Genetic Variation and Acid Tolerance in the Nemouridae (Insecta: Plecoptera)

by

Jonathan Heald Lees

A Doctoral Thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of the Loughborough University of Technology.

September 1987.

ABSTRACT

An electrophoretic survey of 16 enzyme loci in nine of the eleven British Nemouridae (Insecta: Plecoptera) was carried out in order to assess their systematic relationships and to determine the level of genetic variation in these primitive insects. The species screened were : Nemoura cinerea, N. cambrica, N. dubitans, N. avicularis, Nemurella picteti, Protonemura meyeri, P. praecox, Amphinemura sulcicollis and A. standfussi. The Nei genetic distances between all the species are large, ranging from 0.53 to 2.07, and are consistent with the proposed ancient origin of these species. The phylogeny derived using biochemical characters is in agreement with the taxonomic classification of this family based on morphological features. The amount of variation within each species is small, with an overall mean expected heterozygosity per locus of 0.047, mean proportion of polymorphic loci of 0.146 and mean number of alleles per locus of 1.25. A reasonable explanation for these low levels of variability in comparison with most other insect species is that variation has been lost during periodic population bottle-necks. An electrophoretic key for the identification of these species was devised.

The influence of stream water acidity on genetic variation was investigated by comparing allele frequencies at four polymorphic loci (Aminopeptidase - 1, Phosphoglucose Isomerase, Phosphoglucomutase and Hexanol Dehydrogenase - 1) in populations of Nemoura cinerea from streams with different pH's. There was a clear difference at the HDH-1 locus between upland, acid streams and lowland circum-neutral streams with the 90 allele only present in the latter. The results from acute toxicity tests showed that there was no difference in tolerance to low pH of nymphs from alkaline or acid streams. The 96 hour LC-50 ranged from pH 2.96 to pH 3.22. No difference was found between the genotypes of survivors or those killed in the toxicity tests.

Differences in life-history characteristics of two populations of N. cinerea from the same catchment were investigated and appeared to result from differences in the thermal budget at each site rather than a difference in pH.

Low pH did not appear to have any effect on the rate of oxygen uptake of N. cinerea which was around 1000 µl/g dry weight/hr.

CONTENTS

Tables, Plates and Figures

CHAPTER 1

GENERAL INTRODUCTION

In recent years there has been growing public concern as reports of widespread serious pollution caused by acid rain have appeared in the media. However, as Cowling (1980) and others (Last and Nicholson,1982; Franks,1983) have pointed out, this is hardly a new phenomenon, and the term "acid rain" was first used by Smith (1872) in his study on regional variation in precipitation chemistry in Britain.

Generally, "acid rain" is taken to mean precipitation (rain, hail, snow and fog) with a pH of less than 5.6 (the pH of distilled water in equilibrium with atmospheric concentrations of carbon dioxide). Precipitation becomes acidic when moisture in the atmosphere combines with sulphur dioxide (SO_2) and nitrous oxides (NO_x)* to form sulphuric and nitric acids.

Natural sources of sulphur and nitrogen compounds exist which can cause a significant decrease in pH (acidification) of atmospheric water (Charlson and Rodhe,1982). These include hydrogen sulphide, ammonia and nitrous oxides from biological decay and soil reactions, and sulphates from sea salts. Volcanoes also provide a source of sulphur and nitrous oxides.

In the industrial regions of the northern hemisphere, however, anthropogenic emissions can equal those from natural sources, causing further acidification (Dovland, Joranger and Semb,1976). The main producers of SO_2 are power stations, smelters and refineries. Nitrous oxides are also emitted from

* NO and NO_2

these sites, but another main source is from the exhaust fumes of motor engines.

Some complex chemical processes are thought to be involved in the formation of sulphuric and nitric acids in the atmosphere. These include photochemical oxidation and oxidation of the gases dissolved in water droplets or adsorbed to solid particles. Therefore the longer the gases remain in the air, the greater the chance that they will combine with water to form acids and return to earth as "wet deposition". Fall-out of the uncombined gases is termed "dry deposition". This too will cause acidification if it combines with surface water. Dry deposition can often contribute more sulphur and nitrogen than wet deposition (Fowler,1980). Hence the term "acid deposition" is a more accurate description of this pollution phenomenon than "acid rain".

Several researchers in both Europe and North America studied isolated aspects of atmospheric and surface water chemistry during the first half of this century. However it was only in the 1950's that Gorham established that acidity in precipitation was correlated with industrial sources of pollution, and seemed to have influenced the chemistry of lakes, bogs and soils (Gorham,1955,1958 and 1961). Unfortunately, like Smith's work a century earlier, the implications of this were not recognised and acted upon.

It was Oden in 1968 who stimulated wider concern over pollution by acid deposition. His analyses of rainwater chemistry data showed that both precipitation and surface waters in southern Sweden were becoming more acidic. This was attributed to long range transport of sulphur and nitrous oxides emanating from outside Sweden in the industrial

countries of Europe. Oden also hypothesised that the probable ecological consequences would be a decline in fish populations and decreased forest growth.

These conclusions and hypotheses led to an upsurge in research activity in this field both in Europe and North America. The Norwegian government provided the main impetus by establishing the "SNSF Project" in 1972 with an annual budget of over one million pounds.

The results of this project (Overrein et al,1980; Drablos and Tollan,1980) together with other extensive research (noteably the OECD Study on the Long Range Transport of Air Pollutants,1977), have confirmed some of Oden's hypotheses. The transport of pollutants over long distances has been demonstrated in Europe and North America, and precipitation has been shown to have become more acidic over the last two decades in several areas (Dovland et al,1976; Mathews et al,1980; Loucks, 1982). This increase in acidity parallels the dramatic rise in SO_2 and NO_x production that has taken place over the same time period (Dovland and Semb,1980; Brimblecombe and Stedman,1982).

The adverse effects accredited to acid deposition include corrosive damage to buildings and monuments, and the release of copper and lead from water pipes into drinking water supplies (Glass et al,1982). However, the probable effects on aquatic ecosystems and on crop and forest growth have received the greatest attention. In these cases local geology is the principal factor determining which areas are sensitive to acidification. Sensitive areas are ones with weathering resistant rocks such as granites and shales. Here the overlying soils are thin and deficient in bases (calcium

and magnesium) giving poor buffering capacity. Similarly the surface waters have a low ionic concentration and poor resistance to changing pH.

The afforestation of upland catchments has been shown to cause acidification of streams in some areas of Scotland (Harriman and Morrison, 1982). However, it is unclear whether this is attributable to the trees collecting more acid pollutants than natural upland vegetation, the removal of bases from the soil into the vegetation coupled with hydrogen ion production during tree growth and litter decay, or to changes in the hydrology of the catchment brought about by modern planting techniques.

Several reports have been made on the die-back and decline of forests in both Europe and North America (Tomlinson,1983;Johnson and Siccama,1983; Acid News, 1984), where hundreds of thousands of hectares of conifers are affected. This could have serious economic as well as environmental implications. However, a universally accepted mechanism by which acid deposition can cause forest decline has yet to be established.

One widely accepted theory was proposed by Ulrich (1980). He postulated that acid deposition acidifies the soil leading to increased concentrations of aluminium in the soil water. High aluminium concentrations are thought to be toxic to plants, affecting root growth and interfering with the uptake of essential calcium and magnesium ions. However, not all scientists accept that this is the real cause of the observed forest decline. The absence of tree damage in many areas subjected to acid deposition (most noteably Scandinavia) suggests that other factors may be involved. In particular

direct damage to foliage by ozone, produced by photochemical oxidation of NO_x, appears to be more important than was formerly recognised.

The evidence linking acid deposition to disturbances of freshwater ecosystems is far more compelling. Oden's hypothesis on declining fish populations was based on earlier observations of fishery losses in Scandinavia (Dannevig, 1959). Today, thousands of lakes and rivers in Southern Sweden and Norway are thought to have lost their fisheries since the beginning of this century (Overein et al, 1980). Similar losses have been reported from Canada (Harvey, 1980; Watt et al, 1983) and the United States (Schofield, 1976; Pfeiffer and Festa ,1980). In many cases a concurrent reduction in pH has been recorded.

Experimental work has shown that egg hatching and early growth is severely affected at pH's commonly found where fish stocks have been lost (Brown, 1981; Peterson et al,1980; Swarts et al, 1978). This indicates that recruitment failure is the main cause of stock declines.

Studies on adult fish (Dickson, 1978; Baker and Schofield, 1980) and investigations of situations where fish-kills have been observed (Muniz et al, 1975, 1979; Grahn, 1980) show that aluminium toxicity is often a major factor in adult mortality. The aluminium is leached from acidified soils in the catchment (Cronan and Schofield, 1979). Fish die due to mucus production at the gills which interferes with ionic regulation and oxygen uptake (Muniz and Leivestad, 1980).

Acidification of freshwaters has also been shown to cause a reduction in diversity of the plant (Hendrey et al, 1976) and invertebrate communities (Sutcliffe and Carrick,

1973; Hendrey and Wright, 1976; Fiance, 1978; Hall et al, 1980). This is due to the loss of acid sensitive groups such as molluscs and crustaceans, leaving a few tolerant species (Overein et al, 1980). These tolerant species have often only been identified from large scale surveys where presence or absence data is taken to imply tolerance. Relatively few laboratory experiments have been performed to determine tolerance limits in invertebrates (Singer, 1981). However, the studies that have been made indicate that, as in fish, mortality is due to a failure of ionic regulation and, perhaps, oxygen uptake (Vangenechten and Vanderborght, 1980; Havas, 1981; Raddum and Steigen,1981; Alibone and Fair, 1981).

Several researchers have found that different populations of fish differ in their ability to withstand experimental acidification (Gjedrem, 1976; Swarts et al, 1978; Rahel, 1983). These differences in tolerance were shown to be heritable and thus had a genetic basis.

One of the main aims of this study was to look for a similar genetic difference between populations of an aquatic invertebrate, Nemoura cinerea (Insecta: Plecoptera). However, rather than looking for heritable differences in tolerance to low pH, electrophoresis was used to measure genetic variation between populations inhabiting acid or circumneutral streams and thus reveal any correlations between genotype and stream acidity. N. cinerea was chosen because it is widely distributed and can be found in a range of streams with different acidities in Britain (Elton, 1956; Hynes, 1977; pers. obs.)

It was thought that an investigation such as this would be of value to those examining the effects of acid

deposition on aquatic environments since, as Last and Nicholson (1982) point out, few studies have been made in the United Kingdom. However, this part of the investigation will, perhaps, be of greater interest to population geneticists as a survey to determine if genetic variation has been influenced by environmental parameters.

Electrophoresis is a technique for the identification of genetic variation at gene loci coding for proteins, especially soluble enzymes. Mutations causing a change in the net charge carried by a protein molecule can be detected from their different rates of migration through a gel medium (usually starch, agar or poly-acrylamide) when an electric potential is applied across the gel. After several hours, the positions of the variants of individual protein loci can be made visible by staining the gel with the appropriate histochemical stain.

The technique was first used in the 1960's by Lewontin and Hubby (1966) and Harris (1966). These, and subsequent studies, have revealed that high levels of variation are present at the molecular level in most populations of outcrossing species, with 25-50 % of the loci examined being polymorphic per population and 5-15 % heterozygous per individual (Selander, 1976). Generally, invertebrate species have been found to be more variable than vertebrates (Nevo et al, 1984). Electrophoresis detects only those genic substitutions that cause a difference in the ionic charge carried by the proteins. Since only about 26-28 % of substitutions cause a change in charge (Lewontin, 1974), the measures of polymorphism must be an underestimate of the total variation. This has been highlighted further by the

discovery of more extensive 'hidden' variation when gel, buffer, pH and temperature conditions are varied in electrophoresis (Coyne, 1976; Singh et al, 1976).

The discovery of the high levels of molecular variation led to the still unresolved debate over how this variation is maintained within a population, and what is its adaptive significance. The debate is often termed the 'neutralist - selectionist controversy', though proponents of both schools of thought appear to dislike the labels (Lewontin, 1974; Kimura, 1983). It really is an extension of the arguments that had dominated population genetics theory for the previous twenty years. These were categorised by Dobzhansky (1955) as the 'classical' and 'balance' hypotheses. The main point of contention was over the role played by natural selection in evolution.

The classical theory, ascribed to Muller (1950) and his co-workers, saw selection acting in the way proposed by Darwin (1859), purifying the species of deleterious mutations and leading to the fixation of the fittest genotype. As a consequence it was thought that individuals in sexually reproducing populations would be homozygous for a 'wild-type' allele at almost all their loci. In contrast, the balance theory (Dobzhansky, 1955; Wallace, 1958) proposed that natural selection acted not only to remove genetic variation by 'directional selection' against deleterious mutants, but also to promote and conserve variation through 'balancing selection' (heterosis or frequency-dependent selection). This would lead to individuals being heterozygous at nearly all their loci.

The observations from electrophoretic investigations

and from other techniques employed by molecular geneticists, such as amino-acid sequencing of proteins and, more recently, DNA analysis, which have also revealed extensive polymorphisms, appear to support the predictions arising from the 'balance' hypothesis rather than the 'classical' hypothesis. However, proponents of the classical school have maintained their view that balancing selection is relatively unimportant in evolution by questioning the biological significance of the molecular variation revealed by these techniques.

The new hypothesis is based on theoretical arguments developed by a number of authors (Kimura and Crow, 1964; King and Jukes, 1969; and Kimura and Ohta, 1971 a,b,c). They propose that the majority of the molecular variation has no effect on the fitness of an organism. The alternative varieties, or alleles, at any locus are selectively equivalent (neutral) so that their frequency within a population or species is largely determined by mutation rates and random genetic drift. Hence this theory has been termed the neutral mutation - random drift hypothesis, or neutral theory in short (Kimura, 1983), and adherents to this theory are called 'neutralists'. Proponents of the opposing school of thought, the 'balance' school, are now referred to as 'selectionists'.

Much of the debate has centred on mathematical considerations of the predictions made from models proposed by both schools of thought, using data from experimental observations (see Lewontin, 1974 and Kimura, 1983). However, all the models depend on parameters that cannot be measured with sufficient accuracy and thus are difficult to falsify. Neutral theory relies on estimates of mutation rates and

effective population size; selectionist models are greatly influenced by estimates of fitness and migration rates. All depend on estimates of gene frequencies in natural populations which involve sampling errors (Lewontin, 1974). Hence, arguments for both view points can often be made from the same observations giving firm evidence for neither.

The failure of these mathematical attempts to resolve the controversy has led many geneticists to believe that, in the absence of a statistical test with sufficient resolving power, the accumulation of direct experimental evidence is the most useful way to settle the debate (Nevo et al, 1984; Shorrocks, 1978; Clarke, 1975). One way of providing this evidence is to carry out an extensive survey of populations such as the one reported here. Any correlations of genetic variation with environmental parameters would provide a strong indication that selection of some kind has occured and be difficult to interpret from a neutralist view. As shown earlier, acidification of fresh waters can have very severe ecological effects and has already been implicated as the selecting factor producing acid tolerant strains of fish.

A number of studies have revealed significant genotype - environment associations in a range of organisms. For example, Schopf and Gooch (1971) found a cline in the frequency of a leucine-aminopeptidase allele in Bryozoans associated with a 6°C change in August water temperature. Johnson (1971) discovered correlations in an allele frequency cline for lactate dehydrogenase in the crested blenny with temperature, oxygen concentration and the incidence of another blenny. Several studies have found correlations between allele frequencies at the leucine-aminopeptidase locus in Mytilus

edulis and salinity (Koehn et al, 1976; Lassen and Turano, 1978; Koehn et al, 1980). Place and Powers (1979) found an allele frequency cline associated with temperature at the lactate dehydrogenase-B locus in the fish Fundulus heteroclitus on the Atlantic Coast of the USA. Hoffman (1981 b) found that both phosphoglucose isomerase and phosphoglucomutase showed clinal variation in the anemone Metridium senile with an apparent association with temperature. Smith et al (1983) found significant correlations between several loci in the mosquitofish and temperature, flow and elevation.

Correlations between temporal changes in allele frequency and an aperiodic feature of the environment, or repeatable cyclic fluctuations in allele frequency would also provide strong evidence that selection can influence molecular variation (Lewontin, 1974). In one case, Berger (1971) found that the 'slow' allele at the ∝-GPDH locus in Drosophila melanogaster doubled in four populations from June to November in two different years. In the present study, samples of N. cinerea were taken from some sites in the Peak District through-out a three year period in order to detect possible temporal changes in gene frequencies.

Although correlations between allele frequencies and spatial or temporal differences in the environment provide evidence that natural selection rather than random drift has influenced the frequency distribution, they do not in themselves indicate that selection is acting directly on the loci being observed. They may be carried along by linkage to other loci which are the real target of selection (Clarke, 1975). This problem can only be overcome by demonstrating

biochemical and physiological differences between the enzymatic products of the alleles consistent with the proposed selective agent. Koehn et al (1980) demonstrated that allele frequency clines in Mytilus edulis were probably due to functional differences in response to salinity between the aminopeptidase-1 alleles. Hoffman (1981 a) showed that there were significant differences in the activities of phosphoglucose isomerase allozymes in Metridium which could explain the observed frequency distribution in populations along the northeast coast of the United States.

In some cases inter-specific comparisons have been used to test selectionist theories in which the amount of genetic variation that can be supported in any situation is an adaptive strategy to increase the mean population fitness in spatio-temporally heterogeneous environments (Dobzhansky, 1951). Perhaps the most popular of these is the niche-width variation hypothesis (Van Valen, 1965). This expects there to be a positive correlation between niche-breadth and genetic variability. The grain theory (Ludwig, 1950; Levins, 1968; Selander and Kaufman, 1973) has essentially the same predictions. Several electrophoretic comparisons between broad-niched and narrow-niched species have supported the theory (Avise and Selander, 1972; Levinton, 1973; Nevo, 1976; Lavie and Nevo, 1981; Lacy, 1982). However, the expectation that deep-sea fauna from a stable, monotonous environment would have low variation was not fulfilled (Gooch and Schopf, 1972; Ayala and Valentine, 1974). This leads to an alternative explanation, the time divergence hypothesis, which predicts that constancy of environmental parameters, such as trophic resources, promotes allelic diversity. Arrays of morphs are

produced, each best at exploiting a narrow niche, so intra-specific competition is reduced. Genetic uniformity is predicted in unstable environments because a generalised genotype would be better adapted to cope with uncertainty (Valentine, 1971; Ayala and Valentine, 1974; Valentine 1976; Valentine and Ayala, 1975).

Perhaps the main application of electrophoretic techniques has been to measure the amount of genetic differentiation between related species. Phylogenies or species relationships can then be inferred from this information. The advantage that electrophoresis has over classical morphological characters for deriving phylogenies is that proteins, as primary translations of the genetic code, are little influenced by environmental factors, allowing similarities and differences to be treated in a more rigorous, numerical way (e.g. Nei, 1972). Electrophoresis has also proven to be of particular value in the identification of cryptic species that have defied systematic analysis by standard morphological criteria (Hanken, 1983). In some cases a biochemical identification key has been devised (Buth, 1979; Berlocher, 1980). However, the technique is limited to comparisons between taxa of lower status than families as the likely number of gene substitutions accumulated since such distantly related species shared common ancestry is too great (Ayala, 1983).

Whilst the main emphasis of this thesis is on the biochemical genetics of the Nemouridae and especially on variation between populations of Nemoura cinerea, the most widespread species, data from investigations into the broader effects of environmental acidity on the ecology of

streams are also presented. The ability of N. cinerea to tolerate conditions of low pH has been examined by respirometry and acute toxicity tests, providing physiological evidence to complement the genetic studies. By electrophoretically screening the animals used in the toxicity tests, it was hoped that any differential survival of individuals with a particular genotype could be detected. The influence of low pH on the respiration of N. cinerea was investigated because, although a reduction of oxygen uptake leading to eventual mortality at low pH has been demonstrated in fish (Havas, 1981), only one study appears to have been made on the effects of acid per se in invertebrates (Alibone and Fair, 1981).

The main aims of this investigation were, therefore :

i) To determine the amount of molecular genetic variation in the nemourid stoneflies (Plecoptera) in Britain and to examine the genetic relationships between species using enzyme electrophoresis.

ii) To study the effect of stream acidity on genetic variation in Nemoura cinerea, the most widespread species of the British Nemouridae.

iii) To investigate the response of N. cinerea to acid stress through respirometry and acute toxicity tests in the laboratory, and through comparisons of life-history in natural populations.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Experimental Organisms

The Plecoptera form one of the most primitive extant orders of winged insects. In Britain, there are thirty-two species (two other species in Hynes' key (1977) are described as being of uncertain British status). The Nemouridae, with eleven species in four genera, is the largest British 'stonefly' family. The nymphs of species in two of the genera are characterised by the possession of prosternal gills, whilst nymphs of the other two genera are gill-less. The following is a species list of the British Nemouridae :

Nemoura cinerea (Retzius). Plate 2.1.
Nemoura cambrica (Stephens)
Nemoura dubitans (Morton)
Nemoura avicularis (Morton) GILL-LESS
Nemoura erratica (Claassen)
Nemurella picteti (Klapalek)

Protonemura meyeri (Pictet). Plate 2.2.
Protonemura praecox (Morton)
Protonemura montana (Kimmins) GILLED
Amphinemura standfussi (Ris)
Amphinemura sulcicollis (Stephens)

All Nemouridae take one year to complete their life-history in Britain - a 'univoltine' life-cycle. The aquatic nymphal phase is the longest — lived stage and is,

therefore, the most important in their ecology. Generally, nymphs hatch from eggs in late summer/early autumn. They grow steadily through the winter months, moulting many times. The exact number of instars is uncertain, being influenced by temperature and sex (Khoo, 1964). They are herbivorous, feeding mainly on allochthonous material or vegetation growing on the stream bed.

Most nymphs reach maturity in the period extending from March through to August. They crawl out of the water on to the banks or exposed boulders in the stream and, after a final moult, adult stoneflies emerge (Plate 2.3.).

Adults live for a relatively short time (from two to a maximum of fifty days (Hynes,1942)). They are poor fliers and are rarely found far from the water from which they emerged. A few days after mating the females extrude an egg mass which is carried on the under-side of the tip of the abdomen. The eggs are then deposited on the surface of the water and have attachment mechanisms to anchor them to the stream bed where they take several weeks to develop (Khoo, 1964).

The nymphs are intolerant of pollution and are mainly confined to headwater streams where they often dominate the benthic macro-invertebrate fauna. Noteable exceptions are Nemoura avicularis, which is mainly found on the shores of unpolluted lakes or sluggish streams with emergent vegetation (Hynes, 1977), and Nemoura cinerea (and to a lesser extent Amphinemura sulcicollis) which occur in organically rich lowland streams as well as in upland areas (Brinck, 1949, Hynes, 1977; personal observations).

2.2. Collecting Sites and Collecting Methods

2.2.1. The Derbyshire Peak District

The Peak District of Derbyshire around Snake Pass was chosen as the initial sampling area because earlier work had shown that acid streams could be found there (Brown & Martin, 1980). The acid streams drain peat bogs that blanket the flat summit of Kinder Scout and the surrounding peaks. Peat is a very acid soil due to humic and organic acids being formed as vegetation slowly decomposes. This means that rain falling in this area will not be neutralised before entering stream channels and may be acidified further by organic acids being flushed out of the peat. The latter causes the brown discolouration of stream water that is common in this area.

In addition to the natural source of acidity in the peat, the rainfall here is itself acidic (Brown & Martin, 1980; Ferguson & Lee, 1983). This area of the south Pennines lies at the centre of the region where the industrial revolution started over two hundred years ago. Evidence from the dramatic decline of Sphagnum mosses in the bogs, together with records of air and rain pollution measured in the nearby industrial towns since before the start of the century, indicates that this area has probably been exposed to damaging levels of atmospheric pollution for longer than any other area in the world (Ferguson & Lee, 1983). It is, therefore, an obvious place to look for evidence of the impact of acid deposition on the environment.

There are no historical data concerning trends in stream water acidity. Today, however, these headwater streams are typical of acidic streams found in areas where acid

deposition is reported to have caused environmental problems over the last few decades (Drablos & Tollan, 1980). No fish are present and the invertebrate fauna is less diverse than that of more neutral streams, with a few species of Plecoptera being dominant in numbers for most of the year.

2.2.2. Collecting Methods

Invertebrates were collected by the kick-sampling technique (Morgan & Egglishaw, 1965) using a hand held 1 millimetre mesh net. During late summer and autumn, an additional fine mesh net (250 µm) was used to collect early instars. All parts of the stream (riffle, pool, edge, centre and vegetation) were sampled to ensure, as far as possible, that all species present were collected. Adult stoneflies, when present, were collected from the undersides of boulders protruding out of the water, using a pootah. Samples were returned to the laboratory, or temporary base, for sorting and identification. When necessary, animals were stored frozen at -20°C prior to use for electrophoresis.

The results of the early electrophoresis indicated that *N. cinerea* would be the most suitable species for inter-population comparisons, due to its greater electrophoretic variability than the other Nemouridae. Furthermore, it was found in both acid and circumneutral streams in Derbyshire and was reported to be one of the most widespread species (Hynes, 1977). The Peak District streams known to contain *N. cinerea* were kept as regular sampling sites to investigate temporal changes in genetic variation and for life-history comparisons. These streams also provided a

plentiful supply of specimens for laboratory experiments. In addition sampling was extended to encompass other areas of Britain, both to increase the number of populations in the survey, and to collect species not found in the Peak District for an inter-specific electrophoretic comparison within the Nemouridae. Most sampling trips were to locations studied by previous investigators (Hynes, 1952,1961; Elton, 1956; Brown, Cragg & Crisp, 1964; Langford and Bray, 1969; Fawcett, 1971; Casey & Ladle, 1976; Maitland et al, 1981; Wade & Rhodes, 1982) though some were to areas where previous invertebrate surveys have not been reported. The sites which provided specimens for electrophoresis are listed in Table 2.1. Their locations are indicated on Figure 2.1.

2.2.3. Water Analysis

At every site visited, a water sample was collected in a 500 millilitre screw-top plastic bottle. Immediately on returning to the sampling base, the pH and conductivity were measured. The pH was measured using a Radiometer PHM80 pH meter with a GK2401C combined electrode. The conductivity was measured at 20°C using a Radiometer CDM80 conductivity meter with a CDC104 electrode. When possible the samples were analysed further to determine concentrations of calcium, magnesium, sodium and potassium ions using a Hilger and Watts H1170 'ATOMSPEK' atomic absorption spectrometer.

2.3. Annual Growth Patterns

2.3.1. Measurements of Growth

Whilst collecting invertebrate samples in the Peak District, it was noticed that the life-history of *Nemoura cinerea* appeared to be slightly different at two sites in the same catchment. The sites were Thomasons' Hollow and Lady Clough (indicated by arrows in Figure 2.2.). Thomasons' Hollow is a headwater stream at 457 metres above sea level and has a low pH all year round. Lady Clough is a higher order stream, further down the catchment at 321 metres above sea level, and has a varying pH dependent on the conditions of flow (Brown & Martin, 1980). Adult emergence appeared to be delayed at Thomasons' Hollow relative to Lady Clough, so that nymphs could be collected for two to three months after they had disappeared from the bottom fauna at Lady Clough (in June).

A monthly sampling programme was undertaken to study this difference in life-history timing. Each month, twenty nymphs (or as many as possible when the total collected was less) from each sample were measured using a Zeiss binocular microscope fitted with an eye-piece graticule. Three measures were taken (Figure 2.3.) :

a) Total body length (excluding cerci and antennae)

b) Head-capsule width

c) Hind tibial length

For adult flies, the body-length measurement was replaced by the length of the forewing.

2.3.2. Temperature Measurement

Temperature has been found to be an important factor in controlling growth and emergence patterns in Plecoptera (Nebeker & Gaufin, 1967; Nebeker, 1971; Hynes, 1976). Temperature readings were taken, at the time of sampling, with a mercury in glass thermometer. However, such spot readings are of limited value since they cannot be representative of the actual temperature regime of the stream (Macan, 1958a).

To overcome this problem of measuring the long term temperature patterns in remote sites, a method based on a controlled chemical reaction was used (Ashworth, 1980). This employs the acid hydrolysis reaction of potassium ethyl xanthate solution (KEtX) which is strongly temperature dependent.

A known weight of KEtX (0.5 gramme) is allowed to hydrolyse in an aqueous buffer solution (50 millilitres) in a bottle, left in situ for the measurement period. Then nickel sulphate solution is added to precipitate the remaining xanthate as the insoluble nickel salt. After filtering and drying, the weight of the salt is converted to a corresponding mean temperature by reference to a calibration curve obtained at known constant temperatures in the laboratory.

The pH of the phosphate buffer solution was increased from pH 6.2 to pH 7.2 to increase the reaction time from one to ten weeks, in accordance with the recommendations in Ashworth (1980).

Three replicate bottles were firmly anchored to the stream bed at each site, in a position that ensured a constant covering of water for the ten week measuring period.

The reaction mixtures were made up in the laboratory and transferred to and from the Peak District in a cooled ice box to prevent any variations in heating during transportation.

2.4. Electrophoresis Techniques

Whole animals were ground up individually with 1-2 drops of 0.02 M Tris-HCl buffer pH 8.0 in a 24 well perspex block using a hand-held glass rod. The homogenate was either absorbed on to small squares of Whatman No.3 filter paper for starch-gel electrophoresis, or mixed 1:1 with 10 % sucrose solution and loaded on to vertical polyacrylamide gels using a 25 microlitre micro-pipette.

Horizontal starch-gel electrophoresis was carried out using 11 % Connaught starch gels and Shandon equipment. Four buffer systems were used as follows :

1) Continuous tris-citrate (Ward & Beardmore, 1977). Electrode : 0.25 M tris, 0.057 M citric acid, pH 8.0. Gel : 25:1 dilution of electrode buffer. Running conditions : 200 V for 4.5 hours.

2) Discontinuous tris-citrate (modified from Ashton & Braden, 1961). Electrode : 0.3 M boric acid, 0.1 M sodium hydroxide, pH 8.9. Gel : 0.066 M tris, 0.007 M citric acid, pH 8.6. Running conditions : 150 V for 1 hour, the 300 V for 4 hours.

3) Discontinuous tris-citrate (Poulik, 1957). Electrode : 0.3 M boric acid, 0.06 M sodium hydroxide, pH 8.6. Gel : 0.076 M tris, 0.005 M citric acid, pH 8.2. Running conditions : 200 V for 1 hour, then 300 V for 4 hours.

4) Continuous tris-citrate (Ayala et al,1972). Electrode: 0.135 M tris, 0.045 M citric acid, adjusted to pH 7.0. Gel : 15:1 dilution of electrode buffer. Running conditions : 200 V for 4.5 hours.

Vertical polyacrylamide gels (7 % Sigma gelling agent, 0.1 % ammonium persulphate + Temed.) were run in an LKB 2001 vertical electrophoresis unit. The buffer system was :

5) Discontinuous tris-glycine (modified from Davis, 1964). Electrode : 0.05 M tris, 0.383 M glycine, dilute 1:9 buffer:distilled water for use, pH 8.5. Gel : 0.19 tris, 30 millilitres 1 N HCl, 0.15 Millilitres Temed, per litre, pH 8.9. Running conditions : 50 V for 30 minutes, then 300 V for 4 hours.

Forty-four enzyme staining techniques were tried, adapted from Harris and Hopkinson (1976) and Shaw and Prasad (1976).

For the comparisons between populations of Nemoura cinerea, only four polymorphic loci were routinely screened :

Aminopeptidase-1

Hexanol dehydrogenase

Phosphoglucose isomerase

Phosphoglucomutase

2.5. Measurement of pH Tolerance

2.5.1 Apparatus

The tolerance of *Nemoura cinerea* nymphs to low pH was investigated using specially constructed, plastic 'bioassay chambers' (Figure 2.4). Every chamber was divided into three separate compartments, each aerated through an inverted syringe needle. The tops of the chambers reduced evaporative water loss and prevented the escape of emergent adults or roving nymphs.

2.5.2. Experimental Procedure

Acid tolerance was determined using 96 hour acute toxicity tests (APHA,1976; Murphy, 1978; Bulkema *et al*, 1982) to find the concentration that would kill half the test organisms in this time. This is the median lethal concentration (96 hour LC50).

Field samples were returned to the laboratory for sorting to ensure that all the nymphs used in the tests were similar in size. Nymphs were acclimated to experimental conditions for four days prior to the toxicity tests. They were held, unfed, in a 2 litre beaker containing 'artificial' water at pH 6.5 (ionic composition as given in section 2.6.2. except for the addition of calcium ions). The beaker was kept aerated inside a Fisons' growth cabinet at 10°C and 16 hours:8 hours light:dark lighting conditions.

Twenty-four hours before the experiment, the bioassay chambers were filled with 'artificial' water and the pH adjusted using 0.1 M sulphuric acid. These were placed inside the growth cabinet and aerated. A pH range of pH 2.8 - 4.0,

with 0.2 pH unit intervals, was used in all the tests. The control was unadjusted 'artificial' water. All three compartments in each chamber had the same pH.

Ten nymphs were placed in each compartment along with a piece of cotton netting to provide an artificial substratum. This arrangement ensured that there was no overcrowding and made it easier to count the number of survivors. In some tests, a shortage of specimens meant that all three compartments were not used.

The numbers surviving were checked every twelve hours and dead animals removed until the test was finished after ninety-six hours. At each count the pH was checked and adjusted if necessary. If there was greater than 10 % mortality in any of the controls the test was terminated.

In the tests using nymphs from alkaline streams, all the nymphs were frozen either when they died or at the end of the test to determine their HDH-1 genotype later.

Parallel tests in which the ionic composition of the test water was doubled, or the concentration of the calcium ion (added as calcium nitrate solution) altered, were run on three occasions to determine if this could influence tolerance to low pH.

2.6. Respirometry

2.6.1. Apparatus

An open-flow respirometer was constructed to measure the possible influence of low pH on the oxygen consumption of *Nemoura cinerea* nymphs. The design was based on that used by

Nagell (1975) and is shown in Figure 2.5.

The cylindrical vessel A (made of perspex, with a volume of 1.5 litres) contained the experimental water. The glass float B was 3 centimetres deep with a diameter 4 millimetres smaller than the inside of the vessel. The float was weighted so that the lower 2 centimetres were submerged. This created an effective boundary to oxygen diffusion into, or out of, the reservoir, since convective currents within the vessel were minimised by placing the whole apparatus in a water-bath, ensuring even temperature distribution.

Water was drawn out of vessel A by means of the peristaltic pump C. This was electrically driven and could not be submerged in the water-bath. Initial tests with the apparatus showed that water warmed up as it passed through the pump, reducing its oxygen carrying capacity and leading to the formation of air bubbles which could then pass into the rest of the system. To overcome this problem, the pump was enclosed in an insulated box and cooled with a commercial ice-pack.

The water passed from the pump into the respiratory chamber D, which contained the experimental animals. The respiratory chamber was the bulb of a 25 millilitre glass pipette. The animals were prevented from escaping by nylon mesh fixed at the downstream end of the chamber (E), and a removeable plug with nylon mesh at F.

After passing through the respiratory chamber, the water flowed to the oxygen electrode, G . This was a Clark, membrane covered type, manufactured by Rank, with a replaceable, 0.0005 millimetre teflon membrane. The electrode was connected to an MSE 'Spectroplus' to measure the dissolved

oxygen concentration in the water. The 'Spectroplus' was connected to a Philips PM8251 pen recorder to give a continuous record of the oxygen concentration throughout the experiment. The stopper for the electrode, H, was modified to allow water to flow through the system. The flow rate was measured with a 25 millilitre measuring cylinder. The volume of the electrode chamber was 4 millilitres. Water in the chamber was circulated using a magnetic stirrer.

The original dissolved oxygen concentration in vessel A was measured by switching tap I so that only water by-passing the respiratory chamber entered the oxygen electrode. The flow through the respiratory chamber was maintained via outlet J. The speed of the pump was doubled whilst the water was passing along the two routes to keep the flow rate through the electrode constant. The oxygen concentration in water passing through the respiratory chamber was measured by repositioning tap I , reducing the output from the pump, and preventing outflow through J by raising it above the level of the outflow from the oxygen electrode.

The temperature of the water bath, containing the respiratory chamber and vessel A, and of the insulating jacket surrounding the oxygen electrode, was maintained at 10°C with a Churchill 'Thermo-circulator'.

The outlet K, from vessel A, was used to take samples of water to determine the oxygen concentration by the standard Winkler method (Golterman, Clymo & Ohnstad, 1978).

Narrow diameter (1.5 millimetre) 'Portex' nylon tubing was used for inter-connecting the major components of the system to minimise the dead volume.

2.6.2. Experimental Procedure

After sorting, freshly collected nymphs were acclimated to experimental conditions for four days. They were held in a two litre beaker containing well aerated 'artificial' water at pH 6.5, and maintained at 10°C under 16hours : 8 hours, light : dark illumination in a Fisons growth cabinet. Cotton netting was spread in the beaker to provide an artificial substratum.

Twenty-four hours before the experiment, the nymphs were transferred to water with a pH ajusted to either 3.0 using 0.1 Molar sulphuric acid, or to 7.0 using 0.1 Molar sodium hydroxide solution. This allowed acclimation to the experimental pH, and also ensured that the digestive tracts of the nymphs were emptied to prevent the deposition of faecal pellets in the respiratory chamber.

The 'artificial' water used in the experiment was maintained under the same conditions of temperature and aeration prior to the respirometry. The water was made up from a stock solution and diluted with deionised water (10 millilitres stock per litre) when required. The composition of the stock solution was as follows:

Sodium Chloride	3.0	grammes/litre deionised H^2O
Magnesium Sulphate	1.4	"
Potassium Bicarbonate	0.2	"
Ammonium Sulphate	0.1	"

The final ionic concentrations were intended to be similar to those found at Lady Clough in the Peak District (Brown & Martin, 1980).

Animals were introduced into the respiratory chamber after removing plug F. A piece of cotton netting was also

placed inside the chamber. This acted as a substrate for the nymphs and reduced their level of activity.

After sealing the chamber, tap I was positioned so that water flowed directly from vessel A to the oxygen electrode to record the initial oxygen concentration. A 250 millilitre sample was drawn off through K for Winkler titration. Approximately one hour was allowed for the animals to settle and to check the stability of the electrode before tap I was repositioned to determine the oxygen concentration in the water passing through the respiratory chamber.

After six hours the dissolved oxygen concentration in vessel A was re-checked before the water was drained via outlet K and replaced with fresh water of different pH. During this operation the pump was stopped to prevent air being drawn into the system. Fresh water was used, rather than changing the pH of the existing water, because rapid acidification can raise the concentration of dissolved carbon dioxide to levels known to affect the activity of aquatic invertebrates (France, 1982). A 250 millilitre sample was taken from the replenished reservoir for Winkler titration and the oxygen concentrations measured as before.

Regular checks were kept on the temperature and flow rate throughout the experiment. Flow rate tended to decrease gradually as the water level fell in reservoir A. This was caused by the increased effort needed to pump the water as the height difference between the reservoir and pump grew. Unfortunately, the pump control was limited to five pre-set speeds so that fine-scale adjustment was impossible. The problem was overcome by putting the pump on an adjustable platform so that its height could be changed to compensate for

the fall in water level in A.

Previous workers (in Hynes, 1970; Correa et al, 1983) have reported that flow rate can be a major influence on the rate of oxygen uptake in some, though not all, aquatic invertebrates. However, the results of preliminary investigations showed that flow rate had no effect on oxygen uptake in N. cinerea under the slow flow conditions used in these experiments, although the speed of flow through the respiratory chamber did appear to affect the mixing of the water. This resulted in some oscillation of the read-out from the oxygen electrode under the slowest flow conditions (around 45 ml/hr). The rate of flow itself had no effect on the stability of the electrode read-out when the flow by-passed the respiratory chamber. A higher flow rate of around 100 ml/hr was used whenever possible. However, when small nymphs were used, only a small reduction in the dissolved oxygen concentration could be achieved at this flow rate due to problems in collecting a sufficient number of similar sized specimens. According to Nagell (1975) and Forstner (1983), the precision of measurement of the dissolved oxygen concentration is diminished when there is a small difference between the initial and final concentrations. Therefore, the flow rate was reduced to produce a concentration difference of between 5 and 10 %. A similar reduction in dissolved oxygen concentration was used for the larger specimens at the flow rate of around 100 ml/hr. A greater reduction was not used because, although the difference in oxygen concentration could be measured more precisely, nymphs at the downstream end of the respiratory chamber would be subjected to different conditions than those upstream, and they could be under

respiratory stress. Nagell (1975) considered that a reduction of about 10 % was an appropriate compromise between measurement precision and the need for uniform conditions in the respiratory chamber.

A further consideration at the start of each experiment was the speed of the magnetic stirrer in the oxygen electrode. If this was too slow, or so fast that it became erratic, small oscillations were produced in the electrode read-out.

After the respirometry the nymphs were dried at 100°C for 24 hours and weighed.

Table 2.1. Sites where Nemouridae were found.

	Site	O.S. grid ref.	Species 1	2	4	5	6	7	8	9
1	River Alport	SK142896	o	x	-	-	x	x	-	o
2	River Ashop	SK108907	o	x	-	-	x	x	-	o
3	Beacon	SK522147	*	-	-	x	-	-	-	-
4	Birchin Clough	SK109914	o	o	-	-	x	-	-	-
5	Blackstone Edge	SD988184	*x	-	-	-	-	-	-	-
6	Burbage Brook	SK262808	*x	-	-	-	-	-	-	-
7	Nant y Bustuch	SN807528	-	-	-	-	x	-	x	-
8	Cabin Clough	SK072933	*x	x	-	-	o	-	-	-
9	Charnwood	SK518155	-	x	-	-	-	-	-	-
10	Llynn Cwellyn	SH566545	-	-	x	-	-	-	-	-
11	Dalnacarn	NO003628	-	-	-	-	-	-	x	-
12	Dean Clough	SE082063	*	-	-	-	-	-	-	-
13	River Derwent	SK171946	o	x	-	-	x	x	-	x
14	Nant y Fannog	SN811516	o	-	-	-	x	-	x	-
15	Franklyden	NO216294	o	x	-	-	-	-	-	-
16	Glebe Farm	SK739032	*x	x	-	-	-	-	-	x
17	Glen Rait	NO208278	-	-	-	-	-	-	-	x
18	Gordale	SD914638	-	-	-	-	-	-	x	-
19	Grinds Brook	SK123862	-	-	-	-	x	x	-	x
20	Hoby	SK667172	o	-	-	-	-	-	-	x
21	Holden Clough	SK083926	*x	o	-	-	o	-	-	-
22	Knock Ore Gill	NY715310	-	x	-	-	x	-	-	-
23	Lady Clough	SK108907	*	x	-	o	x	x	-	o
24	Lincoln.	TF168919	-	-	-	-	-	-	-	x
25	March Hill	SE003130	*	-	-	-	-	-	-	x
26	Moor House	NY737323	*	-	-	-	-	-	-	x
27	River Noe	SK112847	-	-	-	-	x	x	-	o
28	North Wales 3	SH964245	-	-	-	-	o	-	x	-
29	Odin Sitch	SK142834	-	o	x	-	-	-	-	-
30	Owston	SK782057	*x	-	-	-	-	-	-	x
31	Oyster Clough	SK118902	o	-	-	-	x	-	-	-
32	Rising Clough	SK215875	*x	x	-	-	x	-	-	x
33	Roman Road	SK103928	-	o	-	-	x	-	-	-
34	Scaleber Force	SD841625	-	-	-	-	-	-	x	-
35	Thomasons Holl.	SK096927	*	x	o	-	x	-	-	x
36	Top Lady Clough	SK094928	*	x	-	-	o	-	-	o
37	Nant Trawsnant	SN805489	*	-	-	-	o	-	x	-
38	Thrussington	SK644163	*x	-	-	-	-	-	-	x
39	Wareham 1	SY919856	*x	-	x	-	-	-	-	x
40	River Westend	SK154928	-	-	-	-	x	x	-	-
42	Wymondham	SK737214	*x	-	-	-	-	-	-	x

continued

43	Moor House Mine	NY724319	*	-	-	-	-	-	-	-
44	North Wales 4	SH915228	*	-	-	-	-	-	-	-
45	Ogden	SD981127	*	-	-	-	o	-	-	-
46	Port Erin	SC192684	*	-	-	-	-	-	-	-
47	Shiny Brook	SE055067	*	-	-	-	-	-	-	-
48	Wareham 2	SY924891	*	-	-	-	-	-	-	-
49	Baluain	NN837658	*	-	-	-	-	-	-	-
50	Cressbrook Dale	SK172735	*	-	-	-	-	-	-	-
51	Lutterworth 1	SP598864	*	-	-	-	-	-	-	-
52	Lutterworth 2	SP530859	*	-	-	-	-	-	-	-
53	Millers Dale	SK140735	*	-	-	-	-	-	-	-
54	Nailstone	SK425084	*	-	-	-	-	-	-	-
55	South Beacon	SK508144	*	-	-	-	-	-	-	-

Species: 1, Nemoura cinerea; 2, N. cambrica; 4, N. avicularis; 6, Protonemura meyeri; 7, P. praecox; 8, Amphinemura standfussi; 9, A. sulcicollis.

The other species sampled was: 3, Nemoura dubitans : Cames Farm, South Winterbourne, Dorset, SY712889.

x = species present and screened electrophoretically for species comparisons.

o = species present but not screened electrophoretically for species comparisons

* = samples for inter-population comparisons of Nemoura cinerea

No entry indicates the species was not detected at that site.

Table 2.1.

Plate 2.1. Nemoura cinerea nymphs, × 10

Plate 2.2. Protonemura meyeri nymphs with prosternal gills, × 6

Plate 2.3. Adult Nemoura cinerea, × 10.

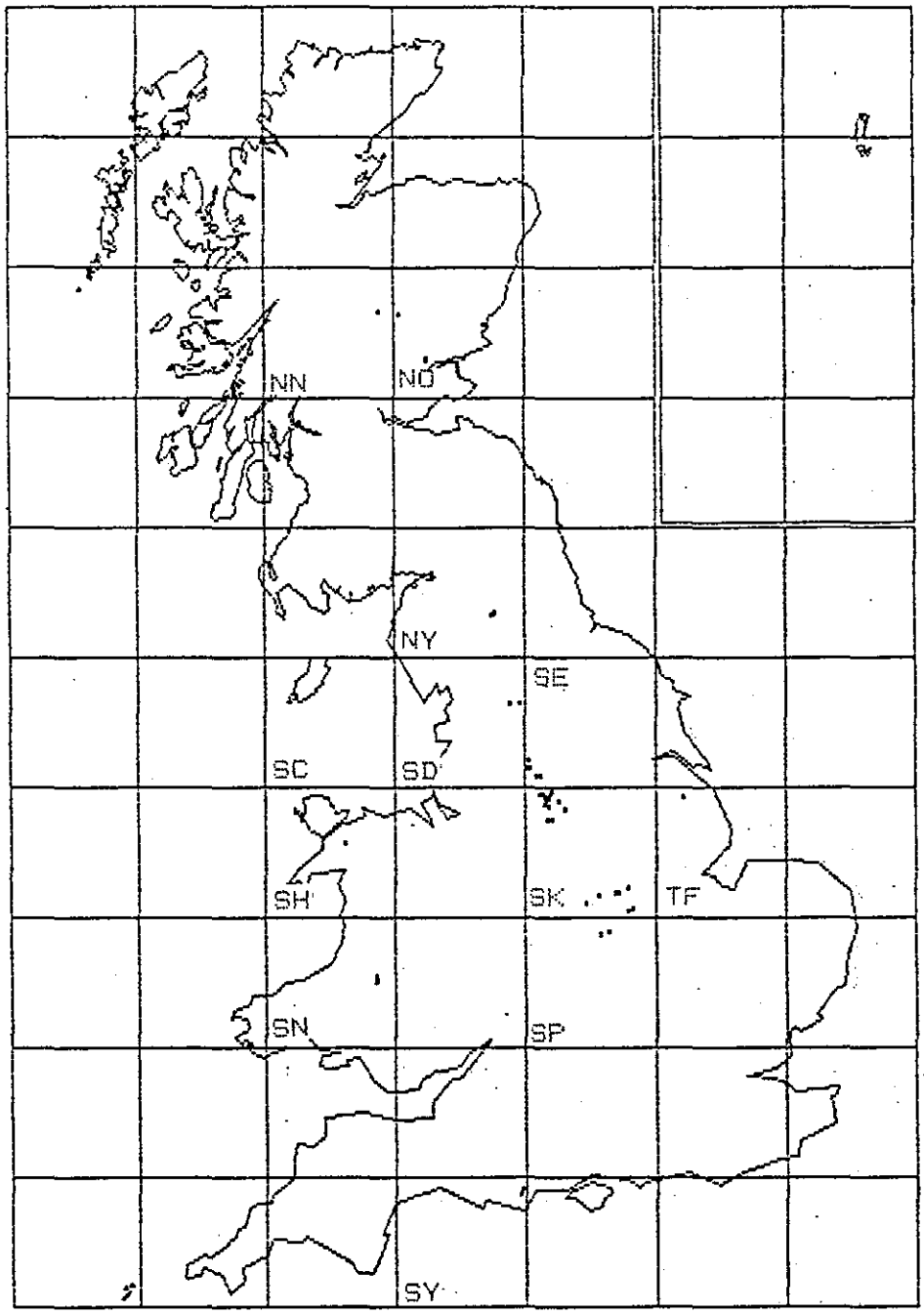


Figure 2.1. Map to show the location of the sample sites listed in Table 2.1.

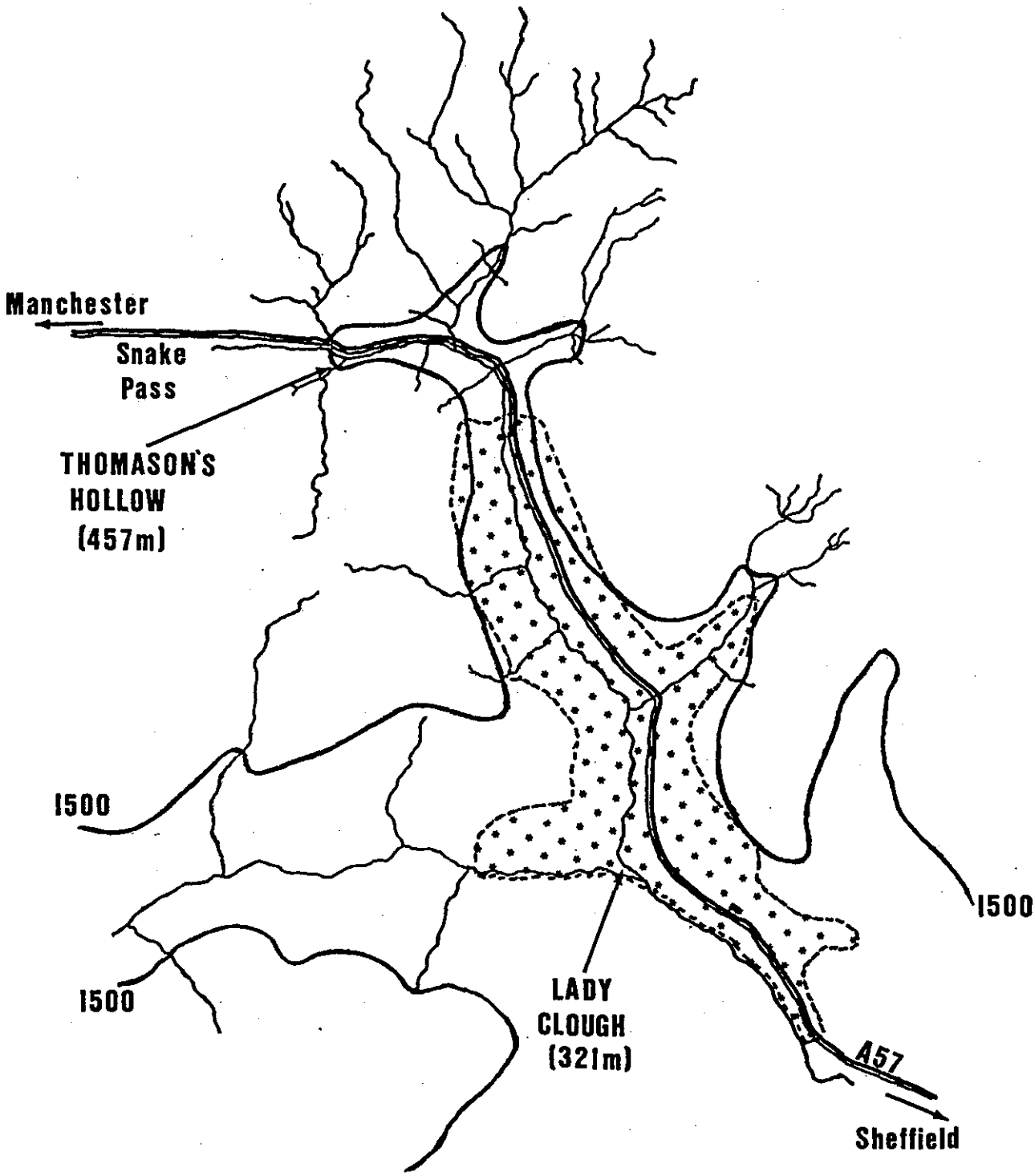


Figure 2.2. The Snake Pass Area of the Derbyshire Peak District Scale 1:50000.

Key

Wooded Area

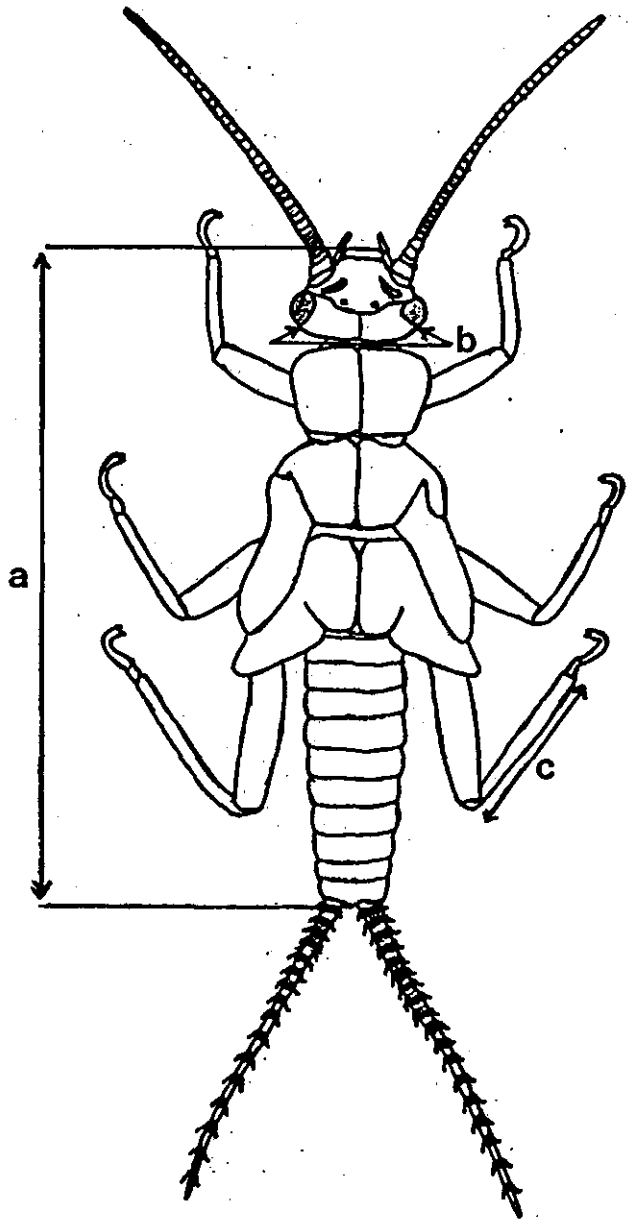


Figure 2.3. Measurements of Nemoura cinerea. a) Total body length, b) Head-capsule width, c) Hind tibia length.

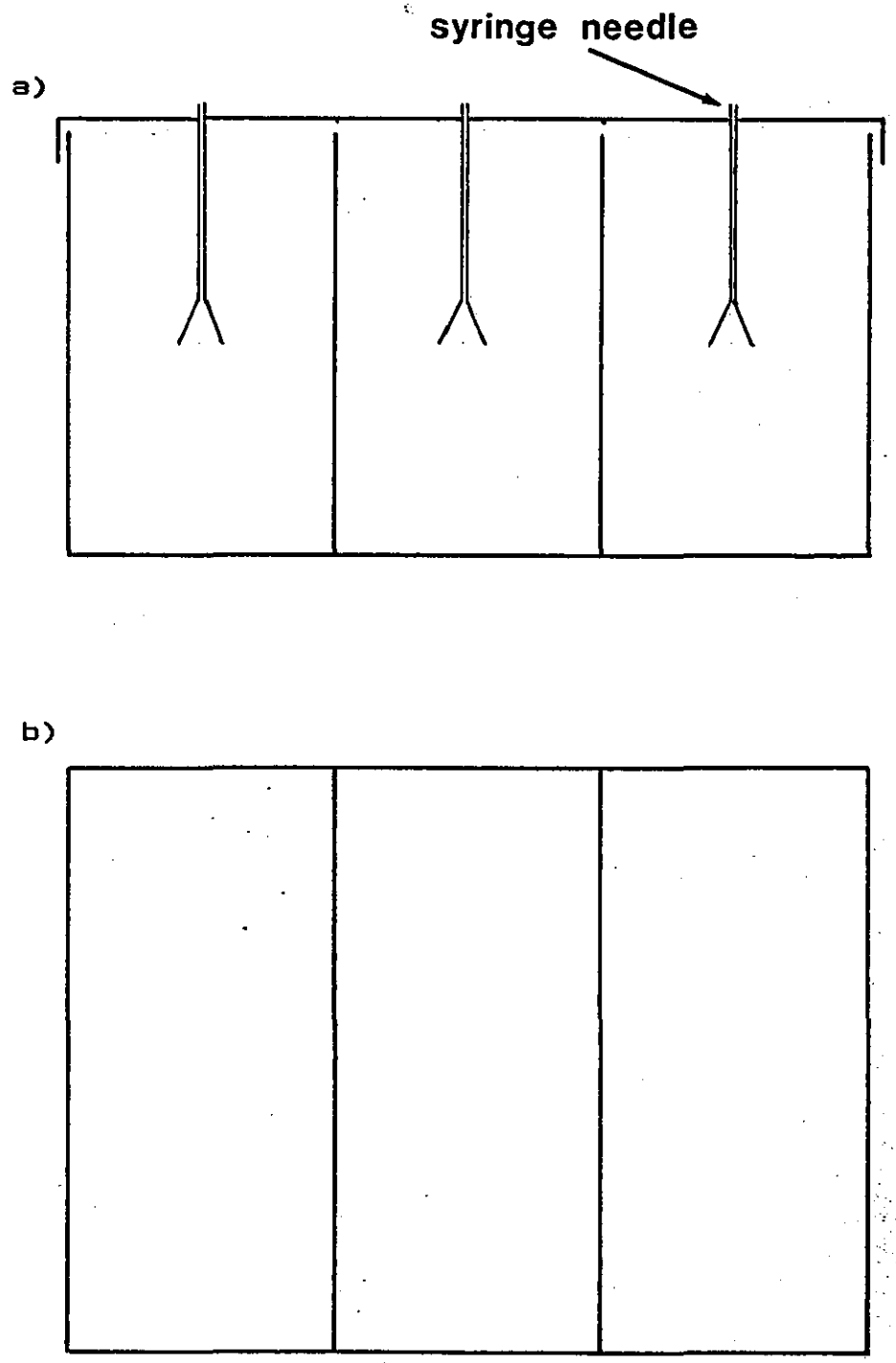


Figure 2.4. Bioassay chamber, a) transverse section, b) plan.

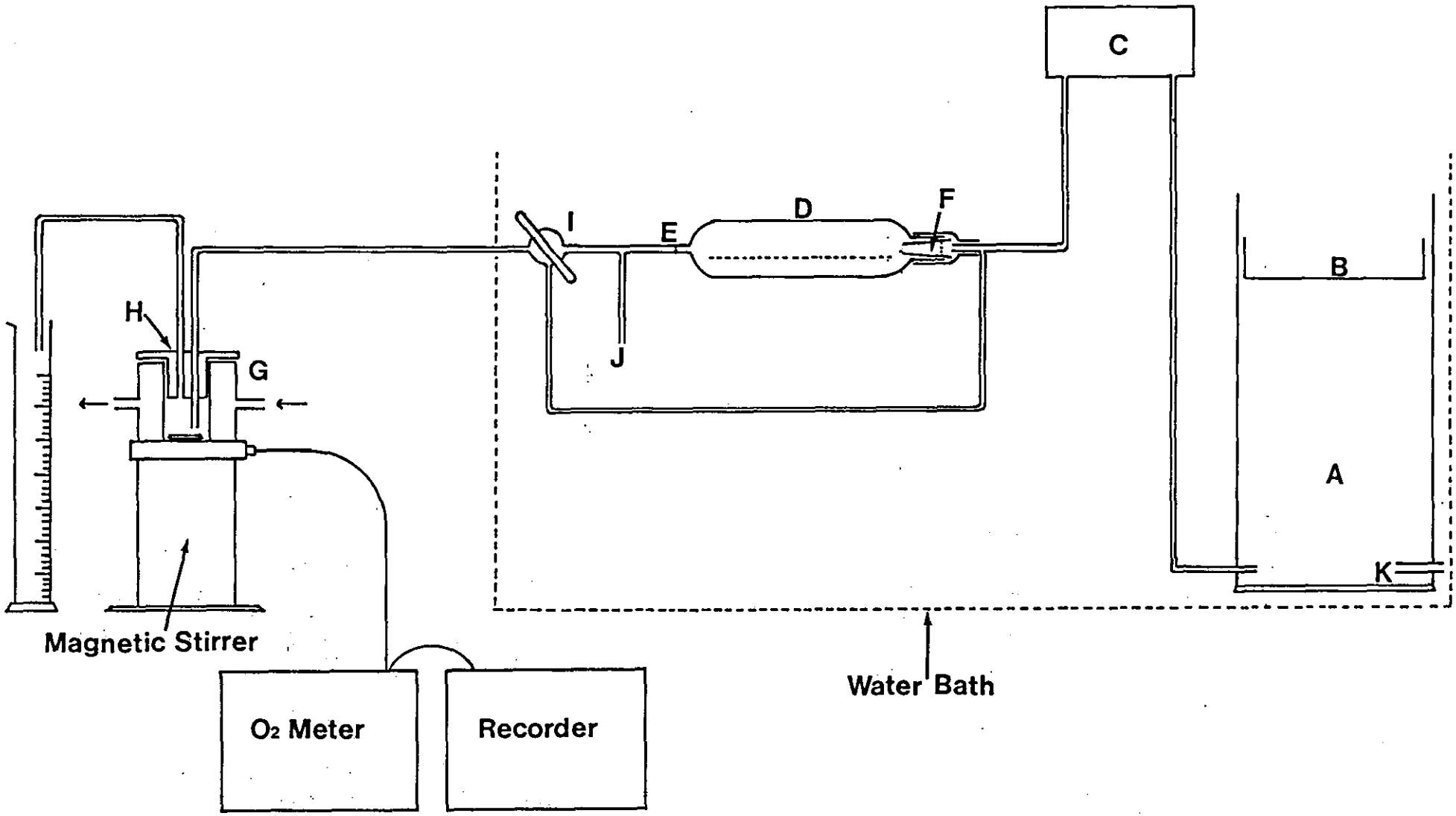


Figure 2.5. The flow-through respirometer.
A. reservoir, B. Float, C. peristaltic pump, D. respiratory chamber, E. mesh, F. plug, G. oxygen electrode, H. stopper, I. tap, J. and K. outlets.

Figure 2.5.

CHAPTER 3

LIFE-HISTORY PATTERNS OF NEMOURA CINEREA FROM TWO STREAMS

3.1. Introduction.

The first requirement of the investigation into the influence of stream water acidity on genetic variation was to determine which species could be found in both acid and circum-neutral streams. The aim of field trips in the early months of this study was, therefore, to locate streams in the Derbyshire Peak District which had a range of different acidities (pH) and to identify their Plecopteran communities.

During this period, an observation was made that the nymphs of some species could be found in the higher altitude streams for over a month after they had emerged as adults from lower altitude streams nearby. This was particularly noticeable in Nemoura cinerea from two sites in the same catchment area. Therefore, a program was initiated to investigate this observation further: to see if it was a recurrent feature in these streams and to identify possible causes.

Similar observations of altitude related differences in life-history have been reported from several areas (e.g. Brink, 1949; Gledhill, 1960; Maitland, 1966; Lillehammer, 1984). In all cases the consistently lower temperature of high altitude streams had been thought to be responsible for the retardation of the life-cycle.

Initial measurements taken at the two Peak District sites did not show any difference in temperature. This was taken as an indication that other factors might be more

important in this case. Previous studies have shown that differences in photoperiod (Nebeker, 1971) or food supply (Ulfstrand, 1968, and Lillehammer, 1974) may influence growth and emergence in Plecoptera, and a decrease in the growth rate of mayfly nymphs occurred following acidification of a stream (Fiance, 1978). It has also been suggested that alterations in the life cycle have resulted from genetic change in different locations (Macan, 1958b,1981; Brittain., 1973; Lillehammer, 1976). However, spot temperature readings have been shown to be poor indicators of the thermal budgets of streams (Macan, 1958a, and Smith and Lavis, 1975). Therefore, a more informative, longer term measure of the temperatures experienced at both sites had to be found in order to assess the importance of this factor.

3.2. Materials and Methods

3.2.1. Description of the sites.

The geographic location of the two streams has already been indicated in Figure 2.2. Both sites were close to the Snake Pass (A57) road in the Peak District. The sampling site on Lady Clough (SK 108907) at 321 metres above sea level is 136 metres lower and approximately 3 kilometres down stream from the site at Thomason's Hollow (SK 096927). The relative sizes of the two streams can be seen from Plates 3.1 and 3.2.

Thomason's Hollow is a headwater stream draining the peat moorland plateau around Featherbed Top (SK 091921). The dominant vegetation of this area is cotton sedge (Eriophorum vaginatum) and grasses such as Festuca ovina and Nardus stricta furnish rough grazing for sheep. The stream is between

0.5 and 1.5 metres wide. The substratum consists of bedrock and medium sized boulders (25 to 50 centimetres in diameter). Moss (Fontinalis) is found growing in the stream in patches where the bottom is stable.

Lady Clough is a much larger stream 5 to 10 metres in width. The gradient of the stream bed is less and the substratum mainly consists of smaller boulders (15 to 30 centimetres in diameter). Here again moss is found growing in patches where the substratum is stable. Figure 2.2 and Plate 3.2 show that the dominant vegetation in the valley is a coniferous woodland plantation.

The pH and conductivity of the streams were measured throughout the study period when invertebrate samples were collected. Figures 3.1 and 3.2 show that both measurements often varied considerably between sampling dates. A similar pattern of fluctuating pH values, particularly from Lady Clough, was reported by Brown and Martin (1980). They found a significant inverse relationship between pH and stream level. Observations made in the present study are in agreement with this; the lowest pH values at Lady Clough all being recorded when stream flow was high following periods of heavy rain and the high pH values at both sites occuring during periods of low flow.

The overall mean pH and conductivity at Thomason's Hollow was pH 4.54 (range 3.65 to 6.2) and 80.7 μS^{-1} (range 52 to 130 μS^{-1}) respectively. The corresponding values for Lady Clough were pH 5.94 (range 4.4 to 7.0) and 101.7 μS^{-1} (range 81 to 140 μS^{-1}).

3.2.2. Calibration of the Potassium Ethyl Xanthate measurement of temperature.

The method for calibrating the rate of hydrolysis of potassium ethyl xanthate (KEtX2) in the buffer solution was basically the same as that described by Ashworth (1980). Two water baths were used to maintain constant temperatures. One was filled with a mixture of water and ethylene glycol and kept at 0°C ± 0.5°C for the ten week measurement period with the aid of a chiller unit. The other water bath was maintained at 20°C ± 0.5°C. Two replicate bottles containing the reaction mixture were placed in each bath and weighted down to ensure that they remained fully submerged.

After exactly ten weeks the reaction was halted by adding excess nickel sulphate solution and the dry weight of the precipitate measured to the nearest milligramme. The weights of the precipitates obtained at 0°C and 20°C could then be used to calculate expected weights at the other temperatures so that a calibration curve could be constructed by substitution into the equation derived by Ashworth (1980).

The above method overcomes the problem of maintaining a series of constant temperature environments in order to construct a calibration curve. However, two additional water baths, one at 12°C ± 1°C and the other at 25°C ± 1°C, were used to compare the results obtained with those predicted by interpolation and extrapolation from the calibrations at 0°C and 20°C.

3.3. Results.

3.3.1. Temperature.

The temperature readings taken on sampling dates between July 1981 and August 1983 are shown in Table 3.1 and Figure 3.3. The monthly air temperature records from the nearest Meteorological Office Station at Buxton, 17 kilometres from the Snake Pass, at an altitude of 307 metres, are included in Figure 3.3 for comparison. It can be seen that stream temperature tracked air temperature quite closely throughout this period. A similar observation was made by Smith and Lavis (1975) in their more detailed investigation of the environmental influences on the temperature of a small upland stream.

The dry weights of the nickel ethyl xanthate (NiEtX2) precipitated after the calibration runs at 0°C and 20°C are given in Table 3.2 together with those at 12°C and 25°C. The values obtained at 0°C and 20°C were substituted into the equation derived by Ashworth (1980) to calculate expected values at other temperatures as follows:

$$\log10(\log e(yo/y)) = -E/2.303\ R\ T + \text{constant} \qquad (1)$$

where, yo = dry weight of NiEtX2 obtained at time zero .

y = dry weight of NiEtX2 after 10 weeks at temperature TK

E = activation energy of the reaction

R = the gas constant

T = temperature in K

At 0°C :

$$\log_{10}(\log_e(.462/.369)) = -E/2.303 * 8.3 * 273.15 + C$$

Therefore, $$-E = -3384.926 - 5221.2349C \quad (2)$$

At 20°C :

$$\log_{10}(\log_e(.462/.087)) = -E/2.303 * 8.3 * 293.15 + C$$

Therefore, $$-E = 1243.424 - 5603.5329C \quad (3)$$

By solving (2) and (3) simultaneously,

$$4628.3506 = 382.298C$$

Therefore, $$C = 12.1067$$

Substituting in (2),

$$E = -66.597 \text{ KJ.mol}^{-1}$$

Using the above values of E (in Joules.mol^{-1}) and C, and equation (1), the dry weight of NiEtX2 expected at other temperatures could be calculated, assuming that E remains constant (Table 3.3), and the theoretical graph of the variation of y with temperature was constructed (Figure 3.4). The curve passes through the values of y obtained at 0°C and 20° C since these were used in it s construction. The values at 12°C and 25°C also fall near the calculated line giving an indication of the accuracy of this method.

The dry weights of NiEtX2 obtained from the bottles left in the streams for 10 week periods, and the corresponding mean temperatures calculated from Figure 3.4. are shown in Table 3.4. Unfortunately, the bottles were lost from one of the sites during the first two measurement periods. However, it can be seen that in the following 30 weeks the mean

temperature at Lady Clough was consistently higher than at Thomason's Hollow. There was a particularly large difference in February and March 1983.

3.3.2. Life-Histories.

The annual growth pattern of N. cinerea in both streams can be seen, for three different body parameters in Tables 3.5 and 3.6 and Figures 3.5 to 3.7. Each point on the growth curves is the mean of the sample measured on that date and the vertical lines indicate the size range of the nymphs. Three different size measurements were used to record the growth of the nymphs to act as controls for each other and to see if any would prove easier or more reliable to use. A straight line relationship was found between the three measurments from a plot of head capsule width and hind tibial length against body length (Figure 3.8.), using the mean figures given in Tables 3.5 and 3.6. The regression equations of the straight lines are given in Table 3.7. together with the correlation coefficients. All four correlations were found to be highly significant ($P < 0.001$).

The morphology of the nymphs was compared between the two streams by a test of the homogeneity of the regression coefficients (Bailey,1959). The null hypothesis Ho : b1 - b2 = 0 was tested where b1 = the coefficient of regression between head capsule width and body length at Lady Clough and b2 = the coefficient of regression of the same measurement at Thomason's Hollow. A similar test was made between the other two regression coefficients. In both cases the null hypothesis was accepted indicating that nymphs had the same morphology throughout the size range in both streams (Table 3.7).

The close correlation of the measurements is reflected in the similarity of the growth curves (Figures 3.5. to 3.7.). Of the three characters, head capsule width was the easiest to measure. The hind leg had to be removed before the tibia could be measured and the abdomen is often distorted and telescoped making body length more difficult to measure.

Figures 3.5 to 3.7 clearly indicate that N. cinerea had a univoltine life history (one complete life cycle per year) in both streams. This is in agreement with previous records both in the United Kingdom (Hynes, 1941, 1961) and continental Europe (Brink, 1949, and Bengtsson, 1984).

The flight period of the adult flies (the dates between which adults were collected from the streams) is shown on Figure 3.7. Although there was some variation between years, there was a consistent pattern of late adult emergence at Thomason's Hollow compared with Lady Clough. In 1981 adults were found from April to July at Lady Clough and from May to August at Thomason's Hollow. In 1982 and 1983 adults emerged from Lady Clough in May, June and July. At Thomason's Hollow adults were collected from June to September in 1982 and from June to the last sampling date on August 8th in 1983.

The first small nymphs were found from early September at both sites, 3 or 4 months after the adults began to emerge. This suggests that the egg incubation period takes about the same length of time, and accounts for the appearance of small nymphs in all samples until the end of the year (3 to 4 months after the last adults were recorded). Previous workers have found egg incubation periods of 3 to 4 months in N. cinerea (Khoo, 1964, and Bengtsson, 1984). The persistence of small nymphs in the spring samples, most noticeably in 1981, could

have resulted from either the slow growth of nymphs hatched in the coldest months of the year, or from the delayed hatching of some eggs. The occurence of an egg diapause has been reported in other Plecoptera (Maitland, 1966; Elliot, 1967, and Hynes, 1970).

The long hatching period of the eggs results in the wide size variations in each sample. The variation can be most easily seen from the size group histograms drawn in Figures 3.9. and 3.10. The length of the hind tibia was used to construct the histograms because, of the three measurements taken, this one showed the greatest difference between adult flies and the nymphs (Figure 3.8), so that the size variations of both adults and nymphs could be clearly shown in the same Figures.

The extended recruitment of the nymphs will reduce the mean size in each sample, making it difficult to assess the actual growth rates. One possible way of overcoming this would be to find the mode in each sample so that the change in the size attained by the greatest number of nymphs can be followed throughout the life cycle (Macan,1958b; Elliot, 1967). However, the small sample sizes in this study meant that there was no obvious modal class on many occasions (Figures 3.9. and 3.10.). Alternatively, the growth rate of the earliest hatched group of nymphs can be inferred from the maximum size attained on each sample date.

The variations between the years and the occurence of unusually low temperatures in the winter of 1981/2 (Figure 3.3.) make it difficult to determine a general pattern. However, if the 1981/2 generation is excluded, it appears that growth was continuous from hatching in autumn through to

emergence in spring or summer (Figures 3.5. to 3.7.). There was some slow down in winter, particularly in Thomason's Hollow, but there was no evidence of a winter diapause unlike the findings of Khoo (1964). Nymphs could be found at Thomason's Hollow for more than a month after they had disappeared from samples taken at Lady Clough in each summer. Late hatching nymphs presumably grew at an accelerated rate as the temperature rose in spring in order to emerge in summer. They also seemed to mature at a faster rate so that they were not as large as the early emerging adults (Figures 3.9. and 3.10). Khoo (1964) showed that in Capnia bifrons there was a similar decrease in the size of emerging specimens as the season progressed. He suggested that this was caused by the increasing day length hurrying nymphs to emerge before growth is completed to avoid high summer temperatures.

In December 1981 and January 1982 Britain experienced extreme winter conditions. Air temperatures below -10°C were recorded at Buxton in both months. Thomason's Hollow was completely frozen over and both anchor and slush (frazil) ice was encountered at Lady Clough. Figures 3.5. to 3.7. show that these conditions resulted in a marked slow down in the growth of the nymphs, especially at Thomason's Hollow, and later adult emergence compared with other years.

3.4. Discussion.

Continuous monitoring of the growth and development of N. cinerea over a three year period has confirmed that each year, adults emerge from Lady Clough a month or more before emergence begins at Thomason's Hollow (Figure 3.7.). Other workers have reported a similar effect of altitude on the life cycles of Plecoptera. Gledhill (1960) reported that Lectra inermis, L. hippopus and Chloroperla torrentium emerged a month earlier and over a shorter period from a lowland stream (altitude 45 metres) than from a high mountain stream (altitude 669 metres). Maitland (1966) found that the nymphs of Amphinemura sulcicollis took longer to develop in the extreme upper reaches of the River Endrick in Scotland. Minshall (1969) determined that nymphs of Capnia vidua, Chloroperla torrentium and Leuctra hippopus persisted longer (by at least a month) at higher elevations in Gaitscale Gill in the English Lake District. Nebeker (1971) found that the emergence of several species was delayed by 4 to 6 months at high elevations in the Rocky Mountains and Lillehammer (1975a) recorded a difference of 76 and 96 days between the first emergence of L. hippopus and Protonemoura meyeri respectively from a coastal stream and a high altitude stream in Norway. Lillehammer (1984) also reported delayed emergence at high altitude in two other stoneflies, Isoperla obscura and Amphinemura standfussi, in Norway. In each case, the lower temperatures at high altitude have been proposed as the most likely explanation for the delayed emergence. However, other factors such as food supply (Ulfstrand, 1968, and Lillehammer, 1975,1984), acidity (Flance, 1978) or photoperiod (Hynes, 1970; Nebeker, 1971) may be of equal importance. The

temperature difference revealed using the KEtX2 method seems to support the view that temperature is an important factor regulating growth and development in aquatic insects.

This study has shown that the KEtX2 method can be a useful and reliable means of monitoring temperatures over extended periods at remote sites. On each occasion the replicate bottles gave similar results and the estimates of mean temperature were in the range expected from the air temperatures and spot readings. The main problem in the field was to anchor the bottles to the stream bed so that they were not lost or damaged during spates or left exposed during periods of low flow.

The loss of bottles from Thomason's Hollow in November 1982 and from Lady Clough in January 1983 meant that mean temperatures could only be compared between the sites from the beginning of February to the end September. Throughout this period the mean temperature was lower at Thomason's Hollow, especially from 1.2.83. to 13.4.83. (Table 3.4.). The northerly aspect of Thomason's Hollow, together with the higher altitude, could account for the difference in temperature for most of the year but the extra reduction in the winter months is probably caused by the persistence of snow in the deep peat groughs on Featherbed Moss which feed into Thomason's Hollow.

This method of temperature estimation gives a more accurate indication of the differences in the thermal budget of the streams than spot temperature readings. The latter gave a useful check on the chemical determination. However, they do not measure the wide diurnal fluctuations that commonly occur in streams or local variations caused by different conditions

of flow (Smith and Lavis, 1975). The chemical method also has an advantage over maximum and minimum thermometers for the estimation of mean temperatures over long periods. The thermometers only indicate the range that has occured since they were last reset. They do not give a continuous measurement of temperature change. In addition the chemical estimate does not risk the loss or damage of expensive instruments left in the field. The major disadvantages of using the KEtX2 method are that there will be recurrent costs for the chemicals, and the time needed to prepare chemicals and weigh the end product may become prohibitive if a large number of streams were to be monitored.

It appears, then, that the delay in adult emergence at Thomason's Hollow can be explained by the consistently lower temperatures relative to Lady Clough. The detail of how this actually occurs is not clear due, in part, to the difficulties of determining rates of growth from the samples measured. However, the greatest temperature difference occurs in the winter when Thomason's Hollow receives inflow from snow filled groughs. This coincides with an apparent slow down in the growth of nymphs in Thomason's Hollow compared with Lady Clough and their development seems to lag behind from then on. This is in accord with Khoo (1964) who demonstrated that the growth of N. cinerea was considerably slowed by cold temperatures in the laboratory. Ward and Stanford (1982) have also found that cold may be responsible for slowing development in stoneflies. Brink (1949) recorded a similar effect of climate on life history. He found that many species had a progressively later emergence from southern to northern Sweden associated with the increasing severity of the winter.

The difference in the timing of adult emergence does not seem to be due to the life cycle beginning earlier in the year at Lady Clough. Small nymphs first appeared in the autumn samples collected at the same time from each site and the similarity in size suggests that the eggs must have hatched at about the same time. The eggs must, therefore, take longer to develop and hatch at Lady Clough since the adult flight period occurs a month or more earlier than at Thomason's Hollow. The delay in egg hatching seems to be caused by the higher summer temperatures at Lady Clough. Khoo (1964) found that eggs of N. cinerea hatched more slowly at room temperatures than at lower temperatures and in Amphinemura standfussi and Brachyptera risi hatching was inhibited at temperatures above those that are commonly found in streams. Similar reductions in the rate of egg development with increasing temperature have been reported for several stoneflies and mayflies (Brittain, 1977,1982, and Brittain and Mutch, 1984).

Further evidence that temperature is a major influence on the life history of N. cinerea came from the effect of the severe winter of 1981/2. Following the period of intense cold in December and January, the growth of the nymphs appeared to have been markedly reduced. This was particularly evident at Thomason's Hollow where there was a decrease in the mean size from 3.51 mm to 2.63 mm body length (Table 3.5) due to the absence of the larger specimens in the January sample (Figures 3.5 to 3.7. and 3.9.). The loss of the biggest nymphs may have occured because they inhabit the undersides of large boulders and riffles in the stream bed where ice is likely to form most quickly. Smaller specimens can live deeper in the sediments where they are protected from ice by the percolation of ground

water (Hynes, 1970). The retardation of growth resulted in late adult emergence at both sites. A similar observation was made by Elliot (1967). Following the unusually cold winter of 1962/3 the emergence of several stonefly species was delayed and mortality was high amongst Protonemoura meyeri and Perlodes microcephala. Also Lillehammer (1975) reported that Diura bicaudata emerged over a month late from a Norwegian lake after a cold winter in 1967 and Macan (1981) found that the emergence of the mayfly Ecdyonurus was later following the persistence of cold weather in 1979.

The discovery of a marked difference in the annual thermal budgets of Thomason's Hollow and Lady Clough, and the observation that emergence was delayed following an unusually cold winter, supports the view that temperature is the most important factor controlling growth and emergence in stream insects. There is no evidence that any other factor is involved here. Differences in photoperiod can be ruled out due to the close proximity of the two sites and a difference in the quantity or quality of food items seems an unlikely explanation for the observed pattern since the organic input from the coniferous trees surrounding Lady Clough is of poor quality and non-seasonal.

Fiance (1978) reported a dramatic (30%) reduction in the growth of the mayfly Ephemerella funeralis in the USA following the addition of sulphuric acid to a stream. This was thought to have been caused by a greater energy demand for ionic regulation in the acidified section. A similar sub-lethal effect of environmental acidification could be influencing the growth of N. cinerea in Thomason's Hollow. Unfortunately, an experiment designed to test this failed due

to poor survival of nymphs under control (neutral) conditions. However, it seems less likely to be an important factor here for two main reasons: first, N. cinerea lives naturally in the conditions found at Thomason's Hollow and so is unlikely to be as stressed as E. funeralis was under the experimental conditions used; and second, there is a smaller difference in the pH between Thomason's Hollow and Lady Clough, particularly during peak flow conditions (Brown and Martin, 1980).

An additional explanation for the difference in life history could be that it has a genetic basis. Brittain (1973) suggested that this could account for differences in the growth rate of Nemoura avicularis in Sweden and Wales, and Lillehammer (1975) reported that Leuctra hippopus from one Norwegian population had different morphological characteristics and emerged earlier than those from other populations, even under controlled laboratory conditions. This indicated that a unique life-cycle had evolved in the unusual environment occupied by this population. However, there is no evidence to suggest that the delayed emergence from Thomason's Hollow is the result of a genetic change. Specimens from both streams are morphologically similar (Figure 3.8,Table 3.7), both populations showed a similar response to extreme winter conditions, and there was a wide variation in the duration of egg hatching and speed of development at both sites. In addition the two populations have very similar allele frequencies at the four polymorphic loci examined in this species (Tables 5.3-6., Figures 5.4-7.) and it is likely that there is appreciable gene flow between these two close sites in contrast with the isolated populations investigated by Brittain and Lillehammer.

TABLE 3.1. Temperature readings taken at Thomason's Hollow and Lady Clough. (°C).

Date	Lady Clough	Thomason's Hollow
5.07.81.	14.5	14.5
3.08.81.	15.4	14.3
8.09.81.	13.7	13.0
6.10.81.	8.0	8.0
10.11.81.	5.5	5.0
13.01.82.	0.8	0.8
26.03.82.	8.0	6.0
14.06.82.	12.0	11.0
6.07.82.	12.5	12.0
19.07.82.	15.0	13.0
13.08.82.	13.0	12.5
26.10.82.	9.0	9.0
1.02.83.	2.5	1.5
8.08.83.	13.5	12.5

TABLE 3.2. The dry weight of NiEtX2 (y) recovered after 10 weeks at controlled temperatures

Mean Temp.(°C) for 10 weeks	Dry weight(g) of NiEtX2 (y)	Mean y (g)
0 [1]	0.367 0.371	0.369
12	0.203 0.206	0.205
20 [1]	0.084 0.090	0.087
25	0.031 0.029	0.030
At 'zero' time (y)	0.464 0.460	0.462

(1) - used to construct calibration curve

TABLE 3.3. Expected dry weights of NiEtX2 calculated using equation (1)

Mean Temp. (°C)	Dry Weight of NiEtX2 (g)
0[1] (273.15K)	0.369
5	0.316
10	0.245
15	0.164
20[1]	0.087
25	0.033
30	0.008

(1) experimental values used to derive C and E in equation (1)

TABLE 3.4. The dry weight of NiEtX2 recovered after 10 week periods in the two streams and their corresponding mean temperatures

Measurement Period	Thomason's Hollow			Lady Clough		
	Dry Wt. (g)	Mean Wt. (g)	Temp. (°C)	Dry Wt. (g)	Mean Wt. (g)	Temp. (°C)
13.09.82.	---	---	---	.227	.227	11.00
to 23.11.82.	---			.224		
	---			.229		
23.11.82.	.294	.290	6.75	---	---	---
to 1.02.83.	.287			---		
	---			---		
1.02.83.	.320	.316	5.00	.272	.265	8.50
to 13.04.83	.311			.263		
	.315			.266		
9.05.83.	.134	.136	16.90	.135	.133	17.00
to 18.07.83.	.135			.129		
	.139			.132		
18.7.83.	.214	.209	12.25	.181	.182	14.00
to 27.09.83.	.204			.183		

TABLE 3.5. The mean size (head capsule width, hind tibial length and body length) of nymphs collected on the dates shown at Thomason's Hollow.

Sample Date	N	Measurement (mm)					
		Length		Capsule		Tibia	
		mean	S.D.	mean	S.D.	mean	S.D.
31.10.80	20	2.46	0.69	0.55	0.09	0.77	0.17
4.01.81	20	3.08	0.83	0.64	0.16	0.92	0.25
4.02.81	20	3.83	0.79	0.72	0.14	1.09	0.25
9.03.81	20	4.01	1.24	0.77	0.23	1.13	0.34
7.04.81	20	4.69	1.64	0.81	0.27	1.18	0.45
12.06.81	16	5.43	1.00	0.94	0.10	1.45	0.23
6.07.81	20	5.28	0.62	1.00	0.08	1.56	0.16
3.08.81	6	6.11	1.02	1.13	0.08	1.85	0.22
8.09.81	9	1.32	0.13	0.26	0.04	0.44	----
6.10.81	20	2.31	0.45	0.52	0.09	0.74	0.14
10.11.81	20	3.51	0.65	0.57	0.12	0.81	0.18
13.01.82	16	2.63	0.41	0.57	0.08	0.75	0.15
19.02.82	20	3.06	0.83	0.66	0.17	0.92	0.23
17.05.82	20	3.15	0.66	0.70	0.14	0.95	0.22
14.06.82	20	4.44	1.07	0.84	0.18	1.25	0.31
6.07.82	20	4.83	0.85	0.94	0.11	1.42	0.21
13.08.82	6	5.70	0.83	1.06	0.11	1.67	0.24
13.09.82	18	1.66	0.29	0.36	0.05	0.44	0.10
26.10.82	16	2.27	0.72	0.48	0.15	0.56	0.31
23.11.82	20	2.48	0.68	0.59	0.06	0.83	0.12
4.02.83	20	3.64	0.61	0.70	0.10	1.00	0.16
25.04.83	20	4.67	0.98	0.93	0.20	1.28	0.31
30.06.83	18	5.51	0.72	1.00	0.11	1.53	0.21

TABLE 3.6. The mean size (head capsule width, hind tibial length and body length) of nymphs collected on the dates shown at Lady Clough.

Sample Date	N	Measurement (mm)					
		Length		Capsule		Tibia	
		mean	S.D.	mean	S.D.	mean	S.D.
31.10.80	15	2.95	0.58	0.58	0.12	0.81	0.18
4.01.81	20	3.54	0.87	0.69	0.15	0.99	0.24
4.02.81	20	4.27	0.91	0.78	0.15	1.15	0.25
9.03.81	20	4.58	1.20	0.78	0.19	1.18	0.32
7.04.81	20	5.28	1.41	0.92	0.22	1.38	0.38
12.06.81	15	5.18	1.94	1.02	0.15	1.42	0.48
8.09.81	12	1.21	0.21	0.29	0.05	0.40	----
6.10.81	20	1.87	0.42	0.43	0.07	0.53	0.14
10.11.81	18	2.34	0.56	0.57	0.11	0.70	0.16
13.01.82	7	2.99	0.45	0.69	0.12	0.95	0.17
19.02.82	8	3.67	0.92	0.77	0.17	1.11	0.26
29.04.82	6	4.33	0.88	0.87	0.12	1.32	0.21
17.05.82	20	5.89	1.26	1.02	0.17	1.53	0.48
6.07.82	9	4.69	0.55	0.93	0.07	1.39	0.11
13.09.82	15	1.36	0.39	0.31	0.07	0.36	0.09
26.10.82	20	1.67	0.43	0.38	0.10	0.50	0.15
7.02.83	20	4.54	1.33	0.86	0.18	1.28	0.28
24.04.83	18	5.72	0.70	1.09	0.08	1.59	0.21

TABLE 3.7. The relationship between head capsule width or hind tibial length and body length of *Nemoura cinerea* from Lady Clough (LC) and Thomason's Hollow (TH). N = number of paired observations.

Site	N	Measurement	Regression Equation	Correlation Coefficient	Homogeneity of regression (t_{3s} values)
Th	22	Head capsule v Body length	$y = 0.117 + 0.163x$	0.98	0.004
LC	17		$y = 0.132 + 0.162x$	0.98	
Th	22	Hind tibia v Body length	$y = 0.025 + 0.277x$	0.98	0.070
LC	17		$y = 0.097 + 0.259x$	0.99	

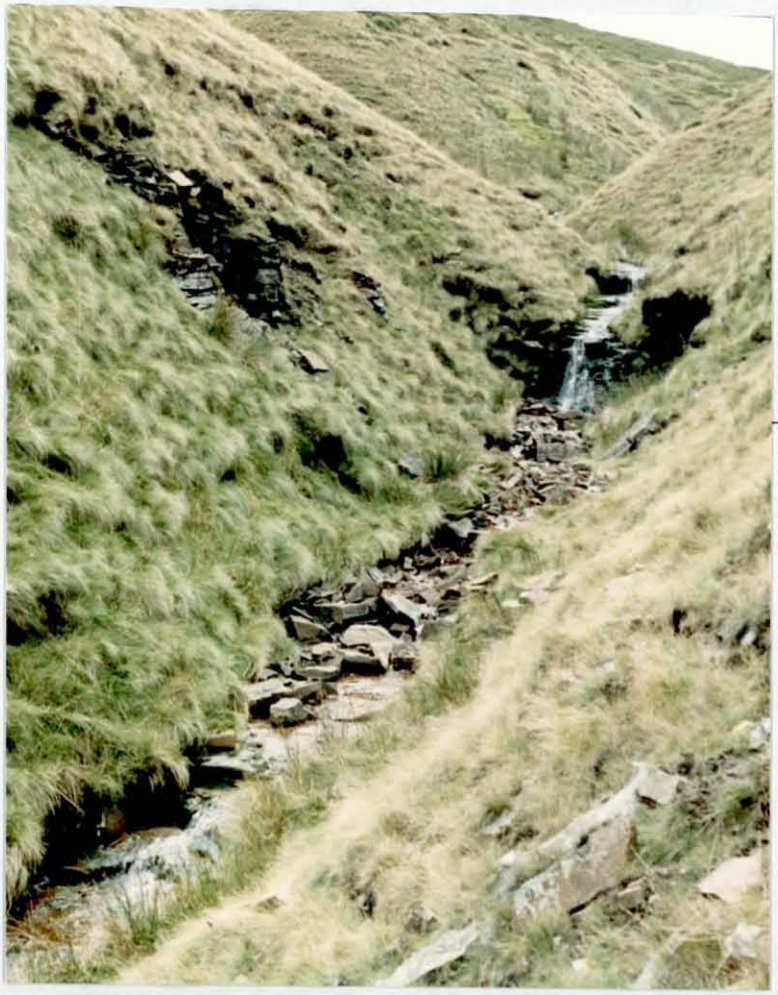


Plate 3.1.
Thomason's
Hollow

Plate 3.2.
Lady Clough

Plate 3.1. and 3.2.

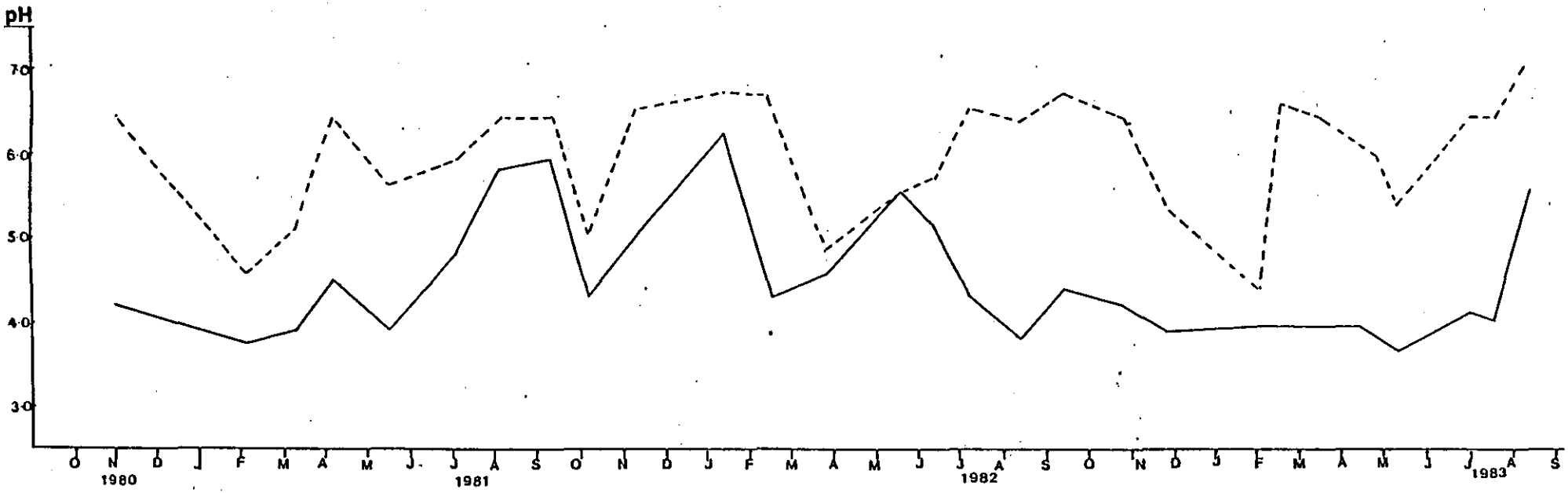


FIGURE 3.1. pH readings taken on each sampling date at Thomason's Hollow (—) and Lady Clough (--)

Figure 3.1.

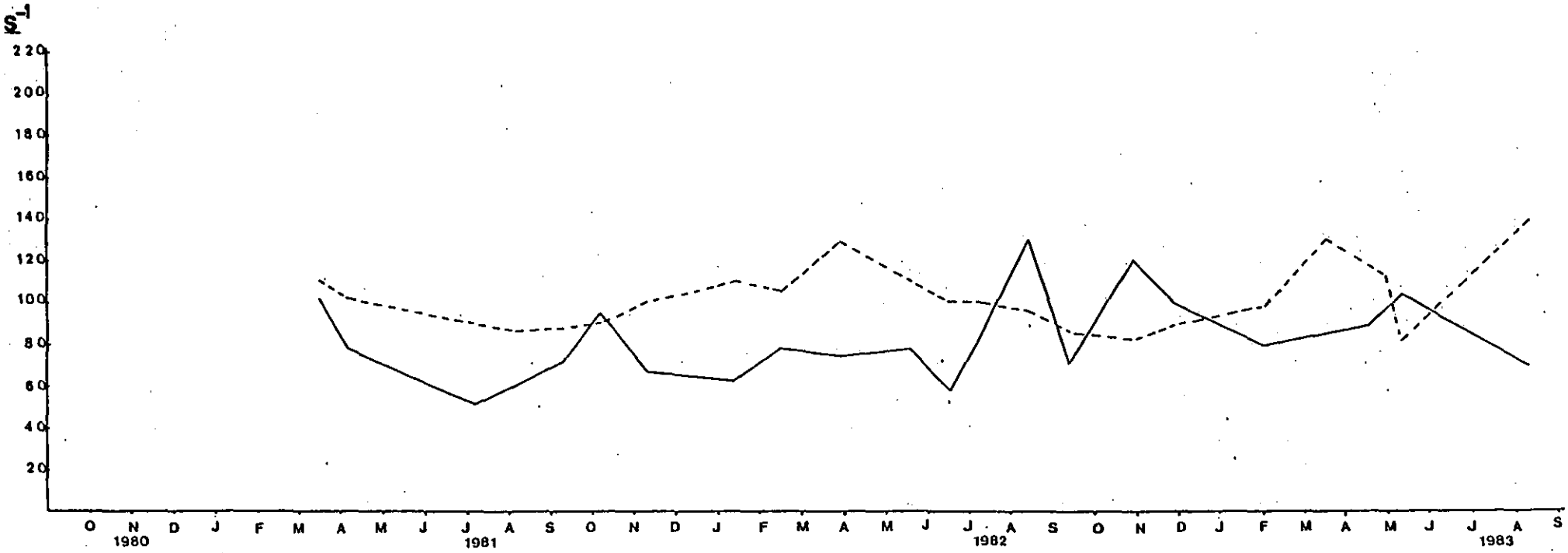


FIGURE 3.2. Conductivity measured at Thomason's Hollow (—) and Lady Clough (--)

Figure 3.2.

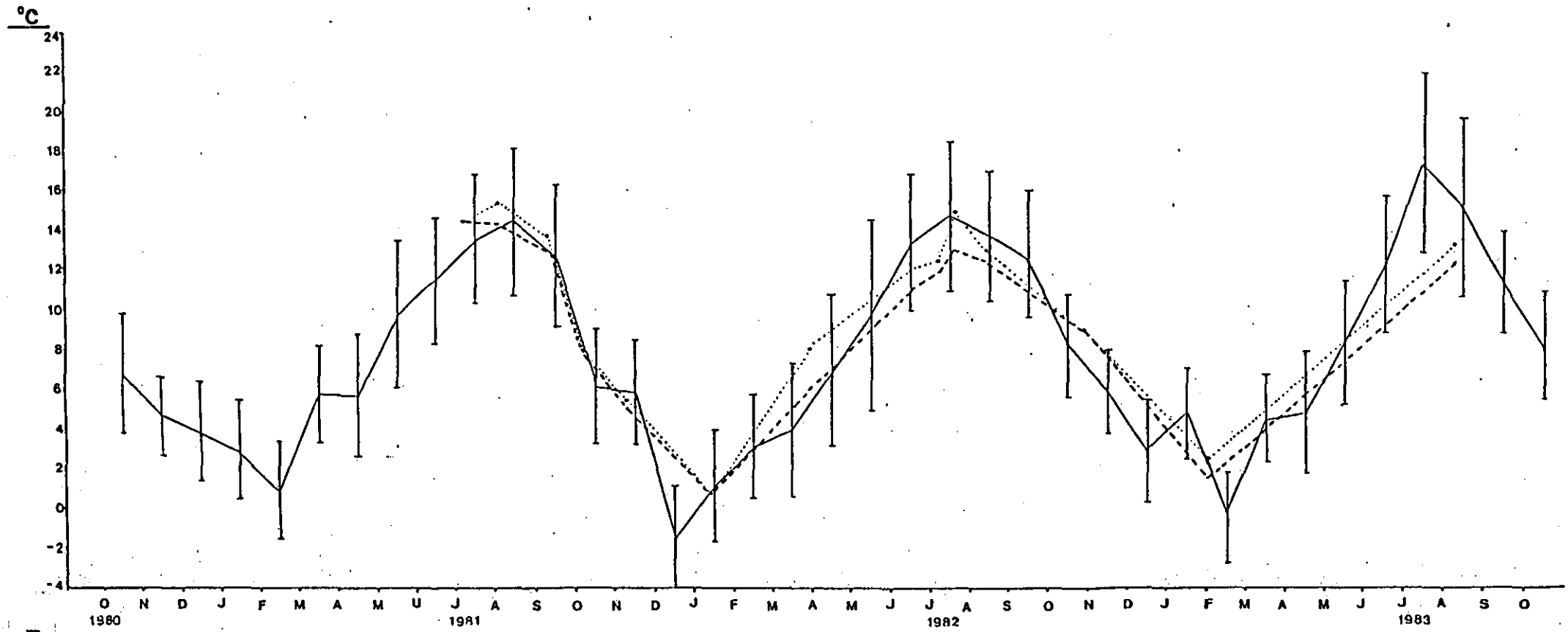


FIGURE 3.3. Spot temperature readings at Thomason's Hollow (---) and Lady Clough (——) together with the monthly mean air temperatures at Buxton, Derbyshire (——). The vertical bars show the range.

Figure 3.3.

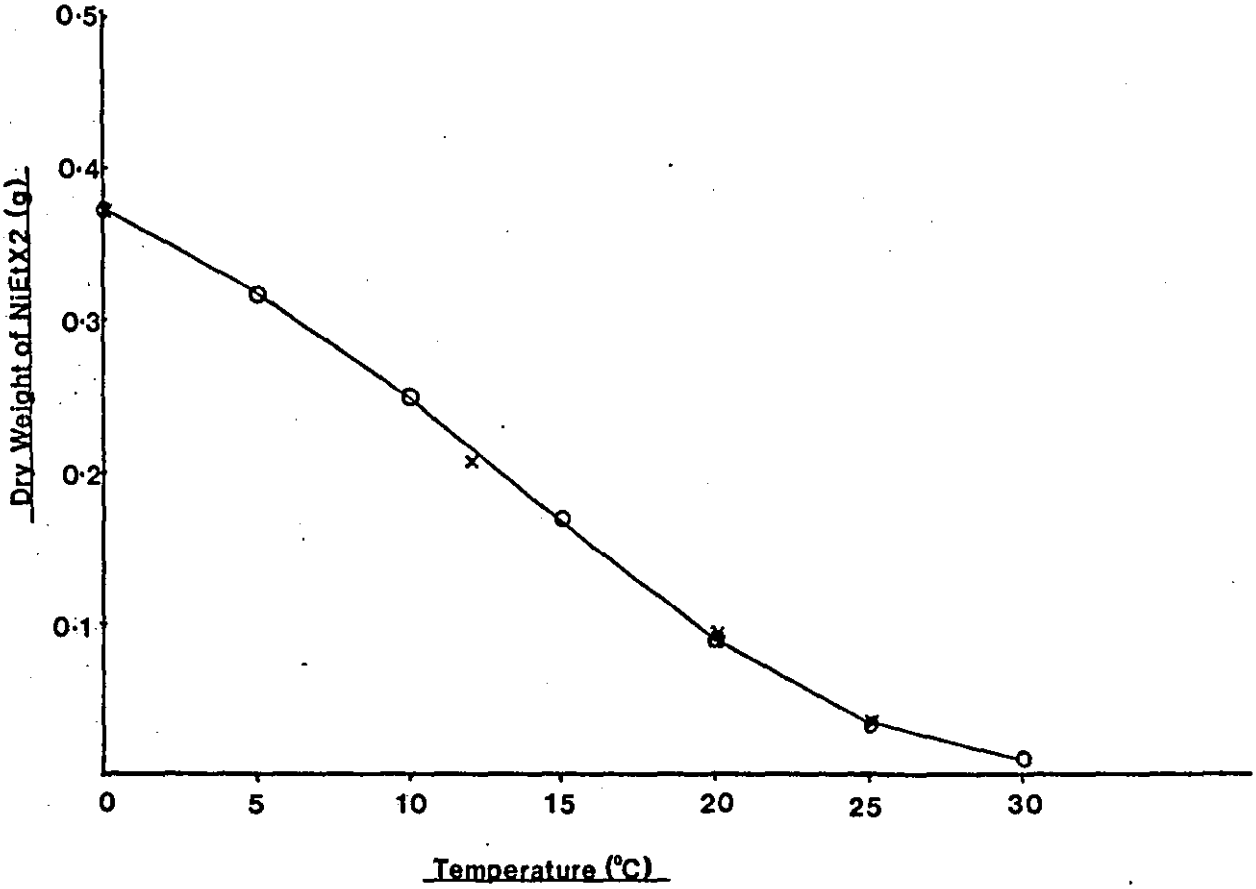


FIGURE 3.4.Calculated calibration curve for KEtX2 method of temperature measurement (from data in TABLE 3.3.) and experimental results (X) from table 3.2.

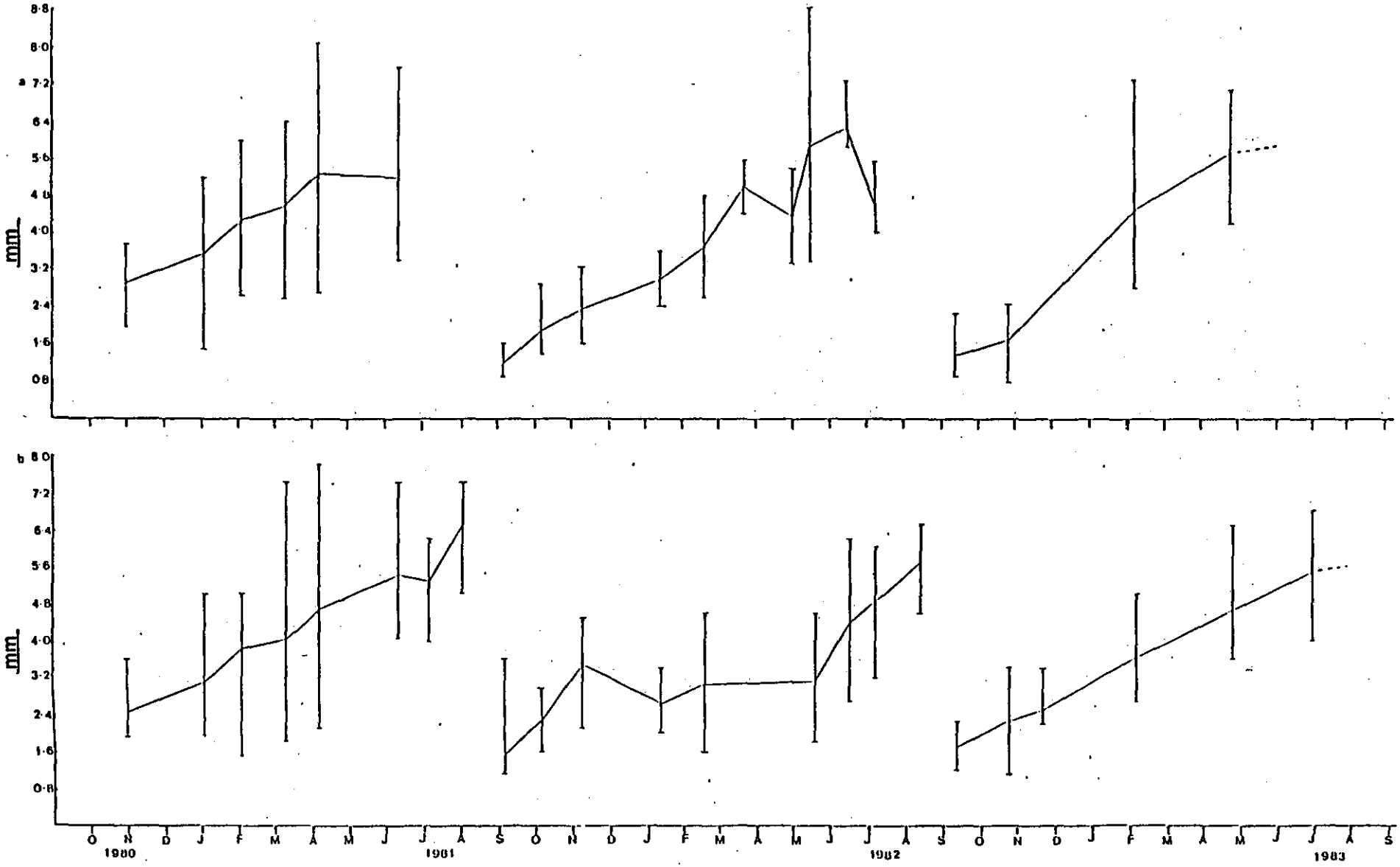


FIGURE 3.5. Growth curves at a) Lady Clough and b) Thomason's Hollow based on body length measurements. The lines join the mean of each sample and the vertical bars show the range.

Figure 3.5.

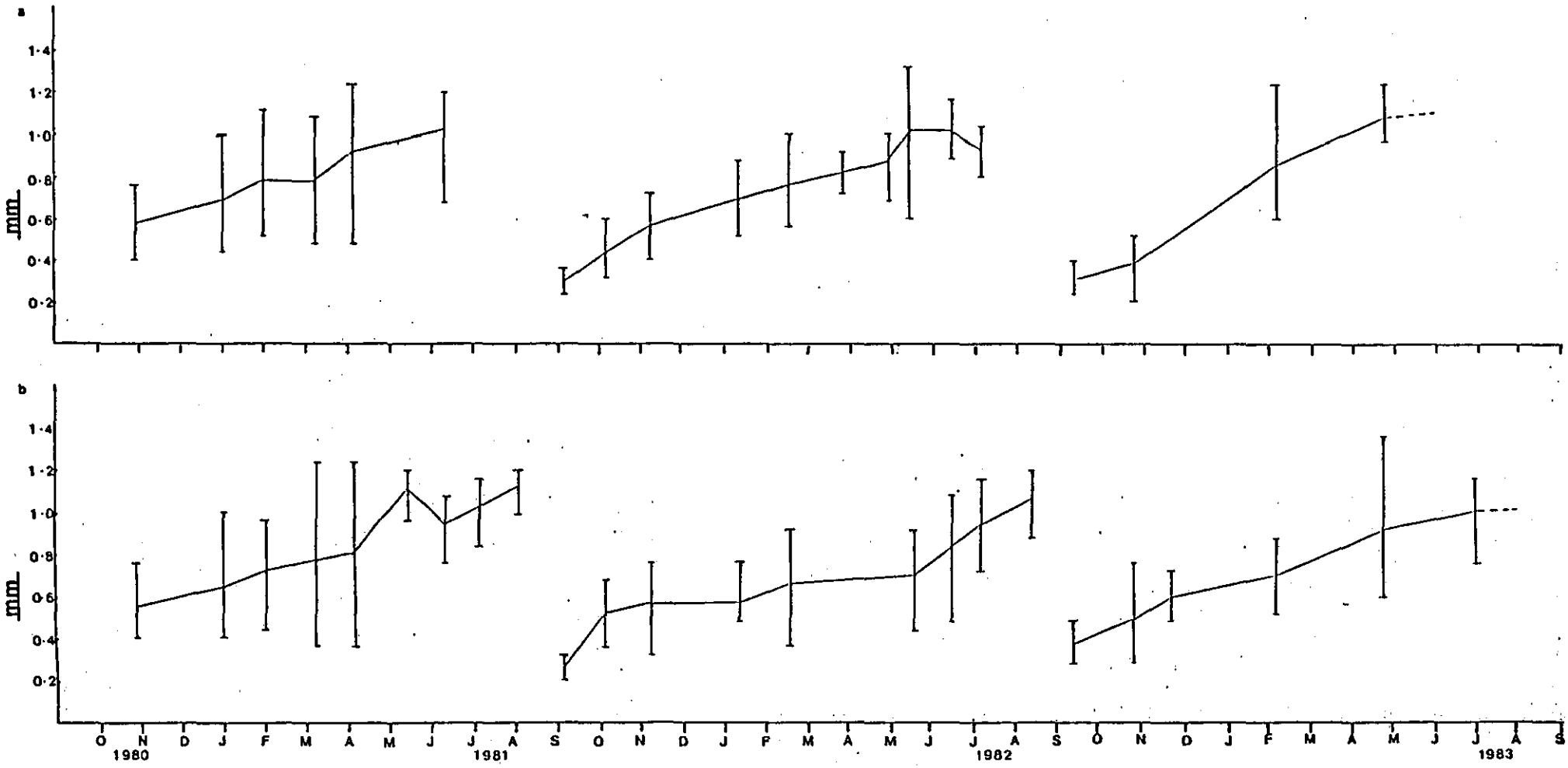


FIGURE 3.6.Growth curves at a) Lady Clough and b) Thomason's Hollow based on head capsule width. The lines join the mean of each sample and the vertical bars show the range.

Figure 3.6.

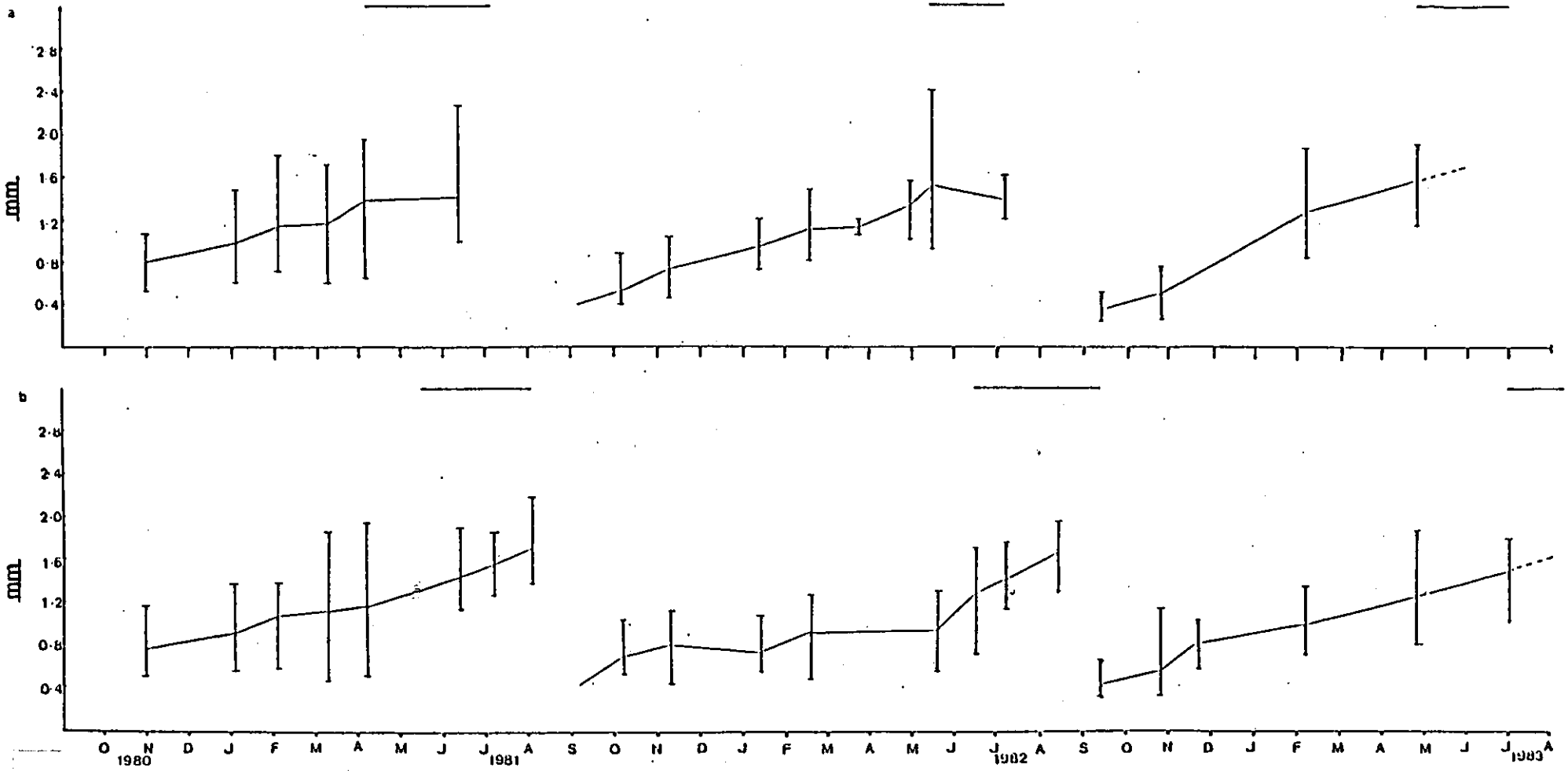


FIGURE 3.7. Growth curves at a) Lady Clough and b) Thomason's Hollow based on hind tibia measurements. The lines join the mean of each sample and the vertical bars show the range. Adult flight period is indicated by the horizontal lines.

Figure 3.7.

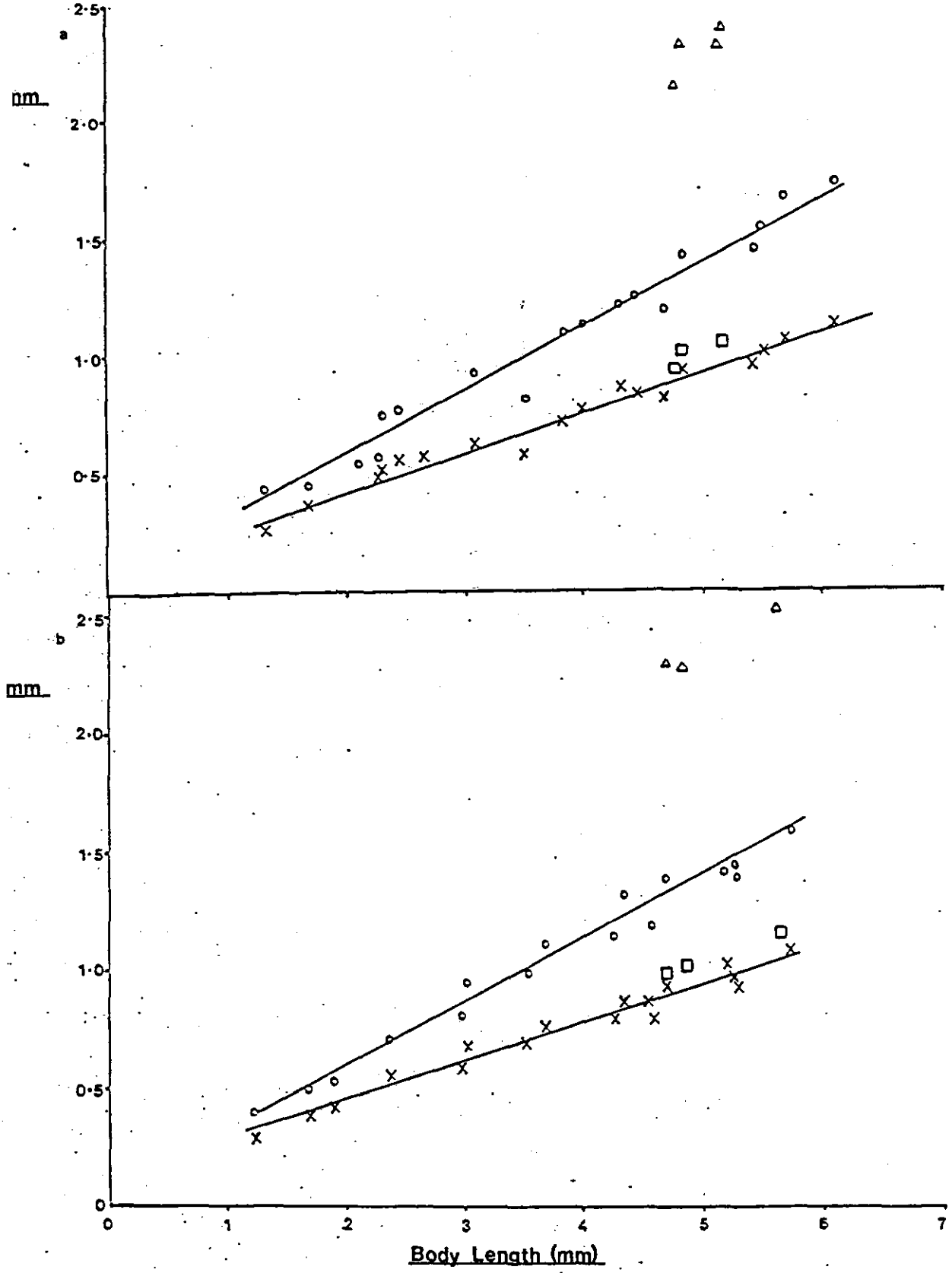


FIGURE 3.8. The relationship of the nymphal head capsule width (X) an hind tibial length (o), and adult head capsule width (□) and hind tibial length (Δ), with body length at a) Thomason's Hollow and b) Lady Clough.

Figure 3.8.

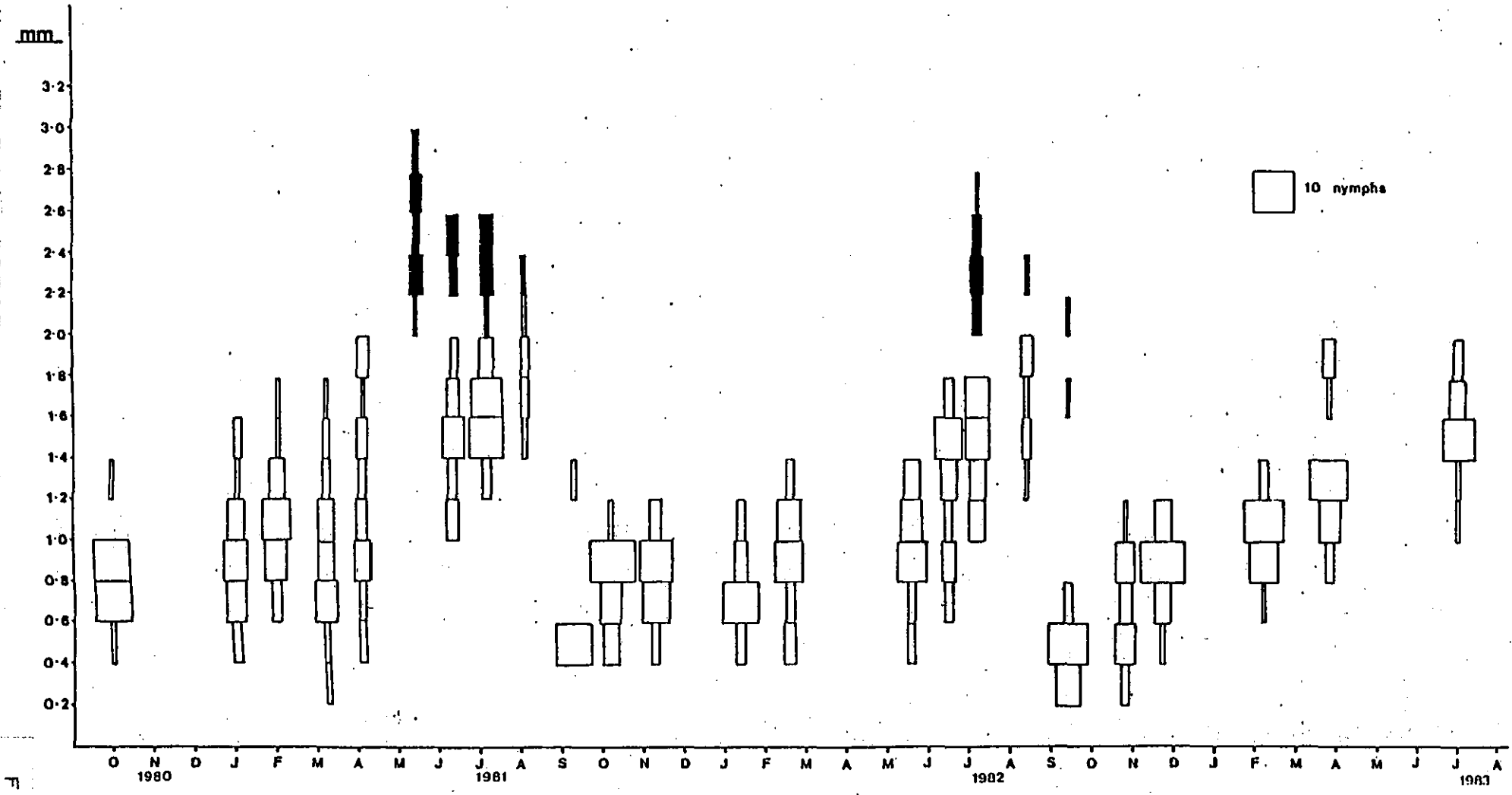


FIGURE 3.9. Population size structure histograms for N. cinerea at Thomason's Hollow. The numbers in each 0.5 mm size group is indicated by the thickness of the bar. Adults are represented by shaded bars.

Figure 3.9.

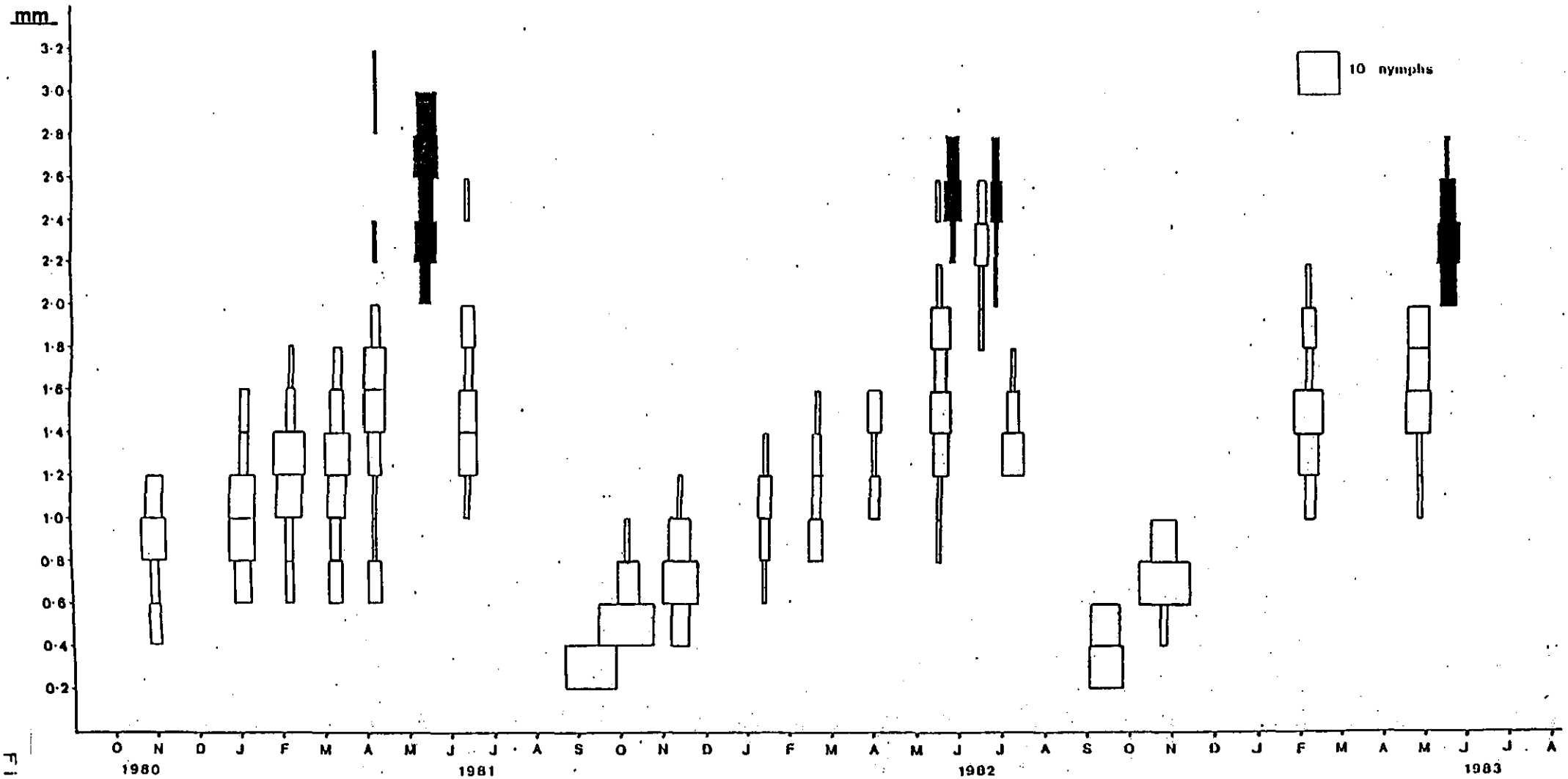


FIGURE 3.10 Population size structure histograms for N. cinerea at Lady clough. The numbers in each 0.5 mm size group is indicated by the width of the bar. Adults are represented by shaded bars.

Figure 3.10.

CHAPTER FOUR

GENETIC VARIATION WITHIN THE NEMOURIDAE

4.1. Introduction

Several species of Plecoptera, mainly from the families Nemouridae and Leuctridae, were found in samples collected from streams in the Derbyshire Peak District. All were used initially to devise electrophoretic methods and to determine which species would give the best results following electrophoresis. The classical key for stone-fly identification (Hynes,1977) is based on the features of well grown, late instar nymphs. Difficulties encountered with the identification of small nymphs, collected in early winter, suggested that an electrophoretic key for identification would be of value. The Leuctridae were excluded because early electrophoresis was less successful with these species. Therefore, one of the main aims of this section was to produce an electrophoretic key for the identification of as many of the British Nemouridae as was possible.

When the electrophoretic mobilities of multiple allozymes have been examined, the phylogeny of species groups can be estimated and compared with the accepted systematics derived from conventional taxonomic characters. The genetic similarity between species is assessed and quantified using such measures as the identity and distance statistics of Nei (1972,1978), Rogers (1972), and Cavalli-Sforza and Edwards (1967).

In addition to its application in systematics studies, electrophoresis provides information on how much genetic

variation is present within a particular population or species. Hundreds of electrophoretic studies have been made on a wide range of organisms since the technique was first used in the 1960's (Lewontin & Hubby, 1966; Harris, 1966). Most investigations have found high levels of variation in almost all populations of outcrossing species. These data have been used as the basis for more global analyses (eg. Ward, 1977; Nei, Fuerst & Chakroborty, 1978; Ward & Skibinski, 1982; Skibinski & Ward, 1981, 1982; Thorpe, 1983; Nevo, Beiles & Ben-Shlomo, 1984). However, from these overviews, it can be seen that, in relation to the number of species within the class, there have been few electrophoretic comparisons between insects other than Drosophilidae.

The restricted population structure of the British Nemouridae raised such questions as whether these species are less genetically variable than more vagile insects, and whether the more widespread stone-flies (Nemoura cinerea and Amphinemura sulcicollis) are more variable than the more local species. The answers to these questions could help to give an insight into the evolutionary significance of the molecular variation detected using electrophoresis. There do not appear to be any previous reports of multi-locus electrophoresis investigations using Plecoptera; Picker (1980) used only one enzyme (esterase) in an examination of the taxonomy of some South African stoneflies.

Part of the work reported in this section has been published (Lees and Ward, 1987).

4.2. Materials and Methods

The sampling sites which provided the animals for this survey are shown in Table 2.1. and Figure 2.1. (Chapter 2). Samples from more than one population were used for electrophoresis in all species except Nemoura dubitans and Nemurella picteti which came from single populations, although not all populations were screened for all the enzymes.

A complete list of the enzyme staining systems investigated is given in Table 4.1. The stains were mostly standard ones adapted from Harris and Hopkinson (1976) and Shaw and Prasad (1976). The stains for trehalase, fructose-6-phosphate dehydrogenase and threonine dehydrogenase were as described by Meredith (1980).

Several other buffer systems were tried in addition to the final ones listed in chapter two, in an attempt to increase the number of loci screened and improve resolution, but these were unsuccessful.

The BIOSYS 1 program (Swofford & Selander, 1981) was used to analyse the data and to generate dendrograms.

4.3. Results

4.3.1. Species Collected

Despite extensive field trips around the United Kingdom (the sites listed in Table 2.1. represent less than one third of the total number of streams sampled), only nine of the eleven British Nemouridae were collected. These were :

Nemoura cinerea (Retz.)

Nemoura dubitans (Mort.)

Nemoura cambrica (Steph.)

Nemoura avicularis (Mort.)

Protonemura meyeri (Pict.)

Protonemura praecox (Mort.)

Amphinemura sulcicollis (Steph.)

Amphinemura standfussi (Ris.)

Nemurella picteti (Klap.)

The two species not found were Nemoura erratica (Claas.) and Protonemura montana (Kim.). P. montana is described as rare in Britain, and has only been found in streams over 500 metres above sea level (Hynes, 1977). Samples were collected from two streams where it has been recorded in the past: Rydal Beck, Cumbria (Kimmins, 1941) and Knock Ore Gill, Cumbria (Brown, Cragg & Crisp, 1964), but the species was not found. N. erratica has been reported in several stream surveys where N. cambrica was found at the same site (Brown, Cragg and Crisp, 1964; Langford and Bray, 1969). Samples were taken from some of these streams but only one species appeared to be present. There were no major biochemical differences in the N. erratica/cambrica samples and there was no other evidence such as an excess of homozygotes at the Est-2 or PGM loci to indicate that two species were present. The identity of N. cambrica nymphs was confirmed from adults collected from the Peak District streams. Therefore, all similar specimens collected from other areas were subsequently classified as N. cambrica. It is difficult to separate the nymphs of N. erratica and N. cambrica using morphological characters (Bird,

1983). It seems likely, therefore, that some previous records may be inaccurate unless the identifications were supported by adult specimens.

4.3.2. Gel Interpretation

Fourteen enzyme staining systems gave satisfactory results, interpreted as the products of sixteen gene loci, that could be scored for all nine species (Table 4.2.). For each locus, the enzyme bands were scored relative to the position of the commonest allozyme of N. cinerea which was designated as '100'. Higher numbers indicate faster migrating (more anodal) bands on the gel.

Stained gels showed only one zone of activity for most enzymes in all the species. The exceptions to this were gels stained for hexanol dehydrogenase, esterase and leucine aminopeptidase. These zones were interpreted as the products of different loci. The most anodal were labeled locus 1 for hexanol dehydrogenase and esterase (Note that a third, slower zone appeared on gels stained for esterase for all species, but this was never interpreted satisfactorily). Gels stained for leucine aminopeptidase displayed two monomorphic bands of activity. The most strongly staining one appeared in identical positions on gels of a variety of buffer systems stained for acid phosphatase and alkaline phosphatase as well as leucine aminopeptidase. This phenomenon has not been reported in other electrophoretic studies. However, in the absence of any variation to help clarify the situation, all these were conservatively assumed to be the product of a single 'phosphatase' locus producing an enzyme with a broad substrate specificity. The faster migrating band, which appeared only on

gels stained for leucine aminopeptidase, is the leucine aminopeptidase of Table 4.3.

Two further points of interest were:

1. Hexanol dehydrogenase appears to have a substrate specificity for hexanol since octanol, ethanol and isopropanol were inactive as substrates.

2. All P. meyeri screened for phosphoglucose isomerase produced a three banded pattern on the zymogram (Plate 4.2.).The bands could be caused by species specific post-translational modifications of the original isozyme or by the hybridisation as dimers of the products of duplicate loci. The absence of any observed genetic variation for the enzyme in this species meant that it was not possible to discriminate between the two explanations. The first interpretation, that a single locus is present, has been used in all the analyses.

The genotypes of the animals screened were determined from their banding pattern on the gels (see Plates 4.1. and 4.2.). Allele frequencies at each locus could then be estimated and are presented in Table 4.3. along with sample sizes and the number of populations used for each locus. Note that not all enzymes were screened in all populations. Allele frequencies were calculated from the sum of the genotypes observed in all populations screened for that enzyme.

As with most studies of this type, the absence of formal breeding data means that the genetic basis for the enzyme banding patterns can only be inferred. However, the appearance of the heterozygotes of the polymorphic loci is consistent with the quaternary structures of the enzymes determined in other surveys, and the lack of significant

deviations from Hardy-Weinberg expectations in individual populations adds further support to a genetic interpretation.

4.3.3. Analysis

Estimates of overall genetic variability (observed and expected mean heterozygosities per locus; proportion of polymorphic loci; and mean number of alleles) are given in table 4.4. All the measures of variability were low. The mean observed heterozygosity per species ranged from 0.005 for Nemurella picteti to 0.067 for N. cinerea and the overall mean observed and expected heterozygosities were 0.036 and 0.047 respectively. The observed heterozygosity is less than expected for most species due to the effect of combining data from several populations, causing a 'Wahlund effect'.* The mean proportion of loci that were polymorphic per species was 0.146, and the mean number of alleles per locus was 1.25.

The BIOSYS1 program calculates genetic distance statistics between populations or species using most measures that have been employed in the past (ie. Cavalli-Sforza and Edwards, 1967; Edwards, 1971, 1974; Nei, 1972, 1978; Rogers, 1972; Wright, 1978). The most widely used statistics in the literature are those of Nei (1972,1978). The main reason for this appears to be that Nei's measures of genetic distance and identity were devised expressly to estimate a biological attribute, the average number of amino acid substitutions per protein, whereas the other measures are based purely on statistical considerations (Nei, 1975). However, Nei's measures have been criticised by Farris (1981) because they do not satisfy the triangle inequality , where, for any three taxa, ABC, distance (A,C) < distance (A,B) + distance (B,C), and are

* A deficit of heterozygotes in a sample caused by the inclusion of individuals from two or more seperate populations where different alleles predominate.

therefore, considered less appropriate for phylogenetic analysis than the other statistics. A matrix of Nei's unbiased genetic distances and identities (1978) is presented in Table 4.5. to facilitate comparisons with other work.

Dendrograms or phylogenetic trees can be constructed from genetic distance matrices to give a clearer presentation of the phylogeny. As with the genetic distance statistics, numerous methods have been used in the literature to estimate phylogenetic trees (eg. Sokal and Sneath, 1963; Cavalli-Sforza & Edwards, 1967; Fitch & Margoliash, 1967; Farris, 1972; Felsenstein, 1981). There is considerable debate over which approach gives the 'best' tree (Farris,1972; Sneath & Sokal, 1973; Nei, 1975; Prager & Wilson, 1978; Swofford, 1981). Trees constructed using two of the more widely used methods, the unweighted pair group method of analysis (UPGMA) of Sokal & Sneath (1963) and the distance-Wagner method of Farris (1972), are presented in Figures 4.1. to 4.6. allowing a direct comparison of the resulting phylogenies to be made.

Nei's genetic distance and identity statistics cannot be used to construct distance-Wagner trees because they do not satisfy the triangle inequality. Distance-Wagner trees were produced using the other distance measures available on BIOSYS1 in conjunction with the alternative branch-length optimisation and rooting procedures. Very similar tree topologies were produced from all the different distance matrices. However, the trees generated using Edward's (1971, 1974) distance measure (Figures 4.3-6., Table 4.6.) had the best fit to the input data as measured by all the tree comparison methods: % standard deviation, Fitch and Margoliash (1967), Farris' (1972) f-statistic, Prager and Wilson (1976) F, and the cophenetic

correlation. The 'goodness of fit' statistics are given in the legends to the dendrograms. Figures 4.3. and 4.5. show the trees that were generated using either addition criterion 1 of Farris (1972) or the multiple addition criterion of Swofford (1981). Figures 4.4. and 4.6. were generated using addition criteria 2 or 3 of Farris (1972). These addition criteria use different methods to determine the order that taxa are added to the dendrogram to minimise the total distance between members of the group. Figures 4.5. and 4.6. were rooted at the mid-point of the longest path. Figures 4.3. and 4.4. were rooted by the outgroup method; Nemurella picteti having been preselected as the outgroup species from the UPGMA trees.

4.3.4. Electrophoretic key

The key shown below is divided into three sections because even small nymphs can usually be ascribed to one of the groups using the number of prosternal gills (Hynes, 1977). All mobilities are scored relative to Protonemura meyeri, since these are the largest Nemouridae in streams at the time of year when most difficulties are likely to be encountered in identifying other species. Many loci are diagnostic between species (Table 4.3.). The four used here, PGI, AP, EST, and PGM, have been chosen because they have good activity in small specimens (the dehydrogenases in particular show weak activity in small nymphs). Also, since the stains for PGM and PGI can be combined, all three enzymes can be typed on a single gel (buffer system 1) sliced into three. This saves time and money and is particularly useful when very small specimens are involved. Note, however, that resolution of EST is better on buffer system 2. Figures 4.7. (PGI), 4.8. (AP-1), 4.9. (EST,

on buffer system1) and 4.10. (PGM) show the banding patterns observed for the species under discussion. Where a locus is polymorphic, all the observed phenotypes are shown in the figures.

SECTION 1

Genus: Protonemura (3 prosternal gills on each side)

PGI (i) three banded pattern .....P. meyeri

(ii) single band .....P. praecox

SECTION 2

Genus:Amphinemura (5 to 8 prosternal gills on each side)

AP-1 (i) mobility less than P. meyeri.....A. standfussi

(ii) mobility greater than P . meyeri.....A. sulcicollis

or

PGM (i) mobility greater or equal to P. meyeri..A. standfussi

(ii) mobility less than P. meyeri.....A. sulcicollis

SECTION 3

Genus: Nemoura or Nemurella (No prosternal gills)

1) PGI (i) mobility approximately equal to P. meyeri 2

(ii) mobility less than P. meyeri.....N. avicularis

(iii) mobility greater than P. meyeri.....N. dubitans

2) AP-1 (i)mobility approximately equal to P.meyeri.N.cambrica

(ii.) mobility greater than P. meyeri.....3

3) ES (i) mobility of ES-1 much slower than P. meyeri, and close to ES-2 (which is faster than ES-2 of P. meyeri).....Nemurella picteti

(ii) ES-1 only slightly slower than P. meyeri, and ES-1 and ES-2 well separated.. .....N. cinerea

An alternative key using electrophoretic markers only is shown below. This may be needed when all specimens are small. Again, all the enzymes can be screened on a single gel using buffer system 1. Here mobilities are scored relative to N. cinerea since this is the most widespread species.

1) AP-1 i) mobility greater than commonest allele in N. cinerea.....2

ii) mobility less than in N. cinerea.....3

2) PGI i) mobility of commonest allele greater than N. cinerea 100 allele.....A. sulcicollis

ii) mobility of commonest allele equal to N. cinerea 100 allele.....Nem. picteti

3) PGI i) mobility of commonest allele about twice N. cinerea 100.....N. dubitans

ii) mobility of commonest allele half of N. cinerea 100.....N. avicularis

iii) mobility equal to N. cinerea 100.....N. cambrica

iv) mobility slightly greater than N. cinerea 100.....A. standfussi

v) mobilty slightly less than N. cinerea 100.....4

4) PGI i) all three banded phenotypes.....P. meyeri

ii) not all three banded.....P. praecox

or

PGM i) mobility greater than N. cinerea.....P. meyeri

ii) mobility less than N. cinerea.....P. praecox

4.4. Discussion

Electrophoretic keys are becoming more common in the literature (eg. Ayala and Powell, 1972; Avise, 1974; Berlocher, 1980). The main disadvantage of using electrophoresis for species identification is the cost of equipping a laboratory and retraining personel. Also, because enzymes are denatured by alcohol or heat, it is necessary to transport material for analysis either live or frozen. However, the capital costs are probably about the same as a research quality dissecting microscope and transport problems can be overcome. The additional recurrent costs of chemicals for the enzyme stains may be more than balanced by the time saved in making a positive identification (Berlocher, 1980). This is particularly true of the keys presented here, where a clear diagnosis can be made using only one starch gel.

The use of electrophoresis for insect identification has several advantages. The lack of clearly distinguishing morphological characters in certain groups has already been mentioned and ,unlike morphology , enzyme mobility is largely unaffected by environmental variables allowing unambiguous classification of species. Another feature of electrophoretic keys is that badly mangled specimens and anomalous morphotypes can be identified.

The key proved its value in identifying some anomalous specimens of P. meyeri and A. standfussi collected near Llyn Brianne in Dyfed, Wales. The Protonemura were completely gill-less and were initially considered to be in the genus Nemoura , whilst the Amphinemura possessed only short gill stumps giving an appearance similar to a typical Protonemura. Also, N. dubitans was only initially detected by electrophoresis. The nymphs of this species resemble those of N. cinerea very closely making it very difficult to distinguish between them. They can be easily identified by electrophoresis, however, and many loci are diagnostic (Table 4.3.).

A feature common to most invertebrate electrophoretic comparisons is the low numbers of loci that have been successfully resolved , relative to vertebrate studies (Nevo et al. 1984). This seems to be mainly due to the lack of the multiple loci which often appear when vertebrates are screened for single enzymes. In this investigation, only gels stained for esterase and hexanol dehydrogenase (and possibly phosphoglucose isomerase in P. meyeri) gave band patterns that could be interpreted as the products of more than one locus. When attempting to quantify genetic similarities between populations, the sizes of the errors attached to the estimates of genetic identity or distance are more dependent on the numbers of loci screened than on numbers of individuals examined (Nei, 1978; Gorman and Renzi, 1979). Fifteen loci is perhaps a minimum acceptable number.

There were clear differences in the topologies of the trees produced by the UPGMA or Distance-Wagner procedures from

the data on 16 enzyme loci (Figures 4.1-6.). The trees generated by UPGMA (Figures 4.1 and 2.) are in agreement with the phylogeny derived using morphological features, with the family comprising four distinct genera : Nemoura, Protonemura, Amphinemura and Nemurella (Hynes, 1977). However, none of the Distance-Wagner trees conform with the classical systematics of the family. In all the Distance-Wagner trees, the genus Nemoura is split and the positions of N. cambrica and N. avicularis varies depending on which addition criterion or method of rooting the trees is used (Figures 4.2-6.). Swofford (1981) also found that different tree topologies and branch-lengths were generated when different addition criteria were used. He suggested the use of his 'multiple addition criterion' (MAC) to circumvent the problem. In the data presented here, however, the trees generated using MAC are identical in topology to those generated using Farris' (1972) addition criterion 1 (Figures 4.3 and 5.)

The debate over which method of generating trees is most appropriate for various biochemical data sets has usually involved the evaluation of alternative trees using 'goodness of fit statistics' (for example, Prager and Wilson, 1978, and Swofford, 1981). A comparison of the UPGMA tree and Distance-Wagner trees derived using Edward's (1971, 1974) genetic distance (Figures 4.2.-6.) shows that the UPGMA tree gave a better fit to the input distance matrix than any of the Distance-Wagner trees (low f-value, low percent standard deviation and high cophenetic correlation). The superior performance (as measured by goodness of fit) of the UPGMA procedure over the Distance-Wagner procedure contrasts with the findings of Farris (1972), Prager and Wilson (1978) and

Swofford (1981), who all recommend the Distance-Wagner method. However, Rohlf and Sokal (1981) re-examined several published comparisons between tree generating methods and found that UPGMA gave the best fit when tests were correctly formulated.

The fit to the input data of the Distance-Wagner trees can be improved by using Swofford's (1981) branch-length optimisation procedure. This method was devised to minimise Prager and Wilson's (1976) F-value, and is claimed to allow a fairer comparisons with the other tree generating methods. This is because Farris' (1972) Distance-Wagner procedure requires all output (branch-length) distances to be greater than or equal to the input distances whereas UPGMA does not allowing an unfair advantage for UPGMA in goodness of fit comparisons (Swofford, 1981). After optimisation, all the Distance-Wagner trees had a better fit to the input data than the UPGMA tree. However, the trees shown in Figures 4.4. and 4.6. have a better fit than those in Figures 4.3. and 4.5. after optimisation, which is the reverse of the outcome using the original, non-optimised method.

The main advantage claimed for the Distance-Wagner (or Fitch-Margoliash) methods over UPGMA is that trees produced using UPGMA can only be regarded as true evolutionary trees if the rate of gene substitution per unit length of time is constant in all phyletic lines and this assumption may not be valid. The other methods do not require this assumption. This may be an advantage for the Distance-Wagner procedure when examining the evolutionary relationships of closely related species. However, in cases such as in the Nemouridae, where the genetic distances between taxa are large, the UPGMA method may perform better than the others because the error

values attached to the distance estimates make it more likely that erroneous trees will be produced by Distance-Wagner methods (Nei, 1975). This seems to be true of the data presented here since, apart from the UPGMA trees having a better fit to the input data than standard Distance-Wagner trees, the branching sequences of the latter trees seem to indicate some unlikely common ancestors. Also when Distance-Wagner trees are rooted at the mid-point of the longest path (Figures 4.5. and 4.6.), equal rates of gene substitution along the two phyletic lines is inferred, negating one of the claimed advantages of this method. However, recent work (Nei et al, 1983) has shown that the accuracy of the topology and branch-length estimates is low for trees generated by any method when less than 20 loci have been examined, though the UPGMA method performed the best.

The intra-generic genetic distances are large (Table 4.5.), which contrasts with the close external morphological similarities of some of the species. Indeed, these intra-generic distances are of a comparable magnitude to inter-generic estimates reported for other invertebrates (Ayala, 1975; Ferguson, 1980). This supports the accepted view that the Plecoptera are an evolutionarily archaic order (Rausser, 1971) and that most species pre-date the Pleistocene glaciation (Illies, 1965). If certain assumptions are made (Nei, 1975), a crude estimate of the elapsed time since two lineages separated can be obtained from the genetic distance measures. Based on the correlation proposed by Maxson and Maxson (1979), in which a Nei distance of 1.0 equals 14 million years, the time elapsed since the divergence of P. meyeri and P. praecox, the two most closely related species,

is about 7.5 million years, and the four genera appear to have diverged around 22-27 million years before present. It should be pointed out, however, that as a consequence of the relatively small numbers of loci screened, the standard errors attached to the D values are large, being around one-third of the actual values.

Within the genus Nemoura, the nymphs of N. cinerea and N. dubitans are the most similar in external morphology and are difficult for the non-specialist to separate on morphological grounds, although they may readily be separated allozymically. This similarity appears to be reflected in the phylogenies shown in Figures 4.2.-6. based on Edward's (1971, 1974) genetic distance. (The distance measures of Cavalli-Sforza and Edward's (1967) and Prevosti (in Wright, 1978) gave the same result but these are not shown). However, the phylogenies derived using Nei's (1972, 1978) genetic distance indicate that N. cinerea is more closely related to N. cambrica. The same result was obtained using Roger's (1972) genetic distance (not shown). Differences in the ordering of species in phylogenetic trees depending on which distance measure was employed have been reported before (Rogers, 1972). Based on the goodness of fit statistics and morphology, it appears that the UPGMA tree produced using Edward's (1971, 1974) distance (Figure 4.2.) is the most accurate representation of the species relationships in the Nemouridae. Nei's distance estimates appear to favour the N. cinerea / N. cambrica cluster because of the fixation of the PGI-100 allele in N. cambrica. This produces a smaller genetic distance than that resulting from 3 shared alleles at the PGM locus between N. cinerea and N. dubitans.

Nemoura avicularis appears to be genetically distant from the other three Nemoura species (Figures 4.3.- 6.). This may be a reflection of adaption to a more specialised habitat; N. avicularis is most common in lacustrine environments whereas the other species mainly occur in small streams. However, the large standard errors attached to the genetic distance estimates mean that conclusions about species relationships can only be tentative.

The amount of overall genetic variability is low in all the species relative to that found in other invertebrate surveys (Ferguson, 1980), and is below the average for non-Drosophila insects in Nevo et al's (1984) study. There, the mean expected heterozygosity per locus over 122 species was 0.089,and the mean proportion of polymorphic loci over 130 species was 0.351, compared with an expected heterozygosity per locus of 0.047 and proportion of polymorphic loci of 0.146 for Nemouridae.

Many different types of explanations have been put forward to account for differences in heterozygosity between species. Non-adaptive or "neutralist" interpretations attempt to explain such variation in terms of variation in demographic parameters, such as effective population size, population histories or rates of migration, whereas adaptive or "selectionist" explanations invoke variation in ecological features such as niche-width or trophic resource utilization.

The most probable explanation for the low levels of variation in Nemouridae is that variation has been lost during periodic population crashes producing bottle-necks in population size. The disjunct distribution patterns and poor mobility means that re-colonisation is likely to have occured

from a few survivors within a particular stream rather than from another population, so that any alleles lost during the population crash would not be replaced when the stream was re-colonized. Drastic fluctuations in fresh-water invertebrate numbers between years is a common observation (Hynes, 1970). An alternative, selectionist, interpretation of the low variability might be that these insects occupy rather narrow niches, where selection might favour single specialised genotypes.

Nemoura cinerea and Amphinemura sulcicollis are the two most variable species within the family when all measures of variation are considered. This appears to conform to the prediction of the niche-width/variation hypothesis, that there is a positive correlation between niche breadth and amount of genetic variation (Somero and Soule, 1974; Levins, 1978) since these two species have the widest temperature tolerances (Brown et al, 1964) and are found in a greater range of environments than the others (Brinck, 1949; Hynes, 1977). This is especially true for N. cinerea which has been termed a "eurycoenic" species from its wide distribution in Europe (Rausser, 1971). A selectionist interpretation of these data would be, therefore, that the greater genetic variation of these species has resulted from adaptation for survival in varied environmental conditions. However, an equally plausible explanation is that species which can survive in the widest range of habitats have the largest effective population sizes, since migration between isolated upland areas via lowland drainages is more probable. This would then conform to a neutralist interpretation of the results since the number of neutral alleles that can be maintained in a population is

dependent upon its effective size (Kimura and Ohta, 1971; Ohta and Kimura, 1973). Unfortunately, the distribution of Plecoptera in most lowland regions of Britain has not been fully investigated (Hynes, 1977), and informed estimates of effective population sizes can not be made.

The high level of genetic variation found in Protonemura praecox seems rather enigmatic. This species is stenothermic (Brown et al, 1964) and its present distribution is more restricted than that of P. meyeri (Hynes,1977). All the populations of P. praecox were from large streams which may provide a wider range of habitat than the head water streams where most of the other species were collected. This would constitute a selectionist interpretation. However, a neutralist could argue that these larger streams have a greater effective population size due to the drift of nymphs down-stream from streams higher up the catchment.

A neutralist explanation for the apparently low level of variation in populations of P. meyeri is more difficult since it is one of the most numerous and widely distributed members of the British Nemouridae. This species has been found to have a habitat preference for patches of moss growing on the stream bed (Hynes, 1941; Brown, Cragg and Crisp, 1964; personal observations). The low amount of genetic variation could, therefore, be taken as support for the niche-width variation hypothesis since, by selecting a particular habitat, P. meyeri may experience a narrower range of environments.

Thus the overall patterns of variation of the Nemouridae appear, as is so often the case, to fit equally well the expectations of both the selectionist and neutralist schools of thought. The biological significance of this

variation is questionable; what is not questionable is that it provides a useful tool for species identification and characterisation.

TABLE 4.1. The enzymes investigated in the Nemouridae

ENZYME	ABBREVIATION	E.C.NUMBER
Acid phosphatase	ACP	3.1.3.2
Acid phosphatase (u.v.)	ACP(u.v.)	
Adenosine deaminase	ADA	3.5.4.4
Adenylate kinase	AK	2.7.4.3
Alcohol dehydrogenase	ADH	1.1.1.1
Aldehyde oxidase	AO	1.2.3.1
Aldolase	ALD	4.1.2.13
Alkaline phosphatase	ALP	3.1.3.1
Amino acid transferase	AAT	2.6.1.1
Aminopeptidase Phe-leu	AP	3.4.11.2
" Phe-pro	AP-D	3.4.13.9
" Leu-tyr		
Amylase	AMY	3.2.1.1
NAD diaphorase	DIA	1.6.2.2
Esterase	ES	3.1.1.1
Fructose-6-phosphate dehydrogenase	F6PDH	4.12.13
Fucose	FUC	
Fumarase	FUM	4.2.1.2
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49
Glutamate dehydrogenase	GDH	1.4.1.2
Glyceraldehyde-phosphate dehydrogenase	G3PDH	1.2.1.12
∝-Glycerophosphate dehydrogenase	GPDH	1.1.1.8
Glyoxalase	GLO	4.4.1.5
Hexanol dehydrogenase	HDH	1.1.1.1
Hexokinase	HK	2.7.1.1
Hydroxybutyrate dehydrogenase	HBDH	
Isocitrate dehydrogenase	IDH	1.1.1.42
Lactate dehydrogenase	LDH	1.1.1.27
Leucine aminopeptidase	LAP	3.4.11
Malate dehydrogenase	MDH	1.1.1.37
Malic enzyme	ME	1.1.1.40
Mannose phosphate isomerase	MPI	5.3.1.8
Nucleoside phosphorylase	NP	2.4.2.1
Octanol dehydrogenase	ODH	1.1.1.1
Phosphoglucose isomerase	PGI	5.3.1.9
Phosphoglucomutase	PGM	2.7.5.1
6-Phosphoglucose dehydrogenase	6PGDH	1.1.1.44
Protein	PT	
Sorbitol dehydrogenase	SDH	1.1.1.14
Tetrazolium oxidase	TO	1.15.1.1
Threonine dehydrogenase	TDH	4.2.1.1.6
Trehalase	TRE	3.2.1.28
Xanthate dehydrogenase	XDH	

Table 4.2. The enzymes succesfully resolved in 9 Nemourid Species

Enzyme	EC No.	No.loci	Structure	Buffer
Aldehyde oxidase	1.2.3.1	1	--	2
a-amylase	3.2.1.1.	1	--	5
Aminopeptidase-1	3.4.11.2	1	dimer	1
Aminopeptidase-D	3.4.13.9	1	--	1
Esterase	3.1.1.1.	2	monomer	2
Hexokinase	2.7.1.1.	1	--	1
Hexanol dehydrogenase	1.1.1.1.	2	dimer	3
Lactate dehydrogenase	1.1.1.27.	1	--	3
Leucine aminopeptidase	3.4.11.	1	--	3
Malate dehydrogenase	1.1.1.37.	1	--	4
Malic enzyme	1.1.1.40.	1	--	4
Phosphoglucose isomerase	5.3.1.9.	1	dimer	1
Phosphoglucomutase	2.7.5.1.	1	monomer	1
Phosphatase	3.3.3.	1	--	3

Note: Aminopeptidases 1 and D used phenylalanyl-leucine dipeptide and phenylalanyl-proline dipeptide respectively as substrates.

Quaternary structures are only given for the polymorphic enzymes, and are interpretations of heterozygote banding patterns.

TABLE 4.3. Allele frequencies at 16 loci in 9 Nemourid Species

		Species								
locus	allele	1	2	3	4	5	6	7	8	9
AO	n	139	55	23	18	15	56	16	38	23
	N	10	6	1	3	1	6	2	3	8
	112	--	--	--	--	1.000	--	--	--	--
	110	--	--	--	--	--	1.000	1.000	--	--
	108	--	--	--	1.000	--	--	--	--	--
	105	--	1.000	--	--	--	--	--	--	--
	102	--	--	--	--	--	--	--	1.000	1.000
	100	1.000	--	1.000	--	--	--	--	--	--
a-AMY	n	63	23	16	17	15	33	18	19	25
	N	10	4	1	1	1	6	2	1	4
	115	--	--	--	--	1.000	--	--	--	--
	110	--	--	--	--	--	--	1.000	--	--
	107	--	--	--	1.000	--	--	--	--	--
	105	--	--	--	--	--	--	--	--	1.000
	102	--	--	--	--	--	--	--	1.000	--
	100	1.000	--	--	--	--	--	--	--	--
	97	--	--	1.000	--	--	--	--	--	--
	95	--	--	--	--	--	1.000	--	--	--
	90	--	1.000	--	--	--	--	--	--	--
AP-1	n	920	140	41	35	57	203	37	28	77
	N	10	9	1	3	1	13	4	9	12
	120	--	--	--	--	1.000	--	--	--	--
	115	0.005	--	--	--	--	--	--	--	--
	110	--	--	--	--	--	--	--	--	1.000
	100	0.904	--	--	--	--	--	--	--	--
	90	0.091	--	--	--	--	--	--	--	--
	55	--	--	--	--	--	1.000	--	--	--
	52	--	--	1.000	--	--	--	--	--	--
	50	--	1.000	--	--	--	--	--	--	--
	47	--	--	--	1.000	--	--	--	--	--
	45	--	--	--	--	--	--	1.000	--	--
	35	--	--	--	--	--	--	--	1.000	--
AP-D	n	338	17	22	32	19	199	34	24	37
	N	11	3	1	3	1	11	4	7	8
	105	--	--	--	--	1.000	--	--	--	--
	100	1.000	--	1.000	--	--	--	--	--	--
	97	--	--	--	--	--	--	1.000	--	--
	95	--	--	--	--	--	--	--	1.000	--
	92	--	--	--	--	--	--	--	--	1.000
	90	--	--	--	--	--	1.000	--	--	--
	85	--	1.000	--	1.000	--	--	--	--	--
ES-1	n	430	69	45	19	50	128	46	62	110
	N	10	4	1	3	1	8	3	4	8
	110	--	--	--	--	--	--	--	--	1.000
	108	--	--	--	--	--	--	--	1.000	--
	107	--	--	--	--	--	--	1.000	--	--
	105	--	--	--	--	--	1.000	--	--	--
	102	--	--	--	1.000	--	--	--	--	--

	100	1.000	--	--	--	--	--	--	--	--
	97	--	1.000	--	--	--	--	--	--	--
	95	--	--	1.000	--	--	--	--	--	--
	85	--	--	--	--	1.000	--	--	--	--
ES-2	n	430	42	45	19	50	138	27	52	73
	N	10	4	1	3	1	6	3	4	8
	106	--	--	--	--	1.000	--	--	--	--
	102	--	--	--	--	--	--	--	0.212	0.260
	100	1.000	--	--	--	--	--	--	--	--
	98	--	--	--	--	--	--	--	0.788	0.596
	97	--	0.024	--	--	--	--	--	--	--
	96	--	--	--	1.000	--	--	--	--	--
	95	--	0.881	--	--	--	--	--	--	--
	94	--	--	--	--	--	0.159	--	--	0.144
	93	--	0.095	--	--	--	--	--	--	--
	92	--	--	--	--	--	0.841	0.407	--	--
	90	--	--	--	--	--	--	0.593	--	--
	88	--	--	1.000	--	--	--	--	--	--
HK	n	287	48	22	16	16	115	24	17	36
	N	12	5	1	2	1	11	4	1	7
	110	--	--	--	--	1.000	--	--	--	--
	105	--	--	1.000	--	--	--	--	--	--
	100	1.000	1.000	--	--	--	1.000	1.000	1.000	1.000
	95	--	--	--	1.000	--	--	--	--	--
HDH-1	n	795	33	20	21	26	21	23	114	101
	N	10	4	1	3	1	5	1	10	10
	110	0.167	--	--	--	--	--	--	--	--
	105	--	1.000	--	--	--	--	--	--	--
	102	--	--	--	--	--	--	--	1.000	1.000
	100	0.765	--	--	--	--	--	--	--	--
	97	--	--	1.000	--	--	--	--	--	--
	95	--	--	--	--	1.000	--	--	--	--
	92	--	--	--	--	--	--	0.413	--	--
	90	0.069	--	--	--	--	--	--	--	--
	88	--	--	--	--	--	1.000	0.587	--	--
	80	--	--	--	1.000	--	--	--	--	--
HDH-2	n	121	33	16	21	26	21	23	114	66
	N	10	4	1	3	1	5	1	10	10
	120	--	--	--	--	--	--	--	1.000	0.326
	110	--	--	--	--	1.000	--	--	--	--
	100	1.000	1.000	1.000	1.000	--	--	--	--	0.674
	98	--	--	--	--	--	1.000	1.000	--	--
LAP	n	140	19	16	15	18	22	17	18	16
	N	9	3	1	2	1	3	1	1	3
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
LDH	n	244	54	18	16	17	144	29	103	104
	N	14	6	1	2	1	9	3	9	11
	120	--	--	--	--	1.000	--	--	--	--
	115	--	--	--	1.000	--	--	--	--	--
	110	--	--	1.000	--	--	--	--	--	--
	105	--	--	--	--	--	--	--	--	1.000
	100	1.000	1.000	--	--	--	--	--	--	--
	98	--	--	--	--	--	1.000	1.000	--	--
	95	--	--	--	--	--	--	--	1.000	--

Table 4.3.

MDH	n	201	65	26	16	17	106	17	20	19
	N	12	6	1	1	1	8	5	1	4
	110	--	--	--	1.000	--	--	--	--	--
	100	1.000	--	--	--	--	--	--	--	--
	80	--	--	1.000	--	--	--	--	--	--
	70	--	1.000	--	--	--	--	--	--	--
	60	--	--	--	--	1.000	--	--	--	--
	50	--	--	--	--	--	1.000	1.000	--	--
	40	--	--	--	--	--	--	--	1.000	1.000
ME	n	214	58	23	26	24	117	39	23	28
	N	11	7	1	2	1	11	4	2	5
	115	--	--	--	--	--	--	--	--	1.000
	110	--	--	--	--	--	--	--	1.000	--
	105	--	--	--	--	--	--	1.000	--	--
	102	--	1.000	--	--	--	--	--	--	--
	100	1.000	--	--	--	--	1.000	--	--	--
	95	--	--	1.000	--	--	--	--	--	--
	90	--	--	--	1.000	--	--	--	--	--
	85	--	--	--	--	1.000	--	--	--	--
PGI	n	1095	171	33	43	64	194	67	77	101
	N	10	9	1	3	1	15	8	9	12
	220	--	--	0.742	--	--	--	--	--	--
	200	--	--	0.227	--	--	--	--	--	--
	150	--	--	--	--	--	--	--	0.188	0.015
	120	0.007	--	--	--	0.039	--	--	0.805	0.970
	100	0.944	1.000	0.030	--	0.961	--	--	0.006	0.015
	90	--	--	--	--	--	1.000	1.000	--	--
	60	0.048	--	--	1.000	--	--	--	--	--
PGM	n	979	124	16	22	45	269	70	82	100
	N	10	9	1	3	1	15	8	9	12
	130	--	--	--	0.068	--	--	--	--	--
	120	0.004	--	--	0.932	--	--	--	--	0.845
	115	--	--	--	--	--	0.993	--	--	0.155
	110	0.372	--	0.438	--	--	--	--	--	--
	105	--	0.573	--	--	1.000	0.007	--	--	--
	100	0.597	--	0.531	--	--	--	--	--	--
	97	--	--	--	--	--	--	--	0.043	--
	95	--	0.407	--	--	--	--	--	--	--
	92	--	--	--	--	--	--	--	0.957	--
	90	0.028	--	0.031	--	--	--	--	--	--
	85	--	0.020	--	--	--	--	--	--	--
	80	--	--	--	--	--	--	0.814	--	--
	70	--	--	--	--	--	--	0.186	--	--
PHOS	n	289	27	16	31	15	120	28	21	20
	N	13	4	1	3	1	12	5	1	4
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Species: 1. Nemoura cinerea; 2. Nemoura cambrica; 3. Nemoura dubitans; 4. Nemoura avicularis; 5. Nemurella picteti; 6. Protonemura meyeri; 7. Protenemura praecox; 8. Amphinemura standfussi; 9. Amphinemura sulcicollis.

n = number of individuals sampled
N = number of populations sampled

Table 4.3.

TABLE 4.4. Measures of genetic variation in 9 Nemourid Species

Species	Mean sample size/locus	Mean no. alleles / locus	% loci[1] polymorphic	Mean heterozygosity Observed	Expected[2]
N. cinerea	417.8 (84.1)	1.63 (0.29)	25.00	0.067 (0.035)	0.073 (0.039)
N. cambrica	61.1 (11.3)	1.25 (0.17)	12.50	0.031 (0.023)	0.045 (0.034)
N. dubitans	24.9 (2.6)	1.31 (0.22)	12.50	0.060 (0.043)	0.062 (0.043)
N. avicularis	22.9 (2.1)	1.06 (0.06)	6.25	0.009 (0.009)	0.008 (0.008)
Nem. picteti	29.6 (4.3)	1.06 (0.06)	6.25	0.005 (0.005)	0.005 (0.005)
P. meyeri	117.9 (18.6)	1.13 (0.09)	6.25	0.010 (0.009)	0.018 (0.017)
P. praecox	32.2 (4.1)	1.19 (0.10)	18.75	0.055 (0.031)	0.081 (0.045)
A. standfussi	50.8 (9.1)	1.25 (0.14)	18.75	0.026 (0.015)	0.046 (0.028)
A. sulcicollis	58.5 (9.1)	1.38 (0.18)	25.00	0.063 (0.032)	0.083 (0.044)
overall mean		1.25 (0.18)	14.58 (7.65)	0.036 (0.025)	0.047 (0.031)

1) 0.99 criterion.
2) unbiased estimate (Nei, 1978)
Figures in parentheses are standard errors.

TABLE 4.5. Nei's (1978) genetic identities (I, above diagonal) and distances (D, below diagonal) between pairs of species.

Species	1	2	3	4	5	6	7	8	9
1. N. cinerea	----	0.39	0.37	0.20	0.19	0.26	0.20	0.20	0.25
2. N. cambrica	0.93	----	0.20	0.26	0.23	0.19	0.20	0.20	0.25
3. N. dubitans	1.00	1.61	----	0.19	0.13	0.13	0.13	0.13	0.18
4. N. avicularis	1.61	1.36	1.64	----	0.13	0.13	0.13	0.13	0.23
5. Nem. picteti.	1.66	1.48	2.03	2.07	----	0.13	0.13	0.13	0.13
6. P. meyeri	1.34	1.64	2.04	2.07	2.06	----	0.59	0.19	0.21
7. P. praecox	1.59	1.61	2.00	2.03	2.03	0.53	----	0.20	0.20
8. A. standfussi	1.61	1.62	2.01	2.05	2.03	1.64	1.61	----	0.51
9. A. sulcicollis	1.38	1.40	1.70	1.48	2.01	1.56	1.59	0.67	----

TABLE 4.6. Edward's (1971, 1974) genetic distances between pairs of species.

Species	1	2	3	4	5	6	7	8
1. N. cinerea	----							
2. N. cambrica	1.04	----						
3. N. dubitans	1.02	1.17	----					
4. N. avicularis	1.17	1.14	1.18	----				
5. Nem. picteti	1.18	1.15	1.22	1.23	----			
6. P. meyeri	1.13	1.18	1.22	1.23	1.23	----		
7. P. praecox	1.17	1.18	1.22	1.22	1.22	0.86	----	
8. A. standfussi	1.17	1.18	1.22	1.22	1.22	1.19	1.18	----
9. A. sulcicollis	1.13	1.14	1.18	1.15	1.21	1.15	1.17	0.91

PLATE 4.1. Gel slice stained for Hexanol dehydrogenase (N. cinerea)

HDH-1 Genotypes

100	110	100	100	110	100	100	110	100	110	110	100	100	110	100
100	110	100	100	100	100	100	110	100	100	100	100	100	100	100
110	110	110	110	100	110	100	100	110	110	110	100	100	100	100
110	110	110	100	100	100	100	100	100	100	100	100	100	100	100

Genotype Frequencies

100/100	100/110	110/110
16	9	5

Allele Frequencies

100 : 32 + 9 = 41/60 = 0.683

110 : 9 + 10 = 19/60 = 0.317

PLATE 4.2. Gel slice stained for Phosphoglucomutase and Phosphoglucose isomerase. Species from left: Nemoura cinerea N. dubitans, N. avicularis, Nemurella picteti, Amphinemura standfussi, N. cambrica, Protonemura meyeri.

Genotypes

PGM	110	110	100	110	110	110	110	110	120	120	120	120
	100	100	100	100	100	90	110	100	120	120	120	120
	105	105	105	105	92	92	92	105	105	105	115	115
	105	105	105	105	92	92	92	95	95	105	115	115
PGI	100	100	100	100	220	220	220	220	60	60	60	60
	100	100	100	100	220	220	220	220	60	60	60	60
	100	100	100	100	120	120	120	100	100	100	90	90
	100	100	100	100	120	120	120	100	100	100	90	90

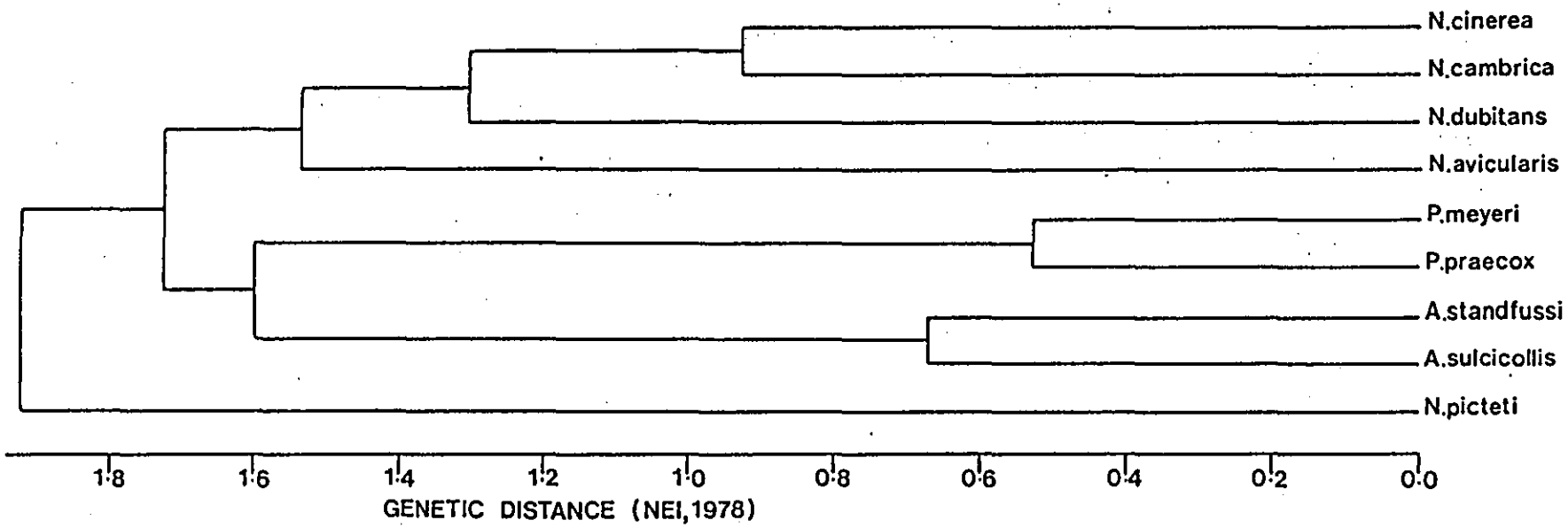


Figure 4.1. Genetic relationships between 9 Nemourid species based on allele frequencies at 16 loci (UPGMA cluster analysis).
Farris (1972) f = 6.196
Prager and Wilson (1976) F = 10.541
Percent Standard Deviation (Fitch & Margoliash, 1967) = 13.91
Cophenetic Correlation = 0.831

Figure 4.1.

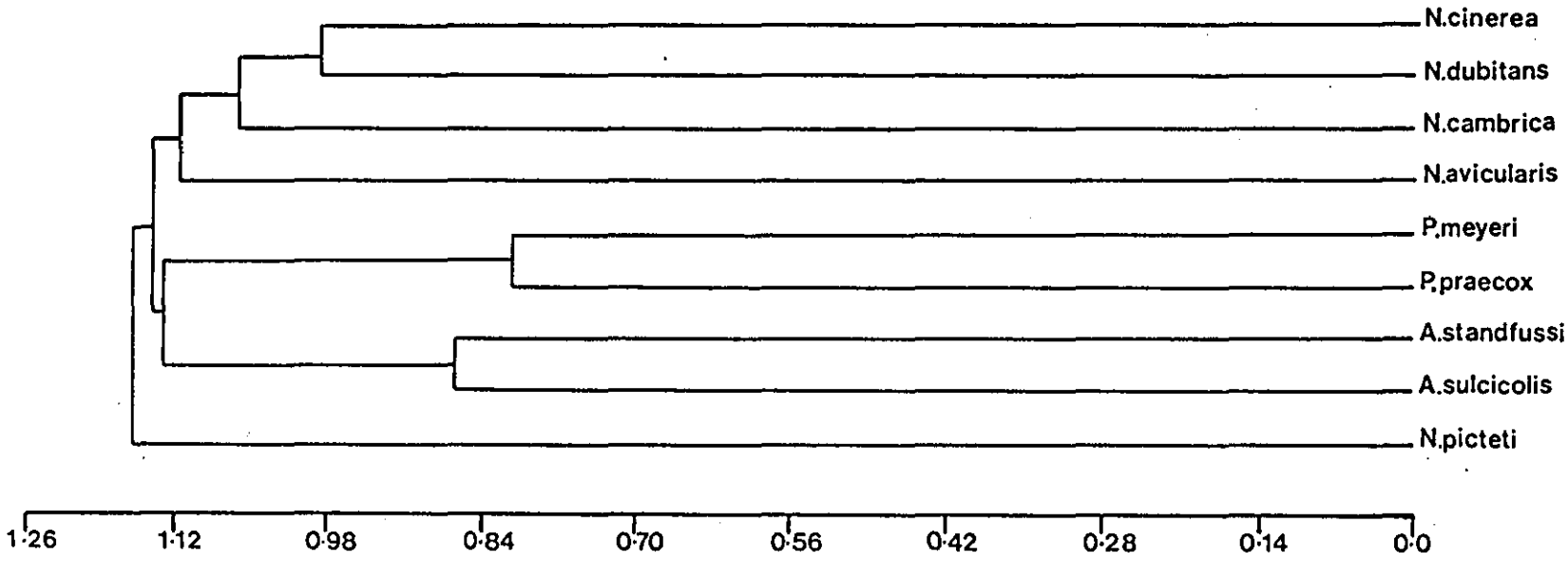


Figure 4.2. Genetic relationships between 9 Nemourid species based on allele frequencies at 16 loci (UPGMA cluster analysis).
Farris (1972) f = 0.851
Prager and Wilson (1976) F = 2.037
% Standard Deviation (Fitch & Margoliash, 1967) = 2.741
Cophenetic Correlation = 0.922

Figure 4.2.

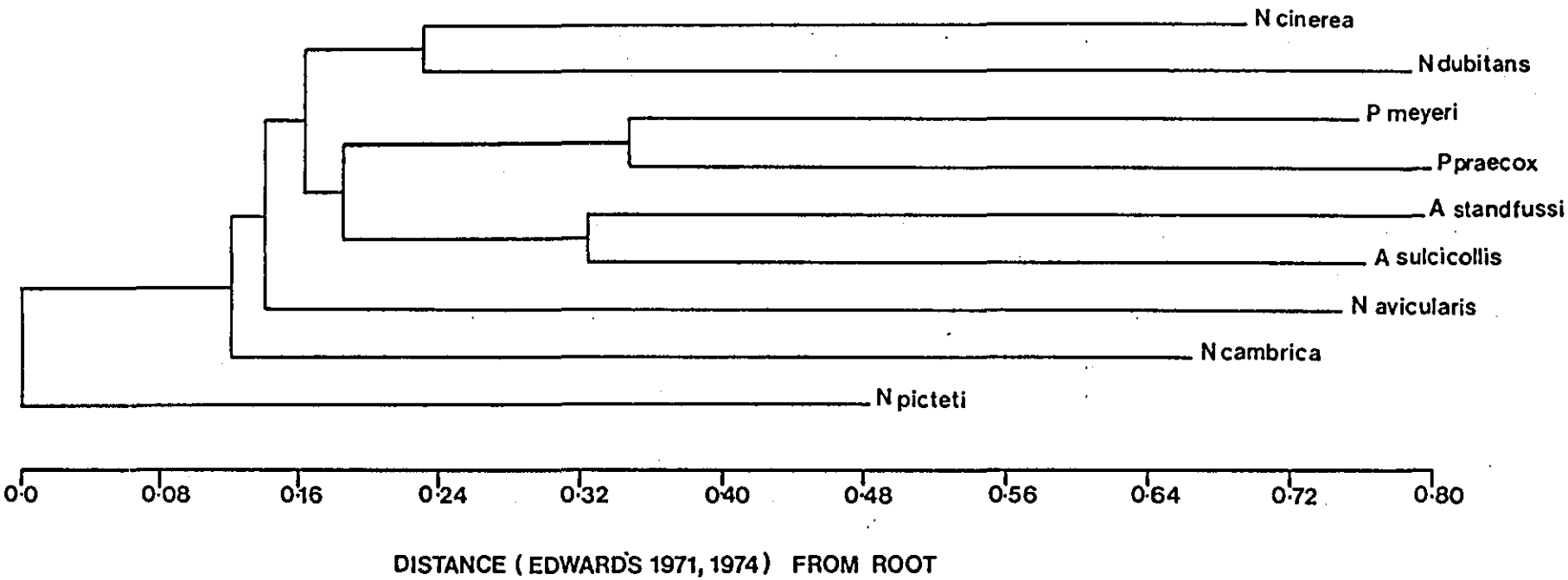


Figure 4.3. Genetic relationships between 9 Nemourid species based on allele frequencies at 16 loci (Farris, 1972 distance-Wagner analysis, addition criterion 1 and MAC). Rooted by outgroup method.
Farris (1972) f = 0.981 (0.403)
Prager and Wilson (1976) F = 2.348 (0.964)
% Standard Deviation (Fitch & Margoliash, 1967) = 3.337 (1.391)
Cophenetic Correlation = 0.957 (0.982)

Figure 4.3.

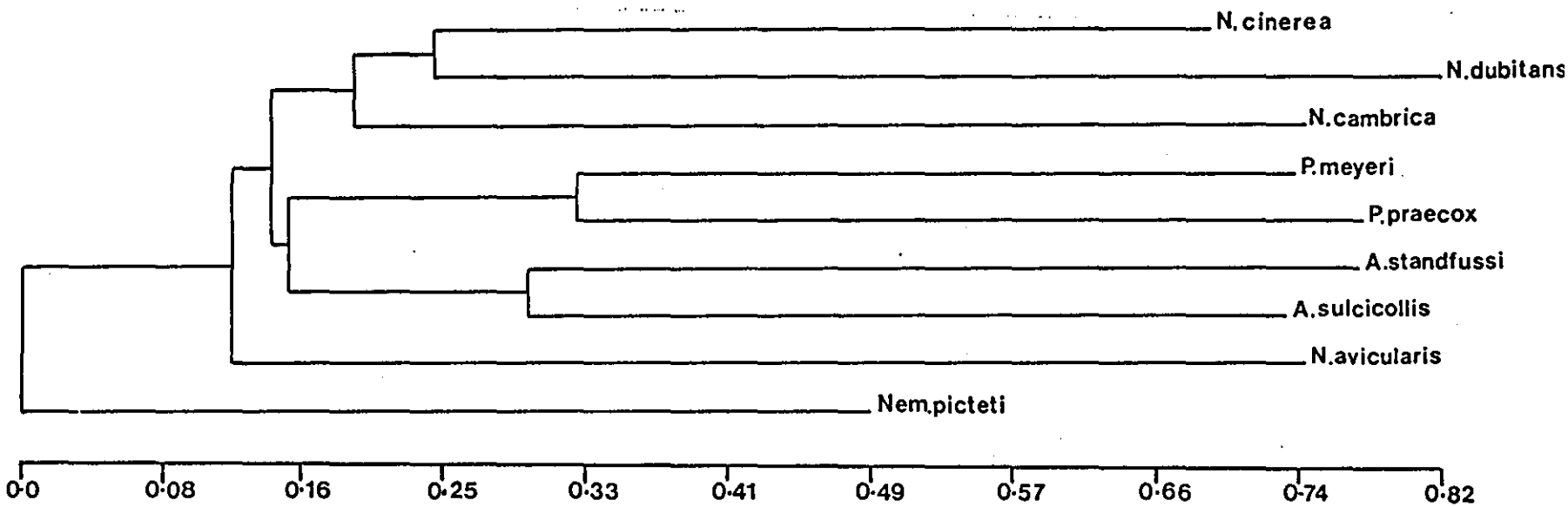


Figure 4.4. Genetic relationships between 9 Nemourid species based on allele frequencies at 16 loci (Farris, 1972 distance-Wagner analysis, addition criterion 2 and 3). Rooted by outgroup method.
Farris (1972) f = 1.194 (0.384)
Prager and Wilson (1976) F = 2.857 (0.919)
% Standard Deviation (Fitch & Margoliash, 1967) = 4.217 (1.327)
Cophenetic Correlation = 0.936 (0.983)

Figure 4.4.

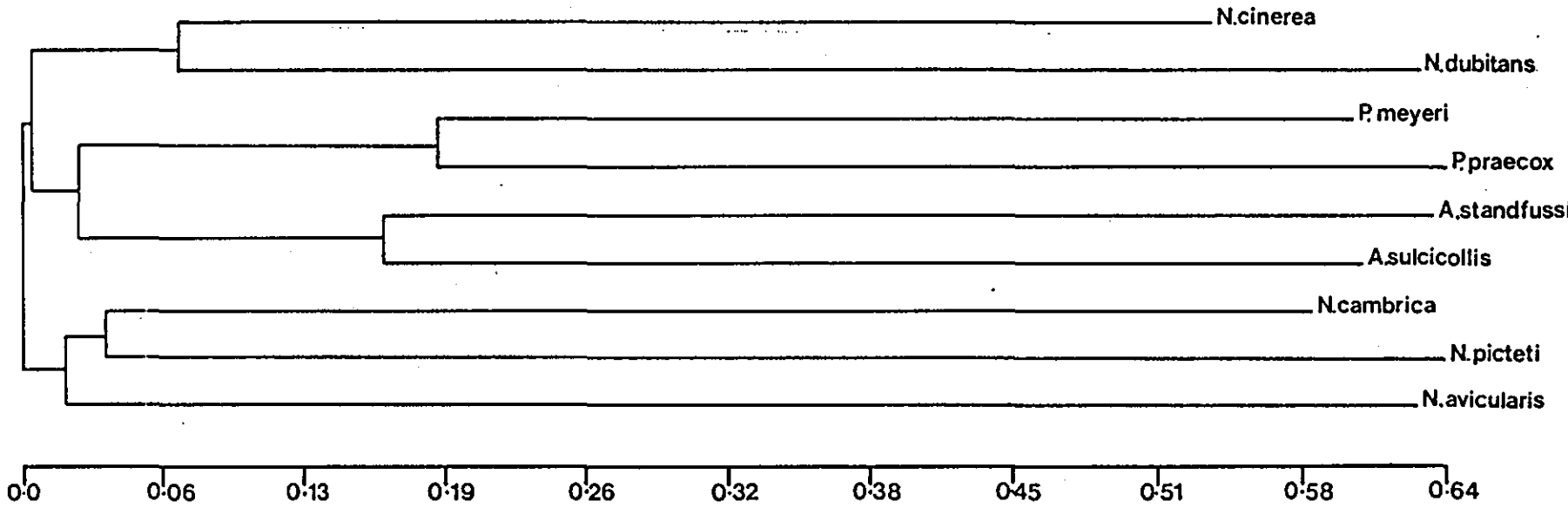


Figure 4.5. Genetic relationships between 9 Nemourid species based on allele frequencies at 16 loci (Farris, 1972 distance-Wagner analysis, addition criterion 1 and MAC). Rooted at mid-point of longest path.
Farris (1972) f =0.981 (0.403)
Prager and Wilson (1976) F = 2.348 (0.964)
% Standard Deviation (Fitch & Margoliash, 1967) = 3.337 (1.391)
Cophenetic Correlation =0.957 (0.982)

Figure 4.5.

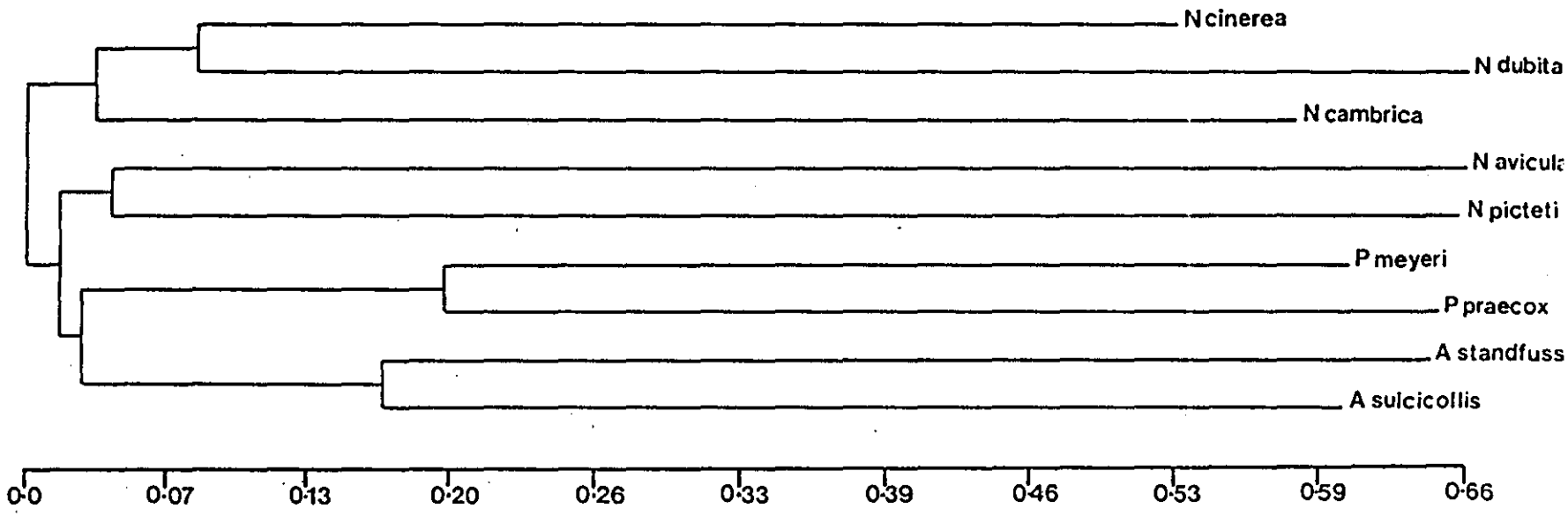


Figure 4.6. Genetic relationships between 9 Nemourid species based on allele frequencies at 16 loci (Farris, 1972 distance-Wagner analysis, addition criterion 2 and 3). Rooted at mid-point of longest path.
Farris (1972) f = 1.194 (0.384)
Prage and Wilson (1976) F = 2.857 (0.919)
% Standard Deviation (Fitch & Margoliash, 1967) = 4.217 (0.936)

Cophenetic Correlation = 0.936 (0.983)

Figure 4.6.

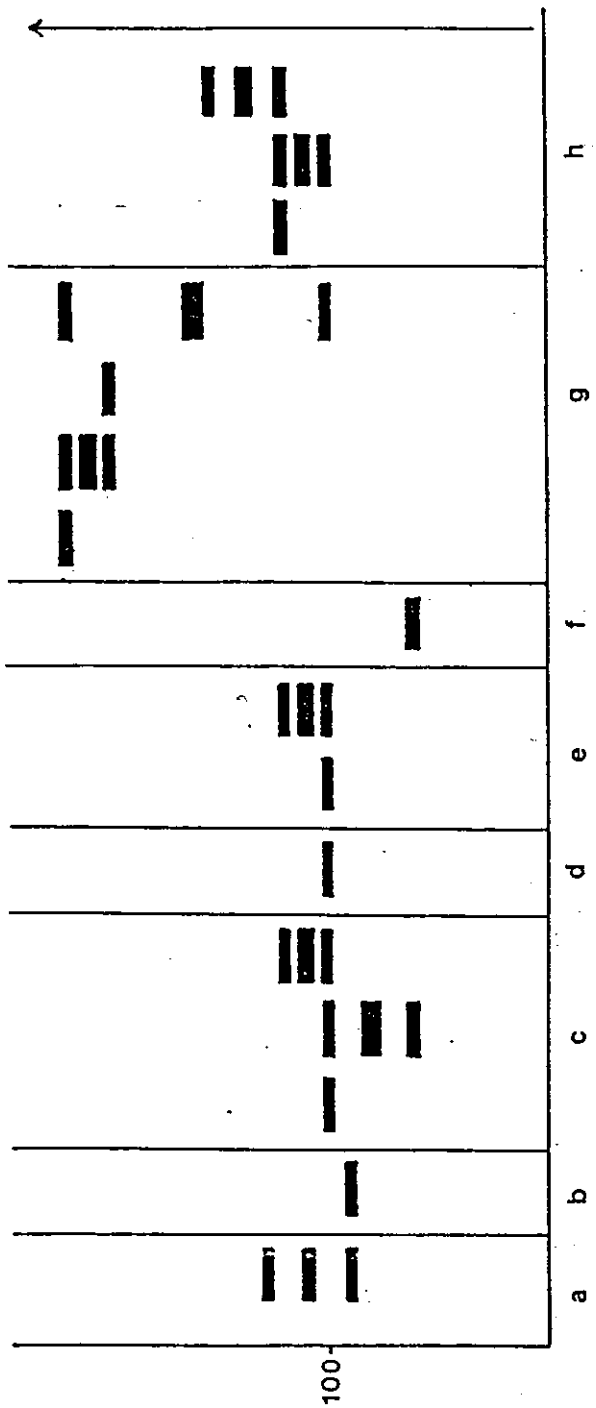


Figure 4.7. Observed phosphoglucose isomerase banding patterns. Genotypes are as follows:

a. P. meyeri 90/90
b. P. praecox 90/90
c. N. cinerea 100/100, 60/100, 100/120
d. N. cambrica 100/100
e. Nem. picteti 100/100, 120/100
f. N. avicularis 60/60
g. N. dubitans 220/220, 200/220, 200/200, 100/220
h. A. standfussi/sulcicollis 120/120, 120/100, 150/120

Figure 4.7

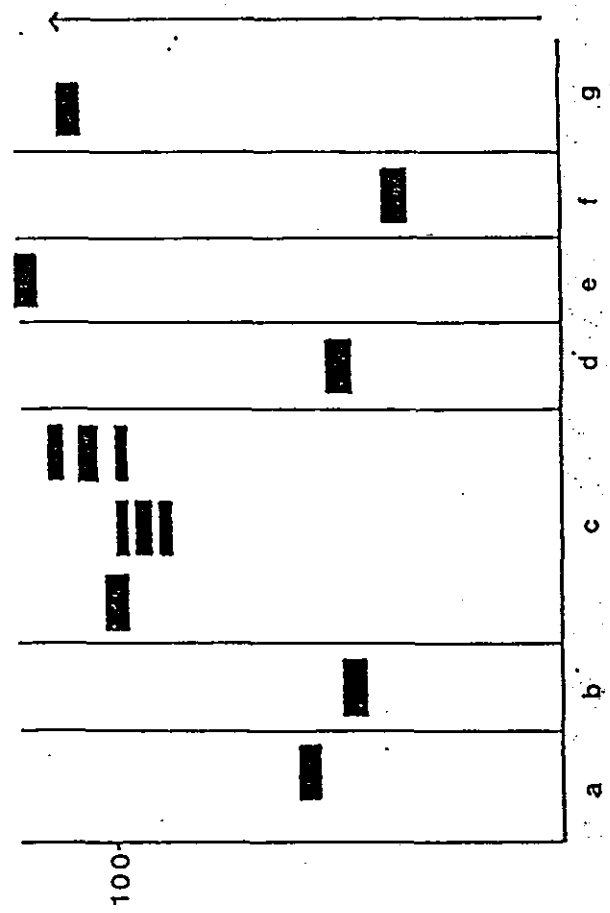


Figure 4.8. Observed aminopeptidase banding patterns.
Genotypes are as follows:
a. P. meyeri 55/55
b. P. praecox 45/45
c. N. cinerea 100/100, 90/100, 100/115
d. N. cambrica 50/50
e. Nem. picteti 120/120
f. A. standfussi 35/35
g. A. sulcicollis 110/110

Figure 4.8.

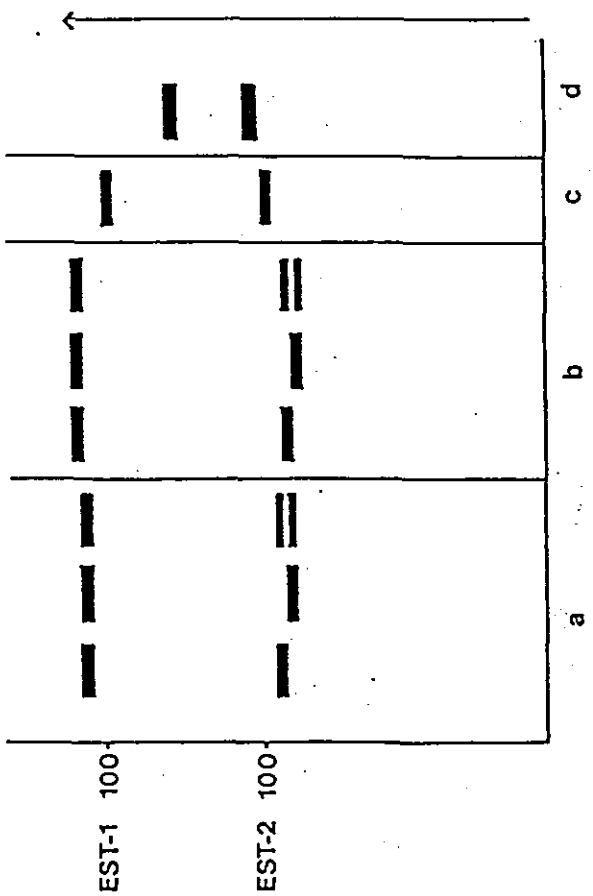


Figure 4.9. Observed esterase banding patterns.
Genotypes are as follows:
a. P. meyeri ES-1 105/105; ES-2 94/94, 92/92, 92/94
b. P. praecox ES-1 107/107; ES-2 92/92, 90/90, 90/92
c. N. cinerea ES-1 100/100; ES-2 100/100
d. Nem. picteti ES-1 85/85; ES-2 106/106

Figure 4.9.

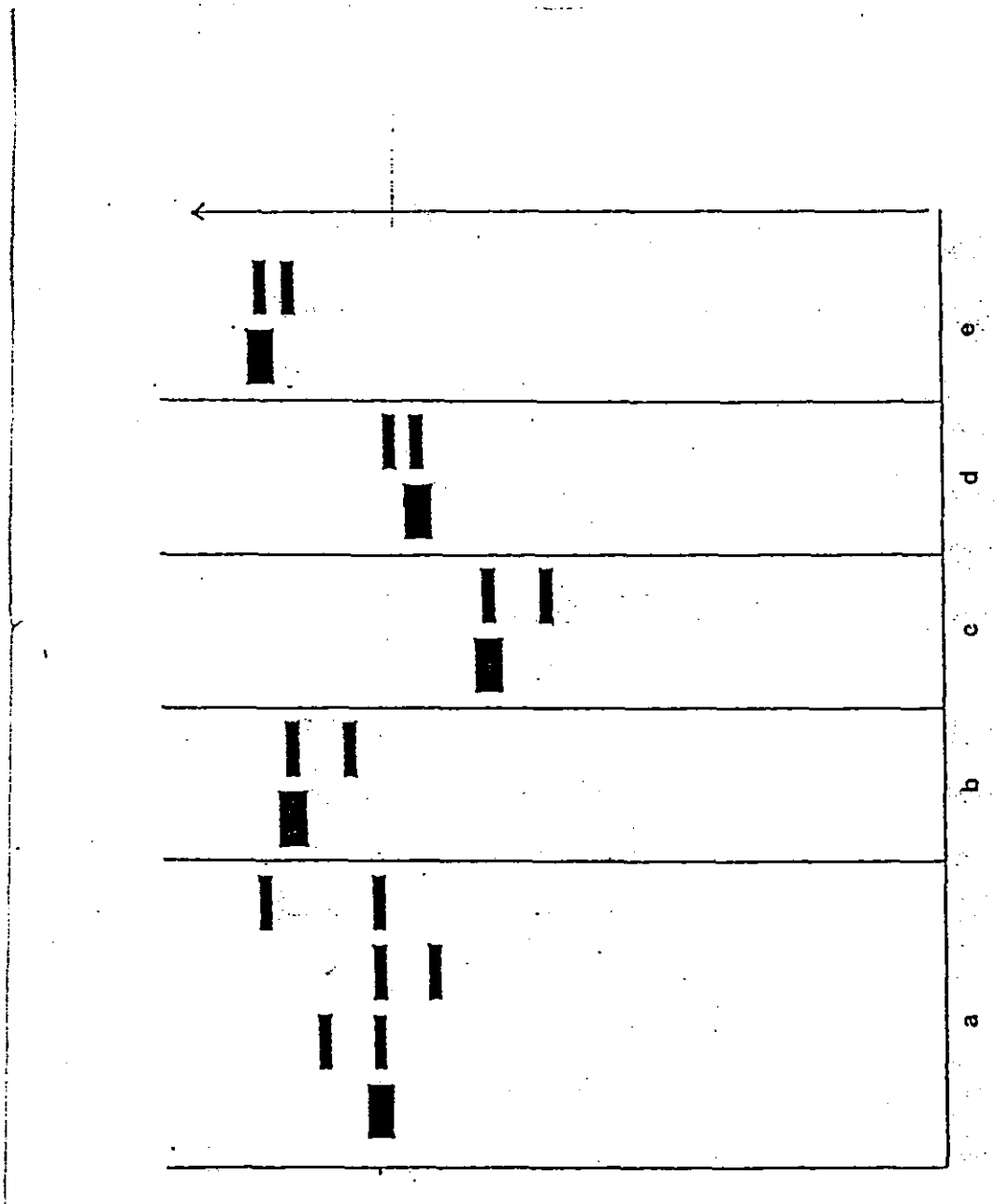


Figure 4.10. Observed phosphoglucomutase banding patterns. Genotypes are as follows:

a. N. cinerea 100/100, 100/110, 100/90, 100/120
b. P. meyeri 115/115, 115/105
c. P. praecox 80/80, 80/70
d. A. standfussi 92/92, 92/97
e. A. sulcicollis 120/120, 120/115

CHAPTER 5

INTER-POPULATION GENETIC VARIATION IN NEMOURA CINEREA

5.1. Introduction

The resolution of the debate over the evolutionary significance of the variation revealed by molecular genetics techniques appears to be almost as far away today as when population genetics theory was originally polarized into the so called 'neutralist' or 'selectionist' schools of thought almost 20 years ago. Neutral theory (or the neutral mutation - random drift hypothesis) proposes that the majority of molecular variation is caused by random fixation of selectively neutral or nearly neutral mutants in a population or species. In contrast, selectionist theory regards the molecular polymorphism as adaptive, and argues that it is maintained in the population or species by some form of balancing selection (Kimura, 1983). Some erosion away from the two opposing standpoints has occurred since the early 1970's, accelerated by more recent findings of DNA sequence variability, so that today most population geneticists believe that both stochastic (neutral) and selective processes can account for the observed molecular variation. The debate has shifted from' either or' to 'how much' of each (Nevo et al, 1983).

Mathematical attempts to answer the question of how much of the observed molecular variation can be explained by either a neutralist or a selectionist interpretation have foundered on the difficulty of measuring any of the factors involved, such as population size, rates of mutation and

migration, and selection intensity, with sufficient accuracy. It has been suggested that a more promising way to answer the question is to identify correlations between gene frequencies and spatial or temporal variations in the environment. This would provide strong evidence that selection can influence genetic variation at the molecular level (Clarke, 1975; Nevo et al, 1984). The accumulated evidence from many similar investigations would give an indication of the relative importance of stochastic or selective forces. The results of an investigation into the effects of stream water acidity on molecular genetic variation in the plecopteran Nemoura cinerea are presented in this chapter.

The acidity of stream water was identified as a potential selective factor from the reports of serious environmental pollution caused by 'acid rain' in Scandinavia, Central Europe and North America, where the ecology of fresh waters has been severely disrupted (Overein et al, 1980; Haines, 1981). The Plecoptera were chosen for study because members of this insect order form the main component of the benthic macro-invertebrate community in many of the acidified upland strams in Britain (Hynes, 1970).

5.2. Materials and Methods.

5.2.1. Experimental Organisms.

This investigation concentrated on inter-population comparisons of Nemoura cinerea. The choice of species was made primarily on the basis of it s reported widespread distribution (Brink, 1949; Hynes, 1977) and high level of genetic variation relative to other members of the Nemouridae

(Chapter 4).

The 31 sites from which collections of N. cinerea were made are indicated in Table 2.1. (Chapter 2). The pH and conductivity were measured at each site and the altitude, latitude and longitude noted.

The temporal stability of allele frequecies was monitored over two or three years in five populations in the Derbyshire Peak District. The five populations were: Thomason's Hollow, Top Lady Clough, Lady Clough, Rising Clough and Miller's Dale.

5.2.2. Electrophoresis.

Only the four polymorphic loci (AP-1, HDH-1, PGI and PGM, Table 4.3.) were routinely screened in all thirty-one populations. The enzyme stains were as follows:

Aminopeptidase (AP) -

Phenylalanyl-leucine	10 mg
Peroxidase	5 mg
L-amino acid oxidase	3 mg
Dianisidine	3 mg
Manganese Chloride	2 mg
Buffer-Tris-HCl pH 8.0	25 ml
(1% Agar Overlay)	25 ml

Hexanol dehydrogenase (HDH) -

Hexanol	1.5 ml
NAD	15 mg
MTT	5 mg
PMS	2 mg

	Magnesium Chloride	20 mg
	Buffer - Tris-HC1 pH 8.0	30 ml

Phosphoglucose isomerase (PGI) -

Fructose-6-phosphate	10 mg
NADP	3 mg
MTT	7 mg
G6PDH	10 λ
PMS	2 mg
Magnesium Chloride	20 mg
Buffer - Tris-HC1 pH 8.0	30 ml

Phosphoglucomutase (PGM) -

Glucose-1-phosphate	50 mg
(cont. 1% Gluc-1,6-diphos)	
NADP	3 mg
MTT	5 mg
Magnesium Chloride	30 mg
G6PDH	10 λ
PMS	2 mg
Buffer -Tris-HC1 pH 8.0	30 ml

Buffer system 1 was used for AP, PGI and PGM, and buffer system 3 for HDH (Chapter 2).

The allele frequencies were calculated from single samples of nymphs from each population.

5.2.3. Analysis.

The MINITAB (Pennsylvania State University) statistics program was used to perform regression analyses between the five environmental characteristics of the sample sites, and between the common allele frequencies at the four polymorphic loci and the environmental features. Regression has been used in many similar investigations into the effects of environment on genetic variation (Nevo et al, 1982; Karlin et al, 1984; and Carvalho and Crisp, 1987) and has been useful in identifying possible associations between allele frequency or heterozygosity and environmental factors. However, the results should always be interpreted with caution when the variables are not normally distributed. Regression is also of value for identifying unusual observations which deviate from the general pattern (Sokal, 1965).

All other analyses of the allozyme data were provided by the BIOSYS1 program of Swofford and Selander (1981).

5.3. Results.

5.3.1. Site Attributes.

The five physical characteristics recorded for all the sampling sites are shown in Table 5.1. and the altitude, pH and conductivity are shown in Figures 5.1 - 3. Water chemistry shows considerable seasonal variation in some streams (Figure 3.1. Chapter 3). However, the pH and conductivity shown in Table 5.1. are values obtained from single samples to avoid any bias that may result from using means from the regularly monitored sites in the Peak District. Latitude was recorded as minutes north of 51°N, longitude as minutes west of 0°.

The correlations between the five physical characteristics of the sites are shown in Table 5.2. Statistically significant associations ($P < 0.05$) were found between the altitude, pH and conductivity. pH and conductivity both decreased in the higher altitude streams. Figures 5.1-3 indicate that the sites can be divided into two categories on the basis of these three characteristics :

CATEGORY 1.) High altitude (>250 metres), high acidity (pH < 6.0) and low conductivity (< 250 $\mu S.cm^{-1}$). Number = 17

CATEGORY 2.) Low altitude (<250 metres), low acidity (pH > 7.0) and high conductivity (> 250 $\mu S.cm^{-1}$). Number = 12

The only exceptions to this were Baluain (site 18) where there was low conductivity with a high pH, and Wareham 2 which is the only low altitude/low pH site. If the data from these two sites are omitted from the analysis, the correlations between altitude, pH and conductivity are improved (Table 5.2.).

5.3.2. Spatial Allele Frequency Variation.

The allele frequencies at the four loci are presented in Tables 5.3 - 6. together with the results of Hardy-Weinberg X^2 analysis and the frequency of heterozygotes for each population. In the X^2 tests for fit to Hardy-Weinberg equilibrium, rare allele frequencies were combined to overcome the problem caused by expected values of less than 5. Heterozygosity was calculated using Nei's (1978) estimate which is unbiased by differences in sample sizes. The mean

heterozygosities (He) over the four loci are included in Table 5.3. Figures 5.4 - 7. give an illustration of the variation in allele frequencies between the populations.

Only 4 out of the 124 comparisons between the observed allele frequencies and those expected assuming Hardy-Weinberg equilibrium showed any significant deviation. The sample from Rising Clough (site 30) had a slight excess of 90 alleles at the AP locus (Table 5.3.). There was a slight excess of 60 alleles at the PGI locus at site 4, Dean Clough (Table 5.5.) and there was a heterozygote deficit at the PGM locus in samples from site 15, Thomason's Hollow and site 16, Top Lady Clough (Table 5.6.)

Contingency X^2 analysis showed that there was significant heterogeneity in allele frequency at all four loci between the populations (Table 5.9.). However, the expected frequency for several classes was low so that the results can only be interpreted with caution.

5.3.2.1. Aminopeptidase

Three alleles were found for this locus though only two (90 and 100) were present in most populations. The rare 115 allele was found, in low frequency, in just three populations from the southern Pennines: Cabin Clough (site 3), Holden Clough (site 5) and Shiny Brook (site 14). Only one population, site 13, Port Erin on the Isle of Man, had no variation, being fixed for the 100 allele. The frequency of heterozygotes ranged from 0.00 to 0.431 with a mean of 0.183.

The distribution of allele frequencies shown in Figure 5.4. does not appear to follow an obvious pattern and regression analysis did not reveal any significant relationships between the allele frequencies at this locus and

any of the environmental factors recorded for each site (Table 5.7.).

5.3.2.2. Phosphoglucose Isomerase.

A total of four alleles was found at this locus. Only two alleles (60 and 100) were present in fifteen of the populations. Four populations: Moorhouse Mine (site 8), Nant-y-trawsnant (site 10), Nailstone (site 25) and Wymondham (site 29) were fixed for the 100 allele. The 140 allele was found in just four scattered populations: North Wales 4 (site 11), Ogden (site 12), Shiny Brook (site 14) and Wareham 1 (site 28). The frequency of heterozygotes ranged from 0.00 to 0.344 with a mean of 0.109.

The most noteable feature of the allele frequency distribution, shown on Figure 5.5., is the high frequency of the 60 allele in the Baluain population (site 18) where, in contrast to the other populations, this was the most common allele. Regression of the frequencies of the 60 and 100 alleles against the five environmental variables indicated that there was a significant relationship between latitude and longitude and the frequency of both alleles (Table 5.7.) The frequency of the 60 allele appears to increase to the north ($r = 0.667$, $p<0.001$) and west ($r = 0.400$, $p<0.05$) whilst the frequency of the 100 allele decreases ($r = -0.66$, $p<0.001$; $r = -0.405$, $p<0.05$, respectively). However, closer analysis of the results showed that the apparent relationship was caused by the inclusion of data from Baluain, Scotland, with its high latitude, westerly position and unusual allele frequencies (Figure 5.5.). When the Baluain data were excluded from the analysis, there was no significant relationship between allele frequency and any of the environmental parameters (Table 5.7.).

5.3.2.3. Phosphoglucomutase

Five alleles were present at this locus. One population, site 8, Moorhouse Mine, was fixed for the 100 allele. The most common alleles were 100 and 110 in all populations. The 80 allele was found in only one population, site 27, Thrussington. Although the 90 allele was present in fourteen populations it was only found at a relatively high frequency in the Derbyshire Peak District, especially in Rising Clough (site 30) and Burbage Brook (site 2) (Figure 5.6.). The heterozygote frequency ranged from 0.00 to 0.529 with a mean of 0.443.

Regression analysis showed a significant association between the frequency of the 100 and 110 alleles and latitude, longitude, altitude and conductivity (Table 5.7.). The 100 allele appears to increase in frequency, and the 110 allele to decrease in frequency, to the north, the west, at high altitude, and in streams with a low conductivity. There was no significant association between allele frequency and pH. However, MINITAB indicated that several populations had an unusually large influence on the analysis. These were : Baiuain (Site 18) and Wareham 2 (Site 17), the two sites with unusual physical characteristics (section 5.3.1.); Nailstone (Site 25) and Nant-y-Trawsnant (Site 10), which had very small sample sizes; and Moorhouse Mine (Site 8), which was fixed for the 100 allele. When these populations were excluded from the regression, the only significant relationship was between conductivity and the frequency of the 110 allele ($r = 0.439$, $p<0.05$).

5.3.2.4. Hexanol dehydrogenase.

There were three alleles present at this locus. Eighteen populations had only two alleles (100 and 110). Thirteen had all three. No population was fixed for one allele and the frequency of heterozygotes ranged from 0.125 to 0.610 with a mean of 0.396.

Figure 5.7. clearly illustrates the division of the populations into two groups on the basis of the presence or absence of the 90 allele. After consulting Table 5.1. and Figures 5.1 - 3. it becomes apparent that all the populations where the 90 allele was present were from category 2 streams i.e. they are all streams from lower altitude with high pH and high conductivity.

An indication of the relationship between the allele frequencies at this locus and the physical attributes was given by regression analysis (Table 5.7.). The 90 allele was missing from over half of the populations and could not, therefore, be included in the analysis shown in Table 5.7. However, it could be included in the regression of the allele frequencies in the category 2 streams against the environmental characteristics (Table 5.8.). There appeared to be a significant correlation between heterozygosity and conductivity in these streams, however, as in the other cases, MINITAB indicated that the Baluain data was exceptional. When this was excluded, the relationship was not significant.

There were no significant relationships between the frequency of the 110 allele and any of the environmental factors, suggesting that the variation in frequency of this allele is unaffected by either the environment or the presence of the 90 allele. Statistically significant proportions of

the inter - population variation of the 100 allele were explained by regression with pH and conductivity ($p < 0.001$), and, to a lesser extent, by longitude ($p < 0.05$). The frequency of the 100 allele increased with lower pH and conductivity, and from east to west. The single locus heterozygosity was also significantly correlated with environmental features : heterozygosity tended to increase with increasing pH and conductivity ($p < 0.001$), and decreased at high latitude ($p < 0.01$) and from east to west ($p < 0.05$). When the two unusual sites (Baluain and Wareham 2) were excluded from the analysis, the correlations were improved and significant associations between the frequency of the 100 allele and altitude ($r = 0.395$, $p<0.05$) and between heterozygosity and latitude ($r = -0.398$, $p<0.05$) were revealed. The main cause of the differences in 100 allele frequency and heterozygosity between the two stream categories was the presence or absence of the 90 allele. The 100 allele was more common and the heterozygosity lower when the 90 allele was missing.

Following the observations from the HDH-1 locus, the populations were divided into the two environmental categories to further analyse the allele frequency data. Wright's (1978) hierarchical f-statistics (Table 5.10.) are a measure of the variance within and between the two categories for each locus. The F-value or 'inbreeding coefficient' is the ratio of the observed variance in allele frequency between populations to the variance that would be observed between isolated populations fixed for alternate alleles. More than fifty percent of the total variance at the HDH-1 locus was accounted for by a difference between the two categories. Between

category variance was relatively unimportant at the other three loci. The value for within category variation at the PGI locus was inflated due to the Baluain population. When this was excluded from the analysis the variance was greatly reduced (Table 5.10.).

Nei's (1978) unbiased genetic distances over the four loci, averaged for the two population categories, are presented in Table 5.11. The values given were calculated with Baluain excluded since this population distorted the overall figures. The mean genetic distance between the Baluain population and the other populations was 0.229 with a range of 0.161 to 0.301. The mean genetic distances presented in Table 5.11. show that there is a statistically significant difference between the category 1 and category 2 populations when they are grouped in this way. The f-statistics show that this is mainly due to the between group difference at the HDH-1 locus.

5.3.3. Temporal Allele Frequency Variation.

Comparisons of the allele frequencies at the five sites sampled in different years are presented in Tables 5.12 - 15. The X^2 values shown are for contingency tables including comparisons between all the samples taken at each site. The actual date of sampling varied between sites and between years. Collections were made from each stream in November/December and June/July to examine allele frequency changes within single generations. The November/December samples and June/July samples are labelled 'Winter' and 'Summer' in Table 5.12. Unfortunately, insufficient adult flies were collected from each site to facilitate a meaningful

adult v. nymph comparison.

Statistically significant changes in allele frequency were found in 4 out of the 17 tests. At the AP locus, the only variation was an increase in the frequency of the 90 allele from 1981 to 1983 over 3 generations in the Thomason's Hollow population. The most significant change occurred between 'summer' 1982 and 'winter' 1982, two different generations (Table 5.12.).

There was variation at the PGI locus in both the Miller's Dale and Lady Clough populations (Table 5.13). In MIller's Dale, there was a significant decrease in the frequency of the 60 allele between the 'winter' and 'summer' samples (the same generation). In Lady Clough, the heterogeneity was caused by the 'winter' 1982 sample where there was a higher frequency of the 60 allele than in the other samples. The allele frequencies were identical in the Winter 1981 and Summer 1983 samples.

For HDH there was an increase in both the 90 and 110 alleles in the generation from 'winter' 1982 to 'summer' 1983 in Miller's Dale (Table 5.15.). There were no significant temporal variations at the PGM locus in any of the populations (Table 5.14.).

5.4. Discussion

5.4.1. Temporal Allele Frequency Variation.

The investigation into temporal allele frequency stability did not provide any clear evidence that natural selection was influencing genetic variation at these four

polymorphic loci. Lewontin (1974) pointed out that temporal changes must be well correlated with some environmental variable to provide good evidence for selection. Only one of the four significant temporal changes in allele frequency showed a sustained pattern. That was at the AP locus in the Thomason's Hollow population. However, no obvious gene/environment interaction was revealed from the spatial variation between populations, (Section 5.3.3.2.) and no parallel change in the environment was recorded during this period which could account for the variation (Chapter 3, Figures 3.1-3).

For the other three cases of temporal variation there was either no obvious pattern (PGI at Lady Clough), or the data was too limited to draw any firm conclusions (PGI and HDH-1 at Miller's Dale). The allele frequencies at the HDH-1 locus were shown to be highly correlated with differences in stream type (Section 5.3.3.4.), but there was no marked seasonal change in stream water chemistry at Miller's Dale which is a well buffered neutral/alkaline stream. Also, both the 90 and 110 alleles increased in frequency and only the 90 allele appears to be selected against in acid streams.

The simplest explanation for the observations of temporal frequency variation is that they have resulted from sampling errors. This seems quite probable in view of the small samples taken on some occasions (Tables 5.12-15.). However, the number of significant changes is greater than expected due to chance factors.

A selectionist explanation for the observations could be that an unmeasured environmental factor changed the allele frequencies through natural selection. However, if the

temporal variation has been caused by environmental selection, similar changes in allele frequency might be expected in adjacent populations (Thomason's Hollow, Top Lady Clough and Lady Clough), as they are likely to experience similar environmental conditions.

A neutralist interpretation could be that the fluctuations in allele frequency have been caused by changes in population size at Thomason's Hollow, Lady CLough and Miller's Dale. This would be in line with the explanation proposed to account for the low levels of genetic variation in the Nemouridae (Section 4.4.). The increase in frequency of the AP 90-allele in the Thomason's Hollow population could be due to migration into the sampling area, either from higher-up or lower down the stream, following a population crash which eliminated this allele before 'Winter' 1981.

It can be seen that these data on temporal allele frequency variation provide no clear evidence for either a selectionist or neutralist interpretation. Without such evidence, speculative, post-hoc hypotheses can not answer the question of how much molecular genetic variation is influenced by natural selection (Lewontin, 1974).

5.4.2. Spatial Allele Frequency Variation.

For three of the four enzyme loci (AP, PGI and PGM), the results of the investigation into spatial variation of allele frequencies in 31 population failed to provide unequivocable support for either the neutralist or selectionist schools of thought. There were no significant correlations between the environmental variables and allele frequencies at these loci with the exception of those resulting from distortions of the analysis caused by atypical samples (sections 5.3.2.2. and 5.3.2.3.). The occurence of

these spurious correlations shows that results should be interpreted with caution when small sample sizes of around 30 are used for regression. However, it does show that regression analysis can be useful for identifying data that is out of the ordinary (eg. Baluain). Data from several populations appeared to upset the regression of allele frequencies at the PGM locus (section 5.3.2.3.). All these had small sample sizes, so it is not surprising that they had unusual allele frequencies at this locus where 5 different alleles were recorded. Whilst the lack of any correlation between allele frequency and the environmental factors examined favours a neutralist explanation for the variation at these loci (the spatial heterogeneity between populations arising from random fluctuations in the frequencies of selectively neutral or nearly neutral alleles), the possibility that natural selection could have caused the inter population variation in allele frequency, due to unique environmental conditions at each site, can not be excluded.

Both selectionist or neutralist interpretations could account for the relatively high frequency of the PGM 90 allele in populations from the Derbyshire Peak District. A selectionist explanation could be that these populations experience similar environmental conditions and, therefore, similar selection pressures. High levels of airborne pollutants from the industrial centres of Manchester and Sheffield could be a possible selecting factor. However, a neutralist explanation would be that either the populations were established from a common founder population in the post-glacial period or that gene-flow has occurred between them. The distance between the sites (Figure 5.8.), coupled with the poor flying ability of adult stoneflies (Hynes, 1977), excludes the possibility of direct migration between

the streams which have the highest frequency of the PGM 90 allele (site 30, Rising Clough, and site 2, Burbage Brook). However, gene-flow could have occurred through populations in intervening water courses. An indication that gene-flow could occur by adult migration between adjacent streams is given by the presence of the rare PGM 120 allele in populations 5, Holden Clough, and 16, Top Lady Clough, two streams draining either side of the Snake Pass watershed (Figure 5.8.).

Similarly, both neutralist or selectionist hypotheses can be devised to account for the anomalous allele frequencies at the PGI locus in the Baluain population (site 18). However, without any firm evidence, they can only be speculative. None the less, this observation indicates that more sites should be sampled in Scotland, to establish whether the 60 allele shows a general increase in frequency at higher latitudes, or whether the Baluain observation is unique or caused by sampling error (the sample size was only 12).

The non-random distribution pattern of the HDH-1 90 allele provides far clearer evidence that selection has influenced the allele frequencies at this locus, either directly or indirectly. The problem that arises from this discovery is to determine which factor, or factors, might be responsible for eliminating the 90 allele from one stream category whilst maintaining it in the other. Only direct experimental evidence can positively identify the factors involved. However, the results from the field survey point to several possibilities. The regression analyses show that water chemistry (pH and conductivity) could be important though other environmental variables associated with stream catchment, or geographic location, may be involved (Table

5.3.).

The altitude, pH and conductivity at each site were closely correlated with each other (Table 5.2.), all three showing sharp differences between the sites where the 90 allele was present and the sites where it was not found (Figures 5.1 - 3). The relationship between altitude, pH and conductivity confirms that geology is the main factor determining which areas are susceptible to acidification. The upland areas sampled were underlain by weathering resistant rocks covered with base poor soils, causing run-off with low ionic concentration (conductivity) and poor buffering capacity. However, it is unclear whether these streams have been affected by acid deposition since they must have been acidic before the pollution problem, due to natural acid production in the organic soils of these areas.

The 90 allele was only found in Category 2 (low altitude, high pH and high conductivity) streams. Most of these streams were located to the south-east of the Category 1 streams in this survey (section 5.3.1.) which gives an impression that allele frequency might be influenced by factors related to geographic position eg. temperature or annual rainfall. A direct causal relationship seems unlikely, however, since all the sites do not follow this pattern, and two steams (site 17, Wareham 2 (Category 1), and site 18, Baluain (Category 2)) are totally opposite to the general trend. Similarly, a direct relationship between altitude and allele frequency seems unlikely. Altitude is known to have a marked influence on the mean air temperature at any location, and temperature has been shown to affect the life-histories of Plecoptera, cooler temperatures delaying the onset of adult

emergence (Elliot, 1967; Nebeker, 1971). However, the 90 allele was found in the Baluain population which probably has the lowest mean annual temperature of all the sites,and the 90 allele was also present at Wareham 1 (site 28) but absent from Wareham 2 (site 17), two low altitude sites separated by less than three miles.

Water chemistry, particularly acidity, has been shown to have a marked influence on the ecology of fresh waters. The acidification of streams and lakes by acid deposition has caused fish losses and an impoverished algal and invertebrate community in many areas of Europe and North America (Overein et al, 1980; Haines, 1981). Stream acidity could influence the allele frequencies at the HDH-1 locus either directly or indirectly.

Hexanol dehydrogenase may have a similar role in Plecoptera to that of alcohol dehydrogenase in Drosophila. Clarke (1975) demonstrated that alcohol dehydrogenase was important in detoxifying the alcohols ingested by Drosophila melanogaster in their diet of rotting fruit. The products of alternate alleles had different activities and appeared to be maintained in the population by their different selective advantages in different environments. Nemoura cinerea feeds mainly on coarse organic material which collects under boulders in streams (Hynes, 1941). Hexanol dehydrogenase may detoxify harmful chemicals produced as this material decomposes allowing it to be safely eaten. Stream acidity could directly select against the 90 allele if intracellular pH is affected by external pH and the product of this allele has a higher pH optimum than the other allozymes. Biochemical studies of in vitro enzyme activity would be needed to

determine whether different HDH-1 allozymes have different pH optima.

An alternative hypothesis could be that selection is not acting directly on the HDH-1 locus. HDH-1 may simply be closely linked to another unidentified locus (or loci) that affects tolerance to low pH conditions. Linkage disequilibria between isozyme loci have been reported several times, particularly in Drosophila (Lewontin, 1974). However, it seems that these are almost always associated with chromosome inversions (Kimura, 1983). It has been possible to identify inversions in Drosophila because of the occurence of giant chromosomes in the salivary glands. No such chromosomes could be found in the Nemouridae so it would be very difficult to recognise inversions in these species.

Another possible way that stream acidity could indirectly affect the HDH-1 locus is through the influence of pH on the breakdown of organic matter. It has been shown that allochthonous material decomposes less quickly in acid waters (Hendrey et al, 1976; Otto and Svenson, 1983). The slow rate of decomposition is caused by a reduction in the numbers and diversity of micro-organisms in acid conditions, and fungi replace bacteria as the main decomposers (Bick & Drews, 1973). The micro-organisms are thought to play an important role in "conditioning" the organic matter before it is eaten by the macro-invertebrates (Cummins & Klug, 1979). The slow decomposition or changed microbial content may cause an increase in toxic organic compounds. If the 90 allele has lower activity than the other allozymes, individuals with this allele would be selected against in acid streams. However, the quality of the diet may be independent of acidity, the nature

of the catchment vegetation being more important. None of the catchments of the Category 1 streams (90 allele absent) contained deciduous woodland upstream from the sample sites. Therefore, the main input of allochthonous material would be from grasses or moss. All the category 2 stream catchments did have deciduous woods which would provide the major annual supply of organic material. Allochthonous food supply has been shown to influence genetic variation in the water hoglouse, Asellus aquaticus in Denmark (Christensen, 1977). Habitat selection appeared to be occuring between different amylase genotypes with one type favouring areas with beech leaves and the other selecting areas where willow was predominant. A difference in ability to metabolise different fungi colonising the leaf-litter was proposed as a possible explanation. However, the inheritance of amylase genotypes has been found to be more complicated than it originally appeared so a complete explanation for the variation has yet to be made (Oxford, 1986).

An indication that catchment vegetation could be more important than microbial conditioning in determining food quality, and hence allele frequencies at the HDH-1 locus, comes from the Beacon population (site 19 sampled in 1983). The 90 allele was present in this woodland stream even though it was found to have a low pH during the winter and an impoverished invertebrate community typical of acid streams (Greenwood et al, 1986). However, this observation does not provide conclusive evidence that leaf litter was the essential factor determining the presence of the 90 allele. N. cinerea was not found at this site in 1980 or 1981, but did occur further downstream where the pH and invertebrate community was

radically altered after passing through a golf course. Therefore, it appears that the 90 allele was present at the Beacon site in 1983 due to upstream migration from the neutral "refuge" further down the catchment.

The discussion so far has concentrated only on the nymphal life-stage. Other possible explanations could be devised to account for the non-random distribution of the 90 allele involving other stages in the life-history. These might include drought resistance or factors affecting hatching in the eggs, or adult food requirements. The problem of identifying a selective factor has arisen because there are several environmental differences between streams where the 90 allele is present and those where it is absent. However, although no direct proof of natural selection was possible, the observation that the distribution of HDH-1 alleles is non-random with respect to stream type is incompatible with neutral theory, and can only be explained by the action of selective forces.

TABLE 5.1. The physical characteristics recorded for the 31 streams sampled. LAT. = latitude as minutes north of 51° N. LONG. = longitude as minutes west of 0° . ALT. = altitude in metres above sea level. COND. = conductivity in $\mu S^{-1}.cm$.

	SITE	LAT.	LONG.	ALT.	pH	COND.
1.	Blackstone Edge	219	121	366	4.35	200
2.	Burbage Brook	200	96	305	3.90	90
3.	Cabin Clough	206	114	381	4.10	147
4.	Dean Clough	213	112	442	4.35	75
5.	Holden Clough	205	113	488	4.20	89
6.	Lady Clough	205	110	321	5.90	120
7.	March Hill	217	119	430	4.90	156
8.	Moorhouse Mine	281	146	686	4.00	48
9.	Moorhouse 2	281	145	590	4.20	38
10.	Nant-y-Trawsnant	127	225	320	5.20	46
11.	North Wales 4	167	216	482	5.20	41
12.	Ogden	216	122	380	4.05	78
13.	Port Erin	244	286	106	n.r.	n.r.
14.	Shiny Brook	213	115	472	3.80	215
15.	Thomason's	205	111	457	4.50	83
16.	Top Lady Clough	206	111	472	4.50	120
17.	Wareham 2	42	126	15	4.25	122
18.	Baluain	406	234	160	7.20	94
19.	Charnwood Beacon	163	74	140	5.90	250
20.	Cressbrook Dale	195	105	190	7.45	523
21.	Glebe Farm	157	54	198	8.00	550
22.	Lutterworth 1	148	67	130	7.90	740
23.	Lutterworth 2	148	74	120	8.00	780
24.	Miller's Dale	195	107	200	8.05	474
25.	Nailstone	159	82	140	7.75	3650
26.	Owston	158	50	167	7.30	540
27.	Thrussington	164	63	65	7.80	810
28.	Wareham 1	40	127	15	7.00	n.r.
29.	Wymondham	167	54	110	7.50	725
30.	Rising Clough	203	101	275	6.00	110
31.	South Beacon	163	75	210	7.20	365

n.r. = not recorded

TABLE 5.2. The correlation coefficients between the 5 environmental variables measured for 31 streams. The degrees of freedom are shown in parentheses.

		Latitude	Longitude	Altitude	pH
Longitude	a)	+0.379[1](29)	***		
	b)	+0.251 (27)			
Altitude	a)	+0.489[2](29)	+0.197 (29)	***	
	b)	+0.698[3](27)	+0.300 (27)		
pH	a)	-0.221 (28)	-0.387[1](28)	-0.742[3](28)	***
	b)	-0.606[3](26)	-0.543[2](26)	-0.846[3](26)	
Conductiv.	a)	-0.361 (26)	-0.663[3](26)	-0.685[3](26)	+0.833[3](26)
	b)	-0.571[2](24)	-0.691[3](24)	-0.824[3](24)	+0.880[3](24)

a) Correlations including all sites.
b) Correlations excluding the Baluain and Wareham 2 sites.

[1] = $P < 0.05$, [2] = $P < 0.01$, [3] = $P < 0.001$

TABLE 5.3. The allele frequencies and single locus heterozygosity (Hu) at the Aminopeptidase locus in 31 populations of Nemoura cinerea together with the results of the X^2 tests of fit to Hardy-Weinberg Equilibrium and the mean expected heterozygosity over the four polymorphic loci (He). The degrees of freedom = 1 in all tests.

	Site	N	Allele Frequency			X^2	P	Hu	He
			90	100	115				
1.	Blackstone	72	.118	.882	--	.003	.955	.210	.280
2.	Burbage Brook	52	.154	.846	--	.821	.365	.263	.294
3.	Cabin Clough	127	.059	.933	.008	.613	.434	.126	.237
4.	Dean Clough	152	.066	.934	--	.253	.615	.123	.253
5.	Holden Clough	143	.101	.874	.024	.862	.353	.226	.255
6.	Lady Clough	50	.070	.930	--	.241	.624	.132	.251
7.	March Hill	79	.038	.962	--	.102	.749	.074	.246
8.	Moorhouse Mn.	24	.063	.938	--	.070	.792	.120	.147
9.	Moorhouse 2	104	.034	.966	--	.108	.743	.065	.342
10.	Nant. Trawsn.	10	.200	.800	--	.450	.502	.337	.268
11.	Nth. Wales 4	24	.271	.729	--	.127	.722	.403	.290
12.	Ogden	219	.021	.979	--	.085	.770	.040	.220
13.	Port Erin	48	--	1.00	--	--	--	0.00	.166
14.	Shiny Brook	326	.054	.943	.003	1.15	.284	.108	.267
15.	Thomason's	69	.080	.920	--	.467	.494	.148	.264
16.	Top Lady Cl.	59	.042	.958	--	.092	.762	.082	.189
17.	Wareham 2	126	.060	.940	--	.469	.493	.112	.246
18.	Baluain	12	.042	.958	--	.021	.880	.083	.292
19.	Charnwood	120	.150	.850	--	.969	.325	.256	.320
20.	Cressbrook	87	.155	.845	--	.644	.422	.264	.396
21.	Glebe Fm.	73	.130	.870	--	.745	.388	.228	.319
22.	Lutterworth 1	177	.093	.907	--	.199	.656	.170	.302
23.	Lutterworth 2	79	.120	.880	--	.009	.923	.213	.339
24.	Miller's Dale	194	.039	.961	--	.292	.589	.075	.320
25.	Nailstone	11	.227	.773	--	.890	.346	.368	.337
26.	Owston	86	.157	.843	--	.752	.386	.266	.347
27.	Thrussington	30	.100	.900	--	.304	.581	.183	.330
28.	Wareham 1	146	.014	.986	--	.021	.885	.027	.253
29.	Wymondham	32	.156	.844	--	.156	.693	.268	.325
30.	Rising Clough	80	.150	.850	--	4.04	.044*	.257	.310
31.	South Beacon	28	.304	.696	--	.181	.670	.431	.377

* denotes a statistically significant ($p < 0.05$) deviation.

TABLE 5.4. The allele frequencies and single locus heterozygosity (Hu) at the Hexanol Dehydrogenase locus in 31 populations of Nemoura cinerea together with the results of the X^2 tests of fit to Hardy-Weinberg Equilibrium. The degrees of freedom = 1 in all tests.

	Sites	N	Allele Frequency			X^2	P	Hu
			90	100	110			
1.	Blackstone	43	--	.884	.116	.524	.469	.208
2.	Burbage Brook	50	--	.820	.180	.195	.659	.298
3.	Cabin Clough	99	--	.828	.172	.364	.546	.286
4.	Dean Clough	102	--	.789	.211	2.33	.127	.334
5.	Holden Clough	139	--	.860	.140	.053	.818	.242
6.	Lady Clough	51	--	.735	.265	.144	.704	.393
7.	March Hill	78	--	.795	.205	.313	.576	.328
8.	Moorhouse Mn.	24	--	.646	.354	.009	.924	.467
9.	Moorhouse 2	91	--	.632	.368	.009	.923	.468
10.	Nant. Traws.	9	--	.611	.389	1.17	.279	.503
11.	Nth. Wales 4	22	--	.864	.136	.448	.503	.241
12.	Ogden	200	--	.877	.122	.394	.530	.216
13.	Port Erin	8	--	.938	.063	0.00	1.00	.125
14.	Shiny Brook	286	--	.808	.192	.328	.567	.311
15.	Thomason's	77	--	.799	.201	.471	.493	.324
16.	Top Lady Cl.	59	--	.907	.093	.715	.398	.171
17.	Wareham 2	140	--	.843	.157	2.81	.094	.266
18.	Baluain	9	.222	.722	.056	1.31	.249	.451
19.	Charnwood	121	.132	.698	.169	.002	.967	.468
20.	Cressbrook	85	.065	.565	.371	.000	.994	.543
21.	Glebe Farm	59	.280	.602	.119	2.25	.133	.550
22.	Lutterworth 1	165	.173	.688	.139	.000	.990	.479
23.	Lutterworth 2	79	.253	.589	.158	1.64	.201	.568
24.	Millers Dale	135	.119	.696	.185	.455	.500	.469
25.	Nailstone	11	.136	.545	.318	1.94	.164	.610
26.	Owston	86	.227	.645	.128	2.42	.120	.519
27.	Thrussington	30	.283	.567	.150	.143	.705	.586
28.	Wareham 1	43	.070	.744	.186	3.39	.065	.411
29.	Wymondham	31	.177	.645	.177	3.03	.082	.529
30.	Rising Clough	48	--	.756	.244	1.99	.157	.373
31.	South Beacon	28	.214	.625	.161	.022	.883	.547

TABLE 5.5. The allele frequencies and single locus heterozygosity (Hu) at the Phosphoglucose isomerase locus in 31 populations of Nemoura cinerea together with the results of the X^2 tests of fit to Hardy-Weinberg Equilibrium. The degrees of freedom = 1 in all tests.

	Sites	N	Allele Frequency				X^2	P	Hu
			60	100	120	140			
1.	Blackstone	76	.132	.868	--	--	.069	.792	.230
2.	Burbage Brook	78	.045	.955	--	--	.147	.702	.086
3.	Cabin Clough	159	.044	.956	--	--	.613	.434	.084
4.	Dean Clough	151	.086	.914	--	--	4.06	.044*	.158
5.	Holden Clough	143	.035	.958	.007	--	.251	.617	.081
6.	Lady Clough	55	.009	.991	--	--	0.00	1.00	.018
7.	March Hill	79	.044	.949	.006	--	.170	.680	.097
8.	Moorhouse Mn.	24	--	1.00	--	--	--	--	0.00
9.	Moorhouse 2	100	.180	.800	.029	--	.003	.960	.329
10.	Nant. Traws.	10	--	1.00	--	--	--	--	0.00
11.	Nth. Wales 4	29	.052	.931	--	.017	.117	.732	.132
12.	Ogden	286	.054	.937	.005	.003	.011	.917	.119
13.	Port Erin	60	.058	.942	--	--	.196	.658	.111
14.	Shiny Brook	386	.078	.904	.014	.004	.787	.375	.176
15.	Thomason's	69	.051	.948	--	--	.168	.682	.097
16.	Top Lady Cl.	59	.017	.983	--	--	.009	.926	.034
17.	Wareham 2	143	.042	.944	.014	--	.887	.346	.107
18.	Baluain	12	.792	.208	--	--	.831	.362	.344
19.	Charnwood	178	.037	.961	.003	--	.276	.599	.076
20.	Cressbrook	85	.176	.824	--	--	.103	.748	.292
21.	Glebe Farm	76	.033	.967	--	--	.070	.791	.064
22.	Lutterworth 1	177	.028	.966	.006	--	.199	.655	.066
23.	Lutterworth 2	79	.038	.962	--	--	.102	.749	.074
24.	Millers Dale	310	.166	.834	--	--	.387	.534	.278
25.	Nailstone	10	--	1.00	--	--	--	--	0.00
26.	Owston	86	.047	.942	.012	--	.293	.588	.111
27.	Thrussington	30	.017	.983	--	--	0.00	1.00	.033
28.	Wareham 1	236	.023	.962	.013	.002	.350	.554	.074
29.	Wymondham	31	--	1.00	--	--	--	--	0.00
30.	Rising Clough	105	.043	.933	.024	--	.495	.482	.127
31.	South Beacon	28	.018	.982	--	--	0.00	1.00	.036

* denotes a statistically significant ($p < 0.05$) deviation.

TABLE 5.6. The allele frequencies and single locus heterozygosity (Hu) at the Phosphoglucomutase locus in 31 populations of Nemoura cinerea together with the results of the X^2 tests of fit to Hardy-Weinberg Equilibrium. The degrees of freedom = 1 in all tests.

	Site	N	Allele Frequency				X^2	P	Hu
			90	100	110	120			
1.	Blackstone	74	--	.622	.378	--	.055	.814	.474
2.	Burbage Brook	75	.180	.640	.180	--	.092	.762	.529
3.	Cabin Clough	139	.014	.676	.306	.004	3.10	.078	.451
4.	Dean Clough	102	.005	.730	.265	--	1.83	.176	.398
5.	Holden Clough	140	.004	.650	.329	.018	.533	.465	.471
6.	Lady Clough	151	.030	.669	.301	--	.327	.567	.462
7.	March Hill	77	--	.597	.403	--	.029	.865	.484
8.	Moorhouse Mn.	24	--	1.00	--	--	--	--	0.00
9.	Moorhouse 2	99	.005	.475	.520	--	1.80	.179	.507
10.	Nant. Traws.	8	--	.875	.125	--	.077	.782	.233
11.	Nth. Wales 4	23	--	.761	.217	.022	.067	.795	.382
12.	Ogden	206	.002	.546	.447	.005	2.56	.110	.503
13.	Port Erin	15	--	.733	.267	--	1.95	.163	.405
14.	Shiny Brook	322	.008	.630	.362	--	4.78	.029*	.472
15.	Thomason's	67	.015	.627	.351	.007	3.93	.048*	.487
16.	Top Lady Cl.	59	.008	.678	.271	.042	1.42	.234	.469
17.	Wareham 2	143	--	.528	.472	--	.577	.448	.500
18.	Baluain	12	--	.833	.167	--	1.92	.166	.290
19.	Charnwood	139	--	.601	.399	--	1.10	.295	.481
20.	Cressbrook	87	.006	.609	.385	--	1.63	.220	.483
21.	Glebe Farm	72	--	.688	.312	--	.258	.612	.433
22.	Lutterworth 1	177	.003	.596	.390	.011	3.24	.072	.494
23.	Lutterworth 2	79	--	.576	.411	.013	.001	.970	.502
24.	Millers Dale	262	.002	.643	.355	--	1.63	.201	.461
25.	Nailstone	11	--	.773	.227	--	.735	.391	.368
26.	Owston	86	--	.570	.430	--	.122	.726	.493
27.	Thrussington	30	--	.550	.433	--	.001	.970	.518
28.	Wareham 1	224	.002	.424	.569	.004	.483	.487	.497
29.	Wymondham	34	--	.544	.456	--	.537	.464	.504
30.	Rising Clough	76	.132	.684	.184	--	1.73	.189	.484
31.	South Beacon	28	--	.589	.411	--	.098	.754	.493

* denotes a statistically significant ($p < 0.05$) deviation.

TABLE 5.7. The correlation coefficients of the common allele frequencies and heterozygosity at four polymorphic loci in *Nemoura cinerea* with the 5 environmental variables. a) Correlations including data from all sites. b) Correlations exclusive of the Baluain and Wareham 2 sites. c) Correlations of PGM exclusive of Baluain, Wareham 2, Nailstone, Nant-y-Trawsnant and Moorhouse Mine.

LOCUS	ALLELE		LAT.	LONG.	ALT.	pH	COND.
AP	90	a)	-0.276	-0.214	-0.122	+0.307	+0.182
		b)	-0.339	-0.165	-0.193	+0.328	+0.145
	100	a)	+0.274	+0.217	+0.110	-0.290	-0.170
		b)	+0.334	+0.166	+0.178	-0.311	-0.133
	Hu	a)	-0.276	-0.260	-0.112	+0.296	+0.203
		b)	-0.335	-0.211	-0.183	+0.320	+0.166
HDH	100	a)	+0.130	+0.412[1]	+0.318	-0.722[3]	-0.632[3]
		b)	+0.319	+0.455[1]	+0.395[1]	-0.721[3]	-0.634[3]
	110	a)	+0.045	+0.000	+0.305	-0.100	-0.210
		b)	+0.155	+0.117	+0.269	-0.075	-0.282
	Hu	a)	-0.152	-0.462[2]	-0.388[1]	+0.794[3]	+0.683[3]
		b)	-0.398[1]	-0.536[2]	-0.462[1]	+0.788[3]	+0.699[3]
PGI	60	a)	+0.667[3]	+0.400[1]	+0.063	-0.122	-0.145
		b)	+0.332	+0.191	+0.150	-0.067	-0.096
	100	a)	-0.660[3]	-0.405[1]	-0.055	+0.114	+0.182
		b)	-0.314	-0.211	-0.175	+0.091	+0.136
	Hu	a)	+0.495[2]	+0.282	+0.104	-0.045	-0.150
		b)	+0.303	+0.228	+0.174	+0.108	+0.153
PGM	100	a)	+0.451[1]	+0.482[2]	+0.378[1]	-0.152	-0.409[1]
		c)	+0.368	+0.379	+0.306	-0.249	-0.386
	110	a)	-0.439[1]	-0.421[1]	-0.391[1]	+0.214	+0.456[1]
		c)	-0.340	-0.287	-0.296	+0.315	+0.439[1]
	Hu	a)	-0.345	+0.195	+0.177	-0.077	-0.176
		c)	-0.197	+0.221	+0.169	-0.027	-0.106
He		a)	-0.180	-0.491[2]	-0.411[1]	+0.670[3]	+0.593[3]
		b)	-0.361	-0.545[2]	-0.470[2]	+0.669[3]	+0.594[2]

1) = $p < 0.05$, 2) = $p < 0.01$, 3) = $p < 0.001$

Degrees Freedom : LAT., LONG. and ALT .a) = 29 b) = 27 c) = 24
(n - 2) pH a) = 28 b) = 26 c) = 23
COND. a) = 26 b) = 24 c) = 22

TABLE 5.8. The correlation coefficients between the allele frequencies and Heterozygosity at the Hexanol dehydrogenase locus and the environmental factors for the category 2 stream populations only.

ALLELE	LATITUDE	LONGITUDE	ALTITUDE	PH	CONDUCTIVITY
d.f.	11	11	11	11	9
90	+0.236	-0.216	+0.113	+0.341	+0.259
100	+0.113	+0.476	-0.205	-0.441	-0.591
110	-0.300	-0.183	+0.064	+0.048	+0.194
Hu	-0.081	-0.519	+0.209	+0.452	+0.646* (+0.494)[1]

1 Correlation exclusive of Baluain (d.f. 8)
* = p <0.05

TABLE 5.9. X^2 Contingency analysis of the variability between the allele frequencies at four polymorphic loci in 31 populations of Nemoura cinerea

Locus	No. Alleles	X^2	d.f.	P
AP	3	320.4	60	< 0.001
HDH	3	821.6	60	"
PGI	4	533.7	90	"
PGM	5	975.2	120	"

TABLE 5.10. Wright's (1978) Hierarchical F- statistics to show the variation of the populations within and between the two stream categories. The figures in parentheses are the f-values for PGI exclusive of the Baluain data.

Locus	F-Value		Total
	Within Categories	Between Categories	
AP	0.042	0.001	0.043
HDH	0.028	0.031	0.058
PGI	0.261 (0.045)	0.009 (-0.003)	0.255 (0.042)
PGM	0.052	0.002	0.053

TABLE 5.11. Nei's (1978) mean genetic distance between populations of Nemoura cinerea in the two stream categories.

Category	No. Pops.	1	2
1	18	0.017 [a] (0.000-0.096)	
2	12	0.026 [b] (0.000-0.111)	0.012 [c] (0.000-0.058)

t - test a - b $t_{28} = -3.837$ $P < 0.001$
b - c $t_{21} = -5.127$ $P < 0.001$
a - c $t_{18} = 1.093$ $P = 0.276$

TABLE 5.12. Temporal allele frequency variation at the Aminopeptidase locus in 5 populations of *Nemoura cinerea* from the Derbyshire Peak District.

Site	Date	N	Allele Frequency 90	Allele Frequency 100	X^2	d.f.	p
Thomason's	S 1981	12	--	1.000			
	W 1981	29	0.018	0.982			
	S 1982	48	0.021	0.979	10.63	4	0.031*
	W 1982	59	0.102	0.898*			
	S 1983	69	0.080	0.920			
Miller's	W 1982	117	0.030	0.970	0.40	1	0.526
	S 1983	97	0.041	0.959			
Top Lady Cl.	S 1982	25	0.060	0.940			
	W 1982	72	0.042	0.958	0.32	2	0.853
	S 1983	59	0.042	0.958			
Lady Cl.	W 1981	22	0.045	0.955			
	S 1982	16	0.063	0.937	0.32	3	0.956
	W 1982	25	0.060	0.940			
	S 1983	50	0.070	0.930			
Rising Cl.	S 1982	23	0.152	0.848	0.07	1	0.786
	S 1984	41	0.171	0.829			

S = 'summer', W = 'winter'
* denotes a statistically significant ($p < 0.05$) change.

TABLE 5.13. Temporal allele frequency variation at the phosphoglucose isomerase locus in 5 populations of *Nemoura cinerea* from the Derbyshire Peak District.

Site	Date	N	Allele Frequency 60	100	120	X^2	d.f.	p
Thomason's	S 1981	16	--	1.000	--			
	W 1981	41	0.037	0.963	--			
	S 1982	62	0.065	0.935	--	4.10	4	0.392
	W 1982	76	0.026	0.974	--			
	S 1983	68	0.044	0.956	--			
Miller's	W 1982	213	0.204	0.796	--	14.26 *	1	<0.001
	S 1983	97	0.082	0.918	--			
Top Lady Cl.	S 1982	50	0.020	0.970	0.010			
	W 1982	71	0.049	0.930	0.021	6.10	4	0.192
	S 1983	59	0.017	0.983	--			
Lady Cl.	W 1981	54	0.009	0.991	--			
	S 1982	35	--	1.000	--	11.11 *	3	0.011
	W 1982	48	0.063	0.927	--			
	S 1983	55	0.009	0.991	--			
Rising Cl.	S 1982	29	0.052	0.914	0.034	0.77	2	0.681
	s 1984	40	0.050	0.938	0.013			

* denotes a statistically significant ($p < 0.05$) change.

TABLE 5.14. Temporal allele frequency variation at the phosphoglucomutase locus in 5 populations of Nemoura cinerea from the Derbyshire Peak District.

Site	Date	N	Allele Frequency 90	100	110	X^2	d.f.	p
Thomason's	S 1981	22	--	0.795	0.205			
	W 1981	26	--	0.788	0.212			
	S 1982	43	--	0.651	0.349	10.91	8	0.207
	W 1982	65	0.023	0.646	0.331			
	S 1983	66	0.015	0.629	0.356			
Miller's	W 1982	188	0.003	0.646	0.351	0.46	2	0.794
	S 1983	74	--	0.635	0.365			
Top Lady Cl[1]	S 1982	38	0.013	0.658	0.329			
	W 1982	65	--	0.685	0.300	3.52	6	0.741
	S 1983	56	0.009	0.714	0.268			
Lady Cl.	S 1982	33	0.015	0.682	0.303			
	W 1982	25	--	0.580	0.420	1.35	3	0.510
	S 1983	22	--	0.659	0.341			
Rising Cl.	S 1982	20	0.125	0.700	0.175	0.07	2	0.966
	S 1984	39	0.141	0.679	0.179			

1) Note the 120 allele frequency not shown

TABLE 5.15. Temporal allele frequency variation at the Hexanol dehydrogenase-1 locus in 2 populations of Nemoura cinerea from the Derbyshire Peak District.

Site	Date	N	Allele Frequency 90	100	110	X^2	d.f.	p
Thomason's	S 1982	25	--	0.920	0.080	2.06	1	0.152
	S 1983	58	--	0.840	0.160			
Miller's	W 1982	40	0.100	0.825	0.075	10.49	2	0.005*
	S 1983	95	0.126	0.642	0.232			

* denotes a statistically significant change ($p < 0.05$).

Figure 5.1. Altitude (metres above sea level) of the thirty-one sample sites

Figure 5.1.

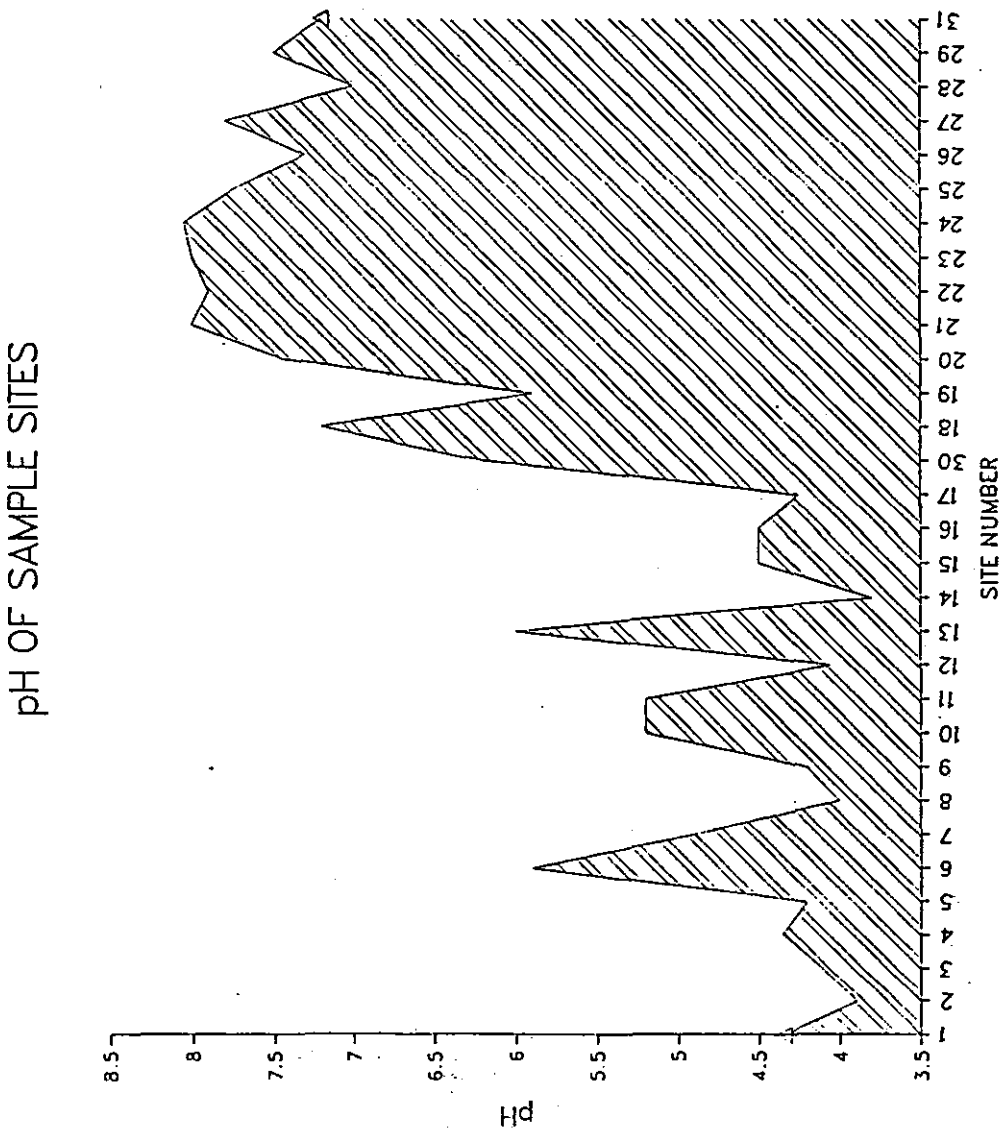


Figure 5.2. pH at the thirty-one sample site

Figure 5.2.

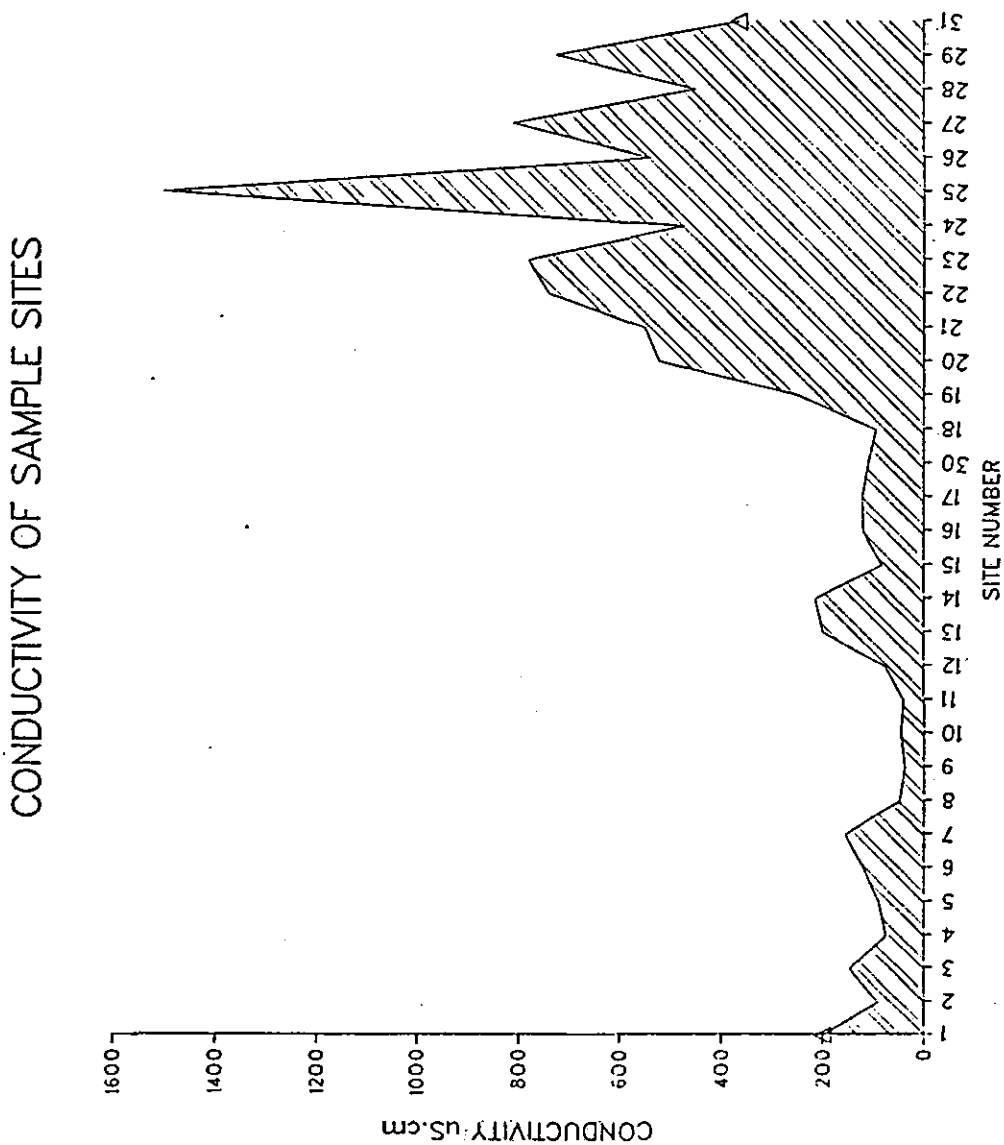


Figure 5.3. Conductivity ($\mu S.cm^{-1}$) at the thirty-one sample sites

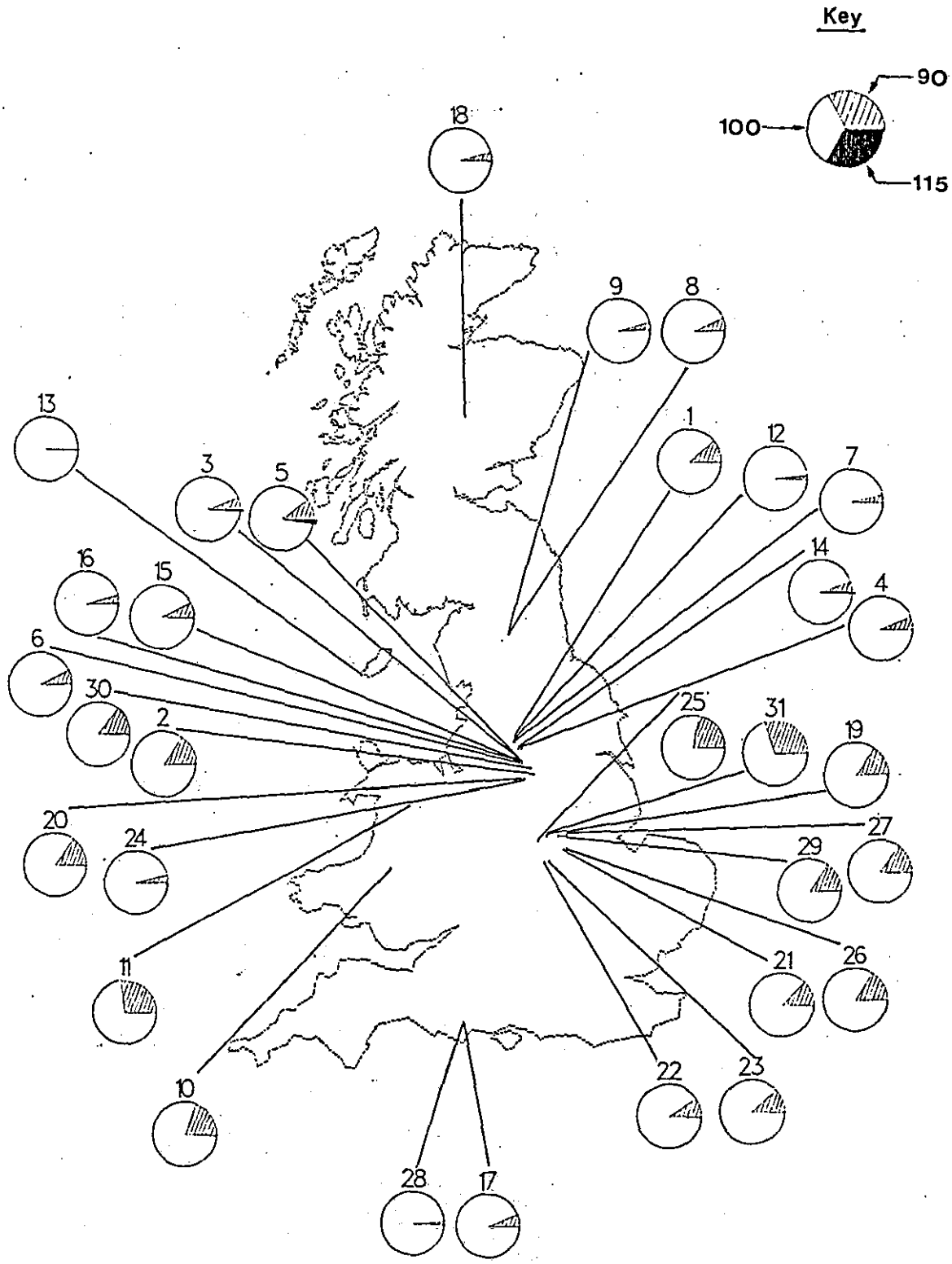


Figure 5.4. Allele frequencies at the AP-1 locus for thirty-on populations of N. cinerea

Figure 5.4.

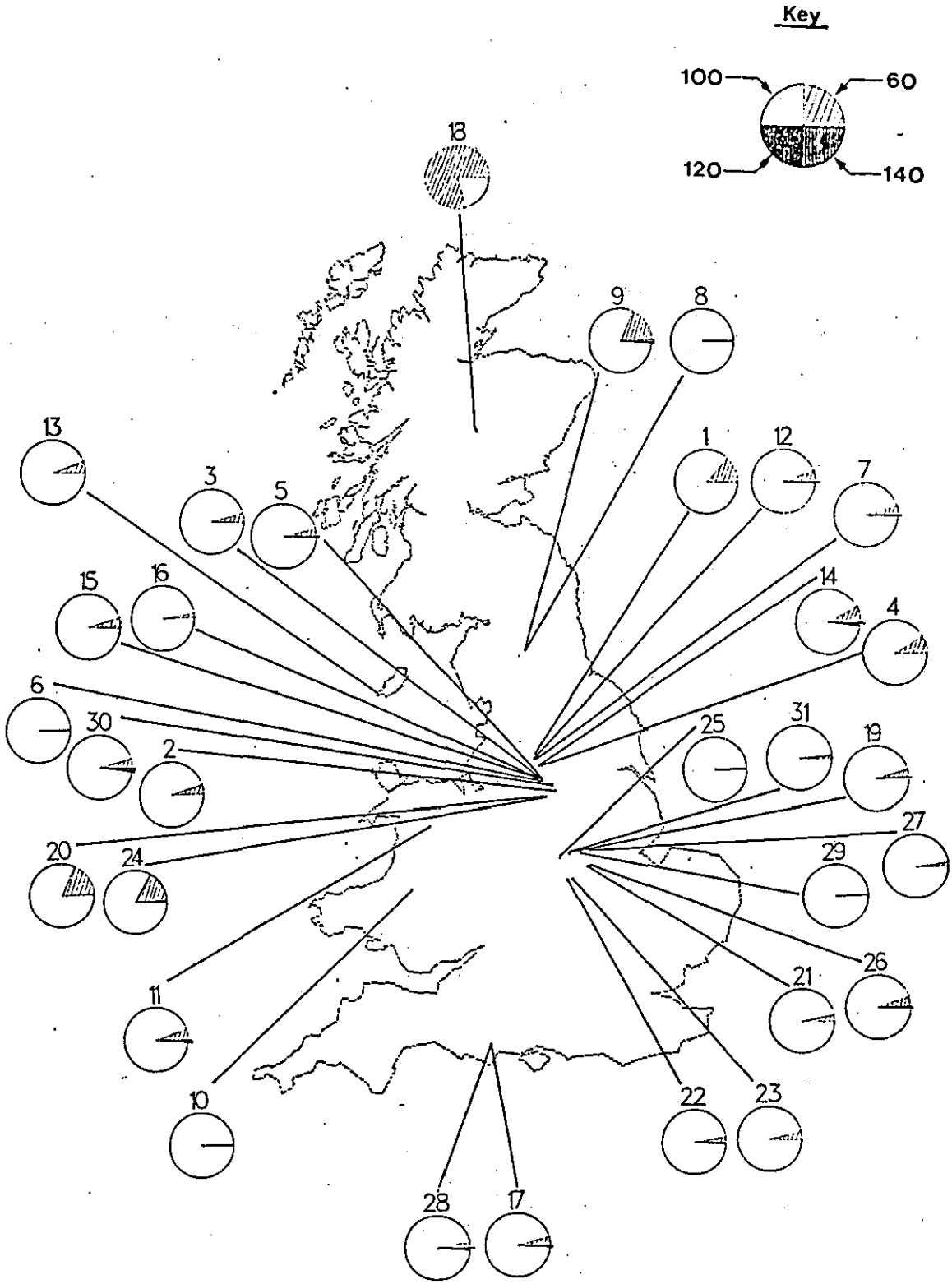


Figure 5.5. Allele frequencies at the PGI locus for thirty-or populations of N. cinerea

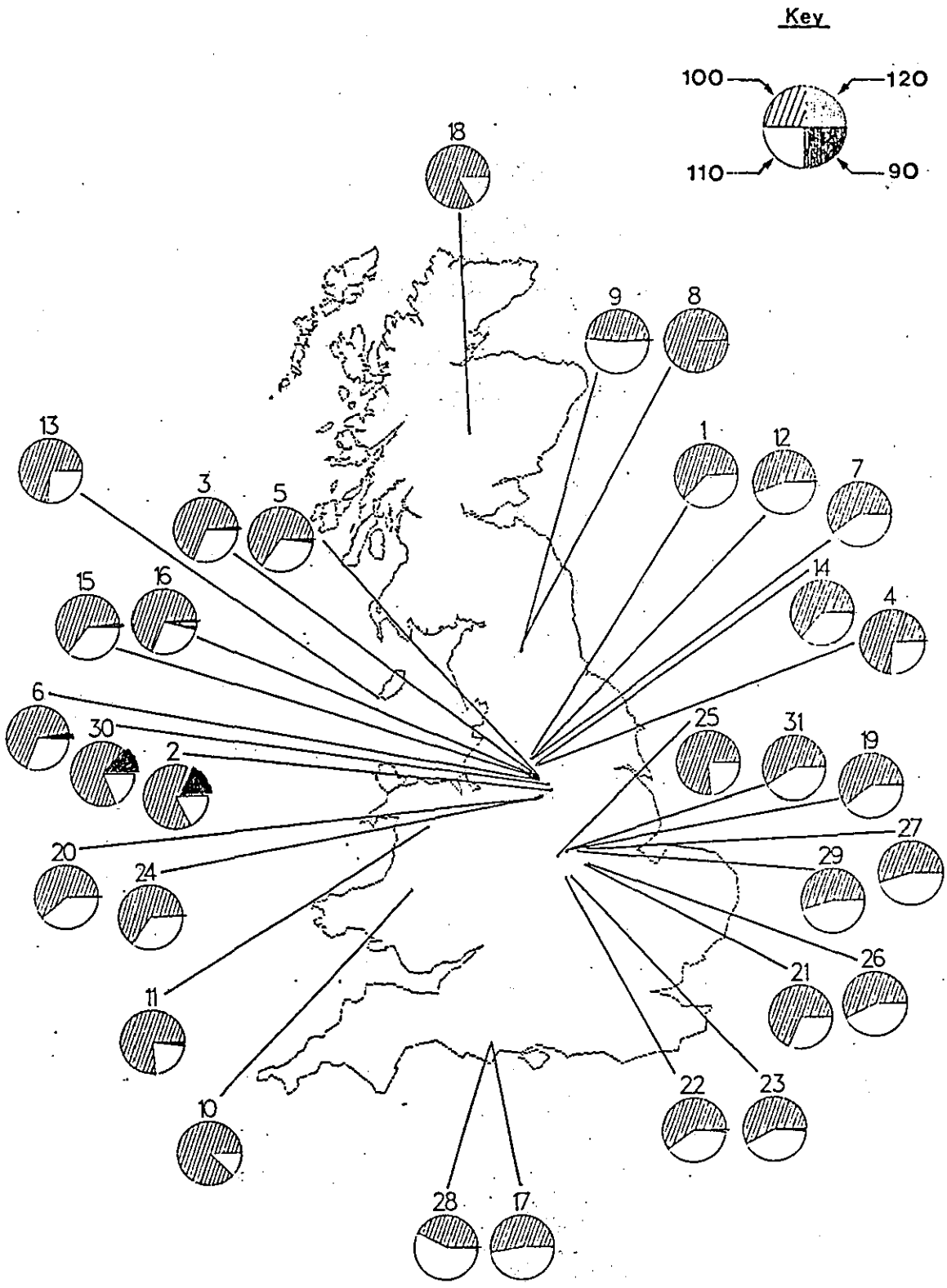


Figure 5.6. Allele frequencies at the PGM locus for thirty-o populations of N. cinerea

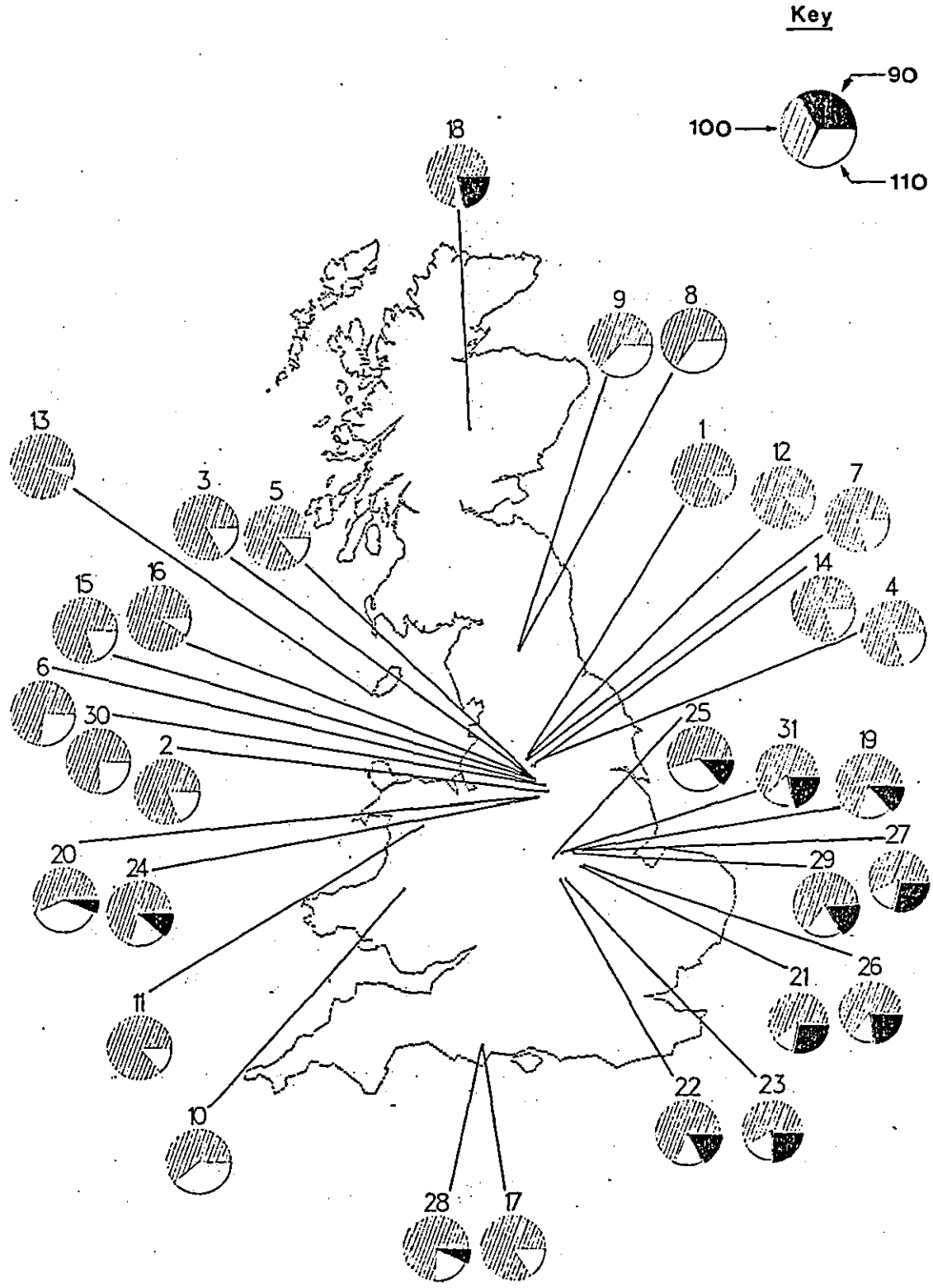
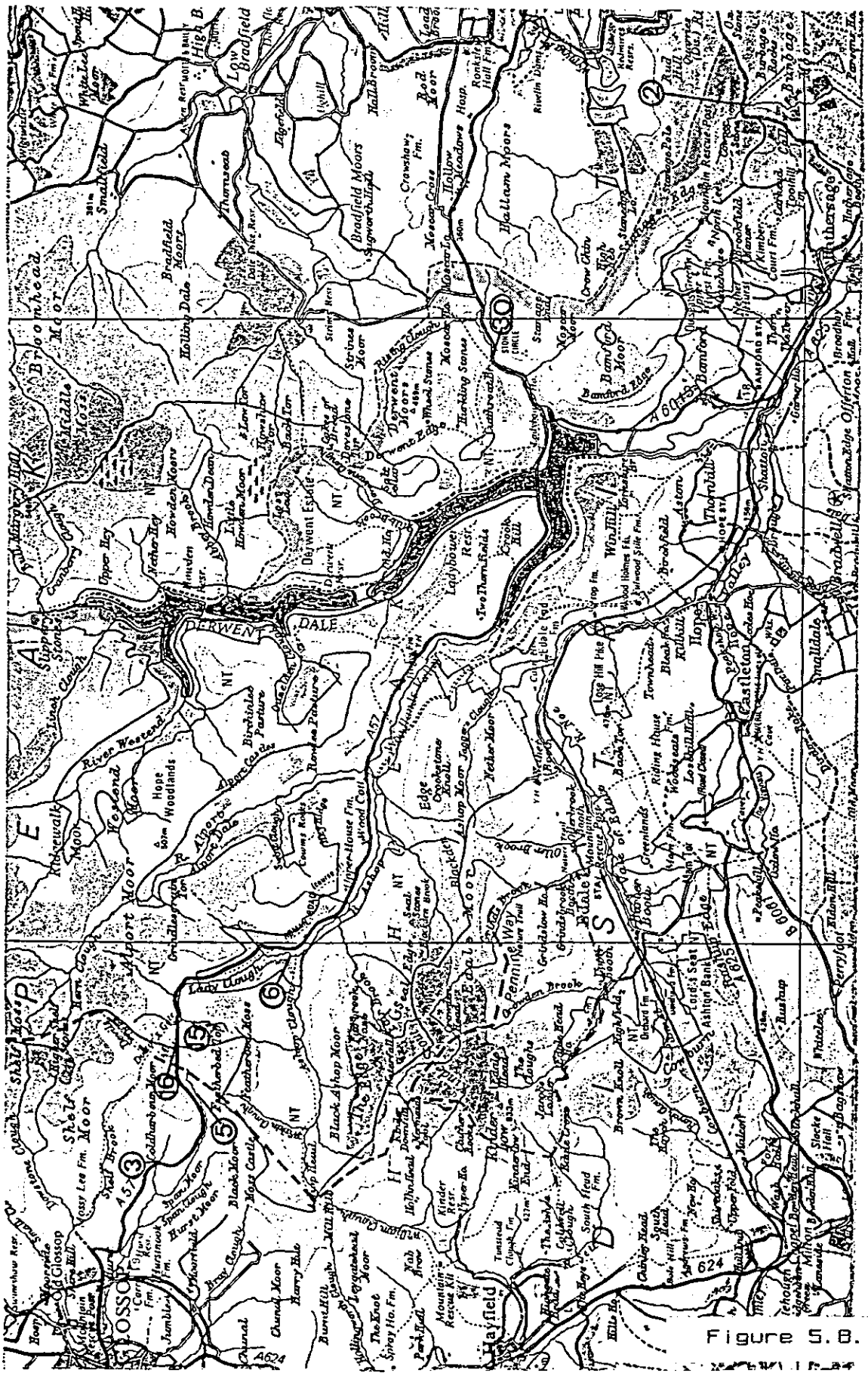


Figure 5.7. Allele frequencies at the HDH locus for thirty-or populations of N. cinerea

Figure 5.7.

FIGURE 5.8. The location of the streams in the Derbyshire Peak District with a high frequency of the PGM 90 allele.
Scale 1 : 100000.



CHAPTER 6

THE TOLERANCE OF NEMOURA CINEREA TO ACUTE ACIDITY STRESS

6.1. Introduction

The results of the investigation into inter-population genetic variation in N. cinerea (Chapter 5) revealed a clear association between the distribution of the HDH-1 90 allele and stream 'type'. The 90 allele was only present in lower altitude streams with a high pH and high conductivity. The most likely explanation for this non-random distribution is that the 90 allele, or another closely linked gene or genes in disequilibrium with it, has been removed from higher altitude, low pH and low conductivity streams through the action of natural selection. Stream water chemistry, especially acidity (pH) was proposed as a possible selecting agent because regression analysis revealed a strong relationship between pH and the frequency of the HDH-1 100 allele and between pH and heterozygosity. However, although correlations between gene frequency and a presumed selective agent provide evidence that natural selection rather than random drift has influenced the distribution of a particular allele, only direct cause-effect laboratory experiments can unambiguously relate changes in gene frequency to a selective agent (Clarke, 1975; Nevo et al, 1980). Therefore, toxicity tests were carried out to determine the tolerance of N. cinerea to acidification, and to investigate whether nymphs from naturally acidic streams were more tolerant than those from neutral or alkaline streams. Differential survival or fitness of any HDH genotype could be determined by screening the nymphs used in toxicity tests on populations from alkaline streams.

6.2. Materials and Methods.

Details of the apparatus and experimental procedure are given in section 2.5. (Chapter 2).

6.2.1. Experimental Organisms.

The nymphs used in these 96 hour acute toxicity tests came from Thomason's Hollow, an acid stream (pH 4.5) in the Derbyshire Peak District (site 15 in Chapter 5) and Glebe Farm, an alkaline stream (pH 8) in Leicestershire (site 21 in Chapter 5).

6.2.2. Analysis.

The 96 hour LC-50 values and their confidence limits (Fiducial Limits) from each test were determined by probit analysis (Finney, 1971). Differences in the LC-50's were compared using the nomographic relative potency analysis of Litchfield and Wilcoxon (1949). Chi-squared contingency tables were used to compare the gene and genotype frequencies of the nymphs killed in the toxicity tests with those of the survivors from acidified chambers and non-acidified controls.

6.3. Results.

The mortalities observed after 96 hours in seven toxicity tests are shown in Table 6.1. The percentage of the animals killed at each pH was plotted on a probability (probit) scale against hydrogen ion concentration on a logarithmic scale (pH). (Figures 6.1 - 6.4). The regression lines and 96 hour median lethal concentrations (96 hour

LC-50) were calculated by probit analysis. The 96 hour LC-50's and their fiducial limits are shown in Table 6.2. together with the regression coefficients (b). (Fiducial limits are used in the analysis of toxicity data rather than the more common variance and confidence limits because the variance should only be used to describe direct observations or statistics calculated from the observations. Fiducial limits are a statement that there is 95% chance that the LC-50 value falls within the specified limits (Finney,1971)). The LC-50's ranged from pH 2.960 to 3.221 which converts to a hydrogen ion concentration of 1096 to 602 micro equivalents per litre.

The regression lines and LC-50's were compared using the method of Litchfield and Wilcoxon (1949) to determine if the responses to pH were significantly different for the two populations and whether doubling the concentration of dissolved salts or adding calcium ions (20 mg.l^{-1}, as Calcium nitrate) could alter the toxic response. These results are shown in Table 6.3. There were statistically significant differences ($P < 0.05$) between most of the toxicity estimates with either the slope of the regression line or the LC-50 (or both) being different. Non-parallelism of regression lines indicates that differences in the toxic response were inconsistent over the range of doses used in the tests being compared, so that a simple comparison of LC-50's could be misleading (eg. between tests 2 and 7, and between tests 4 and 5). These differences might have been predicted, since the paired comparisons were between different populations (Thomason's Hollow or Glebe Farm), different water conditions (no added salts or double concentration or calcium ions added), or nymphs of different ages or from different years

(see site/sample date in Table 6.2).

The gene and genotype frequencies from two toxicity tests on nymphs from the Glebe Farm site are shown in Table 6.4 together with the results of X^2 contingency analysis. No significant differences were found between the genotypes or gene frequencies of those that were killed, the survivors or the controls. Figure 6.5 shows the gene frequencies of the nymphs from two tests.

Data from the tests starting on 16.5.83. could not be used for determining LC-50's due to an excess of deaths in one control chamber. This must have been contaminated in some way. Two other tests from June 1983 were not used to determine LC-50's because nymphs emerged as adults in the test chambers. This invalidated the tests because the adults were no longer exposed to acid stress. However, these tests did show that adults could successfully emerge from water with a pH of 3.0. The genotypes of these adults are shown in Table 6.5.

6.4. Discussion.

The 96 hour LC-50's for *N. cinerea* ranged from pH 2.96 to pH 3.22. These values are similar to those reported by Lechleitner *et al.* (1985) for three North American stoneflies: *Tallaperla maria* and *Pteronarcys proteus* (pH 2.8), and *P.dorsata* (pH 3.3). However, most 96 hour LC-50's reported for stoneflies are higher. In Murphy's (1978) review of toxicity test data, the range for eight North American species was from pH 3.23 to pH 5.33. This gives an indication that *N. cinerea* is probably one of the most acid tolerant stonefly species and provides laboratory evidence to explain why this species is often one of the dominant invertebrates in Europe's acid

streams (Otto and Svensson ,1983).

The comparison of the acid tolerance of two different populations did not give the result that might have been predicted. Nymphs collected from the alkaline stream at Glebe Farm, where the water chemistry was very different from the test conditions, proved to be equally, or more, tolerant to acid than those from Thomason's Hollow (Table 6.2). This contrasts with reports of consistent differences in acid tolerance between populations of fish (Gjedrem,1976, Swarts et al, 1978 and Rahel, 1983) and aquatic insects (Jernelov et al,1980) collected from naturally acidic or neutral/alkaline waters. In all these cases, organisms from alkaline streams (or from hatcheries) were more sensitive to acid stress. The results of the toxicity tests on N. cinerea from Glebe Farm show that this species has a remarkable ability to survive large changes in external pH, at least for short periods (96 hours).

Many investigations into the toxic mode of action of low pH have found that ionic regulation is adversely affected by increases in the external hydrogen ion content (see Fromm, 1980, and Havas, 1981 for reviews). Evidence for this has come, in part, from observations that survival can sometimes be prolonged at low pH by adding sodium or calcium ions to the test water (Packer and Dunson, 1970, Leivestad et al 1976), and that natural fish populations tend to be lost first from dilute lakes (Schofield, 1976). The effect of adding ion supplements was not examined in detail in the present study. The results from the three occasions when extra salts were added were not consistent, however. The ion supplements significantly reduced mortality in the two tests on nymphs

from Thomason's Hollow but there was no change in the test involving nymphs from Glebe Farm (Tables 6.2 and 3). Also, a comparison of the toxicity curves shows that, in the first experiment on nymphs from Thomason's Hollow, when 20 mg.l^{-1} Calcium was added, the line was parallel with the non-supplement curve. This indicates that calcium reduced toxicity over the whole range of pH's used. In the experiment where the total salt concentration was doubled, there was a significant increase in the gradient of the line ($P < 0.001$). This indicates that survival was only improved at pH's greater than 3.0 and, in fact, there was increased mortality at pH 3.0 (Table 6.1). These inconsistencies mean that no firm conclusions can be drawn about the importance of ionic regulation in pH induced mortality in N. cinerea.

One reason why these experiments failed to give clear evidence that mortality was caused by a failure of the osmoregulatory system could be that the ion supplements were not sufficiently concentrated to reduce mortality. Several workers have reported that only very high concentrations (more than 200 times those used here) have a noticeable effect on fish mortality (in Havas,1981). However, lower concentrations have been effective in invertebrates (Greenwood et al, 1986), and McWilliams & Potts (1978) showed that 20 mg.l^{-1} of calcium reduced gill permeability to hydrogen ions in brown trout by 43% compared to solutions with no added calcium. A further explanation could be that the ion supplements can only reduce mortality for a short time (hours) so that the effect was missed over 96 hours.

The analysis of the HDH-1 genotypes of the nymphs used in the tests did not suggest that low pH was directly

responsible for the removal of the 90 allele from acid stream populations (Table 6.4.). Individuals with the 90 allele appeared to be equally tolerant to acid stress as those with the 100 or 110 alleles. This result is consistent with the finding that nymphs from alkaline streams are as tolerant to low pH as those from acid streams. It appears, therefore, that another environmental factor (or factors) may be responsible for the non-random distribution of the HDH-1 90 allele amongst populations of N. cinerea in Britain. One possibility, food quality, has already been proposed in Chapter 5. However, it is not possible to completely rule out low pH as a selective agent. Different life stages may have different sensitivities to acid stress. Bell (1971) showed that the pH for the successful emergence of 50% of adults was usually 1 unit higher than the 96 hour LC-50 for the nymphs, and several workers have shown that egg hatching and early growth in fish is particularly sensitive to low pH (Fromm, 1980). It is possible that acidity could directly remove individuals with the HDH-1 90 allele at these critical stages. However, there was no evidence that there was a difference in the genotypes of the emergent adults screened in these tests (Table 6.5). Unfortunately, attempts to hatch N. cinerea eggs in the laboratory were unsuccessful. It is also possible that any differential survival of HDH-1 genotypes would only be apparent, either at even lower pH's than those used here, or over a longer time period. Attempts were made to carry out 30 day LC-50 tests but these were unsuccessful due to poor survival of nymphs in both test and control conditions.

Thus the evidence presented in this chapter is not supportive of a direct mechanism of acid selection at the

HDH-1 locus in N. cinerea. There was no difference in the LC-50s for acid or alkaline stream populations and no differential survival of HDH-1 genotypes was detected. However, only resistance to short term acute acidity was measured. It is possible that different HDH-1 genotypes have different long term resistances to low pH, including differences in fecundity in acid conditions.

Table 6.1. The number of mortalities observed at each pH in the 96 hour acute toxicity tests.

Site/ Sample Date	Additions	No. per chamber	Number Dead after 96 Hours 2.8	3.0	3.2	3.4	3.6
1. TH 6.12.82.	----	30	30	24	6	0	0
2. TH 6.12.82.	20mg. l^{-1} Ca	30	30	15	4	0	0
3. TH 26.4.83.	----	30	30	15	6	0	0
4. TH 26.4.83.	salts X 2	30	30	27	4	0	0
5. GF 30.5.82.	----	20	20	17	6	2	0
6. GF 27.4.84.	----	30	*16	13	3	-	0
7. GF 27.4.84.	20mg. l^{-1} Ca	30	*14	10	6	-	0

TH = Thomason's Hollow, GF = Glebe Farm.
*N = 20 per chamber

Table 6.2. The LC-50's (as μ.eq. l^{-1} of hydrogen ions and pH), fiducial limits and regression coefficients (b) calculated using Finney's (1971) probit analysis for the 96 hour acute toxicity tests.

Test No.	Site/ Sample Date	Additions	N	LC-50 μ.eq. l^{-1}	pH	Fiducial Limits	b
1	Thomason's 6.12.82.	----	180	794	3.10	3.057-3.143	8.8
2		20 mg. l^{-1} Ca	180	935	3.03	2.959-3.098	7.7
3	Thomason's 26.4.83.	----	150	601	3.22	3.141-3.279	5.7
4		Salts X 2	150	798	3.10	3.061-3.134	12.4
5	Glebe Fm. 30.5.83.	----	120	700	3.15	3.096-3.215	6.8
6	Glebe Fm. 27.4.84.	----	140	1096	2.96	2.885-3.019	5.4
7		20 mg. l^{-1} Ca	140	1086	2.96	2.906-3.012	3.7

Table 6.3. The probabilities of accepting the null hypothesis (no difference) for comparisons between the slopes of the regression lines (below diagonal) and LC-50's (above diagonal) of the 96 hour acute toxicity tests (from the nomographs of Litchfield and Wilcoxon, 1949).

Test	1	2	3	4	5	6	7
1	----	<0.05	<0.001	N.S.	N.S.	<0.01	<0.01
2	N.S.	----	<0.001	<0.05	<0.01	N.S.	N.S.
3	<0.05	N.S.	----	<0.01	N.S.	<0.001	<0.001
4	N.S.	<0.01	<0.001	----	N.S.	<0.001	<0.01
5	N.S.	N.S.	N.S.	<0.01	----	<0.001	<0.001
6	N.S.	N.S.	N.S.	N.S.	N.S.	----	N.S.
7	<0.01	<0.05	N.S.	<0.001	<0.05	N.S.	----

N.S. = no significant differance

Table 6.4. a.) The genotype numbers and b.) the gene frequencies at the HDH-1 locus in N. cinerea nymphs from the Glebe Farm site screened following 96 hour acute toxicity tests (d.f. = degrees of freedom, P = Probability, cont = control group).

a.)

Test Date	Group	Genotype Numbers 90 90	90 100	90 110	100 100	100 110	110 110	X^2	d.f.	P
	cont	1	11	3	9	2	1			
30.5.83.	dead	2	11	4	17	3	1	7.93	10	0.64
	live	3	8	1	11	7	1			
	cont	1	6	4	8	3	1			
16.5.83.	dead	4	18	5	28	11	8	9.17	10	0.52
	live	2	24	3	30	8	3			

b.)

Test Date	Group	Allele Frequencies 90	100	110	X^2	d.f.	P
	cont	0.296	0.574	0.130			
30.5.83.	dead	0.250	0.632	0.118	1.04	4	0.90
	live	0.242	0.587	0.161			
	cont	0.261	0.543	0.196			
16.5.83.	dead	0.209	0.574	0.216	5.44	4	0.25
	live	0.221	0.657	0.121			

Table 6.5. The genotypes of the adult N. cinerea (from Glebe Farm) that successfully emerged from the test chambers during 96 hour acute toxicity tests.

Test Date	Genotype numbers 90 90	90 100	90 110	100 100	100 110	110 110
June 1983	2	9	3	11	5	--

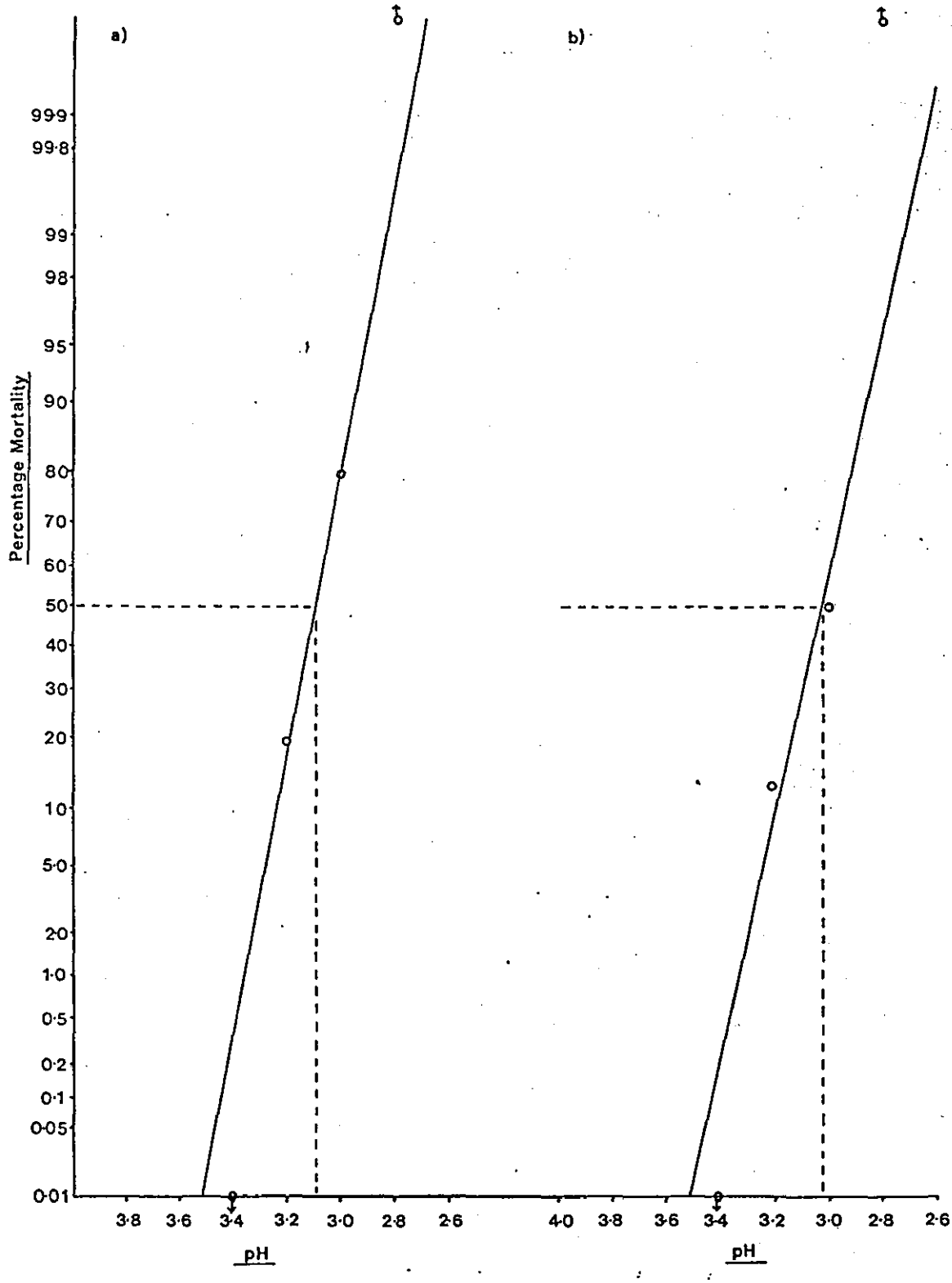


Figure 6.1. The percentage mortality of N.cinerea nymphs in the 96 hour acute toxicity test on 6.12.82. a.) no added salts, b.) 20 mg.l^{-1} calcium added. Line fitted by probit analysis.

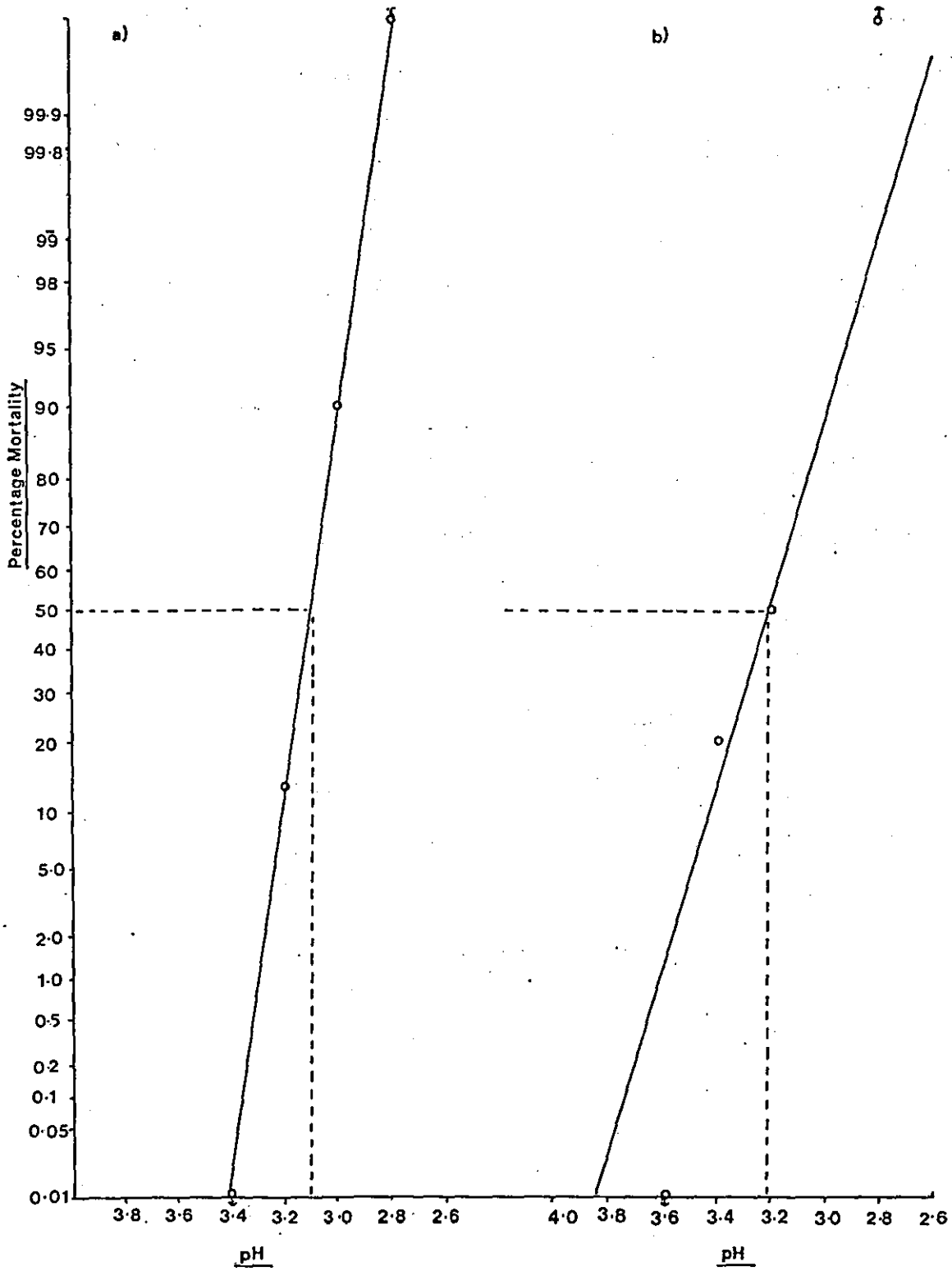


Figure 6.2. The percentage mortality of N.cinerea nymphs in the 96 hour acute toxicity test on 26.4.83. a.) no added salts, b.) salt concentration doubled. Line fitted by probit analysis

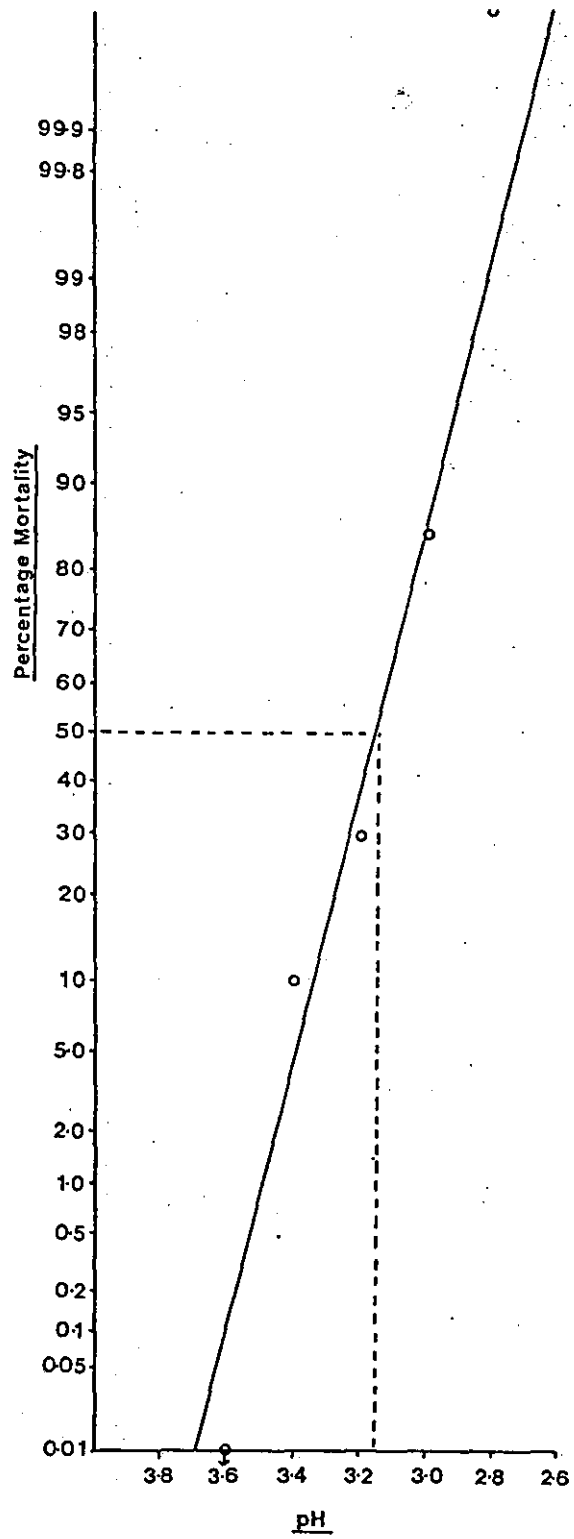


Figure 6.3. The percentage mortality of N.cineres nymphs in the 96 hour acute toxicity test on 30.5.83. Line fitted by probit analysis.

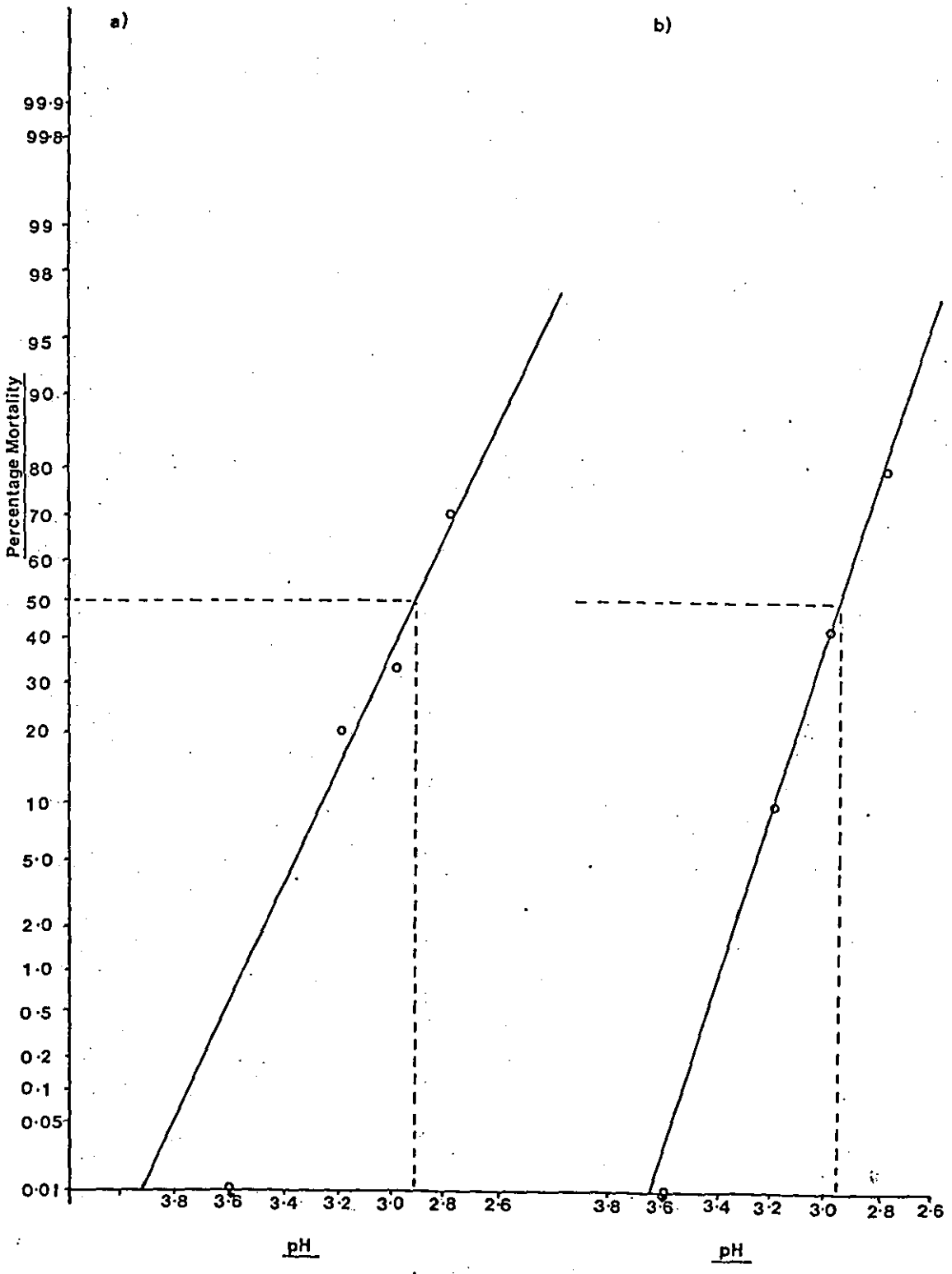


Figure 6.4. The percentage mortality of N.cinerea nymphs in the 96 hour acute toxicity test on 27.4.84. a.) 20 mg.l^{-1} calcium added, b.) no added salts. Line fitted by probit analysis.

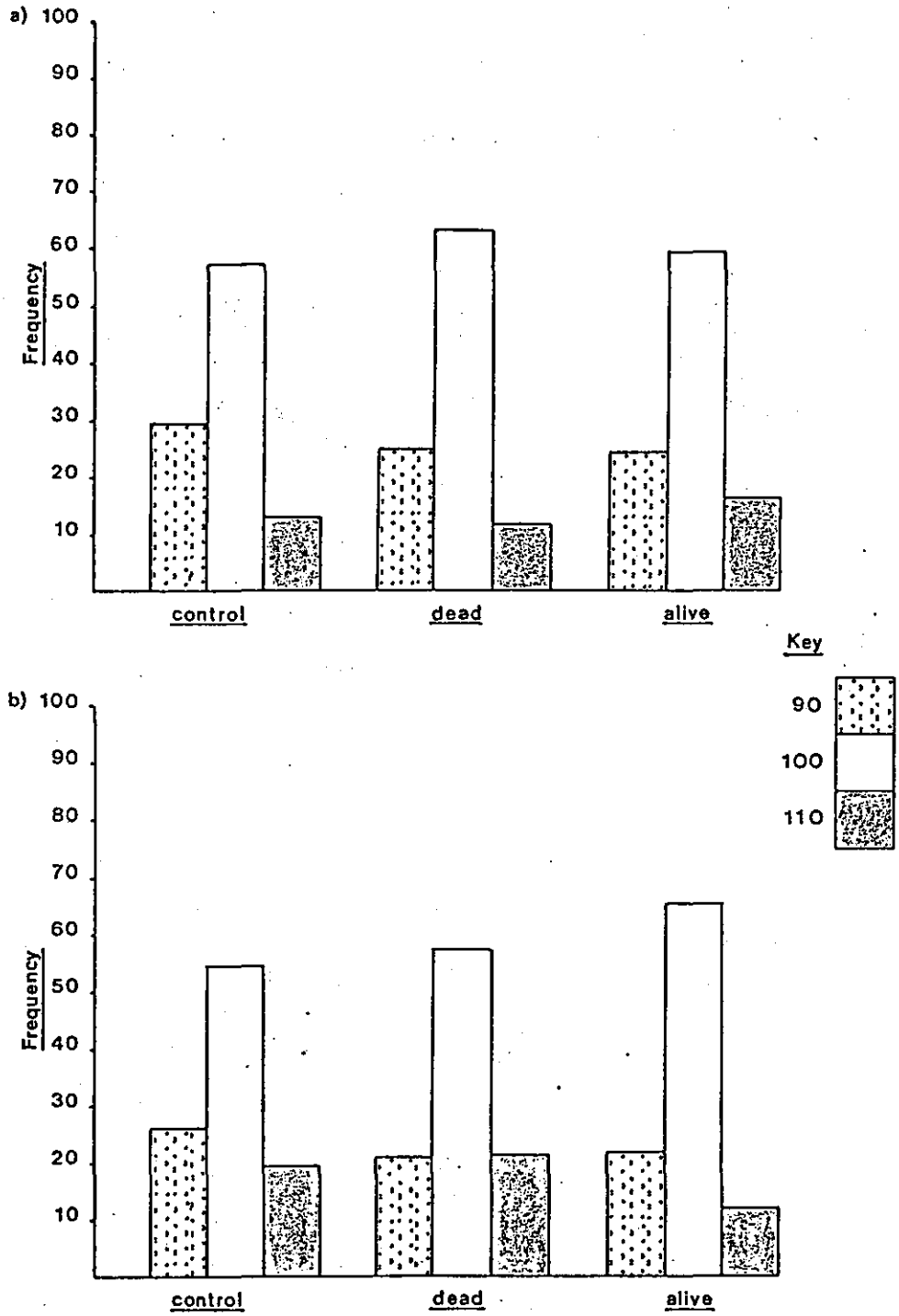


Figure 6.5. The gene frequencies of the N.cinerea nymphs screened following 96 hour acute toxicity tests. a.) From 30.5.83. b.) From 16.5.83.

CHAPTER 7

THE EFFECT OF LOW PH ON THE RATE OF OXYGEN CONSUMPTION IN NEMOURA CINEREA

7.1. Introduction.

Many investigations into the toxic mode of action of acidity have been carried out on aquatic organisms, though most work has centered on fish (Fromm, 1980; Havas, 1981). From these studies it appears that at least four interrelated physiological functions may be affected by low pH. These are : acid-base balance, sodium regulation, calcium regulation and respiration. Their relative importance is thought to depend on the ambient hydrogen ion concentration. However, a complete understanding of the process is hampered by the variable responses of different organisms to acid stress.

Of the four functions, the influence of low pH on respiration has received the least attention in invertebrates. Only two reports relating to this subject could be found in the literature. Doherty and Hummon (1980) found that acid mine water did not consistently alter the respiratory rates of two mayfly species and one stonefly species. In contrast, Alibone and Fair (1981) found that low pH values severely depressed the oxygen uptake rates in the crustacean Daphnia magna. This difference in response could be a reflection of their different tolerances to acidification. However both investigations had methodological deficiencies which may limit the general applicability of their findings: Doherty and Hummon used Warburg manometers to determine oxygen consumption. No substratum was used to reduce disturbances

caused by the sixty strokes per minute shaking motion, and the long measurement period (3 hours), together with the small volume (2 ml) of test fluid used, may have resulted in a considerable reduction in the oxygen concentration. No details were given however. In a critical review of Alibone and Fair's (1981) paper, France (1982) pointed out that they really measured the effects of carbon dioxide rather than pH on oxygen uptake. The carbon dioxide arose as an artifact of their method of acidifying the test water immediately prior to measurement in a closed oxygen electrode system.

The problems of maintaining a constant ambient oxygen concentration and of minimising physical disturbances to test organisms during the addition of a specific pollutant can be overcome by using a flow-through respirometer (Maki et al, 1973; Nagell 1975, and Gnaigner, 1983). An investigation into the effects of low pH on respiration in Nemoura cinerea under the more natural conditions produced by a flow-through respirometer is reported in this chapter.

7.2. Materials and Methods.

Details of the apparatus and experimental procedure are given in section 2.6. (Chapter 2).

7.2.1. Experimental Organisms.

With the exception of the experiment conducted on 25.1.83, all the nymphs used in the studies on oxygen uptake came from an acid stream, Thomason's Hollow, in the Derbyshire Peak District. The nymphs used on 25.1.83 came from an alkaline site at Glebe Farm in Leicestershire.

7.3. Results.

An example of the output from the chart recorder from three days of experiments is presented in Figure 7.1. From this it can be seen that the recording of the oxygen concentration from the respiratory chamber was more variable than that from the reservoir. This may have been partly caused by changes in the respiratory activity of the nymphs but the main reason was that mixing of the water was affected by the pulsating flow produced by the peristaltic pump.

The raw data taken from recordings similar to those shown in Figure 7.1 were used to calculate the mean rate of oxygen consumption per animal (nl/hr) and weight specific rate of oxygen consumption (µl/g/hr) at each pH (Table 7.1) The details of each experiment are also included in the table. The weight specific rate of oxygen consumption was calculated for comparison with similar published data.

The relationship between respiration (R) and mean dry weight (W), under both acid and neutral conditions, is shown in Figure 6.2. The regression equations of the lines have the form : $\log R = a + b(\log W)$ with R in $nl0^2/hr$ and W in mg dry weight.

At pH 7.0 the regression equation was: $\log R = 3.0174 + 0.9006 \log W$ (the correlation coefficient $r = 0.99$) At pH 3.0 the regression equation was: $\log R = 2.9950 + 0.8507 \log W$ ($r = 0.99$) In both cases the regression coefficient b was significantly different from zero ($t_8 = 27.6$ at pH 7 and $t_7 = 13.7$ at pH 3, $P < 0.001$). This, together with the high correlation coefficients ($P < 0.001$), gives an indication of the strong mass dependence of the rate of oxygen uptake.

The rate of oxygen uptake was compared at pH 7 and pH 3 for all sizes of nymphs by a test of the homogeneity of the regression coefficients (Bailey, 1959). The null hypothesis H: b1 - b2 = 0 was tested where b1 = the coefficient of regression at pH 7 and b2 = the coefficient of regression at pH 3. No significant difference was found between the coefficients (t_{15} = 0.19, P>> 0.10) which indicates that there was no difference in the rates of oxygen uptake at pH 7 and pH 3.

7.4. Discussion.

The flow-through respirometer seemed to perform very well throughout this series of experiments. The read-out from the oxygen electrode was stable in each experiment, and the results were consistent between years, and over a range of animal sizes. The values obtained for the respiration rate of Nemoura cinerea at 10°C (about 1000 µl/g/hr) were close to those obtained by Nagell (1973). He used a similar flow-through respirometer to determine the effects of reduced oxygen concentration on the rate of oxygen uptake in one mayfly and three stonefly species, including Nemoura cinerea. There was considerable variation in the oxygen consumption values he obtained. However, he reported an average of around 750 µl/g/hr for 'full grown' N. cinerea nymphs at 8°C and a dissolved oxygen concentration around 10 mg/l. 'Full grown' nymphs were used in the tests carried out in July 1982 and 1983 here.

One of Nagell's findings influenced the experimental procedure used here. He showed that, for all the species examined, the rate of oxygen consumption reached a plateau at

about 6 mg O^2/l and was independent of the ambient concentration above this. Hence, the initial dissolved oxygen concentration of the water in the reservoir was not closely controlled before the start of the experiments to determine the effect of low pH on respiration. A comparison of the rates of oxygen consumption of equal sized nymphs at slightly different initial oxygen concentrations (Table 7.1) is in agreement with Nagell's conclusions that small changes in the dissolved oxygen concentration do not affect uptake rate when initial levels are high.

The values for the rate of oxygen consumption of N. cinerea also compare favourably with those obtained by Knight and Gaufin (1966). They used Warburg apparatus to investigate the respiratory metabolism of several North American stonefly species. The species most similar to N. cinerea , in terms of size, were Nemoura cinctipes and N. californica with an average dry weight of 1 to 2 mg. Their oxygen consumption rates were found to be 1144 and 1783 µl/g/hr respectively, at 10°C. Knight and Gaufin (1966) also investigated the effect of temperature on oxygen consumption and found temperature coefficients (Q_{10}) of about 2.2 in the 5 to 10°C temperature range. If N. cinerea is assumed to have a similar Q_{10}, Nagell's (1973) results can be adjusted to give an average oxygen consumption of 915 µl/g/hr at 10°C, results very similar to those described here.

Both Nagell (1973) and Knight and Gaufin (1966) reported that the rate of oxygen consumption was influenced by body size. Nagell (1973) simply stated that small specimens of N. cinerea and the mayfly Cloeon dipterum were more efficient in taking up oxygen at low concentrations and had a

higher consumption than larger ones at the same concentration. Knight and Gaufin (1966) made a fuller investigation of the relationship between body size and respiration rate. They found that, for nymphs of Acroneuria pacifica and Pteronarcys californica weighing less than 60 and 100 mg respectively, the coefficents of the regression of log. oxygen uptake on log. dry weight were 0.72 and 0.90. These are similar to the values obtained for N. cinerea in this study (0.85 and 0.90).

The regression coefficent is the gradient of the log.-log. plot of oxygen uptake rate against body weight. This has been found to have a value of about 0.75 for most species examined (Hemmingsen, 1960). This means that although the rate of oxygen consumption per individual is greater in large animals, their weight specific rate of oxygen consumption will be lower than that of small ones. Various explanations have been proposed to explain this phenomenon though none seems to be universally accepted (Prosser, 1973, Banse, 1982).

The regression coefficient for N. cinerea is slightly higher than the generally quoted value for the mass dependence of oxygen uptake. This may have been caused by the temperature used in the experiments to determine the respiratory rates. Several workers have reported an inverse relationship between the regression coefficent and temperature in invertebrates (in Banse, 1982). The value of 0.75 was derived from experiments conducted at 20°C whereas the rate of oxygen uptake in N. cinerea was determined at 10° C. However, high coefficients of mass dependence of between 0.80 and 0.95 have also been reported from other studies of the respiratory physiology of small invertebrates (1µg to 40mg dry weight). This has led to

the conclusion that these organisms do not follow the same pattern of mass dependent reduction in respiration rate as the larger organisms used to give a value of 0.75 (Prosser, 1973).

Numerous physiological and toxicological studies on fish have shown that acidification can cause a significant reduction in the rate of oxygen uptake leading to eventual death by hypoxia (see Fromm, 1980 and Havas, 1981 for reviews). This, together with the observation that several insect species that rely on atmospheric oxygen are common in acid environments (Hultberg and Grahn, 1975; Havas and Hutchinson, 1981), has led to the inference that invertebrates may be similarly affected (Havas, 1981, Alibone and Fair , 1981). However, the results of this study, together with those of Doherty and Hummon (1980), indicate that acid pollution does not affect the respiration of aquatic insects. No significant change could be detected in the rate of oxygen uptake of N. cinerea even at a pH of 2.5 which was found to be toxic to 50% of the test organisms within 24 hours in preliminary acute toxicity tests (Chapter 6).

The apparent lack of involvement of the respiratory system in the toxic effect of acid pollution does not, on reflection, seem too surprising. In fish, respiration is affected only at pH's below which problems with ionic regulation and acid-base balance are known to occur. The reduction in oxygen uptake is thought to be caused by the reduced carrying capacity of haemoglobin as the blood pH falls (Fromm, 1980). Nemoura cinerea and the species studied by Doherty and Hummon (1980) do not rely on respiratory pigments to transport oxygen and seem, therefore, to be unaffected by low pH. It may be speculated that invertebrates that possess

respiratory pigments might show a similar respiratory response to acidification as fish.

In the absence of any respiratory pigment to cause a reduction in the rate of oxygen uptake at low pH, it was thought that the opposite reaction might occur in N. cinerea. Internal ionic balance is maintained in all freshwater organisms by the active uptake of sodium ions against a concentration gradient. Low pH interferes with this process, mainly by increasing the rate of sodium loss, so that more energy is needed to replenish it by active uptake (Havas, 1981). The increase in the energy demands for ionic regulation could be met by an increase in the rate of oxygen uptake when organisms are exposed to acid stress. A similar hypothesis was proposed to account for the reduction in growth rate of a mayfly in an experimentally acidified stream in North America (Fiance, 1978) and the reduction in the calorific content of stoneflies, mayflies (Raddum, 1979) and the waterlouse Asellus (Steigen and Raddum, 1981) exposed to low pH. However, as mentioned above, no significant change was recorded in the rate of oxygen uptake in N. cinerea after acidification.

At least two explanations can be proposed to account for the lack of a respiratory response to acid conditions in N. cinerea. The first is that the oxygen electrode was not sensitive enough to detect subtle changes in oxygen uptake. This seems unlikely however, since the electrode did respond to minor changes caused by poor mixing or small alterations in flow rate through the respiratory chamber (Figure 6.1). The second explanation is that ionic regulation was unaffected under the conditions used or within the time limits of the experiments. Aquatic insects, including the Plecoptera, are

known to use varying amounts of organic compounds for osmoregulation (Sutcliffe, 1962, 1963) which may explain their tolerance to low pH . Several aquatic insects including Diptera (Havas, 1981) and the waterbug, Corixa punctata (Vangenechten and Vanderborght, 1980) have been shown to be capable of maintaining constant haemolymph sodium concentrations for several days at pH 3. The acidified water used in the respirometer was found to be toxic to N. cinerea nymphs with the 96 Hour LC-50 lying between pH 2.96 and pH 3.22 (Chapter 6). However, it appears from the data presented here, that acidification does not cause respiratory stress, in the first few hours of exposure.

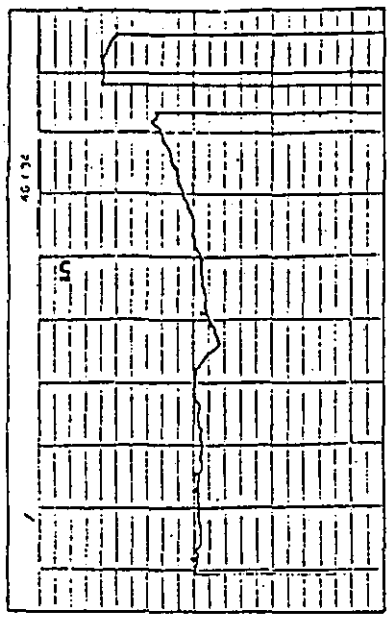
TABLE 7.1. Experimental conditions (pH, oxygen concentration, flow rate, number and dry weight of nymphs) on the dates shown, and the rates of oxygen consumption at each pH.

Date/ Location	pH	O^2Conc (mg/l)	Flow (ml/Hr)	N	Mean Dry Wt. (mg)	Mean O^2consumption /animal (nl/Hr)	/g dry wt (µl/g/Hr)
23.04.82	7.0	9.4	45	90	0.41	460.4	1123
Thomason	3.0	9.4	43	90	0.41	453.9	1107
26.04.82	7.0	9.9	50	80	0.40	454.0	1135
Thomason	3.0	9.9	43	80	0.40	432.0	1080
9.07.82	7.0	10.2	130	60	0.60	588.6	981
Thomason	2.8	10.2	130	60	0.60	572.4	954
21.07.82	7.1	10.9	110	77	0.70	744.8	1064
Thomason	2.5	10.8	106	77	0.70	769.3	1099
30.11.82	7.0	11.3	48	210	0.06	74.6	1244
Thomason	3.0	11.3	40	210	0.06	93.2	1554
1.12.82	7.0	10.9	45	220	0.06	79.6	1327
Thomason	3.0	10.9	35	220	0.06	88.0	1467
25.01.83	7.0	10.7	59	160	0.08	126.0	1575
Glebe Fm	3.0	10.6	62	160	0.08	118.5	1481
	7.0	10.7	110	80	0.36	421.2	1170
5.07.83 Thomason	3.0	9.8	105	67	0.69	698.3	1012
6.07.83 Thomason	7.1	9.8	101	67	0.69	877.0	1271
7.07.83	7.0	9.8	103	67	0.69	701.0	1016
Thomason	3.0	10.1	108	67	0.69	817.0	1184

N = number of nymphs in the respirometer

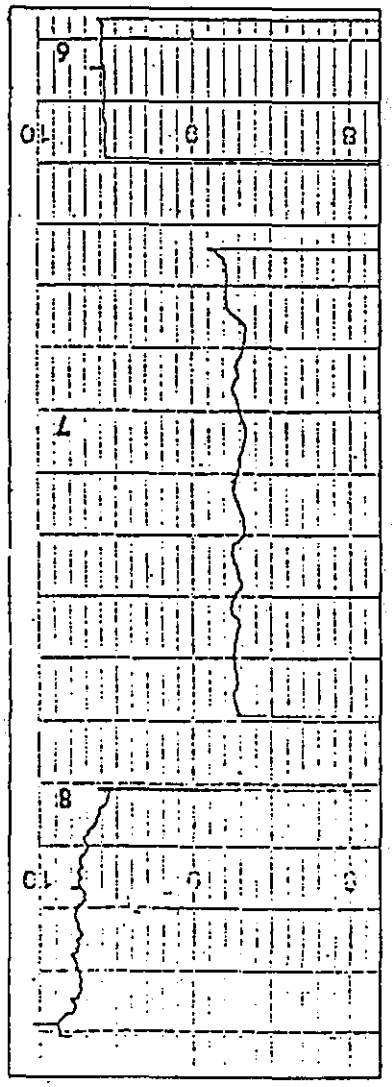
5.7.83.

Start 09.30
Reservoir : 9·8 mg $O_2.l^{-1}$
pH 3·0



6.7.83.

Start 09.00
pH 7·1
Reservoir: 9·8 mg $O_2.l^{-1}$



7.7.83.

Start 08.30
Reservoir : 9·8 mg $O_2.l^{-1}$
pH 7·0

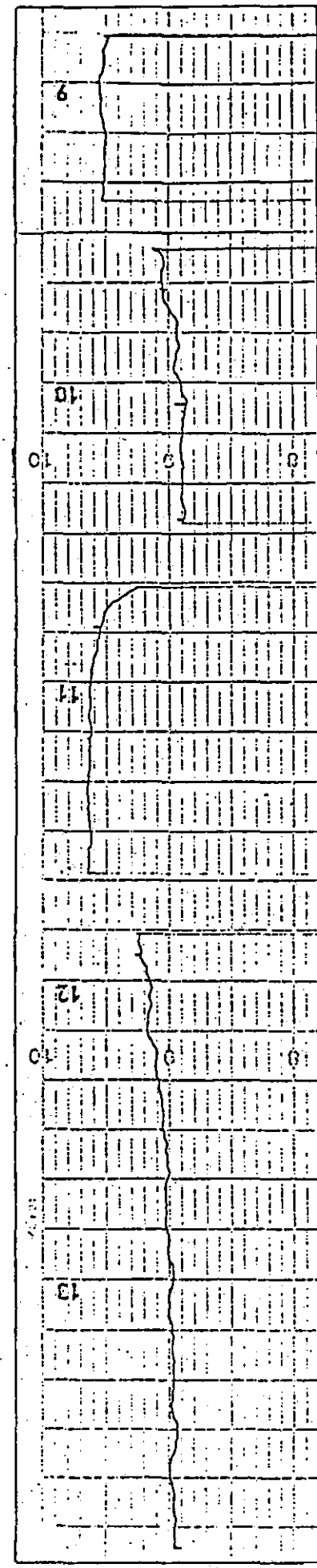


FIGURE 7.1. Chart recorder output from experiments carried out

Figure 7.1.

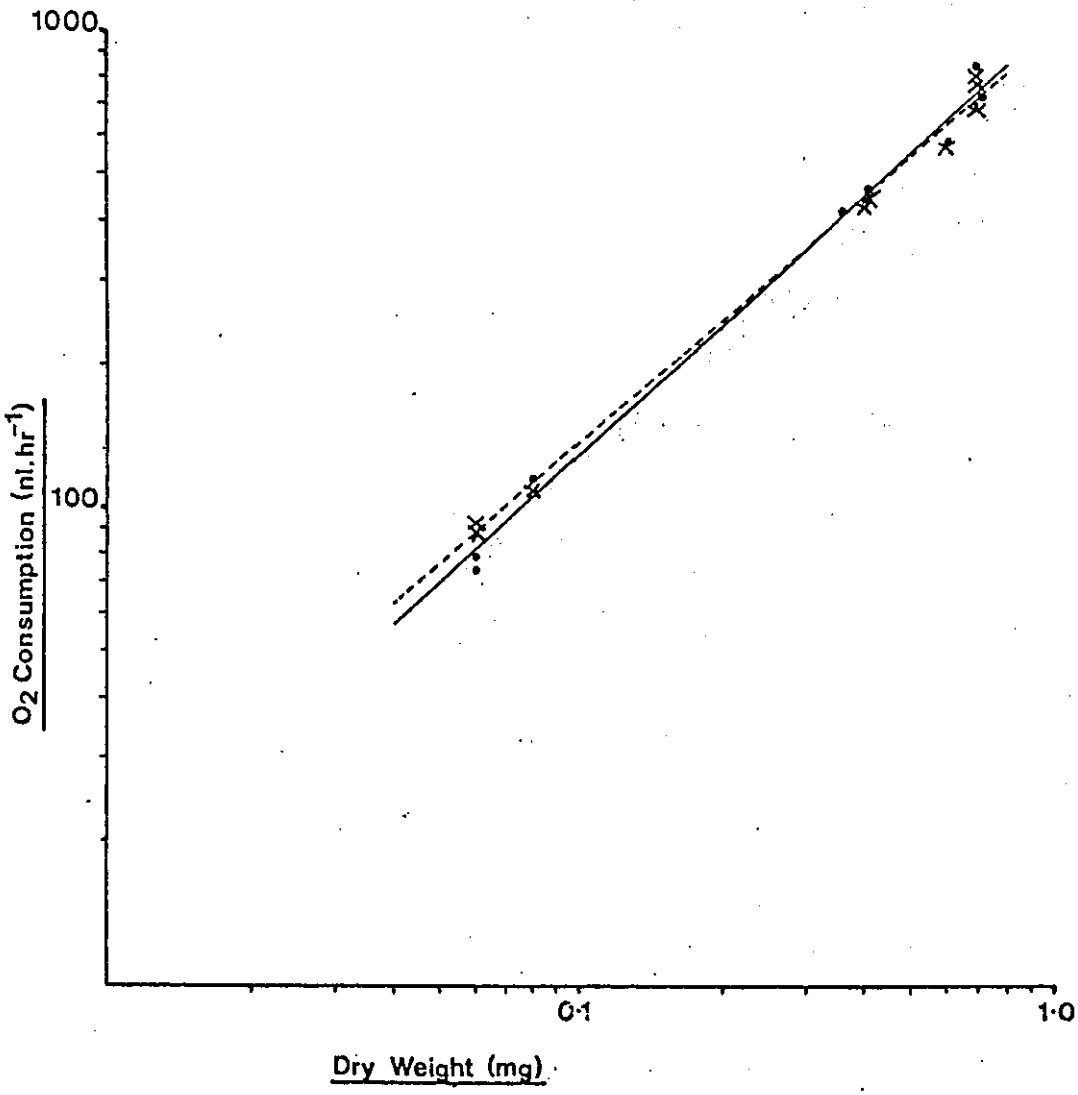


FIGURE 7.2. The relationship between the rate of oxygen consumption ($nl.hr^{-1}$) and body weight (mg. dry weight) in Nemoura cinerea at pH 7 (. ,———) and pH 3 (x ,------). The regression equations are given in the text.

Figure 7.2.

CHAPTER 8

General Discussion

The investigation into genetic variation and acid tolerance in the Nemouridae had three main stages. In the first stage electrophoretic techniques had to be refined through trial and error to establish a program for the characterisation of enzyme variation in stoneflies as no previous multi-locus electrophoresis had been reported in this family. A range of species could then be screened to assess how much genetic variation was present in each one (Chapter 4). Nemoura cinerea proved to have the most scoreable polymorphic loci of the species examined, so this was used for the second, central stage; a survey of inter-population genetic variation and its relationship to environmental changes (Chapter 5). The clear association between stream 'category' and the non-random distribution of the HDH-1 90 allele indicated that natural selection could be influencing variation at this locus and stream water chemistry was identified as a possible selecting agent. However, Clarke (1975) and Koehn (1978) have pointed out that a comprehensive explanation for observed gene frequency patterns depends upon a demonstration that the allozymes differ in biochemical reactivity or stability and that these result in physiological differences that could be affected by environmental factors. Therefore, in the third stage, an attempt was made to fulfil part of this program through investigations to determine whether acidity could have a direct effect on growth, survival or respiration in N. cinerea (Chapters 3,6 and 7).

During the first stage, electrophoresis was most successful with members of the Nemouridae, so all further work was concentrated on this group. Data were accumulated to allow a comparison between nine of the eleven British species at 16 enzyme loci. This number of inter - locus comparisons is low, relative to many similar investigations on vertebrates, but is comparable with other invertebrate studies (Nevo, et al, 1984). This suggests that, either invertebrates have low activity for the enzymes that are commonly used in electrophoresis making them difficult to score, or that difficulties are often encountered in making tissue preparations from invertebrates. Low numbers of inter - locus comparisons can cause some problems for data analysis and interpretation since the errors attached to estimates of genetic identity and distance are increased (Nei, 1978; Gorman and Renzi, 1979). However, 16 loci allows species to be compared with reasonable accuracy (Ferguson, 1980).

The inter-specific genetic distances within the Nemouridae were large in comparison with values reported for some other insect families (Table 8.1.). This was especially true of the genus Nemoura. Thorpe (1983) suggested that the critical value for distinguishing between species and genera is about D = 1.05, since 85 % of comparisons between congeneric species fall below this value. The mean genetic distance between Nemoura species exceeds the mean for inter-generic comparisons in Thorpe's (1983) survey (Table 8.1.). The distance values within the other two genera, Amphinemura and Protonemura, are lower than those for Nemoura but they are at the upper end of the range of values reported from other surveys. These high genetic distance values are in

agreement with the view that the Plecoptera are an archaic order (Rauser, 1971) and that most species pre-date the Pleistocene Glaciation (Illies, 1965). A crude estimate of the time since two lineages separated can be obtained from the genetic distance measures. Using the correlation proposed by Maxson and Maxson (1979), in which a Nei distance of 1.0 equals 14 million years, the two most closely related species, Protonemura meyeri and P. praecox, diverged about 7.5 million years before present.

The high genetic distance measures, coupled with the low number of inter-locus comparisons, caused some difficulties in estimating the phylogeny of this family. Different phylogenetic trees were produced depending on which distance measure and which tree generating method was used. Nei (1975) has shown that trees generated by the unweighted pair group method of analysis (UPGMA) give the best performance with data such as this and the UPGMA dendrograms gave the best fit to the input data here. This classification is in agreement with the accepted systematics of the Nemouridae, derived from morphology, with the family comprising four distinct genera : Nemoura, Protonemura, Amphinemura and Nemurella.

One of the most useful findings of this survey was that each species could be easily identified from unique band patterns on gels stained for several enzymes, and an electrophoretic key was devised to assist species identification. This proved to be of value for identifying anomalous morphotypes and for differentiating Nemoura cinerea and N. dubitans nymphs. These two species are very similar in external morphology and are difficult for the non-specialist

to separate on morphological grounds. Yet many diagnostic loci enable them to be unequivocably identified by electrophoresis. This resolving power means that electrophoresis would be an ideal tool to establish whether the two species that were not included in this survey can still to be found in Britain. Both species are described as rare by Hynes (1977), although Nemoura erratica has been reported from far more locations than Protonemura montana, and both are similar in external morphology to other species (P. montana resembles P. meyeri and P. praecox; N. erratica resembles N.cambrica) causing identification problems (Bird, 1983). Collections were made from streams where both species have been reported in the past, without success. Failure to collect these two species could have been caused by patchy distribution in the streams or it is possible that these species, particularly P. montana, have become extinct in the years since they were last recorded (Kimmins, 1941; Brown et al, 1964). However, it also seems likely that some records are inaccurate unless identifications were confirmed from adult specimens.

The amount of overall genetic variability was low in all species relative to other invertebrates (Ferguson, 1980; Nevo, et al, 1984), though N. cinerea and A. sulcicollis were the two most genetically variable species in the Nemouridae. Many different types of explanation have been put forward to account for differences in heterozygosity between species but in most cases both neutralist or selectionist interpretations could account for the observations so that there is conclusive support for neither. The low genetic variability in Plecoptera would have been anticipated by followers of the neutral theory of evolution, since the drastic population fluctuations

between years that have commonly been observed in aquatic invertebrates (Hynes, 1970) would produce bottle-necks in effective population size. The disjunct distribution patterns and poor adult mobility in these species means that re-colonisation is likely to have occurred from a few survivors within a stream rather than from another population, so that alleles lost during the population crash might not be replaced as the numbers recover. An alternative, selectionist, interpretation could be that these insects occupy rather narrow niches where selection might favour single specialised genotypes. This explanation arises from the view that genetic diversity is an adaptive strategy to exploit temporally or spatially heterogeneous environments. This 'niche-width variation hypothesis' (Somero and Soule, 1974; Levins, 1978) proposes that there is a positive correlation between genetic variability and environmental heterogeneity because the number of specifically adapted genotypes increases when more types of niche become available. Support for this hypothesis has come from several electrophoretic surveys in vertebrates, invertebrates and plants (eg. Lavie and Nevo, 1981; Lac y, 1982; Wallis and Beardmore, 1984; Nevo *et al*, 1986).

The higher genetic variability of *N. cinerea* and *A. sulcicollis* appears to conform with the expectations of the niche-width variation hypothesis. These two species have the widest temperature tolerances (Brown *et al*, 1964) and are found in a greater range of environments than the other nemourids (Brinck, 1949; Hynes, 1977). This is especially true of *N. cinerea* which was described as a 'eurycoenic' species from its wide distribution in Europe (Rausser, 1971). However, an equally plausible explanation is that species which can

survive in the widest range of habitats have the largest effective population sizes, since migration between isolated upland areas via lowland drainages is more probable. This would constitute a neutralist interpretation since the number of neutral alleles that can be maintained in a population is dependent on its effective size (Kimura and Ohta, 1971). Unfortunately, the distribution of Plecoptera in most lowland regions of Britain has not been fully investigated (Hynes, 1977) and informed estimates of population size can not be made. Thus, as is so often the case, the overall patterns of genetic variation in the Nemouridae fit equally well with both neutralist or selectionist interpretations and do not answer the question over the relevance of genetic variation at loci coding for enzymes to evolutionary adaptation.

The investigation into inter-population genetic variation in N. cinerea did provide clear evidence that molecular variation can be influenced by natural selection. The HDH-1 90 allele was only present in neutral/alkaline (category 2) streams across the whole of Britain, an observation that is incompatible with neutral theory. This discovery adds to the growing body of evidence, from numerous similar studies on a diverse range of organisms, that at least some loci coding for soluble enzymes do appear to affect fitness (eg. Koehn and Rasmussen, 1967; Schopf and Gooch, 1971; Clarke, 1975; Koehn et al 1976, 1980; Hoffman, 1981b; Nevo et al, 1982; Smith et al, 1983). The gene frequency - environment correlations revealed through these ecological genetics surveys have frequently suggested a clear relationship between an environmental factor, such as temperature or salinity and further laboratory work has

furnished a mechanistic explanation of this variation.

The classic example of this approach is the work done by Koehn (1969) following up a discovery of an allele frequency cline for esterase with latitude, in the fish Catostomas clarkii from the Colorado River (Koehn and Rasmussen, 1967). Temperature was thought to be the most likely selective factor, and in vitro biochemical studies revealed that the product of the allele that reached its highest frequency in the north was more active than the alternative form at low temperatures. The 'southern' form was more active at higher temperatures. This combined ecological and biochemical strategy is now accepted to be the best way of helping to understand the action of natural selection at particular loci (Clarke, 1975; Koehn, 1978). However, although a strong case for selection has been made through following this program in several of the studies cited above, there is still a need for caution when extrapolating from the results of in vitro studies to explain in vivo observations. This is most clearly illustrated through the work on the alcohol dehydrogenase enzyme (ADH) in Drosophila melanogaster. It had been widely accepted, following biochemical studies and genotype / environment correlations, that high ADH activity was selectively advantageous in allowing detoxification and utilisation of alcohol in the natural environment (Clarke, 1975). However, more recent work (Oakeshott and Gibson, 1981) has contradicted this view. The product of the ADH reaction, using ethanol as substrate, is acetaldehyde which is more toxic than ethanol, so high ADH activity might be disadvantageous in high alcohol environments.

Water chemistry (acidity and conductivity) appeared to

be the most important environmental factor in relation to variation at the HDH-1 locus in N. cinerea. However, other features such as altitude and the nature of the catchment could also be important.

No in vitro biochemical studies were made to discover whether the three HDH-1 allozymes were functionally different. This should be the next step to fulfil the criteria proposed by Clarke (1975) and Koehn (1978) to give a complete explanation for allele frequency variation. However, investigations were carried out to ascertain the physiological responses of N. cinerea to a proposed selecting agent (acidity). These studies showed that N. cinerea is highly tolerant to acid stress with a 96 hour LC50 of 2.96 to 3.22. This helps to explain why N. cinerea is often one of the dominant invertebrates in Europe's acid streams (Otto and Svensson, 1983). However, no difference in acid tolerance was detected between specimens collected from an acid stream (site 15, Thomason's Hollow, mean pH 4.5), where the 90 allele was absent, and an alkaline stream (site 21, Glebe Farm, pH 8.0), where the 90 allele was in high frequency, and there was no differential survival of any particular genotype (Chapter 6). Also, low pH did not appear to affect the rate of oxygen uptake when test water was acidified from pH 7.0 to pH 3.0 (Chapter 7). The difference in life-history between two sites in the same catchment seemed to result from a difference in annual thermal budget rather than different pH conditions (Chapter 3).

Although these experiments failed to show any relationship between variation at the HDH-1 locus and pH, acidity can not be completely ruled out as a possible

selecting agent. Other life stages, such as egg hatching or adult emergence, may be more sensitive to low pH than nymphs (Bell, 1971) so selection could act through these stages. However, no evidence of differential adult emergence was observed in the toxicity tests where emergence occured, and attempts to culture eggs in the laboratory were unsuccessful. Perhaps future work would profit through concentrating on these stages.

Acute toxicity tests have been successfully used to demonstrate differential survival among genotypes in other organisms (Nevo et al, 1980; Burton and Feldman, 1983). However, acute toxicity tests use rather extreme conditions so that small differences in fitness may be masked. This seems probable when selection differences often appear to be less than 1% from laboratory studies (Lewontin, 1974). It was, therefore, thought that longer term chronic tests might be more useful for the detection of differences in survival. However, these were unsuccessful due to poor survival in the test conditions. Also, the fitness difference of the genotypes may not be related to mortality at all but may involve fecundity in some way.

The close correlation between the altitude, pH and conductivity of the streams reflects that geology is the main factor which determines the nature of a stream and its catchment. All the acid streams, with the exception of site 17, Wareham 2, were in upland areas, where weathering resistant rocks have produced base-poor soils and run-off with a low conductivity and low buffering capacity. The Wareham 2 stream drained an acid heath area. The altitude and poor soils meant that deciduous woodland was absent from all the acid

stream catchments, the only significant stands in some areas being coniferous plantations. In contrast, the neutral/alkaline streams were in areas where the geological conditions have produced soils rich in calcium and magnesium so that run-off has a high pH and buffering capacity. The close association between these three factors makes it more difficult to isolate any one factor from the others. Multiple regression gives further evidence that these three factors are all inter-related, since the correlation coefficient for the three combined factors together is not significantly higher than that for acidity alone ($r = 0.72$ for acidity/HDH-1 frequency, $r = 0.73$ for the combination, $p < 0.001$).

The relationship between the environmental factors, and the failure of the physiological investigations to provide any evidence that acidity acts as a selecting agent, means that a complete mechanistic explanation for the variation at the HDH-1 locus is not possible. Several hypotheses were discussed in chapter 5. However, the most plausible, at this stage, appears to be that acidity has an indirect effect through altering food quality, assuming that HDH has a similar detoxifying role in N. cinerea to ADH in Drosophila. This seems likely because the coarse organic material that forms the basis of the diet of N. cinerea breaks down more slowly in acid conditions due to a reduction in the numbers and diversity of micro organisms (Bick and Drews, 1973; Hendrey et al, 1976; Otto and Svensson, 1983), and the food may be less 'conditioned' and of poorer nutritive value (Cummins and Klug, 1979). However, a similar difference in food quality may arise from a difference in catchment vegetation. There may be a greater and better quality input of allochthonous material from

deciduous woodland into category two streams. Diet has been found to have a marked influence on allozyme variation in several invertebrates. In Drosophila melanogaster significant differences in the frequency of amylase alleles were found between populations reared on different carbohydrates (DeJong, et al, 1972) and the ethanol content of adult food caused differential mortality between alcohol dehydrogenase genotypes (in Clarke, 1975). In the Lepidopteran, Heliothis virescens, significant differences in allele frequencies were observed at three loci between larvae reared on different beans (Sluss, et al, 1978) and in the water hoglouse, Asellus aquaticus differences in leaf litter quality appeared to account for habitat selection by different amylase genotypes (Christensen, 1977).

Further study of the Charnwood Beacon stream (site 19) may help to determine whether lack of 'conditioning' or the absence of deciduous trees is most important. The 90 allele was present in this woodland stream even though it has a low pH during the winter and an impoverished invertebrate community, typical of other acid streams (Greenwood et al, 1986). (Note that it did have a higher pH and conductivity when the sample was collected for electrophoresis.) This could be taken as an indication that leaf litter is essential for the survival of individuals with the 90 allele. However, the situation is not so simple. N. cinerea was not found at this site in 1980 or 1981, but it did occur further down stream where the pH and invertebrate community was altered after passing through a golf course. Therefore, it is possible that the 90 allele was only present in 1983 due to upstream migration from the neutral 'refuge' further down the

catchment.

Thus, a comprehensive explanation for the pattern of allele frequency variation at the HDH-1 locus in N. cinerea is not possible at this stage. Further work to establish whether the allozymes are functionally different and, perhaps, to extend the number and range of populations in the survey, is needed before the picture can be completed. However, the observation that the distribution of HDH-1 alleles is non-random with respect to stream type indicates that natural selection can influence genetic variation at loci coding for soluble enzynes, and adds weight to the argument that much of this type of variation is not neutral.

Investigations into the effect of pollution on evolutionary change have frequently shown that genetic variation is sensitive to environmental change. The classic examples are industrial melanism in the moth, Biston betularia (reviewed by Kettlewell, 1973) where the frequency of melanic forms showed a rapid increase in smoke-polluted areas, and the selection for heavy-metal tolerance in plants inhabiting old mine workings (Bradshaw, 1970). There is a growing body of evidence from electrophoretic studies that enzyme diversity is important in the adaptation of organisms to polluted environments (Nevo, 1986). Much of this work has used a 'physiological stress' approach, where differential fitness of allozymes is detected by screening organisms following acute toxicity tests. Differential survival has been found in a range of invertebrates including barnacles, crustaceans and marine gastropods subjected to thermal and chemical pollution. These results were in agreement with the observed allele frequency patterns in nature (Nevo, 1986). The physiological

effects of these genetic changes were not examined. However, the sensitivity of allozyme frequencies to pollution suggests that they could be used as indicators and monitors of pollution (Beardmore, 1980; Beardmore, et al, 1980; Nevo, 1986). Allele frequencies at the HDH-1 locus in N. cinerea appear to be related to stream water chemistry, though the mechanism of selection is unclear. It is possible, therefore, that this species could be used as an indicator of acidification. However, further experimental work would be necessary to establish a more definite link between acidity and HDH-1 variation before it could be accepted as a reliable marker.

Conclusions

1) Electrophoresis has shown that the Nemouridae have low levels of genetic variation in comparison with other invertebrates. There was no clear evidence for either a neutralist or selectionist explanation of this variation though it seems likely, from ecological reports and a study of temporal genetic change (Section 5.4.), that variation has been lost during periodic population bottle-necks.

2) Nemoura cinerea can be found in a wide range of stream habitats, ranging from very acid, soft-water, moorland streams to alkaline, hard-water, lowland drainages. This species is acid tolerant with a 96 hour LC-50 of pH 2.96 - 3.22. Low pH did not appear to have any effect on the rate of oxygen uptake in N. cinerea which was around 1000 µl/g dry weight/hour.

3) Inter-population comparisons of the allele frequencies at four polymorphic loci in N. cinerea gave strong evidence that natural selection rather than random stochastic processes has influenced variation at the HDH-1 locus. The 90 allele was found only in neutral/alkaline streams.

TABLE 8.1. The mean genetic distances (Nei, 1972) between congeneric insect species and the overall mean from a range of vertebrate, invertebrate and plants. N = number of species compared, n = number of loci, * = comparisons between genera.

Taxa	N	n	Distance		Reference
GENERAL					
Species	824[1]	--	0.616		Thorpe, 1983
Genera	160[1]	--	1.298	*	"
PLECOPTERA					
Nemoura	4	16	1.358		Present Study
Amphinemura	2	16	0.530		"
Protonemura	2	16	0.670		"
Nemouridae	9	16	1.822	*	"
DIPTERA					
Hawaiian Drosophila	8	31	0.618		Ayala, 1975
D. mulleri group	5	17	0.252		Zouros, 1973
D. willistoni group	5	31	1.056		Ayala et al, 1974
Aedes mariae complex	2	26	0.283		Cianchi, et al, 1978
LEPIDOPTERA					
Speyeria	10	16	0.182		Brittnacher, et al 1978
Heliothis	2	19	0.874		Sluss, et al, 1978
ORTHOPTERA					
Gryllus	5	18	0.140		Harrison, 1979
Troglophilus	2	17	0.374		Sbordoni, et al, 1981
COLEOPTERA					
Platynus	2	20	0.822		Liebherr, 1986
Dendroctonus	10	18	0.720		Bentz & Stock, 1986
EPHEMEROPTERA					
Epeorus	4	17	0.559		Zurwerra, et al, 1984

1 = number of paired comparisons.

ACKNOWLEDGEMENTS

I wish to acknowledge the help and advice given by my supervisor Dr. R.D. Ward throughout my research. Mr. J.A.B. Bass and colleagues at the Freshwater Biological Association River Laboratory, Dorset,and Dr.P.S. Maitland of the Institute of Terrestrial Ecology, Edinburgh, gave advice on the collection of some samples, and Neil Billington, Barbara Hilton and Andrew Lees gave valuable help on several trips. I also wish to thank the Nature Conservancy Council for permission to sample in the Moor House National Nature Reserve, and the Forestry Comission for permission to work in the Hope Woodlands, Derbyshire. Excellent technical assistance was provided by Mrs. M. Marsh, Mrs. A. Pacynko and Mr. P. Lockwood and Mr. G. Gerrard gave advice on using the BIOSYS 1 computer program.

I would also like to express my gratitude to the Natural Environment Research Council (U.K.) for the award of a research studentship from 1980 to 1983.

REFERENCES

Alibone, M.R. and Fair, P. 1981. The effects of low pH on the respiration of Daphnia magna (Straus). Hydrobiologia 85:185-188.

APHA 1976. Standard methods for the examinations of water and wastewater. 15th Edition America Public Health Association, American Water Works Association, and Water Pollution Control Federation. Washington.D.C.

Ashton, G.C. and Braden, A.W.H. 1961. Serum B-globulin polymorphism in mice. Australian Journal of Biological Sciences 14: 248-253.

Ashworth, J. 1980. Gravimetric determination of mean temperature by xanthate hydrolysis. J.App.Ecol. 17: 227-233.

Avise, J.C.,1974. Systematic value of electrophoretic data. Systematic Zoology 23: 465-481.

Avise, J.C and Selander, R.K. 1972. Evolutionary genetics of cave dwelling fishes of the genus Astyanax. Evolution 26: 1-19.

Ayala, F.J., 1975. Genetic differentiation during the speciation process. In Evolutionary Biology. Edited by Dobzhansky, Th., Hecht, M.K. and Steere, W.C., pp. 1-78. New York. Plenum Press.

1983. Enzymes as taxonomic characters. In : Oxford, G.S. and Rollinson, D. (Eds.) Protein Polymorphism: Adaptive and Taxonomic Significance. Academic Press.

Ayala, F.J.& Powell, J.R., 1972. Allozymes as diagnostic characters in sibling species of Drosophila. Proc. Nat. Acad. Sci. USA 69: 1094-1096.

* Acid News, 1986. A newsletter from the Swedish and Norwegian NGO SEcretariats on Acid Rain. No. 3/4.

Ayala, F.J and Valentine, J.W. 1974. Genetic variability in the cosmopolitan deepwater Ophiuran Ophiomusium lymani. Mar.Biol. 27:51.

Ayala, F.J., Powell, J.R., Tracey, M.L., Mourao, C.A. & Perez-Salas, S. 1972. Enzyme variability in the Drosophila willistoni group. 4: Genic variation in natural populations of D.willistoni. Genetics 70: 113-139.

Bailey, N.T.J. 1959. Statistical methods in Biology. Hodder and Stoughton.

Baker, J. and Schofield,C. 1980. Aluminium toxicity to fish as related to acid precipitation and Adirondack surface water quality. pp 292-293. In Drablos and Tollan 1980.

Banse, K. 1982. Mass-scaled rates of respiration and intrinsic rates of growth invery small invertebrates. Mar. Ecol. Prog. Ser. 9: 281 297.

Beardmore, J.A. 1980. Genetical considerations in monitoring effects of pollution. Rapp P -v Reun. Cons. Int. Explor. Mer. 179: 258 - 266.

Beardmore, J.A., Barker, C.J., Battaglia, B., Payne, J.F. and Rosenfield, A. 1980. The use of genetical approaches to monitoring biological effects of pollution. Raap P -v Reun. Cons. Int. Explor. Mer. 179: 299 - 305.

Bell, H.L. 1971. Effect of low pH on the survival and emergence of aquatic insects. Water Research 5: 313-319.

Bengtsson, J. 1984. Autecological studies of Nemoura cinerea (Retz.) Plecoptera. Arch. Hydrobiol. 100: 299-309.

Bentz, B.J. and Stock, M.W. 1986. Phenetic and phylogenetic relationships among ten species of Dendroctonus bark beetles (Coleoptera: Scolytidae). Ann. Entomol. Soc. Am. 79: 527 - 534.

Berger, E.M.1971. A temporal survey of allelic variation in natural and laboratory populations of Drosophila melanogaster. Genetics 67: 121-36.

Berlocher, S.H. 1980. An electrophoretic key for distinguishing species of the genus Rhagoletis (Diptera:Tephritidae) as larvae, pupae, or adults. Ann. Ent. Soc. Am. 73: 131-137.

Bick, H and Drews, E.F.1973. Self purification and silicate communities in an acid milieu (model experiments). Hydrobiologia 42: 393-402.

Bird, L.M.1983. Records of stoneflies (Plecoptera) from rivers in Great Britain. Entomologists Gazette. 34: 101-111.

Bradshaw, A. 1970. Pollution and plant evolution. New Scientist 17: 497 - 500.

Brimblecombe.P, and Stedman, D.H. 1982. Historical evidence for a dramatic increase in the nitrate component of acid rain. Nature 298: 460-2.

Brinck, P. 1949. Studies on Swedish stoneflies. Opuscula Entomologica Suplementum 11: 1-250.

Brittain, J.E. 1973. The biology and life cycle of Nemoura avicularis. Morton (Plecoptera).Freshwater Biol. 3: 199-210.

Brittain, J.E. 1977. The effect of temperature on the egg incubation period of Taeniopteryx nebulosa (Plecoptera). Oikos 29: 302-305.

Brittain, J.E. 1982. Biology of Mayflies. Ann. Rev. Entomol. 27: 119-47.

Brittain, J.E. and Mutch, R.A. 1984. The effect of water temperature on the egg incubation period of Mesocapnia oenone (Plecoptera) from the Canadian Rocky Mountains. Can. Ent. 116: 549-554.

Brittnacher, J.G., Sims, S.R. and Ayala, F.J. 1978. Genetic differentiation between species of the genus Speyeria (Lepidoptera: Nymphalidae). Evolution 32: 199 - 210.

Brown, D.J.A. 1981. Effect of calcium and aluminium concentrations on the survival of brown trout (Salmo trutta) at low pH. Bull. Environ. Contam. Toxicol.

Brown, D.J.A. and Martin, A. 1980. Aspects of the chemistry of rain water and stream water in the Derbyshire Peak District. CERL. Job. No.VJ.420.

Brown, V.M., Cragg, J.B. & Crisp, D.T. 1964. The Plecoptera of the Moor House National Nature Reserve, Westmorland. Transactions of the Society for British Entomology 16: 123-134.

Buikema Jr, A.L, Niederlehner, B.R. and Cairns, Jr. J. 1982. Biological monitoring part IV - toxicity testing. Water Res. 16: 239-262.

Burton, R.S. and Feldman, M.W. 1983. Physiological effects of an allozyme polymorphism: glutamate-pyruvate transaminase and response to hyperosmotic stress in the copepod Tigriopus californicus Biochem. Genet. 21: 239 - 251.

Buth, D.G. 1979. Biochemical Systematics of the Cyprinid genus Notropis 1. The subgenus Luxilus. Biochemical Systematics and Ecology 7: 69-79.

Carvalho, G.R. and Crisp, D.J. 1987. The clonal ecology of Daphnia magna (Crustacea : Cladocera) 1. Temporal changes in the clonal structure of a natural population. J. Anim. Ecol. 56 : 453 - 468.

Casey, H. and Ladle, M. 1976. Chemistry and biology of the South Winterbourne, Dorset, England. Freshwater Biol. 6: 1-12.

Cavalli-Sforza, L.L. and Edwards, A.W.F. 1967.Phylogenetic analysis: models and estimation procedures. Amer. J.Hum.Genet. 19: 233-57.

Charlson, R.J. and Rodhe, H. 1982. Factors controlling the acidity of natural rainwater. Nature 295 : 683-685.

Christensen, B. 1977. Habitat preference among amylase genotypes in Asellus aquaticus (Isopoda: Crustacea). Hereditas 87: 21 - 26.

Cianchi, R., Urbanelli, S., Coluzzi, M. and Bullini, L. 1978. Genetic distance between two sibling species of the Aedes mariae complex (Diptera: Culicidae). Parassitologia 20: 39 - 46.

Clarke, B. 1975. The contribution of ecological genetics to evolutionary theory: Detecting the direct effects of natural selection on particular polymorphic loci. Genetics 79:191-113.

Correa, M. Coler, R.A. and Damon, R.A. 1983. Oxygen consumption by nymphs of dragonfly Samatochlora cingulata (Odonata: Anisoptera). J. Freshwat. Ecology 2: 109-116.

Cowling, E.B. 1980. A historical resume of progress in scientific and public understanding of acid precipitation and it s biological consequences. In: Research Report 18/ 80, SNSF - project, Oslo, Norway.

Coyne, J. 1976. Lack of genic similarity between two sibling species of Drosophila as revealed by varied techniques. Genetics 84: 593-607.

Cronan, C.S. and Schofield, C.L. 1979. Aluminium leaching response to acid precipitation: Effects on high elevation watersheds in the Northeast. Science, 204: 304-6.

Cummins, K.W. and Klug, M.J. 1979. Feeding ecology of stream invertebrates. Ann. Rev. Ecol. Syst. 10: 147-172.

Darwin, C. 1859. The origin of species by means of natural selection. London: John Murray.

Dannevig, A. 1959. The influence of precipitation on the acidity of water courses and on fish populations. Jager og Fisker 3: 116 - 118.

Davis, B.J. 1964. Disc electrophoresis. 2. Method and application to human serum proteins. Annals of the New York Academy of Science 121: 404-427.

DeJong, G.A., Hoorn, J.W., Thorig, G.E.W. and Scarloo, W. 1972. Frequencies of amylase variants in Drosophila melanogater. Nature 238: 453 - 454.

Dickson, W. 1978. Some effects of the acidification of Swedish Lakes. Internationale Vereinigung fuer Theoretische and Angewordte Limnologie Verhandhingen. 20: 851-6.

Dobzhansky, Th. 1951. Genetics and the origin of species 3rd Edn. Columbia University Press.

Dobzhansky, Th. 1955. A review of some fundamental concepts and problems of population genetics. Cold Spring Harbor Symp. Quant. Biol. 20:1-15.

Doherty, F.G. and Hummon, W.D. 1980. Respiration of aquatic insect larvae (Ephemeroptera, Plecoptera) in acid mine water. Bull. Environ. Contam. and Toxicol. 25: 358-363.

Dovland, H. Joranger, E, and Semb,A. 1976. Deposition of Air Pollutants in Norway. In: Impact of Acid Precipitation on Forest and Freshwater Ecosystems in Norway. Ed. Braekker, F. SNSF. Report 6, Oslo, Norway.

Dovland, H. and Semb, A. 1980. Atmospheric transport of pollutants. In: Drablos, D. and Tollan, A. (Eds.) Ecological impact of acid precipitation. p. 14 - 21. SNSF.

Drablos, D. and Tollan , A. 1980. Eds. Ecological Impact of Acid Precipitation Proc. Int. Conf. Sandefjord, Norway. SNSF.

Edwards, A.W.F. 1971. Distances between populations on the basis of gene frequencies. Biometrics 27:873 - 881.

1974. Distance measures for phylogenetic trees. In : Crow, J.F. and Denniston, C. (Eds.), Genetic Distance. p. 41 - 43. Plenum Press, New York.

Elliot, J.M. 1967. The life histories and drifting of the Plecoptera and Ephemeroptera in a Dartmoor Stream. J. Anim. Ecol. 36: 343-62.

Elton, C.S. 1956. Stoneflies (Plecoptera, Nemouridae), a component of the aquatic leaf litter fauna in Wytham Woods, Berkshire. The Entomologists Monthly Magazine :231-236.

Farris, J.S. 1972. Estimating phylogenetic trees from distance matrices. Amer. Nat. 106: 645-68.

Farris, J.S. 1981. Distance data in phylogenetic analysis. pp.1-23. In: Advances in Cladistics. Proc. 1st meeting Willi Hennig Society. Ed. Funk, V.A. and Brock, D.R. Publ. NY Botanical Garden.

Fawcett, J.E. 1971. Leicestershire Stoneflies. Trans. of the Leicester Literary and Philosophical Soc. Vol. LXV, 33-48.

~~Felsenstein, J., 1976. The theoretical population genetics of variable selection and migration. Annual Review of Genetics 10 : 253-280.~~

Felsenstein, J. 1981. Evolutionary trees from gene frequency and quantitative characters: finding maximum likelihood estimates. Evolution 35: 1223-1242.

Ferguson, A. 1980. Biochemical systematics and evolution. Glasgow and London. Blackie & Son.

Ferguson, P. and Lee, J.A. 1983. Past and present sulphur pollution in the southern Pennines. Atmospheric Environment 17: 1131-1137.

Fiance, S.B. 1978. Effects of pH on the biology and distribution of Ephemerella funeralis (Ephemeroptera). Oikos 31: 332-339.

Finney,D.J. 1971. Probit Analysis 3rd. Ed. Cambridge University Press.

Fitch, W.M. and Margoliash, E. 1967. Construction of phylogenetic trees. Science 155: 279-84.

Forstner, H. 1983. An automated multiple - chamber intermittent flow respirometer. Chp. 2.1 In Gnaigner and Forstner 111-126.

Fowler, D. 1980. Removal of sulphur and nitrogen compounds from the atmosphere in rain and by dry deposition. In: Drablos, D and Tollan, A. SNSF, Oslo.

France, R.L. 1982. Comment on Daphnia respiration in low pH water (The importance of CO2 in laboratory acidification experiments. Hydrobiologia 94: 195-198.

Franks, J. 1983. Acid Rain. Chemistry in Britain 19: 504-9.

Fromm, P.O. 1980. A review of some physiological and toxicological responses of freshwater fish to acid stress. Env. Biol. Fish 5: 79-93.

Gjedrem, T. 1976. Genetic variation in tolerance of brown trout to acid water. Acid Precipitation - Effects on Forest and Fish Project. SNSF Report 5. Norway.

Glass, N.R, Arnold, D.E. Galloway, J.N. Hendrey, G.R: Lee, J.J: McFee, W.W: Norton, S.A: Powers, C.F: Rambo, D.L: Schofield, C.L. 1982. Effects of acid precipitation. Env. Sci and Technol. 16: 162A-169A .

Gledhill, T. 1960. The Ephemeroptera, Plecoptera and Trichoptera caught by emergence traps in two streams during 1958. Hydrobiologia 15: 179-188.

Gnaiger, E. 1983. The twin flow microrespirometer and simultaneous calorimetry. Chp. 2.3 in Gnaiger and Forstner.

Golterman, H.L, Clymo, R.S and Ohnstad, M.A.M. 1978 2nd. Ed. Methods for physical and chemical analysis of freshwaters. IBP Handbook No.8. Blackwell Scientific Publicators.

Gooch, J.L. and Schopf, T.J.M. 1972. Genetic variability in the deep sea: relation to environmental variability. Evolution 26: 545-552 .

Gorham, E. 1955. On the Acidity and Salinity of Rain. Geschimica et Cosmochimica Acta 7: 231-239.

1958. Free acid in British soils. Nature 181: 106.

1961. Factors influencing supply of major ions to inland waters, with special references to the atmosphere. Geol. Soc. Amer. Bull. 72: 795 - 840.

Gorman, G.C. and Renzi, J.J. 1979. Genetic distance and heterozygosity estimates in electrophonetic studies: effects of sample size. Copia 1979: 242-49.

Grahn, O. 1980. Fish kills in two moderately acid lakes due to high aluminium concentrations 310-311 In: Drablos and Tollan.

Greenwood, M.T, Black, V.J. and Peters, P.D. 1986. The relationship between acidification and invertebrate communities in a freshwater system. unpublished.

Haines, T.A 1981. Acidic precipitation and it's consequences for aquatic ecosystems: A review. Transactions of the American Fisheries Soc. 110: 669-707.

Hall, R.J. Likens, G.E., Fiance, S.B. and Hendrey, G.R. 1980. Experimental acidification of a stream in the Hubbard Brook experimental forest, New Hampshire. Ecology 6 (4): 976-89.

Hanken, J. 1983. Genetic variation in a dwarfed lineage, the Mexican salamander genus Thorius (Amphibia: Plethodontidae): taxonomic, ecologic and evolutionary implications. Copeia 1983 (4): 1051 - 1073.

Harriman, R. and Morrison, B.R.S. 1982. Ecology of streams draining forested and non-forested catchments in an area of central Scotland subject to acid precipitation. Hydrobiologia 88: 251-263.

Harris, H. 1966. Enzyme polymorphisms in man. Proc. Roy. Ser. B 164: 298-310.

Harris, H and Hopkinson, D.A. 1976. Handbook of enzyme electrophonesis in Human Genetics. North Holland. Amsterdam.

Harrison, R.G. 1979. Speciation in North American field crickets: evidence from electrophoretic comparisons. Evolution 34: 1009 - 1023.

Harvey, H 1980. Widespread and diverse changes in the biota of North American lakes and rivers coincident with acidification 93-98. In Drablos and Tollan 1980.

Havas, M. 1981. Physiological response of aquatic animals to low pH. In: Effects of acidic precipitation on benthos. Proc. Symp. Acidic Precipitation on benthos, 1980, N.American Benthological Soc. Hamilton, N.Y. 1981. Ed. Singer, R.

Havas, M. and Hutchinson, T.C. 1982. Aquatic invertebrates from the Smoking Hulls, N.W.T., effect of pH and metals on mortality. Can. J.Fish and Aquat. Sci. 39: 890-903.

Hemmingsen, A.M. 1960. Energy metabolism as related to body size and respiratory surfaces, and it's evolution. Rep. Steno Meml. Hosp. 9: 1-110.

Hendrey, G.R., Baalsrud, K.,Traaen, T.S. Laake, M and Raddum, G. 1976. Acid Precipitation: some hydrobiological changes. Ambio 5: 224-227.

Hendrey, G and Wright, R. 1976. Acid Precipitation in Norway: effects on aquatic fauna. Journal of Great Lakes Research 2 (1): 192-207.

Hoffmann, R.J. 1981a. Evolutionary Genetics of Metridium senile. I. Kinetic differences in phosphoglucose Isomerase Allozymes. Biochem. Genet. 19:129-143.

Hoffmann, R.J. 1981b. Evolutionary Genetics of Metridium senile. II. Geographic patterns of Allozyme Variation. Biochem. Genet. 19: 145-154.

Hultberg, H. and Grahn, O. 1975. Effects of acid precipitation on macrophytes in oligotrophic Swedish Lakes. 208-221. In: Proc. 1st. Spec. Symp. Atmospheric Contributions to the chemistry of Lake Waters, Int.Assoc. Great Lakes Res.

Hynes, H.B.N. 1941. The taxonomy and ecology of the nymphs of British Plecoptera with notes on the adults and eggs. Transactions of the Royal Entomological Society of London. 91 :459-557.

1942. A study of the feeding of adult stoneflies (Plecoptera). Proc. R. ent. Soc. Lond. A. 17: 81-2.

1952. The Plecoptera of the Isle of Man. Proc. R.Ent.Soc. Lond. (A) 27: 71-76.

1961. The invertebrate fauna of a Welsh Mountain Stream. Arch. Hydrobial 57: 344-88.

1970. The ecology of running waters. Liverpool. Liverpool University Press.

1976. Biology of Plecoptera. A.Rev.Ent. 21: 135-153.

1977. A key to the adults and nymphs of British stoneflies (Plecoptera). 3 rd. Edition. Freshwater Biological Association. Scientific Publication Number 17.

Illies, J. 1965. Phylogeny and zoogeography of the Plecoptera. Anual Revue of Entomology. 10: 117-140.

Jernelov,A, Nagell, B and Svenson, A. 1981. Adaptation to an acid environment in Chironomus riparius (Diptera, Chironomidae) from Smoking Hills, N.W.T, Canada.Holarctic Ecology, 4: 116-119.

Johnson, A.H, and Siccama 1983. Acid deposition and forest decline. Environ. Sci. Technol. 17: 294A-305A.

Johnson, M.S. 1971. Adaptive lactate dehydrogenase variation in the crested blenny, Anoplorchus. Heredity 27: 205-26.

Karlin, A.A., Guttman, S.I. and Rathbun, S.L. 1984. Spatial autocorrelation analysis of heterozygosity and geographic distribution in populations of Desmognathus fuscus (Amphibia : Plethodontidae). Copeia 1984(2) : 343 - 356.

Kettlewell, H.B.D. 1973. The Evolution of Melanism. Oxford. Clarendon Press.

Khoo, S.G. 1964. Experimental studies on diapause in stoneflies. University of Liverpool Ph.D. Thesis.

Kimmins, D.E. 1941. A new species of Nemouridae (Plecoptera). J. of the soc. for Brit. Entomology. 2: 89-93.

Kimura, M. 1979. The neutral theory of evolution. Scientific American 241: 98-126

Kimura, M. 1983. The neutral theory of molecular evolution 367pp. Cambridge University Press.

Kimura, M. and Crow, J.F. 1964. The number of alleles that can be maintained in a finite population. Genetics 49: 725-38.

Kimura, M. and Ohta, T. 1971a. Theoretical aspects of population genetics. Princeton University Press.

1971b. Protein polymorphism as a phase of molecular evolution. Nature. 229: 467-9.

1971c.On the rate of molecular evolution. J.Mol.Evol. 1:1-17.

King, J.L. and Jukes, T.H. 1969. Non-Darwinian evolution. Science 164: 788-798.

Knight, A.W. and Gaufin, A.R. 1966. Oxygen consumption of several species of stoneflies (Plecoptera). J. Insect Physiol. 12: 347-55.

Koehn, R,K. 1969. Esterase heterogeneity: Dynamics of a polymorphism. Science. 163 : 943 -

1978. Physiology and biochemistry of enzyme variation : The interface of ecology and population genetics. In : Brussard, P.F. (Ed.) Ecological genetics : The interface. Springer-Verlag. New York.

Koehn, R.K. Milkman, R and Mitton, J.B. 1976. Population genetics of marine pelecypods 1V. Selection, Migration and genetic differentiation in the blue mussel, Mytilus edulis.Evolution 30: 2-32.

Koehn, R.K, Newell, R.I.E, and Immermann, F. 1980. Maintainance of an aminopeptidase allele frequency cline by natural selection. Proc. Natl. Acad. Sci. U.S.A. 77 : 5385-89.

Koehn, R.K. and Rasmussen, D.I. 1967. Polymorphic and monomorphic serum esterase heterogeneity in catostomid fish populations. Biochem. Genet. 1 : 131.

Lacy, R.C. 1982. Niche breadth and abundance as determinants of genetic variation in populations of Mycophagous Drosophilid flies (Diptera: Drosophilidae). Evolution 33: 1265-1275.

Langford, T.E. and Bray, E.S. 1969. The distribution of Plecoptera and Ephemeroptera in a Lowland Region of Britain (Lincolnshire). Hydrobiologia 34: 243-271.

Lassen,H.H. and Turano, F.J. 1978. Clinal variation and heterozygote deficit at the LAP locus in Mytilus edulis. Mar. Biol. 49: 245-54.

Last, F.T. and Nicholson, I.A. 1982. Acid Rain. Biologist 29: 250-52.

Lavie, B. and Nevo, E. 1981. Genetic diversity in marine molluscs: a test of the niche-width variation hypothesis. Mar. Ecol. 2: 335-342.

Lechleitner, R.A. Cherry, D.S., Cairns, J.Jr. and Stetler, D.A. 1985. Ionoregulatory and toxicological responses of stonefly nymphs (Plecoptera) to acidic and alkaline pH. Arch. Environ. Contam. Toxical. 14: 179-185.

Lees, J.H. and Ward, R.D. 1987. Genetic variation and biochemical systematics of British Nemouridae. Biochem. Syst. Ecol. 15: 117-125.

Leivestad, H., Hendrey, G., Muniz, I.P. and Snekvik, E. 1976. Effects of acid precipitation on freshwater organisms 86-111. In: Braekke, F.H. (Ed) Impact of acid precipitation on forest and freshwater ecosystems in Norway. SNSF Report 6/76.

Levins, R. 1968. Evolution in changing environments. Princeton University Press.

Levinton, J.S. 1973. Genetic variation in a gradient of environmental variability: marine bivalvia (Mollusca). Science 180: 75-76.

Lewontin, R.C. 1974. The genetic basis of evolutionary change. Columbia University Press.

Lewontin, R.C. & Hubby, J.L. 1966. A molecular aproach to the study of genic heterozygosity in natural populations. 2: Amount of variation and degree of heterozygosity in natural populations of Drosophila pseudoobscura. Genetics 54: 595-609.

Liebherr, J.K. 1986. Comparison of genetic variation in two Carabid Beetles (Coleoptera) of differing vagility. Ann. Ent. Soc. Am. 79: 424 - 433.

Lillehammer, A. 1974. Norwegian Stoneflies II: Distribution and relationship to the environment. Norw. J. Ent. 21: 195-250.

1975. Norwegian Stoneflies III. Field studies on ecological factors influencing distribution. Norw. J.Ent. 22: 71-80.

1976. Norwegian Stoneflies V: Variations in morphological characters compared to differences in ecological factors. Norw. J.Ent. 23: 161-172.

1984. Distribution, seasonal abundance and emergence of stoneflies (Plecoptera) in the Ovre Heimdal area of the Norwegian Jatunheimen Mountains. Fauna Norv. Ser.B. 31: 1-7.

Litchfield, J.T. and Wilcoxon, F. 1949. A simplified method of evaluating dose effect experiments. J. Pharmacol. 95: 99-113.

Loucks, O.L. 1982. The concern for acidic deposition in the Great Lakes Region. In : D'Itri, F.M. (Ed.) Acid precipitation effect on ecological systems. p. 21 - 41. Ann Arbor Science.

Ludwig, W. 1950. Zur Theorie der Konkurrenz. Die Annidation (Einnischung) als funfter Evolutionsfaktor. Neue Ergebn. Probl. Zool. 516-537.

Macan, T.T. 1958a. The temperature of a small stony stream. Hyrobiologia 12: 89-106.

1958b. Causes and effects of short emergence periods in insects. Verh. int. Verein. theor. angew. Limnol. 13: 845-9.

1981. Life Histories of some species of Ecdyonurus (Ephemeroptera) in the River Lune, North Western England. Aquatic Insects. 3: 225-232.

McWilliams, P.G. and Potts,W.T.W. 1978. The effects of pH and calcium concentrations on gill potentials in the brown trout, Salmo trutta. J. comp. Physiol. 126:277-286.

Maitland, P.S. 1966. The distribution, life-cycle and predators of Amphinemura sulcicollis (Stephens) (Plecoptera) in the River Endrick, Scotland. The Entomologist 99: 72-81.

Maitland, P.S, Smith, I.R., Jones, D.H., East, K., Morris, K.H. and Lyle, A.A. 1981. The freshwaters of Tayside. ITE Project 117 Final Report to the NCC.

Maki, A.W., Stewart, K.W. and Silvey, J.K.G. 1973. The effects of Dibron on Respiratory activity of the stonefly, Hydroperla crosbyi, Hellgrammite, Corydalus cornutus and the golden shiner, Notomigonus crysoleucas.Trans.Amer. Fish.Soc. 102: 806-15.

Mathews, R.O., McCaffrey, F. and Hart, E 1980. Acid Rain in Ireland. Irish J. of Env. Sci. 1: 47-50.

Maxson, L.R. & Maxson, R.D. 1979. Comparative albumin and biochemical evolution in plethodontid salamanders. Evolution 33: 1057-1062.

Meredith, S.E.O. 1980. A study of methods for the identification of members of the Simulium (Edwardsellum) damnosum complex. Ph.D. Thesis University of Liverpool.

~~Minshall, G.W. 1969. The Plecoptera of a Headwater Stream. Arch. Hydrobiol. 65: 494-514.~~

Morgan, N.C. and Egglishaw, H.J. 1965.A survey of the bottom fauna of stream in the Scottish Highlands. Part 1. Composition of the fauna. Hyrobiologia 25: 181-211.

Muller, H.J. 1950. Our load of mutation. Amer. J.Hum. Genet. 2: 111-76.

Muniz, I.P. and Leivestad, H. 1980. Toxic effects of aluminium on the brown trout, Salmo trutta L. 320-321 In: Drablos and Tollan. 1980.

Muniz, I.P., Leivestad, H. and Bjerknes, V. 1979. Fishkill in the River Nidelva, spring 1979. SNSF-project TN48/79. 29 p.

Muniz, I.P., Leivestad, H., Gjessing, E., Joranger, E. and Svalastag, D. 1975. Fishkill during snowmelt in the Tovdal River catchment, southern Norway, spring 1975. SNSF-project IR13/75, 60 p.

Murphy, P.M. 1978. Manual for toxicity tests with freshwater macro-invertebrates and a review of the effects of specific toxicants. UWIST. Dep. of the Environ. Contract D.GR/480/486.

Nagell, B. 1973. The oxygen consumption of mayfly and stonefly larvae at different oxygen concentrations. Hydrobiologia 42: 461-489.

1975. The open-flow respirometric method : Precision of measurement in general and description of a high precision respirometer for aquatic animals. Int. Revue. ges. Hydrobiol. 60:655-667.

Nebeker, A.V. 1971. Effect of temperature at different altitudes on the emergence of aquatic insects from a single stream. J. Kans. Ent. Soc. 44: 26-35.

Nebeker, A.V. and Gaufin, A.R. 1967. Factors affecting wing length and emergence in the winter stonefly Capnia nana. Entomological News 78: 85-92.

Nei, M. 1972. Genetic distance between populations. The American Naturalist 106: 283-292.

1975. Molecular population genetics and evolution. New York. North-Holland American Elsevier.

1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Nei, M. Fuerst, P.A. and Chakroborty, R. 1978. Subunit molecular weight and genetic variability of proteins in natural populations. Proc. Natl. Acad. Sci, USA. 75: 3359-3362.

Nei, M.; Tajima, F. and Tateno, Y. 1983. Accuracy of estimated phylogenetic trees from molecular data II. Gene frequency data. J. Mol. Evol. 19: 153-170.

Nevo, E. 1976. Genetic variation in constant environments. Experientia 32: 858-859.

1986. Pollution and genetic evolution of marine organisms: theory and practice. Environmental Quality and Ecosystem Stability 3: 841 - 847.

Nevo, E.; Bar-el, C. and Bar, z. 1983. Genetic diversity, climatic selection and speciation of Sphincterochila landsnails in Israel. Biol. J. Linn. Soc. 19: 339-373.

Nevo,E., Beiles, A. & Ben-Shlomo, R. 1984. The evolutionary significance of genetic diversity: Ecological, demographic and life history correlates. In: Lecture notes in biomathematics 53: Evolutionary dynamics of genetic diversity. Edited by Mani, G.S. Springer-Verlag. Berlin.

Nevo, E. Beiles, A., Kaplan, D., Golenberg, E.M., Olsvig-Whittaker,L. and Naveh, Z. 1986. Natural selection of allozyme polymorphisms: a microsite test revealing ecological genetic differentiation in wild barley. E volution 40: 13 - 20.

Nevo, E.; Golenberg, E.; Beiles, A.; Brown, A.H.D. and Zohary, D. 1982. Genetic diversity and environmental associations in wild wheat, Triticum dicoccoides, in Israel. Theor. Appl. Genet. 62 : 241 - 254.

Nevo, E.; Perl, T.; Beiles, A.; Wool, D. and Zoller, U. 1980. Genetic structure as a potential monitor of marine pollution. Ves. Journees. Etud. Pollutions. 61-68. Cagliari, C.I.E.S.M.

Oakeshott, J.G. and Gibson, J.B. 1981. Is there selection by environmental ethanol on the alcohol dehydrogenase locus in Drosophila melanogaster ? In Gibson, J.B. and Oakeshott, J.G. (Eds.) Genetic studies of Drosophila Populations. Australian National University Press, Canberra, pp 103 - 120.

Oden, S. 1968. Ekologikommitteen. Bull. no. 1. Stockholm.

OECD. 1977. Programme on the long range transport of air pollutants. Paris. OECD

Ohta, T. and Kimura, M. 1973. A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. Genet. Res. 22: 201-204.

Otto, C. and Svensson, B. 1983. Properties of acid brown water streams in south Sweden. Arch. Hydrobiol. 99: 15-36.

Overein, L.N.; Seip, H.M. and Tollan, A. 1980. SNSF final report. Acid precipitation -effects on forest and fish.

Oxford, G.S. 1986. Multiple amylase loci in Asellus aquaticus (Crustacea: Isopoda): genetics and linkage. Heredity 56: 105 - 110.

Packer, R.K. and Dunson, W.A. 1970. Effects of low environmental pH on blood pH and sodium balance of brook trout. J. Exp. Zool. 174: 65-71.

Pearce, F. 1982. The menace of acid rain. New Scientist 95: 418-424.

Peterson, R.; Daye, P. and Metcalfe, J. 1980. The effects of low pH on hatching of Atlantic salmon eggs. 338. In: Drablos and Tollan. 1980.

Pfeiffer, M. and Festa, P. 1980. Acidity status of lakes in the Adirondack region of New York in relation to fish resources. New York Department of Environmental Conservation Report FW.168. Albany, New York.

Place, A.R. and Powers, D.A. 1979. Genetic variation and relative catalytic efficiencies: lactate dehydrogenase B allozymes of Fundulus heteroclitus. Proc. Nat. Acad. Sci. USA 76: 2638 - 2642.

Picker, M. 1980. Neoperla spio (Plecoptera): a species complex? Systematic Entomology 5: 185-198.

Poulik, M.D. 1957. Starch gel electrophoresis in a discontinuous system of buffers. Nature 180: 1477-79.

Prager, E.M. and Wilson, A.C. 1976. Congruency of phylogenies derived from different proteins. Amolecular analysis of the phylogenetic position of the cracid birds. J. Mol. Evol. 9: 45 - 47.

1978. Construction of phylogenetic trees for proteins and nucleic acids: comparison of alternative matrix methods. J.Mal. Evol 11: 129-142.

Prosser, C.L. 1973. Oxygen: respiration and metabolism. In Prosser, C.L. (Ed) Comparative Animal Physiology 3rd Ed. Saunders, Philadelphia.

Raddum, G.G. 1979. Effects of low pH on insect larvae. SNSF Project Report IR 45/79.

Raddum, G.G. and Steigen, A.L. 1981. Reduced survival and calorific content of stoneflies and caddisflies in acid water. In: Singer, R. (ed.) Effects of acidic precipitation on benthos.

Rahel, F.J. 1983. Population differences in acid tolerance between yellow perch, Perca flavescens, from naturally acidic and alkaline lakes. Can.J.Zool. 61: 147-152.

Rauser, J. 1971. A contribution to the question of the distribution and evolution of plecopterological communities in Europe. Acta Faunistica Entomologica Musei Nationalis Pragae 14: 33-63.

Rogers, J.S. 1972. Measures of genetic similarity and genetic distance. Studies in Genetics VII: 145-153. University of Texas.

Rohlf, F.J. and Sokal, R.R. 1981. Comparing numerical taxonomic studies. Syst. Zool. 30: 459 - 490.

Sbordoni, V., Allegrucci, G., Caccone, A., Cesaroni, D., Cobolli Sbordoni, M. and de Matthaeis, E. 1981. Genetic variability and divergence in cave populations of Trogolophilus cavicola and T. andreinii (Orthoptera: Rhaphidophoridae). Evolution 35: 226 - 233.

Schofield, C. 1976. Acid Precipitation: effects on fish. Ambio 5: 228-230.

Schopf, T.J. and Gooch, J.L. 1971. Gene frequencies in a marine ectoproct. A cline in natural populations related to sea temperature. Evolution 25: 286-89.

Selander, R.K. 1976. Genic variation in natural populations. 21-45. In: Molecular Evolution Ed. Ayala, F.J. Sunderland: Sinauer.

Selander, R.K. and Kaufman, D.W. 1973. Genic variability and strategies of adaptation in animals. Proceedings of the National Academy of Science. U.S.A. 70: 1875-77.

Shaw, C.R. & Prasad, R. 1970. Starch gel electrophoresis of enzymes - a compilation of recipes. Biochemical Genetics 4: 297-320.

Shorrocks, B. 1978. The genesis of diversity. Hodder and Stoughton.

Singer, R. 1981. Effects of Acid Precipitation on Benthos. Proc. Symp. Acidic Precipitation on Benthos, 1980, North American Benthological Society, Hamilton, New York.

Singh, R.S., Lewontin, R.C. and Felton, A.A. 1976. Genetic heterogeneity within electrophonetic 'alleles' of xanthine dehydrogenase in Drosohila pseudobscura. Genetics 84: 609-629.

Skibinski, D.O.F. & Ward, R.D. 1981. Relationship between allozyme heterozygosity and rates of divergence. Genetical Research 38: 71-92.

1982. Correlations between heterozygosity and evolutionary rate of proteins. Nature 298: 490-492.

Sluss, T.P., Sluss, E.S., Graham, H.M. and Dubois, M. 1978. Allozyme differences between Heliothis virescens and H. zea. Ann. Ent. Soc. Am. 71: 191 - 195.

Sluss, T.P., Rockwood-Slus, E.S., Patana, R. and Graham, H.M. 1978. Dietary influenced allozyme differences between laboratory populations of Heliothis virescens. Ann. Ent. Soc. Am. 71: 367 - 371.

Smith, K. and Lavis, M.E. 1975. Environmental influences on the temperature of a small upland stream. Oikos 26: 228-236.

Smith, M.W, Smith, M.H. and Chesser, R.K. 1983. Biochemical genetics of mosquitofish I. Environmental correlates and temporal and spatial heterogeneity of allele frequencies within a River Drainage. Copeia. 1983 (1): 182-193.

Smith, R.A. 1872. Air and Rain. The beginnings of a Chemical Climatology. London. Longmans, Green and Co.

Sneath, P.H.A. and Sokal, R.R. 1973. Numerical taxonomy: the principles and practice of numerical classification. Freeman, San Francisco.

Sokal, R.R. 1965. Statistical methods in systematics. Biol. Rev. 40 : 337 - 391.

Sokal, R.R. and Sneath, P.H.A. 1963. Principles of Numerical Taxonomy. San Francisco. Freeman.

Somero, G.N. and Soule, M. 1974. Genetic variation in marine fishes as a test of the niche-variation hypothesis. Nature 249 : 670 - 672.

Steigen, A.L. and Raddum, G.G. 1981. Effects of acidified water on behaviour and energy content in the water-louse Asellus aquaticus (L). In: Effects of Acidic Precipitation on Benthos Ed. Singer, R.

Sutcliffe, D.W. 1962. The composition of haemolymph in aquatic insects. J.Exp. Biol. 39: 325-343.

1963. The chemical composition of haemolymph in insects and some other arthropods in relation to their phylogeny. Comp. Biochem. Physiol 9: 121-135.

Sutcliffe, D.W. and Carrick,T. 1973. Studies on mountain streams in the English Lake District. 1 pH, calcium and the distribution of invertebrates in the River Duddon. Freshwater Biol. 3: 437-62.

Swarts, F.A., Dunson, W.A. and Wright, J.E. 1978. Genetic and environmental factors involved in increased resistance of brook trout to sulfuric acid solutions and mine acid polluted waters. Trans. Am. Fish. Soc. 107: 651-677.

Swofford, D.L. 1981. On the utility of the distance Wagner procedure. In: Advances in Cladistics. Proc. 1st. meeting of Willi Hennig Society. Publ. New York Botanical Garden, Bronx, New York.

Swofford, D.L. & Selander, R.B. 1981. BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. Journal of Heredity 72: 281-283.

Thorpe, J.P. 1983. Enzyme variation, genetic distance and evolutionary divergence in relation to levels of taxonomic separation. In: Systematics Association Special Volume 24 Protein polymorphism: Adaptive and taxonomic significance. Edited by Oxford, G.S. & Rollinson, D.London and New York. Academic Press.

Tomlinson, G.H.II, 1983. Air pollutants and forest decline. E.S. and T. 17: 246A-256A.

Ulfstrand, S. 1968. Life cycles of benthic insects in Lapland streams (Ephemeroptera, Plecoptera, Trichoptera, Diptera Simulidae). Oikos 19: 167-190.

Ulrich, B. 1980. The predicted development of the forests of central Europe based on a study of environmental pollution and the associated theoretical risks. Allegemeine Forst Zeitschrift, 44: 1198-1202.

Valentine, J.W. 1971. Resource supply and species diversity patterns. Lethaia 4: 51 - 61.

1976. Genetic strategies of adaptation. In: Molecular Evolution. Ed. Ayala, F.J. Sinauer, Mass.

Valentine, J.W. and Ayala, F.J. 1975. Genetic variation in Frieleia halli, a deep-sea brachiopod. Deep-sea Res. 22: 37 - 44.

Vangenechten,J.H.D and Vanderborght, O.L.J. 1980. Effects of acid pH on sodium and chloride balance in an inhabitant of acid freshwaters: the waterbug Corixa punctata (Illig.) (Insecta: Hemiptera) 342-343. In: Drablos and Tollan 1980.

Van Valen, L. 1965. Morphological variation and width of ecological niche. The American Naturalist 99: 377-390.

Wade, K.R. and Rhodes, T 1982. A study of the macroinvertebrates and algae (Llyn Briamme Fishery Protection Scheme). In: Biological Investigations in the area administered by the Welsh W.A. Proceedings of a seminar held on 9th March 1982 at Brecon. Ed. Brooker, M.

Wallace, B. 1958. The role of heterozygosity in Drosophila populations. Proc. Int. Congr. Genet. 10th. 1: 408-19.

Wallis, G.P. and Beardmore, A. 1984. Genetic variation and environmental heterogeneity in some closely related goby species. Genetica 62: 223 - 237.

Ward, J.V. and Stanford, J.A. 1982. Thermal responses in the evolutionary ecology of aquatic insects. A.Rev. Ent. 27: 97-117.

Ward, R.D. 1977. Relationship between enzyme heterozygosity and quaternary structure. Biochemical Genetics 15: 123-135.

Ward, R.D. and Beardmore, J.A. 1977. Protein variation in the Plaice, Pleuronectes platessa L. Genetical Research 30: 45-62.

Ward, R.D. & Skibinski,D.O.F. 1982. Interlocus allozyme mobility correlations and species divergence. Experientia 38: 654-655.

Watt, W.D.; Scott, C.D. and White, W.J. 1983. Evidence of acidification of some Nova Scotian rivers and its impact on Atlantic salmon Salmo salar. Can. J. Fish. Aquat. Sci. 40: 462-473.

Wright, S. 1978. Evolution and the genetics of populations, volume 4, Variability within and among natural populations. University of Chicago Press.

Zouros, E. 1973. Genetic differentiation associated with early stages of speciation in the mulleri subgroup of Drosophila Evolution 27: 601 - 621.

Zurwerra, A., Tomka, I. and Lanpel, G. 1984. Application of the scanning electron microscope and the enzyme gel electrophoresis to solve taxonomical problems: the European species of the genus Epeorus sensu Tshernova (1981) (Ephemeroptera: Heptageniidae). Proc. 4th Int. Conf. Ephemeroptera. (Eds. Landa, V., et al). 213 - 218.