator

$$\chi^2 = 2 \sum O \ln \frac{O}{E}$$

particularly where low expected numbers, (less than 5) are encountered. The maximum likelihood estimator was used for calculating all χ^2 values in this thesis. In some cases, where several expected classes were less than 5, χ^2 values are probably inflated. Where this results in a significant deviation from the null hypothesis, the statistic is somewhat unreliable. Insignificant deviations from the null are, nevertheless, made more robust by low expected values.

IV - 3 Variation in P. jenkinsi A

TABLE 23: Comparison of electrophoretic and morphological phenotypes of Potamopyrgus jenkinsi populations.

Population No. (see Appendix for locality)	Number Electrophoresed	Electrophoretic Phenotype(s)	Morphological Strain
1	52	G	B
2	30	A	A
3	30	A	A
4	30	A	A
5	30	A	A
6	30	A	A
7	30	A	A
8	34	A	A
9	30	A	A
10	30	A	A
11	30	A	A
12	1	A	A
13	15	A	A
14	30	A	A
15	11	A	A
16	30	A	A
17	30	A	A
18	31	A, B, D	A
19	30	A	A
20	30	A, E	A
21	25	A	A
22	30	A	A
23	30	A	A
24	30	A	A
25	28	A	A
26	30	A, F	A
27	30	A	A
28	30	G	B
29	30	A	A
30	30	A, H	27A 3C
31	30	A	A
32	30	A	A
33	12	A	A
34	30	A	A
35	30	H	C
36	14	A	A
38	2	A	A

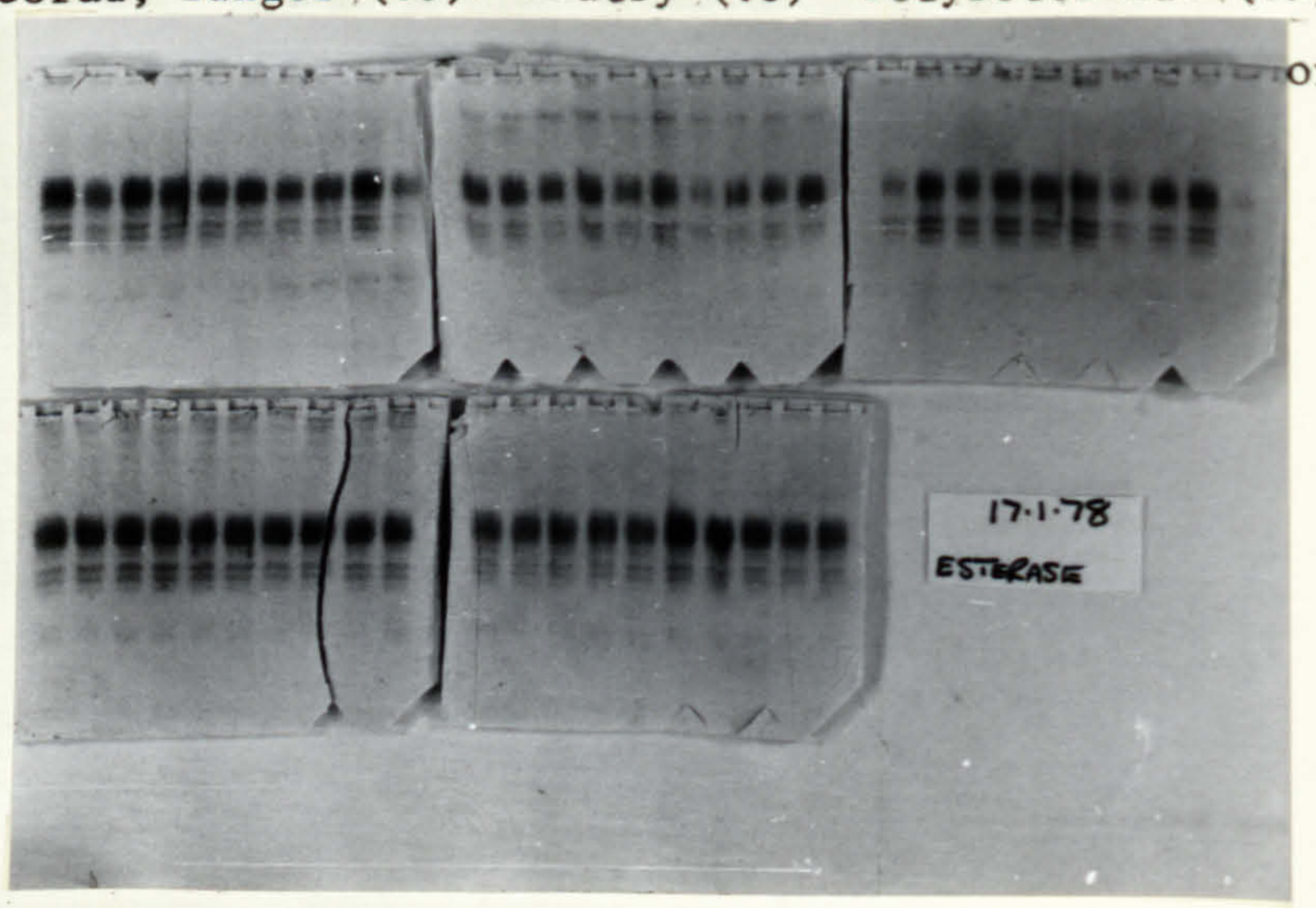


Plate 9 : Esterase phenotypes of individuals from five populations of P.jenkinsi A. Banding patterns are identical except at the Es - 1 locus in populations 19 and 20. Staining intensity varies between gels due to slight differences in the size of snails from different populations.

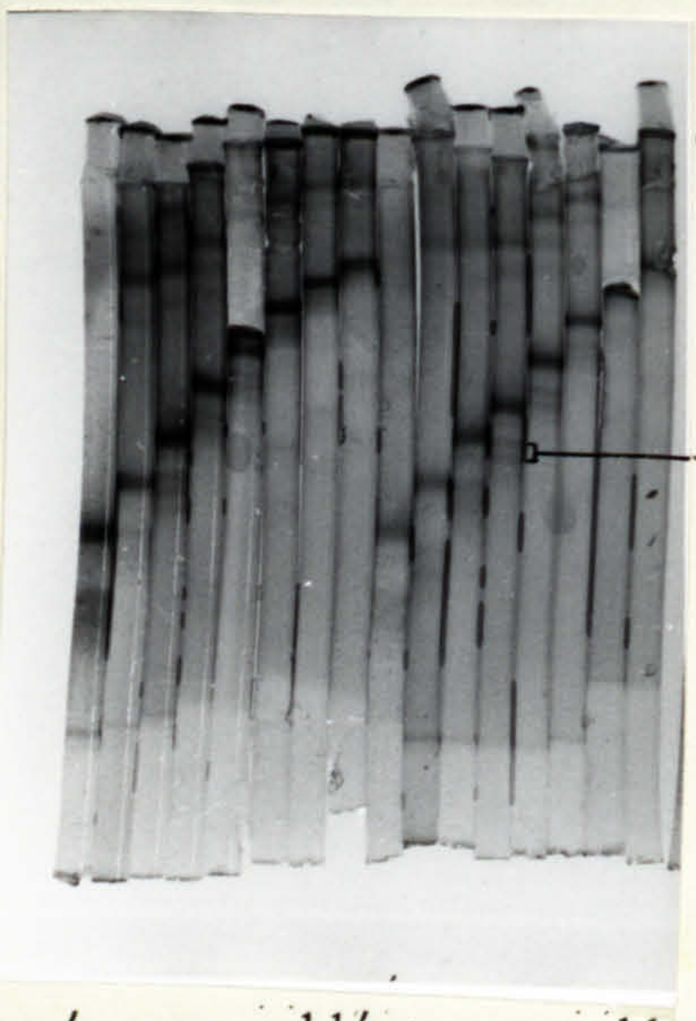
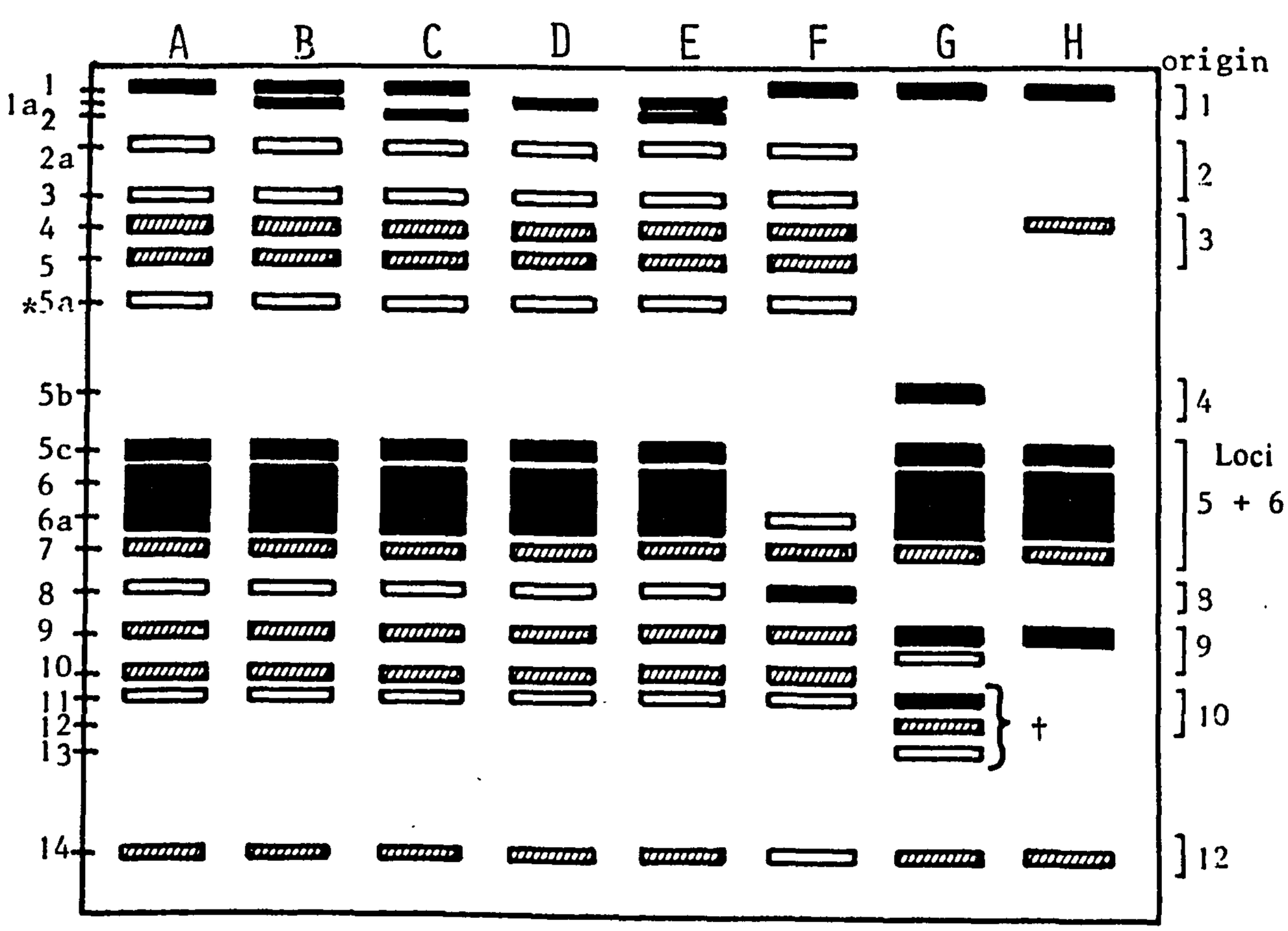


Plate 8 : Replicate sets of disc gels (4 to 11%) used for relative molecular weight determinations; P.jenkinsi A stained for esterase using ∝- n - butyrate. Esterases 9 and 10 remain equidistant in all gel concentrations showing that they differ in charge only and not in molecular size. The 6% gel is missing from the set on the left.

TABLE 24: Variation at the Es-1 locus in two populations of Potamopyrgus jenkinsi A :

Population	N		GENOTYPE DISTRIBUTION						BAND FREQUENCY		
			$^{1}/_{1}$	$^{1}/_{2}$	$^{1a}/_{2}$	$^{1a}/_{1a}$	$^{2}/_{2}$	$^{1}/_{1a}$	1	1a	2
CROSE MERE (Popln. 18)	31	O	0·	27	4	0	0	0	0.435	0.065	0.500
		E	5.88	13.5	2.0	0.13	7.75	1.74			
POLYBOTTS LANE (Popln. 20)	30	O	24	-	-	3	-	3	0.85	0.15	-
		E	21.68	-	—	7.64	-	0.68			

Fig.18 Esterase phenotypes of *Potamopyrgus jenkinsi* populations; 7.5% acrylamide pH 9.5 continuous buffer stained α-n-a, α-n-p, β-n-a.



A, B, C, D, E, F — *P.jenkinsi A*

G – *P.jenkinsi B*

H – *P.jenkinsi C*

* Est.5a was sometimes present in snails electrophoresed straight from the wild but was absent from laboratory stocks (see section V-2-2 for details)

† Esterases 11, 12 and 13 were not normally visible in the Hull population of *P.jenkinsi B* (see section V-3-2)

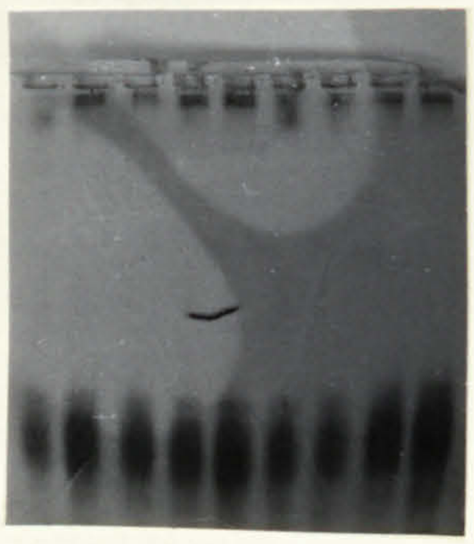
Es-1 [ origin

Plate 10 : Variation at the Es 1 locus in the Leicester (Polybotts) population (popln.20) of *P.jenkinsi A.*

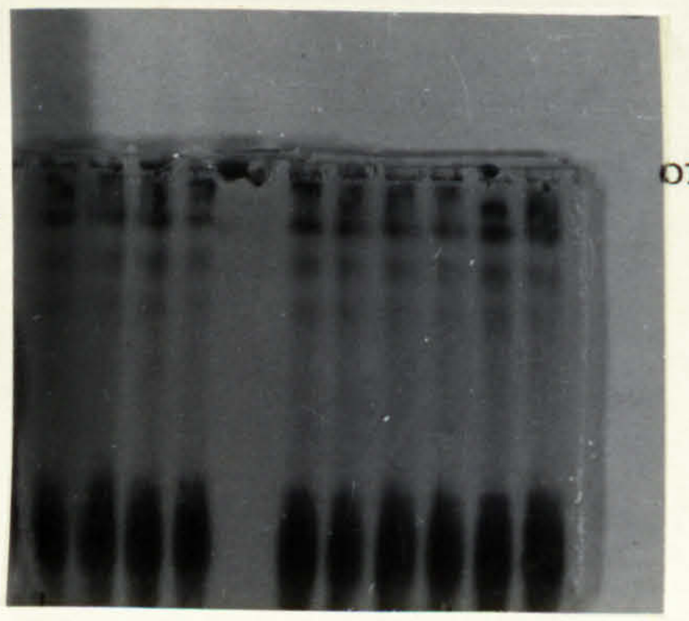
Es-1 [ origin

Plate 11 : Variation at the Es 1 locus in the Crose Mere population (popln.18) of *P.jenkinsi A.*

IV - 3 - 1 Esterases

P. jenkinsi A populations were remarkably monomorphic; only three populations (18, 20 and 26) were found to exhibit variation at esterase loci. With the exception of these, all individuals had phenotype A (Fig. 18, Plate 9 and Table 23).

In two populations (18 and 20) variation was suspected at the Es-1 locus although the small differences in R_m values of these bands (Est. 1, 1a and 2) on 7.5% gel run for four hours made resolution difficult. The extracts were, therefore, re-run on 7.5% gels for eight hours. The results of these runs are shown in Plates 10 and 11. Variation was found to be due to the presence of bands Est. 1 and 1a in popln. 20 and Est. 1, 1a and 2 in popln. 18. The distribution of genotypes in these populations is shown in Table 24. The possibility that variation at the Es-1 locus in these populations was due to segregation is unlikely as no males were present in the samples and no segregation had occurred at other loci thought to be heterozygous. It is, therefore, pointless to compare the distribution with that expected from Hardy-Weinberg assumptions as one of the major assumptions (random mating) is clearly inapplicable.

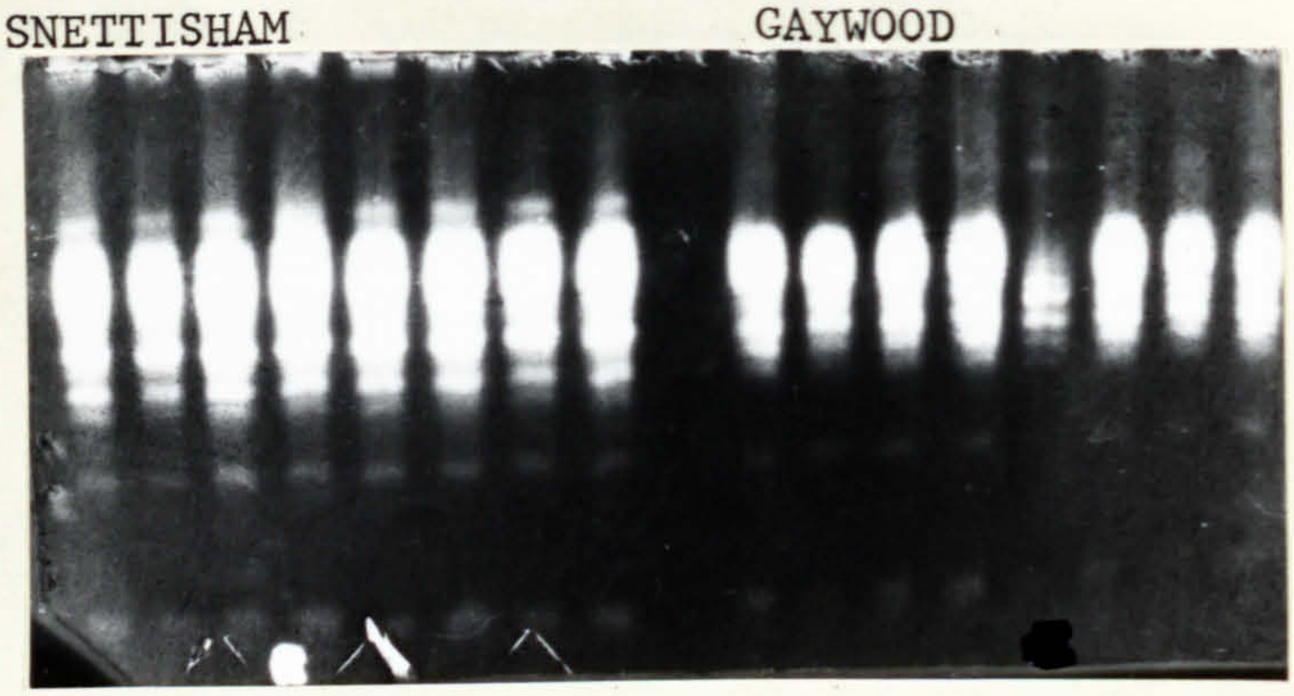
It is likely that these genotypes (which represent genetically distinct clones) have arisen by mutation at the Es-1 locus giving rise to viable heterozygotes. This does not, however, adequately explain the evolution of the 1a/1a clone in popln. 20 as two similar mutational events would be needed to give this genotype. It is possible that the 1a/1a assumed homozygote is, in fact, a 1a/null heterozygote.

In the sample taken from the Gaywood River, Norfolk population (popln. 26), one individual was found in which Est. 5c and Est. 6 were undetectable (phenotype F on Fig. 18 and Plate 12). Est. 8 was heavily staining in this individual and two slower bands (Est. 7 and ?6a) were visible.

This was the only individual having phenotype F found in the entire survey and clearly lacked two highly active esterase bands. Est. 14 was faintly staining in this individual, although the snail was the same size as others in the sample and the protein concentration in the extract was therefore similar. If it is assumed that the Es-12 locus is normally homozygous for Est. 14, then pheno-

P.jenkinsi B SNETTISHAM | *P.jenkinsi A* GAYWOOD

origin

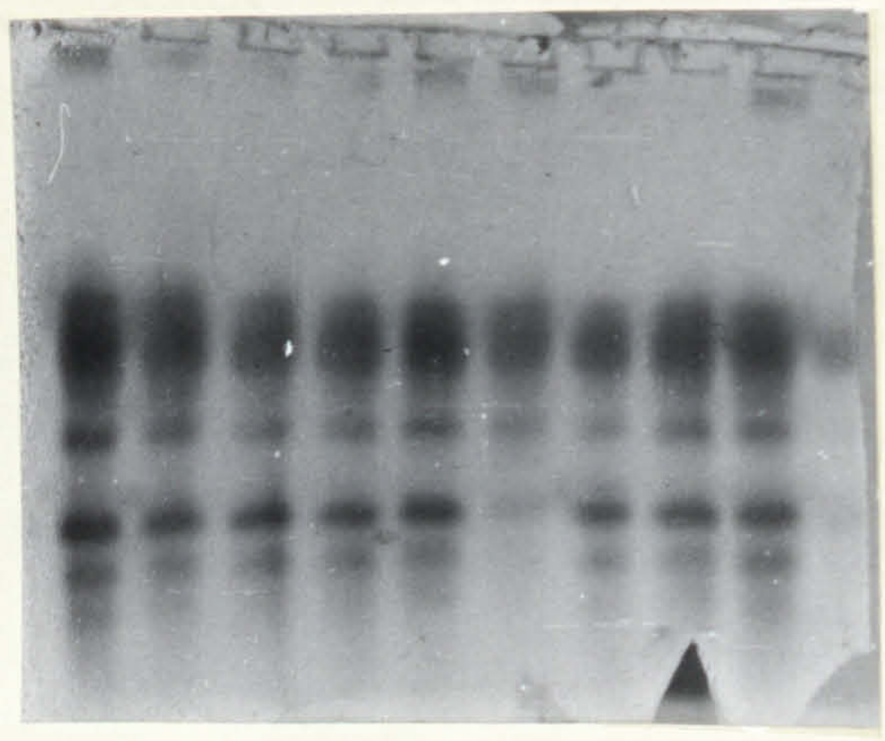


phenotype G G G G G G G A A A A F A A A

Plate 12 : Esterase phenotypes of individuals from two populations of *P.jenkinsi*. Phenotype F is missing bands 5c and 6 seen in phenotype A.

origin

11
12
13



F1 (------F2 progeny--------)

Plate 16 : Esterase phenotypes of individuals from the Hull population of *P.jenkinsi B* showing the 'mutant' (F1) found in laboratory stocks and eight of its progeny. Bands 11, 12 and 13 are not normally detectable in snails from this population.

type F may represent the Est. 14/null heterozygote. Phenotype F differed from phenotype A at possibly four loci (Es-5, Es-6, Es-8 and Es-12) and may represent a new clone of P. jenkinsi. There were no obvious morphological differences between snails in this population and the individual showing phenotype F had the usual P. jenkinsi Mdh and Ac.Ph. phenotypes. A larger survey of the Gaywood River population might establish whether two viable strains (A and F) co-exist or whether F was a unique mutant.

Variation in the staining intensity of Est. 5a on disc gels was frequently found between snails from a single population. A band thought to correspond to Est. 5a was also seen on some slab gels. Est. 5a was present in some snails but absent or faint in others when population samples were electrophoresed immediately after collection from the wild. The band was virtually undetectable in snails which had been kept for over a month on the laboratory diet of boiled and dried lettuce.

In view of the phenotypic modification of some esterases by diet in Cepaea nemoralis and C. hortensis reported by Oxford[2], an experiment was set up to investigate possible dietary effects on the esterases of P. jenkinsi A.

A clone of 200 adult snails was reared from a single specimen of P. jenkinsi A taken from the Bielby Beck population (popln. 11) and fed solely on boiled and dried lettuce as described in section I - 1.

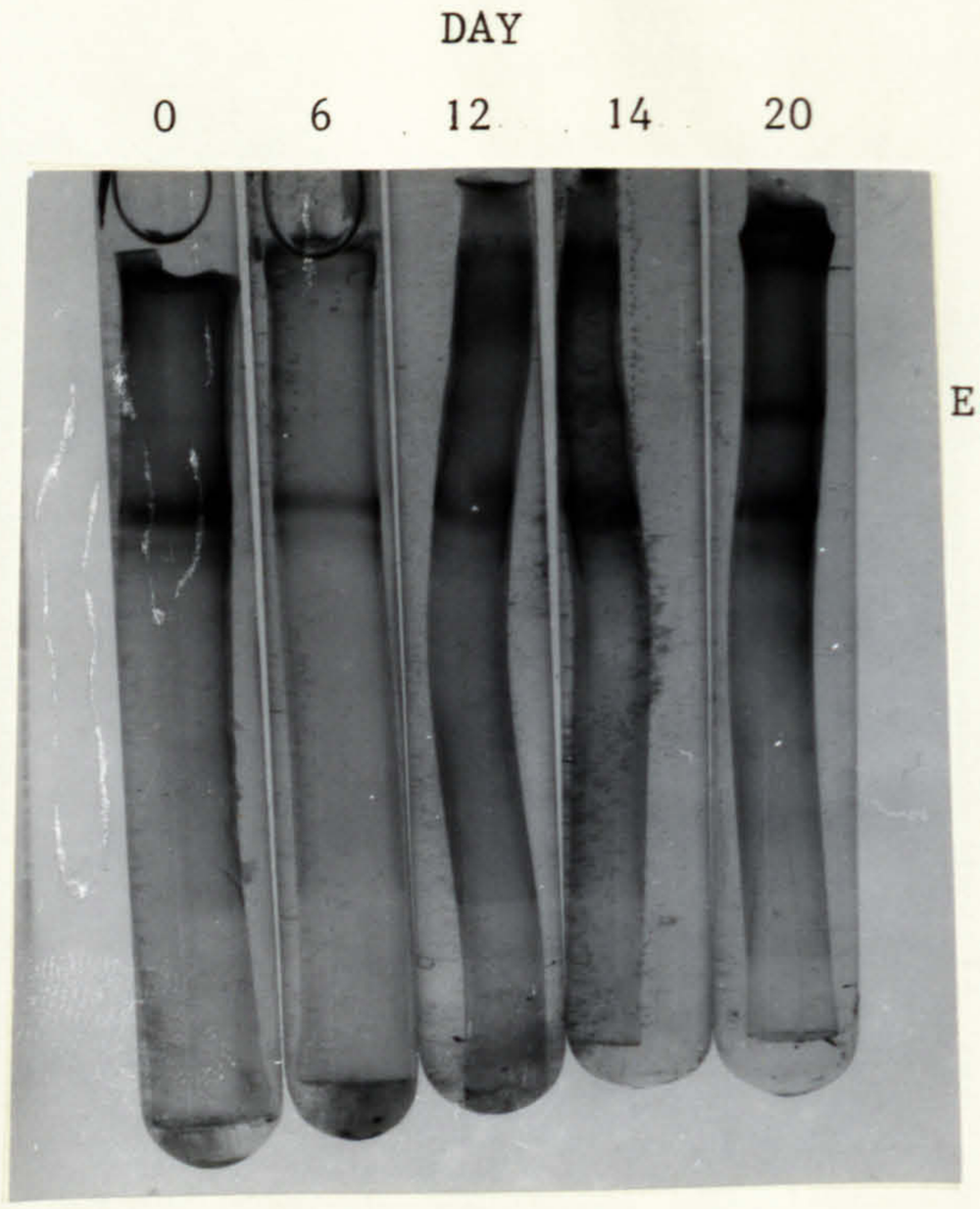
Fifty individuals were placed in each of four plastic boxes (14 cm x 8 cm x 5 cm deep), fitted with lids to prevent evaporation and each containing 400 ml of pre-aerated (10 days) tap water. The boxes were aerated using a capillary tube aerator and kept in the dark (to prevent algal growth) at room temperature (approx. 22°C).

The following treatments were chosen in an attempt to represent different aspects of the natural diet.

Box 1 Benthic detritus from the Bielby Beck (probably the natural diet).

Box 2 boiled and dried lettuce, representing a nutritious component of the diet.

Box 3 dried and ground autumn maple leaves, representing a non-nutritious component of the diet.



Est 5a

Plate 13 : The induction of Est. 5a in P.jenkinsi A. Individuals sampled from a clone fed solely on ground, dried maple leaf for 20 days; disc gels stained for general esterase.

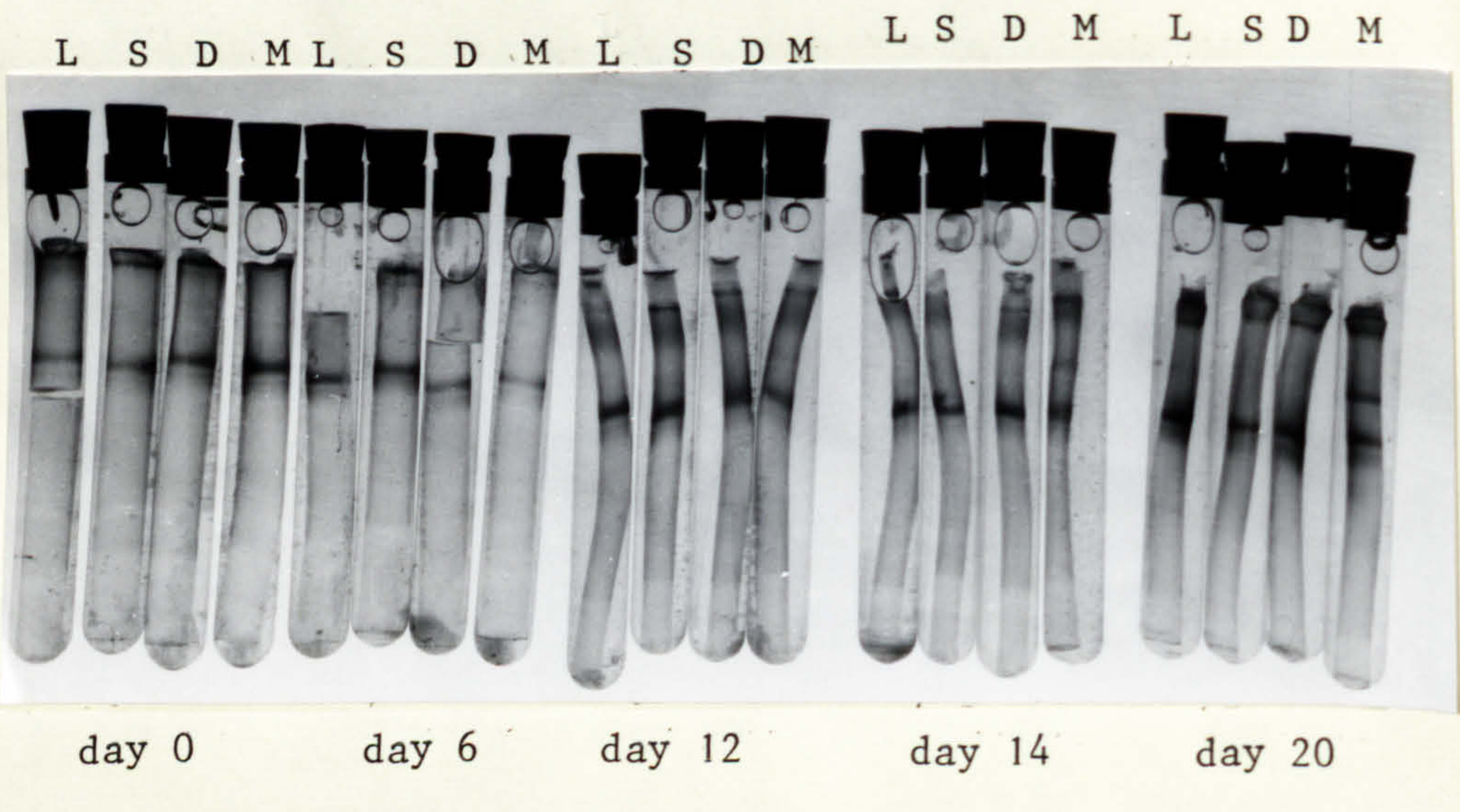


Plate 14 : Sets of disc gels prepared from snails (P.jenkinsi A) maintained on four different feeding regimes for 20 days.

L - Boiled, dried lettuce leaf

S - Starved

D - Detritus from the habitat

M - Ground, dried maple leaf

Box 4 snails in this box were starved. Starvation probably occurs in the wild during periods of inactivity caused, for example, by extremes of temperature.

In the first three treatments, sufficient material was added to form a layer approximately 2 mm thick on the bottom of the box.

Four individuals were removed from each box at the start of the experiment and subsequently at the periods shown on Plate 13. The snails were electrophoresed on disc gels which were stained for general esterase (section I - 5 - 1).

The banding patterns obtained are shown in Plates 13 and 14. All four individuals sampled from each treatment at each time gave the same banding patterns. At the start of the experiment snails from all boxes showed similar banding patterns with Est. 5a staining very faintly at R_f 0.24. Samples taken on day 14, however, showed that Est. 5a was relatively strongly staining in those snails kept on maple leaves. The band was faintly staining in snails taken from the other boxes. By day 20 Est. 5a was even more strongly staining in the snails kept on maple leaves but remained faint in the other treatments (see Plates 13 and 14). Electrophoresis on day 46 revealed continued high activity of Est. 5a in maple leaf-fed snails. The experiment was discontinued shortly afterwards due to heavy mortality of snails in this treatment.

Murray and Solomon[3] report that prey esterases can be detected in extracts of predatory mites and bugs for up to 31 hours after prey ingestion. As the maple leaf layer in the box would have had an associated 'flora' of bacteria and fungi, assimilation of esterases produced by these organisms might explain the high activity of Est. 5a in snails from this treatment. Electrophoresis of a sample taken from the detritus in the box on day 30 revealed no esterase activity. It is therefore unlikely that Est. 5a was being produced in this way.

Hebert[4] has reported the modification of Ldh molecules in the water-flea Daphnia magna by a period of starvation resulting in 'new' Ldh bands on gels. Oxford[5] has found a similar effect on some esterases of Cepaea nemoralis and C. hortensis fed on a diet of nettle,

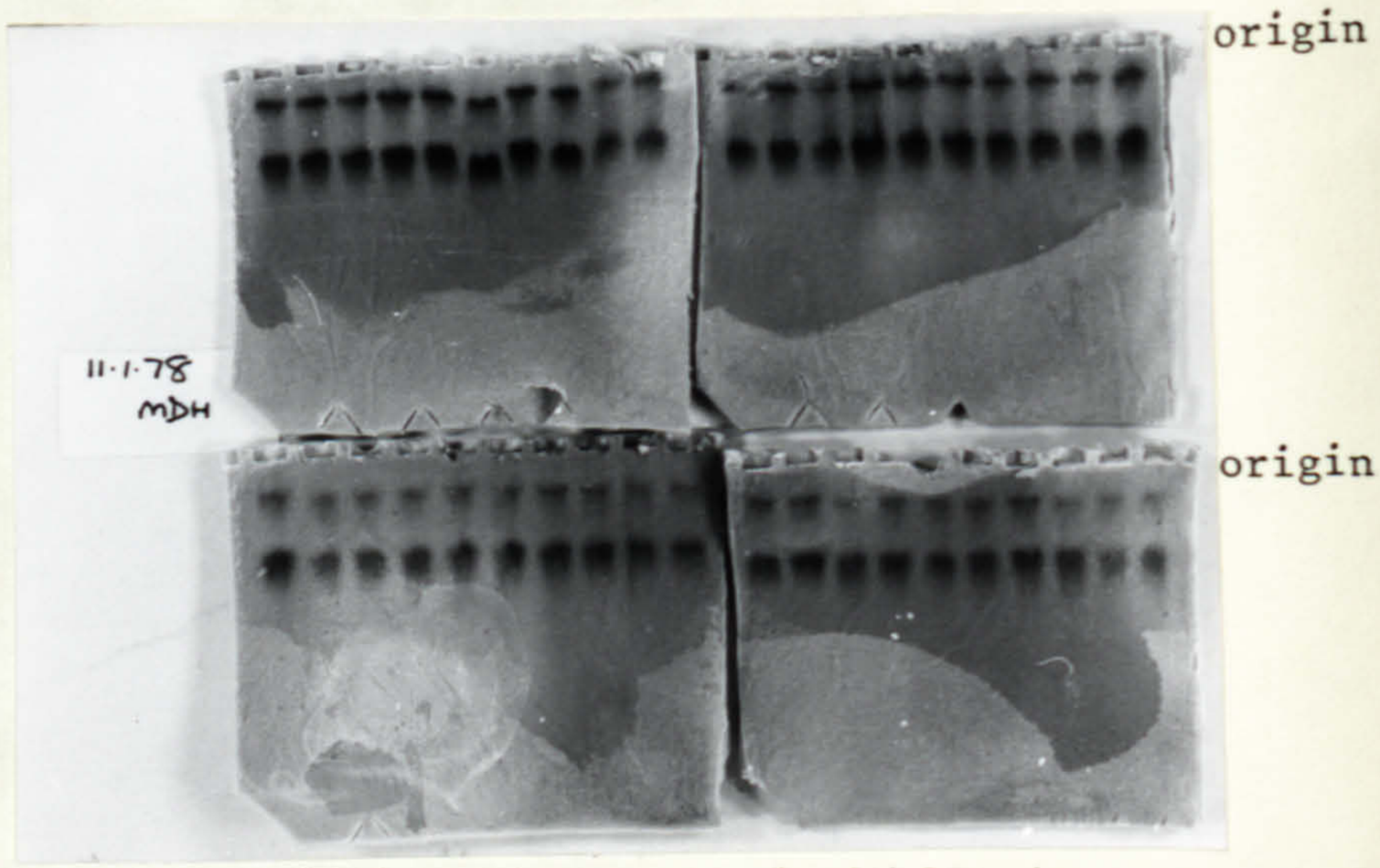


Plate 15 : Mdh phenotypes of ten individuals from each of four populations of P.jenkinsi A. The shift in mobility of the bands of individual 6 in popln. 21 is an artifact due to the presence of an air-bubble beneath the sample wick.

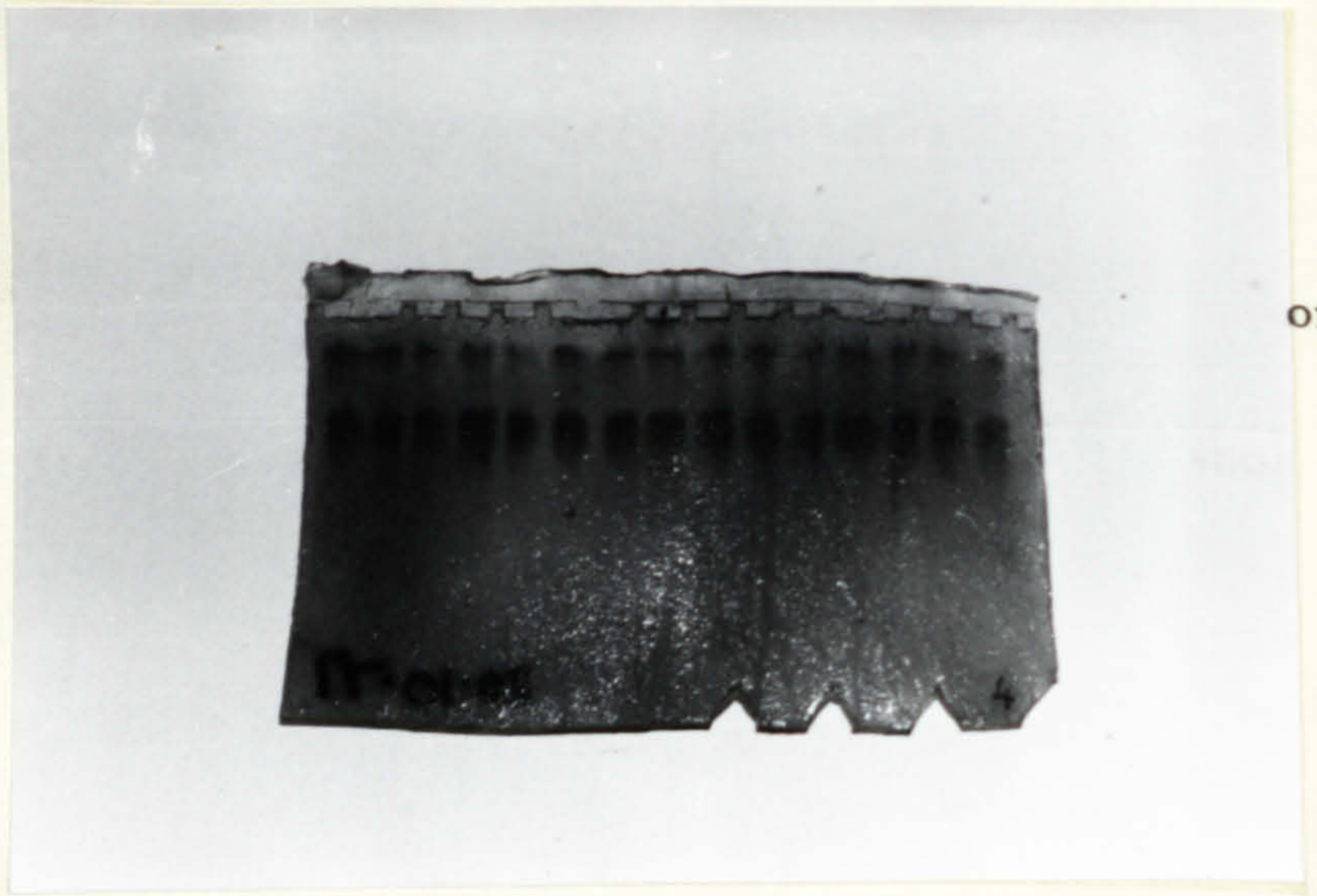


Plate 17 : Mdh phenotypes of fifteen individuals from the Waikato River population (45) of P.antipodarum.

Urtica dioica. Both these phenomena seem to result from post-translational modification of the enzyme (by proteolysis in Daphnia and, possibly, alteration of the gut flora in Cepaea) causing the "disappearance" of a pre-existing band simultaneous with the appearance of the modified isozyme at a different mobility. In the present study, the intensification of Est. 5a did not coincide with the disappearance of another band suggesting that Est. 5a is not a product of post-synthetic modification of another isozyme.

A more likely explanation is that Est. 5a represents the induction of an esterase required by the snail to deal with some aspect of a maple leaf diet. Similar mechanisms have been proposed for the appearance of temperature-induced isozymes in the sea-urchin Arbacia puntulata by Marcus[6], for a food-induced esterase zone in the helicid snail, Monacha cantiana by Oxford,[5] and for non-genetic variation of isozymes in Cepaea nemoralis by Gill.[7]

IV - 3 - 2 Malate dehydrogenase (Mdh)

No Mdh variants were found in any population. Gels of ten individuals from each of four populations are illustrated on Plate 15. The faster zone consists of two bands (Mdh 2 and 3) which are frequently difficult to distinguish due to their close proximity on 7.5% gel. Extracts run on 5% gel gave Mdh banding patterns in which Mdh 1 and Mdh 2 were relatively further apart than on 7.5% gel but Mdh 2 and Mdh 3 remained equidistant, indicating that Mdh 2 and 3 have the same molecular weight (see section III - 3 - 6). It is assumed that Mdh 2 and Mdh3 were at the Mdh-2 locus and that the single slow band, Mdh-1, represented the homozygous state of the Mdh-1 locus.

IV - 3 - 3 Acid phosphatase (Ac.Ph.)

All individuals showed the same phenotype viz. bands Ac.Ph.1 and the faster zone Ac.Ph.2. The latter zone was found to consist of two close bands Ac.Ph.2a and Ac.Ph.2b forming a diffuse zone on all but the most lightly stained gels. The allelic nature of Ac.Ph.2a and 2b was suggested by running extracts on 5% gel in the same way as in section IV - 3 - 2. In the same way Ac.Ph.1 and Ac.Ph.2 were shown to have different molecular weights and are probably non-allelic.

The banding pattern was assumed to be produced by two

loci Ac.Ph.-1 (genotype $1^1/1^1$) and Ac.Ph.-2 (genotype $2^1/2^2$).

IV - 4 Variation in P. jenkinsi B

IV - 4 - 1 Esterases

Individuals within each population of P. jenkinsi B had the same esterase phenotypes although there were clear differences between the two populations (popln. 1 and popln. 28). Individuals from Snettisham (popln. 28) had an extra three bands (11, 12 and 13) which were absent from the Stone Creek (Hull) population. In the course of a cloning experiment described below, a 'mutant' which also possessed Est. 11, 12 and 13 appeared in laboratory stocks of P. jenkinsi B from Stone Creek.

The cloning experiment was set up by rearing ten 2-week old (3-whorled) individuals from Stone Creek in isolation. After eight weeks, each isolated snail produced a number of progeny (labelled F_1 for convenience) parthenogenetically. Each parent and eight of her progeny were electrophoresed (slab method) and five of the remaining offspring were reared in isolation. After a further eight weeks 24 of these F_1 individuals produced progeny ('F_2'). When these progeny had grown to the 4-whorled stage, both F_1 and F_2 individuals were electrophoresed and stained for general esterase. Gels from 23 of these families showed that most F_1 parents and progeny had identical phenotypes to the original parents (i.e. Est. 11, 12 and 13 absent). In one case, however, the F_1 and its F_2 progeny clearly possessed Est. 11, 12 and 13; bands previously only known from the Snettisham population. The original parent of this line displayed the usual Hull phenotype (Est. 11, 12 and 13 absent: see Plate 16).

Est. 11 and 12 are probably allelic (on the grounds that they have the same biochemical properties - see Table 22) and Est. 13 (a faint band) may be a 'shadow' band associated with these alleles. The simplest explanation for their sudden appearance in laboratory stocks is that a mutation or chromosomal re-arrangement had occurred affecting the expression of the locus (Es-11) coding for these enzymes. This implies that the Es-11 locus in the Hull population is normally 'silent'. The two populations of P. jenkinsi B may, therefore, possess the same structural esterase loci but normally differ in their regulatory loci. Alternatively, it is possible that

the Es-11 locus in the Hull population is normally fixed for the null allele and the 'mutant' produced an active esterase. This hypothesis requires that Est. 11, 12 and 13 are, in fact, produced by one allele (? Est. 11) and the three bands result from the addition of one and two charged molecules to Est. 11 giving Est. 12 and Est.13 respectively. The addition of carbohydrate molecules such as sialic acid is known to give rise to multiple amylase bands in man.[8] Incubation of the extracts with neuraminidase (which removes sialic acid) did not alter the electrophoretic patterns but, although sialic acid addition is not involved, other charged units (e.g. naturally occurring esters) may be responsible for the production of Est. 11, 12 and 13. In preparing estimates of heterozygosity and genetic distance it was assumed that both populations were identical at the structural Es-11 locus.

IV - 4 - 2 Malate dehydrogenase and acid phosphatase

Banding patterns at these loci were the same as those for P. jenkinsi A. No variants were found in either the Snettisham or the Hull populations. The genetics of the bands was assumed to be the same as that of P. jenkinsi A (sections IV - 3 - 2 and IV - 3 - 3).

IV - 5 Variation in P. jenkinsi C

IV - 5 - 1 Esterases

All 30 individuals from the Burgh Castle population and three from the Brittany population were found to possess phenotype H. (Fig. 18).

IV - 5 - 2 Malate dehydrogenase (Mdh) and acid phosphatase (Ac.Ph.)

All P. jenkinsi C individuals had the same Mdh and Ac.Ph. phenotypes as those of P. jenkinsi A and B.

IV - 6 Variation in P. antipodarum

IV - 6 - 1 Esterases

Nine electrophoretic phenotypes were identified in 43 individuals from the Waikato River population (popln. 45) and three

Fig.19 : Esterase phenotypes of two populations of *Potamopyrgus antipodarum*

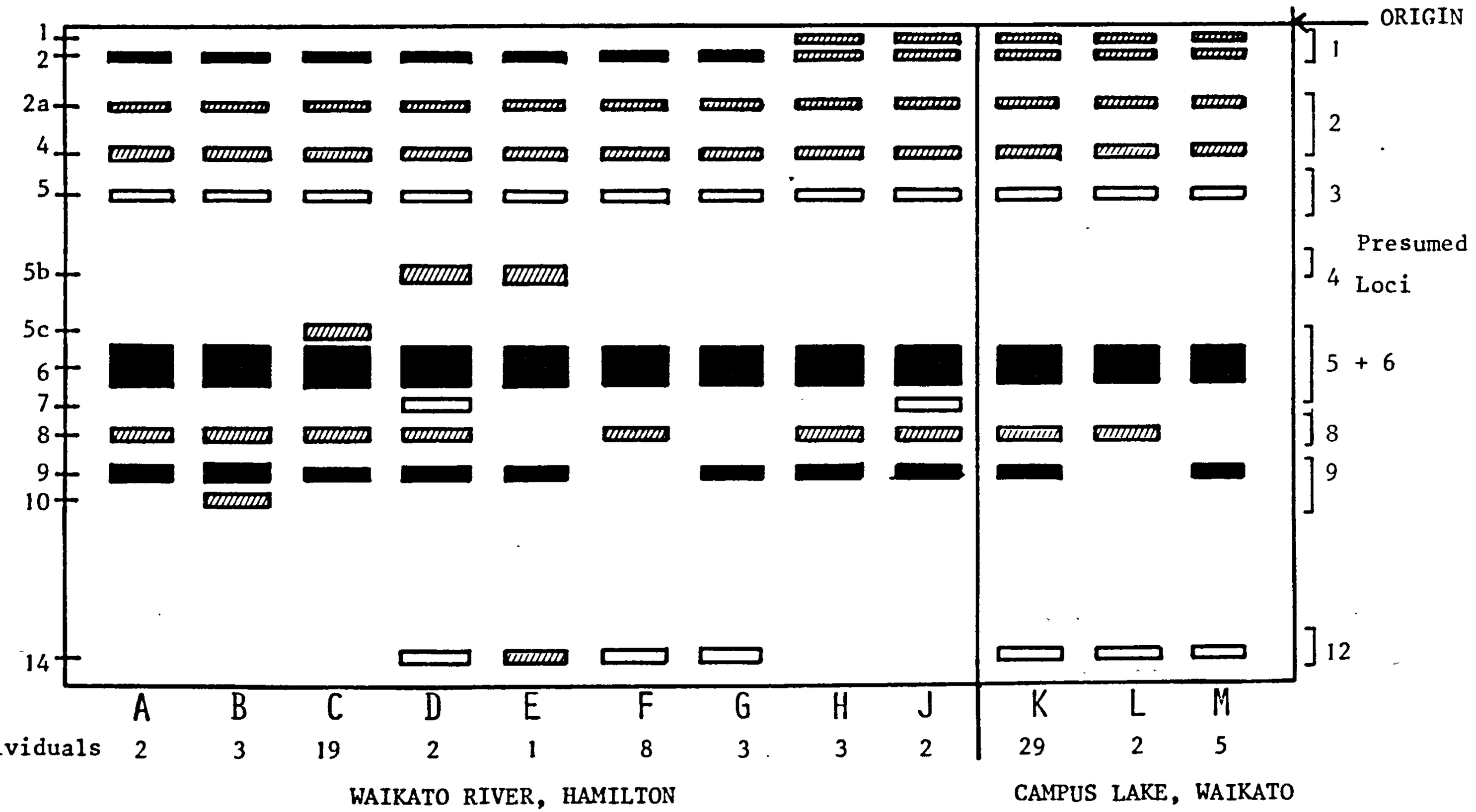
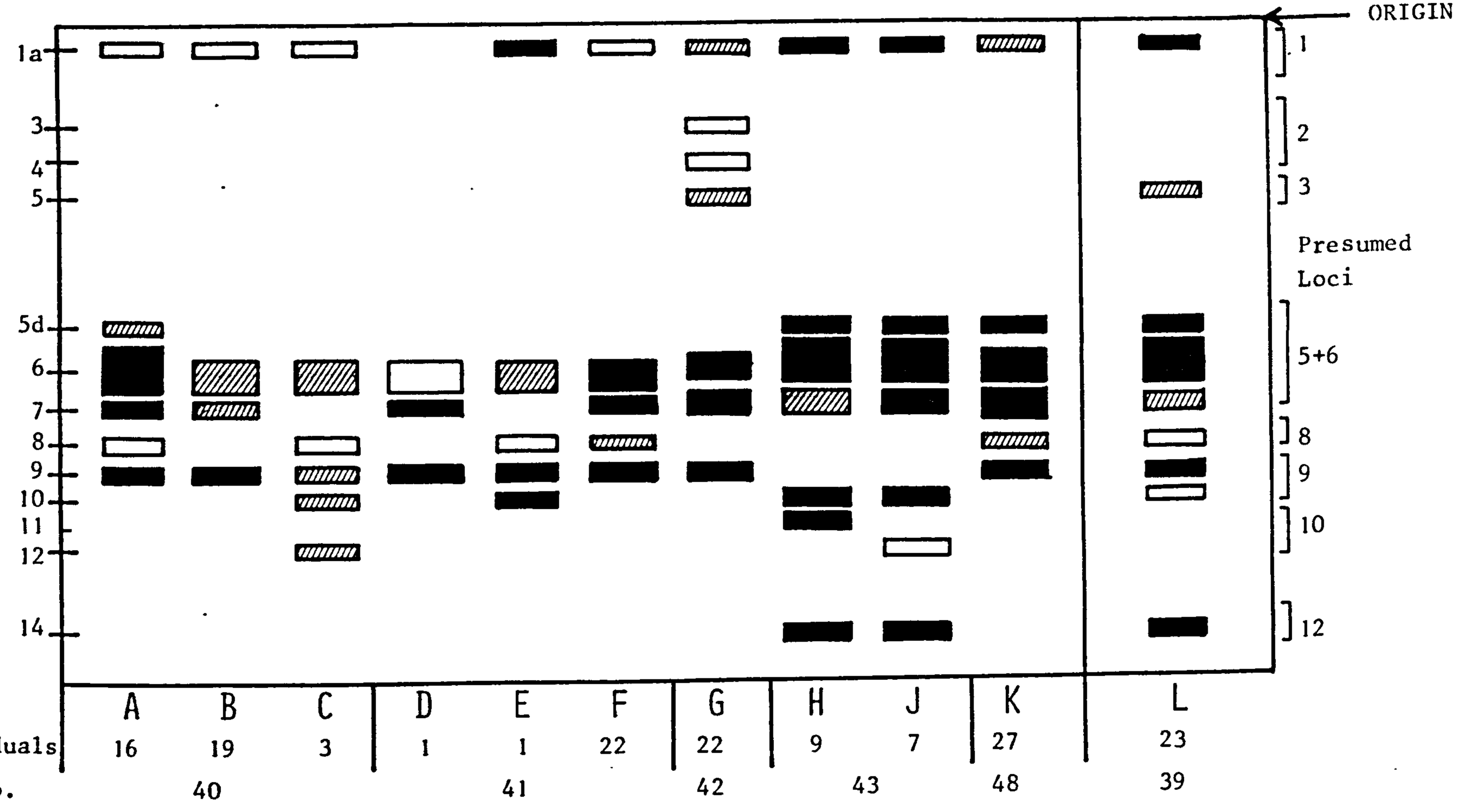


Fig.20 : Esterase phenotypes of six *Potamopyrgus nigra* populations



phenotypes in 36 individuals from the Campus Lake population, (popln. 46). The number of snails showing each phenotype and diagrammatic representations of each are given in Fig. 19. The most common phenotype differed in each population (C and K in Fig. 19) and several bands (Est. 5b, 5c, 7 and 10) found in the Waikato River population were unrepresented in the Campus Lake population. The overall staining intensity was similar in both populations so that "between gel effects" cannot account for these differences. The significance of this variation is discussed in relation to the breeding systems in these populations in section IV - 9. Bands were assigned to loci according to the homologies between them described in section III - 3 - 7.

IV - 6 - 2 Malate dehydrogenase (Mdh)

All individuals from both populations had the phenotype illustrated in Plate 17. The faster zone consisted of two bands (Mdh 2 and 3). The phenotype was identical to that found in all P. jenkinsi individuals. Mixtures of P. jenkinsi A and P. antipodarum extracts gave the same banding pattern. It was therefore assumed that the loci Mdh-1 and Mdh-2 were represented by genotypes Mdh $1^1/1^1$ and Mdh $2^1/2^2$ in all individuals.

IV - 6 - 3 Acid phosphatase (Ac.Ph.)

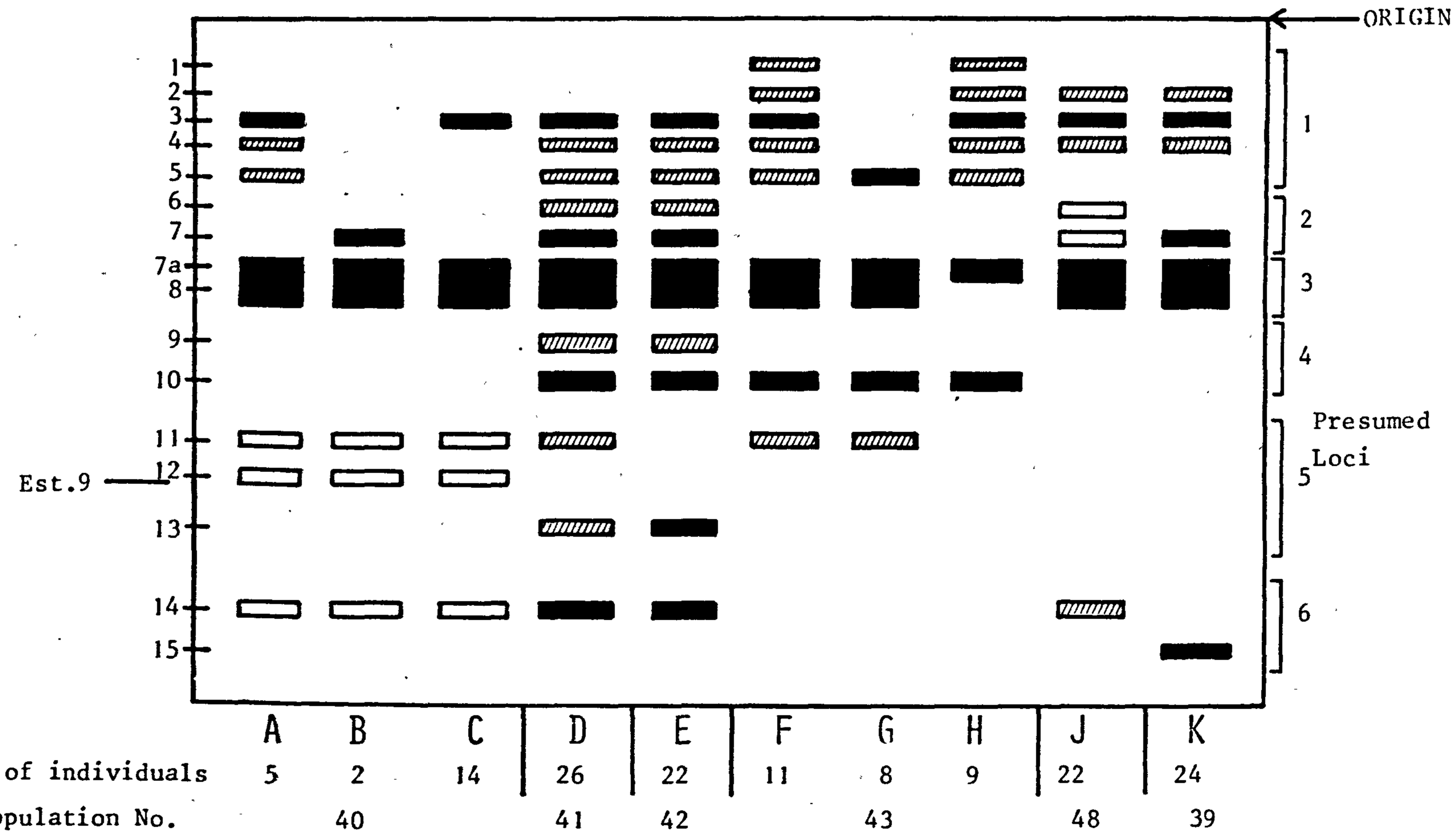
All individuals of P. antipodarum had the same phenotype as P. jenkinsi individuals. Bands were assumed to be homologous to those of P. jenkinsi and to represent the Ac.Ph $1^1/1^1$ homozygote at the Ac.Ph.-1 locus and Ac.Ph. $2^1/2^2$ heterozygote at the Ac.Ph.-2 locus.

IV - 7 Variation in P. nigra

IV - 7 - 1 Esterases

Eleven electrophoretic phenotypes were recognised in 150 individuals from six populations of P. nigra (Fig. 20). Three populations (39, 42 and 48) were each monomorphic for a different phenotype. Populations 40, 41 and 43 were polymorphic although few phenotypes were included in each. Similar instances of a restricted distribution of phenotypes have been reported for parthenogenetic populations of weevils and moths by Suomalainen and Saura[9], Lokki et. al.[10] and Saura et. al.[11]

Fig. 21 : Mdh phenotypes of six *Potamopyrgus nigra* populations



Wallace[12] has recently reported a very low (less than 5%) incidence of males in many populations of P. nigra from New South Wales and Victoria and this was the case in the populations used for the present study (poplns. 39, 41 and 42 were all-female and less than 5% males occurred in populations 40, 43 and 48 - see Appendix I). Hardy-Weinberg assumptions of random mating clearly do not apply to populations of P. nigra which probably consist of a number of different clones. Gene flow between these clones may occur due to occasional sexual matings but appears to be insufficient to generate a large number of clones per population. The distribution of pheno-types in populations of P. nigra (and P. antipodarum) is more fully discussed in section IV - 9.

IV - 7 - 2 Malate dehydrogenase (Mdh)

In contrast to the other Potamopyrgus species, P. nigra populations contained individuals having different Mdh phenotypes (Fig. 21); up to 11 bands were present in an individual snail. Four populations (39, 41, 42 and 48) were each monomorphic for a different phenotype. Significantly, these were the same populations which were monomorphic for esterase phenotypes (except popln. 41 - see IV - 7 - 1).

Six loci were thought to be responsible for the banding patterns and these are shown on Fig. 21. It was not possible to confirm that the assignment of bands to these loci was correct as no crosses could be set up (the snails were received in poor condition - see I - 1 - 2). However, by running the extracts on 5% gels (see III - 3 - 2) bands 7a and 8 and 11 and 13 were shown to have the same molecular weight suggesting that they are alleles at the Mdh-3 and Mdh-5 loci respectively.

Bands at the presumed Mdh-1 locus were rather confusing and seemed to represent a multimeric system. For heterozygosity estimates it was assumed that phenotypes C and G were homozygous for Bands 3 and 5 whilst the other phenotypes represented heterozygotes. Phenotype B was assumed homozygous for a null allele at Mdh-1 i.e. band 7 was the Mdh $2^2/2^2$ homozygote.

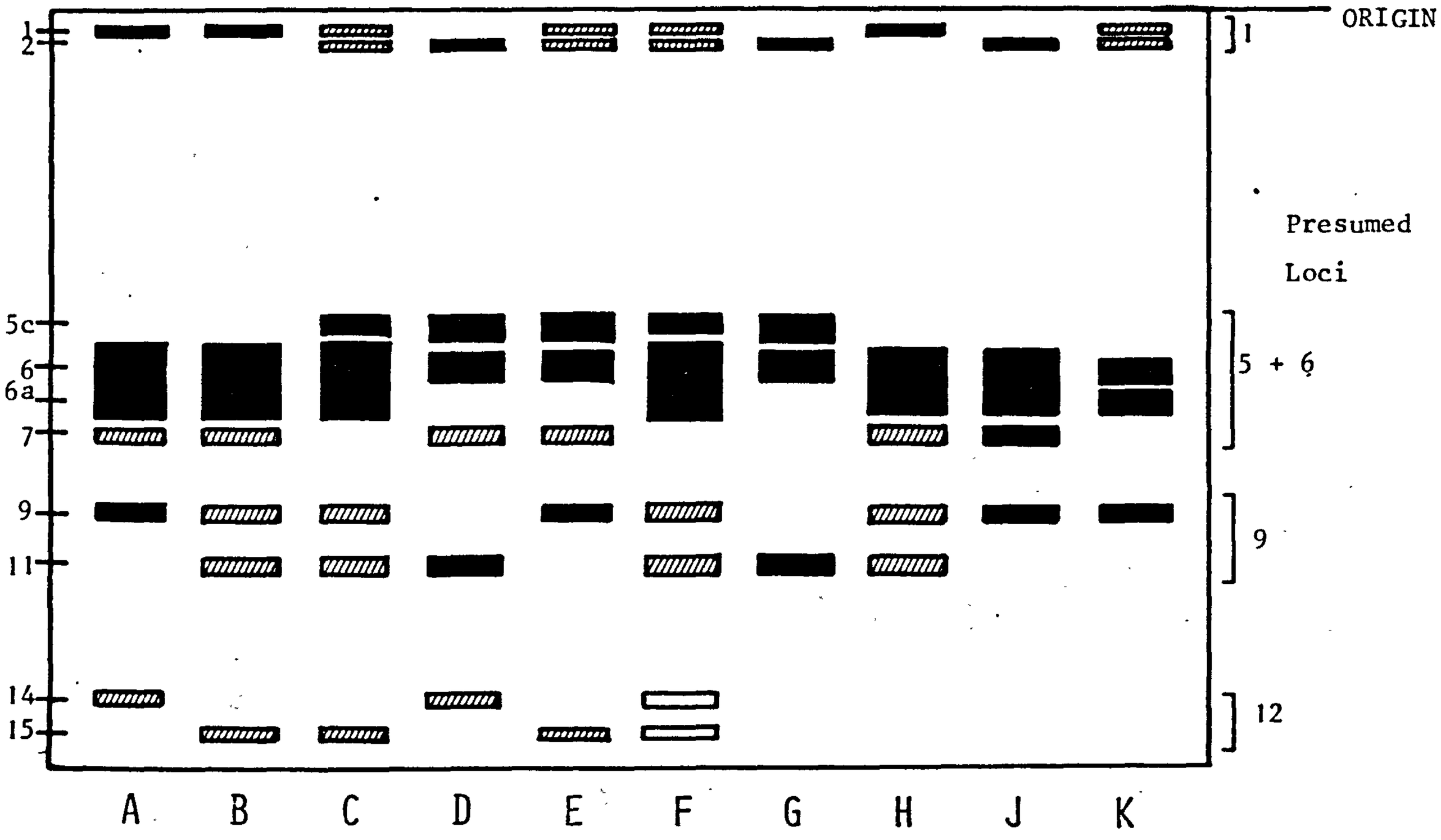
IV - 7 - 3 Acid phosphatase (Ad.Ph.)

Three regions of acid phosphatase activity were detected

TABLE 25: Variation at five esterase loci of Potamopyrgus estuarinus

LOCUS	N		GENOTYPE DISTRIBUTION				ALLELE FREQUENCIES			χ^2 (df)	p	he
ES - 1	14	O	1/1	1/2	2/2		1	2		0.29 (1)	0.60 ns	0.5
			4	6	4		0.5	0.5				
		E	3.5	7	3.5							
ES - 5	14	O	5c/5c	5c/6	6/6		5c	6		1.65 (1)	0.20 ns	0.337
			0	6	8		0.214	0.786				
		E	0.64	4.72	8.64							
ES - 6	14	O	6a/6a	6a/7	7/7		6a	7		0.22 (1)	0.70 ns	0.49
			5	6	3		0.57	0.43				
		E	4.57	6.86	2.57							
ES - 9	14	O	9/9	9/11	11/11		9	11		0.44 (1)	0.50 ns	0.436
			7	5	2		0.679	0.321				
		E	6.45	6.10	1.45							
ES - 12	14	O	14/14	14/15	15/15	null	14	15	null	8.46 (2)	** 0.01	
			3	1	4	6	0.250	0.321	0.429			
		E	0.88	2.247	1.446	9.427						
							$\bar{H}_o$ = 0.4107				$\bar{H}_e$ = 0.44	

Fig. 22 : . Esterase phenotypes of *Potamopyrgus estuarinus*



in P. nigra extracts but the zones were too diffuse to interpret with certainty. The faster zone (Fig. 8) migrated to approximately the same position as the Ac.Ph.-2 locus in P. jenkinsi but no clear bands were visible in this region. Several individuals in populations 40 and 41 appeared to lack the slow zone but the genetic interpretation of this was not clear. As a result of this uncertainty, data for acid phosphatase loci of P. nigra were not included in estimates of heterozygosity or genetic distance.

IV - 8 Variation in the Esterases of P. estuarinus

The esterase phenotypes of fourteen individuals are shown diagrammatically in Fig. 22. The snails were all the same size resulting in approximately the same protein concentration in individual extracts. Ten distinct phenotypes were recorded and simple variation involving two alleles was assumed for the Es-1 and Es-9 loci. The relative mobility of the Est. 5c band was significantly different to Est. 5c in P. jenkinsi when mixed extracts of each were run on the same gel.

A genetic interpretation of the region from Est. 5c to Est. 7 was attempted by assuming that Est. 5c and Est. 6 were alleles at a different locus (Es-5) to Est. 6a and Est. 7 (at the Es-6 locus). At least one null allele was present at the Es-12 locus (coding for Est. 14 and 15). Neither of these alleles at this locus was found in any other Potamopyrgus population.

As P. estuarinus is an obligate sexually-reproducing species genotype frequencies were compared to those expected from Hardy-Weinberg assumptions, (Table 25). Significant deviation occurred only at the Es-12 locus probably as a result of the presence of an unknown number of null alleles. An estimate of heterozygosity (H_e) was made for the four loci which could be scored with some certainty (see also section IV - 9 and Table 26).

IV - 9 Polymorphism in Potamopyrgus populations

Estimates of the proportion of heterozygous loci per individual are usually made by computing the mean expected heterozygosity (H_e) from estimates of genotype frequencies given by the Hardy-Weinberg expansion.

TABLE 26: Electrophoretic polymorphism in Potamopyrgus species: Summary of Data

SPECIES	N (N')	LP	P	LOCI (D)	TOTAL LOCI	No. Heterozygotes	$\bar{H}$	Ho/He	Prop of Males
P.jenkinsi A (all poplns)	890 (34)	0.002	0.93	7120 Est (8) 1780 Mdh (2) 1780 AcPh(2)	10680	OBS 7983 EXP 3164	0.747 0.296	2.52	0
P.jenkinsi B (popns 1 & 28)	82 (2)	0.045	1.00†	574 Est (7) 164 Mdh (2) 164 AcPh(2)	902	OBS 492 EXP 246	0.545 0.273	2.00	0
P.jenkinsi C (popns 35 & 30)	33 (2)	0.00	1.00	198 Est (6) 66 Mdh (2) 66 AcPh(2)	330	OBS 132 EXP 66	0.400 0.200	2.00	0
P.antipodarum (both popns)	79 (2)	0.31	0.37	790 Est(10) 158 Mdh (2) 158 AcPh(2)	1106	OBS 353 EXP 215	0.319 0.194	1.64	0.16
Popln. 47 P.antipodarum (Waikato)	43 (1)	0.54	0.44	430 Est(10) 86 Mdh (2) 86 AcPh(2)	602	OBS 173 EXP 125	0.287 0.208	1.38	0.30
Popln. 46 P.antipodarum (Campus Lake)	36 (1)	0.08	0.81	360 Est(10) 72 Mdh (2) 72 AcPh(2)	504	OBS 180 EXP 90	0.357 0.179	1.99	0
P. nigra (all poplns.)	150 (6)	0.16	0.18	1350 Est (9) 858 Mdh (6)	2208	OBS 740 EXP 418	0.335 0.189	1.77	0.03
P. nigra (poplns. 39)	24 (1)	0.00	1.00	207 Est (9) 144 Mdh (6)	351	OBS 94 EXP 47	0.268 0.134	2.00	0
P. nigra (popln. 40)	28 (1)	0.40	0.50	342 Est (9) 126 Mdh (6)	468	OBS 179 EXP 110	0.382 0.235	1.62	0.01
P. nigra (popln. 41)	26 (1)	0.27	0.92	216 Est (9) 156 Mdh (6)	372	OBS 150 EXP 81	0.403 0.218	1.85	0.05
P. nigra (popln. 42)	22 (1)	0.00	1.00	198 Est (9) 132 Mdh (6)	330	OBS 110 EXP 55	0.333 0.167	2.00	0
P. nigra (popln. 43)	28 (1)	0.27	0.56	144 Est (9) 168 Mdh (6)	312	OBS 87 EXP 64	0.279 0.205	1.36	0.10
P. nigra (Popln. 48)	27 (1)	0.00	1.00	243 Est (9) 132 Mdh (6)	375	OBS 120 EXP 60	0.320 0.160	2.00	0.03
P.estuarinus (popln. 49)	14 (1)	1.00	0.14	56 Est (4)	56	OBS 23 EXP 25	0.41 0.44	0.932	0.47

N Maximum number of individuals (not all gels were scored for all loci)
N' Number of populations
LP Proportion of loci polymorphic per population.
P Proportion of individuals having the most common esterase phenotype
D Number of different loci
$\bar{H}$ Mean proportion of loci heterozygons per individual
† See section IV – 4 – 1.

$$\bar{H}_e = \frac{\text{Estimated number of heterozygous loci}}{\text{Total number of loci in the sample}}$$

For sexually-reproducing small populations, $\bar{H}_e$ has been shown by Nei and Roychoudhury[13] to have inherently better statistical properties than the mean observed heterozygosity ($\bar{H}_o$). In populations where there is a significant degree of apomixis, however, Hardy-Weinberg assumptions are clearly inapplicable and it is inappropriate to use $\bar{H}_e$. A better estimate of the heterozygosity in these populations is simply H_o; large differences between $\bar{H}_e$ and $\bar{H}_o$ have been used by Wright,[14] Suomalainen[15] and Selander[16], to demonstrate the genetic consequences of different breeding systems. As $\bar{H}_e$ equals $\bar{H}_o$ only if Hardy-Weinberg assumptions of random mating and selective equilibrium apply, differences between these estimates may also result from selection.

Table 26 presents $\bar{H}_e$ and $\bar{H}_o$ estimates for Potamopyrgus populations together with estimates of the proportion of polymorphic loci per population. The proportion of individuals having the most common electrophoretic phenotype (P) is included as this parameter gives an indication of the distribution of variation within populations.

Estimates of heterozygosity are subject to several sources of potential error:-

i) The sample of snails may not be representative of the total population. Clearly, the larger the sample the more representative it should be.

ii) The loci assayed may not be representative of all loci in the genome. Electrophoresis only reveals soluble proteins and only separates those which are charged. The isozymes used in this study are a sub-set of this sub-set and it is known that, e.g. esterases in Drosophila are significantly more variable than those metabolising glucose (Mdh, Idh, etc.).[17] Powell[18] has shown that 'regulatory' enzymes are less variable than non-regulatory and variable-substrate enzymes (categories put forward by Johnson[19]) and Koehn[20] has postulated that large enzyme molecules may be less variable than small enzymes. The data presented in this thesis were not formally analysed in order to investigate the above theories as it was felt that the number of

loci examined was too small to allow statistical comparison.

iii) Alleles may have been assigned to the wrong loci; a particular problem with rare bands which were not included in the study of biochemical and physical properties (chapter III).

iv) Null alleles may not have been detected (see section III - 3 - 7).

Comparisons between species were considered justified on the grounds that similar loci were assayed in each and most alleles were assigned to loci (at least in Potamopyrgus) on the basis of their biochemical and/or physical properties.

Variation in P. antipodarum and P. nigra is similar to that found in populations of apomictically reproducing weevils by Suomalainen[9], Saura[11] and Lokki,[10] colonising apomictic cockroaches by Parker et. al.[21] the aphid Myzus persicae by Baker,[22] Daphnia magna by Hebert[23] and Aphis fabae by Baranek and Berry.[24] Variation in these largely apomictic species is distributed between populations such that individual populations are either monoclonal or weakly polyclonal (i.e. few phenotypes per population). P. nigra populations were monoclonal in three cases and weakly polyclonal in populations 40, 41 and 43. Similarly, the Campus Lake population (popln. 46) of P. antipodarum was weakly polyclonal with over 80% of individuals having the same phenotype. In contrast, the Waikato River population was moderately polymorphic with nine esterase phenotypes represented in the population sample, although one clone made up 44% of the population.

Parthenogenesis is widespread in populations of P. antipodarum and P. nigra.[25,26] The proportion of males in each population was determined by sexing approximately 100 randomly chosen adults (see Appendix I). The majority (over 95%) of snails in P. antipodarum and P. nigra populations were female; exceptions were the Waikato River population of P. antipodarum (29% males) and population 43 of P. nigra (10% males). The proportion of males in the small sample of P. estuarinus was 0.47.

These last three populations had a lower proportion of excess heterozygotes ($\overline{H}_o/\overline{H}_e$ in Table 26) and were generally more variable than populations with a higher proportion of parthenogenetic

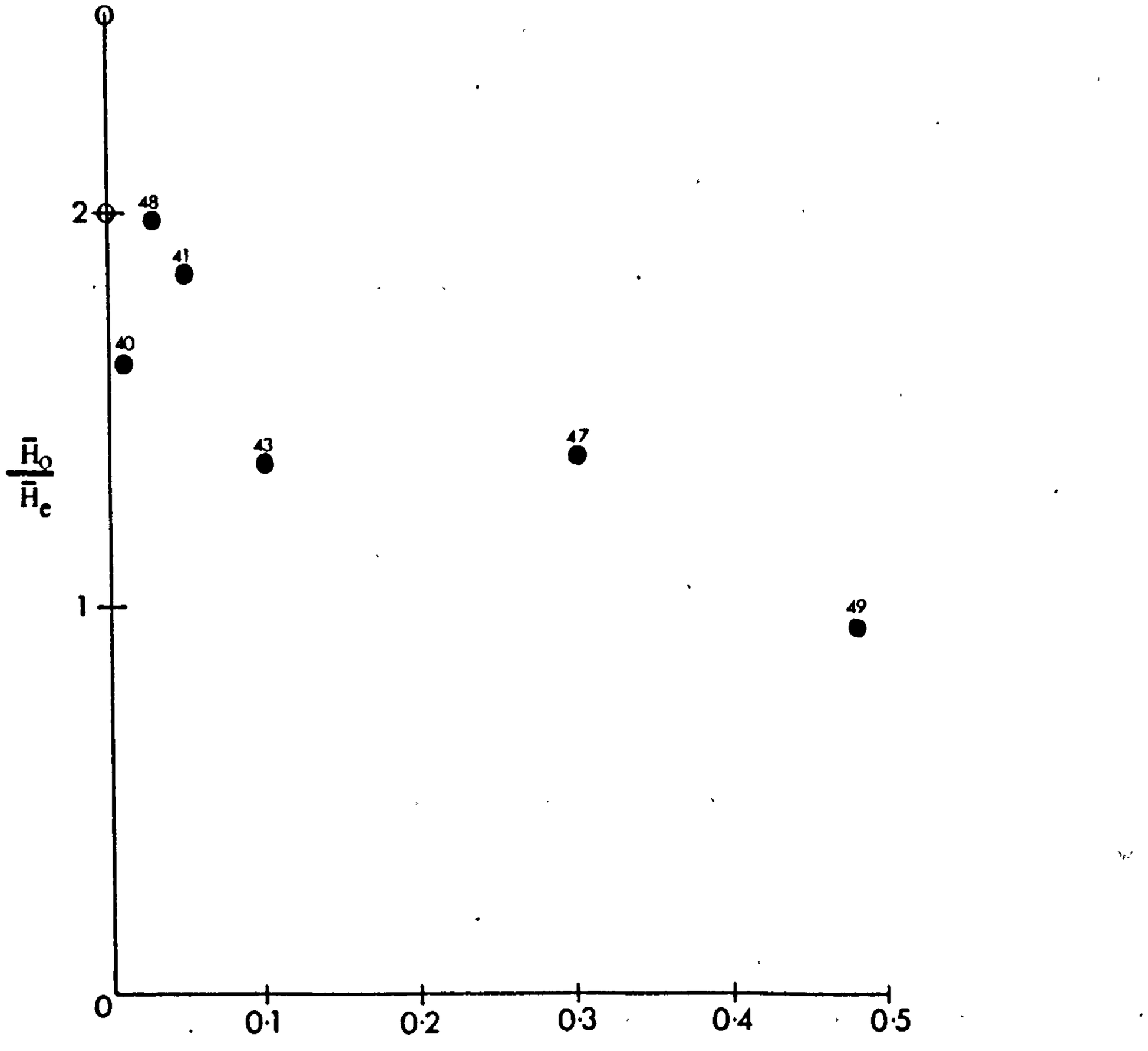


Fig 23: The excess of heterozygotes in Potamopyrgus species plotted against the proportion of males in these populations.
(regression b = -2.24, a = 2.00, $t_{(10)}$ = 4.64, p = 0.001, r = -0.83)

● Australasian species

O Potamopyrgus jenkinsi

females. Fig. 23 shows the excess of heterozygotes plotted against the proportion of males in each Potamopyrgus population. Although the relationship may not be fully linear, the regression ($y = 2.24x + 2$) is highly significant ($r = 0.83$, $p = 0.001$).

An excess of heterozygotes might be expected in parthenogenetic populations for two reasons:

i) Parthenogenetic clones may originate from hybridisation between sexual individuals (often from different species) which are fixed for different alleles at many loci.

Parker and Selander[27] consider that the high heterozygosity found in parthenogenetic lizards may be due to their hybrid origin. For example, mean heterozygosity ($\bar{H}_e$) in diploid hybrids of Cnemidophorus tigris and C. septemvitlatus was 0.56 and in triploids from C. tessalatus and C. sexlineatus crosses was 0.714.

Similarly, parthenogenetic fish in the genus Poeciliopsis are frequently of hybrid origin[28] and have high (up to 0.43) heterozygosity. Suomalainen[15] has suggested that the high heterozygosity found in some apomictic weevils may be partly due to their hybrid origin (but see below).

It is possible that parthenogenetic populations of Potamopyrgus result from hybridisation between individuals from different populations giving some highly heterozygous progeny. If these progeny exhibit heterosis and are facultative parthenogens then they may become locally dominant over the sexual individuals.

ii) Theoretically, apomixis allows the accumulation of mutations and chromosomal structural re-arrangements; the absence of segregation and recombination means that recessives are not exposed to selection (except in the rare event of a double mutation). Polyploidy (P. jenkinsi may be tetraploid - see section VI - 3) allows an even greater accumulation of mutations.

Suomalainen[29] suggests that mutations may accumulate to the point where homologous chromosomes are so dissimilar that they can no longer be regarded as diploid in the genetic sense. At this point, barriers to the expression of accumulated recessives may be removed and this should lead to clonal diversity.

However, electrophoretic clonal diversity should be detectable in the early stages of this process as dominance is not usually a feature of electrophoretic alleles. Thus an accumulation of mutations in apomictic organisms should result in an increase in the heterozygosity of electrophoretic loci and an increase in the clonal diversity of a population.

One or both of the above processes may be operating to produce the high levels of heterozygosity shown in Fig. 23. Accumulation of heterozygosity in P. jenkinsi A has clearly not occurred to any great extent since its (probable) introduction into Britain as populations are virtually genetically uniform. It is likely, therefore, that P. jenkinsi A (and possible B and C) represent highly heterozygous propagules from an ancestral population (probably P. antipodarum - see chapter VI).

Although the highest levels of excess heterozygosity occur in populations where males are absent (or rare), there is a substantial excess in populations where males are present together with parthenogenetic females. In P. estuarinus, where asexual females are absent, the level of heterozygosity is approximately in accordance with Hardy-Weinberg expectations. ($\bar{H}_o/\bar{H}_e$ = 0.93).

The presence of males in populations of P. nigra and P. antipodarum is not a transient phenomenom; Wallace has recently reported that sex ratios in many populations were constant over five years. This suggests that some sexual mating takes place within these populations, and that the proportion of sexual mating is, perhaps, constant from year to year. Small proportions of males occur in largely parthenogenetic populations of other molluscs, notably species in the genera Melanoides[39] and Campeloma.[31]

P. jenkinsi, P. antipodarum and P. nigra females are probably facultative parthenogens since the reproductive system in all three species has an apparently functional bursa copulatrix and receptaculum seminis. All three species also possess a sperm groove leading to these organs. Winterbourn[32] argues that the capacity to store sperm has been lost in parthenogenetic females of P. antipodarum although there is no structural difference between these and females from sexual populations of this species.

It is perhaps more likely that Potamopyrgus females are capable of mating but 'choose' whether to produce sexual or asexual progeny in response to some environmental stimulus such as 'harshness' (e.g. low food supply and/or adverse physical conditions). If the local environment is temporally and spatially stable and benign toa female, then it may be an advantage (to the genome) to produce parthenogenetic progeny on the assumption that environmental conditions are going to remain stable for at least one more generation.

Conversely, if the environment is 'harsh' it may be advantageous to mate in the hope that at least some of the genetically dissimilar progeny will survive. If the habitat occupied by a population is sufficiently heterogeneous to always include some proportion that is 'harsh' (e.g. in this context, seasonal drying out of river and lake margins), snails in this part will breed sexually. The proportion of males in a population may, therefore, be a reflection of the proportion of 'harshness' in the habitat.

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CHAPTER V : ELECTROPHORETIC VARIATION IN BRITISH POPULATIONS OF HYDROBIA

In contrast to the largely asexual Potamopyrgus species described in chapter IV, the three British species in the genus Hydrobia (H. ventrosa, H. ulvae and H. neglecta) are all obligate sexual outcrossers.

As such, they provide a convenient opportunity to compare the level and distribution of genetic variation between species having different breeding strategies. In the absence of sufficient numbers of sexually reproducing Potamopyrgus species, we can at least compare species within the family Hydrobiidae by electrophoretically surveying similar loci in both genera.

Also, although genetic distances between species (and more closely related taxa) are well documented (for a review see Nei[1]), few electrophoretic studies have investigated the phylogenetic relationships between genera within a family. For this reason, and in view of the possibility that H. ventrosa is closely related to P. jenkinsi (see Introduction p13), an electrophoretic survey of populations of Hydrobia species is needed for the phylogenetic analysis described in chapter VI.

Besides being considered taxonomically and reproductively distinct from P. jenkinsi A, the Hydrobia species have a different geographic distribution (in Britain) from that of P. jenkinsi A. All three Hydrobia species are restricted to coastal (brackish or marine) habitats and are never found in fresh-water.[2] In view of the current interest in the relationship between levels of genetic variation and ecological strategy, comparative studies of genetic variation in closely related species confined to different habitats are of some interest.

In this chapter, an attempt is made to survey loci coding for classes of isozyme that are similar to those used in the electrophoretic surveys of Potamopyrgus populations described in chapter IV.

V - 1 Materials and Methods

V - 1 - 1 Electrophoresis

Extracts of individual snails sampled from various populations of H. ventrosa, H. ulvae and H. neglecta were electrophoresed on 7.5% slab gels stained for general esterase (section I - 5 - 1), acid phosphatase (section I - 5 - 2) and Mdh (section I - 5 - 3).

V - 1 - 2 Breeding experiments

In chapters III and IV it has been shown that electrophoretic banding patterns are frequently complex and can be misinterpreted due to the possible presence of over-lapping bands, null alleles, incorrect assignment of bands to loci and epigenetic effects. As the British species of Hydrobia are sexually reproducing, crosses were set up in order to investigate the formal genetics of bands.

One of the disadvantages of using electrophoretic genetic markers in small invertebrates is that the animals must be killed (or at least severely injured) in order to score the markers. Crosses must, therefore, be set up between parents of unknown electrophoretic phenotype and markers scored after the production of progeny. Where the required marker is rare, either large numbers of crosses must be set up (in the hope that some crosses containing the marker are fortuitously included) or crosses may be set up using progeny from parents known to carry the rare marker. Where significant mortality occurs during the maintenance of laboratory stocks (e.g. in some cultures of H. ulvae and H. neglecta) it is perhaps best to adopt the former method as only one generation has to be reared.

Immature (3-whorled) individuals of H. ventrosa, H. ulvae and H. neglecta were sexed (the penis was just visible) and pairs set up in water of the appropriate salinity. Several 3-whorled females of each species were isolated and as these did not produce young after maturation, it was assumed that 3-whorled individuals were virgin (and that these species were obligate outcrossers). Pairs of snails were maintained on the standard diet (section I - 1) in 10 cm x 5 cm x 3 cm plastic boxes fitted with lids.

TABLE 27: Esterase phenotype ratios obtained from *H. ventrosa* breeding experiments.

Pair	Parental Esterase Phenotype Male	Parental Esterase Phenotype Female	Number of Progeny Scored	Progeny Esterase Phenotype	Expected Phenotype Ratio	χ^2	df	P
3+7	3/4	3/4	7	3/3	1:2:1	0.996	2	0.6
			13	3/4				
			4	4/4				
12	4/4	3/4	7	3/4	1:1	0.334	1	0.6
			5	4/4				
13	3/3	4/4	18	3/4	All 3/4	-	-	-
14	3/3	3/4	6	3/4	1:1	1.020	1	0.35
			3	3/3				
16	2/3	2/3	5	2/2	1:2:1	0.092	2	0.95
			7	2/3				
			3	3/3				
18	3/3	4/4	12	3/4	All 3/4	-	-	-
10	5/5	5/5	6	5/5	All 5/5	-	-	-

When the snails had matured, mating was seen to occur in all three species. Only H. ventrosa females subsequently produced viable progeny. H. ulvae and H. neglecta females rarely produced eggs (which in these species were laid in a gelatinous mass on the floor of the box) and subsequent dissection of some of the barren individuals revealed a high level of Trematode parasitism of the ovary and digestive gland. Parasitism was thought to be responsible for infertility in H. ulvae by Rothschild,[3] who reported infection of both male and female reproductive organs.

Eggs of H. ulvae and H. neglecta hatched into active veliger larvae after about 20 days; a similar development time for the eggs of H. ulvae was found by Fish and Fish.[4] The larvae, however, never survived for more than ten days and usually died during or shortly before metamorphosis, possibly as a result of having insufficient food reserves to survive the process. Veliger larvae are known to feed on planktonic diatoms[5] and the lack of a suitable food organism in laboratory cultures may have been responsible for the failure of these progeny.

Pairs of H. ventrosa (from population 50) produced eggs about eight weeks after reaching maturity (5 whorls). Eggs were deposited singly on the floor of the box and hatched into veliger larvae within three days of laying. The larvae settled and underwent torsion within eight days from hatching. After the progeny had grown to the 3-whorled stage, parents were electrophoresed. Progeny grew to the 5-whorled stage within three months and were then electrophoresed.

V - 1 - 3 Results of laboratory crosses of H. ventrosa

The segregation ratios obtained for Est. 2, 3, 4 and 5 are given in Table 27. The ratios did not differ significantly from those expected assuming the bands to be Mendelian alleles at the Es-2 locus. Conclusive evidence for allelism of Est. 5 was not obtained as no parents were 2/5, 3/5, 4/5 heterozygotes. Allelism of Est. 5 was, however, suggested by running heterozygotes on 5% and 7.5% gel; Est. 5 had the same relative molecular weight as Est. 3.

All parents and their progeny possessed Est. 1 suggesting that parents and progeny were fixed for Est. 1 at the

TABLE 28: Acid phosphatase (AP) phenotype ratios obtained from H. ventrosa breeding experiments

Pair(s)	Parental AP Phenotypes Male	Parental AP Phenotypes Female	Number of Progeny Scored	Progeny AP Phenotype		Ac. Ph. - 1 Expected Phenotype Ratio	χ^2	df	p
12+14+18 + 10	3/3	3/3	41	3/3	5/5	All 3/3	-	-	-
	5/5	5/5							
13	2/3	3/3	7	2/3	5/5	1:1	0.25	1	0.60
	5/5	5/5	9	3/3	5/5				
16	2/3	2/2	10	2/3	5/5	1:1	0.53	1	0.40
	5/5	5/5	7	2/2	5/5				

TABLE 29: Variation at esterase loci of H. ventrosa

SITE	N		GENOTYPE DISTRIBUTION Es-1	Es - 2										χ^{2*}	df	p	ALLELE FREQUENCIES					H_o (Es-2)	H_e (Es-2)
			$1^1/1^1$	2/2	2/3	3/3	3/4	4/4	2/4	2/5	3/5	4/5	5/5				1^1	2^2	2^3	2^4	2^5		
Little Humber Farm (Popln 50)	78	O	78	0	1	44	11	1	0	0	14	0	7	2.21	1	0.15 ns	1.0	0.006	0.73	0.077	0.179	0.333	0.429
		E	78	0	0.7	41.6	9.7	0.4	0.1	0.2	20.5	2.3	2.5										
Kirton Marsh (Popln 57)	51	O	51	0	5	22	9	8	7	0	0	0	0	3.68	1	0.07 ns	1.0	0.12	0.57	0.31	-	0.412	0.568
		E	51	0.7	6.8	16.3	18.4	5	3.8	0	0	0	0										
Bleak House (Popln 59)	45	O	45	0	2	25	12	3	3	0	0	0	0	0.21	1	0.65 ns	1.0	0.06	0.71	0.23	-	0.378	0.438
		E	45	0.1	3.6	22.8	14.9	2.4	1.2	0	0	0	0										

* Due to the presence of rare alleles, genotype classes have been merged i.e. for popln. 50, 2+3 = 3 and 4+5 = 5 and for poplns. 57 and 59, 2+3 = 3, 4 = 4.
The χ^2 value refers to Es - 2 only.

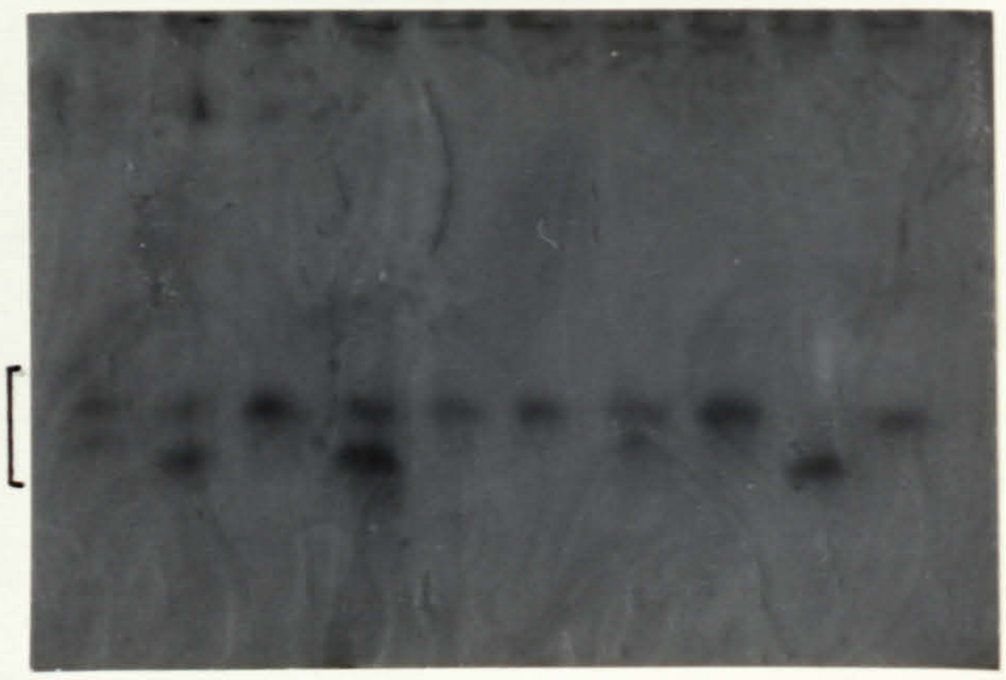

Plate 18 : Variation at the Es 2 locus in the Little Humber Farmh population (popln.50) of *Hydrobia ventrosa*.

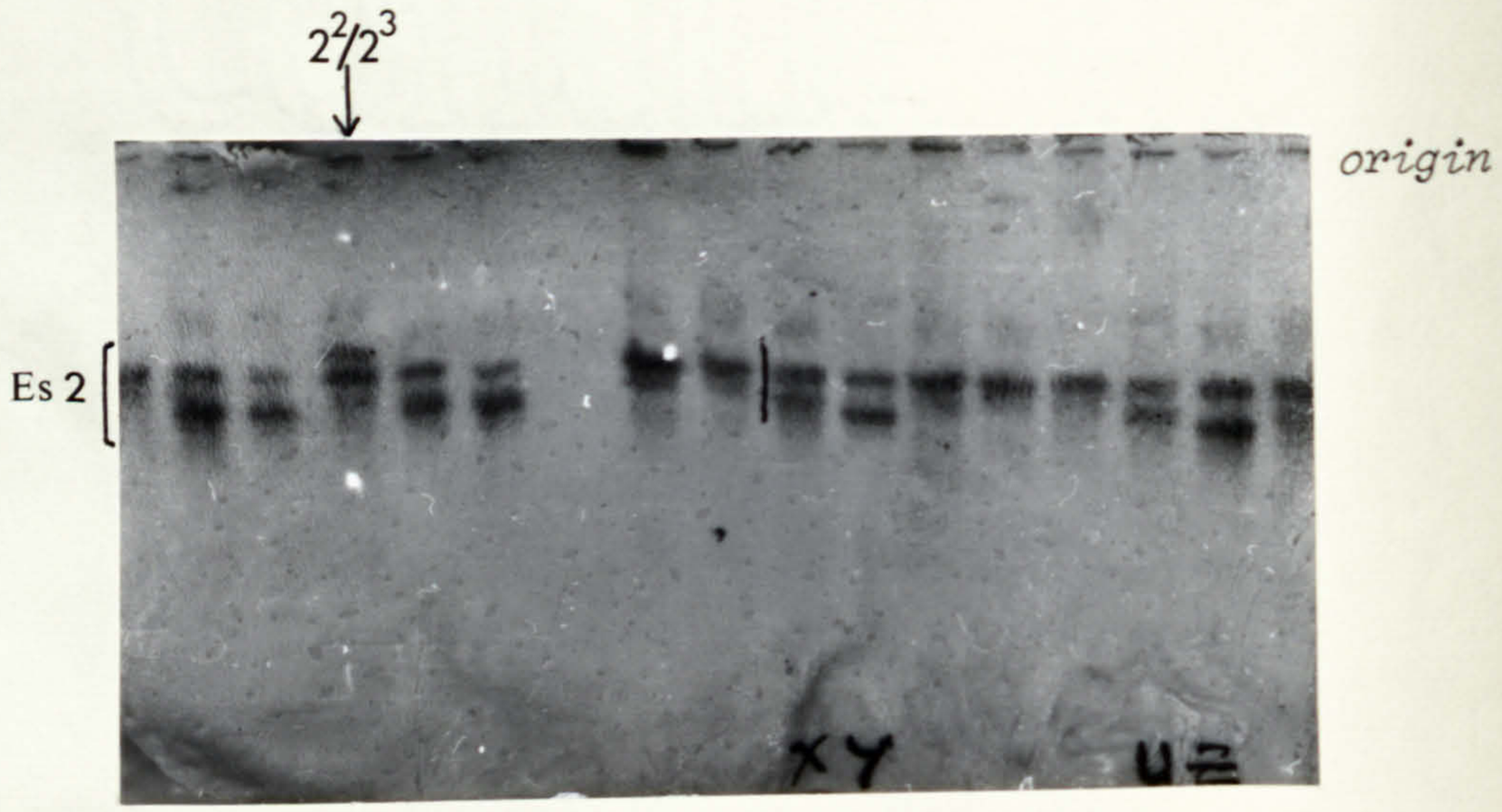

Plate 19 : Variation at the Es 2 locus in the Little Humber Farm population of *Hydrobia ventrosa* showing the rare $2^2/2^3$ heterozygote.

Es-1 locus.

Several of the crosses were scored for acid phosphatase. The results are shown in Table 28 and indicate that two loci are involved; Ac.Ph.-1 coding for bands Ac.Ph.2 and 3 and Ac.Ph.-2 coding for Ac.Ph.5. Unfortunately, the results of the crosses only provide conclusive evidence for allelism of Ac.Ph.2 and 3. Extracts run on 5% and 7.5% gels showed that Ac.Ph. 5 had a different molecular weight to bands 2 and 3, suggesting that the interpretation of the number of loci is correct. The crosses show that the Ac.Ph.5 band in the parents represents the fixed allele at the Ac.Ph.-2 locus and not the Ac.Ph.5/null heterozygote. During subsequent population surveys, one other band (Ac.Ph.4) was scored in this region but was shown to be a rare allele at the Ac.Ph.-2 locus (see section V - 2 - 2).

V - 1 - 4 Interpretation of other banding patterns

Banding patterns for which no breeding data were available were interpreted by a combination of recognising typical hetero- and homozygote patterns (see section III - 3 - 7) and running extracts on both 5% and 7.5% gels in order to obtain information on the relative molecular weights of bands thought to be allelic. The latter technique is fully described in sectiom III - 3 - 6.

V - 2 Variation in H. ventrosa populations

H. ventrosa was found in sufficient numbers at Hull (popln. 50) and at two sites in the Wash area (poplns. 57 and 59) to enable variation at electrophoretic loci to be quantified. Individuals were electrophoresed on slab gels which were sliced and stained, one slice for esterase, the others for acid phosphatase and/or Mdh.

V - 2 - 1 Esterases

Table 29 shows the results of the survey of the Es-1 and Es-2 loci in these populations together with the expected genotype ratios calculated from Hardy-Weinberg assumptions. Plates 18 and 19 show esterase variation in the Hull population.

All individuals were fixed for the $Es.1^{1}$ allele. The most common allele at the Es-2 locus was $Es.2^{3}$ in all three populations. The $Es.2^{5}$ allele was found only in the Hull population and

TABLE 30 : Variation at acid phosphatase loci of H. ventrosa.

SITE	N		GENOTYPE DISTRIBUTION AP - 1					AP - 2	2	df	p	ALLELE FREQUENCIES			AP - 1	
					3/3	3/2	2/2	5/5				1^2	1^3	2^5	H_o	H_e
Little Humber Farm (Popln. 50)	49	O			39	8	2	49	2.21	1	0.15 ns	0.122	0.878	1.0	0.163	0.215
		E			37.7	10.57	0.73	49								
Kirton Marsh (Popln. 57)	37	O			33	3	1	37	2.63	1	0.12 ns	0.068	0.932	1.0	0.081	0.128
		E			32.1	4.73	0.17	37								
Bleak House (Popln. 59)	41	O			37	4	0	41	0.21	1	0.65 ns	0.049	0.951	1.0	0.098	0.093
		E			37.1	3.8	0.1	41								

Es.2^2 was rare in all three populations.

The 'lumped' χ^2 values given in Table 29 (genotype classes were combined due to very low expected values) show that genotype frequencies in these populations conform to Hardy-Weinberg expectations. This is, perhaps, not surprising since H. ventrosa is sexually reproducing and populations are large.

V - 2 - 2 Acid phosphatase (Ac.Ph.)

Approximately 40 individuals from each of the populations scored for esterase loci were scored for acid phosphatase. Two alleles (Ac.Ph. 1^2 and 1^3) were found at the Ac.Ph.-1 locus in all three populations. Several individuals possessed the Ac.Ph.4 band (see Plate 20) which was shown to have a different molecular weight to bands at the Ac.Ph.-1 locus and to Ac.Ph.5 by the usual method of running extracts on 5% and 7.5% gel. For this reason, it is likely that Ac.Ph.4 represents a visible allele at a third, normally silent locus. All individuals in the three populations were fixed for Ac.Ph.5 at the Ac.Ph.-2 locus.

Table 30 shows the results of scoring for acid phosphatase in these populations. Deviations from Hardy-Weinberg expectations are not significant even though the expected numbers of $1^2/1^2$ genotypes were small (in some cases less than one) due to the low allele frequency of Ac.Ph.2.

V - 2 - 3 Malate dehydrogenase (Mdh)

The individuals scored for esterase in section V - 2 - 1 were also scored for Mdh. All showed only one band in the same position as the Mdh.1 band in P. jenkinsi. Extracts run on 5% gel still only showed one band and it is, therefore, likely that all individuals were fixed for the Mdh 1^1 allele.

V - 3 Variation in H. neglecta.

V - 3 - 1 Esterases

The esterases of 153 individuals from three populations (poplns. 57, 58 and 62) of H. neglecta were scored from slab gels stained for general esterase.

TABLE: 31 Variation at the Es - 2 Locus of H. neglecta

SITE	N		GENOTYPE DISTRIBUTION 2/2	2/3	3/3	3/4	2/4	4/4	χ^2	df	p	ALLELE FREQUENCIES 2^2	2^3	2^4	H_o	H_e
Kirton Marsh (Popln 57)	76	O	1	6	0	42	19	8	38	2	*** <.001	0.18	0.32	0.50	0.88	0.61
		E	2.4	8.5	7.6	24.3	13.7	19.5								
Gedney Marsh (Popln 58)	23	O	-	-	1	17	-	5	6.9	1	* 0.01		0.41	0.59	0.74	0.49
		E	-	-	3.9	11.2	-	7.9								
Guernsey (Popln 62)	54	O	-	-	9	33	-	12	2.71	1	0.10 ns		0.47	0.53	0.61	0.50
		E	-	-	12	27	-	15								

TABLE 32: Distribution of esterase phenotypes in six populations of H. ulvae

Popln.	N	ESTERASE PHENOTYPE (SEE FIG. 24 FOR DETAILS) 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	P
52	25	-	-	-	-	-	-	-	-	-	-	-	8	-	-	-	7	-	-	-	-	-	-	3	-	-	-	7	0.32
53	30	-	-	-	1	-	-	-	6	-	-	-	-	1	-	-	4	-	-	-	-	-	-	-	-	-	18	-	0.60
5454	27	-	-	-	1	-	-	2	-	-	5	-	2	2	-	-	3	-	1	-	-	4	-	-	-	-	7	-	0.259
56	40	-	-	-	6	-	-	-	-	-	-	-	10	-	-	-	3	-	-	-	-	-	-	-	-	-	-	21	0.525
60	36	1	1	1	1	1	2	2	2	1	3	1	1	2	1	2	1	1	1	1	1	1	1	2	1	2	1	1	0.083
61	16	-	-	-	2	-	2	-	-	-	-	-	3	-	-	-	-	-	-	-	-	9	-	-	-	-	-	-	0.5625

$\bar{P}$ 0.39

P = proportion of individuals having the most common esterase phenotype

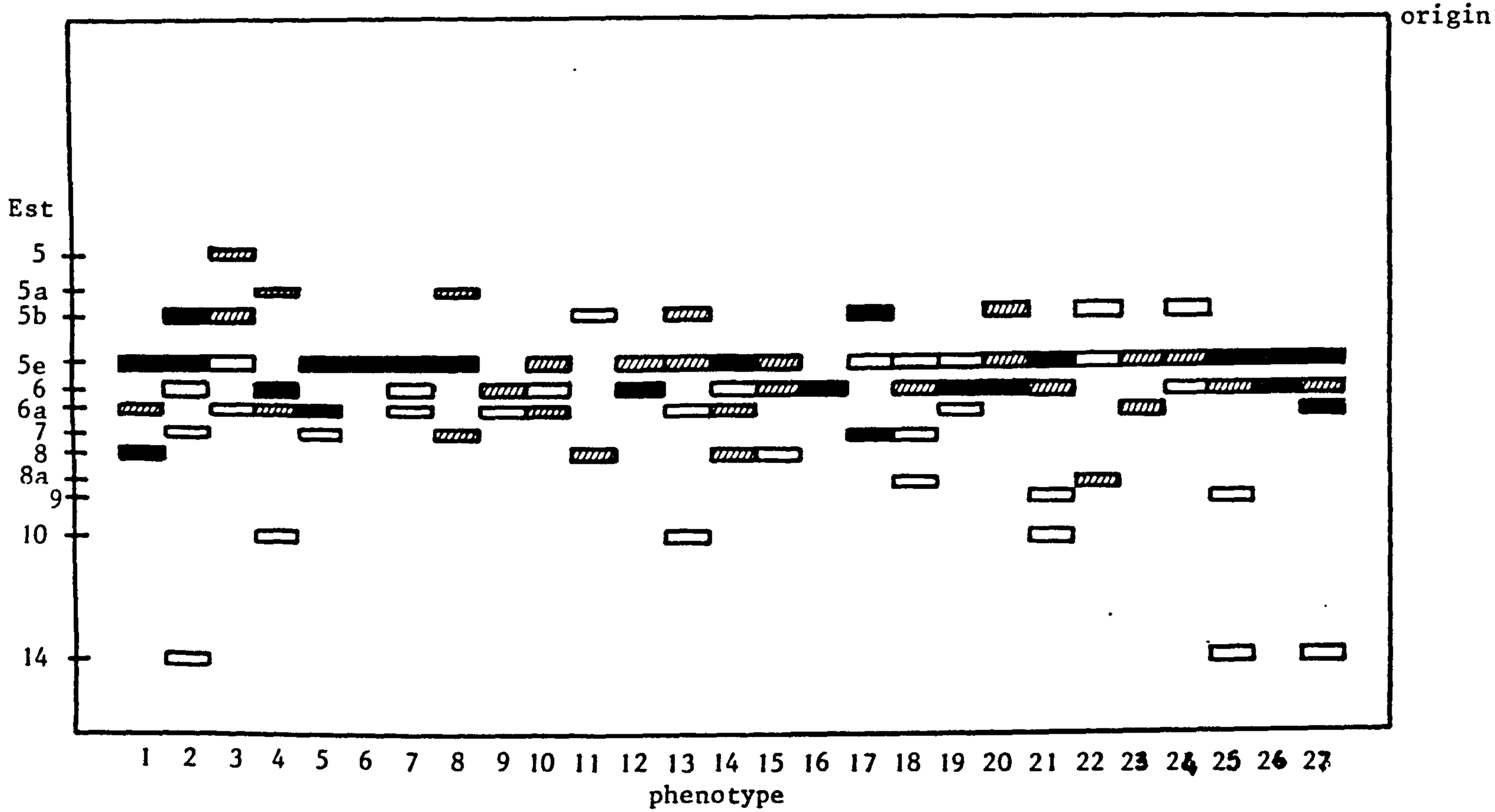

Fig. 24 : Esterase phenotypes found in six populations of Hydrobia ulvae; 7.5% acrylamide slab gel stained ∝ - n - acetate, ∝- n - proprionate and β- n - acetate.

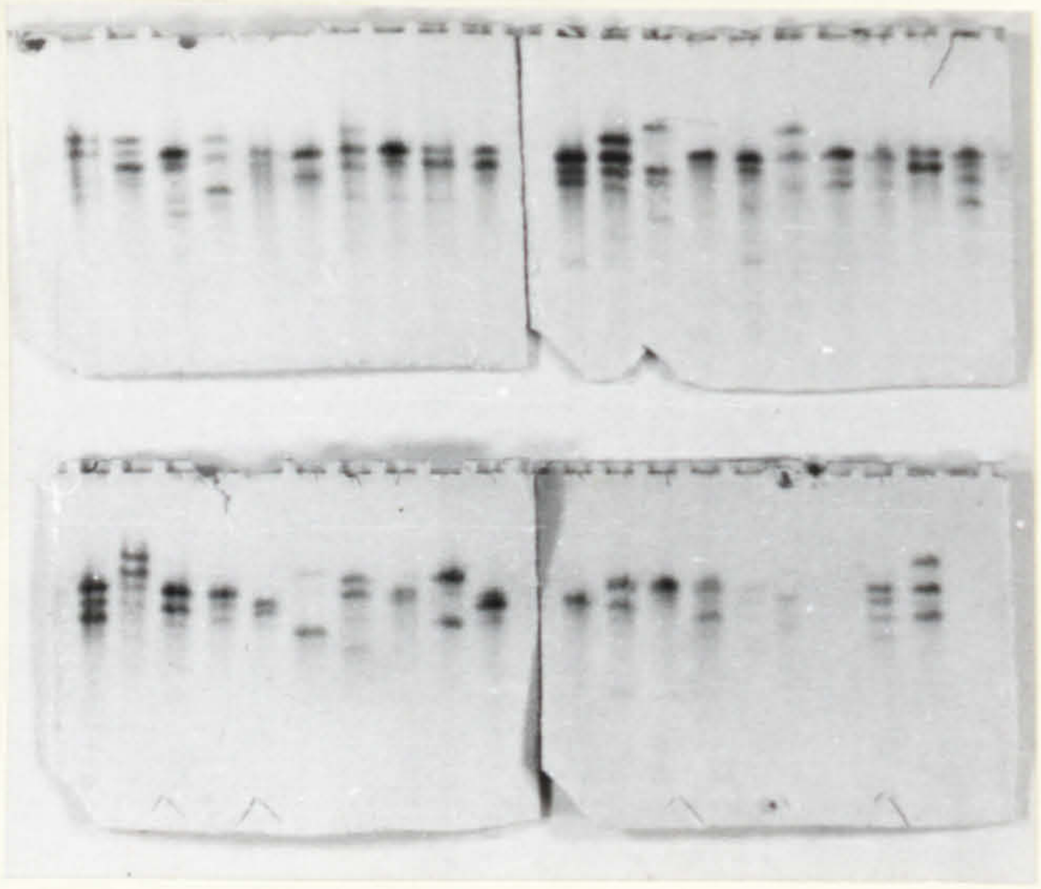

Plate 21 : Esterase phenotypes found in the Snettisham Lake population of *Hydrobia ulvae*.

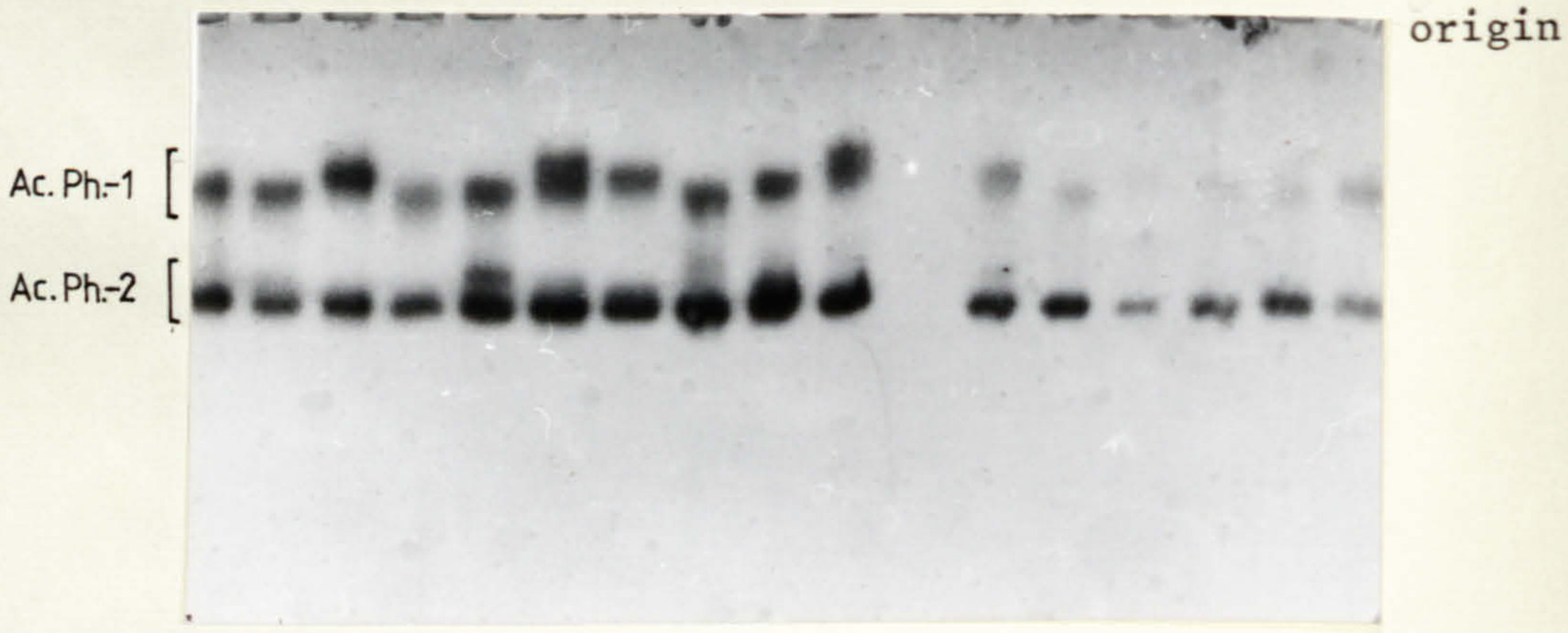

Plate 20 : Acid phosphatase phenotypes of individuals from the Little Humber Farm population of *Hydrobia ventrosa*.

In the absence of breeding data (see section V - 1 - 2) the assignment of bands to loci was based on assessment of their relative molecular weights (by running extracts on 5% and 7.5% gels) and by visual estimates of the relative staining intensities of supposed homozygotes and heterozygotes (see section III - 3 - 7).

All the strongly staining bands (Est. 2, 3 and 4) appeared to be alleles segregating at the Es-2 locus. A slower-running faintly staining banding system was detected (?Es-1) but could not be scored with certainty.

Table 31 gives the distribution of esterase genotypes at the Es-2 locus. In all three populations there is an excess of heterozygotes; significantly in populations 57 and 58 ($p < 0.001$ and $0.05 > p > 0.01$ respectively) and not significantly in population 62 ($p = 0.3$). Est. 2 was absent from populations 58 and 62.

V - 3 - 2 Mdh and Ac.Ph.

Mdh and acid phosphatase bands in this species were too diffuse to score with certainty. This was probably due to the presence of overlapping multiple bands as several distinct bands at different R_m positions were sometimes seen on lightly stained gels.

At least three Mdh phenotypes were visible on gels run using individuals from the Wash populations (poplns. 57 and 58) and at least five in the Guernsey population (popln. 62).

V - 4 Variation in H. ulvae

V - 4 - 1 Esterases

About 30 individuals from each of populations 52, 53, 54, 56, 60 and 61 were electrophoresed on slab gels and stained for general esterase.

The number of phenotypes found in each population ranged from four in poplns. 16 and 25 to 27 in popln. 60 (see Plate 21 and Fig. 24). Table 32 shows the distribution of phenotypes within each population.

The assignment of bands to loci presented a problem that was difficult to solve without breeding data. Banding patterns were complex; at least three loci were involved with several alleles at each locus. The possible presence of overlapping bands and null alleles makes the situation even more complicated.

Clearly the Snettisham population (popln. 60) is considerably more variable than the other five populations (all adults from long-established populations). The Snettisham population consisted entirely of young snails (+ four-whorled) which had been swept into the lake during a severe storm. No adults were found when the snails were collected (11 days after the storm); adults were either not present or were in deeper water. It is possible that snails were sorted according to size by wave action during the storm.

Population 54 was collected from a site only 38 km from Snettisham and in the same Wash system of mudflats. It is interesting to contrast variation in populations 54 and 60 as this may represent variation before (popln. 60) and after (popln. 54) selection has operated via juvenile mortality. Later samples from Snettisham may confirm this hypothesis.

V - 4 - 2 Acid phosphatase (Ac.Ph.)

Samples of approximately 20 snails from each of the six populations (section V - 4 - 1) were scored for acid phosphatase on slab gels. Variation was virtually absent, most individuals possessing only one band (Ac.Ph.2).

One individual from the Spurn Point population (popln. 56, n = 21) was found to have a slower band (Ac.Ph.1) in addition to Ac.Ph.2. Ac.Ph.1 remained equidistant from Ac.Ph.2 in 5% gel and it is likely that this individual was heterozygous for the rare Ac.Ph.1 allele and that all other individuals were fixed for Ac.Ph.2 at the Ac.Ph.-1 locus.

V - 4 - 3 Malate dehydrogenase (Mdh)

Two bands, Mdh. 1 and Mdh. 2, were found in H. ulvae. Both remained equidistant on 5% and 7.5% slab gels, suggesting that they were alleles at the Mdh-1 locus. Heterozygote patterns

TABLE 33: Distribution of Mdh Phenotypes in six populations of H. ulvae.

SITE	N		GENOTYPE DISTRIBUTION 1/1	1/2	2/2	χ^2	df	P	ALLELE FREQUENCIES 1^1	1^2	H_o	H_e
Popln. 52	24	O	0	5	19	2.77	1	0.10 ns	0.181	0.819	0.208	0.296
		E	0.79	7.11	16.10							
Popln. 53	18	O	1	8	9	0.28	1	0.60 ns	0.297	0.703	0.444	0.417
		E	1.59	7.51	8.90							
Popln. 54	13	O	0	2	11	5.19	1	<0.05 *	0.246	0.754	0.154	0.375
		E	0.79	4.87	7.39							
Popln. 56	24	O	2	13	9	0.84	1	0.35 ns	0.354	0.646	0.542	0.458
		E	3.01	10.98	10.01							
Popln. 60	33	O	3	18	12	1.08	1	0.30 ns	0.364	0.636	0.545	0.463
		E	4.36	15.27	13.37							
Popln. 61	22	O	0	6	16	1.88	1	0.15 ns	0.092	0.908	0.273	0.167
		E	0.19	3.67	18.14							

TABLE 34: Electrophoretic polymorphism in Hydrobia species; Summary of Data.

SPECIES	(N')	P	LOCI (D)	TOTAL LOCI	NUMBER OF HETEROZYGOTES	H	Ho/He
H. ventrosa	174 (3)	0.517	348 Est (2) 254 Ac.Ph (2) 174 Mdh (1)	776	O 79 E 101.3	0.102 0.131	0.779
H. neglecta	153 (3)	0.63	153 Est (1)	153	O 117 E 84.7	0.764 0.554	1.379
H. ulvae	134 (6)	0.39	134 Mdh (1) 134 Ac.Ph (1)	268	O 52 E 49.41	0.194 0.184	1.054

Abbreviations as Table 26.

suggested that Mdh was dimeric.

Mdh. 2 was the most common band in all six populations and genotype frequencies conformed to Hardy-Weinberg expectations (Table 33) except in the case of popln. 54 where the deviation was probably due to the small number of snails electrophoresed.

V - 5 Polymorphism in Hydrobia populations: A Discussion

Table 34 summarises the electrophoretic data obtained for the Hydrobia species.

The levels of heterozygosity in populations of H. ventrosa and H. ulvae, although based on few loci, are comparable with thoss given by e.g. Selander and Kaufman[6] and Powell[7] in their reviews of electrophoretic variation in invertebrate species. The estimate of $\bar{H}e$ for H. neglecta ($\bar{H}_e$ = 0.55) is extremelyhigh due to the significant excess (see Table 31) of heterozygotes found at the Es-2 locus in populations 57 and 58.

In contrast to the Potamopyrgus populations (with the exception of P. estuarinus), variation in Hydrobia species is distributed evenly not only between (as in Potamopyrgus) but also within populations. This pattern of polymorphism is typical of that found in other obligate sexually-reproducing populations of invertebrates, [6,7,8] notably in populations of Drosophila.

Due to the difficulties experienced in the resolution and interpretation of variation at the esterase loci of H. ulvae (section V - 4 - 1) and the Mdh and acid phosphatase loci of H. neglecta (section V - 3 - 2), only the H. ventrosa data is included in the estimates of genetic distance described in chapter VI.

CHAPTER V : REFERENCES

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CHAPTER VI : PHYLOGENETIC RELATIONSHIPS IN THE BRITISH AND AUSTRALASIAN HYDROBIIDAE

VI - 1 Estimation of Evolutionary Relationships

VI - 1 - 1 Taxonomic techniques

Since P. jenkinsi first appeared in Britain its taxonomic status has been the subject of much debate. The species is thought to be either a parthenogenetic form of H. ventrosa[1] or an introduced clone or clones of Australasian[2] or North American[3] species of Potamopyrgus.

Perhaps the most useful taxonomic feature of the Hydrobiidae is the morphology of the penis[4] and, since only one male of P. jenkinsi has been found,[5] (but see Discussion and Conclusions for recent work), it is hardly surprising that there is confusion over the taxonomy of this species. Shell character and mantle pigmentation have been used by Winterbourn[6] in an attempt to deduce the morphological relationships between P. jenkinsi and the New Zealand species P. antipodarum. His studies demonstrate that P. antipodarum is highly variable for these characters and that this variation includes that of P. jenkinsi.

In his description of P. estuarinus, Winterbourn[7] reported clear differences in female reproductive anatomy and shell shape between P. estuarinus and P. jenkinsi. He has also recently[7a] compared the morphology of the Australian species P. nigra with P. jenkinsi and considers P. nigra to be an equal contender (with P. antipodarum) for the ancestral population of P. jenkinsi. The snails used in these morphological studies came from fresh-water and were, therefore, probably P. jenkinsi A.

The problem with taxonomic studies of molluscs based on morphology is that phenotypic variation may, to an unknown extent, be environmentally induced. For example, keeling in P. jenkinsi is thought by Warwick[8] to be environmentally induced; the presence or absence of a keel, therefore, is an unreliable taxonomic feature.

Electrophoretic markers are generally unaffected by environmental factors and are useful taxonomic tools. Most are also simply inherited and do not usually exhibit dominance - unlike morphological markers. Davis[9] has produced a full review of the use

of electrophoretic markers in molluscan taxonomy:

One of the attractions of using such markers in taxonomy is that the genetic distance between populations may be estimated by computing an index of identity based on the probability of choosing identical alleles when randomly sampling the gene pools.

Several indices have been proposed by Malacot[10] (co-efficient of kinship), Lewontin[11], Hedrick[12], Cavalli-Sforza and Edwards[13], Balakrishnan and Sanghri[14], Rogers[15] and Nei[16]. Not all of these indices are easy to interpret biologically but Nei's is clearly related to evolutionary distance since it estimates the accumulated number of gene substitutions per locus. It is also independent of the breeding systems and ploidy levels of the populations being compared. Nei's index is also the most widely used and its use in the present study enables comparison with other studies to be easily made.

Nei defined the normalised identity of alleles (at a single locus) between two populations X and Y as:

$$Ij = jxy / \sqrt{jx \; jy}$$

where $jx = \sum x_i^2$ (x_i is the frequency of the ith allele in population X), $jy = \sum y_i^2$ and $jxy = \sum x_i \, y_i$. Ij is unity when the two populations have identical alleles at the same frequency and is zero when they have no common alleles at the locus.

The mean normalised identity, $\bar{I}$ (i.e. the proportion of alleles shared by the two populations) over a loci is then the geometric mean:

$$\bar{I} = Jxy / \sqrt{Jx \; Jy}$$

where $J = j/n$

The genetic distance D is defined as

$$D = - \log_e \bar{I}$$

When calculating $\bar{I}$ over several different loci in populations, it is assumed that the rate of gene substitution is similar for each locus. This may not be strictly true but is probably a reasonable assumption in the case of a comparison where similar classes of enzyme are used for each species (this is largely

the case in the present study).

A serious shortcoming of estimates of genetic distance based on electrophoretic data is that the absolute value obtained is probably biased towards the subset of enzymes that are detectable by electrophoresis. Electrophoresis only detects those proteins which are soluble, have different charges and are enzymatically active. There is reason to believe that these enzymes are more variable than, say, structural proteins and electrophoretic estimates of genetic distance may be too high as a result of this bias.

Again, the absolute values may lead to confusion due to the relationship between regulatory and structural loci in the genome. This aspect of genetic distance is more fully discussed later in this chapter (p.159).

When calculating genetic distance from electrophoretic data in this chapter, two other assumptions have been made:

i) where no bands occur at a 'locus' in two species (the locus is represented in a third, closely related species) then Ij is not included in the overall mean I value. This is probably reasonable if it is assumed that no (or different) null alleles are present at the locus. It is possible that the same null alleles are present at such loci but this is unlikely.

ii) In cases where no bands occur at a locus in one species but at least one band in another, Ij is assumed to be zero. Once again, the assumption may be erroneous due to the presence of the same null alleles in each species but this is probably unlikely.

Other sources of error may be due to the incorrect assignment of alleles to loci, particularly in the case of bands for which no data is available other than their mobility on 7.5% gel (e.g. some Mdh bands in P. nigra).

However, having pointed out the multifarious pitfalls in estimating genetic distance, it is fair to say that estimates for invertebrates at least show remarkable consistency. Examples of such

TABLE 35: Genetic Identities (Ij's) of British and Australasian Hydrobiid Snails

A – P. jenkinsi A: B – P. jenkinsi B: C – P. jenkinsi C: AN – P. antipodarum: N – P. nigra: V – H.ventrosa

LOCUS		AXAN	AXN	BXAN	BXN	CXAN	CXN	AXB	AXC	BXC	ANXN	AXV	BXV	CXV	ANXV	NXV
Es –	1	0.347	0.999	0.331	1.00	0.331	1.00	1.00	1.00	1.00	0.331	0.999	1.00	1.00	0.331	0.968
	2	0.500	0.	0.	–	0.	–	0.	0.	–	0.	0.	–	–	0.	–
	3	0.707	0.279	0.	0.	0.	0.	0.	0.707	0.	0.394	0.	–	0.	0.	0.
	4	0.	–	–	–	–	–	–	–	–	0.	–	–	–	–	–
	4a	0.	–	0.038	0.	0.	–	0.	–	0.	0.	–	0.	–	0.	–
	5	0.796	0.662	0.796	0.662	0.796	0.662	1.00	1.00	1.00	0.927	0.	0.	0.	0.	0.
	6	0.725	0.999	0.725	0.999	0.725	0.999	1.00	1.00	1.00	0.743	0.	0.	0.	0.	0.
	8	0.989	0.613	0.	0.	0.	0.	0.	0.	–	0.901	0.	–	–	0.	0.
	9	0.734	0.707	0.707	0.707	0.999	0.968	0.500	0.707	0.707	0.999	0.862	0.675	0.955	0.965	0.955
	10	0.	0.035	–	0.049	–	0.	0.	0.	–	0.	0.	–	–	–	0.
	11	–	–	0.	0.	–	–	0.	–	0.	–	–	0.	–	–	–
	12	0.847	0.331	0.847	0.331	0.847	0.331	1.00	1.00	1.00	0.783	0.	0.	0.	0.	0.
Mdh –	1	1.00	0.808	1.00	0.808	1.00	0.808	1.00	1.00	1.00	0.808	1.00	1.00	1.00	1.00	1.00
	2	–	0.	–	0.	–	0.	–	–	–	0.	–	–	–	–	0.
	3	1.00	0.998	1.00	0.998	1.00	0.998	1.00	1.00	1.00	0.998	0.	0.	0.	0.	0.
	4	–	0.	–	0.	–	0.	–	–	–	0.	–	–	–	–	0.
	5	–	0.	–	0.	–	0.	–	–	–	0.	–	–	–	–	0.
	6	–	0.	–	0.	–	0.	–	–	–	0.	–	–	–	–	0.
Ac. Ph –	1	1.00	–	1.00	–	1.00	–	1.00	1.00	1.00	–	0.	0.	0.	0.	–
	2	1.00	–	1.00	–	1.00	–	1.00	1.00	1.00	–	0.	0.	0.	0.	–
I j		9.645	6.431	7.444	5.554	7.698	5.766	8.500	9.414	8.707	6.884	2.861	2.675	2.955	2.296	2.923
n		15	15	14	16	13	14	15	13	12	17	13	11	10	13	14
$\bar{I}$		0.643	0.429	0.532	0.347	0.592	0.412	0.614	0.724	0.726	0.405	0.220	0.243	0.296	0.177	0.209
$\bar{D}$		0.442	0.847	0.652	1.058	0.524	0.887	0.488	0.323	0.321	0.9040	1.514	1.414	1.219	1.734	1.566

estimates are given later in this chapter (p.157). Estimates of distance based on electrophoretic data also generally agree with morphological divergence estimated by classical taxonomic methods. In this chapter, estimates of genetic distance between the Potamopyrgus species and H. ventrosa are calculated using Nei's index from the electrophoretic data presented in chapters IV and V. In this way, the phylogenetic relationships between the species are determined and compared with the morphological taxonomy of the Hydrobiidae. Two further sections are included describing mass extract electrophoresis and chromosomal techniques used in an attempt to add to the information obtained from the genetic distance estimates.

VI - 1 - 2 Estimates of Genetic distance - results and Discussion.

Table 35 shows Ij values for pairwise comparisons of the Potamopyrgus species and H. ventrosa. The data for H. ulvae and H. neglecta are not included as an insufficient number of loci could be scored in these species (see sections V - 3 - 2 and V - 4 - 1). Estimates of the mean normalised identity ($\bar{I}$) and the mean genetic distance ($\bar{D}$) between pairs of species are also presented in Table 35.

There is clearly a close relationship between the three strains of P. jenkinsi with strain A less closely related to B and C than B and C are to each other. All three strains are more closely related to P. antipodarum than to P. nigra with strain A more closely related to P. antipodarum than B and C.

P. nigra and P. antipodarum share 41% of common alleles, a similar proportion to that shared by P. jenkinsi (aggregate) and P. nigra (39.6%). Winterbourn[7a] and Ponder[17] consider P. antipodarum and P. nigra to be good morphological species and if this is so then the electrophoretic evidence suggests that P. jenkinsi and P. nigra are different species.

The three strains of P. jenkinsi share an average of 69% of their alleles, nearly double that of alleles shared by P. antipodarum and P. nigra. This suggests that P. jenkinsi A, B and C are more closely related than species within the genus Potamopyrgus and may be sibling species or races. Although this may be so in an electrophoretic sense, since they are obligate parthenogens they are (by definition) genetically isolated entities

the 'agamospecies' of White.[18]

The genetic distance between P. jenkinsi (aggregate) and P. antipodarum, D = 0.529, is higher than that between strains within P. jenkinsi (D = 0.374).

If we assume that, as Winterbourn[6] suggests, P. jenkinsi strains are parthenogenetic propagules from the array of genotypes making up the species P. antipodarum, then the difference between these values must be accounted for.

The genetic distance between parthenogenetic propagules and the ancestral population may be related to:

i) sampling effects. The genetic distance between propagules and the ancestral population must initially reflect variation within the latter. If successful propagules represent selection for a particular class of genotype (say, those which are heterozygous at many loci), then the genetic distance between propagules may well be less than that between the propagules and the ancestral population.

ii) the divergence of propagules from the ancestral population since isolation. Theoretically divergence can occur in apomictic propagules by the accumulation of mutations (including chromosomal re-arrangements) within clones. Lokki[19] gives a full account with models of this phenomenon in his review. Since populations of P. jenkinsi are often large (the number of individuals in the Snettisham population was estimated to be 2×10^6) one might well expect populations to consist of mixed clones. In fact, the only electrophoretic evidence for this in P. jenkinsi A was the individual variant in the Gaywood River and the small amount of variation at the Es-1 locus described in section IV - 2 - 1. Populations were remarkably uniform, suggesting that divergence due to accumulation of mutations has contributed little to the genetic distance between P. jenkinsi and P. antipodarum.

It is likely, therefore, that the genetic distance between strains in P. jenkinsi and between these strains and P. antipodarum is largely composed of sampling effects. The fact that the genetic distance between P. jenkinsi strains is less than that between P. jenkinsi and P. antipodarum may be evidence for biased sampling from P. antipodarum populations, although the population samples of P. antipodarum used in the present study may not be wholly representative of the ancestral population of P. jenkinsi. Equally, P. jenkinsi may be more closely related to some other species of Potamopyrgus not used in this study.

Hydrobia ventrosa is clearly not closely related to the Potamopyrgus species, sharing an average of only 23% of common alleles with them. The mean genetic distance between the Potamopyrgus species and H. ventrosa is 1.474. These estimates can only be regarded as a first approximation of the true genetic distance between these genera as, apart from the usual shortcomings of genetic distance estimates, no information from H. ulvae and H. neglecta is included. Nevertheless, it is of interest that the genetic identity between genera is about half of that between species within the genus Potamopyrgus ($\bar{D}$ = 0.921, $\bar{I}$ = 0.400).

The question of how genetically distinct populations have to be in order to be regarded as sub-species, species or genera in a morphological sense is particularly relevant to the taxonomy of populations where each individual is a genetically isolated agamospecies. Ayala et. al.[20] have quantified electrophoretic variation of populations in the Drosophila willistoni group and report the following genetic distances between morphological taxa:

	$\bar{I}$	$\bar{D}$
Non-sibling species	0.35	1.06
Sibling species	0.52	0.75
Subspecies	0.80	0.23
Semispecies	0.80	0.23
Geographic populations	0.97	0.03

Comparable estimates have been made for species in the D. obscura and D. repleta complexes by Ayala and Dobzhansky[21] and Zouros[22] respectively. The only real exception is that of the non-sibling species D. mulleri and D. aldrichi (in the D. repleta group)

TABLE 36: Estimates of genetic identity and distance (calculated using Nei's index of normalised identity) between five Potamopyrgus 'species' and Hydrobia ventrosa.

	A	B	C	AN	N	V
A	-	0.488	0.323	0.442	0.847	1.514
B	0.614	-	0.321	0.632	1.058	1.414
C	0.724	0.726	-	0.524	0.887	1.219
AN	0.643	0.532	0.592	-	0.904	1.734
N	0.429	0.347	0.412	0.405	-	1.566
V	0.220	0.243	0.296	0.177	0.209	-

Values above the diagonal ($\bar{I} = 1$, $\bar{D} = 0$) are estimates of genetic distance and those below are estimates of genetic identity.

TABLE: 37: Nei's indices of genetic identity and distance calculated for different taxonomic levels within the Hydrobiidae.

	$\bar{I}$	$\bar{D}$
Strains within the species Potamopyrgus jenkinsi	0.688	0.374
Species within the genus Potamopyrgus	0.398	0.921
Genera (Potamopyrgus and Hydrobia) within the family	0.229	1.474

where $\bar{D}$ = 0.124.

The electrophoretic distances between different morphological taxa in the Hydrobiidae are given in Tables 36 and 37 and have been calculated by computing means within taxa.

The genetic distance between the non-sibling species P. antipodarum and P. nigra ($\bar{D}$ = 0.904) is remarkably similar to that generally found between Drosophila non-sibling species. If the Drosophila estimates are comparable with those for the Hydrobiidae, then the strains of P. jenkinsi are sufficiently distinct to be regarded as subspecies (within P. antipodarum?).

The genetic distance between the genera Hydrobia and Potamopyrgus ($\bar{D}$ = 1.474) is similar to those found by Hedgecock and Ayala[23]) between salamanders in the genera Taricha and Notopthalmus ($\bar{D}$ = 1.2) and (reported by Avise[24]) between genera of kangaroo rats ($\bar{D}$ = 1.8).

There are, however, some notable exceptions to these general relationships. For example, the genetic distance between minnow genera[25] has been estimated to be D = 0.53 and King and Wilson[26] consider chimpanzees and men to be (electrophoretically) sibling species!

Clearly, an understanding of the relative roles of structural and regulatory loci in determining the phenotype is urgently needed in order to assess the significance of electrophoretic estimates of genetic divergence compared with morphological and behavioural divergence.

VI - 2 Comparisons of Mass Extracts

Electrophoresis of a mass extract prepared by homogenising a large number of snails from a population should produce a banding pattern which is related to the frequencies of bands in the population. Bands which are common in the population should be easily detectable on the gel while rare bands will be diluted (see section III - 1 - 2) to suchan extent that they may be undetectable.

The staining intensity of a band in a mass extract zymogram is a function of its hydrolysis rate (or absorption rate of Coomassie Blue if a general protein) and its frequency in the population

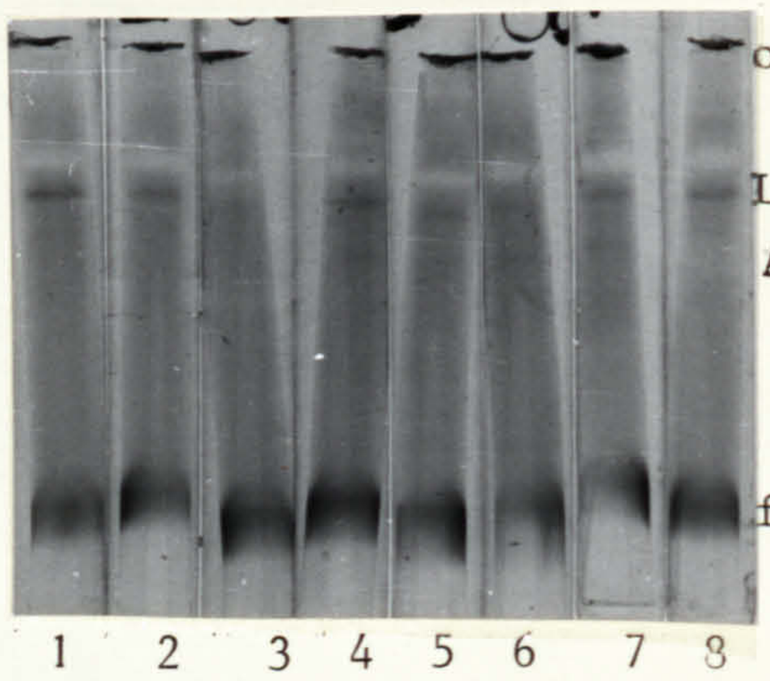

Plate 22 : TO and Ldh in mass extracts of Potamopyrgus ssp. ; disc gels stained in vacuo.

1 & 2 - P.jenkinsi A
3 - P.jenkinsi B
4 - P.jenkinsi C
5 & 6 - P.antipodarum
7 7 8 - P.nigra

The dark region at the front is due to the bromophenol blue marker and the faint band below Ldh is Adh. Adh often appeared on dehydrogenase-stained gels stored in 30% ethanol.

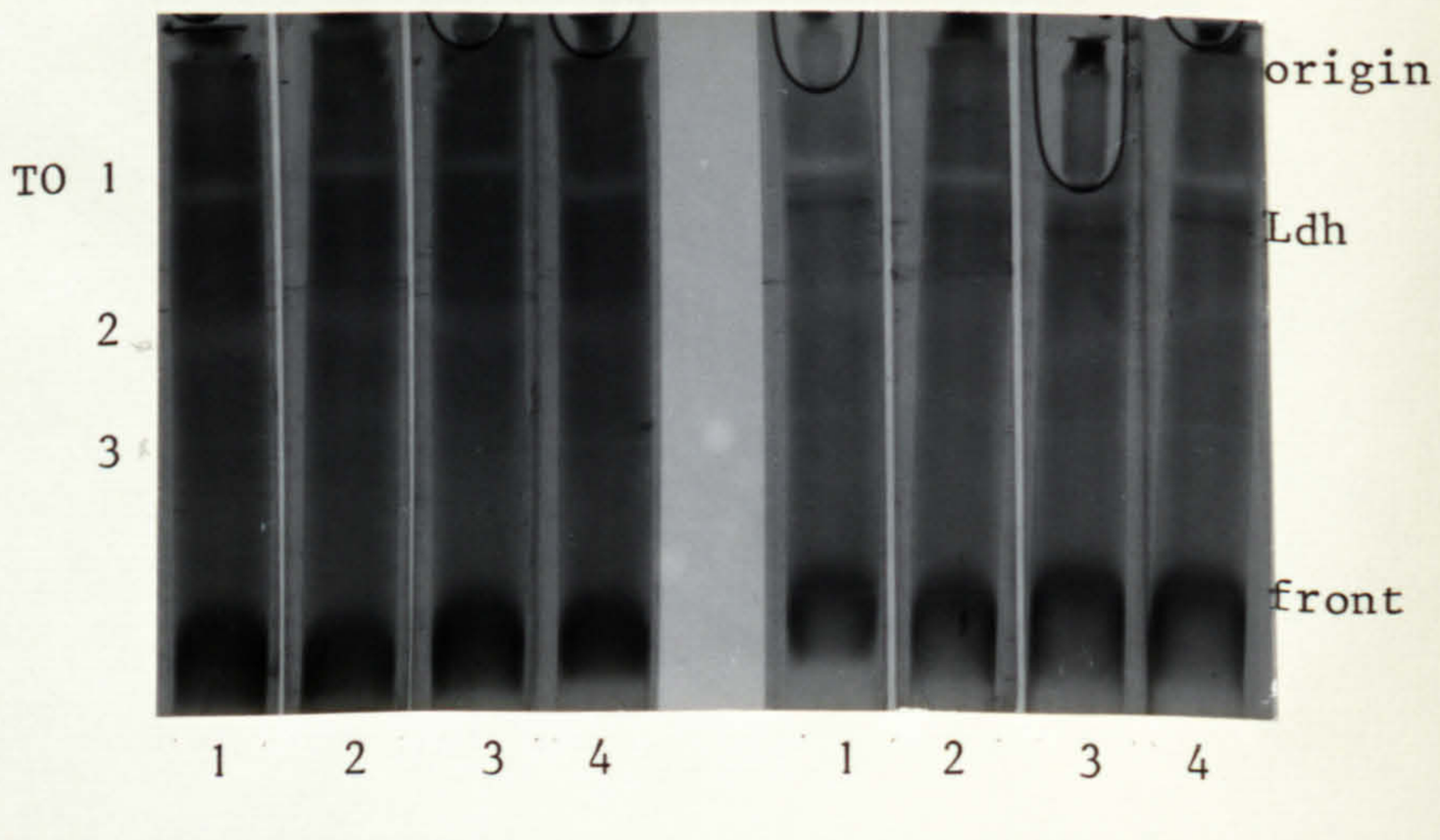

Plate 23 : TO and Ldh in mass extracts of four Potamopyrgus ssp. The left-hand set of gels was stained using no substrate in order to test for the possible presence of 'nothing dehydrogenases'. The set on the right were stained using lactic acid substrate.

1 - P.jenkinsi A
2 - P.jenkinsi B
3 - P.antipodarum
4 - P.nigra

(x_i and y_i in section VI - 1 - 1). Assuming the hydrolysis (or absorption) rates of homologous bands in two populations to be similar, then the banding pattern is determined by the relative frequencies of bands within the populations.

A visual comparison of banding patterns derived from mass extracts is approximately similar, therefore, to a probablistic index of identity prepared from a survey of individuals from different populations. It is reasonable to suppose that, if mass extracts of two populations give similar banding patterns, then they are essentially identical at the loci producing the bands.

VI - 2 - 1 Electrophoresis and staining

Mass extracts (prepared as described in section I - 2 - 2) of P. jenkinsi A, B, C, P. antipodarum and P. nigra were run on disc gels and stained for alcohol dehydrogenase (Adh - section I - 5 - 3), lactate dehydrogenase (Ldh - section I - 5 - 3), tetrazolium oxidase (TO - section I - 5 - 4), peroxidase (section I - 5 - 6), amylase (section I - 5 - 5), and general protein (section I - 5 - 7).

In view of the large number of bands found on gels stained for general protein, extracts were run on gels of different concentrations (as described in section III) in order to identify possible homologies between bands in different extracts.

VI - 2 - 2 Adh, Ldh, TO, peroxidase and amylase

Sets of gels stained for these enzymes revealed the same banding patterns in all five extracts. The patterns are shown diagrammatically in Figs. 4 to 12; Plates 22 and 23 show sets of gels stained for TO and Ldh.

VI - 2 - 3 General protein

Extracts of P. jenkinsi A, B, C and P. antipodarum gave the same banding patterns. Plate 24 shows mass extracts of P. jenkinsi A and P. antipodarum run on different gel concentrations and clearly demonstrates that the similarities between bands are true homologies with respect to molecular size and charge; homologous bands migrate to similar positions in different gel concentrations (see section III - 3 - 2).

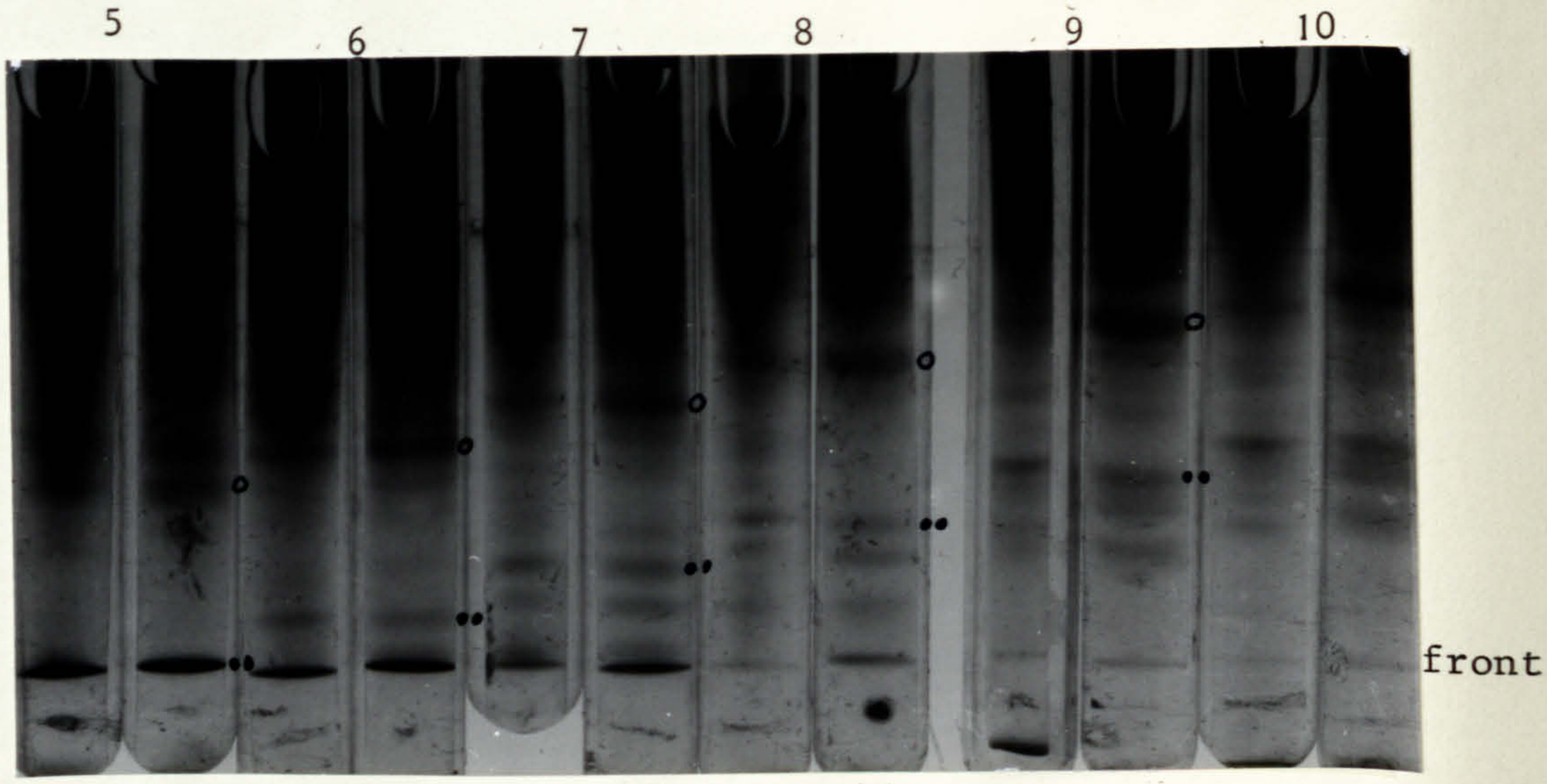

Plate 24 : Mass extracts of P.jenkinsi A (popln. 11) and P.antipodarum electrophoresed on 5 to 10% disc gels stained for general protein using Coomassie Blue/TCA.

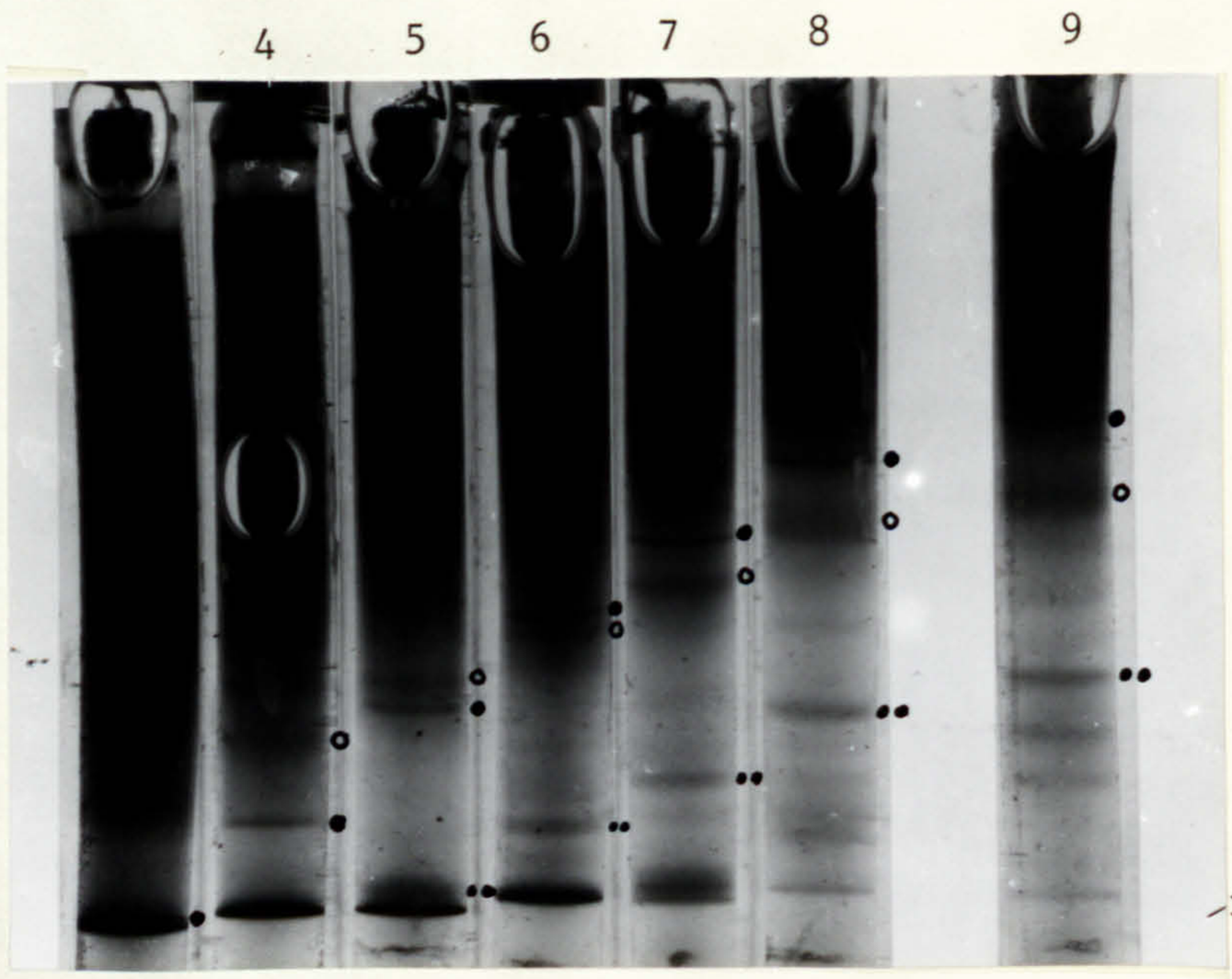

Plate 25 : A mass extract of P.nigra (populations 42 + 40) electrophoresed on 4 to 9% disc gels stained for general protein using Coomassie Blue/TCA.

A set of gels prepared using an extract of P. nigra is shown on Plate 25. Although there are similarities between these patterns and those in Plate 24, the P. nigra gels show at least one extra band (marked with a single dot) for which there was no equivalent in the P. jenkinsi and P. antipodarum extracts. Bands marked with an open circle and those marked with two dots are thought to be homologous in all the Potamopyrgus extracts.

VI - 2 - 4 Phylogenetic significance of mass extract studies

The results of the mass extract comparisons are consistent with the hypothesis that P. jenkinsi strains are closely related to each other and to P. antipodarum and that P. nigra is less closely related to these species.

However, since the only difference between these species was apparent on the general protein gels, it may be that the estimates of genetic distance computed in section VI - 1 - 2 are too high. The lack of detectable difference between mass extracts of these species stained for the isozymes other than general protein may, however, be due to the lower sensitivity of mass extract comparisons relative to genetic distance calculations based on surveys of individuals within populations.

VI - 3 Chromosomal relationships between the Potamopyrgus species

A commonly used taxonomic technique is comparison of chromosome numbers and chromosomal structure.

The earliest attempt to count the chromosomes of P. jenkinsi was made by Rhein[27], using European material, who considered that P. jenkinsi was diploid with a chromosome number of 20 - 22. Sanderson,[28] in a later study using British P. jenkinsi, reported that this race was tetraploid (4n = 36-44). Suomalainen[29] and Patterson[30] consider that this count needs re-investigation and certainly the evidence for tetraploidy in P. jenkinsi offered by Sanderson is far from convincing.

Winterbourn[7] has shown that both P. antipodarum and P. estuarinus have a diploid chromosome number of 2n = 24. He used testis tissue to obtain the counts since he found the interpretation

of squashes prepared from ovarian material 'difficult'.

In view of the dubious nature of the previous work on P. jenkinsi, about 100 squashes were made by myself in the laboratory of (and with the advice of) Dr. G. Hewitt of the University of East Anglia - where squashes of insect material are routinely made. Despite using various techniques (including that used by Sanderson[28]) and tissues (ovary, digestive gland and foot muscle) none of the squashes resulted in a countable metaphase plate. Difficulty was experienced with:

i) the presence of silica particles (insoluble in nitric and hydrochloric acid) which prevented coverslips from pressing on nuclei. This was a particular problem with ovarian and digestive gland material.

ii) the absence of meiosis; no evidence of meiosis (e.g. smaller nuclei) was found during examination of ovarian squashes. Metaphase in mitotically-dividing cells (spindle formation was stopped with colchicine) was characterised by ill-defined condensed chromatin strands often appearing to be linked together forming a complex web. Separation of these structures proved to be impossible even when the squashes were annealed with 10% HCl for 10 minutes at 80°C - treatment considered by Dr. Hewitt to be optimal.

Preparations of apomictic individuals of P. antipodarum and P. nigra were similarly unsuccessful, although control squashes prepared from grasshopper ovaries resulted in easily-countable metaphase plates.

Separation of nuclei by ultracentrifugation is clearly needed in order to overcome the problem of silica particles but even then separation of the chromosomes may prove to be difficult. It is possible that cell division in P. jenkinsi is, in some way, unusual and that the connections between the chromatin strands are functionally important in cell division in this species.

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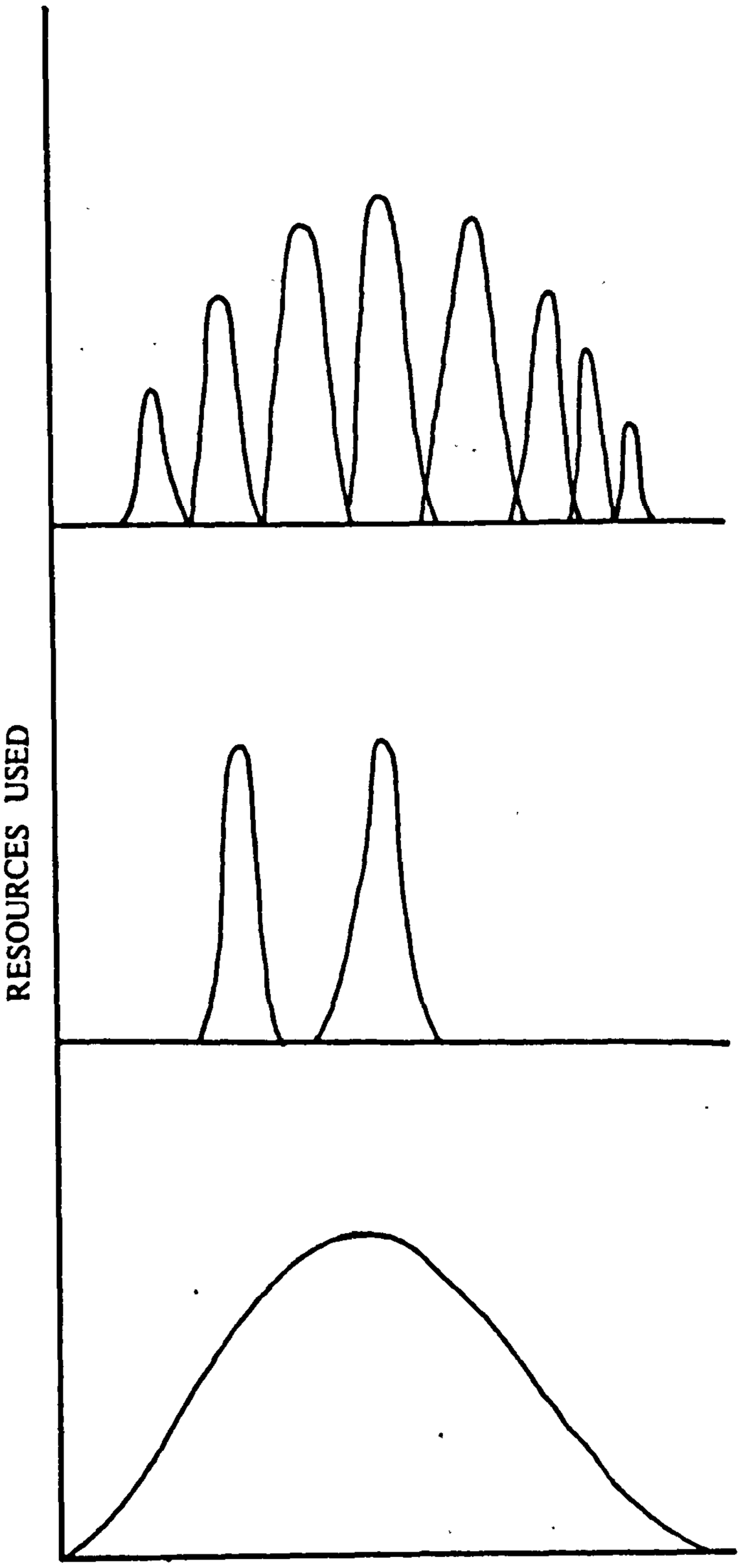


a) Between-phenotype exploiter

b) Within-phenotype exploiters: low biochemical flexibility

c) Within-phenotype exploiter; high biochemical flexibility

Fig.25 : Theoretical exploitation of environmental resources by populations whose component individuals differ in their phenotypic plasticity.

CHAPTER VII : FACTORS AFFECTING THE BEHAVIOUR AND SURVIVAL OF SOME HYDROBIID SNAILS

In the previous chapters it has been shown that there are differences in levels of heterozygosity and in the distribution of variation between and within populations (and species) of Hydrobia and Potamopyrgus. In view of this, it is tempting to speculate on how these differences might affect or determine the ecological strategy pursued by a particular population or species.

For example, Van Valen[1] and, more recently, Roughgarden[2] have suggested that polymorphic and monomorphic populations may differ in their ability to exploit available resources. Van Valen divides populations into two extreme categories:

i) 'between-phenotype' exploiters are (polymorphic) populations made up of an array of different phenotypes each of which has the capacity to exploit a unique part of the resource continuum. The resource spectrum (the proportion of the resource continuum exploited) of such a population is the sum of the resource spectra of the individual 'specialist' phenotypes (Fig. 25a).

ii) 'within-phenotype' exploiters are (monomorphic) populations in which all individuals have a similar phenotype and all, therefore, have the capacity to exploit the whole resource spectrum of the population (Fig. 25b and c). Pianka[3] argues, largely on intuitive grounds, that such individuals would be 'generalists' in the sense of their being able to exploit more of the available resources than individuals in a 'between-phenotype' population.

The resource utilisation capability of an individual is limited by the biochemical flexibility of its gene products.

If biochemical flexibility is low, due for example, to gene fixation or some aspect of gene regulation, then one might expect monomorphic populations (where the resource utilisation capability of the individual equals that of the population) to be more specialised than polymorphic populations (Fig. 25b).

However, individuals within a monomorphic population might be selected for (or accumulate) high levels of heterozygosity and become heterotic due to increased biochemical flexibility. The population (see Fig. 25c) would then be near that envisaged by Pianka as a "population composed of pure generalists with each member exploiting the entire range of resources".

Providing that differences in electrophoretic variation between populations determine differences in phenotypic variation, populations of the Hydrobiidae present an opportunity to investigate the above hypotheses. In fact, Winterbourn[4] presents evidence that morphological variation in P. jenkinsi populations (which are electrophoretically monomorphic) is very much less than morphological variation in partly sexual populations of P. antipodarum (which are electrophoretically polymorphic), suggesting that, at least in these species, levels of electrophoretic variation are positively correlated with levels of morphological variation.

The experiments described in this chapter were initially designed to compare the resource utilisation spectra of populations of P. antipodarum and P. nigra since both monomorphic and polymorphic populations occur within these species. Unfortunately, insufficient numbers of individuals were available for such experiments and so populations of the British Hydrobiidae were used instead; P. jenkinsi (A and B) populations to represent monomorphic populations and Hydrobia populations to represent polymorphic populations. Although the species used are from different genera, the snails are all approximately the same size and, at least in some habitats (see below), co-exist.

Two resource dimensions, temperature and salinity, were used; resource utilisation was estimated by assessing the mortality and activity of snails exposed to various temperatures and salinity levels. The results of these experiments are discussed in relation to the differences in electrophoretic variation between the populations and also in the context of the distribution of the British Hydrobiidae.

VII - 1 Materials and Methods

VII - 1 - 1 The main experiments

A range of six salinities (from 0 to 100% sea-water (SW))

TABLE 38: Salinities of the dilutions of sea-water used in the mortality and activity experiments.

Nominal % S.W.	mg/l NaCl by titration
0	52
20	7821
40	13802
60	21725
80	28905
100	37602

was prepared by diluting sea-water with aerated (10 days) tapwater. The chloride concentration of each dilution was estimated by titration against standardised silver nitrate solution (see Table 38).

Adult snails were obtained from populations 11 (P. jenkinsi A); 1 (P. jenkinsi B); 50 (H. ventrosa); 56 (H. ulvae) and 57 (H. neglecta). Snails were kept in the laboratory in habitat water at room temperature for two days before each experiment was started. For each species, a fixed number of snails (see Tables 40 - 49 for numbers used) was placed in 200 ml of water of each salinity in polystyrene boxes (10 cm x 10 cm x 10 cm) fitted with lids to prevent evaporation. Food in the form of boiled and dried lettuce was provided; the boxes were then randomly arranged in trays and placed in constant temperature rooms having a 16 hour light/8 hour dark cycle. Three temperature regimes were chosen as approximations of winter (4^{o}), summer (32^{o} - typical of habitats that were drying out) and mean (19^{o}) conditions in the native habitats.

After 72 hours' exposure to the experimental conditions, the number of snails which were extended and crawling in the undisturbed boxes were scored and classed as 'active'.

To score mortality, all snails in each box were then removed and placed, aperture uppermost, in a test-box containing water of the salinity in which they were found in the field. Test-boxes were kept at room temperature (about 22^{o}C). Those snails which had not turned over and started crawling within 30 minutes were classed as 'effectively dead' (see the following section VII - 1 - 2 for an evaluation of this method). Both the above behavioural criteria had to be met before a snail was classed as alive: occasionally a snail would be able to turn over but would remain stationary, seemingly unable to crawl, and was therefore classed as effectively dead.

VII - 1 - 2 Scoring of effective death: rationale and evaluation

One of the problems encountered in scoring mortality in operculate prosobranch snails is knowing when mortality has occurred. A recently-dead snail with the operculum closed looks much the same as a dormant snail. There are no reliable external indicators of death such as the visible cessation of respiratory function in pulmonate animals.

TABLE 39 : THE NUMBERS OF SNAILS DEAD AFTER HAVING BEEN KEPT IN THE WATER IN WHICH THEY WERE FOUND FOR 30 DAYS AT ROOM TEMPERATURE

Source of Snails	No. Used	No. Dead at 30 days	χ^2 (2 df)
P. jenkinsi A			
Scored			
'Dead'	30	29	1.72
'Alive'	30	5	
From wild	50	4	
H. ventrosa			
Scored			
'Dead'	30	27	1.41
'Alive'	30	1	
From wild	50	4	
H. ulvae (from treatments other than 4°C.)			
Scored			
'Dead'	30	30	0.40
'Alive'	30	3	
From wild	50	6	

BACKGROUND MORTALITY FOR ALL 'WILD' SNAILS = $14/150 = 0.093$

Fraenkel[5] has suggested a way of surmounting this problem based on the premise that normal physiological functioning of the organism may be characterised by the ability to complete certain behavioural patterns. Experimentally-induced stress may irreversibly damage the organism such that this ability is lost; the organism is then 'effectively dead' in the sense that it would have a much lower life expectancy in the field than an organism which had not been stressed.

In the present study, the ability both to <u>turn over</u> and <u>start crawling</u> were used as criteria for normal physiological functioning. Snails which, when placed, aperture uppermost, in water of the habitat salinity at room temperature (approx 22°C), failed to meet both criteria within 30 minutes were classified as 'effectively dead'.

The predictive power of the technique (in terms of lowered life expectancy) was evaluated in the following way:

At the end of the 72-hour experimental period (see section VII - 1 - 1), 30 snails scored as 'alive' and 30 'effectively dead' snails of each species were taken randomly from all treatments. Each group was maintained separately in the water in which the population was found for a further 30 days at room temperature (about 22°C). Food in the form of boiled and dried lettuce was provided at regular intervals. As a control, 50 adult snails of each species were collected from the wild and treated in a similar way.

At the end of this period the numbers still alive were rescored by using the behavioural critera; most dead individuals had decomposed by this time. The results are presented in Table 39.

Comparisons (using a 'corrected' χ^2 test[6]) of the mortalities of snails scored as 'alive' in the main experiment with those of the live snails collected from the wild showed no significant differences (probabilities for each comparison are given in Table 39). The 'background' mortalities in these classes were low (8 - 12%) during the 30 day evaluation period.

In contrast, the mortality of those snails classified as 'effectively dead' in the main experiments was high (90 - 100%) during the 30 day evaluation period. The method of scoring effective death is clearly a reliable way of assessing irreversible damage caused by

TABLE 40: The numbers of P.jenkinsi A effectively dead after 72 hours. N = 10

	% Sea-water											
	0		20		40		60		80		100	
T^0C	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
4°	10	10	0	0	10	3	10	10	10	10	10	10
mean	10		0		6.5		10		10		10	
19°	6	10	0	0	0	1	10	10	10	10	10	10
mean	8		0		0.5		10		10		10	
32°	10	10	0	0	0	1	10	10	10	10	10	10
mean	10		0		0.5		10		10		10	

TABLE 41: The numbers of H. ventrosa effectively dead after 72 hours. N = 12

	% Sea-water											
	0		20		40		60		80		100	
T^0C	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
4°	0	0	1	0	0	0	0	0	3	2	3	2
mean	0		0.5		0		0		2.5		2.5	
19°	8	8	0	0	0	0	0	0	0	0	0	1
mean	8		0		0		0		0		0.5	
32°	12	6	0	0	0	0	0	0	0	0	7	8
mean	9		0		0		0		0		7.5	

TABLE 42: The numbers of H. ulvae effectively dead after 72 hours. N = 20

	% Sea-water											
	0		20		40		60		80		100	
T^0C	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
4°	0	0	0	2	2	3	0	0	0	0	0	0
mean	0		1		2.5		0		0		0	
19°	0	0	0	3	7	3	0	0	0	0	0	0
mean	0		1.5		5		0		0		0	
32°	17	17	17	16	3	3	0	1	0	0	0	0
mean	17		16.5		3		0.5		0		0	

TABLE 43: The numbers of P. jenkinsi B effectively dead after 72 hours. N = 10 (1 replicate)

	% Sea-water					
	0	20	40	60	80	100
4°	0	0	3	7	9	10
19°	0	0	1	2	5	9
32°	2	0	1	10	10	10

TABLE 44: The numbers of H. neglecta effectively dead after 72 hours. N = 20.

	% Sea-water											
	0		20		40		60		80		100	
T°C	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
4°	0	0	3	2	3	0	0	0	0	1	1	0
mean	0		2.5		1.5		0		0.5		0.5	
19°	0	3	3	3	0	0	1	0	3	1	5	0
mean	1.5		3		0		0.5		2		2.5	
32°	3	10	8	5	4	0	7	1	6	1	12	1
mean	6.5		6.5		2		4		3.5		6.5	

TABLE 45: The numbers of P. jenkinsi A active after 72 hours. N = 12

	% Sea-water											
	0		20		40		60		80		100	
T°C	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
4°	0	0	5	2	0	2	0	0	0	0	0	0
mean	0		3.5		1.5		0		0		0	
19°	0	0	12	12	12	12	2	6	0	0	0	0
mean	0		12		12		4		0		0	
32°	0	3	12	12	12	12	0	0	0	0	0	0
mean	1.5		12		12		0		0		0	

TABLE 46: The numbers of H. ventrosa active after 72 hours. N = 12.

	% Sea-water											
	0		20		40		60		80		100	
$T^{o}C$	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
4°	0	0	3	0	3	2	1	0	0	0	0	0
mean	0		1.5		2.5		0.5		0		0	
19°	0	0	12	12	12	12	12	12	12	12	12	12
mean	0		12		12		12		12		12	
32°	0	0	12	12	12	12	12	12	12	12	11	10
mean	0		12		12		12		12		10.5	

TABLE 47: The numbers of H. ulvae active after 72 hours. N = 20.

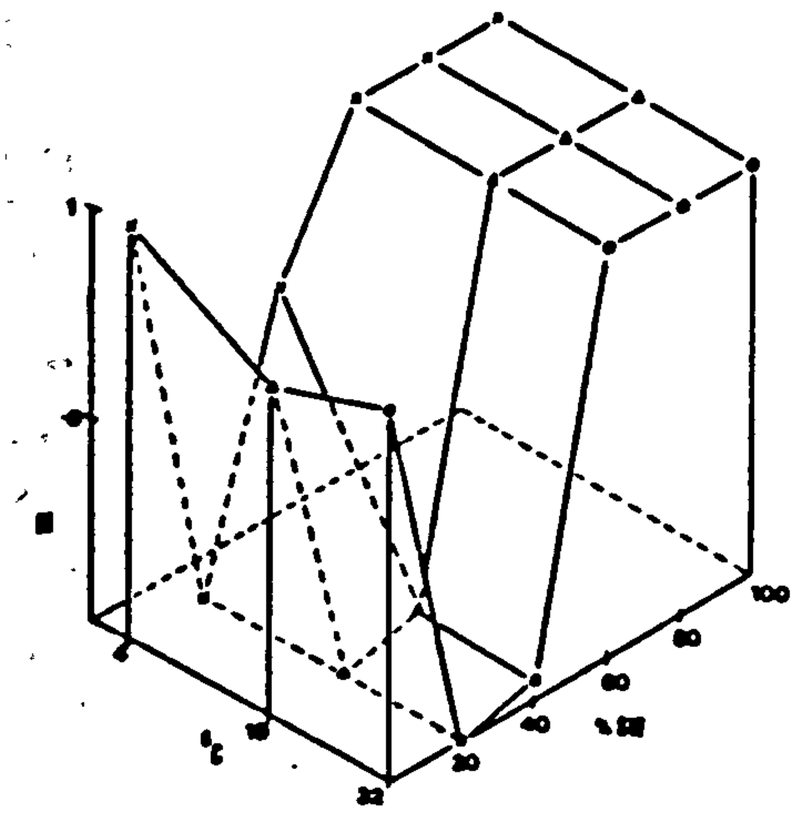
	% Sea-water											
	0		20		40		60		80		100	
$T^{o}C$	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
4°	0	0	0	0	0	0	5	5	8	10	20	14
mean	0		0		0		5		9		17	
19°	0	0	0	0	20	19	20	20	20	19	20	20
mean	0		0		19.5		20		19.5		20	
32°	0	0	0	0	20	17	20	19	20	20	20	18
mean	0		0		18.5		19.5		20		19	

TABLE 48: The numbers of P.jenkinsi B active after 72 hours. N = 10. (1 replicate).

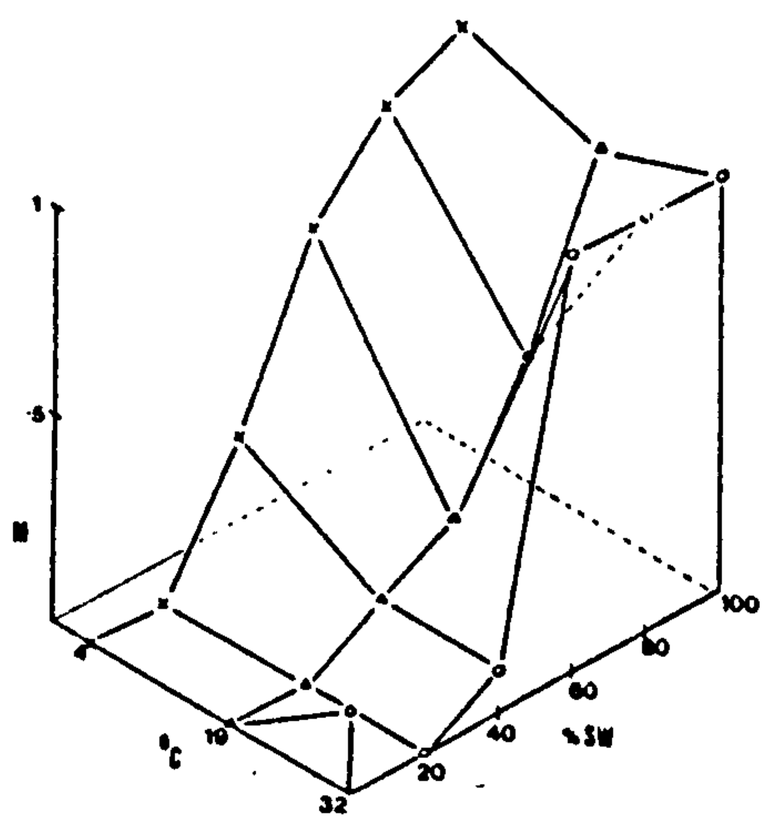
	% sea-water					
	0	20	40	60	80	100
4°	10	10	7	3	1	0
19°	10	10	9	8	5	1
32°	8	10	10	0	0	0

TABLE 49: The numbers of H. neglecta active after 72 hours. N = 20.

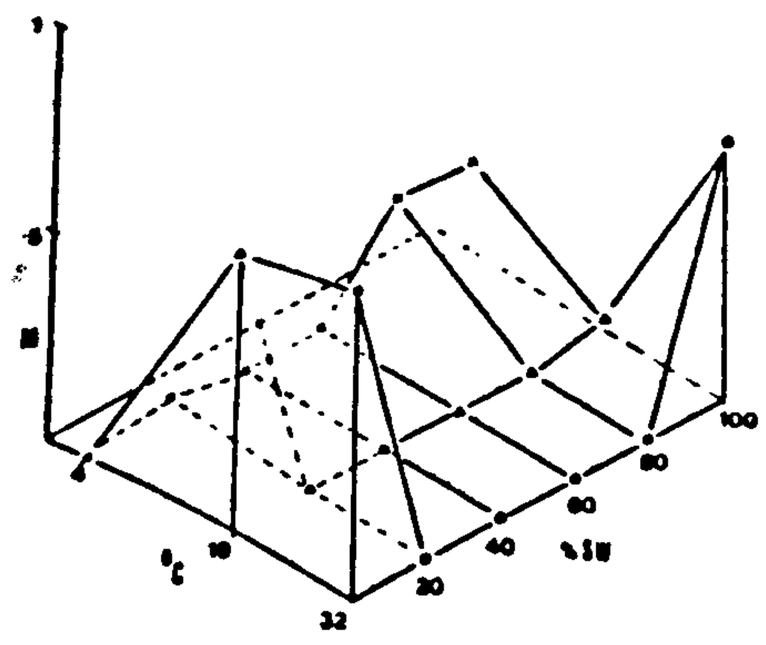
	% Sea-water											
	0		20		40		60		80		100	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
4°	0	0	9	6	16	17	20	18	19	19	14	19
mean	0		7.5		16.5		19		19		16.5	
19°	0	1	13	18	20	19	17	19	20	19	20	19
mean	0.5		15.5		19.5		18		19.5		19.5	
32°	0	0	4	18	12	20	9	19	11	19	13	19
mean	0		11		16		14		15		16	



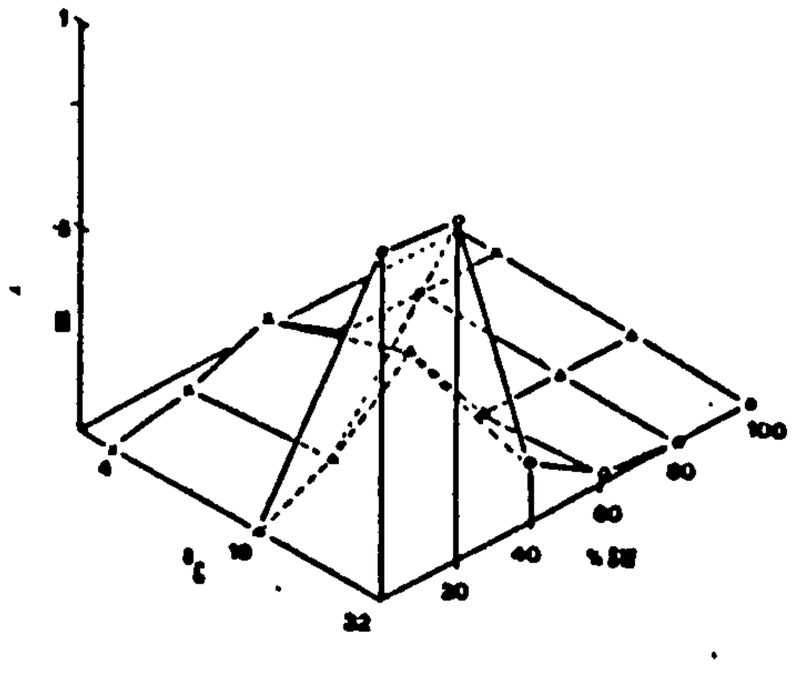
P.jenkinsi A



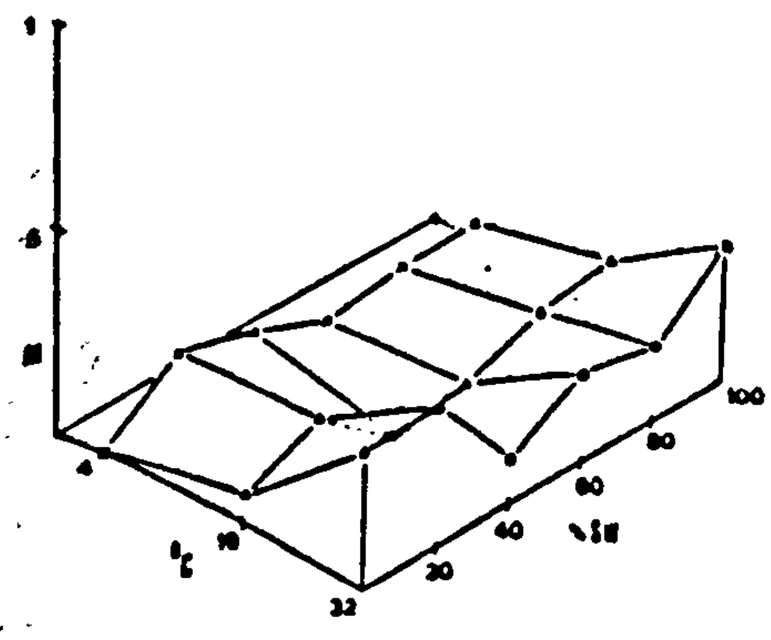
P.jenkinsi B



H.ventrosa



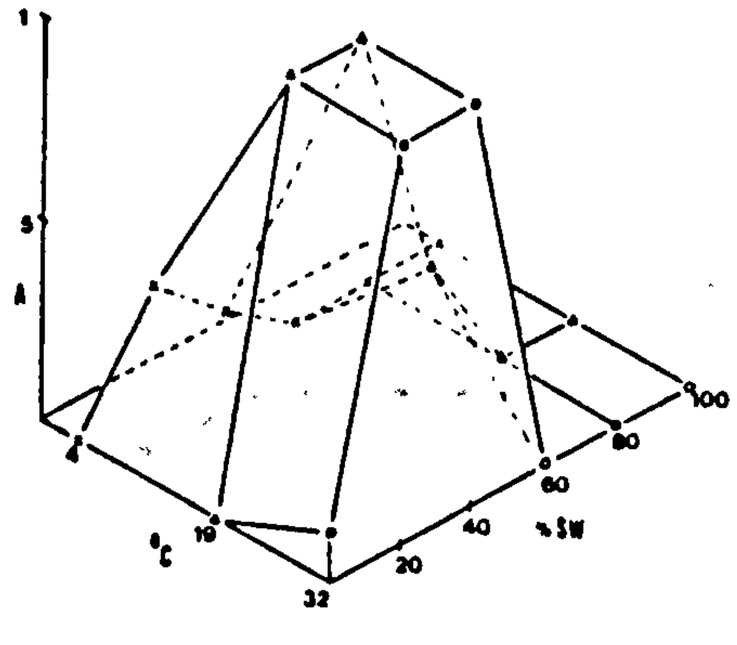
H.ulvae



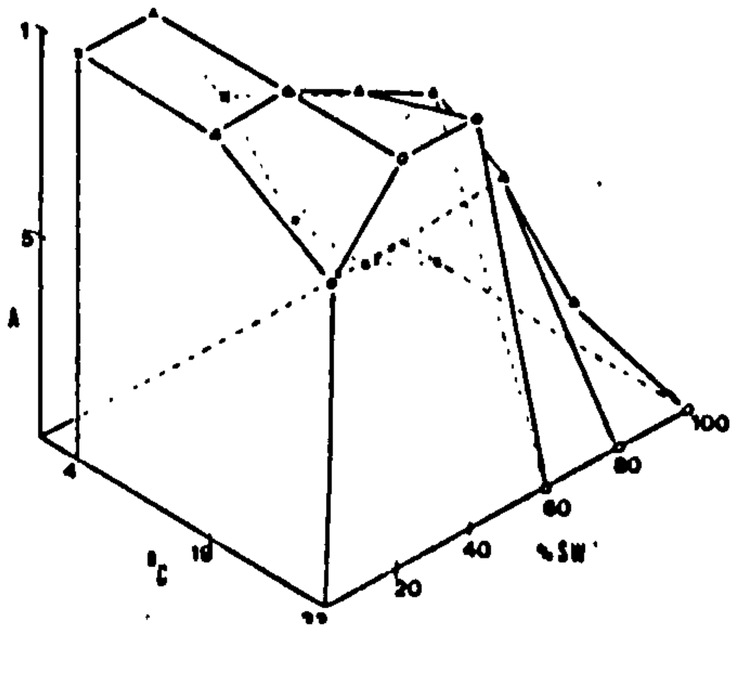
H.neglecta

Fig. 26
Perspective plots of the mortality of five 'species' of Hydrobiid at 18 combinations of temperature and salinity.

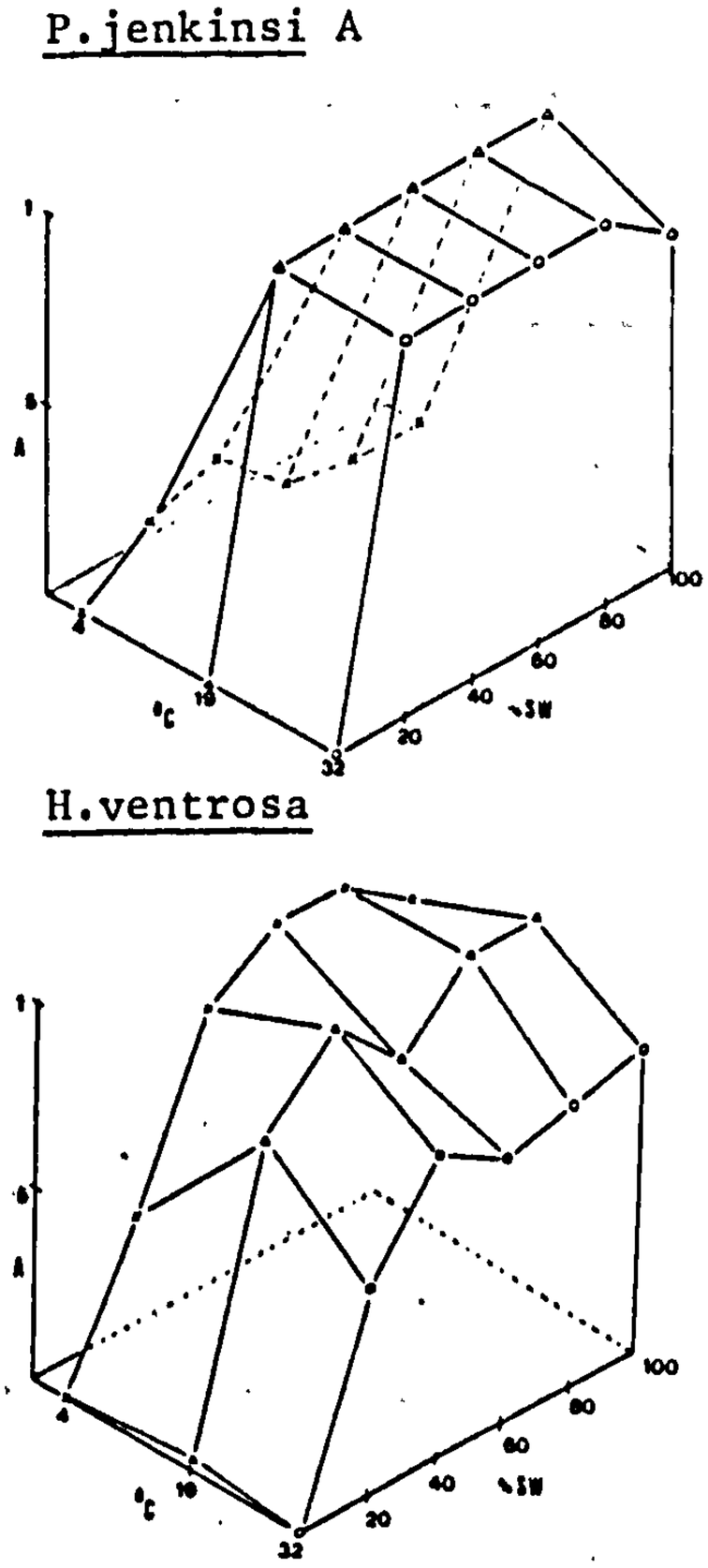
P.jenkinsi A



P.jenkinsi B



H.ventrosa



H.ulvae

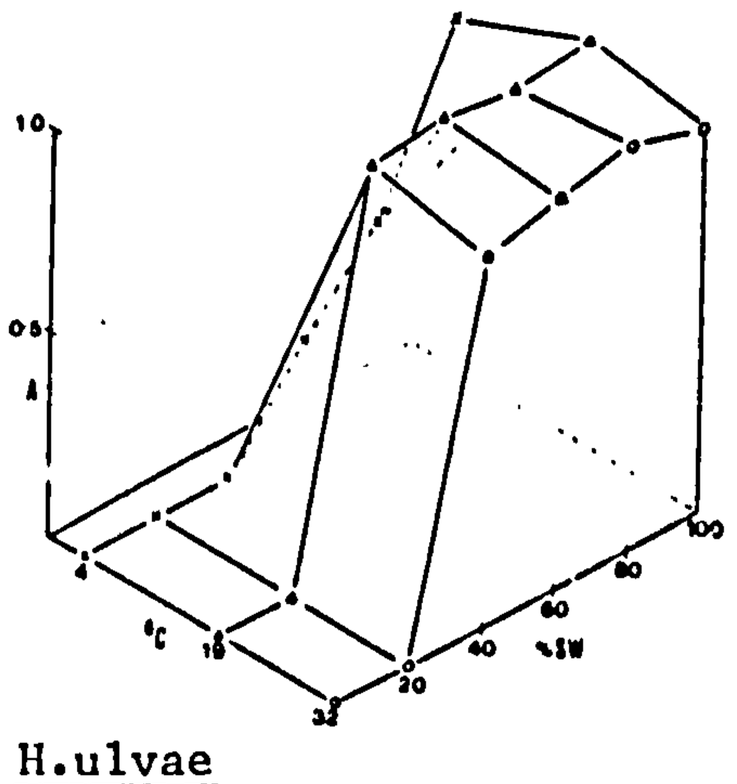
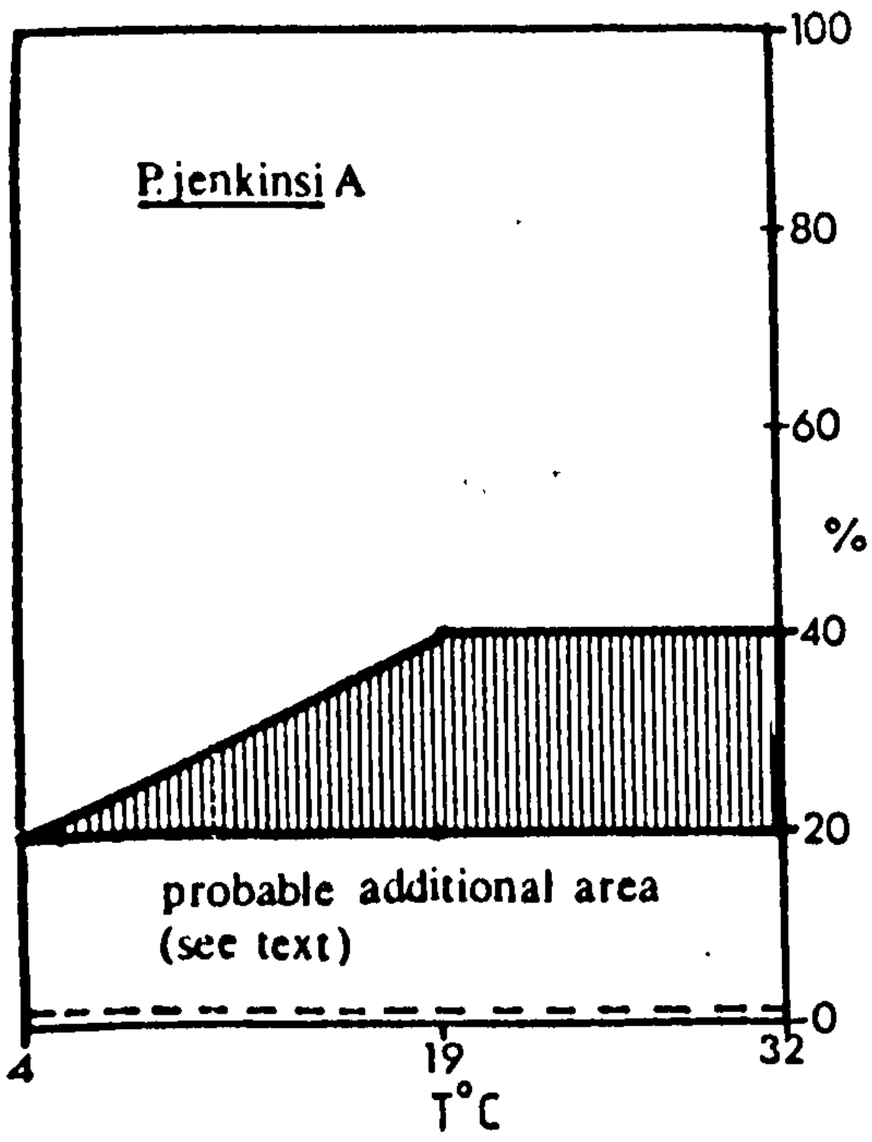
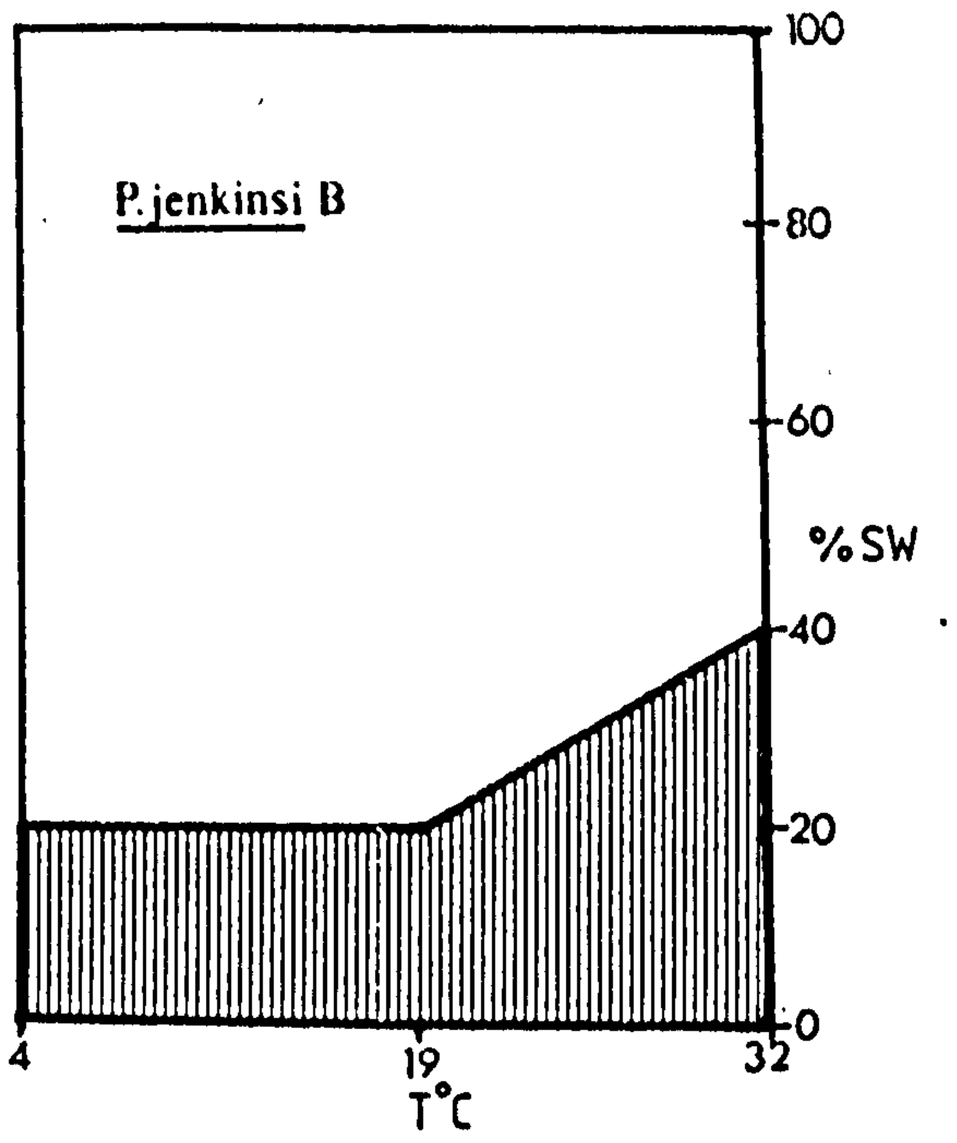


Fig.27
Perspective plots of the activity of five 'species' of Hydrobiid at 18 combinations of temperature and salinity.

H.neglecta





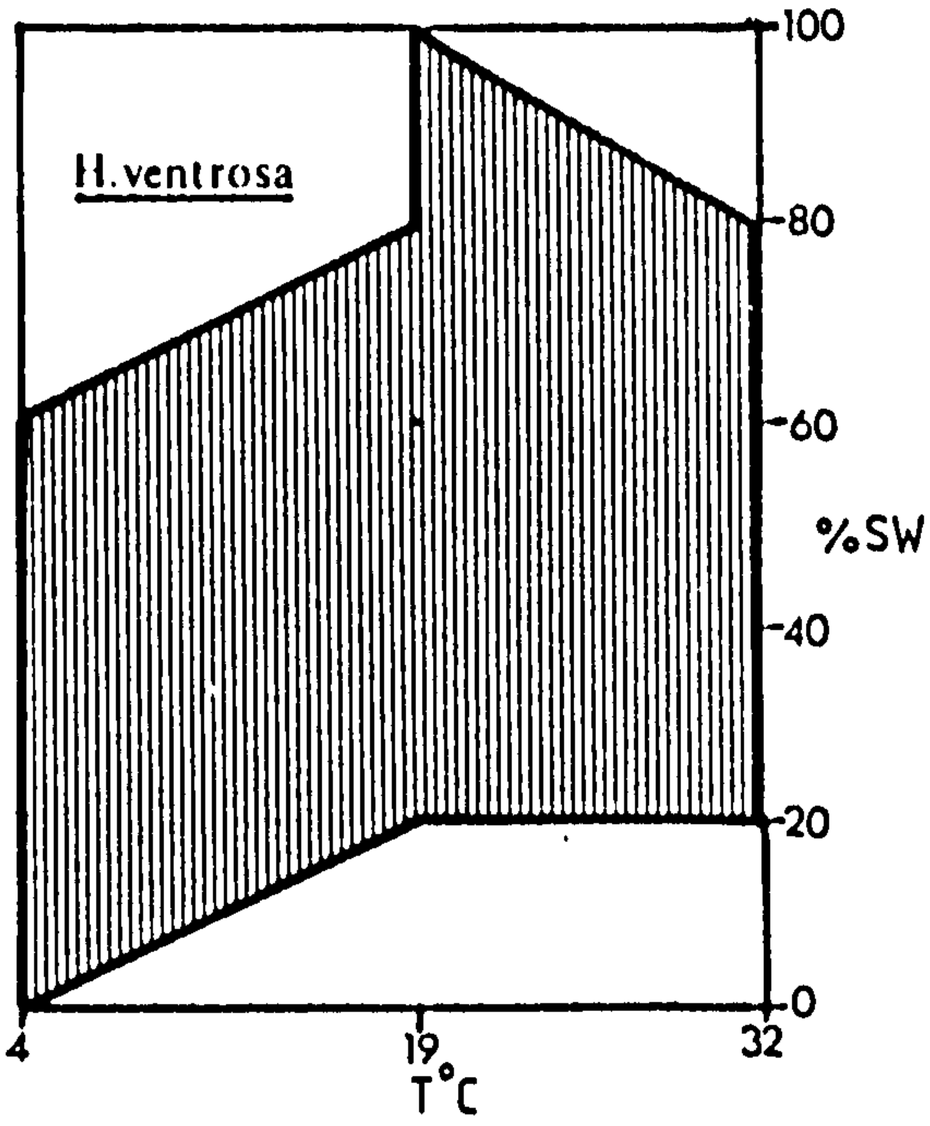
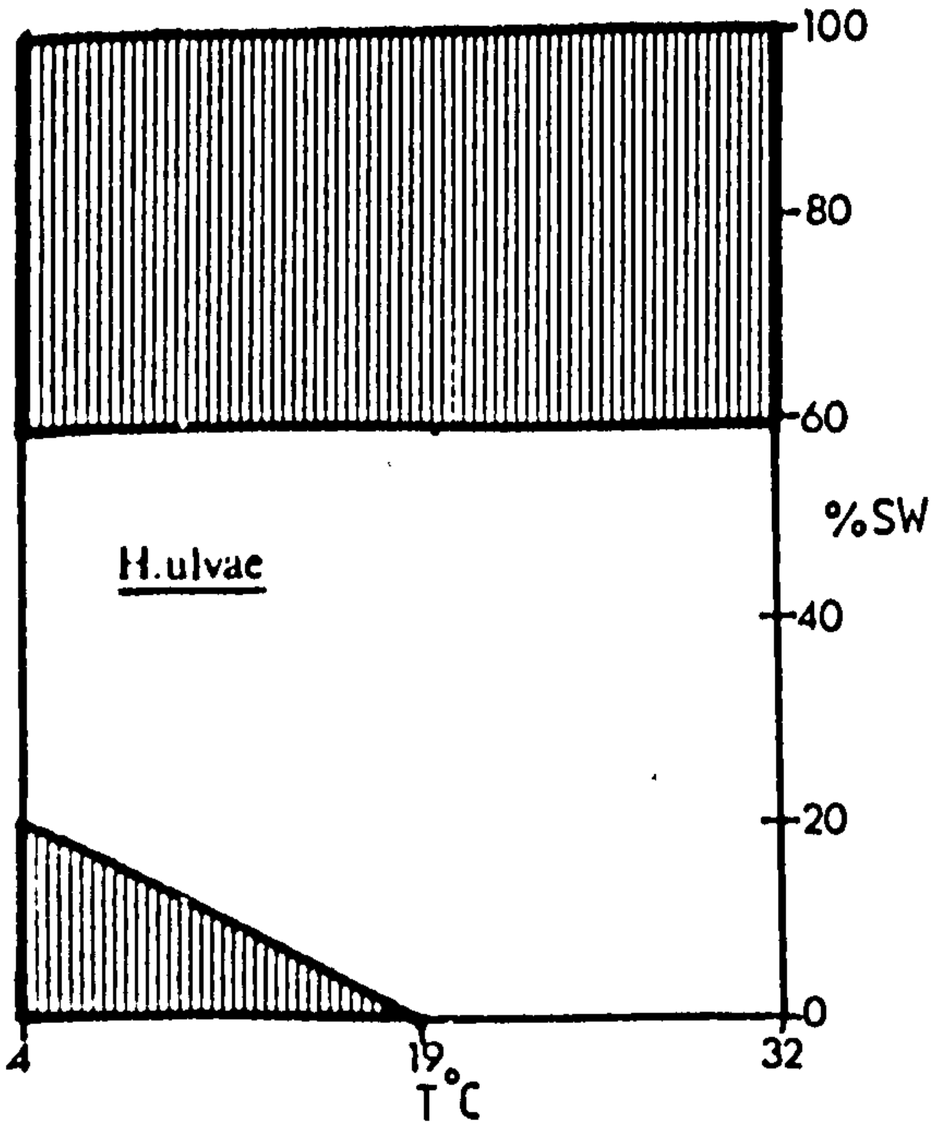
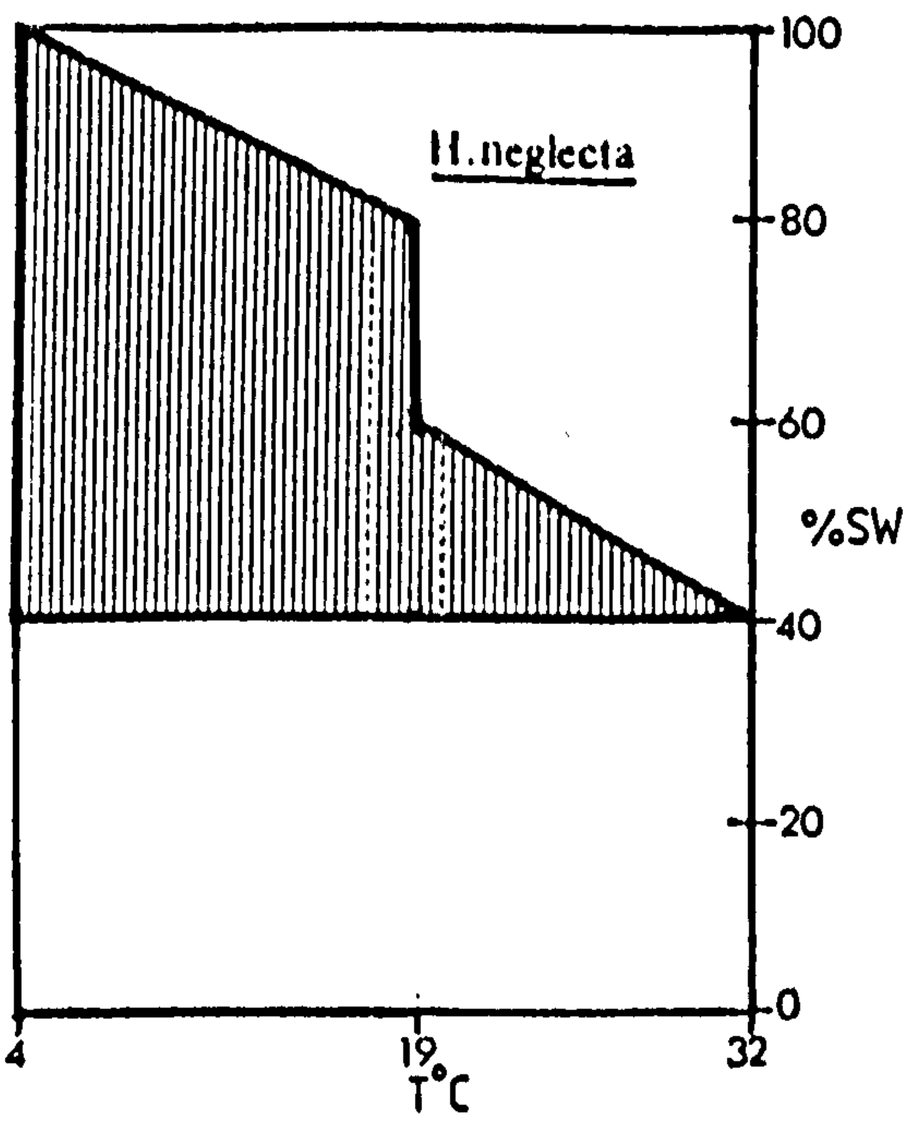


Fig. 28 : Physiological optima obtained from mortality experiments : the zero mortality plane.





experimental stress.

The above method of evaluating the effective death criteria relies on the assumption that the 60 snails sampled randomly from all treatments for each species is an unbiased sample. It is possible that, in some treatments, snails scored as effectively dead were, in fact, dormant and that these individuals were not included in the sample.

VII - 2 Results of main experiments

The raw data obtained for the mortality and activity scores are presented in Tables 40 to 49. The mean proportions of dead or active snails have been used to construct the perspective plots shown in Figs. 26 and 27. Broken lines on the plots represent parts of the surface which are obscured by the foreground (solid lines). The plots for P. jenkinsi B were made from a single set of data as insufficient numbers of snails were available to obtain a replicate set.

All the plots show some part of the surface where mortality is insignificant (zero or less than the 'background' mortality described in section VII - 1 - 2). These areas are shown in Fig. 28 and give some indication of the physiological optima of these species. The actual optima are probably larger than the shaded areas since the mortality between salinity points is unknown. For example, the mortality of P. jenkinsi A between 0% and 20% SW is probably much lower than the plots indicate. If this were not the case then it is difficult to understand how this P. jenkinsi A population persists in its habitat salinity of 171 mg/l Cl^- (approx 0.57% SW). The chloride concentration of the experimental 0% SW was 52 mg/l Cl^- and, therefore, mortality must increase greatly between these values.

Snails in this population were active and feeding in their native habitat and so, using the above argument, the approach to maximum activity from 0% SW to 20% SW should be much steeper than that shown in Fig. 27.

The above example is, however, an extreme case as no other experimental optima are in apparent conflict with snail behaviour in the habitat conditions.

VII - 3 Discussion

VII - 3 - 1 Electrophoretic variation and resource utilisation

Populations of P.jenkinsi A and B have been shown in chapter IV to be virtually momomorphic whereas Hydrobia populations (chapter V) are highly polymorphic.

The reults presented in this chapter (Figs. 26 and 27) show that in terms of the relative exploitation of physical resources available in these experiments, P.jenkinsi populations have narrower physiological ranges than the Hydrobia populations.

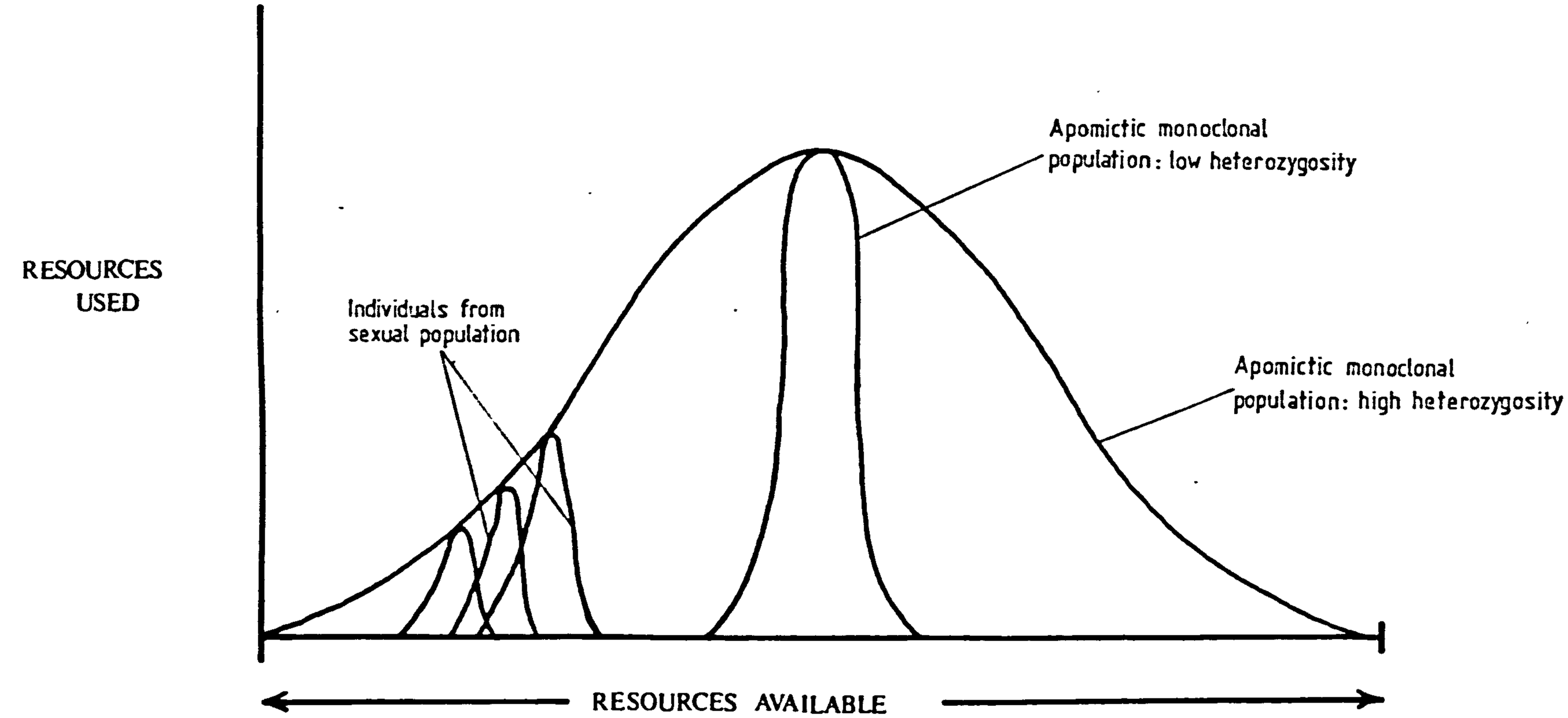
It is tempting to suggest that these results provide support for Van Valen's contention that phenotypes of individuals from monomorphic populations are capable of exploiting a wider range of available resources than individuals from polymorphic populations.

However, since it is impossible to distinguish between the resource spectra of individuals in the polymorphic Hydrobia populations, it may be that each individual has a wider tolerance than P.jenkinsi individuals.

Since Van Valen's contention depends on the relative resource exploitation of individuals within populations and these experiments were not designed to investigate this specific aspect, then the data obtained shed no light on the problem of polymorphism and resource utilisation.

Furthermore, the entire physiological range of temperature and salinity tolerance of some species was not determined fully and so the physiological optima in Fig. 28 can only be regarded as approximations of the finite ranges. This shortcoming should also be recognised in the discussion section VII - 3 - 3.

Fig 29: Possible Relationships of Heterozygosity to Resource Utilisation



VII - 3 - 2 Heterozygosity and resource utilisation

Although P. jenkinsi populations are considerably more heterozygous than Hydrobia populations (see chapters IV and V), increased heterozygosity does not appear to confer an ability to exploit a greater proportion of the resources available. Whilst this may be true where populations are compared, it is nevertheless possible that highly heterozygous individuals (e.g. of P. jenkinsi) in monomorphic populations are capable of greater resource utilisation then individuals of 'normal' heterozygosity such as those found in polymorphic outcrossers. Theoretical resource spectra of individuals differing in heterozygosity are illustrated in Fig. 29. Since the experiments were not designed to investigate individual plasticity, this aspect of the effects of heterozygosity is unresolved.

The relevance of heterozygosity to colonising ability in the Hydrobiidae is discussed in the General Discussion and Conclusions (p.198).

VII - 3 - 3 The determination of species distribution: A discussion

If the experimental physiological ranges determined in this chapter define the species' responses to temperature and salinity in the field (this may not be so due, for example, to behavioural avoidance of extreme conditions), then we may make certain predictions with respect to the distribution of the Hydrobiidae.

Firstly, P. jenkinsi A and B should be confined to fresh or brackish-water habitats whereas the Hydrobia species should be found in brackish to marine habitats. Since the activities of the Hydrobia species are low in low salinity (i.e. they are incapable of feeding) they will be excluded from habitats with persistent low salinity.

Ellis[8] using sites in Sussex, Robson[9] using Essex locations and Fenchel[10] by examining populations in Denmark have estimated the habitat salinity ranges (in % SW) of the three species of Hydrobia and of P. jenkinsi to be:

	Ellis[8]	Robson[9]	Fenchel[10]
P. jenkinsi	up to 1	0 - 45	1.5 - 27
H. ventrosa	*68	18 - 61	9 - 74
H. ulvae	35 - 95	30 - 100	21 - 97
H. neglecta	-	30 - 73	32 - 92

*one population only

None of these authors provides sufficient detail to allow certain identification of the strain or strains of P. jenkinsi to which these ranges apply. All agree that salinity alone is a poor predictor of distribution in the field but that P. jenkinsi generally has a narrower physiological range and is found in less saline water than the Hydrobia species. Fenchel found P. jenkinsi to be the dominant species in habitats with a salinity of less than 3% SW.

In the above studies, only one resource dimension, salinity, was measured in the field; usually by taking a few spot samples from each habitat. This is clearly inadequate for any meaningful comparisons of physical resource partitioning to be made for these species since aquatic habitats are in physical terms, neither unidimensional nor stable.

The experimental results presented in this chapter are largely in agreement with the field observations described above.

Secondly, P. jenkinsi A should be excluded from habitats which have a salinity of more than 30% SW (20% in low temperature) whereas P. jenkinsi B can, at median temperatures, tolerate brackish conditions at least for short periods.

Ecological separation of the strains of P. jenkinsi was suggested by Warwick[11] who described strains B and C as coastal in their distribution and strain A as the typical fresh-water form. In the present work, no population of P. jenkinsi A was found in water of greater than 10% SW and no P. jenkinsi B populations were found in water of less than 20% SW. However, the experimental evidence presented here suggests that strain B is not physiologically excluded from fresh-water. Factors possibly contributing to this apparent exclusion of P. jenkinsi B from fresh-water are discussed below.

Thirdly, on physiological grounds, we might expect to

find mixed populations of Hydrobia species in mesohalinous habitats where median temperatures prevail since there is considerable overlap between the experimental ranges in this region. H. ventrosa is the least tolerant with moderate mortality occurring at extremes of temperature and salinity. H. ulvae and H. neglecta have the widest ranges; H. ulvae is less tolerant of low salinities and high temperatures than H. neglecta. Hylleberg,[12] using Danish populations, has recently investigated the faecal egestion rates (roughly equivalent to the activity scores in the present work) of the Hydrobia species in 35 different combinations of temperature (5° - 35°) and salinity (30% - 100% SW). The surfaces he obtained for H. ulvae and H. ventrosa are similar to those reported in this study, whereas he found that the H. neglecta surface was similar to that of H. ulvae in Fig. 27 i.e. with optimum activity at high temperature and high salinity.

In support of this prediction that the three Hydrobia species should co-exist in sheltered mesohalinous areas, Fenchel[10] and Hylleberg[12] report that in Danish fjords where these physical conditions prevail, mixed-species populations of H. ulvae, H. ventrosa and H. neglecta are commonly found. Of 50 sites examined by Hylleberg, 43 were occupied by mixed-species populations.

In contrast to the Danish fjords, mixed-species populations of Hydrobia were rarely found in the collections made for the present work; occasionally mixed-species populations were encountered in man-made dykes (see poplns. 57 and 58 in Appendix I).

This raises the question of whether the physiological tolerances of these species have a role in determining the species composition of a particular habitat and, if this is so, can we account for the differences in species compositions of British coasts and Danish fjords?

Perhaps the most obvious difference in the physical nature of these habitats lies in their stability. Danish fjords have little tidal range, are largely mesohalinous and are relatively sheltered. The eastern British coast, however, is subject to violent fluctuations in these physical conditions. For example, summer conditions at the Little Humber Farm site (where the sample of H. ventrosa was collected) were up to 36°C and 85% SW. In winter the ditch was almost fresh-water (less than 5% SW) for periods of several

weeks and was frequently frozen over. Similarly, salinity at Stone Creek (where P. jenkinsi B was collected) was measured on several occasions and varied from 85% SW at high tide to under 10% SW at low tide.

If these fluctuations are sufficiently large, then some species may be excluded or extirpated from particular habitats, leading to allopatric (single-species) populations. Evidence for extinction was fortuitously provided during the study at Stone Creek where the population of P. jenkinsi B went extinct during a period of drought (summer 1976), and at Snettisham where the P. jenkinsi B population was totally extirpated by an influx of sea-water during a severe storm in January 1978. The population was replaced overnight by H. ulvae carried into the lake by the sea-water.

Hylleberg[12] argues, from experimental evidence and field observations, that environmental heterogeneity and unpredictability allows co-existence of congeneric species by conferring advantages fluctuating in time and space to each. He concludes that when the environment is stable and predictable only one species is 'really successful' (due to competitive exclusion) and allopatry results. However, the experimental evidence presented by Hylleberg and in the present work, together with the field observations both in Denmark and in Britain tend to refute this argument. Moreover, Fenchel[13] has clearly and elegantly demonstrated (by measuring the sizes of ingested substrate particles) that where species of Hydrobia co-exist, competition for food results in character displacement rather than exclusion. Competitive exclusion has been demonstrated in the laboratory in the Hydrobiidae[14] and in several other experimental systems using invertebrates[15,16,17,18] but in these systems there may be insufficient time (number of generations) or insufficient genetic variation in experimental populations to allow the evolution of character displacement.[19,20]

Where competing populations are monomorphic however, little, if any, character displacement can occur; in this case competition may well result in exclusion of a species. For example, P. jenkinsi B, although physiologically able to exploit both brackish and fresh-water habitats was only found in brackish coastal waters (see above). It is possible that this strain of P. jenkinsi is competitively excluded from fresh-water by P. jenkinsi A.

In summary, if we assume, in contrast to Hylleberg,[12] that competitive exclusion of Hydrobia species rarely occurs in the field then, due to their overlapping ranges, mixed-species populations may co-exist in mesohalinous, sheltered (in terms of temperature extremes) habitats; competition results in a divergence of resource partitioning between species rather than competitive exclusion of species. Conversely, in habitats where these physical conditions fluctuate between extremes, exclusion or extinction of species may occur, leading to allopatry.

The availability and stability of physical conditions such as temperature and salinity may therefore largely determine the Hydrobiid species composition of a particular habitat.

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DISCUSSION AND CONCLUSIONS

A short discussion has been provided at the end of each of the preceding four chapters (IV, V, VI, VII). In this concluding section I shall discuss the results of the present work in the context of current population genetics and ecological theory in order to develop a model to explain the widely different patterns of polymorphism found in Hydrobiid populations. I shall also discuss the relationships of this model to the adaptive strategies of such populations with particular reference to their colonising ability and evolutionary persistence.

Since much of the following discussion is concerned with levels of electrophoretic variation it is useful to point out initially that, for wholly or partly asexual populations, variation cannot be expressed by using an estimate of heterozygosity, the usual practice when considering variation in sexual populations. Heterozygosity is an estimate of variation only in populations where fully random segregation occurs, allowing the expression of heterozygous genomes in succeeding generations. In wholly or partly apomictic populations heterozygosity is not directly related to the amount of variation within a population. This is clearly demonstrated in the present work where the most heterozygous populations of Potamopyrgus are also the least variable in terms of the number of different electrophoretic phenotypes within a population (see Chapter IV). In contrast, populations of Hydrobia with lower heterozygosities than Potamopyrgus ssp. contain many more phenotypes and are thus more variable. Hence in the discussion below 'level of variation' is used to describe the array of phenotypes detected in a population. Relationships between heterozygosity and other variables such as reproductive strategy are discussed as a separate entity later in this chapter.

Let us first consider the evidence presented in the foregoing chapters in the light of the neutralist/selectionist controversy referred to in the Introduction (pp. 8-10).

The following evidence extracted from the preceding chapters is of critical importance:

1. Levels of electrophoretic variation are clearly related to reproductive strategy.
   In all six Potamopyrgus species examined, except the sexually reproducing P. estuarinus, a large proportion of females in the

population is correlated with high heterozygosity per individual and a small array of phenotypes per population.

2. The levels and patterns of variation in the obligate sexually reproducing populations of Hydrobia ssp. and P. estuarinus are typical of those found in the majority of sexually reproducing invertebrates (see Chapters IV and V).

3. Variation within and between populations of P.jenkinsi appears to be virtually absent despite the presence of very large populations of this species in Britain for at least one hundred years (see Chapter IV).
Due to the inherent limitations of electrophoretic techniques and sampling error, this estimate of variation in P.jenkinsi may be erroneous in a finite sense but is certainly valid relative to levels of variation estimated in the Hydrobia and Australasian Potamopyrgus populations used in this study.

4. Levels of variation within Hydrobiid populations may be adaptive, at least in terms of the physical variables used in the laboratory experiments described in Chapter VII.

Can we explain these differences in patterns of polymorphism between asexual and sexual Hydrobiid populations by means of the 'neutral' theory of Crow and Kimura?[1]

According to neutral theory the age of a population should be related to its variability since one might expect older populations to have accumulated more neutral electromorphs than say a recently colonising population or one that has been through a recent numerical 'bottle-neck'.

In addition to age-related variation within a population, neutral theory also predicts that the size of a population should influence its variability. Using an extension of the neutral theory, the mutation-equilibrium hypothesis, Nei et. al.[2] have predicted that small sexual populations have lower equilibrium heterozygosities than large populations but achieve genetic equilibrium faster. They have also predicted that the rate of increase of heterozygosity in asexual populations should be proportional to population size.

Soulé in a review[3] of largely electrophoretic data from invertebrate and vertebrate populations has demonstrated that, although direct relationships between the sizes or ages of populations and electrophoretic variability certainly exist, such relationships depart substantially from those predicted by neutralist theory. It appears that the rates of incorporation of mutations into sexual populations are much too low to lend support to neutral theory.

In some cases, notably in small vertebrate populations that have passed through a population size 'bottle-neck' (e.g. the elephant seal[4]) or have experienced long-term inbreeding (e.g. the Canadian elk[5]), a lack of electrophoretic variation has been found but, in contrast, small populations of Drosophila willistoni in Hawaii have similar levels of variation to the large populations of D. willistoni on the North American mainland which are thought to be of similar age[6].

It is difficult to test for age/variation relationships due to our scant knowledge of the age of wild populations. Somero and Soulé's work[7] on marine fish and Ayala's study[8] of electrophoretic variation in Hawaiin Drosophila populations of apparently different ages fail to demonstrate a relationship between variation and population age. Moreover, populations of the King Crab, Limulus, a species having an ancient evolutionary lineage[9], are as polymorphic as some recently evolved species of Drosophila[8].

The overall impression gained from reviews of electrophoretic variation in vertebrate and invertebrate populations is that such variation does not appear to be correlated with the age of a population in the manner predicted by neutral theory.

A further prediction of neutral theory is that stochastic divergence should occur between genetically isolated populations causing a loss of genetic identity increasing with time. The problem with testing this prediction is that geographically isolated populations may not be genetically isolated; gene flow between them may occur sporadically thus maintaining their genetic identity. Ayala et. al[10] have shown that small geographically isolated populations of Drosophila appear to maintain their genetic identity (suggesting strong stabilising selection); Slatkin[11] has produced evidence suggesting that in this particular situation, gene flow is unlikely to be sufficient to account for the observed lack of stochastic divergence.

In the present work neutral theory predicts that isolated populations of P.jenkinsi A which differ in size and age (in terms of the time since their introduction to different areas) should show different levels of variation and that stochastic clonal divergence should have occurred in the British population. A crude estimate of the extent of this divergence might be made by assuming the following parameters:

i) P.jenkinsi A populations are often made up of greater than 10^6 individuals. This is a low estimate of the numbers observed in the field.

ii) The generation time in the field is twelve months. Three generations per annum were obtained in laboratory cultures used in the present work.

iii) P.jenkinsi A has been widespread for at least one hundred years (see Hubendick[12] for confirmation of this estimate).

iv) The mutation rate at electrophoretic loci is approximately $= 10^{-5}$.

v) Roughly 10% of mutant alleles are detectable by electrophoresis. Assumptions (iv) and (v) are supported by Mukae and Cockerham's[13] experimental estimation of the spontaneous mutation rate to electrophoretically detectable alleles in Drosophila of $= 1.8 \times 10^{-6}$.

vi) Gene flow between populations is negligible: for apomictic aquatic molluscs this is probably a reasonable assumption since outcrossing can be discounted and large-scale migration is unlikely.

Using these approximations and ignoring back-mutations, the number of electrophoretically detectable alleles per locus per population (N_m) is given by:

$N_m = D\ Npt\mu$ where D = proportion of mutant alleles detectable by electrophores.
Np = No. individuals.
t = No. generations
μ = mutation rate.

Hence:

$N_m = 0.1 \times 10^6 \times 10^2 \times 10^{-5}$

$= 10^2$

This is only a crude estimate of the number of mutations per locus that have arisen in the population since its introduction. As Crow and Kimura[1] have pointed out, the vast majority of these will have been eliminated within a few generations by stochastic events such as drift and migration. It is surprising therefore that several mutations were detected during the P.jenkinsi A survey. For this reason the results of the survey do not appear to contradict neutralist theory.

The patterns of polymorphism shown by the Australasian species P.antipodarum and P.nigra provide some evidence against neutralism of electrophoretic alleles since variation appears to be distributed 'patchily' among the populations studied: one clone tends to dominate each particular geographic area (see chapter IV). Again, levels of variation within populations of these species are similar to many other apomictic species,i.e. lower than those predicted by neutral theory (see below).

As a result of the failure of neutral theory to account satisfactorily for patterns of electrophoretic variability in the majority of wild populations, recent attempts have been made to correlate levels of variation with physical and trophic resource stabilities. This selectionist approach differs from that of the neutralists in that levels of variation are regarded as adaptive phenomena and not as the result of stochastic drift.

Using data from literature surveys, Levins[14] concludes that populations in unstable environments have higher genetic variability than those living in stable environments. In contrast, Valentine[15] suggests that for some populations the reverse may be true; such populations may pursue a 'fine-grained' strategy (see Introduction) in an unstable environment and may have lower genetic variability than those pursuing a 'coarse-grained' strategy. The crux of these models is the 'perception' of the environment by the organism, a factor apparently related to its size and mobility. Selander and Kaufman[16] have clearly demonstrated that small relatively immobile species (which perceive their environment as coarse-grained) are more variable electrophoretically than large and mobile fine-grained strategists. Powell[17] has lent experimental support to such 'resource stability' models by demonstrating that Drosophila laboratory cultures kept in unstable environments retain more electrophoretic variability than similar cultures maintained in stable conditions.

A major problem with such adaptive theories concerning genetic variation is that when different phylads are compared metabolic regulation probably differs between them both qualitatively and quantitatively. Since electrophoresis detects only structural gene products and tells us little of their relative contribution to the overall phenotype upon which selection acts, it is difficult to establish confidently a causal relationship between levels of electrophoretic variation and environmental parameters.

It is probably reasonable to assume that metabolic regulation of structural gene products is more uniform within a taxonomic family than between different phyla. The evidence presented in Chapter VII suggesting that levels of variation in the Hydrobiidae are adaptive may therefore be more robust than some previous studies[16, 17] using data from different phyla.

In summary then, the above evidence (except the apparent genetic uniformity of isolated P.jenkinsi A populations previously referred to) provides some grounds for invoking selection to explain patterns of polymorphism in Hydrobiid populations.

Stabilising selection for genetic uniformity has been suggested for two invertebrate examples of exceptionally low electrophoretic variation. Suomalainen et al[18] report that a single genotype dominates Scandinavian populations of the apomictic chrysomelid beetle Adoxus obscurus; Selander and Kaufman[19] report a similar pattern of polymorphism in populations of the self-fertilising land snail Rumina decollata which has recently colonised North America. Since these species appear to be the only reported instances of genetic uniformity among geographically widespread populations it seems that this extreme pattern of polymorphism is unusual in both apomictic and sexual invertebrates.

In the majority of apomictic species that have been examined electrophoretically (for reviews see Suomalainen et al.[18] on animals and Berry[20] on plants), populations consist of several different clones often with one clone dominant in a particular geographical area. This pattern of polymorphism is found particularly in apomictic species which have long been established in an area (e.g. weevils,[18] dandelions,[21] and Lady's Mantles[22]); lower clonal diversity is found in recently colonised areas.

This suggests that genetic variation does, as neutral theory predicts, accumulate with time. However, it is the rate of accumulation of variation which is at issue here since, as Soule[3] points out, we would expect a very much higher rate of accumulation than that observed in these populations if electrophoretic alleles were largely selectively neutral.

Such patterns of polymorphism most probably result from selective 'fine-tuning' adaptation to ecologically and climatically different environmental patches. In some cases (e.g. Alchemilla vulgaris agg.[22]) such ecotypic adaptation may result in clones which are good morphospecies, giving rise to the possibility that clonal divergence is an important factor in sympatric speciation.

This is of particular significance in those species which are facultative parthenogens since long periods of apomixis may result in clones so divergent that potential interclonal fertility is lost. Although many species are recognised as being apomictic upon close examination most exhibit a small but significant degree of sexual reproduction (e.g. weevils,[18] the Australasian Potamopyrgus ssp.[23] and the wall cress Arabidopsis thaliana[24]) thus maintaining a degree of genetic flexibility greater than that produced solely by mutation rate.

No direct evidence of sexual reproduction in P.jenkinsi was obtained during the present work: no males were found and no evidence of segregation was obtained for heterozygous loci. This suggests that if sexual reproduction occurs in this species it is restricted to a very low level, perhaps being confined to particular areas. Wallace[25] has recently investigated the occurrence of males in various British and Dutch populations of P.jenkinsi A and has found a low frequency (less than 11%) in several areas, mainly confined to North Wales. This lends further support to the suggestion that P.jenkinsi is a facultative parthenogen (Chapter IV).

If a low frequency of sexual reproduction occurs in P.jenkinsi A populations, the variation released by such matings should intensify clonal divergence, particularly if selective neutrality of electromorphs is assumed. No evidence of this was obtained during the present study so that either intense stabilising selection is operating on the progeny of sexual matings or sexual reproduction does not occur in the populations examined, none of which coincided with those sampled by Wallace.

Nothing is known of sex-determining mechanisms in Potamopyrgus; the presence of males may not necessarily mean that sexual reproduction occurs. Electrophoresis of the North Wales populations examined by Wallace and of the progeny of laboratory crosses would establish whether segregation at heterozygous loci is commonplace in this area.

Turning now to the distribution of heterozygosity in populations of Potamopyrgus and Hydrobia, the level of heterozygosity in a population appears to be related to reproductive strategy. Specifically, heterozygosity is highest in apomictic populations (see Fig. 23).

The simplest explanation of these results is to assume that highly heterozygous individuals are more fit than those with lower heterozygosity. If highly heterozygous individuals exhibit generalised heterosis (i.e. a phenotype capable of relatively high fitness in a wide range of environmental conditions) we can go some way towards a model to explain the patterns of polymorphism found in the Hydrobiidae.

Experimental and observational support for heterosis (overdominance) as a mechanism for the maintenance of genetic variation is widespread and deeply entrenched in population genetics and hybridisation techniques involving heterosis are widely used in plant breeding. Overdominance of visible traits has been extensively reported by, for example Haldane,[26] Ford,[27] Sheppard,[28] and many other authors. The heterotic maintenance of electrophoretic variation has been reported by e.g. Koehn,[29] Marshall and Allard,[30] Koehn and Mitton,[31] Beardmore and Shami,[32] and Beardmore and Ward[33].

Heterotic models of the maintenance of genetic variability in sexual populations contain the intrinsic problem of segregational load since, at many loci, homozygous segregants will have lower fitnesses than heterozygotes. Many authors[34] have argued that a substantial proportion of polymorphisms cannot be maintained by heterosis for this reason; the genetic load would be unbearable. Such load resulting from segregation has been shown theoretically by Kimura and Ohta[35] to be overcome by increased fecundity only if selective co-efficients are low. Tracey and Ayala,[36] in a study of the fitnesses of Drosophila homozygous for a complete chromosome, have demonstrated that fitness differentials between these populations and normal wild flies are more than adequate to overcome the effects of segregational load. Previous studies of Drosophila by

Sved,[37] Sved and Ayala,[38] and Mourão et. al.[39] in which the fitness of flies homozygous for 'quasi-normal' chromosomes was compared with that of wild heterozygotes, also indicate that heterosis is not overcome by load.

Although for many loci in Drosophila (up to 1258 according to Tracey and Ayala[36]) load does not seem to preclude the possibility of heterotic maintenance of variation, there may well be many other loci where this is not so. Nei[40] and Lewontin[41] point out that until selection co-efficients are better known, heterosis is still a scientifically valid model of genetic variation in sexual populations.

Recently, Levins[42], Dobzhansky[43], and Lewontin[41], have advanced an alternative model which avoids the problem of load. The model is based on ecological niche selection in which alternative homozygotes achieve optimum fitness in different environmental patches although gene flow is maintained between patches.

Direct observational and experimental support for this niche selection model has been provided by Christensen[44] who demonstrated that different amylase homozygotes of an Asellus population preferred different detritus food types and this determined their distribution within a pond. Furthermore, he has shown that for another parameter, temperature, heterozygotes have lower fitnesses than homozygotes although the biochemical mechanisms responsible for this fitness differential are unknown.

In the Hydrobiidae, Fenchel[45] has demonstrated a special case of the niche selection model in which closely related species of Hydrobia undergo character displacement of feeding mechanisms where they occur sympatrically; the environmental patches in this instance are found in the food dimension (for more details see section VII - 3 - 3). Other similar cases of character displacement have been reported by Grant[46] and Pianka.[47]

If segregational load is a major obstacle to heterosis, fitness of heterozygotes in facultative parthenogens may be substantially increased by heterotic individuals reproducing apomictically; segregation is thus avoided. Since these individuals are potentially twice as fit as sexual individuals in the population (see Introduction p. 4) we might expect these mixed reproductive strategy populations to become dominated by these highly fit apomictic clones; this is indeed the case in Australasian Potamopyrgus populations (with the exception of the obligate sexually

reproducing P.estuarinus). Dominant clones differ between populations of Potamopyrgus; this may reflect selection for the clone having the highest fitness in each habitat patch.

Wide environmental fluctuations within an environmental patch (e.g. a river estuary) may not allow the evolution of apomixis particularly if such fluctuations are stochastic and sporadically outside the physiological tolerances of even the most heterozygous individuals. Sexual reproduction in the Hydrobia ssp. and P.estuarinus populations may therefore function in the 'immediate' sense of Williams[48], continually to break up previously successful genotypes and so avoid extinction by maintaining a wide range of variation in progeny.

A combination of facultative parthenogenesis and heterotic clonal dominance is put forward as a model to account for patterns of polymorphism in Potamopyrgus populations since it also provides a basis for the colonising strategy characteristic of the genus.

P.jenkinsi is a successful coloniser of a wide range of aquatic habitats in Britain and Europe[49]; the species is most commonly found in man-made ecologically immature habitats such as ditches and canalised streams although it is sometimes encountered living at low population densities in mature freshwater ecosystems. Winterbourn[50] reports that the Australasian Potamopyrgus ssp. are typically colonisers of ecologically immature aquatic habitats.

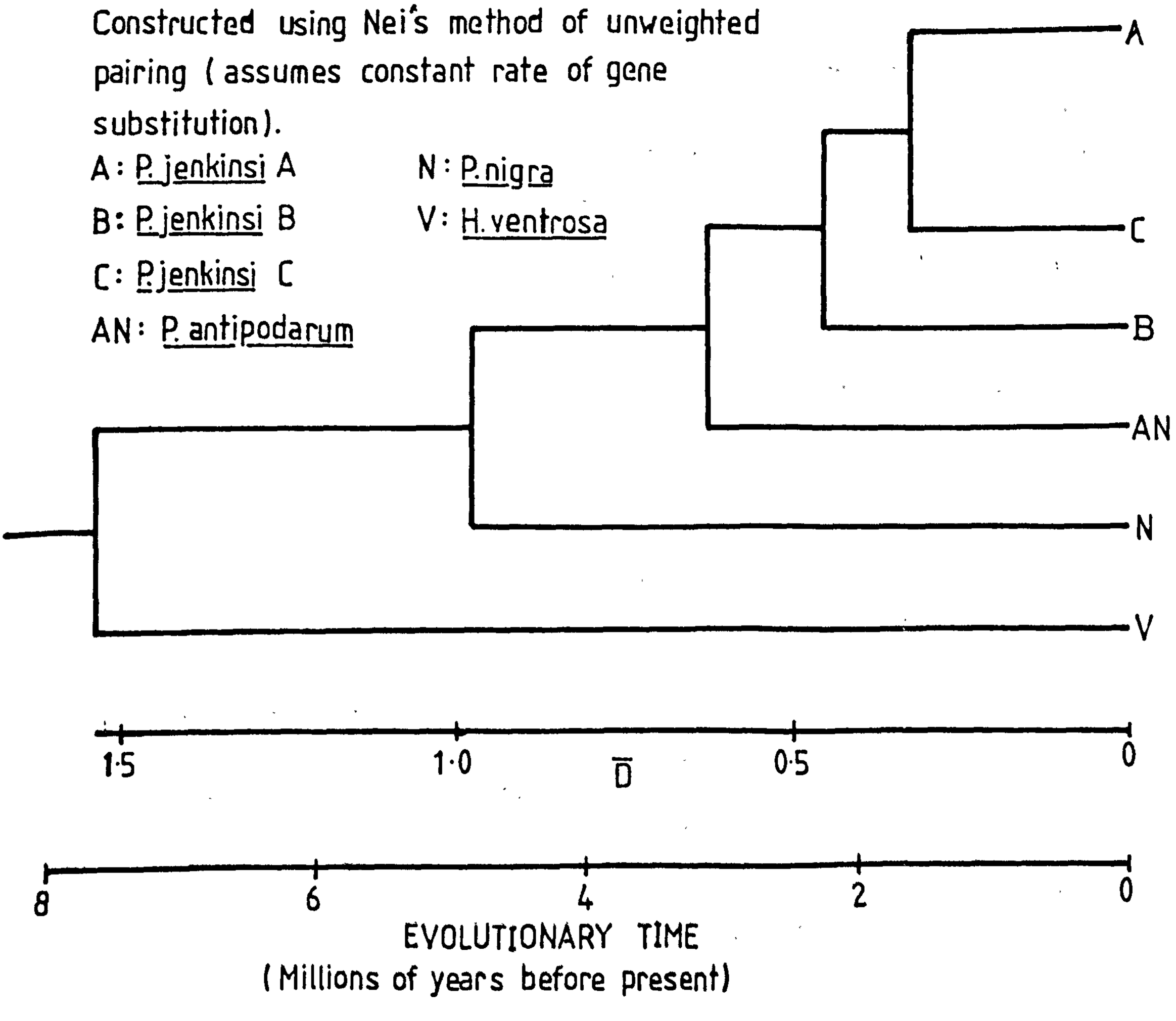
Populations of Potamopyrgus containing highly heterozygous (and heterotic) facultative parthenogens are inherently good colonisers since propagules may start from a single individual in any stage of development and may expand rapidly due to the high intrinsic rate of increase characteristic of parthenogenesis. Furthermore, if high heterozygosity confers wide physiological plasticity then the probability of extinction of a propagule may be low.

The virtual confinement of Potamopyrgus populations to ecologically immature ecosystems is interesting since it may be explained in terms of the low variability and reproductive strategy of such populations. In mature ecosystems it is possible that competition from other fauna may exclude apomictic Potamopyrgus propagules since the avoidance of such competition by the evolution of character displacement is not possible

Fig. 30 Phylogenetic tree of six 'species' of Hydrobiid.

Constructed using Nei's method of unweighted pairing (assumes constant rate of gene substitution).

A: P. jenkinsi A
B: P. jenkinsi B
C: P. jenkinsi C
AN: P. antipodarum
N: P. nigra
V: H. ventrosa



(see section VII - 3 - 3).

Similarly a new clone of P.jenkinsi arising sympatrically might not become established if it is competing with the parental clone. Since neither clone can evolve character displacement, that with lower fitness (in the sense of competitive ability) might go extinct. Such a model explains the scarcity of mixed clone populations observed in the present collections of P.jenkinsi A.

Whether such stabilising selection has reduced the clonal diversity of P.jenkinsi since its introduction must remain rather speculative since we have no direct information on the number of initial propagules in Britain. Possible alternatives are:

i) A large number of genetically different clones were introduced and only the fittest have persisted.

ii) Fewer clones were introduced and most have survived.

iii) A single clone was introduced and has diverged since the mid-nineteenth century.

Alternative (iii) appears to be unlikely if we examine the probable phylogeny of P.jenkinsi A, B and C using the genetic distance data obtained in Chapter VI. Nei[55], by assuming the rate of gene substitution to be constant, has calculated the temporal equivalent to his genetic distance parameter, $\bar{D}$. Fig. 30 represents a phylogenetic tree constructed in this way where the divergence times for the species investigated in Chapter VI are indicated by vertical lines. If we accept the assumptions and errors on which this figure is based, then the three dominant British clones of P.jenkinsi diverged over one million years before present i.e. before their introduction into the Northern Hemisphere. It must be emphasised, however, that this exercise is subject to considerable imprecision (for a review of the validity of the assumptions see Berry[20]). Alternatives (i) and (ii) remain equally possible.

It has long been argued by, for example, Fisher,[52] Darlington,[53] and White,[54] that parthenogenesis is an evolutionary 'dead-end'; it is therefore worthwhile examining the likely evolutionary fate of P.jenkinsi in the light of both this view and of the results presented in the present study.

According to Fishers Fundamental Theorem of Natural Selection (see Introduction p.2) P.jenkinsi should have little or no ability to adapt to its surroundings and is therefore evolutionarily unlikely to persist. However, P.jenkinsi appears to be extremely well adapted to its surroundings; it is particularly successful in immature man-made habitats where competition from other species may be relatively low.

In the context of evolutionary persistence the important question toanswer is not whether P.jenkinsi is adapted to its present surroundings but whether the gene pool of the species contains sufficient variation to allow adaptation to a change in its surroundings.

Two lines of evidence have been presented which suggest that there is insufficient variability in P.jenkinsi populations to allow them to persist in the face of environmental change:

i) In the present work (section VII - 3 - 3), two examples of the rapid and complete extinction of large populations of P.jenkinsi B have been given. In one of these (the Stone Creek population) a population of the sexually reproducing H.ventrosa in a tributary dyke survived the environmental extremes.

ii) Fenchel[45] has shown that P.jenkinsi populations do not undergo character displacement when living sympatrically with Hydrobia species. In contrast, sympatric populations of sexually reproducing Hydrobia ssp. appear to be able to avoid competition for food particle size by evolving character displacement.

In view of this evidence, it would appear that P.jenkinsi populations rely on having relatively stable resource dimensions for their persistence.

Suomalainen et. al.[18] and Lokki[55] have demonstrated convincingly that apomictic populations accumulate genetic variation; given enough time many become as variable as sexual species, although variation is distributed differently. It is thought that such variation accumulates during periods of habitat diversification when newly arisen mutant clones are able to colonise previously unexploited niches. Populations of

P.jenkinsi may be expected to follow this pattern only if there is sufficient habitat stability during the period when variation is accumulating; rapid and large shifts in resource dimensions might lead to extirpation of the species during this period.

Only if such environmental conditions are fulfilled over sufficient time will P.jenkinsi accumulate enough variation to become an evolutionarily persistent member of the European fauna.

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APPENDIX I : LOCALITIES OF POPULATIONS USED IN THE STUDY

Date	Popln. No.	Site	Habitat Description	Grid Ref.	Collector (Coll.)	Species (Sp.)	Strain (Str.)	No. of Snails Collected (N)
21. 6.75	1	Stone Creek, Paull, Humberside.	Brackish Marsh Drain (5000 mg/1 Cl)	TA 235190	B.R.J.	P.jenkinsi	B	2000
29. 4.75	2	Muriau Farm Carnarfon	Freshwater stream - fast flowing	-	G.S.	P.jenkinsi	A	500
28.10.76	3	Hendre Farm Llangefni Anglesey	Clear fresh water stream - fast flowing	-	B.R.J.	P.jenkinsi	A	700
19.10.76	4	Cardiff	Drainage Channel (Reen) (100 mg/1 Cl) Callitriche sp.	ST 392873	J.M.	P.jenkinsi	A	500
18. 6.75	5	Longniddry Golf Course Edinburgh	Drainage Ditch	-	T.W.	P.jenkinsi	A	38
10. 6.75	6	R. Ure Thornton Br., Fife	Small fresh water river	291969 Sheet 56	J.S.	P.jenkinsi	A	35
10. 6.75	7	Burnhead Fife	Eutrophic Drainage Ditch	-	J.S.	P.jenkinsi	A	32

Date	Popln. No.	Site	Habitat Description	Grid Ref.	Coll.	Sp.	Str.	N
10. 6.75	8	River Tay Inchyra Tayside	Mud Flats Max. Salinity 5%	184204 Sheet 55	J.S.	P. jenkinsi	A	34
23.4.77	9	Primrose Valley Filey N. Yorkshire.	Small fast-flowing stream freshwater Gravel bed	-	B.R.J.	P. jenkinsi	A	58
14. 9.75	10	Gorad Stream, Bangor	Small fresh-water stream on sandy beach	725574	T.B.R.	P. jenkinsi	A	Approx 100
23. 9.75	11	Bielby Beck Bielby Humberside.	Shallow canalised chalk stream fresh-water (171 mg/1 Cl^-)	SE 785452	B.R.J.	P. jenkinsi	A	2000
12. 4.77	12	River Severn Stourport	Lowland river (54 mg/1 Cl^-)	SO 79 72	G.S.O.	P. jenkinsi	A	1
11. 4.77	13	River Leam Leamington Spa Warwickshire	Small river freshwater (62 mg/1 Cl^-)	SP 322656	G.S.O.	P. jenkinsi	A	31 (including juveniles)
9. 5.77	14	River Severn Dingle Bridge Shropshire	-	SO 273967	G.S.O.	P. jenkinsi	A	35

Date	Popln. No.	Site	Habitat Description	Grid Ref.	Coll.	Sp.	Str.	N
9. 5.77	15	Pye Brook Peaton Bridge Shropshire	-	SO 530850	G.S.O.	P. jenkinsi	A	11
24.11.76 14.1.77	16	River Rye Nunnington North Yorks.	Fast flowing trout stream Rich flora	-	G.S.O. B.R.J.	P. jenkinsi	A	35
30. 4.77	17	River Derwent Forge Valley North Yorks.	Fast-flowing chalk stream	-	B.R.J.	P. jenkinsi	A	68
21.11.76	18	Polybotts Lane Leicester.	Drainage ditch.	-	B.R.J.	P. jenkinsi	A	83
21.11.76	19	Ratby, near Leicester.	Small stream	-	B.R.J.	P. jenkinsi	A	47
1. 6.77	20	Crose Mere Shropshire	Stony shore Eutrophic lake	-	J.O.Y.	P. jenkinsi	A	38
11.5.77	21	Grand Union Canal	Radford Semele Lock. Eutrophic Canal	SP 352679	G.S.O.	P. jenkinsi	A	30
22. 9.77	22	Janetstown Thurso	Quarry (Slate) Pool on stones	ND 093665 Sheet 39	R.M.	P. jenkinsi	A	48

Date	Popln. No.	Site	Habitat Description	Grid Ref.	Coll.	Sp.	Str.	N
20. 5.77	23	Lewes Brooks Sussex	Flowing water Groenlandra sp. & Callitriche " sp.	TQ 424053	A.R.	P. jenkinsi	A	45
27. 6.77	24	River Mole Near Dorking Surrey	On stepping stones in fast flowing small river	TQ 172512	M.S.	P. jenkinsi	A	60
18.10.76	25	River Ure Boroughbridge North Yorks.	Medium flow river	-	B.R.J.	P. jenkinsi	A	39
30.11.77	26	A.149 Bridge Gaywood River Kings Lynn, Norfolk.	Fast flowing canalised eutrophic lowland stream	-	B.R.J.	P. jenkinsi	A	100
6. 5.77	27	River Conder Galgate, Lancashire.	Fast flowing fellside stream	-	B.R.J.	P. jenkinsi	A	40
30.11.77	28 †	Snettisham Lakes, Norfolk	Brackish (11,000 mg/l Cl^-) ponds on shingle ley	648332	B.R.J.	P. jenkinsi	B	2000
10.10.77	29	River Laune Killorglin Co. Kerry, Ire.	Fast flowing eutrophic river	-	B.R.J.	P. jenkinsi	A	50

† Population extirpated by salt water 12.1.78 (see VI-4-1)

Date	Popln. No.	Site	Habitat Description	Grid Ref.	Coll.	Sp.	Str.	N
15.4.77	30	Stream in Brittany, France	Coastal small stream	-	M.J.W.	P. jenkinsi P. jenkinsi	A C	37 8
30.11.77	31	River Till Lincs. Lincoln - Gainsborough Road.	Small lowland river, medium flow (Canalised)	-	B.R.J.	P. jenkinsi	A	35
30.11.77	32	Brook at Gainsborough Lincs.	Fresh water (100 mg/l Cl^-) brook. Clean R. eutrophic	699436	B.R.J.	P. jenkinsi	A	100
12. 4.76	33	River Yare Norwich.	Lowland canalised river	-	J.B.	P. jenkinsi	A	14
22. 9.77	34	River Thurso Mall footbridge Thurso.	Oligotrophic fast flowing peaty river	ND 115679 Sheet 39	R.M.	P. jenkinsi	A	35
15.12.76	35	Burgh Castle E. Suffolk	-	-	T.W.	P. jenkinsi	C	134 (from lab population)
19.6.77	36	Kenilworth Near Warwick.	Drainage ditch.	-	G.S.O.	P. jenkinsi	A	14

Date	Popln. No.	Site	Habitat Description	Grid Ref.	Coll.	Sp.	Str.	N
20.6.77	37	Grand Union Canal at M.1 junction	Eutrophic canal	-	G.S.O.	P. jenkinsi	A	15
-	38	River Cam Grantchester Meadows, Cambridge	Lowland river	-	G.S.O.	P. jenkinsi	A	1
19. 4.77	39	Creek at Wakehurst Parkway Narrabeen, New South Wales	Freshwater Creek above tidal influence	-	P.H.C.	P. nigra	all female	100
15.10.77	40	Brownhill Creek (Mitchem), Adelaide N.S.W.	-	Adelaide 163678	W.D.W.	P. nigra	1 male in 100	200
15.10.77	41	Deep Creek Castambul NSW.	-	Adelaide 174692	W.D.W.	P. nigra	5% males	200
15.10.77	42	Back Creek Melrose Pt. Germain	-	484297	W.D.W.	P. nigra	all female	200
15.10.77	43	Congertina Creek, Southern Fleurieu Pen. South Australia	-	623613	W.D.W.	P. nigra	10% male	200

Date	Popln. No.	Site	Habitat Description	Grid Ref.	Coll.	Sp.	Notes	N
15.10.77	44	Severnhills Creek South of Clare, South Australia 100 km N Adelaide	-	-	W.D.W.	P. nigra	- snails distressed on arrival	800
6.10.77	45	Waikato River Hamilton N.Z.	Large medium flow river	-	C.W.	P. anti	29% male	500
6.10.77	46	University Campus Lake, Waikato University, N.Z.	Artificial lake	-	C.W.	P. anti	All female (100 examined)	500
9.11.77	47	Waikato River, Porana Park, Hamilton, N.Z.	cf. popln 42	-	C.W.	P. anti	30% male	200
15.10.77	48	Coolawang Creek South Australia	-	642603	W.D.W.	P. nigra	3 males in 100	200
-	49	Brooklands Lagoon near Christchurch New Zealand	Saline lagoon	-	M.J.W.	P.estuarinus	- 8 male 9 female	17
4. 5.75	50	Drain at Little Humber Farm, Paull, Humberside	Brackish ((11,000 mg/l Cl^-) enter- omorpha ssp.	TA 208222	B.R.J.	H. ventrosa	About 35% males	2000
19. 5.75	52	Maltreath Salt Marsh, Anglesey.	Brackish Pools (18,000 mg/l Cl^-)	-	B.R.J.	H.ulvae	-	200

Date	Popln. No.	Site	Habitat Description	Grid Ref.	Coll.	Sp.	Notes	N
23.11.75	53	Ythan Estuary	Unpolluted estuary	-	S.F.	H. ulvae	-	63
28. 5.75	54	Saltmarsh near Wareham Norfolk	Muddy saltmarsh	TF 963449	B.J.	H. ulvae	-	200
-	55	Hayling Island Portsmouth	Muddy foreshore		C.T.	H. ulvae	-	31
6. 4.77	56	Spurnpoint Humberside	Muddy saltmarsh	TA 419148	B.R.J.	H. ulvae	50% males	200
29.11.77	57	Drainage ditch Kirton Marsh, Lincs.	Tidal brackish ditch (20,000 mg/l Cl^-) ulva lactuca	349368	B.R.J.	H. neglecta H. ventrosa	-	200 55
29.11.77	58	Drain at Gedney Marsh Lincs.	Brackish ditch (15,500 mg/l Cl^-) Palesmonetes sp.	469284	B.R.J.	H. ventrosa H. neglecta	-	7 131
29.11.77	59	Drain at Bleak House Farm, Gedney Marsh, Lincs.	Brackish ditch (6,200 mg/l Cl^-)	456297	B.R.J.	H. Ventrosa	-	68

Date	Popln. No.	Site	Habitat Description	Grid Ref.	Coll.	Sp.	Notes	N
23. 1.78	60	Snettisham Lake N. Norfolk.	Same site as Popln. 28, but after inundation by sea during severe storm (25,000 mg/l Cl^-)	648332	B.R.J.	Juvenile (3 - 4 whorled) H. ulvae All P.jenkinsi B Dead	-	Approx 200
11. 9.75	61	Foreshore at Aber Near Bangor, North Wales.	Mud near high tide level - Saline	730642	T.B.R.	H. ulvae	-	23
20.11.77	62	Baie Des Pulias Vale, Guernsey.	Salt water lagoon	-	G.L.	H. neglecta	-	37

APPENDIX II

BARTLETT'S TEST

If s_1^2, s_2^2, s_3^2 —— $s_,^2$ are k estimates of variance with n_1, n_2, n_3, —— n_k degrees of freedom and s^2 is the pooled estimate of variance with n degrees of freedom, then, if the estimates s_1^2, s_2^2, s_3^2 —— s_k^2 are homogenous, the value of $\frac{M}{C}$ where $M =$

$$(n \log s^2 - n_1 \log s_1^2 - n_2 \log s_2^2 \text{ —— } n_k \log s_k^2)$$

and $C = 0.4343 \left[1 + \frac{1}{3(k-1)} \left(\frac{1}{n_1} + \frac{1}{n_2} + \text{——} + \frac{1}{n_k} - \frac{1}{n} \right) \right]$

is distibuted as a Chi-square with k - 1 degrees of freedom.

E.g. Esterase 9 in five Potamopyrgus species

		SS	df	df.log MS
P.jenkinsi	A	0.959221	44	-73.107496
P.jenkinsi	B	0.647297	23	-35.664358
P.jenkinsi	C	0.427490	15	-23.177478
P.antipodarum		0.938196	42	-69.340140
P.nigra		0.941329	37	-58.995031
		3.913533	161	-260.2845

$M = (\Sigma df) (\log MS) - \Sigma(df \log MS)$

$= -259.89536 - (-260.2845)$

$= 0.389143$

$C = 0.4343 \left[1 + \frac{1}{3(4)} \left(\Sigma \frac{1}{df} - \frac{1}{\Sigma df} \right) \right]$

$= 0.4343 \quad 1 + \frac{1}{12} \left(0.1837088 - \frac{1}{161} \right)$

$= 0.440724$

$\chi^2_{(4)} = M/C = 0.88296$ (n.s. - data are homogenous)

COMPARISON OF REGRESSION COEFFICIENTS

$$\text{Variance Ratio} \quad (F) = \frac{\text{difference between slopes MS}}{\text{pooled residual MS}}$$

$$\text{Pooled residuals} = 7.14713 \times 10^{-3}$$

$$\text{Pooled total MS} = 2.4308 \times 10^{-2}$$

$$\text{Difference MS} = 1.7161 \times 10^{-2}$$

$$F_{(5,156)} = \frac{1.7161 \times 10^{-2}}{7.14713 \times 10^{-3}}$$

$$= 2.4011$$

i.e. the regression coefficients do not differ significantly ($p = 0.95$).