Geographical and temporal variation of biochemical and colour-pattern polymorphisms in the European moth, *Noctua pronuba* (L.).

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Abstract:

Many small, numerically abundant animals of low trophic position exhibit polymorphisms for colour and pattern. This variation is assumed to be adaptive and maintained by frequency-dependent selection, the fitness of phenotypes being negatively correlated with their frequency in the population.

Noctua pronuba is an abundant moth species of the western Palaearctic and is now established in north eastern America. When the moth is at rest it is apparently cryptically coloured with the visible surfaces polymorphic in colour and pattern. The limitation of this variation to the exposed surfaces of the moth suggests that the variation may be maintained by visual selection by predators.

The forewing polymorphism in *N.pronuba* can be split into three distinct phenotypes: rufous, ochre and silver, with the variation probably controlled by a single locus, with three alleles in a dominance hierarchy. The expression of these alleles is influenced by sex with females lighter in colour than males. The aim of this work was to establish whether natural selection maintains variation in forewing colour and pattern to give a balanced polymorphism, using the null hypothesis that the variation was neutral to selection and non-adaptive. This has been approached by experiment and the analysis of temporal and geographic variation in forewing phenotype frequencies.

Background resting experiments failed to show different phenotypes adopting different backgrounds on which to rest but experimental conditions did affect the behaviour of individuals. Selection acting in opposite directions on males and females has been suggested as a mechanism maintaining the forewing polymorphism. Little evidence has been found to substantiate this claim with phenotype frequencies in light trap samples similar in males and females. No systematic changes in phenotype frequencies were observed in an analysis of temporal variation over a twenty-five year period. Only the ochre allele varied significantly but the variation is minimal suggesting that the polymorphism is temporally stable.

Geographically there was remarkably little variation in phenotype frequencies with only samples from Finland, Scotland and N.Ireland having significantly differentiated phenotype frequencies.

A study of polymorphic allozymes suggested that large amounts of gene flow occur in the species. The consequence of this gene flow will be to unite geographically separate populations into one panmictic unit. High levels of gene flow, in conjunction with the local abundance of the species, mean that the effective population size will be large.

Previous authors have considered that as crypsis is an adaptive trait, variation in the colour and pattern of a cryptic species must also be adaptive, and maintained by selection. This is not necessarily true, and there may be a number of colour patterns that are equally cryptic in the same habitat. It is hypothesised that the large population size and magnitude of gene flow in *N.pronuba* gives the forewing polymorphism inherent stability both temporally and geographically, without the need to invoke balancing selection.

Chapter 1: Polymorphism:

1.1: Polymorphism:

Polymorphism is the coexistence of two or more dissimilar genetic forms within the same population, or as Ford (1940) originally defined: "the occurrence together in the same locality of two or more discontinuous forms of a species in such proportions that the rarest of them cannot be maintained by recurrent mutation."

Population geneticists define a gene locus as polymorphic if the commonest allele at that locus has a frequency of 0.95 or less (Hartl & Clarke 1989). The frequency of 0.95 is an arbitrary cut-off point but it does exclude those loci with rare alleles at low frequency. This is in accordance with Ford's original definition as loci cannot be polymorphic solely because of recurrent mutation, unless the mutation rate is extraordinarily high.

Historically, within the fields of genetics and ecology, polymorphic variation has received much attention from researchers. This interest has arisen from single locus and two loci models being used to theoretically predict the effect of a range of parameters on gene and genotype frequencies in populations. Before the advent of protein electrophoresis only visible polymorphic characteristics were open to ecological investigation and comparison with models of population structure. Early examples of such investigations are: colour and banding polymorphisms in *Cepaea* spp. (Cain & Sheppard 1954); wing coloration polymorphisms in *Panaxia dominula* (Fisher & Ford 1947), and *Biston betularia* (Kettlewell 1957, 1958); and chromosome inversion polymorphisms in *Drosophila* spp. (Wright & Dobzhansky 1946).

Three processes can explain the existence of polymorphisms. These are:

1) A balance between selection and mutation.

2) Genetic drift acting on neutral alleles.

3) A balance of selective forces.

The existence of a polymorphism may indicate a balance of selective forces maintaining the two or more forms in the population. This is only true if the polymorphism *is* balanced, and Ford (1975) has drawn a distinction between balanced polymorphisms and transient polymorphisms. A mutation that produces a gene with a selective advantage over other alleles at that locus will spread throughout a population until it reaches fixation (has a frequency of 1) and all the other alleles are displaced. There will be a time when the population is polymorphic, the length dependent upon the selective advantage of the mutant allele over the normal allele.

Ford (1975) considered transient polymorphisms to be rare, as advantageous genes will have already been incorporated into the organisms genome. A mutant allele, with a small selective advantage which is completely recessive to the wild type, will take many generations to increase in frequency. Consequently some polymorphisms thought to be balanced may in fact be transient and, over the time period usually studied, gene frequencies may be imperceptibly changing.

Transient polymorphisms have been essentially ignored by ecological geneticists and the first assumption made in many theoretical and experimental studies is that the allele or morph frequencies are at equilibrium. This simplification has been criticised by Lewontin (1974) and he considered the "ecological genetics" school to be " frankly partisan in their belief that polymorphism is in general balanced." If one is considering the maintenance of polymorphisms within a population it is important to establish whether it is in fact at equilibrium and not at a transitory stage.

1.2: Selective mechanisms that maintain polymorphisms:

If the polymorphism is at a stable equilibrium there must be a balance of selective forces maintaining the different alleles within the population (Cook 1971). Stable equilibria are gene frequencies that if perturbed return to the stable equilibrium position, alternatively neutrally stable equilibria are gene frequencies that if perturbed do not return to the previous equilibrium position but remain at the perturbed gene frequency. Balanced polymorphisms (*sensu* Ford) are maintained by selection and so are thought to be stable equilibria. To maintain these stable equilibria a special type of selective force is needed to maintain this variation, namely one where there is a conflict between two forces acting on the same locus. There are several categories of selection that fulfil this criterion.

1.2.1: Heterozygote advantage:

This is an interaction between selection favouring the heterozygote and the process of Mendelian segregation which throws up the relatively less fit homozygotes (Fisher 1930). This mechanism has been proposed to maintain the banding and colour polymorphism in *Cepaea* (Cain & Sheppard 1954) and the melanic and typical forms of *Biston betularia* (Haldane 1956, Ford 1975). Unfortunately, in both of the above examples the heterozygote is not visible as there is complete dominance. Any explanations involving heterosis in polymorphisms that are completely dominant should be treated with care as the identification of the heterozygotic class is not possible.

The greater relative fitness of the heterozygote is still the most common mechanism proposed to maintain polymorphisms even though there is little direct evidence to substantiate this. Endler (1986), in his comprehensive survey of natural selection, found only six examples where heterosis was the major factor maintaining polymorphisms in wild populations: the butterfly

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Colias philodice; the grouse *Dendragapus obscurus*; humans, *H.sapiens*; pocket gophers, *Geomys* spp.; barley *Hordeum vulgare* and the monkey flower *Mimulus guttatus* (Endler 1986 and references therein).

It is possible, though, when all the fitness components throughout the life history of an organism are considered. that there is an overall *net* heterozygote advantage (Lerner 1954, Mitton & Grant 1984). The study of life-time fitness is difficult and often only a few age classes are investigated. Most studies of the adaptive significance of heterozygosity have involved correlations with characters that are deemed to affect life time fitness.

Eanes (1978, 1981) showed that the monarch butterfly, *Danaus plexippus*, when heterozygous for one of six metabolic enzymes, had lower variance estimates for two morphological characters (forewing length and forewing spot) than homozygotes. Other studies have shown that heterozygotes are more symmetrical (Leary *et al.* 1984) and have faster growth rates (Koehn & Shumway 1982). In contrast, studies have shown an apparent independence between protein heterozygosity and life-history traits (Hutchings & Ferguson 1992, Elliot & Pierce 1992, Whitlock 1993). Whether overall heterozygosity generally affects fitness is still debatable.

1.2.2: Heterogeneous environments:

Polymorphisms may be maintained by selection in a variable environment, where the fitness of each genotype is dependent on the environmental patch it is found in. The relationship between a species lifetime mobility and patch size is of utmost importance; the environment must be coarse-grained, rather than fine-grained, relative to the mobility of the species. A coarse-grained habitat is one where patch size is large relative to the mobility of an individual. In such an environment, individuals mainly encounter one patch type throughout their lifetime. In fine-grained environments all individuals

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experience, on average, the same set of different environmental patches and so fitness would be constant between patches. The distinction between coarse-grained and fine-grained habitats can be alternatively explained in terms of the variance in patch heterogeneity. Two environments may have the same variance, but in a fine-grained environment the majority of the variance would be within the area occupied by an individual; whereas in a coarse-grained environment it would be between areas occupied by individuals.

Polymorphisms can be maintained by variable environments, but the type of selection acting on populations determines the relative importance of the parameters involved. Natural selection can be split into two types: hard and soft (Wallace 1968). Soft selection is density-dependent; the population size remaining constant and competition occurring between individuals. In contrast, hard selection leads to variable population size and does not imply density-dependence. Dempster (1955) showed that under hard selection, environmental heterogeneity only protects a polymorphism, giving a stable equilibrium point, when the heterozygote is of greater fitness than both homozygotes, the fitnesses averaged (arithmetically) across all patches. Thus the outcome does not differ significantly from heterozygote advantage as a mechanism maintaining polymorphisms.

The soft selection model of Levene (1953) produced quite different results. To maintain a polymorphism it does not require the arithmetic mean fitness of the heterozygote to be greater than those of both homozygotes. For the equilibrium to be stable, unless the fitness advantages per locus are large, the fitness is required to be adjusted to the niche size. This means that if the relative niche sizes vary the fitnesses must also vary. For small or intermediate selection advantages (<10%) the range of niche sizes over which the polymorphism is stable is small. This lack of robustness, coupled

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with its assumption of soft selection, may mean that selection in variable environments is not a common mechanism maintaining polymorphisms (Maynard-Smith 1966, Maynard-Smith & Hoekstra 1980).

Models that include variable selection in space, habitat selection, and limited gene flow, may escape the above criticisms, but the stability of the equilibrium produced by such processes is still questionable; it may only lead to a neutral equilibrium. This delays the loss of the polymorphism, rather than prevents it and unless some other selective factor acts to maintain the polymorphism it is lost by random genetic drift (Endler 1988).

Experimental work with *Drosophila* shows that heterogeneous environments do lead to either a maintenance of genetic variability or, at least a reduction in its rate of loss (Jones & Probert 1980, Powell 1971). It is possible that a stable equilibrium only results from selection in a heterogeneous environment if it is coupled to frequency-dependent predator behaviour (Cook 1986).

Bishop and Cook (1975) proposed a situation that might operate on *Biston betularia.* In some locations, where there is a polymorphic population, the lichens may be distributed in patches and individual moths settle and move onto the background against which they are least conspicuous. If there is competition for resting sites (i.e. density is a critical factor determining the degree of camouflage) then this model would maintain a polymorphism without the need to incorporate heterosis. When the frequency of one form or the other has diverged from the equilibrium frequency, the moth form in excess would be forced to take up positions which render it conspicuous and so be eliminated by predation. Hence the equilibrium would be restored. This is similar to Levene's soft selection model but is more robust because of habitat selection by the moths; typical *B.betularia* choose to rest on lichen

covered backgrounds while *carbonaria* rest on dark backgrounds (Kettlewell 1955).

The proposed lichen resting behaviour of typical *B.betularia* has recently been criticised (Grant & Howlett 1988). However, the above model does not rely on lichens, just that melanic and typical *B.betularia* choose backgrounds, whatever they are, on which they are cryptic, and that these backgrounds are limiting.

1.2.3: Frequency-dependent selection:

Many models of natural selection assume that fitnesses are constant; this is unlikely to be true. A more realistic approach is to assume that fitness is dependent on the frequency of the gene in the population. If the fitness increases as the gene frequency decreases then a polymorphism can be maintained without the need for heterosis. This type of relationship produces stable equilibria and is potentially a potent mechanism for maintaining polymorphisms, even when the heterozygote has a lower relative fitness than both homozygotes.

The maintenance of a visual polymorphism by a frequency-dependent system directly involving the visual characters and the action of predators was first clearly outlined by Clarke (1962a), although others had alluded to it before. His theory, based on the colour and banding polymorphism in *Cepaea,* is supported by previous experimental and theoretical work by Reigard (1908), Fisher (1930), and Popham (1941, 1942). Frequency-dependent selection had been provisionally suggested by Poulton (1884) much earlier with respect to the green-brown polymorphism in geometrid larvae (*Cyclophora* spp.). He hinted that the rare forms may be overlooked while the common types are predated heavily.

Clarke (1962a) re-analysed Popham's (1941) work on the predation of corixid bugs (*Sigara distincta*) by rudd (*Scardinius erythrophthalmus*). The re-analysis showed that selection varied with the frequency of prey type and it was different from that expected if the morphs were eaten in proportion to their frequency. The phenotypes underwent increased selection at high relative frequencies and reduced at low relative frequencies, these results being statistically significant (Clarke 1962a).

Clarke (1962a) pointed out three important conclusions from these data:

(1) The predators' behaviour could maintain a polymorphism without the aid of heterosis, the most common morph being the most cryptic.

(2) The colour variation in Popham's experiments was solely tonal, therefore a visually monochromatic predator, such as a small mammal, may inflict a similar type of selection and maintain a tonal polymorphism.

(3) The inclusion of another prey type enhanced the magnitude of frequency-dependent selection. This may mean that a polymorphic population is capable of taking in more colour types and becoming more complex over evolutionary time.

Clarke (1962a) called these cryptic, non-mimetic polymorphisms apostatic polymorphisms, as there is a selective advantage to those individuals that stand out from the norm. The advantage of rare phenotypes over common ones has been shown using artificial pastry prey that differ in colour presented at different frequencies to passerine birds (Allen & Clarke 1968, Allen 1988). However, at high densities selection is anti-apostatic so the rarer phenotypes are preferentially selected by predators (Allen & Anderson 1984).

A similar theory was independently proposed by Moment (1962) as an explanation of the enormous variation in the North Atlantic brittle star, *Ophiopholis aculeata* and the butterfly clam, *Donax variabilis*. These species are so variable that a sample of hundreds is required before identical specimens are found. Moment commented that examples of diversity similar to these have been dismissed as being the result of neutral mutation. His alternative explanation is that the variation provides a degree of protection against visual predators, such as teleosts and birds, the limitless variation making it very difficult for a predator to learn that all the morphs are in fact edible prey. This type of selection was termed reflexive selection as "the frequency of any one type is determined by a feedback relationship with all the other types" (Moment 1962).

Frequency-dependent selection can act on variation other than colour and pattern. Clarke and his co-workers (Clarke 1979, Clarke *et al.* 1988) have suggested that because parasites and parasitoids are adapted to the modal host they are at a disadvantage if they encounter a rare host-type. As a consequence rare host types are at a selective advantage, with this advantage being frequency-dependent. According to Clarke *et al.* (1988) the host-type could be dependent on variation in continuous characters, such as hydrogen ion, metabolite and hormone concentrations.

1.2.4: Frequency-dependent mating:

Frequency-dependent sexual selection occurs whenever a departure from random mating is a function of the frequency of genotypes within the population (Ayala & Campbell 1974). This could maintain a polymorphism if rare genotypes are more likely to be selected as mates than the more common genotype.

Most of the experiments revolve around male mating success and this phenomenon is known as "the rare male effect" (RME) (Partridge & Hill 1984). This effect has been shown in many organisms differing in numerous traits, but the majority of studies have involved *Drosophila* spp. (Partridge 1988). The traits considered include enzyme variants, inversion karyotypes, geographical races, and visual differences in morphology. One such study was on the two spot ladybird, *Adalia bipunctata*, which showed RME acting on the melanic and typical forms (Majerus *et al.* 1982).

1.3: Random processes:

Stochastic processes act on all genetic variation in all populations, but the effects can be obscured by selection over-riding changes in gene frequencies caused by genetic drift. The important parameter is the magnitude of selection acting on a particular gene. A gene at intermediate frequency and under strong positive selection will almost always increase in frequency; however, if the selection coefficient is small (tending to zero) then genetic drift will be the most important process affecting its frequency.

Polymorphisms that are neutral to selection (have selection coefficients of 0), and only influenced by stochastic processes, are transitory rather than balanced as there is no stable equilibrium point. The polymorphism will persist until it either drifts to fixation or is lost from the population altogether. In a large population frequency changes due to drift will be small thus making the polymorphism seem balanced when in fact it is transitory.

Whether a polymorphism is subjected to selection (selection coefficient > 0) or not (selection coefficient = 0) is part of a much larger debate as to whether the majority of polymorphisms are maintained by selection at all (Kimura 1983). The debate between the neutralists and the selectionists is

concerned with evolution at the molecular level and the existence of molecular polymorphisms (Wills 1981). The neutralists argue that the majority of changes at the molecular level, changes in DNA base substitution, are neutral to selection and that only a fraction of such changes are adaptive and maintained by some sort of balancing selection. In contrast, some proponents of the neutral theory draw a distinction between visible and molecular polymorphisms and conclude that the basic mechanism for adaptive evolution is natural selection acting on variations produced by chromosomes and genes (Kimura 1983).

Early researchers of visible polymorphisms believed that most intraspecific variation was selectively neutral. Lamotte (1951), in a study of *Cepaea* in France, concluded that the geographical structure of the polymorphism could be explained by Wright's (1948) random drift model as he found no relationship between the polymorphism and habitat. This explanation was criticised by Cain and Sheppard (1954) on account of the high rates of mutation (10^{-4} compared to the usual 10^{-6}) that were needed to fit the data to the mutation / random drift model. In fact, almost any distribution of phenotype or genotype frequencies can be explained by Wright's model if subtle adjustments are made to the mutation rate, migration rate, and population size (Jones, Leith, & Rawlins 1977).

There are an increasing number of studies that, under certain circumstances, suggest that stochastic events may influence phenotype frequencies rather than these just being the result of natural selection. Studies of *Cepaea* (Goodhart 1962, 1973, Cameron & Dillon 1984), *Enoplognatha ovata* (Oxford 1989), and *Philaenus spumarius*, (Brakefield 1990) have highlighted the importance of random genetic drift in determining local phenotype frequencies.

Random processes are more effective in species with low vagility, and it can be shown theoretically that even a small amount of long distance movement will reduce divergence between populations caused by random genetic drift (Crow & Kimura 1970). It seems unlikely that a species such as *Cepaea nemoralis* will undergo long distance dispersal as adults, although individuals may move further than originally thought, their eggs attached to birds' legs and even adults being transported by gusts of wind (Rees 1965). Consequently random processes such as, founder effects, historical accidents and population "bottlenecks", may have an influence on the distribution of *Cepaea* phenotypes as the effective population size is small and the probability of inbreeding within isolated demes is high.

When studying polymorphisms it is prudent to consider the neutral theory as the null hypothesis of any investigation rather than, as too often, immediately assuming the polymorphism is balanced and that selection must be maintaining the variation. The use of neutrality as the null hypothesis has been criticised (Beatty 1987) and the opposite, that is natural selection as the null hypothesis, has been proposed as an alternative (Mayr 1983). In my view the neutral hypothesis is the only tenable null model. All genetic variation will be influenced by mutation, gene flow and genetic drift but not all will be influenced by selection. This being true, it is important to consider what temporal and geographic patterns would be expected if polymorphisms are neutral to selection. If the observed patterns do not hold with the neutral model then selection can be invoked (Selander 1985).

1.4: Populations:

Much of the study of polymorphisms has been conducted at the population level. An ecological population may be the individuals within a defined geographic area, or belonging to a specific age class, whereas a genetic

population will reflect the structure of the gene pool and the magnitude of gene flow (Crawford 1984). In habitat specific species with disjunct ranges, where suitable habitat patches are isolated from others by unsuitable habitat, it is much easier to constitute what is a population than in non-habitat specific, vagile species. The subject of this thesis, the moth *Noctua pronuba,* is wide ranging and not habitat specific. This makes the identification of populations less easy. Throughout this thesis a population of *N.pronuba* refers to the body of moths represented by a sample taken at a particular geographic location.

Chapter 2: *Noctua pronuba*:

2.1: Species range:

Noctua pronuba is a medium sized (50-60 mm wing-span) moth belonging to the sub-family Noctuinae. It occurs over the whole of western Europe, Iceland, North Africa, and the Middle East and is most common in lowland, especially disturbed, habitats (Heath & Emmet 1979).

In 1979, the moth was first recorded from the American continent with a single individual being identified in Halifax, Nova Scotia (Neil 1981). Since that time the species has undergone a dramatic increase in numbers and by 1985 had expanded its range to include Prince Edward Island, Newfoundland, and New Brunswick in Canada and the state of Maine in the U.S.A. (Wright & Neil 1983, Neil & Specht 1987, Wright 1987). The precise route by which *N.pronuba* reached Canada is unclear.

2.2: Life cycle:

In northern Europe the female lays eggs during late summer on the undersides of leaves of the larval food plant. Each female lays 1000-1500 eggs during its lifetime and the eggs are arranged in regular batches of 200-300 (Singh & Kevan 1956). They hatch after about two weeks but the duration of development has been shown to be temperature-dependent, ranging from 5 days at 27°C to 43 days at 10°C (Madge 1962). The larvae consume the egg case immediately after hatching and develop through seven instars. Early instars are photo-positive, while the later instars have cutworm-like behaviour, spending the day buried just under the soil surface and emerging on warm evenings to feed (Madge 1964a & b).

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The larvae are polyphagous feeding on a wide range of wild and cultivated grasses and herbs (Skinner 1984, Spitzer *et al.* 1984) and have been recorded as a pest on a wide variety of agricultural crops (Emmet 1984, Rahn 1983, Madge 1964a &b). The final instar larvae pupate in the late spring and the adults emerge two or three weeks later, with the precise length of the pupal period being temperature-dependent (Singh 1962).

2.3: Phenology:

In northern Europe the moth seems to be univoltine but in southern Europe and the middle East it has been described as bivoltine (Sannino *et al.* 1991, Ghanim *et al.* 1979). Investigations into the adult activity of *N.pronuba* suggest summer aestivation and that this aestivation is triggered by the length of the photoperiod (Novak & Spitzer 1972). In the laboratory it has been shown that adult females do not oviposit if there is a photoperiod of 16/8 hours (light/dark) but do when subjected to photo phases shorter than 15/9 (Novak & Spitzer 1975). However, even under these shorter photo phases there is still an obligatory pre-oviposition period of 22 days (mode = 22 days, range 14-26 days).

Novak and Spitzer (1975) suggested that aestivation is an adaptation to the dry summers of the central Palaearctic and that the *N.pronuba* population may be split into two sub-populations. The larger part of the total population is made up of autochthons that aestivate, while there is a smaller migratory sub-population. There is, however, no direct evidence for this population division.

Whether the species is univoltine or bivoltine in central Europe may be confounded by adults aestivating through the hottest and driest periods of

the year, giving the impression that there are two generations a year rather than one.

2.4: Evidence for long distance dispersal:

Most insect species that disperse large distances have a prolonged pre-oviposition period due to the antagonism between reproduction and flights of long duration (Johnson 1969, Wilson & Gatehouse 1992, Han & Gatehouse 1993). Newly emerged female *N.pronuba* have no fully developed eggs and, as described above, have a modal pre-oviposition period of 22 days.

Indirect evidence for long distance dispersal comes from the sudden appearance of large numbers of *N.pronuba* in light-traps on the coast of Britain. An example of this comes from Spurn Head, where on the 19/07/82 a single mercury vapour trap caught 2,081 *N.pronuba* (Spence 1991). This is in contrast to the second highest catch of 1982 which numbered only 156 individuals. It was thought that these moths were of continental European, rather than local, origin and the occurrence of *Trichoplusia ni* (a continental European species and a first record for Yorkshire) in the same trap supports this view.

There is some evidence to suggest that the species undergoes movement in specified directions. Mark-release-recapture studies conducted in French Alpine valleys showed that the majority of movement was in a northerly direction in spring and in the opposite direction in the autumn (Poitout *et al.* 1974).

Other evidence for the migratory ability of the species comes from two studies on the elemental composition of *N.pronuba* (Bowden *et al.* 1979, Bowden *et al.* 1984). Two females, thought on biological grounds to be

immigrants, were distinguished from other, presumably resident females, by X-ray spectrometry of trace elements (Bowden *et al.* 1979). Subsequent work showed that both host plants and soil type affected the trace element composition of individuals. Bowden *et al.* (1984) concluded that this method is unlikely to separate different source populations of *N.pronuba* because the larvae are polyphagous.

There is considerable evidence that *N.pronuba* is wide ranging and may belong to a class of migrants that are nomadic over a large home range (Rankin & Burchsted 1992).

2.5: Polymorphism for forewing colour and pattern:

Tutt (1892) recognised 10 morphs [see table 2.i](3 female and 7 male), Heath and Emmet (1979) 7 (4 male and 3 female) and Skinner (1984) described the species as being variable but did not name any of the variants. Tutt (1892) also suggested that the species is sexually dimorphic, with males darker than females.

None of the above authors described the variation as polymorphic or made reference to its genetic basis. The first reference to the variation being polymorphic (*sensu* Ford 1940, 1955, 1975) was by Poitout and Bues (1976). They categorised the species into 6 different phenotypes, 3 male and 3 female [table 2.i]. Their phenotypic classification seems based on that of Tutt (1892), the three female phenotypes being the same, although not in name, while the number of male phenotypes was reduced by grouping like phenotypes together. The additional phenotypes recognised by Tutt (1892) but not recognised by Poitout and Bues (1976) reflect a degree of continuous variation within each of the distinct phenotypes. Independently, Cook and Sarsam (1981) classified the morphs in the same way as Poitout and Bues

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(1976) although with different names [table 2.i]. It is their (Cook & Sarsam 1981) classification and terminology which I adopt here. Both sets of authors recognised that there is a sexual dimorphism in the species and Cook and Sarsam (1981) suggested that the polymorphism is sex limited.

The polymorphism in *N.pronuba* is not strictly sex-limited as the variation is not limited to one sex. A better description of the polymorphism in *N.pronuba* is sex-influenced, as the sex of an individual influences the expression of the genotype rather than limits the polymorphism to one sex.

Table 2.i: A comparison of the phenotypic classifications used by Tutt (1892), Poitout & Bues (1976), and Cook & Sarsam (1981).

		Phenotype classifications	
Sex	**Tutt(1892)**	**Poitout & Bues (1976)**	**Cook & Sarsam (1981)**
Female	*rufa*	marrons foncé (F)	rufous (R)
Female	*ochrea*	fauve clair (C)	ochre (O)
Female	*caerulescens*	gris (G)	silver (S)
Male	*innuba*	marrons foncé (F)	rufous (R)
Male	*brunnea*	marrons foncé (F)	rufous (R)
Male	*ochreainnuba*	fauve clair (C)	ochre (O)
Male	*ochreabrunnea*	fauve clair (C)	ochre (O)
Male	*griseabrunnea*	gris (G)	silver (S)
Male	*griseainnuba*	gris (G)	silver (S)
Male	*distinctacaerulescens*	gris (G)	silver (S)

2.5.1: Description of the phenotypes:

The dorsal surface of the hindwing, and the ventral surfaces of both the forewing and the hindwing are not polymorphic for either colour or pattern and are relatively constant between individuals. This contrasts with the polymorphic variation found on the dorsal surface of the forewing and on the tegula and patagium of the thorax. It is variation in these areas (dorsal forewing, tegula and patagium) that is used to classify individual moths [plate 1].

The six phenotypes classified by Cook and Sarsam (1981) are:

rufous:

1: male: unicolourous dark, reddish-brown.

2: female: unicolorous red-brown, but lighter than the male.

ochre:

3: male: dark, reddish-brown ground colour with an ochre-coloured costa and patagium, with pronounced ochre transverse fascia

4: female: as above but the ground colour is much lighter.

silver:

5: male: similar pattern to *ochre* but the ochre scales are replaced with silver-grey scales.

6: female: as above but ground colour lighter.
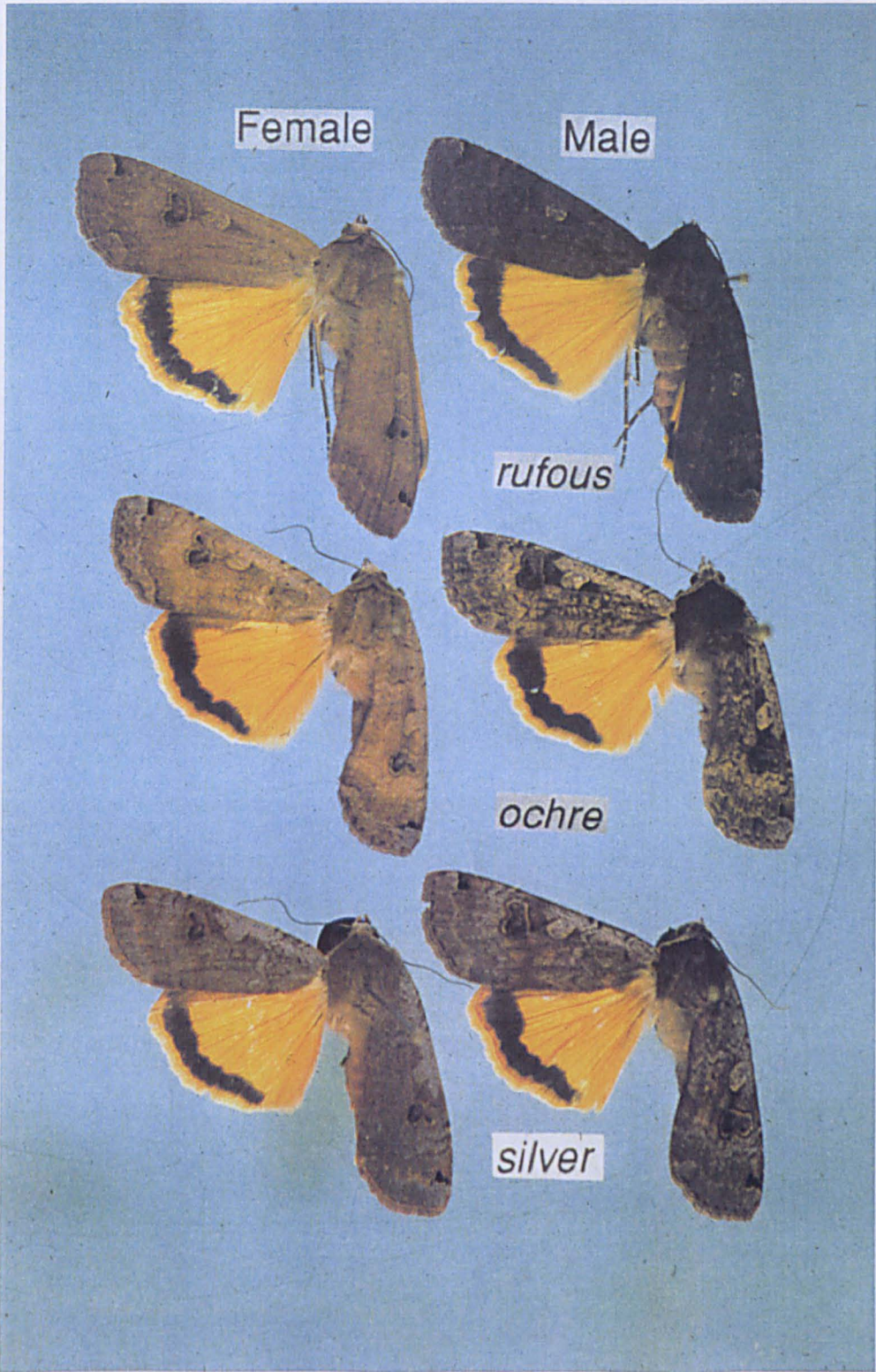
Plate 1: Phenotypes of N.pronuba using the classification of Cook & Sarsam (1981); left column females, right column males.

2.5.2: Genetic models:

Both Poitout and Bues (1976) and Cook and Sarsam (1981) reported the results of controlled breeding experiments and proposed genetic mechanisms that might control the polymorphism. Poitout and Bues (1976) used an autosomal single locus model with three alleles in a dominance hierarchy, r (*rufous* allele) was dominant to both s (*silver* allele) and o (*ochre* allele), and s was dominant to o, and all their crosses supported this model. Cook and Sarsam's breeding lead them to propose an alternative autosomal two loci model with two alleles at each of the loci. At one locus r was dominant to o, while at the other s was recessive to n^s (*non-silver*). The two loci were deemed to be unlinked, or at most loosely linked, but they proposed an epistatic relationship between the two loci; silver only expressed in non-rufous individuals.

As there is confusion over the precise mechanism that controls the forewing polymorphism, I attempted a series of brood rearing and breeding experiments. The moths were reluctant to mate in captivity, perhaps due to the restricted space available for pairing, so it proved impossible to obtain mating of known parentage. Instead, broods derived from wild caught females of known phenotype were successfully reared and the methods and full results can be found in appendix 1.

All broods produced phenotype ratios that fit with the single locus model of Poitout and Bues (1976). Two ochre females produced ochre offspring only, suggesting that they had mated with ochre males. This is expected to be the most common mated type, provided that mating is random with respect to forewing phenotype, as ochre is the most common morph in the majority of populations. Oddly, Poitout and Bues (1976), in a total of 62 successful crosses of known parentage, did not have results from a cross of this type. My results, although the females were wild caught and the paternal

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phenotype unknown, do suggest that ochre is the bottom recessive and that ochre individuals are homozygous at the locus controlling forewing colour and pattern.

The data do not provide absolute confirmation of the genetics underlying the forewing colour and pattern polymorphism as the paternal phenotypes are not known but support the single locus model of Poitout and Bues (1976), rather than the alternative two locus model first outlined by Cook and Sarsam (1981).

2.5.3: Previous studies of the colour and pattern polymorphism:

Three previous surveys of the forewing polymorphism have been published, each with different conclusions:

1) Poitout and Bues (1976) found significant geographic variation in phenotype frequencies between sample sites in the south west and south east of France.

2) Cook & Sarsam (1981) considered the phenotype frequencies to be "remarkably constant throughout Britain and between years", but there were significant differences in phenotype frequencies between the sexes. They proposed that differential selection acting in opposite directions on males and females may maintain the polymorphism.

3) Soli & Andersen (1990), in a survey of the polymorphism in Norway, found no difference in phenotype frequencies between males and females. There was geographical variation, however, with a clinal change in morph frequency in south eastern Norway.

The results and conclusions of the three studies differ. The differences in geographic variation could be explained by variations in the amount of gene

flow between populations, however, no attempt has been made to investigate the extent of gene flow by other methods nor have differences in the resting behaviour of the different phenotypes been studied. In light of these discrepancies, the colour and pattern polymorphism, extent of gene flow and background resting behaviour in *N.pronuba* have been investigated.

2.6: Aims:

The aims of this study were to:

- investigate whether background selection helps maintain the forewing polymorphism.

- consider the evidence for the forewing polymorphism being maintained by selection acting in opposite directions on males and females.

- examine the stability or transience of the forewing polymorphism through time.

- obtain estimates of gene flow between populations based on allozyme variation.

- use gene flow estimates to explain the geographic variation in forewing phenotype frequency.

The results gained from this study of the forewing polymorphism in *N.pronuba* were used to make general conclusions about the maintenance of colour and pattern polymorphisms in other species.

Chapter 3: Background resting behaviour:

3.1: Introduction

Many species rely on crypsis as a primary defence mechanism against predation (Cott 1940, Edmunds 1974). Crypsis is not a property of an individual's colour and pattern alone, but depends on the background it is resting on. Endler defines a pattern as cryptic or inconspicuous if it "resembles a random sample of the background perceived by predators at the time and age, and in the microhabitat where the prey is most vulnerable to visually hunting predators" (Endler 1978, 1980). Implicit in this definition is that any departure of the potential prey item's colour and pattern from the random sample of the background, in terms of pattern, size, shape, colour, brightness and the distribution of these elements, will make a pattern conspicuous. This relationship between the colour of an animal and its background raises is particularly interesting when a species, such as *N.pronuba*, is polymorphic for colour and pattern and also relies on crypsis as a primary defence mechanism.

Crypsis is presumed to have evolved by individuals that best resemble their background being, on average, fitter than those that do not. As most animal species are mobile it is plausible that individuals that can actively select a background on which they are maximally cryptic, given the range of backgrounds available, are at an evolutionary advantage over those that cannot. Extension of this argument means that, for species polymorphic for colour and pattern, there may well be a selective advantage for different phenotypes to choose different backgrounds on which to rest. This choice of a background that matches an individual's colour can be considered to be habitat selection. Theoretically it has been shown that habitat selection may be important in the maintenance of polymorphisms as it promotes stable

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equilibria when there is selection in heterogeneous environments (Cook 1986, see introduction).

Moths are good subjects for testing theories of crypsis and background matching and they have been the subject of many studies (Kettlewell 1955, Sargent & Keiper 1969, Boardman *et al.* 1974, Endler 1984). Many species are inactive during the day but are thought to rely on crypsis as their primary method of defence against predators. The best method to study crypsis in moths is to search for them in suitable habitats and record them in their natural resting posture. This is a time consuming exercise and, even with a well studied species such as *B.betularia*, few records have been published (Howlett & Majerus 1987).

The paucity of data on natural resting positions has been circumvented by the use of choice experiments. In these, either wild caught or reared moths are introduced into enclosed chambers which contain a number of different backgrounds on which the moths can take up resting positions. The backgrounds tested range from different colours of paper (Kettlewell 1955, Sargent & Keiper 1969, Sargent 1969) to more natural backgrounds such as dead logs, saplings, long grass and short grass (Boardman *et al.* 1974).

Kettlewell (1955) presented typical and melanic forms of *B.betularia* with a choice of resting on either black or white surfaces and found a significant difference between the behaviour of the two morphs; typical moths preferred white backgrounds while the dark melanic morph preferred the black substrate. Replication of these experiments has proved contradictory with some authors providing supporting evidence (Boardman *et al.* 1974) while others have shown that all three phenotypes (typical, *carbonaria* and *insularia*) prefer to rest on black substrates (Howlett & Majerus 1987, Grant & Howlett 1988).

Sargent (1966) and Sargent and Keiper (1969) showed, using a similar experimental set-up to Kettlewell's (1955), that different species of moths prefer to rest on backgrounds that have similar reflectances to their forewings. They also provided evidence that tactile stimuli may play a role in the selection of final resting positions, showing that the matching of forewing colour with substrate colour is not the only important factor in the choice of background.

Boardman *et al.* (1974) tested 29 British species of moth, including *N.pronuba*, using an experimental design similar to that of Kettlewell (1955). In addition they tested 30 species, including *N.pronuba*, in a 2m x 2m x 2m arena which contained a variety of natural backgrounds such as: dead leaves, fresh leaves, short grass, long grass and dead logs. Their results from the black and white backgrounds agreed with both Kettlewell's (1955) and Sargent's (1966) results, confirming the correlation between the reflectance of the forewings and the reflectance of the background. The natural resting backgrounds results are more complicated but Boardman *et al.* were able to divide the species up into preferred microhabitat types: e.g. bark-resting and ground-resting species.

In Boardman *et al.'s* (1974) experiments *N.pronuba* and its congener *N.comes* were predominantly ground-resting, being found most often on dead leaves, bare soil and short grass. In contrast *N.pronuba* showed no preference for either black or white when presented with these two colours to rest on. This highlights an important methodological point: the experimental set up, especially the type of background available, can influence the behaviour of the moth species. *N.pronuba* "appeared to rest at random with respect to background colour" when presented with a black and white background but did not rest at random when presented with a more natural array of substrates (Boardman *et al.* 1974).

3.2: Aims:

Boardman *et al.* (1974) did not investigate whether there were differences in background resting behaviour between the six phenotypes of *N.pronuba*. Differences in the behaviour of polymorphic forms of *Cepaea nemoralis* have been reported (Jones 1982) and habitat selection is possibly important in the maintenance of polymorphisms. In view of this, I designed an experiment to investigate whether the six phenotypes (3 male and 3 female) preferred different backgrounds and whether the time of day the moths were tested influenced their behaviour.

3.3: Materials and methods:

Between 27 July and 12 August 1992 the following experiment was performed.

Four nylon mesh cages were erected, each measuring 80cm long by 70cm wide and 60cm in height. All four cages were positioned in the corners of a shaded greenhouse, measuring 200cm by 200cm by 200cm and each cage was covered by a clear perspex sheet. Each cage had a tunnel entrance in the side so the moths could be introduced.

Eight plastic seed trays measuring 24cm x 38cm were positioned within each of the cages so there were few gaps around the edge where moths could hide; gaps that were found were filled with peat. The eight trays in each cage were filled with one of four backgrounds, so each background was represented twice in each cage. The backgrounds available for the moths to rest on were: long grass (>15cm), short grass (3-5 cm), dried leaves, and bare soil.

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The experimental moths had been caught, at light, at one of two sites in Oxfordshire (Long Wittenham or Bullingdon Green) on the previous night and kept in a cold room at 5°C. On each day of the trial between nine and fifteen moths, of known phenotype and sex, were introduced into each cage and then scored after a set time period for their background resting position. The moths did not always adopt resting positions on one of the required substrates but often on the struts of the cage or on the mesh itself. Rather than ignoring these moths they were also scored. The following backgrounds were recognised: upright-struts, horizontal-struts, and nylon mesh.

Every moth was tested under two experimental regimes.

1. The moths were introduced into the cage during the late afternoon and left overnight. They were scored the following morning before 10am.

2. The moths were introduced into the cages and left for a period of two hours through the day before being scored.

Whether the moths were first tested under regime 1 or 2 was altered each day. This was to reduce the chance that moths new to the cage situation behaved differently from those acquainted with the cage.

After each moth was scored for the first time it was placed into a small nylon cage until the particular experimental cage was empty. Once completely empty the moths were re-released through the side tunnel for the next trial. Occasionally a moth would escape during the scoring period while the perspex cage lid was removed. This explains the discrepancy in totals for the two experiments in tables 3.i and 3.ii.

After each trial the plastic seed trays in each cage were moved around, to negate positional effects in each of the cages. The corners of the cages

may have been more shaded than the centres of the cages and this could lead to moths selecting backgrounds on the basis of shade rather than on type of substrate.

Individual moths were tested once using both experimental regimes so the totals in tables 3.i-ii correspond to the number of individuals tested rather than the number of observations.

3.4: Results:

For each of the two experiments there were results from four cages and these data were pooled together [table 3.i and 3.ii]. The experiment was designed to minimise the possibility of heterogeneity, since strictly the results from the four individual cages should have been tested for heterogeneity This was not possible due to the large size (6 x 7) of the contingency tables.

The raw data suggest that *N.pronuba* does actively select particular backgrounds. If the moths were randomly choosing backgrounds one would expect equal numbers of moths on each of the four backgrounds presented in seed trays (short grass, long grass, bare soil, and dried leaves) as each cage and background had the same area available.

In all statistical tests the data for the background "bare soil" were omitted as few moths rested there. The object of the experiment was to test for behavioural differences between the morphs in their adoption of specific resting sites. All phenotypes, under both experimental procedures, were tested against one another using a G test of independence [tables 3.iii-v]. None of the tests between phenotypic classes from experiment 1 (overnight) was significant [table 3.iii].

Table 3.i: Results from experiment 1 where moths were left to settle overnight; data summarised from all four cages. Numbers in brackets are the % frequency of a particular phenotype found on a particular background; those not in brackets the number of individuals recorded on a particular background.

Background Type	Male Rufous	Male Ochre	Male Silver	Female Rufous	Female Ochre	Female Silver	Tot
Short Grass	7 (7.3)	7 (6.1)	1 (1.8)	3 (5.5)	3 (4.6)	1 (3.0)	22
Long Grass	60 (62.5)	67 (58.8)	36 (63.2)	29 (52.7)	39 (60.0)	19 (57.5)	250
Bare Soil	1 (1.0)	3 (2.6)	- (0.0)	-. (0.0)	- (0.0)	1 (3.0)	5
Dried Leaves	14 (14.6)	12 (10.5)	10 (17.5)	5 (9.1)	6 (9.2)	5 (15.2)	52
Upright Struts	4 (4.2)	12 (10.5)	3 (5.3)	8 (14.5)	7 (10.8)	3 (9.1)	37
Horizontal Struts	5 (5.2)	11 (9.6)	5 (8.8)	4 (7.3)	8 (12.3)	3 (9.1)	36
Nylon Mesh	5 (5.2)	2 (1.8)	2 (3.5)	6 (10.9)	2 (3.1)	1 (3.0)	18
Total:	96	114	57	55	65	33	420

Table 3.ii: Results from experiment 2 where moths were left to settle for two hours during daylight; data summarised from all four cages. Numbers in brackets are the % frequency of a particular phenotype found on a particular background; those not in brackets the number of individuals recorded on a particular background.

Background Type	Male Rufous	Male Ochre	Male Silver	Female Rufous	Female Ochre	Female Silver	Tot
Short Grass	11 (9.7)	1 (0.9)	3 (5.3)	4 (7.3)	5 (8.1)	1 (3.2)	25
Long Grass	33 (29.2)	26 (24.1)	18 (31.6)	16 (29.1)	24 (38.7)	9 (29.0)	126
Bare Soil	2 (1.8)	3 (2.8)	- (0.0)	- (0.0)	- (0.0)	- (0.0)	5
Dried Leaves	9 (8.0)	9 (8.3)	10 (17.5)	1 (1.8)	4 (6.5)	4 (12.9)	37
Upright Struts	28 (24.8)	32 (29.6)	8 (14.0)	17 (30.9)	12 (19.4)	4 (12.9)	101
Horizontal Struts	18 (15.9)	25 (23.1)	15 (26.3)	8 (14.5)	9 (14.5)	9 (29.0)	84
Nylon Mesh	12 (10.6)	12 (11.1)	3 (5.3)	9 (16.4)	8 (12.9)	4 (12.9)	48
Total:	113	108	57	55	62	31	426

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Table 3.iii: G tests of independence results for experiment 1 against experiment 1. All tests had five degrees of freedom as the results for dead leaves were omitted.

	Male Ochre	Male Silver	Female Rufous	Female Ochre	Female Silver
Male Rufous	7.116 n.s.	3.666 n.s.	7.877 n.s.	6.623 n.s.	2.736 n.s.
Male Ochre		5.140 n.s.	7.105 n.s.	0.813 n.s.	1.216 n.s.
Male Silver			8.028 n.s.	3.997 n.s.	0.768 n.s.
Female Rufous				4.252 n.s.	3.456 n.s.
Female Ochre					1.067 n.s.

n.s. = not significant

Table 3.iv: G tests of independence results for experiment 2 against experiment 2. All tests have five degrees of freedom as the results for dead leaves were omitted.

	Male Ochre	Male Silver	Female Rufous	Female Ochre	Female Silver
Male Rufous	11.829*	9.544 n.s.	4.607 n.s.	2.082 n.s.	5.975 n.s.
Male Ochre		11.853*	10.092 n.s.	11.841*	4.870 n.s.
Male Silver			17.361**	8.492 n.s.	1.982 n.s.
Female Rufous				4.215 n.s.	9.748 n.s.
Female Ochre					4.997 n.s.

n.s. = not significant

* = 0.05 > p > 0.01

** = 0.01 > p > 0.001

*** = p < 0.001

Table 3.v: G tests of independence results for experiment 1 against experiment 2. All tests had five degrees of freedom as the results for dead leaves were omitted.

	Male Rufous	Male Ochre	Male Silver	Female Rufous	Female Ochre	Female Silver
Male Rufous	39.719***	61.789***	23.670***	41.129***	21.213***	20.361**
Male Ochre	29.334***	46.986***	15.850**	32.379***	15.657**	16.558**
Male Silver	31..253***	37..759***	14.955*	34.667***	18.570**	14.188*
Female Rufous	10.453 n.s.	20.871***	12.064*	7.892 n.s.	3.756 n.s.	9.182 n.s.
Female Ochre	19.165**	29.243***	10.841 n.s.	22.306***	9.688 n.s.	10.924 n.s.
Female Silver	12.611*	20.030**	7.793 n.s.	19.480**	8.981 n.s.	8.957 n.s.

n.s. = not significant

* = $0.05 > p > 0.01$

** = $0.01 > p > 0.001$

*** = $p < 0.001$

For tests between phenotypes in experiment 2, 4 of the 15 tests were significant, three at $p < 0.05$, and one at $p < 0.01$ [table 3.iv]. Out of 36 tests between all the phenotypic classes of both experiments, 26 are significant with 14 at $p < 0.001$, 8 at $0.01 > p > 0.001$, and 4 at $0.05 > p > 0.01$ [table 3.v].

3.5: Discussion:

There is no difference between the behaviour of different phenotypes in their choice of background resting site in experiment 1. Night active moths rest in the day and must adopt a resting position during the morning where they remain inactive during daylight hours. More than 50% of all individuals were found resting in long grass. Against this substrate the moth is cryptic to the human eye, however, the moths were observed resting at the base of the grass stems, partially hiding from view rather than relying solely on a resemblance to the background.

Similarly there seems to be little difference in the behaviour of different phenotypes when placed in the more unnatural conditions of experiment 2 [tables 3.ii, & 3.iv]. The only real differences in behaviour are seen in the comparison of phenotypes from the two experimental set ups [table 3.v]. This, taken in conjunction with the other G-test results, points to the experimental conditions having a much greater effect on behaviour than variation in background choice between the six phenotypes of N.pronuba.

The experimental conditions were artificial, and a problem of this experiment, and of other background choice experiments, is how relevant they are to natural situations. It is possible that under natural conditions the phenotypes do adopt different resting sites but the range of resting sites available under

the experimental conditions did not allow this differential behaviour to be observed.

Boardman *et al.* (1974) recorded the position of moths on 9 different substrates, including long and short grass, bare soil and dead leaves. When the results from my study are compared to those of Boardman *et al.* there is considerable disagreement. From 518 observations in Boardman *et al.'s* study, 67.2% rested on bare soil and short grass combined (35.1% on soil; 32.1% on short grass); in my study; 6.2% in experiment 1, and 7% in experiment 2 (based on results for all the phenotypes and both the sexes combined) were found on these two substrates combined. Boardman *et al.* (1974) only allowed the moths 30 minutes to take up resting positions and the experiment was conducted during the morning after the moths had been collected from a light trap.

Although I have no direct evidence, it seems probable that resting sites occupied by individuals during the early morning, after a period of night time activity, more accurately reflect the true day resting positions of moths. This is not only important for *N.pronuba* but may also call into question the results Boardman *et al.* gathered for 29 other species.

The difference in daytime resting behaviour and nocturnal resting behaviour is outlined by Endler (1984). He suggested that some species with unpredictable behaviours may be less concerned about what background they rest on if disturbed in the day, compared with where they rest after nocturnal activity. This may be especially true for *N.pronuba* as it has bright yellow hindwings which are thought to elicit a startle response when disturbed. From personal observations and those of D.F.Owen (*pers. comm.*), *N.pronuba* takes off quickly if disturbed, flies for a short duration, and then dives into the vegetation. It seems unlikely that, under these

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circumstances, it is actively searching for the background on which it is most cryptic. Movement is integral to crypsis. If a species' major anti-predator defence is looking like its background it should stay still, as any movement will attract predators. As stated above, the species has a remarkable ability to take off when disturbed, this suggests that crypsis may be of limited importance as a defence mechanism.

All of the *N.pronuba* phenotypes could be considered cryptic although the pattern does not look like any specific background. When tested in background resting experiment 1 (the moths left to take up resting positions over night) the most favoured background was the long grass. From my casual observations moths on this substrate were not visible from above. This was not due to crypsis, but because they were obscured by vegetation. The overall impression from these observations and results, is that the moth does not rely on crypsis alone, but uses a number of defence mechanisms: e.g. hiding from predators, crypsis and rapid take-off coupled with flash coloration.

The theoretical mechanism of background resting site selection and its ability to maintain polymorphisms is, in my view, problematic and illustrates the potential importance of factors that at first may seem totally unrelated to the polymorphism in question.

If particular phenotypes choose habitat patches, heterogeneous environments may maintain polymorphisms. The background resting experiments indicated that there is no observable difference in the background resting selection. These results must be treated with caution as the experimental cage conditions and possibly elevated population densities may have affected behaviour. If, in the wild, the phenotypes do adopt resting positions that maximise their cryptic qualities this will not necessarily lead to

a balanced polymorphism. For a polymorphism to be balanced the relationship between the specific background and the phenotype that rests on that background needs to be density-dependent (see Introduction). Once a phenotype becomes so numerous that all the potential resting positions, on which that phenotype is maximally cryptic, are taken up, phenotypes will have to adopt less cryptic backgrounds on which to rest. These individuals will be selected against and so the frequency of the phenotype will decrease relative to others.

This density-dependence seems unlikely to occur in nature as there are many other factors, totally unrelated to a moth's colour and pattern, that limit population size and hence density. Unoccupied resting sites are likely to be always available, so the polymorphism will not be balanced by selection for background matching. This will lead to a neutrally stable equilibrium. This idea can be demonstrated with a hypothetical example; if a dimorphic species is at a density of 2 individuals per m² and each phenotype occupies a particular habitat patch, selection in heterogeneous environments can potentially maintain this dimorphism. If the two habitats are also available at 2 patches per m² then the whole population of the species can be incorporated in either patch type; no density-dependence exists and the phenotypes will drift in frequency over evolutionary time until one is fixed and the other is lost. In this model the factors that constrain the population density influence the dynamics of the polymorphism.

In conclusion the results of the background resting experiments do not aid understanding the mechanisms that may maintain the polymorphism in *N.pronuba* but they do call into question the validity of approaching background selection by choice experiments conducted in cages. For experimental approaches to provide useful information on ecological and evolutionary processes the results must not only be replicated, but also the

experiment performed in near natural conditions and with a variety of different densities. In all, four experiments have been conducted on the resting behaviour of *N.pronuba*, two by myself and two by Boardman *et al.* (1974), all of which gave different results.

Chapter 4: Variation in morph frequencies between males and females at the same site:

4.1: Introduction:

Cook and Sarsam (1981) suggested that the polymorphism for forewing colour and pattern in *N.pronuba* may be maintained by selection acting differently on males and females. The evidence they provided for this was twofold. Firstly, breeding results did not show any imbalance in the inheritance of particular alleles; so the frequency of rufous, ochre and silver phenotypes in males and females was the same and there was no evidence for differences in segregation of alleles in the sexes (results from Poitout and Bues (1976) and from reared broods in this study showed similar patterns). Secondly, female morphs are lighter in colour than the corresponding male morphs and in their survey of phenotype frequencies in the wild; the light ochre morph was at a higher frequency in females than in males, whereas the dark rufous morph was at a higher frequency in males compared to females. Cook and Sarsam argued that the frequencies of the morphs in the sexes are equal at emergence but change because light females have a higher relative fitness than dark females and dark males have a higher fitness than light males. Thus over the flight season the frequency of male rufous and female ochre increase so the phenotype frequencies of the sexes diverge. On mating, which is assumed to be random with respect to phenotype, the allele frequencies will return to their original frequency.

Poitout and Bues (1976) and Soli and Andersen (1990) used the same phenotype classification but did not report differences in phenotype frequencies between the sexes. Poitout and Bues discussed the results of a large survey of more than 57,000 individuals of *N.pronuba* but without reference to males and females separately. Although they tested for

differences between sample sites, and within and between years at the same site, they did not test for variation in morph frequencies between the sexes.

Soli and Andersen (1990) tested for differences between the sexes in 12 samples where there were enough females to allow analysis. They found no differences between the frequency of rufous individuals in all of the 12 samples tested.

4.2: Aims:

The evidence for selection acting in opposite directions on males and females is equivocal. The aim of this study was to compare the phenotype proportions in males and females and review the evidence for selection, acting in opposite directions on males and females, as a mechanism that may maintain the forewing polymorphism.

4.3: Methods:

Samples of *N.pronuba* were collected at light from sample sites throughout Europe and a single sample from Canada. They were collected as part of a study of geographical variation in phenotype frequencies (see chapter 6). In addition samples from the Rothamsted light trap survey, analysed initially for a study of the temporal stability of the polymorphism, were used (see chapter 5). In every case the moths were sexed by examining the structure of the frenulum and retinaculum, and classified into the phenotypes described by Cook and Sarsam (1981).

4.4: Results:

4.4.1: Comparison of phenotype frequencies in males and females:

The sex ratio in light traps is biased towards males and some samples had too few females to be analysed. Where numbers would permit analysis the proportions of each morph were analysed using a χ^2 test of association, the null hypothesis being no difference in the proportion of the three morphs between males and females [table 4.i][1]. Five samples had too few females to allow the comparison of all three morphs and so the *ochre* and *silver* phenotypes were combined giving 1 degree of freedom in the χ^2 analysis.

Three out of twenty nine tests with two degrees of freedom, and two out of five tests with one degree of freedom, were significant at $p<0.05$ or less. The results indicate that there is little difference between the distribution of morph frequencies between the sexes. The majority of tests were in agreement with the findings of Soli and Andersen (1990), where no differences between phenotype frequencies in males and females were found, but five agreed with Cook and Sarsam (1981).

Cook and Sarsam's (1981) mechanism makes specific hypotheses about the way in which phenotype frequencies should differ; that is the frequency of rufous should be higher in males than females and the frequency of ochre should be lower in males than in females. In the three populations in which differences were observed the departures were not consistent with the mechanism [figure 4.i]. The frequency of male rufous was slightly higher but the frequency of male ochre was also higher. The largest difference was in the frequency of silver, this being higher in females.

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[1]Sample sizes and phenotype frequencies used in these tests can be found in tables 6.i-ii.

Table 4.i: χ^2 results from comparisons between phenotype frequencies in males and females collected at the same location.

Location of sample	Year	χ^2	d.f.	p=
Headington, Oxford	1990	2.009	2	0.336 n.s.
Long Wittenham, Oxford	1991	2.490	2	0.288 n.s.
Long Wittenham, Oxford	1992	2.282	2	0.320 n.s.
Bullingdon Green, Oxford	1991	2.499	2	0.287 n.s.
Bullingdon Green, Oxford	1992	3.030	2	0.220 n.s.
Dymchurch, Kent	1991	1.686	2	0.431 n.s.
Dymchurch, Kent	1992	3.737	2	0.154 n.s.
Forton, Shropshire	1990	13.314	2	0.001 ***
Forton, Shropshire	1992	5.594	2	0.061 n.s.
York, Yorkshire	1992	1.077	2	0.583 n.s.
Newquay, Cornwall	1991	1.487	2	0.476 n.s.
Isle of Wight	1992	0.754	2	0.686 n.s.
West Bexington, Dorset	1992	2.313	2	0.315 n.s.
Aberdeen, Scotland	1992	0.313	2	0.855 n.s.
Glasgow, Scotland	1992	0.020	2	0.990 n.s.
Wailly-Beauchamp, France	1992	1.693	2	0.484 n.s.
Stockholm, Sweden	1992	9.792	2	0.008 **
Ticino, Switzerland	1992	3.011	2	0.222 n.s.
Napf-Cebiet, Switzerland	1992	0.420	2	0.810 n.s.
Ramoos-Napf, Switzerland	1992	10.780	2	0.005 **
Ramoos-Napf, Switzerland	1993	0.995	2	0.608 n.s.
Ceské Budejovice, Czech	1992	1.941	2	0.379 n.s.
Cochem, Mosel, Germany	1991	0.678	2	0.713 n.s.
Kentville, Nova Scotia, Canada	1992	0.503	2	0.778 n.s.
Rothamsted, Hertfordshire	1976	4.509	2	0.105 n.s.
Rothamsted, Hertfordshire	1980	3.792	2	0.150 n.s.
Rothamsted, Hertfordshire	1984	1.051	2	0.591 n.s.
Rothamsted, Hertfordshire	1985	0.270	2	0.874 n.s.
Rothamsted, Hertfordshire	1989	1.063	2	0.588 n.s.
Rothamsted, Hertfordshire	1986	0.893	1	0.941 n.s.
Glasgow, Scotland	1992	0.006	1	0.941 n.s.
Armagh, N.Ireland	1992	2.204	1	0.138 n.s.
Miskoloc, N.Hungary	1992	6.403	1	0.011 *
Fogars, Spain	1978-1980	6.612	1	0.010 **
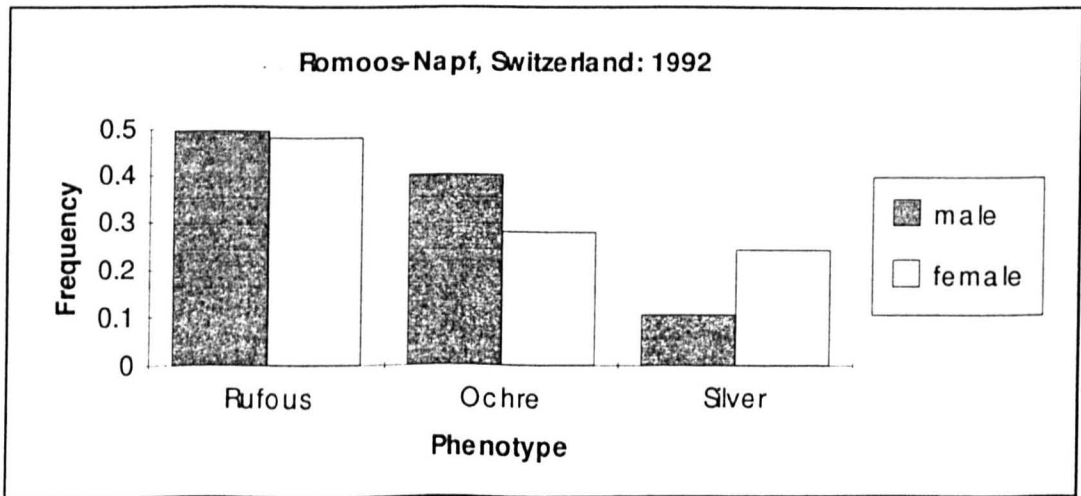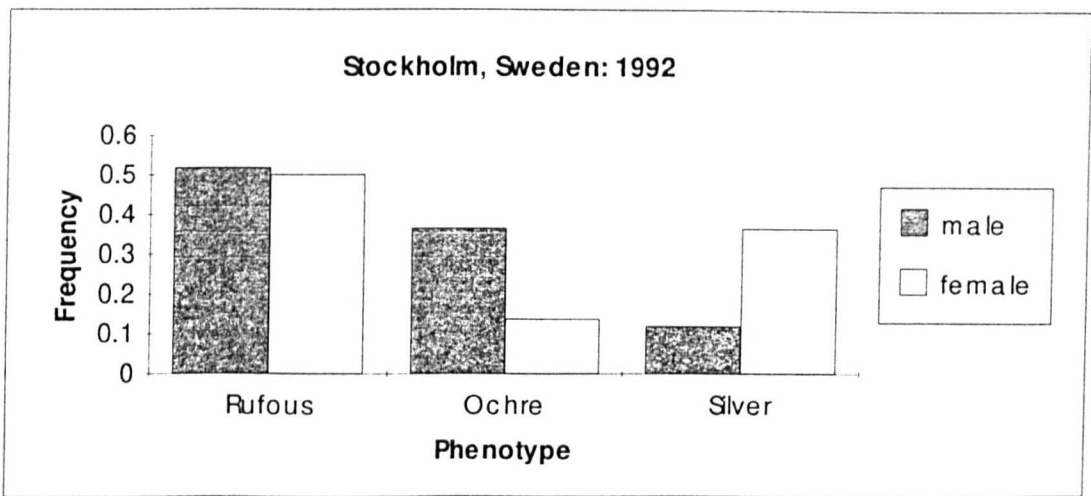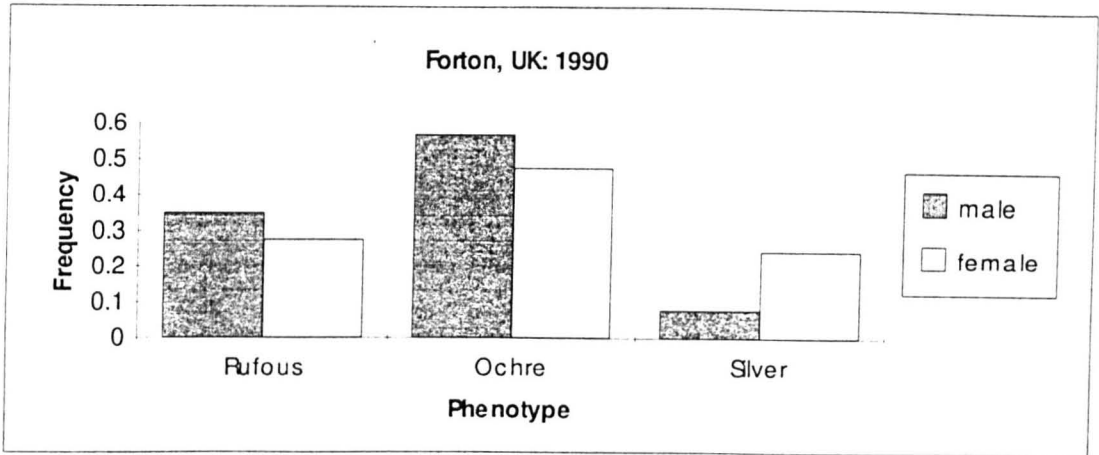
Figure 4.i: Statistically significant phenotype frequency differences between males and females. In all three samples the frequency of ochre is higher in males than in females, this is inconsistent with the mechanism proposed by Cook & Sarsam (1981).

4.4.2: Variation between sexes over the flight season:

Cook and Sarsam's model of selection acting on males and females differently implies that the morph frequencies in both sexes are equal when the moths emerge. This equality of phenotype frequencies at emergence is supported by the breeding data in Cook and Sarsam (1981) and the results from broods reared in the present study. Selection then proceeds to alter the frequencies and causes an increase in the frequency of male rufous relative to female rufous and *vice versa* for ochre.

If the model is correct, samples of males and females collected at the beginning of the flight season should have more equal morph frequencies than samples caught later in the flight season. In the later part of the flight season there should be a higher frequency of rufous in males, compared to females, and a higher frequency of ochre in females, compared to males. Graphs of morph frequency for each sex should show a gradual divergence in frequencies over time. Pivotal to this prediction is the timing of selection. If selection occurs when the imagines emerge the prediction will not hold as the phenotype frequencies will always be different in samples of males and females caught at light. The prediction of diverging phenotype frequencies in males and females relies on selection being spread out over the life time of the moth.

Three samples, Rothamsted 1989, Bullingdon Green 1992, and Dorset 1992 were of sufficient size, with a sufficient number of females, and with samples spread over the duration of the flight period, to allow meaningful comparisons. The data were originally collected as frequencies per trap night, however on some nights only a few individuals were trapped and so days were grouped together to give samples of sufficient size. Ideally the time intervals should have been equal but the low numbers of individuals

caught in the early and late part of the flight season meant that a larger number of weeks needed to be grouped to give representative sample sizes.

Figures 4.ii to 4.iv show the variation in morph frequencies over time for both species from the three sample sites. Each point has standard error bars as the sample size, from which the frequencies were calculated, varies both with time and between the sexes. To overcome this, error bars were calculated using the binomial standard error formula:

$$S.E. = \sqrt{\frac{p(1-p)}{n}} \qquad (4.1)$$

where p = phenotype frequency
n = total sample size

In all three figures the frequency of male rufous and female rufous does not show any systematic change as predicted. At Rothamsted the frequencies were approximately equal at the beginning of the flight season, diverged and at the end were approximately equal again [figure 4.ii]. At Bullingdon Green [Figure 4.iii] the difference between male and females was largest during the first period, the frequencies converging during the middle two periods and finally diverging. For the Dorset sample [figure 4.iv] the difference in the frequency of rufous between males and females remained approximately constant.

The changes in the ochre phenotype also failed to show systematic changes that would bolster the proposed mechanism. The difference in males and females was essentially the same at the beginning and at the end of the flight season at Rothamsted [figure 4.ii], whereas in the Bullingdon Green sample [figure 4.iii] the greatest difference in frequency was at the beginning of the flight season with the frequency of ochre converging towards the end.

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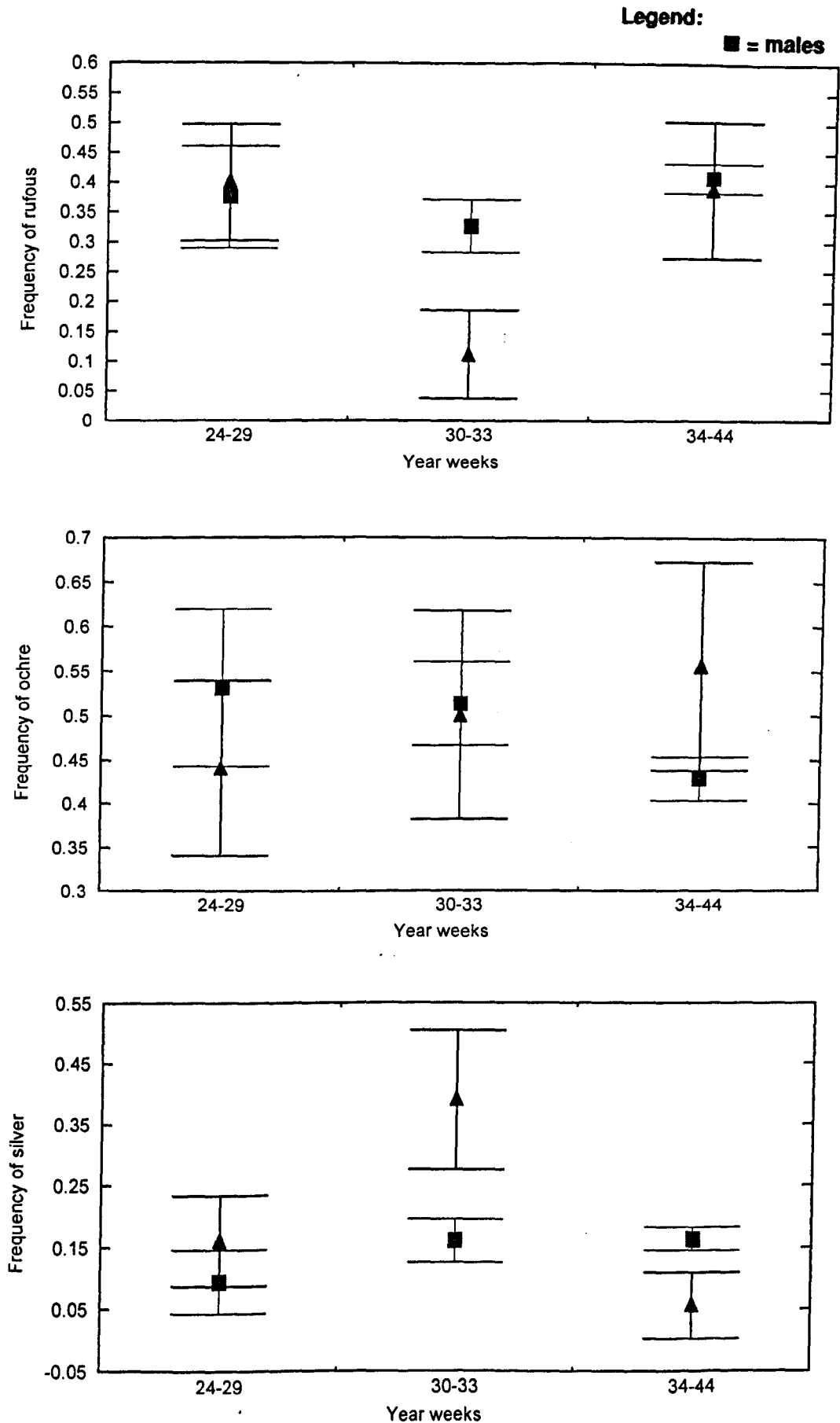
Figure 4.ii: Variation in phenotype frequencies between males and females over a single flight season: Geescroft trap, Rothamsted 1989.

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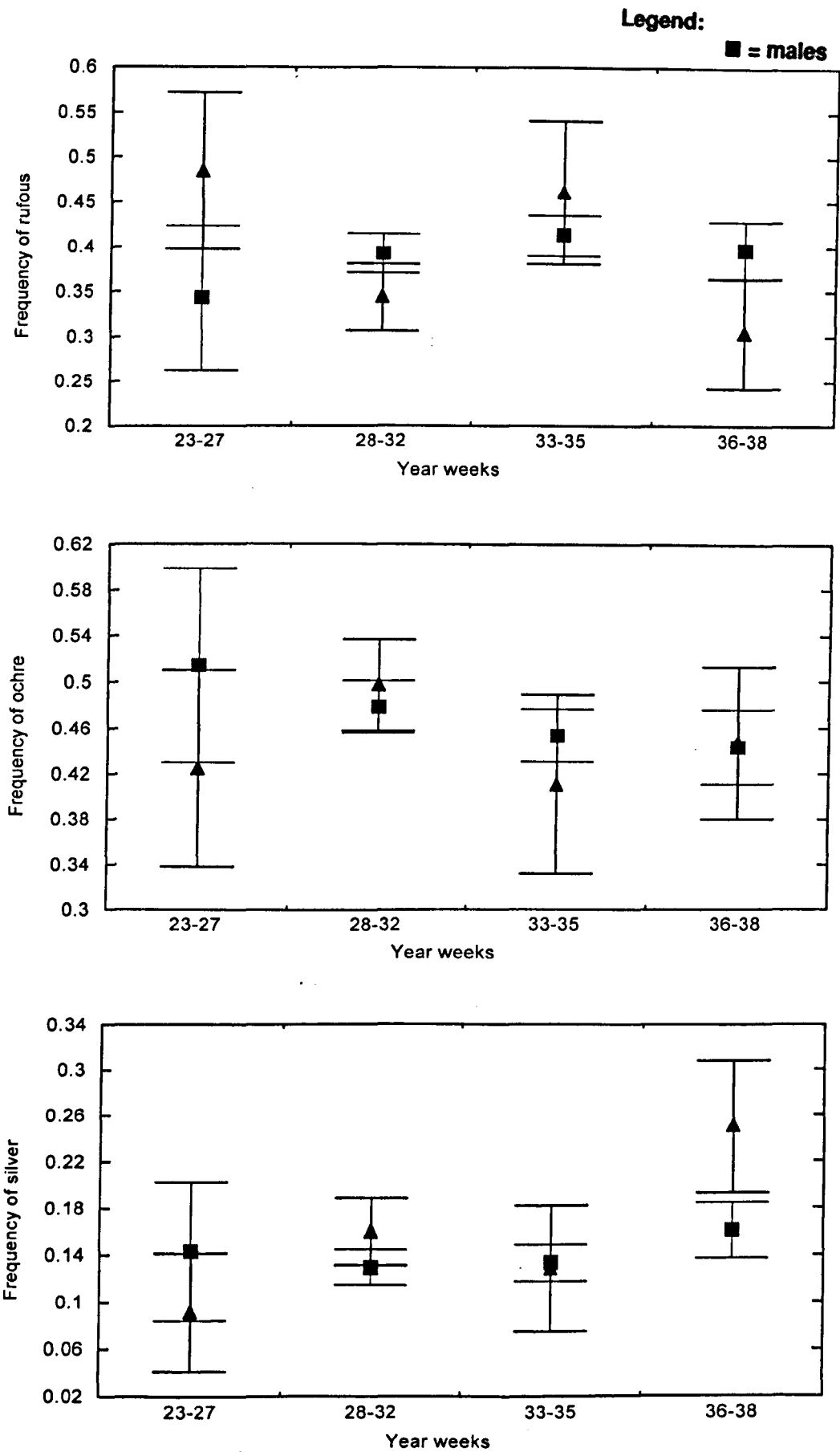
Figure 4.iii: Variation in phenotype frequencies between males and females over a single flight season: Bullingdon Green 1992.
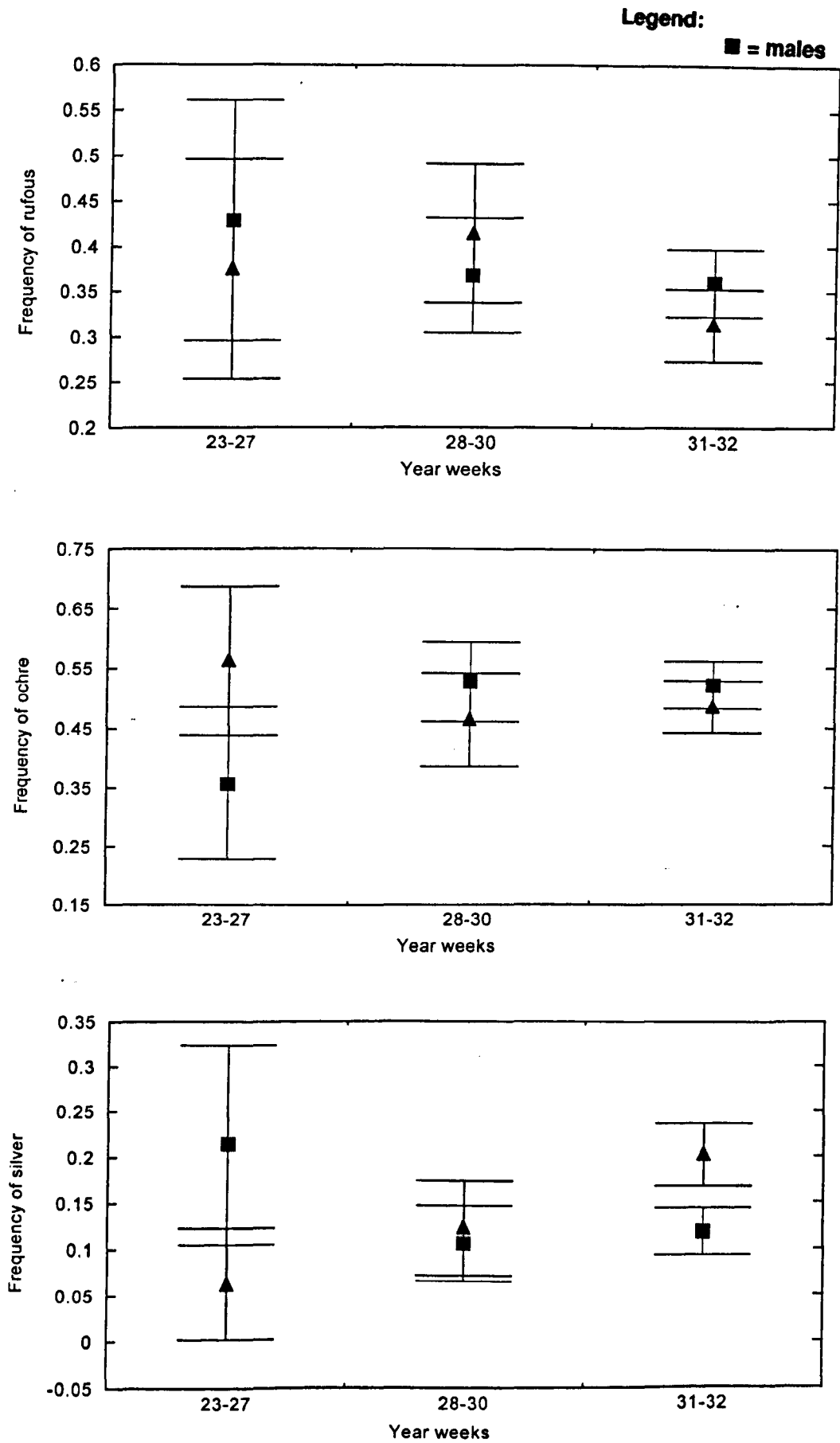
Figure 4.iv: Variation in phenotype frequencies between males and females over a single flight season: Dorset 1992.

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For the silver phenotype two contrasting patterns are seen. At Bullingdon Green and Dorset [figures 4.iii and 4.iv] female silver was lower in frequency at the beginning of the flight season but rose so at the end it was higher in females than in males. The Rothamsted sample [figure 4.ii] showed the opposite trend.

4.5: Discussion:

The discrepancy between Soli and Andersen's data (1990), which did not show differences in phenotype frequencies between males and females and Cook and Sarsam's (1981) which did, might be explained by the surveys being undertaken in different parts of the moths' range. This cannot explain the discrepancy between Cook and Sarsam's data and the data from Britain in the present study. Most of the samples from Cook and Sarsam's were collected in 1970,1971, and 1972, about twenty years before the samples in the present study were collected but it seems unlikely that the selective mechanism they proposed would cease to exist.

Selection acting differently on each sex and maintaining a polymorphism has been proposed before. In *Danaus chrysippus* two alleles, from two of the three loci involved with the determination of dorsal forewing and hindwing pattern, are thought to be influenced by selection operating in different directions in each sex (Smith *et al.* 1993). Smith *et al.* suggested that " ...selection pressures, favouring alternative alleles in the two sexes, must result in balanced polymorphisms....." This is an overstatement as the selective differences between sexes need not lead to a stable equilibrium. Theoretically it has been shown that fitness differences between the sexes need to be quite large to lead to a balanced polymorphism (Haldane & Jayakar 1963, Li 1967). These conditions are similar to those outlined for

the maintenance of polymorphisms in heterogeneous environments (see introduction).

A similar model for the dimorphic Müllerian mimic, *Heliconius numata* was discussed by Turner (1968). In his mathematical treatment he also came to the conclusion that differences in selection could lead to polymorphism. Given that to maintain polymorphisms by differential selection on each of the sexes the selective differentials between the sexes need to be large, the fate of such polymorphism must be the linkage of the trait to the sex chromosomes. Turner (1968) drew attention to another species, *Heliconius nattereri* where the phenotypes seen in *H.numata* are restricted to one sex; the species being sexually dimorphic but not polymorphic in the true sense. This, he suggested, is the fate of the polymorphism in *H.numata* (Turner 1968).

The *Heliconius* example has direct relevance to *N.pronuba*. *N.pronuba* is both polymorphic and sexually dimorphic and if selection does operate on the sexes in different ways we might expect the moth to proceed towards sexual dimorphism over evolutionary time. Other members of the genus are variable in colour also. Of the six species of *Noctua* in Britain *N.fimbriata* and *N.comes* are variable for forewing coloration. Neither are polymorphic in the same way as *N.pronuba* and the variation does not correspond directly to those found in *N.pronuba*. This contrasts with phenotypes in *Cepaea nemoralis* and *C.hortensis* where analogues of each phenotype exist in each species. *N.fimbriata* and *N.comes* are sexually dimorphic, with males being darker than females. The existence of sexual dimorphism in other species of the genus *Noctua* implies that this variation predates speciation events. Given the ancestral nature of the dimorphism it is surprising that there is no evidence from reared broods for even weak linkage to the sex chromosomes.

Cook and Sarsam (1981) suggested that "selection on adults of the two sexes must differ: the premium is on survival during egg maturation in the mated female but on passing the maximum number of gametes in males." This is undoubtedly true and the co-occurrence of the sexual dimorphism in the three species does imply that selection acts differently on males and females.

Mark-recapture data that I have collected (see appendix ii) showed males are less likely to be recaptured than females. In mark-recapture experiments at two locations, Long Wittenham and Little Wittenham, there was a significant difference between the proportions of recaptured individuals in males and females. At Long Wittenham there was a 7% recapture of males compared to a 20% recapture in females ($\chi^2=4.81$; d.f.=1; p=0.028), while at Long Wittenham there was a 10% recapture of males compared to a 21% recapture of females ($\chi^2=5.32$; d.f.=1; p=0.021). The results are consistent between the two mark-recapture experiments and imply that males are more likely to disperse than females.

These mark-recapture studies and the heavily male biased light trap catches suggest that males and females do behave differently at least in terms of dispersal. To suggest that selection differs on males and females is, in my view, a truism; the problem is whether these differences cause differential selection which acts to maintain the polymorphism in N.pronuba.

Cook and Sarsam's mechanism could potentially maintain the polymorphism but providing evidence of its existence is problematic. There were five samples, in this study, where the phenotype frequencies of males and females were different and this is more than would be expected by chance. The independent study in Norway failed to show any examples of divergent frequencies between males and females (Soli & Andersen 1990). In addition

to the low incidence of frequency divergence between the sexes, predicted patterns of temporal change, based on the proposed mechanism of maintenance, are not revealed by the observed data. In conclusion, the existence of a balanced polymorphism that is maintained by selection favouring different alleles in each of the sexes must be treated with caution, although it cannot be totally discounted.

Chapter 5: Temporal variation in phenotype frequencies:

5.1: Introduction:

A method of detecting selection operating on a polymorphism is the analysis of trait frequency distributions over an extended period of time (Manly 1985, Endler 1986). In the absence of selection the frequency of phenotypes would be expected to vary at random, only influenced by genetic drift. If selection is operating the effect will depend upon whether it is directional or stabilising. Directional selection results in a consistent directional change of phenotype frequencies over time, and if stabilising the phenotype frequencies should remain at an equilibrium point with the polymorphism being balanced.

Long-term studies of natural populations are uncommon in evolutionary biology although such studies have been invaluable (Cain 1983, Cowie 1992). Long-term studies of wild populations of Lepidoptera, such as *Panaxia dominula* (Jones 1989), *Biston betularia* (Kettlewell 1973), and *Danaus chrysippus* (Smith *et al.* 1993), have provided good evidence for micro-evolutionary changes in phenotype frequencies, although the *P.dominula* study has been re-assessed in the light of new observations and experiments (Owen & Clarke 1993, Owen & Goulson 1994).

The study of industrial melanism in the moth *Biston betularia* is a good example of the benefit of such long-term approaches (Kettlewell 1973). Even though much of the work documenting the rise in frequency of the melanic morph was done in the 1950s (Kettlewell 1957,1958) recent work has shown there has been a fall in the frequency of melanics over the past 25 years. This fall coincides with the imposition of smoke control legislation

and so provides a selective agent that may have caused the rise and subsequent fall in melanic frequency over the last 150 years (Cook *et al.* 1986).

Lepidoptera are open to long-term investigation as they have been collected by entomologists for decades and museum collections can provide supplementary data. Museum collections may not be random samples of the species in question as many collectors have concentrated on rare, or aberrant forms. This presents a problem when trying to compare samples caught under known circumstances with museum specimens. To investigate the temporal dynamics of a polymorphism a series of samples, collected over a number of years, using the same methods is essential.

5.2: Aims:

The aim of this part of the study was to gain information on the temporal dynamics of the forewing colour and pattern polymorphism in *N.pronuba*. This information was used to assess whether the forewing polymorphism is maintained by balancing selection, is transitory, or fluctuates randomly affected only by genetic drift.

5.3: Methods:

It was possible to obtain a series of samples of *N.pronuba* that had been caught at the same site between 1965 and 1989. Rothamsted Experimental Station has for many years run light traps with the aim of investigating the diversity of macro-Lepidoptera in agricultural habitats both on Rothamsted farm and over the whole of Britain as part of the Rothamsted Insect Survey (Woiwod & Stewart 1990, Taylor 1986, Taylor *et al.* 1978).

One trap was sited at Geescroft Wilderness, Rothamsted Farm. Geescroft is a 1.3 hectare area of land which was under cultivation until 1888. Since that time it has been left and is now considered to be stable secondary woodland (Taylor *et al.* 1978). The trap was run every day of the year, regardless of weather, although the samples of *N.pronuba* in 1971 and 1982 had been removed for another study.

Samples from Rothamsted caught in 1973 and 1989 were of sufficient size, with daily samples spaced throughout the flight season, to allow analysis of variation throughout the flight season (see chapter 4). In addition to these, two other sites, one from West Bexington, Dorset 1992 and one from Bullingdon Green, Oxford were analysed. The samples from Dorset and Oxford were collected as part of a study of the geographic variation in phenotype frequencies (see chapter 6).

5.4: Results:

5.4.1: Variation throughout the flight season:

Four flight seasons were analysed in males, while only three were in females [tables 5.i-5.v]. All of the seven χ^2 analyses were insignificant at p=0.05 or less. In conclusion the frequencies of the three phenotypes, in both males and females, are homogeneous within flight seasons.

Table 5.i: Phenotype frequency variation in males and females throughout the 1992 flight season at Bullingdon Green, Oxford.

Time Intervals	male			female		
	rufous	ochre	silver	rufous	ochre	silver
1:6:92 - 12:7:92	22	28	9	19	24	6
	(0.37)	*(0.48)*	*(0.15)*	*(0.39)*	*(0.49)*	*(0.12)*
13:7:92 - 2:8:92	134	173	42	52	57	20
	(0.38)	*(0.50)*	*(0.12)*	*(0.40)*	*(0.44)*	*(0.16)*
3:8:92 - 23:8:92	146	170	54	16	20	6
	(0.39)	*(0.46)*	*(0.15)*	*(0.38)*	*(0.48)*	*(0.14)*
24:8:92 - 5:9:92	162	166	54	21	22	13
	(0.42)	*(0.44)*	*(0.14)*	*(0.38)*	*(0.39)*	*(0.23)*
6:9:92 - 3:10:92	37	43	14	6	15	3
	(0.39)	*(0.46)*	*(0.15)*	*(0.25)*	*(0.62)*	*(0.13)*

Male χ^2 [d.f.=8] = 3.593   p = 0.8918   n.s.

Female χ^2 [d.f.=8] = 6.155   p = 0.630   n.s.

Table5.ii: Phenotype frequency variation for males and females throughout the 1992 flight season at West Bexington, Dorset.

Interval	male			female		
	rufous	ochre	silver	rufous	ochre	silver
7/6/92 - 17/6/92	6	5	3	6	9	1
	(0.43)	(0.36)	(0.21)	(0.38)	(0.56)	(0.06)
7/7/92 - 29/7/92	21	30	6	17	19	5
	(0.37)	(0.53)	(0.10)	(0.41)	(0.46)	(0.12)
31/7/92	32	40	11	21	30	13
	(0.39)	(0.48)	(0.13)	(0.33)	(0.47)	(0.20)
7/8/92 - 8/8/92	26	44	8	21	35	14
	(0.33)	(0.56)	(0.10)	(0.30)	(0.50)	(0.20)

Male χ^2 [d.f.=6] = 3.138   p = 0.791   n.s.

Female χ^2 [d.f.=6] = 3.767   p = 0.708   n.s.

Table 5.iii: Variation in phenotype frequency for males throughout the 1973 flight season at Geescroft, Rothamsted.

Interval	Male Rufous	Male Ochre	Male Silver
1/6/73 - 31/7/73	9	10	4
	(0.39)	(0.43)	(0.17)
1/8/73 - 31/8/73	20	34	9
	(0.32)	(0.54)	(0.14)
1/8/73 - 31/10/73	8	7	5
	(0.40)	(0.35)	(0.25)

χ^2 [4] = 2.742   p = 0.602   n.s.

Table 5.iv: Variation in phenotype frequency for males throughout the 1989 flight season at Geescroft, Rothamsted.

Interval	rufous	ochre	silver
1/6/89 - 31/7/89	23	28	6
	(0.40)	(0.49)	(0.10)
1/8/89 - 31/8/89	66	77	24
	(0.40)	(0.46)	(0.14)
1/8/89 - 31/10/89	123	131	56
	(0.40)	(0.42)	(0.18)

$\chi^2_{[4]} = 6.219$ p = 0.183   n.s.

Table 5.v: Variation in phenotype frequency for females throughout the 1989 flight season at Geescroft, Rothamsted.

Time Interval	rufous	ochre	silver
1/6/89 - 31/7/89	12	18	9
	(0.31)	(0.46)	(0.23)
1/8/89 - 31/10/89	6	12	2
	(0.30)	(0.6)	(0.1)

$\chi^2_{[2]} = 1.714$ p = 0.425   n.s.

5.4.2: Variation over a twenty five year period:

There were low numbers of females caught in the trap [figure 5.i]. During this period the frequency of females averaged 0.072 (standard deviation 0.037). This sex ratio bias towards males prohibited the comparison of phenotype frequencies between years in females. The variation in the three male phenotypes between 1965 and 1989 is shown in figures 5.ii-iv. Each bar chart is plotted along with standard errors as there was a large range of sample size from year to year [figure 5.v].

The three bar charts [figures 5.ii-5.iv] show that the frequency of each phenotype looks stable over the 25 year period for which samples were available. The only exceptional result was that for 1966. In this year the frequency of male *ochre* individuals was high (0.636) while the frequency of *silver* was lower than usual (0.023). To statistically test for differences in the frequency of the three male phenotypes between years, the yearly frequencies were tested using a χ^2 contingency test. There was no significant heterogeneity over the 25 year period ($\chi^2=55.234$, d.f.=44, p=0.119).

The important question to ask of such temporal data, as provided by the Geescroft trap is as follows; can the fluctuations in frequencies from year to year be accounted for by genetic drift alone or is selection responsible?
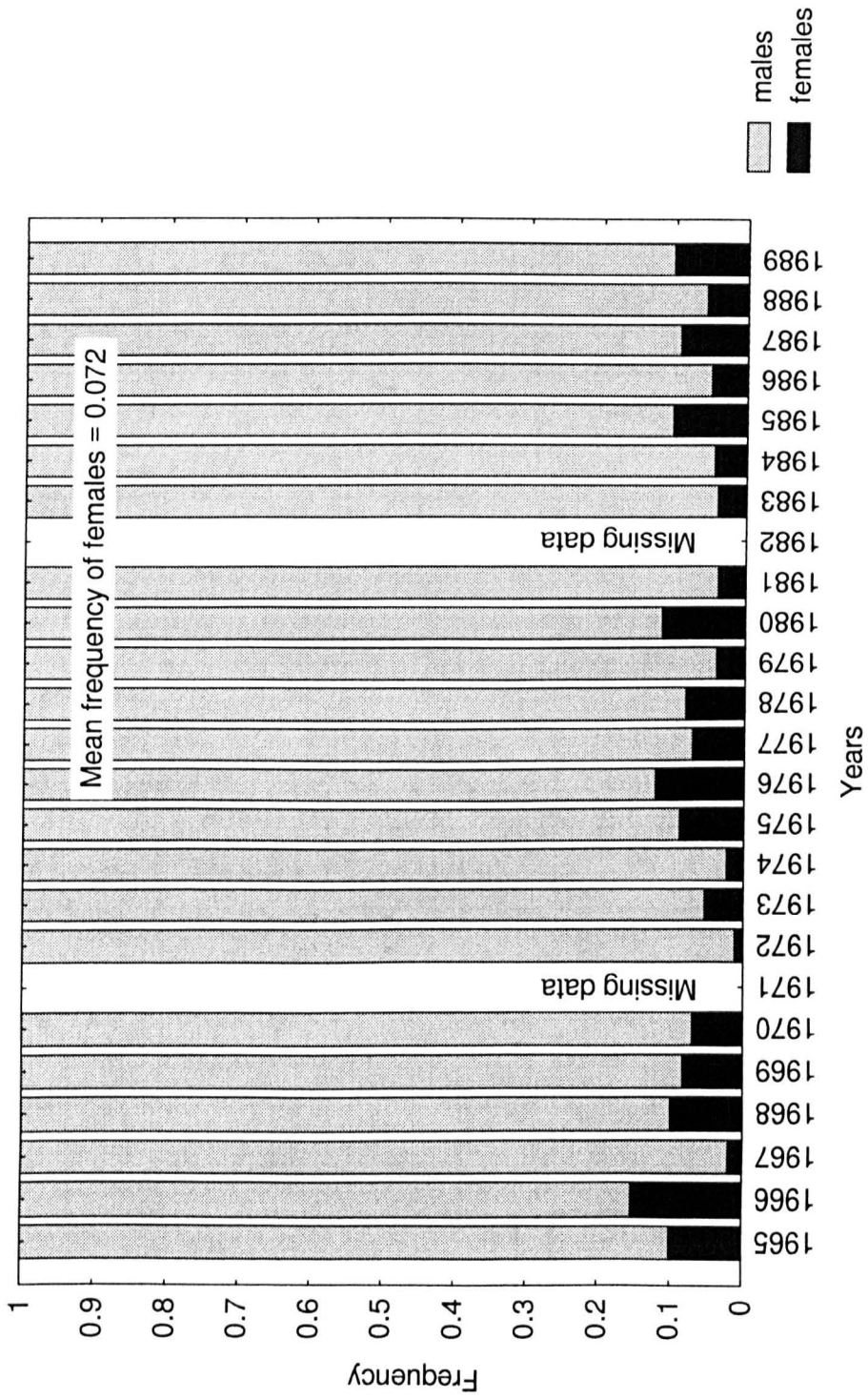
Figure 5.i: Variation in the frequency of males and females caught at Rothamsted Experimental Station.
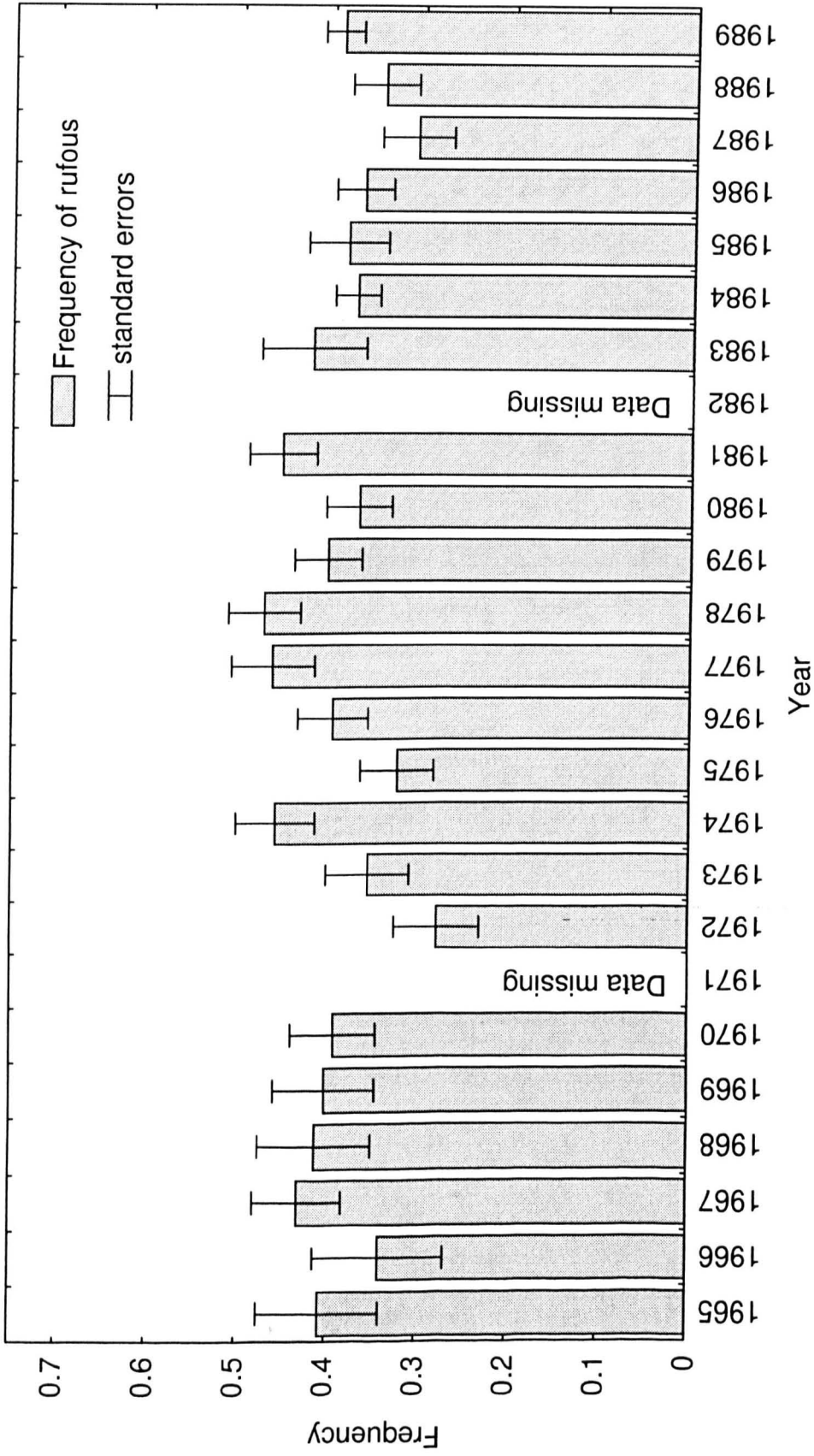
Figure 5.ii: Temporal variation in the frequency of the rufous phenotype in males caught at Rothamsted.
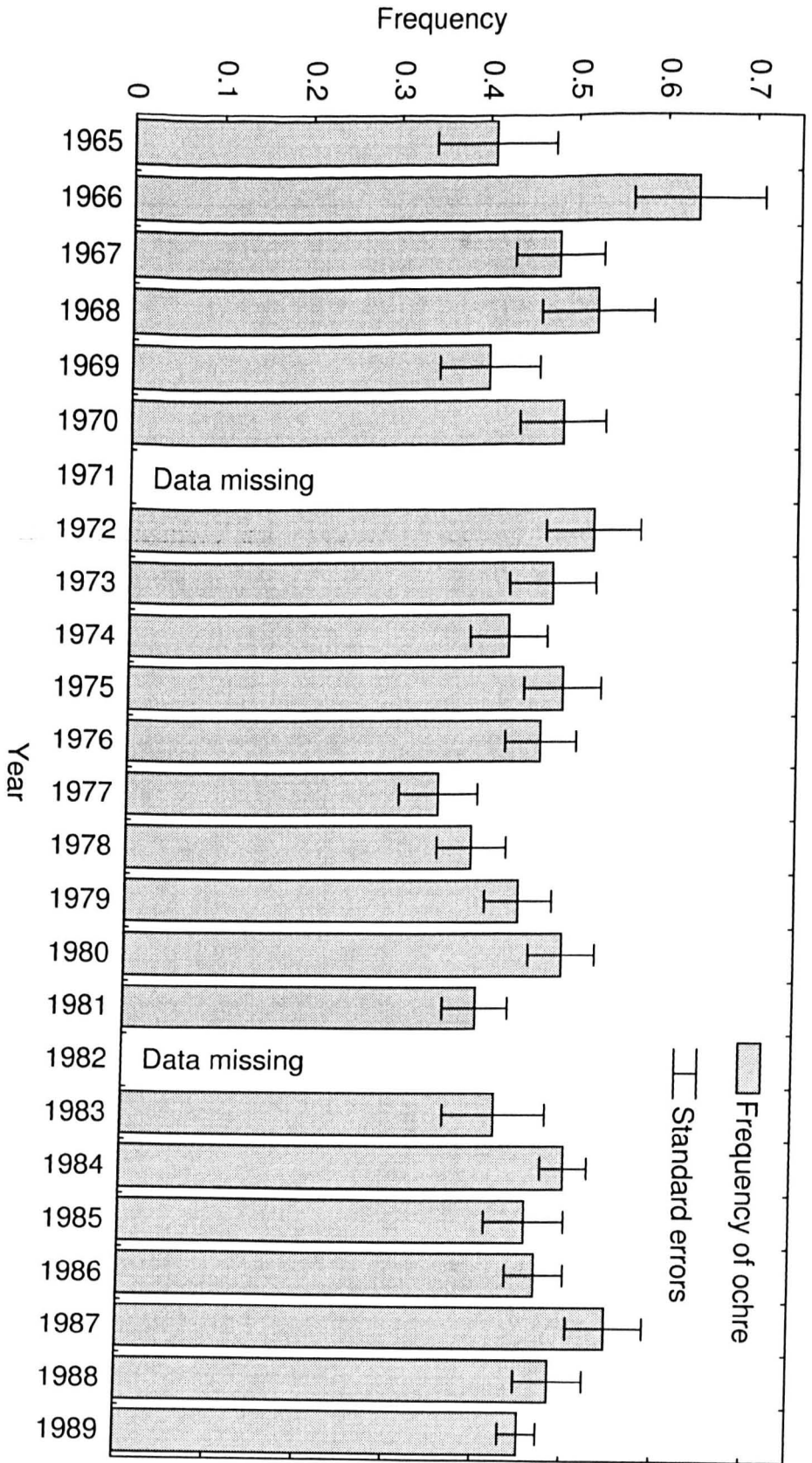
Figure 5.iii: Temporal variation in the frequency of the ochre phenotype in males caught at Rothamsted.
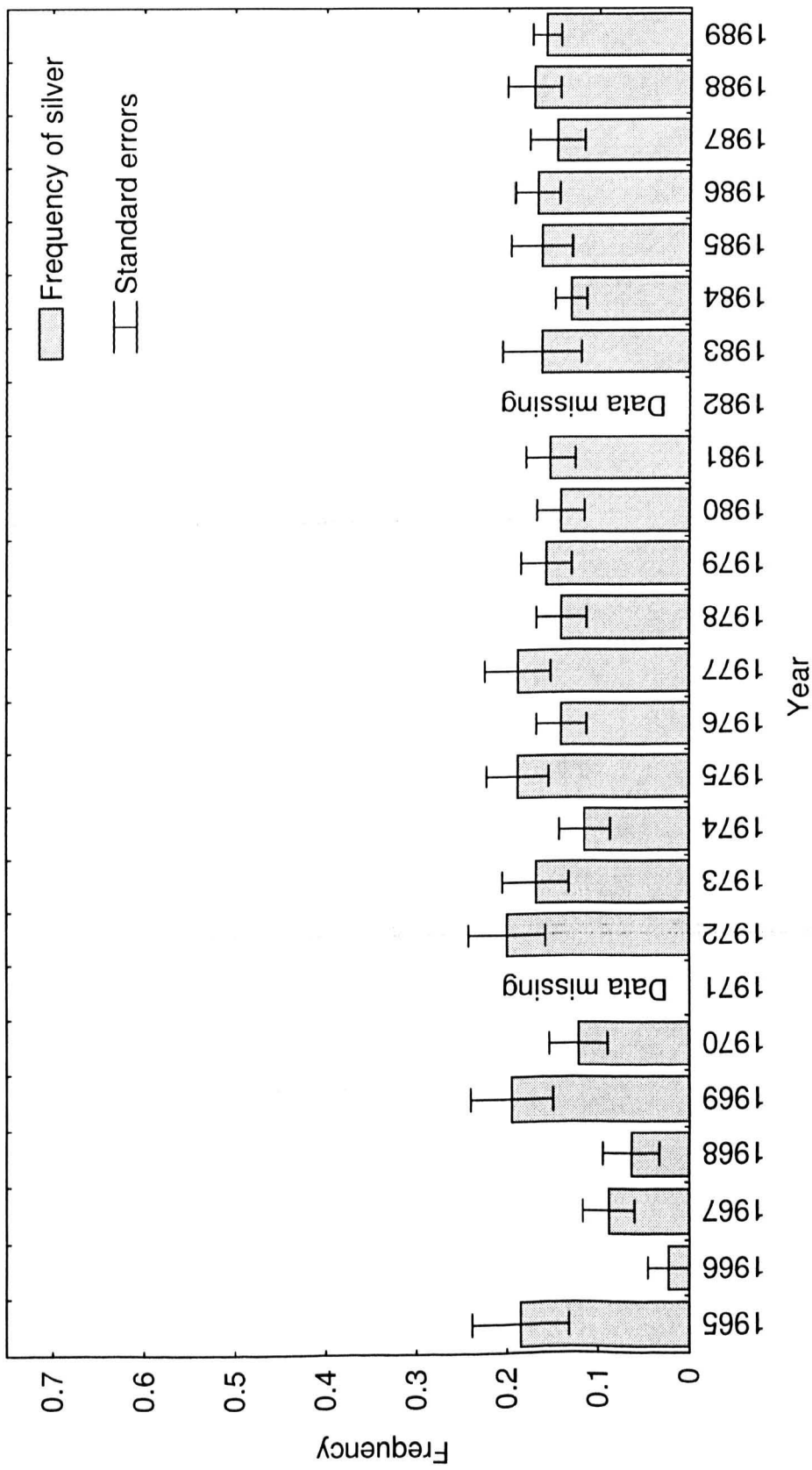
Figure 5.iv: Temporal variation in the frequency of the silver phenotype in males caught at Rothamsted.
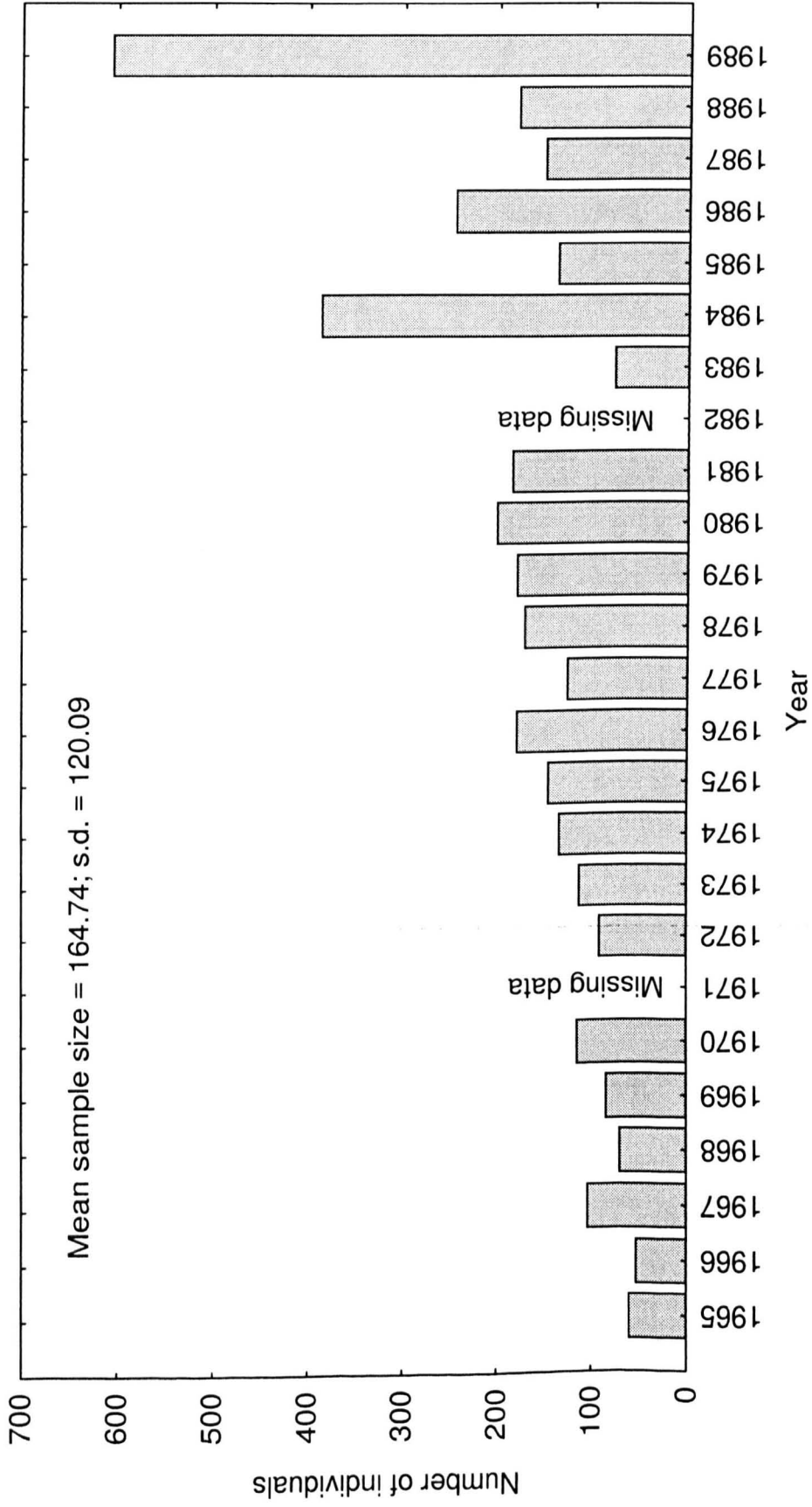
Figure 5.v: Temporal variation in total trap catches from Rothamsted Experimental Station.

This is the same question that interested R.A.Fisher and E.B.Ford in their long running study of *Panaxia dominula* at Cothill, Oxfordshire (Fisher & Ford 1947). In their 1947 paper a test for the presence or absence of selection was described, but the sampling regime at Cothill differed from that at Rothamsted as it relied on sampling with replacement rather than sampling with removal. This original model has been modified (Schaffer *et al.* 1977, Manly 1985) to enable the analysis of sampling with removal data. The test was devised to work with gene frequencies rather than phenotype frequencies. The genotype frequencies were estimated from the phenotype frequencies using the formulae given by Cook (1971). It was assumed that there were three alleles, in a dominance hierarchy, and that these alleles were at Hardy-Weinberg equilibrium.

Phenotype	*rufous*	*silver*	*ochre*
Gene frequency	p	q	r

phenotype numbers

observed	b	c	d
estimated	$(p^2 + 2pq + 2pr)n$	$(q^2 + 2qr)n$	$r^2 n$

Genotype frequency

estimated

$$p = 1 - \sqrt{\frac{c+d}{n}} \qquad (5.1)$$

$$q = \sqrt{\frac{c+d}{n}} - \sqrt{\frac{d}{n}} \qquad (5.2)$$

$$r = \sqrt{\frac{d}{n}} \qquad (5.3)$$

where n = total number of males caught

The model requires that sampling of the kind described in figure 5.vi is used. This method of sampling incurs two errors; (i) the genetic sampling error,

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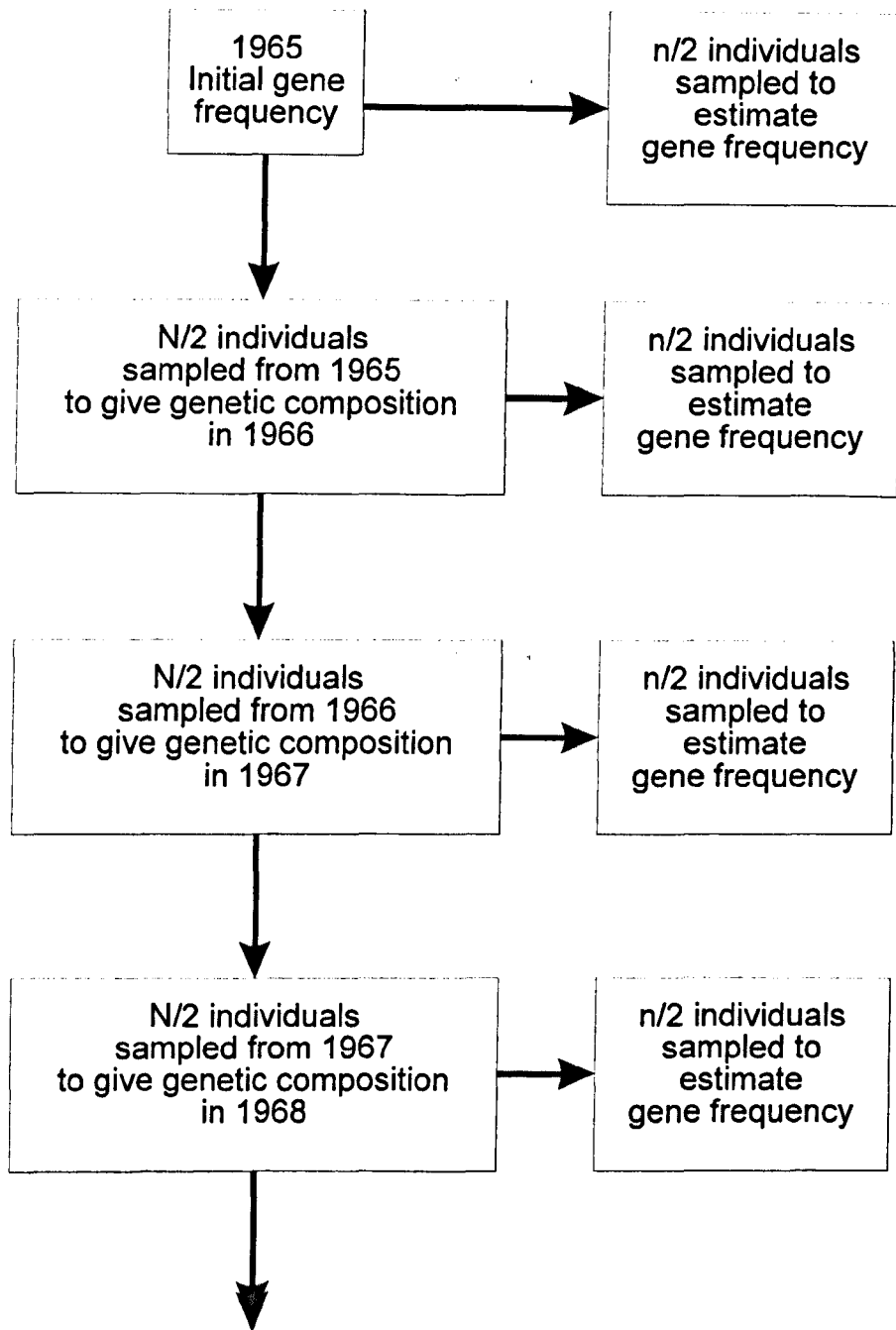
Figure 5.vi: Sampling regime used at Rothamsted to estimate forewing allele frequencies. Where N=population size and n=sample size in terms of genes in a diploid genome.

genetic drift, caused by the breeding population of *N.pronuba* being finite in size, (ii) the experimental sampling error as the samples caught at Rothamsted to estimate the phenotype frequencies were finite as well. In addition the phenotype frequency in each year will be dependent upon the frequency in the preceding year.

The data collected at Rothamsted do not provide any indication of the population size as no mark-recapture studies were performed so it is difficult to estimate the importance of sampling error between years (genetic drift). The variance due to sampling errors between years is proportional to $1 - \dfrac{1}{N}$ and so when N is large this term tends to 1. In consideration of this the formulae given by Schaffer *et al.* (1977) were modified so the sampling error term was ignored and the population sampled was considered to be infinite in size (V.Simonite *pers comm*).

The variance of the gene frequency estimates is dependent on gene frequency itself not just the sample size and is maximal at *p,q* or *r* = 0.5. To overcome this, each gene frequency was arcsine transformed to give a variate that was measured in radians; this approach follows that of Schaffer *et al.* (1977), and Fisher and Ford (1947).

The gene frequencies were transformed as follows:

$$y_i = 2\sin^{-1}(\bar{p}^{1/2}) \qquad\qquad (5.4)$$

where $(\bar{p}^{1/2})$ is the gene frequency of p, q or r from equations (5.1), (5.2) and (5.3)

The transformed gene frequencies were then tested to see if the variance was greater than that expected by chance. The test statistic *T* is compared

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with a χ^2 distribution with s-1 degrees of freedom (where s = the number of generations):

$$\mu = \frac{\sum y_i n_i}{\sum n_i} \qquad (5.5)$$

$$T = \sum n_i (y_i - \mu)^2 \qquad (5.6)$$

Where:
y_i = transformed gene frequency in radians.
n_i = sample size for that year.

Table 5.vi gives the values of the test statistic T calculated for each of the three genes when all twenty three years, for which data is available, and with 1966 excluded.

Table 5.vi: Results of tests of significance for temporal variation in phenotype frequencies. The null hypothesis is that variation in phenotype frequencies is caused by the finite sample sizes used to estimate phenotype frequencies.

	Rufous male	Ochre male	Silver male
all years d.f.=23	T=23.727 n.s.	T=63.787 p<0.005	T=12.321 n.s.
1966 excluded d.f.=22	T=23.395 n.s.	T=52.697 p<0.005	T=9.497 n.s.

The frequencies of two of the genes, rufous and silver, showed changes over the study period that are compatible with the null hypothesis i.e. the population gene frequency was constant and any variation between years was caused by the use of finite samples to estimate the population gene frequency.

The ochre gene does give a significant test statistic, even when data from 1966 is removed. The variation in the frequency of ochre is too large to be accounted by the sample size used to estimate the phenotype frequency.

In the above analysis the effects of genetic drift between years were ignored and it was assumed that it did not occur. This is a simplification as the population size of *N.pronuba* does vary with time.

If genetic drift accounts for the fluctuations in allele frequency the largest changes in allele, and hence phenotype frequency, should occur when the population size is small. As described above, there were no attempts to estimate population size at Rothamsted and this is why its effect was ignored in the analysis. As the Geescroft trap was run every day throughout the flight season of *N.pronuba* the total trap catches must reflect the actual population size. It has been demonstrated that the weather alters the size of light trap catches (Bowden 1982), but over the long flight season of *N.pronuba* these weather effects should even out. Assuming this is true the large sample sizes (e.g. 1989, n=544) and the small sample sizes (e.g. 1966, n=44) represent high and low population sizes. If fluctuations in population size accounted for the allele frequency shifts there should be a negative correlation between allele frequency change and population size. Unfortunately the sample size determines the statistical confidence of the original phenotype frequencies and hence allele frequencies (see equation 4.1) in addition to providing a relative measure of the population size.

To overcome this problem the effect of sample size on the estimation of phenotype frequency estimates was eliminated by standardising the sample sizes across all years. Phenotype frequencies were calculated by re-sampling technique. The random integer generator in Excel (function RANDBETWEEN) produces a flat distribution of randomly chosen integers

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between two specified limits. The limits were set from 1 to the total sample size so each integer represented an individual; e.g. for 1989 the limits were 1 to 544, as the sample comprised of 212 rufous males, 246 ochre males and 86 silver males, the integers 1-212, 213-458 and 459-544 were designated as representing rufous, ochre and silver individuals. From this function 44 random integers were produced and phenotype frequencies calculated by assigning the integers a phenotype depending upon which range they fell into; e.g. the following randomly generated integers: 3, 543, 345, 231, 43 would be classified as: rufous, silver, ochre, ochre, rufous.

To investigate the potential effect of fluctuating population size, the difference in phenotype frequencies between two years was regressed against the harmonic mean sample size for those two years. The difference in phenotype frequency was calculated as the positive modulus of the sum of the differences between the numbers of the three phenotypes [see table 5.vii for an example]. This distance was regressed against the harmonic mean, rather than the arithmetic mean, because the harmonic mean is dominated by small population sizes. Genetic drift has greater effect on allele frequencies in small populations and this is reflected in the harmonic mean (Hartl & Clark 1989). The relationship between phenotypic difference and the relative mean population size was not significant ($r^2=0.022$; $p=0.512$) [figure: 5.vii].
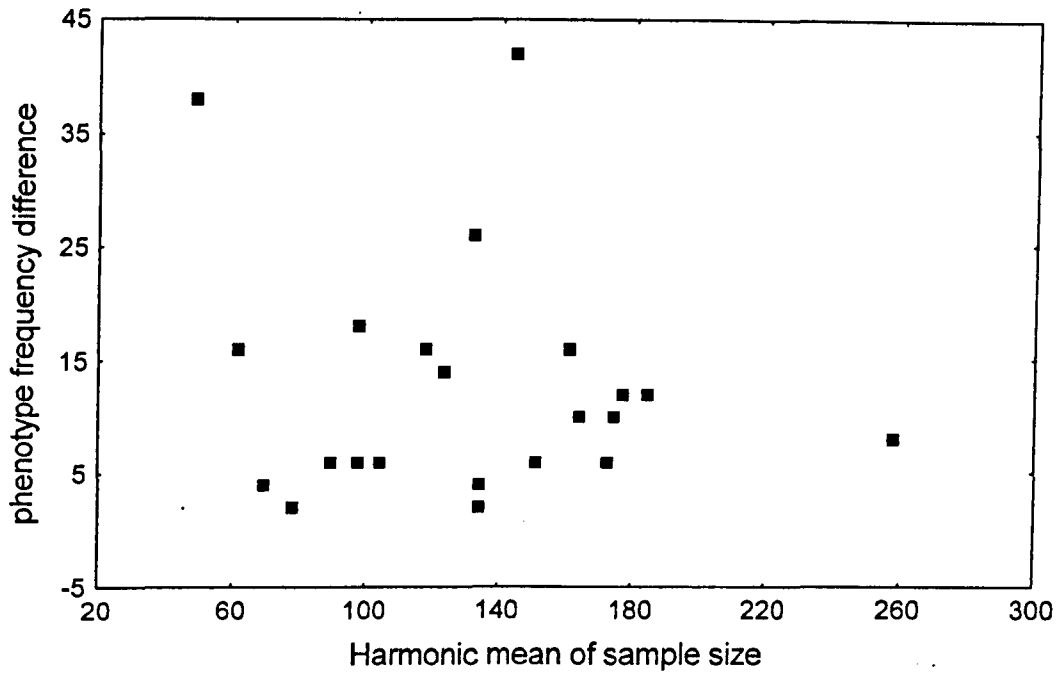
Figure 5.vii: Variation of phenotype frequency change with a measure of relative population size.

Table 5.vii: an example of a calculation of phenotype frequency differences between two years data at Rothamsted. The data was obtained by randomly resampling the observed data to negate the effects of sample size.

Year	rufous	ochre	silver
1965	20	10	12
1966	14	30	0
positive difference	6	20	12
Σ positive modulus			38

5.5: Discussion:

The data show that the polymorphism was stable throughout single flight seasons and there was no divergence in phenotype frequencies as the flight season proceeded. This suggests that there were no differences in longevity and emergence between the morphs and that, if selection is operating, it does not vary throughout the flight season.

The statistical analysis of the 25 year variation gives different results. The χ^2 test results suggest that the phenotype frequencies are homogeneous over time but using the test based on that formulated by Schaffer *et al.* (1977) the variation in the frequency of ochre is too great to be accounted for by chance.

The gene frequency test is more specific and tests directly the questions which the data raise. In consideration of this, what causes this significant variation? One plausible explanation is that the forewing phenotype is under selection and this is manifested in the data as changes in the frequency of the ochre phenotype. Examination of graphs of phenotype frequency with

time (figures 5.ii-5.iv) do not show any directional changes in phenotype frequency but the significance of the statistical tests could be explained by fluctuating selection; i.e. selection in one direction, followed by selection in the opposite direction.

The precise mechanism that causes this significant shift in allele frequency is unclear. It may be explained by the ochre allele being the bottom recessive at the forewing locus and it being at high frequency (mean 0.68). All phenotypically ochre individuals are homozygous and have two copies of the ochre allele, while phenotypically rufous and silver individuals can be heterozygotes. The relatively low frequencies of rufous and silver alleles (mean allele frequency; rufous=0.218; silver=0.103) means that individuals homozygous for those alleles will be rare (genotype frequency; rufous=0.048; silver=0.01). Equal changes in phenotype frequency will cause different changes in allele frequency, the greatest difference being in ochre.

Other studies of colour and pattern polymorphisms have failed to detect phenotype frequency changes over time. Cowie (1992) described a colour and pattern polymorphism in the helicid snail, *Theba pisana*. Over a period of 13 years there was no temporal change in phenotype frequency of the 6 studied populations, although the phenotype frequencies of the 6 populations differed from one another.

Reports of the decline in the frequency of the *carbonaria* form of *B.betularia* are in total contrast to the patterns of stability observed in *N.pronuba*. At West Kirby, on the Wirral, the frequency of *carbonaria* has declined from 0.933 in 1959 to 0.230 in 1993 (Clarke *et al.* 1994). This rapid change has been explained by the impact of smoke control legislation in the 1960s; interestingly the rapid decline in Britain is mirrored by North American

samples (D.F.Owen *pers comm*) that once had high frequencies of melanics (Owen 1961).

Dramatic increases and decreases in phenotype or allele frequencies are indicative of selective processes. Stability, however, can be brought about by a number of different processes that are difficult, if not impossible to distinguish between. *N.pronuba* is an abundant moth species in Europe and it is capable of flying large distances; this suggests that the effective population size is large. Cowie (1984) estimated that neighbourhood sizes in *Theba pisana* ranged up to 4130 individuals and that, in a population of this size, genetic drift would be insignificant (Cowie 1992). The population size of *N.pronuba* is possibly an order of magnitude larger than that observed in *T.pisana* and so it is doubtful whether genetic drift could account for the significant variation observed in male phenotype frequencies.

If selection does fluctuate and cause the observed variation in the frequency of ochre; the evidence for it is elusive. Surveys of similar length to this study but on *Cepaea nemoralis* have also failed to distinguish selection from stochastic effects (Cain & Cook 1989, Cain *et al.* 1990). This has led Cain and Cook (1989) to speculate that longer studies (250 years) are necessary to distinguish between directional changes in phenotype frequency, the stability of phenotype frequencies due to selection and stochastic fluctuations. The length of time needed to distinguish between random drifting of allele frequencies and selection causing the systematic changes is dependent upon the rate of change.

The recent decline in melanic *B.betularia* shows that dramatic changes of phenotype and allele frequency caused by selection are observable over short time periods. This is only possible when selection coefficients are large. It is possible that most selection coefficients, of evolutionary

importance, may be of such a size to preclude temporal analysis over twenty-five year periods, as described here. If a polymorphism is maintained by weak selection, and the population size is large the disentanglement of stochastic and deterministic processes may only be possible over thousands of generations.

Chapter 6: Geographic variation in the forewing polymorphism:

6.1: Introduction:

It is difficult to observe and record evolutionary changes over time unless the selective differences between genotypes are large and the study is over a protracted period. The widely quoted study of the increase in the melanic form of *Biston betularia* benefited from both of these points and it is still one of the best documented examples of natural selection causing the increase, and the subsequent decrease, in the frequency of a gene within a natural population (Cook *et al.* 1986).

Other examples of polymorphism are not so open to interpretation as the selective differences between genotypes are not as pronounced and long term data are not available for examination. The study of such polymorphic systems presents a problem. A method that overcomes such barriers is to use variation on a geographical scale instead of temporal changes in the characters of interest, as populations separated by geography are in essence separated temporally by a common ancestor (Singh & Long 1992).

Geographic variation is found in almost all groups of organisms and this variation can be in morphological, behavioural, life history and genetic traits (Endler 1977). Study of such variation has proved to be a successful method for gathering information on the selective processes that may affect the traits in question (Endler 1986).

Patterns of geographic variation in the forewing colour pattern polymorphism in *N.pronuba* have been investigated on three occasions prior to this study. The data from the studies are comparable as the same scoring regime was used in each. Poitout and Bues (1976) analysed samples from the south

west and south east of France from 1971-1974. These were collected from six regions: Aquitaine, région de Poitiers, Basse-vallée du Rhône et Mont-Ventoux, Pyrénées Occidentales and Alpes de Savoie. The results showed some geographic variation, the frequency of ochre increasing to the west. Although small, the frequency differences between regions were statistically significant.

Cook and Sarsam (1981) sampled 80 populations throughout England, Scotland and Wales, with all but two of the samples collected between 1970 and 1972. In contrast to Poitout and Bues, the results show little geographical variation in phenotype frequencies. In fact the authors comment "the frequencies are remarkably constant throughout the country."

The most recent survey was conducted in Norway. Soli and Andersen (1990) analysed 10623 individuals from 38 localities, 9 samples from south eastern Norway, 29 from western Norway. The samples from western Norway showed little geographic variation whereas in south eastern Norway, along the Skagerrak coast, there was a clinal change; the frequency of rufous increasing towards the north east.

6.2: Aims:

The results of the previous surveys provide contrasting information on the geographical structure of the polymorphism. In view of these discrepancies I decided to undertake a wider survey of phenotype frequencies. The aims were to categorise the geographic variation over a wider geographic area and to compare the geographic structure of the polymorphism in Britain in 1990-1993 with that 20 years before, when Cook and Sarsam conducted their survey (Cook & Sarsam 1981).

6.3: Materials and Methods:

All samples of *N.pronuba* were collected at light, using either a Robinson mercury vapour trap or a Heath actinic trap. I collected the samples from Shropshire and Oxford, while the samples from throughout the U.K., continental Europe, and Canada were collected by a network of professional and amateur Lepidoperists (see Acknowledgements for a full list of collectors).

Once caught and killed, with a suitable solvent such as ethyl acetate, the adult moths were packed in cotton wool. This prevented the movement of specimens removing the forewing scales and rendering them difficult to score. The packages were sent by post to Oxford.

Some of the samples used to score forewing phenotypes were also used in a survey of allozyme variation. The details of these samples and the methods used to transport them are given in chapter 7.

The samples were scored for forewing phenotype, using the classification and naming system of Cook and Sarsam (1981). Sex was determined by the presence or absence of a frenulum and retinaculum (Chapman 1969).

In total 46 samples were collected from 40 locations, 39 in Europe and one from Kentville, Nova Scotia, Canada [Figure 6.i[2]]. In all, a total of 9348 individuals were scored.

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[2]The numbers in Figure 6.i correspond to the place names in Tables 6.i and 6.ii. Sample 40 from Kentville, Nova Scotia, Canada is not illustrated in figures 6.i-6.iii.
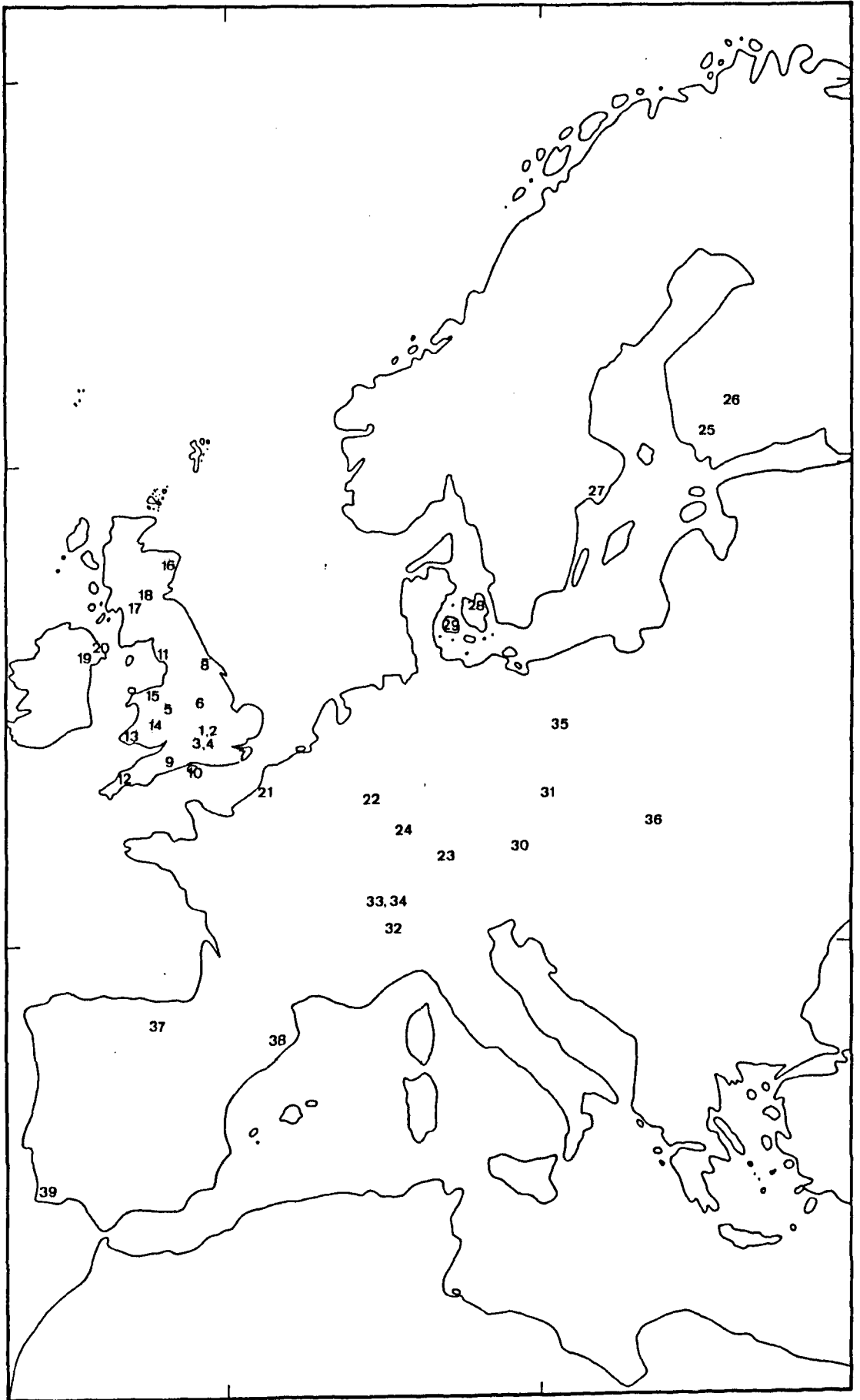
Figure 6.i: Locations of N.pronuba collection points.
Numbers refer to those in tables 6.i and 6.ii.

6.4: Results:

Males were more common in all 46 samples, with the proportion of females, relative to males, being 0.242 with all samples combined; one sample, 37, did not contain any females [Tables 6.i, 6.ii]. Samples from the Rothamsted light trap survey were not included in the analysis of geographic variation.

Of the 46 samples of males, all but one contained all three phenotypes; sample 38 lacked the silver phenotype [Table 6.i]. For females, 8 out of the 45 samples did not contain all three phenotypes; sample 11 lacked the rufous phenotype, samples 19, 20, 23, 25, 30, and 38 lacked the silver phenotype and sample 18 lacked both the rufous and silver phenotypes.

Table 6.i: Geographic variation in male phenotype frequencies.

No	Sample site	Year	Latitude	Longitude	No of male			Frequency of male			Total No
					rufous	ochre	silver	rufous	ochre	silver	of males
	England										
1	Headington, Oxfordshire	1990	51.46N	1.15W	81	113	29	0.363	0.507	0.130	223
2	Bullingdon Green, Oxfordshire	1991	51.46N	1.15W	126	160	46	0.380	0.482	0.139	332
	"	1992	51.46N	1.15W	504	582	174	0.400	0.462	0.138	1260
3	Long Wittenham, Oxfordshire	1991	51.38N	1.12W	115	139	42	0.389	0.470	0.142	296
	"	1992	51.38N	1.12W	77	94	28	0.387	0.472	0.141	199
	"	1993	51.38N	1.12W	73	95	20	0.388	0.505	0.106	188
4	Little Wittenham, Oxfordshire	1993	51.38N	1.12W	94	114	33	0.390	0.473	0.137	241
5	Forton, Shropshire	1990	52.47N	2.22W	75	122	17	0.350	0.570	0.079	214
	"	1992	52.47N	2.22W	110	160	34	0.362	0.526	0.112	304
6	Leicester, Leicester	1992	52.39N	1.09W	26	27	7	0.433	0.450	0.117	60
7	Eastbridge, Kent	1991	51.02N	1.00E	37	35	14	0.430	0.407	0.163	86
	"	1992	51.02N	1.00E	120	180	47	0.346	0.519	0.135	347
8	York, Yorkshire	1992	53.58N	1.05W	50	69	19	0.362	0.500	0.138	138
9	West Bexington, Dorset	1992	50.43N	2.43W	85	119	28	0.366	0.513	0.121	232
10	Freshwater, Isle of Wight	1992	50.41N	1.31W	72	86	29	0.385	0.460	0.155	187
11	Grange-Over-Sands, Cumbria	1992	54.12N	2.55W	20	23	9	0.385	0.442	0.173	52
12	Newquay, Cornwall	1991	50.25N	5.05W	20	20	4	0.455	0.455	0.091	44
	Wales										
13	Lamphey, Pembrokeshire	1992	51.40N	4.51W	15	16	5	0.417	0.444	0.139	36
14	Builth Wells,	1992	53.05N	3.24W	29	37	16	0.354	0.451	0.195	82
15	Betws-Y-Coed,	1993	53.05N	3.48W	43	48	22	0.381	0.425	0.195	113
	Scotland										
16	Aberdeen,	1992	57.10N	2.04W	24	50	10	0.286	0.595	0.119	84
17	Glasgow,	1992	55.53N	4.15W	73	144	21	0.307	0.605	0.088	238
18	Falkirk,	1992	56.00N	3.48W	13	29	4	0.283	0.630	0.087	46

No	Sample site	Year	Latitude	Longitude	No of male			Frequency of male			Total No
					rufous	ochre	silver	rufous	ochre	silver	of males
	Northern Ireland										
19	Portadown, County Armagh	1992	54.26N	6.27W	22	34	2	0.379	0.586	0.034	58
20	Bangor, County Down	1992	54.40N	5.40W	52	64	2	0.441	0.542	0.017	118
	France										
21	Wailly-Beauchamp,	1992	50.24N	1.35E	49	77	18	0.340	0.535	0.125	144
	Germany										
22	Cochem, Mosel Valley	1991	50.08N	7.10E	34	28	11	0.466	0.384	0.151	73
23	Steißlingen	1992	47.46N	8.50E	22	12	5	0.564	0.308	0.128	39
24	Mainz,	1992	50.00N	8.16E	18	30	7	0.327	0.545	0.127	55
	Finland										
25	Koski	1992	60.40N	23.10E	105	37	13	0.677	0.239	0.084	155
26	Hattula	1992	61.06N	24.20E	90	25	7	0.738	0.205	0.057	122
	Sweden										
27	Stockholm	1992	59.20N	18.05E	57	40	13	0.518	0.364	0.118	110
	Denmark										
28	Hillerød	1992	55.56N	12.19E	12	20	5	0.324	0.541	0.135	37
29	Kirkenby	1992	55.08N	10.33E	59	61	18	0.428	0.442	0.130	138
	Austria										
30	Scharnstein	1993	47.53N	13.56E	43	36	15	0.457	0.383	0.160	94
	Czech Republic										
31	Ceské Budejovice, Branisovaka	1992	48.58N	14.29E	19	20	11	0.380	0.400	0.220	50
	Switzerland										
32	Ticino	1992	46.20N	8.45E	112	139	31	0.397	0.493	0.110	282
33	Napf-Cebiet	1992	47.00N	7.56E	34	33	12	0.430	0.418	0.152	79
34	Romoos-Napf	1992	47.01N	8.03E	113	91	24	0.496	0.399	0.105	228
	"	1993	47.01N	8.03E	38	51	9	0.388	0.520	0.092	98

No	Sample site	Year	Latitude	Longitude	No of male			Frequency of male			Total No
					rufous	ochre	silver	rufous	ochre	silver	of males
	Poland										
35	Poznan	1992	52.25N	16.53E	8	14	1	0.348	0.609	0.043	23
	Hungary										
36	Miskoloc	1993	48.07N	20.50E	11	7	3	0.524	0.333	0.143	21
	Spain										
37	Burgos	1992	42.20N	3.42W	6	8	2	0.375	0.500	0.125	16
38	Fogars	1978-80	41.25N	2.10E	16	14	0	0.533	0.467	0.000	30
	Portugal										
39	Portimão	1992	37.08N	8.32W	25	27	4	0.446	0.482	0.071	56
	Canada										
40	Kentville, Nova Scotia	1992	48.05N	64.30W	26	17	11	0.481	0.315	0.204	54

Grand total= 7082

Table 6.ii: Geographic variation in female phenotype frequencies.

No	Sample site	Year	Latitude	Longitude	No of female			Frequency of female			Total No of females
					rufous	ochre	silver	rufous	ochre	silver	
	England										
1	Headington, Oxfordshire	1990	51.46N	1.15W	66	68	17	0.437	0.450	0.113	151
2	Bullingdon Green, Oxfordshire	1991	51.46N	1.15W	66	67	29	0.407	0.414	0.179	162
	"	1992	51.46N	1.15W	114	116	48	0.410	0.417	0.173	278
3	Long Wittenham, Oxfordshire	1991	51.38N	1.12W	52	75	30	0.331	0.478	0.191	157
	"	1992	51.38N	1.12W	30	42	8	0.375	0.525	0.100	80
	"	1993	51.38N	1.12W	17	23	7	0.362	0.489	0.149	47
4	Little Wittenham, Oxfordshire	1993	51.38N	1.12W	36	41	21	0.367	0.418	0.214	98
5	Forton, Shropshire	1990	52.47N	2.22W	18	31	16	0.277	0.477	0.246	65
	"	1992	52.47N	2.22W	17	17	10	0.386	0.386	0.227	44
6	Leicester, Leicester	1992	52.39N	1.09W	1	1	1	0.333	0.333	0.333	3
7	Eastbridge, Kent	1991	51.02N	1.00E	38	42	9	0.427	0.472	0.101	89
	"	1992	51.02N	1.00E	36	42	20	0.367	0.429	0.204	98
8	York, Yorkshire	1992	53.58N	1.05W	15	30	8	0.283	0.566	0.151	53
9	West Bexington, Dorset	1992	50.43N	2.43W	65	93	33	0.340	0.487	0.173	191
10	Freshwater, Isle of Wight	1992	50.41N	1.31W	7	5	2	0.500	0.357	0.143	14
11	Grange-Over-Sands, Cumbria	1992	54.12N	2.55W	0	2	1	0.000	0.667	0.333	3
12	Newquay, Cornwall	1991	50.25N	5.05W	15	20	7	0.357	0.476	0.167	42
	Wales										
13	Lamphey, Pembrokeshire	1992	51.40N	4.51W	3	2	1	0.500	0.333	0.167	6
14	Builth Wells,	1992	53.05N	3.24W	2	1	1	0.500	0.250	0.250	4
15	Betws-Y-Coed,	1993	53.05N	3.48W	2	4	1	0.286	0.571	0.143	7
	Scotland										
16	Aberdeen,	1992	57.10N	2.04W	7	19	4	0.233	0.633	0.133	30
17	Glasgow,	1992	55.53N	4.15W	4	8	1	0.308	0.615	0.077	13
18	Falkirk,	1992	56.00N	3.48W	0	3	0	0.000	1.000	0.000	3

No	Sample site	Year	Latitude	Longitude	No of female			Frequency of female			Total No
					rufous	ochre	silver	rufous	ochre	silver	of females
	Northern Ireland										
19	Portadown, County Armagh	1992	54.26N	6.27W	22	16	0	0.579	0.421	0.000	38
20	Bangor, County Down	1992	54.40N	5.40W	6	3	0	0.667	0.333	0.000	9
	France										
21	Wailly-Beauchamp,	1992	50.24N	1.35E	35	50	19	0.337	0.481	0.183	104
	Germany										
22	Cochem, Mosel Valley	1991	50.08N	7.10E	23	21	5	0.469	0.429	0.102	49
23	Steißlingen	1992	47.46N	8.50E	3	4	0	0.429	0.571	0.000	7
24	Mainz,	1992	50.00N	8.16E	4	2	2	0.500	0.250	0.250	8
	Finland										
25	Koski	1992	60.40N	23.10E	12	1	0	0.923	0.077	0.000	13
26	Hattula	1992	61.06N	24.20E	4	1	1	0.667	0.167	0.167	6
	Sweden										
27	Stockholm	1992	59.20N	18.05E	11	3	8	0.500	0.136	0.364	22
	Denmark										
28	Hillerød	1992	55.56N	12.19E	3	1	2	0.500	0.167	0.333	6
29	Kirkenby	1992	55.08N	10.33E	4	4	2	0.400	0.400	0.200	10
	Austria										
30	Scharnstein	1993	47.53N	13.56E	2	1	0	0.667	0.333	0.000	3
	Czech Republic										
31	Ceské Budejovice, Branisovaka	1992	48.58N	14.29E	11	9	2	0.500	0.409	0.091	22
	Switzerland										
32	Ticino	1992	46.20N	8.45E	10	11	6	0.370	0.407	0.222	27
33	Napf-Cebiet	1992	47.00N	7.56E	26	22	11	0.441	0.373	0.186	59
34	Romoos-Napf	1992	47.01N	8.03E	26	15	13	0.481	0.278	0.241	54
	*	1993	47.01N	8.03E	46	43	14	0.447	0.417	0.136	103

No	Sample site	Year	Latitude	Longitude	No of female			Frequency of female			Total No
					rufous	ochre	silver	rufous	ochre	silver	of females
	Poland										
35	Poznan	1992	52.25N	16.53E	10	5	4	0.526	0.263	0.211	19
	Hungary										
36	Miskoloc	1993	48.07N	20.50E	6	3	3	0.500	0.250	0.250	12
	Spain										
37	Burgos	1992	42.20N	3.42W	0	0	0				0
38	Fogars	1978-80	41.25N	2.10E	5	10	0	0.333	0.667	0.000	15
	Portugal										
39	Portimão	1992	37.08N	8.32W	3	4	1	0.375	0.500	0.125	8
	Canada										
40	Kentville, Nova Scotia	1992	48.05N	64.30W	14	13	7	0.412	0.382	0.206	34

Grand total= 2266
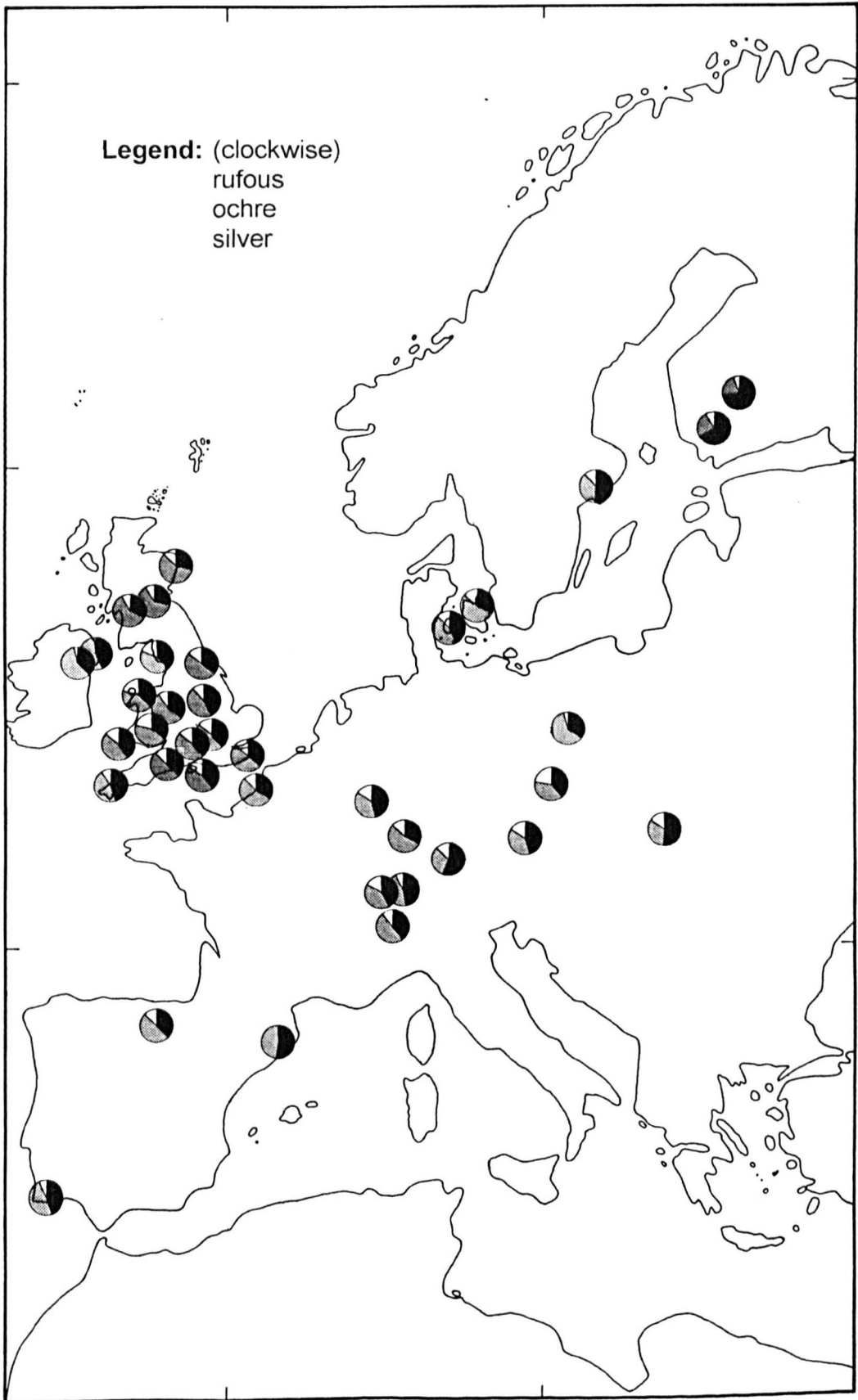
Figure 6.ii: Variation in male N.pronuba forewing phenotype frequencies.
Only those samples with >20 individuals are shown.
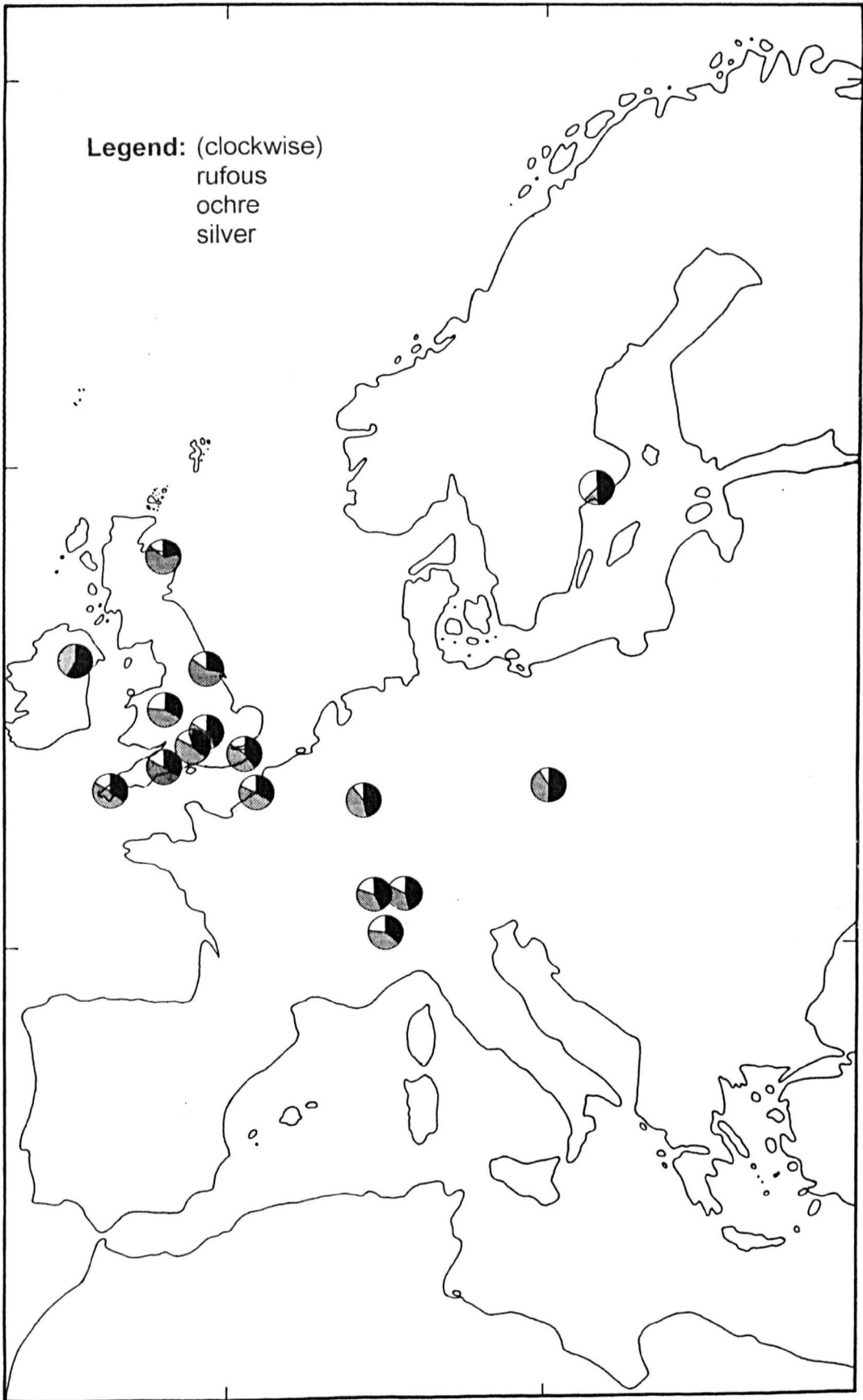
Figure 6.iii: Variation in female N.pronuba forewing phenotype frequencies.
Only those samples with >20 individuals are shown.

6.4.1: Variation in males:

For males, the overall impression from the pie charts is one of stability in phenotype frequencies over the whole geographic area, with slight differentiation at the extremes [figure 6.ii]. The two samples from N.Ireland (19 and 20) had low frequencies of the silver phenotype (0.034 and 0.017), while the two samples from Finland (25 and 26) had high frequencies of the rufous phenotype (0.677 and 0.738) relative to the majority of samples from continental Europe and Britain. The three samples from Scotland (16, 17 and 18) have higher frequencies of the ochre morph (0.595, 0.605 and 0.630) than all samples sites except Poznan (site 35, frequency 0.609). However, the sample size at Poznan is quite small (n=23).

As there are many samples, all the sites from single countries were grouped together and these groups tested for significant heterogeneity using a χ^2 test [table 6.iii]. None of the tests were significant so grouping the data is statistically valid. To test for differences between the phenotypic proportions the groups of sites were tested against one another using a χ^2 contingency test. In each case there were 2 degrees of freedom [table 6.iv]. Thirty eight of the 72 tests were significantly different at p=0.05 or less (52.8%). The phenotype frequencies in Finland were significantly different from all other sample sites, while N.Ireland were significantly different from 11 and Scotland were significantly different from 10 of the 12 comparisons. Samples from England, Wales and central Europe had similar phenotype frequencies with few tests reaching formal significance.

The use of statistical tests repeatedly on the same data set can lead to type 1 errors which leads to the rejection of the null hypothesis when in fact it is correct (Sokal & Rholf 1981). This criticism could be levelled at this analysis, but the probability of the important comparisons, i.e. those between Finland, Scotland, N.Ireland

Table 6.iii:χ^2 *tests of heterogeneity of male phenotype frequencies within countries or regions.*

Country	Number of sample sites	Degrees of Freedom	χ^2	Probability
England	12	22	17.684	0.725 n.s.
Wales	3	4	0.865	0.930 n.s.
Scotland	3	4	0.857	0.931 n.s.
N.Ireland	2	2	1.009	0.604 n.s.
Germany	3	4	6.666	0.155 n.s.
Finland	2	2	1.364	0.506 n.s.
Denmark	2	2	1.383	0.501 n.s.
Central Europe	4	6	7.065	0.315 n.s.
Switzerland	3	4	4.393	0.355 n.s.

The central Europe region includes samples 30 (Scharnstein), 31 (Ceské Budejovice), 35 (Poznan) and 36 (Miskoloc).

Table 6.iv: χ^2 test results for comparisons of male phenotype frequencies between countries, regions or sample sites.

	England	Wales	Scotland	N.Ireland	France	Germany	Finland	Sweden	Denmark	c.Europe	Switzer-land	Portugal
Wales	5.918 n.s.	#										
Scotland	19.818***	19.110***	#									
N.Ireland	18.152***	26.508***	14.388***	#								
France	1.397 n.s.	4.138 n.s.	2.360 n.s.	13.445***	#							
Germany	3.039 n.s.	2.503 n.s.	16.190***	17.813***	4.347 n.s.	#						
Finland	112.148***	55.379***	108.042***	53.326***	52.286***	29.629***	#					
Sweden	8.560*	6.608*	21.142***	17.023***	8.716*	1.500 n.s	11.965**	#				
Denmark	0.412 n.s.	2.196 n.s.	9.862**	15.044*	1.748 n.s.	0.677 n.s	39.534***	3.562 n.s.	#			
c. Europe	4.298 n.s.	1.367 n.s.	19.625***	22.347***	5.136 n.s.	0.333 n.s.	34.952***	2.340 n.s.	1.220 n.s.	#		
Switzer-land	6.814*	9.038*	22.044***	14.644***	4.165 n.s.	1.316 n.s.	59.002***	3.486 n.s.	0.767 n.s.	3.665 n.s.	#	
Portugal	2.122 n.s.	4.389 n.s.	4.882 n.s.	3.429 n.s.	2.494 n.s.	1.906 n.s.	16.307***	2.470 n.s.	1.510 n.s.	2.963 n.s.	0.834 n.s.	#
Canada	6.657*	2.869 n.s.	16.984***	25.964***	7.729*	2.416 n.s.	13.262**	2.115 n.s.	4.125 n.s.	1.695 n.s.	6.240*	5.524 n.s.

n.s. = not significant (p>0.05); * = 0.05>p>0.01; ** = 0.01>p>0.001; *** = p<0.001

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and other sites, are of sufficient magnitude (p<0.001) to cast little doubt on their validity.

6.4.2: Variation in females:

In females this pattern is not as clear because of smaller sample sizes and of 2266 females caught in this survey, 973 were caught in Oxfordshire (sample sites 1,2,3 and 4) [table 6.i, figure 6.iii]. The samples of females from Finland and Scotland were small but Aberdeen (16) had a high frequency of ochre individuals (0.633) relative to the rest of the U.K. while Koski (25), in Finland, had a high frequency of rufous (0.923). The sample from Portadown(19) had no female silver phenotypes which is compatible with the low frequency found in males. Overall the geographic structure seems similar to that displayed by males but the low sample sizes, especially from continental Europe, hinder the statistical analysis of the data.

Samples within England were pooled and were homogeneous (χ^2=14.228; d.f.=14; p=0.433) as were those from Switzerland (χ^2=0.829; d.f.=6; p=0.934). These two pooled samples, along with those of sample sizes greater than 10 were tested for heterogeneity using a χ^2 test, as with males. The results of these tests are given in table 6.v. Blank cells in the table are where more than 20% of the expected values are below 5.

Of the 36 tests that were possible 13 (36.1%) were significant at p<0.05. N.Ireland is significantly different from both England and France but unfortunately no test could be carried out with Scotland. The Finnish sample could only be tested against the French sample and it was significant, while the Scottish sample was homogeneous with England and France. Overall, the three regions which are most different in males (Finland, Scotland and N.Ireland) do not show the same pattern in females [table 6.v].

Table 6.v:χ^2 test results for comparisons of female phenotype frequencies between countries, regions or samples. Blank spaces indicate tests where more than 20% of the expected values were <5.

	England	Scotland	N.Ireland	France	Germany	Finland	Sweden	Czech	Switzer-land	Poland
		Aberdeen	Portadown	Wailly-Beachamp	Cochem	Koski	Stockholm	Ceske-Budejovice		Poznan
Scotland	3.045 n.s.	#								
N.Ireland	12.348**		#							
France	0.088 n.s.	2.174 n.s.	11.230**	#						
Germany	3.600 n.s.	4.431 n.s.		3.127 n.s.	#					
Finland				16.594***		#				
Sweden	10.892**	12.934**	17.542***	9.224**	9.540**		#			
Czech	2.468 n.s.			2.446 n.s.			6.600*	#		
Switzerland	9.572**	7.612*	8.353*	4.083 n.s.	1.885 n.s.		6.790*	1.147 n.s.	#	
Poland	3.534 n.s.			3.349 n.s.	2.289 n.s.				0.942 n.s.	#
Canada	1.106 n.s.	4.042 n.s.		1.025 n.s.	1.751 n.s.		4.303 n.s.	1.356 n.s.	0.177 n.s.	0.864 n.s.

n.s. = not significant (p>0.05); * = 0.05>p>0.01; ** = 0.01>p>0.001; *** = p<0.001

This is in part accounted for by the small sample sizes in females; which not only invalidates some comparisons but reduces the power of χ^2 tests.

6.4.3: Variation in male phenotype frequency with longitude and latitude:

To investigate any underlying patterns in the geographic variation in phenotype frequencies regression analyses were performed on the data for males. Only male data were used as sample sizes of females from outside the U.K. were small.

In all six graphs there is a large amount of scatter with no striking relationships [Figures 6.iv, 6.v].. A problem with such data is that the sample sizes used to make the frequency estimates are unequal, consequently each frequency estimate has different confidence limits. To overcome this a weighted ordinary least squares regression analysis was used, where the following weights were applied (Manly 1985).

$$\text{weight} = \frac{1}{\text{var}(p)}$$

$$\text{where:} \quad \text{var}(p) = \frac{p(1-p)}{n}$$

p=phenotype frequency
n=sample size used to estimate p

Two of the six regressions gave significant relationships, these being between longitude and the frequency of rufous and ochre. It is important to note that ochre and rufous co-vary and cannot be considered to be independent. This covariance is to be expected as they are both controlled by alleles at the same locus; as one increases the other must decrease.

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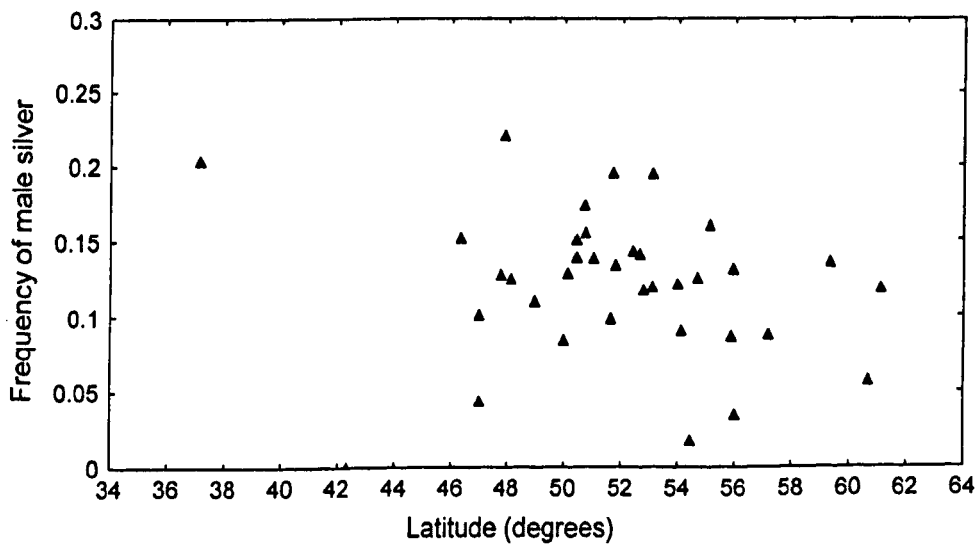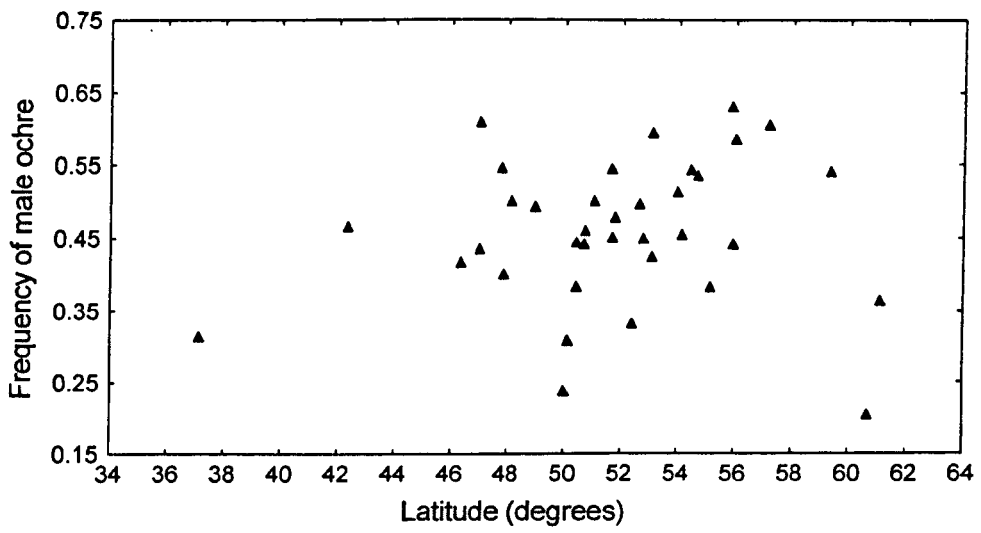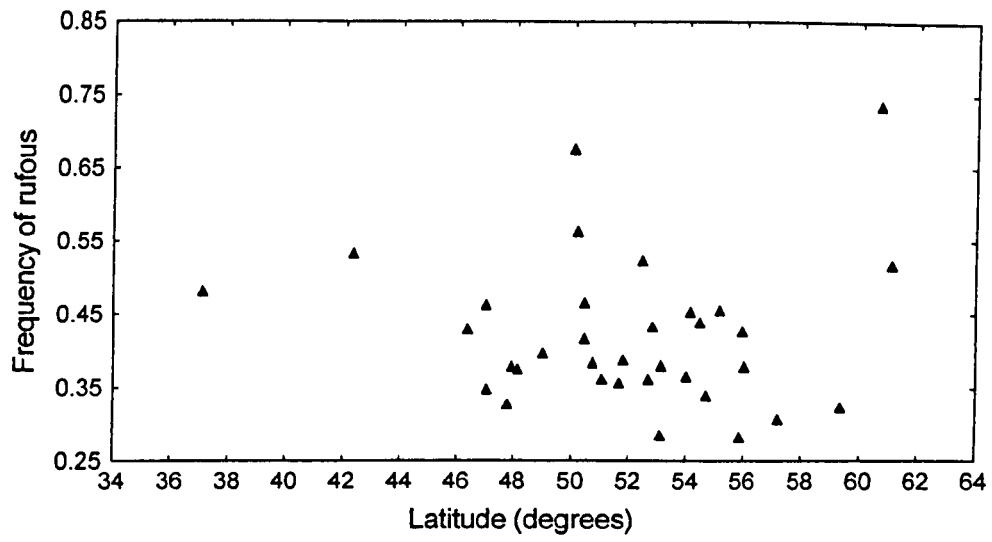
Figure 6.iv: Variation of male phenotype frequency with latitude.

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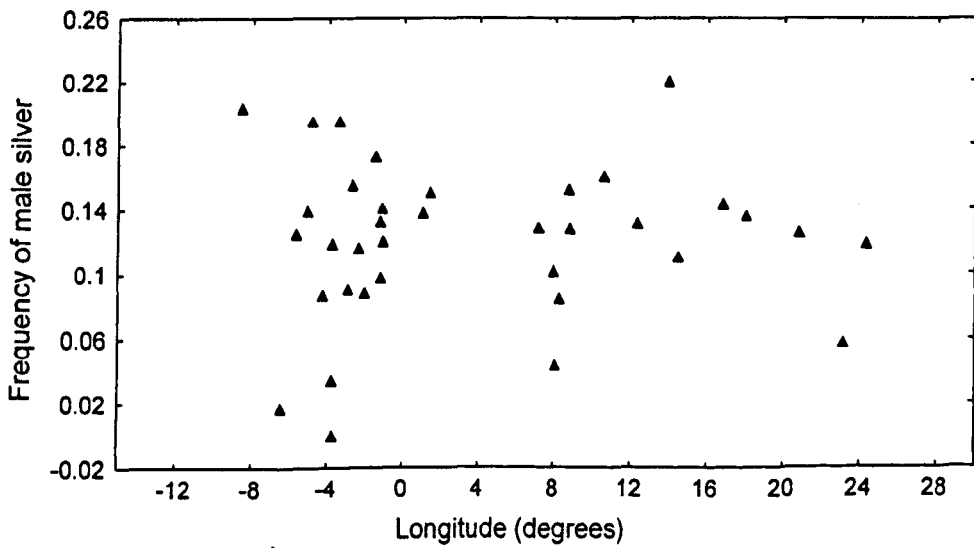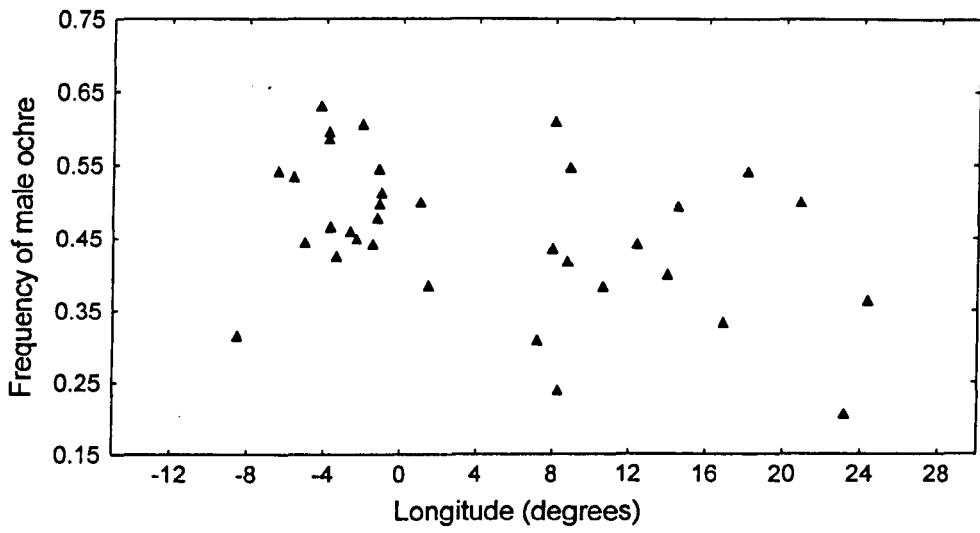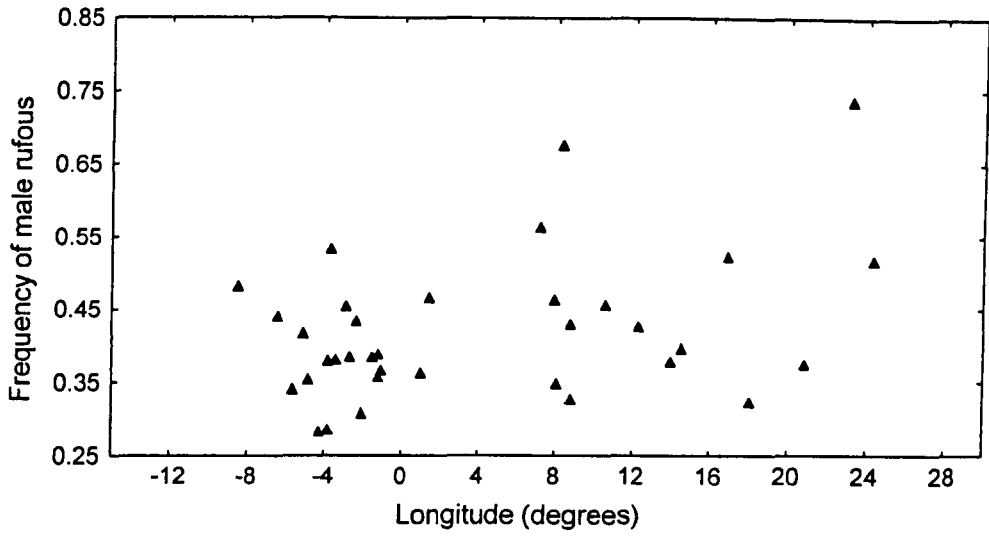
Figure 6.v: Variation of male phenotype frequency with longitude.

Table: 6.vi Weighted ordinary least squares regression results for male phenotype frequencies against longitude and latitude. Weights used were calculated from 1/(variance of the phenotype proportions).

Independent variable - Latitude:

Dependent variable	R^2	F	P	significance
frequency of rufous	0.0391	1.3820	0.2479	n.s.
frequency of ochre	0.0183	0.6331	0.4317	n.s.
frequency of silver	0.0154	0.5311	0.4713	n.s.

Independent variable - Longitude

Dependent variable	R^2	F	P	Significance
frequency of rufous	0.3152	15.6052	0.0004	***
frequency of ochre	0.2600	11.9306	0.0015	**
frequency of silver	0.0151	0.5064	0.4817	n.s.

n.s. = not significant (p>0.05); * = 0.05>p>0.01; ** = 0.01>p>0.001; *** = p<0.001

6.5: Discussion:

To facilitate comparisons between this and previous studies, summaries of published data are provided [Figures 6.vi, 6.vii, and 6.viii]. It must be noted that the frequency data from this study, Cook and Sarsam (1981) and Soli and Andersen (1990) is for males only, while that for Poitout and Bues (1976) is for males and females combined.

6.5.1: Comparisons with Cook & Sarsam (1981)

There are differences in the phenotype frequencies given by Cook and Sarsam (1981) compared to this study [Figure 6.ix]. The frequency of silver males was lower, while the frequency of ochre males was higher in their study compared to this. In contrast the frequency of rufous was similar with the modal frequency within the same range (0.35-0.40) for both studies [figure 6.vi].

For analysis purposes, Cook and Sarsam pooled their samples into 100km grid squares if the samples were homogeneous when tested with a χ^2 test of heterogeneity. To aid comparison, the samples from this survey were grouped in a similar manner. Tests of heterogeneity were performed on 100km grid squares that contained more than one sample site or samples

Table 6.vii: A comparison of Cooks & Sarsam's (1981) data with data from this study. 100Km grid squares common to both surveys were tested for heterogeneity in phenotype frequencies using a χ^2 test.

100Km grid square	Degrees of freedom	χ^2	Probability
Males			
NS	2	15.769	0.0004***
SH	2	26.254	<0.0001***
SK	Expected values too low to perform test		
SP	2	13.365	0.0011**
SU	2	7.903	0.0192*
Females			
NS	Expected values too low to perform test		
SH	Expected values too low to perform test		
SK	Expected values too low to perform test		
SP	2	9.486	0.0087**
SU	2	3.954	0.1385

n.s. = not significant (p>0.05); * = 0.05>p>0.01; ** = 0.01>p>0.001; *** = p<0.001
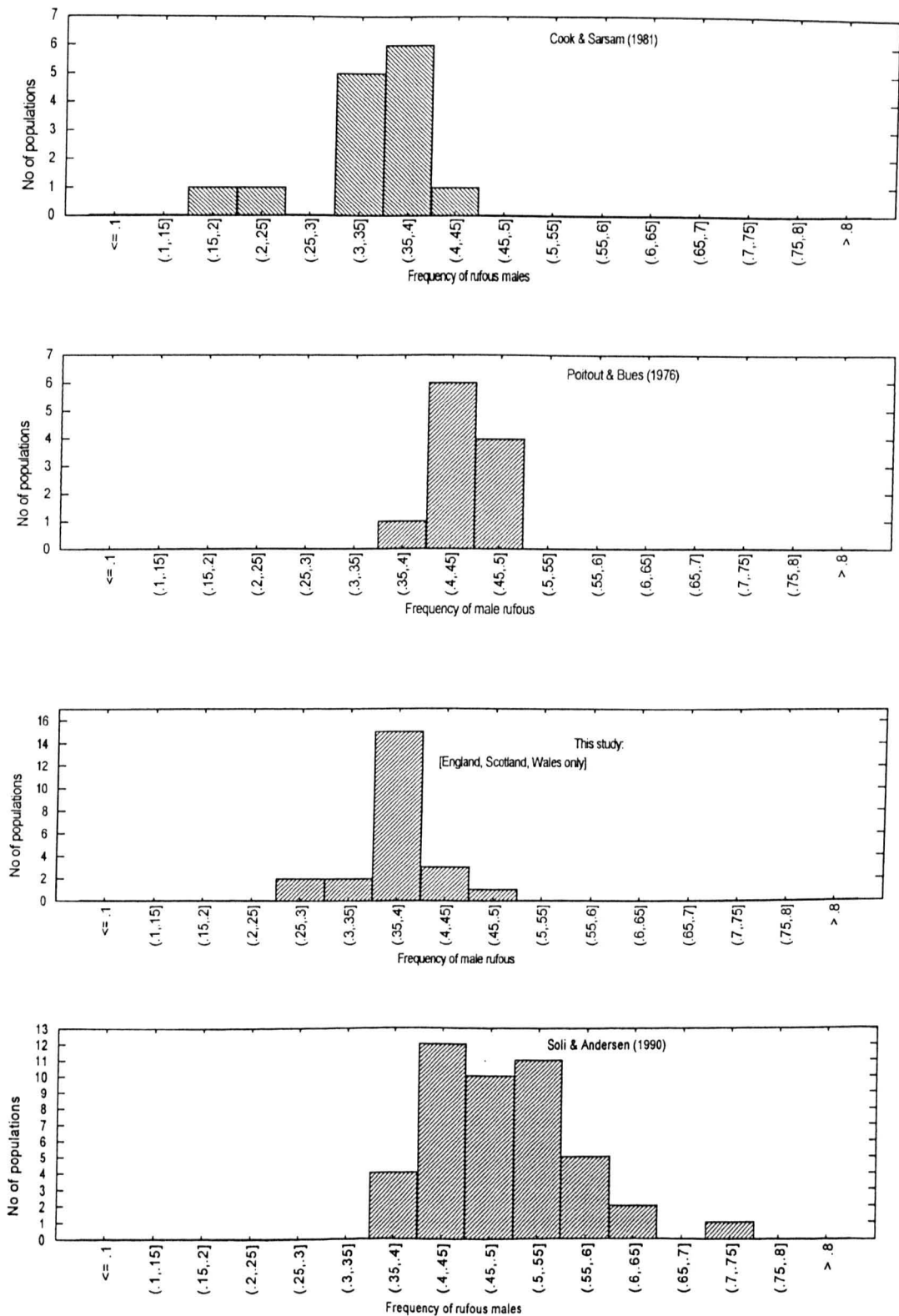
Figure 6.vi: Phenotype frequency distributions for rufous males from this study and all previous studies of geographical variation in phenotype frequencies in N.pronuba. The data from this study only includes samples from England, Scotland and Wales to facilitate comparison with Cook & Sarsam (1981).
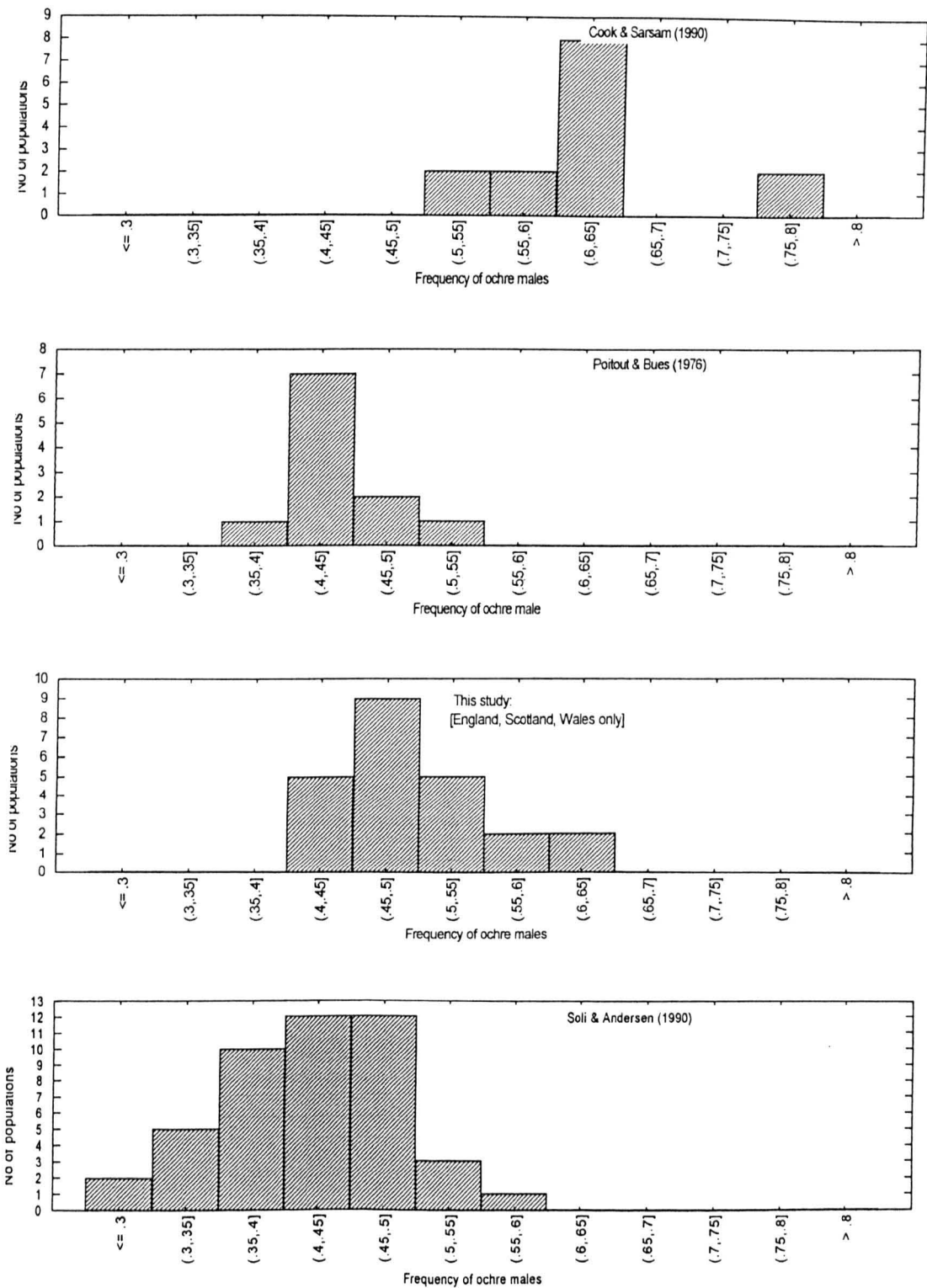
Figure 6.vii: Phenotype frequency distributions for ochre males from this study and all previous studies of geographical variation in phenotype frequencies in N.pronuba. The data from this study only includes samples from England, Scotland and Wales to facilitate comparison with Cook & Sarsam (1981).

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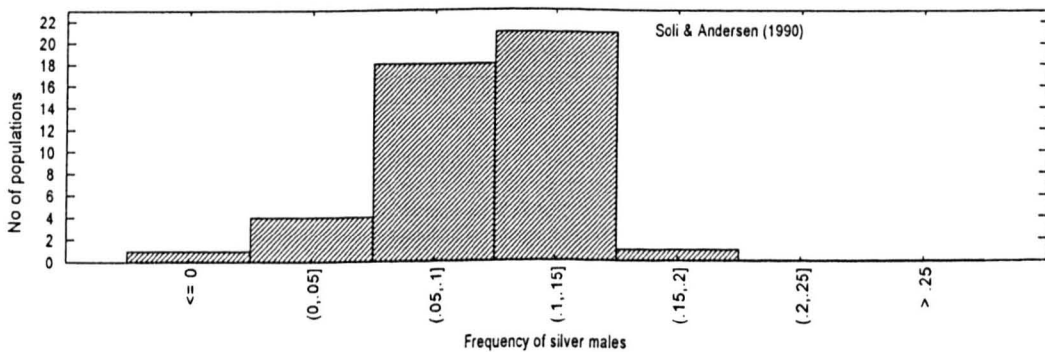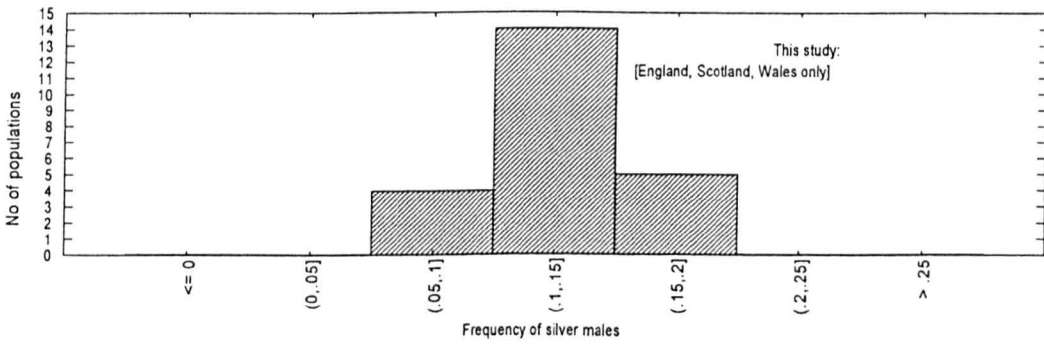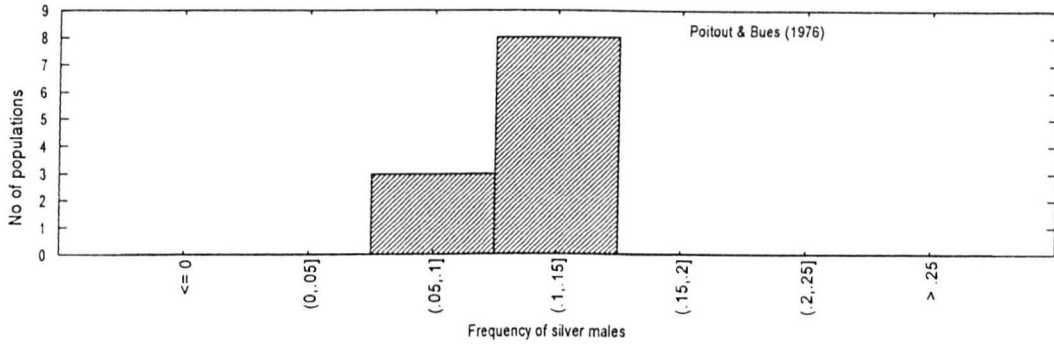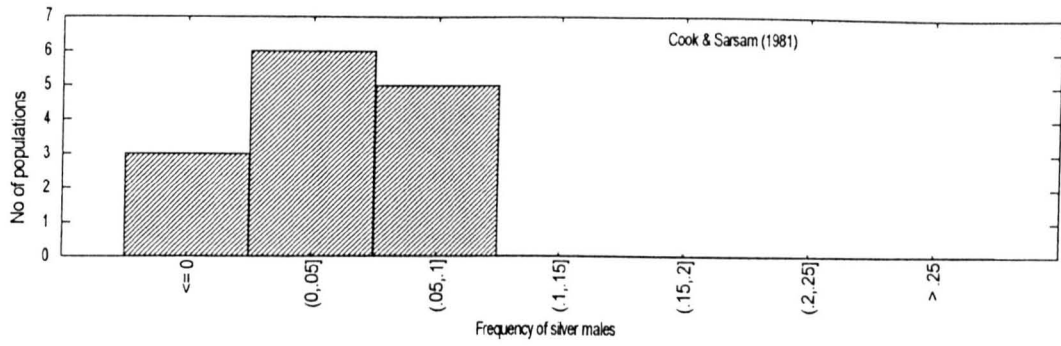
Figure 6.viii: Phenotype frequency distributions for silver males from this study and all previous studies of geographical variation in phenotype frequencies in N.pronuba. The data from this study only includes samples from England, Scotland and Wales to facilitate comparison with Cook & Sarsam (1981).
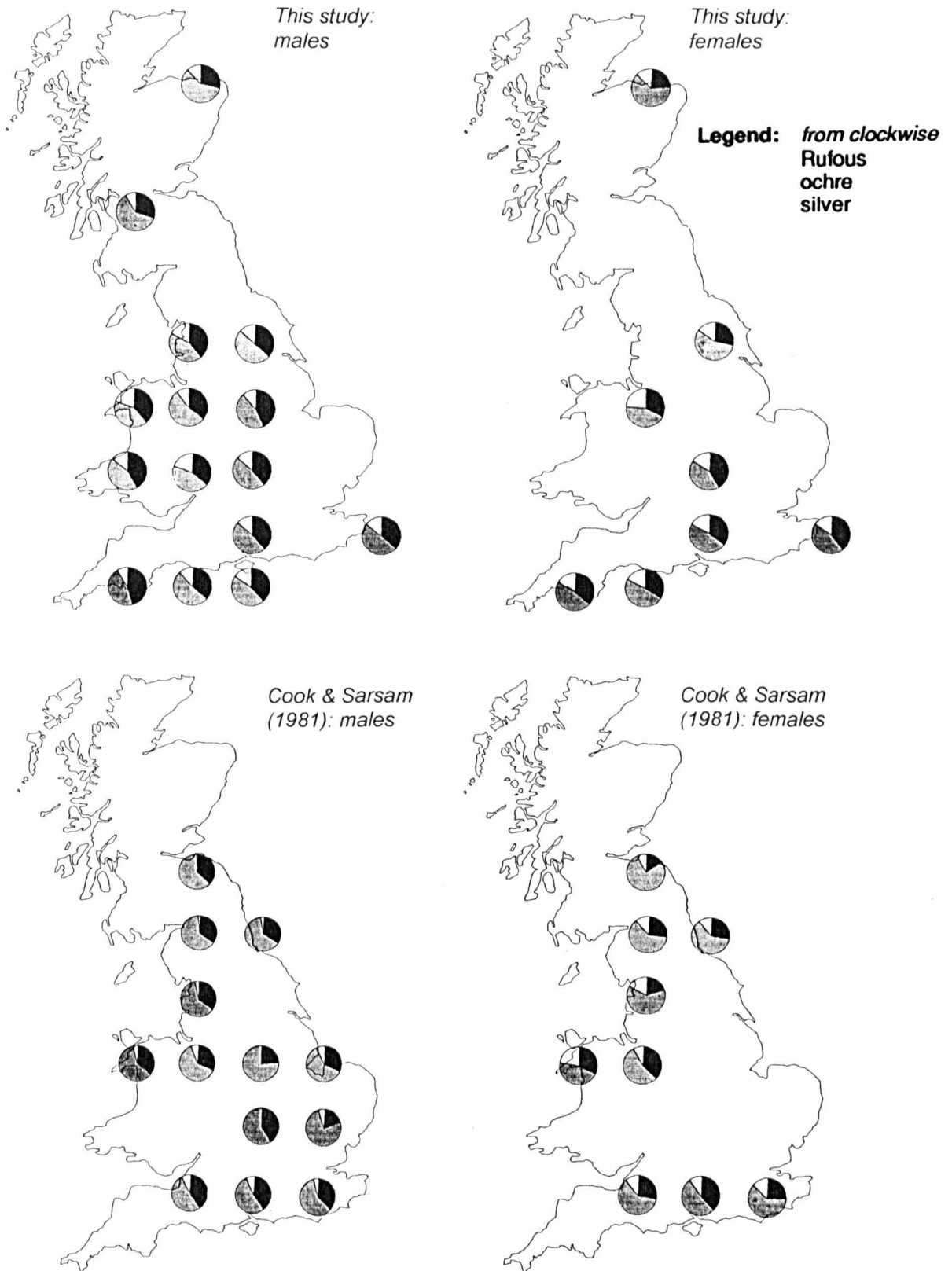
Figure 6.ix: Phenotype frequencies from this study and Cook & Sarsam (1981). Phenotype frequencies are for 100 Km squares; where multiple sample sites were collected from single 100 Km squares, phenotype frequencies were tested for heterogeneity. Pie charts are positioned at the centre of 100 Km grid squares on the National Grid.

collected in more than one flight season. None of the tests of heterogeneity was significant so all samples were pooled.

The pie charts represent the phenotype proportions in the 100km grid squares, with each pie chart positioned in the centre of the grid square [Figure 6.xi]. The data from Cook and Sarsam (1981) are not complete; four samples from grid square SE and four samples from grid square SJ (grid reference 850921) were omitted as there was significant heterogeneity to prevent pooling. These omissions are unimportant as the maps are intended to show graphically the geographic pattern of the forewing polymorphism.

Only five grid squares were common to both surveys, these being NS, SH, SK, SP and SU. The proportions of the three phenotypes in both males and females were compared using χ^2 tests of heterogeneity, each test having two degrees of freedom [table 6.vii]. Out of a possible ten tests only six could be performed, the other four having expected values below five for more than 20% of the contingency table (Seigel 1956). Of these six tests, five are significant at $p<0.05$ or less, indicating differences between phenotype proportions between the two studies.

The reasons for the discrepancies in phenotype frequencies between the two studies could be:

(1) the phenotype frequencies have changed over time, this change mediated by some evolutionary event or process

(2) the phenotypes have been scored differently.

Data from Rothamsted Experimental Station (chapter 5) indicate that the phenotype proportions are fairly stable over time, although there is evidence that the frequency of male ochre varies to a significant degree.

Rothamsted data [see chapter 5] suggest that the average frequency of ochre for 1970 and 1972 (the years that Cook and Sarsam conducted their survey) was 0.504 From their data the frequency of ochre in 100Km square TL, was 0.76. Similarly the differences for silver were considerable. No population in Britain had a frequency of greater than 0.1, in comparison to my data from Rothamsted in 1971-72, where the average frequency was 0.172.

In my view the discrepancies in phenotype frequencies are due to differences in scoring. The frequency of rufous in both studies is similar and rufous is the most easily distinguished form; rufous differs from ochre and silver in colour and pattern, whereas silver and ochre have the same pattern, with one colour change between the two phenotypes. I hypothesise that the differences are most probably caused by scoring errors in the separation of silver and ochre phenotypes. There is also a possibility that part of Cook and Sarsam's data was lost during the 10 years between the collection of the samples and the re-scoring of them; this possibility is admitted by the authors.

6.5.2: Comparisons with Soli & Andersen (1990):

In Norway the same three phenotypes were present and in the majority of samples rufous was the most common phenotype (28 out of 38 samples). In south eastern Norway the data suggested that there was a weak cline as the frequency of rufous tends to increase towards the north east. My data from Scandinavia (samples from Sweden and Finland) also had elevated frequencies of rufous and in general the frequency of rufous was higher at eastern sample sites.

In their paper, Soli and Andersen drew attention to a sample collected from Finland where the frequency of rufous was high (0.67) (Mikkola & Jalas

1977). This agrees with my two samples from Finland, both of which had high frequencies of rufous. The report not only confirms that rufous is more common in Finland than elsewhere in northern Europe, but also that this elevated frequency is stable over a number of generations.

6.5.3: Comparisons with Poitout and Bues (1976):

The data provided by Poitout and Bues (1976) were for males and females combined and so complicates comparisons. For the five regions they report significant differences for six of the possible ten comparisons and conclude that differentiation is caused by the limited influence of dispersion in the distribution area. The differentiation between the regions is small and of comparable magnitude to the differences seen over the U.K in this study. The largest difference in the frequency of rufous reported in Poitout and Bues (1976) is 0.105 while the largest difference within England is 0.089. The statistical significance of these differences is only realised because of the massive sample sizes in the French study. In conclusion the geographic differentiation reported by Poitout and Bues is no greater than that observed on similar scales in this study.

6.5.4: Kentville, Nova Scotia - a recently established population:

The phenotype frequencies of the Canadian population were not incorporated in the regression analysis as the population is of recent origin, being first recorded in 1979 (Neil 1981). The establishment of a new population, that is isolated from the main range of a species, provides an opportunity for founder events to take place. A founder event is caused by the incomplete sampling of the genetic variation within the source population and can lead to changes in gene frequency, heterozygosity and the loss of alleles at loci (Nei *et al.* 1975, Carson & Templeton 1984).

The phenotype frequency data were similar to European samples with all three phenotypes in both sexes recorded. The occurrence of all three phenotypes in the new population is not surprising as the founding population size would have to be small for sampling error to have any great effect on the number of alleles and their frequency. None of the three alleles that control the polymorphism can be considered rare and it is rare alleles that are at most risk of being lost in founder events (Nei et al. 1975). A simulation of the founder event can demonstrate this. Hypothetically, if the source population had the same phenotype frequencies as those recorded at Bullingdon Green (site 2) then the chance of all alleles at the forewing phenotype locus (assuming the single locus model is correct) being present in the founding population, if one mated female founded the new population, is approximately 0.174. This probability increases quickly as the number of founding females increases; with five randomly mated females the approximate probability is 0.615.

6.5.5: General discussion:

This study demonstrates that throughout a wide geographical area the frequencies of the three phenotypes are homogeneous with some differentiation at the extremes.

A number of processes may explain this differentiation. In Finland the rufous phenotype may be at a selective advantage over both ochre and silver and so increases in frequency. It is unlikely to reach fixation because of the constant influx of ochre and silver alleles to the region from elsewhere. The similar process may be happening in Scotland and N.Ireland. An important consideration in this scenario is the amount of gene flow that occurs between the areas with different phenotype frequencies and other regions. N.Ireland and Finland are separated from other sample sites by large tracts of water

(the Irish and Baltic Seas) which may act as barriers to gene flow. Thus the differences may be caused not only by selection but by a reduction in the magnitude of gene flow. On the continent and within England there may be similar differences in selection, but without barriers to gene flow differences caused by selection for alternative morphs may be obscured. This idea is compromised to an extent by the data from Scotland. Here we have significant differentiation in terms of phenotype frequencies but no obvious barrier to gene flow. In Norway a population sampled from the island of Tjøme produced phenotype frequencies that differed markedly from surrounding, mainland populations (Soli & Andersen 1990). These differences were interpreted as being caused by reduced gene flow. Whether the actual changes were caused by genetic drift or by selection was not discussed.

How does the forewing polymorphism in *N.pronuba* compare with other polymorphisms studied in other species? The homogeneity over such a wide area contrasts with other studies of colour and pattern polymorphisms. Many other well studied colour and pattern polymorphisms are much more complicated in their geographic structure. *Cepaea nemoralis* shows that phenotype frequencies can change dramatically over a few metres (Arthur 1978) but there are "area effects" where phenotype frequencies are stable over areas larger than the panmictic unit (Jones *et al.* 1977). Steady clines in phenotype frequencies are superimposed over this complex local structure. Similar geographic patterns are observed in the spider, *Enoplognatha ovata* (Oxford 1985, Oxford & Shaw 1986). In contrast *Biston betularia*, *Gonodontis bidentata*, and *Amathes glareosa* all have areas where particular morphs are at high frequency, and these areas are connected together through a series of clines (Cook *et al.* 1986, Cook & Jacobs 1983,

Kettlewell & Berry 1969, Bishop & Harper 1970) but the local structure often found in *E.ovata* and *C.nemoralis* seems to be absent.

Differences in geographic structure of colour and pattern polymorphisms between moth species have been explained in part by differences in dispersal. The industrial melanics, *Biston betularia* and *Gonodontis bidentata* are characterised by clinal changes in phenotype frequencies (Cook *et al.* 1986, Cook & Jacobs 1983), as is the non-melanic but polymorphic *A.glareosa* (Kettlewell & Berry 1961, 1969). Bishop and Cook (1975) explained the differences in geographic structure between *B.betularia* and *G.bidentata* by differences in dispersal and hence gene flow. From mark-recapture studies it has been shown that *B.betularia* moves more than 1km per night whereas *G.bidentata* moves a maximum of 150m (Bishop 1972, Bishop *et al.* 1978). *N.pronuba* is more vagile than either of these species [see chapter 2] and so there is little local differentiation, and significant differentiation is only seen between regions. Mark-recapture studies performed on *N.pronuba* and other noctuid species in the Alps indicate that they can, on occasion, fly 30km in one hour (Poitout *et al.* 1974). Given its long adult life span, this species can disperse widely.

To gain more insight into the geographic structure of the forewing polymorphism of *N.pronuba* it is important to have some idea of the magnitude of gene flow in this species. Mark-recapture studies are direct ways of gaining estimates of dispersal but the data fall short in three ways.

(1) They can give estimates of dispersal rates but there is no reason why dispersal should equate with gene flow (Endler 1977).

(2) Assuming dispersal equates with gene flow, mark recapture studies only measure gene flow at the time of study, not over time periods of evolutionary importance.

(3) The distribution of dispersal distances for noctuid moths has been shown to be leptokurtic, that is the variance in dispersal distance is not symmetrical about the mean dispersal distance (Schneider *et al.* 1989). Unless a large mark-recapture study is used (Schneider *et al.* released 250,000 individuals), where the density of traps is kept constant with increasing distance from the release point, no data on the shape of this leptokurtic distribution will be gained.

To overcome the short falls of mark-recapture, indirect methods, such as the spatial distribution of allele frequencies and their fit to known population genetic models, can be used to give gene flow estimates (Slatkin 1987). It is this approach that I have used in this study and the results are explained in the next chapter. It is interesting to note that there have not been any attempts to gain estimates of gene flow in other species of moth that are polymorphic for colour and pattern.

Chapter 7: Geographic variation in allozyme frequencies:

7.1: Introduction:

Allozyme electrophoresis is a chromatographic technique that separates protein molecules, usually enzymes, on the basis of net charge and sometimes, depending on the substrate used, size (Hunter & Market 1957). Within a population mutations occur that cause changes in the base composition of DNA. This may cause a change in the amino acid sequence of the protein which alters the net charge on the molecule, so altering the relative mobility of the molecule when placed on a suitable substrate and an electrical potential placed across it.

The charge of a protein molecule only changes if the DNA base mutation causes a change in the amino acid composition the molecule. There are five amino acids that are charged: lysine, arginine and histidine all carry NH_3^+ (basic) groups, while aspartic acid and glutamic acid both carry COO^- (acidic) groups. A mutation that causes:

 i) a charged amino acid to be replaced by a neutral amino acid

 ii) a neutral amino acid to be replaced by a charged amino acid

 iii) a charged amino acid to be replaced by an amino acid of opposite charge

 iv) an addition of a charged amino acid

 v) a deletion of a charged amino acid

changes the overall net charge and alters the molecule's mobility in an electric field (Murphy *et al.* 1990).

Within a population there may be alternative charge forms of the same protein encoded by the same locus. These different allelic forms are known as allozymes. Generally allozymes are inherited in a Mendelian fashion with heterozygotes co-dominant and easily distinguishable from homozygotes. This allows allele frequencies to be counted and population allele frequencies estimated. This is in contrast to classical polymorphisms, such as the colour pattern polymorphism in *N.pronuba*, where heterozygotes cannot be separated because of dominance relationships. In such classical polymorphisms allele frequencies can only be estimated if the inheritance mechanism is known, and the locus in question is assumed to be in Hardy-Weinberg equilibrium.

Studies in evolutionary biology and population genetics have advanced greatly over the past thirty years with the advent, and application of, allozyme electrophoresis and DNA analysis techniques (Lewontin 1974, Avise 1994). Studies of allozyme variation have now been conducted in over a thousand different species, the results showing the majority of species to be variable at a proportion of protein or enzyme loci (Nevo 1984, Singh & Rhomberg 1987).

The ubiquity of this type of polymorphism has been used to gain extensive information on the distribution of genetic variation at different population hierarchies, e.g.

i) within individuals and between individuals

ii) within populations and between populations

iii) within species and between species.

Such information has been used to quantify the extent of inbreeding, out-crossing and gene flow between populations (Slatkin 1987,1994; Berry 1989).

Gene flow is often thought to prevent the adaptation of organisms to their immediate environment (Mayr 1963, Jackson & Pounds 1979). Local adaptations can be disrupted by the influx of migrant individuals, and the genes they carry, from other populations; this tends to promote genetic and ecological homogeneity. This view of gene flow as a constraining force has been criticised. Ehrlich and Raven (1969) suggested that high levels of gene flow were not as ubiquitous as first thought and that strong selection could cause differentiation in the face of gene flow. The degree to which gene flow acts as a constraining force depends upon its magnitude, and allozyme electrophoresis has enabled researchers to gain much insight into its size and importance.

The use of allozymes to study gene flow and the importance of genetic drift in population structure depends upon allozyme variation not being subjected to strong selection. If allozymes are subject to strong selection their frequencies in populations will be determined by selection rather than genetic drift and gene flow. Selection can bring about convergence in allele frequencies. This can give the incorrect impression that two populations are recently derived from a single ancestral population when, in fact, they have been derived from two quite different ancestral populations.

The discovery of large amounts of genetic variability, particularly in allozymes, since the 1960s opened up an ongoing debate as to whether the majority of molecular polymorphisms are neutral to selection (Lewontin 1974, Kimura 1983, Eanes 1987, Koehn & Hilbish 1987). This debate is not resolved; however, demonstrations of selection acting on allozyme variation are few relative to the number of studies that have been conducted. Some have provided convincing evidence for selection acting on individual loci or alleles (DiMichelle *et al.* 1991, Watt 1983, Watt *et al.* 1985, Riddoch 1993)

and it is possible that the paucity of good evidence for selection is the infrequency that researchers study such mechanisms.

7.2: Aims:

The aim of the allozyme survey of *N.pronuba* was to:

i) test for random mating within populations

ii) test for geographic variation in allele frequencies

iii) estimate the levels of gene flow between populations.

The data provided information on the population structure of the species which was compared to the geographical structure of the colour pattern polymorphism. This was used to interpret the colour pattern polymorphism in terms of stochastic and selective processes.

7.3: Materials and methods:

7.3.1: Gel Preparation:

Gels were prepared the evening before use. Twenty-five grams of either Connaught or Sigma hydrolysed potato starch was weighed out into a 500ml Buchner flask. Two hundred and thirty ml of gel buffer was added to the starch and the flask agitated so as to suspend the starch in solution and make sure there were no lumps of solid starch. The flask was heated over a roaring Bunsen flame whilst being swirled continuously to prevent localised heating. Once boiling the suspension was heated and agitated vigorously for a further minute.

After one minute the Buchner flask was corked and placed on a vacuum pump so as to degas the molten starch. Degassing took approximately 10-

15 seconds and the flask was once again agitated continuously. The resulting molten starch was poured into a gel mould and allowed to set.

The gel mould consisted of a clean glass plate onto which a perspex frame had been placed; the internal dimensions of the frame were 18cm x 16cm x 0.5cm. Once the molten starch had been poured into the frame, any air bubbles were removed with a Pasteur pipette and a second glass plate placed on top. If the gel contained many bubbles it was discarded, but this was rare.

The gel was allowed to cool and then wrapped in Cling-Film to prevent desiccation overnight. The following morning the gel was uncovered and cooled at 4°C for 1-2 hours prior to use.

7.3.2: Extract Preparation:

Clean centrifuge tubes were labelled with the run code and a number from 1-30, then 0.75 ml of ice cold homogenisation buffer was added and the tubes placed on crushed ice. Various homogenisation buffers were tried, but the best resolution was given by that of Loxdale *et al.* (1983) (see Appendix 3 for a list of extraction buffers).

All four wings of each moth were removed and placed in small (5cm x 5cm), labelled glassine envelopes, while the abdomens of both males and females were placed in individually labelled centrifugation tubes. The head, thorax and legs were placed in the relevant labelled homogenisation tube. The material was then homogenised for 3-5 seconds with a blunt-ended glass rod attached to an Ultra Turrax homogeniser. This procedure disrupted the body cells to liberate the soluble proteins into solution.

The resulting solution contained many scales from the head, patagium and tegula and so was centrifuged for 10 minutes at 4700 revs/min at a

temperature of 4°C. After centrifugation the supernatant was a yellowish / clear liquid, with the body parts and scales forming a dense pellet at the bottom of the tube.

The supernatant was either loaded onto the gel as described below or, if the moths were from a location with a small sample size, a method similar to that described by Wynne and Brookes (1992) was used. This method involved quickly freezing 30 μl droplets of supernatant by dropping them into liquid N_2 with a Finn pipette. The frozen pellets were then kept in individually labelled tubes, at -75°C, until they were required for analysis. This procedure allowed the same individual to be re-run in the event of mistakes during the running or staining of the gel.

7.3.3: Loading of the starch gels:

The gel was cut parallel to the longest side approximately 5cm from the edge and the resulting strip (the cathodal strip) was drawn back to allow the wicks to be positioned. The supernatant was then loaded on to the gel using wicks of small (7mm x 5mm) rectangles of Whatman No 3 chromatography paper. Each wick was first moistened with supernatant, then blotted on a filter paper to remove excess liquid[3], and finally placed on the gel leaving 2mm between it and the adjacent wick. After each wick had been loaded and positioned on the gel the forceps were cleaned thoroughly to prevent cross contamination between lanes.

The wicks were ordered into groups of five with each group separated by a marker individual. The marker was prepared using the modified version of the method described by Wynne and Brookes (1992). This allowed the

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[3]Overloading of the wicks can produce a smear when the gel is stained

same marker individual to be run repeatedly and reduced the probability of scoring errors between runs. The marker was run after every five individuals as one of the enzymes, MPI, was prone to warping[4].

The two outside wicks were loaded with the tracer dye bromophenol blue rather than homogenate, so that the progress of migration could be monitored throughout the run.

7.3.4: Running the gels:

Once all the wicks were in place the cathodal strip was replaced and two perspex spacers were put between the edge of the gel and the gel mould. This ensured good contact was made between the cathodal strip and the rest of the gel and eliminated the possibility of the two pieces of gel separating as a result of shrinkage.

The gels were run on Shandon electrophoresis tanks. Each tank consisted of two electrode buffer reservoirs, each filled with 250-300ml of the required buffer. The gel was placed on top of the tank and the electrical connection between the two electrodes completed by two pieces of lint that had been soaked in electrode buffer.

During the run there was a tendency for the gel to shrink because of gradual desiccation. This was reduced by wrapping the whole gel in Cling-Film. Finally, an ice tray was placed on top of the gel to cool it and prevent denaturation of the proteins.

The whole apparatus was put into a cold room at a temperature of 4°C and run for 3.5 hours at a constant current of 40mA. After the gel had been

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[4]Warping is the unequal migration of proteins on the same gel. The outside lanes sometimes migrate further then the central ones due to temperature differences across the gel.

running for fifteen minutes the paper wicks were removed. This increased the band resolution, and once the gel was stained aided scoring by preventing continuous migration from the origin.

7.3.5: Slicing the gels:

Once a gel had run for the required time, it was removed from the tank and prepared for staining. The gels were cut using a length of taut fishing line that was run along pieces of 0.9mm Formica veneer. After each cut a level of veneer was removed and the process repeated. Each gel was cut four times and the resulting five slices were stained for different enzyme systems.

Researchers sometimes discard the top slice of a gel as the proteins migrate disproportionately faster on the periphery due to subtle temperature differences. This did not prove to be a problem and all five slices were stained.

7.3.6: Staining the gel slices:

Staining protocols were based on those of Shaw and Prasad (1970), Pasteur et al (1988)[5], Goulson (1991) Antrobus (1992) and Wynne et al. (1992). Those described by Wynne et al. (1992), were found to be particularly useful as, although the recipes were tailored for cellulose acetate systems, the enzymes listed had been found to be polymorphic in insects, particularly moths.

Each gel slice was carefully lifted from the glass plate and placed in a labelled perspex staining tray and the specific staining solution added. All

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5Care must be taken when using this book as it contains many mistakes in the staining recipes.

the stains, except SOD[6], using MTT and PMS were incubated in the dark at 37°C until the enzyme was stained sufficiently to allow accurate interpretation. A few stains, namely PGI and PGM, stained quickly and care had to be taken to avoid over-staining.

7.3.7: Initial screening procedure:

An initial screening was undertaken to ascertain which enzyme protocols produced clear, unambiguous staining with *N.pronuba*. Table 7.i gives a review of the enzymes and buffers initially screened.

7.3.8: Enzymes routinely screened:

After the initial screening five enzyme systems were chosen to be routinely analysed in all samples. Those chosen were PGM, PGI, MPI, GOT, and MDH. These particular systems were chosen because they stained reliably, four were polymorphic (all except MDH) and all could be run successfully using the continuous buffer system of Shaw and Prasad 1 (1970). Each gel was run for 3.5 hours and was sliced so all five enzymes could be stained from one gel.

7.3.9: Allozyme nomenclature:

The allozymes (*sensu* Prakash *et al.* 1969) of polymorphic enzyme systems have historically been labelled by one of three methods:

    i) numbers

    ii) letters

    iii) % mobility of the most common allele in the population.

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[6]See appendix 3 for abbreviations.

Throughout this work the alleles were categorised by method (iii), the most common allele being assigned a mobility of 100% and every other allele identified by its mobility relative to this common allele.

Enzymes	Buffer Systems						
	Histidine Citrate pH 7.0	Tris Citrate pH 8.0	Tris Citrate Borate pH 8.7	Histidine Citrate pH 5.0	Tris Glycine Citrate pH 7.9	Tris Malate pH 7.3	Tris Malate EDTA pH 6.9
IDH	√	√	√		√		√
GPDH	√	√					
PGM		√				√	
PGI		√				√	
MDH	√	√		√			
ADH	√	√					
G6PD							
ME		√					
ACON	√	√	√	√	√	√	√
XDH	√	√	√	√	√	√	√
HBDH	√	√	√	√	√	√	√
MPI		√	√	√		√	
GLD							
LAP	√	√	√	√	√	√	√
HK		√				√	
FK		√			√		
LDH		√					
EST 1,2		√				√	√
EST 3	√	√				√	√
GOT		√				√	
SOD		√				√	
6PGD		√				√	

√ - The enzyme / buffer combination was tried but did not necessarily produce a scoreable zymogram. For a list of abbreviations see appendix 3.

7.3.10: Zymogram descriptions and interpretation:

Five enzymes, controlled by five presumptive loci were screened routinely in all fifteen populations.

Table 7.ii lists the enzymes scored, their EC number specified by the Nomenclature Committee for the International Union of Biochemistry, and their metabolic role.

Table 7.ii: The metabolic role of the routinely screened enzymes.

Enzyme system	EC number	Metabolic role
PGI	5.3.1.9	Glycolysis
PGM	2.7.5.1	Conversion of carbohydrate reserves to glucose-6-phosphate
GOT	2.6.1.1	Amino acid degradation
MDH	1.1.1.37	Krebs cycle
MPI	5.3.1.8	

Malate dehydrogenase (MDH): This enzyme proved to be monomorphic in all populations sampled. Only one MDH locus was visualised, although both MDH-1 and MDH-2 have been recorded in Lepidoptera (Brussard *et al.* 1985).

Phosphoglucose isomerase (PGI): The stained zymogram for PGI reveals triple banded heterozygotes; this pattern is characteristic of a dimeric enzyme. Over all 15 sample sites a total of eight alleles were recorded with three of these (relative mobilities: 133, 100, 46) being found in all of the samples.

Phosphoglucomutase (PGM): The presence of double banded heterozygotes indicated that the enzyme was monomeric. A total of nine

alleles were recorded over all of the sample sites. Of these nine alleles, only three (relative mobilities:115, 100, 88) were present in all populations.

Glutamate Oxaloacetate Transaminase (GOT): Heterozygotes were triple banded as the enzyme is dimeric. In all 15 samples there was a common allele (relative mobility: 100) with five rare alleles recorded in the total survey. Staining revealed one locus for this enzyme system although previous studies on Lepidoptera have revealed two loci (Brussard *et al.* 1985) and three loci (Mallet *et al.* 1993).

Mannose Phosphate Isomerase (MPI): The enzyme is monomeric with two banded heterozygotes. The enzyme was found to be highly variable with more than eight alleles being recorded in the survey.

No allele of MPI was recorded with a frequency greater than 0.5 and so there was no common allele as such and the large amount of variability found in MPI made the scoring of the zymograms difficult. The outside lanes also tended to travel further than the central bands. The use of marker individuals every sixth lane did not help in the interpretation. This warping, along with the high degree of variability, means that the allozymes were difficult to score accurately.

Unfortunately the staining of MPI was not as consistent as the other four routinely screened allozymes and successful zymograms were not obtained from all fifteen locations. This may be because the enzyme was less stable and the protein denatured quickly, losing its catalytic properties.

In view of this only very limited analysis has been performed on this locus. It has not been included in the calculation of F statistics, or genetic distance measures, as the allocation of bands to specific alleles and the difficulty in

comparing mobilities between electrophoretic runs would compromise the reliability of these measures.

7.3.11: Gel fixation and scoring of zymograms:

Once the gel was sufficiently well stained it was fixed using 10% acetic acid. This quenched enzymatic reactions and prevented the gel becoming over-stained and the resolution being reduced. The positions of the bands were recorded by making a precise scale drawing of the gel on graph paper and the genotypes of each individual was scored onto a separate table. The drawn copy of the gel allowed the position of alleles to be checked between runs and rare alleles to be assigned the correct mobility.

7.4: Statistical methods for analysing allozyme data

The allozyme electrophoresis results were analysed using the Fortran program Biosys-1.7 (Swofford & Selander 1981). Using this program the following analyses were performed.

7.4.1: Conformance to Hardy-Weinberg:

Each of the polymorphic loci was tested to see if the frequency of particular genotypes was significantly different from those expected if the sample was at Hardy-Weinberg equilibrium.

The usual method involves the comparison of expected genotype frequencies, given the observed allele frequencies, with the observed genotype frequencies using a χ^2 goodness of fit test (Richardson *et al.* 1986). This test must be performed on individuals from a single sample site as the grouping of two sample sites, that differ in allele frequencies, can

cause spurious results because of the Wahlund Effect[7] (Wahlund 1928). Unfortunately small sample sizes, coupled with large numbers of alleles leads to small expected values in the goodness of fit test. A χ^2 test is liable to increased error when the values in expected classes are below five (Sokal & Rohlf 1981). In consideration of this, rare alleles, those other than the most common allele 100 for each locus, were grouped to give the following genotypic classes:

i) homozygotes of the common allele (100 / 100)

ii) heterozygotes of the most common allele and one of the other alleles (100 / x)[8]

iii) all other genotypes (x/ x).

The conformance to Hardy-Weinberg was tested using the EXACTP option in Biosys-1. This is an exact probability test that is analogous to Fisher's Exact Test. The test is preferable to a χ^2 as it is not susceptible to the problems associated with low expected values (Elston & Forthhofer 1977).

7.4.2: Multilocus associations:

The data from the polymorphic loci were tested to see if there were random gametic associations between alleles. If mating is at random the alleles at two loci should show random associations and so are said to be in gametic equilibrium. If mating is not random, was not random in the recent past, or

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[7]Consider a di-allelic locus, in two populations, both in Hardy-Weinberg equilibrium but with different gene frequencies; in population 1 p=0.2, q=0.8 (2pq=0.32), while in population 2 p=0.6, q=0.4 (2pq=0.48). If these two samples were grouped together and considered as one population (the pooled sample represents 50% from populations 1 and 2). The expected number of heterozygotes is calculated from the average gene frequency and equals p=0.4, q=0.6 (2pq=0.48) while the observed number of heterozygotes is 0.4 ((0.32+0.48)/2). This discrepancy is known as the Wahlund Effect.

[8]Where x is any other allele at a locus other than the most common 100 allele.

natural selection favours particular combinations of alleles at specific loci, the alleles may be non-randomly associated.

Studies of multilocus associations of isozyme alleles usually show the loci to be in gametic equilibrium (Hartl & Clark 1989); however, there have been reports of highly significant associations (Baker 1975, Lack & Kay 1988).

The randomness of associations between alleles was tested using a χ^2 test of association (Lack & Kay 1988). This approach causes similar problems to those outlined in section 4.6.2 The large number of alleles recorded in some populations but not in others leads to contingency tables that have expected results below five. This was overcome in two ways.

(1) The alleles were grouped as in 4.6.2 giving three genotypes for each locus.

(2) Results were pooled between populations where there was no heterogeneity in allele frequencies as tested with a χ^2 test.

Associations between colour and pattern phenotypes and allozyme genotype frequencies were tested using a χ^2 test of heterogeneity. Allozyme genotypes were grouped as in 4.6.2 and males and females were treated separately.

7.4.3: F Statistics.

Wright's standardised analysis of variance (Wright 1951, 1969, 1978) is the most commonly used method for analysing protein electrophoresis data from geographically separated sub-populations (Weir & Cockerham 1984, Cockerham & Weir 1993). Population sub-division has similar effects to inbreeding with a reduction in the proportion of heterozygotes. F statistics rely on the comparison of heterozygosity at different levels of population complexity, namely:

H_I = heterozygosity of an individual in a sub-population

H_S = expected heterozygosity of an individual if the sub population is randomly mating

H_T = expected heterozygosity of an individual if the total population is randomly mating.

The above heterozygosities are used to calculate Wright's F statistics which give measures of: (1) the reduction in heterozygosity within a sub-population because of non-random mating (F_{IS}), (2) the reduction in heterozygosity within a sub-population because genetic drift (F_{ST} - the fixation index) and (3) the inbreeding coefficient (F_{IT}) for the total population. F_{IT} includes the effect of inbreeding contributed by both F_{IS} and F_{ST} and is the heterozygote reduction for an individual relative to the total population.

$$H_I = \sum_{i=1}^{k} \frac{H_i}{k} \qquad (7.1)$$

where: H_i is the heterozygosity in sub-population i
         k is the number of sub-populations

$$H_S = 1 - \sum_{i=1}^{h} p_{i,s}^2 \qquad (7.2)$$

where: h is the number of alleles
         $p_{i,s}$ is the frequency of the i th allele in population s

$$H_T = 1 - \sum_{i=1}^{k} \overline{p}_i^2 \qquad (7.3)$$

where: $\overline{p}_i$ is the frequency of allele i averaged over the
         sub-populations

From these heterozygosities hierarchical F statistics are calculated in the following manner:

$$F_{IS} = \frac{\overline{H_T} - H_I}{H_S} \qquad (7.4)$$

$$F_{ST} = \frac{H_T - \overline{H_S}}{H_T} \qquad (7.5)$$

$$F_{IT} = \frac{H_T - H_I}{H_T} \qquad (7.6)$$

F_{IT}, F_{IS} and F_{ST} are related to one another in the following way:

$$(1 - F_{IS})(1 - F_{ST}) = (1 - F_{IT}) \qquad (7.7)$$

One reason for estimating F_{ST} from allozyme data is to attempt to infer the amount of gene flow that occurs between sub-populations. Wright (1931) showed that for alleles that are neutral to selection:

$$F_{ST} = \frac{1}{(1 + 4Nm)} \qquad (7.8)$$

where N is the size of the local population and m is the average number of individuals that move into a sub-population, given an infinite island population model. Equation (7.8) can be rearranged as follows;

$$N_m = \frac{1}{4}\left(\frac{1}{F_{ST}} - 1\right) \qquad (7.9)$$

where Nm is the sub-population size multiplied by the proportion of migrants; this is the absolute number of individuals that are exchanged between sub-populations (Wright 1951).

The infinite island model assumes a species is split up into discrete sub-populations and that migration can occur between any of the sub-populations. It is the population model that allows for the greatest amount of

gene flow and is considered to be at one extreme on the spectrum of gene flow (Slatkin 1985, Slatkin & Barton 1989).

The following F_{IS}, F_{IT}, and F_{ST}, measures were calculated using the FSTAT step in Biosys 1.7:

(1) for each allele at each locus the weighted averages across all sub-populations. The weights applied follow that of Kirby (1975).

(2) The weighted average across all alleles at a locus.

(3) The mean over all loci.

The mean F_{ST} for all three loci was used to calculated Nm, the number of migrants moving between sub-populations, using equation (7.9).

The F statistics for the weighted average across all alleles at each locus and the mean over all alleles were tested for significance using equations (7.10), (7.11) and (7.12) (Workman & Niswander 1970).

$$\chi^2 = NF_{is}^2 \qquad (7.10) \qquad \text{d.f.} = 1$$

$$t = \left| F_{IT}\sqrt{N} \right| \qquad (7.11) \qquad \text{d.f.} = \text{infinity}$$

$$\chi^2 = 2NF_{ST} \qquad (7.12) \qquad \text{d.f.} = \text{number of sites - 1}$$

Where N is the sample size for that locus or the mean sample size across all loci. The null hypothesis in each case is that the F statistic is equal to zero ($F_{IT} = 0$; $F_{IS} = 0$; $F_{ST} = 0$) (Wright 1978).

7.4.4: Genetic distance:

Genetic distance measures the degree of divergence between pairs of sites and so produces a genetic distance half matrix. This distance matrix can be

subjected to cluster analysis and a dendrogram constructed using a suitable algorithm.

Many genetic distance measures have been formulated. The most commonly used approach in allozyme work is that of Nei (Nei 1972, Nei *et al.* 1983). This utilises Nei's genetic distance D in conjunction with the unweighted pair-group method with arithmetic averaging algorithm (UPGMA clustering). Nei's genetic distance D is based on the standardised genetic identity, which is the probability that two alleles chosen at random, one from each sub-population, will be identical relative to the probability that two alleles chosen at random from the same sub-population will be identical. The identity of alleles in this context does not refer to their genetic history; they do not have to be identical by descent, just indistinguishable from one another.

This method has been criticised because the assumptions of neutrality, stable population size, constant mutation rates, and that once a specific allele has originated by mutation it cannot arise *de novo* again, are generalisations that do not hold for most, if not all, situations (Farris 1985). In spite of these criticisms, the method is widely used, in particular for the study of intraspecific genetic variation (Coates 1992, Vrijenhoek & Graven 1992, Puterka *et al.* 1993). For comparative purposes Nei's genetic distances (Nei 1972) have been calculated.

7.5: Results:

Table 7.iii lists the 15 sample sites, their corresponding population number and latitude and longitude. The geographic positions of the 15 sampling locations is shown in figure 7.i. Samples 1a and 1b were both collected from Bullingdon Green, Oxford in 1992. Sample 1a consisted of individuals

caught early in the flight season (4/6/92 - 30/6/92) whereas sample 1b was caught late in the flight season (6/9/92 - 29/9/92). In all, 16 samples were screened from 15 different locations.

7.4.1: Allele frequencies:

The relative mobilities, allele frequencies and sample sizes for PGM, PGI, GOT and MDH alleles are provided in table 7.iv.

7.4.2: Conformance to Hardy-Weinberg:

Out of 48 exact tests only three were significant at $p<0.05$ [table 7.v]. With the number of tests performed one would expect 2 tests to be significant at $p=0.05$ by chance. From these results it is possible to conclude that all the populations are in, or near, Hardy-Weinberg equilibrium and that each sub-population represents a panmictic population.

7.4.3: Direct-count heterozygosities:

For simple comparison of the genetic variation at each locus table 7.vi lists the direct count heterozygosities (the proportion of heterozygotes relative to the total number of genotyped individuals). The heterozygosities were counted for PGM, PGI and GOT in all populations, while those for MPI are only given where the staining of the gel was sufficiently clear. GOT has the lowest heterozygosity of those allozymes screened with a minimum of 0.040 at Lamphey, Pembrokeshire (5)[9] and maximum of 0.160 at Falkirk (8). MPI is the most variable allozyme locus with direct count heterozygosities ranging from a maximum of 0.760 at Bullingdon Green (1b) to a minimum of 0.560 at Builth Wells (4). For PGM the proportion of heterozygotes ranges from 0.508 (15) to 0.680 (11) and for PGI between 0.207 (6) and 0.444 (11).

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[9]numbers in brackets refer to the population numbers in table 7.i

Table 7.iii: A key to the sample sites and their corresponding population numbers.

Sample number	Sample location	Longitude/ Latitude
1a	Bullingdon Green, Oxford, England	1.15W 51.46N
1b	Bullingdon Green, Oxford, England	1.15W 51.46N
2	Long Wittenham, Oxfordshire, England	1.12W 51.38N
3	Forton, Shropshire, England	2.22W 52.47N
4	Builth Wells, Wales	3.24W 53.05N
5	Lamphey, Pembroke, Wales	4.51W 51.40N
6	Betws-Y-Coed, Wales	3.48W 53.05N
7	Aberdeen, Scotland	2.04W 57.10N
8	Falkirk, Scotland	3.48W 56.00N
9	Grange-Over-Sands, Cumbria, England	2.55W 54.12N
10	Eastbridge, Kent, England	1.00E 51.02N
11	York, England	1.05W 53.58N
12	Leicester, England	1.09W 52.39N
13	Bangor, Northern Ireland	5.40W 54.40N
14	Portimão, Algarve, Portugal	8.32W 37.08N
15	Scharnstein, Austria	13.56E 47.53N
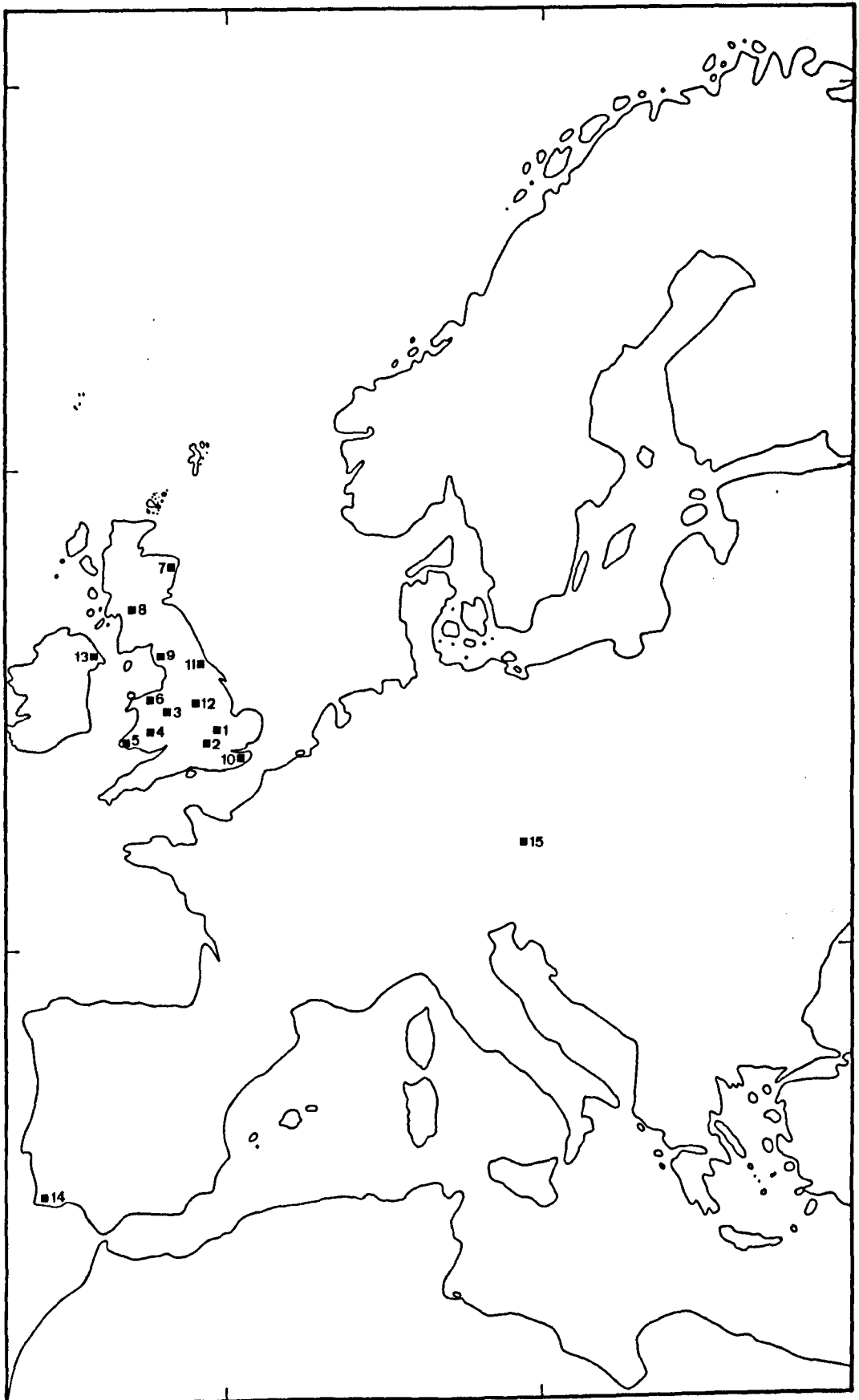
Figure 7.i: Geographic location of collection sites for samples used for allozyme electrophoresis.

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Table 7.iv: Allozyme allele frequencies

Locus	1a	1b	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Tot n
							Sample site (population)										
MDH																	
(n)	*25*	*50*	*60*	*58*	*27*	*25*	*58*	*50*	*25*	*26*	*30*	*45*	*28*	*30*	*30*	*59*	*626*
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
PGI																	
(n)	*25*	*50*	*60*	*58*	*27*	*25*	*58*	*20*	*25*	*25*	*30*	*45*	*28*	*29*	*30*	*59*	*594*
180	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.008	
166	0.000	0.010	0.017	0.000	0.000	0.000	0.017	0.000	0.020	0.020	0.033	0.000	0.000	0.000	0.050	0.008	
133	0.140	0.060	0.142	0.172	0.148	0.200	0.095	0.125	0.100	0.120	0.133	0.189	0.089	0.138	0.150	0.153	
111	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008	
100	0.780	0.880	0.817	0.759	0.815	0.720	0.862	0.800	0.820	0.800	0.800	0.733	0.839	0.845	0.800	0.797	
77	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
46	0.060	0.040	0.025	0.060	0.019	0.060	0.009	0.025	0.060	0.020	0.033	0.078	0.071	0.017	0.000	0.025	
38	0.020	0.010	0.000	0.009	0.019	0.000	0.009	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	

(n) = number of individuals scored

Table 7.iv: (continued)

Locus	Sample site (population)																Tot n
	1a	1b	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
PGM																	
(n)	25	50	60	58	27	25	58	50	25	26	45	75	28	30	29	59	670
125	0.020	0.010	0.025	0.000	0.019	0.000	0.000	0.010	0.020	0.019	0.011	0.013	0.018	0.017	0.000	0.000	
120	0.060	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
115	0.340	0.200	0.200	0.276	0.315	0.200	0.216	0.260	0.280	0.385	0.200	0.307	0.321	0.267	0.293	0.178	
108	0.020	0.020	0.000	0.017	0.000	0.020	0.009	0.000	0.020	0.000	0.000	0.000	0.000	0.017	0.000	0.017	
103	0.000	0.000	0.008	0.000	0.037	0.000	0.000	0.000	0.000	0.000	0.022	0.000	0.000	0.000	0.000	0.008	
100	0.420	0.590	0.567	0.569	0.463	0.620	0.569	0.540	0.440	0.500	0.556	0.540	0.464	0.500	0.621	0.644	
94	0.020	0.010	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
88	0.120	0.160	0.200	0.129	0.167	0.160	0.190	0.170	0.220	0.058	0.200	0.133	0.196	0.200	0.086	0.153	
72	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.020	0.020	0.038	0.011	0.007	0.000	0.000	0.000	0.000	
GOT																	
(n)	25	50	60	58	27	25	58	50	25	26	30	30	28	25	30	59	606
140	0.000	0.000	0.000	0.000	0.000	0.020	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.017	0.000	
128	0.000	0.010	0.008	0.000	0.019	0.000	0.017	0.000	0.020	0.000	0.000	0.000	0.018	0.000	0.000	0.008	
108	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.017	0.000	
100	0.940	0.910	0.950	0.948	0.944	0.980	0.940	0.940	0.900	0.962	0.967	0.967	0.946	0.960	0.950	0.941	
80	0.000	0.030	0.008	0.026	0.019	0.000	0.009	0.000	0.020	0.019	0.000	0.000	0.000	0.000	0.000	0.008	
58	0.060	0.050	0.033	0.026	0.019	0.000	0.017	0.060	0.060	0.019	0.033	0.017	0.036	0.020	0.017	0.042	

(n) = number of individuals scored

7.4.4: Associations between allozyme loci:

Only associations between PGM and PGI could be analysed statistically. This was because those between GOT and the other two polymorphic loci (PGM, PGI) produced low expected values for both the χ^2 tests of heterogeneity between sample sites, and the χ^2 goodness of fit test of random associations between genotypes. The low expected results were because of the low levels of polymorphism at this locus.

The alleles at PGM and PGI were grouped as described in the methods section and tested for heterogeneity between the sample sites. For all 16 sample sites (15 sample locations) included, the PGI genotype classes showed no significant heterogeneity (χ^2 = 27.739; d.f. = 30; p = 0.584). The contingency test had a third of its expected values below 5 but all were >1. The test was repeated with the 100/x and x/x genotype classes combined and gives a non-significant result (χ^2 =16.112; d.f. = 15; p = 0.375). The PGM locus showed significant heterogeneity when all 16 samples (15 sample sites) were included (χ^2 = 50.893; d.f. = 30; p = 0.010). To allow the grouping of sample sites the test was repeated with three sample sites removed (5,6 and 15), these were the sites which contributed the most to the overall χ^2 value. With only 13 sample sites included there was no significant heterogeneity (χ^2 = 29.522; d.f. = 24; p = 0.201). The same three samples were removed from the PGI test for heterogeneity and this modified test was non-significant (χ^2 = 11.203; d.f. = 12;p = 0.5116).

Table 7.vii shows the observed and expected values for the associations between PGI and PGM genotypes the null hypothesis being that the genotypes showed random associations. The χ^2 test of association is not significant (χ^2 = 3.990; d.f. = 4; p = 0.408), so the genotypes at each locus are randomly associated.

Table 7.v: Tests of goodness of fit to Hardy -Weinberg. Exact probability values from goodness of fit tests using EXACTP step of BIOSYS-1.7.

population	PGI	PGM	GOT
1a	1.000	0.687	1.000
1b	0.527	0.146	0.326
2	0.091	0.793	1.000
3	1.000	0.428	1.000
4	1.000	1.000	1.000
5	0.330	1.000	1.000
6	0.060	0.031*	1.000
7	1.000	0.085	1.000
8	0.140	0.420	0.191
9	0.542	0.705	1.000
10	0.307	0.764	1.000
11	1.000	0.036*	1.000
12	0.533	0.142	1.000
13	0.518	1.000	1.000
14	0.560	0.457	1.000
15	0.046*	0.400	0.172

* = significant at $0.05 > p > 0.01$

Table 7.vi: Observed, direct count, heterozygosities.

population	PGI	PGM	GOT	MPI
1a	0.360	0.600	0.120	0.680
1b	0.220	0.520	0.140	0.760
2	0.233	0.583	0.100	0.700
3	0.431	0.603	0.103	-
4	0.333	0.667	0.111	0.560
5	0.400	0.560	0.040	-
6	0.207	0.483	0.121	0.678
7	0.350	0.660	0.120	0.593
8	0.240	0.560	0.160	-
9	0.400	0.615	0.077	-
10	0.300	0.667	0.067	-
11	0.444	0.680	0.067	0.679
12	0.286	0.607	0.107	0.633
13	0.241	0.633	0.080	-
14	0.400	0.621	0.100	-
15	0.288	0.508	0.085	0.690

- = heterozygosities not available because of poor staining of zymograms.

Table 7.vii: Observed and expected values (in brackets) for genotype combinations.

	PGI 100/100	PGI 100/x	PGI x/x
PGM 100/100	88 (83.91)	32 (38.27)	6 (5.67)
PGM 100/x	142 (146.68)	70 (66.90)	13 (9.91)
PGM x/x	67 (66.07)	32 (30.23)	2 (4.46)

Tests of homogeneity between allozyme genotype frequency in each forewing phenotype were performed on PGM and PGI loci; GOT was not tested because low levels of polymorphism precluded analysis. For PGI, the female silver phenotype class was excluded, as when included one expected value was below one. Both tests were non-significant with genotype frequencies homogeneous between the forewing phenotypes (PGM: $\chi^2=8.263$, d.f.=10, p=0.603; PGI: $\chi^2=4.809$, d.f.=8, p=0.778).

7.4.5: F statistics:

The results of the hierarchical F statistic analyses for individual alleles are given in table 7.viii and a summary of the F_{IS} F_{IT} and F_{ST} values are provided in table 7.ix.

The estimated inbreeding coefficients (F_{IS}) are low except for Pgm *120*. With the exception of this allele, the others vary from 0.066 to -0.055, where negative values indicate higher out-crossing than random and positive values indicate higher inbreeding than random. The high F_{IS} value (0.548) for allele *120* is due to the allele only being found in two sub-populations (1a and 1b). This could be caused by a *125* allele or a *115* allele being incorrectly classed as a *120* allele; this is unlikely as both *115* and *125* alleles occurred on the same zymogram as the *120* allele. The average F_{IS} values for all alleles at

each locus [see table 7.ix] were tested against a null hypothesis of $F_{IS}=0$; all the tests were non-significant.

Fixation index values (F_{ST}) varied from 0.049, for the Pgm *120* allele, to 0.007 for the Pgm *125* and Got *100* alleles [table viii]. The mean F_{ST} over all alleles and over the three loci is low with a value of 0.014; this value suggests that only 1.4% of the genetic variation resides between populations. The F_{ST} values for each allozyme locus and for the mean over all three loci were tested against a null hypothesis that $F_{ST}=0$ and all four results were non-significant [table 7.ix].

Assuming that Wright's Infinite Island model of genetic structure holds for *N.pronuba* the mean F_{ST} value was used to calculate the average number of migrants exchanged between populations using equation (7.9). The value of 17.60 is high and suggests there is considerable gene flow between sub-populations.

7.4.6: Genetic distance measures:

The low level of genetic structuring between sub-populations is reflected in the low genetic distance measures [table 7.x]. The greatest distance, 0.014 is found between sample 1a and 15. Due to the low distances between sites no cluster analysis was performed as a dendrogram based on such small differences would suffer from a lack of robustness.

Table 7.viii: F statistics for individual alleles at each variable locus.

Locus:	PGI		
Allele	F(IS)	F(IT)	F(ST)
180	-0.018	-0.003	0.015
166	-0.030	-0.011	0.019
133	0.010	0.020	0.011
111	-0.019	-0.003	0.016
100	0.066	0.076	0.011
77	-0.026	-0.002	0.023
46	-0.055	-0.039	0.015
38	-0.016	-0.005	0.011
Mean	0.027	0.038	0.012

Locus:	PGM		
Allele	F(IS)	F(IT)	F(ST)
125	-0.018	-0.011	0.007
120	0.548	0.570	0.049
115	-0.028	-0.010	0.018
108	-0.019	-0.009	0.010
103	-0.027	-0.005	0.022
100	0.016	0.033	0.017
94	-0.017	-0.003	0.014
88	0.053	0.067	0.014
72	-0.025	-0.007	0.018
Mean	0.012	0.028	0.017

Locus:	GOT		
Allele	F(IS)	F(IT)	F(ST)
140	-0.018	-0.004	0.014
128	-0.016	-0.006	0.010
108	-0.015	-0.003	0.012
100	0.031	0.039	0.007
80	-0.021	-0.009	0.012
58	0.032	0.042	0.010
Mean	0.021	0.03	0.009

Table 7.ix: Summary F statistics for all loci.

Locus	F(IS)	F(IT)	F(ST)
PGI	0.027 n.s.	0.038 n.s.	0.012 n.s.
PGM	0.012 n.s.	0.028 n.s.	0.017 n.s.
GOT	0.021 n.s.	0.030 n.s.	0.009 n.s.
Mean	0.017 n.s.	0.032 n.s.	0.014 n.s.

The significance of F statistics was tested using formulae (7.10), (7.11) and (7.12) in section 7.4.3.

Table 7.x: Nei's (1972) genetic distance measures

	1a	1b	2	3	4	5	6	population 7	8	9	10	11	12	13	14
1b	0.012	*****													
2	0.009	0.003	*****												
3	0.006	0.006	0.003	*****											
4	0.003	0.008	0.005	0.004	*****										
5	0.013	0.009	0.004	0.002	0.010	*****									
6	0.010	0.001	0.001	0.005	0.005	0.007	*****								
7	0.005	0.003	0.001	0.002	0.003	0.005	0.002	*****							
8	0.004	0.006	0.005	0.007	0.003	0.013	0.005	0.003	*****						
9	0.004	0.012	0.011	0.005	0.004	0.013	0.010	0.006	0.008	*****					
10	0.009	0.003	0.000	0.003	0.004	0.004	0.002	0.002	0.005	0.010	*****				
11	0.005	0.010	0.005	0.001	0.004	0.004	0.007	0.003	0.007	0.005	0.005	*****			
12	0.003	0.006	0.005	0.006	0.002	0.012	0.005	0.003	0.001	0.006	0.005	0.005	*****		
13	0.005	0.005	0.002	0.004	0.001	0.007	0.002	0.001	0.002	0.007	0.002	0.004	0.002	*****	
14	0.009	0.006	0.005	0.002	0.006	0.005	0.005	0.004	0.010	0.005	0.005	0.004	0.009	0.006	*****
15	0.014	0.004	0.002	0.003	0.009	0.002	0.003	0.003	0.011	0.013	0.002	0.006	0.011	0.006	0.004

7.5: Discussion:

7.5.1: Allozyme polymorphism:

The enzymes PGM, PGI, GOT and MPI were highly polymorphic with numerous alleles at each locus, while MDH was monomorphic. The variation visualised at these loci is almost certainly an underestimate of the variation that actually exists. Many mutations do not alter amino acid sequences because the redundancy of the genetic code, or cause changes in amino acid sequence that have no effect on charge. These types of changes lead to cryptic variation, that is not visualised by electrophoresis (Kreitman 1983). Studies have shown that as little as a sixth of all changes that cause amino-acid substitutions are detectable by allozyme electrophoresis (Coyne 1982). The high level of variation observed requires explanation. These fall into two categories:

(i) molecular variation is adaptive, maintained by balancing selection (Nevo 1983)

(ii) it is non-adaptive and neutral or nearly neutral to selection (Kimura 1983).

These two points of view are not mutually exclusive but represent extreme points of view.

Selectionists rely on mechanisms such as those described in the introduction. Heterozygote advantage has been implicated with individuals heterozygous at specific loci showing reduced morphological variance (Mitton 1978, Eanes 1978, Zink *et al.* 1985, Strauss 1991) and increased growth rates (Hu *et al.* 1993, Elliott & Pierce 1992, Mitton & Grant 1984). Selection in heterogeneous environments may maintain genetic variation where environmental variation is correlated with genetic variation (Nevo &

Beiles 1988, Powell 1971). Even frequency-dependent selection has been implicated, where rare genotypes may be at a selective advantage as parasites are better adapted to the modal host genotypes (Clarke 1979).

Neutralists consider mutation and genetic drift to be the important processes that influence the degree of polymorphism at the molecular level (King & Jukes 1969, Kimura 1983). This is demonstrated by the full title of the neutral theory; the "neutral-mutation random drift hypothesis." Polymorphisms exist as transient phenomena caused by the drifting of alleles either to fixation or being lost altogether.

Selection is not disputed in this hypothesis but is relegated to a more minor role in molecular evolution; the majority of molecular changes being neutral to selection, rather than all being neutral. Observations of functional and non-functional genes support this view; pseudo-genes have higher variability than functional genes and nucleotide base substitutions at the silent 3rd position of codons are more common than 1st and 2nd position changes (Ohta 1992).

The degree of polymorphism is dependent upon the population size, with more polymorphism in large populations. This is because heterozygosity (in this case the chance of two alleles chosen at random being different) is dependent upon $N_e\mu$, where N_e is the effective population size and μ is the mutation rate (Kimura 1983). The prediction that the amount of polymorphism is dependent upon the population size is borne out by experimental results. *Escherichia coli* is polymorphic at 47% of its loci (Selander & Levin 1980), while the cheetah, a species thought to have been through a severe reduction in population size, is virtually monomorphic at all allozyme loci (O'Brien *et al.* 1983).

Which model does *N.pronuba* fit?. Although relatively few allozyme loci were analysed, and so the sampling variance was high, the majority of loci that produced interpretable results were polymorphic. *N.pronuba* is an abundant moth and the high proportion of polymorphic loci agrees with this. One problem with the neutral theory is that abundant species should be highly polymorphic, with most loci being variable. Levels of polymorphism found in natural populations are less than expected but the discrepancy, between observed and expected levels of polymorphism in large populations, can be explained in two ways:

(i) the effective population size (N_e) is considerably less than the actual population size,

(ii) most polymorphic molecular variation is not strictly neutral but slightly deleterious. In large populations, selection will be effective against slightly deleterious alleles, whereas in small populations genetic drift will be more important.

The effective population size (N_e) of *N.pronuba* may be lower than the current abundance of the species would suggest. Periods of low population size have a disproportional effect on the genetic variation within a population (the mean N_e over time is the harmonic rather than the arithmetic mean). It is likely that *N.pronuba* has not always been as common as it is today. It is most abundant in disturbed lowland habitats, and is an agricultural pest species. These lowland, disturbed habitats are more common now than they ever have been so the genetic variability observed in present day populations may well be influenced by the smaller populations of *N.pronuba* in the past.

It is possible that selection, of the types described above, maintains the allozyme polymorphism. There is no evidence, however, for particular alleles

being at a selective advantage in different parts of the moths range. There are numerous habitat / environmental differences between southern Portugal and Aberdeen but these are not translated into allele frequency differences. Selection may be favouring different alleles in different populations but the magnitude of gene flow in *N.pronuba* may disguise this variation. Differentiation of allele frequencies have been observed in highly vagile species suggesting that strong selection can act to overcome the homogenising effects of drift. *Mytilus edulis* has planktonic larvae so dispersal and gene flow is considerable, however clinal variation in the frequency of LAP has been observed, and the cline coincided with a salinity gradient (Hilbish & Koehn 1985). It is interesting to note that other allozyme loci showed no such pattern. No clinal associations have been found in *N.pronuba* and the uniformity of allele frequencies is congruent across all the alleles studied.

In conclusion the allozyme variability in *N.pronuba* is compatible with the neutral hypothesis, although weak selection cannot be ruled out. The initial controversy that surrounded the formulation of the neutral theory has abated somewhat; this is in recognition of the good fit the theory has to observed data, to quote Bruce Wallace "few persons, if any, would question the success the neutralists have had in demonstrating that their theory is compatible with the data" (Wallace 1993).

7.5.2: Population structure:

The main aim of the allozyme study was to give some idea of the magnitude of gene flow. The data show that, over the range of sample sites studied, the genetic structure is essentially uniform. The overall F_{ST} value indicates that only 1.4% of genetic variation resides between populations compared to 98.6% that resides within populations. Differences between populations in

terms of allele frequencies are, according to the infinite island model, caused by genetic drift. The low levels of genetic variation are thus indicative of high levels of gene flow between populations which is reflected in the high Nm value (17.6). Theoretically it has been shown that an Nm value of 1 or greater, that is a single migrant exchanged between sub-populations, is sufficient to prevent differentiation due to genetic drift. The Nm estimates derived from this electrophoretic data are more than sufficient to negate the effects of genetic drift.

For comparison table 7.xi lists F_{ST} and Nm values for a number of other species of Lepidoptera that have been studied using allozyme electrophoresis. *N.pronuba* has a similar overall F_{ST} to *Pieris rapae* and *Danaus plexippus* which are known migrants. The comparison of F_{ST} values between studies must be treated with care as mutation rates, selection coefficients, the demographic history, population structures and sampling ranges differ between species and / or between studies (Weir & Cockerham 1984). Simulations, however, have shown that F_{ST} values are consistent over a wide range of assumptions of population structure, selection and mutation (Barton & Slatkin 1986) so the main problem with the comparison of F_{ST} is that in most studies the sampling area varies in size; for instance *Maniola jurtina* has a similar F_{ST} value, calculated over a similar number of loci, but the sampling area is much smaller.

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Table 7.xi: A comparison of F_{ST} and Nm values, from previous studies of genetic structure of lepidopteran populations, determined by allozyme electrophoresis.

Species	F_{ST}	Nm	No. of Loci	Sampling area	Reference
Heliothis virescens	0.002	124.75	13	Gulf states USA	Korman *et al.* (1993)
Spodoptera exempta	0.006	41.42	6	Kenya, Tanzania, Zimbabwe	Den Boer (1978)
Danaus plexippus	0.009	27.53	6	E. USA	Eanes & Koehn (1978)
Pieris rapae	0.014	17.61	4	USA	Eanes & Koehn (1978)
Noctua pronuba	**0.014**	**17.61**	**3**	**U.K., Austria, Portugal**	**This study**
Maniola jurtina	0.015	16.42	4	S.E. Britain	Goulson (1993)
Spodoptera frugiperda	0.084	2.73	13	Caribbean, Mexico, E. USA	Pashley *et al.* (1985)
Euphydryas chalcedona	0.090	2.53	8	California	McKechnie *et al.* (1975)
Euphydryas editha	0.118	1.87	8	California	McKechnie *et al.* (1975)

McCauley and Eanes (1987) noted that there is good agreement between the amount of gene flow inferred from F_{ST} values and the proposed vagility of species; sedentary species, e.g. *Euphydryas* spp. have high F_{ST} values, while known migrants, e.g. *Danaus plexippus*, have F_{ST}s close to zero. An exception to this is the extreme population subdivision found in both a winged, *Platynus angustatus,* and a wingless, *Platynus tenuicollis,* species of carabid beetle (Liebherr 1986). The F_{ST} and Nm values of *N.pronuba* are of comparable magnitude with those of species known to disperse widely.

In conclusion, the uniform population structure, suggested by the relative homogeneity of allele frequencies at the three polymorphic allozyme loci, is indicative of high levels of gene flow between the populations sampled.

Chapter 8: Comparison of the geographic structure of allozyme and forewing polymorphisms:

8.1: Introduction:

The geographic structure of the forewing polymorphism of *N.pronuba* is notable because of the uniformity of phenotype frequencies. This uniformity could be caused by high levels of gene flow homogenising allele frequencies at the forewing locus.

An indirect method for assessing whether a particular genetic locus **a** is under the influence of natural selection is to compare the spatial genetic structure of locus **a** with another locus, or group of loci **b**, which is thought to be neutral to selection. The spatial variation of the "neutral" (in this case **b**) locus or loci should be solely dependent on genetic drift, mutation, and gene flow. In contrast, if character **a** is under the influence of natural selection the spatial structure of this character will be dependent on those processes mentioned above and natural selection. This hypothesis is tenable because genetic drift and gene flow act equally, on average, over the whole genome while selection does not.

If samples of the species are taken over a wide geographic range and these geographic locations differ in a number of environmental variables, e.g. temperature, habitat and altitude one can hypothesise that a character that is expected to be influenced by selection will vary between these sampling locations. The character that is not influenced by selection should only reflect the evolutionary history of the species in question. The degree of geographic variation that is observed in the selected character will be affected by:

(i) the strength of natural selection,

(ii) the amount of gene flow between populations.

The use of neutral genetic markers gives an insight into the relative importance of gene flow in the system being studied. Table 8.i summarises the interaction between points 1 and 2 where Nm is the absolute number of migrants that move from one sub-population to another and breed.

The allozyme survey [see chapter 7] shows that for the three polymorphic loci that were reliably scoreable, there is little geographic differentiation and this is reflected in the low F_{ST} value and the consequently high Nm value. The method of comparing the two groups of genetic characters assumes that one group is neutral to selection. In this case it is assumed that the enzyme variants are not subject to selection and the alleles at each locus have equal fitnesses.

In *N.pronuba* the F_{ST} values for each of the three independent loci are similar. This supports the assumption that the variation in PGM, PGI and GOT is not under strong selection. Studies of geographic variation of allozymes that have revealed evidence for selection acting on a locus, are usually characterised by the population structure inferred from the locus under selection being different from other loci surveyed. This is usually seen as (1) high F_{ST} values relative to the other loci studied (Singh & Rhomberg 1987, Gasperi *et al.* 1991), or (2) alleles at a particular locus showing clinal variation while other loci show no such pattern (Christiansen & Frydenberg 1974). None of the loci in *N.pronuba* show such patterns.

Wright's island model assumes that migrant individuals from each sub-population are equally likely to move to any other sub-population (Wright 1943). Thus there should be no evidence for isolation by distance, where sub-populations at the extremes of the sampling area are less similar than

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Table 8.i: The relationship between gene flow (calculated from variation in a neutral genetic marker) and divergence character potentially under the influence of natural selection. Adapted from Singh & Long (1992).

Selected character divergence	Gene flow (Nm value)	
	Low - Nm<<2	High - Nm>>2
High	Selection on the character strong enough to overcome the effects of weak gene flow.	Strong selection causing character divergence even in the face of large amounts of gene flow.
Low	Selection on the character too weak or absent. Even small amounts of gene flow overcome the divergence caused by selection. Geographic variation probably due to genetic drift	Selection for divergence may be considerable but the differentiation swamped by gene flow

those in close geographical proximity. If a genetic locus is under selection we might expect a relationship with genetic distance as the environmental conditions will also be correlated with genetic distance. Sites in close proximity will tend, on average, to be more similar than those further away.

8.2: Aims:

The aim was to test the hypothesis that the forewing colour and pattern polymorphism in *N.pronuba* is influenced by natural selection, and that this selection causes geographical differentiation in spite of high levels of gene flow. This was approached by comparing the geographical structure of the forewing polymorphism with the geographical structure of the allozyme polymorphisms.

8.3: Methods:

Individuals from the sixteen sample sites employed in the allozyme survey [chapter 7] were classified into the three forewing phenotypes. In addition to those individuals used in electrophoresis other individuals were scored to swell the sample sizes. This was necessary as each individual only supplied one data point because forewing genotypes of specific moths could not be elucidated from the phenotypic data. In contrast individuals scored for allozymic variation provide two independent data points as two genes can be visualised (in heterozygotes) or inferred (in homozygotes). The conformance to Hardy-Weinberg equilibrium suggests that mating is random with respect to allozyme genotype and so a sample of 30 individuals represents a random sample of 60 alleles at each locus.

8.4: Statistical analyses:

Rogers' genetic distances were calculated for each allozyme locus and for all three variable loci combined together (Rogers 1972) as follows

$$R = \sqrt{0.5 \sum_{n}^{i} (P_{Ai} - P_{Bi})^2}$$

where:
R = Rogers' genetic distance
n = number of alleles
P_{Ai} = frequency of the ith allele in population A
P_{Bi} = frequency of the ith allele in population B

In addition the geographical distances between sampling sites were measured, to the nearest 10km using the program "map" on a Macintosh computer. Forewing morph frequencies were converted to allele frequencies (Cook 1971) assuming the single locus genetic model (Poitout and Bues 1976). These allele frequencies were used to calculate Rogers' genetic distance using the allele frequency input data step in Biosys 1.7 (Swofford & Selander 1981) (the distance half matrices used in this analysis can be found in appendix iv).

Rogers' genetic distance was used as it does not rely on any genetic assumptions and is purely an observational measure of the difference in allele frequencies between sample sites (Richardson *et al.* 1986). This contrasts with Nei's genetic distance which is based on the probability of two alleles, one picked at random from each sample site, being identical. Nei gave the measure biological significance by viewing it as an estimate of the number of DNA base differences per locus between the sample sites (Nei 1972). Rogers' distance makes no such assumptions and is a better measure for comparing spatial data from independent sources, such as allozyme variation and forewing phenotypes.

The statistical analysis of the relationship between genetic distance and geographic distance presents a problem. The points on the graph are not independent from one another as they represent distances extrapolated from thirteen independent samples, consequently any conventional correlations or regressions performed on such data would be invalid. The problem of the non-independence of these data points can be overcome by using a re-sampling or randomisation procedure (Douglas & Endler 1982, Crowley 1992, Coll et al. 1994).

The test used was the Mantel matrix correlation test (Mantel 1967) which was performed using the randomisation testing computer program RT (Manly 1992). Matrix correlation procedures have been used in a number of studies to test the significance of geographic variation and to see if the observed variation is distributed in a random manner (Sokal 1979, Dillon 1984, Smouse & Long 1992).

The Mantel test compares the elements of two distance matrices using a correlation statistic. This coefficient is then compared to a set of correlation coefficients which have been calculated by holding the rows and columns of one matrix (the dependent matrix) fixed while the elements of the other are randomly reordered (Manly 1986, 1991). In each test the correlation coefficient was compared to a distribution generated from 30,000 randomisations of the independent matrix.

8.5: Results:

Figures 8.i-8.v show relationships between geographic distance and Rogers' genetic distance; figure 8.i shows single locus distances calculated for PGI, PGM and GOT respectively and in figure 8.iv the distances are averaged
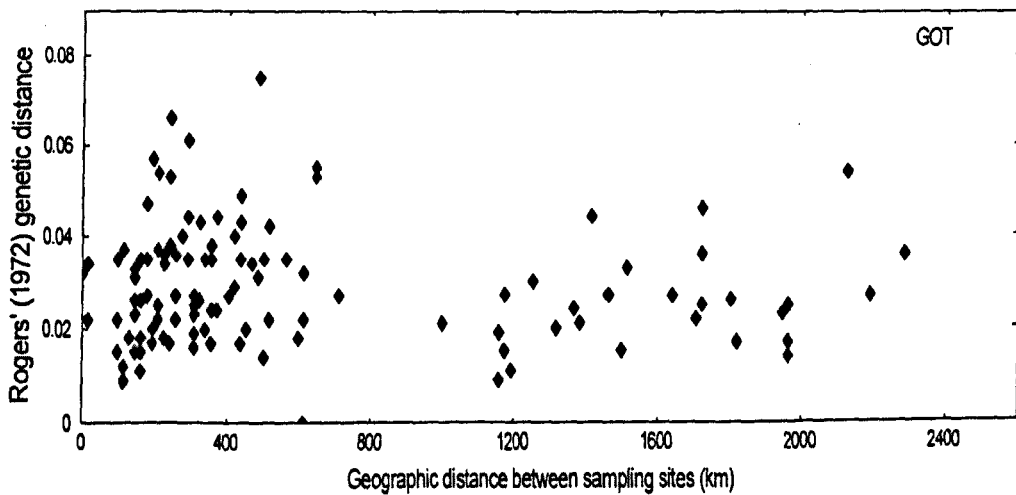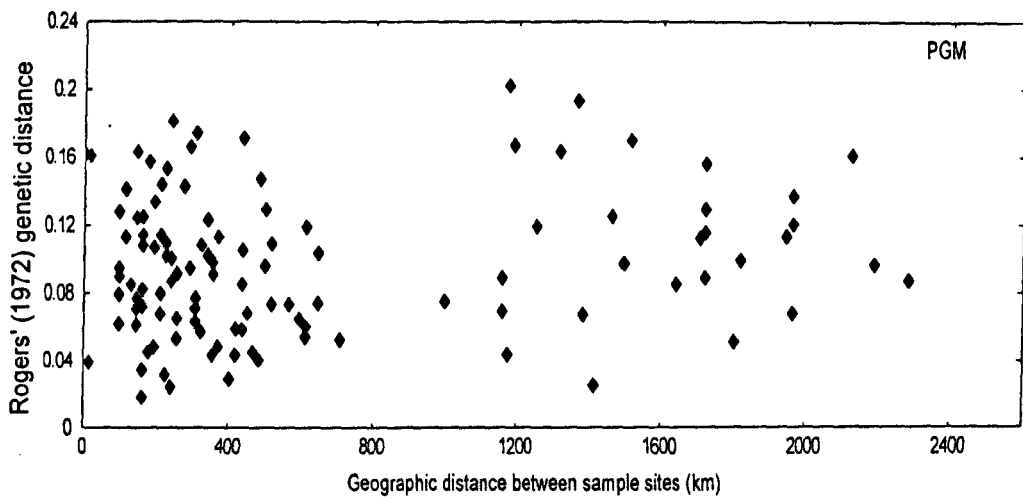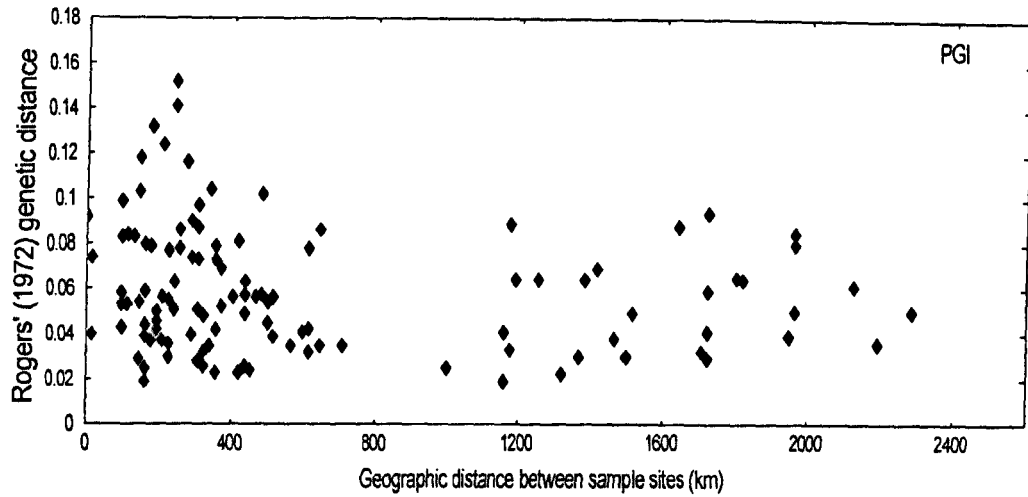
Figure 8.i: Variation of single locus Rogers' genetic distance (1972) with geographic distance between sample sites.
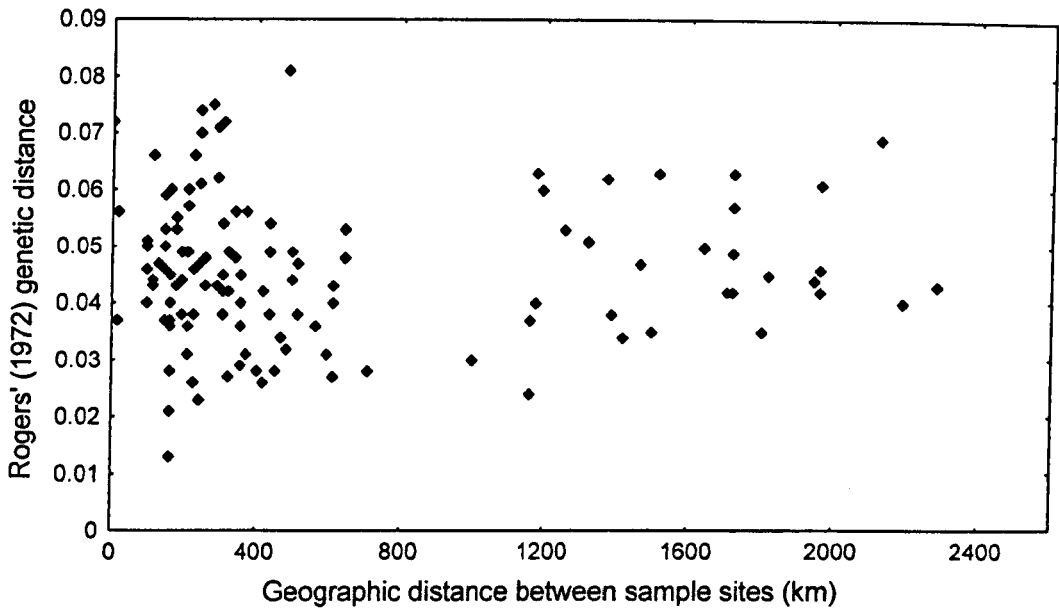
Figure 8.ii: Variation of Rogers' genetic distance (1972), averaged across all three polymorphic loci, with geographic distance between sample sites.
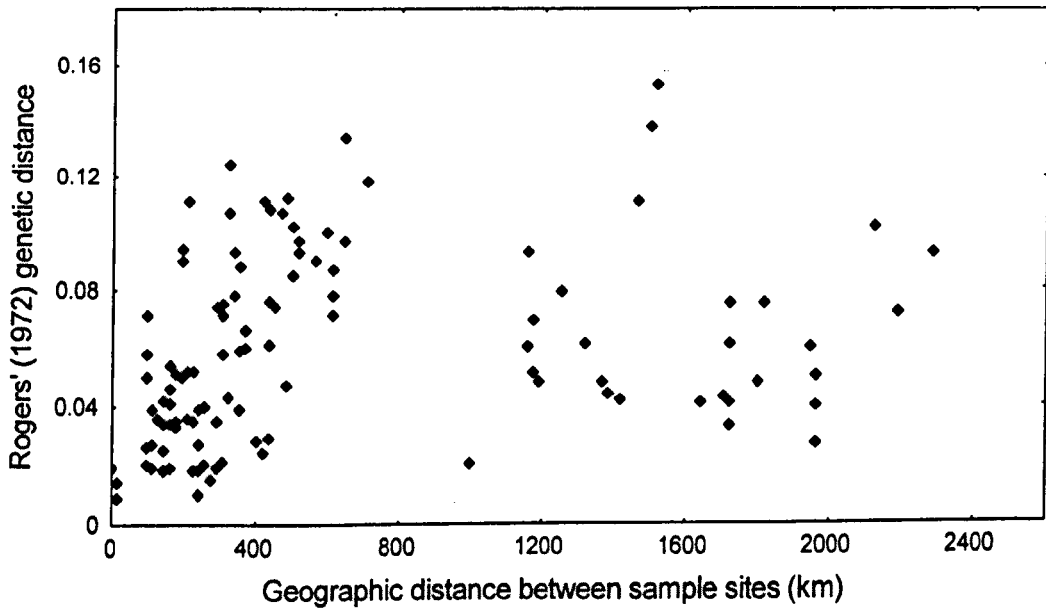
Figure 8.iii: Variation of Rogers' genetic distance (1972), based on the forewing locus, with geographic distance between sample sites.
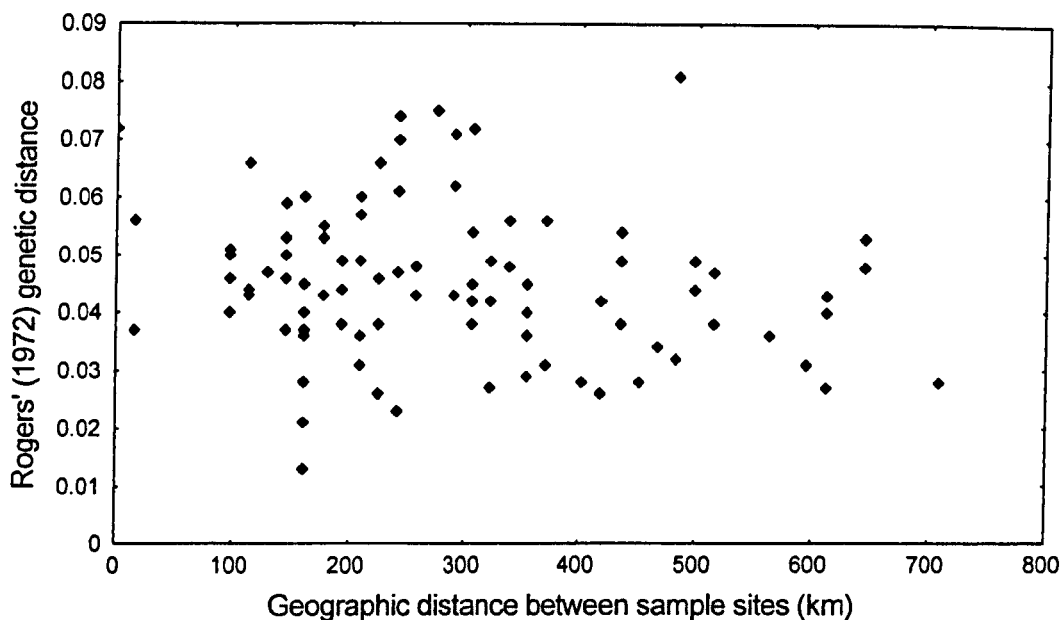
Figure 8.iv: Variation of Rogers' genetic distance (1972), averaged over all three polymorphic loci, with geographic distance between sample sites. Only samples collected within the U.K.
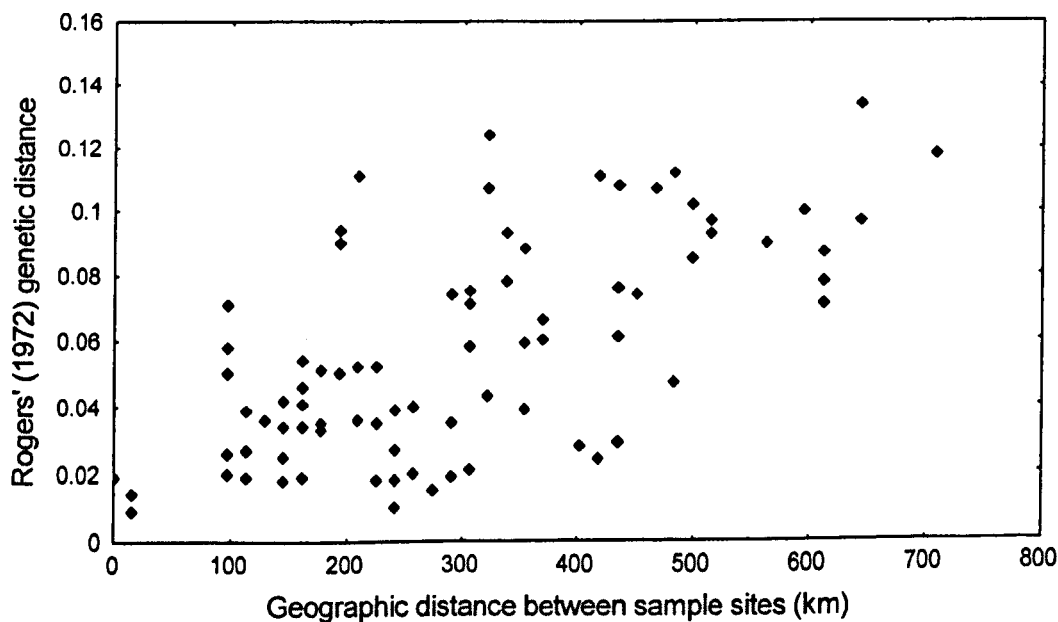
Figure 8.v: variation of Rogers' genetic distance (1972), based on the forewing locus, with geographic distance between sample sites. Only samples collected within the U.K.

Table: 8.ii: Mantel test results; in all cases the independent matrix was the geographic distance between sample sites. The probabilities were estimated by comparing the observed correlation coefficient with a distribution based on 30,000 randomisations of the dependent matrix.

Dependent matrix - Rogers' (1972) genetic distances	Total sum of squares	β_0	β_1	Extra sum of squares	Probability
All 16 sample sites					
PGI	0.890×10^{-1}	0.621×10^{-1}	-0.614×10^{-5}	0.164×10^{-2}	0.1841 n.s.
PGM	0.214	0.884×10^{-1}	0.115×10^{-4}	0.576×10^{-2}	0.0818 n.s.
GOT	0.198×10^{-1}	0.302×10^{-1}	-0.174×10^{-5}	0.132×10^{-3}	0.4404 n.s.
Average over all loci	0.195×10^{-1}	0.452×10^{-1}	0.841×10^{-6}	0.304×10^{-4}	0.7029 n.s.
Forewing	0.123	0.495×10^{-1}	0.134×10^{-4}	0.774×10^{-2}	0.0067**
14 sample sites from U.K. only					
Average over all loci	0.157×10^{-1}	0.495×10^{-1}	0.145×10^{-4}	0.486×10^{-3}	0.1023 n.s.
Forewing	0.935×10^{-1}	0.187×10^{-1}	0.127×10^{-3}	0.369×10^{-1}	<0.0001***

n.s. = not significant; * = 0.05>p>0.01; ** = 0.01>p>0.001; *** = p>0.001.

over all three polymorphic loci and 8.iii are Rogers' genetic distances based on the forewing locus. The geographical distance between samples falls into two distinct groups. Those samples from within the U.K. which are separated by distances of less than 800Km and between the U.K samples and those from Scharnstein, Austria and Portimão, Portugal which are separated by distances between 900Km and 2400 Km.

The three single locus allozyme plots [figure 8.i] for samples within the U.K. indicate that there is no relationship between genetic distance and geographic distance and no support for the isolation by distance model. The Rogers' distance coefficients do not appear to be greater between more distant populations than between populations in close proximity. This is confirmed in the plot [figure 8.ii] for Rogers' genetic distance averaged over the three loci against geographical distance. In contrast the Rogers' genetic distance based on forewing allele frequencies shows a weak relationship with geographic distance: the greater the geographic distance, the greater the genetic distance [figure 8.iii].

As geographic distances between the 14 U.K. samples and the two continental samples (15 and 16) form two distinct distance groups, the two continental samples are omitted in figures 8.iv and 8.v. The graphs show similar relationships to those where all samples were used; there is no relationship between geographic distance and genetic distance for the allozyme loci [figure 8.iv] whereas there is a positive relationship for the forewing polymorphism [figure 8.v].

The significance of these relationships, as tested by Mantel matrix correlation tests, is given in table 8.ii. Only two results, of the seven conducted, are significant at p=0.05 or less, these being for the forewing polymorphism. In conclusion there seems to be no relationship between

geographic distance and genetic distance based on the allozyme polymorphisms; in contrast there is a significant relationship between geographic distance and the forewing polymorphism.

8.6: Discussion:

Previously Mantel tests have been used to compare the relationships between genetic traits and environmental variables in an attempt to understand the underlying selective mechanisms that may affect genotype and gene frequencies (Dillon 1984, Manly 1986). The application of such a method relies on choosing environmental variables that may act directly on the trait.

This study is concerned with gathering evidence for natural selection acting on the forewing polymorphism of *N.pronuba*. As there is little direct evidence to suggest that selection acts on the forewing polymorphism of *N.pronuba*, let alone what type of selection, the choice of environmental variable would be purely arbitrary. As there are an enormous number of environmental variables, e.g. mean summer temperature, mean winter temperature, mean rainfall etc. which can be correlated with the genetic trait, it would be quite possible to get spurious correlations between the forewing polymorphism and environmental variables. To overcome this the approach used in this study is the comparison of the population structure of the two types of polymorphism one of which is thought to be neutral to selection and only influenced by gene flow and genetic drift.

The allozyme polymorphisms do not show any significant relationship with geographic distance and the overall F_{ST} value (see chapter 7) is low (0.014). Both of these results imply that gene flow is considerable and, over the geographic range studied, it is sufficient to overcome divergence due to

genetic drift. In contrast the forewing polymorphism does show significant variation with geographic distance. Given that gene flow is high, the divergence observed in the forewing polymorphism is unlikely to be caused by random genetic drift and may be caused by natural selection acting on the forewing phenotype.

This conclusion must be treated with some caution as the degree of variation in the phenotype, and consequently genotype, frequencies is small; the greatest Rogers' genetic distance is 0.153. In addition the hypothesis of allozymes being neutral to selection has recently been questioned (Karl & Avise 1992). The neutrality of allozymes has been questioned before and there are good examples of selection acting on individual allozyme loci [see chapter 7]. The evidence from a study of the American oyster, *Crassostrea virginica*, is somewhat different in that it provides evidence for balancing selection acting on five, unlinked allozyme loci simultaneously (Karl & Avise 1992). The allozymes indicated that gene flow in *C.virginica* is considerable and that populations from along the north American, Atlantic coast and the Gulf of Mexico are exchanging genes. In contrast, mitochondrial DNA (mtDNA) and single copy nuclear DNA (scnDNA) showed a disjunct pattern of gene flow, with the Atlantic and Gulf populations being separated by shifts in allele frequency and the occurrence of different mtDNA haplotypes. Importantly, the boundary in the geographic structure of these two different genetic markers (mtDNA and scnDNA) coincided.

Karl and Avise's (1992) work is the first convincing study suggesting that balancing selection acts on many different, independently inherited allozyme loci simultaneously. Other studies utilising a number of different types of genetic marker have given contrasting population structures (Palumbi & Baker 1994) The increasing use of a number of genetic markers: e.g. mtDNA, mini-satellites, microsatellites and scnDNA, allows the congruence

of population genetic structures based on the analysis of different parts of the genome to be investigated. If these studies show similar results to Karl and Avise (1992) then the use of allozyme variation as an population genetic marker may have to be questioned.

The finding that selection does seem to act on the forewing phenotype does not explain the existence and persistence of the polymorphism. Cook and Sarsam (1981) considered that a balance between migration and selection, in different directions in different regions, was a possible mechanism that may maintain polymorphisms. They concluded that this would not lead to the constant frequencies observed in *N.pronuba* and introduced their differential selection on the sexes hypothesis (see chapter 4). The reasons for their dismissal of the migration-selection mechanism was the total lack of differentiation throughout Britain. My data do show differentiation at the extremes of the range with N.Ireland, Scotland and Finland being different to other regions. Even with this greater differentiation I am in agreement with Cook and Sarsam and to quote them "it is difficult to visualise conditions....under which this (a migration and selection balance) could lead to the constant frequencies over large areas." However, implicit in their rejection of a migration and selection balance and their adoption of differential selection on the sexes, is a belief that the polymorphism *needs* to be explained in terms of balancing selection. Is there any evidence for selection actively maintaining the colour and pattern polymorphism and do the temporal and geographic patterns of the polymorphism provide indirect evidence for this balancing selection? This question is discussed in the final chapter.

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Chapter 9: Conclusions:

9.1: Temporal and geographic variation - is the stability explained by demographic parameters?

Since the study of evolutionary mechanisms began, the interpretation of polymorphisms, particularly those of colour and pattern, has changed dramatically. Darwin considered that polymorphic variation "in which species present an inordinate amount of variation" was neutral to selection, the variation being "of no service or disservice to the species" (Darwin 1872). A century after the publication of the *Origin of Species*, almost every student of evolution believed that polymorphic variation was adaptive and maintained by some type of balancing selection (Provine 1986). The work of R.A.Fisher and E.B.Ford was particularly influential in the change from viewing intraspecific variation as neutral, to being maintained by balancing selection. Ford's field work on *Maniola jurtina* and *Panaxia dominula* was conducted with the aim of showing the importance of selection in the maintenance of such variation in natural populations.

In the introduction I suggested that the null model, when considering the maintenance of polymorphisms, should be one of neutrality. Too often the mere existence of a polymorphism leads to the variation being explained as balanced, implying the variation is actively maintained by selection. Given this null model what evidence is there that the forewing colour and pattern phenotypes of *N.pronuba* are selectively maintained?

Much of the data collected in this study are of temporal and geographic variation in the forewing polymorphism. Temporally, the phenotype frequencies are stable with only the changes in the frequency of ochre being significantly larger than that due to chance. Similarly, phenotype frequencies are stable over a large part of Europe, with significant differentiation

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occurring only at the northern edge of the sampling area. This stability could be thought of as being a good example of balancing selection maintaining a polymorphism, the phenotype frequencies being a stable equilibrium point. Alternatively, the variation in forewing colour and pattern could be neutral to selection; this is not to say that the character, in this case the colour and pattern of the forewing, is not under selection but that the phenotypes rufous, ochre and silver, are equivalent with respect to natural selection. If the phenotypes were equivalent to one another, is it possible that the demography of the species might explain the temporal and geographic patterns?

N.pronuba is a common species and Poitout and Bues (1976) reported nightly captures of greater than 10,000 individuals. In Norway it is the most commonly caught moth in deciduous forests (Soli 1987) and in the newly established Canadian populations the increase and establishment of large numbers of the species has been dramatic (figure 9.i). Although light trap catches do not solely reflect abundance, movement and attraction also being important, there should be a strong correlation between light trap abundance and population size. In view of this one can, with confidence, consider *N.pronuba* populations to be large.

Gene flow is common in this species. Allozyme variation show low levels of differentiation between populations and consequently estimates of migration, based on the island model of population structure, are large. Kimura and Ohta (1971) argued that the existence of large amounts of gene flow, as found in *N.pronuba*, has the effect of making a series of populations act as one large panmictic population. This effect of gene flow, coupled with the high densities of this species means that the effective population size of *N.pronuba* is large.
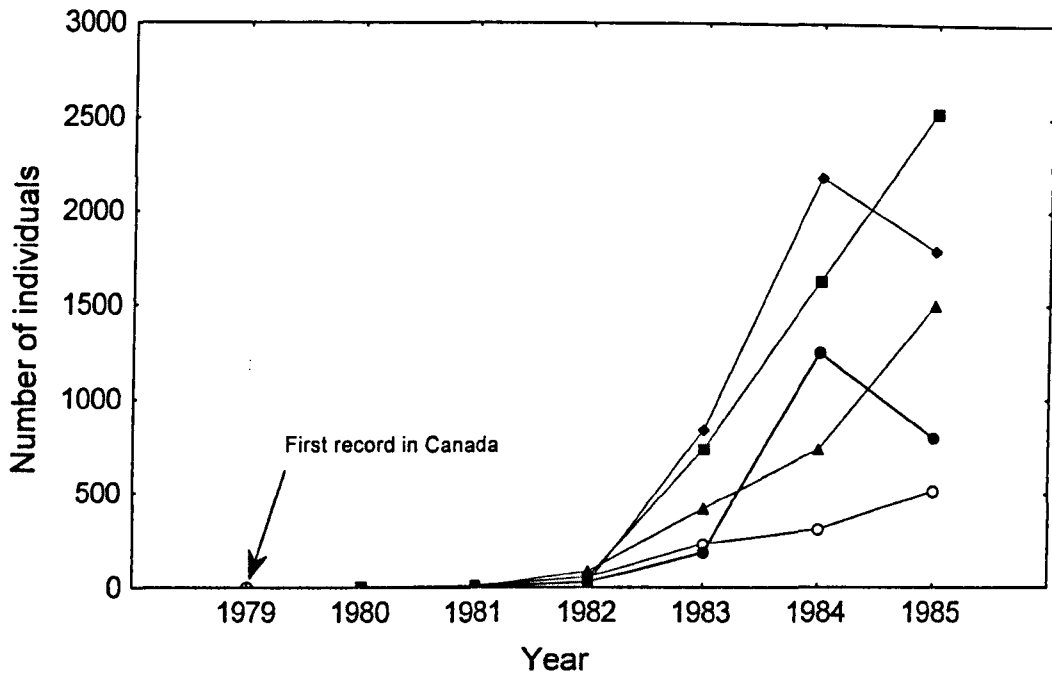
Figure 9.i: Light trap catches per year at five sites in Nova Scotia, Canada. Re-drawn from Wright (1987).

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For neutral alleles the time needed for the replacement of an allele by another has been shown to be approximately equal to $4N_e$ generations, where N_e is the effective population size (Nei 1975). During this period of replacement a transient polymorphism will exist. As the population size is large, the transient polymorphism will exist for many generations. For example, if the effective population size of *N.pronuba* is 1 million there would be a period of polymorphism of 4 million years, given one generation per year. The rate of change per year would be so small that it would be undetectable over the time periods examined in this study; the observed pattern would be one of stability rather than transience.

Thus the large local population sizes, coupled with the large amounts of gene flow demonstrated indirectly by allozyme variation can largely explain both the temporal and geographic stability in phenotype frequencies.

Some evidence for selection acting on the forewing colour and pattern is provided by the differentiation of Scottish and N.Irish populations in terms of their forewing phenotype frequencies compared to the lack of differentiation, in terms of the allozyme polymorphisms. This observation indicates that in some populations, those at the northern edge of the species range, some phenotypes may be favoured over others.

There is no doubt that *N.pronuba* is a highly dispersive moth species with high levels of gene flow. The investigation of the maintenance of colour and pattern variation is complicated by this because gene flow and large population size can explain the seemingly stable phenotype frequencies as well as frequency-dependent selection or any other balance hypothesis. There is insufficient information to discard the null hypothesis of neutrality.

This study of *N.pronuba* has shown that it is not possible to assume that all polymorphisms are balanced. Even though the polymorphism in *N.pronuba*

is both geographically and temporally stable this does not necessarily mean that it is balanced and "the extreme age of certain genetic polymorphisms is not proof that they are subjected to natural selection" (Wallace 1981).

9.2: Possible selective alternatives:

It is difficult to disentangle the relative effects of genetic drift, migration and selection on a polymorphism (Cabe & Alstad 1994). In view of this it is worth considering what selective alternatives might explain the observed patterns of variation in *N.pronuba*.

Heterozygote advantage can potentially maintain polymorphisms but there have been only six studies that have showed this unequivocally (Endler 1986). In a polymorphism that exhibits complete dominance, where heterozygotes cannot be observed, heterosis is difficult to detect. To investigate heterozygote advantage in dominant traits, the heterozygote genotype must be identified so that comparisons between the fitness of heterozygote and homozygote classes can be made. Involved breeding experiments are required to do this, either with the creation of homozygous lines or the use of back crosses to homozygous recessive individuals. Producing lines that are homozygous at the forewing phenotype locus can cause inbreeding, so when two lines are crossed together, giving individuals heterozygous at the forewing locus, the out-breeding effect can produce results that are spuriously compatible with heterozygote advantage. In *N.pronuba* the alleles at the forewing locus are in a dominance hierarchy. Consequently if heterozygote advantage maintains the colour and pattern polymorphism in *N.pronuba* some sort of pleiotropic effect of the alleles at the colour and pattern locus must be assumed. The role of heterozygote advantage in maintaining the polymorphism in *N.pronuba* remains purely

speculative, as does its general importance in maintaining genetic variation in natural populations (Jones *et al.* 1977).

If the attractiveness of the phenotypes to mates was based on colour and pattern, and the degree of attractiveness depended on the frequency of the phenotype in the population, with rare phenotypes being more attractive, frequency-dependent mating could potentially maintain this polymorphism. *N.pronuba* is a nocturnal species which is active and mates during darkness and it is well known that nocturnal Lepidoptera use pheromonal cues rather than visual cues to recognise conspecifics. It is difficult to imagine that males and females would choose mates based on their colour and pattern if this variation was not visible and so, given the presumed mating behaviour of the species, frequency-dependent mating seems an implausible mechanism.

Selection in heterogeneous environments can potentially maintain polymorphisms, but the conditions are restrictive. These conditions broaden if individuals of specific genotypes or phenotypes can choose the habitat where their fitness is maximised. In a colour and pattern polymorphism the habitats may correspond to backgrounds on which a particular phenotype is maximally cryptic. Background resting experiments suggested that different phenotypes of *N.pronuba* do not prefer to rest on different backgrounds, although it must be conceded that the artificial nature of the experiments may have obscured subtle relationships of particular phenotypes with a particular background.

Aldridge *et al.* (1993) provided evidence that different phenotypes of *N.pronuba* select different habitats. Captures from three light traps sited in a garden, a conifer plantation and the boundary between the two habitats, showed significant differences in phenotype frequencies although the distance between the two furthest was only 54 metres. Most of my samples

were collected from gardens but the results from their garden trap data is in disagreement with my findings. Their sample size was small (mean sample size per trap=21, s.d.=8.0), their phenotype classification was different to that specified by Cook and Sarsam (1981) and their phenotypes did not account for the sexual dimorphism in the species. In view of these points their data must be treated with caution.

I have found no evidence for phenotypes selecting backgrounds on which their crypsis is maximised and the geographical stability over a wide area also suggests that there is not differential selection of habitats by phenotypes.

If phenotype fitness decreases as the frequency of the phenotype increases a stable equilibrium occurs and thus a polymorphism will be maintained. As discussed in the introduction there have been a number of investigations using artificial prey and a few on natural polymorphisms that have yielded positive results (see Allen 1988 for a review). Predators searching for food items may form specific search images that could be disrupted by variations in colour and pattern (Endler 1978, Edmunds 1974, Driver & Humphries 1988).

This is one selective mechanism that might explain the existence of the polymorphism in *N.pronuba* but it seems unlikely that frequency-dependent selection would explain the geographic homogeneity of phenotype frequencies over such a wide area.

Many species of polymorphic Lepidoptera, including *N.pronuba,* share a common feature. This is the limitation of colour and pattern polymorphism to the surfaces of the wings that are exposed when the individual is at rest. This has been interpreted as evidence for polymorphisms being adaptive and the variation maintained by visual selection by predators (Owen &

Whiteley 1989). This idea is an extension of a phenomenon first documented by Oudemans (1903). He suggested that there was a clear relationship between the resting posture of a lepidopteran and the distribution of colour patterns on each of the wing surfaces. These observations strongly support crypsis as a mechanism of defence against predators but do not necessarily mean that variation in the colour and pattern is adaptive *per se*. As Endler (1978) has pointed out; "a color pattern is cryptic if it resembles a random sample of the background perceived by predators at the time and age, and in the microhabitat where the prey is most vulnerable to predators." He also stresses that " a polymorphism may be selectively neutral if each color pattern morph appears to predators as a different random sample of the same background pattern." Thus, for any background, one could calculate the variance of particular pattern elements and colours that correspond to a random selection of backgrounds. A mutation that causes a change in colour and pattern that is outside the variance calculated for the background will render the individual conspicuous and be selected against, so crypsis will be under stabilising selection. However, a mutation that changes the wing pattern so that the colour and pattern falls within the background variance will not be selected against, as the pattern will still represent a random sample of the background and so be cryptic. Simply, habitats are complex and heterogeneous and many different patterns are equally cryptic in a particular habitat. Thus variation in colour and pattern could be accommodated rather than actively maintained. It is quite possible that a colour and pattern is adaptive (it is cryptic) but that variation in colour and pattern is not.

Studies on the snail, *Limicolaria martensiana* have shown a correlation between density and the degree of polymorphism, with more dense populations containing more phenotypes (Owen 1963). Owen suggested

that in populations with high densities the diversity of phenotypes confuses potential predators by disrupting the search image, thus rare phenotypes are selectively maintained as they are over-looked by predators. Jones *et al.* (1977) found a positive correlation between species which climb most actively and the degree of polymorphism and also explained this in terms of apostatic selection with there being advantage in phenotypes looking different. Alternatively, the existence of these correlations can be explained by incorporating ideas of background complexity and the broadening of the selective limits on crypsis. More dense populations of snails, or snails that climb higher, may encounter a wider diversity of backgrounds so there may be more ways to be cryptic.

Frequency-dependent selection has been proffered as a potential mechanism maintaining the well-studied shell colour and pattern polymorphisms in *Cepaea* spp. and Clarke first developed the idea of apostatic polymorphisms while studying these helicid snails. Any explanation of the existence of a polymorphism by apostatic selection requires that the phenotypes that make up the polymorphism are sufficiently different from one another for the potential predator (the agent of selection) not to confuse them. In *Cepaea* this is not always the case and there is good evidence to suggest that selection for crypsis brings about convergence between phenotypes. In woodland habitats unbanded brown and pink morphs are common as they are a good match to the background, but other morphs occur which appear dark from above because they are banded and the bands are fused together. Some studies of mixed colonies of *Cepaea hortensis* and *Cepaea nemoralis* have shown that phenotype frequencies are more similar when compared to colonies possessing one species (Carter 1967), again implying apostatic selection is unimportant, but there are exceptions where dramatic differences exist (Clarke 1962b).

Interestingly, cryptic equivalence of phenotypes and frequency-dependent selection are not mutually exclusive. If two phenotypes exist which are cryptically equivalent (they both represent a random sample of the background) a balanced equilibrium can exist provided the fitness of the phenotypes increases with decreasing frequency. Under such a scenario the equilibrium frequency will be an equal frequency of the two phenotypes.

This illustrates an important point about frequency-dependent selection, namely that it is a property of the predator not of the polymorphism. The commonly aired view that colour and pattern polymorphisms disrupt searching images is an unproven one and some authors dispute the existence of the search image mechanism (Guilford & Dawkins 1987). It is quite possible that predators have an intrinsic preference for common phenotypes rather than fail to see rare phenotypes. There is likely to be inter-individual variation in such behaviours and a study of apostatic behaviour of song thrushes (*Turdus philomelos*) predating *Cepaea* spp. showed one bird out of four preferred rare over common phenotypes, a behaviour that would promote monomorphism rather than polymorphism (Tucker 1991). It is feasible to have caged predators, such as song thrushes selecting morphs of *Cepaea* spp. that are presented at different frequencies (Tucker 1991), but it is difficult to do such experiments on highly vagile moth species like *N.pronuba*. The most satisfactory way of detecting frequency-dependent selection in wild, non-captive populations is to manipulate the phenotype frequencies in the field. In *N.pronuba*, which has large population sizes and is highly vagile, it is impossible to manipulate phenotype frequencies as any released individuals would be (i) swamped by the large number of resident individuals and (ii) would disperse from the release point. The study of apostatic selection and its importance to the study of natural polymorphisms is best investigated by studying the predators as they behave

in a frequency-dependent manner, rather than the prey species. It is important to obtain some idea of the preponderance of frequency-dependent behaviour in a large sample of predators which prey upon a known polymorphic species. Only with such studies will the relative importance of frequency-dependent selection in the maintenance of polymorphisms be elucidated.

Over the last thirty years mutation has been essentially ignored in the study of colour and pattern polymorphisms. Until the new synthesis, mutation had been the main explanation of phenotypic variation and polymorphism (Lamotte 1951), but the rise of balancing selection as a mechanism explaining colour and pattern polymorphisms led to its role being under-played. Studies of colour variation in the Florida tree snail, *Liguus fasciatus*, suggest that mutation may be important in the geographic structure and in the maintenance of colour and pattern variation (Hillis 1991). The colour variation in this species is thought to be genetically inherited and many populations are fixed for a particular phenotype which differs from population to population. Thus the variance in colour and pattern is between populations rather than within populations, the converse to that observed in *N.pronuba*. The huge diversity of phenotypes, but the lack of polymorphism, has led to 58 morphological forms being described in Florida alone. Hillis (1991) investigated the degree of genetic variation between populations based on allozyme variation. He found that there was low genetic diversity, with only one locus, PGI, being polymorphic out of a total of 34 loci examined. Most of the populations were fixed for one of the two alleles at the PGI locus. Thus there is a discordance between the levels of variation observed at the allozyme loci and the colour and pattern polymorphism; even the usually highly variable esterases and peptidases (Sarich 1977) were monomorphic. Hillis concluded that the discrepancy arose because of higher

mutation rates at the colour and pattern loci compared to the allozyme loci. The fixation of the colour and pattern alleles occurs by genetic drift, which will be a potent force in a sendentary species that is found in small isolated populations and is self-fertile (Hillis 1989). It seems highly likely that mutation rates at loci that control colour and pattern polymorphisms do vary between species and in some cases may be more important in the maintenance of colour and pattern diversity than current theory would suggest.

9.3: Final Comments:

There is little doubt that selection can act on colour and pattern in species, as the quite remarkable resemblance between models and their mimics suggests. This does not necessarily mean that all colours and patterns are selected for, and especially, that colour and pattern variation is adaptive and maintained by balancing selection. The colour and pattern variation in *N.pronuba* is exemplified by both the temporal and geographical stability of the forewing colour and pattern phenotypes. This stability could be interpreted as evidence for balancing selection. However, the low levels of between population variation in polymorphic allozyme loci suggest that gene flow is of major importance in determining the geographical stability of the forewing polymorphism. In addition, the large population size, coupled with the high levels of gene flow, also explain the temporal stability. The results of this study show the benefits gained from obtaining complementary information from genetic markers which give independent estimates of gene flow. This approach needs to be used more widely when studying the geographic variation in morphological characters, particularly polymorphisms for colour and pattern. It is difficult to dismiss frequency-dependent selection as a selective mechanism that may be important in the overall maintenance

of the polymorphism, but it can be concluded that gene flow is the major mechanism that causes phenotype frequency stability.

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Appendix i: Brood rearing experiments:

Female *N.pronuba* were collected from light traps run at Bullingdon Green, Oxfordshire and Long Wittenham, Oxfordshire. As the females were wild caught it was presumed that they had already mated with at least one male of unknown phenotype. To facilitate oviposition the females were placed in clear perspex boxes measuring 28 cm x 9 cm x 9cm. A piece of nappy liner was put into each box for the moths to oviposit on. In addition a small ball of cotton wool, that had been previously soaked in a dilute honey and water solution, was provided as a food source. The boxes were kept in a growth cabinet between $22^{O}C$ (+ or -$2^{O}C$) and with a photophase of 12 hours. These conditions were chosen as they have been shown to be optimal for oviposition in this species (Novak & Spitzer 1975).

Eggs were removed from the cages every other day and placed into small plastic bottles until they hatched. Once the larvae had hatched and consumed the remnants of the egg casing, they were carefully moved with a camel hair brush to a clear perspex box which was lined with tissue paper and a layer of nappy liner. At the start of the rearing work the larvae were fed on either dock (*Rumex* spp) or dandelion (*Taraxacum* spp). Once the larvae reached the third instar it became impractical to collect enough vegetation to feed them. The diet was switched to an artificial recipe (table A1.1). Before use the diet mixture was autoclaved to reduce the chance of contamination by pathogenic bacteria and yeasts.

As the larvae grew the numbers per box were reduced until at the final instar a maximum of 10 individuals could be kept in the same box. This reduced the numbers of each brood that could be reared as space became a major constraint.

In the final instar the plastic box was half filled with moist peat. This allowed the insects to burrow into the peat and pupate. Once the pupal case had hardened off the pupae were removed and put into a clean plastic box that was lined with tissue. They remained in the box until they emerged from the pupae and were scored for their phenotype.

Artificial *N.pronuba* diet:

For 1 litre of diet the following were mixed together and autoclaved at 100 Kpa for 20 minutes:

Agar	20g
Caesin	35.2g
Wheatgerm	76.8g
Wessons salts	10g
Dried yeast	15.2g
Sucrose	31.2g
Cholesterol	1g
Methyl-4-Hydroxybenzoate	1g
Sorbic acid	1.6g
Water	1000ml
Linseed oil	2ml

After autoclaving the mixture was allowed to cool and the following were added:

Vitamin / antibiotic mix	6g
Choline Chloride	1g

The diet was kept in a refrigerator until use.

Results:

Females oviposited with varying degrees of success. Under these conditions Novak and Spitzer (1975) reported that the median oviposition period was 19 days. The females in this study were wild caught and so it was difficult to

estimate their age. In light of this, the females should have oviposited more quickly than those reported by Novak and Spitzer.

Some females were kept for long periods of time without laying any eggs at all, especially those females that had been caught early in June at the beginning of the flight season. Dissection of these females revealed that they had not mated as no spermatophores were found in the corpus bursae.

The eggs, if fertile, hatched within 2 weeks. As the eggs developed there was an associated change in colour from an initial yellow to purplish grey. This colour change is caused by the development of the head capsule and the dark pigment being visible through the egg casing.

Fifteen of the 25 females caught in June and put to oviposit laid eggs even though they had not been mated. The eggs from these females were laid in small batches (<10 eggs) or singly rather than in the large (200+ eggs) batches that fertile females laid. The unfertilised eggs shrivelled up within a few days of being laid.

Phenotype ratios from reared broods:

Eighteen of the 73 females that were put to oviposit produced broods that were reared to adulthood. The numbers in each brood varied from 1 to 53 with many individuals being lost due to bacterial and viral infections. Table A1.1 shows the phenotype ratios produced by each of the broods.

Table A1.1: Phenotypic ratios from females of known phenotype

Brood code	Maternal Phenotype	Offspring phenotype								sex ratio	total
		rm	om	sm	tot m	rf	of	sf	tot f		
56	Rufous	24			24	20			20	1.20	44
49	Rufous	19	3		22	18	4		22	1.00	44
63	Rufous	11	5		16	11	3		14	1.14	30
68	Rufous	2			2	2	1		3	0.67	5
50	Rufous	3	5		8	4	7		11	0.73	19
53	Rufous	4	1		5	1	1		2	2.50	7
69	Rufous	1		1	2				0		2
43	Rufous	3	2		5	3	2		5	1.00	10
63	Rufous		1		1	1			1	1.00	2
48	Ochre		2	2	4		4	3	7	0.57	11
67	Ochre			1	1				0		1
55	Ochre	24			24	29			29	0.83	53
59	Ochre	1			1	2	1		3	0.33	4
66	Ochre		30		30		23		23	1.30	53
41	Ochre		23		23		20		20	1.15	43
57	Silver		· 1	6	7		3	3	6	1.17	13
71	Silver		6	5	11		7	4	11	1.00	22
73	Silver		10	7	17		11	9	20	0.85	37
					203				197	1.03	400

Appendix ii: Mark-recapture experiments:

Methods:

Three 125 watt, Robinson mercury vapour traps were used in the experiment. Two of the traps were sited in a garden, surrounded by agricultural land, on the outskirts of Long Wittenham village in Oxfordshire, while the other was run in the vegetable / fruit garden of Little Wittenham Manor.

At Long Wittenham the traps were placed as far away as possible (~50m) from one another so as to reduce interference between the two light sources. One trap was run on a table ~0.5m in height (Long W. back) while the other was situated at ground level (Little W. front). At Little Wittenham (Little W.) the trap was positioned on a low bench (~0.3m).

The traps were run every night for ten nights starting from 19/08/93 until 29/08/93 and on each morning (5.30am - 8.00am) the traps were checked for *N.pronuba* individuals. Captured individual were sexed and classified according to phenotype. They were then checked for marks from preceding days and they themselves were marked. The moths were marked on either the dorsal or ventral surface of the hind wing or the ventral surface of the forewing using a permanent marker pen, they were then released into the surrounding vegetation. Each trap day had its own 'day specific' mark and a list of these is given below.

Day specific marks given at each trap:

Date	wing pair / side / surface / colour
19/08/93	hind / right / dorsal / black
20/08/93	hind / left / dorsal / black
21/08/93	hind / right / dorsal / red
22/08/93	hind / left / dorsal / red
23/08/93	hind / right / ventral / black
24/08/93	hind / left / ventral / black
25/08/93	hind / right / ventral / red
26/08/93	hind / left / ventral / red
27/08/93	fore / right / ventral / black
28/08/93	fore / left / ventral / black
29/08/93	fore / right / ventral / red

The hind wing was the only dorsal surface marked as this is not visible when the moth is at rest.

The results of the mark-recapture experiments are provided in tables A2.1-A2.4.

Table A2.1: Long Wittenham - both traps combined: Males:

Date:	Tot Cap	Tot Recap:	Date of last capture;									
			19	20	21	22	23	24	25	26	27	28
19/08/93	16											
20/08/93	45	2	2									
21/08/93	28	2	0	2								
22/08/93	34	0	0	0	0							
23/08/93	17	2	0	0	2	0						
24/08/93	4	2	0	0	0	1	1					
25/08/93	3	0	0	0	0	0	0	0				
26/08/93	11	0	0	0	0	0	0	0	0			
27/08/93	7	2	0	0	0	0	0	0	0	2		
28/08/93	9	1	0	0	0	0	0	0	0	0	1	
29/08/93	11	2	0	0	0	0	0	0	0	0	0	2
Total	185	13										
	% recap =	7.03										

Table A2.2: Little Wittenham: Males:

Date:	Tot Cap	Tot Recap:	Date of last capture;									
			19	20	21	22	23	24	25	26	27	28
19/08/93	22											
20/08/93	55	3	3									
21/08/93	22	7	0	7								
22/08/93	54	4	1	0	3							
23/08/93	16	5	0	1	2	2						
24/08/93	9	2	0	0	0	1	1					
25/08/93	6	1	0	0	0	0	0	1				
26/08/93	28	1	0	0	0	0	0	0	1			
27/08/93	5	2	0	0	0	0	0	0	0	2		
28/08/93	10	0	0	0	0	0	0	0	0	0	0	
29/08/93	14	0	0	0	0	0	0	0	0	0	0	0
Total	**241**	**25**										

% recap = 10.37

Table A2.3: Long Wittenham - both traps combined: Females:

Date:	Tot Cap	Tot Recap:	Date of last capture:									
			19	20	21	22	23	24	25	26	27	28
19/08/93	5											
20/08/93	8	0	0									
21/08/93	9	2	1	1								
22/08/93	4	1	0	0	1							
23/08/93	6	3	0	0	0	3						
24/08/93	2	2	0	0	1	0	1					
25/08/93	1	0	0	0	0	0	0	0				
26/08/93	1	0	0	0	0	0	0	0	0			
27/08/93	1	0	0	0	0	0	0	0	0	0		
28/08/93	1	0	0	0	0	0	0	0	0	0	0	
29/08/93	3	0	0	0	0	0	0	0	0	0	0	0
Total	41	8										

% recap = 19.51

Table A2.4: Little Wittenham: Females:

Date:	Tot Cap	Tot Recap:	Date of last capture;									
			19	20	21	22	23	24	25	26	27	28
19/08/93	5											
20/08/93	27	1	1									
21/08/93	15	6	0	6								
22/08/93	22	4	0	0	4							
23/08/93	8	6	0	1	0	5						
24/08/93	0	0	0	0	0	0	0					
25/08/93	1	0	0	0	0	0	0	0				
26/08/93	13	2	0	0	0	1	0	1	0			
27/08/93	4	2	0	0	0	0	0	0	0	2		
28/08/93	2	0	0	0	0	0	0	0	0	0	0	
29/08/93	1	0	0	0	0	0	0	0	0	0	0	0
Total	98	21										

% recap = 21.43

Appendix iii: Allozyme electrophoresis protocols:

Extraction buffers:

Loxdale *et al* (1983) pH 7.1.

Sucrose 15% (w/v)	
Tris / HCl	50mM
Triton X-100	0.5% (v/v)

Tris / HCl pH 7.6
Tris	12.1g
KCl	0.75g
$MgCl_2$	2.0g
EDTA	0.38g
H_2O	1000ml

adjust to pH 7.6 with HCl

Gel / Electrode buffer systems:

Gel concentration: (10.9%) 25g "Connaught" or "Sigma" hydrolysed potato starch in 230ml of gel buffer.

Tris-Malate pH 7.3: (modified from Pasteur *et al* 1988)
solution 1:
Tris (0.1M)	36.3g
Malic Acid (0.08M)	21.45g
H_2O	3000ml

solution 2:
NaOH	12g
H_2O	3000ml

Electrode buffer: pH 7.3
1200ml of solution 1, then add solution 2 until pH 7.3 (approx 800ml).

Gel buffer: pH 7.3

20ml of running buffer and make up to 800ml with distilled water
(pH checked and if necessary altered with solution 1 or 2).

Tris-Citrate pH 8.0: (modified from Pasteur *et al* 1988)

Electrode buffer:

Tris (0.62M)	75.64g
Citric Acid (monohydrate) (0.14M)	30g
H_20	1000ml

adjust to pH 8.0 with Tris (1M) or Citric acid (1M)

Gel buffer:

1/29 dilution of running buffer.

Tris-Citrate pH 7.0: (Shaw and Prasad 1970)

Electrode buffer pH 7.0:

Tris (0.135M)	16.4g
Citric acid (monohydrate) (0.042M)	9.1g
H_20	1000ml

Gel buffer pH 7.0:

1/29 dilution of running buffer	
electrode buffer	66.7ml
H_20 to make up to	1000ml

Lithium borate pH 8.3: (Pasteur et al 1988)

Electrode buffer pH 8.1:

Lithium hydroxide (0.05M)	2.98g
Boric acid (0.19M)	11.75g

adjust to pH 8.1 with either LiOH (1M) or Boric acid (1M)

Gel buffer pH 8.3:

Tris (0.046M)	5.57g
Citric acid (monohydrate) (0.007M)	1.473g

electrode buffer   100ml
    adjust to pH 8.3 with Tris (1M) or citric acid (1M)

Stain buffers:

Tris A (pH 8.0) (Pasteur *et al* 1988)
    Tris   12.1g
    EDTA   0.2g
    H_2O 500ml
    adjust to correct pH with concentrated HCl

Tris B (pH8.0) (Pasteur *et al* 1988)
    Tris   30.275g
    H_2O 500ml
    adjust to correct pH with concentrated HCl

Tris C (pH 5.5) (Pasteur *et al* 1988)
    Tris   12.1g
    Malic acid   13.4g
    H_2O 500ml
    adjust to correct pH with concentrated NaOH

Phosphate F (pH 6.5)(Pasteur *et al* 1988)
    KH_2PO_4 4.6g
    Na_2HPO_4 2.4g
    H_2O 1000ml

Staining Recipes:

Phosphoglucose isomerase PGI:

Tris A buffer (Pasteur et al 1988)   15ml
Fructose-6-phosphate   20mg
NADP   5mg
$MgCl_2$ 25mg
just before use add
Glucose-6-phosphate dehydrogenase
(Leuconostoc) $6\mu l$

MTT	7mg
PMS	1-2mg
1.5% agarose overlay	15ml

Phosphoglucomutase PGM:

Tris A buffer (Pasteur et al 1988)	15ml
Glucose-1-phosphate	50mg
NADP	5mg
MgCl$_2$	70mg
just before use add	
MTT	7mg
Glucose-6-phosphate dehydrogenase (Leuconostoc)	6µl
PMS	2mg
1.5% agarose overlay	15ml

Mannose phosphate isomerase MPI:

Tris A buffer (Pasteur et al 1988)	15ml
D-Mannose-phosphate	20mg
Pyruvate	20mg
NAD	10mg
NADP	5mg
just before use add	
Glucose phosphate isomerase (Leuconostoc)	6µl
Glucose-6-phosphate dehydrogenase (Leuconostoc)	6µl
MTT	10mg
PMS	3mg
1.5% agarose overlay	15ml

Esterase EST (method 1):

Phosphate F buffer (Pasteur et al 1988)	40ml
α-Napthyl acetate (2% in acetone)	5ml
β-Napthyl acetate (2% in acetone)	5ml

Incubate at room temperature for 15 minutes then add:
Fast garnet GBC   100mg

Esterase EST (method 2):

Phosphate F buffer (Pasteur et al 1988)   40ml
α-Napthyl acetate (2% in acetone)   1ml
β-Napthyl acetate (2% in acetone)   1ml
Incubate at room temperature for 15 mins then add
Fast garnet GBC   100mg

Glutamate oxaloacetate transaminase GOT:

Tris A (Pasteur et al 1988)   40ml
L - Aspartic acid   200mg
α - Ketoglutaric acid   100mg
Pyridoxal - 5 - phosphate   10mg
Incubate at room temperature for 30 minutes then add
Fast Blue BB100mg

Malate dehydrogenase MDH:

Tris A (Pasteur et al 1988)   40ml
Malic acid (2M)   5ml
$MgCl_2$ 30mg
NAD   20mg
just before use add
NBT   10mg
PMS $5\mu l$
MTT $1\mu l$

Fumarase FUM:

Tris A (Pasteur et al 1988)   40ml
Fumaric acid   100mg
Pyruvate   30mg
NAD   20mg
just before use add

Malate dehydrogenase	25µl
NBT	10ml
MTT	5ml

Malic enzyme ME:

$MgCl_2$	150mg
Malic acid (2M)	1ml
NADP	1mg
just before use add	
PMS	1ml
NBT	2mg
MTT	2mg

Isocitrate dehydrogenase IDH:

Tris A (Pasteur et al 1988)	20ml
$MnCl_2$	15mg
$MgCl_2$	30mg
NADP	3mg
just before use add	
NBT	3mg
PMS	3mg
MTT	3mg
incubate with above solution for 30 minutes then add	
Isocitric acid(0.29g in 10ml)	2ml

Hydroxybutyrate dehydrogenase HBDH:

Tris A (Pasteur et al 1988)	40ml
Hydroxybutyrate	100mg
NAD	10mg
just before use add	
MTT	10mg
PMS	10mg

Sorbitol dehydrogenase SORDH:

Tris A (Pasteur et al 1988)	40ml
Sorbitol	250mg
$MgCl_2$	20mg
NAD	20mg
just before use add	
NBT	10mg
MTT	3mg
PMS	5mg

Leucine amino peptidase LAP:

Tris C (Pasteur et al 1988)	40ml
L-leucyl-β naphthylamide	100mg
$MgCl_2$	500mg
Black K salt	30mg

Fructokinase FK:

Tris A (Pasteur et al 1988)	15ml
Fructose	200mg
ATP	20mg
NAD	10mg
just before use add	
Glucose-phosphate isomerase (Leuconostoc)	10μl
Glucose-6-phosphate dehydrogenase (Leuconostoc)	10μl
MTT	5mg
PMS	5mg
1.5% agarose overlay	15ml

Hexokinase HK:

Tris A (Pasteur et al 1988)	15ml
α D$^+$ Glucose	500mg
ATP	80mg
NAD	10mg
MgCl$_2$	100mg
NADP	5mg
just before use add	
Glucose-6-phosphate dehydrogenase	10μl
MTT	10mg
PMS	3mg
1.5% agarose overlay	15ml

Glutamate dehydrogenase GLD:

Phosphate-1 buffer	15ml
H$_2$O	15ml
L-Glutamic acid	13mg
NAD	30mg
Just before use add	
NBT	10mg
PMS	4mg

6-Phosphogluconate dehydrogenase 6PGD:

Tris-A	15ml
MgCl$_2$	0.152g
6-Phosphogluconic acid	20mg
NADP	10mg
Just before use add	
PMS	1mg
NBT	2mg
MTT	2mg

Superoxide dismutase SOD:

Tris A	40ml
$MgCl_2$	0.026g
NAD	10mg
Just before use add	
NBT	10mg
PMS	5mg

The gel was then incubated at 25°C whilst illuminated by a neon tube.

Aconitase ACON:

Tris B	15ml
cis-Aconitic acid	75mg
$MgCl_2$	0.2g
NADP	10mg
Just before use add	
IDH	$10\mu l$
MTT	5mg
PMS	2mg
1.5 % agarose overlay	15ml

Xanthine dehydrogense XDH:

Tris A	40mg
Hypoxanthine	100mg
NAD	10mg
Just before use add	
MTT	10mg
PMS	2mg

Lactate dehydrogenase LDH:

Tris A	35ml
D,L,Lactic acid (0.5M)	6ml
NAD	10mg
Just before use add	

| NBT | 3mg |
| PMS | 5mg |

Glucose-6-phosphate dehydrogenase G-6-PD:

Tris-HCl pH 8.0	40ml
Glucose-6-phosphate	30mg
NADP	5mg
Just before use add	
MTT	7mg
PMS	2mg

Table A4.1: Rogers' (1972) genetic distance - PGI locus

	1a	1b	2	3	4	5	6	population 7	8	9	10	11	12	13	14
1b	0.092	*****													
2	0.040	0.074	*****												
3	0.029	0.118	0.054	*****											
4	0.039	0.080	0.019	0.053	*****										
5	0.063	0.152	0.086	0.037	0.084	*****									
6	0.077	0.036	0.048	0.099	0.053	0.132	*****								
7	0.042	0.078	0.032	0.057	0.035	0.086	0.056	*****							
8	0.045	0.054	0.039	0.069	0.049	0.102	0.048	0.044	*****						
9	0.040	0.074	0.028	0.059	0.030	0.087	0.051	0.035	0.040	*****					
10	0.037	0.079	0.019	0.051	0.032	0.081	0.056	0.035	0.035	0.026	*****				
11	0.051	0.141	0.078	0.025	0.078	0.023	0.124	0.079	0.090	0.083	0.073	*****			
12	0.058	0.043	0.053	0.083	0.060	0.116	0.050	0.056	0.023	0.056	0.055	0.103	*****		
13	0.057	0.063	0.024	0.073	0.026	0.104	0.037	0.042	0.046	0.042	0.041	0.097	0.052	*****	
14	0.059	0.094	0.032	0.065	0.041	0.088	0.064	0.050	0.061	0.039	0.029	0.085	0.080	0.050	*****
15	0.033	0.089	0.019	0.041	0.022	0.069	0.064	0.030	0.049	0.030	0.025	0.064	0.064	0.038	0.036

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Appendix iv: Distance measures used in Mantel Tests:

Table A4.2: Rogers' (1972) genetic distance - PGM locus

	1a	1b	2	3	4	5	6	population 7	8	9	10	11	12	13	14
1b	0.162	*****													
2	0.161	0.039	*****												
3	0.124	0.061	0.077	*****											
4	0.072	0.125	0.114	0.090	*****										
5	0.181	0.024	0.053	0.068	0.141	*****									
6	0.153	0.031	0.027	0.062	0.109	0.046	*****								
7	0.119	0.060	0.054	0.040	0.073	0.074	0.045	*****							
8	0.096	0.129	0.109	0.113	0.058	0.147	0.107	0.082	*****						
9	0.095	0.166	0.174	0.108	0.102	0.175	0.163	0.123	0.144	*****					
10	0.157	0.045	0.018	0.077	0.108	0.059	0.029	0.052	0.103	0.171	*****				
11	0.100	0.087	0.092	0.034	0.065	0.098	0.080	0.043	0.097	0.085	0.091	*****			
12	0.079	0.128	0.113	0.095	0.034	0.143	0.107	0.073	0.043	0.114	0.109	0.071	*****		
13	0.105	0.085	0.068	0.071	0.057	0.102	0.064	0.040	0.048	0.134	0.065	0.063	0.048	*****	
14	0.156	0.089	0.112	0.051	0.129	0.085	0.099	0.087	0.161	0.113	0.115	0.068	0.137	0.120	*****
15	0.202	0.044	0.069	0.089	0.163	0.025	0.067	0.097	0.170	0.193	0.075	0.119	0.167	0.125	0.096

Table A4.3: Rogers' (1972) genetic distance - GOT

	1a	1b	2	3	4	5	6	population 7	8	9	10	11	12	13	14
1b	0.032	*****													
2	0.022	0.034	*****												
3	0.031	0.033	0.015	*****											
4	0.035	0.035	0.015	0.015	*****										
5	0.053	0.066	0.036	0.037	0.037	*****									
6	0.034	0.036	0.017	0.021	0.012	0.035	*****								
7	0.000	0.032	0.022	0.031	0.035	0.053	0.034	*****							
8	0.035	0.014	0.042	0.044	0.043	0.075	0.043	0.035	*****						
9	0.035	0.044	0.016	0.011	0.018	0.027	0.023	0.035	0.054	*****					
10	0.027	0.047	0.014	0.023	0.026	0.029	0.027	0.027	0.055	0.017	*****				
11	0.038	0.053	0.022	0.026	0.027	0.024	0.025	0.038	0.061	0.018	0.017	*****			
12	0.022	0.035	0.009	0.023	0.018	0.040	0.017	0.022	0.040	0.024	0.019	0.026	*****		
13	0.035	0.049	0.020	0.025	0.026	0.020	0.022	0.035	0.057	0.020	0.018	0.019	0.024	*****	
14	0.036	0.046	0.022	0.026	0.025	0.027	0.017	0.036	0.054	0.023	0.024	0.017	0.025	0.014	*****
15	0.015	0.027	0.009	0.019	0.020	0.044	0.021	0.015	0.033	0.024	0.021	0.030	0.011	0.027	0.027

Table A4.4: Rogers' (1972) genetic distance - averaged over all three loci

	1a	1b	2	3	4	5	6	population 7	8	9	10	11	12	13	14
1b	0.072	*****													
2	0.056	0.037	*****												
3	0.046	0.053	0.037	*****											
4	0.036	0.060	0.037	0.040	*****										
5	0.074	0.061	0.043	0.036	0.066	*****									
6	0.066	0.026	0.023	0.046	0.043	0.053	*****								
7	0.040	0.043	0.027	0.032	0.036	0.053	0.034	*****							
8	0.044	0.049	0.047	0.056	0.038	0.081	0.049	0.040	*****						
9	0.043	0.071	0.054	0.045	0.038	0.072	0.059	0.048	0.060	*****					
10	0.055	0.043	0.013	0.038	0.042	0.042	0.028	0.028	0.048	0.054	*****				
11	0.047	0.070	0.048	0.021	0.043	0.036	0.057	0.040	0.062	0.047	0.045	*****			
12	0.040	0.051	0.044	0.050	0.028	0.075	0.044	0.038	0.026	0.049	0.046	0.050	*****		
13	0.049	0.049	0.028	0.042	0.027	0.056	0.031	0.029	0.038	0.049	0.031	0.045	0.031	*****	
14	0.063	0.057	0.042	0.035	0.049	0.050	0.045	0.043	0.069	0.044	0.042	0.042	0.061	0.046	*****
15	0.063	0.040	0.024	0.037	0.051	0.034	0.038	0.035	0.063	0.062	0.030	0.053	0.060	0.047	0.040

Table A4.5: Rogers' (1972) genetic distance - calculated from forewing gene frequencies

	1a	1b	2	3	4	5	6	population 7	8	9	10	11	12	13	14
1b	0.019	*****													
2	0.014	0.009	*****												
3	0.025	0.042	0.034	*****											
4	0.046	0.034	0.033	0.058	*****										
5	0.027	0.01	0.02	0.052	0.039	*****									
6	0.052	0.035	0.039	0.071	0.019	0.035	*****								
7	0.071	0.087	0.078	0.047	0.09	0.097	0.107	*****							
8	0.085	0.102	0.093	0.06	0.108	0.112	0.124	0.019	*****						
9	0.035	0.019	0.021	0.054	0.018	0.022	0.018	0.093	0.11	*****					
10	0.051	0.033	0.041	0.075	0.043	0.024	0.028	0.118	0.134	0.029	*****				
11	0.018	0.028	0.019	0.019	0.04	0.039	0.052	0.059	0.074	0.036	0.06	*****			
12	0.026	0.02	0.027	0.05	0.053	0.015	0.05	0.097	0.111	0.036	0.034	0.043	*****		
13	0.061	0.076	0.074	0.058	0.107	0.078	0.111	0.088	0.09	0.094	0.1	0.071	0.066	*****	
14	0.033	0.041	0.043	0.048	0.075	0.041	0.075	0.093	0.102	0.06	0.061	0.05	0.027	0.04	*****
15	0.069	0.051	0.06	0.093	0.061	0.042	0.044	0.138	0.153	0.048	0.02	0.079	0.048	0.111	0.072

Table A4.6: geographic distance - measured in km

	1a	1b	2	3	4	5	6	Population 7	8	9	10	11	12	13	14
1b	0	0													
2	16	16	0												
3	145	145	145	0											
4	161	161	161	97	0										
5	241	241	257	209	113	0									
6	225	225	241	97	113	177	0								
7	612	612	612	483	563	644	467	0							
8	499	499	515	370	435	483	322	161	0						
9	290	290	306	161	225	306	145	338	209	0					
10	177	177	161	306	322	418	402	708	644	435	0				
11	241	241	257	161	257	354	209	354	290	129	354	0			
12	97	97	113	97	161	274	193	515	418	209	225	145	0		
13	435	435	451	306	322	338	209	354	193	193	595	306	370	0	
14	1722	1722	1706	1802	1722	1641	1819	2285	2124	1947	1722	1963	1963	1963	0
15	1175	1175	1159	1159	1320	1416	1384	1497	1513	1368	998	1255	1191	1464	2189