ABSTRACT OF THESIS

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Title of Thesis "An Investigation of the Alcohol Dehydrogenase Polymorphism in a Laboratory Population of Drosophila melanogaster"

1. The opposing hypotheses of neutral mutation-random drift and neo-Darwinian evolution have been discussed in the context of the finding of ubiquitous protein polymorphism in many species. It has been proposed that the controversy may only be resolved by experimental studies of the forces acting upon contemporary representative polymorphisms.

2. The alcohol dehydrogenase polymorphism in the old-established Kaduna laboratory population of Drosophila melanogaster has been studied to ascertain whether natural selection acts at this locus and what form that selection may take.

3. In vitro assays have shown that the enzymatic activity of alcohol dehydrogenase from FF homozygotes is approximately twice that of SS homozygotes and that heterozygote activity is intermediate. That these functional differences may be of importance in the determination of the fitnesses of the genotypes in certain environments has been demonstrated by the superior survival of FF individuals in ethanol-enriched food media.

4. Although the equilibrium gene-frequency in the population cage remained constant throughout the period of study a consistent weekly oscillation in the genotypic frequencies was found in adult male flies. It is suggested that this finding is incompatible with the view that the Adh alleles are equivalent in the determination of fitness components.

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5. Gene-frequency perturbation experiments indicated that some form of balancing selection maintains the equilibrium gene-frequency in the population. Further, in egg-adult viability tests there was a trend towards the survival of the FF genotype being related to its initial frequency in a culture. This finding lends support to the sypothesis that frequency-dependent modes of selection may be important in the maintenance of the Adh polymorphism.

6. The response of the Adh polymorphism to natural changes in ecological conditions in the population cage and to experimentally manipulated environments was examined. The magnitude and form of differences in fitness between genotypes were found to be markedly dependent upon the environmental conditions under which observations were made, and upon the sex of the carrier of a genotype.

7. It was concluded that the alcohol dehydrogenase polymorphism in Drosophila melanogaster is influenced by the forces of natural selection, and that components of environmental heterogeneity play an important role in the maintenance of the polymorphism.

An Investigation of the Alcohol Dehydrogenase Polymorphism in a Laboratory Population of *Drosophila melanogaster*

by

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CHAPTER I

Introduction

1. The extent of heterozygosity in natural populations

The theory of evolution by gradual change supposes that genetic modification is brought about by the action of natural selection upon pre-existing genic variation in populations. Thus, a fundamental objective of experimental evolutionary and population genetics is the revelation and quantitation of gene-pool variability and the elucidation of the mechanisms which maintain such variation.

The efforts of geneticists during the past fifty years have revealed a great deal about the nature of karyotypic variation such as inversions in Drosophila (Dobzhansky, 1943), about the frequencies of rare visible and lethal mutants at many loci (Ives, 1945) and about striking visible and biochemical polymorphisms, such as the colour-banding polymorphisms in Cepaea (Cain and Sheppard, 1954) and the antigenic polymorphisms in Man (Race and Sanger, 1962). However, the majority of these studies dealt with only one, or a few, polymorphic systems in any one species and were concerned solely with the dynamics of these limited systems. They thus left unanswered the central question, are these variations restricted cases representing only a small proportion of the total number of genetic loci or are they representative of a significant fraction of the genome?

In the absence of techniques capable of yielding unambiguous answers two opposing schools of opinion developed. Consideration of the implications of the mathematical theory of genetic loads led Crow (1961) and, also, Muller (1950) to believe that species were genetically monolithic. In their view the maintenance of more than a few tens of

polymorphic systems by heterozygote advantage was incompatible with the calculated resultant genetic load on the population. The majority of genetic variation available for the action of natural selection was, therefore, considered in terms of extremely low frequencies of alternative alleles maintained by rare but recurrent mutation. The opposing view derived from experimental rather than theoretical considerations, notably from Dobzhansky and his coworkers, (Dobzhansky et al., 1955). These studies convinced Dobzhansky that "wild-type flies are an assemblage of many different genotypes... inwwhich no two individuals are alike and often show their unlikeness by different reactions to the environment". Thus, genetic variability was considered to be intimately involved in the short-term adaptive strategy of the species.

Wallace (1958 : 1963) attempted to resolve the dilemma by examining the effect on viability of radiation-induced heterozygosity in Drosophila lines isogenic for individual second chromosomes. In each of four replicates flies heterozygous for one irradiated and one non-irradiated chromosome were superior in fitness to individuals homozygous for the same non-irradiated chromosome. As the induction of heterozygosity increased viability in the majority of cases, Wallace proposed that more than half the loci in natural populations may be polymorphic. Although these results lent strong indirect support to the concept of widespread heterozygosity they could, alternatively, be interpreted as indicating that most mutations are selectively neutral or mildly deleterious, the observed increase in heterozygous fitness resulting from a small proportion of strongly heterotic loci.

The application of sensitive histochemical stains to the technique of enzyme electrophoresis afforded the first opportunity for investi-

gation of naturally occuring single-locus variation, without recourse to morphological mutants. Early studies (see Shaw, 1965 for review) revealed that in many species some soluble enzymes and proteins existed in two or more distinct forms or allozymes, each form being inherited as a simple, codominant, Mendelian allele. Exploiting the technique further Lewontin and Hubby (1966) investigated a group of enzymes and soluble proteins in five populations of Drosophila pseudoobscura, each protein being chosen solely on the basis of the availability of a reliable electrophoretic assay. Seven out of eighteen systems were polymorphic in more than one population while an additional two systems exhibited restricted local polymorphism. Their data indicated that 39% of the genome was polymorphic over the species; the figure being 30% for any one population. Approximate gene frequencies for each population suggested that an average individual of this species was heterozygous at 12% of its loci.

These values compare remarkably well with those obtained in contemporary studies on human blood enzymes (Harris, 1966). More recent examination of other Drosophilid species reveals that the proportion of polymorphic loci ranges from close to 0% in D. simulans (O'Brien and MacIntyre, 1969; Berger, 1970) to more than 70% in the D. willistoni group (Ayala, Powell and Tracey, 1972). Similar high levels of protein polymorphism are reported for the horseshoe crab Limulus (Selander et al., 1970), Cepaea (Manwell and Baker, 1968), Mus (Selander, Hunt and Yang, 1969) and many other animal species. Plants appear to be equally variable (Hamrick and Allard, 1972).

Since extrapolation of these estimates of heterozygosity to the genome as a whole has provoked considerable controversy, it is worthwhile

to reappraise the inherent biasses of the electrophoretic assay. Electrophoresis and subsequent histochemical staining will resolve only one subset of genes, those coding for soluble protein products, and reveal little about allelic variation at regulatory and polynucleotide-coding loci. Furthermore, only that fraction of soluble proteins and enzymes which are sufficiently robust to withstand extraction, separation and staining with semi-synthetic substrates can be detected. It could be argued that robust enzymes with broad substrate-specificity may be more tolerant of genetic variation than those highly specific enzymes involved in the central metabolism of the organism. Enzymes concerned with central energy metabolism have indeed been shown to be considerably less variable than peripheral enzymes, whose substrates are frequently derived directly from the environment (Gillespie and Kojima, 1968; Kojima, Gillespie and Tobari, 1970). Conversely, electrophoresis may underestimate variability since only 26% of all possible DNA base changes result in a change of nett charge (Lewontin, 1967a). Mutations which do not alter gross tertiary structure or nett charge of the protein are unlikely to be detected by electrophoresis.

Nonetheless, an ingenious historical analysis of the rate of discovery of human blood group antigens has provided a totally independent estimate of 30% polymorphism, indicating that the biasses of the electrophoretic technique are unlikely to be critical (Lewontin, 1967b).

While these results reveal the extent of natural heterozygosity there remains the problem of reconciling the observations with the classical theoretical structure of population genetics which initially

led Muller and Crow to believe that ubiquitous polymorphism was impossible. Again, two schools of opinion have developed. Kimura and his colleagues (Kimura and Ohta, 1971) and King and Jukes (1969) have proposed that the majority of allozymic variation merely represents evolutionary "noise", a series of two or more isoalleles occupying each segregating locus. Isoalleles are considered to be functionally equivalent in physiological, adaptive and evolutionary terms. This view is strongly opposed by Clarke (1970a) and Richmond (1970) who maintain that the observed frequencies of alleles reflect the stable equilibria of selectively balanced polymorphisms which confer adaptive advantage to the population or species.

2. The controversy of selective neutrality

Kimura and Crow (1964) have examined the limits to the amount of variation that may be sustained in a population of finite size N_e by calculating n_e, the effective number of selectively neutral alleles which may be maintained by a balance between the neutral mutation rate (U) and elimination through random genetic drift. Assuming that each mutation gives rise to a unique and novel allele, n_e is found to be the reciprocal of the inbreeding coefficient (F, the probability that two alleles uniting in a zygote are identical by descent). At equilibrium they showed that

$$F = \frac{1}{4N_eU + 1} \qquad \text{and} \qquad n_e = 4N_eU + 1$$

The mean probability of heterozygosity is therefore

$$\bar{H} = (1-F) = \frac{4N_e U}{4N_e U + 1}$$

This model yields an upper limit to the amount of variation that can be attributed to mutation-drift of neutral alleles. However, if the number of allelic states is restricted, or if the frequencies of all the allelic types are not equal, homozygosity will be greater, and n_e much smaller, than the above value. In order to maintain a probability of heterozygosity greater than 0.12 (the value found by Lewontin and Hubby, 1966) N_e must be greater than 10^4 unless the neutral mutation rate is greater than 10^{-6}. This places a strain on the ability of the model to explain the high levels of heterozygosity frequently observed in relatively small populations. However, Maruyama (1970), analysing two-dimensional stepping-stone models of migration between adjacent populations of finite size, has shown that local genetic differentiation is only possible where $N_e m$ is less than unity (where m is the rate per generation at which each colony exchanges individuals with neighbouring colonies). Thus, even extremely small migration rates can render effectively panmictic populations which are apparently discrete. Effective population size, N_e, may therefore be far larger than presumed, allowing high levels of heterozygosity on the Kimura-Crow model.

Kimura has extended his argument by an analysis of the actual rate of amino acid substitution during evolution. Published data of the amino acid sequences of three proteins in several mammalian species (Dayhoff, 1968) furnished details of the amino acid difference between

the sequences. Using this data and the approximate timings of the age of phylogenetic divergence, an average rate of one amino acid substitution per 100-residue polypeptide per 2.8×10^7 years was derived (Kimura, 1968). Combining this rate with Muller's estimate (1958) of 4×10^9 base pairs in haploid human DNA, and adjusting for synonymous mutations, Kimura calculated that the average time taken for one base pair replacement in the mammalian genome was two years. This figure contrasts sharply with Haldane's (1957) estimate of one new allele fixed per 300 generations. The genetic load created by such a high rate would be intolerable unless the majority of substitutions involved effectively neutral alleles ($S \ll \frac{1}{N_e}$) replacing pre-existing alleles by a process of genetic drift.

The argument is susceptible to two major criticisms. The calculation assumes that total genomic DNA codes for protein, an assumption which is now clearly invalid (Britten and Kohn, 1968). If only 10% of the DNA codes for protein the observed rate of substitution is more compatible with Haldane's argument. Furthermore, the calculation of the cost of substitution is based on the multiplicative aspect of fitness in which it is assumed that selection acts independently at each locus and in an additive fashion over loci. This assumption has been severely challenged by a number of authors (eg. Sved, Reed and Bodmer, 1967: see Section 4).

Using estimates derived from palaeontology of the time elapsed since the divergence from common ancestors of several vertebrate groups, Kimura (1969) has compared the rates of amino acid substitution in different evolutionary lines. Zucherkandl and Pauling's (1965) finding, that the rate per year for haemoglobin is remarkably constant

in lines as diverse as those leading to carp and man, has been confirmed. It can be shown (Kimura, 1968) that the rate of substitution of selectively neutral mutants K (the average number of substitutions per unit time) is equivalent to U, the neutral mutation rate per gamete per unit time. Conversely, if selection is responsible for substitution $K = 4N_eSU$ where S and U are, respectively, the selective advantage and mutation rate of advantageous mutants (Kimura and Ohta, 1971). Kimura argues that the probability of the product of N_e, S and U remaining constant over diverse evolutionary lines is extremely small and, therefore, concludes that the constancy of rate of protein evolution is simply a reflection of very similar neutral mutation rates in different lines.

The argument loses much of its strength when other observations are considered. The constancy of rate is far less striking when proteins other than haemoglobin are examined (Crow, 1972) and, furthermore, rates differ markedly between protein species (Ohta and Kimura, 1971a). Further, Clarke (1970a) has argued that the selective advantage of a mutant is determined to a large extent by the genetic environment in which it occurs. This internal "coadaptation" of the genotype will therefore constitute an inertial force sufficient to damp the effects of short term environmental fluctuations and will give rise to a fairly constant product of N_e, S and U.

It is noteworthy that neither the neutral nor the selectionist hypotheses offer an adequate explanation for the rate of substitution being related to the calendar rather than to the biological interval, the generation.

A further array of arguments in support of the neutral mutation-random drift hypotheses has been presented (King and Jukes, 1969). These authors take as a tenet that "natural selection is the editor rather than the composer of the genetic message, one thing the editor does not do is to remove changes it is unable to perceive". They then examine the extent to which current data supports the hypothesis that natural selection fails to detect the majority of mutations which eventually reach fixation.

Due to the degeneracy of the genetic code 25% of single base-change mutations will result in synonymous codons. These represent a potentially neutral class of mutations since they are not expressed in the gene product and are therefore presumed to be protected from the action of natural selection. Thus, the observations of Walker (1968) that species divergence at the DNA level is far greater than at the protein level are interpreted in terms of random synonymous base replacement in the third position of codons. However, even synonymous mutations may be subjected to selective constraints imposed by the availability of nucleotides, specific transfer RNA etc. (Clarke, 1970a). Indeed, in mammals arginine is regularly coded by only two of six possible codons (Subak-Sharpe, Shepherd and Hay, 1966).

A second line of reasoning follows from the finding that the distribution of the number of evolutionary amino acid substitutions per site in a polypeptide chain agreed well with that predicted by the Poisson distribution for a representative sample of proteins. The conclusion that this fit reflected the essential randomness of the substitution process has been challenged. Clarke (1970a) and Uzzel and Corbin (1971) argue that the underlying criteria of the

Poisson distribution (homogeneous probabilities, lack of contagion and independence) are not met by the data. For example, the first criterion cannot be met as of the 190 possible amino acid pairs only 75 are interchangeable by a single-base mutation; 14 require that all three bases are changed to bring about the substitution.

In an elegant extension of his analysis Clarke (1970b) examined the relationship between the observed frequencies of particular one-step amino-acid pair interchanges and the degree of biochemical dissimilarity between the participating pair. The negative correlation between frequency of interchange and Sneaths D value (a purely chemical measure of the dissimilarity between amino acids) was highly significant. This relationship is most likely to be caused by selection acting against mutations causing gross changes in protein structure. However, even this analysis is equivocal as it has been argued (Jukes and King, 1971) that the greater the degree of similarity between two amino acids, the greater is the probability that an interchange between them will be functionally, and therefore selectively, neutral.

The neutral mutation-random drift hypotheses clearly predicts that the average amino acid composition of proteins should to a large extent reflect passively the relative frequencies of the four nucleotides in the DNA, translated through the genetic code. Indeed, when the average frequencies of amino acids in a large number of mammalian proteins were compared with the frequencies expected from random nucleotide permutations a remarkably good agreement was found (King and Jukes, 1969). However, while this is true for averaged proteins, individual proteins differ significantly in their amino acid

content. For example, fibroins are extremely rich in glycine and alanine, collagens in proline and hydroxyproline (Seifter and Gallop, 1966). The argument of King and Jukes may therefore be reversed to say that the genetic code itself has evolved, under the pressure of natural selection, to provide larger numbers of codons for the more commonly required amino acids.

The application of the preceding neutral mutation - random drift hypotheses to the problem of extensive protein polymorphism has been summarised by Kimura and Ohta (1971). Since $K = U$, a new neutral mutation destined to become fixed in the population arises every $\frac{1}{U}$ generations. The mean time between origin and fixation is approximately $4N_e$ generations (Kimura and Ohta, 1969). Thus, if $4N_e$ is large relative to $\frac{1}{U}$ considerable transient polymorphism will be evident if a population is examined at any one point in time. As the speed of substitution of any particular allele is extremely slow, the polymorphism will appear to be stable.

A general problem of the neutral mutation-random drift hypotheses of protein polymorphism is that its predictions are formulated in quantities which are not readily measurable. To test the hypothesis against observed data population size, mutation and migration rates, and the geographical breeding structure must be known. However, Maruyama (Maruyama, 1972; Yamazaki and Maruyama, 1972) has recently derived a relationship between the proportion of heterozygotes, summed over populations, and the global gene-frequency of the allele. This relationship is independent of the population parameters above and expected forms of the relationship can be calculated under the rival hypotheses of selection or neutrality. Preliminary comparisons

between the relationship observed in studies of electrophoretic polymorphism and those predicted from theory suggest that the observed data is compatible with the neutralist hypothesis, although by no means conclusively. Further refinement of this approach should offer a powerful method to test the fit of observed data with the rival hypotheses.

3. Experimental approaches to the controversy

It is clear that a considerable number of theoretical arguments have been interpreted as evidence in support of the hypothesis of neutrality, but that each point has been countered by those workers who maintain a neo-Darwinian concept of evolution. The crucial test of these hypotheses may be found in the elucidation of the stochastic and deterministic processes acting upon presently existing protein polymorphisms. Although data is still sparse, a number of situations have been examined in sufficient depth to allow preliminary conclusions to be drawn. Several questions may be posed. Firstly, do the alternative gene products of segregating alleles display functional differences of sufficient magnitude to allow potential discrimination by natural selection, "the editor"? Secondly, are the temporal and spatial distributions of gene frequencies in natural populations more compatible with the neutral or the neo-Darwinist hypotheses? Finally, what direct evidence is available of directional or balancing selection acting upon specific, representative polymorphisms.

Two points should be clarified at the outset. The neutralist and selectionist hypotheses are not mutually exclusive, the question is rather one of determining the relative contributions of the two processes.

Moreover, any observational or experimental test of the hypotheses must, by its nature, be one-sided. While it may be possible to demonstrate unequivocally the action of selection at a particular locus, it is never possible to prove that selection does not act.

(i) Functional differences between allelic products

Of the many possible random mutations which may alter the electrophoretic mobility of a protein only a proportion may be expected to occur at those sites determining the functional properties of the enzyme. Under the neutral mutation-random drift hypotheses alleles not belonging to this class would have the highest probability of participating in polymorphism. Conversely, a neo-Darwinist interpretation would predict that the majority of observed polymorphisms involve functionally differing alleles.

Harris (1971a; 1971b) has shown that 16 out of 23 human enzyme polymorphisms do exhibit significant activity differences between allelic products. For example, individuals homozygous BB at the red cell acid phosphatase locus exhibit roughly 50% more enzyme activity than AA individuals. Although cytochromes c exhibit no difference in reaction rate, even between species which differ at 26% of the polypeptide sites, recent studies indicate significant differences in ion-binding capacity between species. Similarly, some studies of domesticated animals reveal associations between physiological or production traits and genotype at specific protein loci. For example, the sheep haemoglobin HbA variant renders carriers more resistant to hypoxia and possibly better adapted to upland pastures than does the HbB variant (Tucker, 1971). Thus, there is indeed evidence of functional differences between allelic products in

some protein polymorphisms.

Until the precise relationship between enzyme function and fitness is established for a number of cases, the evolutionary implications of this data must remain conjectural. A clear association between enzyme genotype, physiological function and fitness has, however, been demonstrated for the case of transferrin polymorphism in pigeons (Frelinger, 1972). Maternally produced transferrin acts as a bacteriostat in many avian eggs (Schade and Caroline, 1944). Frelinger found that both whole egg-white and purified transferrin from heterozygous Tf^{A}/Tf^{B} females inhibited microbial growth in culture to a far larger extent than did transferrin from either homozygote. The hatchability of eggs from heterozygous females was significantly higher than those from homozygotes, presumably because embryonic mortality due to pathogens was reduced. This case of polymorphic maintenance through higher fitness, (fecundity), of heterozygotes is also interesting in that it results in no deviation from Hardy-Weinberg expectations in the surviving progeny (Frelinger and Crow, 1973) and hence imposes no formal genetic load.

(ii) Spatial and temporal distribution of gene frequencies

The hypothesis of isoallelic neutrality predicts a random distribution of allele frequencies across the species range, with fixation of different alleles in different localities through random genetic drift. Even in the initial study of Lewontin and Hubby (1966) such a distribution was conspicuously absent. Subsequent reports have amply substantiated the fact that many polymorphisms exhibit remarkable identity of allele frequency across populations (Prakash, Lewontin and Hubby,1969; Lakovaara and Saura, 1971;

Richmond, 1972). For example, the variation of PGM^{A-1} allele frequency between populations of Aedes aegypti from Tanzania and the Far East is only marginally greater than the variation between neighbouring collections within Tanzania (Bullini and Colluzzi, 1972). Even those alleles existing at low frequencies, which should render them highly susceptible to random fixation, are found consistently throughout the species range (Prakash et al., 1969).

However, it may be argued from Maruyama's (1970) calculations that there is sufficient migration between adjacent populations to maintain a roughly uniform gene frequency over the majority of populations. Only in isolated communities would random drift and fixation of alleles be discernible. The Bogata population of Drosophila pseudoobscura is indeed depauperate in allozyme variability, as would be predicted for a marginal population removed from the inflow of genes from the main distribution of the species in North America (Prakash et al., 1969). Nevertheless, selective neutrality and migration cannot account for populations of D. willistoni having very similar allele frequencies at some loci but very different frequencies at others (Ayala, Powell and Dobzhansky, 1971).

A second geographical distribution which has been cited as evidence of the action of natural selection is the gene-frequency cline. This is especially important where gene frequencies can be correlated with some independent measure of environment. A convincing demonstration of such a cline is provided by populations of the freshwater fish Catastomes clarkii, in which the frequency of an esterase allele is correlated with latitude and temperature. Koehn (1970) has demonstrated that the enzyme activities of two

alternative homozygous esterase phenotypes are adapted to the ambient water temperatures at the ends of the cline at which they are most frequent. The heterozygote has maximal enzyme activity at intermediate temperatures. Several other studies using different organisms have found near-significant regressions of allele frequencies on latitude or longitude (Johnson et al., 1969; Prakash et al., 1969; Richmond, 1972).

Relatively little information is yet available on temporal distributions of allelic frequencies. However, surveys of Microtus agrestis populations have revealed regular cycles of Esterase-1 allele frequency which are related to season and population density (Semeonoff and Robertson, 1968). Esterase-1 negative genotypes were found to be favoured during periods of high population density but were at an apparent disadvantage to positive genotypes during the winter. Similar cyclical fluctuations of allele frequency related to season have been found in populations of D. pseudoobscura and D. persimilis (Dobzhansky and Ayala, 1973). The magnitude and timing of these cycles were consistent over two continuous years of study.

Kojima et al. (1972) have conducted a more extensive survey of temporal and spatial allele frequency distributions in populations of D. pavani utilising the analytical methods of Smouse and Kojima (1972). Each of the eight segregating loci studied displayed significant variation in allele frequencies between populations. A significant portion of this variation was assignable to correlations with three indices of environmental variability, namely season, latitude and altitude. Nonetheless, even this sophisticated analysis was unable to unequivocally distinguish whether stochastic or

deterministic processes were responsible. Under the neutral mutation-random drift hypothesis the significant regressions of frequency on environmental variables may be attributed to the fact that neighbouring populations will share a large portion of a common gene pool through migration. They will, incidentally, share many aspects of environment through their close proximity. Thus, the regressions may be spurious in cause-effect terms.

Thus, while the majority of studies of gene-frequency distributions lend some support to the selectionist viewpoint, no clearcut general conclusions can yet be drawn.

(iii) Direct evidence of the action of natural selection

Evidence for the action of natural selection has been obtained from studies of the distribution of isozyme alleles between third chromosome inversions of Drosophila pseudoobscura (Prakash and Lewontin, 1968; 1971). It was found that there were high correlations between inversion type and allelic state at the Amy and Pt-10 loci. Thus, the Santa Cruz phylad of inversions was generally characterized by alleles 1.06 and 0.84 and the Standard phylad by alleles 1.04 and 1.00 at the Pt-10 and Amy loci respectively. This relationship was true regardless of the geographical origin of the chromosomes. All D. persimilis inversion types are derived from an ancestral Standard arrangement, which antedates the speciation of D. persimilis and D. pseudoobscura, and carry predominantly the 1.04 and 1.00 alleles. Thus, the correlation between allelic state and inversion type must be extremely stable as it extends across the present species range and transcends the species boundary. Moreover, the relationship is not explicable as a historical relic of sampling

since alternate alleles are present at low frequencies in the "wrong" chromosome arrangement. It must, therefore, be concluded that natural selection is maintaining the alleles at optimal frequencies for the particular array of genes of which they are a part.

The action of balancing selection through heterozygote advantage has been inferred from the significant excess of heterozygotes detected at the To locus in wild-caught Drosophila paulistorum (Richmond and Powell, 1970). A similar natural excess of Mdh heterozygotes, associated with increased heterozygote fecundity, has been observed to accumulate in autumn populations of the ameiotically parthenogenetic Daphnia magna (Hebert, Ward and Gibson, 1972). However, in neither case can heterozygote excess be unequivocally attributed to heterosis at the locus in question. Heterotic selection may act upon a neighbouring locus, supergene or inversion to which the allozyme alleles are linked in disequilibrium.

Powell (1971) investigated experimentally the influence of environmental variability on the ability of populations to retain heterozygosity. A population of D. willistoni was replicated into 13 cages in some of which the environments were constant, while in others they were varied in either one or three factors (yeast-type, temperature, food). After 15 culture generations the average extent of genetic heterogeneity exhibited by the populations at 22 loci was found to be positively correlated with the number of variable factors in their respective environments. Thus, at least a proportion of the loci examined retained polymorphism by a balancing force acting through environmental heterogeneity, although inversions causing non-independence between loci may have contributed to the observation.

A similar approach, namely environmental manipulation rather than experimental perturbation of gene frequency, was adopted by de Jong *et al.* (1972). Striking changes in amylase phenotype frequencies were recorded when each of four *D. melanogaster* populations, previously cultured on a high-sucrose medium were replicated onto a starch-rich medium. In all cases the allele known to have the highest enzymatic activity on starch (Doane, 1969) increased in frequency. Neither of the preceding findings is easily explained in terms of allelic neutrality.

The classical population genetics technique of gene-frequency perturbation has recently been employed to detect possible balancing selection at polymorphic protein loci. However, this technique suffers from a major handicap of methodology through "associative overdominance" (Ohta and Kimura, 1970 : 1971b). When small numbers of chromosomes are extracted from populations and multiplied to found populations with divergent initial gene frequencies, sampling effects will cause some linkage disequilibrium between truly overdominant loci and neighbouring, potentially neutral loci. Thus, when perturbed Est-6 gene frequencies in *D. melanogaster* were observed to return towards an equilibrium value, it was concluded that linked genes were responsible for the shift and that the Est-6 alleles themselves were selectively neutral (MacIntyre and Wright, 1966). In a more comprehensive study, which involved far larger founder chromosome numbers, no return towards gene-frequency equilibrium values was observed at the Est-5 locus in *D. pseudoobscura* (Yamazaki, 1971). Subsidiary tests similarly failed to detect differences of fitness between genotypes. Clearly the above results

are consistent with the hypothesis of selective neutrality, although it may equally be argued that the selective values were too small to be detectable.

Kojima and his associates have performed a series of gene frequency perturbation experiments utilising the Est-6 and Adh polymorphisms of D. melanogaster (Kojima and Yarbrough, 1967). In all cases a return towards equilibrium gene frequency values was clearly demonstrated and individual components of fitness were found to be subject to frequency-dependent regulation. (These results are discussed in detail in Chapter 4.)

Wills and Nichols (1972) have claimed evidence for single gene heterosis at the octanol dehydrogenase (ODH) locus of D. pseudoobscura. For twelve generations inbreeding by brother-sister mating was performed and only those matings in which both parents were heterozygous were retained at each generation. In this way the genetic background was made more homozygous while the ODH polymorphism was retained. At the twelfth generation the expected 1:2:1 ratio of genotypes was observed in progeny reared in normal medium and in medium to which potassium chloride had been added as a stress factor. However, medium stressed with octanol produced a significant excess of heterozygous progeny. It was concluded that in outbred populations this heterosis was masked by the effect of the heterogeneous genetic background. Furthermore, the heterosis observed after inbreeding was considered to be due solely to the locus in question as octanol, the potential substrate of ODH, was required to elicit the response. This experiment has been criticised (Yamazaki, 1972) on the basis that the inbreeding program reduced heterozygosity at loci linked to

ODH to a smaller extent than that at unlinked loci. Thus, at the time of the viability test ODH homozygotes were considerably more inbred than were heterozygotes. Since octanol is a poison to Drosophila, the apparent heterosis may simply have been a result of the ability of less-inbred individuals to overcome stress.

Studies on the persistence of mutagen-induced acid phosphatase null variants in populations of D. melanogaster have raised a further problem of methodology (Ogah and MacIntyre, 1972). It was suggested that laboratory environments may be so beneficial to the welfare of Drosophila species that some peripheral enzyme systems become redundant. Selectively maintained polymorphisms of these enzymes may therefore become selectively neutral by such a change of habitat. Thus, for example, the apparently neutral Est-5 polymorphism studied by Yamazaki (1971) may be critical in dealing with particular substrates encountered in the wild, but redundant in the laboratory where these substrates are absent.

This argument may be generalised for all types of environment. Enzyme polymorphisms presently observed may represent the relic equilibria of previous selection pressures. Thus, an environment which previously favoured the establishment and maintenance by heterosis of a polymorphism may have altered such that all genotypes are currently equally fit. In this way the polymorphism though currently selectively neutral was established by deterministic rather than stochastic processes.

4. Mechanisms of natural selection

It may be concluded from the evidence cited above that the neutral

mutation-random drift hypotheses is not a sufficient explanation of ubiquitous polymorphism. Attention must, therefore, be focussed upon the problem of determining the nature of the selective mechanisms maintaining at least a portion of this heterozygosity.

Classically heterozygote advantage has been discussed as if it were the only major mechanism whereby stable equilibria may be maintained. However, the total amount of selective death required to maintain heterosis at many loci may be beyond the reproductive potential of any species. Lewontin and Hubby (1966) illustrate this dilemma by considering a population segregating at 2000 loci with symmetrical selection of 10% against both homozygotes at each locus. In this model the fitness of the population is reduced by 5% due to each locus alone, the overall fitness being reduced to $(0.95)^{2000} = 10^{-46}$, an intolerable level. In order to reduce the segregational load to biologically realistic dimensions, selection coefficients must be so small as to render the alleles effectively neutral.

This illustration assumes, however, that selection acts independently at each locus and that individual fitnesses are the product of these independent effects. The concept of multiplicative action of selection has been vigorously challenged (King, 1967; Milkman, 1967; Sved, Reed and Bodmer, 1967) on the grounds that the individual, not the locus, is the unit object of the selective process. Accordingly, individual fitness may be only a function of the multiplicative probability of survival, the overall probability depending, also, on the fitnesses of other genotypes competing for survival in the population. Thus, in Lewontin's example the fitness of each individual is compared with that of the theoretical total heterozygote,

while in competition models the mean fitness of the population is the yardstick. Not only will individuals with maximal heterozygosity be extremely rare, but also there is likely to be a physiological limit to the amount by which the fitness of these individuals may exceed the mean fitness (Sved et al., 1967).

Sved and his colleagues calculated the variance of selective values in a population with N segregating loci maintained by symmetrical heterosis. The variance is surprisingly low, suggesting that individuals with extreme selective values are so rare as to be unimportant in the composition of the population. For example, where $N = 10^4$ and selection coefficients = 0.01 the mean fitness of the population on the multiplicative scale is $(0.995)^{10,000}$, while the fitness of an individual with the mean number of heterozygous loci, (5000), is $(0.99)^{5000}$; both absurdly low values. However, the fitness of the latter individual relative to the mean fitness of the population in which it competes is $(0.99)^{5000}/(0.995)^{10,000} = 0.881$. Similarly, an individual heterozygous at 5242 loci will have a relative fitness of approximately 10. It can be shown from the binomial distribution that fewer than 1 in 10^6 individuals will exceed this number of heterozygous loci. Thus, an arbitrary upper limit may be imposed on the fitness scale. The advantage of the heterozygote at each locus is necessarily reduced by the imposition of this limit, but only to a negligible extent.

In a similar model Milkman (1967) postulated that selection acts by culling those individuals with the lowest number of heterozygous loci. In this case the selection differential (average heterozygosity of survivors minus the mean heterozygosity of the unselected population)

is equal to the average selection pressure at each locus. For example, where selection against homozygotes at each locus equals 0.1, 256 polymorphisms may be maintained when the least heterozygous 86.5% of individuals is culled. This level of selective death is acceptable for many species.

Competitive models may be related to Wallace's (1968) distinction between "hard" and "soft" selection. Hard selective death results from direct interaction between a phenotype and its inanimate environment, while soft selection is considered to act through competitive interactions between individuals. As Milkman points out, even totally homozygous, inbred individuals may be fully viable when cultured apart from their competitively superior heterozygotes. It may be concluded, then, that heterosis is capable of maintaining large numbers of electrophoretic polymorphisms without invoking excessive segregational load.

Some potentiality for single locus heterosis may be deduced from the fact that heterozygote individuals possess both parental (and, in many cases, additional heteropolymer) forms of an enzyme. Variability in the same functional protein may enhance physiological flexibility and hence favour survival of the carrier in a fluctuating environment. Efron (1973) has refined this concept by suggesting that the molecular environment of an enzyme may differ between tissues or developmental stages of an organism. Selection might then favour heterozygotes possessing enzymes which are non-rate-limiting in all tissues at all stages.

The problem of segregational load which stimulated the construction of the models of Sved *et al* (1967), Milkman (1967) and King (1967),

also reawakened interest in an alternative mode of selection, namely frequency-dependent selection. In this mode the selection pressure acting upon a genotype is considered to be a function of the frequency of the genotype in a population, genotypes being favoured when rarer than their equilibrium value and at a disadvantage when in excess. In certain frequency-dependent selection models the fitnesses of all genotypes are equal at gene frequency equilibrium, thereby eliminating segregational load.

Fisher (1930) demonstrated that non-trivial equilibrium gene frequency is related to selective value by $\hat{q} = \frac{b-a}{2b-a-c}$ (where a, b, c represent the selective values of the three genotypes at a locus). The stability of the equilibrium is determined by the sign of the derivative $\frac{d}{dq}[b-a-q(2b-a-c)]$. This relationship has been generalised (Lewontin, 1958) by considering the values a, b, c as weights encompassing both frequency dependent and independent functions rather than as constant properties of the genotypes. In this case it can easily be demonstrated that heterozygote advantage is not a necessary condition for the maintenance of stable equilibria. Indeed, the introduction of frequency-dependent elements into the weights a, b, c may provide stable equilibria even where the heterozygote is at a selective disadvantage.

Clarke and O'Donald (1964) examined models in which the frequency dependent component was determined by a constant relationship between frequency and selective value for all three genotypes ($a = 1-tp^2$, $b = 1-2tpq$ etc.). The action of frequency-dependent selection alone was found to be capable of maintaining stable equilibrium, even when the heterozygote was at a disadvantage.

Inclusion of dominance in the model removed this disadvantage and specified different equilibrium gene frequencies. The interaction of frequency dependent and independent functions in this model can result in three non-trivial equilibria, two of which are stable as determined by the method given above.

Pursuing a different approach Cockerham *et al.*(1972) investigated a general model which specifies the fitness of each genotype when associated with each other type. In a population any one individual of a given genotype is associated with other genotypes in proportion to their Hardy-Weinberg frequencies. Thus, for example, the mean fitness of genotype 2 is given by $\bar{W}_2 = p^2W_{22} + 2pqW_{21} + q^2W_{20}$ (where W_{22}, W_{21}, W_{20} are the fitnesses of genotype 2 when in association with genotypes 2, 1, 0 respectively). The probability of survival of an individual is, therefore, related to population composition and is frequency-dependent. Stable equilibria are again possible under a wide variety of conditions.

Although frequency-dependent selection may maintain stable equilibria with negligible genetic load in an infinite population, stochastic processes, such as the sampling variance of gametes, will induce minor perturbations about the gene-frequency equilibrium in finite populations. The load of genetic deaths necessary to counteract these fluctuations has been explored (Kojima, 1971b) under both heterotic and frequency-dependent modes of selection. In most sizes of population heterosis produced 10 to 100 times more segregational load than did frequency-dependent selection. Thus, frequency-dependent selection avoids many of the theoretical problems associated with maintenance of several alleles at each of many loci.

5. Objectives of the present study

The discovery of ubiquitous polymorphism has raised questions fundamental to the understanding of the evolution and genetic adaptation of natural populations. Despite the considerable debate that has taken place concerning the nature of the mechanisms maintaining this heterozygosity, it is clear that the controversy cannot be resolved until a far larger array of protein polymorphisms has been investigated experimentally in a representative cross-section of species. The present study examines in some depth the alcohol dehydrogenase (Adh) polymorphism in a laboratory population of Drosophila melanogaster.

The Kaduna stock of D. melanogaster was chosen for study as it has been maintained for many years as a large, closed laboratory population. It was therefore considered improbable that any selection observed at the Adh locus would be attributable to random linkage effects arising from recent immigration or bottlenecks in effective population size. In this population the two alleles at the Adh locus were segregating at intermediate frequencies suitable for experimental study. This locus had the further advantage that it specifies the only enzyme which will utilise short-chain alcohols as a substrate. This facilitated the study of functional properties of the three Adh genotypes.

The research program investigated several parameters of the Adh polymorphism.

(i) The activity of the enzymes produced by the three genotypes was assayed to determine whether there were functional differences between them which might be exploited by natural

selection.

(ii) Population and egg-adult viability experiments were conducted to determine whether the Adh polymorphism was stable and whether balancing selection maintained the equilibrium gene frequency. The viability test was designed specifically to test whether selective forces acted in a frequency-dependent manner.

(iii) Experiments were conducted to examine whether fitnesses of the three genotypes were dependent upon environmental variables in the population cage and whether these fitnesses responded to experimental manipulation of the culture conditions.

CHAPTER II

Materials and Methods

(A) Drosophila techniques

(i) The Kaduna Population

The Kaduna stock of *Drosophila melanogaster* is derived from a collection made at Kaduna, Nigeria, and had, at the commencement of the present study, been maintained in the Institute of Animal Genetics, Edinburgh, for over 20 years. Although the initial sample size is unknown, the stock has always been maintained as a large, random-mating population (N_e approximately 10^4) in a laboratory cage at 25°C. It may, therefore, be considered to be adapted to its current environment and to have reached a state of genic and linkage stability.

It is pertinent to later discussions to outline briefly the method of culture of the population. A fresh pot containing roughly 250ml of food medium is introduced into the cage every week and removed three weeks later. Thus, at any one time the cage contains three pots, aged 0-1, 1-2 and 2-3 weeks.

The standard food medium is prepared by boiling maize meal (150gm), molasses (130gm), agar (20gm), flaked brewers yeast (22gm) and fungicides, Nipagin (1gm) and propionic acid (5gm), in 2 litres of water.

(ii) Homozygous lines

Eggs obtained from Drosophila of the Kaduna cage were allowed to develop in bottles under uncrowded conditions. Virgin adults collected from these bottles were randomly pair-mated in 3" x 1" glass vials containing standard food medium. After 6 days of mating and egg laying the parents were removed from the vial and analysed electro-

phoretically to determine their Adh genotype. Progeny from parental flies shown to be homozygous for similar Adh alleles i.e. from FF x FF or SS x SS pairs, were retained as homozygous lines. Thus, each homozygote line has 4 totally independent founder chromosomes.

Fresh homozygous lines were extracted from the population for each experiment, and were used as soon as possible after extraction.

(iii) Egg collection

Where eggs of known genotype were required, male and female Drosophila of known genotype were mated, and the fertilized females yielding eggs of the required genotype were placed in an oviposition chamber. This consisted of a 5cm diameter disposable Petri dish containing food medium smeared with live yeast and fitted snuggly into the mouth of an inverted plastic 100ml beaker. Large numbers of eggs could be obtained in this way. The eggs were easily removed from the surface of the medium by means of a flat-ended needle.

Egg hatchability was estimated directly by counting the number of eggs in a given area of the medium in the Petri dish, and recounting the unhatched eggs in the same area after 48hr at 25°C.

(B) Electrophoresis

Four different supporting media, polyacrylamide, cellulose acetate membrane, agar and starch gel, were tested for use in zone electrophoresis of Drosophila ADH. Horizontal starch gel electrophoresis was found to be eminently suitable and preferable to the other media where accurate determination of the phenotype of large numbers of individual flies was required.

The Poulik system of buffers (Poulik, 1957) was adopted for the majority of the study, but, in the interests of efficiency and economy an EDTA-Boric acid-Tris continuous buffer system was developed for use in later studies.

(i) Electrophoresis using the Poulik system of Buffers

A 10% mass/vol suspension of hydrolysed starch (Connaught, Toronto) in Tris-citric acid buffer (0.075M pH 9.2) was heated to 90°C in a conical flask with mechanical stirring. The flask was then connected to a vacuum pump and the contents allowed to boil under reduced pressure for 30 seconds to remove dissolved gases. The liquid gel was then poured into a gel mould consisting of a perspex former of internal dimensions 18.5 x 10.1 x 0.6cm resting on a clean glass plate. The gel was sealed into the mould by laying a second plate over the top of the former, and was allowed to set for at least 3hr. Before insertion of samples the gel was cooled for an hour and the upper glass plate removed.

Individual Drosophilae were homogenised with a glass rod in a well slide containing 0.02ml distilled water. .5 x .5cm squares of cellulose acetate membrane were impregnated with the homogenate and inserted into a vertical slit in the prepared gel. Twenty-four inserts could be placed down the long axis of the gel in a row positioned 3.0cm from the long edge.

The gel was connected to the electrode vessels by means of filter-paper wicks (8 thicknesses of Whatman 3MM, W. and R. Balston, Ltd) soaked in electrode buffer, (sodium hydroxide - boric acid, 0.3M pH 9.2). Electrophoresis was performed for 1hr with a constant potential gradient of 12.5 volts/cm and 75mA. The gel was cooled either by

maintaining the apparatus at 4°C throughout the run, or by placing sealed polythene bags containing ice over the surface of the gel for the duration of the electrophoresis.

(ii) Electrophoresis using the EBT Buffer system

The EDTA Boric acid-Tris (EBT) continuous buffer system had the economic advantage that two parallel rows of inserts (i.e. 48 individual samples) could be placed 2.0 and 5.0cm across the breadth of the gel. The method of preparation and running of the gel was essentially similar to that previously described, but the electrode buffer was 0.5M EDTA-Boric acid-Tris (15gm EDTA; 100gm Boric acid; 151.5gm Tris (Sigma 7-9) in 2.5 litres distilled water) and the gel was prepared using a one-tenth dilution of this buffer. Electrophoresis was carried out for 1hr at 20 volts/cm, 100mA, and cooling was always achieved by placing an ice-bag over the gel.

(iii) Staining the gel for ADH activity

After electrophoresis the cellulose acetate sample-inserts were removed and the gel was sliced in two horizontally. The cut surface of one slice was developed for ADH activity by application of a staining mixture containing 5ml iso-propanol, 10mgm β-DPN (β-Diphosphopyridine nucleotide, Sigma Chemical Co.), 10mgm NBT (Nitro BT, Koch-Light Labs. Ltd.) and 3mgm PMS(Phenaxine methosulphate, Sigma Chemical Co.) in 100ml of Tris-HCl buffer (0.1M, pH 8.5). After 30 minutes at 25°C the developed zymograms were scored and were sometimes photographed for future reference.

CHAPTER III

The Alcohol Dehydrogenase Polymorphism in D.melanogaster

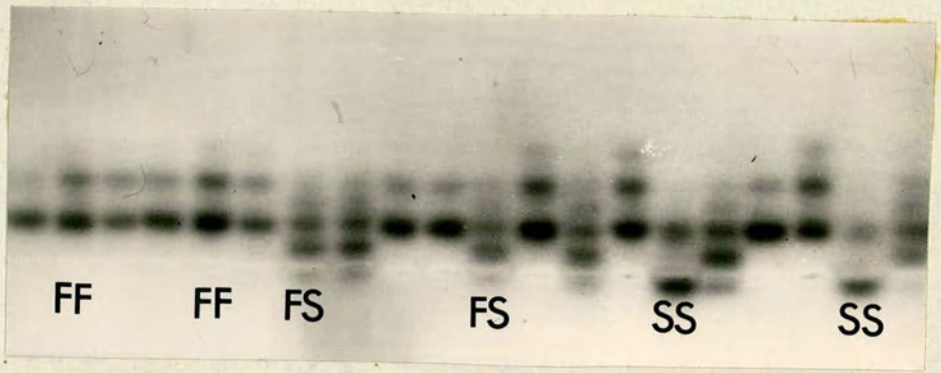
The "nothing dehydrogenases" which reduced tetrazolium to formazan in the apparent absence of substrate were identified as alcohol dehydrogenase (ADH: E.C. 1.1.1.1.) by Shaw and Koen (1965). Electrophoretic variation of the enzyme in D.melanogaster was first demonstrated between inbred lines, some of which displayed a rapid, anodally-migrating form (ADH Fast) while others displayed a slower-migrating form (ADH Slow) (Johnson and Denniston, 1964). These alternatives were soon shown to be genetically determined by two alleles (Adh^F and Adh^S respectively) at a locus positioned at 50.1 on chromosome II. (Grell et al, 1965). In this thesis these alleles are referred to simply as F and S.

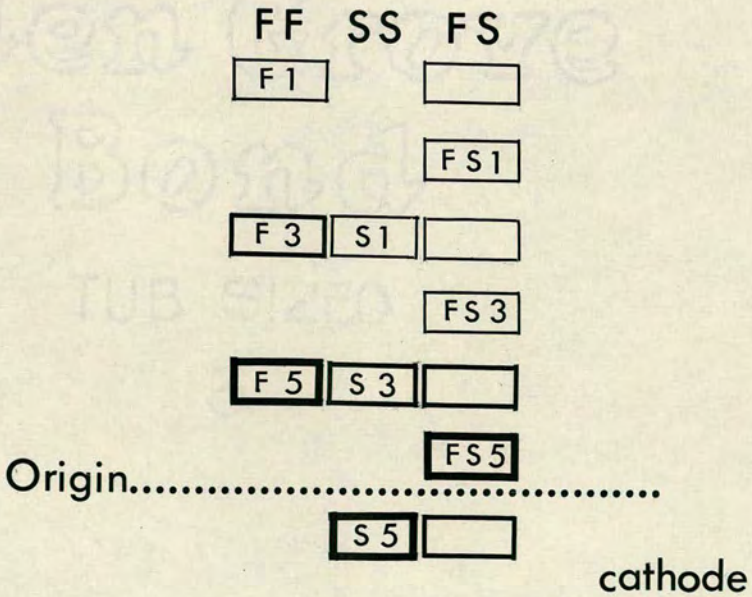
(A) Isoenzymes of Alcohol Dehydrogenase

Both of the electrophoretic systems employed in the present study resolved the ADH from homozygous individuals into a triplet of discrete staining zones, or isoenzymes, on the gel (Fig.III.1). Thus, Adh^{FF} individuals displayed zones F_1, F_3, F_5 in ascending order of cathodal migration and of staining intensity. The Adh^{SS} triplet were likewise designated S_1, S_3, S_5. Under the prevailing separation conditions zones F_5 and S_3 and zones F_3 and S_1 shared identical electrophoretic mobilities. Heterozygous Adh^{FS} zymograms were made up of seven zones of activity, four of which correspond to the parental isoenzymes, the remaining three being unique to the heterozygote. This pattern is consistent with the enzyme being a dimer, the FS_5 zone, for example,

Figure III.1

ADH isoenzymes and alloenzymes of the three Adh genotypes FF, FS, SS revealed by starch-gel electrophoresis using the Poulik system of buffers.





Nomenclature Gibson, 1970

being a heterodimer composed of one F_5 and one S_5 unit.

However, the three isoenzymes extracted from homozygotes are not explicable in terms of random association of two different subunits as dimers, since these individuals are homozygous at the only structural gene producing subunits. These isoenzymes may, therefore, result from a biochemical or conformational alteration of the polypeptide, post-translation, and belong to that class of isoenzymes termed "conformers" (Lutstorf and von Wartburg, 1969). This suggestion is strengthened by the finding that Drosophila ADH isoenzymes from homozygous Adh^{FF} stock, like the isoenzymes of horse liver ADH (McKinley-McKee and Moss, 1965), may be interconverted by the addition of the coenzyme NAD. The ADH_5 conformer, which contains no bound NAD, is converted *in vitro* by addition of NAD into ADH_1, which contains 3.5 moles NAD/mole enzyme (Jacobson, 1968). The fact that the isoenzymes may be interconverted with NAD does not logically require that their fundamental differences are in terms of bound NAD. Indeed, contrary reports of the efficacy of NAD in this process have been presented (Grell *et al* 1968). It is probable, however, that NAD does truly convert one conformer to the other as conversion alters not only the electrophoretic charge but also a kinetic parameter of the conformer. Both natural ADH_1 and "synthetic" ADH_1, derived by conversion of ADH_5, are heat stable while ADH_5 itself is heat sensitive (Jacobson, 1968).

The isoenzymes of *Drosophila melanogaster* ADH have been investigated in considerable detail as a model system for the examination of isoenzymes as a general biochemical phenomenon. Principally, the ADH isoenzymes differ in electrostatic charge, in response to heat, and in kinetic parameters but are identical antigenically (Day and

Needham, 1973). In the present investigation it was observed that inadequate cooling of the gel resulted in a differential loss of the more cathodal isoenzymes. This is entirely consistent with the findings of Day and Needham (1973), that *in vitro* ADH S_3 has a temperature optimum and heat stability considerably greater than ADH S_5. Further, it has been demonstrated that the Michaelis constant $K'_{ethanol}$ of S_3 is greater than that of S_5 and that the specific activity/molecule of S_3 is approximately half that of S_5 (Day and Needham, 1973). Alteration of electrostatic charge, stabilization of the molecules and modification of kinetic parameters may, in theory at least, be attributable to differential binding of the coenzyme NAD.

In conclusion it is worth noting that if the isoenzymes exist *in vivo* adaptational flexibility is gained without the necessity of structural gene heterozygosity or gene duplication. Each individual, regardless of genotype, may possess both a highly stable but kinetically slower form (ADH_1 : ADH_3) and a more active but less stable (ADH_5) form of the enzyme.

(B) Enzymatic Activities of the ADH Allozymes

In Chapter 1 it was proposed that the separate gene products synthesised by alleles at electrophoretically polymorphic loci should exhibit clear biochemical differences if natural selection is to discriminate between alternative alleles. The simplest biochemical parameter for investigation, and probably the most important physiologically, was considered to be the gross specific ADH activity expressed by the three genotypes.

Materials and Methods

Materials and Methods

Lines homozygous for Adh^F and Adh^S were prepared as previously described. Heterozygotes were collected as F_1 progeny from between-line crosses. For each assay approximately 30 4-day old (3rd Instar) larvae reared under optimal conditions were separated from the culture medium, washed and placed on cotton wool moistened with 1% sucrose in distilled water for 24 hours. This time interval allowed for digestion of yeast in the alimentary canal and thereby prevented contamination of the assay by yeast ADH.

The larvae were blotted dry, weighed and homogenized in a ground glass homogenizer with 0.1ml/larva of Tris-HCl buffer (0.1M, pH 8.5) at $0^{\circ}C$. After 30 minutes centrifugation at 1,700g the supernatant was carefully removed, avoiding contamination by the floating lipid fraction. 0.1ml of supernatant, 1.6ml of homogenization buffer and 0.1ml 6×10^{-2} M NAD (β-DPN) in the same buffer, all at $25^{\circ}C$, were added to a 1cm light path quartz cuvette. Endogenous activity (due to traces of ethanol or substrates for other NAD dependent dehydrogenases in the reagents) was assayed by observing the change in OD_{340nm} in a Pye-Unicam SP 800 recording spectrophotometer for two minutes. ADH activity was measured as the initial rate of increase of OD_{340nm} due to NADH formation after addition of 0.1ml 3M Analar ethanol to the assay mixture. The effect of endogenous activity was removed from the readings before calculation of the results.

Results

The activities of 7 FF lines, 7 SS lines and the heterozygotes between them are given in Table III.1. Two assays were performed on separate extracts of each line, the first assay for heterozygotes being

Table III.1: ADH Enzymatic Activities of 7 FF and 7 SS Homozygous Lines (arbitrary units/min/larva).

Line	Genotype					
	FF		FS		SS	
	a	b	a*	b**	a	b
1	7.78	7.64	5.12	4.87	3.90	4.13
2	7.86	6.95	5.43	5.19	4.36	4.64
3	6.08	6.54	5.41	5.33	3.32	3.02
4	7.10	7.36	5.63	5.47	4.41	3.34
5	6.80	6.95	4.84	5.23	3.41	4.05
6	7.50	7.80	5.61	5.17	3.30	3.17
7	7.18	7.20	5.17	5.62	3.80	3.94
Mean	7.195 ± .137		5.316 ± 0.108	5.269 ± 0.091	3.771 ± .138	

* Progeny of ♂ FF x ♀ SS cross.

** Progeny of ♂ SS x ♀ FF cross.

performed on the progeny of a ♂FF x ♀SS cross, the second assay on the progeny of the reciprocal cross. Activities are expressed as arbitrary units/minute/larva as it was considered that activity per larva was most meaningful in terms of natural selection. The more usual units (activity per mgm soluble protein) were discarded since between-line genetic variation in the quantity of soluble protein per larva would have unduly biassed results expressed in this form.

Two main conclusions may be drawn from these values. Firstly, the activity of FF individuals is approximately 1.9 times that of SS individuals. From this it is clear that the allelic products do display functional differences of sufficient magnitude to allow discrimination by natural selection under conditions in which ADH catalytic activity may be related to fitness (see Chapter V). Secondly, the activity of heterozygotes is intermediate between the parental values, although clearly closer to SS than to FF. There is thus no evidence for "molecular heterosis" in the gross specific activity despite the fact that heterozygotes display more electrophoretic isoenzymes than do homozygotes.

Ward and Herbert (1972) have shown that it is incautious to attribute between-line specific activity differences solely to the Adh locus. In a line isogenic for Adh^S, but segregating at other loci, upward and downward selection for ADH activity elicited an immediate response which respectively doubled and halved the initial activity. After three generations of selection plateaux were abruptly reached, suggesting that only one, or a few, modifer loci of large effect were responsible.

In the present study no attempt was made to detect modifiers. It is unlikely, however, that the Kaduna population was segregating for modifier genes as the activities of the homozygous lines (each composed of four second chromosomes of independent origin) were remarkably homogeneous. Greater heterogeneity would be expected if the lines differed, by sampling, at modifier loci with an effect comparable to that found by Ward and Herbert (cf. their Table 1).

Aldehyde oxidase in *D.melanogaster* larvae is largely of maternal origin (Ursprung *et al*, 1968). If this were true of ADH also, the activity of heterozygotes from ♂FF x ♀SS crosses would be lower than that of reciprocal heterozygotes. Examination of Table III.1 indicates that maternal effects on activity are unimportant, at least by the third instar stadium.

The results presented are consistent with previous findings (Rasumson *et al*, 1966) which demonstrated two-fold activity differences between FF and SS inbred lines and intermediacy of their heterozygotes. Subsequent reports have explored further differences between the allelic products. The FF genotype is more sensitive than SS to the *in vivo* induction of enzyme activity by ethanol-rich media (Gibson, 1970). Further, the FF enzyme has a greater temperature coefficient and pH coefficient (between pH 5-9) than the SS enzyme (Day *et al*, 1973). However, 10 minutes at 40°C destroyed 86%, 62% and 46% of the initial activity of FF, FS and SS extracts respectively, indicating that the SS enzyme is more robust than FF (Gibson, 1970).

Discussion

The sum of these findings is in agreement with the conclusion of Harris (1971) that amino acid substitutions resulting in enzyme

polymorphism frequently affect biochemical parameters. The specific relevance of *in vitro* differences to the possible action of balancing selection is unfortunately difficult to assess in the absence of detailed knowledge of the physiology of ADH and of its relationship to fitness.

(C) Ontogeny of Drosophila ADH

Selection pressures are presumably only operative upon enzyme polymorphisms when the genotype at a locus is phenotypically expressed, in this case as ADH activity in the individual. Previous reports (Ursprung *et al*, 1968, 1970; Dunn *et al*, 1969) have shown a characteristic ontogenetic profile of Drosophila ADH. This phenomenon has been reinvestigated in homozygous lines derived from the Kaduna population.

Materials and Methods

Premated females yielding either FF or SS offspring were placed in oviposition chambers for 24 hours to allow retained eggs to be shed. Thereafter eggs were collected every 3 hours and transferred to vials in which to develop under optimal conditions. Individual larvae, pupae or adults were harvested at specific developmental stages and immediately homogenized in glass well slides with 0.1ml of ice-cold Tris-HCl buffer (0.1M, pH8.5). 0.2ml of a staining mixture, containing 0.5mgm/ml each of NBT and β-DPN and 0.05mgm/ml of PMS in 5% iso-propanol in the same Tris-HCL buffer, was added to the homogenate. Histochemical staining was terminated when the least active homogenate (1st instar) showed appreciable formazan formation, usually after 10 minutes at 25°C. The intensity of formazan in each well was assessed subjectively on a relative scale.

Results

Spot-test results from five FF and five SS lines are given in Table III.2. A typical series of wells is shown in Fig.III.2. Formazan formation could be shown to be entirely due to ADH, as control wells containing all reagents except iso-propanol yielded negative readings.

Within the rather broad experimental limits of this technique, all individuals of a given genotype and age yielded identical results, except 1st instar SS larvae which were at the threshold of detection. SS homozygotes displayed a similar developmental profile to FF although their overall activity was lower (as expected from their lower specific activities). The developmental profile obtained was entirely in agreement with those previously reported. No activity was observed in eggs even when 50 eggs were homogenized in a single well, again suggesting that ADH, unlike aldehyde oxidase, is not maternally transmitted to the offspring.

Alcohol dehydrogenase activity is at a maximum in the late 3rd instar larva and, again, in the mature adult when the animals are actively feeding. This finding is consistent with the hypothesis that ADH is primarily concerned with catalysis of substrates of external origin, such as ingested alcohols, and is therefore physiologically important at all life-cycle stages except the embryo and pupa.

From these observations it is not possible to specify any particular developmental stage at which natural selection may preferentially act.

Conclusion

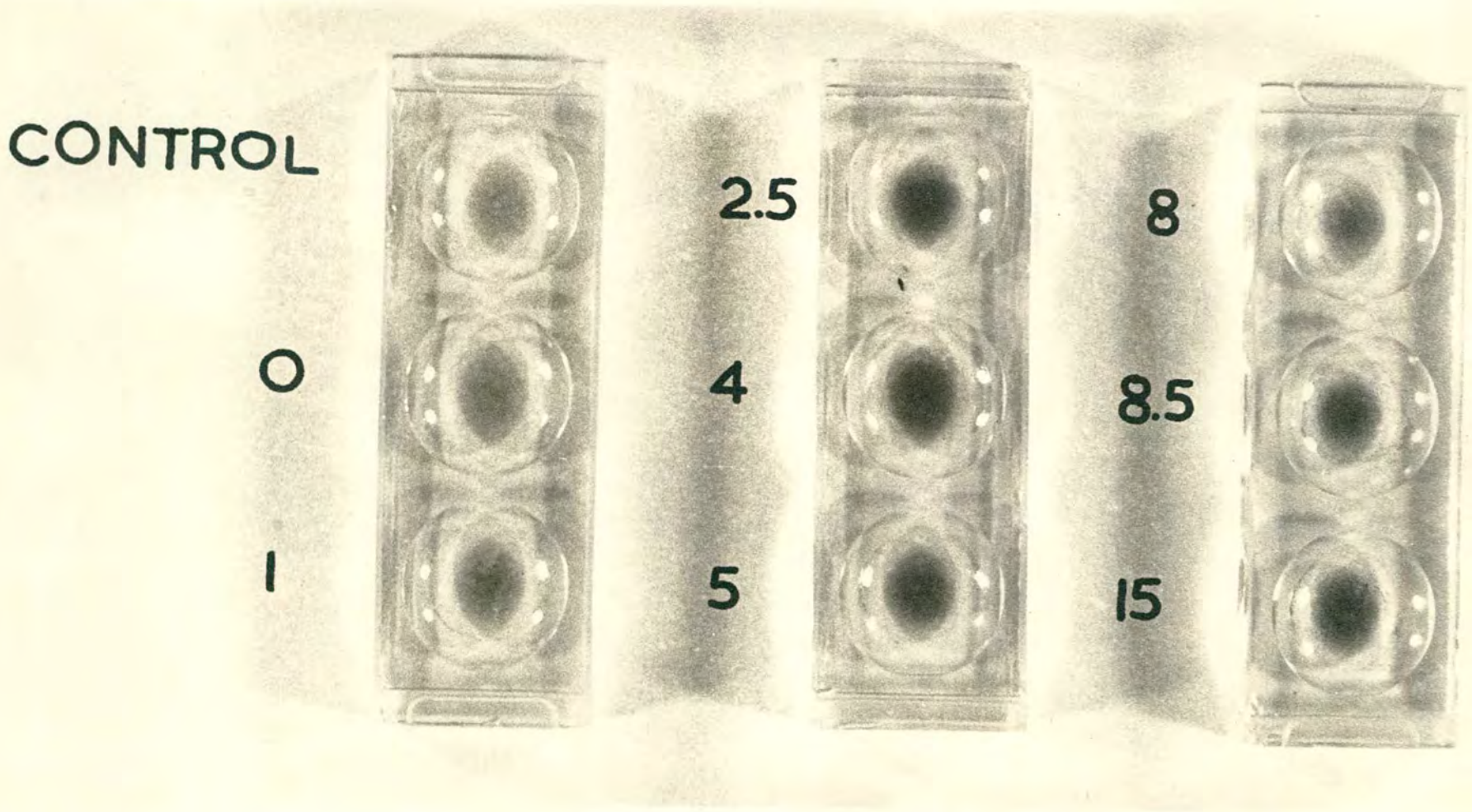
In this chapter some aspects of the Adh polymorphism in D.melanogaster have been discussed. It is clear that functional differ-

Table III.2: Relative staining intensities of ADH during the development of *D. melanogaster*.

Age (Days)	Stage	ADH staining intensity	
		FF	SS
0	Egg	-	-
1	1st instar	+	±
2.5	2nd "	+++	++
4	3rd "	+++++	++++
5	Prepupa	++++	+++
8	Pupa	++	+
8.5	Adult (young)	+++	++
15	Adult (old)	++++	+++

Figure III.2.

Spot-test wells showing the relative activity of ADH at different stages of development of FF individuals. (Numbers beside each well refer to age, in days, of individual homogenised in the well.)


CONTROL
0
1
2.5
4
5
8
8.5
15

ences do exist, both between isoenzymes within a genotype, and between the allelic products of alternative alleles. It remains to be determined whether these differences are in fact detectable by natural selection. Further, as ADH activity is at a maximum during those periods of the life-cycle when feeding occurs, it is postulated that one function of ADH is concerned with the catalysis of substrates derived directly from the environment.

CHAPTER IV

Stability of the Adh Equilibrium Gene-Frequency

In this chapter investigations of the stability of the Adh equilibrium gene frequency in the Kaduna population are reported. Two lines of approach were adopted in the investigation. Firstly the equilibrium gene frequency has been followed over both long and short-term time periods to test the temporal stability of the polymorphism. Secondly the dynamics of the polymorphism have been examined using the classical method of gene-frequency perturbation. A brief examination of possible linkage disequilibrium involving the Adh locus is also reported.

A. Long-term stability of the polymorphism

During the course of the total study samples of eggs were taken at intervals from the population cage and raised under optimal conditions in order to obtain virgin flies for the initiation of homozygous lines (see Chapter II). These samples were always taken at the weekend.

The numbers of each genotype observed among the adults emerging from these egg-samples for seven separate collections are given in Table IV 1. As differences in genotype-frequency between sexes are not significant, the data from the two sexes has been pooled. The gene frequency of the F allele in each sample is shown in Fig. IV 1 together with the binomial standard error of the estimate.

It is evident that the gene and genotype frequencies in the population have remained constant during the period of study ($\chi^2_{(12)}$ for heterogeneity between samples = 6.094; $p > 0.9$). None of the samples differ significantly from any other. Since the seven samples are statistically homogeneous

Table IV.1

Sample Date	Genotype			Total	q(F)
	FF	FS	SS		
11.3.68	109	111	33	253	0.650
3.10.68	15	21	10	46	0.554
28.1.69	58	67	29	154	0.594
25.6.69	21	22	9	52	0.615
29.9.69	34	40	16	90	0.600
6.7.70	64	80	25	169	0.615
14.1.71	58	67	28	153	0.598
Totals	359	408	150	917	0.613
Expected*	345.7	434.7	136.6	917	0.613
	$\chi^2_{(1)}$** = 3.446				

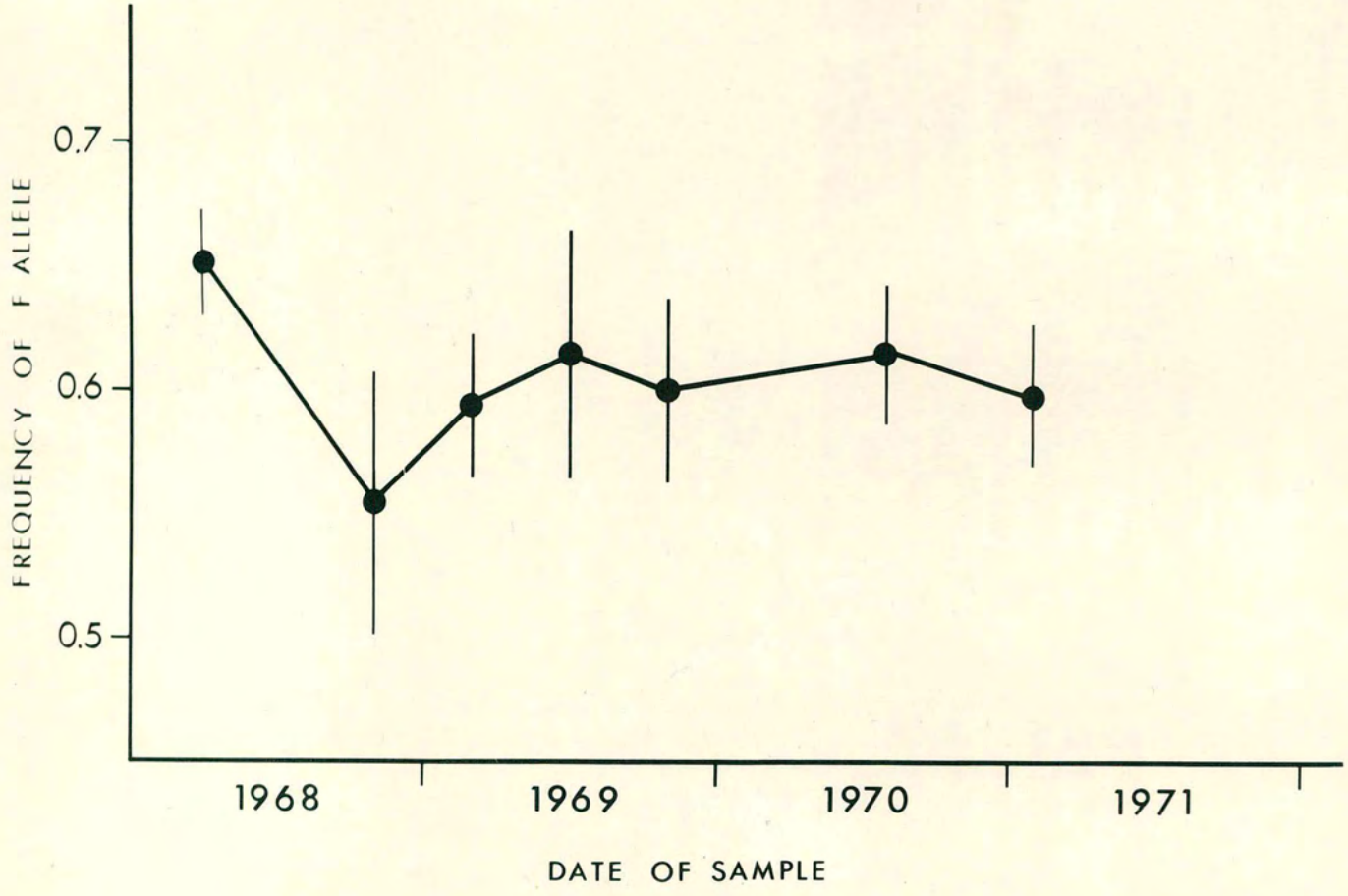
*Expected from Hardy-Weinberg proportions

**Deviation of observed numbers of each genotype from Hardy-Weinberg expectations.

Numbers of each genotype and frequency of F allele in seven egg-samples taken from the Kaduna population over a three-year period.

Figure IV.1

Frequency of the Adh F allele in seven samples of eggs taken from the Kaduna base population over a three year period (± binomial s.e.)


0.7
0.6
0.5
FREQUENCY OF F ALLELE
1968
1969
1970
1971
DATE OF SAMPLE

the data has been pooled and a mean equilibrium gene frequency of the F allele, q(F) = 0.613, has been calculated. The total observed numbers of each genotype do not differ significantly ($\chi^2_{(1)} = 3.446$; $p > 0.05$) from those expected from Hardy-Weinberg proportions for this gene frequency.

B. Short-term stability of the polymorphism

Since the conclusion of the major part of the study reported in this thesis three further samples of eggs have been taken from the Kaduna population. These samples were obtained as previously described with the exception that they were taken during the middle of the week. The observed numbers of each genotype in these samples are given in Table IV.2.

The three samples are not significantly heterogeneous ($\chi^2_{(4)} = 1.678$; $p > 0.7$) and the observed deviations of genotype proportions from Hardy-Weinberg expectations are not significant ($\chi^2_{(1)} = 0.058$; $p > 0.98$). However, the observed genotype frequencies in these three samples differ from those observed (Table IV.1) in the weekend egg-samples of the previous three years ($\chi^2_{(2)} = 14.571$; $p < 0.001$). Nonetheless the gene-frequency observed in one further weekend egg-sample remained at the high level of the previous weekend samples (45 FF: 52 FS: 17 SS; q(F) = 0.622).

This finding prompted a study of the short-term stability of the Adh gene and genotype frequencies in the Kaduna population cage.

Samples of adult flies were removed from the population cage every Monday, Wednesday and Friday over two separate intervals of two weeks duration and electrophoresed to determine Adh genotype (Table IV.3).

Table IV.2:

Date of Sample	Genotype			Total	q(F)
	FF	FS	SS		
19.11.71	30	58	22	110	0.536
22.3.72	36	52	28	116	0.534
10.10.72	167	273	102	542	0.560
Total	233	383	152	768	0.553
Expected*	234.6	379.7	153.7	768	0.553
	** $\chi^2_{(1)} = 0.058$				

* Expected from Hardy-Weinberg proportions.

** Deviations of observed numbers of each genotype from Hardy-Weinberg expectations.

Table IV.3: Observed numbers of each genotype and gene frequency of F allele in samples of adult flies removed from the Kaduna population cage on different days of the week.

Day of week	Sample (Week)	♂ Genotypes				♀ Genotypes			
		FF	FS	SS	q(F)	FF	FS	SS	q(F)
Monday	1	19	23	2	0.693	13	31	8	0.548
	2	27	52	6	0.624	13	28	7	0.562
	3	16	18	3	0.676	16	29	10	0.554
	4	24	40	8	0.611	22	28	12	0.581
	Total	86	133	19	0.640	64	116	37	0.562
Wednesday	1	10	26	12	0.479	17	25	6	0.615
	2	31	41	24	0.536	36	41	19	0.589
	3	12	23	13	0.490	21	31	13	0.562
	4	15	22	11	0.542	18	24	6	0.625
	Total	68	112	60	0.517	92	121	44	0.593
Friday	1	18	20	10	0.583	16	22	10	0.562
	2	17	25	6	0.615	11	28	9	0.521
	3	19	19	10	0.594	19	25	15	0.534
	4	15	25	8	0.573	16	19	10	0.567
	Total	69	89	34	0.591	62	94	44	0.545

$\chi^2_{(2)}$ for heterogeneity in observed genotype numbers between days in male flies.

Monday - Wednesday $\chi^2_{(2)} = 25.09$; $p < 0.001$

Monday - Friday $\chi^2_{(2)} = 9.96$; $p < 0.01$

Wednesday - Friday $\chi^2_{(2)} = 4.56$; $p > 0.1$

The numbers of each genotype observed within a sex are not significantly heterogeneous between samples collected on the same dayoof the week ($\chi^2_{(6)}$ never exceeds 5; $p > 0.5$). The data for each sex has therefore been pooled for each day of the week (totals in Table IV.3). Further, the observed numbers of each genotype in female flies do not differ significantly between days of the week ($\chi^2_{(2)}$ in no case exeeds 2.4; $p > 0.2$). It may therefore be concluded that among female flies the gene and genotype frequencies remain relatively constant over the week (Fig.IV.2).

In contrast a striking pattern is evident in the temporal distribution of genotype-frequencies in male flies (Fig.IV.3). The frequency of the SS homozygote is low in the Monday samples (0.08), rises to a peak (0.25) in Wednesday samples and declines in Friday samples (0.18). The pattern is consistent over all four weeks of study and is highly significant (Table IV.3).

If cycles of genotype frequency among adult flies are to account for the discrepancy between mid-week and weekend egg-samples, the data presented in Table IV.3 would indicate that male flies alone are responsible. On first inspection the cycle in male genotype frequencies appears to be compatible with the observations on egg-samples since the average gene-frequency of males during the middle of the week is lower than at weekends. However, this conclusion requires certain assumptions, primarily the assumption that females laying eggs at any point in time have been fertilised by males present in the adult population at that point in time. This assumption may be unreasonable. It may be more likely that females laying eggs on Wednesdays will have been mated

Figure IV.2

Genotype frequencies among adult female flies sampled from the Kaduna base population on different days of the week. (Each point represents the mean of four separate samples.)

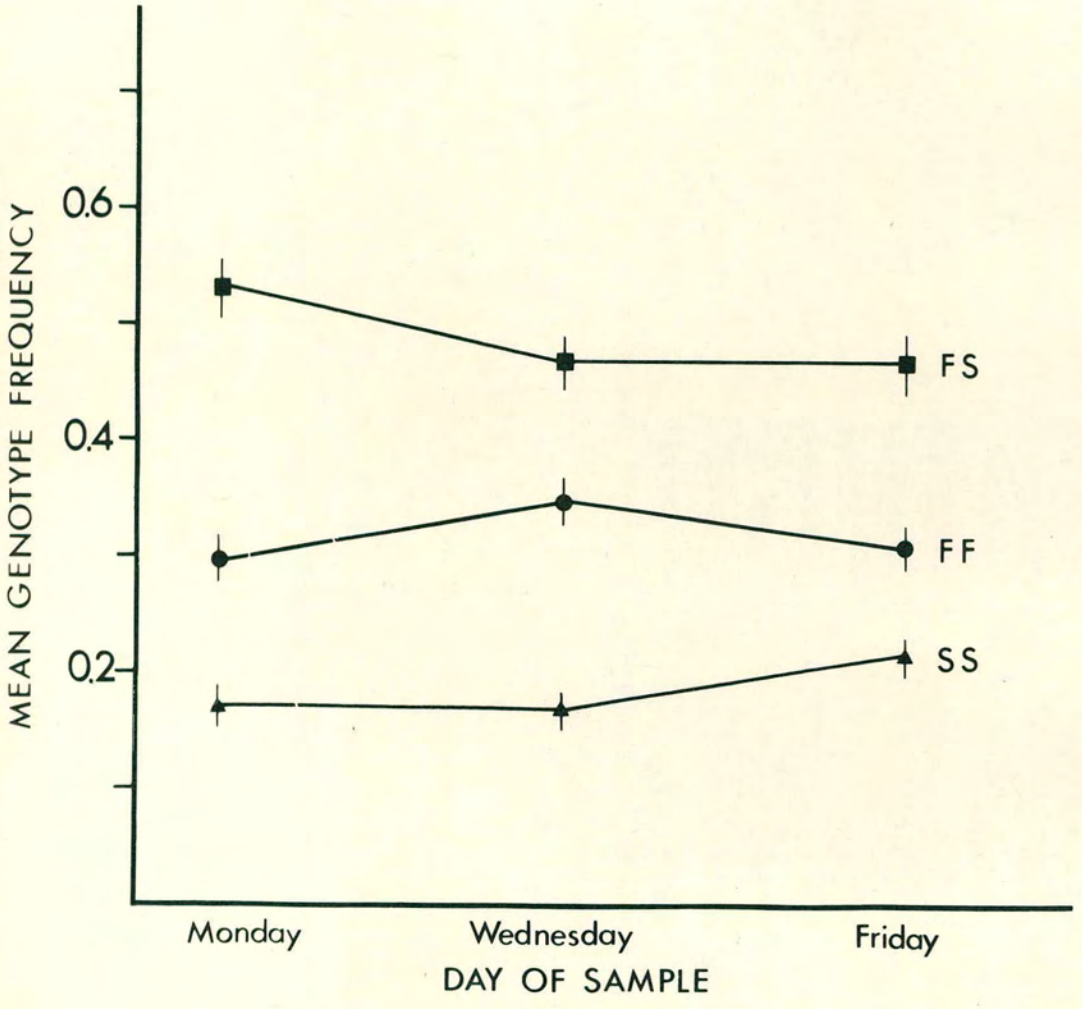
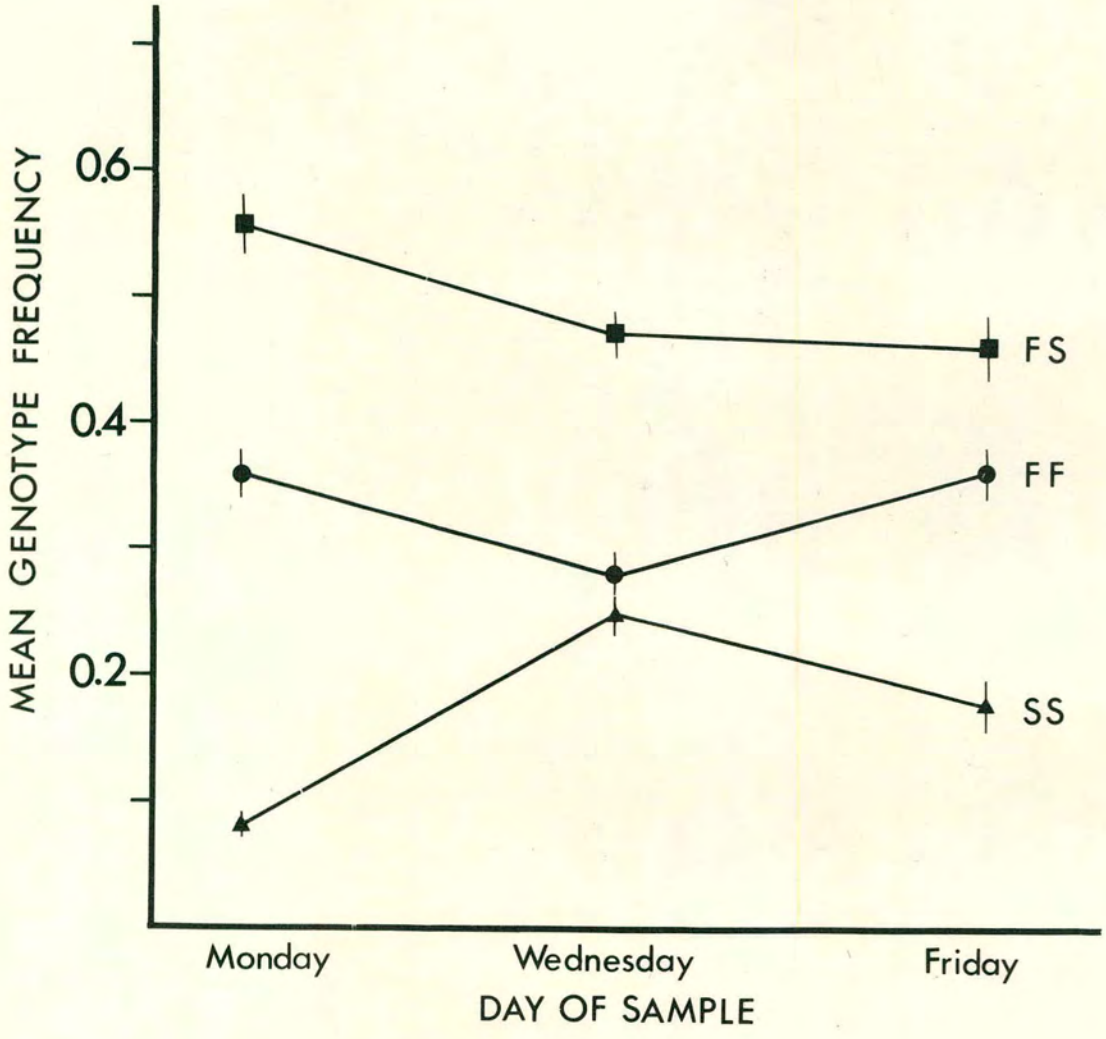

MEAN GENOTYPE FREQUENCY
0.6
0.4
0.2
FS
FF
SS
Monday
Wednesday
Friday
DAY OF SAMPLE

Figure IV.3

Genotype frequencies among adult male flies sampled from the Kaduna base population on different days of the week. (Each point represents the mean of four separate samples.)


MEAN GENOTYPE FREQUENCY
0.6
0.4
0.2
FS
FF
SS
Monday
Wednesday
Friday
DAY OF SAMPLE

over the previous weekend, in which case the frequency of the F allele in their eggs would be high, in contradiction to the observed frequency.

This dilemma can only be resolved if it is assumed that SS males, although relatively rare in the total male population on Mondays, are at a high frequency among those young, sexually-active males which succeed in fertilising females destined to lay eggs on Wednesdays. Males which were young on Mondays may have the highest probability of surviving until the middle of the week, giving rise to the high frequency of SS males on Wednesdays. It is clear, then, that the observed cycle in male genotype frequencies cannot be taken as a sufficient explanation of the significant discrepancy between mid-week and weekend egg-sample gene-frequencies until more is known of the age and sexual structure of the population.

Weekly cycles in genotypic frequencies are not, perhaps surprising in population cages which are maintained by once-weekly food medium renewal. Indeed, similar observations have been made in population cages of the kelp-fly, Coelopa frigida (B.C. Clarke; personal communication). Similarly, Kojima and Tobari (1969) report rhythmical oscillations in Adh allele frequencies in a laboratory population of D.melanogaster although these had a longer periodicity than that observed in the present study.

It is possible that the cycle in males is related to deterioration in the food medium. The peak of SS homozygote frequency occurs on the day immediately preceding the replacement of the oldest food container with fresh medium. Thus the peak may reflect either the superior ability of SS adults to withstand the absence of fresh food, or a relatively high proportion of SS homozygotes in the final emergence of

the oldest food pot. Data pertinent to the latter proposition are given in Chapter V.

Regular fluctuations in gene and genotype frequencies have been cited as evidence against the neutral mutation-random drift hypothesis of protein polymorphism (e.g. Ayala and Dobzhansky, 1973). It is, indeed, difficult to reconcile the significant cycles of genotype frequency in the present study with the hypothesis that fitnesses do not differ between Adh genotypes. However, the potential importance of this phenomenon to the maintenance of the polymorphism is not easy to assess as it is not known whether the differential selection giving rise to the cycle takes place in the pre-reproductive or post-reproductive phase of the life of the males. If the selection is post-reproductive it can play no role in the maintenance of the polymorphism.

C. Gene-frequency perturbation in population cages

In a preliminary attempt to detect the possible action of balancing selection at the Adh locus, population cages were initiated with divergent initial frequencies of the F allele. The rationale behind such an experiment is simple. If the fitnesses of the three genotypes are equal, that is the polymorphism is selectively neutral, changes in gene frequency in the following generations should be random in direction irrespective of the initial frequency. Conversely, balancing selection should bring about directional changes and return gene-frequencies towards an equilibrium value.

Method: Three population cages were founded with initial frequencies of the F allele at 0.75, 0.5 and 0.25 respectively. The 0.75 population was initiated using equal numbers of progeny from each of twelve

independent FF x FS pair matings obtained directly from the Kaduna base population. Thus, the experimental population contained 12 independent S-bearing second chromosomes and 36 independent F-bearing chromosomes. The 0.5 and 0.25 populations were likewise founded with the progeny of 12 FS x FS and 9 FS x SS matings respectively.

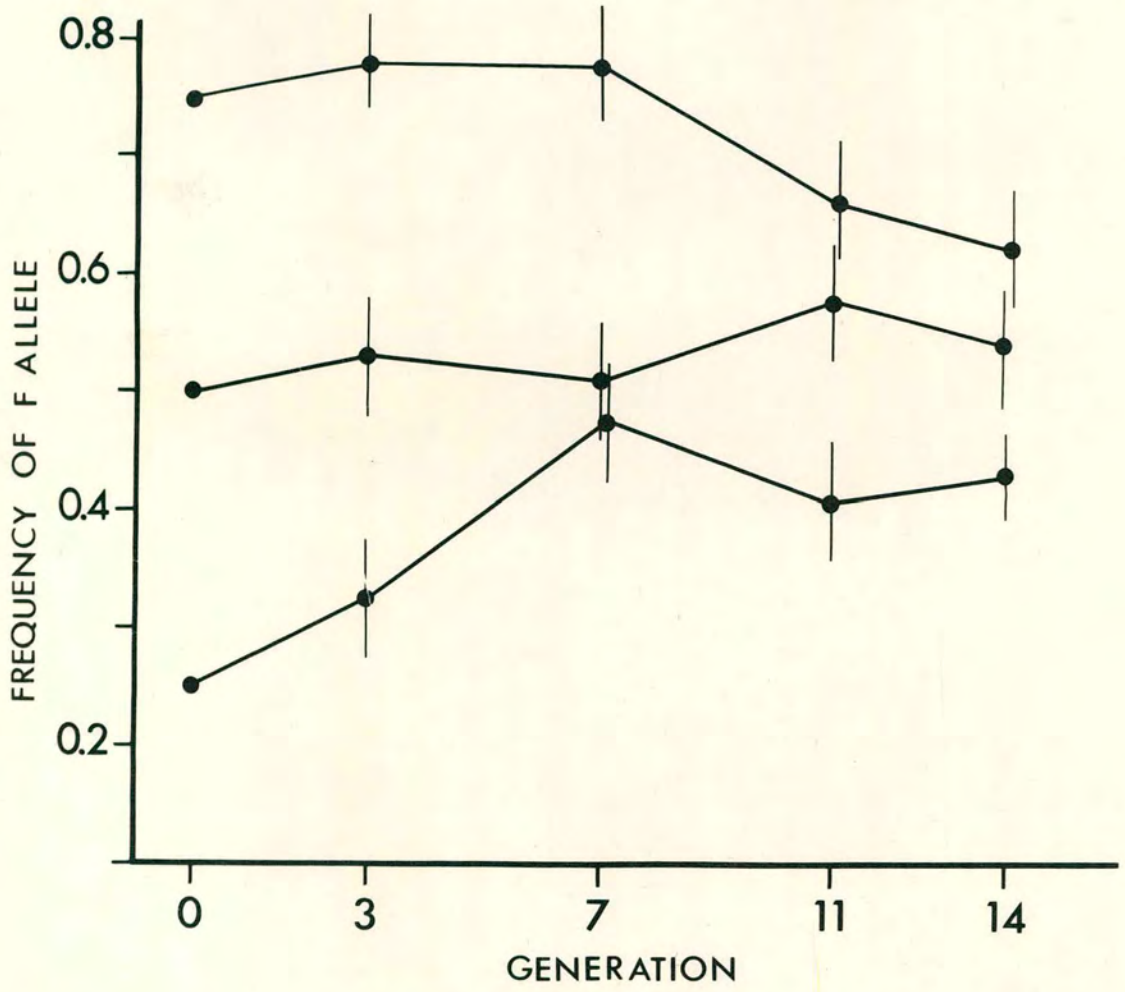
This design had one inherent bias. The minority alleles in the 0.75 and 0.25 populations were represented in only one-quarter of the founder chromosomes. Thus minority homozygotes had a higher probability of carrying two second chromosomes which were identical by descent than did majority-allele homozygotes. It is generally accepted that homozygosity for a whole chromosome leads to a reduction in fitness through the expression of recessive lethal and detrimental genes. However, in the present study this bias would be against the finding of balancing selection as minority alleles would decline in frequency rather than increasing towards an equilibrium value.

The experimental population cages were maintained on the same regime as the base population. The adult population number was constant around 5,000 individuals and generation time was approximately two weeks. Samples of adult flies were assayed for Adh gene frequency after approximately 3, 7, 11 and 14 generations. All three populations were lost at generation 15 due to an incubator failure.

Results: The observed gene frequencies in the populations are shown in Fig.IV.4 together with the binomial standard errors of the estimates. Deviations from Hardy-Weinberg proportions of genotypes were not significant in any samples, although this may be a reflection of the rather small sample sizes examined (96-192 genes per sample).

Figure IV.4

Observed frequencies of the F allele in three population cages founded with different initial frequencies. (± binomial s.e.).


0.8
0.6
0.4
0.2
FREQUENCY OF F ALLELE
0
3
7
11
14
GENERATION

Changes of gene frequency in the 0.75 and 0.25 cages appear to be directional, the frequency of the F allele returning towards an equilibrium value in the range q(F) = 0.5 - 0.6. As discussed above this gene frequency shift is not explicable in terms of differences in the degree of inbreeding between homozygotes. However, Kimura and Ohta (1971) have severely criticised the inference of the existence of balancing selection from perturbation experiments of this type. They argue that when small numbers of chromosomes are used to found experimental populations "associative overdominance" (Ohta and Kimura, 1971) will result from the chance association of truly heterotic loci with the locus under investigation. Thus a spurious balancing selection may be observed at truly neutral loci. It does, indeed, appear from the literature that the strength of balancing selection reported is inversely related to the number of chromosomes used to found the experimental populations. For example, strong selection was observed by MacIntyre and Wright (1966), Berger (1971) and Ayala and Anderson (1973) using small numbers of founder chromosomes, while Yamazaki (1971) using far larger numbers, detected no selection at the Est-5 locus in Drosophila pseudoobscura. Although the number of chromosomes used to found the populations in the present study was smaller than that employed by Yamazaki it exceeded the number commonly used in similar studies.

Two other population perturbation studies using the Adh locus of D.melanogaster are known to the author. Bijlsma and van Delden (1972) observed a return of gene frequencies towards an equilibrium value of q(F) = 0.5 - 0.6 in populations started at four different frequencies of the F allele. The rate of this return was comparable with that in

the present study. Conversely, two experimental populations derived from the Kaduna population remain at their initial frequencies of q(F) = 0.9 and q(F) = 0.1 after nearly five years of laboratory culture in Edinburgh (A. Robertson; personal communication). This finding must cast doubt upon the generality of the inferences drawn from the present study.

The possibility that the Adh locus forms part of selectively maintained blocks of genes in linkage-disequilibrium, and that selection acts upon the entire block rather than upon Adh individually, is examined in the following section.

D. Linkage equilibrium at the Adh locus

Franklin and Lewontin (1970) have examined theoretically the fate of alleles segregating at loci linked on the same length of chromosome. They showed that where heterotic selection acts at each locus, and where heterosis is multiplicative between loci, considerable linkage disequilibrium can be generated. The disequilibrium spreads sequentially until many loci are "crystallised" into a single gene-block or supergene, which may extend the total length of the chromosome. In this case each allele at a locus will be represented in only one major gametic type in the population. Thus, where linkage disequilibrium is present, the fitness values determined experimentally at a locus cannot be attributed to that locus alone, but rather to the entire block of genes surrounding the locus.

Ohta and Kimura (1970; 1971b; Ohta, 1971) have reached a similar conclusion by considering the fate of selectively neutral loci which are linked in disequilibrium to truly heterotic or deleterious loci. Here

the disequilibrium is considered to result from random genetic drift or mutation in finite populations. Thus, where the alleles at a neutral locus are linked in disequilibrium to recessive lethals at different loci an apparent heterosis will be observed experimentally.

Investigations have been conducted to determine whether alleles at the Adh locus (II. 50.1) are linked in disequilibrium with other loci on the second chromosome in the Kaduna population.

The Kaduna population is free from inversions (A. Robertson; personal communication). Thus, associations between Adh alleles and gene-arrangements of the type described by Prakash and Lewontin (1968) could not be investigated. Samples from the population confirmed the finding of O'Brien and MacIntyre (1969) that soluble malic dehydrogenase (Mdh, II 41.2) and alpha-glycerophosphate dehydrogenase (alpha-Gpdh, II 20.5) were not segregating in the population. These two loci, together with Adh, are the only known loci coding for soluble proteins on the left arm of chromosome II. Thus, linkage disequilibrium with other allozyme loci could not be investigated.

Recessive lethals therefore remained the only source of genetic variability which could be tested for association with Adh alleles. Accordingly, a number of second chromosomes were made isogenic using the standard Cy/Pm method (where Cy marks a multiple-inversion crossover-suppressor chromosome). The Adh allele carried by each chromosome was determined by electrophoresis of +/+ flies where the chromosome was homozygous-viable and Cy/+ flies where the chromosome carried a recessive lethal gene. The results are given in Table IV.4.

Table IV.4. Adh alleles and recessive lethal genes in the Kaduna population.

		Number of Chromosomes		
		Viable	Lethal	Total
Adh allele	F	60	4	64
	S	54	6	60
Total		114	10	

$\chi^2_{(1)} = 0.587$

It is evident that there is no association between chromosome lethality and Adh alleles. These results contrast sharply with the finding of highly significant associations between Esterase-6 alleles and recessive lethals on the third chromosome in the same population (Malpica, unpublished redults). In the latter study, the association was of sufficient magnitude to play a part in the dynamics of the polymorphism.

In conclusion it must be pointed out that, although no linkage disequilibrium has been found at the Adh locus, the possibility remains that Adh is integrated into a small block of genes not detectable by present techniques.

E. Frequency-dependent selection: Egg-Adult viability test

The revelation of the extent of genetic heterozygosity in natural populations has led a number of population geneticists, notably Kojima

and his colleagues, to re-examine the possible role of selective mechanisms other than the classical heterozygous advantage model in the maintenance of polymorphism. Such mechanisms propose that the selective values of genotypes are not constants but are dependent upon population density (Clarke, 1972), or genotype frequencies in the population. The major attraction of these modes of selection is that the selective values of all genotypes may be nearly equal at gene-frequency equilibrium. Thus, many polymorphisms may be maintained concurrently without the enormous genetic load inherent in heterosis models.

The concept that selective values are not necessarily constants is not new. Indeed, the notion of frequency-dependent selection maintaining biochemical polymorphism is intrinsic in Haldane's (1949) essay on the evolution of disease-resistance. Models invoking frequency-dependent selection have been explored mathematically (e.g. Wright, 1948; Teissier, 1954a,b; Lewontin, 1958; Clarke and O'Donald, 1964) and it has been shown that stable equilibria may be maintained under a wide range of genetic situations.

The majority of experimental studies to date have been concerned either with large genetic elements such as inversions (e.g. Levene et al, 1954) or with visible mutants. In the latter category recognition of phenotype by members of the same or predatory species plays an important role in the dynamics of the polymorphism. For example, one component maintaining the pattern polymorphism in Panaxia dominula is a mating preference whereby matings between unlike phenotypes are in excess of that expected from random pairing of males and females. Accordingly, a rare male has a strong frequency-dependent advantage (Sheppard and

Cook, 1962). Similarly, Petit (1968) has shown that Drosophila melanogaster males carrying the mutant white are at a sexual advantage over wild-type males when present below a certain frequency in laboratory mixtures. Above this frequency white males are at a disadvantage. A stable equilibrium could be maintained by this mechanism if all other components of fitness were equal between the genotypes.

Predators may develop a tendency to hunt specifically for those prey-types to which they are most accustomed, that is, they develop a "searching-image". Clarke (1962, 1962a; Allen and Clarke, 1968) has indicated that this behaviour will result in predators taking proportionately more of common prey-types than of rare ones. A rare phenotype is then at an advantage until it reaches a sufficient frequency that predators become accustomed to it. This phenomenon may result in balanced polymorphism with many different visible phenotypes. The probability of survival of any individual is dependent upon the frequency of similar phenotypes in the population.

In a number of reports in the literature frequency-dependent selection appears to arise through direct competitive interactions between genotypes. For example, in experimental populations of Triboleum castaneum containing both wild-type and black individuals, the survival of wild-type individuals was found to increase as their initial frequency in the population was reduced (Sokal and Karten, 1964).

Similar studies have been conducted by Kojima and his colleagues (Kojima and Yarbrough, 1967; Yarbrough and Kojima, 1967; Kojima and Tobari, 1969; Kojima, 1971) using the naturally occurring Adh and Est-6 enzyme polymorphisms in D.melanogaster. At both these loci it was found that the probability of survival of a genotype was dependent

upon its frequency in a culture. The egg-adult survival of a genotype was enhanced when it was initially present below its equilibrium frequency and depressed when initially present in excess. Where a series of cage populations were founded with divergent allele frequencies at the Est-6 locus a rapid return towards gene frequency equilibrium was observed. The pattern of gene frequency change was more compatible with the hypothesis of frequency-dependent fitness values than with heterosis (Yarbrough and Kojima, 1967).

Extensions of these studies indicated that the interaction between genotypes was mediated through the environment (Huang, Singh and Kojima, 1971; Kojima and Huang, 1972). Thus, the relative survival of a genotype was lowest when grown on a medium previously exploited by a like genotype and highest when grown on medium "conditioned" by unlike genotypes. It must be concluded that the conditioning genotypes either depleted a specific resource, or left metabolic products, to the detriment of their own genotype.

In the present investigation the egg-adult viability of Adh genotypes has been examined to determine whether fitness changes as a function of initial gene frequency.

Method: Lines homozygous for Adh^F or Adh^S were extracted from the Kaduna population as previously described. The lines were divided into seven independent groups, each group being composed of two FF lines (FF1 and FF2) and two SS lines (SS1 and SS2). Matings were set up within groups to give females laying eggs of the genotypes shown.

	FF1	FF2	SS1	SS2
FF1	-	FF	FS	FS
FF2	FF	-	FS	FS
SS1	FS	FS	-	SS
SS2	FS	FS	SS	-

Sib matings were avoided such that none of the resulting eggs were inbred.

The fertilized females were placed in oviposition chambers and eggs of known genotype were collected every 6-12 hours. From each experimental group three cultures were set up by transferring exact numbers of eggs (Table IV.5) to 3" x 1" shell vials containing 2ml of standard food medium. These cultures simulated exact Hardy-Weinberg proportions for initial frequencies 0.3, 0.5, 0.7 of the F allele.

Table IV.5. Input numbers of eggs of the three genotypes in experimental cultures*

Initial q(F)	Genotypes		
	FF	FS	SS
0.3	18 (0.09)	84 (0.42)	98 (0.49)
0.5	50 (0.25)	100 (0.50)	50 (0.25)
0.7	98 (0.49)	84 (0.42)	18 (0.09)

*Initial genotype frequencies are given in parentheses

The hatchability of the eggs of each genotype was checked (see Chapter II) and was in no case less than 98%.

The cultures were placed in a constant humidity chamber (R.H. 60%) at 25°C and all individuals surviving to adulthood were collected and electrophoresed. Preliminary studies indicated that approximately 60% of the eggs survived to adulthood under these culture conditions.

Results: The numbers of each genotype surviving from each vial are given in Table IV.6. From these figures viability estimates have been derived following the method of Kojima and Yarbrough (1967). Here the

$$\text{viability estimate} = \frac{\text{Proportion of genotype in survivors}}{\text{Proportion of genotype in egg input}}$$ (Table IV.7)

This table illustrates the overall qualitative pattern of the results. It is apparent that there is a trend towards the viabilities of the genotypes being related to the initial gene-frequency of the culture in which they developed. Thus the FF homozygotes are relatively more viable when rare (q(F) = 0.3) than when in excess (q(F) = 0.7) of their equilibrium genotype-frequency. Similarly, SS homozygotes are more viable when rare (q(F) = 0.7) than when in excess. All three genotypes have very similar viabilities at q(F) = 0.5, the nearest frequency to the equilibrium value in the population cage (q(F) = 0.55 - 0.60).

t-test analyses were performed to determine whether the mean absolute viability (mean of six groups excluding (3)) of each genotype differed between initial frequencies. The data were entered as arcsin transformed absolute viabilities where;

$$\text{absolute viability} = \frac{\text{number of genotype surviving}}{\text{input number of eggs of that genotype}}$$

Table IV.6: Numbers of each Genotype observed in Progeny of Experimental Vials

Initial q(F)	Group	Genotypes			Total
		FF	FS	SS	
0.3	1	17	52	47	116
	2	11	61	67	139
	3	16	46	67	129
	4	15	56	71	142
	5	13	59	77	149
	6	14	65	72	151
	7	15	68	49	132
0.5	1	23	40	15	78
	2	25	49	23	97
	*3	-	-	-	-
	4	42	76	39	157
	5	20	72	44	136
	6	39	73	46	158
	7	45	78	30	153
0.7	1	34	34	5	73
	2	44	42	8	94
	3	73	69	16	158
	4	60	66	16	142
	5	37	67	16	120
	6	73	59	17	149
	7	82	67	10	159

* This culture was lost through accident.

Table IV.7: Viability Estimates of the three genotypes.

Initial q(F)	Group	Genotypes		
		FF	FS	SS
0.3	1	1.629	1.067	0.827
	2	0.879	1.045	0.984
	3	1.378	0.849	1.060
	4	1.173	0.939	1.020
	5	0.969	0.943	1.055
	6	1.030	1.025	0.973
	7	1.262	1.227	0.758
	*Pooled	1.171	1.011	0.959
0.5	1	1.179	1.026	0.769
	2	1.031	1.010	0.948
	3	-	-	-
	4	1.070	0.968	0.994
	5	0.588	1.059	1.294
	6	0.987	0.924	1.165
	7	1.176	1.020	0.784
	Pooled	0.996	0.996	1.012
0.7	1	0.951	1.109	0.761
	2	0.955	1.064	0.946
	3	0.943	1.040	1.125
	4	0.862	1.107	1.252
	5	0.629	1.329	1.481
	6	1.000	0.943	1.268
	7	1.052	1.003	0.700
	Pooled	0.919	1.075	1.092

*The pooled viability is a weighted average

The t-tests were performed within genotypes between pairs of initial frequencies e.g. FF(0.3) v. FF(0.5); FF(0.3) v. FF(0.7) etc. Only one of the nine tests showed a significant difference between the mean absolute viability of a genotype at different frequencies (FF(0.3) v. FF(0.7): $t_{(10)} = 2.366$; $p < 0.05$). This significant value reflects the largest observed difference between viability estimates given in Table IV.7. However, the finding of one test out of nine being significant at the 5% level may be expected by chance alone.

The lack of significance in the remaining eight tests is not surprising. It can be seen from Table IV.6 that there were consistent differences in total survival between experimental groups, presumably due to genetic background effects. These differences cause considerable inflation of the variance of absolute viability of a genotype at a particular initial gene frequency and, hence, lower the probability of obtaining a significant result.

A second analysis was therefore performed following the precedent of Kojima and Tobari (1969). The proportions of the three genotypes surviving in all cultures founded at the same gene frequency were shown to be homogeneous by the chi-square test. The data for all groups with a particular initial frequency were therefore pooled and tested by the chi-square test for deviations of the observed numbers of each genotype from those expected on the null hypothesis of no difference in proportional survival between genotypes, (Table IV.8).

Table IV.8. Observed numbers of each genotype and expected numbers on the assumption of no viability difference between genotypes.

Initial q(F)		Genotype FF	FS	SS	No. of groups	$\chi^2_{(2)}$		Final q(F)
0.3	Observed	101	407	450	7	3.39	.2>p>.1	0.318
	Expected	86.2	402.4	469.4				
0.5	Observed	194	388	197	6	0.04	NS	0.498
	Expected	194.7	389.6	194.7				
0.7	Observed	403	404	88	7	5.67	.1>p>.05	0.676
	Expected	438.6	375.9	80.5				

Only one chi-square value approaches formal significance. However, it is interesting to note that the deviations from expectation are again in the direction of frequency-dependent viability values.

Finally, a chi-square analysis was performed within genotypes between frequencies. Here the observed numbers of a genotype (columns in Table IV.9) were compared with the numbers expected on the null hypothesis that the survival of a genotype was independent of the initial gene frequency in the culture in which it developed (Table IV.9).

It is evident that the survival of the FF homozygote is significantly influenced by the initial composition of the culture, although this is clearly not true for the FS heterozygote or the SS homozygote.

Discussion

While the results presented above do not provide unequivocal evidence, there is a strong indication that one component of selection

Table IV.9: Observed and expected numbers of each genotype on the null hypothesis that survival is independent of initial culture composition.

Initial q(F)		Genotype		
		FF	FS	SS
0.3	Observed	101	407	450
	Expected	79.09	396.97	453.42
0.5	Observed	194	388	197
	Expected	188.31	405.06	198.29
0.7	Observed	403	404	88
	Expected	430.6	396.97	83.28
$\chi^2_{(2)}$		8.01	1.096	0.302
p		<0.02	NS	NS

acting upon the Adh polymorphism affects fitness in a genotype-frequency dependent manner. Further, there is no indication that heterozygote advantage has played a role in determining the survival of the genotypes (Table IV.8).

The FF homozygote alone demonstrates a fairly clear change in survival in response to initial culture composition. This genotype has a higher viability estimate when rare than when in excess of its equilibrium frequency in 6 of the 7 independent groups (Table IV.7) and its proportional survival differs significantly between the three initial gene-frequency cultures (Table IV.9). The FS and SS genotypes are apparently less sensitive to alterations of population composition. The egg-adult viability test does not, of course, preclude the possibility that other components of fitness may differ between the genotypes e.g. mating success, fecundity.

In this experiment seven independent groups of genotypes were used to initiate the experimental cultures. This design was chosen such that the repeatability of the effects could be ascertained. It is clear, however, that genetic background effects, resulting from the use of small numbers of chromosomes to found each group, have greatly inflated the overall variability of the results. This can be seen in the consistent differences in total survival between experimental groups. It might, therefore, have been preferable to have pooled the original homozygous lines and to have initiated seven replicates at each frequency from this pool.

It may be unreasonable to expect to detect large differences of fitness between genotypes in laboratory experiments of this type, since the probability of survival of an individual Drosophila will be a function

of its genotype at many loci. As natural populations are segregating at many hundreds of loci, the effect on fitness of any one locus may be small. In this respect it is of interest to compare the results above with those obtained in two studies by Kojima (Kojima and Yarbrough, 1967; Kojima and Tobari, 1969). In these two studies remarkably strong and consistent frequency-dependent selection was detected. The magnitude of the selective coefficients implied by Kojima's findings must raise speculation that loci other than the one directly studied were involved. The Texas experimental population used was founded by crossing wild type stocks and maintaining a large random-mating population thereafter. It is, therefore, possible that linked blocks of genes, marked by Adh and Est-6 and originating from the different stocks, were still present in the population at the time of the viability tests. Further, it is difficult to equate the finding of strong balancing selection at the Adh locus in this population, with the observation that in two replicates of the base population the Adh F allele had reached fixation (Kojima and Tobari, 1969).

Although the Kaduna population is less genetically variable than recently captured Drosophila populations (Malpica, 1973), it has never been inbred. Thus, it should be free of linkage disequilibrium (as was suggested by the limited data presented in the previous section) unless the linkage disequilibrium is itself selectively maintained. Further, it is unlikely that the small number of chromosomes used to found each experimental group would give rise to linkage disequilibria resulting in a consistently frequency-dependent fitness of the FF genotype.

Chapter conclusion

The results presented in this chapter indicate that the Adh equilibrium gene frequency was stable over the period of study. Further, the data are consistent with the hypothesis that the polymorphism is influenced by the forces of natural selection. These forces are reflected in the regular and significant cycle in genotype frequencies among adult males in the base population and in the directional changes in gene and genotype frequencies observed in perturbation experiments in both population cages and vial cultures. The egg-adult viabilities estimated in the latter experiment give a strong, but not conclusive, indication that the viability of one genotype is dependent, at least in part, upon the initial gene-frequency in the culture in which it develops.

CHAPTER V

Environmental manipulation and selection at the Adh locus

The experiments described in the latter part of the previous chapter relied upon the gene-frequency perturbation method to detect the action of natural selection at the Adh locus. In this chapter an alternative approach to the investigation of gene-frequency equilibria, that of environmental manipulation, is examined.

This approach is based upon the assumption that the selective forces acting upon a locus are related either directly or indirectly to components of environmental heterogeneity. Thus the equilibrium gene frequency observed in a population occupying a stable environment may be considered to reflect a balance between the selective forces exerted by particular combinations of environmental factors upon the different genotypes. Changes in the environment, whether cyclical or directional, may therefore be expected to alter the balance of selective forces acting upon a proportion of segregating loci and to result in detectable changes in gene frequency at these loci. Experimentally manipulation of the environment may be achieved by the replication of entire populations into one or more novel environments and the subsequent assay of possible changes in gene or genotype frequencies.

This rationale is, of course, only applicable where polymorphism at a locus is selectively maintained. At a truly neutral locus gene frequency is independent of environment, provided that changes in environment have no effect upon mutation and migration rates, nor upon effective population number. Environmental manipulation therefore offers a possible method of determining whether a

polymorphism is selectively maintained.

A practical difficulty is that of choosing novel environmental conditions which have the highest probability of altering the selective forces which may act at the locus under investigation. This problem may be more easily solved for enzyme polymorphisms than for other classes of polymorphism. For example, it may be expected *a priori* that gene frequencies at the octanol dehydrogenase locus should be sensitive to octanol level in the environment (Wills, 1972). However, it has been argued that the overall flux in a metabolic pathway cannot be sensitive to dramatic changes at individual links in the chain (Robertson, 1967). This homeostasis may buffer the effects of forces acting upon individual polymorphic enzymes such that the final effect upon fitness is extremely small. Extremely strong environmental stimuli may therefore be required to elicit a detectable change in gene frequency.

Environmental manipulation is necessarily a one-sided test. Where a significant gene-frequency change can be correlated with a natural or artificial perturbation of environment, the action of natural selection may be justifiably invoked. If, conversely, no change occurs, it may always be argued that the specific perturbation employed did not sufficiently disturb the balance of selective forces at the locus.

The major advantage of the method is its avoidance of the problems of linkage artefacts inherent in the gene-frequency perturbation method. The genetic structure of the population is not dislocated in the establishment of the experiment as long as large numbers of individuals are used to initiate the replicate population.

Furthermore, the technique more closely reflects the natural fate of polymorphic systems. It seems reasonable to suppose that the major challenges to the stability of a selectively maintained polymorphism arise through changes of environment rather than from sudden perturbations in gene-frequency in a stable environment. The latter may only occur when population size is extremely low or when massive immigration occurs. In these circumstances random drift and chance linkage effects are likely to predominate over specific selective forces in the determination of gene frequency at a single locus.

In this chapter further aspects of the Adh polymorphism in the Kaduna population have been examined in two novel environments and an attempt has been made to determine whether the survival of the genotypes differs over time in response to a natural environmental cycle in the base population cage. Thus this chapter deals with attempts to localise the environmental factors which could give rise to the apparent natural selection acting upon the Adh locus indicated in the previous chapter.

A. The effect of ethanol on Egg-Adult viability

Alcohol is toxic to many animals but may also be utilised as an energy source through providing [H] during its primary oxidation and through supplying acetyl coenzyme A to the citric-acid cycle. It was shown in Chapter III that alcohol dehydrogenase enzyme from FF individuals has approximately twice the *in vitro* alcohol oxidising capacity of ADH from SS homozygotes, and that the heterozygote activity was intermediate. It was therefore predicted that perturbation of the standard medium supplied to the Kaduna population

by the addition of ethanol should shift gene frequencies at the Adh locus in favour of the F allele.

Methods: A random sample of virgin females and males were collected from the standard Kaduna population cage and pair-mated individually. After several days of egg laying the parent flies were electrophoresed to determine Adh genotypes, and the progeny of FF x SS or SS x FF matings were retained. The 36 progeny groups so obtained were divided at random into 9 sets and virgin adults were collected and allowed to mate at random within each set. Thus 9 independent groups of FS flies were obtained, each group being derived from four original pair matings.

Standard food medium was remelted, boiled, divided into two 93 ml aliquots and allowed to cool to 50°C. To one aliquot 7 ml of absolute ethanol were added (ethanol medium), to the other 7 ml of distilled water (control medium). 10 ml of ethanol medium were dispensed into each of 9 sterile shell vials (25 x 100 mm), 9 control vials received 10 ml each of control medium.

Each group of fertilised FS female flies was divided equally between an ethanol and a control vial. After 6 days of egg-laying the parent flies were discarded and all offspring surviving the crowded conditions were collected and electrophoresed for Adh genotype. The expected ratio of genotypes in the offspring was 1:2:1; FF:FS:SS.

Results: The total numbers of each genotype surviving from each of the 9 pairs of ethanol and control vials are given in Table V.1 together with the final gene frequency in each vial. Total survival was lower on the ethanol medium than on control medium in all nine

Table V.1: Numbers of each genotype among progeny from ethanol-treated and control medium for 9 independent groups of FSxFS matings

(Expected ratio 1 FF : 2 FS : 1 SS)

Group	Treatment	♂ Genotypes				♀ Genotypes				Pooled q(F)	Final pH
		FF	FS	SS	q(F)	FF	FS	SS	q(F)		
1	Ethanol	14	38	12	0.516	24	40	16	0.55	0.535	6.35
	Control	25	49	18	0.538	25	61	21	0.519	0.528	6.4
2	Ethanol	14	26	7	0.574	15	36	9	0.550	0.561	6.6
	Control	6	33	18	0.395	14	43	25	0.433	0.417	5.8
3	Ethanol	17	46	19	0.490	18	43	8	0.570	0.526	5.7
	Control	26	66	28	0.492	33	64	31	0.508	0.500	6.2
4	Ethanol	24	41	22	0.511	22	41	17	0.531	0.521	6.5
	Control	21	34	18	0.521	23	43	29	0.468	0.491	6.2
5	Ethanol	24	39	16	0.551	13	42	17	0.472	0.513	6.1
	Control	25	47	17	0.545	13	56	20	0.461	0.500	5.85
6	Ethanol	13	18	10	0.537	11	21	8	0.537	0.537	6.3
	Control	20	37	27	0.458	19	32	6	0.614	0.521	7.4
7	Ethanol	19	38	19	0.500	23	40	26	0.483	0.491	5.75
	Control	20	49	11	0.556	25	62	30	0.479	0.510	5.3
8	Ethanol	19	33	19	0.500	15	53	10	0.532	0.517	6.20
	Control	20	86	17	0.521	14	46	26	0.430	0.472	5.5
9	Ethanol	20	37	9	0.583	16	24	6	0.609	0.594	6.0
	Control	18	37	22	0.474	16	54	19	0.483	0.479	5.45

groups.

In eight of the nine independent groups the frequency of the F allele, q(F), was higher in the survivors of ethanol medium than in the survivors of control medium. This result is significant ($p < 0.05$) on the binomial test (Siegel, 1956). Similarly, the mean gene frequency of the F allele in ethanol cultures is significantly higher than in control vials ($t_{(16)} = 2.846$; $p < 0.02$, using arcsine transformed gene-frequencies). It may be of interest that the majority of this effect is attributable to the distribution of genotypes among female flies. Examination of Table V.1 reveals that there was no trend in gene frequencies in male flies, in fact, the gene frequency of the F allele in male flies is higher in control vials than in ethanol vials in five of the nine groups.

Further analysis of this data was complicated by two artefacts of the experimental design.

(i) The FS flies used to initiate each replicate pair are derived from 8F and 8S bearing chromosomes from the Kaduna population. Therefore, relative to heterozygotes, homozygotes in the progeny had a 12.5% probability of being inbred for the whole second chromosome, relative to heterozygotes, (assuming random mating between FS individuals and ignoring recombination in FS females). The effect of inbreeding is clear in 13 of the 18 cultures, where an overall excess of heterozygotes was found. Comparisons between genotypes within vials are thus unreliable. Nonetheless, comparisons within genotypes between control and ethanol vials in a pair are valid as both vials in the pair were founded by the same stock of FS parents. In 8 of the 9 ethanol vials the genotypic frequency of FF was greater, and the genotypic frequency

of SS less, than in the corresponding control vial ($p < 0.05$ on the binomial test). The frequency of FS individuals showed no clear response to ethanol treatment.

(ii) It was observed at the end of the experiment that the colour of the remaining food medium varied considerably between control vials. The colour of ethanol medium was a consistent orange-brown. It was speculated that the colour differences between control vials were the response of natural indicators in the medium to differences of pH between vials. This proved to be the case, paler food media having a lower pH (5-6) than the deeper, chocolate-brown media (pH 6-7.5). The pH of the ethanol vials was considerably less variable ($\sigma^2 = 0.097$) than that of control vials ($\sigma^2 = 0.413$).

Analyses were therefore performed to determine whether the frequencies of surviving genotypes in control vials were correlated with the pH of the medium in which they developed (Table V.2). A pattern is discernible in these results. Higher survival of female FF individuals is significantly correlated with high pH. In males the situation may be reversed, high pH favouring higher frequency of SS individuals, although this correlation is not significant. No significant correlations were detected in ethanol vials.

It had been hoped that the only factor influencing the survival of genotypes in control vials would have been the inbreeding effect. Had this been the case the observed genotypic frequencies in ethanol cultures could have been corrected for inbreeding, and relative fitnesses of the three genotypes calculated. The introduction of the pH effect makes such a correction factor impossible.

Table V.2: Correlations between Ahd genotypic frequencies among survivors and the final pH of the medium in which they developed (control vials only).

Final pH	Genotype Freq. ♂♂			Genotype Freq. ♀♀			Group
	FF	FS	SS	FF	FS	SS	
6.4	.27	.53	.20	.23	.57	.20	1
5.8	.11	.58	.32	.17	.52	.31	2
6.2	.22	.55	.23	.26	.50	.24	3
6.2	.29	.47	.25	.24	.45	.31	4
5.85	.28	.53	.19	.15	.63	.22	5
7.4	.24	.44	.32	.33	.56	.11	6
5.3	.25	.61	.14	.21	.53	.26	7
5.5	.27	.49	.23	.16	.53	.31	8
5.45	.23	.48	.29	.18	.61	.21	9
Correlation	+.077	-.536	+.431	+.843	-.079	-.668	
ρ	NS	NS	NS	<0.01	NS	<0.05	

It is possible that the differences in final pH between control cultures resulted from the adherence to semi-sterile techniques during the preparation of the medium. In this case the few bacteria and yeasts which did infect the cultures may have differed in type between vials. The final pH may, therefore, have reflected differences in microflora between cultures. The observation that ethanol treated medium was less variable in final pH is compatible with this hypothesis as the growth of microorganisms was severely retarded by the alcohol.

Discussion: Incautious experimental design has considerably diminished the value of the results presented in Table V.1. The design was originally chosen to maximise the probability of detecting the effect of ethanol stress by initiating each culture with exact proportions of the three genotypes. However, the resultant inbreeding effects have prevented a detailed analysis of the response. In retrospect it would have been preferable to have used adult flies direct from the population cage to initiate the cultures.

Nonetheless, the experiment achieved its primary objective in demonstrating that manipulation of the environment by the addition of a substrate specific to alcohol dehydrogenase did exert a differential stress on the survival of the three Adh genotypes. Further, the direction of the differences in survival between genotypes in control and ethanol cultures was consistent with predictions made solely on the basis of *in vitro* differences in enzymatic activity.

This data is consistent with a recent report of Bijlsma and van Delden (1972) which demonstrates that cultures homozygous for

the Adh S allele become extinct more rapidly than FF cultures when grown on an ethanol-supplemented medium.

It may be argued that 7% ethanol is an unnatural stress and that resistance to alcoholic environments is not an important physiological role of alcohol dehydrogenase in natural populations. Three points may be cited against this view. Firstly, individual Drosophila homozygous for Adh null-mutants die when exposed to ethanol-rich media (Grell, 1967). Thus ADH is important in the detoxification of alcohol. Secondly, in studies subsequent to those reported in this thesis, M.M. Malpica and the author have examined natural populations of D. melanogaster which breed in wine containing up to 14% ethanol. These populations have extremely high frequencies of the F allele, and in one population a novel electrophoretic allele has been found which, when homozygous, has twice the enzymatic activity of the F allele. This again suggests that ADH is important in the physiology of Drosophila inhabiting alcoholic environments. Finally, it has been shown that yeast fermentation of the food medium does produce appreciable quantities of ethanol in the Kaduna population cage. Up to 1.5% ethanol has been detected (using a Boehringer Blood-Alcohol Test Kit) in the medium during the fourth to seventh day of the life of the food pot.

It may be concluded from this section that the three Adh genotypes respond differentially to the presence of alcohol in their environment and that this response is predictable from a knowledge of the enzymatic activities of the three genotypes. It is further argued that the response may play an important role in the maintenance of the polymorphism in the Kaduna, and wild, populations.

B. pH changes and genotype emergence-patterns in the Kaduna population cage

In the previous section data was presented which indicated a possible association between the viability of FF homozygotes and the final pH of the medium in which they developed. These observations were not sufficient to draw firm conclusions but it was considered worthwhile to investigate the phenomenon further.

(i) Initially a survey was conducted to determine whether the pH of food medium altered over the three week period it was present in the population cage. A small sample of medium (approximately 2 gm) was removed from each food pot at half-weekly intervals over a period of five weeks, mixed with 2 ml of distilled water and its pH measured. The sample was taken from the top 2 cm depth of the medium in which larvae were actively feeding. The results of this survey are given in Fig. V.1.

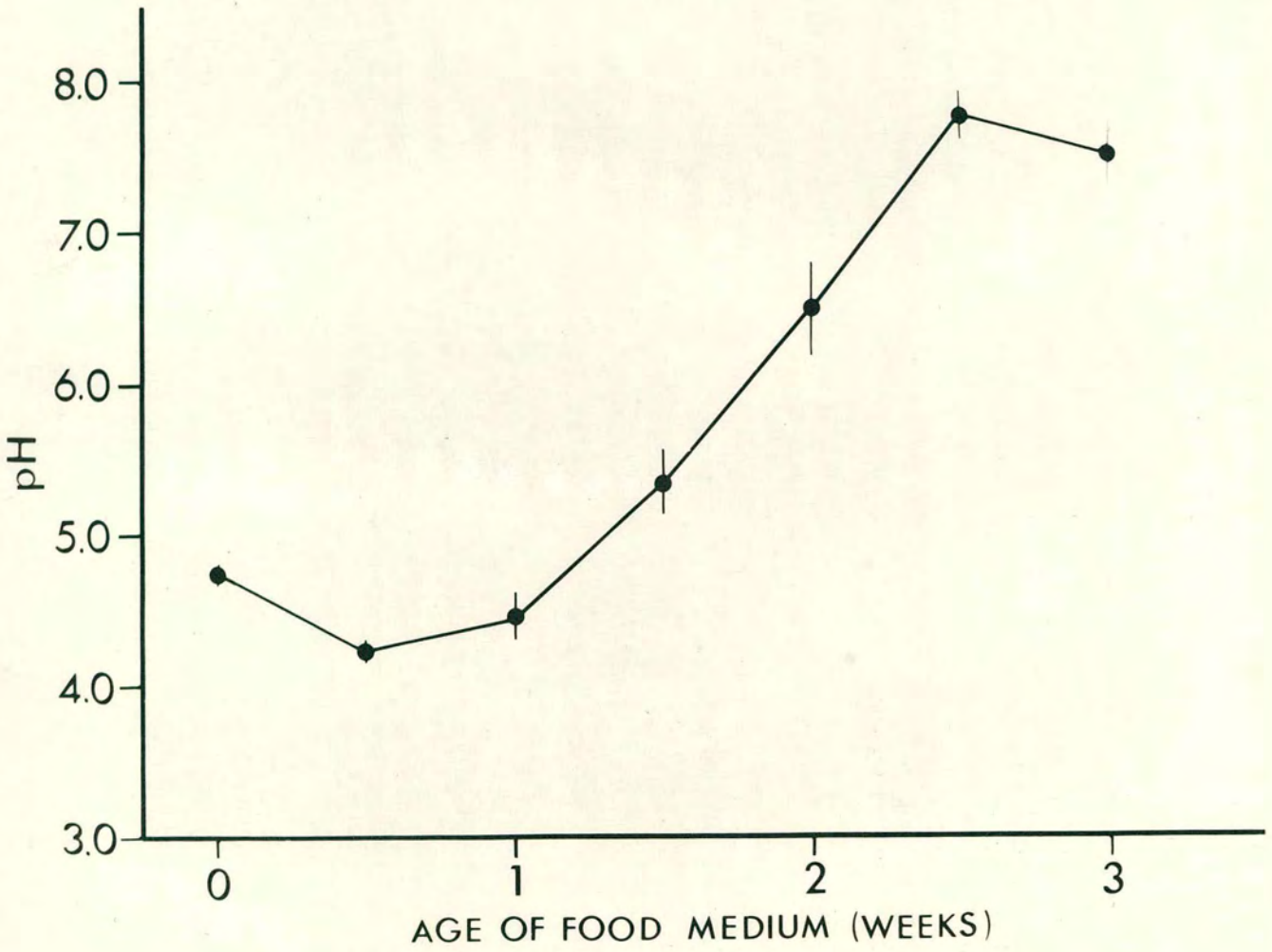
It is evident that the food medium undergoes a regular change in some parameter which is reflected in pH. The initial decrease in pH corresponds to the period of time when young larvae are breaking up the surface of the food medium and when yeast growth and alcohol production are at a maximum. The rise in pH after the first week probably reflects the accumulation of nitrogenous wastes from the larvae and the replacement of yeast by bacteria as the predominant microflora.

From this observation it seems reasonable to suppose that the environment encountered by early-developing larvae may differ radically from that encountered by later developing individuals. Such a regular pattern of environmental change may be capable of

Figure V.1

The pH of food medium in pots in the standard Kaduna cage measured at half-weekly intervals.

(Pots are removed from the cage after three weeks.)


8.0
7.0
6.0
5.0
4.0
3.0
pH
0
1
2
3
AGE OF FOOD MEDIUM (WEEKS)

maintaining genetic polymorphism if one homozygote is favoured in the "early" environment while the alternative homozygote is equally favoured in the "late" environment. However, the resulting gene-frequency equilibrium will only be stable and robust to perturbation if the pattern of environmental change is regular over generations and if the heterozygote exhibits marginal overdominance when the effects of selection in both environments are pooled.

(ii) A small experiment was therefore initiated to determine whether pH *per se* exerted a differential selection pressure upon the three Adh genotypes. Food medium at pH 11 and pH 3 were prepared by adding 1.3 gm sodium hydroxide or 2 ml N hydrochloric acid respectively to 100 ml of standard food medium in bottles. Fifty virgin females and fifty males from the Kaduna population were placed in each of three high pH bottles and each of three low pH bottles. After three weeks the progeny from each bottle were tipped-over into a new bottle prepared as above. After five generations of this method of culture samples of adult flies from each of the six bottles were examined for Adh genotype. The results are given in Table V.3.

It is clear from this Table that manipulation of the pH of the culture medium had no detectable effect upon the frequency of the F allele, nor upon the frequencies of the three genotypes emerging at the fifth generation.

However, this experiment is not a sufficient test of the hypothesis that temporal changes in the condition of the food medium affect Adh genotypes differentially. The observed changes in the pH of the food medium were probably themselves the result of more fundamental alterations in environmental conditions.

Table V.3: Observed numbers of each genotype emerging after five generations of culture in low and high pH media.

pH	Replicate	♂ Genotypes				♀ Genotypes				Pooled q(F)
		FF	FS	SS	qF	FF	FS	SS	qF	
3	1	10	15	8	0.530	11	18	4	.606	0.568
3	2	20	21	6	0.649	12	26	10	.521	0.584
3	3	14	26	8	0.562	13	24	10	.532	0.547
11	1	17	24	7	0.604	14	23	11	.531	0.567
11	2	13	27	8	0.552	17	22	9	.583	0.567
11	3	11	25	12	0.490	16	22	9	.575	0.532

Note: Gene and genotype frequencies do not differ significantly between pH 3 and pH 11 cultures

(iii) A more comprehensive study of the survival of the three Adh genotypes at different stages in the deterioration of the medium was therefore undertaken. This study was performed under conditions which simulated the population cage environment as closely as possible.

Method: Two standard food pots were placed in the Kaduna population cage at the same time (Thursday) as a regular weekly food pot. After five days the two pots were transferred to a clean replicate cage at 25°C. The number of eggs layed upon the medium in each pot was estimated to be 60,000-80,000 as determined by counting the number of eggs and shed egg-cases in representative areas of the surface of the medium. All adult flies emerging from these two pots were removed from the cage at approximately 18-hour intervals over the entire emergence period. Seventy-two flies of each sex were taken at random from each 18-hour sample, weighed individually on a torsion balance and electrophoresed to determine Adh genotype. At the beginning and at the end of the emergence period the total numbers emerging were less than 144; in these samples all individuals were analysed.

On the basis of previous results the following pattern of genotype emergence was anticipated.

(a) Adult flies emerging during the early phase of the eclosion period develop in medium with a low pH in which alcohol produced by yeast fermentation reaches 1.5%. Environmental conditions in which pH is low apparently favour the survival of SS female individuals (if the correlation between pH and survival of genotypes given in Table V.2 are valid), but alcohol favours FF females (Table V.1).

The survival of male Adh genotypes is relatively insensitive to both pH and environmental alcohol. Thus the genotype frequencies in the early phase of eclosion were not expected to depart significantly from expected Hardy-Weinberg proportions.

(b) Later emerging adults experience an environment in which pH is high and alcohol is no longer detectable. High pH environments are apparently beneficial to the survival of FF females (Table V.2) and detrimental to the survival of SS females. It was therefore anticipated that the genotypic frequency of FF females would rise, and the frequency of SS females decline, towards the end of the total emergence period.

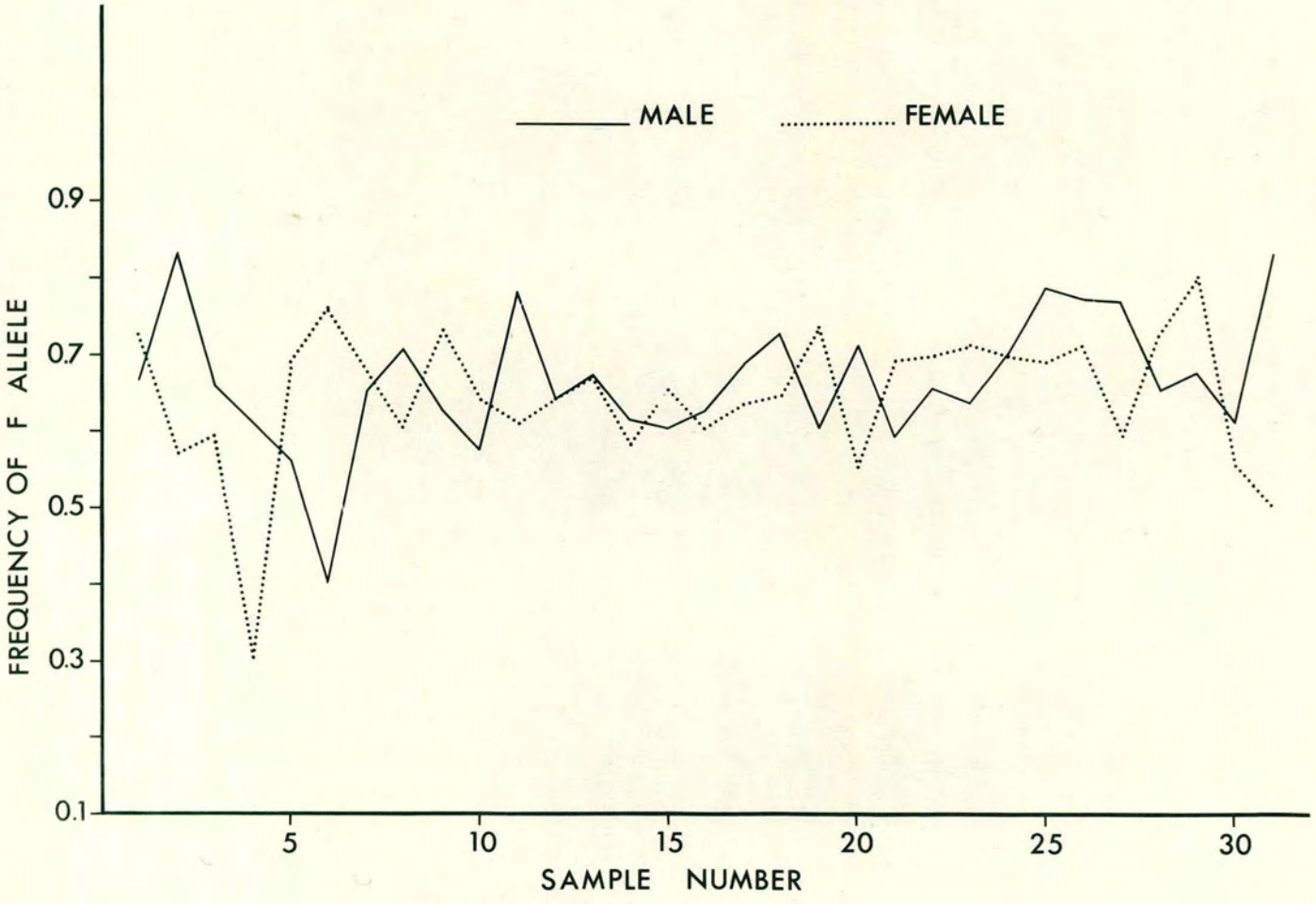
The weight of each fly was recorded as an additional measure of fitness. As body-weight and fecundity are positively correlated in Drosophila (Robertson, 1957) it was speculated that changing environmental conditions may have had a differential effect on the fitnesses of the Adh genotypes through the determination of final body weight and hence genetic contribution to the next generation of zygotes.

Results: The gene frequency of the F allele, genotype frequencies, and mean weights of each genotype in each 18-hour sample are given in Figs. V.2-V.6.

The first adult flies emerged from the food pots eight days after the initiation of the experiment. The total numbers of flies in the first seven samples were 43, 13, 38, 23, 19, 16 and 32 respectively. Thereafter the total number per collection exceeded 200 until the final six collections which contained 75, 57, 27, 18, 14 and 5 adults respectively. It should be noted that under the regular maintenance

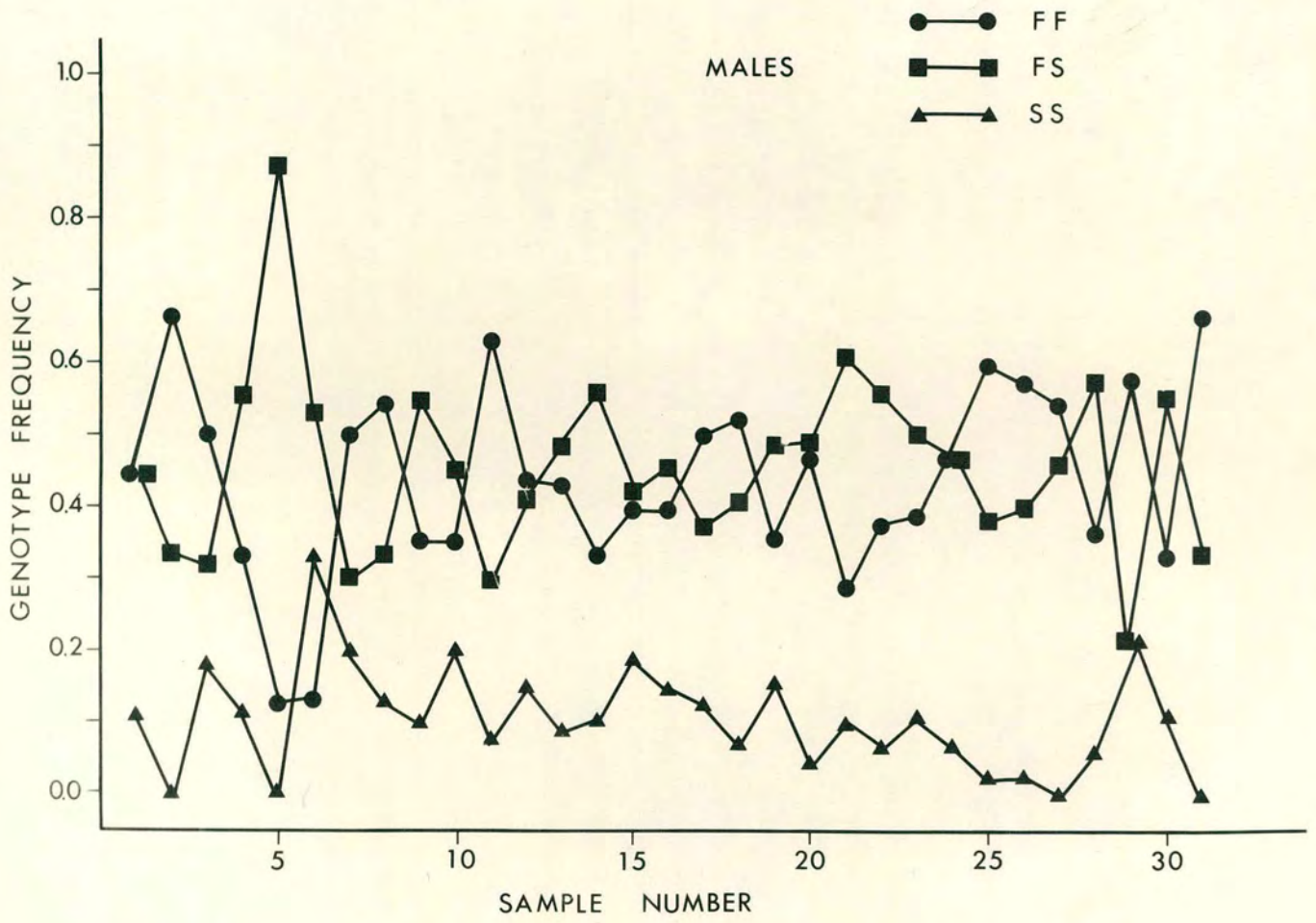
Figure V.2

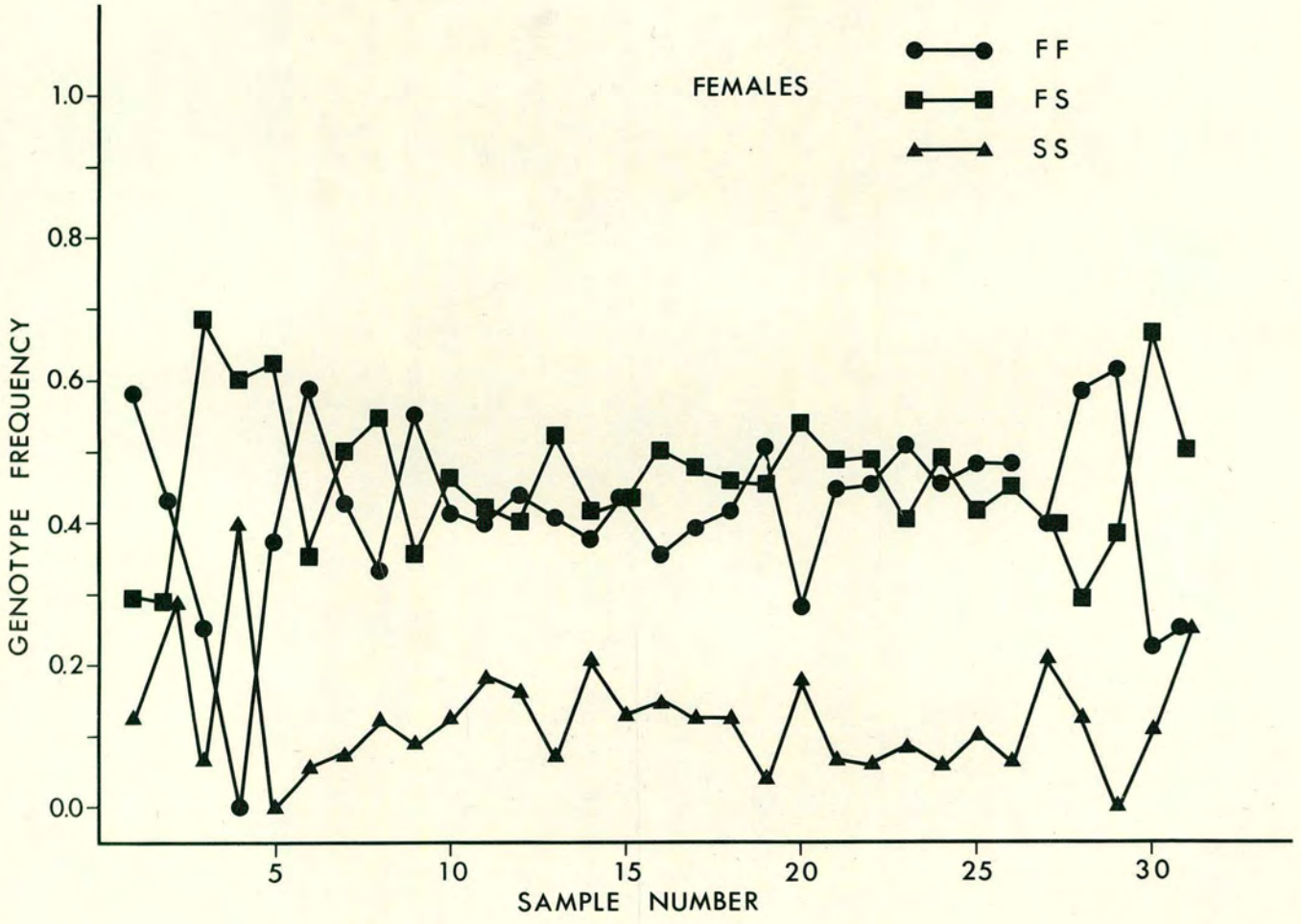
Frequency of the F allele in male and female flies over the entire eclosion period from standard food pots. Samples were taken at 18-hour intervals.


MALE
FEMALE
FREQUENCY OF F ALLELE
0.9
0.7
0.5
0.3
0.1
5
10
15
20
25
30
SAMPLE NUMBER

Figures V.3 and V.4.

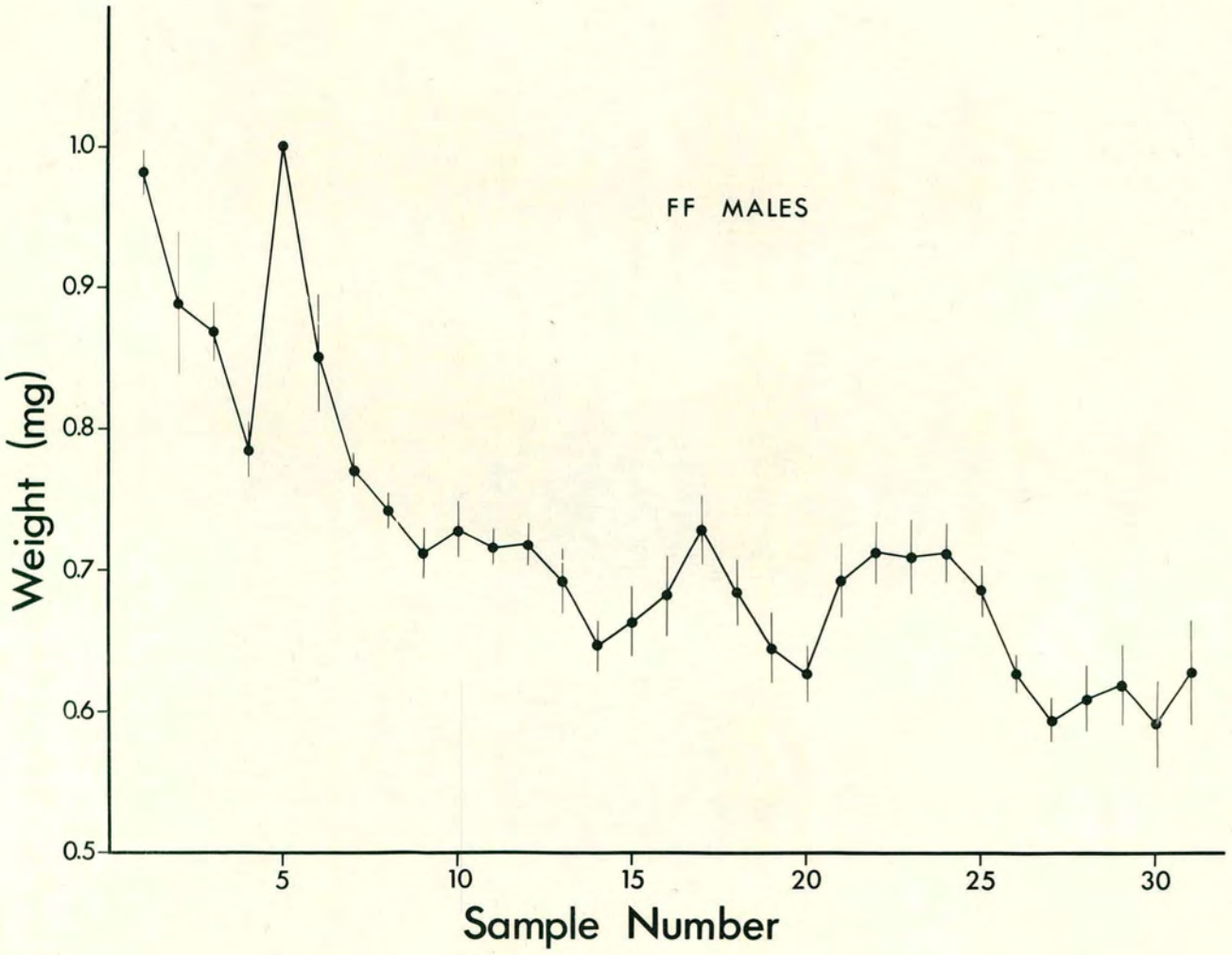
Frequencies of the three Adh genotypes in male (Fig. V.3) and female (Fig. V.4) flies over the entire eclosion period from standard food pots. Samples were taken at 18-hour intervals.

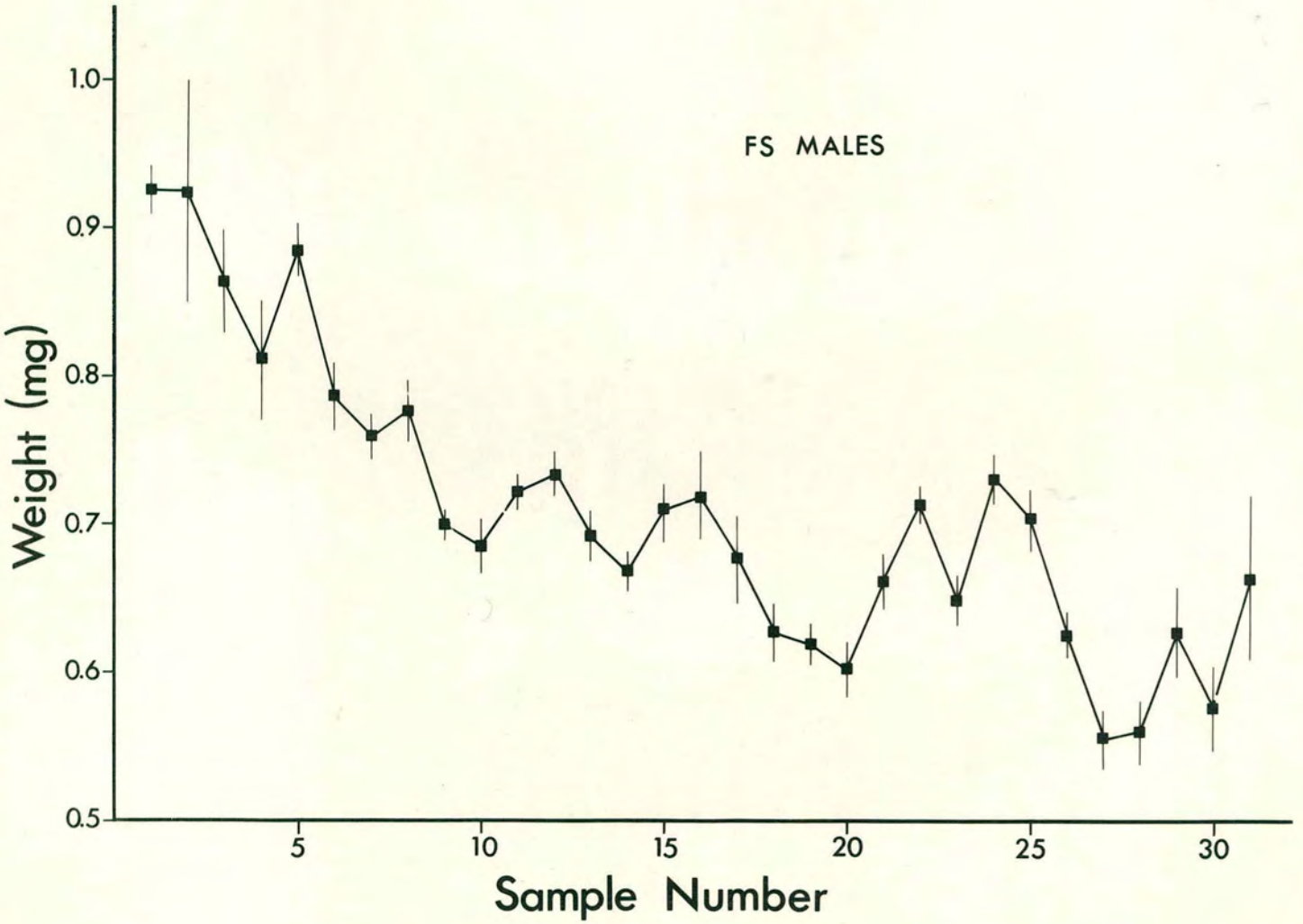

MALES
FF
FS
SS
GENOTYPE FREQUENCY
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
25
30
SAMPLE NUMBER

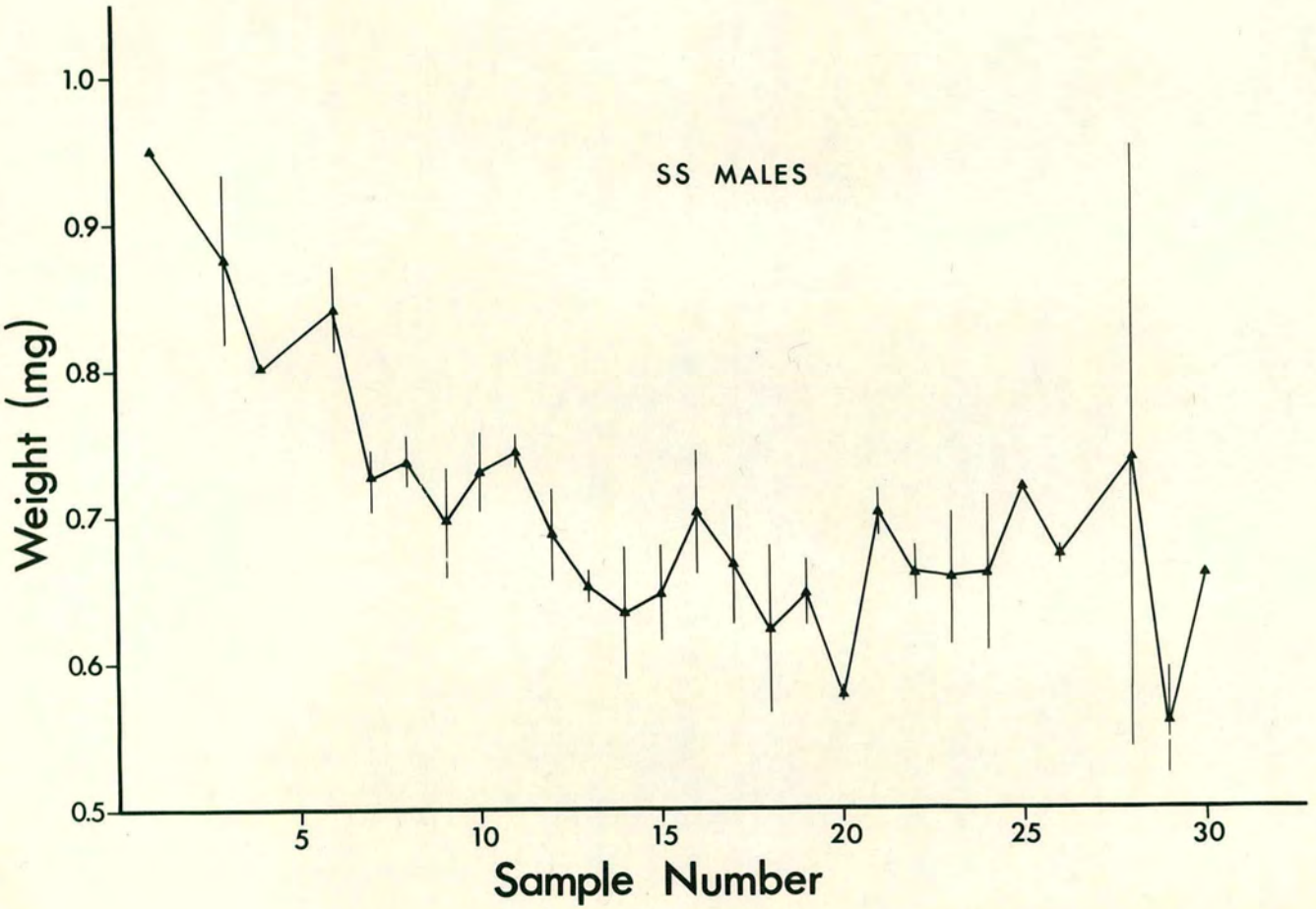

FEMALES
FF
FS
SS
GENOTYPE FREQUENCY
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
25
30
SAMPLE NUMBER

Figures V.5 a, b, c.

Individual weights (± s.e.) of male flies of the three Adh genotypes in 18-hour samples taken over the entire eclosion period from standard food pots.

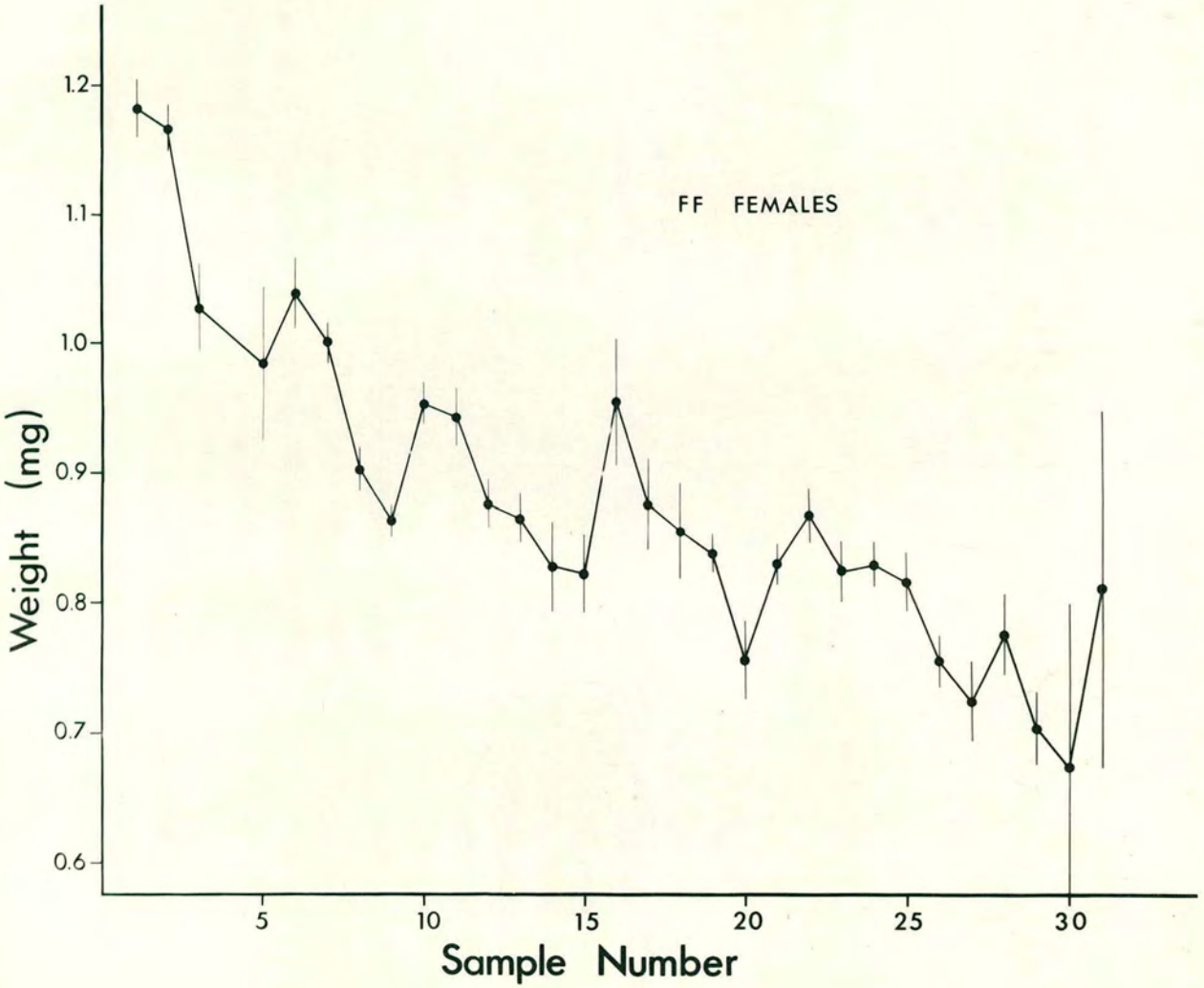

FF MALES
Weight (mg)
1.0
0.9
0.8
0.7
0.6
0.5
5
10
15
20
25
30
Sample Number

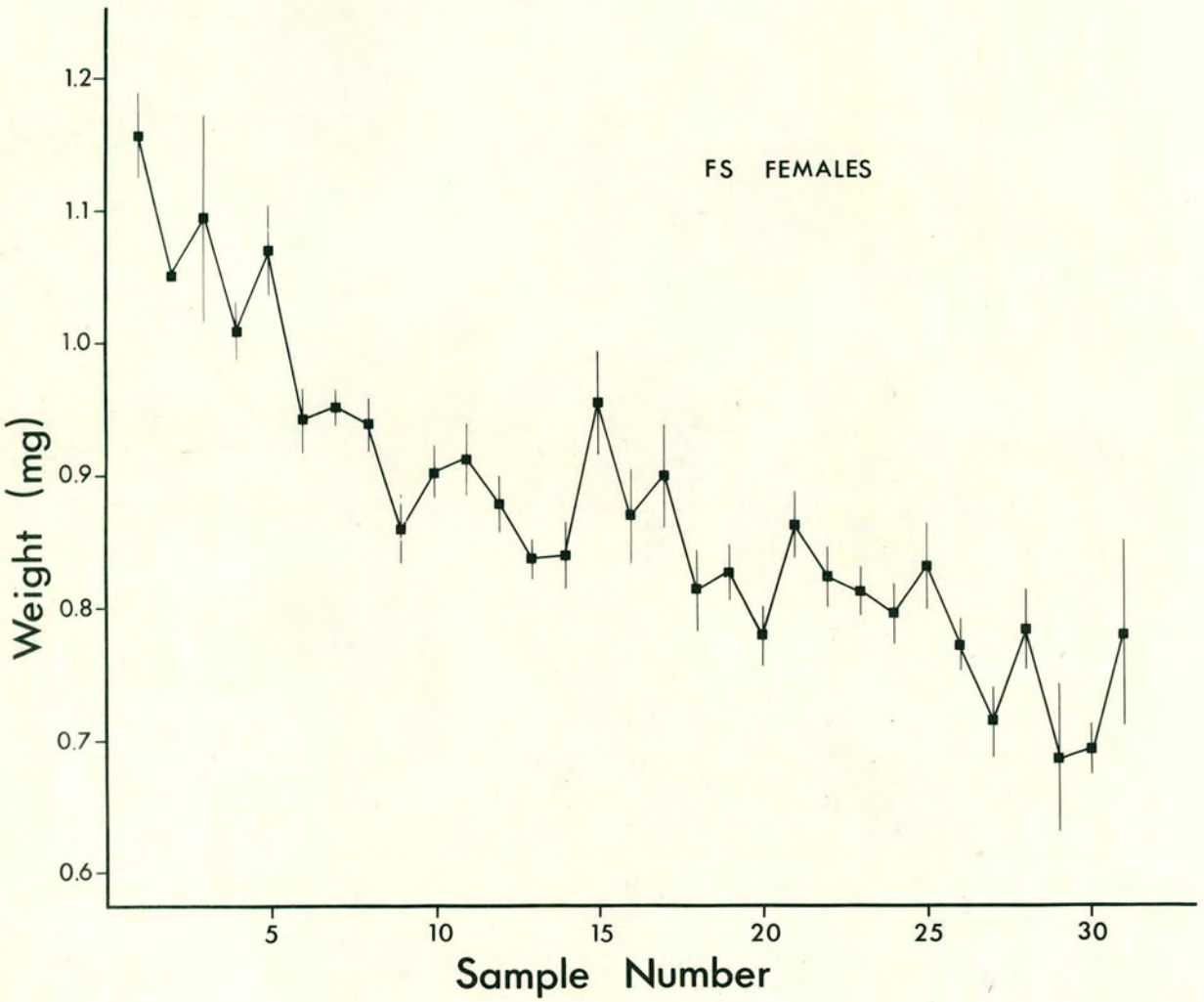

FS MALES
Weight (mg)
1.0
0.9
0.8
0.7
0.6
0.5
5
10
15
20
25
30
Sample Number

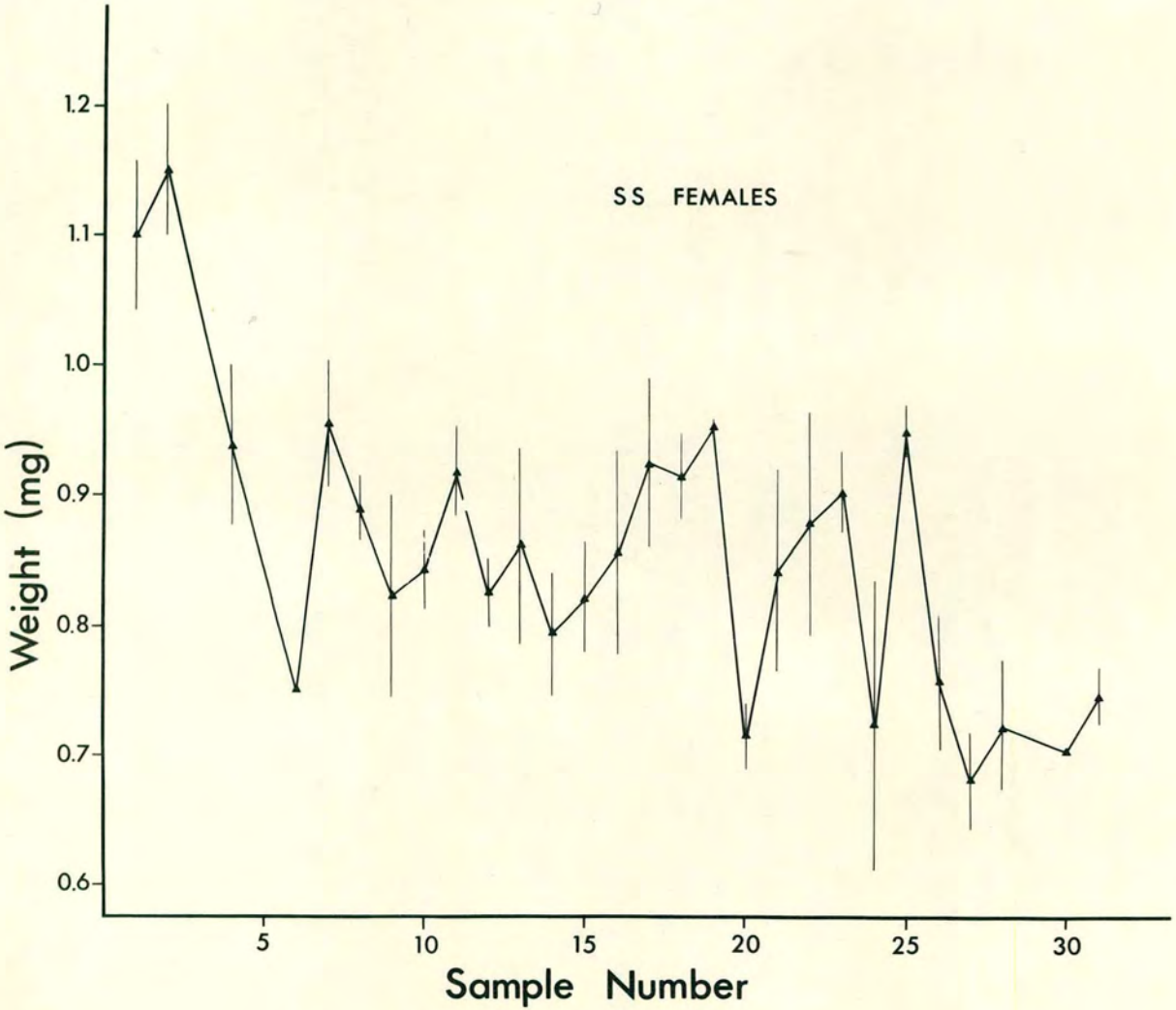

SS MALES
Weight (mg)
1.0
0.9
0.8
0.7
0.6
0.5
5
10
15
20
25
30
Sample Number

Figures V.6 a, b, c.

Individual weights (± s.e.) of female flies of the three Adh genotypes in 18-hour samples taken over the entire eclosion period from standard food pots.


FF FEMALES
Weight (mg)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
5
10
15
20
25
30
Sample Number


FS FEMALES
Weight (mg)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
5
10
15
20
25
30
Sample Number


SS FEMALES
Weight (mg)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
5
10
15
20
25
30
Sample Number

regime a food pot is removed after 21 days in the population cage. Therefore only those adults emerging in samples 1-17 would normally contribute to the next generation.

The results of this experiment were disappointing since they in no way fulfilled the *a priori* expectations given above.

The gene frequency of the F allele declined during the first samples to a low point in sample 4 in females and sample 6 in males. This decline is not significant and is most likely to be a reflection of the small numbers of individuals in these samples. In the remaining samples the frequency of the F allele fluctuated at random about a mean value of $q(F) = 0.66$. Similarly, genotype frequencies showed departures from Hardy-Weinberg expectations only in those samples where total numbers were small. The slight downward trend in ♂ SS genotype frequency in later samples (Fig. V.3) is not significant. As the gene and genotype frequencies did not alter significantly over time the data for each sex has been pooled (Table V.4).

The total gene and genotype frequencies do not differ significantly between sexes. Further, the deviations of observed numbers of each genotype from Hardy-Weinberg expectations are extremely small, although in both sexes there is a very slight excess of heterozygotes. There is thus no evidence of differential egg-adult viability between the three Adh genotypes when cultured in a standard food pot. This conclusion is consistent either with the hypothesis that selection does not act upon the Adh polymorphism in this environment or, alternatively, with the hypothesis that the polymorphism is maintained by a frequency-dependent mode of selection such that the viabilities

Table V.4: Total Numbers of each genotype surviving from food pots, all collections pooled.

	♂♂ Genotypes			Total	q(F)
	FF	FS	SS		
Observed	713	731	171	1615	0.6678 ±0.0083
Expected*	720.2	716.6	178.2	1615	
	♀♀ Genotypes				
Observed	703	739	184	1626	0.6596 ±0.0083
Expected*	707.4	730.2	188.4	1626	

* Hardy-Weinberg expectations.

of the three genotypes were equal in this equilibrium culture (see Chapter IV.[E]).

The mean frequency of the F allele among the survivors is significantly higher than that usually observed in the adult population in the cage (Chapter IV.[B]). This difference may be attributable to the fact that adults sampled at random from the cage represent the survivors of post-eclosion mortality. In the present study adults were collected and typed for Adh within 18 hours of emergence and therefore represent the pre-mortality adult population.

The mean weights of both male and female flies declines in a regular manner in later samples (Figs. V.5 a-c; V.b a-c). However, there is no significant difference between the weights of the three genotypes at any stage in the emergence period.

The problem remains of equating the observed negative results with the expectations formulated on the basis of previous experiments. These expectations were based upon experiments conducted in vial cultures under conditions of crowding. Kinross (personal communication) has shown that nearly 40% of the eggs layed on a food pot during its first day in the population cage survive to adulthood. It may therefore be argued that competition in a food pot is not as severe as in vials, and that viability differences observed in vials will be too small to measure in food pots.

Finally, it must be pointed out that the food pots studied in the present experiment differed from those in the standard population cage in one important respect. In the population cage adult flies continue to feed and lay eggs on the medium for most of its time in the cage, whereas in the present study the food pots were removed

from these influences after only five days. It is possible that these influences may be of importance to the relative survivals of the Adh genotypes.

C. Selection at the Adh locus under an alternative culture regime

During the course of a study of possible stabilising selection acting upon sternopleural-chaeta number J.G.C. Speirs replicated a large number of adults from the standard Kaduna population into an identical population cage in which only the regime of food supply was altered. In this population (designated Kaduna II) the weekly food pot is replaced by the addition every second day of two 3 x 1 inch vials each containing 5 ml of medium. Exhausted food vials are removed from the population cage after 3 weeks. Under this culture regime competition between larvae is severe, body size is reduced and the population size is between 2,500-3,000 adults.

This population cage provided material for the study of possible selection at the Adh locus in the Kaduna population in an environment which differed radically from that of the standard cage.

Approximately one year after the founding of the Kaduna II population adult flies were sampled from the cage and analysed for Adh genotype. The numbers of each genotype observed in this sample and four subsequent samples taken at monthly intervals are presented in Table V.5.

Between-sample heterogeneity within sexes is not significant (males $\chi^2_{(8)} = 6.1$; $p > 0.5$: females $\chi^2_{(8)} = 8.9$; $p > 0.3$). However, gene and genotype frequencies differ markedly between males and females. This difference is consistent over all five samples and is highly

Table V.5: Numbers of each genotype observed in samples from the Kaduna II population, taken at monthly intervals.

Sample	Adh Genotype							
	♂				♀			
	FF	FS	SS	q(F)	FF	FS	SS	q(F)
1	14	32	26	0.417	22	34	16	0.542
2	16	48	32	0.417	40	44	16	0.620
3	8	20	17	0.400	14	25	6	0.589
4	36	79	52	0.452	36	60	29	0.528
5	11	28	9	0.521	15	28	5	0.604
Pooled	85	207	136	0.440	127	191	72	0.571
Exp.*	82.9	210.9	134.2	0.440	127.2	191.1	71.7	

* Numbers expected from Hardy-Weinberg proportions for the gene frequency observed within each sex.

Note: Although the male/female sex ratio in all samples was approximately 2.0 roughly equal numbers of each sex were used for electrophoresis.

significant ($\chi^2_{(2)} = 23.5$; $p < 0.001$, comparing pooled maled with pooled females). It is noteworthy that the contribution made to chi-square by between-sex differences in heterozygote frequency is extremely small (0.015). Thus the selection which gives rise to the differences between males and females must act differentially only upon homozygotes. The final row in Table V.5 reveals that selection acts in such a way that the genotype frequencies within a sex simulate Hardy-Weinberg proportions for the gene frequency within that sex.

The results presented in Table V.5 are surprising both in their magnitude and consistency. It is clear that the new environment and extreme larval competition imposed by this regime of maintenance have revealed novel selective forces acting upon the Adh polymorphism which are not evident under standard cage conditions.

On the basis of these observations a simple algebraic model has been constructed to represent the action of natural selection upon the Adh locus in the Kaduna II population. Three factors were taken into account in the formulation of this model.

(i) Adh is an autosomal locus. It was therefore assumed that zygotic genotype frequencies were identical in the two sexes.

(ii) Sample 4 in Table V.5 was obtained by collecting, over a period of one week, adult flies as they eclosed from the food vials in the population cage. As this sample is homogeneous with the other four samples which were taken by sampling the flying adult population, it was concluded that selection acted between fertilisation and eclosion.

(iii) In order to be compatible with the observed data the mode of selection must act such that post-selection genotype frequencies

within a sex mimic Hardy-Weinberg proportions for the gene frequency within that sex. Further, selection must act such that the post-selection genotype frequency of heterozygotes does not differ significantly between sexes.

The outlines of the simplest model which fulfils these requirements are given in Table V.6. The zygotic genotype frequencies, although equal in the two sexes, do not necessarily obey the Hardy-Weinberg law as gametic gene-frequencies may differ between sexes. Fitness functions are additive within a sex but reversed in direction in the two sexes. Thus the model is symmetrical when the sexes are pooled and can be shown to maintain a single stable equilibrium point of $q(F) = 0.5$ for the population, with an excess of heterozygotes in zygotes. The observed mean value of $q(F) = 0.5055$ in the population agrees well with this predicted equilibrium value, (where the observed mean value is simply the arithmetic mean of the gene frequencies in adult males and females).

Computation of the model using the Edinburgh Multi-Access System facility confirms the stability of the gene-frequency equilibrium (Fig. V.7).

In this figure the return of gene frequency towards the equilibrium point is illustrated in two theoretical populations started at $q(F) = 0.60$ and $q(F) = 0.40$ respectively. It can be seen that the return towards the equilibrium value is relatively slow as the only force driving the gene frequencies is the small excess of heterozygotes produced at fertilization each generation.

At equilibrium $\Delta x = 0$ and, since the model is symmetrical $x + y = 1$. Substitution of these values into equation (1) (Table V.6)

Table V.6: A model of the action of natural selection in the Kaduna II population.

	♂♂			♀♀		
	FF	FS	SS	FF	FS	SS
Zygotic frequency	xy	x(1-y) + y(1-x)	(1-x)(1-y)	xy	x(1-y) + y(1-x)	(1-x)(1-y)
Fitness	1-2S	1-S	1	1	1-S	1-2S

$$\Delta x = \frac{-2Sx^2 + x(1+S) + y(S-1)}{2(1-S(x+y))} \qquad (1)$$

$$\Delta y = \frac{2Sy^2 - y(2S-1) + x(S-1)}{2(1-S(2-x-y))} \qquad (2)$$

where x = gametic frequency of F allele in ♂♂

y = " " " " " " ♀♀

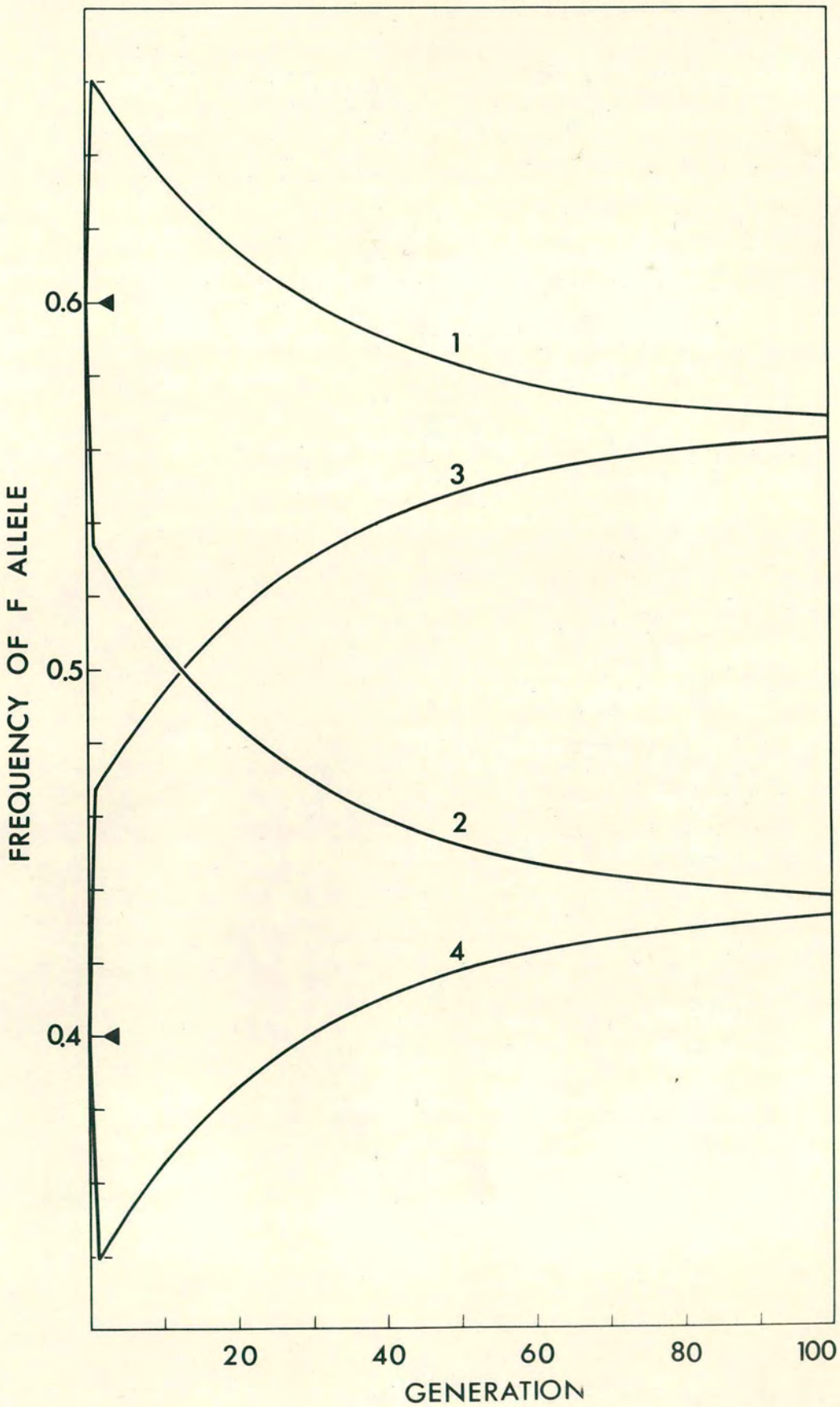
Fig. V.7

Computation of the return of gene-frequencies towards equilibrium values on the model of selection proposed for the Adh locus in the Kaduna II population.

S = 0.21

1. Frequency of F allele in ♀♀. Initial frequency 0.6
2. " " " " ♂♂. Initial frequency 0.6
3. " " " " ♀♀. Initial frequency 0.4
4. " " " " ♂♂. Initial frequency 0.4

Note For simplicity genotype frequencies in both sexes were assumed to be equal, and in Hardy-Weinberg proportions, at generation 0.


FREQUENCY OF F ALLELE
0.6
0.5
0.4
1
2
3
4
20
40
60
80
100
GENERATION

allows the derivation of an expression for the value of S required to maintain a particular departure of post-selection male gene frequency from the equilibrium value of q(F) = 0.5 at each generation:

$$S = \frac{1-2x}{1-2x^2}$$

In the Kaduna II population x = 0.44, thus S is approximately 0.2. Computation of numerical examples using this value of S and observed values of x and y confirms that post-selection genotypic frequencies within a sex mimic closely the expected Hardy-Weinberg frequencies for the gene frequency observed within that sex. Similarly this mode of selection results in the post-selection frequency of heterozygotes being equal in males and females. Thus, this simple model adequately describes a mode of selection capable of maintaining the Adh polymorphism which is compatible with the observations made in the Kaduna II population.

Although this mode of selection agrees remarkably well with the observations, the biological validity of its assumptions are open to question. The model assumes that the gametic pool which founds the next generation reflects exactly the gene frequency in the adult population, i.e. it assumes that the genetic contribution of each genotype is strictly in proportion to its frequency in the adult population. This assumption is open to challenge until the sexual structure of the population has been determined. The assumption of additivity of selective values may also be challenged. However, it would seem plausible that selective values are additive at those polymorphic enzyme loci where the heterozygote displays an enzyme

activity midway between that of the homozygotes (Chapter III).

The magnitude of the selective coefficients invoked in this study are surprisingly large. A genetic death of approximately 40% of the disadvantaged homozygote is required in order to maintain the observed difference between sexes. This level of genetic death may be sustained in a population such as Kaduna II where larval competition is extreme, but must again raise the question whether a single locus or selectively-maintained blocks of genes marked by the Adh alleles, is under investigation.

It is only possible to speculate upon the possible environmental factors which gave rise to this mode of selection. That intense larval crowding exerts a differential stress upon the two sexes is evidenced by the observation that the male/female sex ratio in this population is approximately 2. This is true both for the samples of adults taken at random from the cage and for the single sample (number 4) obtained by collecting adults as they eclosed from the food vials. Further, the crowding in the food medium accelerated the normal course of environmental change as reflected in pH. The pH of the medium during the first six days of the life of a food vial remained at the low level characteristic of medium in food pots (pH 4.25; Fig. V.1), but rose rapidly to pH 7.6 between days 6 and 7. Thus all individuals surviving to adulthood must experience conditions of high pH at least during late-larval and pupal stages. It is therefore of interest to note that the correlations between pH and the relative survival of the three genotypes (Table V.2) are in the same direction as the results presented here; high pH favouring FF females and SS males.

Chapter Conclusion

In this chapter the Adh polymorphism has been studied in two experimentally manipulated environments (ethanol medium and altered culture regime) and an attempt has been made to correlate egg-adult survival of the genotypes with a natural temporal change of environment in the base Kaduna population cage. The latter attempt failed to detect any differences in overall survival, emergence time or body weight between Adh genotypes raised in standard food pots. However, both of the experimentally manipulated environments elicited significant responses in gene or genotype frequencies. Under conditions of high environmental alcohol a directional shift in favour of the F allele was observed, as was predicted on the basis of a knowledge of the enzymatic activities of the three genotypes. Conditions of intense larval competition elicited an alteration of the equilibrium gene frequency and revealed a selective mechanism capable of maintaining the polymorphism which was not evident under the normal culture conditions of the Kaduna population.

These results highlight the necessity of accurately defining the environmental conditions under which the stability of a polymorphism is investigated. Further, the results suggest that a polymorphism may be apparently selectively neutral in some environments and yet demonstrate clearly the effects of the action of natural selection in alternative environments.

General discussion

Although the neutral mutation-random drift hypothesis of protein evolution was derived largely from *a posteriori* analyses of the substitution rates of alleles, it is generally accepted that studies of contemporary protein polymorphisms offer the best opportunity of discriminating between this hypothesis and the opposing neo-Darwinist concept of evolution. In Chapter I the contrasting predictions of the two hypotheses were condensed, somewhat arbitrarily, into three main questions which may be asked in any study of protein polymorphism. The present study of the alcohol dehydrogenase polymorphism in the Kaduna population of *Drosophila melanogaster* was broadly-based in an attempt to answer these questions. Firstly, do the alternative allo-enzymes display significant functional differences, secondly, are there consistent patterns in the temporal distribution of the genotypes, and thirdly, is there direct evidence of the action of natural selection and what form does that selection take?

In general the results presented favour the "selectionist" viewpoint and are incompatible with the hypothesis that protein polymorphisms merely represent selectively neutral isoalleles drifting at random through populations on their way to fixation or elimination.

The finding of significant differences in kinetic parameters between Adh allo-enzymes (Chapter III) provides strong evidence against the belief that the amino-acid substitutions involved in protein polymorphism are insignificant in terms of functional parameters. Further, it has been demonstrated that enrichment of the food medium with ethanol favours the survival of individuals carrying the allele which specifies

the ADH enzyme with the highest *in vitro* alcohol-oxidising activity (Chapter V.A). It must be concluded that the Adh alleles do differ sufficiently that natural selection, "the editor", (King and Jukes, 1969) is capable of discriminating between alternative genotypes.

More direct evidence of differences in fitness components between Adh genotypes has been provided by the consistent temporal patterns of genotypic frequency in male flies in the base population (Chapter IV.B), by the return towards an equilibrium gene-frequency value in perturbation experiments and the trend towards frequency-dependent viabilities in the egg-adult survival test (Chapter IV.C and E), and, most dramatically, by the differential selection between the sexes in the Kaduna II population (Chapter V.C).

While these results indicate that the Adh locus is subject to the action of natural selection the mode of selective maintenance of the polymorphism appears to be complex. The magnitude of the selective forces, and, indeed, the form of selection, were found to be heavily dependent upon the environmental conditions under which the observations were made. Thus, while no evidence of the action of natural selection was obtained from a study of the genotypes surviving from standard food pots (Chapter V.B), vial cultures provided some evidence of differences in the egg-adult viability of the three genotypes (Chapter IV.E). Further, ethanol-enriched medium exerted a stress which significantly favoured the F allele (Chapter V.A) and the high-competition conditions in the Kaduna II population cage revealed a novel mode of selection which was not evident in the standard population cage environment (Chapter V.C). In addition to these environmental parameters, Bijlsma and van Delden (1972) have shown that high relative humidity favours

the survival of individuals carrying the S allele.

In the context of environmental heterogeneity the sex of the carrier of a particular Adh genotype may be considered to represent a major discontinuity in the "environment" of that genotype. This hypothesis is supported by the observations that the temporal cycle in genotypic frequencies was restricted to males, while responses to ethanol, and possibly to an environmental parameter associated with pH, were more pronounced in female flies. In the high-competition Kaduna II population the S allele was favoured in male individuals but the F allele was favoured in females.

The operation of selection through ecological factors has been demonstrated in a number of other polymorphisms. For example, the frequencies of alleles at the amylase locus in Drosophial melanogaster populations were found to be dependent upon the amount of starch in the food medium supplied to the populations (de Jong et al, 1972), while heterosis at the octanol dehydrogenase locus of D.pseudoobscura was evident only when octanol was included in the food medium (Wills, and Nichols, 1972). Similarly, Lewontin (1958a) and Powell (1971) have attributed the loss of genetic variability, observed when natural populations are transferred to population cages, to the reduction of environmental heterogeneity in the laboratory environment.

Recently, Ogah and MacIntyre (1972) have speculated that some enzyme polymorphisms may be maintained by environmental conditions which are only periodically encountered. In this situation selection will be intermittent with intervening periods of apparent selective neutrality. The strength and direction of the forces acting during the selective phase may well depend upon the exact nature of the

environment encountered at that time.

These considerations suggest that it may be unreasonable to expect that the selective mechanisms maintaining particular polymorphisms will conform neatly to a single model of selection. Rather, the magnitude and direction of differences in fitness between genotypes may change markedly from time to time or from place to place according to the specific ecological conditions encountered by each population. Ecological factors may be especially important in the maintenance of polymorphisms which involve enzymes, such as alcohol dehydrogenase, which are thought to derive their substrates directly from the environment.

It has been assumed throughout this study that the alcohol dehydrogenase polymorphism in the Kaduna population of Drosophila melanogaster is a representative example of the general phenomenon of biochemical polymorphism. There are reasons for questioning this assumption.

The Kaduna population has been maintained for many years as a large, closed laboratory stock in a relatively constant environment. It is therefore possible that the only polymorphisms retained in this population are those which are selectively maintained in the laboratory environment. The removal of immigration may have resulted in the loss of those selectively neutral polymorphisms which might normally be preserved by random genetic drift within populations and migration between populations. This view is partially substantiated by the recent finding of J.M. Malpica and the author that old laboratory populations are less genetically variable than wild populations, but that the Adh and Esterase-6 loci are invariably still segregating in these populations. These two loci may therefore be exceptional cases but are, unfortunately, among the most intensively studied examples of

protein polymorphism in *Drosophila*.

The magnitude of the selective forces implicated by this study pose a dilemma. In the Kaduna II population selection coefficients of approximately 0.4 against one homozygote are required to preserve the observed difference in gene-frequency between adult males and females at each generation. Very few polymorphisms with selection coefficients of this order could be maintained in a population unless the coefficients are associated with blocks of genes linked in disequilibrium rather than with single loci. This hypothesis can only be tested for the Adh locus when sufficient natural marker loci close to the locus become available (see Chapter IV.D). Alternatively it may be argued that, in the relatively homogeneous genetic background of the Kaduna population, the effects upon fitness of the few remaining segregating loci may be disproportionately large.

Despite these reservations the results presented, together with those of similar studies reported in the literature, do indicate that at least a proportion of biochemical polymorphisms are under the influence of the forces of natural selection, and may contribute to the flexibility of response of populations to changing, and often extreme, environments.

SUMMARY

1. The opposing hypotheses of neutral mutation-random drift and neo-Darwinian evolution have been discussed in the context of the finding of ubiquitous protein polymorphism in many species. It has been proposed that the controversy may only be resolved by experimental studies of the forces acting upon contemporary representative polymorphisms.

2. The alcohol dehydrogenase polymorphism in the old-established Kaduna laboratory population of Drosophila melanogaster has been studied to ascertain whether natural selection acts at this locus and what form that selection may take.

3. In vitro assays have shwon that the enzymatic activity of alcohol dehydrogenase from FF homozygotes is approximately twice that of SS homozygotes and that heterozygote activity is intermediate. That these functional differences may be of importance in the determination of the fitnesses of the genotypes in certain environments has been demonstrated by the superior survival of FF individuals in ethanol-enriched food media.

4. Although the equilibrium gene-frequency in the population cage remained constant throughout the period of study a consistent weekly oscillation in the genotypic frequencies was found in adult male flies. It is suggested that this finding is incompatible with the view that the Adh alleles are equivalent in the determination of fitness components.

5. Gene-frequency perturbation experiments indicated that some form of balancing selection maintains the equilibrium gene-frequency in the population. Further, in egg-adult viability tests there was a trend towards the survival of the FF genotype being related to its initial frequency in a culture. This finding lends support to the hypothesis that frequency-dependent modes of selection may be important in the maintenance of the Adh polymorphism.

6. The response of the Adh polymorphism to natural changes in ecological conditions in the population cage and to experimentally manipulated environments was examined. The magnitude and form of differences in fitness between genotypes were found to be markedly dependent upon the environmental conditions under which observations were made, and upon the sex of the carrier of a genotype.

7. It was concluded that the alcohol dehydrogenase polymorphism in Drosophila melanogaster is influenced by the forces of natural selection, and that components of environmental heterogeneity play an important role in the maintenance of the polymorphism.

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